A STUDY OF THE
REDUCING SUBSTANCES OF URINE

by

MARY ELIZABETH TENNEY

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

December 1936

APPROVED:

[redacted]

Major Adviser

[redacted]

For the Graduate Committee

of the Medical School

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A STUDY OF THE REDUCING SUBSTANCES IN URINE

Introduction

Progress in the study of the carbohydrate-like reducing substances of urine and the factors affecting their excretion has necessarily been governed by the methods of analysis used.

The use of unwashed yeast in fermentation procedures, the incomplete removal of interfering non-sugar reducing materials, and the use of sugar methods incapable of determining the minute quantities of reducing substances present in normal urine have resulted in a diversity of opinion regarding the amount, nature, and source of these substances.

In 1918 Benedict and Osterberg (2) studied the effect of diet and starvation upon the excretion of total reducing substances by the dog. Prior to this time, treatment with the mercuric nitrate-sodium hydroxide reagent of Patein and Dufau (19) had been used to remove non-sugar reducing substances. With this alkaline reagent there was the danger of sugar oxidation and the further disadvantage of a high salt concentration in the filtrate. Benedict and Osterberg used a modification of this reagent, replacing the sodium hydroxide with sodium

bicarbonate, and improving the reagent somewhat by reducing its alkalinity. The fermentation procedures used involved incubation with unwashed yeast for 18-24 hours at 38° C. The long incubation periods used with unwashed yeast often resulted in the addition of so much reducing material that the removal of reducing substances by the yeast could not be detected. In addition, bacterial contamination at times resulted in the removal of reducing materials and the consequent over-estimation of fermentable reducing substances. Determination of fermentable substances under such conditions can not be considered accurate. The picrate method (1) of sugar determination was used.

Benedict's and Osterberg's results indicated that excretion was highest on a carbohydrate diet, appreciably decreased on a protein diet, and markedly decreased during starvation. They found the maximum excretion to occur four to five hours after a meal and the minimum about fifteen hours after a meal. Output of reducing substances was found to have no relation to volume. These workers, with Neuwirth (4), also studied the excretion of reducing substances, both non-fermentable and fermentable, of two normal men as affected by diet, and came to the conclusion that both fermentable and non-fermentable

excretion is higher on a high carbohydrate diet than on a high protein diet. On the assumption that fermentable sugar is always present in normal urine, they advanced the idea that there is a continued "glycuresis" of blood sugar into the urine.

In 1922 Folin and Berglund (6) studied the total reducing substances of urine and blood following the ingestion of large quantities of carbohydrates. Filtrates were prepared with Lloyd's alkaloidal reagent, and reducing substances were determined by the Folin and Wu copper colorimetric method (7). Treatment with Lloyd's reagent leaves large quantities of non-sugar reducing materials in the filtrates. They did not determine the fermentable fraction. When their results showed an increase in total sugar when passing from a high protein diet to a mixed diet, and no increase in output following the ingestion of pure carbohydrates, Folin and Berglund concluded that no glycuresis occurs, but that urine sugar represents moieties of the unassimilable carbohydrates of food, formed naturally or by heating.

Höst (16) in 1923, after experiments on many individuals, using the methods of Benedict and Osterberg (2), concluded that bread is responsible for a large part of urine "sugar", and that glucose is not present in normal urine.

Greenwald, Gross, and Samet (9) in 1924-25 studied the effect of diet on the excretion of total reducing substances by both dog and man, using mercuric nitrate-sodium bicarbonate filtrates and determining sugar by the two picrate methods of Benedict and Osterberg (1,3) and by the copper methods of both Folin and Wu (7) and Shaffer and Hartman (20). Fermentable reducing substances were not determined. They considered that urine "sugar" consists of both difficultly assimilable carbohydrates and sugar derived from food protein and endogeneous sources. Glucose was believed not to be present. In 1927 Greenwald, Gross, and McGuire (8) carried out other experiments, using Nagle's fermentation procedure (5), in which unwashed yeast is used and the incubation period shortened to forty minutes at 38° C. The results of these experiments indicated to them that the fraction of urine sugar arising from heat altered carbohydrates is small, and that the small quantities of fermentable reducing material found is a sugar other than glucose. They suggested the possibility that bacterial action in the intestines may be responsible for some urine sugar.

