CCLXXVI. STUDIES ON VITAMIN E

I. THE ISOLATION OF SOME CRYSTALLINE ALCOHOLS FROM THE UNSAPONIFIABLE MATTER OF RICE AND WHEAT GERM OILS

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THE unsaponifiable matter from various cereal germ oils is known to possess vitamin E activity, but the majority of investigators appear to have employed solely wheat germ as starting material in attempts to isolate the antisterility factor.

Chemical investigation of rice germ oil was first recorded by Kimm & Noguchi [1933] who identified melissyl alcohol, ergosterol, dihydrositosterol, γ -sitosterol and a new sitosterol, M.P. 156° , as constituents of the unsaponifiable matter from this oil. Later Kimm [1935, 1, 2] employed rice germ oil in an effort to isolate vitamin E. Following the procedure employed by Evans & Burr [1927] in experiments with wheat germ oil, he claimed to have obtained from the unsaponifiable matter an oil which when tested biologically by the Evans method on rats was active in a single dose of 3 mg. This oil on treatment with β -naphthoyl chloride in pyridine yielded four crystalline naphthoates: (a) M.P. 168° , (b) M.P. 156° , (c) M.P. 158° , and (d) M.P. 134° . Of these (b) was reported to yield on hydrolysis an alcohol showing full vitamin E activity in a single dose of 0.5 mg. Beyond the fact that these esters had colour reactions similar to those of sterol derivatives and that analysis suggested a formula $C_{29}H_{48}O$ for the parent alcohol of (b) no further details were recorded.

Some time later Evans *et al.* [1936] reported the isolation from wheat germ oil of two alcohols α - and β -tocopherol. These substances, themselves oils, were separated from the sterol-free unsaponifiable matter of the wheat germ oil as allophanates. The more active substance, α -tocopherol, characterized as its *p*-nitrophenylurethane and allophanate showed vitamin E activity in a dosage of 3 mg., and analysis indicated an approximate formula $C_{29}H_{50}O_2$ in which one oxygen was present in a hydroxyl group and the other perhaps in an ether linkage.

The relatively low activity of α -tocopherol was surprising in view of the results of Drummond *et al.* [1935], who by applying chromatographic analysis to the unsaponifiable matter of wheat germ oil, had earlier obtained vitamin E-containing oils active in dosages of 0.5–1 mg.

In view of these discrepancies in activity and particularly of the difference of an oxygen atom in the formula of "vitamin E" in rice and wheat, we decided to carry out a series of parallel investigations with rice germ and wheat germ oils with a view to the final isolation of the vitamin and elucidation of its structure. The similar order of activity recorded for the preparations of Kimm and Drummond *et al.* led us, in the first place, to concentrate mainly on the use of

the purification process of the latter authors followed by acylation with various reagents. The results of these earlier experiments, concerning which a preliminary note has already been published [Todd *et al.* 1937], are described in this paper.

A number of crystalline alcohols, including Kimm's alleged active alcohol, were isolated from the oils in this way, but they were found to be uniformly devoid of vitamin E activity when tested on rats by the method of Evans. During the progress of our work Karrer & Salomon [1937], reported the isolation from wheat germ oil of compounds of the same type, to which they gave the names α - and β -tritisterol. These authors separated the tritisterols from the unsaponifiable fraction of the oil by precipitating them from 95% alcoholic solution with digitonin, as amorphous, rather unstable digitonides. We have also employed this method in the cases of both rice and wheat with success, the same substances being obtained as by direct acylation without digitonin precipitation.

The compounds from rice, which we designate for convenience as orysterols, are very similar in their properties to the tritisterols of wheat, and indeed one of them may be identical with α -tritisterol. It is however difficult to be quite certain as to the identity of these substances, and in view of the fact that many supposedly homogeneous sterols have been shown to be mixtures we wish to record that, despite the available evidence, we consider it not impossible that the substances we describe may be shown by future work to be in reality mixtures of closely related compounds. As yet we have not investigated the detailed structure of any of the orysterols or tritisterols.

The properties of the various alcohols are described in the experimental part of the paper. All show similarities to the sterols and are characterized by a remarkable tendency to separate from solution in the form of gels even when highly purified; on standing the gels slowly change to a crystalline mass.

