

Vitamin E and stress

4.* The metabolism of D- α -tocopherol during nutritional hepatic necrosis in the rat and the effects of selenium, methionine and unsaturated fatty acids

By J. GREEN, A. T. DIPLOCK, J. BUNYAN,
I. R. MUTHY AND D. McHALE

Walton Oaks Experimental Station, Vitamins Ltd, Tadworth, Surrey

(Received 30 November 1966—Accepted 16 January 1967)

1. Liver necrosis was produced in rats given diets deficient in selenium and vitamin E and the metabolism of [5-Me-¹⁴C]D- α -tocopherol was studied during the development of the disease.
2. When a torula yeast diet (containing sufficient of the yeast to provide essential fatty acids) was used, the addition of 5% cod-liver oil fatty acid methyl esters produced only a slight decrease in the time taken to produce the disease, compared to controls given methyl oleate; methionine had little protective effect. The metabolism of the radioactive tocopherol was unaffected by dietary lipid, Se or methionine and was not influenced by the development of necrosis.
3. When a casein diet (devoid of fat and containing insufficient Se to prevent liver necrosis) was used, the addition of small amounts of linoleic acid to the diet (as 2% maize oil fatty acid methyl esters) decreased the time taken to produce the disease and increased its incidence. However, the metabolism of the radioactive tocopherol was independent of dietary lipid or Se.
4. It would appear that either Se or vitamin E may be necessary for effective utilization of the rat's requirement for linoleic acid.

In preceding papers (Green, Diplock, Bunyan, McHale & Muthy, 1967; Diplock, Bunyan, McHale & Green, 1967; Diplock, Green, Bunyan, McHale & Muthy, 1967; Bunyan, Murrell, Green & Diplock, 1967) it was shown that the metabolism of small amounts of radioactive α -tocopherol was not affected by several nutritional stresses imposed on the vitamin E-deficient rat and chick and that there was no direct evidence for an increase in 'lipid peroxidation' in vitamin E-deficient animals. In particular, it was shown that the dietary stress of polyunsaturated fat in the rat and the chick was without influence on the metabolism of tocopherol or of other labile hydrogen donors such as sulphhydryl compounds and ascorbic acid. It was suggested that the biological antioxidant theory was inadequate to explain the role of vitamin E.

In the vitamin E-deficient chick, a dietary stress of polyunsaturated fatty acids quickly leads to signs of disease. This is not so in the rat, in which the development of clear pathological signs may take many months (Witting & Horwitt, 1964). However, if the diet of the weanling rat is deficient in selenium as well as vitamin E there is a rapid development of hepatic necrosis, with a high incidence of mortality. Thus the withdrawal of Se from the diet of the vitamin E-deficient rat can be regarded as a powerful stress, precipitating a fatal form of vitamin E deficiency disease. It has been suggested by several workers (Hamilton & Tappel, 1963; Shimazu & Tappel, 1964; Caldwell & Tappel, 1965; Tappel, 1965; Witting & Horwitt, 1964) that the role of Se is simply that of a special biological antioxidant. If this is so, the production of liver

* Paper no. 3: Br. J. Nutr. (1967), 21, 115.

necrosis by withholding Se from the vitamin E-deficient rat must be caused by the proliferation of 'lipid peroxidation'. As discussed previously (Green *et al.* 1967), it should be possible to detect such a process *in vivo*, if it occurs, by measuring the destruction of small amounts of radioactive tocopherol.

The part played by dietary unsaturated fat in the production of nutritional liver necrosis has been subject to some dispute (McLean & Beveridge, 1952, 1954) but it is clearly of importance. If the development of the disease is indeed related to uncontrolled lipid peroxidation in the liver it could be expected to be markedly affected by an increase in tissue unsaturated lipids; hence an increase in dietary unsaturated fatty acids should exacerbate the disease and, in the process, lead to an acceleration of antioxidant destruction.

A study of the metabolism of small amounts of [^{14}C]D- α -tocopherol in liver necrosis in the rat has been made and the effects of Se, methionine and unsaturated fatty acids have been examined.

