J. Biol. Chem., 1950, v184, 71.

3. Friedman, M., Byers, S. O., and Michaelis, F., *Am. J. Physiol.*, 1951, v164, 786.

4. Friedman, M., Byers, S. O., and Shibata, E., *J. Exp. Med.*, 1953, v98, 107.

5. Byers, S. O., and Biggs, M. W., *Arch. Biochem.*, 1952, v39, 301.

6. Friedman, M., Byers, S. O., and Gunning, B., *Am. J. Physiol.*, 1953, v172, 309.

7. Byers, S. O., Friedman, M., and Gunning, B., In preparation.

8. Gilbert, A., and Jomier, J., *Arch. de med. exp.*, 1908, v20, 156.

9. Jaffé, R. H., and Levinson, S. A., *Am. Rev. Tbc.*, 1928, v11, 217.

10. LeQuire, V. S., Gray, M. E., and Cobb, C. A., *Circ. Res.*, 1953, v1, 523.

Received February 19, 1954. P.S.E.B.M., 1954, v85.

Inhibition of Hyaluronidase Activity by Factors Associated with Blood Clotting. (20942)

KARL L. ROTH. (Introduced by D. R. Meranze.)

From the Department of Research, Albert Einstein Medical Center, Southern Division, Philadelphia, Pa.

The interaction of hyaluronidase with the clotting mechanism has been suspected for some time. Attempts have also been made to identify complement (C′) with the nonspecific serum hyaluronidase inhibitor and also with prothrombin(1-5). This suggests a possible relationship between the prothrombin complex and inhibitor. Baserga *et al.*(6) noted that hyaluronidase interferes with the prothrombin consumption in sera. The inhibition of the agglutination of platelets by this enzyme was observed by Quattrin(7). Fiala *et al.*(8,9) have shown that dialyzed bovine hyaluronidase acts as an anticoagulant *in vitro*, by virtue of its capacity to counteract the thromboplastic effect of platelets. This interaction between platelets and hyaluronidase was postulated as representing a competition between the two, for a specific plasma protein(9). The latter was found to be stable on storage, and to have the same adsorption affinities as prothrombin, but was nevertheless present in a prothrombin free serum. Fiala has further stated(10) that tissue thromboplastin inhibits the enzymatic degradation of hyaluronic acid. Studies made in this laboratory on the interaction between hyaluronidase and the clotting mechanism have revealed that this phenomenon is more complex than can be explained merely as a competitive inhibition between platelets and enzyme. The data to be presented below suggest that the nonspecific serum (plasma) inhibitor of hyaluronidase may play a specific role in the mechanism of blood coagulation.

Materials and methods. Platelets were isolated by differential centrifugation from plasma containing Sequesterine® (ethylenediamine-tetraacetic acid), and collected in siliconized vessels. The final centrifugate was washed 4-fold with saline, resuspended in distilled water and then shaken for 5 minutes with aid of a mechanical shaker. The shattered and agglutinated platelets were separated by centrifugation at low speed, and the supernatant "platelet extract" poured off. The sediment was washed once by shaking with one-third volume distilled water and the wash water combined with the supernate. The final sediment was discarded. This platelet extract was found to correct the clotting

TABLE I. The Effect of Proconvertin Concentration on "Anticoagulant" Activity of Hyaluronidase.

Proconvertin eluate added (ml)	0	.1	.2	.3	.4	.5	Control
Clotting time on recalcification (sec.)	∞	1800	1450	1100	1000	1040	1060

Each tube contained 0.5 ml plasma with 3×10^8 platelets, 0.1 ml hyaluronidase and saline plus proconvertin eluate, total 1.1 ml. In the control saline replaced enzyme. 0.5 ml (0.025M) $CaCl_2$ added after 10 min. incubation at 37°C. Clotting times are the avg of 3-4 determinations.

TABLE II.

Plasma added:	0	Dic. 1	Dic. 2	Dic. 3	Normal	Dic. 1 Dic. 2 only	Control (no enz.)
Clotting time on recalcification (sec.)	∞	3300	2900	2500	2100	∞	1700
Prothr. times of added plasmas (sec.)		28.2	21.4	17.7	12.4		12.4

Each tube contained .2 ml platelet poor test plasma, .1 ml enzyme and .2 ml correcting plasma or saline to make a volume of .5 ml. Calcium, and additional saline, where necessary, added after 10 min. incubation at 37°C. Final volume: 0.9 cc.

defect of a platelet-free plasma. The activity of the preparation was resistant to boiling for 5 minutes and to treatment with 10% trichloracetic acid, and subsequent neutralization with NaOH. Whenever intact platelets were required, the washed saline suspension, kept in siliconized test tubes, was used. Any desired platelet number was achieved by simple dilution of the suspension with additional saline.

