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Anticoagulant and Fibrinolytic Properties of Salivary Proteins from the Leech *Haementeria ghilianii*[1] (41271)

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Abstract. The salivary glands of the blood-sucking leech *Haementeria ghilianii* contain an anticoagulant that not only inhibits the clotting of human and bovine plasma, but also dissolves previously formed fibrin clots. This anticoagulant activity is attributable to an enzyme, for which the name hementin is proposed. Hementin catalyzes the proteolytic degradation of fibrinogen and fibrin, even in the presence of the inhibitors of proteases occurring in human plasma. The enzyme has the same affinity for human fibrinogen and fibrin. In human fibrinogen cleaves the Aα chain initially and then the γ chain to yield characteristic fragments of high molecular weight that are different from the fragments resulting from the digestion of fibrinogen by plasmin. The salivary extracts do not contain any appreciable amounts of an activator of human plasminogen or an inhibitor of human or bovine thrombin. Thus, *H. ghilianii* prevents coagulation of its host blood through a fibrinogenolytic mechanism that is entirely different from that of hirudin, a thrombin-inactivating polypeptide present in the saliva of another leech, *Hirudo medicinalis*.

Blood-sucking animals, such as leeches, mosquitoes, and vampire bats, usually contain in their saliva substances that prevent the host blood from coagulating as it is being withdrawn and after it is ingested. Such anticoagulant substances fall into two general categories. One consists of inhibitors of thrombin or other clotting factors (1, 2). The other consists of promoters of fibrinolysis (3–6), whose action directly or indirectly results in the cleavage of fibrinogen, rendering it unclottable. Interest in these substances arises both from their high specificity of action and from their potential clinical use in thromboembolic diseases.

The best-understood of these anticoagulants is hirudin, secreted by the leech *Hirudo medicinalis*. Hirudin is a polypeptide of known amino acid sequence (7) which binds irreversibly to human thrombin (8). The saliva of the vampire bat, *Desmodus rotundus*, contains an anticoagulant of the second category, namely an activator of mammalian plasminogen (4). An anticoagulant has been reported in a leech, *Haementeria lutzi* Pinto 1920, native to Brazil. This anticoagulant did not have any direct proteolytic activity on casein, fibrinogen, or fibrin and was promoting fibrinolysis by activation of host plasminogen (9).

The work to be reported here aimed to elucidate the nature of the anticoagulant found in the saliva of yet another leech, the giant *Haementeria ghilianii*, native to French Guyana (10, 11). The study of the properties of extracts made from leech salivary glands showed that the anticoagulant of *H. ghilianii* belongs to the second category. Its action results from the presence of a fibrinolytic enzyme, to be called hementin, that prevents the formation of fibrin clots by proteolysis of host fibrinogen.

Materials and Methods. *Reagents.* Analytical reagents, human fibrinogen, and α-thrombin were the same as in the preceding paper (12). Human and bovine plasma were obtained by centrifugation of 9 vol of blood mixed with 1 vol of 0.135 *M*

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sodium citrate. Human plasmin (10.2 CTA[2] units/ml; 0.8 mg/ml) was kindly supplied by Dr. David L. Aronson, Bureau of Biologics, FDA, Rockville, Maryland. Human plasminogen was obtained from fresh citrated plasma by affinity chromatography on lysine–Sepharose (14). Streptokinase (Varidase, Lederle Laboratories, Pearl River, N.Y.) and urokinase (Abbott Laboratories, North Chicago, Ill) were commercial preparations.

Salivary gland extracts. The anterior and posterior salivary glands of *H. ghilianii* were collected separately and extracted with 0.15 *M* Tris–HCl buffer, pH 7.8, as described in the preceding paper (12). The extracts were centrifuged at 100,000*g* for 60 min to remove the remaining particulate matter.

Clotting time was assayed using plasma or human fibrinogen at a concentration of thrombin-clottable protein at 2.5 mg/ml. A buffer containing 0.05 *M* sodium Veronal, 0.1 *M* sodium chloride, pH 7.75, was used for dilutions. For testing, 0.1 ml of the substrate was mixed with 0.1 ml of extract (or buffer when the extract was omitted) and 0.1 ml of 0.02 *M* calcium chloride (or buffer when calcium ions were omitted). The mixture was incubated at 37° for 8 min and 0.1 ml of human α-thrombin (100 u/ml) was added. The control clotting time was approximately 10 sec. The clotting time was expressed as a ratio of sample to control clotting time.

