Patients with severe uremia may have a bleeding tendency associated with a prolonged bleeding time and an adequate platelet count. We have tested the effects on platelet aggregation of three compounds that are found in increased concentration in the blood of patients with renal failure. The addition of urea was followed by an immediate, but transient, increase in optical density of platelet-rich plasma. This precluded the use of turbidimetric techniques for the measurement of platelet aggregation after addition of urea, until the optical density became stable. Adenosine diphosphate-, collagen-, and norepinephrine-induced platelet aggregation were shown to be inhibited 1 hr after urea was added to platelet-rich plasma to produce urea nitrogen increments of 100–300 mg/
100 ml. Increasing concentrations of creatinine by 10 or 20 mg/100 ml did not inhibit platelet aggregation. Guanidinosuccinic acid, in a concentration in the range found in uremic plasma, also had no effect on platelet aggregation. Through inhibition of platelet aggregation, elevated blood concentrations of urea may be one of the causes of the bleeding tendency of uremic patients.

BLEEDING TENDENCY may occur in uremic patients whose bleeding times are frequently prolonged when their platelet counts are at a level usually associated with adequate hemostasis. Such patients may have decreased retention in glass bead filters of platelets in either platelet-rich plasma (PRP) or whole blood. Salzman and Neri reported that thrombin-induced platelet aggregation was decreased, but adenosine diphosphate (ADP)-induced platelet aggregation was normal in the PRP of uremic patients. Hutton and O'Shea also reported normal ADP-induced platelet aggregation, but five other groups of investigators found it to be decreased in the PRP of some uremic patients. Morris and Heslop reported decreased irreversible glass bead-induced platelet aggregation in the blood of uremic patients.

There have been conflicting reports as to whether urea or creatinine added in vitro inhibits platelet adhesion to glass or ADP-induced aggregation, and to...
our knowledge the effects of these compounds on collagen-induced platelet aggregation have not been previously investigated. Salzman and Neri\textsuperscript{7} reported no inhibition of ADP-induced aggregation when urea was incubated with PRP. On the other hand, Somer et al.\textsuperscript{17} stated that urea initially inhibits and then enhances ADP-induced aggregation. Hellem et al.\textsuperscript{4} found that the addition of urea, but not the addition of creatinine, to normal PR decreased ADP-induced retention of platelets in glass bead filters. Stewart and Castaldi\textsuperscript{13} stated that creatinine added in vitro had no deleterious effect on ADP-induced platelet aggregation. Praga and Cortellaro\textsuperscript{18} reported that their preliminary results seemed to suggest a direct inhibitory effect of exogenous creatinine on platelet adhesiveness. These reports suggested a need for further investigation of the possible effects of urea and creatinine on platelet aggregation. Guanidinosuccinic acid was also investigated because its concentration is elevated in the sera of uremic patients,\textsuperscript{19} and it was reported to inhibit ADP-induced platelet factor 3 activation\textsuperscript{20} and platelet aggregation induced by ADP, epinephrine, or connective tissue.\textsuperscript{21}

MATERIALS AND METHODS

Blood Samples

Blood was obtained from fasting men with plasma concentrations of urea nitrogen and creatinine no more than 20 mg/100 ml and 1.2 mg/100 ml, respectively. In a few instances we used blood from male hospital workers under 35 yr of age without determining plasma urea nitrogen or creatinine concentrations.

After mixing 0.9% sodium chloride solution (saline) and each test compound in saline with PRP to achieve the highest concentration of each test compound reported in this paper, there was no more than 0.1 pH unit difference between the pH of the saline control mixture and any of the mixtures containing a test substance.

Test Compounds

Test compounds were of reagent grade. Urea and creatinine were purchased from Calbiochem, Los Angeles, Calif., and guanidinosuccinic acid from Mann Research Laboratories, New York. Purification of urea with a mixed bed resin (AG501-X8, 20–50 mesh, of Bio-Rad Laboratories, Richmond, Calif.) just before use in the PRP of two men did not reduce the inhibition of ADP-, collagen-, or norepinephrine-induced platelet aggregation, indicating that the inhibition was not due to contamination of urea with cyanate or other ions.

The urea used in all the experiments on collagen-induced platelet aggregation at 37°C was treated with the resin just before use. Untreated urea was used in all other experiments.

