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Platelet Damaging Factor, a Fifth Activity of Staphylococcal α-Toxin[1]

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Crude and purified staphylococcal α-toxin were used to demonstrate that the platelet-damaging effect of crude α-toxin represents a fifth activity of the α-toxin molecule. The homogeneity of the purified toxin employed was demonstrated by ultracentrifugation, Ouchterlony, and immunoelectrophoretic methods. Continuous-flow electrophoretic migration studies demonstrated under a variety of conditions that the platelet-damaging and the α-hemolytic activities migrated as a unit. Fractionation studies with the use of Sephadex G-100, carboxymethyl cellulose, and diethylaminoethyl cellulose failed to separate these two activities. Further, when α-toxin of demonstrated purity and crude toxin were adjusted to the same hemolytic activity, they possessed the same platelet-damaging activity. In addition, heat-reactivation studies with crude α-toxin revealed that the platelet-damaging effect was inactivated and reactivated in parallel with α-hemolytic activity. Comparable studies with purified α-toxin showed parallel inactivation of both activities at 60 C. Additional heating at 100 C failed to reactivate either activity. Electron micrographs revealed that purified α-toxin produced distinct degenerative changes in rabbit platelets. These studies also provided definite evidence that purified α-toxin has a damaging effect on human platelets. Monovalent α-antisera prevented platelet damage.

Gengou (3) was the first to study the effects of crude staphylococcal toxin on blood platelets. He reported that a turbid suspension of rabbit blood platelets cleared on exposure to crude staphylococcal toxin.

A comprehensive study by Siegel and Cohen (13) demonstrated beyond doubt that a component(s) of crude staphylococcal toxin damaged human blood platelets. These workers demonstrated that human platelets exposed to crude staphylococcal toxin (concentrated by ammonium sulfate precipitation) showed distinct degenerative changes, as evidenced by microscopic examination and by an increase in the packed platelet volume. Staphylococcal toxin also shortened the clotting time and inhibited clot retraction. In addition, toxin-treated platelets showed a rapid loss of potassium and nicotinamide adenine dinucleotide.

Bernheimer and Schwartz (1), using a staphylococcal α-toxin which they characterized as being 70% pure, demonstrated that this preparation produced damage to rabbit blood platelets. These workers used the decrease in turbidity of a toxin-treated platelet suspension as an index of platelet damage. They ruled out platelet aggregation as a cause of this decrease in turbidity.

Contrariwise, Jeljaszewicz et al. (5) stated that α-toxin caused aggregation of platelets which led to platelet destruction.

MATERIALS AND METHODS

The methods used for the preparation of α- and β-hemolysins and of crude α-antitoxin, measurement of α- and β-antitoxin, titration of α-, β-, and δ-hemolysins, and purification of α-toxin, and the methods used for immunoelectrophoresis were identical to those employed by Manohar et al. (9).

Preparation of monovalent α-antisera. Monovalent α-antisera were prepared by the method of Kumar et al. (8).

Rabbits. Adult New Zealand white rabbits were used in these studies. All rabbits used were free from α- and β-antihemolysins.

Glassware. All glassware used for platelet studies was washed and siliconized by the method of Martin and Green (10).

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Hemolytic activity. The hemolytic activity of α-toxin eluted from Sephadex G-100, diethylaminoethyl (DEAE) cellulose, and carboxymethyl (CM) cellulose was measured in hemolytic units according to the method of Cooper et al. (2). The hemolytic activity of electrophoretic eluates was expressed as hemolytic titers, according to the method of Kumar and Lindorfer (7).

Exposure to heat. Crude or purified α-toxin, which had been dialyzed against 0.145 M sodium chloride, was divided into several 1-ml samples in rubber-stoppered tubes (100 by 12 mm). These samples were placed in a water bath for the indicated times and temperatures. After heat exposure, the tubes were immediately placed in an ice bath until the various activities were measured.

