CCLXXVII. STUDIES ON VITAMIN E

II. THE ISOLATION OF β -TOCOPHEROL FROM WHEAT GERM OIL

By ALEXANDER ROBERTUS TODD, FRANZ BERGEL
AND THOMAS SPENCE WORK

From the Biochemical Department, Lister Institute, London

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THE isolation from wheat germ oil of two isomeric oily alcohols α - and β -tocopherol both showing vitamin E activity has been reported by Evans *et al.* [1936]. These workers purified the unsaponifiable matter of their oil by partition between pentane and methyl alcohol, followed by removal of sterols by freezing out. The concentrated oil thus obtained, active in a dose of 10 mg. on rats, gave on saturation with cyanic acid in benzene solution a mixture of allophanates from which three crystalline esters were separated (a) M.P. 250°, (b) M.P. 158–160° and (c) M.P. 135–138°. The ester, M.P. 250°, was thought to be β -amyrin allophanate, while (b)—the main product—gave on hydrolysis an oil, α -tocopherol, showing vitamin E activity in a dosage of 3 mg. and (c) yielded a somewhat less active oil, β -tocopherol, on similar treatment.

The tocopherols appeared to be isomeric monohydric alcohols of approximate formula $C_{29}H_{50}O_2$, the second oxygen atom being presumably present in an ether linkage. No yields were given.

Our early experiments on the isolation of vitamin E (see preceding paper) from rice and wheat, having convinced us of the inaccuracy of Kimm's [1935] results and of the impracticability of isolating the vitamin by *p*-nitrobenzoylation or naphthoylation of concentrates, we turned our attention to allophanation in the hope that the results of Evans *et al.* [1936] might be confirmed. At first we endeavoured to repeat the procedure described by these authors, but failed to obtain any definitely crystalline product other than the allophanate M.P. 256–257° (Evans *et al.* give M.P. 250°) derived from an alcohol $C_{30}H_{50}O$. This failure we ascribe to the fact that we were quite unable to obtain, by their method, concentrates from wheat germ oil having anything like the activity they reported. The purified oil of the American authors was active in a dosage of 10 mg.; our similarly treated concentrates were never active in a dosage less than 30 mg. It was therefore clear that further purification would be necessary before application of the allophanate isolation method.

We were able by hydrolysis of the oily esters left after removal of the tritosteryl *p*-nitrobenzoates from distilled wheat germ oil concentrates (see preceding paper) to obtain oils from which poor yields of β -tocopheryl allophanate and an allophanate M.P. 158–159° (apparently not identical with α -tocopheryl allophanate) could be obtained on treatment with cyanic acid. Similar poor yields were obtained by Drummond & Hoover [1937] from their wheat germ oil concentrates. We have since worked out a more satisfactory purification process for the unsaponifiable matter of wheat germ oil, which yields regularly oils showing vitamin E activity in a dosage of about 15 mg. Details of this process are given in the experimental section; it involves partition, chromatographic analysis and

precipitation of tritisterols with digitonin. The oils obtained have the property of reducing ammoniacal methyl alcoholic silver nitrate in the cold with formation of a silver mirror. This property may indeed be used during the later stages of the purification process to determine in which fractions the vitamin occurs, since it is always associated with vitamin E in our experience. On saturating benzene solutions of the active oils with cyanic acid a complex mixture of allophanates is obtained, from which products similar to those described by Evans *et al.* can be isolated.

For the isolation of β -tocopheryl allophanate and the allophanate of M.P. 256–257° simple fractionation with solvents is very unsatisfactory, being attended by considerable loss of material. The isolation of the crystalline allophanates is fairly readily effected, however, by taking advantage of the fact that they are much more strongly adsorbed on activated aluminium oxide (Merck) than the other constituents of the mixture. Elution of the adsorbates with a mixture of benzene, acetone and methyl alcohol, gives a somewhat sticky product from which the crystalline allophanate, M.P. 256–257°, can readily be separated by reason of its very sparing solubility in acetone. Crystallization of the more soluble portion from methyl alcohol yields a trace of an allophanate, M.P. 158–160°, corresponding in M.P. to the α -tocopheryl allophanate of Evans *et al.* [1936] and a very much larger amount of β -tocopheryl allophanate, M.P. 138–139°. Further crystallization of the latter substance yields a product, M.P. 143·5–144·5°, crystallizing in beautiful colourless laths (Fig. 1). A remarkable feature

