NOTES ON SUGAR DETERMINATION.

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I.

Influence of Alkalinity on Copper Reduction.

In the course of the past few years we have had the experience that whenever acid-glucose solutions—as represented for instance by hydrolysates of glycogen—were neutralized with sodium carbonate or bicarbonate, the values obtained by copper reduction have been somewhat inconsistent. On closer investigation upon pure glucose solutions it has been found that added bicarbonate, even in slight amounts, causes appreciable increase of the copper reduction values. This is in line with the well known fact that a lowering of the alkalinity of copper solutions entails an increase in reduction values. Shaffer and Hartmann (1) observed an increase of 10 per cent with their carbonate-citrate copper reagent as compared with the reduction given by the more alkaline Fehling solution. It is in accord also with the observation of the same authors that when they have substituted Rochelle salt for tartaric acid in their carbonate-tartaric acid copper reagent the resulting solution had about 10 per cent lower reduction values. This difference is probably due to the bicarbonate formed (and consequent decrease of alkalinity) when the free tartaric acid is neutralized by sodium carbonate.

These facts prompted us to determine more accurately the effect of varying alkalinity upon the reduction values in the system, glucose-alkaline copper solution. To this end a series of nine copper reagents was prepared in all of which Rochelle salt was used instead of tartaric acid; the reagent of highest alkalinity contained 40 gm. of sodium carbonate per liter, the other eight solutions were

made up with buffer mixtures containing varying amounts of sodium carbonate and bicarbonate. The rest of the components were identical with those of the original Shaffer-Hartmann reagent. The reduction values of the nine reagents were determined on pure glucose solutions of concentrations of 0.04, 0.03, 0.01, and 0.00252 per cent, following the standard procedure, the actual amounts of glucose in the 5 cc. portions used for each determination being 2, 1.5, 0.5, and 0.126 mg. respectively.

In Table I are presented the sodium carbonate-bicarbonate ratios of the several reagents and the respective reduction values,

TABLE I.

Reduction Values of Shaffer-Hartmann Reagents with Varying Amounts of Carbonate and Bicarbonate.

No. of reagent.	Molar concentration of:		Apparent pH of Na_2CO_3 mixture.	Cc. of iodine consumed by				Mg. of Cu reduced by			
				2.0	1.5	0.5	0.126	2.0	1.5	0.5	0.126
	$NaHCO_3$	Na_2CO_3		mg. of glucose.				mg. of glucose.			
I	0.400	0.100	9.45	15.60	11.07	3.02		4.96	3.52	0.95	
II	0.375	0.125	9.55	16.40	11.35	3.10	0.22	5.22	3.60	0.99	0.07
III	0.350	0.150	9.68	16.80	12.03	3.25	0.32	5.34	3.83	1.03	0.101
IV	**0.325**	**0.175**	**9.80**	**17.0**	**12.45**	**3.45**	**0.34**	**5.40**	**3.96**	**1.10**	**0.108**
V	**0.300**	**0.200**	**9.90**	**17.0**	**12.45**	**3.45**	**0.36**	**5.40**	**3.96**	**1.10**	**0.115**
VI	0.275	0.225	10.00	16.40	12.08	3.40	0.39	5.22	3.84	1.08	0.117
VII	0.250	0.250	10.10	16.0	11.90	3.13	0.27	5.09	3.79	1.00	0.086
VIII	0.100	0.350	10.60	14.3	10.6	2.83	0.23	4.55	3.37	0.90	0.075
IX	0	0.400	11.60	12.35	9.4	2.50	0.24	3.93	2.99	0.80	0.076

expressed in quantities of 0.005 N iodine consumed in reoxidation of the cuprous oxide formed.

