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

II. (a) THE EFFECT OF CERTAIN "TYPE MEALS" ON THE "HYDROLYSABLE SUGAR" IN URINE.

(b) SOME FURTHER EVIDENCE AS TO THE NATURE OF THE HYDROLYSABLE SUGAR IN URINE.

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In the preceding paper Harding *et al.* [1936] presented evidence to show that fasting urine contained non-fermentable substances which on hydrolysis yielded glucose and small amounts of galactose and of fructose and/or mannose. Some evidence was presented which indicated that at least a portion of the hydrolysable sugar was reducing sugar but nothing more regarding the nature or source of the hydrolysable sugar was determined. West *et al.* [1932] showed that all types of food produced some increase in the hydrolysable carbohydrates excreted, the amounts of the hydrolysable sugar excreted being similar with all types of foods except those containing caramelised carbohydrates, these latter causing a very much greater increase in the excretion of fermentable, non-fermentable and hydrolysable sugars than any of the others. These authors believed that the hydrolysable sugar in urine was non-reducing as did Laug and Nash [1935]. Harding and Selby [1931] showed that the nature of the midday meal had a marked effect on blood sugar curves determined 4 hours after the ingestion of a "type meal" of carbohydrate, protein or fat. There was no effect on the fermentable sugar excreted in the urine for a 4-hour period following the meal. The effects of these "type" meals on the excretion of hydrolysable sugar in the urine were not studied. It was thought that an investigation of the effect of such meals on the amounts of non-fermentable hydrolysable sugars excreted in the urine and on the proportions of the various fermentable sugars released on acid hydrolysis might yield further information as to the source of the hydrolysable sugar.

A comparison of the amount of sugar produced by the hydrolysis of "Lloyd's" and " $\text{HgSO}_4\text{-BaCO}_3$ " [West *et al.*, 1929] filtrates was made in order to determine whether or not any large proportion of fermentable sugar was contained in compounds (possibly nitrogenous in nature) which were precipitable by mercury but not by Lloyd's reagent.

More direct evidence as to the nature of the hydrolysable sugar was sought by subjecting one portion of urine to the action of hypiodite before hydrolysis and comparing the fermentable sugars produced on hydrolysis of the portion so treated with those liberated from another portion which had not undergone the

hypoiodite treatment. By this means it was possible to estimate what proportion of the fermentable material liberated from the hydrolysable material was so combined that it presented a free aldehyde group.

Analytical methods.

In order to get sufficient urine for analysis the urines of two individuals were combined in each of the series of tests made. The individuals ate a light breakfast of orange juice, cereal, bacon, toast and coffee. At noon the "type" meal was taken. The "fat", "starch" and "protein" meals were similar to those described by Harding and Selby [1931]. The "fruit" meal consisted of mixed fresh fruits—oranges, grapefruit and apples—to give about one-half the amount of total carbohydrate present in the "carbohydrate" meal. In addition, tests were made on urines collected when no noon meal was taken and in the morning, 12 hours after the last meal. The urine passed in the 4 hours following the taking of the "type" meal was collected. In the case of the 12-hour fasting specimen, the urine was collected between 7 and 11 a.m. When no noon meal was taken the urine was collected between noon and 4 p.m. The combined urines were made up to a volume of 500 ml. thus facilitating calculations by giving a constant volume to the 4-hour specimen. "Fermentable" sugar was removed by incubating 325 ml. of the urine for 10 min. at 38° with 10 g. wet weight of baker's yeast (Fleischmann's) prepared according to Somogyi's [1927] directions. After incubation the yeast was removed by centrifuging. 100 ml. of the fermented urine specimen were cleared with Lloyd's reagent as described by Harding and Selby [1931] and 200 ml. were cleared by the use of basic lead acetate¹ followed by HgSO_4 - BaCO_3 , using the same proportions of reagents to urine and the same methods of removing the excess reagents from solution as were specified in the preceding paper. In both instances the urines were diluted 1 : 2 in the process of clearing. Each filtrate was then treated in an identical way. The largest convenient aliquot was again diluted 1 : 2, sufficient 5.0 *N* H_2SO_4 being added in the course of dilution to give *N* concentration. The acidified filtrate was boiled for 4 hours under a reflux condenser. After cooling the solution was neutralised with CaCO_3 . The precipitated CaSO_4 was removed by suction filtration and the filtrate again

