LI. SOME PROPERTIES OF THE REDUCING MATERIAL IN CERTAIN FRACTIONS OF NORMAL URINES.

I. THE NATURE OF THE "FREE" FERMENTABLE SUGARS AND THE FERMENTABLE SUGARS PRODUCED ON HYDROLYSIS IN "FASTING" URINES.

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THE problem of the occurrence of glucose in "fasting" urine was investigated recently as far as available methods would permit by Harding and Selby [1931; 1933] and by West *et al.* [1932]. Both agreed that human "fasting" urine contained small amounts of reducing material removable by baker's yeast. West and Steiner [1932] differed from Harding and Selby in believing that the fermentable substance was all glucose. Recently Harding and Nicholson [1933] developed a method for quantitative determination of small amounts of sugars in solution by specific fermentation. It was thought that the use of this method, which for brevity has been called "mycological" (although the term is not strictly applicable), might throw further light on the nature of free "fermentable" sugar in fasting urine and also on the nature of the "fermentable" sugar produced on hydrolysis.

West *et al.* [1932] have emphasised the prime importance of separating non-carbohydrate reducing substances of urine as completely as possible from the carbohydrates; by using $\text{HgSO}_4\text{-BaCO}_3$ they obtained filtrates almost entirely free from nitrogenous interfering substances. The mycological method of sugar analysis was applied to urines treated in this way but the filtrates contained nitrates which interfered with the estimation of glucose by *Proteus vulgaris*.¹ Other unidentified substances which interfered with the mycological method of analysis when applied to concentrated filtrates were found in some urines. For these reasons a further fractionation of the urine was necessary. By applying a modification of the copper-lime method [Salkowski, 1879; Van Slyke, 1917] to urines cleared with basic lead acetate followed by $\text{HgSO}_4\text{-BaCO}_3$ [West *et al.*, 1929], solutions were obtained which gave satisfactory results.

¹ The action of *P. vulgaris* converted the nitrates into nitrites. The latter would later oxidise the KI used with the Harding-Downs reagent, liberating free iodine and thus giving reducing values which were much too low.

Analytical methods.

Reagents: Basic lead acetate solution [Plimmer, 1915].

Mercuric sulphate [West *et al.*, 1929].

Barium carbonate.¹

Proteus vulgaris

Monilia krusei

Monilia tropicalis

} [Harding and Nicholson, 1933].

Saccharomyces marxianus [Harding *et al.*, 1933].

Sugar reagent—modified Shaffer-Hartmann [Harding and Downs, 1933].

20% Copper sulphate solution.

8% Calcium hydroxide suspension.

All determinations of reducing material were done by the method of Harding and Downs [1933] with the modification that the time of boiling was increased to 20 min. in accordance with the recommendation of Shaffer and Somogyi [1933] for similar copper solutions. In order to get a sufficient amount of solution and to obtain "average" results "composite" fasting urine specimens were used. The urine excreted by six normal individuals between 8 a.m. and 12 noon (the last food having been taken at 6 p.m. the previous day) was mixed and made up to 1500 ml. with distilled water.

Preliminary clearing.

To this solution 185 ml. of basic lead acetate solution [Plimmer, 1915] were added and the volume made up to 3000 ml. After standing for half an hour with occasional shaking the precipitate was removed by centrifuging. Excess Pb was removed by precipitation with H_2S and filtration. Excess H_2S was then removed by aeration.² To every 250 ml. of this filtrate 100 ml. of $HgSO_4$ reagent [West *et al.*, 1929] were added. Distilled water was added to make the final volume twice that of the basic lead acetate filtrate used. Powdered $BaCO_3$ was added with constant shaking until the mixture was slightly alkaline. The precipitate was removed by suction filtration. The filtrate was made distinctly acid with concentrated H_2SO_4 and the Hg remaining in solution³ was removed by precipitation with H_2S .⁴ After suction filtration the excess H_2S was removed by aeration

¹ The barium carbonate was prepared either by passing a stream of CO_2 through a hot concentrated $Ba(OH)_2$ solution or by adding a hot solution of Na_2CO_3 to a hot solution of $BaCl_2$. After washing and drying the $BaCO_3$ was finely pulverised by passing through the finest mesh grinder of a meat chopper.

² The conditions for obtaining the optimum "clearing" of urines with basic lead acetate, without loss of fermentable sugar, were determined by Mr S. H. Jackson working in this laboratory. A more complete discussion of the method is given in the second paper of this series [Harding, Nicholson and Jackson, 1936].

