APPLICATIONS OF DIPHENYLAMINE IN THE DETERMINATION OF LEVULOSE IN BIOLOGICAL MEDIA

I. THE DETERMINATION OF INULIN

II. THE DETERMINATION OF LEVULOSE IN SMALL AMOUNTS OF BLOOD

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The Determination of Inulin

The formation of a blue compound of diphenylamine and levulose in the presence of concentrated HCl was applied semi-quantitatively to urine by Jolles (1) and quantitatively to blood filtrates by van Creveld (2), whose method for blood levulose has since been variously modified (3-7). In the van Creveld method and its modifications, the reaction was carried out in an aqueous medium and the water-insoluble blue product extracted into an organic solvent for color comparison. This disadvantage was removed by Herbert (8) by the addition of sufficient alcohol to keep both diphenylamine and colored product in solution during and after the reaction. The method here described is an application of Herbert's method to the determination of inulin, the final determination being made in the single cell, compensating photoelectric colorimeter described by Evelyn (9). Glucose and levulose present in the material tested are removed by fermentation with yeast. The inulin is hydrolyzed by the strong acid in which the reaction occurs.

Reagents—

1. Precipitating media (10). (a) 1.25 per cent ZnSO₄·7H₂O, in 0.03125 N H₂SO₄; (b) 0.75 N NaOH.

Although these concentrations of reagents are those recommended by Somogyi (10) for whole blood rather than plasma,
they have been found quite satisfactory and are employed because of their general use in this laboratory. The weaker reagents recommended for plasma may be substituted without affecting this method.

2. Stock diphenylamine solution. 20 per cent diphenylamine in alcohol.

The stock solution is kept in a cool place in a dark bottle. Diphenylamine which precipitates out at ice box temperatures is easily redissolved as the solution is brought to room temperature. The diphenylamine used was obtained from the Eastman Kodak Company.

3. Acid alcohol-diphenylamine reagent. 95 per cent ethyl alcohol, 70 parts; concentrated HCl, 50 parts; stock 20 per cent diphenylamine solution, 6 parts.

This solution is also kept in a cool place in dark bottles. Although it is stable for at least 1 month (8), we have prepared it weekly.

4. Thoroughly washed bakers' yeast.

Bakers' yeast (obtainable from Standard Brands, Inc.) is washed by centrifugation with distilled water until the supernatant is clear for two washings.

**Apparatus**

1. Water bath with removable tube rack.
2. Tall resistance glass test-tubes graduated at 25 cc.
3. Photoelectric colorimeter of the type described by Evelyn (9), equipped with a light filter transmitting at a maximum of about 620 m\(\mu\).1

**Procedure**

*Serum or Plasma Inulin*—(a) 1 part of serum or plasma is added to 8 parts of \(\text{ZnSO}_4\) solution, mixed, and the protein precipitated by addition of 1 part of 0.75 \(\text{N NaOH}\). The volume of filtrate obtained from small samples (0.5 or 1.0 cc.) of plasma or serum may be increased by centrifuging the mixture before filtration. After filtration, the protein-free filtrate is placed in a centrifuge tube which contains about 1/3 volume of thoroughly

1 The photoelectric colorimeter, light filters, and absorption tubes may be obtained from the Rubicon Company, Philadelphia.
packed yeast. These tubes are prepared by adding the required volume of 1:1 suspension of washed yeast in water to a centrifuge tube, centrifuging at 2800 R.P.M. for 20 minutes, pouring off the supernatant, and allowing the tubes to dry during inversion in a rack. The water is then removed from the lips of the tubes, and droplets which may have clung to the tube wall removed with clean gauze.

(b) The yeast is stirred into the plasma filtrate with a clean dry rod and the mixture allowed to stand with occasional shaking for 15 to 30 minutes. The tubes are then centrifuged at the rate and for the length of time used in packing the yeast from its water suspension. The supernatant fluid is then decanted through small filter papers.

