THE DETERMINATION OF GLUCOSE, GALACTOSE, AND

LACTOSE IN MIXTURES OF ALL THREE SUGARS

by

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THE DETERMINATION OF GLUCOSE, GALACTOSE, AND LACTOSE IN MIXTURES OF ALL THREE SUGARS

INTRODUCTION

For some years the attention of biochemists has been drawn to the desirability of being able to determine quantitatively individual sugars in mixtures. Several methods have been worked out for different combinations of sugars. In this thesis there will be presented a procedure whereby glucose, galactose, and lactose can be determined in mixtures of the three sugars.

Harding and coworkers have published a system for the analysis of a series of sugars including, among others , glucose, galactose, and lactose. This will be discussed in some detail later. These workers used differential fermentation by special yeasts and bacteria combined with acid hydrolysis and reduction. The scheme to be developed here depends on fermentation by baker's (Fleischmann's) yeast together with hydrolysis and copper reduction procedures.

Sugar analysis has developed along widely divergent lines. The more common quantitative procedures are the polariscopic, refractive index, and reduction methods. The first two methods are purely physical and often well suited to industrial control work. This is especially true in cases where but one sugar is present. On the other hand, the third depends usually on the reduction of such heavy metal ions as cupric and mercuric, and also the ferricyanide ion.

The reducing action of sugars in alkaline solutions is apparently dependent upon the process of enolization brought about by the action of the alkali. This process has been studied especially by Nef (1) and Gustus and Lewis (2). According to the views of these workers the series of reactions which takes place in alkaline solutions of sugars may be represented by the following:
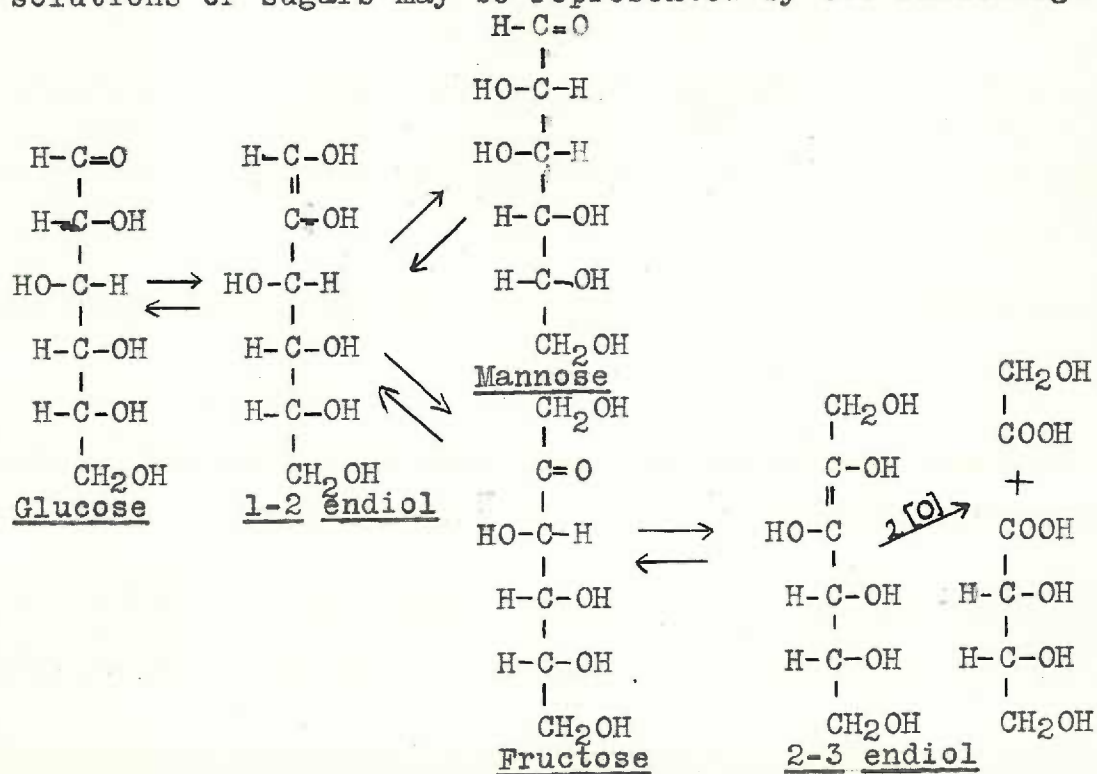
The enols formed in the process are powerful reducing
agents and reduce the oxidizing ion with the formation
of various acids. In the very alkaline solutions such
as Fehling's, intramolecular oxidation-reduction follows
enolization to some extent with the formation of
saccharinic acids, especially lactic. These latter
compounds do not reduce the copper reagent. This accounts
for the low sensitivity of the very alkaline reagents.
By using well buffered carbonate solutions of low
hydroxyl ion concentration the enols formed are not
destroyed by the intramolecular reaction. In this way
more sensitive reagents are obtained.

HISTORICAL DISCUSSION

Sugar Methods

Since the beginning of sugar chemistry it has been
known that alkaline solutions of copper are reduced by
some sugars. In 1841, Trommer(3) used an alkaline
solution of copper sulfate to distinguish grape sugar
from cane sugar. In 1844, Barreswil (4) improved
Trommer's reagent by the addition of potassium tartrate
which increased the stability of the solution. To make
the method quantitative, he added the unknown sugar
solution to boiling copper reagent previously standardized
against a known glucose solution. Fehling (5), in 1848,
studied the proportions of the components in the reagent

and published the details of an improved method which
is essentially the one now bearing his name. The copper
solution he used contained copper sulfate, neutral
potassium tartrate, and sodium hydroxide. This solution
decomposed rapidly upon standing. Soxhlet (6) avoided
this by using two solutions: (A) a water solution of
copper sulfate, (B) Rochelle salt and sodium hydroxide
in water. Solutions A and B are mixed immediately
preceding use.

