LII. A STUDY OF THE UNSAPONIFIABLE FRACTION OF WHEAT GERM OIL WITH SPECIAL REFERENCE TO VITAMIN E.

By JACK CECIL DRUMMOND, ELEANOR SINGER
AND ROY JAMES MACWALTER.

From the Department of Biochemistry, University College, London.

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PREVIOUS chemical studies of vitamin E have shown it to be a substance which can readily be concentrated in the unsaponifiable fraction of certain oils. The properties of these concentrated preparations indicate that the active substance possesses a relatively high degree of stability. The original investigations of Evans and Burr [1927, 1] showed that the vitamin is not readily destroyed by aeration at 100°, treatment with 20 % hydrochloric acid, subjection to temperatures up to 250° or hydrogenation with palladium at 75°. Exposure to strong ultra-violet radiation seemed to cause slight inactivation after 20 minutes, whereas after 45 minutes much activity was lost. Treatment with alcoholic hydrochloric acid or bromination in glacial acetic acid caused inactivation and, more surprisingly, evidence that acetylation was also destructive was obtained.

Little had been added to this knowledge of the chemical behaviour of vitamin E concentrates until Olcott and Mattill recently began to report the results of their investigations [1934; Olcott, 1934, 1, 2].

In the main the observations of Evans and Burr were confirmed. A striking exception was a failure to bring about inactivation by acetylation and an important addition was successful reactivation of chlorinated and brominated material by boiling with zinc dust and hydrochloric acid in methyl alcoholic solution. Destruction by ozone, perbenzoic acid, potassamide and, somewhat unexpectedly, potassium ethoxide was recorded.

Investigators are agreed that the vitamin is volatile. Evans and Burr found on refractionating that activity was associated with material collected in the narrow range 225–30°/0.01 mm., but Olcott and Mattill recorded a much greater volatility (190–220°/0.1 mm.).

An important question concerns the absorption bands shown by concentrates of vitamin E. Evans and Burr remarked "these measurements did not point to any definite curves for vitamin A or E which would help in following the concentration of the substance". Broad but diffuse bands, becoming much sharper at the temperature of liquid air, were however detected by Bowden and Moore [1933, 1] and additional maxima were recorded by Morton and Edisbury [1933]. Correlation of these data with biological activity was first attempted by Bowden and Moore [1933, 2] who pointed out that some part of the absorption in the range 320–285 $m\mu$ might be related to vitamin E.

Edisbury [1933] also reported a detailed consideration of the spectroscopic data obtained from the examination of vitamin E concentrates, with the object of tracing a correlation with the chemical behaviour of the vitamin.¹ He also

¹ We are greatly indebted to Dr R. A. Morton for his kindness in bringing this report of Dr Edisbury's work to our attention and for putting unreservedly at our disposal the results of his own spectrographic examinations of vitamin E concentrates.

reached the conclusion that the bands in the region indicated by Bowden and Moore should be further studied and drew attention to the three maxima, 291, 271, and 256.5 $m\mu$. The work of Olcott and Mattill [1934] tended however to weaken the belief that the vitamin might be a substance showing marked selective absorption in the ultra-violet, because their highly concentrated fractions showed only general absorption. Recently, however, a preliminary communication by Martin *et al.* [1934] announced that a well-defined absorption band was exhibited by a vitamin E concentrate. The band in question had a maximum at 294 $m\mu$; the strongest absorption recorded being $E_{1\text{ cm.}}^{1\%} = 27$. This material gave a positive biological test on one female rat when a single dose of 16 mg. was administered.

Olcott [1934, 2] has also recorded this band in the examination of concentrates from wheat germ oil and cottonseed oil. Whereas Martin and his colleagues are undecided whether the band can be ascribed to the vitamin or to accompanying substances, Olcott concludes tentatively that it is not related directly to the vitamin because certain treatments (AgNO_3 , acetylation) change or obliterate the band but do not destroy biological activity.

Chemical analyses of biologically active fractions have been made but the results have been put forward with reservations regarding the homogeneity of the material. These figures will be referred to later.

EXPERIMENTAL.

The raw material employed for the investigation now being recorded was the unsaponifiable fraction of wheat germ oil. The oil was prepared by cold extraction of a commercial wheat germ preparation (Bemax) with purified trichloroethylene. Ordinary commercial wheat germ is seldom a good raw material because it usually contains a large proportion of bran and other milling products and, moreover, may often be badly rancid. These facts are often ignored. On the one hand, some recorded estimations of the vitamin B content of wheat germ are low because of the unsuspected presence of bran; on the other, it is reasonable to attribute the low vitamin E activity of some preparations of wheat germ oil to the post-milling changes which rapidly occur in the crushed embryo. In this connection the work of Evans and Burr [1927, 2] on the influence of rancidity on vitamin E is of interest. The preparation (Bemax) chosen for this experiment is practically pure germ, has a negligible degree of free acidity and no trace of other products associated with rancidity.

