PRECIPITATING REAGENTS

FOR

BIOLOGICAL FLUIDS

by

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A Thesis

Presented to the Department of Biochemistry
and the Graduate Faculty of the University of Oregon
in partial fulfillment
of the requirements for the degree of
Master of Arts

June, 1956

APPROVED:

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Major Adviser

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For the Graduate Committee

of the University of Oregon

TABLE OF CONTENTS

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INTRODUCTION

The purpose of this research was to find an improved precipitating substance to remove as high a percentage as possible of the non-fermentable reducing substances of urine. By this procedure the author hoped to provide a faster, simpler and possibly more accurate estimation of the fermentable and non-fermentable reducing substances of urine. The writer also held the hope of discovering some reagent which would totally remove all the non-fermentable reducing substances of urine, thereby eliminating the fermentation procedure heretofore necessary in the determination of true fermentable sugar. Because the work has been done with the sole aim of formulating the most desirable precipitating reagent and because the actual reducing determinations followed recognised and tested procedures, the author does not feel particularly obligated to go into the discussion of the evolution and evaluation of reducing reagents used in the past and present. The discrepancies bound to arise in the evolution of the most desirable and accurate sugar determining methods undoubtedly played, however, an important role in throwing research on precipitating reagents into the background. Until writers could get comparable figures on known sugar solutions, there was little point in trying to " split hairs " and determine what amount of reduction in biological fluids was really sugar and what was not. The complexities of this subject are clearly illustrated by the historical development of the study of precipitating

reagents and the realization of the presence of non-sugar reducing substances in blood, urine and tissues.

In any procedure for the determination of fermentable and non-fermentable reducing substances, the following factors must be given consideration:

1. Desirable sugar reagent
2. Satisfactory fermentation procedure
3. Precipitating reagent which will remove a large proportion of the non-fermentable reducing substances but not the fermentable
4. Procedure which is fast, accurate, adds little electrolytes and allows satisfactory pH adjustment.

As regards the first point in question, it is readily seen that the proportion of fermentable and non-fermentable reducing substances is going to depend on the sensitivity of the sugar reagent. In 1926 Benedict (1) had noted that when he added 100 mg. percent of glucose to previously fermented blood filtrates, he recovered his sugar within plus or minus 3 mg.percent. Still before the addition of the sugar these same fermented filtrates had shown with the same reagent a residual reduction corresponding to from 6-14 mg.percent. He postulated, therefore, that:

1. Either the sum of the non-fermentable substance and added sugar was not completely determined
2. In the presence of sugar (though not in its absence) the non-fermented substances are inactive toward the reagent, and the result represents only the added glucose or true sugar.

He, himself, favored the latter assumption but made no attempt to

explain the mechanism. Then, too, one finds that Benedict's reagent averages about 15.8 mg.percent lower than the Folin-Wu reagent although both were standardized against glucose and the decrease in reduction by fermentation should be the same. The question arises as to whether this is:

1. Not glucose but some other sugar having different reducing properties with the two reagents;

2. Benedict's reagent is not sensitive to non-sugars.

Just which is the case has not been decided, though about one-half as much (in terms of glucose) non-sugar is oxidized by Benedict's as by the Folin-Wu reagent. It is the opinion of the writer that though the two sugar reagents may both have been standardized to glucose, there is no reason to assume that they will give the same determinations on the non-fermentable material, particularly when no definite conclusion, as yet, has been reached as to just what constitutes non-fermentable reducing material. One must consider, too, variations in identical precipitation procedures when done by different workers and pH variations toward the alkaline side, especially when mercuric salts are used for precipitation. Here a shift to the alkaline side may cause the oxidation of some of the sugar by the mercuric ion.

In 1931 Harding and Selby (2) working on fermentable sugar in normal urine, decided that the problem of true sugar in blood had received a satisfactory solution. The solution to urine sugars

remains fairly well hidden. Methods involved to date include yeast fermentation, osazones and differential fermentation by bacteria. Some author's reports indicate no sugar in urine (Lumi and Wolf (3); Patterson (4); Eagle (5)); others have found variable amounts (Van Slyke and Hawkins (6); Greenwald-Gross-Maguire (7); Peterson and Nest (8)). Harding and Selby concluded that the occurrence of fermentable sugar in normal urine was a variable phenomenon depending on a number of complex factors.

The modified Shaffer-Hartmann copper reagent has been used by the author. The suitability of such reagents for the determination of low concentrations of sugar has been shown also by Harding and Downs (9); Harding and Van Nostrand (10); Harding and Selby (11). By using this reagent exclusively, the comparative efficiency of various precipitating reagents should be accurate within the limits of experimental error.

Even though the argument as to whether normal urine contains glucose still waxes and wanes, the author has determined the fermentable reducing substance on the assumption of its being glucose and the non-fermentable reducing substance on the glucose factor of the reagent, although it probably consists of lactose, galactose, uronic acids, phenolic substances, modified non-utilizable carbohydrates and nitrogenous substances.

While to the casual observer the use of yeast to ferment the so-called fermentable sugars in urine might seem a simple and fool-proof method, it has been fraught with innumerable arguments as to method of preparing the yeast, length of time of fermentation period necessary and temperature at which fermentation should be carried on. Older attempts to obtain true sugar values were based on Otto's (12) original method of determining the residual reduction after yeast fermentation of blood and subtracting this from the apparent sugar (total reduction). This led to confusing results, residual reductions varying from 0 to 60 mg.percent. One cause, of course, was the different sugar reagents used by various authors. The main source of error, however, seems to have been that variable amounts of reducing substances were given up by the yeast in the course of the fermentation, even though pure glucose solutions and pure cultures of yeast were used.

Van Slyke et al (13) overcame this difficulty by drastic abbreviation of the fermentation period. They found that no reducing substances were produced in the short periods of time which were sufficient to ferment completely the sugar in blood. They incubated 20 minutes at body temperature. Correction for reduction due to yeast impurities was made by running a blank on the yeast. Somogyi (14) then found that 15 minutes incubation

at room temperature with a yeast suspension was sufficient to give consistent fermentable sugar results for blood. In 1928 he modified his yeast procedure to the method now used by the author. He stressed again the careful washing of the yeast. The impurities in ordinary yeast are a variable source of error and probably played a major role in the confusion of the problem of residual reduction. Eagle (15) in 1927 had reached the conclusion that there were certain substances in urine that must be removed before fermentation by clearing with Lloyd's reagent, else the results of fermentation were obscured.

In 1931 Harding and Selby (16) found that a minor but by no means negligible error can be introduced by yeast in either urine or blood filtrates by insufficient centrifuging. A very small number of yeast cells remaining in the supernatant liquid and thus being pippetted into the modified Shaffer-Hartmann reagent, causes a marked increase in reduction. In 1933 (17) these same authors reported on work in which they used yeast on diluted urine previous to treatment with mercuric sulfate-barium carbonate or the sulfuric acid-Lloyd's procedure. Their fermentable sugar results were very confusing and variable. It will be noted that Eagle had encountered this same difficulty and had come to the conclusion that urines must be given preliminary clearing before fermentation.

West and Steiner (18) in discussing the failure of Lund and Wolf (5) to obtain carbon dioxide in their fermentation of normal

urines, considered one of the factors to be the presence in untreated urine samples of a non-sugar material which might inhibit yeast action on small quantities of sugar.

Laug and Nash (19) used the mercury reagent of Best (20) and fermented both before and after precipitation. Here they found lower values on fermentation before precipitation than after. The difference they concluded could be interpreted in at least two ways:

1. An interfering substance in urine which prevents complete action of yeast; (This interpretation agrees with Best and Steiner (18))
2. The acidity of the precipitating reagent forms extra fermentable material by hydrolysis.

The authors discounted the first suggestion because in control experiments glucose added to urine was completely determined.

Harding, Nicholson and Archibald (21) discount the above hydrolysis explanation as they point out that Harding and Selby (17) found fermentable sugar in human urines and that they fermented before precipitation. However, it is to be noted that while Harding and Selby fermented before clearing, they also obtained variable and confusing results. As previously stated, Eagle concluded that fermentation must be carried out following clearing.