EXPERIMENTAL AND RESULTS

General methods and materials. These followed closely those described by Green *et al.* (1967). Animals used for experiments involving radiochemical analyses were housed in tube cages to prevent coprophagy. Cod-liver oil methyl esters (CLOME), maize oil methyl esters (MOME) and methyl oleate (OLME) were as described previously. The preparation of [5-*Me*- ^{14}C]D- α -tocopherol, the determination of radioactive tocopherol and its metabolites in tissues, general methods of scintillation counting, and other analytical methods used have all been described (Green *et al.* 1967).

Animals and diets. Rats of the Norwegian hooded strain were used. Dams were given the vitamin E-deficient diet A10Y3 (Bunyan, McHale & Green, 1963) when their litters were born. The young rats were kept on this diet until the experiments started. The torula yeast diet used in Expt 1 had the percentage composition: torula yeast (Lake States Yeast and Chemical Division of St Regis Paper Co., Rhinelander, Wisconsin, USA) 30, sugar 48.4, glucose 18, salt mixture (Bunyan, Green, Diplock & Robinson, 1967) 3.2, vitamin mixture (Bunyan, Green *et al.* 1967) 0.4. The casein diet used in Expt 2 had the percentage composition: casein ('vitamin-free'; Nutritional Biochemicals Corp., Cleveland, Ohio, USA) 10, salt mixture (Diplock, Green *et al.* 1967) 0.4, sugar 62.3 and glucose 20. Vitamin A, 11 i.u./g diet, was added to make the total vitamin A content 22 i.u./g.

Expt 1. Liver necrosis was produced in rats by giving them diets based on the torula yeast diet. Young rats of both sexes were given this diet, supplemented with 10 i.u. vitamin A per g, from 21 to 32 days of age. Then the supplement was withdrawn and each rat was given 1000 i.u. vitamin A palmitate by mouth, and 24 h later the rats, weighing 33–46 g, were each given by mouth 253.9 μg (6460 disintegrations/sec (dps)) [^{14}C]D- α -tocopherol emulsified with Tween 80 in 0.2 ml water. The rats were then allocated at random to six groups to receive the torula yeast diet, with the following substitutions in place of sugar: group 1, 5% OLME; group 2, 5% OLME and 0.31% DL-methionine; group 3, 5% OLME and 0.05 ppm Se (as sodium selenite); group 4, 5% CLOME; group 5, 5% CLOME and 0.31% DL-methionine; group 6,

5% CLOME and 0.05 ppm Se (as sodium selenite). Diets were stored at 4°, those containing CLOME being kept under N₂. Food left in the feeding trays was discarded daily. The rats received these diets for 9 days, after which they were killed and their livers and carcasses (without alimentary canal) were analysed for [¹⁴C]α-tocopherol and its radioactive fat-soluble metabolites.

The experiment was carried out in three parts. In part 1, each group contained six rats, whose tissues were combined in pairs for analysis. In part 2, each group contained three lots of three rats, there being an interval of 1 week between starting each lot on the experiment. Each lot was killed, as in part 1, 9 days after the tocopherol dose, and the tissues of each lot were combined for analysis. In part 3, the rats were given 250 μg D-α-tocopherol, allocated to the dietary groups (except group 3), and housed in ordinary cages in groups of five. These rats were observed until 58 days of age, when the survivors were killed and the incidence of necrosis was recorded. The results of all three parts of the experiment are given in Table 1.

In part 3, groups 1, 2, 4 and 5 (i.e. those that did not receive Se) all showed a high incidence of liver necrosis. Methionine showed little protection. CLOME (given to groups 4 and 5) appeared to accelerate death; but this may have been due to an additional toxic stress, for the weight gains (not shown) of the CLOME-supplemented groups were considerably less than those for the OLME-supplemented controls. When the rats in tube cages (parts 1 and 2) were killed at the age of 41 days, visual examination of their livers indicated that, at this stage of development of the disease, methionine had delayed the necrotic process in rats given OLME (groups 1 and 2), but not in rats given CLOME (groups 4 and 5). The incidence of early necrotic change seemed slightly greater in rats given CLOME than in rats given OLME.

