# Lipid peroxidation status as an index to evaluate the influence of dietary fats on vitamin E requirements of young pigs

BY YAO H. WANG[1], J. LEIBHOLZ[2], W. L. BRYDEN[3] AND D. R. FRASER[3]

[1] *CSIRO Division of Human Nutrition, Adelaide, SA 5000, Australia*
[2] *Willawong, Murringo, NSW 2586, Australia*
[3] *Department of Animal Science, University of Sydney, Camden, NSW 2570, Australia*

The aims of the present study were to establish a suitable criterion for estimating the vitamin E requirement for young pigs, and to investigate the influence of dietary fats on the requirement of this nutrient. In Expt 1 weaned pigs were given a semi-purified diet supplemented with 0, 20, or 100 mg DL-$\alpha$-tocopheryl acetate/kg. Pigs in Expt 2 were fed on diets containing 10 g sunflower oil (SO) stripped of vitamin E/kg in diets 1 and 2, 100 g SO/kg in diets 3 and 4, and 100 g tallow/kg in diet 5. Diets 2, 4 and 5 were supplemented with DL-$\alpha$-tocopheryl acetate at 20 mg/kg for 0–28 d and 50 mg/kg for 29–56 d of the experiment respectively. Results showed that vitamin E concentrations in plasma and tissues reflected dietary levels of vitamin E. No apparent clinical signs of vitamin E deficiency were observed, but pigs fed on diets without vitamin E supplementation showed a higher ($P < 0.05$) lipid peroxidation status as indicated by thiobarbituric acid-reactive substances in erythrocytes, and ethane and pentane levels in exhaled gases than those fed on supplemental diets. This indicated that the former was deficient in vitamin E. Data in Expt 1 suggested that supplementation with 20 mg DL-$\alpha$-tocopheryl acetate/kg is adequate for young pigs when the diet contains 30 g lard/kg. However, results in Expt 2 showed that this level was inadequate when the diet contains 100 g SO/kg or its equivalent to 70 mg polyunsaturated fatty acids (PUFA)/g. Vitamin E and lipid peroxidation status of pigs were affected by both dietary vitamin E and dietary PUFA. This demonstrates that the requirement for vitamin E in young pigs increases as PUFA levels in the diet increase. The present study shows that lipid peroxidation response of pigs is a suitable index to evaluate vitamin E requirements.

Lipid peroxidation: Vitamin E requirement

It is well known that vitamin E functions as a biological antioxidant. The fat-soluble nature of this vitamin enables it to reside in biological membranes and to function as a free-radical quencher protecting membrane phospholipids from peroxidation damage. Vitamin E cannot be synthesized by animals and, therefore, its presence in animal tissues reflects dietary origin. Deficiencies of vitamin E result in a wide variety of pathological changes in animal tissues. The main manifestations in pigs of vitamin E deficiency and Se deficiency are dietetic microangiopathy or Mulberry heart disease, hepatosis dietetica, and nutritional myopathy (Moir & Masters, 1979). Death from vitamin E and Se deficiencies, which is most likely to be seen in weaned pigs, is sporadic but can reach 5–10 % of the herd (Van Vleet & Kennedy, 1989). It is suggested that mulberry heart disease is particularly related to vitamin E deficiency (Moir & Masters, 1979; Rice & Kennedy, 1989).

Vitamin E requirements of pigs have been extensively studied, and the presently recommended dietary requirement varies from 9 to 30 mg/kg for young pigs (Agricultural Research Council 1981; Jensen *et al.* 1988). In many studies establishing requirements, clinical signs of deficiency or mortality are the main assessment criteria, and most

recommended levels are based on that amount of vitamin E needed to prevent gross signs of deficiency. Nevertheless, the occurrence of clinical signs of deficiency in pigs may be variable in different experiments because variations in the experimental conditions and in the genotypes of pigs could affect the estimated requirement. Plasma vitamin E concentration has been shown to be a reliable indicator of dietary intake (Jensen *et al.* 1988). However, plasma vitamin E is not always closely associated with the actual lipid peroxidation values (Jensen *et al.* 1983; Duthie *et al.* 1989) and, therefore, does not accurately indicate the extent of vitamin E deficiency. A better approach, based on the relationship between vitamin E and lipids, may be the measurement of lipid peroxidation status in pigs as an index for assessing the degree of vitamin E protection against lipid peroxidation damage and requirement for this vitamin.

The fatty acid profile of animal tissues is affected by both quantity and quality of dietary fat (Marchello *et al.* 1983; Lin *et al.* 1989). Changes in fatty acid composition may be accompanied by changes in susceptibility of tissue lipids to free-radical attack. Polyunsaturated fatty acids (PUFA), an important component of biological membranes, are susceptible to such attack because their unsaturated double bonds are inherently unstable. Previous studies indicated that vitamin E deficiency in pigs was accelerated by the addition of highly unsaturated fat to the diet (Lindberg & Orstadius, 1961; Nafstad & Tollersrud, 1970). Relatively large amounts of fat (> 50 g/kg) are frequently added to diets to achieve a high energy content for rapidly growing, young pigs.

The objectives of the present study were to determine whether lipid peroxidation status measured as thiobarbituric acid (TBA)-reaction values in erythrocytes (RBC) and hydrocarbon levels in exhaled gases can be used as indices to assess the requirement of vitamin E for pigs, and to investigate the effect of different levels of dietary fats on vitamin E requirements and lipid peroxidation status of young pigs.

## MATERIALS AND METHODS
### Animals and housing

Cross-bred pigs (Landrace × Large White) were born to sows fed on a diet without vitamin E supplementation from 1 month before farrowing until the piglets were weaned at 21 d of age in Expt 1 and 17 days in Expt 2. At weaning, four piglets were allocated to each of three treatment groups in Expt 1, and five treatment groups in Expt 2. Each treatment group contained two males and two females with similar mean body weights. All pigs were reared in individual cages throughout the experimental period of 63 d.

