that the cholesterol levels were significantly lowered, the hens were sacrificed and blood collected for lipid analysis. The aorta, about a quarter gram of heart muscle and a similar mass of liver were individually and accurately weighed, then macerated with sand and 1 to 3 ether alcohol solution. The extract of each organ was analyzed and the amounts of total cholesterol and cholesterol esters in 100 g of each tissue calculated. Twenty old hens fed as simultaneous controls were also sacrificed and the blood and tissues likewise treated. The results are set down as controls in Table I.

It is evident that the total blood cholesterol and cholesterol ester levels definitely decreased and the phospholipids rose after the old hens had been given 0.5 g methionine daily for about 3 weeks. After 5 to 7 weeks treatment, significantly lower levels of the blood cholesterol and cholesterol esters were established, and the aorta, heart, and liver showed significantly lower lipid levels than those of the control series. Methionine therefore, seems to act as a decholesterolizing agent in old hens.

15771

Fibrinogenolytic Demonstration of Activation and Inhibition of Tryptase in Plasma Protein Fraction-I ("Antihemophilic Globulin").*

JOHN H. FERGUSON, BURTON L. TRAVIS, AND EARL B. GERHEIM.

From the Department of Physiology, University of North Carolina, Chapel Hill, N.C.

Previous experience has shown the superiority of lysis of fibrinogen[1] over fibrinolysis[2] in the assay of tryptic enzymes. Recent revival of the idea[3] that the natural blood-clotting system involves the same protease (serum-tryptase) that is responsible for fibrinolysis[4] and other proteolytic phenomena is based upon experimental analogies with crystalline pancreatic trypsin. The ability of trypsin to restore or improve the clotting of hemophilic blood *in vitro*[5] and *in vivo*[6] suggests a plasma tryptase deficiency in hemophilia, for which not-altogether-conclusive evidence is afforded by data[7,8] on proteases demonstrable by the Delezenne and Pozerski (1903) chloroform-activation method, and by similar finding[9] of such proteases in certain "globulin" plasma protein fractions, which the Harvard investigators[10] have developed in application to the clinical treatment of hemophilia. The present observations employ a fibrinogenolytic reaction[1] to establish some fundamental considerations with respect to the development of "tryptase" activity in fibrinogen-rich human plasma fractions, as compared with similar materials of bovine plasma origin.

Reagents. 1. H.F.: human plasma Fraction I, courtesy of Drs. Cohn, Minot, Edsall, and colleagues (Harvard Medical School);[10-12]

* This work is the first of a series of investigations on "Enzymes and enzyme-inhibitors, in relation to blood coagulation and hemorrhagic diseases," aided by a grant from the John and Mary R. Markle Foundation.

1 Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 243.

2 Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 73.

3 Ferguson, J. H., *Science*, 1943, **97**, 319.

4 Nolf, P., *Medicine*, 1938, **17**, 381.

5 Ferguson, J. H., *Am. J. Physiol.*, 1939, **126**, 669.

6 Tagnon, H. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 45.

7 Feissly, R., *Schweiz. Med. Wschr.*, 1942, **72**, 648.

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

9 Kaplan, M. H., Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1942, **21**, 533.

10 Lewis, J. H., Tagnon, H. J., Davidson, C. S., Minot, G. R., and Taylor, F. H. L., *Blood*, 1946, **1**, 166.

TABLE I.
Clotting-times in Relation to Concentration of Plasma Fraction.*
Clotting-times (sec.) at 25°C for 0.5 cc F (strengths* cited) + 0.5 cc T (1%).

F.	Plasma fraction	Strength* of solution (% dry weight of original fraction): 2.0	1.0	0.5	0.2	0.1	0.05	0.02	0.01
		sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
1.	H.F.	7	6	6	8	14	35	115	±
2.	H.Fb	6	5	5	8	13	34	136	±
3.	B.F.	6	5	5	6	7	10	47	115
4.	B.Fb.	7	6	5	6	8	18	126	±

* Fractions I contain about 60% coagulable fibrinogen, whereas the fibrinogens are about 90% coagulable.

