Brit. J. Nutr. (1962), **16**, 519

Exudative diathesis and lipid peroxidation in the chick

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(*Received* 28 *December* 1961—*Revised* 25 *April* 1962)

The mode of action of vitamin E is still not fully understood. One hypothesis, however, is that it is a physiological antioxidant, preventing the accumulation of undesirable metabolic oxidation products in animal tissues. These products are usually supposed to be lipid peroxides, which, in the absence of vitamin E or other antioxidant, can break down to give free radicals that can react at random with sensitive cell structures. This hypothesis is supported by the observations that oxygen consumption by mitochondria and tissue homogenates is increased in the absence of vitamin E and that malonaldehyde production in incubated tissue homogenates (usually regarded as signifying that lipid peroxidation has taken place) is greater in those from vitamin E-deficient animals than in controls (Tappel & Zalkin, 1959; Zalkin, Tappel & Jordan, 1960; Bieri & Anderson, 1960; Bieri, Dam, Prange & Søndergaard, 1961). The nature of this lipid peroxidation remains obscure, and Green, Diplock, Bunyan, Edwin & McHale (1961) have drawn attention to several anomalies that appear to make the hypothesis unacceptable in its present form. A new factor has been introduced into discussions of the antioxidant hypothesis by the discovery that selenium is an essential trace element for several species (Schwarz & Foltz, 1957) and that, in certain circumstances, whose precise nature is still under investigation, it can apparently function as a partial alternative to tocopherol and prevent some, though not all, signs of vitamin E deficiency. The effective amounts of Se are minute compared with those of vitamin E. The chemical form in which Se functions is at present unknown. However, Se is known to be incorporated into amino acids, and McConnell & Wabnitz (1957) found significant amounts in non-protein fractions of tissues. In order to account for the way in which Se resembles vitamin E in its nutritional role, several workers have tried to explain it also by the antioxidant hypothesis and have suggested that Se functions by forming an amino acid or protein that is a powerful in vivo antioxidant (Tappel, Zalkin & Knapp, 1960; Bieri, 1959; Bieri *et al.* 1961).

Two criticisms can be made of experiments that have been claimed to demonstrate a relationship between the protective effect of vitamin E against the deficiency state in the chick (exudative diathesis, encephalomalacia or muscular dystrophy, depending on the nature of the diet) and its inhibition of tissue peroxidation (Machlin, Gordon & Meisky, 1959; Bieri *et al.* 1961). First, not all tissues show such a relationship (indeed, some show the opposite effect) and much emphasis is placed upon those that do, without any justification for it in terms of the pathology of the deficiency state. The second criticism, which is perhaps more important, is that, in those experiments in which tissue peroxidation has been markedly inhibited, the dietary concentrations of

vitamin E used have invariably been considerably greater than the minimal needed to protect completely against the nutritional deficiency. These experiments, therefore, leave unanswered the real question: is there a decrease in tissue peroxidation when the dietary concentration of vitamin E is the minimum required to prevent overt deficiency and, if so, is this decrease great enough to be physiologically significant? Further, can the effect of vitamin E be correlated quantitatively with that of other antioxidants or of Se?

In our experiments we have studied exudative diathesis, as did Bieri *et al.* (1961). We have, however, investigated the effects of several dietary levels of vitamin E, Se and the antioxidant, ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline, Monsanto Chemical Co.), which has been shown by several workers (e.g. Machlin *et al.* 1959) to prevent signs of vitamin E deficiency in the chick. We also tested DPPD (*N*,*N'*-diphenyl-*p*-phenylenediamine) at 250 p.p.m., since Scott, Hill, Norris, Dobson & Nelson (1955) found this antioxidant relatively ineffective against exudative diathesis, but did not report its effects on tissue peroxidation.