The germ yielded 7.2 % of oil possessing the following characteristics:

Sap. value	...	...	...	...	182
Iodine value	...	...	...	...	131
Refract. index (20°)	...	...	...	...	1.4773
Unsap.	...	...	...	...	5.76 %
Iodine value of unsap.	...	...	...	...	97.6

The oil was found to be biologically active in daily doses of 23 mg. In our experience extraction with ether may sometimes cause inactivation. One sample prepared from the same bulk of germ failed to protect 5 rats from resorptions when given in daily doses of 46 mg. Preparation of the unsaponifiable fraction was made by treatment with boiling alcoholic KOH. Two saponifications of 30 minutes' duration were found to serve best. In the light of our experience it is surprising to learn that Olcott [1934, 2] has found inactivation after treatment with potassium ethoxide.

The unsaponifiable fraction thus obtained was, at room temperature, a hard yellow wax. It was found to be active in doses of 2.5 mg. It usually contained about 60 % of sterols precipitable by digitonin.

The bulk of the sterols was removed by cooling a saturated solution in methyl alcohol to -25° by the direct addition of powdered CO_2 . This treatment effectively removed between 80 and 90 % of the sterols present. The mother-liquor from the crystallisation of the sterols yielded on removal of the methyl alcohol a red-orange coloured, soft wax.

Crude sterol fraction.

As was suspected from reference to the observations of Anderson and Nabenhauer [1924, 1], the greater part of the crude sterol mixture was found to consist of sitosterol and dihydrositosterol. Using their method of separation [1924, 2; Anderson *et al.* 1926-27], a preparation of the saturated constituent was obtained possessing the following characteristics: M.P. $143-5^{\circ}$; $[\alpha]_D^{18} + 21.9^{\circ}$; acetate, M.P. 141° ; $[\alpha]_D^{18} + 13.29^{\circ}$. All the sterol fractions showed absorption bands at $261 m\mu$ and usually well-marked bands at 254 and $244 m\mu$ in addition to the triple system characteristic of ergosterol. Similar findings have been reported by Morton and Edisbury [1933] and by Edisbury [1933]. It is probable that certain of these bands are related to ergosterol D, but unidentified sterols may also be concerned.

Fractional adsorption experiments.

Preliminary experiments having shown that more promising fractionation of the unsaponifiable material after removal of the greater part of the sterols was effected by adsorption on aluminium oxide than by distillation in high vacua or by partition between solvents, the former method was adopted. A typical experiment is described. 33 g. of the viscous red-orange oil obtained after removal of the crude sterols were dissolved in 500 ml. light petroleum. A small amount of dark brownish red material remained undissolved and was filtered off. More of this dark red material separated from the petroleum solution on standing overnight. The combined weight of the two insoluble fractions was 0.67 g. It proved to be slightly impure lutein (xanthophyll). Recrystallisation from methyl alcohol gave typical crystals, M.P. 194.5° .

The material soluble in light petroleum was passed slowly through a column of "Aluminium oxydatum anhydricum, nach Brockmann" (Merck), long-continued washing with the solvent being maintained. Two fractions of unadsorbed material were separated. The first represented that which passed through the column without any delay (fraction A). The second corresponded with a pale yellow coloured zone which slowly passed down the column as the washing proceeded. Eventually this pigmented substance passed out into the receiving flask completely, the washings once again becoming colourless (fraction B).

At this stage the experiment was usually discontinued and the column of adsorbent was broken up into four sections. The uppermost layer containing the most strongly adsorbed substance was of a deep brownish yellow tint (fraction F). Next came a paler, sandy coloured zone (fraction E), below which was another coloured region but definitely darker in tint (fraction D). At the bottom of the column came a deep zone showing in general little colour but marked by more than one indefinite band of slightly more pronounced yellow or sandy colour (fraction C).

It must be emphasised that this description refers only to one experiment. In

every case the behaviour was similar so far as the separation of fractions *A* and *B* was concerned but the appearance of the column itself at the end of the experiment was not always the same.

To what causes these differences, not always of a minor character, are to be ascribed we do not know, but with experience it was found possible to recognise and separate the important sections of the column. The fractions of oxide were separately eluted with 80/20 methyl alcohol-ether.

The amount and characteristics of the fractions obtained in the actual experiment we have described are given below in Table I.

Table I.

Fraction	Appearance	Wt. (g.)	Iodine value	Biological activity	Spectroscopic examination max. at $m\mu$
<i>A</i>	Clear, pale lemon-yellow oil	6.23	64	—	388, 362, 337, 323, 289
<i>B</i>	Orange oil	3.97	235	—	485, 453, 362, 320, 289
<i>C</i>	Rich orange oil	6.97	200	Active 1 mg., slightly active 0.2 mg.	492, 303, 295
<i>D</i>	Soft red wax	6.16	216	Active 2 mg., slightly active 1 mg.	475, 445, 295
<i>E</i>	Dark brown-red crystalline mass	4.60	101	Slight activity 2.5 mg.	344, 327, 303, 297, 284, 273
<i>F</i>	Dark red, thick oil containing crystals	3.34	105	—	475, 445, 416, 347, 335, 320, 297, 284, 273

Fraction A. Superficial examination of this mixture suggested that it consisted for the greater part of hydrocarbons. It contained material precipitated by digitonin, the amount usually being about 3.5 %. This was removed before further fractionation was attempted by passing again through aluminium oxide. The latter treatment removed by adsorption a small amount of material with a high iodine value (see fraction *B*) and gave clear, pale yellow oils with iodine values in the range 40–50. This material seeming to be suitable for purification by distillation the latter was carried out (*a*) by the usual procedure at a pressure of 0.05 mm. and (*b*) by use of the Burch [1929] molecular still.¹ Even at the higher pressure (*a*) the material distilled readily. A typical distillation gave the following fractions:

(*a*) 2.1 g. distilled at 0.05 mm.

