Equine Exercise Physiology 3: 315–322, 1991

The Effect of Vitamin E on Membrane Integrity during Submaximal Exercise

K. H. PETERSSON,[1] H. F. HINTZ,[1,2] H. F. SCHRYVER[2] and G. F. COMBS, Jr[3]

[1]*Department of Animal Science,* [2]*Department of Clinical Sciences,* [3]*Department of Poultry and Avian Sciences, Cornell University, Ithaca, New York 14853, USA*

ABSTRACT. The effect of vitamin E (VE) on membrane integrity in horses was studied. Eight Standardbred horses were assigned to either exercise (3 times wk^{-1}) or non-exercise groups. All horses were fed low dietary levels of VE for 4 months (Period 1) followed by 1 month (Period 2) of VE supplementation. Blood was taken weekly for determination of glutathione peroxidase, red blood cell hemolysis, VE, ascorbic acid, creatine kinase, aspartate aminotransferase and total lipids. Muscle was analyzed weekly for VE and thiobarbituric acid reactive substances (TBARS). Expired air was analyzed for ethane and pentane. Standardized exercise tests (SET) were conducted prior to the start of the trial, between periods and at the end of the trial. No clinical signs of a VE deficiency were observed. Plasma VE was repressed over time during Period 1 in the exercised horses ($p<0.01$). Ascorbic acid levels were lower ($p<0.05$) in the exercised horses throughout. No breath ethane was detected. Pentane measurements varied considerably between horses. A diet low in VE did not compromise performance on SET.

Key words Horses; ascorbic acid; pentane; lipid peroxidation; exercise.

INTRODUCTION

Increased metabolic activity, such as occurs with physical activity, has been suggested to increase production of destructive oxygen radical species (free radicals), and thus increase demands upon cellular antioxidant systems which include free radical scavenging enzymes, water- and lipid-soluble antioxidants.[16] Vitamin E (VE) is the only antioxidant that can scavenge free radicals within cellular membranes. Vitamin E deficient liver and muscle mitochondria are more susceptible to losses in transmembrane potential, electron transport and respiratory control, as well as increased lipid peroxidation.[17]

The cellular requirements for VE in the horse have not been determined. The objective of this study was to determine if submaximal exercise would compromise membrane integrity and subsequent performance in horses fed a low VE diet.

METHODS AND MATERIALS

Design

Ten adult Standardbreds were assigned to an exercise or non-exercise (control) group. The exercised horses were pre-conditioned to the required work load. The control horses were not previously conditioned. In the first period (4 months), all horses were fed a low VE diet. During the second period (1 month), the diet was supplemented with VE. Horses in the exercised group were worked 3 times per week for 30 min at an average speed of 7 m s^{-1}. Blood was taken weekly via jugular puncture from fasted animals every Monday between 0800–0900 hours. Whole blood was analyzed for selenium-dependent glutathione peroxidase (SeGSHpx) and red blood cell hemolysis. Plasma content of VE, creatine kinase (CK), aspartate aminotransferase (AST), ascorbic acid, cholesterol, triglycerides and phospholipids was determined. Breath samples were taken weekly, starting

Table 1. *Composition of basal concentrate*

	%
Autoclaved oats	59.0
Autoclaved soybean meal	20.7
Fat[a]	7.9
Molasses	9.9
Vitamin/Mineral mix[b]	2.5

[a] Booster fat; animal fat specially prepared without antioxidants (Balanced Energy Co., Clinton, IA).

[b] Per kg concentrate: vitamin A, 2660 IU; vitamin D, 399 IU; thiamin, 6.65 mg; riboflavin, 2.66 mg; folacin, 2.2 mg; sodium selenite, 0.423 mg; limestone, 5.26 g.

with Week 13 of Period 1, for the analysis of ethane and pentane, indices of lipid peroxidation. Muscle was analyzed weekly for VE and thiobarbituric reactive substances (TBARS). At the start of the trial, between periods and at the end of the trial, standardized exercise tests (SET) were conducted to measure any changes in performance. The test that each horse performed at the start of the trial was repeated in the following tests.

