Inhibition of Sulfhydryl-Dependent Platelet Functions by Penetrating and Non-Penetrating Analogues of Parachloromercuribenzenzene

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With the technical assistance of Patricia Santillo

IN 1959 BETTEX-GALLAND AND LUSCHER1 demonstrated the presence in human platelets of a contractile protein, thrombosthenin. This protein is held to be responsible for clot retraction. They,2 as well as Grette,3 demonstrated that this protein had Ca\(^{2+}\)- and/or Mg\(^{2+}\)-activated adenosine-triphosphatase (ATPase) activity as well as displaying the characteristic viscosity changes of actomyosin upon the addition of ATP. The existence of a separate, distinguishable Na\(^{+}\), K\(^{+}\)-dependent ATPase activity in the platelet has been implied by the work of several investigators.4 This evidence suggests that maintenance of a high intracellular K\(^{+}\) content by platelets and the control of platelet volume depend upon energy production via anaerobic and oxidative glycolytic pathways.7

The present investigation was undertaken to study the effects of two agents which inhibit sulfhydryl (SH\(^{-}\)) dependent enzymic reactions on the ATPase activities of osmotically lysed platelets and on certain other properties of intact platelets. Parachloromercuribenzoate\(^{-}\) (PCMB) and parachloromercuribenzenesulfonate (PCMBS), have been shown\(^{8-10}\) to react in identical fashion with soluble proteins such as hemoglobin and with human erythrocyte ghosts which are readily permeable to both compounds. Because of the highly polar sulfonic acid moiety of PCMBS, this compound is markedly hydrophilic and
consequently penetrates intact red cells at a much slower rate than PCMB. Thus, in contrast to the inhibition of physiologic parameters in the red cell produced by the more rapidly penetrating compound PCMB, immediate inhibition of any physiologic cell function by PCMBS indicates inhibition of a function localized to the outer surface of the cell.

The corollary of this is that failure of PCMBS to rapidly inhibit a SH-dependent cell function may indicate that the function is dependent upon sulfhydryl groups localized beneath an outer permeability barrier. In the present studies with PCMB and PCMBS, these observations have been applied to human platelets by comparing ADP-induced aggregation, platelet cationic content, platelet volume and ability to support clot retraction at intervals after exposure to both compounds.

**METHODS**

Freshly drawn blood was collected into silicone-coated glassware using 4.45 mM Na$_2$EDTA/L of whole blood as the anticoagulant. The blood was centrifuged immediately at 300 x g. for 15 minutes at room temperature. The resultant platelet-rich EDTA plasma was used for the separation of platelets for studies relating to the measurement of ATPase activity. Sodium citrate, 1.29 x 10$^{-2}$M/L of whole blood, was used as the anticoagulant for studies of ADP-induced platelet aggregation, clot retraction, effects of organic mercurials on platelet function, cationic content and platelet volume.

Changes in platelet volume were measured by use of tubes especially prepared for the measurement of cell volume changes in dilute cell suspensions (Blaessig Glass Specialties, Rochester, New York). These tubes contained a total volume of 3 ml. and had a narrowed stem containing 0.3 ml. with 0.01 ml. subdivisions. By use of these tubes for measurement of platelet volume in conjunction with platelet counts, mean values for platelet volume were found to be 6.1 ± 0.5 µm$^3$. This value is similar to the 5.8 µm$^3$ value obtained by Bull and Zucker. Platelet content of Na$^+$ and K$^+$ was measured as follows: Initial centrifugation of platelet-rich plasma was followed by washing twice with choline chloride (6 C.) and resuspension of the platelets in deionized, distilled water to lyse them. Digestion of the intact platelet button with HNO$_3$ was found to yield identical Na$^+$ and K$^+$ values confirming the adequacy of distilled water lysis for Na$^+$ and K$^+$ determinations. The Na$^+$ and K$^+$ content of centrifuged but unwashed platelets, corrected for trapped plasma by use of $^{131}$I albumin, indicated that no loss of Na$^+$ or K$^+$ resulted from the choline washes. The Na$^+$ and K$^+$ ion concentrations were measured with an Instrumentation Laboratories flame photometer (Instrumentation Laboratories, Inc., Boston, Massachusetts). Platelet cell water was measured by introducing into 1 ml. conical centrifuge tubes 0.5 ml. volumes of platelet suspensions containing C$^{14}$ labeled sucrose. The suspensions were weighed, dried in a Precision Scientific circulating air chromatography oven at 100 C. for 12 hours and reweighed, correcting for the extracellular water by quantification of the C$^{14}$ sucrose present. With this technique, triplicate determinations were made on platelets from seven individuals. The mean normal platelet cell water was found to be 65.6 ± 1.3 percent (S.E.) by weight and the normal platelet Na$^+$ and K$^+$ concentrations expressed as mM/L of platelet water were 19.7 ± 1.3 (S.E.) and 135. ± 1.0 (S.E.), respectively. These K$^+$ values are consistent with those published previously by Hartmann et al.

