THE EFFECTS OF VITAMIN E IN PLATELET FUNCTION

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ABSTRACT

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By

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Two factorial experiments involving 14 young pigs were conducted to evaluate the effects of vitamin E on platelet function. The pigs were fed on 4 different diets: 1) basal ration (deficient in vitamin E and selenium), 2) basal ration plus intramuscular (I.M.) injections of vitamin E, 3) basal ration plus I.M. injections of selenium, and 4) basal ration plus I.M. injections of vitamin E and selenium.

Normal values for clot retraction (43-90%) and platelet factor three availability (24.4 $\pm$ 7.4 sec.) were established for the pig. Platelet ultrastructural changes following hydrogen peroxide aggregation indicated that aggregation was followed by destruction of the platelet. Platelet factor three availability and hydrogen peroxide aggregation were two measurable parameters of platelet function which were altered in pigs fed a basal ration. The pig appeared to be a suitable experimental animal for determining the effects of vitamin E on platelet function.

In addition, *in vivo* experimentation on human platelets indicated that vitamin E neither inhibited nor augmented platelet aggregation.

THE EFFECTS OF VITAMIN E IN PLATELET FUNCTION

By

Jerry Alan Holmberg

A THESIS

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DEDICATION

To my wife, Rebecca Laura Holmberg, for her love and encouragement during my graduate study

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INTRODUCTION

Platelets are a key component of hemostasis in that they plug the leaks in vessel walls and initiate the intrinsic clotting system (57). When the cellular layers of the vessel wall are ruptured and blood escapes through the interstitial spaces, hemostasis must arrest the bleeding before the animal hemorrhages to death (18). Inappropriate quantities of platelets or platelet dysfunction can lead to hemorrhage and death. Hypercoagulability and/or venous stasis, on the other hand, may result in the formation of a thrombus. Vascular occlusion caused by thrombosis can restrict blood flow in an area and lead to focal necrosis (53). Occlusion of a coronary artery can lead to myocardial infarction and death (32). Increased lipid peroxidation, as occurs in vitamin E deficiencies (23,45,55), can alter platelet function to an extent to cause these pathologic lesions (39-41,50,13,14,15).

Since the middle of the twentieth century, vitamin E has been suggested for the treatment of cardiovascular disorders (37,38,42,46). It has been thought to have anticoagulant properties (21,56). Hemostatic disorders have been reported in vitamin E deficient animals, indicating that vitamin E is necessary for the maintenance of hemostasis (10,30,52).

In order to study the effects of vitamin E deficiency on platelet function, an animal model was needed that resembled man. The pig was chosen on the basis of its physiological similarities to man, its platelet activity and the volume of specimen needed (8).

Since hemostasis is important in man and animal, research was undertaken to determine the role of vitamin E in platelet function, using the young pig as the experimental model.

LITERATURE REVIEW

The literature on platelet function is voluminous. This literature review pertains primarily to the effects of lipid peroxidation on platelet function. In addition, the role of vitamin E as an anticoagulant, some hemostatic disorders associated with vitamin E deficiency, and its possible role at the cellular level will be reviewed.

Lipid peroxidation of platelets

Lipid deterioration caused by peroxidation has occurred in the platelet (13-15,39-41,50). Aged platelets accumulate lipid peroxides and appear to lose their cellular defense mechanism against peroxidation (39). Lipid peroxides accumulate in stored platelets at 4C and this accumulation is even more marked at 20C. In the absence of vitamin E and its protective effect, this peroxidation may be enhanced.

Lipids make up an integral part of the platelet and influence its function and maintenance. The lipid content of platelets is 2.98 per cent (19). Seventy-six per cent of the total lipids are phospholipids, 20 per cent are neutral lipids, and four per cent are lipoproteins (27). Platelets are unique in that they are capable of *de novo* fatty acid synthesis, utilizing the malonyl CoA pathway to maintain the phospholipids and membrane lipids (20,26).

In the absence of the protective mechanism of vitamin E, the accumulation of lipid peroxides can alter platelet aggregation. Platelet aggregation is dependent on the free sulfhydryl (SH) groups in the platelet membrane (43). Tateishi *et al.* (50) believed that it is the oxidation of the free membrane thiol (SH) by lipid peroxidation that alters its aggregation. The availability of platelet factor three (PF3), a phospholipoprotein which is thought to be located on the membrane (57), is greatly decreased after lipid peroxidation (50). Its activity is not detectable in the circulating platelets but occurs after exposure to adenosine diphosphate (ADP) or aggregation. Platelet factor three is necessary for the initiation of the intrinsic clotting system (57). The decrease in activity after lipid peroxidation indicates either peroxidation of the membrane-bound phospholipoprotein or membrane change due to the oxidation of the free SH group.

Okuma *et al.* (41) have reported that neither ADP nor epinephrine causes increased lipid peroxidation. They concluded that aggregation as such does not stimulate lipid peroxidation of the platelet. However, they did report an increase in lipid peroxides in aggregation resulting from thrombin, glutathione (SH) inhibitors, polystyrene latex particles and platelet antibodies.

Circulating peroxides, on the other hand, appear to enhance platelet aggregation. Peroxidized arachidonic acid enhances platelet aggregation in contrast to unperoxidized arachidonic acid (31). Platelet aggregation stimulated by lipid peroxides might contribute to thromboembolic phenomena. Polymorphonuclear leukocytes produce

peroxides (1,2) and can be part of a thrombus or line the periphery of it (7). Hydrogen peroxide has recently been used as an aggregating agent (13-15,22). Higashi *et al.* (15) have suggested that peroxide caused permeability changes and/or membrane disruption which would release ADP and in turn cause platelet aggregation.

Vitamin E as an anticoagulant

Vitamin E has been thought to act as an anticoagulant and has been advocated for the treatment and prevention of thrombosis (38,42,46). Zierler *et al.* (56) reported that administration of alpha tocopherol phosphate altered the clotting mechanism. Kay *et al.* (21) suggested that alpha tocopherol phosphate may be a normal circulating antithrombin. It was later reported that alpha tocopherol phosphate had antithrombin properties but that alpha tocopherol acetate did not. Monkhouse (33), in 1963, stated that the antithrombic activity was not due to the alpha tocopherol but to the phosphate ion.

Role of vitamin E in hemorrhage

Hemorrhagic lesions and sudden deaths have been reported in vitamin E deficiencies (10,30,52). The most prominent lesion reported has been the hemorrhagic necrotic liver and occasionally hemorrhagic lymph nodes. Grant (10), in an extensive study, was able to produce dietetic microangiopathies (MAP) in swine. The diets were high in unsaturated fat and, therefore, oxidatively unstable fatty acids, an inadequacy of tocopherol (vitamin E) and selenium. In dietetic MAP the red, hemorrhagic lesions of the heart gave it the appearance

of a "mulberry." These MAP lesions were located in the capillaries and small muscular vessels of the myocardium. The lesions contained a periodic acid-Schiff (PAS) positive material. There were also extracardiac MAP lesions in the lung, kidney, skeletal muscles, colon, stomach, abdominal wall, liver, uterus and the adrenals. Grant has suggested that the vascular changes, the hemolysis and the thrombocytopenia of MAP in swine resemble the pathologic changes seen in thrombotic thrombocytopenic purpura (TTP) in man. A possible explanation for the hemorrhages and the vascular changes could be alterations in platelet function.

Vitamin E, a cellular function

The cellular function of vitamin E may include the stabilization of membranes, the breaking of free-radical chain reactions and participation in heme synthesis.

It has been suggested by Lucy (23) that membrane phospholipids might form a stable complex with vitamin E in order to control membrane permeability and stability. The cis double bond of the arachidonyl acid forms a pocket in which the fourth and eighth carbons (C_4 and C_8) of tocopherol fit. This structural complex of vitamin E in the biological membrane may act to inhibit oxidative destruction of polyunsaturated fatty acids (PUFA) in the cell and its membrane.

Peroxidation of lipids has been seen in association with damage to cellular structures, proteins, enzymes and metabolic pathways (47-49,51). Cellular membranes may be broken by lipid peroxidation permitting their contents to leak out. In addition to the

membrane of the cell, the organelles may be affected. Tappel (48), who has done extensive work with antioxidants, has mentioned that lysosomal membranes may be damaged. This would allow the digestive enzymes to leak into the cell's cytoplasm. The mitochondria and the microsomes may also be damaged. The mitochondria contain approximately 25 per cent unsaturated phospholipids and the microsomes 30-40 per cent. Vitamin E is the only known antioxidant in the mitochondria and the microsomes. Zalkin and Tappel (55) have stated that the membrane and the cytochromes are labile to lipid peroxidation. They concluded that it was this membrane change that resulted in the swelling of the mitochondria and altered metabolic activity. Schwartz (45) believed that vitamin E has a specific role in the mitochondria, that of preventing respiratory decline.

The biochemistry of vitamin E enables it to act as an antioxidant in preventing free radical lipid peroxidation (48,49). Tappel, an authority on vitamin E, explained that if lipid peroxidation were permitted to proceed unchecked, it would advance in a free-radical chain reaction (Figure 1). When lipid peroxidation is initiated, a lipid free radical (R·) is generated. This lipid free radical combines with molecular oxygen (O_2) to form a lipid peroxyl radical (ROO·). The lipid peroxyl radical is now capable of continuing the peroxidative cycle by removing a hydrogen from another lipid source (RH), generating a lipid peroxide (ROOH) and another lipid free radical (R·). The lipid antioxidant, vitamin E, functions to supply hydrogen to the lipid peroxyl radical (ROO·) and break the chain reaction. The oxidized form of alpha tocopherol, alpha

