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# THE INFLUENCE OF DIETARY HISTORY ON THE PRODUCTION OF FREE RADICALS IN RAT LIVER MICROSOMES

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Free radicals have been trapped in microsomal fractions from rat livers by the use of α-(4-pyridyl-l-oxide)-N-*tert*-butylnitrone (4-POBN) and detected by ESR spectroscopy. Using this technique we have investigated the effects of low dietary concentrations of vitamin E and selenium (Se) on free radical production. When the microsomal fractions were incubated with Fe, ADP and NADPH significantly greater amounts of radicals were initially trapped when rats were deficient in vitamin E (whether combined with Se deficiency or not) than from Se-deficient animals or Se- and vitamin E-supplemented controls. After prolonged incubation (ca. 30–45 mins) the free radicals trapped from the microsomes from the Se-deficient animals rose to amounts comparable to those seen in the vitamin E-deficient microsomes. Over the same period of incubation approximately half this quantity of radicals were trapped in liver microsomes from Se- and vitamin E-supplemented rats. The effects of 4-POBN on the levels of peroxidation in the various microsomal fractions were assessed by measuring the thiobarbituric acid reactive substances (TBARS). The presence of 4-POBN significantly reduced the amounts of TBARS formed on incubation and there was a clear distinction between the groups on the basis of the vitamin E status of their diets.

KEY WORDS: Free radicals, liver microsomes, ESR, vitamin E, Se deficiency.

## INTRODUCTION

Adequate supplies of the trace nutrients selenium (Se) and vitamin E are essential for the health of animals. Both nutrients participate in the antioxidant systems of the cell; vitamin E scavenges free radicals in lipid fractions and Se is an essential component of the cytosolic enzyme glutathione peroxidase (Se-$GSH_{px}$), which metabolises hydrogen peroxide and lipid hydroperoxides.[1] Peroxides in the cell may be sources of free radicals via reactions catalyzed by transition metal ions. Free radical mediated reactions have been proposed to cause several diseases, although it is not always clear whether the radicals initiate or are formed as a consequence of the disease.[2]

In a previous paper[3] we have described the use of the spin trap α-(4-pyridyl-l-oxide)-n-*tert*-butylnitrone (4-POBN) to monitor the produciton of free radicals in heart homogenates from Se- and vitamin E deficient rats. The amounts and rates of formation of free radicals in homogenates containing Fe, adenosine diphosphate (ADP) and reduced nicotinamide adenine-dinucleotide phosphate (NADPH) were higher in animals with vitamin E deficiency than in controls or Se-deficient animals and higher still in those animals with combined Se and vitamin E deficiency. The present work describes the use of similar procedures to investigate the formation of unstable radical species in liver microsomal fractions from rats deficient in Se or vitamin E or both Se and vitamin E.

TABLE I
Liver microsomal vitamin E concentration and $GSH_{px}$ activity[a]

| | +Se + vit E | −Se + vit E | +Se − vit E | −Se − vit E |
|---|---|---|---|---|
| Vit E (μg/mg protein) | 0.48 ±0.02 | 0.52 ±0.06 | 0.015 ±0.005 | 0.028 ±0.020 |
| $GSH_{px}$ (units/mg protein) | 0.090 ±0.020 | <0.002 | 0.087 ±0.016 | <0.002 |

[a]results are expressed as means ± SEMs.

## EXPERIMENTAL

### *Animals and Diets*

From weaning, four groups of 6 male Hooded Lister rats of the Rowett Institute strain were fed semisynthetic diets[3] containing the following Se and vitamin E concentrations:- Basal diet (− Se − vit E), <0.01 mg Se/kg and < 1 mg α-tocopherol/kg; Se-deficient diet (− Se + vit E), basal diet supplemented with 200 mg α-tocopherol/kg as α-tocopherol acetate; Vitamin E deficient diet (+Se − vit E), basal diet supplemented with 0.1 mg Se/kg as $Na_2SeO_3$; control diet (+Se + vit E), basal diet supplemented with Se and vitamin E as above. After 8 weeks rats were anaesthetised with ether, whereupon their livers were perfused with 0.15 M KCl to removed residual blood, and immediately frozen in liquid nitrogen.

