protein pattern following the freezing has been demonstrable so far. It may be, therefore, that the two activities are functions of different parts of the same molecule. An association between antithrombin and antiplasmin has been reported by other workers.[16,17]

While the main antithrombin activity is associated with the alpha globulins, it has been shown that some antithrombin activity is associated with preparations of alpha macroglobulins.[18] This antithrombin apparently does not inhibit the esterase activity of antithrombin.[19] The main antithrombin, that associated with the alpha globulins, inactivates both the clotting activity and the esterase activity of thrombin.

The exact relationship of these closely associated proteolytic activities cannot be settled until more chemically pure fractions are available. To obtain this purity without loss of activity is proving extremely difficult.

[16] N. Heimburger, *First International Symposium on Tissue Factors in the Homeostasis of the Coagulation-Fibrinolysis System. Florence, Italy, May, 1967* p. 353.
[17] H. Gans and B. H. Tan, *Clin. Chim. Acta* **17**, 111 (1967).
[18] M. Steinbuch, C. Blatrix, and F. Josso, *Rev. Franc. Etudes Clin. Biol.* **13**, 179 (1968).
[19] M. Steinbuch, C. Blatrix, and F. Josso, *Nature* **216**, 500 (1967).

[69] Hirudin as an Inhibitor of Thrombin

By FRITZ MARKWARDT

Introduction

The salivary glands (also called neck glands or pharyngeal glands) of the leech *Hirudo medicinalis* contain a substance with anticoagulant properties which has been named hirudin. During leeching, the blood sucker secretes this anticoagulant into the wound in order to keep the blood from clotting. Hirudin was first isolated in 1955 and identified as a polypeptide.[1,2] The following amino acid composition was found (number of residues per mole): 10 Asp, 13 Glu, 6 Cys, 4 Ser, 9 Gly, 4 Thr, 1 Ala, 3 Val, 4 Leu, 2 Ile, 3 Pro, 2 Phe, 2 Tyr, 1 His, and 4 Lys.

The amino acid sequence from the C-terminal end of the molecule is -Ala-Gly-Ser-Glu-Leu.[3] The molecular weight based on the amino acid composition was 10,800. This is in agreement with a value of 9060 obtained by measurements with the analytical ultracentrifuge. The sedi-

[1] F. Markwardt, *Naturwissenschaften* **42**, 587 (1955).
[2] F. Markwardt, *Hoppe-Seylers Z. Physiol. Chem.* **308**, 147 (1957).
[3] P. de la Llosa, C. Tertrin, and M. Jutisz, *Biochim. Biophys. Acta* **93**, 40 (1964).

mentation constant of hirudin ($s^0_{20,w}$) was 0.98, the diffusion constant ($D^0_{20,w}$) was 10.8. The electrophoretic mobility at pH 5.0 (ionic strength 0.1) was 6.6×10^{-5} cm²/V/second. The isoelectric point was found to be pH 3.9.[4] The partial specific volume ($\bar{V}$) was 0.741 ml/g, the sedimentation coefficient at zero concentration being 0.98 S, the diffusion coefficient 10.8 F, and the molar frictional ratio 1.42.[5]

Hirudin is a specific inhibitor of thrombin.[6-8] For its effect, it does not require the presence of other coagulation factors or plasma constituents. Hirudin inhibits blood coagulation by blocking the end product of the first stage of clotting, thrombin, and thereby prevents the conversion of fibrinogen to fibrin.

The reaction of thrombin with hirudin is faster than the reaction of thrombin with fibrinogen. Like an ionic reaction, the time interval of the thrombin–hirudin reaction is too fast to be measured accurately. When thrombin reacts with hirudin, a complex is formed which can be identified by means of electrophoresis and chromatography. Hirudin can be dissociated from this inactive complex by denaturation with heat or acids. The inactive thrombin–hirudin complex is only poorly dissociable, as seen in Fig. 1. The noticeable deviation from the stoichiometric reaction indicates the point of dissociation. From these data a dissociation constant (K_m) of 0.8×10^{-10} (pH 7.4, 20°) was calculated. This dissociation constant is so small that for all practical purposes the complex can be regarded as nondissociable.

