SCREENING METHOD FOR GLUCOSE OF BLOOD SERUM UTILIZING GLUCOSE OXIDASE AND AN INDOPHENOL INDICATOR

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Approximately 80,000 blood specimens are submitted to this bureau annually for syphilis serology testing, and it was proposed¹ that these specimens be further employed for purposes of diabetic screening. To carry out this type of program, it was desirable to devise a rapid method for the mass determination of glucose levels in blood serum. This paper describes such a method.

It is well known that established methods which require a protein-free filtrate are generally unsuited for the estimation of blood glucose on a large scale. Wilkerson and Heftmann (1), however, have devised a screening method which, with the aid of special apparatus, has been successfully applied to the routine examination of large numbers of specimens.

In recent years, the enzyme, glucose oxidase, has been employed toward a more accurate appraisal of glucose in biological fluids (2). Through the manometric measurement of oxygen uptake, Keilin and Hartree (3) have demonstrated that the oxidation of glucose proceeds specifically and quantitatively in the presence of this enzyme. The hydrogen peroxide liberated in the reaction has been utilized by a recently marketed paper tape to yield a chromogenic oxidation product, the color intensity of which is semiquantitatively proportional to glucose concentrations in urine and blood plasma (4).

The method described herein is based upon our independent finding that the oxidation-reduction indicator, 2,6-dichlorobenzenone-indophenol, can replace molecular oxygen as the hydrogen acceptor in the glucose-glucose oxidase reaction. The literature discloses, however, that this finding in fact had only confirmed earlier observations (5). In demonstrating that under anaerobic conditions a quantitative as well as a qualitative relationship exists between glucose oxidized and indophenol reduced, a new method is evolved which is particularly applicable to the mass screening of glucose levels in blood sera.

¹ Proposed by Dr. Walker L. Loving, Director, Bureau of Laboratories.

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SCREENING METHOD FOR GLUCOSE

EXPERIMENTAL

Materials and Method

Reagents—

1. Stock indicator. 100 mg. of sodium 2,6-dichlorobenzene-indophenol (Eastman 3463) are accurately weighed and diluted to 100 ml. with dilution reagent given below (1 ml. = 1000 γ of indophenol).²

2. Dilution reagent. 250 ml. of an aqueous solution are prepared to contain 40 per cent v/v propylene glycol, 1 per cent v/v ethyl alcohol, and 0.2 per cent w/v sodium bicarbonate.

3. Screening indicator. This solution is made up in accordance with the day's requirements. A preparation from standardized stock is given under "Procedure," Step II.


5. Stock buffer, pH 4.6. 14.0 gm. of citric acid·H₂O and 16.6 gm. of anhydrous disodium hydrogen phosphate are diluted to 100 ml. with water and mixed with slight warming until dissolved.

6. Buffer-enzyme mixture. By volume, 6 parts of stock buffer, pH 4.6, are mixed with 3 parts of water and 1 part of stock enzyme.

7. Stock glucose standard. An accurately prepared 1 per cent w/v glucose solution in 0.6 per cent w/v sodium fluoride.

8. Glucose working standards. Aliquots of stock glucose are diluted with 0.6 per cent sodium fluoride to give desired concentrations.

9. Light petroleum oil.

Apparatus—

Test tubes (125 mm. X 15 mm.) are used and into each is placed a small glass stirring rod (about 20 mm. X 3 mm.) for mixing purposes.

Procedure

Step I: Standardization of Stock Indicator; Leuco Point at 100 Mg. per 100 Ml. Glucose Level—A series of five test tubes is arranged in a metal rack with dimensions suitable for keeping the tubes in a vertical position. To each tube is added 0.5 ml. of glucose working standard (100 mg. per 100 ml.), followed by 1 ml. of light petroleum oil. To the first tube is added 0.60 ml. of stock indicator. Successively increasing amounts, in increments of 0.04 ml., are placed in the four succeeding tubes, resulting in the concentrations of indophenol which range from 600 γ through 760 γ. Each tube now receives an appropriate volume of dilution

* Formula weight, 340 (sodium 2,6-dichlorobenzene-indophenol·2.78 H₂O) as determined iodometrically.
reagent so that the combined volume of indicator and dilution reagent in each instance totals 1.5 ml. 0.5 ml. of buffer-enzyme mixture is finally added to each of the five tubes.

