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The Preparation and Stability of DL-α-Tocopherol Labelled with Tritium at High Specific Activity

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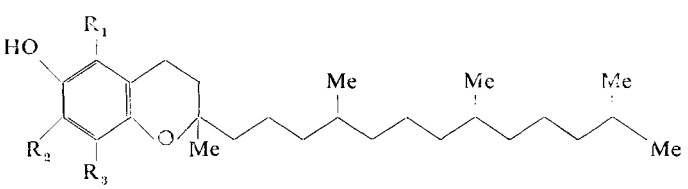
SUMMARY

Hydrogen-tritium exchange labelling methods proved to be unsatisfactory for the preparation of tritiated DL-α-tocopherol at high specific activity. The preparation of α-tocopherol specifically labelled in the 5-methyl hydrogen positions is described : the method permits specific activities in the curies per millimole range to be readily achieved. The synthesis is also suitable for preparing tocopherol doubly labelled with tritium and carbon-14. The stability of the tritiated vitamin on storage is discussed.

The existence of the antisterility fat soluble vitamin, α-tocopherol (vitamin E), was first recognised more than forty years ago [1, 2]. It was isolated in 1936 [3], its structure (1) elucidated in 1937 [4] and first synthesised by Karrer *et al.* [5] in 1938. The naturally occurring ' tocopherols ' are predominantly a mixture of three compounds differing only in the number and position of methyl groups. Separation of these similar compounds is readily achieved by thin layer chromatography [6–8], a technique which is made use of in the purification of the labelled tocopherol.



	R_1	R_2	R_3	*Name*	*Occurrence*
(1)	Me	Me	Me	α-Tocopherol	Wheat germ oil
(2)	Me	H	Me	β-Tocopherol	Wheat germ oil
(3)	H	Me	Me	γ-Tocopherol	Maize

Vitamin E cannot be synthesised by the tissues of animals i.e. there is no endogenous synthesis, it must therefore be absorbed through the gastro-intestinal tract. The mode of absorption of the vitamin, its distribution and localisation in tissues, and in blood levels, are of fundamental importance in any attempt to study the mode of action (pharmacology) of tocopherol. For any such investigations the use of tocopherol labelled with a radioisotope has many advantages but in spite of various investigations using both carbon-14 [9, 10, 13] and tritium labelling [11–13], the mechanism of the action of vitamin E is still obscure. As a possible aid to further studies we have therefore developed a method for the preparation of tritiated DL-α-tocopherol at very high specific activity.

Isler *et al.*[13] have reviewed various approaches for the synthesis of tocopherol labelled with carbon-14 in the methyl groups of the ring skeleton, for example by a Mannich reaction using formaldehyde-C14, or with tritium by the hydrogenation of unsaturated intermediates such as the $\Delta^{1',2}$- or the $\Delta^{3,4}$-tocopherols.

We first attempted to generally label the vitamin by a modified Wilzbach irradiation, by mixing the tocopherol with an equal weight of 10 % platinum on charcoal [14] and exposing this mixture to 60 curies of tritium gas at room temperature for 7 days. Many radioactive products were produced but a negligible amount of the radioactivity was associated with the purified tocopherol. The platinum catalysed exchange in tritiated water gave better results [15], as might have been expected [16], and a summary of the labelling conditions and the specific activities achieved are given in the table I.

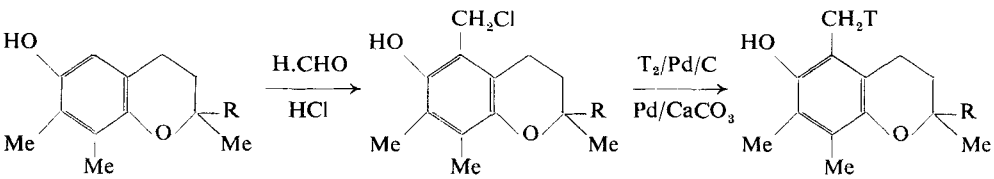
TABLE I. DL-α-Tocopherol labelled by platinum catalysed exchange in THO [15]

Weight of DL-α-Tocopherol mg	Weight[a] of Catalyst mg	Tritiated water		Reaction		Chemical yield %	Specific activity mC/mM
		Volume ml	Specific activity mC/mM	Temp. °C	Time hours		
750	200	3	1080	130°	16	38	51
700	200	2	3600	130°	16	43	26
500	200	2	3600	145°	16	67	35

[a] Weight of platinum oxide (Adams' catalyst) pre-reduced in hydrogen.