Investigations into which groups of the hirudin molecule might be important for the binding of thrombin revealed that esterification of the carboxyl groups of hirudin destroyed its activity. Besides free carboxyl groups, hydroxyl groups of phenol or imidazole groups also could be important for the binding. However, chemicals that reacted with both of these groups did not display an effect on the biological activity of hirudin. In contrast, oxidation of the disulfide bridges by performic acid destroyed the capability of hirudin to bind thrombin.

In order to further elucidate the mode of action of hirudin, its reaction with a chemically modified form of thrombin was of special interest. The acetylation of the free amino groups of thrombin results in esterase–thrombin which has lost its specific proteolytic action on the conversion

[4] F. Markwardt and P. Walsmann, *Hoppe-Seylers Z. Physiol. Chem.* **348**, 1381 (1967).
[5] H. Triebel and P. Walsmann, *Biochim. Biophys. Acta* **120**, 137 (1966).
[6] F. Markwardt, *Arch. Exptl. Pathol. Pharmakol.* **229**, 389 (1956).
[7] F. Markwardt and P. Walsmann, *Hoppe-Seylers Z. Physiol. Chem.* **312**, 85 (1958).
[8] F. Markwardt, "Blutgerinnungshemmende Wirkstoffe aus blutsaugenden Tieren." Fischer, Jena, 1963.

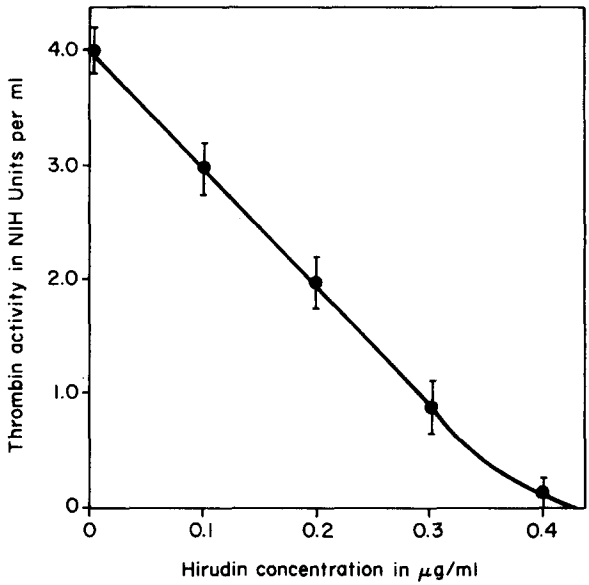

FIG. 1. Inhibition of the clotting activity of thrombin by hirudin.

of fibrinogen to fibrin. Such an esterase–thrombin did not bind hirudin, and therefore, the esterolytic activity of this enzyme cannot be inhibited by hirudin. In contrast, diisopropylphosphoryl-thrombin (DIP-thrombin) in which the serin residue in the active enzyme center has been phosphorylated by diisopropylphosphofluoridate (DFP), is like the native form of thrombin bound by hirudin.

When thrombin reacts with hirudin, which leads to the immediate neutralization of the enzymatic activity of thrombin, the active surface of the enzyme is apparently occupied by hirudin. It has recently been suggested[9] that when thrombin reacts with fibrinogen, the fibrinopeptides, due to their polyanionic characteristics, are bound to positively charged sites on the surface of the enzyme. This brings the fibrinogen molecule into a position where its fibrinopeptides can be cleaved. From the finding that DIP-thrombin still binds hirudin, it can be concluded that the hydrolytically active groups of thrombin are not involved in the binding of hirudin. Apparently the binding sites for hirudin can be found in the cationic groups of the active surface of the enzyme which binds the fibrinopeptide portion of the fibrinogen substrate. Acetylation of these

[9] L. Lorand, "Anticoagulants and Fibrinolysis" (R. L. MacMillan and J. F. Mustard, eds.), p. 333. Pitman, London, 1961.

groups, as in esterase-thrombin, blocks the binding of hirudin and thrombin.

Methods of Assay

For the standardization and control of hirudin activity, its inhibitory effect can be determined in biochemical test systems. A known amount of thrombin is added to a hirudin solution with unknown activity and the remaining thrombin activity is measured. Thrombin activity can be tested on fibrinogen (clotting activity) or on synthetic substrates (esterolytic activity) (Fig. 1).

