Sodium Arachidonate Can Induce Platelet Shape Change and Aggregation Which Are Independent of the Release Reaction

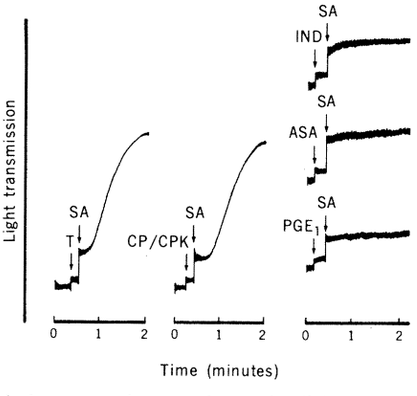
Abstract. *Sodium arachidonate causes shape change and aggregation of rabbit or human platelets that have been washed and then degranulated by treatment with thrombin. Since these platelets do not contain releasable adenosine diphosphate (ADP) and the aggregation is not inhibited by the creatine phosphate–creatine phosphokinase system, sodium arachidonate must be able to cause aggregation that is independent of the release of ADP. Since aggregation of these platelets induced by sodium arachidonate is inhibited by acetylsalicylic acid or indomethacin, it seems likely that products (such as prostaglandin G_2) formed from sodium arachidonate are responsible for aggregation. Thus, sodium arachidonate–induced shape change and aggregation of platelets may be caused (i) by the release of ADP by products of sodium arachidonate metabolism and (ii) directly by the products of sodium arachidonate metabolism, independently of released ADP.*

When sodium arachidonate is added to platelets in platelet-rich plasma it induces aggregation and the release of platelet granule contents, particularly serotonin, adenosine triphosphate (ATP), and adenosine diphosphate (ADP) (*1*, *2*). Several investigators (*2*–*4*) have concluded that arachidonate is converted to endoperoxides and thromboxanes by platelets, and that these labile compounds are responsible for the release reaction and aggregation. It has also been suggested that production of endoperoxides from platelet arachidonate may be responsible for the release reaction induced by collagen, or by ADP and epinephrine under some conditions (*2*, *5*). Malmsten *et al.* (*5*) have reported observations from which they concluded that the mechanism responsible for aggregation induced by the endoperoxide prostaglandin G_2 (PGG_2) was the release of ADP, which caused the platelets to aggregate. Further work by this group indicates that thromboxane A_2 formed from PGG_2 is the unstable factor responsible for this effect (*3*). We report here evidence that sodium arachidonate can also cause shape change and aggregation of platelets without release of ADP. The experiments were done with human or rabbit platelets that had first been treated with thrombin to remove their releasable ADP.

Suspensions of washed human or rabbit platelets were prepared (*6*, *7*), and thrombin was used to release 99 percent of the serotonin in their amine storage granules as described (*8*). These thrombin-degranulated platelets show a normal or increased sensitivity to ADP-induced aggregation, shape change and aggregation in response to collagen which is not dependent on ADP, and no response to thrombin (*8*). Rabbit platelets degranulated by thrombin survive for a normal length of time when they are infused into rabbits (*9*).

The addition of sodium arachidonate (45 μM) to a suspension of human or rabbit platelets degranulated by thrombin induced shape change and aggregation (Fig. 1). Creatine phosphate with creatine phosphokinase (CP/CPK) in concentrations that inhibit ADP at a final concentration of 10 μM by converting it to ATP, did not block sodium arachidonate–induced shape change or aggregation, whereas indomethacin (8 μM), acetylsalicylic acid (ASA) (4.6 mM), or prostaglandin E_1 (8 μM) did (Fig. 1). Prostaglandin E_1, but not ASA, also blocks ADP- and inophore (A23,187)-induced platelet aggregation (*8*, *10*); therefore, it is possible that prostaglandin E_1 is inhibiting a fundamental mechanism in platelet aggregation.

