CLXVIII. GLUCOSE IN NORMAL URINE.

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INTRODUCTION.

IN spite of considerable investigation, the question whether small amounts of glucose may occur in normal urine has not yet been satisfactorily answered. The present state of indecision is partly due to the absence of a specific test for this sugar and partly to the fact that it normally occurs in very small quantities.

Apart from reduction tests the detection of glucose has generally been based on (1) fermentation with yeast, and (2) the phenylhydrazine reaction. That the reaction is not specific for glucose is not the only drawback as far as yeast fermentation is concerned, for yeast [von Lipmann, 1904] fails, sometimes, to ferment sugar when the concentration of the latter is as low as 0.1 g. per 100 cc. During the present investigation this observation was confirmed. It was possible to obtain glucosazone from urines to which 0.1 g. glucose per 100 cc. had been added even after 3 days' incubation at 37° with a pure yeast culture [see also Patterson, 1926].

Objections have often been raised against the use of yeast for the detection of glucose in normal urine. The laborious technique, which the use of pure cultures of yeast necessitates, has unfortunately led to the employment of ordinary yeast instead. This practice has no doubt weakened the evidence for the existence of glucose, though in the majority of urines thus treated fermentation did occur [Benedict and his co-workers, 1918; Sumner, 1921; Neuwirth, 1922]. But, as has frequently been suggested, such fermentation might be due to the bacteria introduced with the yeast. To attempt to inhibit bacterial growth by making the urine slightly acid or by adding a little volatile antiseptic like toluene is of doubtful value especially if, as is customary, fermentation with ordinary yeast is allowed to go on for 17 to 24 hours or more. In spite of starting with a p_H varying between 5.5 and 6.8, Höst [1923] found that some of the urines undergoing fermentation with ordinary yeast became strongly ammoniacal. Much reliance cannot, therefore, be placed on positive evidence obtained in experiments with yeast if bacteria were present.

The osazone reaction properly carried out is free from such serious drawbacks, and it may, therefore, be expected to furnish more reliable information regarding the question of glucose in normal urine. As has been pointed out

by Höst [1923], the only sugar capable of giving an osazone identical with that of glucose is laevulose, but the optical behaviour of normal urine seems to show that laevulose is most probably not one of the urinary sugars. Moreover, observations reported by Folin and Berglund [1922] suggest that, unlike galactose and lactose, laevulose ingested even in large quantities is retained in the body.

The belief that glucose is a constituent of normal urine arose from observations made by Baisch [1894] and confirmed by Lemaire [1895] and by Pavy and Siau [1901]. These investigators isolated from normal urines an osazone apparently identical with glucosazone. Recently, however, Geelmuyden [1915] and Höst [1923], who applied the same test to small quantities of urines, were unable to confirm the results arrived at by these earlier investigators, though many normal urines were examined.

Dissatisfied with the methods so far employed, Folin and Berglund [1922] attempted to throw fresh light on the problem by studying the relation between sugar elimination and the concentration of sugar in the blood. The blood-sugar and the urine-sugar were determined before and after hydrolysis under various conditions and it was found that giving as much as 200 g. of pure glucose to normal persons was not followed by any glucosuria, whereas definite "glycuresis" was obtained after every ordinary carbohydrate meal. These observers ascribe the increase in the reducing substances of the urine after an ordinary meal to the excretion of foreign unassimilable carbohydrates and carbohydrate decomposition products produced during the preparation of the food. According to them glucose is probably not one of the urinary sugars. Similar conclusions were arrived at by Höst [1923] and by Greenwald and his co-workers [1925].

The present paper records certain observations which were made while applying the osazone test to a number of urines mainly from healthy Egyptian students and which provide confirmation of the older view that glucose may be a constituent of normal urine.

EXPERIMENTAL.

1. *The preparation of the osazones of the urinary sugars.*

Normal urines contain substances that interfere with the proper crystallisation and consequently with the identification of the osazones of the urinary sugars. Even when the concentration of the sugars is so large that recognisable crystalline osazones come down when the urine is treated with phenylhydrazine, the crystals are, as a rule, ill-formed, dirty and often buried in an amorphous deposit of brown particles and oily drops. The substances which thus interfere with the formation and crystallisation of osazones can be removed quite readily by treating the urine with adsorbent charcoal before applying the phenylhydrazine test.

