

## AN ULTRASTRUCTURAL STUDY OF THE MECHANISMS OF PLATELET-ENDOTOXIN INTERACTION\*

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Many species of animals develop thrombocytopenia following intravenous injection of endotoxin (1-3), and low platelet counts are commonly seen in patients with Gram-negative bacteremia (4, 5). Relevant *in vitro* reactions have been reported. Light microscopic studies have demonstrated platelet aggregation, fusion, and fragmentation when endotoxin-platelet interaction occurs (6, 7). The release of platelet serotonin, histamine, and coagulant activity [platelet factor three (PF3)] has been shown to accompany these morphologic changes, both *in vitro* and *in vivo* (8-10).

The present studies were undertaken to determine the ultrastructural changes accompanying platelet-endotoxin interaction, and they have led to three lines of evidence suggesting that an immune mechanism is involved: (1) The reaction requires a plasma cofactor, presumably antibody, which can be adsorbed out by endotoxin. (2) It is complement-dependent. (3) Endotoxin particles adhere to primate red cells and nonprimate platelets, a pattern consistent with immune adherence (11, 12).

### *Materials and Methods*

*Preparation of Platelet-Rich Plasma.*—Male and female New Zealand rabbits, weighing 3-5 kg, were bled from the central artery of the ear or by direct cardiac puncture. Guinea pigs and Sprague-Dawley rats were bled by cardiac puncture. Blood was obtained from mongrel dogs and baboons<sup>1</sup> by femoral arteriotomy, and from normal human subjects by venipuncture. The blood, anticoagulated with  $\frac{1}{10}$ th volume of 3.8% sodium citrate, was collected and processed in Nalgene tubes. Platelet-rich plasma (PRP) was prepared by centrifugation at 200 g for 25 min at 4°C in an International centrifuge, Model PR-2 (International Equipment Co., Needham Heights, Mass.).

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*Reagents.*—The experiments were performed with commercially prepared Boivin type endotoxins, obtained from Difco Labs., Detroit, Mich. *E. coli* 0127:B8 was used for most experiments. In a few studies *E. coli* 0126:B8, 055B:5, 0128:B15 or *S. enteritidis* were used. Sodium iodoacetate, 2,4-dinitrophenol, potassium cyanide, and sodium fluoride were purchased from Fisher Scientific Co., Pittsburgh, Pa. Zymosan was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and bovine serum albumin (BSA) from Pentex Inc., Kanakakee, Illinois.

The endotoxin was suspended in imidazole-buffered saline (IBS) in a concentration of 1 mg/ml. Buffered saline was prepared by adding  $\frac{1}{10}$ th volume of imidazole buffer, pH 7.2, made according to the method of Langdell et al. (13), to 0.9% sodium chloride.

*Antigen-Antibody Complexes.*<sup>2</sup>—Rabbit antibody to BSA (anti-BSA) was obtained from New Zealand rabbits which had been immunized with three weekly intramuscular injections of 2 mg BSA in Freund's adjuvant. The animals were bled 1 wk after the last injection. Optimal proportions of anti-BSA and BSA were calculated from the precipitin reaction and Kjeldahl nitrogen determination (14). 1.2 mg of anti-BSA was incubated with 0.2 mg of BSA at 37°C for 1 hr. The antigen-antibody precipitate was removed by centrifugation at 39,000 g for 30 min at 4°C in the Sorvall RC-2 centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The precipitate was then washed three times in cold saline and resuspended in 1 ml of ice cold saline.

*Incubation and Fixation.*—The mixtures of PRP and endotoxin were agitated in 50 ml Nalgene tubes in a Dubnoff metabolic incubator. The incubated specimens were fixed in suspension with two parts ice cold 5% glutaraldehyde in phosphate buffer, pH 7.4. After 10 min of fixation, a platelet button was obtained by centrifugation at 1500 g for 20 min at 4°C. The supernatant was discarded, and additional buffered glutaraldehyde was added for 30 min. The specimens were post-fixed in Dalton's chrome-osmium (15) for 1 hr. The fixed pellet was loosened from the bottom of the test tube with a wooden applicator stick, and was processed like a block of tissue, in preparation for electron microscopic examination. After dehydration in alcohol, the blocks were embedded in Epon (16), sectioned on a Porter-Blum MT-2 ultramicrotome (Sorvall) and stained with both uranyl acetate (17) and lead citrate (18). Specimens were examined with either a Siemens 1A (Siemens America, New York, N.Y.) or an RCA EM 3F (Radio Corp. of America, Camden, N.J.) electron microscope.

## RESULTS

### *Effect of Endotoxin on Rabbit Platelets.*—

1 ml of either an homogeneous endotoxin suspension or control IBS was added to 4 ml of rabbit citrated PRP, and the mixtures were incubated for 2 hr at 37°C.

Control rabbit platelets washed and suspended in citrated plasma appeared intact after incubation with buffered saline (Fig. 1 *a*), and were essentially similar to fresh and unwashed cells. Maintenance of the normal discoid platelet shape was suggested by the oval contour found with the more common tangentially cut sections. A rounded configuration was seen only in the occasional platelet which was presumably cut in cross section. The electron-dense cytoplasm, mitochondria, glycogen granules, and microtubules all appeared normal. Two types of platelet granules were seen: a strongly osmiophilic type, and

<sup>2</sup> Antigen-antibody complexes were kindly prepared by Dr. Edward E. Fischel, Bronx-Lebanon Hospital Center, Bronx, N. Y.

a dark granule. The latter was separated from the surrounding membrane and is presumably the site of serotonin concentration (19). All of the organelles were well separated from the plasma membrane.

Platelets exposed to endotoxin underwent marked structural alterations (Fig. 1 *b*). Most of the cells appeared to be round, indicating that platelet swelling and sphering had occurred. The cytoplasm was pale and only a few organelles remained. This electron microscopic picture of degranulation was the characteristic finding obtained when rabbit platelets interacted with endotoxin. Curled, three-layered structures were seen in close association with the altered platelets. Several observations indicated that these structures were endotoxin and not of platelet origin. (1) Electron microscopy of the *E. coli* 0127:B8 endotoxin revealed structures identical to those seen in association with the degranulated platelets (Fig. 2 *a*). (2) A *Salmonella enteritidis* preparation was found to be incompletely disrupted by the Boivin extraction procedure. Examination of this endotoxin revealed cross sections of the relatively intact bacteria with a characteristic three-layered cell wall, structurally similar to the *E. coli* endotoxin (Fig. 2 *b*). (3) When degranulation of rabbit platelets occurred on exposure to the *S. enteritidis* preparation, the bacteria, not the curled structures, were visualized (Fig. 2 *c*). (4) Increasing doses of endotoxin in the incubating mixture resulted in a greater number of electron microscopically visualized particles, but platelet degranulation remained uniform. Thus, the structure under discussion evidently did not derive from degranulated platelets. This morphological association of endotoxin and platelets will be referred to as platelet-endotoxin adherence in the subsequent presentation.

Additional evidence that the endotoxin activity was associated with the three-layered structure was provided by the following experiment.

*E. coli* endotoxin was suspended in IBS at a concentration of 2 mg/ml. 10 ml of this suspension was centrifuged at 65,000 *g* for 9 hr in a Spinco Model L ultracentrifuge. 4 ml of the supernatant was added to 4 ml of rabbit PRP. The sediment was resuspended in 1 ml of IBS and added to 4 ml of PRP. Both mixtures were incubated for 2 hr at 37°C.

Only those platelets exposed to the sediment degranulated and endotoxin particles were clearly present in these specimens. Platelets reacting with the supernatant remained intact, and particulate endotoxin was not seen. This finding is in agreement with previous work indicating that the sedimentable lipopolysaccharide component of enterobacterial cell walls contains the endotoxic properties (20).

#### *Effect of Endotoxin Dosage and Incubation Time.*—

Concentrations of endotoxin ranging from 0.5 to 300  $\mu\text{g}/100$  ml were incubated with rabbit PRP at 37°C for 2 hr. The degree of degranulation was estimated in the following manner: Platelets containing two or less organelles were considered degranulated. Those with more than two granules or mitochondria were counted as intact even though cytoplasmic altera-

tions were present. One thousand platelets were counted at each dosage level and the per cent of those degranulated was calculated.

As noted in Table I, incubation with lower doses of endotoxin resulted in little platelet alteration; at doses of 100  $\mu\text{g}$  or more, virtually all of the platelets degranulated.

Rabbit PRP was incubated with endotoxin (100  $\mu\text{g}/\text{ml}$ ) for 2 hr at 37°C. Aliquots of the incubating mixture were examined at time intervals of 0 to 120 min. The degree of degranulation was estimated as above.