Harding et al (22) by differential fermentation with bacteria found that urine contained glucose and galactose, and that some urines contained small amounts of fructose or mannose.

The discussion of the most desirable precipitating reagent and procedure is unfolded in the historical and analytical sections.

HISTORICAL

While the actual chemical identification of the substances of urine has developed within the last 200 years, the inspection of urine as an aid to diagnosis in disease dates back as far as 4000 B.C. when Sumerian and Babylonian physicians noted various changes in the color and constituents of urine. Diseases were classified according to the gross appearance of the urine sample. Hindu medicine also included the physical examination of urine as an aid to diagnosis in disease during the pre-Greek period (4000 B.C. to 700 B.C.). The realization of the sugar content of urine began somewhere in the period between 700 B.C.-200 A.D. when Charaka and Susurata, Hindu writers, observed the sweet taste of diabetic urine. These authors noted that long trains of black ants were attracted by sweet urine, and these insects became recognized as a means of diagnosis. Hippocrates (400 B.C.); Galen (150 A.D.); Paulus Aeginata (latter 17th century); students of the School of Salerno (200-1200 A.D.) all employed and advocated the careful inspection of urine samples in medical diagnosis.

Thomas Willis (1621-75) by remoting the fact that diabetic urine had a characteristic sweetish taste, started the long chain of research on the reducing properties of urine. Lorenzo Bellini (1643-1704) by first evaporating urine discovered the important fact that the change in color, taste and odor were due to changes in the relative proportion of water and solids present. He concluded

that urine was composed of water, salt and tasteless earth or tartar. Dobson of Liverpool (1776) made an amazing experiment on diabetic urine. He evaporated the urine and found a cake of white crystalline residue which he was unable to distinguish from ordinary sugar by taste or smell. In 1798 Cruickshank studied the action of nitrous acid on the evaporated extract of diabetic urine and on milk sugar. He concluded that the two sugars were dissimilar. In 1836 Rees recorded a method for the analysis of the sugar of diabetic urine by the process of evaporation and incineration. In 1838 Bouchardat and Peligot identified the sugar of diabetic urine as grape sugar. In 1841 Trommer published the first work on the determination of grape sugar. He showed that an alkaline copper salt solution gave a distinctive precipitation of cuprous oxide when boiled with a solution of grape sugar containing but one part of sugar to 100,000 parts of water.

It is interesting to note that the use of copper as a test for sugar presumably dates back to the Egyptian era when a mixture used for the application to wounds and tumors was noted to change color on heating. The constituents of the mixture were verdigris (impure copper carbonate), honey and vinegar. It was not until 1815, however, when Vogel of Paris first offered an explanation of this color change. He said that the reddish or brownish colored precipitate was due to the action of the sugar in the honey on the copper salt.

In 1844 Barriswell suggested an improvement on the Trommer method,

This improvement was the addition of potassium tartrate to the solution to prevent decomposition on heating. In the same year Heller advanced the caustic potash test for sugar in urine. In 1847 Harnick in his Handbook on Urinalysis gave the procedures for the determination of sugar as the yeast test and the copper test of Trommer. In 1848 Fehling published his work on the quantitative test for sugar in urine. Fehling's contribution consisted merely in working out with greater care the details of Trommer's method. In 1886 Otto (12) published the original procedures for the determination of the residual reduction after yeast fermentation of blood. In 1887 Johnson (23) introduced bechloride of mercury as a preliminary clearing agent in the determination of urine sugar. Whereas previous authors (Pavy (24); Salkowski (25)) had attributed various proportions of the reducing action of urine to sugar and glycuronic acids, respectively, Johnson came to the conclusion that the total amount of reduction effected by normal urine was accounted for by the uric acid and creatinin which it contained. In 1902 Patein and Dufau (26) used acid mercuric nitrate neutralized with an alkali for the precipitation of blood proteins preliminary to analysis for blood sugar. In 1913 Michaelis (27) precipitated blood proteins with colloidal iron hydroxide and heat. In 1914 Shaffer (28) precipitated the proteins of blood with gentle heat, a few drops of acetic acid, colloidal iron and sodium sulfate. He then centrifuged and determined the sugar in the supernatant liquid. He also noted that precipitation of the proteins

with methyl alcohol did not give true sugar values and that the work of Bang (29) on precipitation with ethyl alcohol gave even greater error; that is, the results were low, presumably due to inclusion of some of the sugar in the alcohol precipitates. In 1915 Plimmer (30) used basic lead acetate as a preliminary urine clearing agent. In 1918 Benedict and Osterberg (31) used mercuric nitrate and sodium bicarbonate to precipitate the non-fermentable reducing substances of urine. They removed the excess mercury with zinc dust and determined the fermentable sugar by fermenting at 35-38° C. for 18-20 hours and determining the residual reduction colorimetrically by picrate-picric acid solution against a picramic acid standard. In 1919 Folin and Su (32) published a method for the determination of blood sugar which still stands as one of the most used blood protein precipitation methods. These workers precipitated with 10% sodium tungstate and 2/3 normal sulfuric acid. In 1922 Folin and Berglund (33) precipitated urine non-fermentable reducing substances with Lloyd's reagent. In 1926 Folin and Svedberg (34) modified the Lloyd's procedure by precipitating urine with 0.05 N oxalic acid and Lloyd's reagent, shaking, filtration and subsequent addition of permutit. In 1926-27 Eagle (15) in studying urine sugar found that if he fermented the untreated urine first and then used 0.1 N sulfuric acid and Lloyd's that he obtained very confusing results. If he precipitated first and then fermented, his results were more consistent. In 1927 Somogyi (14) published a

procedure to eliminate the previous inconsistencies in the determination of fermentable sugars by the yeast fermentation technique. Whereas it had previously been the custom to run reductions on the yeast samples themselves and correct the final result, Somogyi now suggested taking weighed amounts of fresh commercial yeast (Fleischmann's) suspended in 5-10 parts of water, centrifuging and decanting the water. This was repeated until the supernatant liquid was practically clear and colorless and the washings gave a zero reduction with copper reagent. In 1928 Van Slyke and Hawkins (35) devised a gasometric method for the determination of reducing sugars applicable to analysis of blood and urine. They precipitated their bloods by the Folin-Wu method; the urine was not given a preliminary clearing as diabetic urines were run, and the non-fermentable reducing substances were ignored. In the same year Benedict (36) reported some work on the determination of blood sugar. He used Folin-Wu filtrates and determined the sugar by a colorimetric procedure. Benedict and Newton (37) used tungstomolybdic acid in the precipitation of the proteins of blood. They claimed no loss of non-protein constituents of blood by this method. Somogyi (38) stated that the fermentation of the filtrate instead of whole blood obviated one source of occasional error. He found that some pathological bloods when mixed with yeast reacted to produce reducing substances even in the few minutes required for the short fermentation procedure. Hoat et al (39) found that the precipitation of blood proteins with mercuric sulfate-barium carbonate technique with the modified Shaffer-Hartmann reagent

gave these sugar values which were practically identical with the sugar values obtained by fermentation. Because the procedure was faster, less laborious, more accurate and left no salt error, they recommended it not only for the determination of true blood sugar but for pretreatment of urine, hydrolysed tissues, etc.. Van Slyke and Hawkins (40) again using a gasometric determination of the fermentable sugar of blood, precipitated their blood with a modified Folin-Wu tungstic acid procedure. Harding and Selby (2) determined the fermentable sugar in normal urine by precipitating with the Folin-Berglund (38) procedure and fermentation per Somogyi (14). Somogyi (41) reported the use of cupric salts and ferric salts neutralised with sodium hydroxide in the preparation of protein-free blood filtrates. In 1932 Nest et al (42) found that ferric sulfate was satisfactory in the preparations of blood, plasma, spinal fluid and milk filtrates. They stated, however, that iron could not be satisfactorily substituted for mercury in the pretreatment of urine and hydrolysed tissue. They also advanced the point that Somogyi's ferric salt procedure did not remove the iron completely and further treatment with sodium carbonate was necessary. These workers used 2% ferric sulfate and neutralised with barium carbonate; solid thorium sulfate neutralized with barium carbonate. Nest and Peterson (43) in work on the sugars of urine made a rather thorough and critical discussion of past precipitation procedures. According to them the Benedict and Osterberg mercuric nitrate-sodium bicarbonate method