Orysterols from rice germ oil

The initial fractionation of the unsaponifiable matter of rice germ oil, after removal of most of the sterols by freezing out, was carried out by chromatographic analysis on activated aluminium oxide (Merck). The rice chromatograms resemble in their general characteristics those obtained from correspondingly treated wheat oils, but show a greater number of individual coloured bands, evidence that rice oil is a much more complex mixture than wheat oil. Further purification was effected by removal of normal sterols by digitonin precipitation and distillation of the oil in a high vacuum. We have found that similar results are obtained when distillation is omitted; vitamin E activity survives distillation and lower-boiling hydrocarbon impurities can be removed by this procedure. By acylation of the purified oils with *p*-nitrobenzoyl chloride or β -naphthoyl chloride we obtained mixtures of esters which could be fractionated to give apparently homogeneous compounds yielding on hydrolysis three alcohols which we provisionally designate α -, β - and γ -orysterols; particulars of these substances are given in Table I.

Table I

	M.P.	$[\alpha]_D$	<i>p</i> -Nitrobenzoate M.P.	β -Naphthoate M.P.
α -Orysterol	121–122°	+49°	187–189°	174–177°
β -Orysterol	113–114°	+51.3°	227–228°	166°
γ -Orysterol	119–120°	+51.9°	233–234°	157°

All these substances are unsaturated and have properties similar to those of the tritisterols of Karrer & Salomon [1937] and all are biologically inactive. They

appear to be isomeric alcohols of formula $C_{30}H_{50}O$, but, owing to the extreme difficulty of determining a molecular formula of this nature with complete accuracy, we consider that this formula must await fuller confirmation before it is finally accepted. β - and γ -orysterols as well as their esters show no definite depression in M.P. when mixed with each other although depression is always observed with α -orysterol. In our opinion, however, they are distinct substances, since the crystalline form of the β -naphthoate is quite distinct in the two cases and we were unable to find any evidence in favour of dimorphism as an explanation of the difference. γ -Orysteryl β -naphthoate appears from its M.P. and crystalline form to be the substance isolated by Kimm [1935, 2], but we are quite unable to confirm his claim that the parent alcohol has vitamin E activity.

Neither β - nor γ -orysterol shows a depression in M.P. when mixed with α -tritisterol (M.P. 113–114°) from wheat. Unfortunately we have had insufficient α -tritisterol as yet to permit of definite identification, but from the properties of α -tritisteryl β -naphthoate it seems at least possible that α -tritisterol and γ -orysterol may be identical. It is hoped in further work to settle this point and to ascertain the structure of the orysterols. These substances represent only a small fraction of the rice germ oil (0.12%). Work on the vitamin E-containing oils left behind after removal of the orysterols will be reported in a separate communication.

Tritisterols from wheat germ oil

As starting material for experiments on wheat germ oil we used a preparation of the unsaponifiable matter from which most of the sterols had been removed by freezing out. This material was purified by partition between light petroleum and methyl alcohol [Evans *et al.* 1936] followed by chromatographic analysis after the manner of Drummond *et al.* [1935] and Karrer & Salomon [1937]. Vacuum distillation of the purified oils was also employed as in the case of our rice experiments. Acylation with *p*-nitrobenzoyl chloride furnished a mixture of oily and crystalline esters. The latter were fractionated with great difficulty as the available quantity was small, but yielded finally on hydrolysis β -amyryn, α -tritisterol, M.P. 113–114°, and a small amount of a third alcohol, M.P. 174–175°, as well as an oily alcohol giving a nitrobenzoate, M.P. 185°. None of these substances showed biological activity. β -Amyryn was isolated by Drummond *et al.* [1935] from wheat germ oil and α -tritisterol was obtained by Karrer & Solomon [1937]. We did not obtain the β -tritisterol described by the latter authors, but this was probably due to the fact that the pre-treatment of our starting material had removed most of the tritisterols present originally, and that we obtained insufficient amounts to permit of complete separation of the various alcohols. The alcohol of M.P. 174–175° may possibly be identical with the alcohol of M.P. 162–163° obtained in traces by Karrer & Salomon [1937], but there was insufficient material to permit of proper characterization.

