

The effect of vitamin E deficiency on some platelet membrane properties

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Abstract The effects of α -tocopherol (vitamin E) deficiency on membrane properties of platelets were studied to determine if vitamin E has a measureable stabilizing role in biological membranes. Three groups of rats and three of mice were studied: two groups consisted of Fisher strain rats and one of Sprague-Dawley rats fed a Draper corn oil diet with and without high levels of supplementary vitamin E. The mice were two groups of BALB/c animals maintained on an 8% hydrogenated coconut oil diet, and one group of CBA/J mice on an 8% lard diet, in each case either deficient in or supplemented with vitamin E. The relative content of fatty acids obtained from both rat platelets and erythrocytes was unchanged by vitamin E deficiency. Depletion of vitamin E had no effect on the degree of fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene-labeled rat platelets. No changes in hematocrit values were seen in any of the studies. The platelet count of only the vitamin E-deficient Sprague-Dawley rats was elevated with respect to vitamin E-supplemented counterparts; the others remained constant. Platelet reactivities, as measured by ADP- and thrombin-induced platelet aggregation and by the thrombin-induced changes in platelet transmembrane potential, were unaffected by vitamin E deficiency in all three groups of rats. Our results indicate that a membrane stabilizing effect of vitamin E on rat platelet or erythrocyte membrane fatty acids or on platelet response to external stimuli could not be demonstrated, nor was elevation in platelet count a general phenomenon associated with vitamin E deficiency.—Whitin, J. C., R. K. Gordon, L. M. Corwin, and E. R. Simons. The effect of vitamin E deficiency on some platelet membrane properties. *J. Lipid Res.* 1982. 23: 276–282.

Supplementary key words membrane fluidity • platelet fatty acids • erythrocyte fatty acids • fluorescence polarization

Although the specific biochemical function of vitamin E (α -tocopherol) has not yet been elucidated, deficiency of vitamin E in animals leads to readily recognizable symptoms of cell membrane damage, such as cystic fibrosis and muscle degeneration, necrosis of the liver and of the testes, and erythrocyte hemolysis (1). Therefore, while it is agreed that the absence of vitamin E has a membrane destabilizing effect, there are at least two hypotheses for its mechanism: the biological antioxidant properties of vitamin E, since similar membrane stabi-

lization can be achieved with some synthetic antioxidants which are structurally unrelated to vitamin E (1) or the ability of vitamin E to intercalate in the membrane, its conformation being complementary to that of the polyunsaturated fatty acids in the membrane phospholipids (2). There may be a synergistic relationship between vitamin E and selenium; glutathione peroxidase, which requires selenium, eliminates lipid hydroperoxides and other peroxides, while vitamin E may prevent the formation of these lipid hydroperoxides (3). Deficiency in either selenium or vitamin E could thus be compensated by the presence of the other, but deficiency in both could be and is life threatening.

The possible involvement of vitamin E deficiency in thrombotic episodes was evoked by some investigators while others have shown that platelet suspensions supplemented with vitamin E showed decreased aggregability (4). Studies of prolonged vitamin E deficiencies in the rat have indeed demonstrated a variety of hematological effects, i.e., elevated platelet and reticulocyte counts, normocytic anemia, increased in vitro platelet aggregation, and peroxide-induced erythrocyte hemolysis (5, 6). If the deficiency is expressed in the blood cells as reported by Machlin and co-workers (6), one would expect differences not only in platelet number and aggregability, but also in the fatty acid content of their membrane lipids as well as in parameters that are sensitive to subtle environmental changes such as membrane permeability to cations, in receptor response to aggregation stimuli, and in overall membrane fluidity. We have therefore performed, and report here, a series of such studies on two different strains of rats, one inbred (Fisher), the other not (Sprague-Dawley), as well as on two strains of mice. The parameters evaluated for rats were blood erythrocyte and platelet fatty acid content, erythrocyte and platelet counts, platelet membrane potential, platelet membrane fluidity, and platelet aggregability, while only platelet counts were monitored for the

Abbreviation: DPH, 1,6-diphenyl-1,3,5-hexatriene.

mice whose blood quantity is too low to permit the other tests to be performed without using a large number of animals.

