THE DEPENDENCE ON DIETARY SELENIUM AND VITAMIN E OF OXIDANT-LABILE LIVER MICROSOMAL NON-HAEM IRON

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1. Introduction

Diplock et al. [1] showed that a significant proportion of the ^{75}Se in a dose of $Na_2{}^{75}SeO_3$ given to rats was converted to an acid-labile form found in liver subcellular organelles, which has been identified (Diplock et al. [2]) as selenide. The selenide is particularly associated with mitochondria and smooth endoplasmic reticulum, is protein-bound, and its presence is dependent on the presence of vitamin E in the animal's diet (Caygill et al. [3]). It has been suggested that a function of vitamin E is to protect the selenide from oxidation *in vivo*, and that the selenide may form part of the active centre of a class of non-haem iron proteins (Diplock and Lucy [4]).

In this paper, results are presented in which the presence of non-haem iron in rat liver microsomes was investigated using an established technique; the influence of the addition of antioxidants *in vitro*, and the presence or absence of dietary vitamin E and selenium, were also examined. It was found that the detection of non-haem iron in microsomes from normal rats was dependent upon the presence of antioxidants in the media used for the subcellular fractionation and subsequent measurement of the non-haem iron. When vitamin E was removed from the diet of the animals, the oxidant-sensitive non-haem iron was replaced by a form of non-haem iron that was not readily oxidisable.

2. Materials and methods

2.1. *Animal diets and dosage*

The vitamin E-deficient casein diet and the *Torula* yeast diet have been described previously [1]. Table 1 shows the 12 dietary treatments and the number of rats given each treatment.

2.2. *Microsomal preparation*

The rat livers were removed, half were placed in ice-cold 0.01 M phosphate buffer pH 7.4 containing 0.25 M sucrose. The other half were placed in the same buffer containing 5 mM mercaptoethanol and 100 μg/ml DL-α-tocopherol [1]. Subsequent preparation of the microsomes was done in solutions with or without the antioxidants as appropriate. A 10% homogenate was prepared using the homogeniser described before [3] and centrifuged at 12 000 rpm for 12 min. The supernatant fluid (20 ml) was layered, to remove haemoglobin [5], onto a sucrose gradient consisting of 0.4 M sucrose (4 ml) and 0.3 M sucrose containing 0.23 M NaCl (10 ml). This was then centrifuged at 30,000 rpm for 1.5 hr. The resulting microsomal fraction was resuspended in 0.01 M phosphate buffer pH 7.4, the protein concentration estimated [6], and the suspension diluted to give a final concn. of approx. 35 mg protein/ml. The microsomal fraction prepared in the presence of antioxidants was resuspended in the phosphate buffer to which 5 mM mercaptoethanol alone was added. α-Tocopherol was omitted because it interfered with subsequent spectrophotometric measurements.

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Table 1
Dietary treatment and dosage.

Dietary treatment	Description	No. of rats used
A	Adequate diet: 41 Oxoid B pellets.	6
B	Adequate diet: phenobarbitone (1 mg/ml) given in the drinking water for the 5 days prior to death	3
C	Vitamin E-deficient casein diet*: rats given both 3 mg per day α-tocopherol orally and phenobarbitone in the drinking water for 5 days prior to death	3
D	Vitamin E-deficient casein diet: rats given phenobarbitone in the drinking water for 5 days prior to death	3
E	Vitamin E-supplemented casein diet* (100 mg per kg α-tocopherol acetate)	3
F	Vitamin E-deficient casein diet: rats given 3 mg per day vitamin E orally for the 5 days prior to death	4
G	Vitamin E-deficient casein diet	3
H	Vitamin E and Se-deficient *Torula* yeast diet supplemented with 100 mg/kg α-tocopherol acetate and 0.1 ppm of Se as Na_2SeO_3.	9
I	Vitamin E- and Se-deficient *Torula* yeast diet supplemented with 100 mg/kg α-tocopherol acetate	9
J	Vitamin E and Se-deficient *Torula* yeast diet*	9
K	Vitamin E and Se-deficient *Torula* yeast diet: rats given 3 mg per day α-tocopherol orally for the 5 days prior to death	12
L	Vitamin E and Se-deficient *Torula* yeast diet: rats given 3 mg per day α-tocopherol for 5 days and 8 μg Se as Na_2SeO_3 intraperitoneally for 3 days prior to death	9

* Ref. [1].

All rats used were given the diets for 3–6 months except those on the vitamin E and Se-deficient diets, which were given the casein diet for 1 week after weaning and the vitamin E and Se-deficient diet for a further 14–20 days.

The non-haem iron and total iron contents of the microsomal suspensions were measured in duplicate on each sample of microsomes by the methods of Doeg and Ziegler [7]. Succinate 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium reductase was measured as before [3].

