REDUCING SUBSTANCES IN NORMAL AND DIABETIC URINE

by

WILLIAM M. BURGET

A THESIS

Presented to the Department of Biochemistry
and the Graduate Division of the University of Oregon Medical School
in partial fulfillment
of the requirements for the degree of
Master of Science

September 1944

APPROVED:

[redacted]

(Professor in Charge of Thesis)

[redacted]

(Dean, Graduate Division)

Sept 6, 1944

TABLE OF CONTENTS

INTRODUCTION

The purpose of this study was to investigate the reducing substances in urine and, more particularly, the reducing substances of diabetic urine as compared with those of the normal. Reducing substances in normal urine are not confined to carbohydrates but are composed of a complex mixture including nitrogenous substances such as creatinine, creatine, uric acid, a little glucose, some pentose, anhydro-sugars, and complex carbohydrate which breaks down on hydrolysis to form reducing material. Many investigations have been undertaken as to the nature of the reducing substances of normal urine, but very little work has been done on diabetic urine, especially with reference to the non-fermentable fraction of the reducing substances and the effects of hydrolysis upon both the non-fermentable and fermentable fractions.

It has been known since the time of Hippocrates that the urine of some diabetic patients contain a sugar-like substance. This fact initiated a long chain of research on the reducing properties of urine. In 1772, Dobson of Liverpool evaporated diabetic urine and obtained a crystalline material which he was unable to distinguish from sugar. Bouchardt and Peligot identified the sugar of diabetic urine as glucose in 1838. In 1841, Traumer published his method of sugar determination using an alkaline-copper solution which has been the basis for many methods of sugar analysis up to the present time. It was considered by all of these workers, and many since that time, that

glucose in normal urine exists as a pathological substance. In 1907, MacLean (1), determining what he considered to be fermentable sugar with safranin, found fermentable material in every urine he examined, between 0.03 and 0.07 per cent. He believed this fermentable material was glucose. In addition, he found that urine contained some non-fermentable reducing substances which exist approximately in the proportion of three to one of the fermentable material. Schultz (2), in 1910, reported small amounts of glucose in all urine samples analyzed as did Gilbert and Boudouin (3) in 1911.

Benedict and co-workers (4), (5), (6), in 1918, published several articles upon carbohydrate metabolism in which they consistently found small amounts of fermentable material in urine. By fermenting the urine and then precipitating with mercuric nitrate and sodium bicarbonate, they determined the amount of reducing substances present colorimetrically using a picric acid reagent. They advanced what was at that time a new concept--that there is a renal threshold for sugar below which there is a minimal but constant passage of sugar from the blood into the urine. This process was called glycuresis. In addition, there was non-fermentable reducing material present in all urines in the proportion of three to one of glucose.

However, Shaffer and Hartmann (7), (1920), using a copper potassium iodide reagent, were unable to detect any fermentable sugar in urine.

In 1922, Folin and Berglund (8) in a similar study also found no glucose or fermentable material. They cleared the urine with Lloyd's alkaloidal reagent and determined the reducing substances by the method of Folin and Wu. Their conclusions were that reducing

materials are consistently present in urine, that the amounts of material are independent of the blood sugar level, and that no glucose is present. They suggested that urine sugar is composed of carbohydrate materials rendered non-utilizable by cooking processes. However, Benedict and Osterburg (9) maintained that Folin and Berglund had misinterpreted their experimental results. But Host (10) (1923), using the methods of Benedict and Osterburg (11); Lund and Wolfe (12), measuring the CO_2 formed by fermentation of urine; and Patterson (13) (1926), and Eagle (14) (1927) were unable to detect fermentable material. Greenwald, Gross, and Samet (15), (16) (1924), applied two picrate methods, the Shaffer-Hartmann procedure and the Folin and Wu method to filtrates of urine prepared by mercuric nitrate and sodium bicarbonate and corroborated the previous work of Folin and Berglund (17). They denied the existence of a process such as glycuresis. In a later paper (18), they found a reducing fermentable substance, but did not believe it to be glucose.

Van Slyke and Hawkins (19) (1929), using a ferricyanide-gasometric method of sugar determination after preliminary precipitation with oxalic acid and Lloyd's reagent, found fermentable substances in all urines in amounts from 9 to 23 mg. per cent. They used the fermentation method of Somogyi (20) with washed yeast.

Everett and Sheppard (1930) (21), as a result of treating urine with bromine, which destroys the reducing power of aldoses, decided that a major proportion of the reducing material in normal urine is a ketose--perhaps a pentose. Later work (22) by the same investigators based upon the reaction of reducing substances in urine with hypo-iodite as well as bromine also suggested the presence of a ketose.

West (23) (1931), determined the fermentable and non-fermentable reducing substances in mercuric sulfate-barium carbonate filtrates (24), (25) of normal urines and found fermentable material in all cases, the amounts of which he related to pancreatic activity and the general carbohydrate metabolism. Harding and Selby (26) (1936) also investigated the problem of urine sugars. They cleared the urine with sulfuric acid and Lloyd's reagent before determining the reducing substances by a modification of the Shaffer-Hartmann technique. They also used the Somogyi method of fermentation (27). They found fermentable sugar in all but fasting urines, and concluded that there are, undoubtedly, a number of variable factors involved in determining the presence and amount of fermentable material.

West, Lang, and Peterson (28) (1932), in a study of the effect of diet on fermentable and non-fermentable sugar elimination, concluded that a considerable proportion of the non-fermentable fraction is derived from carbohydrate-containing foods which have been subjected to high temperatures in cooking and that this non-fermentable fraction is not increased by a high nucleo-protein diet, is decreased in starvation, is increased by intestinal stasis, and is little changed by hydrolysis. Fermentable sugar was found in all normal urines and was presumed to be glucose, which had passed from the blood into the urine through the renal tubules as suggested by Benedict in 1918 (29). They also noted that following hydrolysis of urine there was an increase in the amount of fermentable sugar which they suggested to be due to the breakdown of some non-reducing polysaccharide. In a long series of determinations, Peterson and West (30) found an average of 142 mg. of fermentable sugar and an average

of 395 mg. of non-fermentable sugar per 24-hour sample of normal urine. In these determinations, they used the mercuric sulfate-barium carbonate precipitation, which they had described previously, a modified Shaffer-Hartmann copper reagent and Somogyi's fermentation method.