Endotoxin did not cause immediate degranulation of rabbit platelets in citrated plasma (Table II). During the first 15 min period, most of the platelets remained intact. At 1 hr 30% were degranulated; at 2 hr there was uniform

TABLE I  
*Dependence of Platelet Degranulation on Endotoxin Dosage*

Dose of endotoxin ( $\mu\text{g}/100\text{ ml}$ )	Per cent degranulation
IBS	2
0.5	4
1.0	5
5.0	16
10.0	30
50.0	38
100.0	97
300.0	99

degranulation. These morphologic observations paralleled published biochemical studies which indicated that platelet damage produced by endotoxin is both dose- and time-dependent (8, 9).

*Requirement of Complement.*—Previous studies have shown that the platelet-endotoxin induced release of PF3 and serotonin depends upon the reaction temperature, calcium, and a heat-labile plasma factor (8, 9). An incubation temperature of 5°C, anticoagulation with 1% disodium ethylenediaminetetraacetate (EDTA), and heating the suspending plasma to 56°C for 30 min—all have been shown to inhibit endotoxin activity. With structural alterations as a measure of endotoxin-induced platelet damage, experiments were designed to determine whether or not complement was required for these reactions.

Inactivation of the complement system in the suspending plasma was performed by a variety of procedures. The effect of endotoxin on rabbit platelets suspended in this complement depleted plasma was then noted.

- (1) PRP was prepared from rabbit blood collected in  $\frac{1}{10}$ th volume of 1% EDTA.
- (2) A citrated PRP-endotoxin mixture was incubated at 5°C for 2 hr instead of the usual 37°C.

(3) Platelet-poor plasma (PPP) was prepared by centrifugation of citrated rabbit PRP in the Lourdes LCA-1 centrifuge (Lourdes Instrument Corp., Old Bethpage, N. Y.) at 4°C for 30 min at 9000 *g*. The platelet pellet was saved at 25°C and the supernatant PPP collected and processed by one of the following procedures:

(a) *Control*: PPP was incubated at 37°C for 90 min.

(b) *Plasma heating*: The plasma was heated to 56°C for 30 min and the precipitate was removed by centrifugation for 30 min at 9000 *g* in the Lourdes LCA-1 centrifuge.

(c) *Adsorption with Zymosan* (14): Zymosan was added to the PPP in a concentration of 2-4 mg/ml. The mixture was placed in a 37°C incubator and continually agitated for 90 min in an upright circular rotator (Baltimore Biological Labs., Baltimore, Md.). Zymosan-free PPP was prepared in the Lourdes LCA-1 by centrifugation at 9000 *g* for 30 min at 5°C.

(d) *Ammonium hydroxide incubation* (14): 0.25 ml of 0.15 N NH<sub>4</sub>OH was added to each milliliter of PPP. This mixture was agitated for 90 min as described in the above experiment with zymosan, and was then neutralized to pH 7.4 with 0.15 N HCl.

TABLE II  
Dependence of Platelet Degranulation on Incubation Time  
with 100 µg/ml Endotoxin

Incubation time (min)	Per cent degranulation
0	2.0
5	0.3
15	0.6
30	19
45	42
60	74
90	91
120	99

(e) *Adsorption with washed antigen-antibody complexes* (14): 1 ml of washed antigen-antibody precipitate (BSA anti-BSA), containing 1.5 mg of antibody nitrogen, was incubated for 1 hr with 16 ml of rabbit PPP. Following incubation, the antigen-antibody complex was removed by centrifugation at 4°C for 39,000 *g* and 1 hr in the Sorvall RC-2.

The original platelet button was washed and resuspended in PPP which had been processed by one of these five methods. 4 ml of treated plasma was added to the platelet button, which was then gently dislodged from the test tube wall with wooden applicator sticks. A homogeneous platelet suspension was obtained by manual mixing. This PRP was centrifuged at 4°C for 20 min at 9000 *g*. The supernatant plasma was discarded and the platelets were resuspended in another 4 ml aliquot of plasma. Following the third wash, the button was suspended in 8 ml of PPP. 4 ml portions of this treated PRP were then incubated with 1 ml of either IBS or endotoxin (200 µg/ml) for 2 hr at 37°C.

A reaction temperature of 5°C, anticoagulation with 1% EDTA, with removal of divalent cation (21), and heating the suspending plasma to 56°C for 30 min all inhibited platelet degranulation and platelet-endotoxin interaction (Table III and Figs. 3 *a*, *b*, *c*, *d*). Treatment of the suspending plasma with ammonium hydroxide appeared only to partially inhibit degranulation (Fig. 3

f). However, the presence of identical changes in the control as well as in the endotoxin-treated platelets suggests that the apparent degranulation was a direct effect of ammonium ion (Fig. 3 e). Adsorption with zymosan (Fig. 3 g) and washed antigen-antibody complexes (Fig. 3 h) completely inhibited both degranulation and platelet-endotoxin adherence. Since each of these six procedures inactivates different complement components (14), the common interference with platelet-endotoxin interaction suggests a dependence upon complement.

*Requirement for a Plasma Cofactor.*—Involvement of antibody is suggested by previous studies (8), which have demonstrated the requirement of a plasma

TABLE III  
*Effect of Complement Inhibition on Platelet-Endotoxin Interaction*

Treatment of suspending plasma	Incubation temperature	Complement components inhibited	Effect of incubation with endotoxin	
			Adherence	Degranulation
	(°C)	(R)		
Citrated	37	None	+	+
Citrated	5	C'3	0	0
EDTA	37	C'1, C'2	0	0
Heated to 56°C for 30 min	37	C'1, C'2	0	0
Zymosan adsorbed	37	C'3	0	0
NH <sub>4</sub> OH incubated	37	C'4	0	0
Adsorbed with washed antigen-antibody complexes (BSA-anti-BSA)	37	C'1, C'4, C'2	0	0

cofactor, specific for endotoxin. This observation has been verified by present techniques.

Endotoxin (300 µg/ml) was incubated with PPP for 1 hr. The particles were then removed by centrifugation at 18,000 g for 9 hr in a Spinco Model L ultracentrifuge. Intact rabbit platelets, washed once and resuspended in this adsorbed plasma, were then incubated with fresh endotoxin for 2 hr at 37°C. Controls consisted of platelets suspended in plasma which had been incubated with IBS instead of endotoxin for 1 hr, and had been centrifuged in a similar manner.

Platelets suspended in endotoxin-adsorbed plasma showed significant loss of reactivity to endotoxin. This requirement of an endotoxin-specific plasma component may well be due to participation of antibody, since "naturally occurring antibodies" to lipopolysaccharides are present in a wide variety of species (22, 23).

*Immune Adherence and Species Differences.*—The data presented thus far have shown the effect of endotoxin on rabbit platelets, in which degranulation

invariably accompanies platelet-endotoxin adherence. The effect of endotoxin on platelets from other species was tested.

Citrated PRP from the rat, guinea pig, dog, baboon, and human was incubated with 200  $\mu\text{g}/\text{ml}$  of endotoxin for 2 hr at 37°C. The platelet pellets were studied as above.

Three distinct patterns of reaction were found (Figs. 4 *a, b, c, d*). The rat was like the rabbit in that its platelets underwent both adherence and degranulation. Platelets from dog and guinea pig showed striking adherence with endotoxin, which appeared to line up along the platelet plasma membrane. In spite of this intimate contact, degranulation was not seen. Platelets from primates, on the other hand, showed neither adherence nor degranulation.

A 0.5% red cell suspension of human or baboon red cells in their respective PRP was incubated with 300  $\mu\text{g}/\text{ml}$  of *E. coli* endotoxin for 2 hr at 37°C.

Figure 5 *a* shows adherence of endotoxin particles to baboon red cells, and the findings were similar with human red cells. This is in striking contrast to the lack of interaction with respective primate platelets in the same specimen. On the other hand, interaction of endotoxin was seen with platelets but not erythrocytes in the nonprimate species tested (Fig. 5 *b*).

Nelson (24) has shown that bacterial antigens, sensitized with antibody and complement, adhere to primate red cells and nonprimate platelets. This phenomenon has been termed immune adherence. The interaction of endotoxin (bacterial cell wall) with primate red cells and nonprimate platelets conforms to the pattern of immune adherence.

