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On the Determination of Reducing Sugars

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For a number of years one of us (J. B.) has been working on the enzymatic decomposition of starch. Gradually as the work progressed it was found that the methods usually employed for the determination of the reducing sugars were inadequate. Though the prospects of getting better results seemed doubtful, we nevertheless felt compelled to undertake a testing of known methods, perhaps developing a new one better suitable for our purpose. When looking back on it now we feel almost tempted to say with Jessen-Hansen[1] that »if we had foreseen the difficulties which later appeared in this investigation it is perhaps doubtful that we should ever have undertaken it».

DETERMINATION OF ALDOSES BY MEANS OF IODINE

In alkaline solution aldoses are oxidized by iodine to carboxylic acids. Since the reaction is not sufficiently rapid to make a direct titration possible, the sugar solution is mixed with an excess of iodine and alkali. After standing for some time the solution is acidified and the unconsumed iodine determined. One molecule aldose consumes 2 equivalents of iodine:

$$C_6H_{12}O_6 + I_2 + 3\ OH^- \longrightarrow C_6H_{11}O_7^- + 2\ I^- + 2\ H_2O \qquad (1)$$

This method is originally described by Romijn[2] who realized that the reaction has to be carried out in a weakly alkaline medium. Twenty years later Bougault[3] developed a method that proved better in practice. As base he

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used partly mixtures of Na_2CO_3 and $NaHCO_3$, partly Na_2CO_3 alone. The oxidation of the aldoses proceeded in the presence of the buffer quantitatively in 1 hour, with soda in 30 minutes. Auerbach and Bodländer[4] investigated the effect of the hydrogen ion concentration more systematically. The best results were obtained with equimolecular parts of $NaHCO_3$ and Na_2CO_3. Linderstrøm-Lang and Holter[5] arrived at the result that a more basic carbonate buffer was preferable. Finally, we shall mention Willstätter and Schudel's method[6], which employs NaOH as base. The sugar solution is mixed with about twice the necessary amount of 0.1 *N* iodine; drop by drop there is added 1 ½ time the theoretical amount of 0.1 *N* NaOH; acidifying after 12—15 minutes, and titration of the excess of iodine. Goebel[7] demonstrated that this method, owing to the formation of iodate, only gives good results if the base is added very slowly. This fact is not sufficiently emphasized in the original description and is undoubtedly the reason why satisfactory results have not always been obtained. Myrbäck and Örtenblad[8] prescribe the dropwise addition of 1 $^1/_2$ time the theoretical amount of 0.1 *N* NaOH in the course of not less than 4 minutes.

Table 1. Reagents.

		g/l	mol/l	ml/ analysis	molarity during reaction	pH in blank
I	Iodine 0.1 *N*			10.00	0.033	
II a	$NaHCO_3$	42.0	0.50	10.0	0.167	8.3
II b	Na_2CO_3	10.6	0.10	10.0	0.033	9.3
	$NaHCO_3$	33.6	0.40		0.133	
II c	Na_2CO_3	26.5	0.25	10.0	0.083	9.7
	$NaHCO_3$	21.0	0.25		0.083	
II d	Na_2CO_3	42.4	0.40	10.0	0.133	10.2
	$NaHCO_3$	8.4	0.10		0.033	
II e	Na_2CO_3	53.0	0.50	10.0	0.167	10.6
III	H_2SO_4		1.0	10.0		
IV	$Na_2S_2O_3$ 0.1 *N*					
V	Centrifuged solution of soluble starch					

Method

The reagents employed are listed in table 1. For the measurement of the iodine solution a 10 ml automatic pipette is used, and for titration a 10 ml burette, graduated in 1/50 ml divisions. The reaction flasks are 100 ml flasks as used in the determination of the iodine number. The sugar or the sugar solution is weighed in or pipetted into the flasks — at the most 0.35 millimole in not more than 10 ml; then follows the addition of 10.00 ml of iodine solution, 10 ml buffer solution, and water, to a total of 30 ml. For the blanks a mixture of 10.00 ml of iodine and 10 ml of buffer solution are mixed with 10 ml of water. The flasks are equipped with glass stopper, shaken and put away, protected against direct sunlight. After 30 minutes 10 ml of sulphuric acid are added, and the excess of iodine titrated with thiosulphate and starch as indicator. The difference: blank experiment — main experiment gives the amount of iodine consumed, and 1 ml of 0.1 *N* iodine corresponds to 9.005 mg of monosaccharide (anhydrous), resp. 17.11 mg of disaccharide (anhydrous). When nothing else is said, the experiments are carried out with 0.25 millimole aldose, which corresponds to an iodine excess of 100 % as well as buffer IId.

Results

Table 2. Influence of pH on oxidation of glucose.

pH in blank	8.3	9.3	9.7	10.2	10.6
Reaction time, min	%	%	%	%	%
15	13.4	78.5	98.7	100.7	99.8
30	20.0	89.9	100.5	100.9	101.0
45	24.6	94.1	100.9	101.0	100.7
60	28.6	96.3	101.3	101.2	101.3

The influence of the acidity on the oxidation of glucose is shown in table 2. The reaction velocity rises with increasing pH. pH is measured in the blank. pH must be about 10 before the reaction proceeds quantitatively in 30 minutes. Since the determinations most frequently involve sugar in weakly acid solution, and since OH^- ions are consumed during the reaction itself (1), one safeguards against a drop of pH below 10 by using a more basic buffer. Like Linderstrøm-Lang and Holter we use in the following method as well as in the definitive method buffer IId. With this buffer pH decreases during the oxidation of the aldose from 10.2 to 10.0. Na_2CO_3 (IIe) — which we have previously used in innumerable experiments — gives also good results, in complete agreement with Bougault. As table 3 shows, galactose, maltose and lactose can be determined with the same accuracy as glucose. In the case of all

Table 3. Influence of reaction time on oxidation of various sugars.

