Brit. J. Nutr. (1963), **17**, 391

The vitamin E activity of α-tocopherylquinone and α-tocopherylhydroquinone in the rat

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(*Received* 6 *February* 1963)

The vitamin E activity of α-tocopherylquinone and its corresponding hydroquinone has remained a subject of interest. The quinone has been shown to be present in animal tissues (Morton & Phillips, 1959; Diplock, Green, Edwin & Bunyan, 1960), and Csallany, Draper & Shah (1962) have identified it as a metabolite of α-tocopherol in rat liver. Either substance, or both, may play a part in the biological function of vitamin E; a scheme typical of several suggested is that of Slater (1961). Golumbic & Mattill (1940) found the quinone inactive in preventing foetal resorption in the rat when given orally, and Issidorides & Mattill (1951) found a single 25 mg dose of the hydroquinone, given intravenously, also ineffective. However, both substances prevent creatinuria and loss of weight in the muscular dystrophy of vitamin E-deficient rabbits (Mackenzie & McCollum, 1940; Mackenzie, 1942; Mackenzie, Rosenkrantz, Ulick & Milhorat, 1950): the activity of the hydroquinone was much enhanced when it was given intravenously. More recently, Mackenzie & Mackenzie (1960) found that tocopherylhydroquinone, given in daily doses of 5 mg (either intraperitoneally or intravenously) during pregnancy, prevented resorption in the rat. Green, Diplock, Bunyan, Edwin & McHale (1961) reported that tocopherylquinone, given in daily 10 mg doses from the 7th to the 13th day of pregnancy, was also effective.

Two considerations suggested that a more detailed investigation of the problem should be made. Examination of the literature revealed that several workers who have prepared α-tocopherylquinone have commented that the preparation always contained a small, unidentified, amount of material that, like tocopherol, was reducing to ferric chloride. Mackenzie & Mackenzie (1960) found that their tocopherylquinone (their methods of preparation and purification were not mentioned) contained about 0·6% of reducing material, which they considered was not α-tocopherol. We have examined the product obtained when α-tocopherol is oxidized with ferric chloride (a preparative method is given by Mackenzie *et al.* 1950) and have also found a similar amount of reducing material present. Paper chromatography showed that, in fact, some of the reducing material was α-tocopherol (see p. 392). Secondly, although the median fertility dose of α-tocopherol for the rat is of the order of 1 mg, it became apparent during our trials that a much smaller amount of α-tocopherol could prevent resorption if it was divided into a series of doses and given daily over the first 2 weeks of pregnancy. It therefore seemed possible, in view of the fairly large amounts of tocopherylquinone or hydroquinone that seem to be required to prevent resorption, that the activity found for these substances might have been due to their containing

significant quantities of α-tocopherol. In the trials described here, this possibility was examined.

The biological activity of α-tocopherol has been attributed to—or is thought to be closely related to—its effect in vivo on lipid peroxidation. Green *et al.* (1961) showed that both α-tocopherylquinone and its hydroquinone inhibited lipid peroxidation in liver homogenates in vitro, although these compounds were only about one-tenth as effective as α-tocopherol. We have therefore carried out some experiments on the effect of the quinone (given orally to rats) on lipid peroxidation of homogenates and further tests on its activity in vitro. We also thought it appropriate to report here the finding of measurable reduced nicotinamide–adenine dinucleotide-linked reductase activity for tocopherylquinone in tissues of several species, since evidence for such activity was found by Rosenkrantz (1956) but not confirmed by other workers (Deul, Slater & Veldstra, 1958; Wosilait, 1960; Frimmer, 1960).