Temp. range	Wt. of fraction g.	Iodine value	Refractive index 18°	C %	H %
122–148°	0.861	39.4	1.5002	86.49	12.35
148–165°	0.779	37.7	1.5062	86.78	12.42
165–200°	0.359	46.7	1.5088	86.09	12.05
above 200°	0.079	69.0	—	—	—

(*b*) A fractionation of 4.35 g. in the molecular still (pressure ca. 0.00001 mm.).

Temp. range	Wt. of fraction g.	Iodine value	Refractive index 18°	C %	H %
55–69°	1.75	43.7	1.5009	86.64	12.16
69–120°	1.25	73.0	1.5059	86.06	12.44
above 120°	0.70	71.0	—	—	—

¹ We are much indebted to Dr F. H. Carr and Mr Jewell of B.D.H. Ltd. for carrying out this and a number of other fractionations in the molecular still. We greatly appreciate their valuable and ready help.

Inspection of these figures suggests a relatively low-boiling hydrocarbon contaminated with a less volatile oxygen-containing impurity possessing a higher degree of unsaturation and a higher refractive index. We have succeeded in obtaining after more than one distillation preparations of the former which gave satisfactory analysis.

The best material had the following properties:

B.P.	...	120–140° (0.05 mm.)
Iodine value	...	42
C %	...	87.02, 87.05
H %	...	12.82, 12.96
Mol. wt. (camphor)	...	258, 249
Refract. index 18°	...	1.5009
Absorption bands	336	$m\mu$ medium intensity
	326.5	„ very narrow
	301	„ inflection
	288	} „ very weak and narrow
	282	
	277	
	257	
		„ broad and strong

The analyses would agree with a formula such as $C_{18}H_{32}$ (C, 87.09; H, 12.91 %).

Some or all of the members of this group of absorption bands appear to be always shown by these hydrocarbon fractions and it is of interest to note that they were also exhibited by a similar fraction obtained from olive oil by Thorbjarnarson and Drummond [1934]. It is possible that those with maxima at 336, 326.5 and 301 $m\mu$ may be associated with dihydroergosterol, although these bands do not correspond closely with the published figures.

Enough of this liquid hydrocarbon has not yet been available to enable us to examine its properties more fully.

In view of the discovery in fraction *B* of a highly unsaturated hydrocarbon of the squalene type it was thought at one time that the iodine value of preparations from fraction *A* might be due to contamination with this substance. An effort was made to separate the suspected contaminant by bromination and by treatment with HCl (see fraction *B*), but without appreciable success. Only very minute amounts of solid derivatives separated and on removing the halogens from the residues the iodine values remained in the neighbourhood of 35–40.

It would be inadvisable to comment further on this compound until larger quantities have been prepared and examined. The fraction was inactive as a source of vitamin E.

Fraction B. The constituents of this fraction passed slowly through the adsorbing column, visibly as a pale yellow zone, during the washing with light petroleum. The pigment was characterised by absorption bands in the visible spectrum at 453 and 485 $m\mu$. The position of these bands and the behaviour during adsorption suggest that the colour is due to kryptoxanthin¹ [Kuhn and Grundmann, 1933]. Absorption in the ultra-violet zone was indicated by maxima at 362, 325 and 289 $m\mu$. The intensities were usually of approximately the same order as that obtained in the examination of fraction *A*. The significance of these bands is as yet unknown.

This fraction was characterised by high iodine values, the range encountered in different experiments being 215–266. This fact, together with the low adsorbability of the fraction, suggested the presence of a highly unsaturated hydrocarbon. Attempts were made therefore to prepare halogenated derivatives. Bromination in dry ether at 0° gave a moderately good yield of a white micro-

¹ In a preliminary report of this work the pigment was described as β -carotene, the absorption bands of which correspond closely with those of kryptoxanthin [Drummond *et al.*, 1934].

crystalline bromide resembling the bromides of the squalene type of hydrocarbon. This derivative darkened at 165° and showed definite signs of decomposing at 180° . It contained 69.2 % of bromine. Treatment with dry HCl gas in acetone at -10° gave crystalline derivatives, the form of which usually recalled the prismatic plates of squalene hydrochloride. In a few cases however the typical plates did not appear and the form was that of bunches of very small fine needle-shaped crystals, more like the hydrochloride of the hydrocarbon isolated by Channon and Marrian [1926] from pig liver fat. Sometimes both types of crystals have appeared. Preparations of the hydrochloride recrystallised from acetone showed melting-points within the range $107-116^{\circ}$, but insufficient was available to enable an adequate fractionation to be carried out. Recent evidence [Thorbjarnarson and Drummond, 1934] supports the belief of earlier investigators that several isomeric squalene hydrochlorides with different melting-points exist.