In one experiment we applied the digitonide isolation process of Karrer & Salomon to our oils but the only product we could identify with certainty was α -tritisterol.

EXPERIMENTAL

(a) Experiments on rice germ oil

Starting materials. In early experiments we used the crude oil extracted from rice germ with peroxide-free ether. This material was kindly placed at our disposal by Messrs Hoffmann-La Roche, Basle, to whom we wish to express our

gratitude. The same firm subsequently supplied us with the carefully prepared unsaponifiable matter from the oil, thereby greatly facilitating our experiments, as the saponification of the large amounts of oil necessary for the work was impracticable with the apparatus at our disposal.

Isolation method I for α - and β -orysterols. 2 kg. rice germ oil were hydrolysed (in 500 g. portions) by refluxing for 5 hr. with 15% methyl alcoholic KOH. After removal of the fatty acids by precipitation as barium soaps, the unsaponifiable matter was extracted with ether. In this way 37.9 g. of a brownish wax were obtained from which 22.7 g. of mixed sterols were removed by crystallization from methyl alcohol at 0°. From the remaining wax a further 2 g. of sterols were removed as crystalline digitonides by precipitation with digitonin from 90% alcoholic solution. The total yield of sterol-free oil was 13.2 g.

On submitting the residual oil thus obtained (11 g.) to distillation under a pressure of 0.1–0.2 mm. the following fractions were obtained: (a) 1.0 g., B.P. below 190°; (b) 7.1 g., B.P. 190–275°; (c) 2.4 g. distillation residue. From the boiling-point range fraction (b) was assumed to contain any vitamin E present [cf. for example Drummond *et al.* 1935].

Fraction (b) was dissolved in light petroleum (750 ml., B.P. 40–60°) and subjected to chromatographic analysis using a column of activated aluminium oxide (31 × 3.5 cm. diam.). The chromatogram was developed with light petroleum (B.P. 40–60°) containing a trace of benzene. Viewed in ultraviolet light, a number of distinct fluorescent layers were visible in the column; these were separately eluted with a mixture of methyl alcohol and peroxide-free ether (4:1). A considerable amount of a yellowish mobile oil (*ca.* 2 g.) was not adsorbed in the column. The various layers in the column starting at the top had the following characteristics: (1) yellowish brown 2.3 cm. thick giving 0.22 g. eluate, (2) absorbent and greenish fluorescent 11 cm. thick giving 3.74 g. eluate, (3) bright blue fluorescent 5.5 cm. thick giving 0.6 g. eluate, (4) blue fluorescent 11.5 cm. thick giving 0.33 g. eluate.

Fraction 2. The dark brown oil (3.74 g.) dissolved in pyridine (50 ml.) was refluxed for 2 hr. with *p*-nitrobenzoyl chloride (6 g.). After standing overnight, the solution was poured on ice and acidified with sulphuric acid. The somewhat sticky precipitate was collected, dried *in vacuo* over phosphorus pentoxide and extracted with boiling light petroleum (500 ml., B.P. 40–60°). The insoluble residue was now extracted with peroxide-free ether (500 ml.).

Ether extract. After removal of *p*-nitrobenzoic acid with alkali, drying and evaporation, the residue set to a mass of crystals, M.P. 223–224°. The ester, after several recrystallizations from acetone, formed colourless crystals, M.P. 227–228°. (Found: C, 77.7; H, 9.2; N, 2.9%. $C_{30}H_{49}O.C_7H_4O_3N$ requires: C, 77.2; H, 9.3; N, 2.4%.)

Hydrolysis of the above *p*-nitrobenzoate was effected by refluxing with 10% butyl alcoholic KOH during 2 hr., the use of this reagent being rendered necessary by the very low solubility of the ester in methyl alcohol. After removal of the butyl alcohol by steam-distillation the flocky precipitate was taken up in ether. Evaporation of the extract left a hard glassy mass, which could not be crystallized from alcohol, acetone or mixtures of these solvents with each other and with water; in every case a stiff gel was produced. Crystallization was finally effected from a mixture of alcohol (50%), water (10%) and acetic acid (40%). Once crystallized in this way β -orysterol could be further purified by recrystallization from absolute methyl alcohol. Even the purest samples, however, separate from solution initially as gels which slowly set to a mass of colourless needles.