EXPERIMENTAL

Materials. It is difficult to obtain α-tocopherylquinone free of measurable reducing material. The primary product obtained after oxidation of α-tocopherol with ferric chloride for 24 h by the method of Mackenzie *et al.* (1950), as determined spectrophotometrically, contained about 90% quinone and 0·3–2·0% reducing material (measured as tocopherol). Paper chromatography on a reversed-phase system (liquid paraffin, 95% (v/v) ethanol) revealed the presence of four reducing spots, one of them due to α-tocopherol. Tocopherethoxide (this name has recently been proposed by Vasington, Reichard & Nason (1960) for the substance previously designated, erroneously, α-tocopheroxide) was also present. Oxidation with excess of gold chloride in ethanol gave, after several hours at room temperature, the quinone in 96–98% yield, but 0·2% of reducing material (some of which was α-tocopherol) was present. The presence of tocopherethoxide was also readily demonstrable by an inflexion at 240 mμm in the u.v. spectrum. More prolonged oxidation with gold chloride gave a red product (*o*-quinone?) whose i.r. spectrum was different from that of tocopherylquinone. The purest specimen of α-tocopherylquinone was prepared by the method of Tishler & Wendler (1941) and Mackenzie *et al.* (1950). The quinone (gold chloride preparation) was reduced to the hydroquinone with sodium dithionite, and the hydroquinone was crystallized several times from light petroleum (b.p. 40–60°) at −40° until it had m.p. 66–67° (Tishler & Wendler (1941) describe the hydroquinone as a waxy solid; Rosenkrantz & Milhorat (1950) give m.p. 59–61°). Oxidation with Ag_2O in diethyl ether gave the quinone as a pale yellow oil, with $E^{1\%}_{1\,cm}$ 427 at λ_{max} 268 mμm (in hexane). Several different figures are given in the literature for the $E^{1\%}_{1\,cm}$ of α-tocopherylquinone, but the solvent, whose nature affects the extinction, has usually not been reported. Tishler & Wendler (1941) found 418, Issidorides (1951) 422, Frampton, Skinner & Bailey (1954) 419, Kofler (1947) 425 (light petroleum), Mackenzie *et al.* (1950) 450 and 455 (figures considerably higher than the others quoted, but again the solvent was not mentioned).

The crystalline hydroquinone was examined in the gestation–resorption test by

dissolving the required daily dose in a solvent vehicle under nitrogen immediately before administration. The quinone was stable in solution. Titration with ferric chloride showed it to contain less than 0·1% of a reducing substance, probably the hydroquinone. When 700 μg were chromatographed, α-tocopherol could not be detected on the paper (less than 1 μg present).

Diets. The rat diet A40Y contained dried brewer's yeast (Marmite Ltd) 25, casein, 'low vitamin content' (Genatosan Ltd) 10, sucrose 59, lard 1 and salt mixture (Bunyan, Green & Diplock, 1963) 5%, and was supplemented with vitamin A, 40 i.u./g, and vitamin D_3, 2·5 i.u./g. Diet A40Y3 consisted of A40Y with the lard content raised to 3% at the expense of sucrose. Diet A10Y3 consisted of A40Y3 with the vitamin A content decreased to 10 i.u./g, and diet A10Y3+E consisted of diet A10Y3 supplemented with 100 mg D-α-tocopheryl acetate/kg. The stock colony diet SD 1 was that described by Edwin, Diplock, Bunyan & Green (1961).

The soya-based diet for chicks was deficient in vitamin E and contained ADM assay protein C-1 (Archer-Daniels Midland Co., Ohio, USA) 30, stripped lard (Distillation Products Industries Ltd, Rochester, NY, USA) 4, salt mixture (Bunyan, Diplock, Edwin & Green, 1962) 6, glucose 58·2, vitamin mixture (Bunyan *et al.* 1962) 0·2, choline dihydrogen tartrate 0·4 and DL-methionine 0·3%, and was supplemented with vitamin A, 20 i.u./g, vitamin D_3, 2 i.u./g and chlortetracycline 0·2 p.p.m.

Fertility tests. Female rats (Norwegian hooded) were depleted of vitamin E by rearing them on diet A40Y from 13 days of age and were used at 4–7 months. Doses of the substances under test were given by the oral, intramuscular or intraperitoneal route at the times described in Table 1. Whenever possible, quinone and hydroquinone were examined together, and the test always included a positive control group given D-α-tocopheryl acetate or D-α-tocopherol and many negative controls. Following Mason (1942), the criterion of a positive response was taken as the presence of at least one live foetus at full term or at least two foetuses at the 17th or 18th day of pregnancy.

Lipid peroxidation. For the tests in vivo, 5-month-old vitamin-E depleted male rats were given three daily oral doses of the test substance, dissolved in ethyl oleate, and killed 24 h later. Liver homogenates were prepared and the malonaldehyde present was measured, after incubation for 1 h, by the thiobarbituric acid method as described by Bunyan *et al.* (1962). For the tests in vitro, the test substance was suspended in 1 ml phosphate buffer pH 7·4 (0·09 M) with the aid of 1 mg Tween 80 (polyoxyethylene sorbitan mono-oleate; Honeywill & Stein Ltd, London) and incubated with 4 ml homogenate at 37° for 1 h with shaking. Malonaldehyde formation was determined as in the in vivo test. Potencies of the quinone and hydroquinone relative to D-α-tocopherol were calculated by the procedure of Bunyan, Green, Edwin & Diplock (1960). Dahle, Hill & Holman (1962) showed that malonaldehyde formation cannot exactly indicate the degree of oxidation of a lipid unless its pattern of unsaturation is known. Hence, after an oral or dietary supplement, a change in amount of malonaldehyde formed could be due to a change either in degree of oxidation or in lipid composition or in both. However, this criticism has less force for the in vitro test, since a change in lipid composition is less likely to occur in so short a test period (1 h).