The results of the radiochemical analyses are also given in Table 1. There were no significant differences between groups in part 1 of the experiment, nor in part 2, even when allowance was made for differences between the three lots of rats in each dietary group.

Expt 2. The purpose of this experiment was to follow the important work of Valberg, Young & Beveridge (1959), who found that the addition of even small amounts of linoleic acid to a lipid-free yeast diet low in vitamin E and Se greatly accelerated the development of liver necrosis. It seemed particularly relevant to inquire whether such a dramatic effect could be associated with the acceleration of lipid peroxidation *in vivo* and, as a consequence, an increase in the destruction of small doses of α-tocopherol.

The yeast used by Valberg *et al.* (1959) was not available to us, so we used a lipid-free low-casein diet to produce liver necrosis. This diet contained casein manufactured in the USA. We have been unable to produce liver necrosis in our rats with a similar diet using casein of low vitamin content (Genatosan Ltd) made in the United Kingdom, which we normally include in our vitamin E-deficient diet A10Y3. Linoleic acid was added, where appropriate, as MOME, which contains about 50% methyl linoleate.

The general plan of the experiment was similar to that of Expt 1. Young rats of both sexes were given the diet A10Y3 from 21 to 36 days of age. They were then

Table 1. *Expt 1. Effect of selenium deficiency on the metabolism of [¹⁴C]D-α-tocopherol in weaning rats given a necrogenic torula yeast diet, with and without supplements of methyl oleate (OLME), cod-liver oil methyl esters (CLOME) and DL-methionine*

(The experiment was carried out in three parts. In Part 1, six rats of each group were each given 253.9 μg (6460 dps) [¹⁴C]D-α-tocopherol, housed individually in tube cages for 9 days, and combined in pairs for analysis. The results are given as means with standard deviations. Part 2, in which the rats were treated as in part 1, was carried out in three sections, each with groups of three rats whose tissues were subsequently combined for analysis. The results are given as the means and standard deviations of the triplicate analyses (each of three rats). In part 3, groups of five rats were housed in cages and the incidence of necrosis and survival time were measured. Survivors were killed at 58 days of age)

Group	Dietary supplement	Part 1		Part 2		Part 3						
		Liver	Carcass	Liver	Carcass	Incidence of necrosis†	Survival time‡ (days)	No. of survivors at 58 days§				
		Wt (g)	[¹⁴ C]α-tocopherol (dps/g)	Wt (g)	[¹⁴ C]α-tocopherol (dps/g)	Wt* (g)	[¹⁴ C]α-tocopherol (total dps)					
1	5% OLME	2.15 ± 0.23	4.7 ± 2.0	4.4 ± 1.1	3.55 ± 1.75	2.92 ± 0.48	9.4 ± 5.7	35.6 ± 8.8	292 ± 169	4/5	52.4 ± 8.2	3/5
2	5% OLME + 0.31% methionine	2.53 ± 0.12	4.7 ± 1.5	5.3 ± 2.2	3.27 ± 0.91	3.33 ± 0.84	8.7 ± 7.2	41.6 ± 11.4	383 ± 235	4/5	54.2 ± 5.0	2/5
3	5% OLME + 0.05 ppm Se	2.17 ± 0.06	7.7 ± 2.9	4.5 ± 3.3	3.90 ± 1.35	3.31 ± 0.39	8.0 ± 5.0	35.4 ± 7.9	312 ± 191	—	—	—
4	5% CLOME	2.06 ± 0.14	8.7 ± 4.9	4.5 ± 3.3	4.45 ± 1.80	2.93 ± 0.87	13.8 ± 6.5	31.6 ± 8.6	385 ± 122	5/5	42.2 ± 6.6	0/5
5	5% CLOME + 0.31% methionine	2.15 ± 0.28	5.2 ± 4.5	4.1 ± 1.1	2.83 ± 1.71	3.43 ± 0.68	7.8 ± 3.8	36.4 ± 10.3	426 ± 79	5/5	47.6 ± 1.5	0/5
6	5% CLOME + 0.05 ppm Se	1.60 ± 0.13	10.9 ± 3.4	3.9 ± 1.7	3.95 ± 1.31	2.86 ± 0.70	9.5 ± 2.4	32.6 ± 6.9	341 ± 188	1/5	56.8 ± 2.7	4/5

