THE EFFECT OF ACETYLSALICYLIC ACID ON PLATELET FUNCTION*

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(Received for publication 13 June 1968)

We have shown previously that phenylbutazone and sulfinpyrazone inhibit the response of platelets to particulate matter and surfaces, and that this is associated with impairment of hemostasis and prolongation of platelet survival (2, 3). Since the pyrazole compounds have anti-inflammatory properties, it seemed possible that other anti-inflammatory compounds might have similar effects on platelets.

Acetylsalicylic acid (aspirin) is associated with hemorrhagic episodes in man. Beaumont and Willie (4) found that it caused a prolongation of the bleeding time in adults with cardiac diseases. Recently it has been shown that the administration of acetylsalicylic acid to subjects with hemorrhagic disorders also causes a considerable prolongation of the bleeding time (5), and Gast (6) has reported that it caused a decrease in platelet-stickiness. Bounameaux and van Cauwenberge (7) demonstrated in rats that administration of sodium salicylate also decreased platelet adherence to glass. Weiss and Aledort (8) have reported that aspirin inhibits collagen-induced platelet aggregation, and we have shown that inhibition of the platelet-collagen reaction impairs hemostasis (3). In this paper some effects of acetylsalicylic acid on platelet function in vitro and in vivo are described.

Materials and Methods

Tyrode's Solutions.—Tyrode's solution was prepared as specified by Parker (9). Modified Tyrode's solution (containing no calcium or magnesium), Tyrode-albumin solution, Tyrode-albumin-EDTA, and Tyrode-gelatin solution, were prepared as previously described (3).

Acetylsalicylic Acid (ASA).—(Fisher Scientific Co., Toronto, Ont.) was dissolved in modi-

*This work was supported by the Ontario Heart Foundation and the Medical Research Council of Canada (MT 1309, MA 2629). Presented in part at the Oak Ridge Platelet Symposium, 23 June 1967 (1).

fied Tyrode's solution and the pH adjusted to 7.35 with sodium hydroxide. This preparation was used in the in vitro experiments and was also sometimes given intravenously. Acetylsalicylic acid (aspirin, Bayer Company, Ltd., Aurora, Ont.) was used as the oral preparation in the other in vivo studies.

Sodium Salicylate.—(Fisher Scientific Co.) was dissolved in modified Tyrode's solution and the pH adjusted to 7.35 with hydrochloric acid.

Adenosine Diphosphate (ADP) and Monophosphate (AMP).—These phosphates (Sigma Chemical Co., St. Louis, Mo.) were dissolved in modified Tyrode's solution to the required concentrations.

Collagen Suspension.—2 g of commercial collagen (Sigma Chemical Co.) were suspended in 100 ml of modified Tyrode's solution at 0°C and homogenized in a Waring blender for a total of 5 min. The mixture was centrifuged at 810 *g* for 15 min to remove coarse particulate matter. The supernatant suspension was then diluted with modified Tyrode's solution to a concentration which would produce maximum aggregation of the platelets being tested, but on further dilution would cause less than maximum aggregation.

Antigen-Antibody Complexes.—Antigen-antibody complexes were prepared (10). The concentration used to induce platelet aggregation was adjusted by dilution with modified Tyrode's solution, as with the collagen suspension (see above).

Polystyrene Coated with Gamma Globulin.—This suspension was prepared from a 30% polystyrene latex suspension (British Drug Houses, Toronto, Ontario) and a human gamma globulin solution (Connaught Medical Research Laboratories, Toronto, Ontario) as reported earlier (3). The coated polystyrene particles were suspended in modified Tyrode's solution at a concentration of 0.1%. The concentration used to induce platelet aggregation was adjusted by dilution with modified Tyrode's solution, as with the collagen suspension.

Thrombin.—Crude bovine thrombin (Parke Davis & Co., Detroit, Mich.) was dissolved in modified Tyrode's solution to the required concentration.

Animals.—Pigs and rabbits were lightly anesthetized with pentobarbital sodium (approximately 20 mg/kg) for cannulation of an external jugular vein or carotid artery and, in the case of the rabbits, for examination of hemostatic plug formation in the mesentery and for the in vivo experiments with an extracorporeal shunt.

Glassware.—Glassware used to contain platelets was coated with silicone (General Electric dry film SC 87 in carbon tetrachloride).

Platelet-Rich Plasma (PRP).—Blood was taken through a carotid artery or external jugular vein cannula (Intramedic Polyethylene Tubing, Clay Adams Inc., New York, PE 190 for rabbits and PE 320 for pigs) into 3.8% trisodium citrate or 2% EDTA-saline (1 part anticoagulant to 9 parts of blood) and centrifuged at 77 *g* for 15 min at room temperature. The supernatant PRP was removed with silicone-coated Pasteur pipettes. Human blood was taken from a forearm vein using a 19 gauge thin-walled needle and a plastic syringe.

Platelet Suspensions.—PRP from EDTA blood was centrifuged at 650 *g* for 15 min at 4°C. The platelets were resuspended and washed twice with cold Tyrode-albumin-EDTA, recovered each time by centrifugation, and finally resuspended in Tyrode-gelatin solution to a platelet concentration of 500,000–600,000 per mm^3. The platelet suspension was stored in an ice water bath.

Platelets Counts.—These were done on a Coulter counter (11).

Platelet Aggregation.—This was studied by the turbidimetric method (12).

