Vitamin E Activity and Metabolism of *N*-Methyltocopheramines*

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ABSTRACT: The synthesis of new tocopherol derivatives (Schwieter, U., Tamm, R., Weiser, H., and Wiss, O. (1966), *Helv. Chim. Acta 49*, 2297) has initiated reconsideration of structure–activity relationships of vitamin E active compounds. In two different bioassays with the chick, *dl*-α-tocopheramine, *dl*-*N*-methyl-β-tocopheramine, and *dl*-*N*-methyl-γ-tocopheramine were as active on a molar basis as *dl*-α-tocopherol. Considerably less active were *dl*-β-tocopheramine and *dl*-γ-tocopheramine. When the compounds were fed to rats, blood levels of *N*-methyl-β- and *N*-methyl-γ-tocopheramines were one-half that of α-tocopherol but in liver there was twice the concentration of the *N*-methyltocopheramines as α-tocopherol. The amounts of β- and γ-tocopheramines in blood and liver were considerably lower than those of α-tocopheramine and the *N*-methyl derivatives. The distribution of α-tocopherol, β-tocopheramine, and *N*-methyl-β-tocopheramine in liver cellular fractions was similar. Neither the tocopheramines nor *N*-methyltocopheramines, when administered to rats, gave rise to detectable amounts in the tissues of their corresponding tocopherols. The compounds were recovered from liver and identified by their ultraviolet absorption spectra and gas chromatographic retention times.

N-Methyl-β-tocopheramine had antoxidant activity equal to that of α-tocopherol in stabilizing methyl linolenate emulsions, while β-tocopheramine was one-half as active.

Although the amine analogs of the tocopherols have been known for many years, little data regarding their biological activities have been reported. Smith *et al.* (1942) synthesized α-tocopheramine·HCl and stated that H. M. Evans had found "on a molecular basis it was approximately as active biologically as α-tocopherol itself." Farber *et al.* (1953) reported that α-tocopheramine would cure muscle dystrophy in the rabbit but its relative activity was not tested. Other tocopheramines (β and δ) were synthesized (Quaife, 1950) but no information on their biological activities appeared.

Recently, Schwieter *et al.* (1966) have synthesized a number of *N*-alkyl derivatives of α-, β-, γ-, and δ-tocopheramines, and have tested their vitamin E activity in the rat erythrocyte hemolysis test. The metabolism of some of these derivatives was also investigated by these workers (Gloor *et al.*, 1966). They found *N*-methyl-β-tocopheramine and *N*-methyl-γ-tocopheramine to be slightly more active than α-tocopherol. Since the hemolysis test is partially an *in vitro* assay it was considered important to determine the vitamin E activity of these derivatives in preventing some of the classical deficiency symptoms of this vitamin. In the present study, it is shown that these two *N*-methyltocopheramines, and also α-tocopheramine, have biological activity in two bioassays in the chick equal to that of α-tocopherol. The distribution of various tocopheramines and *N*-methyltocopheramines in blood and liver is also reported.

Materials and Methods

Compounds. *dl*-α-Tocopherol, *dl*-α-tocopheramine, *dl*-β-tocopheramine, *dl*-γ-tocopheramine, *dl*-*N*-methyl-β-tocopheramine, and *dl*-*N*-methyl-γ-tocopheramine were kindly supplied by Dr. O. Wiss, Hoffmann-La-Roche, Ltd., Basle. The structural relationship of several of these compounds to α-tocopherol is shown in Figure 1. The compounds used in feeding experiments were furnished as gelatin-coated beadlets (1.1–1.6% active compound) in order to prevent oxidative destruction. Analyses[1] of these preparations were repeated at the end of the studies, after 1 year of storage at −20°, by three procedures: fluorescence spectra, ultraviolet absorption, and reducing activity ($FeCl_3$–bipyridyl reaction). Except for γ-tocopheramine, which had undergone considerable decomposition, the preparations all contained at least 90% of their original potency. *d*-α-Tocopherol used in the antioxidant experiment was from Distillation Products Industries.

