CCLIII. A NEW METHOD FOR THE ISOLATION OF α - AND β -TOCOPHEROLS

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MOST attempts to isolate the antisterility factor from various oils have made use of saponification as the first stage, thereby effecting a concentration, at least theoretically, of about fourteen times in the case of wheat germ oil. In view of the unexpected sensitivity of the tocopherols to alkali [Moss *et al.* 1938], it seemed to us that a method avoiding the use of the latter would provide information as to the substances responsible for the biological activity of the natural oil, and might also give a better yield. Our experience has been that there are considerable losses during the saponification, even when the treatment is carried out with special precautions.

When it became apparent that the hydroxyl group of the tocopherol molecules was phenolic, it seemed reasonable to think that these compounds might be present in the original oil in the free state although it had usually been assumed, for no very clear reason, that they were esterified. Accordingly, the simple experiment was tried of adsorbing the tocopherols directly from a solution of the original oil. It was entirely successful.

Adsorption of wheat germ oil on Merck's alumina from solution in light petroleum has been found to effect a concentration of the active factor of about fourteen times, both biological and spectroscopic evidence indicating that the fraction recovered from the column contains the whole of the activity of the original oil. The experiments were performed on wheat germ oils from various sources and the results obtained have been uniform both qualitatively and quantitatively.¹

The chromatogram shows three sets of bands. The first is a pinkish yellow system which moves rapidly down the column. The second is a yellow system which moves slowly downward and divides into upper and lower sections as more material becomes adsorbed; whilst the third is a deep orange yellow band which tends to stay near the top of the column. The tocopherols are associated with the second, slow-moving band system.

Since the great majority of the other constituents of wheat germ oil pass through the alumina column, it was found possible to use much larger amounts of oil for a given size of column than is the usual practice in the adsorption of unsaponifiable material. The maximum amount possible for a 45×4.5 cm. column was found to be 150 g. The limiting factor is the development of the chromatogram toward the bottom of the column, a process occurring more rapidly under the influence of a 10 % solution of oil in light petroleum than with the pure solvent. This circumstance is advantageous in that the usual prolonged washing with large volumes of solvent is unnecessary, the maximum possible

¹ Since this paper was submitted for publication our attention has been drawn by Prof. A. R. Todd to a German patent by the I.G. Farbenindustrie taken out on 30 Jan. 1936 which covers a process of extraction very similar to our own.

separation between the bands occurring during the feeding of the column with the solution. It is then only necessary to wash the column with a small volume of solvent until the filtrate is colourless and practically free from fat, a column of the size mentioned above requiring 300–500 ml.

Eluted separately with mixtures of ether, benzene and methyl alcohol, containing about 80 % of ether, the two yellow bands of the slow-moving system yield yellow oils of which approximately 93 % appears to be triglyceride and esterified sterol. Examined spectroscopically in alcohol, the unsaponifiable materials of these fractions exhibit selective absorption in the ultra-violet, the upper one having a maximum at 295 $m\mu$, and the lower at 292 $m\mu$. Below the lower of the two bands there usually appears, during the washing of the column, a reddish-pink band with which is associated a substance having an ultra-violet absorption with a maximum at 288 $m\mu$.

An attempt was then made to separate the tocopherol from the lower fraction by direct allophanation of the material eluted from the column. Spectroscopic examination before allophanation revealed selective absorption having a rather broad maximum between 286 and 289 $m\mu$. It is reasonable to suppose that this maximum represents the absorption at 292 and 288 $m\mu$ shifted toward shorter wave-lengths by the presence of unsaponified fat.

The usual procedure was adopted in the allophanation and after removal of the cyamelide and benzene, three crystalline compounds were isolated from the oil by freezing the solution, first in acetone and then in light petroleum. All three substances were transparent to ultra-violet light and were not further examined. The uncrystallizable residues were adsorbed on alumina from light petroleum, yielding an upper fraction showing absorption with a maximum at 281–283 $m\mu$; and a lower fraction having absorption with a maximum at 277–279 $m\mu$. Readsorption of the two fractions produced no apparent alteration in the physical properties of the mixtures. It is known from the work of John [1937] and of Bergel, Todd & Work [1938] that allophanation causes a shift in the position of the maxima of ultra-violet absorption of 8–9 $m\mu$ toward shorter wave-lengths, and it therefore appeared likely that the absorbing substances in these two fractions were the allophanates of compounds having absorption maxima at 288 and 292 $m\mu$; a hypothesis supported by the expected movement of these bands toward the near ultra-violet on alkaline hydrolysis. The hydrolysate of the lower fraction was re-allophanated and the maximum was observed to return to 279 $m\mu$. This substance is being further investigated.

Since the tocopherols of the oily fraction from the original chromatographic absorption seem to be susceptible of allophanation it seems certain either that the hydroxyl groups are free in the original oil, or that they are in combination so labile as to be broken by contact with alumina.