(c) A 1 cc. portion of filtrate is transferred to a tall test-tube graduated at 25 cc., or, if deposits from the water bath can be excluded, into a colorimeter absorption tube (8) which has been graduated at this mark. The sides of the tube are washed down

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**Fig. 1.** The ordinate represents color density (L value (9)); abscissa: Curve A, inulin determination, levulose equivalent in mg. per 100 cc. of protein-free filtrate or water; Curve B, levulose determination, levulose in mg. per 100 cc. of blood; Curve C, levulose determination of glucose equivalent in mg. per 100 cc. of blood.
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during the addition of 5 cc. of acid alcohol-diphenylamine reagent and the tubes at once placed in a boiling water bath for exactly 15 minutes. The rack of tubes is then hastily removed from the water bath and cooled in an ice bath for about 5 minutes, at which time they are made up to 25 cc. with alcohol. The compensating blank is prepared from an identically treated sample of plasma or serum taken before the administration of inulin.

(d) The color comparison is made against the compensating blank with Filter 620 (transmission 595 to 660 mµ). The inulin content is found by reference to a calibration chart which has been prepared from samples of pure levulose in precipitating media which have been treated exactly as described in the preceding paragraph (c) (Fig. 1, Curve A).

Urine Inulin—Urine inulin may be determined by this method when present in a concentration of about 2.5 per cent. The urine sample, or the mixture of urine diluted in bladder washings, is diluted in distilled water to at least 1000-fold of the original urine. Although urine protein in concentrations as high as 5 per cent does not interfere with the reaction at dilutions higher than 1 part in 500, it may be removed before dilution by treatment of the urine with the precipitating media described above. The diluted urine sample is treated exactly as the blood filtrates in paragraphs (b), (c), and (d) above, with the exception that the compensating blank is prepared from the distilled water in which the urine was diluted.

Results

The factors affecting the reaction are as follows:

Duration of Heating—At 15 minutes the color obtained is equivalent to 88 per cent of that obtained at 30 minutes of heating. The shorter period has been used because the color developed follows Beer's law throughout the range equivalent to 2.5 to about 15.0 mg. per cent of material tested. The deeper color obtained with longer heating shows a deviation from linearity at lower concentrations. Because the maximum color has not been developed by the shorter period of heating, care must be taken to avoid prolonged contact of reagent and sample before heating and to cool the tubes immediately and thoroughly at the end of heating.
Chromogen Present in Blood Plasma—The chromogen remaining in plasma filtrates after treatment with yeast amounts to the equivalent of about 1 mg. per 100 cc. of plasma inulin and is canceled in the determination by the use of the compensating blank prepared from plasma obtained before the injection of inulin.

Chromogens Present in Urine—Nitrates and nitrites yield blue colors with this reagent. Consequently, the reaction cannot be attempted in undiluted urine, although dilution to 1 part in 1000 or more practically abolishes the color obtained from the normal dog or human urine. There is no perceptible blank obtained from urine to which nitrates have been added to a concentration of 1 part in 1000 or nitrate to a concentration of 1 part in 100, the samples subsequently having been diluted a thousand times. These concentrations of urine nitrate and nitrite will rarely be reached in the urine of the intact animal.

Recovery—The levulose used in the preparation of the calibration chart (Fig. 1, Curves A and B) had a specific rotation of $-91.5^\circ$ at 20° (average of two determinations by different observers).2 The inulin used (Pfanstiehl, c.P.) was dissolved in water by heat, passed through a Seitz filter, thrice reprecipitated from water solution with alcohol, and carefully dried in a vacuum desiccator over CaCl$_2$. The recovery of inulin from blood plasma and from solutions in water and precipitating medium averaged 99 per cent by weight and varied from 98 to 101 per cent at concentrations equivalent to 5 to 15 mg. per 100 cc. of plasma filtrate, precipitating medium, or water (average of 60 determinations).

The same inulin sample was found to have a levulose equivalent of 94 per cent by weight after acid hydrolysis and determination of hydrolyzable reducing substance by the Shaffer-Somogyi method (Reagent 50, 5 gm. of KI, 150 cc. of 0.1 n iodate per liter) (11). Hydrolysis was carried out by addition of 0.2 cc. of 1 n H$_2$SO$_4$ to 5 cc. of inulin solution; the tubes were heated in a boiling water bath for 15 minutes and the reducing substance determined after neutralization. An 8-fold increase in the concentration of acid did not increase the yield of reducing substance. The whole of the reducing substance thus obtained was fermented by yeast.