These copper reduction methods may be made quanti-
tative in either of two general ways, by filtering off
and weighing the cuprous oxide, or by determining
volumetrically excess cupric salt.

Since strong alkalies are known to be very destruc-
tive to glucose through enolization and subsequent
fragmentation, many suggestions have been made for
reducing the alkalinity of the copper reagent.
Soldiani (7) used potassium becarbonate in place of
sodium hydroxide. Ost (8) added potassium carbonate
to Soldiani's solution.

Bang (9) used three solutions: (A) copper sulfate,
(B) Rochelle salt and anhydrous sodium carbonate;
(C) potassium sulfocyanate. In the presence of reducing
sugar, chalk white cuprous sulfocyanate is formed instead
of the red oxide. Excess cupric copper is determined
by titration to colorless with standard hydroxylamine.

Hydroxylamine is unstable, and as a whole the method proved inconvenient.

Benedict (10), in 1907, suggested a solution similar to Bang's. He added the unknown sugar from a burette into a boiling mixture of solutions A, B, and C with extra carbonate added. The end point was the disappearance of the blue color. In 1908, Benedict (11) suggested citrate to replace Rochelle salt, with the advantage that the solutions A, B, and C could be mixed without the subsequent decomposition which occured with the Rochelle salt solution. At the same time the reagent became more sensitive to glucose. Later, (1911) , Benedict (12) found that a solution which contains copper sulfate, sodium carbonate, sodium or potassium citrate, potassium thiocyanate and potassium ferrocyanide is stable for an indefinite length of time. White cuprous thiocyanate separates upon reduction and the potassium ferrocyanide keeps any cuprous oxide in solution. Into the known amount of boiling reagent, the unknown sugar is added from a burette. Loss of blue color, indicating complete reduction of the copper, is easily seen since the precipitate is white.

Bang (13), in 1913, secured the reductions of cupric ions in a solution which was nearly saturated with potassium chloride. This held the cuprous ions

in solution, and they were determined by titration with dilute iodine solution. The disadvantage of this method is that some of the cuprous ions upon contact with the air may precipitate as cuprous oxide and thus escape titration.

In 1915, Scales (14) introduced a volumetric method for the determination of the cuprous oxide in reduced Fehling's solution. The cuprous oxide without filtration from the solution was converted into cuprous chloride and pipetted into a measured amount of a standard dilute iodine solution. Excess iodine was determined by titration with sodium thiosulfate.

Maclean (15), in 1916, used a carbonate-bicarbonate solution of copper sulfate and added potassium iodide and potassium iodate to that solution. The solution after boiling ten minutes with the unknown sugar, was cooled, concentrated hydrochloric acid added, liberating iodine, and the excess iodine was titrated with thio-sulfate.

Bang (16), in 1918, again revised his method. He added a known excess of iodate before boiling and an excess of potassium iodide after cooling and acidifying; afterward he titrated the liberated iodine with thio-sulfate and calculated the iodate used up. From this sugar was calculated. This method eliminated the oxidation of the cuprous ions during the titration.

Benedict and coworkers (17, 18, 19) developed a colorimetric procedure in which the sugar solution is boiled with sodium carbonate-sodium picrate solution and compared against a permanent standard of picramic acid or potassium dichromate. In 1928, Benedict (20) suggested a copper method which is used at the present time. His copper solution contains alanine in place of some of the tartrate. Sodium nitrate replaces the sodium citrate because the increase of sodium ions depresses the ionization of sodium carbonate and yields a reagent of high potential alkalinity but low hydroxyl ion content. A trace of sodium sulfite added increases the reducing action of the sugar at the relatively low pH of the solution. A standard solution of glucose is treated along with the unknown and, after boiling and cooling, phosphomolybdic acid is added to each for color development. The standard and unknown are compared in a colorimeter. Benedict claims this reagent to be less affected by non-sugar reducing substances in blood filtrates and consequently to give more nearly true sugar values.

Folin and Wu (21, 22, 23) made up a copper solution containing, besides copper sulfate, tartaric acid and sodium carbonate. The sugar solution is heated with the alkaline copper reagent using a special tube to prevent re-oxidation. The cuprous oxide formed is

treated with an acid molybdate solution, the resultant blue color being compared in a colorimeter with that of a standard. In 1926, (24) he revised his copper solution by adding sodium bicarbonate to further reduce the alkalinity. He also showed that citrate containing reagents, as Benedict's, give lower sugar reduction as a result of the powerful depressing effect of the citrate upon reduction.

Folin, McEllroy, and Peck (25, 26) have presented a titration method which depends upon an alkaline copper reagent in which the usual tartrates or citrates are replaced by a phosphate.

For 0.1 cc. of blood, Folin and Malmros (27) used alkaline potassium ferricyanide which is reduced by the sugar to ferrocyanide. The latter, after being converted to Prussion blue, can be determined colorimetrically. The yellow color of the excess ferricyanide is compensated for by using a yellow light filter.

In Sumner's method (28) urine is heated with a dinitrosalicylic acid reagent (containing in addition, phenol, sodium hydroxide, sodium bisulfite, and Rochelle salt) which is reduced by the sugar and the resultant color compared with standard glucose treated similarly.