Analyses of Tissues. An unsaponifiable extract of blood serum was analyzed by one-dimensional thin layer chromatography (tlc) on silica gel G containing sodium fluorescein as described previously (Bieri and Prival, 1965). The compounds were detected by their quenching against the fluorescent background under ultraviolet light. With the solvent system used (benzene–ethanol, 99:1), all of the compounds studied (α-, β-, and γ-tocopheramines and *N*-methyl-β- and *N*-methyl-γ-tocopheramines), migrated considerably slower (R_F values of 0.13–0.20) than the tocopherols (α = 0.50, β and γ = 0.39).

For the analysis of liver, 0.5–1.0 g of chopped frozen tissue in 2 ml of ethanol containing 2% pyrogallol was saponified with 0.5 ml of 11 N KOH at 65° in a nitrogen

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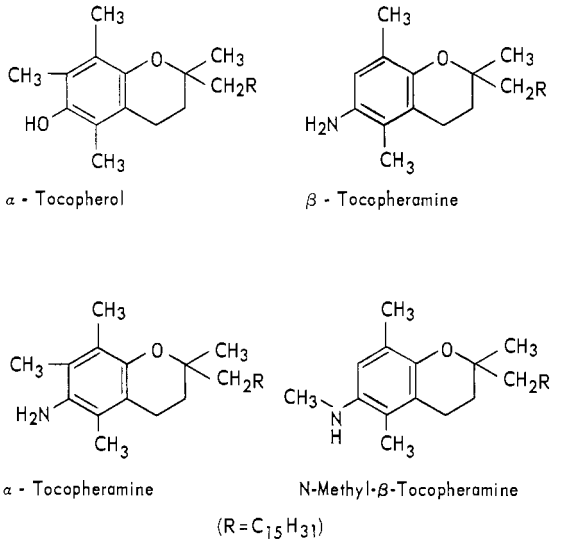

FIGURE 1. Structural relationships of tocopheramines and *N*-methyltocopheramines to α-tocopherol. In the tocol series, α is the 5,7,8-trimethyl, β is the 5,8-dimethyl, γ is the 7,8-dimethyl-, and δ is the 8-methyltocopherol.

atmosphere. The unsaponifiable extracts were analyzed by one-dimensional tlc except in the case of α-tocopherol. This compound was separated by two-dimensional tlc (solvent 1, benzene–ethanol, 99:1; solvent 2, hexane–ethanol, 9:1) to eliminate interference from ubichromenol. In some experiments, extracts containing β- and γ-tocopheramines were analyzed in one dimension in benzene–ethanol (98:2) in order to achieve a better separation of these compounds from retinol. The one-dimensional analyses of tocopheramines were occasionally checked by two-dimensional tlc.

The areas of silica gel were scraped off and eluted by mixing with 1.2–2 ml of 0.02% bipyridyl in ethanol. After centrifuging, 1 ml of the supernatant solution was transferred to a 1-ml cuvet and 0.1 ml of 0.1% $FeCl_3$ was added with mixing. The relative absorbancies at 520 mμ after a 1-min reaction time, in the Beckman DU spectrophotometer, were: α-tocopherol = 1.00; α-tocopheramine, 0.75; β-tocopheramine, 0.76; γ-tocopheramine, 0.96; *N*-methyl-β-tocopheramine, 0.82; and *N*-methyl-γ-tocopheramine, 0.86. Identification of the compounds from the thin layer plates was made in two ways: (1) the ultraviolet absorption curve and (2) the retention time when analyzed by gas chromatography on a 1% SE-30 column as described previously for tocopherols (Bieri and Andrews, 1963).

Bioassays. Two different vitamin E deficiency syndromes in the chick, produced independently, were used. In the first, exudative diathesis, a diet deficient in both vitamin E and selenium must be fed (Schwarz *et al.*, 1957). Chicks develop massive subcutaneous hemorrhages in 12–18 days which are preventable by either α-tocopherol or selenium. In the second, encephalomalacia, a diet relatively high in linoleic acid must be used and only vitamin E, but not selenium, will prevent the brain damage and death which occur in 14–21 days.