β -Orysterol has m.p. 113–114°. (Found: C, 84.3; H, 12.3%; mol. wt. (Rast), 382. $C_{30}H_{50}O$ requires: C, 84.4; H, 11.8%; mol. wt. 426.) In alcoholic solution it is dextrorotatory, $[\alpha]_D^{25} + 51.3^\circ$, and shows only feeble general ultraviolet absorption. In the Liebermann sterol reaction, the free alcohol and its esters show only a reddish yellow colour which rapidly changes to brown, the solution acquiring a green fluorescence. With digitonin in 96% alcohol in the cold, an amorphous precipitate is formed which dissolves on warming. This digitonide breaks up into its components on boiling with absolute alcohol. Tested on rats β -orysterol had no vitamin E activity in a dosage of 5 mg.

β -Orysteryl β -naphthoate. The alcohol (60 mg.) dissolved in pyridine (5 ml.) was refluxed for 3 hr. with β -naphthoyl chloride (0.4 g.), and the solution, after cooling, poured on ice and acidified with sulphuric acid. The precipitate was collected, dried and extracted with boiling light petroleum (b.p. 40–60°). The extract was freed from acid by shaking with sodium carbonate, dried and evaporated. The residue contained, in addition to the desired ester, a considerable amount of β -naphthoic anhydride, m.p. 133–137°, which however was more soluble in acetone than β -orysteryl β -naphthoate; the latter crystallized from acetone or alcohol in colourless plates. (Found: C, 84.2; H, 9.7%. $C_{30}H_{48}O \cdot C_{11}H_7O$ requires: C, 84.4; H, 9.7%.)

A remarkable property of the ester is its behaviour on heating. In an ordinary melting-point apparatus it sinters slightly at 164° and melts sharply to a cloudy, liquid crystalline mass acquiring on further heating a red colour, which at 191° suddenly disappears with formation of a colourless transparent liquid. In thin layers (under the microscope) the appearance of the liquid crystalline phase is somewhat different; at 166° the melt has a lilac colour and on further heating becomes bluish green and strongly iridescent. In this state it is doubly refractive, the clear melt formed at 191° being isotropic. All these changes are observed in the reverse order on cooling and are common to the β -naphthoates of β - and γ -orysterols and of α -tritisterol (see later).

β -Orysteryl acetate. On heating β -orysterol for 90 min. with acetic anhydride, an acetate, m.p. 104°, was obtained which crystallized with difficulty from methyl alcohol.

Light petroleum extract. The extracted material was separated into a more soluble gelatinous fraction and a sparingly soluble fraction, m.p. 193–205° (consisting of a mixture of α -, β - and possibly also γ -orysterol). The residual oily material from the mother-liquors of these fractions was combined with ester from the chromatogram fraction 3.

α -Orysterol. The gelatinous, readily soluble fraction (1.13 g.) of the above extract was dissolved in a mixture of light petroleum and benzene (9:1) and adsorbed on a column of activated aluminium oxide (Merck), the chromatogram being developed with ca. 1 l. of the same solvent. The middle section of the column, characterized by strong ultraviolet absorption, was eluted with peroxide-free ether and yielded 0.95 g. of a slightly yellowish semi-crystalline mass. The small, brown, top layer of the column was discarded as were the blue fluorescent lower layer and the washings.

This semi-crystalline product was not homogeneous and yielded on crystallization from alcohol a rather gelatinous ester, m.p. 182–189°, as well as some uncrystallizable oil.

The above product (0.8 g.) was hydrolysed by refluxing for 2 hr. with methyl alcoholic KOH (10%), the solution diluted with water, extracted with ether and the extract dried and evaporated. The residual oil crystallized on standing. Recrystallization was difficult as the substance tended to separate from most

solvents as an oil. Crystallization was effected ultimately by slow concentration of an alcoholic solution at room temperature over phosphorus pentoxide. α -Orysterol was thus obtained as small colourless crystals, m.p. 121–122°, after slight sintering at 118°. (Found: C, 84.7; H, 11.6%; mol. wt. (Rast), 390. $C_{30}H_{50}O$ requires: C, 84.4; H, 11.8%; mol. wt. 426.) α -Orysterol gives with digitonin an amorphous precipitate which is moderately soluble in hot 96% alcohol. It shows only feeble ultraviolet absorption with no characteristic maxima, and in alcoholic solution is dextrorotatory, $[\alpha]_D^{25} + 49^\circ$. In the Liebermann test the substance gives a yellow colour which soon changes to brown, the solution at the same time acquiring a green fluorescence.