Diet

The low VE diet consisted of autoclaved oats and soybean meal (18 h, 104°C) supplemented with fat, vitamins (except E) and minerals (Table 1). Wheat straw was fed free choice. The horses had previously been fed sweet feed and timothy hay. Analyses are shown in Table 2.

Exercise program

The objective of the exercise program was to maintain the horses at their current level of fitness. To equalize workouts between horses, a standard work effort was calculated

Table 2. *Chemical composition of concentrates and forages fed prior to and during the trial*[a,b]

	Pre-trial		Depletion		Repletion	
	Grain	Hay	Grain	Straw	Grain	Straw
DM, %	91.6	89.3	90.7	95.5	89.5	95.1
DE, Mcal kg^{-1}[c]	3.30[b]	1.59	3.31	1.48	3.31	1.48
CP, %	14.4	8.5	18.2	4.1	16.3	4.3
Ca, %	0.38	0.43	0.35	0.34	0.41	0.32
P, %	0.39	0.21	0.42	0.11	0.40	0.10
Mg, %	0.17	0.12	0.18	0.08	0.16	0.10
K, %	0.75	1.56	0.95	0.53	0.81	0.84
Na, %	0.237	0.005	0.033	0.015	0.043	0.015
Fe, ppm	145	57	98	87	136	67
Zn, ppm	63	18	44	21	40	12
Cu, ppm	8	5	13	7	11	5
Mn, ppm	45	35	43	52	44	85
Se, ppm[d]	0.30	0.05	0.26	0.04	0.36	0.07
Vit. E[e], IU kg^{-1}	13.1	53.0	7.1	17.7	76.6	16.2

[a] As fed basis.

[b] Analysis conducted by New York State Dairy Herd Improvement Cooperative, Ithaca, NY.

[c] NRC, Nutrient Requirements of Horses, 1989.

[d] Analysis conducted by Poultry Science, Cornell University, Ithaca, NY.

[e] Analysis conducted by Hoffman LaRoche, Nutley, NJ.

Table 3. *Various parameters related to vitamin E status in horse*[a]

	Period 1[b]	Period 2[c]
Plasma vitamin E ($\mu g\ ml^{-1}$)	1.81 ± 0.08	2.59 ± 0.09
Plasma vitamin E ($mg\ g^{-1}$ lipid)	0.66 ± 0.03	0.89 ± 0.03
Ascorbic acid ($\mu g\ ml^{-1}$)	3.84 ± 0.16	3.75 ± 0.14
Total lipids ($mg\ dl^{-1}$)	283.0 ± 9.9	298.6 ± 6.0
Creatine kinase ($U\ l^{-1}$)	110 ± 5	102 ± 5
Aspartate aminotransferase ($U\ l^{-1}$)	153 ± 5	137 ± 3
Glutathione peroxidase ($U\ g^{-1}$ Hb)	32.65 ± 0.91	39.00 ± 0.91
TBARS (pmoles mg^{-1} muscle)	202 ± 44	134 ± 20
Muscle vitamin E ($\mu g\ g^{-1}$)	2.09 ± 0.13	3.03 ± 0.17
Expired pentane (pmoles l^{-1} air)	383 ± 49	297 ± 26

[a] Mean ± SEM.
[b] Low vitamin E.
[c] Vitamin E supplemented.

at the start of the trial using the average weight of the exercised horses and a speed that all horses could maintain for the designated 30 min. Each exercise day the horses were weighed prior to their workout and the speed of the treadmill (Säto, Uppsala, Sweden) was adjusted to achieve the desired work effort.

Standardized exercise test

The test began with a 3 min warm-up period ($4\ m\ s^{-1}$). In the last minute of the warm-up period the treadmill was inclined to 5°. Speed was then increased each minute until the horses could no longer maintain treadmill speed. Heart rate was taken continuously (Heartwatch Model 8799, Computer Instruments Corp., Hempstead, NY). Blood was taken via jugular catheter during the last 15 seconds at each speed for lactate determination.