Clot retraction was estimated following the addition of 20 units of thrombin (The Upjohn Company, Kalamazoo, Michigan—supplied as topical bovine thrombin) to 1 ml. of citrated platelet-rich plasma in siliconized tubes. The clotted plasma then was incubated for 1 hour at 37 C. and evaluated for clot retraction. Fresh thrombin solutions were prepared before each experiment.

Varying concentrations of adenosine diphosphate, sodium salt (ADP) (Sigma Chemical
Company, St. Louis, Missouri) were added to citrated platelet-rich plasma to produce platelet aggregation. After agitation of the tubes, aggregation was evaluated by gross observation as well as by examination with the phase contrast microscope.

Osmotically lysed platelets were prepared by the successive osmotic lysis technique described by Hillier and Hoffman for the preparation of red blood cell ghosts. Red cell ghosts prepared in this fashion lose 97 percent of their cellular protein. In contrast, osmotically lysed platelets liberate only one-third of their cellular protein. Further washes of lysed platelets with 30 mOsm buffer did not solubilize more cellular protein. Platelets lysed in this manner undoubtedly contain some of their intracellular membranous components.

Protein determinations were carried out on all platelet preparations using the biuret method with albumin as the standard.

The assay employed for platelet ATPase determination was a modification of the methods of Tosteson, Cook and Blount and Tal, Dickstein, and Sulman for red blood cell ATP and ATPase activity. Consumption of ATP by the lysed platelets was assayed as follows: Approximately 4 x 10^10 lysed platelets (0.075 mg. of platelet protein) were incubated at 37 C. for 30 minutes in the presence of 10^-5 M ATP, 10^-3 M Mg++, and either 80 mM Na+ and 17 mM K+ or 97 mM K+. The final volume was 2.5 ml. and the pH was maintained at 7.2 by 0.1 M Tris buffer. At the end of the 30 minute incubation, the tube containing the incubation mixture was placed in boiling water for one and one-half minutes to inactivate ATPase activity.

In the present study, total ATPase activity is defined as the moles of ATP consumed per milligram of platelet protein per minute in the presence of 1 mM Mg++, 80 mM Na+ and 17 mM K+. The Na+, K+ dependent ATPase activity is calculated as the difference between the ATPase activity in the presence of Mg++, Na+ and K+ and that in the presence of Mg++, and K+. Parachloromercuribenzoate and PCMBS were used both as the unlabeled chemical (Sigma Chemical Company, St. Louis, Missouri) and as Hg2O3-labeled (Radiochemical Centre, Amersham, England) compounds. The uptake of the labeled agents by both intact platelets and lysed platelets was determined by methods described previously. The labeled compounds were added to platelet-rich suspensions in isotonic, buffered NaCl containing 1.29 x 10^-2 M citrate and, after appropriate intervals, radioactivity remaining in the supernatant was measured. Because of the high affinity of the mercurial agents for sulfhydryl groups of plasma proteins, when the agents were used in plasma the uptake by the platelets was only a small percentage of the total present in this system. Under the plasma conditions, uptake was measured by counting radioactivity of the platelets directly after washing them free of plasma with isotonic choline chloride.