Continuous-flow electrophoresis. A Spinco (model CP) continuous-flow curtain electrophoretic apparatus was used to fractionate crude α-toxin under two different conditions. In one fractionation, lactate buffer at a *p*H of 3.6, ionic strength of 0.02, and 98 ma was used. The second fractionation of crude α-toxin employed Veronal buffer at a *p*H of 8.6, ionic strength of 0.02, and 98 ma. Each of the 32 eluates was then titrated for its α-hemolytic and platelet-damaging activity.

Sephadex fractionation. Sephadex G-100 (lot no. T02946; particle size, 40 to 120 microns; U.S. Standard; Pharmacia, Uppsala, Sweden) was suspended in water for 3 days. The fine particles were removed with the supernatant fluid, and the slurry was washed with phosphate-buffered saline (*p*H 7.0) five times. A column (85 by 4 cm) was packed and allowed to equilibrate with the above buffer for 3 days. The void volume and the uniformity of packing were determined by use of 0.01% blue dextran in the above buffer. The fractionation of a 10-ml sample of crude α-toxin was carried out at room temperature with a flow rate of 21 ml/hr. Each eluate contained 4 ml. The α-hemolytic and platelet-damaging activity of each eluate was tested.

DEAE cellulose column chromatography. The DEAE cellulose used was obtained from Schleicher and Schuell Co., Keene, N.H., and had an exchange capacity of 0.95 meq/g. The method of packing and washing the column was that of Peterson and Sober (11). A column (36 by 2 cm) was packed and washed with the starting buffer [tris(hydroxymethyl)aminomethane (Tris) chloride, 0.005 M, *p*H 7.2] until the effluent reached a *p*H of 7.2. A 5-ml sample of crude α-toxin which had been equilibrated against the starting buffer was applied to the column. A linear gradient was established by use of 0.7 M NaCl. Elution was carried out with a flow rate of 16 ml/hr. Eluates of 4 ml were collected. Fractionation was carried out at room temperature. The α-hemolytic and platelet-damaging activity of each eluate was measured.

CM cellulose column chromatography. The CM cellulose used was obtained from Schleicher and Schuell Co., Keene, N.H., and had an exchange capacity of 0.72 meq/gram. The method of Peterson and Sober (11) was used to prepare the column. A column (36 by 2 cm) was washed with starting buffer (citrate-phosphate, 0.0075 M, *p*H 5.0) until the effluent had a *p*H of 5.0. A 5-ml sample of crude α-toxin which had been equilibrated against the starting buffer was applied to the column at 4 C. A linear gradient was established by use of phosphate buffer, 0.2 M, at *p*H 7.0. The flow rate of the column was 11 ml/hr and 4-ml eluates were collected. The α-hemolytic and platelet-damaging activity of each eluate was measured.

Preparation of platelets for clot retraction, platelet volume, and per cent light transmission studies. The platelets used in these studies were prepared by the method of Siegel and Cohen (13). Rabbit platelets were used in studies which involved the use of clot retraction and per cent light transmission measurements. Human platelets were used in studies which employed platelet volume for the measurement of platelet damage.

Clot retraction. Platelet-free plasma and an unwashed platelet suspension were used in this test. Crude or purified α-toxin (0.1 ml) was mixed with 0.1 ml of the unwashed platelet suspension in a small tube (75 by 7 mm) and incubated for 30 min at 37 C. After incubation, 0.8 ml of platelet-free plasma and 0.2 ml of 0.1 M calcium chloride were added, and the tubes were reincubated at 37 C. The tests were observed at intervals for clot retraction. A platelet control containing 0.1 ml of modified Hanks solution instead of toxin and a medium control containing 0.1 ml of a saline extract of Leonard and Holm medium instead of toxin were used in each experiment.

