CCCI. FURTHER OBSERVATIONS ON THE CONSTITUENTS OF THE UNSAPONIFIABLE FRACTION OF WHEAT GERM OIL WITH PARTICULAR REFERENCE TO VITAMIN E.

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The hydrocarbons of wheat germ oil.

In a previous paper [1935] we described hydrocarbons separated from wheat germ oil unsaponifiable matter in fractions which either were not adsorbed or but slightly adsorbed by aluminium oxide from solution in light petroleum. One fraction seemed to consist mainly of a compound of molecular weight about 250, B.P. 120–140°/0.05 mm., probably saturated.

Larger quantities of this material have now been prepared and examined. When first obtained the iodine values of this fraction are usually about 30–40, but that a highly unsaturated contaminant is present is shown by bromination in dry ethereal solution at -18° when an insoluble bromide is deposited. The properties of this derivative suggest its identity with the bromide of the hydrocarbon ($C_{45}H_{76}$ or $C_{50}H_{84}$) isolated from mammalian livers by Channon and Marrian [1926] and Channon *et al.* [1934]. Considerable amounts of this highly unsaturated hydrocarbon are found in an adjacent wheat germ oil fraction. Channon *et al.* [1934] believe that under appropriate conditions the preparation of the bromide is almost quantitative and that it is not significantly inhibited by the presence of other substances. Bromination of our fractions under the conditions they describe did not remove more than half the unsaturation. Other attempts to separate the unsaturated contaminant, such as by partition between solvents, were no more successful and we were seldom able to reduce the iodine value below 15–20. Efforts were then made to purify the saturated hydrocarbon fraction by distillation.

In every case the course of the distillation indicated that the material was a mixture. Unfortunately the amounts available were insufficient to permit satisfactory refractionation, but strong indications were obtained that two hydrocarbons predominated. Thus in one redistillation of 4.3 g. at 0.03 mm. three fractions were obtained: (i) 118–130°, 1.4 g.; (ii) 130–150°, 0.12 g.; (iii) 150–165°, 1.6 g. Several preparations of the lowest-boiling fractions were obtained in the form of a colourless limpid oil which solidified just below zero. A number of the elementary analyses were unsatisfactory as it was found very difficult to prepare material entirely free from an oxygen-containing impurity. Four acceptable analyses gave the figures: C, 84.89, 85.17, 85.18, 85.45%; H, 14.85, 14.72, 14.81, 14.42%; Mean values. C, 85.12%; H, 14.45%; mol. wt. 254, 274, 261, 251. Mean 260.

These figures would satisfy the formula $C_{18}H_{38}$ (mol. wt. 254; C, 85.04%; H, 14.96%). We thought at first that the substance might be identical with

isooctadecane (pristane) isolated by Toyama [1923] from a number of shark-liver oils; an interesting possibility because of association in both instances with hydrocarbons of the squalene type. There are however differences which seem to point to the compounds being distinct. For example, our hydrocarbon has a refractive index n_D^{40} 1.4722, higher than that recorded for pristane. The purified hydrocarbon is optically inactive and shows no absorption bands in the ultra-violet. The bands recorded for the impure preparations described in our earlier paper are now known to be due to traces of substances of low volatility, probably of the sterol family.

The higher-boiling material (iii) of this hydrocarbon fraction has also proved difficult to free from oxygen-containing impurities sufficient in amount to disturb the analytical figures. The fractions were all optically inactive and spectroscopically transparent.

Four satisfactory analyses gave the figures: C, 86.48, 86.45, 86.53, 86.39 %; H, 13.21, 13.51, 13.34, 13.23 %; mol. wt. 261, 274, 260, 294. Mean values. C, 86.46 %; H, 13.32 %; mol. wt. 272; n_D^{40} 1.4729. These fractions invariably gave iodine values of the order of 30–40. It is uncertain at present whether this material is a saturated hydrocarbon contaminated with a small proportion of a highly unsaturated hydrocarbon which, under the conditions employed by us, is not precipitated on bromination, or whether a considerable proportion of a less unsaturated compound is present. In this connection the observations of Channon *et al.* [1934] on the presence in pig-liver of hydrocarbons of rather similar character are of interest.