MATERIALS AND METHODS

Diets

Weanling male Fisher or Sprague-Dawley rats (Charles River Breeding Laboratories) were fed modified Draper diets (8 or 10% corn oil) with or without 250 U/kg of supplemental D, α -tocopheryl acetate (7). Simultaneous selenium and vitamin E deficiency effects were evaluated on weanling male Fisher rats fed a Torula yeast diet (5% lard) with or without 240 U/kg of supplemental D, α -tocopheryl acetate (8).

Blood

Platelet counts on whole blood (obtained from severed rat or mouse tails) were determined visually under phase contrast microscopy using Becton-Dickinson platelet dilution Unipettes (9). Hematocrits were determined in heparinized capillary tubes after centrifugation at 6300 *g* for 5 min in a Clay-Adams Readacrit Centrifuge.

Larger volumes of blood were obtained by cardiac puncture immediately after N₂-induced narcosis. The onset of vitamin E deficiency was evaluated via the erythrocyte hemolysis test (10). Platelets were chromatographically washed in modified Tyrodes' buffer as previously described (11).

Fatty acid determination

One-ml aliquots of cell suspensions (10⁷–10⁸ cells) were subjected to alcoholic digestion (12). Methyl esters of the extracted fatty acids were formed using BCl₃ in methanol according to the procedure of Metcalf, Schmitz, and Pelka (13). Fatty acid determinations were performed on a Bendix Gas Chromatograph equipped with a 6-foot Chromasorb 80/100 W AW, 10% DEGS-PS column (Supelco).

Plasma tocopherol levels

Fluorescence determinations of plasma vitamin E levels (λ_{ex} = 302 nm, λ_{em} = 330 nm) were performed on a Perkin-Elmer MPF-2A according to the method of Duggan et al. (14).

Determination of fluorescence polarization (P)

Fluorescence polarization measurements were made on intact platelets suspended and stirred in albumin-free Tyrodes' buffer. The technique, using 1, 6-diphenyl-

1,3,5-hexatriene (DPH) as a probe, has been previously described (15–18). The polarization, *P*, was calculated from the fluorescence intensities measured when the emission and excitation polarizers are parallel (*I_{vv}*) and perpendicular (*I_{vh}*) respectively:

$$P = \frac{(I_{vv} - I_{vh})}{(I_{vv} + I_{vh})}$$

Aggregometry

Platelet aggregation was measured according to the method of Michal and Born (19) as previously reported (11, 20).

Transmembrane potentials

The fluorescence of the cyanine dye diSC₃-(5) (3,3'-dipropylthiodicarbocyanine), a probe of platelet transmembrane potentials, was monitored in a Perkin-Elmer MPF-2A as previously described (11, 20). We are very grateful to Dr. Allan Waggoner for the dye.

Statistical analysis

Data expressed in Tables 1–3 are given as mean \pm standard deviation. Differences between values are considered as significant when *P* \leq 0.05.

RESULTS

There were no discernible differences in weight and general appearance between the vitamin E-deficient and vitamin E-supplemented animals throughout the course of all the studies, i.e., for 12 to 52 weeks with rats or mice. Vitamin E deficiency was detected via the peroxide hemolysis test for erythrocytes within 2 weeks after the animals began eating the diets. The animals receiving the vitamin E-deficient diet in all study groups exhibited 100% erythrocyte hemolysis in the peroxide hemolysis test after 2 weeks on the diet. Controls (erythrocytes from vitamin E-supplemented animals) exhibited approximately 25% hemolysis under identical conditions, even though normal plasma vitamin E levels (16–20 μ g/ml) were maintained.

