Biochemical Pharmacology, Vol. 27, pp. 2131–2138.

0006-2952/78/0901-2131$02.00/01

EFFECT OF ARACHIDONIC ACID ON SOME INHIBITORS OF THE HUMAN PLATELET RELEASE REACTION*

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(*Received* 5 *December* 1975; *accepted* 18 *February* 1977)

Abstract—The inhibitory action of many agents on platelet aggregation and 5-hydroxy-tryptamine release inducible by epinephrine, comprising several non-steroidal anti-inflammatory drugs (NSAA)—indomethacin, acetylsalicylic acid, sodium salicylate, phenylbutazone and sulfinpyrazone—and other non-anti-inflammatory agents (NAA)—prostaglandin E_1, adenosine, dipyridamole, methysergide and cyproheptadine—can be prevented, and under special circumstances reversed, by the prior addition to human platelet rich plasma of the essential fatty acid, 5,8,11,14 eicosatetraenoic (arachidonic) acid. Although this agent, and only its all *cis* stereosiomeric form, can per se induce aggregation and release, its protective action against the platelet-toxic drugs is attributed to its role as the key substance essential for prostaglandin enzymatic biosynthesis. The mechanism of NSAA action is evidently different from that of the non-anti-inflammatory agents.

The platelet release reaction induced by several well-known agents—thrombin, adenosinediphosphate, epinephrine and collagen—is inhibited by acetylsalicylic acid (ASA,‡ or aspirin) and other non-steroidal anti-inflammatory agents (NSAA) [1]. Many block prostaglandin synthesis in a variety of cells [2, 3]. We have briefly reported that arachidonic acid (5,8,11,14 eicosatetraenoic acid, C20: 4ω6 [E-tetral]) could prevent the inhibition by ASA [4]. Moreover, among many analogues and related congeners studied, this effect was unique to E-tetra, and particularly to its all *cis* isomer [5]. Furthermore, among several fatty acids, saturated or unsaturated, E-tetra was the only one that *per se* did not inhibit to some degree aggregation and 5-hydroxytryptamine release. In addition, E-tetra is the only polyunsaturated fatty acid that *per se* can induce aggregation and release [6, 7]. Indeed, some unsaturated fatty acids actually inhibit release. As to the saturated fatty acids, they actually aggregate platelets [6, 8].

Since E-tetra is the precursor of prostaglandin (PG) E_2 and $F_{2\alpha}$ formed in many cells [9], we postulated that its effects were perhaps due to the production of these platelet prostaglandins [4, 6]. This synthesis was subseqently demonstrate [7, 10], and some of the intermediates as well as the final products were shown to play a fundamental role in aggregation and release.

This report presents more detailed and extended evidence indicating that E-tetra can not only prevent the inhibitory effects of several NSAAs and other platelet-toxic agents, but also that under certain conditions it can reverse the untoward effect of some.

* Supported by USPHS grant 09011.

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‡ Abbreviations used in the text are: ASA, acetylsalicylic acid; NSAA, non-steroidal anti-inflammatory agents; NAA, non-anti-inflammatory agents; E-tetra, 5,8,11,14 eicosatetraenoic acid, C20: 4w6; PRP, platelet-rich plasma; and PG, prostaglandin.

MATERIALS AND METHODS

Materials. Eicosatetraenoic acid (all *cis*, Schwarz-Mann, New York, NY, >99 per cent pure; and the Hormel Institute, MN, high purity), dissolved in dimethyl-sulfoxide (DMSO, Sigma Chemical Co., MO, grade 1), was tested at a final concentration in PRP of 1×10^{-3} M. The final dimethylsulfoxide concentration was 0.01 M.

A stock solution of epinephrine (1×10^{-3} M) (free base, Eastman Organic Chemicals, NY, reagent grade) was converted to the bitartrate by combining equimolar concentrations with tartaric acid. The solution was rendered anaerobic and slightly acidic by gassing with 95% N_2, 5% CO_2 (Matheson Gas Co., NJ, "high purity grade") and was subdivided into 1-ml aliquots which were kept at −60° until used. These were protected against light, and discarded if not used within 3 months.

A stock solution of 1×10^{-3} M adenosinediphosphate disodium salt (Sigma, grade 1) in saline was divided into 1-ml aliquots which were kept at −60° until used.

Bovine thrombin (EC 3.4.4.13) (Parke-Davis, MI, topical thrombin) was purified by the method of Lundbland [11]. The material was dissolved in saline at a concentration of 40 i.u./ml; the solution was subdivided into 1-ml aliquots and kept at −60°.