The specific activity of the tocopherol which could be achieved by the catalysed exchange in solution method, even when using several hundred curies of tritiated water and a fairly high reaction temperature, was limited and far too low for studies involving (say) tissue and cellular localisation by high resolution autoradiography or for administering physiological doses of suitable radioactive content. Because of the failure of these exchange procedures we therefore directed our attention towards synthetic methods.

In 1963 Suishchuk and Tikhomirova [17] prepared DL-α-tocopherol-5-*methyl*-^{14}C from labelled paraformaldehyde. DL-γTocopherol (3) reacts with paraformaldehyde-^{14}C and HCl to yield 5-*chloromethyl*-^{14}C-tocopherol which is reduced by activated zinc in hydrochloric acid to DL-α-tocopherol-5-*methyl*-^{14}C. When tritiated paraformaldehyde was used in the chloromethylation reaction, most of the tritium was lost by exchange and a poor yield of DL-α-tocopherol-5-*methyl*-T at low specific activity was obtained. However, we modified this reaction sequence by reducing the unlabelled chloromethyl compound (4) in dioxan with tritium gas using a mixed catalyst consisting of



(3) (4)

(R = Phytyl—)

equal parts of 10 % palladium on charcoal and 10 % palladium on calcium carbonate. This catalysed halogen-tritium replacement reaction yielded DL-α-tocopherol-5-*methyl*-T at very high specific activities but it was necessary to purify the compound by preparative thin-layer chromatography on alumina in benzene and on silica gel in chloroform. Specific activities in excess of 2 curies/mM were readily obtained by this route; doubly labelled tocopherol was prepared by using 5-*chloromethyl*-^{14}C-tocopherol in the reduction stage with tritium gas. Only poor yields (0-5 %) were obtained when (4) was reduced with zinc in hydrochloric acid (or tritiated hydrochloric acid), in contrast with other investigators [17].

Stability

Tritiated DL-α-tocopherol at high specific activity was found to be very sensitive to decomposition by self-radiolysis and some results and storage conditions are shown in the table II.

TABLE II. Self-radiolysis of DL-α-tocopherol-5-methyl-T

Specific activity mC/mM	Storage conditions	Radio-active concen-trations mC/g	Time of storage months	Temper-ature °C	Energy absorbed Rads	G(-M)[20]	Decom-position %
2150	Ethanol *(in vacuo)*	1.8	0.75	−40°	1.2×10^4	4.6	6
2150	Ethanol *(in vacuo)*	1.8	2	−40°	3.2×10^4	4.7	17
2150	Ethanol *(in oxygen)*	1.8	2	−40°	3.2×10^4	7.3	25
872	Ethanol *(in vacuo)*	0.8	2.5	−40°	1.8×10^4	5.5	10
872	Ethanol *(in vacuo)*	0.8	2.5	−196°	1.8×10^4	2.6	5
1720	Benzene *(in vacuo)*	0.57	5	−20°	2.6×10^4	3.7	25
1720	Benzene *(in vacuo)*	0.57	6	20°	3.1×10^4	0.8	7

G(—M) values were calculated as discussed by Evans and Bayly [20]. Ethanol and benzene were investigated as solvents, and of the storage conditions studied a temperature of —196 °C. in the absence of air was found to be the most satisfactory when the compound was stored in ethanol solutions. In benzene solution the compound stored best above the freezing point of the solvent i.e. at room temperature (20 °C) and storage in benzene proved more effective than storage in ethanol. These results are in general agreement with observations made for other tritiated compounds at high specific activity [16,18]. The use of tocopherols as antioxidants is well known and it is therefore not surprising to note the sensitivity of the labelled compound to oxygen, which is clearly seen from table II.

Self-radiolysis of α-tocopherol-T in ethanol was found to give four main radioactive decomposition products, whose proportions varied according to whether oxygen was present. This is seen from the radiochromatogram scans of the thin-layer chromatograms in the figures 1 and 2. These products were not identified although it is possible that they may be similar to the products produced by gamma irradiation or by oxidation with ferric chloride, which are well known [19]. Two main decomposition products are formed in benzene solution of which one appears to be different in character to any of the products produced in ethanol solution (Fig. 3).

