Platelet Factor 3 in Normal Subjects and Patients with Renal Failure

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ABSTRACT Two tests were used to differentiate abnormalities in release of platelet factor 3 (PF3) from quantitative deficiencies of this factor in normal subjects and in patients with renal failure. The first test was an assay which determined availability of PF3 (PF3-A time) and involved the use of a mixture of patient's platelet-rich plasma (PRP) and normal platelet-poor plasma (PPP) in a fixed ratio (1:8). The second test was similar but used "frozen and thawed" platelets to obtain a quantitative estimate of PF3 (PF3-F time). An abnormal PF3-A time was found in approximately three-quarters of 55 patients with renal insufficiency; 43 of these had chronic and 12 had acute renal failure. This abnormality was present both in patients with and without hemorrhagic manifestations, although it was slightly more common in bleeders. The PF3-F test was abnormal in approximately one-third of the bleeding patients and one-quarter of the non-bleeders. The PF3-A time returned to normal or was significantly shortened 24–48 hr after peritoneal or hemodialysis. Studies on patients who were not dialyzed showed no statistically significant correlations between the PF3-A time and either the serum urea nitrogen, creatinine, calcium, or phosphorus. Furthermore, the PF3-A time was not affected by guanidosuccinic or guanidinoacetic acids. We therefore conclude that the demonstrable platelet abnormality in patients with uremia is produced by an unknown dialyzable material.

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The mean plasma clot retraction of uremic patients was also significantly different from the mean plasma clot retraction of normal controls. However, unlike the PF3-A time, the abnormality of plasma clot retraction was not immediately affected by dialysis. Correction of plasma clot retraction did occur after repeated dialyses.

INTRODUCTION

Shortening of the clotting time of intact platelet-rich plasma (PRP) by incubation with Celite or kaolin is believed to result from activation of the Hageman and plasma thromboplastin antecedent (PTA) coagulation factors and from release of platelet factor 3 (PF3). It is possible to control the test system so that it is sensitive only to the increase in platelet coagulant activity (1–3). Studies of the platelet coagulant activity which has been termed platelet factor 3 availability (PF3-A) have revealed abnormalities in a variety of congenital and acquired conditions (2–7).

It is the purpose of this paper to report a study of platelet function in 55 patients with uremia, employing techniques for assay of PF3-A and total platelet factor 3 activity (PF3-F) which, because of their insensitivity to plasma coagulation factors, are highly specific and reproducible. With these specific tests, we have been able to demonstrate abnormal platelet function in a considerable number of patients with uremia. Furthermore, these abnormalities were corrected after the patient had been subjected to hemodialysis or peritoneal dialysis. Finally, the relationship of the platelet abnormalities to clot retraction and to

some of the biochemical abnormalities of uremia were assessed.

METHODS

PF3-A and PF3-F times. All blood samples were collected by venipuncture with 20-gauge disposable needles and 5-10-ml plastic syringes. To avoid stasis, the tourniquet was removed and the 2 syringe technique employed. Blood from the second syringe was transferred to 100 × 13 mm plastic test tubes and mixed with sodium citrate in a ratio of blood-citrate, 9:1 (v/v). The sample was immediately placed in an ice bath until tested.

The blood was first centrifuged at 5°C at 30 *g* for 10 min (Sorvall RC-2). After the central layer of PRP was removed, the remainder was recentrifuged at 12,100 *g* for 15 min to obtain platelet-poor plasma (PPP). The samples of plasma were handled in plastic material (test tubes and pipettes) and kept cool in an ice bath until tested. Platelet counts on PRP were performed by phase contrast microscopy (8) and, when necessary, platelet concentration was adjusted to 400,000-500,000/mm³ by dilution with PPP from the same blood. Celite (Filter-Cel, Johns-Manville, Lampoc, Calif.) was suspended in buffered saline at a concentration of 15 mg/ml. Buffered saline was prepared by the addition of 1 part 0.2 M imidazole buffer, pH 7.35 (9), to 20 parts normal saline.

1 part of PRP from subject to be tested was added to 8 parts of PPP from a normal individual. This addition resulted in a final platelet concentration of approximately 50,000/mm³. To test PF3-A time, 0.1 ml of this mixture was added to an equal volume of Celite reagent in a glass test tube and incubated at 37°C for exactly 20 min. The clotting time, after recalcification with 0.1 ml of 0.035 M CaCl₂, was then determined with the aid of a stop watch. In preliminary experiments, varying incubation times of the plasma-Celite mixture were tested. A control was performed daily.