The rack is now rotated in a horizontal plane to insure mixing of the reagents. It is placed in a water bath at 37–40° and again mixed at 15 minute intervals. After a 30 minute reaction period, the point of delineation between colored and colorless (leuco) solutions is observed. Henceforth, this demarcation will be referred to as the leuco point. It represents the maximal amount of indophenol that is visibly reduced to the leuco state by 0.5 ml. of glucose standard under specified conditions. In our experiments the leuco point for a glucose concentration of 100 mg. per 100 ml. was established as requiring 0.64 ml. of indicator or 640 γ of indophenol. By starting with lesser or greater amounts of stock indicator, the leuco points of lower or higher concentrations of glucose can be similarly established.

**Step II: Preparation of Screening Indicator at 100 Mg. per 100 Ml. Glucose Level**—A screening indicator solution is prepared so that each 1.5 ml. aliquot contains 60 γ of indophenol in excess of the established leuco point. Consequently, for purposes of screening at the 100 mg. per 100 ml. of glucose level, the stock indicator is diluted with dilution reagent to an indophenol concentration of 700 γ per 1.5 ml. With this volume added to each tube, only sera containing glucose in excess of the screening level will exhibit colorless reaction products. In a like manner a screening indicator can be prepared for any desired glucose level.

**Step III: Screening at 100 Mg. per 100 Ml. Level**—For the actual test a single tube is employed for each specimen. For maximal efficiency, forty to fifty specimens should be screened. The mechanics are essentially the same as in Step I. Specimens and reagents are added in the following order: 0.5 ml. of serum, 1 ml. of light petroleum oil, 1.5 ml. of screening indicator, and 0.5 ml. of buffer-enzyme mixture.

At the end of the reaction period, the tubes are scanned for changes in color which may range from a practically unchanged deep blue to light blue, to colorless, indicating glucose levels below 100 mg. which approximate 100 mg., and above 100 mg. per 100 ml., respectively.

**Results**

**Quantitative Relationship between Glucose Oxidized and Indophenol Reduced**

The leuco points of eight glucose standards with concentrations ranging from 40 through 180 mg. per 100 ml. were determined at increments of 20 mg. On plotting micrograms of glucose oxidized against micrograms of indophenol reduced to the leuco state by each of the eight standards, a
linear quantitative relationship, as shown by Fig. 1, Curve I, becomes apparent. The curve further indicates that, for every increment of 100 parts of glucose oxidized, there is a corresponding increment of 180 parts of indophenol reduced.

Whereas 1 mole of glucose (formula weight 180) will theoretically reduce 1 mole of sodium 2,6-dichlorobenzenone-indophenol (formula weight 340) on plotting their stoichiometric relationship, the slope of the resulting curve (Fig. 1, Curve II) is found to be nearly identical with the curve obtained experimentally. From this close resemblance, it is inferred that the incremental ratio between glucose oxidized and indophenol reduced corresponds to the stoichiometric ratio.

Concordant results were obtained on substituting glucose serum standards\(^3\) for aqueous standards. It seems, therefore, that the protein of blood serum does not affect the over-all reaction between indophenol and glucose. Accordingly, for screening blood sera, a protein correction is not required when aqueous glucose standards are applied to the standardization of the indophenol indicator.

\(^3\) Prepared by diluting appropriate amounts of stock glucose standard with a pooled glucose-free serum.
Reduction of Indophenol by Serum Substances Other Than Glucose

70 human blood sera were tested to determine the amount and extent of indophenol-reducing substances other than glucose in different specimens. Except for the omission of glucose oxidase, this examination was conducted under conditions of the actual screening method. The screening indicator was adjusted to contain 40 μ of indophenol per 1.5 ml. The amount of indophenol remaining after the reaction period was ascertained by comparisons with previously prepared indicator solutions containing from 5 μ through 40 μ of indophenol at intervals of 5 μ. The difference between the amount of indophenol originally added and the quantity remaining became a measure of the indophenol reduced. Because glucose oxidase was not used, any reduction of indophenol was, in this instance, attributed to substances other than glucose.