2. H.Fb.: human fibrinogen, purified, courtesy of Harvard workers; 3. B.F.: bovine plasma Fraction I, courtesy of Dr. Lesh (Armour Laboratories);† 4. B.Fb.: fibrinogen, repptd. × 2 from B.F. by ¼ sat. $(NH_4)_2SO_4$; 5. Strep.: streptokinase (miscalled "streptococcal fibrinolysin") prepared according to the methods of Tillett and Garner.[13] Extraction of 100 mg with 10 cc borate buffer[1] yields a clear supernatant which is removed from the insoluble residue for use in the tests; 6. T: thrombin, enzyme-free, in the form of "rabbit hemostatic globulin," courtesy of Dr. Parfentjev (Lederle Labs).[14] The dried material is soluble in 1.0% solution in borate buffer; 7. Hep.: Sod. heparinate (Lederle), 1 mg = 100 Toronto units, 1% soln.; 8. T.I.: crystalline trypsin-inhibitor, from pancreas, courtesy of Dr. Kunitz (Rockefeller Institute, Princeton);[15] 9. S.B.I.: crystalline trypsin-inhibitor, from soybean, courtesy of Dr. Kunitz;[16] 10. Egg: commercial egg-albumen ("albumin, egg, soluble powder," Merck), a preparation in laboratory stocks for many years; 11. E.I.: a purified inhibitor from egg-white, sent us by Dr. E. Weichselbaum (Washington Univ., St. Louis) 6 years ago; 12. Buffer: borate buffer,[1] pH = 7.7, used as solvent and diluent throughout.

Clotting-time in relation to concentration of plasma fraction (Table I). Percentage concentration is in terms of dry weight of original material. It is not corrected for small amounts of insoluble matter and for somewhat significant contamination with non-coagulable protein. Fractions I yield about 60% true fibrin, whereas fibrinogens are about 90% clottable. The data in Table I vary slightly from experiment to experiment but the values do afford a fair approximation of the amounts of fibrinogen equivalent to the various clotting-times (c.t.) throughout the present studies. Solutions of 0.5-1.0% give optimal clotting-times and are used throughout. Such concentrations are admittedly high for maximal sensitivity of fibrinogenolytic tests but are preferred for testing their content of proteolytic enzyme system.

Fibrinogenolytic tests for tryptase in plasma fractions (H.F., H.Fb., B.F., and B.Fb.) There is no demonstrable fibrinogenolysis in these 4 plasma fractions in 24-48 hr. Clotting tests of 0.5% solutions, sampled at intervals and added to 0.5 cc T, gave c.t. of 5-6 sec. except in H.F. (48 hr.) and B.Fb. (24 hr.) where 10″ and 14″ c.t., respectively, were, due to removal of some fibrinogen by "spontaneous" clotting, of very minor importance. That minute traces of tryptase are present, nevertheless, in the human fractions is indicated by lysis of their fibrin clots on overnight incubation at 40°C. Bovine ma-

11 Cohn, E. J., *et al.*, *J. Am. Chem. Soc.*, 1946, **68**, 459.

12 Edsall, J. T., Ferry, R. M., and Armstrong, S. H., Jr., *J. Clin. Invest.*, 1944, **23**, 557.

† Lesh prepares bovine plasma fractions on the principles worked out at Harvard.

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

14 Parfentjev, I. A., Goodline, M. A., and Clapp, F. L., *J. Lab. Clin. Med.*, 1943, **28**, 1465.

15 Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, 1936, **19**, 991.

16 Kunitz, M., *J. Gen. Physiol.*, 1946, **29**, 149.

TABLE II.
Fibrinogenolysis by Varying Strength of Streptokinase Solution.
L = 5 cc H.F. + 0.5 cc buffer + 0.5 cc strep. (strengths cited), incubated at 40°C for periods stated. Clotting-times (sec.) at 25°C for 0.5 cc L + 0.5 cc T (1%).

