

# Vitamin E

## AN INHIBITOR OF THE PLATELET RELEASE REACTION

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**ABSTRACT** Formation of lipid peroxides rises sharply when platelets undergo the release reaction. In this study the *in vitro* effect of vitamin E on platelet aggregation was investigated.  $\alpha$ -Tocopherol, an antioxidant of known inhibitory action on lipid peroxidation, was added to platelet suspensions in concentrations up to 1.5 mM. A dose-dependent reduction in platelet aggregation was observed, with complete inhibition of the secondary wave of aggregation at  $\geq 0.9$  mM  $\alpha$ -tocopherol. The inhibitory effect of  $\alpha$ -tocopherol on the platelet release reaction was further documented by the decrease in aggregation-induced release of [ $^{14}$ C]5-hydroxytryptamine from prelabeled platelets and by the reduction of *N*-acetylglucosaminidase activity released into the medium. The sharp rise in lipid peroxides normally associated with platelet aggregation was markedly reduced by  $\alpha$ -tocopherol and also by acetylsalicylic acid, a known inhibitor of the platelet release reaction.

*In vivo* studies examined the effect of oral vitamin E administration (1,200–2,400 IU daily) on plasma and platelet levels of  $\alpha$ -tocopherol. Up to 1,800 IU daily, increasing dosages of vitamin E resulted in increasing concentrations of  $\alpha$ -tocopherol in plasma and platelets, but intake of vitamin E in excess of this dosage failed to show any further increase in plasma or platelet levels.

### INTRODUCTION

Aggregation is one of the most important functions of the platelet, by which it exerts its hemostatic effectiveness under normal conditions, and which may be crucial for the development of thrombosis under pathologic circumstances. Whether platelet aggregation is a reversible event or not is primarily a function of the platelet release reaction (1).

A previous study from this laboratory indicated that the platelet release reaction is associated with a sudden

*Received for publication 2 September 1975 and in revised form 18 November 1975.*

increase in lipid peroxidation (2). Cause and mechanism of this phenomenon have recently been elucidated primarily by the studies of Hamberg et al. (3, 4), Smith et al. (5), and Willis et al. (6, 7). The rapid synthesis of prostaglandins during platelet aggregation (8) involves the transient formation of endoperoxide intermediates, extremely potent inducers of platelet aggregation. Whether these metabolites are causally involved in the platelet release reaction is not yet resolved. Support for such a hypothesis comes from the observation that acetylsalicylic acid, a potent inhibitor of the platelet release reaction (9) and of prostaglandin synthesis (10, 11), irreversibly inhibits cyclo-oxygenase (4, 12), the enzyme responsible for the conversion of arachidonic acid into endoperoxide prostaglandin  $G_2$ , and also blocks the thrombin-induced burst in oxygen uptake (13) apparently necessary for the formation of these oxygenated products.

The inhibitory effect of antioxidants on lipid peroxide formation is well known (14), and the efficacy of  $\alpha$ -tocopherol in reducing the rate of lipid peroxidation in stored platelet suspensions has been shown in a previous investigation (15). A growing body of evidence suggests that  $\alpha$ -tocopherol exerts its biological function by associating with and binding to membranes of cells and subcellular organelles (16), possibly through specific physicochemical interaction between its phytyl side chain and the fatty acyl chains of polyunsaturated phospholipid, particularly those derived from arachidonic acid (17). We considered it thus of great interest to determine whether  $\alpha$ -tocopherol can inhibit platelet aggregation, which is the subject of this report.

### METHODS

Platelets were isolated from acid-citrate-dextrose (ACD)<sup>1</sup> or 3.8% sodium citrate-anticoagulated blood of normal,

<sup>1</sup> *Abbreviations used in this paper:* ACD, acid citrate dextrose; 5-HT, 5-hydroxytryptamine; MA, malonaldehyde; *N*-A-Glu, *N*-acetyl-glucosaminidase; PRP, platelet-rich plasma, TBS, Tris-HCl buffer containing NaCl.