In view of the failure to isolate allophanates by direct treatment of the fraction containing unsaponified fat, it was decided to saponify before allophanation; the comparatively small bulk of the fractions from the column allowed of shorter and more careful treatment during hydrolysis than is possible in handling the larger quantities of the original oil.

It is always found that the upper and lower fractions are each contaminated with a small proportion of the other, so that it is advisable, though not essential, to readsorb the unsaponifiable material before allophanation. This readsorption serves in addition, to remove most of the sterol set free in the hydrolysis; the remainder being precipitated out, as far as possible, with digitonin.

From the material absorbing at 295 $m\mu$, β -tocopherol allophanate was isolated. The orange-coloured oil resulting from the allophanation, dissolved in

light petroleum (B.P. 40–50°), deposited on cooling to 0° a voluminous crystalline precipitate which was free from oil, and which carried with it only a small proportion of colour. Solution of this precipitate in a small volume of cold acetone removed a minute amount of an insoluble flocculent material which was probably a mixture of tritosterol allophanates. The small amount of colour remaining in the acetone-soluble fraction was then removed with blood charcoal from boiling methyl alcoholic solution. The product was recrystallized from anhydrous methyl alcohol, in which it had a very satisfactory temperature coefficient of solubility. At first the substance separated as small, compact, spherical aggregates, but after three recrystallizations as glistening feathery masses of lath-like crystals identical in appearance with those described by Todd *et al.* [1937], M.P. 144.5–145.5°. On anaerobic hydrolysis the allophanate yielded a colourless oil showing, in alcohol or hexane, an ultra-violet absorption with a maximum at $295\text{ m}\mu$ $E_{1\text{ cm.}}^{1\%}$ 82 ± 2 ; persistence 10.

From the lower fraction showing the absorption at $292\text{ m}\mu$, α -tocopherol allophanate was isolated. The flocculent material mentioned above was much more abundant in this fraction, separating along with the allophanate and a good deal of coloured matter from alcoholic solutions. Acetone was employed for the separation; the α -allophanate being moderately soluble and the flocculent material almost entirely insoluble. The same solvent was used for the subsequent recrystallizations, as it was found to keep the oily contaminants and colour in solution in a very satisfactory manner. The final product had M.P. 158.5–159.5°, and gave on hydrolysis a colourless oil which in hexane had selective ultra-violet absorption with the double maximum at $292\text{ m}\mu$ and $298\text{ m}\mu$ as shown in the curve published by Emerson *et al.* [1937] with $E_{1\text{ cm.}}^{1\%}$ $298\text{ m}\mu$ 80 ± 2 ; persistence 10. In solution in ethyl alcohol the curve showed only a single peak at $292\text{ m}\mu$; $E_{1\text{ cm.}}^{1\%}$ 70 ± 2 .

In contrast to the findings of Evans *et al.* [1936] since confirmed by other workers, the α -tocopherol allophanate isolated by the above process was found to show a small optical activity, $\alpha_D^{25} = +4.8$. As it is likely that the α -tocopherol of the original oil is optically active, becoming racemized under the somewhat drastic conditions of the usual saponification, this figure may represent an incomplete racemization under the milder conditions employed for the saponification of our concentrates.

From 2 kg. of wheat germ oil approximately 1 g. of α -tocopherol allophanate and 0.75 g. of β -tocopherol allophanate were isolated.

In a previous paper, from the results of film measurements we postulated the existence in the molecule of a third ring. This was incompatible with the evidence on structure provided by the recent work of Fernholz, Karrer and Todd, from which it seems certain that both α - and β -tocopherols consist essentially of trimethyl-coumaran or -chroman rings bearing hydrocarbon side chains. We have been considerably disturbed by the discrepancy, particularly because the examination of a tocopherol prepared by Dr A. R. Todd and his associates which Danielli recently reported, indicated the area of 30 sq. Å., apparently more compatible with the simpler ring structure.

We were alive to the possibility that some of the preparations previously examined in our laboratory might contain traces of impurities or oxidation products which might give misleading measurements indicating a larger area; and we therefore examined the allophanates and tocopherols described in this paper, having confidence in their purity. To our surprise we obtained values of about 60 sq. Å. for all four compounds. Samples of pure α - and β -tocopherol allophanates, obtained through the kind co-operation of Dr A. R. Todd also

gave measurements indicating areas of about 60 sq. Å. Dr Danielli then very kindly re-examined for us the synthetic compound 5-hydroxy-4:6:7-trimethyl-2-*n*-heptadecylcoumarone for which data had previously been reported by him in a footnote to the publication of Bergel, Jacob *et al.* [1938]. He obtained the value of 51 sq. Å. Hence it appears that the second coumaran or chroman ring is pulled down into the surface, increasing the area to that of a 2-ringed structure. The oxygen atom of these rings must possess a much greater attraction for water than has hitherto been suspected.

Reviewing the foregoing findings we are satisfied now that the film measurements are compatible with the types of formulae recently suggested.

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