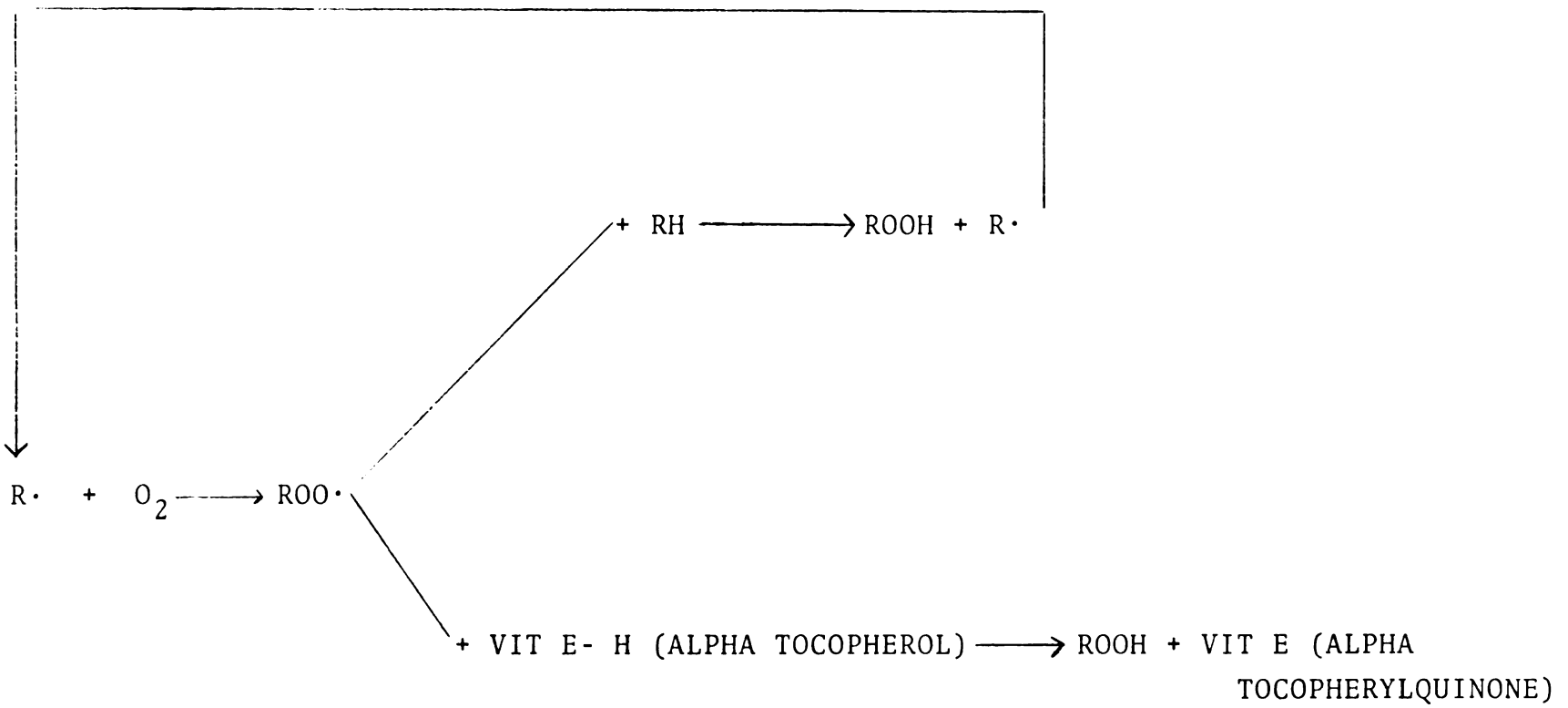

Figure 1. Lipid peroxidation, free-radical chain reaction.

tocopherylquinone, is the main product formed. It appears that one of the main functions of vitamin E is its reaction with free radical intermediates of lipid peroxidation and peroxides.

In addition to its roles in membrane stabilization and control of lipid peroxidation, recent studies have indicated that vitamin E participates in heme synthesis (36,4). The first step in heme synthesis is the condensation of glycine and succinyl CoA within the mitochondria to form delta (δ)-aminolevulinic acid (ALA). Two molecules of δ-ALA are condensed to form porphobilinogen outside the mitochondria. Both of these steps are controlled by the enzymes ALA synthetase and ALA dehydratase. Four molecules of porphobilinogen then combine through a series of reactions to form protoporphyrin IX. Protoporphyrin IX incorporates iron within the mitochondria to form heme. It is thought that vitamin E regulates the ALA synthetase and dehydratase at an inducer-repressor genetic site (36). Vitamin E may regulate heme synthesis somewhere between the translation and transcription of the inducer messenger ribonucleic acid (mRNA) by affecting the activity of the repressor. In the rat, vitamin E deficiencies result in a decrease in ALA synthetase activity in the bone marrow and a decrease of ALA dehydratase in the liver (4). The bone marrow in vitamin E deficiencies is usually hyperplastic with many red cell precursors, the majority being multinucleated (30,34,35). In addition, the megakaryocytes contain ten to twenty nuclei and have normal granular cytoplasm. There are two possible explanations for the anemia: destruction of red cells and decreased synthesis of heme. The red cell membranes in vitamin E deficiency are increasingly

sensitive to hydrogen peroxide (17). Jacobs *et al.* (17) explained this by postulating that the oxidation of the sulfhydryl groups (SH) in the membrane accompanied the hemolysis by hydrogen peroxide.

There are other pathologic changes associated with vitamin E deficiency that appear to be related to excessive lipid peroxidation and membrane damage. These include a form of muscular dystrophy (36,25), cellular degeneration including liver necrosis, testicular degeneration, encephalomalacia, prematurity, fetal loss and malabsorption syndromes (29).

In summary, there appear to be two mechanisms by which peroxides affect platelet function: an external and an internal reaction. Tateishi *et al.* (50) indicated that accumulation of lipid peroxides within the platelet causes platelet dysfunction through membrane and organelle damage. External stimulation of platelets by lipid peroxides caused a hypercoagulative response (13-15,31,22). It appears that vitamin E is needed to stabilize the platelet membrane and protect it from lipid peroxidation.

OBJECTIVES

The general objective of this research was to investigate platelet function in vitamin E-selenium deficiency, using the pig as the experimental animal. The specific objectives were:

1. To determine if a basal ration that induces a vitamin E-selenium deficiency would cause platelet dysfunction.

2. To determine if a basal ration that induces a vitamin E-selenium deficiency would cause enhanced platelet function.

3. To determine if a basal ration that induces a vitamin E-selenium deficiency would impair platelet production.

MATERIALS AND METHODS

Animals

Hampshire and Yorkshire-cross pigs were used as the experimental animals. In the first experiment, experiment A, eight litter mates and one non-litter mate were obtained from the Michigan State University Swine Research Farms. In the second experiment, experiment B, eight pigs from different litters, but the same boar, were obtained from the Richard Chaney Farm of Mason, Michigan. All pigs had iron injections prior to the experimentation.

Housing

All animals were housed in individual metal metabolism cages in a heated room.

Ration

The pigs were fed the ration given in Table 1, twice daily. The basal ration used to induce a vitamin E-selenium deficiency was the same as that used previously by numerous workers and is described by Michel (30). The torula yeast was low in selenium and a protein source. The cod liver oil was used as the source of unsaturated fats to enhance the vitamin E-selenium deficiency. Water was available at all times in a separate bowl.

Table 1. Basal ration for experiments A and B

Torula yeast	32%
Cod liver oil	5%
Mineral mix*	6%
Vitamin mix**	2%
Cerelose	55%

*Mineral mix: percentage of the total mineral mixture

KCl	10.0
KI	0.002
$FeSO_4 \cdot H_2O$	0.4
$MgCO_3$	2.0
$NaHCO_3$	25.0
$CaHPO_4 \cdot 2H_2O$	36.0
$CaCO_3$	12.5
Cerelose	13.098

**Vitamin mix:

Thiamin	0.3 gm
Riboflavin	0.6 gm
Nicotinic acid	4.0 gm
Pantothenate	3.0 gm
Pyridoxine HCl	0.2 gm
PABA (para-aminobenzoic acid)	1.3 gm
Ascorbic acid	80.0 gm
Inositol	13.0 gm
Choline	130.0 gm
Folic acid	26.0 mg
d-biotin	5.0 mg
Cyanocobalamin	10.0 mg
Menadione Na bisulfite	6.5 mg

Animals maintained on the basal ration plus vitamin E were given supplements of vitamin E as dl-alpha-tocopherol acetate (Hoffman La Roche, Nutley, N.J.). In experiment A, 100 I.U. of vitamin E were injected I.M. weekly after blood specimens were collected. In experiment B, 200 I.U. of vitamin E were injected I.M. weekly.

Animals maintained on the basal ration plus selenium were given supplements of 0.25 mg of selenium weekly.

Animals fed the basal ration were not given supplements, and their ration was deficient in both vitamin E and selenium.

Experimental design

In experiment A, the eight pigs were divided into four groups (Table 2). In this experiment the platelet aggregation, platelet counts, platelet factor three availability, clot retraction, hematocrit, white blood counts, erythrocyte osmotic fragilities, lactic dehydrogenase (LDH) and serum glutamate-oxalacetate transminase (SGOT) were determined in blood samples at selected intervals.

Table 2. Experimental design and pig numbers

Ration	Experiment A	Experiment B
Basal	6,1	4,5,6,7,8
Basal plus vitamin E	8,4,9	3
Basal plus selenium	7,0	1
Basal plus vitamin E, selenium	3,5	2

In experiment B, eight pigs were divided into a similar set of four groups, in order to examine the hydrogen peroxide red cell hemolysis and the hydrogen peroxide aggregation (Table 2). In addition, all of the laboratory parameters monitored in experiment A were repeated in this experiment. Two of the eight pigs died from respiratory infections before the first bleeding. One pig was fed from the basal diet group and one fed basal diet plus vitamin E and selenium.

Blood specimens were collected from the anterior vena cava once a week for the period of the experimentation. The disodium salt of ethylenediaminetetraacetic acid (EDTA) was used as the anticoagulant in the hematologic studies (white cell counts, platelet counts, hematocrits and red cell fragilities). Sodium citrate was used as the anticoagulant in the platelet studies (aggregation, platelet factor three availability and platelet counts of the platelet rich plasma). Immediately after a specimen was drawn in a plastic syringe, 6% sodium citrate for pig platelets (12) and 3.8% sodium citrate for human platelets was added in a ratio of 1 part citrate to 9 parts blood.

Tissue specimens were obtained at necropsy from three animals that died and two that were sacrificed at the end of the experimentation and were examined by light microscopy.

Analyses

A. Platelet counts

Platelets were counted manually using a phase contrast microscope on both anticoagulated whole blood (EDTA) and platelet rich plasma (PRP). A unipet capillary (Becton-Dickinson, Rutherford, N.J.)

was filled with the specimen (EDTA or PRP). This was then diluted in the unipet reservoir containing 1% ammonium oxalate.

B. Clot retraction

The clot retraction of whole blood was determined using a modification of the method described by Didishiem and Bunting as described by Bowie *et al.* (3). Four milliliters of whole blood was allowed to clot at room temperature and was then incubated at 37C for one hour. After an hour the clot was removed and described morphologically. The serum and cells remaining in the tube were centrifuged. The volume of the sedimented red cells was then subtracted from the total volume to obtain a true serum volume. The clot retraction was calculated as follows:

$$\text{Clot retraction (\%)} - \frac{\text{serum volume/blood value x plasmacrit)}}{\text{plasmacrit = 100 - hematocrit}}$$

The normal value for man is 40-94% clot retraction. As the normal for pigs has not been established, the value for man was used.

C. Packed cell volume

Microhematocrits were determined by centrifugation. Capillary tubes were filled and sealed with clay. They were then centrifuged (International Equipment Co., Needham Heights, Mass.) for four minutes. The percentage of packed red cells to volume was recorded.

D. Platelet factor three availability

Platelet factor three availability was determined using a modification of the procedures of Hardisty and Ingram (11). Two-tenths milliliter of an equal mixture of platelet rich plasma (PRP)

and platelet poor plasma (PPP) was mixed with 0.2 ml of Kaolin (J. T. Baker, Phillipsburg, N.J.). This was then incubated for 20 minutes with an occasional mixing. At the end of 20 minutes, the specimen was recalcified with 0.2 ml of 0.035 M $CaCl_2$ and clot formation was timed in seconds.

E. Platelet aggregation

All labware used was plastic unless otherwise noted. The citrated specimen was centrifuged at 1360 rpm for 5 minutes to remove the red blood cells. The supernatant, citrated platelet rich plasma (PRP), was removed to another test tube until needed.

Platelet aggregation studies were monitored using a commercial aggregometer (Chrono-Log Corp., Broomal, Pa.) with a strip chart recorder. Five-tenths (0.5 ml) milliliter of PRP was equilibrated in the aggregometer at 37C for a minimum of one minute. Then 20 lambda (0.02 ml) of the test aggregating agent was added to 0.5 ml of PRP; the concentration of the aggregating agent was then expressed as the final concentration.

