An Enzymatic Mechanism for the Antithrombotic and Antihemostatic Actions of Aspirin

Abstract. *Aspirin-type drugs may inhibit irreversible clumping of human blood platelets by blocking the enzymatic conversion of arachidonic acid to a hitherto undiscovered factor, labile aggregation-stimulating substance, which triggers aggregation and to prostaglandin E_2, which sensitizes the platelets to its aggregatory effects.*

Smith and Willis (*1*) reported that the formation and release of prostaglandins (PG's) E_2 and $F_{2\alpha}$ which occurs in thrombin-stimulated platelets is inhibited by very small concentrations of indomethacin and aspirin (the former being more potent), but that sodium salicylate has only a weak effect. Phenylbutazone has activity intermediate between aspirin and sodium salicylate, and acetaminophen (4-acetamidophenol, paracetamol) is inactive (*1*). Such in vitro findings can also be observed when these drugs are orally ingested (*1, 2*). To a remarkable degree, these results parallel the relative ability of the drugs to suppress the second phase or irreversible type of platelet aggregation and the accompanying release of platelet constituents (*3*) known as the platelet release reaction (*4*). In addition, Kocsis *et al.* (*2*) have shown that, after oral administration of aspirin or indomethacin, duration of the inhibitory effects on aggregation parallels their effects upon platelet prostaglandin production.

These data point to a role for platelet PG synthesis in irreversible aggregation. However, it is improbable that any of the known prostaglandins are true mediators of aggregation (although that is not to say that they do not have some modulatory function). Prostaglandins of different structure may enhance or inhibit aggregation of platelets from rat or man (*5*). Nevertheless, PG's cannot themselves induce platelet aggregation, nor can they surmount the inhibitory effects of aspirin (*5, 6*).

To explain these anomalies, Willis (*7*) suggested that during biosynthesis of PG's in the platelets, free radical or peroxide intermediates transiently accumulate in the cell and cause irreversible aggregation and the release reaction. The feasibility of this hypothesis is demonstrated by the following data.

To test whether proaggregating material was generated during PG biosynthesis, a phenol-activated enzyme from sheep vesicular glands was used (*8, 9*). However, to eliminate the possibility of studying a phenomenon peculiar to vesicular gland, washed homogenates of human platelets were also used, and similar results were obtained (*6, 9*). Arachidonic acid or sodium arachidonate (*6*), at 25 to 50 μg, was stirred (1000 rev/min) at 37°C with 50 μl of the vesicular gland preparation (375 μg), which, as assayed (*10*), contained 122 μg of protein. Incubations were carried out at the bottom of the siliconized tube used in the platelet aggregometer (Chrono-Log Corp.). Citrated platelet-rich plasma (PRP) was then added (to a total volume of 1 ml), and transmission of light through the PRP was recorded immediately.

Aggregation of the platelets, indicated by increases in light transmission (*11*), could be induced by incubation mixtures containing the enzyme and arachidonate (*12*) and was attributable to the presence in them of a hitherto undiscovered factor, termed labile aggregation-stimulating substance (LASS). This factor was transitory in appearance. It was present in maximal amounts after 45 seconds of incubation and had