Sufficient acetylsalicylic acid (Sigma, crystalline) was solubilized in saline by the addition of 0.10 N NaOH to produce a 1.0 M solution at pH 7.0. One-ml aliquots were kept at −60°, and used within 1 month. At 5×10^{-4} M in PRP it inhibited platelet aggregation that was otherwise inducible by all aggregating agents studied.

A 0.1 M solution of sodium salicylate (Eastman Organic Chemicals, NY, reagent grade) in saline was subdivided into 1-ml aliquots, and kept at −60°.

A 0.02 M solution of adenosine (Sigma) in saline was subdivided into 1-ml aliquots, and these were stored at −60° for a maximum of 1 month.

A 1.0 M solution of ethylenediaminotetraacetic acid (Eastman Organic Chemicals, NY, practical grade) in saline was adjusted with NaOH to pH 7.0, and stored at 4°. When used, 0.01 ml was added to the 1 ml of sodium citrate anticoagulant (see below) to give a final whole blood tetraacetic acid concentration of 10 mM.

We thank the following investigators and companies for their generous supply of the following agents: PGE_1, Dr. J. Pike, Upjohn Co., MI; dipyridamole, sulfinpyrazone and phenylbutazone, Dr. T. Kaicher, Ciba-Geigy Pharmaceuticals, New York, NY; methysergide, Mr. S. Wahram, Sandoz Pharmaceuticals, NJ; and cyproheptadine and indomethacin, Dr. C. Stone, Merck, Sharp & Dohme, PA. Dissolved in saline, or as directed by the manufacturer, these were stored in aliquots at −60° until used.

Methods. Venous blood (9 ml into 1 ml sodium citrate, 0.31% final concn) was obtained at the end of a donation of ~450 ml blood from voluntary, random, presumably healthy, non-fasting donors at the New York Blood Center. As far as could be ascertained, they had not knowingly taken any medication in the preceding 5–7 days. The blood was promptly centrifuged at room temperature for 15 min at 150 *g*. The supernatant PRP's from six to ten donors were pooled, and studied within 1-hr after blood collection.

Normal platelets readily take up 5-hydroxytryptamine and release it upon proper stimulation. In the present study [^{14}C]-5-hydroxytryptamine uptake and release were monitored according to the method of Zucker and Peterson [1], modified as described previously [6].

"*Prevention*" *study.* The following procedure was used to study the ability of E-tetra to protect against certain inhibitors of platelet release: PRP was incubated at room temperature for 30 min with a 75% ethanol solution of radio-labeled hydroxytryptamine (final concn, 0.016 μCi/ml, Amersham-Searle, IL, 35 mCi/m-mole; less than 0.1% ethanol final concn). Subsequently, the fatty acid was dissolved in dimethylsulfoxide, was mixed with the PRP (0.01 M final concn of DMSO), the mixture was further incubated for 30 min without stirring, then placed in a Chronolog platelet aggregometer cuvette (Chrono-Log Corp., PA), warmed at 37°, stirred with a magnetized bar at 1000 rev/min, and aggregation was measured. Another plasma sample containing the E-tetra was concurrently monitored when admixed with epinephrine, adenosine diphosphate or thrombin added to the plasma–fatty acid mixture 1 to 2 sec after placing in the aggregometer. When a platelet-toxic agent was studied, it was added approximately 5 sec prior to placing the combination in the aggregometer. Immediately thereafter, the release inducer (epinephrine, adenosinediphosphate or thrombin) was added. After 8–10 min in the aggregometer the cuvette contents were centrifuged at room temperature for 30 min at 1000 *g* (Adams Serofuge, Clay Adams, NJ), and 0.1 ml of the supernatant solution was pipetted into 10 ml of Aquasol (New England Nuclear, MA) scintillation-counting solution. The ratio of the radioactivity in the supernatant, in counts/min (Packard Tri-Carb Liquid scintillation counter model 3375, Packard Inst. Co., IL), to that originally added to the PRP, multiplied by 100, gives the per cent of 5-hydroxytryptamine released. Throughout these studies the uptake was approximately 90 per cent of that added.

"*Reversal*" *study.* After the PRP was incubated for 30 min with [^{14}C]-5-HT, the action of the various inhibitors was assayed. The agent was mixed with the plasma, the combination was left without stirring at room temperature for exactly 2.5 min, then placed in the aggregometer, and the inhibitory action on [^{14}C]-5-HT release was examined. Epinephrine, or E-tetra, or adenosinediphosphate, or thrombin was added immediately thereafter, and the mixture was placed in the aggregometer. When, however, combinations of two release inducers were studied, they were added individually approximately 1 sec apart. After 8–10 min the cuvette contents were centrifuged and the supernatants assayed for radioactivity, as described above.