Blood analyses

Plasma CK, AST, cholesterol, triglycerides and phospholipids were assayed using commercially available kits. Cholesterol, triglycerides and phospholipids were summed together to obtain an estimate of total lipid in each sample in order to analyze plasma VE on a lipid basis. Plasma lactate was determined using an automated blood chemistry analyzer (YSI, Yellow Springs, OH, Model 27). Plasma ascorbic acid was measured by the dinitrophenylhydrazine method.[3] Plasma VE was determined fluorometrically using high performance liquid chromatography[7] without the use of detergent in the extraction. Wavelength settings were 295 nm and 330 nm for excitation and emission, respectively. The mobile phase utilized was methanol and water (98:2), with a flow rate of $2\ ml\ min^{-1}$. A reverse phase C-18 column was used. Red blood cell fragility was deter-

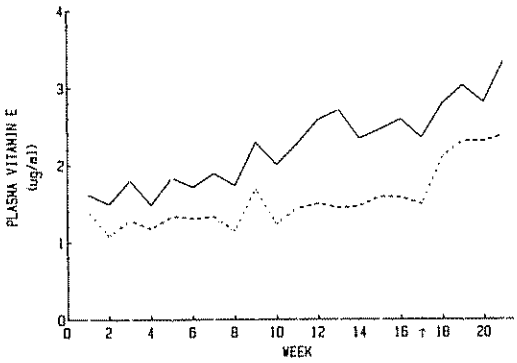

Fig. 1 Plasma vitamin E over time ($p<0.01$) in exercised (---) and non-exercised (—) horses during Period 1 and Period 2 (arrow indicates the start of period 2). SEM for Period 1 exercised (0.13), control (0.10); Period 2 exercised (0.15), control (0.12).

mined by the erythrocyte peroxide hemolysis test.[11] Whole blood SeGSHpx was determined using established procedures.[15]

Breath analysis by gas solid chromatography

Exhaled breath was collected weekly into 5 l gas tight sampling bags. One liter of air was loaded onto a pre-column.[8] Nitrogen was used as the carrier gas. The injector temperature on the gas chromatograph was 150°C, the detector temperature was 200°C. The pre-column was heated for 8 min (180°C), during which the oven maintained a temperature of 40°C. The heater was then turned off and the oven temperature was raised to 190°C at a rate of 15°C min^{-1}. Ethane and pentane were detected at 6.25 and 27.5 min, respectively. Standards for the hydrocarbons were N-paraffins in nitrogen (Supelco, Bellafonte, PA). Quantification of each sample was determined using the peak height.

Muscle analyses

Muscle biopsies were taken weekly from the gluteus medius muscle[9] and stored at −70°C. The lipid extract for VE analysis was prepared according to established procedure.[17] A modification of the procedure of Uchiyama and Mihara[19] was used for the thiobarbituric acid test for determination of TBARS. Modifications were as follows: tissue was homogenized in 0.174 M KCL, 0.025 M Tris-HCL, pH 7.4. A 15 min preincubation with 1.5 mM ascorbic acid at a ratio of 1 part homogenate: 2 parts ascorbic acid in buffer: 3 parts buffer was used to stress the tissue. A zero time control for the pre-incubation was determined.

Statistical analysis

Data were analyzed using Analysis of Variance for repeated measures with least squares analyses of the means. Each period was analyzed separately. Exercise was considered the treatment. Differences due to treatment were tested using the horse by treatment interaction as the error term. The interaction of interest was the time by treatment interaction. Data presented are least square means with the standard errors of the least square means.

RESULTS

No data were used from two horses from the exercised group removed from the trial due to injuries unrelated to the study. Table 3 contains the mean of all observations during each period for parameters measured. All parameters were analyzed at the start of the trial. CK activities and weight were found to be significantly different between groups ($p<0.05$).