Proconvertin. In some experiments aged serum was used as a source of proconvertin (11). The addition of thromboplastin, as suggested by Owren, was omitted. For most work, however, a concentrate prepared by adsorption of aged serum with $BaSO_4$ or $Ca_3(PO_4)_2$ and elution with sodium citrate (0.02 M) was used. This is referred to as proconvertin eluate. In the studies of the effect of proconvertin on the depolymerizing activity of hyaluronidase, the eluate was dialyzed not against saline, as in the clotting studies, but against tap water. Human brain thromboplastin, prepared for routine use in this laboratory, served as thromboplastic reagent. Commercial preparations of hyaluronidase from Armour & Co. (Chicago, Ill.), G. D. Searle & Co. (Chicago, Ill.), Ortho Pharmaceutical Co. (Raritan, N. J.), and Wyeth Inc. (Philadelphia, Pa.) were used. Hyaluronic acid was obtained from Armour & Co.* and General Biochemical, Inc. (Chagrin Falls, O.). The enzyme solution was employed after dialysis against distilled water or saline. These materials were assayed viscosimetrically with an Ostwald type viscosimeter (vol. 5 cc) at 37°C. Oxalated plasmas were used in clotting studies. Sodium citrate was found to inhibit to some extent the anticoagulant effect of hyaluronidase.

Results. A platelet poor plasma, on incubation with hyaluronidase in a concentration of one mg/ml of plasma, becomes incoagulable. This defect can be corrected by addition of either tissue thromboplastin or platelets (9). Furthermore, it will be shown that addition of proconvertin will also correct the clotting defect. A normal plasma also loses its ability to clot on recalcification if a sufficiently high concentration of hyaluronidase is added to it. It has already been demonstrated that this "anticoagulant" effect of the enzyme appears to be inversely proportional to the number of platelets in the plasma(8). However, our experiments show (Table I) that if the platelet concentration is kept constant, and the level of proconvertin varied, the "anticoagulant" effect of hyaluronidase appears to be related to the concentration of proconvertin. The proconvertin effect is also illustrated in Table II, in which the behavior of 3 dicoumarolized plasmas (proconvertin poor plasmas) is compared with that of a normal plasma, relative to their capacity toward correcting the hyaluronidase clotting defect. The data given in Table II show that the decreased level of proconvertin in dicoumarolized plasmas is able to rectify only in part the clotting inhibition by hyaluronidase, the degree of rectification being inversely related to the prothrombin time of the plasmas.† If hyaluronidase is initially incubated with platelets(9), tissue thromboplastin or proconvertin eluate, its anticoagulant activity is lost; simultaneously the clotting factors also lose part or all of their activity.

* We are indebted to Dr. L. L. Lachat for generous supplies of hyaluronic acid.

† Preliminary experiments show that the level of the nonspecific serum hyaluronidase inhibitor is depressed in patients receiving dicoumarol. The inhibitor level tested in sera of two hemophiliacs was found to be normal.

TABLE III. Reciprocal Inactivation of Clotting Factors and Hyaluronidase.

Factor present:	0	Hyaluronidase	Platelet extract	Proconv. eluate	T'plastin (1:100)
A. Recalcified clotting times/sec. *before* incubating factors with hyaluronidase	800	2100	95	610	35
B. Recalcified clotting times *after* incubating factors with enzyme	—	—	890	880	760
C. After boiling 1 min.	—	—	98	—	70*

A.—0.1 ml of given factor or enzyme added to 0.1 ml of platelet poor test plasma and the volume made up to 0.3 ml with saline. After bringing temperature to 37°C, 0.1 ml of 0.025M $CaCl_2$ was added and the clotting time determined.

B.—Equal volumes of enzyme and factors were mixed, then 0.2 ml added to 0.1 ml plasma. After a short incubation at 37°C recalcified clotting times were determined as in A.

C.—(*) The activity of a 1:200 saline dilution of above thromboplastin preparation was 68, 72 and 69 sec. after 1 min. boiling.

This reaction between the enzyme and clotting factors occurs very rapidly, without an irreversible change of platelets or thromboplastin; for when the mixture of either one of these 2 factors is boiled for one minute, the activity of the factors is recovered. (Table III). With proconvertin, however, the reaction with enzyme gives rise to an irreversible alteration of the proconvertin, as reflected by a loss of specific proconvertin activity, the alteration proceeding at a slow, but definite rate, and marked by the recovery of the anticoagulant effect of the enzyme. To demonstrate this, hyaluronidase was incubated with a small excess of proconvertin eluate. Initially the mixture did not exhibit any anticoagulant effect when added to a test plasma,