The analytical figures for the hydrochlorides are also unsatisfactory. Squalene hydrochloride, $C_{30}H_{50} \cdot 6HCl$, has 33.9 % Cl; the hydrochloride of the liver hydrocarbon is probably $C_{45}H_{78} \cdot 8HCl$ containing 31.2 % Cl [Channon *et al.*, 1934]. Various preparations of our hydrochloride gave 31.34, 31.39, 31.99, 32.08, 32.24, 32.79, 33.34 % Cl.

Until material is available for a more thorough study it is unwise to draw conclusions from these figures but they suggest the possibility that more than one hydrocarbon of the squalene type is present. It is not unreasonable to think that the liver hydrocarbon might be present in plant materials, particularly since squalene has recently been identified as an important constituent of olive oil.

Analysis of the crude fraction *B* having shown that considerable amounts of oxygen-containing substances were also present, even after removal of all material precipitable by digitonin, efforts were made to separate them from the hydrocarbon by the use of solvents and by fractional distillation. Neither was successful. Fractions were obtained with higher iodine values, the highest being 306 (squalene has i.v. 371), but analyses and the yields of hydrochloride and bromide showed that no effective concentration had been achieved.

Since it appeared that at least one of the oxygen-containing substances was highly unsaturated attempts were made to separate it on the assumption that it was alcoholic in character by acetylation and benzylation. Evidence that reaction occurred was obtained, but no real success in separation was attained; once again our efforts being limited by the small scale on which we were obliged to work. Fraction *B* was found to be biologically inactive as a source of vitamin E.

Fraction C. The material yielded by elution of the lower zone of the adsorption column was in all experiments a deep orange-yellow viscous oil. The amount was usually of the order of one-fifth of the original material submitted to adsorption. When sterols had not been completely removed before the adsorption was performed as much as 15 % of material precipitated by digitonin was found in this zone. In such cases it was quantitatively removed before further fractionation was attempted. The sterol-free material behaved on general examination as if it were composed to a large extent of unsaturated alcohols of high molecular weight. The degree of unsaturation was indicated by iodine values ranging from 170 to 200. This fraction invariably showed a well-marked absorption band with a maximum at $294 m\mu$ (Curve *A*, Fig. 2), to which we are inclined to attach importance. Related to this maximum is a minimum displayed at $267.5 m\mu$, but in the examination of some preparations, particularly in the early stages of fractionation, this may be masked by general absorption. For a typical preparation of sterol-free material the measurements gave $E_{1\text{ cm}}^{1\%} 294 m\mu = 27.8$;

267.5 $m\mu$ = 17.3. Definite biological activity is found in this fraction, a daily dose of 0.2 mg. producing satisfactory litters. Since 1 mg. of the material before adsorption exerted about the same biological activity this indicates satisfactory concentration without significant loss.

Adsorption having proved so helpful and other methods of fractionation having given such disappointing results it was decided to carry the concentration as far as possible by use of aluminium oxide. Fraction *C* was therefore re-adsorbed from light petroleum solution, on appropriately smaller columns of the oxide. One unexpected observation was made. Whereas in the initial adsorption the biological activity was associated with the fractions more lightly adsorbed, in subsequent re-adsorptions it was usually found in fractions located higher up the column. After a third re-adsorption it appeared to be almost wholly concentrated in the top zone of the oxide and held there in spite of prolonged washing with solvent (see Fig. 1). Without doubt the position taken up by any sub-

Fig. 1. General scheme of fractionation of wheat germ oil unsaponifiable. The "arrow-heads" indicate the fractions which contained the substance responsible for the 294 $m\mu$ band. An attempt to indicate the quantitative distribution has been made by using one to four "arrow-heads". In general the distribution of the vitamin is similar.

stance in these adsorption columns is much influenced by the presence of other substances. In our experience it was the removal of the highly unsaturated substance described as being found in fraction *B* which rendered the vitamin more readily adsorbed by the oxide.

Biological activity having also been detected in fraction *D* (see later) and refractionation of this material being decided upon, it became necessary to

combine certain fractions from the main fractions *C* and *D*. The scheme of separation is shown in Fig. 1.

From Fig. 1 it will be seen how curiously the active material changes in its behaviour from being adsorbed at the bottom of the column to being readily held up by the uppermost layers of the oxide.

At the stage of fractionation represented by the fractions 75 *C_id* and 75 *C_{ii}d*, the biological activity was found to be represented by the minimum daily dose of 0.1 mg. This corresponds satisfactorily with the factor of concentration represented by the material yields. Further attempts at concentration were

Fig. 2. Influence of 12 hours' short range irradiation by a mercury vapour lamp on the absorption band of an active vitamin E concentrate. Little change in the absorption at the maximum at 294 $m\mu$ but a great decrease of the "persistence" are regarded as evidence of considerable reduction in the concentration of the substance responsible for the band (see p. 465). (A) Original material. (B) Irradiated material.

disappointing. Fractions 75 *C_id₂* and 75 *C_id₃* were not found active in 0.1 mg. but a hydrogenated preparation of the former was found active in doses of 0.2 mg.; unfortunately, this material was not tested in smaller doses.

A general parallel was noted between biological activity and the intensity of absorption in the ultra-violet region at 294 $m\mu$, particularly when this was measured in relation to the minimum at 267.5 $m\mu$.