Platelet volume. These measurements were carried out with washed human blood platelets. The platelets were washed three times with modified Hanks solution. The washed platelet suspension (0.1 ml) was mixed with serial twofold dilutions of crude or purified α-toxin prepared in Veronal buffer. These mixtures were incubated for 15 min at 37 C. A sample from each dilution was drawn into a capillary tube which was then sealed on one end by flame. After centrifugation in a model MB microcapillary centrifuge, the packed platelet volume was measured. Buffer and media controls were employed in these studies.

Per cent light transmission. The platelet-damaging activity of the eluates obtained from electrophoretic, Sephadex, DEAE, and CM cellulose fractionations of crude α-toxin was measured by changes produced in light transmission. In these studies, 0.5 ml of each eluate was mixed with 0.5 ml of a washed platelet suspension. After incubation of the mixtures at 37 C for 10 min, the light transmission of the suspensions was measured at 540 mμ in a Coleman Jr. spectrophotometer (model 6D). Phase microscopy revealed that the platelet suspensions used in these studies did not aggregate when treated with α-toxin.

Electron microscopy. A 45-ml amount of rabbit blood was mixed with 5 ml of 1.5% ethylenediaminetetraacetate (EDTA) or with 5 ml of 3.8% sodium citrate. The blood was centrifuged at 120 × *g* for 15 min at 4 C. The supernatant fluid was removed and recentrifuged at 810 × *g* for 12 min at 4 C. The platelet button was resuspended in phosphate-buffered saline (PBS), *p*H 7, and washed three times. The final platelet button was suspended in 2 ml of PBS. The

purified α-toxin used in these studies was dialyzed against PBS and contained 186 hemolytic units (HU)/ml. A 1-ml amount of the washed platelet suspension was mixed with 1 ml of purified α-toxin and incubated at 25 C for 20 min. The platelets were then fixed for 1 hr in 2% glutaraldehyde (in 0.1 M phosphate buffer, *p*H 7.4). After centrifugation, the platelets were post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (*p*H 7.4). The platelets were then dehydrated in a graded series of ethyl alcohol, passed through two changes of toluene, and embedded in Maraglas (15). Sections obtained by means of glass knives (16) were mounted (unsupported) on copper grids and stained with lead citrate (12). Sections were examined with an RCA-EMU-3F electron microscope.

RESULTS

Fractionation studies. Attempts were made to separate the α-hemolytic and platelet-damaging activities of crude staphylococcal toxin by various fractionation procedures. Continuous-flow curtain electrophoresis, Sephadex G-100, DEAE cellulose, and CM cellulose fractionation methods were used.

The first attempts to separate these two activities used continuous-flow electrophoretic methods. Figure 1 shows the results of fractionation with lactate buffer at a *p*H of 3.6. It will be noted that the α-hemolytic and platelet-damaging activities migrated as a unit under these conditions. Figure 2 shows the results of an electrophoretic separation by use of Veronal buffer at a *p*H of 8.6. Again, it may be seen that the two activities are inseparable.

The fractionation of crude staphylococcal toxin with the use of a Sephadex G-100 column gave the results depicted in Fig. 3. Both activities appeared well beyond the void volume of the column and both appeared in the same eluates.

Figure 4 also demonstrates that these two activities could not be separated by use of DEAE cellulose column fractionation.

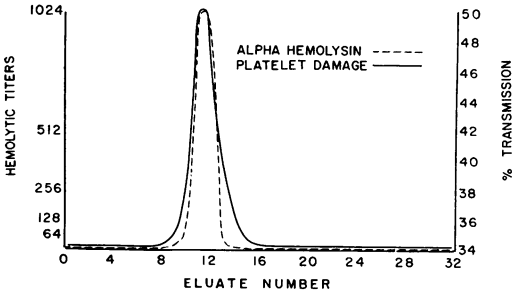

FIG. 1. *Electrophoretic migration of the α-hemolytic and the platelet-damaging activities of crude α-toxin. This separation used lactate buffer, pH 3.6, 0.02 ionic strength, 98 ma.*