Measurement of reductase activity. Tissues were obtained from freshly killed hooded and albino male rats and male Rhode Island Red × Light Sussex chicks. Homogenates (10%, w/v) were prepared by means of a nylon–glass homogenizer in ice-cold phosphate buffer pH 7·0 (0·08 M) and then centrifuged at 10000 *g* for 15 min at 0°. Vitamin K_1 and tocopherylquinone were dissolved in a solution of BRIJ-35 (polyoxyethylene lauryl alcohol: Honeywill & Stein Ltd) as described by Wosilait (1960). Reductase activities of the crude supernatant solution were determined with NADH as electron donor, as described by Bunyan, Green, Diplock & Edwin (1961).

RESULTS AND DISCUSSION

The results of all the fertility tests are given in Table 1. It is clear that both the route of administration and solvent vehicle affected vitamin E activity. The oral route was less effective than the intraperitoneal, and activity was enhanced considerably (by both routes) if the solvent vehicle was a Tween-based aqueous emulsion. As tests 2 and 3 show, the median fertility dose of α-tocopherol could be as little as 200 μg or less if it was given intraperitoneally, as an aqueous emulsion or in propylene glycol containing 10% ethanol, and spread over the first 2 weeks of pregnancy. We encountered difficulties in testing tocopherylhydroquinone in daily doses of 5 mg or more since toxic signs were observed and pregnancy was usually suppressed. The results with negative controls (Table 1) showed that 4·2% of implantations resulted in live foetuses at the 17th or 18th day of pregnancy, even without vitamin E dosing.

In view of the relatively low activity of both quinone and hydroquinone, it is difficult to make an accurate assessment of their potency. The optimal dose of hydroquinone seemed to be 2·5 mg in aqueous emulsion intraperitoneally: 5 mg/day for 9 days was effective, but was toxic when given for longer periods. If 2·5 mg hydroquinone are approximately as potent as 16 μg α-tocopherol, as the results suggest, then the former has 0·6% of the latter's activity. The quinone at 10 mg/day orally was less effective than 60 μg α-tocopherol, and it is doubtful whether the results show any activity for the quinone by the oral route. There were some toxic signs on giving 5 mg intraperitoneally, which were not reversed by adding menaphthone (4 p.p.m.) to the diet. Considering together all the results of intraperitoneal injections of quinone, we found that (apart from the 1 mg dose) they produced four positive responses out of eleven—say a mean response of 4/11 at a mean dose of 4 mg. This is less than the response to 16 μg α-tocopherol (3/5). The maximum potency of the quinone was therefore about 0·4% that of D-α-tocopherol, rather less than that of the hydroquinone, a finding that agrees with earlier ones in muscular dystrophy by Mackenzie & Mackenzie (1959). These experiments lead us to believe that tocopherylquinone and tocopherylhydroquinone are slightly effective in preventing resorption and that their activity is not due to the presence of a reducing impurity (even if it had the same potency as α-tocopherol itself).

Table 2 shows the effect of three daily oral doses of tocopherylquinone on the lipid peroxidation of liver homogenates. The quinone significantly lowered malonaldehyde production. Graphical estimation showed that it has about 5–10% of the activity of

Table 1. *Assessment of vitamin E activity of α-tocopherylquinone and α-tocopherylhydroquinone by gestation–resorption tests on vitamin E-deficient rats*