Male chicks (1-day old) were obtained from the Arbor Acres Hatchery, Glastonbury, Conn. Details of the feeding procedure are given in the tables. In the assays using the prevention of exudates, diet C34 (Bieri, 1964) was fed, while in the assays based on the prevention of encephalomalacia, a casein–gelatin-type diet (Bieri *et al.*, 1957) was used. Both diets contained glucose as the carbohydrate and had adequate amounts of minerals and vitamins, except vitamin E. A feeding test of the gelatin matrix, used in preparing the beadlets, revealed that at twice the highest dietary concentration used in the assays, there was no biologically active selenium that could have influenced the assays for preventing exudates.

Chicks were inspected daily and the incidence of symptoms was recorded. The per cent of chicks protected from the vitamin E deficiency symptoms at each dosage level was expressed as probits and plotted against the logarithm of the dose (Bliss and György, 1951). From the parallel lines obtained the potencies relative to *dl*-α-tocopherol were calculated.

Distribution Studies. In the first analyses of liver unsaponifiable extracts by tlc, it was found that retinol, which had an R_F only slightly less than that of the β- and γ-tocopheramines, often would not separate distinctly from these compounds. To eliminate this interference, in subsequent experiments the retinol in the diets was replaced with retinoic acid (10 mg/kg) since the latter compound permits normal growth but is not stored in the liver (Dowling and Wald, 1960). Chicks (1-day old) were depleted of their α-tocopherol reserves by feeding the casein–gelatin diet with 8% stripped corn oil, but with the addition of 0.015% of 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (ethoxyquin, Monsanto Chemical Co.). This antioxidant prevents encephalomalacia (Machlin *et al.*, 1959) and the birds develop normally.

In the rat experiments, rats were depleted of α-tocopherol with a diet containing 20% of vitamin-free casein, 4% stripped lard, and sucrose, with adequate minerals and vitamins. Vitamin A was supplied as retinoic acid (3 mg/kg). Details are given in Table IV. The rats were divided into groups of similar weight distribution and each rat was given 10 g of food each night containing the test compound. This amount of food was 2–3 g less than any of the rats had been consuming daily up to this time, thus the dietary supplements were entirely consumed.

Antioxidant Activities. The relative antioxidant activities of two tocopheramines in preventing the autoxidation of methyl linolenate (Hormel Institute) emulsions was performed essentially as described by Lea and Ward (1959). A mixture of methyl linolenate, tocopheramine in ethyl laurate, and buffer (Figure 2) in a 25-ml cellulose acetate tube was emulsified for 10 sec with a Branson ultrasonic apparatus. The tubes were held in an ice bath and flushed with nitrogen during the emulsification. Relative protection factors were calculated according to Lea and Ward (1959) from a plot of the oxygen uptake *vs.* time. The protection factor is the ratio of the induction periods in the presence and absence of antioxidant

