ALTERATION OF THE RESPONSE OF PLATELETS TO SURFACE STIMULI BY PYRAZOLE COMPOUNDS*

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Plates 7 and 8

(Received for publication 7 February 1967)

Previous studies have shown that sulfinpyrazone (one of the pyrazole group of compounds) prolongs platelet survival in man and rabbits (1, 2, 3). This could mean that some aspects of platelet function and metabolism are altered or that the mechanisms removing platelets from the circulation are changed. One of the characteristics of platelets is their response to surface stimuli (4) such as collagen (5, 6, 7), antigen-antibody complexes (8, 9), and gamma globulin–coated surfaces (10, 11). When such a surface is presented to platelets they adhere to it[1] and may release a number of substances including adenine nucleotides (AMP, ADP, and ATP) (12), serotonin (13), and a factor or factors which increase vessel permeability (14). The released ADP is believed to cause platelet aggregation (12). Platelets may also phagocytose particulate matter (9, 15–17). In may be the participation of the platelets in these reactions which influences their survival in the circulation. If the action of the pyrazole drugs in prolonging platelet survival is primarily on the platelets' response to surface stimuli, then it would be anticipated that the platelets from animals or subjects given these drugs would show an altered response. We have, therefore, examined the effect of the pyrazole compounds on some of the factors which induce platelet aggregation.

Materials and Methods

Pyrazole Compounds (18).—Sulfinpyrazone (Anturan, Geigy Pharmaceuticals, Montreal, Quebec) 10% solution G-28315-R2206 was used in the in vivo experiments and sulfinpyrazone

* Presented in part at the Annual Meeting of the Canadian Federation of Biological Societies, Vancouver, 10 June 1966. Supported in part by grants from the Medical Research Council of Canada (MT-1309), the Ontario Heart Foundation, and Geigy Pharmaceutical Co.

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[1] Hovig, T., L. Jørgensen, A. Senyi, M. F. Glynn, and J. F. Mustard. A study of platelet adherence to different surfaces and subsequent platelet aggregation. In preparation.

powder 18057 (solubilized in water with NaOH in equimolar amounts) in the in vitro experiments.

Sulfinpyrazone placebo: the solvent in which the 10% solution of Anturan was prepared.

Phenylbutazone (Butazolidin, Geigy Pharmaceuticals) 20% solution G-13871, 6670464 was used in the in vivo experiments and phenylbutazone powder G-15137 (solubilized in water with NaOH in equimolar amounts) in the in vitro experiments.

Butazolidin placebo: the solvent in which the 20% solution of Butazolidin was prepared.

Tyrode's Solutions.—

Tyrode's solution was prepared as described by Parker (19). Modified Tyrode's solution was 8 g NaCl, 0.2 g KCl, 1 g $NaHCO_3$ 0.05 g $NaH_2PO_4 \cdot H_2O$ and 1 g glucose per liter, pH adjusted to 7.35 with NaOH. (This solution does not contain calcium or magnesium.)

Tyrode-albumin: modified Tyrode's solution plus 1.4 g bovine serum albumin per liter (Calbiochem, Los Angeles, Calif., fraction V).

Tyrode-albumin-EDTA: 5 parts Tyrode-albumin plus 1 part EDTA–saline solution (2% disodium ethylenediaminetetraacetate, 0.33% NaCl).

Tyrode-gelatin solution: Tyrode's solution plus 2.5 g gelatin per liter.

Adenine Nucleotides.—ATP, ADP, and AMP (Sigma Chemical Co., St. Louis, Mo.) were dissolved in Tyrode's solution to the required concentrations.

Thrombin.—Crude bovine thrombin (Parke, Davis & Co., Detroit, Mich.) was dissolved in Tyrode's solution to the desired concentrations, usually 5 NIH units per milliliter.

Gamma Globulin.—The crude gamma globulin fraction (16%) (Connaught Medical Research Laboratories, Toronto, Ontario) was dialyzed against two changes (each of 4000 ml) of Tyrode's solution at 4°C for 12 hr, diluted with Tyrode's solution to 1.6%, and centrifuged at 110,000 *g* for 30 min to remove any insoluble material.

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

Immune Complexes.—Antigen-antibody complexes were prepared as previously described (9). The concentration used to induce platelet aggregation was adjusted by dilution with Tyrode's solution, as described for the collagen suspension.

Latex Coated with Gamma Globulin.—A 30% polystyrene latex suspension (British Drug Houses, Toronto, Ontario) was diluted to 0.1% with Tyrode's solution. 1 part was then added to 9 parts of a gamma globulin solution (prepared as described above) and allowed to stand at room temperature for 15 min. The coated latex particles were recovered by centrifugation at 40,000 *g* for 20 min at 4°C and washed twice with Tyrode's solution. They were finally resuspended in Tyrode's solution at a concentration of 0.1%. The concentration used to induce platelet aggregation was adjusted by dilution with Tyrode's solution, as described for the collagen suspension.

Animals.—Pigs and rabbits were anesthetized with sodium pentabarbitol (1 gr/2.5 kg) for cannulation of a carotid artery and for the examination of hemostatic plug formation in the mesentery of the rabbits.

Glassware.—Glassware which was 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 cannula into 3.8% trisodium citrate (1–9 parts of blood) and centrifuged at 77 *g* for 15 min at room temperature. The supernatant PRP was removed with siliconed Pasteur pipettes. In some experiments, citrated human blood was taken from a vein in the forearm using an 18 gauge needle and a plastic syringe.

Platelet Suspensions.—Blood was taken from pigs into 2% EDTA-saline (1–9 parts of blood) either through a carotid artery cannula or by collection in a polyethylene pail at a slaughterhouse (Canada Packers, Ltd., Toronto, Ontario). PRP was prepared either by centrifuging (as described above) or by allowing the blood to stand for 2 hr at room temperature. The PRP was centrifuged at 650 *g* for 15 min at 4°C. The platelets were gently 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.

Platelet counts were done on a Coulter counter as previously described (10).

Platelet aggregation was studied by the turbidimetric method previously described (20).