while probably the most efficient method in the past, left too high a salt concentration in the filtrate and if too much bicarbonate were added, resulted in the oxidation of sugar by the mercuric ion. They also reached the conclusion, which has been confirmed by the author, that treatment with Lloyd's reagent alone left much non-fermentable reducing substance. Best et al (39) had previously found in 1928 that with the use of mercuric sulfate-barium carbonate there was no appreciable addition of electrolytes and no loss of sugar. These workers determined their reducing substances with the modified Shaffer-Hartmann copper reagent with decreased KI. Yeast fermentation with washed yeast according to Somogyi (14) was employed. In 1934 Grasser et al (44) employed the Somogyi (41) technique of using zinc sulfate-sodium hydroxide in their precipitations for tissue sugar determinations. Laug and Nash (18) maintained that the mercuric sulfate-barium carbonate procedure hydrolysed substances depending on the length of time the mixture stood with the mercuric sulfate and was therefore subject to unavoidable errors. Harding et al (45) used lead subacetate as a primary clearing agent and followed by treatment with mercuric sulfate-barium carbonate in their determination of urine sugars. They found more complete removal of non-fermentable reducing substances by this method. Miller and Van Slyke (46) precipitated blood proteins with cadmium sulfate in 1.0 N sulfuric acid and neutralized with sodium hydroxide and barium carbonate.

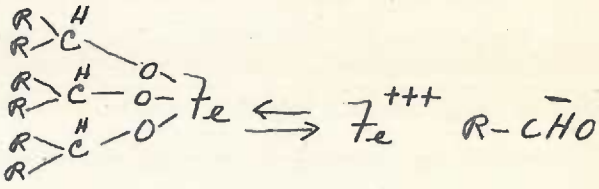
Curtis, Lane and West (47) in unpublished work made the following additions to the study of precipitating reagents. A comparison on the precipitating efficiency of mercuric sulfate (West et al-39); 2% ferric sulfate; 2% ferric sulfate-2% mercuric sulfate in 1.0 N sulfuric acid; above ferric sulfate-mercuric sulfate with Lloyd's; above ferric sulfate with Lloyd's was made. All these reagents were neutralized with barium carbonate. They found that the mercury-iron and the mercury-iron-Lloyd's were the most efficient in the removal of urea, creatine and creatinine; the latter being slightly better for the removal of creatinine. Both reagents, they concluded, were far superior to the old mercuric sulfate-barium carbonate. It was found that the mercury-iron-Lloyd's was slightly more efficient in removing non-fermentable reducing substances from urine, hydrolyzed muscle and liver than was plain mercury-iron. They were both, however, superior to the old mercuric sulfate-barium carbonate. Iron-Lloyd's was generally a little better than mercuric sulfate-barium carbonate in removing non-fermentable reducing substances from urine.

THEORY OF THE ACTION OF PRECIPITATING AGENTS

As to the actual mechanism of the reaction between the precipitating reagents and non-fermentable reducing substances not much is known aside from the postulation that certain insoluble " metallic complexes " are formed with the non-fermentable substances. Because the exact knowledge of just what constitutes the non-fermentable reducing substances is lacking, the problem is even more baffling. Best et al (39) base the efficiency of their mercuric sulfate-barium carbonate procedure on the effective removal of interfering nitrogenous substances from biological fluids. Johnson (23) had said that the total reduction of normal urine was due to uric acid and creatinine. The writer, however, will later present evidence that would lead one to believe that the efficiency of the reagent is not indicated by the percentage removal of nirtogenous substances such as creatinine, urea, etc..

Chapin in his " Second Year College Chemistry " (1926) states that organic compounds which contain the secondary alcohol group tend to form complexes with metals.

1. Secondary Alcohol Groups



$(R\text{-}CHO\text{-}Fe)^{+}$ ppt. in alkaline solution $\rightarrow (R\text{-}CHO\text{-}Fe)(OH)$

2. Amino Groups

$[Fe\text{-}(NH_2)_3]^{+++}$ ppt. by basic OH $\longrightarrow [Fe\text{-}NH_2](OH)_3$

Analogous to $Ag(NH_3)_2^{+} \longrightarrow Ag(NH_3)_2(OH)$

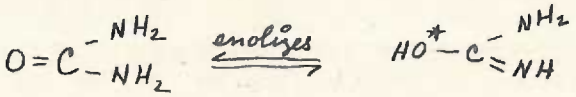
There is the possibility, then, of these two factors acting here:

1. Thru secondary alcohol groups;

2. Thru amine groups ($-NH_2$ or $-NH-$)

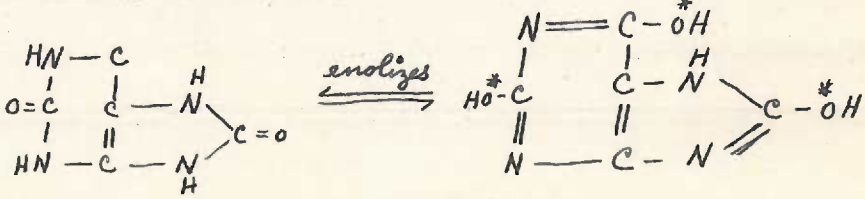
If we now consider the formulae for the various known nitrogenous constituents of urine, it may be demonstrated how either of these theories may work.

Urea



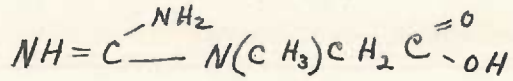
Urea is the least effectively removed from solution by any precipitating reagents used by the author.

Uric Acid



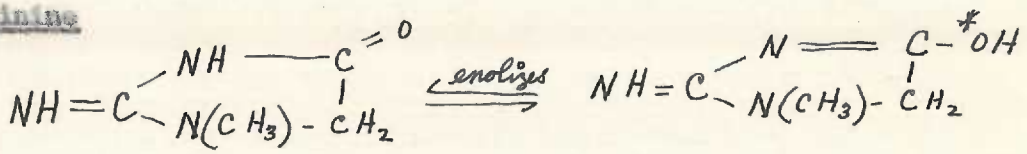
This would theoretically give 3 secondary alcohol groups which should increase the possibility of complex formation. The metallic salt is probably formed.

Creatine



In this case there are no secondary alcohol groups. The carboxyl group is probably an active spot.

Creatinine



This is one of the most efficiently removed substances; yet it possesses but one potential secondary alcohol group and two reactive nitrogen groups.

Looking back over these formulae to compare them with the efficiency of their removal as shown by the author and Best et al (47), one notes that the degree of efficient removal reads: 1. Uric Acid; 2. Creatinine; 3. Creatine; 4. Urea. The uric acid not only has the most potential secondary alcohol groups but also the most -NH- groups. Creatinine has two -NH- groups but only one potential secondary alcohol group. Creatine has one -NH- group, no potential secondary alcohol groups but one carboxyl group. Urea has one potential -NH- group and one potential secondary alcohol group. The author is inclined to disregard the NH_2 groups as urea, which has more than any of the rest, is most poorly precipitated. When we extend our field to cover the sugars which are in solution and which therefore must be reckoned with, we note that sugars contain a large number of secondary alcohol groups. It becomes at once obvious that now we must either disregard the secondary alcohol group as a factor in precipitation or else give recognition to the fact that although

certain complexes form, there is a differential solubility factor which undoubtedly plays a part. The writer favors the latter point of view. It is obvious that though a certain compound has more possibility of complex formation, its solubility may be such that the actual percentage removal may be below what one might theoretically expect. Urea is noted for its great solubility. Creatine and creatinine are more difficultly soluble. It is possible that the solubility of urea might offset any insolubility produced by the complex ion formation.

As will be noted many precipitating procedures employ the use of heavy metal salts which are precipitated in an alkaline solution. The complex between the non-fermentable reducing substances and the metals are undoubtedly precipitated as insoluble hydroxides.

The author is inclined to believe that the secondary alcohol groups are the important factors in precipitation but that the solubility of the metallic complexes thus formed is materially influenced by the original solubility of the substance to be precipitated.