### Experimental procedure and diets

All procedures involving animals were approved by the University of Sydney Animal Care and Ethics Committee before commencement of the experiments, and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Pigs in Expt 1 were given the basal diet shown in Table 1. The diet was supplemented with either 0, 20 or 100 mg DL-$\alpha$-tocopheryl acetate (F. Hoffman-La Roche Co Ltd, Basel, Switzerland)/kg for treatment groups 1, 2 and 3 respectively. Pigs in Expt 2 were given diets containing 10 g sunflower oil (SO)/kg (diets 1 and 2), 100 g SO/kg (diets 3 and 4) or 100 g tallow/kg (diet 5; Table 2). The diets were supplemented with vitamin E at either 0 (diets 1 and 3) or 20 mg (diets 2, 4 and 5) DL-$\alpha$-tocopheryl acetate/kg on days 0–28 and either 0 or 50 mg/kg respectively on days 29–56 of the experiment. In both experiments pigs were allowed *ad lib.* access to feed and water for 56 d. After this time $Cr_2O_3$ at concentrations of 10 g/kg in Expt 1 and 2 g/kg in Expt 2 was included in the diets which were then fed hourly to pigs in equal portions for 6 d.

Table 1. *Expt 1. Composition (g/kg) of the basal diet for pigs**

| Ingredients | |
|---|---|
| Maize starch | 630 |
| Casein | 170 |
| Soyabean meal | 100 |
| Dicalcium phosphate | 35 |
| Lard | 30 |
| Calcium stearate† | 20 |
| Vitamin+mineral premix‡ | 15 |

* The basal diet contained 0·5 mg α-tocopherol/kg diet, and the diet was supplemented with 0, 20 or 100 mg DL-α-tocopheryl acetate/kg.
† Calcium stearate was added to the diet as a pelleting agent.
‡ Vitamin+mineral premix (mg/kg diet): retinyl acetate 894 $\mu$g, cholecalciferol 6·5 $\mu$g, vitamin K 0·6, cyanocobalamin 21 $\mu$g, pyridoxine 1·8, riboflavin 4·2, folic acid 0·36, niacin 18, pantothenic acid 12, thiamin 1·2, biotin 60 $\mu$g, choline 0·6 g, NaCl 2 g, $FeSO_4 . H_2O$ 0·31 g, KCl 5·6 g, $ZnSO_4 . 7H_2O$ 0·5 g, MgO 0·7 g, $Na_2SeO_3$ 0·63, NaI 0·19, $CuSO_4 . 5H_2O$ 23·8, $MnSO_4 . H_2O$ 1·25.

Table 2. *Expt 2. Composition and nutrient content (g/kg) of diets fed to pigs**

| Experimental period (d)... | 0–28 | | | 29–56 | | |
|---|---|---|---|---|---|---|
| Diet no.... | 1 and 2 | 3 and 4 | 5 | 1 and 2 | 3 and 4 | 5 |
| Ingredient | | | | | | |
| Maize starch | 535 | 445 | 445 | 555 | 465 | 465 |
| Casein | 100 | 100 | 100 | — | — | — |
| Skim milk | 120 | 120 | 120 | 50 | 50 | 50 |
| Meat meal | 100 | 100 | 100 | 100 | 100 | 100 |
| Soyabean meal | 100 | 100 | 100 | 250 | 250 | 250 |
| Sunflower oil | 10 | 100 | — | 10 | 100 | — |
| Tallow | — | — | 100 | — | — | 100 |
| Calcium stearate | 20 | 20 | 20 | 20 | 20 | 20 |
| Vitamin+mineral premix† | 15 | 15 | 15 | 15 | 15 | 15 |

* The diets were supplemented with DL-α-tocopheryl acetate at (mg/kg): 0 diets 1 and 3, 20 diets 2, 4 and 5 from day 0 to day 28 then supplementation increased from 20 to 50 mg/kg from day 29 to day 56.
† For details, see Table 1.

SO used for Expt 2 was stripped of vitamin E by a method based on that of Mohri *et al.* (1983). The α-tocopherol concentration of SO was reduced from about 400 mg/l to between 20 and 30 mg/l.

*Sample collections*

*Faeces and urine.* In Expt 1 faeces and urine samples were collected daily for 5 d at days 8–12, 29–33 and 50–54 of the experiment. The faeces was obtained from a sloping tray which allowed the urine to drip into a bucket containing 25 ml 6 M-HCl. The urine was weighed and approximately 60 ml retained. All faeces and urine were stored at −20° immediately after collection. The bulked faecal sample was subsequently dried in a fan-forced oven at 105°, ground through a 1 mm screen and a 20 g subsample was taken and stored at room temperature for analysis.

*Blood.* Blood samples were collected by jugular vein puncture, with sodium heparin used as an anticoagulant. Duplicate whole-blood samples were used for the TBA-reaction test

and for the determination of haemoglobin (Hb) and enzymes. The remainder of each sample was centrifuged at 3000 rev./min for 10 min. The plasma was then separated and stored at $-20°$.

*Tissues.* At the end of each experiment, pigs were anaesthetized and then killed. Samples (approximately 20 g) of liver, heart, *longissimus dorsi* muscle, kidney and fat tissue from back fat were excised from each pig and stored at $-20°$ for later vitamin E analysis.

*Exhaled gases.* In the first experiment the exhaled-gas collecting apparatus consisted of a polyvinyl mask, unidirectional inspiratory valve (CIG Medishield, Melbourne, Australia), $CO_2$ absorber (CIG Medishield), 500 ml sample bottle (Alltech Inc, Deerfield, USA) and connecting tubing. When a sample was being collected the pig was held still and the mask sealed around its snout by hand. The unidirectional inspiratory valve was attached to the mask and allowed exhaled gases to pass through the $CO_2$ absorber and sample bottle, and outside air to go to the pig. $CO_2$ and water in exhaled gases were removed by soda lime in the $CO_2$ absorber. The unidirectional inspiratory valve to the $CO_2$ absorber and from the $CO_2$ absorber to the sample bottle were connected with 20 mm × 1 m latex tubing and 5 mm × 100 mm polyvinyl tubing respectively. The other end of the sample bottle was immersed in water. The gas-sample collection time was exactly 3 min after the first bubble appeared from the sample bottle, and then the sample was immediately analysed for hydrocarbons.