A word has to be said regarding the pH values in the table: these are by no means actual pH values of our copper reagents, not even those of our carbonate-bicarbonate buffer mixtures but are cited from the data by Auerbach and Pick (2) on sodium carbonate-bicarbonate mixtures of the same ratios but lower concentration (0.2 molar solutions). We have not attempted to determine the actual pH for optimum copper reduction by glucose. This would not be accomplished even if the pH values of the reagents were accurately known, as presumably a considerable

shift in reaction occurs during the 15 minutes warming, due to acid formation from the sugar oxidation, and from the loss of carbonic acid from the bicarbonate. However, the order of *actual differences* between the pH values of the individual reagents

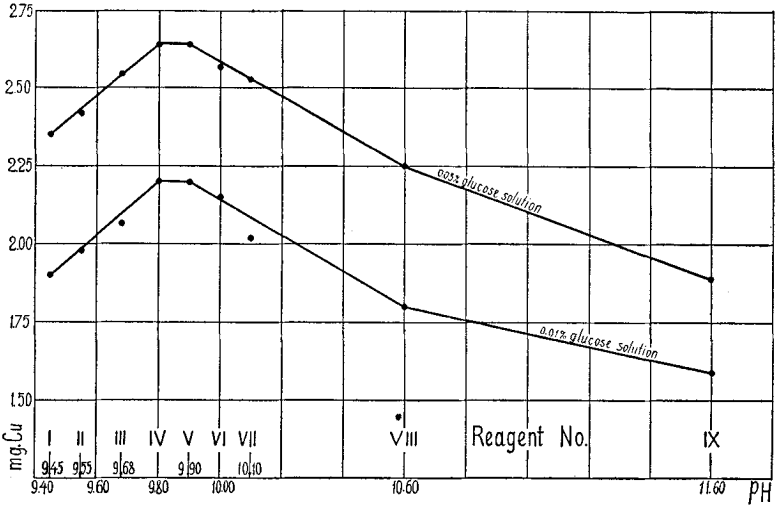

FIG. 1.

in our series is indicated by the figures cited, and this will suffice to emphasize the fact that *comparatively small changes in alkalinity produce considerable differences in reduction values.*[1]

[1] After this paper had been sent to press, Visscher (3) reported his observation that the addition of acid phosphate to sugar solutions in determinations by the Shaffer-Hartmann macro method effected a lowering of the reduction values by about 20 per cent as compared with results obtained on the addition of a similar amount of dibasic phosphate. Since the total concentration of the phosphate in Visscher's experiments is constant apparently the shift in hydrogen ion concentration alone is responsible for the change in reduction values. I am emphasizing in my present paper that for the determinations by alkaline copper reagents in general the sugar solutions ought to be neutral or at least approximately so, and must not possess sufficient buffer value to change materially the alkalinity of the copper reagent. Where such buffer action is unavoidable it is necessary to determine anew the reducing value of the copper reagent in the presence of the buffer, as done by Visscher, instead of using the published tables of copper:sugar values.

As shown in the table, Reagents IV and V—with $\frac{[Na_2CO_3]}{[NaHCO_3]}$ ratios $\frac{7}{13}$ and $\frac{2}{3}$ respectively—*furnish the highest reduction values.* (In our electrometric measurements we have found for the carbonate-bicarbonate buffer mixture, No. IV, pH = 9.40, for No. V, pH = 9.55.) Departure in either direction from this quite narrow range of optimum alkalinity causes a decline of the reduction values, the change being relatively greater in the direction of increasing alkalinity.

For better illumination of these relations two curves are given in Fig. 1 showing the alterations of reduction values with changing alkalinity. The curves are based on data in Table I. Against the "apparent" pH values on the abscissa are plotted on the ordinate corresponding values of the ratio $\frac{\text{copper reduced}}{\text{glucose}}$ = mg. of copper reduced by 1 mg. of glucose. From the fairly close parallelism of the two curves it can be seen that the effect of variations in alkalinity is much the same for different concentrations of glucose, regardless of the well known fact that the ratio $\frac{\text{copper reduced}}{\text{glucose}}$ suffers a rapid decline with decreasing concentration of sugar.