RESULTS

E-tetra *per se* induces rapid platelet aggregation (Fig. 1c), even to the point of masking the secondary wave, confirming preliminary findings [4]. When preincubated with E-tetra for 30 min, followed by aspirin added 5 sec before epinephrine, the usually observed inhibition of the second wave of epinephrine-induced aggregation (Fig. 1b) was obviated (Fig. 1d). Our earlier studies [5, 6] had indicated that the all *cis* E-tetra isomer was unique among eight fatty acids studied, including saturated and unsaturated as well as *trans* forms of E-tetra. Indeed, except for all *cis* E-tetra, all the other fatty acids to some extent inhibited epinephrine-, adenosinediphosphate-, thrombin- or collagen-induced release.

The facts (1) that only E-tetra could trigger release; (2) that only E-tetra, when combined with the other releasing agents, did not inhibit release; and (3) that only E-tetra blocked inhibition by acetysalicylic acid, prompted investigation of the effect of E-tetra on other inhibitors. Before this was done, however, it was deemed important to determine whether a combination of two release-inducing agents (not including E-tetra), or a comparable double concentration of a single agent, could obviate inhibition by aspirin. The data in Table 1 show that the release induced by some combinations was about the same as that produced by a single one. When ADP was incubated for 30 min, followed by a supplement of the same amount of nucleotide or epinephrine, release was slightly lower than that produced by either agent alone. As evident in Table 1B, the release triggered by E-tera, followed immediately by epinephrine, exceeded that induced by E-tetra that was first incubated for 30 min and then followed by epinephrine. Although this difference may not be statistically significant *per se*, preliminary observations suggested that this might be related to metabolic alterations in E-tetra after its uptake by the platelet [5]. Furthermore, only E-tetra preincubated with plasma can prevent inhibition by aspirin of various releasing agents. It should be emphasized

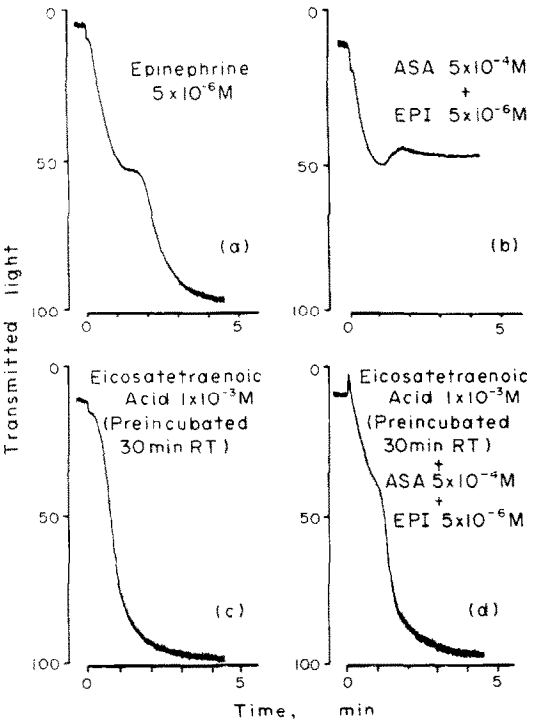

Fig. 1. Platelet aggregation response in pooled PRP (five to ten donors) to agents indicated. Typical of >30 experiments. Concentrations are final, as added to PRP. The E-tetra concentration (1×10^{-3} M) is the cut-off point below which per se it fails to induce release. It should be noted that this concentration is far greater than that of epinephrine or adenosinediphosphate, and it is the minimal concentration at which it protects against platelet-toxic agents (see text). However, the relative added concentration may be notably different from what obtains physiologically in view of different binding strengths of E-tetra, epinephrine and adenosinediphosphate to albumin.

that doubling the dose of epinephrine, or adding a combination of two release-inducing agents, or incubation of one release inducer with subsequent addition of another, could not prevent or overcome inhibition by aspirin (Table 1A and 1B). Only E-tetra combined with epinephrine, adenosinediphosphate or thrombin could prevent, but not overcome, the action of acetysalicylic acid (Table 1B).

The question arose as to whether E-tetra was acting uniquely by its co-presence with another release inducer, or whether two orthodox inducers, e.g. epinephrine and adenosinediphosphate, would act similarly in obviating inhibition by the NSAA. First examined was the release induced by epinephrine and adenosinediphosphate, singly or in combination, on a small group of inhibitors. Aspirin, idomethacin, phenylbutazone and sulfinpyrazone inhibit release induced by epinephrine, adenosinediphosphate or both together (Table 2). The results obtained with a combination of the two indicated that it failed to obviate inhibition by the NSAA. This was in striking contrast to the action of E-tetra combined with either one of the other inducers—adenosinediphosphate or epinephrine.