Since thrombin activity is standardized in National Institute of Health units, the activity of hirudin can easily be related to this international standard. The hirudin activity is expressed in antithrombin units (AT-U), whereby one antithrombin unit is the amount of hirudin which neutralizes one NIH unit of thrombin (fibrinogen assay). One antithrombin unit (AT-U) corresponds to 0.1 μg of pure hirudin.[9]

Hirudin Titration with Thrombin

Based on the specific, rapid, and stoichiometric reaction between hirudin and thrombin, hirudin activity can be quantitatively determined by titration with a standardized thrombin solution.[6] The following principle is involved: A fibrinogen solution to which hirudin has been added will not clot until enough thrombin is added to neutralize all of the hirudin present. An excess amount of thrombin can be noted within seconds by the appearance of a fibrin clot.

Procedure. Aliquots of 0.01–0.1 ml of a hirudin solution with unknown activity are added to 0.2 ml of a 0.5% fibrinogen solution in Tris-NaCl buffer, pH 7.4. After mixing, a standardized thrombin solution (100 NIH units/ml) is gradually added at room temperature. Since the formation of fibrin takes some time, the thrombin cannot be added as quickly as reagents are added when performing chemical titrations. Therefore, thrombin is added in 0.005-ml aliquots (corresponding to 0.5 NIH units) in minute intervals. The thrombin is added with micropipettes and immediately mixed with the fibrinogen–hirudin mixture. Only if the approximate amount of thrombin to be added is already known (repeat titration or after preliminary titration), can thrombin initially be added faster in larger quantities, but as the expected final concentration is approached, small amounts of thrombin are added in minute intervals. The end point of titration is reached when the fibrinogen coagulates within 1 minute. Instead of purified fibrinogen solutions, citrated plasma also can be used as the source of fibrinogen. The standard deviation for this method is $\pm$0.5 AT-U.

Use of Hirudin in Coagulation Studies

The effect which hirudin displays on thrombin can be employed for the determination of the activity of this enzyme. The practical application of this procedure has revealed certain advantages over other techniques. The determination of thrombin and those reactions which are closely related to the formation of thrombin can be easily handled in a rather simple procedure. The developed techniques require, however, pure or standardized hirudin which can either be obtained commercially (VEB Arzneimittelwerk, Dresden, German Democratic Republic) or purified by the technique outlined below.

Determination of Thrombin[10]

Principle. After knowing that thrombin activity is neutralized by hirudin, hirudin can be employed for the determination of thrombin activity by titrating a thrombin solution of unknown activity against a known concentration of pure or standardized hirudin. In principle, the procedure is similar to the one already described for the determination of hirudin activity.

Procedure. An aliquot of 0.1 ml hirudin solution (containing 10 AT-U hirudin) is mixed with 0.1 ml of a 0.5% fibrinogen solution or citrated plasma. After mixing, small quantities of thrombin (from 50 to 200 NIH units) are added at room temperature and the clotting of fibrinogen is recorded. The clotting of the substrate indicates that 10 NIH units of thrombin have been added. The standard deviation is $\pm$ 0.2 NIH units of thrombin.

Determination of Prothrombin[11]

Prothrombin can be assayed only after it is completely converted to thrombin. One unit of prothrombin is defined as the amount of prothrombin which gives rise to 1 NIH unit of thrombin. In order to correctly determine prothrombin, one must be certain that its conversion to thrombin is complete.

Principle. Freshly drawn blood is mixed with an excess and known amount of hirudin. By adding tissue thromboplastin, a complete conversion of prothrombin to thrombin is achieved, but the generated thrombin will be immediately neutralized by the hirudin. The remaining amount of hirudin in the blood sample is next titrated by adding known quantities of standardized thrombin, until the blood sample clots. The

[10] F. Markwardt, *Arch. Pharm.* **290/62,** 281 (1957).
[11] F. Markwardt, *Arch. Exptl. Pathol. Pharmakol.* **232,** 487 (1958).

prothrombin content is calculated from the consumption of hirudin during the coagulation of the blood.

