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Dietary Antioxidants in Young Swine[1,2]

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Abstract

Young swine obtained by hysterectomy were fed purified diets low in vitamin E and supplemented with d-α-tocopheryl acetate and ethoxyquin (Santoquin [R]).

It was demonstrated that with very low levels of polyunsaturated fatty acids (PUFA) in the diet, both tocopherol and Santoquin protected the tissues of the pig from increased thiobarbituric acid (TBA) values and from increased hemolysis usually associated with low vitamin E status. When the dietary PUFA were increased to levels over 5%, the supplements of tocopherol and Santoquin protected against increased TBA values of tissue homogenates, but not against increased hemolysis of erythrocytes, even when blood serum showed substantial amounts of tocopherol.

Some of the interrelationships of dietary PUFA and α-tocopherol were demonstrated. It was shown that for each 1% of peroxidized corn oil added to the diet above 4%, roughly 100 mg of d-α-tocopheryl acetate was necessary to protect the pigs from erythrocyte hemolysis.

The failure to reach a "zero" TBA value in vitamin E-deficient swine tissue homogenates substantiated the theory of *in vivo* lipid autoxidation, and the increased TBA values of incubated tissue homogenates demonstrated *in vitro* lipid autoxidation in tissues not protected by a biological antioxidant.

Introduction

DIETARY ANTIOXIDANTS have been used in several species of animals for several years to replace vitamin E. The results have varied, depending upon the antioxidant used, the species of animal, and the symptom studied. Several investigators (1,2,3,4) reported varying success in substituting methylene blue and NN'-diphenyl-p-phenylenediamine (DPPD) in the diet of the rat in place of tocopherol, and Draper et al. (5) were able to carry rats through two generations using DPPD in place of vitamin E. The antioxidant DPPD has been shown also to prevent muscular dystrophy in lambs (6). Shull et al. (7) found DPPD and Monsanto's Santoquin to be partially effective in preventing muscular dystrophy in guinea pigs. Studies with the vitamin E-deficient chick have shown a protective action of DPPD and Santoquin against encephalomalacia (8,9), and a protective action with Santoquin against exudative diathesis and muscular dystrophy. Selenium has been shown also to protect the vitamin E-deficient chick from exudative diathesis (10,11).

In the past 10 yr, several workers have described the *in vitro* hemolysis of erythrocytes from vitamin E-deficient animals of several species (12,13,14,15,16). The degree of hemolysis of erythrocytes of animals has been considered an indication of (and actual assay for) their vitamin E status (17). Those animals whose erythrocytes showed a high degree of *in vitro* hemolysis, either by H_2O_2 or by dialuric acid, were considered to be vitamin E-deficient.

More recently, it has been found that certain symptoms of vitamin E deficiency are either more readily apparent or appear only under the stress of adding significant amounts of PUFA to the diet (18,19,20,21, 22). Studies involving dietary PUFA have shown that tissue extracts from vitamin E-deficient animals show large increases in TBA reactant material (malonaldehyde) in *in vitro* incubation, in contrast to tissues from normal controls, which show very little or no increase in TBA value (23,24). This criterion has been assumed to indicate a high level of lipid autoxidation in vitamin E-deficient tissues, and that vitamin E prevents this autoxidation *in vivo* and *in vitro*. It has been shown in this laboratory that TBA values measure malonaldehyde release from the autoxidation of PUFA with three or more double bonds, but not from linoleic acid (25).

Experimental

The experiments to be described here are the results of several years' work on the relationship of vitamin E and other antioxidants, mainly ethoxyquin (or Santoquin) in the nutrition and blood characteristics of young swine.

[1] Presented at the AOCS meeting in Toronto, Canada, 1962.
[2] Supported by grants from the Monsanto Chemical Co., St. Louis, Mo., and The Hormel Foundation.

