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Carbenicillin and Penicillin G Inhibit Platelet Function In Vitro by Impairing the Interaction of Agonists with the Platelet Surface

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ABSTRACT Carbenicillin or penicillin G administered in large doses can cause a bleeding diathesis as a result of platelet dysfunction. These antibiotics also inhibit platelet aggregation in vitro, although several-fold larger concentrations of drug are required to demonstrate this effect. We wondered whether these antibiotics might impair platelet function by interfering with the initial step of platelet activation: the binding of agonists to their specific receptors on the platelet surface.

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These studies demonstrate that carbenicillin and penicillin G inhibit platelet function in vitro by impairing the interaction of several agonists with their specific receptors on the platelet surface membrane. If this were mechanism operative in vivo, it could account for the hemorrhagic as well as the potential anti-thrombotic effects of these antibiotics.

INTRODUCTION

Large doses of the antibiotics, carbenicillin, and penicillin G, can cause a bleeding diathesis in patients (1, 2). These drugs presumably do so by impairing platelet function because they prolong the bleeding time of human volunteers (3, 4). Moreover, platelets from individuals taking these antibiotics aggregate poorly in response to several aggregating agents (3, 4) and they agglutinate poorly in response to ristocetin (2). Platelets incubated with carbenicillin or penicillin G in vitro also respond poorly to a variety of platelet agonists, although larger concentrations of antibiotic are required to demonstrate this effect (5). The mechanism by which these antibiotics impair platelet function is unknown.

Platelet adhesion, aggregation and secretion are initiated by the binding of agonists to their specific receptors on the platelet membrane (6, 7). We wondered, therefore, whether carbenicillin or penicillin G might inhibit platelets in vitro by interfering with the binding of agonists to the platelet surface. We studied two agonists for platelet aggregation, epinephrine, and ADP, as well as the plasma protein, von Willebrand factor, which participates in the adhesion of platelets to the vascular subendothelium (8). Epinephrine-induced platelet function is an α-adrenergic event (9, 10). Therefore, platelet α-adrenergic receptors were studied by measuring the binding of the alpha adrenergic antagonist, [3H]dihydroergocryptine, to platelets (11). ADP binding was assessed by measuring the covalent incorporation of an ADP affinity analogue, 5'-p-fluoro-
aggregation and for that of concentrations. Concentrations of cillin or penicillin (for penicillin) to control for the concentrations of cations in the antibiotic solutions. Platelet aggregation and [3H]serotonin release at pH 7.5 were studied in response to (-)-epinephrine (Winthrop Laboratories, Sterling Drug Co., New York), or ADP (Sigma Chemical Co., St. Louis, Mo.) as previously described (14). To study the release of [3H]serotonin induced by the calcium ionophore, A23187 (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.), platelets in plasma were labeled with [3H]serotonin, gel-filtered in Tyrode's-albumin buffer that contained 2 mM EDTA (16), and stimulated with A23187 for 2 min at 37°C. The release reaction was stopped by placing samples in an ice bath and then centrifuging samples at 4°C in preparation for counting 4C.

Alpha-adrenergic binding assays. Platelet-rich plasma was centrifuged at 2,000 g for 10 min. The resulting platelet pellet was resuspended and washed twice at 16,000 g for 10 min at 4°C in an incubation buffer that contained 50 mM Tris, 100 mM NaCl, and 5 mM EDTA, pH 7.4. Platelets were resuspended in the incubation buffer to a platelet count of 1 x 10^9/ml and mixed with 1/10 vol of an aqueous solution of either carbenicillin or penicillin G, or with NaCl or KCl as controls. α-adrenergic binding sites on intact platelets were quantitated as described previously (17, 18). Briefly, intact platelets (3 x 10^8 cells) were incubated with 1/10 vol of [3H]dihydroergocryptine (3H-DHE)* (26–39 Ci/mmol, New England Nuclear, Boston, Mass.) in duplicate 250 μl aliquots at 37°C for 50 min in equilibration studies and for various periods of time in kinetic studies. Binding reactions were stopped with 4 ml incubation buffer that contained 10 μM phenolamine at 37°C. Samples were then filtered over glass fiber filters, and the filters were washed within 20 s with 20 ml incubation buffer at 37°C (17, 18). Non-specific binding, determined by incubation of platelets simultaneously with 3H-DHE and 10 μM phenolamine, accounted for 20–40% of the total amount of radioactive label bound. All data are presented as specific 3H-DHE binding, defined as total minus non-specific binding. To monitor the stability of 3H-DHE in the presence of the antibiotics, 3H-DHE was incubated at 37°C with 50 mM carbenicillin or penicillin for 2 h. The radioligand was extracted with ethanol and migrated identically as native 3H-DHE on thin-layer chromatography (18).

