Inhibition of Adenosine Diphosphate-Induced Secondary Aggregation and Other Platelet Functions by Acetylsalicylic Acid Ingestion* (32737)

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Macmillan (1) observed two phases of aggregation—primary and secondary—when adenosine diphosphate (ADP) was added to stirred citrated platelet-rich plasma at 37°C. Secondary aggregation (SA) was irreversible and appeared to be caused by ADP liberated from the platelets. Studies in our laboratory (2) showed that serotonin release and activation of platelet factor 3 (PF-3) accompanied SA and were absent in subjects failing to show SA. We observed that 0.325 gm acetylsalicylic acid (ASA) abolished SA and diminished serotonin release in one individual although primary aggregation was still evident. This observation seemed worth pursuing, since Quick (3) has found that 1.3 gm of ASA prolonged the bleeding time slightly, and Mills and Roberts (4) have reported that other drugs (chlorpromazine, imipramine and related compounds) added *in vitro* abolished SA. In addition, Packham and co-workers (5) noted that the anti-inflammatory drugs sulfinpyrazone and phenylbutazone added *in vitro* inhibited platelet aggregation produced by collagen and other particles, probably by interfering with the release of ADP which is thought to be responsible for connective tissue-induced aggregation (6, 7).

Materials and Methods. Citrated platelet-rich plasma (PRP) was prepared as described by Macmillan (1) and used within 75 min. Platelet aggregation induced by ADP was recorded by a method reported by Skoza *et al.* (8) but the PRP was maintained at 37°C instead of room temperature, and one-hundredth rather than one-tenth volume of ADP (Sigma Chemical Co., St. Louis, Mo.) or a 1:10 suspension of connective tissue (CT) (9) was added. The stated concentrations of ADP refer to the final concentrations in PRP. Platelet factor-3 activity was estimated from the Russell Viper Venom clotting time (RVV) of aliquots of PRP taken from the cuvette immediately before and 5 min after ADP was added. The venom (Burroughs Wellcome & Co., Tuckahoe, N.Y.) was diluted 1:30,000, mixed with an equal volume of 0.02 *M* $CaCl_2$, kept at 37°C, and used within about 30 min. Two-tenths ml was added to 0.1 ml PRP and the clotting time recorded. Control times ranged from 30 to 40 sec.

Serotonin release was studied at 37°C using a separate sample of PRP in which the platelets had been labeled with serotonin-^{14}C (9); 0.05 ml of either 10 μM of ADP, 100 μM of ADP, a CT suspension, or highly purified human thrombin (9) (about 30 units/ml) was added to 0.45 ml of labeled PRP at 37°C. The samples were shaken for 2 min, incubated at 37°C for 13 min, and then centrifuged. Release of radioactivity was determined as previously described. In two experiments, acid phosphatase activity of the supernatant was also measured using nitrophenylphosphate (10).

The subjects were five male and five female laboratory workers 21–47 years of age. They were not taking drugs regularly and had had none, including ASA, for at least 2 days. The first blood sample was drawn about 11:30 a.m. and about 1 hour later 650 mg of ASA was ingested. The dose was repeated at about 1:30 p.m., and an hour later the second blood sample was drawn.

Results. Figure 1A shows the changes in optical density (OD) in a typical experiment in which SA was induced. Eight of the 10 subjects had this response with a critical concentration of ADP: 1.0 μM in three instances, 1.5 μM in one, and 2.0 in four. Table I indicates the average maximum decreases in OD. After the critical concentration of ADP was added, the OD reached its lowest value within 1 min during the primary phase and at about 5 min during the secondary phase. With lower levels of ADP (0.8, 1.0, or 1.5 μM),

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TABLE I. Average Decreases in Optical Density and Russell Viper Venom Clotting Time (RVV) Produced by ADP in Normal Subjects Before and After Ingestion of Acetylsalicylic Acid.