The highly unsaturated hydrocarbon fraction (B) previously separated by us from wheat germ oil unsaponifiable has also been further studied. The fraction is that which can be washed out of the aluminium oxide column by light petroleum and it is characterised by high iodine values, 180–240. In our former study we detected squalene in this material and also thought that it might contain the hydrocarbon ($C_{45}H_{76}$ or $C_{50}H_{84}$) which Channon and Marrian [1926] first isolated and which appears to be of the squalene type. This we can now confirm. The amounts of squalene seem to vary in different specimens of wheat germ oil but in most cases they are small compared with those of the other hydrocarbons. In some cases indeed it was difficult to separate the characteristic hydrochloride of the former compound. The fraction as a whole usually gave a yield of hydrochloride or bromide corresponding to about 25 % of its weight as $C_{45}H_{76}$. Both hydrochloride and bromide are micro-crystalline and the latter is unusually difficult to purify. Typical analyses are:

Hydrochloride. m.p. 116–120°; Cl, 31.56, 31.34, 31.16, 31.39 %. Calculated for $C_{45}H_{76}, 8HCl$; Cl, 31.2 %.

Bromide darkening about 120° and becoming black with decomposition at 160–180°. Br, 67.7, 67.34, 67.54, 67.4 %. Calculated for $C_{45}H_{76}, 16Br$; Br, 67.7 %.

It is doubtful whether the unsaturation shown by fraction B can be entirely attributed to these hydrocarbons. In the first place, in view of the fact that Channon and his colleagues obtained quantitative precipitation of $C_{45}H_{76}$, our yields of bromide are too small. Moreover, as was tentatively suggested in our former paper, there is evidence of the presence of rather highly unsaturated substances containing oxygen in these fractions. Fractions readily soluble in methyl alcohol have been prepared. These have iodine values as high as 200 and are now being investigated.

From a number of examinations of wheat germ oil unsaponifiable we estimate that the saturated C_{18} and the unsaturated $C_{45}H_{76}$ hydrocarbons are present to the extent of approximately 3 and 7 % respectively.

The vitamin E fraction.

Larger quantities of this fraction prepared as described in our earlier paper have been investigated. We have confirmed the occurrence of β -amyrin, but find that the amount associated with the vitamin may vary considerably with preparations from different oils. It is without effect when administered to vitamin E-deficient rats.

A new sterol.

The vitamin fraction contains a sterol-like substance which does not appear to have been described before. It escaped notice in our previous investigation because we unwisely assumed that the material removed from the vitamin fraction by precipitation with digitonin was the common "sitosterol mixture", of which so much is found in the original unsaponifiable matter. Having in this case a larger amount of the digitonide precipitate the substance in question was recovered by xylene treatment. With a reservation prompted by knowledge of the great difficulty of purifying small quantities of sterols we are inclined to believe that it consists almost entirely of one compound. Crystallised from ethyl or, more satisfactorily, methyl alcohol, it separated as a curious gelatinous mass; a behaviour recalling that of lanosterol. On microscopic examination the solid was found to consist of very minute spherulites of needle-shaped crystals. Recrystallisation did not raise the melting point beyond 105–106°, but the amounts did not permit exhaustive fractionation. It was optically active $[\alpha]_D^{20} + 58.5^\circ$ (benzene) and spectroscopically transparent. The acetate, which crystallises more satisfactorily in clusters of fine needles from methyl alcohol solution, melts at 98–100° and shows $[\alpha]_D^{20} + 65.7^\circ$ (benzene).

The dinitrobenzoate darkened at 90°, contracted at 130°, and finally melted fairly sharply at 156–158°; $[\alpha]_D^{20} + 56.9^\circ$ (benzene).

The mean figures given by seven determinations of elementary composition were: C, 81.66%; H, 11.4%; mol. wt. 425; Acetate: C, 78.86%; H, 10.52%; mol. wt. 462.

These figures correspond fairly well with a compound of formula $C_{28}H_{48}O_2$, (C, 81.3%; H, 11.21%; mol. wt. 428), giving a monoacetate (C, 79.15%; H, 10.64%; mol. wt. 470). The values obtained in determinations of acetyl groups also confirm that one is present. The iodine value found, 109, points to two ethylenic bonds; calculated value 118. We are, however, not entirely satisfied with the formula suggested.