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Treatment of the fermented hydrolysate with diphenylamine reagent gave a color equivalent to from 5 to 7 per cent of the inulin used (three determinations). This chromogen, as determined by the average difference of the two methods, was equal to 6 per cent by weight of the inulin sample and is apparently identical with the fructose anhydride described by Jackson and Goergen (12), which amounted to 5 per cent of their inulin sample. Our recovery of 99 per cent inulin by weight suggests that the inulin used had a non-chromogenic aldose content of about 1 per cent, and thus that the hydrolyzable levulose content of our inulin was 93 per cent, a value which agrees well with recoveries of 92 and 94 per cent reported in the literature (12, 13).

SUMMARY

A photocolorimetric method for the determination of small amounts of inulin in blood and urine is described. The method depends upon the color produced by levulose and probably by levulose anhydrides in contact with strongly acid diphenylamine.

The Determination of Levulose in Small Amounts of Blood

The method here described is a convenient modification of Herbert's method (8) which requires only 0.2 cc. of blood.

Reagents—
1. Precipitating media (modified from Hagedorn and Jensen (14)). (a) 4.5 per cent \(\text{ZnSO}_4\cdot7\text{H}_2\text{O}\); (b) 0.2 \(\text{N NaOH}\).
2. Stock diphenylamine solution.
3. Acid alcohol-diphenylamine reagent.

Apparatus—
1. Resistance glass test-tubes graduated at 10 cc.
2. Water bath, tube rack, and photoelectric colorimeter.

Procedure

(a) Precipitation of Blood Protein—0.2 cc. of blood is delivered from a pipette calibrated "to contain" into a centrifuge tube containing 1 cc. of \(\text{ZnSO}_4\) solution and the pipette rinsed back into the tube. 1 cc. of 0.2 \(\text{N NaOH}\) is added and the tube placed in a boiling water bath for 4 minutes, when it is removed and centrifuged at about 2800 R.P.M. for 5 minutes.

(b) Development of Color—1 cc. of the supernatant fluid is taken
into a pipette whose tip is guarded by a wisp of washed cotton. The cotton is removed and the protein-free filtrate transferred to a test-tube graduated at 10 cc., or, if deposits from the water bath can be excluded, to a colorimeter absorption tube graduated at this mark. 5 cc. of acid alcohol-diphenylamine reagent are added and the tubes at once placed in a boiling water bath for exactly 15 minutes. When the tube is removed from the water bath, it is cooled in an ice bath for about 5 minutes and made up to 10 cc. with alcohol. The compensating blank is prepared from an identically treated sample of blood taken before administration of levulose.

(c) Color Determination—Color comparison is made with reference to the compensating blank with Filter 620. The levulose content is found by reference to a calibration chart prepared from samples of pure levulose in precipitating media treated exactly as in paragraphs (b) and (c), p. 603, (Fig. 1, Curve B).

Results

Effect of Blood Glucose—The color given by solutions of glucose and reagent heated for 15 minutes varies slightly in its levulose equivalent. The average color obtained in terms of blood glucose is represented in Fig. 1, Curve C, in which 100 mg. per 100 cc. of blood glucose are equivalent to about 2.5 mg. per cent of levulose. Error from this source is abolished by the compensating blank when blood glucose is constant, and the error amounts to only 0.5 mg. of levulose per 100 cc. with glucose concentrations varying 20 mg. per 100 cc. When there is reason to suspect that wider variations will occur, total blood sugar may be separately determined by some micromethod. A close approximation of true levulose may then be made by subtraction from total blood sugar of the apparent levulose to yield approximate true glucose. The levulose equivalent of the approximate blood glucose is found by reference to the calibration chart (Fig. 1, Curve C) and this value applied to correct the apparent blood levulose.

The recovery of levulose added to blood averages 100 per cent at concentrations varying from 5 to 50 mg. per 100 cc. of blood. The range of error amounts to about 0.5 mg. of levulose per 100 cc. (average of thirty determinations).

The method is applicable to the study of levulose metabolism
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in small animals and has been satisfactorily used in the clinical
determination of levulose tolerance.

SUMMARY

A method is presented for the determination of levulose in
0.2 cc. of blood.

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