EXPERIMENTAL

Chicks. Six batches of 1-day-old Rhode Island Red × Light Sussex cockerels were purchased from a commercial breeder, reared in electrically heated wire-floored cages and provided with food and water *ad lib.*

Diets. The basal diet had the percentage composition: torula yeast (Lake States Yeast & Chemical Division of St Regis Paper Co., Rhinelander, Wisconsin, USA) 60, lard 4, salt mixture 6·75, glucose 27·25, vitamin mixture 0·2, choline tartrate 0·4, DL-methionine 0·4 and glycine 1. Vitamin A, 20 i.u./g, vitamin D_3, 2 i.u./g, and chlortetracycline, 0·2 p.p.m., were also added. The salt mixture supplied (g/kg diet) $CaCO_3$ 22, K_2HPO_4 9, $NaH_2PO_4.2H_2O$ 7·3, $CaH_4(PO_4)_2.2H_2O$ 14, NaCl 8·8, $MgSO_4.7H_2O$ 5, ferric citrate 0·4, $MnSO_4.4H_2O$ 0·4, KI 0·04, $ZnCO_3$ 0·02, $CuSO_4.5H_2O$ 0·02 and $CoCl_2.6H_2O$ 0·004. The vitamin mixture supplied (mg/kg diet) thiamine 8, riboflavin 8, calcium pantothenate 20, nicotinic acid 100, pyridoxine 8, biotin 0·3, folic acid 3, menaphthone sodium bisulphite 1 and cyanocobalamin 0·02.

Deficiency signs. Exudative diathesis occurred from 20 days onwards and was observed externally as a green discoloration of the abdomen, which on dissection was seen to be due to an exudate—usually coagulated—under the skin.

Haemorrhages were seen in the breast and leg muscles and on the viscera of many birds with exudates, and a few of them had white striations of the breast muscle.

Selection of birds for tissue analysis. Nearly all the chicks of Expts 1–4 and 6 were killed in pairs from days 22 to 36, 20 to 30, 22 to 28, 21 to 27 and 21 to 30, respectively. The pairs were selected to contain chicks apparently affected to about the same degree, those most seriously affected being taken first. Birds that were apparently normal were selected in pairs at random. Some chicks (four or five) in each of these five experiments were examined individually when suitable pairs could not be found. In Expt 5 all examinations were carried out on single birds. The chicks were killed by breaking their necks, and each analysis of tissue, whether from one or two chicks, was carried out at once in duplicate.

Determination of peroxides in tissues. A sample of each tissue (or pair of tissues from two chicks) was finely chopped and made into a 20 mg/ml homogenate in ice-cold phosphate buffer (pH 7·4) by means of a nylon-glass homogenizer. Peroxidation was studied *in vitro* by shaking 5 ml of the homogenate at 100 excursions/min for 60 min in a water bath at 37°. After addition of 35 % (w/v) trichloroacetic acid solution (5 ml), cooling and filtration, a portion of the filtrate was added to an equal volume of 0·5 % (w/v) thiobarbituric acid solution in water, and the mixture was heated in boiling water for 10 min. After the solution had cooled in ice-water for 3 min, the optical density of the pink colour was read in an EEL (Evans Electroselenium Ltd) long-cell absorptiometer with an Ilford filter 604 ($\lambda_{max.}$ 520 mμ). The instrument was calibrated with crystalline malonaldehyde bis-bisulphite disodium salt prepared by the method of Saslaw & Waravdekar (1957). Results were recorded as μg malonaldehyde/g tissue.

Determination of peroxides in diets. The thiobarbituric acid method of Machlin *et al.* (1959) was used.

Experiments. Six batches of chicks were allocated at random to the groups described below. They received supplements as shown.

Expt 1 (fifty-three chicks):

Group A, no supplement.
Group B, D-α-tocopheryl acetate, 10 p.p.m.
Group C, D-α-tocopheryl acetate, 20 p.p.m.
Group D, D-α-tocopheryl acetate, 30 p.p.m.
Group E, D-α-tocopheryl acetate, 50 p.p.m.

Expt 2 (fifty-two chicks):

Group A, no supplement.
Group B, sodium selenite to give 0·15 p.p.m. Se.
Group C, sodium selenite to give 0·50 p.p.m. Se.
Group D, sodium selenite to give 1·0 p.p.m. Se.

Expt 3 (forty-three chicks):

Group A, no supplement.
Group B, ethoxyquin, 100 p.p.m.
Group C, ethoxyquin, 200 p.p.m.
Group D, ethoxyquin, 500 p.p.m.
(Group E was given a commercial chick mash).