Platelet Aggregation at Room Temperature

The decrease in optical density (OD) at 600 ms of citrated PRP after agitation with an aggregating agent was used as an index of platelet aggregation. Our method was modified from that of Born and Cross\textsuperscript{22} and differs from theirs in that aggregation occurred at room temperature (about 25°C) and was not continuously recorded. It has been described in detail elsewhere.\textsuperscript{23}

With the exception of the experiments reported in the last two rows of Table 1 (where 0.8 ml of PRP and 0.2 ml of additive were used), 0.9 ml of PRP was mixed with 0.1 ml of a solution of the test compound in saline or with the same volume of saline as
Table 1. Effects of Urea, Creatinine, and Guanidinosuccinic Acid on Platelet Aggregation Induced by 1.7 μM ADP at Room Temperature

<table>
<thead>
<tr>
<th>Urea Nitrogen (mg/100 ml)</th>
<th>Creatinine</th>
<th>Guanidinosuccinic Acid</th>
<th>Mean of Mean Maximum Decrease* in OD (% of Change Produced by Centrifugation)</th>
<th>ρ</th>
<th>% Difference From Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Saline Control</td>
<td>Test Substance in Saline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>0</td>
<td>49.0 46.5</td>
<td>&lt; 0.01</td>
<td>- 5</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>0</td>
<td>49.0 48.7</td>
<td>&lt; 0.7</td>
<td>- 1</td>
</tr>
<tr>
<td>200</td>
<td>0</td>
<td>0</td>
<td>61.2 54.0</td>
<td>&lt; 0.001</td>
<td>- 12</td>
</tr>
<tr>
<td>0</td>
<td>20</td>
<td>0</td>
<td>61.2 59.7</td>
<td>&lt; 0.25</td>
<td>- 3</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>4.5</td>
<td>54.1 54.5</td>
<td>&lt; 0.80</td>
<td>+ 1</td>
</tr>
<tr>
<td>160</td>
<td>0</td>
<td>0</td>
<td>53.8 50.2</td>
<td>&lt; 0.001</td>
<td>- 7</td>
</tr>
<tr>
<td>160</td>
<td>0</td>
<td>0</td>
<td>53.8 49.4</td>
<td>&lt; 0.001</td>
<td>- 8</td>
</tr>
</tbody>
</table>

*Each value represents mean of duplicate tests with 20 different PRP, with exception that guanidinosuccinic acid was tested with 10 PRP.
†Instead of urea in saline, urea in aqueous solution isosmotic with saline was used.

Table 2. Effects of Urea, Creatinine, and Guanidinosuccinic Acid on Platelet Aggregation Induced by Collagen and Norepinephrine at Room Temperature

<table>
<thead>
<tr>
<th>Aggregating Agent</th>
<th>Increment (mg/100 ml)</th>
<th>Urea Nitrogen</th>
<th>Creatinine</th>
<th>Guanidinosuccinic Acid</th>
<th>% Difference From Control</th>
<th>No. of Plasmas Tested</th>
<th>ρ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-16</td>
<td>20</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Collagen</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>4.5</td>
<td>+ 4</td>
<td>10</td>
<td>&lt; 0.50</td>
</tr>
<tr>
<td>Collagen</td>
<td>0</td>
<td>0</td>
<td>6.8</td>
<td>0</td>
<td>- 6</td>
<td>20</td>
<td>&lt; 0.025</td>
</tr>
<tr>
<td>Collagen (half-strength)</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-21</td>
<td>8</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Collagen (half-strength)</td>
<td>0</td>
<td>20</td>
<td>4.5</td>
<td>0</td>
<td>+ 3</td>
<td>8</td>
<td>&lt; 0.70</td>
</tr>
<tr>
<td>Norepinephrine (5.4 μM)</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>4.5</td>
<td>-24</td>
<td>10</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Norepinephrine (5.4 μM)</td>
<td>0</td>
<td>20</td>
<td>4.5</td>
<td>0</td>
<td>- 3</td>
<td>10</td>
<td>&lt; 0.40</td>
</tr>
<tr>
<td>Norepinephrine (5.4 μM)</td>
<td>0</td>
<td>0</td>
<td>4.5</td>
<td>0</td>
<td>- 3</td>
<td>10</td>
<td>&lt; 0.50</td>
</tr>
</tbody>
</table>
PLATELET AGGREGATION

Fig. 1. Changes in OD at 600 m\(\mu\) of PRP following addition of saline and of urea in saline. Superimposed tracings were made by a Gilford 2000 Multiple Sample Absorbance Recorder linked to a Beckman DU Spectrophotometer. Value of zero was arbitrarily assigned to OD of PRP before additions were made.

