Simplified Microestimation of Fibrinogen and Seromucoid in Plasma

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Simplified methods for the microestimation of fibrinogen and seromucoid in plasma are described, and their relationship is discussed. The normal range corrected for age and sex is given.

Measurement of fibrinogen and seromucoid in plasma is used to detect tissue damage and necrosis. A chemical review of fibrinogen in abnormal conditions has been published by Ham and Curtis (1), and of seromucoid by Keyser (2). No reports have appeared on the relationship between the two in miscellaneous diseases.

The present study was made to delineate normal values, using two simplified micromethods for the analyses, and to survey the relationship between the two substances in a variety of diseases.

Methods and Material

Specimens

Routine heparinized plasma samples, selected at random, were analyzed on the day of receipt. After 100 specimens for each sex had been collected, some further specimens were analyzed for fibrinogen alone; for these assays, patients were under 40 or over 80 years of age.

Seromucoid

Seromucoid was measured by a modification of the protein method of Lowry et al. (3).

Reagents

*Stock copper solution* 2.5 gm. CuSO$_4$ · 5 H$_2$O, and 5 gm. sodium potassium tartrate were diluted to 500 ml. with 0.2 N NaOH.
Perchloric-boric acid reagent 2.5 gm. of boric acid was dissolved in 400 ml. of water, and 100 ml. of 60% (v/v) HCIO₄ added.

Protein standard 350 mg. of bovine albumin (Armour) was dissolved in 100 ml. of saturated benzoic acid solution. A further weighed aliquot of powder was dried at 100° overnight, and the weight corrected for moisture content.

Working Folin-Ciocalteau reagent A commercially purchased stock solution was adjusted to normality by our adding 5 ml. to 20 ml. of water in a flask, adding a minute amount of dry phenolphthalein, and titrating with N NaOH. Larger amounts of this reagent are then prepared by using the figures derived from this titration.

Working copper reagent 50 gm. of anhydrous Na₂CO₃ was dissolved in 90 ml. of 4N NaOH. Water was added to 960 ml., and then 40 ml. of stock copper solution.

The working Folin-Ciocalteau reagent is stored at 4° and the other reagents at room temperature.

Procedure

Pipette 1 ml. of perchloric acid-boric acid reagent into conical centrifuge tubes. Rapidly add 0.05 ml. of standard or plasma. The blank needs no addition. The solution is mixed immediately by tapping of the tubes, and then thoroughly by a mechanical tube mixer. Centrifuge the test samples (but not the blank and standard) at 3000 rpm for 15 min. Decant only the test samples into another set of tubes by inverting sharply and draining thoroughly. Add 5 ml. of working copper reagent to each tube including the standard and blank, and mix. Heat in a 37° water bath for 10 min.; add 1 ml. of Folin-Ciocalteau reagent and mix immediately. Allow to stand for 10 min., and measure the blue color at 650 m\(\mu\). Subtract the blank readings, and calculate the protein concentration by the usual mathematical formula for colorimetric measurements.

Since decantation does not give complete transfer of the tube contents, and the standard is not decanted, there is a small constant error in the estimation of the seromucoid content of the plasma; this error has been incorporated into the normal range given. The main hazard with the above method is the possible carryover of particles of the protein precipitate. This has been minimized by using conical tubes and adequate centrifugation.

Fibrinogen

Reagents

Protein reagents The same reagents are used as in the seromucoid method.
**Ethylene diamine tetraacetic acid solution** 4 gm. K₂EDTA, dissolved in 100 ml. of water.

**Octan-2-ol (caprylic alcohol)**

0.6 M *Acetic acid* 36 ml. glacial acetic acid is made to 1 L. with water.

0.6 M *Sodium acetate* 49.2 gm. anhydrous sodium acetate is dissolved in saturated benzoic acid and made up to 1 L.

**Urea solution: 20% (w/v)** 20 gm. urea is dissolved in water and diluted to 100 ml.

**Working diluent** Dissolve 100 gm. anhydrous sodium sulphate in about 800 ml. of water. Add 10 ml. of 0.6 M acetic acid, 90 ml. of 0.6 M sodium acetate, 1 ml. K₂EDTA, and 0.5 ml. caprylic alcohol. Dilute to 1 L. with water. The pH should be 5.3–5.4.

