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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Studies on Vitamin E

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

of compounds as ϵ -tocopherol and is not identical with 5:7-dimethyltolcol.

In the present investigation the activities of tocol, the seven methylated tocols and ϵ - and ζ -tocopherol were compared. In addition, the effects of several antioxidants, detergents and metal ions were studied.

EXPERIMENTAL

Materials. The α -, β -, γ -, δ - and η -tocopherol, 5-methyltolcol, 5:7-dimethyltolcol and tocol were synthetic specimens. The ϵ - and ζ -tocopherol were natural isomers, isolated from wheat bran.

Vitamin E-deficient diet. Vitamin-free casein (Genatosan Ltd.), 25%; sucrose, 50%; pure Danish lard (Wilts. United Dairies Ltd.), 10%; dried brewer's yeast (Marmite Food Extract Co.), 10%; McCollum's salt mixture, 5%; vitamin A palmitate, 40 i.u./g. of diet; vitamin D₃, 2.4 i.u./g. of diet.

Animals. The animals used were female rats of the Norwegian hooded strain. Sensitivity to dialuric acid-induced haemolysis developed after a few weeks on the diet, but the rats actually used were all 3–12 months old, having been depleted of vitamin E since weaning.

Dialuric acid-haemolysis test. The technique employed was essentially that of György (1951) with slight modifications. Reagents were: iso-osmotic (0.9%) sodium chloride solution, sodium chloride-citrate solution (0.9 g. of sodium chloride and 0.6 g. of sodium citrate/100 ml.), phosphate buffer, pH 7.4 (0.2M-KH₂PO₄, 50 ml., and 0.2M-NaOH, 39.34 ml. made up to 200 ml. with water) and dialuric acid solution (12 mg. of dialuric acid dissolved in 5 ml. of buffer at 60° and diluted with 20 ml. of buffer at room temperature). The dialuric acid solution became inactive after about 30 min. Cleaning of the reaction tubes with detergents and soaps was avoided, only water being used.

Tests in vivo

Collection and testing of blood. The rat's tail was washed in hot water and dried. Blood (0.1 ml.) was collected from a fresh cut at the tip, transferred to 2 ml. of the sodium chloride-citrate solution and mixed gently. The suspension was centrifuged at 2500 rev./min. for 2 min. and the supernatant liquid removed by suction. The cells were resuspended in 2 ml. of 0.9% sodium chloride and again centrifuged and the supernatant removed. Finally, the cells were made into an approximately 5% suspension by gentle mixing with 1 ml. of 0.9% sodium chloride solution. From each suspension 0.25 ml. was pipetted into each of three tubes A, B and C. To the three tubes were added respectively 0.25 ml. of buffer, 0.25 ml. of dialuric acid solution and 8.5 ml. of water. Tubes A and B were kept at 37° for 15 min., at room temperature for a further 45 min. and then the contents were centrifuged. From each supernatant 0.2 ml. was pipetted into 3.3 ml. of water and the extinctions of the resulting solutions (A_1 , B_1) and of the control solution (C) were measured on an EEL long-cell absorptiometer, with Ilford filter 605 (maximum transmission at 550 m μ), and with tube A_1 set at zero. The percentage haemolysis was calculated from

$$\frac{\text{Reading } B_1 \times 100}{\text{Reading } C}.$$

Dosage methods in vivo. After preliminary investigation it was decided to use only those rats that showed 80% or more of dialuric acid-induced haemolysis on a preliminary bleeding. The rats were dosed orally by micrometer syringe with olive-oil solutions of the test substances at 4.30 p.m. on one day and bled again at 10.0 a.m. the next day. From each pair of results the 'percentage protection' was calculated:

$$100 \left[\frac{\% \text{ haemolysis before dosage} - \% \text{ haemolysis after dosage}}{\% \text{ haemolysis before dosage}} \right].$$

Dosage groups were decided by random selection from suitable rats of approximately the same age, and doses of DL- α -tocopherol acetate were given in parallel with the test doses. Rats could be used again after a rest period of about 14 days.

