A common solvent impurity resembling a tocopherol

Many tocopherols and similar chromanols are present in plant tissues; they include α, β, γ and δ-tocopherols[1,2], the corresponding tocotrienols[1] and plastochromanol[2,3]. Recently POWLS AND REDFEARN[4] have isolated a menachromanol from *Chlorobium thiosulphaticum*; in contrast to the other chromanols the menachromanol showed a strong blue fluorescence in ultraviolet light.

During studies on α-tocopherol in various plant tissues a fluorescent compound was located which resembled menachromanol in many respects but proved to be an artifact originating in the solvents. In certain chromatographic systems this impurity moves with α-tocopherol, in others it moves with plastoquinone A. Moreover, since it has reducing properties it interferes with estimations of α-tocopherol. It is widespread and occurs even in Analytical Reagent grade acetone, consequently awareness of this impurity is of importance in this field of lipid biochemistry.

Experimental

Plant tissues. Broad beans (*Vicia faba* L. var. Giant Windsor) were grown in a growth chamber at 21° in a regime of 13 h light and 11 h dark. Leaves were harvested from plants, aged between 15 and 30 days, immediately before use.

Pelargonium zonale plants were grown in a growth chamber at 20° in a regime of 12 h light and 12 h dark. Other tissues were collected from the field.

Chloroplast isolation. Chloroplasts were isolated from broad bean leaves (75 g) at pH 7.3 in 0.3 *M* sucrose containing 0.075 *M* tricine–sodium hydroxide buffer[5] by a method essentially that of WALKER[6]. The supernatant from the chloroplasts was then centrifuged at 15,000 × *g* for 1 h in the 6 × 90 ml head of a Griffin-Christ Omikron centrifuge to produce a final supernatant and a precipitate containing chloroplast fragments and mitochondria.

Lipid extraction. The lipids were extracted by the method of BUCKE *et al.*[7]. Chlorophyll was estimated in 80 % acetone by the method of ARNON[8].

Chromatography. Thin layers of Silica Gel G (Macherey Nagel) were prepared by the application of a 1:2 slurry to glass plates 20 cm square using a Shandon spreader set at 0.25 mm thickness.

Some lipid extracts were chromatographed on columns of neutral alumina (Woelm) deactivated with 5.0 % water. Other total lipid extracts and fractions from alumina columns were dissolved in cyclohexane and applied to thin layers. For purification of α-tocopherol from plant lipid samples aliquots were applied as bands together with separate spots of authentic α-tocopherol; after development the marker α-tocopherol was located by covering the remainder of the plate and spraying with 0.2 % ethanolic ferric chloride + 0.5 % ethanolic α,α-dipyridyl (50 : 50) (EMMERIE-ENGEL reagent[9]). The band corresponding to the marker α-tocopherol was scraped off and eluted twice with 5 ml of spectroscopically pure ethanol and the α-tocopherol estimated by the method of the Analytical Methods Committee[10].

Ultraviolet spectra were determined in 1 cm cells with a Unicam S.P. 800 recording spectrophotometer.

Results

Table I summarises the apparent intracellular distribution of α-tocopherol in broad bean leaves when the α-tocopherol was purified from total lipid extracts by

TABLE I

DISTRIBUTION OF α-TOCOPHEROL IN INTRACELLULAR FRACTIONS OF BROAD BEAN LEAVES

For methods of isolation, extraction and estimation of α-tocopherol see text. Leaves from plants aged 18 days.

Fraction	*α-Tocopherol (μmoles)*	*Chlorophyll (μmoles)*	*α-T:CHL × 10³*
Chloroplasts	0.880	53.47	16.5
15,000 × *g*	0.326	5.72	57.0
Supernatant	0.490	0.125	392.0

chromatography on Silica Gel G using 20% di-isopropyl ether in light petroleum (b.p. 40–60°) as solvent. It appeared that a considerable amount of the cell α-tocopherol was located in the cytoplasm but during the estimation it was noted that a longer time was needed for the maximum absorption to be attained in the supernatant sample than in the chloroplast sample. Further investigation showed that each sample contained a substance which had an intense blue fluorescence in ultraviolet light and which reduced EMMERIE-ENGEL reagent slightly more slowly than α-tocopherol. This substance proved to be identical with a substance previously partially purified from alumina fractions from extracts of *Pelargonium zonale* and ivy (*Hedera helix* L.) In these fractions the substance co-chromatographed with plastoquinone A on Silica Gel G using 20% heptane in benzene as solvent.

The unknown was purified on Silica Gel G using a two-dimensional solvent system, 40% heptane in benzene in the first dimension and 10% di-isopropyl ether in light petroleum (b.p. 40–60°) in the second. Fig. 1 shows the location of the unknown on a typical chromatogram in relation to known compounds. Besides reducing EMMERIE-ENGEL reagent the unknown produced a blue colouration with diazotised

