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2. REVERSAL OF RESPIRATORY DECLINE IN NUTRITIONAL LIVER NECROSIS BY INTRAPORTAL ADMINISTRATION OF TOCOPHEROLS AND OTHER SUBSTANCES

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The onset of overt symptoms in dietary liver necrosis is preceded by a metabolic lesion that impairs the ability of the liver to maintain its respiratory rate (Chernick, Moe, Rodnan & Schwarz, 1955). By comparing the rates of respiration of liver slices at two different time intervals, the intensity of the defect could be assessed. Chernick *et al.* showed that dietary supplements of vitamin E or factor 3 (naturally occurring selenium compounds) prevented or delayed both the necrotic degeneration and the biochemical lesion. Later, Rodnan, Chernick & Schwarz (1956) described the effects of intraportal administrations of α -, β -, γ - and δ -tocopherol on the metabolic defect and showed that, although these substances did not markedly alter the initial rate of respiration, they arrested the respiratory decline. The four tocopherols showed considerable differences in their activity, α -tocopherol being the most active. It was of interest to extend this study to other tocopherols that have since been isolated and, in view of other work on the connexion between metal ions and vitamin E deficiency (King, Lee & Visscher, 1955; Bunyan, Edwin & Green, 1958; Bunyan, Green, Edwin & Diplock, 1960), to investigate the effect of metal ions.

MATERIALS AND METHODS

Rats. Norwegian hooded rats of both sexes were reared on a stock diet until they were about 45–60 g. in weight. They were fed on the necrogenic diet until the respiratory decline developed, usually in a further 35–55 days, when the rats weighed 60–120 g., depending on their age and weight when started on the necrogenic diet.

Necrogenic diet. This consisted of 45% of sucrose, 10% of glucose, 5% of McCollum's salt mixture, 5% of pure lard (Wilts. United Dairies Ltd.), 30% of dried baker's yeast and 5% of a vitamin B mixture in glucose, with oral doses of vitamins A, D and K to supply respectively 1400 i.u., 100 i.u. and 4 μ g./rat/week. The vitamin B mixture supplied to each kg. of diet was: 9 mg. of thiamin, 18 mg. of riboflavin, 90 mg. of nicotinic acid, 9 mg. of pyridoxine, 90 mg. of calcium pantothenate, 2 mg. of folic acid, 0.3 mg. of cobalamin, 90 mg. of inositol, 90 mg. of *p*-aminobenzoic acid and 900 mg. of choline tartrate. The salt mixture included ferric citrate, potassium iodide and potassium alum.

Tocopherols. The 5:7:8-trimethyltolcol (α -tocopherol), 5:8-dimethyltolcol (β -tocopherol), 7:8-dimethyltolcol (γ -tocopherol), 8-methyltolcol (δ -tocopherol), 7-methyltolcol (η -tocopherol), 5-methyltolcol, 5:7-dimethyltolcol and tococ were synthetic specimens. The ϵ -tocopherol was the natural substance, prepared from the unsaponifiable fraction of wheat-bran oil.

Intraportal injections. The procedure of Rodnan *et al.* (1956) was followed, with certain modifications. Rats were

anaesthetized by intraperitoneal injection of sodium pentobarbitone. An incision was made in the abdomen and a small piece of the left lobe of the liver was removed and placed in cold Krebs-Ringer phosphate buffer, pH 7.4 (Umbreit, Burris & Stauffer, 1957). The substance to be tested was dissolved in 0.9% sodium chloride, if it was soluble, or (as was the case with the tocopherols) suspended in Tween 80 emulsion and injected into the portal vein. After 1 min. the needle was withdrawn, bleeding staunch and the incision closed. After 20 min. the rat was killed and the rest of the liver removed and placed in the Krebs-Ringer phosphate buffer.