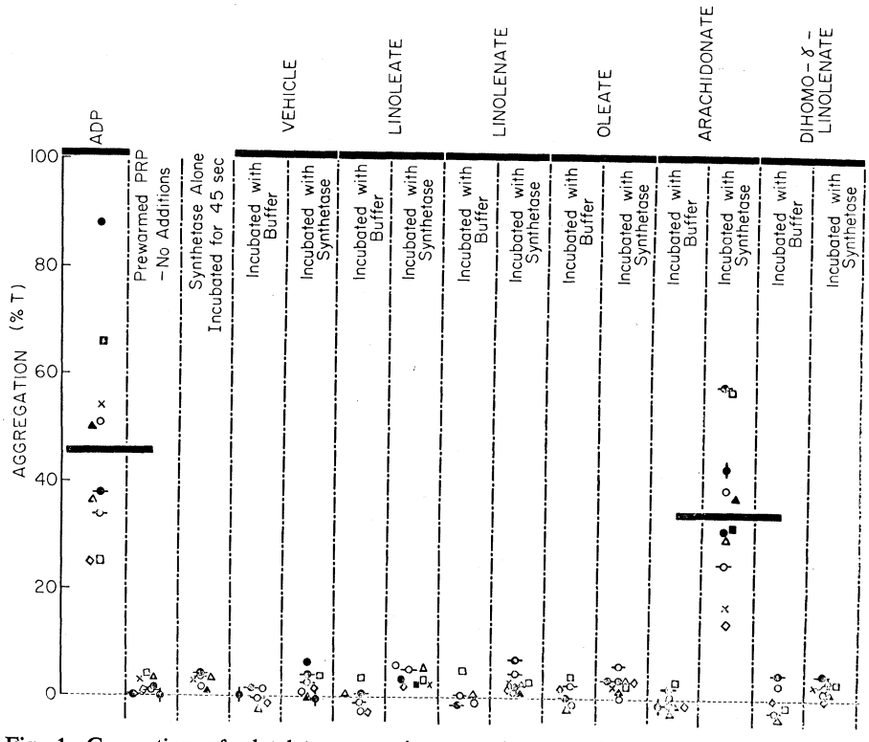

Fig. 1. Generation of platelet aggregating material (LASS) from arachidonate but not from closely similar unsaturated fatty acids. Shown are the maximal changes in light transmission (*T*; indicative of aggregation) during a 2- to 3-minute period of stirring which followed addition of warmed PRP (0.95 ml) to the aggregometer tube. The tube either contained nothing, or various incubation mixtures (as indicated). Fatty acids or appropriate vehicle (*12*) were stirred (1000 rev/min) at 37°C with either vesicular gland microme synthetase (122 μg of protein in 50 μl of tris phenol buffer) or else with the buffer alone. In some experiments, the effects of synthetase only were also examined. An incubation time of 45 seconds was used throughout, as peak LASS activity is present at this time (*12*). The vesicular gland preparation generated LASS only from arachidonate and not from the other fatty acids tested. Until incubated together, neither arachidonate nor the vesicular gland synthetase caused aggregation of the platelets. Basal changes in light transmission are indicated by the effects of stirring warmed PRP with no additions for up to 3 minutes. Susceptibility of the platelets to aggregation induced by 4 μM ADP is also shown. Mean values for aggregation induced by LASS and ADP are indicated by the black bars. Symbols represent observations with the platelets from 11 normal healthy donors, with platelet counts of 262,000 to 440,000 cell/mm^3. Females, circles; males, rectangles, triangles, and crosses.

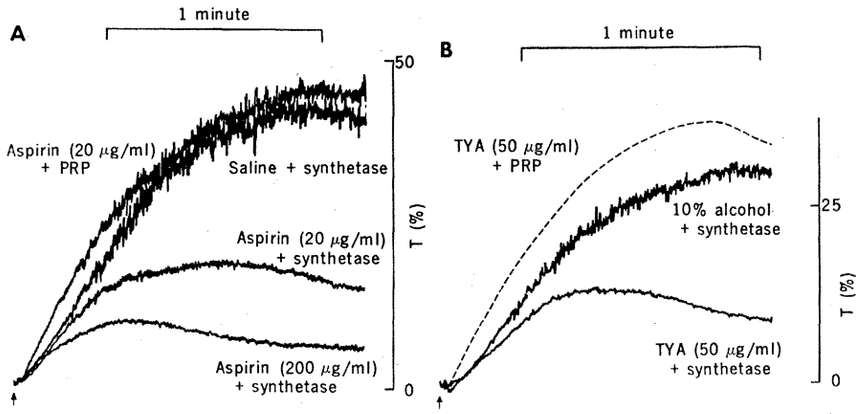

Fig. 2. Inhibition of LASS generation by inhibitors of PG synthetase. (A) Conditions were as described for Fig. 1 and the incubation time was 45 seconds. The aspirin or saline vehicle were incubated with the synthetase at 25°C for 4 minutes before the arachidonate was added. No aggregation was produced by the arachidonate or synthetase alone. The donor (female) platelet count was 430,000 cell/mm^3. (B) The ammonium salt of TYA (5 to 500 μg/ml) also produced graded reductions in generation of LASS when present in the synthetase. The TYA was dissolved in 10 percent (by volume) ethanol in 0.9 percent sodium chloride solution. The effects of the 50 μg/ml concentration of ammonium tetraynoate are illustrated. Addition of TYA (50 μg/ml as the ammonium salt) to the PRP only caused some potentiation of aggregation. The donor (male) platelet count was 404,000 cell/mm^3. TYA (200 μg/ml) used as the free acid was capable of complete suppression of LASS generation when present in the incubation mixture. The vehicle (95 percent ethanol) was evaporated under nitrogen before the enzyme was added.