In the same year, West and Steiner (31), (32) proved by measuring the rates of fermentation of various sugars in the Warburg apparatus that the fermentable sugar of normal urine is glucose. This work was corroborated by Harding and Selby (33) in 1933 using essentially the same methods as West (34). Harding and Nicholson (35), utilizing a mycological method of specific fermentation, confirmed the presence of glucose in normal urine and also detected a small amount of galactose and occasionally some fructose and mannose.

Everett and Edwards (36) (1934), continuing their work on bromine and hypo-iodite oxidation products, postulated the presence of a substance which they called uroketose, which they believed is responsible for most of the reducing power of urine. Laug and Nash (37) (1935), using the methods of West (38) and confirming his work (39), suggested that the values obtained for fermentable sugar using the acid mercuric sulfate-barium carbonate precipitation procedure are too high because the high acidity of the precipitating agent hydrolyzes non-fermentable precursors of fermentable reducing substances. They proposed a mechanism for the stepwise production by hydrolysis of a reducing fermentable substance from a non-reducing, non-fermentable substance.

Harding, Nicholson, and Jackson (40) (1936), in a series of similar experiments, found that all foods except protein cause increases in the amounts of substances giving rise to fermentable sugar upon hydrolysis and that these increases

are almost entirely due to glucose-producing substances. By hypo-iodite oxidation of the sugars produced by acid hydrolysis, these workers showed that about one third of the hydrolyzable material has reducing properties and is glucose. They also concluded that the galactose released on hydrolysis of urine is combined as a polysaccharide through its reducing group. They suggested that the reduction in the amount of non-fermentable reducing material of urine filtrates which is to be expected following acid hydrolysis and yeast fermentation may be masked by galactose liberated if the fermentation organism does not remove galactose.

Harding, Nicholson, and Archibald (1936) (41), using fractionated urine filtrates, showed by their mycological fermentation method the presence of glucose and galactose both before and after hydrolysis, and that most of the fermentable sugars produced on hydrolysis are derived from the non-fermentable reducing fraction. They also suggested that the postulate of a non-reducing precursor of fermentable sugar (as by Laug and Nash) (42) is unnecessary if galactose is included in the fraction removed by yeast.

Nicholson and Archibald (43) (1939), using basic lead acetate-mercuric sulfate precipitation of urine followed by treatment with copper and lime, obtained material which was free from nitrogen and contained a high percentage of true sugars. Sodium hypo-iodite oxidation of solutions of this material showed that 75 per cent of it behaved as aldose.

Dittebrandt, Tenney, and West (44) (1944) used ferric sulfate-barium carbonate-Lloyd's reagent and ferric sulfate-lead carbonate-Lloyd's reagent for precipitation of urine and found that these

filtrates give lower values for fermentable substances than do mercury filtrates. This was probably due to the lower acidity of the iron as compared with the mercury reagents (Lang and Nash) (45). They found that hydrolyzed filtrates and filtrates prepared from hydrolyzed urine showed a distinct increase in both the fermentable and non-fermentable fractions. The increase was usually more marked in the filtrates prepared from hydrolyzed urine. They also analyzed similarly several samples of diabetic urine and noticed that there was no increase in fermentable sugar in the filtrates prepared from hydrolyzed urine. This accidental finding initiated the present study. It must be remembered, however, that these samples were limited in number and were from only one patient. Since no investigations similar to those undertaken on normal urine had been applied to diabetic urine, it may be supposed that previous workers considered that the only difference between diabetic and normal urine lay in the amount of glucose present, which is not necessarily a logical assumption inasmuch as the disease diabetes mellitus involves much of carbohydrate metabolism. There may be other differences in the products of carbohydrate metabolism found in diabetic urine.

METHODS

1. Preparation of Urine Filtrates

A short discussion of precipitation methods to indicate their purpose and mode of action should perhaps preface this section. The purpose of precipitation is to remove reducing substances which are not of a carbohydrate nature and, therefore, mask the true sugar values. These substances as indicated previously are largely of a nitrogenous nature. Also, precipitation procedures eliminate substances which may interfere with the fermentation processes used to determine fermentable sugar.

In 1902, Patein and Dufau (46) used acid mercuric nitrate and sodium hydroxide as a precipitating agent and found that it gave urine filtrates containing much less reducing material than the original urine.

Benedict and Osterburg (47) (1918) changed this slightly by using acid mercuric nitrate and sodium bicarbonate. Shaffer and Hartmann (48) (1920) and Patterson (49) (1926) used the same procedure. Folin and Berglund (50) in 1922 cleared the urine with Lloyd's reagent preliminary to analysis. Van Slyke and Hawkins (51) (1939) cleared the urine with Lloyd's reagent and oxalic acid.

West, Scharles, and Peterson (52) used mercuric nitrate neutralized with barium carbonate but preferred mercuric sulfate neutralized with barium carbonate because it was simpler and left very little electrolyte in the filtrate. This method was also used by Harding and Downs (53). In 1932, Steiner, Urban and West (54) used ferric

sulfate neutralized with barium carbonate. This method was later modified by treatment of urine with ferric sulfate and Lloyd's reagent previous to neutralization with barium carbonate (Curtis, Lane, and West) (55), and was found to give somewhat lower non-fermentable reducing values than the mercuric sulfate-barium carbonate method. It has the advantage of simplicity, removal of most of the electrolyte, and of providing essentially neutral filtrates.

In 1944, Dittebrandt, Tenney, and West (56) modified this latter method by neutralizing the ferric sulfate with lead carbonate, giving still lower values for non-fermentable substances and thus approaching more closely actual sugar values. These last two methods of preparing urine filtrates were the ones used in the work reported in this thesis.

2. Fermentation Procedure

Before Somogyi's paper in 1927 (57), the fermentation methods used gave widely varied results. These differences were due to impurities in the yeast, prolonged fermentation, which added products of yeast metabolism causing too high values for reducing substances, and fermentation before clearing or precipitating the urine. Somogyi, however, introduced the use of washed yeast at room temperature and greatly reduced fermentation time. With this method, results are constant and all glucose is removed.

