Protective Effect of Vitamin E in a Rat Model of Focal Cerebral Ischemia*

M. Stohrer[a], Andrea Eichinger[a], M. Schlachter[b], M. Stangassinger[a]

[a] Institute for Physiology, Physiological Chemistry and Animal Nutrition, University of Munich, Veterinärstr. 13, 80539 Munich, Germany
[b] F. Hoffmann-La Roche Ltd, Department of Vitamins and Fine Chemicals, 4070 Basel, Switzerland

Z. Naturforsch. **53c,** 273–278 (1998); received December 19, 1997/January 22, 1998

Vitamin E, Middle Cerebral Artery Occlusion, Rat, Brain Infarct Volume, Oxygen Radicals

Under certain pathological conditions such as cerebral ischemia and reperfusion the occurrence of free radicals is remarkably increased. However, only very little information is available on their quantitative relevance for the pathophysiology and final outcome of diseases. The aim of the present study was to evaluate the contribution of oxygen radicals in the pathogenesis of a stroke. For this purpose a rat model for stroke was used. Two of three vitamin E deficient groups were repleted with different dosages of DL-α-tocopherylacetate. No signs of vitamin E deficiency could be observed. However, the weight gain during repletion was increased in the vitamin E repleted groups. Brain infarction was created by occlusion of the right middle cerebral artery (MCAO) for two hours. After 24 hours the measurements of infarct volumes were taken. The infarct volume of the group with the highest repletion dosage was significantly reduced by 81%. This was also expressed in a higher rate of gait disturbances after MCAO of the deficient animals. The control of vitamin E status exhibited a similar repletion-dependent level in plasma and brain. These results strongly support the hypothesis that the generation of oxygen radicals occurring during reperfusion is an important aspect of the pathophysiological mechanism in brain infarction.

Introduction

Strokes are one of the three major causes of death in our civilization. The available treatment of patients is only symptomatic. Recently, some pathophysiological mechanisms determining the pathogenesis and final outcome are becoming more and more evident. One of these mechanisms is the generation of oxygen radicals. It is evident that free radicals cause tissue damage by reacting with polyunsaturated fatty acids in cellular membranes, critical sulfhydryl bonds in proteins and nucleotides in ribonucleic acids (Machlin and Bendich, 1987). There are various pathological conditions under which radical damage is described. Asphyxia (Pourcyrous *et al.,* 1990), cerebral ischemia and reperfusion (Armstead *et al.,* 1988; Dietrich, 1994; Hall *et al.,* 1995), seizures (Armstead *et al.,* 1989), cerebral contusion (Wei *et al.,* 1989), acute hypertension (Kontos, 1981) and alcohol abusus (Mufti *et al.,* 1993) induce significant generation of oxygen radicals. However, their quantitative relevance for the pathophysiology and final outcome is obscure.

The objective of the present study was to evaluate the contribution of oxygen radicals in the development of strokes indirectly by changing the antioxidative status of membranes. For this purpose a rat model was used to study experimentally induced stroke. The significance of oxygen radicals for tissue damage was evaluated by comparing study groups which differed in their antioxidant protection by vitamin E.

Materials and Methods

Animals and diets

All experiments were performed on adult male Ibm RORO rats (350–450 g), a specific pathogen free strain from the Biological Research Laboratories Ltd., CH-4414 Fuellinsdorf, Switzerland. Animals were divided into three experimental groups receiving different vitamin E doses. It is known that

* This contribution was presented at a Workshop on the occasion of the 20th anniversary of the "Oxygen Club Munich", organized by M. Saran and E. F. Elstner.

Reprint requests to Dr. M. Stohrer.
Telefax: 0049–89/344937.
E-mail: stohrer@tiph.vetmed.uni-muenchen.de.

