Specific Enzymatic Determination of Glucose in Blood and Urine Using Glucose Oxidase

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Adequate methods for the determination of glucose in biologic fluids based upon its reducing ability are well established. These methods, however, lack specificity since they will detect reducing substances other than glucose. Improved specificity has resulted from procedures designed to remove interfering reducing substances. These measures have been applied most successfully to blood and plasma.

The procedure for the precipitation of proteins introduced by Somogyi[1] eliminates the great majority of interfering reducing substances likely to be present in blood, such as proteins, amino acids, glutathione, phosphorylated hexoses, and others. This, however, does not make it possible to distinguish glucose from other hexoses (such as fructose or galactose) or from pentoses and from such substances as glyceraldehyde.[2] For fluids such as urine where many of the interfering reducing substances cannot be removed by available technics the need for a specific determination is obvious. Fermentation, formation of osazones, and chromatography have proved their usefulness, but being rather cumbersome and difficult to quantitate, have not found access to routine laboratories.

Muller in 1928[3] and Coulthard and co-workers in 1942[4] isolated an enzyme from *Aspergillus niger* and *Penicillum notatum* which had the unique property of specifically catalyzing the conversion of beta-glucose to gluconic acid. This enzyme, which was given the name of glucose oxidase, has been studied extensively by Bentley and Neuberger[5] and by Keilin and Hartree.[6] Glucose oxidase was shown to be a flavoprotein with a molecular weight of 152,000 and an alloxazine-adenine dinucleotide as a prosthetic group.[7]

The reactions involved in the conversion of beta-glucose to gluconic acid are shown in table 1. Beta-glucose is oxidized to gluconolactone while the prosthetic group of glucose oxidase is reduced. Gluconolactone is then hydrated to gluconic acid, which is the end product of the reaction. Molecular oxygen serves as a hydrogen acceptor for the reoxidation of the reduced flavoprotein, and hydrogen peroxide is formed. The accumulation of hydrogen peroxide would inhibit the further course of the reaction and catalase is therefore needed to catalyze its decomposition to oxygen and water. The over-all reaction results in the incorporation of one atom of oxygen into each glucose molecule, and glucose loses thereby its reducing properties.

TABLE 1
Oxidation of glucose by glucose oxidase

β-glucose + glucose oxidase $\rightleftarrows$ gluconolactone + glucose oxidase H_2
Gluconolactone + $H_2O \rightleftarrows$ gluconic acid
Glucose oxidase H_2 + 2 O $\rightleftarrows$ glucose oxidase + H_2O_2
H_2O_2 + catalase $\rightleftarrows$ H_2O + O
Over-all reaction:
Glucose + ½ $O_2 \rightarrow$ gluconic acid

It is apparent that in order to get complete oxidation of glucose, which in solution is usually present as a constant mixture of 36 per cent alpha-glucose and 64 per cent beta-glucose, glucomutarotation of the alpha to the beta form must occur. This procedure is speeded up by the addition of buffer, an elevation in temperature, and more specifically by an additional enzymatic

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factor, present in many glucose oxidase preparations, which has been named glucomutarotase.[8]

Glucose oxidase has been experimentally used for glucose determinations by Keilin,[9] Mann,[10] and Needham[11] in England and by Ashmore and others[12] in this country. In most instances oxygen uptake during the reaction has been used to quantitate the amount of glucose present. Ashmore, however, used the decrease in reducing ability after glucose oxidase action to estimate the amount of glucose present in fluids used for the incubation of liver slices. Recently, Eli Lilly and Co. has perfected a practical semiquantitative indicator paper for quick appraisal of glucose in urine, based on the formation of hydrogen peroxide during the reaction. We have attempted to develop a quantitative procedure applicable to urine and blood and particularly suitable for the specific determination of small amounts of glucose.

REAGENTS USED

Two commercial glucose oxidase preparations were used (Sigma Corporation, St. Louis, and the Takamine Laboratories, Inc., Clifton, New Jersey). Both preparations contained approximately 1,500 glucose oxidase units per gram of dry crude extract and variable amounts of catalase and glucomutarotase. Each new batch of enzyme was evaluated by incubating it with a known concentration of glucose under standardized conditions to insure the presence of adequate amounts of these two catalytic agents.

