Utilization of Ascorbic Acid During Platelet Aggregation[1] (34717)

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In 1936, Stephens and Hawley (1) noted high levels of ascorbic acid in the white cell layer of blood. Later Butler and Cushman (2) found that ascorbic acid was more highly concentrated in the "upper buffy coat" layer of whole blood (centrifuged at a slow speed) that contained predominantly platelets. Other workers (3, 4) have confirmed and extended these observations. Crandon, Lund, and Dill (5) have shown that the drop in buffy coat ascorbic acid closely parallels the onset of clinical scurvy; whereas, the serum values are low-to-absent long before this time. Other observations (6, 7) on buffy coat and serum ascorbic acid concentrations agree with this.

Recently, a platelet functional defect has been noted in scorbutic patients (8) as well as in guinea pigs (9). This confirms the original report by Cetingil *et al.* (10) of a defect in platelet agglutinability and adhesion in a scorbutic patient that was corrected by ascorbic acid therapy. Born and Wright (11) showed that adenosine diphosphate-induced platelet aggregation is diminished in scorbutic guinea pigs. In the present investigation we have attempted to determine whether ascorbic acid is (i) released from platelets into the plasma [as in the release reaction described by Grette (9)], (ii) utilized in metabolic processes within the platelet during aggregation, or (iii) merely transported to sites for tissue utilization.

Materials and Methods. Fifteen fasting human subjects with no knowledge of prior disease (6 women and 9 men, ranging from 23 to 57 years of age) participated in the study. Blood was drawn from an antecubital vein, using a 20-gauge needle and plastic syringe, and immediately placed in siliconized glassware containing either acid citrate (henceforth referred to as citrated blood or citrated plasma) or ethylenediaminetetraacetic acid (EDTA) as previously described (12). Platelet-rich and platelet-poor plasma (PRP and PPP) fractions were obtained by differential centrifugation at room temperature and appropriately diluted to adjust the platelet concentration to $400 \times 10^3/mm^3$. The PRP had approximately 1 white cell/30,000,000 platelets and 1 red cell/50–100,000 platelets.

Blood from each donor was collected in three separate aliquots. The first aliquot was anticoagulated with citrate and after the PRP was separated, 2 ml of PRP was stirred with 0.1 ml of an aggregating agent or isotonic saline at 37° in an EEL[4] titrator (aggregometer). At 2 min, the maximum optical density change was recorded. At 8 min, the platelets were separated from the plasma by centrifugation (4° at 3000 rpm for 20 min) and ascorbic acid analysis was begun on both plasma and platelets. This procedure was carried out with the following aggregating agents: thrombin,[5] collagen suspension, epinephrine, and adenosine diphosphate (ADP) with the final concentrations shown in Table I. In this aliquot we measured any net change of ascorbic acid in platelets or in plasma and compared these data with the maximum degree of platelet aggregation.

[1] Supported by Research Grants HE-02754 and Research Career Award IK6 HE-21, 835 (P. M. A.) from the National Heart Institute, and by Training Grant 5-TI-AM-5103 from the National Institute of Arthritis and Metabolic Diseases.

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[4] Evans Electroselenum Ltd., England.

[5] Thrombin, Topical (Bovine origin), Parke, Davis & Co.

TABLE I. The Net Changes in Total Ascorbic Acid in Platelets and in Corresponding Cell-Free Plasma after the Addition of 0.1 ml of the 4 Aggregating Agents.

Separate values for ascorbic acid after addition of collagen or epinephrine are given for the six subjects who had ingested aspirin and nine subjects who had not had any aspirin for 7 days.