0939–5075/98/0300–0273 $ 06.00

the kinetics of α-tocopherol uptake and depletion is dose, time and tissue dependent and it is almost impossible to saturate brain tissue with α-tocopherol (Machlin and Gabriel, 1982; Goss-Sampson and Muller, 1988; Clément *et al.*, 1995). Therefore, the content of vitamin E in various organs is highly variable between individual rats. In order to achieve a standardized basic status, the animals were fed a vitamin E-free diet over a 6 month period and were subsequently repleted by a daily p.o. application of DL-α-tocopherylacetate diluted in a triglyceride oil, for a duration of 6 weeks.

Animals of the first group, DR_0 (**d**epleted rats **r**epleted with **0** mg vitamin E / kg body weight) were not repleted. They received only the vehicle (the triglyceride oil without vitamin E). The rats of the second group, DR_1, were repleted daily with **1** mg / kg body weight (according to the minimal recommended dose of 30 mg vitamin E / kg dry food; Scott, 1978) for the 6 week period. The last group, DR_{100}, received **100** mg vitamin E / kg body weight. All animals had free access to food and tap water.

Operative and experimental procedures

After fasting the animals overnight, brain infarction was generated by transient occlusion of the right middle cerebral artery (MCAO), by a modified intraluminal filament occlusion model as described by Memezawa et al (1992). Anesthesia was induced by inhalation of isoflurane in oxygen in spontaneously breathing animals. Body temperature was monitored and a normal value was kept constant with a heated operating table. Blood pressure and body temperature were monitored continuously. A catheter was inserted into a tail artery in order to record blood pressure (via connection to a pressure transducer, model P 23 XL, Ohmeda Medical Devices Division, Oxnard CA, USA), and to sample blood for blood gas and vitamin E analysis. Arterial blood gases and pH (AVL 990 – S, AVL Medizintechnik GmbH, Bad Homburg, Germany) were determined immediately upon the initiation, after 1 hour and then again after 2 hours of MCAO. A surgical midline incision was made to expose the right common, internal and external carotid arteries. The external carotid and the occipital arteries were ligated. The common carotid artery and the internal carotid artery were closed by microvascular clips. A small incision was then made in the external carotid artery, and the MCA-occluding device (prolene suture; 1.5 metric) was inserted and pushed forward 19 mm via the internal carotid artery. The occluder filament was advanced to close the origin of the MCA. Following a two hour period of ischemia, the filament was withdrawn to allow recirculation of the ischemic focus. 24 hours later the measurements of infarct volumes were completed.

Measurement of α-tocopherol

Vitamin E in blood plasma was measured according to the method of Vuilleumier *et al.* (1983). Plasma samples were extracted with n-hexane, evaporated under nitrogen and resolved in methanol / ethanol (80:20; v/v). The HPLC quantification was performed using a reversed phase C 18 column Lichrosorb RP-18, 5 μm, 125 x 4 mm. Pure methanol was used as mobile phase and a Merck F 1000 fluorometer for signal detection (Darmstadt, Germany; with excitation at 290 nm and emission at 330 nm). Concentration of vitamin E in brain tissues was determined by a modified method of Westerberg *et al.* (1981). The tissue was homogenized with a mixture of aqua bidest. and ethanol (50:50; v/v) containing butylated hydroxytoluene (0.004%). After extraction with n-hexane, evaporation under nitrogen and resolving in methanol / ethanol (80:20; v/v), the samples were analyzed with the HPLC system described above. The only difference was that the mobile phase contained 3% water in methanol.

Measurement of infarct volume

After 24 hours of reperfusion the animals were reanesthetized by isoflurane inhalation and killed by cervical dislocation. The brain was immediately removed and chilled in ice-cold PBS (phosphate buffered saline). A tissue slicer was used to cut fifteen 1 mm coronal slices (beginning 1 mm behind the anterior pole) which were then immersed in PBS containing 1% 2,3,5-triphenyltetrazolium chloride at 37 °C for 20 minutes. The infarct areas remained unstained by this procedure. Contact copies of the stained slices were prepared (Fig. 1). The infarct volume was calculated by summing up these areas of each slice, which were quantified

