Relationship between Antioxidant and Antihemolytic Activities of Vitamin E Derivatives *In Vitro*

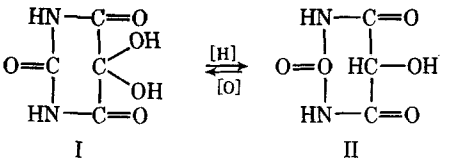
W. A. SKINNER*, H. L. JOHNSON, M. ELLIS, and R. M. PARKHURST

Abstract □ Various derivatives of vitamin E and other antioxidants were evaluated *in vitro* for their antihemolytic effects on the blood from vitamin E-deficient rats. These results were compared with the activities of some of these compounds as antioxidants in protecting β-carotene from air oxidation in corn oil. The lack of a good correlation between these effects would indicate that different mechanisms are involved in their antihemolytic and antioxidant effects.

Keyphrases □ Vitamin E derivatives—antioxidant, antihemolytic activities, relationship □ Antihemolytic effects, vitamin E derivatives—correlated with antioxidant activities □ Antioxidants, vitamin E derivatives—*in vitro* evaluation of antihemolytic activities, correlation

During an investigation of the relation of dietary factors to the development of diabetes in the alloxan-treated rat, Rose and György (1) discovered that vitamin E-deficient rats experienced marked hemolysis after administration of the alloxan. Further studies led to the finding that, *in vitro*, alloxan (I) was not active in hemolyzing red blood cells but that its reduction product, dialuric acid (II), was quite active (Scheme I).

Friedman *et al.* (2) studied the dialuric acid-induced hemolysis as a bioassay method for vitamin E, pointing out some precautions necessary for increased accuracy of the determination. The relative *in vivo* potency of α- and γ-tocopherol was also studied. The statement was made that α-tocopherol was active by addition



I ⇌ II

Scheme I

to the blood of deficient rats *in vitro* but that the biological specificity was lost in this test because the several tocopherols exerted the same quantitative effects.

Rose and György (3) found that the activity of various tocopherols evaluated *in vivo* for prevention of this blood hemolysis varied, with α-tocopherol being the most active.

Deficiency of vitamin E in premature infants results in increased susceptibility of their erythrocytes to hemolysis *in vitro* by dilute hydrogen peroxide solutions (4). Thus, vitamin E deficiency in various animal species results in susceptibility of the blood to hemolysis *in vitro* in the presence of either H_2O_2 or dialuric acid.

An interesting review of the biological effects of the tocopherols and of various antioxidants summarized the relative effectiveness of the tocopherols in the *in vitro* hemolysis test using dialuric acid (5). The results derived from studies by Rose and György (3) and Bunyan *et al.* (6) indicated that α-tocopherol was most effective while diisooctylhydroquinone was of com-

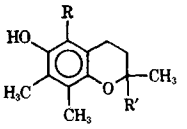


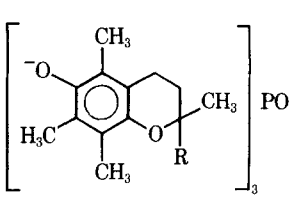
Table I—Antioxidant Activity and Antihemolytic Activity of Vitamin E Analogs *In Vitro*

R	R′	Antioxidant Activity (Half-Life), hr.[a]	Antihemolytic Activity (Median Effective Dose), mcg.[a]
H	$CH_3(C_{16}H_{33})$	394 (>570)	0.5–1 (1–5)
CH_3	$CH_3(C_{16}H_{33})$	375 (570)	0.5 (0.2–0.3)
CH_2OH	$CH_3(C_{16}H_{33})$	244	5–10 (1–5)
$CH_2OCH_2C_6H_5$	$CH_3(C_{16}H_{33})$	179 (263)	100 (25–50)
$CH_2OC_2H_5$	$CH_3(C_{16}H_{33})$	171 (225)	1–5 (10–25)
CH_2OCH_3	$CH_3(C_{16}H_{33})$	169 (221)	5–10 (10)
$CH_2OCOC_6H_5$	CH_3	66	>100
CHO	CH_3	62	5–10
Orthoquinone	CH_3	41	5
	$CH_3(C_{16}H_{33})$	39 (58)	50 (>100)
	$CH_3(C_{16}H_{33})$	38 (37)	50–100 (50–100)
$CH_2N(CH_3)_2$	$CH_3(C_{16}H_{33})$	35 (24)	10 (2.5–5)
$CH_2SC(NH)NH_2 \cdot HCl$	CH_3	27	1–5
$CH_2SC(NH)NH_2 \cdot HCl$ (acetate)	CH_3	—	10–25
$CH_2SC_2H_5$ (acetate)	$CH_3(C_{16}H_{33})$	—	10 (>100)
CH_2Cl (acetate)	$CH_3(C_{16}H_{33})$	< 12	>100 (>100)
Control		12	—

