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

III. SOME OBSERVATIONS ON THE NATURE OF THE NON-FERMENTABLE REDUCING SUBSTANCES IN "FASTING" URINE

BY THOMAS FREDERICK NICHOLSON
AND REGINALD MACGREGOR ARCHIBALD¹

*From the Department of Pathological Chemistry, University of Toronto,
Toronto, Canada*

(Received 21 January 1939)

In the two preceding papers of this series [Harding *et al.* 1936, 1, 2] the nature of the "free fermentable sugar" in urine and of the substances which yield fermentable sugar on hydrolysis was discussed. No attempt was made at that time to determine the nature of the reducing substances which were non-fermentable even after hydrolysis. Everett & Sheppard [1930; 1931] and Everett *et al.* [1934, 1] presented evidence indicating the presence of a substance which they named uroketose. It is precipitated by neither Hg^{++} nor Ba salts in neutral solutions. It is resistant to bromination at room temperature and gives rise to 2-furfuraldehyde when heated with acid. Everett claims that this substance, which differs from ketoxylose, constitutes the greater part of the reducing substance of normal urine.

It was thought that further information as to the nature of the residual reducing substances in normal human urine might be gained by the application of the modification of Everett *et al.* [1934, 1] of the Votocek and Nemecek technique of Br oxidation and a modified NaOI oxidation method to urine fractions obtained by the copper-lime method [Archibald, 1935; Harding *et al.* 1936, 1] which gives solutions of the urine sugars free from nitrogen and phenols. These procedures were therefore first tested on dilute sugar solutions of about the same concentration as the reducing substances found in the urine fractions and then applied to the urine fractions.

Analytical methods

Composite fasting urines were collected, diluted and treated with Pb acetate [Harding *et al.* 1936, 1, 2], HgSO_4 [West *et al.* 1929] and copper-lime [Harding *et al.* 1936, 1, 2].² Further fractionation was accomplished by acid hydrolysis and NaOI oxidation according to the steps illustrated in the diagram.

¹ Part of the data embodied in this paper was taken from a thesis submitted by one of the writers (R. M. A.) in partial fulfilment of the requirements for the degree of Ph.D. in the University of Toronto.

² In view of the fact that urine may contain considerable amounts of diastase, starch-free filter papers were used throughout these experiments as well as in those previously reported.

Dilutions reported are based on the assumption that 100 ml. of urine per 4 hr. corresponds to Dilution 1.

Hydrolyses were carried out by boiling in $N H_2SO_4$ under a reflux condenser for 1 hr. NaOI oxidations were done by the method described by Harding *et al.* [1936, 2] except that the final dilutions were varied and the small amount of KI left after HgSO₄ treatment was precipitated as AgI by the addition of a slight excess of powdered Ag₂SO₄, the excess Ag being removed by the addition of a slight excess of HCl. The copper-lime filtrates required increased amounts of I₂ solution and of NaOH.

All fractions were adjusted to pH 6.5. "Reducing values" were determined by the Harding & Downs [1933] reagent and are expressed as mg. glucose per single 4 hr. specimen. Br oxidation curves were determined on Fractions B, C, D, E and F by the method of Everett *et al.* [1934, 1], using the Harding & Downs reagent to determine changes in reducing value. The quantity of furfural-yielding material was determined in Fractions B, C, D and E by McCance's [1926] technique, separating the two layers by centrifuging for 30 sec. instead of by standing, and the free sugar by differential fermentation [Harding & Nicholson, 1933] in Fractions B, C, E, H and J.

Discussion of methods

Although most of the solutions used were obtained by regenerating the precipitates after a single treatment with copper-lime and therefore contained small amounts of nitrogenous material [Archibald, 1935], determinations done on N-free solutions obtained by reprecipitating the material [Archibald, 1935; Harding *et al.* 1936, 1] gave parallel results. It is therefore certain that none of the phenomena observed can be due to N-containing substances.

NaOI rapidly destroys all the "reducing power" of common aldohexoses and aldopentoses and the furfural-producing properties of the latter; it is therefore a more satisfactory method than Br oxidation for differentiating ketose from aldoses such as mannose, which are oxidized but slowly by the Br. Everett *et al.* [1934, 1] report that using Kolthoff's method of NaOI oxidation in urines, 30 % of the uroketose and added fructose is destroyed. It has been found, however, that the reducing power of fructose towards the Harding-Downs reagent, both in water solution and when added to urine, is almost unchanged by the NaOI or Br procedures used here. NaOI oxidation also completely removes the "reducing values" of free glucuronic acid, glucuronic acid monobenzoate, and borneol glucuronate both before and after hydrolysis as well as after 48 hr. treatment with Br.

The estimation of the amounts of furfural-producing substances present was of necessity very inaccurate because of the great differences in furfural yield between even such closely related substances as pentoses, e.g. arabinose yields three times as much furfural as does xylose.

