EFFECTS OF BACTERIAL ENDOTOXIN ON RABBIT PLATELETS*

I. Platelet Aggregation and Release of Platelet Factors in Vitro

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There is much to suggest that certain of the biologic effects of endotoxins of Gram-negative bacteria are consequences of a reaction involving blood platelets. Thrombocytopenia occurring after injection of endotoxin is well documented (1, 2) and is associated with the appearance of platelet aggregates in pulmonary arterial blood (3). Concomitantly 5-hydroxytryptamine (5HT), which normally circulates virtually completely bound to platelets in an inactive form, appears in plasma in appreciable quantities (4–6). These observations suggest that the interaction of endotoxin and platelets *in vivo* results in platelet aggregation, trapping of these aggregates in vascular beds, and a transfer of pharmacologically active platelet factors, including 5HT, from platelets to plasma. The studies to be reported herein indicate that platelet aggregation and transfer of 5HT from platelets to plasma can also be observed *in vitro* during the course of incubation of platelet-rich rabbit plasma with endotoxin. In addition, two other platelet factors, platelet phospholipid and a heat-stabile bactericidin active against *Bacillus subtilis*, are liberated from platelets during incubation with endotoxin.

Materials and Methods

All glassware was freshly siliconized by immersion in a 1:1000 dilution of Dow Corning Z4141 for 1 hour and was baked for 2 hours at 170°C prior to use. An *Escherichia coli* endotoxin of the Boivin type (Difco *E. coli* endotoxin lot 0127:B8) suspended in saline solution to a concentration of 2 mg per ml was used in all experiments. Several preparations bearing different control numbers of this lot of endotoxin were used and were found to vary somewhat in activity.

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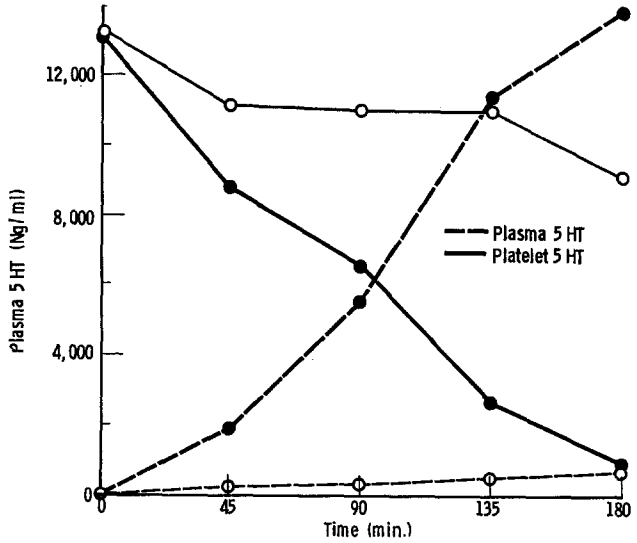
Assay of 5HT.—5HT content of plasma and platelets was determined by the estrus rat uterus bioassay system of Erspamer (7) using a freshly prepared low calcium (20 mg per liter) Ringer's solution in a 10 ml muscle bath. Amounts of 5 HT required for threshold response varied from 15 to 50 NG (1 NG = 0.001 μg) and maximum responses occurred with from 50 to 200 NG. Both speed of onset and amplitude of contraction were measured and in almost all instances varied directly with each other. In the present studies, amplitude was used as the criterion of response. Differences as small as 10 NG could be measured with accuracy by bracketing the test response with responses to known quantities of 5HT. Standards of 5HT were prepared from frozen stock solutions containing 1 mg serotonin creatinine sulfate (Sandoz Corporation, Hanover, New Jersey) per ml of Ringer's solution. Stock 5HT solutions were kept no longer than 1 month. When specimens for assay contained large amounts of 5HT, a considerable error was introduced by the large dilution factor necessary to bring the 5HT concentration within the relatively narrow range of the assay.

The specificity of the assay for 5HT was determined as follows: (*a*) The uterus-stimulating activity in plasma and platelets was completely blocked by prior treatment of the test uterus with 20 μg of the 5 HT antagonist, 1-benzyl 2,5-dimethyl serotonin in 10 ml of Ringer's solution for 15 minutes (8). (*b*) Incubation of test plasmas with chymotrypsin, 1 mg per ml, for 15 minutes at 37°C, a procedure which inactivates the uterus-stimulating activity of polypeptides (9), did not decrease the activity of the test plasmas and platelet residues. (*c*) Plasma potassium concentrations were 2 to 4 meq per liter, far below the level required to produce uterus stimulation (10) and did not change during incubation of platelet-rich plasma with endotoxin. (*d*) Inclusion of atropine in the muscle bath in a concentration which completely inhibits the uterus response to acetylcholine (10) was without effect on the response to the test plasmas. (*e*) The activity which developed in the test plasmas was stable over a 24 hour period at 4°C indicating that labile uterus-stimulating factors appearing during surface activation of human plasma by glass beads were not involved (11). (*f*) Increase in uterus-stimulating activity in plasma was accompanied by reciprocal decrease in the activity remaining with the platelets. (*g*) Histamine has no effect in this assay system (10).

Preparation of Plasma.—Rabbit blood was obtained from a freely bleeding marginal ear vein or by cardiac puncture and delivered directly into siliconized tubes containing anticoagulant. Except as specifically indicated the anticoagulant used was sodium citrate in a final concentration of 0.38 per cent. Heparin in a final concentration of 1 per cent or disodium ethylenediaminetetraacetate (EDTA) in a final concentration of 0.2 per cent was used in certain experiments. Platelet-rich plasma (PRP) was prepared by centrifugation of anticoagulated whole blood in a 15 ml graduated centrifuge tube at 1000 RPM (225 *g* at the bottom of the tube) for 20 to 30 minutes at 4°C in a refrigerated centrifuge (International, model 2, with an 8 place head, catalog No. 269, and 50 ml cups, catalog No. 320). The supernatant plasma from citrated or EDTA blood contained from 400,000 to 800,000 platelets per c. mm and negligible numbers of white and red cells. PRP prepared from heparinized blood contained substantially fewer platelets, apparently because of increased tendency to spontaneous platelet clumping. The total 5HT content of PRP was determined after lysis of the platelets by three cycles of freezing and thawing. Centrifugation of PRP at 3000 RPM (2000 *g* at the bottom of the tube) for 30 minutes yielded platelet-poor plasma which contained only small amounts of 5HT, usually less than 50 NG per ml. 5HT content of the platelet residue, determined after resuspension of the platelets in a volume of saline equal to that of the plasma from which they had been sedimented and lysis of the suspension by freezing and thawing, ranged from 6,000 to 18,000 NG per ml. Mixtures of PRP and endotoxin were incubated at 37°C in a standard bacteriology incubator, and during incubation were constantly rotated at 15 RPM on a motor-driven Spinnerette (model ST, New Brunswick Scientific Co., New Brunswick, New Jersey).