In Vitro ^{14}C-Serotonin Labeling of Platelets.—The labeled compound used was 5-hydroxytryptamine-3′-^{14}C-creatinine sulfate (^{14}C-5-HT) (Radiochemical Centre, Amersham, England). ^{14}C-5-HT was added to EDTA platelet-rich plasma and allowed to incubate for 30 min at 37°C. The platelet-rich plasma was then centrifuged at 650 *g* for 15 min at 4°C. Suspensions of washed platelets were prepared as described above for studies of the release of ^{14}C-serotonin.

Release of AMP, ADP, and ^{14}C-Serotonin.—1 ml of platelet suspension was mixed with 0.1 ml of a solution of acetylsalicylic acid (diluted to the desired concentration with modified Tyrode's solution). In the control experiments, 0.1 ml of modified Tyrode's solution was substituted for the solution of acetylsalicylic acid. In order to induce platelet aggregation and release platelet constituents, 0.1 ml of a suspension either of collagen, antigen-antibody complexes, gamma globulin–coated polystyrene, or a thrombin solution was added, and the mixture was agitated constantly for 10 min at 37°C. The concentrations of ADP and AMP in the platelets and in the ambient fluid were determined, and the amounts of ^{14}C in the ambient fluid and platelets measured by methods reported elsewhere (3, 11).

Whole Blood Clotting Time; One-Stage Prothrombin Time; Partial Thromboplastin Time.—These studies were carried out with whole blood, or plasma prepared from blood taken into citrate, by methods given in detail in earlier publications (13, 14).

Thrombin Clotting Time.—Freeze-dried human fibrinogen (Connaught Medical Research Laboratories) was dissolved in distilled water (0.5 g/100 ml) and dialyzed against 0.85% NaCl for 12 hr at room temperature. The fibrinogen solution was adsorbed with aluminum hydroxide (moist gel, British Drug Houses), 1 part Al $(OH)_3$ to 5 parts fibrinogen solution, incubated at 37°C for 10 min, centrifuged at 4000 *g* for 15 min). To 0.2 ml of the fibrinogen solution was added 0.2 ml of M/40 $CaCl_2$, 0.1 ml of the ASA solution, and 0.2 ml of the thrombin solution. The clotting time of the fibrinogen solution after addition of the thrombin was determined at 37°C.

Hemostasis.—The technique for evaluation of hemostasis by cutting small mesenteric vessels has been described (15). In these experiments, the acetylsalicylic acid was given orally to animals starved for 12 hr. The duration of time from the onset of bleeding to its arrest was recorded by direct observation of the vessel. The total duration of any renewed bleeding was also observed during the 30 min following transection of the vessel.

Statistical Considerations (16).—The studies on hemostasis were done on 72 animals which were randomly assigned to 12 groups of 6 each. On each of 4 days, three groups were studied; one group acting as controls, one receiving 100 mg/kg of ASA, and one 200 mg/kg. Thus there are four possible sources of variation in the results:

1. There may be a variation attributable to differences produced by treatment with aspirin. This will be referred to as the dosage effect. Since there are three dosage levels, there are $3-1=2$ degrees of freedom associated with it. This source of variation may in turn be partitioned into a linear component (which implies a proportionality between prolongation of bleeding time and dosage) and a quadratic component (which implies that there is a prolongation related to the square of dosage). The complete experiment explores the model

$$y_i = a + bx_i + cx_i^2 + \epsilon_i$$

where x_i is a particular dosage, y_i the bleeding time, ϵ_i a random error and a, b, and c constants. If $c=0$, there is a simple linear relationship; if $c=b=0$, effect is quite unrelated to dosage. The tests whether c and b are zero are orthogonal, i.e. statistically independent, and one of the degrees of freedom is associated with each.

2. There may be variation attributable to difference in days caused by a variety of unspecified causes. This is denoted by "day effect." With it are associated $4-1=3$ degrees of freedom.

3. While on each day the bleeding time may be affected by dosage, that is in the above equation either b or c or both are non-zero, the values b and c take may vary from day to day. This component of variance is designated "interaction." There are $(3-1)(4-1) = 6$ degrees of freedom associated with it. A significant interaction would indicate that on some days, the drug has more effect than on others.

4. Even under identical conditions and on the same dosage, animals differ in their responses

to treatment. This source of variation is termed "error." For each of the 12 groups of 6, one parameter (the mean) is estimated and therefore one degree of freedom lost. Thus in all there are $12 \times (6-1) = 60$ error degrees of freedom.

For the purposes of this experiment, the error mean square is created as the irreducible component of variation and provides the denominator for all F tests. However, if the interaction mean square is significantly greater than the error mean square, principal effects (days and dosage) should be tested not against error but against interaction. The resulting F tests will therefore have 6 rather than 60 degrees of freedom associated with their denominators.

Platelet Survival Studies on Rabbits.—These were carried out by a modification (2, 17) of the method of Leeksma and Cohen (18). Acetylsalicylic acid–treated rabbits were given the drug twice daily in doses ranging from 50–400 mg/kg per day. The control animals received placebo tablets. On the 4th day of this regimen, the animals received intravenously diisopropyl-(1,3-T) phosphorofluoridate (in sterile solution) (^{3}H-DFP) obtained from the Radiochemical Center. The specific activity of the labeled DFP was 2000 μc/mg and each rabbit received 50 μc. The dose of labeled DFP was therefore less than 0.01 mg/kg body weight. The rabbits were bled daily for 7 days from ear veins into plastic syringes containing 2% EDTA-saline. Platelets from each 10 ml blood sample were isolated and their radioactivities measured. The platelets were separated (2), put on a small piece of tared aluminum foil, dried, and weighed. The dried platelets were then placed in a counting vial to which 0.25 ml NCS (Nuclear-Chicago Corp., Des Plaines, Ill.) was added. 12 hours later a toluene-fluor solution (PPO, POPOP) was added and the radioactivity measured in a liquid scintillation counter. The radioactivity is expressed as cpm/mg dried platelets. The platelet half-life value for each animal was calculated by the iterative, nonlinear, unweighted least squares method (19). Platelet turnover (in platelets/mm^3 per day) was calculated as:

$$\frac{0.693 \times \text{average number of platelets per mm}^3}{\text{Half-life in days}}$$

Assay of Acetylsalicylic Acid in Plasma.—This was carried out by the method of Trinder (20) (Extraction of plasma from untreated animals gave a variable blank value between 1 and 3 mg per 100 ml of plasma.)