In experiments measuring the rate of dissociation of 3H-DHE from platelets, 3H-DHE (2–4 nM) was incubated with platelets for 50 min at 37°C at which time 10 μM phenolamine or a 100-fold excess of incubation buffer was added. Incubations were continued at 37°C, and the amount of specific binding was determined at intervals for the next 60 min. In all kinetic experiments, the concentration of 3H-DHE relative to that of the receptor was >10:1.

It should be noted that platelet function studies with (-)-epinephrine (epinephrine) were performed in plasma, whereas binding studies with 3H-DHE were performed in protein-free buffer. Nonetheless, it is assumed that at the antibiotic concentrations used, most of the antibiotic in plasma was not bound to protein (19).

5'-p-fluorosulfonlbenzoyl [3H]adenosine incorporation into platelet membranes. Intact, gel-filtered platelets (16) or isolated platelet membranes (20) were incubated with carbenicillin (36–180 μM) or NaCl (72–360 μM) at 25°C for 15 min. 100 μM 5'-p-fluorosulfonlbenzoyl [3H]adenosine in dimethylformamide was then added and the incubations were continued for an additional 15 min at 37°C. The incorporation reaction was stopped by the addition of excess buffer at 4°C and the platelets or membranes were washed twice at 4°C (12). After washing, the intact, labeled platelets were lysed and their membranes isolated (20). Labeled membranes were dissolved in 10% sodium dodecyl sulfate, 0.2 M dithiotheritol, and 8 M urea and dialyzed for 19 h to remove unincorporated label. 300 μg membrane protein was applied to 5% polyacrylamide, 0.1% sodium dodecyl sulfate disk gels. After electrophoresis, the gels were sliced, and the labeled proteins were eluted from the gel slices and counted for tritium (12).

Interaction of von Willebrand factor with platelets. Platelets in plasma were fixed in 1% formalin, washed, and resuspended to 5 x 10^9/ml in Tris-buffered saline (21). 780 μl of carbenicillin, penicillin G, or control NaCl or KCl was added. Von Willebrand factor was assayed by a macroscopic agglutination method (22), with 0.1 ml fixed, washed platelets, 0.1 ml normal plasma pooled from 10 male donors, and 0.05 ml of 0.8 μg/ml serotonin (Sigma Chemical Co., Arlington, Va.; Pacific Hemostasis Laboratories, Bakersfield, Calif.). In separate experiments, platelets were agglutinated with the snake venom from Bothrops neuwiedii (0.62–40 μg/ml final concentration) (Sigma Chemical Co.) instead of ristocetin (23). Fixed platelets were also agglutinated with bovine factor VIII, using a bovine fibrinogen preparation (0.06–4 mg/ml final concentration) (Sigma Chemical Co.) as the source of bovine factor VIII (24). Factor VIII antigen levels were quantitated by the Laurell quantitative immunoelectrophoretic technique as modified by Zimmerman et al. (25). Factor VIII coagulant activity was measured by a standard one-stage assay (26). Two separate experiments were performed to rule out a fluid-phase interaction of the von Willebrand factor and penicillin. First, sucrose density gradient ultracentrifugation of von Willebrand factor (human cryoprecipitate) and [14C]penicillin G (Amersham Corp., Arlington Heights, Ill.) was performed on a 10–40% continuous sucrose gradient by the method of Moore et al. (27). Amounts loaded onto 5 ml gradients were: 250 μl of [14C]penicillin G (1 x 10^6 cpm; 31 mM), and 250 μl cryoprecipitate (100 μg/ml factor VIII antigen). Second, potential binding of antibiotic to von Willebrand factor was assessed by gel filtration (28), 400 μl of phosphate-buffered saline (pH 7.4) that contained factor VIII antigen (24.9 u/ml) and [14C]penicillin (10 mM; 4 x 10^6 cpm/ml) was applied to a 30 x 0.8 cm Sephadex G-25 (course) column equilibrated with the buffer that contained [14C]penicillin.