Haemolysis tests in vitro

Blood was taken from one or more vitamin E-deficient rats and mixed into 5% suspensions as described above. Immediately before each test an emulsion of the test substance was freshly prepared by first mixing it with about four times its weight of Tween 80 and then making up in 0.9% sodium chloride to the required strength. Portions of the suspension of the test substance were mixed with 1 ml. quantities of the blood-cell suspension. Sufficient of a dilute Tween 80 solution in 0.9% sodium chloride was then added to each tube to make up the total amount of Tween 80 to 2 μ g., to rule out variations in the amount of Tween 80 caused by taking differing portions of the test substance. Finally, 0.9% sodium chloride was added to produce, in each tube, a total volume of 2 ml. The tubes were held at 37° for 1 hr. and then centrifuged. The supernatant was removed and each batch of 'protected' cells was mixed with 1 ml. of 0.9% sodium chloride to give a suspension to be tested for dialuric acid-induced haemolysis by the method described above for the technique *in vivo*. Tocopherols and the other substances under examination were compared directly with DL- α -tocopherol, each test thus being internally controlled to check such (rare) occurrences as spontaneous haemolysis or inactivity of the dialuric acid solution. Tests in any way unsatisfactory were rejected.

RESULTS

Potencies in vivo

The relative potencies *in vivo* of the compounds tested are set out in Table 1. A twin crossover design was used for the test of 5:8-dimethyltolcol, a 14-day interval being allowed between doses, and the potency and limits of error were calculated by the method of Smith, Marks, Fieller & Broom (1944). In fact the crossover showed little advantage over a straightforward dilution assay. The potency of 7:8-dimethyltolcol was estimated from a four-point assay evaluated by the method of B.S. 911:1940. With 8-methyltolcol, a five-point assay resulted in non-parallel lines and so only an approximate estimate of potency is given. The 5:7-dimethyltolcol and 7-methyltolcol were tested by

giving a graded series of doses of each in comparison with doses of DL- α -tocopheryl acetate: for the calculation, variance was estimated from the sum of the deviations about the standard and the test lines. Since 5-methyltocol and natural ϵ - and ζ -tocopherol were only available in small quantities at the time the present work was done, their potencies were assessed by reference to the DL- α -tocopheryl acetate regression line (relating percentage protection to log dose).

The antioxidants *NN'*-diphenyl-*p*-phenylenediamine (DPPD), 2:6-ditert.-butyl-4-methylphenol (BHT) and propyl gallate were administered orally in single doses varying between 1 and 50 mg. (the larger doses by stomach tube) and methylene blue was administered in aqueous solution by stomach tube at the same dosage. BHT, propyl gallate and methylene blue were almost inactive at all doses, an oral dose of 50 mg. giving only about 5% protection. However, DPPD was about 3% as active as α -tocopherol, giving complete protection at the 50 mg. dose.

Potencies in vitro. The relative potencies *in vitro* of the tocopherols and tocols are set out in Table 2.

Table 1. *Relative potencies in vivo of tocopherols and other compounds against dialuric acid-induced haemolysis*

Substance	Potency	Limits of error (<i>P</i> 0.95)
α -Tocopherol acetate	100	—
5:7-Dimethyltocol	66	29–260
5:8-Dimethyltocol	27	16–49
ζ -Tocopherol (natural)	25	21–42
7:8-Dimethyltocol	20	7–39
5-Methyltocol	3–14	—
ϵ -Tocopherol (natural)	1–5	—
7-Methyltocol	1	0.5–2.0
8-Methyltocol	0.3	—
Tocol	1–5	—
DPPD	About 3	—
BHT	About 0.5	—
Propyl gallate	About 0.5	—
Methylene blue	About 0.5	—

Table 2. *Relative potencies in vitro of tocopherols against dialuric acid-induced haemolysis*

Substance	No. of assays	Potency	Limits of error (<i>P</i> 0.95)
ϵ -Tocopherol (natural)	4	133	114–156
5:7-Dimethyltocol	4	121 (± 19)*	See text
ζ -Tocopherol (natural)	2	106	97–118
5:7:8-Trimethyltocol	—	100	—
7-Methyltocol	8	88	80–101
7:8-Dimethyltocol	2	67	51–86
5-Methyltocol	1	67	57–77
5:8-Dimethyltocol	2	54	39–62
8-Methyltocol	1	26	20–35
Tocol	2	34	26–42

* S.E.

