Heat Stability Studies of Clotting Factors in "Thromboplastic" Preparations. (21108)

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"Thromboplastin" in spite of being widely employed is not well characterized and defined. Generally, "thromboplastin" has come to embrace diverse tissue preparations which accentuate the formation of thrombin from prothrombin-containing systems, regardless of their mode of action. In practice "thromboplastic" activity is usually judged by the ability of a given preparation to accelerate the clotting of recalcified citrated or oxalated plasma. In addition, differences in nomenclature and definitions have caused a great deal of confusion with regard to the functional role of tissue preparations in the mechanism of blood coagulation. Following the nomenclature proposed by Milstone(1), thrombokinase is defined in this paper as an agent which has the capacity of directly converting prothrombin to thrombin.* The term thrombokinase was first introduced by Morawitz(2). Thromboplastin is defined in this paper as an accessory factor, which by itself cannot directly activate prothrombin but first reacts with another plasma factor, such as proconvertin, in the presence of calcium ions to yield a thrombokinase-like agent. The concept of a thromboplastic substance was first introduced by Nolf(3). The findings of Owren(4) made it evident that it is necessary to use thrombokinase and thromboplastin as distinct terms. Jensen, Gray, and Schaefer (5) reported that the clotting times of a $BaSO_4$-eluate from normal oxalated plasma with either platelets and $CaCl_2$ or with a commercial preparation of "thromboplastin," heated for 5 minutes at 70°C, are similar in both one-stage and 2-stage tests. These results indicate a similarity in the role of platelet factor and thromboplastin in the clotting mechanism.

In the present study the nature of the clotting factors present in brain extract was further investigated.

Materials and methods. Preparation of plasma. Rabbit blood was collected by cardiac puncture using the multiple syringe technic. The blood was drawn into an ice-cooled, silicone-coated syringe containing 1/10 volume of 0.1 M $K_2C_2O_4$ solution. Blood and oxalate were well mixed, delivered into ice-cooled, silicone-coated conical centrifuge tubes and centrifuged at 3000 rpm for 30 minutes at 0 to 5°C. Plasma was drawn off with a pipette, care being taken to minimize contamination of the plasma with platelets.

Preparation of platelets from rabbit blood. Blood was drawn by cardiac puncture as described above using 1/10 volume of 1% Di-sodium versenate, pH 7, as anticoagulant. Blood was delivered into ice-cooled, silicone-coated conical centrifuge tubes and centrifuged at 550 rpm for 25 minutes at 0 to 5°C to remove the erythrocytes. The platelet-rich supernatant plasma was drawn off with an ice-cooled, silicone-coated syringe. This plasma was centrifuged in ice-cooled, silicone-coated conical centrifuge tubes at 1700 rpm for 30 minutes at 0 to 5°C to pack the platelets. The supernatant plasma was decanted and the packed platelets were washed 2 times with volumes of 0.9% NaCl equal to the original plasma volume. After the second wash, the packed platelets were frozen. Before use, the platelets were thawed, suspended in 0.9% NaCl to the desired concentration and homogenized in a glass homogenizer. Commercial "thromboplastin"† was made up according to the manufacturer's directions. "Thromboplastin" was prepared from rabbit brain tissue according to the method of Quick(6). Resin (Rohm and Haas IR-105 (Na)) was employed to remove calcium ions. The essential details of representative individual experi-

* According to Milstone(1) conversion of prothrombin to thrombin by thrombokinase can also take place in the absence of calcium ions.

† Simplastin, Chilcott Laboratories, Inc., Morris Plains, N. J.

TABLE I. Effect of Heat on Ability of Thromboplastin* and of Rabbit Brain Tissue† to Clot Oxalated Rabbit Plasma.

Test mixture‡			Clot formation times .066 mg $CaCl_2$	.56 mg $CaCl_2$
0.2 ml oxalated plasma			NC20′	5′37″
A. Thromboplastin susp. incubated 10 min. at 37°C, then filtered through resin:				
No treatment			30″	19″
Heated 5 min. at 56°C			50″	22″
	20	60	NC20′	41″
	5	70	1′11″	29″
B. Thromboplastin filtered through resin:				
No treatment			38″	23″
Heated 5 min. at 56°C			53″	26″
	20	60	NC20′	48″
	5	70	1′50″	33″
C. Brain tissue susp. in 0.9% NaCl incubated 10 min. at 37°C:				
No treatment			15″	18″
Heated 5 min. at 56°C			21″	24″
	20	60	NC20′	1′26″
	5	70	47″	45″

* Simplastin, Chilcott Laboratories, Morris Plains, N. J., made according to manufacturer's directions.