The collection procedure used in Expt 1 was stressful and was difficult to undertake when handling bigger pigs. Therefore, the procedure used in the second experiment was modified. Pigs were placed in an air-tight box constructed from 5 mm-thick Perspex and sealed by adhesives. The size of the box was 400 mm wide × 600 mm long × 400 mm high for pigs from 5 to 13 kg body weight and 500 mm wide × 900 mm long × 680 mm high for pigs over 13 kg. A small electric fan (150 mm diameter) was installed underneath the top side of the box to circulate air. A perforated Perspex platform sustained by two plastic plates was provided to separate the animal from urine and faeces. Two access ports were present in the box: an outlet port in front of the animal and inlet port behind. Air from the outlet port of the box was passed through a series of traps containing granular drieritte (0·8–2 mm, for water, Drieritte Company, Ohio, USA), 50 ml $H_2SO_4$/l (for $NH_3$) and 100 ml KOH/l (for $CO_2$). The box and traps were connected with polyvinyl tubing which allowed flexibility in the system and was relatively impermeable to hydrocarbons. A Clements vacuum pump (H. I. Clements Pty Ltd, Sydney, Australia) circulated the air through the box and a series of traps. A 'Y'-shape connector was placed in the tubing line at the inlet to the box to allow $O_2$ to enter from a glass bottle ($O_2$ reservoir) and also to maintain a closed system at ambient pressure. In this closed system it was found that leakage of ethane and pentane was about 2% in 2 h and 5% in 4 h. The temperature inside the box was maintained at 22–25° by spreading ice on the top of the box. A 500 ml sample bottle was placed in the tubing line between the vacuum and the drieritte container. The sample bottle was removed 15–30 min after placing the pigs in the box, and the sample was immediately analysed for hydrocarbons.

### Analyses

*Vitamin E.* α-Tocopherol concentrations in plasma, tissues and feeds were determined by HPLC with fluorescence detection after extraction of samples.

The plasma sample (1 ml) was mixed with an equal volume of absolute ethanol in a Pyrex tube, and light petroleum (b.p. 40–70°) was then added. The mixture was shaken for 3 min, allowed to settle for 10 min, and shaken again for 3 min. After settling, a portion of the light petroleum phase was measured accurately and evaporated to dryness under vacuum. The residue was dissolved in a suitable volume of methanol (0·5–1 ml).

The extraction of vitamin E from tissue samples was based on the method described by Bayfield & Romalis (1979). The procedure to extract vitamin E from feed samples was the same as that for tissues, but before the addition of the saponification reagent the finely-ground feed samples (0·5 g) were moistened with distilled water (1 ml).

Analysis of $\alpha$-tocopherol was carried out by injecting a portion (10 $\mu$l) of methanol solution onto a 3·9 mm $\times$ 150 mm Nova-pak® $C_{18}$ reverse-phase column (Millipore® Co., Milford, USA). Methanol was used as the eluting solvent at a flow-rate of 2 ml/min for isocratic elution. The HPLC system consisted of a Waters Millipore liquid chromatography 6000A pump model, fitted with a Waters WISP 710B Auto Sampler (Millipore® Co.). The effluent was monitored using a Hitachi F1000 Fluorescence Spectrophotometer (Hitachi Co., Mito, Japan) with excitation and emission wavelengths of 295 and 340 mm respectively. The duration of the chromatographic analysis was about 6 min. The chromatographic peak was identified by comparison of the retention time with a pure standard of $\alpha$-tocopherol (F. Hoffman–La Roche Co. Ltd, Basel, Switzerland) and the concentration of vitamin E in samples was calculated from peak height with the known-concentration standard solution as the reference. The standard solution was run in triplicate before a new batch of samples and, thereafter, once every five samples.

*TBA reaction assay of erythrocytes and feed samples.* The assay of TBA-reactive substances in porcine RBC was based on the method of described by Fontaine & Valli (1977), with minor modification. Freshly-collected blood (0·5 ml) was used for preparing RBC suspensions. The TBA-reactive substance was expressed as $\mu$mol malondialdehyde (MDA)/100 g Hb.

For quantitative determination of TBA-reactive substances, 1,1,3,3-tetraethoxypropane (TEP) was used as the external standard. To prepare the external standard, stock TEP (97–98 %, purity, Sigma Chemical Co., St Louis, MO, USA) was diluted with distilled water to make a standard solution containing 61 $\mu$mol TEP/l. For each assay a standard curve was made using a few tubes containing the same amounts of fresh blood, as those for the TBA-reaction assay and these were subjected to the same assay procedure. Before the incubation, various amounts of the standard solution (0·05, 0·1, 0·2 and 0·4 ml respectively) were added and mixed with the RBC suspension. After reading the results a standard curve for the MDA complex was prepared. The contents of TBA-reactive substances in RBC induced by $H_2O_2$ were calculated as MDA equivalents from the standard curve.

Since pigs on diet 3 of Expt 2 were observed to eat less and grow more slowly than other pigs, samples of diets in Expt 2 were collected and dietary oxidative values were measured according to the method described by Tarladgis *et al.* (1960). Rancidity values of feeds were expressed as $\mu$mol MDA/kg diet.

*Ethane and pentane in exhaled gases.* A Shimadsu GC-7A gas chromatograph (Shimadsu Co., Kyoto, Japan) with an ionization detector and fitted with a six-way gas sample valve was employed for chromatographic analysis of hydrocarbon gases. A stainless-steel column (32 mm $\times$ 1·8 m) filled with activated alumina (80–100 mesh) was used with $N_2$ carrier gas, flow-rate 28 ml/min. The chromatographic peaks of samples were recorded using a Spectro-Physics integrator (Model SP4270; Spectro-Physics, California, USA). The temperature of the detector and injector was 265°, and the column temperature was programmed as 50° for 1 min and then raised by 16°/min to 250° which was held for 5 min. Between sample application and after each day's analyses the column and alumina sample loop, which is described later (pp. 85–86), were held at 250° to remove any high-molecular-weight hydrocarbons.

Since the concentrations of ethane and pentane were very low in gas samples, it was necessary to concentrate the sample before injecting it onto the column. A length of brass tubing (3·2 mm $\times$ 125 mm) was attached with an adaptor fitted to the inlet port of a six-way

gas sample valve which was mounted on the side of the gas chromatograph. A stainless-steel sample loop ($3 \cdot 2$ mm × 105 mm) containing activated alumina (80–100 mesh) was connected to the brass tubing. During sample transfer from the sample bottle this loop was immersed in an icy slush of acetone–liquid $N_2$ until the sample was injected into the gas chromatograph. Perspex tubing (25 mm × 90 mm) containing granual drieritte ($0 \cdot 8$–$2 \cdot 0$ mm mesh) was connected to the other end of the sample loop by polyvinyl tubing (5 mm × 50 mm). The outlet port of the sample valve was attached to a small Clements vacuum pump (H. I. Clements Pty Ltd) with adjustable evacuation pressure.