Thrombin activity was measured using a chromogenic substrate D-Phe-Pip–Arg-NH-C_6H_4-NO_2·2HCl (S-2238, Ortho Diagnostics Inc., Raritan, N.J.) at a final concentration of 0.667 m*M*. The reaction was done in 0.15 *M* Tris–HCl buffer, pH 7.4, at 37°. The initial rate of *p*-nitroaniline release was determined by measuring the absorbance change at 405 nm (15) in a recording spectrophotometer (Model 230, Gilford, Oberlin, Ohio). A molar absorption coefficient of 10,600 liters·mole^{-1} was used for *p*-nitroaniline at this wavelength and pH (16).

Amidolytic activity of hementin and activated plasminogen was measured using a chromogenic substrate D-Val–Leu–Lys-NH-C_6H_4-NO_2·2HCl (S-2251, Ortho Diagnostics Inc.) at a final concentration of 0.6 m*M*. The reaction was carried out in 0.05 *M* Tris–HCl buffer containing 0.01 *M* sodium chloride, pH 7.4, at 37° for 3 min and stopped by the addition of 0.1 vol of 50% acetic acid and the absorbance was determined at 405 nm (17).

Euglobulin clot lysis was used for the measurement of the fibrinolytic activity (18). Euglobulins were obtained from 1 vol of citrated plasma mixed with 10 vol of cold acetic acid (3.67 m*M*). The precipitate was collected by centrifugation, dissolved in 0.7 vol of 0.05 *M* sodium phosphate buffer containing 0.1 *M* sodium chloride, pH 7.7, and stored at −20°. For testing, euglobulins were diluted with the buffer to 2.5 mg/ml protein concentrations and 0.1 ml of the substrate was mixed with 0.1 ml of gland extract and 0.1 ml of human α-thrombin of 10 u/ml. The clots were incubated at 37° and time of complete dissolution of the clots was measured.

Fibrin strips were prepared as described in the preceding paper (12).

Labeling of human fibrinogen was done using the iodine monochloride method (19): 5 mg of fibrinogen (14.7 nmole) in 0.35 ml of 0.3 *M* glycine–NaOH buffer, pH 8.2, was mixed with 500 μCi of carrier-free $Na^{125}I$ (New England Nuclear, Boston, Mass.) and 10 μl of 0.003 *M* iodine monochloride (30 nmole). After 60 sec the reaction was stopped by the addition of 0.1 ml of 0.01 *M* sodium metabisulfite and the labeled fibrinogen was purified by column (0.5 × 20 cm) gel filtration on Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, Calif.). The labeled protein contained approximately 0.8 atom of iodine per molecule and had a specific radioactivity on the order of 100 μCi/mg protein.

Polyacrylamide gel electrophoresis was carried out in 7% gels containing 0.1% SDS (20) and stained with Coomassie brilliant blue R-250 (21)

Results. *Anticoagulant activity.* The thrombin clotting time of either human or bovine plasma and human fibrinogen was

[2] Abbreviations used: SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; CTA units, Committee on Thrombolytic Agents units (13); Pip, pipecolyl.

prolonged by the presence of extracts from the anterior or posterior salivary glands (Fig. 1). All plots of extract concentration versus clotting time were concave upward, a feature associated with the fact that at high extract concentrations the substrates became incoagulable. The specific anticoagulant activity was higher in the posterior than in the anterior gland extract. To assess the effect of calcium ions on anticoagulant activity similar experiments were performed in the presence of 5 m*M* calcium chloride (Fig. 1). Calcium ions did not have any pronounced effect on anticoagulant properties of either gland extract. A scatter diagram of clotting time in the presence of calcium ions versus that in the absence was linear and the correlation coefficient was 0.87. Linear regression analysis showed no significant difference ($P > 0.1$) between the clotting time in the presence and absence of calcium ions.