![Image](http://www.jbc.org/)

**TABLE I**

*Amount and Extent of Indophenol Reduced by Substances Other Than Glucose in 70 Individual Blood Serum Specimens*

<table>
<thead>
<tr>
<th>No. of specimens</th>
<th>Indophenol added (μ)</th>
<th>Indophenol remaining (μ)</th>
<th>Indophenol reduced (μ)</th>
<th>Specimens of total per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>40</td>
<td>40</td>
<td>0</td>
<td>22.9</td>
</tr>
<tr>
<td>7</td>
<td>40</td>
<td>35</td>
<td>5</td>
<td>10.0</td>
</tr>
<tr>
<td>42</td>
<td>40</td>
<td>30</td>
<td>10</td>
<td>60.0</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>25</td>
<td>15</td>
<td>1.4</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>20</td>
<td>20</td>
<td>5.7</td>
</tr>
</tbody>
</table>

On referring to Table I, it may be noted that 93 per cent of the specimens reduced 10 μ or less of indophenol, whereas the remaining 7 per cent reduced a maximum of 20 μ. In terms of glucose, the maximal error due to reducing substances other than glucose is approximately 2 mg. per 100 ml. and the most probable error only 1 mg. per 100 ml.

**Screening of Blood Sera**

Preliminary investigation revealed that bloods for serosyphilitic examination were usually drawn 18 to 24 hours before testing. During this interval, it was observed that glycolysis occurred in normal bloods to the extent that their serum glucose values fell below 100 mg. per 100 ml. On the other hand, blood from known diabetics, with original glucose concentrations of 160 mg. per 100 ml. or more, still yielded, after 24 hours at room temperature, serum glucose values exceeding 100 mg. per 100 ml. Our choice for screening at the 100 mg. glucose level is based on these observations. The method, it may be emphasized, can be easily adapted to screen at any other desired level of glucose.
Of the 187 specimens screened, 89 per cent showed a decidedly blue color (negative, <100 mg. per 100 ml.), 6 per cent light blue (borderline, 100 mg. per 100 ml. ±10), and 5 per cent colorless (positive, >100 mg. per 100 ml).

For purposes of comparison with the Somogyi-Nelson method (6, 7), random samples were chosen from specimens showing negative reactions. Those specimens showing borderline or positive results, however, were taken in their entirety for analysis.

From data given in Table II, the method is observed to be reliable to within 10 mg. of the designated screening level. Every tube which exhibited color possessed a glucose concentration which in no instance exceeded the screening level by more than 7 mg. All colorless tubes had glucose concentrations which, in every case, were greater than the screening level.

Plasma, from a limited number of oxalated whole bloods, was screened in the same manner as serum. Our findings indicate that the method is equally applicable to blood plasma.

**DISCUSSION**

With the exception of substituting indophenol for molecular oxygen as the hydrogen acceptor, the reactions involved in the foregoing experiments are believed to be similar to those proposed by earlier investigators.

The reactions may be represented as follows: glucose + glucose oxidase = gluconolactone + glucose oxidase H₂; gluconolactone + water = gluconic acid; glucose oxidase H₂ + indophenol = glucose oxidase + reduced indophenol; over-all reaction = glucose + indophenol + water = gluconic acid + reduced indophenol.

Reagents containing indicator, enzyme, or glucose should be stored at refrigerator temperature. Because there is a slight degradation of the
stock indicator over a period of about 2 weeks, it is advisable to determine the leuco point before each day's run. After an elapse of a month, this reagent should be freshly prepared.

At pH 4.6, the indicator assumes a decidedly red-violet hue which in the presence of serum changes to a strong blue-violet. The difference in color is believed to be due to a loose attachment of the dye to the protein of the serum.

Because no further color changes could be discerned at the end of 30 minutes, this time was taken as the period of completed reaction.

Having determined the leuco point for the desired screening level, an individual may easily screen forty serum specimens in 0.5 hour of working time.

SUMMARY

1. It has been demonstrated that a direct quantitative linear relationship exists between glucose oxidized and indophenol reduced. The inter-relation is such that for every increment of 100 parts of glucose oxidized there is a corresponding increment of 180 parts of indophenol reduced. This incremental ratio approaches stoichiometric proportions.

2. A method is described which is particularly applicable to the mass screening of blood serum glucose. The operational step of removing protein is eliminated. Glucose concentrations of serum or plasma can be directly classified, with a reliability of 10 mg. per 100 ml., as being above or below any predetermined screening level.

BIBLIOGRAPHY

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