In an attempt to estimate PF3 content rather than availability (PF3-F time), PRP was frozen and thawed three times by placing the test tube alternately in dry ice in acetone and in a water bath at 37°C. An aliquot of this suspension was then added to PPP (1:8) and the test performed in the same way as the PF3-A time, except that the mixture was incubated with Celite for only 4 min before recalcification.

Prothrombin time and partial thromboplastin time. The prothrombin time was performed by a modification of Quick's method (10), and a commercial preparation of thromboplastin-calcium (Simplastin, General Diagnostics, Warner-Chilcott Laboratories, Morris Plains, N. J.). The activated partial thromboplastin time (PTT) was performed as follows. A suspension of 20 mg of Celite per ml in buffered saline was mixed with equal parts of 3.8% inosithin diluted 1:35 in imidazole buffer. 0.1 ml of this mixture was added to 0.1 ml of citrated plasma and 0.1 ml of normal saline in a glass test tube. After 4 min of incubation at 37°C, 0.1 ml of 0.025 M calcium chloride was added and the clotting time determined by tilting the test tube every 5 sec.

Plasma clot retraction. Plasma clot retraction was performed by a modification of the method of Benthous (11) using citrated PRP with the platelet count adjusted as described for PF3-A time (platelet count 400,000-500,000/mm³). 0.2 ml of 0.1 M calcium chloride and 0.1 ml of Simplastin were pipetted into 13 × 100 mm glass test tubes, and saline was added to bring the volume to 4.5 ml. To this solution was added 0.5 ml of PRP and the solution mixed by repeated inclination of the test tube. When the plasma clotted, usually within 3 min, the fibrin clot was carefully detached from the test tube wall by rotating the tube around its long axis. After 3 hr, clot retraction was estimated by measuring the length of the plasma clot in millimeters.

Preparation of adsorbed plasma. Adsorbed plasma was prepared by mixing 1.0 ml of PPP with 0.1 ml of aluminum hydroxide gel (Wyeth Laboratories, Inc., Philadelphia, Pa.) at 37°C for 3 min and then centrifuging the mixture at 12,000 *g* for 30 min. The supernatant-adsorbed plasma was then carefully aspirated.

Miscellaneous chemicals and reagents. Guanidinosuccinic acid (GSA) and guanidinoacetic acid (GAA) were obtained in purified form from Dr. Samuel Natelson, Director of Biochemistry, Michael Reese Hospital and Medical Center, Chicago, Ill. (12). Adenosine 5'-diphosphate (ADP)-Na was obtained from Nutritional Biochemical Corporation, Cleveland, Ohio, and used in a final concentration of 2.06×10^{-5} mole/liter. Stypven brand Russell's Viper Venom was obtained from Burroughs Wellcome & Co., Tuckahoe, N. Y. This chemical was first reconstituted in the solvent supplied by the company to a concentration of 100 µg/ml and then further diluted 1:10 (v/v) in buffered saline.

Control and patient population. Control subjects consisted of the authors, residents, technicians, and secretary of the Division of Clinical Hematology. Patients were all clinic or in-patients at Michael Reese Hospital and Medical Center. Patients on warfarin were all hospitalized, the majority having had myocardial infarcts.

Statistical methods. The *t* test (13) was used to determine the significance of differences between: PF3-A and PF3-F times of controls vs. patients on warfarin; PF3-A and PF3-F times of controls vs. patients with renal failure; plasma clot retraction of controls vs. patients with renal failure; and, effects of CA⁺⁺ and GSA on PF3-A time. The paired *t* test (14) was employed to analyze the effect of dialysis on the PF3-A time, PF3-F time, and plasma clot retraction. Other statistical methods were correlation coefficient (15), and, where standard deviations were greatly different, means were compared by use of normal distribution of SE of difference of means for large samples (16).

RESULTS

Factors affecting assay of PF3

PREINCUBATION WITH CELITE

Preincubation of PRP with Celite resulted in progressive shortening of the clotting time over

a 20 min period. In our system, with normal PRP and PPP [1:8 (PF3-A time)], there was a linear relationship on log-log paper between clotting time and the duration of preincubation with Celite (Fig. 1). When platelets, disrupted by freezing and thawing, were studied in an identical manner, maximum shortening of clotting time occurred after 4 min, and there was no significant change with continued preincubation with Celite.