Another method which is in common use at the present time is that of Hagedorn and Jensen (29). The reagent consists of a sodium carbonate-potassium

ferricyanide solution. Potassium ferricyanide is reduced by the sugar to ferrocyanide. Excess ferricyanide is determined iodometrically according to the following reaction, the iodine being titrated with standard thiosulfate:

$$2 \, H_3Fe(CN)_6 \; + \; 2 \, HI \longrightarrow 2 \, H_4Fe(CN)_6 \; + \; I_2$$
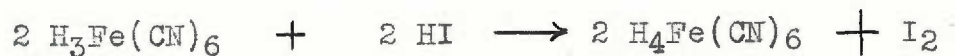
To prevent reversal of the reaction, zinc sulfate is added to precipitate the ferrocyanide as the zinc salt.

In 1920, Shaffer and Hartmann (30) made a thorough study of the equilibria in the reaction between copper sulfate and potassium iodide, which is used in several methods of sugar determination. Bang, Maclean, and Scales determined the amount of cuprous oxide by its reoxidation to cupric salt by a known amount of standard iodine, excess iodine being titrated with thiosulfate. Another procedure used in some sugar methods was to filter off the cuprous oxide and determine cupric ions by converting them to cuprous iodide in the presence of excess potassium iodide, with liberation of free iodine equivalent to the copper reduced. Thus, both cupric and cuprous salts may be determined iodometrically by means of the reversible reaction

$$Cu^{++} + \; I^- \; \rightleftharpoons \; Cu^+ \; + \; \tfrac{1}{2}I_2$$

"For the determination of cupric salts potassium iodide must be added to give a final concentraion of about

0.25 M, while in the determination of cuprous salts the
solution must be so diluted that the final concentration
of copper and of iodide does not exceed about five
millimolar each."

Shaffer and Hartmann, on the basis of the foregoing
study, proposed two methods for determining sugar, one
based on "cupric titration" and one on "cuprous titration".
The latter is preferred, and a modification of the pro-
cedure is made use of in the analyses reported in this
paper. If complete oxidation of cuprous ions is to occur,
means must be found to keep the concentrations of cupric
and iodide ions low. Dilution is possible but limits
the determination to a small amount of cuprous iodide
and a correspondingly low sugar concentraion. Oxalate
was found to combine with cupric ions forming a slightly
dissociated compound. Consequently, by adding oxalate
the cupric ion concentration was lowered sufficiently
to prevent the reverse reaction.

They boiled their sugar solution with the Soxhlet-
Fehling reagent, and to determine cuprous copper, added
excess iodate-iodide solution, acidified with sulfuric
acid and after complete solution of the cuprous oxide
added potassium oxalate and titrated with thiosulfate
using starch indicator. The acid must be added rapidly
and mixed quickly since hypoidite in alkaline solution
oxidizes the organic acids present, thus using some

of the iodine.

In a **reagent** for micro determination of sugar, Shaffer and Hartmann added potassium iodide, iodate, and oxalate to copper sulfate, tartaric acid, and sodium carbonate. If Rochellesalt or citrate is substituted for tartaric acid in the above, the reagent gives lower reduction values. The carbonate-citrate reagent gives ten percent higher reduction than the very alkaline Fehling's solution.

The conditions of the determination must be standardized if results are to be comparable. Slight differences in duration and temperature of heating, composition and amount of copper reagent, volume of the reaction mixture, and even the size and shape of the vessel containing the solution cause variations in the amount of reduction for a given amount of glucose. Some reoxidation of cuprous oxide occurs because of the oxygen dissolved in the solution. With care to minimize contact of the solution with air during the boiling and until cooling is complete, this factor of reoxidation remains constant. Unnecessary agitation of the tubes during heating and cooling must be avoided. Large amounts of salts in the reaction mixture increase reduction values.

In a study of the influence of alkalinity on copper reduction, Somogyi (31) tested nine variations

of the Shaffer-Hartmann reagent with different ratios
of carbonate to bicarbonate. He found that comparatively
small changes in pH gave large differences in reduction
values. The pH range, 9.40--9.55, gave optimum reduction.
This study served to emphasize the idea that a certain
low amount of alkalinity produced oxidizable fragments
of the sugar molecule, while high alkalinity served to
counteract the oxidation by favoring intramolecular oxi-
dations and condensations between the fragments forming
non-oxidizable acids. At the optimum pH range, the first
of the two reactions is favored. Somogyi modified the
Shaffer-Hartmann reagent by using Rochelle salt instead
of tartaric acid and adjusting the amounts of carbonate
and bicarbonate to give a buffered solution of the proper
pH. The reagent gives higher reduction values and so
extends the use to sugar solutions of lower concentration.
Since the reagent has been brought to the proper alkalinity
it becomes evident that acid solutions from fermentations
or hydrolyses should be neutralized before adding the
copper reagent. This point should be kept in mind when
using all similar sugar reagents.

In 1933, Shaffer and Somogyi (32) thoroughly
studied the different components of the Shaffer-Hartmann
reagent. Different carbonate to bicarbonate ratios
were tried. The lower the alkalinity, that is, the
lower the carbonate to bicarbonate ratio, the slower

is the reduction but the more copper is finally reduced.
For low sugar concentrations a high amount of total car-
bonate but a low ratio are desirable; heating must be
continued for a longer period of time. It has been
found that, when heating is shorter than required to
nearly complete the reaction, results are poorly re-
producible since minor variations in conditions of
boiling play a greater role.

The iodate in the reaction mixture plays no part
during reduction. It was thought that the iodide
solution was inactive also until acidification. However,
it exerts a two fold effect. It has been found to markedly
decrease reduction when present to the extent of several
grams per liter. If the iodide is omitted completely
and added before acidification, the reagent gives much
more reduction per unit of sugar, in other words it is
more sensitive. In fact, without any added iodide it
becomes so sensitive and unstable that it auto-oxidizes
and precipitates cuprous oxide. However, as little as
one gram of potassium iodide per liter of reagent
interferes very little with the sensitivity of the
reagent and at the same time renders it relatively
stable when kept in Pyrex glass. With such reagents
excess iodide must be added before acidification. The
oxalate has also been found to diminish reduction and
is preferably added before acidification with the iodide.