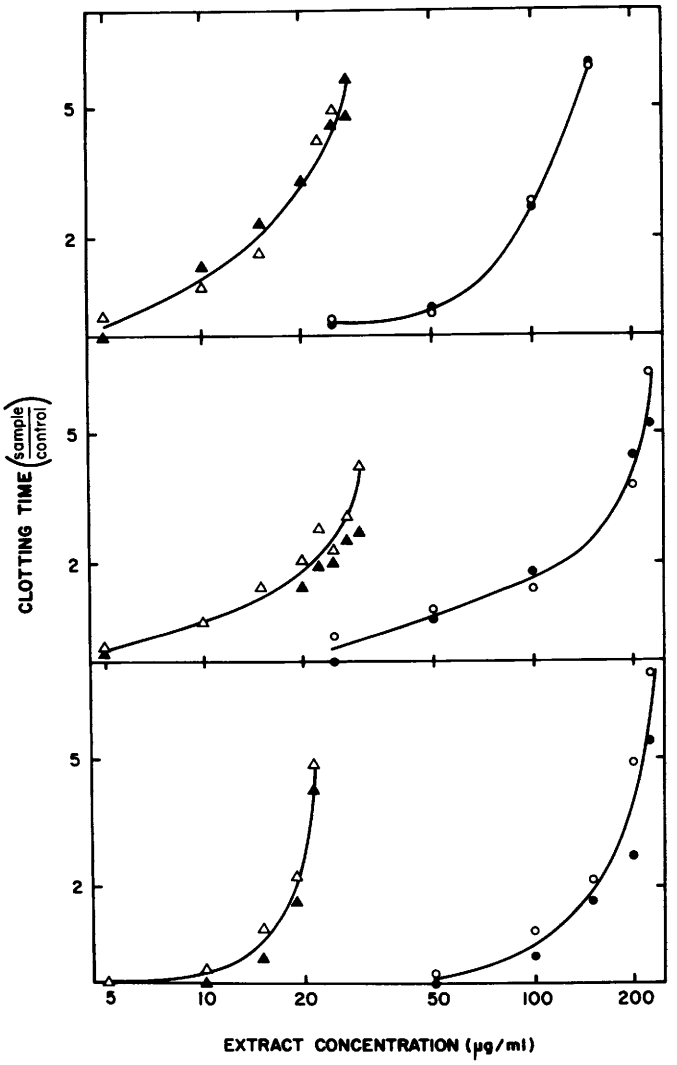


FIG. 1. Anticoagulant effect of the anterior (circles) and posterior (triangles) salivary gland extracts on human plasma (top), human fibrinogen (middle), and bovine plasma (bottom). The thrombin clotting time was measured either in the absence of calcium ions (open symbols) or in the presence of 5 m*M* calcium chloride (closed symbols). A ratio of sample clotting time to control clotting time is plotted on ordinate. Each point represents a mean value of six determinations.

The anticoagulant activity was not due to the inactivation of thrombin by the extracts, since preincubation of human α-thrombin with the extracts at 37° for 10 min did not result in any additional prolongation of the clotting time in the subsequent tests. Further evidence for the absence of a potent direct antithrombin from the gland extracts was obtained from measurements of amidolytic activity of human α-thrombin (Table I). The amidolytic activity of thrombin remained unchanged after incubation with amounts of either anterior or posterior gland extract that render fibrinogen unclottable.

Fibrinolytic activity. Fibrin clots were formed by the action of thrombin on human or bovine euglobulins. The substrates were used at a protein concentration of 2.5 mg/ml and the anterior gland extract at 1 mg/ml. The clots were incubated at 37° in the presence of calcium chloride (5 m*M*). The clots did not lyse spontaneously while incubated without gland extracts for 24 hr. The lysis time, defined as the period between the addition of extract and complete clot lysis, was for human and bovine clots 51 and 47 min, respectively, indicating similar susceptibility of fibrin from both species to dissolution by the anterior gland extracts. A similar fibrinolytic activity was found in the posterior salivary gland extracts when tested at a concentration of 0.2 mg/ml. Under the same conditions, plasmin (0.2 CTA μ/ml) gave a lysis time of 60 and 1000 min with human and bovine clots, respectively.

The fibrinolytic activity of the extracts from both anterior and posterior salivary glands was confirmed by the use of the fibrin strip method. Upon incubation of fibrin strips to which a 1-μl sample of extract had been applied, a circular zone of lysed fibrin developed. The area of the lysed zone (or the square of its diameter), and hence the fibrinolytic activity (Fig. 2), increased with increasing concentration of the salivary extracts, according to the relation

$$\log D^2 = k \log aC,$$

where D is the diameter of the lysed zone, C the concentration of the extract and a and k are constants. Both extracts show a similar value for k, namely, about 0.7, as does plasmin, for which control values are also shown in Fig. 2. The fact that k has a value of 0.7 indicates that the area of the lysed zone increases only with 0.7th power of the amount of fibrinolysin applied to the fibrin strip. However, the value of a, and hence the specific fibrinolytic activity, is 3.6 times higher for the posterior than for the anterior gland extract.

