Heparin fractionation in the study of lytic activity

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The precipitation of fibrinogen from plasma by means of heparin was reported by Smith (1955), and the formation of complexes between acidic mucopolysaccharides and protein (Anderson, 1963) has been used in a range of studies, for example, in β lipoprotein (Burstein and Samaille, 1958) and on desoxyribonucleoprotein (Kent, Hichens, and Ward, 1958). The observation of fibrinogenolysis and fibrinolysis in one of the heparin complexes prepared from fresh human plasma at low ionic strength (Green, 1959) has been adapted for clinical study. Lysis in this system is more rapid than any hitherto reported, and it was felt that an isolating technique would be of value in defining variable factors and in applying objective methods of measurement (Green and Thomson, 1962).

METHODS

Fractionation was originally carried out by the step-wise addition of either acid to diluted heparinized plasma or heparin to diluted plasma at pH 6.8. For routine purposes it is carried out in one standard procedure, by adding 1 volume of plasma to 2 volumes of 6×10^{-3} N HCl containing 12.5 units of heparin per millilitre. The complex so produced is spun down, washed rapidly, and re-dissolved in buffer.

ESTIMATION OF VISUAL LYSIS ACTIVITY The precipitate is dissolved in 1 volume of barbitone-NaCl buffer (Owren, 1947), pH 7.5, and 0.25 ml. aliquots made up to 0.5 ml. with buffer and clotted at room temperature with 0.5 ml. thrombin solution (10 N.I.H. units/ml.). The resultant clot is incubated in a water bath at 37°C., and the lysis time recorded as the time required for complete disappearance of the clot.

ESTIMATION OF PROTEOLYTIC ACTIVITY Anderson's (1962) technique has been modified to study fibrinogenolysis and fibrinolysis. In the latter case, 1 ml. of the above re-dissolved fraction is clotted with 0.2 ml. thrombin solution (20 N.I.H. units/ml.) and incubated for two hours at 25°C. or one hour at 37°C. Then 1 ml. of 40% urea is added, and, when any remaining fibrin has dissolved, 1.8 ml. 10% trichloroacetic acid (T.C.A.) is also added. The control 1 ml. sample receives 1 ml. 40% urea, 0.2 ml. thrombin, and 1.8 ml. T.C.A. immediately. The increase in tyrosine and tryptophane peptides in the supernatant is measured at 280 μ m. on a Unicam model SP 500 and expressed as μ mol tyrosine.

MEASUREMENT OF ESTERASE ACTIVITY The precipitate from 1 volume of plasma is redissolved in 0.1 M Tris buffer, pH 7.5, 0.33-1.0 volume, as required, and incubated for 10 minutes at 37°C. to allow activation. Amounts each of 1 ml. are incubated as above with 1 ml. of 20 μ mol/ml. tosyl arginine methyl ester or lysine ethyl ester, precipitated with 2 ml. 10% T.C.A., and the residual ester measured as the ferric hydroxamate (Brown, 1960).

OTHER PROCEDURES Total protein and total minus fibrin can be measured by the biuret method of Wolfson, Cohn, Calvary, and Ichiba (1948). Lysis is inhibited by using 1×10^{-3} M ϵ -amino-caproic acid.

RESULTS

The fraction varies in composition and solubility with the anticoagulant employed, but will contain on average 50 to 75% of plasma fibrinogen and about 20% of the plasminogen. Electrophoresis of the re-precipitated fraction on cellulose acetate at pH 8.6 shows a main fibrinogen band, some β globulin, and virtually no other fractions (Fig. 1).

The reproducibility of activity in this system depends upon three main factors. (1) The constancy of the ratio of decalcifying agent to plasma (variation can be minimized by using a three- or four-fold excess of E.D.T.A., i.e., 1 ml. M/20 E.D.T.A. to 10 ml. blood). (2) The control of the temperature of manipulation, the extreme lability of the system has required operation as near 0°C. as possible to minimize loss of activity in shed blood and activation once the precipitate is formed. Reducing the pH of blood below 7.0, and the incorporation of lysine methyl ester in the anticoagulant diminish the lability in shed blood but these measures have not been fully tried. (3) Control of pH; 7.5 has been used for all measurement of activity, although the optimum for visual lysis is around pH 8.2. Other requirements are constancy in speed of centrifuging blood samples, in the amount of thrombin used, and in the elimination of calcium. Any sign of clotting in blood sampling invalidates the estimation.

VISUAL LYtic ACTIVITY Clotting times in the above systems range from 12 seconds to over three minutes, and are inversely related to lysis times.

FIG. 1. Electrophoresis on cellulose acetate in 0.06M Barbitone buffer pH 8.6.

Top strip Normal plasma for reference.