Reaction time, min	Glucose, %	Galactose, %	Maltose, %	Lactose, %
15	100.7	99.8	100.3	99.5
30	100.9	100.0	100.6	100.3
45	101.0	100.2	100.9	101.1
60	101.2	100.3	101.0	101.0

sugars the oxidation must be regarded as completed in 30 minutes. There is no distinct difference between the velocities at which mono- and disaccharides are oxidized. The reaction between aldose and iodine is not altogether confined to the stoichiometric oxidation of the aldehyde group to carboxylic acid, but is accompanied by a secondary reaction, though an insignificant one, which according to Bougault should consist of a slow oxidation of the alcohol groups. Auerbach and Bodländer try to limit this reaction by using a buffer with low pH and by working in a substantially more dilute reaction medium. In order to limit the reaction time to 2 hours, however, they reduce pH only to 10.1, corresponding to equivalent amounts of Na_2CO_3 and $NaHCO_3$. Our investigations on the influence of the reaction volume on the oxidation af glucose (table 4) show that the secondary reaction is not suppressed by dilution, but that this dilution only slows up the process.

Table 4. Influence of reaction volume on oxidation of glucose.

Reaction time, min	Reaction volume 30 ml	Reaction volume 60 ml
30	100.9	98.9
45	101.0	—
60	101.2	100.4
120	—	101.2

The following experimental series include also dextrins. Since the equivalent weight of dextrins is unknown we shall in the following tables put the iodine consumption under standard conditions (30 min. buffer II d) for each sugar at 100.0 %. While the oxidation of glucose, maltose and maltodextrin already is complete in about 15 minutes, the same (table 5) is not true of the residual- and amylodextrin which are of substantially higher molecular weight. Whether this is due to a slower oxidation of the aldehyde groups or is caused

Table 5. Influence of reaction time on oxidation of glucose, maltose and various dextrins.

Reaction time, min	Glucose, %	Maltose, %	Maltodextrin, %	Residual dextrin, %	Amylodextrin, %
15	99.8	99.7	99.3	93.3	75.8
30	100.0	100.0	100.0	100.0	100.0
45	100.1	100.3	100.8	104.8	108.4
60	100.3	100.4	101.9	108.6	114.6
Equivalent weight, calculated (30 min.)	90	171	420	abt. 1600	abt. 2800

by an oxidative attack on the other groups can not be decided. When considering that the residual dextrin contains about 20, the amylodextrin about 35 glucose residues for each free aldehyde group, it is understood that there is ample opportunity for oxidation of alcohol groups or rupture of glucoside bonds. The amylodextrin belongs to the so-called erythrodextrins, *i. e.*, dextrins containing groups or bonds which add iodine under red colour reaction. It is perhaps these which are attacked oxidatively. Dilution to twice the original volume does not (table 6) have any substantial effect on the iodine consumption, except in the case of amylodextrin where dilution causes an increase in the consumption of iodine. This can be explained by assuming that the above mentioned bonds or groups in the dilute solution occupy less iodine than in the strong solution, so that in the dilute solution there is more iodine available for oxidation of the aldehyde group. The ratio: iodine/sugar (table 7) is without any essential influence on the oxidation of glucose, maltose and maltodextrin. But it is of decisive importance to the oxidation of residual dextrin and amylodextrin. The just mentioned phenomena may give rise to difficulties in the determination of the activity of amylase preparations. In the very first stages of the hydrolysis of the starch the reaction mixture still contains so many dextrins that bind iodine so powerfully (the solution turning blue or red) that the oxidation of the free aldehyde groups in the presence of an insufficient excess of iodine does not proceed quantitatively. On the other hand, iodine in alkaline solution is so strong an oxidizing agent that certain bonds in the degradation products of the starch can be split oxidatively.

Table 6. Influence of reaction volume on oxidation of glucose, maltose and various dextrins.

Reaction volume, ml	Glucose, %	Maltose, %	Maltodextrin, %	Residual dextrin, %	Amylo-dextrin, %
30	100.0	100.0	100.0	100.0	100.0
60	98.0	97.2	98.6	99.2	113.7

Table 7. Influence of quantity of sugar on oxidation of glucose, maltose and various dextrins.

Quantity of sugar % of standard	Glucose, %	Maltose, %	Malto-dextrin, %	Residual dextrin, %	Amylo-dextrin, %
50	100.5	100.4	100.4	107.0	133.1
100	100.0	100.0	100.0	100.0	100.0
150	99.0	98.7	98.4	90.1	69.3

The Mechanism of the Reaction

By hydrolysis of iodine, hypo-iodous acid and iodine ion are formed:

$$I_3^- + OH^- \rightarrow HIO + 2\ I^- \tag{2}$$

Hypo-iodous acid is an extremely weak acid. Appreciable amounts of IO^- are not formed until higher $[OH^-]$:

$$HIO + OH^- \rightleftarrows IO^- + H_2O \tag{3}$$

Hypo-iodous acid is a very unstable compound which rapidly is decomposed to iodide and iodate. According to Förster and Gyr[9] this reaction depends on the simultaneous presence of free acid and ion, and may summarily be described by the following equation:

$$IO^- + 2\,HIO + 2\,OH^- \rightarrow IO_3^- + 2\,I^- + 2\,H_2O \tag{4}$$

More recent investigations by Bray[10] have shown, however, that the course of the reaction is much more complicated.

It is well known that the oxidative properties of inorganic, oxygen-containing compounds like chlorate, bromate, iodate, nitrate, nitrite, do not appear in alkaline or neutral solution. The oxidative ability is not displayed until the solution has been acidified, *i. e.*, until the formation of the free acids. Assuming that the same is true of hypo-iodite then it is only the free acid that possesses oxidative properties. The formation of iodate is then explained by oxidation of IO^- with HIO to IO_3^- (4), while the oxidation of an aldose proceeds analogously:

$$R{-}CHO + HIO + 2\ OH^- \rightarrow R{-}COO^- + I^- + 2\ H_2O \tag{5}$$

If we now look at table 8, with these points of view in mind, the displacement of the pH of the buffer by mixing with iodine potassium iodide is explained by reaction (2). With $NaHCO_3$ the displacement is much smaller than with Na_2CO_3, owing to the formation of increasing amounts of hypo-iodous acid with increasing pH (2). Rising concentration of hypo-iodous acid is accompanied by an increase in the rate at which the aldoses are oxidized, table 2, (5). In the oxidation of aldoses OH^- is consumed; this is distinctly seen from the pH-measurements in the blanks and in the analyses, *i. e.*, before and after the reaction has occurred (5). That the fall in pH during the

Table 8. pH in buffer, blank and analysis after reaction.