³ Owing to the presence of urea, this filtrate contained a much greater quantity of mercuric salt than would be present if urea were absent. This indicates that urea unites with Hg^{++} to form a compound which is moderately soluble in alkaline solution. Supposedly, Hg^{++} is in equilibrium with the almost insoluble $HgCO_3$ and the urea complex which is only slightly dissociated in mildly alkaline solutions. This explains why, after the use of a certain quantity of $HgSO_4$ reagent, the use of additional amounts fails to remove appreciably greater quantities of urea. The reducing power of the filtrate is even less affected than the nitrogen content by the use of increased amounts of $HgSO_4$.

⁴ West *et al.* [1929] recommend the use of H_2S instead of zinc for the removal of $HgSO_4$ because the use of zinc would result in the production of H_2O_2 . We have found that another disadvantage in the use of zinc is that it reduces nitrate to nitrite which interferes with the determination of sugar in the manner described in the footnote on page 326.

(Filtrate = Fraction A). The filtrate (about 3945 ml.) was adjusted to p_H 6.5 and concentrated by evaporation under reduced pressure at 37° to a final volume of 150 ml.

Copper-lime procedure.

The concentrated filtrate was chilled to 0° in a large centrifuge bottle. 4 ml. of 20% CuSO_4 and 8 ml. of 8% fine suspension of Ca(OH)_2 were added and stirred intermittently (at 0°) for 30 min. The copper-lime precipitate was then centrifuged at about 2000 r.p.m. The supernatant fluid was poured off and termed the copper-lime filtrate. The inside of the bottle was rinsed and the precipitate thoroughly mixed with four successive portions of 5 ml. of cold saturated Ca(OH)_2 solution. After the addition of each 5 ml. portion the precipitate was centrifuged and the washings added to the filtrate. After the fourth washing the precipitate was suspended in 50 ml. of water and the sugar regenerated by acidification with a slight excess of concentrated H_2SO_4 .

The mixture of copper-lime filtrate and washings was then treated again under the same conditions with the same quantities of CuSO_4 and Ca(OH)_2 . This procedure was repeated until four precipitations had been effected. Each precipitate was washed four times as above. The four regenerated precipitates were combined. Cu^{++} was removed by precipitation as CuS with H_2S . After filtering, the H_2S was removed by aeration, Ca^{++} with KH_2PO_4 and MgO by the same technique as was used in removal of NH_4^+ [Harding and Downs, 1933]. The resulting solution was called the first copper-lime precipitate or Fraction B and contained all the free fermentable material. The filtrate was acidified and cleared of Cu^{++} and Ca^{++} by the same methods. This was the first copper-lime filtrate or Fraction F. Water was added to both fractions to make them up to known suitable dilutions.

A portion of Fraction B was removed for differential sugar analysis by the method of Harding and Nicholson [1933]. The remainder was incubated for 30 min. at 37° with *S. marxianus* (0.5 g. packed wet weight per 10 ml. of solution) to remove the free sugar including galactose. The yeast was removed by centrifuging and the supernatant solution (Fraction C) divided into two portions, one of which was hydrolysed by boiling with 0.5 *N* H_2SO_4 for one hour under a reflux condenser (Fraction D). The other portion was hydrolysed by boiling with 0.1 *N* H_2SO_4 for 6 hours under similar conditions (Fraction E). After neutralising the hydrolysate with BaCO_3 the sugar produced by hydrolysis was analysed by the mycological method. This "fermentable" sugar produced on hydrolysis will for convenience be referred to as the "hydrolysis sugar".

In two instances Fraction C was hydrolysed continuously for 6 hours with *N* H_2SO_4 , small portions being removed at intervals for differential sugar analysis. The first copper-lime filtrate (Fraction F) was hydrolysed by boiling for 1 hour with 0.5 *N* H_2SO_4 , neutralised, the ammonia produced by hydrolysis of urea removed by the use of KH_2PO_4 and MgO and the free sugar produced removed by the copper-lime method. The sugar precipitated (Fraction H) was analysed as before. The filtrate from this precipitation was hydrolysed further by boiling with *N* H_2SO_4 for 6 hours, neutralised, cleared of ammonia and the free sugars precipitated with copper-lime. The recovered sugars (Fraction J) were analysed mycologically.

Before treatment with the micro-organism all solutions were adjusted to p_H 6.5 (approx.) [Harding and Nicholson, 1933].

The various steps in the fractionation procedure are illustrated in the diagram.

Discussion of methods.

