On the Mechanism of Platelet Function Inhibition by Acetylsalicylic Acid[1,2] (34533)

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Acetylsalicylic acid (ASA, aspirin) can interfere with certain *in vitro* and *in vivo* platelet functions. Exposure of platelets to this drug results in a reduced capacity to aggregate in response to connective tissue fragments (collagen) (1), although the primary adhesion reaction is unaffected (2). Platelets obtained from donors who have ingested aspirin show normal primary aggregation in response to adenosine diphosphate (ADP), but the secondary wave of aggregation together with ^{14}C-serotonin release and platelet factor 3 activation by a critical concentration of ADP are abolished (3). ASA similarly inhibits the secondary wave of aggregation produced by epinephrine (4). These aspirin effect on platelets can be observed when the usual therapeutic doses of drug are ingested or when it is incubated with platelets *in vitro*.

The laboratory abnormalities of platelet function produced by aspirin are accompanied by corresponding hemostatic defects. Quick (5) demonstrated a small but significant prolongation of the bleeding time in normal subjects 2 hr after ingesting 650 mg of aspirin, and similar findings were subsequently obtained by Weiss *et al.* (6). When patients with coagulation disorders were studied, the effect of aspirin was considerably greater, leading to the suggestion that an "aspirin tolerance test" might be helpful in detecting some of these conditions (7). Antithrombotic effects of aspirin have been reported in rabbits. There was reduction of deposits formed in extracorporeal shunts (8), and the generalized Shwartzman reaction was inhibited (9).

The mechanism and site of action of aspirin on platelets are not known. O'Brien (4) and Weiss *et al.* (6) reported that platelets can remain abnormal for many days after the ingestion of a single dose of aspirin. This suggests that platelets are permanently affected by the drug, and restoration to normal probably represents the appearance of a new platelet population. In the study to be reported the mechanism and site of action of aspirin on human platelets were investigated.

Materials and Methods. Blood was obtained from healthy donors who had taken no medication for at least 6 days; the anticoagulant was 0.1 vol of 3.8% sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation at 300*g* for 15 min in plastic (Nalgene) tubes; all processing was accomplished at room temperature. The average platelet count of the PRP was about 400,000/mm^3; occasional leukocytes and erythrocytes were present.

The effect of aspirin on platelet function was compared to that of another acetylating compound, acetic anhydride. Each compound was dissolved in isotonic saline, and the pH raised to 6.0 with 0.5 *N* NaOH. One-tenth volume of reagent was added to PRP and the final pH was adjusted to 7.6. Tests were performed after 15 min of incubation at 37°. Control PRP was similarly treated except

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TABLE I. Radioactively Labeled Compounds Used.

Compound	Specific activity (mCi/mmole)	Dose used (μCi/1 ml PRP)	Supplier
1-Acetyl-1-^{14}C salicylic acid	2.4	0.05–0.1	Merck Sharp & Dohme Research Labs., Rahway, New Jersey
2-Carboxyl-^{14}C acetylsalicyclic acid	3.26	0.1	Nuclear Chicago, Inc., Chicago, Illinois
3-Acetic 1-^{14}C-anhydride	55	0.1	International Chemical & Nuclear Corp., California
4-Sodium 1-^{14}C-acetate	40	0.05–0.1	Nuclear Chicago, Inc., Chicago, Illinois

that isotonic saline was used in place of the active compounds. The final pH, volume, and platelet count were equated in all specimens tested. The final concentration of aspirin and acetic anhydride was 20 μM. In another experiment acetic anhydride was preincubated for 15 min in platelet-poor plasma (PPP) at a final concentration of 40 μM. One-tenth volume aliquot of this mixture was then incubated for 15 min at 37° with PRP, and the same tests were performed.

Platelet aggregation was estimated by modification of the optical-density method described by Born and Cross (10), with 0.1 vol of epinephrine at 20 μM final concentration, adenosine diphosphate (ADP) at 2 μM final concentration, or connective tissue fragments (collagen) as previously described (11), as the aggregating agents. Platelet factor 3 release was measured by the method described by Zucker and Peterson (3), in which Stypven® times were performed on samples taken from the aggregation cuvettes during the course of optical-density studies. Tests were performed just prior to and 5 min after addition of the aggregating agents.