A significant interaction of treatment by time during Period 1 was found whether VE was analyzed on a volume basis (μg ml^{-1}) ($p<0.01$) or on a lipid basis (mg g^{-1} lipid) ($p<0.0001$) (Figs. 1 and 2). No significant differences were found between treatments during Period 2. Exercised horses had lower ($p<0.05$) plasma ascorbic acid values during Periods 1 and 2 (Fig. 3).

CK activities were different ($p<0.05$) between groups at the start of the trial (control, 128 ± 6 U l^{-1}; exercise, 183 ± 8 U l^{-1}). This difference was not present prior to the preconditioning period (control, 113 ± 14 U l^{-1};

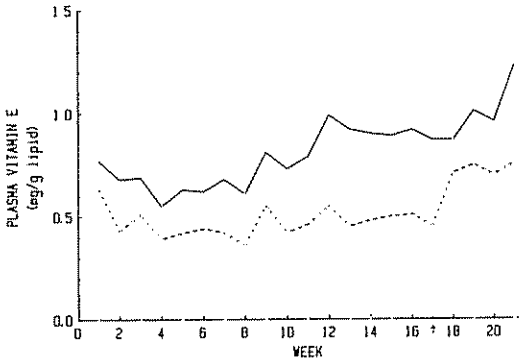

Fig. 2. Plasma vitamin E over time ($p<0.0001$). Legend as in Fig. 1. SEM for Period 1 exercised (0.05), control (0.04); Period 2 exercised (0.05), control (0.04).

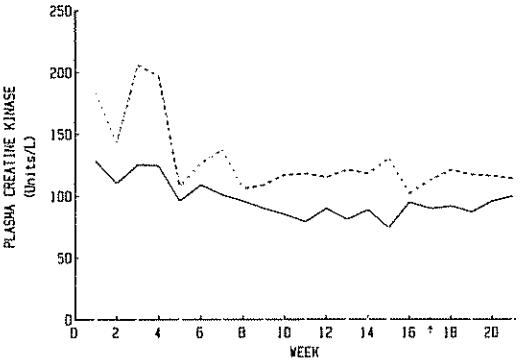

Fig. 4. Plasma creatine kinase between treatments ($p<0.05$), over time ($p<0.0001$). Legend as in Fig. 1. SEM for Period 1 exercised (8), control (6); Period 2 exercised (9), control (7).

exercise, 124 ± 18 U l^{-1}). CK activities of the exercised group were higher than the controls during Periods 1 and 2 ($p<0.05$) (Fig. 4). Treatment by time interaction was significant ($p<0.0001$) during Period 1. There was a significant interaction ($p<0.01$) in treatment by time in AST values (Fig. 5). No difference was found in Period 2.

No evidence of red blood cell hemolysis was found during Period 1 or 2. No changes occurred in SeGSHpx or in total lipid concentrations. No significant differences due to exercise were found in muscle VE or TBARS in either period. When muscle VE levels were plotted with TBARS a trend towards an inverse relationship was observed during Period 2 (Fig. 6).

Breath pentane measurements were not found to be significantly different between groups in either period. No ethane was detected in the breath samples of the horses. The mean pentane level in environmental air was 80 pmoles l^{-1} of air.

Lactate concentrations at 8 m s^{-1} in the first SET (10.55 ± 0.06 mmol l^{-1}) were greater ($p<0.01$) than the second (8.05 ± 0.43 mmol l^{-1}) and third SET (8.06 ± 0.43 mmol l^{-1}).

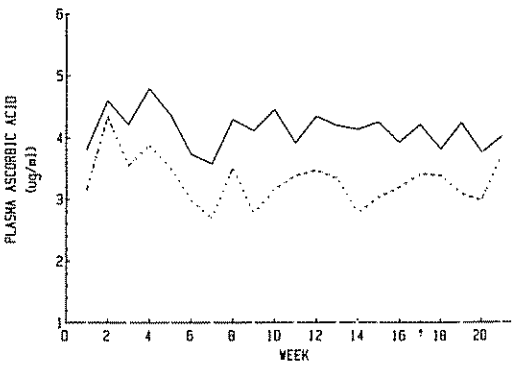

Fig. 3. Plasma ascorbic acid over time ($p<0.05$). Legend as in Fig. 1. SEM for Period 1 exercised (0.26), control (0.20); Period 2 exercised (0.22); control (0.17).