	Secondary platelet aggregation (8 subjects)		No secondary platelet aggregation (2 subjects)	
ADP level	Max. OD decr.	RVV decr.[a] (sec)	Max. OD decr.	RVV decr. (sec)
Critical				
Primary	0.190 (0.119–0.300)[b]	—	0.205, 0.230[c]	—
Secondary (5 min)	0.317 (0.185–0.420)	12 (10–15)	—	5, 4
Below critical	0.158 (0.080–0.330)	3 (0–6)	0.075, 0.175	3, 0
Above critical	0.350 (0.290–0.460)	11 (9–15)	0.241, 0.245	5, 6
Critical after ASA	0.189 (0.110–0.240)	4 (0–9)	—	—

[a] Decrease in RVV time 5 min after ADP in the five subjects with data for all four tests.
[b] Numbers in parentheses represent the range.
[c] Critical ADP concentration taken as 2.0 μM.

the maximum decrease occurred within 1 min and the OD then rose toward its initial value, whereas with higher levels (1.5, 2.0, or 3.0 μM) the OD continued to fall for at least 5 min. In the two subjects who failed to show SA, the OD decrease after the addition of 2.0 μM ADP resembled the primary response of the other eight to the critical concentration of ADP, and the OD changes with a higher concentration of ADP (3.0 μM) were less than those for any of the eight "reactors".

Also shown in Table I are the changes in RVV times. The clotting time decreased 6 sec or less with ADP concentrations below the critical value in subjects showing SA or with any concentration tested in the two nonreactors. On the other hand, in the five reactors tested with all the appropriate concentrations of ADP, the clotting time decreased at least 9 sec with the critical concentration of ADP or higher levels.

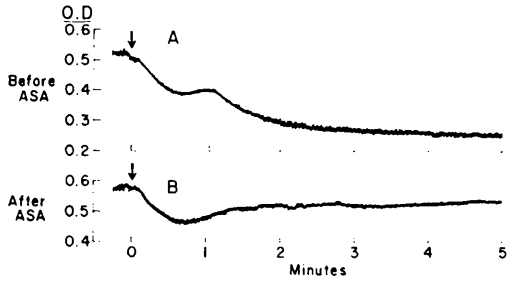

FIG. 1. A. Optical density changes of citrated platelet-rich plasma after addition of 1 μM of ADP. B. Same after ingestion of 1.3 gm acetylsalicylic acid.

After ASA ingestion, the previously critical ADP concentration failed to cause SA but merely resulted in an OD decrease about equal to the primary response before ASA (Fig. 1B). The maximum decrease in the RVV time was only 9 sec (Table I). The ADP concentrations up to 4.0 μM also failed to produce SA and decreased the OD only slightly more. In contrast to OD changes, the aggregation rate measured from the steepest slope of the OD curve did not consistently reflect the different responses to ADP or the effect of ASA.

In five subjects who showed SA, the OD changes in response to CT averaged 0.345 as compared with 0.085 after ASA. In one participant (R.G.) who did not show SA, CT produced an OD decrease of 0.300.

Platelet serotonin-^{14}C was released by ADP,

TABLE II. Release of Platelet Serotonin-^{14}C in Normal Subjects Before and After Ingestion of Acetylsalicylic Acid (ASA).

	Secondary platelet aggregation			No secondary platelet aggregation (2 subjects)
	No. of subjects	Av release (%) before ASA	Av release (%) after ASA	Release (%) before ASA
ADP 10 μM	8	41 (11–54)[a]	5 (0–24)	5, 7
ADP 1 μM	7	37 (0–56)[a]	2 (0–8)	1, 2
CT	4	59 (30–77)	7 (0–19)	55, 79
Thrombin	6	82 (40–106)	67 (30–102)	75, 86

[a] The low values were from one individual. If they are omitted, the average value for 10 μM ADP is 45% (34–54) and the average value for 1.0 μM ADP is 43% (30–56).

CT, and thrombin in the persons showing SA (Table II). With 1.0 μM ADP, platelet serotonin was liberated in all but one of the seven subjects even though 1.5 or 2.0 μM ADP was required to produce SA in four of them. Use of different agitation methods and incubation times for studying serotonin release and SA probably accounts for this discrepancy. In our earlier studies (2) serotonin release was measured in aliquots removed from the cuvette 5 min after the addition of ADP and was observed only in samples showing SA and a considerably shortened RVV time. The ADP, CT, and thrombin did not release acid phosphatase although seven times more enzyme activity was noted in PRP which had been frozen and thawed than in platelet-poor plasma. After ASA ingestion, serotonin release by ADP and CT was almost abolished, whereas release induced by thrombin was relatively unaffected (Table II). In the two subjects who showed no SA, the platelets failed to release serotonin with ADP, but release was excellent with CT and thrombin.

Studies were repeated within several weeks on the two young male subjects who had not exhibited SA or associated changes. One subject (R.B.) showed SA, but unlike the eight reacting subjects, required 4.0 μM ADP and the RVV time became only 8.0 sec shorter. The SA was also evident with 5.0 μM ADP and RVV became 11.0 sec shorter. The other subject (R.G.) showed no SA, and RVV became only slightly shorter (3, 6, 6, 8, and 10 sec with 1.5, 2, 3, 4, and 5 μM ADP, respectively). There was no serotonin release with 1.0 μM ADP in either subject, but 25% and 28% of platelet serotonin was released, respectively, with 10 μM ADP.