Possible binding of aspirin to platelets was studied by means of radioactively labeled reagents, and several compounds were compared. The characteristics of the compounds used are given in Table I. In each case, 0.1 ml of the test compound at specified concentration was added to 1.0 ml of PRP. Specimens were incubated as indicated below, after which platelet buttons were collected by centrifugation for 20 min in a Clay Adams Sero-Fuge. These buttons were washed three times by resuspension in isotonic saline and subsequent centrifugation as before. Determination of radioactivity was performed in a Nuclear Chicago liquid scintillation counter.

To determine the anatomic site of binding of aspirin to platelets, subcellular fractionation of platelets heavily labeled with acetyl-1-^{14}C-salicylic acid was performed by methods previously described (12). In each of these experiments PRP obtained from 1 unit of freshly drawn blood was used. The usual three fractions: soluble (containing soluble enzymes and proteins), membrane (containing plasma membranes), and granular (containing the cytoplasmic organelles) were obtained and tested for radioactivity. All experiments were performed at least in duplicate, and usually repeated three times or more.

Results. Normal, untreated PRP showed optical density (OD) response similar to those reported by earlier investigators, after addition of aggregating agents (1, 3, 4, 6). Epinephrine of ADP produced a biphasic curve, with a primary, and then a secondary wave of aggregation. The response to connective tissue fragments was that of a progressive drop in OD without evidence of biphasic response. When PRP was incubated with either aspirin or acetic anhydride, similar abnormalities were observed. Although the primary OD fall was unaffected, the secondary phase of aggregation in response to epinephrine or ADP was abolished, and the response to connective tissue fragments was greatly reduced. These observations are illustrated in Figs. 1, 2, and 3. In this system acetic anhydride appeared to be about as active as aspirin on a molar basis. Neither compound inhibited the

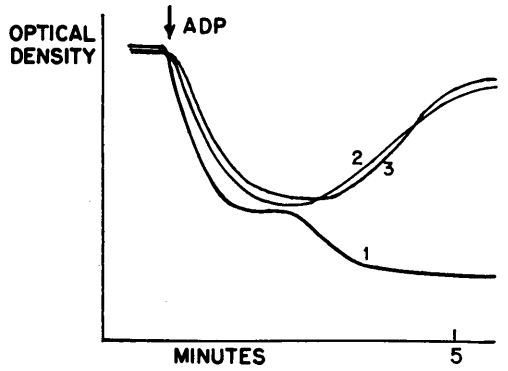

FIG. 1. Platelet aggregation with ADP: (1) Control specimen showing the biphasic response. After incubation with acetic anhydride (2) or aspirin (3) the secondary wave was abolished.

response of PRP to larger concentrations of ADP. In normal untreated PRP, platelet factor 3 activity appeared after addition of each aggregating agent. As shown in Fig. 4, this response was similarly blocked in specimens incubated with either aspirin or acetic anhydride. PPP incubated with acetic anhydride failed to affect platelet aggregation or factor 3 activity when added to PRP. An action through acetylated intermediates in the plasma thus seems unlikely.

The above observations suggested that the mechanism of action of aspirin on platelets might be through acetylation, since another known acetylating agent, acetic anhydride, produced similar functional defects. An attempt was therefore made to demonstrate

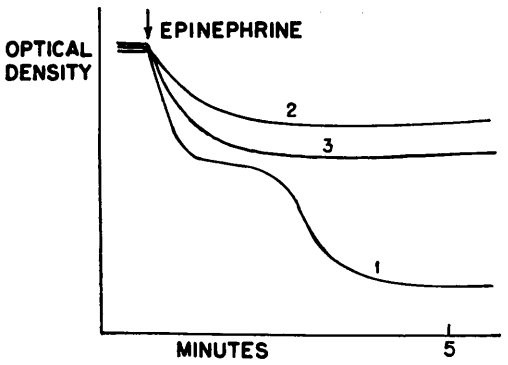

FIG. 2. Platelet aggregation with epinephrine: (1) Control specimen showing the typical biphasic response. After incubation with acetic anhydride (2) or aspirin (3) the secondary wave was abolished.