Measurement of Available Platelet Phospholipid (Product I Substrate Time).—The amount of platelet phospholipid available to the clotting process was determined by adding preformed product I and calcium to the test plasma and determining the clotting time of this mixture (product I substrate time) (12). Stable product I was prepared from rabbit instead of human blood reagents according to the method of Spaet (13). The clotting system consisted of 0.1 ml of a dilution of stable product I, 0.1 ml 0.025 M $CaCl_2$, and 0.1 ml of test plasma in the platelet-rich state. The dilution of product I was adjusted to give a minimal product I substrate time of 10 seconds or less when tested with plasma in which cephalin in optimum concentrations substituted for platelet phospholipid (14). This dilution was usually 1:10 or 1:20.



TEXT-FIG. 1. Concentration of 5HT in plasma (broken line) or platelets (solid line) during incubation of 100 μg per ml of *E. coli* endotoxin (●) or saline (○) with platelet-rich rabbit plasma.

When cephalin plasma was serially diluted in platelet-poor plasma, a straight line log-log plot relating product I substrate time to phospholipid concentration was obtained indicating that the test assayed available phospholipid.

Measurement of Bactericidal Activity of Plasma.—Plasma bactericidal activity against *B. subtilis* was determined by a modification of the method of Hirsch (15). Test plasma specimens were first centrifuged to remove platelets and then serially diluted in twofold steps in 0.5 ml sterile physiological saline solution containing 0.05 per cent bovine albumin (Armour Laboratories, Chicago, Illinois). An 18 hour growth of *B. subtilis* on trypticase–soy agar was suspended in physiological saline solution containing 0.05 per cent bovine albumin and turbidometrically standardized to contain approximately 2000 viable units per ml. Each dilution of test plasma (0.5 ml) was mixed with 0.5 ml of bacterial suspension and incubated for 1 hour at 37°C. A mixture of equal parts of bacterial suspension and saline-albumin diluent served as a control. Pour plates were then prepared with 0.1 ml aliquots of the plasma–*B. subtilis* mixture and melted trypticase–soy agar. Colonies were counted after 24 hours' incubation, and the titer of bactericidal activity was expressed as the reciprocal of the highest dilu-

tion of plasma which effected a 50 per cent or greater reduction in bacterial count as compared with control plates.

EXPERIMENTAL

Release of 5HT during Incubation of Platelets with Endotoxin.—Repeated experiments have demonstrated that incubation of citrated rabbit PRP with endotoxin is associated with a progressive transfer of 5HT from platelets to plasma.

TABLE I

Effect of Endotoxin Concentration on Release of Platelet 5HT in Citrated PRP

Material added	Time incubated	Platelet aggregation*	Concentration of 5HT	
			Plasma	Platelet residue
	min.		*NG/ml*	*NG/ml*
Saline	0	0	<50	18,500
	180	0	225	20,000
Endotoxin, 1 μg/ml	0	0	<50	–
	180	0	275	–
Endotoxin, 10 μg/ml	0	0	<50	–
	180	+	225	–
Endotoxin, 100 μg/ml	0	0	<50	20,000
	180	++++	15,000	1,000

* 0 to ++++ indicates degree of platelet aggregation.

Endotoxin was added to rabbit PRP to a final concentration of 100 μg per ml of plasma and a second portion of the same PRP was inoculated with a similar volume of saline. Both mixtures were rotated in an incubator at 37°C. Aliquots of each were removed at intervals, centrifuged to sediment platelets, and 5 HT concentrations of the plasma supernate and platelet residue determined.

As is shown in Text-fig. 1, in the specimen of PRP to which saline had been added only a small transfer of 5HT from platelets to plasma occurred. In contrast, in the specimen containing endotoxin a marked transfer was seen as shown by a progressive increase in plasma 5HT content and a reciprocal decrease in the concentration of 5HT in the platelet residue. In many instances this transfer was nearly complete after 3 hours' incubation.

Changes in Platelet Morphology Induced by Endotoxin.—PRP which had been incubated with endotoxin appeared less opalescent in the gross than control PRP incubated with saline, and microscopic observations confirmed the notion

that platelet aggregation accounted for this clearing. Phase microscopic inspection of control preparations (Fig. 1A) revealed intact evenly dispersed platelets. However, in the endotoxin–PRP mixtures (Fig. 1B) a process of aggregation and fusion of platelets had occurred to a degree roughly parallel to the release of 5HT. Aggregation and fusion of platelets could be differentiated with considerable confidence from simple clumping in which individual cells remained distinguishable. These morphologic changes are similar to those termed viscous metamorphosis by others (16).

Influence of Concentration of Endotoxin and Type of Anticoagulant on the

TABLE II

Effect of Graded Doses of E. coli Endotoxin on Release of 5HT from Platelets in Heparinized PRP

Material added	Time of incubation	Platelet aggregation*	5HT	
			Plasma	Per cent released‡
	min.		*NG/ml*	
Saline	0	0	35	2
Saline	120	0	40	2
Endotoxin, 1 μg/ml	120	++	1,000	61
Endotoxin, 10 μg/ml	120	++++	1,250	77
Endotoxin, 100 μg/ml	120	++++	1,250	77

* 0 to ++++ indicates degree of platelet aggregation.