TABLE I

Semipurified Diets Used in Swine Experiments

	Exp. 17	Exp. 19	Exp. 20	Exp. 22	Exp. 24
			(Amount per kg of diet)		
Cerelose (glucose)	609 g	661 g	658 g	638 g	444 g
Vit-free casein	200 g	200 g	200 g	200 g	.....
Gelatin	(Used 4% in place of cerelose 1st 8 wk, Exp. 17–22)				.....
Alpha-cel	50 g	50 g	50 g	50 g	50 g
Methionine hydroxy analogue	2.18 g	.....	.....	.....	1 g
Aureomycin	100 mg.	100 mg	100 mg	100 mg	100 mg
Oil	100 g	50 g	50 g	70 g	(Varied—see Fig. 4)
Dried skim milk	.....	.....	.....	.....	500 g
Minerals [a]	36.55 g	36.55 g	36.55 g	36.55 g	3.55 g [b]
Vitamins:					
Chlorine chloride			2 g in all diets—		
B-Vitamins [c]			224 mg in all diets—		
Vit A acetate			9000 I.U. in all diets—		
Vit D_2			900 I.U. in all diets—		

[a] Mineral mix contained per kg of diet: $CaHPO_4$ 20 g; KCl, 5 g; NaCl, 5 g; $MgSO_4 \cdot 7H_2O$, 5 g; $FeSO_4 \cdot 7H_2O$, 1 g; $MnSO_4 \cdot 4H_2O$, 250 mg; $ZnSO_4 \cdot H_2O$, 200 mg; KI, 40 mg; $CuSO_4 \cdot 5H_2O$, 25 mg; $CoSO_4 \cdot H_2O$, 0.1 mg; sodium selenite, 0.1 mg.

[b] Mineral mix as in a, with $CaHPO_4$, KCl, and NaCl deleted, and $MgSO_4 \cdot 7H_2O$ used at 2 g/kg.

[c] Vitamin mix contained per kg of diet: Biotin, 0.5 mg; thiamine · HCl, 11 mg; calcium pantothenate, 55 mg; riboflavin, 22 mg; pyridoxine · HCl, 22 mg; niacin, 110 mg; folacin, 2 mg; vitamin B_{12}, 110 mcg; vitamin K (klotogen F), 2 mg.

The animals used in these experiments were miniature baby pigs obtained by hysterectomy 4 or 5 days preparatum and reared in isolation units. They were fed fortified milk diets for periods varying from 1–4 wk, and then fed experimental semi-purified diets made up largely of vitamin-free casein, cerelose, alphacel, gelatin, minerals, and vitamins. The diets were mixed twice weekly and stored at −20C. The diets used in the early experiments were similar to those shown in Table I for experiments 17–22, with only the fat source varied. The pigs were bled periodically, and serum tocopherol estimated by the Farber et al. (26) modification of the method of Quaife et al. (27). Erythrocyte hemolysis was determined with H_2O_2 by the method of György et al. (28). The alkaline isomerization method of Holman and Hayes (29) was used for PUFA determination. Serum cholesterol was estimated by the method of Abel et al. (30).

Tissue extracts were analyzed for TBA reaction and the malonaldehyde was estimated by a modification of the Zalkin and Tappel procedure (24). The tissues were excised immediately upon exsanguination of the pigs under CO_2 anesthesia, and frozen on solid CO_2. One gram samples were macerated with 20 ml of $n/10$ NaCl diluent in a Servall Omni-Mixer in an ice bath. The sample was then divided into two equal portions, one of which was incubated with shaking at 37C for 1 hr, and the other treated immediately with trichloroacetic acid. An equal portion (10 ml) of 10% trichloroacetic acid was added, the samples filtered, and 4 ml of the filtrate was added to 1 ml of 0.67% TBA. The samples were heated in a boiling water bath for 15 min, cooled, and the optical density determined against appropriate blanks at 535 mμ in the spectrophotometer. 1,1,3,3-tetraethoxypropane was used for a standard curve All TBA values are expressed as mμ moles malonic dialdehyde per gram of fresh tissue.

One of the earlier experiments, using a diet containing 4% stripped lard, showed the effect on erythrocyte hemolysis when the low-E diets were supplemented with levels of d-α-tocopheryl acetate of 5,7.5, 10, or 100 mg/kg, or with 0.1% methylene blue. Figure 1 shows the results of the 100 mg/kg level of the tocopheryl acetate addition. It was found that the serum tocopherol level of the supplemented pigs, although variable, remained at levels of 150–400 mcg/100 ml. The unsupplemented pigs showed very low serum tocopherol levels. The % hemolysis of the supplemented pigs remained very low, while that of the unsupplemented pigs varied widely and remained over 40% throughout the 20 wk.

