Platelet Dysfunction Induced by Parenteral Carbenicillin and Ticarcillin

Studies of the Dose-Response Relationship and Mechanism of Action in Dogs

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Sequential studies of platelet function were performed in dogs receiving continuous intravenous carbenicillin (CARB) or ticarcillin (TIC). Dose- and time-dependent platelet dysfunction was uniformly observed during the administration of CARB or TIC, 250 to 1000 mg/kg/24 hr. ADP-induced primary and secondary platelet aggregation was markedly inhibited within 24 to 48 hours in dogs receiving 750 or 1000 mg/kg/24 hr, but maximum impairment of aggregation did not occur until 3 to 5 days in dogs receiving 250 or 500 mg/kg/24 hr. Platelet glass bead column retention was abnormal in all dogs studied, and platelet factor 3 availability was impaired in 91%. Collagen-induced platelet aggregation was consistently impaired and the bleeding time was prolonged only during the infusion of ≥ 750 mg/kg/24 hr. Plasma fibrinogen concentrations and thrombin times remained normal. CARB and TIC infusions resulted in inhibition of ^{14}C-serotonin release and slightly decreased platelet ADP, while serotonin, ATP, and ultrastructure remained unchanged. The mutual correction of abnormal platelet aggregation by mixing CARB or TIC platelets with aspirin-treated platelets suggested that CARB and TIC inhibited the platelet release reaction by a mechanism other than inhibition of platelet cyclo-oxygenase. The platelet inhibitory properties of CARB and TIC demonstrated in this study suggest that they may be useful antithrombotic agents. (Am J Pathol 91:85–106, 1978)

HEMORRHAGIC PHENOMENA occurring in patients receiving carbenicillin (α-carboxybenzyl penicillin) (CARB) first suggested that impaired hemostasis resulted from treatment with this drug.[1-4] The initial observations were made in patients with renal failure,[1,2] but, shortly thereafter, other patients with normal renal function were reported to exhibit cutaneous and mucous membrane hemorrhagic phenomena during the administration of large doses of CARB.[3,4] Abnormal platelet function was implicated as the cause of bleeding.[3] Platelet aggregation in response to adenosine diphosphate (ADP) was found to be abnormal in patients receiving CARB, 500 to 750 mg/kg/24 hr, and the majority had

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prolonged bleeding times.[3] Subsequent *in vitro* studies confirmed the human platelet aggregation inhibitory properties of CARB.[3,5,6] The platelet release reaction was impaired following the *in vitro* incubation of platelets with high concentrations of CARB and penicillin G.[5] CARB and ticarcillin (α-carboxy-3-thienylmethyl penicillin) (TIC) administered to human volunteers in doses comparable to those commonly used to treat serious infections uniformly resulted in impaired platelet function.[7,8] A dose-response relationship between the amount of drug administered and the degree of platelet dysfunction induced was suggested by these studies. In addition, the bleeding time was not maximally prolonged until after several days of drug administration.[7,8] Preliminary studies conducted in this laboratory[9] also suggested that CARB-induced platelet dysfunction was dose- and time-dependent. To further define the relationship of drug dose and duration of administration to the severity of platelet dysfunction induced and to investigate the responsible mechanisms, the effect of parenteral CARB and TIC on dog platelet function was observed.

Materials and Methods

Animals

Nineteen adult mongrel dogs weighing 15 to 23 kg were selected from a larger sample of randomly chosen animals. These 19 dogs were chosen for study because they had blood platelets which aggregated maximally with a final ADP concentration of at least 21.2 μM prior to drug administration.

Drug Administration

CARB and TIC (kindly supplied by Beecham Laboratories, Bristol, Tenn.) are semisynthetic penicillins whose structures differ in regard to the side chain attached to the penicillin (6-aminopenicillanic acid) nucleus. CARB is disodium α-carboxybenzyl penicillin and TIC is disodium α-carboxy-3-thienylmethyl penicillin.[10] They have a similar spectrum of antimicrobial activity.[11,12]

CARB and TIC were administered intravenously (IV) via a polyethylene catheter inserted in an extremity vein of each dog anesthetized with sodium pentobarbital using a modification of the method of Dudrick et al.[13] Following the insertion of the catheter, 5% dextrose in water was infused at a rate of 500 ml 24 hr for 3 to 7 days. CARB and TIC were diluted with 5% dextrose and administered at a constant rate of 500 ml 24 hr by means of an infusion pump for intervals varying from 24 hours to 3 weeks, without apparent adverse effects on the animals.

Studies Performed

Thirty-five sequential studies were performed in 15 dogs receiving continuous IV infusions of CARB or TIC. The following number of studies was performed at the specified dose in animals receiving IV CARB or TIC: 250 mg/kg/24 hr, 7; 500 mg/kg/24 hr, 7; 750 mg/kg/24 hr, 6; 1000 mg/kg/24 hr, 12; 1500 mg/kg/24 hr, 1; 2000 mg/kg/24 hr, 2.

Serum Antibiotic Concentration

Serum concentrations of CARB and TIC were determined by Beecham Laboratories, Bristol, Tenn., using a petri dish assay method.[14] *Pseudomonas aeruginosa* was the test organism. A standard curve was constructed by measurement of zones of inhibition surrounding wells containing known concentrations of drug.

Blood Drawing Technique

Blood was obtained by jugular venipuncture using a two-syringe technique. Blood was drawn in plastic syringes and immediately mixed with the appropriate anticoagulant in plastic tubes.

Plasma Preparation

Platelet-rich plasma (PRP) was obtained by centrifugation at 200*g* for 8 minutes. Platelet-poor plasma (PPP) was obtained by centrifugation at 2000*g* for 20 minutes.

Platelet Count

Platelets were counted in EDTA anticoagulated plasma following gravity sedimentation of erythrocytes utilizing an electronic particle counter (Coulter Model B) and the method of Bull et al.[15]

Platelet Aggregation

Platelet aggregation was performed in a Payton aggregometer using a modification of the method of Born and Cross.[16] PRP anticoagulated with 3.8% sodium citrate (v/v, 9:1) was diluted to 200,000/cu mm with PPP prior to the addition of aggregating agents. PPP was used to define the maximum change in optical density obtainable for each specimen. Platelet aggregation was measured as a percent of this maximum change in optical density. ADP in final concentrations varying from 1.06 to 212 μM was added to PRP diluted to 200,000/cu mm, and maximum aggregation was recorded. Prior to treatment, most normal dogs studied had a maximal aggregation response to 5.3 or 10.6 μM, but some required 21.2 μM. Dog tendon collagen prepared from a saline extract of a crude homogenate of Achilles tendon was also used as an aggregating agent. A normal control dog whose aggregation response was well documented was studied along with each drug-treated animal to document the potency of the aggregating agents.