Extracorporeal Shunts.—The design of the shunt used has been specified in detail (2). In these experiments, ASA was given intravenously to rabbits at a dosage of 200 mg/kg body weight over a period of 10 min. Control animals were given modified Tyrode's solution. Blood flow through the extracorporeal shunts was begun 30 min after the intravenous infusion and continued for 20 min.

In these experiments, the bifurcation of the shunt was coated with gamma globulin (3). The bifurcation was filled with the solution, emptied, and rinsed three times with modified Tyrode's solution prior to its insertion into the shunt.

A Note on Transformations.—To meet more closely the assumptions underlying analysis of variance (principally that the error terms be normally distributed and have a uniform variance), it is sometimes desirable to use not the measurments in their natural state but to take some mathematical function of these, such as the logarithms. This procedure, while not destroying the information in the values, modifies it somewhat. It should be regarded as a simple computational device which may or may not have an interpretable biological meaning. It is convenient in expressing means or confidence limits to convert them back to the original system of measurement.

RESULTS

In Vitro Studies.—The addition of acetylsalicylic acid (ASA) to citrated human PRP suppressed platelet aggregation induced by collagen but did not

influence aggregation induced by ADP (Fig. 1). Similar results were observed with pig and rabbit PRP. Shown in Fig. 2 is the effect of different concentrations of ASA on platelet aggregation induced by collagen. Acetylsalicylic acid also inhibited thrombin-induced platelet aggregation in human and rabbit citrated PRP (Fig. 3). This effect was demonstrable only with the minimum doses required to produce platelet aggregation. Because the high concentration of thrombin necessary to induce platelet aggregation in citrated pig PRP

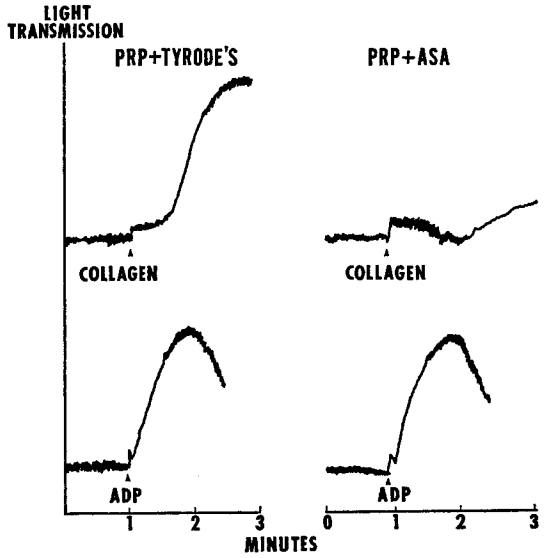

FIG. 1. Platelet aggregation in citrated platelet-rich human plasma (PRP) induced by the addition of a collagen suspension or ADP (final concentration 5×10^{-7} M). Increased light transmission indicates platelet aggregation. The recordings on the left show the response of the platelets without the addition of ASA; on the right, the response of the platelets in the presence of ASA (final concentration 1 mg/ml). Only collagen-induced aggregation was suppressed.

caused the rapid onset of fibrin formation, it was not possible to study the effect of acetylsalicylic acid on thrombin-induced platelet aggregation in pig plasma.

To examine the influence of this drug in a system in which the effects of antigen-antibody complexes and gamma globulin–coated surfaces could be explored, we used suspensions of washed human, pig, or rabbit platelets. Acetylsalicylic acid inhibited the action of collagen, antigen-antibody complexes, and gamma globulin–coated polystyrene in inducing platelet aggregation in these suspensions (Fig. 4). In the washed platelet suspension, the effect of thrombin in low concentrations was also inhibited by acetylsalicylic acid (Fig. 5). However, although ASA inhibited thrombin-induced platelet aggregation, it did not inhibit the clotting of fibrinogen by thrombin (Table I).

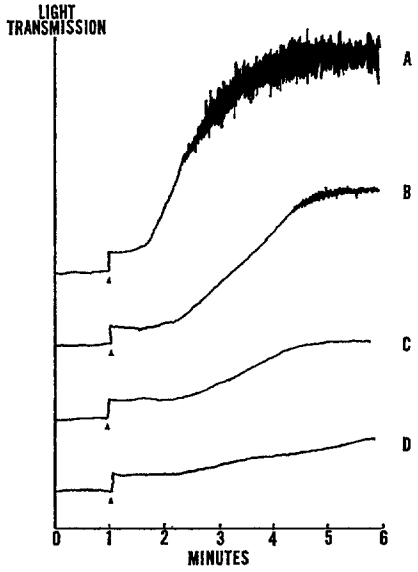

FIG. 2. Effect of ASA on collagen-induced platelet aggregation in citrated platelet-rich pig plasma. Final concentrations of ASA; A, 0; B, 0.1 mg/ml; C, 0.5 mg/ml; D, 1 mg/ml.