Test no.	Substance	Solvent	Route of administration	Daily dose (mg)	Duration of dosing (days of pregnancy)	No. of rats	Result	Live foetuses/total implantations
1	Quinone	Ethyl oleate	Oral	10	6–13	5	All negative	0/51
		Ethyl oleate	Oral	5	6–13	5	All negative	0/50
		Tween 80–water	Oral	10	6–13	5	One positive	8/31
	D-α-tocopheryl acetate	Ethyl oleate	Oral	0·5	6–13	5	All positive	41/52
2	Quinone	PGE	IP	5	0–17	4	One positive, one negative, one rat died, one not pregnant	3/10
	Hydroquinone	PGE or Tween 80–water	IP	5	0–8 or 0–13	7	Toxic effects. Some rats died with haemorrhage and internal inflammation. No pregnancies	0/0
	D-α-tocopherol	Ethyl oleate	IM	0·6	7 and 8	3	All negative	0/32
		Ethyl oleate	IP	0·7	6, 7 and 8	3	All negative	0/21
		PGE	IP	0·100	0–13	3	All positive	30/30
		PGE	IP	0·050	0–13	5	All positive	48/49
		PGE	IP	0·015	0–13	4	Three positive, one negative	24/34
3	Quinone	Tween 80–water	IP	5·0	0–14	5	One positive, two negative, one rat died, one not pregnant	7/11*
		Tween 80–water	IP	3·8	0–14	3	One positive, one negative, one rat not pregnant	4/17
		Tween 80–water	IP	2·5	0–13	4	One positive, three negative	6/38
		Tween 80–water	IP	1·0	0–13	6	All negative	0/53
	Hydroquinone	Tween 80–water	IP	5·0	0–14	4	Toxic signs, no rat pregnant	0/0*
		Tween 80–water	IP	5·0	0– 9	2	Two positive	12/19
		Tween 80–water	IP	2·5	0–13	6	Four positive	25/51
		Tween 80–water	IP	1·0	0–13	4	All negative	0/39
	D-α-tocopherol	Tween 80–water	Oral	0·06	0–13	2	Both positive	9/11
				0·033	6, 7 and 8	4	All negative	0/46
				0·007	0–13	4	All negative	0/31
		Tween 80–water	IP	0·016	0–14	5	Three positive	20/44
				0·008	0–14	11	All negative	0/99
				0·004	0–14	4	All negative	0/34
Negative controls in all tests given similar doses of diluent only						110	Nine positive, remainder negative	40/959

* Menaphthone (4 p.p.m.) in diet in an attempt to suppress haemorrhagic signs.
IP, intraperitoneal; IM, intramuscular; PGE, propylene glycol with 10% ethanol.
Tween 80 was used at four times the concentration of the substance dissolved.

D-α-tocopheryl acetate in this test. This perhaps provides a further example of disparity between effects on lipid peroxidation in vivo and vitamin E activity in the nutritional and biological sense (cf. Bunyan *et al.* 1962, 1963).

Table 2. *Effects of oral doses of α-tocopherylquinone on lipid peroxidation of liver homogenates of vitamin E-deficient 6-month-old male rats receiving diet A 40 Y 3* (*see p.* 393)

Test no.	No. of rats	Substance	Daily dose for 3 days (mg)	Malonaldehyde (μg/g liver) (mean with standard deviation)
1	4	None	—	26·4 ± 0·8
	4	Quinone	5·8	23·7 ± 4·0
	4	D-α-tocopheryl acetate	0·9	17·5 ± 4·5
2	4	None	—	30·2 ± 6·4
	4	Quinone	11·6	21·2 ± 4·6
	4	D-α-tocopheryl acetate	0·9	23·1 ± 5·4

Consideration of the results of both tests, regardless of the amount of quinone given, shows that α-tocopherylquinone significantly decreased production of malonaldehyde ($P < 0·05$).

Table 3. *Effects of α-tocopherylquinone on lipid peroxidation in vitro of liver homogenates of vitamin E-deficient 6-month-old male rats receiving diet A 40 Y 3* (*see p.* 393)

Substance	Amount added to 4 ml homogenate (moles × 10^8)	Percentage decrease* in malonaldehyde formation			Relative potency
		Test 1	Test 2	Test 3	
D-α-tocopherol	20	—	60	57	100
	10	50	—	—	—
	4	—	56	41	—
	2	14	—	27	—
α-tocopherylquinone	100	69	—	—	45 (29–126)†
	50	—	69	67	—
	20	50	—	—	—
	10	—	44	42	—
	5	—	—	27	—

* In terms of control homogenates with only solvent added (see Table 1).
† Limits of error, $P = 0·95$.

Inhibition of lipid peroxidation in vitro by tocopherylquinone is shown in Table 3. The activity of the substance relative to that of D-α-tocopherol was estimated as 45%, which is greater than thought previously (Green *et al.* 1961). These authors also suggested that quinones active in this system may be reduced to quinols in vitro; indeed, such enzymic reduction has been shown for tocopheronolactone, the water-soluble metabolite of α-tocopherol (Bunyan *et al.* 1961). Further work now described has shown that tocopherylquinone is also reduced enzymically with NADH as hydrogen donor in the soluble fraction of several animal tissues, and these results are set out in Table 4, together with parallel results for other substrates.