3. Results and discussion

Fig. 1 shows that where the dietary treatment of the animals was adequate in both α-tocopherol and selenium (A, E, F, H and L), only very small amounts of non-haem iron could be detected when the microsomes were prepared in the absence of antioxidants. However, the level of non-haem iron detected in microsomes prepared from the same livers in the presence of antioxidants was significantly higher, and of the same order of magnitude in molar terms as the cytochrome P_{450} in microsomes from animals given similar dietary treatments [8]. Where the dietary treatment was deficient in α-tocopherol (G) or selenium (I and K) or both (J), there was no difference in the levels of non-haem iron whether the microsomes were prepared in the presence or absence of antioxidants. The detection of non-haem iron that is labile in the absence of antioxidants *in vitro* thus depends on the presence of selenium in the diet, as well as α-tocopherol, in contrast to the non-haem iron that is unaffected by antioxidants, which is present when either selenium or selenium and α-tocopherol, are absent from the diet. This is shown in particular by comparing dietary treatment J with K and L; it was only when selenium was re-fed (L) that the oxidant-labile non-haem iron appeared. In other experiments not reported here we have shown that this occurs within 24 hr of giving selenium.

It has been shown that selenium can replace *in vitro* the acid labile sulphur in putidaredoxin [9,10], parsley ferredoxin [11] and adrenodoxin [12], to give biologically active products with an altered iron–selenide active centre. It is possible, therefore, that the liver microsomes from rats adequate in both α-tocopherol and selenium contain a biologically active antioxidant-dependent non-haem iron protein with selenide at its active centre. However, when α-tocopherol or selenium, or both, are removed from the animals' diet a different type of non-haem iron protein,