‡ Total 5HT of heparinized plasma was 1625 NG per ml.

Release of Platelet 5HT.—The interaction of endotoxin and PRP is dose-dependent and is strikingly influenced by the type of anticoagulant.

E. coli endotoxin in graded doses of 100, 10, and 1 μg per ml was added to PRP prepared with citrate, heparin, or EDTA. Control flasks of PRP were treated with similar volumes of saline solution. At intervals varying from 90 to 180 minutes in different experiments the specimens were centrifuged to remove platelets and concentrations of 5HT in the supernatant plasmas determined. The proportion of 5HT released to total 5HT was determined by measuring the 5HT concentration remaining in the platelet residue or the total 5HT content in an aliquot of untreated PRP.

As is illustrated in Table I, endotoxin in a concentration of 1 or 10 μg per ml caused almost no platelet aggregation or release of 5HT in citrated PRP, whereas endotoxin in a concentration of 100 μg per ml evoked a nearly complete release of 5HT and marked platelet aggregation. In other experiments the

intermediate dose of endotoxin (10 μg per ml) demonstrated slight activity but always substantially less than the larger dose.

Table II illustrates the effects of incubation of endotoxin with heparinized PRP. Platelets in heparinized plasma exhibit spontaneous platelet aggregation not found in either sodium citrate or EDTA plasma, and heparinized PRP contained substantially lower numbers of platelets and total 5HT. Nevertheless, platelet aggregation and a substantial transfer of 5HT from platelets to plasma occurred at endotoxin concentrations of 1 μg per ml (61 per cent of platelet 5HT transferred to plasma) or 10 μg per ml (77 per cent of platelet

TABLE III

Effect of Temperature on the Interaction of Endotoxin and Platelets

Temperature	Time of incubation	PRP—saline* mixture (control)		PRP—endotoxin‡ mixture	
		Platelet aggregation§	Plasma 5HT	Platelet aggregation§	Plasma 5HT
°C	*min.*		*NG/ml*		*NG/ml*
4	0	0	—	0	10
4	90	0	22	0	37
4	180	0	325	0	62
37	0	—	—	0	22
37	90	0	45	+++	10,000
37	180	0	65	+++	8,750

* Saline, 0.1 ml per ml of PRP.

‡ *E. coli* endotoxin, 100 μg per ml PRP.

§ 0 to ++++ indicates degree of platelet aggregation.

5HT transferred to plasma) of heparinized PRP. As noted in the preceding paragraph, 10 μg of endotoxin caused only slight platelet aggregation or release of 5HT in citrated PRP.

When EDTA was used as an anticoagulant, no morphologic change or 5HT release could be demonstrated at any of the three endotoxin concentrations tested.

Effect of Temperature on the Endotoxin-Platelet Interaction.—

Citrated PRP was divided into four aliquots, two of which were inoculated with endotoxin, 100 μg per ml, and two with saline solution. One endotoxin-containing and one saline-containing aliquot were slowly rotated at 37°C and the other two specimens were rotated at 4°C. Specimens were removed at intervals and concentrations of plasma 5HT determined after separation of plasma and platelets by centrifugation.

As is seen in Table III, platelet aggregation and release of platelet 5HT occurred when endotoxin was incubated with PRP at 37°C, whereas neither

morphologic change nor release of 5HT occurred when the reaction was carried out at 4°C. The spontaneous platelet aggregation observed by others (17) to occur at 4°C did not interfere with these microscopic observations which were made after the slides had remained several minutes at room temperature.

Other Substances Active in Releasing 5HT from Platelets.—Heat-killed *E. coli* in concentration of 10^8 cells per ml of PRP was found to be quite active in releasing platelet 5HT. Similar activity was demonstrated by equal concentrations of heat-killed staphylococci, pneumococci, and streptococci, but polysterene latex particles of coccal size were without effect.

TABLE IV

The Effect of Endotoxin on Platelets Suspended in Previously Heated Plasma

Material added	Time incubated	Platelets in previously heated* plasma			Platelets in unheated plasma		
		Platelet aggregation‡	Plasma 5HT	Platelet residue 5HT	Platelet aggregation	Plasma 5HT	Platelet residue 5HT
	min.		*NG/ml*	*NG/ml*		*NG/ml*	*NG/ml*
Endotoxin, 100	0	0	15	–	0	20	–
μg/ml	45	0	60	–	++	4200	–
	90	+	90	7350	+++	5850	2200
Saline solution, 0.1	0	0	10	–			
ml/ml	45	0	225				
	90	+	90	–			

* 56° for 30 minutes.

‡ 0 to ++++ indicates degree of platelet aggregation.

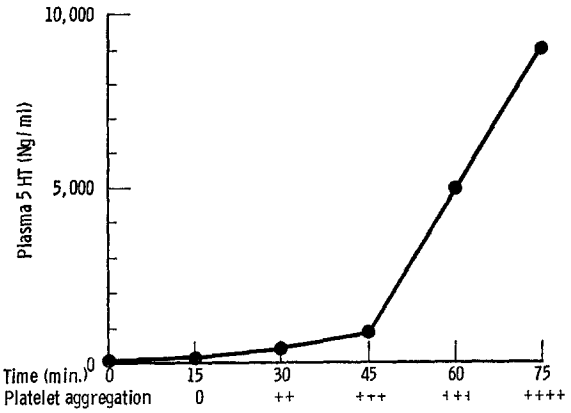
Platelet aggregation and release of 5HT as described by others (18–20) in the presence of antigen-antibody reactions could also be observed in the system used in these experiments. These changes were observed after addition of human gamma globulin and rabbit anti-human gamma globulin to a non-immunized animal's plasma or after addition of human gamma globulin to PRP obtained from an animal immunized with human gamma globulin.