In 1929 Van Slyke and Hawkins (24), using filtrates prepared with Lloyd's alkaloidal reagent, and their own gasometric ferricyanide reduction method (23) for determination of reducing substances, reported the finding of

significant amounts of fermentable reducing materials in urine. They used the greatly improved fermentation procedures of Somogyi (22), in which washed yeast is used and the incubation period shortened to fifteen minutes at room temperature.

Harding and Selby (14) in 1931 prepared filtrates with Lloyd's reagent, used washed yeast, and determined reducing substances with a Shaffer-Hartman micro-reagent. They concluded that fermentable reducing substances are to be found in normal urine, but are absent from normal fasting urine.

In 1932 West and Peterson (27) prepared filtrates by treatment with mercuric sulfate and barium carbonate (28). This treatment not only removes a large percentage of non-sugar reducing materials, but also has the advantage of leaving a very low salt concentration in the filtrate. Washed yeast was used, and reducing substances were determined with a sensitive Shaffer-Hartman reagent. Haug and Nash (17) suggested that treatment with the acid solution of mercuric sulfate might lead to an increase in reducing substances due to hydrolysis of a non-fermentable precursor. West and Peterson reported an average output of 142 mg. fermentable and 395 mg. non-fermentable reducing substances in the urine of fifty-five normal individuals. West, Lange, and Peterson (26), using the same

methods, determined factors affecting the excretion of these substances. No diet was found under which no excretion of non-fermentable substances occurred, but a fairly constant level was maintained when only certain foods such as white bread, meat proteins, eggs, milk, many common fruits and vegetables were found in the diet. The ingestion of heat-altered carbohydrates, as found in dried fruits, dark Karo syrup, etc. was found to increase the non-fermentable reducing substances markedly.

These workers found that the metabolism of nucleoproteins was unrelated to sugar excretion. Excretion of non-fermentable reducing substances was found to occur in fasting urines at a somewhat lower level. In the dog, intestinal stasis was found to increase the excretion of non-fermentable substances, furnishing corroborative evidence for the theory of Greenwald et al (8) that bacterial action in the intestine is responsible for some sugar excretion in the urine. The non-fermentable portion of the sugar was thought to be little changed by hydrolysis. Fermentable sugar was found in all normal urines and in decreased amounts during fasting. The excretion of fermentable sugar was shown to be related to the activity of the pancreas and the general carbohydrate metabolism of the body, supporting Benedict's

(2) idea of a glycuresis. The excretion of fermentable sugar in the urine was markedly decreased after the ingestion of large amounts of carbohydrates, while it was decidedly increased upon taking ordinary meals after short periods of starvation. These workers noted an increase of fermentable sugar upon hydrolysis of filtrates and attributed it to the breakdown of non-reducing polysaccharide material.

In a third paper, West and Steiner (29) presented evidence that the fermentable sugar of both normal and starvation urine is glucose. Their evidence consisted in showing the fermentation rate of the fermentable urine sugar to be similar to that of glucose and different from the fermentation rates of fructose and mannose. The fermentation rates were determined in a Warburg manometric apparatus.

In 1933 Harding and Selby (15), using mercuric sulfate-barium carbonate, Lloyd's, and KH_2PO_4-MgO filtrates, confirmed the presence of fermentable sugar in fasting urines, but concluded that a yeast-removable substance other than glucose is present.

In 1935 Laug and Nash (17) made a study of reducing substances in normal dog urine. They prepared urine filtrates by the acid mercuric sulfate-barium carbonate procedure of West and Peterson (27) and used the sensi-

tive Shaffer-Hartman Reagent 50 (21) for determination of reducing materials. By determining the reducing substances after hydrolysis in each of a series of filtrates with increasing residual N content, these workers found that the amount of fermentable reducing substance yielded upon hydrolysis is not affected by the amounts of nitrogenous substances present. Indirect evidence that the hydrolyzable fractions of urine are not nitrogenous in nature was thus presented. Laug and Nash agreed with West and coworkers (26) that the fraction of non-fermentable reducing substances is little changed by hydrolysis, yielding little or none of the fermentable reducing compounds produced by hydrolysis. All of the reducing substances produced by hydrolysis were thought to be fermentable. In a later paper (18), Laug and Nash, using the same methods, presented the variations in urinary reducing substances of two normal dogs maintained on bread diets. The kind of bread diet (crust, whole wheat, etc.) was found to affect the production of reducing substances in the urine.