Platelet Aggregation at 37°C

The method used was essentially that of Born and Cross. Mixtures in which platelet aggregation was tested contained 0.8-0.9 volume of PRP and 0.1-0.2 volume of saline or test substance in saline. Mixtures (0.4 ml) of test compounds or saline with PRP were incubated for 55 min at room temperature and 5 min at 37°C. We used a Chrono-Log Platelet Aggregometer linked to a potentiometric recorder to record light transmission continuously at 37°C. A magnetic stirrer was set at maximum speed (approximately 1800 rpm). After stirring for approximately 1 min, 0.04 ml of one of the aggregating agents described above was added.

Statistical Methods

Each test substance was used an equal number of times immediately before and immediately after its saline control to compensate for any effects of aging of the PRP and aggregating agents. The t test for paired samples was used in analysis of the data.

RESULTS

Preliminary Studies

Figure 1 shows that when urea was added to PRP there was a rapid but transient increase in OD. The upper tracing shows the effect on OD of increasing the concentration of urea nitrogen by 200 mg/100 ml when one part of urea in saline was added to nine parts of PRP. The lower tracing shows the result of adding one part of saline to nine parts of PRP. The final OD of each of the two mixtures was essentially the same and was lower than the base line as expected because of dilution. By phase-contrast microscopy platelets appeared unaggregated after addition of the urea solution, and we were unable to detect a difference in their size or shape from those to which saline had been added. The transient increase in OD observed after addition of the urea solu-
Fig. 2. Superimposed tracings of platelet aggregation at 37°C show effects of incubation of PRP with increment of urea nitrogen of 300 mg/100 ml for 1 hr. ADP was added at arrow to obtain final concentration of 1.7 μM. Tracing of control PRP begins below and reaches peak above that of PRP to which urea was added. There is more rapid platelet disaggregation in PRP with added urea.

Addition to PRP required the presence of platelets and was not observed after addition of the urea solution to platelet-poor plasma.

When an increase of osmolarity of PRP equal to that produced by the urea solution was obtained by adding solutions of sodium chloride or potassium chloride in saline, there was an immediate increase in OD approximating in magnitude that produced by the urea solution, but differing in that it was sustained. When one part of water was mixed with nine parts of PRP there was a rapid and sustained decrease in OD. The decrease in OD produced by water was much greater than that produced by substituting an equivalent volume of saline for water. By phase-contrast microscopy we were unable to differentiate PRP to which water had been added from PRP to which saline had been added.

The above experiments led us to conclude that the OD of PRP was sensitive to osmotic changes. Therefore, we did not attempt to use changes in OD of PRP as a monitor of platelet aggregation until after the osmotic effects of urea had stabilized.

PRP from four men was stirred for 1 min without an aggregating agent immediately after the addition of urea in saline had caused an increment of urea nitrogen of 200 mg/100 ml and 1 hr after the urea solution was mixed with PRP. By phase-contrast microscopy we could not distinguish these PRP from their controls that had been agitated with saline. Thus, we could not detect an effect of urea on spontaneous aggregation of platelets in stirred PRP.

ADP-Induced Aggregation at Room Temperature

Table 1 shows that increasing urea nitrogen of PRP by 100 or 200 mg/100 ml inhibited ADP-induced platelet aggregation and that increasing creatinine by 10 or 20 mg/100 ml or guanidinosuccinic acid by 4.5 mg/100 ml had no demonstrable effect. The last two horizontal rows of figures in Table 1 compare the effects on platelet aggregation of adding urea in saline and urea in aqueous solution isomotic with saline. Since urea in saline did not have a greater effect, inhibition of platelet aggregation by urea at this concentration was not due to increasing the osmolarity of PRP.

In other experiments (not shown in Table 1), PRP from ten men were tested to determine whether creatinine or guanidinosuccinic acid could enhance the inhibitory effect of urea. Increments of creatinine (20 mg/100 ml) or of guanidinosuccinic acid (4.5 mg/100 ml), together with an increment of urea nitrogen (200 mg/100 ml), were no more effective than urea alone.
Fig. 3. Superimposed tracings of platelet aggregation at 37°C show effects on collagen-induced platelet aggregation of incubation of PRP with increment of urea nitrogen of 200 mg/100 ml and increment of guanidinosuccinic acid of 4.5 mg/100 ml for 1 hr.

ADP-Induced Aggregation at 37°C

The effects of urea and guanidinosuccinic acid were tested in duplicate in the PRP of six men. The mean of the mean increases in per cent transmission was 24.9 for the saline control, 20.8 for a urea nitrogen increment of 300 mg/100 ml \((p < 0.05, 16\%\) inhibition), and 24.6 for a guanidinosuccinic acid increment of 6.8 mg/100 ml. More rapid disaggregation usually occurred in the presence of urea (Fig. 2). Disaggregation curves in the presence of guanidinosuccinic acid did not appear different from the controls.