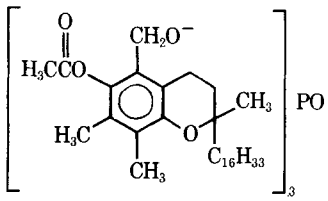
[a] Values in parentheses are for the corresponding compounds in which R′ is the normal tocopherol side chain ($C_{16}H_{33}$) in place of CH_3.

Table II—Antihemolytic Activities of Vitamin E Analogs *In Vitro*

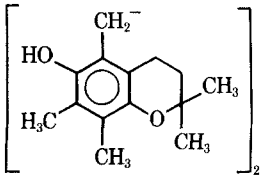
Compound	Antihemolytic Activity (Median Effective Dose), mcg.
6-Hydroxy-2,2,5,7-tetramethylchroman	>100
2,2,6,7-Tetramethylchroman-5,8-quinone	50
1,2-Dihydro-6-ethoxy-2,2,4-trimethylquinoline	1–5
Compound 1	>100
Compound 2	100
Compound 3	>100
2,2,5,7,8-Pentamethylchroman-6-phosphate	0.5–1
Tocopherolquinone model	>100
2,2,7,8-Tetramethyl-5-hydroxymethyl-6-phosphate	50
Compound 4	0.5–1
Compound 5	1–5
Methylene blue	5–10
Butylated hydroxytoluene	1–2.5
Butylated hydroxyanisole	2.5–5
α-Tocopherol nicotinate	>100
4,4′-Methylenebis(2,6-di-*tert*-butylphenol)	50
4,4-Thiobis(6-*tert*-butyl-*o*-cresol)	1
1,3,5-Trimethyl-2,4,6,tris[3,5-di-*tert*-butyl-4-hydroxybenzyl]benzene	>100
N,*N*′-Diphenyl-*p*-phenylenediamine	0.5–1
Pyrogallol	1
Ascorbic acid	>100



(1) R = $C_{16}H_{33}$
(2) R = CH_3



(3)



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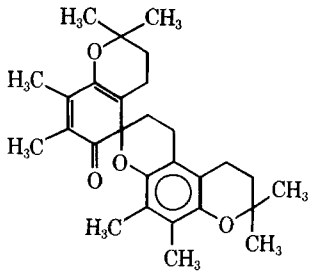

(5)

parable potency and *N*,*N*′-diphenyl-*p*-phenylenediamine was quite affective but less potent. Moore and Sharman (7) reported that when *N*,*N*′-diphenyl-*p*-phenylenediamine, methylene blue, 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline[1], or sodium selenide was administered to vitamin E-deficient rats, only *N*,*N*′-diphenyl-*p*-phenylenediamine offered significant protection against blood hemolysis induced by dialuric acid. Similar tests conducted by Bunyan *et al.* (6) failed to show correlations in protective efficacy in tests conducted *in vivo versus* those conducted *in vitro*.

More recently, Gloor *et al.* (8) reported that α-tocopheramine (6-amino-5,7,8-trimethyltocol) was as active as α-tocopherol in the blood hemolysis test, whereas γ-tocopherol had only 10% of the activity of α-tocopherol. *N*-Methyl-γ-tocopheramine(6-*N*-methylamino-7,8-dimethyltocol) was 13 times more active than γ-tocopherol in this test.

Because it was felt that the biological activity of vitamin E could be related to its antioxidant effect, a study was initiated to determine the ability of certain antioxidant compounds to afford protection against the dialuric acid-induced hemolysis of the blood from vitamin E-deficient rats. This activity was compared with antioxidant activities of these compounds as measured *in vitro*. Most of the compounds were derivatives of α-tocopherol or of 6-hydroxy-2,2,5,7,8-pentamethylchroman.