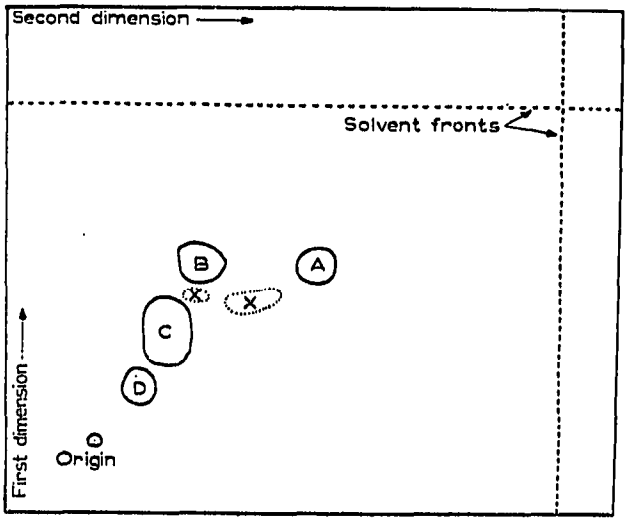

Fig. 1. Two-dimensional chromatogram on Silica Gel G of an aliquot of a fraction (2% diethyl ether in light petroleum (b.p. 40–60°)) from the chromatography on alumina of an extract of ivy leaves. A = PQ A, B = the blue-fluorescing unknown, C = α-tocopherol, D = γ-tocopherol, X = unknown. Solvent in first dimension = 40% heptane in benzene, in second dimension 10% di-isopropyl ether in light petroleum (b.p. 40–60°). Quinones detected in ultraviolet light (254 mμ) after spraying with 0.01% ethanolic fluorescein, tocopherols by spraying with EMMERIE-ENGEL reagent.

o-dianisidine[10], suggesting that it might be phenolic and have an unsubstituted position adjacent to the hydroxy group. These data and the similar chromatographic behaviours of the unknown and menachromanol suggested that the unknown might be the chromanol of a demethyl vitamin K[11]. In addition the ultraviolet spectrum (Fig. 2) did not disagree with this theory. Oxidation with gold chloride by the method of DILLEY AND CRANE[12] replaced this spectrum by a peak at 263 mμ. This latter spectrum, however, was not altered by the addition of sodium borohydride,

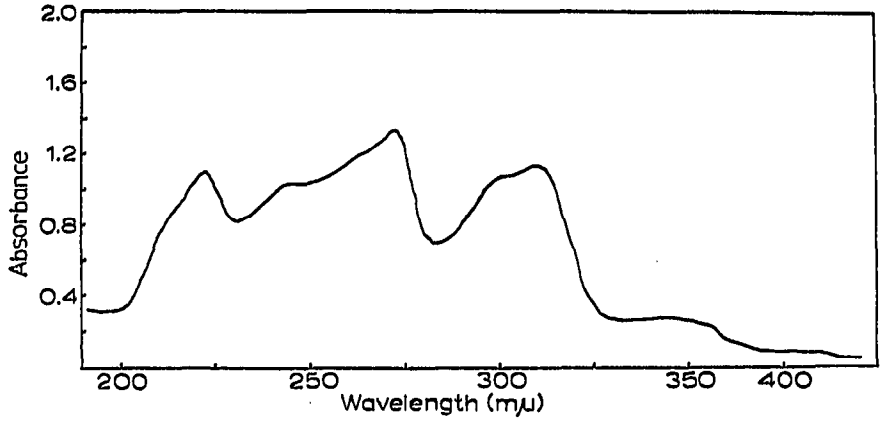

Fig. 2. Ultraviolet spectrum in ethanol of the blue-fluorescing compound purified from the fraction used in Fig. 1.

consequently the oxidation product was not a quinone and the unknown was not a chromanol. The nature of the compound was not investigated further.

Later experiments showed that the amount of the unknown in cell fractions varied greatly and randomly. Eventually it was found that the unknown, together with other fluorescent compounds was present in large amounts in reagent grade acetone and in smaller amounts in analytical grade acetone. Treatment of acetone with activated charcoal followed by distillation removes these compounds almost entirely.

Table II shows the distribution of α-tocopherol in intracellular fractions of broad bean leaves using purified acetone and chromatography using 20 % heptane in benzene as solvent to purify the α-tocopherol. Now the distribution pattern is completely different; the α-tocopherol is clearly associated with the chlorophyll and is absent from the supernatant.

TABLE II

DISTRIBUTION OF α-TOCOPHEROL IN INTRACELLULAR FRACTIONS OF BROAD BEAN LEAVES

For methods of isolation, extraction and estimation of α-tocopherol see text. Leaves from plants aged 25 days.

Fraction	*α-Tocopherol (μmoles)*	*Chlorophyll (μmoles)*	*α-T:CHL × 10³*
Chloroplasts	0.729	13.23	42.33
15,000 × *g*	0.191	3.17	41.43
Supernatant	N.D.	N.D.	—

Discussion

The fluorescent impurity isolated from acetone is probably very similar to the "substance IV" isolated from various solvents by CROSBY AND AHARONSON[13]. CAIN AND MORTON[14] purified a compound with a very similar ultraviolet spectrum from various liver oils using techniques which did not involve acetone and showed that it was similar to a substance isolated from New Zealand butter[15]. They also isolated the compound from chromatographic alumina and concluded that it was a laboratory contaminant. Recently BROWN AND KING[16] isolated a lipid with a similar ultraviolet spectrum from various bacteria; their methods also did not involve the use of acetone. There are probably many other unpublished instances of the isolation of this annoying compound which is widespread enough to suggest that it might be atmospheric in origin.

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Separation of aflatoxins by two-dimensional thin-layer chromatography

Thin-layer chromatography (TLC) of the aflatoxins is routinely performed using 250 μ layers of Macherey-Nagel Silica Gel G-HR and a suitable solvent system, *i.e.*, methanol–chloroform[1] (3:97, v/v), acetone–chloroform[2] (1:9, v/v) or benzene–ethanol–water[3] (46:35:19, v/v). Separated toxins are detected on the plates by their fluorescence under ultraviolet light. The preceding methods of chromatography provide a generally satisfactory separation of aflatoxins B_1, B_2, G_1 and G_2. However