3. Sugar Method and Reagent

The method of sugar determination used was that of Shaffer and Hartmann with one modification. Shaffer-Hartmann sugar reagent in

itself is well-known and needs no discussion. Shaffer and Somogyi (58) in 1933, in discussing micro-sugar reagents, showed that the addition of sodium sulfate to the reagent slows the rate of sugar oxidation but increases the maximum amount of reduction obtained. Also, these workers showed that the sensitivity of the reagent is inversely proportional to the amount of potassium iodide included in the reagent.

Somogyi (59) (1937) described a micro-copper reagent saturated with sodium sulfate capable of giving good accuracy even at very low sugar concentrations, but with a limited upper range. The work reported in this thesis required a reagent giving good accuracy with small amounts of sugar as well as being applicable to larger amounts of sugar since both normal urines containing very little sugar and diabetic urines containing much sugar were to be analyzed. In order to meet these requirements, it was found satisfactory to modify the regular Shaffer-Hartmann reagent No. 50 by the addition of 20 per cent anhydrous sodium sulfate. The reagent is satisfactory for the determination of sugar between the limits of 0.025 mg. and 2.0 mg. per 5 cc., the titration difference at 0.025 mg. being 0.31 cc. A curve plotted for this reagent is a straight line function from 2.0 to 0.2 mg. per 5 cc. sample, but this is not quite true as zero is approached (Figs. 1 and 2). Figure 2 is an enlarged projection of segment A B in Figure 1, the segment in which the deviation from normal occurs. C B is a Projection of Figure 1 above 0.2 mg., in which region the slope is constant, extended to 0 so as to indicate the amount of deviation from constant slope in the lower ranges.

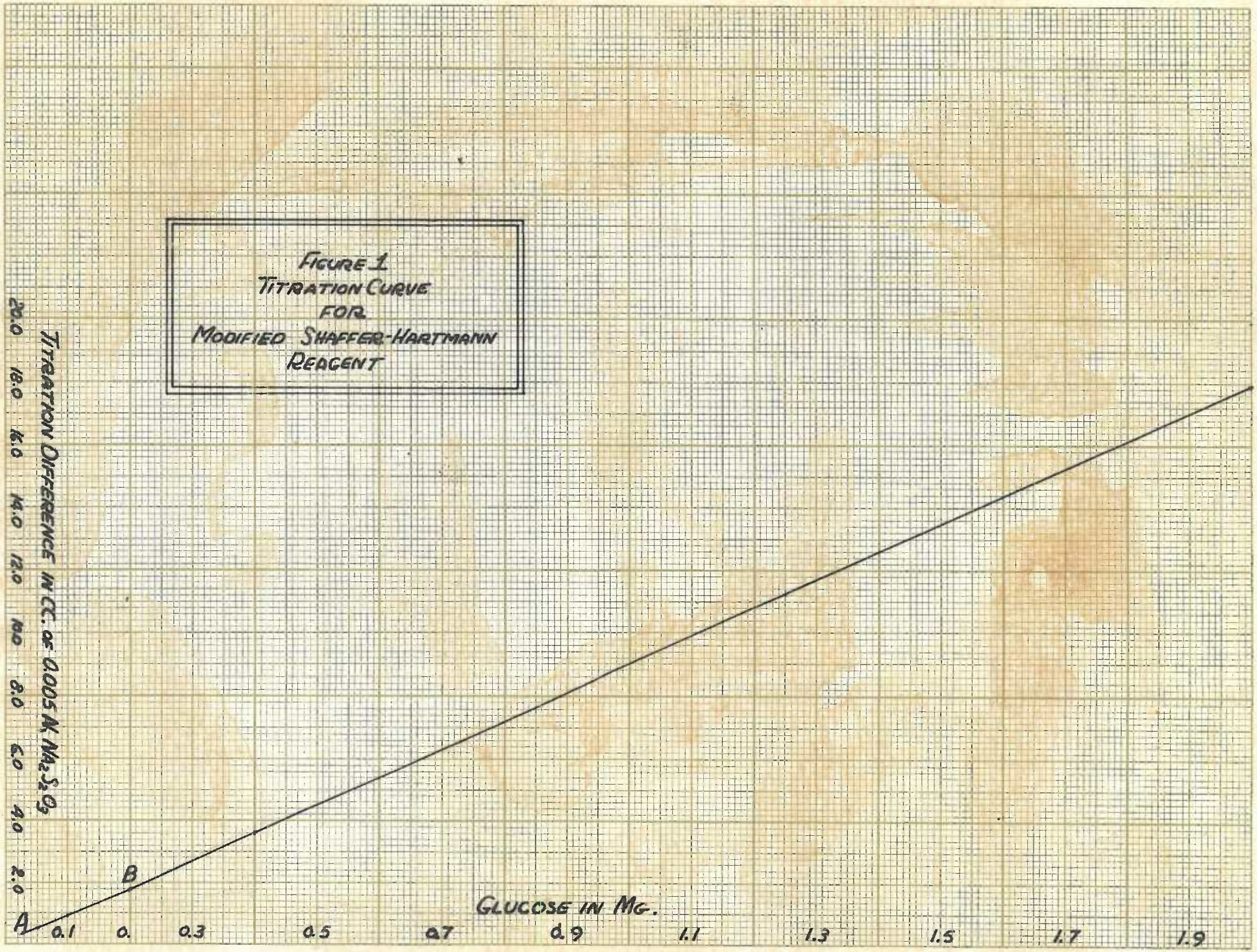
FIGURE 1
TITRATION CURVE
FOR
MODIFIED SHAFFER-HARTMANN
REAGENT
TITRATION DIFFERENCE IN CC. OF 0.005 N. $Na_2S_2O_3$
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
A
B
GLUCOSE IN MG.
0.1
0.
0.3
0.5
0.7
0.9
1.1
1.3
1.5
1.7
1.9