Three aggregating agents were used:

(1) A final concentration of 1×10^{-4} M adenosine 5' diphosphate (ADP) was used as the stock solution. This stock solution was prepared by dissolving 11.8 mg of ADP disodium salt from equine muscle (Sigma, St. Louis, Mo.) in 10 ml of imadazole buffered saline (28). Various concentrations (1×10^{-5} M and 1×10^{-6} M) were made by dilutions from the stock solution.

(2) Ristocetin (Abbott Laboratories, North Chicago, Ill.) was dissolved in saline to give a concentration of 104 mg/ml. This gave

a final concentration of 4 mg/ml. Various concentrations (1, 2 and 3 mg/ml) were made by dilutions of the stock solution.

(3) A 1% solution of hydrogen peroxide was prepared by dilution from 30% hydrogen peroxide (Analytical Reagent, Mallinckrodt Chemical Works, St. Louis, Mo.). This gave a final concentration of 0.4%.

F. Enzyme determination

Lactic dehydrogenase (LDH) assays utilizing the reverse reaction of Wroblewski and LaDue (54) were performed. LDH-P Stat Packs (Calbiochem, La Jolla, Cal.) were used. Nicotinamide-adenine dinucleotide (reduced NADH) was used to activate the substrate, pyruvate. The change in the absorbance at 340 nanometers (nm) was determined for one minute.

Serum glutamate-oxalacetate transaminase (SGOT) activities were determined using GOT Stat-Pack (Calbiochem, La Jolla, Cal.). The substrates employed were aspartic acid and alpha ketoglutarate, activated with NADH. The change in the absorbance at 340 nm was determined for 5 minutes.

Lactic dehydrogenase isoenzymes were separated on cellulose acetate plates by electrophoresis at 180 volts for 25 minutes in high resolution buffer, pH 8.6 (Helena Laboratories, Beaumont, Tex.). The cellulose acetate plates were then stained with Helena LDH reagent (nitrozolium blue) for 30 minutes at 37C. After color development, the plates were fixed in 5% acetic acid for 5 minutes and then optically scanned on the Densicord (Photovolt Corp., New York, N.Y.).

G. Red cell sensitivity

Red cell osmotic fragilities were determined in experiment A according to the method of Dacie as described by Lynch *et al.* (24). A series of graded strength buffered sodium chloride solutions was used which ranged in concentration from 0.1% to 0.8%. Five milliliters of each concentration of sodium chloride were mixed with 0.02 ml of anticoagulated (EDTA) blood. The tubes were incubated for 30 minutes and centrifuged at 2000 rpm for 10 minutes. The 0.8% sodium chloride was used as the blank. The remaining tubes were measured against the blank at 540 nm in a spectrophotometer (Bausch and Lomb Spectronic 20, Rochester, N.Y.).

The hydrogen peroxide hemolysis test used in experiment B utilized the modification of Rose and Gyorgy (44). A 5% suspension of washed red cells was made in 0.9% sodium chloride. Three tubes were prepared, each containing 0.25 ml of the 5% red cell suspension. To the first was added 0.25 ml of phosphate buffer. This served as the blank. To the second and third tubes were added 0.20 ml of phosphate buffer and 0.05 ml of 4 M hydrogen peroxide (Mallinckrodt, St. Louis, Mo.). All tubes were mixed, incubated for 15 minutes at 37C and then allowed to stand at room temperature for 2 hours and 45 minutes.

At the end of three hours, 5 ml of saline-phosphate buffer was added to the blank and to the second tubes. Five milliliters of distilled water was added to tube three. All tubes were centrifuged for 10 minutes at 2000 rpm. Absorbances were determined for both tubes 2 and 3. The per cent hemolysis due to hydrogen peroxide sensitivity was determined by the following formula:

$$\text{Per cent hemolysis} = \frac{\text{Absorbance saline-phosphate (tube 2)}}{\text{Absorbance water (tube 3)}} \times 100$$

H. White blood cell counts

In experiment A, white blood cell counts (WBC) were determined using an electronic particle counter (Coulter Counter, model B, Hialeah, Flor.). Coincidence corrections were made for all counts over 10,000 WBC/cu.mm.

In experiment B, WBC counts were determined utilizing a unipet reservoir (Becton-Dickinson, Rutherford, N.J.). A 1:100 dilution was obtained using EDTA blood. Four square millimeters of a hemacytometer with a chamber depth of 0.1 mm were counted and the WBC count was calculated as follows:

WBC = cells counted (100/4 sq.mm x 0.1 mm depth)

Vitamin E and its influence on platelets in man

One human volunteer was used throughout the experiment. During this time no other medication was taken. Platelet aggregations were begun on the PRP after a week during which the volunteer consumed alpha tocopherol acetate (100 I.U. for 6 days and 400 I.U. for 7 days). In addition to the *in vivo* experimentation, specimens were incubated *in vitro* with alpha tocopherol acetate (0.81 mg/ml).

RESULTS

Normal values

Normal values (Table 3) for platelet factor three availability, platelet counts, LDH and SGOT were established from values obtained from the positive control (vitamin E and selenium supplemented) pigs. In both experiments for all pigs, the mean clot retraction and range were established.

Table 3. Normal values

Procedure	X	± 2 S.D.*
Platelet factor 3 (8)	24.4 sec.	7.4
Clot retraction (37)	71.9%	19.7 Range 43-90%
Platelet counts (10)	624,050/cu.mm	258,974
LDH (8)	781 mU/ml	246
SGOT (9)	23 mU/ml	11

( ) indicates number of analyses

*standard deviation

A high concentration of ADP (1×10^{-4} M) was needed to sustain the primary wave of aggregation. Other concentrations were used (1×10^{-5} M and 1×10^{-6} M); however, a small initial wave was followed by dissasociation of the platelet aggregate. No secondary wave was observed in pig platelets.

Ristocetin at final concentrations of 1, 2, 3 and 4 mg/ml of PRP were used. A final concentration of 4 mg/ml was needed for pig platelets. Not only did this concentration cause platelet aggregation but it also caused the conversion of fibrinogen to fibrin. Upon the addition of this concentration, there was a drop in light transmission from the ristocetin itself. This was followed by a rise in light transmission as a result of aggregation. Pig platelets produced a one wave tracing with this concentration of ristocetin.

During experiment A, various concentrations (0.1% and 1%) of hydrogen peroxide were added to PRP and platelet changes observed. The 0.1% hydrogen peroxide (final concentration 0.004%) did not cause visible platelet aggregation. When 1% (final concentration 0.4%) hydrogen peroxide was added to equilibrated PRP in the aggregometer, there was a slight rise in the light transmission. This was followed by a sharp drop in light transmission, then an equally sharp rise in light transmission with much bubble formation. Transmission electron microscopy (TEM) was used to study the changes in platelets. These specimens were from pig 9 of experiment A (vitamin E supplemented), and are illustrated (Figures 2 and 3). It was observed that after the addition of 1% hydrogen peroxide, the light transmission increased 3%, plateaued, then increased another 9%. Transmission

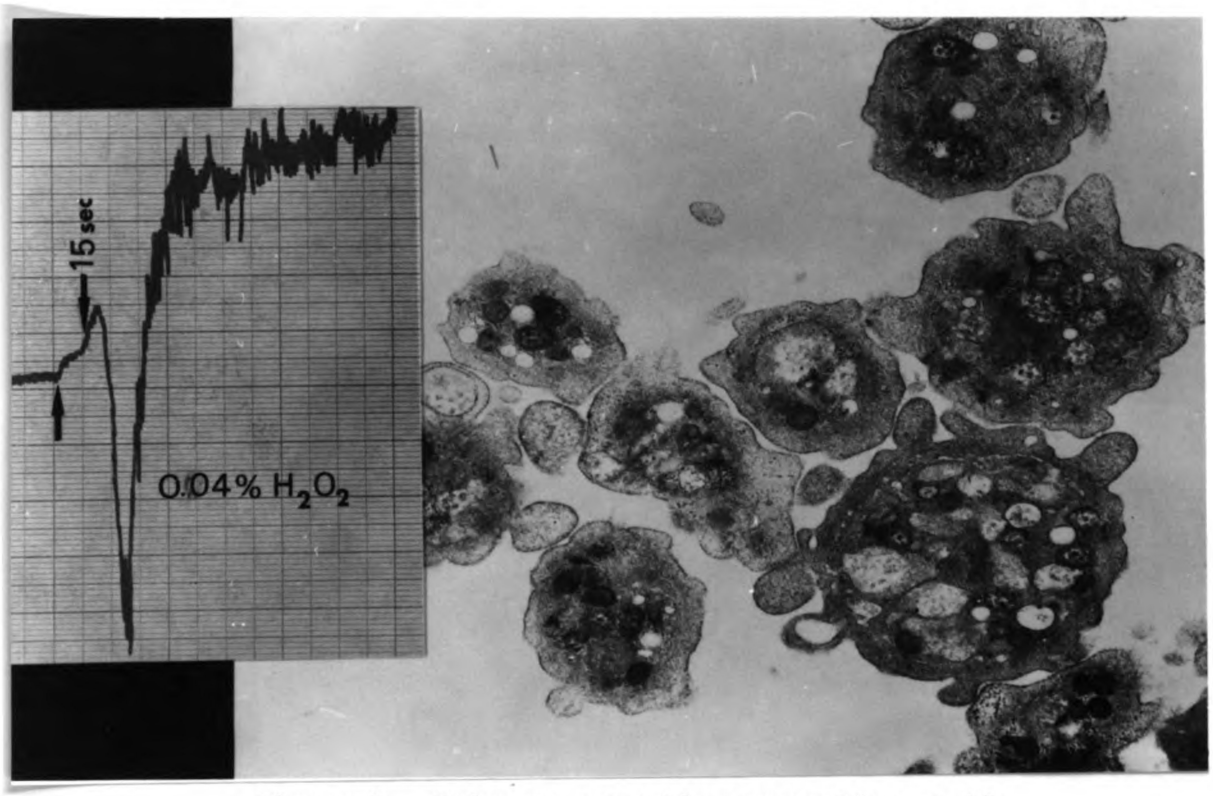

Figure 2. Hydrogen peroxide aggregation at 15 seconds. x25,000.

