COPPER-IODOMETRIC REAGENTS FOR SUGAR DETERMINATION

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(Received for publication, August 8, 1932)

For the determination of reducing sugars in pure solution or in biological fluids (after suitable pretreatment to remove interfering substances and supplemented where needed by the use of washed yeast), one of the most accurate and convenient methods is the use of copper reagents of the type utilizing iodometric titration of cuprous oxide (Shaffer and Hartmann (1), Somogyi (2)). The delicacy and accuracy attainable in the iodometric titration are such that the error of analysis depends chiefly upon the composition of the copper reagent and the conditions which affect its sensitiveness and reproducibility during the sugar oxidation. With the object of better defining the optimum composition of reagents, the influence of various factors has been studied on the rate of copper reduction and on the maximum reduction equivalent per unit of sugar.

For determination of other sugars than glucose or of mixtures of sugars, especially if quite dilute solutions are to be analyzed with maximum accuracy, reagents and directions designed for glucose are usually not suitable, because of wide differences in the rates of copper reduction by different sugars. Instances might be cited in the literature in which neglect of these considerations—the use of reagents and conditions designed for glucose, with other sugars and mixtures—has led to error and confusion. The information here reported, although fragmentary, should permit more intelligent choice of composition of reagents suited for particular purposes.

The conditions adopted with the Shaffer-Hartmann micro-reagent for blood sugar estimation, namely 5 cc. of reagent and 5 cc. of sugar solution in 25 × 200 mm. Pyrex test-tubes, covered
by glass bulbs and heated by immersion in a rapidly boiling water
bath, have been found convenient and satisfactory for nearly all
purposes, and have been adhered to in the experiments here
reported. Other conditions and volumes may of course be used
if standardized for the sugar to be analyzed.

Effect of Alkalinity of Copper Reagent—The greater sensitiveness
of copper carbonate solutions, compared with the alkalinity of
alkali hydroxide, first noted by Soldiani in 1876 and by Ost in

TABLE I

Variation of Rate of Copper Reduction and of Total Reduction Equivalence of
Glucose with Carbonate Ratio and Content of Reagents

<table>
<thead>
<tr>
<th>Total carbonate</th>
<th>Molar ratio, Na₂CO₃:NaHCO₃</th>
<th>Maximum per cent of maximum reduction in heating periods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂CO₃ + NaHCO₃</td>
<td></td>
<td>5 min.</td>
</tr>
<tr>
<td>0.1 (NaOH)</td>
<td>No carbonate</td>
<td>5.4</td>
</tr>
<tr>
<td>0.47</td>
<td>1</td>
<td>9.2</td>
</tr>
<tr>
<td>0.6</td>
<td>10</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>No carbonate</td>
<td>11+</td>
</tr>
<tr>
<td>0.7</td>
<td>0.7</td>
<td>10.0</td>
</tr>
<tr>
<td>1.0</td>
<td>3</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>10.7</td>
</tr>
</tbody>
</table>

1890 (3), has led to the adoption of alkali carbonates in most of
the sugar reagents designed since that time. The effect of varying
the concentration and ratio of the carbonates on the rate and
maximum reduction by glucose has been studied with copper
solutions by Somogyi (2) and with ferricyanide solutions by Van
Slyke and Hawkins (4). To extend this information the following
experiments were performed.

A series of copper tartrate sodium carbonate-bicarbonate solu-
tions was prepared, of the compositions shown in Table I, differing only in the total carbonate concentration and in the ratio of carbonate to bicarbonate (with exceptions noted). For reasons stated later iodide and oxalate, components of the Shaffer-Hartmann reagents and necessary for the cuprous iodometric titration, were omitted from the solutions and added after the heating period. With these reagents the amounts of copper reduced by known amounts (usually 1 or 2 mg.) of Bureau of Standards dextrose in

![Graph](http://www.jbc.org/)

**Fig. 1.** Rate of copper reduction by glucose as influenced by carbonate to bicarbonate ratio. Reagents contained 0.6 M total carbonate. \( R \) represents molar ratio, \( \text{Na}_2\text{CO}_3: \text{NaHCO}_3 \).

different periods of heating, under the standard conditions, were determined by iodometric titration of the cuprous oxide. The salient results with a few of the solutions used are shown in Table I.