† 1 mg brain tissue/0.1 ml suspension.

‡ 0.1 ml thromboplastin-$CaCl_2$ or brain tissue-$CaCl_2$ mixture + 0.2 ml oxalated plasma. When 0.9% NaCl was substituted for $CaCl_2$, no clotting occurred.

ments are included in the various tables.

Results. The data presented in the various tables constitute average values of several identical experiments. The results presented in Table I indicate that heating of a "thromboplastin" preparation or of a rabbit brain tissue suspension at 60°C for 20 minutes destroyed the ability of both preparations to produce a clot when added to oxalated normal plasma in the presence of an amount of $CaCl_2$ not sufficient to produce any recalcification effect. Heating of these "thromboplastic" preparations at 56°C or 70°C for 5 minutes caused a partial destruction of this activity. In the presence of an amount of $CaCl_2$ sufficient for recalcification, clotting times were obtained which were much shorter than the plasma recalcification time.

The data in Table II show that the ability of brain extract to clot oxalated plasma in the presence of $CaCl_2$ not sufficient to produce any recalcification is lost after being incubated at 60°C for 10 minutes. In the presence of $CaCl_2$ sufficient for recalcification the activity also decreased somewhat on heating but the clotting times obtained were greatly reduced when compared with the plasma recalcification time. However, it is obvious that the clotting activity with regard to oxalated plasma in the presence of $CaCl_2$ not sufficient for recalcification is much more labile to heat than the clotting activity in the presence of $CaCl_2$ sufficient for recalcification. Heating of brain extract at 64°C for various lengths of time gave results similar to those obtained at 60°C. A comparison of the data of Tables II and III shows that a small amount of brain tissue will not clot oxalated plasma in the presence of an amount of $CaCl_2$ insufficient to produce any recalcification effect.

The data in Table III indicate that the heat (60°C) stability of thromboplastic activity of brain tissue and platelets is of about the same magnitude. The brain tissue and platelets were employed in comparable amounts with regard to their ability to accelerate the clotting of recalcified plasma. According to Soulier and Larrieu(7) 3 minutes at 64°C are sufficient to destroy completely the antihemophilic activity in a suspension of platelets, and 30 minutes destroys, in addition, a large part of a semithermolabile thromboplas-

TABLE II. Effect of Heat (60°C) on Rabbit Brain Tissue.*

Test mixture†	Min. incubation	Clot formation times .066 mg $CaCl_2$	.56 mg $CaCl_2$
0.2 ml oxalated plasma		NC20′	6′ 7″
0.1 ml brain tissue-$CaCl_2$	0	12″	12″
mixture + 0.2 ml	5	2′17″	21″
oxalated plasma	10	NC20′	29″
	17	″	47″
	25	″	1′ 8″
	30	—	1′10″
	40	—	1′27″
	50	—	1′28″
	60	—	2′ 4″
	70	—	2′ 9″

* 1 mg brain tissue/0.1 ml suspension.

† Brain tissue-$CaCl_2$ mixtures incubated at 60°C. At time intervals shown, 0.1 ml mixture added to 0.2 ml oxalated plasma.

TABLE III. Effect of Heat (60°C) on Ability of Rabbit Brain Tissue* and of a Platelet Homogenate† to Clot Oxalated Rabbit Plasma.

Test mixture		Min. incubation at 60°C	Clot formation times .066 mg $CaCl_2$	Clot formation times .56 mg $CaCl_2$
.2 ml oxalated rabbit plasma + .1 ml saline		0	NC20′	8′31″
+ brain		0	″	1′50″
+ saline		10	—	9′40″
+ brain	+ .2 ml $CaCl_2$	10	—	6′ 1″
+ saline		20	—	10′13″
+ brain		20	—	7′21″
+ saline		30	—	9′36″
+ brain		30	—	7′13″
.2 ml oxalated rabbit plasma + .1 ml saline		0	NC20′	7′35″
+ platelets		0	″	2′19″
+ saline	+ .2 ml $CaCl_2$	10	—	8′ 7″
+ platelets		10	—	4′50″
+ saline		20	—	7′53″
+ platelets		20	—	5′13″

* .00625 mg/.1 ml suspension.
† 6× blood concentration.

tic lipoprotein, leaving a thermostable platelet factor.