*Mechanism for Degranulation of Rabbit Platelets.*—The mechanism for degranulation was investigated by examination of platelets exposed to endotoxin for varying periods of time. As already noted, no significant changes were observed during the initial 15 to 30 min. At 30 min, some platelets contained aggregates of organelles and clear spaces within the cytoplasm (Fig. 6 *a*). Specimens at 60 and 90 min (Fig. 6 *b, c, d*) showed loss of cytoplasmic osmophilia, with organelles in direct apposition to the plasma membrane. This peripheral zone is normally free of these structures. Large numbers of granules appeared empty, suggesting lysosomal membrane lysis, with loss of contents into the cytoplasmic substance. In addition, there were platelets which exhibited lysis of the plasma membrane (Fig. 6 *d, e*), with release of cell contents into the surrounding medium, so that intact granules and mitochondria were seen extracellularly (Fig. 6 *f*). There were also platelets which seemed to have engulfed the endotoxin (Fig. 6 *g, h*). Granular material was present within the vacuole containing the endotoxin (*E. coli* or *S. enteritidis*) and was not seen in the surrounding media. This observation suggested that apparent phagocytosis of endotoxin was not due to a sectioning artifact. It therefore seemed possible that phagocytosis was a prerequisite for degranulation. In contrast to

the granulocyte which was capable of digesting phagocytized material, the nonnucleated platelet may have responded by degeneration with membrane lysis and cell death. Thus, the role of phagocytosis was further investigated.

It has been shown that inhibitors of the glycolytic cycle can interfere with leukocytic phagocytosis (25). Recently Movat et al. (26) were able to inhibit the platelet uptake of latex particles by preincubation of PRP with a combination of aerobic and anaerobic blockers. These same agents therefore should inhibit endotoxin-induced degranulation if this process is a result of phagocytosis. Experiments were therefore performed attempting to inhibit platelet degranulation by preincubation with metabolic inhibitors.

TABLE IV  
*Effect of Metabolic Blocking Agents on Degranulation of Rabbit Platelets Induced by Endotoxin*

Agent added	Type of inhibitor	Incubation times	Degranulation
Buffered saline	None		+
Iodoacetate $10^{-4}$ M	Anaerobic	15 min, 2 hr, 10 hr	+
Dinitrophenol $10^{-4}$ M	Aerobic	15 min, 2 hr, 10 hr	+
Iodoacetate and dinitrophenol $10^{-4}$ M	Combination	15 min, 2 hr, 10 hr	+
Sodium fluoride $10^{-2}$ M	Anaerobic	15 min, 30 min	+
Potassium cyanide $10^{-2}$ M	Aerobic	15 min, 30 min	+
Sodium fluoride and potassium cyanide $10^{-2}$ M	Combination	15 min, 30 min	+

The aerobic and anaerobic inhibitors employed and their concentrations are listed in Table IV. 0.4 ml of agent was incubated with 3.6 ml of rabbit PRP for the indicated times. Following this procedure, *E. coli* endotoxin at a final concentration of 100  $\mu$ g/ml was added, and the mixture was incubated for an additional 2 hr at 37°C.

As noted in Table IV, inhibition of aerobic, or anaerobic cell metabolism, or both in combination, had no effect on platelet degranulation.

*In Vivo Studies.*—Three New Zealand rabbits, weighing 4 to 5 kg were prepared for repeated blood sampling by the method of Spaet et al. (27). 5 mg of *E. coli* endotoxin suspended in 3 ml of sterile saline was injected into the marginal ear vein. Blood samples were taken from the right atrium at time intervals of 1–90 min. These specimens were anticoagulated with  $\frac{1}{10}$  volume of 1% EDTA in Nalgene tubes. PRP was immediately prepared and fixed in suspension.

Following these lethal doses of intravenous *E. coli* endotoxin (28), the platelet counts fell to levels of approximately 2000 to 25,000 in 2 or 3 min. Electron micrographs of the 3 and 5 min specimens revealed large numbers of altered



platelets in clumps (Fig. 7 *a*). Endotoxin particles were seen in close association with the degranulated platelets, and occasionally engulfment was visualized (Fig. 6 *g*). By 90 min, when the platelet count had reached levels of 80,000–150,000, circulating thrombocytes appeared normal (Fig. 7 *b*).

#### DISCUSSION

The present electron microscopic studies have shown that endotoxin produces morphological changes in rabbit platelets resembling those following exposure to collagen (29), thrombin (30, 31), or fibrin (32, 33). In vitro incubation of the platelets with bacterial lipopolysaccharide resulted in degranulation characterized by loss of cytoplasmic structures: There was disappearance of granules, glycogen, and mitochondria and lysis of lysosomal and plasma membranes. The reactive component of endotoxin appeared to be particulate, and was seen as characteristic three-layer curled structures. Davis (34) also reported these structures in studies with 10 mg/ml of endotoxin in heparinized rabbit PRP, but he concluded that they were of platelet origin. Evidence for their bacterial rather than platelet origin is (1) that they could be identified in endotoxin preparations in the absence of platelets; (2) that their concentration in platelet preparations was proportional to the amount of endotoxin added and uncorrelated with the degree of degranulation; and (3) that no such structures appeared when platelets were degranulated by partially intact bacteria. An additional difference between our findings and those of Davis is that he found considerably less degranulation, probably because of the low dose of endotoxin used.

Experiments on rabbits made thrombocytopenic by lethal doses of endotoxin suggest an *in vivo* significance of these *in vitro* observations. Electron microscopic examination of platelets remaining in the circulation 2 and 5 min after parenterally administered endotoxin revealed that both degranulation and platelet–endotoxin interaction were present. The marked platelet damage noted is in agreement with the radioisotope studies of Cohen et al. (3), which showed that platelet destruction occurred in experimental endotoxemia. Morphologic association of endotoxin particles and circulating platelets correlates with the observations that <sup>51</sup>Cr-labeled endotoxin is found circulating in the platelet fraction of the buffy coat (28, 35).

Several observations in the present studies suggest that the effects of endotoxin on platelets were mediated through immune mechanisms. When rabbit platelets were suspended in plasma which had been depleted of various complement components, endotoxin adherence and degranulation were absent.

Removal of divalent cation by anticoagulation with EDTA (21), reaction temperature at 5°C, and heating the suspending plasma to 56°C for 30 min all abolished endotoxin–platelet interaction. Pretreatment with ammonium hydroxide, inactivating C'4 (14), at least partially inhibited platelet degranula-

tion. Zymosan adsorption, removing C'3 (14) more dramatically inhibited endotoxin effects. Finally, adsorbing with washed antigen-antibody complexes, a maneuver which "fixes" complement (14), also interfered with endotoxin-platelet interaction and degranulation.

These findings extend the observations of Spink and Vick (36), who reported that a heat-labile factor was important for *in vivo* endotoxin effect, and of Gilbert and Maude (37), who found low serum complement levels in rabbits given intravenous endotoxin. Gocke and Osler (38) have recently shown that antigen-antibody complexes release serotonin from rabbit platelets by a complement-dependent mechanism quite analogous to the requirement we have shown to be necessary for endotoxin-induced platelet damage.

On the other hand, Ream et al. (39), employing saline-washed human platelets, suspended in a variety of deficient plasmas, concluded that coagulation factor V was the heat-labile plasma factor involved in endotoxin-induced platelet aggregation. Some residual uncertainty remains concerning the heat-labile component. Other workers have found that endotoxin action as measured by serotonin release was not restored by addition of fresh guinea pig complement to platelets suspended in heat-inactivated plasma (8). However, heating to 56°C may have destroyed naturally occurring heat-labile antibody to endotoxin (23), so that replacement of complement alone did not supply all of the requisite factors. Additionally, the amount of complement added may have been inactivated by heat-aggregated  $\gamma$ -globulin (40).

Des Prez et al. (8) have shown that endotoxin damages platelets through a mechanism dependent upon calcium ion, the reaction temperature, and a heat-labile plasma factor. More recent studies have demonstrated that the release of serotonin from rabbit platelets by endotoxin required significantly smaller quantities of  $\text{Ca}^{++}$  than did the release produced by soluble antigen-antibody complexes (21). Des Prez therefore suggested that the different  $\text{Ca}^{++}$  requisites reflected separate modes of action, with endotoxin not dependent upon complement. It would appear from the present studies that no explanation other than complement activity is required to account for the  $\text{Ca}^{++}$  requirement.

An immune mechanism is suggested also by the endotoxin-specific plasma cofactor demonstrated by Des Prez et al. (8) and confirmed by us. Naturally occurring antibodies to Gram-negative organisms have been identified in several animal species and man (22, 23). The endotoxin-specific plasma component may be the naturally occurring antibody to lipopolysaccharides. However, since bacterial lipopolysaccharides react with serum to remove the C'3 component (41), the inhibition noted with endotoxin adsorption may be primarily a result of complement depletion rather than antibody adsorption.

The role of complement and antibody is further suggested by studies on the interaction of endotoxin with platelets from different species. The endotoxin particles adhered selectively to nonprimate platelets and to primate red cells.