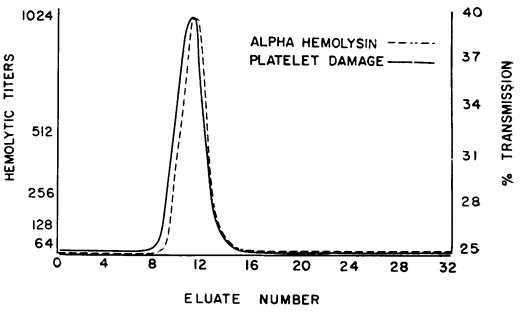

FIG. 2. *Electrophoretic migration of the α-hemolytic and the platelet-damaging activities of crude α-toxin. This separation used Veronal buffer, pH 8.6, 0.02 ionic strength, 98 ma.*

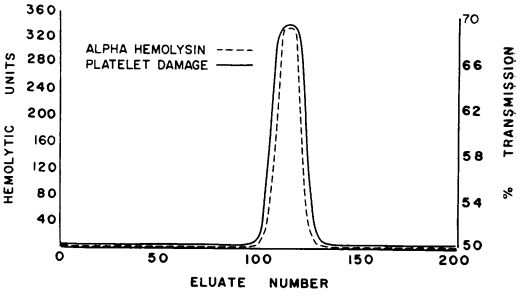

FIG. 3. *Fractionation of crude α-toxin with Sephadex G-100. Buffer = phosphate-buffered saline (pH 7.0); sample volume = 10 ml; flow rate = 21 ml/hr; eluate volume = 4 ml.*

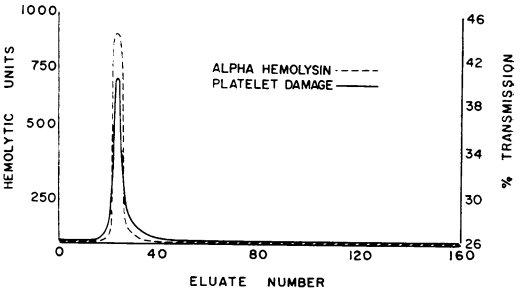

FIG. 4. *Fractionation of crude α-toxin with DEAE cellulose. Exchange capacity = 0.95 meq/g; column size = 36 by 2 cm; flow rate = 16 ml/hr; eluate volume = 4 ml; starting buffer = Tris chloride, 0.005 M, pH 7.2; linear gradient elution with 0.7 M NaCl started after 40th eluate.*

The results of attempts to separate the two activities by use of a CM cellulose column are shown in Fig. 5. Note that both activities were eluted from the column in parallel.

Heat-reactivation studies on crude toxin. It has been demonstrated (9) that the α-hemolytic,

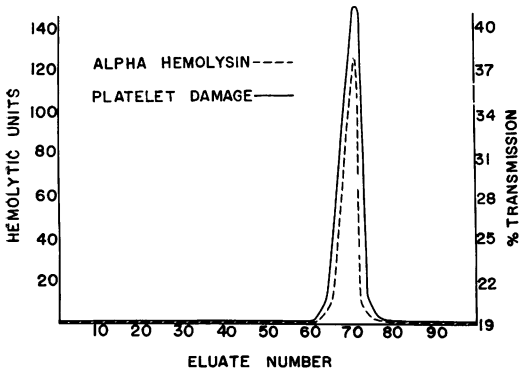

FIG. 5. *Fractionation of crude α-toxin with CM cellulose. Exchange capacity = 0.72 meq/g; column size = 36 by 2 cm; flow rate = 11 ml/hr; eluate volume = 4 ml; starting buffer = phosphate-citrate, 0.005 M, pH 5.0; linear gradient elution with phosphate buffer, 0.2 M, pH 7.0, started after 35th eluate.*

