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Proteolytic Activity of Hemophilic Plasma.

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Tagnon, Davidson, and Taylor[1] reported that the proteolytic activity of hemophilic plasma was lower than normal, but subsequently this group of investigators found no such difference.[2] In both studies the proteolytic enzyme was activated by treatment with chloroform; proteolytic activity was then measured by the rate of destruction of fibrinogen and fibrin[1] or by the rate of digestion of casein.[2]

Human blood plasma contains the inactive proteolytic enzyme precursor, plasminogen,*[3] which can be activated partially at least by chloroform treatment; activation with streptokinase appears to be much more effective. Plasma also contains an antiproteolytic factor[7-9] that must be removed or destroyed in order to obtain a true measure of the enzyme activity. In the procedure herein described the plasminogen has been separated from the antiproteolytic material by an alcohol fractionation similar to that devised by Cohn *et al.*[10,11] for the separation of plasma proteins. The enzyme was then activated with streptokinase,[12] and the plasmin activity was estimated from a measurement of fibrinogenolysis time.[1,13] A comparison of hemophilic and normal plasmas showed little if any difference in plasminogen content.

Experimental. Reagents. 1. 50% alcohol. 500 ml of 95% ethanol were diluted with distilled water to 950 ml.

2. Acetate buffer, pH 4. 12 ml of 1 M sodium acetate and 72 ml of 1 M acetic acid were mixed and diluted to 1 liter with distilled water.

3. Phosphate buffer, pH 10.1, with sodium citrate. 13.4 g of $Na_2HPO_4 \cdot 7H_2O$ and 29.4 g of $Na_3C_6H_5O_7 \cdot 2H_2O$ were dissolved in approximately 500 ml distilled water. Normal sodium hydroxide was added to adjust the pH to 10.1, and the solution was then diluted to 1 liter.

4. Phosphate buffer, pH 7.2, with sodium citrate and sodium chloride. 4.14 g of $NaH_2PO_4 \cdot H_2O$, 8.82 g of $Na_3C_6H_5O_7 \cdot 2H_2O$ and 8.91 g of NaCl were dissolved in approximately 500 ml distilled water. The pH of the solution was adjusted to 7.2 with normal sodium hydroxide, and the solution was then diluted to 1 liter.

5. Streptokinase. A crude preparation of this enzyme was prepared from a β-hemolytic streptococcus medium by the alcohol precipitation method of Garner and Tillett.[14] The precipitate was dried from the frozen state

[1] Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1943, **22**, 127.

[2] Lewis, J. H., Davidson, C. S., Minot, G. R., Soulier, J. P., Tagnon, H. J., and Taylor, F. H. L., *J. Clin. Invest.*, 1946, **25**, 870.

* Several proposals for the nomenclature of the active enzyme and its precursor have been made. They are a) plasmin and plasminogen;[3] b) fibrinolysin and profibrinolysin;[4] c) serum tryptase and tryptogen;[5] d) serum protease and lytic factor[6] respectively.

[3] Christensen, L. R., and MacLeod, C. M., *J. Gen. Physiol.*, 1945, **28**, 559.

[4] Loomis, E. C., George, C., Jr., and Ryder, A., *Arch. Biochem.*, 1947, **12**, 1.

[5] Ferguson, J. H., *Science*, 1947, **105**, 488.

[6] Kaplan, M. H., *J. Clin. Invest.*, 1946, **25**, 331.

[7] Christensen, L. R., *J. Gen. Physiol.*, 1946, **30**, 149.

[8] Grob, D., *J. Gen. Physiology*, 1943, **26**, 405.

[9] Kaplan, M. H., *J. Clin. Invest.*, 1946, **25**, 337.

[10] Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, **68**, 459.

[11] Edsall, J. T., *Advances in Protein Chemistry*, 1947, **3**, 383.

[12] Christensen, L. R., *J. Gen. Physiol.*, 1945, **28**, 363.

[13] Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 243.

[14] Garner, R. L., and Tillett, W. S., *J. Exp. Med.*, 1934, **60**, 239.

TABLE I.
Fibrinogenolytic Activity of the Plasmin Fraction of Normal and Hemolytic Plasmas Activated with Streptokinase.

	Subject	Amt of streptokinase added to 5 ml of solution (mg)	Fibrinogenolysis time: % conc. of plasma 100 Min.	50 Min.	12.5 Min.
Normal Plasma Preparations	1	25	4		21
	2	25	5		15
	3	25	4		8
	4	25	8		35
	5	25	4		18
Hemophilic Plasma Preparations	1	25	7		18
	2	25	6		19
	3	25	No act.		41
		75	—	7	—
		200	6		—
Beef fibrinogen		25	Good clot formation after 21 hr		
		200	Good clot formation after 21 hr		

and resuspended in the phosphate buffer just before use.