Addition of sodium arachidonate to suspensions of normal, untreated, and washed human or rabbit platelets induced aggregation and the release reaction (Fig. 2). Under these circumstances CP/CPK caused partial inhibition of sodium arachidonate–induced platelet aggregation and of the platelet release reaction (Fig. 2). With these untreated platelets, aggregation is attributable to the combined effects of sodium arachi-



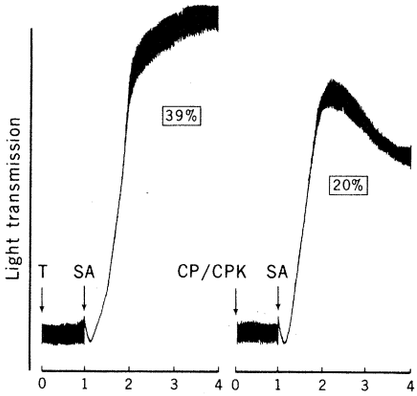


Fig. 1 (left). Sodium arachidonate–induced aggregation of degranulated, washed, human platelets suspended in Tyrode solution containing 0.35 percent albumin, 0.1 percent fibrinogen treated with diisopropyl fluorophosphonate, and apyrase (*6*, *12*). Before the response to sodium arachidonate was studied, the platelets had been induced to release 99 percent of the contents of their amine storage granules by exposure to thrombin (2 unit/ml); the recovered individual platelets were washed and resuspended at a platelet count of 500,000/mm^3 (*8*). Increase in light transmission through the stirred suspension of platelets indicated platelet aggregation. (The small increase upon addition of each reagent was caused by dilution of the platelet suspension.) The initial oscillations of light transmission through the suspension was due to the disk shape of the platelets; when the platelets changed to a more rounded form, the oscillations no longer occurred. Additions are indicated by arrows. Final concentrations after all additions: sodium arachidonate (*SA*), 45 μM; creatine phosphate (*CP*), 4.2 mM; creatine phosphokinase (*CPK*), 8.3 units; indomethacin (*IND*) (Merck), 8 μM; acetylsalicylic acid (*ASA*), 4.6 mM; and prostaglandin E_1 (*PGE*$_1$) (Upjohn), 8 μM. Tyrode solution (*T*) was added to control samples to maintain constant volume. Results shown are typical of two experiments with human platelets and five with rabbit platelets. Sodium arachidonate was prepared from arachidonic acid (5,8,11,14-eicosatetraenoic acid) [grade L, 99 percent purity (Sigma)]; arachidonic acid was dissolved in benzene at a concentration of 50 mM; 1-ml portions were stored at −20°C; immediately before use, the benzene was removed from a portion by evaporation and the residue was dissolved in 10 percent ethanol in 0.85 percent saline; Na_2CO_3 was added to bring the *p*H to 8.2, and the volume was adjusted to 10 ml with saline; 10-μl portions were used to induce aggregation. Fig. 2 (right). Sodium arachidonate–induced aggregation of washed human platelets suspended in the same medium as described in the legend of Fig. 1. These platelets had not been exposed to thrombin and hence retained their granule contents. These platelets were labeled by incubation in the first washing fluid with [^{14}C]serotonin (*12*), which was concentrated in their amine storage granules. During aggregation induced by 45 μM sodium arachidonate (*SA*), radioactivity was released from the amine storage granules into the suspending medium. The radioactivity in the supernatants prepared by centrifuging the platelets 3 minutes after the addition of SA has been calculated as percentages of the total [^{14}C]-serotonin in the unstimulated platelets. These percentages are shown in the boxes beside the aggregation curves. Tyrode (*T*) and CP/CPK were added as described (Fig. 1). Results shown are typical of three experiments with human platelets and five with rabbit platelets.

donate and the ADP that it releases from the platelets; CP/CPK inhibits only the contribution made by ADP. The inhibitory effect of CP/CPK on the release of [^{14}C]serotonin from the previously labeled platelets is probably related to its inhibition of the synergistic effect of ADP on the release reaction (*11*).