This pattern of reaction, termed immune adherence by Nelson (42), has been shown to be dependent upon antigen, antibody, and complement. The adherence of relatively large antigens such as bacteria, spirochetes, zymosan granules, and tissue cells to human red cells is readily visible by light microscopy. Immune adherence involving smaller antigens has been detected by indirect methods such as hemagglutination, use of labeled antigens, clearance of small microorganisms from suspension, and macroscopic platelet agglutination. Electron microscopy has provided an additional method for studying immune adherence involving small antigens. With this technique, the cell wall fragments of endotoxin were noted to surround the interacting cells. Mediation of endotoxin-platelet interaction by immune adherence clarifies a number of previous observations on primate platelets. Aggregation of human platelets by endotoxin is highly variable (38) as is PF3 activation (43). Thrombocytopenia occurs in only a fraction of human patients with Gram-negative septicemia (4) and does not occur in Rhesus monkeys given large doses of endotoxin (44).

Although all tested nonprimate platelets showed *in vitro* endotoxin adherence, a striking variability in resulting degranulation was noted. All rabbit platelets degranulate, whereas only 30% of rat platelets were altered following similar exposure. In contrast, guinea pig and dog platelets remained intact, in spite of striking adherence. It is not clear as yet whether these findings stem from plasma or platelet differences.

The nature of the endotoxin interaction leading to rabbit platelet degranulation was investigated by both morphologic and metabolic methods. One possibility is that degranulation could have followed phagocytosis, a process known to be facilitated by immune adherence (42). Indeed, the presence of some platelets showing apparent engulfment of endotoxin would fit with such a view. This type of reaction has been identified in the case of leukocytic phagocytosis in which degranulation occurs by release of lysosomal material into phagocytic vacuoles by membrane fusion (45). Cell integrity is thus maintained as digestive enzymes remain in the phagocytic vacuole, which may eventually be extruded. This phagocytic response is accompanied by increase cell metabolic activity, and measures which prevent this also inhibit phagocytosis (46).

The nonviable-appearing platelets seen in these studies suggested that a mechanism other than phagocytosis was operative. Zucker-Franklin (47) has shown that leukocytes exposed to streptolysin O degranulate as a result of lysosomal and cell membrane lysis without ingestion of particulate material. Our findings of empty intracellular organelles and plasma membrane breaks associated with extracellular granules and mitochondria, are similar to those seen in leukocytes acted upon by this bacterial lysin. The empty, spheroid, apparently nonviable platelets resulting from exposure to endotoxin are the anticipated result of membrane lysis. The failure of metabolic inhibitors to interfere with endotoxin action provides further support for this interpretation.

It is concluded that the close association of particulate endotoxin to platelets, provided by immune adherence, is the prerequisite for degranulation. These studies have not determined whether platelet lysis is directly a result of complement or endotoxin action.

#### SUMMARY

Electron microscopy has confirmed previous studies and has provided much new information on the mechanism of endotoxin-platelet interaction. The Boivin lipopolysaccharide preparation is particulate, and on electron microscope examination appears as a three-layered structure, morphologically similar to bacterial cell wall.

In vitro and in vivo experiments have demonstrated that these endotoxin particles adhere to platelets. In some species, particularly the rabbit, this is associated with loss of platelet contents, due to lysosomal and cell membrane lysis, resulting in platelet sphering and apparent cell death. Serial observation of degranulating platelets and metabolic studies indicate that although some platelet engulfment of endotoxin occurs, degranulation is not dependent upon phagocytosis.

Several observations suggest that these endotoxin effects are mediated through immune mechanisms: (1) Inactivation of complement in the suspending plasma by heating to 56°C, anticoagulation with EDTA, a reaction temperature of 5°C, ammonium hydroxide incubation, and adsorption with either zymosan or washed antigen-antibody complexes, inhibits both endotoxin adherence and platelet degranulation. (2) The reaction requires a plasma cofactor, possibly antibody, which can be adsorbed out by endotoxin. (3) Endotoxin adheres selectively to nonprimate platelets and primate red cells, a pattern conforming to immune adherence, a phenomenon requiring antigen, antibody, and complement.

It is suggested that endotoxin-induced platelet damage is dependent upon the intimate contact provided by immune adherence. We have not established whether degranulation is an endotoxin or complement effect. The species variation in susceptibility to endotoxin also merits further investigation.

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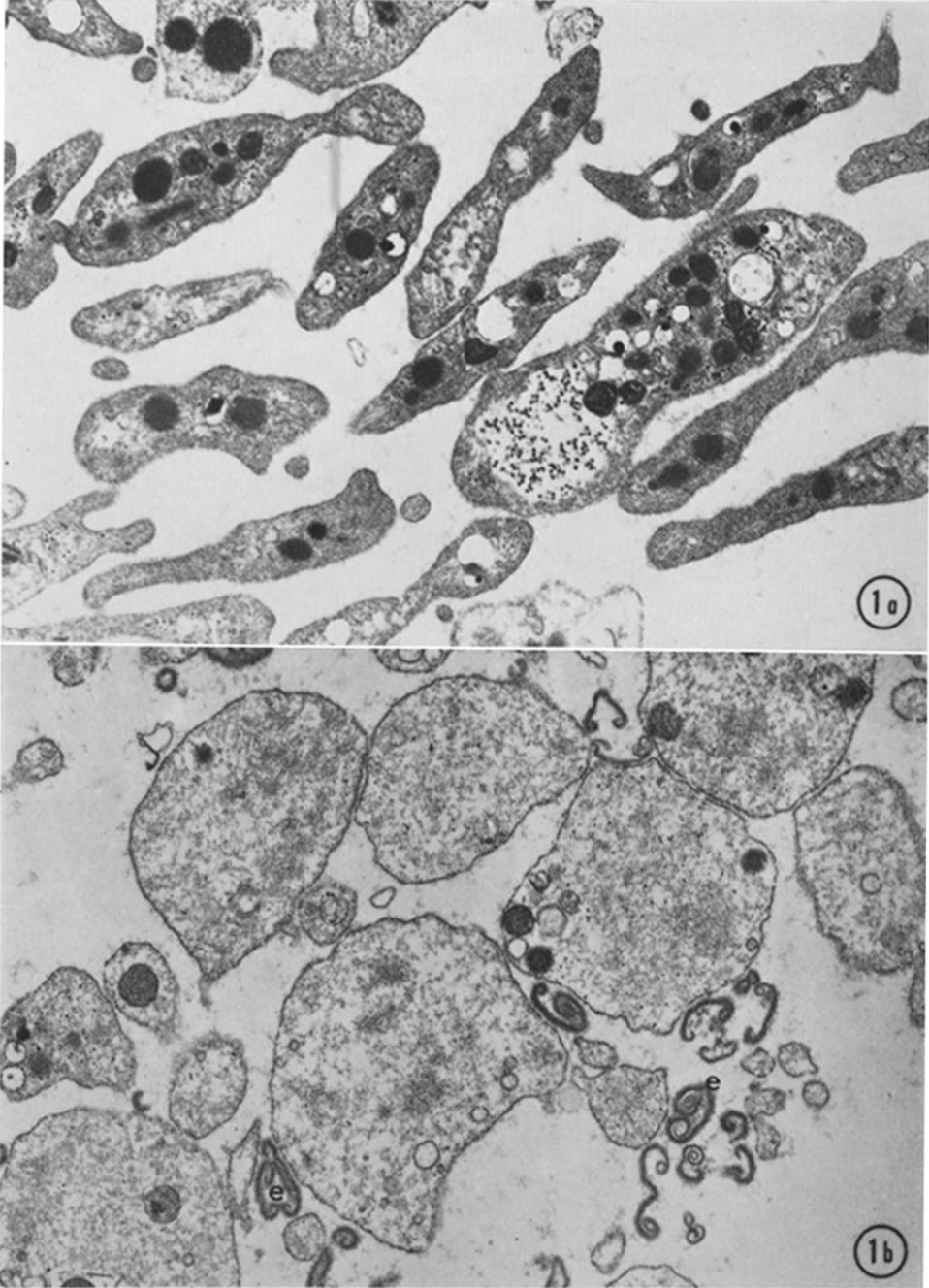
## EXPLANATION OF PLATES

## PLATE 10

FIG. 1 *a*. Control rabbit platelets.  $\times 22,000$ .

FIG. 1 *b*. Degranulated rabbit platelets associated with endotoxin. *E. coli* 0127:B8 endotoxin (e).  $\times 22,000$ .