When the sample was analysed, one side of the 500 ml sample bottle was connected to the Perspex tubing, and the other side was immersed in water. The gas sample in the sample bottle was drawn into the sample loop by the vacuum pump. A 100 ml flask containing 90 ml $H_2SO_4$ (50 ml/l) was placed between the Perspex tubing and the sample bottle to trap any $NH_3$ in the sample. After the sample bottle was filled with water the acetone–liquid $N_2$ was removed from the sample loop, the $N_2$ flow to the gas chromatograph was diverted through the loop via the six-way sample valve, and a hot water-bath (about 90°) was placed around the loop for a 3 min injection period. The $N_2$ flow was then diverted from the sample loop directly onto the chromatographic column. Drieritte in the Perspex tubing and the $H_2SO_4$ solution were replaced for each sample.

Standard ethane ($1 \cdot 1$ $\mu$l/l) and pentane ($2 \cdot 7$ $\mu$l/l) gases balanced in $N_2$ were purchased from CIG Ltd, Melbourne, Australia. A standard curve was obtained by injecting various amounts of standard gases ($0 \cdot 5$–3 ml) onto the column via the sample bottle. Chromatographic peaks of samples were identified by comparison with the retention time of the standard gases. Before and after collection each day, duplicate 500 ml air samples from the pig house were collected and analysed to determine the background level of hydrocarbon gases. Ethane and pentane contents in exhaled samples were calculated by measuring the peak area, and adjusting for background levels. Although the same chromatographic procedure was used for both experiments, the pentane peak in most samples for Expt 2 was unreadable due to the interference by the baseline rising simultaneously with the pentane peak. Therefore, pentane values are not reported for Expt 2.

The collection procedure for exhaled gases in Expt 1 used an open system with a defined period of time (3 min). The ethane and pentane concentrations were expressed, therefore, as pmol/l. In Expt 2 a closed system was used for the collection of exhaled gases, which allowed the production rate of exhaled hydrocarbons of pigs to be measured. The collection period of time varied for different pigs (from 15 to 30 min after placing the pigs in the system). Accordingly, for Expt 2 hydrocarbon production rate was expressed as pmol/kg body weight per min.

*Haemoglobin.* The Hb concentration of whole blood was determined in duplicate using a Haemoglobin Test-Combination Kit (Boehringer Mannheim, Mannheim, Germany).

*Total plasma lipid.* The total lipid in plasma was determined calorimetrically in duplicate using a diagnostic kit (Boehringer Mannheim).

*Nitrogen.* The N contents of feed ($0 \cdot 5$ g), urine (1 ml) and dried faeces ($0 \cdot 5$ g) samples were determined using a Kjel-Foss automatic analyser (Foss Electric, Hillerod, Denmark). The protein contents of samples were calculated using the factor of $6 \cdot 25$.

*Enzymes.* The activity of glutathione peroxidase ($EC$ 1.11.1.9; GSH-Px) in whole blood (100 $\mu$l), creatine phosphokinase ($EC$ 2.7.3.2; CK) and aspartate aminotransferase ($EC$ 2.6.1.1; ASAT) in plasma (10 $\mu$l) were measured using a Roche Cobas Mira automatic system (F. Hoffman–La Roche Co. Ltd). GSH-Px activity was expressed as $\mu$mol NADPH oxidized/min per g Hb, and CK and ASAT as unit/ml.

## Statistical procedures

The experiment was using a randomized complete block design as described by Steel & Torrie (1980). The data were analysed by ANOVA, and the means were compared by least significant difference for a probability of $P < 0.05$.

## RESULTS

### Expt 1

*Production performance and health state.* Apparent digestibilities of dry matter, body-weight gains and feed efficiencies were similar for pigs on the various treatments. N retention was lower ($P < 0.05$) in pigs consuming vitamin E-supplemented diets during the early experimental period, but the differences were not significant after 29 d on the experimental diets. No clinical signs of vitamin E deficiency were observed in the pigs during the experimental period or at necropsy. The values for plasma CK and ASAT were 743·4 (SE 121·4) and 52·6 (SE 6·5) unit/ml respectively at 56 d of experiment. These values were within the normal range.

*Plasma vitamin E concentrations.* Plasma $\alpha$-tocopherol concentrations at weaning were similar for all groups and the mean concentration was 1·35 (SE 0·34) $\mu$g/ml. Values then significantly declined in all treatment groups ($P < 0.05$) 7 d after weaning (Fig. 1). The magnitude of the decline in the first week post-weaning was less for pigs supplemented with either 20 or 100 mg vitamin E/kg diet than for the pigs not receiving the supplement. In pigs receiving no vitamin E supplement, plasma $\alpha$-tocopherol fell continuously to 0·1 $\mu$g/ml within 21 d and remained low thereafter. Pigs given the vitamin E supplement showed elevated plasma $\alpha$-tocopherol concentrations when compared with controls, after reaching minimum circulating levels of 0·31 $\mu$g/ml with diet 2 and 0·36 $\mu$g/ml with diet 3. The time-course and rate of increase in plasma $\alpha$-tocopherol concentrations were affected by the level of dietary supplementation.

*Tissue vitamin E concentrations.* Table 3 shows the distribution of $\alpha$-tocopherol in tissues at day 63 of the experiment. The $\alpha$-tocopherol concentrations of tissues differed significantly ($P < 0.05$) between treatment groups, reflecting the supplementation level. With all analysed tissues the highest tocopherol value was found in adipose tissue, while the lowest value was in kidneys.

*Thiobarbituric acid-reactive substances of erythrocytes.* Data in Table 4 shows that pigs not receiving a vitamin E supplement had greater levels of TBA-reactive substances ($P < 0.05$) than pigs given the supplemented diet. There was no difference between the two supplemented groups.