disappeared within 6 to 10 minutes, although at these times PG production (up to 90 percent PGE_2) was near maximal. Differences and similarities between time courses for generation of LASS, PGE_2, and material that causes contractions of rabbit aorta have been described (*12*). The transitory appearance of LASS was not artifactual. For instance, the time-dependent decrease in proaggregating activity in the incubation mixtures was not due to accumulation of substances that either inhibited the formation of LASS or its proaggregatory effects (*6*). No significant aggregation was produced by the enzyme preparation or arachidonate before they were incubated together. Arachidonate could, however, aggregate platelets suspended in tris-buffered (*p*H 7.4) saline solution (*1, 12*)—a finding similar in essence but different in detail to those of Ingerman *et al.* and of Vargaftig and Zirinis (*13*) that arachidonate may cause aggregation of platelets in plasma.

Arachidonate was apparently unique as a precursor for the generation of LASS (Fig. 1). This factor could not be formed from the sodium salts of dihomo-γ-linolenic acid (precursor for the biosynthesis of PGE_1), oleic acid, linoleic acid, and linolenic acid. This finding compares with the apparently unique ability of arachidonate to prevent the inhibitory effects of aspirin and 5,8,11,14-eicosatetraynoate (TYA) on platelet aggregation (*14, 15*).

The effects of known inhibitors of PG synthetase were then examined. Typically, they inhibited generation of LASS from arachidonate in a dose-dependent manner. They were, however, ineffective when they were present only in the platelet-rich plasma (that is, they could not inhibit the actions of LASS once it had been formed). The effects of three such inhibitors (*8, 16*)—indomethacin, aspirin, and the ammonium salt of TYA—were examined. Average concentrations of these drugs causing 50 percent reduction in generation of LASS by vesicular gland microsomes were of the order of 3, 20, and 80 μg/ml, respectively (four to six experiments in each case). The effects of aspirin and TYA are shown in Fig. 2. When platelet enzyme was used, aspirin was more potent, a concentration of 20 μg/ml causing virtually complete suppression of LASS production. This finding is parallel to the known high activity of aspirin in suppressing platelet prostaglandin production induced by thrombin (*1*).

The aggregation produced by LASS was (after a brief phase of platelet swelling) immediate in onset and was not blocked by adenosine (10 m*M*) or by prolonged exposure of the PRP to 90 m*M* adenosine diphosphate (ADP) at 25°C. Both these treatments block the aggregating effects of ADP (*11, 17*). The aggregation response to LASS was, however, reduced at least 60 percent by ethylenediaminetetraacetate (EDTA) at 5.8 m*M*, which also suppressed responses to standard aggregating agents such as ADP or connective tissue extract. It would thus appear that aggregation of the platelets in response to LASS is not exerted through liberation of platelet ADP, but may involve a calcium-dependent process.

Because prostaglandins can greatly influence aggregation induced by various agents, their effects on aggregation induced by LASS were examined. As was expected (*5*), when PGE_1 (1 to 5 μg/ml) was first incubated with the PRP for 3 to 4 minutes at 37°C, little or no aggregation could be induced by either LASS or the standard aggregating agents.

In contrast, PGE_2 (1 μg/ml) caused a potentiation of the aggregation response to LASS (mean potentiation with PRP from four donors of 176 percent, range 27 to 315 percent). There was also a significant release of platelet-bound serotonin (*6*). Smaller amounts of PGE_2 (< 1 μg/ml) can also potentiate aggregation induced by standard aggregating agents (*5*) or LASS (*18*). Prostaglandin $F_{2\alpha}$ had a much weaker potentiating effect on aggregation produced by LASS (less than 50 percent increase in aggregation response with a PG concentration of 1 μg/ml).

These data support the likelihood that LASS is a mediator of irreversible platelet aggregation, for they provide an explanation for the known ability of PGE_2 (and to a lesser extent $PGF_{2\alpha}$) to potentiate irreversible aggregation (and the release reaction) induced by aggregating agents such as collagen or ADP (*5*). The combined effects on the platelets of LASS and PGE_2 are the antithesis of the effects of aspirin-type drugs.

Thus aspirin-type drugs and TYA (*15*) may exert their inhibitory effects on platelet aggregation by blockade of enzymes that convert arachidonate [made available by platelet phospholipases (*19*)] to LASS and to PG's E_2 and $F_{2\alpha}$. LASS thus acts as a trigger to irreversible aggregation, while the PG's sensitize the platelets to its aggregatory effects. A similar explanation

may be possible for the inhibitory effects of aspirin and TYA (*3, 15*) on the platelet release reaction, because sodium arachidonate induces a substantial release of platelet-bound [^{14}C]serotonin from platelets resuspended (*1*) in a saline medium. This release can be as high as 80 percent with 25 μg of arachidonate (*6*).