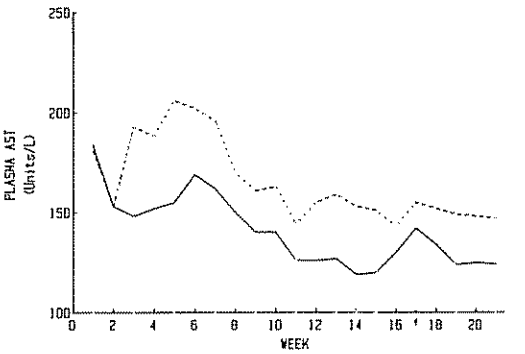

Fig. 5. Plasma aspartate aminotransferase over time ($p<0.01$). Legend as in Fig. 1. SEM for Period 1 exercised (8), control (6); Period 2 exercised (4), control (3).

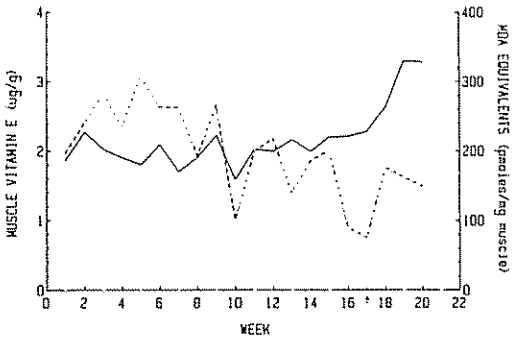

Fig 6. Muscle vitamin E (– – –) and TBARS (——) over time (arrow indicates start of period 2) SEM for vitamin E in Period 1 (0.14) and Period 2 (0.18). SEM for TBARS in Period 1 (45) and Period 2 (17).

DISCUSSION

Our objective was to determine if a diet low in VE fed for an extended period of time would compromise membrane integrity in the exercised or non-exercised horse and if so, would compromise the horses' overall performance.

SETs were utilized to monitor any performance changes that might occur over the course of the study. If the low VE diet alone or in combination with exercise had compromised the horses overall performance the lactate levels of the SET following Period 1 might be expected to be higher, than either the first or the third SET. An effect due to training or acclimatization to the treadmill were ruled out. All horses were previously trained to the treadmill as well as having undergone a stress test four months prior to the start of the study. The first SET took place in January. The second and third SETs occurred in May and June, respectively. The treadmill room temperature was consistent from test to test however the environmental temperatures differed considerably from SET 1 to SETs 2 and 3. Perhaps the elevation in lactate concentration during the SET in January was due to animals living in a cold environment performing strenuous work in a warm environment whereas in May and June environmental temperatures and treadmill room temperatures were equivalent.

The diet in Period 1 contained much lower levels of VE than what the horses had been fed previously. The dietary VE concentration during Period 1 is considered inadequate according to NRC recommendations.[13] The plasma VE values in this study were similar to previously reported values of horses fed a VE insufficient diet.[10,18]

A drop in plasma and tissue VE would be expected in horses fed a diet totally deficient in VE. If a VE deficient diet was coupled with exercise, a further decline in VE might occur as was seen in VE deficient, endurance trained rats.[1] The exercise program in this study was not rigorous. However, the cumulative effect of the exercise over 4 months of low VE intake was seen in the significantly different responses in plasma VE between the control and exercised group over time. With the exception of two horses in the control group, all horses had been off pasture for 1.5 years prior to the start of the study. The mean plasma VE value at the start of the study was 1.54 $\mu g\ ml^{-1}$ or 0.72 $mg\ g^{-1}$ lipid, still considered to be in the deficient range. A previous VE depletion/repletion study reported that the plasma VE of horses during a 2.5 month depletion period did not fall below 0.50 $mg\ g^{-1}$ lipid.[18] The corresponding muscle VE content during the depletion was approximately 4 $\mu g\ g^{-1}$, twice that observed in this study. These data indicate that plasma VE might not be a good indicator of muscle VE stores. Perhaps the horses, prior to the start of the study, had depleted their muscle VE stores to the extent that when the dietary level was reduced further there was no corresponding decline in either plasma or VE status.