Evidence for the Participation of Plasma Factors in the Interaction of Platelets and Endotoxin.—

Three aliquots of PRP were centrifuged and the sedimented platelets set aside. Two of the plasma supernates were heated at 56°C for 30 minutes, and the third was kept at room temperature. After the heated specimens had returned to room temperature, the platelets were resuspended in the plasmas from which they had been removed. Endotoxin in a concentration of 100 μg per ml was added to one of the suspensions of platelets in previously heated plasma, and to the platelets suspended in plasma which had been kept at room temperature. As a control, saline was added to the remaining aliquot of platelets in previously heated plasma.

The three aliquots were then incubated at 37°C and samples were removed at intervals for determination of content of 5HT in plasma and platelets.

Heating plasma at 56°C for 30 minutes inactivates or removes some factor necessary for the endotoxin-platelet interaction. As shown in Table IV, endotoxin in a concentration of 100 μg per ml produced marked platelet aggregation and release of 5HT when incubated with platelets resuspended in unheated normal plasma, but had no apparent effect on platelets in plasma previously heated at 56°C for 30 minutes. The plasma factor that is



TEXT-FIG. 2. Time of appearance of platelet aggregation and release of 5HT after addition of 100 μg per ml of *E. coli* endotoxin to platelet-rich rabbit plasma. 0 to ++++ indicates degree of platelet aggregation.

removed or inactivated by heating at 56°C for 30 minutes is not restored by addition of fresh pooled guinea pig serum.

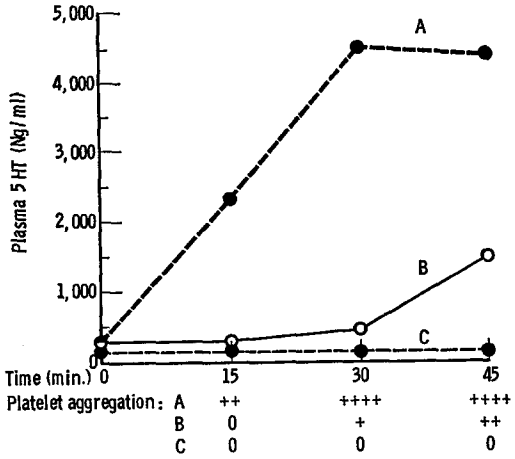
A lag period of 30 to 60 minutes has been consistently observed between addition of endotoxin to PRP and the occurrence of platelet aggregation and release of 5HT. This is illustrated in the experiment depicted in Text-fig. 2 in which observations were made every 15 minutes. Morphologic changes in platelets were not observed until 30 minutes after addition of endotoxin and appreciable amounts of plasma 5HT did not appear until 60 minutes after endotoxin had been added.

It is of interest that the lag period between addition of endotoxin and release of platelet 5HT could be shortened by incubation of endotoxin with plasma prior to addition of platelets.

Three aliquots of PRP were centrifuged and the sedimented platelets set aside. Endotoxin in a concentration of 100 μg per ml was added to the supernatant plasma from the first aliquot, saline solution was added to the second aliquot, and nothing was added to the third. The three

aliquots of plasma were rotated at 37°C for 90 minutes. The platelets were then resuspended in the plasma aliquots from which they had been removed, and at this time 100 μg per ml of endotoxin was added to the third previously untreated aliquot. All specimens were again rotated at 37°C and samples removed at intervals, spun to remove platelets, and the supernatant plasma assayed for 5HT content.

When platelets and endotoxin were added to plasma which had been incubated at 37°C for 90 minutes, the usual lag period between addition of endotoxin and the appearance of platelet aggregation or release of platelet 5HT



TEXT-FIG. 3. Time of appearance of platelet aggregation and release of 5HT (*A*) after resuspension of platelets in plasma previously incubated with 100 μg per ml of *E. coli* endotoxin at 37°C for 90 minutes, (*B*) after addition of 100 μg per ml of *E. coli* endotoxin to platelets resuspended in plasma previously incubated at 37°C with 0.1 ml saline per ml for 90 minutes, and (*C*) after resuspension of platelets in plasma previously incubated with saline solution. 0 to ++++ indicates degree of platelet aggregation.

was observed (Text-fig. 3). In contrast, the specimen of plasma previously incubated with endotoxin demonstrated moderate platelet aggregation and a high concentration of plasma 5HT as soon as 15 minutes after resuspension of platelets and maximum changes had occurred by 30 minutes. No change in plasma 5HT occurred in the saline control over the period of observation.

Platelet aggregation and release of 5HT induced by addition of *E. coli* or staphylococci to rabbit PRP could be inhibited by prior absorption of plasma with bacterial cells.

Platelets were removed from four aliquots of PRP by centrifugation. Two aliquots of supernatant plasma were incubated at 37°C for 90 minutes with thrice washed heat-killed *E. coli* in a concentration of 10^8 cells per ml, and the other two aliquots were incubated with saline solution as controls. The plasmas were centrifuged to remove the bacterial cells, and the platelets then resuspended in their original plasmas. Heat-killed *E. coli* in a con-

centration of 10^8 cells was then added to one of the *E. coli* absorbed and one of the control specimens, and a similar concentration of heat-killed *S. aureus* was added to the other *E. coli* absorbed and the other control specimen. All four suspensions were then reincubated at 37°C for 90 minutes. Platelet morphology and plasma 5HT concentrations were determined immediately after the final addition of *E. coli* and again after 90 minutes' incubation.

Prior absorption of plasma with *E. coli* greatly diminished the response of resuspended platelets to subsequent chalenge with homologous or heterologous bacterial cells (Table V).

Release of Platelet Phospholipid during Incubation of PRP with Endotoxin.—

TABLE V

The Effect of Absorption of Plasma with Heat-Killed E. coli on Response of Resuspended Platelets to Homologous or Heterologous Bacterial Cells

Challenge*	Time	Platelets in plasma absorbed/with *E. coli*‡		Platelets in plasma treated with saline§	
		Platelet aggregation‖	Plasma 5HT	Platelet aggregation‖	Plasma 5HT
	min.		*NG ml*		*NG/ml*
E. coli	0	++	700	+	225
E. coli	90	+	2,250	++++	10,500
Staphylococci	0	+	825	+	275
Staphylococci	90	No platelets seen	3,350	No platelets seen	13,500

* 10^8 heat-killed bacterial cells per ml.