*Ethane and pentane concentrations in exhaled gases.* The effect of dietary vitamin E on ethane and pentane concentrations in exhaled gases at the end of the experiment is illustrated in Fig. 2. The mean values for ethane and pentane in the unsupplemented group were 177·2 (SE 17·3) and 149·3 (SE 24·0) pmol/l respectively. The addition of either 20 or 100 mg vitamin E/kg to the diet significantly depressed the concentration of hydrocarbons ($P < 0.05$). Although pigs on the diet supplemented with 20 mg vitamin E/kg had somewhat higher ethane and pentane concentrations than those supplemented with 100 mg vitamin E/kg, the difference was not statistically significant ($P > 0.4$ for ethane and $P > 0.2$ for pentane).

*Total plasma lipid concentrations and blood glutathione peroxidase activity.* There was an increase in plasma lipid concentrations ($P < 0.05$) with age up to 42 d on the experimental diets. However, vitamin E supplementation of diets did not affect the plasma lipid level. The activity of GSH-Px also increased significantly with age ($P < 0.05$) during the first 21 d
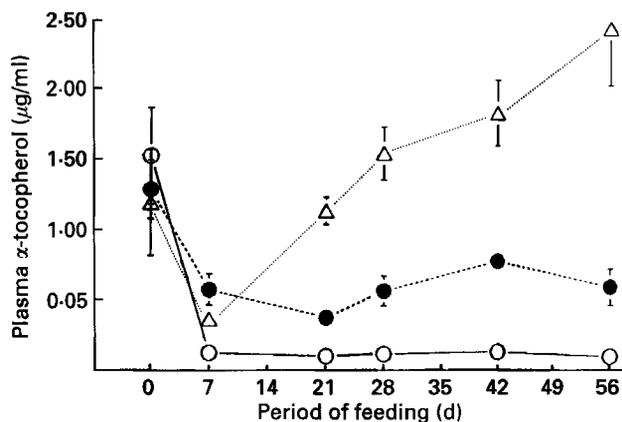
Fig. 1. *Expt 1.* Plasma α-tocopherol concentrations of pigs fed on diets supplemented with three levels of vitamin E. (○), Without vitamin E; (●), 20 mg DL-α-tocopheryl acetate/kg; (△), 100 mg DL-α-tocopheryl acetate/kg. For details of diets and procedures, see Table 1 and pp. 82–85. Values are means with their standard errors represented by vertical bars.

Table 3. *Expt 1. Tissue α-tocopherol concentrations (μg/g wet tissue) at day 63 of the experiment for pigs fed on diets supplemented with three levels of vitamin E**

| | Tissue α-tocopherol concentration (μg/g) | | | |
|---|---|---|---|---|
| Vitamin E supplement (mg/kg diet)... | 0 | 20 | 100 | SEM |
| Liver | 0·13[a] | 1·41[b] | 5·24[c] | 0·21 |
| Heart | 0·39[a] | 3·20[b] | 8·60[c] | 0·31 |
| Adipose tissue | 1·21[a] | 5·11[b] | 16·89[c] | 0·23 |
| Skeletal muscle | 0·32[a] | 0·97[b] | 3·22[c] | 0·23 |
| Kidney | nd | 0·55[a] | 1·89[b] | 0·04 |

[a,b,c] Means within a row with different superscript letters were significantly different ($P < 0.05$).
nd, Not detected.
* For details of diets and procedures, see Table 1 and pp. 82–87.

Table 4. *Expt 1. Concentrations of thiobarbituric acid (TBA)-reactive substances for erythrocytes from pigs fed on diets supplemented with three levels of vitamin E**

| | TBA-reactive substances (μmol MDA/100 g Hb) | | | |
|---|---|---|---|---|
| Day of experiment... | 35 | 42 | 49 | 56 |
| Vitamin E supplement (mg/kg diet) | | | | |
| 0 | 15·81[b] | 12·42[b] | 11·84[b] | 14·23[b] |
| 20 | 8·39[a] | 8·65[a] | 9·21[a] | 9·14[a] |
| 100 | 9·21[a] | 9·23[a] | 8·26[a] | 9·25[a] |
| SEM | 1·06 | 0·48 | 0·40 | 0·80 |

[a,b] Means within columns with different superscript letters were significantly different ($P < 0.05$).
MDA, malondialdehyde; Hb, haemoglobin.
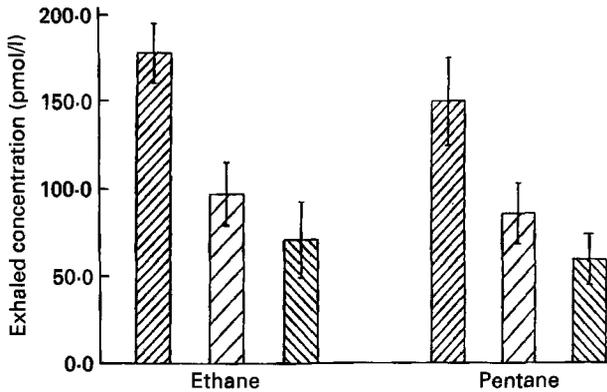* For details of diets and procedures, see Table 1 and pp. 82–87.

Fig. 2. *Expt 1*. Ethane and pentane production in exhaled gases at day 56 for pigs fed on diets supplemented with three levels of vitamin E. (▨), Without vitamin E; (▧), 20 mg DL-α-tocopheryl acetate/kg; (◩), 100 mg DL-α-tocopheryl acetate/kg. For details of diets and procedures, see Table 1 and pp. 82–87. Values are means with their standard errors represented by vertical bars.
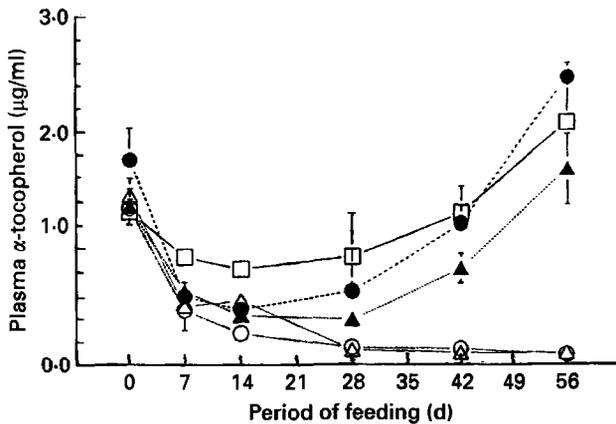