The effects of low-level tocopheryl acetate supplementation on % hemolysis is shown in Figure 2. All pigs had high hemolysis at the start, having been fed low-E diets for some time. The 100 mg/kg level of tocopheryl acetate abruptly reduced the hemolysis to a minimum at 2 wk. At 3 wk the supplement was withdrawn, and the hemolysis rose in 4 wk to over 40%. Pigs fed low-E diets were supplemented with

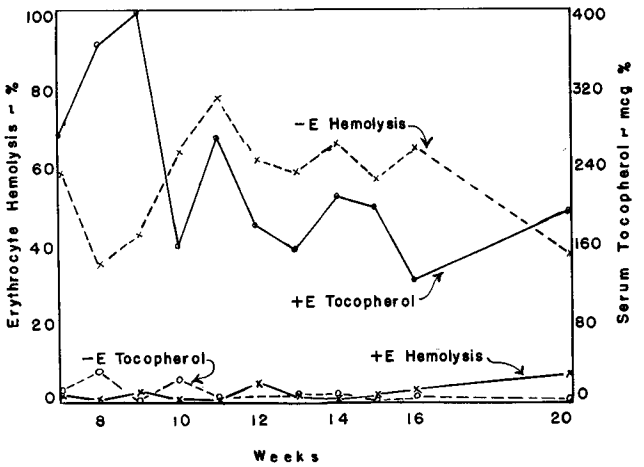

FIG. 1. Erythrocyte hemolysis and serum tocopherol of young pigs fed purified diets low and high (100 mg d-α-tocopheryl acetate/kg) in vitamin E.

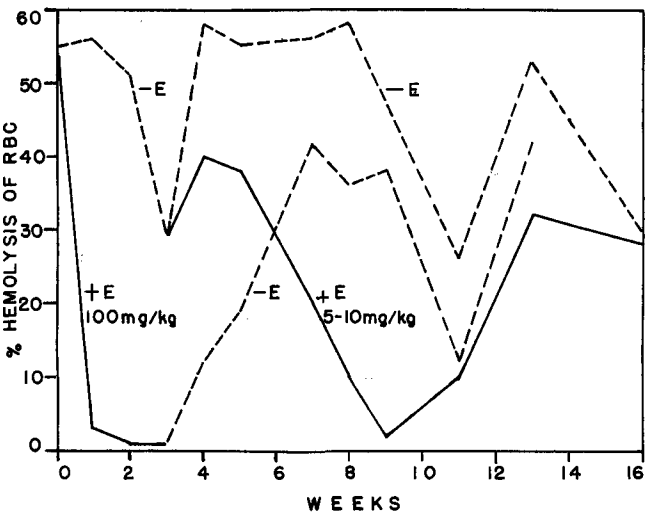

FIG. 2. Effect of low-level supplementation of d-α-tocopheryl acetate on erythrocyte hemolysis.

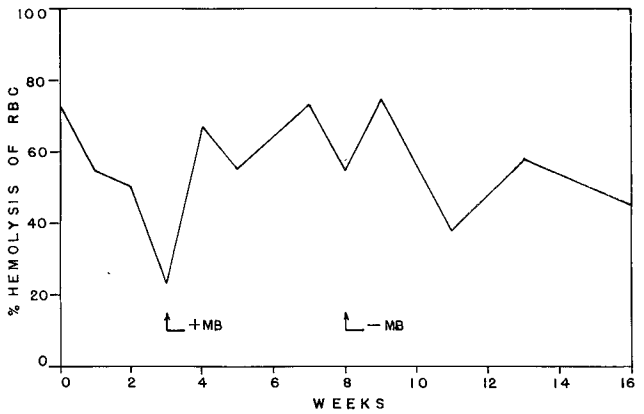

FIG. 3. Effect of methylene blue on hemolysis of erythrocytes of swine fed low-E diets.