In all the experiments recorded in this paper, the urines were treated in this manner and evidence was obtained that charcoal removes these interfering substances without appreciably lowering the concentration of the urinary sugars. The charcoal employed was usually unpurified Merck's decolorising charcoal. The following method was found suitable for the preparation of osazones from normal urines.

25 cc. urine were well mixed with 2 g. dry charcoal and filtered after standing for about 5 minutes. About 15–17 cc. clear colourless filtrate was usually obtained, and to this was added 1 g. of a mixture of pure phenylhydrazine hydrochloride and sodium acetate (prepared by mixing well in a mortar one part of pure phenylhydrazine hydrochloride and two parts of sodium acetate just before use). The filtrate was now placed in a boiling water-bath for about 5 minutes or just until the reagents were dissolved and then filtered into another clean tube. The final filtrate was put back in the bath for 2 hours and then allowed to cool gradually in the bath and the deposit examined for crystals 12–15 hours later.

As described above the reaction is not only sensitive but seldom fails to yield clean and well-crystallised osazones. Occasionally urines of low specific gravity fail to give crystals at first, but most of these do so on longer standing at a temperature of 3–5°.

Sometimes the crystals are contaminated with variable amounts of orange-brown oily drops which, whilst in no way interfering with the delicacy of the test, may tend to obscure their appearance under the microscope. This impurity can be effectively removed by washing the deposit two to three times with a little ether after the removal of the supernatant liquid.

The sensitivity of the osazone test.

To test the delicacy of this reaction for the detection of glucose, it was applied, in the form described above, to solutions of glucose in water and in urine.

Glucose dissolved in water. From aqueous solutions of glucose it was a simple matter to obtain typical glucosazone crystals when the concentration of glucose was 2.5 mg. per 100 cc. This confirms the observations of Höst [1923] who employed a modified Neumann's method. The reaction is, however, more sensitive than he imagined, for by cooling the tubes to 3–5° for a few hours, aqueous solutions containing as little as 1 mg. glucose per 100 cc. were found to give crystals of glucosazone.

Glucose added to urine. The presence in urine of substances which interfere with the osazone test, as well as the fact that all urines examined yield osazones, make it difficult to determine the delicacy of the test for the detection of added glucose until the urinary sugars have been removed. This was accomplished by bacterial fermentation in the following way.

The urine is made just alkaline with ammonia, inoculated with a few drops from another urine undergoing active alkaline fermentation and incubated at

37° for 10–12 hours. It is then acidified slightly with acetic acid, a sufficient quantity of washed kieselguhr added to assist the flocculation of the bacteria and brought up to the boil. After cooling, it is filtered and the clear filtrate is used for the test. Such urine will not give an osazone.

Tests on such urines with added glucose demonstrated that as little as 1 mg. per 100 cc. can be detected by the osazone test.

The nature of the osazones derived from normal urine.

The crystalline forms of the osazones obtained from normal urines are very varied (Plate V, Figs. 1–7). Nevertheless, it can be shown that they usually consist of mixtures of at least two osazones which can, in most cases, be separated by simple means. The crystals may be composed entirely either of fine needles or of broad flat needles, but more frequently both kinds are combined to form one crystal mixture (Figs. 2, 3 and 4). In some, however, their mixed nature is not quite so clear. Union between the two osazones apparently takes place before crystallisation and the resulting crystal possesses new characters, but these are always intermediate between those of the components (Fig. 5). A more complex type of crystal is frequently met (Fig. 6) but this is simply an aggregate of crystals belonging to the two classes of the crystal mixture just described.

The appearance of crystals in Figs. 2, 3, 4 and 6 indicates that they are a mixture of the two osazones represented by Figs. 1 and 7. This can be proved by fractional crystallisation, since these two osazones differ markedly in solubility.