At a concentration close to maximal solubility, sulfinpyrazone inhibits least. It is of interest that each of the above NSAA inhibits approximately to the same degree. On the other hand, the non-anti-inflammatory agents, dipyridamole and PGE_1, markedly inhibit the action of epinephrine or adenosine diphosphate alone, but have considerably less effect on the combination (Table 2). PGE_1 inhibits epinephrine action essentially completely, and adenosinediphosphate by ~82 per cent (Table 2). PGE_1 is far more inhibitory than dipyridamole but less effective than the NSAA against the release triggered by the epinephrine–adenosinediphosphate combination. Thus, all the agents which inhibit epinephrine- or adenosinediphosphate-induced release also inhibit a combination of the two, except for dipyridamole, which was ineffective.

As already mentioned, E-tetra *per se* is a release inducer. The cardinal question, whether this property underlies the observed "protective" effect against NSAA and NAA inhibition, was examined in more detail. Acetylsalicylic acid completely inhibits both E-tetra- and epinephrine-induced release (Table 3). When PRP is preincubated with E-tetra, followed even 30 min later by the addition of aspirin, the fatty acid-induced 5-hydroxytryptamine release (~76 per cent) is reduced to ~20 per cent (Table 3, "prevention" column). Furthermore, when added 2.5 min before, aspirin also markedly inhibits the release induced by the epinephrine–E-tetra combination (Table 3, "reversal" column). When, however, PRP is preincubated with E-tetra for 30 min, inhibition by acetylsalicylic acid is greatly reduced (Table 3, prevention column). Thus, aspirin inhibition of epinephrine-induced release can be prevented but not reversed. These data and those in Table 1 further support our concept that E-tetra plays a unique role in protecting the release reaction.

At equimolar concentrations, sodium salicylate is a weaker anti-inflammatory agent than ASA [1]. Notwithstanding, it completely inhibits epinephrine-induced release whereas it is a weaker inhibitor of E-tetra (Table 3), in this respect comparable to its weakness as an anti-inflammatory agent. Also, at best it is negligibly weak in its inhibition of the E-tetra–epinephrine combination. When PRP is incubated with E-tetra for 30 min at room temperature, followed by sodium salicylate (5×10^{-3} M), and subsequently stirred at 37°, 5-hydroxytryptamine release is somewhat inhibited (Table 3). When epinephrine is added, however, release is perhaps less affected. Even though sodium salicylate alone completely inhibits epinephrine-induced release, it has little effect on E-tetra-induced release, extending our earlier report [12].

Indomethacin is one of the most powerful release inhibitors [1]. Our findings (against epinephrine) are in accord (Table 3). It was also the most potent inhibitor of E-tetra-induced release. Like aspirin, indomethacin inhibition of epinephrine-induced release cannot be reversed by prompt addition of both E-tetra and epinephrine (Table 3, "reversal" column). This drug almost totally inhibited the preincubated 30-min E-tetra-induced release, but when epinephrine was added 2.5 min after indomethacin to the E-tetra–PRP combination, the release was ~70 per cent. Thus, preincubation with E-tetra obviates inhibition by indomethacin, similar in this respect to its block of inhibition by aspirin.

Table 1. ASA inhibition of platelet [^{14}C]-5-HT release induced by various agents, singly or in combination*

A

Treatment	Epi	2 × Epi	Epi (30 min)	Epi (30 min) + Epi	Epi (30 min) + ADP	ADP	ADP (30 min) + ADP	ADP (30 min) + Epi	Epi + ADP	Epi + thr	ADP + thr	thr
No ASA	40 ± 6	39 ± 6	42 ± 5	40 ± 2	40 ± 2	30 ± 6	35 ± 12	28 ± 3	43 ± 7	47 ± 2	42 ± 7	40 ± 11
ASA	0	0	0	0	0	0	0	0	2.5 ± 2.5	8 ± 5	7 ± 1	3 ± 3

B

Treatment	E-tetra	E-tetra + Epi	E-tetra (30 min)	E-tetra (30 min) + Epi	E-tetra (30 min) + ADP	E-tetra (30 min) + thr
No Asa	35 ± 6	48 ± 7	31 ± 7.5	40 ± 7	37 ± 6	42 ± 4
ASA	2 ± 2	1 ± 3	8 ± 7	36 ± 3	32 ± 4	36 ± 6

* Conditions are as follows: PRP was stirred in an aggregometer at 37°. ASA (5×10^{-4} M), aggregating agent (epi, 5×10^{-6} M; ADP, 1×10^{-5} M; or thrombin, 0.4 I.U.), and, when indicated, E-tetra, were added (final concn 1×10^{-3} M). When agents were incubated at room temperature for 30 min, the sequence of addition to the PRP in the aggregometer was ASA, followed by the other agent. Number of observations: Epi, E-tetra and Epi–E-tetra experiments (50); other E-tetra combination experiments (10–15); all other observations were made on 6–10 samples of pooled PRP derived from five to ten donors. Values represent the mean per cent ± S.D. of labeled 5-HT that was released, using the labeled 5-HT uptake as 100 per cent.