RESULTS

PCMB and PCMBS Uptake

Figure 1 compares the uptake of 10^-4 M PCMB and PCMBS from citrate-containing NaCl by intact platelets. It is clear that the uptake of PCMB continued for at least one hour while PCMBS exhibited immediate binding but little further platelet uptake over the same period of time. This suggests that uptake of PCMBS was limited by a permeability barrier, presumably the platelet membrane, and that immediate binding presumably was to the outer surface. At 10^-5 M PCMBS, after a lag period of 30 minutes, the uptake approached that of PCMB suggesting that at this concentration PCMBS was able to enter the cell, either because of damage to the membrane caused by the agent itself or because of the increased concentration gradient from outside to inside. When the mercurial compounds were employed at 2.5 x 10^-3 M in platelet-rich plasma, considerable binding to plasma proteins occurred, and the concentration of free mercurial was probably very close to the 10^-4 M
Fig. 1.—Uptake of $10^{-4}$ M ($10^{-7}$ moles/ml. or $10^{-7}$ moles/1.81 mg. of platelet protein) PCMB, and PCMBS by platelets suspended in saline.

In the absence of plasma. The uptake patterns obtained using $2.5 \times 10^{-3}$ M in platelet-rich plasma were similar to those seen in Figure 1.

In contrast to the patterns of uptake of these two mercurial agents by intact platelets, osmotically lysed platelets rapidly bind $1.56 \times 10^{-16}$ M/platelet of both agents. The striking difference in uptake patterns indicates that osmotically lysed platelets have become permeable to both PCMB and PCMBS as was true with red cell ghosts.

**ATPase Activities**

In the present investigation, two different ATPase activities have been characterized in osmotically lysed human platelets. One ATPase activity is Na$^+$, K$^+$ dependent and comprises 24 percent of the total ATPase activity of the osmotically lysed platelets. By analogy to other well studied cellular systems, this ATPase activity may be presumed to be responsible, in part, for the maintenance of platelet cellular cation content. Ouabain, PCMB and PCMBS all inactivate the Na$^+$, K$^+$ ATPase activity in osmotically-lysed platelets, establishing sensitivity of the Na$^+$, K$^+$ ATPase to cardiac glycosides and to sulfhydryl inhibitors. In the intact platelet, however, this Na$^+$, K$^+$ dependent ATPase activity is not essential for clot retraction or for ADP induced aggregation as shown by the failure to ouabain to influence these processes.

Table 1 summarizes the relative ATPase activities and patterns of inhibition of these activities measured in the lysed platelets. ATPase activity is expressed
Table 1.—Human Platelet Membrane ATPase* (× 10^{-9} M/mg./min.)

<table>
<thead>
<tr>
<th>1) Total Activity</th>
<th>5.1 ± 1.2</th>
<th>(100%)</th>
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<tbody>
<tr>
<td>(Mg^{++}, Na^{+}, K^{+})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2) (Total) Mg^{++}, K^{+}</td>
<td>3.9 ± 1.3</td>
<td>(76%)</td>
</tr>
<tr>
<td>3) Na^{+}, K^{+}</td>
<td>1.2 ± 0.4</td>
<td>(24%)</td>
</tr>
<tr>
<td>4) Mg^{++}, Na^{+}, K^{+} plus</td>
<td>3.8 ± 1.1</td>
<td>(74%)</td>
</tr>
<tr>
<td>5) PCMB (10^{-6} M)</td>
<td>0</td>
<td>(0%)</td>
</tr>
<tr>
<td>6) PCMBS (10^{-6} M)</td>
<td>0</td>
<td>(0%)</td>
</tr>
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*Mean ± 1 SD (based on 8 separate experiments). Each determination was carried out in quadruplicate.

†By subtraction of 2 from 1.