It is known that thrombin, collagen, antigen-antibody complexes, and gamma globulin–coated surfaces cause the release of platelet constituents such as serotonin, ADP, and AMP into the ambient fluid. Accordingly, we examined the effect of ASA on the release of these compounds from pig platelets. The

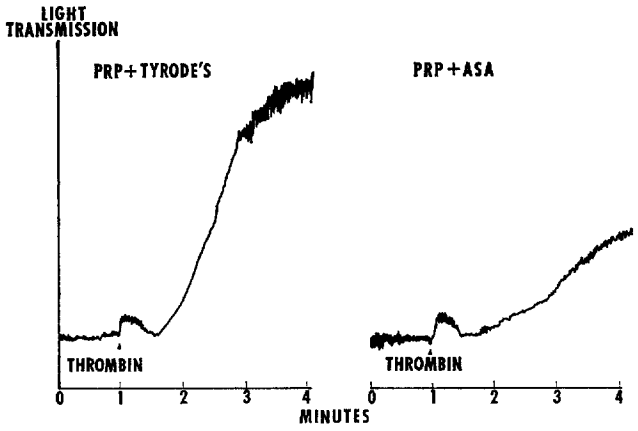

FIG. 3. Effect of ASA (final concentration 1 mg/ml) on thrombin-induced platelet aggregation in citrated platelet-rich rabbit plasma (PRP)

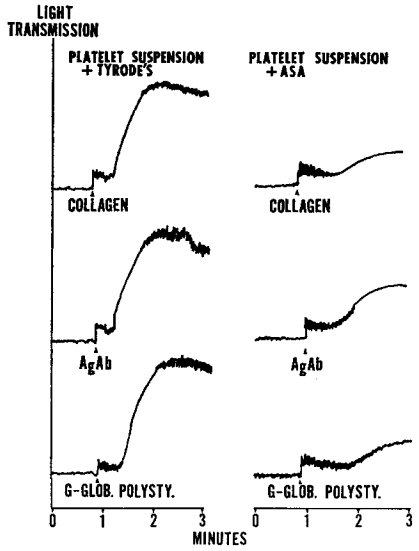

FIG. 4. Effect of ASA (final concentration 1 mg/ml) on platelet aggregation in a suspension of washed human platelets. Aggregation was induced by either a collagen suspension, antigen-antibody complexes (AgAb), or polystyrene particles coated with gamma globulin (G-GLOB. POLYSTY.) ASA suppressed the response of the platelets to all these stimuli.

addition of ASA to a platelet suspension reduced the amount of ADP and AMP released by a collagen suspension (Table II).

Pig platelets were labeled in vitro with ^{14}C-serotonin. In the presence of ASA, they released less radioactivity into the ambient fluid when they were

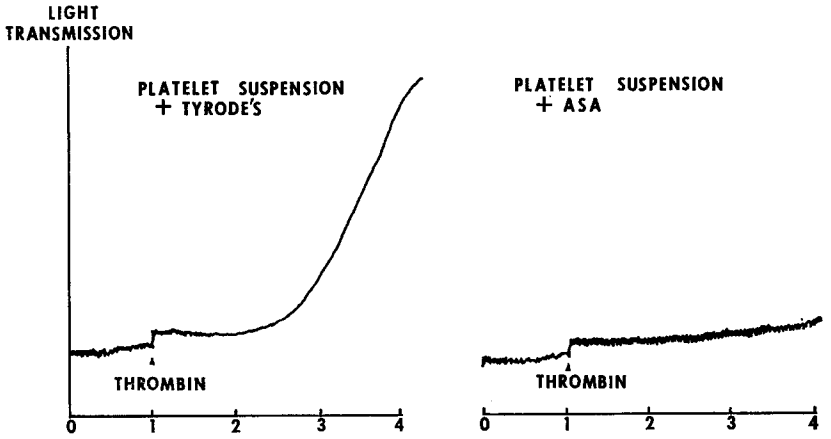

FIG. 5. Effect of ASA (final concentration 1 mg/ml) on thrombin-induced platelet aggregation in a suspension of washed human platelets. Final concentration of thrombin, 0.1 units/ml.

stimulated with collagen, antigen-antibody complexes, or gamma globulin–coated polystyrene (Table III). The effect of low concentrations of thrombin on the release of platelet constituents from a suspension of washed pig platelets was inhibited by acetylsalicylic acid (Table IV).

TABLE I

The Effect of Acetylsalicylic Acid on the Clotting of Fibrinogen by Thrombin

Experiments	Thrombin, final concentration	Acetylsalicylic acid, final concentration	Clotting time of fibrinogen solution
	units/ml	*μg/ml*	*sec*
1	0.6	0	35
	0.6	140	34
	0.6	640	36
	1.4	0	24
	1.4	140	23
	1.4	640	24
2	0.14	0	62
	0.14	646	65
	0.29	0	35
	0.29	640	36
	1.4	0	22
	1.4	640	24

TABLE II

Effect of Acetylsalicylic Acid on Collagen-Induced Release of Platelet ADP and AMP

Material added to platelet suspension	Final concentration of acetylsalicylic acid in platelet suspension	Platelet ADP and AMP after release reaction		ADP and AMP in ambient fluid after release reaction	
		ADP	AMP	ADP	AMP
	μg/ml	*nanomoles/ml of platelet suspension*			
Collagen suspension	0	50	13	64	23
" "	1	61	17	56	20
" "	10	76	15	56	20
" "	50	78	14	44	17
Tyrode's solution	0	86	15	26	7

Sodium salicylate was also found to suppress platelet aggregation induced by collagen and low concentrations of thrombin but did not affect ADP-induced platelet aggregation in citrated PRP. The addition of sodium salicylate to a suspension of washed ^{14}C-serotonin–labeled pig platelets inhibited the release of radioactivity and nucleotides induced by collagen, antigen-antibody complexes, and gamma globulin–coated polystyrene (Table V).

