Micromethod for the Estimation of Plasma Fibrinogen

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Fibrinogen is quantitatively precipitated from plasma by 1.20 M phosphate buffer. The amount of precipitated fibrinogen is determined turbidimetrically at 450 µm. The method requires 0.25 ml. of plasma for the blank and sample. Reliability of the method has been established by comparison with an ammonium sulfate turbidimetric method and a colorimetric method, demonstration of specificity, variation in incubation temperature, demonstration of optimal molarity and pH, and replicate analyses.

The determination of fibrinogen is most useful in hemorrhagic emergencies. For this purpose, a method which combines rapidity, accuracy, precision, small sample requirements, and reagent integrity would be of obvious value to the clinical laboratory. It was believed that the turbidimetric technic was the only one extant which would satisfy these criteria.

Several workers (1–3) have used ammonium sulfate to flocculate fibrinogen prior to its turbidimetric determination. Aull and McCord (4) described the fractionation of serum protein with potassium acid phosphate and the determination of the fractions by turbidity. These authors did not apply the technic to the specific determination of fibrinogen in their paper, nor did they specify the pH of the working phosphate reagents. They did specify the pH of their stock solution. No attempt was made in that presentation to show that calcium in the serum does not interfere by the formation of insoluble calcium phosphate.

It is the purpose of this paper to show that the application of the phosphate turbidity method of Aull and McCord (4) to the estimation of plasma fibrinogen is not only feasible but highly desirable. It will also be demonstrated that, contrary to popular belief, reinforced by

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the review article by Reiner and Cheung in a recent publication (5), the
turbidity methods can actually define borderline values as well as one
of the more elaborate, time-consuming, but theoretically more accu-
rate procedures (6).

Reagents

**Fibrinogen reagent (1.20 M Phosphate [0.60 M KH$_4$PO$_4$ and 0.60
M Na$_4$HPO$_4$])**  In a 250-ml. volumetric flask dissolve 20.414 gm. of
reagent-grade KH$_2$PO$_4$, 21.297 gm. of reagent-grade Na$_2$HPO$_4$, and
0.25 gm. of reagent-grade potassium sorbate in distilled water. Add
the distilled water with constant shaking to prevent as much of the
Na$_2$HPO$_4$ as possible from caking on hydration. Dilute to the 250 ml.
mark with distilled water and mix thoroughly until all solute is dis-
solved. The pH, checked with a glass electrode, will be 6.5 ± 0.02. The
pH is not critical within the range 6.4–7.5. The reagent is stable at least
2 years at room temperature.

**Sodium chloride 0.9% (w/v)**  Dissolve 9.0 gm. of reagent-grade
NaCl in 1 L. of distilled water.

**Standard fibrinogen**  Commercially available fibrinogen* may be
used when first assayed for nitrogen by a Kjeldahl method. The factor
6.00, may be used to convert nitrogen to fibrinogen (7, 8). The solution
is prepared according to the manufacturer’s directions. It is stable
approximately 12 hr. in the refrigerator (approximately 5°) and at
least 24 hr. at room temperature.

Procedure

**Preparation of Calibration Curve**

Into each of 2 test tubes (10 × 75 mm.), pipet 0.5 and 1 ml. of standard
fibrinogen. Add 1.5 and 1 ml., respectively, of 0.9% (w/v) sodium
chloride. Mix thoroughly. Undiluted standard fibrinogen is used as
the third working standard. These solutions are equivalent to one-
fourth, one-half, and 1 times the assay value obtained by Kjeldahl
analysis of the standard fibrinogen. Use 0.25 ml. of each solution in
place of plasma in the routine assay that follows. Use 0.25 ml. of 0.9%
(w/v) sodium chloride in the blank. Prepare a graph relating gm./100
ml. of fibrinogen to absorbance. This should be checked at regular in-
tervals. A typical calibration curve is shown in Fig. 1.

**Assay**

At room temperature (20°–33°), pipet 0.25 ml. of unhemolyzed plas-
ma into each of two 19-mm. round cuvets. Mark one cuvet “sample”

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*General Diagnostics Division, Warner-Chilcott Division, Morris Plains, N. J.
and the other "blank." Add to the sample cuvet 3.0 ml. of fibrinogen reagent. To the blank cuvet add 3.0 ml. of 0.9% (w/v) sodium chloride. Promptly mix by gentle lateral shaking. Readings are made in a Coleman Junior Spectrophotometer, Model 6D* at 450 m\(\mu\) against the blank adjusted to 100% transmittance. (In order to permit readings on as little as 3 ml. of solution in the 19-mm. round cuvets, cut a slice of rubber from a solid rubber stopper of sufficient thickness to elevate 3 ml. of solution, contained in the cuvet, into the optical path when the rubber slice is placed at the bottom of the cuvet adapter.) The turbidity remains optically constant up to 9 min. The concentration of fibrinogen in the plasma in gm./100 ml. is read from the calibration curve. For values over 0.30 gm./100 ml., the plasma must be diluted with saline and the test repeated.

**Experimental**

**Comparison of Methods**

In order to validate this turbidimetric method, the present method was compared with that of Fowell (3), which employs ammonium sulfate as the protein precipitant, and with the colorimetric method of Reiner and Cheung (6). The results are shown in Table 1.