To test for the presence of an activator of human plasminogen in the salivary extracts, fibrin strips were used in which the substrate was either enriched in or depleted of plasminogen. Since the extracts promoted the same rate of fibrinolysis on plasminogen-rich strips as on plasminogen-depleted strips, it was concluded that neither the anterior nor the posterior salivary gland contains plasminogen activator.

Amidolytic activity. Human plasminogen

TABLE I. THE EFFECT OF GLAND EXTRACTS ON THE AMIDOLYTIC ACTIVITY OF THROMBIN

	Concentration[a] (μg/ml)	Amidolytic activity[b] (pNA μmole/min)
Thrombin	1	12.1 ± 2.7
Anterior extract	250	1.0 ± 0.4
Posterior extract	50	0.28 ± 0.09
Thrombin	1	
+ Anterior extract	250	12.8 ± 1.5
Thrombin	1	
+ Posterior extract	50	11.1 ± 1.8

[a] The concentration of α-thrombin and extracts are final in the reaction mixture.

[b] Amidolytic activity was determined with the chromogenic substrate S-2238 (D-Phe-Pip–Arg-NH-C_6H_4-NO_2) by spectrophotometric determination at 405 nm of the released *p*-nitroaniline (pNA).

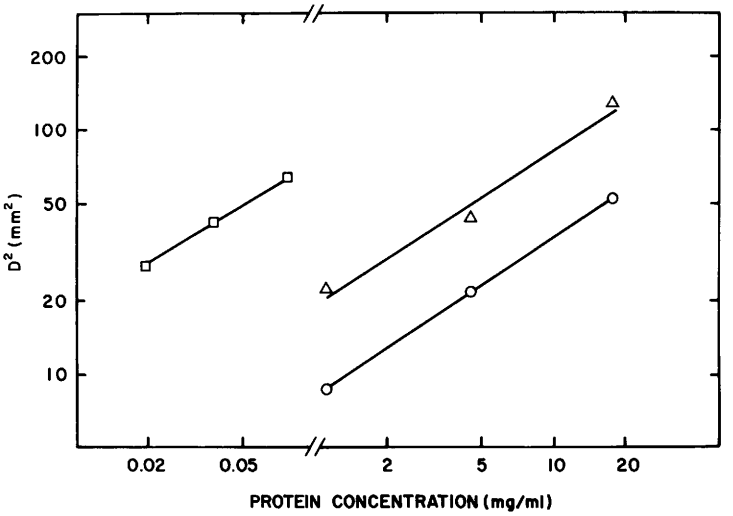


FIG. 2. Dependence of fibrinolytic activity on the concentration of the anterior (○) and posterior (△) salivary gland extracts, and of plasmin (□). The activity, expressed as the second power of the diameter (D^2) of the lysis zone on fibrin strips incubated at 37° for 24 hr. is plotted against protein concentration, both on a logarithmic scale. Each point represents a mean value of six determinations.

incubated with streptokinase was activated and gained amidolytic activity (Table II). However, when the same plasminogen preparation was incubated with either the anterior or posterior gland extract, only a small fraction of that activity was measured, and it was entirely accountable for the amidolysis induced by the extracts themselves. The result supports the conclusion that the anterior and posterior gland extracts do not convert human plasminogen to an active enzyme.

The rate of *p*-nitroaniline cleavage and specific amidolytic activity of the anterior

TABLE II. THE EFFECT OF GLAND EXTRACTS ON THE AMIDOLYTIC ACTIVITY OF PLASMINOGEN[a]

Sample	Activator	Amidolytic activity (pNA μmole/min)	Specific activity (pNA μmole/min)	
			Per mg of plasminogen	Per mg of gland extract
Plasminogen, 3.68 μg/ml	Buffer	0	—	—
Plasminogen, 3.68 μg/ml	Streptokinase, 1000 units/ml	20.0	5.43	—
Plasminogen, 3.68 μg/ml	Anterior extract, 200 μg/ml	2.08	0.57	0.0104
Buffer	Anterior extract, 200 μg/ml	2.06	—	0.0103
Plasminogen, 3.68 μg/ml	Posterior extract, 200 μg/ml	0.88	0.24	0.0044
Buffer	Posterior extract, 200 μg/ml	0.77	—	0.0039

[a] The concentration of human plasminogen, streptokinase, and extracts are final in the reaction mixture. Amidolytic activity was determined using a chromogenic substrate S-2251 (D-Val–Leu–Lys-NH-C_6N_4-NO_2) and spectrophotometric determination of the cleaved *p*-nitroaniline (*p*NA).