To prepare the enzyme solution glucose oxidase was dissolved in acetate buffer (20 mg. enzyme per ml. of acetate), shaken, and centrifuged. The clear yellowish supernatant was used for glucose determinations. If kept in solution in the cold or frozen, little activity was lost, but reducing substances were liberated from the enzyme preparation, causing high enzyme blank values. For this reason a freshly prepared solution was used for each set of determinations. Powdered, dry, glucose oxidase preparations were kept for as long as one year without appreciable loss of activity. They should be protected from excess light.

The other reagents used were as follows:

Acetate buffer, *p*H 5.6 (19 parts 0.154 M acetic acid and 181 parts 0.154 M sodium acetate).
Lloyd's reagent (Hartman-Leddon Co.).
Zinc sulfate, 5 per cent.
Sodium hydroxide, 0.5 normal.
Alkaline copper tartrate and arsenomolybdate reagent according to Nelson.[13]

DETERMINATION OF GLUCOSE IN URINE

Refrigerated urine specimens are collected using toluene as a preservative. Urines giving a blue Benedict's may be used undiluted. Others must have appropriate dilutions made.

One milliliter of urine is pipetted into each of two Wassermann tubes and a drop of caprylic alcohol is added to prevent foaming during aeration. To one tube is added 1 ml. of acetate buffer (tube A) and to the other 1 ml. of enzyme solution (tube B). All B tubes are then aerated for 20 minutes at room temperature, preferably not below 22°C. For the delivery of oxygen a glass manifold is used, fitted with rubber tubing in which are inserted #25 hypodermic needles. Polyethylene tubing is threaded over the needles in appropriate length to reach the bottom of each tube, thereby insuring thorough mixing and oxygenation. Two milliliters of distilled water and approximately 300 mg. of Lloyd's reagent are added to both A and B tubes and the mixture is filtered. One milliliter of the clear filtrate is then analyzed for reducing substances according to the method of Nelson.[13] The values obtained from the A tubes represent total reducing substances, while those from the enzyme tubes represent only nonglucose reducing substances.

An enzyme blank containing 1 ml. of distilled water and 1 ml. of enzyme solution, as well as a 0.4 mg. per ml. glucose standard, are carried through the same procedure. The reading of the enzyme blank must be subtracted from the readings of the enzyme tubes, thereby correcting for any reducing substances in the enzyme solution which were not entirely removed by the Lloyd's reagent. The standard will serve as an indication of the completeness of the glucose oxidation and therefore as a daily check on the activity of the glucose oxidase preparation used. By subtracting B from A, a measure of true glucose is obtained. One person can easily handle 30 determinations per day.

DETERMINATION OF GLUCOSE IN BLOOD

Blood is drawn and preserved in bottles containing sodium oxalate and sodium fluoride. One milliliter of fluorinated blood is laked by the addition of 2 ml. of water. One milliliter of laked blood is treated with 1 ml. of enzyme solution and another milliliter of laked blood is treated with 1 ml. of acetate buffer. One drop of caprylic alcohol is added to each tube and the tubes containing enzyme are aerated with oxygen for 20 minutes at room temperature. Deproteinization of both tubes is then carried out with 1 ml. of zinc sulfate

and 1 ml. of sodium hydroxide. After centrifugation, the supernatant is used for determination of reducing substances according to the method of Nelson.[13] An enzyme blank and a glucose standard of 0.4 mg. are again similarly treated.

Corrections and calculations are identical with those described for the determination of glucose in urine. When determinations must be carried out on capillary blood, the following procedure is recommended. One-tenth milliliter of blood collected from the fingertip is added to each of two tubes containing 0.9 ml. of dilute sodium fluoride solution. Two-tenths milliliter either of glucose oxidase or of acetate buffer is added to each tube, and after incubation deproteinization is carried out with 0.2 ml. of zinc sulfate and 0.2 ml. of sodium hydroxide. Five-tenths milliliter of the clear supernatant is then used for the determination of reducing substances, proper adjustments being made in the final volume of the solution for colorimetry to insure adequate optical density.

EVALUATION OF THE METHOD

The results of the incubation of glucose oxidase with varying amounts of glucose and for different periods of time at room temperature are shown in table 2. One milligram of glucose was oxidized within 20 minutes, whereas 5 and 10 mg. of glucose required two hours of incubation at room temperature for completion of the reaction. From this and similar experiments it was concluded that glucose concentrations greater than 1.0 mg. per ml. of incubation sample should be avoided when using an incubation period of 20 minutes at room temperature. A small quantity of reducing substances, probably glucose, amounting to between 3 and 4 per cent of the initial glucose incubated with the enzyme was regularly found to remain at the end of incubation. By raising the temperature 37°C., completion of glucose oxidation could be achieved (table 3). On the other hand, the incubation at lower temperature resulted in a marked increase in the non-oxidized glucose remnant. It is important therefore to control the specific conditions in each instance by incubating a known amount of glucose along with the unknown samples. When maximal accuracy is needed, incubation should be carried out at 37 to 40°C., when glucose oxidation can be expected to go to completion.