dps, disintegrations/sec.
 * Wt without liver or alimentary tract.
 † Total no. of rats in denominator; no. with necrosis in numerator.
 ‡ Results, including values of 58 days for survivors, given as means with standard deviations.
 § Total no. of rats in denominator; no. of survivors in numerator.

Table 2. *Expt 2. Effect of selenium deficiency on the metabolism of [¹⁴C]D-α-tocopherol in weanling rats given a fat-free necrogenic casein diet, with and without supplements of methyl oleate (OLME) and maize oil methyl esters (MOME)*

(The rats used for analysis were housed individually in tube cages. There were six in each group and each rat received 52.5 μg (6412 dps) [¹⁴C]D-α-tocopherol. They were killed after 11 days on the experimental diets and combined in pairs for analysis (three such analyses per group). Results are given as means with standard deviations. The rats used for survival and necrosis studies were housed in groups in cages)

Group	Dietary supplement	Liver		Carcass			Survival time (days)	Incidence of necrosis*	No. of survivors at 78 days†
		Wt (g)	[¹⁴ C]-tocopherol (dps/g)	Wt (g)	[¹⁴ C]-tocopherol (total dps)	[¹⁴ C]-metabolites (total dps)			
1	None	4.17 ± 0.70	9.2 ± 2.4	62.4 ± 15.3	641 ± 112	418 ± 68	3‡/15	13/15	
2	0.05 ppm Se	4.46 ± 0.61	8.1 ± 3.9	65.0 ± 15.6	527 ± 121	349 ± 184	—	—	
3	2 % OLME	4.16 ± 0.46	10.0 ± 2.5	63.7 ± 17.7	509 ± 119	368 ± 66	0/8	8/8	
4	2 % OLME + 0.05 ppm Se	4.17 ± 0.55	8.3 ± 2.0	63.8 ± 15.7	508 ± 128	395 ± 74	—	—	
5	2 % MOME	3.93 ± 0.85	9.7 ± 1.9	64.9 ± 15.4	594 ± 111	303 ± 49	7§/16	10/16	
6	2 % MOME + 0.05 ppm Se	4.47 ± 0.38	6.6 ± 1.3	63.7 ± 14.1	526 ± 41	352 ± 105	0/7	7/7	

dps, disintegrations/sec.

* No. of rats in group in denominator; no. with necrosis in numerator.

† No. of rats in group in denominator; no. of survivors in numerator.

‡ Three other rats in this group had post-necrotic scarring.

§ One other rat in this group had post-necrotic scarring.

housed in tube cages and given a single oral dose of $52.5 \mu\text{g}$ (6412 dps) [^{14}C]p- α -tocopherol. After 24 h they were allotted to six groups, each of six males and six females, to receive the casein diet with the following substitutions in place of sugar: group 1, none; group 2, 0.05 ppm Se (as sodium selenite); group 3, 2% OLME; group 4, 2% OLME and 0.05 ppm Se (as sodium selenite); group 5, 2% MOME; group 6, 2% MOME and 0.05 ppm Se (as sodium selenite). The rats were killed after 11 days on these diets, and their livers and carcasses were combined in pairs for analysis. Other rats were housed in ordinary cages in groups, given $52.5 \mu\text{g}$ D- α -tocopherol each, and then given the diets described for groups 1, 3, 5 and 6. They were kept for 80 days and the incidence of necrosis and mortality was recorded.

