47. THE SPECTROSCOPIC DETECTION OF VITAMIN E IN THE TISSUES OF THE RAT¹

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(Received 29 January 1940)

SPECTROSCOPIC studies of concentrates of vitamin E prepared from wheat germ and cotton seed oils led to the detection of an absorption band at about 294 m μ [Bowden & Moore, 1933; Olcott, 1934; Martin *et al.* 1934; Drummond *et al.* 1935]. This band was later found to be characteristic of pure tocopherol whether natural [Evans *et al.* 1936] or synthetic [Karrer & Demole, 1938].

The detection and estimation of vitamin E in tissues by the spectroscopic method is, however, complicated by several difficulties. The extraction of the vitamin from the tissues may involve destruction or loss, particularly if saponification is included in the procedure [Emmerie & Engel, 1939]. On the other hand, if saponification is omitted substances other than vitamin E which contribute substantial absorption at 294 m μ are included. Moreover, when tocopherol is acetylated its absorption is decreased in intensity, with alteration of the maximum to about 285 m μ . The presence of tocopherol in the tissues in esterified form, a possibility which cannot be ruled out, might therefore make its spectroscopic detection more difficult. Finally the absorptions of α -tocopherol ($E_{1\text{ cm}}^{1\%}$, 294 m μ = ca. 77) and α -tocopherol acetate ($E_{1\text{ cm}}^{1\%}$, 285 m μ = ca. 42) are much less intense than that of vitamin A ($E_{1\text{ cm}}^{1\%}$, 328 m μ = ca. 1800). The strong absorption of vitamin A would make the spectroscopic detection of vitamin E impossible in the liver without the careful removal of the vitamin A by processes which might cause loss of vitamin E.

The rats used in the present work received diets containing vitamin A in amounts so adjusted as to permit maintenance, while being inadequate to allow storage in the liver. Spectroscopic examinations were made on tissues from animals deficient in vitamin E, and for comparison from others which had been given large amounts of wheat germ or synthetic tocopherol. The results obtained indicate that vitamin E may be detected spectroscopically in the fat of specially prepared animals. At its present stage of development, however, the method cannot be recommended for routine estimations.

EXPERIMENTAL

Material. Tissues were examined from rats under the following conditions: (1) After restriction to a diet deficient in vitamin E (light white caseinogen 25 %, rice starch 50 %, lard 20 %, salt mixture 5 % + dried yeast 15 %, radiostol (vitamin D) one drop per week). The casein gave enough vitamin A for maintenance or slow growth, but not enough to allow storage in the liver. (2) After

¹ A brief report of some of the results described in this paper was included in a communication by Moore *et al.* [1939] at the recent Symposium on Vitamin E held by the Food Group of the Society of Chemical Industry.

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restriction to the diet deficient in vitamin E followed by dosing with synthetic *dl*- α -tocopherol. (3) After maintenance for long periods on a diet containing wheat germ (casein, alcohol-extracted 20 %, wheat germ 60 %, arachis oil 15 %, salts 5 % + dried yeast 10 %). Even this large amount of wheat germ did not cause measurable storage of vitamin A in the liver.

Methods. The rats were killed by coal gas. The tissues were dissected and digested by mincing and heating with 5 % aqueous KOH. Digestion was usually complete in about 2 hr. Alcohol was then added to the digest and the fat extracted with ether. The extract was washed, evaporated, saponified for about 1 min. with hot alcoholic KOH and the unsaponifiable fraction separated in the usual way. Two precautions were necessary to prevent undue spectroscopic absorption in blank experiments. Ether of sp. gr. 0.730 (B.D.H.) which is satisfactory for use in vitamin A determinations by the SbCl_3 method [Davies, 1933] had to be redistilled. The use of anhydrous Na_2SO_4 in drying the ethereal extracts caused considerable absorption and was abandoned. In some experiments the absorption was measured before saponification of the fat. In a few instances duplicate determinations were done on extracts prepared by grinding the tissues with anhydrous Na_2SO_4 and extracting by the Soxhlet method, the extracts being carefully washed to remove Na_2SO_4 before evaporation.