Procedure. In a test tube, 0.05 ml hirudin (containing 30 AT-U) is mixed with exactly 0.2 ml freshly drawn blood and 0.1 ml thromboplastin solution. After 30 minutes of incubation, a thrombin solution of known activity is slowly added using a micropipette, as described for the assay of hirudin. The coagulation of the blood sample is recorded. The amount of thrombin that had to be added to facilitate clotting is substracted from the amount of hirudin added (30 AT-U), the difference being the amount of thrombin generated from the prothrombin in the blood sample by tissue thromboplastin. Since one unit of prothrombin equals 1 NIH unit of thrombin, the exact amount of prothrombin can be calculated.

The use of hirudin in other coagulation procedures, such as the hirudin tolerance test, has been described by Markwardt.[8,12,13]

Isolation of Hirudin[3,14]

Preparation of the Animals and Extraction of the Inhibitor

The glands containing hirudin are located in the region of the body of the leeches which is next to the head region or suctorial disc or, more specifically, in body segments VII, VIII, and IX. These comprise the so-called neck region of the leeches. Since a clean anatomical dissection of the glands for the purpose of obtaining hirudin is not practical, the entire corresponding body parts are separated. For this purpose, leeches of more than 1.5 g body weight, who have not been leeching for at least 3 months, are sacrified by placing them in a 96% ethanol solution. After 24 hours, the frontal portion of the leeches is separated by dissecting this portion from the body approximately 5 mm before the anterior (male) genital orifice. The sections are once more placed into 96% ethanol and dehydrated for an additional 24 hours. The still-wet head sections are next chopped into small pieces and twice extracted for 30 minutes under stirring with a 10-fold volume of a 40% acetone–water solution. The extracts are combined and diluted with ½ volume of an 80% acetone–water solution. By adding glacial acetic acid, the pH is lowered to 4.3–4.5 and the resulting precipitate removed by centrifugation. The supernatant is mixed with a diluted ammonia solution until a pH of 6.0 is obtained. The volume is reduced to 1/10 of the original volume by placing the solution in a vacuum at 40°. Next, the pH is adjusted to 1.8 by adding 10% trichloroacetic acid. The raw hirudin

[12] F. Markwardt, *Klin. Wochschr.* **37**, 1142 (1959).
[13] R. Schmutzler and F. Markwardt, *Klin. Wochschr.* **40**, 796 (1962).
[14] F. Markwardt, G. Schäfer, H. Töpfer, and P. Walsmann, *Pharmazie* **22**, 239 (1967).

PURIFICATION OF HIRUDIN

Procedure	Dry weight (mg)	Total activity (10^3 AT-U)	Specific activity (AT-U/mg)
1. 1000 leech heads dehydrated in 96% ethanol, chopped to small pieces, extracted with 40% acetone, separation of impurities	900	450	500
2. Fractionation with ethanol	270	405	1,500
3. Adsorption on cation-exchange resin	80	360	4,500
4. Gel filtration	35	295	8,400
5. Chromatography on anion-exchange resin	20	208	10,400

product is precipitated from the 10-fold dilution with acetone, washed with acetone, and the solvents are removed in the vacuum. The raw hirudin preparations have a specific activity of approximately 500 AT-U/mg. (See the table.)

Fractionation with Ethanol

Approximately 1.0 g of raw hirudin is dissolved in 30 ml distilled water and chilled to 0°–5°. Over a period of 30 minutes, 54 ml of 96% ethanol are slowly added. The resulting precipitate is removed by centrifugation. The main bulk of hirudin remains in solution. In order to extract additional hirudin, the precipitate is extracted with ethanol two more times as described above. The combined supernatants are cooled to 0° and cold (—10°) ethanol is slowly added until a concentration of 85% (v/v) is obtained. For more complete precipitation, 0.5% ammonium acetate was added to the ethanol. The precipitate is collected by centrifugation and after washing with ethanol, it is dried in a dessicator under vacuum.

These hirudin preparations contain 10–15% pure hirudin. In addition, inert material and small amounts of a trypsin inhibitor are present.[15] These preparations are useful for experimentally inhibiting coagulation and for certain blood clotting tests.

