Vitamin E and stress

6*. Iron overloading and the metabolism of D-α-tocopherol in the rat

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1. The effects of iron overloading and unsaturation of dietary lipid on the metabolism of α-tocopherol in the rat were studied.

2. Young adult male vitamin E-deficient rats were given 1000 i.u. of vitamin A and 100 μg of [^{14}C-5-*Me*]D-α-tocopherol and then given diets containing 5% methyl oleate or 5% cod-liver oil fatty-acid methyl esters. Rats from each group were given intramuscular injections of iron-dextran (50 mg Fe/kg rat) at 48 h intervals for 15 days, and compared with controls given dextran. After this time, liver, kidney and the remainder of the carcass were analysed for [^{14}C]α-tocopherol, and liver and kidney were also analysed for vitamin A.

3. There was no evidence that Fe overloading caused any increase in the destruction of either tocopherol or vitamin A in vivo, whether or not the diet contained polyunsaturated fatty acids. Indeed, treatment with Fe significantly decreased the metabolism of the radioactive tocopherol dose in all three tissues studied.

4. These experiments show that the stress effect of Fe in the vitamin E-deficient animal is unrelated to an increase in oxidative reactions. They provide further evidence that 'lipid peroxidation' is not causally concerned in 'anti-vitamin E' stress conditions and that α-tocopherol does not function, in vivo, as an antioxidant.

In earlier papers we have shown that the metabolism of small amounts of [^{14}C]D-α-tocopherol given to vitamin E-deficient rats or chicks is not affected by a number of stress agents; their action was not considered to be due to their ability to promote or accelerate peroxidation in vivo (Green, Diplock, Bunyan, McHale & Muthy, 1967; Diplock, Bunyan, McHale & Green, 1967; Diplock, Green, Bunyan, McHale & Muthy, 1967; Cawthorne, Diplock, Muthy, Bunyan, Murrell & Green, 1967). We were thus especially interested in the phenomenon of iron overloading, which produces toxic effects, preventable by vitamin E, in the rat and other species. Fe in the ferric form is a powerful oxidant, reacting rapidly with α-tocopherol in vitro. It is also, in ionic and covalent forms, a powerful catalyst for autoxidizing lipid systems in vitro (Tappel, 1953, 1954). If the protective role of vitamin E against stress is concerned with its prevention of peroxidation in vivo, it might be expected that Fe overloading would be a particularly useful system in which to study any direct effect of the stress agent on the postulated antioxidant.

Golberg and his colleagues have, in a comprehensive series of papers, described and elucidated the mechanisms of Fe overloading toxicity, and they first drew attention to certain similarities between the effects of Fe and some manifestations of vitamin E deficiency (Golberg, Smith & Martin, 1957; Golberg & Smith, 1958, 1960*a*, *b*; Golberg, Martin & Smith, 1960; Baker, Golberg, Martin & Smith, 1961;

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Muir & Golberg, 1961*a*, *b*). They showed that dietary unsaturated fatty acids exacerbated the toxicity of Fe, and they were thus led to regard at least some of the phenomena of Fe overloading as due to the pro-oxidant effects of the metal in vivo.

In this paper we present the results of a study of Fe toxicity in relation to the metabolism of α-tocopherol and the biological antioxidant theory.

EXPERIMENTAL

Materials and methods

Animals and diets. Male Norwegian hooded rats were given the vitamin E-deficient diet G 15 F (Cawthorne *et al.* 1967), which contained 15 % casein and 5 % lard, from weaning until they were $3\frac{1}{2}$ months of age, when they were used for the experiment.

Materials. The preparation of iron-dextran used was Iron Dextran Injection (Boots Pure Drug Co. Ltd). It contained 10 % (w/v) Fe. The dextran control preparation was Dextraven (Benger Laboratories Ltd). Methyl oleate (OLME) and cod-liver oil fatty-acid methyl esters (CLOME) were prepared free from tocopherol as described by Green *et al.* (1967). The [^{14}C-5-*Me*]D-α-tocopherol was prepared and administered orally to rats as described by Green *et al.* (1967).

Analyses. The analysis of tissues for [^{14}C]α-tocopherol and its fat-soluble metabolites was carried out as previously described (Green *et al.* 1967). Vitamin A analyses in liver and kidney were carried out on the non-saponifiable extracts by the usual antimony trichloride colorimetric method, measurements of blue colour being made in a spectrophotometer.