In 1936 Harding, Nicholson, and Archibald (12) applied a modification of the Salkowski copper-lime method for the precipitation of carbohydrates to urines cleared with basic lead acetate. They employed the specific fermentation methods of Harding and Nicholson (11) and determined reducing substances by the method of Harding

and Downs (10). Composite fasting urines were used for these experiments, and were fermented before clearing.

Differential fermentation indicated the presence of glucose and galactose both before and after hydrolysis of filtrates. Very small amounts of fructose and mannose were found in some urines. Fermentation by bakers' yeast alone as used by previous workers delegates galactose to the non-fermentable fraction of reducing substances. According to these workers, the fermentable reducing substances produced by hydrolysis are derived from the non-fermentable reducing fraction of urine and the shift of galactose to the fermentable fraction eliminates the necessity of postulating a non-reducing precursor. By repeated copper-lime precipitation a nitrogen-free fraction containing most of the reducing substances of urine was obtained, which constitutes more direct evidence than presented by Laug and Nash (17) that the reducing substances of urine are non-nitrogenous in character.

The effect of type meals on the hydrolyzable sugars of urine was presented in a second paper by Harding, Nicholson, and Jackson (13). Lloyd's-lead acetate and mercuric sulfate-barium carbonate urine filtrates were hydrolyzed and then precipitated with copper-lime. Increase in hydrolyzable reducing substances was produced by each of the type meals of fat, starch, and fruit, but

not by the protein meal. The increase was due almost entirely to glucose-producing substances.

A study of the reducing substances in urine was undertaken in which improved methods of precipitation were employed. The substances in urines and various urine filtrates which give fermentable and non-fermentable reducing substances upon hydrolysis were investigated in an attempt to determine whether such materials arise from the non-fermentable substances or possibly from non-reducing non-fermentable compounds of a polysaccharide character. The following pages give the methods used and results obtained in this study.

Experimental Work

Reagents

Ferric sulfate, 21% in water.

Mercuric sulfate, saturated (30%) in 2 N H_2SO_4. Solution decanted from precipitate of basic sulfate.

Ferric chloride, 20%, and mercuric chloride, 15%, in water.

Lloyd's alkaloidal reagent.

Barium carbonate.

Lead carbonate.

Yeast, Fleischmann's, washed and kept as recommended by Somogyi (22).

Sugar reagent, Shaffer-Somogyi Reagent 50, with 1 gm. KI (21).

	Gm. per liter
Na_2CO_3 (anhydrous)	25.
$NaHCO_3$	20.
Rochelle salts	25.
$CuSO_4.5H_2O$ (75 cc. 10% solution)	7.5
KIO_3 (20 cc. N solution)	
KI	1.0

Urine samples, preserved with a little toluene and kept in ice box.

Analytical procedures

Preparation of filtrates. Ferric sulphate-Lloyd's (25): The filtrates in a dilution of 1:7 were prepared by placing 10 cc. urine, 45 cc. water, and 15 cc. $Fe_2(SO_4)_3$ reagent in a 500 cc. Erlenmeyer flask. 4 gm. Lloyd's

reagent were added, and the mixture allowed to stand three or four minutes before neutralization. After the addition of 25-30 gm. $BaCO_3$, the flask was stoppered and shaken cautiously, the CO_2 formed being released at intervals. When the mixture no longer reddened blue litmus paper, it was filtered with light suction. The filtrate was acidified to congo red paper with concentrated H_2SO_4 (2 drops) and filtered to remove a slight precipitate of $BaSO_4$.

Mercuric sulphate (27): Essentially the same procedure was followed in the preparation of mercuric sulphate filtrates. Lloyd's reagent was not used, and 35-40 gm. $BaCO_3$ were necessary for neutralization. After acidification the solution was treated with H_2S for a few minutes, and the H_2S removed by aeration. The precipitated HgS and $BaSO_4$ were then filtered off.

Ferric chloride-mercuric chloride-Lloyd's[1]: In this case a 1:8 filtrate was prepared by placing 10 cc. urine, 60 cc. water, and 10 cc. $FeCl_3$-$HgCl_2$ in a 500 cc. Erlenmeyer flask, adding 4 gm. Lloyd's reagent, and allowing the mixture to stand three or four minutes. 70 gm. $PbCO_3$ were added, and the flask stoppered and shaken until all CO_2 had escaped. After filtering with suction,

1. Under consideration as a precipitating agent in West's laboratory at the time the work was done.

1 gm. $BaCO_3$ was added to the filtrate, and the solution saturated with H_2S. After again filtering with suction, 0.4 cc. concentrated H_2SO_4 were added to the filtrate, and excess H_2S was blown off with a current of moist air. The rather heavy $BaSO_4$ precipitate was filtered off with suction. 5 drops of 0.02% phenol red were added to the filtrate, followed by concentrated NaOH to near neutrality. The change in total volume was so small that it was disregarded in the calculations.