Breast muscle of chicks on the soya diet deficient in vitamin E showed little reductase activity towards any substrate, but liver and kidney showed more, that for tocopheryl-

quinone being less than that for vitamin K_1. In livers of rats on various diets there was about the same activity for these two substrates, it being less than for the other three substrates tested: tocopheronolactone, menaphthone and *p*-benzoquinone.

Rosenkrantz (1956) suggested that rabbit adrenal contained an oxidation–reduction system for tocopherylquinone and its hydroquinone, relating these substances to steroid production, but the test we did on the adrenals from thirteen rats did not reveal much tocopherylquinone reductase activity, at least not in the supernatant liquid.

Table 4. *Reduced nicotinamide–adenine dinucleotide reductases of various tissues*

Animals	Age (months)	Diet (see p. 393)	Tissue	Reductase activity* (units/mg protein; mean with standard deviation) with substrate: Toco-pheryl-quinone	Vitamin K_1	Toco-pherono-lactone	Menaphthone	*p*-Benzo-quinone
Male hooded rats	4–6	A 10 Y 3	Liver	51 ± 14 (17, 6)	62 ± 11 (17, 6)	120 ± 30 (21, 9)	122 ± 36 (21, 9)	290 ± 68 (20·8)
	4–6	A 10 Y 3 + E	Liver	54 ± 25 (5, 3)	60 ± 30 (5, 3)	159 ± 70 (8, 5)	162 ± 72 (8, 5)	376 ± 51 (8, 5)
	4–6	SD 1	Liver	100 (2, 1)	125 (2, 1)	343 ± 96 (8, 5)	330 ± 63 (8, 5)	677 ± 106 (7, 4)
Male albino rats	3–6	SD 1	Adrenal	8 (13, 1)	12 (13, 1)	79 (13, 1)	91 (13, 1)	288 (13, 1)
Male chicks	1½	Soya diet	Liver	13 ± 3 (2, 2)	27 ± 5 (2, 2)	15 (2, 2)	30 ± 4 (2, 2)	165 ± 28 (2, 2)
	1½	Soya diet	Kidney	6 ± 5 (2, 2)	12 ± 17 (2, 2)	10 ± 1 (2, 2)	15 ± 3 (2, 2)	105 ± 4 (2, 2)
	1½	Soya diet	Breast muscle	3 ± 3 (2, 2)	0 (2, 2)	2 ± 2 (2, 2)	2 ± 2 (2, 2)	7 ± 5 (2, 2)

Figures in parentheses show numbers of animals followed by number of tests carried out.

Tocopherylquinone and vitamin K_1 were dissolved in a solution of BRIJ-35 (Wosilait, 1960). The other substrates were dissolved in water with the aid of 2–3 % ethanol.

* Activity of crude supernatant solution after centrifuging homogenate at 10 000 *g* for 15 min at 0°.

Wosilait (1960) could not find such activity in dog liver, using a preparation in BRIJ-35 similar to ours. Frimmer (1960), using a suspension of tocopherylquinone in Tween 80, found no activity in pig liver. However, our figures for tocopherylquinone and vitamin K_1 reductases in rat liver approximate to that for vitamin K_1 reductase found by Wosilait (1960) in dog liver, full allowance having been made by us for the different units employed and for coenzyme–enzyme and coenzyme–substrate reactions.

SUMMARY

1. α-Tocopherylquinone and α-tocopherylhydroquinone have been examined for activity in the gestation–resorption test in the rat and found to have about 0·4 and 0·6% respectively of the potency of D-α-tocopheryl acetate. This amount is more than can be accounted for by traces of α-tocopherol that might have been present in the products.

2. α-Tocopherylquinone, given orally to rats, significantly inhibited the production of malonaldehyde by liver homogenates in vitro. It had about 5–10% of the activity of D-α-tocopheryl acetate.

3. α-Tocopherylquinone inhibited malonaldehyde formation in vitro in liver homogenates of vitamin E-deficient rats, having about 45% of the activity of D-α-tocopherol.

4. Reduced nicotinamide–adenine dinucleotide-linked reductase activity for tocopherylquinone was found in rat liver and adrenal and in chick liver, kidney and breast muscle.

We acknowledge the technical assistance of the Misses M. Burbage, R. J. Castle and J. M. Taylor.

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Printed in Great Britain