In attempting to find a correlation between the biological activities of our concentrates and their absorption measurements we found it important to consider this relation rather than the individual values for $E'_{1\%1\text{cm.}}$. In general, the substance responsible for the selective absorption at 294 $m\mu$ will be contaminated by other absorbing materials. Removal of these latter often causes a decrease in the measured maximum of absorption, but also causes a decrease (to a proportionately greater extent) in the absorption at the minimum of the band.

We have therefore been concerned with two criteria in considering the effect of concentration of the substance responsible for the band: (i) increase in absorption at the maximum and (ii) "persistence", which we describe as the ratio of maximum to minimum absorption. We are inclined to consider that this ratio provides a better estimate of the effect than the difference between maximum and minimum absorption.

Certain data for four of the more powerfully absorbing fractions obtained are set out in Table II.

Table II.

Fraction	Iodine value	Absorption $E_{1\text{ cm.}}^{1\%}$		Ratio 294/ 267.5	Optical rotation approx. 1.5 % in benzene $[\alpha]_D^{18^\circ}$
		294 $m\mu$	267.5 $m\mu$		
75 $C_{ii}d$	167	42.6	20.0	2.13	+20.0°
75 C_{id_s}	177	39.6	20.4	1.96	+16.2
75 $C_{id_{3b}}$	164	40.9	12.8	3.18	+22.6
75 $C_{id_{3c}}$	141	45.0	17.6	2.56	+18.0

Investigation of the nature of these active fractions led in due course to acetylation which immediately revealed that they were not homogeneous. Variable amounts of a beautifully crystalline acetate were obtained, some of the higher yields being as much as 15 %. The acetate was slightly soluble in methyl and ethyl alcohol, more readily in ether and chloroform. Recrystallised from acetone or ethyl alcohol it melted sharply at 236°. (Found: C, 81.59, 81.24; H, 11.10, 11.26 %; mol. wt. 442, 455; $[\alpha]_D^{18^\circ} + 97.3^\circ$.) Determination of acetyl value showed the presence of one acetyl group. The parent alcohol crystallised in fine needles from ethyl alcohol, m.p. 190°. (Found: C, 84.57, 84.52; H, 11.66, 11.70 %; mol. wt. 427, 430; $[\alpha]_D^{18^\circ} + 97.7^\circ$.) It appeared certain from this data that the substance was β -amyrin, $C_{30}H_{50}O$.¹ Mixed melting-point determination with pure specimens of the parent substance and the acetate showed no depression. Spectroscopic examination showed that β -amyrin exhibits only very slight absorption in the vicinity of 284 $m\mu$ ($E_{1\text{ cm.}}^{1\%} = 1.0$) so that this substance is not responsible for the selective absorption shown by the active fraction. The acetate is no more transparent. It was also without biological activity in doses of 0.1 mg. daily. It is as yet too early to dismiss the possibility that β -amyrin is related to the vitamin E activity but animal tests on this substance itself have given negative results.

Acetylation of our active fraction also threw light on another interesting matter concerning the vitamin. It will be recalled that other investigators are not in agreement regarding the effect of this treatment. Olcott did not confirm Evans and Burr's observation that destruction of biological activity followed treatment with acetic anhydride. He noted, however, that it caused a change of the absorption in that the maximum moved from 294 to 281 $m\mu$. He does not mention the relative intensities but he is inclined to use these observations as support for concluding that the former band is not connected with the vitamin content.

We have found that selective absorption disappears on acetylation and reappears on hydrolysis but biological activity is unaffected. We have as yet insufficient data to give us confidence, but we are inclined to believe that immediately after hydrolysis the 294 $m\mu$ band may reappear with an intensity greater than that shown by the material before acetylation. The position of the band and its intensity are not incompatible with the substance being a polycyclic ketone. Apart from this question the disappearance and regeneration of the band suggest that the responsible structure may be one capable of undergoing tautomeric change.

¹ Mr F. A. Askew has very kindly examined this compound by the surface film technique and is satisfied that it behaves in a manner similar to what would be expected from a very slightly impure β -amyrin [Askew, 1935].

Susceptibility to ultra-violet radiation. If the absorption band at $294 m\mu$ is a property of the vitamin, it would be expected that radiation at the appropriate frequency would result in photochemical change, and that changes occurring in the absorption spectrum would be accompanied by corresponding changes in vitamin activity. Evans and Burr [1927, 1] found signs of loss of biological activity after short range exposure to ultra-violet radiation for 20 minutes at 0° and definite destruction after 45 minutes at ordinary temperature. Our preliminary experiments with a relatively crude preparation (55 F) indicated an even greater degree of stability. This preparation before radiation was biologically active in doses of 0.2 mg. daily. 0.33 g. of the preparation contained in a quartz flask and sealed under nitrogen was exposed to the direct rays of a mercury arc lamp (atmospheric type) at a distance of 3 inches for 13.75 hours. The activity was not completely destroyed, since 5 out of 6 rats produced litters on a daily dose of 1 mg. A dose of 0.4 mg. was inactive.

A definite change in absorption spectrum was observable. The intensity of the original material at $294 m\mu$ was $E_{1\text{ cm.}}^{1\%} = 48.2$ and the persistence (see p. 463) 1.74. After irradiation the intensity maximum remained constant ($E_{1\text{ cm.}}^{1\%} = 47.8$) but the persistence had decreased to 1.33.