The question arises here, in what manner is the process of oxidation-reduction in this system affected by the degree of alkalinity: by an alteration in the condition of the copper complex, or in that of the glucose, or perhaps both. Two experimental facts seem to indicate that the variations are to be ascribed solely to changes in the reactions of glucose with changing alkalinity. First, we have found that the reduction values are subject to variations of the same nature and the same order if iron is taking the place of oxidizing agent, as in the Hagedorn-Jensen method. A second evidence is furnished by the behavior of alkaline copper solutions in which sodium citrate is substituted for Rochelle salt. Such reagents exhibit markedly depressed reduction values as compared with tartrate reagents, all other conditions in both being the same. Shaffer and Hartmann (1) found 10 per cent lower reduction with citrate in place of tartrate, and recently Folin (4) emphasizes this phenomenon speaking of "the antireducing effect of citrates" brought about by "a powerful depressive action

on the oxidative properties of dissolved copper compounds." In our present experiments we have found as much as 33 per cent drop in reduction *at optimum* alkalinity if citrate be substituted for Rochelle salt, and we agree with Folin that this is probably due to a change in the condition of the copper complex. But concurrently we have found that through variations of alkalinity, the reduction values of citrate reagents undergo changes in the same direction and of the same relative magnitude as those of tartrate reagents.

Thus we are confronted by two factors of distinctly different character, both altering the reduction values in the system: glucose-alkaline copper solution. The powerful depressing effect of citric acid is apparently due to a diminished oxidative intensity of the copper complex. The degree of alkalinity, on the other hand, seems to exert its effect upon reduction values by influencing the reactions of the sugar alone.

The curves in Fig. 1 might be interpreted as representing the resultant of two types of reactions in alkaline glucose solutions: one is to produce oxidizable fragments of the glucose molecule, the other to counteract the oxidation—by intramolecular oxidations, condensations, etc., between the fragments—before their oxidation is accomplished. Both these reactions are accelerated in speed by increasing alkalinity but there is a range of pH—represented by the short ridge of the curves—which favors the first of the two types of competitive reactions more than the second. Further increase of alkalinity then accelerates the velocity of the reactions of the second type which thus gradually get the upper hand and lead to diminished reduction values.

II.

Modified Shaffer-Hartmann Reagent.

The bearing of the foregoing upon the methods of sugar determinations by copper-reduction methods in general is obvious. In the light of it we understand why Folin (4) found it necessary to neutralize his tungstate filtrates for blood sugar determinations when using his modified copper reagent; and we obtain an explanation for the findings by Duggan and Scott (5) who have observed that the reduction values obtained by them with the Shaffer-

Hartmann method were slightly but definitely lower than those given by the authors of the method. In this laboratory, too, it had been noticed during the past few years that various batches of the Shaffer-Hartmann carbonate-tartaric acid reagent showed in their reduction values deviations ranging from 2 to 5 per cent. While no particular importance was attributed to this for ordinary clinical work, every new preparation has been checked with pure glucose before use in investigations.

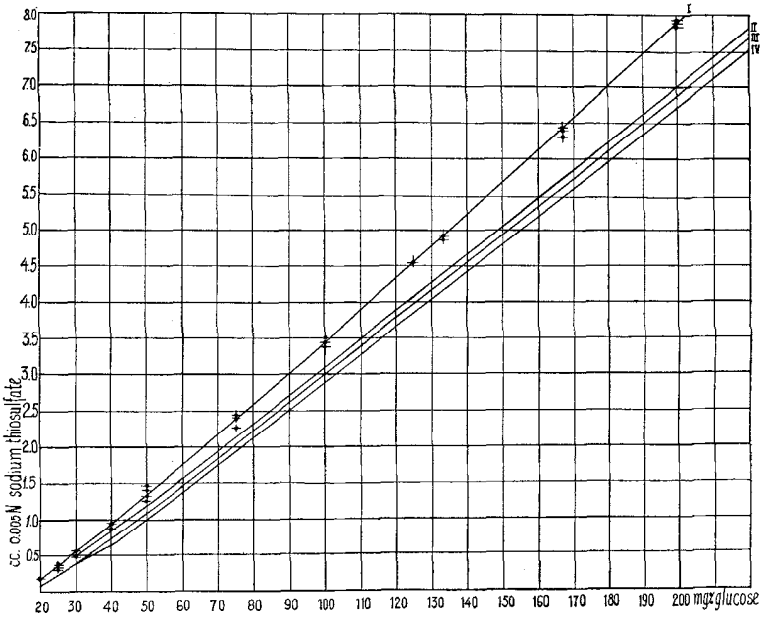

FIG. 2.