	Buffer	Blank	Analysis
ml buffer	10	10	10
ml water	20	10	—
ml 0.1 *N* iodine	—	10	10
ml sugar solution	—	—	10
II a	8.33	8.29	7.50
II b	9.32	9.26	8.60
II c	10.0	9.7	9.4
II d	10.6	10.2	10.0
II e	11.9	10.6	10.5

reaction is much smaller at pH = 10.6 than at pH = 9.3 is due to the circumstance that in the first instance we approach the optimal buffer capacity of the Na_2CO_3—$NaHCO_3$ buffer (pH = about 10.1) while the buffer II b only has a relatively low capacity.

In the soda-iodine-potassium iodide mixture there is still only relatively little IO^- and relatively much HIO, (3). The rate at which the sugar is oxidized is therefore so much greater than the rate at which hypo-iodous acid is converted into iodate that the oxidation of the aldoses proceeds quantitatively. Upon complete oxidation it is without importance whether the iodate formation continues, since the same amount of iodine as that used for this purpose again is formed by acidifying before titration:

$$IO_3^- + 8\ I^- + 6\ H^+ \rightarrow 3\ I_3^- + 3\ H_2O \tag{6}$$

In the mixture of sodium hydroxide, iodine and potassium iodide the amount of the IO^- is increased relatively to HIO, and the velocity of the two competing reactions is displaced so much in favour of the iodate formation that the oxidation of the aldoses may be incomplete.

When using NaOH one may by slow addition of this base achieve quantitative oxidation of the aldose. Measurement with glass electrode showed that the oxidation, and hence the OH^- consumption, is so rapid that pH up to the time when the theoretical amount has been added only exceeds the value 9—10 immediately after the addition of the base. If the base is added rapidly, however, or too much of it is used, the formation of iodate will get so favourable conditions that the iodine only suffices for a partial oxidation of the aldose. These difficulties are avoided when using carbonate or carbonate-bicarbonate buffer, for which reason this procedure is preferable.

DETERMINATION OF REDUCING SUGARS BY MEANS OF FERRICYANIDE

According to Hagedorn and Jensen[11] the sugar solution is heated with an excess of a weakly alkaline solution of potassium ferricyanide whereby the sugar reduces $\mathrm{Fe(CN)_6^{---}}$ to $\mathrm{Fe(CN)_6^{----}}$. The excess of ferricyanide is determined by titration with thiosulphate, according to the reaction:

$$2\ \mathrm{Fe(CN)_6^{---}} + 2\ \mathrm{I^-} \rightarrow 2\ \mathrm{Fe(CN)_6^{----}} + \mathrm{I_2} \qquad (7)$$

To complete the conversion, $\mathrm{Fe(CN)_6^{----}}$ is precipitated by $\mathrm{Zn^{++}}$ and $\mathrm{K^+}$ as $\mathrm{K_2Zn_3\,[Fe(CN)_6]_2}$. Since the reaction between sugar and ferricyanide does not proceed stoichiometrically, the amount of sugar is found in empirically established tables or calculated by means of factors.

Method

Table 9. Reagents.

		g/l	mol/l		ml/analysis	molarity during reaction		pH in blank
I	K_3FeCy_6	32.9	0.1		10.00	0.04		
II a	Na_2CO_3	53.0	0.5		10.0	0.2		9.8
	$NaHCO_3$	42.0	0.5			0.2		
II b	Na_2CO_3	53.0	0.4	CO_3^{--}	10.0	0.16	CO_3^{--}	10.5
	HCl 1 *N*	100 ml	0.1	HCO_3^-		0.04	HCO_3^-	
II c	Na_2HPO_4, 12 H_2O	179.1	0.25	HPO_4^{--}	10,0	0.10	HPO_4^{--}	11.9
	NaOH 2.5 *N*	100 ml	0.25	PO_4^{---}		0.10	PO_4^{---}	
III	KI	83.0	0.5		5.0			
	Na_2CO_3	0.5						
IV	$ZnSO_4$, $7H_2O$	72.0	0.25		10.0			
	H_2SO_4, conc.	98.0	1.0					
V	$Na_2S_2O_3$	0.1 *N*						
VI	Centrifuged solution of soluble starch							

The reagents employed are listed in table 9. The ferricyanide solution is measured by a 10 ml automatic pipette, and the titration is carried out with a 10 ml burette graduated in 1/50 ml divisions. The reaction flask is a 25 ml volumetric flask, the titration flask a 150 ml Erlenmeyer. Solutions I and II may be mixed. The sugar solution is weighed in or pipetted off into the volumetric flask. I and II are added. The solution is made up with water to the mark and shaken. The flasks are placed in a boiling water bath. When the heating is completed they are cooled in running water. The contents of the flasks are washed quantitatively over into the titration flasks. III and IV are added, and the liberated iodine titrated with thiosulphate. Volumetric flasks are chosen as reaction flasks because this procedure reduces the deviations in double determinations. The reason may be a more uniform heating or exclusion of atmospheric oxygen (*vide* copper methods).