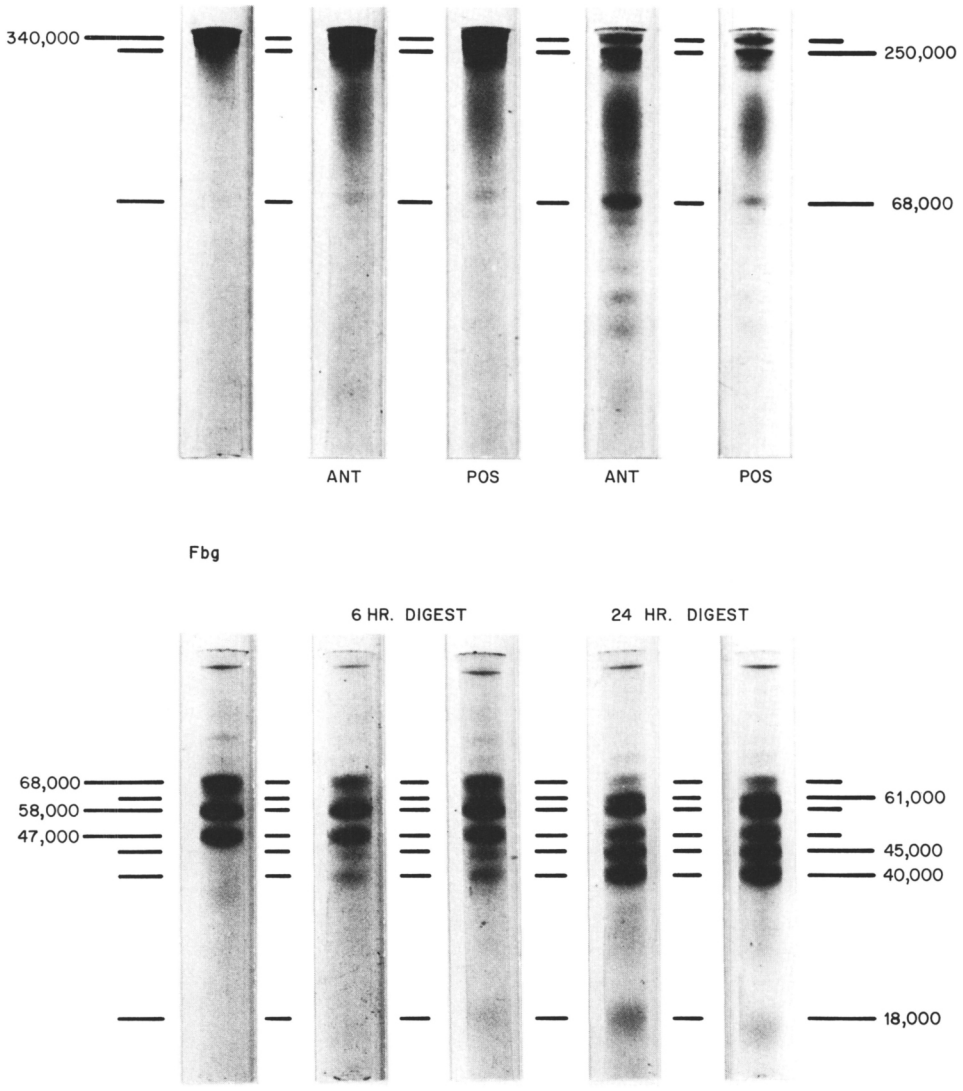


FIG. 3. Electrophoretic patterns of purified human fibrinogen incubated with either the anterior (ANT) or posterior (POS) gland extracts. Equal volumes of fibrinogen (2.5 mg/ml) in 0.15 *M* Tris–HCl buffer, pH 7.8, and the anterior (0.1 mg/ml) or posterior (0.02 mg/ml) gland extracts in the same buffer were incubated at 37° for 6 and 24 hr. A 6 *M* urea solution containing 3% SDS was added to stop the reaction and the digests were analyzed in 7% polyacrylamide gels under nonreduced (top) and reduced (bottom) conditions. The starting fibrinogen (Fbg) is shown on the left. The numbers on the left margin denote the accepted values of molecular weight of fibrinogen (340,000) and its polypeptide chains Aα (68,000), Bβ (58,000), and γ (47,000). The numbers on the right margin show the calculated molecular weights of predominant fragments.

gland extract were higher than those of the posterior gland. This observation may reflect the fact that the chromogenic substrate S-2251 is a rather poor one for both extracts since on fibrin strips (Fig. 2) the posterior gland extract has a specific fibrinolytic activity higher than that of the anterior gland extract. Presumably, the chromogenic substrate S-2251 is cleaved by proteolytic enzymes other than hementin present in the extracts.