For each test, several doses of the test substance were compared with a series of doses of DL- α -tocopherol. A number of such tests were carried out on each substance, the α -tocopherol usually being in the range 0.05–0.40 μ g.

Each separate test was evaluated by the standard formulae for interpreting 'parallel-line assays', i.e. assays in which the response is linearly related to the logarithm of the dose. If X and Y are the mean differences (standard—test) between preparations for the logarithm of the dose and for response respectively, and if B is the pooled estimate for the two preparations of the slope of the log (dose)—response line, then the logarithm of the potency ratio (test to standard) is estimated as

$$L = X - Y/B.$$

Also, if N_1 and N_2 are the numbers of responses obtained to the standard and test preparations, s^2 with n degrees of freedom the estimate of the error variance for a single response, t the 5% level of Student's variate for n degrees of freedom, and s^2/P the estimated variance of B , then (Fieller, 1940) the 95% fiducial (or confidence) limits for the true potency ratio are $L' \pm ts'_L$,

$$L' = X - CY/B,$$

$$\text{where } s'_L{}^2 = \frac{Cs^2}{B^2} \left[\frac{1}{N_1} + \frac{1}{N_2} + \frac{C}{P} \left(\frac{Y}{B} \right)^2 \right],$$

$$\text{and } C = B^2/[B^2 - t^2 s^2/P].$$

(Analysis of variance showed no significant departures from parallelism in any of the tests.)

Successive tests on the same preparation exhibited significant variations in s^2 and B ; it was accordingly necessary to use an approximate and somewhat empirical method for combining the several results. Each individual estimate L of the logarithm of the potency ratio was accorded a weight $w_L = 1/s'_L{}^2$, so that the pooled estimate was $\bar{L} = (\sum w_L L)/\sum w_L$, and the values of L were checked for concordancy by entering the χ^2 table with

$$\chi^2 = \sum w_L L^2 - \bar{L}^2 \sum w_L$$

and degrees of freedom one fewer than the number of tests to be combined. The corresponding 'limits of error' were calculated as $\bar{L}' \pm ts'_L$, where

$$\bar{L}' = [\sum w_L (X - CY/B)]/\sum w_L$$

$$\text{and } s'_L{}^2 = 1/\sum w_L.$$

The degrees of freedom for t were taken to be the average of those for the individual tests. These formulae are not claimed to be exact (except when the number of assays reduced to one), but would clearly be reasonable, if each assay were so precise that C could be taken as unity and L treated as normally distributed.

Successive assays of 5:7-dimethyltocol were not concordant and so the mean of the four potency

Table 3. *Activity in vitro of non-tocopherol compounds against dialuric acid-induced haemolysis*

Substance	Amounts tested ($\mu\text{g.}$)	Estimated median effective dose ($\mu\text{g.}$)
α -Tocopherol (for comparison)	0.1-0.5	0.2-0.3
Phytol	5, 10, 50	25
DPPD	0.2, 2	1
BHT	1-20	10
Menaphthone	2-20	10-20
Menaphthone sodium bisulphite	2, 20	> 20
Vitamin K ₁	0.3-14	2
Vitamin B ₁₂	10-200	60-100
Teepol	2, 20, 200	Variable (see text)
Ubiquinone 50	2-160	No activity found
Methylene blue	2, 20	
Vitamin A palmitate	2, 20	
Choline tartrate	2, 10, 50	
Linoleic acid	1, 2, 10	
Cetylpyridinium bromide	2, 10, 20	

Table 4. *Ions showing protection in vitro against dialuric acid-induced haemolysis*