Discussion. The effect of the "thromboplastin" preparations plus $CaCl_2$ (an amount not sufficient to produce any recalcification effect) on normal oxalated plasma is probably due to a direct activation of prothrombin, that is, to a typical thrombokinase action. In those cases in which an amount of $CaCl_2$ sufficient for recalcification is employed, tissue preparations react with another plasma factor forming a thrombokinase. When heated for 10 to 20 minutes at 60°C, the "thromboplastin" preparations apparently lost their thrombokinase activity since they failed to clot oxalated plasma in the presence of $CaCl_2$ not sufficient for recalcification. However, in the presence of $CaCl_2$ sufficient for recalcification, clotting times were obtained which were definitely shorter than the plasma recalcification times. The thrombokinase factor is obviously more labile to heat than the thromboplastic factor.

Quick, Hussey, and Epstein(8) found that the addition of rabbit brain extract to hemophilic blood will cause good consumption of prothrombin. When such an extract was heated to 60°C for 20 minutes, its ability to convert prothrombin to thrombin in hemophilic blood was lost, but it retained the ability to cause consumption of prothrombin in normal platelet-poor plasma. Biggs, Douglas, and MacFarlane(9) have expressed the view that human brain extract itself is not capable of converting prothrombin to thrombin but can form a thrombokinase-like substance upon reaction with factors V‡ and VII.‡

The data presented here and the observations of Quick and his associates(8) indicate that the indirect effect of thromboplastin must be distinguished from the direct action of thrombokinase on prothrombin in order to understand the action of brain tissue extracts. The clotting times obtained with tissue extracts may vary with their mode of preparation. The question of whether the presence of thrombokinase in brain extracts is derived from the blood trapped in the brain tissue, or is actually a constituent of the tissue, cannot be answered at present. The nature and degree of activity of tissue extracts may vary with the species of animal from which it is obtained.

The heat stable component of brain extracts apparently exerts a function in the clotting mechanism similar to that of the platelet factor. Both factors require the cooperation of another factor, such as proconvertin, before they are capable of converting prothrombin to thrombin. The similarity in action of platelet extract and brain extract

‡ Factor V is the labile factor and factor VII of Koller(10) is proconvertin.

(heated to 60°C for 20 minutes) has also been pointed out by Quick and his associates(8). Whether the platelet factor and the thromboplastic factor of brain tissue are chemically identical has still to be determined.

Summary. 1. On the basis of heat stability studies, the clotting properties of brain tissue extracts are due to 2 components: a) a thrombokinase, which has the ability to convert prothrombin directly to thrombin, and b) a thromboplastin, which is an accessory factor and must react first with another plasma factor, such as proconvertin, before it is capable of converting prothrombin to thrombin. 2. The indirect effect of thromboplastin must be distinguished from the direct action of thrombokinase on prothrombin if the mode of action of so-called "thromboplastic" brain tissue extracts is to be understood.

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Levels of Serum Protein Fractions in Diabetic Patients with *Retinitis proliferans.** (21109)

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There has been much speculation as to the etiology of the degenerative vascular lesions seen in long-term diabetes. The question has been raised as to what role, if any, shifts in serum protein and, in particular, serum mucoproteins and lipoproteins, play in the development of such abnormalities(1-3). Changes in the major serum protein fractions in diabetes have been reported. Schneider, Lewis, and McCullagh(4) described changes in the albumin and β-globulin fractions of the serum from 31 diabetic patients with retinitis. The reduction in albumin and the elevation of the β-globulin fraction were more marked in the retinitis cases than similar changes observed in uncontrolled diabetics. That these changes may not represent the same thing was indicated by a difference in response to treatment. Rifkin and Petermann(5) have reported the results of an electrophoretic study of the serum proteins of patients with clinically established diabetic glomerulosclerosis. A decrease in the albumin and a significant elevation of the α_2-globulin were found in all 10 cases. The β-globulin was normal or only slightly elevated. Changes in the serum lipoproteins in diabetics with vascular degenerative changes have been reported by Keiding *et al.*(6) and by Engelberg *et al.*(7). The elevation of the S_f 12-20, S_f 21-35, and S_f 35-100 lipoproteins was found to correlate with the presence of both diabetic retinopathy and nephropathy. Changes in the serum mucopolysaccharides and mucoproteins in patients with vascular degenerative disease have been investigated systematically both by Berkman *et al.*(8) and by Keiding and Tuller(9). However, during the work of the latter authors it was evident that it would be of interest to follow changes in the major serum protein fractions along

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