Discussion. Our results confirm the findings of Macmillan (1) and of Mills and Roberts (4) that ADP can cause secondary aggregation of platelets, presumably through release of endogenous platelet ADP. In common with other investigators (1, 4), we found evidence of SA only at a critical concentration of ADP. This lay between 1.0 and 2.0 μM in our experiments, whereas Macmillan (1) reported a range of 0.1–10.0 μM. In this and one other study (2), liberation of serotonin and development of PF-3 activity were found to be associated with SA. At ADP concentrations above the critical level, serotonin release and PF-3 activation occurred but SA was not evident, presumably because primary and secondary aggregation were fused, as Macmillan suggested (1). At ADP concentrations slightly below the critical value, PF-3 was only slightly activated, no serotonin was released, and aggregation was quickly reversed. Hardisty and Hutton (11) had previously reported a marked decrease in RVV time when

platelet aggregation was induced by adding as little as 0.5 μM ADP to stirred citrated PRP at 37°C. They did not observe SA, perhaps because their recording of OD was compressed.

These ADP-induced reactions have several interesting features: PF-3 activation (11) and serotonin release (2) are closely associated with aggregation, being absent when ADP is added to unstirred PRP; the level of ADP is very critical, an all-or-none response; and SA, at least, occurs at 37°C but not at room temperature.

Macmillan's and our own studies revealed SA in only 70–80% of normal persons. When our subjects who did not show SA and associated changes were again studied they exhibited some aspects of the ADP-induced release reaction but fewer changes than those seen in the other subjects. One (R.B.) had had several teeth extracted without unusual bleeding. The other (R.G.) experienced mild oozing from a tooth socket for 6 hours following an extraction 2 years earlier, but underwent tonsillectomy as a child without excessive hemorrhage. The bleeding time (Ivy method) is normal in both and they do not bruise easily. Neither has had any major operations. The cause and significance of the defective release reaction produced by ADP in some subjects is not known. It is of interest that addition of CT caused a normal serotonin release from the platelets of both nonreacting subjects and a normal decrease in OD of PRP in the one subject tested in this respect (R. G.).

While our study was in progress, Evans *et al.* (12) reported that the addition of 0.5 mg of ASA per ml of human citrated PRP prevents platelet aggregation by collagen. Although using techniques similar to ours,[1] they did not observe SA in normal subjects. They, too, found no change in primary aggregation with ASA. Considered with our results, it appears that ASA should be added to the list of drugs which prevent secondary aggregation induced by ADP (4) and primary aggregation induced by collagen (4, 5). These drugs presumably prevent collagen and ADP from producing the profound changes that cause platelets to release an aggregating agent and serotonin, develop PF-3 activity, and undergo irreversible aggregation. It is noteworthy that the changes are produced by ADP, which is soluble, and also by particulate material as previously described (5). Inhibition of this release reaction by sulfinpyrazone, phenylbutazone, and ASA may provide a clue to the mechanism underlying their anti-inflammatory activity. Our results and those of others (4, 5) indicate that these drugs do not influence the release reaction induced by thrombin.

In our experiments, ASA proved effective *in vivo* when the amounts ingested were well within the normal clinical range. Weiss and Aledort (13) observed independently that blood drawn from subjects given 3 gm of ASA daily for 2.25 days exhibited poor aggregation with CT and a diminished release of platelet ADP by kaolin. All their subjects had a "normal" response to ADP after ASA but since they studied aggregation only at room temperature they observed no SA before or after ASA ingestion. Thus, there is no discrepancy between their results and ours.

The variety and ubiquity of drugs which inhibit the ADP- and CT-induced release reaction in platelets make it difficult to obtain reliable results in patients receiving numerous medications, or to interpret data already reported in the literature. For example, the rapid reversal of ADP-induced aggregation and poor response to collagen noted by Hardisty and Hutton in 13 patients with mild bleeding symptoms (14) are similar to changes produced *in vivo* by ASA and possibly by other drugs. Their findings would be significant only if the patients were not currently receiving medication.