The behaviour of this compound with some colour reagents for the sterols recalls that of lanosterol. *Salkowski*; after a time the acid layer acquires a pale orange-yellow colour with green fluorescence; *Liebermann-Burchard*; slow development of brown-red colour with strong green fluorescence; *Whitby's B*; slow development of pinkish red colour; *methyl sulphate*; no colour in the cold but a pale brownish red on gentle warming; *benzoylperoxide*; pale straw-yellow with very slight green fluorescence.

We are investigating the chemical nature of this interesting compound further but meanwhile it is important to record that it was found of no value in counteracting vitamin E-deficiency in female rats.

Further investigation of vitamin E.

In our previous study we described the preparation from this fraction of concentrates of the vitamin of which the most potent protected rats in daily doses of 0.1 mg. Evans and Burr [1927] showed that the physiological effect of a single dose of a vitamin E concentrate given at, or shortly after, the time of positive mating is indistinguishable from that produced by the same amount given in a

number of doses spread over the whole period of pregnancy. Our own experiments support this view. The total dose of the concentrate referred to can therefore be placed as 2 mg. In the present communication all dosages given are "total" doses.

It was reported that the active fractions showed a well marked absorption with a maximum at $294 m\mu$ and a minimum at $267 m\mu$, and that during fractionation and concentration the vitamin activity tended to run parallel with the intensity and persistence of the band. From the behaviour on acetylation it was tentatively suggested that the substance giving rise to the band might be a cyclic ketone capable of showing keto-enol change.

Attempts were therefore made to separate ketonic material from the concentrate by employing the methods of Girard [1933; Girard and Sandulesco, 1934] and of Beall and Marrian [1934]. As reported in our previous paper the ketonic fractions separated in this manner did not show the selective absorption in the ultraviolet. We can now report that they did not exercise the biological activity of vitamin E.

During the examination of these fractions for the presence of ketonic substances it was observed that they gave positive responses to colour tests such as that with *m*-dinitrobenzene [von Bittó, 1897; Zimmermann, 1935]. Efforts were therefore made to isolate an oxime or a semicarbazone but without success, although definite evidence of combination with these reagents was obtained. As far as could be judged about 8% of the material combined and it is interesting that the ketonic fractions separated by the use of the reagent of Girard and of Beall and Marrian represented about the same proportion (8–10%).

With 2:4-dinitrophenylhydrazine sulphate in acid alcohol a small quantity of a scarlet coloured derivative was obtained but the amount was only sufficient for one recrystallisation from methyl alcohol and for one elementary analysis. The product was micro-crystalline and its colour indicated an unsaturated ketone. It melted at $118-120^\circ$ and had C, 69.92%; H, 8.46%; N, 9.43%. These values would correspond with a molecule of the cholestenone type.

The material left behind after separation of the dinitrophenylhydrazone and removal of the excess of reagent showed the characteristic band at $294 m\mu$, although in diminished intensity. These observations were sufficient to dispel the view that the substance responsible for the band contains an active ketonic grouping. In attempting to carry the concentration further the next step was to remove the β -amyirin, which is often rather closely associated in adsorption experiments with the vitamin fraction. In the earlier experiments this was effected by acetylation, β -amyirin acetate being so sparingly soluble in alcohol that separation is almost quantitative. It was found however on hydrolysing the residue by means of hot alcoholic potash that the intensity and persistence of the band at $294 m\mu$ were lower than before acetylation. There was also evidence that the biological activity had been reduced. This observation occasioned surprise because in our experience we had not found the vitamin to be injured by such treatment, recovery from the products of saponification of wheat germ oil having always been satisfactory. Recently however Evans *et al.* [1935] have published convincing evidence that treatment with hot alkalis may under some conditions inactivate the vitamin; previous work in their laboratory had aroused a suspicion that unexpectedly large losses sometimes occurred during saponification [Evans, 1932].

Instead of removing β -amyirin by acetylation therefore we had recourse to fractional distillation at low pressure (<0.0001 mm.). For this we employed an apparatus originally designed for the separation of vitamin A [Heilbron *et al.*, 1932]. Although this apparatus enables satisfactory fractionations to be carried

out it failed to give the desired results in that investigation because the vitamin A molecule underwent a change, probably, it is thought, of the nature of a cyclisation as a consequence of exposure to a relatively large surface of heated glass.

In the present case we at first concluded that no such change occurred from the fact that satisfactory concentrations of the substance responsible for the $294m\mu$ band were obtained.

The course of the distillation and the yield of the various fractions indicated that there was no appreciable loss of the substance responsible for the band.