dermonecrotic, and lethal activities of crude α-toxin all exhibit the "Arrhenius effect" (heat reactivation). The concurrent heat reactivation of the platelet-damaging activity would indicate that this activity is a function of α-toxin. The results in Figure 6 show that the original crude toxin possessed an α-hemolytic titer of 1:4,096, and that this original preparation damaged platelets to the extent that they were incapable of initiating clot retraction. Note that after exposure of crude toxin to 60 C for 30 min, the α-hemolytic titer dropped to 0, and the platelets initiated clot retraction. Further, it may be seen that additional heating for 10 min at 100 C reactivated both activities, as evidenced by the return of an α-hemolytic titer of 1:1,024, and by the fact that clot retraction was again inhibited. Finally, it is evident that an additional 50 min of heating at 100 C resulted in the complete loss of both activities.

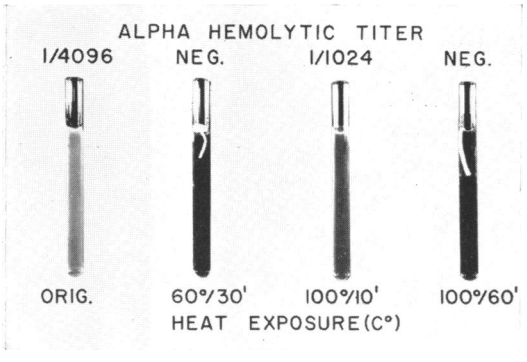

FIG. 6. *Heat reactivation of α-hemolytic and platelet-damaging activities of crude α-toxin. The original sample was heated to 60 C for 30 min. It was then heated for an additional 60 min at 100 C. Platelet and medium controls showed clot retraction.*

Heat-inactivation studies on purified toxin. The procedure used for the purification of α-toxin should have removed the inhibitor(s) responsible for heat reactivation (9). Therefore, purified toxin should not show reactivation of either activity. Figure 7 shows that both the α-hemolytic and the platelet-damaging activities failed to reactivate. In addition, it may be seen that, when the α-hemolytic activity was inactivated, the platelet-damaging activity was also inactivated. These results also show that α-toxin of demonstrated purity damaged rabbit platelets.

Effect of crude and purified α-toxin on human platelets. The ratio between the α-hemolytic and the platelet-damaging activities should be the

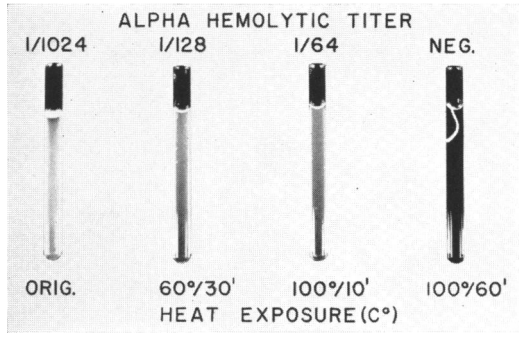

FIG. 7. *Heat inactivation of α-hemolytic and platelet-damaging activities of purified α-toxin. The original sample was heated to 60 C for 30 min. It was then heated for an additional 60 min at 100 C. Platelet and medium controls showed clot retraction.*

TABLE 1. *Effect of crude and purified α-toxin on the volume of human blood platelets*[a]

Reciprocal of toxin dilution	Platelet vol (%)	
	Crude toxin	Purified toxin
40	6	6
80	6	6
160	5	4.5
320	5	3.6
640	4.5	2.8
1,280	2.8	2.8
2,560	2.8	2.8

[a] Buffer and medium controls, 2.8% platelet volume. Hemolytic titers of crude and purified toxin, 1/320.