Platelet Glass Bead Column Retention

A modification of the method of Bowie et al [17] was used. Nine parts whole blood was added to 1 part 3.8% sodium citrate containing 10 units of heparin in polycarbonate plastic tubes. After mixing by inverting gently six times, the blood was immediately passed through a glass bead column containing 5.2-g glass microspheres (Superbrite, 3M Company, St. Paul, Minn.) at a speed of 3.9 ml/min. Each study was done in duplicate, and the mean of the two determinations was used for comparison. Counting the platelets in the fourth milliliter to emerge from the column, normal platelet retention was found to be 92 $\pm$ 4.9% (mean $\pm$ 1 SD) in 100 determinations performed on blood from 28 normal dogs.

Bleeding Time

The bleeding time was determined by a modification of the template method of Mielke et al.[18] A No. 11 surgical blade fixed in a blade holder and guided by a template was used to make an incision 1 cm long and 1.5 mm deep in the clean-shaven skin of the lateral distal foreleg of the dog. A blood pressure cuff was inflated to 40 mmHg around the proximal leg,

and the pressure was maintained during the duration of bleeding. The mean bleeding time (three incisions) for normal dogs was 2.7 ± 0.9 (mean ± 1 SD) in 117 determinations performed on 25 normal dogs. The range was 1.5 to 5 minutes.

Platelet Factor 3 Availability

The method of Hardisty and Hutton [19] was modified so that the concentration of platelets in the PRP was adjusted to 50,000/cu mm by the addition of PPP. Two-tenths milliliter of this dilute PRP (PRP- D) was incubated for 20 minutes with 0.2 ml of kaolin (5 mg/ml) suspended in buffered saline. After the addition of 0.1 ml of calcium chloride (0.035 M), the clotting time was determined. Each study was done in duplicate and control studies were performed with each determination. The control tubes contained either PRP-D plus buffered saline or PPP plus kaolin. The clotting time for the kaolin incubated PRP-D (PF-3 clotting time) was 31.0 ± 3.7 seconds (mean ± 1 SD) in 100 determinations performed on blood from 27 normal dogs.

Platelet Ultrastructure

Platelet ultrastructure was studied before and after the administration of CARB or TIC by the method of White.[20]

Platelet Adenine Nucleotides

Total platelet adenine nucleotides, ADP, and ATP were measured by the firefly luciferase method of Holmsen et al,[21] using the extraction procedure of Rao et al.[22]

Platelet Serotonin Content

Endogenous platelet serotonin was measured by the perchloric acid extraction method of Rao et al.[22]

Platelet ^{14}C-Serotonin Release

The release of ^{14}C-labeled serotonin following exposure of platelets to aggregating agents *in vitro* was studied by the method of Jerushalmy and Zucker [23] modified by White et al.[24]

Platelet Mixing Studies

Equal numbers of platelets obtained from dogs treated with CARB or TIC and from dogs treated with oral aspirin were mixed with normal platelets and with each other, and their aggregation response to ADP and collagen was observed. Aggregation responses and release of ^{14}C-serotonin were studied by the method of Gerrard et al.[25]

In Vitro Studies

The *in vitro* effects of CARB and TIC on platelet aggregation were evaluated after the addition of either drug to PRP. Platelet aggregation in response to ADP and collagen was observed after incubation at 37 C for up to 2.5 hours. The aggregation responses obtained with platelets incubated with drug were compared with those of PRP incubated at 37 C for the same period.

Fibrinogen

Plasma fibrinogen was determined before and during drug administration in 10 studies by the thrombin clottable method of Swaim and Feders.[26] Pretreatment fibrinogen values ranged from 178 to 462 mg/100 ml.

Thrombin Time

The thrombin time (TT) was determined before and during drug administration in 10 dogs by a modification of the method of Penner.[27] The normal TT for this method was 13.7 ± 1.69 seconds (mean ± 1 SD) in 54 normal dogs.

Statistical Methods

The significance of differences between mean values was determined by the Student *t* test.[28] *P* values of 0.05 or less were considered significant.

Results

Control Infusions

Prior to the administration of CARB or TIC, platelet count, platelet glass bead column retention, and platelet aggregation studies were performed at 24- to 48-hour intervals for 3 to 7 days during the infusion of 5% glucose until platelet function studies were normal and stable.

Serum Antibiotic Concentration

The mean concentrations of drug found in the dogs' plasma during continuous IV infusion of several doses are recorded in Table 1. Serum drug concentrations were found to be proportional to the dose of drug administered. Although the serum concentrations attained during the infusion of TIC were consistently higher than those found with the same dose of CARB, equivalent effects on platelet function were observed when equal doses of the two drugs were administered. Therefore, the data obtained from studies done with equivalent drug doses were pooled for all the subsequent analyses.

Table 1—Serum Drug Concentration During Intravenous Administration of CARB and TIC

Drug	Dose (mg/kg/24 hr)	Duration (hr)	No. of studies	Drug concentration (μg/ml)	
				Mean	Range
Carbenicillin	250	4–24	4	22.1	7.4–40.7
	500	4–24	4	63.9	42.6–76.5
	750	4–24	4	83.7	49.1–109.7
	1000	4–24	5	154.9	80.0–367.8
	1500	72	1	360.0	360.0
Ticarcillin	250	4	2	65.0	52.9–77.1
	500	4	2	100.7	75.1–126.4
	750	4–24	3	122.2	53.7–173.6
	1000	4–48	5	183.1	82.4–270.5

Platelet Count

The peripheral blood platelet count remained in the normal range during all but 3 of 35 studies. Three dogs receiving CARB or TIC, $\geq$ 1000 mg/kg/24 hr, had nadir platelet counts of 99,000, 92,000, and 28,000/cu mm. Prompt recovery occurred when the drug was discontinued.

Platelet Function Studies

ADP-Induced Aggregation

Platelet aggregation in response to ADP was decreased in all 35 studies and in all 15 animals receiving CARB or TIC. The degree of inhibition induced and the rate at which the defect developed varied with the dose administered within the range of 250 to 1000 mg/kg/24 hr (Table 2, Text-figure 1). Impaired ADP-induced aggregation was noted in individual studies as early as 4 hours after the drug infusion was begun. Compared with pretreatment values, mean ADP-induced aggregation was significantly decreased at 24 hours during the infusion of 500 to 1000 mg/kg/24 hr and at 48 hours during the infusion of 250 mg/kg/24 hr. Thereafter, mean ADP-induced aggregation became progressively more decreased with continued drug administration. Following administration of 1000 mg/kg/24 hr, maximum inhibition of the aggregation response was observed at 24 to 48 hours, although it did not occur until 5 to 7 days when 250 mg/kg/24 hr was administered (Table 2, Text-figure 1). Doses of 250 and 500 mg/kg/24 hr did not result in significantly different aggregation responses up to 5 days, and doses of 750 and 1000 mg/kg/24 hr did not result in significantly different aggregation responses at 48 hours. Therefore, the data were pooled for low-dose (250 and 500 mg/kg/24 hr) and high-dose (750 and 1000 mg/kg/24 hr) studies, and their effects on ADP-induced aggregation were evaluated. The differences between the mean ADP-induced aggregation response of the high- and low-dose groups were significant at 24 hours, 48 hours, and 72 hours, but at 5 days the mean aggregation responses of the two groups were not significantly different.