Stage of fractionation	$E_{1\%}^{1\text{ cm.}}$		Ratio
	$294m\mu$	$267m\mu$	
2nd adsorption on Al_2O_3	33	15.5	2.1
1st distillation	39	10.7	3.65
2nd distillation	55	15.2	3.6
3rd distillation	59	17.0	3.5

Three such concentrations by distillation have been carried out with sufficient material to enable a separation to occur and in each case the final product has had approximately the same spectroscopic properties. $E_{1\%}^{1\text{ cm.}}$ $294m\mu$ ca. 55. Ratio $E_{1\%}^{1\text{ cm.}} \frac{294}{267} = 3.6-3.8$.

As this attempt to isolate the absorbing substance was being made in the belief that it was probably the vitamin these results seemed to indicate that success might be near. The results of the biological tests were however disconcerting as the following results will illustrate.

Fraction	$E_{1\%}^{1\text{ cm.}}$		Ratio	Total rat dose mg.	Activity
	$294m\mu$	$267m\mu$			
45	30	16.6	1.8	2	+
				1	+
76/2	40	10.8	3.7	3	+
				2	+
				1	+
				0.5	+
72/c	43	11	3.9	2	+
				1	?
80/3	55	15.2	3.6	2	-
				1	-
				0.5	-

Fractions 45 and 76/2 represent stages in the concentration by means of adsorption on aluminium oxide; 72/c and 80/3 further concentration by distillation. As 4-6 animals were used in the testing of each dosage these results make it impossible to retain the view that the vitamin E potency can be measured by the intensity of absorption at $294m\mu$. Olcott [1934] has already cast grave doubts on the implied significance of the band because he finds (a) that the maximum is shifted by acetylation and entirely disappears after oxidation with silver nitrate without a corresponding loss of biological activity, and (b) that concentrates prepared from palm oil may show the band without being of any value as a source of the vitamin [1935].¹

The possibility of loss as a result of distillation will be referred to in the discussion (p. 2520).

¹ Additional evidence against accepting a relation between the band and vitamin potency has recently been provided by Olcott [1935, 2]. It must be pointed out that measurements of $E_{1\%}^{1\text{ cm.}}$ $294m\mu$ alone may be misleading. We have stressed the importance of measuring $E_{1\%}^{1\text{ cm.}}$ $267m\mu$ at the same time [1935].

Properties of vitamin E fractions.

The most potent fractions we have prepared have been those corresponding with the highest concentration effected by adsorption on aluminium oxide of the substance showing the 294 $m\mu$ band. These preparations have evoked positive responses in vitamin E-deficient does when given in doses of 1–0.5 mg. In view of the fact that distillation at very low pressures separates such materials into two or possibly three fractions, in one of which there is a corresponding concentration of the 294 $m\mu$ -absorbing substance but in none of which has a similar concentration of the active substance been demonstrated, it is necessary to proceed with great caution in attempting to throw light on the properties of the vitamin by a study of the properties of the fractions themselves. The original investigations of Evans and Burr [1927] first indicated that the vitamin is an alcohol, and additional evidence in support of this view has since been recorded [Olcott, 1935, 2]. To it can now be added an observation made during an attempt to adsorb the vitamin directly from wheat germ oil by passing solutions in light petroleum through columns of aluminium oxide. As would be expected, the adsorbent removed uncombined sterols and hydroxylated lipochromes, amounting in all to about one-third or less of the unsaponifiable matter, but it failed to retain the vitamin. This might be interpreted as indicating that the vitamin is present as an ester in the oil.

Hydrogenation. We have repeatedly confirmed that vitamin E concentrates can be hydrogenated without loss of biological activity. Olcott [1934; 1935, 1, 2] has reported that hydrogenation did not fully saturate these materials. This also we can confirm, although in our experience it is possible to effect considerable reduction of the iodine value without using such drastic methods as those which he employed. By treatment with hydrogen at 3 atmospheres at room temperature in the presence of Adam's platinum oxide catalyst concentrates of vitamin E will take up hydrogen, not rapidly it is true, but steadily, so that an original iodine value of 170 or 180 falls to about 55 in 48 to 72 hours. Beyond this we have not succeeded in carrying the reduction. The time of treatment is longer than that mentioned in our earlier paper; it will be recalled that we then reported reduction to iodine values as low as 26. It has been found that the different behaviour of these preparations was due to the presence of a readily reducible component unrelated to the vitamin. The reduced compound can be separated as a white, waxy, crystalline substance and is fully saturated. Enough was not obtained to purify it satisfactorily but the crude material once recrystallised from methyl alcohol melted between 67° and 70°. This crystalline material did not separate from the more potent concentrates hydrogenated in the course of the present investigation. It is worth recording that in our experience vitamin E concentrates are highly resistant to hydrogenation when much impurity is present, particularly so when contaminated with the resinous products derived from the saponification of the original oil. Thus, for example, concentrates from unsaponifiable matter prepared industrially will often not reduce at all until freed from the dark coloured resinous contaminants, preferably by distillation.