Recently, Wood (33) has done a nice piece of work in a physicochemical study of the reducing action of glucose. He studied the reduction of alkaline ferri-cyanide solutions potentiometrically. His findings undoubtedly are applicable to copper reagents. According to Wood, the oxidation of glucose is dependent upon the following factors:

1. "<u>pH of the solution</u>. An increase in acidity causes a slowing of the reaction and an increase in the final amount of oxidant reduced, following a linear reaction. $A = -1.5 \text{ pH} + C$

2. "<u>Temperature</u>. A lowering of temperature brings about the same qualitative result as does lowering of pH.

3. "<u>Salt content of the solution</u>. Increasing the salt content likewise retards the reaction and increases the final quantity of oxidant reduced.

4. "<u>Concentration of glucose</u>. The final quantity of oxidant reduced is directly proportional to the concentration of glucose (within the limits of blood sugar determinations) and the greater the concentration of glucose, the longer is the reaction time.

5. "<u>Concentration of oxidant</u>. Increasing the concentration of the oxidant slightly increases the final amount of oxidant reduced."

Preparation of Biological Materials for Sugar Analysis

Since sugar reagents react with many non-sugar reducing substances, it is an essential feature of biochemical sugar analysis to clear the solution as much as possible of non-sugar reducing materials.

In 1887, Johnson (34) precipitated urine with mercuric salts (mercuric chloride) to remove non-sugar reducing substances. (Creatinine, glycuronates, phenols, uric acid etc. as well as protein in urine interfere.) Patein and Dufau (35), in 1899, used a solution of mercuric nitrate neutralized by sodium hydroxide. Picric acid has been used for blood precipitation but is not as effective as some later reagents. Colloidal iron (and heat) for deproteinization was introduced in 1913 by Michaelis (36). Benedict and Osterberg (18), in 1918, modified the mercuric nitrate technique by treatment with excess sodium bicarbonate and removal of the mercury with zinc dust. In 1919, Folin and Wu (22) precipitated proteins from blood with tungstic acid. These tungstate filtrates are very commonly used for blood precipitation but give results on sugar determinations ten to twenty-five milligram percent too high. By fermentation of tungstate filtrates it can be proved that some non-sugar reducing substances remain because there is still reduction after fermentation.

Benedict and Newton (37) introduced a tungstomolybdic acid method similar to that of Folin and Wu which they claimed to remove more non-sugar reducing substances.

Filtrates from mercury, zinc, iron, or copper hydroxide precipitation of blood yield true sugar values. According to the work of West, Scharles, and Peterson (38) and of West and Peterson (39), mercuric nitrate can be neutralized by barium carbonate, or, better still, mercuric sulfate can be substituted; and upon neutralization with barium carbonate, carbon dioxide is blown off and barium sulfate is precipitated, so ridding the solution of excess salt. Zinc dust and a drop of saturated sodium sulfate solution rids the mixture of mercury. Filtrates from the mercuric sulfate procedure give true sugar values for blood and low non-sugar reducing values for urine. It has the advantage of automatic neutralization without danger of the solution becoming alkaline (with oxidation of the sugar by the mercuric ion), and also of removing the precipitating salts from the solution.

Somogyi (40) deproteinized blood with zinc hydroxide, and, in 1931, (41) he investigated ferric sulfate plus sodium hydroxide to replace the old colloidal iron method. He found further that copper salts are preferable to iron salts.

Steiner, Urban, and West (42) found that ferric

sulfate neutralized with barium carbonate is an excellent precipitating agent, yielding correct results on blood with a rapid, fairly inexpensive, and simple procedure suitable for routine use. Thorium sulfate and barium carbonate give filtrates similar to those of the ferric sulfate-barium carbonate. By these methods the solutions are automatically neutralized and the precipitating salts are removed.

Lately cadmium has been used with values similar to those after zinc and iron precipitation.

EXPERIMENTAL

Methods for the determination of individual sugars in a mixture are rather limited, yet at times such procedures are highly desirable in biochemical work. Harding and coworkers (43, 44, 45) have devised methods based upon selective fermentation by various yeasts and bacteria for the analysis of sugar mixtures. They require the use of materials not readily available. These workers proposed a method for the determination of glucose, galactose, and lactose in a mixture of the three sugars based upon fermentation of glucose by M. krusei, of galactose by S. marxianus, followed by hydrolysis of the lactose with 1 N sulfuric acid and determination of either the glucose (by Fleischmann's yeast) or galactose (by "galac" yeast). A modified Shaffer-Hartmann method was used to determine reductions before and after the fermentations. The procedure used gave only about 70% hydrolysis of the lactose, and its determination was not generally satisfactory.

Because of interest in the determination of these three sugars in a mixture, an attempt has been made to provide a relatively simple and accurate method of analysis. It is believed that such a method has been developed and the experimental details relating to it are presented in this thesis.

According to the new method the total reducing power of the sugar mixture is first determined by a Shaffer-Hartmann reagent. An aliquot of the mixture is then fermented with washed baker's yeast (Fleischmann's) according to the directions of Somogyi (46), after which its reducing power is determined. The difference in reducing power before and after fermentation represents glucose in the mixture. An aliquot of the original unfermented sample is then hydrolyzed in 1 N sulfuric acid, completely converting lactose to one molecule of glucose plus one molecule of galactose. The glucose formed by hydrolysis is determined by fermentation and reduction, and from this the lactose content calculated. The remaining non-fermentable sugar is composed of the galactose from the lactose and the original galactose in the solution. The latter is calculated by subtracting from the total galactose that corresponding to the lactose.