A more highly refined preparation (75 $C_{ii}d$), originally active in 0.1 mg. dose, lost at least half its activity after direct exposure under similar conditions for 12 hours. In this experiment the change in absorption spectrum was considerable (see Fig. 2), the band having disappeared almost completely after irradiation.

Influence of hydrogenation on vitamin E activity. Evans and Burr [1927, 1] found that wheat germ oil and extracts prepared therefrom retained their vitamin E activity after hydrogenation with palladium at 75° . Olcott [1934, 1, 2] found that active fractions withstood drastic catalytic hydrogenation at 200° and 200 atmospheres but were still unsaturated, as indicated by an iodine value of 70.

Our own experience confirms the survival of biological activity after catalytic hydrogenation but we found no difficulty in obtaining a more satisfactory uptake of hydrogen. One of our most active fractions (75 $C_{ii}d$) was reduced with palladium at atmospheric temperature and pressure, the concentrate being dissolved in absolute alcohol. In one experiment 138 mg. of this golden yellow viscous oil took up hydrogen readily under these conditions, the iodine value falling from 166 to 26 in about 5 hours with one renewal of the catalyst. Difficulty was encountered in carrying the hydrogenation further and it is possible that the residual iodine value represented an unsaturated impurity resistant to reduction. The amounts of material available did not enable us further to investigate this matter. The hydrogenated material was a soft white wax with an indefinite melting-point about 35° . The biological activity was unaffected by the reduction, good litters being obtained with a dose of 0.2 mg. The examination of the material before and after reduction showed that the absorption band had been but slightly changed, apart from a very slight shift towards the more refrangible region, the maximum and minimum being now at 296.5 and $271 m\mu$ respectively (Table III).

Table III.

Condition	Iodine value	Absorption $E_{1\text{ cm.}}^{1\%}$	Ratio	Positive rat dose
Original	166	294 42.6	2.13	0.2 mg.
		267 20.0		
Partly reduced	70	296 40.2	2.58	—
		268.5 15.6		
Maximum reduction achieved	26	296.5 37.8	2.52	0.2 mg.
		271 11.0		

Unfortunately, we have not had sufficient reduced material to make it possible seriously to attempt chemical fractionation but a few preliminary trials have given encouraging results.

Chemical composition of the active fractions. We have thought it worth while to make a few analyses of our most active fraction although we are fully aware that they may have been far from homogeneous. These materials are yellow and somewhat viscous oils. The analytical data are given below:

	C %	H %	Mol. wt. (camphor)	Iodine value
	80.77	11.44	460	164
	81.12	11.66	430	179
	81.16	11.56	420	166
	80.62	11.61	445	162
	80.82	11.64	—	167
Mean	80.89	11.58	439	167

The material acetylates with the introduction of one acetyl group. It will be remembered that we found that the band at $294\text{ }m\mu$ disappeared as a result of this change and was again exhibited by the material after hydrolysis. Analyses of the acetylated product gave:

	C %	H %	Mol. wt. (camphor)	Iodine value
	78.33	10.70	480	160
	78.55	10.85	440	156
	78.36	10.41	476	—
Mean	78.41	10.65	465	158

Determination of acetyl groups.¹

(a) 0.1197 g. contains 0.223 milliequiv. acid.

(b) 0.113 g. " 0.230 "

On the assumption that our material is homogeneous these analytical figures would agree reasonably well with a polycyclic structure related to the sterol or amyrin class.

	C %	H %	Mol. wt.	Iodine value (3 double bonds)
$\text{C}_{28}\text{H}_{48}\text{O}_2$	80.76	11.53	416	183
$\text{C}_{30}\text{H}_{50}\text{O}_2$	81.44	11.31	442	172

It will be recalled that the analyses of the distilled fraction of Evans and Burr [1927, 1] fitted the formula $\text{C}_{36}\text{H}_{64}\text{O}_2$. Regarding the degree of unsaturation it may be noted that the volume of hydrogen absorbed was of the order required to saturate three double bonds. Again assuming homogeneity, the formulae indicate the presence of two oxygen atoms in the molecule. On the spectroscopic evidence one of these might represent a ketonic grouping capable of tautomerising and yielding an acetylated derivative on treatment with acetic anhydride. If so, the second oxygen is present either as a relatively stable ketonic group or in another form not reacting to acetylation.

Again with reservations regarding purity the view that a polycyclic ketone may be concerned is not at variance with the evidence derived from a study of surface films which is presented by Askew [1935]. His data suggest that if the substance contains two polar groups they are probably close together in the molecule.

¹ We are greatly indebted to Dr A. H. Palmer for making these determinations.

In order to test for the presence of ketonic substances in the active concentrates two methods of fractionation were investigated. One has been devised by Girard [1933] but as the nature of his reagent has not yet been fully revealed this could not be investigated without his assistance. Dr Girard was kind enough to carry out for us an extraction depending upon the use of his "Reagent T". He informs us that under the conditions of his reaction all ketones except the "aromatic nuclear ketones" are converted into water-soluble derivatives which can be readily separated from impurities soluble in ether and other fat solvents. When ketones are thus abstracted in water-soluble form the solutions give a precipitate with mercuric iodide; the reaction being so sensitive that it will detect 1 or 2 γ per ml. of oestrone. Approximately 1 g. of sterol-free wheat germ concentrate was sent to Dr Girard for treatment. He reported that the extraction and separation of the ketonic fraction did not proceed altogether smoothly. From the behaviour with HgI_2 he was inclined to think that only small amounts of ketonic substances were present. "If the molecular weight of the ketones present is about the same as that of oestrone I would consider the total amount of the ketones as not greater than 10 to 15 mg." Actually, the extracted material, a pale yellow, soft wax, weighed 10 mg. (1.0 %).