EXPERIMENTAL

An adaptation of the methods of Bunyan *et al.* (6) and Friedman *et al.* (2) was used for the hemolysis studies. Test compounds were preincubated with saline suspensions of washed red cells from vitamin E-deficient rats (37°, 1 hr.). These treated red cells were resuspended in saline–phosphate buffer (pH 7.4) and incubated with dialuric acid (15 min. at 37° and then 45 min. at room temperature). After centrifugation and dilution of the supernatant, the optical density of the latter was determined at 415 nm. This value was compared with values obtained for samples not exposed to dialuric acid and for samples completely hemolyzed with distilled water (2). The red cells used exhibited 80–100% hemolysis by dialuric acid in the absence of protecting agents. Compounds were evaluated at 100-, 50-, 25-, 10-, 5-, 1.0-, 0.5-, 0.25-, and 0.1-mcg. levels.

Synthetic and antioxidant data were previously reported by Skinner and Parkhurst (9), who determined the protection of β-carotene in corn oil against air oxidation.

RESULTS AND DISCUSSION

A comparison of the *in vitro* antioxidant activities with the *in vitro* antihemolytic activities of these vitamin E analogs (Table I) indicates some overall correlation between the two activities, but notable exceptions are present. For example, the thiourea derivative protects β-carotene only 27 hr. (half-life) but is quite active in protecting blood cells from hemolysis. The same is true of the 5-dimethylaminomethyl derivatives. The orthoquinone and the 5-aldehyde are also better antihemolytic agents than the antioxidant results indicate. The 5-hydroxymethyl and 5-methoxymethyl derivatives are quite active in both tests.

It is obvious from a study of Table II that all antioxidants are not equally effective in preventing the dialuric acid-induced hemolysis of red blood cells of vitamin E-deficient rats. None of the compounds evaluated was as effective as α-tocopherol, but some of the model chroman derivatives were quite active; *e.g.*, 2,2,5,7,8-pentamethylchroman-6-phosphate; Compound 4; Compound 5; 2,2,5,7,8-pentamethyl-6-hydroxychroman; 2,2,7,8-tetramethyl-6-hydroxychroman; 5-ethoxymethyl-2,2,7,8-tetramethyl-6-hydroxychroman; and 5-thioureiodomethyl-2,2,7,8-tetramethyl-6-hydroxychroman hydrochloride. The most active of the nontocopherol antioxidants were pyrogallol, *N*,*N*′-diphenyl-*p*-phenylenediamine, butylated hydroxytoluene, 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline, and 4,4′-thiobis(6-*tert*-butyl-*ortho*-cresol). Ascorbic acid, which is an excellent antioxidant in certain systems, was inactive in preventing hemolysis.

It would be of interest to compare the most active of these derivatives for their *in vivo* effectiveness against dialuric acid-induced hemloysis of red blood cells of vitamin E-deficient rats.

REFERENCES

(1) C. S. Rose and P. György, *Blood*, **5**, 1062(1962).

(2) L. Friedman, W. Weiss, F. Wherry, and O. L. Kline, *J. Nutr.*, **65** (1), 143(1958).

(3) C. S. Rose and P. György, *Amer. J. Physiol.*, **168**, 414 (1952).

[1] Santoquin.

(4) H. H. Gordon and J. P. DeMetry, *Proc. Soc. Exp. Biol. Med.*, **79**, 446(1952).
(5) v. G. Brubacher and H. Weiser, *Deut. Ges. Ernaehr., Wissenschaftl. Veroeffenslich.*, **16**, 50(1967).
(6) J. Bunyan, J. Green, E. E. Edwin, and A. T. Diplock, *Biochem. J.*, **75**, 460(1960).
(7) T. Moore and I. M. Sharman, *Int. Z. Vitaminforsch.*, **34** (3), 317(1964).
(8) v. U. Gloor, F. Weber, and O. Wiss, *Deut. Ges. Ernaehr., Wissenschaftl. Veroeffenslich.*, **16**, 66(1967).
(9) W. A. Skinner and R. M. Parkhurst, *Lipids*, **5** (2), 184(1970).