Assessment of respiratory declines. Three slices were taken from both the pre-injection and post-injection liver samples, the same (left) lobe being used wherever possible and areas free from necrosis were chosen. Slices were prepared with as uniform thickness as possible and, although after the early trials they were not weighed, test slices were generally of the order 80 ± 30 mg. In the earlier phases of the study the slices were weighed and oxygen consumptions were calculated (Table 1). However, since the determination of a substance's activity in reversing the decline in respiration can be made by measurements involving only the rate of fall in oxygen consumption, slices were later used unweighed and the calculations made from the actual volumes of oxygen taken up by each slice. Oxygen uptake was measured by incubation of the slices in Warburg vessels with 3 ml. of oxygenated Krebs-Ringer phosphate buffer containing 0.01 M-glucose. The gas phase was oxygen and, after equilibration for 25 min. at 37.5° , readings were taken at 0.5 hr. intervals for 2 hr. The percentage decline in respiration for each slice was calculated as the ratio of the difference between the volume of oxygen consumed during the first half hour (a) and the fourth half hour (b) to the volume consumed in the first half hour:

$$(\% \text{ decline} = [a - b]/a \times 100).$$

The percentage reversal of respiratory decline is given by the ratio of the differences between mean pre-injection and post-injection declines to the mean post-injection decline. This calculation is a reduction of that of Chernick *et al.* (1955), which includes individual slice weights and gives the same results.

Livers that gave initial pre-injection declines of less than 30% were regarded as not having reached the true pre-necrotic state, in which the biochemical lesion exists, since declines of up to 30% were occasionally observed even in very young animals on the necrogenic diet. Livers giving initial declines of over 80% were regarded as being beyond the pre-necrotic state. Although slices from both these types of liver usually responded to α -tocopherol, only results from livers showing initial declines of between 30 and 80% were included in the data used for the statistical evaluation of potencies.

RESULTS

Tocopherols

In the tables presented, the doses given are uniformly reported as $\mu\text{g.}/100$ g. body wt. of rat and this unit was used for all the subsequent calculations. Table 1 gives some examples of initial oxygen consumptions by liver slices before and after intraportal injections. In agreement with Rodnan *et al.* (1956) no difference was observed whether the injected Tween 80 emulsion contained α -tocopherol or not. Table 2 gives some examples from a series of experiments on α -tocopherol and shows pre- and post-injection declines, together with the percentage reversals of decline.

Table 3 presents the data from which the relative potencies of the nine substances under test were calculated. The potencies relative to α -tocopherol were calculated by plotting percentage reversal against log dose and inspecting for departure from linearity at 0 and 100% response. The best pair of parallel lines to pass through the points from α -tocopherol and each substance in turn was calculated by the method of least squares. Since some doses were given only once and not repeated, error variance was estimated from sums of squares of deviations from the parallel lines. Linearity was

Table 1. Initial oxygen consumption by liver slices before (a) and after (b) intraportal injections

Substances injected and amount/100 g. body wt.	Fresh slice wt.* (mg.)	$q_{O_2}^\dagger$	Mean q_{O_2}
Tween 80 (3 mg.)	(a)	82.6	256
		127.0	
		86.7	
	(b)	87.5	278
		57.5	
		48.2	
Tween 80 (3 mg.) and α -tocopherol (800 $\mu\text{g.}$)	(a)	87.7	252
		73.0	
		52.1	
	(b)	98.0	267
		70.0	
		60.9	

* Fresh wt. of slices was obtained from the dry weight of slices after the measurement of oxygen uptake and the moisture content of the liver, determined on a separate sample.

† Oxygen (mm.³) consumed/hr./100 mg. fresh wt. of slice, calculated from the first 30 min. incubation period.

Table 2. *Effect of intraportal injections of α -tocopherol on respiratory decline of liver slices*

The tocopherol was used either in Tween 80 emulsion or in the olive oil-glycerol monostearate mixture of Rodnan, Chernick & Schwarz (1956). When Tween 80 was used the amount was about three to four times the quantity of α -tocopherol in the emulsion.