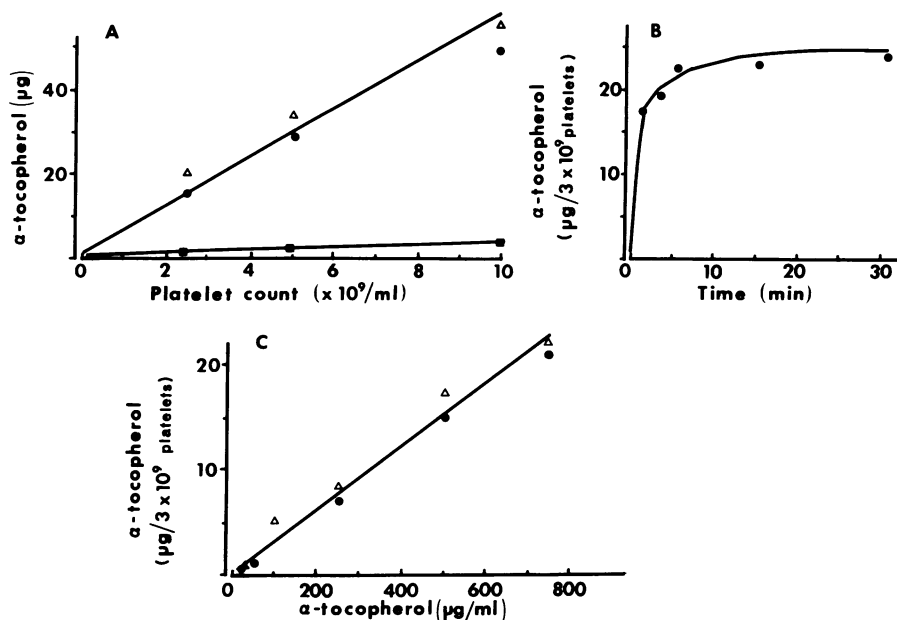


FIGURE 1 Uptake of  $\alpha$ -tocopherol by human platelets. (A). Effect of platelet concentration on platelet  $\alpha$ -tocopherol levels. Portions of platelet suspension of the desired cell concentration were incubated for 30 min at 37°C either with ethanolic  $\alpha$ -tocopherol solution at a concentration of 500  $\mu$ g/ml (full circles and open triangles) or with an equal volume of 95% ethanol (full squares). (B). Effect of incubation time on  $\alpha$ -tocopherol uptake by platelets. Platelet suspension containing  $2.5 \times 10^9$  cells/ml were incubated at 37°C with 500  $\mu$ g  $\alpha$ -tocopherol/ml. At the indicated times the reactions were stopped, the platelets were washed, and platelet  $\alpha$ -tocopherol levels were measured as described in Methods. (C). Effect of  $\alpha$ -tocopherol concentration in the medium on platelet  $\alpha$ -tocopherol levels. Platelet suspension of  $4 \times 10^9$  cells/ml were incubated at 37°C for 30 min with  $\alpha$ -tocopherol of the indicated concentrations. Controls lacking  $\alpha$ -tocopherol were treated identically. Incubations were terminated, platelets were washed, and  $\alpha$ -tocopherol was assayed as described in Methods. Full circles and open triangles represent the results with platelets from two different donors performed in duplicate.

healthy volunteers who had not taken any medication during the 96 h preceding the blood collection. Platelets were separated at room temperature as described previously (18). Red and white cell contamination was carefully removed by repeated brief centrifugations.

DL- $\alpha$ -Tocopherol or DL- $\alpha$ -tocopherol acetate was dissolved in 95% ethanol at a concentration  $\leq 400$ -fold in excess of that desired in the platelet suspensions. The  $\alpha$ -tocopherol preparation used was found to be  $\leq 95\%$  pure when assayed by thin layer chromatography (19).

For experiments designed to determine various parameters of  $\alpha$ -tocopherol uptake, platelets were suspended in autologous plasma at concentrations ranging from  $2.5$  to  $4 \times 10^9$  platelets/ml. The platelet suspensions were incubated with ethanolic  $\alpha$ -tocopherol or with an equal volume ( $\leq 5 \mu$ l/ml) of 95% ethanol at 37°C for varying periods of time. Incubations were terminated by addition of 10 vol 0.02 M Tris-HCl buffer, pH 6.8, containing 0.15 M NaCl (TBS), and immediate centrifugation. The platelets were washed once with the above buffer containing 15% ACD (TBS + ACD), and then resuspended in TBS + ACD, and  $\alpha$ -tocopherol was measured according to the method of Kayden et al. (19) with minor modifications, including a tripling of the volume of  $\text{FeCl}_3$  solution used in the color reaction of the extracted  $\alpha$ -tocopherol.