Until there is evidence to show what proportion of these concentrates is represented by the vitamin it cannot even be assumed that a fall of iodine value on hydrogenation indicates a reduction of the active substance. The same reservation applies to Olcott's [1934] interesting observations that the vitamin will survive treatment of concentrates with chlorine and bromine and subsequent removal of the halogen by means of zinc and acid. If however these reactions

cover the vitamin itself the results indicate the presence of two reactive ethylenic groupings. Our observations of the behaviour of the vitamin in adsorption columns and Olcott's experience with oxidising reagents [1934] might also support the idea that the molecule is unsaturated to this extent. The possibility of the vitamin being of the same general chemical nature as the substance responsible for the selective adsorption at $294m\mu$ will be referred to later (p. 2520).

Vitamin E in other vegetable oils.

(a) *Cottonseed oil.* Olcott [1934] has found that crude, refined and even hydrogenated cotton-seed oils contain vitamin E in amounts sufficient to make these raw materials as satisfactory as wheat germ oil for the preparation of concentrates. This is supported by our investigation of a sample of unrefined cotton-seed oil of Brazilian origin.¹ From 35.3 g. of unsaponifiable matter, after removal of most of the solid sterols by freezing a methyl alcoholic solution, there were obtained as a result of two fractional adsorptions on aluminium oxide 4 g. of a concentrate of surprisingly high biological potency. Two of three rats treated with a single dose of 0.5 mg. produced normal litters.

This material was then distilled at 0.0001 mm. and gave the following fractions:

Fraction	Wt. g.	Temp. °C.	n_D^{20}	I.V.	$E_{1\text{cm.}}^{1\%}$	
					294 $m\mu$	267 $m\mu$
77/1	0.007	50	—	—	—	—
77/2	1.16	120–140	1.4925	—	Transparent	
77/3	1.12	140–160	1.4977	94.5	47	15
77/4	1.41	160–180	1.4950	68.0	59	18

The separation of the fractions was quite sharp. The most volatile (77/1) was a minute amount of crystalline material which collected at the coolest end of the tube. Unfortunately, there was not sufficient for a test on rats, so the possibility that it was active cannot be dismissed but, on the other hand, in view of what is known of the behaviour of the vitamin on distillation it is most improbable that activity would have been shown by so volatile a compound. Fraction 77/2 was a white crystalline solid of low melting point. It crystallised from methyl alcohol fairly well at 0° but proved difficult to separate satisfactorily. The melting point was about 28°. It appeared to be an alcohol of mol. wt. about 270 giving a liquid monoacetate, but its examination is incomplete. It was spectroscopically transparent and administered to rats in doses of 2 mg. failed to prevent typical resorptions. Fractions 77/3 and 77/4 showed the $294m\mu$ band to a degree that encouraged us at the time to expect good potency. Doses of 1 mg. proved inactive, this being another example of unexpected loss of potency as a result of distillation. Attempts are now being made to ascertain whether reconstituting the former mixture will restore the biological activity.

The properties of fraction 77/3 were very similar to those of the corresponding fractions from wheat germ oil and they will be referred to again in the next section of this paper.

(b) *Linseed oil.* A sample of unrefined oil gave 1% unsaponifiable matter. After removal of the bulk of the sterols by recrystallisation from methyl alcohol the residue was fractionated by adsorption from light petroleum solution on aluminium oxide. The "vitamin E fraction" was inactive in rat tests in doses of 4 mg. The material was spectroscopically transparent. It may be of interest to

¹ We wish to thank Mr J. Hanley, F.I.C., of Messrs Bibbys Ltd., Liverpool, for this material and for other samples of vegetable oils investigated.

record that a highly unsaturated hydrocarbon of the squalene type is present in linseed oil. Good yields of an insoluble bromide were readily prepared from the fraction which was not retained by the aluminium oxide. Evans and Burr [1927] showed that flaxseed oil is not a source of vitamin E.