Thrombin-degranulated platelets have little if any releasable ADP, and CP/CPK has no effect on their shape change or aggregation induced by collagen or the ionophore A23,187 (*8*). In contrast, with intact platelets, CP/CPK inhibits aggregation induced by the ADP released by collagen, thrombin (*11*), or sodium arachidonate. Our observations show that sodium arachidonate can induce shape change and aggregation of platelets that no longer have releasable constituents in their amine storage granules. Thus sodium arachidonate can cause aggregation that is not mediated by released ADP. It appears that sodium arachidonate has effects that are somewhat similar to the action of ADP and the ionophore A23,187 in that sodium arachidonate can induce shape change and aggregation of platelets whether or not ADP is released from the platelet granules.

However, the mechanisms by which ADP and the ionophore A23,187 induce shape change and aggregation of thrombin-degranulated platelets appear to be different from that involving sodium arachidonate since indomethacin and ASA inhibit the sodium arachidonate–induced platelet changes but do not inhibit aggregation induced by ADP or the ionophore A23,187 (*8*, *12*).

Platelet aggregation induced by sodium arachidonate has been attributed to the formation of the endoperoxide PGG_2 (*5*) or thromboxane A_2 (*3*); evidence has been presented that the cyclooxygenase (*13*) responsible for the formation of PGG_2 is inhibited by ASA and indomethacin (*5*). Roth and Majerus (*14*) have shown that ASA acetylates cyclooxygenase; this may be responsible for the inhibitory effects of ASA on the formation of PGG_2 and the thromboxanes. Sodium arachidonate–induced aggregation of thrombin-degranulated platelets is probably due to endoperoxides or thromboxanes.

R. L. KINLOUGH-RATHBONE
H. J. REIMERS
J. F. MUSTARD
Department of Pathology,
McMaster University,
Hamilton, Ontario L8S 4J9, Canada
M. A. PACKHAM
Department of Biochemistry,
University of Toronto,
Toronto, Ontario M5S 1A1, Canada

References

1. M. J. Silver, J. B. Smith, C. Ingerman, J. J. Kocsis, *Prostaglandins* **4**, 863 (1973); B. B. Vargaftig and P. Zirinis, *Nature (London) New Biol.* **244**, 114 (1973).
2. J. B. Smith, C. Ingerman, J. J. Kocsis, M. J. Silver, *J. Clin. Invest.* **53**, 1468 (1974).
3. M. Hamberg, J. Svensson, B. Samuelsson, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2994 (1975).
4. A. L. Willis and D. C. Kuhn, *Prostaglandins* **4**, 127 (1973); B. B. Vargaftig, *Agents Actions* **3**, 357 (1973).
5. C. Malmsten, M. Hamberg, J. Svensson, B. Samuelsson, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1446 (1975).
6. N. G. Ardlie, D. W. Perry, M. A. Packham, J. F. Mustard, *Proc. Soc. Exp. Biol. Med.* **136**, 1021 (1971).
7. J. F. Mustard, D. W. Perry, N. G. Ardie, M. A. Packham, *Br. J. Haematol.* **22**, 193 (1972).
8. R. L. Kinlough-Rathbone, A. Chahil, M. A. Packham, H.-J. Reimers, J. F. Mustard, *Thromb. Res.* **7**, 435 (1975).
9. H.-J. Reimers, M. A. Packham, R. L. Kinlough-Rathbone, J. F. Mustard, *Br. J. Haematol.* **25**, 675 (1973).
10. R. L. Kinlough-Rathbone, M. A. Packham, J. F. Mustard, *ibid.* **19**, 559 (1970).
11. M. A. Packham, M. A. Guccione, P.-L. Chang, J. F. Mustard, *Am. J. Physiol.* **225**, 38 (1973).
12. J. F. Mustard, D. W. Perry, R. L. Kinlough-Rathbone, M. A. Packham, *ibid.* **228**, 1757 (1975).
13. M. Hamberg and B. Samuelsson, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3400 (1974).
14. G. J. Roth and P. W. Majerus, *J. Clin. Invest.* **56**, 624 (1975).