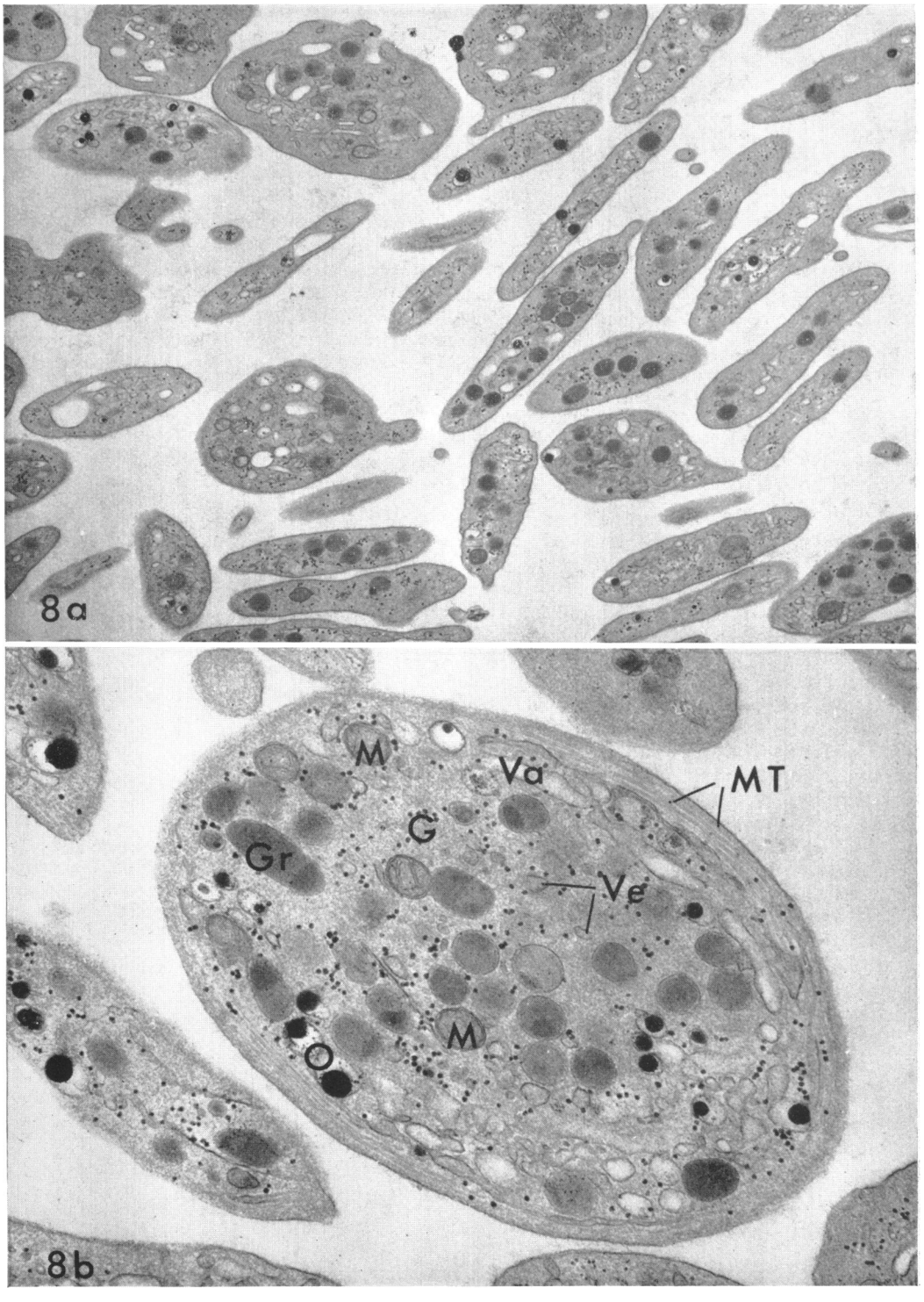

FIG. 8a. *Survey electron micrograph of normal rabbit platelets.* × *10,000.*

FIG. 8b. *Electron micrograph of normal rabbit platelet. Mitochondria* (*M*), *microtubules* (*MT*), *glycogen* (*G*), *vesicles* (*Ve*), *vacuoles* (*Va*), *granules* (*Gr*), *and dense osmiophilic granules* (*O*). × *35,000.*