Tocopherol injected ($\mu\text{g./100 g.}$ body wt.)	Injection vehicle	Mean pre-injection decline (%)	Mean post-injection decline (%)	Reversal of decline (%)
None	Tween*	64.7	61.8	4.6
5	Tween	65.9	61.0	7.5
50	Tween	65.8	40.3	38.8
100	Tween	50.8	9.6	81.0
500	Tween	56.4	8.2	85.5
500	Olive oil-glycerol monostearate	69.2	46.4	33.0

* In many control experiments without tocopherol, amounts of Tween 80 up to 37 mg. were used without any significant effect being observed.

Table 3. *Regression lines for intraportal-potency tests*

y , Reversal (%); x , log dose.

Substance	No. of dosages	Range of doses ($\mu\text{g.}$)	Regression equation	Potency
5:7:8-Trimethyltolcol	8	5-625	$y = 45.8x - 19.6$	100
5:7-Dimethyltolcol	4	25-200	$y = 68.4x - 70.2$	60
5:8-Dimethyltolcol	10	11-1780	$y = 33.6x - 8.0$	55
7:8-Dimethyltolcol	10	20-13 800	$y = 15.5x - 2.0$	5
5-Methyltolcol	3	50-200	$y = 41.9x - 66.1$	7
7-Methyltolcol	4	540-1000	$y = 30.2x - 82.7$	0.4
8-Methyltolcol	8	25-2000	$y = 16.7x - 18.2$	4
Tocol	4	200-1000	$y = 4.4x + 6.8$	1
ϵ -Tocopherol	4	25-200	$y = 70.8x - 96.7$	23

thus assumed and could not be tested for statistically. Analysis of variance showed no significant departures from parallelism in the assays of 5:7-dimethyltolcol, 5:8-dimethyltolcol, 5-methyltolcol, 7-methyltolcol and ϵ -tocopherol. For these substances, potency estimates and limits were calculated by the method of Fieller (1940) and are given in Table 3. This procedure could not be applied in the assays of 7:8-dimethyltolcol, 8-methyltolcol and tocol because of significant departures from parallelism. In these assays, potency was estimated from the difference between the mean log dose for the test material and the mean log of α -tocopherol doses giving corresponding responses.

Effect of metal ions

The following metal ions were administered intraportally at doses varying between 5 and 1000 $\mu\text{g.}$ (depending on solubility and toxicity effects): TeO_3^{2-} , $\text{Mo}_7\text{O}_{24}^{6-}$, Mn^{2+} , Co^{2+} , VO_3^- , SeO_3^{2-} and CrO_4^{2-} . The Mn^{2+} , Co^{2+} , SeO_3^{2-} and CrO_4^{2-} ions were also administered in admixture with small quantities of α -tocopherol in order to investigate possible synergistic effects; SeO_3^{2-} ion was, in addition, administered with larger amounts of α -tocopherol in order to observe any antagonistic effect of this ion on the activity of the tocopherol,

Table 4. *Effect of Co^{2+} , Mn^{2+} and SeO_3^{2-} ions in arresting respiratory decline*

Ion	No. of rats	Dose ($\mu\text{g./100 g.}$ body wt.)	Mean reversal (%)
Co^{2+}	1	10	26
	1	25	36
	3	100	60
	2	200	60
Mn^{2+}	1	5	0
	1	10	0
	1	50	9
	1	100	7
	1	200	13
SeO_3^{2-}	4	10	8
	1	20	10
	1	50	5
	3	600	10
	2	1000	8

such as observed by Bunyan *et al.* (1960) in erythrocyte-haemolysis tests. Finally, the Co^{2+} ion was further investigated in admixture with 5:7-dimethyltolcol and 5:8-dimethyltolcol. Of the ions in the first group, only Co^{2+} , Mn^{2+} and SeO_3^{2-} showed activity in inhibiting the decline of respiration, and these results are given in Table 4. The effects observed with Mn^{2+} and SeO_3^{2-} ions were

slight, even at high doses, but the response appeared definite. The effect of the Co^{2+} ion was reproducible and approximately proportional to the dose administered, and was equivalent to that produced by about half the same weight of α -tocopherol. In a number of trials no synergistic or antagonistic effects of the ions investigated could be clearly demonstrated: if present, they would seem to be small and concealed by the large variation in the test system.