31 October 1975; revised 17 February 1976

Human Bloodstains: Individualization by Crossed Electroimmunodiffusion

Abstract. *Crossed electroimmunodiffusion was evaluated as a means for establishing the individuality ("fingerprinting") of human bloodstains. In ten separate examinations on stains from each of ten persons there was at least one peak with a unique range in height so that individualization was possible. The heights of certain peaks showed statistically significant female-male differences.*

Individualization or "fingerprinting" of human bloodstains is probably the primary unrealized goal of forensic serology. Improved and extended typing of erythrocytes (*1*) can approach this goal but is inherently incapable of reaching it because no human is unique in any one or combination of the known types.

We reasoned that if serologic individualization is possible it will come from use of a technique that goes beyond the qualitative distinctions made by typing and includes as well a quantitative aspect. Since crossed electroimmunodiffusion (CEID) is among the most sensitive techniques with these characteristics, we chose to evaluate it as a means for individualization. An earlier preliminary report by Whitehead *et al.* (*2*) established that eluted bloodstain antigens are reactive and apparently distinguishable by CEID.

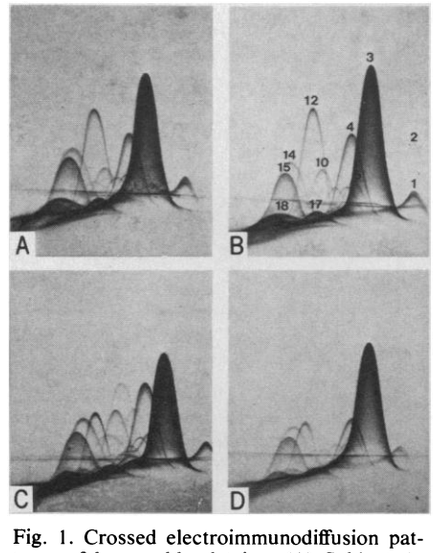


Fig. 1. Crossed electroimmunodiffusion patterns of human bloodstains. (A) Subject A, day 24, (B) Subject A, day 41. Those peaks identified as particular proteins are: 1, α_1-lipoprotein; 2, prealbumin; 3, albumin; 4, α_1-antitrypsin; 5, Gc globulin; 10, ceruloplasmin; 12, haptoglobin; 14, hemopexin; 15, transferrin; 17, α_2-macroglobulin; and 18, immunoglobulin A. (C) Subject B, day 57. (D) Subject G, day 48.

Blood for stains was obtained by finger puncture on ten different occasions over a 4-month period from five female and five male college students, all apparently healthy and ranging in age from 22 to 35 years. Blood was collected on pieces of a cotton bed sheet, allowed to dry for 48 hours, and then eluted by placing sections (6 mm in diameter) of the bloodstained sheet into a 2-ml beaker containing 0.05 ml of 0.85 percent NaCl, buffered to *p*H 7.2 with 0.02*M* sodium phosphate (PBS). After 5 minutes blood was withdrawn and diluted with PBS to a concentration of 10 mg of protein per milliliter (*3*). Antiserum to whole human serum was prepared in each of two rabbits by making 21 subcutaneous injections of 0.3 ml of serum in 0.3 ml of complete Freund's adjuvant over a 14-week period. Rabbits were then exsanguinated; the serums were pooled, dispensed into 7-ml portions, and stored at −20°C. The CEID was conducted on microscope slides (5 by 8 cm) as previously described (*4*). Initial experiments, in which antigen (bloodstain) and antibody were varied, led to the choice of 0.01 ml of antigen and 0.3 ml of antibody as optimum for routine assay. With these conditions as many as 22 antigens, some of which