Now it is evident that these variations result from slight differences in the alkalinity of the copper reagents originating from the mode of their preparation. Namely, when, according to the directions, a solution of tartaric acid and copper sulfate is poured into a solution of sodium carbonate, carbonic acid is generated giving rise to the formation of *sodium bicarbonate* but *in every case allowing a part of the carbonic acid to escape.* The quantity lost will vary according to the temperature of the alkaline and acid solutions when poured together, and will be influenced

by the method of stirring and possibly by other small factors. This can readily be seen if we prepare two carbonate-tartaric acid reagents with the sole difference in the procedure that in one of them the carbonate and acid are united at room temperature while in the other a warm solution of tartaric acid is poured into a warm solution of sodium carbonate. In Fig. 2, Curve II represents the reduction values for the reagent prepared at room temperatures (which agree perfectly with the data by Shaffer and Hartmann, except for sugar solutions of concentrations below 8.3 mg. per cent); Curve IV is plotted from reduction values of the reagent prepared at higher temperature. The lower reduction values of the latter are obviously due to a greater loss of carbonic acid and—as a result of it—higher alkalinity. Curve III is plotted from the data of Duggan and Scott; it permits the inference that in the preparation of their reagent they have lost less carbonic acid than we in our preparation with warm solutions, however more than in our sample made up at room temperature.

It is obvious that, in order to rule out these variations in alkalinity, one has to eliminate the indirect formation of bicarbonate by using Rochelle salt instead of free tartaric acid, and adjusting the alkalinity by direct addition of sodium bicarbonate. A glance at Table I and Fig. 1 will indicate the proper choice of the $\frac{[Na_2CO_3]}{[NaHCO_3]}$ ratio. It is evident without much comment that Solution V, in which the ratio is $\frac{2}{3}$, best responds to our requirements. With this reagent the reduction is about 10 to 15 per cent greater than with the Shaffer-Hartmann reagents, as shown by Curve I in Fig. 2. Not only is the alkalinity of the modified reagent in the range of maximum reduction, but due to its buffer action this high reduction is maintained when slightly acid sugar solutions are added to it. An equal volume of a 0.025 N acid would shift its alkalinity only to that of Reagent IV which possesses the same reduction value; and this amount of added acid is $12\frac{1}{2}$ times that of the Folin-Wu blood filtrate. (Folin found that 10 cc. of filtrate require 0.2 cc. of 0.1 N alkali for neutralization to phenolphthalein, or are equivalent to 0.002 N acid.)

The composition of the modified tartrate-carbonate copper reagent is as follows:

Final concentration.		gm. per liter
0.026 M	Copper sulfate (crystalline)	6.5
0.06 "	Rochelle salt	12
0.2 "	Sodium carbonate (anhydrous)	20
0.3 "	" bicarbonate	25
0.023 N I_2	Potassium iodide	10
	" iodate	0.80
0.1 M	" oxalate	18

Dissolve the Rochelle salt, sodium carbonate, and sodium bicarbonate in about 500 cc. of water, and into this pour with stirring the copper sulfate dissolved in about 100 cc. of water; then add the solution of the other constituents and dilute to 1 liter. (Only the potassium iodate has to be weighed accurately to cgm.)

This reagent, besides furnishing perfectly consistent reduction values and possessing a buffer effect for slightly acid sugar solutions, has as a third advantage,—a greater sensitiveness at the lowest concentrations of glucose and hence permits reliable determinations of blood sugar values as low as 0.020 per cent.