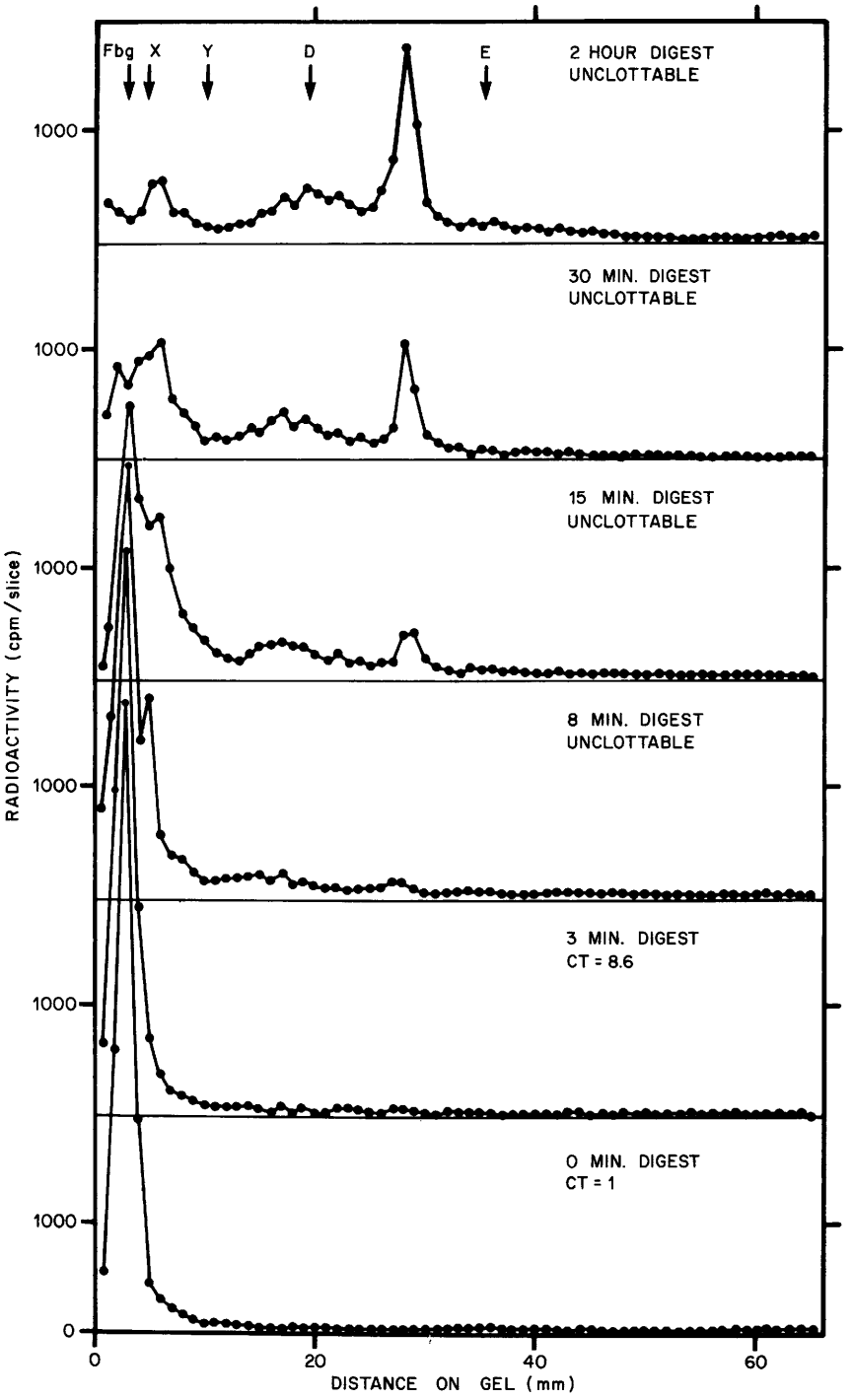
Degradation of fibrinogen. The anti-

coagulant effect of the salivary extracts on plasma and fibrinogen can be explained by a direct action of hementin that converts fibrinogen into unclottable derivatives. To test this possibility purified human fibrinogen was digested with an extract from either the anterior or posterior gland for various times. The digestion products were characterized by polyacrylamide gel electrophoresis under either nonreduced or reduced conditions. The digestion conditions were selected so as to degrade completely the fibrinogen added to the reaction mixture (2.5 mg/ml) after 24 hr of incubation at 37° (Fig. 3). It is evident that the anterior and posterior gland extracts gave the same digestion pattern, provided that the concentration of the latter is one-fifth of the former. The close similarity of fibrinogen degradation products indicates the same enzymatic specificity in both salivary glands. There was no evidence of the formation of fibrinogen degradation products resembling the well-characterized plasmic derivatives: Fragments X (MW 250,000), Y (MW 150,000), D (MW 103,000) and E (MW 45,000) (22). Instead, groups of several fibrinogen derivatives were formed with molecular weights in the range 130,000–300,000 with a predominant species of MW 250,000. A terminal product of MW 68,000 was consistently present after long digestion. The $A\alpha$ polypeptide chain of fibrinogen was evidently cleaved first by hementin, followed by the degradation of the γ chain as demonstrated by electrophoresis in reduced conditions (Fig. 3). Four major polypeptide chain fragments of MW 61,000, 45,000, 40,000, and 18,000 were observed in the digests. This fibrinogen degradation pattern was found to be the same even if human plasminogen or calcium chloride (5 m*M*) was added to the digestion mixture. However, the digestion proceeded slower in the presence of calcium ions.

The fibrinogen preparation used in these experiments was contaminated with plasma fibronectin, which is seen as a band near the top of the reduced gels (Fig. 3). The electrophoretic mobility of fibronectin did not seem to change after digestion by salivary gland extracts.