The osazone deposit from 200 cc. of a 24 hours' fresh urine was washed with distilled water and then with ether, 10 cc. of 20 % alcohol were then added and after stirring well, the mixture was centrifuged in a corked tube. The clear orange-brown supernatant liquid was then drained off and kept aside and the residue examined microscopically. This residue was dissolved in a little hot absolute alcohol and distilled water carefully added to the solution until a slight permanent turbidity occurred. The solution was then boiled to drive off some of the alcohol and on cooling fine long needles were obtained, many of which remained single, others formed broom-like aggregates. Microscopically these crystals were identical with glucosazone.

The clear supernatant liquid was treated with a little ether, and the top ether-alcohol layer pipetted off. This on recrystallisation gave a mixture of crystalline forms in which were recognised (a) a small glucosazone sheaf, (b) a larger irregular mixed osazone sheaf, and (c) two separate broad flat needles.

Crystals containing comparatively little glucosazone give after such treatment a fair yield of the osazone which crystallises out in broad flat needles.

The fractional crystallisation of many similar crystals obtained from urine gave only the following two simple osazones.

Osazone I. This is characterised by crystallising in broad flat orange-yellow needles of which the ends usually taper gradually, or in some cases form blunt spear-shaped points. The needles tend to form aggregates usually in the form of large irregular rosettes. Microscopically the crystals sometimes resemble maltosazone, but their melting-point is much lower, 152–153°. This osazone is soluble in hot water and in 20 % alcohol (Fig. 1).

Osazone II. Glucosazone. This is characterised by its fine long dark yellow needles which occur singly or more often in sheaves, brooms and rosettes. When formed from urine this osazone tends to float to the surface of the liquid forming a woolly mass in which crystals belonging to other types may be found entangled. If the amount is small, this osazone remains at the bottom among the deposit formed by the other crystals. When purified by solution in hot absolute alcohol and recrystallisation from dilute alcohol (about 20 %) this osazone melts at 202–204°, but when first obtained from urine its melting-point is much lower, 188–198°. The purified osazone is insoluble in hot water and in cold absolute alcohol but dissolves in the latter on heating. It appears to be identical with glucosazone in melting-point, colour, crystalline appearance and solubility. Fig. 7 represents a sheaf obtained from a normal urine voided 1½ hours after an ordinary meal.

The crystal mixtures have no fixed melting-point, solubility or crystalline form. These characteristics depend entirely on the amount contributed to the crystal by each osazone. This peculiarity might explain why so many investigators succeeded in obtaining from normal urines osazones with various melting-points and crystalline forms. But it is quite significant that the melting-point of all these new osazones lies between those of the two osazones already described. It is, therefore, highly probable that all such osazones are of the same nature as the crystal mixtures. The osazone isolated by Patterson [1926] seems to be a good example, for its crystalline form resembles closely certain crystal mixtures which can be easily prepared by dissolving a mixture of osazone I and osazone II in a little hot absolute alcohol and then fractionating it by simply diluting with water. As the alcohol becomes weaker, two to three well-formed precipitates come down. On examination the appearance and properties of these fractions will be found to be intermediate between those of the components.

The principal object of this investigation being to throw some light on the long-discussed question of the occurrence of glucose in normal urines, little attention has been given to the identity of osazone I. Baisch [1895], however, isolated from urines fermented with yeast an osazone which had m.p. 152–154°, and Lemaire [1895] later obtained a similar product of m.p. 150–151°. The former investigator believed his osazone to be identical with Fischer's *iso*-maltosazone both in crystalline appearance and melting-point, but apparently there has been no further evidence provided regarding the identity of Baisch's product. It is uncertain whether the product is really *iso*-maltosazone or not. The melting-point of the product isolated by Baisch and Lemaire agrees with

that given for osazone I, but the crystalline appearance of osazone I is totally different. For, while the osazone isolated by these observers crystallises in small rosettes composed of very fine needles, much finer than those of glucosazone, the needles of osazone I are broad, flat and large. This, however, may be due to differences in the method of preparation. An osazone prepared from a specimen of *iso*-maltose kindly supplied by Prof. Ling gave crystals similar to those described by Baisch and Lemaire, and when glucosazone was added to it and then recrystallised, crystal mixtures were obtained, but none of the crystals resembled osazone I.

Crystals similar to those described here for osazone I have also been obtained by Geelmuyden [1915] from normal urines and from urines of "sugar-free" diabetics.

The detection of glucose added to ordinary urine.