Determination of reducing substances. The filtrates prepared according to the above procedures were divided into two parts, and one part fermented. 5 cc. portions of a 15% suspension of bakers' yeast were centrifuged, the supernatant liquid decanted, and the sides of the centrifuge tubes dried with a small roll of filter paper, to avoid dilution of the filtrate. 8-10 cc. filtrate was added to each of two yeast tubes, and allowed to ferment at room temperature 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, 4 drops 0.02% phenol red added, and 0.5 N NaOH (3-6 drops) until the indicator turned red. Blanks of distilled water with phenol red were also run. After

the addition of 5 cc. 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 cc. of a 4% KI-5% $K_2C_2O_4$ solution was added to each, followed by 5 cc. N H_2SO_4, blown in quickly. Each tube was shaken, bulb still in place, until the precipitate of Cu_2O had dissolved. After standing a few minutes, the sides of the tube and the covering bulb were washed down with a fine stream of water from a wash bottle. Titrations were made with 0.005 N $Na_2S_2O_3$, 5 drops of a 0.5% starch solution being added near the endpoint. Usually titrations checked within 0.02 cc. Calculations were made in terms of glucose reducing equivalent. 1 cc. thiosulphate titration is equivalent to 0.113 mg. glucose. The difference between the titrations of the fermented samples and the blank multiplied by a factor (15.8 in case of a 1:7 dilution) indicates the mg. non-fermentable reducing substance (as glucose) per 100 cc. urine. Similarly, the difference between the titrations of the fermented samples and the non-fermented samples multiplied by the factor indicates the mg. fermentable reducing substance per 100 cc. urine.

Hydrolysis. Hydrolysis, whether of the urine directly or of the filtrate, was carried out in an approxi-

mately 1 N solution of H_2SO_4 for three hours.

When the urine was hydrolyzed before precipitation, 20 cc. urine and 20 cc. 2 N H_2SO_4 were placed in a pyrex sugar tube fitted with a rubber stopper carrying a 6 inch piece of capillary tubing, and heated three hours in a boiling water bath. The contents of the tube were then transferred to a 500 cc. Erlenmeyer flask, 70 cc. of water added (including that used to rinse out the tube), and the filtrate prepared as usual, using double amounts of all reagents.

When the sample was hydrolyzed after precipitation, a double amount of filtrate was prepared, in a 1:8 dilution, so that sugar determinations both before and after hydrolysis could be made on the same filtrate. 60 cc. of the filtrate prepared in the usual way were placed in a hydrolysis tube, and 1.7 cc. concentrated H_2SO_4 added to make the solution approximately 1 N. (A correction was made for this slight change in volume when calculating results.) After hydrolyzing three hours in a boiling water bath, the solution was neutralized with $BaCO_3$ (about 10 gm.), filtered, and the filtrate acidified to congo with concentrated H_2SO_4. It was then aerated to remove traces of H_2S from sulfides in the $BaCO_3$ and filtered to remove $BaSO_4$. The determination of reducing substances in this filtrate was carried out as given above.

Comparison of Reducing Substances in $Fe_2(SO_4)_3$-Lloyd's and $HgSO_4$ Filtrates of Normal Urines

Determinations of reducing substances in both $Fe_2(SO_4)_3$-Lloyd's-$BaCO_3$ and $HgSO_4$-$BaCO_3$ filtrates of a number of normal urines were made in an effort to detect any hydrolytic effect of the more acid precipitating reagent ($HgSO_4$), as was found by Laug and Nash (17).

