# A Simplified Quantitative Enzymatic Procedure for Glucose in Urine

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SUMMARY

A simplified resin treatment for removal from urine of substances which inhibit the glucose oxidase peroxidase system of analysis of glucose is described. The original technic designed for AutoAnalyzer use has been modified for a manual procedure. The cost of enzyme reagents and resin has been minimized by use of micro proportions.

The enzymatic principle for determination of glucose in blood, serum and plasma is well established. Application of the glucose oxidase procedure to urine has been more limited, mainly due to the necessity of removing inhibitory substances from urine. Removal of these substances by adsorption, using a mixture of activated charcoal and Lloyd's reagent (hydrated aluminum silicate), has been found more satisfactory than the use of either component singly.[1] The ingredients must be carefully adjusted because it is known that charcoal itself adsorbs glucose.[2,3] Treatment of urine with resin has been described by Salomon and Johnson,[3] and has been simplified for use with the enzymatic procedure on the AutoAnalyzer in our laboratory.[4] Although automated procedures are becoming more widespread in use, there is still, and undoubtedly always will be, a need for simplified manual methods. Consequently the technic for the removal of inhibitory substances by means of a mixed bed resin has been investigated for adaptation to a manual glucose oxidase method. In this investigation we have found that the proportion of specimen to enzyme reagent may be adjusted so that ultramicro quantities of specimen may be assayed efficiently and conveniently. Precalibrated capillary tubes, 20 lambda volume, were extremely convenient to use and gave reproducible results. An economical amount of enzyme reagent (1 ml.) gave satisfactory photometric sensitivity. This simplification of the enzymatic procedure should help to make more practicable a method specific for urinary glucose. DIABETES *15*:127-30, February, 1966.

## MATERIALS AND METHODS

*Specimens* of urine* were obtained from the Urinalysis Laboratory of the Ottawa Civic Hospital. A number of "laboratory" specimens were prepared from normal urine (showing no glucose) with added amounts of glucose and uric acid. Uric acid present in the normal urine was first removed by treatment with resin.[4]

Added glucose was allowed to stand at least two hours for mutarotation of α- to β-glucose. Uric acid to give concentrations ranging from 5 to 200 mg. per 100 ml. was put into solution by addition of lithium carbonate in concentration of 60 mg. per 100 ml. resin-treated urine.

Commercially packaged *enzyme reagents* were obtained from Worthington Biochemical Corporation, Freehold N.J., "Glucostat"; from Dade Reagents Incorporated, Miami, Fla., "Glucose Oxidase Reagent"; and from Clinton Laboratories, Los Angeles, California, "Glucose Reagent."

Enzyme reagent was made in the laboratory from ingredients procured from Worthington Biochemical Corporation as follows: Glucose oxidase 125 mg., peroxidase 5 mg., dissolved and made up to 100 ml. volume with M/15 phosphate buffer, *p*H 7, including the incorporation of 1 ml. o-dianisidine solution (100 mg. o-dianisidine dissolved in 10 ml. absolute methyl or ethyl alcohol).

A mixed bed *resin** was prepared for use as described previously.[4]

*Glucose standards* were prepared in saturated benzoic acid (50, 100, 200 mg. glucose per 100 ml.). It was found that the buffering capacity of the enzyme preparation was adequate to take care of the acidity of the benzoic acid in the standards in the proportions used.

For measurement of specimen "Microcaps," 20 lambda size, disposable *micropipettes* made from precision bore tubing, labeled accuracy ±1 per cent stated volume, Drummond Scientific Company, Broomall, Pa., were used. Sahli hemoglobin pipettes of suitable accuracy, ±1 per cent, could be used but are considerably less convenient.

*Urine was treated with resin* by (a) "macro" method, as described by Logan and Haight,[4] and (b) "micro"

*Preservative tablet: F. B. Kingsbury Formula, R. P. Cargille Laboratories, Inc., 117 Liberty St., New York, N. Y.

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*La Motte Chemical Products Co., Chestertown, Maryland.

method, as follows:

A volume of 20 lambdas resin-treated specimen was found to give suitable color development with reasonable economy of enzyme reagent. Trials of small quantities of resin and urine led to the final recommendation of 0.2 gm. resin with ten drops of urine. Polystyrene AutoAnalyzer sample cups were used because they were readily available; any small dish or watch glass could be substituted. Portions of 0.2 gm. resin were measured into a number of containers ready for use as required. Ten drops of urine (measured by a dropper from which twenty drops delivered 1 ml.) were added to the resin, mixed by stirring or by shaking for twenty seconds and allowed to stand for five minutes. Thorough mixing of resin with urine was found to be essential. A waiting time of five minutes was found to be a generous allowance for making certain that the adsorption had been completed. The resin was then pushed to one side of the container to allow the clear liquid to be withdrawn with a pipette.

*Glucose oxidase procedure.* Specimens were tested qualitatively before analysis with enzyme paper Tes-Tape* or with a copper reduction tablet Clinitest.† Specimens which rated +++ or ++++ were diluted 1:5 or 1:10 with distilled water before resin treatment.