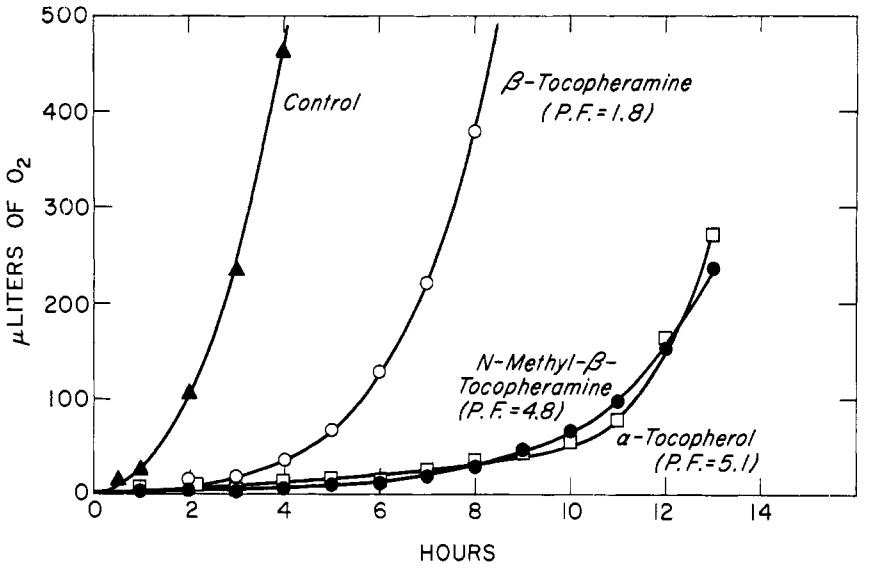

FIGURE 2: Antioxidant activity of *d*-α-tocopherol, *dl*-β-tocopheramine, and *dl*-*N*-methyl-β-tocopheramine in preventing the autoxidation of methyl linolenate. Each Warburg flask contained 3.0 ml of an emulsion of 210 μmoles of methyl linolenate, 10.7 mμmoles of antioxidant, and 70 mμmoles of ethyl laurate in 0.1 M phosphate buffer (pH 7.4) containing 1% Tween 40 (polyoxyethylene sorbitan monopalmitate, Atlas Powder Co.). Center well contained 0.2 ml of 10% KOH. Incubated with shaking at 47.5° in air. P.F. = protection factor (Lea and Ward, 1959).

minus one. (A factor of one means that the induction period has been doubled.) A value of 80 μl of oxygen for the induction time was selected in the experiment described in Figure 2.

Results

The relative biological activities of several tocopheramines and *N*-methyltocopheramines in preventing exudative diathesis in chicks as determined in three separate experiments are shown in Table I. α-Tocopheramine appeared to be slightly more active than α-tocopherol but the limits of error in such assays make this difference of doubtful significance. The other two tocopheramines (β and γ) had about one-half and one-third the activity of α-tocopherol, respectively. The *N*-methyl-β- and *N*-methyl-γ-tocopheramines, however, had essentially full vitamin E activity. When three of these compounds were tested for their activity in preventing encephalomalacia (Table II), α-tocopheramine and *N*-methyl-β-tocopheramine were again as effective as α-tocopherol. β-Tocopheramine was considerably less active in this assay than in the previous one.

The concentration of α-tocopheramine in the serum of chicks was slightly higher than that of α-tocopherol while *N*-methyl-β-tocopheramine was about one-fourth lower (Table III). In the liver, this situation was reversed, in that the concentration of *N*-methyl-β-tocopheramine was twice that of α-tocopherol while α-tocopheramine was intermediate. In this experiment, the compounds were fed continuously to groups containing four chicks each (weights ranged from 357 to 452 g) so that the amount of compound ingested by each chick was variable.

A second experiment was carried out with rats in which the amount of compound ingested was kept uniform. The data (Table IV) show that the blood concen-

TABLE I: Relative Biological Activities of Tocopheramines in Preventing Chick Exudative Diathesis.[a]

Compound	Expt 1[b]	Expt 2[c]	Expt 3	Av
α-Tocopherol	100	100	100[c]	100
α-Tocopheramine	112	109	—	110.5
β-Tocopheramine	—	—	50[d]	50
γ-Tocopheramine	—	—	38[d]	38
N-Methyl-β-tocopheramine	86	116	—	101
N-Methyl-γ-tocopheramine	90	87	—	88.5

[a] Chicks (1-day old) were depleted of vitamin E for 6 days on a 60% Torula yeast diet containing 4% stripped lard (Distillation Products Industries). Each compound was then fed for 18 days to duplicate groups of eight chicks. The incidence of exudative diathesis in unsupplemented chicks was 100%. [b] Dietary levels were 11.6, 17.4, and 26.1 μmoles/kg. [c] Dietary levels were 13.9, 20.9, and 31.3 μmoles/kg. [d] Dietary levels were 70 and 140 μmoles/kg.