REAGENTS

Glucose, U.S. Bureau of Standards # 41

Galactose, Merck and Co., pure

Lactose, Pfanstiehl's lactose hydrate

The glucose and galactose were stored in a dessicator between weighings. One percent stock solutions of each of the sugars, with toluene as a preservative, were

kept in the refrigerator. From these all dilutions were made.

Yeast, Fleischmann's yeast cakes washed according to the directions of Somogyi (46). A 15% suspension, frequently washed, was kept in the refrigerator.

Soluble starch, Merck's , according to Lintner

Sodium thiosulfate, Baker's C.P. From a 0.1050 N stock solution with a small amount of alkali added to increase its stability, 0.005 N solution was made daily.

Barium carbonate, Mallinckrodt's analytical reagent

The salts used in the preparation of the sugar reagent were of C.P. or analytical reagent grade.

COMPOSITION AND CHARACTERISTICS OF A SHAFFER-HARTMANN REAGENT SUITABLE FOR THE DETERMINATION OF GLUCOSE, GALACTOSE, AND LACTOSE

Galactose and lactose are slowly reducing sugars toward alkaline copper reagents as contrasted with glucose and fructose. A reagent of low carbonate to bicarbonate ratio and content may be employed quite satisfactorily for the latter sugars but not for the former. However, it was found that by increasing the total carbonate content and making the carbonate to bicarbonate ratio equal to three, a reagent satis-factory for all three sugars was obtained. This reagent

is described in the paper by Shaffer and Somogyi (32).

Composition of the Reagent

	gm./liter
Sodium carbonate, anhydrous	79.5
Sodium bicarbonate	21.0
Rochelle salt	25.0
Copper sulfate (5 H_2O) (37.5 cc. 20% solution)	7.5
Potassium iodide	1.0
Potassium iodate (1 N as to I_2)	20.0 cc.

The sodium carbonate and Rochelle salt are dissolved in about 600 cc. of distilled water in a liter volumetric flask. Through a funnel with the stem resting on the bottom of the flask, the 20% copper sulfate solution is added slowly with careful shaking to dissolve the copper carbonate and to avoid loss of carbon dioxide. The sodium bicarbonate and potassium iodide are next added, and when they are dissolved, the iodate solution is accurately measured in. The whole is made to volume, mixed thoroughly, and filtered into a Pyrex flask for storing. No evidence of autoreduction was manifested during a period of two months although the reagent was kept at room temperature and in the light.

SUGAR TITRATION CURVES

In a fixed period of boiling, each sugar shows a

characteristic total reduction and uses a correspondingly great or small amount of sodium thiosulfate in the titration of the excess iodine. Then, for each sugar there can be determined a curve in which varying sugar concentrations are plotted against their respective thiosulfate requirements. The curves for lactose, galactose, and glucose are all very nearly straight lines. (see Figure I) The amount of sugar represented by 1 cc. of thiosulfate is therefore practically constant and has been considered as such in this work.

DETERMINATION OF GLUCOSE AND GALACTOSE IN A MIXTURE OF THE TWO SUGARS

When Harding and van Nostrand (47) used large amounts of baker's yeast as recommended by Somogyi, they often experienced a loss of galactose. Schrumf (48) met with difficulties when he attempted to separate a mixture of glucose and galactose. It seemed that the presence of a trace of glucose caused the yeast which he employed to ferment galactose although pure galactose solutions apparently were not attacked.

Therefore an attempt was made th establish suitable conditions for the quantitative separation of galactose and glucose by Fleischmann's yeast. The analysis was tried in neutral and acid solutions. Most satisfactory

Sugar Titration Curves – Fig. I
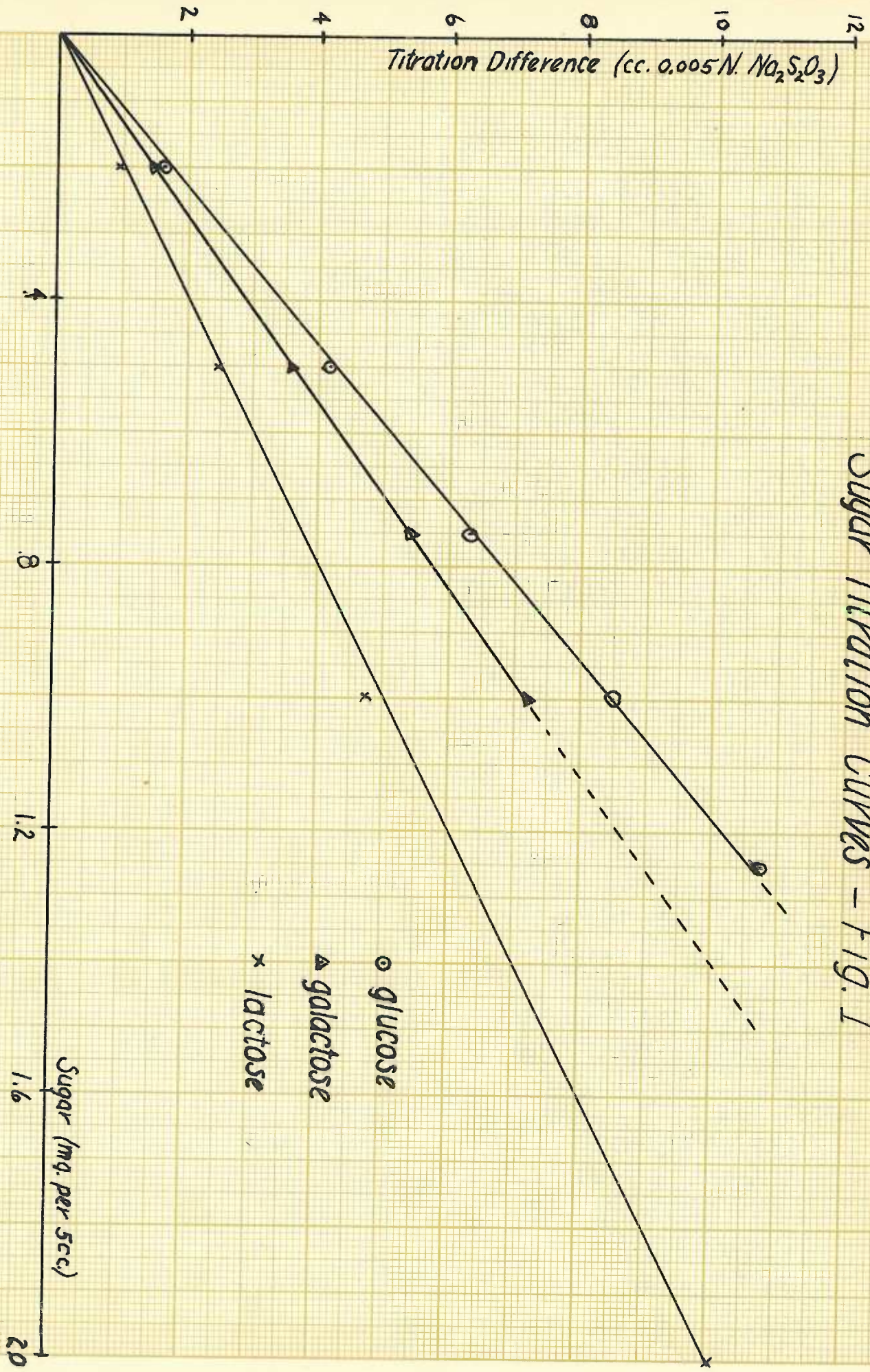
Titration Difference (cc. 0.005 N. Na₂S₂O₃)