(c) *Sunflower seed oil*. This specimen gave about 0.6% of unsaponifiable which was fractionated in the usual manner. No biological activity was found in doses of 4 mg., nor was selective absorption at $294m\mu$ detected.

The substance responsible for selective absorption at $294m\mu$.

Although discrepancies to which Olcott [1934; 1935, 1, 2] has drawn attention and others which are described in this paper have greatly weakened and may in some minds have entirely dispelled the view that the compound responsible for the $294m\mu$ band is the vitamin, further investigation of its nature is of importance because frequent association with the active substance during fractionation may be significant. In our former publication the maximum intensity for this band recorded was $E_{1\text{cm.}}^{1\%} 294m\mu = 49$; that of the minimum at $267m\mu = 15.4$, giving a persistence ratio of 3.18. We have since prepared material of greater intensity of absorption from cotton-seed oil and from wheat germ oil concentrates. The highest values obtained were $E_{1\text{cm.}}^{1\%} 294m\mu = 59$, with a ratio of 3.6, but we have had several specimens showing $E_{1\text{cm.}}^{1\%} 50-56$ and persistence ratios of 3.1-3.6. Much higher values for $E_{1\text{cm.}}^{1\%} 294m\mu$ were often recorded for less pure material but invariably the persistence ratio was low (1.5-2.7), indicating the presence of substances showing general absorption in this region. It is of interest to compare the properties of three of the richest fractions, all of which were clear, almost colourless, rather viscid oils.

No.	$E_{1\text{cm.}}^{1\%}$		Ratio E_{294} E_{267}	n_D^{40}	I.V.	$[\alpha]_D^{20}$ (benzene)	C %	H %	Mol. wt.
	$294m\mu$	$267m\mu$							
77/3	59	18	3.3	1.4977	—	+ 6.4	81.45	11.86	399
80/3	55	15	3.6	1.5000	180	+ 12.2	81.77	11.64	416
73/2	49	13.4	3.65	1.4992	178	+ 12.0	81.26	11.64	409
87/3	50	15	3.3	1.4990	172	+ 10.8	81.54	11.62	424

In all cases the fractions which had been purified by distillation condensed at a temperature range of $130-160^\circ$ at about 0.0001 mm. It is still uncertain whether these preparations are mixtures or whether they consist largely of one substance. One has a superficial impression that they are reasonably homogenous because there is no marked change of their properties on redistillation, partition between solvents or reabsorption on aluminium oxide. Failure to prepare crystalline derivatives has greatly hampered attempts at purification and it must again be admitted that the question of purity must be left open.

Mr F. A. Askew who has again kindly assisted us by examining these materials by the film-spreading technique has expressed the following opinion regarding fraction 80/3 and a closely related material 80/2: "If, as is probable, the concentrates contain more than one molecular species, then either (a) the two species are very similar in surface properties, or (b) if they are dissimilar they must be present in nearly constant proportions, or the proportion of one must be small, *i.e.* 10% or less."

A shift in the position of the maximum from 290 to $281m\mu$ on acetylation was noted by Olcott [1934]. This change is sometimes obscured if other substances showing absorption in the vicinity are present but it is readily observed with the purer preparations. The new band is weaker than the original one at

294 $m\mu$ and on hydrolysis of the acetate the latter band usually reappears with somewhat diminished intensity. The hydrogenated fractions (see later) show similar behaviour. Typical figures are:

Fraction	I.V.	$E_{1\text{ cm.}}^{1\%}$		
		294 $m\mu$	282 $m\mu$	267 $m\mu$
80/3	180	55	—	15
Acetate 80/3	—	—	35	—
Regenerated 80/3	—	45.4	—	21.5
Reduced 80/3	55	47	—	7.8
Acetate 80/3	—	—	27.5	—
Regenerated reduced 80/3	—	42	—	18

The position of the maximum of the band does not change on hydrogenation but there is a marked increase in the persistence ratio, values as high as 6.7 having been recorded, which would be consistent with a decrease in absorption of some component in the region of 267 $m\mu$. There is a fall both in intensity and persistence of the band when the reduced product is regenerated from its acetate by hydrolysis with hot alcoholic potash. This recalls the similar behaviour of the parent substance.