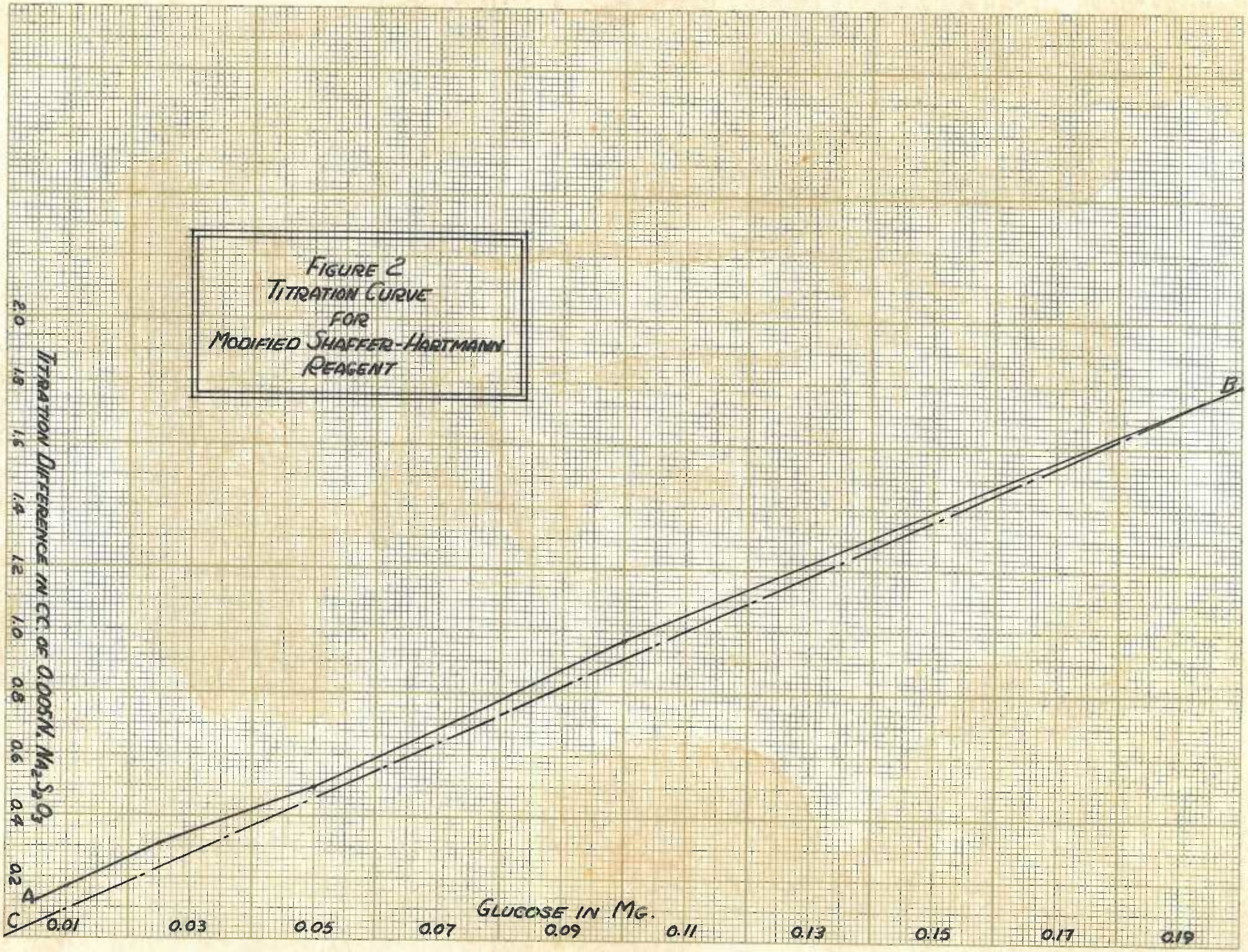
FIGURE 2
TITRATION CURVE
FOR
MODIFIED SHAFFER-HARTMANN
REAGENT
TITRATION DIFFERENCE IN CC. OF 0.005N. $Na_2S_2O_3$
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
A
B
C
GLUCOSE IN MG.
0.01
0.03
0.05
0.07
0.09
0.11
0.13
0.15
0.17
0.19

REAGENTS

1. Lloyd's alkaloidal reagent
2. Barium carbonate--technical
3. Lead carbonate--pure
4. Fleishman's yeast--commercial
5. Shaffer-Hartmann reagent No. 50 containing 20 per cent sodium sulfate (below)
6. Potassium iodide-oxalate reagent (2% potassium iodide--2.5% potassium oxalate)
7. 1 N. sulfuric acid
8. Ferric sulfate--30 per cent aqueous solution
9. Urine--preserved with toluene and kept in a cool place

PREPARATION OF SUGAR REAGENT

25 Gm. of anhydrous sodium carbonate and 25 Gm. of Rochelle salts are dissolved in about 850 cc. of warm water. 7.5 Gm. copper sulfate is dissolved in about 50 cc. of warm water and is added slowly with stirring to the above. 20 Gm. of dry sodium bicarbonate, 1 Gm. potassium iodide, and 200 Gm. of anhydrous sodium sulfate are added. The solution is heated to boiling and immediately removed from the flame, allowed to cool, and when room temperature is reached, the solution is filtered. After filtering, 20 cc. of 1 N. potassium iodate is added and the solution diluted to one liter with distilled water.

PREPARATION OF YEAST (SOMOGYI)

Fleishman's commercial grade yeast is suspended and mixed in 5 to 10 parts of water, centrifuged, and the supernatant fluid discarded. 5 to 10 parts of water are added and the yeast resuspended by stirring with a glass rod, avoiding the material in the bottom of the centrifuge bottle, which may contain some starch. The resuspended yeast is decanted off, and the material in the bottom is discarded. The yeast suspension is centrifuged, and the above process is repeated 4 to 5 times, or until the supernatant liquid is colorless. The yeast is kept in about 7 to 10 parts of water in the refrigerator and may be used for several weeks, if the supernatant fluid is changed occasionally.

METHODS OF PREPARATION OF FILTRATES

1. 50 to 60 cc. of urine is aerated to remove all toluene. 10 cc. of this urine is measured into a 500 cc. Erlenmyer flask. 15 cc. of 30 % ferric sulfate solution is added and the mixture diluted to 100 cc. with water. 4 Gm. of Lloyd's reagent is added, the mixture shaken, and allowed to stand for 3 or 4 minutes with frequent shaking. About 30 Gm. of dry barium carbonate is added to the mixture, the flask stoppered and shaken, carbon dioxide being allowed to escape occasionally. A little more barium carbonate is added, the shaking continued until no more carbon dioxide is evolved, and the mixture does not redden blue litmus. The mixture is rapidly filtered in a Buchner funnel. The precipitate is discarded and the filtrate is

made acid to congo red paper with a drop of concentrated sulfuric acid. The barium sulfate is removed by filtering, and the filtrate is ready for analysis.