acetylation of platelets by radioactive tracer techniques. Figure 5 demonstrates that label became irreversibly bound to platelets when PRP was incubated with aspirin labeled with ^{14}C at the acetyl position, whereas no such binding was detected with carboxyl-labeled aspirin. Binding of the acetyl group was progressively greater with increasing incubation times. Failure of carboxyl-labeled compound to impart radioactivity to the washed platelet button was evidence against nonspecific trapping. ^{14}C-labeled acetic anhydride was bound to platelets similarly to acetyl-labeled aspirin. Incubating PRP with un-

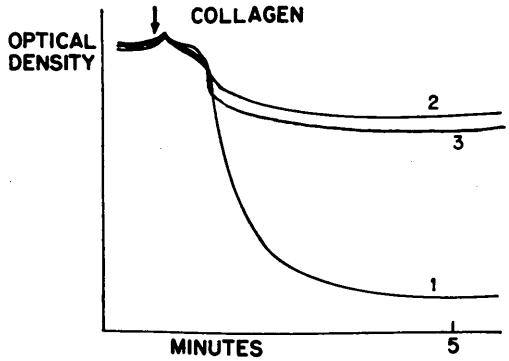

FIG. 3. Platelet aggregation with collagen: (1) Control. Aggregation was inhibited after incubation with acetic anhydride (2) or aspirin (3).

labeled aspirin or unlabeled acetic anhydride (50 μM) inhibited the binding of acetyl ^{14}C-salicylic acid by washed platelet by about 24% and 57%, respectively.

When platelets labeled with acetyl 1-^{14}C salicylic acid were subjected to subcellular fractionation, the label was detected in all three subcellular compartments, as illustrated in Fig. 6. The acetyl group of aspirin thus appears to react with more than one site in or on the platelet.

Since aspirin, and acetic anhydride as well, hydrolyze in water to form free acetate radicals, the observed binding might have been the result of acetate incorporation into platelet compounds by normal metabolic pathways (13). A series of experiments was performed, designed to exclude this possibility. This proved not be possible, and the results are summarized briefly.

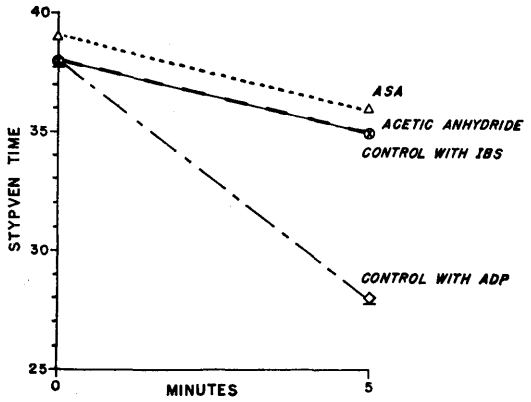

FIG. 4. Platelet factor 3 release with ADP in a control specimen, incubated with imidazole-buffered saline (IBS), and after incubation with acetic anhydride or aspirin. Similar results were obtained when collagen or epinephrine was the releasing reagent.

Sodium ^{14}C-acetate incubated with PRP showed incorporation of label into platelets comparable to that produced by similar incubation with acetyl 1-^{14}C salicylic acid. Both compounds produced labeling of lipid and nonlipid fractions of the platelets, and when unlabeled "cold" aspirin or sodium acetate was added to incubation mixtures at equimolar to 100-fold concentration either compound inhibited platelet binding of the acetyl-labeled aspirin or labeled acetate to a degree roughly proportional to its concentration. When PRP was incubated with the labeled compounds at 4° instead of 37°, uptake

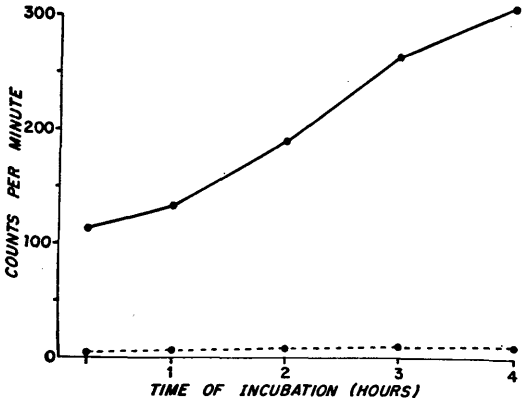

FIG. 5. Platelet uptake of ^{14}C-labeled aspirin. The curves show the platelet-bound radioactivity after incubation of PRP with acetyl-^{14}C aspirin (solid line or carboxyl-^{14}C (broken line).