L.	Strength strep. %	15 sec.	¼ hr	½	¾	1	2	2½	3	4	8	18 hr
		sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
1.	0.05	6	7	8	11	13	25	35	45	55	360	∞
2.	0.1	6	9	13	21	37	174	300	∞			
3.	0.5	6	16	46	105	∞						
4.	1.0	6	19	65	186	∞						

TABLE III.
Fibrinogenolytic Tests for Tryptogen in Plasma Fractions.
L = 5 cc cited fractions + 0.5 cc strep. (1%) incubated at 40°C for periods stated. Clotting times (sec.) for 0.5 cc L + 0.5 cc T (1%).

L.	Fraction	15 sec	¼ hr	½	¾	1	1¼	1½	2	5	21 hr
		sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
1.	H.F.	7	9	10	55	420	∞				
2.	B.F.	5	5	5	5	5	5	5	5	5	5
3.	H.F. B.F.	5	5	5	9	25	100	∞			
4.	H.Fb.	6	24	∞							
5.	B.Fb.	5	5	5	5	5	5	5	5	5	5
6.	B.Fb. H.F.	5	5	5	10	45	260	∞			

terials, on the other hand, are remarkably stable. B.F. remains clear and gives identical c.t., with T, for 7-10 days at room temperature. The fibrin clots show no visible change in 10-14 days at 40°C.

Fibrinogenolysis by varying strengths of streptokinase solution. (Table II). The clear supernatant from extracting 100 mg crude streptokinase with 10 cc buffer solution is decanted and filtered through glass-wool to yield a solution designated "1%," from which dilutions are made for the tests of Table II. Concentrations of 0.5-1.0% are about optimal and weaker solutions give slower fibrinogenolysis in H.F. The streptokinase extraction for use in these tests requires fresh preparations, since solutions rapidly lose potency and are inactive within 24-48 hours even at ice-box temperature. Solutions of 0.01% (or less) show no fibrinogenolysis but continue to give the same (6″) c.t. as controls (buffer only) in tests extending over 18 hours at 40°C.

Fibrinogenolytic tests for tryptogen in plasma fractions. (Table III). Recent workers[17-19] have shown that streptokinase is not, as long believed,[13] itself the proteolytic agent, but merely the kinase or activator of enzyme-precursor (tryptogen) in plasma. The extent to which one-tenth volume of 1% streptokinase can activate the tryptogen in the present plasma fractions is brought out by the fibrinogenolytic tests of Table III. The high content of tryptogen in H.F. and H.Fb. is clearly shown as compared with the absence of tryptase development in B.F. and B.Fb. That the failure to lyse B.F. and B.Fb. is due to lack of enzyme precursor rather than to presence of inhibitors in the bovine material is shown in Exp. 3 (and 6), where a mixture of H.F. and B.F. (or B.Fb.) shows complete lysis of *all* the fibrinogen, bovine as well as human, in very little longer time than the human alone (L_1). These data also rule out any strict species specificity and some similar experiments on dog fibrinogen add further evidence on this point.

[17] Milstone, H., *J. Immunol.*, 1941, **42**, 109.

[18] Kaplan, M. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 40.

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

TABLE IV.

Fibrinogenolytic Tests of Effects of Heparin on Tryptase Formation and Stability. Lytic mixtures at 40°C for periods stated. All reagents 0.5%. Clotting-times (sec.) at 25°C for 0.5 cc L + 0.5 cc T (1%). L_1 (*without* heparin): tests 3-6. L_2 (*with* heparin): tests 7-10.