Recovery experiments are illustrated in table 4. Twenty, 50, 100, and 200 mg. of glucose were added to one liter of each of two urine specimens which were found to contain 40 and 36 mg. per liter. The recoveries of these small amounts of glucose varied from 86 to 107 per cent. The errors, expressed in milligrams per liter, ranged from 8 to 16 mg. Thirty milligrams per liter, or 3 mg. per 100 ml., can therefore easily be detected and an increase of as little as 20 mg. per liter above the base level can be considered significant.

TABLE 2

Glucose oxidation during incubation with glucose oxidase at 25°C.

Glucose added	Per cent glucose oxidized after: 20 min.	60 min.	120 min.
1 mg. per ml.	94	96	95
5 mg. per ml.	86	93	97
10 mg. per ml.	54	84	97

TABLE 3

Glucose oxidation during incubation with glucose oxidase at varying temperatures

Incubating temperature	Per cent glucose remaining after incubation of 1 mg. with glucose oxidase for 20 minutes
5°C.	9.2
25°C.	4.0
37°C.	1.0 0 0.5

TABLE 4

Recovery of glucose added to 1,000 ml. of urine

Sample	Glucose added (mg.)	Glucose expected (mg.)	Glucose measured (mg.)	Per cent recovered
Urine A	None	—	36	—
	20	56	48	86
	50	86	80	93
	100	136	146	107
Urine B	None	—	40	—
	200	240	248	103
			252	105
			248	103
			256	106

The great specificity of this enzyme for beta-glucose has been studied extensively by Keilin and Hartree.[14] In good agreement with these investigators we detected slight activity only toward mannose and xylose. At the end of an incubation period of 20 minutes at room temperature, 6.5 per cent of 0.4 mg. of mannose and 4.2 per cent of 0.4 mg. of xylose were oxidized (table 5). Raising the temperature to 37°C. had no appreciable effect on the oxidation of mannose and xylose.

The total absence of activity toward fructose and galactose should be stressed and is further illustrated by the blood values obtained during a fructose tolerance test (table 6). Fructose was measured with resorcinol

TABLE 5

Specificity of enzyme preparation used

	Per cent of sugar (0.4 mg. per ml.) oxidized after 20 minutes aeration
Hexoses	
Glucose	96
Fructose	<1
Galactose	<1
Rhamnose	<1
Sorbose	<1
Mannose	6.5
Pentoses	
Xylose	4.2
Ribose	<1

TABLE 6

Fructose tolerance test (0.5 gm. per kilogram over 10 minutes) in a normal subject

Time (minutes)	Glucose oxidase method		Resorcinol method
	Glucose (mg. per ml.)	"Fructose" (mg. per ml.)	Fructose (mg. per ml.)
0	46	0	0
10	49	153	157
20	40	65	68
30	49	38	39
40	46	27	25
60	43	19	20

according to Higashi's modification of Roe's method[15, 16] and by the present method using glucose oxidase. In the latter instance it was assumed that under fructose tolerance conditions the nonglucose reducing substances present in blood after glucose oxidase action would mainly represent fructose. The values obtained for fructose by both methods are in good agreement; the advantage of the glucose oxidase method resides in the simultaneous measurement of glucose and fructose. The same applies to galactose and may prove of particular value in this instance, since convenient methods for the determination of galactose are not available.

PRACTICAL APPLICATIONS

The method has been extensively applied in this laboratory and glucose determinations have been carried out in over two thousand urine and blood samples. The daily excretion of glucose by normal subjects and by patients without anomalies of carbohydrate metabolism was found to vary within surprisingly narrow limits.