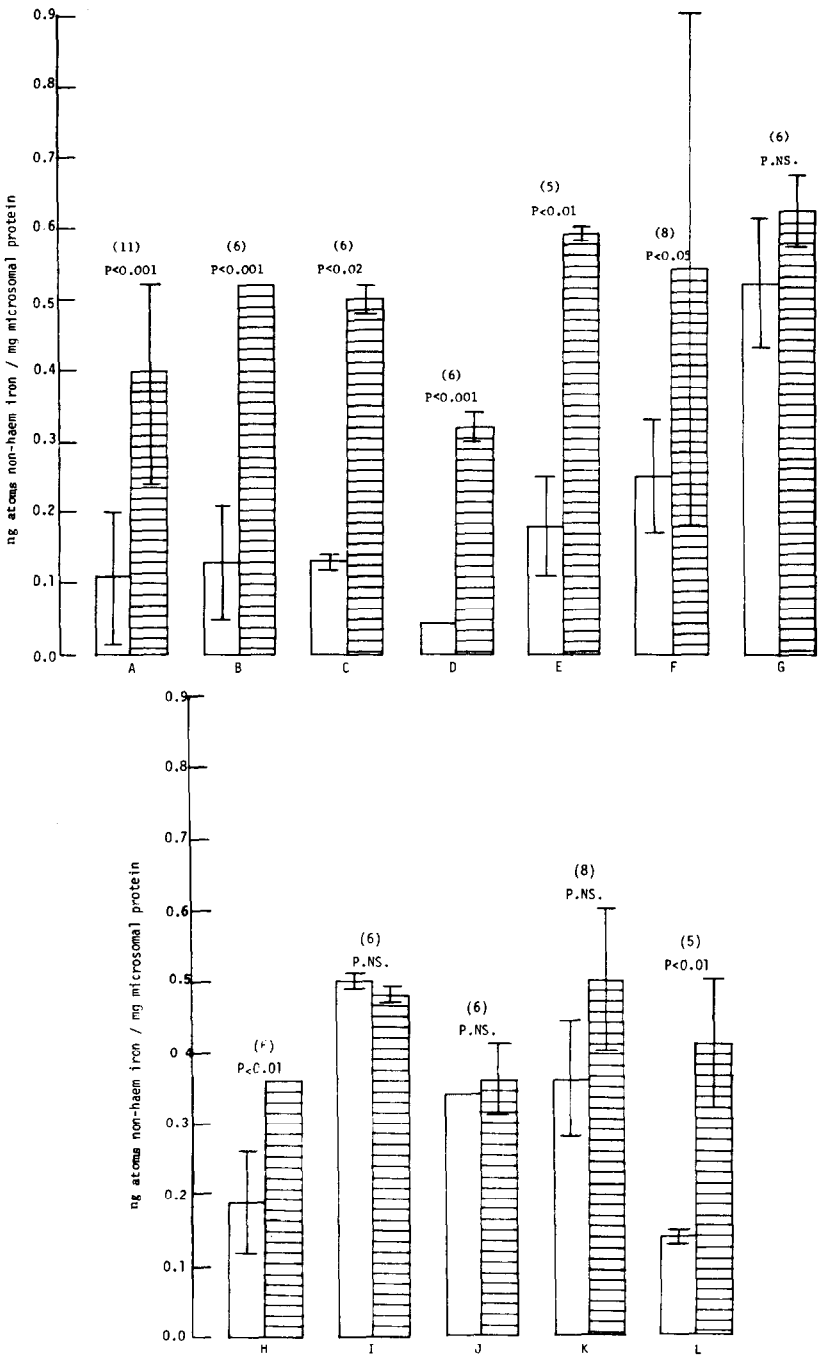

Fig. 1. Non-haem iron content of microsomal fractions prepared from livers of rats under different dietary conditions. (□): No antioxidants *in vitro*; (■): with antioxidants *in vitro*. Values given are mean values with std. deviations represented by the bars. The number of observations is given in parentheses. Significance of the difference between values obtained in the presence or absence of antioxidants was assessed by the Student's *t* test.

possibly containing sulphur in place of selenium, may be formed, that does not require protection by antioxidants and is formed as a response to the dietary restriction of selenium. Recent EPR studies by Hoffström et al. [13] have shown the presence of an iron–sulphur protein in rat kidney cortex microsomes, but earlier investigations [14, 15] failed to show the presence of a similar protein in rabbit liver microsomes. Baron et al. [16] have also shown by an immunological technique that there is no sulphur protein in rat liver microsomes similar to the adrenodoxin of the adrenal mitochondrial cytochrome P_{450}-containing enzyme system. In adequately fed animals however, the presence of antioxidants *in vitro* may be necessary to preserve the selenide in a labile non-haem iron protein that may normally be present in rat liver microsomes. Further experiments are now being undertaken with Dr. E. Slade of the University of Keele to study the EPR characteristics of antioxidant-treated rat liver microsomes in an attempt to elucidate this question.

Phenobarbitone administration is known to induce the formation of the cytochrome P_{450}-containing mixed function oxidase system of rat liver microsomes [13], and thus its effect on the non-haem iron was also investigated (dietary treatments B, C and D). As can be seen from fig. 1, it has no effect on either the level of non-haem iron or its dependence on antioxidants in rats fed a diet adequate in α-tocopherol and selenium. However, when the animals' diet was deficient in α-tocopherol phenobarbitone caused the conversion of antioxidant-independent non-haem iron (G) to an antioxidant-dependent non-haem iron (D).

Table 2 shows the total iron content of liver microsomes from rats given dietary treatments A–L. These are independent of antioxidants and are a non-specific measure of the haemoprotein content and residual haemoglobin in the microsomes.

The possibility that the presence of non-haem iron in microsomes was due to mitochondrial contamination was considered. However, no contamination of our preparations by the mitochondrial marker enzyme succinate-2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium reductase could be detected except in those microsomes prepared from livers of rats fed the unsupplemented *Torula* yeast diet (J). Here trace amounts of mitochondrial contamination were detected, but only enough to account for 1–5% of the total non-haem iron; this is probably due to the increased fragility, that we have observed previously [3], of liver mitochondria from rats given diets deficient in both vitamin E and Se. The possibility of contamination by fragments of mitochondria that do not show marker enzyme activity is, however, still open.

Table 2
Total iron content of microsomal fractions prepared from livers of rats under different dietary conditions.

Dietary treatments	Total iron content ± std. deviation (ng atoms/mg protein)	
	+ Antioxidants	– Antioxidants
A	6.20 ± 0.18	6.06 ± 0.11
B	4.54 ± 1.40	4.74 ± 1.57
C	11.43 ± 6.37	12.30 ± 5.45
D	5.27 ± 0.47	6.07 ± 0.54
E	3.81 ± 0.14	3.63 ± 0.06
F	9.94 ± 4.09	9.13 ± 4.05
G	7.60 ± 0.50	7.04 ± 0.71
H	4.43 ± 0.15	5.14 ± 0.10
I	5.23 ± 0.84	4.77 ± 0.72
J	3.24 ± 1.06	3.38 ± 0.67
K	2.48 ± 0.10	2.66 ± 0.20
L	2.67 ± 0.99	2.47 ± 0.78

The results presented here are considered to support our view that there may be a selenide-containing non-haem iron protein that is functional in the microsomal electron transport system. Furthermore, it would appear from the results that this protein is normally protected from oxidation *in vivo* by vitamin E and that in the absence of vitamin E it is possible that it is replaced by a more stable sulphide-containing protein.

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