In Vivo Studies.—Four aspects of the in vivo effects of acetylsalicylic acid were studied in rabbits: the suppression of collagen-induced platelet aggregation following oral administration of the drug, the effects of oral administration on hemostasis, the effect of the drug on platelet survival, and the effect of intravenous administration of the drug on the formation of deposits in the bifurcation of an extracorporeal shunt.

TABLE III

Effect of Acetylsalicylic Acid on the Release of Radioactivity, ADP, and AMP from ^{14}C-Serotonin-Labeled Platelets Exposed to Collagen, Antigen-Antibody Complexes or Gamma Globulin-Coated Polystyrene

Material added to platelet suspension	Final concentration of acetylsalicylic acid in platelet suspension	^{14}C		ADP and AMP in ambient fluid after release reaction	
		Ambient fluid	Platelets	ADP	AMP
	μg/ml	*cpm/ml*	*cpm/mg*	*nanomoles/ml*	
Experiment I					
Collagen suspension	—	26,675	13,450	14	14
" "	10	18,787	13,650	11	12
" "	100	14,014	16,800	8	10
Tyrode's solution	—	10,440	16,080	10	7
" "	10	8,198	16,800	10	5
" "	100	8,621	16,660	8	7
Experiment II					
Antigen-antibody complexes	—	37,852	7,780	22	19
" " "	10	23,415	8,220	19	14
" " "	100	2,916	19,400	8	5
Tyrode's solution	—	721	20,300	6	2
" "	10	2,199	18,800	6	2
" "	100	1,329	21,400	5	2
Experiment III					
Gamma globulin-coated polystyrene	—	7,898	11,150	10	7
" " " "	10	3,798	16,700	6	8
" " " "	100	2,762	14,520	6	5
Tyrode's solution	—	1,642	19,290	7	3
" "	10	1,886	17,000	5	4
" "	100	2,242	16,480	5	3

The effect of in vivo administration of acetylsalicylic acid: Platelet aggregation in vitro in citrated PRP prepared from blood taken from rabbits before and 30 min after the intravenous infusion of ASA (200 mg/kg) was similar to that seen in the in vitro experiments. The response of the platelets in citrated platelet-rich plasma to collagen and thrombin, but not to ADP, was suppressed after the infusion compared with the preinfusion response.

Collagen-induced platelet aggregation was studied 2 hr after the oral administration of ASA to rabbits at different dosage levels (100 mg/kg of ASA produced a plasma level of 38 mg/100 ml). Aggregation induced by this stimulus was inhibited (Table VI). In these experiments, the oral administration of ASA had no detectable effect on the platelet count, the whole blood-clotting time in glass and silicone-coated tubes, the one-stage prothrombin time, or the partial thromboplastin time (Table VII).

TABLE IV

Effect of Acetylsalicylic Acid on Thrombin-induced Release of Platelet Constituents

Material added to platelet suspension*	Final concentration of acetylsalicylic acid in platelet suspension	^{14}C		ADP and AMP in ambient fluid after release reaction	
		Ambient fluid	Platelets	ADP	AMP
	μg/ml	*cpm/ml*	*cpm/mg*	*nanomoles/ml*	
Thrombin 1 unit/ml	0	110,941	3,970	37	29
	10	97,390	5,870	31	30
	50	91,573	5,620	25	19
	100	88,718	5,880	21	19
Thrombin 0.2 units/ml	0	35,228	16,800	19	15
	10	35,228	17,000	12	14
	50	18,877	23,100	15	21
	100	8,847	28,000	9	7
Tyrode's solution	0	6,089	27,200	6	5
	10	5,712	27,900	6	5
	50	5,854	24,700	6	5
	100	6,644	24,800	2	5

* 0.1 ml added to 1.1 ml of platelet suspension containing acetylsalicylic acid.

The effect on hemostasis: Acetylsalicylic acid was given orally to rabbits in the dosage of 100 or 200 mg/kg. It can be seen (Table VIII and IX) that, upon transection of the mesenteric vessels 2 hr after administration of the drug, hemostasis was impaired. The rabbits given ASA showed a considerable lengthening of the time taken for the formation of a platelet plug capable of arresting hemorrhage. There was considerable renewed bleeding through the platelet plugs indicating that they were unstable. The total time of bleeding represents the combined time for primary arrest and the duration of the renewed bleeding. In comparison, control animals showed little renewed hemorrhage through the plugs and the total time of bleeding rarely exceeded 5 min. The extent of the prolongation is dose dependent. Over the dosage range used in these experiments, the relationship appears to be linear. No significant quadratic component was demonstrated. While the effect was consistent in the few days of study, the regression slope of prolongation on dosage varied appreciably from day to day.