Fig. 2.—Effects of PCMB, PCMBS and ouabain added to platelet-rich plasma on intracellular K^{+} of platelets.

as 10^{-9} moles of ATP hydrolyzed per milligram of platelet protein per minute. The Na^{+}, K^{+} ATPase activity constitutes an average of 24 percent of the total activity. Ouabain at 10^{-4} M, which inhibits Na^{+}, K^{+} dependent ATPase activity in human erythrocytes, inhibits total ATPase activity to an extent equal to the estimation of Na^{+}, K^{+} activity. This is based on subtraction of Mg^{++}, K^{+} activity from total activity. In the lysed platelet, all ATPase activity is inhibited by PCMB and PCMBS. No difference in ATPase activities could be demonstrated if sodium citrate was used as an anticoagulant.

Platelet Cation Content

Figure 2 shows the effect of ouabain, PCMB and PCMBS on the K^{+} concen-
The addition of ouabain at $10^{-4}$ M resulted in a mean loss of 18 percent of the platelet K+ over thirty minutes of incubation. Both PCMB and PCMBS, at $2.5 \times 10^{-3}$ M in plasma produced a loss of intracellular K+ of 40 percent over thirty minutes. No K+ was lost from control platelets incubated for the same period.

**Effect on Platelet Volume**

Figure 3 illustrates the effects of ouabain, PCMB and PCMBS on the volume of platelets in plasma. The addition of ouabain at $10^{-4}$ M led to no change in platelet volume. This observation is in agreement with that of Buckingham and Maynert. The failure of ouabain to change platelet volume raises the question of whether platelet volume control may be related to another mechanism. Pump II of erythrocytes described by Hoffman, is not inhibited by ouabain and could represent such a mechanism. In contrast to ouabain, both PCMB and PCMBS at $2.5 \times 10^{-3}$ M in plasma produced an increase in platelet volume of 40 percent over a thirty minute period of incubation. Since this concentration of the mercurials produced marked loss of K+, the increase in platelet volume strongly implies a greater intracellular gain in Na+ and water.

**ADP-Induced Platelet Aggregation**

Both PCMB and PCMBS, at $2.5 \times 10^{-3}$ moles/L of plasma, inhibited ADP aggregation immediately. This inhibition of aggregation was manifest also at concentrations of ADP as high as $10^{-2}$ M. At intervals following platelet ex-
Table 2.—Effects of PCMB, PCMBS, and Ouabain on ADP-induced Platelet Aggregation and Thrombin-induced Clot Retraction, in Platelet-rich Plasma

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>$2.5 \times 10^{-3}$M PCMB</th>
<th>$2.5 \times 10^{-3}$M PCMBS</th>
<th>$10^{-9}$M Ouabain</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP Aggregation</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Clot Retraction</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
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Exposure to the mercurials, cysteine (Sigma Chemical Company, St. Louis, Missouri) at concentrations equal to that of the mercurials, was added to the platelet suspension. This sulfhydryl-containing compound is able to compete with the cellular sulfhydryl sites for the mercurial compounds. The cysteine experiments were done to evaluate reversibility of the inhibition of ADP-induced aggregation. Cysteine, when added up to one hour following mercurial exposure, reversed the inhibition of ADP aggregation. Thrombin induced platelet aggregation similarly is inhibited by these two mercurials and also is reversed by cysteine. Cysteine alone did not cause platelet aggregation. 

Ouabain, which blocks the Na$^+$, K$^+$ ATPase activity, did not interfere with ADP or thrombin induced aggregation (Table 2).

DISCUSSION

Previous work by others has shown that several aspects of normal platelet function can be altered by sulfhydryl inhibitors. Fantl, Ebbels and Nelson$^{17}$ demonstrated that PCMB inhibited the ability of human platelets to induce retraction of fibrin gels. Betex-Galland and Lüscher$^2$ showed that Salyrgan inactivated the ATPase activity of the purified contractile protein, thrombosethenin, obtained from human platelets. Robinson, Mason and Wagner$^{18}$ have shown that N-ethylmaleimide and PCMB interfere with the ability of pig platelets to aggregate with ADP and thrombin. Buckingham and Maynert$^6$ noted that platelets treated with PCMB lost K$^+$. Since sulfhydryl groups are known to be vital for the functional integrity of many enzyme proteins, it is not surprising that penetrating sulfhydryl inhibitors interfere with a variety of intracellular platelet functions. Alterations in a variety of membrane functions similarly can be expected from interference with surface sulfhydryl integrity. It should be emphasized, however, that sulfhydryl inhibition does not necessarily imply that the sulfhydryl groups are involved at the active center of in-
hindered enzymes. For example, sulphydryl inhibition may produce changes with resultant secondary allosteric effects as illustrated by Riggs' studies of hemoglobin and the Bohr effect.