Adsorption on Cation-Exchange Resins

About 0.3 g of the ethanol-precipitated hirudin is dissolved in 90 ml of a 0.01 *M* ammonium acetate buffer, pH 4.6. Amberlite IRC-50 (80 ml) in its hydrogen form is added to this solution under stirring until the inhibitor is completely adsorbed into the resin. The resin is collected

[15] H. Fritz, K.-H. Oppitz, M. Gebhardt, I. Oppitz, and E. Werle, *Hoppe-Seylers Z. Physiol. Chem.* **350**, 91 (1969).

by filtration and washed three times with 100 ml of distilled water. For elution of the hirudin, the resin is suspended in 50 ml of a 1 *M* ammonium acetate solution. The pH is adjusted to pH 7.0 by adding a 5% ammonia solution. The eluate is removed by filtration. The resin is once more suspended in 50 ml 1 *M* ammonium acetate solution and the pH adjusted to 8.0 by adding a 5% ammonia solution. The eluates are combined and concentrated to a volume of 50 ml by placing them in a vacuum at 20°–30°. Next, the hirudin is precipitated in the cold from the eluate by adding a 10-fold volume of 95% ethanol containing 0.5% ammonium acetate. After storage for several hours, the hirudin-containing precipitate is collected by centrifugation, washed with cold acetone, and dried in a vacuum dessicator.

Instead of bulk adsorption column chromatography can be employed and Amberlite IRC-50, equilibrated with 0.05 *M* ammonium formate, pH 4.9, or CM-Sephadex C-50 can be used. First eluation is performed with the equilibration buffer, followed by a 1 *M* ammonium formate solution, pH 7.0. The obtained preparations contain 40–50% pure hirudin.

Gel Filtration on Sephadex G-50

Approximately 100 mg hirudin (40–50% pure) are dissolved in 1 ml of a 0.1 *M* NaCl solution and placed on a Sephadex G-50 column (1 × 130 cm), previously equilibrated with 0.1 *M* NaCl, and eluated with 0.1 *M* NaCl. The eluate is collected in 3-ml aliquots. The tubes containing active material are combined, concentrated in a vacuum, desalted on Sephadex G-25 and lyophilized. These preparations contain about 15–20% impurities.

Chromatography on Anion-Exchange Resins

About 40 mg hirudin (80–90% pure) are dissolved in water and placed on a DEAE-Sephadex A-25 column (0.7 × 20 cm) which has been equilibrated with a 0.05 *M* pyridine acetate solution, pH 7.4. Hirudin is eluted by establishing a linear gradient with 0.05 *M* pyridine acetate buffer, pH 7.4 and 1 *M* NaCl in 0.5 *M* pyridine acetate buffer, pH 6.9. The tubes containing active material are combined, concentrated in a vacuum over P_2O_5 and potassium hydroxide at 4° and desalted by filtering on Sephadex G-25. The salt-free solution contains pure hirudin and is dried from the frozen state.

Criteria of Purity

Hirudin is stable in dried form. In water (preservative added) it is stable for 6 months at room temperature. It is also stable when heated for 15 minutes at 80°. The heat stability decreases with increasing pH

values of the solvent, and in contrast, decreasing the pH increases the heat stability. Hirudin is also stable for 15 minutes at 20° in 0.1 *N* HCl or 0.1 *N* NaOH. Trypsin and α-chymotrypsin do not destroy the hirudin activity. The resistance against these proteolytic enzymes is probably due to the tertiary structure of the peptide. This assumption is supported by the finding that oxidized hirudin is readily destroyed by trypsin. Papain, pepsin, and subtilopeptidase A destroy hirudin completely. Using paper electrophoresis, several ninhydrin-positive spots could be identified in the digestion products. Since hirudin does not contain arginine residues, the presence of arginine will identify proteins that contaminate hirudin. Also interesting is the absence of tryptophan and methionine residues in hirudin.

The purity of the hirudin preparations was investigated by means of ultracentrifugation and electrophoresis, using a Tiselius electrophoretic apparatus. With both techniques hirudin was homogeneous. Homogeneity was also observed with other electrophoretic techniques. The staining properties of the spots with ninhydrin or amido black 10 b coincided with the antithrombin activity.