The results of the survival test (Table 2) in general confirmed those of Valberg *et al.* (1959), although a lower incidence of liver necrosis was found. The casein diet used by us may have contained marginally more Se than the yeast diet of Valberg *et al.* (1959). The animals kept in tube cages, which were killed for analysis after 11 days, did not show any signs of liver necrosis, irrespective of the dietary regimen. There were no significant differences in the concentrations of [^{14}C] α -tocopherol or its metabolites in the livers of the six groups, nor in the total recovery of [^{14}C] α -tocopherol and its metabolites from the carcasses of the six groups (Table 2).

DISCUSSION

The nutritional relationships of vitamin E and Se are now reasonably well understood for a number of species, and the two substances clearly exert a similar role in the prevention of several diseases, such as liver necrosis in the rat, exudative diathesis in the chick, and muscular dystrophy in sheep and cattle. The nature of the relationship at the metabolic level is, however, still obscure. The simplest explanation is that the two substances have essentially identical actions biochemically and can replace each other molecularly. This approach is broadly that of the biological antioxidant theory and is, indeed, implicit in that theory. Se (in its biological form) and α -tocopherol are both considered to be simple antioxidants, in the absence of which widespread 'lipid peroxidation' is thought to occur *in vivo*, with resultant metabolic damage. Since both substances are considered to have the same function, they can simply substitute one for another (Witting & Horwitt, 1964) and spare each other at the molecular level. Schwarz (1965), however, has analysed the synergistic and potentiating relationships between α -tocopherol, Se and sulphur amino acids in the prevention of nutritional liver necrosis in the rat and has concluded that Se and α -tocopherol have no mutually potentiating effect but probably have independent metabolic roles.

If Se and α -tocopherol can substitute for each other at the molecular level, it might be expected that, in rats made deficient in both substances (as by a necrogenic diet), dietary supplementation with Se would lead to an increase in the tocopherol content of the tissues. There is little evidence of this, and quantitative analyses of small amounts of tocopherol in vitamin E-deficient tissues are difficult to obtain with the required degree of accuracy. A much more confident answer can be given by studying the effect of Se on the metabolism of a small amount of radioactive α -tocopherol in rats

given a basal necrogenic diet, as we have done here. The results of the two experiments carried out show that, in fact, Se does not affect the metabolism of tocopherol during the critical 9–11 days in which the necrosis is developing. Se evidently does not spare tocopherol molecularly, as is required if both exercise a simple antioxidant function. The results rather support the suggestion of Schwarz (1965) that the two substances have independent roles and they agree with our previous finding that Se does not affect the metabolism of tocopherol in the vitamin E-deficient chick (Diplock, Bunyan *et al.* 1967). The results of Expt 1, moreover, provide no evidence that the necrotic process is causally related to 'lipid peroxidation' *in vivo*. As we have shown previously (Green *et al.* 1967), peroxidation must result in the destruction of the antioxidant. This was not found. Furthermore, the metabolism of a 250 μg dose of [^{14}C] α -tocopherol was unaffected, during the development of the necrosis, by the administration of polyunsaturated fatty acids (CLOME).

Calvert, Nesheim & Scott (1962) have studied the relationship between Se and α -tocopherol in the chick and have provided evidence that the two substances have a mutually potentiating action in preventing muscular dystrophy. Desai & Scott (1965) have suggested that Se supplementation increases the plasma tocopherol level of chicks given 10 ppm α -tocopherol in their diet. They gave chicks oral doses (5 mg/chick) of [^3H]D- α -tocopheryl acetate and found that concomitant administration of 0.5–1.0 mg Se increased the retention of tritium in serum collected 48 h later, although they did not identify the tritiated compound. It may have been tocopherol as they suggested, but we have found (unpublished results) that serum is a major carrier of tocopherol breakdown products, especially α -tocopherolquinone. We have shown that Se in physiological amounts does not affect the metabolism of a single small amount of [^{14}C]D- α -tocopherol given to chicks (Diplock, Bunyan *et al.* 1967). The results of Desai & Scott (1965) are, however, not necessarily incompatible with ours, for their experimental conditions were quite different. In the chick, large amounts of Se may indeed affect the transport and retention of tocopherol, especially under conditions in which a small dietary supply of the latter is continuously available. Erwin, Sterner, Gordon, Machlin & Tureen (1961) have suggested that Se may affect the transport of tocopherol in the dystrophic lamb. Nevertheless, it seems that such a role for Se cannot be a major one as Se has an extraordinarily high activity in preventing exudative diathesis in the chick, even in the absence of significant amounts of tocopherol (Bieri, 1964), and, in the rat, it is highly effective in preventing necrosis in the absence of measurable quantities of vitamin E.