Solutions of the extracts in alcohol or cyclohexane were examined with a Hilger H 290 spectrophotometer. The colorimetric method of Emmerie & Engel [1938], using FeCl_3 and α - α -dipyridyl, was also applied during the later stages of the investigation.

Calculation of results. Absorption data have been calculated on the weight of fresh tissue, without reference to the fat content, and are expressed as $E_{1\text{ cm.}}^{100\%}$ instead of the usual $E_{1\text{ cm.}}^{1\%}$ in order to adjust the decimal point to a convenient position. Results are given to two figures, although the second is of doubtful significance if the first is high.

Comprehensive dissections. In early experiments the dissections included brain, heart, lung, kidneys, liver, spleen, intestines, muscle, bone, blood, thymus, suprarenals, pituitary, testes, seminal vesicles, uterus, ovaries, fat deposits, urine and faeces. Such experiments were done on young rats reared for 2 months on the diet deficient in vitamin E, on a similar rat killed 48 hr. after receiving 60 mg. of synthetic *dl*- α -tocopherol, and on an adult rat which had received the diet containing 60 % of wheat germ for 2 years. Extractions were by the standard method including saponification. Even with the rat given wheat germ, which showed the absorption band of vitamin E in its fat deposits, the absorption of most of the small organs was too low to be measured. The absorption of large organs, with the possible exception of muscle, gave no indication of the presence of vitamin E. In all these rats, however, absorption due to unidentified substances was shown by some tissues, usually as an inflexion at about 280 $m\mu$. The intestinal tract, cleared of its contents, showed an absorption band at 275–280 $m\mu$. Faeces sometimes showed much general absorption, but the intensity varied greatly even with rats receiving the same diet. With faeces much higher results were obtained by extracting the fat by the Soxhlet method than by our usual procedure of digestion with alkali.

In later experiments, attention was usually confined to the fat deposits and liver. This organ was selected as being the main site of storage for some other vitamins and as yielding the next most highly absorbing extracts after fat and faeces.

Storage fats. Spectroscopic and colorimetric data on storage fat are given in Table 1. Absorption bands at 294 $m\mu$ were seen in 3 out of 4 fats taken from

Table 1. *Spectroscopic and colorimetric data on the unsaponifiable fractions of the fat deposits of rats given diets with and without vitamin E*

No. of rat	Treatment	Site of deposits	$E_{1.0\%}^{1.0\text{ cm.}}$		Nature of spectroscopic absorption	Max. tocopherol mg. per 10 g. by spectroscopic method	Tocopherol mg. per 10 g. by Emmerie's method
		I.P. = intra-peritoneal S.C. = subcutaneous	294 m μ	270 m μ			
Diet deficient in vitamin E							
1	Deficient diet for 2 months	I.P.	0.88	1.28	Inflexion 275 m μ (Fig. 1)	—	—
2	Deficient diet for 4 months	I.P.	0.35	0.44	Inflexion 290–270 m μ	0.45	0
Diet deficient in vitamin E with doses of tocopherol							
3*	Tocopherol acetate, 3 mg. per week for 4 months	I.P.	0.37	0.43	Slight inflexions 290 and 330 m μ	—	—
4	Tocopherol acetate, 25 mg. over 5 days	I.P.	0.93	1.4	? Slight inflexion 294 m μ	1.2	0.06
		S.C.	1.1	1.9	No inflexion	1.4	0.3
5	Killed 48 hr. after single dose 64 mg. tocopherol	I.P.	0.91	1.8	No inflexion	—	—
		S.C.	1.2	2.0	No inflexion	—	—
Diet with 60% of wheat germ							
6†	Wheat germ diet for 2 months	I.P.	0.27	0.35	Slight inflexion 280 m μ	—	—
		S.C.	2.0	2.6	Slight inflexion 280 m μ	2.6	4.1
7–11 mixed	Wheat germ diet for 3 months	I.P.	1.1	0.81	Shallow band 294 m μ	1.4	0.81
12‡	Wheat germ diet for 2 years	I.P.	5.6	3.2	Sharp band 294 m μ (Fig. 1)	—	—
13	Wheat germ diet for 2 years	I.P.	3.0	3.2	Inflexion 294 m μ	—	—
		S.C.	2.5	1.7	Band at 294 m μ	—	—