3 levels of tocopheryl acetate (5,7.5, and 10 mg/kg). The 3 groups reacted similarly and the means of the groups are shown. These pigs showed a gradual decrease reaching a minimum in 6 wk but gradually rose again, indicating these levels of tocopheryl acetate were not enough to prevent hemolysis of erythrocytes after 10 wk. In this same trial, it was found that 0.1% methylene blue had no effect on erythrocyte hemolysis (Fig. 3).

The use of tocopheryl acetate, Santoquin, and essential fatty acid (EFA) supplementation of an extremely low-fat diet was studied (Table II) with 11 baby pigs fed purified diets beginning at 3 wk of age. These pigs were all fed the low-E, low-EFA basal diet for 8 wk, following which groups 2 and 3 were supplemented, as shown, for an additional 11 wk. Growth rates were not significantly different, although the +E, +EFA group was slightly larger at the end of the experiment. Serum cholesterol levels were not different. The % hemolysis was reduced some by the Santoquin or the vitamin E supplements. The incubated TBA values were vastly different, showing protection against autoxidation *in vitro* by both the Santoquin and the vitamin E. The PUFA data reveal that the pigs were in an EFA deficiency state, as observed by elevated trienoic acid levels in tissue lipids, combined with low dienoic and tetraenoic acid levels. The EFA supplement of 1 g ethyl linoleate/kg of diet was obviously not enough to correct this situation. The dietary linoleate requirement of swine has since been found to be 2% of calories, or ca. 1% of the diet (31), which was not met in this experiment. However, even in this condition of high excess of trienoic acid in tissue lipids, the vitamin E and Santoquin protected the tissue lipids from *in vitro* autoxidation as measured by TBA values.

An attempt was made to determine peroxide in erythrocytes or tissues of vitamin E-deficient pigs by

TABLE II

Effect of Dietary Antioxidant in Swine Fed Low-Vitamin E, Low-Fat Diet for 11 wk

	−E −EFA	−E −EFA +.1% santoquin	+E (100 mg/kg) +EFA (1 gm/kg)
No. of pigs	4	4	3
Serum cholesterol, mg%	79	74	76
Serum tocopherol, γ%	0	0	400
Hemolysis of RBC, %	50	36	36
Incubated liver TBA[a]	245	8	6
PUFA in liver lipids, %:			
Dienoic	0.99	1.51	2.76
Trienoic	9.03	9.95	6.48
Tetraenoic	1.76	1.87	5.21

[a] TBA values expressed as mμ moles malonic dialdehyde/g fresh tissue.

TABLE III

TBA of Swine Liver Tissue (Low Vit E) on Storage [a]

Weeks in storage at −20C	No. of samples	Initial	Incubated 60 min at 37C
0	11	18	75
1	9	30	171
2	10	35	222
3	10	41	189
4	10	42	201
5	11	38	170
6	12	40	174
7	12	36	117
8	12	33	111

[a] TBA value expressed as mμ moles malonic dialdehyde/g tissue.

both chemical (32) and thin layer chromatography methods. We were unable to show evidence of tissue peroxides by these methods. These procedures suggest that lipid peroxides, if present, existed at such low levels they could not be detected by these means. This did not rule out, however, the event of *in vivo* lipid autoxidation in vitamin E-deficient animals, since peroxides could be quickly decomposed on tissue storage. The tissues had been stored frozen for some time.

The failure of these methods led us back to the more sensitive TBA analysis.

The effect of storage of tissues on TBA values of liver was studied (Table III). The initial values increased some for about 3 wk, and then leveled off, while the incubated samples increased markedly for 2 wk, and then gradually declined. This showed that tissues must be analyzed quickly for best results.

These background studies led to our investigation of the effect of higher levels of PUFA in the diets. Two types of peroxidized oils were used, one heat treated below 90C to a peroxide value of 50, the other warmed with lauryl peroxide to 100C, and cooled immediately, resulting in peroxide values ranging from 5–20 (33). It was expected that higher levels of PUFA in the diet would place a stress on the animals that could be detected by the measurements of TBA of tissue homogenates and % hemolysis of erythrocytes.