	Net change in ascorbic acid			
	Platelets ($\mu g/10^{10}$ platelets)		Cell-free plasma (μg/100 ml)	
Adenosine diphosphate (1×10^{-5} *M*)	-20.44 ± 2.7		-0.22 ± 1.8	
Thrombin (0.1 NIH units/ml)	-21.81 ± 3.5		-0.47 ± 0.5	
	No aspirin	Aspirin	No aspirin	Aspirin
Collagen suspension (0.1 ml)	-23.5 ± 2.8	$+5.5 \pm 2.65$	-1.5 ± 0.27	-0.66 ± 3.6
Epinephrine (4.5×10^{-4} *M*)	-18.7 ± 2.6	$+15.2 \pm 5.6$	-1.3 ± 0.5	-9.0 ± 1.9

The second aliquot of blood was anticoagulated with EDTA. Platelet-rich plasma was separated and stirred for 6 min at 37° after the addition of freshly prepared thrombin. The supernatant plasma was immediately separated by centrifugation and was analyzed for ADP and adenosine triphosphate (ATP). Some of these specimens were stored for up to 6 weeks at —70°. Nucleotides were measured by the firefly bioluminescence method of Holmsen *et al.* (13) except that a Turner fluorometer was used.[6] In this aliquot we attempted to compare the release of nucleotides in EDTA plasma after the addition of thrombin with the degree of aggregation observed in the same donor's citrated PRP.

The third aliquot of blood was also anticoagulated with EDTA and PRP was stirred in the titrator for 8 min with either 0.1 ml of thrombin (0.1 μ/ml, final concentration) or 0.1 ml of saline. Again after centrifugation the supernatant plasma and platelet button were separated and analyzed for ascorbic acid. In this last aliquot we attempted to measure ascorbic acid release from platelets.

Ascorbic acid was measured as total ascorbic acid (1-ascorbic acid, 1-dehydroascorbic acid and diketo-1-gulonic acid) by the method of Bessey, Lowry, and Brock (14).

Results. The mean concentration of ascorbic acid in platelets was 62.8 ± 5.6 (SEM) $\mu g/10^{10}$ platelets and the value in PPP was 115.2 ± 10.8 (SEM) μg/100 ml. Since the platelet count was kept constant at 400,000 mm^3, the value for 10^{10} platelets represents platelets from 25 ml PRP. Therefore, the corresponding volume of PPP is actually 115.2/4 or 29 $\mu g/25$ ml. For convenience, concentrations of ascorbic acid in PPP will be expressed as μg/100 ml. The reproducibility of platelet and plasma ascorbic acid values varied within 5% error. In citrated PRP there was a highly significant coefficient of correlation between the net loss of ascorbic acid from platelets (Table I) and the degree of platelet aggregation induced by ADP ($r = -0.86$), epinephrine ($r = -0.90$), thrombin ($r = -0.89$), and collagen ($r = -0.86$). In Fig. 1 the linear relationship between the loss

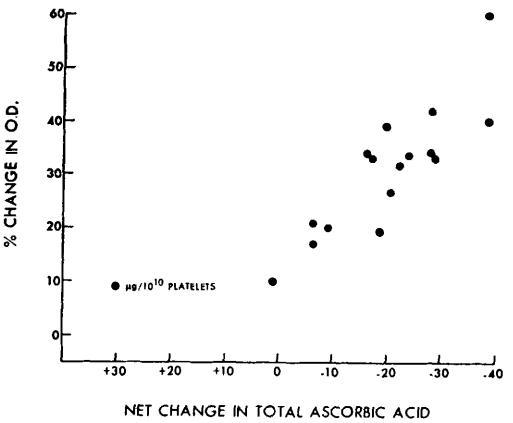

FIG. 1. The net change in total platelet ascorbic acid ($\mu g/10^{10}$ platelets) correlated with the degree of platelet aggregation [as percentage change of optical density (OD)] after the addition of ADP in a final concentration of 1×10^{-5} *M*.

[6] G. K. Turner Assoc., East Palo, California.