Fig. 1. β -Tocopheryl allophanate ($\times 80$).

of our results is the virtual absence of α -tocopheryl allophanate from our products. From 250 g. of a concentrate of the unsaponifiable matter of wheat germ oil (fully active in a dose of 125 mg.) we obtained at most a few mg. crude " α -tocopheryl allophanate" of doubtful identity, and nearly 1 g. crude β -tocopheryl allophanate. There can be little doubt as to the homogeneity of our β -tocopheryl allophanate, which yields on hydrolysis an alcohol showing full

vitamin E activity in a dosage of 5 mg. It should be emphasized that this is not necessarily the minimum dose which will show activity; owing to the protracted nature of the biological test method the minimum dose for activity will be reported later.

After removal of the crystalline allophanates the residual oil was hydrolysed in a nitrogen atmosphere. The product did not reduce ammoniacal methyl alcoholic silver nitrate solution; moreover, it showed no vitamin E activity in doses of 50 mg. and 100 mg. In view of these facts we are of the opinion that "vitamin E" in our wheat germ oil concentrates consists almost entirely of β -tocopherol.

β -Tocopheryl allophanate appears to have the formula $C_{31}H_{52}O_4N_2$ or $C_{31}H_{50}O_4N_2$, i.e. it is the allophanate of a monohydric alcohol, β -tocopherol, $C_{29}H_{48-50}O_2$, in agreement with the results of Evans *et al.* [1936]. On the available evidence, of course, a C_{30} formula for β -tocopherol cannot be excluded. The allophanate is dextrorotatory in chloroform solution $[\alpha]_{5461}^{21} + 7.4^\circ$; we have examined several specimens at different stages of purification and from different isolations and think it unlikely that this rotation is due to impurity.

From certain fractions of the purified oils obtained in the course of our work we obtained by the action of cyanic acid a crystalline allophanate, m.p. $73.5-74.5^\circ$, which, from its analytical values, appeared to be the allophanate of a mono-unsaturated alcohol $C_{20}H_{40}O$ containing 3-4 side-methyl groups. Phytol being of common occurrence in nature it seems possible that this substance may be phytyl allophanate.¹

The allophanate of m.p. $256-257^\circ$ has not yet been thoroughly investigated. It derives from an alcohol of probable formula $C_{30}H_{50}O$; since β -amyrin allophanate has m.p. $272-273^\circ$ (Drummond & Hoover [1937] give m.p. 266°) it is unlikely that this alcohol is β -amyrin. We have not obtained the allophanate m.p. $230-232^\circ$ reported by Drummond & Hoover; this may have been derived from one of the tritisterols.

The chemical nature of β -tocopherol will form the subject of a later communication.

EXPERIMENTAL

Isolation of β -tocopheryl allophanate. As starting material we employed a concentrate of the unsaponifiable matter of wheat germ oil supplied by Messrs Glaxo Laboratories, Ltd., and showing vitamin E activity in a dosage of 125 mg. Throughout our experiments all operations involving heating above room temperature were carried out in a nitrogen atmosphere. The concentrate (250 g.) was dissolved in light petroleum (2 l., b.p. $100-120^\circ$) and washed with 92% methyl alcohol. Partition between light petroleum and absolute methyl alcohol was then carried out in the manner described by Evans *et al.* [1936], 5 l. of methyl alcohol (in portions of 500 ml.) being used. The oil obtained from the methyl alcohol fraction in the partition process was redissolved in absolute methyl alcohol (750 ml.) and allowed to stand at 0° during 24 hr. The dark brown semi-crystalline mass (40 g.) which separated was collected, and the filtrate yielded on evaporation a dark brown oil (58 g.). After setting aside 1 g. for various tests, the oil was divided into three equal portions and submitted to chromatographic analysis on activated aluminium oxide (Merck) using a column 35 cm. $\times$ 4.5 cm. diam. the solvent used being light petroleum ($40-60^\circ$). The chromatograms, after developing with light petroleum (ca. 5 l.), all had a similar

¹ Since the above was written we have established the identity of this substance with phytyl allophanate (m.p. 78°). Repeated recrystallization raises the m.p. to $77-78^\circ$, undepressed on admixture with synthetic phytyl allophanate.

appearance and, viewed in ultraviolet light, showed six distinct zones: (1) narrow, orange layer at top; (2) yellow; (3) sandy, strongly absorbent; (4) large white layer; (5) orange-yellow; (6) blue-fluorescent; the non-adsorbed material was also blue-fluorescent. The column in each case was divided into four sections each eluted separately with benzene-acetone-methyl alcohol (8 : 1 : 1): (A) zone 1 (2.5 cm.), (B) zones 2 and 3 (6 cm.), (C) zones 4 and 5 (14 cm.), (D) zone 7 and washings. The eluates from corresponding fractions were combined giving finally: (A) dark gummy oil (6.7 g.), (B) dark gummy oil (14.7 g.), (C) pale yellow oil (25.5 g.), (D) pale yellow oil (*ca.* 18 g.).