Expt 4 (fifty chicks):

Group A, no supplement.
Group B, tocopheronolactone, 2 mg/day, given by mouth.
Group C, tocopherylquinone, 5 mg/day, given by mouth.
Group D, D-α-tocopheryl acetate, 10 p.p.m.
Group E, D-α-tocopheryl acetate, 150 p.p.m.
Group F, sodium selenite to give 0·15 p.p.m. Se.
Group G, sodium selenite to give 1·0 p.p.m. Se.

Expt 5 (fifty-two chicks):

Group A, no supplement.
Group B, D-α-tocopheryl acetate, 10 p.p.m.
Group C, D-α-tocopheryl acetate, 20 p.p.m.
Group D, sodium selenite to give 0·15 p.p.m. Se.
Group E, ethoxyquin, 100 p.p.m.
Group F, ethoxyquin, 200 p.p.m.

Expt 6 (seventy-four chicks):

Group A, no supplement.
Group B, sodium selenite to give 0·03 p.p.m. Se.
Group C, sodium selenite to give 0·06 p.p.m. Se.
Group D, sodium selenite to give 0·10 p.p.m. Se.
Group E, sodium selenite to give 0·15 p.p.m. Se.
Group F, DPPD, 250 p.p.m.

RESULTS

Table 1 shows the incidence of deficiency signs in the 324 chicks used and also gives the results of examining the tissues of 300 of them.

Expt 1. In this experiment, small stepwise additions of vitamin E to the diet were made to see if, at the minimum concentration preventing exudative diathesis completely, peroxidation in liver would also be prevented completely. The results show clearly that it was not. Additions of vitamin E linearly decreased peroxides in liver. At the lowest dietary concentrations, however, although the effect on peroxides was small and not significant at the 0·05 level, the incidence of exudative diathesis was greatly decreased. Further, although the breast muscles of the control chicks often showed haemorrhagic lesions, no significant differences in peroxidation were observed between these tissues and the breast muscles of any of the groups given supplements. Indeed, peroxidation in all breast muscles was uniformly low, the amounts of malonaldehyde being between 1 and 3 μg/g (cf. the values for other tissues in Table 1).

Expt 2. In this experiment the effects of different levels of dietary Se on exudative diathesis and on the amounts of peroxide in liver, spleen and kidney were studied. Even the lowest level of Se in the diet decreased the incidence of exudative diathesis greatly from 12/13 to 1/13. The amounts of peroxides were significantly decreased in all three tissues, but the higher levels of Se had no greater effect than the lower. Samples of venous blood were taken from three chicks of group A, which had exudates, and from three chicks of group C, which had not. The blood was made into 17% (v/v) suspension in saline–citrate solution (0·9% (w/v) NaCl, 0·6% (w/v) sodium citrate solution). When tested in vitro for peroxide production, they both showed low peroxide values ($\simeq$ 3 μg malonaldehyde/g). Peroxidation in blood is clearly not concerned in the deficiency state.

Expt 3. In this experiment, the effects of adding ethoxyquin to the basal diet were studied. Increasing concentrations appeared to produce a roughly parallel decrease in the occurrence of exudative diathesis and in peroxidation in liver and kidney. Lipid

peroxidation in spleen, however, was not significantly decreased. Four chicks in this test were reared on a commercial chick mash: their tissues showed low peroxidation.

Expt 4. In this experiment, some of the previous tests with vitamin E and Se were repeated and were extended by using a still higher dietary concentration of vitamin E.

Table 1. *Lipid peroxidation in tissues and the incidence of exudative diathesis in chicks. Effects of supplementing the torula-yeast diet with vitamin E, selenium, ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) or DPPD (N, N'-diphenyl-p-phenylenediamine)*