of label from both compounds was similarly inhibited; repeated freezing and thawing of PRP resulted in depressed uptake of label from aspirin or acetate about equally. Although labeled acetate thus appeared to bind to platelets indistinguishably from the acetyl group of aspirin, sodium acetate did not inhibit platelet aggregation in response to the agents of the present study, even at concentrations of 1 m*M*.

Discussion. Earlier studies have provided data suggesting that the acetyl group of aspirin is responsible for the drug's effects upon platelet function. Quick (5) showed that aspirin prolonged the bleeding time of normal volunteers, whereas sodium salicylate was ineffective in this test, a finding confirmed by Weiss and associates (6). O'Brien (4) reported that sodium salicylate was a relatively ineffective inhibitor of *in vitro* platelet aggregation as compared to aspirin, and we have obtained similar data. Pinckard *et al.* (14) demonstrated that aspirin can acetylate a wide variety of biological materials including serum proteins, nucleic acids, and hormones. The above observations, together with the prolonged effect of aspirin upon platelets (4), suggest that the alteration in platelet function produced by aspirin might represent acetylation of one or more platelet reactive sites.

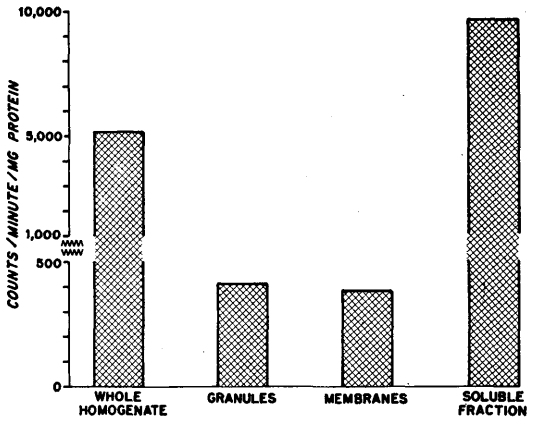

FIG. 6. Radioactivity in the platelet homogenate, and its distribution in the subcellular fractions.

The present data support the acetylation hypothesis by demonstrating that another

acetylating agent has a functional effect on platelets similar to that of aspirin. Acetic anhydride, as aspirin, selectively eliminated the secondary wave of aggregation produced by ADP or epinephrine, and it depressed the aggregation response to connective tissue fragments (collagen). Acetic anhydride also prevented platelet factor 3 release in response to these aggregating agents, another aspirin effect.

Attempts to identify acetylation of platelets by means of acetyl-1-^{14}C-salicylic acid were unsuccessful. Incubation of PRP with this compound was associated with platelet binding of the label, and nonspecific trapping was excluded by lack of uptake with labeling in the carboxyl position. However, the observed platelet labeling could not be distinguished from metabolic acetate incorporation despite a number of maneuvers. Perhaps simultaneous acetate incorporation and acetylation occurred, and the fraction of binding from acetylation was too small to detect.

Were acetylation of platelets demonstrable, two important considerations would emerge: A tool for dissection of platelet reactive sites might become available; and a basis for synthesizing pharmacologically useful platelet inhibitors as antithrombotic agents could emerge.

Summary. Aspirin produces alteration of platelet function characterized by loss of secondary aggregation in response to ADP or epinephrine, and inhibition of aggregation by collagen. In addition, platelet factor 3 release by these reagents is reduced. A similar platelet lesion was produced by an acetylating agent, acetic anhydride, supporting earlier concepts that aspirin activity against platelets may be a consequence of acetylation. An attempt to identify specific acetylation of platelets with acetyl-1-^{14}C salicylic acid was unsuccessful. Although platelet uptake of the label was demonstrated, metabolic incorporation of acetate could not be excluded.

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