Compound	Ion	Range tested ($\mu\text{g.}$)*	Estimated median effective dose ($\mu\text{g.}$)
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	Co^{2+}	1-20	7
$\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$	Sn^{2+}	2-50	7
$\text{K}_2\text{Cr}_2\text{O}_7$	$\text{Cr}_2\text{O}_7^{2-}$	4-20	10
K_2CrO_4	CrO_4^{2-}	2-50	33
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	Mn^{2+}	2-50	40

* The figures in this column and corresponding figures in succeeding Tables refer to the weights of the ions, not of the salts.

estimates is quoted together with the appropriate standard error. Potency estimates for 5-methyltocol and 8-methyltocol were derived from single assays. In this way potencies ranging from 26 to 133 % of DL- α -tocopherol were estimated.

In Table 3 the activities of several other substances are summarized. Of the non-ionic compounds tested, only DPPD approached the tocopherols in activity, but some protection was found with BHT, phytol, menaphthone, menaphthone sodium bisulphite, vitamin K₁ and vitamin B₁₂ at higher doses. The anionic detergent Teepol was found to have activity in the test but the median active dose varied considerably from batch to batch. One sample showed 50 % protection at 20 $\mu\text{g.}$ whereas another showed this activity only at 200 $\mu\text{g.}$ The cationic detergent Cetrinide (cetylpyridinium bromide) was inactive.

Effects of metal ions in vitro. The study of the effects of ions *in vitro* in the haemolysis test arose from a number of observations of the effects of such ions in other systems sensitive to vitamin E deficiency. The ions tested and their various effects are presented in Tables 4-7. They fall into three

groups. In the first group (Table 4), three cations, Co^{2+} , Mn^{2+} and Sn^{2+} , and two anions, CrO_4^{2-} and $\text{Cr}_2\text{O}_7^{2-}$, had full protective activity, the median effective doses ranging from 7 $\mu\text{g.}$ for Co^{2+} and Sn^{2+} ions to 40 $\mu\text{g.}$ for Mn^{2+} ion. Further studies (Table 5) revealed that the protective effects of

Table 5. *Additive effects of ions and tocopherol on dialuric acid-induced haemolysis in vitro*

See footnote to Table 4.

Test no.	Tocopherol or ion used ($\mu\text{g.}$)	Protection (%)	
		By single substance	By tocopherol + ion
1	Co^{2+} (2.0)	7	—
	α - (0.14)	0	61
	α - (0.20)	14	88
	α - (0.26)	50	97
2	Co^{2+} (2.0)	0	—
	β - (0.2)	0	60
	β - (0.3)	68	92
3	Co^{2+} (2.0)	0	—
	δ - (0.3)	0	11
	δ - (0.4)	0	32
	δ - (0.5)	0	54
	δ - (0.6)	0	78
4	Mn^{2+} (10.0)	0	—
	α - (0.20)	0	83
	α - (0.23)	5	91
	α - (0.25)	0	93
5	Sn^{2+} (4.0)	2	—
	α - (0.24)	4	8
	α - (0.28)	52	76
	α - (0.32)	76	82
6	$\text{Cr}_2\text{O}_7^{2-}$ (20.0)	45	—
	α - (0.18)	10	66
	α - (0.21)	30	73
	α - (0.24)	39	66
7	CrO_4^{2-} (4.0)	11	—
	α - (0.23)	59	82
	CrO_4^{2-} (8.0)	3	—
	α - (0.23)	59	83

these ions were additive with the protection due to α -tocopherol. The additive action of Co^{3+} ion was also clearly demonstrated with the less-active β - and δ -tocopherol. With Co^{2+} and Mn^{2+} ions these additive effects were so great as to suggest synergism between the ions and tocopherol. However, it is difficult to calculate the magnitude of the purely additive effect because of the large difference between the slopes of the respective log dose-response lines, and so synergism is difficult to prove. Another consequence of this difference between slopes is that, in experiments designed to show the additive effects of the weakly active ions CrO_4^{2-} and $\text{Cr}_2\text{O}_7^{2-}$, choice of doses is critical. A second group of ions that included Cu^{2+} , Cu_2^{2+} , La^{3+} , Zn^{2+} , SeO_3^{2-} , AuCl_4^- and MnO_4^- exerted a pronounced antagonistic effect on the action of α -tocopherol (Table 6). The Cd^{2+} and Cr^{3+} ions also had a slight antagonistic activity, but noticeably less than the other ions in this group. The antagonistic action of the SeO_3^{2-} ion was powerful enough