This relatively high degree of hemolysis in control erythrocytes may be attributable to the high polyunsaturated fatty acid content of the diets which was designed to exacerbate any vitamin E deficiencies; in support of such an explanation, we have found that erythrocytes from mice fed diets with high saturated fatty acid content do not hemolyze with peroxide even if severe vitamin E deficiency exists (data not shown). After 6 weeks on the corn oil diets, a consistent difference in the rat plasma

TABLE 1. Erythrocyte, platelet, and tocopherol values

Animal ^a		Weeks on Diet	Hematocrit ^b	Platelet Count ^b	Plasma Tocopherol ^b
			%	$\times 10^5 \mu\text{L}^{-1}$	$\mu\text{g} \mu\text{L}^{-1}$
Fisher rat	+Vitamin E	12	50.1 \pm 2.8 (6)	8.64 \pm .68 (4)	15.5 \pm 4.8 (5)
	-Vitamin E		50.9 \pm 2.6 (6)	9.04 \pm .25 (6)	0.8 \pm 0.4 (5)
Sprague-Dawley rat	+Vitamin E	18	48.6 \pm 3.4 (7)	9.6 \pm 1.1 (7) ^c	21.7 \pm 2.9 (3)
	-Vitamin E		45.5 \pm 2.3 (7)	17.6 \pm 2.0 (7) ^c	1.4 \pm 0.4 (4)
CBA/J mouse	+Vitamin E	26	43.6 \pm 1.5 (3)	14.8 \pm 0.3 (2)	
	-Vitamin E		45.3 \pm 2.5 (3)	15.6 \pm 0.3 (2)	
BALB/c mouse	+Vitamin E	52	42.5 \pm 0.7 (2)	12.2 \pm 1.7 (4)	
	-Vitamin E		49.3 \pm 3.9 (2)	13.6 \pm 2.3 (4)	

^a Rat diets contained 8–10% corn oil; mouse diets, 8% saturated fats (lard or hydrogenated corn oil).

^b Data are expressed as mean \pm standard deviation with the number of animals studied in parentheses.

^c A significant difference in Sprague-Dawley platelet counts appeared after 6 weeks as evaluated using the Student's *t*-test, where *P* \leq 0.05 is significant. The values shown are for the final experiment at 18 weeks.

vitamin E levels was attained, approximately 20 $\mu\text{g}/\text{ml}$ and 1 $\mu\text{g}/\text{ml}$ in the vitamin E-supplemented and vitamin E-deficient animals, respectively. There were no further changes in these plasma vitamin E levels. Rat hematocrits were determined at 2-week intervals, those of mice less frequently. In no case were any significant differences between the hematocrits of vitamin E-deficient and of vitamin E-supplemented animals observed. Varying the degree of unsaturation of fat, from 8% corn oil to 8% lard and 8% hydrogenated coconut oil, did not alter these observations. The same independence of diet held for the platelet count of the animals, the sole exception being the Sprague-Dawley rats. In the latter, a consistently higher count (Table 1) increasing with time on diet, was detected in the vitamin E-deficient rats. This finding did not hold true in Fisher rats or in two species of mice, and is therefore not a general phenomenon associated with vitamin E deficiency.

The relative fatty acid compositions of both platelets and erythrocytes in the three rat studies were also determined (Tables 2 and 3). The membrane content of the various fatty acids was not significantly different in the vitamin E-deficient and vitamin E-supplemented animals in any of the studies, indicating that vitamin E deficiency does not affect the relative unsaturated fatty acid content of these blood cells.

The response of vitamin E-deficient and vitamin E-supplemented platelets to aggregating stimuli was unperturbed in platelet-rich plasma (PRP) or in washed platelets. They aggregated upon addition of ADP or of thrombin (Figs. 1 and 2) at the same rate and to the same extent for both the vitamin E-deficient and vitamin E-supplemented animals. The change in washed platelet transmembrane potential in response to a range of α -thrombin concentrations occurred at the same rate and to the same extent in both vitamin E-deficient and vi-

