supplying nutritional substances, and/or by decreasing permeability.

Loeb(6) suggested that the starfish egg normally becomes unfertilizable after a damaging amount of aerobic oxidation takes place, and believed that the slowing down of this action (through use of potassium cyanide and low oxygen) was responsible for prolonging fertilizability in these eggs. The writer(2) has suggested that cobalt prevents deterioration of fertilizability by uniting with (and preventing oxidation of) thiol groups at the egg surface. The action of Vit. B_{12} in preserving fertility may well be through protection of the egg surface from destructive oxidations. It seems not unlikely that B_{12} combines with surface proteins in much the same way that Co^{60} Vit. B_{12} combines with serum proteins (alpha and beta globulins) and the protein of cerebro-spinal fluid(7). The preserving action of benzyl alcohol alone may be in the retardation of the processes (oxidations, increased permeability, etc.) which normally lead to the decay of fertility.

Summary. Vitamin B_{12} (100-6.25 μg/cc) in the presence of 0.15% benzyl alcohol extends fertilizability in the eggs of *Dendraster excentricus*. Benzyl alcohol alone also has some preserving action. It is suggested that both substances prolong fertility by inhibiting destructive processes that occur with aging of the eggs.

1. Rulon, O., *Anat. Rec.*, 1960, v137, 390.
2. Rulon, O., *ibid.*, 1960, v138, 381.
3. Schechter, V., *Biol. Bull.*, 1937, v72, 366.
4. Whitaker, D. M., *J. Exp. Zool.*, 1937, v75, 155.
5. Runnström, J., *Symposia of Society for Experimental Biology*. Cambridge Univ. Press, 1952, v6, 39.
6. Loeb, J., *The Mechanistic Conception of Life*, Univ. of Chicago Press, 1912, 155.
7. Meyer, L. M., Bertcher, R. W., Mulzac, C., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 607.

Received July 14, 1961. P.S.E.B.M., 1961, v108.

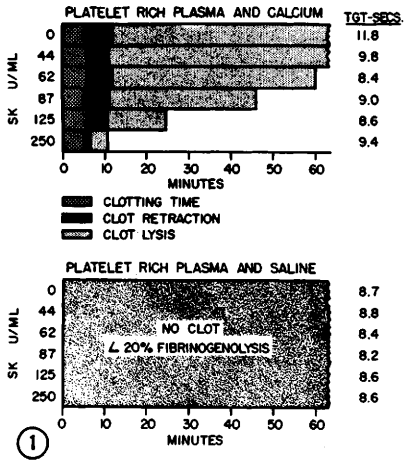
Effects of Clotting and Clot Lysis on Platelet Thromboplastic Activity.* (26946)

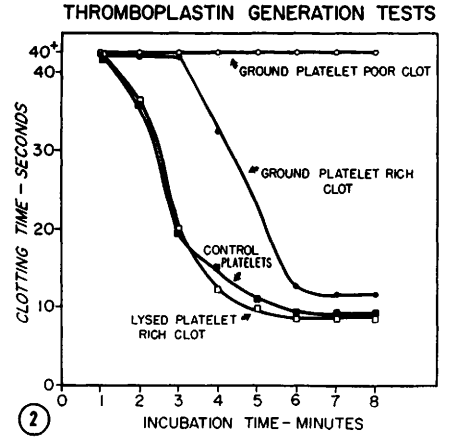
JESSICA H. LEWIS AND CAROL L. JEFFREYS
Department of Medicine, University of Pittsburgh, Pittsburgh

During coagulation of shed blood the majority of platelets undergo clumping, "viscous metamorphosis",(1,2) and, eventually, disappear from the liquid phase. Presumably these platelets are either trapped in the fibrin meshwork of the clot or disintegrated. The few platelets which remain in the liquid phase have been called "serum platelets" and their properties described by Alexander(3). Platelets contain a thromboplastic factor (platelet factor 3) which is essential to normal blood coagulation. These studies were undertaken to determine what happens to this factor during and after clotting. To avoid interference from red cells, platelet-rich plasma was used. To free platelets from enmeshing fibrin network, clots were either chopped and ground or lysed.