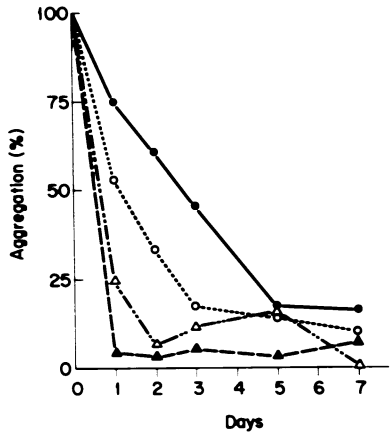
In 7 dogs receiving 250 to 1000 mg/kg/24 hr the infusions were continued for 9 to 21 days. ADP-induced aggregation responses were not significantly different from those observed at 5 or 7 days.

Abnormal ADP-induced aggregation developed in three stages. The first stage, noted within 4 to 24 hours, was a change from the usual single-phase response observed in dogs to a two-phase response similar to that seen with human platelets. The second stage, usually observed within 24 hours, was a slight to moderate decrease in aggregation, apparently due to inhibition of the secondary phase of ADP-induced aggregation. Deaggre-

Table 2—Mean Platelet ADP-Induced Aggregation (Percent of Pretreatment Maximum) During Intravenous Administration of CARB and TIC

Dose (mg/kg/24 hr)	ADP concentration	Mean aggregation (%) 12 hr	24 hr	48 hr	72 hr	5 day
250	Minimum*		75.0 ± 14.5†(6)	60.7 ± 13.8(6)	45.5 ± 25.6(4)	17.4 ± 4.4(5)
	Minimum × 4		92.9 ± 3.09(6)	97.2 ± 1.7(6)	80.2 ± 9.9(4)	87.6 ± 7.8(5)
500	Minimum		53.6 ± 11.4(8)	33.2 ± 11.0(5)	15.5 ± 21.9(2)	14.8 ± 4.6(5)
	Minimum × 4		80.0 ± 7.7(8)	87.5 ± 8.8(6)	100.0(2)	73.6 ± 12.5(5)
750	Minimum		24.5 ± 14.8(2)	7.0 ± 5.0(4)	12.0 ± 17.0(2)	16.0 ± 32.0(2)
	Minimum × 4		90.0 ± 14.2(2)	66.4 ± 15.2(5)	74.0 ± 2.8(2)	—
1000	Minimum		4.3 ± 2.5(7)	3.5 ± 3.8(6)	5.5 ± 3.1(8)	3.2 ± 2.7(6)
	Minimum × 4		73.1 ± 14.4(8)	80.8 ± 11.3(6)	25.2 ± 12.7(8)	26.8 ± 12.0(7)
2000	Minimum	53 (1)	0(1)	0(1)		
	Minimum × 4	82 (1)	0(1)	0(1)		
Significance of differences between means observed with minimum ADP	250 vs 500		$P < 0.040$	$P < 0.20$		$P > 0.50$
	250 vs 1000		$P < 0.001$	$P < 0.005$	$P < 0.05$	$P < 0.025$
	750 vs 1000			$P < 0.50$		

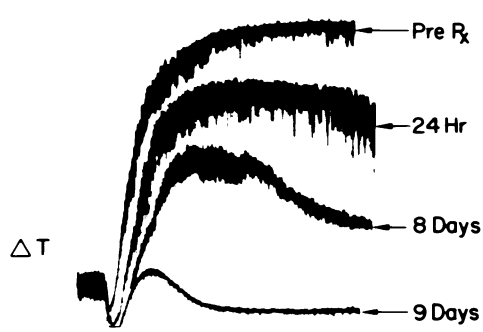
* Minimum concentration of ADP required to induce maximum aggregation prior to treatment
† SEM
No. of observations is indicated in parentheses.



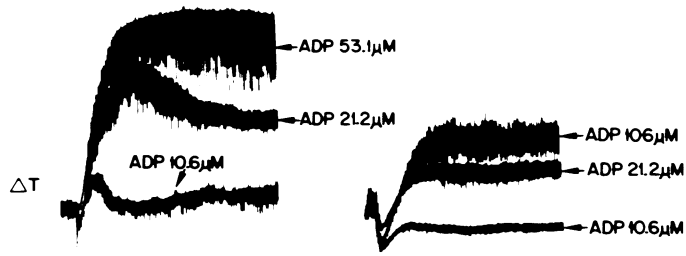
TEXT-FIGURE 1—ADP-induced platelet aggregation in dogs receiving CARB or TIC intravenously (*solid circles*, 250 mg/kg 24 hr [7 studies]; *open circles*, 500 mg kg 24 hr [8 studies]; *open triangles*, 750 mg kg 24 hr [6 studies]; *solid triangles*, 1000 mg kg 24 hr [12 studies]). Aggregation is expressed as a percent of the maximum pretreatment response obtained with a minimum concentration of ADP. Each point represents the mean of two to seven determinations calculated from pooled CARB and TIC studies.

gation usually followed this suboptimal response (Text-figure 2). The third stage, observed at 24 hours and thereafter, was characterized by marked inhibition of the secondary phase of aggregation and variable degrees of inhibition of the primary phase (Text-figure 2). This sequence developed more rapidly with higher drug doses, but the second and third stages were observed consistently.

Although a comparable degree of inhibition of the platelet response to the minimum concentration of ADP (required to produce maximum aggregation prior to treatment) was achieved by lower (250 and 500 mg/kg/24 hr) and higher (750 and 1000 mg/kg/24 hr) doses of drug after 5 days (Table 2), a more severe defect was induced by higher doses. Higher concentrations of ADP were able to overcome the aggregation inhibition induced by lower doses of drug (250 and 500 mg/kg/24 hr), but 4 to 10 times the minimum concentration of ADP resulted in suboptimal aggregation following treatment with 750 and 1000 mg/kg/24 hr (Table 2, Text-figure 3).



TEXT-FIGURE 2—Sequential studies of ADP-induced (21.2 μM final concentration) platelet aggregation in 1 dog receiving TIC (500 mg/kg 24 hr) intravenously for 9 days. Δ *T* = change in light transmission.



TEXT-FIGURE 3—Sequential studies of ADP-induced platelet aggregation in 1 dog receiving CARB, 250 mg kg 24 hr (*left*), compared with ADP-induced platelet aggregation in 1 dog receiving CARB, 1000 mg kg 24 hr (*right*), intravenously for 5 days. The final ADP concentration used to induce aggregation is indicated for each curve. ΔT = change in light transmission.