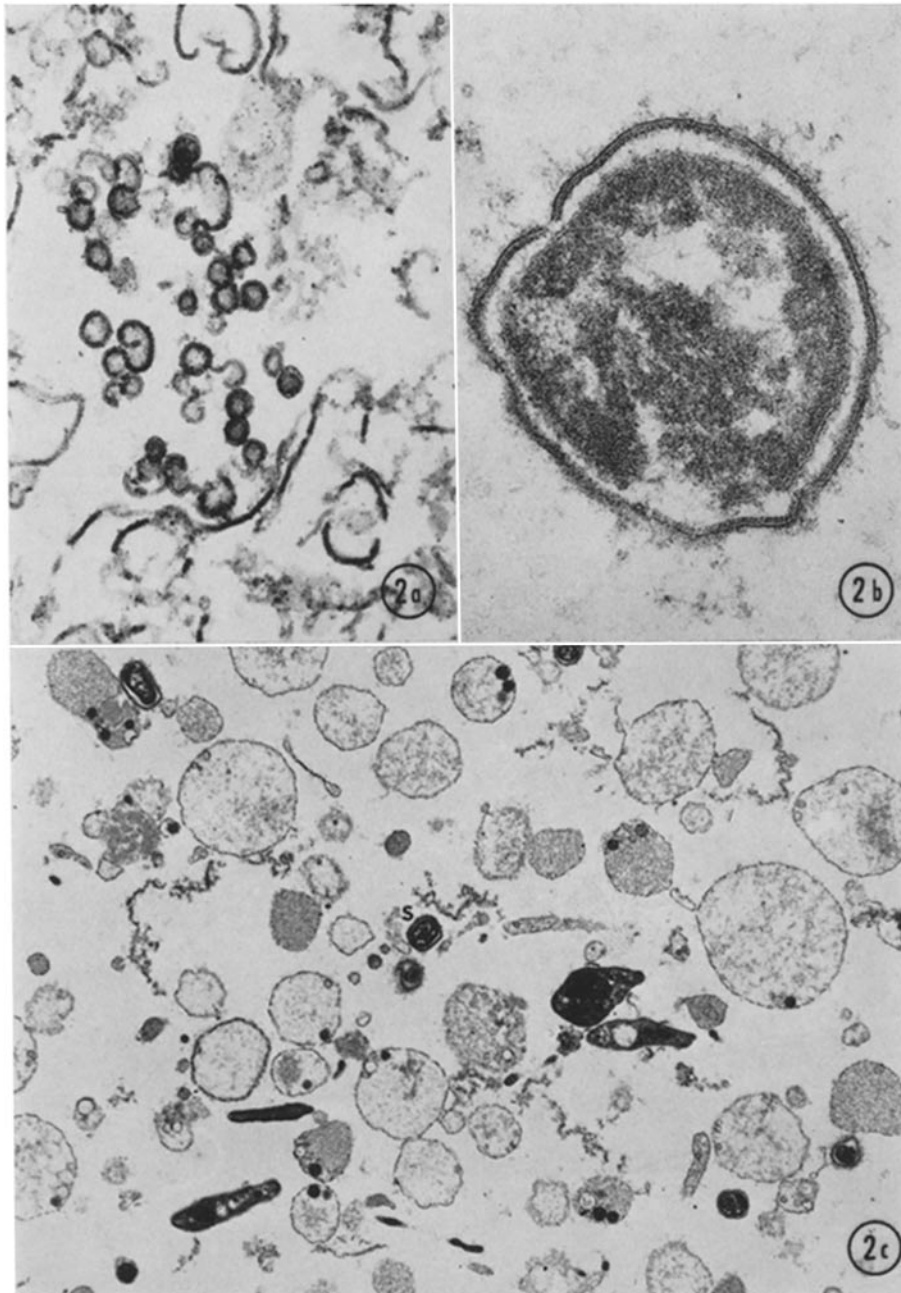
(Spielvogel: Mechanisms of platelet-endotoxin interaction)

PLATE 11

FIG. 2 *a*. Bovine *E. coli* endotoxin 0127:B8.  $\times 40,000$ .

FIG. 2 *b*. Bovine *Salmonella enteritidis* endotoxin.  $\times 92,000$ .

FIG. 2 *c*. Rabbit platelets with *S. enteritidis* endotoxin. (S).  $\times 7500$ .



(Spielvogel: Mechanisms of platelet-endotoxin interaction)

PLATE 12

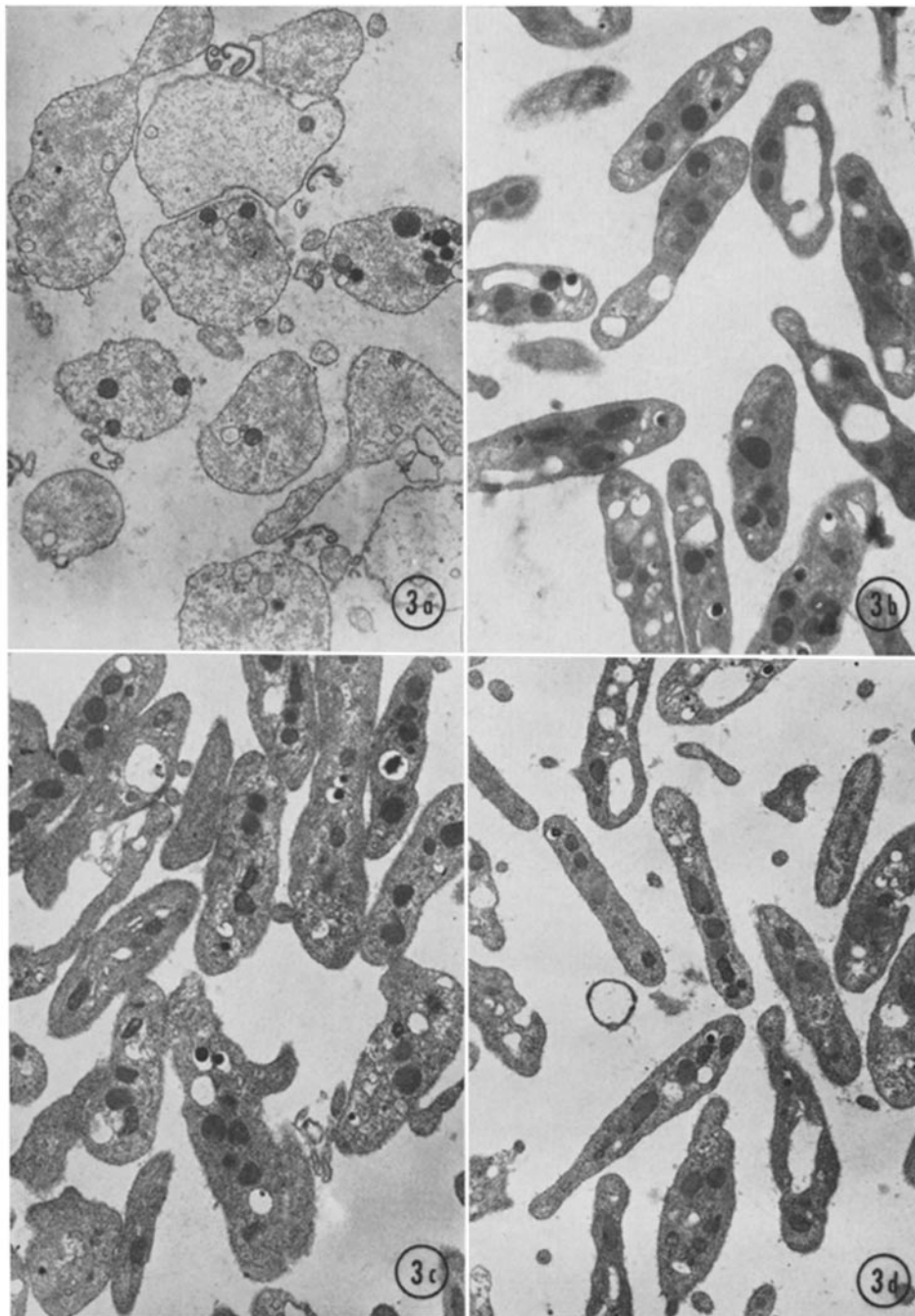
FIG. 3. Effect of complement inactivation on rabbit platelet-endotoxin interaction.

FIG. 3 *a*. Platelets in citrated plasma with endotoxin.  $\times 15,000$ .

FIG. 3 *b*. Platelets incubated with endotoxin at 5°C.  $\times 15,000$ .

FIG. 3 *c*. Platelets in versenated plasma (EDTA) with endotoxin.  $\times 15,000$ .

FIG. 3 *d*. Platelets washed and suspended in heated plasma with endotoxin.  $\times 13,000$ .