Sugar (mg. per 5cc.)

⊙ glucose
▲ galactose
✕ lactose

results were obtained with the use of a solution made barely
acid to congo red, i.e., one drop of concentrated sulfuric
acid to about 25 cc. of solution. The reduction of the
solutions was determined before and after fermentation.
Non-fermentable reduction was calculated as galactose
and the fermentable reduction as glucose. Table I shows
the separations in solutions containing varying amounts
of glucose and galactose.

THE HYDROLYSIS OF LACTOSE

The length of time necessary for complete hydrolysis
of lactose is a function of the acidity and the temperature
of the hydrolysis mixture. Sulfuric acid was chosen as
the acidifying agent since by neutralization with barium
carbonate, barium sulfate is precipitated and carbon
dioxide is expelled, thus freeing the mixture from excess
salts. Since even the purest barium carbonate often
contains sulfides, the solution was acidified to congo
red with a drop of concentrated sulfuric acid and air
passed through to remove any hydrogen sulfide. For
analysis 5 cc. samples of the solution were accurately
pipetted into sugar tubes, 2-3 drops of phenol red (0.04
percent in water) added and 0.5 N NaOH added drop by
drop with shaking to the alkaline color of the indicator.
5 cc. of sugar reagent was then added and the analysis
continued in the usual way.

TABLE I

ANALYSIS OF GALACTOSE AND GLUCOSE

Galactose			Glucose		
Calc. mg./5cc	Experim. mg./5cc.	Error %	Calc. mg./5cc.	Experim. mg./5cc.	Error %
1.50	1.49	-0.6	5.00	----	----
1.50	1.49	-0.6	2.50	----	----
1.50	1.51	0.6	1.00	----	----
1.50	1.53	2.0	0.50	0.50	0.0
1.50	1.51	0.6	0.25	0.28	12.0
1.25	1.25	0.0	2.50	----	----
1.25	1.29	3.0	0.50	0.48	-4.0
1.25	1.26	0.8	0.25	0.26	4.0
1.00	1.01	1.0	2.50	----	----
1.00	0.99	-1.0	1.00	1.02	2.0
1.00	1.04	4.0	0.25	0.23	-8.0
0.50	0.494	-1.2	2.50	----	----
0.50	0.493	-1.4	1.00	1.03	3.0
0.50	0.493	-1.4	0.25	0.26	3.0
0.25	0.27	8.0	5.00	----	----
0.25	0.27	8.0	1.00	1.006	0.6
0.25	0.25	0.0	0.25	0.246	-1.0
0.625	0.639	2.0	0.370	0.373	0.8
0.313	0.336	8.0	0.313	0.300	-4.0
0.219	0.236	7.0	0.219	0.214	-2.1
0.150	0.131	-13.0	0.317	0.317	0.0

Time-percent hydrolysis curves were determined for lactose in sulfuric acid concentrations of both 0.5 and 1.0 N. In cases of incomplete hydrolysis, percent hydrolysis was calculated from fermentable sugar (glucose); otherwise, it was figured from both glucose and galactose. It was found that the 0.5 N acid curve rose very slowly; at one hour, hydrolysis was only 70% complete. This agrees with results of Harding and Grant (45) who found 72% hydrolysis with 0.5 N sulfuric acid solution in one hour of heating. Using their procedure, we were able to reproduce their results in our laboratory. On the other hand, the 1 N solution gave complete hydrolysis in two hours, and upon longer periods of heating (even at six hours) there was no destruction of the glucose or galactose. The hydrolysis curves are shown in Figure II.