6. Beef fibrinogen was prepared according to Ware, Guest, and Seegers.[15] A 0.4% solution was made up in the phosphate buffer, pH 7.2. This fibrinogen was free of any plasminogen that could be activated by streptokinase.

7. Thrombin. Sufficient dried Harvard Fraction III-2† was dissolved in 0.85% sodium chloride so that 0.2 ml clotted 0.2 ml of a 0.4% fibrinogen solution in approximately 1 minute.

Plasma Fractionation Procedure. Citrated blood (12 ml whole blood and 1 ml 4% sodium citrate) was centrifuged; 5 ml of the plasma were measured into a 50 ml centrifuge tube and cooled to 1°C in an ice bath. 2.5 ml of cold acetate buffer were added, giving a pH of approximately 6.8. 12.5 ml of the cold ethanol solution were then added slowly from a pipette with constant stirring to give a precipitate containing the fibrinogen, plasminogen, antiproteolytic material and other proteins. After standing in an ice bath for 30 minutes the precipitate was removed by centrifuging in the cold (3-5°C), and the supernatant solution was discarded.

The precipitate was then suspended in 0.3 ml of phosphate buffer, pH 10.1, and the suspension was dissolved by the addition of 5 ml of cold water. To the solution were added 10 ml of cold water and 15 ml of cold ethanol solution. The pH of this medium was about 7.6. It was cooled in an ice bath for 30 minutes, centrifuged in the cold, and the supernatant solution (containing the antiproteolytic material) was discarded. The precipitate was redissolved in phosphate buffer, pH 7.2, and the final volume was adjusted with buffer to 5 ml. The buffer was prewarmed to 38°C, and was added just before the sample was treated with streptokinase.

This solution, containing the original plasma concentration of plasminogen and fibrinogen, was tested at both 100% and 12.5% of the original plasma concentration. In testing the undiluted solution (100%) the fibrinogen of the original plasma served as the substrate. In testing at 12.5% concentration, 1 ml of the solution was diluted with 7 ml of the beef fibrinogen substrate. By including cit-

[15] Ware, A. G., Guest, M. M., and Seegers, W. H., *Arch. Biochem.*, 1947, **13**, 231.

† The fraction III-2 was obtained through the courtesy of Dr. John T. Edsall. It was prepared under a contract between the Office of Scientific Research and Development and Harvard University from human blood collected by the American Red Cross.

rate in the buffer reagents the fibrinogen did not clot until thrombin was added.

Activation and Test for Proteolytic Activity. 5 ml of the solution to be tested were placed in a constant temperature bath at 38°C for 5 minutes; 0.2 ml of the streptokinase suspension was then added. Aliquots of 0.2 ml were removed at one-minute intervals following the streptokinase addition, and these were immediately tested for clotting power by the addition of 0.2 ml of thrombin solution. The time at which thrombin no longer clotted the test solution was recorded as the fibrinogenolysis time. This was the time required for the streptokinase to activate the enzyme and for the latter to digest the fibrinogen substrate to the point where it no longer coagulated.

Results. Table I shows the fibrinogenolytic activities of 3 hemophilic plasmas and of 5 normal human plasmas.

The fraction obtained from hemophilic patient No. 3 showed no activity when the 100% solution was treated with 25 mg of streptokinase. However, good proteolytic activity was demonstrated in this plasma when the relative proportion of streptokinase to plasma was greatly increased. This suggests that the fraction from this plasma contained a high concentration of antistreptokinase[16] which could be overcome by treatment with large amounts of streptokinase.

Discussion. By the precipitation of plasminogen from plasma under the conditions described, its activation by streptokinase and its proteolytic activity could de demonstrated without interference from the antiproteolytic factor. When whole plasma was treated with streptokinase in the same proportion as was used with fractionated plasma, this rapid digestion of fibrinogen was not observed. Neither could a solution of the first alcohol precipitate of plasma be rapidly activated by streptokinase.

Christensen and MacLeod[3] and Kaplan[17] have shown that the chloroform activated and the streptokinase activated proteolytic enzymes are identical. Since the streptokinase activated proteolytic activity of hemophilic plasma in this experiment was found to be comparable to that of normal human plasma, the results confirm the conclusions of Lewis, *et al.*,[2] that the proteolytic activity of hemophilic plasmas is not impaired.

Summary. 1. A method is described by which the proteolytic activity of human normal and hemophilic plasmas may be compared.

2. The proteolytic activity of 3 hemophilic plasmas was found to be comparable to that of normal plasmas.

Gratitude is expressed to Dr. Tyree C. Wyatt and Dr. John Houpis of the Pediatrics Departments, Syracuse University College of Medicine and Syracuse Memorial Hospital for their cooperation in securing the bloods from the hemophilic patients.

[16] Kaplan, M. H., in collaboration with the Commission on Acute Respiratory Diseases, *J. Clin. Invest.*, 1946, **25**, 347.

[17] Kaplan, M. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 1940.