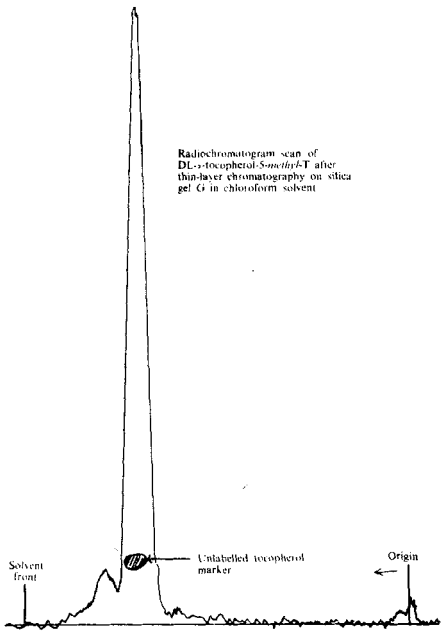


FIG. 1. DL-α-Tocopherol-5-*methyl*-T stored in ethanol solution *(in vacuo)* 2 months at −40 °C (Specific activity 2,150 mC/mM.

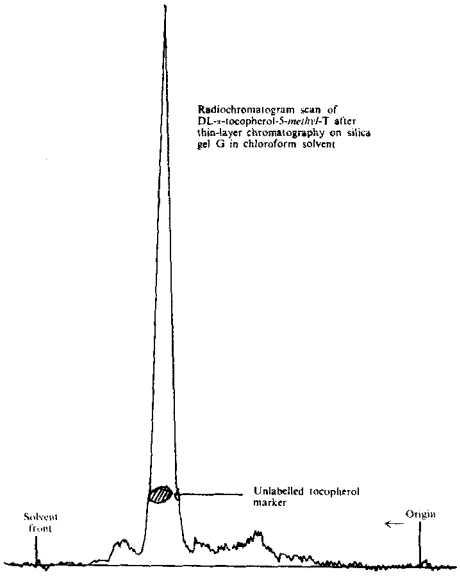


FIG. 2. DL-α-Tocopherol-5-*methyl*-T stored in ethanol solution (in oxygen) 2 months at −40 °C. (Specific activity 2,150 mC/mM.)

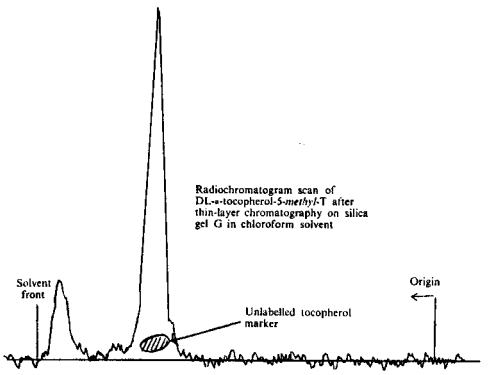


FIG. 3. DL-α-Tocopherol-5-*methyl*-T stored in benzene solution *(in vacuo)* 5 months at −20 °C. (Specific activity 1,720 mC/mM).

EXPERIMENTAL

Radioactivity was measured by β-liquid scintillation counting using a Nuclear Chicago 727 instrument. A Unicam S. P. 500 instrument was used for ultra-violet spectral analyses.

A. — EXCHANGE METHODS.

1. — *Modified Wilzbach Labelling.* DL-α-Tocopherol (0.1 g Koch-Light and Co. Bucks) was mixed with 10 % platinum-on-charcoal (0.1 g) and exposed to tritium gas (60 curies, 98 % isotopic abundance) for 7 days at room temperature in the dark. The tritiated products were extracted with ethanol, the catalyst filtered and washed with ethanol, and the solvents removed by distillation *in vacuo*. The crude viscous residue was chromatographed on thin-layer plates on silica gel G using chloroform as the solvent. Several radioactive products were present but insufficient radioactivity corresponded with the tocopherol to warrant further investigation of the product.

2. — *Catalysed Exchange in Tritiated Water.* DL-α-Tocopherol (0.5 g) was heated (with shaking) in a sealed tube containing a platinum catalyst (0.2 g platinum oxide pre-reduced in hydrogen) and tritiated water (2 ml, 400 curies), for 16 hours at 145 °C. The tritiated solvent was recovered by distillation *in vacuo* and aqueous alcohol (25 ml, 50 % *v/v*) distilled from the crude products to remove readily labile tritium. The crude tocopherol (450 mC) was extracted with ethanol (20 ml), the solvent distilled and the brown viscous residual oil distilled in a short path distillation apparatus at 210-230 °C (bath temp.)/0.1 mm. The radiochemical purity of the pale yellow distillate varied between 50-90 %, and the product was further purified by

thin-layer chromatography on silica gel G in benzene/methanol (98 : 2) yielding radiochemically pure DL-α-tocopherol-T(G) (334 mg, 67 %, 27 mC) at 35 mC/mM. Other results are shown in table I.