Since selenite is only slightly active intraportally but is able to prevent both hepatic necrosis and the biochemical lesion if incorporated into the necrogenic diet, the effect of adding cobalt and manganese salts to the diet was investigated. Three groups of six rats were used, one group on the necrogenic diet, the other two groups on the diet plus 1 and 10 p.p.m. of Co^{2+} and Mn^{2+} ion respectively (as sulphates). The mean decline in the respiration of slices from livers of rats on the basic necrogenic diet was 41 %, compared with 63 and 55 % for slices from rats on the diet plus cobalt and manganese respectively. Analysis of variance showed that the increase due to cobalt was significant ($P < 0.05$), but that the increase due to manganese was not significant.

DISCUSSION

In common with Rodnan *et al.* (1956) we have found α -tocopherol to be the most active of the tocopherols in reversing respiratory decline in preneurotic-liver slices. There are, however, two important differences between the results of these workers and the present results. For α - and β -tocopherol we have obtained significant reversals with much lower doses than was found necessary by Rodnan *et al.* These authors found 190 μg . of DL- α -tocopherol and 533 μg . of D- β -tocopherol necessary for 50 % reversal of respiratory decline, whereas from our calculations the amounts needed are approximately 27 and 60 μg . respectively. This may be due to a better dispersion when the substances are injected in Tween 80 emulsion rather than in olive oil-glycerol monostearate emulsion (see, for example, the increased activity found by those workers for the water-soluble 'D- α -tocopherol polyethylene glycol-1000 succinate').

Although our estimated potency for β -tocopherol agrees fairly well with the estimate of Rodnan *et al.* the relative potencies of γ - and δ -tocopherol in the present series differ widely from their figures (83 and 17 % respectively of the activity of α -tocopherol). The specimens used by us were synthetic, whereas Rodnan *et al.* used the natural D-isomers, but this fact would appear to be insufficient to account for the differences, since these authors found no difference in the activities of D- α -toco-

pherol and DL- α -tocopherol. The potencies found by us are in agreement with the usual low activities found for γ - and δ -tocopherol in other biological tests. The high activity of 5:7-dimethyltolcol is in agreement with the relatively high biological potency of this substance in other tests (see Bunyan, Green, Mamalis & Marcinkiewicz, 1957; Ward, 1958; Bunyan *et al.* 1960). The low activity of 5-methyltolcol is in accord with the activities of other monomethyl tolcols in this and other tests *in vivo*.

ϵ -Tocopherol has been shown by Green, McHale, Marcinkiewicz, Mamalis & Watt (1959) not to be identical with 5-methyltolcol, which structure had previously been assigned to it. Recent work in these Laboratories has shown it to be, in fact, 2-farnesyl-2:5:8-trimethylchroman-6-ol, the unsaturated analogue of β -tocopherol (Green, Mamalis, Marcinkiewicz & McHale, 1960). The appreciable activity of ϵ -tocopherol in arresting respiratory decline parallels the high activity found for it by Bunyan *et al.* (1960) in dialuric acid tests both *in vitro* and *in vivo*.