**Fibrinogen standard** Dissolve about 400 mg. of commercial fibrinogen (British Drug Houses) in 20 ml. of 20% urea solution, and add 0.2 ml. of 4% K₂EDTA. This yields a faintly turbid solution which is stable at 4° for about 8 weeks.

The other solutions are all stable at room temperature.

**Calibration of Standard**

Place 10 ml. of working diluent in each of 4 15-ml. conical centrifuge tubes. Add 0.1 ml. of fibrinogen standard directly to the diluent in the tubes. Mix well and allow to stand for 30 min. Centrifuge at 2000 rpm for 15 min. Decant and drain; then add 0.4 ml. of 20% urea solution. Mix thoroughly and repeatedly until the precipitate is dissolved. To 2 other tubes add 0.4 ml. of 20% urea solution, and then 0.1 ml. of protein standard to 1. The second tube of this series is the blank. To all tubes add 5 ml. of working copper solution and mix. Incubate in a 37° bath for 10 min. Add 2 ml. of Folin-Ciocalteau reagent and mix immediately. Allow to stand for 30 min. and then transfer the colored solution to a cuvet with a 0.5-cm. light path and measure the color at 650 mμ. Calculate the precipitated protein from the fibrinogen standard.

**Apparatus**

An E.E.L. Nephelometer with Unigalvo* was used for measuring turbidity.

**Method**

In 15-ml. conical centrifuge tubes, place 10 ml. of diluent; add 0.1 ml. of plasma or standard directly to the diluent, and mix immediately by

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*Obtained from Evan's Electroselenium, St. Andrew's Works, Halstead, Essex, Great Britain.
inversion. The blank needs no addition. Allow to stand 30 min. and read. Set 100% transmission on the galvanometer, using the green filter (540 m\(\mu\)) and the perspex standard supplied; set zero scattering, using the reagent blank. Calculate the fibrinogen content by simple proportionality, using the values obtained from the standard calibration for the standard value.

If the plasma is obviously lipemic, transfer the solution after reading to a centrifuge tube, and centrifuge for 15 min. at 200 rpm. Read the supernatant turbidity, and deduct this value from the original reading.

The method contains a systematic error due to nonspecific turbidity in plasma. In nonlipemic specimens, this error is equivalent to 10–30 mg./100 ml. The error is included in the normal range as calculated. The method has been checked by using serum from blood which had been given ample time to clot. Such specimens gave values of 10–30 mg./100 ml., after correction for nonspecific turbidity.

**Statistical Methods**

For males under 30 and females under 40 years of age, the fibrinogen values segregated into two groups with significantly different means \((P < 0.001)\). When elevated values were excluded, and the remaining values for males (under 420 mg./100 ml.) were used, they yielded a significant correlation with age. The frequency distribution of the deviation from the regression was skewed toward positive values. A gaussian curve was superimposed on this frequency distribution, with the modal value used as origin, as described elsewhere \((4, 5)\). Values outside the 95% confidence limits were eliminated, and the regression recalculated; the same procedure was applied once more. An identical technic was used for the female group, for all those with values less than 370 mg./100 ml. In both sexes, the scatter of the data was more accurately described with a constant percentage used as standard error, rather than a constant weight; so the 95% confidence limits were defined in this manner.

For seromucoids, a superimposed gaussian curve centered on the mode was applied directly to the male distribution. All figures under 150 mg./100 ml. were used. From this group, all those with elevated fibrinogen levels were rejected, and the correlation with age was calculated for the remainder. The female subjects showed a rather irregular seromucoid distribution, and a variety of technics failed to yield a significant correlation with age. The normal values were finally demarcated by the use of all values under 150 mg./100 ml. and the superimposition of a gaussian curve on the calculated mode.
Results

The frequency distributions for all values, and the relationships of the lower values and age are shown in Fig. 1–4.