With one sample of material the colour in the Liebermann test was at first red and changed rapidly through blue to greenish brown, but this was probably due to an impurity.

α -Orysterol is unsaturated towards bromine or potassium permanganate and in biological tests it was found to be devoid of vitamin E activity in doses up to 5 mg.

The m.p. of α -orysterol is depressed on admixture with β - or γ -orysterol.

The acetate was obtained as an oil which has not yet been crystallized.

The small amount of oily material remaining in the crystallization mother-liquors of α -orysterol was also inactive in vitamin E tests (dosages up to 4 mg.). Esterification with *p*-nitrobenzoyl chloride in the usual manner gave an ester, m.p. 190–194°.

α -Orysteryl *p*-nitrobenzoate. Esterification of pure α -orysterol with *p*-nitrobenzoyl chloride in pyridine gave an ester, m.p. 187–189°, which tended to form gels from all solvents tried and was only crystallized from alcohol with great difficulty. (Found: C, 77.2; H, 9.0; N, 2.8%. $C_{30}H_{49}O.C_7H_4O_3N$ requires: C, 77.2; H, 9.3; N, 2.4%.)

α -Orysteryl β -naphthoate. On heating with β -naphthoyl chloride in pyridine, α -orysterol gave a β -naphthoate crystallizing from alcohol or acetone in needles, m.p. 174–177°; an unstable liquid crystalline phase could be obtained by supercooling the melt. (Found: C, 84.1; H, 9.0%. $C_{30}H_{49}O.C_{11}H_7O$ requires: C, 84.8; H, 9.7%.) Mixed with the β -naphthoates of β - or γ -orysterol marked depression in m.p. was observed.

Fraction 3. The oily eluate was *p*-nitrobenzoylated but the ester mixture could not be crystallized. It was combined with the oily nitrobenzoate obtained in the working up of fraction 2 (above), hydrolysed and β -naphthoylated. By direct crystallization of the product a naphthoate, m.p. 149–152°, showing a liquid crystalline phase (till 180°) on heating was obtained. The mother-liquors of this substance were submitted to chromatographic analysis on activated aluminium oxide (Merck) previously treated with phenol to reduce its alkalinity; in this way a naphthoate, m.p. 124–128°, was obtained. Owing to the small amounts, these products were not further investigated.

Isolation method II for β - and γ -orysterols. The starting material in this case was a preparation of the unsaponifiable matter of rice germ oil from which a large amount of crystalline sterol had been removed by crystallization. The oil showed full vitamin E activity in rats in a dosage of 125 mg.

The oil (25 g.) was dissolved in light petroleum (500 ml., b.p. 40–60°), filtered, adsorbed on a column of activated aluminium oxide (Merck) and developed with ca. 2 l. of the same solvent. The chromatogram was divided into three portions: (a) brown layer at top, (b) sand coloured main fraction, showing ultraviolet absorption, (c) lower yellowish layer. (a) and (c) were neglected as was also the non-adsorbed oil (6.2 g.). The main fraction (b) was eluted with

ether-methyl alcohol (1 : 4); on concentrating the solution obtained, a quantity of sterol (3.9 g.) separated and was filtered off (M.P. 120–130°). The filtrate at this stage had a volume of 220 ml.; it was divided into two equal portions which were worked up separately as follows:

A. The first portion ($\approx$ 12.5 g. starting material) was precipitated with digitonin (350 ml. of 1% solution in 90% alcohol). Only crystalline digitonides (3 g.) separated. On evaporating the filtered solution and extracting the residue with light petroleum an oil was obtained which distilled entirely at 230–260°/0.2 mm. (3.7 g.). The entire distillate was esterified with β -naphthoyl chloride in pyridine. The crude ester (ca. 2 g.) crystallized on trituration with acetone and was pressed thoroughly on a filter to get rid of oil.