TABLE V

Effect of Sodium Salicylate on the Release of Radioactivity, ADP, and AMP from ^{14}C-Serotonin-labeled Pig Platelets Exposed to Collagen, Antigen-Antibody Complexes or Gamma Globulin-Coated Polystyrene

Material added to platelet suspension	Final concentration of sodium salicylate in platelet suspension	^{14}C		ADP and AMP in ambient fluid after release reaction	
		Ambient fluid	Platelets	ADP	AMP
	μg/ml	*cpm/ml*	*cpm/mg*	*nanomoles/ml*	
Collagen suspension	0	3,496	13,920	20	11
" "	10	1,277	19,000	17	9
" "	100	789	19,500	14	7
Antigen-antibody complexes	0	29,242	6,790	29	24
" " "	10	6,720	14,850	24	17
" " "	100	4,984	16,500	15	9
Gamma globulin–coated polystyrene	0	34,918	3,960	29	14
" " " "	10	12,025	9,970	25	10
" " " "	100	5,602	14,600	16	10
Tyrode's solution	0	902	15,300	10	3
" "	10	869	16,700	3	2
" "	100	583	17,480	2	2

TABLE VI

Effect of Oral Administration of Acetylsalicylic Acid to Rabbits on the Aggregation of Their Platelets to a Collagen Suspension*

Oral dosage of ASA	Maximum platelet aggregation (Per cent of control)‡
mg/kg	
0	100
50	95
100	76
200	20
300	0

* ASA was given 2 hr before the sample of blood was taken for aggregation studies. Aggregation was measured in citrated PRP.

‡ Maximum light transmission through the PRP during platelet aggregation was assigned a value of 100% for the control sample (taken before the administration of ASA). The maximum light transmission obtained in the presence of ASA was calculated as a percentage of this. These values are dependent on the collagen concentration and represent relative values only with a given collagen suspension. The values shown are the means of three experiments.

This is reflected in the interaction term in the analysis of variance which exceeds the error term to a highly significant extent. Accordingly, as has been noted above, the effect of dosage has to be tested against the interaction mean square rather than that for error.

The effect on platelet survival: Platelet survival studies were done on rabbits receiving, orally, various doses of acetylsalicylic acid (Table X). Control animals were given placebo tablets. On the 4th day of the regimen the animals were injected with ^{3}H-DFP, platelets were isolated daily thereafter for 7 days and their radioactivity measured. Platelet survival follows a normal distribution curve sufficiently closely for accurate analysis (21). In a previous paper (2), however, it was noted that the standard deviation of mean survival is sometimes proportional to the mean, a relationship which is abolished if the logarithms of

TABLE VII

Effect of Acetylsalicylic Acid on Platelet Count and Clotting Tests

Test*	Mean values before administration of ASA		Mean values 2 hr after oral administration of ASA (200 mg/kg)
Whole blood clotting time, glass, *min*	4.4		4.6
Significance of difference between mean values		$t = 0.9$ $P < 0.4$	
Whole blood clotting time, silicone, min	8.8		9.1
Significance of difference between mean values		$t = 0.7$ $P < 0.5$	
Prothrombin time, *sec*	17.0		18.3
Significance of difference between mean values		$t = 1.42$ $P < 0.2$	
Partial thromboplastin time, *sec*	39.9		37.5
Significance of difference between mean values		$t = 0.74$ $P < 0.5$	
Platelet count, No./mm^3 $\times$ 10^3	464		472
Significance of difference between mean values		$t = 0.69$ $P < 0.5$	

* The values shown are the means from studies in 10 rabbits.

the values are used. This relationship was evident in the present study and to stabilize the variance a logarithmic transformation was used on the individual estimates of the means before the regression line on dosage was fitted. In spite of some irregularity in results which may be due to the small size of some of the treatment groups, there is considerable increase in mean survival with increasing ASA intake, and this is significant at the 1 in 100 level. There is a corresponding decrease in platelet turnover. Since the variance for this value was naturally stable, no logarithmic transformation was required.

Formation of deposits of blood materials in the bifurcation of extracorporeal shunts: We have reported earlier (22) that by using the logarithms of the weights of the deposits, the distribution of the weights is normalized and the variance which is ordinarily closely related to the mean is stabilized. All the calculations are therefore carried out on the logarithms of the dry weights of deposits but

mean values have been converted back into the original scale for display in Table XI. These values are accordingly geometric means. It can be seen that administration of acetylsalicylic acid (200 mg/kg) intravenously 30 min before blood flow was started in the shunt produced a significant diminution in the amount of deposit on a bifurcation coated with gamma globulin. There was no significant difference in mean rates of flow between the two groups of animals.

TABLE VIII

Effect of Oral Administration of Acetylsalicylic Acid on Primary Bleeding Time in Rabbits

Treatment group	Study*				Mean primary bleeding time for all groups
	1	2	3	4	
	Mean primary bleeding time				
	sec	*sec*	*sec*	*sec*	*sec*
Control	144	270	274	284	243
100 mg/kg	315	441	508	402	417
200 mg/kg	320	660	957	930	717

Analysis of variance

Source	Degrees of freedom	Sum of squares	F	*P*
Days	3	1,092,486	3.17	<0.25
Dosage	2	2,757,446	12.02	<0.01
Linear	1	2,693,269	23.47	<0.001
Quadratic	1	64,178	0.56	<0.5
Interaction (dosage × days)	6	688,428	28.78	<0.0001
Error	60	239,200		
Total	71	4,777,560		

* There were 6 animals in each group for each study.