2. 10 cc. of urine is measured into a 500 cc. Erlenmyer flask, 15 cc. of 30% ferric sulfate added, the mixture diluted to 100 cc. and treated with Lloyd's reagent as before. To this is added with shaking about 30 to 35 Gm. of lead carbonate. Carbon dioxide is allowed to escape from the stoppered flask, and small amounts of lead carbonate are added until no more carbon dioxide is evolved when the mixture is shaken and it does not redden blue litmus paper. Then the mixture is filtered in a Buchner funnel, and the filtrate is acidified to congo red paper with a drop of concentrated sulfuric acid. The filtrate is saturated with hydrogen sulfide to precipitate the lead, then filtered, and the filtrate aerated to remove the excess of hydrogen sulfide. Fermentation or sugar determination, as the case may be, is performed upon this filtrate.

FERMENTATION PROCEDURE

5 cc. of yeast suspension is spun down in a 15 cc. centrifuge tube, the supernatant liquid removed, and the tube dried with filter paper. About 12 cc. of the iron filtrate is added and the yeast is resuspended by stirring with a glass rod. The suspension is left at room temperature for 15 minutes, then centrifuged and filtered. It is essential that the centrifuging be complete since the presence of any yeast organisms will cause an error in the results. After filtering, the amount of reduction may be determined.

DETERMINATION OF REDUCTION

To 5 cc. of filtrate in an 8-inch test tube is added 5 cc. of the modified Shaffer-Hartmann reagent. These are mixed thoroughly. The tube is covered with a glass bulb and inserted in a boiling water bath for 20 minutes, after which the tubes are cooled to approximately 30 degrees or to room temperature. 2 cc. of potassium iodide-oxalate reagent are added and followed by 5 cc. of 1 N. sulfuric acid blown in rapidly with shaking. The iodine which is liberated is titrated with 0.005 N. sodium thiosulfate, which is prepared daily from a stock 0.1 N. solution. Water blanks in duplicate are run simultaneously with each series of determinations. Each determination is run in duplicate. Titration results usually agreed within 0.03 cc.

METHOD OF HYDROLYZING URINE

20 cc. of urine and 20 cc. of 2 N. sulfuric acid are mixed, and the mixture hydrolyzed in 8-inch test tubes covered with glass marbles in a boiling water bath for at least three hours. This is cooled, filtered, and precipitation carried out as described above.

METHOD OF HYDROLYZING FILTRATES

To 30 cc. of filtrate prepared by one of the foregoing precipitation methods, 30 cc. of 2 N. sulfuric acid is added and the mixture, in 8-inch test tubes covered with glass marbles, is hydrolyzed in a boiling water bath for at least three hours. After cooling and add-

ing 2 cc. of 30 per cent ferric sulfate solution, it is diluted to 100 cc. in a volumetric flask. The solution is neutralized with barium carbonate or lead carbonate, as the case may be, after the manner described above, and sugar determinations are made on the filtrate.

CONTROLS

Sugar determinations performed on water obtained after centrifugation of yeast suspension which had been left for 15 minutes at room temperature showed reduction equivalent to 0.05 to 0.07 cc. of 0.005 N. sodium thiosulfate, which, according to Figure II, is equivalent to less than 0.005 mg. of glucose, which may be considered negligible.

Blanks on the reagents and filter paper were also run. 10 cc. of distilled water was substituted for the 10 cc. of urine used in the precipitation procedure, and the determination was carried out in detail as though urine were being analyzed. A portion of the filtrate was hydrolyzed, and the amount of reduction determined as though it were a urine filtrate. Sugar estimations were run in duplicate. Table I shows the amount of reduction obtained in a cc. of 0.005 N. sodium thiosulfate. It may be seen that a slight amount of reduction was obtained in some cases. However, the amount is below that which may be taken as the lower limits of the accuracy of the reagent.

Table I

	Fermentable reducing material		Non-fermentable reducing material	
	Barium	Lead	Barium	Lead
Filtrate	.06	0	0	0
Hydrolyzed filtrate	.06	.11	0	0

Reduction expressed in cc. of 0.005 N. sodium thiosulfate

EXPERIMENTAL RESULTS

1. Normals

Table No. II indicates in mg. of glucose per 24 hours the amount of reduction obtained after the various procedures were carried out. In these cases, the ferric sulfate-barium carbonate-Lloyd's reagent and ferric sulfate-lead carbonate-Lloyd's reagent filtrates were used. In further discussions the terms "lead" and "barium" will refer to these two precipitation methods. In each case, the two procedures (lead and barium) were run simultaneously in order to further compare their effectiveness as precipitating agents.

The results, averaged, differ somewhat from those reported previously (60) in that there is no increase in non-fermentable reducing material in hydrolyzed filtrates or in filtrates of hydrolyzed urine which were precipitated by ferric sulfate-lead carbonate-Lloyd's reagent. Value for non-fermentable reducing substances are lower when the urine is treated with lead. With both types of precipitation procedures, there is a marked increase in fermentable sugar in hydrolyzed filtrates and filtrates from hydrolyzed urine. This increase was most marked in the filtrates of hydrolyzed urine treated with barium and in the hydrolyzed filtrates precipitated by lead. Also contrary to expectation, the amount of fermentable sugar in the lead filtrates is slightly higher than it is in the barium filtrates. One almost constant finding is a decrease in the non-fermentable fraction observed in filtrates prepared from hydrolyzed urine precipitated by either method. This corroborates the findings of

Table II

Subject	BARIUM						LEAD					
	Filtrate		Hydrolysed Filtrate		Filtrate of Hydrolysed Urine		Filtrate		Hydrolysed Filtrate		Filtrate of Hydrolysed Urine	
	Ferm.	Non-Ferm.	Ferm.	Non-Ferm.	Ferm.	Non-Ferm.	Ferm.	Non-Ferm.	Ferm.	Non-Ferm.	Ferm.	Non-Ferm.
WB	44	241	208	254	241	204	34	309	61	501	112	381
WB	24	186	354	44	127	34	49	308	303	210	210	282
RG	116	117	435	200	517	33	133	0	534	26	414	0
RG	58	289	339	382	366	239	58	166	486	0	351	58
RG	41	216	143	459	426	41	82	74	519	0	410	0
Av.	58	209	295	268	335	110	71	170	380	147	299	144

Expressed in mg. of glucose per 24-hr. sample.