TABLE 2. Relative platelet fatty acid content^a

	Fisher (8% Corn Oil Diet) ^a		Sprague-Dawley (10% Corn Oil) ^a		Fisher (Selenium Deficient) ^a	
	+Vit. E	-Vit. E	+Vit. E	-Vit. E	+Vit. E	-Vit. E
12:0	0.5 \pm 0.1	1.0 \pm 0.1	n.d. ^b	n.d.	n.d.	n.d.
14:0	1.3 \pm 0.6	1.6 \pm 0.6	1.9 \pm 0.6	2.9 \pm 1.1	2.5 \pm 0	1.8 \pm 0.7
14:1	0.8 \pm 0.1	2.1 \pm 1.0	n.d.	n.d.	n.d.	3.9 \pm 0.6
16:0	19.2 \pm 2.3	18.5 \pm 2.3	25.4 \pm 2.8	28.5 \pm 0.8	26.1 \pm 5.7	29.1 \pm 1.3
16:1	3.7 \pm 1.0	2.7 \pm 0.8	3.7 \pm 0.4	3.8 \pm 0.3	4.3 \pm 0.3	8.3 \pm 0.8
16:2	1.2 \pm 0.7	3.1 \pm 1.7				
18:0	6.8 \pm 0.8	6.8 \pm 1.0	16.9 \pm 2.0	16.8 \pm 0.6	16.6 \pm 3.1	17.7 \pm 3.1
18:1	21.4 \pm 1.7	19.8 \pm 1.8	15.2 \pm 3.8	15.8 \pm 3.3	26.1 \pm 5.2	24.5 \pm 1.1
18:2	34.7 \pm 2.8	31.9 \pm 3.0	14.7 \pm 2.4	13.5 \pm 2.4	14.0 \pm 5.7	8.9 \pm 0.7
20:4	10.1 \pm 2.1	10.4 \pm 1.7	19.5 \pm 2.5	18.0 \pm 2.3	10.4 \pm 1.8	12.9 \pm 4.2

^a Expressed as the mean percentile \pm standard deviation where *n* = 2. The values shown are for the final experiment in each study. There were no significant differences to *P* = 0.05 as evaluated using the Student's *t*-test.

^b n.d., non-detectable.

TABLE 3. Relative erythrocyte fatty acid content^a

	Fisher (8% Corn Oil Diet) ^a		Sprague-Dawley (10% Corn Oil) ^a		Fisher (Selenium Deficient) ^a	
	+Vit. E	-Vit. E	+Vit. E	-Vit. E	+Vit. E	-Vit. E
12:0	0.9 ± 0.3	1.5 ± 1.4	n.d. ^b	n.d.	n.d.	n.d.
14:0	1.4 ± 0.9	2.2 ± 0.9	1.0 ± 0.4	0.7 ± 0.1	0.9 ± 0.1	0.7 ± 0.1
14:1	2.1 ± 0.4	2.2 ± 0.4	1.8 ± 0.4	1.9 ± 0.3	2.2 ± 0.4	1.9 ± 0.3
16:0	26.0 ± 1.1	34.0 ± 5.2	27.8 ± 1.1	27.1 ± 0.7	31.1 ± 1.3	27.1 ± 0.7
16:1	2.3 ± 1.0	2.7 ± 0.9	2.3 ± 0.7	1.9 ± 0.3	3.6 ± 0.6	1.9 ± 0.3
16:2	1.9 ± 0.7	1.5 ± 0.6	n.d.	1.8 ± 0.6	n.d.	1.9 ± 0.6
18:0	12.6 ± 2.1	11.7 ± 2.7	16.4 ± 1.3	17.6 ± 1.1	17.2 ± 2.3	17.6 ± 1.1
18:1	10.8 ± 1.6	12.1 ± 1.8	11.4 ± 1.1	11.8 ± 1.1	14.4 ± 1.8	11.8 ± 1.1
18:2	10.3 ± 3.0	9.3 ± 1.3	11.4 ± 0.7	10.2 ± 1.0	7.5 ± 1.3	7.5 ± 0.3
20:4	28.2 ± 2.7	22.1 ± 6.4	27.4 ± 2.1	28.1 ± 1.1	27.2 ± 0.7	28.1 ± 1.1

^a Expressed as the mean percentile ± standard deviation where n = 2. The values shown are for the final experiment in each study. There were no significant differences to $P = 0.05$ as evaluated using the Student's *t*-test.