Release of AMP and ADP.—1 ml of platelet suspension was mixed with 0.1 ml of a solution of the pyrazole drug being studied (diluted to the desired concentration with Tyrode's solution). In the control experiments, 0.1 ml of Tyrode's solution replaced the solution of the pyrazole drug. In order to induce platelet aggregation and release platelet constituents, 0.1 ml of a suspension of either collagen, antigen-antibody complexes, gamma globulin–coated latex, or a thrombin solution was added and the mixture was continuously shaken or stirred for 10 min at 37°C. Then 0.2 ml of 2% EDTA-saline was added to prevent conversion of ADP to AMP, and AMP to adenosine. (The EDTA does not interfere with the analysis of ADP and AMP.) The suspension was chilled and centrifuged at 2000 *g* for 10 min at 4°C. For AMP and ADP analysis, the supernatant was removed and mixed with an equal volume of cold 6% perchloric acid. The precipitate was removed by centrifugation and the supernatant analyzed for ADP and AMP by the Boehringer test kit method as described by Adam (21) and by Glynn et al. (10). For AMP and ADP analysis, the platelet button was resuspended in 0.25 M sucrose (equal in volume to the final platelet suspension). 1 ml of this suspension was mixed with 1 ml of cold 6% perchloric and the assay continued as described above for the supernatant. (Phenylbutazone at a final concentration of 50 μg/ml did not affect this assay.)

Serotonin-^{14}C Labeling of Platelets.—The method of labeling in vivo (10) has been described. The labeled compound used was 5-hydroxytryptamine-3′-^{14}C creatinine sulphate supplied by the Radiochemical Centre, Amersham, Buckinghamshire, England. Each rabbit was given 3.5 ml containing 10 μc (350 μg).

Release of Serotonin-^{14}C from Platelets.—Citrated platelet-rich plasma or platelet suspensions were prepared from the blood of rabbits given serotonin-^{14}C. The release of platelet constituents and the separation into platelet button and supernatant were carried out as described above. In experiments in which the AMP and ADP concentrations were determined, samples of the supernatants were removed for counting before the addition of perchloric acid. The platelet buttons were washed three times with Tyrode-albumin-EDTA before addition of Hyamine and transfer to counting vials. Radioactivity was determined in a liquid scintillation counter as previously described (10).

Hemostasis.—The technique for evaluation of hemostasis by cutting small mesenteric vessels will be described elsewhere (22). The length of time from the onset of bleeding to its arrest was recorded by direct observation of the transected vessels. The vessels were observed for 30 min in order to note any renewed bleeding.

Platelet survival studies on rabbits (3) were carried out by a modification (23) of the method of Leeksma and Cohen (24) using diisopropyl phosphorofluoridate-^{32}P (sterile solution) (DFP-^{32}P) obtained from the Radiochemical Centre. The specific activity of the labeled DFP at the time of its shipment was between 200 and 240 μc/mg of DFP. It was used for platelet survival studies up to 3 wk after shipment. The dose of labeled DFP ranged from 0.03 to 0.12 mg/kg body weight. The rabbits were bled from ear veins with siliconized syringes containing 2% EDTA-saline (1–9 parts of blood). With the exception of three animals, matched pairs of phenylbutazone-treated and nontreated rabbits were used. The phenylbutazone-

treated animals received 100 mg of phenylbutazone (0.5 ml of 20% solution) twice daily, intramuscularly, for 3 days before, and also during, the platelet survival study. The non-treated animals received the same volume of the Butazolidin placebo solution. The platelet half-life value for each animal was calculated by the method of least squares. Platelet turnover was calculated as

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

This must not be interpreted as meaning that we believe that platelets disappear from the circulation in an exponential manner. We have used this as a means of providing an objective index of platelet survival (1).

Assay of phenylbutazone and sulfinpyrazone in plasma was carried out as described by Burns et al. (25, 26). (Extraction of plasma from untreated animals gave a variable blank value between 1 and 5 mg per 100 ml of plasma.)

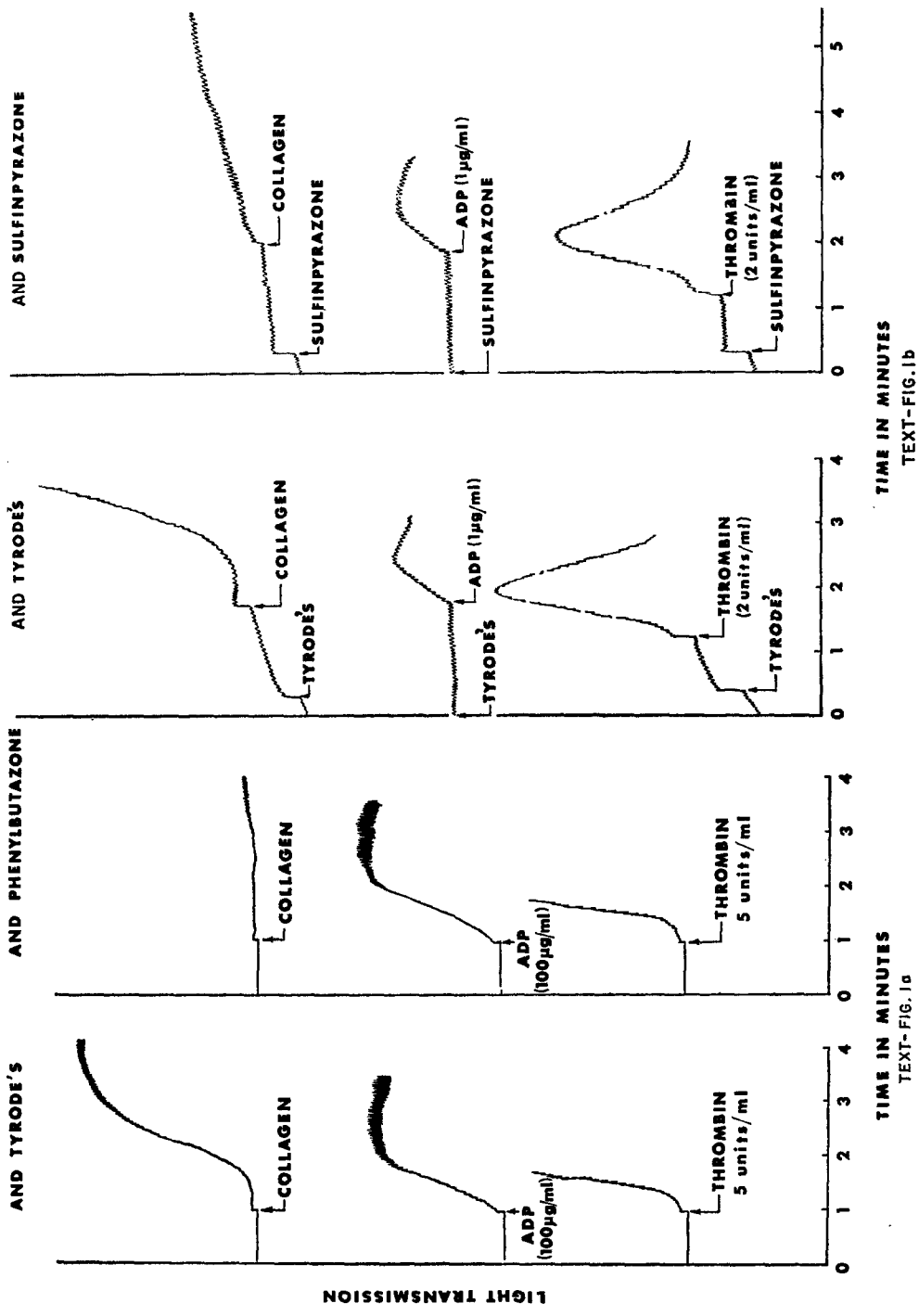
Specimens for electron microscopy were immediately added to cold 1% osmium tetroxide in Tyrode solution (pH 7.4). After 1.5–2 hr in the osmic acid solution, the tissue was dehydrated in increasing concentrations of ethanol, and treated in propylene oxide for 0.5–1 hr. The specimen was then placed in a mixture of equal parts of Epon 812 and propylene oxide, for 8–12 hr, and then embedded in Epon 812. 0.5 μ thick sections were cut on a Porter-Blum microtome and stained with azure II for orientation by light microscopy. A suitable area was selected and ultrathin sections cut and stained with lead hydroxide or with lead hydroxide and uranyl acetate. The sections were picked up on uncoated grids and examined in either a Philips 200 or RCA EMU-3F electron microscope.