Collagen-Induced Aggregation at Room Temperature

Table 2 shows that a urea nitrogen increment of 200 mg/100 ml inhibited platelet aggregation induced by either a standard or a one-half strength collagen preparation. A guanidinosuccinic acid increment of 4.5 mg/100 ml did not inhibit aggregation induced by either preparation. A higher concentration of guanidinosuccinic acid had a small inhibitory effect. A creatinine increment of 20 mg/100 ml had no demonstrable effect.

In other experiments (not shown in Table 2) PRP from ten men were tested to determine whether creatinine could enhance the inhibitory effect of urea. An increment of creatinine (20 mg/100 ml) together with an increment of urea nitrogen (200 mg/100 ml) was no more effective than urea alone.

Collagen-Induced Aggregation at 37°C

The effects of urea and guanidinosuccinic acid on one-half strength collagen-induced platelet aggregation were tested in the PRP of eight men. The mean increase in per cent transmission was 37.0 for the saline control, 25.8 for a urea nitrogen increment of 200 mg/100 ml \((p < 0.01, 30\%\) inhibition), and 37.9 for a guanidinosuccinic acid increment of 4.5 mg/100 ml. An example is shown in Fig. 3.

Norepinephrine-Induced Aggregation at Room Temperature

Table 2 shows that urea inhibited norepinephrine-induced platelet aggregation and that guanidinosuccinic acid did not potentiate this inhibition. Neither guanidinosuccinic acid nor creatinine demonstrably inhibited norepinephrine-induced aggregation.

Norepinephrine-Induced Aggregation at 37°C

Guanidinosuccinic acid increments from 4.5 to 9.1 mg/100 ml had no effect on platelet aggregation curves (Fig. 4).
Large second phase of platelet aggregation occurred at 37°C in PRP that had been incubated with increment of guanidinosuccinic acid of 9.1 mg/100 ml for 1 hr. Norepinephrine was added at arrow to obtain final concentration of 5.4 μM. Saline control aggregation curve is not shown, as it is almost identical to one shown for guanidinosuccinic acid.

DISCUSSION

Our data show that incubation of PRP for 1 hr with a urea nitrogen increment of 200 mg/100 ml inhibited platelet aggregation induced by ADP, norepinephrine, or collagen by means of 12–30%. These data contrast with the reports of Salzman and Neri7 who stated that urea did not inhibit ADP-induced platelet aggregation, and Somer et al.17 who stated that urea initially inhibits and later enhances platelet aggregation. A likely explanation of this apparent discrepancy is that those investigators, who either simultaneously mixed urea and ADP with stirred PRP17 or did not state the period of incubation of urea with PRP,7 attempted to use OD changes of PRP as an index of platelet aggregation at a time when the OD of PRP was unstable for another reason. Figure 1 shows that the mere addition of a urea solution to PRP immediately altered the OD curve and temporarily invalidated its use as an indicator of platelet aggregation.

Lovett26 reported that when bacteria (which approximate the size of platelets) “are mixed with solutions of urea they undergo an initial osmotic dehydration which produces an increase in light scattering and then, as urea enters the cell, osmotic equilibrium is regained.” Fantl27 found a transient increase in the OD of PRP after urea was added in much higher concentrations than were used in our experiments. Stewart and Castaldi13 observed shrinkage of platelets by phase-contrast microscopy when the concentration of urea in PRP was approximately ten times the 71 mM increment used in our experiment shown in Fig. 1. Lundberg28 found that platelet volume decreased 45% when osmolarity increased 400%. If this relationship is linear down to the approximately 25% increase in osmolarity that we produced by raising the plasma urea nitrogen 200 mg/100 ml, we might expect only approximately 3% decrease in platelet volume, which could explain our failure to detect it by phase-contrast microscopy. It seems reasonable to conclude that the transient increase in OD that occurred after urea in saline was added to PRP (Fig. 1) was probably due to osmotic shrinkage.