This material gave disappointing results on spectroscopic examination for no evidence of significant concentration of the substance responsible for the 294 $m\mu$ band was obtained.

Material before treatment	Inflection 294 $m\mu$ $E_{1\text{ cm.}}^{1\%} = 46$
"Ketonic" fraction	,, 294 $m\mu$ $E_{1\text{ cm.}}^{1\%} = 45$
Residue after removal of "ketonic" fraction	,, 294 $m\mu$ $E_{1\text{ cm.}}^{1\%} = 30.8$

The extracted material has not yet been tested biologically.

Another attempt was made to separate ketones by the method fully described by Beall and Marrian [1934]. This was applied to two active fractions:

(a) a sample of the sterol-free unsaponifiable fraction similar to that which had been treated by Dr A. Girard;

(b) a more highly concentrated preparation (75 $C_{ii}d$).

In both cases the yield of ketonic substances was small, whilst the spectroscopic examination gave no evidence of effective concentration (Table IV):

Table IV.

	Material	Wt. g.	$E_{1\text{ cm.}}^{1\%}$ 294 $m\mu$	$E_{1\text{ cm.}}^{1\%}$ 268 $m\mu$	Remarks
a	Before treatment	2.268	46.9	32.7	Persistence (ratio) 1.4
	"Ketonic" fraction	0.206	21.6	—	Inflection slightly better defined than in "non-ketonic" fraction
	"Non-ketonic" residue	1.997	26.8	—	Very weak inflection
b	Before treatment	0.098	42.6	20	Persistence (ratio) 2.13
	"Ketonic" fraction	0.0035	43	—	Inflection only
	"Non-ketonic" residue	0.086	35	—	Bands of low persistence

Whether these "ketonic" extracts contain a high concentration of the vitamin has not yet been ascertained, but it is difficult to reconcile the relevant spectroscopic data with the view that the active substance is a polycyclic ketone normally showing a broad strong absorption band at 294 $m\mu$.

The pigment responsible for the yellow colour of fraction *C* was not satisfactorily identified. The band at $492\text{ m}\mu$ (alcohol) indicates a lipochrome; probably a trace of γ -carotene or rubixanthin.

The substance responsible for the maximum at $303\text{ m}\mu$ was not traced.

Fraction D. Spectroscopic examination of this soft red wax showed the presence of a lipochrome pigment resembling lutein. The only other important absorption was at $295\text{ m}\mu$ in the ultra-violet. Since this fraction showed biological activity its investigation proceeded in the main side by side with that of fraction *C* (see Fig. 1). It contained about 33 % of the familiar sterol mixture in all cases in which this material had not been removed before adsorption. In the examination of this fraction some evidence of the presence of phytol was obtained by the preparation of phthalates but the amounts of silver derivatives isolated were too small to purify and analyse.

Fraction E. In all cases in which sterols were not completely removed by digitonin before adsorption this fraction contained the highest proportion of these substances. The appearance of the material in such cases clearly indicated its composition, as it usually resembled a crude preparation of phytosterol coloured with a brownish yellow pigment. The latter was identified as lutein. Quantitative estimations showed from 80 to 89 % of sterol precipitable by digitonin.

By recrystallisation from methyl alcohol or light petroleum clean preparations of crystalline sterol mixtures (phytosterol) were readily made. Spectroscopic examination of this material revealed bands at 342, 328, 294, 283, 272 and $262\text{ m}\mu$; that at 283 being strong. Edisbury [1933] has recorded rather feeble maxima at 312, 323 and $343\text{ m}\mu$ in sterol fractions and thought they might be attributed to dehydroergosterol. Only one of these is recorded in our measurements. Other maxima are probably attributable to ergosterol. Fractional crystallisation of the sterol from methyl alcohol gave crystalline preparations from the more soluble fractions which showed clearly the ergosterol group of bands and the other triplet believed to be due to ergosterol D (254, 243 and $235\text{ m}\mu$). In the less soluble fractions, as in the original material, general absorption tended to mask selective absorption in the shorter wave-length range.

Fraction F. This material, the most firmly adsorbed, was but superficially examined as it did not possess vitamin E activity. The pigment was almost certainly lutein. Considerable amounts of sterol (35 %) and the associated absorbing substances were also present.

Behaviour of the active fractions on distillation.

Some of the later stages of fractional adsorption having given disappointing results efforts were made to carry the concentration a step further by distillation in high vacuum. Unfortunately, the quantities of material available were so small that satisfactory fractionation could not be expected. The following results refer to the distillation of 1.35 g. of fraction 75 C_1d_3 which was believed to be biologically active in doses of 0.1 mg. daily. Dr Carr and Mr Jewell, who very kindly carried out the fractionation of this material in a Burch molecular still, reported that "no sign of a constant-boiling fraction could be observed".