The characteristic feature is that the lower the alkalinity, or the ratio \( \text{Na}_2\text{CO}_3: \text{NaHCO}_3 \), the slower is the sugar oxidation, but the higher is the final amount of copper reduced. On plotting the time of heating against the amount of copper reduced there is obtained for any one series of reagents of constant total carbonate
content a family of curves which cross in the manner illustrated in Fig. 1. The curves show that the reducing equivalent with any one reagent depends upon the time of heating selected. If short periods of heating are chosen high alkalinity appears to give higher reduction; while if the maximum reduction of each solution is compared, the reverse relation is seen to exist. The maximum reduction per mg. of glucose in carbonate-bicarbonate solutions is 40 to 100 per cent greater than in 0.1 N NaOH, and is increased about one-half within the range of alkalinity covered by changing the ratio of Na₂CO₃:NaHCO₃ from 10 to 0.3.

The influence of changing the carbonate to bicarbonate ratio upon the rate of oxidation and upon the maximum reduction equivalent is presumably a function of hydroxyl ion activity. It is not feasible to determine the pH of the solutions at the temperature of the water bath, but it may be assumed that the pH varies as the log of the carbonate to bicarbonate ratio. When the maximum reduction per unit of glucose is plotted (as in Fig. 2) against the logs of these ratios (as added),¹ the points for solutions

¹ The actual ratios of Na₂CO₃:NaHCO₃ in the reagents differ from that of the added salts because of the reaction, CuSO₄ + 2Na₂CO₃ + 2H₂O →
of the same total carbonate content fall about a straight line, which indicates that the maximum reduction varies inversely with the logarithm of the carbonate ratio. Increasing the total carbonate content gives a rise in the maximum reduction, and is thus equivalent to a decrease of the carbonate ratio.

The rate of copper reduction conforms roughly with the first order equation and may be treated as a pseudomonomolecular reaction. Time curves were plotted for each reagent and from these the time was read at which the reduction was 80 per cent of the maximum, this being in the region of least error. On plotting the values of $1/t$ against the logs of $(\text{Na}_2\text{CO}_3)/(\text{NaHCO}_3)$ for each reagent, the points fall about straight lines as shown in Fig. 3. Since $1/t$ varies as $k$ when $a/(a - x)$ is fixed in the first order equation, this relation indicates that the velocity of copper reduction (and of sugar oxidation) is likewise a linear function of the log of the carbonate ratio, and presumably of pH. The linear relation holds only with constant total carbonate content; as indicated in Fig. 3, an increase of total carbonate slows the rate of reduction,

$$\text{Cu(OH)}_2 + \text{Na}_2\text{SO}_4 + 2\text{NaHCO}_3.$$  

The extent to which this takes place is uncertain, and has been neglected. Using the ratios of the salts as added obviously limits the values in the relations described to the particular concentrations of copper and tartrate as well as of total carbonate used, and to the particular conditions during the heating period.
and is thus equivalent to a decrease of the carbonate ratio. The addition of sodium sulfate similarly slows the rate of oxidation and increases the maximum reduction. The same relations hold also with solutions containing ferricyanide instead of copper as the oxidizing agent.

From these facts it is evident that for maximum sensitiveness toward very low sugar concentrations a low ratio and high total carbonate content would be desirable; but this combination gives a slow reagent which requires correspondingly long periods of heating. When the heating period adopted is shorter than required for nearly maximum reduction, the results are poorly reproducible, and the proportionality between sugar oxidized and copper reduced is less constant, probably because minor variations in the rates of temperature rise on placing tubes in the water bath and consequent differences in the effective duration of the heating period lead to variations in the completeness of sugar oxidation. These errors are avoided when the heating period is long enough to reach a point beyond the steep portion of the time curve (Fig. 1) of the reagent for the sugar or mixture analyzed. Heating much longer than necessary to reach nearly complete oxidation is undesirable because of slow reoxidation of cuprous oxide and of continued reduction by other substances which may be present.