The metal ions investigated were chosen because of certain relationships with α -tocopherol in vitamin E-deficiency syndromes, such as previously observed by other workers: their activity was particularly interesting in view of the concurrent tests, described by Bunyan *et al.* in the next paper, on their effects in reversing erythrocyte haemolysis. The activity of Co^{2+} ion is outstanding in both sets of experiments whereas Mn^{2+} ion shows some, but much less, activity in the two tests. This 'tocopherol-like' effect of Co^{2+} and Mn^{2+} ions has now been observed in several different and apparently unconnected biochemical manifestations of vitamin-E deficiency. Thus Caputto, McCay & Carpenter (1958) have found that ascorbic acid synthesis is curtailed in the livers of vitamin E-deficient rats, and can be reversed by the addition of Co^{2+} and Mn^{2+} ions to the enzyme preparations (the former ion apparently having synergistic effects also). Corwin & Schwarz (1959) have shown that Mn^{2+} ion has a tocopherol-like effect in restoring succinate oxidation in mitochondria prepared from the livers of vitamin E-deficient rats. In contrast with the effect of Co^{2+} ions in arresting respiratory decline when administered intraportally liver slices from rats fed in diets containing 1 p.p.m. of Co^{2+} ion showed greater respiratory declines than those from similar rats fed on the normal necrogenic diet. (Our results indicate that further work may well demonstrate the same effect with Mn^{2+} ions.) This contrasts with the phenomenon observed with SeO_3^{2-} ion, which, added to rat diets, completely protects against the onset of liver necrosis and prevents the decline in respiration of liver slice (Chernick *et al.* 1955), but,

administered intraportally, has only a marginal effect on respiratory decline.

SUMMARY

1. Eight tocopherols and one other naturally occurring tocopherol have been compared for their activity in reversing the decline in respiration of liver slices, when administered by injection through the portal vein half an hour before taking the test slices.

2. β -Tocopherol (5:8-dimethyltocol) had 60% and 5:7-dimethyltocol had 55% of the activity of α -tocopherol; but, contrary to results of previous workers, we have found the activity of γ - and δ -tocopherol to be very small. Of the monomethyl tocopherols 5-methyltocol was the most active. ϵ -Tocopherol, which is now known not to have the tocopherol structure, has 23% of the activity of α -tocopherol.

3. The Co^{2+} ion was found to have high activity in this test. Mn^{2+} and SeO_3^{2-} ions had low activity. No antagonistic or synergistic effects could be clearly demonstrated by intraportal administration of mixtures of these ions with tocopherols.

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3. THE RELATIVE ACTIVITIES OF TOCOPHEROLS AND SOME OTHER SUBSTANCES *IN VIVO* AND *IN VITRO* AGAINST DIALURIC ACID-INDUCED HAEMOLYSIS OF ERYTHROCYTES

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Rose & György (1950) discovered the haemolytic action of dialuric acid on erythrocytes from vitamin E-deficient rats and showed that the haemolysis could be prevented by vitamin E in the diet and also by incubating the erythrocytes *in vitro* with α -tocopherol. The same authors (1952) evaluated the relative potencies of α -, β -, γ - and δ -tocopherol in preventing haemolysis both by dosage *in vivo* to vitamin E-deficient rats and by addition *in vitro* to a suspension of erythrocytes from a deficient animal. Friedman, Weiss, Wherry & Kline (1958) have described a bioassay procedure to measure the protective effects of doses of vitamin E *in vivo* and have also reported on the relative potencies of racemic and optically active forms of α - and γ -tocopherol and their esters.

Seven tocopherols have now been described as occurring in Nature. Eggitt & Ward (1953) discovered ϵ -tocopherol and Eggitt & Norris (1956) offered considerable evidence that the substance was 5-methyltocol. Green, Marcinkiewicz & Watt (1955) found ζ -tocopherol in wheat bran and considered it to be 5:7-dimethyltocol. Green & Marcinkiewicz (1956) found η -tocopherol in rice and identified it as 7-methyltocol. Green, McHale, Marcinkiewicz, Mamalis & Watt (1959) have shown that ϵ -tocopherol is not 5-methyltocol, and more recent work has shown it to be 2-farnesyl-2:5:8-trimethylchroman-6-ol, the unsaturated analogue of β -tocopherol (Green, Mamalis, Marcinkiewicz & McHale, 1960). The ζ -tocopherol in wheat appears to be the trimethylated member in the same series