Collagen-Induced Aggregation

Platelet aggregation in response to dog tendon collagen was much less inhibited by CARB or TIC than was ADP-induced aggregation. At doses of 250 and 500 mg/kg/24 hr, collagen-induced aggregation was generally unchanged or only slightly decreased (Table 3, Text-figure 4). Usually the interval between the addition of collagen and the onset of aggregation was slightly prolonged, and occasionally aggregation was slightly decreased. More variable responses were noted with 750 mg/kg/24 hr, but 1000 mg/kg/24 hr usually resulted in marked inhibition of aggregation (Table 3, Text-figure 4). Mean collagen-induced aggregation at 48 and 72 hours was not significantly different from pretreatment values when doses of 250, 500, and 750 mg/kg/24 hr were administered. From 48 hours to 5 days, however, mean collagen-induced aggregation was significantly impaired during the infusion of $\geq$ 1000 mg/kg/24 hr (Table 3). Collagen-induced aggregation was decreased to less than 50% of pretreatment aggregation in 8 of 11 dogs receiving $\geq$ 1000 mg/kg/24 hr for 3 to 7 days. Bleeding times were determined in 6 of these 8 dogs. Five of the six had prolonged bleeding times (>5 minutes). Drug-induced inhibition of aggregation was more readily overcome by increasing the concentration of collagen than it was with ADP (Table 3).

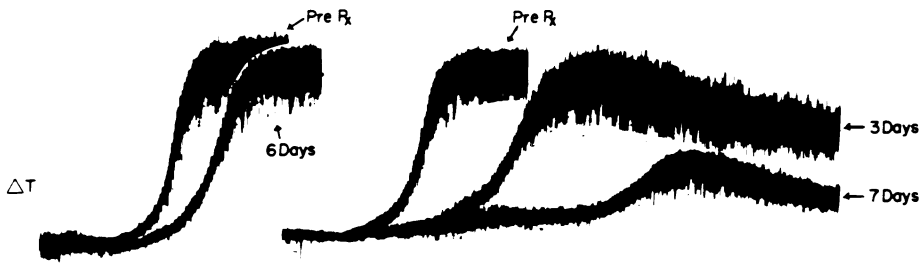
Platelet Glass Bead Column Retention

Platelet retention in glass bead columns was decreased during all studies. Abnormal platelet retention was observed as early as 4 hours, and within 24 hours after the initiation of the drug infusion the mean platelet retention was significantly decreased (70% or less) in each of the four drug dose groups (Text-figure 5). As with platelet aggregation, the inhibition of platelet retention was dose- and time-dependent. Although these relation-

Table 3—Mean Platelet Collagen-Induced Aggregation (Percent of Pretreatment Maximum) During Intravenous Administration of CARB and TIC

Dose (mg/kg/24 hr)	Collagen concentration	Mean aggregation (%) 24 hr	48 hr	72 hr	5 days
250	Minimum*	89.6 ± 2.7†(6)	95.6 ± 2.9(5)	90.2 ± 6.6(4)	95.8 ± 3.5(5)
500	Minimum	83.9 ± 6.6(8)	91.0 ± 6.6(6)	91.0 ± 12.7(2)	76.7 ± 12.0(3)
750	Minimum	95.7 ± 5.3(3)	69.4 ± 20.5(5)	86.5 ± 19.1(2)	84.0 ± 22.6(2)
1000	Minimum	50.3 ± 14.6(8)	24.2 ± 18.1(5)	17.3 ± 4.3(9)	45.5 ± 16.9(3)
	Minimum × 2	90.6 ± 4.5(5)	65.3 ± 17.3(3)	76.8 ± 8.9(8)	88.0 ± 5.17(2)
2000	Minimum	47.0(1)	14.0(1)		
	Minimum × 2	91.0(1)	58.0(1)		
Significance of differences between means observed with minimum collagen concentrations	250 vs 500	$P > 0.50$	$P > 0.50$	$P > 0.50$	$P < 0.10$
	250 vs 1000	$P < 0.05$	$P < 0.005$	$P < 0.001$	$P < 0.025$
	750 vs 1000	$P < 0.10$	$P < 0.20$		

* Minimum concentration of collagen required to induce maximum aggregation prior to treatment
† SEM
No. of observations is indicated in parentheses.

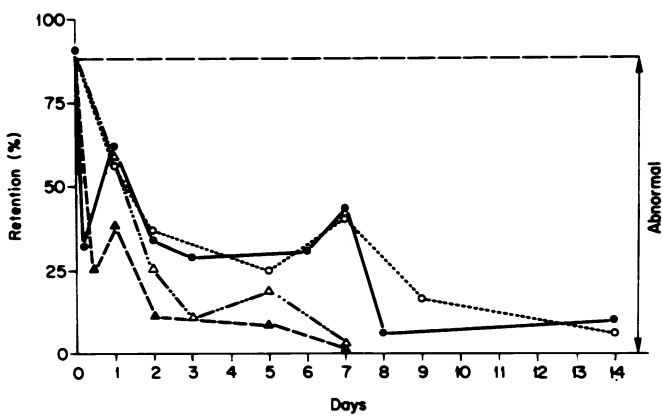


TEXT-FIGURE 4—Sequential studies of collagen-induced aggregation in 1 dog receiving CARB, 500 mg kg 24 hr (*left*), compared with collagen-induced aggregation in 1 dog receiving CARB, 1000 mg kg 24 hr (*right*), intravenously. The same final concentration of collagen was used for the pretreatment and posttreatment studies. ΔT = change in light transmission.

ships were more variable than those observed with ADP-induced aggregation, in general, higher drug doses (750 and 1000 mg/kg/24 hr) resulted in lower platelet retention which developed more rapidly than when lower doses (250 and 500 mg/kg/24 hr) were administered. The mean differences between the low-dose and high-dose groups were significant at 48 hours and 7 days. However, the maximum degree of impairment of platelet retention attained after 7 days was similar for each drug dose (Text-figure 5).

Bleeding Time

The template bleeding time was determined sequentially during 20 studies in which the platelet count remained normal. Prolongation of the



TEXT-FIGURE 5—Platelet glass bead column retention in dogs receiving CARB or TIC intravenously (*solid circles*, 250 mg kg 24 hr [6 studies]; *open circles*, 500 mg kg 24 hr [7 studies]; *open triangles*, 750 mg kg 24 hr [6 studies]; *solid triangles*, 1000 mg kg 24 hr [6 studies]). Each point represents the mean of two to six determinations calculated from pooled CARB and TIC studies.

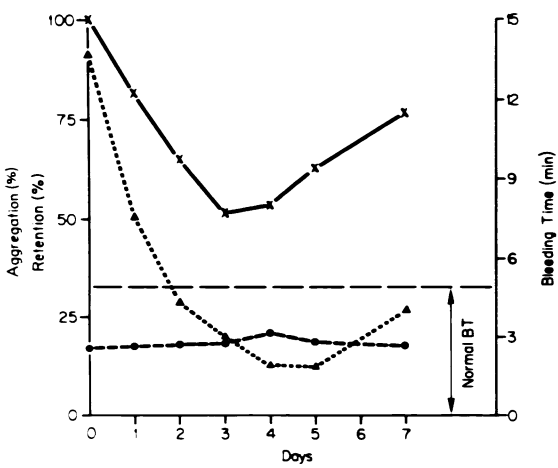
bleeding time (>5 minutes) was observed in 2 of 7 dogs receiving CARB or TIC, 250 or 500 mg/kg/24 hr; 6 of 12 dogs receiving 750 or 1000 mg/kg/24 hr had a prolonged bleeding time. One dog receiving 2000 mg/kg/24 hr also had a prolonged bleeding time. With two exceptions the template bleeding time was prolonged only when collagen-induced aggregation was markedly impaired (<50% pretreatment aggregation) (Text-figures 6 and 7). Although mean platelet glass bead column retention was slightly lower in the dogs that developed prolonged bleeding times, it was significantly decreased in both groups (Text-figures 6 and 7). Prolongation of the bleeding time was related primarily to inhibition of collagen-induced aggregation produced by higher drug dosage.