to oppose even the combined protective effect of both α -tocopherol and cobalt (other antagonist ions were not studied with this combination). Certain substances had a direct haemolytic effect on the erythrocytes during the first incubation in the absence of dialuric acid: these were potassium permanganate, chrome alum, auric chloride, cadmium chloride and sodium tellurite. For the studies on protection and antagonism, these substances were used below their effective haemolytic concentrations. Finally, Table 7 lists the third group of ions, which had no activity when tested.

DISCUSSION

The potencies *in vitro* of tocol and its seven methylated derivatives form groups dependent on the number and position of the substituent methyl groups. The range of potency is not great, tocol itself having about one-third of the activity of the fully methylated α -tocopherol. Methyl groups at

Table 6. *Compounds opposing the protective action of α -tocopherol in vitro in the haemolysis test*

See footnote to Table 4.

Compound	Ion ($\mu\text{g.}$)	Protection (%)	
		α -Tocopherol alone	With ion
Na_2SeO_3	SeO_3^{2-} (16)	11-91	0
Na_2SeO_3	SeO_3^{2-} (3)	3-86	5-15
		(with 2 $\mu\text{g.}$ of Co^{2+})	
$\text{Zn}(\text{CH}_3 \cdot \text{CO}_2)_2 \cdot 2\text{H}_2\text{O}$	Zn^{2+} (10)	14, 75	6, 3
KMnO_4	MnO_4^- (4)	88, 96	0
$\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$	AuCl_4^- (3.4)	22-97	0
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	Cu^{2+} (2)	65, 79	6, 0
Cu_2Cl_2	Cu_2^{2+} (5)	74, 94	9, 3
$\text{La}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$	La^{3+} (10)	77, 90	16, 28
$\text{Cr}_2(\text{SO}_4)_3 \cdot \text{K}_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$	Cr^{3+} (10)	66, 77	53, 64
CdCl_2	Cd^{2+} (5)	66, 77, 90	38, 66, 84

Table 7. *Compounds found to be ineffective in the haemolysis test*

See footnote to Table 4.

Compound	Ion	Amounts tested ($\mu\text{g.}$)	
		Alone	With α -tocopherol
$\text{Na}_2\text{SeO}_4 \cdot 10\text{H}_2\text{O}$	SeO_4^{2-}	4-90	18
$\text{H}_2\text{TeO}_4 \cdot 2\text{H}_2\text{O}$	TeO_4^{2-}	3-30	15
Na_2TeO_3	TeO_3^{2-}	3-28	0.1, 0.7
$\text{Ce}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$	Ce^{3+}	2-20	10
$(\text{NH}_4)_2\text{Ce}(\text{NO}_3)_6$	Ce^{4+}	2-20	10
$\text{VOCl}_2 \cdot 2\text{H}_2\text{O}$	VO^{2+}	3, 13	3
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$	$\text{Mo}_7\text{O}_{24}^{6-}$	16	16
$\text{NiSO}_4 \cdot 7\text{H}_2\text{O}$	Ni^{2+}	2-50	2
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	Fe^{2+}	2-10	2, 4
OsO_4	OsO_5^{2-}	14-70	14
$\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	UO_2^{2+}	2-50	2
TiNO_3	Ti^+	2-50	10
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	Mg^{2+}	2-20	—
KCl	K^+	2-50	10
K_2SO_4	K^+	2-50	10
$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$	Ca^{2+}	2-50	10
NaNO_2	NO_2^-	1-30	7
$\text{Na}_2\text{S}_2\text{O}_4$	$\text{S}_2\text{O}_4^{2-}$	1-37	7