DISCUSSION

The results from this study show that ASA inhibits platelet aggregation in citrated PRP or in suspensions of washed platelets induced by collagen, antigen-antibody complexes, or gamma globulin–coated polystyrene, but does not inhibit aggregation induced by ADP. In addition, ASA inhibits platelet aggregation induced by small doses of thrombin. There is evidence that thrombin and particulate stimuli cause platelet aggregation by releasing ADP from platelets (23). Since doses of ASA which inhibited platelet aggregation induced by thrombin or by the particulate stimuli also inhibited the release of platelet constituents, it seems likely that the effect of ASA on platelet aggregation can be

attributed to inhibition of the release of platelet ADP. Weiss and Aledort (8) have come to a similar conclusion concerning the effect of ASA in inhibiting collagen-induced platelet aggregation. We originally thought that ASA did not influence thrombin-induced platelet aggregation (1, 24) but we have subsequently found that ASA will inhibit thrombin-induced platelet aggregation

TABLE IX

Effect of Oral Administration of Acetylsalicylic Acid on Total Bleeding Time in Rabbits

Treatment	Study*				Mean total bleeding time for all groups
	1	2	3	4	
	Mean total bleeding time				
	sec	*sec*	*sec*	*sec*	*sec*
Control	211	269	290	288	263
100 mg/kg	987	1163	704	774	897
200 mg/kg	1517	1638	1144	1301	1391

Analysis of Variance

Source	Degrees of Freedom	Sum of squares	F	*P*
Days	3	0.3075	2.14	<0.25
Dosage	2	8.6544	90.44	<0.0001
Linear	1	8.3744	175.02	<0.0001
Quadratic	1	0.2800	5.85	<0.1
Interaction (days × dosage)	6	0.2871	16.99	<0.0001
Error	60	0.1689		
Total.....	71	9.4180		

N.B. To stabilize the variance the transformation $Y = \log (X + 600)$ is used where X is the number of seconds. The figures in the analysis of variance refer to this transformation. However, the mean values calculated from this transformation have been converted back into the original scale by the reverse process, i.e., $X = \text{antilog } (Y) - 600$. The mean values given above are in this form.

* There were 6 animals in each group for each study.

if the concentration of thrombin used is adjusted to that which will just produce platelet aggregation. In earlier studies (3), we similarly failed to demonstrate an effect of the pyrazole compounds on thrombin-induced platelet aggregation, because the concentration of thrombin used was too high (25).[1]

ASA is converted to sodium salicylate in vivo, and our in vitro results indicate that sodium salicylate also is effective in inhibiting platelet aggregation induced

[1] Packham, M. A., and J. F. Mustard. Unpublished observations.

by the particulate stimuli, probably by the same mechanism of reducing the extent of the release of platelet constituents. There is evidence from other studies (7, 26) that sodium salicylate decreases platelet stickiness and interferes with hemostasis.

TABLE X

Effect of Oral Administration of Acetylsalicylic Acid on Platelet Economy in Rabbits

Dose per day	No. of animals studied	Mean platelet half-life	Mean platelet turnover
mg/kg		*days*	*No./ mm³ per day × 10³*
0	16	1.33	359
50	5	3.07	263
100	12	3.18	223
150	2	1.40	258
200	5	4.23	158
300	2	1.42	305
400	2	3.89	101
Intercept		0.43579*	324.90
Regression		0.0024303*	0.58807
t		2.842	2.804
P		<0.01	<0.01

* These values refer to the logarithms of the platelet half-life in days.

TABLE XI

Effect of Acetylsalicylic Acid on the Formation of Deposits in Gamma Globulin-Coated Bifurcations Connected to Extracorporeal Shunts

	Untreated rabbits (6)	Rabbits given 200 mg/kg of ASA (6)
Mean flow rate, *ml/min*	117.3	92.8
Significance of difference between means	$t = 1.20$, $P < 0.3$	
Geometric mean dry weight of deposit, *mg*	22.80	6.37
Significance of difference between means	$t = 7.57$, $P < 0.001$	

The results from the in vivo experiments substantiate the in vitro studies. The concentrations of ASA necessary to inhibit platelet aggregation were of the same magnitude as those in the in vitro studies.

There is considerable evidence (15) that the exposed connective tissue at the end of a transected vessel provides a stimulus for the formation of a platelet mass. In the present experiments, the administration of ASA in doses which inhibited collagen-induced platelet aggregation caused defective hemostasis

manifested by a delay in the primary arrest of bleeding and by increased susceptibility to breakdown of the platelet plug with renewed bleeding through it. The action of ASA on hemostasis could be due to its effects on collagen and thrombin-induced platelet aggregation. Since in the acute experiments, ASA did not affect blood coagulation, and we could show no effect of ASA on the conversion of fibrinogen to fibrin by thrombin, it seems reasonable to attribute the hemostatic defect to inhibition of platelet aggregation.

The function of the platelet is important in determining its survival (2, 3, 25). The results from this study indicate that ASA prolongs platelet survival. Since the interaction of platelets with stimuli such as collagen, antigen-antibody complexes, or viruses can induce the release of platelet constituents and cause aggregation, it is possible that ASA increases platelet survival by suppressing the response of platelets to such stimuli.

In the experiments with the extracorporeal shunts we used surfaces which had been exposed to gamma globulin. This provided a surface which, in vitro, produced adherence of platelets to the surface and to each other with extensive release of ADP (3). When the bifurcation is coated with gamma globulin it produces more than four times the deposit found in an uncoated bifurcation (27). The results of the present experiments show that ASA produces a significant diminution in the amount of material deposited when blood flows past such a surface.

Addendum.—Since completion of this manuscript, M. B. Zucker and J. Peterson (1968, *Proc. Soc. Exptl. Biol. Med.* **127**:547) have reported that acetylsalicylic acid abolishes the secondary aggregation of platelets induced by critical concentrations of adenosine diphosphate (ADP) as well as inhibiting platelet aggregation induced by connective tissue particles. J. R. O'Brien (1968. *Lancet.* **1**:894) has shown that the oral administration of acetylsalicylic acid to human subjects suppressed the secondary response of these platelets to adrenaline and ADP as well as inhibiting the effect of collagen. He did not find any effect on thrombin-induced platelet aggregation.