Platelt Factor 3 (PF-3) Availability

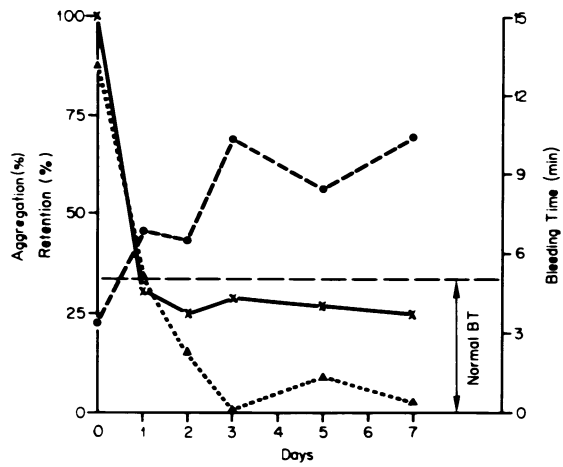
The PF–3 clotting time was prolonged (>5 seconds beyond pretreatment time) during 91% of the 22 studies in which this test was performed. PF–3 clotting times greater than 2 standard deviations beyond the mean of normal dogs occurred during 8 of 12 CARB studies and 3 of 8 TIC studies.

Platelet Ultrastructure

The ultrastructure of platelets from 4 dogs receiving CARB or TIC, 250 to 1000 mg/kg/24 hr, was examined. Platelet aggregation defects typical of those described for animals receiving low- and high-dose antibiotic were observed in these dogs. The ultrastructure of platelets from the CARB- or TIC-treated animals did not differ from that of untreated control dogs.



TEXT-FIGURE 6—Bleeding time (*circles*), collagen-induced platelet aggregation (Xs), and platelet glass bead column retention (*triangles*) in 9 dogs receiving CARB or TIC (500 to 1000 mg kg 24 hr) intravenously, whose bleeding time remained normal during treatment. Each point represents the mean of two to nine determinations calculated from pooled CARB and TIC studies.



TEXT-FIGURE 7—Bleeding time (*circles*), collagen-induced platelet aggregation (Xs), and platelet glass bead column retention (triangles) in 9 dogs receiving CARB or TIC (500 to 2000 mg kg 24 hr), whose bleeding time was prolonged during treatment. Each point represents the mean of two to nine determinations calculated from pooled CARB and TIC studies.

Platelet Serotonin, ADP, and ATP

Although a slight to moderate decrease in platelet ADP (15 to 61%) was observed during 4 of 5 studies following the administration of CARB or TIC for 3 to 7 days, only 1 dog receiving TIC, 750 mg/kg/24 hr, had a decrease in ADP of more than 50%. Platelet serotonin and ATP did not change significantly compared with pretreatment values (Table 4). The platelet ATP/ADP ratio and serotonin content remained within the normal range observed in our laboratory during all studies. No relationship between platelet aggregation responses and platelet serotonin or adenine nucleotides was suggested by these studies (Table 4).

^{14}C-Serotonin Release

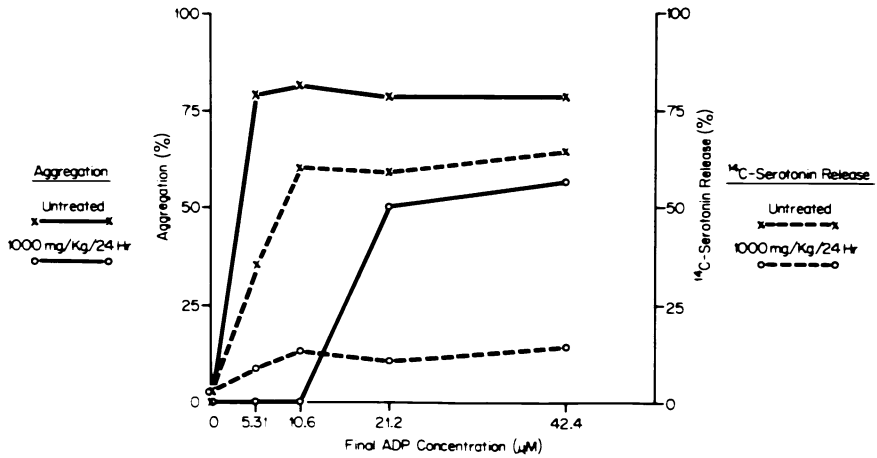
The release of radioactivity from platelets incubated with ^{14}C-serotonin before exposure to aggregating agents was markedly inhibited by CARB or TIC, 1000 mg/kg/24 hr, administered to 3 dogs for 3 to 6 days (Text-figure 8). Inhibition of ^{14}C-serotonin release was accompanied by marked inhibition of ADP aggregation in response to lower concentrations of ADP (5.31 to 10.6 μM), but suboptimal aggregation (60 to 70% of pretreatment aggregation) could be induced with higher concentrations of ADP (21.2 to 42.2 μM) in spite of markedly impaired ^{14}C-serotonin release (Text-Figure 8).

Mixing of CARB or TIC and Aspirin Platelets

Dog platelets rendered unresponsive to aggregating agents following the administration of CARB or TIC and aspirin were mixed in equal pro-

Table 4—Serotonin (5HT), ADP, and ATP Content of Platelets Before and After Intravenous Administration of CARB or TIC

Dog	Dose (mg/kg/24 hr)	Duration (days)	Aggregation (%)		5HT (ng/10^9)	ATP (μM/10^{11})	ADP (μM/10^{11})	ATP/ADP ratio
			ADP	Collagen				
1	None	—	100	100	1436	2.99	0.58	5.26
	TIC 250	4	39	80	1930	2.80	0.49	5.79
1	None	—	100	100	2449	2.63	0.78	3.36
	CARB 250	4	39	100	2141	2.18	0.58	3.81
2	None	—	100	100	2162	1.88	1.17	1.62
	TIC 750	4	0	60	2324	1.50	0.46	3.25
3	None	—	100	100	1856	2.96	1.24	2.41
	TIC 750	3	7	100	1284	2.11	0.78	2.77
1	None	—	100	100	2449	2.63	0.78	3.36
	CARB 1000	7	18	66	2690	2.83	0.76	3.72
Normal (7 dogs)								
Mean					1937			3.09
Range					1245-2449			1.62-5.26