The reduced product is liquid at room temperature but can be obtained as a micro-crystalline solid by cooling a solution in methyl alcohol to -10° . The melting point of the solid appears to be about 8° , but the small amount of material available made purification by this means difficult. Fractional sublimation at reduced pressures (0.001 mm.) was also disappointing.

Three preparations of the reduced material purified to a certain extent by one or other of these methods gave the following data:

No.	$E_{1\text{ cm.}}^{1\%}$	Persistence	$[\alpha]_D^{20^\circ}$	C %	H %	Mol. wt.
	294 $m\mu$	ratio				
R 88/2	36	6.7	—	81.3	12.71	366
R 88/3	43	6.3	+10.8	81.52	12.69	390
R 80/3	47	6.2	+12.7	81.37	12.44	398

Attempts to prepare crystalline derivatives by reaction with the hydroxyl group failed, the products proving to be in all cases either oils or soft waxy solids at room temperature. During the preparation of the 3:5-dinitrobenzoate there separated out on two occasions a small amount of a crystalline material. Only sufficient for one recrystallisation from alcohol and a melting point determination was obtained. It melted sharply at 102° . It was not a derivative of the substance responsible for the 294 $m\mu$ band. Hydrolysis of the oily dinitrobenzoate of the main fraction resulted in a reduction both of intensity and persistence of the band similar to but greater than that noted when the acetate was hydrolysed.

Action of sodium in amyl alcohol. Absorption at 294 $m\mu$ fell by nearly 80 % as a result of boiling for 3.5 hours with sodium in amyl alcohol. No reduction occurred.

Condensation with maleic anhydride. A preparation showing $E_{1\text{ cm.}}^{1\%} = 55$ and persistence ratio 3.6 was used for this experiment. 0.48 g. was dissolved together with 0.41 maleic anhydride in 25 ml. of ether and allowed to stand in the dark for 9 days. After removal of the solvent the material was separated in the manner described by Windaus and Lüttringhaus [1931] into "neutral" and "acidic" fractions. Both were similar in appearance to the original product.

	Wt. g.	$E_{1\text{ cm.}}^{1\%}$	Persistence ratio
"Neutral"	0.296	40.7	2.1
"Acidic"	0.184	54.5	2.3

There is evidence that the rate of combination with maleic anhydride is slow at ordinary temperatures, for the "neutral" fraction thus obtained was further separated after a second treatment into about the same proportion of "neutral" and "acidic" fractions. The falling off in intensity and persistence of the band is attributed to the action of the hot alkali employed in the later stages of the separation.

Colour reactions. Various preparations showed consistent behaviour in the following reactions:

Salkowski. Pale straw colour darkening on standing, with development of green fluorescence.

Liebermann-Burchard. Gradual development of a brownish red colour with strong green fluorescence.

Whitby B. Straw colour.

Benzoyl peroxide. (Oxycholesterol reaction.) Port wine-red.

No colour was given with antimony trichloride, dimethyl sulphate or trichloroacetic acid.

Nature of the substance responsible for the 294m μ band.

Accepting for the time being the evidence, admittedly inadequate, that the preparations consist mainly of one compound, a few suggestions can be offered regarding its nature. The colour reactions, the behaviour during adsorption fractionations and the data from film-spreading experiments which Mr F. A. Askew has very kindly placed at our disposal¹ seem to point to a constitution based on a reduced polycyclic structure of the sterol type. The physical properties and those of the reduced compound indicate a fairly long side chain, branched and unsaturated. It might be isoprenic in character. On the evidence of the maleic anhydride experiment two of the ethylenic linkages are probably conjugated, and there may be a third which is less reactive. These tentative deductions are supported by the results of a preliminary investigation of the products of oxidation which will be reported later. Estimation of C-methyl by oxidation with chromic acid gave figures equivalent to a yield of 3·3·3 mols. of acetic acid.² Elementary analyses indicate two oxygen atoms in the molecule. One is present as a non-phenolic hydroxyl. The opinion which we formerly expressed [1935] on the basis of the spectroscopic evidence that a keto-enol grouping might be present seems no longer tenable.

DISCUSSION.