The ascorbic acid levels in the exercised horses were consistently lower than the non-exercised horses throughout the study. Since horses have the necessary enzyme, galunolactone oxidase, required to synthesize ascorbic acid, a depletion in levels over time would not be expected unless the system was so stressed that intrinsic production of the

vitamin was inadequate to meet needs. Active tocopherol can be regenerated by ascorbic acid[2,14] *in vitro*, therefore it is tempting to theorize that a portion of ascorbic acid synthesized by the horse was being used to recycle VE in the exercising horse thus offsetting a potential decline in plasma Ve status.

Whole blood levels of glutathione peroxidase were monitored to evaluate selenium status because of the relationship between selenium, as a component of SeGSHpx and VE as a cellular antioxidant. Glutathione peroxidase activity in tissues has been shown to be a reliable indicator of selenium status.[4]

The thiobarbituric acid test was used to assess changes in the muscle tissue by measuring the potential for peroxidation in the weekly tissue samples. Vitamin E can quench the formation of TBARS.[12] Whether TBARS would decline to insignificant levels if VE status increased requires further research.

Despite the relatively low plasma and muscle VE values, a clinical deficiency of VE was not apparent as there was no evidence of red blood cell hemolysis, nor clinically abnormal levels of CK or AST. CK and AST are indicators of damage occurring in the muscle and liver, respectively. If the increased CK and AST in the exercised horses represent modest levels of ongoing membrane destruction then they would correlate with the modest levels of pentane and TBARS measured.

This is the first report of pentane and ethane measurement in horses. Expired hydrocarbons increase in conjunction with lipid peroxidation[20] and VE deficiency.[6] A decrease in expired pentane occurred in exercising humans after VE supplementation.[5] The pentane values in the horse, although not significantly affected by treatment, were still above environmental concentrations of pentane, but there was considerable variation between horses and among weeks. Perhaps refinement of techniques could reduce the variation and pentane could be used as an index of lipid peroxidation in the horse. Thus, further studies are needed to determine if pentane would be produced regardless of the VE status of the horse or whether a complete quenching of pentane (i.e. lipid peroxidation) could be accomplished by VE or antioxidant supplementation. Since lipid peroxidation has been implicated in the aging process one questions what impact even basal levels of lipid peroxidation would have over an animal's or, more importantly, a human's lifetime.

This study has demonstrated that horses can tolerate low dietary levels of VE for at least 4 months without any clinical signs of a deficiency. A repressive effect of exercise on VE levels was shown, however, plasma VE was shown to be an unreliable indicator of muscle VE stores. Exercise significantly depressed plasma ascorbic acid. Further research needs to be conducted in order to determine the VE requirement of horses at the cellular level.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the financial support of the Harry M. Zweig Memorial Fund for Equine Research, Hoffman LaRoche Inc., Purina Mills Inc., and Agway Inc. The technical assistance of Vincent Soderholm was very much appreciated.

REFERENCES

1 Aikawa, K. M., Quintanilha, A. t., deLumen, B. O., Brooks, G. A. and Packer, L. (1984). Exercise endurance-training alters vitamin E tissue levels and red-blood-cell hemolysis in rodents. Bioscience Reports 4, 253–257.

2 Bascetta, E., Gunstone, F. D. and Walton, J. C. (1983). Electron spin resonance study of the role of vitamin E and vitamin C in the inhibition of fatty acid oxidation in a model membrane. Chem. Phys. Lipids 33, 207–210.

3 Bessey, O. A., Lowry, O. H. and Brock, M. J. (1947). The quantitative determination of ascorbic acid in small amounts of white blood cells and platelets. J. Biol. Chem. 168, 197–205.