It is interesting to note that Van Slyke (48) demonstrated that in normal blood the sum of the reduction equivalent of uric acid and creatinine does not exceed the equivalent of 3-5 mg. per-cent of glucose; therefore it was safe to assume that some other substance or substances were responsible for the greater part of the reduction derived from non-fermentable reducing substances.

The same would undoubtedly apply to urine. The author will

later show data that indicates that although the precipitation reagent is relatively less efficient in removing nitrogenous reducing substances than others recently reported (West et al-47), still the reagent removed all the non-fermentable reducing substances from several urine samples and in others reduced the values far lower than had ever previously been determined.

With recent work indicating that normal urines contain lactose and galactose also and in view of the fact that these two sugars are rather heavily removed from sugar solutions by the author's reagents, it is possible that this may be a portion of the clue to the efficiency of these reagents on urine samples. Just why a high percentage recovery of glucose can be obtained from a stock solution by a precipitating procedure while the same procedure removes relatively large quantities of lactose and galactose, the author is unable to explain to her own satisfaction. Presumably it has to do with the ability to form metallic complexes and also the relative solubility of these complexes when formed. If a large part of the non-fermentable reducing substances consists of these two sugars, the author has fritted away a good many hours of work. All precipitating reagents were run on standard solutions of glucose, galactose and lactose; and the percentage recovery of all taken as one indication of the desirability of the reagent.

To test the theory that galactose might be an important factor in the non-fermentable reducing substances, the author devised the following experiment: A urine sample was precipitated with the ferric sulfate-Lloyd's-lead carbonate reagent (later referred to) in the usual manner and the fermentable and non-fermentable reducing substances determined. To this urine sample was then added weighed quantities of both glucose and galactose and the precipitation procedure repeated. By this method the author sought to show the percentage recovery of both glucose and galactose from urine solutions and also the effect on the non-fermentable of the addition of galactose. The results were as follows:

0.057 gm. glucose and 0.069 gm. galactose were added to 10 cc. of a urine sample. The urine before the addition of the sugars ran 0.20 mg. fermentable reducing substance and 0.20 mg. non-fermentable reducing substance/10 cc.. Theoretically, after the addition of the above sugars, the fermentable obtained would be 0.059 gm. and the non-fermentable 0.071 gm. presupposing no removal of either during the precipitation. Actually after precipitation the fermentable ran 0.055 and the non-fermentable 0.050. There was thus a 10% loss of glucose and a 30% loss of galactose determined as non-fermentable. The effect of the addition of galactose to the non-fermentable figure is readily shown. The 30% removal of galactose by this method is to be compared with the 0% removal of galactose by the ferric sulfate-

Lloyd's-barium carbonate procedure, this latter having been used always in conjunction with the new reagent as a check for fermentable sugar recovery. A duplicate experiment was run with essentially the same results. The author feels that this points rather strongly to the removal of galactose as one explanation for the efficiency of the ferric sulfate-Lloyd's-lead carbonate reagent.

Experimental Work

Reagents

Lloyd's Alkaloidal Reagent

Lead Carbonate-Mallinckrodt's pure

Barium Carbonate-Mallinckrodt's technical precipitated;dried

Yeast-Fleischmann's yeast washed and kept as recommended by Somogyi (14)

Sugar reagents: 1. Modified Shaffer-Hartmann containing 1.0 gm. KI per liter and kept in a pyrex flask. (#50)

2. Modified Shaffer-Hartmann for galactose, lactose and glucose. (49)

3. Copper reagent for the determination of very small amounts of sugar-Somogyi. (50)

Urine-Samples preserved with a little toluene and kept in the ice box.

Mercury sulfate-15%: ferric sulfate-50%: cadmium sulfate-30% in 0.5 N sulfuric acid.

Mercury sulfate-21.5%: ferric sulfate-46% in 0.25 N sulfuric acid. (new Hg-Fe)

Mercury sulfate-25%: ferric sulfate 21% in 1 N sulfuric acid. (old Hg-Fe)

Mercury sulfate-17.5%: ferric sulfate 30% in 0.5 N sulfuric acid.

Ferric chloride-20%: mercuric chloride-15% in aqueous solution.

Ferric sulfate-21% in aqueous solution.

Composition of Copper Reagents Used in This Work

Shaffer-Somogyi #50	Gm. per liter
Sodium Carbonate (anhydrous)	25.0
Sodium Bicarbonate	20.0
Rochelle Salt	25.0
Copper Sulfate.5 H_2O	7.5
KIO_3 (0.1 N as to I_2)	100.0 cc.
KI	1.0

Somogyi Modification of Shaffer-Somogyi #50 For Determination of Small Amounts of Sugar	
Sodium Carbonate (anhydrous)	25.0
Rochelle Salt	25.0
Copper Sulfate.5 H_2O	4.0
Sodium Bicarbonate	20.0
Sodium Sulfate (anhydrous analytical)	200.0
KI	1.5
N KIO_3	6.0 cc.

The above reagents were used for all standard glucose, urine and blood determinations; the #50 being used in all determinations except on urines as in Table XII where dilutions of 24 hour volumes were made. Somogyi's modified reagent with sodium sulfate was used in the latter case.

Copper Reagent for the Determination of Galactose and Lactose

	Gm. per liter
Sodium Carbonate (anhydrous)	79.5
Sodium Bicarbonate	21.0
Rochelle Salt	25.0
Copper Sulfate, 5 H_2O (37.5 cc. of 20% solution)	
KI	1.0
KIO_3 (1 N as to I_2)	20.0 cc.

This reagent was used for all determinations on standard galactose and lactose solutions.

Preparation of Precipitating Reagents

Mercury-Iron Reagents:

All mercury-iron reagents were prepared by dissolving the required quantity of ferric sulfate in a portion of warmed sulfuric acid of the proper normality. After solution was effected, the material was cooled to 0-5° C. in an ice bath and the mercuric sulfate incorporated a little at a time. The flask was shaken thoroughly after each addition to insure complete solution. The whole was then made up to 100 cc. volume by adding the necessary amount of sulfuric acid. Upon cooling to room temperature, a certain portion of the mercuric sulfate separated out as the basic salt. This was filtered off, washed, dried and weighed. The correct percentage of mercuric sulfate in solution was determined from this data.

Mercury-Cadmium-Iron:

All mercury-cadmium-iron reagents were prepared exactely as the mercury-iron with the exception that after the ferric sulfate was in solution, the cadmium sulfate was first incorporated with the aid of morter and pestle and then this mixture was cooled as above. Procedure from here was identical with the above.

Mercuric Chloride-Ferric Chloride:

An aqueous solution of the desired strength of ferric chloride was prepared and to it was added the required amount of mercuric sul-

chloride. No difficulty was encountered in putting the mercury into solution at room temperature, and likewise no mercury salt separated from the solution on standing. The writer has found it easily possible to ibtain a 30% solution of mercuric chloride by using as little as 5% ferric chloride to aid solution.

Notes on other chemicals tried:

Attempts to use chromium sulfate, zinc sulfate, aluminum sulfate, copper sulfate, cobalt sulfate, nickel sulfate were unsuccessful as either the metallic ions were very incompletely precipitated by barium carbonate or the whole mass set to a solid in the process of neutralization.

Analytical Procedures

Preparation of filtrates:

The ferric chloride-mercuric chloride filtrates were prepared as follows: To 60 cc. of distilled water 10 cc. of urine was added and 10 cc. of ferric chloride-mercuric chloride reagent. To this solution 4.0 gm. of Lloyd's reagent was added and the mixture was shaken thoroughly. This mixture was then neutralized with 70.0 gm. of lead carbonate and shaken until no more carbon dioxide was evolved. The solution was filtered on a Buchner funnel with light suction. To the filtrate 1.0 gm. of barium carbonate was added and hydrogen sulfide gas bubbled through until a black precipitate formed and the solution was saturated with hydrogen sulfide. This solution was then filtered with light suction. To the filtrate 0.4 cc. of concentrated sulfuric acid was added and air bubbled through until all excess hydrogen sulfide was removed. The solution was filtered by gravity, the filtrate returned through the filter paper until it was clear.

