Application and Modification of the Momose-Ohkura Fluorometric Determination of Blood Glucose

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Results of the fluorometric determination of normal and abnormal blood glucose by the method of Momose and Ohkura (2) are lower in many instances than those obtained by reduction methods. The sensitivity of the method was greatly increased by readings at an activation of 470 m\(\mu\), a previously unreported maximum for the reaction product, in 20% sulfuric acid. A modified method is presented which uses a 1:1000 dilution of 2 \(\mu\)l of blood. Protein-free supernate equivalent to 0.5 \(\mu\)l of blood is reacted. Final volume of fluorophore is 10 ml.

The study and possible application of fluorometric assay in the area of ultramicro blood glucose determination appears warranted, as previously pointed out by Udenfriend (1). In 1958, Momose and Ohkura (2) reported a method for blood glucose using the reaction of 5-hydroxytetralone with glucose to give the fluorescent product, benzonaphthenone. Blood sugar values of eight normal individuals were similar by this fluorometric assay and the method of Hagedorn. Recently, Towne and Spikner (3) described a reaction of carbohydrates with o-phenylenediamine to give a product which exhibits fluorescence.

The present paper describes our experience with the Momose-Ohkura fluorometric determination of normal and abnormal blood sugars, modified only in the instrumentation and wavelengths used in

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quantitation. In addition, a modification is presented which uses 2 μl. of blood instead of 20 μl., allowing triplicate determinations on the supernate obtained from the deproteination of 2 μl. of blood; the volume of the final solution is maintained at 10 ml. in both methods. Supernate equivalent to 0.5 μl. of blood is reacted in the modified test.

Experimental

Somogyi filtrates were prepared from fasting bloods and from specimens during glucose tolerance procedures. The samples were anticoagulated with fluoride and oxalate, and assayed for glucose by the automated iron reduction method (4) at the Clinical Chemistry Laboratories of Jackson Memorial Hospital. Second Somogyi filtrates were prepared in our laboratory in duplicate within 24–48 hr. and each filtrate was analyzed in duplicate also by the copper reduction method (5). Simultaneously, duplicate analyses were made using the procedure of Momose and Ohkura except for instrumentation. All readings were made on the Aminco Bowman spectrofluorometer (SPF) at 470 mμ (activation) and 550 mμ (fluorescence), the uncorrected maxima (6) of the product in the final 20% sulfuric acid solution (Fig. 1). Standards were prepared according to Momose and contained 0.210% benzoic acid before dilution. The fluorescence intensities of the reacted standards were much greater than those commonly employed in fluorescence assay (Fig. 2); linear response up to 200 mg./100 ml. (equivalent to 5 μg. glucose in the reaction mixture or 0.5 μg./ml. of final solution) was confirmed (Fig. 2, curve A). However, 1:10 dilution of the

![Fig. 1. Activation and fluorescence spectra of final reaction mixture in 20% sulfuric acid: 5 μg. glucose (equivalent to 100 mg./100 ml. blood sugar) and reagent II were used.](image-url)
final solutions with 20% sulfuric acid increased linearity to at least 300 mg./100 ml. (Fig. 2, curve B).

The effects of reagent and glucose concentrations on reaction rates were studied in an effort to obtain satisfactory conditions for a method using less blood and consequently a higher dilution.

More than 1000 determinations have been made. Our precision seemed to increase with improved technic. The 2-μl. pipets gave reproducible measurements (within ±1% when checked by fluorometry of quinine solutions prepared with 2 μl. of quinine solution added to 5 ml. of 0.1N sulfuric acid). In our early studies, quadruplicate determinations were routinely used. Duplicate determinations are recommended as a check for contamination.