For measuring platelet aggregation, 0.4-ml portions of platelet-rich plasma (PRP) were magnetically stirred at

1,000 rpm in the heated (37°C) well of an aggregometer (Chrono-Log Corp., Broomall, Pa.). Changes in optical transmission were traced with a 10-mV recorder (20).

The platelet release reaction was monitored by the release of [<sup>14</sup>C]5-hydroxytryptamine (5-HT) from platelets prelabeled with this amine (21) and by the release of *N*-acetylglucosaminidase (EC 3.2.1.30) (*N*-A-Glu). Portions of PRP, 0.5 ml in volume, were incubated at 37°C with constant stirring (1,000 rpm) for up to 5 min. At 1-min intervals, the release reaction was stopped by rapid separation of the platelets at 7,000 *g* for 3 min at 4°C. The release of *N*-A-Glu activity and of [<sup>14</sup>C]5-HT was measured in the supernatant solution and expressed as a percentage of the activity present in nonaggregated platelets. *N*-A-Glu activity was determined essentially according to the method of Li (22). The enzyme assay was modified by changing the incubation time with substrate to 30 min at 30°C. Total activity of the enzyme was determined by treating nonaggregated platelets with Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.) (final concentration 0.1%).

Lipid peroxide formation was estimated by quantitative determination of malonaldehyde (MA) with thiobarbituric acid reagent (15). Platelets were suspended at a concentration of  $2 \times 10^9$  cells/ml in a medium consisting of 9 vol Tyrode's solution modified to contain 0.5 mM  $\text{Ca}^{2+}$ , 0.35 g/dl bovine serum albumin, and 1 vol of autologous plasma.

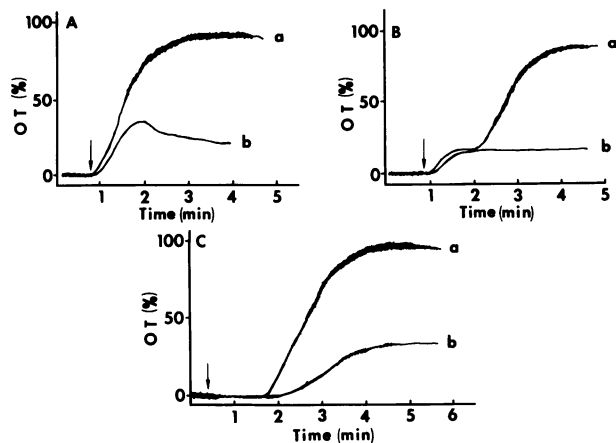


FIGURE 2 Inhibition by  $\alpha$ -tocopherol of platelet aggregation induced by  $2.5 \mu\text{M}$  ADP (A),  $2.5 \mu\text{M}$  epinephrine (B), and  $70 \mu\text{g}$  collagen (C). Aggregation is indicated by an increase in optical transmission (OT). To 0.4-ml portions of PRP were added under agitation (10 s)  $2.5 \mu\text{l}$  95% ethanol (A) or  $2.5 \mu\text{l}$  95% ethanol containing  $\alpha$ -tocopherol (final concentration  $0.9 \text{ mM}$ ) (B). These platelet suspensions were allowed to stand for 10 more min at room temperature before platelet aggregation was tested. The arrows indicate the time of addition of aggregating agents.

In vivo experiments were performed to determine the correlation between vitamin E intake and  $\alpha$ -tocopherol levels in plasma and platelets. Five healthy volunteers, three men and two women, age 23–48, were placed on 1,200–2,400 IU of vitamin E as DL- $\alpha$ -tocopherol acetate administered in form of chewable tablets (Roche Laboratories, Div. of Hoffmann La-Roche, Inc., Nutley, N. J.) in three divided doses with meals. All subjects continued on their usual diet, and base-line levels of  $\alpha$ -tocopherol were measured before vitamin E supplementation was begun. Each dosage level of vitamin E was continued for 1 wk before  $\alpha$ -tocopherol was assayed in plasma and platelets. None of the subjects experienced any untoward side effects while taking the vitamin.