¹ The maximum amount of basic lead acetate that may be used without the removal of fermentable sugar is 1 ml. of 0.5 *N* solution to 8 ml. of urine. The time that the basic lead acetate is in contact with the urine is of importance. If the precipitate is filtered off immediately, a considerable loss of fermentable sugar occurs, nor can added sugars be completely recovered. If on the other hand the precipitate and filtrate are allowed to remain in contact (with occasional shaking) for 30 min. no loss of fermentable sugar occurs and added sugars may be recovered quantitatively. Some sugar is evidently occluded in the precipitate and later diffuses out into the filtrate. The use of this procedure before the HgSO_4 - BaCO_3 decreases the total nitrogen of such urine filtrates by from 45 to 55 %, lowers the total reducing value of the filtrate by from 15 to 20 % and completely removes the substance which is responsible for the secondary removals by successive yeast treatments, as in the Van Slyke and Hawkins [1929] phenomenon which was observed in concentrated HgSO_4 - BaCO_3 filtrates of urine by Harding *et al.* [1936]. The decrease of nitrogen in the lead-mercury filtrates as compared with the filtrates obtained by the use of the HgSO_4 - BaCO_3 alone was due to a decrease in the urea content of the filtrate as neither filtrate contained any organic non-urea nitrogen. Since basic lead acetate itself has little effect on urea this reduction in urea content was probably due to the action of the lead acetate in removing non-urea-nitrogen and thus allowing more complete precipitation of the urea by the HgSO_4 - BaCO_3 procedure. Similar reductions in the urea content of the filtrates may be obtained by a second treatment with HgSO_4 - BaCO_3 but this decreases the amount of filtrate very markedly and has no effect on the substance which gives the successive removals on repeated yeast fermentation.

treated with $\text{HgSO}_4\text{-BaCO}_3$, 20 ml. of the HgSO_4 solution being used for every 100 ml. of filtrate, and a further dilution of 1 : 2 being effected, to remove the charred material produced during the hydrolysis.

The reaction of the filtrate was adjusted to p_{H} 6.5 with a drop of 50 % KOH and the filtrate concentrated by evaporation under reduced pressure at a temperature of 37° to about 25 ml. The concentrate was subjected to the modified Salkowski [1879] copper-lime precipitation procedure [Archibald, 1935] described in the preceding paper.

The regenerated copper-lime precipitate which represented a dilution of 1 : 1.5 of the original 500 ml. 4-hour specimen was analysed by the mycological method of Harding and Nicholson [1933] to determine the amounts of the various sugars that had been liberated by hydrolysis.

Another group of combined urines passed in the 4-hour period from noon to 4 p.m. by individuals who had taken their ordinary breakfast and lunch and a further group collected from 8 a.m. to noon following a breakfast consisting solely of a quart of skimmed milk were cleared by the basic lead acetate- $\text{HgSO}_4\text{-BaCO}_3$ method. The filtrate from each "combined" urine was then divided to give two 100 ml. portions. One portion was treated in the same manner as the other lead acetate-mercury filtrates. The other portion was neutralised with 40 % NaOH and 15 ml. of 0.05 *N* I solution (made up in as dilute KI as possible) was added. 0.1 *N* NaOH was added drop by drop, with stirring, at such a rate that not more than 0.5 ml. was added per min. The addition was continued until the solution was almost colourless (about 15 ml. were usually required). After 10 min. the solution was acidified with 3 ml. of 5 *N* H_2SO_4 and aerated vigorously for 2 hours to drive off as much of the free iodine as possible. The remaining iodine and any iodides or iodates were removed by the addition of 10 ml. of the HgSO_4 reagent. The volume was then made up to 200 ml. and the Hg removed with BaCO_3 followed by H_2S . The resulting solution was then hydrolysed and treated in the same manner as the other filtrates.

All determinations of reducing material were made with the modified Shaffer-Hartmann reagent of Harding and Downs [1933] using a 10-min. boiling period.

The effect of "type meals" on the hydrolysable sugar in urine.

Tables I and II give typical results obtained after hydrolysis of "Lloyd's" and "lead acetate-mercury" filtrates. All the meals produced marked increases in hydrolysable sugar over the fasting specimen but the true effects of the type meals are seen by comparison with the urines collected in the afternoon when a normal breakfast had been taken but no noon meal given. There was a noticeable increase in the excretion of substances giving rise to fermentable sugar on

Table I. *Fermentable sugar produced on hydrolysis.*

Sugar as mg. glucose per 4 hours.