Whether the enzymatic pathways involved in the formation of LASS and PG's from arachidonate are identical has yet to be established (the microsomal preparations used in this study must have contained several enzymes). If they are identical, then LASS is probably one of the chain of intermediates of prostaglandin production, such as the recently characterized cyclic endoperoxide (*20*). To support this assumption, we have found that LASS behaves similarly to the endoperoxide (*6*). For instance, it is inactivated by stannous chloride and is extractable as an acid lipid. Also, it can be isolated on silica gel when subjected to thin-layer chromatography in a solvent system consisting of toluene and dioxan (1 : 1); its mobility is midway between that of PGE_2 (which remains near the origin) and arachidonate (which travels near the solvent front).

The above hypothesis thus explains the relation which apparently exists between inhibition of platelet prostaglandin production (*1, 2, 15*), inhibition of platelet aggregation, and prolongation of bleeding time (*3, 21*) observed for drugs such as indomethacin, aspirin, acetaminophen, and sodium salicylate because platelet aggregation is a primary event in hemostasis. Also, some bleeding disorders might involve defects in the ability of platelets to produce LASS or to respond to it.

Finally, irreversible aggregation of platelets is thought to be a terminal pathway in the many processes involved in the etiology of arterial thromboembolism. Furthermore, studies in laboratory animals (*22*) imply that aspirin could be clinically useful in the prophylactic treatment of arterial thrombosis. It is therefore feasible that production of LASS is a causal event in thrombotic episodes. If this is so, there is now a biochemical basis for the development of prophylactic therapy.

ANTHONY L. WILLIS
Department of Pharmacology,
Hoffmann—La Roche, Inc.,
Nutley, New Jersey 07110