## RESULTS

The uptake of  $\alpha$ -tocopherol by platelets was measured in relation to time of incubation, number of platelets, and concentration of the antioxidant (Fig. 1). The rate of uptake was fast, and equilibration of  $\alpha$ -tocopherol between platelets and medium was reached within 10–15 min. A linear relation between the concentration of  $\alpha$ -tocopherol in the medium and its level in platelets was observed up to  $750 \mu\text{g}$   $\alpha$ -tocopherol/ml. The  $\alpha$ -tocopherol content of control platelets was found to be  $0.82 \text{ nmol}/10^9$  cells (mean of five experiments).

Platelet aggregation induced by ADP, collagen (soluble rat skin collagen), and epinephrine in control and  $\alpha$ -tocopherol-treated platelet suspensions is shown in Fig. 2. At a concentration of  $\geq 0.9 \text{ mM}$ ,  $\alpha$ -tocopherol completely inhibited the second wave of aggregation, indicating that  $\alpha$ -tocopherol inhibited the platelet release reaction. To examine this effect in greater detail, plate-

TABLE I  
Effect of  $\alpha$ -Tocopherol Concentration on the Release of  $[^{14}\text{C}]5\text{-HT}$  from Platelets

Aggregating agent	DL- $\alpha$ -Tocopherol concentration	$[^{14}\text{C}]5\text{-HT}$ released
	mM	% of control
ADP, $5 \mu\text{M}$	0	$43.0 \pm 5.2$
	0.12	39.2
	0.24	35.4
	0.45	30.1
	0.9	19.3
	1.8	12.8
Collagen, $70 \mu\text{g}$	0	$80.2 \pm 10.1$
	0.12	65.0
	0.24	52.3
	0.45	42.1
	0.9	21.0
	1.8	15.7
Epinephrine, $2.5 \mu\text{M}$	0	$52.4 \pm 6.3$
	0.12	45.0
	0.24	40.2
	0.45	31.5
	0.9	20.6
	1.8	13.8

Platelet suspensions prelabeled with  $[^{14}\text{C}]5\text{-HT}$  were treated with  $\alpha$ -tocopherol as described in Methods, except for varying the concentration of the ethanolic  $\alpha$ -tocopherol solution. The results presented are the means of three or four experiments for  $\alpha$ -tocopherol-containing platelet suspensions, and the means of eight experiments  $\pm 1$  SE for controls not treated with  $\alpha$ -tocopherol.

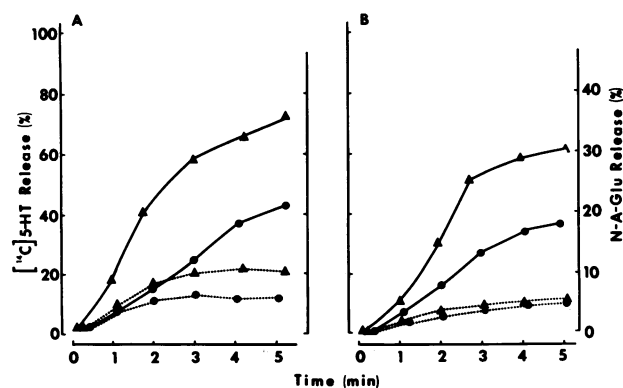


FIGURE 3 Inhibition by  $\alpha$ -tocopherol of the platelet release reaction (triangles,  $[^{14}\text{C}]5\text{-HT}$  release; circles,  $N\text{-A-Glu}$  release) induced by  $70 \mu\text{g}$  collagen (A) and  $2.5 \mu\text{M}$  epinephrine (B). Aliquots of PRP either prelabeled with  $[^{14}\text{C}]5\text{-HT}$  or not prelabeled were treated with ethanol or ethanolic  $\alpha$ -tocopherol (final concentration  $1.5 \text{ mM}$ ) as described in Methods. The respective aggregating agents were added at 0 min to control (solid lines) and  $\alpha$ -tocopherol-containing (interrupted lines) platelet suspensions. This experiment is representative of three performed.