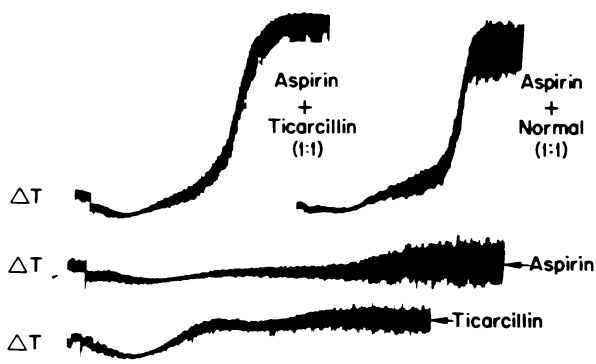


TEXT-FIGURE 8—ADP-induced platelet aggregation and ^{14}C-serotonin release in 3 dogs before and during the administration of CARB or TIC (1000 mg kg 24 hr) intravenously for 3 to 6 days. Each point represents the mean of two to four determinations calculated from pooled CARB and TIC studies.

portions and observed following the addition of ADP and collagen. Mutual correction of aggregation defects was observed (Text-Figure 9). CARB platelets were as effective as normal platelets in correcting the ADP and collagen aggregation defect of aspirin platelets, but ^{14}C-serotonin release following the addition of ADP was greater when normal platelets were mixed with aspirin platelets than when CARB platelets were mixed with aspirin platelets (Table 5).

In Vitro Studies

PRP was incubated at 37 C with CARB or TIC, 250 to 1000 μg/ml, for intervals of up to 2.5 hours. Fourteen studies were performed. No inhibi-



TEXT-FIGURE 9—Mutual correction of collagen-induced aggregation by mixing an equal number of platelets from aspirin- and TIC-treated dogs. The aggregation response using the same concentration of collagen (1:200 final dilution) was equal to that obtained by mixing platelets from aspirin-treated and normal dogs. ΔT = change in light transmission.

Table 5—Correction of the Defect in ADP-Induced Release of ^{14}C-Serotonin From the Platelets of 2 Aspirin-Treated (AT) Normal Dogs When the AT Platelets Were Mixed With Platelets From Normal and CARB-Treated Dogs

	Pellet ($\times 10^3$ cpm)	Supernatant ($\times 10^3$ cpm)	^{14}C release (%)
Normal platelets	28.3	1.85	6.0 ± 0.05*
AT platelets +			
ADP 5.3 μM	28.6	1.20	3.9 ± 0.20
ADP 21.2 μM	27.9	1.73	5.7 ± 1.00
AT platelets and normal platelets +			
ADP 5.3 μM	21.5	8.0	27.0 ± 0.80
ADP 21.2 μM	15.6	14.2	47.7 ± 0.40
AT platelets and CARB platelets +			
ADP 5.3 μM	26.6	3.1	10.4 ± 0.80
ADP 21.2 μM	22.0	7.9	26.3 ± 0.40

* SEM

tion of platelet aggregation in response to ADP or collagen was observed when compared with control platelets following incubation for 1 hour with any of the drug concentrations studied. After 2 hours of incubation drug concentrations of ≥700 μg/ml resulted in slight inhibition of the secondary phase of ADP-induced aggregation, but no drug effect on collagen-induced aggregation was observed.

Plasma Fibrinogen

Plasma fibrinogen was determined before and during treatment in 10 dogs receiving CARB or TIC, 500 to 2000 mg/kg/24 hr. Nine of these 10 dogs received ≥1000 mg/kg/24 hr. No significant change in plasma fibrinogen was noted in dogs receiving 500 to 1500 mg/kg/24 hr. In 1 dog the administration of CARB, 2000 mg/kg/24 hr, was associated with a fall in plasma fibrinogen from 352 to 53 mg/100 ml after 48 hours.

Thrombin Time

No significant change was noted in the thrombin time during the administration of CARB or TIC, 1000 mg/kg/24 hr, to 10 dogs for 3 to 9 days.

Recovery of Platelet Function

Sequential studies of recovery of platelet aggregation were carried out over a period of 7 to 21 days following 23 studies. Full recovery of pretreatment aggregation responses was observed at 72 hours to 21 days following 19 studies. Platelet aggregation returned to normal within 7 days in 18 of these 19 studies. Platelet aggregation in 1 dog was signifi-

cantly improved at 4 days, but full recovery did not occur until 21 days. Four dogs were improved, but they had not attained pretreatment aggregation responses when observations were discontinued after 7 to 12 days. Collagen-induced aggregation improved more rapidly than ADP-induced aggregation.

Discussion

The results of this study indicate that clearly defined platelet dysfunction occurs uniformly in dogs receiving parenteral CARB or TIC. The type of platelet dysfunction observed in dogs receiving CARB or TIC is nearly identical to that observed following the administration of these antibiotics to humans.[7,8] Inhibition of ADP-induced aggregation, the most sensitive indicator of the effects of CARB [7] and TIC [8] on human platelets, was observed in all dogs studied. The secondary wave of ADP-induced aggregation was uniformly impaired, and marked inhibition of the primary wave of ADP-induced aggregation was observed following the administration of higher drug doses, a phenomenon observed in humans receiving TIC.[8] Collagen-induced aggregation was much less inhibited than ADP-induced aggregation in the dogs, as it is in humans.[7,8] Platelet retention in glass bead columns, possibly related to the depressed "platelet adhesiveness" to glass observed by McClure et al,[3] was also uniformly inhibited in dogs receiving CARB or TIC. Platelet factor 3 availability was decreased in 91% of dogs receiving CARB or TIC, a finding of similar significance to the frequently shortened serum prothrombin times observed in humans receiving CARB [7] and TIC.[8] Although prolongation of the bleeding time was observed in dogs only when collagen-induced aggregation was significantly impaired, a relationship not observed in humans, the bleeding time was more often prolonged with higher drug doses in both dogs and humans.[7,8] The striking similarity of the platelet functional abnormalities observed in the dog to those previously described in humans indicates that the observations made in the present study are applicable to human platelet dysfunction induced by CARB and TIC.

The cumulative effects of CARB and TIC on platelet function observed in the present study precisely define the time and dose-response relationships only suggested by previous observations in humans. Brown et al [7,8] administered CARB and TIC to human volunteers. They observed a higher frequency of prolongation of the bleeding time and a longer duration of bleeding in those who received higher doses of either drug (CARB, 600 mg/kg/24 hr; TIC, 300 mg/kg/24 hr) compared with those who received lower doses (CARB, 300 mg/kg/24 hr; TIC, 100 mg/kg/24 hr). Prolongation of the bleeding time did not occur until after 2 to 7 days

of treatment, and maximum prolongation occurred after 3 days. The primary wave of ADP-induced platelet aggregation was decreased following the administration of TIC, 300 mg/kg/24 hr, to human volunteers, but doses of 100 and 200 mg/kg/24 hr did not inhibit the primary wave of aggregation.[6] Inhibition of clot retraction was more marked following 400 mg/kg/24 hr of CARB than 300 mg/kg/24 hr.[7] Studies of the effect of parenteral penicillin G [29-31] and ampicillin [30] on human platelets similarly suggested that platelet dysfunction induced by penicillins is dose-dependent, but none of these studies defined a precise dose-response relationship.