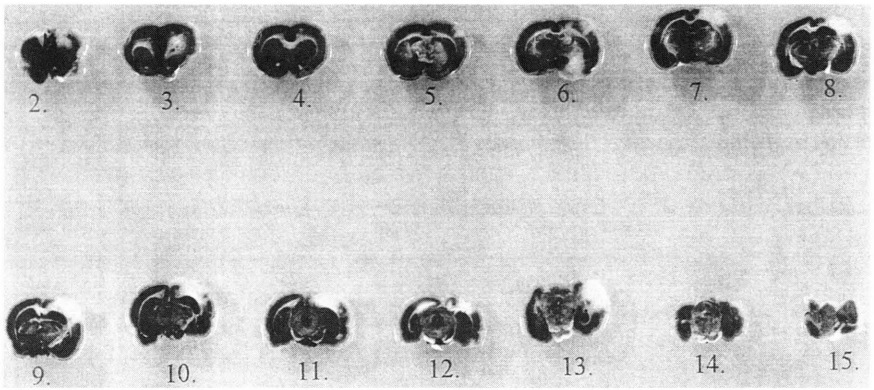

Fig. 1. Contact copy of serial coronal brain slices of a vitamin E deficient rat after 2,3,5-triphenyltetrazolium chloride staining. The numbers indicate the sequence of the slices from rostral to occipital without cerebellum. The large unstained white regions in cortex and striatum are infarct areas, which were measured with an image analysis system to calculate the infarct volumes.

with a computer image analyzer (KS 400, Kontron Elektronik, Munich, Germany).

Statistical analysis

The statistical analysis of the data was performed on a computer employing the GraphPad InStat™ program (GraphPad software, version 1994). The null hypothesis which assumed that medians of all groups were equal was checked using the Kruskal-Wallis test. If this could be rejected ($p < 0.05$), Dunn's Multiple Comparisons test was performed to ascertain which groups were significantly different from the deficient group (DR_0; $p < 0.05$ = *; $p < 0.01$ = **; $p < 0.001$ = ***).

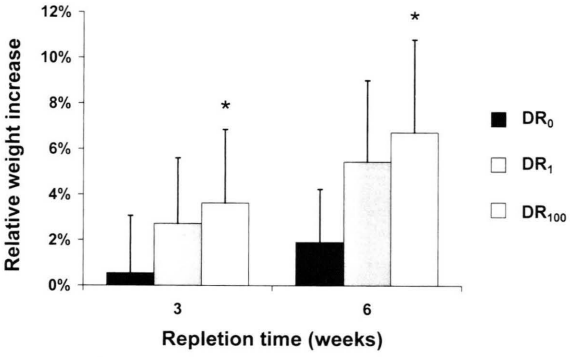

Fig. 2. Relative weight increase of the rats during the repletion time (mean ± standard deviation; *n* between 11 and 13). Due to vitamin E supplementation, there was an augmented weight gain (weight increase in relation to the weight at the beginning of the vitamin E repletion) after 3 and once again after 6 weeks. The difference between the animals of group DR_0 (**d**epleted rats **r**epleted with **0** mg vitamin E / kg body weight) and DR_{100} (**d**epleted rats **r**epleted with **100** mg vitamin E / kg body weight) was significant ($p < 0,05$).

Results and Discussion

Vitamin E status and condition of animals

During the repletion phase, weight development was controlled at the beginning, after 3 weeks and 6 weeks. Whereas the animals in the vitamin E deficiency phase (DR_0) exhibited only a modest weight gain (2.1 ± 2.3% after 6 weeks; weight increase in relation to the weight at the beginning of the vitamin E repletion), the vitamin E supplemented groups showed a distinct weight increase of 5.4 ± 3.6% (DR_1) or 6.7 ± 4.1% (DR_{100}) during 6 weeks, respectively (Fig. 2). The described diseases due to vitamin E deficiency are degenerations in the neuromuscular system (Goss-Sampson and Muller, 1988), encephalomalazia of chicken, muscle dystrophy of rabbits and guinea pigs (Pappenheimer and Goettsch, 1931; Goettsch and Pappenheimer, 1931), steatitis of pigs (Robinson and Coey, 1951), as well as steatitis of minks (Stowe

and Whitehair, 1963). Clinical symptoms of vitamin E deficiency are hypo- or areflexia, cerebellar ataxia, retinopathia and muscle weakness (Sokol, 1988). Although the rats used for this study were definitely vitamin E deficient, after more than 5 months of depletion, we observed no clinical signs.