RESULTS

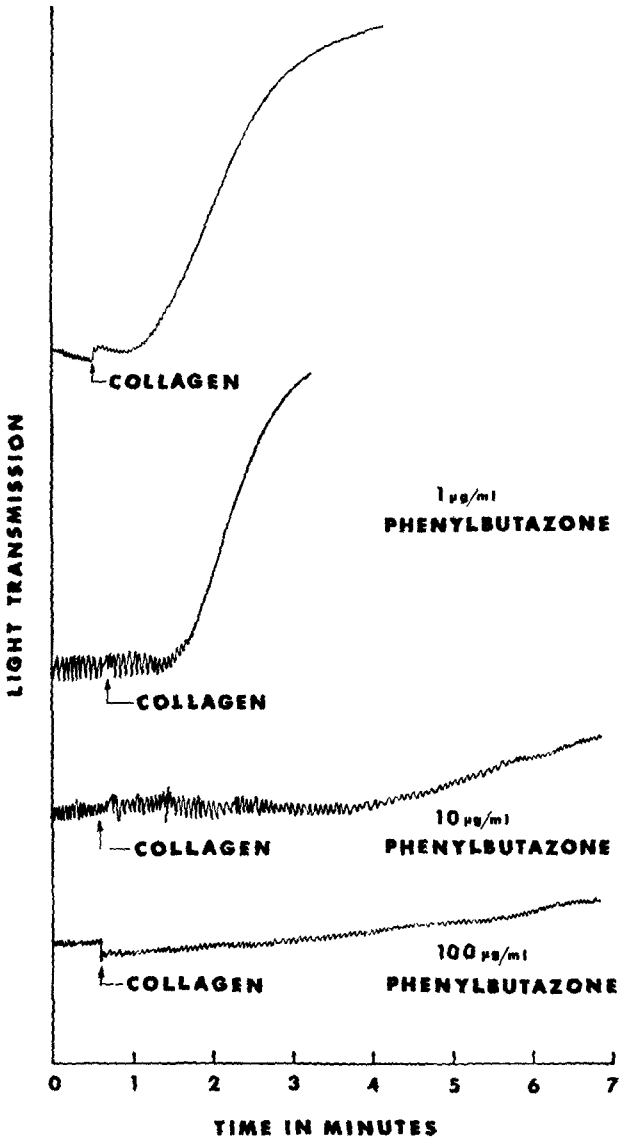
In Vitro Studies.—It was found that when sulfinpyrazone, or phenylbutazone, was added to citrated platelet-rich plasma, platelet aggregation induced by collagen was suppressed. However, platelet aggregation induced by ADP or thrombin was not affected. The results of experiments with phenylbutazone and sulfinpyrazone are shown in Text-figs. 1 *a* and 1 *b*. Inhibition of platelet aggregation by the pyrazole compounds was found with pig, rabbit, or human PRP. Text-fig. 2 shows the effect of different concentrations of phenylbutazone on platelet aggregation induced by collagen.

To examine the influence of these drugs in a system free of plasma proteins and also one in which the effect of antigen-antibody complexes and gamma globulin-coated surfaces can easily be explored, we studied the action of these compounds in a suspension of washed platelets. It was found that sulfinpyrazone and phenylbutazone inhibited the action of collagen, antigen-antibody complexes, and gamma globulin–coated latex in inducing platelet aggregation in a washed platelet suspension (Text-fig. 3). The action of thrombin, however, was not inhibited. We could not examine ADP in this system because most of our washed platelet suspensions do not aggregate when ADP is added.

Collagen, antigen-antibody complexes, and gamma globulin–coated surfaces are known to cause the release of platelet constituents such as serotonin, ADP, and AMP. We therefore examined the effect of the pyrazole compounds on the release of nucleotides from the platelets. It was found that when phenyl-