In vitro studies have also indicated a relationship between the concentration of CARB, TIC, or penicillin G to which platelets are exposed and the resulting degree of platelet aggregation inhibition,[5,29,32] but the significance of these observations is questionable since the drug concentrations required to inhibit platelet aggregation in these studies are far in excess of those found in humans with normal renal function receiving therapeutic doses.

The present study clearly establishes and defines the dose- and time-dependent nature of CARB- and TIC-induced platelet dysfunction suggested by previous studies. Within the dose range of 250 to 1000 mg/kg/24 hr administered by continuous IV infusion, the degree of platelet dysfunction and the rapidity with which it develops is proportional to the dose of CARB or TIC. The *in vitro* drug effects observed in the present study also indicate that inhibition of aggregation is dose-related; however, drug concentrations 15 to 45 times the mean concentrations found in the plasma of dogs with significant aggregation inhibition were required to produce slight inhibition of ADP-induced aggregation *in vitro*. The fact that aggregation inhibition was not observed until after 2 hours of incubation confirms the time-related nature of the drug effect observed *in vivo*. The failure of studies of short-term infusions of CARB[6] and TIC[32] and short-term incubations of CARB *in vitro*[7] to demonstrate impaired platelet aggregation may be explained by the fact that the drugs were not administered or incubated with platelets for a sufficient period.

The total drug doses administered to dogs in this study ranged from below to significantly above the usual therapeutic dose of CARB used to treat human *Pseudomonas* infections.[33] However, the serum drug concentrations attained were predictably lower than the peak levels observed in humans given similar total doses administered by the usual rapid intermittent infusions.[7] Continuous infusions of CARB or TIC yield serum drug concentrations approximately 30 to 75% of those attained by rapid IV infusion of the same total dose.[10,34-36] The range of serum drug concen-

trations observed in this study during the continuous IV administration of CARB in doses of 500 and 750 mg/kg/24 hr (42.6 to 109.7 μg/ml) is comparable to that observed 1 to 2 hours after rapid IV infusion in humans receiving CARB in standard therapeutic doses of 300 and 400 mg/kg/24 hr (39.1 to 142.8 μg/ml).[7] Since the serum drug concentrations of CARB or TIC observed in this study were directly proportional to the total drug doses administered by continuous IV infusion, and since the severity of platelet dysfunction and the rapidity with which it developed were also proportional to drug dose, it is likely that the serum drug concentration is an important determinant of the severity of the platelet functional deficit induced.

The observations made in this study regarding the mechanism of platelet dysfunction induced by CARB or TIC indicate that these drugs administered in therapeutic doses significantly inhibit platelet ^{14}C-serotonin release. Although similar observations have been made following exposure of human platelets to penicillin G and CARB and rabbit and pig platelets to penicillin G,[5] extremely high drug concentrations were required to inhibit release in washed platelets. The failure of *in vitro* incubation of platelets with concentrations of CARB or TIC equal to those obtained following parenteral drug administration to inhibit platelet aggregation in the present study confirms similar observations in humans.[7] In contrast, the current study indicates that inhibition of the platelet release reaction occurs with normal therapeutic drug concentrations when CARB and TIC are administered parenterally.

The mechanism by which CARB and TIC inhibit the release reaction is not defined by the present study. Although the impairment of the primary phase of ADP-induced aggregation may have resulted in inhibition of the release reaction, markedly decreased ^{14}C-serotonin release was documented despite normal primary aggregation in response to 21.2 and 42.4 μM ADP. Penicillin G binds to erythrocyte membranes,[37,38] and both penicillin G and ampicillin are strongly bound to phospholipids,[39] prominent constituents of platelets.[40] Although the penicillins have not been demonstrated to bind to platelet membranes, it has been postulated that penicillin G, CARB, and TIC inhibit platelet responsiveness to aggregating agents by coating platelets and blocking sites on the platelet surface, where aggregating agents exert their effects.[5,8] However, the aggregation responses observed with high concentrations of ADP, in the present study, which occurred despite marked inhibition of ^{14}C-serotonin release, suggest that the mechanism is more complex than simple inhibition of membrane receptors.

Although inhibition of platelet cyclo-oxygenase [41] could explain CARB-

and TIC-induced impairment of ^{14}C-serotonin release, the mixing experiments performed with aspirin-treated platelets in the present study do not provide support for such a mechanism. Decreased platelet ADP following CARB or TIC administration could contribute to impaired platelet aggregation, but the modest and inconsistent degree of decrease in platelet ADP observed in the present study is unlikely to account for the marked inhibition of aggregation observed. Similar modest decreases in platelet ADP were observed infrequently following the administration of ampicillin and methicillin to human volunteers.[30] The normal platelet serotonin, ATP, and ATP/ADP ratio and ultrastructure observed following CARB and TIC administration in the present study indicate that acquired storage pool deficiency[42] did not result from exposure to these drugs.

Prompt recovery of function occurred in some dogs after CARB or TIC was discontinued, but the majority did not recover fully until 7 to 21 days. Whatever the mechanism of CARB- and TIC-induced platelet dysfunction, this pattern of recovery suggests an irreversible alteration of platelet function and possibly an effect on megakaryocytes.[7,8]

The data obtained in the present study indicate that CARB and TIC impair platelet function by a complex dose- and time-dependent mechanism which is similar for both drugs. This mechanism remains incompletely defined. Decreased responsiveness to aggregating agents may be a result of drug binding to the platelet membrane, but decreased sensitivity to aggregating agents does not appear to be fully responsible for the platelet dysfunction observed. The mechanism by which CARB and TIC inhibit the platelet release reaction appears to be different from that induced by aspirin; therefore, it is of considerable theoretic interest. Further studies designed to elucidate the mechanisms of CARB- and TIC-induced platelet dysfunction are in progress.

Although high concentrations of CARB result in impaired conversion of fibrinogen to fibrin,[43-45] the observations made in the present study support the contention of Brown et al[7,8] that hemorrhage in humans receiving CARB may result from impaired platelet function in the absence of impaired fibrin formation.

The predictable impairment of platelet function which follows CARB and TIC administration and the dose-response relationship demonstrated in the present study suggest that these and other penicillins may be useful antithrombotic agents. Since they appear to inhibit platelet function by a mechanism other than inhibition of platelet cyclo-oxygenase, further study of their antithrombotic potential is warranted.