References and Notes

1. J. B. Smith and A. L. Willis, *Nat. New Biol.* **231**, 235 (1971); A. L. Willis and J. B. Smith, in preparation.
2. J. J. Kocsis, J. Hernandovich, M. J. Silver, J. B. Smith, C. Ingerman, *Prostaglandins* **3**, 141 (1973).
3. H. J. Weiss and L. M. Aledort, *Lancet* **1967-II**, 495 (1967); J. R. O'Brien, *ibid.* **1968-I**, 894 (1968); G. Evans, M. A. Packham, E. E. Nishizawa, J. F. Mustard, E. A. Murphy, *J. Exp. Med.* **128**, 877 (1968); M. B. Zucker and J. Peterson, *J. Lab. Clin. Med.* **76**, 66 (1970).
4. H. Holmsen, H. J. Day, H. Stormorken, *Scand. J. Haematol. Suppl. 8* (1969).
5. J. Kloeze, in *Prostaglandins*, S. Bergström and B. Samuelsson, Eds. (Almqvist and Wiksell, Stockholm, 1967); p. 241; N. Chandra Sekhar, *J. Med. Chem.* **13**, 39 (1970); H. Shio and P. W. Ramwell, in *Prostaglandins in Cellular Biology*, P. W. Ramwell and B. B. Pharriss, Eds. (Plenum, New York, 1972), p. 77; H. Shio and P. W. Ramwell, *Nat. New Biol.* **36**, 45 (1972).
6. A. L. Willis, *Prostaglandins*, in press; unpublished observations.
7. ———, in *Symposium on Analgesics*, Cornell University Medical College, 26 and 27 April 1972, A. Van Poznak, Ed. (Cornell Univ. Press, New York, in press).
8. W. L. Smith and W. E. M. Lands, *J. Biol. Chem.* **246**, 6700 (1971).
9. Routinely an acetone-pentane powder of sheep vesicular gland microsomes was used (provided by Dr. J. Paulsrud of the Roche Department of Biomedical Nutrition). Human platelet synthetase was prepared as follows: Fresh human platelet-rich plasma (with acid citrate dextrose as anticoagulant) was obtained from a local blood bank. The platelets were isolated and resuspended (*1*), then homogenized, frozen, thawed, and sonicated to completely disrupt the platelet granules. A preparation containing microsomes was then obtained by centrifuging the sonicated granules at 105,000*g* for 1 hour in an ultracentrifuge (Beckman) and resuspending the pellet in the tris phenol buffer. This suspension was further centrifuged and resuspended in fresh buffer to yield the platelet synthetase, which was largely free of ADP or other proaggregating material, but was capable of synthesizing LASS and PG-like material from arachidonate. Such an enzyme preparation containing 148 μg of protein in 50 μl of tris phenol buffer, generated approximately the same amounts of LASS activity from arachidonate (25 μg) as did 122 μg of the vesicular gland enzyme. Precautions taken in preparation of the arachidonate, and other fatty acids have been described (*12*).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
11. G. V. R. Born and M. J. Cross, *J. Physiol. (Lond.)* **168**, 178 (1963).
12. A. L. Willis, *Proceedings of the IVth International Congress on Thrombosis and Haemostasis* (1973) (Abstr. 47), p. 79; ——— and D. C. Kuhn, *Prostaglandins* **4**, 127 (1973); A. L. Willis, *Circulation* **48** (Suppl. 4), 55 (1973).
13. C. Ingerman, J. B. Smith, J. J. Kocsis, M. J. Silver, *Fed. Proc.* **32**, 219 (Abstr.) (1973); B. B. Vargaftig and P. Zirinis, *Nat. New Biol.* **244**, 114 (1973).
14. R. G. Leonardi, B. Alexander, F. White, *Fed. Proc.* **31**, 248 (Abstr.) (1972).
15. A. L. Willis, D. C. Kuhn, H. J. Weiss, *Science* **183**, 327 (1974).
16. J. R. Vane, *Nat. New Biol.* **231**, 232 (1971); D. G. Ahern and D. T. Downing, *Biochim. Biophys. Acta* **210**, 456 (1970).
17. B. Nunn, *Br. J. Pharmacol.* **46**, 579P (1972).
18. The concentration (1 μg/ml) of PGE_2 used routinely to demonstrate its potentiating effect upon aggregation is some 10 to 100 times greater than those detected after PG production by platelets, induced by thrombin (*1, 2*) or by collagen and epinephrine [J. B. Smith, C. Ingerman, J. J. Kocsis, M. J. Silver, *J. Clin. Invest.* **52**, 965 (1973); also (*15*)]. However, in several experiments we have observed significant potentiating effects on LASS-induced aggregation with concentrations of PGE_2 or between 1 to 100 ng/ml. The effects with small concentrations of the PG are most noticeable on rate of aggregation and size of the aggregates. In addition, during platelet prostaglandin production (which is extremely rapid), local concentrations of PGE_2 inside and closely adjacent to the platelets may be considerably higher than in the plasma. Also, PGE_2 content of inflamed tissue can reach 100 ng/ml (*19*) and, after infarction, the injured tissue could produce these quantities of prostaglandins.
19. A. L. Willis, P. Davison, P. W. Ramwell, W. E. Brocklehurst, B. Smith, in *Prostaglandins in Cellular Biology*, P. W. Ramwell and B. B. Pharriss, Eds. (Plenum, New York, 1972), p. 227.
20. M. Hamberg and B. Samuelsson, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 899 (1973).
21. A. H. Sutor, E. J. W. Bowie, C. A. Owen, *Mayo Clin. Proc.* **46**, 178 (1971).
22. C. A. Danese, C. D. Voleti, H. J. Weiss, *Thromb. Diath. Haemorrh.* **25**, 288 (1971).
23. I thank G. Hawthorne of the Roche Dispensary for drawing the blood samples.

25 June 1973; revised 11 September 1973

Acetylenic Analog of Arachidonate That Acts Like Aspirin on Platelets

Abstract. *Development of irreversible platelet aggregation and the accompanying release of platelet-bound serotonin and production of prostaglandins is suppressed by 5,8,11,14-eicosatetraynoic acid (TYA). These findings may be explained by an ability of TYA to inhibit the enzymatic conversion of arachidonate to a newly recognized factor, labile aggregation-stimulating substance, which induces platelet aggregation, and to prostaglandins E_2 and $F_{2\alpha}$.*

Like aspirin (*1*) 5,8,11,14-eicosatetraynoic acid [TYA, Ro 3-1428 (Roche)] inhibits production of prostaglandins (PG's) at many sites and potently inhibits conversion of arachidonate to PG's E_2 and $F_{2\alpha}$ in broken cell preparations (*2, 3*). Also, as has been shown (*4*), it acts like aspirin in suppressing the enzymatic generation of a labile aggregation-stimulating substance (LASS) from arachidonate. However, aspirin inhibits other enzymes, such as platelet phosphodiesterase (*5*). Similar properties have not been reported for TYA, and, because of its structure (it is an acetylenic analog of arachidonic acid), one might expect it to compete directly with the substrate in arachidonate-utilizing enzyme systems. We have used TYA to