‡ Plasma was absorbed with 10^8 heat-killed *E. coli* per ml, 37°C for 90 minutes, centrifuged; platelets then resuspended in absorbed plasma.

§ Plasma was mixed with 0.05 ml of saline solution per ml of plasma, incubated at 37°C for 90 minutes, centrifuged; platelets then resuspended in plasma.

‖ 0 to ++++ indicates degree of platelet aggregation.

The intrinsic or blood coagulation process is classically represented in three stages, thromboplastin generation, conversion of prothrombin to thrombin, and conversion of fibrinogen to fibrin. Each successive stage is catalyzed in part by the product of the preceding one. When fully formed blood thromboplastin (21) is supplied and prothrombin and fibrinogen are present in normal amounts, the second and third stages proceed to completion in about 10 seconds or less. Any time beyond this period necessary for normal blood or plasma to coagulate is a measure of the time required for the first stage to proceed to completion under the experimental circumstances. The first stage is thought to involve two parallel processes which interact at their completion. On the one hand an interaction of several first stage factors forms a powerful coagulation activity termed product I (21), and on the other hand intact platelets make phospholipid available to the clotting process (22). Blood thromboplastin

results from the interaction of product I and available platelet phospholipid in the presence of factor V and calcium (21).

McKay and associates (23) and Robbins and Stetson (17) have demonstrated that bacterial endotoxin shortens the coagulation time of recalcified citrated plasma or whole blood in siliconized tubes. This coagulation acceleration occurred only when platelets were present, and was necessarily a function of a shortened first stage. The acceleration of the first stage of coagulation induced by endotoxin could be mediated through an acceleration of product I formation, or through an effect on platelets to make phospholipid available. If the coagulation-accelerating effect of endotoxin should obtain when preformed product I

TABLE VI

The Effect of E. coli Endotoxin on Product I Substrate Time (Available Phospholipid) of PRP

Time of incubation	Product I substrate time			
	PRP + endotoxin 100 μg/ml	PRP + endotoxin 10 μg/ml	PRP + saline	Plasma + cephalin + endotoxin 100 μg/ml
min.	*sec.*	*sec.*	*sec.*	*sec.*
0	30.0	32.0	31.3	10.7
15	22.8	28.3	30.1	
30	19.8	26.3	27.6	
45	13.0	19.2	25.5	
60	10.0	17.4	25.0	
180	13.9	10.2	23.0	10.4

is supplied in excess, this would indicate that the acceleration is consequent to an effect on platelets which increases the availability of phospholipid for the clotting process.

PRP was incubated at 37°C with endotoxin in concentrations of 100 and 10 μg per ml. Aliquots were removed at intervals and assayed for product I substrate activity (available phospholipid) by determining the clotting time after addition of product I and calcium. Product I substrate times were also determined on specimens of platelet-poor plasma containing brain cephalin as a phospholipid substitute during the course of incubation with endotoxin.

Addition of preformed product I and calcium to plasma in which added cephalin provided freely available phospholipid resulted in clotting in approximately 10 seconds, and incubation of the mixture of plasma and cephalin with endotoxin did not further accelerate coagulation after addition of product I and calcium (Table VI). However, when product I and calcium were added to plasma in which intact platelets served as the only source of phospholipid, clotting was delayed approximately 20 seconds, this prolongation representing the time necessary for platelets to release phospholipid. The product I substrate time of PRP incubated with 10 or 100 μg per ml of *E. coli* endotoxin

progressively decreased to an extent proportional to the duration of incubation, and by 60 minutes sufficient phospholipid had become available so that its concentration was no longer the rate-limiting factor. Participation of surface-activated factors seemed excluded by the finding that maximal surface activation (agitation of PRP with 25 per cent by volume Celite powder, Johns Manville) did not appreciably alter the product I substrate time.

Release of Platelet Bactericidins during Incubation of PRP with Endotoxin.— Hirsch (15) and others (24) have presented evidence for a bactericidal system contained in platelets which is active against certain Gram-positive organisms,

TABLE VII

Morphologic Change and Release of B. subtilis Bactericidins and 5HT During Incubation of PRP with E. coli Endotoxin

Material added	Time of incubation	Platelet aggregation*	Titer of bactericidal activity in plasma‡	Plasma 5HT
	min.			*NG/ml*
Saline	0	0	8	10
	90	0	8	10
	180	0	8	57
Endotoxin, 100 μg/ml	0	0	8	10
	90	+	128	7,500
	180	++	128	11,500

* 0 to ++++ indicates degree of platelet aggregation.

‡ Reciprocal of highest twofold dilution of plasma producing more than 50 per cent kill of *B. subtilis* in a concentration of about 2000 viable units per ml.

notably *B. subtilis*. Incubation of endotoxin with rabbit PRP is associated with a progressive transfer of bactericidal activity from platelets to plasma.

An aliquot of PRP was incubated with endotoxin, 100 μg per ml at 37°C. A control aliquot was incubated with saline solution. Samples of PRP were removed at zero, 90, and 180 minutes after incubation and centrifuged to remove platelets. The titer of bactericidal activity against *B. subtilis* in the plasma supernate was determined by a serial dilution technique described under Methods.

No change in titer of plasma bactericidal activity was observed in the saline-treated aliquots (Table VII). However, in the aliquot of PRP incubated with endotoxin the bactericidal activity in the plasma supernate increased more than 16-fold and 5HT appeared in the plasma in increasing amounts. A decrease in bactericidal activity in the platelet residue occurred concomitantly with the increase in bactericidal activity of plasma. Additional experiments have shown that the endotoxin-induced release of *B. subtilis* bactericidins is also influenced by dose of endotoxin, temperature of incubation, and anti-

coagulant, and is dependent on the presence of heat-labile plasma factors in a manner quite similar to that previously described for the release of 5HT.