For the sake of convenience we give here the procedure of sugar determination, largely quoted from Shaffer and Hartmann's communication.

Measure 5 cc. of the reagent into a large test-tube (250 × 25 mm.) and add 5 cc. of the sugar solution containing not less than 0.1 mg. and not more than 2.0 mg. of glucose. Mix by gentle shaking, cover the tube with small funnel or bottle cap or glass bulb, and keep it in a boiling water bath for 15 minutes. Cool by placing in a shallow dish of water until temperature is lowered to 35–40°C. Add with agitation 1 cc. of 5 N H_2SO_4 (or equivalent amount) and see that all Cu_2O is promptly dissolved. After about 2 minutes titrate with 0.005 N sodium thiosulfate. A blank titration on 5 cc. of the reagent is determined after heating with an equal amount of water.

The difference between the blank and the titration of a determination is equivalent to the copper reduced and thus to the sugar. The corresponding amounts of sugar, in mg. per 100 cc. of blood, are given in Table II. (For its elaboration U. S. Bureau of Standards glucose was used.)

Example.—The blank titration on 5 cc. of the reagent was 22.65 cc. of 0.005 N thiosulfate, the titration of a determination 18.22 cc.;

22.65 − 18.22 = 4.43; Table II gives for 4.40 cc. 121 mg., for 4.50 cc. 124 mg. per cent sugar, thus the result of the determination is 122 mg. in 100 cc. of blood.

The table is calculated for the conventional 1:10 dilutions of blood. For other cases the actual amount of glucose, in the 5 cc.

TABLE II.

Amounts of Glucose Corresponding to Titration Values when 5 Cc. of 1:10 Blood Filtrate and 5 Cc. of Copper Reagent (Modified) Are Heated in Water Bath for 15 Minutes.

Cc. of 0.005 N thiosulfate.	Tenths of 1 cc. of 0.005 N sodium thiosulfate.									
	0	1	2	3	4	5	6	7	8	9
	Mg. of glucose in 100 cc. of blood.									
0			21	23	26	29	31	34	36	39
1	41	44	46	49	51	53	56	58	61	63
2	65	68	70	72	75	77	80	82	84	86
3	89	92	94	97	99	101	103	106	108	110
4	113	115	117	119	121	124	126	128	130	132
5	135	137	139	141	143	146	148	150	152	154
6	157	159	161	163	165	168	170	172	174	176
7	179	181	183	185	187	190	192	194	196	199
8	201	203	205	207	210	212	214	216	218	221
9	223	225	227	230	232	234	237	239	241	243
10	245	248	250	252	254	256	259	261	263	265
11	267	270	272	274	276	279	281	283	285	288
12	290	292	294	296	299	301	303	305	308	310
13	312	314	316	318	321	323	326	328	330	332
14	334	337	339	341	343	345	347	350	352	354
15	356	359	361	363	365	367	370	372	374	376
16	378	381	383	386	388	390	392	394	396	398
17	400									

solution used for a determination, is computed by dividing the values in the table by 200. In the above example the actual amount of sugar in 5 cc. of solution is $\frac{122}{200} = 0.61$ mg.

III.

Determination of Sugar in 0.2 Cc. of Blood.

"When only blood sugar values are desired, as in following blood sugar curves in testing carbohydrate tolerance, it is prefer-

able to avoid unnecessary venous puncture and to draw the blood from the tip of a finger or the ear lobe. In this way 0.2 to 0.6 cc. can readily be obtained, amounts which are ample for determination of blood sugar (and for hemoglobin, blood counts and, with special precautions, for pH). The use of the Shaffer-Hartmann method with 0.2 cc. blood is as follows.