PLATELET CONCENTRATION OF FINAL MIXTURE

To test the effect of platelet concentration on the PF3 assay, the platelet concentration was adjusted by adding varying amounts of patient's PPP. As indicated in Table I, clotting time shortened progressively as the concentration of normal platelets in final test system increased. After 20 min of incubation, similar clotting times were obtained with both 98,000 and 49,000 platelets mm^3 .

PLASMA COAGULATION FACTORS

It was shown by the following experiments that when a ratio of PRP-PPP of 1:8 was employed, alterations in plasma factors did not significantly affect the PF3 assay.

Studies with adsorbed plasma. A series of PF3 assays was performed on 0.1 ml of normal PRP plus 0.8 ml of PPP prepared from varying proportions of $\text{Al}(\text{OH})_3$ adsorbed and nonadsorbed plasmas (Table II). Platelet concentration in all final mixtures was 53,700/ mm^3 . Coagulation studies on adsorbed plasma revealed *no demonstrable Factor II, 47% Factor V, 25% Factor VIII, 1.5% Factor IX, and 4.8% Factor XI.*

FIGURE 1 Shortening of recalcification time by incubation of normal platelet-rich plasma (PRP) with Celite. Platelet-rich plasma/platelet-poor plasma = 1/8 recalcification after incubation with celite at indicated times, at 37°C. Dots represent mean clotting times. Limits denote 1 sd. Numerals denote number of determinations.

TABLE I
Effect of Platelets in Final Test System
on Clotting Time (sec)

No. of platelets/ mm^3 in final test system	Incubation (Min)				
	4	8	12	16	20
790,000	60.0	52.5	42.5	34.0	27.0
396,000	75.0	57.0	41.0	32.5	28.0
176,000	86.0	77.5	57.5	41.5	31.0
98,000	95.0	81.0	73.0	62.5	57.0
49,000	97.0	83.0	77.5	65.0	57.5

These multiple deficiencies resulted in prolongation of both prothrombin time (from 11.1 to 64.0 sec) and PTT (from 50 to 1,080 sec). Despite these severe abnormalities, the PF3-A time obtained with the mixture of 1 part adsorbed to 8 parts nonadsorbed plasma was essentially the same as the time obtained with all nonadsorbed plasma. It is important to note that there was incomplete correction with 1:1 and 1:4 mixtures.

In another experiment, the platelet button from normal plasma was resuspended in either normal adsorbed or normal nonadsorbed PPP, and 0.1 ml of these suspensions were tested, employing 0.8 ml normal PPP. The PF3-A time of PRP, prepared with adsorbed plasma, was not significantly different from that obtained with PRP prepared with nonadsorbed plasma (57.5 vs. 56.5 sec).

PF3-A time of patients with acquired or congenital coagulation defects. When the 1:8 ratio was employed, as described (patient's PRP:normal PPP = 1:8), the PF3-A time was performed on three patients with severe hemophilia A, two patients with Von Willebrand's disease, and one patient with Factor XII (Hageman) deficiency.

TABLE II
Adsorbed Platelet-Poor Plasma in Test Mixture

Ratio of adsorbed:non-adsorbed normal PPP in test mixture	PF3-A time
	sec
All adsorbed	105
1:1	72
1:4	62
1:8	57
All nonadsorbed	56

PPP, platelet-poor plasma; PF3-A, platelet factor 3 availability.

Results obtained were within normal limits (normal mean ± 2 SD). These studies are presented on Table III where, for comparison, the results using patient's PPP instead of normal PPP also are indicated.

PF3-A and PF3-F times in normal subjects

MEAN $\pm$ SD

The mean PF3-A time of 116 determinations on 28 normal subjects was 55.4 ± 3.0 (SD) sec. This SD is calculated from all 116 determinations, since the SD of all 116 was 3.0, whereas SD of 28 repeated studies on one subject on different days was 2.1. The mean PF3-F time of 92 examinations on normal subjects was 51.6 ± 2.7 (SD) sec. This SD is calculated from all 92 determinations, since the SD of all 92 was 2.7, whereas the SD of 20 repeated studies on one subject on different days was 2.6.