Expt no.	Addition to basal diet	Incidence* of exudative diathesis	No. of deaths	No. of chicks examined for peroxides	Malonaldehyde (μg/g tissue) (group mean with standard deviation) Liver	Spleen	Kidney
1	None	11/11	1	10	39±12	—	—
	D-α-tocopheryl acetate: 10 p.p.m.	2/11	1	11	34±7	—	—
	20 p.p.m.	1/11	0	11	32±9	—	—
	30 p.p.m.	1/10	0	10	29±11	—	—
	50 p.p.m.	0/10	1	9	24†±6	—	—
2	None	12/13	0	13	48±9	24±4	14±4
	Se: 0·15 p.p.m.	1/13	0	13	33‡±9	19†±1	9†±1
	0·50 p.p.m.	0/13	0	13	33‡±9	17†±1	9†±1
	1·0 p.p.m.	0/13	0	13	31‡±6	18†±3	9†±1
3	None	8/9	1	8	41±19	18±2	12±3
	Ethoxyquin: 100 p.p.m.	9/11	2	9	29±5	19±5	7†±3
	200 p.p.m.	6/10	1	9	30±5	16±2	5‡±2
	500 p.p.m.	2/9	0	7	21†±3	16±4	3‡±2
	Commercial chick mash given	0/4	0	4	0‡	2‡±1	5‡±5
4	None	8/9	3	6	42±10	19±3	13±2
	None (tocopheronolactone, 2 mg/day orally)	6/6	0	6	38±10	21±8	10±5
	None (tocopherylquinone, 5 mg/day orally)	5/6	2	4	33±11	23±9	12±4
	D-α-tocopheryl acetate: 10 p.p.m.	0/8	0	8	30±5	16±2	11±2
	150 p.p.m.	0/4	0	4	4‡±3	2‡±0	6†±0·5
	Se: 0·15 p.p.m.	0/9	0	9	27†±5	20±3	10±3
	1·0 p.p.m.	0/8	1	7	30†±11	20±4	10±2
5	None	8/9	0	9	31±7	—	8±2
	D-α-tocopheryl acetate: 10 p.p.m.	1/9	0	9	26±9	—	9±3
	20 p.p.m.	0/8	1	8	23±11	—	8±4
	Se, 0·15 p.p.m.	1/8	1	7	22±10	—	8±2
	Ethoxyquin: 100 p.p.m.	8/9	0	9	31±6	—	7±3
	200 p.p.m.	4/9	0	9	28±9	—	6±5
6	None	12/13	1	12	33±11	—	10±2
	Se: 0·03 p.p.m.	4/13§	0	13	48†±21	—	10±2
	0·06 p.p.m.	2/12§	1	11	26±11	—	6‡±3
	0·10 p.p.m.	0/12	0	12	22±8	—	5‡±3
	0·15 p.p.m.	0/13	3	8	22±12	—	5‡±3
	DPPD, 250 p.p.m.	11/11	2	9	27±9	—	6†±2

* Total no. of chicks in denominator; no. with exudative diathesis in numerator.
† Significantly different from control value, $P < 0.05$.
‡ Significantly lower than control value, $P < 0.01$.
§ See p. 524.

In addition, some chicks were given tocopheronolactone and tocopherylquinone dissolved in ethyl oleate, both by mouth. This dietary addition of vitamin E at 10 p.p.m. afforded full protection against exudative diathesis. The decrease in liver peroxides was only just significant ($P < 0.05$, one-tailed test), and there were no significant effects on spleen and kidney. When, however, vitamin E was included in the diet at 150 p.p.m., peroxide formation in the three tissues was decreased to low values: for liver this result was consistent with the trend found in Expt 1. The two dietary concentrations of Se gave results that were similar to those of Expt 2, although the effects on kidney and spleen were smaller. Neither tocopheronolactone nor tocopherylquinone had any significant effect.

Expt 5. In this experiment, the effects of two dietary concentrations of ethoxyquin (100 and 200 p.p.m.) were compared with those of the lowest concentration of Se and of the two lowest concentrations of vitamin E used in previous tests. Vitamin E and Se gave results much like those in Expts 1 and 2, although there was less peroxidation in the tissues of the control chicks. Ethoxyquin was almost as effective as before in decreasing the incidence of deficiency signs, but had less effect on tissue peroxidation.