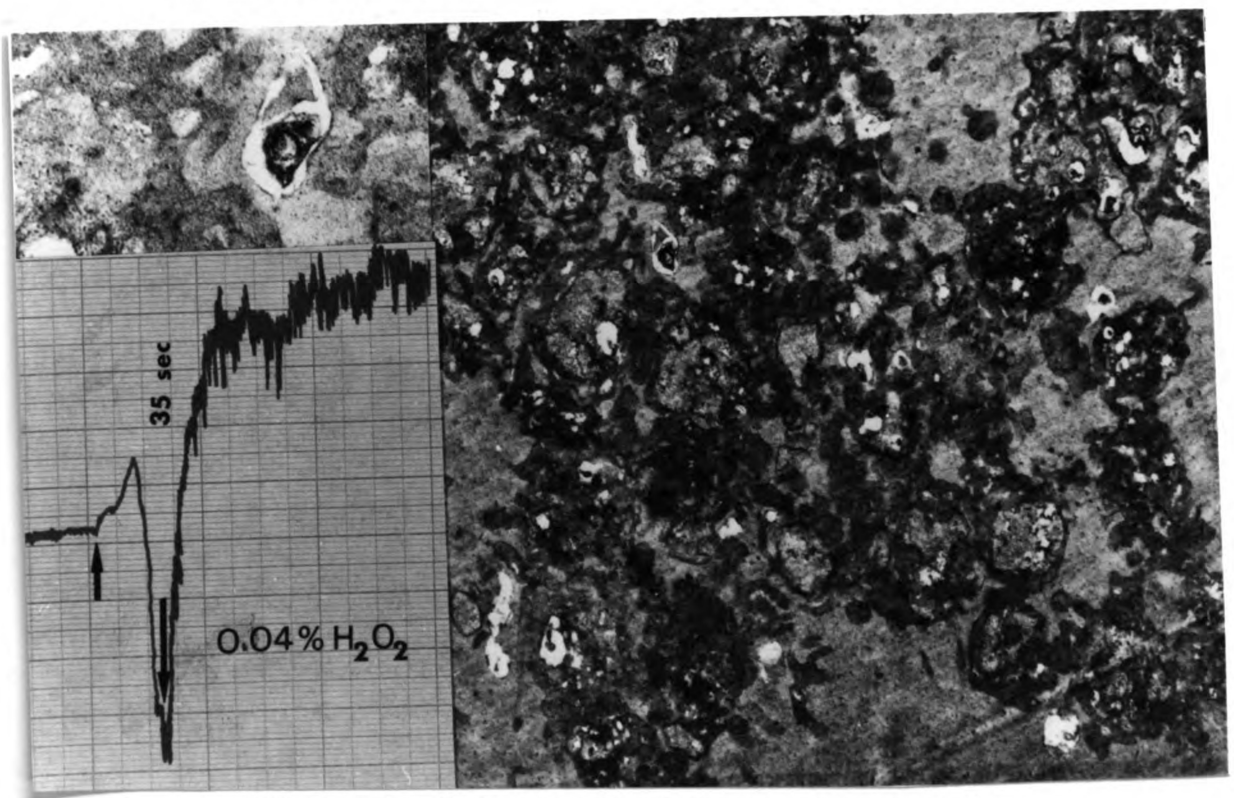

Figure 3. Hydrogen peroxide aggregation at 35 seconds. x17,500 insert x50,000.

electron microscopy revealed that the platelets were activated 15 seconds after the addition of hydrogen peroxide (Figure 2). At 35 seconds, TEM revealed (Figure 3) that the platelets were destroyed as evidenced by the presence of myelin figures. Another specimen was fixed for TEM 45 seconds after the addition of hydrogen peroxide; however, no specimen was recovered.

Platelet rich plasma from pig 9 was then incubated with 0.81 mg/ml of alpha tocopherol acetate for 2 hours. The hydrogen peroxide aggregation tracing appeared the same as described in the previous paragraph.

Polylysine (2 mg/ml) and epinephrine did not cause pig platelets to aggregate. Efforts to demonstrate platelet aggregation with my own preparation of bovine and pig collagen were unsuccessful; however, it has been reported in the literature that collagen does aggregate pig platelets (6).

Platelet counts

Platelet counts were sporadically abnormal in all groups except specimens from pigs injected with selenium. The platelet counts never decreased below 12,000/cu.mm in any pig (Table 4).

On bone marrow examination of a vitamin E deficient pig, megakaryocyte production was not suppressed and the marrow appeared slightly hyperplastic. There was excessive multinucleation of the megakaryocytes.

Table 4. Platelet counts averaged for each group

Day	Basal	Basal + vitamin E	Basal + selenium	Basal + vitamin E/selenium
3*	197,500 (1)	162,500 (2)	400,000 (2)	482,500 (2)
9**	532,488 (4)	508,400 (1)	756,450 (1)	---
10*	490,000 (1)	280,000 (2)	408,750 (2)	303,750 (2)
15**	561,250 (4)	902,500 (1)	977,500 (1)	---
17*	412,500 (2)	502,500 (2)	545,000 (2)	632,750 (2)
23**	466,667 (3)	760,000 (1)	482,500 (1)	---
25*	575,000 (1)	410,000 (2)	501,250 (2)	683,750 (2)
29**	471,875 (4)	430,000 (1)	772,500 (1)	---
32*	---	307,500 (2)	478,750 (2)	540,000 (2)
37**	367,500 (3)	550,000 (1)	495,000 (1)	---
40*	---	500,000 (2)	508,750 (2)	797,500 (2)

( ) indicates number of pigs

*experiment A

**experiment B

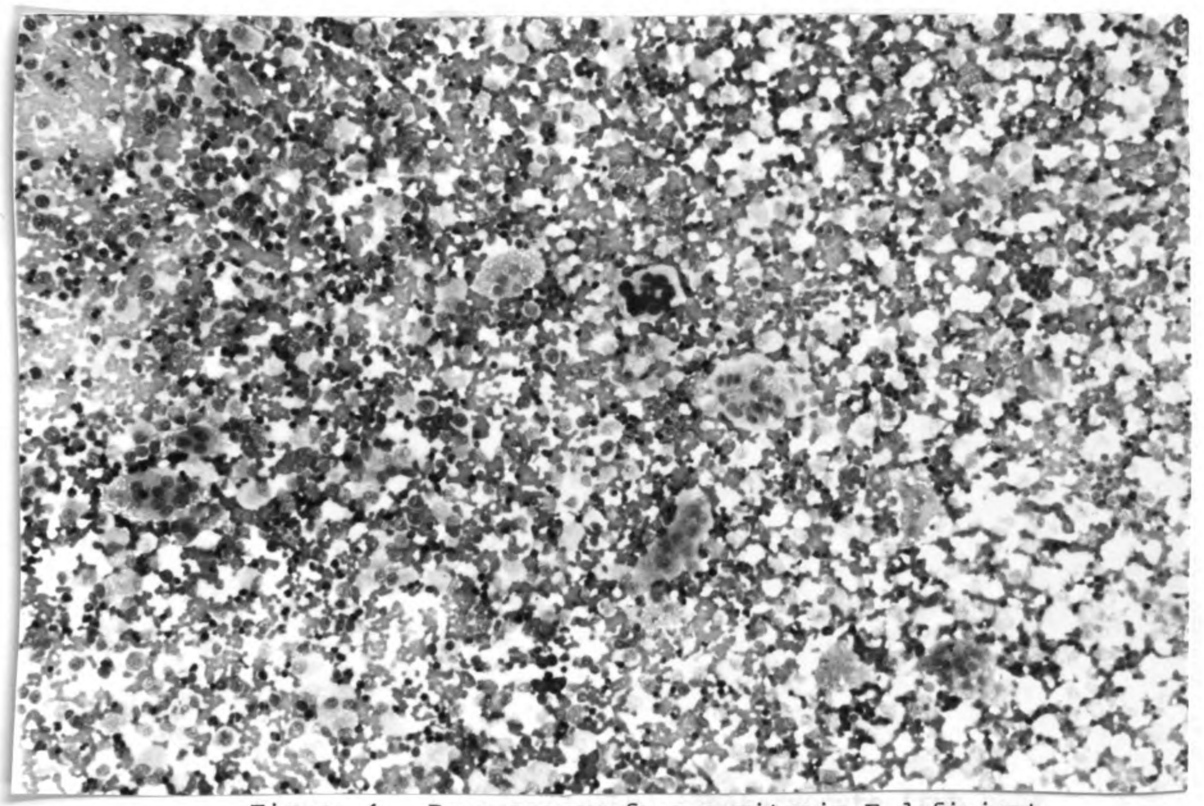

Figure 4. Bone marrow from a vitamin E-deficient pig. x45.

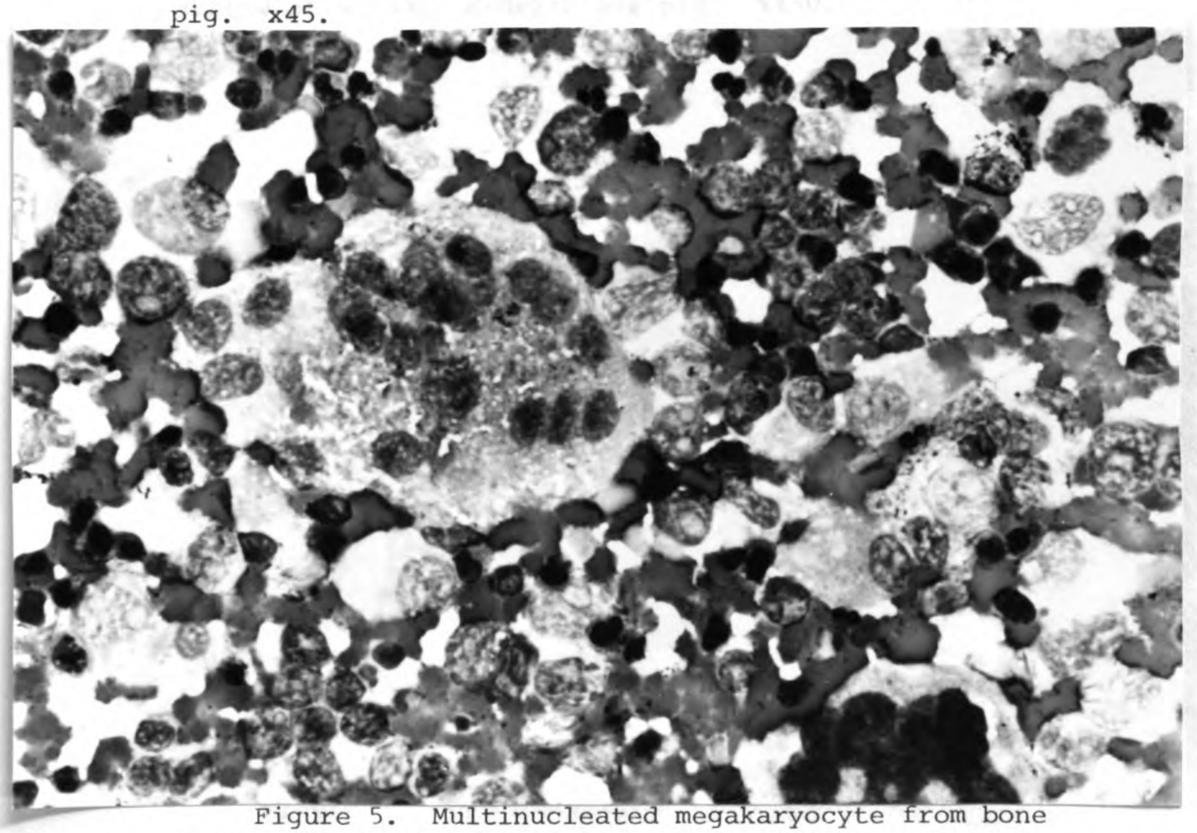

Figure 5. Multinucleated megakaryocyte from bone marrow of vitamin E-deficient pig. x202.5.