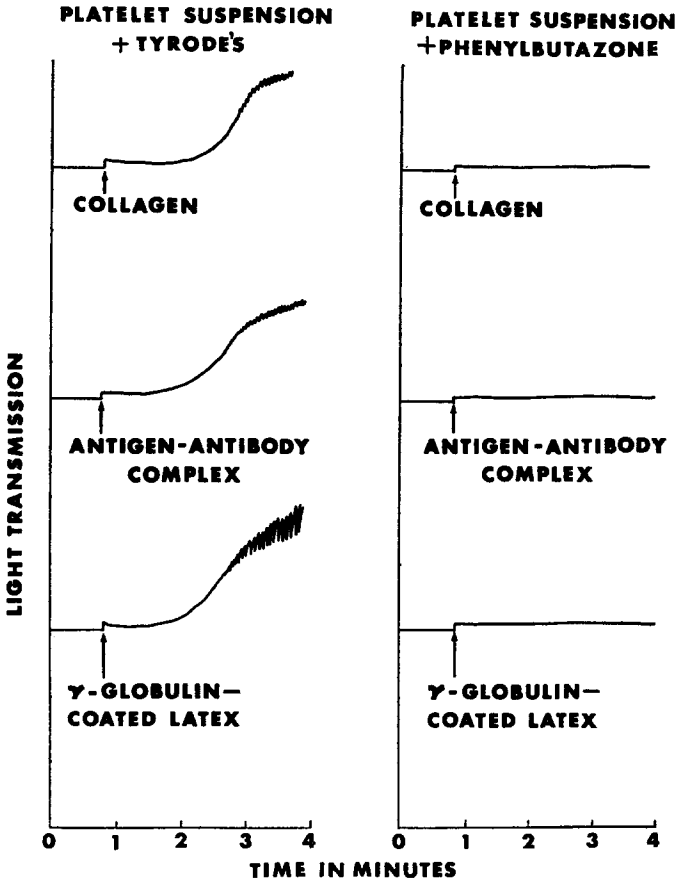


TEXT-FIG. 1. Platelet aggregation in citrated platelet rich plasma induced by collagen, ADP, and thrombin. Upward deflection of the tracing represents aggregation; the oscillations are caused by large aggregates interrupting the light beam. (*a*) Effect of phenylbutazone (final concentration 100 µg/ml) on pig platelets. (*b*) Effect of sulfinpyrazone (final concentration 1 mg/ml) on human platelets. Only collagen-induced aggregation is suppressed by these pyrazole drugs.



TEXT-FIG. 2. Suppression of platelet aggregation in a suspension of pig platelets by increasing concentrations of phenylbutazone. No phenylbutazone was added to the upper sample. The final concentrations of phenylbutazone in the other samples are shown with the corresponding curves.

butazone or sulfinpyrazone was added to a platelet suspension in a final concentration of 100 μg/ml, the drugs diminished the amount of nucleotides in the form of ADP and AMP released into the ambient fluid (Table I). In Table



TEXT-FIG. 3. Platelet aggregation in a suspension of washed pig platelets, showing suppression by phenylbutazone of the effect of collagen, antigen-antibody complex, and gamma globulin-coated latex particles.

II it can be seen that, coincident with the inhibition of release of nucleotides into the ambient fluid, the platelet levels of these nucleotides were higher and the effect was proportional to the amount of pyrazole compound added to the suspension. In Table III the inhibition of the release of AMP and ADP in the presence of different concentrations of sulfinpyrazone is shown to be related to the degree of suppression of aggregation which occurred in the platelet suspension. It was also found, in other experiments, that the fall in platelet ATP usually induced by the stimuli was diminished by the presence of the pyrazole compounds. Platelets labeled in vivo with 5-hydroxytryptamine-^{14}C showed less release of radioactivity into the ambient fluid in the presence of the pyrazole drugs when stimulated with collagen, antigen-antibody complexes, or gamma globulin-coated latex (Table IV).

TABLE I

Effect of Pyrazole Drugs on Surface-Induced AMP and ADP release from Platelets

Stimulus*	Solution added to platelet suspension			AMP and ADP in ambient fluid after release reaction (nanomoles/ml platelet suspension)		
	Tyrode's solution	Phenylbutazone (final conc'n)	Sulfinpyrazone (final conc'n)	AMP‡	ADP	AMP + ADP
		100 μg/ml	*100 μg/ml*			
Collagen	+			30.6	32.0	62.6
		+		29.2	19.5	48.7
	+			36.2	32.7	68.9
			+	20.8	20.9	41.7
Collagen	+			14.6	11.1	25.7
		+		12.2	7.0	19.2
Collagen	+			16.0	15.3	31.3
		+		10.4	9.0	19.4
Collagen	+			34.1	36.9	71.0
		+		26.4	32.7	59.1
Gamma globulin–coated latex	+			46.3	48.0	94.3
		+		35.8	28.5	64.3
	+			43.5	50.8	94.3
			+	43.5	36.9	80.4
Gamma globulin–coated latex	+			35.5	23.7	59.2
		+		24.4	11.8	36.2
	+			24.7	22.3	47.0
			+	17.7	13.2	30.9
Antigen-antibody complex	+			34.5	30.6	65.1
		+		20.5	21.6	42.1
	+			24.4	20.9	45.3
			+	12.9	13.2	26.1
Antigen-antibody complex	+			30.6	20.9	51.5
		+		16.0	12.5	28.5
	+			34.8	22.3	57.1
			+	22.6	13.9	36.5

* The results between the horizontal lines are from experiments carried out on the same day.

‡ These results have not been corrected for possible AMP contamination of the NADH used in the enzyme assay. In addition, the control platelet suspension usually showed some release of AMP and ADP into the ambient fluid and this has not been subtracted from the reported levels.

TABLE II

Effect of Pyrazole Drugs on AMP and ADP Levels in Platelets Following Reaction with Collagen

Stimulus	Final concentration of pyrazole compound in platelet suspension		AMP and ADP remaining in platelet button after release reaction (nanomoles/ml platelet suspension)		
	Phenylbutazone	Sulfinpyrazone	AMP*	ADP	AMP + ADP
	μg/ml	*μg/ml*			
Collagen	0		32.5	60.6	93.1
"	1		60.9	76.6	137.5
"	10		68.6	77.9	146.5
"	100		69.9	93.3	163.2
Collagen		0	51.5	53.6	105.1
"		1	52.2	71.7	123.9
"		10	66.8	70.9	137.7
"		100	64.7	74.5	139.2

* It must not be concluded that this represents the AMP level of fresh platelets. See note under Table I.

TABLE III

Relationship Between the Degree of Inhibition of AMP and ADP Release and Suppression of Platelet Aggregation

Final concentration of sulfinpyrazone in platelet suspension	AMP and ADP in ambient fluid following release reaction* (nanomoles/ml of platelet suspension)		Suppression of aggregation (% of control)§
	AMP‡	ADP	
μg/ml			
0	36.2	32.7	0
0.01	37.9	32.7	1
0.1	33.1	30.6	26
1.0	31.3	25.7	30
10	24.0	20.9	92
100	20.8	20.9	92

* Aggregation was produced by a collagen suspension.

‡ See note under Table I.

§ Maximum vertical deflection on the tracing during aggregation was assigned a value of 100% for the control sample (no sulfinpyrazone). The maximum deflections obtained in the presence of sulfinpyrazone were calculated as percentages of this and subtracted from 100% to give "suppression of aggregation." These values are dependent on the collagen concentration and represent relative values only with a given collagen suspension.

In contrast to the effect of the pyrazole drugs on the release of platelet constituents induced by particulate stimuli, there was no inhibition of thrombin-induced release of serotonin-^{14}C, AMP, or ADP from platelets (Table V).