Limitations and characteristics of the model

Standardization and reproducibility of stroke volume after MCAO are the most important preconditions to test the protective effect of vitamin E. Consequently, we tried to keep all relevant experimental conditions and performance as constant and optimal as possible. During surgery for MCAO the physiological parameters body temperature, pH, pCO_2 and pO_2 in arterial blood and mean arterial blood pressure (Table I) were monitored in order to correct them and to collect criteria for later assessment of the experimental results. Experimental data of Minamisawa *et al.* (1990) and Dietrich *et al.* (1994) demonstrated that instabilities in body temperature play a significant role in final determination of infarct volume. Body temperature decline causes a reduction of heart rate and consequently a lower blood pressure. This results in a decreased blood perfusion in penumbra and finally in an enlarged stroke volume. But reduced brain temperature is neuroprotective. A rise of brain temperature can lead to increased vascular permeability and neuronal damage via leukocyte endothelial interactions. The continuous registration of mean arterial blood pressure was important because of its influence on brain perfusion as above described. In addition, this also provided the possibility to monitor the depth of anesthesia, since a side effect of isoflurane is blood pressure reduction and the anesthetic may be neuroprotective. In this study, the mean arterial blood pressure could be kept between 103 and 108 mmHg, therefore, influences of pressure variations on the measured infarct volumes are unlikely.

Table I. Physiological parameters recorded during the middle cerebral artery occlusion as a control for the quality of experimental conditions and performance. No relevant differences between the groups could be observed.

Physiological parameters	DR_0	DR_1	DR_{100}
Body temperature (°C)	37.8±0.3	37.7±0.2	37.7±0.2
Blood pressure (mm Hg)	106±5	103±5	108±8
pH	7.30±0.01	7.30±0.03	7.30±0.04
pCO_2 (mm Hg)	49.7±3.2	50.3±4.3	50.9±7.8
pO_2 (mm Hg)	244.7±80.3	215.8±53.8	204.4±52.2

From the elevated pCO_2 (50 instead of a normal value of 38 mmHg) combined with the decreased pH values (7.30 instead of 7.40) in arterial blood, the existence of a respiratory acidosis as a consequence of the depressive effect of the anesthetic gas on the breathing center can be concluded. This side effect cannot be avoided without artificial ventilation, which itself could then have additional disadvantages like disturbances in the vegetative nervous system. The carbon dioxide rise induces vasodilatation and, via impairment of blood perfusion, modifies possibly the final infarct volumes. However, there are only minor differences between the animals. Therefore it can be concluded, that comparisons between the experimental groups are still valid. All measured arterial oxygen partial pressures were above the physiological range due to the inhaled anesthetic gas mixture. Since hemoglobin was saturated with oxygen, the partial pressure rise in arterial blood from 100 to 200 mmHg was probably irrelevant.

After regaining consciousness, there were symptoms of neurological deficits. In the DR_0 group 77% of the rats exhibited such typical signs, as contralateral circling and walking to the contralateral site, but only one rat in each repleted group showed these gait disturbances. After 24 hours of reperfusion these symptoms were eliminated. The gait disturbances are probably direct consequences of the respective extents of the infarcts and typical for the experimental model, as described by e.g. Memezawa *et al.* (1992) and Kiyota *et al.* (1993).