	Total fermentation		Glucose		Fructose and/or mannose		Galactose	
	Lloyd's	Pb Ac. HgSO_4	Lloyd's	Pb Ac. HgSO_4	Lloyd's	Pb Ac. HgSO_4	Lloyd's	Pb Ac. HgSO_4
12-hr. fasting	7.3	4.0	5.5	2.6	0.3	0.0	1.5	1.4
No noon meal	20.8	15.1	15.1	9.6	0.0	0.0	5.7	5.5
Fat	25.0	24.8	18.0	18.2	2.0	1.6	5.0	5.0
Fruit	28.9	27.0	21.0	19.6	3.3	3.0	4.6	4.4
Starch	25.2	20.2	17.5	13.6	3.1	2.0	4.6	4.6
Protein	20.6	15.4	13.0	8.8	3.1	1.8	4.5	4.8

Table II. *The fermentable sugars produced by hydrolysis before and after hypiodite oxidation of "fermented" filtrates of urine passed after mixed meal.*

		Sugars as mg. glucose per 4 hours		
		Direct hydrolysis	Hydrolysis after hypiodite oxidation	Loss of sugar due to hypiodite oxidation
A	Fructose and/or mannose	4.8	3.8	1.0
	Glucose	21.6	14.4	7.2
	Galactose	5.1 (7.0)*	5.7 (7.5)*	—
B	Fructose and/or mannose	1.7	2.2	—
	Glucose	15.4	8.1	7.3
	Galactose	2.1 (2.9)*	1.7 (2.4)*	0.5

* mg. galactose per 4 hours as galactose.

hydrolysis (shown by the increase in fermentable sugar produced on hydrolysis) in all instances except after the protein meal. These substances will be referred to for convenience as "hydrolysable sugar". The increase in all cases was due principally to an increase in the glucose-containing portion but there was also a definite excretion of some fructose-containing substance which was not present in the 12-hour fasting or in "no noon meal" specimens. The amounts of fructose found did not vary markedly although there was slightly more after the "fruit" meal than after any of the others. The amount of galactose found was fairly constant, there being little change in the amount excreted after any of the meals as compared with the afternoon specimen collected when no noon meal was given. There was however a very considerable increase over the fasting value. The amount of galactose found evidently depended on the morning meal and was probably due to excretion of a portion of the lactose taken in the milk.

When fermentable sugars resulting from the hydrolysis of the Lloyd's and the lead acetate- HgSO_4 - BaCO_3 filtrates are compared, it is seen that the use of the lead-mercury method of clearing resulted in a definite decrease in the amount of glucose produced on hydrolysis in the "12-hour fasting" and "no noon meal" specimens and following the "starch" and "protein" meals. The difference in the cases of the "fat" and "fruit" meals was very small or non-existent. Hydrolysis of the lead-mercury filtrates gave rise to a smaller amount of fructose than did hydrolysis of the Lloyd's filtrates. The difference was small but definite and was observed in the urines passed after every type of meal. The galactose figure did not vary with the method of clearing used. It is evident that urine contains some fermentable sugar in substances (possibly nitrogenous in nature or more probably dextrin-like and corresponding to a portion of the alcohol-precipitable carbohydrate of Lustig and Landau [1932]) which are precipitable by lead acetate and mercury but not by Lloyd's reagent.

The immediate effects of the various meals on the total amount of hydrolysable sugar excreted were not great but "fat" and "fruit" meals had marked effects on the excretion of the lead-mercury-precipitable substance. There was almost complete suppression of the excretion of this substance for the 4-hour period following the ingestion of these two types of meals. It was remarkable that the glucose produced on hydrolysis of the specimen collected after the fat meal showed such a definite increase over that of the "no meal" specimen. This could not be due to specific dynamic action since protein, which has a much greater specific dynamic action, failed to increase any of the hydrolysable sugars. This effect might however be connected with the very striking effect of the fat meal on the glucose tolerance which was noted by Harding and Selby [1931]. The fact

that fruit, in which the sugars occur mainly as monosaccharides or simple polysaccharides that are readily hydrolysed in the digestive tract, increased the excretion of "hydrolysis" glucose even more than did complex polysaccharides such as starch, was also of considerable interest. The ingestion of protein had no immediate effect on the excretion of hydrolysable carbohydrate material, the total and the amount of lead-mercury-precipitable substance found being the same for the 4-hour period following the protein meal as for the similar period when no noon meal was taken.

Further evidence as to the nature of the hydrolysable sugar in urine.

West *et al.* [1932] and Laug and Nash [1935] have stated that the fermentable reducing sugar produced on hydrolysis of lead-mercury filtrates of urine was derived entirely from non-reducing polysaccharides. Harding *et al.* [1936] suggested that a part at least of the "hydrolysable" material was reducing in nature and that the failures of West *et al.* and Laug and Nash to detect any reduction of the non-fermentable portion of the urine filtrates after hydrolysis were due to the fact that liberation of galactose, which is not fermented by baker's yeast, masked the reduction in the non-fermentable reducing substances.