Animal experiment

Twenty-four vitamin E-deficient adult male rats were allotted at random to four groups and each rat was given a single oral dose of 1000 i.u. vitamin A palmitate in 0·1 ml ethyl oleate. After 24 h, each rat was given 98·0 μg (11613 disintegrations per sec (dps)) [^{14}C]D-α-tocopherol by mouth. After another 24 h had elapsed, groups 1 and 2 were given the basal diet G 15 F from which the vitamin A was omitted and to which 10 % OLME had been added at the expense of the lard and 5 % sugar. Groups 3 and 4 were given the diet supplemented similarly with 10 % CLOME. On the same day, rats in groups 2 and 4 were given a single intramuscular injection of the iron-dextran preparation in the right leg, so that each rat received 50 mg Fe/kg body-weight. The rats in groups 1 and 3 were given an equivalent injection of dextran. The dietary regimens were continued for 15 days and, on each alternate day, the intramuscular dosage was repeated (seven injections in all), as far as possible in the same site of injection. The rats were killed on the 15th day, 24 h after the last injection. Liver, kidney, and the remainder of the carcass, after removal of the intestinal tract, were taken for analysis.

RESULTS

The results are given in Table 1. There was a significant depression of average weight gain in rats given Fe or CLOME, or both together, compared to rats given OLME. Neither Fe nor dosage with CLOME, nor both together, accelerated the

destruction of [^{14}C]α-tocopherol in the whole animal, or in kidney and liver, tissues that are known to be specially susceptible to damage by Fe overloading (Golberg & Smith, 1958). In fact Fe administration led to a significant increase in the amounts of [^{14}C]α-tocopherol found in liver, irrespective of the nature of the dietary lipid. In kidney and carcass, significance could not be demonstrated for group 2 *v.* group 1, or group 4 *v.* group 3; but, taking the groups all together, significantly more [^{14}C]α-tocopherol was found in groups 2 and 4 than in groups 1 and 3. Some significant differences in [^{14}C]metabolites were also found and are shown in Table 1. There were no significant effects of either CLOME or Fe on vitamin A in kidney and liver.

Table 1. *Metabolism of* [^{14}*C*]α*-tocopherol in vitamin E-deficient rats given cod-liver oil methyl esters* (*CLOME*) *or methyl oleate* (*OLME*) *in the diet, and the effects of iron-dextran given intramuscularly*

(Twenty-four adult male rats were each given 98·0 μg (11613 dps) [^{14}C-5-*Me*]D-α-tocopherol and 24h later were divided into four groups. Groups 1 and 2 received the vitamin E-deficient diet with 10% OLME and groups 3 and 4 the diet with 10% CLOME. Groups 2 and 4 were given iron-dextran (50 mg Fe/kg body-weight) every other day for 15 days, controls in groups 1 and 3 being given dextran. The tissues from two animals in each group were combined for each analysis and there were three analyses per group. The results are given as means with standard deviations)

	Group			
Treatment	1 OLME	2 OLME+Fe	3 CLOME	4 CLOME+Fe
Initial rat weight (g)	162±39	180±39	163±45	160±31
Weight gain* (g)	13±9	0±10	−1±9	−16±8
Liver				
Weight (g)	7·8±1·1	8·4±0·9	6·9±0·8	6·3±0·6
Vitamin A (total i.u.)	609±243	823±319	698±269	604±172
[^{14}C]tocopherol†(total dps)	80±13	102±12	66±3	100±9
[^{14}C]metabolites (total dps)	25±2	27±1	23±6	29±3
Kidney				
Weight (g)	1·5±0·1	1·7±0·1	1·6±0·05	1·3±0·1
Vitamin A (total i.u.)	7·0±1·0	7·2±0·6	7·1±0·4	6·7±0·8
[^{14}C]tocopherol‡ (total dps)	28±1	36±3	33±7	38±1
[^{14}C]metabolites (total dps)	11±1	11±3	13±5	16±6
Carcass				
[^{14}C]tocopherol‡ (total dps)	2430±500	3070±380	2050±350	2470±344
[^{14}C]metabolites† (total dps)	920±53	1250±172	850±141	1220±96

* Both Fe and CLOME significantly decreased weight gain ($P < 0.02$).
† Fe significantly raised these values, with each lipid ($P < 0.05$).
‡ Values for Fe-treated rats (both groups taken together) were greater than for the controls ($P < 0.05$).

DISCUSSION

The similarity between the effects of Fe overloading and vitamin E deficiency is of particular interest, for the relationship between Fe and tocopherol in vivo would seem superficially to be that of oxidant and antioxidant. The pattern of the stress condition is familiar, resembling in many of its aspects the picture described for several other types of stress condition in the rat. Typical signs are the presence of ceroid, browning of the uterus, renal autolysis, production of so-called 'lipid peroxides' in adipose

tissue, accentuation of the stress condition by feeding cod-liver oil, and finally prevention of the condition by (usually massive) dosing with vitamin E and, perhaps, other 'antioxidants' (Golberg & Smith, 1958). The muscular lesions and creatinuria often associated with vitamin E deficiency are not found in the Fe-overloaded rat; but, as Golberg & Smith (1958) have stated, Fe toxicity experiments are usually carried out with adult animals, whereas vitamin E depletion experiments are usually undertaken with young animals. Lannek, Lindberg & Tollerz (1962) refer to waxy degeneration of muscle in piglets given Fe preparations.