(Spielvogel: Mechanisms of platelet-endotoxin interaction)

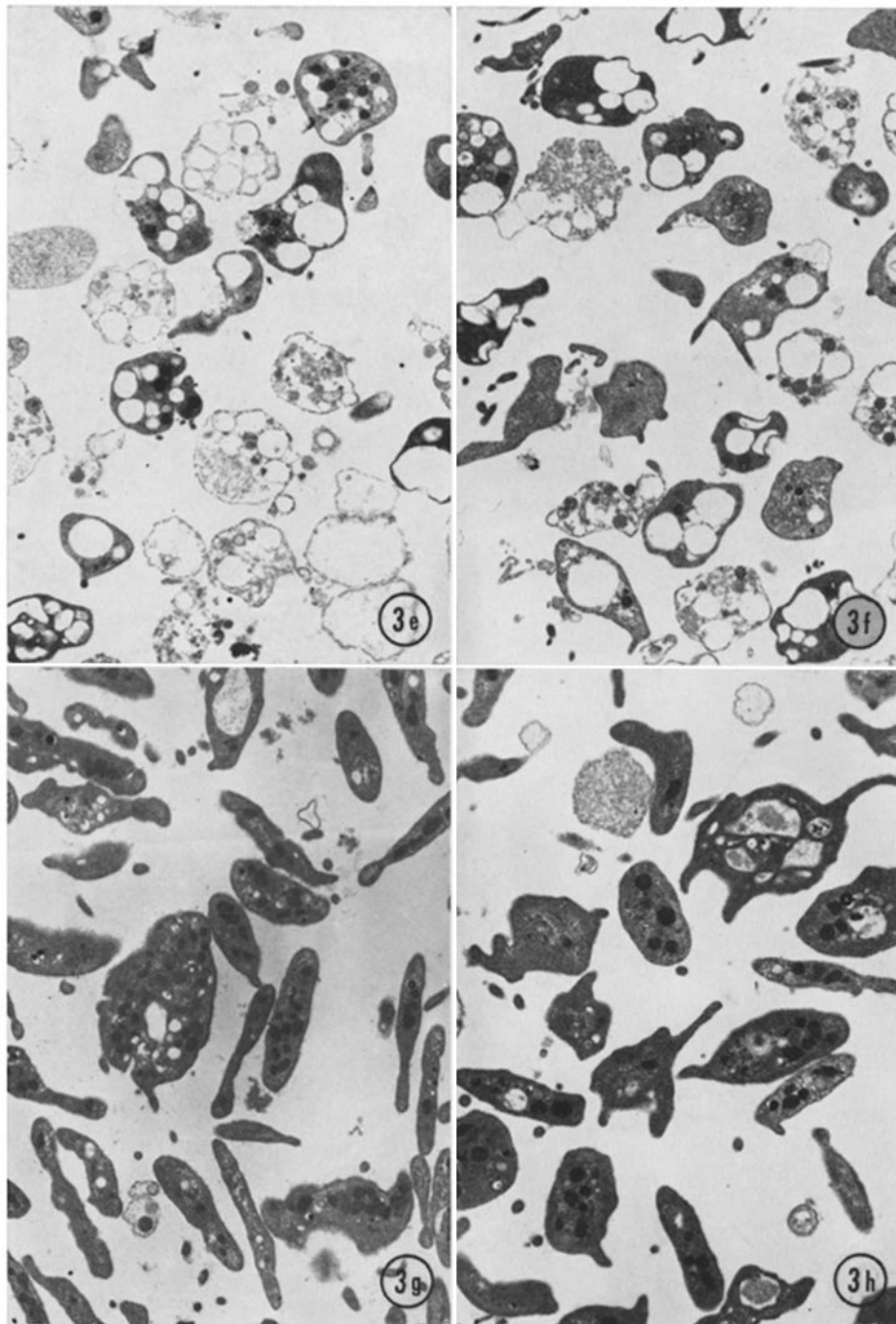
PLATE 13

FIG. 3 *e*. Platelets washed and suspended in ammonium hydroxide treated plasma with endotoxin.  $\times 8,000$ .

FIG. 3 *f*. Platelets washed and suspended in ammonium hydroxide treated plasma without endotoxin.  $\times 8000$ .

FIG. 3 *g*. Platelets washed and suspended in zymosan adsorbed plasma with endotoxin.  $\times 9000$ .

FIG. 3 *h*. Platelets washed and suspended in antigen-antibody adsorbed plasma with endotoxin.  $\times 9000$ .



(Spielvogel: Mechanisms of platelet-endotoxin interaction)

PLATE 14

FIG. 4. Effect of species variation on platelet-endotoxin interaction.

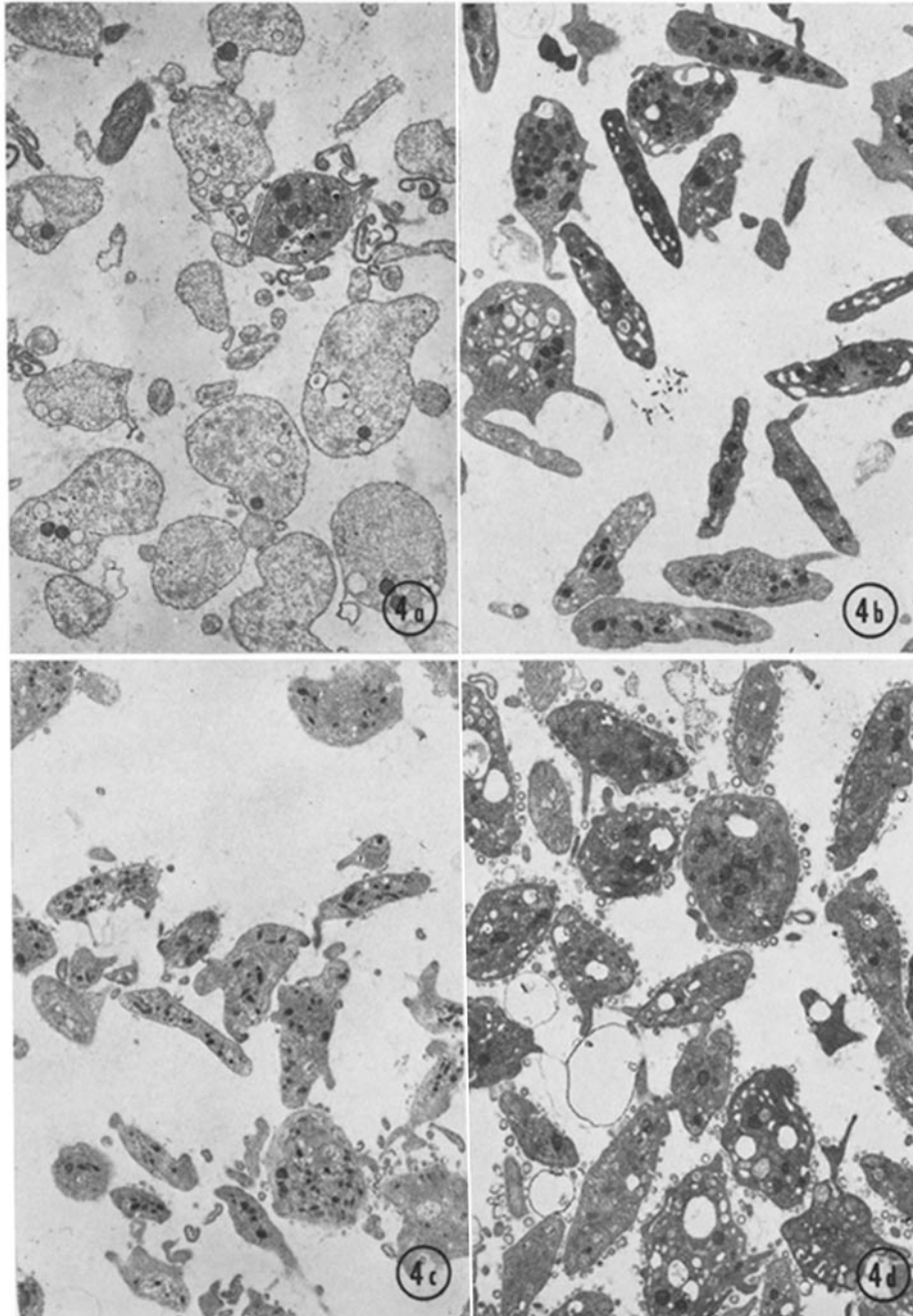
FIG. 4 *a*. Rabbit platelets.  $\times 11,000$ .

FIG. 4 *b*. Primate platelets.  $\times 9000$ .

FIG. 4 *c*. Guinea pig platelets.  $\times 8000$ .

FIG. 4 *d*. Dog platelets.  $\times 10,000$ .