A general procedure for mercury-iron and mercury-cadmium-iron reagents was as follows: To 45 cc. of distilled water 10 cc. of urine was added and 15 cc. of the precipitating reagent. If Lloyd's reagent was to be used, 4.0 gm. of it were added now. The mixture was neutralized to litmus with 30-40 gm. of barium carbonate and agitated until no more carbon dioxide was evolved. This solution was filtered with light suction. The filtrate was made acid to congo red paper by the

addition of 3-4 drops of concentrated sulfuric acid and treated with hydrogen sulfide gas until the solution was saturated. Air was bubbled through this saturated solution to remove excess hydrogen sulfide and the solution filtered by gravity.

The procedure for the use of ferric sulfate-lead carbonate was as follows: To 55 cc. of distilled water 10 cc. of urine was added and 15 cc. of 21% ferric sulfate solution. 4.0 gm. of Lloyd's reagent were added and shaken in thoroughly. This mixture was then neutralized with about 40.0 gm. of lead carbonate and shaken until no more carbon dioxide was evolved. The solution was filtered with light suction. To the filtrate concentrated sulfuric acid was added until an acid reaction was obtained with congo red paper. This solution was treated with hydrogen sulfide gas until saturated. Air was bubbled through this solution until all excess hydrogen sulfide had been removed and then filtered by gravity.

Determination of Reducing Substances

The filtrates were prepared and divided into two parts, and one part was fermented. 5 cc. portions of a 15% yeast suspension were centrifuged, the supernatant liquid decanted and the sides of the centrifuge tubes dried with a small roll of filter paper. 8-10 cc. of filtrate was added to each of two yeast tubes and allowed to ferment at room temperature (23-24° C.) for 15 minutes with

occasional stirring. After centrifugation, the filtrate was decanted through a small filter.

5 cc. portions of both fermented and non-fermented filtrates, in triplicate, were pipetted into 25 X 200 mm. pyrex tubes, 5 drops of 0.02% phenol red added, and 0.5 N sodium hydroxide until the indicator turned red. Blanks of distilled water with phenol red were also run. After the addition of 5 cc. of sugar reagent to the tubes, they were covered with glass bulbs and heated in an actively boiling water bath for 15 minutes. The tubes were then cooled to about 30° C. and 1.0 cc. of 4% KI-$K_2C_2O_4$ solution added to each, followed by 5 cc. of 1.0 N sulfuric acid blown in quickly. (5 cc. 2.0 N sulfuric acid were added when using galactose-lactose sugar reagent.) Each tube was shaken, bulb in place, until the precipitate of cuprous oxide had dissolved. After standing a few minutes (5-10), the sides of the tubes and the covering bulbs were washed down with a fine stream of water from a wash bottle. Titrations were made with 0.005 N thiosulfate, 5 drops of a 0.5% starch solution being added near the end point. Titrations checked within 0.02 cc.. Calculations were made in terms of glucose reducing equivalents. 1.0 cc. of thiosulfate titration is equivalent to 0.115 mg. glucose. With the use of the galactose-lactose-glucose sugar reagent 1.0 cc. of thiosulfate is equivalent to 0.115 mg. of glucose; 0.136 mg. of galactose;

0.200 mg. of lactose. The difference between the titrations of the fermented samples and the blank multiplied by the factor (dilution x 20 x 0.113) indicates the mg. of non-fermentable reducing substance (as glucose) per 100 cc. of urine. Similarly, the difference between the titrations of the fermented and non-fermented samples multiplied by the factor indicates the mg. of fermentable reducing substance per 100 cc. of urine.

TABLE 1

Determination of the Percentage Recovery of Standard Glucose Solutions after Precipitation with Various Reagents

Reagent	Standard	Standard-$BeCO_3$	Standard-Reagent	Error
Cd-Hg-Fe[1] (10 cc.)	1.47	1.44	1.42	-3%
	0.72	0.73	0.70	-2%
	0.73	0.73	0.69	-5%
Old Hg-Fe[2] (10 cc.)	1.45	1.38	1.35	-7%
	0.72	0.69	0.69	-5%
New Hg-Fe[3] (10 cc.)	1.45	1.36	1.35	-6%
	0.70	0.70	0.65	-7%
	0.68	0.67	0.63	-7%

1. Cd-Hg-Fe reagent contains 30% $Fe_2(SO_4)_3$-30% $CdSO_4$-18% $HgSO_4$ in 0.5 N H_2SO_4
2. Old Hg-Fe contains 21% $Fe_2(SO_4)_3$-25% $HgSO_4$ in 1.0 N H_2SO_4
3. New Hg-Fe contains 46% $Fe_2(SO_4)_3$-21.5 % $HgSO_4$ in 0.25 N H_2SO_4

TABLE 11

Comparison of Recovery from Standard Glucose Solutions Containing 0.69 mg./5 cc. Using Various Precipitation Reagents

$BaCO_3$	Hg-Fe[1]	Old Hg-Cd-Fe[2]	New Hg-Cd-Fe[3]
98%	91%	98%	———
100%	96%	97%	———
100%	94%	100%	———
97%	93%	97%	———
98%	—	—	96%
95%	97%	—	———
100%	95%	—	100%
99%	95%	—	97%

1. Hg-Fe reagent contains 30% $Fe_2(SO_4)_3$-17.5% $HgSO_4$ in 0.5 N H_2SO_4

2. Old Hg-Cd-Fe contains 30% $Fe_2(SO_4)_3$-30% $CdSO_4$-18% $HgSO_4$ in 0.5 N H_2SO_4

3. New Hg-Cd-Fe contains 30% $Fe_2(SO_4)_3$-26% $CdSO_4$-18% $HgSO_4$ in 0.5 N H_2SO_4

TABLE III

Comparison of Recoveries from Standard Galactose and Lactose Solutions Using Various Precipitation Reagents* (Shaffer-Somogyi # 80)

Galactose
Concentration of 0.70 mg./5 cc.

Reagent	CC. Reagent	Percentage Recovery
Ferric Sulfate-20%-Lloyd's-Barium Carbonate	15 cc.	100%
" " "	15 cc.	100%
Saturated Mercuric Sulfate in 10% Sulfuric Acid-Barium Carbonate	10 cc.	100%
" " "	10 cc.	100%
Ferric Sulfate-20%-Mercuric Sulfate-2% in .25 N Sulfuric Acid-Barium Carbonate	15 cc.	90%
" " "	15 cc.	88%
Ferric Sulfate-20%-Cadmium Sulfate-15% in .25 N Sulfuric Acid-Barium Carbonate	15 cc.	84%
" " "	15 cc.	86%
" " plus Lloyd's	15 cc.	92%
" " "	15 cc.	94%
New Mercury-Cadmium-Iron	15 cc.	95%
" " plus Lloyd's	15 cc.	102%
New Mercury-Iron	15 cc.	78%
" " plus Lloyd's	15 cc.	82%

TABLE III-a

Continuation of Table III

Reagent	CC. Reagent	Percentage Recovery
Old Mercury-Cadmium-Iron	15 cc.	98%
" " "	15 cc.	94%
Ferric Sulfate-40%-Mercuric Sulfate 17% in .25 N Sulfuric Acid-Barium Carbonate	15 cc.	93%
" " plus Lloyd's	15 cc.	86%
New Mercury-Cadmium-Iron	15 cc.	92%
" " plus Lloyd's	15 cc.	91%
New Ferric Chloride-20%; Mercuric Chloride-15%-Lloyd's-Lead Carbonate	10 cc.	82%

Lactose

Concentration of 0.60 mg./5 cc.

New Mercury-Cadmium-Iron	15 cc.	90%
" " plus Lloyd's	15 cc.	93%
10 cc. Ferric Sulfate-21%; 5 cc. Mercuric Acetate-25%-Barium Carbonate		104%
Ferric Chloride-20%; Mercuric Chloride 15%-Lloyd's-Lead Carbonate	10 cc.	73%

* In these determinations dilutions of 1:7 were used with the exception of those with the ferric chloride-mercuric chloride reagent, in which case a 1:8 dilution was used.