THE DETERMINATION OF GLUCOSE, GALACTOSE, AND LACTOSE IN MIXTURES OF ALL THREE

General Procedure

Hydrolysis of lactose. 50 cc. of the sugar solution is diluted in a 100 cc. volumetric flask with 2 N sulfuric acid making 100 cc. of a 1 N acid solution for hydrolysis. The solution is placed in three 25 x 200 mm. Pyrex test tubes (as used in reduction determinations). Rubber stoppers carrying about 12 cm. of capillary

Lactose Hydrolysis – Fig. II
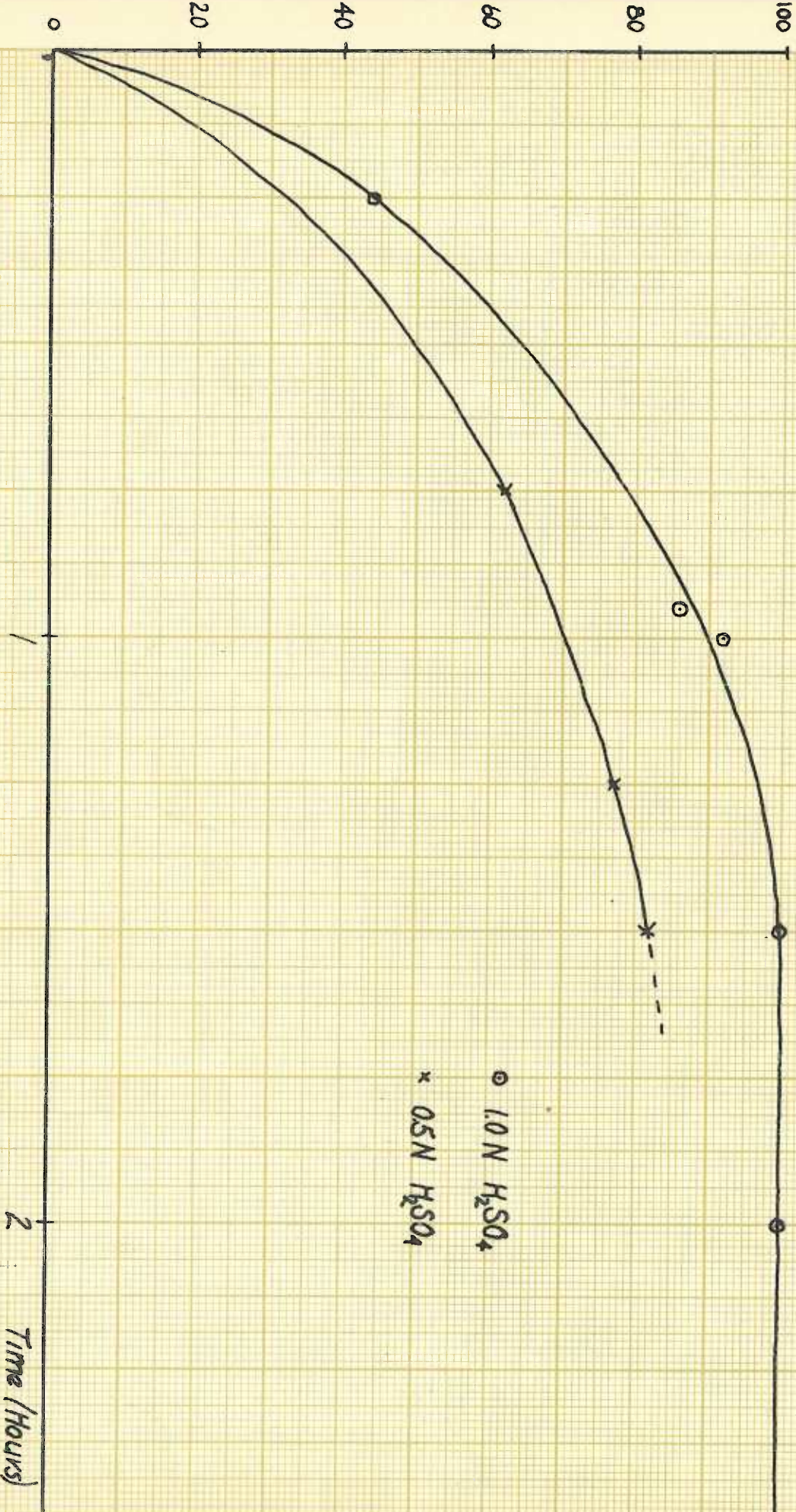
tubing to allow for expansion during heating and to minimize evaporation are placed in the mouths of the test tubes. The tubes are put in a boiling water bath for $2\frac{1}{2}$ hours. After hydrolysis the tubes are cooled, the contents mixed and poured into a 500 cc. Erlenmeyer flask. Neutralization of the acid is accomplished by adding 30 gm. of barium carbonate. Since much carbon dioxide is blown off, the mixture should be thoroughly shaken before it is stoppered. Caution is necessary, and the stopper should be loosened often and carefully. The mixture is shaken until no further pressure is developed and the solution does not redden blue litmus. It is filtered by light suction. Concentrated sulfuric acid is added to the filtrate drop by drop until it is just acid to congo red paper (4-5 drops); moist air is bubbled rapidly through to clear out any hydrogen sulfide, and the slight precipitate of barium sulfate is filtered off. The solution is now ready for fermentation and reduction tests.

Fermentation and reduction. Into each of four centrifuge tubes is measured 5 cc. of a 15% suspension of washed yeast, the cells are centrifuged down, the supernatant fluid poured off, and excess moisture absorbed from the walls with a roll of filter paper. An aliquot of the original sugar solution (unhydrolyzed) made just acid

to congo (1 drop conc. H_2SO_4 per 25 cc.) is placed in
two tubes of yeast; and hydrolyzed solution is placed
in the remaining two tubes. The solutions are permitted
to ferment at room temperature for fifteen minutes. The
yeast is stirred up with a slender glass rod at the
beginning of fermentation and several times during the
period. The cells are centrifuged down again and the
supernatant fluid filtered through a small paper with
care not to stir up the yeast.