The right-hand ordinate of Fig. 3 indicates the heating periods required to reach 98 per cent of maximum reduction with glucose for reagents of different carbonate ratios and different total carbonate concentrations. For example, 15 minutes is a suitable heating period with a reagent containing 0.6 M total carbonate, with a carbonate to bicarbonate ratio of 1. A 15 minute period is too short for accurate results with lower ratios, or higher total carbonate concentration than 1 M. When less total carbonate is used, the velocity for any given ratio is greater and the heating period may be shorter. It must be emphasized that the particular values of the relations shown are applicable only with glucose, with reagents of the composition stated, and with the particular conditions during the heating period; but the principle is perhaps generally applicable.

The Folin reagent (1926, 1929) (5), the carbonate ratio of which is only about 0.28, with 0.33 M total carbonate (1929 reagent 0.17 M), when used under the same conditions with glucose, re-
quires about 25 minutes heating to attain 98 per cent of maximum reduction. The heating period advised by Folin, 10 to 15 minutes (2 cc. of reagent + 2 cc. of solution), attains only 75 to 90 per cent completion. For more slowly reacting sugars than glucose the Folin reagent should be modified by increasing its alkalinity or should be heated for longer periods. The reagent recently described by Harding and coworkers (6) is also less alkaline than optimum, under the prescribed conditions, for the slowly reacting sugars for which it was designed.

The relative rate of reduction by various sugars with a single copper reagent (Reagent 50 with 5 gm. of KI) is shown in Fig. 4. With this reagent a heating period of 15 minutes is satisfactory for fructose or glucose, but is not optimum for other sugars, which require either a more alkaline reagent or a longer heating period.2

2 The rate of reduction by different sugars is (in pure solution) quite characteristic and is sometimes a useful supplementary means of identification. For this purpose a reagent of low alkalinity is desirable, since the differences
Table II gives optimum heating periods and the reduction with various sugars and several different copper reagents.

Copper—The copper content of the reagent obviously limits its oxidizing capacity, but so long as a considerable excess is present copper concentration has relatively slight effect on the rate of oxidation or upon the reduction equivalent. In the solutions here described the copper content, 7.5 gm. per liter (0.03 M), provides for the oxidation of amounts up to 2.5 mg. of glucose by 5 cc. of reagent. With equal volumes of reagent and sugar solution, this limits the maximum sugar concentration to 0.05 per cent (of glucose or equivalent reducing capacity) in the solution analyzed (corresponding to blood sugar values of 500 mg. per cent when 1:10 blood filtrates are used). Reagents of higher capacity (greater copper content) may be prepared for stronger sugar solutions, but we usually find it more convenient to dilute the sugar solutions to within the range of the microreagents. Within the range of 1.0 to 2.5 mg. of glucose in 5 cc. (0.02 to 0.05 per cent) the error should not exceed about 1 per cent.

Iodate—Iodate is used only to provide a known amount of iodine (liberated when the solution is acidified) to reoxidize the cuprous ion; it does not react in alkaline solution and has no rôle in the sugar oxidation. The amount of iodate added in preparing the reagent may be varied, depending upon the range of sugar concentration over which it is to be used. An excess of iodine over the amount required to oxidize the cuprous oxide is necessary, and the amount of iodate (and iodide) therefore limits the capacity of the reagent. The amount of the excess is immaterial except as regards convenience of titration; time is saved if large excess be avoided. For the upper range of reagents containing 7.5 gm. of copper sulfate, 0.025 N KIO₃ is a suitable concentration

In rates are thereby magnified. Failure to realize the slow rate of reduction by mannose and the necessity of prolonging the heating period to approach completion if reproducible results are to be obtained, explains the failure of the Shaffer-Hartmann reagent with mannose in the hands of Moore, Lloyd, and Burget (7). With the first Shaffer-Hartmann microreagent and a heating period of 25 minutes, mannose determination in proper solutions presents no difficulties, as shown by experiments carried out for us by Mr. Ray D. Williams. But even with a suitable copper reagent and heating time, dependable results could not be expected with untreated intestinal contents as used by these authors.
### TABLE II
Data for Construction of Curves or Tables for Copper Reagents

