one may think that the intracellular fluids are not suitable for an active multiplication of Brucella, exception is made of zones where the tissues have been subject to considerable damage. In these zones one may find extracellular Brucella in large numbers. One would consider that the organism could be placed together with true intracellular parasites, but this is not the case since Brucella have its own enzyme systems with which it is able to use rather ordinary material for its metabolism.

Considering as likely that in man Brucella grow in a manner similar to that which has been observed in the experimental infection, the pathogenesis of the disease seems to us better understood.

15775

Thromboplastic Action of Plasma Protease (*Tryptase*).*

JOHN H. FERGUSON, BURTON L. TRAVIS, AND EARL B. GERHEIM.
From the Department of Physiology, University of North Carolina, Chapel Hill, N.C.

Indirect evidence, based on experimental analogy with trypsin, papain, etc. was reviewed[1] in support of the theory that blood coagulation normally involves natural plasma protease (*tryptase*[1] or *plasmin*[2] or *fibrinolytic enzyme*[3]) apparently identical with the agent responsible for fibrinogenolysis, fibrinolysis, and other proteolytic phenomena. Plasma tryptase resembles pancreatic trypsin in many respects, especially relevant to these problems, but does differ in 1. origin,[4] 2. kinase-specificity,[3] and 3. other important ways.[2] The following experiments confirm some similarities and lead to *direct* evidence that plasma tryptase can participate as a "thromboplastic" factor in the blood-clotting system.

Reagents. In the first paper[5] of this series, on the activation and inhibition of the fibrin(ogen)olytic protease (tryptase) in certain plasma fractions, are described: 1. Borate buffer, pH = 7.7, *viz.* 45 vol. 2.5% H_3BO_3, 45 vol. 0.5% NaCl, 10 vol. 4% $Na_2B_4O_7$, $12H_2O$, used as solvent and diluent (to constant volume) throughout; 2. Harvard (courtesy Drs. E. J. Cohn, J. T. Edsall, and colleagues) human plasma Fraction-I (H.F.), which contains about 60% fibrinogen, a trace of active protease (tryptase) and considerable enzyme-precursor (tryptogen) that can be activated by a suitable kinase (Table I, A); 3. Armour's (courtesy Dr. J. B. Lesh) enzyme-free[5] bovine plasma Fraction-I (B.F.), of about the same fibrinogen content as H.F.; 4. Thrombin (THR.), a 1% soln. of lyophilized rabbit "hemostatic globulin," Lederle's (courtesy Dr. I. A. Parfentjev); 5. Streptokinase (STREP.), the streptococcal material (Garner and Tillett, 1934) of which a 1% extract is an excellent kinase-type activator of the plasma protease; 6. Crystalline trypsin-inhibitors, protein (or polypeptide) in nature, from pancreas (T.I.)

* This report is the second 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.

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.

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

2 Christensen, L. R., and Macleod, C. M., *J. Gen. Physiol.*, 1945, **28**, 559.

3 Kaplan, M. H., *J. Clin. Invest.*, 1946, **25**, 331; 337, *et seq.*

4 Tagnon, H. J., *Arch. internat. physiol.*, 1942, **51**, 472.

5 Ferguson, J. H., Travis, B. L., and Gerheim, E. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 285.

TABLE I.

Thromboplastic Action of Plasma Protease (Tryptase) Compared with Pancreatic Trypsin.

A. *Preparation of Activators. Timing of Fibrinogenolysis.* Clotting-time test at 24°C.

	Mixture (cc)	¼ min.	¼	½	¾	1	1½ hr (incub. at 39°C for lysis)
		sec.	sec.	sec.	sec.		
A.	4.0 H.F. 0.4 STREP.	6	15	45	85	+	∞
B.	4.0 H.F. 0.4 TRYP. (20 unit)	6	21	51	90	+	∞
C.	4.0 H.F. 0.4 buffer	heat-defibrination at 45°C for 3 min.; filtered through glass wool.					

B. *Prothrombin Activation Tests.* Recalcified PRO.-A.

Clotting-times (c.t.), in seconds, at 24°C, pH = 7.7 (borate buffer) for 0.5 cc B. F. + 0.25 cc T, sampled at stated incubation periods (i.t.).