Fig. 3. *Expt 2*. Effects of dietary vitamin E and fats on plasma α-tocopherol concentrations of pigs. (○), 10 g sunflower oil (SO)/kg, without vitamin E (diet 1); (●), 10 g SO/kg + vitamin E (diet 2); (△), 100 g SO/kg, without vitamin E (diet 3), (▲), 100 g SO/kg + vitamin E (diet 4); (□) 100 g tallow/kg + vitamin E (diet 5). Diets were supplemented with DL-α-tocopheryl acetate at (mg/kg): 0 diets 1 and 3, 20 diets 2, 4 and 5 from day 0 to day 28, and then supplementation was increased from 20 to 50 mg/kg from day 29 to day 56. For details of diet composition and procedures, see Table 2 and pp. 82–85. Values are means with their standard errors represented by vertical bars.

of the experiment. No differences in GSH-Px activities were found between various treatment groups.

## Expt 2

*Production performance*. Pigs fed on 100 g SO/kg diet without vitamin E supplementation had lower feed intakes and body-weight gains ($P < 0.05$) than the other treatment groups. This may be due in part to the high oxidative value of this diet, since results showed that both diets 3 and 4 had been partially oxidized for the first 14 d, and the oxidation of diet 3 was more than 2-fold greater than that of diet 4 (the value for diet 4 was also 2-fold higher than other diets). Higher oxidation values were found also in diets 3 and 4 compared with diet 5 when sampled on other occasions. Increased oxidation values may result in decreased palatability of the feed. No clinical signs of vitamin E deficiency were observed in pigs during the experimental period, or at necropsy.

Table 5. *Expt 2. Effects of dietary vitamin E and fats on tissue α-tocopherol concentrations for pigs at day 63 of the experiment*\*

| | Tissue α-tocopherol concentration ($\mu$g/g wet wt) | | | | | |
|---|---|---|---|---|---|---|
| Diet no.†.... | 1 | 2 | 3 | 4 | 5 | SEM |
| Liver | 0·47[a] | 12·43[c] | 0·34[a] | 9·87[b] | 10·35[bc] | 0·82 |
| Heart | 0·61[a] | 12·70[b] | 0·41[a] | 10·45[b] | 10·42[b] | 0·74 |
| Adipose tissue | 1·20[b] | 13·13[c] | 0·62[a] | 8·71[b] | 11·58[c] | 0·92 |
| Muscle | 0·29[a] | 4·49[c] | 0·19[a] | 3·01[b] | 3·23[b] | 0·28 |
| Kidney | nd | 4·32 | nd | 3·60 | 3·73 | 0·47 |

[a,b,c] Means within a row with different superscript letters were significantly different ($P < 0.05$).
nd, Not detectable.
\* For details of diet composition and procedures, see Table 2 and pp. 82–87.
† Diets were supplemented with DL-α-tocopheryl acetate at (g/kg): 0 diets 1 and 3, 20 diets 2, 4 and 5 from day 0 to day 28, and then supplementation was increased from 20 to 50 mg/kg from day 29 to day 56. Diets 1 and 2 contained 10 g sunflower oil (SO)/kg, diets 3 and 4 100 g SO/kg, diet 5 100 g tallow/kg.

*Concentrations of α-tocopherol in plasma and tissues.* The plasma α-tocopherol concentrations of pigs in various treatment groups are presented in Fig. 3. At the beginning of the experiment they were almost identical for all treatment groups, and then declined during the first 14 d after weaning. Subsequently, plasma tocopherol concentrations rose in pigs supplemented with vitamin E, but not in the pigs fed on 100 g SO/kg diet when vitamin E was supplemented at 20 mg/kg. Vitamin E supplementation at 50 mg/kg in diets resulted in a significant increase in plasma α-tocopherol concentrations in pigs in all groups. Pigs on 10 g SO/kg diet with vitamin E (diet 2) had significantly higher ($P < 0.05$) plasma α-tocopherol concentrations after 42 d of the experiment than those on the diet containing 100 g SO/kg (diet 4). For pigs not receiving the vitamin E supplement, plasma α-tocopherol concentrations declined throughout the experiment.

The α-tocopherol concentration in tissues was significantly higher ($P < 0.05$) in pigs on the vitamin E-supplemented diets than in those fed on unsupplemented diets (Table 5). When diets were supplemented with vitamin E the α-tocopherol concentrations in liver, fat and muscle were significantly greater in the pigs on 10 g SO/kg than in those on 100 g SO/kg. In pigs fed on the diet containing 100 g tallow/kg, tissue α-tocopherol concentrations tended to be higher than that for pigs fed on the diet containing 100 g SO/kg, and the difference was significant ($P < 0.05$) for adipose tissue. In pigs fed on the unsupplemented diets, tissue α-tocopherol concentrations tended to decrease with increasing SO intake, and a significantly lower concentration was found in adipose tissue in the pigs on 100 g SO/kg compared with those on 10 g SO/kg ($P < 0.05$).

*Lipid peroxidation status.* During the first 28 d of the experiment the levels of TBA-reactive substances of RBC were significantly lower ($P < 0.05$) in pigs supplemented with 20 mg vitamin E/kg diet than in pigs receiving unsupplemented diets (Table 6). However, the levels of TBA-reactive substances were not depressed by dietary vitamin E supplementation at the level of 20 mg/kg diet when the diet contained 100 g SO/kg. When diets contained 50 mg vitamin E/kg no significant difference in TBA-reactive substances was found between groups. For pigs on the unsupplemented diets, TBA-reactive substances increased continuously with age, and pigs on the diet with 100 g SO/kg (diet 3) had greater ($P < 0.05$) levels of TBA-reactive substances than those on the 10 g SO/kg diet (diet 1) after 42 d of the experiment.