In 48 hr. Br neither hydrolyses nor oxidizes appreciable quantities of glucuronic acid monobenzoate but it does hydrolyse 40 % of borneol glucuronate and destroys the reducing properties of the material thus liberated. Repeated Br oxidations on sugars 100 times as dilute as those used by Everett *et al.* [1934, 1] gave curves identical with those obtained by these authors. The Br oxidations gave results which fitted very well into a smooth curve. Little difficulty was experienced in repeating the results with a given fraction if the conditions influencing the rate of oxidation were carefully controlled.

The nature of the non-fermentable reducing substances of "fasting" urine

Typical results for the changes in reducing value towards the Harding-Downs reagent induced by NaOI oxidation and for the fermentable sugar in the various urine fractions are given in Table I. Fig. 1 represents the Br oxidation

Table I. *The fermentable sugars and the reducing substance destroyed by NaOI in various urine fractions*

	B	C	Increase	E	Increase	H	J
Total reduction	26.8	32.3	5.5	9.7	2.8	6.1	4.3
Fermentable sugar (including galactose)	10.3	16.6	6.3	3.0	3.0	1.8	1.0
Reducing material destroyed by NaOI	19.9	28.8	8.9	—	—	—	—

All results are expressed as mg. glucose/4 hr.

Fig. 1. The effect of Br oxidation on the reducing power of different urine fractions. Fraction B, $\bullet$ — $\bullet$; Fraction C, $\times$ — $\times$; Fraction D, $\times$ — $\times$; Fraction E, $\circ$ — $\circ$; Fraction F, $\circ$ — $\circ$; HgSO₄-KOH filtrate, $\times$ — $\times$.

Fig. 1a. The effect of Br oxidation on the reducing power of glucuronic acid monobenzoate.

curves plotted over a period of 14 days and the curves obtained during the first 16 hr. of oxidation are recorded on a larger scale in Fig. 2. Although these are results obtained with male urines, composite specimens of female urine gave similar results.

Fig. 2. For description see Fig. 1.

NaOI oxidation decreases the reducing values of Fraction B from 26.8 mg./4 hr. to 6.9 mg./4 hr. and of Fraction C from 32.3 mg./4 hr. to 3.5 mg./4 hr. Of the reducing material destroyed by NaOI in Fraction B, 50% can be accounted for by the fermentable sugar present and of that destroyed in Fraction C, 57%. This is a much greater proportionate drop in the reducing power than that observed by Everett *et al.* [1934, 1], but the difference between the two observations is to be expected when the much more drastic clearing procedure used here is considered, the copper-lime precipitation giving a fraction which is much more nearly a solution of carbohydrate only. Furfural-yielding materials represent a considerable portion of the aldose-like substance, the percentage depending on their reducing power. Expressed in terms of xylose the total furfural-producing material in B represents about 7 mg./4 hr. of which about two-thirds are destroyed by NaOI. The nature of the furfural-producing material has not been definitely determined. Although some of it may be glucuronic acid which French workers [see Cammidge, 1913; 1923] have long maintained is responsible for the non-fermentable reducing material of urine, a considerable part of it at least is probably pentose, since three yeasts, *M. tropicalis*, *M. pinoyi* and *M. metalondinensis*, which were found to have the ability to remove small amounts of pentose (xylose, arabinose, ribose and rhamnose), removed reducing material from urine fractions which had been freed from fermentable hexoses by *S. marxianus*, which does not attack pentose. Fraction G (the copper-lime filtrate) could not be analysed by mycological methods as it contained small amounts of material which were removed erratically by all the yeasts including baker's yeast.¹ However, yeast treatment of Fractions H and J shows "pentose"-like material which is equal in amount in the two fractions. That NaOI treat-

¹ This finding supports the conclusion of Harding & Selby [1933] that normal urine contains small amounts of reducing substances other than glucose which are removable by yeast.

ment did not destroy this material in Fraction G may be due to ketonic properties or to protection from oxidation by combination with some hydrolysable radical. Van Ekelen *et al.* [1933], Birch [1934] and many later workers have presented evidence showing the presence of ascorbic acid in urine, but the alkalinity of the clearing reagents and the low recovery of reducing material after copper-lime precipitation of ascorbic acid preclude the possibility that any appreciable fraction of the furfural-producing material in our fractions can be derived from vitamin C. That hydrolysis of Fraction G liberates reducing materials which on separation by copper-lime precipitation show properties similar to those in Fraction B suggests that the precursors are unfamiliar substances since the higher polysaccharides are removed by the preliminary clearing and mono- and di-saccharides are precipitated by copper-lime. The resistance of part of the furfural-producing material to NaOI is not due to protection from oxidation by a hydrolysable radical as Fraction F gives rise to fully as much furfural as does Fraction D. It is probable therefore that the furfural comes from ketonic material. Of the NaOI-resistant material a small amount, representing in most urines less than 1.0 mg./4 hr., can be accounted for by the presence of small amounts of fructose, as revealed by mycological analysis. A considerable portion of the remainder, over 2 mg./4 hr. as xylose, is furfural-producing material.

