CCIII. THE SUGARS OF URINE.

I. DETERMINATION OF THE REDUCING SUGARS OF URINE.

BY EDWARD STAUNTON WEST
AND VERNON LESLIE PETERSON.

*From the Laboratory of Biological Chemistry, Washington University
School of Medicine, Saint Louis, Missouri.*

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HARDING AND SELBY [1931] have recently published extensive observations on the output of fermentable sugar in normal urine. Their report prompts us to record some findings made in this laboratory during recent years. Our plan has been to study the daily excretion of both fermentable and non-fermentable sugar by normal individuals and with these data for comparison to investigate various factors which influence the excretion.

As will be noted from the introduction of the paper by Harding and Selby the question of a suitable method of analysis is paramount in an investigation of this kind. This is especially true when determination of non-fermentable as well as fermentable sugar is involved. Here the efficient pre-treatment of the urine to remove non-sugar reducing substances is imperative. Precipitation with mercuric nitrate-sodium bicarbonate according to Benedict and Osterberg [1918] is probably the most efficient treatment which has been reported in the past, yet this method has the disadvantage of leaving a high salt concentration in the filtrate and there is danger of adding too much bicarbonate with resulting oxidation of sugar by the mercuric ion. Treatment with Lloyd's reagent which was introduced by Folin and Berglund [1922] and which has been used in various ways by different workers, according to our experience, leaves much non-sugar reducing material. In order that maximum accuracy be obtained it is also advisable to use a sugar method capable of estimating small quantities.

Preliminary to their studies, the writers have developed a procedure of analysis for fermentable and non-fermentable sugar in urine which seems to them to represent an improvement over former methods. It is based upon three procedures. Filtrates of urine are prepared by the HgSO_4 - BaCO_3 technique of West, Scharles and Peterson [1929] which has been found effective in removing interfering nitrogenous substances from biological fluids without introducing appreciable amounts of electrolytes and without loss of sugar.

The reducing substances are determined in these filtrates before and after yeast fermentation, using modified Shaffer-Hartmann copper reagents, made much more sensitive than the original due mainly to omission (or decrease) of KI. The reagents used are the outgrowth of unpublished work by Drs Shaffer and Somogyi. The suitability of such reagents for the determination of low concentrations of sugar has been shown also by Harding and Downs [1929], Harding and Van Nostrand [1930] and Harding and Selby. Yeast fermentation is carried out with washed yeast prepared according to Somogyi's [1927] directions. We believe that this combination gives more nearly correct reducing values for fermentable and non-nitrogenous non-fermentable reducing substances (possibly of carbohydrate nature), than has been achieved by other procedures.

This paper reports in detail the analytical methods used and the daily excretion of fermentable and non-fermentable sugars in the urines of a number of normal human subjects. A second paper has to do with some of the factors which influence the excretion of these substances.

Reagents.

Mercuric sulphate. A saturated solution in 10 % H_2SO_4 prepared by dissolving 27 g. HgSO_4 per 100 cc. of cold acid (5–10°). The solution is decanted from the basic sulphate which separates as the solution comes to room temperature.

*Barium carbonate*¹. Mallinckrodt's technical precipitated BaCO_3 , dried.

Yeast. Fleischmann's yeast washed and kept as recommended by Somogyi.

Sugar reagent. Modified Shaffer-Hartmann containing 1 g. KI per litre, kept in pyrex flasks².

Urine. Urine samples were preserved with a little toluene and kept in the cold room.

Procedure of analysis.

Preparation of filtrates. 10 cc. of urine are pipetted into a 500 cc. Erlenmeyer flask, together with 45 cc. of water, and 15 cc. of mercuric sulphate reagent are added from a burette. To the mixture are added 35–40 g. of BaCO_3 with vigorous rotation for a few seconds until most of the CO_2 has escaped. The flask is tightly closed with a rubber stopper, shaken hard and the pressure released. This is repeated until no more pressure develops. If the mixture reddens blue litmus, a little more BaCO_3 is added and the shaking repeated. After neutralisation the contents of the flask are poured upon a rapid filter and the filtrate (about 25 cc.) acidified with 1 large drop of conc. H_2SO_4 .