T.	Activator	¼ min.	½	1	2	6	18	24 hr	4 day	7 day	(i.t.)
		sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	
1.	None	268	228	195	163	110	72	55	28	13	(c.t.)
2.	Mixture C	307	247	207	172	98	68	55	28	12	
3.	" A	324	132	86	62	45	36	28	20	12	
4.	" B	335	58	40	27	13	8½	7	5	5	
5.	40-unit TRYP.	—	5	5	5	6	15	19	100	—	

and soybean (S.B.I.), 0.2%, courtesy Dr. M. Kunitz (Rockefeller Institute, Princeton). The present studies also use 7. a Harvard (courtesy Dr. J. T. Edsall) human plasma protein fraction (III-3), 0.1%, possessing proteolytic and thrombic properties; 8. Purified prothrombin (PRO.), 0.2%, from lyophile-dried bovine preparations supplied through the courtesy of Dr. W. H. Seegers[6] (Wayne Univ., Detroit): PRO.-A being stated to yield 15,200 thrombin units per mg tyrosine N, while PRO.-B is somewhat less purified (see below); 9. Pancreatic trypsin (TRYP.), from 2% stock solution (in glycerol-borate) prepared from commercial (Fairchild Bros. and Foster) trypsin, and diluted to 40 or 20 "unit" strength (1 cc ≡ 1 mg = 100 "units," by fibrinogenolytic test);[7] 10. Rabbit brain thromboplastin (tpln.), 0.5%, from a commercial (Difco) preparation, devoid of significant proteolytic activity; 11. M/10 $CaCl_2$(Ca).

Prothrombin. Data on the properties, especially activation and stability, of Seegers' purified prothrombin preparations will be reported in detail elsewhere, but it is relevant to note: 1. some active thrombin is present and increases on standing at room temperature in borate buffer solutions (Table II, 6); 2. there is a very slow (increased) activation to thrombin on adding calcium salt (Table I, B, 1; Table II, 1), which, in comparison with the prompt and complete activation by Ca + tpln. (Table II, 2), shows either an ultimate reaching of the same optimum in 2-7 days (PRO.-B) or incomplete activation even after a week (PRO.-A), although still capable of reaching the optimum on the subsequent addition of thromboplastin. The obvious suggestion is that the prothrombins are contaminated with small but significant traces of thromboplastic factor (? phospholipid): 3. activation by 1/10 vol. of 40-unit trypsin (and Ca) to optimal thrombic activity in about ½ hr., followed by weak thrombinolysis (Table I, B, 5).

Fibrinogenolysis test. When the natural tryptase in H.F. (human plasma fraction-I) is activated by streptokinase and the fibrinogenolysis[5] followed by the lengthening clotting-times of 0.5 cc samples added to 0.5 cc thrombin (THR.), after successive incubation periods of the lytic mixture, as illustrated in Table I, A, mixture A, the data show close similarity to lysates of H.F. and weak pancreatic trypsin (1/10 vol. of 15-20

[6] Seegers, W. H., Loomis, E. C., and Vandenbelt, J. M., *Arch. Biochem.*, 1945, **6**, 85.

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

TABLE II.
Inhibition of Tryptase-Activation of Prothrombin.

5 cc vol. of thrombic mixtures (T), containing 2 cc PRO.-B + 0.25 cc Ca and activators ± inhibitors noted, incubated at 24°C, pH = 7.7, for periods stated (i.t.). Clotting-times (sec.): 0.25 cc T + 0.5 cc B.F.

T.	Activator	Inhibitor	¼ min.	¼	½	1	2	6	24 hr	7 day (i.t.)
			sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
1.	(Ca only)	0	218	167	133	87	45	30	13	4 (c.t.)
2.	tpln. (0.25 cc)	0	78	4	4	4	4	4	4	4
3.	III-3 (2.0 ")	0	77	20	14	10	10	9	8	5
4.	III-3 (2.0 ")	T.I. (0.75 cc)	71	61	39	25	18	11	9	6
5.	III-3 (2.0 ")	S.B.I. (0.75 cc)	75	92	100	108	194	220	150	45
6.	PRO + buffer (No Ca or III-3)		800	610	555	360	210	125	58	12
7.	III-3 + Ca (No PRO.)		78	—	—	—	—	—	87	—

unit TRYP.), as in mixture B. The lysates from these mixtures, as used in the subsequent prothrombin activation studies, obviously represent very small amounts of proteolytic enzyme.

Tryptic activation of prothrombin (Table I, B). Thrombic mixtures (T) consisting of 2 cc PRO.-A, 0.25 cc Ca, and cited activators (to 5 cc vol.) are sampled after successive incubation periods (i.t.), the 0.25 cc samples being added to 0.5 cc fibrinogen (B.F.) and the clotting-times (c.t.) noted. The shortening c.t. denotes increasing amounts of thrombin formation. This method has long been used in our laboratories for quantitative and qualitative study of the first phase of blood-clotting.[8]

The ¼ min. test, in all series, shows the trace of thrombin originally present in the particular prothrombin solution used. It is of very little significance even compared to the very slow and incomplete activation by Ca-salt alone, as shown in T_1. Series T_3 and T_4, on the other hand, show a definite "thromboplastic" acceleration of thrombin formation by the lysates containing tryptase (A) and trypsin (B), respectively. To rule out the possibility that H.F. might contain some thromboplastic factor other than tryptase, mixture C was prepared by simple heat (56°C) defibrination. Series T_2 corresponds closely to the T_1 control.