The determination of fermentable sugar in normal fasting urine by the use of ordinary yeast can be made quite satisfactorily on Lloyd's or HgSO₄-BaCO₃ filtrates when the urine is diluted 1:7 or more. At these concentrations the mycological method of differential sugar analysis can be employed if the filtrate is treated with H₂S after the action of *P. vulgaris* to reduce the nitrites formed (see footnote, page 326) to ammonia. At these concentrations however it is impossible to say with certainty whether any sugar, other than glucose, is present. In some specimens galactose and in a small number fructose appears to exist, but the amounts found are so close to the limits of error of the methods of analysis used that it is evident that a greater concentration of the urine filtrate is necessary. This was accomplished by vacuum evaporation. No phosphate was added and the *p*_H remained constant. West and Steiner [1932] noted a considerable loss of sugar during vacuum evaporation of HgSO₄-BaCO₃ urine filtrates in the presence of added phosphate. Under our conditions no loss of "fermentable" or "non-fermentable" reducing material was noted during the process and no reducing material was found in the distillate. The concentrates were diluted to give a dilution of from 1:1.5 to 1:3 on the original urine. At these concentrations the amount of the non-fermentable reducing substances was so high that it almost exceeded the limits of accuracy of the copper reagent used and all the yeasts, including baker's yeast, showed a continuous removal on repeated fermentations similar to that observed by Van Slyke and Hawkins [1929] on uncleared urines. The urea concentration is also quite high, producing much ammonia on hydrolysis. The substance giving the secondary yeast removal could be taken out of solution by preliminary treatment with basic lead

acetate, which also decreased the non-fermentable reducing substances and organic nitrogen to a slight extent (see footnote, p. 326), but the amounts of non-fermentable reducing substances and of urea in the concentrated filtrates were still too great. Another difficulty was the presence of a considerable amount of nitrate which interfered with the use of *P. vulgaris* for the estimation of glucose (see footnote, p. 326). The use of the modified copper-lime procedure overcame these difficulties. By this method it was possible to remove all the "free" fermentable sugar from urine with a maximum error of 3%. Added sugars were recovered with the same degree of accuracy. No appreciable Lobry de Bruyn conversion occurred, the added sugars being recovered unchanged and identified by hypoiodite titrations and the mycological method of differential analysis [Archibald, 1935]. The regenerated precipitates were free from nitrates and organic nitrogen. Some of the substance giving the secondary yeast removals was precipitated by the copper-lime method from the $\text{HgSO}_4\text{-BaCO}_3$ filtrates, necessitating the use of the preliminary treatment with lead acetate. By the procedure used, quantitative determinations of the "free" fermentable sugars and the "hydrolysis" sugars in both the copper-lime precipitate and copper-lime filtrate portions of the lead acetate- $\text{HgSO}_4\text{-BaCO}_3$ urine filtrates could be made. These represented the total "free" fermentable and "hydrolysis" sugar present in such urine filtrates. No sugars were considered to be present unless they were in the urine fractions in concentrations sufficient to give a reduction equivalent to 0.50 mg./100 ml. glucose (a titration figure of 0.08 ml. of 0.005 N $\text{Na}_2\text{S}_2\text{O}_3$). The dilutions of the different fractions varied from 3.0 to 0.5. Titrations were done in triplicate and usually agreed within 0.02 ml.

The use of the two strengths of acid and the two periods of hydrolysis was found advisable because, although sucrose, maltose and lactose were hydrolysed completely by refluxing for 1 hour with 0.5 N H_2SO_4 , the urine filtrate contained some substance which was very slowly hydrolysed. The milder treatment was used first because of the greater destructive action of the more prolonged hydrolysis with the stronger acid on some of the reducing substance found in urine. The 6-hour refluxing of urine filtrates with normal acid caused some destruction of both fermentable and non-fermentable carbohydrate and the production of some furfuraldehyde and charring. Ordinary sugars under similar conditions did not char. The charring was probably the result of reaction of urine phenols with furfuraldehyde liberated by acid destruction of urine sugar. The charring and furfuraldehyde production on hydrolysis were not nearly as marked in either of the copper-lime fractions as in Fraction A, probably owing to the fact that most of the furfuraldehyde-producing compounds were in Fraction B whilst the phenols were largely in the copper-lime filtrate. Although Everett *et al.* [1927] showed that appreciable amounts of fructose were destroyed during acid hydrolysis, no destruction of fructose occurred under the conditions of milder hydrolysis used here. Considerable amounts of fructose were destroyed by the more prolonged hydrolysis. Glucose was affected very little by either procedure. Harding and Grant [1931] demonstrated that prolonged hydrolysis of lactose resulted in an alteration of the ratio of measurable glucose and galactose. After 6 hours' hydrolysis of lactose with N H_2SO_4 , analysis with *M. tropicalis* and *M. krusei* showed theoretical values for glucose and a slightly raised figure for galactose.