That our demonstration of inhibition of platelet aggregation by urea in vitro may be relevant to in vivo situations is suggested by the results of two groups of investigators. Hellem et al.4 reported that the intravenous infusion of 150 g of urea into a subject with normal hemostasis prolonged the bleeding time and decreased platelet adhesiveness. Eknoyan et al.3 found that the bleeding time increased and platelet adhesiveness decreased in normal volunteers.
who ingested urea. Both Castaldi et al.\(^2\) and Cronberg,\(^5\) who reported the administration of urea to be without effect on platelet function\(^2\) or adhesiveness,\(^5\) raised the serum urea nitrogen concentration of normal subjects to less than 100 mg/100 ml. Hellem et al.\(^4\) presumably achieved higher concentrations as they administered more than twice as much urea intravenously as was administered orally by Cronberg,\(^5\) who measured platelet adhesiveness after only 1 hr of experimental azotemia. Eknayan et al.\(^3\) found that the bleeding times of four volunteers did not increase after 8–10 hr of experimental azotemia, but the mean bleeding time of six other volunteers increased more than 50% after 24 hr of experimental azotemia. The failures of Cronberg\(^5\) and Castaldi et al.\(^2\) to demonstrate an effect of urea administration may have been due to failure to maintain an inhibitory concentration of urea long enough before testing platelet function.

Several guanidino compounds have been shown to inhibit ADP-induced platelet aggregation in citrated PRP.\(^{29,30}\) Their inhibitory effect may be antagonized by calcium that appears to compete with the guanidino compounds to form a complex with ADP.\(^30\) Horowitz et al.\(^{20}\) have reported inhibition of ADP-induced platelet factor 3 activation by 0.2 mM (3.5 mg/100 ml) guanidinosuccinic acid. This concentration is within the range found in the sera of uremic patients,\(^{19}\) but the inhibition of ADP-induced platelet factor 3 activation was completely reversed by 2.5 mM calcium\(^{20}\) and presumably would not occur in vivo, as this addition of calcium was insufficient to allow coagulation to occur. Rabiner and Hrodek\(^{31}\) found that 75% of their uremic patients had decreased platelet factor 3 activation. In their assay systems utilizing celite, guanidinosuccinic acid did not inhibit platelet factor 3 activation.

Horowitz et al.\(^{21}\) recently reported that 0.1 mM (1.75 mg/100 ml) guanidinosuccinic acid blocked the second phase of platelet aggregation induced by 2.5 \(\mu\)M ADP or 1.4 \(\mu\)M epinephrine and inhibited aggregation induced by a connective tissue suspension. We found no inhibition of either first or second phase of platelet aggregation induced by 1.7 \(\mu\)M ADP after incubation with increments of guanidinosuccinic acid as large as 6.8 mg/100 ml. Increments as large as 9.1 mg/100 ml did not inhibit either phase of norepinephrine-induced aggregation (Fig. 4). We found no inhibition of collagen-induced aggregation by increments of guanidinosuccinic acid of 4.5 mg/100 ml and slight inhibition by increments of 6.8 mg/100 ml (Table 2). The highest concentration of guanidinosuccinic acid reported in the sera of 15 uremic patients was 5.4 mg/100 ml.\(^{19}\)

The reason for the apparent conflict of our results with those of Horowitz et al.\(^{21}\) is not clear. It may be related to their inclusion of an inhibitor of platelet aggregation in their test system as a buffer. It appears from their description of methods\(^{20,21}\) that they dissolved both guanidinosuccinic acid and aggregating agents in imidazole-buffered saline, bringing the final concentration of imidazole to 0.5 mM. Recent experiments in our laboratory have shown inhibition of collagen-induced platelet aggregation by 0.5 mM imidazole and of the second phase of norepinephrine- and ADP-induced aggregation by 1 mM imidazole.\(^{32}\)
Hemostasis depends upon adherence of circulating platelets to subendothelial substances such as collagen, basement membranes, and microfibrils exposed by vascular injury. ADP released from the adherent platelets brings about platelet aggregation and formation of a hemostatic platelet plug. It has been suspected that catecholamines, which in low concentrations potentiate ADP-, collagen-, and thrombin-induced platelet aggregation in vitro, may play a role in hemostasis. Since our experiments have shown that urea (in concentrations found in uremic patients) inhibited human platelet aggregation induced in vitro by collagen, ADP, and norepinephrine and that guanidinosuccinic acid, a recently discovered metabolite of urea, did not inhibit platelet aggregation in vitro in concentrations found in the sera of uremic patients, it appears likely that the increased bleeding times, found by Eknoyan et al. and Hellem et al. after administration of urea to people, were due to urea itself, and not to its metabolite, guanidinosuccinic acid. Their in vivo data coupled with the in vitro data of the present paper also make it seem likely that the prolonged bleeding times and mucosal bleeding frequently seen in patients with severe uremia are due at least in part to inhibition of platelet aggregation by urea, which also inhibits the oxidative metabolism of platelets.

ACKNOWLEDGMENT

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REFERENCES

Guanidinosuccinic Acid on Human Platelet Effects of Exogenous Urea, Creatinine, and Aggregation In Vitro

James W. Davis, James R. McField, Phyllis E. Phillips and Barbara A. Graham