The fractions were examined with the following results (Table V).

On the assumption that the persistence of the $294\text{ m}\mu$ is a measure of vitamin activity fraction 2 from the distillation should have been the most potent material we have possessed. Unfortunately, the biological tests were inconclusive owing to a large proportion of the animals showing infertile matings. Two

Table V.

Fraction	Temp.	Wt. g.	Iodine value	$E_{1\text{ cm.}}^{1\%}$		Ratio 294/ 267
				294	267	
1	76–103°	0.3	169	13.5	8.16	1.65
2	96–127°	0.45	164	40.9	12.8	3.18
3	123–180°	0.45	151	45.0	17.6	2.56
4	>180°	0.1	—	—	—	—

typical resorptions on a daily dose of 0.1 mg. of fraction 2 make us fear, however, that the material was not as potent as our interpretation of the spectroscopic data had led us to expect.

DISCUSSION.

Most of the work reported in this paper must be regarded as of a preliminary nature and at this stage it is impossible to consider the results from the standpoint of the chemical identity of vitamin E. If we were certain that the intensity of selective absorption at 294 $m\mu$ were a measure of vitamin E potency the problem would be greatly facilitated, but unfortunately at the moment there are discrepancies which make us hesitate to conclude that such is the case. In general we have found that biological potency and absorption at 294 $m\mu$ run parallel during the fractionation, and the results with irradiation and hydrogenation can be reconciled with the view that the vitamin is a substance showing selective absorption at this region of the ultra-violet.

There are, however, other observations, such, for example, as the apparently low biological activity of a distilled fraction showing relatively high intensity of absorption, which make us cautious. The difficulties are in many respects similar to those which were encountered in the early days of the investigation of vitamin A and the most serious one is the absence of any criteria of purity. The information we have obtained does not enable us to say whether our most highly active fractions represent nearly pure vitamin E or whether they are composed of a biologically inert substance in a state of comparative purity contaminated with a small amount of highly active vitamin.

We are inclined to think that our most potent fractions consist to a large extent of one substance. It is true that the evidence from fractional distillation experiments does not support this view, but the amounts subjected to fractionation by this method were so small that too much weight must not be attached to the results.

The analytical data recorded for this active fraction must also be accepted with reserve, until more definite information is forthcoming to show whether or not the material is reasonably homogeneous.

Our studies provide further confirmation of the relative stability of vitamin E. Of its chemical nature we can say little beyond expressing an opinion that it may prove to possess a polycyclic nucleus. We are inclined to dismiss the suggestion put forward by Euler *et al.* [1933] that it may be related to xanthophyll. The vitamin is probably a colourless substance or one showing very slight absorption in the visual spectrum.

SUMMARY.

1. An examination of the unsaponifiable fraction of wheat germ oil has been made with the object of throwing light on the chemical nature of vitamin E.
2. The following substances have been isolated from or detected in this material: sitosterol, dihydrositosterol, ergosterol, dihydroergosterol, lutein, kryptoxanthin, β -amyrin, squalene, a second hydrocarbon of the squalene type,

possibly identical with that found by Channon and Marrian in mammalian livers, a new liquid hydrocarbon which may have the composition $C_{15}H_{32}$ and a lipochrome which may be γ -carotene or rubixanthin. None of these substances appears to be related directly to the vitamin activity.

3. Fractional adsorption on aluminium oxide was found to effect a considerable concentration of vitamin E. The most potent fraction obtained was physiologically active in rat tests in doses of 0.1 mg. daily.

4. The most active fractions were pale yellow and somewhat viscous oils. In the absence of any criteria of purity it is worth recording that analyses of several such fractions show reasonably good agreement, the analytical data indicating a polycyclic structure possibly related to the sterols or amyryns. Two oxygen atoms are present in a molecule of mol. wt. about 440. One of these forms an acetyl derivative but the spectroscopic evidence suggests that the reacting grouping may be keto-enol. If so, the second oxygen is present in a form which does not react with acetic anhydride. The reactions with iodine and hydrogen suggest that three reacting double bonds are present. Whether these observations concern the vitamin itself or another substance which forms the greater part of the active fractions is as yet unknown.

5. The active fractions show a well-marked absorption band with a maximum at 294 $m\mu$ and a minimum at 267 $m\mu$. So far as this investigation has progressed it has shown that the vitamin activity tends to run parallel with the intensity of this band or, more particularly, with its "persistence". It is not yet finally determined whether the band is a characteristic of the vitamin or of an associated substance, but it is significant that with ultra-violet irradiation the reduction in "persistence" is accompanied by loss of biological activity, whereas treatment with hydrogen and palladium, which effects almost complete saturation of the material of the active fraction, does not appreciably lower either the "persistence" of the band or the value as a source of vitamin E.

6. The absorption band at 294 $m\mu$ disappears on acetylation but is shown again by the hydrolysed acetate. As other investigators have noted, biological activity is unaffected by acetylation. The position of the absorption band and its disappearance on acetylation are compatible with the substance containing a ketonic grouping capable of exhibiting tautomeric change.

7. Attempts were made to separate the ketonic substances present in the active fractions in the form of water-soluble mercury complexes. Small yields of "ketonic" material were obtained. These showed no concentration of the substance responsible for the 294 $m\mu$ band.

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