TABLE II: Relative Biological Activities of Tocopheramines in Preventing Chick Encephalomalacia.[a]

Compound	Act.
α-Tocopherol[b]	100
α-Tocopheramine[b]	98
β-Tocopheramine[c]	20
N-Methyl-β-tocopheramine[b]	110

[a] Chicks (1-day old) were depleted of vitamin E for 8 days on a 20% casein–8% gelatin diet containing 8% stripped corn oil (Distillation Products Industries). Each compound was then fed for 18 days to duplicate groups of seven chicks. The incidence of encephalomalacia in unsupplemented chicks was 100%. [b] Dietary levels were 7, 9.9, and 13.9 μmoles/kg. [c] Dietary levels were 9.9, 13.9, and 19.6 μmoles/kg.

TABLE III: Blood Levels and Liver Storage of Tocopheramines in Chicks.[a]

	Serum (μg/100 ml)	Liver (μg/g)
α-Tocopherol	1071 ± 92	23.0 ± 1.3
α-Tocopheramine	1228 ± 59	31.5 ± 4.3
N-Methyl-β-tocopheramine	734 ± 102	53.5 ± 6.8

[a] Chicks depleted of vitamin E for 22 days, then fed the compounds (as beadlets) at a level of 100 mg/kg diet for 4 days. Values are means plus and minus standard errors for four chicks.

tration was highest for α-tocopherol while the values for α-tocopheramine, *N*-methyl-β-, and *N*-methyl-γ-tocopheramines were about one-half to one-third that of α-tocopherol. The β- and γ-tocopheramine concentrations were one-third those of their corresponding *N*-methyl derivatives.

In liver, *N*-methyl-β- and *N*-methyl-γ-tocopheramines were deposited in greatest amount. Intermediate were α-tocopherol and α-tocopheramine while β- and γ-tocopheramines were lowest. These relationships were true whether the storage was expressed as concentration or total amount in the liver. The considerably greater deposition of *N*-methyl-β-tocopheramine than α-tocopherol agrees with the results from the chick experiment.

The intracellular distribution in rat liver of β-tocopheramine and *N*-methyl-β-tocopheramine compared with that of α-tocopherol is shown in Table V. The distribution of all three compounds was very similar with about one-half in the nuclei plus debris fraction, about one-fourth to one-third in both the mitochondrial and microsomal fractions, and no significant amount in the supernatant fraction. It is apparent that there is no specificity in uptake of the three compounds once they enter the cell. The greater deposition of *N*-methyl-β-tocopheramine noted in the previous experiments (Tables III and IV) was confirmed in this study.

It was of interest to know if the difference in biological activities of β-tocopheramine and *N*-methyl-β-tocopheramine might be related to their activities as antioxidants. In three separate experiments in which the inhibition of autoxidation of methyl linolenate was studied, *N*-methyl-β-tocopheramine consistently was superior to β-tocopheramine. From the results of one experiment (Figure 2), it was determined that *N*-methyl-β-tocopheramine and α-tocopherol had similar protection factors which were about 2.5 times that of β-tocopheramine.

In the tlc analyses of liver there was no evidence that any of the unsubstituted tocopheramines were converted to their corresponding tocopherols. When the compounds eluted from the tlc plates were examined for their ultraviolet absorption spectra and also for their retention times by gas chromatography, both criteria indicated no change from the compounds that were fed. Quantitative analyses of several tlc eluates by gas chromatography agreed well with the reducing activity determined on an aliquot by the $FeCl_3$–bipyridyl assay.

When *N*-methyl-β-tocopheramine was fed, however, all tlc analyses of both rat and chick livers revealed that in addition to the quenching spot of the compound itself, there was always another quenching spot (termed spot "X") lower on the plate with an R_F similar to that of β-tocopheramine. This material reacted quickly with $FeCl_3$–bipyridyl. The ultraviolet absorption curve was similar to that of β-tocopheramine (symmetrical peak with λ_{max} 300 in ethanol) but on analysis by gas chromatography two incompletely separated peaks were obtained, one of which had the same retention time as β-tocopheramine (Table VI). This is considered to be only suggestive evidence that *N*-methyl-β-tocopheramine may be demethylated to β-tocopheramine in the liver.