Results

Since we have found in experiments with copper methods that the pH of the reaction mixture has a decisive influence on the reduction equivalents (RE) of the sugars, buffers are added to fix the pH of the reaction mixture. We choose buffers IIa, IIb and IIc mentioned above. In the experiments on the influence of the time of heating (table 10), the sugar concentration is kept constant, corresponding to a consumption of about one-half of the amount of ferricyanide added. Blank determinations were made for each time of heating, though it was found that this time does not affect the value of the blank. The individual determination is encumbered with considerable uncertainty. In the case of both sugars, RE increases with the heating time and decreases with increasing pH. Under the same conditions RE of the maltose is substantially higher than that of the glucose. The reason may be that »maltose by heating with the alkaline ferricyanide is broken down to glucose», as assumed by Linderstrøm-Lang and Holter [5]. In favour of this idea is also the fact that the quotient: RE-maltose/RE-glucose increases with increasing pH. Since the rate at which sugar is oxidized increases with increasing pH we have in the investigation on the influence of the sugar concentration, (table 11) at pH = 9.8 used the time of heating 45 minutes, at pH = 10.5 and 11.9 15 minutes which is the heating time usually employed. At pH = 9.8 and 11.9

Table 10. Influence of time of heating at various pH on the RE of glucose and maltose. Constant sugar concentration.

pH	9.8			10.5			11.9		
Time of heating, min	Glucose 0.067 mmol	Maltose 0.059 mmol	$\frac{\text{Maltose}}{\text{Glucose}}$	Glucose 0.073 mmol	Maltose 0.070 mmol	$\frac{\text{Maltose}}{\text{Glucose}}$	Glucose 0.077 mmol	Maltose 0.074 mmol	$\frac{\text{Maltose}}{\text{Glucose}}$
10	6.94			6.69			6.02		
15	7.73	10.77	1.39	6.72	10.39	1.55	6.03	9.47	1.57
20	7.93			6.97			6.40		
30	8.07	11.53	1.43						
45	8.18	11.96	1.46						

Table 11. Influence of sugar concentration at various pH on the RE of glucose and maltose. Constant time of heating.

pH	9.8		10.5		11.9	
Time of heating, min	45		15		15	
abt. mmol	Glucose	Maltose	Glucose	Maltose	Glucose	Maltose
0.02	9.41	12.80		10.29		
.03					7.47	10.93
.04			6.60			
.05	8.53	12.06		10.28		9.86
.06					6.44	
.07	8.26	11.93	6.72	10.39		9.47
.08					6.17	
.10	8.21		6.74		6.08	
.12					5.87	

the RE of the glucose and of the maltose decreases strongly with increasing sugar concentration. In the case of both sugars RE varies the least at pH = 10.5, and less for maltose than for glucose. This is in agreement with Hanes [13] who, with Na_2CO_3 and 15 minutes of heating, reaches constant RE for maltose and with increasing amount of sugar only slightly decreasing RE for glucose.

Table 12. Modifications of the method of Hagedorn, Jensen.

	The reaction mixture Molarities		pH	Time of heating,	Glucose	Maltose	Maltose in % of
	$FeCy_6^{---}$	Alkali	abt.	min	RE	RE	glucose
Hagedorn, Jensen [11]	0.0007	0.014 Na_2CO_3	11.7	15	5.08		
Pucher, Finch [12]	0.0007	0.014 »	11.7	15	5.08 (calc.)	7.65 (calc.)	151
Hanes [13]	0.0125	0.05 »	11.7	15	5.24	8.26 K	158
Sobotka, Reiner [14]	0.0125	0.05 »	11.7	15	5.30	8.73	165
Nørby [15]	0.006	0.024 »	11.7	20		7.94 K	
Blisch [16]	0.0333	0.13 »	11.7	20		8.50 K	
Fujita, Iwatake [17]	0.003	0.13 K_3PO_4 0.53 K_2HPO_4	11	15	5.98 K		
Blom, Rosted	0.04	0.2 Na_2CO_3 0.2 $NaHCO_3$	9.8	15	7.73	10.77	139
» »	0.04	0.16 Na_2CO_3 0.04 $NaHCO_3$	10.5	15	6.72	10.39	155
» »	0.04	0.1 Na_3PO_4 0.1 Na_2HPO_4	11.9	15	6.03	9.47	157

»K« indicates that RE is constant. Calculation by means of a factor.
In all other cases RE is not constant. Calculation by means of a table.

Since Hagedorn and Jensen published the ferricyanide method in 1923 various modifications have been introduced, some of which are listed in table 12. In most instances Na_2CO_3 is employed. Fujita and Iwatake [17] use a phosphate buffer with pH about 11. In the different methods the RE of the glucose lies between 5 and 7.7 and the RE of the maltose between 7.5 and 10.8, which is about 1 $^1/_2$ times as high as the RE of the glucose. As was to be expected, the RE of the maltose as well as that of the glucose increase with increasing $[Fe(CN)_6^{---}]$ under the same conditions, *i. e.*, same pH and same time of heating. Our ferricyanide concentration of the reaction mixture is the highest, which must account for the strong oxidation of the sugar. However, the ratio between the RE of the maltose and the RE of the glucose is not substantially displaced from 3 : 2. Experiments with dextrins are mentioned in the last chapter.

DETERMINATION OF REDUCING SUGARS BY MEANS OF COPPER TARTRATE

When heated, alkaline solutions of cupric salts oxidize reducing sugars with precipitation of Cu_2O. In the alkaline medium Cu^{++} can only be kept in solution through complex formation. Most frequently oxy-acids are used for this purpose, in most instances tartaric acid as in Fehling's solution. Alkaline copper tartrate solutions are not stable, owing to their large contents of organic substance in a strongly alkaline medium. Hence Fehling's solution is divided into two components which are not mixed until the agent is to be used: I, $CuSO_4$, and II, Seignette's salt + NaOH. Even solution II is not really stable and may, when stored for a longer period of time, be a cause of error, Josephson [18]. Nor is a solution of Seignette's salt stable, being a good substrate for microorganisms. Hence Kjeldahl [19] mixes the components immediately before the analysis, adding Seignette's salt in solid form.