DAY-TO-DAY VARIATION AND REPRODUCIBILITY

The day-to-day variation of 28 determinations of PF3-A time on the same normal subject over a 9 month period was 56.3 ± 2.1 (SD) sec. The mean of 20 PF3-F times performed on the same normal subject over a 6 month period was 52.3 ± 2.6 (SD) sec.

In order to assess reproducibility, the PF3-A time was performed on 10 separate mixtures of PPP with PRP obtained from one sample. A mean value of 54.1 ± 0.7 (SD) sec was obtained. PF3-A times were performed on a series of 10 patients receiving warfarin. All of these patients had abnormal prothrombin times (mean = 24.6 ± 6.0 (SD) sec). The mean PF3-A time of this group of patients was 56.1 ± 7.4 (SD) sec. This mean is not significantly different from normal ($P > 0.60$).

TABLE III
PF3-A Time of Patients with Congenital
Coagulation Defects

Diagnosis	PPP	
	Normal	Patient
		sec
1 Hemophilia A	49.0	>200
2 Hemophilia A	51.0	>200
3 Hemophilia A	48.5	>200
4 Von Willebrand's disease	55.0	>200
5 Von Willebrand's disease	58.5	> 66.5
6 Factor XII deficiency	52.1	>200

When patient's PPP was used, instead of normal PPP, clotting times were markedly prolonged, reflecting the coagulation defects induced by warfarin (mean = 116.8 ± 41.5 (SD) sec).

PF3-A and PF3-F time in patients with renal failure

DESCRIPTION AND RESULTS FROM ALL PATIENTS STUDIED

Of the 55 patients with renal insufficiency, 43 had chronic and 12 acute renal failure. Included among the patients with chronic renal failure were 13 patients with chronic renal disease of undetermined origin, 18 with glomerulonephritis, four with arteriolar nephrosclerosis, two with systemic lupus erythematosus, two with pyelonephritis and four with other diseases, including polycystic kidneys, postnecrotic cirrhosis with renal failure, polyarteritis nodosa, and carcinoma of the urinary bladder with chronic obstruction. Among the patients with acute renal failure were four with urinary retention due to prostatic enlargement, four with tubular necrosis, and four with miscellaneous disorders including a gunshot wound of the abdomen, pheochromocytoma, microangiopathy, and postoperative disorders. The clinical diagnosis was confirmed by renal biopsy or autopsy in 22 patients.

124 PF3-A and PF3-F times were performed on the above 55 patients. Multiple studies were performed on 28 of the 55 patients. A prolonged (greater than normal mean plus 2 SD) PF3-A time was found in 73% of all 55 patients with renal failure. Of the 40% of patients with hemorrhagic manifestations, 82% had abnormal PF3-A tests, as compared to 67% of nonbleeders. The PF3-F test was prolonged in 29% of the entire group, 36% of patients with hemorrhagic manifestations, and 24% of nonbleeders. The mean PF3-A time for all 55 patients with renal failure was 67.0 ± 9.1 (SD) sec, while the mean of PF3-F time was 54.2 ± 3.8 (SD) sec. The average of replicate values were used for these calculations. Although prolongation of PF3-A time of these patients is greater than PF3-F abnormality, both are significantly longer than normal ($P < 0.001$). The correlation between PF3-A and PF3-F times in patients with renal failure [$r = 0.559 \pm 0.06$ (SE)] is clearly significantly greater than 0 but does not

reach a level that would be useful for predicting the one measure from the other.

The differences in PF3-A and PF3-F times between normal subjects and patients with uremia cannot be attributed to platelet number in the final test system, since these values were similar: $44,968 \pm 8,072$ (SD) mm^3 for patients vs. $48,580 \pm 7,699$ (SD) mm^3 for controls. Nor can the abnormalities be attributed to plasma coagulation defects, since the test was found not to be affected by such abnormalities. In addition, there was no significant correlation between PF3-A time and either prothrombin time ($r = 0.008$) or partial thromboplastin time [$r = 0.17 \pm 0.13$ (SE)]. Furthermore, more abnormal PF3-A and PF3-F times were obtained in many patients with both normal prothrombin times and PTT's, and studies on 10 patients with both abnormal PF3-A and PTT revealed that there was correction of PTT by a 1:8 mixture of patients with normal PPP.

An example of progressive prolongation of PF3-A time in a patient with chronic glomerulonephritis is presented in Fig. 2. This patient was never subjected to dialysis. The initial PF3-A time was normal; however, as the clinical state of the patient deteriorated, progressive prolongation of PF3-A time occurred.

