Proteolytic and Fibrinolytic Activity of Serum
Activation by Streptokinase and Staphylokinase Indicating Dissimilarity of Enzymes

By Eugene E. Clifftone, M.D. and Dolly A. Canamela, B.S.

It has been known, at least since the report of Dastré, that blood usually contains a material which results in lysis of clots at the normal pH of blood. This material has been shown to be present in the blood primarily as an inactive precursor called plasminogen which can be activated by many materials and methods in vivo and in vitro. This enzyme, or a material with similar properties found in the euglobulin fraction of serum, has also been shown to have a proteolytic activity which is similar to, but not identical with, trypsin. Most investigators, studying this enzyme system, have indicated their belief that the fibrinolysis effect is simply a means of demonstrating the activity of the proteolytic enzyme. They explain the more obvious fibrinolytic effect on the basis of the necessity for breakage of only a single peptide bond of the fibrin to give a positive test. At least two investigators have suggested that the fibrinolytic and proteolytic effects may be due to different enzymes; Mole, on the basis of the lysis of blood postmortem and Halse, because of variations in heparin effect. Christensen himself suggested that the lysin factor in rabbit serum may be qualitatively different as shown by his tests. It may also be significant that the fibrinolysin found in human prostatic secretion was not fibrinogenolytic (proteolytic), whereas that of dog prostatic secretion was found to be more fibrinogenolytic than fibrinolytic according to Huggins and Neal.

Among plasminogen activators mentioned briefly above, bacterial products have been prominent, and the two most consistent and powerful activators used in experimental work have been Streptokinase (SKP) and Staphylokinase (STA). In the course of experiments to determine the effects of these activators on the plasminogen from serum of a number of different species of animals, as measured by the digestion of casein (proteolytic) we noted that the results with staphylokinase did not correspond in all cases with those published by Gerheim and Ferguson, using the fibrinolytic method. The results to be reported were those obtained using both fibrinolytic and proteolytic tests with both activators, and on sera from the animal species previously tested plus a few new species.

Principal Materials

Plasminogen: prepared by the previously reported modification of Milstone's method. See methods.

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Streptokinase (SKP) crude: the filtrate of a 24 hour culture of group A, type II beta hemolytic streptococci grown in beef heart infusion broth with 0.05 per cent added dextrose. In later tests a special streptokinase—streptodornase preparation containing 100,000 units SKP/cc. was used.

Staphylokinase (STA): prepared according to the method of Lewis and Ferguson\(^6\) from a culture of a virulent Staphylococcus aureus.

Casein: a 5 per cent solution of soluble casein (Eimer and Amend), adjusted to a final pH of 7.35 to 7.45. (Free of proteolytic activity).

Veronal buffer: pH of 7.35.

Fibrinogen: a 0.5 per cent solution of Armour Fraction I, bovine fibrinogen, essentially lysozime free.

Thrombin: prepared from Armour beef plasma globulin ppt. pH 5.1, by a method previously reported by Clark.\(^7\)

**Methods**

**Proteolytic Method**

The euglobulin fraction containing plasminogen is precipitated from serum by adding 2 ml. serum to 38 ml. of .016 per cent acetic acid inverting once to mix and centrifuging at 2000 R.P.M. for 15 minutes.

The supernatant is discarded and the tubes are inverted, allowing the acetic acid solution to drain. The precipitate is then dissolved in an amount of veronal buffer, (2.0 cc.) equal to the volume of serum used. Of this solution, 0.5 cc. is placed into each of three test tubes. To the first 0.4 ml. streptokinase and 0.1 ml. veronal buffer is added. To the second 0.4 ml. of a 2 per cent staphylokinase solution and 0.1 ml. veronal buffer are added. To the third 0.5 ml. veronal buffer is added. This tube represents spontaneous activity. A control tube for each of the three tubes is then prepared by substituting veronal buffer for the plasminogen solution and adding like quantities of activators.

To each of the tubes 5 ml. of a 5 per cent casein solution is added, and mixed by inversion and immediately placed in an incubator at 37 C. for 24 hours.

After incubation, a 2.5 ml. aliquot from each tube is placed in a test tube and 5 cc. of 16 per cent trichloroacetic acid, is added and mixed. After standing 10 minutes, each sample is filtered through Whatman no. 3 paper. A 2.5 ml. aliquot of the filtrate is added to 5 ml. of 1 N NaOH in a 50 ml. Erlenmeyer flask.

To each flask in turn, 1.5 ml. of diluted phenol reagent (1:2) is added as rapidly as possible and the contents of the flasks are transferred to spectrophotometric cuvettes. Exactly 10 minutes after the phenol reagent was added to the first flask the per cent transmission of each sample is determined in turn using the Coleman senior spectrophotometer set at 675 \(\mu\). To negate the color produced by the reagents, the blank of each series is used as reference, by setting the galvanometer index at 100 per cent transmittance or 0 concentration. The per cent transmittance is then read directly and converted to mg. per cent tyrosine formed, by reference to a standard curve.