SUMMARY

Acetylsalicylic acid (ASA, aspirin) and sodium salicylate inhibit platelet aggregation induced by collagen, antigen-antibody complexes, gamma globulin–coated particles or thrombin. These compounds suppress the release of platelet constituents, such as adenosine diphosphate (ADP) and serotonin, induced by such stimuli. Since ASA and sodium salicylate do not inhibit ADP-induced platelet aggregation, it appears that their effect on the action of the other stimuli is due to a decrease in the amount of ADP released. The administration of ASA to rabbits (in doses which inhibited collagen-induced platelet aggregation) impaired hemostasis, prolonged platelet survival, and diminished the amount of deposit formed in an extracorporeal shunt.

BIBLIOGRAPHY

1. Evans G., E. E. Nishizawa, M. A. Packham, and J. F. Mustard. 1967. The effect of acetylsalicylic acid (aspirin) on platelet function. *Blood.* **30:**550.
2. Mustard, J. F., H. C. Rowsell, H. A. Smythe, A. Senyi, and E. A. Murphy. 1967. The effect of sulphinpyrazone on platelet economy and thrombus formation in rabbits. *Blood.* **29:**859.
3. Packham, M. A., E. S. Warrior, M. F. Glynn, A. S. Senyi, and J. F. Mustard. 1967. Alteration of the response of platelets to surface stimuli by pyrazole compounds. *J. Exptl. Med.* **126:**171.
4. Beaumont, J. L., and A. Willie. 1955. Influence sur l'hémostase, de l'hypertension artérielle, des antivitamines K, de l'héparine et de l'acide acétyl salicylique. *Sang.* **26:**880.
5. Quick, A. J. 1966. Salicylates and bleeding: the aspirin tolerance test. *Am. J. Med. Sci.* **252:**265.
6. Gast, L. F. 1964. Influence of aspirin on haemostatic parameters. *Ann. Rheumatic Diseases.* **23:**500.
7. Bounameaux, Y., and H. van Cauwenberge. 1954. Action de la cortisone, de l'A.C.T.H. et du salicylate de soude sur les thrombocytes et la coagulation sanguine. *Sang.* **25:**889.
8. Weiss, H. J., and L. M. Aledort. 1967. Impaired platelet connective tissue reaction in man after aspirin injection. *Lancet.* **2:**495.
9. Parker, R. C. 1961. Methods of Tissue Culture. Harper and Row, New York. 3rd edition. 57.
10. Movat, H. Z., J. F. Mustard, N. S. Taichman, and T. Uriuhara. 1965. Platelet aggregation and release of ADP, serotonin, and histamine associated with phagocytosis of antigen-antibody complexes. *Proc. Soc. Exptl. Biol. Med.* **120:** 232.
11. Glynn, M. F., H. Z. Movat, E. A. Murphy, and J. F. Mustard. 1965. Study of platelet adherence and aggregation with latex particles. *J. Lab. Clin. Med.* **65:**179.
12. Mustard, J. F., B. Hegardt, H. C. Rowsell, and R. L. MacMillan. 1964. Effect of adenosine nucleotides on platelet aggregation and clotting time. *J. Lab. Clin. Med.* **64:**548.
13. Murphy, E. A., and J. F. Mustard. 1960. Dicumarol therapy. Some effects on platelets and their relationship to clotting tests. *Circulation Res.* **8:**1187.
14. Mustard, J. F., H. C. Rowsell, F. Lotz, B. Hegardt, and E. A. Murphy. 1966. The effect of adenine nucleotides on thrombus formation, platelet count and blood coagulation. *Exptl. Mol. Pathol.* **15:**43.
15. Hovig, T., H. C. Rowsell, W. J. Dodds, L. Jørgensen, and J. F. Mustard. 1967. Experimental hemostasis in normal dogs and dogs with congenital disorders of blood coagulation. *Blood.* **30:**636.
16. Cochran, W. G., and G. M. Cox. 1957. Experimental Designs. John Wiley Inc., New York. 2nd edition.
17. Murphy, E. A., and J. F. Mustard. 1961. Dicumarol therapy and platelet turnover. *Circulation Res.* **9:**402.

18. Leeksma, C. H. W., and J. A. Cohen. 1966. Determination of the life span of human blood platelets using labelled di-isopropyl-fluorophosphonate. *J. Clin. Invest.* **35**:964.
19. Rao, C. R. 1952. Advanced Statistical Methods in Biometric Research. John Wiley Inc., New York.
20. Trinder, P. 1954. Rapid determination of salicylate in biological fluids. *Biochem. J.* **57**:301.
21. Mustard, J. F., H. C. Rowsell, and E. A. Murphy. 1966. Platelet economy (platelet survival and turnover). *Brit. J. Haematol.* **12**:1.
22. Mustard, J. F., E. A. Murphy, H. C. Rowsell, and H. G. Downie. 1962. Factors influencing thrombus formation in vivo. *Am. J. Med.* **33**:621.
23. Mustard, J. F., M. F. Glynn, E. E. Nishizawa, and M. A. Packham. 1967. Platelet surface interactions: relationship to thrombosis and hemostasis. *Federation Proc.* **26**:106.
24. Evans, G., M. A. Packham, and J. F. Mustard. 1967. Spontaneous bruising. *Lancet.* **2**:724.
25. Packham, M. A., E. E. Nishizawa, and J. F. Mustard. 1968. The response of platelets to tissue injury. *Biochem. Pharmacol. Suppl.* 171.
26. Hofmann, H. 1956. Experimentelle Untersuchungen zur Frage der Blutungszeitveränderung durch Salizylate. *Med. Monatsschr.* **10**:84.
27. Evans, G., and J. F. Mustard. 1968. Platelet-surface reaction and thrombosis. *Surgery.* **64**:273.