Expt 6. The results of the earlier tests on dietary Se showed that the concentrations used exceeded the minimum required to prevent exudative diathesis. In this experiment, therefore, we tested concentrations as low as 0·03 p.p.m., which is less than the minimal amount (0·05 p.p.m.) that Bieri (1960) found protective. None of the chicks receiving Se at 0·10 and 0·15 p.p.m. developed exudates, although the amounts of malonaldehyde formed in liver and kidney homogenates were, respectively, about two-thirds and one-half of the control values. Out of twelve chicks given 0·06 p.p.m. Se, only two showed slight haemorrhage of leg muscle, and no exudates were seen. Tissue peroxidation in this group was slightly lower than in the controls, only the effect in kidney being significant. The results for the lowest concentration of Se, 0·03 p.p.m., were remarkable in that peroxidation in liver was significantly greater than in controls, although few chicks developed signs of deficiency, two of them having exudates and two others having slight haemorrhage of leg muscle. Peroxidation in kidney was not affected. There was a slight incidence of encephalomalacia in this experiment: eight of the fifty chicks receiving supplements of Se showed ataxia, head twisting and paralysis. In agreement with Scott *et al.* (1955), we found DPPD, 250 p.p.m., to be completely ineffective against exudative diathesis, but it did inhibit peroxidation in kidney significantly.

Expts 1–6, *considered together*. Thirty-two groups of chicks received the basal diet with and without supplements. When the percentage incidence of exudative diathesis (Y) in each group was compared with the group mean peroxide value for liver (X), a significant regression relationship was found:

$$Y = 3{\cdot}03X - 54 \quad (P < 0{\cdot}01). \tag{1}$$

The results for the group of chicks given vitamin E at 150 p.p.m. in the diet were omitted from this calculation, because this concentration of vitamin E greatly exceeded the minimum required to prevent the occurrence of the deficiency syndrome, and the marked inhibition of peroxidation that it produced might thus have biassed the overall

results in favour of a regression effect. A similar regression relation was not found for spleen and kidney.

The regression relation (1) was then investigated for vitamin E, Se and ethoxyquin separately. In order to do so, it was desirable to allow for the variation in peroxides of control tissues from experiment to experiment and the extinction readings were therefore converted into percentages of the corresponding control reading. In this way, the following regression for vitamin E (10–50 p.p.m.) was found:

$$Y = 2{\cdot}75X - 197 \quad (P < 0{\cdot}001). \tag{2}$$

For Se, the equation was found to be:

$$Y = 2{\cdot}75X - 187 \quad (P \ll 0{\cdot}001). \tag{3}$$

The result for Se, 0·03 p.p.m., has been omitted from this calculation because it was clearly anomalous.

Table 2. *Comparison of the effects of low dietary concentrations of vitamin E and selenium and high concentrations of ethoxyquin* (6-*ethoxy*-1,2-*dihydro*-2,2,4-*trimethyl*-*quinoline*) *and DPPD* (N,N′-*diphenyl*-p-*phenylenediamine*) *on lipid peroxidation and the incidence of exudative diathesis in chicks*

	Percentage decrease* in		
	Peroxides		Incidence of exudative diathesis
Addition to basal diet	Liver	Kidney	
D-α-tocopheryl acetate, 10 p.p.m.	16	0	88†
Se, 0·03 p.p.m.	−44‡	4	67‡
Se, 0·15 p.p.m.	29†	21§	92†
Ethoxyquin, 500 p.p.m.	49‡	75§	75‡
DPPD, 250 p.p.m.	18	38‡	0

* Compared to controls not given supplements.
† Significantly lower than control value, $P < 0{\cdot}001$.
‡ Significantly different from control value, $P < 0{\cdot}05$.
§ Significantly lower than control value, $P < 0{\cdot}01$.

The equations (2) and (3) are remarkably similar, and they show that, when either vitamin E or Se was added to the diet in the minimum amount to prevent exudative diathesis completely, the peroxides in the liver were decreased in amount by only about one-third, namely 33 and 35 %, respectively. In the same way, a regression equation was found for peroxides in kidney, which showed a decrease of 52 % when deficiency signs were prevented completely by minimal addition of Se to the diet:

$$Y = 1{\cdot}53X - 90 \quad (P < 0{\cdot}01). \tag{4}$$

When the results for peroxides in liver of chicks receiving ethoxyquin were treated in the same way, the best line of fit was:

$$Y = 1{\cdot}02X - 17{\cdot}8. \tag{5}$$

The slope of this line was not quite statistically significant ($F = 6{\cdot}22$, cf. $F = 6{\cdot}61$ at $P = 0{\cdot}05$), but it does indicate that the least amount of ethoxyquin to prevent exudative diathesis decreased the amount of peroxides in liver by about 85 %. This reduction is in strong contrast with that of one-third associated with minimal concentrations of vitamin E or Se.