TABLE I

Reducing Substances in $Fe_2(SO_4)_3$-Lloyd's and $HgSO_4$ Filtrates

Sample		Ferric Sulfate-Lloyd's		Mercuric Sulfate	
		Nonf.	Ferm.	Nonf.	Ferm.
MET	11-17-35	247	49	276	50
ESW	11-17-35	385	139	411	155
ESW	11-18-35	394	140	333	180
ESW	11-19-35	360	132	348	158
ESW	11-20-35	415	87	384	94
ESW	11-21-35	331	142	376	181
ESW	11-22-35	381	110	372	108
JES	11-21-35	365	92	462	161
LFN	11-20-35	592	63	694	104
	Averages	386	106	406	132

The results in Table I show definitely higher values for fermentable reducing substances after treatment with the mercury reagent. Results vary widely as would be expected,

since the amount of hydrolysis under such conditions would depend upon the nature of the hydrolyzable material present in the urine and upon the time in contact with the acid reagent before neutralization with $BaCO_3$. Averages obtained were 386 mg. non-fermentable reducing substances and 106 mg. fermentable reducing substances from the $Fe_2(SO_4)_3$-Lloyd's filtrates and 406 mg. non-fermentable reducing substances and 132 mg. fermentable reducing substances from the $HgSO_4$ filtrates. The average increase of 26 mg. in fermentable reducing substance is probably due to the hydrolytic effect of the acid reagent. Although the averages of the non-fermentable reducing substances show a 20 mg. increase with the mercury reagent, this variation is not consistent in the individual samples. Differemges in this respect may be attributed to the difference in the action of the two precipitating reagents upon the varying concentrations and kinds of materials present.

The Effect of Hydrolysis Before and After Precipitation upon the Reducing Substances of Urine

Determinations of reducing substances in a number of urines were made both before and after hydrolysis. In each case the urine was hydrolyzed before treatment with $Fe_2(SO_4)_3$. As will be shown later the true values for

TABLE II

Hydrolysis before precipitation. Ferric sulfate filtrates.

Sample	Before Hydrolysis		After Hydrolysis	
	Nonf.	Ferm.	Nonf.	Ferm.
MET 1-17-36	211	65	257	129
MET 1-27-36	194	70	304	145
MET 1-28-36	253	64	362	147
MET 1-29-36	272	55	332	168
MET 1-30-36	300	48	392	71
MET 2-13-36	384	56	619	417
MET 2-14-36	176	60	325	108
MET 2-17-36	215	35	234	82
MET 2-19-36	255	47	295	100
KLM 2-9-36	106	52	176	135
JES 2-18-36	331	117	571	241
ESW 4-5-36	358	119	259	485
Averages	255	66	344	187
Hof.[1] 1-22-36	298	31	431	330
Hof. 1-23-36	216	21	158	382
Hof. 1-24-36	236	46	572	62
Hof. 1-25-36	270	63	578	82
Averages	255	40	460	214

1. Subject with 3 feet of small intestine.

hydrolyzable sugar can not be obtained using this method of hydrolysis. Table II shows the results for a series of urines. A consistent increase in both non-fermentable and fermentable reducing substances after hydrolysis is observed. West and coworkers (26) and Laug and Nash (17) noted no appreciable change in the non-fermentable fraction after hydrolysis of mercury filtrates of urine.

The last four results shown in Table II were made on samples from an individual with a short gut. Increases in both non-fermentable and fermentable reducing substances are noted after hydrolysis. Table III also shows increases in both fractions in two second day starvation samples, and

TABLE III

Effect of Starvation and a Milk Diet upon the Reducing Substances of Urine

Hydrolysis before precipitation. Ferric sulfate filtrates.

Sample		Before Hydrolysis		After Hydrolysis	
		Nonf.	Ferm.	Nonf.	Ferm.
JES[1]	2-26-36	189	59	164	115
CW[1]	2-26-36	179	44	193	82
MET[1]	3-8-36	125	75	156	87
	Averages	164	59	171	95
WZ[2]	3-31-36	222	57	284	231

1. Second day starvation samples.

2. Milk diet sample.

an increase in only the fermentable fraction of a third sample. In all cases these increases are markedly smaller than those noted in normal urines. These results apparently point to materials absorbed from the intestine as precursors of much of the reducing substances formed by hydrolysis of urine.