Successive crystallizations from ethyl acetate, alcohol and acetone gave γ -orysteryl β -naphthoate, M.P. 157°. The mother-liquors from these crystallizations furnished on concentration and further purification β -orysteryl β -naphthoate, M.P. 166°.

γ -Orysterol. The above naphthoate, M.P. 157°, hydrolysed by refluxing with 10% butyl alcoholic KOH, yielded crude γ -orysterol, M.P. 104–106°. Like the β -compound γ -orysterol usually separates initially from solution as a gel which quickly crystallizes; crystallization was effected in the first instance from acetic acid-alcohol, and further recrystallization from methyl alcohol gave a final M.P. 119–120°. (Found: C, 83.7; H, 11.9%. $C_{30}H_{50}O$ requires: C, 84.4; H, 11.8%.) γ -Orysterol resembles β -orysterol closely in all its properties and no definite depression in M.P. is observed on mixing the two substances. In vitamin E tests on rats γ -orysterol showed no activity in doses up to 5 mg. In alcoholic solution it has $[\alpha]_D^{24.3} + 51.9^\circ$.

γ -Orysteryl β -naphthoate. The β -naphthoate (see above) has M.P. 157°, with liquid crystalline phase up to 187°. (Found: C, 84.6; H, 9.3%; mol. wt. (Rast), 520. $C_{30}H_{49}O.C_{11}H_7O$ requires: C, 84.8; H, 9.7%; mol. wt. 580.)

It crystallizes in needles and shows no clear depression in M.P. when mixed with β -orysteryl β -naphthoate (plates, M.P. 166°); despite numerous efforts, no evidence that these substances were dimorphous forms of one compound could be obtained.

γ -Orysteryl *p*-nitrobenzoate. Esterification of γ -orysterol with *p*-nitrobenzoyl chloride in pyridine gave an ester crystallizing from acetone in leaflets, M.P. 232–233°. (Found: C, 77.3; H, 9.1; N, 2.8%. $C_{30}H_{49}O.C_7H_4O_3N$ requires: C, 77.2; H, 9.3; N, 2.4%.) No depression in M.P. was observed on mixing with β -orysteryl *p*-nitrobenzoate (M.P. 227–228°).

B. The second portion of 110 ml. solution was evaporated to dryness and the residue distilled in a high vacuum. The main bulk of the oil distilled with some decomposition at 210–250°/0.1–0.2 mm.

The distillate (5.9 g.) was dissolved in hot 96% alcohol (100 ml.) and 1% digitonin solution (300 ml.) added, following the procedure used by Karrer & Salomon [1937] for the tritosterols. The crystalline sterol digitonides were filtered off from the hot mixture and the filtrate on cooling deposited a mass of amorphous digitonides which was collected and washed with absolute alcohol. By repeated concentration of the filtrate and addition of further amounts of digitonin (in all a further 5.7 g.) all the orysterols were precipitated.

The non-precipitable fraction, which we later found to contain all the vitamin E activity, was a brownish waxy mass which could not be crystallized, nor could the oil obtained from it on β -naphthoylation, despite repeated efforts at fractional adsorption and precipitation.

The above amorphous digitonides (8.1 g.) were decomposed by dissolving in

warm pyridine (30 ml.) and precipitating the digitonin with peroxide-free ether (350 ml.) [cf. Schoenheimer & Dam, 1933].

The digitonin-free solution was evaporated and the non-crystalline residue esterified with β -naphthoyl chloride in pyridine. From the mixture of crystalline esters produced β - and γ -orysteryl β -naphthoates could be separated as well as traces of a higher-melting ester (M.P. *ca.* 168°) which may have been largely α -orysteryl β -naphthoate.

Isolation method III. By using methods I and II described above, but omitting the distillation of the concentrates, the same substances were isolated.

(b) *Experiments on wheat germ oil*

As starting material for experiments on wheat germ oil we employed a preparation of the unsaponifiable matter from which most of the sterols had been removed by freezing out. This oil, which was kindly supplied by Messrs Glaxo Laboratories, Ltd., to whom we express our thanks, showed full vitamin E activity when tested on rats in a dosage of 125 mg.