DISCUSSION

A reaction between endotoxins of Gram-negative bacteria and platelets leading to platelet aggregation and release of platelet factors, as demonstrated by the *in vitro* studies described in this report, may be of importance in the pathogenesis of certain biologic events observed in the intact host after injection of endotoxin.

Platelet aggregates have been detected in the circulating blood of rabbits inoculated intravenously with endotoxin (3), and it is reasonable to postulate that these aggregates as well as altered individual platelets are trapped in small blood vessels in vascular beds. The characteristic granulocytopenia observed after injection of endotoxin has been attributed to sequestration of white cells within pulmonary blood vessels and spleen (1, 25), and it is likely that the mechanism of thrombocytopenia has many similarities. Platelet and leukocyte thrombi in small blood vessels have been observed after single injections of endotoxin, and are among the most prominent pathological findings in the local and generalized Shwartzman phenomena, occurring prior to overt vascular rupture (25).

The transfer of 5HT from platelets to plasma during incubation of platelet-rich plasma with endotoxin *in vitro* closely parallels the extent of platelet aggregation. As mentioned previously, Davis, Meeker, and McQuarrie (3) have presented evidence that platelet aggregates appear in circulating blood of dogs after injection of endotoxin. Other studies (4–6) have clearly shown that increases in plasma 5HT concentrations also occur following endotoxin injections in dogs and rabbits. Although the increase in plasma 5HT concentration is small and transient, the concomitant fall in whole blood 5HT content is much more extensive, at times exceeding 80 per cent of control whole blood 5HT values (6). This decrease in whole blood 5HT is probably due both to transfer of 5HT from platelets to plasma with subsequent rapid enzymatic degradation, and to removal of platelet-bound 5HT as the sequestration of platelets develops.

Thomas and associates (26), as part of an extended investigation of the mechanisms underlying the Shwartzman phenomena, have demonstrated that both 5HT and endotoxin greatly enhance the necrotizing and vasomotor effects of epinephrine and norepinephrine on small blood vessels. These observations led to the hypothesis that part of the effect of endotoxin may be to make 5HT available in or around the walls of blood vessels for an action in concert with epinephrine. Although speculative, this idea blends well with the evidence that endotoxin-platelet interactions *in vitro* and *in vivo* result in release of 5HT, and also with the protective action of antiserotonin compounds against the lethal effects of endotoxin (27).

A recent review by Gilbert (28) has compiled a large body of experimental evidence indicating that profound vasomotor changes and injury to the microcirculation occur after injection of bacterial endotoxin. These changes are rapid in onset, and include vasoconstriction followed by vasodilatation, profound systemic hypotension, and in-

crease in portal and pulmonary vascular resistance and organ weight in certain species. Hinshaw and associates (29), in studying the effects of endotoxin on the isolated perfused dog lung, noted that the increase in organ weight and vascular resistance which occurred when endotoxin was perfused in whole blood or cell-rich plasma did not obtain when the endotoxin-containing perfusate was cell-poor plasma, dextran, or gelatin. Since 5HT itself causes entirely similar changes in pulmonary vascular resistance and lung weight (30–32), it seems possible that the substance in blood or cell-rich plasma necessary for this activity of endotoxin may be platelet 5HT.

The prominence of platelet-leukocyte thrombi in the local and generalized Shwartzman phenomena and their occurrence after single injections of endotoxin (25) have led several investigators to study endotoxin-induced changes in the coagulation system. Among these, McKay and associates (23, 25) and Robbins and Stetson (17) demonstrated that *in vivo* injections of endotoxin and *in vitro* incubation of whole blood or plasma with endotoxin were followed by shortening of the coagulation time as measured in siliconized tubes. These *in vitro* changes occurred only when platelets were present, and are compatible with the concept that the endotoxin effect was mediated by release of platelet phospholipid, as demonstrated in the present studies. In this regard, Spaet (33) has demonstrated that intravenous injection of crude phospholipid in rabbits causes a transient but marked hypercoagulable state. This systemic change might be of great local importance if vascular injury, vasospasm, and platelet aggregates are also present to favor the development of intravascular thrombosis.

The rather constant lag period between addition of endotoxin and release of 5HT from platelet-rich plasma *in vitro* is discordant with the immediate changes in plasma and whole blood 5HT following endotoxin injections *in vivo*. This disparity may be more apparent than real, since all the anticoagulants used in *in vitro* studies inhibit platelet changes to one degree or another. Moreover, in the *in vivo* systems it is likely that initial platelet changes result in the appearance of thrombin which itself is an extremely active promoter of platelet viscous metamorphosis and might lead to an autocatalytically accelerated reaction.

The findings presented herein are compatible with the concept that the effect of endotoxin on platelets involves immune mechanisms. Antigen-antibody reactions have been demonstrated to produce platelet aggregation, release of histamine, and release of 5HT *in vitro* (18, 19). Two groups (20, 19, 34) have reported that injections of antigen in sensitized animals are followed by changes in whole blood and plasma 5HT content entirely similar in magnitude and time of onset to those observed after injection of endotoxin. Two recent studies of the action of antigen-antibody reactions on platelets have been reported, one (35) measuring platelet aggregation and the other (36, 37) release of platelet histamine. Both of these studies presented evidence that plasma factors were necessary, but differed as to whether these factors were components of complement or represented an analogous but separate lytic system. The present studies do not allow a decision as to whether the plasma

factor involved is complement. Heating plasma at 56°C for 30 minutes resulted in loss of this plasma activity and addition of guinea pig complement did not restore it. However, fibrinogen is precipitated by heating at 56°C for 30 minutes, and Jackson has presented evidence that fibrinogen is necessary for thrombin-induced platelet aggregation (38).

SUMMARY

Incubation of platelet-rich rabbit plasma with *E. coli* endotoxin at 37°C results in platelet aggregation and transfer of platelet 5-hydroxytryptamine to plasma. Release of 5HT is influenced by dose of endotoxin, type of anticoagulant, and temperature of incubation. A heat-labile plasma factor is necessary for the platelet-endotoxin interaction. Additional studies have shown that incubation of endotoxin with platelet-rich rabbit plasma also results in release of platelet phospholipid and bactericidins active against *B. subtilis*.