A series of determinations of reducing substances was run on a number of urines to determine the difference between hydrolyzing urine directly before treatment with $Fe_2(SO_4)_3$ and hydrolyzing a filtrate of the urine. As

TABLE IV

Effect of Hydrolysis Before and After Precipitation upon the Reducing Substances of Urine

Ferric sulfate-Lloyd's filtrates prepared in all cases

1 Sample		2 Before Hydrolysis		3 Hydrolysis Before Precipitation		4 Hydrolysis After Precipitation	
		Nonf.	Ferm.	Nonf.	Ferm.	Nonf.	Ferm.
MET	5-31-36	342	112	456	278	511	403
MD	6-3-36	251	49	312	109	333	231
MET	6-9-36	255	107	319	271	344	299
LFN	6-11-36	325	117	405	293	483	448
RSW	6-11-36	320	131	410	286	453	333
WFA	6-17-36	284	708	348	750	380	852
	Averages	296	204	375	331	417	428

TABLE V

Ratios Expressing Relation of Fermentable Reducing Substances Produced by Hydrolysis to Non-fermentable Reducing Substances

Sample		R_1	R_2
MET	5-31-36	0.85	1.62
MD	6-3-36	0.72	2.22
MET	6-9-36	0.75	2.16
LFW	6-11-36	1.02	2.10
RSW	6-11-36	0.63	1.52
WFA	6-17-36	0.51	1.5

shown in Table IV the yield of additional reducing substances, both fermentable and non-fermentable is markedly increased when the filtrate is hydrolyzed. It is probable that the lower values obtained by hydrolyzing urine before precipitation are due to interaction of the reducing substances formed by hydrolysis with other material (removable by the precipitating agents) in the urine leading to destruction of the reducing power.

In order to ascertain if possible a relation between the non-fermentable reducing substances and the increase in fermentable after hydrolysis calculations for the ratio:

$$\frac{\text{Fermentable after hydrolysis} - \text{fermentable before hydrolysis}}{\text{Non-fermentable before hydrolysis}}$$

were made respectively for the above series. The values

calculated from the figures of columns 2 and 4 of Table IV are given under R_1 in Table V. The variations in the ratios are sufficient to indicate that the non-fermentable reducing substances in the filtrates of the urine bear no direct relation to the increase in fermentable reducing substances caused by hydrolysis. The ratios of the increase in fermentable to the increase in nonfermentable reducing substance upon hydrolysis are given under R_2 in Table V. There is a somewhat closer correlation in this case than for R_1 which may indicate the simultaneous production of fermentable and non-fermentable reducing substances by hydrolysis of a common precursor.

TABLE VI

Effect of a Meat Diet on Reducing Substances of Urine

Sample	Before Hydrolysis		After Hydrolysis	
	Nonf.	Ferm.	Nonf.	Ferm.
I	37	9.4	42.6	24.8
275 gm. lean boiled beef.....				
II	60.5	10.3	72.6	22.1
III	59.9	16.2	62.6	22.3

Ferric sulfate filtrates. Hydrolysis after precipitation. Sample I--four hour control collected eight hours following preceding meal. Samples II and III--collected during the two four hour periods following the beef meal.

To determine the effect of a meat diet upon the reducing substances of urine, samples were collected before and after a meal consisting only of lean boiled beef. According to the values shown in Table VI, the increase in fermentable reducing substances after hydrolysis is proportionately greater than the increase in non-fermentable reducing substances. The amounts of reducing materials, both fermentable and non-fermentable, produced by hydrolysis in the urine collected after the beef meal are similar to those amounts found in the control sample. These results indicate that little or no additional production of hydrolyzable material follows a meat meal.

Determinations of reducing substances in the urine of a diabetic individual were made. Although the urine was hydrolyzed directly, so that the true values for hydrolyzable reducing substances were not obtained, the difference between these values (Table VII) and the values obtained in the same way from normal urines (Table II) is marked. There was no increase in fermentable reducing substances after hydrolysis of these samples. In order to discover if acetone present in diabetic urine might interfere with the sugar determinations, 0.5 cc. acetone were added to 100 cc. glucose solution containing 0.5 mg. glucose in 5 cc. solution, and determinations made. There was no effect evident. Glucose added to one sample of the diabetic urine was recovered quantitatively.

TABLE VII

Reducing Substances in the Urine of a Case of Diabetes on a Controlled Diet

Sample	Before Hydrolysis		After Hydrolysis	
	Nonf.	Ferm.	Nonf.	Ferm.
KC 2-18-36	262	136	319	141
KC 2-19-36	251	123	364	134
KC 2-20-36	362	321	487	298
KC 3-3-36	304	319	415	334
Averages	295	225	396	227

2-15-36 Diet: CH-35, P-55, F-220 $\frac{\text{Fatty acid}}{\text{Glucose}}$-2.5

Fasting blood sugar--194. Dextrose tolerance curve high and fell slowly (typical diabetes). Sample high in acetoacetic acid and acetone.