Table 2. Action of several inhibitors of platelet 5-HT release induced by Epi, ADP and an Epi–ADP combination*

Inhibitors†	Per cent [^{14}C]-5-HT released (mean ± S. D.) Epi 5×10^{-6} M	Epi 5×10^{-6} M ADP 1×10^{-5} M	ADP 1×10^{-5} M
Control	40 ± 6	43 ± 7	30 ± 6
ASA (5×10^{-4} M)	0	2 ± 2	1 ± 1
Indomethacin (5×10^{-5} M)	0	0	0
Phenylbutazone (5×10^{-4} M)	0	0	1 ± 1
Sulfinpyrazone (1×10^{-3} M)	12 ± 9	16 ± 6	12 ± 10
Dipyridamole ($\times 10^{-3}$ M)	0.5 ± 0.7	35 ± 12	3 ± 3
PGE_1 (2.5 ± 10^{-7} M)	1 ± 1	23 ± 3	8 ± 3

* The inhibitor was incubated with PRP at room temp for 2.5 min prior to placing the plasma in the aggregometer. The PRP was then stirred at 37° and a specific aggregating agent was added. Number of observations are: Epi, 50; all others, 10 to 15.

† Figures in parentheses indicate final concentrations in PRP.

Phenylbutazone [1, 13] was also studied. As expected, it promptly and completely inhibited both epinephrine- and E-tetra-induced release (Table 3). This could be blocked, however, by the almost instantaneous addition of E-tetra, followed by epinephrine. Incubation of PRP with E-tetra alone resulted in ~76 per cent release. When, however, phenylbutazone was added to a plasma–E-tetra mixture, release was completely inhibited. No release occurred when epinephrine was added to a mixture comprising PRP, the drug and E-tetra. Thus, unlike aspirin and indomethacin, phenylbutazone inhibition can be circumvented by a combination of E-tetra and epinephrine (Table 3, reversal column) but cannot be prevented by prior

Table 3. Effect of inhibitors on Epi- and E-tetra-induced platelet 5-HT release*

Inhibitor	PRP + inhib. + Epi† (%)	Reversal of inhibition† PRP + inhib. + E-tetra (%)	Reversal of inhibition† PRP + inhib. + E-tetra + Epi (%)	Prevention of inhibition‡ PRP + E-tetra 30 min incub. + inhib. (%)	Prevention of inhibition‡ PRP + E-tetra 30 min incub. + inhib. + Epi (%)
Control	100	86 ± 15	120 ± 19	76 ± 19	100 ± 17
ASA (5×10^{-4} M)	0	5 ± 4	6 ± 8	20 ± 18	90 ± 6
Na salicylate (5×10^{-3} M)	0	69 ± 7	91 ± 6	66 ± 18	78 ± 16
Na salicylate (1×10^{-2} M)	0	61 ± 5	75 ± 8	—	—
Indomethacin (5×10^{-5} M)	0	4 ± 2	3 ± 3	2 ± 1	70 ± 26
Phenylbutazone (5×10^{-4} M)	0	8 ± 9	67 ± 27	3 ± 2	4 ± 5
Sulfinpyrazone (1×10^{-3} M)	30 ± 23	19 ± 4	61 ± 6	24 ± 10	32 ± 15
PGE_1 (2.5×10^{-7} M)	3 ± 2	4 ± 1	80 ± 6	3 ± 2	78 ± 18
Dipyridamole (1×10^{-3} M)	1 ± 2	5 ± 4	70 ± 12	5 ± 2	56 ± 13
Cyproheptadine (1×10^{-4} M)	0			90 ± 10	98 ± 19
Methysergide (1×10^{-4} M)	0	94 ± 10	100 ± 16	75 ± 16	83 ± 11

* Data are expressed as mean per cent of [^{14}C]-5-HT released ± S.D. Control data are mean of 50 observations on pooled PRP obtained from six–ten donors. For all other observations the data represent the mean of a minimum of 10–15 observations on pooled PRP.

† PRP stirring at 37° in aggregometer, followed by addition of inhibitor; the other agent was added 2.5 min later (chart paper movement).