If glucose is added to ordinary urines the quantity that gives typical glucosazone will, as Höst [1923] pointed out, vary. Höst obtained typical glucosazones from ordinary urines after adding about 10 mg. glucose per 100 cc. and in some cases not before the addition of 20 mg. The causes of these variations may be appreciated from what has already been stated regarding interfering substances. There is little doubt that the tendency of the osazones of the urinary sugars to form crystal mixtures is responsible for these variations. Variable crystalline forms with mixed types of crystals are also obtained when aqueous solutions of mixed sugars are examined. From an aqueous solution containing glucose and lactose it sometimes happens that nothing but typical glucosazone is obtained. But this glucosazone though typical microscopically is a crystal mixture from which the lactosazone can be recovered by fractional crystallisation. This behaviour explains why the majority of diabetic urines give only glucosazone crystals, and also explains the disappearance of the "physiological osazone" when the amount of glucose added by Höst to ordinary urines was adequate to produce "typical" glucosazone. The melting-point of such a typical glucosazone obtained from urine is generally much lower than that of pure glucosazone, because of the other osazone incorporated in it. The elimination of the latter osazone, by fractional crystallisation, leads to a marked rise in the melting-point. In a particular case the melting-point went up from 188° to 202°, or just two degrees less than that of pure glucosazone. It is not always easy to separate osazone I in a pure state from this typical glucosazone, but this point may be said to have been satisfactorily proved, if the rise in the melting-point follows the elimination of an osazone whose characters are unmistakably different from those of pure glucosazone.

It appears, therefore, that the phenylhydrazine reaction is less sensitive for the detection of glucose added to ordinary urines. This is, however, not entirely correct for small quantities of added glucose may be detected by watching the minute changes in the crystal mixtures, as little as 2.5 mg.

glucose per 100 cc. producing a visible increase in the glucosazone element in some of the crystal mixtures.

The phenylhydrazine reaction employed by Höst for the detection of glucose in normal urine.

Höst [1923] employed a modified Neumann's phenylhydrazine reaction and no precautions were taken to remove interfering substances from the urine. The osazones obtained by Höst from normal urines differ enormously in appearance from those described here. When his method was applied to 14 urines, no glucosazone whatever was obtained, though two of these urines gave typical glucosazone by the method described here. Some of the "physiological osazones" described by Höst were subjected to fractional crystallisation and in many cases it was possible to split them into glucosazone and another osazone very similar to osazone I. It, therefore, seems that Höst's failure to obtain glucosazone from normal urine was not due to the absence of glucose but to the method employed.

The occurrence of glucose in the urines of healthy individuals.

The osazone test in the form described here was applied frequently to the urines of about 700 Egyptians. These were mostly students whose ages varied between 17 and 27 years. Every urine gave osazone crystals that differed in detail in different persons and even in the same person at different times. Great variety of crystal shapes was encountered but nevertheless they were all mixtures of the types already described. The frequency with which typical glucosazone crystals were obtained was rather striking. Of urines voided 4-5 hours after a mixed meal 12-15 % give typical glucosazone crystals as well as other crystals. The remaining urines give crystal mixtures in which the glucosazone element varies greatly. In urines voided 1-2 hours after an ordinary meal the percentage is practically doubled (20-30 %) and the crystal mixtures in all the urines show a definite increase in the glucosazone element over what is usually obtained 4-5 hours after a meal. After fasting for about 12 hours the urines of two out of 30 persons gave typical glucosazones.

The question now arises as to the state of the carbohydrate metabolism in those whose urine gives typical glucosazone. Two points should be grasped before this question is discussed. Urines giving typical glucosazone do not reduce Fehling's reagent or Benedict's reagent in the ordinary sense. Such urines may give a reaction similar to that given by normal concentrated urines if equal parts of the reagent and the urine are boiled for 20 seconds and allowed to stand for 10-30 minutes. Again every subject examined passed at times a urine that gave typical glucosazone. The relation of this to meals was not always clear for it happened even after a 12 hours' fast.