¹ One or two samples of BaCO_3 have been found to contain something which inhibits the action of yeast. The interfering substance is not removed by H_2S in acid solution.

² Details of a number of improved reagents will be published shortly in a joint paper by Drs Shaffer and Somogyi. The reagent used by the writers had the following composition in g. per litre: Na_2CO_3 (anhydrous), 25.0; NaHCO_3 , 20.0; Rochelle salt, 25.0; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 7.5; KI, 1.0; KIO_3 , 20.0 cc. of *N* sol. per litre.

The filtrate, in a 300 cc. Erlenmeyer flask, is treated with H_2S for several seconds and the H_2S removed by a rapid current of moist air. The precipitated HgS and BaSO_4 are filtered off on a small paper and the filtrate received in a large test-tube. The funnels and tubes used must not be contaminated by previous use with solutions containing mercury salts. It is convenient to keep a supply of funnels and tubes especially for this filtration to prevent poisoning the yeast with Hg -contaminated vessels. The time required for the preparation of filtrates is only a little greater than that consumed in making Folin-Wu blood-filtrates. The filtrations proceed rapidly. The urine-filtrates so obtained represent a dilution of 1 : 7. Greater concentration cannot well be achieved. Dilutions up to 1 : 12 give quite satisfactory results when the urines themselves are not too dilute.

Determination of reducing substances. The filtrate obtained by the above method is divided into two approximately equal portions (12–14 cc. each). About 7 cc. of a 15 % suspension of washed yeast are placed in a 15 cc. pointed centrifuge-tube and the yeast centrifuged down firmly. Supernatant liquid is poured off and the walls of the tube dried with a roll of filter-paper. One portion of filtrate is added to the yeast, mixed and allowed to ferment for 10 minutes at room temperature, the yeast being occasionally stirred. After centrifuging the yeast the fermented and unfermented filtrates are analysed for reducing substances as follows. 5 cc. portions, in duplicate, are measured into 25×200 mm. pyrex tubes, a drop of 0.05 % aqueous phenol red added to each, and 0.5 *N* NaOH drop by drop with shaking to the red colour of the indicator. 5 cc. of copper reagent are added, the tubes covered with glass bulbs, and heated in a steady rack in an actively boiling water-bath for 15 minutes. The tubes are then cooled to about 30° , 1 cc. of a solution containing 4 % KI and 5 % potassium oxalate is added followed by 5 cc. of *N* H_2SO_4 . Upon acidification the tubes are thoroughly shaken (bulbs still in place), until all Cu_2O is dissolved, and permitted to stand for 10 minutes. They are then rinsed down, including sides of covering bulbs, with a fine stream of H_2O from a wash-bottle (3–5 cc.) and titrated with 0.005 *N* thiosulphate, adding a solution of starch towards the end. Heated blanks are run on distilled water (containing phenol red). The latter does not affect the reduction and improves the end-point of the iodimetric titration for most people.

The method of calculating results will be illustrated by an example. Suppose the sugar reagent used was such that 1 cc. of 0.005 *N* thiosulphate titration was equivalent to 0.113 mg. of glucose. The total concentration of reducing substances (as glucose) in mg. per 100 cc. for a titration difference of 3.10 cc. of 0.005 *N* thiosulphate on the unfermented filtrate would be: $3.10 \times 0.113 \times 7 \times 20 = 49$. A titration difference of 2.0 cc. on the fermented filtrate would represent: $2.0 \times 15.8 = 32$ mg./100 cc. total non-fermentable reducing substances (as glucose). $49 - 32 = 17$ mg. per 100 cc. fermentable (as glucose).

Discussion of the method.