References

1. Lurie A, Ogilvie M, Townsend R, Gold C, Meyers AM, Goldberg B: Carbenicillin-induced coagulopathy. Lancet 1:1114–1115, 1970

2. Gordon DH: Carbenicillin in renal failure. Lancet 2:422–423, 1970
3. McClure PD, Casserly JG, Monsier C, Crozier D: Carbenicillin-induced bleeding disorder. Lancet 2:1307–1308, 1970
4. Waisbren BA, Evani SV, Ziebert AP: Carbenicillin and bleeding. JAMA 217:1243, 1971
5. Cazenave J-P, Packham MA, Guccione MA, Mustard JF: Effects of penicillin G on platelet aggregation, release, and adherence to collagen. Proc Soc Exp Biol Med 142:159–166, 1973
6. Lederer DA, Davies T, Connell G, Davies JA, McNicol GP: The effect of carbenicillin on the haemostatic mechanism. J Pharm Pharmacol 25:876–880, 1973
7. Brown CH III, Natelson EA, Bradshaw MW, Williams TW Jr, Alfrey CP Jr: The hemostatic defect produced by carbenicillin. N Engl J Med 291:265–270, 1974
8. Brown CH III, Natelson EA, Bradshaw MW, Alfrey CP Jr, Williams TW Jr: Study of the effects of ticarcillin on blood coagulation and platelet function. Antimicrob Agents Chemother 7:652–657, 1975
9. Johnson GJ, White JG: Platelet dysfunction induced by parenteral administration of carbenicillin and ticarcillin. Thromb Diath Haemorrh 34:341–342, 1975 (Abstr)
10. Lynn B: Administration of carbenicillin and ticarcillin-pharmaceutical aspects. Eur J Cancer 9:425–433, 1973
11. Bodey GP, Deerhake B: In vitro studies of α-carboxyl-3-thienylmethyl penicillin, a new semisynthetic penicillin. Appl Microbiol 21:61–65, 1971
12. Neu HC, Garvey GJ: Comparative in vitro activity and clinical pharmacology of ticarcillin and carbenicillin. Antimicrob Agents Chemother 8:457–462, 1975
13. Dudrick SJ, Wilmore DW, Vars HM, Rhoads JE: Long-term total parenteral nutrition with growth, development, and positive nitrogen balance. Surgery 64:134–142, 1968
14. Vann R: Personal communication
15. Bull BS, Schneiderman MA, Brecher G: Platelet counts with the Coulter Counter. Am J Clin Pathol 44:678–688, 1965
16. Born GVR, Cross MJ: The aggregation of blood platelets. J Physiol (Lond) 168:178–195, 1963
17. Bowie EJW, Owen CA Jr, Thompson JH Jr, Didisheim P: Platelet adhesiveness in von Willebrand's disease. Am J Clin Pathol 52:69–77, 1969
18. Mielke CH Jr, Kaneshiro MM, Maher IA, Weiner JM, Rapaport SI: The standardized normal Ivy bleeding time and its prolongation by aspirin. Blood 34:204–215, 1969
19. Hardisty RM, Hutton RA: The kaolin clotting time of platelet-rich plasma: A test of platelet factor-3 availability. Br J Haematol 11:258–268, 1965
20. White JG: Fine structural alterations induced in platelets by adenosine diphosphate. Blood 31:604–622, 1968
21. Holmsen H, Storm E, Day HJ: Determination of ATP and ADP in blood platelets: A modification of the firefly luciferase assay for plasma. Anal Biochem 46:489–501, 1972
22. Rao GHR, White JG, Jachimowicz AA, Witkop CJ Jr: An improved method for the extraction of endogenous platelet serotonin. J Lab Clin Med 87:129–137, 1976
23. Jerushalmy Z, Zucker MB: Some effects of fibrinogen degradation products (FDP) on blood platelets. Thromb Diath Haemorrh 15:413–419, 1966
24. White JG, Rao GHR, Estensen RD: Investigation of the release reaction in platelets exposed to phorbol myristate acetate. Am J Pathol 75:301–314, 1974
25. Gerrard JM, White JG, Rao GHR, Krivit W, Witkop CJ Jr: Labile aggregation stimulating substance (LASS): The factor from storage pool deficient platelets correcting defective aggregation and release of aspirin treated normal platelets. Br J Haematol 29:657–665, 1975
26. Swaim WR, Feders MB: Fibrinogen assay. Clin Chem 13:1026–1028, 1967
27. Penner JA: Blood coagulation. Laboratory Manual. The University of Michigan

Medical Center, Simpson Memorial Institute, 1973
28. Snedecor GW, Cochran WG: Sampling from a normally distributed population. Statistical Methods, Sixth edition. Ames, Iowa, The Iowa State University Press, 1967, pp 32–65
29. Houbouyan L, Stoltz JF, Goguel A: Influence of penicillin G on platelet aggregation in vitro and in vivo. Platelets. Recent Advances in Basic Research and Clinical Aspects. Proceedings of the International Symposium on Blood Platelets, Istanbul, Turkey, August 24–27, 1974. Edited by ON Ulutin. Amsterdam, Excerpta Medica, 1975, pp 381–387
30. Brown CH III, Bradshaw MW, Natelson EA, Alfrey CP Jr, Williams TW Jr: Defective platelet function following the administration of penicillin compounds. Blood 47:949–956, 1976
31. Andrassy K, Scherz M, Ritz E, Walter E, Hasper B, Storch H, Vömel W: Penicillin-induced coagulation disorder. Lancet 2:1039–1041, 1976
32. Drouet FH, Davies T, Lederer DA, McNicol GP: The effect of ticarcillin on the haemostatic mechanism. J Pharm Pharmacol 27:964–966, 1975
33. Bodey GP, Whitecar JP Jr, Middleman E, Rodriguez V: Carbenicillin therapy for pseudomonas infections. JAMA 218:62–66, 1971
34. Rodriguez V, Inagaki J, Bodey GP: Clinical pharmacology of ticarcillin (α-carboxyl-3-thienylmethyl penicillin, BRL-2228). Antimicrob Agents Chemother 4:31–36, 1973
35. Klastersky J, Henri A, Daneau D: Ticarcillin, a new semisynthetic penicillin active on *Pseudomonas aeruginosa: In vitro* activity and blood levels in man. J Clin Pharmacol 14:172–175, 1974
36. Bodey GP, Rodriguez V, Stewart D: Clinical pharmacological studies of carbenicillin. Am J Med Sci 257:185–190, 1969
37. Josephson AS, Kaplan AP: Interaction of penicillin and erythrocytes. J Immunol 98:293–302, 1967
38. Ley AB, Harris JP, Brinkley M, Liles B, Jack JA, Cahan A: Circulating antibody directed against penicillin. Science 127:1118–1119, 1958
39. Padfield JM, Kellaway, IW: The interaction of penicillins with phospholipids. J Pharm Pharmacol 24:Suppl:171P, 1972
40. Marcus AJ, Ullman HL, Safier LB: Lipid composition of subcellular particles of human blood platelets. J Lipid Res 10:108–114, 1969
41. Hamberg M, Svensson J, Samuelsson B: Prostaglandin endoperoxides. A new concept concerning the mode of action and release of prostaglandins. Proc Natl Acad Sci USA 71:3824–3828, 1974
42. Zahavi J: Acquired "storage pool disease" of platelets. Thromb Haemostas 35:501–507, 1976
43. Grotz RT, Fox A, Forman WB: Carbenicillin inhibition of fibrinogen-fibrin conversion. Clin Res 21:556, 1973 (Abstr)
44. Lurie A, Ogilvie M, Gold CH, Meyer AM, Goldberg B: Carbenicillin-induced coagulopathy. S Afr Med J 48:457–461, 1974
45. Andrassy FK, Weischedel E, Ritz E, Andrassy T: Bleeding in uremic patients after carbenicillin. Thromb Haemostas 36:115–126, 1976

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