According to Kjeldahl, the sugar solution is boiled on a water bath with an excess of Fehling's solution for 20 minutes while hydrogen is led through. The precipitated Cu_2O is sucked off, reduced to Cu and weighed. The amount of sugar is found in empirically prepared tables. This method is undoubtedly more carefully worked out and gives more exact results than any other method employing Fehling's solution. But being rather elaborate it has not been widely used.

Bertrand's [20] method is a very popular copper method. The sugar solution is boiled over open fire with an excess of Fehling's solution for 3 minutes. Cu_2O is sucked off and determined after dissolving in sulphuric acid containing Fe^{+++} by titration with $KMnO_4$ of the Fe^{++} formed. This method is more rapid when using a special filter stick, Blom [21]. In this form we have applied Bertrand's method to thousands of analyses.

In the direct methods Cu_2O is sucked off. Since Cu^+ can remain in solution by complex formation with NH_3, amino acids, peptones, or the like, Neuberg [22], and thus not be included in the determination, direct methods can give too low values. This source of error is avoided in the indirect methods. In these the sugar solution is boiled with an excess of alkaline copper tartrate solution and the amount of unconsumed Cu^{++} determined by titration. Such methods are encumbered with the fundamental errors of a difference determination, but since the methods are very rapid and especially suited for serial analyses they are widely used.

In order to temper the conditions for the reaction, and thereby reduce the hydrolytic cleavage of oligo- and polysaccharides, we have replaced the strong alkali by a Na_2CO_3—$NaHCO_3$ buffer in the proportion 1 : 1. In spite of very comprehensive experiments (table 13) we failed, however, in developing a method of analysis on this basis that gave results which were reproducible with the desired accuracy. Within each individual series of experiments it was possible to reproduce the results satisfactorily, but when a new reagent was prepared the results might show a displacement of 2 %. Even when the individual components of the reagent were mixed immediately before use it was not possible to eliminate these fluctuations, just as we could not find their cause. Similar difficulties led Kjeldahl to start his well known investigations. Maquenne [23] and Jessen-Hansen [1] encountered the same problem, which also made us, at last abandon the copper tartrate methods in favour of the copper method described in the following chapter.

Table 13. The reduction equivalent (RE) of various sugars with alkaline solutions of copper tartrate.

Method	Molarity of Cu^{++} in mixture	Alkali	pH	Time of heating, min	Glucose	Galactose	Maltose	Lactose
Bertrand	0.053	NaOH	>14	3	5.4	5.2	5.9	7.4
Kjeldahl	0.083	NaOH	>14	20	6.1	5.5	6.9	8.3
Blom, Rosted	0.080	Na_2CO_3 $NaHCO_3$	9.9	45	8.2	—	8.2	—

DETERMINATION OF REDUCING SUGARS BY MEANS OF POTASSIUM COPPER CARBONATE

Concentrated solutions of potassium bicarbonate, potassium carbonate or mixtures of these salts dissolve freshly precipitated copper carbonate under the formation of complex potassium copper carbonate. Soldaini [24] was the first to recommend potassium copper carbonate as reagent for reducing sugars, and Ost [25] was the first to use it in quantitative determinations. While Soldaini's reagent contains only bicarbonate, Ost's contains also carbonate. According to Ost, solutions containing only the bicarbonate react so slowly that they are not suitable for sugar determinations. In his »Investigations on the reaction of the sugars to alkaline copper solutions», Kjeldahl [19] presents a comprehensive experimental material on the determination of different sugars by means of Ost's reagent, without however giving definite sugar tables and without any final appraisal of the method.

Our reason for starting the present investigations was that potassium copper carbonate presents several obvious advantages in relation to Fehling's solution. Cu^{++} remains complexly in a solution of well defined inorganic compounds which at the same time constitute components of a weakly alkaline buffer. Because of the lower pH the reagent does not decompose oligo- and polysaccharides to the same extent as does Fehling's solution.

Method

The sugar solution is heated on a boiling water bath with an excess of potassium copper carbonate. Cu^{++} is reduced by the sugar to Cu^{+} which is precipitated as Cu_2O. The excess is determined iodometrically. A blank experiment is carried out under the same conditions. From the difference between the blank and the main experiment the amount of sugar is calculated by multiplication by a factor.

Reagents: I: The copper sulphate is dissolved in a 1 liter beaker in 200 ml of boiling water. After cooling K_2CO_3 I is added cautiously while stirring. When CO_2 has escaped, which is accelerated by stirring, there are added 400 ml of water, pulverized $KHCO_3$ and K_2CO_3 II. When the main part of the salts is dissolved, the total contents are washed over into a 1 liter volumetric flask and diluted with water to the neck of the flask. K_2CO_3 is easily dissolved, $KHCO_3$ with difficulty. Large crystals of $KHCO_3$ must therefore be pulverized very carefully. Gradually everything dissolves when the flask is shaken, but at the same time K_2SO_4 appears in fine crystals. After dilution to the mark and shaking, the flask is left overnight for continued precipitation of K_2SO_4, whereupon the content is filtered. The solution does not keep indefinitely, as there slowly separate bluish salts, probably consisting of copper carbonates.

II: Na_2CO_3 stabilizes the solution.

III: The lead acetate is dissolved in about 20 ml of water, to which a few drops of glacial acetic acid are added, before addition of the hydrochloric acid.

Table 14. Reagents.

		g/l	mol/l	ml/ analysis	mmol/ analysis
I	$CuSO_4$, $5H_2O$	41.6	0.167	15.00	2.5
	K_2CO_3 I	23.0	0.167		—
	$KHCO_3$	166.9	1.67		25.0
	K_2CO_3 II	230.3	1.67		25.0
II	KI	33.2	0.2	5.0	1.0
	Na_2CO_3	1.0	—		
III	HCl, conc.	333 ml	4.0	25.0	100.0
	Pb-acetate	1.0	—		
	Glacial acetic acid	4—5 drops	—		
IV	KCNS	194	2.0	5.0	10.0
V	$Na_2S_2O_3$		0.1		
VI	Centrifuged solution of soluble starch				

V: The solution of 2 % of soluble starch is centrifuged in order to remove particles which blur the end-point.