L.	Lytic mixture	15 sec	¼ hr	½	¾	1	2	4	6	17	24 hr
		sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
1.	10 cc H.F. + 1 cc buffer + 1 cc Strep.	6	16	46	105	∞					
2.	10 cc H.F. + 1 cc Hep. + 1 cc Strep.	25	±	∞							
3.	5 cc B.F. + 1 cc L_1 (15′ old)	6	6	6	—	6	7	11	20	480	∞
4.	5 " " + 1 " " (25′ ")	6	6	6	—	6	6	8½	10	100	165
5.	5 " " + 1 " " (50′ ")	6	6	6	—	6	6	7	8	35	55
6.	5 " " + 1 " " (100′ ")	6	6	6	—	6	6	6	6	14	16
7.	5 " " + 1 " L_2 (15′ ")	12	12	13	—	14	18	28	40	180	340
8.	5 " " + 1 " " (25′ ")	12	12	12	—	13	16	20	28	140	195
9.	5 " " + 1 " " (50′ ")	12	12	12	—	12	14	16	21	52	66
10.	5 " " + 1 " " (100′ ")	12	12	12	—	12	13	14	17	34	38

Instability of activated tryptase and effects of heparin. (Table IV). Some preliminary tests showed that 1 cc of (H.F. + strep.) lysate had a stronger effect in lysing 5 cc of fresh H.F. if added at (or before) the time of complete lysis in the original mixture than if added ¼-½ hour later. Because of the finding that heparin apparently enhances the potency of the tryptase, and because this might possibly be due to some stabilization of the enzyme, the stability tests of Table IV were conducted with and without heparin. At incubation periods of 15, 25, 50, 100 minutes, 1 cc samples were removed from (H.F. + strep.) lytic mixtures (a) without (L_1) and (b) with (L_2) one-tenth volume of 0.5% heparin (v. reagents). On addition of the 1 cc samples to 5 cc B.F. (0.5%), and observation of the rather slow fibrinogenolysis, the activity differences are clearly brought out. The instability of tryptase occurs in both series, so there is no evidence that heparin is stabilizing the enzyme. The best explanation of the *apparent* improvement of tryptase activity in the heparinized mixtures is that there is some immediate antithrombic action of heparin.[20,21] While this merely increases the clotting-time from 6″ to 12″ while the fibrinogen is at, or close to, full strength, it would become more significant as the fibrinogen is weakened by tryptic lysis. In support of this explanation, mixtures of B.F. diluted to contain in 0.5 cc volume 0.5, 0.2, 0.1, 0.05% of protein, and all treated with 0.05 cc of heparin (diluted to give the identical concentration of the above tests) and then clotted with the usual 0.5 cc T, give clotting-times: 12″, 18″, 35″, 100″ as compared with controls (without heparin): 5½″, 6″, 7″, 10″. Obviously, the antithrombic effect of heparin is much stronger with weaker fibrinogen concentrations.

Fibrinogenolytic tests of trypsin inhibitors. (Table V). The effects of known inhibitors (of pancreatic trypsin) on the natural plasma tryptase system are illustrated by selected tests, using both H.F. and H.Fb., in the first instance, on the original protein + streptokinase mixture. Inhibition is readily revealed by the fibrinogenolytic test method and is demonstrated for (a) crystalline trypsin-inhibitors, both from pancreas (T.I.) and from soybean (S.B.I.), and (b) egg-albumen, crude (Egg.) and fractionated for trypsin-inhibitor (E.I.).

In a second approach, in confirmation of the ability of these inhibitors to inactivate fully-formed tryptase, not merely to prevent its formation (possible antikinase action), additional tests were made on mixtures: 5 cc B.F. + 0.5 cc fresh lysate (H.F. + strep. incub. 35 min.) + 0.5 cc inhibitor solution. The control (buffer only) lysed in 12 hours, at which time there was no lysis

[20] Ferguson, J. H., and Glazko, A. J., *Am. J. Physiol.*, 1941, **134**, 47.

[21] Glazko, A. J., and Ferguson, J. H., *Am. J. Physiol.*, 1941, **134**, 54.