^b n.d., non-detectable.

tamin E-supplemented rats in all three studies (Fig. 3); none were performed on mice. A thrombin dose-dependent increase in the depolarization of the platelet transmembrane potential ensued.

The temperature dependence of the polarization of 1,6-diphenyl-1,3,5-hexatriene-labeled intact platelets from rats was calculated at various stages of the extended studies, always pairing animals of equal age and time on the diets. It should be noted that intact platelets were utilized for these studies since these are more indicative than are membrane preparations of the properties of whole platelets. The probe is highly lipophilic and may

be reporting on changes in the canalicular system and, to a limited extent (since it is not soluble in the cytoplasm), in organellar membranes. The observed polarization is hence an average value, but should be as representative an average for vitamin E-deficient as for vitamin E-replete platelets. Representative data are shown in Fig. 4, in which the polarization of DPH was identical in platelets from vitamin E-deficient and vitamin E-supplemented Sprague-Dawley rats fed the diets for 6 to 10 weeks. All four curves appear to show the same temperature dependence of *P*; their differences in *P* are not statistically significant in view of the experimental error, ±3%, in the measurement of fluorescence

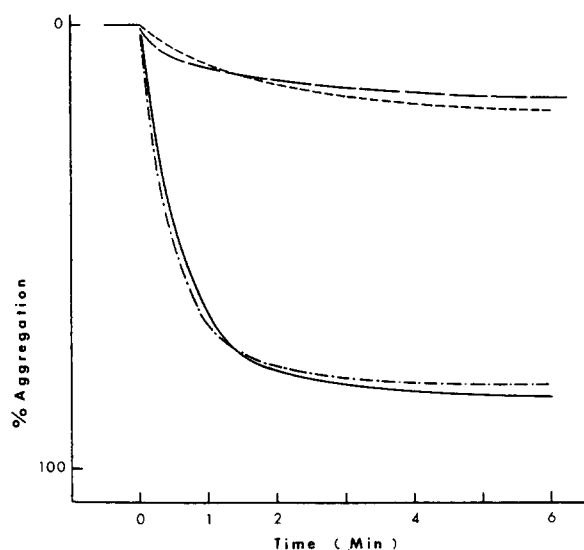


Fig. 1. Aggregation of platelet-rich plasma in response to ADP. Platelet aggregation from vitamin E-supplemented rats initiated by addition of ADP to a final concentration of 10^{-5} M, (—); 5×10^{-7} M, (----). Platelet aggregation from vitamin E-deficient rats initiated by addition of ADP to a final concentration of 10^{-5} M, (- · - · -); 5×10^{-7} M (---).

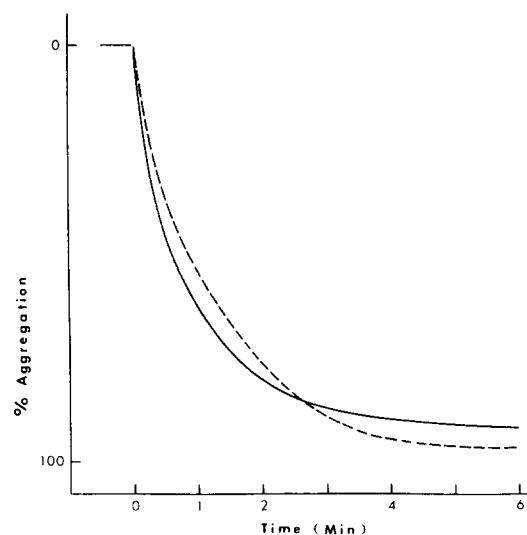


Fig. 2. Aggregation of washed platelets in response to α -thrombin. Platelet aggregation from vitamin E-supplemented rats initiated by addition of α -thrombin to a final concentration of 0.05 U/ml (maximum response), (—). Platelet aggregation from vitamin E-deficient rats treated in an identical manner, (---).