Materials and methods. Platelet-rich plasma. One unit of ACD blood, drawn in a plastic bag, was centrifuged at 400 g for 25 min.; the plasma was transferred to plastic tubes and recentrifuged at 300 g a number of times until essentially free from red cells. *Streptokinase* (SK), Varidase, was kindly supplied by Dr. Ruegsegger of Lederle Laboratories. *Platelet-thromboplastic factor* was estimated by 2 methods: (a) a standard TGT test(4) in which equal volumes of normal $BaSO_4$ adsorbed plasma (dil. 1-5), normal serum (dil. 1-10), platelets and 0.025 M $CaCl_2$ were mixed and tested for thromboplastic activity every minute by adding 0.1 ml to 0.2 ml recalcified normal substrate plasma. Minimum clotting time of the latter is reported.

* This investigation was supported by research grants from Division of Research Grants, Nat. Inst. Health, U.S.P.H.S., Bethesda, Md.





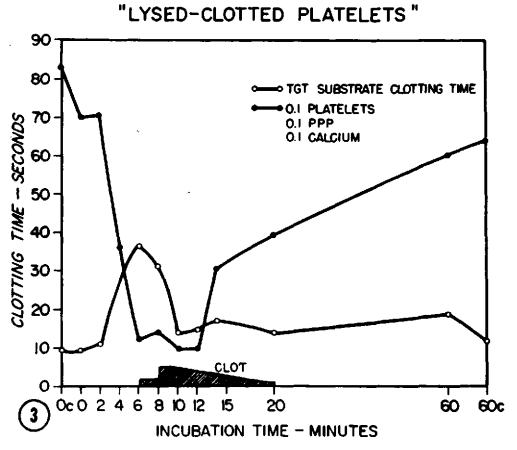


FIG. 1. TGT substrate clotting times from mixtures containing platelet sediments from platelet-rich plasma with varying amounts of streptokinase, with and without calcium.

FIG. 2. Effects of control platelets, clot-lysed platelets, ground platelet-rich clot and ground platelet-poor clot on thromboplastin generation

(b) The second method recorded the clotting time of a mixture of 0.1 ml platelet-poor plasma (normal plasma centrifuged 60 min. at 20,000 g) plus 0.1 ml platelets or saline plus 0.1 ml 0.02 M $CaCl_2$.

Results. Effects of streptokinase concentration and clotting on platelet-thromboplastic factor. Fig. 1 shows the results of an experiment in which platelet-rich plasma was distributed in 4 ml amounts in 12 new glass 13 × 100 mm tubes; 0.1 ml appropriately diluted streptokinase was added to each tube to give the final concentrations shown in the figure, and 0.1 ml 0.8 M $CaCl_2$ was added to the first 6 tubes and 0.1 ml saline to the last 6. Clotting time, clot retraction time and clot lysis time of the first 6 tubes were recorded. After 60 min. all were centrifuged at 20,000 g for 15 min., the sediments washed twice and suspended in 0.4 ml saline. Sediments from tubes 1 and 2 were chopped and ground. All sediments were diluted 1-10 for TGT, the results of which are shown in the last column.

Streptokinase alone had no effect upon the activity of platelets in the TGT test and caused little fibrinogenolysis of the mother plasma. Clotting and subsequent clot-lysis also had little or no effect on TGT time, although the sediments from tubes 1 and 2 were very difficult to suspend smoothly.

Fig. 2 illustrates another experiment in which the TGT curves of control platelets, sediment from a lysed platelet-rich clot, sediment from a ground platelet-rich clot and sediment from a ground platelet-poor clot are compared. The "clot-lysed" platelets appear entirely similar to the control unclotted platelets. The ground washed platelet-rich clot contained significant amounts of thromboplastic factor, although again it proved difficult to prepare a fine suspension. The ground platelet-poor clot showed no platelet-thromboplastic activity.