4 Caple, I. W., Edwards, S. J. A., Forsyth, W. M., Whiteley, P., Selth, R. H. and Fulton, L. J. (1978). Blood glutathione peroxidase activity in horses in

relation to muscular dystrophy and selenium nutrition. Aust. Vet. J. 54, 57–60.
5. Dillard, C. J., Litov, R. E., Savin, W. M., Dumelin, E. E. and Tappel, A. L. (1978) Effects of exercise, vitamin E, and ozone on pulmonary function and lipid peroxidation. J. Appl. Physiol. 45, 927–932.
6. Dillard, C. J., Dumelin, E. E. and Tappel, A. L. (1977). Effect of dietary vitamin E on expiration of pentane and ethane by the rat. Lipids 12, 109–114.
7. Lang, J. K., Gohil, K. and Packer, L. (1986) Simultaneous determination of tocopherol, ubiquinols and ubiquinones in blood, plasma, tissue homogenates and subcellular fractions. Anal. Biochem. 157, 106–116.
8. Lawrence, G. D. and Cohen, G. P. (1982). Ethane exhalation as an index of *in vivo* lipid peroxidation: concentrating ethane from a breath collection chamber. Anal. Biochem. 122, 283–290.
9. Lindholm, A. and Piehl, K. (1974). Fibre composition, enzyme activities and concentration of metabolites and electrolytes in muscle of Standardbred horses. Acta Vet. Scand. 15, 287–309.
10. Liu, S. K., Dolensek, E. P., Adams, C. R. and Tappe, J. P. (1983). Myelopathy and vitamin E deficiency in six Mongolian wild horses. J. Am. Vet. Med. Ass. 183, 1266–1268.
11. Lubin, B. H., Baehner, R. L., Schwartz, E., Shohet, S. B. and Nathan, D. G. (1971) The red cell peroxide hemolysis test in the differential diagnosis of obstructive jaundice in the newborn period. Pediatrics 48, 562–565.
12. Nakamura, T. and Hishinima, I. (1978). Protective effect of tocopherol on the formation of TBA-reactive materials on rat liver. *In* deDuva, C. and Hayarishi, O. (eds.): *Tocopherol, Oxygen and Biomembranes*. Elsevier/North Holland Biomedical Press, pp. 95–108.
13. N. R. C. (1989) *Nutrient Requirements of Horses*, 5th rev. ed., National Academy Press, Washington, D.C.
14. Packer, J. E., Slater, T. F. and Willson, R. L. (1979) Direct observation of a free radical interaction between vitamin E and vitamin C. Nature 278, 737–738.
15. Paglia, D. E. and Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 70, 158–169.
16. Quintanilha, A. T. and Packer, L. (1983) Vitamin E, physical exercise and tissue oxidative damage. *In Biology of Vitamin E*. Proc. Ciba Foundation Symp. 101, 56–59.
17. Quintanilha, A. T., Packer, L., Davies, J. M. S., Racanelli, T. L. and Davies, K. J. A. (1982) Membrane effects of vitamin E deficiency: Bioenergetic and surface charge density studies of skeletal muscle and liver mitochondria. *In* Lubin, B. and Machlin, L. J. (eds.): *Vitamin E: Biochemical, Hematological and Chemical Aspects*. Ann. N.Y.A.S. 393, 32–46.
18. Roneus, B. O., Hakkarainen, R. V. J., Lindholm, C. A. and Tyopponen, J. T. (1986) Vitamin E requirements of adult Standardbred horses evaluated by tissue depletion and repletion. Equine Vet. J. 18, 50–58.
19. Uchiyama, M. and Mihara, M. (1978) Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal. Biochem. 86, 271–278.
20. Wade, C. R. and van Rij, A. M. (1985) *In vivo* lipid peroxidation in man as measured by the respiratory excretion of ethane, pentane and other low-molecular-weight hydrocarbons. Anal. Biochem. 15, 1–7.