### *Analytical Procedures*

Microsomal fractions were prepared from the livers using a calcium precipitation technique.[4] Vitamin E concentrations and Se-$GSH_{px}$ activity in the microsomes were determined as described previously.[3]

For the ESR measurements microsomal suspensions (1.0 ml) were transferred to 5 ml "Reacti-vials" (Pierce U.K. Ltd., Cambridge, U.K.), open to the atmosphere and continually stirred at ambient temperature (21–23°C). Peroxidation was initiated by addition of 1 ml Fe-ADP (30 μM $FeSO_4$, 10 mM ADP) and 0.4 ml NADPH (2.6 mM) with 2 ml of the spin trap 4-POBN (100 mM) being added simultaneously. All solutions were prepared in potassium phosphate buffer (0.05 M, pH 7.4). Aliquots (0.4 ml) of the microsomal incubations were taken at 5 min intervals and their ESR spectra recorded at ambient temperature in a flat quartz cell on a Varian E104 X-band spectrometer, operating at ca. 9.5 GHz with 100 KHz modulation frequency, 10 mW microwave power and 0.2 mT modulation amplitude.

Products of peroxidation were determined as thiobarbituric acid reactive substances (TBARS) in samples incubated under the same conditions as in the ESR spin trapping studies but maintained at 37°C. Samples (0.25 ml) were removed from incubations after 0, 5, 10 and 15 mins, immediately mixed with 2.5 ml 15% trichloracetic acid and TBARS determined as reported previously.[4]

## RESULTS AND DISCUSSION

Microsomal Se-$GSH_{px}$ activities in rats given the − Se diets were < 3% of those in the +Se animals. The liver microsomal vitamin E concentrations in rats given the − vit E diet were 6% of values in the + vit E animals (Table I).