TABLE IV

Effect of Phenylbutazone on Serotonin-^{14}C Release from Platelets by a Collagen Suspension

Platelets in 1 ml of	Tyrode's solution	0.1 ml of Phenylbutazone solution	Collagen suspension	Supernatant radioactivity	Platelet button radioactivity cpm/5 × 10^8 platelets
	ml		*ml*	*cpm/0.1 ml*	
Citrated PRP	0.2	—	—	192	48,504
" "	0.1	—	0.1	3,110	8,772
" "	—	1 mg/ml	0.1	1,920	26,620
" "	—	100 μg/ml	0.1	2,854	17,000
" "	—	10 μg/ml	0.1	3,548	9,352
Suspension	0.2	—	—	206	117,392
"	0.1	—	0.1	2,907	86,011
"	—	1 mg/ml	0.1	593	111,158
"	—	100 μg/ml	0.1	1,460	69,321
"	—	10 μg/ml	0.1	2,838	56,027

TABLE V

Effect of Phenylbutazone on the Release of Serotonin-^{14}C, ADP, and AMP from Platelets by Thrombin

Volume of platelet suspension	Tyrode's solution	0.1 ml of Phenylbutazone solution	Thrombin solution (Units/ml)			Supernatant radio-activity	AMP and ADP in ambient fluid after release reaction (Nanomoles/ml of platelet suspension)	
			50	5	1		ADP	AMP
ml	*ml*		*ml*	*ml*	*ml*	*cpm/0.1 ml*		
1	0.2	—	—	—	—	166	2.78	2.78
1	0.1	—	0.1	—	—	3,003	13.90	11.10
1	0.1	—	—	0.1	—	2,949	12.51	11.45
1	0.1	—	—	—	0.1	3,162	9.73	8.33
1	—	1 mg/ml	0.1	—	—	2,976	11.12	9.37
1	—	1 mg/ml	—	0.1	—	3,040	12.51	8.33
1	—	1 mg/ml	—	—	0.1	3,064	7.65	8.33
1	—	100 μg/ml	0.1	—	—	2,999	14.60	8.68
1	—	100 μg/ml	—	0.1	—	3,008	15.29	6.25
1	—	100 μg/ml	—	—	0.1	3,045	7.65	7.98
1	0.1	1 mg/ml	—	—	—	106	1.39	3.47
1	0.1	100 μg/ml	—	—	—	138	2.09	1.74

In Vivo Studies.—Three aspects of the in vivo effects of these drugs were also studied: the suppression of the aggregating action of collagen on platelets isolated following intravenous infusions; the effect of intravenous infusions on hemostatic plug formation; and the effect of the drugs on platelet survival.

*1. The effect of in vivo administration of pyrazole drugs:*Platelet aggregation in citrated PRP taken from rabbits before and after infusion of 25 mg/kg of phenylbutazone showed a pattern similar to that in the in vitro experiments. The response of the platelets in citrated platelet-rich plasma to collagen, but not to ADP or thrombin, was suppressed after the infusion, compared with the

TABLE VI

Effect of Phenylbutazone Infusions into Rabbits on Plasma Levels of Phenylbutazone and Platelet Aggregation to Collagen

Amount of phenylbutazone infused	Phenylbutazone per 100 ml plasma (20 min after infusion)	Suppression of aggregation* (% of preinfusion sample)
mg/kg	*mg*	
0	1.10	0
5	3.30	64
5	2.27	100
12.5	8.93	100
25	5.90	100
25	11.0	64
25	14.9	91
50	18.8	42
50	21.6	100
75	32.5	87
100	59.2	93
100	41.6	100
100	30.6	100
150	41.1	100
Placebo*	3.7	0
12‡	5.34§	69§
15‡	8.2	51
15‡	8.8§	31§

See notes under Table III.

* 6 repeated studies with the placebo solution showed no suppression of aggregation.

‡ Pigs.

§ 60 min values.

preinfusion response. Table VI summarizes the results of infusions of various amounts of phenylbutazone into rabbits and pigs, and Table VII gives the results of infusions of sulfinpyrazone into rabbits. The plasma levels of the drugs are tabulated, as well as an indication of the amount of aggregation occurring in response to collagen. The extent of aggregation was dependent on the concentration of the collagen suspension used and, because the effect of the pyrazole drugs could be overcome by using an excess of collagen, a suitable dilution of the collagen suspension had to be determined empirically for the platelets being tested in any one series of experiments. (Because only two or three animals

could be studied on one day, and because it was not possible to have a collagen suspension which would give uniform results from day to day, the variability in the aggregation results is to be expected.) Infusion of the pyrazole compounds had no detectable effect on the platelet count. Suppression of the aggregating effect of collagen was somewhat dependent on the plasma level of the drug.

2. Effect on hemostatic plug formation: Phenylbutazone was administered intravenously to rabbits at a dosage which the previous experiments had indicated would cause a consistent and marked suppression of collagen induced aggregation. Fig. 1 shows a motion-picture frame sequence of the normal arrest of bleeding from a transected mesenteric vessel. Vessels were transected both before and after phenylbutazone infusions (or infusion of the placebo solution).

TABLE VII

Effect of Sulfinpyrazone Infusions into Rabbits on Plasma Levels of Sulfinpyrazone and Platelet Aggregation to Collagen

Sulfinpyrazone infused	Sulfinpyrazone per 100 ml plasma (60 min after infusion)	Suppression of aggregation* (% of preinfusion sample)
mg/kg	*mg*	
0	4.5	0
25	3.5	0
50	21.1	36
75	27.0	49
100	33.1	69
150	39.5	88

* See note under Table III.

It can be seen that after transection of the mesenteric vessels there was a gradual accumulation of material at the ends of the cut vessels. Detailed study of the structure of this material showed that it was primarily composed of platelets (Fig. 2) and that there was a marked alteration in the morphology of the platelets in contact with collagen at the end of the transected vessel. Even as the plug was forming, blood could be seen streaming through parts of it (Fig. 1). Finally, the plug became sufficiently solid to arrest bleeding. In the rabbits given no treatment or infused with the placebo material, there was sometimes renewed bleeding through the plug but this usually lasted for less than 2 min. The rabbits infused with phenylbutazone showed a considerable lengthening in the time for the initial arrest of bleeding, and in some instances there was no arrest during the 30 min period of observation. When an initial arrest of bleeding did occur, there was frequently rebleeding through the plug, usually lasting 4 min or more. In some instances, rebleeding was continuous throughout the observation period. Table VIII summarizes the data from these experiments.