Titration differences, in cc. of 0.005 N thiosulfate, after heating 5 cc. of reagent + 5 cc. of sugar solution for the period stated, in a boiling water bath, in tubes covered with glass bulbs.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Reagent 50, with 1 gm. KI</th>
<th>Reagent 50, with 5 gm. KI</th>
<th>Shaffer-Hartmann micro Reagent</th>
<th>Shaffer-Hartmann reagent, except only 1 gm. KI</th>
<th>40 gm. Na₂CO₃ (no bicarbonate) otherwise like Reagent 50 with 1 gm. KI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Fructose</td>
<td>Arabinose</td>
<td>Mannose</td>
<td>Xylose</td>
</tr>
<tr>
<td>Heating period, min.</td>
<td>15</td>
<td>10</td>
<td>15</td>
<td>35</td>
<td>30</td>
</tr>
<tr>
<td>Mg. sugar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.50</td>
<td>22.80</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.00</td>
<td>18.35</td>
<td>17.90</td>
<td>15.42</td>
<td></td>
<td>16.90</td>
</tr>
<tr>
<td>1.50</td>
<td>13.65</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td>9.10</td>
<td>8.79</td>
<td>7.76</td>
<td>8.27</td>
<td>8.80</td>
</tr>
<tr>
<td>0.50</td>
<td>4.45</td>
<td>4.25</td>
<td>3.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>2.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td>1.72</td>
<td>1.68</td>
<td>1.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>0.85</td>
<td>0.84</td>
<td>0.77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>0.42</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.025</td>
<td>0.20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Reagents for Sugar Determination

(0.893 gm. of KIO₃ per liter). This permits analysis of solutions up to 0.05 per cent glucose, or 1:10 blood filtrates of hyperglycemic bloods up to 500 mg. per cent of blood sugar. For normal and hypoglycemic bloods, and for blood filtrates of 1:20 or higher dilution, it is rather more convenient to use reagents with less iodate (0.01 or 0.015 N), merely to reduce the time required in titration.

Rochelle Salt—The amount of Rochelle salt must be sufficient to keep the copper in solution; about 3 times the weight used of copper sulfate appears to be optimum. (The amount prescribed in the first Somogyi modification (2) proved too small in that it permits the separation of some copper carbonate.) A large excess of tartrate, as noted by Folin (1929), somewhat decreases the rate of copper reduction.

The effect of substituting citrate for tartrate was studied. Citrate considerably decreases the amount of copper reduced, and decreases greatly the sensitiveness of reagents for high dilutions of glucose, as found by Folin (5). In comparison with tartrate, citrate does not, according to our experiments, render copper reagents selective in the sense of depressing the relative reducing power of non-fermentable substances in either urine or blood filtrates.

Potassium Oxalate—This constituent is best omitted from the reagents and added before titration, as described in a later section. Its presence during the heating period, as noted by DeLong (8), decreases the reduction.

Potassium Iodide—In the Shaffer-Hartmann reagent KI was incorporated for convenience and was assumed to be inert up to the time of acidification before the iodometric titration. It has been found, however, that the iodide fills two distinct rôles which require explanation.

If the amounts of copper reduced (cc. of thiosulfate titration difference) by known amounts of glucose, with either the Shaffer-Hartmann reagent or the Somogyi modification, be plotted on coordinates, the points above about 0.2 mg. of glucose in 5 cc. of solution lie on a line (different for each reagent) which is almost straight, extension of which to zero reduction bisects the base not at the point of origin (zero sugar) but at a distance removed. The curve corresponds not to simple proportionality, but to $y = ax -$
$b$, in which $y$ is the copper reduced or titration difference, $x$ the sugar present, $a$ the proportionality constant, and $b$ another constant representing an amount of sugar which is apparently lost. It is the loss of this quantity which is responsible for the lack of sensitiveness of the Shaffer-Hartmann reagent toward very dilute solutions (less than 0.003 per cent glucose) noted by Hiller and coworkers (9). When the total reduction approaches the amount “lost” the results are variable and unreliable. When hypoglycemic bloods came to be analyzed repeated attempts were made to correct this defect and to obtain a simple proportionality, but without much success until DeLong (8) noted that the amount of copper reduced is increased on merely omitting the iodide. In view of the fact that with Bang’s microsugar reagent which contained much KCl, no cuprous oxide precipitates, and heating in an atmosphere of inert gas is necessary to avoid oxidation of cuprous salt, the effect of iodide should have been recognized earlier. Iodide, much more effectively than chloride, holds cuprous oxide in solution readily accessible to reoxidation, and the greater the amount of iodide present, the greater the loss of cuprous oxide proves to be.