Abbreviations used in this paper: 3H-DHE, [3H]dihydroergocryptine; epinephrine, (-)-epinephrine.
Platelet membrane fluidity. The fluidity of platelet membrane lipids was assessed with the hydrophobic fluorescent probe 1,6-diphenyl-1,3,5-hexatriene by measuring fluorescence polarization and calculating fluorescence anisotropy (29).

Statistics. Mean values are expressed ±1 SEM. The difference between means was assessed by Student's \( t \) test for paired or unpaired data. Equilibrium binding of \(^3\)H-DHE with platelets was analyzed by the method of Scatchard (30). The inhibition constant (\( K_i \)) for epinephrine was calculated from the concentration of epinephrine that inhibited specific \(^3\)H-DHE binding by 50% (31). The association and dissociation rate constants for \(^3\)H-DHE binding to platelets were calculated as described by Newman et al. (11). Von Willebrand factor activity was determined by using log-log plots of percent plasma vs. platelet agglutination time (32).

RESULTS

Epinephrine-platelet interactions. Addition of carbenicillin or penicillin G (1–50 mM) to platelet-rich plasma resulted in the immediate inhibition of platelet function induced by 2 \( \mu \)M epinephrine. Platelet function was inhibited whether measured as the initial rate of platelet aggregation, as the amplitude of aggregation or as [\(^{14}\)C]serotonin release. The degree of inhibition was related to the concentration of antibiotic (Fig. 1). Carbenicillin inhibited the initial rate of aggregation half-maximally at ~24 mM, the aggregation amplitude half-maximally at 17 mM (Fig. 1), and [\(^{14}\)C]serotonin release half-maximally at 8 mM. Penicillin G was more potent than carbenicillin in that it inhibited the initial rate of aggregation half-maximally at 14 mM, the aggregation amplitude at 2.5 mM (Fig. 1), and [\(^{14}\)C]serotonin release at 2 mM. However, the inhibitory effects of each concentration of carbenicillin or penicillin G could be reversed by increasing the concentration of epinephrine (Fig. 2).

Epinephrine initiates platelet function by binding to platelet \( \alpha \)-adrenergic receptors. Because carbenicillin and penicillin G inhibited all phases of platelet function induced by epinephrine, we examined the effect of these antibiotics on platelet \( \alpha \)-adrenergic receptors by measuring the binding of the nonselective \( \alpha \)-adrenergic antagonist, \(^3\)H-DHE. \(^3\)H-DHE binds at equilibrium to a single class of binding sites on intact platelets and this binding demonstrates the saturability, high affinity, reversibility, and stereoselectivity char-
The studies described above document the effects of carbenicillin and penicillin G on the binding of an \( \alpha \)-adrenergic antagonist to the platelet, but they do not address the issue as to whether these antibiotics affect the binding of the \( \alpha \)-adrenergic agonist, epinephrine. Therefore, the affinity of epinephrine for platelet \( \alpha \)-adrenergic receptors was assessed by determining the potency with which epinephrine competed for \( ^3 \)H-DHE binding sites (31). Just as with \( ^3 \)H-DHE itself, the binding affinity of \( ^3 \)H-DHE sites for epinephrine was decreased twofold by carbenicillin \( (P < 0.02) \) and sixfold by penicillin G \( (P < 0.001) \) (Table I). Thus, the binding of \( ^3 \)H-DHE and epinephrine to platelet \( \alpha \)-adrenergic receptors was competitively inhibited by concentrations of antibiotic that competitively inhibited epinephrine-induced platelet aggregation and \( [^{14}C] \) serotonin release. Penicillin G was more potent than carbenicillin both in inhibiting the binding of the agonist and in inhibiting platelet function. The inhibitory effects of these antibiotics on platelet function and binding could be reversed either by a single washing of platelets or by gel filtration in antibiotic-free buffer (data not shown).

**ADP-platelet interactions.** Carbenicillin inhibited the initial rate of ADP-induced (2 \( \mu \)M) platelet aggregation half-maximally at 26 mM, the aggregation amplitude half-maximally at 14 mM, and \( [^{14}C] \) serotonin release half-maximally at 2 mM. Inhibition could be reversed by increasing the concentration of ADP 20-fold (Fig. 4).