3. Effect on platelet survival: Platelet survival studies were done with rabbits receiving intramuscular injections of 100 mg of phenylbutazone twice daily for 3 days before and also during the survival measurements. The platelets were labeled with DFP-^{32}P in vivo. At daily intervals for 7 days, the platelets were isolated and their radioactivity determined. The results for two pairs are shown in Text-Fig. 4. Table IX gives the mean results for 12 phenylbutazone-treated rabbits and 12 control rabbits given the placebo solution and illustrates that platelet survival (estimated as the half-life) was significantly prolonged by this drug. There was no effect on the platelet count but platelet turnover was significantly reduced.

TABLE VIII

The Effect of Phenylbutazone on Hemostasis in Transected Rabbit Mesenteric Vessels

Material infused	No. of rabbits* tested	Total No. of vessels observed‡	Initial bleeding time		Frequency of renewed bleeding					
			Mean	SE	None		Once		Twice or more	
			sec	*sec*	*No.*	%	*No.*	%	*No.*	%
None	11	64	209	17.7	34	53	20	31	10	16
Placebo	13	74	216	20.1	40	54	16	22	18	24
Phenylbutazone (200 mg)	9	36	470§	87.0‖	7	19	5	14	24	67

* Rabbits were all about 2 kg in weight.

‡ Vessels transected were 150–200 μ in diameter.

§ 4 of the 36 vessels bled continuously during the 30 min of observation and these are included in the group of 24 vessels listed as showing renewed bleeding two or more times.

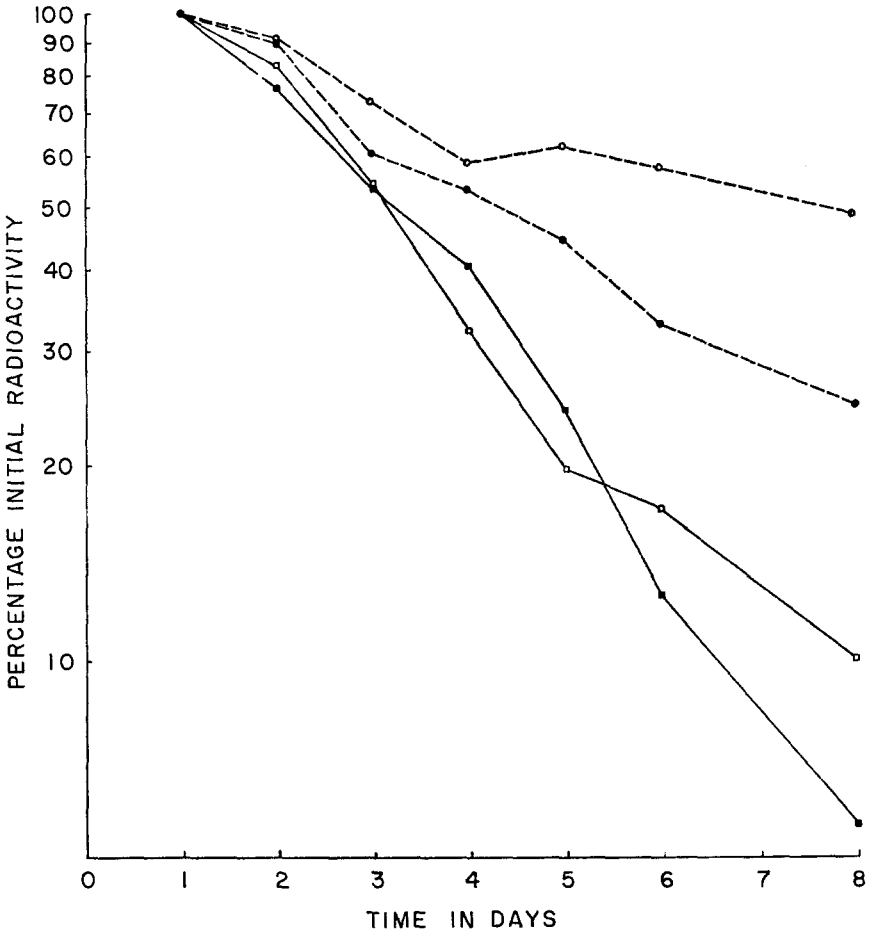
‖ Significance of the difference between the mean initial bleeding time after phenylbutazone infusion and the mean initial bleeding time after placebo infusion, $P < 0.001$.

DISCUSSION

These studies show that platelet aggregation induced by collagen, antigen-antibody complexes, or gamma globulin–coated latex (polystyrene) can be blocked by phenylbutazone and sulfinpyrazone. The mechanism does not appear to be a direct inhibition of the action of ADP because ADP can cause aggregation in the presence of these pyrazole drugs. They may interfere with the adherence of platelets to surfaces or with reactions which occur at the platelet membrane.

The evidence from the present studies indicates that in a platelet suspension the pyrazole drugs diminish the amount of ADP released from the platelets by surface stimuli. It is assumed on the basis of the available evidence (12, 13) that collagen, antigen-antibody complexes, and gamma globulin–coated surfaces induce aggregation through the release of platelet ADP. However, it is also

known[2] (as was the case with the suspensions in these experiments) that washed platelets in suspension do not usually aggregate upon the addition of ADP. It would seem, therefore, in the case of the platelet suspension, that the surface



TEXT-FIG. 4. The effect of phenylbutazone on platelet survival as estimated with labeled DFP in rabbits. To establish a standard origin for all the curves, the radioactivity values (as cpm/mg platelets) have been expressed as a percentage of the initial value. ----------, phenylbutazone administered;————, placebo solution administered.

stimuli, if they are inducing platelet aggregation through the release of ADP, must also be making available another factor involved in aggregation. This could occur through the release of such a factor from the platelets or by alteration of the surface characteristics of the platelets. Possibly, the pyrazole drugs

[2] Mustard, J. F. Unpublished observation.

exert their inhibiting effect by influencing this aspect of platelet aggregation. It seems most reasonable, however, that the primary action was in inhibiting the release of nucleotides, particularly ADP. It was found in the present experiments that the degree of inhibition of aggregation was proportional to the inhibition of nucleotide release. Furthermore, the evidence from the studies with citrated PRP indicates that ADP-induced platelet aggregation is not inhibited by the pyrazole drugs, thus supporting the suggestion that inhibition of ADP release by surface stimuli is the most likely mechanism.

The observation that the pyrazole drugs inhibit the release of serotonin-^{14}C from platelets stimulated with collagen suggests that the drugs exert their effect by diminishing the interaction of platelets with surface stimuli.