The figures obtained in 30 normal subjects are shown in table 7. It is of interest that glucose represents a relatively small percentage (averaging 14 per cent) of the total reducing substances present in urine after treatment with Lloyd's reagent. The nonglucose reducing substances, expressed as glucose equivalents, ranged from 218 to 809. The range of glucose excretion from day to day in a single metabolically normal patient is shown in figure 1. During 78 days, while on diets with different carbohydrate content, glucose excretion varied from 10 to 239 mg. per day, with a mean of 108 mg. and a standard deviation of ± 52.6. A glucose infusion of 25 gm. over eight hours was without effect on glucose excretion. In contrast, very significant changes were observed when carbohydrate active adrenal steroids were added to the infusion (figure 2). When 200 mg. of hydrocortisone was infused over eight hours the urinary excretion of glucose during the corresponding 24-hour period rose to 1,900 mg. Fifty milligrams of Meticortandrolone and 10 mg. of Fluorometicortandrolone produced an increase in the urinary excretion of glucose to approximately 1,000 mg. for the corresponding 24-hour period. These results are presented at this time to illustrate the possible usefulness of this para-

TABLE 7

Twenty-four-hour urinary glucose excretion in 30 normal subjects

	"Glucose" as measured by conventional methods (mg.)	Glucose as measured by glucose oxidase method (mg.)	Nonglucose reducing substances (mg.)
Mean	511	72	439
Range	242-845	16-132	218-809

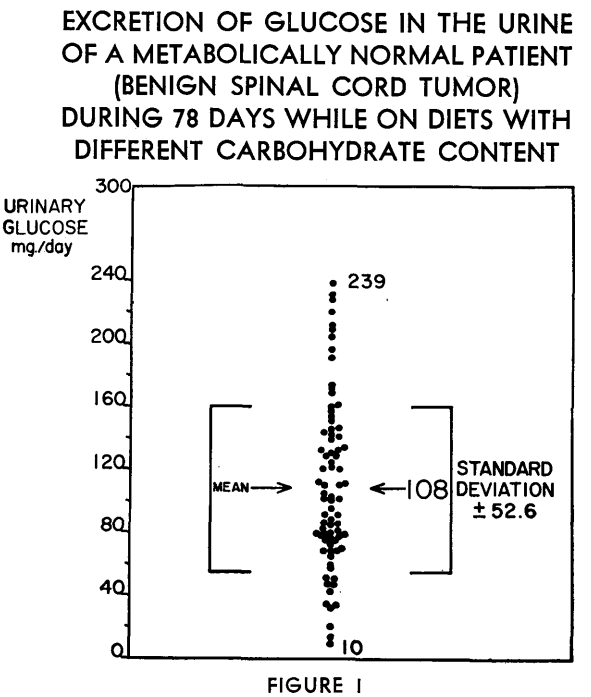

FIGURE 1

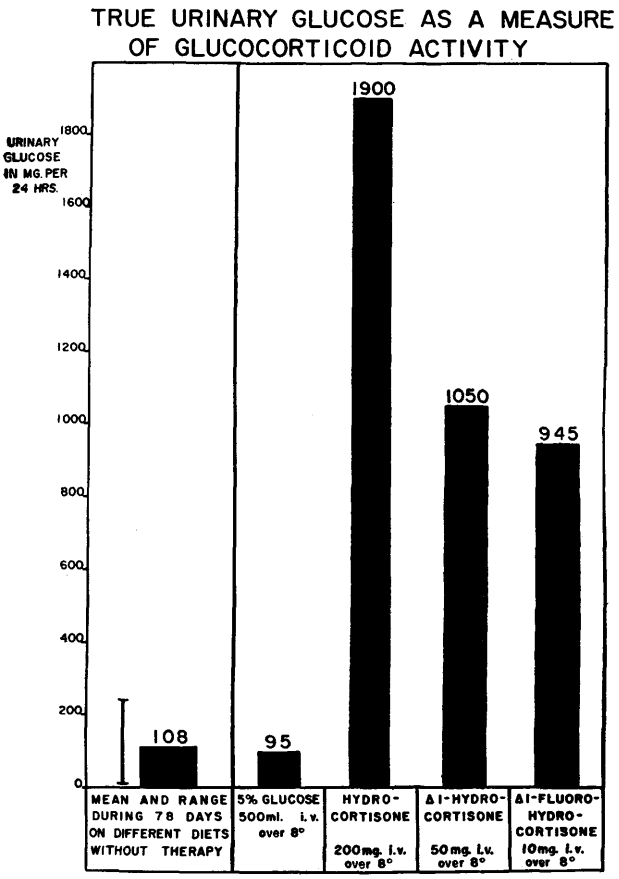

FIGURE 2

meter in the evaluation of hormonal and other effects on carbohydrate metabolism. Such studies are at present being carried out.