The diets used in these experiments (Table 1) were all similar except for experiment 24, which used dried skim milk. The major variable was the level of oil used. It has been demonstrated more recently, selenium may have some effect on *in vitro* lipid peroxide (34,35) or peroxides as measured by TBA. These diets would not be expected to be deficient in selenium because the basic ingredient was casein, but selenium was added to all diets to avoid any possibility of having the results confounded by another variable. We had found earlier that selenium had no effect on hemolysis of erythrocytes of pigs fed low-fat diets low in vitamin E.

The composition of the peroxidized oils fed is shown in Table IV. The safflower oil methyl esters contained 77% linoleate, and the corn oil contained 58% linoleate. The safflower oil methyl esters prepa-

TABLE IV

Analysis of Peroxidized Oils by Gas Chromatography

Key	Fatty acid	Methyl esters of safflower oil [a]	Corn oil [b]
		%	%
16:0	Palmitate	6.68	10.91
18:0	Stearate	2.65	1.63
18:1	Oleate	13.17	27.21
18:2	Linoleate	77.35	58.23
18:3	Linolenate	trace, <0.5	1.46
20:4	Arachidonate	0	0.14

[a] Heated to 90C until PV rose to 50. Time averaged 10 hr.
[b] Peroxidized with Lauryl Peroxide. Average PV <25.

TABLE V

Hemolysis of Erythrocytes of Swine Fed Low-E Diets Supplemented with Tocopherol [a] and Santoquin [b]

Experiment	% Oil in diet	% Hemolysis			
		−E−S [c]	+E−S	−E+S	+E+S
17	10	90	57	68	[d]
19	5	59	84	93	81
20	5	83	75	78	52
22	7	100	[d]	100	[d]

[a] d-α-tocopheryl acetate fed at 100 mg/kg diet.
[b] Santoquin fed at .1% (Exp. 17,19) and .05% (Exp. 20,22).
[c] E and S refer to the presence or absence of d-α-tocopheryl acetate or Santoquin, respectively, in the diet.
[d] Not tested.

ration and the edible grade corn oil were obtained from commercial sources.

The composite results of all the hemolysis analyses of experiments 17,19,20, and 22 are shown in Table V. No differences are discernible in the % hemolysis of erythrocytes of any group within any experiment. The failure of Santoquin at either the 0.05% or 0.1% level, or tocopheryl acetate at the 100 mg/kg level, to protect the erythrocytes from H_2O_2 hemolysis, was evident in all 4 experiments in which the level of oil added was above 5%.

The liver, heart, muscle, and brain tissues from each animal in all 4 experiments were analyzed for TBA activity. In experiment 17, we used 10% peroxidized methyl esters of safflower oil, and supplements of 0.1% Santoquin and 100 mg d-α-tocopheryl acetate/kg of ration. The baby pigs were fed the fat-free basal diet for 2 wk, and the test diets for 18 wk (Table VI). This experiment showed that both tocopherol and Santoquin protected the liver tissue and heart tissue from increased TBA values on *in vitro* incubation. While the muscle tissue was protected by both the antioxidants, the brain tissue was not so protected, and resulted in similar high TBA values after incubation for all groups. The PUFA analyses of these tissues provided a clue to this behavior, in that the liver, heart, and muscle tissue were high in dienoic and tetraenoic, but very low in trienoic, pentaenoic, and hexaenoic fatty acids. In contrast, the brain tissue was low in dienoic and trienoic fatty acids, but high in tetraenoic, and very high in pentaenoic and hexaenoic fatty acids. These higher PUFA would be expected to provide very high TBA values if they autoxidized to any extent.

In order that we might reduce the level of PUFA, and with it the stress upon the autoxidative capacity of the animal, the next experiment (number 19) was conducted using only 5% peroxidized methyl esters of safflower oil (Table VII). The conditions of experiment 19 were similar to those of experiment 17. In experiment 19, the liver tissue was protected by both tocopherol and Santoquin. The heart tissue did not

TABLE VI

TBA Values of Swine Tissue Homogenates from Pigs Fed Diets Containing 5% Peroxidized Methyl Esters of Safflower Oil (Exp. 17)

Tissue	Sample conditions	TBA value [a]		
		−E−S [b]	+E−S	−E+S
Liver	Initial	32	12	11
	Incubated [c]	261	17	14
Heart	Initial	14	10	12
	Incubated	57	11	19
Muscle	Initial	12	7	8
	Incubated	69	8	14
Brain	Initial	7	6	7
	Incubated	83	36	82

[a] TBA values expressed as mμ moles of malonic dialdehyde/g fresh tissue.
[b] E and S refer to the presence or absence of dietary supplements of d-α-tocopheryl acetate (100 mg/kg) or Santoquin (0.1%), respectively.
[c] Incubated 60 min at 37C.