To clear up this point, the blood-sugar and the osazones of the urine-sugars were simultaneously studied in 28 student volunteers, just before and after giving each 50 g. pure glucose dissolved in 100 cc. water by the mouth after

fasting for about 12 hours. Samples of urine and blood were taken just before administering the glucose and at half hourly intervals afterwards. The fasting urine of two students gave typical glucosazone, all the others gave crystal mixtures. After the glucose none of the urines gave typical glucosazone even in the case of the two students whose fasting urine gave typical glucosazone. The behaviour of the blood-sugar was apparently quite normal, for the fasting levels varied from 0.078 to 0.120 g. % and the maximum from 0.120 to 0.198 g. % and the return to the fasting level occurred from 1½ to 2½ hours after taking the glucose.

It is justifiable to believe, then, that when urines give typical glucosazone the condition is not always due to an abnormal carbohydrate metabolism. This statement applies to specimens of urine taken at random during the day, for it has always been observed that a mixed 24 hours' urine of a healthy individual will only give crystal mixtures but no typical glucosazone. Under normal conditions the balance between the urine-sugars in a mixed 24 hours' urine appears to be such that glucose does not have a chance of forming typical glucosazone.

It is interesting to note that the excretion of glucose after 50 g. pure glucose seems to be less than that which follows an ordinary mixed meal.

DISCUSSION.

The failure of certain investigators, in particular Geelmuyden [1915] and Höst [1923], to obtain glucosazone from normal urines is probably due to the fact that the test is not sufficiently delicate to detect minute amounts of glucose unless certain precautions are observed. The most important of these is the removal by adsorption on charcoal of certain substances which interfere with the formation of the osazones. The tendency of different osazones to form crystal mixtures is another disturbing factor, which must be borne in mind when glucosazone is looked for. With the exception of crystals (*a*) and (*b*) in Geelmuyden's figure, and the few detached broad needles lower down in the diagram and also crystal (*m*), Geelmuyden's other crystals appear to be all of the mixed variety.

The results presented in this paper leave little doubt that glucose is a constituent of normal urine. Evidence for this can also be obtained from Höst's own results, for he observed that urines fermented with yeast required the addition of more glucose in order to give typical glucosazone crystals than unfermented urines. Höst himself suspected that this result might have the interpretation given to it here.

The increase in the reducing power of urines after ordinary meals seems to be due, at least in part, to an increased excretion of glucose and not, as has been suggested by Folin and Berglund [1922], to the presence of unassimilable carbohydrates and degradation products arising from carbohydrates during the preparation of the food. The paradoxical behaviour of the kidney towards glucose taken alone and that absorbed during the digestion of a mixed meal,

may have a great deal to do with the other digestion products absorbed with the glucose. Hamburger observed that changing the inorganic composition of a perfusate altered the permeability of the frog's kidney towards glucose.

SUMMARY.

1. A trustworthy method for the preparation of the osazones of the urine-sugars is described.

2. When this method is applied to aqueous solutions of glucose or to glucose dissolved in urines from which the normal sugars have been removed, glucosazone is obtained even when the concentration is as low as 1 mg. per 100 cc. In suitable cases glucose added to ordinary urines can be detected when the amount added is as low as 2.5 mg. per 100 cc.

3. Normal urines give numerous types of crystal mixtures, but these have been shown to be formed of a mixture of two simple osazones, one of which is identical with glucosazone and the other appears to correspond with Baisch's *iso*-maltosazone, as regards melting-point, but differs markedly in crystalline appearance.

4. The urines of over 700 persons have been examined under different conditions, and it has been found that typical glucosazone crystals are given by 20–30 % of the urines voided 1–2 hours after an ordinary meal; the percentage drops to 12–15 in urines passed 4–5 hours after meals, and it falls further to approximately 7 % after a 12 hours' fast. That this is, in most cases, not due to an abnormal carbohydrate metabolism, has been shown by examining the tolerance of 28 students to 50 g. glucose.

5. Evidence is given to show that some of Höst's "physiological osazones" are impure crystal mixtures. It is also suggested that the same applies to some of Geelmuyden's osazones.

6. The old view that glucose in small quantities is a constituent of normal urine is confirmed.

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Fig. 1. Osazone I. m.p. 152-153°.
Figs. 2-6. Crystal mixtures.
Fig. 7. Glucosazone "impure."