‡ PRP + E-tetra, no stirring at room temp for 30 min, followed by stirring at 37° in aggregometer and addition of agent under study.

incubation with E-tetra (Table 3, prevention column). The explanation for this striking difference—phenylbutazone vis-à-vis aspirin and indomethacin—will be considered below.

Sulfinpyrazone, a structural analogue of phenylbutazone, also inhibits release [1, 14], although much more weakly, requiring about five times the concentration. If E-tetra replaces epinephrine, sulfinpyrazone inhibits by ~80 per cent (Table 3). When, however, it is added prior to the addition of an E-tetra–epinephrine combination, inhibition is approximately halved (Table 3, reversal column). It thus appears that sulfinpyrazone inhibition of the E-tetra–epinephrine combination is not strikingly different from sulfinpyrazone inhibition of the epinephrine–adenosinediphosphate combination (Table 2). When E-tetra is preincubated with PRP, followed by the drug, the release is markedly reduced (~24 per cent). This remains essentially unchanged when epinephrine is added. Thus, inhibition by sulfinpyrazone, like phenylbutazone, cannot be prevented by E-tetra, but can be reversed only if added within 2.5 min.

Another, and extremely potent, inhibitor is PGE_1 [15, 16]. When added 2.5 min prior to epinephrine, release was completely blocked (Table 3). PGE_1 also inhibits E-tetra-induced release. If added, however, 2.5 min prior to the E-tetra–Epi combination, inhibition is obviated. When PGE_1 was added to a 30-min-incubated plasma–E-tetra combination, and the mixture left undisturbed for 2.5 min, and then stirred, E-tetra-induced release was blocked. This effect on epinephrine- and E-tetra-inducible release can be circumvented by the almost simultaneous addition of the E-tetra–epinephrine combination. Substantial delay results in irreversibility. In contrast to the 50 per cent inhibition produced by PGE_1 on the Epi–ADP combination (Table 2), this effect points up the unique action of E-tetra.

Dipyridamole completely inhibits the epinephrine- or E-tetra-induced release (Table 3) [1, 17]. Inhibition by the drug was, however, markedly reduced by the almost simultaneous addition of epinephrine or E-tetra. Similar results were obtained in the prevention study. Here again, dipyridamole inhibited E-tetra-induced release (Table 3, prevention column). This was halved by the addition of epinephrine 2.5 min after the dipyridamole. Thus, although this drug effectively inhibits epinephrine- or E-tetra-induced release, it is considerably less effective when promptly challenged by the combination. In this respect the results resemble those obtained with a brace of other releasing agents, i.e. adenosinediphosphate and epinephrine (Table 2), as mentioned above.

Although cryoheptadine and methysergide inhibit epinephrine-induced release [18], they do not affect release triggered by E-tetra alone, or by E-tetra combined with epinephrine (Table 3).

Ethylenediaminotetraacetic acid completely inhibits epinephrine-, adenosinediphosphate- and thrombin-induced release, although in some instances [19, 20]

Table 4. Inhibitory action of EDTA on Epi- and E-tetra-induced [^{14}C]-5-HT release*

Agent	Control	EDTA (10 mM)
Epi (5×10^{-6} M)	100	2.0 ± 2
E-tetra	86 ± 15	2.5 ± 2
Epi + E-tetra	120 ± 19	44.0 ± 7

* PRP was incubated at 37°, stirred at 1000 rev./min for 30 sec prior to the addition of EDTA (final concn, 0.01 M). After 1 min, either Epi, E-tetra or both were added. The data represent the mean of six experiments on pooled PRP from five to ten donors ± the standard deviation.

first phase aggregation may occur. At 1×10^{-2} M the chelator completely blocks epinephrine- or E-tetra-triggered release, whereas inhibition of the E-tetra–epinephrine combination is considerably less (Table 4). These data suggest that the release occasioned by each, albeit requiring Ca^{2+}, occurs via otherwise different mechanisms. Inhibition was not greater when chelator concentration was doubled.

Another highly specific inhibitor is adenosine [16]. Because it prevents platelet clumping caused by several agents, it has been postulated that the corresponding nucleotide (adenosinediphosphate) is central to the mechanism of aggregation [19]. Adenosine completely inhibits both adenosinediphosphate- and E-tetra-induced release (Table 5). It also blocks release even when the E-tetra is preincubated with the plasma for 30 min. Adenosine inhibition of nucleotide-induced release was, however, prevented (~72 per cent) by preincubation of the plasma with E-tetra. These data, also indicate that the protective action of E-tetra against the platelet-toxic agents is not an artifact associated with its inherent release-inducing property, but is related to its conversion to platelet prostaglandins. This is in accord with recent work showing that E-tetra is not only the precursor of platelet PG's and other oxygenase products [21–27], but also protects against progressive irreversible destruction of tissue PG oxygenase by certain fatty acids and certain NSAA [28].