Golberg & Smith (1958, 1960*a*, *b*) and Golberg, Martin & Batchelor (1962) have discussed the problems of Fe overloading and the relationships between the toxic signs observed in this condition and the general picture of vitamin E deficiency in the rat. Their carefully balanced argument draws attention to the analogies between the two states but also cautions against taking a superficial view in identifying them one with the other. Thus, quoting Hove (1955), they state that 'reliance on pathologic similarity as an indication of vitamin E deficiency or antagonism may be misleading'. Nevertheless, in several ways they were perforce led to discuss the mechanisms involved in Fe toxicity in terms of classical antioxidant theory. Thus, Golberg & Smith (1958), considering the problem of ceroid formation (common to Fe overloading and vitamin E deficiency), showed that addition of cod-liver oil to the diet led to a marked increase in polymer formation in rats given iron-dextran, compared to controls not given Fe. However, simultaneous administration of α-tocopherol hardly affected this increase, the cod-liver oil overcoming 'the protective effect of the vitamin'. Although they suggested that Fe overloading might lead to increased pro-oxidation of lipid, they were unable to show that ferritin (the main physiological form of Fe) was a pro-oxidant for unsaturated fatty acids. Treatment with Fe was found to increase the peroxide content of adipose tissue (Golberg & Smith, 1958) and the formation, in vitro, of 'thiobarbituric acid reactants' of skin and muscle (Golberg *et al.* 1962), and these findings were regarded as evidence for the pro-oxidative effect of Fe in vivo. However, the significance of such tests must be viewed with caution in the light of the recent work of Woodford, Böttcher, Oette & Ahrens (1965) and Bunyan, Murrell, Green & Diplock (1967).

Golberg & Smith (1960*b*) discussed in great detail some of the possible mechanisms of Fe toxicity and their relation to hepatic vulnerability. They gave due place to a consideration of peroxidation hypotheses current at that time, suggesting that 'bound ferric iron might be expected to display non-specific activity in direct oxidations of sulphydryl groups, tocopherol or adrenalin'. However, they were cautious about certain mechanistic implications of lipid peroxidation in vivo, suggesting that the 'methionine-vitamin E relationship must rest on some basis other than antioxidant activity'. They suggested that ceroid production might be, in the words of Himsworth (1947), 'an incidental result of the diets used'. They used the term 'equilibrated swamping' to describe the condition of the Fe-loaded rat and suggested that 'while under optimal circumstances this state is compatible with health and prolonged survival, it renders the animal particularly susceptible to nutritional, toxic and other metabolic hazards'. Perhaps, however, the converse is more nearly true: that it is the

nutritional deficiency (of vitamin E) that renders the animal particularly susceptible to Fe toxicity and other metabolic hazards.

The results of the experiment described here do not support the suggestion that Fe overloading causes an increase in peroxidative reactions—unless it is possible to envisage such reactions as being unaffected by the presence of the powerful lipid anti-oxidant, α-tocopherol. As Table 1 shows, the tissues of Fe-treated rats in fact contained more tocopherol than those of controls (cf. our previous results (Diplock *et al.* 1967) with silver toxicity in the rat). Furthermore, in agreement with Golberg & Smith (1960*a*), we found no effect of Fe overloading on vitamin A metabolism in liver or kidney. Although the 'stress' period used in our experiment was only 15 days (in contrast to the period of several weeks used by Golberg & Smith, 1958), there is no doubt that this time-interval is fully sufficient for the recognition of biochemical and early histological changes induced by Fe. Thus Baker *et al.* (1961) described significant histological changes in rats 1 week after two injections of iron-dextran, and Golberg *et al.* (1962) described biochemical changes in tissues of rats within 2 weeks.

As Golberg & Smith (1960*b*) have demonstrated, the organism has a remarkable propensity for binding Fe to specific proteins by covalent forces, and it is doubtful whether ionic Fe has more than a transient existence in vivo (Muir & Golberg, 1961*a*; Baker *et al.* 1961). If Fe toxicity is not related to a catalytic pro-oxidant action in vivo, why is it affected by vitamin E? Does tissue siderosis change the intracellular oxidation–reduction potential, as postulated by Golberg & Smith (1958)? Does the 'pro-oxidant' Fe ever come into contact with lipid structures? This is in doubt, for Muir & Golberg (1961*b*), in an electron-microscope study of sarcomata induced in mice by iron-dextran injections, found that neither iron-dextran nor ferritin was in proximity to the lipid droplets that accumulate in the tumour cells. The problem of the role of vitamin E in protecting against Fe overloading must be viewed against the whole background of the relationship between this vitamin and states of stress. In the light of our findings in the experiment described here and of our previous work on the relationships between vitamin E and stress (cf. earlier papers in this series) it would now seem essential to ask the wider question: do random oxidation or peroxidation reactions ever take place to a pronounced degree in vivo, whether vitamin E is present or not? If they occur, what substances are attacked and what are the products?

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