Apparatus: Volumetric flasks, 25 ml, marked with the weight in decigrams, width of neck 8—9 mm, surface about 0.6 cm^2. Small rings with numbers. A wire basket with compartments for the flasks. A pot, the water level of which is kept above the marks of the measuring flasks by regulating with the aid of a Mariotte bottle. Burette, 25 ml, graduated in 1/20 ml divisions.

Procedure: The sugar solution is weighed in or pipetted into the flasks which are equipped with rings. The solution must not occupy more than 10.0 ml. 15.00 ml of reagent I are added. Into 2—3 volumetric flasks there are pipetted off 15.00 ml for blank determinations. The flasks are filled to the mark, shaken and placed in the boiling water bath which must not cease boiling during the operation. Splashing must be avoided. The time is noted. After 45 minutes the flasks are cooled in running water. 300 ml Erlenmeyer flasks are used for the titration. 5 ml of reagent II are measured off in the flask. The reaction mixture is transferred and the measuring flask rinsed 3 times with distilled water. No attention is paid to any Cu_2O that may have stuck in the volumetric flask. In the blanks one will often find a ring of CuO in the neck of the flask. The cupric oxide is dissolved in a few ml of reagent III and washed over into the titration flask. 25 ml of reagent III are cautiously added. Effervescence! The flask is shaken while CO_2 escapes. Then 5 ml of reagent IV are added. Titration with $Na_2S_2O_3$ follows immediately. The starch solution is not added until the titration is almost finished. The colour shift is from blue to white-yellow for small amounts of Cu^{++} and from blue to pink for larger amounts. The end-point is rather sharp. When the sun is not shining, the end-point is best observed in the light of a daylight lamp.

This procedure was adopted for the following reasons: In common with all methods employing Cu^{++} as oxidizing agent, the potassium copper carbonate method has the drawback that the amount of precipitated Cu_2O is dependent on the extent to which air has access to the mixture during the heating. Kjeldahl, who was the first to call attention to this fact, drove out the oxygen by leading hydrogen through the reaction mixture. According to Kjeldahl, the influence of the atmospheric oxygen depends on the size of the surface of the solution, and can, practically speaking, be eliminated by using flasks of suitable size with narrow necks, filling the flasks clear to the neck. For practical reasons this was the procedure adopted. The heating must be carried out in boiling water, which at the same time makes it possible to obtain a better control of the conditions of the reaction than in the case of direct heating. Just as in the copper tartrate methods, the analysis itself may involve a determination of the Cu^+ formed or the Cu^{++} not consumed. We have chosen the indirect method because it is much more rapid and relatively accurate. The excess of Cu^{++} is determined according to a modified iodometric method with thiocyanate and iodide (Bruhns[26]). A disadvantage of this method is that one has to use many different reagents. According to Kühnel Hagen[27], an addition of a little Pb^{++} causes the end-point to be sharp, which enables one to make a copper determination with a standard error of 0.2—0.3 % (relatively).

Results

The stability of the potassium copper carbonate complex is dependent on the total as well as the relative carbonate- and bicarbonate concentrations. Potassium copper carbonate is stable only at very high concentrations of carbonate and/or bicarbonate. Upon dilution, basic copper carbonates are precipitated. Potassium copper carbonate in K_2CO_3-solutions is unstable when heated; in $KHCO_3$-solutions it can stand boiling. Solutions like Soldaini's reagent, which contains only bicarbonate, are, according to Ost, not suitable for quantitative sugar determinations since they react too slowly. The composition of a suitable reagent is therefore limited to mixtures of $KHCO_3$ and K_2CO_3, and even in that case only within a relatively narrow range. Experiments with buffer mixtures (table 15) show that pH has a very great influence on the reduction equivalent (RE) of the different sugars. The definitive choice

Table 15. Influence of pH and time of heating on RE, pH and molarity of salts in reaction mixture (25 ml).

$CuSO_4$ mol/l	0.1		0.1		0.1	
K_2CO_3 »	0.5		1.0		1.0	
$KHCO_3$ »	1.0		1.0		0.5	
pH	9.4		9.9		10.2	
Time of heating, min	RE		RE		RE	
	Glucose	Maltose	Glucose	Maltose	Glucose	Maltose
10	8.61		9.43			
15	9.20		10.05			
20	10.11	8.79	10.17	9.45	9.21	8.64
30	10.42	9 58	10.24	9.99	9.16	8.85
40	10.54	9.92	10.31	10.22	9.28	9.06
50	10.53	10.27	10.31	10.43	9.33	9.05
60	10.62	10.53	10.34	10.61	9.35	9.45

of a reagent was therefore a solution containing equimolecular amounts of $KHCO_3$ and K_2CO_3. This solution has maximal buffer capacity and thus is the best one for fixing pH and the reduction equivalent. By selecting 25 ml volumetric flasks and mixing 15 ml of reagent with 10 ml of sugar solution the reaction mixture obtains the composition:

Total Cu^{++} : 0.1 molar
» HCO_3^- : 1 »
» CO_3^{--} : 1 »

The blank proved to be constant. This was a great advantage in comparison with Fehling's solution, the tartrate content of which causes the autoreduction to increase with the time of heating.

For the same heating time the RE of the glucose decreases with increasing pH, while RE of the maltose has a maximum at pH = 9.9 (table 15). At this pH the RE of the glucose and that of the maltose reach the same value after 40—50 minutes of heating. This is yet another essential reason for the final choice of the composition of the reagent as well as of the heating time. In investigations of mixtures of glucose and maltose, as they occur, for example, after enzymatic degradation of starch, it is a great advantage to have an analytical method in which the two sugars have the same RE.

All experimental series dealing with the effect of the heating time on the reduction equivalents of different sugars (table 16) show that the increase in RE becomes slower and slower with the time of heating, although RE does not reach a constant value. Galactose has a lower RE than glucose. The same is observed in oxidation with Fehling'ss olution. This can be deduced from Kjeldahl's and Bertrand's tables. Sobotka and Reiner[14] found similar results for ferricyanide.