TABLE IV

Recovery from Standard Glucose Solutions with Ferric Sulfate-Lloyd's-Lead Carbonate (1:16 dilution)

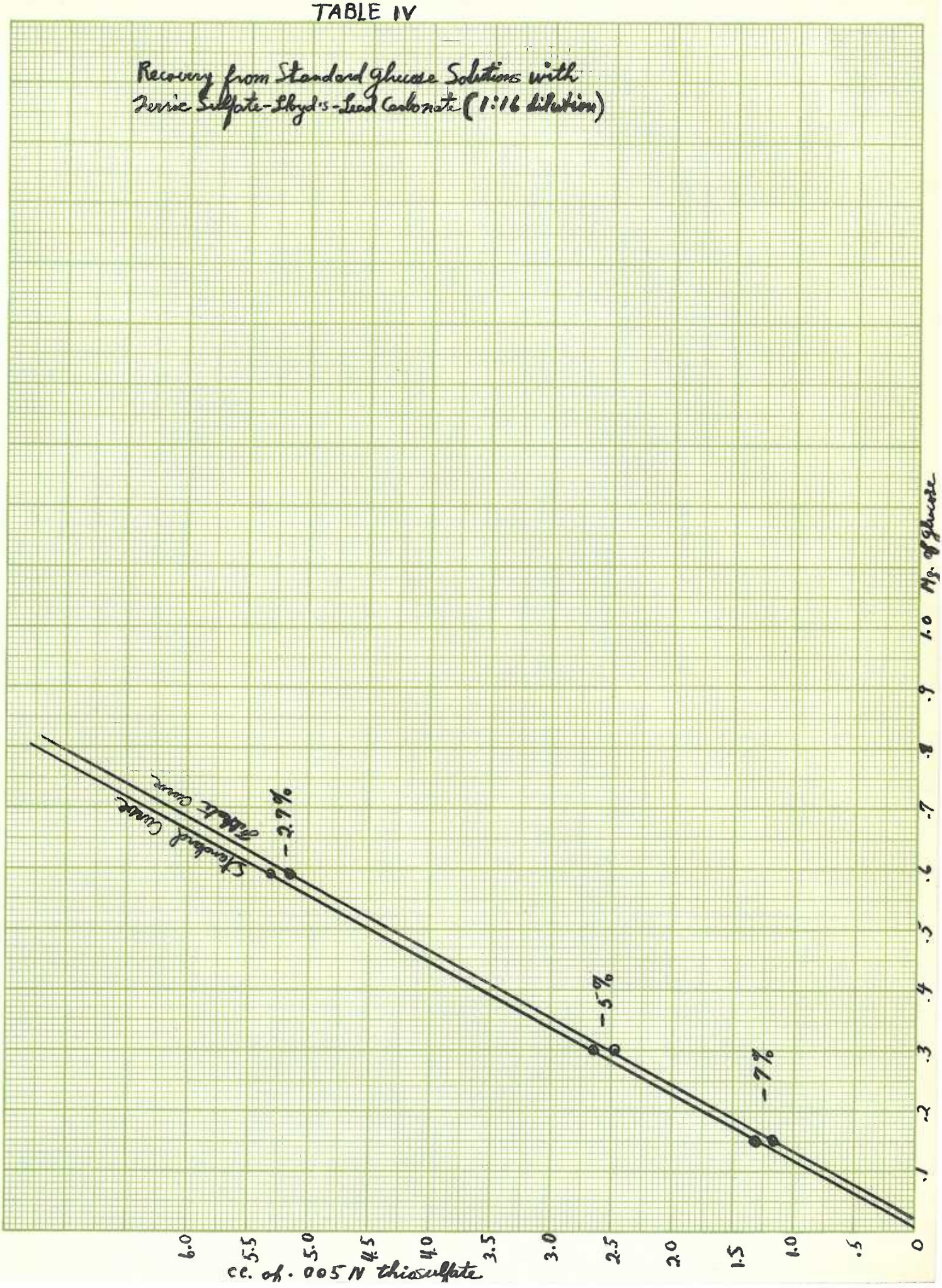


TABLE V

Percentage of Sugar Recovered from Standard Solutions Precipitated in a Final Volume of 80 cc. with Ferric Sulfate-Lloyd's-Lead Carbonate.

GLUCOSE

Concentration per 80 cc.	Reduction of first filtrate	Reduction of washings	Total reduction	Percentage error
39.64	39.02	0.91	39.93	+ 0.7%
40.46	35.86	5.44	41.70	+3%
40.60	36.00	4.00	40.00	-1%
11.62	7.92	2.88	10.80	-6%

GALACTOSE

105.19	89.72	15.72	105.44	-1%
98.28	85.56	15.30	100.86	+2%

LACTOSE

193.18	167.18	27.69	194.87	+0.8%
184.08	158.68	24.15	183.01	-0.5%

In the above experiment precipitation was carried out in the usual manner and reductions run on the filtrates. The precipitation mass on the Buchner funnel was broken up and dissolved in 80 cc. of distilled water and the resulting mixture filtered. Reductions were then run on these filtrates and the results added to those from the first filtrate to determine the total reduction.

TABLE VI

Showing the Percentage Removal of Urea, Creatine and Creatinine by Different Precipitating Agents (West, Lane and Curtis [47])

Mg N	$HgSO_4$	$HgSO_4$ Lloyd's	$Fe_2(SO_4)_3$	$Fe_2(SO_4)_3$ Lloyd's	$HgSO_4$ $Fe_2(SO_4)_3$	$Fe_2(SO_4)_3$ $HgSO_4$ Lloyd's
UREA						
270	48.5	60.7	4.1	4.8	57.0	73.3
135	78.6	78.8	15.0	11.0	77.3	88.8
67.5	96.1	92.4	18.4	19.4	92.8	95.8
CREATINE						
120	50.0	55.8	10.0	15.0	77.5	72.5
60	23.8	45.0	----	38.0	81.7	80.0
30	24.0	27.5	11.7	38.5	82.3	84.0
CREATININE						
120	42.7	95.0	19.0	81.6	92.8	97.2
60	55.0	82.8	20.0	81.0	96.0	97.0
30	78.5	91.5	25.0	90.0	95.0	100.0

TABLE VII

Determination of the Percentage Removal of Urea, Creatine and Creatinine

Mg. N	$FeCl_3$-$HgCl_2$-Lloyd's-$PbCO_3$	$Fe_2(SO_4)_3$-Lloyd's-$PbCO_3$
Urea		
270	15.0	8.0
135	11.0	20.0
67.5	13.0	17.0
Creatine		
120	75.0	64.0
60	85.0	61.0
30	95.0	70.0
Creatinine		
120	92.0	86.0
60	98.0	87.0
30	99.0	76.0

TABLE VIII

Comparison of Fermentable and Non-fermentable Reducing Substances in 24-hour Urine Samples when Precipitated by Ferric Chloride-Mercuric Chloride-Lloyd's-Lead Carbonate and by Ferric Sulfate-Lloyd's-Barium Carbonate; Values Express Total Mg. in 24-hour Samples

	Ferric Chloride-Mercuric Chloride		Ferric Sulfate	
	F.	N.F.	F.	N.F.
J.S.	64	72	59	189
M.T.	80	nil	75	125
E.C.*	321	117	319	304
M.Z.	55	82	57	222
E.W.	120	205	119	358
M.D.	69	nil	49	251
M.T.	68	165	65	211
M.D.	75	nil	52	227
M.A.*	555	145	424	552
M.A.#	552	164	—	—

* Controlled diabetics

Preliminary clearing with lead subacetate as per Harding et al (45). 1.0 cc. of 0.5 N solution of acetate was used to 8.0 cc. of urine. The mixture was allowed to stand for 30 minutes with occasional shaking. Filtration and subsequent treatment with ferric chloride-mercuric chloride-Lloyd's-lead carbonate gave results which showed no definite improvement over our method.