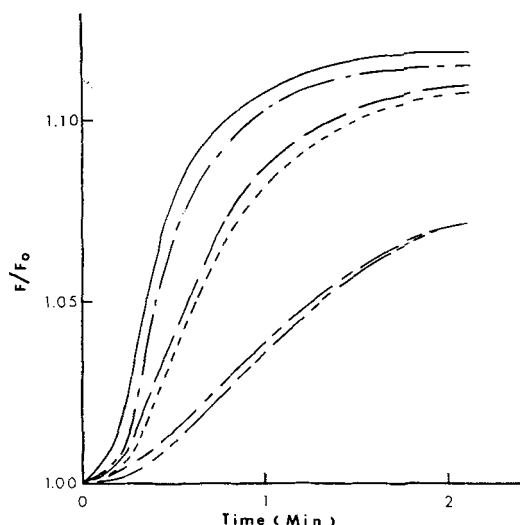


Fig. 3. Membrane potential changes of washed platelets upon α -thrombin stimulation. Platelets from: vitamin E-supplemented animals stimulated with 0.05 U/ml α -thrombin, (—); 0.02 U/ml α -thrombin, (---); 0.005 U/ml α -thrombin, (- - -); vitamin E-deficient animals stimulated with 0.05 U/ml α -thrombin, (- - -); 0.02 U/ml α -thrombin, (---); 0.005 U/ml α -thrombin, (—). F_0 = initial fluorescence; F = fluorescence at time t ; F/F_0 = relative fluorescence.

intensities. The reasons for the apparent change in slope at 36° are unknown.

Thus the increased number of platelets in a single strain, the Sprague-Dawley rat, was the sole difference attributable to nutritionally induced vitamin E deficiency in any of the parameters we have examined.

DISCUSSION

Vitamin E has long been implicated as a possible preventive of thrombotic events (21), with one plausible mechanism being its putative inhibition of platelet aggregation (22). The latter property is still in dispute, at least in humans (23). Another possibility has been that vitamin E deficiency is associated with increased thrombosis via enhancement of the platelet number.

With this in mind, Machlin and his colleagues (5, 24) have shown that nutritional vitamin E depletion leads to an increased platelet number and ensuing enhanced platelet aggregability in Sprague-Dawley rats. The implication in terms of prevention of cardiovascular disease and of possible beneficial effect of vitamin E on mammalian systems led us to investigate whether this is a general phenomenon.

It was of interest to know whether vitamin E deficiency led to an altered lipid content or protein environment in the cell's membrane. A change in the lipid composition of the platelet membrane or in the extent of hydropho-

bicity of its embedded proteins would be expected to change the polarization of a membrane-embedded probe(15-18). For example, it has been reported that altering the thermal properties of membranes through cholesterol enrichment or depletion (25) or insulin addition (17) affects the accessibility of membrane proteins. Different thermal membrane properties have also been demonstrated in synthetic liposomes containing varied amounts of polyunsaturated fatty acids (15). The latter are the specific membrane components that might be protected from damaging autoxidation by the antioxidant properties of vitamin E. One might have expected changes in lipid composition or in the polarization of DPH-labeled platelets, but no such changes were found. In fact, there were no differences in any of the measured parameters even in the doubly deficient (selenium and vitamin E) rats in experiments performed just before necrotic liver-induced death of these animals.

The results shown here demonstrate that enhanced platelet levels accompany vitamin E deficiency in only one of five species studies, i.e., Sprague-Dawley rats, and that this is therefore a species-specific rather than a general finding. Furthermore, it should be noted that there is an important difference between our results in Sprague-Dawley rats and the previously published reports using