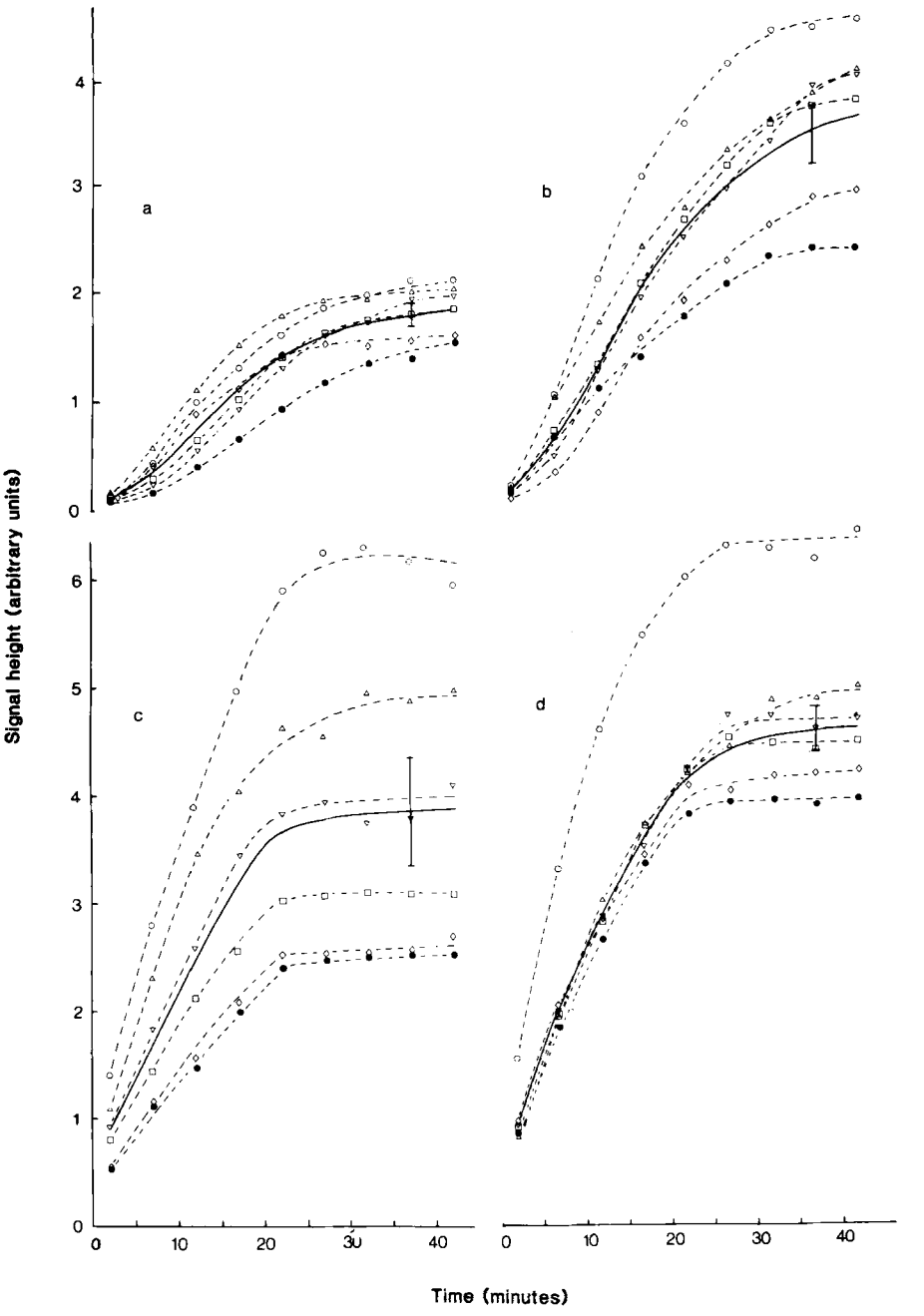


FIGURE 1 Time course for the development of the ESR spectra for microsomal fractions from rat liver incubated with Fe/ADP, NADPH and 4-POBN as described in the Experimental section, (a) +Se + vit E, (b) −Se + vit E, (c) +Se − vit E and (d) −Se − vit E groups. In each group the curves for individual samples are indicated by dashed lines and the mean curve by a bold solid line with its SEM at 35 mins.