Neutralisation with a salt which forms an almost insoluble sulphate was necessary. Otherwise the salt concentration (7% Na_2SO_4 if NaOH be used to neutralise N H_2SO_4) was sufficient to inhibit completely the action of *P. vulgaris*. Partial inhibition of the action of *P. vulgaris* occurs in solutions containing 3% or

more of sodium salt. Such salt concentrations also increase the reducing value of the non-fermentable fractions to a very considerable extent (10–15 %), although they have no effect on the fermentable fraction.

It has been shown that yeast juice acting on sugar produces a polysaccharide [Cremer, 1899; Harden and Young, 1913; Naganishi, 1926]. It was thought possible that some of the “hydrolysis” sugars might have been produced in this way by the action of the organism (*M. tropicalis* or *S. marxianus*) used to remove the “fermentable” sugars from the solution before hydrolysis. That this did not occur however was shown by the fact that hydrolysis of blanks consisting of fermented glucose solution of about the same concentration as the fermentable sugars in the urine fractions gave no reducing substance on hydrolysis, and the “hydrolysis” sugars in the fermented urine fractions did not differ appreciably from the “hydrolysis” sugar calculated by subtracting the fermentable sugars found in the unhydrolysed urine fraction from the total “fermentable” sugars found after hydrolysis of the “unfermented” fraction.

The “free” fermentable sugar and fermentable sugar produced on hydrolysis in fasting human urine.

Since the micro-organisms were used only on urines which had undergone some clearing, those carbohydrates or carbohydrate derivatives which were completely precipitated by the clearing reagent lay entirely outside the scope of this study. In the case of those substances which are only partly precipitated by the clearing reagent, the amounts reported present in the filtrates do not represent the amounts present in whole urine. The reducing fraction lost in clearing would include the oxyproteic fraction examined by Gwozdz [1933].

Typical results obtained by the differential sugar analysis of the various urine fractions are shown in Tables I and II and in Fig. 1. The results are expressed as mg. per 4 hours per single urine specimen. For simplicity in calculating

Table I. *Sugars of composite fasting male urines as mg. of glucose per 4 hours.*

	Fraction B	Fraction D	Fraction E	Fraction H	Fraction J
Total sugar	28.5	25.9	26.8	2.7	4.8
Glucose	6.0	4.7	7.0	0.5	1.6
Fructose and/or mannose	1.6	0.9	0.3	—	—
Galactose	1.7	3.2	3.6	0.2	0.3
Non-fermentable	19.2	17.1	15.9	2.0	2.9

Table II. *Sugars of composite fasting female urines as mg. of glucose per 4 hours.*

	Fraction B	Fraction D	Fraction E	Fraction H	Fraction J
Total sugar	19.2	22.0	23.8	2.4	4.0
Glucose	4.4	3.6*	7.5*	0.3	2.1
Fructose and/or mannose	0.0	—	—	—	—
Galactose	1.5	1.1	3.6	0.3	0.0
Non-fermentable	13.3	17.3*	12.6*	1.7	1.9

* The determinations of “hydrolysis” sugars of female urines were done before the effects of salt concentration on the action of *P. vulgaris* and on the reducing power of the non-fermentable fraction were observed. No differentiation of glucose from fructose and/or mannose was possible and the non-fermentable fractions were presumably 10–15 % too high.

“non-fermentable” by subtracting “total fermentable” from “total” sugar the amounts of all sugars are expressed in terms of glucose. Glucose accounts for by far the largest portion of the “free” fermentable reducing substance present in

urine filtrates.¹ Very small amounts of fructose and/or mannose are found in some but not all urines. Galactose occurs in appreciable amounts in some urines, being proportionately higher in female urines. This sugar however would not be included in the fermentable sugar reported by other workers since baker's and brewer's yeasts do not remove galactose. After hydrolysis of the fermented first copper-lime precipitate (Fraction D) considerable amounts of galactose were found. The glucose found after the prolonged hydrolysis was always considerably greater than that found after the milder hydrolysis. In a few cases, particularly in the female urines, the amount of galactose also was increased on prolonged hydrolysis. When the proportion of fermentable to non-fermentable substance produced on hydrolysis of the copper-lime precipitate (Fraction C) was observed at frequent intervals during the course of hydrolysis (Fig. 1), it was