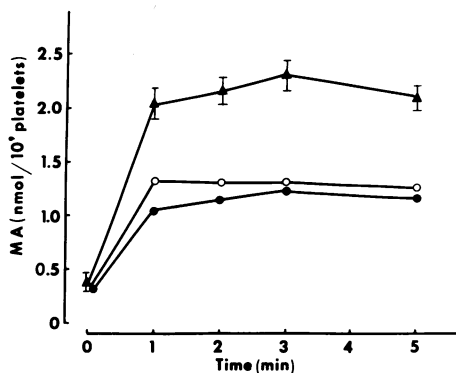


FIGURE 4 Formation of MA-yielding lipid peroxides in platelets aggregated by collagen. Control (triangles) and  $\alpha$ -tocopherol-containing (solid circles) platelet suspensions were prepared as described in Methods. Those containing acetylsalicylic acid (open circles) were prepared by incubation with 0.1 mM acetylsalicylic acid for 10 min at 22°C. Portions of the respective platelet suspensions were then incubated up to 5 min at 37°C with constant stirring (1,000 rpm). Aggregation was induced by addition of 70  $\mu$ g collagen at 0 min. The reactions were stopped by addition of thiobarbituric acid reagent and MA was then determined (5).  $\pm 1$  SE is indicated for controls.

lets were labeled with [ $^{14}$ C]5-HT. In addition, to assess the release from  $\alpha$ -granules, *N*-A-Glu activity was measured in the platelet suspension medium after aggregation. The temporal release profile of [ $^{14}$ C]5-HT and *N*-A-Glu from normal and  $\alpha$ -tocopherol-treated platelets exposed to collagen or epinephrine is shown in Fig. 3. The marked depression in the release of both [ $^{14}$ C]5-HT and *N*-A-Glu from platelets preincubated with  $\alpha$ -tocopherol is in agreement with the absence of a secondary wave of aggregation. It should be remarked that the epinephrine-induced release of *N*-A-Glu, a consistent finding in our experiments, was greater than expected from the currently held belief that  $\alpha$ -granule release is very limited with ADP and epinephrine (23). It should be remembered, however, that release from  $\alpha$ -granules is not an all-or-nothing phenomenon and that it is the strength of the initial stimulus that determines the degree of  $\alpha$ -granule release. A dose-dependent reduction of the release process could be demonstrated (Table I). Complete inhibition was obtained at  $\alpha$ -tocopherol levels of 0.9–1.8 mM, depending on the individual donor's platelets.

To determine whether  $\alpha$ -tocopherol exerted its inhibitory action on the platelet release reaction through its inhibition of the autocatalytic propagation of free radical formation, we measured the rate of lipid peroxide production in  $\alpha$ -tocopherol-treated platelets exposed to aggregating agents (Fig. 4). In normal, non- $\alpha$ -tocopherol-treated platelets there is a four- to fivefold increase in lipid peroxides when the release reaction is initiated by aggregating agents. Inhibition of the release

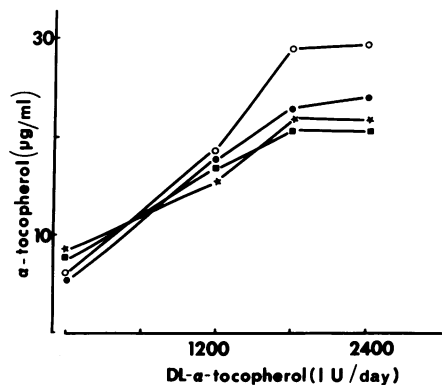


FIGURE 5 Plasma levels of  $\alpha$ -tocopherol in four subjects on oral vitamin E supplementation. Each dosage level of vitamin E (DL- $\alpha$ -tocopherol acetate) was administered for 1 wk before plasma levels were determined. Each assay was carried out in duplicate and the mean values were plotted.

reaction by acetylsalicylic acid (9) markedly reduced this sudden rise in lipid peroxide formation, an observation also reported by Stuart et al. (24). Preincubation of platelet suspensions with  $\alpha$ -tocopherol had a similar effect on the aggregation-associated lipid peroxidation.