Sediments were also tested for complete prothrombinase (or plasma thromboplastin) activity by testing in TGT systems deficient

using standard $BaSO_4$ plasma and serum.

FIG. 3. Effects of incubation before decalcification of mother tube on platelet-thromboplastic activity.

in $BaSO_4$ plasma, in AHF (using Hemophilic $BaSO_4$ plasma), in serum and in PTC (using PTC deficient serum). All tests showed no generation of plasma-thromboplastin or prothrombinase unless both normal $BaSO_4$ plasma and normal serum were present. Sediments were also tested for presence of thrombin by adding fibrinogen solution. No clots were formed in 6 hours. Recalcification times of platelet-poor plasma to which 0.1 ml full-strength platelets or "clot-lysed" platelets were added were similar.

In another experiment (Fig. 3), an attempt was made to stop coagulation at various intervals after recalcification by mixing 0.1 ml 1 M sodium citrate into the coagulation mixtures containing 4 ml platelet-rich plasma, 0.1 ml streptokinase (1000 units) and 0.1 ml 0.8 M $CaCl_2$. The tubes were chilled and centrifuged immediately after addition of citrate. The sediments were washed twice, suspended in 0.4 ml saline and tested full strength in the recalcification test and at a 1-10 dilution in the TGT. Tubes 0_c and 60_c were not recalcified. As shown in Fig. 3 there was a rapid decrease in the platelet-poor plasma recalcification time employing sediments from tubes which had been recalcified from 2 to 12 min. earlier. Thereafter activity decreased, nearly reaching control levels 60 min. after the platelet-rich plasma had been recalcified. This apparent increase in "thromboplastic activity" of the platelets (or sediment) occurred simultaneously with beginning clot formation in the mother tube. It was due, at least in part, to the presence of thrombin, as, even after two washings, these platelets clotted (in 60 secs. or longer) fibrinogen or plasma *without* the addition of calcium. At the same time what appeared to be a paradoxical lengthening of the TGT substrate clotting time was often observed. In one experiment the control platelets produced a 9.8 sec. TGT clotting time and the 6 min. platelets a 36.8 sec. time.

In another experiment, shown in Table I, platelets from the unclotted SK control tube and a 7 min. clot-lysed tube were diluted to various strengths and then used in the standard TGT test. The substrate clotting times after 1 min. and 6 min. incubation of the TGT mixtures are shown. The 100% platelet dilution was that in which the platelets were suspended in a volume of saline equal to that of the platelet-rich plasma from which they were obtained. As expected, when control platelets were diluted the substrate clotting times after both 1 min. and 6 min. of incubation became longer. Rather surprisingly, the platelets from the 7 min. clot-lysed tube acted in a reverse fashion, the TGT substrate clotting times becoming progressively shorter with dilution down to 25%. This experiment has been repeated a number of times with similar results.

TABLE I. TGT Substrate Clotting Times 1 and 6 Min. after Incubation of Dilutions of Control and 7 Min. Clot-Lysed Platelets in Standard TGT Mixtures.

	TGT substrate clotting time (sec.)			
	Control		7 min. clot-lysed	
Dilution, %	TGT incubation time (min.) 1	6	1	6
100	18.2	7.6	18.2	16.2
75	27.0	8.6	11.8	9.2
50	30.2	9.6	10.8	7.8
25	40+	10.8	10.0	8.0
10	40+	12.8	11.8	11.2
5	40+	18.6	19.6	14.6

Platelets from the control tube (Table I) did not contain thrombin and were unable to clot plasma without additional calcium. Those from the 7 min. tube clotted plasma or fibrinogen in approximately 60 sec. in dilution similar to that in the TGT mixture. In an attempt to demonstrate a possible TGT inhibiting effect of thrombin, platelets were incubated with various dilutions of bovine thrombin (250 to 0.1 unit), washed twice, and tested in a standard TGT test. Although the 1 min. substrate clotting times were shorter than that with the untreated platelets, the 6 min. times were identical.