Table 16. Influence of time of heating on RE at constant pH. K_2CO_3 *1.0* M, $KHCO_3$ *1.0* M, *pH = 9.9.*

Time of heating, min	Galactose	Invert sugar	Glucose	Maltose	Lactose
10	7.94	9.63	9.43	—	—
15	8.44	10.17	10.05	—	—
20	8.71	10.23	10.17	9.45	8.69
30	8.76	10.30	10.24	9.99	9.25
40	8.86	10.39	10.31	10.22	9.67
50	8.89	10.48	10.31	10.43	9.84
60	—	—	10.34	10.61	10.05

The most suitable heating time is that at which Δ RE/Δt is so small that unavoidable variations in the time of heating have but an insignificant effect on the definitive result. From the experiments in table 16 it is seen that this time, practically speaking, is reached for the sugars investigated in the course of 40—50 minutes. A very large number of determinations showed that RE within certain limits is independent of the amount of sugar. If the determinations are limited to this range it is thus unnecessary to prepare any tables. The calculations are then based on the factor given in table 17 for each sugar (molecular weight / reduction equivalent).

Table 17. Range, RE and factors for calculation.

Sugar	Determinations	Range mg sugar	RE average	Factor $\frac{MW}{RE}$
Galactose	21	10—33	8.86	20.33
Invert sugar	17	10—25	10.48	17.18
Glucose	98	10—33	10.34	17.48
Maltose	37	25—60	10.31	33.19

$$\text{mg sugar} = (\text{blank} - \text{main experiment}) \times n \times \text{factor}.$$

The factors are calculated for the anhydrous sugars. The relative standard error is 0.8 % (based on 240 determinations). The RE of the lactose is not constant, but increases with increasing excess of copper. Hence we have omitted both factor and table.

COMPARATIVE VALUATION OF THE DIFFERENT METHODS

The determination of sugar in pure solutions presents many difficulties. In the case of determinations in solutions that contain other oxidizable organic compounds — and these are the ones to be dealt with in practice — the difficulties increase.

Methods for the determination of reducing sugars are generally based on non-specific oxidation reactions. Since the total consumption of oxidizing agent is computed as sugar one must in principle prefer the method in which the reaction with non-sugar substances is the least. The oxidizing agent which is just capable of oxidizing the reducing carbohydrates must be regarded as the ideal one. And even in that case the non-sugar substances which are more easily oxidized than sugar will enter into the calculations as sugar.

A measure of the strength of the oxidizing agent is to be found in the oxidation-reduction potential. Oxidation-reduction measurements in the reaction mixtures employed in the iodine-, ferricyanide- and potassium copper carbonate methods (table 18) show that iodine is the most powerful oxidizing agent. Ferricyanide is almost as strong, but potassium copper carbonate is much weaker. Measurements were carried out with the electrode arrangement of Jørgensen [28]. A saturated calomel electrode was used as comparison electrode. In the case of potassium copper carbonate one-half of Cu^{++} is, by heating with the estimated amount of glucose, converted into Cu_2O. The potentials are stable and reproducible. Since hydrogen ions do not enter into the equilibrium, the potentials become independent of pH. Displacements in the dissociation, salt error, formation of complexes, sparing solubility of one of the components, etc., do not, however, make the potentials much different from the normal ones for the particular oxidation-reduction systems: $I^- \rightleftarrows I$, $Fe(CN)_6^{----} \rightleftarrows Fe(CN)_6^{---}$, and $Cu^+ \rightleftarrows Cu^{++}$.

Table 18. Oxidation-reduction potentials of various reaction mixtures. t = 20.0°.

Method	[Ox]		[Red]		$[HCO_3^-]$	$[CO_3^{--}]$	pH	E_h Volt
Iodine	I_2	0.03	I^-	0.05	0.03	0.13	10.2	+ 0.57
Ferricyanide	$Fe(CN)_6^{---}$	0.02	$Fe(CN)_6^{----}$	0.02	0.20	0.20	9.8	+ 0.49
Potassium copper carbonate	Cu^{++}	0.05	Cu^+	?	1.0	1.0	9.9	+ 0.01

Iodine is so active that the oxidation of aldoses proceeds at room temperature. This is an advantage, but the readiness to react will at the same time mean that the presence of reactive non-sugar substances makes the determinations problematic, so much more so as the equivalent weight of the sugar according to the iodine method is very high. Even though ferricyanide is almost as powerful an oxidizing agent as iodine, it nevertheless requires heating before the reaction with the sugar proceeds at a reasonable rate. Copper methods have the same drawback. In the choice between ferricyanide and copper methods the latter must, in principle, be preferable since ferricyanide oxidizes many non-sugar substances that copper does not attack at all. On the basis of experiments on »Bestimmung des echten Blutzuckers ohne Hefe», Fujita and Iwatake [17] arrived at the same conclusion: »Das Reaktionsvermögen der Nichtzuckersubstanzen ist bei der Ferricyanidmethode bedeutend grösser als bei der Cuprimethode».

But non-sugar substances are not alone in causing trouble. Both »reducing» and »non-reducing» oligo- and polysaccharides may make the determination unreliable. This is probably due to a partial, oxidative rupture of the glucosidic bonds and a substantial oxidation of the cleavage products. This reaction is dependent on the oxidation-reduction potential of the oxidizing agent (table 19). The experiments are carried out with solutions containing mixtures of mono-, oligo- and polysaccharides, as formed by partial hydrolysis of starch by means of α-malt-amylase. %T.M. denotes the degree of hydrolysis in % of the theoretically possible amount of maltose from total hydrolysis of starch. The values obtained with iodine is about 10 %, with ferricyanide about 25 % higher than those obtained with the copper methods. In this connection it should be remembered, however, that the oxidation with iodine is carried out at room temperature. Other experiments, which will not be mentioned in detail, show that the difference almost solely is due to the dextrins. In practice these facts play a part in the determination of diastatic power in grain, malt and enzymatic malt preparations where the value often is determined by the enzymatic activity. Concordant results cannot be obtained by the different methods. Hence it is necessary along with the result to state the method employed. In principle, however, one can say that the method employing the oxidizing agent with the lowest oxidation-reduction potential must give the most correct, if not *the* correct, result. The copper methods must therefore be preferable.