By omitting the iodide, reoxidation is greatly reduced and (in covered tubes) a nearly constant proportionality between copper reduced and sugar oxidized holds throughout the range covered by the reagent. The experimental points fall on a line which is curved only slightly upward and which almost exactly bisects the origin. Iodide-free reagents are very serviceable where the utmost sensitiveness is desired, and in private correspondence we have recommended such solutions to several workers. The iodide (and oxalate) are added after heating and cooling, just before acidifying for the titration.

The omission of the iodide, however, brought out a second unsuspected rôle it has when present in the reagents; namely, its stabilizing effect. Reagents containing 5 to 10 gm. of KI (and 25 gm. of Rochelle salt) per liter do not show any autoreduction either at room temperature or upon heating, the titration of heated and cold blanks being identical and unchanged for long

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*The values in the tables (pp. 380, 381) of the paper by Shaffer and Hartmann (1) for amounts of glucose less than 0.5 mg. of glucose in 5 cc. are erroneous.
periods. Reagents without iodide on the other hand separate small amounts of cuprous oxide at room temperature and show additional autoreduction upon heating, which increases with age of the solution. The separation of cuprous oxide at room temperature appears to cease after a week or two since if the solution is decanted no more is deposited if stored in Pyrex glassware. (Some continues to deposit if stored in ordinary glass bottles.)

Another objection to reagents without iodide is their greater sensitiveness to non-sugar reducing substances. With tungstic acid blood filtrates the results are 5 to 10 mg. per cent higher than by reagents containing iodide. Even with zinc or iron filtrates which contain none or barely detectable amounts of non-fermentable reducing substances, the iodide-free reagents give results which are often a few mg. per cent too high. Fortunately these objections are removed by the use of a small amount of iodide.

With 1 gm. of iodide per liter, the autoreduction of the reagents at room temperature is prevented (no cuprous oxide separates), while the sensitivity to low sugar concentration is almost as great as without iodide. The sensitiveness to non-sugars in blood filtrates is also less, in zinc or iron filtrates being negligible. Consequently, we prefer to add this small amount of iodide for its stabilizing effect, and have largely discarded reagents without iodide. 1 gm. of iodide is not, however, enough to produce maximum stability. The autoreduction of heated water blanks increases very slowly with the age of the solution, making it necessary that such blanks be determined regularly or at intervals.

With 5 gm. of KI per liter the reagents are quite stable and for some purposes are to be preferred. We, therefore, describe both reagents containing 1 gm. of KI and those containing 5 gm. of KI. Except as noted below either is equally applicable to solutions containing more than 2 mg. per cent of glucose or the equivalent.

For solutions less than about 2 mg. per cent (0.1 mg. in 5 cc.) the reagent containing 1 gm. of KI should be used because with more iodide the amount of cuprous oxide formed may not be sufficient to give dependable titrations. This point is important in work involving determinations in fermented blood filtrates in which the presence of reducing substances may escape detection by less sensitive reagents.
When used with blood filtrates, the method of precipitation and the dilution of the filtrate as well as the range of blood sugar concentration will determine which of the two reagents may be used. With 1:10 tungstic acid filtrates of laked blood the reagent containing 5 gm. of KI is applicable and somewhat preferable because it is less sensitive to the non-sugars present. But with both reagents the results are too high by 17 to 25 mg. per cent. Although approximate results are obtained by subtracting 20 mg. per cent from the determined value on tungstic acid filtrates (of laked blood), it is preferable to use either zinc (10) or iron-barium carbonate (11) filtrates with the iodometric copper reagents. In most instances these filtrates contain no detectable non-fermentable reducing substance according to results with the iodide-free reagent or the reagent containing 1 gm. of KI and according to the Folin (1929) colorimetric method. With the reagent containing 1 gm. of KI and 1:10 filtrates the reduction corresponding to 5 mg. per cent of sugar in the blood (0.025 mg. of glucose in 5 cc.) gives a titration difference of 0.2 cc. of 0.005 N thiosulfate. After fermentation with washed yeast, zinc or iron filtrates usually show the same titration as the heated blanks, sometimes from 0.1 cc. less to 0.1 cc. more, and only occasionally as much as 0.2 cc. more. Glucose corresponding to 5 to 10 mg. per cent of blood sugar added to fermented filtrates is determinable by the iodometric reagents within 2 mg. per cent or better. We believe that the results obtained in zinc or iron filtrates with these reagents are as accurate as at present attainable for blood glucose, and that no correction for non-sugars is necessary.4