C₍₅₎ and C₍₇₎ enhance the activity of the tocol molecule, whereas a methyl group at C₍₈₎ has a depressing effect. The most active substance in the tocol series is thus 5:7-dimethyltolcol, with 121 % of the activity of α -tocopherol. Natural ϵ -tocopherol, which is not a tocol, was found to have an even greater potency and this is in accord with the belief of Green *et al.* (1959) that this substance has a lower molecular weight than the tocols. Voth & Miller (1958) have postulated that the binding of tocopherol to certain proteins such as serum albumin can be accounted for by the formation of hydrogen bonds between the hydroxyl group of the tocopherol and the carboxyl group of the protein. They point out that the three methyl groups in α -tocopherol, by depressing ionization of the hydroxyl, would enhance the possibility of hydrogen-bonding. If the relative potency of the tocols in the dialuric acid test depended solely on their degree of bonding with some protein of the erythrocyte membrane, it would be expected that the potency would be enhanced by methyl groups in any position, since *ortho*-, *meta*- and *para*-methylated phenols are all less ionized than phenol itself. The depressive effect of the C₍₈₎ methyl group cannot be accounted for by this explanation, and some other factor must be concerned. Nevertheless, the special effect of the *ortho*-methyl groups in enhancing potency might be related to hydrogen-bonding, since 2:6-xyleneol is less ionized than 3:5-xyleneol.

Rose & György (1952), working with the D-isomers, estimated the potencies of β -, γ - and δ -tocopherol *in vitro* as respectively 40, 30 and 20 % of α -tocopherol. The potencies found by us in the present series (in which the synthetic compounds were used) were respectively 54, 67 and 26 %, the activity of γ -tocopherol being considerably higher than was found by Rose & György. The potencies of the substituted tocols *in vivo* are clearly dependent on factors other than structure, probably absorption and transport effects. The most active compound *in vivo* is α -tocopherol. The other tocols form two groups, the dimethyl compounds having intermediate activity and the monomethyl tocols having low activity. Rose & György estimated the potencies *in vivo* of β -, γ - and δ -tocopherol as 20, 4 and 3 % respectively of α -tocopherol. These may be compared with the potencies found here, which are, respectively, 27, 20 and 0.3 %. The figure for γ -tocopherol is again much higher than was found by Rose & György but is in good agreement with the figure of 21.7 % found in a series of tests by Friedman *et al.* (1958). Of four antioxidants tested for their protective effects *in vivo*, only DPPD had appreciable activity. This is in agreement with the findings of Gitler, Sunde & Boumann (1958). These authors and also Friedmann *et al.* (1958) found no

protection *in vivo* with BHT and methylene blue, but their dosage regimen was different from ours.

In any discussion of the mechanism of the haemolytic effect and its inhibition, all the foregoing factors must be considered. The most widely held theory of the mechanism of dialuric acid-induced haemolysis (Rose & György, 1950, 1952) relates it to the presence of a peroxidic intermediate formed during the reversible oxidation-reduction of dialuric acid and alloxan. In accordance with this view, tocopherols and other antioxidants protect by removing such an active intermediate. Nitowsky & Tildon (1956) concluded that α -tocopherol might function by inhibiting the oxidase action of haemoglobin on the unsaturated fatty acids of the cell membrane (previously demonstrated by Collier & McRae, 1955). These workers studied hydrogen peroxide-induced haemolysis and reported an inverse relationship between catalase activity and percentage haemolysis in erythrocytes. They found menaphthone almost as active as α -tocopherol in their tests *in vitro*, whereas in the present work there is a big difference in the activity of the two substances. This supports the suggestion of Rose & György (1952) that hydrogen peroxide is not the haemolysing agent in the dialuric acid test.