The first point to be considered is whether the association between vitamin E activity and selective absorption with a maximum at 294m μ which has been reported by Martin *et al.* [1934] and by us [1935] can any longer be regarded as significant. Olcott [1935, 1, 2] is inclined to attach no importance to it because he has observed a disappearance of the band or a shift in the position of its maximum as a result of chemical treatment which did not appreciably reduce the biological activity, and, furthermore, he has detected the absorption band in examining

¹ Mr F. A. Askew has informed us that preparation 80/3 showed a limiting area at zero pressure of 67 sq. Å. The corresponding measurement for the reduced product R 80/3 was about 54–55 sq. Å. The surface potential of the reduced form is about 80–90 mv. smaller than that of the parent material. The general shape of the curves closely resembles those recorded in the paper by Askew [1935].

² Analyses by Dr Hubert Roth (Heidelberg): 6·99 and 6·43 mg. of the material required respectively 5·88 and 5·48 ml. N/100 NaOH.

concentrates from palm oil which were inactive when given to vitamin E-deficient rats. In our experience we have not encountered a case in which material giving a positive response in a rat failed to exhibit the absorption band, provided of course that a stage of purification had been reached which permitted selective absorption to be detected, but, on the other hand, we now have records of preparations which failed to exercise a biological effect such as might have been expected from the spectroscopic measurements. As we have remarked, this is noticeably true of the material which has been distilled. Evans [1932], referring to experiments made in his laboratory at pressures a good deal higher than those which we employed (0.5–0.01 mm.), comments on “considerable decomposition” observed during the distillations and points to a failure to effect by this means any great concentration of the active material. By employing much lower pressures we had hoped to prevent loss but it now appears that our distilled fractions have considerably lower biological value than was expected. There remains a possibility that the band at $294m\mu$ is shown by the vitamin and that some changes which the molecule may undergo render it of no value to the vitamin E-deficient doe but do not materially affect the absorption. Olcott’s objections make this improbable. On the other hand the gradual loss of biological potency which occurs on exposure of concentrates to ultraviolet light seems to run parallel with the photochemical decomposition of the substance absorbing at $294m\mu$ [Drummond *et al.*, 1935]. This destruction of the vitamin must mean that it absorbs radiations in the ultraviolet unless the explanation is that it is involved in secondary reactions. Olcott has himself shown [1934] that ozone will slowly inactivate the vitamin, whilst there is a curious assortment of evidence to show that inactivation may also occur as a result of secondary reactions associated with the early phases of “oxidative rancidity” of fats [Cummings and Mattill, 1931; Evans, 1932; see also Olcott and Mattill, 1934]. A simple experiment with monochromatic irradiation in an inert atmosphere would throw valuable light on this very important matter. The most potent fractions we have obtained have effected a cure of vitamin E deficiency in female rats when given in doses of 0.5–1 mg. If the substance absorbing at $294m\mu$ has no direct relationship with the vitamin the actual physiological dose must be much smaller. The most active material described by Evans and his colleagues produced positive tests in doses of 5 mg. [1932]¹ whilst that mentioned in Olcott’s records is about twice as potent. A comparison of the properties of these materials with those of our most active preparations is given below.

Preparation	Rat dose mg.	C %	H %	Mol. wt.	I.V.	R.I.	Optical rotation
Evans and Burr [1927] from wheat germ oil	5	81.7	12.2	400	220	N^{20° 1.5009	$[\alpha]_D^{18^\circ} + 7.3^\circ$
Olcott [1934] cotton-seed oil	2–3	81.6	11.5	—	90	N^{20° 1.5090	—
Our material wheat germ oil	1–0.5	81.7	11.8	400	180	N^{40° 1.5006	$[\alpha]_D^{20^\circ} + 8.6^\circ$

It is impossible to say how much importance can be attached to the superficial resemblance which the figures reveal, not only in so far as these three preparations are concerned but also when the comparison includes the data for

¹ In a footnote to this paper Evans reports that Cornish has prepared fractions from wheat germ oil which effected cures of typical vitamin E-sterility when given in doses of 1 mg. No details of the preparation and properties of this material have yet appeared, so far as we are aware.

materials of lower biological activity given on p. 2517. If the substance absorbing at $294m\mu$ is biologically inert so far as the cure of vitamin E-deficiency is concerned we can either think that the proportion of vitamin in such materials is small, which is quite likely, or if we imagine the active substance as forming a reasonably large part of the material we can entertain the idea that it is chemically similar in character to the absorbing substance. That also is likely.

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