The effect of vitamin E supplementation of the diet on the concentration of  $\alpha$ -tocopherol in plasma and platelets was determined in volunteers. The plasma levels of four subjects who took vitamin E in increasing dosage from 1,200 to 2,400 IU daily are shown in Fig. 5. Plasma  $\alpha$ -tocopherol increased linearly with the level of dietary vitamin E supplementation up to 1,800 IU daily. A higher dietary intake of this vitamin did not result in further elevation of its plasma level. A similar correlation was noted between dosage of dietary supplementation and  $\alpha$ -tocopherol content of platelets (Fig. 6).

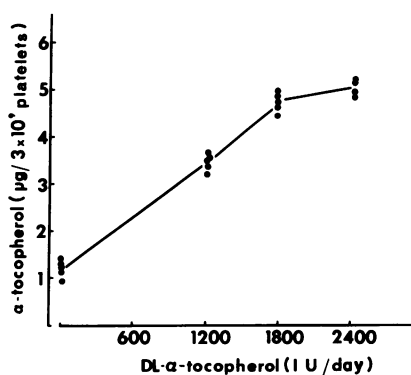


FIGURE 6  $\alpha$ -tocopherol content of platelets from five subjects on oral vitamin E supplementation. Each dosage level of vitamin E (DL- $\alpha$ -tocopherol acetate) was administered for 1 wk before platelet  $\alpha$ -tocopherol was measured. Each determination was carried out in duplicate and the mean values are plotted.

## DISCUSSION

These findings demonstrate that  $\alpha$ -tocopherol is an effective in vitro inhibitor of the platelet release reaction. Inhibition by  $\alpha$ -tocopherol, as acetate or nicotinate, of platelet aggregation induced by hydrogen peroxide has been reported (25). Also, in vitamin E-deficient rats, platelet aggregation induced by collagen was found to be increased compared to that of animals on a normal diet (26). Whether the effect of vitamin E on platelet aggregation is the result of its antioxidant potential or, more likely, of its association with membrane structures, possibly through physicochemical interaction with polyunsaturated fatty acyl chains (17), remains to be determined. It has recently been demonstrated that compared to plasma and red cells, platelets have a relatively high content of  $\alpha$ -tocopherol, only partially explained by their high concentration of polyunsaturated fatty acids (27). While the ratio of  $\alpha$ -tocopherol to unsaturated fatty acids is normally 1:200 in platelets, complete inhibition of the release reaction by  $\alpha$ -tocopherol was noted at a ratio of 1:20. Although only a small portion of the total peroxidized unsaturated fatty acids yields MA, particularly those containing three or more double bonds (28), it is the latter group of fatty acids which contains the precursors for prostaglandin synthesis.

The in vivo studies correlating dosage of vitamin E supplementation with  $\alpha$ -tocopherol levels of platelets and plasma showed definite saturation characteristics above 1,800 IU daily. Although these results do not indicate the reason for this saturation, it is clear that oral tocopherol administration has a definite dosage limit, above which no further increase in plasma or platelet levels of this vitamin occurs. Comparing the respective plasma and platelet levels demonstrates that in vivo platelets accumulate considerably more  $\alpha$ -tocopherol for a given plasma concentration than in vitro. The reason for this is not clear but differences in intracellular distribution may play a role. Finally, the in vivo studies show that although the concentrations of  $\alpha$ -tocopherol in platelets attainable by oral administration of vitamin E are not high enough to completely inhibit the platelet release reaction, they may be sufficient to cause a 40–50% reduction of collagen-induced release at an intake of 1,800 IU daily.

In conclusion, the results of this study not only point to the physiological importance of lipid peroxidation for irreversible platelet clumping, but also demonstrate that  $\alpha$ -tocopherol, a vitamin the role of which in man is still uncertain, has a definite inhibitory action on the platelet release reaction. While vitamin E supplementation of the diet alone cannot be expected to completely suppress platelet aggregability, the administration of this vitamin together with other anti-aggregating

agents may prove to be a possible combination worth further studies.

## ACKNOWLEDGMENTS

A generous supply of vitamin E tablets was provided by Dr. L. J. Machlin of Hoffmann La-Roche, Inc., Nutley, N. J.

This research was supported by Atomic Energy Commission contract AT(11-1)-3234 and by a grant from the Rhode Island Heart Association.

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