RELATIONSHIP BETWEEN PF3-A TIME AND OTHER ABNORMALITIES

45 PF3-A times were performed on 30 of the 55 patients with renal failure who were either never

dialyzed or were tested before their first dialysis. These results were analyzed to determine the relationship between this platelet function test and other abnormalities associated with renal insufficiency. It was found that the correlations between the PF3-A time and the serum urea nitrogen [$r = 0.216 \pm 0.13$ (SE)], serum calcium [$r = 0.114 \pm 0.21$ (SE)] and serum phosphorus [$r = 0.180 \pm 0.22$ (SE)] were not significantly different from 0 and only of borderline significance in the case of creatinine [$r = 0.307 \pm 0.13$ (SE)].

The relationship between PF3-A time and plasma clot retraction, another test of platelet function, was also determined. The mean value of 124 determinations of plasma clot retraction on all uremic patients was 19.1 ± 3.6 (SD) mm, which is significantly larger ($P < 0.001$) than the mean value of 14.3 ± 1.7 (SD) mm obtained from 71 determinations on normal subjects, although the correlation between PF3-A and plasma clot retraction in patients with renal failure was greater than 0 [$r = 0.435 \pm 0.07$ (SE)], it did not reach a statistical level that would be useful for predicting the one value from the other.

EFFECT OF DIALYSIS ON PF3-A AND PF3-F TIMES

79 PF3-A and PF3-F times were performed on 25 patients before, during, and (or) after peritoneal or hemodialysis. Both the immediate (within 48 hr) effect of dialysis and the effect of repeated

FIGURE 2 Repeated determinations of platelet factor 3 availability (PF3-A) time on patient with chronic renal failure. Shaded area includes normal range for PF3-A.

dialyses over 2–6 months, on platelet function were investigated.

Immediate effect of dialysis on PF3-A and PF3-F times. The immediate effect of dialysis (within 48 hr) was studied on 12 occasions in 11 patients. Both the PF3-A and PF3-F times shortened significantly ($P < 0.001$) after dialysis. A summary of this data, plus other measured effects of dialysis, is presented in Table IV.

The effect of dialysis is best illustrated by its effect on PF3-A time, since this test was abnormal in the majority of patients studied. After dialysis there was shortening of the PF3-A time in every instance (Fig. 3). Complete correction (within normal mean ± 2 SD) occurred in five instances, whereas in seven cases, although significant improvement of the PF3-A time followed dialysis, normal values were not achieved. An example of correction of PF3-A after a single dialysis in a patient with acute renal failure is illustrated in Fig. 4. The patient was a 19 yr old boy who had been operated on to remove a large pheochromocytoma. The initial PF3-A time was markedly prolonged, but this was partially corrected by peritoneal dialysis. The value for PF3-A again increased rapidly to abnormal levels but improved gradually during the subsequent 2.5 months and eventually reached normal limits.

The immediate effect of dialysis on plasma clot retraction was tested concurrently, using aliquots of PRP from samples obtained for above study.

TABLE IV
The Immediate Effect (within 48 Hr) of Dialysis

Test	Before dialysis			After dialysis		
	Mean	SD	No.	Mean	SD	No.
PF3-A time, sec	76.8	8.7	12	62.5	5.2	12
PF3-F time, sec	56.9	3.4	12	53.7	2.0	12
Platelets*	42,033	6,745	12	44,033	4,708	12
Clot retraction, mm	20.9	4.5	12	18.3	2.3	12
Prothrombin, sec	12.6	1.1	12	12.9	1.7	12
PTT, sec	57.1	8.0	12	61.9	14.6	12
Serum urea N, mg/100 ml	135.6	25.9	12	85.9	30.6	12
Creatinine, mg/100 ml	18.0	6.9	12	12.3	5.3	12
Potassium, mEq/liter	4.6	0.9	12	4.4	0.9	12
Sodium, mEq/liter	136.0	7.4	12	139.3	5.8	12
Chlorides, mEq/liter	97.6	4.1	12	98.9	4.5	12
CO ₂ , mmol/liter	21.3	4.8	12	24.4	4.5	12
Calcium, mg/100 ml	7.3	0.8	8	8.9	0.6	7
Phosphorus, mg/100 ml	8.6	2.4	4	6.7	2.7	4

* Per mm³ in final test system.

PF3-F, platelet factor 3 using "frozen and thawed" platelets; PTT, partial thromboplastin time.