of ascorbic acid from the platelets and the degree of aggregation induced by ADP is shown. The concentration of ascorbic acid in cell-free plasma deviated only slightly from that in the preaggregation plasma samples and did not account for the loss of platelet ascorbic acid during aggregation. These small changes in plasma ascorbate (see Table I) are even less significant since, as previously mentioned, they are expressed as μg/100 ml of plasma; whereas, the value for platelets represents only 25 ml of plasma. The largest reductions in platelet ascorbic acid concentration occurred in the reactions with thrombin and ADP (20.44 and 21.81 μg of ascorbic acid/10^{10} platelets, respectively). The reason for the lesser reactions to collagen and epinephrine became apparent when it was found that 6 of the 15 subjects had ingested aspirin within 7 days of donating blood. It is well known that platelet aggregation by these 2 agents is inhibited while aggregation by thrombin and high molar ADP is not affected by aspirin (15). In Table I the data for the responses of those who had ingested aspirin and those who had not are separated. In the 6 subjects who had ingested aspirin and who therefore had only a first wave response to epinephrine there was a net gain of ascorbic acid of $+15.2 \pm 5.6$ (SEM) μg/10^{10} platelets. In these same subjects, there was a net loss of -9.0 ± 1.9 μg/100 ml of ascorbic acid from the plasma during incubation with epinephrine. No significant change occurred in the concentration of ascorbic acid in cell-free plasma in the subjects who had not ingested aspirin (net change -1.3 ± 0.5 μg/100 ml).

The mean values for nucleotide release induced by thrombin were 17.9 ± 2.5 for ADP and 30.2 ± 2.9 (SEM) nanomoles/10^9 platelets for ATP, respectively. These data agree with previously published reports (16, 17). The correlation coefficient between nucleotide release in EDTA PRP and platelet aggregation in citrated PRP was highly significant ($r = +0.72$) (Fig. 2). Thrombin did not induce platelet aggregation in PRP anticoagulated with EDTA (as calcium or magnesium ions are required for this reaction) nor did it induce any significant change in the concentration of ascorbic acid in platelets or in cell-free plasma.

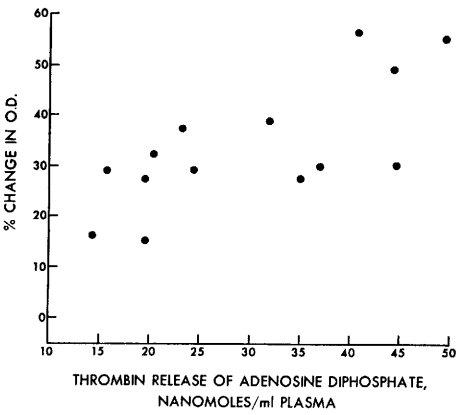

FIG. 2. The release of ADP correlated with the degree of platelet aggregation after the addition of thrombin, 0.1 units/ml, final concentration.

Discussion. Relatively high levels of ascorbic acid in normal platelets and white blood cells were reported by Barkhan and Howard (3) and Denson and Richards (4). Our results confirm the high concentrations of ascorbic acid in the normal human platelet fraction. In addition to the platelet defect in scurvy already mentioned, phagocytosis by leukocytes has been reported defective (18) and studies of peritoneal exudates in guinea pigs show that phagocytic activity of leukocytes bears a direct relationship to their ascorbic acid content (19). Our results show a direct relationship between the degree of platelet aggregation and the ascorbic acid utilized by these platelets. When aggregation by epinephrine is rendered minimal or absent by ingestion of aspirin, platelets take up ascorbic acid from the plasma during incubation at 37°.

Conclusion. In 15 human subjects the concentration of ascorbic acid in platelets was 62.8 ± 5.6 (SEM) μg/10^{10} platelets and the level in cell-free plasma was 115.2 ± 10.8 (SEM) μg/100 ml. A direct correlation was found between the degree of platelet aggregation induced by thrombin, epinephrine, collagen, and ADP and the net loss of ascorbic acid from the platelets. In subjects who had ingested aspirin, platelet aggregation by epinephrine was minimal or absent and there

was either no loss or a net gain in ascorbic acid. In platelets from plasma anticoagulated with EDTA and incubated with thrombin, aggregation of platelets did not occur and the concentration of ascorbic acid did not change even though ADP and ATP were released. Ascorbic acid is not released into the plasma during platelet aggregation induced by various agents and is not merely present for tissue transport. It appears to serve some definite metabolic function in platelet aggregation.

We thank Mrs. Evelyn Garner and Miss Maria Reisz for technical assistance.

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Received Sept. 8, 1969. P.S.E.B.M., 1970, Vol. 134.