On a larger series, involving 30 serums analyzed in duplicate, the standard deviation, calculated by the formula (9)

\[
\text{S.D.} = \sqrt{\frac{\sum (x_1 - \bar{x})^2}{N - 1}}
\]

was 0.018 for the Fowell method, 0.018 for the Reiner-Cheung method and 0.016 for the present method.

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*Coleman Instruments Corporation, Maywood, Ill.
These data would indicate that the choice of method revolves around considerations of the convenience and rapidity desired.

**Spectral Characteristics of the Reaction Mixture**

Absorption spectra were determined on a plasma sample in the "high normal" range and on one in the "low normal" range. Figure 2 is a graph of the results. No clearly defined absorption maximum is observed. The wave length of 450 m\(\mu\) was chosen as a compromise between maximum sensitivity and the ability to adjust the blank to 100% transmittance. Since plasma is used in the blank, interference from plasma pigments is largely compensated.

**Specificity**

Thirty serum samples, derived from the same subjects from which the plasma samples were obtained for the elucidation of standard deviation, were "analyzed" by the phosphate turbidity method. The calcium values in these serums ranged from 8.8 mg./100 ml. to 12.3 mg./100 ml. In no case did a turbidity develop which was capable of being detected by the spectrophotometer. However, the fibrinogen reagent will readily precipitate calcium from aqueous solutions of CaCl\(_2\).

**Stability of Fibrinogen Reagent**

Two batches of fibrinogen reagent, both preserved with potassium sorbate and stored in clear glass bottles at room temperature, remained completely stable for at least 2 years. There was no evidence of mold growth. The reagents were used at weekly intervals. Without potassium sorbate preservation, one batch of the reagent, stored and used in the same fashion as the preserved reagent, exhibited mold.

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<th>Table 1. Assay Results with 2 Turbidimetric Methods and 1 Colorimetric Method for Plasma Fibrinogen</th>
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growth in about 8 months. It is suggested that potassium sorbate be retained as a preservative.

Some batches of phosphate apparently contain impurities which cause a small amount of dark-colored sediment to form in the bottles (10). This does no harm unless pipetted into the cuvet.

**Optimal Phosphate Molarity and pH**

It should be noted that the final phosphate concentration in the reaction mixture is 1.11 M. This has been found to be optimal.

No difference in turbidity was found when the pH of the fibrinogen reagent was varied from pH 6.4 to 7.2. Below and above these values, turbidity intensity diminished. A pH of 6.5 was chosen for the fibrinogen reagent in order to integrate with the serum protein fractionation by phosphate turbidity reported by Aull and McCord (4) and to insure greater stability of the reagent.

**Effect of Temperature Variation**

Four tests were done on the same plasma in which both the plasma and the reagents were maintained, by water bath, at temperatures of 20°, 25°, 30°, and 33°. No statistically significant difference in the results was obtained. For practical purposes, therefore, the influence of room temperature variation may be ignored.

**Normal Values**

Thirty fasting plasma samples were obtained from hospital personnel having no signs of illness. To standardize on the effect of posture on protein levels, as reported by Aull and McCord (11), all blood samples were obtained while the subjects were in the supine position. It is
suggested that blood from patients be similarly drawn. Both sexes were represented about equally in this group, and ranged in age from 22 to 55 years. The fibrinogen determinations were performed on these plasma samples within 1 hr. after collection of the blood at room temperature (approximately 25°).

The 95 per cent limits for fibrinogen were 0.11–0.43 gm./100 ml. No statistically significant difference between male and female levels was found.

Choice of Anticoagulant

For all comparative testing, plasma was derived from blood which was oxalated as described by Reiner and Cheung (6). For the present method it was shown that, in addition, ammonium heparin, sodium citrate or the disodium salt of ethylenediamine tetraacetic acid may be used as anticoagulants with no change in results.

Stability of Fibrinogen in Blood and Plasma

Fibrinogen was found stable in anticoagulated whole blood in the refrigerator (approximately 5°) for at least 3 days but not 4 days. In separated plasma, fibrinogen was found stable up to 1 day at room temperature (approximately 25°) and up to 3 days in the refrigerator (approximately 5°).

Discussion

The experimental evidence indicates that the phosphate turbidity technic of Aull and McCord (4) can be profitably applied to the estimation of plasma fibrinogen. It affords certain important advantages over the ammonium sulfate turbidity technics of Parfentjev et al. (2) and Powell (3). These include somewhat better sensitivity, considerably better pH control of the stable fibrinogen reagent, which constitutes a true buffer, much less need for ambient temperature control, and better adherence to Beer's law of the resulting turbidities with plasma samples. Further, the resulting turbidity is somewhat more stable than when fibrinogen is precipitated with ammonium sulfate. Phosphate turbidity affords a degree of accuracy and precision as good as the more elaborate and time-consuming method of Reiner and Cheung (6) but with greater assay facility. This is not to disparage the latter technic, which definitely serves as an excellent reference method. It does, however, indicate that, at times, greater methodologic sophistication may be self-defeating.

In order to obtain reproducible turbidities, it is necessary to adhere scrupulously to the directions with respect to order of addition of the
reagents and mixing. These precautions are germane to all turbidimetric assays.

This procedure can be easily integrated with the fractionation of the various serum protein components by the phosphate turbidity method of Aull and McCord (4).

References

10. Aull, J. C., personal communication.