FIGURE 3 Effect of dialysis on PF3-A time. PF3-A time was performed before and 16–48 hr after dialysis. Indicated are clotting times, and shaded area represents normal limits (2 SD).

Although there was an appreciable improvement in one patient (33–18 mm), the improvement in the remaining 11 patients was minimal, and the mean values before and after dialysis were not significantly different ($P > 0.10$).

Effect of repeated dialyses on PF3-A and PF3-F times. In four patients with chronic renal failure, PF3-A and PF3-F times were performed repeatedly before dialysis over a period of 2–6 months. Complete or almost complete restoration of the values of both tests to normal followed the repeated dialyses. This improvement in platelet function was sustained, despite biochemical evidence of renal insufficiency, e.g., elevated serum urea nitrogen and creatinine. The results in two cases are presented in Figs. 5 and 6. The patient in Fig. 5 had chronic glomerulonephritis. The first value for PF3-A time was slightly prolonged and this time became more prolonged as her clinical condition deteriorated. 24 hr after a hemodialysis there was return of PF3-A time to normal. After a series of four hemodialyses performed at 5–7-day intervals, the PF3-A time, before the last dialysis, became abnormal again. But, when the interval between dialyses was shortened, six PF3-A times, obtained just before dialyses, were found to be at normal or near normal values.

FIGURE 4 Repeated determinations of PF3-A time on patient with acute renal failure. Shaded area includes normal range for PF3-A. Dialysis is signified by arrow.

The patient in Fig. 6 had chronic glomerulonephritis and was hemodialyzed 34 times before the first study, which was started 8 days after the last dialysis. The PF3-A time was prolonged but shortened after reinstitution of dialysis twice a week.

The effect of repeated dialyses on plasma clot

retraction was determined in all patients simultaneously with the determinations of PF3-A and PF3-F times. Although there was no immediate effect on clot retraction, the plasma clot retraction gradually improved after repeated dialyses and returned to normal limits (twice the SD) in all instances.

FIGURE 5 Effect of chronic dialysis on PF3-A time. Shaded area includes normal range for PF3-A. Dialyses are signified by arrows.

FIGURE 6 Effect of chronic dialyses on PF3-A time. Shaded area includes normal range for PF3-A. Dialyses are signified by arrows.

CALCIUM CONCENTRATION AND GSA

Effect on PF3-A time. To test the effects of added calcium on the PF3-A time, a series of tests were run in the usual way, except that recalcification was performed with added calcium. Results on PF3-A time of normal subjects are shown in Table V and on patients with renal failure in Table VI. There was no demonstrable effect of increased calcium on PF3-A time.

TABLE V
The Effect of Calcium Concentration on
PF3-A Time (sec) in Normal Subjects

Subject	Molarity of calcium chloride			
	0.035	0.040	0.045	0.050
1	60.5	61.5	62.5	63.5
2	50.6	59	—	59.2
3	58.5	58	60.5	62
4	50.5	52	53.5	51.5
5	58.4	56	58.2	58.2
6	59.6	59	59	58
7	60	60	61	62.2
8	55.5	54.5	57.5	58.
9	56	55	55.5	57.5
Mean	56.6	57.2	58.5	58.9
SD	3.83	3.04	2.97	3.55
Compared with 0.035 mole/liter				
"p"		0.347	1.064	1.246
P		>0.70	>0.50	>0.20

The effect of GSA on PF3-A time was also investigated, since this substance, which is increased in urine of uremic patients (17, 18), has been reported to interfere with PF3-A (19). Varying concentrations of GSA dissolved in buffered saline were added to the mixture of PRP and PPP, before the addition of Celite, to obtain a final concentration of 0.03–1.6 mmole/liter. The values for PF3-A times, as compared with controls (buffered saline), are shown in Table VII. The addition of GSA did not significantly prolong the PF3-A time, nor did the preincubation of PRP and PPP for 1 hr with GSA have a significant effect on PF3-A test ($P > 0.050$).