Table 6. *Expt 2. Effect of dietary vitamin E and fats on thiobarbituric acid (TBA)-reactive substances in erythrocytes and ethane production in exhaled gases of pigs**

| Day of experiment | Diet no.† | | | | | SEM |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | |
| | TBA-reactive substances ($\mu$mol MDA/100 g Hb) | | | | | |
| 14 | 18·70[b, c] | 3·45[a] | 5·07[a] | 27·43[c] | 7·48[ab] | 3·88 |
| 28 | 24·46[b] | 7·07[a] | 25·56[b] | 28·88[b] | 5·77[a] | 4·78 |
| 42 | 29·15[b] | 9·08[a] | 42·96[c] | 10·66[a] | 9·32[a] | 2·59 |
| 56 | 27·79[b] | 8·80[a] | 40·87[c] | 9·49[a] | 9·53[a] | 0·89 |
| | Ethane level in exhaled gases (pmol/l per kg body wt per min) | | | | | |
| 14 | 4·64[b] | 2·35[a] | 4·45[b] | 8·19[c] | 3·38[ab] | 0·68 |
| 28 | 5·20[c] | 2·47[a] | 6·24[c] | 4·45[bc] | 2·48[a] | 0·61 |
| 42 | 5·78[b] | 1·86[a] | 6·07[b] | 2·12[a] | 2·34[a] | 0·25 |
| 56 | 3·08[b] | 1·64[a] | 3·72[b] | 1·20[a] | 1·18[a] | 0·27 |

[a, b, c] Means within a row with different superscript letters were significantly different ($P < 0.05$).
MDA, malondialdehyde; Hb, haemoglobin.
* For details of diet composition and procedures, see Table 2 and pp. 82–87.
† Diets were supplemented with DL-$\alpha$-tocopheryl acetate at (g/kg): 0 diets 1 and 3, 20 diets 2, 4 and 5 from day 0 to day 28 and then supplementation was increased from 20 to 50 mg/kg from day 29 to day 56. Diets 1 and 2 contained 10 g sunflower oil (SO)/kg, diets 3 and 4 100 g SO/kg, diet 5 100 g tallow/kg.

Data in Table 6 show that there were significant differences ($P < 0.05$) in ethane production in exhaled gases of pigs fed on the different diets. Ethane production was significantly higher ($P < 0.05$) in pigs when diets were not supplemented with vitamin E compared with vitamin E-supplemented diets. A high level of ethane production was found also in pigs given a 100 g SO/kg diet supplemented with 20 mg vitamin E/kg (diet 4). When supplementation of vitamin E was increased from 20 to 50 mg/kg diet, dietary fat had no effect on ethane production.

## DISCUSSION

At weaning, pigs are most susceptible to the development of vitamin E deficiency. Thus, knowledge of the requirement of pigs for vitamin E at this stage is particularly important. Data in both Expts 1 and 2 showed that the plasma vitamin E status in piglets declined soon after weaning. This finding may help to explain why diseases associated with vitamin E deficiency often occur in weaners. A similar trend has been demonstrated also by other investigators (Mahan & Moxon, 1980; Meyer *et al.* 1981; Bonnette *et al.* 1990). The decrease in plasma vitamin E concentrations may be associated with low feed intake (Peplowski *et al.* 1981) and/or poor absorption efficiency for vitamin E. Results in Expt 1 indicated that plasma lipid concentrations increased with age up to 42 d after weaning, which suggests that digestibility or absorption of dietary fat at weaning may not be as effective as at a later age. Because of the fat-soluble characteristic of vitamin E the absorption of this vitamin is dependent on the animal's ability to digest and absorb fat (Wiss *et al.* 1962). When the diet was supplemented with vitamin E, plasma vitamin E concentrations rose in proportion to the dietary intake. However, it appears that even with a dietary vitamin E supplement as high as 100 mg/kg diet it took about 14–21 d before the full effect on vitamin E concentrations in plasma was seen. This suggests that

supplementation of weaner diets with vitamin E does not effectively prevent a decline in plasma vitamin E in weaners.

In many studies of vitamin E requirements of pigs, clinical signs of deficiency or mortality are the usual assessment criterion, and most recommended levels are based on the amount of vitamin E needed to prevent gross signs of deficiency. However, the occurrence of clinical signs of deficiency may vary according to the experimental conditions and the genotype of the pig, which may in turn indicate different requirements. Although the National Research Council (1988) recommended that supplements of 11–16 mg vitamin E/kg diet are adequate for preventing mortality and deficiency lesions and for supporting the normal performance in growing pigs, Jensen et al. (1988) observed that 16 mg vitamin E/kg diet could not prevent deficiency lesions and mortality of pigs, and they suggested that the supplement level should exceed 30 mg/kg diet. Interestingly, pigs under the present experimental conditions did not show any clinical vitamin E deficiency lesions, even when fed on the diet containing less than 2 mg vitamin E/kg and 100 g SO/kg. In fact, most rations for weaners contain at least 40 mg/kg and frequently considerably more, but the spontaneous incidence of vitamin E deficiency diseases is still reported (E. T. Thornton, personal communication; Nielson et al. 1989). Thus, it seems that the assessment of vitamin E requirements for pigs based on clinical signs or mortality alone may not be appropriate.

Van Vleet (1982) reported that mortality and lesions of vitamin E deficiency developed when vitamin E concentrations in plasma are below 0·4 $\mu$g/ml, and this value has been used by other investigators as a threshold for deciding whether pigs are deficient in vitamin E (Rammell et al. 1988). However, in the study of Jensen et al. (1988) it was reported that the mortality and lesions of vitamin E deficiency still occurred in pigs with plasma concentrations above 1 $\mu$g/ml. By contrast, pigs in the present study did not show any clinical deficiency signs, although plasma vitamin E concentrations were below 0·2 $\mu$g/ml. In view of these observations, vitamin E concentrations in plasma may not be a sensitive indicator of the adequacy of dietary vitamin E intake.

The primary function of vitamin E is believed to be the protection of unsaturated lipids in biological membranes from peroxidation. Inadequate protection from vitamin E may result in a chain reaction of lipid peroxidation leading to disruption of normal membrane function, with eventual dissolution of cellular compartmentation. The degraded products of lipid peroxidation include ethane, pentane, MDA, fluorescent substances and other chemiluminescent substances (Slater, 1984). Based on the relationship between vitamin E and lipid peroxidation, it appears that the measurement of lipid peroxidation status in pigs could provide a reliable and accurate index of the vitamin E protection of the animal and, thus, would be a means for establishing the requirement for this nutrient.