The ADP affinity analogue, 5'-p-fluorosulfonylbenzoyl \([^{14}C] \) adenosine, inhibits ADP-induced platelet shape change as it is incorporated into a 100,000 mol wt polypeptide on the surface membrane of human platelets (12). Incorporation of this analogue is inhibited by ADP or ATP but not by other nucleotides or aggregating agents (12). Because many of the features of incorpora-

<table>
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<tr>
<th>Effect of Carbenicillin and Penicillin G on the Equilibrium Binding of ( ^3 )H-DHE to Human Platelets</th>
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<tr>
<td>( ^3 )H-DHE dissociation constant</td>
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<td>sites/platelet</td>
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<td>Control (9)*</td>
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<td>Carbenicillin, 36 mM (5)*</td>
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<td>Penicillin G, 31 mM (4)*</td>
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* Number of experiments performed.

**Table II**

<table>
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<tr>
<th>Effect of Penicillin G on the Kinetic Constants of ( ^3 )H-DHEDH Dihydroergocryptine Binding to Platelets</th>
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<tr>
<td>Association rate constant</td>
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<td>( M^{-1} \ min^{-1} )</td>
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<td>Control (6)*</td>
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<td>Penicillin G, 31 mM (6)*</td>
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* Number of experiments performed.
concentration as agglutination and this manner, ristocetin-induced platelet agglutination by penicillin inhibited its GCillin agglutination addition of carbenicillin Willebrand factor (12). polypeptides, than analogue ADP of the was decreased. To localize the site of action of carbenicillin to the platelet surface, gel-filtered platelets were incubated with carbenicillin for 15 min followed by the addition of the ADP analogue and incubation for a further 15 min. The extent of incorporation of the ADP analogue into the 100,000 mol wt membrane polypeptide was decreased (Fig. 5). When the ADP analogue is incubated with isolated platelet membranes rather than intact platelets, it is incorporated into four polypeptides, one of which is the 100,000 mol wt polypeptide (12). When isolated membranes were incubated with carbenicillin and the ADP analogue, incorporation of the analogue into all four membrane polypeptides was decreased.

Von Willebrand factor–platelet interactions. Von Willebrand factor binds to platelets in vitro upon the addition of ristocetin (33), and this results in platelet agglutination (13). The effect of carbenicillin and penicillin G on the interaction of von Willebrand factor with its platelet receptor was determined by measuring ristocetin-induced platelet agglutination in the presence of several concentrations of the antibiotics. Platelet agglutination induced by ristocetin (0.8 mg/ml) was inhibited by penicillin G in a concentration-dependent manner, and this effect was observed at a penicillin concentration as low as 0.6 mM (Figs. 6 and 7). Similarly, carbenicillin decreased ristocetin-induced platelet agglutination at a carbenicillin concentration as low as 2.6 mM. These inhibitory effects could be overcome by increasing the concentration of ristocetin. Von Willebrand factor also is known to mediate the platelet agglutination stimulated by certain snake venoms (23) or by bovine factor VIII alone (24). Carbenicillin and penicillin G inhibited platelet agglutination either in the presence of human plasma plus venom from the snake, Bothrops neuwiedi, or in the presence of bovine factor VIII alone (data not shown).

Three experiments were performed to exclude an interaction of von Willebrand factor with penicillin G or carbenicillin, independent of platelets. First, each antibiotic (36 mM) was incubated with normal pooled plasma for 30 min at 37°C. Neither antibiotic inhibited factor VIII coagulant activity or the level of factor VIII antigen present in this plasma. Second, [14C]penicillin and von Willebrand factor (assessed by measurement of factor VIII antigen) were subjected to sucrose density gradient ultracentrifugation. [14C]Penicillin added onto the gradient alone equilibrated at the top of the gradient, whereas factor VIII antigen added alone equilibrated near the bottom. Their location on the gradient was unaffected by the prior addition of ristocetin (0.8 mg/ml) or by preincubation of the [14C]penicillin G with the factor VIII antigen before their addition onto the gradient. Third, no binding of [14C]penicillin to factor VIII antigen was detected after gel filtration on Sephadex.
Thus, inhibition of ristocetin-induced platelet agglutination by these antibiotics appears to occur at
the platelet surface rather than in the fluid phase.