In the range of extreme hypoglycemia with 1:10 filtrates (below 20 mg. per cent), and with filtrates of 1:20 or higher dilution, as when cutaneous blood is used, the reagent containing 1 gm. of KI is preferable because of its greater sensitiveness at low sugar concentrations. With 1:40 filtrates values of 80 mg. per cent and above are satisfactorily determinable with the solution containing 5 gm. of KI; it is only with hypoglycemic bloods or with fermented filtrates that the distinction needs to be made.

4 Benedict (12) finds in fermented zinc filtrates reduction corresponding to 4 to 7 mg. per cent of blood sugar, while MacKay (13) states that his zinc hydroxide filtrates were entirely free from non-fermentable reducing substances toward the iodometric copper reagent used by him.
With filtrates prepared by mercury precipitation, if zinc dust be used to remove the excess Hg, traces of H$_2$O$_2$ are formed. This introduces error with the reagent containing 1 gm. of KI, but not with that containing 5 gm. of KI. If it is desired to take advantage of the more sensitive reagents with 1 gm. or no iodide (to measure rest reduction in mercury filtrates), the excess Hg must be removed by H$_2$S, followed by aeration.

Reagents

The solutions described below have been used for some years for glucose in blood and other biological material. Many other equally good or perhaps better combinations might be selected, by consideration of the factors already discussed. The solutions contain approximately 0.5 M total carbonate, with a ratio of carbonate to bicarbonate of 1, which is about optimum for glucose, with a heating period of 15 minutes. For most sugars other than glucose either longer heating periods or reagents with higher alkalinity are necessary.

**Composition of Copper-Iodometric Reagent 50**

<table>
<thead>
<tr>
<th>Component</th>
<th>Grams per liter</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$CO$_3$ (anhydrous)</td>
<td>25</td>
<td>0.236</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>20</td>
<td>0.238</td>
</tr>
<tr>
<td>Rochelle salt</td>
<td>25</td>
<td>0.089</td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>7.5</td>
<td>0.03</td>
</tr>
<tr>
<td>KIO$_4$, 0.1 N as to I$_4$, cc.</td>
<td>50, 100, 200, or 250</td>
<td></td>
</tr>
</tbody>
</table>

* The reagent containing 5 gm. of KI is made by adding to the preparation 5 gm. of KI per liter; that containing 1 gm. of KI by adding 1 gm. of KI per liter.

Reagent 60 for Slowly Reacting Sugars—This has the same composition as Reagent 50 except that 40 gm. of Na$_2$CO$_3$ per liter are used and bicarbonate omitted. For greater stability at the higher alkalinity it may be preferable to use 5 gm. of KI with this solution. If that be done the reducing values given in Table II for mannose will not hold; the values stated are for the reagent with 1 gm. of KI.

Preparation—In order to retain all CO$_2$, the solutions should be made up as follows: The Na$_2$CO$_3$ and Rochelle salt are dissolved in about 500 cc. of distilled water. The CuSO$_4$ solution (75 cc., 10 per cent) is then added by pipette or funnel extending
well below the surface of the liquid, the solution being stirred. The dry NaHCO₃ is next added and dissolved by stirring, followed by the KI. The solution is rinsed into a liter volumetric flask, the desired quantity of 0.1 N iodate is added, and the whole diluted to the mark and mixed. Filter through washed (and dried) paper. If kept in a stoppered Pyrex flask or Pyrex bottle protected from strong light, the solutions remain unchanged for a year or two.