The oil (125 g.) was dissolved in light petroleum (1 l., B.P. 100–120°), washed with 92% methyl alcohol and then partitioned between light petroleum and absolute methyl alcohol as described by Evans *et al.* [1936]. The methyl alcohol fraction was concentrated and freed from sterols as far as possible by allowing to stand at 0° for several hours and filtering off the precipitated solid. The resulting oil (35 g.), after removal of methyl alcohol, was distilled in a high vacuum (a) B.P. 160–210°; pale yellow oil (10 g.) and (b) B.P. 210–255°; orange viscous oil (19 g.). Fraction (a) consisted mainly of hydrocarbons and was neglected.

Fraction (b) (19 g.) was dissolved in 90% alcohol (1 l.) and the sterols giving crystalline digitonides (*ca.* 8 g.) removed by precipitation with 1% digitonin solution. The solution was evaporated, and the residual oil, freed from digitonin, dissolved in light petroleum (B.P. 40–60°) and adsorbed on a column of activated aluminium oxide (Merck). The chromatogram was developed with the same solvent, and when complete was similar in appearance to the analogous chromatograms of Drummond *et al.* [1935] and Karrer & Salomon [1937]. It was divided into four sections as follows and eluted with a mixture of benzene-acetone-methyl alcohol (8:1:1) this being found the most effective solvent for elution.

Fraction	Appearance	Eluate
1	Greenish yellow	Dark brown oil (1.2 g.)
2	Sandy	Pale yellow oil (3.0 g.)
3	Yellow	Pale yellow oil (4.2 g.)
4	Colourless	Colourless oil (3.5 g.)
Non-adsorbed	Colourless (3.1 g.)	—

The oil from fraction 2 was found to possess vitamin E activity in a dosage of 25 mg. For further work fractions 2 and 3 were combined.

Isolation method I. The above oil from the combined fractions 2 and 3 (4 g.) was esterified by refluxing (2–3 hr.) with *p*-nitrobenzoyl chloride in pyridine solution and the mixture was poured on ice and acidified with sulphuric acid. The precipitate was collected, dried, extracted with boiling light petroleum and the extract shaken with sodium hydroxide to remove acidic material. After drying, the light petroleum solution was concentrated to *ca.* 50 ml. vol.; on standing a quantity of crystalline material separated, and further concentration yielded a second crop. The combined crystalline fractions were recrystallized from acetone (fraction A). The oil left on evaporating the light petroleum mother-liquor was dissolved in a mixture of equal volumes of alcohol and acetone

(ca. 20 ml.). On standing, a crystalline ester separated and was recrystallized from alcohol (fraction B). A further quantity of this ester was obtained by dissolving the oily part in light petroleum and adsorbing on a column of activated aluminium oxide (Merck) previously treated with phenol to reduce alkalinity. The chromatogram was developed with light petroleum containing 2–3% of benzene; apart from a very small colourless fraction (eluate 0.1 g.) at the base and a narrow brown ring at the top (eluate 0.3 g.), the column consisted of two main fractions when viewed in ultraviolet light: (1) an upper colourless layer (eluate 1.3 g.) and (2) a lower highly absorbent layer (eluate 1.0 g.). A quantity of non-adsorbed material passing through the column was discarded.

The eluate from (1), on dissolving in alcohol and allowing to stand, gave a further quantity of fraction B, while the eluate from (3) left for a time in alcohol-acetone solution deposited a small quantity of a third crystalline ester (fraction C). The main bulk of oily esters remaining after removal of these three fractions could not be crystallized.

Fraction A. The substance (140 mg.) crystallized from acetone in colourless needles, m.p. 234–235°. (Found: C, 77.2; H, 9.3; N, 2.7%. $C_{30}H_{49}O \cdot C_7H_4O_3N$ requires: C, 77.2; H, 9.2; N, 2.4%.) It was found on hydrolysis that this apparently pure substance is really a mixture.