TABLE IX

Determination of Removal of Non-fermentable Reducing Substances from a 24-hour Sample

Reagent	Non-fermentable Reducing Substances
Ferric Chloride-20% Mercuric Chloride-16% Lloyd's-Lead Carbonate	nil
Ferric Sulfate-21% Lloyd's-Barium Carbonate	227
Ferric Sulfate-21% Barium Carbonate	556
Saturated Mercuric Sulfate in 2 N Sulfuric Acid-Lloyd's-Barium Carbonate	249
Saturated Mercuric Sulfate in 2 N Sulfuric Acid-Barium Carbonate	278
Ferric Sulfate-21% Mercuric Sulfate-25% in 1 N Sulfuric Acid-Barium Carbonate (Old Mercury-Iron)	142
Old Mercury-Iron with Lloyd's	100
Ferric Chloride-88%-Lloyd's- Lead Carbonate	nil
Untreated diluted urine	805

The efficiency of the ferric chloride-Lloyd's and ferric chloride-mercuric chloride-Lloyd's is clearly demonstrated by the above table. The addition of Lloyd's reagent to the ferric sulfate reagent gives greater additional removal than the addition of Lloyd's to the saturated mercuric sulfate or to the old mercury-iron reagent.

TABLE I

Grams of Fermentable and Non-fermentable Reducing Substances as Determined in Feces Samples Using Different Precipitating Agents *

Sample	Old Hg-Fe(15 cc.)		New Hg-Fe(15 cc.)		New Hg-Fe(15 cc.)	
	F	N.F.	F.	N.F.	F.	N.F.
19	2.0	1.3	1.9	1.0	2.0	1.2
20	1.5	0.9	1.5	0.8	1.6	1.4
21	3.1	2.1	3.3	1.9	3.7	2.0
22	0.7	0.5	0.7	0.4	0.7	0.5
23	3.0	2.1	2.8	1.7	----------	
24	1.3	0.7	1.4	0.6	----------	
25	1.6	0.8	1.7	0.8	----------	

* These determinations were run on collected feces samples from a patient with only 36 inches of small intestine remaining after a series of resections.

New Hg-Fe contains ferric sulfate-48%; mercuric sulfate-21.5% in .25 N sulfuric acid.

TABLE II

Determinations of Fermentable and Non-fermentable Reducing Substances in Urine Samples Using Different Precipitating Reagents

Sample	Reagent	F.	N.F.
E.W.	15 cc. $FeCl_3$-$HgCl_2$-$PbCO_3$ (1)	134	1056
"	" " (2)	121	205
"	15 cc. $Fe_2(SO_4)_3$-$BaCO_3$ (3)	129	344
"	" "	137	428
"	15 cc. $FeCl_3$-$PbCO_3$ (4)	128	214
M.D.	10 cc. $Fe_2(C_2O_4)_3$-$BaCO_3$ (5)	162	252
L.N.	15 cc. $Fe_2(SO_4)_3$-$BaCO_3$ (6)	125	456
"	15 cc. $FeCl_3$-$PbCO_3$(7)	109	209
M.T.	10 cc. $Fe_2(SO_4)_3$-5 cc. $Hg(C_2H_3O_2)_2$-$BaCO_3$(8)	56	210
"	15 cc. $Fe_2(SO_4)_3$-$BaCO_3$ (9)	65	211
"	5 cc. $Fe_2(SO_4)_3$-5 cc. $Hg(C_2H_3O_2)_2$-5 cc. $Pb(C_2H_3O_2)_2$ $BaCO_3$ (10)	62	277
"	10 cc. $Fe_2(SO_4)_3$-5 cc. $Hg(C_2H_3O_2)_2$-$ZnCO_3$ (11)	60	285

TABLE XI-a .

The reagents used in Table XI were of the following strengths:

(1) Ferric Chloride-6%; Mercuric Chloride-30%

(2) Ferric Chloride-20%; Mercuric Chloride-15%

(3) Ferric Sulfate-21%

(4) Ferric Chloride-35%

(5) Ferric Oxalate-20%

(6) As per 5

(7) As per 4

(8) Ferric Sulfate-20%; Mercuric Acetate-25%

(9) As per 5

(10) Ferric Sulfate-20%; Mercuric Acetate-25%; Lead Acetate-25%

(11) As per 5

Lloyd's reagent was used in all these determinations.

TABLE XII

A Comparison of the Fermentable and Non-fermentable Reducing Substances in Urine by Two Procedures

Sample	Volume cc.	$Fe_2(SO_4)_3$-Lloyd's-$BaCO_3$ Ferm.	$Fe_2(SO_4)_3$-Lloyd's-$BaCO_3$ Non-Ferm.	$Fe_2(SO_4)_3$-Lloyd's-$PbCO_3$ Ferm.	$Fe_2(SO_4)_3$-Lloyd's-$PbCO_3$ Non-Ferm.
1. H.S.	2420	116	286	109	nil
2. H.F.	1180	59	518	57	169
(A) "	dil.1:1	55	220	114	nil
3. K.B.	1120	0	90	0	20
4. Starvation Urine	720	15	28	15	nil
5. K.W.	1240	57	377	67	245
(A) "	dil.1:1	65	575	87	200
(B) " "	dil.1:2	370	740	387	nil
6. H.D.*	2680	59	159	52	nil
7. P.H.*	2570	95	185	85	68
8. K.N.*	1270 dil.1:2	145	192	125	nil

* Neu Somogyi Copper Reagent used in these determinations.

Sample 5 B. of K.W. is not the same as 5 and 5 A..

TABLE XIII

Determination of Feces Reducing Substances
Using $Fe_2(SO_4)_3$-Lloyd's-$PbCO_3$ Procedure

Sample	Volume of Dilution	Fermentable(gm.)	Non-fermentable(gm.)
3-2-38	3000	1.86	2.06
3-5-38	3000	1.44	1.79
3-4-38	3000	1.46	1.64
3-5-38	3000	2.78	3.57
3-6-38	3000	2.09	4.90
3-7-38	1000	0.89	0.85
3-8-38	1000	1.09	1.80
3-9-38	3000	1.06	1.28
3-10-38	3000	1.75	3.61
3-9-38*	3000	1.31	1.59
3-10-38*	3000	2.85	2.91

* Ferric Sulfate-Lloyd's-Barium Carbonate Procedure

These samples were run on the patient of Table X. 25 cc. of feces dilution were placed in a large test tube with a capillary tube in the stopper and heated in a boiling water bath for 5 hours for hydrolysis. 10 cc. of hydrolysate were precipitated in the same manner as for urine.

TABLE XIII-a

Determination of the Total Nitrogen in the Feces Samples of Table XIII

Sample	Volume of Dilution	Total Nitrogen (gm.)
5-2-38	3000	3.17
5-3-38	3000	5.93
5-4-38	3000	5.37
5-5-38	3000	6.36
5-6-38	3000	8.35
5-7-38	1000	3.20
5-8-38	1000	4.38
5-9-38	3000	4.35
5-10-38	3000	8.11

TABLE XIV

Determination of Urine Reducing Substances
Using $Fe_2(SO_4)_3$-Lloyd's-$PbCO_3$ Procedure

Sample	Volume	Fermentable Mg.	Non-fermentable Mg.
3-3-38	3700	111.0	74.0
3-3-38	5150	152.0	82.0
3-4-38	2950	56.0	88.0
3-5-38	3100	95.0	62.0
3-6-38	2600	45.0	65.0
3-7-38	4200	126.0	67.0
3-8-38	2675	152.0	11.0
3-9-38	3230	77.0	64.0
3-10-38	3480	139.0	nil

These samples are from the patient of Table X.