Effects of carbenicillin and penicillin G on platelet membranes. Neither carbenicillin nor penicillin G is
strongly lipophilic (19). However, penicillin G is more potent than carbenicillin in inhibiting platelet function
and agonist binding, and it is more lipophilic than carbenicillin (19). The interaction of lipophilic com-
ounds with membrane lipids can be monitored by hydrophobic fluorescent probes (34). The fluidity of
the platelet membrane lipid bilayer was assessed by labeling membranes with the fluorescent probe, 1,6-
diphenyl-1,3,5-hexatriene, to see whether either of the antibiotics interacted with membrane lipids. In three
experiments, neither antibiotic (1–50 mM) affected the fluorescence anisotropy of this probe within platelet
membranes, whether measured at 10°, 25°, or 37°C.

The platelet release reaction is believed to be
mediated by calcium ion fluxes between platelet mem-
branes and intracellular, nonmembranous cell compo-
ents. Such calcium ion fluxes and serotonin release
can be stimulated directly by the calcium ionophore,
A23187. To test whether carbenicillin or penicillin
G had inhibitory effects in addition to their effects on
the binding of agonists, the release of [3H]serotonin by
gel filtered platelets in response to A23187 was studied.
These studies were carried out in Tyrode’s-albumin
buffer in the presence of 2 mM EDTA and without
stirring to prevent platelet aggregation. When present
in concentrations that had inhibited epinephrine or
ADP-induced serotonin release by 70–100%, neither
antibiotic inhibited ionophore-induced [3H]serotonin
release (Table III).

DISCUSSION

These studies demonstrate that both carbenicillin and
penicillin G impair the interaction of several platelet
agonists with their specific receptors on the platelet
membrane. In the case of epinephrine, direct radio-
ligand binding studies demonstrate that these anti-
biotics cause a reduction in the apparent affinity
of platelet α-adrenergic receptors for [3H]DHE and for
epinephrine. In the case of ADP, carbenicillin inhibits
the covalent incorporation of an ADP affinity analogue,
5′-p-fluorosulfonylbenzoyl [3H]-adenosine, into a
platelet membrane polypeptide which may be the ADP
receptor. In both cases, the concentrations of anti-
biotic that inhibit binding also inhibit platelet aggrega-
tion and serotonin release. In addition, carbenicillin
and penicillin G appear to inhibit the interaction of von
Willebrand factor with the platelet surface, whether
this interaction is stimulated by ristocetin or by venom
from the snake, Bothrops neuwiedii. Taken together,
these data are consistent with the concept that im-
pairment of the interaction of agonists with the platelet
surface is causative of the platelet function abnor-
malities produced by these antibiotics in vitro.

The mechanism(s) whereby these antibiotics inhibit
the binding of agonists to the platelet surface is not
clear. The ADP receptor may be a surface membrane
glycoprotein with a mol wt of 100,000 (12), whereas the

![Figure 6](image-url)  
**Figure 6** Inhibition of ristocetin-induced platelet agglutination by penicillin G. In this example, control platelets
were mixed with 31 mM KCl (bottom line) and test platelets
were mixed with 0.6 mM penicillin G (closed circles) or with
32 mM penicillin G (open circles). The platelets were then
added to various dilutions of pooled normal plasma as a source
of von Willebrand factor and then stimulated with 0.8 mg/ml
ristocetin. The data are plotted as the agglutination time of
platelets versus the percent of normal pooled plasma. For
any given amount of plasma (von Willebrand factor), the
amount of time required for platelets to agglutinate was
increased in the presence of penicillin G.

![Figure 7](image-url)  
**Figure 7** Effect of penicillin G on apparent von Willebrand
factor activity. Apparent von Willebrand activity was quan-
titated from curves such as those shown in Fig. 5 (32). Each
experimental curve was compared to a control curve generated
on the same day. The von Willebrand factor activity of con-
trol platelets varied less than 10% from day to day.
platelet receptor for von Willebrand factor appears to be a glycoprotein of mol wt 150,000 (35). The platelet membrane component that binds epinephrine has not yet been characterized. Thus, receptors for agonists appear to include several different proteins or glycoproteins that are presumably embedded within the membrane's lipid bilayer and also exposed, in part, to the platelet's aqueous exterior. Theoretically, carbenicillin G might interfere with the binding of agonists to their receptors by interacting directly with these membrane proteins or by interacting with the lipid milieu in which these proteins are embedded (36).