B. — CHEMICAL SYNTHESES.

1. — *From Tritiated Paraformaldehyde.*

Paraformaldehyde (184 mg) was added to tritiated paraformaldehyde (17.5 mg, 66.5 mC). This mixture was added to anhydrous ether (12 ml) containing γ-tocopherol (0.1 g) and anhydrous zinc chloride (0.2 g). Dry hydrogen chloride was bubbled through the solution for 1 hour and the solution left at room temperature overnight. Water (*ca.* 10 ml) was added slowly, the ether layer separated and washed with water (5-10 ml). The total radioactivity in the aqueous layers was 32.5 mC. The ether extract was dried (Na_2SO_4), filtered and the ether removed in a stream of nitrogen. The residue (5.7 mC) was dissolved in dioxan (3 ml) and hydrogenated at atmospheric pressure using 10 % palladium on calcium carbonate as catalyst (50 mg). Hydrogen (6 ml) was absorbed, the catalyst was filtered, washed with a few ml of methanol and the solvents removed by distillation *in vacuo*. The residual viscous oil was chromatographed first on alumina thin-layer plates in benzene and then on silica gel G in chloroform yielding DL-α-tocopherol-5-*methyl*-T (15 mg, 14.6 %, 563 μC) at 16.2 mC/mM. Ultra-violet light absorption in ethanol : $\lambda_{MAX.}$ 2920 Å, $\varepsilon = 3\,300$.

When this experiment was repeated and the reduction of the chloromethyl compound carried out with hydrochloric acid (1 ml) and activated zinc (1 g), only impure tritiated DL-α-tocopherol-5-*methyl*-T was obtained.

2. — *DL-α-Tocopherol-5-methyl-T at High Specific Activity.*

Dry hydrogen chloride was passed into a mixture of γ-tocopherol (0.2 g), paraformaldehyde (0.4 g) and powdered anhydrous zinc chloride (0.4 g) in dry ether (25 ml) during 1 hour. The solution was left overnight and water (*ca.* 25 ml) added slowly to the mixture keeping the temperature at about 5 °C. The ether layer was separated, washed with water (10 ml) and dried (anhydrous Na_2SO_4). The solution was filtered, the ether evaporated in a stream of nitrogen and the crude 5-chloromethyl compound used for the next stage. Half the product was reduced in concentrated hydrochloric acid (1 ml) and tritiated water (1 ml, 50 curies) with activated zinc (1 g). Isolation of the tocopherol by thin-layer chromatography yielded no radioactive tocopherol. The other half of the chloromethyl product was dissolved in dioxan (2 ml), 10 % palladium on charcoal (25 mg) and 10 % palladium on calcium carbonate (25 mg) were added and the mixture hydrogenated with tritium (98 % isotopic abundance). Approximately 2 ml of gas (5 curies) were used up during 2 hours. The solution was filtered and the catalyst washed with methanol (20 ml). The

solvents were distilled *in vacuo* and unlabelled α-tocopherol (50 mg) was added. The crude tritiated products were purified by preparative thin-layer chromatography first on alumina in benzene and then on silica gel G in chloroform, yielding DL-α-tocopherol-5-*methyl*-T (43 mg) at 2150 mC/mM. Ultraviolet light absorption in ethanol : $\lambda_{MAX.}$ 2920 Å., $\varepsilon = 3340$, and radiochemical purity 98-99 %. After storage in ethanol for 3 weeks, the radiochemical purity by reverse isotope dilution analysis as tocopherol 3,4-dintrobenzoate was 94 %.

3. — DL-α-TOCOPHEROL-5-METHYL-^{14}C, T.

5-Chloromethyl-^{14}C-tocopherol was prepared from γ-tocopherol (0.1, g) paraformaldehyde-^{14}C (202 mg, 1 mC) and zinc chloride (0.2 g), as described above. The crude product was hydrogenated with tritium and the labelled tocopherol isolated and purified by thin-layer chromatography as previously described, yielding DL-α-tocopherol-5-*methyl*-T, ^{14}C (29 mg) at 7450 mC/mM (tritium) 400 μC/mM (carbon-14). Ultra-violet light absorption in ethanol : $\lambda_{MAX.}$ 2,920 Å., $\varepsilon = 3300$. A radiochemical purity of 98-99 % was established by thin-layer chromatographic analysis on silica gel G in chloroform, and in *cyclo*hexane : chloroform : acetic acid (3 : 2 : 1) as solvents.

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