In the present studies of organic mercurial compounds, the different inhibitory effects of the penetrating agent, PCMB, when compared to the relatively nonpenetrating PCMBS (Fig. 1) suggest an intracellular localization of the protein necessary for clot retraction. The failure of PCMBS to inhibit clot retraction at a concentration which produces functional platelet membrane alterations, and at which PCMB does inhibit clot retraction, suggests that clot retraction activity lies beneath an outer permeability barrier, presumably the platelet plasma membrane.

Adenosine diphosphate induced aggregation is blocked by both PCMB and PCMBS at equimolar concentrations. The physiologic role of ADP aggregation, described by Gaarder and associates in vivo hemostasis remains uncertain. Some authors have suggested that ADP aggregation and thrombin induced clot retraction are interdependent and sequential events while McLean, Maxwell and Hertler has suggested that they are independent processes. Although PCMB and PCMBS both interfere with ADP aggregation, PCMBS at the same concentration does not inhibit clot retraction. This information, coupled with our observation that PCMB irreversibly inhibits clot retraction at a time when ADP aggregation still can be restored by cysteine, indicates that ADP aggregation and clot retraction can exist as independent processes.

**SUMMARY**

The effects of PCMB and PCMBS on cation permeability, ADP aggregation, platelet volume and clot retraction have been studied in intact human platelets and have been compared to the effects of ouabain on the same functions. The influence of these agents on Mg\(^{2+}\)-dependent ATPase activity and Na\(^{+}\), K\(^{+}\) activated ATPase activity of osmotically lysed platelets also has been evaluated. (a) Ouabain, which produces inhibition of Na\(^{+}\), K\(^{+}\) dependent ATPase activity, has no influence on ADP-induced aggregation or clot retraction. (b) Both PCMB and PCMBS inhibit all ATPase activities in osmotically lysed platelets and inhibit ADP aggregation by intact platelets. Cysteine reverses the inhibition of ADP aggregation produced by both PCMB and PCMBS at a time when PCMB induced inhibition of clot retraction no longer is reversible. (c) Clot retraction is inhibited by the penetrating agent, PCMB, but is not affected by identical concentrations of PCMBS. These observations suggest that ADP aggregation and clot retraction are independent phenomena.

**SUMMARIO IN INTERLINGUA**

Le effectos de PCMB e de PCMBS super le permeabilitate cationic, le aggregation a ADP, le volume thrombocytic, e le retraction del coagulo esseva studiate in intacte thrombocytos human. Illos esseva comparate con le effectos de ouabaina super le mesme parametros. Le influentia del mentionate agentes super le Mg\(^{2+}\)-dependente activitate de ATPase e super le Na\(^{+}\), K\(^{+}\)-activate activitate de ATPase in omsomaticamente lysate thrombocytos esseva etiam evalutate.

(a) Ouabaina, que effectua le inhibition de Na\(^{+}\), K\(^{+}\)-dependente activitate de ATPase exerce nulle influentia super le ADP-inducite aggregation o retraction de coagulo.

(b) Tanto PCMB como etiam PCMBS inhibi omne activitate de ATPase in omsomaticamente
lysate thrombocytes etiam inhibi le aggregation de ADP per intacte thrombocytes. Cysteina reverte le inhibition del aggregation de ADP producite per PCMB o PCMBS quando le PCMB-inducite inhibition del retraction del coagulo ha cessate esser reversibile.

(c) Le retraction del coagulo es inhibite per le agente penetratori PCMB sed non es afficte per PCMBS al mesme concentrationes. Iste constatationes suggestione que le aggregation de ADP e le retraction del coagulo es mutualmente independente phenomenos.

REFERENCES


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