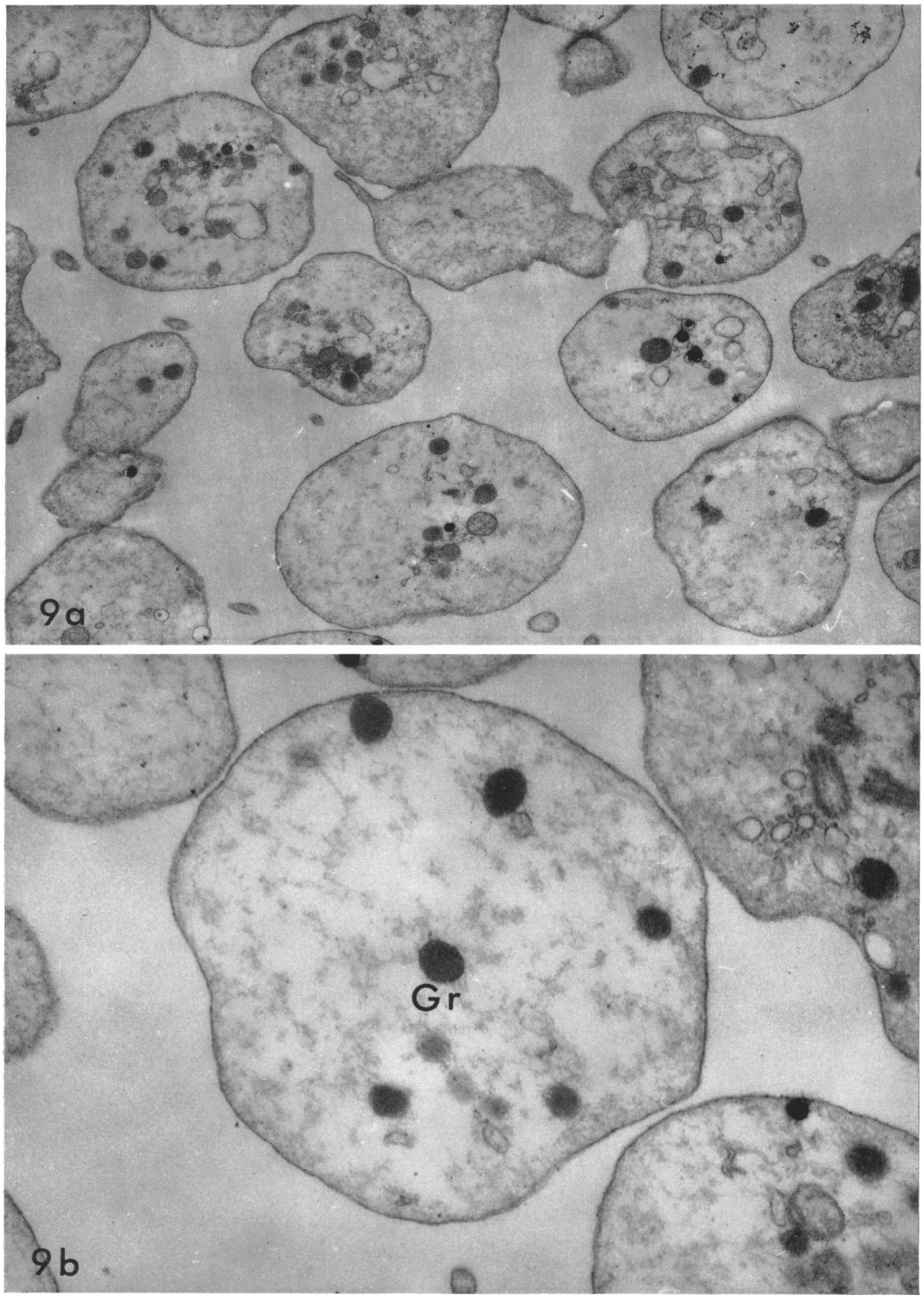

FIG. 9a. *Survey electron micrograph of α-toxin-treated rabbit platelets.* × *10,000.*