In view of the fact that a small loss was experienced in the above procedure, the aluminium oxide from section B in each column was extracted in a Soxhlet apparatus with acetone-methyl alcohol (1 : 1) and the extract (about 0.1 g. in each case) compared with the main eluate of the corresponding section. The loss due to incomplete elution is probably of no significance in the ordinary way. Sections A, B and C from the chromatogram were examined separately, section D being neglected as far as isolation of vitamin E was concerned.

Section A. This oil had little reducing power and was therefore not further examined for vitamin E. On standing in light petroleum (50 ml., B.P. 40–60°) for several weeks a red carotenoid colouring matter (15 mg.) separated. This substance, as yet unidentified, had m.p. 194° after recrystallization from benzene.

Section B. The oil (14.7 g.) was dissolved in methyl alcohol (100 ml.) and allowed to stand at room temperature during 24 hr.; some crystalline sterol (1.4 g.) was filtered off and the methyl alcoholic solution evaporated. The residual oil was dissolved in 90% alcohol (250 ml.) and the tritisterols removed by complete precipitation with digitonin (14 g. in all) according to Karrer & Salomon [1937]. The clear solution remaining after this treatment was evaporated to dryness *in vacuo*, taken up in benzene, filtered from digitonin and again evaporated. The residual brown oil (9.5 g.) showed vitamin E activity in a minimum dosage of 15 mg.

Allophanation of tritisterol-free oil from section B. The brown oil (9.0 g.) was dissolved in dry benzene (250 ml.) cooled to 0–5° and saturated with cyanic acid (from 15 g. cyanuric acid heated in a slow stream of CO₂). The benzene solution was kept at 5° for 48 hr., then heated to boiling and filtered from cyamelide, the latter being washed repeatedly with hot benzene. The combined filtrate and washings were evaporated and the residue triturated with cold acetone (100 ml.). A quantity of the allophanate, m.p. 256–257°, was left undissolved and was filtered off. The acetone solution was evaporated and the residual oil dissolved in light petroleum (B.P. 60–80°). On attempting to run this material through a column of activated aluminium oxide the column choked owing to separation of solid material; this difficulty may be overcome by using higher boiling petroleum (B.P. 100–120°), but the following method using the lower boiling solvent worked quite satisfactorily. The solution was shaken with a small quantity of activated aluminium oxide (Merck) (*ca.* 30 g.), filtered and then passed through a column of the same adsorbent (30 × 2 cm. diam.) and developed with light petroleum (B.P. 60–80°). The chromatogram showed three zones in daylight: (a) pale brown zone at top (12 cm.), (b) orange zone (3 cm.), (c) colourless zone (12 cm.). The column was divided into three sections (2, 3 and 4) from the top downwards, 7.5, 7.5 and 12 cm. long respectively, which were separately eluted with benzene-acetone-methyl alcohol (8 : 1 : 1). The alumina used in the preliminary treatment by shaking was also eluted (fraction 1). The weights of the eluted oils were as follows: (1) 3.7 g., (2) 1.65 g., (3) 1.25 g. Fraction (4) gave an

oil which was united with the non-adsorbed material and set aside as containing negligible amounts of active substance.

Fraction (1) was dissolved in methyl alcohol (50 ml.) and allowed to stand in the ice-chest. The brownish solid which separated was collected and stirred with cold acetone. A quantity of insoluble allophanate of M.P. about 250° was filtered off, the solution evaporated and the residue dissolved in warm methyl alcohol. On standing, a trace of a product separated having M.P. about 150–154° which was possibly α -tocopheryl allophanate. After filtering this off the mother-liquor deposited a yellowish crystalline mass (550 mg.), M.P. 137–142° (β -tocopheryl allophanate).