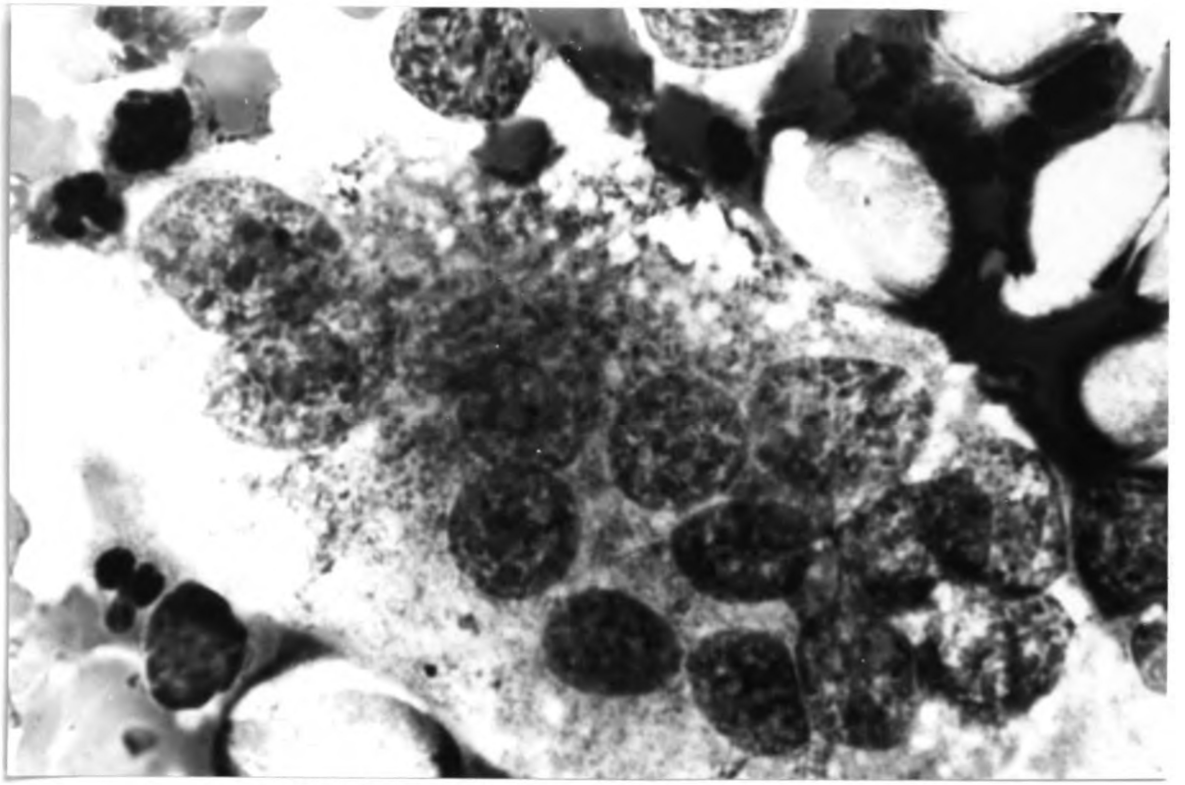

Figure 6. Multinucleated megakaryocyte from bone marrow of vitamin E-deficient pig. x450.

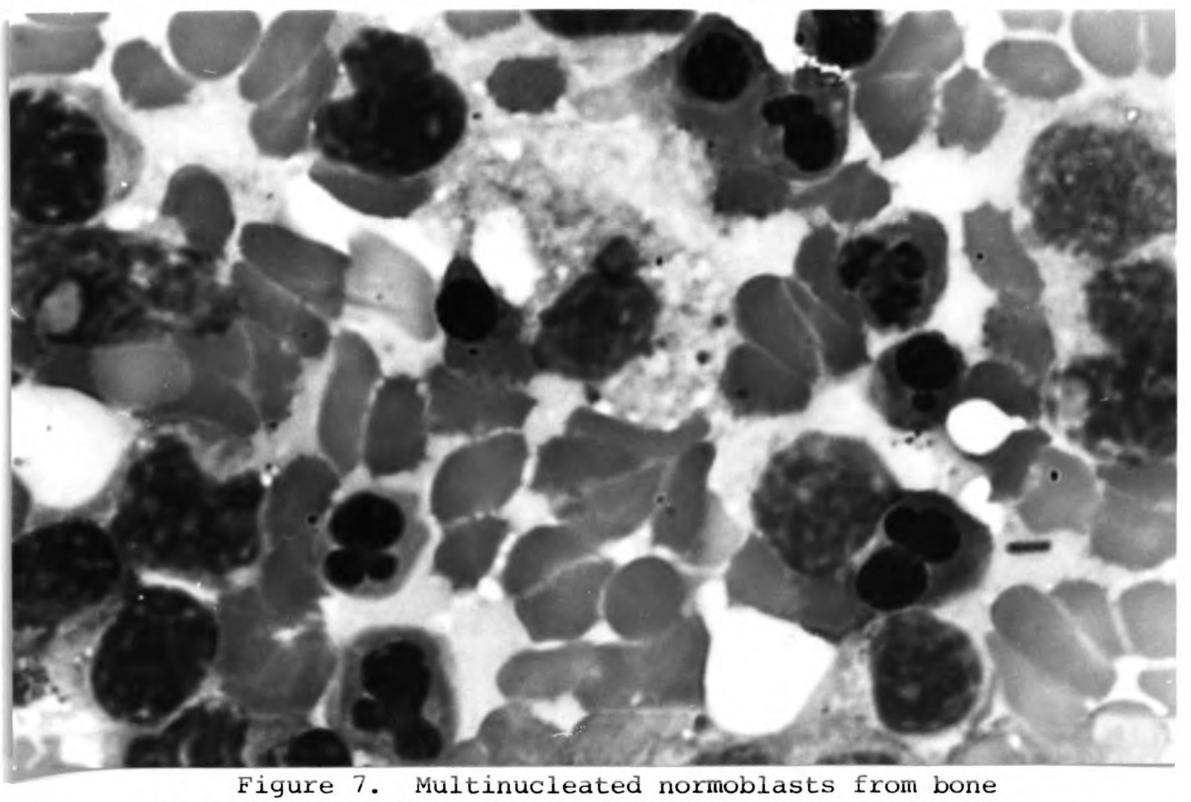

Figure 7. Multinucleated normoblasts from bone marrow of vitamin E-deficient pig. x450.

Clot retraction

The clot retraction was rarely abnormal in any pig (Table 5). In both experiments the mean of all groups was 71.9% with a range of 43-90% retraction. The values are comparable to normal values in man with a range of 40-94% retraction (3).

Platelet factor three availability

Platelet factor three availability was sporadically abnormal in all groups throughout the entire experiment. The pigs injected with both vitamin E and selenium had a lower incidence of abnormal platelet factor three availability (Table 6).

Aggregation

A. ADP aggregation was sporadically abnormal in all groups throughout the experiments.

B. Hydrogen peroxide aggregation was abnormal in all groups except the vitamin E supplemented group.

C. Ristocetin caused platelet aggregation in all pigs; however, the conversion of fibrinogen to fibrin was sporadically inhibited in all groups.

Enzymes

LDH: The LDH was slightly elevated in all pigs partially as a result of hemolysis of the specimens. The LDH elevation became increasingly marked as the pig approached the basal state. In experiment A, the LDH returned to normal after initiation of the supplemental injections (vitamin E and/or selenium). Electrophoresis revealed

Table 5. Clot retraction averaged for each group

Day	Basal	Basal + vitamin E	Basal + selenium	Basal + vitamin E/selenium
3*	74.5% (1)	81% (2)	82.5% (2)	79% (2)
9**	68.5% (4)	43% (1)	72% (1)	---
10*	64.5% (1)	53.5% (2)	58% (2)	75% (2)
15**	77.25% (4)	75% (1)	70% (1)	---
17*	82.5% (2)	68.5% (2)	56.5% (2)	75% (2)
23**	71.5% (3)	90% (1)	66% (1)	---
25*	84% (1)	73% (2)	74% (2)	75% (2)
29**	74.75% (4)	77% (1)	51% (1)	---
32*	---	73% (2)	74.5% (2)	72.5% (2)
37**	60.3% (3)	77% (1)	77% (1)	---
40*	---	78.5% (2)	72% (2)	84% (2)

( ) indicates number of pigs

*experiment A

**experiment B

Table 6. Platelet factor three availability averaged for each group

Day	Basal	Basal + vitamin E	Basal + selenium	Basal + vitamin E/selenium
3*	23.3 (1)	24.7 (2)	24.4 (2)	22.8 (2)
9**	38.3 (4)	37.0 (1)	38.5 (1)	---
10*	16.6 (1)	30.5 (2)	25.5 (2)	33.3 (2)
15**	22.5 (4)	22.5 (1)	26.7 (1)	---
17*	29.0 (2)	27.2 (2)	24.6 (2)	26.9 (2)
23**	54.5 (3)	28.0 (1)	26.2 (1)	---
25*	24.0 (1)	25.0 (2)	22.5 (2)	20.0 (2)
29**	27.7 (4)	30.5 (1)	30.0 (1)	---
32*	---	33.3 (2)	23.9 (2)	20.2 (2)
37**	42.8 (3)	24.0 (1)	33.6 (1)	---
40*	---	23.1 (2)	27.4 (2)	25.5 (2)

( ) indicates number of pigs

* experiment A

** experiment B

that porcine LDH isoenzymes are composed of five fractions. The fifth fraction was elevated in relation to liver damage.

SGOT: The SGOT was sporadically elevated in all pigs.

Red cell sensitivity

Osmotic fragilities were normal in all groups. Initial hemolysis appeared at 0.60-0.65% NaCl and was complete at 0.35-0.40% NaCl.

Hydrogen peroxide hemolysis increased in all pigs fed a diet deficient in vitamin E.

White blood counts

White blood counts demonstrated no specific pattern throughout the experiments.

Tissue changes

Three pigs on the basal ration (deficient in vitamin E and selenium) died during the experiments. On necropsy, each had somewhat different morphologic changes.

In gross examination of pig 8 (experiment A), the major abnormality seen was a markedly patchy, necrotic liver. All organs and muscles were pale. The gastrointestinal tract was normal and no hemorrhagic lesions were present in the heart, striated muscles or parenchymal organs. Microscopically, the liver demonstrated evidence of marked necrosis, lobular collapse and mononuclear cell infiltration. Some small vessels of the heart contained platelet aggregates. The bone marrow was hypercellular and contained abundant, excessively multinucleated megakaryocytes in which signs of platelet production

were evident. In addition, many normoblasts were multinucleated. The kidneys appeared congested.

LDH was increased in pig 6 (experiment A) by the second week. It remained elevated until the pig died during the fourth week of the experiment. At necropsy the muscles were pale but no hemorrhages or petechiae were noted. There were no gross abnormalities of the liver. The most striking lesion was an ulcer approximately 3 cm in diameter at the esophageal-gastric junction. The intestines contained coffee ground-like material. Microscopically the liver appeared normal except for scattered mononuclear infiltration in the sinusoids. Occasionally, there were some polymorphonuclear cells. In the heart there were platelet aggregates in venous spaces. The kidneys were congested but otherwise appeared normal.

Pig 6 (experiment B) died suddenly and unexpectedly. Necropsy was performed shortly after death. Grossly, the major abnormalities seen were a hemorrhagic liver and focal petechiae in the cortex of the kidney. There was no ulceration of the gastrointestinal tract. There were no hemorrhagic lesions in the heart or striated muscles. Microscopic focal hemorrhages were seen in the liver; the kidneys had marked vascular congestion and stasis. No microthrombi were found in the heart, striated muscles, liver, or kidneys.