"Measure into a small test tube 2 cc. of $\frac{1}{15}$ normal (0.0667 N) H_2SO_4. Measure the blood in a special 0.2 cc. pipette, deliver it into the acid and rinse the pipette several times with the liquid in the test tube. Mix the contents of tube well and after a few moments add exactly 0.8 cc. 2.5 per cent sodium tungstate. Shake well, cover the tube by cork, tin foil or rubber cap and centrifugate. With a small thread or rubber band attach a small tuft of absorbent cotton over the end of a 2 cc. pipette, and with it draw up carefully 2 cc. of the clear blood filtrate from the test tube. Deliver it into another clean test tube (16 × 150 mm.), add exactly 2 cc. of the Shaffer-Hartmann reagent, mix by gentle shaking, cover the tube with a glass bulb, and heat in boiling water for 15 minutes. Cool to about 35°C. Add 1 cc. 2 N H_2SO_4, shake to dissolve the cuprous oxide completely, and after a minute or two titrate very carefully with 0.005 N thiosulphate in a micro burette. Titrate also a blank on 2 cc. reagent. The calculation of the result is made taking account of the fact that the 2 cc. blood filtrate represent two-thirds of 0.2 cc. or 0.133 cc. blood."[2]

In Table III are given the blood sugar values in mg. per 100 cc. of blood, corresponding to titration values.

In our experience this micro procedure furnishes very satisfactory results, in fact for all practical purposes as accurate as when greater amounts of blood are used. Of course, great care has to be applied to the handling of volumetric utensils, as the accuracy of the method largely depends on the exactness of measurements.

IV.

A few remarks concerning *technical details* may be added in conclusion.

[2] This technique was developed by Dr. P. A. Shaffer and is quoted from his Notes on biological chemistry, Washington University School of Medicine, 1926 (a mimeographed laboratory guide for the use of medical students).

Thiosulfate Solutions.—A 0.005 N thiosulfate solution cannot be kept unchanged for more than a few days. We prefer to keep a stock of carefully standardized 0.1 N solution and prepare 1:20 dilutions every day or two. When making the dilution the normality factor may be taken into consideration so as to obtain exactly 0.005 N solutions. For example, to make up 500 cc. of 0.005 N solution out of a 0.10045 N stock solution, pipette accurately 25 cc. of stock solution into a 500 cc. volumetric flask, fill up to mark with water, then from a graduated pipette add 2.3 cc. more water.

Cooling.—Any agitation of the test-tubes, from the beginning of heating in the water bath up to the addition of acid before titration,

TABLE III.

Amounts of Glucose Corresponding to Titration Values when 2 Cc. of 1:15 Blood Filtrate and 2 Cc. of Copper Reagent (Modified) Are Heated in Water Bath for 15 Minutes.

Cc. of 0.005 N thiosulfate.	Tenths of 1 cc. of 0.005 N sodium thiosulfate.									
	0	1	2	3	4	5	6	7	8	9
	Mg. of glucose in 100 cc. of blood.									
0			42	53	63	74	83	91	100	108
1	117	125	134	142	150	159	168	176	185	193
2	202	210	219	227	236	245	253	262	270	279
3	288	296	305	313	322	330	339	347	355	364
4	373	381	390	399	407	416	424	433	441	450
5	458									

should be avoided to minimize reoxidation of cuprous oxide by air. We use test-tube racks, holding eight to twenty tubes, which maintain the tubes erect and stationary during the heating and subsequent cooling.

It is undesirable to cool below 30°C. If the 5 cc. solution for a determination contains 1 mg. or more glucose, cooling too far may cause low results, in consequence of incomplete oxidation of the reduced copper by iodine. This reaction is quite rapid until all but 3 to 5 per cent of the cuprous copper is oxidized, but is completed rather slowly at lower temperatures. If the temperature be kept (or raised) between 30 and 40°C. until the acid is added, the oxidation is complete within 2 to 3 minutes. In the case of

sugar solutions of lower concentration (around the normal level of blood sugar) this precaution is of less importance, as the considerable excess of iodine present presses the oxidation quickly to completion even at lower temperatures.