Filtrates prepared from ordinary urines by $\text{HgSO}_4\text{-BaCO}_3$ precipitation contain about 2–3 mg. of N per 100 cc. of filtrate so that the N content of samples for analysis amounts to 0.1–0.15 mg. Urea, which does not interfere with the sugar determinations, represents nearly all the nitrogen. Urines from high protein diets may give mercury filtrates containing many times this amount of nitrogen as a result of the great increase in urea. Such filtrates show normal values for both fermentable and non-fermentable substances. Pure urea solutions were treated with $\text{HgSO}_4\text{-BaCO}_3$, and the nitrogen of the filtrates determined. The precipitations were made at a volume of 70 cc. as in the case of urine, the amount of material specified being present. 2.76 mg. of N remained in the filtrate of a solution originally containing 45 mg. of urea-N, 8.26 mg. in one containing 90 mg. and 16.7 mg. in the filtrate of a solution to which 270 mg. of urea-N had been added. Creatinine, creatine and uric acid removal were tested similarly. Using 3.75 mg. of creatinine-N (10.16 mg. creatinine) no detectable nitrogen was present in the filtrate (10 cc. digested). 7.5 and 15 mg. of creatinine-N gave filtrates containing respectively 0.68 and 1.88 mg. of unprecipitated N. Of 4.23, 8.46 and 12.69 mg. of creatine-N in solution precipitated with $\text{HgSO}_4\text{-BaCO}_3$, 0.71, 1.94 and 4.7 mg. respectively were found in the filtrates. In a like manner 0.23 and 0.29 mg. N remained in filtrates of solutions containing originally 2 and 3.6 mg. of uric acid-N respectively. As would be expected, uric acid and creatinine are practically undetectable in $\text{HgSO}_4\text{-BaCO}_3$ filtrates of urine.

It is probable that mercuric sulphate and mercuric nitrate precipitations are about equally efficient as far as the removal of interfering substances is concerned, when BaCO_3 is used as a neutralising agent, though the $\text{Hg}(\text{NO}_3)_2\text{-BaCO}_3$ filtrates of urine give somewhat higher titration values than do the $\text{HgSO}_4\text{-BaCO}_3$ filtrates, probably owing merely to the influence of their high electrolyte concentration on the oxidising equivalence of the copper reagent. Benedict and Osterberg [1918] reported practically no nitrogen in their $\text{Hg}(\text{NO}_3)_2\text{-NaHCO}_3$ filtrates. We have found it impossible to determine the organic nitrogen in such filtrates. In the Kjeldahl process of digestion the nitrous acid formed from the nitrate liberates free N_2 from amino-groups and ammonium salts. We have observed complete loss of N in the analysis of urea and glycine caused by adding nitrate during the digestion. It is probable, however, that the nitrogen contents of the $\text{Hg}(\text{NO}_3)_2$ and HgSO_4 filtrates are of the same order. It should be pointed out that BaCO_3 is superior to NaHCO_3 as a neutralising agent for HgSO_4 not only because it yields filtrates of very low electrolyte content, but also because with it there is no danger of developing local alkalinity within the solution being neutralised with consequent loss of sugar as a result of oxidation by the mercuric ion.

The effectiveness of the $\text{HgSO}_4\text{-BaCO}_3$ treatment in removing interfering uric acid and creatinine from solution and giving filtrates which show true

sugar values was demonstrated in the following way. A mixture containing 90 mg. of urea-N, 3.75 mg. of creatinine-N, and 2 mg. of uric acid-N in a volume of 70 cc. gave, without pre-treatment, reduction equivalent to a total of 4.3 mg. of glucose. 7.0, 3.5 and 1.4 mg. of glucose were added to this mixture and precipitated at a volume of 70 cc. as in the case of urine. By analysis 7.1, 3.6 and 1.66 mg. of fermentable sugar respectively were recovered. Titrations of the fermented filtrates were identical with those of the blanks on water. As has long been known, it is essential to remove creatinine and uric acid from urine in order to obtain accurate reduction values for non-nitrogenous non-fermentable substances. $\text{HgSO}_4\text{-BaCO}_3$ precipitation effectively removes sulphate and phosphate, which may be found advantageous in certain analyses. Glucose added to $\text{HgSO}_4\text{-BaCO}_3$ filtrates is quickly and quantitatively fermented by yeast.

Sugar added to urine is quantitatively recovered by analysis of the $\text{HgSO}_4\text{-BaCO}_3$ filtrates.