It may be noteworthy that four of the eight subjects in our series who showed SA developed hematomas at the site of the second or both venipunctures. Quick (3) and Weiss and Aledort (13) reported that the bleeding times of normal subjects increased slightly following ASA ingestion. Frequent marked increase in bleeding time and occasional hemorrhagic episodes were observed when 30–60 mg of ASA per kg was given daily for 3–6 days to patients receiving anticoagulant drugs (15) or with hemorrhagic diatheses (16) such as von Willebrand's disease (3), hemophilia

[1] Personal communication.

(16) or leukemia (16). In animals, ASA (12, 17), phenylbutazone (5, 17), and sulfinpyrazone (5) prolonged the bleeding time of transected mesenteric blood vessels, and a very severe hemostatic defect was evident when these drugs were given to rabbits receiving oral anticoagulants (17) or dogs with Factor IX deficiency (18).

Summary. Secondary aggregation of platelets was produced in 8 of 10 normal subjects by a critical concentration of adenosine diphosphate (ADP) ranging from 1.0–2.0 μM. Shortening of the Russell Viper Venom clotting time and release of serotonin-^{14}C were also observed with the critical concentration of ADP or with higher levels, but did not occur in the two subjects who showed only primary aggregation in response to ADP. Ingestion of 1.3 gm of acetylsalicylic acid in a divided dose 1 and 2 hours prior to testing abolished secondary aggregation, serotonin release, and platelet factor-3 activation induced by ADP, as well as aggregation and serotonin release induced by connective tissue particles, but did not affect primary ADP-induced aggregation.

Addendum. O'Brien (Lancet **1**, 204, 1968) has recently shown that ingestion of as little as 0.15 gm of aspirin can abolish secondary aggregation induced by epinephrine for as long as 6 days.

1. Macmillan, D. C., Nature **211**, 140 (1966).

2. Zucker, M. B. and Peterson, J., Blood, **30**, 556 (1967).

3. Quick, A. J., Am. J. Med. Sc. **252**, 265 (1966).

4. Mills, D. C. B. and Roberts, G. C. K., Nature **213**, 35 (1967).

5. Packham, M. A., Warrior, E. S., Glynn, M. F., Senyl, A. S., and Mustard, J. F., J. Exptl. Med. **126**, 171 (1967).

6. Hovig, T., Thromb. Diath. Haemorrhag. **9**, 264 (1963).

7. Spaet, T. H. and Zucker, M. B., J. Appl. Physiol. **206**, 1267 (1964).

8. Skoza, L., Zucker, M. B., Jerushalmy, Z., and Grant, R., Thromb. Diath. Haemorrhag. **18**, 713 (1967).

9. Jerushalmy, Z. and Zucker, M. B., Thromb. Diath. Haemorrhag. **15**, 415 (1966).

10. Zucker, M. B. and Borrelli, J., J. Clin. Invest. **38**, 148 (1959).

11. Hardisty, R. M. and Hutton, R. A., Brit. J. Haematol. **12**, 764 (1966).

12. Evans, G., Nishizawa, E. E., Packham, M. A., and Mustard, J. F., Blood, **30**, 550 (1967).

13. Weiss, H. J. and Aledort. L. M., Lancet **2**, 495 (1967).

14. Hardisty, R. M. and Hutton, R. A., Lancet **1**, 983 (1967).

15. Beaumont, J. L., Willie, A., and Lenègre, J., Bull. Soc. Med. Hôp. Paris **71**, 1077 (1955).

16. Beaumont, J. L., Caen., J., and Bernard, J., Bull. Soc. Med. Hôp. Paris **71**, 1087 (1955).

17. Evans, G., Packham, H. A., Nishizawa, E. E., and Mustard, J. F., J. Clin. Invest. **46**, 1053 (1967).

18. Mustard, J. F., Glynn, M. F., Nishizawa, E. E., and Packham, M. A., Federation Proc. **26**, 106 (1967).

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Gallbladder Damage and Prolonged Excretion of Cholera Vibrios in *Erythrocebus patas* (32738)

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According to Pollitzer (1), Pirogoff, Kulescha, and Gregg were the first students of the relationship of cholera and the gallbladder, but it remained for Snijders to assert that this organ is invaded by vibrios from the intestinal tract rather than the lymphatic channels or the bloodstream. Valk (2) was perhaps the first to relate prolonged excretion of cholera vibrios and their survival in the gallbladder. Gohar (3), Barua (4), Gangarosa *et al.* (5) and Wallace *et al.* (6) used duodenal intubation or oral administration of magnesium sulfate to detect gallbladder carriers of cholera vibrios. Hasan *et al.* (7) found the biliary system also involved in experimentally-infected rhesus monkeys.