BIBLIOGRAPHY

1. Stetson, C. A., Studies on the mechanism of the Shwartzman phenomenon. Certain factors involved in the production of the local hemorrhagic necrosis, *J. Exp. Med.*, 1951, **93,** 489.
2. Shimamoto, T., Yamazaki, H., Ohno, K., Uchida, H., Konishi, T., and Iwahara, S., Effect of bacterial endotoxins on platelets and release of serotonin (5-hydroxytryptamine). I. Bacterial endotoxin on the number of circulating platelets, *Proc. Japan Acad.*, 1958, **34,** 444.
3. Davis, R. B., Meeker, W. R., and McQuarrie, D. G., Immediate effects of intravenous endotoxin on serotonin concentrations and blood platelets, *Circulation Research*, 1960, **8,** 234.
4. Shimamoto, T., Yamazaki, H., Sagawa, N., Iwahara, S., Konishi, T., and Maezawa, H., Effect of bacterial endotoxins on platelets and release of serotonin (5-hydroxytryptamine). II. Appearance of platelet-agglutinating substance and serotonin (5-hydroxytryptamine) in the plasma of rabbit by administration of bacterial endotoxin, *Proc. Japan Acad.*, 1958, **34,** 450.
5. Davis, R. B., Meeker, W. R., and Bailey, W. L., Serotonin release after injection of *E. coli* endotoxin in the rabbit, *Fed. Proc.*, 1961, **20,** pt. 1, 261.
6. Des Prez, R. M., Horowitz, H. I., and Hook, E. W., unpublished observations.
7. Erspamer, V., Pharmacology of indole alkylamines, *Pharmacol. Rev.*, 1954, **6,** 425.
8. Woolley, D. W., and Shaw, E., Differentiation between receptors for serotonin and tryptamine by means of the exquisite specificity of antimetabolites, *J. Pharmacol. and Exp. Therap.*, 1957, **121,** 13.
9. Lewis, G. P., Formation of plasma kinins by plasmin, *J. Physiol.*, 1958, **140,** 285.
10. Armstrong, D., Jepson, J. B., Keele, C. A., and Steward, J. W., Pain-producing substance in human inflammatory exudates and plasma, *J. Physiol.*, 1957, **135,** 350.
11. Margolis, J., Initiation of blood coagulation by glass and related surfaces. *J. Physiol.*, 1957, **137,** 95.

12. Horowitz, H. I., Des Prez, R. M., and Hook, E. W., Mechanism of endotoxin-induced coagulation acceleration (abstract), *J. Clin. Inv.*, 1961, **40,** 1050.
13. Zucker-Franklin, D., Javid, J., and Spaet, T. H., Characterization of blood coagulation product I, *Fed. Proc.*, 1961, **20,** pt. 1, 53.
14. Bell, W. N., and Alton, N. G., A brain extract as a substitute for platelet suspensions in the thromboplastin generation test, *Nature*, 1954, **174,** 880.
15. Hirsch, J. G., Comparative bactericidal activities of blood serum and plasma serum, *J. Exp. Med.*, 1960, **112,** 15.
16. Zucker, M. B., and Borelli, J., Platelet viscous metamorphosis produced by connective tissue particles, *Fed. Proc.*, 1961, **20,** pt. 1, 61.
17. Robbins, J., and Stetson, C. A., Jr., An effect of antigen-antibody interaction on blood coagulation, *J. Exp. Med.*, 1959, **109,** 1.
18. Humphrey, J. H., and Jaques, R., The release of histamine and 5-hydroxytryptamine (serotonin) from platelets by antigen-antibody reactions (*in vitro*), *J. Physiol.*, 1955, **128,** 9.
19. Waalkes, T. P., Weissbach, H., Bozicevich, J., and Udenfriend, S., Serotonin and histamine release during anaphylaxis in the rabbit, *J. Clin. Inv.*, 1957, **36,** 1115.
20. Johansson, S.-A., Inhibition of thrombocytopenia and 5-hydroxytryptamine release in anaphylactic shock by heparin, *Acta Physiol. Scand.*, 1960, **50,** 95.
21. Bergsagel, D. E., and Hougie, C., Intermediate stages in the formation of blood thromboplastin, *Brit. J. Haematol.*, 1956, **2,** 113.
22. Horowitz, H. I., and Spaet, T. H., Generation of coagulation product I and its interaction with platelets and phospholipids, *J. Appl. Physiol.*, 1961, **16,** 112.
23. McKay, D. G., Shapiro, S. S., and Shanberge, J. N., Alterations in the blood coagulation system induced by bacterial endotoxins. II. *In vitro*, *J. Exp. Med.*, 1958, **107,** 369.
24. Jago, R., and Jacox, R. F., Cellular source and character of a heat-stable bactericidal property associated with rabbit and rat platelets, *J. Exp. Med.*, 1961, **113,** 701.
25. McKay, D. G., and Shapiro, S. S., Alterations in the blood coagulation system induced by bacterial endotoxin. I. *In vivo* (generalized Shwartzman reaction), *J. Exp. Med.*, 1958, **107,** 353.
26. Thomas, L., Zweifach, B. W., and Benacerraf, B., Mechanisms in the production of tissue damage and shock by endotoxins, *Tr. Assn. Am. Physn.*, 1957, **70,** 54.
27. Des Prez, R. M., Fallon, N., and Hook, E. W., Modification of the lethal effect of bacterial endotoxin by substances altering the metabolism of 5-hydroxytryptamine, *Proc. Soc. Exp. Biol. and Med.*, in press.
28. Gilbert, R. P., Mechanisms of the hemodynamic effects of endotoxin, *Physiol. Rev.*, 1960, **40,** 245.
29. Hinshaw, L. B., Kuida, H., Gilbert, R. P., and Visscher, M. B., Influence of perfusate characteristics on pulmonary vascular response to endotoxin, *Am. J. Physiol.*, 1957, **191,** 293.
30. Borst, H. G., Berglund, E., McGregor, M., and Waithe, P. E., The effects of pharmacologic agents on the pulmonary circulation in the dog. Studies on epinephrine, nor-epinephrine, 5-hydroxytryptamine, acetylcholine, histamine, and aminophylline, *J. Clin. Inv.*, 1957, **36,** pt. 1, 669.