Expt 2 was undertaken in order to study the effect of small quantities of linoleic acid on the development of liver necrosis in the rat and on the metabolism of [^{14}C] α -tocopherol. The role of dietary lipid in the genesis of liver necrosis has, in the past, been the subject of some disagreement. Goettsch (1951) first investigated the problem, using a diet containing a low level of casein (7.5–10.0%). This diet was highly necrogenic and the casein she used must have been Se-deficient. Goettsch (1951) found that the addition of up to 20% lard to the basal diet did not affect the time taken to produce the disease or its incidence. However, the basal diet used was not fat-free, as it contained 2% cod-liver oil. McLean & Beveridge (1952) used a brewer's yeast diet,

containing lard, that gave a high incidence of necrosis when the yeast component was varied between 5 and 20% of the diet but no necrosis when the fat was omitted. The time taken to produce the disease was much more than that found by Goettsch (1951) or that usually found with torula yeast diets (compare the average time for the development of the lesion (64-90 days) in the experiments of McLean & Beveridge (1952) with the times given in Expt 1, this paper). It seems clear that the basal diet of McLean & Beveridge (1952) must have been only marginally deficient in Se or tocopherol, and this is borne out by the fact that, when they used 30% of the yeast in the basal diet, the incidence of necrosis was reduced to zero. Dam & Granados (1951) also observed no necrosis in rats given a 10% casein diet without fat, but found that the incidence of disease could be progressively increased by increasing the amount of unsaturated fat in the diet. Nevertheless, McLean & Beveridge (1954), attempting to re-investigate the problem and now using a brewer's yeast that had been extracted with 95% ethanol, observed in four well-controlled experiments a high incidence of liver necrosis in rats even when their diet was devoid of fat. It is interesting that much earlier, Schwarz (1944) had presented results indicating that the incidence of liver necrosis in rats given a diet based on alkali-extracted casein was essentially the same whether the diet was fat-free or contained 20% butterfat. Harris & Mason (1956), in their classical review of the vitamin E problem, considered that liver necrosis could occur in the absence of dietary lipid, although they agreed that its development could be accelerated by dietary fat. Valberg *et al.* (1959) obtained no necrosis in rats given a fat-free yeast diet, but observed a high incidence of necrosis when unsaturated fatty acids were added. The addition of 10% oleic acid produced a 50% incidence of disease within a short time. They showed, furthermore, that linoleic acid was highly active in producing necrosis, and 100% incidence was found with as little as 0.25% linoleic acid in the diet, with an average survival time (from the time of adding the lipid) of only 10 days.

The results of the survival tests in Expt 2 are not wholly satisfactory, as we did not obtain the expected incidence of necrosis. Nevertheless they generally support those of Valberg *et al.* (1959) in showing that small amounts of linoleic acid (about 1% of the diet in Expt 2, as MOME contains about 50% methyl linoleate) increase the incidence of necrosis and may be necessary for its induction. However, in agreement with our findings in Expt 1, there was no effect of dietary lipid stress on the metabolism of [^{14}C] α -tocopherol.

The nature of the relationship between dietary lipid (and especially linoleic acid) and Se remains one of the most significant features of the problem. The evidence as to whether liver necrosis is absolutely dependent on a dietary supply of lipid must necessarily still be regarded as inconclusive. Much depends on the significance to be attached to the experiments of McLean & Beveridge (1954) in which they clearly obtained necrosis without dietary fat. They themselves were unable to explain why those experiments should have given a different result from their earlier ones (McLean & Beveridge, 1952), and, because they observed that the incidence of necrosis increased in a series of successive experiments with the fat-free diet, they suggested that an alteration in the rat's intestinal flora might be involved. They were thus led to

adopt the earlier suggestion of György, Stokes, Goldblatt & Popper (1951), who attributed the antinecrogenic action of aureomycin to a suppression of bacterial metabolites 'with which the liver, in the absence of vitamin E or of sulphur—containing amino acids as detoxifying agents, is unable to cope'.