Sugar determinations with the Shaffer-Hartmann
reagent are carried out on aliquots of the original
mixture, the original mixture fermented, the hydrolyzed
mixture, and the hydrolyzed mixture fermented. A distilled
water blank is run simultaneously. Three tubes of each
solution are run for checks. The sugar solutions and the
water are carefully measured with a 5 cc. Ostwald-Folin
blood pipette into the Pyrex test tubes, 2-3 drops of
phenol red indicator are added, and to those solutions
which are still acid to congo, 0.5 N NaOH is added drop
by drop until they are just alkaline to phenol red.
Into each tube 5 cc. of the copper reagent is carefully
measured and mixed by lateral shaking. Each tube is
covered with a sealed glass bulb (blown from glass
tubing to a size large enough to be supported by the
rim of the tube), and placed in a metal rack. The
latter is put in a boiling water bath for a period of

fifteen minutes, measured by an interval timer. At the
end of the heating the tubes are plunged immediately
into a bath of cold water and are cooled to about 30
degrees (2-3 min. cooling). To each tube is added 1 cc.
of 4% potassium iodide-5% potassium oxalate solution;
and then 5 cc. of 2 N sulfuric acid is blown in rapidly,
mixed quickly, and the tube is shaken until all traces
of cuprous oxide have dissolved. The excess iodine is
titrated after five minutes with 0.005 N sodium thio-
sulfate, a few drops of starch indicator being added
near the endpoint.

CALCULATIONS

A. Original solution

B. Original solution fermented

C. Hydrolyzed solution

D. Hydrolyzed solution fermented

A.   glucose, galactose, lactose

B.   -------- galactose, lactose

C.   glucose, galactose, <u>glucose, galactose</u>
                                    from lactose

D.   -------- galactose, -------- <u>galactose</u>
                                     from lactose

In finding C and D it must be remembered that the original solution was diluted with an equal volume of acid for hydrolysis.

1. (A-B) x glucose factor = original glucose in 5 cc. of
                                           solution

2. (C-D) x2 x glucose factor = total glucose (original
                                     glucose plus glucose
                                     from lactose)

3. total glucose - original glucose = glucose from lactose

4. glucose from lactose ÷ 0.53 = original lactose
        (0.53 mg. glucose from 1 mg. lactose)

5. D x 2 x galactose factor = original galactose plus
                                galactose from lactose
                                (total galactose)

6. galactose from lactose = glucose from lactose

7. total galactose - galactose from lactose = original
                                        galactose

Illustration

Let A. = 13.06 cc. of 0.005 N thiosulfate (titration
difference = blank - sugar titration)

    B. = 8.61 cc.

    C. = 8.33 cc.

    D. = 3.80 cc.

In a 5 cc. aliquot:

1. 13.06 - 8.61 = 4.45   4.45 x 0.115 = 0.51 mg. glucose

2. (8.33 - 3.80) x 2 x 0.115 = 1.04 mg glucose total

3. 1.04 - 0.51 = 0.53 mg. glucose from lactose

4. 0.53 ÷ 0.53 = 1.00 mg. lactose

5. 3.80 x 2 x 0.136 = 1.03 mg galactose total

6. galactose from lactose = 0.53 mg.

7. 1.03 - 0.53 = 0.50 mg. galactose

RESULTS AND DISCUSSION

Table II shows the results of eighteen determinations on known solutions containing glucose, galactose, and lactose. It is interesting to notice on Table II the average percent recovery, average deviation, and standard deviation for each of the three sugars. It should be noted that the order of increasing deviation is glucose, lactose, and galactose; and this is the order of the complexity of their determination. These calculations show that the degree of accuracy is sufficient to make the method valuable in biochemical analysis.

As a check on the range of usefulness of the procedure, sugar solutions with varying concentrations and proportions of the three were determined. Results show that the method is accurate when the concentration of each sugar is as low as 0.5 mg. per 5 cc. (10 mg. percent). This compares favorably with the sensitivity of contemporary methods. Any high concentration of sugar solution can be brought down within the capacity of the reagent by dilution.

Further investigation may profitably be done in applying the method to biological fluids. It should be especially useful in lactose solutions where there

TABLE II

GLUCOSE, GALACTOSE, LACTOSE MIXTURES

Sugar in mg./5cc			Deviation from 100% recovery		
glucose	galactose	lactose	glucose	galactose	lactose
1.00	1.00	1.00	103%	99%	107%
			104	101	108
0.50	0.50	0.50	100	102	100
			100	100	102
			96	100	112
1.00	1.00	1.00	100	97	110
0.50	0.50	0.50	98	104	102
			99	112	98
			98	94	106
0.50	0.50	1.00	102	100	100
			99	114	100
			100	102	100
0.50	1.00	0.50	104	108	99
			102	104	98
1.00	0.50	0.50	98	109	102
			102	104	96
1.25	1.25	1.25	101	100	98
			103	98	97
Average percent recovery			100.4	102.6	101.9
Average deviation (from mean)			1.9	4.0	3.7
Standard deviation			2.3	5.1	4.6

is a possibility of hydrolysis. Under the direction
of Dr. E.S. West research is now in progress to perfect
a precipitating agent which will be suitable for
special biological fluids involving sugar determinations.

SUMMARY

1. The history and development of reduction methods
    of sugar analysis with special emphasis upon the
    Shaffer-Hartmann reagent has been outlined.

2. A brief sketch of protein precipitation methods
    has been given.

3. A system involving fermentation by Fleishmann's
    yeast, hydrolysis, and reduction procedures has
    been developed for the determination of glucose,
    galactose, and lactose in mixtures of all three
    sugars.

4. Data is included to show the accuracy of the
    method.

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