Methods based on two commonly used procedures were tried, viz: stopping the enzyme reaction with a dilute solution of acid, final color yellow[1,5] and stopping the reaction with fairly concentrated acid, final color pink.[6-8] It was shown by using a Beckman DB spectrophotometer in conjunction with a Sargent Recorder that the peak absorption for the pink color of the o-dianisidine reaction with 7 N $H_2SO_4$ was at 524 m$\mu$ and for the yellow color with N $H_2SO_4$, 396 m$\mu$. Consequently, optical density readings in two spectrophotometers, a Beckman DB and a Bausch and Lomb Spectronic 20, were made at these wave lengths. It was found that the stronger acid (7N $H_2SO_4$) resulted in a more pronounced intensity of color and a wider range of optical density values than was the case with these same glucose solutions with the weaker acid (N $H_2SO_4$) e.g. O.D. 0.19, 0.39, 0.75 c.f. 0.11, 0.22, 0.43 respectively.

The method subsequently used for quantitative estimation was as follows: 20 lambdas of resin-treated specimen were measured in a "microcap." This was dropped into a tube containing 1 ml. enzyme—dye preparation (glucose oxidase, peroxidase, o-dianisidine in phosphate buffer *p*H 7). With parafilm over the end of the tube, the contents were shaken vigorously to mix thoroughly, then placed in a water bath at 37° C. At the end of one hour, 5 ml. 7 N $H_2SO_4$ was added, and the tube was brought to room temperature prior to reading of optical density at 524 m$\mu$. The reaction progresses at a rate such that after fifty to sixty minutes the comparative length of timing of tubes of specimen and standard is not critical, i.e., a difference in timing of five minutes may not be significant. On the other hand, after shorter times such as ten minutes the accurate timing of specimen and standard is critical; a difference of a few seconds may be significant.

Each of the commercial enzyme preparations tested gave satisfactory results. It was noted that it was necessary sometimes to adjust the final volume of the prepared reagent, for example, Glucostat X4 was made up to 300 ml. rather than 360 ml. in order to yield colors which were sufficiently intense for optimal spectrophotometric readings, without increasing the volume of the specimen. This was particularly noticeable with the yellow colors produced with dilute acid.

## RESULTS AND DISCUSSION

Comparison of the automated glucose oxidase procedure[4] with the procedure described above, using the "macro" method of resin treatment, showed no significant difference between the two methods; $P>0.05$, and no significant difference in the precision.[9]

Quantitative analyses of seven hospital specimens using the "macro" resin treatment and the "micro" methods (0.2 gm. resin) with ten, twelve and fifteen drops urine showed no significant difference in the precision of the methods and no significant difference in the results as evidenced by the F test. The ten drops quantity was adopted for convenience in all later testing.

Comparison of macro and micro methods of resin treatment on a series of seventy-four specimens, hospital and normal urines with added glucose and uric acid, showed no significant difference by the *t* test, $P>0.7$.

Results from quantitative analyses of "laboratory" specimens i.e., normal urine with added quantities of uric acid (five concentrations) and glucose (four concentrations) are listed in table I. Appreciable inhibition of the enzyme reaction by uric acid is noticeable, particularly with smaller amounts of glucose (50 and 200 mg.) and with increasingly large amounts of uric acid (20, 50 and 100 mg. per 100 ml.). After treatment with resin there was satisfactory recovery of glucose regardless of the

*Eli Lilly and Company (Canada) Ltd., Toronto.
†Ames Company of Canada, Ltd., Toronto.

TABLE 1

*Glucose recovery per cent*: Normal urine with added glucose and uric acid*

| Glucose added (mg./100 ml.) | Uric acid added (mg./100 ml.) | | | | |
|---|---|---|---|---|---|
| | 5 | 10 | 20 | 50 | 100 |
| 50 | 100 | 79 | 70 | 37 | 21 |
| 200 | 90 | 87 | 80 | 89 | 38 |
| 500 | 92 | 92 | 92 | 89 | 76 |
| 1,000 | 95 | 92 | 98 | 92 | 87 |

Specimens as above, i.e., glucose 50 to 1,000 mg./100 ml., uric acid .5 to 100 mg./100 ml. resin treated; glucose recovery 101 per cent, S.D. ± 7 per cent, n = 93.

*Original glucose in urine, nil: original uric acid in urine removed by pretreatment with resin.

quantity of uric acid which had been present originally or of the concentration of glucose.

The cost of the enzyme reagents and of the resin for the manual micro method is less than 1/2 and 1/30 respectively that of the AutoAnalyzer method: i.e., for AutoAnalyzer 8.5 cents per specimen; for micro method 0.8 cents per specimen. When the disposable precalibrated capillary tubes are used for measurement of the specimen, approximately 4.5 cents per test must be added to the cost. When allowance is made for the saving of time in measurement and in washing the more conventional Sahli type pipette it is probable that the expense is justified.

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