**Standard Iodate Solutions.** Solution A—3.567 gm. of pure KIO₃ in 1 liter. When treated with an excess of KI and H₂SO₄, this solution liberates iodine equivalent to its volume of 0.1 N. The solution is used to standardize the 0.1 N thiosulfate, and may be used also as a constituent in preparing the copper reagents. (3.250 gm. of KH(IO₃)₂ dissolved in water, neutralized by 83.3 cc. of 0.1 N NaOH, and diluted to 1 liter may be used instead.)

Solution B—An iodate solution equivalent to 0.01 N I₂ is prepared at intervals by accurate dilution of Solution A. The 0.01 N solution is used for standardization of the dilute thiosulfate.

Thiosulfate—An approximately 0.1 N stock solution is used. The addition of about 10 cc. of 0.1 N NaOH per liter greatly increases its stability. (When alkali is added it is essential that all solutions to be titrated with the slightly alkaline thiosulfate be previously acidified.) The thiosulfate is standardized by titration of the standard KIO₃ solution (to 25 cc. of KIO₃ add about 50 cc. of water, 1 gm. of KI (iodate-free), and 5 cc. of N H₂SO₄). To prepare 0.005 N thiosulfate, calculate from the normality factor of the 0.1 N solution the amount required to give 500 cc. or a liter of exactly 0.005 N solution. If about 10 cc. of 0.1 N NaOH are added (before dilution) the solution retains its titer for some days; if not alkaline, it must be prepared fresh each day. 10 cc. of the 0.01 N KIO₃ solution (with 1 cc. of N H₂SO₄ and 2 cc. of 2.5 per cent KI) should titrate exactly 20 cc. of the 0.005 N thiosulfate; 5 cc. of the copper reagent should titrate the same as the volume of normal iodate added per liter (20 cc. for 0.020 N iodate).

**Details of Technique**

Since the ratio of copper reduced to sugar oxidized depends not only upon the composition of the reagent but upon all conditions during the heating period, it is necessary to standardize these conditions and to calibrate the reagent with known amounts of the
sugar under the exact conditions used in analysis. If changes are introduced, the reagents must be calibrated under the new conditions. The procedure used in our laboratories is the following.

With an accurate pipette measure 5 cc. of the sugar solution into a Pyrex test-tube (25 × 200 mm.), followed by 5 cc. of the copper reagent added in a manner to rinse the sugar solution from the walls of the test-tube. Both require careful measurement. Daily, for highest accuracy, or at longer intervals, measure also one or two 5 cc. portions of reagent for blanks, adding water instead of sugar solution. The solutions are mixed by gentle shaking and the tubes are covered by sealed glass bulbs (blown from heavy walled glass tubing and of such size as to be supported on the rim of the test-tube). Covering the tubes is essential to avoid convection currents and oxidation by air during heating and cooling. The tubes are placed in metal racks holding snugly (to avoid agitation during boiling) eight to twelve tubes, and placed in a vigorously boiling water bath for 15 minutes (or other period) an interval timer being used. At the end of this period the rack and tubes are removed to a pail or pan of cold water.

When cooled (preferably only to about 30°), the following is added to each tube, depending upon the reagent used and its iodate content. With the reagent containing 5 gm. of KI, the iodate equivalent to 0.015 N I₂ or less (giving a blank titration of 15 cc. or less), merely acidify with 5 cc. of N H₂SO₄; no additional KI and no oxalate are necessary. When the reagent containing 5 gm. of KI contains above 0.015 N iodate (blank titration more than 15 cc.) and with any reagent containing 1 gm. of KI, add first to each of the tubes 2 cc. of a solution containing 2.5 per cent each of KI and potassium oxalate. (This keeps for a week in a dark glass bottle.) Next add 5 cc. of N H₂SO₄.⁶ Replace bulbs and mix well