Vitamin E and its influence on platelets in man

Baseline aggregation studies of human platelets demonstrated that upon the addition of 1% hydrogen peroxide (0.04% final concentration), the light transmission rose slightly (2%) for approximately 10 seconds. This was followed by a precipitous drop in transmission

of 25% for approximately 25 seconds. There was then an equally sharp rise of 20-25% above the initial baseline. Bubble formation accompanied these changes. This was the same pattern that occurred in pigs fed the basal ration supplemented with vitamin E and selenium.

Vitamin E was ingested, 100 I.U. for 6 days and then 400 I.U. for the following 7 days. The aggregation tracings appeared the same as the baseline except that the final phase of the tracings rose only to the baseline. Alpha tocopherol acetate was incubated *in vitro* with specimens from the week when the daily ingestion was 100 I.U. The tracings were the same as the *in vivo* tracings.

In comparison to the baseline ADP aggregation (5×10^{-6} M) and ristocetin (1 mg/ml) aggregation, consumption of 100 or 400 I.U. alpha tocopherol acetate did not alter platelet aggregation. *In vitro* incubation with vitamin E neither inhibited nor augmented the aggregation response to ADP or ristocetin.

DISCUSSION

The young pig appeared to be a suitable experimental animal for research on platelet physiology and function. It consumed the basal ration and tolerated the torula yeast diet well. Deficiency occurred in some groups within 10 days.

The hematologic and circulatory systems are relatively similar to those in man (8). Pig platelets are similar in appearance to human platelets. However, they require higher concentrations of aggregating agents for stimulation. The size of the pigs allowed the collection of an adequate volume of blood for the analyses. The pig was occasionally difficult to bleed, especially when less than three weeks of age. Hemolysis and an increase in tissue thromboplastin from traumatic punctures presented problems in some blood specimens.

New parameters of pig platelets were evaluated. Reports on values for clot retraction and platelet factor three availability normals were not found in the literature. Clot retraction in the pig (43-90%) is comparable to human normals (40-94%) (3). Platelet factor three availability (24.4 sec. $\pm$ 7.4) is also comparable to man (less than 30 sec.) (11).

Platelet precursors in a vitamin E-deficient pig indicated that there was an interference in megakaryocyte division. The multinucleation seen in many normoblasts was another indication that vitamin E deficiency interferes with cellular division. Even though the megakaryocyte from the vitamin E-deficient pig had alterations in cellular division, the marginal budding of platelets appeared normal. The quantitative platelet counts were adequate in all groups indicating that the deficiency does not decrease the quantity of circulating platelets.

The platelet function of clot retraction was rarely abnormal. This suggested that the function of retraction was not impaired in the deficiency. The release of the phospholipid, platelet factor three, was abnormal in all groups but to a lesser extent in those pigs injected with vitamin E. Platelet factor three is a phospholipoprotein, thought to be located on the membrane of the platelet (57), which may undergo peroxidation in the absence of the antioxidant. Therefore, it appears that vitamin E is necessary in the stabilization of platelet factor three and its availability.

It is postulated that a deficiency of vitamin E might enhance platelet aggregation and produce a state of hypercoagulation. This could explain the production of microthrombi described by Grant in vitamin E-deficient pigs (10). If vitamin E deficiency can enhance aggregation, one might suspect that vitamin E supplementation would be capable of inhibiting aggregation. Indeed, this has been postulated by Schute, who uses vitamin E therapeutically in coronary patients (46). There was no evidence that dietary supplementation

of vitamin E inhibits ADP aggregation. Furthermore, in none of the pigs fed the basal diet was there any evidence of enhanced aggregation. In contrast, subnormal ADP aggregation was observed in each group. The poor response of platelets to ADP stimulation could reflect previous stimulation by ADP released from hemolyzed red cells or hydrogen peroxide or lipid peroxides produced in the absence of vitamin E protection.

Ristocetin is a relatively new tool as an aggregating agent. It is used to evaluate the interaction between factor VIII and the platelet membrane. Ristocetin was used in these experiments to determine if excessive lipid peroxidation altered the membrane-factor VIII interaction. In man aggregation is initiated at a low concentration (0.5-1.6 mg/ml); the conversion of fibrinogen to fibrin is seen only at a higher concentration (greater than 1.6 mg/ml) in both PRP and platelet poor plasma (PPP) (16). In these experiments, 4 mg/ml of ristocetin was needed to aggregate platelets. The platelets would form large aggregates, then ball up in a fibrin clump. In every group there were some atypical ristocetin curves. Visibly, the platelets in these specimens would form large aggregates but would fail to ball up in the fibrin clump. This would indicate that the platelets were functional and adequate to interact with factor VIII at the membrane. The problem appeared to be the level of fibrinogen available for conversion to fibrin. Liver injury due to vitamin E deficiency might decrease the production of fibrinogen.

It is postulated that, if hydrogen peroxide caused *in vitro* aggregation, then *in vivo* circulating peroxides could also cause

intravascular aggregates. The hydrogen peroxide aggregation tracing reflects a complex and poorly understood series of events. Hydrogen peroxide produces an initial small increase (H) followed by a precipitous drop in light transmission. This is followed by an equally sharp return of the curve to at least the baseline (Figure 2). These three components of the curve are designated as phases 1, 2 and 3 of hydrogen peroxide aggregation. Ultrastructural studies show the first phase to represent platelet aggregation. The sharp decline (phase 2) in light transmission is due to platelet disruption with release of platelet content. It is in this phase that platelet disruption is discernible as myelin figures. The cause of the final rise in light transmission is unclear as recovery of platelets for TEM was impossible. It was postulated that the excessive liberation of oxygen, as evidenced by bubble formation, in combination with the total platelet destruction seen on electron microscopy, caused entrapment in platelet debris. The bubbles apparently carried the debris to the surface leaving the specimen more transmissible to light.

Evaluation of hydrogen peroxide aggregation was based on the first phase of the tracing (H). A rise in the first phase indicated aggregation. All groups of pigs except those injected with vitamin E demonstrated inhibition of this small wave of aggregation.

Tissue enzymes (LDH and SGOT) were used to monitor vitamin E deficiency. Normal values were established for these experiments. SGOT appeared to be the best indicator of tissue changes. The LDH results were often elevated. This could have been due to hemolysis. Lack of information in the literature hampered adequate correlation

between isoenzymes and specific organ damage. I was able to demonstrate that pig LDH can be separated into five fractions by electrophoresis. An increase in the fifth isoenzyme appeared to be associated with liver damage. This would be comparable to similar changes seen in man.

Red cell sensitivity, monitored by osmotic fragility, was not a good indicator in increased lipid peroxidation. The results did indicate that porcine red cells have an increased fragility ranging from initial hemolysis at 0.60-0.65% NaCl to complete hemolysis at 0.35-0.40% NaCl. The hydrogen peroxide hemolysis test, which was used in experiment B, was the better test in monitoring lipid peroxidation.

Increased peroxide sensitivity was seen in pigs in all groups except those that were injected with vitamin E. This confirms previous reports in the literature (44).

Alpha tocopherol acetate and, to a greater extent, alpha tocopherol nicotinate have been reported to inhibit hydrogen peroxide and ADP aggregation (22). It was thought that if vitamin E can inhibit aggregation *in vitro* it might have similar effects *in vivo*. This could have great clinical significance in the treatment and prevention of thrombosis. Neither oral consumption nor *in vitro* incubation with alpha tocopherol acetate had any effect on platelet response to hydrogen peroxide, ristocetin and ADP. It is concluded that vitamin E does not alter the platelet release to ADP. This is in keeping with the recent reports by the American Heart Association (9) on the effect of vitamin E on ADP and epinephrine aggregation.

Although no conclusive evidence was obtained in this research to link platelet dysfunction to the hemorrhagic lesions reported in pigs, there was evidence that vitamin E deficiency caused alteration in two measurable parameters of platelet function, platelet factor three availability and hydrogen peroxide aggregation. This information can now be the basis for more sophisticated research into the interaction between platelets and vitamin E. Specifically, the effects of prostaglandin and lipid peroxides can be evaluated using these two sensitive tests. Some exciting areas of platelet research today concern the intraplatelet synthesis of prostaglandin and its intermediates such as LASS (labile aggregating stimulating substance). These substances are peroxides and their synthesis is blocked by aspirin. It would be worthwhile to determine if vitamin E promotes or inhibits prostaglandin in the platelet.

CONCLUSION

Two factorial experiments involving 14 young pigs were conducted to evaluate the effects of vitamin E on platelet function. The pigs were fed on four different diets: 1) basal ration (deficient in vitamin E and selenium), 2) basal ration plus intramuscular (I.M.) injections of vitamin E, 3) basal ration plus I.M. injections of selenium, and 4) basal ration plus I.M. injections of vitamin E and selenium.

Normal values for clot retraction (43-90%) and platelet factor three availability (24.4 $\pm$ 7.4 sec.) were established for the pig. Platelet ultrastructural changes following hydrogen peroxide aggregation indicated that aggregation was followed by destruction of the platelet. Platelet factor three availability and hydrogen peroxide aggregation were two measurable parameters of platelet function which were altered in pigs fed a basal ration. The pig appeared to be a suitable experimental animal for determining the effects of vitamin E on platelet function.

In addition, *in vivo* experimentation on human platelets indicated that vitamin E neither inhibited nor augmented platelet aggregation.

APPENDICES

APPENDIX A

PREPARATION OF REAGENTS

APPENDIX A

PREPARATION OF REAGENTS

Platelet factor three availability

1. Kaolin. A suspension of 4 gm of kaolin (J. T. Baker, Phillipsburg, N.J.) in 160 ml of 0.85% NaCl.

2. 0.035 M calcium chloride. 3.9 gm of anhydrous $CaCl_2$ (Mallinckrodt, St. Louis, Mo.) was dissolved in distilled water and diluted to a liter.

Osmotic fragility of RBC

1. Buffered salt stock solution (10%). 180 gm of NaCl, 27.31 gm of dibasic sodium phosphate (Na_2HPO_4) and 4.86 gm of monobasic sodium phosphate (NaH PO $\cdot 2H_2O$) were dissolved in distilled water and diluted to 2 liters.

Hydrogen peroxide hemolysis test

1. 0.9% sodium chloride. 9.0 gm of analytical grade (NaCl) was dissolved in distilled water and diluted to 1 liter.

2. 4 Molar hydrogen peroxide. Mix 1 part of 30% hydrogen peroxide and 1.5 parts of phosphate buffer.

3. Fifteenth Molar monobasic potassium phosphate (KH_2PO_4). 9.078 gm of monobasic potassium phosphate was dissolved in distilled water and diluted to 1 liter.

4. Fifteenth Molar dibasic sodium phosphate (Na_2HPO_4). 11.876 gm of dibasic sodium phosphate was dissolved in distilled water and diluted to a liter.

5. 0.05 M phosphate buffer, pH 7.4. 61.2 ml of 1/15 M Na_2HPO_4 and 14.6 ml of 1/15 M KH_2PO_4 were mixed with 24.2 ml distilled water.