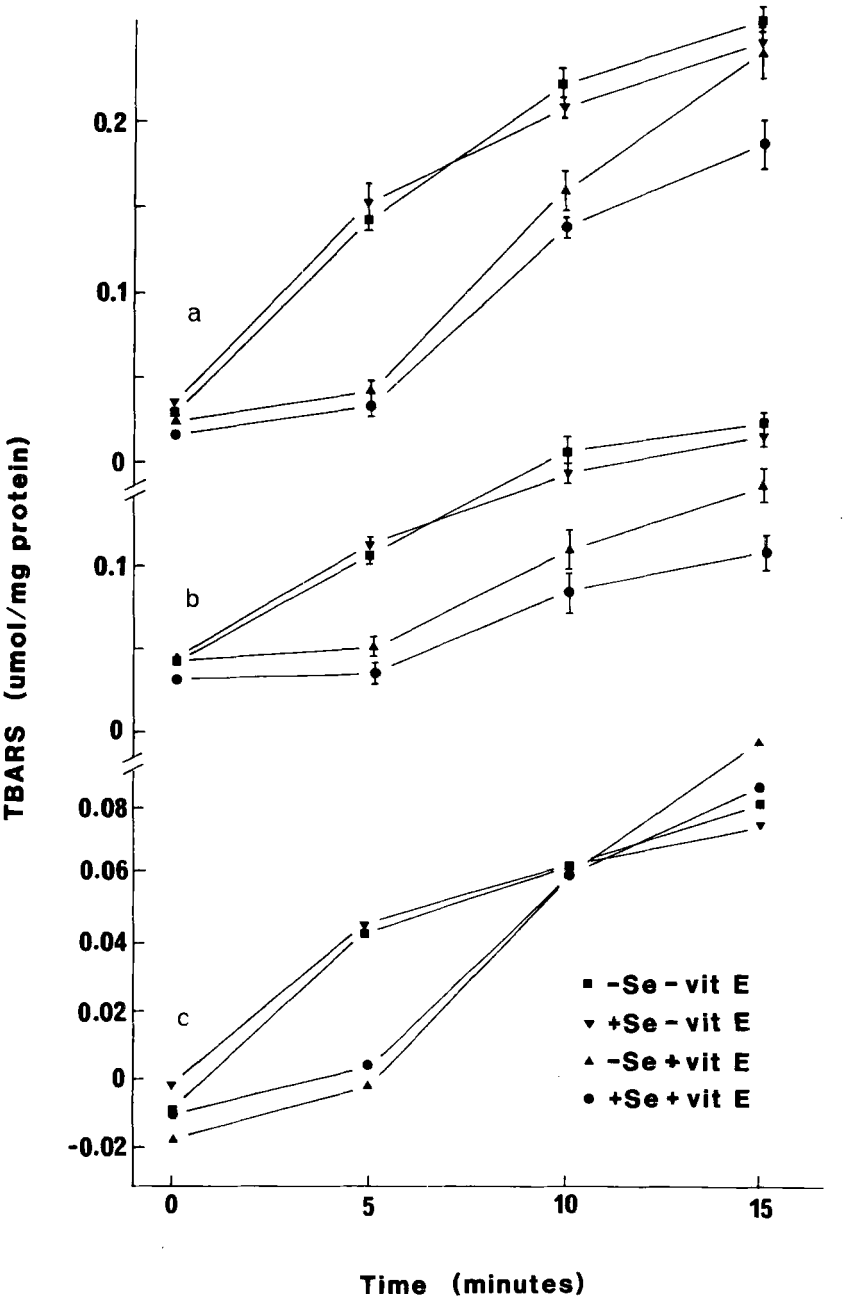


FIGURE 2 Time course of development of TBARS production (normalized for protein content) for the microsomal fractions from rat livers in the absence (a) and the presence (b) of 4-POBN as described in the Experimental section. Curves represent the means from six animals and SEMs are indicated by error bars. (c) represents the difference in curves (a) and (b) and thus illustrates the amount of peroxidation not prevented by 4-POBN for the various microsomal fractions.

When liver microsomes were incubated with 4-POBN in the presence of Fe-ADP and NADPH, ESR spectra characteristic of a free radical/spin trap adduct were obtained. The spectra consisted of a triplet of peaks from interactions of the unpaired electron with a $^{14}N$ nucleus, further split into doublets by interaction with a single $^{1}H$. The hyperfine coupling constants were $A(^{14}N) = 1.56\,mT$ and $A(^{1}H) = 0.253\,mT$, which are identical to those observed previously with heart homogenates.[3] ESR signals were not obtained when the microsomal fraction was omitted from the full incubation mixture. The time course of formation of the 4-POBN adduct obtained from the microsomal preparations within each group is shown in Figure 1. At all stages of sampling the control group (+Se + vit E) gave significantly lower amounts of adduct formation compared to the three deficient groups. Indeed the steady state concentration of adduct obtained in the later stages of the control group experiments were < 50% of the values obtained from all of the other microsomal fractions. The results obtained from the +Se − vit E group were not significantly different from those obtained with the −Se − vit E group, although the spread of results was considerably greater. The −Se + vit E group in the earlier stages of the experiment was significantly different to the −Se − vit E and +Se − vit E groups, and at the outset was close to the starting point for the control group. However, in the later stages of the experiment it rapidly approached the plateau level of the +Se − vit E group and could no longer be regarded as significantly different.

TBARS formation for the various microsomal fractions incubated for up to 15 mins with and without 4-POBN are shown in Figure 2(a) and (b). In each case the initial production of TBARS was greater for the vitamin E-deficient animals than for the −Se + vit E and +Se + vit E groups. However, after 15 mins the TBARS level for the −Se + vit E group had risen to be comparable to those for the vitamin E-deficient cases. For all groups the presence of 4-POBN significantly reduced the amounts of TBARS formed in incubation, thus demonstrating that the spin trap is able to scavenge a radical that is responsible for peroxidation in microsomes. The effects of dietary nutrient deficiencies on the amounts of free radicals not trapped by 4-POBN are shown in Figure 2(c), where the curves represent the differences between the + and −4-POBN curves for each diet. There is thus a clear distinction between the groups on the basis of the vitamin E status of their diets.

These experiments show clearly that nutrient deficiencies in Se or vitamin E increased susceptibility to free radical formation in the microsomal fractions of rat livers. With vitamin E deficiency there is an initial rapid rise in the amounts of free radicals produced, which may be explained by the absence of antioxidant capacity attributable to vitamin E in the lipid-containing membrane. With Se-deficiency there is a much lower initial free radical production, although the amounts of trapped radicals eventually approach those of the vitamin E-deficient groups. The increased build-up in radical contents in the −Se + vit E compared to +Se + vit E rat is surprising since it was expected that the Se-$GSH_{px}$, which is a water-soluble enzyme, would not have significant activity in + Se microsomal fractions. However Table I shows that there was some residual Se-$GSH_{px}$ activity present in the +Se + vit E microsomal fractions, which could be the result of incomplete separation of the microsomes from the other cell components.

The effect of Se-deficiency on free radical production is in contrast to the result obtained previously with heart homogenates,[3] where the effects of Se deficiency alone on the amounts of trapped radicals could not be distinguished from the control diets. Presumably in whole homogenates there was sufficient activity of other antioxidant

systems to prevent any effect of the Se deficiency being detected. Thus the microsomal incubation system may prove valuable in the identification of free radicals which may occur in Se-deficient tissues but not be readily detected in presence of other antioxidants.

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