Fraction (2) gave on similar treatment a further quantity of β -tocopheryl allophanate (130 mg.), M.P. 137–140°, together with a little of the allophanate of M.P. ca. 250°.

The non-crystalline residues from sections (1) and (2) were combined, dissolved in light petroleum (B.P. 100–120°) and adsorbed on a column of activated aluminium oxide (30 × 2 cm. diam.). After developing with the same solvent the chromatogram was similar in appearance to the previous one, the upper pale brown zone being divided into two portions: (a) 6 cm. yielding 1.35 g. oil, and (b) 6 cm. yielding 1.35 g. oil. From (b) β -tocopheryl allophanate (250 mg.), M.P. 134–137°, was isolated by crystallization.

Section C. The oil (26 g.) dissolved in light petroleum (B.P. 40–60°) was submitted to chromatographic analysis on a column of activated aluminium oxide (Merck) (40 × 5 cm. diam.). After developing with ca. 3 l. of light petroleum the column—which showed seven distinct zones in ultraviolet light—was divided into four sections: (1) yellowish layer (3 cm.) not eluted, (2) absorbent (7 cm.) giving 4.45 g. viscous oil, (3) colourless (17 cm.) giving 12.9 g. limpid yellow oil, (4) orange-yellow and blue fluorescent (7 cm.) giving 6.05 g. yellow oil.

The oil from section (2), on standing in methyl alcoholic solution, deposited some crystalline sterol (0.15 g.); after removing this, tritisterols were removed with digitonin (4 g.) and the remaining oil (3.5 g.) treated with cyanic acid in the usual way and the allophanate mixture fractionated by chromatographic analysis; from the upper layers of the chromatogram β -tocopheryl allophanate (150 mg.), M.P. 137–142°, was obtained together with some allophanate of M.P. about 250°.

The oil (12.9 g.) from section (3) in the above chromatogram was freed from tritisterols with digitonin (5.3 g.) in the usual way and the residual oil treated with cyanic acid as before. From the oily product some allophanate (0.5 g.), M.P. about 250°, was separated by trituration with cold acetone. The acetone solution was evaporated and the residue dissolved in warm methyl alcohol. On cooling some gummy material separated; after filtering the mother-liquor was concentrated to about 50 ml. volume and left in the ice-chest. The oil which separated crystallized from a fresh quantity of methyl alcohol in small crystals (700 mg.), M.P. 70°.

The total yields of the various crude allophanates from 250 g. starting material in the above experiment were thus as follows: allophanate, M.P. ca. 250°, ca. 1 g.; α -tocopheryl allophanate (?), ca. 2 mg.; β -tocopheryl allophanate, ca. 1.08 g.; allophanate, M.P. 70°, ca. 700 mg.

α -Tocopheryl allophanate (?). The total available amount of this material was so small that it was impossible to identify it properly. It may have been identical with the substance of similar M.P. isolated from the wheat concentrates freed from crystalline tritisterols by acylation (see below). Recrystallized, it had M.P. 158–160°.

β -Tocopheryl allophanate. The combined crude allophanate isolated above had a rather indefinite m.p. 137–140° and separated from methyl alcohol in small needle- or rod-shaped crystals. It still contained a small amount of the allophanate, m.p. 250° (ca. 0.1 g.), which could be separated by renewed treatment with acetone. *β -Tocopheryl allophanate* of m.p. 139–140° is readily obtained by a simple recrystallization from methyl alcohol. Repeated recrystallization from methyl alcohol and acetone yields finally a product of m.p. 143.5–144.5° (Evans *et al.* [1936] give m.p. 138°) crystallizing in beautiful colourless laths (Fig. 1); the purification is, however, accompanied by considerable loss as the substance is rather soluble in methyl alcohol. As the crystallization mother-liquors yield, on working up, only further quantities of the same material, we are of the opinion that the material of m.p. 139–140° is already practically pure.

β -Tocopheryl allophanate analyses in agreement with a formula $C_{31}H_{50}O_4N_2$ or $C_{31}H_{52}O_4N_2$. (Found: C, 72.0; H, 9.9; N, 5.6%. $C_{31}H_{52}O_4N_2$ requires: C, 72.0; H, 10.2; N, 5.4%. $C_{31}H_{50}O_4N_2$ requires: C, 72.3; H, 9.7; N, 5.5%.) In chloroform solution ($c=2.2$; $l=2.2$) it is dextrorotatory, $[\alpha]_{5461}^{21} + 7.4$; various samples were examined and the lowest specific rotation observed was about +6°.