It would appear probable that the reducing material destroyed by the NaOI would be similarly affected by Br oxidation after 9–16 hr. Br treatment however causes an increase in the reducing power of, or the production of new reducing substances from, certain components of the urine fractions. This is quite apparent when the curve produced by Fraction B is compared with that from Fraction D, where all the free aldose has been destroyed by NaOI. In Fraction B there is a rapid drop in reducing power followed by a steady rise but in Fraction D no drop occurs, but only a rise which is very rapid in the first few hours and which reaches completion at the end of 8 days. Everett *et al.* [1934, 1] have ascribed the rise in the reducing value of whole urine produced by bromination to acid hydrolysis, but this could not be an important factor in the urine fractions studied here because no decrease in pH occurred and the rise in Cu reducing value took place in Fraction F where the sugars released by vigorous acid hydrolysis had been destroyed. It should also be noted that all the substances liberated by such acid hydrolysis acted as aldose towards NaOI and Br, so that if acid hydrolysis were to occur during bromination the products would be promptly destroyed. It has also been observed that with lactose and maltose, and also in the case of acid-hydrolysable urine sugar, both Br and NaOI oxidize the free reducing groups and subsequent acid hydrolysis liberates the protected reducing group. In the case of maltose and lactose such reducing sugar is liberated in theoretical amounts by acid hydrolysis after Br oxidation and is identified by mycological analysis as glucose and galactose respectively. Sucrose is not affected by either Br or NaOI.

It seems that during bromination one or probably more reducing substances resistant to oxidation by Br are formed or liberated, either by the action of Br on some substance which previously had no reducing value or by an increase in the reducing power of some substance originally only feebly reducing. The reducing value is increased to approximately $2\frac{1}{2}$ times the reduction due to substances left after NaOI, as can be seen by examination of the Br oxidation curve of Fraction D, in which all the free aldose has been destroyed by NaOI. Comparison of Fractions D and F shows that there are two reactions tending to increase the reducing substances, one which is relatively rapid and which is almost completed in 24 hr. and one which is much slower and which is not

complete until after 1 week. The second reaction does not take place in Fraction F. That is, hydrolysis permits the NaOI to destroy the substance which on Br treatment gives rise to a slow increase in reducing power. This, and the fact that acid hydrolysis (Fraction C) does not give rise to this substance would indicate that the increase in reducing power is more likely to be due to the production of a new compound than to the liberation of one already present.

Presumably these reactions are taking place in Fractions B, C and E during the first 6 hr. of Br treatment, but their effect is obscured by the much more rapid destruction of aldose. The shape of the rising curve after 12 hr. suggests that by the extension of this latter part back to zero time the curve would fall in the case of Fraction B near the starting-point of D, and in the case of C, near that of F. It is probable that a considerable part of the initial rise observed by Everett *et al.* in whole urines is due to the same compound which causes the rise in reduction in Fractions D and F and that this latter reaction is not caused by acid hydrolysis. That an initial rise does not occur in cleared urines is due at least partly to the fact that such solutions are much more readily saturated with Br than are whole urines, which contain large amounts of halogen-absorbing materials. Whereas the increase in copper-reducing ability of the Br-resistant fraction is almost as rapid in low concentration of Br as in a saturated solution, the oxidation rate of aldoses is affected greatly by Br concentration. Similar increases in reducing power were observed in filtrates from individual urines cleared with $\text{HgSO}_4\text{-KOH}$ according to the directions of Everett *et al.* [1934, 2]. An extreme example of this is shown in Fig. 1. Other samples gave much smaller rises and a few gave curves similar to those described by Everett *et al.* [1934, 1].

As in the case of NaOI, Br oxidation leaves appreciable amounts of furfural-producing material.

It is evident that NaOI and Br oxidations are not of equal value in determining the ratio of aldose to non-aldose reducing substances in urine, but that owing to the fact that Br treatment gives rise to new reducing substances the latter type of analysis may give a false impression of the amount of non-oxidizable material. It would appear probable that the Br-resistant reducing material termed by Everett *et al.* [1934, 1] uroketose is not a single substance but is composed, in so far as those portions which are precipitated by copper-lime are concerned, of (a) Br- and NaOI-resistant reducing substances already present in urine and (b) new reducing material produced by the action of Br. It is probable that a part at least of (a) is a ketopentose, as evidenced by the production of furfural, considered with the action of the pentose-removing yeasts.

SUMMARY

1. By the use of basic Pb acetate, HgSO_4 and a copper-lime precipitation method, a fraction of urine free from N and containing a high percentage of true sugars was obtained.

2. The changes in reducing power induced by NaOI and Br oxidations were observed.

3. Br oxidation gives rise to the production of new reducing substances resistant to further Br and NaOI oxidation and to yeast fermentation.

4. NaOI oxidation gives rise to little of any such substance and shows that 75 % of the reducing material behaves as does aldose.

5. Evidence is presented which suggests the presence of small amounts of pentose in urine, some of which is ketonic in nature.

The writers wish to express their gratitude to the late Prof. V. J. Harding for helpful suggestions and criticism during the early stages of the work.

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