![Graph](image-url)

Fig. 2. Standard curves: Fluorescence intensities of left ordinate were used for Curve A, and those of right ordinate for other curves. Curve B was obtained after 1:10 dilution of final product with 20% sulfuric acid. The remaining curves were obtained with standards and reagents diluted 1:10 before reaction. See text.
Results

Fasting blood sugars from hospitalized patients were 6–20 mg./100 ml. lower by fluorometric analyses, in 80% of 40 cases, than by reduction methods; fasting blood sugars of laboratory personnel ranged 8–9 mg./100 ml. lower except for one subject whose blood sugar was 25 mg./100 ml. lower (81–56) on fluorometric assay. In this case, the difference appeared related to a high ascorbic acid blood level (5 times normal) due to extradietary intake of 300 mg. daily for 2 years. Abstinence from extradietary ascorbic acid for one week caused the difference to drop by one-third. A direct significant influence of ascorbic acid on the reaction of standard glucose with 5-hydroxytetralone could not be demonstrated at concentrations reasonable for blood, when added in 80% sulfuric acid to the reaction mixture. However, addition of ascorbic acid to fresh blood resulted in somewhat lower fluorometric values.

Representative glucose tolerance curves of the 20 studied are presented in Fig. 3, 4, and 5. In some instances the fluorometric values are much lower than those obtained by reduction methods; the latter values agree very well in most cases. The clinical interpretation of the curves obtained by the three methods is identical.

Figures 6 and 7 represent typical rate curves obtained with low concentration of standard and two different concentrations of reagent. Lower concentrations of reagent were studied but results showed inadequate reaction. It had been noted earlier (Fig. 2) that complete reaction also was not obtained when both standard and reagent were further diluted 1:10 and the reaction volume was maintained at 2.5 ml. of 80% sulfuric acid. By selecting a heating period of 20 min. and reagent concentration of 0.0125% (Fig. 7), it was possible to obtain a linear response to 800 mg./100 ml. (equivalent to 4.0 µg. glucose in the reaction step or 0.4 µg./ml. final solution) (Fig. 8). The use of these conditions and a practical method of preparing a 1:1000 dilution of blood resulted in the following modification: To tubes containing 0.1 ml. distilled water, add 2 µl. of whole blood and standard, respectively, and mix. Add 0.10 ml. 5% w/v trichloroacetic acid (TCA) and mix. Wait 5 min., add 1.8 ml. distilled water, mix, and then centrifuge. To 0.5 ml. of supernatants and of water (for blank), add 2.0 ml. 0.0125% (w/v) 5-hydroxytetralone (in concentrated sulfuric acid) with ice cooling. Mix, then heat 20 min. in a boiling-water bath. Cool in an ice water bath. Add 7.5 ml. distilled water. Allow to cool before mixing by inversion. Bring to room temperature in a water bath. Read flu-
orescence at 470/550 m\textmu . The modification gives fluorescence intensity values in a reasonable range. It differs from our use of the original method of Momose and Ohkura in that it uses one-tenth the amount of blood and one-fifth the amount of water and trichloroacetic acid.

Figs. 3 (top) and 4 (bottom). Glucose tolerance curves: \( \times \) fluorescence assay; \( \circ \) iron reduction; \( \bullet \) copper reduction.
(TCA), but maintains the dilution to 2.0 ml. with water, which in this case represents a 1:1000 dilution of blood, rather than 1:100. The heating time is reduced to 20 min. Reagent concentration is one-half that of the original method. The optimal reagent concentration may vary with the source and purity of the reagent and should be checked when new sources become available. The excess of reagent to glucose in the modification ranges from approximately 500-fold for 100 mg./100 ml. blood glucose (0.5 μg. in the reaction) to 63-fold for 800 mg./100 ml. glucose (4.0 μg. in the reaction).