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COMMUNICATIONS

Direct Gas Chromatographic Analysis of Long-Chain Alcohols and Alkyltrimethylammonium Bromides

Keyphrases ☐ Alkyltrimethylammonium bromides—analysis ☐ Alcohols, long chain—analysis ☐ TLC—analysis ☐ GLC—analysis

Sir:

Quaternary ammonium compounds have numerous industrial applications [for example, see Schwartz *et al.* (1)]. In pharmacy, alkyltrimethylammonium halides are important both for their antibacterial action, which may be linked to their micellar properties (2–4), and when used in combination with long-chain fatty alcohols to form mixed emulsifiers (5–8). We are studying the self-bodying action of mixed emulsifiers, including those of the alkyltrimethylammonium bromide–long-chain alcohol type (5, 6).

Commercial alkyltrimethylammonium bromides, including cetrimide BP, and alcohols, such as cetostearyl alcohol BP, are mixtures of homologs, and it is desirable to have a simple, direct method of analysis suitable for all materials.

Link and Morrissette (9) described the analysis of long-chain alcohols by GLC, using a nonpolar substrate on a solid support treated with alkali. However, there is some controversy over the analysis of quaternary compounds in alkaline conditions. Metcalfe (10) used a similar column to analyze long-chain quaternary compounds; under the conditions described, the corresponding tertiary amines were produced. Analysis of these provided a measure of the total homolog composition of the original mixture. No peak had a retention time that corresponded to any of the postulated higher olefins which would result from a Hofmann degradation.

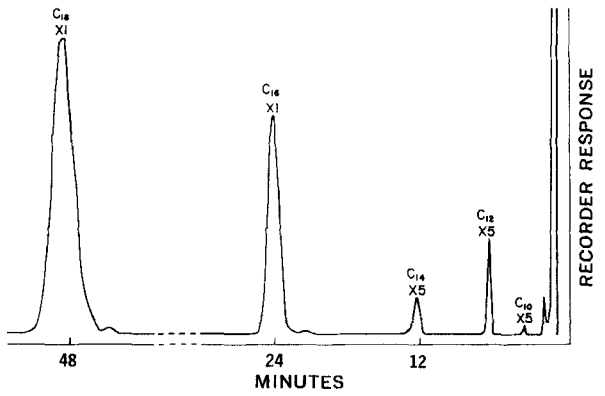

Figure 1—*Chromatogram of cetostearyl alcohol BP.*

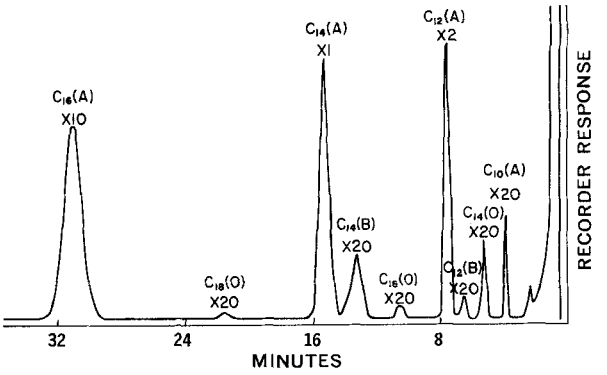

Figure 2—*Chromatogram of cetrimide BP. Key: (A) = tertiary amine; (O) = olefin; and (B) = alkyl bromide. For certain peaks, detector sensitivity increased by the factor shown.*

Laycock and Mulley (11) were unable to reproduce Metcalfe's (10) experiments, and thus they modified the column and analyzed quaternary ammonium compounds in the form of the hydroxides. Under their conditions, each compound decomposed quantitatively into a mixture of tertiary amine and olefin.

Neither paper reported checks on the original quaternary compounds for tertiary amine content which, if present, would make the analyses inaccurate.

We have investigated the homolog composition of a series of alcohols and alkyltrimethylammonium bromides using a similar column to that described by Metcalfe (10). A Perkin-Elmer F.11 chromatograph with flame-ionization detector was used. The column was stainless steel, 2 m. long, 0.3175 cm. (0.125 in.) o.d., packed with acid-washed Chromosorb W (60–80 mesh) coated with 10% w/w potassium hydroxide. The flow rate of the carrier gas, nitrogen, was 25 ml./min. The injection and column temperature for 4% w/v solutions of the alcohols dissolved in *n*-heptane were 225–230