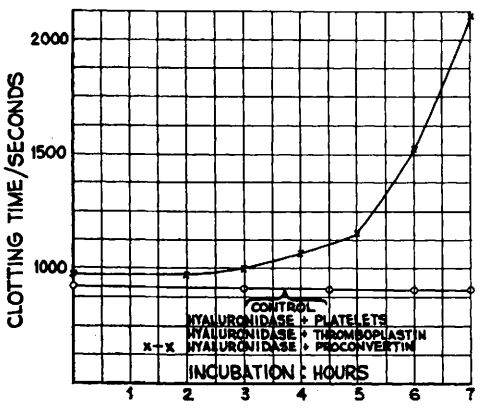

FIG. 1. Effect of prolonged incubation of hyaluronidase with factors on clotting time of a normal plasma. Mixtures were those described in Table III (B). At noted time intervals aliquots (0.2 cc) were added to 0.1 ml test plasma and clotting time determined after a short incubation.

but after the third hour of incubation the mixture caused a marked prolongation of the clotting time, showing the gradual recovery of the hyaluronidase effect (Fig. 1). On the other hand, loss of proconvertin potency was demonstrated by the fact that whereas a 1:10 dilution of tissue thromboplastin in saline easily overcomes the anticoagulant effect of hyaluronidase (shown by the normal prothrombin times), after several hours of incubation of enzyme with plasma, a gradual prolongation of prothrombin times was observed. Addition of as little as 0.05 cc of a proconvertin eluate brought the prothrombin times back to normal. Control tubes showed that only a small fraction of the prothrombin time prolongations was to be attributed to a possible decrease in ACG level (Fig. 2). The capacity of proconvertin preparations and of tissue thromboplastin to inhibit hyaluronidase activity was also demonstrated by viscosimetric studies. 0.1 mg of hyaluronidase‡ in 0.1 ml H_2O and 0.1 ml of clotting factor were added to 3.0 ml of hyaluronic acid in the viscosimeter at 37°C. After suitable intervals the flow time of the mixture was determined to give a measure of the hyaluronidase activity present. As can be seen in Fig. 3, tissue thromboplastin and proconvertin inhibit the depolymerizing action of the enzyme. Boiled thromboplastin possessed some inhibitory activity also. Curiously, a mixture of platelets and proconvertin was a more effective inhibitor than proconvertin alone, even though platelets alone were inactive. It was also

‡ Approx. 20 V.U.

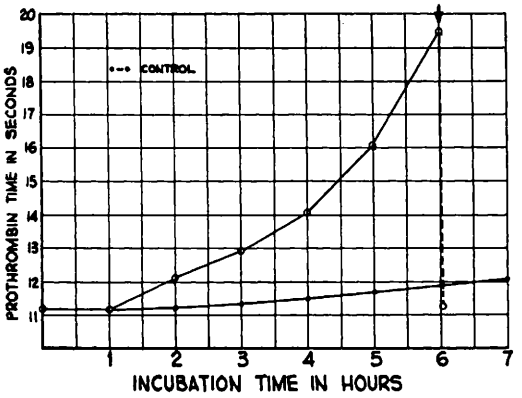

FIG. 2. Alteration of prothrombin time of a test plasma on prolonged incubation with hyaluronidase. 2 ml plasma were incubated with 0.4 ml hyaluronidase. Aliquots were taken at intervals indicated and prothrombin times determined with a 1:10 saline dilution of tissue thromboplastin-Ca. At time shown by arrow, 0.2 cc proconvertin eluate was added to the remaining plasma.

observed that sera freed of all proconvertin activity by adsorption on $Ca_3(PO_4)_2$, $BaSO_4$, $Mg(OH)_2$ or inactivated at 56°C for 30 minutes, possess no inhibitory activity.

Discussion. It is generally agreed that nonspecific serum (plasma) hyaluronidase inhibitor is a factor (or factors) present in the normal circulatory system. Glick *et al.*(12) find the inhibiting property to be "inherent in various products of liver metabolism" . . . It is established that hyaluronidase inhibitor is stable on storage, that it has adsorption affinities similar to prothrombin but is, however, present also in a prothrombin free serum. Its blood level seems to be decreased by dicoumarol administration. Furthermore, all active proconvertin preparations are able to inhibit hyaluronidase. These observations are consistent with the conclusion that the inhibitor and proconvertin may be one and the same substance.

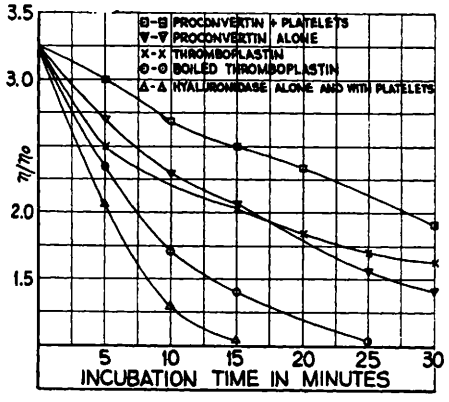

FIG. 3. Effect of various factors on rate of depolymerization of hyaluronic acid.