TABLE VII

TBA Values of Swine Tissue Homogenates from Pigs Fed Diets Containing 5% Peroxidized Methyl Esters of Safflower Oil (Exp. 19)

Tissue	Sample conditions	TBA values [a]			
		−E−S [b]	+E−S	−E+S	+E+S
Liver	Initial	13	12	13	16
	Incubated [c]	193	15	17	23
Heart	Initial	7	4	6	5
	Incubated	7	6	10	6
Muscle	Initial	10	7	7	6
	Incubated	10	5	13	6
Brain	Initial	9	9	11	11
	Incubated	96	65	180	145

[a] TBA values expressed as mμ moles of malonic dialdehyde/g fresh tissue.
[b] E and S refer to the presence or absence of dietary supplements of d-α-tocopheryl acetate (100 mg/kg) or Santoquin (0.1%), respectively.
[c] Incubated 60 min at 37C.

exhibit high TBA values for any group. The muscle tissue showed low TBA values for all groups. The brain tissue again exhibited a tendency to autoxidize readily, with no protection afforded by either supplement.

By changing to corn oil with a lower linoleic acid content, and by using milder peroxidizing conditions for the oil, we felt that perhaps the stress would not be so great. The TBA results of the liver tissue and heart tissue in experiment 20 are shown in Table VIII. This experiment was conducted for 13 wk. Similar TBA observations were observed here as in earlier experiments. The liver tissue and heart tissue both showed protective action of the supplements. The

TABLE VIII

TBA Values of Swine Tissue Homogenates from Pigs Fed Diets Containing 5% Peroxidized Corn Oil (Exp. 20)

Tissue	Sample conditions	TBA values [a]			
		−E−S [b]	+E−S	−E+S	+E+S
Liver	Initial	22	19	21	13
	Incubated [c]	92	15	25	23
Heart	Initial	4	6	6	6
	Incubated	19	8	10	10
Muscle	Initial	14	5	5	2
	Incubated	49	5	12	6
Brain	Initial	12	9	11	9
	Incubated	244	176	223	174

[a] TBA values expressed as mμ moles of malonic dialdehyde/g fresh tissue.
[b] E and S refer to the presence or absence of dietary supplements of d-α-tocopheryl acetate (100 mg/kg) or Santoquin (0.5%), respectively.
[c] Incubated 60 min at 37C.

muscle tissue was protected by the supplements, but not the brain tissue.

Following these studies, we conducted a withdrawal experiment. The pigs in one group were fed Santoquin for 9 wk, then fed with no Santoquin for an additional 9 wk. Table IX shows the TBA results of the tissues. Here we see only a very little residual effect of the Santoquin fed the first 9 wk. Santoquin

TABLE IX

TBA Values of Swine Tissue Homogenates from Pigs Fed Low-E Diets Containing 7% Peroxidized Corn Oil (Exp. 22—Withdrawal Experiment)

Tissue	Sample conditions	TBA values [a]			
		−S [b] (18 wk)	+S; −S (9 wk) (9 wk)		+S (18 wk)
Liver	Initial	36	28		19
	Incubated [c]	350	203		22
Heart	Initial	10	9		9
	Incubated	13	10		10
Muscle	Initial	32	9		9
	Incubated	52	28		16
Brain	Initial	15	18		19
	Incubated	221	219		232

[a] TBA values expressed as mμ moles malonic dialdehyde/g fresh tissue.
[b] S refers to the presence or absence of dietary supplement of Santoquin (0.05%).
[c] Incubated 60 min at 37C.