The normal ranges (in mg./100 ml.) and significant correlations were found to be as follows, for males: fibrinogen, 143 plus 1.9 times the age in years, with an S.E. of ± 13% (p < 0.001); seromucoid, 81 plus 0.5 times the age in years, with an S.E. of ± 13 mg. (p < 0.001). For females the values were: fibrinogen, 177 plus 1.3 times the age in years, with an S.E. of ± 15% (p < 0.001); seromucoid, 104 (mean) with an S.D. of ± 15 mg.

Fibrinogen correlated significantly with age in both sexes; seromucoid correlated significantly with age only in males. Fibrinogen correlated significantly with seromucoid in males, but when age was included, the partial correlation was no longer significant.

Diagnostic Correlations

About two-thirds of the subjects had abnormal values for one or both substances: 8 males and 31 females had raised seromucoid with normal
fibrinogen levels. These cases showed no discernible diagnostic pattern. The reverse (raised fibrinogen and normal seromucoid levels) was found for 16 males and 7 females: 8 of the males had hypercholesterolemia due to a variety of causes; apart from this there was no diagnostic pattern. Of 12 patients with malignant disease, 6 had normal fibrinogen levels, 3 had normal seromucoid levels, and 2 had both. There were 11 patients with recent injury: 3 had normal fibrinogen values and none had normal seromucoid values. Four cases of low fibrinogen levels and 4 of low seromucoid levels showed no diagnostic pattern.

Fig. 3. Fibrinogen (lower values only) versus age, for males (left) and females (right). Also shown are normal-value regression line and 95% confidence limits (based upon a constant percentage). Fig. 4. Seromucoids (lower values only) versus age, for males (left) and females (right). Also shown are regression line and 95% confidence limits for normal males; mean and 2 S.D. for females.
Discussion

Both fibrinogen and seromucoid are alleged to be relatively sensitive indicators of inflammation. Under these circumstances, the concept of normality usually employed in medicine becomes even more nebulous. Minor inflammatory conditions such as rhinitis, sinusitis, bronchitis, osteoarthritis, or gastritis are widespread in any unselected population. Therefore any definition of normality must necessarily be arbitrary; the most that can be done is to make it objective.

The age-dependence of fibrinogen has recently been demonstrated by Ogston and Ogston (6), in confirmation of an earlier German report. The present results are not strictly comparable to theirs since the employed methods were different, but both series demonstrate that the age effect is more marked in males. For both sexes between the ages of 20 and 80, this study gave an over-all 95% confidence limit of 131–390 mg./100 ml., with a mean of 260 mg./100 ml. This compares reasonably well with Stirland’s (7) figure of 270 mg./100 ml. and 95% confidence limits of 160–380 mg./100 ml. Since there were very few subjects under the age of 20, extrapolation to this group is not justified.

The seromucoid method used in our laboratories gives rather higher values than previous technics. Winzler et al. (8) in their initial report showed that extraction was incomplete even at high dilutions of serum. Incorporation of borate in the present method increased extraction by approximately 20%. The extracted material had a nonamino sugar content of about 15%. Allowing for the increased extraction, the mean and range agree fairly well with Winzler’s figures. Cameron and his colleagues (9) found a rising value with age, and in the present study this finding is confirmed for males, although not for females.

In this survey, raised levels of fibrinogen and seromucoid were associated but not correlated. It seems that the use of both procedures gives more effective screening for organic disease. No other test performed in this laboratory gives abnormal results in two-thirds of cases. Even so, there were 5 female subjects diagnosed (respectively) as having lumbago, pyrexia, pyelitis, mono-arthritis, and cerebral hemorrhage, and 2 male subjects diagnosed as having uveitis and phlebitis, all of whom had normal values for both substances. Screening by these procedures is not therefore completely effective. The rising values with age might themselves be due to degenerative pathologic changes, and might be analogous to the rise with age of serum cholesterol or urea levels or the age-dependent increase in blood pressure. If this were so, it should be most useful clinically to compare the patient with others of his own sex and age group.
References