6. Saline-phosphate buffer. Equal parts of the stock saline and the phosphate buffer solutions were mixed.

APPENDIX B

LABORATORY DATA FROM PIGS FED BASAL RATION, EXPERIMENT A

BASAL DIET

PIG 6	Clot Re	H'crit	PLATELETS EDTA	PLATELETS PRP	Plat F3	WBC	LDH	SGOT	RBC SEN	ADP	H_2O_2	RISTO	WEIGHT
9-30	75%	----	----	200,000	23.2	----	----	29	N	S	-----	ok	4260
10-7	66%	----	----	450,000	20.7	----	1610	28	N	S	-------	ok	5186
10-14	86%	42%	582,500	770,000	25.1	40,300	1563	29	N	S	-------	poor	5675

PIG 1	Clot Re	H'crit	EDTA	PRP	PLT F#3	WBC	LDH	SGOT	RBC SEN	ADP	H_2O_2	RISTO	WEIGHT
9-30	74%	43%	197,500	567,500	23.3	20,200	----	25	N	S	-----	ok	3935
10-7	63%	37%	490,000	----	12.5	14,900	657	31	N	----	----	------	4922
10-14	79%	36%	242,500	227,500	33.0	25,100	11196	97	N	S	-------	ok	5675
10-21	84%	28%	575,000	642,500	24.0	8,800	4223	107	N	S	-------	ok	-----

APPENDIX B. Laboratory data from pigs fed basal ration, experiment A.

APPENDIX C

LABORATORY DATA FROM PIGS FED BASAL RATION, EXPERIMENT B

PIG 4	Clot Re	H'crit	PLATELETS EDTA	PRP	Plat F3	WBC	LDH	SGOT	RBC SEN	ADP	H_2O_2	RISTO	WEIGHT
11-20	26%	34%	403,850	537,100	40.6	12,500	1156	21	none	S	H base	ok	3850
11-26	75%	35%	585,000	952,500	15.6	31,200	1221	29	none	S	h a	-----	4449
12-4	76%	34%	370,000	455,000	37.2	34,200	1020	30	60.7%	S	H a	ok	----
12-10	72%	34%	330,000	380,000	24.4	26,100	1129*	35*	52.8%	S	h b	poor	5766
12-18	71%	37%	415,000	482,500	26.6	16,600	1169	65	-----	S	h a	ok	7128
12-20													7809
PIG 6	Clot Re	H'crit	EDTA	PRP	PLT F#3	WBC	LDH	SGOT	RBC SEN	ADP	H_2O_2	RISTO	WEIGHT
11-20	82%	27%	686,750	471,500	33.3	15,800	972	23	none	S	H b	ok	3465
11-26	79%	29%	660,000	767,500	17.8	21,200	1181	30	none	S	H a	ok	3814
12-4	63%	29%	-----	------	32.3	-----	1106*	40	34.4%	----	-----	-----	------
12-10	78%	29%	522,500	465,000	22.6	14,100	840	28	19.7%	S*	h a*	ok*	4676
12-18	-----	-----	-----	------	-----	-----	----	----	------	-----	------	------	------
12-20	----	----	----	-----	----	-----	------	-----	-----	-----	------	------	-------
PIG 7	Clot Re	H'crit	EDTA	PRP	PLT F3	WBC	LDH	SGOT	RBC SEN	ADP	H_2O_2	RISTO	WEIGHT
11-20	80%	31%	424,350	401,800	30.0*	27,300	1142	24	23.4%	poor*	H b*	ok*	3185
11-26	80%	32%	415,000	532,500	21.6*	26,200	1011	29	none	S*	h b*	ok*	3723
12-4	82%	30%	440,000	457,500	>120*	23,800	1057*	38	45.9%	poor*	h b*	ok*	-----
12-10	77%	32%	535,000	625,000	28.4*	18,800	801	26	36.0%	S*	h a*	poor*	4585
12-18	70%	32%	435,000	327,500	75.0*	20,100	998*	33	-----	poor*	h b*	poor*	6038
12-20	-----	-----	------	-----	----	-----	-----	----	-----	----	----	----	6311
PIG 8	CLot Re	H'crit	EDTA	PRP	PLT F3	WBC	LDH	SGOT	RBC SEN	ADP	H_2O_2	RISTO	WEIGHT
11-20	86%	33%	615,000	635,500	49.2	13,900	1050	33	13.9%	---	-----	-----	3096
11-26	75%	30%	585,000	670,000	34.9	29,400	1076	29	none	poor	H base	ok	3677
12-4	65%	27%	590,000	737,500	28.5	31,200	1045	37	46.8%	S	H a	poor	----
12-10	72%	27%	500,000	562,500	35.4	14,200	604	18	33.5%	S	H a	ok	4041
12-18	40%	25%	252,500	292,500	26.7	17,500	578	20	----	S	h a	ok	4812

APPENDIX C. Laboratory data from pigs fed basal ration, experiment B. (* = hemolysis)

APPENDIX F

LABORATORY DATA FROM PIGS FED BASAL RATION PLUS INJECTIONS OF VITAMIN E, EXPERIMENT A

VITAMIN E

PIG 9	Clot Re	H'crit	EDTA	PRP	PLT F3	WBC	LDH	SGOT	RBC SEN	ADP	H_2O_2	RISTO	WEIGHT
9-30													
10-7													
10-14	56%	34%	527,500	342,000	26.7	11,800	750	22	N	S *	-----	ok *	3450
10-22	80%	28%	335,000	382,500	26.0	9,700	741	27	N	S *	------	ok *	4131
10-29	72%	27%	495,000	432,500	21.8	21,800	773	36	N	S	------	ok	4086
11-6	76%	28%	495,000	427,500	20.6	13,400	632	34	N	S	------	ok	4722

PIG 4	Clot Re	H'crit	PLATELETS EDTA	PRP	Plat F3	WBC	LDH	SGOT	RBC SEN	ADP	H_2O_2	RISTO	WEIGHT
9-30	86%	44%	200,000	282,500	22.8	22,800	----	33	N	S	-----	ok	4630
10-7	65%	42%	400,000	435,000	19.4	18,400	668	31	N	S	------	poor	5670
10-14	81%	41%	477,500	510,000	27.7	27,800	886	32	N	S	-----	ok	6084
10-22	66%	36%	485,000	542,500	23.9	17,800	802	32	N	S	-----	ok	7945
10-29	74%	40%	120,000	340,000	44.7 *	49,800	841	53	N	poor *	-----	poor *	7536
11-6	81%	35%	505,000	550,000	25.6	18,100	644	22	N	S	-----	ok	9216

PIG 8	Clot Re	H 'crit	EDTA	PRP	PLT F#3	WBC	LDH	SGOT	RBC SEN	ADP	H_2O_2	RISTO	WEIGHT
9-30	76%	42%	125,000	252,500	26.6	14,300	----	24	N	S	------	ok	3280
10-7	42%	35%	160,000	147,500	41.5	9,200	14,529	254	N	poor	------	poor	3563

APPENDIX F. Laboratory data from pigs fed basal ration plus injections of vitamin E, experiment A. (* = hemolysis)

APPENDIX E

LABORATORY DATA FROM PIGS FED BASAL RATION PLUS INJECTIONS OF SELENIUM, EXPERIMENT B

SELENIUM

PIG 1	Clot Re	H'crit	PLATELETS EDTA	PRP	Plat F3	WBC	LDH	SGOT	RBC SEN	ADP	H_2O_2	RISTO	WEIGHT
11-20	72%	34%	756,450	1,092,650	38.5	13,100	840	21	3.6%	S	Hbase	ok	4225
11-26	70%	29%	977,500	797,500	26.7	27,900	893	33	11.6%	S	h a	ok	4585
12-4	66%	29%	482,500	980,000	26.2	19,200	996 *	42 *	50.1%	S	H a	ok	------
12-10	51%	36%	772,500	757,500	30.0	19,500	906 *	33	31.3%	S *	h below*	poor *	6583
12-18	77%	35%	495,000	557,500	33.6	20,750	917 *	38	-----	S *	h a *	ok *	7854
12-20													8762

APPENDIX E. Laboratory data from pigs fed basal ration plus injections of selenium, experiment B. (* = hemolysis)

VITAMIN E

PIG 3	Clot Re	H'crit	EDTA	PRP	PLT F#3	WBC	LDH	SGOT	RBC SEN	ADP	H_2O_2	RISTO	WEIGHT
11-20	43%	36%	508,400	719,550	37.0	23,600	1156	29	6.1%	S	H a	ok	3850
11-26	75%	35%	902,500	1,007,500	22.5	26,100	1510 *	36	none	S *	H below *	ok *	4449
12-4	90%	40%	760,000	752,500	28.0	34,500	1300	32	3.2%	poor	H base	ok	-----
12-10	77%	37%	430,000	702,500	30.5	26,900	1576 *	60 *	5.0%	poor	H base	ok	5766
12-18	77%	32%	550,000	520,000	24.0	18,100	1024	27	-----	S *	H a *	ok *	7128
12-20													7809

APPENDIX G. Laboratory data from pigs fed basal ration plus injections of vitamin E, experiment B. (* = hemolysis)

APPENDIX H

LABORATORY DATA FROM PIGS FED BASAL RATION PLUS INJECTIONS OF BOTH VITAMIN E AND SELENIUM, EXPERIMENT A

VITAMIN E and SELENIUM

PIG 3	Clot Re	H'crit	PLATELETS EDTA	PRP	Plat F3	WBC	LDH	SGOT	RBC SEN	ADP	H_2O_2	RISTO	WEIGHT
9-30	77 %	48%	497,500	390,000	24.4	16,500	----	20	N	S	----	poor	3240
10-7	81%	38%	142,500	125,000	41.2	14,800	14,469	467	N	poor	---	poor	3785
10-14	71%	35%	575,500	827,000	26.5	30,300	2,529	43	N	S	-----	ok	4313
10-22	74%	32%	732,500	887,500	20.8	9,500	854	26	N	S	----	ok	5312
10-29	72%	24%	480,000	510,000	20.8	10,400	932	30	N	S	-----	poor	5221
11-6	80%	31%	775,000	867,500	25.2	49,100	659	14	N	S *	------	ok *	6311

PIG 5	Clot Re	H'crit	EDTA	PRP	PLT F#3	WBC	LDH	SGOT	RBC SEN	ADP	H_2O_2	RISTO	WEIGHT
9-30	81%	41%	467,500	622,500	21.2	14,100	----	20	N	S	------	ok	5080
10-7	69%	38%	465,000	752,000	25.3	16,700	766	31	N	S	------	poor	6142
10-14	79%	40%	690,000	732,500	27.3	11,700	875	25	N	S	------	ok	6719
10-22	76%	38%	635,000	695,000	19.2	10,300	862	25	N	S	------	ok	7945
10-29	73%	38%	600,000	682,000	29.6	24,200	727	19	N	S	------	ok	7854
11-6	88%	32%	820,000	977,500	25.9	18,400	571	21	N	S	-------	ok	9307

APPENDIX H. Laboratory data from pigs fed basal ration plus injections of both vitamin E and selenium, experiment A. (* = hemolysis)

LITERATURE CITED

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1. Baehner, R. L., Gilman, N., and Karnovsky, M. L.: Respiration and Glucose Oxidation in Human and Guinea Pig Leukocytes: Comparative Studies. J. Clin. Invest., 49: 692, 1970.

2. Baehner, R. L., Nathan, D. G., and Castle, W. B.: Oxidant injury of Caucasian Glucose-6-Phosphate Dehydrogenase-Deficient Red Blood Cells by Phagocytosing Leukocytes During Infection. J. Clin. Invest., 50: 2466, 1971.