In another experiment, Table II, platelets from the unclotted SK control tube and a 7 min. clot-lysed tube were heated for 10 min. in a boiling water bath or frozen-thawed before testing. Heating and freezing both shortened the minimal substrate clotting time obtained after 7 min. incubation of the TGT mixture. In addition heating of the clot-lysed platelets resulted in a longer 1 min.

TABLE II. TGT Substrate Clotting Times (Sec.) 1 and 7 Min. after Incubation of Untreated, Heated and Frozen Control and 7 Min. Clot-Lysed Platelets.

	Untreated		100°C, 10 min.		Frozen-thawed	
	TGT incubation time (min.)					
Platelets	1	7	1	7	1	7
Control	40+	9.8	40+	10.0	40+	9.8
7 min. clot-lysed	23.0	13.0	40+	10.0	21.0	10.8

TGT substrate clotting time, perhaps due to destruction of contaminating thrombin.

It was also noted that the platelets from the early phases of clotting did not contain full plasma-thromboplastin activity as both $BaSO_4$ treated plasma and serum were necessary for their full activity in the TGT test.

Comment. These experiments show that "platelets", even after clotting, retain their full platelet-thromboplastic or factor 3 activity. After clotting and clot lysis the platelets have a shaggy appearance but are still recognizable with the phase microscope. It is possible that the thromboplastic activity is present in small granules outside of the platelet shell which sediment at the same speed, although such granules could not be distinguished microscopically. The chopped ground clot sediment had no ability to retract clots. The sediment from the lysed clotted samples, even after many washings, caused lysis of plasma clots. Additional experiments showed that human platelets contain a streptokinase cofactor (? proactivator) and when added with streptokinase to bovine profibrinolysin produce an active fibrinolysin.

Platelets obtained from recalcified platelet-rich plasma in the early stages of clotting retain traces of thrombin. They are able to clot platelet-poor plasma without added calcium and, in the presence of calcium, produce very rapid clotting. The same platelets when used in a TGT test promote lessened evolution of plasma thromboplastin unless they are first diluted or heated. This apparent inhibition is reminiscent of the poor evolution of plasma thromboplastin observed by Spaet(4) when very concentrated platelets were used. The anticoagulant activity of Spaet's platelet lipid was stable at 100°C., while that found in defatted platelets was destroyed by heat. The apparent anticoagulant activity of platelets separated from lysed samples soon after recalcification is destroyed by 100°C, leaving only the coagulant activity.

Summary. Sediments obtained from platelet-rich plasma allowed to clot for 1 hour contain full platelet-thromboplastic or factor 3 activity. Sediments obtained from recalcified platelet-rich plasma in the early phase of clotting contain thrombin and appear to be inhibitory to normal thromboplastin generation.

1. Sharp, A. A., *Brit. J. Hemat.*, 1958, v4, 28.
2. Zucker, M. B., Borrelli, J., *J. Appl. Physiol.*, 1959, v14, 575.
3. Czernobilsky, H., Alexander, B., *Blood Platelets*, 1961, Little, Brown and Co., Boston, Mass., p565.
4. Spaet, T. H., *J. Appl. Physiol.*, 1957, v11, 119.

Received July 24, 1961. P.S.E.B.M., 1961, v108.

The Cytostat, An Apparatus for Automatically Controlled Continuous Propagation of Suspended Cells.* (26947)

BERTIL BJÖRKLUND, VIVEKA BJÖRKLUND AND JAN-ERIC PAULSSON
Immunology Research Laboratory, State Bacteriological Laboratories, Stockholm, Sweden

Designs for continuous cultivation of cells in suspension have been introduced particularly for the study of bacteria, algae and

* This work was supported in part by the Wallenberg Foundation and in part by Research Grant from Nat. Cancer Inst., Nat. Inst. Health, P.H.S.