To provide direct supportive evidence that the anticoagulant effect originates from the degradation of fibrinogen, human plasma was supplemented with ^{125}I-labeled human fibrinogen, incubated with the extracts for various time, then the clotting time was measured and the distribution of radioactive fragments was analyzed by polyacrylamide gel electrophoresis (Fig. 4). The same electrophoretic patterns and the same sequence of fibrinogen degradation was found with the anterior and posterior extracts when used a final concentration of 0.2 and 0.045 mg/ml, respectively. The degradation of fibrinogen correlated with prolongation of the clotting time. Digestion for 3 min resulted in an 8.6-fold increase of the clotting time and was accompanied by a small but distinct change of fibrinogen electrophoretic mobility. After 8 min of digestion plasma became uncoagulable by thrombin and this fact was paralleled by further degradation of fibrinogen. The cleavage of the labeled fibrinogen gave rise to several intermediate fragments and the electrophoretic patterns of selected radioactive degradation products were very similar to those obtained by the digestion of unlabeled human fibrinogen. The patterns

FIG. 4. Degradation of fibrinogen in plasma by salivary gland extracts. One hundred and fifty microliters of human citrated plasma was mixed with 15 μg of ^{125}I-labeled human fibrinogen (= 5×10^5 cpm total) and 150 μl of either anterior (0.4 mg/ml) or posterior (0.09 mg/ml) gland extract. The mixture was incubated at 37° and after 3, 8, 15, and 30 min and 2 hr, 40-μl subsamples were withdrawn, mixed with 210 μl of 6 *M* urea and 3% SDS to arrest the reaction, and 50-μl aliquots were electrophoresed on 7% SDS–polyacrylamide gels under nonreduced conditions. The gels were sliced into 1-mm sections and counted. In a parallel experiment 15 μg of unlabeled human fibrinogen was substituted for the labeled counterpart. The thrombin clotting time was determined at the end of each incubation interval and its ratio (CT) to control clotting time is shown. The absence of a clot after 15 min is denoted as "unclottable." The electrophoretic mobility of fibrinogen (Fbg) and its Fragments X, Y, D, and E obtained by proteolysis with plasmin, is shown at the top of the figure.