Centre strip Heparin fraction from citrated plasma, twice precipitated at pH 6.8 ionic strength 0.05.

Lower strip Single fractionation from E.D.T.A. plasma incompletely washed, as used in routine estimations. In addition to fibrinogen and β globulin, traces of albumin and γ globulin are seen.

Lysis times range from zero to two and a half hours but for experimental purposes a range of 10 to 90 minutes is desirable. There is good general correlation with values obtained by the Fearnley, Balmforth, and Fearnley (1957) method.

PROTEOLYSIS A measurable increase in optical density at $280 \mu\text{m}$ has been found in all clotted samples when prepared from fresh plasma, ranging from 0.05 to $1.0 \mu\text{mol}$ tyrosine/ml. Negative results have been obtained when aged plasma has been used, or when lysis has been inhibited by ϵ -amino-caproic acid. Results have consistently paralleled changes in visual lytic activity in the same subject, but correlation between subjects is not exact. This is to be expected, since the amount of substrate in the fraction varies with the subject's plasma fibrinogen level, and is insufficient to ensure that a single reading at a fixed time interval will be on the linear slope of the sigmoid curve (Derechin, 1961). For example, with a low substrate concentration, the amount of peptide released would be relatively low and visual lysis times relatively rapid.

Values in unclotted specimens are significantly lower, and the difference appears to be related to the adequacy of clotting as such rather than to any other effect of thrombin on the system.

ESTERASE ACTIVITY A significant increase in esterase activity towards tosyl arginine methyl ester and lysine ethyl ester has been found only in association with high visual lytic activity. When excess E.D.T.A. is used as decalcifying agent for the original plasma, the normal range of values in the fraction is 2.4 μmol tosyl arginine methyl ester and $<0.5 \mu\text{mol}$ lysine ethyl ester hydrolysed per millilitre plasma volume in one hour at 37°C . Virtually, any lysine esterase activity appears to be significant; its detection can be enhanced by forming an adequate clot with thrombin, but the main value of esterase methods has lain at higher levels of activity where this would be precluded. With less efficient decalcification of the original plasma, using citrate, or less

FIG. 2. Changes in visual lysis, proteolysis, and esterase activity following intravenous adrenaline in a patient with the carcinoid syndrome.

than 1 ml. M/50 E.D.T.A. to 10 ml. blood, fractions show widely varying esterase activity, which does not correlate with lytic activity between cases, and is present in fractions prepared from aged plasma.

In the fibrinolytic response to adrenaline or Complamin (Johan Wölfing, Dusseldorf) an increase in esterase activity has been found in fractions from both E.D.T.A. and citrate plasma, and the ratio of activity towards the two substrates corresponds roughly to that of plasmin.

Figure 2 shows the results obtained by the above techniques in following the fibrinolytic response to 0.5 µg. intravenous adrenaline in a patient with the carcinoid syndrome.

EFFECT OF KNOWN ACTIVATORS OF PLASMINOGEN The addition of streptokinase, urokinase, or α-chymotrypsin to plasma which had been inactivated by ageing yields fractions which show increased lysis, and proteolytic and esterolytic activity. They may,

FIG. 3. Changes in visual lysis times, proteolytic and esterolytic activity in fractions prepared from aged E.D.T.A. plasma to which urokinase was added.

of course, be added directly to the inactive fraction itself. Preliminary studies with urokinase show that 50 to 70% of urokinase added to plasma can be found in the heparin fraction.

Figure 3 shows the effect of adding 0.5 to 20 units urokinase (Leo) per millilitre to aged E.D.T.A. plasma followed by immediate fractionation. This also provides a good general picture of the relationship between the three techniques of assessment.

DISCUSSION

These techniques have been developed and tested by following brief sequential changes in the same subject. The activity of the system has not been fully developed, owing to the need to preserve the subject's fibrinogen as substrate before testing, but the potential activity, together with the electrophoretic pattern, suggests that the fraction is relatively free from inhibitors.

Inhibition by $1 \times 10^{-3}M$ ε-amino-caproic acid, the increase in proteolytic and esterolytic activity on clotting, and the lability of activity in shed blood suggest that plasma activator is being measured. Evidence of activation as such, however, has been obtained only from esterase studies in highly active specimens.

While the conditions of fractionation are empirically chosen, the objective methods of end-point determination offer a precision which is lacking in visual lytic methods. Esterase methods appear to be capable of quantitation but at the moment they are insensitive to 'normal' values. The proteolytic method is simple and flexible enough to cover a wide range of values but is dependent upon fibrinogen levels. For comparative purposes additional fibrinogen could be added as substrate (Anderson, 1962), or alternatively, urokinase might be used to standardize the values obtained at varying substrate levels in the fraction. These possibilities are being explored.

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