In Table 2, a comparison is made of the various effects found for low dietary concentrations of vitamin E and Se and for high dietary concentrations of ethoxyquin and DPPD. Ethoxyquin, 500 p.p.m., reduced both tissue peroxides and exudative diathesis significantly, whereas DPPD, 250 p.p.m., decreased peroxide in kidney, but was completely ineffective against exudative diathesis. Vitamin E, 10 p.p.m., in contrast to both ethoxyquin and DPPD, combined a dramatic effect on signs of deficiency with a lack of effect on tissue peroxide. Se significantly protected against exudative diathesis at both 0·03 and 0·15 p.p.m., but peroxides in liver were affected differently at the two dietary concentrations, being increased at 0·03 p.p.m. and decreased at 0·15 p.p.m.

Peroxides in diets. In tests carried out at various times during and after the experiments, no peroxidation was found even in the unsupplemented diet.

DISCUSSION

The experiments with low dietary concentrations of vitamin E (10 and 20 p.p.m.) suggest that the protection it affords against exudative diathesis in the chick is not correlated with its action in reducing peroxidation, at least in any tissue we have studied. Bieri *et al.* (1961) used a considerably higher concentration of vitamin E (100 p.p.m.), sufficient not only to prevent exudative diathesis but also to reduce peroxidation substantially, and they were thus led to believe that the two effects are causally related. Machlin *et al.* (1959) used four times our lowest level of tocopherol in a study of encephalomalacia in the chick, as a result of which they also postulated a causal relationship between peroxidation and the onset of deficiency signs. Conclusions cannot be drawn from the recent study of Zalkin *et al.* (1960), as the amounts of vitamin E used in their supplemented diets were not stated. Table 2 shows clearly that, at the smallest concentration of vitamin E sufficient to prevent exudative diathesis, the two effects can be separated. From the regression lines for the results on vitamin E we find that the minimum dietary concentration preventing exudative diathesis completely, under the conditions of these experiments, decreased peroxidation in liver and spleen by only one-third and by about one-half in kidney. It might be argued—as it has been by Zalkin & Tappel (1960)—that some of the thiobarbituric acid reactants in tissues do not represent lipid peroxidation products; however, as we have shown, the residual two-thirds of the 'peroxides' could be readily decreased by further additions of vitamin E to the diet. This lack of correlation between exudative diathesis and tissue peroxidation is shown still more clearly by comparing the effects of vitamin E with those of ethoxyquin. In our experiments, the lowest dietary concentration of ethoxyquin required to prevent exudative diathesis decreased peroxidation in liver by 85 %. It would follow from the regression line that, if sufficient of this substance were added to the diet to reduce liver peroxidation by one-third, that is all the

reduction 'necessary' if vitamin E were added, then exudative diathesis would still occur in half the birds. Further, DPPD significantly inhibited peroxidation in one tissue, kidney, without influencing the course of the deficiency disease. These facts suggest that the role of vitamin E in preventing exudative diathesis is not causally related to any antioxidant function it may possess in vivo.

In our experiments, the minimum protective dietary concentration of Se appeared to be between 0·06 and 0·10 p.p.m., in fair agreement with the figure of 0·05 p.p.m. given by Bieri (1960). At 0·03 p.p.m., Se reduced the incidence of exudative diathesis significantly, although not completely, but it also allowed the amount of liver peroxide to rise significantly above the control figure. At higher concentrations, peroxidation in liver and kidney was reduced by about one-third, but this effect was maximal at 0·10 p.p.m. and no greater at 1·0 p.p.m. From the regression equation (3), it is apparent that complete prevention of deficiency signs is accompanied by a decrease of 35 % in peroxidation in liver. However, the results obtained with 0·03 and 0·06 p.p.m. show that a high degree of protection can be afforded without such a decrease in peroxidation. Bieri *et al.* (1961), in similar experiments on Se, found a greater decrease in liver peroxide than we did. They suggested that Se may be incorporated in a new protein with remarkable antioxidant properties and accounted for the failure of Se—in contrast with known antioxidants—to reduce peroxidation below a certain level by the inability of the tissues to accumulate sufficient of it in biologically active form even when increased amounts are given in the diet. In support of this idea, Søndergaard Bieri & Dam (1960) found that chicks given 70 μg Se (1 p.p.m. in the diet) during the 1st week of life developed exudative diathesis after a further 7 days, whereas Bieri (1960) had found that continuous supplementation of the diet with 0·05 p.p.m. Se was protective. These findings were considered to indicate that the chick is unable to store biologically active Se in appreciable amounts.