TABLE IX

The Effect of Phenylbutazone on Platelet Survival and Turnover in Rabbits

Treatment	No. of observations	Platelet count		Platelet half-life*		Platelet turnover‡	
		Mean	SD	Mean	SD	Mean	SD
		No./mm³	*No./mm³*	*days*	*days*		
Phenylbutazone	12	565,000	145,000	4.03	2.05	121,500	62,200
Placebo	12	525,000	122,000	1.78	0.39	226,500	119,000
Significance of difference between means		$t = 0.74$ $P < 0.5$		$t = 3.73$ $P < 0.001$		$t = 2.70$ $P < 0.02$	

* Calculated by the method of least squares.

‡ Number/mm^3 per day.

One of the factors in platelet aggregation induced by thrombin is the release of ADP (27). In these studies, thrombin-induced aggregation (both in PRP and in suspension) was not inhibited by the pyrazole drugs. This indicates that the mechanisms involved in platelet aggregation induced by thrombin may be, at least in the initial stages, different from those concerned in platelet aggregation induced by the surface stimuli.

The results from the in vivo experiments substantiate those from the in vitro studies. The plasma concentrations of the pyrazole drugs which produce inhibition of aggregation were of the same order of magnitude as those producing inhibition in the in vitro studies. The greatest inhibition occurred with the highest plasma concentrations.

The effect of the inhibition of platelet aggregation (induced by surface stimuli) on platelet function in vivo could be important in a number of biological processes. It could influence the effect of antigen-antibody complexes on platelets, and possibly the effect of microorganisms such as bacteria and viruses. In addition, it seems likely that it would affect processes in which collagen is an important stimulus in the formation of the platelet mass. It is thought that in

the formation of a hemostatic plug at the end of a transected vessel, the stimulus provided by the exposed connective tissue is one of the necessary factors (13, 22, 28, footnote 1). In the present experiments, the infusion of high doses of phenylbutazone caused a clearly demonstrable change in the arrest of bleeding from the transected vessels in the mesentery of rabbits. This change consisted mainly of a delay in the primary arrest of bleeding and an increased susceptibility of the hemostatic plug to breakdown, with rebleeding. Because we did not find in any of these experiments that platelet aggregation induced by thrombin or ADP was inhibited by these pyrazole drugs, nor were we able to show in other studies (2) an effect on blood coagulation, it is likely that the phenylbutazone influences hemostasis by inhibition of collagen-induced platelet aggregation. In studies of hemostasis in dogs with congenital defects in coagulation,[2] we found that abnormalities in factor VIII or IX caused a similar pattern of deficient hemostasis. In factor IX–deficient dogs, the administration of phenylbutazone produced a further aggravation of the hemorrhagic disorder. All this evidence strongly suggests that both the surface stimulus of exposed connective tissue (collagen) and blood coagulation are important in the production of a stable hemostatic plug.

We have previously shown (1, 2) that sulfinpyrazone administration in adequate doses prolongs platelet survival in man and rabbits. In the present study also, phenylbutazone was found to prolong platelet survival in rabbits. It is likely that the action of the pyrazole drugs on platelet survival is related to the platelets' response to surface stimuli. It seems that platelet survival, as well as being controlled by the age of the platelet, may also be affected by the functions of the platelet.

SUMMARY

Sulfinpyrazone and phenylbutazone block the aggregating action of collagen, antigen-antibody complexes, and gamma globulin–coated surfaces on blood platelets. These drugs do not block the action of ADP or thrombin. Inhibition of surface-induced aggregation appears to be the result of a decreased response of the platelets to surface stimuli, giving rise to diminished release of platelet constituents, such as ADP and serotonin. The intravenous infusion of these drugs produced results similar to those found in the in vitro experiments. Administration of phenylbutazone in doses sufficient to produce marked suppression of the platelet-collagen reaction impaired hemostatic plug formation at the ends of transected mesenteric vessels in rabbits. Since platelet function is considered a factor influencing platelet survival, the effect of phenylbutazone on platelet survival was examined. It was found that phenylbutazone prolonged platelet survival to more than twice the normal time and reduced platelet turnover by nearly 50%. These studies show that drugs which suppress platelet response to surface stimuli alter platelet function in vivo.

The technical assistance of Mrs. C. Fagerstroem, Mrs. M. Pedjasi, J. Willis, and J. Schlamp is gratefully acknowledged.

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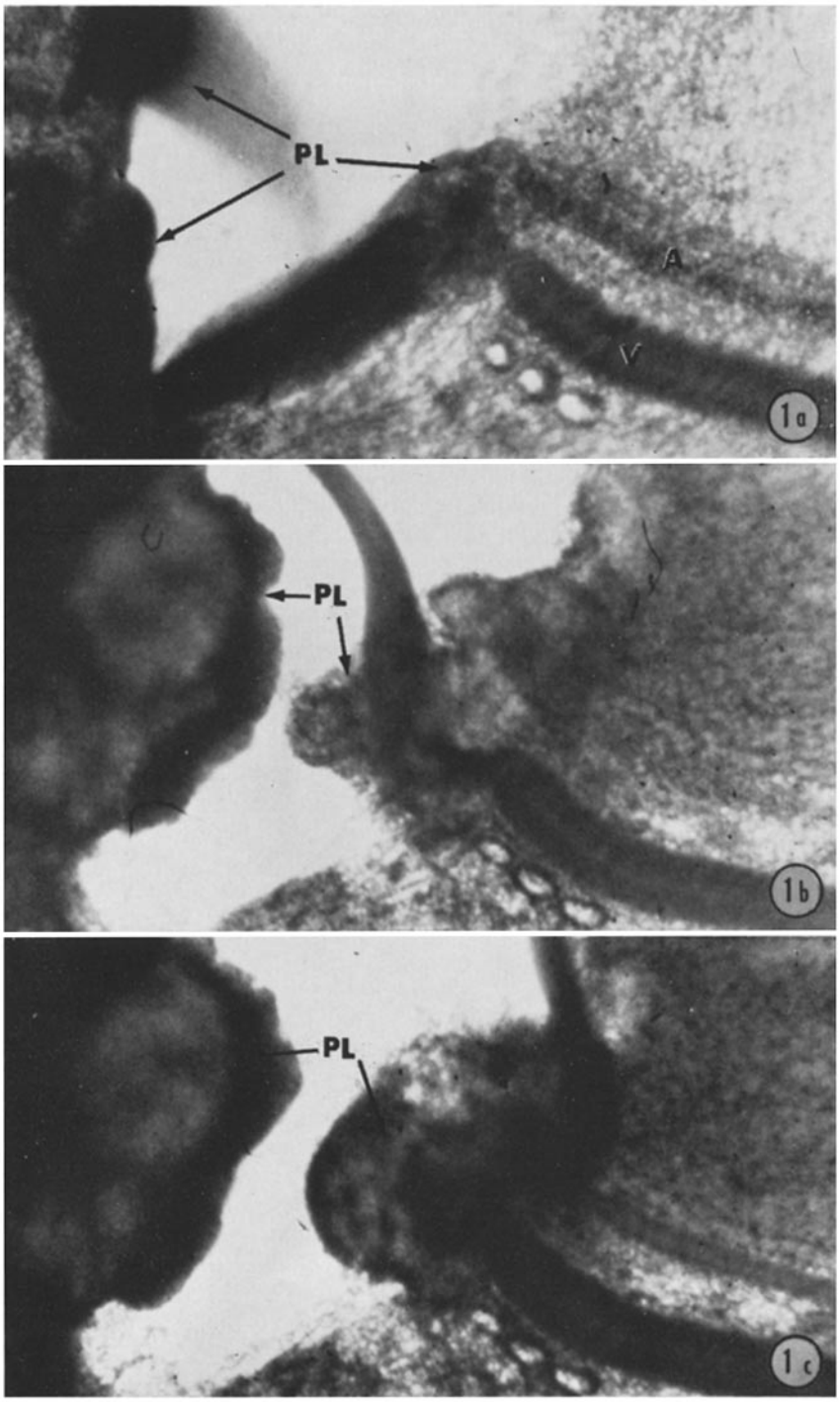
EXPLANATION OF PLATES