3. Bowie, E. J. W., Thompson, J. H., Didisheim, P., and Owen, C. A.: Test of Platelets and Blood Vessels, in *Mayo Clinic Laboratory Manual of Hemostasis*, pp. 29-33. W. B. Saunders, Philadelphia, 1971.

4. Caasi, P. J., Hauswirth, J. W., and Nair, P. P.: Biosynthesis of Heme in Vitamin E Deficiency. Ann. N.Y. Acad. Sci., 203: 93-102, 1972.

5. Call, F. L., II, Lucas, C. T., and Williams, W. J.: Phospholipid Synthesis in Human Platelets. J. Clin. Invest., 48: 13a, 1969.

6. Cazenave, J. A., Packham, M., and Mustard, J. F.: Adherence of Platelets to Collagen-Coated Surface: Development of a Quantitative Method. J. Lab. Clin. Med., 82(6): 978-990, 1973.

7. Chander, A. B.: The Anatomy of a Thrombus, in *Thrombosis*, edited by S. Sherry, K. M. Brinkhous, E. Genton, and J. M. Stengle, pp. 279-299. National Academy of Sciences, Washington, D.C., 1969.

8. Douglas, W. R.: Of Pigs and Men and Research: A Review of Applications and Analogies of the Pig, *sus scrofa*, in Human Medical Research. Space Life Sciences, 3: 226-234, 1972.

9. Gomes, J. A., Venkatachalapathy, D., and Haft, J. I.: The Effect of Vitamin E on Platelet Aggregation. Circ. 49-50 (supp. 3): 278, 1974.

10. Grant, C. A.: Morphological and Aetiological Studies of Dietetic Microangiopathy in Pigs "Mulberry Heart". Acta Vet. Scand., 2(3): 1-107, 1961.

11. Hardisty, R. M., and Ingram, G. I. C.: Laboratory Methods, in *Bleeding Disorders*, pp. 272-274. Blackwell Scientific Publications, Oxford (England), 1965.

12. Hewitt, E. W.: Certain Chemical and Morphologic Phases of the Blood of Normal and Cholera-Infected Swine. Iowa State Coll. Jour. Sci., 6: 143, 1932.

13. Higashi, O., Kikuchi, Y., and Konno, K.: A Case of Thrombasthenia with a Study of Platelet Aggregation by Hydrogen Peroxide. Tohoku J. Exp. Med., 106: 399-409, 1972.

14. Higashi, O., Kikuchi, Y., and Konno, K.: Effects of Metabolic Inhibitors on the Platelet Aggregation Induced by Hydrogen Peroxide. Tohoku J. Exp. Med., 109: 245-250, 1973.

15. Higashi, O., and Kikuchi, Y.: Effects of Vitamin E on the Aggregation and Lipid Peroxidation of Platelets Exposed to Hydrogen Peroxide. Tohoku J. Exp. Med., 112: 271-278, 1974.

16. Howard, M., and Firkin, B. G.: Ristocetin, a New Tool in the Investigation of Platelet Aggregation. Throm. Diath. Hemorrh., 26: 362, 1971.

17. Jacobs, H. S., and Lux, Samuel E., IV: Degradation of Membrane Phospholipid and Thiol in Peroxide Hemolysis: Studies in Vitamin E Deficiency. Blood, 32: 549, 1968.

18. Johnson, S. A.: Platelet in Hemostasis and Thrombosis, in *The Circulating Platelet*, edited by S. A. Johnson, pp. 355-393. Academic Press, New York, 1971.

19. Karpatkin, S.: Heterogeneity of Human Platelets. I. Metabolic and Kinetic Evidence Suggestive of Young and Old Platelets. J. Clin. Invest., 47: 1073, 1969.

20. Karpatkin, S.: Metabolism of Platelets, in *Hematology*, edited by W. J. Williams, E. Beutler, A. J. Erslev, and R. W. Rundles, p. 1010. McGraw-Hill, New York, 1972.

21. Kay, J. H., Hutton, S. B., Weiss, G. N., and Ochsner, A.: Studies on an Antithrombin, III. A Plasma Antithrombin Test for the Prediction of Intravascular Clotting. Surgery, 28: 24, 1950.

22. Kurokawa, I., Toshiyki, K., Nagai, T., and Kamimura, M.: The Effect of Vitamin E on the Adenosine Diphosphate Induced Platelet Aggregation. J. Vitaminalogy, 17: 181-184, 1971.

23. Lucy, J. A.: Functional and Structural Aspects of Biological Membrane: A Suggested Structural Role for Vitamin E in the Control of Membrane Permeability and Stability. Ann. N.Y. Acad. Sci., 203: 4-11, 1972.

24. Lynch, M. J., Raphael, S. S., Mellor, L. D., Spare, P. D., and Inwood, M. J. H.: Red Cell Studies, in *Medical Laboratory Technology and Clinical Pathology*, pp. 675-676. W. B. Saunders, Philadelphia, 1969.

25. MacDonald, P., Edwards, R. A., and Greenhalgh, J. F. D.: *Vitamins in Animal Nutrition*, pp. 68-71. Hafner Press, New York, 1973.

26. Majerus, P. W., Smith, M. B., and Clamon, G. H.: Lipid Metabolism in Human Platelets. I. Evidence for a Complete Fatty Acid Synthesis System. J. Clin. Invest., 48: 156, 1969.

27. Marcus, A. J., Ullman, H. L., and Safier, L. B.: Lipid Composition of Subcellular Particles of Human Blood Platelets. J. Lipid Res., 10: 108, 1969.

28. Mattson, J. C., and Mueller, L.: *Platelet Aggregation Manual*, Michigan State University, 1973.

29. Melhorn, D. K., and Gross, S.: Vitamin E-Dependent Anemia in the Premature Infant. I. Effects of Large Doses of Medicinal Iron. J. Pediatr., 79: 569-580, 1971.

30. Michel, R. L., Whitehair, C. K., and Keahey, K. K.: Dietary Hepatic Necrosis Associated with Selenium-Vitamin E Deficiency in the Swine. J.A.V.M.A., 155(1): 50-59, 1969.

31. Mickel, H. S., and Horbar, J.: The Effect of Peroxidized Arachidonic Acid Upon Human Platelet Aggregation. Lipid, 9: 68-71, 1974.

32. Mitchell, J. R. A.: The Role of Thrombosis in Myocardial Infarction, in *Thrombosis*, edited by S. Sherry, K. M. Brinkhous, E. Genton, and J. Stengle, pp. 117-125. National Academy of Sciences, Washington, D.C., 1969.

33. Monkhouse, F. C.: The Influence of Inorganic Salt on Plasma Antithrombin Activity. Thromb. Diath. Haemorrh., 9: 387, 1963.

34. Nafstad, I.: Studies of Hematology and Bone Marrow Morphology in Vitamin E-Deficient Pigs. Path. Vet., 2: 277-287, 1965.

35. Nafstad, I., and Nafstad, H. J.: An Electron Microscopic Study of Blood and Bone Marrow in Vitamin E-Deficient Pigs. Path. Vet., 5: 520-537, 1968.

36. Nair, P. P.: Vitamin E and Metabolic Regulation. Ann. N.Y. Acad. Sci., 203: 53-61, 1972.

37. Ochsner, A.: Letter to the Editor. N.E.J.M., 271: 211, 1964.

38. Ochsner, A., Kay, J. H., Decamp, P. T., Hutton, S. B., and Balla, G. A.: Newer Concepts of Blood Coagulation with Particular Reference to Post Operative Thrombosis. Ann. Surg., 131: 652, 1950.

39. Okuma, M., Steiner, M., and Baldini, M.: Lipid Peroxidation in Aging Platelets. Blood, 34(5): 712-716, 1969.

40. Okuma, M., Steiner, M., and Baldini, M.: Studies on Lipid Peroxides in Platelets. I. Method of Assay and Effect of Storage. J. Lab. Clin. Med., 75: 283-296, 1970.

41. Okuma, M., Steiner, M., and Baldini, M.: Studies on Lipid Peroxides in Platelets. II. Effect of Aggregation Agents and Platelet Antibody. J. Lab. Clin. Med., 77: 728-742, 1971.

42. Olson, R. E.: Vitamin E and Its Relation to Heart Disease. Circ., 48: 179-184, 1973.

43. Robinson, C. W., Mason, R. G., and Wagner, R. H.: Effect of Sulfhydryl Inhibitors on Platelets Agglutinability. Proc. Soc. Exp. Bio. Med., 113: 857-861, 1963.

44. Rose, C. S., and Gyorgy, P.: Specificity of Hemolytic Reaction in Vitamin E-Deficient Erythrocytes. Am. J. Physiol., 168: 414-420, 1952.

45. Schwarz, K.: The Cellular Mechanisms of Vitamin E Action: Direct and Indirect Effects of Alpha-Tocopherol on Mitochondrial Respiration. Ann. N.Y. Acad. Sci., 203: 45-52, 1972.

46. Shute, E. V.: Alpha Tocopherol in Cardiovascular Disease. The Summary, 25: 5-9, 1973.

47. Tappel, A. L., and Zalkin, H.: Inhibition of Lipid Peroxidation in Mitochondria by Vitamin E. Arch. Biochem. Biophys., 80: 333-336, 1959.

48. Tappel, A. L.: Vitamin E as the Biological Lipid Antioxidant. Vitamin Horm., 20: 493-509, 1962.

49. Tappel, A. L.: Vitamin E and Free Radical Peroxidation of Lipids. Ann. N.Y. Acad. Sci., 203: 12-28, 1972.

50. Tateishi, I., Steiner, M., and Baldini, M.: Studies on Lipids Peroxides in Platelets. III. Effect on Physiologic Platelet Function. J. Lab. Clin. Med., 75: 283-296, 1970.

51. Wasserman, R. H., and Taylor, A. N.: Metabolic Roles of Fat Soluble Vitamin D, E and K. Ann. Rev. Biochem., 41: 179-202, 1972.

52. Whitehair, C. K.: Nutritional Deficiencies, in *Diseases of Swine*, edited by H. W. Dunne, pp. 1015-1044. Iowa State University Press, Ames, 1970.

53. Williams, W. J.: General Effects of Disorders of Hemostasis, in *Hematology*, edited by W. J. Williams, E. Beutler, A. J. Erslev, and R. W. Rundles, pp. 1120-1123. McGraw-Hill, New York, 1972.

54. Wroblewski, F., and La Due, J. S.: Lactic Dehydrogenase Activity in Blood. Proc. Soc. Exp. Biol. Med., 90: 210-217, 1955.

55. Zalkin, H., and Tappel, A. L.: Studies on the Mechanism of Vitamin E Action. IV. Lipid Peroxidation in the Vitamin E-Deficient Rabbit. Arch. Biochem. Biophys., 88: 113-117, 1960.

56. Zierler, K. L., Grob, D., and Lilienthal, J.: On the Antithrombic and Antiproteolytic Activity of Alpha Tocopherol Phosphate. Am. J. Physiol., 153: 127-132, 1948.

57. Zucker, M. B.: Platelet Function, in *Hematology*, edited by W. J. Williams, E. Beutler, A. J. Erslev, and R. W. Rundles, pp. 1014-1022. McGraw-Hill, New York, 1972.

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