**Fibrinolytic Method**

Plasminogen is obtained as above. To three sterile 13 x 100 mm. tubes, 5 ml. of reconstituted plasminogen solution is added. To the first, 0.1 ml. streptokinase solution, to the second, 0.1 ml. 2 per cent staphylokinase solution, and to the third, 0.1 buffer are added, and they are placed in a 37 C. water bath for 3 minutes. Simultaneously a second set of test tubes is prepared, substituting buffer for the plasminogen and adding the same reagents in the same proportion as in the first series. These are the controls.

After activation, 0.3 ml. fibrinogen and after that 0.1 ml. of thrombin are added. The tubes are corked and placed in a 37 C. water bath. The time after the addition of thrombin to complete dissolution of the clot is the lysis time. A lysis time of over 18 hours is open to doubt although all tubes were read till complete lysis occurred.
PROTEOLYTIC AND FIBRINOLYTIC ACTIVITY OF SERUM

Results

The proteolytic activity of the plasminogen obtained from sera of animals was found to show the same species variations as reported previously. Using SKP as the activator the order of activity of the various species was essentially as shown in table 1, with activity of individual sera overlapping in some instances. In general, the activity shown by sera of the last five species, chicken, pig, cow, horse, and rat can be said to be minimal or absent since most fell within the range of error of the method. This was certainly true of the rat.

Using STA as the activator there was an obvious difference (table 2) since the monkey and rabbit showed the maximal activity in all sera tested while the dog and guinea pig showed maximal activity in at least half and high activity in the remaining sera. It will be noted from figures 4, 5, and 6 that in these four species proteolytic activity with STA far outstripped the activity with SKP. On the other hand, the human sera (figs. 3 and 4) showed much greater

Table 1.—Proteolytic Activity of Serum

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
</tr>
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<tbody>
<tr>
<td>SKP</td>
<td>STA</td>
</tr>
<tr>
<td>Dog</td>
<td>Monkey</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Human</td>
<td>Dog</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Guinea pig</td>
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<tr>
<td>Monkey</td>
<td>Human</td>
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<td>Chicken</td>
<td>Pig</td>
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<td>Pig</td>
<td>Chicken</td>
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<tr>
<td>Cow</td>
<td>Horse</td>
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<tr>
<td>Horse</td>
<td>Rat</td>
</tr>
</tbody>
</table>

The animals are listed in the order of the proteolytic activity of their plasmin as activated by SKP (streptokinase) and STA (staphylokinase). The most active at the top.

Table 2.—Fibrinolytic Activity

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
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<tbody>
<tr>
<td>SKP</td>
<td>STA</td>
</tr>
<tr>
<td>Dog</td>
<td>Dog</td>
</tr>
<tr>
<td>Human</td>
<td>Guinea pig</td>
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<tr>
<td>Guinea pig</td>
<td>Rabbit</td>
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<tr>
<td>Rabbit</td>
<td>Human</td>
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<td>Monkey</td>
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<td>Pig</td>
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<td>Rat</td>
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<td>Chicken</td>
<td>Chicken</td>
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<tr>
<td>Cow</td>
<td>Pig</td>
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</tbody>
</table>

The animals are listed in the order of fibrinolytic activity of their plasmin as activated by SKP and STA.
Figs. 1-7.—Proteolytic activity in mg. per cent tyrosine as indicated by the figures along the vertical column to the left. The higher the column, the greater the activity. This is the opposite of the fibrinolytic side where greater activity is indicated by lower columns.

Fibrinolytic activity is measured by the hours or portion thereof necessary for complete lysis. The hours were indicated by the figures in the vertical column in the middle of the figures. Figures over 14 hours are not considered significant but for completeness are shown at the tops of the columns. In the fibrinolytic columns, the greater activity is indicated by the lower column in contrast to the proteolytic graphs.

Table 3.—Activation with Staphylokinase (STA)

<table>
<thead>
<tr>
<th></th>
<th>Fibrinolytic activity</th>
<th>Proteolytic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Horse</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Monkey</td>
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<td>+</td>
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<tr>
<td>Human</td>
<td>+</td>
<td>Minimal</td>
</tr>
<tr>
<td>Pig</td>
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<td>Minimal</td>
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<td>Cow</td>
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<td>Rat</td>
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The presence of definite activity is indicated by a plus sign (+). No attempt is made to indicate degree of activity. Note that only the horse and monkey show absolute differences and the human shows marked variations. All other animals show either both types of activity or practically no activity.

proteolytic activity with SKP than with STA activation. Indeed with several patients no activity was observed with STA. With the last five animals (pig, chicken, cow, horse, and rat) once again the activity was minimal or absent with STA as with SKP.
Using fibrinolytic activity as the test, the order of activity using SKP as the activator was found to be essentially the same as for proteolytic activity (table 2A). Since the lysis time for sera of the last five animals (horse, pig, rat, chicken, and cow) was over 21 hours, the variations cannot be considered of great significance and indicate minimal or absent activity.