We have used varying quantities of yeast for fermentation and failed to note absorption of non-fermentable reducing substances from $\text{HgSO}_4\text{-BaCO}_3$ filtrates of urine. Van Slyke and Hawkins [1929] report absorption from Lloyd's reagent filtrates which were analysed by their ferricyanide-gasometric method. Harding and Van Nostrand [1930] found no absorption from such filtrates using essentially the same sugar reagent as we have employed. The results of Van Slyke and Hawkins are perhaps due to their ferricyanide reagent oxidising yeast-absorbable substances which are unaffected by the copper reagent.

Comparison with Folin's method.

It is conceivable that yeast may remove some iodine-absorbing substance which does not reduce copper and which is not sugar, yet, by the method used, would be calculated as fermentable sugar. Accordingly, it was considered desirable to establish the presence of copper-reducing material, utilisable by yeast, by a method not involving iodimetric titration and also to secure data for a comparison of our method with another. It should be pointed out in this connection that $\text{HgSO}_4\text{-BaCO}_3$ filtrates do not absorb iodine from an acid solution, which in itself suggests the improbability of such an error.

Fermentable and non-fermentable reducing substances were determined on both $\text{HgSO}_4\text{-BaCO}_3$ and Lloyd-permutit filtrates of urine by the sensitive Shaffer-Hartmann reagent and by Folin's [1929] reagent. The Lloyd-permutit filtrates were prepared according to Folin and Svedberg [1926]. Table I summarises the data obtained on 24-hour samples of 11 normal individuals, the fermentable and non-fermentable substances being expressed as mg. per 24-hour sample (glucose reducing equivalent). The first column also includes determinations on diluted (1:10; 1:15 for very concentrated urine) untreated urines by the Shaffer-Hartmann method for comparison.

It will be observed that the average non-fermentable reducing material was 1246 mg. (as glucose) in untreated urine, 581 mg. in Lloyd-filtrates, and

Table I. *Fermentable and non-fermentable reducing substances of urine in untreated urine, $\text{HgSO}_4\text{-BaCO}_3$ and Lloyd-permutit filtrates, determined by Shaffer-Hartmann and Folin methods.*

Results expressed in terms of mg. glucose per 24-hour urine.

Sample No.	Shaffer-Hartmann						Folin			
	Diluted untreated urine		$\text{HgSO}_4\text{-BaCO}_3$		Lloyd		$\text{HgSO}_4\text{-BaCO}_3$		Lloyd	
	Ferm.	Non-ferm.	Ferm.	Non-ferm.	Ferm.	Non-ferm.	Ferm.	Non-ferm.	Ferm.	Non-ferm.
1	123	1895	116	370	125	800	61	362	140	425
2	72	1310	66	304	85	560	66	270	60	406
3	55	1005	73	265	80	496	46	111	50	264
4	82	1270	72	387	66	675	59	351	53	492
5	86	770	118	244	121	428	106	210	94	286
6	41	1163	89	240	84	502	91	211	72	300
7	212	722	104	250	135	545	170	148	138	327
8	85	1465	94	278	119	585	105	237	95	353
9	41	1245	91	260	73	545	39	283	47	351
10	176	1682	119	407	130	813	128	382	164	516
11	52	1180	61	202	72	443	78	144	78	278
Min.	41	722	61	202	66	428	39	111	47	264
Max.	212	1895	119	407	135	813	170	382	164	516
Av.	93	1246	91	292	99	581	86	246	90	362

292 in the $\text{HgSO}_4\text{-BaCO}_3$ filtrates, all by the Shaffer-Hartmann method. Lloyd treatment removed 53 % of the total non-fermentable reducing material and $\text{HgSO}_4\text{-BaCO}_3$ treatment removed 76 %. An interesting point is that, whereas the average difference in reduction equivalent for non-fermentable reducing substances in mercury filtrates by the Shaffer-Hartmann and Folin methods is 16 % (292-246) the difference in Lloyd filtrates is 38 % (581-362). This suggests a decided chemical difference between the reducing material not removed by Lloyd treatment but taken out by mercury and that remaining in the mercury filtrates. If the latter is essentially carbohydrate in character, the former is probably not, and Lloyd filtrates are not to be recommended in studies of the non-fermentable sugar of urine.