The activation maximum in 20% sulfuric acid is at 470 mμ (Fig. 1). This was not apparent from the ultraviolet absorption curve of benzonaphthocone (the reaction product) in alcohol, reported by Momose and Ohkura (8). Minor peaks or shoulders are evident at 280, 365, and

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**Fig. 5.** Glucose tolerance curves: (X) fluorescence assay; (O) iron reduction; (●) copper reduction.
420 m\(\mu\) (Fig. 1) and agree with absorption data obtained by Momose and Ohkura for alcoholic solutions of benzonaphthenone and a product elegantly separated from the reaction mixture of glucose and 5-hydroxytetralone. On the basis of this, Udenfriend (1) suggested 420

\[ \text{Fig. 6. Rate curves obtained with standards diluted 1:10 before reaction with Reagent I at concentration used in unmodified procedure.} \]

m\(\mu\), rather than 365 m\(\mu\) used by Momose and Ohkura, for the activation setting. The activation spectrum (SPF) of benzonaphthenone* in 20% sulfuric acid, and the absorption spectrum (Beckman DU spectrophotometer) in alcohol and in 20% sulfuric acid, were determined. In agreement with previous data (8) we found no maximum absorption at wavelengths beyond 420 m\(\mu\) in alcohol solution. However, in 20% sulfuric acid, larger peaks, 470-502 m\(\mu\), dependent on concentration, were found with the Beckman DU and Amineo-Bowman SPF. Regardless of concentration, the fluorescence peak was 550 m\(\mu\) (uncorrected). The activation and fluorescence maxima obtained were the same with the original Momose and Ohkura method and the modified method, using either reagents I or II reacted with standard glucose or blood supernates. These experiments support the proposal that benzo-
naphthenone is indeed the product of the reaction of glucose and 5-hydroxytetralone. The increased sensitivity obtained using the maxima of the product is obvious.

Discussion

The fluorometric assay of glucose appears to be extremely sensitive, precise, and accurate when proper precautions and optimal wave-

Fig. 7. Rate curves (modified method) obtained with standards diluted 1:10 before reaction with Reagent II at one-half the concentration used in unmodified procedure.

lengths are employed. The tendency for lower values of blood glucose by the fluorometric analyses is of interest and may reflect the inability of other substances reduced by copper or iron to react with 5-hydroxytetralone (6). Whether or not their presence in blood is related to ascorbic acid intake or to previous dietary history cannot be answered here. However, the probable difference in dietary history of the subjects involved may explain the good agreement obtained by Momose and Ohkura in their comparison (2) of 8 fasting normal bloods. Undoubtedly, ascorbic acid intake in our area is greater than in Japan due to the availability here of fruits high in the vitamin, such as lime and
guava. The amount of lime juice added to the tolerance test glucose solutions was not controlled, making it difficult to assess the ascorbic acid intake in the patients undergoing the glucose tolerance tests.

We have observed that substances that do not react with 5-hydroxy-

tetralone, e.g., benzoic acid, will affect the kinetics of the reaction with glucose in 80% sulfuric acid if present in adequate concentrations. In the modified method presented, an additional 1:10 dilution of blood and standards minimizes any effect due to other nonproteinaceous substances in the supernate (TCA). Both the original and the modified method agree within 1% in a limited comparison with the glucose oxidase method (7), when applied to analyses of commercial control sera and to chicken blood. The obvious advantage of the fluorometric assay is the use of one stable reagent after protein precipitation with TCA. Further use of the modified method by other workers is required before final evaluation.

Fig. 8. Standard curve (modified method) obtained with 0.5 to 4.0 µg. glucose using 0.0125% Reagent II.
Contamination of glassware or reagents by lint, fingerprints, etc. will introduce appreciable errors (2). Despite this drawback, it is felt that laboratories adapted to sensitive ultramicro techniques should find the modified method of great value, especially in pediatrics or in research on small laboratory animals or embryos. As noted earlier, 0.5 ml. of a 1:1000 dilution of blood was used in the modification presented. This probably represents the least quantity ever used in ultramicro blood sugar determinations with a final volume of 10 ml., which is greater than required for readings. However, further dilutions of starting material with resulting lesser concentration of final reaction mixture are not recommended.

Various modifications are possible to take advantage of equipment available in some clinical laboratories. Also, an automated, or semi-automated, closed system might be developed to utilize the principle of the reaction but to minimize contamination errors.

References
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