Laug and Nash (61), who determined the fermentable and non-fermentable reducing substances in normal urine after hydrolyzing the urine for varying periods of time. They found a slight increase in non-fermentable reducing material after about one hour's hydrolysis, but when the samples were hydrolyzed for longer periods of time, there was noted a progressive decrease in the amount of the material. After 3 to 4 hours, the amount of non-fermentable reducing substance became constant at a level below that of the unhydrolyzed sample.

It should be especially noted that samples obtained from any one subject behaved similarly under both methods of treatment (barium and lead), but there may be wide differences in the manner in which samples from different persons react. These differences are particularly marked when the effects of hydrolysis upon the non-fermentable reducing fraction are studied. In the samples obtained from R. G. frequently no non-fermentable reducing material could be demonstrated, even after hydrolysis. These facts suggest that the personal variations which may be related to the type of food and drink ingested may be very important.

2. Diabetic Urines

These samples of diabetic urine were obtained at random from patients hospitalized at the Multnomah County Hospital. They were portions of 24-hour urine samples kept under toluene. Table III shows in mg. of glucose per 24 hours the amount of reduction found before and after hydrolysis of filtrates obtained by treatment with lead and with barium. Since the samples were taken at random and some patients were controlled more strictly than others, many showed relatively large amounts of sugar in the urine, while others excreted

Table III

Subject	BARIUM						LEAD					
	Filtrate		Hydrolyzed Filtrate		Filtrate of Hydrolyzed Urine		Filtrate		Hydrolyzed Filtrate		Filtrate of Hydrolyzed Urine	
	Ferm.	Non-Ferm.	Ferm.	Non-Ferm.	Ferm.	Non-Ferm.	Ferm.	Non-Ferm.	Ferm.	Non-Ferm.	Ferm.	Non-Ferm.
M	961	119	1065	283	974	201	974	48	989	158	1009	110
M	2690	212	2470	508	2418	308	2618	116	2837	120	2442	199
L	0	64	50	112	0	108	40	8	50	0	10	128
C	2859	347	4165	2569*	5448	603	2905	297	6092	432	5528	429
L	0	311	484	124	533	180	0	262	434	61	492	352
L	0	167	167	197	304	57	19	76	243	114	311	19
	340	147	417	108	524	144	442	91	412	96	512	153
L	0	97	0	139	69	129						
	328	639	255	925	346	316						
	129	278	407	426	444	347						
	1756	595	2770	1058			1920	368	1035	352		
	6	188	205	382			82	121	154	142		
Average	756	247	1038	578	1106	239	1000	154	1360	149	1472	197

very little. For this reason, averages of the results are not too significant. It is better that each case should be noted individually. In general, however, certain observations may be made.

First, contrary to the experience of Dittebrandt, Tenney, and West (62), in almost every case there is an increase in the amount of fermentable sugar following hydrolysis of both lead and barium filtrates and urine. The amounts of fermentable sugar in lead and in barium filtrates is comparable, perhaps slightly higher in the lead filtrates. The increase in fermentable sugar following hydrolysis is approximately the same in both procedures. Again it must be noticed that urine samples obtained from one patient seem to react similarly to precipitation and to hydrolysis. In the four samples obtained from L., no fermentable sugar could be detected in the filtrate following barium precipitation. The amounts of non-fermentable reducing substances in the filtrates, in hydrolyzed filtrates, and in filtrates prepared from hydrolyzed urine are all in the same general range and seem to have no relationship to the amount of fermentable sugar which is present. There is one exception to the above, noted by the asterisk. This figure is not included in the averages since it is not compatible with the other results, and is probably in error. Following barium precipitation there was a marked increase in the non-fermentable reducing portion of the hydrolyzed filtrate and a lesser increase in this fraction of the filtrate of hydrolyzed urine. These increases are less marked following precipitation with lead. The non-fermentable reducing portion is slightly lower in amount following lead precipitation than barium.

The patients included in Table IV are insulin sensitive; that is, they cannot be kept sugar-free by treatment with insulin without bringing about insulin reactions or hypoglycemia. They react to insulin as do other diabetics up to the point at which they approach normal blood sugar levels. At this point, however, they abruptly go into insulin shock. For this reason, urine from these cases contains greater amounts of sugar than do the previously discussed cases. Because of this peculiar reaction, it was thought that some difference in the excretory products might be found. However, no constant variation can be found in the results of this study. Only the lead precipitation method was utilized. There is an increase in fermentable sugar in most of these cases following hydrolysis as has been shown above for diabetic patients who can be controlled satisfactorily with insulin. The unusually large amount of non-fermentable reducing material in the urine from F. is possibly an error in analysis, since it does not agree with the other values. In two cases there is a decrease in non-fermentable reducing substances after hydrolysis--the value being approximately halved, and in the other two--an increase--the value being doubled. This permits no conclusions to be drawn. Further study of these patients would have been desirable, but, unfortunately, they were discharged from the hospital at this time, and no others were available.

Table IV

Subject	Filtrate		Hydrolyzed Filtrate		Filtrate of Hydrolyzed Urine	
	F.	N.F.	F.	N.F.	F.	N.F.
Robert	8880	416	13840	944	13200	984
Robert	8272	308	10912	646	8976	642
Robert	3622	379	3984	116	3928	167
Fenton	20232	3055	21312	1101	16072	957
Average	10251	1039	12512	701	10544	687

DISCUSSION

The primary consideration of this study is with the effects of hydrolysis on diabetic as compared with normal urine. Secondarily, some information may be gained by comparing reducing values obtained with two methods of precipitation--the ferric sulfate-barium carbonate-Lloyd's reagent and the ferric sulfate-lead carbonate-Lloyd's reagent. Few theoretical conclusions can be drawn from the latter inasmuch as the differences in mode of action are not understood. Indeed, little enough is known of the nature of the non-fermentable fraction itself. Again we must recall that average values in the series may be misleading and that more information may be gained by considering the cases individually. However, for this discussion the conclusions will be made as general as possible. First, it will be noted that in filtrates of hydrolyzed urine and in hydrolyzed filtrates there is, generally, a noticeable increase in fermentable reducing material, showing the probable presence of polysaccharides or other carbohydrate compounds convertible into fermentable sugars. This fact has been definitely shown. However, the problem becomes more complex in attempting to analyze the results of hydrolysis upon the non-fermentable fraction. In the normal series the non-fermentable portion is lowered in the hydrolyzed urine. This is often true in barium-precipitated hydrolyzed diabetic urine, but there is more often a slight increase in this fraction in the lead-precipitated diabetic samples. In the normal and in the diabetic, the hydrolyzed filtrate gives higher values for non-fermentable reducing material than do filtrates of hydrolyzed urine.