Fig. 1. Change in total, fermentable and non-fermentable sugars on hydrolysis for different lengths of time. A, when galactose is included in the non-fermentable portion. B, when galactose is included in the fermentable portion. Ordinates: mg. of reducing material per 4-hr. specimen (expressed in terms of glucose). Abscissae: Hours of hydrolysis.

found that there was a constant decrease of the non-fermentable portion of the filtrate. If the fermentable and non-fermentable fractions were determined by the use of baker's yeast this decrease might be masked during the first hours of the procedure by the production of galactose which would be included in the non-fermentable portion. Mild hydrolysis of the copper-lime filtrate (Fraction H) gave no appreciable increase in total reducing power and produced extremely small amounts of fermentable material. The prolonged hydrolysis (Fraction J) produced a slight increase in reducing value. The fermentable sugar produced was glucose.

The nature of the precursor of the sugar produced on hydrolysis is still unknown. That some portion of it is possibly lactose is shown in the next paper [Harding *et al.* 1936]. The remaining portion is also for the most part non-

¹ It has been suggested by Laug and Nash [1935] that the fermentable sugar found in $\text{HgSO}_4\text{-BaCO}_3$ filtrates might be formed by the hydrolytic action of the strong acid in the reagent on some precursor of the fermentable sugar. Even if this were true for dog's urine, it does not seem at all probable that the fermentable sugar found in human urine originates thus, in view of the findings of Harding and Selby [1933]. These workers found significant amounts of fermentable sugar in fasting human urine when they used the $\text{HgSO}_4\text{-BaCO}_3$ method to prepare their urine filtrates. This fermentable sugar could not however have been produced by any hydrolytic action on the part of the clearing reagent since the fermentations were carried out on the diluted urines before clearing. In the same paper one comparison was made between the fermentable sugar found when the fermentation was done before the urine was cleared and that found when the fermentation was done on cleared urine. No significant difference was noted when $\text{HgSO}_4\text{-BaCO}_3$ was the clearing reagent used.

nitrogenous since the copper-lime precipitate, which is nitrogen-free, contains by far the larger portion of the substances giving fermentable material on hydrolysis.

Recently Laug and Nash [1935] suggested that this precursor of fermentable sugar was non-reducing and non-fermentable and that on hydrolysis it produced first a non-fermentable reducing material and on further hydrolysis a fermentable reducing substance. The relationship between total, fermentable and non-fermentable reducing substances shown in 6-hour hydrolysis curves, the presence of an initial rise in the non-fermentable portion and the fact that the total non-fermentable did not in most cases drop below the level found before hydrolysis were the basis for the theory. Nash presumably determined the amount of fermentable sugar present in the hydrolysates by the use of baker's or brewer's yeast, which does not remove galactose. This sugar would therefore be included in the non-fermentable fraction. An entirely different explanation of the results becomes evident when galactose is included in the fermentable portion. In Fig. 1 are shown curves obtained by determining glucose (including small amounts of fructose and/or mannose), galactose and non-fermentable reducing substances. If galactose is included in the non-fermentable fraction curves similar to those of Laug and Nash are obtained. Otherwise there is in at least 50 % of the urines examined a steady decrease in the non-fermentable portion throughout the period of hydrolysis. It is probable therefore that a considerable proportion of the fermentable sugar produced on hydrolysis comes from the non-fermentable reducing material.

Everett *et al.* [1934] and Laug and Nash [1935] present indirect evidence favouring the view that most of the sugar in urine filtrates is non-nitrogenous. Since repeated copper-lime precipitation gave a nitrogen-free fraction containing most of the reducing material, we are able to support the conclusions of these workers with evidence which is somewhat more direct.

SUMMARY.

1. By application of a modification of the Salkowski copper-lime method for the precipitation of carbohydrates to filtrates of "composite fasting" urine treated with basic lead acetate and mercuric sulphate-barium carbonate, concentrated solutions of the carbohydrate material were prepared.

2. Differential fermentation of these solutions showed the presence of glucose and galactose both before and after hydrolysis. Very small amounts of fructose or mannose were found in some urines.

3. It is probable that the greater part, if not all, of the "fermentable" sugar produced on hydrolysis is derived from the non-fermentable reducing fraction of the urine. The postulation of a non-reducing precursor is unnecessary if galactose is included in the "fermentable" fraction.

4. By far the greater part of the "sugar" in urine is non-nitrogenous.

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