TABLE V.

Fibrinogenolytic Tests of Trypsin Inhibitors.

Lytic mixture (L) = 5 cc H.F. (0.5%) or H.Fb. (1%) + 0.5 cc strep. (1%) + 0.5 cc cited inhibitor, incubated at 40°C for periods stated. Clotting-times (sec.) at 25°C for 0.5 cc L + 0.5 cc T (1%).

L.	Fraction	Inhibitor	15 sec	¼ hr	½	¾	1	1¼	1½	2	2¼	2½	3	8	12	24 hr
			sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
1.	H.F.	none	5	13	27	64	95	320	∞							
2.	H.F.	egg (1%)	5	7	10	13	19	29	37	83	123	∞				
3.	H.F.	S.B.I.(1%)	6	6	6	6	6	6	6	6	6	6	6	6	6	6
4.	H.Fb.	none	6	24	∞											
5.	H.Fb.	E.I. (1%)	5	8	12	16	20	27	35	53	—	—	95	∞		
6.	H.Fb.	T.I. (1%)	6	6	6	6	6	6	6	6	—	—	6	6	6	6

(original 7″ clotting-time) in T.I., mere trace (10″ c.t.) in S.B.I., and advanced lysis (480″ c.t.) in E.I. The last, therefore, acted weakly but the crystalline inhibitors were very effective. T.I. showed no lysis whatever in 48 hours, whereas S.B.I. gave clotting-times 25″, 114″, 168″ at 24, 36 and 48 hours, respectively, showing that the tryptase action finally broke through the inhibition. It is perhaps noteworthy that tryptase activity is demonstrable after such long periods, despite the evidence (Table IV) of a definite degree of instability.

Streptokinase, etc. In the absence of plasma protease (tryptase) or its precursor (tryptogen), the streptokinase lacks proteolytic, thrombic, or thromboplastic effects. It is possible, of course, to obtain tryptase in fibrinogen-free plasma fractions. The very important thromboplastic action of this plasma protease will be reported in a subsequent communication.

Nomenclature. Similarities to pancreatic trypsin, *e.g.* 1. alkaline pH optimum, 2. variety of proteins attacked (fibrin, fibrinogen, prothrombin, gelatine, casein, hemoglobin, etc.), 3. effects of known trypsin-inhibitors, and 4. clot-aiding actions, favor continued characterization of the plasma protease as a *"tryptase"* or trypsin-like enzyme.[1] The new term *plasmin*[19] is acceptable but hardly necessary and, pending isolation, perhaps somewhat premature. The ambiguous term *"fibrinolysin,"* which has been used both for the tryptokinase[22] and for the active tryptase[23] should obviously be avoided.

Summary. The present study is based upon the use of a fibrinogenolytic (rather than fibrinolytic) technic for demonstration of a natural plasma protease *(tryptase)* and its precursor *(tryptogen)* in the fibrinogen-rich plasma fractions. The value of *streptokinase* (miscalled "streptococcal fibrinolysin") for activation of the enzyme precursor is confirmed and tests are presented to show that failure to secure proteolytic effects *e.g.* in bovine plasma Fraction I, is due to lack of tryptogen (tryptase precursor) rather than to any significant presence of tryptase-inhibitor.

Heparin, unaided, shows no enzyme-inhibitory effects in the cited tests. *Antitryptase* effects of (a) crystalline inhibitors, from pancreas and soybean, and (b) crude and fractionated egg-albumen, favor the characterization of the plasma protease as a "tryptase."

Confirmation of the existence of tryptogen, together with a trace of active tryptase, in H.F. ("antihemophilic globulin") is further, but still inconclusive, evidence that the source of the coagulation defect in hemophilia is to be sought for in the complexities of the plasma protease system.

The human plasma fractions used in this work were prepared from blood collected by the American Red Cross under a contract between the Office of Scientific Research and Development and Harvard University.

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

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