In almost all cases, both normal and diabetic, non-fermentable values are lower with lead than with barium. However, fermentable values in both normal and diabetic are higher in lead than in barium filtrates. In uncontrolled diabetics the non-fermentable fraction is generally a little higher than in controlled diabetics or normals.

The differences in non-fermentable reducing fractions of diabetic and normal urines suggest small differences in composition. Diet must play a large part in urinary composition (63), (64), and as none of these subjects' diets were controlled, differences may be expected. Also, there may be present inherent individual variations, as noted before, with both normal and diabetic subjects. There seems to be no more plausible explanation than this for the consistent absence of or lowering of either fermentable or non-fermentable sugar in an individual case.

It has been shown that the non-fermentable reducing materials of urine represent a complex group of substances, including some nitrogenous compounds, such as creatinine, creatine, and uric acid. Since the precipitation methods used in this research remove much of this group of nitrogenous reducing substances, the reducing materials concerned must have been composed largely of carbohydrates or their derivatives. The role of lactose and galactose is uncertain, but may be important in some cases. The range of excretion of these substances is not known definitely. Dittebrandt (65) has shown that they are not removed by precipitating agents, although recovery is less than that of glucose. Lactose may be hydrolyzed to galactose and glucose, and thus give rise to an increase in fermentable sugar almost always found

following hydrolysis of both normal and diabetic urine. Another possibility is the presence of complex carbohydrate structures so changed by the high temperatures of cooking processes that they cannot be utilized and are excreted in the urine. These may conceivable be broken down by hydrolysis to fermentable and non-fermentable units. No such compounds have thus far been isolated. Therefore, it may be seen that at least three systems may contribute to the non-fermentable reducing fraction of urine. The importance of each may vary with diet and method of treatment of the urine before sugar determination

It has also been suggested that the dilution factor may be important in determining the efficiency of precipitating agents. If this were the case, it would be necessary to introduce still another variable into the picture. None of the urine volumes in this series was larger than 2000 cc., but non-fermentable reducing values were somewhat lower in cases with large urine volumes.

Hydrolysis of filtrates and hydrolysis of urine before precipitation brings out differences which are difficult to understand and are in no way constant. Much of the non-fermentable substances should have been eliminated by precipitation before hydrolysis, but often these hydrolyzed filtrates of both normals and diabetics showed larger amounts of non-fermentable material than filtrates which had not been hydrolyzed. This suggests the presence of substances not precipitated from urine and present in the filtrates which yielded non-fermentable reducing substances upon hydrolysis. Normal urines which were hydrolyzed before precipitation showed non-fermentable

values below those of filtrates of unhydrolyzed urine, suggesting decomposition of non-fermentable precursors. In diabetic urines there were more often increases in these values, particularly following treatment with lead.

More to the point in this study, it may be seen that no startling differences between normal and diabetic urine are evident as far as reducing materials are concerned. It is difficult to account for the variations which do occur, in the light of our limited knowledge concerning the identity of the carbohydrate materials of urine.

CONCLUSIONS

1. Fermentable and non-fermentable reducing materials have been determined in iron filtrates prepared from urine and hydrolysed urine. Also these substances have been estimated in the hydrolysed iron filtrates of urine. Urines from both normal and diabetic subjects have been analysed.

2. Filtrates of hydrolysed urine and hydrolysed filtrates of urine of both normal and diabetic subjects contained more fermentable reducing substances than did unhydrolyzed filtrates.

3. The differences observed in non-fermentable reducing substances in normal and diabetic urines before and after hydrolysis are variable and may be related to diet, urine volume, inherent individual variation, and the degree of control with which the diabetic patient is maintained.

4. Ferric sulfate-lead carbonate-Lloyd's reagent filtrates gave lower values for non-fermentable substance than did ferric sulfate-barium carbonate-Lloyd's reagent filtrates in most cases.

5. Ferric sulfate-lead carbonate-Lloyd's reagent filtrates in general gave slightly higher values for fermentable sugar than did ferric sulfate-barium carbonate-Lloyd's reagent filtrates.

BIBLIOGRAPHY

1. MacLean, H. On some applications of safranin as a test for carbohydrates. Biochem. J., vol. 2, pp. 431-442, 1907.

2. Schultz, 1910. Cited in #16.

3. Gilbert and Baudouin. J. Physiol. et Path. Gen, vol. 8, p. 596, 1911. Cited in #16.

4. Benedict, S. R. and Osterburg, E. A method for the determination of sugar in normal urine. J. Biol. Chem., vol. 34, pp. 195-201, 1918.

5. Benedict, S. R. and Osterburg, E. Studies in carbohydrate metabolism. I. A preliminary report on the sugar elimination in the urine of the normal dog. J. Biol. Chem., vol. 34, pp. 209-216, 1918.

6. Benedict, S. R. Studies in carbohydrate metabolism. II. A study of the urinary sugar excretion in two normal men. J. Biol. Chem., vol. 34, pp. 217-262, 1918.

7. Schaffer, P. A. and Hartmann, A. The iodometric determination of copper and its use in sugar analysis. II. Methods for the determination of reducing sugar in blood, urine, milk, and other solutions. J. Biol. Chem., vol. 45, pp. 365-389, 1920.

8. Folin, O. and Berglund, H. Some new observations and interpretations with reference to the transportation, retention, and excretion of carbohydrates. J. Biol. Chem., vol. 51, pp. 213-273, 1922.

9. Benedict, S. R. and Osterburg, E. Sugar elimination after the sub-cutaneous injection of glucose in the dog. J. Biol. Chem., vol. 55, pp. 769-794, 1923.

10. Host. J. Metab. Research, vol. 4, p. 315, 1923. Cited in #16.

11. <u>Op. cit.</u>, 4.