The average values for fermentable sugar by the Shaffer-Hartmann method were 93 mg. in untreated urine, 91 mg. in mercury filtrates and 99 mg. in Lloyd filtrates. By the Folin method the averages were 86 mg. in mercury and 90 mg. in Lloyd filtrates. The averages are rather close together but it will be noted that the individual results on mercury filtrates by the Shaffer-Hartmann method show less range of variation than those by any of the other combinations. We have found it possible to duplicate our results by this method more satisfactorily also.

Reducing substances in normal human urine.

Determinations of the daily output of fermentable and non-fermentable reducing substances have been made on a large number of medical students and laboratory workers. The maximum output of fermentable in 55 cases

was 596 mg., the minimum 23 mg. and the average 142 mg. per day calculated as glucose. The average concentration was 12 mg. per 100 cc. with an average urine volume of 1216 cc. The diet was not controlled. The amount of fermentable material varies widely yet it is probably always present. Our results agree, in general, with those of Van Slyke and Hawkins, and Harding and Selby in so far as the results can be compared. These workers analysed samples of urine taken at definite periods during the day and after the ingestion of various foods and did not report the results on 24-hour samples. Harding and Selby observed the absence of fermentable sugar from urine after short periods of starvation. By our method we have always found fermentable sugar in starvation urine though in decreased amounts. The failure of Harding and Selby to find fermentable sugar in starvation urines is probably due to the fact that they used more highly diluted urines, a smaller sample for analysis (2 cc. instead of 5 cc.), a somewhat less sensitive sugar reagent and a shorter time of heating than did the writers. To illustrate, assuming a minimum dilution of 1 : 10 as used by these workers (their maximum dilution was 1 : 20), a urine containing 6 mg./100 cc. of glucose would give a titration difference of about 0.08 cc. of 0.005 *N* thiosulphate, and if the urine dilution had been 1 : 20 the titration difference would be only 0.04 cc., very close to their normal error of titration. The same urine analysed by our procedure would give a titration difference of about 0.37 cc. of 0.005 *N* thiosulphate, nearly five times that by the procedure of Harding and Selby using a 1 : 10 dilution. Other points relating to fermentable sugar are considered in the second paper.

The advisability of using a sensitive sugar reagent in detecting the presence of fermentable sugar in urine seems to be borne out by some of our experiments. At first we used the Somogyi modification of the Shaffer-Hartmann reagent [1926] with 10 g. of KI per litre for $\text{HgSO}_4\text{-BaCO}_3$ filtrates. In 20 normal cases 5 showed the absence of fermentable sugar. Using exactly the same technique, with the exception that the sensitive iodide-free sugar reagent was employed, fermentable sugar was found present in the urines of 57 normal individuals in a group of 58. Many urines analysed since this series was run have shown fermentable sugar without exception, including those from fasting subjects.

The maximum output of non-fermentable substances in 58 cases, was 554 mg. (as glucose), the minimum 273 mg., and the average 395 mg. with an average urine volume of 1208 cc. The average concentration was 33 mg. per 100 cc. The results of Van Slyke and Hawkins for non-fermentable substances were much higher, probably owing to the use of Lloyd-filtrates and the more powerfully oxidising ferricyanide reagent. It should be pointed out, however, that a comparison of non-fermentable reducing substances as obtained by different sugar methods, even with the same filtrate is meaningless as long as the nature of the material and its reducing power toward the different reagents are unknown.

SUMMARY.

An improved method for the determination of the fermentable and non-fermentable sugars of urine is presented. It is based upon the preparation of $\text{HgSO}_4\text{-BaCO}_3$ filtrates followed by determination of fermentable and non-fermentable sugar using washed yeast and a sensitive Shaffer-Hartmann reagent.

The writers have found fermentable sugar present in almost all 24-hour samples of normal urine examined, an average of 142 mg. (as glucose) in 55 cases.

The average non-fermentable sugar (calculated as glucose) was 395 mg. in 58 24-hour samples of normal urine.

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