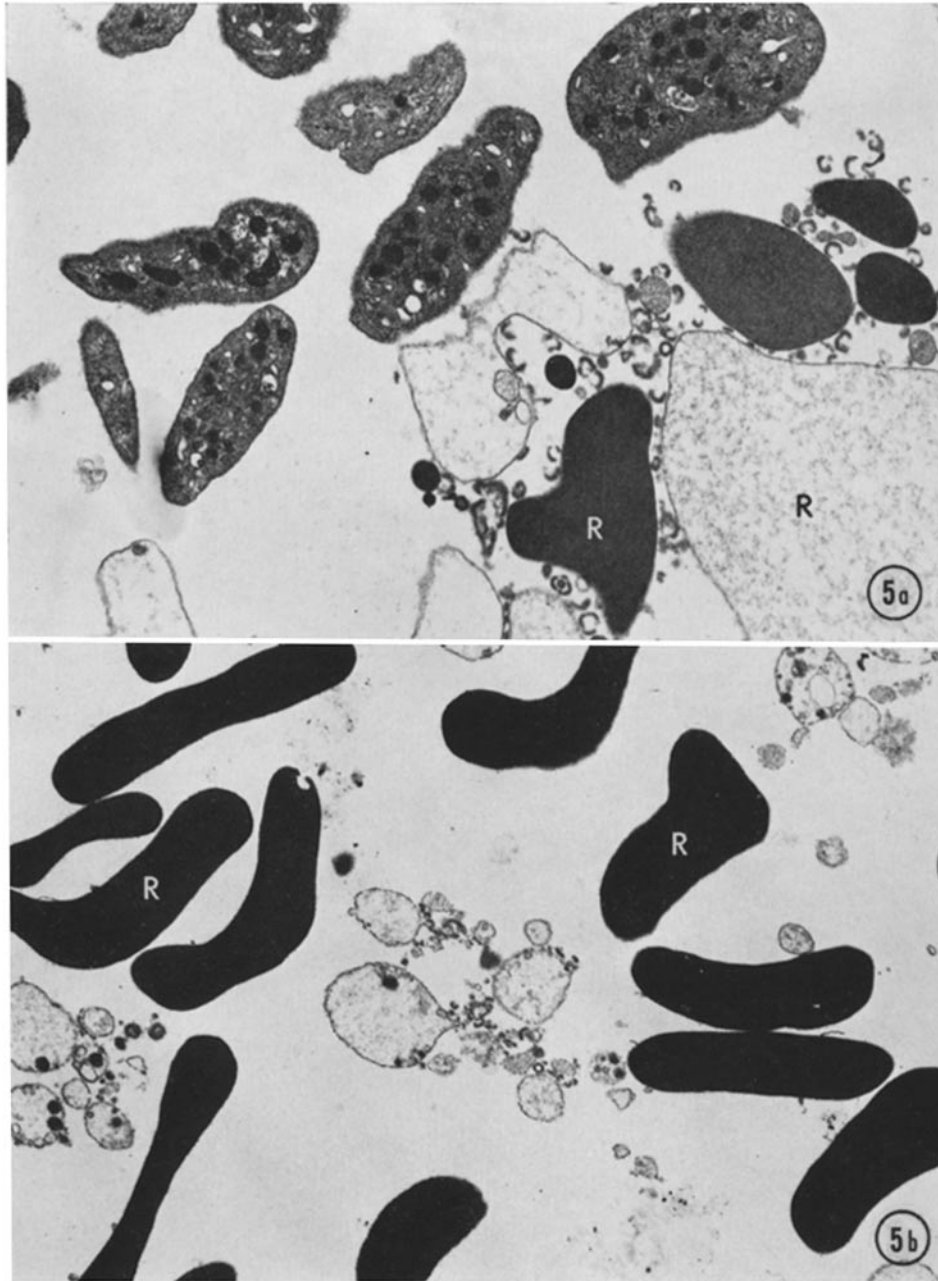




(Spielvogel: Mechanisms of platelet-endotoxin interaction)

PLATE 15

FIG. 5 *a*. Endotoxin incubated with baboon red blood cells (R) and platelets.  $\times 17,000$ .  
FIG. 5 *b*. Endotoxin incubated with rabbit red blood cells (R) and platelets.  $\times 9000$ .



(Spielvogel: Mechanisms of platelet-endotoxin interaction)

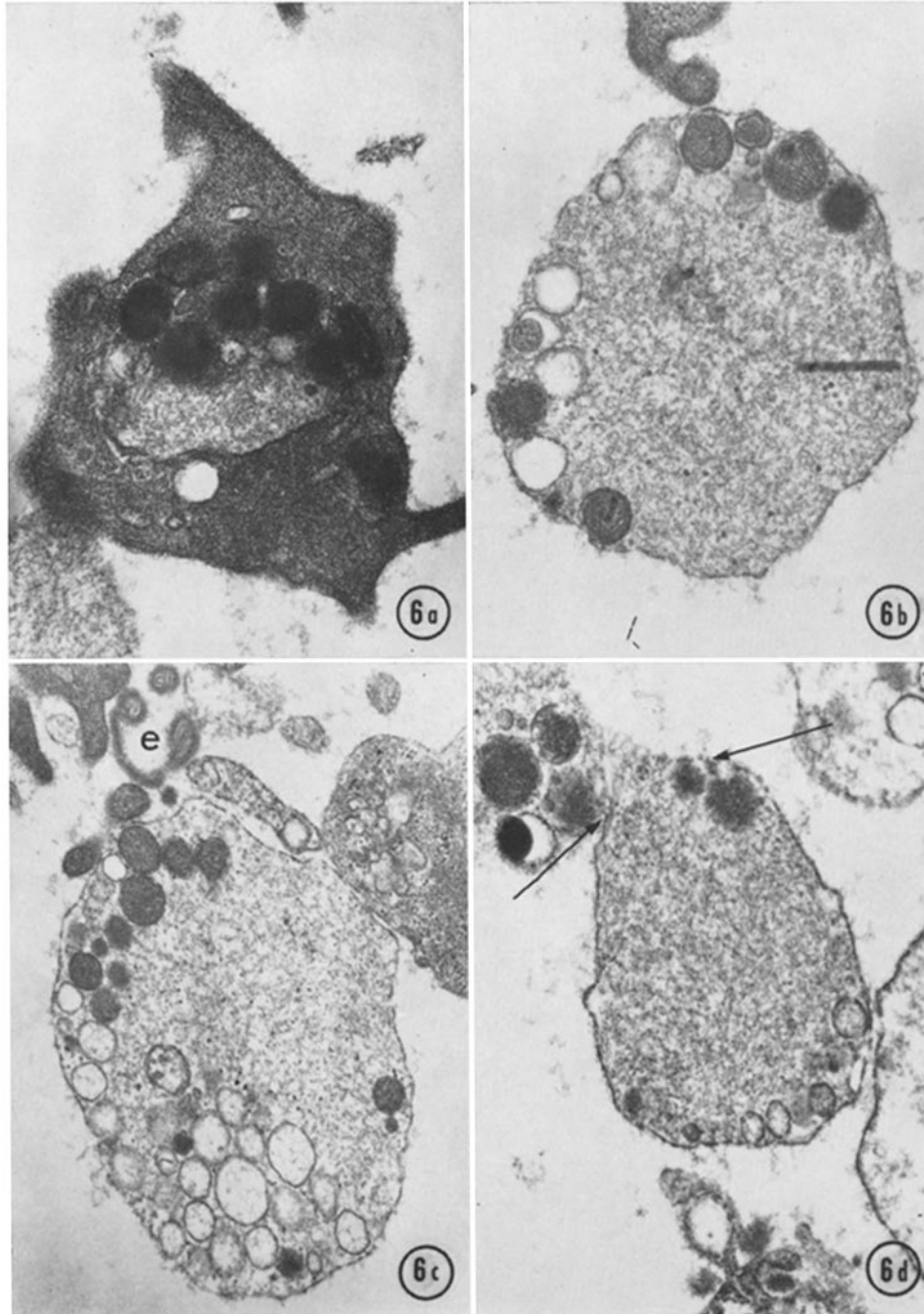
PLATE 16

FIG. 6. A timed in vitro study of rabbit platelets incubated with endotoxin.

FIG. 6 *a*. A platelet at 30 min, showing clumped organelles and cytoplasmic clear spaces  $\times 35,000$ .

FIG. 6 *b, c*. Platelets at 45 and 60 min with decreased cytoplasmic staining, peripheralization of organelles, and empty intracellular granules and mitochondria.  $\times 30,000$ .

FIG. 6 *d, e*. Platelets at 45 and 60 min exhibiting cell membrane lysis (arrows) with release of contents into the surrounding media.  $\times 37,000$ .