DISCUSSION

Platelet PGE_2 and $F_{2\alpha}$ are synthesized during clotting, and during platelet aggregation and release induced by thrombin, adenosinediphosphate, epinephrine or collagen [29–33]. The hypothesis that these changes are vital to certain platelet functions is supported by the following: ingested ASA inhibits release of platelet components, platelet PG elaboration [34], and the physiologic response to small vessel trauma—the bleeding time—is prolonged [35]. E-tetra, the "essential" fatty acid immediate platelet PG precursor, also induces both aggregation and release (see Fig. 1), as well as platelet PG synthesis [7]. In striking constrast, none of the other closely related fatty acids, including even the E-tetra *trans* isomers [5, 7], do this.

Some intermediates and end products formed during platelet PG synthesis are reported to be physiologically active [3, 21–23], and can trigger aggregation. Moreover, some are precursors of PGE_2 and $F_{2\alpha}$ [24–27] which are required for optimal release [21, 36]. Since aspirin and indomethacin act by blocking PG synthesis, and, as we [6] and others [34] have found, they block E-tetra-induced aggregation and release, it is reasonable to assume that E-tetra protects by virtue of its conversion to the platelet prostaglandins.

Whether the "specific" protective effect of E-tetra against the platelet-toxic agents is only apparent, attributable to its inherent release-inducing property, or truly related to its function as the essential source nutrient for PG synthesis, was a question requiring rigorous resolution. We believe this has been done, in favor of the latter, based upon the following: (a) Acetylsalicylic acid, as an example, inhibits release that is induced individually by epinephrine, adenosinediphosphate, thrombin, collagen and E-tetra. (b) Acetylsalicylic acid inhibits the release induced by the following combinations: epinephrine plus adenosinediphosphate; epinephrine plus thrombin; epinephrine plus collagen; adenosinediphosphate plus thrombin; and adenosinediphosphate plus collagen. In studies of the nucleotide plus collagen-induced release, Packham and Guccione [37] similarly found this true of aspirin, as well as of sulfinpyrazone. (c) But aspirin does not inhibit the following combinations: epinephrine plus E-tetra; adenosinediphosphate plus E-tetra; and thrombin plus E-tetra. We have found this also true of collagen plus E-tetra (data not shown).

Thus, in contrast to other release inducers, E-tetra is unique in its ability to block aspirin and other inhibitors when the fatty acid is co-present with other

Table 5. Effects of E-tetra on adenosine inhibition of adenosinediphosphate-induced platelet release reaction

Agent		5-HT release (% of [^{14}C]-5-HT taken up)	
		No adenosine	Adenosine†
Control		12 ± 2	11 ± 2
ADP	(1×10^{-5} M)	100‡	0 ±
E-tetra	(Not incubated)	120 ± 20	11 ± 6
E-tetra§	(30 min) (1×10^{-3} M)	92 ± 11	10 ± 7
E-tetra	(30 min) + (ADP)	95 ± 3	72 ± 12

* The data represent the mean of six experiments on pooled PRP from five to ten donors ± the standard deviation.

† Adenosine (final concn: 5×10^{-4} M) was preincubated for 5 min at room temperature before the addition of adenosinediphosphate or E-tetra (not incubated).

‡ The 5-hydroxytryptamine obtained with adenosinediphosphate is arbitrarily considered at 100 per cent.

§ E-tetra was incubated at room temperature for 30 min with PRP. Subsequently, either adenosine was added (when indicated), and the PRP was left at room temperature for an additional 5 min, or adenosinediphosphate was added to the PRP at 37° while stirring at 1000 rev./min, or both were added (as indicated), and the response was measured.

release inducers, whereas, when alone, its release-inducing property is blocked by aspirin as well as by other pharmacologically related and non-related inhibitors.

In yet another way E-tetra is uniquely different as a release inducer, *per se*. When PRP is combined at room temperature with epinephrine or adenosinediphosphate and kept non-stirred for > 30 min and then stirred at 37°, there is no release. On the other hand, with E-tetra under the same conditions, release ensues.