On the other hand, in the course of a routine experiment it was observed that storage of a proconvertin preparation for several days at —20°C, resulted in the loss of approximately 50% of its initial hyaluronidase inhibiting activity, without any parallel loss in its blood clotting (proconvertin) activity. A similar phenomenon was observed by Fulton *et al.*(4) in their investigation of properties common to hyaluronidase inhibitor and Complement (C′). Intact complement complex was required for hyaluronidase inhibition. However, on lyophilization, guinea pig serum was found to lose its inhibitory activity without suffering any loss in its complement titer. With both complement and proconvertin preparations, the hyaluronidase inhibiting activity lost on freezing can be promptly restored by the addition of inactivated serum, which possesses neither complement nor proconvertin, and very little, if any, inhibitory activity. This suggests that full inhibition depends on the presence of a heat stable second factor, a co-factor, which is present in inactivated serum, but is destroyed by freezing. Such a cofactor has already been postulated by Fulton *et al.*

Furthermore, the experimental results presented in Fig. 3 show that a proconvertin eluate, previously mixed with platelet material, has a slightly higher inhibitory activity than the proconvertin preparation untreated. This activity is stable on storage at room temperature. Platelets alone are inactive.

Thus, it appears that although hyaluronidase inhibition is intimately associated with proconvertin activity, present evidence suggests that inhibitor and proconvertin are closely related but not chemically identical. The inhibitor may be a complex; composed of proconvertin, a heat stable co-factor and another factor, perhaps of platelet origin.

It has been shown that both platelets and tissue thromboplastin are able to counteract

the anticoagulant effect of hyaluronidase, even after boiling for 5 minutes. However, only thromboplastin is able to inhibit the degradation of hyaluronic acid by hyaluronidase, boiled preparations being slightly less active than unboiled. Inactivation by incubation with "Thromboplastinase"(13) of a thromboplastin preparation, to the extent of reducing its clotting potency from 12″ to 160″, also did not affect its inhibitory activity. Even a crude alcohol-ether extract of acetone dried brain tissue was active.

Thus, the level of inhibiting material present in tissue thromboplastin is not affected, and remains essentially unchanged, despite chemical alteration of the thromboplastin to the degree that its plasma coagulation capacity is greatly decreased.

Present understanding of the phenomena involved do not permit a decision as to whether this hyaluronidase inhibiting component present in tissue thromboplastin complex is similar to the hyaluronidase inhibitor in blood. But the observations reported in this paper do show that an intimate relationship exists between some of the components of the blood coagulation reaction chain and the enzyme, hyaluronidase.

Summary. 1. Proconvertin preparations were found to function as hyaluronidase inhibitors. The inhibition of hyaluronic acid depolymerization by proconvertin requires a cofactor and is enhanced by prior incubation with platelet material, which, when tested alone, is inactive. 2. Tissue thromboplastin is also shown to inhibit hyaluronidase. This inhibitory activity seems to be inherent mainly in the lipid moiety, and although always associated with the fraction containing the thromboplastic principle, is independent of its activity. 3. It is suggested that the nonspecific serum hyaluronidase inhibitor is associated with a part of the thromboplastic complex of the blood.

Appreciation is expressed to Dr. D. R. Meranze, Director of Research, for his kind encouragement and interest.

This work was supported by grants No. RG 2927C from the National Institute of Health and No. S-23 from the Albert Einstein Medical Center, Philadelphia.

1. Wilander, O., *Scand. Arch. f. Physiol.,* 1939, Suppl. XV.

2. Felix, Pendel, Roka, *Hoppe Seyler Z. f. Physiol.,* 1949, v284, 198.

3. Mann, Hurn, PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 83.

4. Fulton, Marcus, Robinson, *N. Y. Acad. Sci.,* 1950, v52, 7.

5. Marcus, Fulton, *Fed. Proc.,* 1948, v8, 407.

6. Baserga, de Nicola, Vahi, *Experientia,* 1950, v6, 12.

7. Quattrin, *Il Farmaco,* Napoli, 1950.

8. Fiala, Meranze, Roth, *Science,* 1952, v115, 2996.

9. Fiala, Roth, *Fed. Proc.,* 1952, v11, part 1 (*ibid.* oral communication)

10. ———, *Arch. Int. Physiol.,* 1953, v61, 205.

11. Owren, *Scand. Journ. Clin. and Lab. Inv.,* 1951, v3, 3.

12. Snively, Glick, *J. Clinical Inv.,* 1950, v29, 1087.

13. Gollub, Feldman, Schecter, Kaplan, Meranze, PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 853.

Received February 26, 1954. P.S.E.B.M., 1954, v85.