Fbg
X
Y
D
E
2 HOUR DIGEST
UNCLOTTABLE
30 MIN. DIGEST
UNCLOTTABLE
15 MIN. DIGEST
UNCLOTTABLE
8 MIN. DIGEST
UNCLOTTABLE
3 MIN. DIGEST
CT = 8.6
0 MIN. DIGEST
CT = 1
1000
1000
1000
1000
1000
0
RADIOACTIVITY (cpm/slice)
0
20
40
60
DISTANCE ON GEL (mm)

of fibrinogen degradation provide evidence that hementin is not inactivated by plasma inhibitors of proteolytic enzymes during the course of the reaction, since a clear-cut progress of degradation is evident between each time interval.

Degradation of fibrin. To compare the affinity of hementin for fibrinogen and fibrin two sets of experiments were performed. In one, the degradation of fibrinogen in plasma was assayed; in the other, after addition of thrombin lysis of a plasma clot was tested. Human plasma was supplemented with ^{125}I-labeled human fibrinogen and eight different concentrations were varied between 0.98 and 55 μM either by dilution with a heat-defibrinogenated (60° for 3 min) plasma or by the addition of nonlabeled fibrinogen. From each fibrinogen concentration, subsamples were withdrawn at 0, 5, 10, 15, and 20 min, precipitated with 1.23 M ammonium sulfate, and centrifuged, and the radioactivity of pellets and supernates was counted and the amount of degraded fibrinogen calculated. A double reciprocal plot of fibrinogen concentration in plasma versus initial rate of substrate cleavage showed the same line for fibrinogen and fibrin. A level of 50% of the maximum reaction rate was attained at 16×10^{-6} M and 5×10^{-6} M of the substrate for the anterior and posterior gland extract, respectively. Thus, hementin has the same high affinity for fibrinogen and fibrin.

Discussion. The salivary glands of the leech *H. ghilianii* contain an anticoagulant that prevents the host blood from clotting during feeding and after ingestion. As has been shown in the present work, the mechanism of action of this anticoagulant is direct fibrinogenolysis. On the basis of the evidence provided here that *H. ghilianii* contains a hitherto undescribed enzyme we propose the name hementin for the enzyme.

The mechanism of blood anticoagulation by *H. ghilianii* is entirely different from that of *H. medicinalis*. *Hirudo* produces hirudin arresting blood clotting by inactivation of thrombin (7). The saliva of *H. ghilianii* does not affect thrombin (Table I) but attacks the clot-forming substrate rendering it uncoagulable. These two different mechanisms of blood anticoagulation indicate an early evolutionary divergence of *Haementeria* and *Hirudo*. These mechanisms may be related to different feeding patterns of the two leech species. *Haementeria* is a Rhynchobdellid which is characterized by a proboscis, whereas *Hirudo* is an Arhynchobdellid equipped with jaws.

Hementin is present in both the anterior and posterior salivary glands (Figs. 1 and 2). This is somewhat surprising, in view of the fact that these two glands have a completely different protein composition (12) and that they are composed of very different cell types (23). The specific activity of hementin is approximately four times higher in extracts from the posterior gland. It is not excluded, however, that either or both glands contain protease inhibitors similar to the bdellins identified in the salivary secretions of *H. medicinalis* that inhibit plasmin, trypsin, and chymotrypsin (24). Hementin has a direct proteolytic effect on fibrinogen (Figs. 3 and 4) and fibrin (Fig. 2). No evidence was found that the salivary extracts of *H. ghilianii* contain an inactivator of thrombin (Table I) or an activator of human plasminogen (Table II). Thus, hementin cannot be the anticoagulant substance described by Kellen and Rosenfeld (9), who reported a plasminogen activator in salivary glands of *H. lutzi* Pinto 1920. Moreover, these authors reported also that calcium ions potentiate the activation of the human fibrinolytic system by plasminogen activator from that leech. By contrast, as shown here, calcium ions do not promote the anticoagulant action of hementin (Fig. 1).

The finding that the anticoagulant effect of hementin is expressed similarly in human and bovine plasma and in a solution of purified human fibrinogen (Fig. 1). had important implications. First, it indicates that hementin evolved with a broad specificity to facilitate the feeding on the blood of a variety of animals living around tropical swamps; this enzyme does not restrict the leech to feed on ungulates as was thought in the past. Second, this observation indicates the insensitivity of hementin to the inhibitors of proteolytic enzymes present in plasma (Figs. 1 and 4). This insensitivity of hementin, unique among known fibrinolytic

enzymes, endows it with desirable property from the aspect of thrombolytic agents useful for therapeutic dissolution of blood clots in patients (25).

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