⁶ Our water baths are heavy walled copper pails, 17.5 cm. in diameter and 20 cm. high, supplied with a constant level overflow and connected by pet-coc with a constant slow water supply. The water level is 10 cm. from the bottom and stands 9 cm. from the bottom of the glass tubes when the rack is in place. The bath is supported on a tripod and heated by a large size Meker or Fischer burner so as to maintain vigorous boiling with slight interruption when the rack and tubes are put in place. These details are not unimportant because they influence the rate of rise of temperature, and the effective duration of the heating period.
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to dissolve completely the cuprous iodide which sometimes precipitates, especially when the reduction is considerable or if the Cu$_2$O has settled from long standing before titration. After standing (covered) 5 to 10 minutes, with occasional agitation so long as undissolved cuprous oxide or iodide is visible (important), rinse the bulb and walls and titrate with 0.005 N thiosulfate, adding about 1 cc. of 1 per cent starch solution (Merck’s Lintner) toward the end. The end-point may be set with split drops. If the titration is less than 1 cc., the result is doubtful because of approach to the capacity of the reagent; the determination should be repeated with a greater dilution of the sugar solution.

When oxalate is not used, the solutions become crystal-clear and remain so. When oxalate has been added a precipitate of copper oxalate often forms but does not affect the results.

For stirring the solution during titration, a rod made of glass tubing, sealed and flared at the lower end to form a button-like foot, is convenient. We use high grade 25 cc. burettes with glass stop-cocks, graduated in 0.05 cc., and read to hundredths. The burettes are filled from a reservoir by suction or pressure.

The titration value is subtracted from the heated blank titration and the titration difference corrected by factor if the thiosulfate is not exactly 0.005 N. With the reagent containing 1 gm. of KI the titration difference in cc. of 0.005 N multiplied by 0.113 gives (within about 3 to 5 per cent) mg. of glucose in 5 cc. of the solution analyzed; or with 1:10 blood filtrates multiplied by 22.6 gives (with the same error) mg. per cent of blood sugar. Calculation by factor is sufficiently accurate for many purposes. The proportionality is, however, not quite linear throughout, and for the maximum accuracy the result should be read from a large scale curve or table prepared from analyses of known pure glucose or other sugar solutions, or from the data given in Table II. For reagents containing more than 1 gm. of KI, calculation by factor is not permissible, and a table or curve for that reagent and sugar must be used.

Pretreatment of Sugar Solutions—The accuracy of results obtained with iodometric copper reagents will be influenced by the amount increase in the acid is necessary. With 1 M carbonate reagents use 3 cc. of 5 N acid.
Reagents for Sugar Determination

of other non-sugar reducing substances present in the solutions analyzed. The reagents are not selective in the sense of being insensitive to substances which may be oxidized either by copper in alkaline or by iodine in acid solution. It is not the purpose of this paper to consider methods of removing interfering substances, but the following procedures have been found satisfactory in our laboratories preliminary to use of the iodometric copper reagents. Whole blood is precipitated by zinc (Somogyi (10)) or iron (Steiner, Urban, and West (11)) hydroxides, the filtrates of which appear to give “true” sugar values. For serum, iron precipitation is preferable. For urine and for hydrolyzed or unhydrolyzed tissue extracts precipitation by HgSO₄-BaCO₃ (West et al. (14)) is serviceable, but the filtrates contain other copper-reducing substances, and yeast must usually be resorted to for determination of fermentable sugar. With urine of low glucose content determination in the untreated diluted urine, before and after yeast fermentation, appears to give substantially the same results as by yeast in mercury filtrates; when protein is present iron (11) or mercury (14) precipitation, followed by yeast, should be used. For the most accurate results glycogen hydrolysates are determined before and after yeast fermentation with the more sensitive copper reagents containing 1 gm. of KI. By adequate dilution of the neutralized solutions errors due to salt concentration become negligible.

SUMMARY

In order to learn the optimum composition of iodometric copper reagents for glucose and other reducing sugars the effect of the ratio of sodium carbonate to bicarbonate and of total carbonate concentration on the rate of copper reduction was studied. A comparison of the rates of reduction by different sugars was made. The influence of iodide and other constituents is considered. No one reagent is optimum for all sugars. Several reagents are described.

BIBLIOGRAPHY