Table 19. Comparative determinations by various methods.

	Potassium copper carbonate	Copper tartrate	Iodine	Ferricyanide
E_h Volt	0.01	—	0.57	0.49
% T.M.	23.4	24.1	26.1	29.2
% T.M.	34.3	34.9	36.8	42.0

The best one of the different copper methods must be the one the reaction mixture of which has the lowest pH. At the lowest [OH^-] the rate at which the hydrolytic cleavages, for instance, of glucoside bonds, proceeds will be reduced to a minimum. Add to this that the lowering of pH is connected with a more thoroughgoing oxidation of the sugars whereby their equivalent weights become smaller. This means that the oxidation of the non-sugar substances has less influence on the result. However, since the reaction velocity declines strongly with decreasing pH it is not expedient to make pH substantially less than 10.

The use of Cu^{++} in alkaline medium presupposes that Cu^{++} is kept complexly in solution. For this purpose tartaric acid, as in Fehling's solution, is most frequently used. This means poor keeping qualities, an autoreduction dependent on the heating time, and dependence on the degree of purity of the Seignette's salt. These drawbacks are avoided by the use of potassium copper carbonate which contains only inorganic components. But potassium copper carbonate too has its defects. It cannot be produced in solutions stronger than about 0.167 molar with respect to Cu^{++}, and the reagent cannot be substantially diluted without hydrolysis of the copper complex. The boiling time of 45 minutes is an inconvenience in the case of single analyses, but is partly offset in serial determinations because the waiting time here can be employed with titration of preceding analyses.

SUMMARY

The methods most generally employed in quantitative determinations of reducing sugars have been tested and a new method has been developed.

The determination of aldoses by means of *iodine* gives reliable results when using a buffer that ensures a pH of about 10.2. The method should only be used in the case of pure or almost pure sugar solutions, because of the high reactivity of iodine with many organic substances. An explanation is given of the pH-dependence of the reaction. HIO must be regarded as the actual oxidizing agent.

The determination of reducing sugars by means of *ferricyanide* is rapid. The titration is accurate, but the oxidation of the sugar varies somewhat.

The determination of reducing sugars by means of *copper tartrate* gives serially well reproducible results. With new chemicals, displacements occur in the results, the cause of which is not yet understood. It is to be assumed, that a fundamental reason for this phenomenon is the high content of organic substances of the reagent.

A new method has been developed for the determination of reducing sugars by means of *potassium copper carbonate*. The reagent consists of nothing but inorganic salts which can be procured in a highly purified state. The reagent has a well defined pH and a very high buffer capacity. The procedure involves heating on water bath in filled-up volumetric flasks, practially eliminating the influence of the air. The actual determination is based on iodometric titration. The method is particularly suitable for serial determinations. The relative uncertainty of a single determination is 0.8 %.

The advantages and the drawbacks of the methods are discussed. Owing to the low oxidation-reduction potential of the system $Cu^{+} \rightleftharpoons Cu^{++}$ relative to $Fe(CN)_6^{----} \rightleftharpoons Fe(CN)_6^{---}$, copper methods are, in principle, the better ones.

APPENDIX

Preparation of the sugars employed

Glucose, galactose, sucrose and *lactose* were purified by recrystallization of the purest commercial products. The recrystallization was continued to constant $[\alpha]_D$.

Glucose	$[\alpha]_D^{20} = 53.07^\circ$	$(p = 17.34)$
Tollens[29]	$[\alpha]_D^{17} = 53.01^\circ$	$(p = 17.3)$
Galactose	$[\alpha]_D^{21} = 80.3^\circ$	$(p = 12.22)$
Riiber, Minsaas, Lyche[30]	$[\alpha]_D^{20} = 80.5^\circ$	
Sucrose	$[\alpha]_D^{20} = 66.42^\circ$	$(p = 16.70)$
Frühling, Spengler[31]	$[\alpha]_D^{20} = 66.50^\circ$	$(p = 17)$
Lactose hydrate	$[\alpha]_D^{20} = 52.59^\circ$	$(p = 12.16)$
Kjeldahl[19]	$[\alpha]_D = 52.50^\circ$	$(p = 12—13)$
Schmoeger[32]	$[\alpha]_D = 52.53^\circ$	

Invert sugar. Since reversion products are formed by inversion of cane sugar, the inversion was carried out in highly diluted solution.

Maltose was prepared by enzymatic hydrolysis of starch. The action of α-amylase yields, besides the maltose, low-molecular dextrins, trisaccharides and glucose, which only with great difficulty can be separated from the maltose. β-amylase gives only maltose and a high-molecular dextrin which easily is separated from the maltose. Inasmuch as commercial preparations of maltose ordinarily are produced with malt extracts which contain both α- and β-amylase, we preferred, like Baker and Day[33], the more elaborate procedure with β-amylase from barley.

Maltose hydrate	$[\alpha]_D^{20} = 129.8^\circ$	$(p = 4.78)$
Hudson, Yanovski[34]	$[\alpha]_D = 129.5^\circ$	

Maltodextrin was produced by hydrolysis of potato starch by means of amylase from bacteria, and repeated fractionation with alcohol.

Residual dextrin was produced by hydrolysis of potato starch by means of α- + β-amylase from malt to about 80 % T.M. After fermentation, fractionation with alcohol.

Amylodextrin (Nägeli) was prepared by treatment of non-gelatinized potato starch with about 12 % HCl for 4 months, Brown, Morris[35]. The undissolved part was filtered off and dissolved in water from which it crystallized out as spherical crystals. The »recrystallization» was repeated.

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