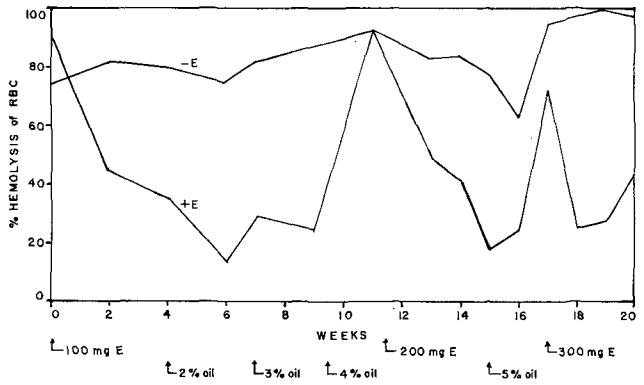

FIG. 4. Effect of varying levels of peroxidized corn oil and d-α-tocopheryl acetate supplements to the diet upon the rate of hemolysis of erythrocytes of young swine (Exp. 24).

fed the entire 18 wk protected liver, heart, and muscle tissues from autoxidation. The pigs themselves appeared all alike, and no visual external symptoms of antioxidant deficiency were observed.

In an attempt to determine what level of vitamin E was necessary to overcome the effects of high levels of PUFA in causing increased hemolysis of erythrocytes, a biological titration experiment was conducted (experiment 24). Here we fed 6 young pigs vitamin E-low diets with 1% peroxidized corn oil until all showed high hemolysis rates. The results of adding alternately more oil or more vitamin E to the diet are shown in Figure 4. It is seen that the % hemolysis could be controlled by adding more vitamin E whenever the oil level became great enough to lower the hemolysis of vitamin E supplemented pigs. Two pigs were left on the basal diet as comparisons.

Table X shows the concomitant TBA values of erythrocytes throughout the experiment. It is seen that the TBA values for the non-supplemented pigs rapidly rose and stayed high, while the TBA values of vitamin E-supplemented pigs gradually fell and remained low, regardless of the hemolysis value. The hemolysis values and TBA values of erythrocytes did not correlate in this experiment. This indicated that the red blood cells were continually protected by vitamin E against lipid autoxidation as measured by TBA, but were not protected against a high degree of hemolysis. Since the sera continually showed tocopherol levels of 300–500 μg/100 ml, the hemolysis assay for vitamin E may have to be reevaluated in the presence of high amounts of PUFA in the diet.

Attempts were made to obtain a tissue homogenate that would show a minimum TBA value. The question of whether or not *in vivo* peroxides are actually formed has not been satisfactorily answered. The data here indicate (Table IX) that we were unable to get a zero value for TBA measurements, but we did homogenize some of the tissue from the last Santoquin-fed animal in EDTA solution, which resulted

TABLE X

TBA Value of Erythrocytes after 60 min at 37C—Exp. 24[a]

Weeks	−E	+E
0	13	32
7	29	9
9	23	13
11	41	11
17	49	10
18	65	20
19	57	16
20	60	14

[a] TBA value expressed as mμ moles malonic dialdehyde/1.066 ml packed cells

TABLE XI

TBA of Swine Liver Homogenates (Low-E Milk Diet)

Pig No.	Age in days	Treatment	% Hemolysis of RBC	TBA value[a] Initially	TBA value[a] Incubated
1	24	None	78	11	35
4	24	20 mg EDTA inj. I.V.	82	15	30
6	24	20 mg EDTA inj. I.M.	75	12	27
5	32	450 mg EDTA inj. I.V. (100 mg/lb body wt.)	98	11	11
2	39	0.1% Santoquin fed last 7 days	62	11	15
3	39	0.1% Santoquin fed last 7 days. 120 mg EDTA inj. I.V. (22.5 mg/lb body wt)	93	6	9

[a] TBA values expressed as mμ moles of malonic dialdehyde/g fresh tissue.

in a TBA value of less than 1 (OD of 0.002), which would be as near zero as the accuracy of our instruments allow. This pig, of course, would not be expected to show a high TBA value. Tissues from vitamin E-deficient pigs still gave positive TBA values even when homogenized in trichloroacetic acid. These tissues homogenates show, however, great differences in TBA value, depending upon the presence or absence of tocopherol or other antioxidants in the diet.

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