Before the experiments were completed, a pilot study was conducted to test the precision of the method. The variation coefficient of infarct volume leveled out at 37%, which was in the range of the published values of 22% to 100% (Fisher *et al.*, 1995; Schäbitz *et al.*, 1997; Belayev *et al.*, 1995). There are numerous possible reasons determining these differences in precision such as rat strain, duration of ischemia and reperfusion or completeness of the occlusion of the middle cerebral artery. In our main experiments the variation coefficients ranged between 54% and 83 %. This inferior precision may be mainly caused by the prolonged

vitamin E deficiency. In conclusion, the relevant experimental conditions could be sufficiently controlled since it was still possible to obtain significant results.

Vitamin E status and infarct volumes

The vitamin E status of the animals was controlled by HPLC determination of vitamin E in blood plasma at the beginning of the MCAO. In blood plasma of the deficient group, vitamin E was not detectable (n = 13). Plasma of the supplemented animals contained 7.7 ± 2.6 µmol / l (DR_1; mean ± SD; n = 11) or 35.2 ± 7.8 µmol / l (DR_{100}; n = 12) vitamin E (Fig. 3). Nevertheless, more relevant for the neuroprotective function of vitamin E is its concentration in brain tissue. Vitamin E content was very low in the brain of the deficient group (1.8 ± 0.5 nmol / g; n = 13), on the other hand, the repleted animals exhibited 12.4 ± 1.8 nmol / g (DR_1; n = 11) or 39.8 ± 8.5 nmol / g (DR_{100}; n = 11). The vitamin E deficient rats (DR_0) showed a stroke volume of 121 ± 76 mm^3 (mean ± SD, n = 13), whereas the infarct volume of rats repleted with vitamin E was significantly reduced to 41 ± 22 mm^3 (n = 11) or 23 ± 19 mm^3 (n = 12) in the DR_1 or DR_{100} group, respectively.

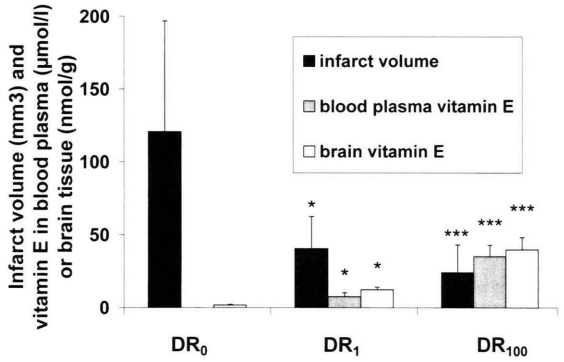

Fig. 3. Vitamin E content in brain tissue and blood plasma together with infarct volumes (mean ± standard deviation; n between 11 and 13). Differences of means were tested for significance against the deficient group. In the deficient group, vitamin E in blood plasma was not detectable. With increased vitamin contents, the repleted groups exhibited significantly reduced stroke volumes (DR_1: $p < 0{,}05$; DR_{100}: $p < 0{,}001$). This interconnection confirms indirectly the noxious role of oxygen radicals in the development of final infarct volume. For DR_1 and DR_{100} see legend of Fig. 2.

Ischemia and reperfusion lead to increased generation of oxygen radicals (Armstead *et al.*, 1988; Dietrich, 1994; Hall *et al.*, 1995). The brain contains large amounts of membranes especially sensible to lipid peroxidation (Halliwell and Gutteridge, 1986; Shivakumar *et al.*, 1991). Therefore, radical scavengers have protective effects in models of cerebral ischemia (Siesjö, 1993). 3-Methyl-1-phenyl-pyrazolin-5-one (MCI-186), a potent scavenger of hydroxyl radicals, can reduce infarct volume by 12% (Kawai *et al.*, 1997). Inhibition of NO-synthase expression was shown to have an effect of reducing the stroke volume by 22% (Iadecola *et al.*, 1995).