The important role of dietary vitamin E in retarding lipid peroxidation was indicated by the elevated levels of TBA-reactive substances of RBC from pigs fed on the diet without vitamin E supplementation. Although it is believed that the value for the lipid peroxidation product MDA may be overestimated in the TBA-reaction assay, numerous studies have clearly demonstrated that this assay is useful for measuring the peroxidative state of biological systems (Buckingham, 1985; Lee & Csallany, 1987). Results of other studies in pigs indicate that levels of TBA-reactive substances only change in response to a dietary vitamin E supplement, but not Se (Fontaine & Valli, 1977; Jensen et al. 1983). In the first experiment of the present study the reactive substances did not differ between the two vitamin E-supplemented groups, implying that dietary supplementation with vitamin E at 20 or 100 mg/kg provided equivalent protection for pigs against lipid peroxidation damage. In the second experiment, pigs not supplemented with vitamin E had higher levels of TBA-reactive substances when fed 100 g SO/kg compared with 10 g SO/kg, which suggests the lipid peroxidation is not only affected by dietary vitamin E, but also by the

level of PUFA in the diet, since SO contains about 700 mg PUFA/g (Cera *et al.* 1988). This was anticipated, as the fatty acid profile of animal tissues is influenced by both quality and quantity of fat fed to animals (Lin *et al.* 1989); as the level of dietary PUFA increases, more lipid peroxidative products will be generated in tissues if the protection by vitamin E is inadequate.

The data from both experiments of the present study showed that an increase in exhaled hydrocarbons was found in pigs not supplemented with vitamin E and the outputs of these gases decreased with vitamin E supplementation. This finding followed the pattern of levels of TBA-reactive substances. Since an open system was used for the collection of hydrocarbons in Expt 1 and a closed system in Expt 2, the results from these two experiments were not strictly comparable. The values for exhaled hydrocarbons, together with those for TBA-reactive substance, indicate that pigs fed on unsupplemented diets are deficient in vitamin E. It could be argued that the method of collection of exhaled gases used in the present study may be confounded by background gases from the gastrointestinal tract. Studies in humans (Lemoyne *et al.* 1987) and rats (Kivits *et al.* 1981) do not support this view. In the present study the background gases were measured before and after each days's assay, and concentrations of ethane or pentane were calculated by subtracting the background levels. In fact the background level of hydrocarbon gases was constant during the experimental period. Although the procedure for measuring hydrocarbons in exhaled gases was non-invasive, there were some drawbacks involved in the procedures of sample collection and analysis, including complicated collection and sampling apparatus, the storage of sample and the effect of a large volume of sample on the efficiency of the gas-chromatography column. Improvements in these aspects would be an advantage in measuring hydrocarbons in exhaled gases as the index of the state of lipid peroxidation and the protection by antioxidants.

Meydani *et al.* (1987) reported that mice fed on a diet supplemented with unsaturated fat maintained lower plasma and tissue tocopherol concentrations than those fed on diets containing fat with a lower degree of unsaturation. Results in Expt 2 showed that tissue $\alpha$-tocopherol concentrations were highest in the group given 10 g SO/kg diet, followed by those given 100 g tallow/kg, and then those given 100 g SO/kg diet. This reflected the differences in the PUFA level between diets as SO and tallow contain PUFA concentrations of about 700 and 200 mg/g respectively (Cera *et al.* 1988; Thi-Dinh *et al.* 1990). Since vitamin E ($\alpha$-tocopherol) concentrations in these diets were almost identical, the lower plasma and tissue $\alpha$-tocopherol concentrations in the pigs fed on the 100 g SO/kg diet were not due, therefore, to oxidative loss of tocopherol in the diet, but to enhanced post-absorptive utilization of vitamin E in the pigs. Absorption studies indicate that the absorption of vitamin E in pigs is not influenced by the dietary source and amount of fat when the diet contains more than 10 g fat/kg (Wang & Leibholz, 1990). Thus, enhanced turnover of vitamin E in pigs is the mechanism for decreased vitamin E status in pigs with an increased PUFA intake.

Values for both TBA-reactive substances and exhaled hydrocarbons gases in Expt 1 suggest that a supplement of 20 mg DL-$\alpha$-tocopheryl acetate/kg diet is adequate for pigs under the present experimental conditions. However, pigs on the diet supplemented with this level of vitamin E and 100 g SO/kg in Expt 2 showed a significant increase in TBA-reactive substances and ethane exhalation compared with pigs given the diet containing 10 g SO/kg or 100 g tallow/kg. Plasma $\alpha$-tocopherol concentrations in this group of pigs also showed a continuous decline after 14 d on the experimental diet. These results suggest that dietary vitamin E at this level is inadequate for pigs fed on diets containing 100 g SO/kg. Supplementation with 50 mg vitamin E/kg diet, however, provided adequate protection against the lipid peroxidation damage.

It should be noted that SO used in Expt 2 were partially stripped of vitamin E, particularly α-tocopherol. Since vegetable oils are rich sources of vitamin E it could be argued that the increased requirement for vitamin E with an increase in dietary PUFA is often met, at least in part, from the oils themselves. Nevertheless, a number of factors need to be considered if the contribution of vitamin E from oils is to be counted in the dietary vitamin E content. First, the vitamin E content in oils of different origins varies considerably (Speek *et al.* 1985). Second, the natural form of vitamin E in diets is not stable and significant losses often occur during processing and storage. Recent studies by Dove & Ewan (1990, 1991) reported that excessive addition of trace minerals in pig diets caused rapid destruction of natural forms of vitamin E (α- and γ-tocopherols), and the presence of fat in the diet accelerated the loss of this vitamin. Third, some oils contain mainly vitamin E isomers other than α-tocopherol (Speek *et al.* 1985; Ball, 1988), and these isomers may not make a significant contribution to the total dietary biological activity of vitamin E for pigs (Bieri & McKenna, 1981).

In conclusion, the present study demonstrated that lipid peroxidation status of pigs is a sensitive index with which to evaluate vitamin E requirements. The vitamin E and lipid peroxidation status of pigs are not only affected by dietary levels of vitamin E, but also by dietary PUFA. The requirement for vitamin E in young pigs increases as PUFA levels in the diet increase; a level of 20 mg vitamin E/kg diet is inadequate when the diet contains 100 g SO (70 mg PUFA/g)/kg.

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