SUMMARY AND CONCLUSIONS

A quantitative and relatively simple method for the specific determination of glucose in biologic fluids, using the enzyme glucose oxidase, has been presented. Specificity results from the exclusive oxidation of beta-glucose to gluconic acid by this enzyme. Slight interference might be expected only from mannose and xylose, toward which glucose oxidase shows some activity. The method was found to be especially suitable for the accurate determination of small amounts of glucose in urine and for the simultaneous measurement in blood of glucose and other hexoses such as fructose or galactose during tolerance tests with these substances.

Glucose excretion as measured in 30 normal subjects ranged from 16 to 132 mg. per day, averaging 72 mg. It seemed to be relatively independent of the dietary carbohydrate intake. Significant increases in glucose excretion were observed after administration of carbohydrate active steroid. Glucose excretion as measured by this specific method might prove to be a sensitive index of alterations in carbohydrate metabolism.

SUMMARIO E CONCLUSIONES IN INTERLINGUA

Es presentate un relativemente simple methodo quantitative pro le determination specific de glucosa in fluidos biologic per medio de oxydase de glucosa. Le specificitate del resultatos es conditionate per le facto que iste enzyma effectua un oxydation exclusive de glucosa beta in acido gluconic. Leve incorrectiones pote resultar de mannosa e xylosa, relative al quales oxydase de glucosa monstra un certe activitate. Iste methodo se provava specialmente utile in le determination exacte de parve quantitates de glucosa in urina e in le determination simultanee in sanguine de glucosa e altere hexosas, como fructosa o galactosa, durante tests de toleration executate con iste substantias.

Le excretion de glucosa, mesurate in 30 normal subjectos, variava ab 16 a 132 mg per die, con un valor median de 72 mg. Illo pareva esser relativemente independente del ingestion dietari de hydratos de carbon. Augmentos significative in le excretion de glucosa esseva observate post le administration de steroide active a hydrato de carbon. Le excretion de glucosa mesurate per iste specific methodo va possibilemente monstrar se un sensibile indice de alterationes in le metabolismo de hydratos de carbon.

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DISCUSSIONS

DAVID ADLERSBERG, M.D., (*New York*): Was a comparison made of the effects on these urinary sugars between cortisone, hydrocortisone, and the delta compounds of these hormones?

DR. FROESCH: We have begun such a study, but it is not as yet completed. Preliminary results would indicate that Meticorten exerts four to five times as much glucocorticoid activity as hydrocortisone, while 9-alpha-fluorometicorten is twenty to twenty-five times as potent as hydrocortisone.

RANDALL G. SPRAGUE, M.D., (*Rochester, Minnesota*): I should like to congratulate Dr. Froesch on an excellent presentation.

One question, Dr. Froesch. Did the increased excretion of glucose in response to hydrocortisone reflect an increase in blood glucose; or was it a renal glycosuria?

DR. FROESCH (*closing*): We have tried to assess whether the corticoid-induced enhancement of urinary excretion of glucose is due to an increase in blood glucose level or to changes in the handling of glucose by the kidney. Inulin clearance was measured before and during the acute administration of high doses of cortisone. Tubular glucose reabsorption was found to be slightly depressed and glomerular filtration rate somewhat increased under the influence of cortisone. It would appear that glycosuria results from the combined effects of increased renal glucose load (due to higher postprandial blood glucose levels and a slightly increased glomerular filtration rate) and of decreased tubular glucose reabsorption.

Food Idiosyncrasies

Personal idiosyncrasy will explain many instances in which food is digested with apparent difficulty. This has been repeatedly emphasized by Alvarez. It must be assumed, as this author suggests, that many persons are peculiarly sensitive to some chemical substance carried by an offending food or to its physical state. Else why should canteloupe, for example, disagree as it sometimes does with a person with otherwise excellent digestion, while it gives comfort to another with apparently poor digestion? . . .

The recognition of the particular food to which a person is allergic is not always easy. Cutaneous tests are a disappointment and are seldom to be relied upon. The same applies to other forms of food idiosyncrasy and in this as in many other fields there is no short cut to diagnosis. The use of elimination diets is often of help; but as a rule, painstaking questioning of the patient who has been prompted to intelligent observation brings the best results.

From the book *Nutrition and Diet in Health and Disease* by James S. McLester, M.D., and William J. Darby, M.D., Ph.D. Philadelphia, W. B. Saunders Co., 1952, 6th ed., pp. 141-44.