The effects of the ions tested are difficult to correlate; it is not even certain that all the ions that have activity exert their effect by the same mechanism. The Co²⁺ and Mn²⁺ ions are of special interest since they not only have considerable activity in the test *in vitro* but have pronounced additive and possibly synergistic activity in combination with α -tocopherol. In the preceding paper, Green, Edwin, Bunyan & Diplock (1960) have described the effects of Co²⁺ ions in reversing the respiratory decline in liver slices from rats on a necrogenic diet, and it is tempting to postulate a connexion between the two phenomena. Corwin & Schwarz (1959) have established a relationship between the biochemical lesion in necrotic livers (deficient both in vitamin E and selenium) and the regulation of succinate oxidation in liver mitochondria. They have, in fact, found Mn²⁺ ions to have a vitamin E-like effect in restoring succinate oxidation in mitochondria prepared from the livers of vitamin E-deficient rats. Another manifestation of vitamin E deficiency was studied by Caputto, McCay & Carpenter (1958), who found that enzymic synthesis of ascorbic acid in the livers of vitamin E-deficient rats and rabbits was severely curtailed and that the effect could be reversed by adding Co²⁺ and Mn²⁺ ions to the enzyme preparations. They also observed a large synergistic effect when Co²⁺ ions were added to the enzyme preparations from vitamin E-containing livers but found no such effect with Mn²⁺ ions; this somewhat parallels our findings on the relative activity of Co²⁺ and

Mn²⁺ ions in reversing dialuric acid haemolysis. Caputto *et al.* also commented on the slight inhibitory effects of Zn²⁺ and Cd²⁺ ions on ascorbic acid synthesis.

It does not seem possible to correlate the various effects of the ions with their charges, although the activity of Teepol indicates that charge alone may play some part. It appeared important to try to discover whether haemolysis was produced by a surface effect on the erythrocyte membrane (in which ionic charge might be expected to be influential) or whether haemolysis was the culmination of a more deep-seated interference with the metabolism of the cell. Some measurements of erythrocyte mobility showed that, compared with normal cells, those from vitamin E-deficient rats had a slightly higher negative charge, with a greater population variance, and this variance was noticeably reduced when the cells were incubated with α -tocopherol. (This variability of charge from cell to cell may help to explain how it is possible to get, say, 50 % haemolysis and not an all-or-none response.)

Two metal-containing anions, CrO₄²⁻ and Cr₂O₇²⁻, and the Sn²⁺ cation were as effective as Co²⁺ and Mn²⁺ ions in inhibiting haemolysis. The Sn²⁺ ion is difficult to keep in solution and part of the effect of this ion may have been due to deposition of an insoluble salt on the surface of the cells. It is difficult to fit the action of the powerfully oxidizing CrO₄²⁻ and Cr₂O₇²⁻ anions into the same picture as the Co²⁺ and Mn²⁺ cations. The only enzymic connexion of CrO₄²⁻ ion, as far as we are aware, is its catalysis of the hydration of carbon dioxide to carbonic acid, which action it shares with carbonic anhydrase and the ions of several other oxy-acids (Roughton & Booth, 1938).

The group of substances that oppose the protective action of α -tocopherol *in vitro* includes both cations and anions, and again it is difficult to correlate their activities with any particular chemical or electrical properties. The group includes the reducing anion SeO₃²⁻ and the oxidizing anion MnO₄⁻, the reducing cation Cu₂²⁺ and the oxidizing cation Cu²⁺. In contrast with the effect of SeO₃²⁻, the SeO₄²⁻ ion is without activity. The antagonistic action of selenite is of particular interest in view of the vitamin E-like activity of selenite in protecting against dietary liver necrosis in rats (Schwarz & Foltz, 1957), the exudative diathesis in chicks (Dam, Nielsen, Prange & Søndergaard, 1957) and white-muscle disease in lambs (Muth, Oldfield, Remmert & Schubert, 1958). In contrast, Green, Edwin *et al.* (1960) found that intraportal selenite has practically no effect in arresting respiratory decline of necrotic-liver slices. Roughton & Booth (1938) find selenite to be more active than chromate in catalysing the hydration of carbon dioxide to carbonic acid.