The eventual elucidation of 'factor 3' and the discovery of the involvement of Se deficiency in nutritional liver necrosis in the rat (Schwarz & Foltz, 1957) directed attention away from the problem of the influence of toxic substances in nutritional liver necrosis. However, it is now known that both vitamin E and Se can protect rats against a variety of hepatotoxic agents; such agents may well influence the development of necrosis in the presence of marginally sufficient Se. Nevertheless, the results of McLean & Beveridge (1954) and their contrast with the earlier ones of McLean & Beveridge (1952) and the later ones of Valberg *et al.* (1959) could have been due to differences in the Se content of the diets used. Se is fully active at 0.05 ppm, and the amounts present in the basal necrogenic diets used are in fact unknown. There may be a critical dietary level of Se at which its protective effect may be counterbalanced by natural antagonistic factors, which may include certain metals and traces of unsaturated lipid (almost impossible to remove entirely), and the total necrogenicity of the diet may depend on the interplay of these factors. This critical concentration of Se may be effective in protecting rats against liver necrosis in the absence of additional stress or toxic factors. The addition of fat to the diet might, under these circumstances, have a marked effect on the development and incidence of necrosis. Indeed, McLean & Beveridge (1954) observed a clear acceleration of necrosis in their rats on adding 5% lard to the basal diet. This observation is in striking contrast to that of Goettsch (1951) who, working with the highly necrogenic casein diet, found no effect of fat on the development or incidence of disease. We have found (unpublished results) that the addition of up to 20% fat to highly necrogenic diets based on torula yeast likewise has no effect on liver necrosis in the rat.

Consider now another feature of the problem. Let us assume that the results of McLean & Beveridge (1954) can be explained in terms of a particular combination of marginal Se deficiency and the presence of unidentified toxic factors arising from their fat-free diet, and that the later results of Valberg *et al.* (1959) are essentially valid. Valberg *et al.* (1959) produced a high incidence of mortality within 10 days by giving their rats amounts of linoleic acid equivalent to about 25 mg per day. In our Expt 2, we gave about 100 mg per day. These quantities, in fact, represent closely the range of the rat's daily requirement for linoleic acid (Hove & Harris, 1946). It would seem to follow, therefore that the rat is unable to deal with its own essential fatty acid requirement in the absence of vitamin E and Se. Hove & Harris (1946) found that the efficacy of linoleic acid, if it was given in amounts below requirement to rats already deficient in essential fatty acids, was increased by vitamin E supplementation.

It seems clear that a great deal more investigation is necessary before the nature of the relationship between Se, vitamin E and dietary lipid is understood. An essential prerequisite for future studies would appear to be the use of diets, the Se and lipid contents of which are much more accurately known than at present. It seems certain, however, that this relationship and the problem of liver necrosis cannot be under-

stood in terms of theories of 'lipid peroxidation' and simple biological antioxidants. The results of the experiments described here provide no evidence that α -tocopherol has a role in controlling 'lipid peroxidation' or that the latter is a process that is accelerated in the Se-deficient animal. Bunyan, Murrell *et al.* (1967) have shown that the true lipid peroxide content of rat liver is unchanged during the development of necrosis. The antioxidant theory would seem singularly ill-fitted, moreover, to explain the quantitative aspects of the relationship between Se and dietary lipid, several of which we have discussed above. The special relationship with linoleic acid metabolism needs much more study, and especially the mechanism by which the rat's own essential fatty acid supply can apparently precipitate the necrosis.