Discussion

The observation that *N*-methyl-β-tocopheramine and *N*-methyl-γ-tocopheramine, as well as α-tocopheramine, have *in vivo* activity equal to that of α-tocopherol is in general agreement with the activities found by Schwieter *et al.* (1966) using an *in vitro* hemolysis test. Although α-tocopheramine had earlier been reported to have high vitamin E activity, the *N*-methyl-β- and *N*-methyl-γ-tocopheramines are the first known derivatives of the less active tocopherols to have full biological activity, *i.e.*, equal to that of α-tocopherol.

Several possible explanations of why *N*-methylation increases the biological activity of β- and γ-tocopheramines can be offered. Fieser (1920) showed that *N*-methylation of aniline reduced the oxidation potential from 1.135 to 1.053 v, thus, the *N*-methyltocopheramines might be expected to be better antioxidants. The

TABLE IV: Blood Levels and Liver Storage of Tocopheramines in Rats.[a]

Compound	Serum (μg/100 ml)	Liver		
		μg/g	μg/Organ	Storage (%)[b]
α-Tocopherol	784 ± 45	23.0 ± 1.3	149 ± 7	3.7
α-Tocopheramine	316 ± 23	28.0 ± 4.5	179 ± 12	4.5
β-Tocopheramine	98 ± 16	17.2 ± 4.5	106 ± 15	2.7
γ-Tocopheramine	88 ± 11	14.9 ± 2.8	96 ± 18	2.5
N-Methyl-β-tocopheramine	358 ± 51	55.5 ± 7.2	377 ± 60	9.4
N-Methyl-γ-tocopheramine	239 ± 29	40.9 ± 1.8	259 ± 9	6.5

[a] National Institutes of Health Sprague–Dawley weanling male rats were depleted of vitamin E for 19 days (weights, 115–135 g), then fed the compounds, 2.33 μmoles (0.97–1.0 mg)/day for 4 days. Values are means plus and minus standard errors for four rats. Three control, unsupplemented rats had 0–0.3 μg of α-tocopherol/g liver, and 0–60 μg of α-tocopherol/100 ml of serum. [b] Amount of compound found in liver/amount ingested × 100.

TABLE V: Distribution of Tocopheramines in Rat Liver Cellular Fractions.[a]

Compound	Whole Liver (μg/g)	Nuclei (%)	Mitochondria (%)	Microsomes (%)	Supernatant (%)	Total Found in Fractions (μg/g)
dl-α-Tocopherol	7.3	52	24	23	1	7.0
dl-β-Tocopheramine	4.5	43	34	19	4	3.9
dl-*N*-Methyl-β-tocopheramine	13.8	48	35	16	1	13.1

[a] Male rats (280–330 g) depleted of vitamin E for 8 weeks were fed 2.33 μmoles of each compound daily for 4 days. Liver (2 g) was homogenized in ten volumes of 0.25 M sucrose containing 1 mM $CaCl_2$. After a 10-min centrifugation at 2000*g* (nuclei), the supernatant fluid was centrifuged for 10 min at 12,000*g* (mitochondria). The resulting supernatant fluid was centrifuged 90 min at 100,000*g* (microsomes). Values are averages of two rats.

experiment on the autoxidation of methyl linolenate demonstrated that *N*-methyl-β-tocopheramine had at least twice the antioxidant activity of β-tocopheramine. Attempts over many years by numerous investigators to correlate antioxidant activity of the tocopherols with their biological activity have not been very successful, so that the difference in antioxidant properties between β-tocopheramine and its methylated derivative is probably a minor factor in explaining their *in vivo* activities.