In striking contrast to its protective effects, E-tetra cannot reverse the action of aspirin and indomethacin. Under special conditions, but only within certain limits, however, it could reverse the inhibition by phenylbutazone, sulfinpyrazone and others. These data suggest that the mechanism of aspirin and indomethacin action differs from that of phenylbutazone and the others. Many investigators [3, 20, 38–40] have proposed that aspirin and indomethacin act on the first stage of the PG synthetase enzyme system, probably the dioxygenase step, by blocking elaboration of the cyclic endoperoxide intermediates. Moreover, the synthetase system (in sheep vesicular gland) can be protected *in vitro* from the action of aspirin and indomethacin by *o*-pehanthroline [40], a synthetic reversible inhibitor of the enzyme(s). Conceivably, an extraneous supplement of E-tetra may similarly increase the intermediates formed, thus protecting the labile dioxygenase against inhibition by aspirin or indomethacin. We have substantial evidence that labeled E-tetra added to PRP rapidly enters the platelet, and that the label becomes preponderantly concentrated in the granular portion [5].

Phenylbutazone, on the other hand, apparently acts on a later enzymatic step in PG synthesis, at least in the bovine seminal vesicle [3]. Indomethacin and aspirin inhibit four products produced from E-tetra by PG synthetase whereas phenylbutazone can only inhibit elaboration of two, namely PGE_2 and F_{2^α} [3]. Thus, phenylbutazone apparently blocks conversion of the formed cyclic endoperoxide intermediates to the PG's. Indeed, it has been shown [32–34] that when platelets are incubated with E-tetra, some intermediates are formed as well as PGE_2 and F_{2^α}. The reason why E-tetra can reverse, but not prevent, the action of phenylbutazone is, however, still obscure.

Further support of the unique nature of the action of E-tetra derives from the data obtained with the diverse inhibitory drugs that are not NSAA, namely PGE_1, dipyridamole, cyproheptadine, methysergide, and adenosine—all powerful inhibitors of epinephrine-induced release, and, except for cyproheptadine and methysergide, strong inhibitors of E-tetra-triggered release. In all cases E-tetra could "reverse," as well as prevent, inhibition. It must be strongly emphasized, however, that the sequential order of reagent addition, and the experimental conditions—i.e. interval of interaction as well as stirring—are extremely important in assessing the data. How the agents affect the PG synthetic machinery at the molecular level remains obscure.

E-tetra is apparently unique in yet another respect. Willis [21] and Silver *et al.* [7] have reported that E-tetra added to PRP induces a phenomenally large burst of platelet PG synthesis, in contrast to the relatively small increases associated with thrombin-, epinephrine- or adenosinediphosphate-induced release. Whether the protective effect of E-tetra combined with various release-inducing agents is related to a potential synthesis of PG intermediates as well as PGE_2 and PGF_{2^α}, will require determinations of PG formation concurrent with 5-hydroxytryptamine release. The action of E-tetra apparently involves its conversion to PGE_2 and F_{2^α} as well as to certain intermediates [24–26]. Our data are consonant with the concept that E-tetra conversion to platelet PG's is the common pathway eventuating in aggregation and release phenomena.

E-tetra induces aggregation and release *in vitro* [6, 25] as well as *in vivo* [41, 42]. This action has been considered to have pathological implications. This, however, is under challenge [43].

E-tetra, which binds to serum albumin [43], is also present in the platelet membrane glycerophosphatides [44, 45]. Deficiency of the fatty acid, its destruction, or platelet inability to utilize it, apparently compromises platelet function. This concept gains support from the work of Okuma *et al.* [46], correlating functional impairment during platelet storage with increased fatty acid peroxide formation. Furthermore, anti-oxidants which preclude peroxide formation should theoretically preserve platelet aggregative response [47], all the more so if oxidation of any of the four double bonds of E-tetra, which are critical, is minimized. This avenue is being explored in studies in our laboratory, aimed at extending platelet viability and function. It should be mentioned in this connection, however, that at relatively high concentrations, some anti-oxidants can prevent PG synthesis [39]. Be that as it may, this concept is under study.

In summary, E-tetra is uniquely capable of preventing the inhibitory effects *in vitro* of several NSAA and NAA on human platelet aggregation and 5-hydroxytryptamine release. Evidence is present to show that this property is not referable to its ability to induce aggregation and release, *per se*. Under special circumstances, E-tetra can also reverse the untoward effects of some of the platelet-toxic agents. The action of E-tetra is attributed to its role of being the essential precursor of platelet PGE_2 and PGF_{2^α}.

The data also suggest that the mechanism of action of indomethacin and acetylsalicylic acid differs from that of phenylbutazone and sulfinpyrazone. Moreover, the mechanism of inhibition by the NAA studied appears to differ from that of the non-steroidal anti-inflammatory agents.

The unique action of E-tetra indicates that this essential fatty acid plays a fundamental role in platelet release phenomena, and warrants further investigation as to its potential value in platelet processing and preservation.

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