TABLE VI
Abnormal PF3-A (Renal Failure)

Subject	Molarity of calcium chloride			
	0.035	0.040	0.045	0.050
1	73	72	74	76
2	72	70.6	—	75
3	65.5	68	66	67.5
4	68	68	67.5	70
5	77	75	75.5	80
Mean	71.1	70.7	70.8	73.7
SD	4.48	2.95	4.70	4.97
Compared with 0.035 mole/liter				
"p"		0.149	0.086	0.777
P		>0.80	>0.90	>0.40

TABLE VII
Effect of GSA in Different Concentration
on PF3-A Time (without ADP)

Buffer			Conc.	GSA		
n	Mean	SD		n	Mean	SD
	sec				sec	
3	59	3.7	0.03-0.04	3	58.3	2.8
1	63.2	—	0.2	1	65	—
3	60.1	2.8	0.4	3	59.3	3.4
8	57.0	5.8	1.6	8	58.0	5.3
15	58.4	4.8	0.03-1.6	15	58.8	4.6

ADP, adenosine diphosphate; n = number of subjects
GSA, guanidinosuccinic acid.

Effect on PF3-A assay with Stypven. In Table VIII are presented the effects of both calcium concentration and GSA (final concentration = 1.6 mmole/liter) on normal PF3-A assay performed as PF3-A time, except that the clotting time was performed with a 0.2 ml of a mixture of Stypven and calcium (Stypven time). No significant effect of GSA or increased calcium concentration was found.

Effect of GSA on ADP-induced PF3-A. In Fig. 7 a series of experiments is depicted where PRP was preincubated for 45 min with adenosine diphosphate (ADP) and either buffered saline or GSA (final concentration 0.4 mmole/liter). After preincubation, 1 part of the mixture was added to 8 parts of either PPP plus Celite, not preincubated, PPP preincubated with Celite for 20 min, or PPP preincubated with Celite for 45 min. After the indicated incubation time, both recalcification and Stypven times were performed. No demonstrable effect resulted from the addition of GSA. However, preincubation of PPP with Celite resulted in

TABLE VIII
Effect of Calcium Concentration and GSA on
Platelet Factor 3 Availability

Ca ⁺⁺ mole	Recalcification time*		Stypven time	
	Buffer	GSA	Buffer	GSA
	sec		sec	
0.025	59.7	59.9	29	30
0.035	58.9	58.4	32	31
0.040	59.1	58.5	31	31
0.050	58.1	58.2	33	33

*Mean of two values.

FIGURE 7 Effect of guanidinosuccinic acid (GSA) and Celite on platelet factor 3 availability. In all experiments PRP plus ADP plus either saline or GSA are preincubated for 45 min. Then 1 part of mixture is added to 8 parts of (1) PPP plus Celite, no preincubation (2) PPP plus Celite, preincubated for 20 min., (3) PPP plus Celite, preincubated for 45 min. Then, after indicated incubation times, either recalcification time (1, 2, 3) or Stypven time (1S, 2S, 3S) is performed.

progressive shortening of both recalcification and Stypven times.

DISCUSSION

The addition of kaolin or Celite to PRP results in liberation of PF3 and activation of plasma coagulation factors to form a substance which converts prothrombin to thrombin in the presence of calcium ions (20). This series of reactions is involved in all methods designed to assay PF3. With normal levels of plasma factors, the quantity of PF3 liberated into the plasma can be estimated by degree of shortening of clotting time which occurs after the addition of calcium alone or calcium plus Stypven. When calcium is employed alone, the test system requires normal amounts of all coagulation factors except Factor VII. When Stypven is added in addition to calcium, the test is not dependent on normal levels of Factors VIII, IX, and XII (4), but Factors V and X are necessary.

To avoid the influence of plasma factors on assay of PF3, Hardisty, Dormandy, and Hutton suggest recalcification of a 1:1 mixture of patient's PRP with normal PPP (1, 2). With this system, they were able to demonstrate normal PF3 in patients with hemophilia and related disorders.

With our method, optimum correction of severe coagulation abnormalities was obtained with a 1:8 mixture of PRP-PPP. This mixture resulted in complete correction of single as well as multiple severe abnormalities, so that a normal PF3-A time was obtained in patients with acquired or congenital coagulation defects and in artificial mixtures with adsorbed plasma.

Spaet and Cintron reported an assay of PF3 employing Stypven (3). Such an assay is sensitive to a deficiency of Factor X which would interfere with the interpretation of studies on newborn infants (21-23), patients with liver diseases (24, 25), and individuals receiving warfarin (26). This sensitivity may contribute to the considerable variability in the results obtained by Spaet and Cintron in normal subjects, which resulted in difficulty in interpreting results on uremic patients (3).