A second possibility is that *N*-methylation may increase the utilization of β- and γ-tocopheramines, *i.e.*, facilitate their absorption, deposition, and retention in the tissues. The experiments with both rats and chicks do not permit an accurate evaluation of these various aspects of utilization. The continuous feeding of the compounds gives an estimation of the steady-state condition since the compounds were ingested over a 4-day period. Blood levels indicated that *N*-methylation either increased the efficiency of absorption of β- and γ-tocopheramines or delayed removal from the circulation. Although it is not possible to tell which mechanism may predominate, the results of Gloor *et al.* (1966) with γ-tocopherol, a compound with low biological activity similar to that of γ-tocopheramine, provide evidence that absorption is not greatly different from that of α-tocopherol and *N*-methyl-γ-tocopheramine. More direct evidence on the rates of absorption of these compounds is necessary to clarify these factors.

The liver storage data indicate that *N*-methylation of β- and γ-tocopheramines greatly improved their deposition and retention; in fact, these methyltocopheramines were retained much better than α-tocopherol. Gloor *et al.* (1966), in their comparison of the uptake by liver of a single dose of ^{14}C-labeled α-tocopherol and *N*-methyl-γ-tocopheramine, found the latter compound to be higher at all time intervals up to 4 days. From our analyses of the intracellular distribution of these compounds, it is apparent that the differences in liver retention were not due to preferential deposition of the compounds in any of the subcellular compartments, *i.e.*, despite considerable differences in the total liver content, the compounds were distributed similarly among the cell fractions.

No evidence was found that the tocopheramines or

TABLE VI: Gas Chromatography Retention Times of Various Tocopherol Derivatives.[a]

Compound	Retention Times
Cholestane (standard)	1.00
β-Tocopherol quinone	1.84
β-Tocopheramine	1.93
Cholesterol	2.00
γ-Tocopheramine	2.01
N-Methyl-β-tocopheramine	2.16
N-Methyl-γ-tocopheramine	2.21
α-Tocopherol	2.28
α-Tocopherol quinone	2.45
α-Tocopheramine	2.68
Spot "X"	1.80, 1.93

[a] A column (200 cm × 4 mm) of 1% SE-30 on Chromosorb W. Column temperature, 225°; flash heater and detector temperatures, 275°. Argon ionization detector (900 v) with gas pressure, 30 psi.

N-methyltocopheramines were converted to their corresponding tocopherols, as noted also by Gloor *et al.* (1966). By means of the isotope dilution technique, these investigators were able to find radioactivity in the corresponding tocopherol quinones and Simon metabolites (Simon *et al.*, 1956) after administering labeled tocopheramines. We were unable to find evidence for either of these metabolites by a combination of tlc and gas chromatography. The sensitivity (*ca.* 0.2 μg) of this technique, however, is considerably less than that of isotope dilution. Preliminary evidence for a metabolite of *N*-methyl-β-tocopheramine was found by tlc analysis of liver but insufficient material was obtained to permit characterization other than that two compounds were found by gas chromatography.

The fact that *N*-methyl-β- and *N*-methyl-γ-tocopheramines have vitamin E activity in the animal equal to that of α-tocopherol has significant implications regarding the biochemical action of such compounds. Although various unnatural antioxidants are well known to be capable of substituting for α-tocopherol in preventing many deficiency symptoms in a variety of animal species, none of these compounds has activity on a molar basis approaching that of α-tocopherol. Although some investigators have explained the lower biological activity of the unnatural antioxidants by their poorer absorption and retention by tissues (Wiss *et al.*, 1962) the difference in activities has often been interpreted as evidence that α-tocopherol has a specificity at the molecular level not met by the less active tocopherols (β, γ, and δ) and the unnatural antioxidants. The full activity of *N*-methyl-β- and *N*-methyl-γ-tocopheramines is evidence that a trimethyl chroman structure, as in α-tocopherol, is not a requisite for optimal biological activity. Accordingly, the formation of an α-tocopheryl radical by oxidation of the 5-methyl group, with subsequent dimerization or phosphorylation (Goodhue and Risley, 1964) cannot be considered to be a functional pathway for α-tocopherol. Furthermore, it is evident that the hydroxyl group of α-tocopherol can be replaced by an amino group (in the case of α-tocopheramine) and also by an *N*-methylamino group (in the β- and γ-tocopheramines).

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