With STA as the activator in the fibrinolytic test, however, there was a marked change in the order of activity (table 2B) as compared to the proteolytic reaction (table 1B). From a study of these tables and figures 1 to 7 it becomes
apparent that although there were many slight shifts in position and degree of activity there were two animals which showed marked differences in their fibrinolytic and proteolytic activity. These were the monkey and the horse. Plasminogen from the serum of monkeys (fig. 1) showed a maximal proteolytic activity when activated with STA, while the same plasminogen activated by the same STA showed very little if any increase of fibrinolytic activity over that with spontaneous activation. One animal only (B, fig. 1) showed increased fibrinolytic activity and even this not of a degree comparable to the proteolytic
activity. Plasminogen of sera from the horses tested showed exactly the opposite phenomenon. There was practically no proteolytic activity evidenced with STA but there was significant fibrinolytic activity (lysis in 4 hours in two cases and in 2 hours in the third) shown by the same sera (fig. 2).

![Graph](https://example.com/graph1.png)

**Fig. 6.** (See legend to fig. 1)

![Graph](https://example.com/graph2.png)

**Fig. 7.** (See legend to fig. 1)

Human sera (figs. 3 and 4) showed a significant differentiation between fibrinolytic and proteolytic activity with STA, although this was not so absolute as with the horse and monkey. All patients except one (P. S., fig. 4) showed much less proteolytic activity with STA than with SKP and much greater fibrinolytic activity with STA than with SKP.

In the other animals chicken and cow (fig. 2), dog (fig. 5), and rabbit and
guinea pig (fig. 6) the proteolytic and fibrinolytic activities with both SKP and STA were roughly comparable (table 3).

**Discussion**

The results of these experiments suggest that fibrinolytic and proteolytic activity of the serum, as measured by these tests, are not identical. Indeed the results indicate that these activities may be due to distinct enzymes. This is especially likely since the results with the monkey and horse are exactly opposite with STA activation. The former showed maximal proteolytic activity with practically no fibrinolytic activity, the latter marked fibrinolytic activity with no significant proteolytic activity.

It is possible that these variations were due to variation in inhibitors. If so, this would imply that beef fibrinogen or thrombin contained a specific anti monkey plasmin inhibitor and that casein contained a specific anti horse plasmin inhibitor since the plasminogen and staphylokinase used were identical. Although the animals studied were few, the results of proteolytic tests closely simulate results obtained in other series of animals previously reported. The possibility of error was further diminished by repeating all tests on several occasions. It is also conceivable that a specific antifibrinolysin as an inhibitor distinct from antitrypsin,18 may affect only the fibrinolysin activity of serum amid may vary from one species to another.

Other recent work on the effect of adrenal cortical hormones, tends to support the impression that different enzymes were being studied. In a recently published paper19 we reported our finding of a marked increase of spontaneous proteolytic activity of serum following the use of ACTH amid Cortisone. True-love,20 using a fibrinolytic method, failed to find any change. Since we were unable to demonstrate any significant change in antitryptic activity this could not explain the difference. However changes in a specific antifibrinolysin could explain this difference.

Considering the known variability of proteolytic enzymes as tested by specific substrates, it does not seem too inconceivable that serum may contain different fibrinolytic and proteolytic enzymes. This does not preclude the possibility that the proteolytic enzyme may also be fibrinolytic as a part of its activity. One enzyme may by choice continue to act on the single bond of multiple fibrin molecules necessary to result in lysis of the clot. The other may by preference continue the breakdown of the protein component of the small portion of fibrin first affected, thus not resulting in clot lysis till much later.

There are undoubtedly many other possible explanations that will come to mind. These may lead to experiments which will disprove this thesis and support the more commonly held view that proteolytic and fibrinolytic activity are due to the same enzyme.

**Summary**

1. The fibrinolytic and proteolytic activity of serum plasminogen, activated by Streptokinase and Staphylokinase and measured by clot lysis and casein digestion, was studied in the following species of animals: human, cow, dog, horse, monkey, pig, rabbit, rat, and chicken.
2. A significant difference in fibrinolytic and proteolytic activity of serum plasminogen (activated by staphylokinnase) was observed in three animal species: monkey, horse, and human. The significance of this observation is briefly discussed.

REFERENCES

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