PLATE 7

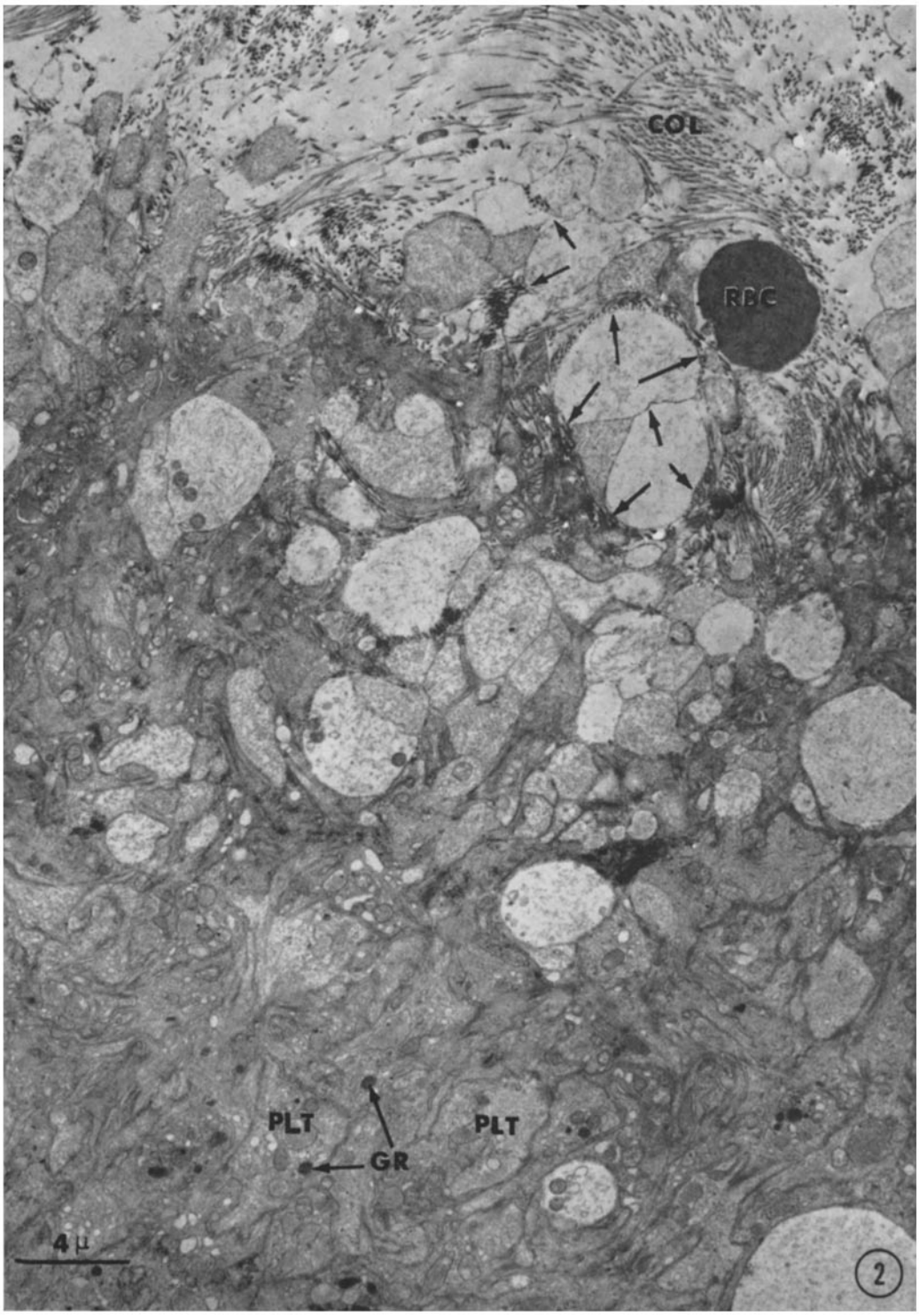
FIG. 1*a–c*. This is a motion-picture frame sequence of bleeding from transected vessels [arteriole (A), venule (V)] in the mesentery of a rabbit. Fig. 1 *a* shows the early formation of platelet plugs (PL) at the ends of the cut vessels (1 min after transection). Fig. 1 *b* shows the extensive platelet plug formation around the cut vessels with arrest of bleeding from the vessels on the left side of the picture (2 min). In Fig. 1 *c*, taken at 3 min, bleeding from the right hand vessel is not yet arrested. × 40.



(Packham et al.: Platelet response and pyrazole compounds)

PLATE 8

FIG. 2. An electron micrograph of a platelet plug at the end of a transected mesenteric blood vessel. The platelets (arrows) in contact with the exposed collagen (COL) of the vessel wall appear swollen, have lost much of their internal structure, have breaks in their membranes, and are less electron-dense than the platelets (PLT) which are remote from the collagen. These platelets are adherent to each other but have retained most of their granules (GR). A red blood cell (RBC) is apparent near the upper right hand corner of the picture.



(Packham et al.: Platelet response and pyrazole compounds)