The haemolysis of vitamin E-deficient erythrocytes may be a more useful system for investigating some of the metabolic functions of α -tocopherol than has hitherto been recognized. It is possible that the role of vitamin E in preventing haemolysis may be related to enzymic or other type of catalytic process in the cell and that a simple theory of combination with peroxide may not be sufficient to account for the phenomenon. It is perhaps of some interest that Vincent & Blackburn (1958), in a study of the effect of several metal ions on normal human erythrocytes, divided the ions into two groups: those that caused a loss of K⁺ ion from the cells, which included Au³⁺, Cu²⁺, Cd²⁺ and Zn²⁺ ions, and those that did not cause a loss of K⁺ ion, which included Mn²⁺ and Co²⁺ ions.

SUMMARY

1. The potencies *in vitro* and *in vivo* of eight tocols and two other naturally occurring tocopherols in preventing dialuric acid-induced haemolysis in rat erythrocytes have been studied, and some observations made on the relationship between structure and activity. The potencies *in vitro* cannot be clearly related to structure, although it appears that the presence of methyl groups *ortho* to the hydroxyl group in the tocol structure enhances activity somewhat. Hydrogen-bonding, as postulated by Voth & Miller (1958), would not seem to account sufficiently for the differences in potency. *In vivo*, the dimethyl compounds all have considerably greater activity than the monomethyltocols.

2. The activities of a number of other substances in the test have been recorded.

3. The Co²⁺, Mn²⁺, Sn²⁺, CrO₄²⁻ and Cr₂O₇²⁻ ions have been found to be active in preventing haemolysis *in vitro* and the action of these ions is additive to that of α -tocopherol. With Co²⁺ and Mn²⁺ ions the combined effect is so great as to suggest synergism.

4. The Zn²⁺, Cu²⁺, Cu₂²⁺, La³⁺, Cr³⁺, Cd²⁺, MnO₄⁻, AuCl₄⁻ and SeO₃²⁻ ions antagonize the action of α -tocopherol.

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Interrelationships between Folic Acid and Cobalamin in the Synthesis of Methionine by Extracts of *Escherichia coli*

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Gibson & Woods (1960) showed that washed suspensions of various strains of *Escherichia coli* synthesize methionine from homocysteine; serine was an effective source of the methyl group. With *p*-aminobenzoic acid and cobalamin auxotrophs they also showed these vitamins to be essential for the reaction. Parallel inhibition by sulphathiazole of both the synthesis of methionine from homocysteine and of folic acid from *p*-aminobenzoic acid gave strong, though indirect, evidence that *p*-aminobenzoic acid must first be converted into some form of folic acid before it exerts its function. However, no folic acid derivative tested was able to replace *p*-aminobenzoic acid for methionine synthesis by the intact organisms.

Enzymic extracts of an auxotroph of *E. coli* requiring serine or glycine for growth were obtained by Cross & Woods (1954) by grinding with alumina and by Szulmajster & Woods (1955, 1960) by extracting acetone-dried organisms. Both preparations synthesized methionine from homocysteine and serine and there was, among other

factors, a partial requirement for an extract of heated *E. coli*. The last-named authors partly purified the heated extract and obtained evidence that it contained a folic acid derivative. With neither source of enzyme could the heated extract be replaced by either tetrahydropteroylglutamic acid or its *N*⁵-formyl derivative (leucovorin). This was surprising, because although tetrahydropteroylglutamic acid may not be the natural folic acid coenzyme (Wright, 1958) it is effective in replacing it in all other C₁-transfer reactions so far studied.

The object of the present work was to investigate this point further and to try to find conditions under which tetrahydropteroylglutamic acid could be used. Since cobalamin is known to be required for methionine synthesis in *E. coli* by growing cultures (Davis & Mingioli, 1950), washed suspensions (Gibson & Woods, 1952, 1960) and enzymic extracts (Helleiner & Woods, 1956) of cobalamin auxotrophs, special attention was paid to the effect of this vitamin. It was found that growth in the presence of added cobalamin permitted the utilization of tetrahydropteroylglutamic acid by enzymic extracts of acetone-dried organisms. Brief reports of some of the results have been given (Kisliuk & Woods, 1957, 1959).

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