Effect of Salts.—It was an early observation of the authors of the Shaffer-Hartmann method that reasonably large amounts of salts in the reaction mixture increase the reduction values, probably due to a decrease of dissolved air, thus diminishing the extent of reoxidation of reduced copper. In many cases one has to deal with sugar solutions containing more or less salts, which at high concentrations may introduce gross errors. For example if 6 per cent sodium sulfate is added to a pure glucose solution, the reduction values are increased 5 to 6 per cent, while the addition of 10 per cent sodium sulfate effects a rise of almost 10 per cent in the reduction. Still higher percentage differences were found at low concentrations (0.040 per cent) of glucose.

Especial attention has been paid to the influence of sodium nitrate. In this laboratory we have used for the precipitation of proteins the Patein-Dufau reagent (6) which represents an acid solution of about 33 per cent mercuric nitrate. On addition of sodium hydroxide, carbonate, or bicarbonate to slightly alkaline reaction, without material change of volume, a solution of about 18 per cent sodium nitrate is produced. For removal of proteins and their cleavage products after direct acid hydrolysis of tissues, as much as half volume of the acid mercuric nitrate solution may be required, thus giving solutions containing about 6 per cent sodium nitrate.

In order to learn the influence of the nitrate, pure glucose solutions were submitted to the entire process of precipitation using varying amounts of mercuric nitrate. The results, given in Table IV, show that plus errors of 4 to 10 per cent may be the consequence of additions of mercuric nitrate precipitant in quantities greater than 1 to 4 volumes of glucose solution. This error can be eliminated either by an adequate dilution of the protein-free filtrate, or—if low concentrations of glucose make a further dilution disadvantageous—a special table of reduction values has to be prepared, using known pure glucose solutions with addition of the amount of salts encountered in the determinations.

Neutralization of Acid Sugar Solutions.—Acid solutions—hydrolysates of glycogen for instance—must be neutralized before use for glucose determinations. Without comment it is evident that carbonates and bicarbonates must not be used for neutralization. Phenol red may be used as a convenient indicator for neutraliza-

TABLE IV.

Effect of Added Salts on Glucose Determinations.

Ratio of $Hg(NO_3)_2$ solution in the mixture.	Actual amount of glucose.	Glucose found.	Difference due to added salt.
	per cent	*per cent*	*per cent*
1:3	0.0200	0.0221	+10
1:3	0.0267	0.0282	+5.6
1:3	0.0250	0.0265	+6
1:3	0.0167	0.0176	+5.4
1:3	0.0255	0.0274	+7.6
1:4	0.0200	0.0208	+4
1:4	0.0100	0.098	−2
1:5	0.0200	0.0200	0
1:6	0.0125	0.0125	0
1:6	0.0200	0.0202	+1
1:7	0.0255	0.0255	0
1:10	0.100	0.0102	+2
Added Na_2SO_4.			
per cent			
6	0.040	0.045	+12.5
10	0.040	0.047	+17
6	0.080	0.085	+6
6	0.200	0.210	+5
6	0.300	0.320	+6.5
10	0.300	0.328	+9
6	0.400	0.427	+6
10	0.400	0.436	+9

tion with sodium hydroxide as its color in acid solution does not interfere with the sharpness of the end-point at titration. On the contrary it makes the end-point more distinct, and for this reason a few drops may be added before titration by those who have difficulties in observing the disappearance of the color of the iodine-starch in the presence of copper compounds.

SUMMARY.

1. The great sensitiveness of the reduction values to variations of pH in the system, glucose-alkaline copper solution, is shown.

2. A modification of the Shaffer-Hartmann carbonate-tartrate copper reagent is offered which (1) has a more constant degree of alkalinity, (2) gives higher reduction values, (3) extends the usefulness of the method to lower concentrations of glucose.

3. An adaptation of the Shaffer-Hartmann method for 0.2 cc. of blood is described.

4. Attention is directed to a few details which may cause errors in determinations by these methods.

The author wishes to express his indebtedness to Dr. P. A. Shaffer for his interest and valuable suggestions in the course of this work.

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