The achieved effect of 81% in this study seems to be enormous. However, this may be also a consequence of the use of vitamin E deficient animals, which have an increased susceptibility to free radicals, in this experiment. Accordingly the highest efficacy of α-tocopherol was observed between the groups DR_0 and DR_1. A high prophylactic vitamin E supply for patients with an increased risk of stroke can be expected to ameliorate the consequences of brain infarction.

Conclusions

To our knowledge this is the first experimental study demonstrating the neuroprotective effect of vitamin E in cerebral ischemia. The range of different vitamin E statuses of the experimental rats permits predictions particularly for patients with poor vitamin E supply. The results strongly support the hypothesis that the occurence of oxygen radicals during reperfusion is an important pathophysiological mechanism in brain infarction.

Acknowledgements

We thank F. Hoffmann-La Roche Ltd for valuable scientific advice, for providing us with the vitamin E-deficient rats, with the vitamin E-free diet and for financial support. The expert technical assistance of Sieglinde Lutz is thankfully acknowledged. Gratefully thanks are also due to Prof. Dr. W. Rambeck, Institute for Physiology, Physiological Chemistry and Animal Nutrition, for support in vitamin E measurements and Prof. Dr. W. Schmahl and Dr. P. Schmidt, Institute of Veterinary Pathology, LMU Munich, for scientific advice and making their image analyzer equipment available.

Armstead W. M., Mirro R., Busija D. W. and Leffler C. W. (1988), Postischemic generation of superoxide anion by newborn pig brain. Am. J. Physiol. **255,** H401-H403.

Armstead W. M., Mirro R., Leffler C. W. and Busija D. W. (1989), Cerebral superoxide anion generations during seizures in newborn pigs. J. Cereb. Blood Flow Metab. **9**, 175–179.

Belayev L., Busto R., Zhao W. and Ginsberg M. D. (1995), HU-211, a novel noncompetetive N-methyl-D-aspartate antagonist, improves neurological deficit and reduces infarct volume after reversible focal cerebral ischemia in the rat. Stroke **26 (12)**, 2313–2320.

Clément M., Dinh L. and Bourre J.-M. (1995), Uptake of dietary RRR-α-tocopherol and RRR-γ-tocopherol by nervous tissues, liver and muscle in vitamin-E-deficient rats. Biochim. Biophys. Acta **1256**, 175–180.

Dietrich W. D. (1994), Morphological manifestations of reperfusion injury in brain. In: Cellular, biochemical, and Molecular Aspects of Reperfusion Injury (DAS D. K., ed.). New York Acad Sciences, New York. Ann. N. Y.Acad. Sci. **723**, 15–24.

Fisher M., Meadows M. E., Do T., Weise J., Trubetskoy V., Charette M. and Finklestein S. P. (1995), Delayed treatment with intravenous basic fibroblast growth factor reduces infarct size following permanent focal cerebral ischemia in rats. J. Cereb. Blood Flow Metab. **15 (6)**, 953–959.

Goettsch M. and Pappenheimer A. M. (1931), Nutritional muscular dystrophy in the guinea pig and rabbit. J. Exp. Med. **54**, 145–165.

Goss-Sampson M. A. and Muller D. P. R. (1988), Studies on the neurochemistry and neurophysiology of experimental vitamin E deficiency. Biochem. Soc. Trans. **16**, 466–467.

Hall N. C., Carney J. M., Cheng M. S. and Butterfield D. A. (1995), Ischemia / reperfusion-induced changes in membrane proteins and lipids of gerbil cortical synaptosomes. Neuroscience **64**, 81–89.

Halliwell B. and Gutteridge J. M. C. (1986), Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. Arch. Biochem. Biophys. **246 (2)**, 501–514.

Iadecola C., Zhang F. and Xu X. (1995), Inhibition of inducible nitric oxide synthase ameliorates cerebral ischemic damage. Am. J. Physiol. **268**, R286–292.

Kawai H., Nakai H., Suga M., Yuki S., Watanabe T. and Saito K.-I. (1997), Effects of a novel free radical scavenger, MCI-186, on ischemic brain damage in the rat distal middle cerebral artery occlusion model. J. Pharmacol. Exp. Ther. **281 (2)**, 921–927.