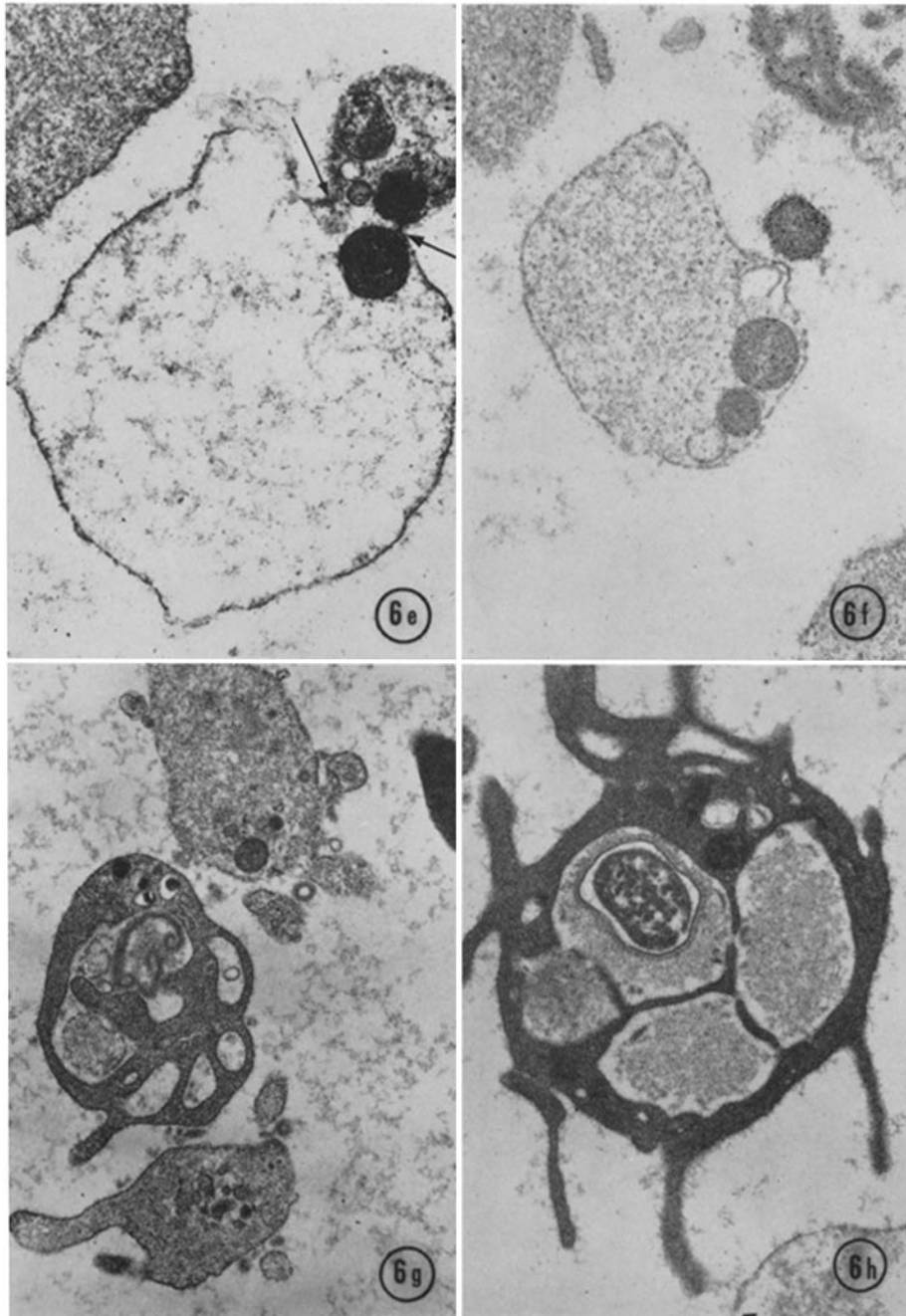
(Spielvogel: Mechanisms of platelet-endotoxin interaction)

PLATE 17

FIG. 6 *f*. An extracellular mitochondrion.  $\times 38,000$ .

FIG. 6 *g*. Rabbit platelet with phagocytosis of an *E. coli* endotoxin particle (in vivo).  $\times 33,000$ .

FIG. 6 *h*. Rabbit platelet phagocytizing *S. enteritidis* endotoxin (in vitro).  $\times 30,000$ .



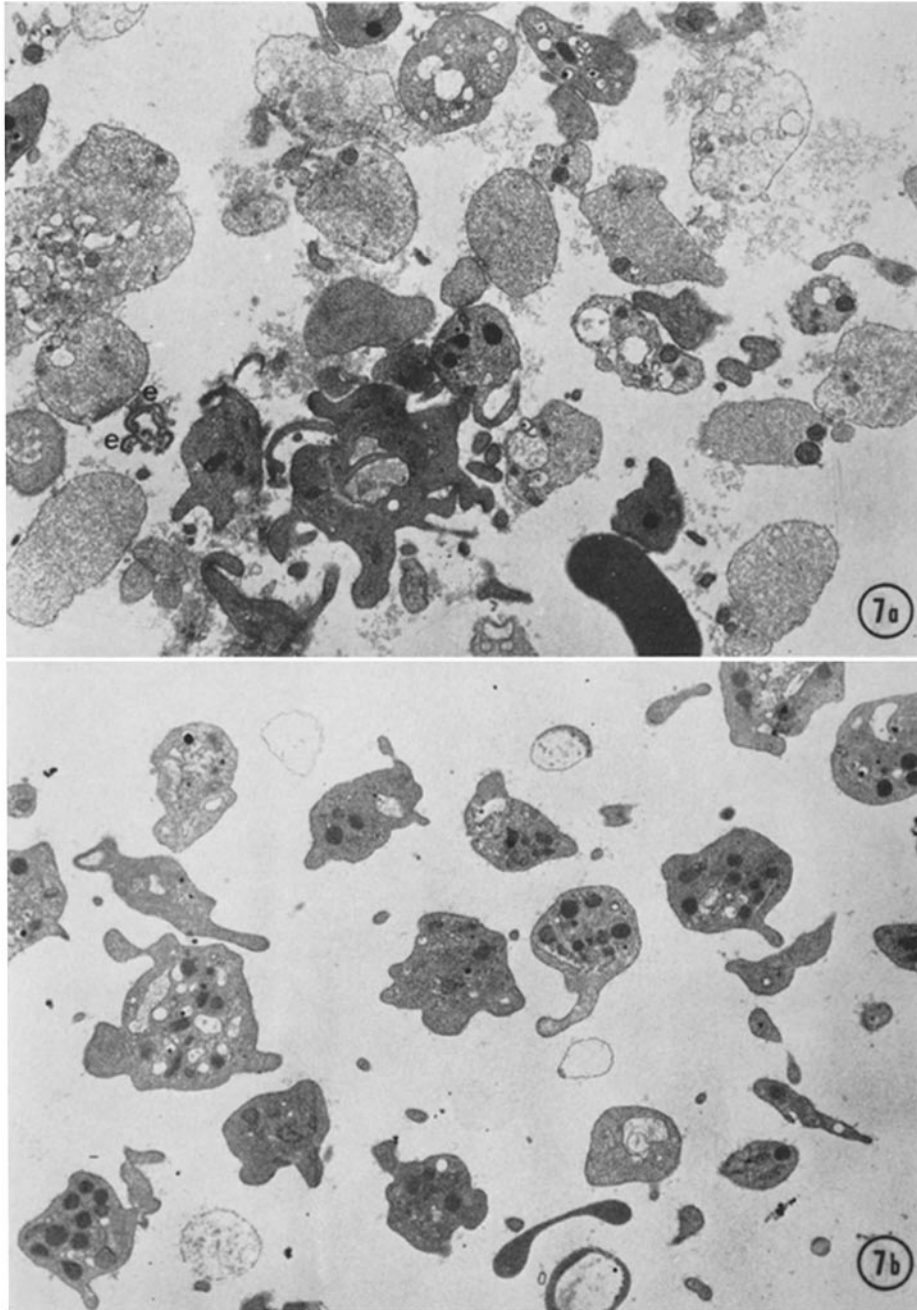
(Spielvogel: Mechanisms of platelet-endotoxin interaction)

PLATE 18

FIG. 7 *a*. Rabbit platelets circulating 2-5 min after parenteral administration of a lethal dose of endotoxin (e).  $\times 10,000$ .

FIG. 7 *b*. Platelets circulating 90 min after parenteral endotoxin.  $\times 10,000$ .





(Spielvogel: Mechanisms of platelet-endotoxin interaction)