The last result in Table IV, also from the urine of a diabetic individual, shows the same tendency, although in this case a slight increase in fermentable reducing substances was evident after hydrolysis. These results may indicate a difference in absorption, metabolism, or production of hydrolyzable precursors of fermentable reducing substances by the diabetic individual.

A comparison of results obtained by determining reducing substances of both ferric sulfate and ferric chloride-mercuric chloride filtrates is shown in Table VIII. The values for fermentable sugar as shown in columns 2 and 6 check closely. However, as a comparison of columns 1 and

TABLE VIII

Reducing Substances in Ferric Sulfate and Ferric Chloride-Mercuric Chloride Filtrates

Hydrolysis after precipitation.

Sample	$Fe_2(SO_4)_3$				$FeCl_3$-$HgCl_2$			
	Before Hydrolysis		After Hydrolysis		Before Hydrolysis		After Hydrolysis	
	1 Nonf.	2 Perm.	3 Nonf.	4 Perm.	5 Nonf.	6 Perm.	7 Nonf.	8 Perm.
MET 5-31-36	342	112	511	403	187	120	160	307
MD 6-3-36	251	49	333	231	___	52	___	94
MET 6-9-36	255	107	344	299	42	126	112	172
LFH 6-11-36	325	117	483	448	126	99	203	319
RSW 6-11-36	320	131	453	333	51	141	151	230
WFA 6-17-36	284	708	380	852	77	674	110	645
Averages	296	204	417	428	81	202	123	295

TABLE IX

Ratio: Columns $\frac{4 - 8}{1 - 5}$ of Table VIII

Sample	R_3
MET 5-31-36	0.62
MD 6-3-36	0.59
MET 6-9-36	0.60
LFH 6-11-36	0.65
RSW 6-11-36	0.38
WFA 6-17-36	1.

5 shows, treatment with ferric chloride-mercuric chloride is more efficient in the removal of non-fermentable reducing substances. In one case all of the non-fermentable reducing substances were removed by this treatment.

R_3 in Table IX shows the ratio:

$$\frac{\text{Fermentable difference after hydrolysis } (Fe_2(SO_4)_3 \text{ filtrate} - FeCl_3 \cdot HgCl_2 \text{ filtrate})}{\text{Non-fermentable difference before hydrolysis } (Fe_2(SO_4)_3 \text{ filtrate} - FeCl_3 \cdot HgCl_2 \text{ filtrate})}$$

This statement indicates the proportionality of the decrease in fermentable sugar upon hydrolysis to the fraction of non-fermentable reducing material which is removed by $FeCl_3$-$HgCl_2$ and not removed by $Fe_2(SO_4)_3$.

Values for the first four samples check closely, indicating that a part of the fermentable reducing substance produced by hydrolysis possibly has its origin in a part of the non-fermentable reducing fraction, as Harding and his coworkers (12) found.

Summary

1. A study of reducing substances in urine has been made, using improved methods of precipitation.

2. An increase in fermentable reducing substances during treatment with an acid precipitating agent ($HgSO_4$) has been found to occur, indicating hydrolysis during treatment.

3. Hydrolysis of urine before precipitation leads to destruction of some of the fermentable and non-fermentable reducing substances. This is probably due to interaction of these materials with unknown urine constituents.

4. Some of the data obtained may indicate the presence in urine of a substance or substances which yield both fermentable and non-fermentable reducing compounds upon hydrolysis.

5. A considerable proportion of the materials in urine which yield fermentable and non-fermentable reducing substances when hydrolyzed apparently are represented by compounds absorbed from the intestine and not metabolized in the body.

6. A meal of beef muscle was found to contribute little to the substances of urine which produce reducing compounds upon hydrolysis.

7. Urines from a case of diabetes on a controlled diet gave no appreciable increase in fermentable sugar after hydrolysis. There was some increase in non-fermentable reducing substances.

8. Precipitation by $FeCl_3$-$HgCl_2$ is more efficient than $Fe_2(SO_4)_3$ in removing non-fermentable reducing substances from urine.

9. A possibility that the non-fermentable reducing compounds of urine may yield a part of the fermentable reducing substances produced on hydrolysis is suggested.

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