Kiyota Y., Pahlmark K., Memezawa H., Smith M.-L. and Siesjö B. K. (1993), Free radicals and brain damage due to transient middle cerebral artery occlusion: the effect of dimethylthiourea. Exp. Brain Res. **95**, 388–396.

Kontos H. A., Wei E. P., Dietrich W. D., Navari R. M., Povlishock J. T., Ghatak N. R., Ellis E. F. and Patterson Jr, J. L. (1981), Mechanism of cerebral arteriolar abnormalities after acute hypertension. Am. J. Physiol. **240**, H511-H527.

Machlin L. J. and Gabriel E. (1982), Kinetics of tissue α-tocopherol uptake and depletion following administration of high levels of vitamin E. Ann. N. Y. Acad. Sci. **393**, 48–59.

Machlin L. J. and Bendich A. (1987), Free radical tissue damage: protective role of antioxidant nutrients. FASEB J. **1**, 441–445.

Memezawa H., Minamisawa H., Smith M.-L. and Siesjö B. K. (1992), Ischemic penumbra in a model of reversible middle cerebral artery occlusion in the rat. Exp. Brain Res. **89**, 67–78.

Minamisawa H., Smith M.-L. and Siesjö B. K. (1990), The effect of mild hyperthermia and hypothermia on brain damage following 5, 10, and 15 minutes of forebrain ischemia. Ann. Neurol. **28**, 26–33.

Mufti S. I., Eskelson C. D., Odeley O. E. and Nachiappan V. (1993), Alcohol-associated generation of oxygen free radicals and tumor promotion. Alcohol Alcoholism **28 (6)**, 621–638.

Pappenheimer A. M. and Goettsch M. (1931), A cerebellar disorder in chicks, apparently of nutritional origin. J. Exp. Med. **53**, 11–16.

Pourcyrous M., Leffler C. W., Mirro R. and Busija D. W. (1990), Brain superoxide anion generation during asphyxia and reventilation in newborn pigs. Ped. Res. **28**, 618–621.

Robinson K. L. and Coey W. E. (1951), A brown discoloration of pig fat and vitamin E deficiency. Nature (London) **168**, 997–998.

Schäbitz W. R., Schwab S., Spranger M. and Hacke W. (1997), Intraventricular brain-derived neurotrophic factor reduces infarct size after focal cerebral ischemia in rats. J. Cereb. Blood Flow Metab. **17**, 500–506.

Scott M. L. (1978), in: Handbook of Lipid Research (DeLuca, H. F., ed.), **Vol. 2.** Plenum Press, New York, 133.

Shivakumar B. R., Anandatheerthavarada H. K. and Ravindranath V. (1991), Free radical scavenging systems in developing rat brain. Int. J. Dev. Neurosci. **9 (2)**, 181–185.

Siesjö B. K. (1993), A new perspective on ischemic brain damage? Progress in Brain Res. **96**, 1–9.

Sokol R. J. (1988), Vitamin E deficiency and neurologic disease. Ann. Rev. Nutr. **8**, 351–373.

Stowe H. D. and Whitehair C. K. (1963), Gross and microscopic pathology of tocopherol-deficient mink. J. Nutr. **81**, 287–300.

Vuilleumier J. P., Keller H. E., Gysel D., and Hunziker F. (1983), Clinical chemical methods for the routine assessment of the vitamin status in human populations. Int. J. Vit. Nutr. Res. **53**, 265–272.

Wei E. P., Dietrich W. D., Povlishock J. T., Navari R. M. and Kontos H. A. (1989), Functional, morphological, and metabolic abnormalities of the cerebral microcirculation after concussive brain injury in cats. Circ. Res. **46**, 37–47.

Westerberg E., Friberg M. and Akeson B. (1981), Assay of brain tocopherols using high performance liquid chromatography. J. Liq. Chromatogr. **4 (1)**, 109–121.