The ester was hydrolysed by refluxing with methyl alcoholic KOH in a nitrogen atmosphere. The product, which had the tendency to gel formation noted in the case of the orysterols could be separated by fractional crystallization from methyl alcohol into two components. The less soluble substance formed colourless needles, m.p. 174–175° (3 mg. from 60 mg. ester). On account of the small quantity available it was not possible to characterize the substance more closely or to purify it further. (Found: C, 83.0; H, 11.7%. $C_{30}H_{50}O$ requires: C, 84.4; H, 11.8%.) It showed no vitamin E activity in doses of 3 mg. The Liebermann colour test was similar to that of α -tritisterol.

The more soluble main fraction gave finally a crystalline alcohol, m.p. 113–114°, having the properties recorded for α -tritisterol. (Found: C, 83.7; H, 11.7%. $C_{30}H_{50}O$ requires: C, 84.4; H, 11.8%.) The acetate had m.p. 105–106° (Karrer & Salomon give m.p. 107–108°) and the β -naphthoate formed needles, m.p. 158–159° indistinguishable from those of γ -orysteryl β -naphthoate. Mixed with the latter compound or with β -orysteryl β -naphthoate no depression in m.p. was observed. The Liebermann test gave the same coloration as with the orysterols. No vitamin E activity was shown in doses up to 5 mg. α -Tritisteryl β -naphthoate has also been obtained by Drummond & Hoover [1937] from a wheat germ oil concentrate, although they failed to crystallize the parent alcohol, which they reported as being biologically inactive. They assume the identity of their product with the β -naphthoate of Kimm's active alcohol from rice.

Fraction B. The ester crystallized from alcohol had m.p. 213–215°. (Found: C, 77.1; H, 9.0; N, 2.8%. $C_{30}H_{49}O \cdot C_7H_4O_3N$ requires: C, 77.2; H, 9.3; N, 2.4%.)

This ester appeared not to be homogeneous, as hydrolysis afforded a small amount of a gelatinous alcohol in addition to a main product which, recrystallized first from alcohol and then from light petroleum, had m.p. 197°. (Found: C, 83.7; H, 11.6%; mol. wt. 426. $C_{30}H_{50}$ requires: C, 84.4; H, 11.8%; mol. wt. 426.) That this substance was β -amyrin was confirmed by a mixed m.p. with an authentic specimen (m.p. 199°). It yielded an acetate, m.p. 236–237°, undepressed by admixture with authentic β -amyrin acetate (kindly supplied by Dr F. S. Spring to whom we express our thanks).

Fraction C. By recrystallization from acetone an ester, m.p. 185°, was obtained. (Found: C, 77.6; H, 9.2%. $C_{30}H_{49}O \cdot C_7H_4O_3N$ requires: C, 77.2;

H, 9.3 %.) Hydrolysis of the ester gave an oily alcohol which had no vitamin E activity in doses of 5 mg.

Isolation method II. The remaining oil from chromatogram fractions 2 and 3 was dissolved in 96 % alcohol and the tritisterols precipitated with digitonin according to Karrer & Salomon [1937]. After decomposing the digitonides the sticky mass of alcohols was esterified with β -naphthoyl chloride in pyridine solution. By repeated fractionation of the crystalline ester, α -tritisteryl β -naphthoate was obtained as colourless needles, m.p. 158–159°, showing the liquid crystalline phase to 186° noted for γ -orysteryl β -naphthoate. (Found: C, 84.0; H, 9.3 %. $C_{30}H_{49}O \cdot C_{11}H_7O$ requires: C, 84.8; H, 9.7 %).

From the mother-liquors various fractions were isolated with m.p. ranging from 134 to 160° but no other sharp-melting product could be obtained.

SUMMARY

1. Methods are described for the isolation of three apparently homogeneous alcohols: α -orysterol, m.p. 121–122°; β -orysterol, m.p. 113–114°; γ -orysterol, m.p. 119–120°, from the unsaponifiable matter of rice germ oil. These substances appear to be isomeric and to have an approximate formula $C_{30}H_{50}O$.

2. By similar methods β -amyrin, α -tritisterol, m.p. 113–114°, and a third alcohol, m.p. 174–175°, were obtained from wheat germ oil.

3. All the substances isolated were devoid of vitamin E activity, the activity remaining in the uncrystallizable fractions of the oils.

4. The supposed "vitamin E" of Kimm [1935, 2] corresponds in its properties to γ -orysterol, which is quite inactive.

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