The hypothesis that Se might function by being incorporated in some protein with exceptionally powerful antioxidant properties has given rise to several studies in vitro on the antioxidant properties of seleno amino acids. The resultant findings hardly lend credence to the hypothesis, if they are considered in relation to the amounts of Se known to be present in tissues. Thus, Zalkin *et al.* (1960) examined several seleno amino acids and found them to be more active, but only up to ten times more active than their sulphur analogues. In order to explain the high biological activity of Se in terms of the antioxidant hypothesis, therefore, they were led to postulate—in contrast to Søndergaard *et al.* (1960)—that Se must be able to accumulate in amounts similar to those of tocopherol in the tissues. As evidence for this possibility, they cited McConnell & Wabnitz (1957), who found 1 μg Se/g protein in the liver of a dog given Se parenterally. Kuttler, Marble & Blincoe (1961) have also shown that Se concentrations can be readily increased in tissues. However, even if the concentrations were of the same order as tocopherol concentrations, they would still be 10^4 times less than that of S in the form of methionine and cystine (Block & Weiss, 1956). Further, the torula-yeast diet used in our experiments contained about 0·26 % S (as sulphur amino acids), which is about 5×10^4 times as much as the effective Se concentration (0·05 p.p.m.) used by Bieri (1960). In view of the findings of Zalkin *et al.* (1960) on the relative

potencies of Se and S analogues, it is difficult to see why the presence of seleno amino acids in relatively minute amounts should have any significant antioxidant effect compared with that of the overwhelming amounts of S-containing amino acids present.

In another study, Olcott, Brown & Van der Veen (1961) compared methionine with seleno methionine as an antioxidant for triolein, lard and menhaden oil. Although methionine was almost inactive, seleno methionine inhibited oxidation of the triolein and lard, but only when the concentration was at least 5 μmoles/g ($\simeq$ 1 mg/g). In menhaden oil, even ten times this concentration was ineffective. It is instructive to consider also the work of Marcuse (1960), who found that several amino acids containing no S, such as lysine, tryptophan and histidine, were much more powerful antioxidants than methionine.

There would thus appear to be three main objections to interpreting the biological action of Se in terms of the antioxidant hypothesis. The amounts of Se in tissues stand in sharp contrast with the amounts of any known seleno amino acid that would be necessary, on the basis of antioxidant studies in vitro, to produce the observed effects in vivo. Secondly, even the amounts that Zalkin *et al.* (1960) had to postulate as accumulating would be minute compared with the overwhelming proportions of S-containing and other amino acids present, and there is no reason why—if in vitro comparisons are to be made—their antioxidant potential should be ignored. Thirdly, the fact that inhibition of lipid peroxidation by Se reaches a maximum, leaving considerable amounts of 'peroxides' still unaffected by further dietary supplementation, is uncharacteristic of any known antioxidant. This phenomenon occurs in spite of the fact (as was noted above) that Se concentrations in tissues can be readily increased (Kuttler *et al.* 1961).

It would seem, from these experiments, that the functions of both tocopherol and Se can be better understood if they are dissociated from any antioxidant properties either of them may possess. The known facts would seem to be better explained by assuming that both substances act independently on alternative pathways of intermediary metabolism. Schwarz (1961*a*) has presented this point of view with clarity. It is also appropriate here to note that Dam, Nielsen, Prange & Søndergaard (1958) produced exudative diathesis in chicks by giving them a diet almost free from polyenoic fatty acids and found no peroxides in the fat tissue of nineteen out of twenty-three chicks with exudates. They suggested that peroxidation in vivo may not itself be the cause of the exudation process, but rather the result of the loss of vitamin E from the tissues, and that peroxidation may be accelerated by haematin compounds released during the haemorrhagic phase of the condition. They also suggested that the effect of dietary Se on peroxidation is an indirect one, following on a direct effect on the exudation process. What, then, is the significance of the 'lipid peroxidation' that occurs in the tissues of deficient animals and that can be, to a certain extent, decreased by dietary supplementation with either substance? It must be emphasized that the tests for 'lipid peroxidation' by the thiobarbituric acid method, as applied by Bieri *et al.* (1961) and many other workers, including ourselves, are believed to measure only the amount of malonaldehyde produced in the tissue homogenates during incubation in