Also of importance in the assay system of PF3-A is the additional effect of Celite on initiation of blood coagulation by activation of Factor XII. Our data indicate that this effect is not circumvented by employing Stypven (Fig. 7). It is therefore critical to time the incubation period carefully whether or not Stypven is employed for PF3 assay.

With the described technique, we have been able to obtain a high degree of reproducibility and minimal day-to-day variation in the PF3-A and PF3-F times. The normal range of the PF3-A time is relatively small and has remained unchanged in our laboratory over a period of 2 yr. The interpretation of the significance of an abnormal PF3-A time has also been abetted by the addition of the PF3-F time, which is performed after disruption of platelets by freezing and thawing. Although freezing and thawing is not related to the physiological mechanism by which platelet factors are released, this procedure does seem to provide a quantitative estimate of PF3. By the combination of techniques, one using intact platelets (PF3-A) and the other disrupted platelets (PF3-F), it seems possible to differentiate abnormalities in release of platelet factor 3 from quantitative deficiencies of this factor.

In the present study, a significant abnormality in PF3-A time was demonstrable in approximately three-quarters of patients with renal failure. This abnormality was present in patients with and without hemorrhagic manifestations, although a slightly

higher incidence was found in patients with bleeding. The abnormal PF3-A time was corrected after peritoneal or hemodialysis, and this correction was regularly demonstrable 24-48 hr postdialysis. With patients on chronic dialysis, correction of PF3-A time could be maintained over long periods of time.

An abnormal PF3-F time was only found in approximately one-quarter of uremic patients. It would therefore appear that the abnormality in PF3-A found in the majority of patients with renal failure is most often due to impairment of release of this factor but, in addition, in a significant number of patients, quantitative defects of PF3 may coexist.

The correction of abnormal PF3-A time after dialysis suggests that the defect is due to a dialyzable substance in uremic plasma. The lack of correlation between PF3-A and serum urea, creatinine, calcium, and phosphorus also indicates that these substances are not responsible for the abnormality. In addition GAA, a substance found to be increased in urine of uremic patients (17), had no effect on PF3-A time.

Nor does the abnormality of PF3-A and PF3-F times appear to be due to a circulating anticoagulant: there was no correlation between PF3-A test and PTT; an abnormal PF3-A time was frequently found with a normal PTT and prothrombin time; in patients where both PF3-A and PTT were abnormal, the PTT repeated on a 1:8 mixture of patient's PPP with normal PPP returned to normal.

Another test of platelet function, plasma clot retraction, was also found to be abnormal in 83.6% of uremic patients. This finding confirms observations of other workers with a similar technique (6). Although both were abnormal in the majority of patients, there was poor correlation between the two tests in patients who had never been subjected to dialysis. In addition, whereas the PF3-A time was promptly corrected by peritoneal or hemodialysis, plasma clot retraction did not improve 12-48 hr after a single dialysis but gradually responded to multiple dialyses. The differences in PF3-A and clot retraction in uremic patients suggest that either the two abnormalities are produced by separate factors or that their sensitivities differ.

Before development of specific assay systems similar to the one used in this study, PF3 was

estimated by a variety of techniques. There were conflicting opinions concerning PF3 activity in uremic patients. The first report which described such an abnormality was by Lewis, Zucker, and Ferguson in 1956 (27). Although there were later reports of similar abnormalities in patients with renal insufficiency (28-39), some investigators did not find any qualitative disturbance of platelet phospholipids in uremics (40-41). Even with the newer, more specific techniques, there has been difficulty in definitely establishing the incidence of PF3 deficiency in these patients (3).

The specificity and reproducibility of the assay systems employed in this study permit us to definitely establish the following. There is an abnormality in Celite-induced PF3-A time in the majority of patients with uremia. There is also a quantitative deficiency in PF3-F time in a smaller percentage of patients with uremia. Both abnormalities are caused by unknown dialyzable material(s).

An abnormality in ADP-induced PF3-A was reported in azotemic patients at the 9th Annual Meeting of the American Hematological Society and has recently been published (19). It is probable that this abnormality differs from the one described in this study, since they report correction by increased CA^{++} , while this increase had no effect in our system. Furthermore, they found that GSA inhibited ADP-induced PF3 release, whereas this had no effect on PF3-A time. The fact that we could not find an effect of GSA on ADP-induced PF3-A may be due to differences in technique, since we added Celite to the final incubation mixture.

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