On oxidation with chromic acid (Kuhn-Roth method) *β -tocopheryl allophanate* gave 3.2 mol. acetic acid. It does not reduce ammoniacal methyl alcoholic silver nitrate solution. Hydrolysed in an atmosphere of nitrogen by heating for 45 min. with 4% methyl alcoholic potassium hydroxide it yields *β -tocopherol* as a slightly yellowish oil, which readily reduces ammoniacal methyl alcoholic silver nitrate. Tested on rats *β -tocopherol* (from allophanate, m.p. 139–140°) showed full vitamin E activity in a dosage of 5 mg. We are indebted to Dr E. L. Smith (Glaxo Laboratories, Ltd.) for the following spectrographic data: *β -tocopherol* in alcohol shows a single absorption maximum at 295 m μ ($E_{1\text{ cm}}^{1\%}=87$).

Allophanate m.p. 250°. Recrystallized from acetone the substance formed small colourless crystals, m.p. 256–257°. (Found: C, 74.7; H, 9.9; N, 5.5%. $C_{32}H_{52}O_3N_2$ requires: C, 74.9; H, 10.2; N, 5.5%.) It has not yet been further examined but does not appear identical with *β -amyrin allophanate* (m.p. 272–273°).

Allophanate m.p. 70°. Further recrystallization from methyl alcohol gave a product, m.p. 73.5–74.5°, separating in small matted granules. (Found: C, 69.8; H, 10.7%. $C_{22}H_{42}O_3N_2$ requires: C, 69.2; H, 10.9%.) Oxidation with chromic acid (Kuhn-Roth method) gave 2.6 mol. acetic acid (i.e. 3–4 side methyl groups). The substance decolorizes bromine instantaneously.

Oily allophanates. After removal of the crystalline allophanates all the oily fractions were combined and hydrolysed by refluxing in a nitrogen atmosphere with 4% methyl alcoholic KOH. The resulting oil had no reducing action on ammoniacal methyl alcoholic silver nitrate. Moreover, it showed no vitamin E activity in doses of 50 and 100 mg.

Preparation of crystalline allophanates from oils obtained after removal of tritosterols by p-nitrobenzoylation

The uncrystallizable oily esters (6.8 g.) left after removal of crystalline *p*-nitrobenzoates in the wheat germ oil isolation method I described in Part I (preceding paper) were hydrolysed by refluxing for 3 hr. with 7% methyl alcoholic KOH. The oil (5.0 g.) was then treated with cyanic acid and the resulting oily allophanate mixture fractionated by adsorption and crystallization in a manner similar to that described above. In this way were isolated (a) the allophanate of m.p. ca. 250° (100 mg.); (b) an allophanate of m.p. 158–159°, corresponding in appearance and m.p. to α -tocopheryl allophanate (ca. 8 mg.);

(c) β -tocopheryl allophanate, m.p. 138–139° (180 mg.), and (d) the allophanate of m.p. 70° (20 mg.).

Allophanate m.p. 158–159°. This material crystallized from methyl alcohol in small granules having an appearance similar to that described by Evans *et al.* for α -tocopheryl allophanate (m.p. 158–160°); the crystals, however, were anisotropic. The analytical values for this material indicate that it is not α -tocopheryl allophanate and is derived from an alcohol of approximate formula $C_{30}H_{50}O$. (Found: C, 75.0; 75.3; H, 10.2, 10.4%. $C_{32}H_{52}O_3N_2$ requires: C, 74.9; H, 10.2%.)

SUMMARY

1. β -Tocopheryl allophanate, m.p. 143.5–144.5°, has been obtained from the unsaponifiable matter of wheat germ oil by an improved method. It is optically active and analysis indicates a formula similar to that suggested by Evans *et al.* The parent alcohol, β -tocopherol, shows an absorption maximum at 295m μ .

2. The vitamin E activity of our concentrates appears to be due to β -tocopherol, which, when tested on rats, shows full activity in a dose of 5 mg.

3. Although traces of an allophanate of m.p. 158–160° were obtained, it is at least doubtful whether this is the α -tocopheryl allophanate of Evans *et al.* The high-melting allophanate of these authors (m.p. 256–257°) was also obtained, together with an allophanate of m.p. 73.5–74.5° derived from an unsaturated alcohol $C_{20}H_{40}O$.

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