TABLE XIV-a

Determination of the Total Nitrogen in the Urine Samples of Table XIV

Sample	Volume	Total Nitrogen (gm.)
5-2-38	3700	15.6
5-3-38	3150	15.5
5-4-38	2930	14.4
5-5-38	3100	14.7
5-6-38	3800	14.6
5-7-38	4200	15.5
5-8-38	2675	15.3
5-9-38	3220	18.3
5-10-38	5480	12.8

Hawk and Bergeim in their " Practical Physiological Chemistry " consider the total nitrogen of urine to represent approximately the following constituents, although each of these may vary with the type of diet ingested:

Urea----------80 to 90% of the total nitrogen

Ammonia----------2.5 to 4.5% of the total nitrogen

Creatinine----------an average excretion of 1.0-1.25 gm/24 hours

Uric Acid----------an average excretion of 0.70 gm./24 hours

TABLE XV

A Comparison of Sugar Determinations on Blood Precipated by Ferric Sulfate-Lloyd's-Lead Carbonate and Ferric Sulfate-Lloyd's-Barium Carbonate

Sample	$Fe_2(SO_4)_3$-Lloyd's-$PbCO_3$	$Fe_2(SO_4)_3$-Lloyd's-$BaCO_3$
L.N.	76 mg.%	76 mg.%
M.D.	90 mg.%	93 mg.%
E.F.	84 mg.%	85 mg.%

These filtrates were prepared according to the method of Steiner, Urban and West (42) using 50 cc. of water, 5 cc. of 21 % ferric sulfate and sufficient carbonate to neutralize.

DISCUSSION

One is somewhat puzzled when attempting to discuss and summarize this type of work. The author has felt particularly inadequate in interpreting the possible theories underlying precipitation procedures as herein used. It is interesting to note that while the determination of reducing substances in urine dates back some one hundred years or so, very few workers have attempted to explain why certain precipitating reagents remove certain reducing substances from biological fluids and not others. The author has previously quoted West et al (42) as saying that the mercuric ion was an essential factor in such precipitations and also that the nitrogenous constituents; mainly, creatine, urea, and creatinine were responsible for a major part of the non-fermentable reducing substances in urine. However, in the unpublished work of Curtis, Lane and West (47) they reach the conclusion, which the author has also found to be true, that the ferric sulfate-Lloyd's-barium carbonate is generally superior to mercuric sulfate-barium carbonate in removing non-fermentable reducing substances from urine. The author has conclusively shown that the ferric sulfate-Lloyd's-lead carbonate reagent is the most efficient remover of non-fermentable reducing substances that has been tried to date. This would tend to rule out rather definitely the necessity in urine precipitants of the mercuric ion. The method is equally as fast as any previously used and leaves no soluble salt in the filtrate.

Although West et al (42) judged the efficiency of their precipitating reagents on the percentage recoveries of added glucose and percentage removal of creatine, creatinine and urea, the author definitely feels that the latter determination is not of great prognostic value. If one compares the tables given in this paper on the percentage removal of these three substances, it will be seen that the author's ferric sulfate-Lloyd's-lead carbonate procedure is rather inferior in the removal of these substances as compared with removal of these by some of the precipitating reagents listed by West et al. One must bear in mind, though, that these workers and others have never been able at any time to remove all of the non-fermentable reducing substances from urine or approach the low values obtained by the author. This, then, would point to the fallacy of considering removal of these three substances as an absolute indication of the efficiency of the precipitating reagent for urine.

Another factor of extreme interest to the writer was that urines of rather large volume when precipitated by either ferric chloride-Lloyd's-lead carbonate or ferric sulfate-Lloyd's-lead carbonate showed complete removal of the non-fermentable reducing substances. This led the author to theorize that in large volume urines (2500-3500) the precipitating reagent was more efficient due presumably to dilution of the non-fermentable reducing substances; presupposing, however, that a fairly constant non-fermentable excretion is maintained by an individual.

To test this theory, smaller volume 24 hour samples were diluted 1:1 or 1:2 bringing the total volumes up to 2400-3500 cc.. Here again it was found that the non-fermentable was either completely removed or markedly reduced. It may be argued at this point that the fermentable sugar being correspondingly diluted would be so low that accurate determinations would not be possible. However, with the new Somogyi micro-copper reagent (50) which is capable of determining quantities as low as 0.01 mg. this argument becomes less valid. There may be those, too, who will maintain that possibly the Somogyi reagent is not as sensitive to non-fermentable reducing substances. Reference to Table XII determinations 2 and 2A and 5 and 5A will show that this is not necessarily true, for in these samples the Shaffer-Somogyi reagent #50 was used on diluted and undiluted urine and a change in the amount of non-fermentable is still noted.

In the first part of the thesis the author presented evidence which she considers highly significant as to one of the reasons for the efficient removal of non-fermentable reducing substances from urine by the ferric sulfate-Lloyd's-lead carbonate. This was the removal of the non-fermentable sugar, galactose. Now as to just what constitutes the average 24 hour excretion of galactose has not been determined. Harding et al (45) ran their determinations of galactose on composite fasting 4 hour urine specimens from six individuals. This procedure makes the estimation of the normal 24 hour excretion of galactose rather difficult. Their results showed a total galactose of

9.0 mg. (both hydrolyzed and unhydrolyzed) in men and 6.5 mg. in women. The amount of galactose in the non-hydrolyzed fractions was 1.7 mg. for men and 1.5 mg. for women. Naturally one would suspect that which they found to be true; namely, that on subsequent hydrolysis of various fractions of the original, they obtained more glucose and galactose (presumably from hydrolysis of lactose). The total glucose on all fractions for men was 10.6 mg. and galactose 9.0 mg. while the non-fermentable reducing substance was 57.1 mg.; still a considerable amount showing that galactose and lactose removal is undoubtedly not the whole picture in the author's work. Harding et al precipitated their urines first with lead subacetate and followed with the mercuric sulfate-barium carbonate procedure of West et al (39). It is possible that some preliminary hydrolysis occurred by treatment with the acid mercuric sulfate reagent; such results have been shown by Laug and Nash (19) and Mary Tenney (51). One might speculate then that the average galactose obtained in a non-hydrolyzing precipitating procedure might be much less with a correspondingly larger amount of precursor, presumably lactose.

West et al (42) have obtained 100% recovery of glucose from standard solutions with the ferric sulfate-Lloyd's-barium carbonate procedure. While the recovery of glucose by the writer showed 97% when there was a concentration of 0.598 mg./5 cc.; 98% when there was 0.299 mg./5 cc.; 95% when there was 0.146 mg./5 cc., it will

be seen by comparing the fermentable sugar determinations on the charts herein presented that , by and large, the results by the ferric sulfate-Lloyd's-barium carbonate and the ferric sulfate-Lloyd's-lead carbonate are probably as close as the limits of experimental error will allow. The fact that some of the fermentable determinations run a little higher, some a little lower while some duplicate the Nest procedure points to the fact that any variation is probably experimental to a great degree.

The blood sugar determinations check very closely.

Another fact of interest to the writer was that when precipitating standard sugar solutions, if the precipitate mass on the Buchner funnel were washed with distilled water and the mixture refiltered, the removed sugar could be recovered. This suggests the possibility that:

1. Sugar is adsorbed and subsequent washing of the precipitate removes it;
2. The organo-metallic complex formed in the precipitation is unstable and readily broken down by the addition of water.

CONCLUSIONS.

1. The precipitation reaction is probably due to the formation of organo-metallic complexes either on the secondary alcohol groups or the $-NH_2$; $-NH$ groups
2. The solubility of these organo-metallic complexes is a variable thing and may be influenced by the original solubility of the substance to be precipitated.
3. The organo-metallic complex formed may be unstable and readily broken down by the addition of water. Sugars removed from standard solutions may be recovered by this method.
4. While creatine, creatinine, and urea probably contribute much to the non-fermentable reducing substances of urine, the author also feels that a large proportion of non-fermentable reducing substance is due to galactose, lactose, etc and non-utilizable carbohydrate material derived from food.
5. The mercuric ion is not essential to satisfactory precipitation of urine and other biological fluids.
6. All or most of the non-fermentable reducing substances can be removed from urines with 24-hour volumes of 2500-3500 cc.; smaller volume urines can be diluted 1:1 or 1:2 with subsequent total removal of non-fermentable reducing substances.
7. The ferric sulfate-Lloyd's-$PbCO_3$ method is satisfactory for the determination of true blood sugar.
8. The fermentable sugars of urine samples by the West (42) procedure and by the author's agree within the limits of experimental error in most cases.

9. Galactose and lactose are more readily removed from solutions by several precipitating reagents than is glucose.

10. The precipitation method with ferric sulfate-Lloyd's-$PbCO_3$ is by far the best procedure for urine precipitation to date. With proper dilution of 24-hour urine samples the fermentable sugar can be determined directly without the necessity of the fermentation by yeast.

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