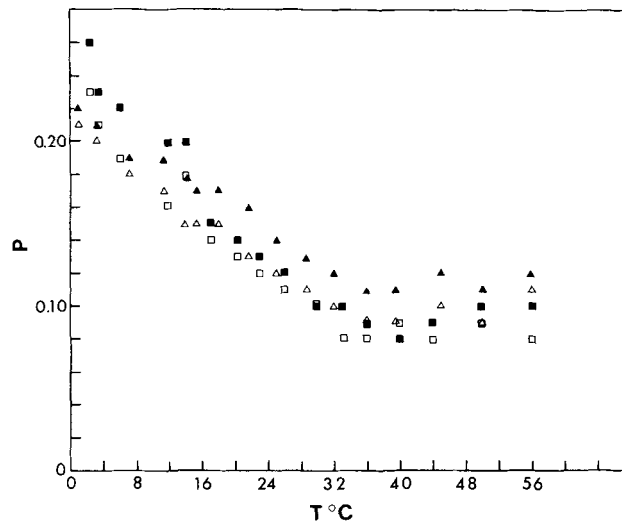


Fig. 4. Fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene-labeled washed platelets. Washed rat platelets (100,000/ μ l) were incubated in presence of 10^{-6} M 1,6-diphenyl-1,3,5-hexatriene for 60 min at 37°C in the dark. The temperature dependence of P was then determined where $P = \frac{(I_{vv} - I_{vh})}{(I_{vv} + I_{vh})}$ where I_{vv} and I_{vh} are the fluorescence intensities when the emission polarizer is oriented parallel and perpendicular, respectively, to the polarized excitation beam. $\lambda_{exc} = 360$ nm; $\lambda_{em} = 426$ nm with a 390 nm cutoff filter in the emission path. Sprague-Dawley rats at 6 weeks: ▲, vitamin E-supplemented; △, vitamin E-deficient. Sprague-Dawley rats at 10 weeks: ■, vitamin E-supplemented; □, vitamin E-deficient.

the strain (5, 6). Our experiments indicate that the platelet counts of vitamin E-supplemented animals are comparable to literature values for "normal" rats (26) and that those of the deficient animals are distinctly higher, while previous reports (5, 6) imply that the platelet counts of vitamin E-deficient and vitamin E-supplemented animals are normal and decreased, respectively, when compared to these same literature values. The reason for this discrepancy is not yet clear.

We had anticipated that if, as our data indicated, vitamin E deficiency has no effect on platelet number, it might nevertheless promote thrombosis by enhancing the platelet response to its stimuli (5, 6). We were unable to document increased aggregability in either platelet-rich plasma or washed platelets in any of our experiments. It should be noted that all of our investigations of platelet properties were performed at constant counts achieved by appropriate dilution (100,000 platelets μl^{-1} for aggregation studies and 55,000 μl^{-1} for membrane potential studies). Thus differences in the extent of aggregation or of membrane potential change attributable to a major difference in platelet concentrations were avoided.

Measuring changes in platelet transmembrane potential in response to stimuli is a sensitive technique for studying membrane receptor responses and membrane permeability, since changes in the environment of ion pumps and channels and stimulus receptors might be detectable as a perturbation of the extent and/or of the rate of change of the potential. Our results indicate that there is no change in the ability of platelets of vitamin E-deficient rats to respond to thrombin, and that the platelets from such animals therefore appear to be functionally identical in this regard to platelets from vitamin E-supplemented rats when these properties are tested on platelet samples at identical concentration.

The results reported here, therefore, show that in vivo induced vitamin E deficiency increases the number of circulating platelets in Sprague-Dawley rats. However, in spite of nutritional stress applied via high levels of corn oil, no effect of vitamin E-deprivation on membrane permeability to cations, membrane potential, or the fluorescence polarization of a membrane-embedded probe could be detected. It should be re-emphasized that this is a lack of effect of nutritional vitamin E-deficiency except for Sprague-Dawley rat platelet number. In contrast, comparison between normal platelets and platelets enriched by incubation with vitamin E in vitro, to levels up to 20 times higher than normal, shows inhibition of aggregation (4) and alteration of membrane fluidity (27). Observations of platelets enriched with vitamin E in vitro, but never previously depleted of vitamin E, may have a very different explanation (e.g., solubility in the mem-

brane at such high concentrations), but observations under such conditions are not relevant to our study. ■■

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