The role of sulphur amino acids, such as methionine, in nutritional liver necrosis is still not clear, and is complicated by the fact that they contain an unknown, and possibly variable, amount of Se. The latter accounts for only a small part of the activity, however. Schwarz (1965) has shown that methionine may exert most of its activity through a pronounced potentiating effect on small amounts of α -tocopherol. The effects of methionine in combination with traces of Se may be more additive than potentiating (Schwarz, 1965). The protective effect of methionine, therefore, would be expected to be weak when it is used with diets highly deficient in vitamin E and Se (Expt 1). Our results do not support the suggestion of Schwarz (1965) that methionine has a 'sparing' effect on α -tocopherol at the molecular level. It may affect the repair of damaged tissue and may perhaps play an important part in protecting small amounts of vitamin E from destruction in the intestinal tract.

We wish to thank Miss J. Helsdon and Miss L. Dennis for their technical assistance.

REFERENCES

- Bieri, J. G. (1964). *Biochem. Pharmac.* **13**, 1465.
 Bunyan, J., Green, J., Diplock, A. T. & Robinson, D. (1967). *Br. J. Nutr.* **21**, 147.
 Bunyan, J., McHale, D., & Green, J. (1963). *Br. J. Nutr.* **17**, 391.
 Bunyan, J., Murrell, E. A., Green, J. & Diplock, A. T. (1967). *Br. J. Nutr.* **21**, 475.
 Caldwell, K. A. & Tappel, A. L. (1965). *Archs Biochem. Biophys.* **112**, 196.
 Calvert, C. C., Nesheim, M. C. & Scott, M. L. (1962). *Proc. Soc. exp. Biol. Med.* **109**, 16.
 Dam, H. & Granados, H. (1951). *Acta pharmac. tox.* **7**, no. 2, p. 181.
 Desai, I. D. & Scott, M. L. (1965). *Archs Biochem. Biophys.* **110**, 309.
 Diplock, A. T., Bunyan, J., McHale, D. & Green, J. (1967). *Br. J. Nutr.* **21**, 103.
 Diplock, A. T., Green, J., Bunyan, J., McHale, D. & Muthy, I. R. (1967). *Br. J. Nutr.* **21**, 115.
 Erwin, E. S., Sterner, W., Gordon, R. S. Machlin, L. J. & Tureen, L. L. (1961). *J. Nutr.* **75**, 45.
 Goettsch, M. (1951). *J. Nutr.* **44**, 443.
 Green, J., Diplock, A. T., Bunyan, J., McHale, D. & Muthy, I. R. (1967). *Br. J. Nutr.* **21**, 69.
 György, P., Stokes, J. Jr, Goldblatt, H. & Popper, H. (1951). *J. exp. Med.* **93**, 513.
 Hamilton, J. W. & Tappel, A. L. (1963). *J. Nutr.* **79**, 493.
 Harris, P. L. & Mason, K. E. (1956). *Int. Congr. Vitam. E*. III. Venice, 1955, p. 1.
 Hove, E. L. & Harris, P. L. (1946). *J. Nutr.* **31**, 699.
 McLean, J. R. & Beveridge, J. M. R. (1952). *J. Nutr.* **47**, 41.
 McLean, J. R. & Beveridge, J. M. R. (1954). *J. Nutr.* **52**, 499.
 Schwarz, K. (1944). *Hoppe-Seyler's Z. physiol. Chem.* **281**, 101.
 Schwarz, K. (1965). *Fedn Proc. Fedn Am. Socs exp. Biol.* **24**, 58.
 Schwarz, K. & Foltz, C. M. (1957). *J. Am. chem. Soc.* **79**, 3292.
 Shimazu, F. & Tappel, A. L. (1964). *Science, N.Y.* **143**, 369.
 Tappel, A. L. (1965). *Fedn Proc. Fedn Am. Socs exp. Biol.* **24**, 73.
 Valberg, L. S., Young, R. A. & Beveridge, J. M. R. (1959). *Can. J. Biochem. Physiol.* **37**, 493.
 Witting, L. A. & Horwitz, M. K. (1964). *J. Nutr.* **82**, 19.