The use of hypiodite oxidation combined with acid hydrolysis gave more direct evidence as to the nature of the hydrolysable substances. In Table II are recorded two sets of results obtained on urine specimens following a mixed meal. Comparison of the amounts of the various sugars produced by acid hydrolysis before and after hypiodite oxidation shows that glucose was the only sugar affected. The galactose figure remained constant. It is evident that about $\frac{1}{3}$ to $\frac{1}{2}$ of the glucose was combined with the aldehyde group free whereas all the galactose was present in the form of galactosides. It seems quite possible that the galactose was present in the form of lactose. Lactose however could not account for all the glucose which might be combined to form a reducing material because the amount of galactose produced on hydrolysis did not in one instance (Table II B) equal more than about $\frac{1}{3}$ of the glucose oxidised by the hypiodite. In some urines therefore a very considerable proportion of the glucose produced on hydrolysis from reducing non-fermentable carbohydrates came from some source other than lactose.

The ingestion of a large amount of skimmed milk did not result in any increase in the total hydrolysable sugar but the proportion of galactose produced on hydrolysis was increased considerably. In Table III are recorded the findings following two "milk breakfasts". The galactose was again unaffected by the

Table III. *The fermentable sugars produced by hydrolysis, before and after hypiodite oxidation, of "fermented" filtrates of urines passed after "milk" meals.*

		Sugar as mg. glucose per 4 hours		
		Direct hydrolysis	Hydrolysis after hypiodite oxidation	Loss of sugar due to hypiodite oxidation
A	Fructose and/or mannose	2.8	1.3	1.5
	Glucose	15.3	6.7	8.6
	Galactose	8.0 (11.1)*	8.0 (11.1)*	—
B	Fructose and/or mannose	3.0	3.5	—
	Glucose	17.6	8.8	8.8
	Galactose	8.5 (11.8)*	8.2 (11.4)*	—

* mg. galactose per 4 hours as galactose.

hypoiodite oxidation, as much having been produced on hydrolysis of the hypoiodite-treated filtrates as by hydrolysis of the untreated filtrates. The amount of galactose liberated exceeded the amount of glucose destroyed by a small but significant amount. Some galactose was therefore excreted combined through its reducing group in some sugar complex other than lactose.

These results confirm the conclusion of Harding *et al.* [1936] that, when fermentations are carried out with baker's yeast, the liberation of galactose may mask reductions in the non-fermentable reducing substances which occur on hydrolysis. If the figures in Table II A are examined it will be seen that the liberation of galactose could maintain the non-fermentable figure at the same level as before the urine filtrate was hydrolysed. On the assumption that all the glucose with its reducing group free was combined as lactose there would be 13.7 mg. of lactose present in the filtrate which would give a titration figure equivalent to 5.3 mg. of glucose. As the galactose liberated (7.2 mg.) would be equivalent to 5.2 mg. glucose there would be no change in the reducing value of that portion of the filtrate which was not fermentable by baker's yeast.¹ Following a milk meal, assuming again that all the glucose oxidised by the hypoiodite was combined as lactose, there would be an actual rise in the non-fermentable reducing substances on hydrolysis of the filtrate if the galactose produced were not fermented.

SUMMARY.

1. All of the various "type" meals—"fat", "fruit", "starch" and "protein" (with the exception of the last-named)—caused increases in the amounts of the substances giving rise to fermentable sugar on hydrolysis excreted in the urine during 4 hours after the meals.
2. The increase was due almost entirely to glucose-producing substances.
3. A small amount of material which produces glucose on hydrolysis is precipitated by lead acetate- HgSO_4 - BaCO_3 treatment but not by Lloyd's reagent. This substance is present in urines collected after 12 hours' fasting as well as in non-fasting urines.
4. The excretion of the lead acetate- HgSO_4 - BaCO_3 -precipitable substance does not occur after "fat" or "fruit" meals.
5. An examination of the effect of hypoiodite oxidation of the urine filtrates on the sugars produced by acid hydrolysis shows that about one-third of the hydrolysable material excreted is reducing in nature, the reducing group being in the glucose molecule.
6. All the galactose released on hydrolysis is combined through its reducing group, possibly in the form of lactose.
7. The reduction in the non-fermentable portion of the urine filtrates which would be expected following acid hydrolysis and yeast fermentation may be masked by the galactose liberated if the fermentation organism does not remove galactose.

¹ These figures refer to reductions obtained with the Harding-Downs reagent using a 10-min. boiling period (see Table I, Harding and Downs [1933]). Using the Shaffer-Somogyi [1933] reagent 50 containing 1 g. of KI per litre with a 15-min. boiling period, as employed by West *et al.* and by Laug and Nash, the results would be very similar, since lactose, under these conditions, gives only 57% of the reduction given by galactose.

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