12. Lund, G. S. and Wolf, C. G. The glucose content of normal urine. Biochem. J., vol. 19, pp. 538-540, 1926.

13. Patterson, J. The carbohydrate of normal urine. Biochem. J., vol. 20, pp. 651-655, 1926.

14. Eagle, H. S. On the nature of urine sugars. J. Biol. Chem., vol. 71, pp. 481-495, 1927.

15. Greenwald, I., Gross, J., and Samet J. On the nature of sugar in normal urine. I. A comparison of the glucose equivalent of various sugars in different methods for the determination of glucose. J. Biol. Chem., vol. 62, pp. 397-399, 1924.

16. Greenwald, I., Gross, J., and Samet, J. The nature of sugar in normal urine. II. The sugar excretion upon various diets and the influence of diet upon glucose tolerance with some remarks on the nature of the action of insulin. J. Biol. Chem., vol. 62, pp. 401-434, 1924.

17. Op. cit., 8.

18. Greenwald, I., Gross, J., and McGuire, G. Observations on the sugar of normal urine. J. Biol. Chem., vol. 75, pp. 491-508, 1927.

19. Van Slyke, D. D. and Hawkins, J. A. Gasometric determination of fermentable sugar in blood and urine. J. Biol. Chem., vol. 83, pp. 51-70, 1929.

20. Somogyi, M. Reducing non-sugars and true sugars in human blood. J. Biol. Chem., vol. 75, pp. 33-43, 1927.

21. Everett, M. R. and Sheppard, F. Sugar of normal urine. J. Biol. Chem., vol. 87, p. XXXV, 1930.

22. Everett, M. R. and Sheppard, F. The ketose of normal urine. J. Biol. Chem., vol. 92, p. XXV, 1931.

23. West, E. S. Reducing substances in urine. J. Biol. Chem., vol. 92, p. XXIV, 1931.

24. West, E. S., Scharles, F. H., and Peterson, V. L. The determination of true sugar in blood. J. Biol. Chem., vol. 82, pp. 137-153, 1929.

25. Peterson, V. L. and West, E. S. Reducing fermentable and non-fermentable substances in normal and pathological urine. Am. J. Phys., vol. 90, p. 470, 1929.

26. Harding, V. J. and Selby, D. L. Fermentable sugar in normal urine. Biochem. J., vol. 25, pp. 1815-1838. 1931.

27. Op. cit., 20.

28. West, E. S., Lang, A. C., and Peterson, V. L. The sugars of urine. II. Factors affecting the excretion of fermentable and non-fermentable sugars in urine. Biochem. J., vol. 26, pp. 1728-1741, 1932.

29. Op. cit., 6.

30. West, E. S. and Peterson, V. L. The sugars of urine. I. Determination of the reducing sugars of urine. Biochem. J., vol. 26, pp. 1720-1727, 1932.

31. West, E. S. and Steiner, A. The sugars of urine. III. Nature of fermentable sugar of normal and starvation urine. Biochem. J., vol. 26, pp. 1742-1749, 1932.

32. West, E. S., Norris, P. C., Steiner, A. Nature of the fermentable sugar in normal urine. J. Biol. Chem., vol. 97, p. LXXXII, 1932.

33. Harding, V. J. and Selby, D. L. Fermentable sugar in fasting urine. Biochem. J., vol. 27, pp. 1598-1608, 1933.

34. Op. cit., 30.

35. Harding, V. J. and Nicholson, T. F. The use of some micro-organisms in sugar analysis. Biochem. J., vol. 27, pp. 1082-1094, 1933.

36. Everett, M. R., Edwards, B. G., Sheppard, F. The nature of sugar of normal urine. II. The behavior of carbohydrates in bromine water and the ketose of normal urine. J. Biol. Chem., pp. 11-38, 1934.

37. Lang, E. P. and Nash, T. P. Observation on the nature of reducing sugars in dog urine. J. Biol. Chem., vol. 108, pp. 479-486, 1935.

38. Op. cit., 30.

39. Op. cit., 28.

40. Harding, V. J., Nicholson, T. F., and Jackson, R. M. Effect of meals on hydrolyzable sugar and evidence as to the nature of hydrolyzable sugar. Biochem. J., vol. 30, pp. 335-341, 1936.

41. Harding, V. J., Nicholson, T. F., and Archibald, R. M. Nature of free fermentable sugars and fermentable sugars produced on hydrolysis in fasting urines. Biochem. J., vol. 30, pp. 326-334, 1936.

42. Op. cit., 37.

43. Nicholson, T. F. and Archibald, R. M. Observations on the nature of non-fermentable reducing substances in fasting urine. Biochem. J., vol. 33, pp. 516-522, 1939.

44. Dittebrandt, B. M., Tenney, M. and West, E. S. Reducing substances in urine. J. Biol. Chem., vol. 152, pp. 395-400, 1944.

45. Op. cit., 37.

46. Patein, E. and Dufau, E. J. Pharm. et Chem., vol. 15, p. 221, 1902. Cited by M. B. Dittebrandt. Unpublished M. A. thesis, U. of Oregon Medical School, 1938.

47. Op. cit., 4.

48. Op. cit., 7.

49. Op. cit., 13.

50. Op. cit., 8.

51. Op. cit., 19.

52. Op. cit., 24.

53. Harding, V. J. and Downs, C. E. Notes on a Shaffer-Somogyi copper reagent. J. Biol. Chem., vol. 101, p. 487-492, 1933.

54. Steiner, A. Urban, F., and West, E. S. Iron and thorium precipitation of biological fluids for sugar and other analyses. J. Biol. Chem., vol. 98, pp. 289-297, 1932.

55. Curtis, G. H., Lane, R. A., and West, E. S. Precipitating agents for use in the estimation of sugars in biological materials. J. Biol. Chem., vol. 109, pp. XCVII-XCVIII, 1935.

56. Op. cit., 44.

57. Op. cit., 20.

58. Shaffer, P. A. and Somogyi, M. Copper-iodometric reagents for sugar determinations. J. Biol. Chem., vol. 100, pp. 695-713, 1933.

59. Somogyi, M. A reagent for the copper-iodometric determination of of very small amounts of sugar. J. Biol. Chem., vol. 117, pp. 771-780, 1937.

60. Op. cit., 44.

61. Op. cit., 37.

62. Op. cit., 44.

63. Op. cit., 28.

64. Op. cit., 40.

65. Dittebrandt, M. B. Unpublished M. A. Thesis. U. of Oregon Medical School, 1938.