31. McGaff, C. J., and Milnor, W. R., Effects of serotonin on pulmonary blood volume and flow in the dog, *Fed. Proc.*, 1960, **19,** 96.
32. Kabins, S. A., Molina, C., and Katz, L. N., Pulmonary vascular effects of serotonin (5-OH-tryptamine) in dogs: its role in causing pulmonary edema, *Am. J. Physiol.*, 1959, **197,** 955.
33. Spaet, T. H., personal communication of unpublished data.
34. Waalkes, T. P., Weissbach, H., Bozicevich, J., and Udenfriend, S., Further studies on release of serotonin and histamine during anaphylaxis in the rabbit, *Proc. Soc. Exp. Biol. and Med.*, 1957, **95,** 479.
35. Siqueira, M., and Nelson, R. A., Jr., Platelet agglutination by immune complexes and its possible role in hypersensitivity, *Immunology*, 1961, **86,** 516.
36. Barbaro, J. F., The release of histamine from rabbit platelets by means of antigen-antibody precipitates. I. The participation of the immune complex in histamine release, *Immunology*, 1961, **86,** 369.
37. Barbaro, J. F., The release of histamine from rabbit platelets by means of antigen-antibody precipitates. II. The role of plasma in the release of histamine, *Immunology*, 1961, **86,** 377.
38. Jackson, D. P., Schmid, H. J., and Gaintner, J. R., Mechanism of action of thrombin on platelets, *J. Clin. Inv.*, 1961, **40,** 1050.

EXPLANATION OF PLATE 88

FIG. 1. Morphology of platelets after incubation for 180 minutes with saline solution (A) or 100 μg per ml of *E. coli* endotoxin (B). Phase contrast. × 1250.

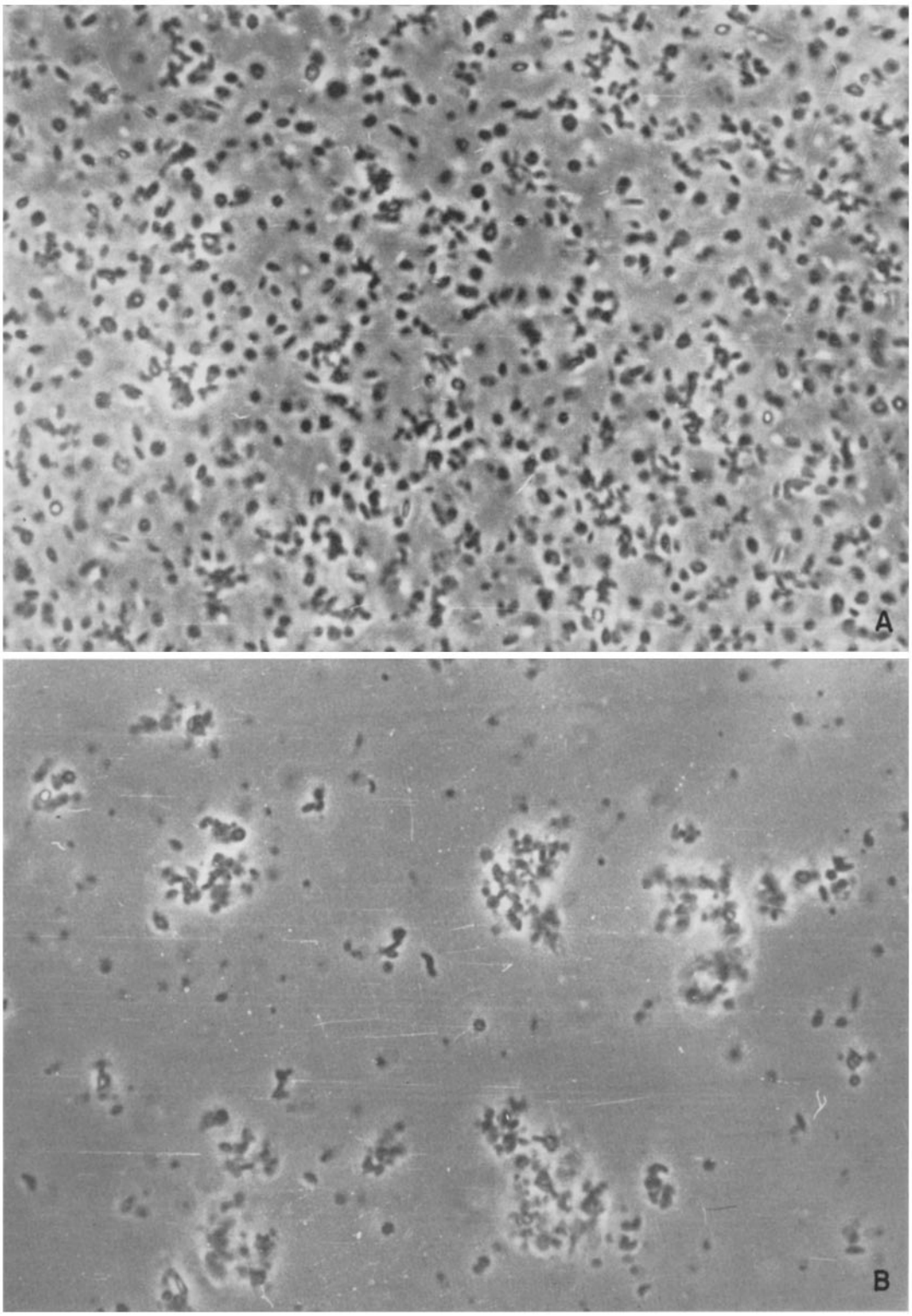


FIG. 1

(Des Prez *et al.*: Bacterial endotoxin and rabbit platelets. I)