vitro. Whether tissues of adequately fed and deficient chicks contain different amounts of malonaldehyde in vivo is open to question. Caputto, McCay & Carpenter (1961) were in fact unable to detect such a difference in the tissues of several species when tissues were homogenized directly in trichloroacetic acid solution, and we have confirmed this finding for chick liver. When malonaldehyde was given to rats by intraperitoneal injection, Caputto *et al.* (1961) were unable to find more than half in the tissues after 10 min. They concluded that the thiobarbituric acid test could not detect lipid peroxidation in vivo even if it occurred. Although, therefore, it is true that, under the artificial conditions of aerobic incubation, there is often a difference in the behaviour of tissue homogenates from adequately fed and deficient animals, the precise relationship of this phenomenon to the situation in the living animal remains obscure. The effect of Se on lipid peroxidation is small in the chick and cannot be adequately correlated with the properties of any of its known derivatives or with its nutritional role, as we have shown. Nor does the effect manifest itself consistently in all tissues, as Bieri *et al.* (1961) have shown; in the rat, in which the nutritional role of Se in preventing necrotic liver degeneration is established, the effect cannot be observed at all (Corwin, 1960). Our experiments with tocopherol have suggested that it is essential to work with low dietary concentrations, if its action is to be distinguished from that of antioxidants such as ethoxyquin and DPPD. Rather than α-tocopherol functioning as an antioxidant, it may be that some antioxidants can function like vitamin E, as Schwarz (1961*b*) has suggested.

SUMMARY

1. Three hundred and twenty-four day-old cockerels were reared on diets containing torula yeast with supplements of vitamin E, sodium selenite, ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) or DPPD (*N*,*N*′-diphenyl-*p*-phenylenediamine). A high proportion (92%) of the chicks given the unsupplemented diet developed exudative diathesis after 20–30 days.

2. Lipid peroxidation occurring in vitro in homogenates of liver, spleen and kidney was measured by the thiobarbituric acid method.

3. Vitamin E as D-α-tocopheryl acetate, at from 10 to 50 p.p.m. in the diet, linearly decreased the amount of peroxides in liver. At about 10 p.p.m. in the diet, vitamin E markedly reduced the number of chicks that developed exudates, but peroxidation in liver was inhibited only slightly. At 150 p.p.m., peroxidation in liver and spleen was almost completely inhibited.

4. Se, at 0·03 p.p.m., significantly decreased the incidence of exudative diathesis, but also significantly increased peroxidation in liver. Higher dietary concentrations of Se decreased the amount of peroxides in liver, spleen and kidney but never by more than one-third in liver and one-half in kidney. The effect of Se on peroxidation was maximal at 0·10–0·15 p.p.m. When Se supplementation was increased to 1 p.p.m. peroxidation was not affected further.

5. Ethoxyquin, at from 100 to 500 p.p.m. in the diet, linearly decreased peroxidation in liver and kidney, and the incidence of exudative diathesis was decreased in parallel.

6. DDPD, tested at 250 p.p.m. only, significantly decreased the amount of peroxides in kidney but was completely ineffective against exudative diathesis.

7. At the lowest dietary concentrations of vitamin E, Se and ethoxyquin needed to protect almost completely against exudative diathesis, peroxidation in liver was decreased by 33, 35 and 85%, respectively, suggesting a fundamental dissimilarity between the nutritional effects of vitamin E and Se and their effects on lipid peroxidation.

8. These experiments suggest that Se and vitamin E do not function in vivo solely as lipid antioxidants.

We thank the Misses R. J. Castle, J. Malein and J. M. Taylor for technical assistance.

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Printed in Great Britain