Penicillin and carbenicillin are known to bind either covalently or reversibly to several bacterial membrane proteins (37). No information is available as to whether these antibiotics bind to the platelet. However, if they do bind to the platelet membrane, their binding affinity must be very low because washing or gel filtration of platelets reverses their inhibitory effects. Penicillin is also known to interact hydrophobically with artificial phospholipid bilayers (38). Thus, it could associate with platelet membrane lipids. In fact, penicillin, which is more lipid soluble than carbenicillin (19), was more inhibitory of platelet function as well. Neither antibiotic affected bulk platelet membrane lipid fluidity as determined by fluorescence polarization of a hydrophobic probe. However, a negative result with the fluorescent probe, which reports on the average motion of all membrane lipids, does not totally exclude a more localized yet significant interaction of the antibiotics with membrane lipids.

The decrease in apparent affinity of alpha adrenergic receptors for 3H-DHE in the presence of penicillin appears to be explained by a slower rate of association of 3H-DHE with the receptor, rather than an increased rate of dissociation of the ligand from the receptor. A similar change has been reported in the kinetics of binding of insulin to its receptors on cultured human lymphocyte membranes in the presence of the membrane perturbant, dimethyl sulfoxide (39).

Little is known concerning the sequence of cellular events that follows agonist binding and results in platelet aggregation and secretion. The synthesis of prostaglandin endoperoxides and thromboxane A2, as well as internal calcium shifts within the platelet appear to play a role (40, 41). An effect of carbenicillin or penicillin G on agonist binding does not exclude other potential mechanisms of platelet inhibition distal to binding. However, Johnson and co-workers (42) have reported that carbenicillin does not affect platelet prostaglandin synthesis and that it inhibits platelets by a mechanism different than aspirin. In this study, platelet [14C]serotonin release induced by the calcium ionophore, A23107, was not inhibited by carbenicillin or penicillin G. This suggests that a mechanism of inhibition unrelated to the process of internal calcium shifts.

There are two important differences between the inhibition of platelet function observed with these antibiotics in vitro and that observed in vivo. First, these antibiotics inhibit platelets immediately in vitro, whereas they appear to prolong the bleeding time only after 12–24 h of parenteral administration (2–4). Second, the peak serum concentration of either antibiotic achieved when high doses are given to man (1 mM) is several-fold lower than that necessary to inhibit platelet aggregation and secretion in vitro (Figs. 1 and 4). Therefore in man, either prolonged contact of platelets with lower concentrations of antibiotic or a metabolite of the antibiotic may be necessary for inhibition. Whatever the reason for these differences, the pattern of functional platelet abnormalities in individuals receiving carbenicillin or penicillin G is similar to the pattern produced in vitro: inhibition of the platelet aggregation and secretion induced by several agonists, this inhibition being reversed by larger concentrations of agonist. Nonetheless, the present in vitro studies do not directly establish the mechanism of platelet inhibition for these antibiotics in vivo.

The demonstration that ristocetin-induced platelet agglutination is decreased by these antibiotics in vitro suggests that impaired interaction of this plasma protein with the platelet might account, in part, for the prolonged bleeding time produced by these drugs. Indeed, we have confirmed the report of Andrassy et al. (2) demonstrating decreased ristocetin-induced platelet agglutination associated with prolonged bleeding times in several patients receiving large doses of carbenicillin or penicillin G.² It is recognized that the process of ristocetin-induced platelet agglutination does not necessarily reflect the complex process of von Willebrand factor-platelet-subendothelium interaction in vivo (43).

² Shattil, S. J. Unpublished observations.

### TABLE III

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<tr>
<th>Effect of Carbenicillin and Penicillin G on A23187-induced [14C]Serotonin Release from Gel-filtered Platelets</th>
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* Mean of four experiments.
Nonetheless, impairment of such interaction by carbencillin or penicillin G could explain the reported antithrombotic activity of these antibiotics in dogs (44, 45) as well as the failure of penicillin-treated platelets to correct the bleeding time of thrombocytopenic rabbits (46).

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