FIG. 9b. *Electron micrograph of α-toxin-treated platelet showing loss of organelles and the appearance of flocculent material. Note that the few remaining granules (Gr) are more electron-dense, and that the platelet membrane is still intact.* × *29,000.*

same in crude and purified toxin, if both activities are functions of one molecule. The results in Table 1 show that crude and purified toxin adjusted to the same α-hemolytic titer caused the same increase in platelet volume. When crude and purified toxins were diluted to 1:160, they both began to show a decrease in platelet volume. Table 1 also shows that α-toxin of demonstrated purity caused an increase in human platelet volume, which is accepted as an indicator of platelet damage (13).

Electron microscopy. The ultrastructure of normal rabbit platelets was recently described in detail by Silver (14). He confirmed the presence of all of the organelles described by earlier authors, and in addition demonstrated the presence of microtubules and dense osmiophilic granules. These osmiophilic granules were eccentrically located in a larger, less dense, membrane-bounded structure. Our electron micrographs (Fig. 8) confirmed all his findings.

When rabbit blood platelets were exposed to purified α-toxin, several degenerative changes occurred (Fig. 9). The platelets became swollen and globular in shape. The number of vesicles and mitochondria decreased. The microtubules, the dense osmiophilic granules, and the glycogen granules disappeared. The granules seemed to be reduced in number, and their membranes became less distinct. However, the remaining granules were more electron-dense than they were in normal platelets. An amorphous flocculent material appeared throughout the platelets, whereas the platelet membrane seemed to remain intact. Monovalent α-antisera prevented all of the aforementioned changes.

DISCUSSION

This study has demonstrated by several methods that the α-hemolytic and platelet-damaging activities of crude staphylococcal toxin are both functions of α-toxin. If the α-hemolytic and platelet-damaging activities of crude staphylococcal toxin are both functions of one molecule, these activities should be inseparable. The continuous-flow electrophoretic migration studies at *p*H 3.6 and 8.6 showed clearly that both activities migrated as a unit. The column fractionation methods, in which Sephadex G-100, CM cellulose, and DEAE cellulose were used, all demonstrated that the two activities were eluted from these columns in parallel. The probability that two distinct entities would react identically under such a wide variety of conditions seems remote.

Additional evidence that these two activities are both functions of α-toxin was provided by heat inactivation and reactivation studies. If the α-hemolytic and platelet-damaging activities of crude α-toxin are both expressions of one molecule, both activities should exhibit the "Arrhenius effect" (heat reactivation). The concurrent reactivation of these activities would be most apt to occur if the same active sites were responsible for both activities. Experiments with crude α-toxin showed that both activities were inactivated when heated at 60 C for 30 min, and that both activities were reactivated when the preparation was reheated at 100 C for 10 min. The probability is low that two distinct substances would show this unusual reactivation.

The heat inactivation studies with α-toxin of demonstrated purity showed that purified toxin possessed both activities and that these two activities were inactivated in parallel.

These studies also provide experimental evidence which supports the suggestion of Siegel and Cohen (13) that α-toxin damages human platelets. In addition, these results showed that the ratio between the α-hemolytic and platelet-damaging activities was the same in purified toxin as it was in crude toxin. Such a result would occur if both activities were functions of one molecule.

Electron microscopy revealed the presence in normal rabbit platelets of all of the organelles reported in earlier studies plus the microtubules and dense osmiophilic granules reported by Silver (14). Electron microscopy also provided visual evidence of extensive platelet damage produced by purified α-toxin.

The fact that monovalent α-antisera prevented the platelet damage caused by purified α-toxin provides additional evidence that platelet damage is produced by α-toxin.

It has been demonstrated previously (4, 6, 8, 9) that the alpha hemolytic, dermonecrotic, lethal, and leukocidal activities are functions of α-toxin. It is now concluded that platelet-damaging activity represents a fifth activity of α-toxin.

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