Other data. STREP. *(streptokinase)*, itself, in repeated tests, is found to be devoid of proteolytic, thromboplastic, or thrombic effects. Several other protease-containing plasma products, some of dog and bovine, as well as human, origin, also show the "thromboplastic" action noted in Table I, B. One Harvard human plasma fraction (III-3) is a potent protease but also possesses thrombic activity. This complicates the experimental analysis but, with appropriate controls, serves to show essentially the same phenomena, as illustrated in Table II. Included in these data are some typical results of the addition of known *trypsin-inhibitors* (T.I. and S.B.I.)

Inhibition of "thromboplastic" action of plasma protease III-3 (Table II). PRO.-B, used in these tests, forms thrombin "spontaneously" (T_6) at a somewhat greater rate than the purer product (PRO.-A), and Ca (T_1) gives maximal activation in 7 days, the 4-sec. clotting-time ultimately reached being identical with the stable value attained in 10-15 min. in the additional presence of brain thromboplastin (T_2). The protease III-3 is markedly "thromboplastic" (T_3), as shown by comparison with controls T_1 and T_7. The last shows only a weak initial thrombic action of III-3 alone which, if anything, is lessened after 24 hr. Pancreatic trypsin-inhibitor (T.I.) is clearly (T_4) able to inhibit a considerable amount of the thromboplastic effect of the protease. Soybean trypsin-inhibitor (S.B.I.) is even more effective (T_5), but the later tests suggest that part of this inhibition may be directed not only against the protease (III-3) but also against the natural thromboplastic factors undoubtedly operating in T_1 and T_6.

[8] Ferguson, J. H., *Ann. N. Y. Acad. Sci.*, 1947, in press.

Fibrinolysis, etc. Lysis of the fibrin clots in A and B (Table I, A) occurred overnight, as did T_2 (Table I, B) and T_3 (Table II). This confirms the fibrinolytic actions of natural plasma protease. Fibrinolysis was absent, even after 7 days at 37°C, in the enzyme-free controls and in the presence of S.B.I., while it was delayed for 3-4 days by the cited amounts of T.I. Thrombinolysis requires further study, but did not occur with the proteases used in the present study, except trypsin (Table I, T_5). This could be a matter of enzyme strength, however.*

The absence of significant prothrombinolysis (*cf.*[9]), due to choice of very weak enzyme preparations, is apparently the key to the successful demonstration of these important "thromboplastic" effects.

Summary. The natural protease *(tryptase)* in several plasma protein fractions resembles a weak pancreatic trypsin in its fibrinogenolytic, fibrinolytic, and "thromboplastic" effects. These actions are inhibited by crystalline trypsin-inhibitors from pancreas and soybean. The clear demonstration of thromboplastic action in these quantitatively-controlled tests is direct proof of participation of plasma tryptase in the blood-clotting system.

* More recent tests show that a sufficiently potent tryptase is thrombinolytic.

9 Seegers, W. H., and Loomis, E. C., *Science*, 1946, **104**, 461.

15776

Chronic Oral Toxicity of Alpha-Naphthyl Thiourea.*

O. GARTH FITZHUGH AND ARTHUR A. NELSON.

From the Division of Pharmacology, Food and Drug Administration, Federal Security Agency, Washington, D.C.

Richter[1] proposed the use of alpha-naphthyl thiourea (usually abbreviated ANTU) as a specific poison for the control of Norway rats because of its high toxicity to rats and its relatively low toxicity to all other species tested. Its emetic property protected dogs in most cases. Although Richter showed that animals became tolerant to acute doses of ANTU, the experiments of McClosky and Smith[2] indicated a cumulative action with successive doses to rabbits and cats. In experiments conducted for short periods of time the chronic effects of ANTU paralleled closely those obtained with thiourea and 2-thiouracil.[1] Because of Richter's report of tolerance to ANTU the present study was undertaken to determine the effects of small amounts of ANTU ingested during the lifetime of the rat.

Experimental. Groups of 18 male weanling rats (21 days) from our colony of Osborne-Mendel strain were started on each of 7 diets containing respectively 0, 50, 100, 200, 400, 600 and 800 ppm ANTU. Ground commercial rat biscuits with 1% added cod liver oil served as the basic diet. The ANTU was mixed with the basic diet by means of a rotary batch mixer. Litter mates were selected and assigned to the various groups according to a randomized design of experiment (balanced incomplete blocks).[3] All

* A portion of the funds used in this investigation was supplied by a transfer between the Committee on Medical Research of the Office of Scientific Research and Development and the Division of Pharmacology, Food and Drug Administration, and also between the Office of the Surgeon General, War Department, and the Division of Pharmacology.

1 Richter, C. P., *J. A. M. A.*, 1945, **129**, 927.

2 McClosky, W. T., and Smith, M. I., *Pub. Health Rep.*, 1945, **60**, 1101.

3 Fisher, R. A., and Yates, F., *Statistical Tables for Biological, Agricultural, and Medical Research*, Oliver and Boyd, Edinburgh, 1938.