As a guide to the nature of the absorption curves data for $E_{1.0\%}^{1.0\text{ cm.}}$ at 294 and 270 m μ are given. The sharpness of absorption due to vitamin E is indicated by the degree of excess of E 294 over E 270 m μ .

* Rat No. 3 differed from all others in receiving a basal diet containing the same components in slightly different proportions and supplemented by 1 drop of halibut liver oil per week.

† Preliminary extraction of rats by Soxhlet extraction with ether. Colour test done on untreated subcutaneous fat, solution rather turbid.

‡ Muscle from rat No. 12 showed a marked inflexion at 294 m μ . This may have been due to adhering fat.

rats which had been given wheat germ. The sharpest and most intense band was found in the intraperitoneal fat of a rat which had been given wheat germ for about 2 years (Fig. 1). In fat from rats which had received the diet deficient in vitamin E, even when supplemented by tocopherol, the absorption in the region of 294 m μ was marked by no more than a slight inflexion. It may be noted that rat No. 3 was given tocopherol for a prolonged period, but at a level much lower than in the diet with wheat germ. Rats Nos. 4 and 5 were given large doses of tocopherol, but only for short periods.

The colorimetric method of Emmerie & Engel was described at a late stage in this investigation, and unfortunately was not available at the time of killing the rats (Nos. 12 and 13) which showed the highest reserves of vitamin E by the spectroscopic method. With the mixed intraperitoneal fat of rats Nos. 7–11 the colour reaction was positive. These rats had received wheat germ for 3 months. The reaction was also positive with the subcutaneous fat of rat No. 6, which had been given wheat germ for 2 months. No reaction was obtained with the

intraperitoneal fat of a rat (No. 2) which had received a diet deficient in vitamin E. The reaction was also insignificant with the intraperitoneal fat of a similar rat (No. 4) which had received large doses of tocopherol over a short period, although the subcutaneous fat of this animal gave a slight reaction.

In general the numerical agreement between the maximum values given by the spectroscopic method and results given by the colorimetric method was poor, indicating the presence of a considerable amount of non-specific absorption in most instances. In the one case (rats Nos. 7-11) in which both a definite absorption band and a positive colour reaction were observed, however, the results by the two methods were reasonably consistent, on the assumption that vitamin E contributed slightly more than half of the spectroscopic absorption. This would be in agreement with the shallow shape of the absorption band. With rat No. 6 the fact that the tocopherol content of the subcutaneous fat found by the colorimetric method exceeded the maximum value that could be obtained by the spectroscopic method may have been due to a slight cloudiness of the solution in the colour test.

In some instances the fats were examined spectroscopically before saponification. The absorption was always much greater than that of the unsaponifiable fractions, being at least 3 times greater in the region of 294 $m\mu$. This position is at the foot of the steep incline in absorption with decreasing wave-length which is seen in most fats. A distinct inflexion at about 275 $m\mu$ was present in some instances. By the colour reaction the mixed untreated fat of rats Nos. 7-11 gave a value of 0.88 mg. per 10 g. of tissues, which is only slightly higher than the 0.81 mg. found for the unsaponifiable matter.

Liver. Spectroscopic and colorimetric data for the unsaponifiable matter of liver fat are given in Table 2. In no instance was there any evidence of selective absorption at 294 $m\mu$. The general absorption of the extracts was possibly slightly greater for rats given wheat germ or tocopherol than for rats receiving the diet deficient in vitamin E.

Colour reactions were given by all livers examined. The differences between results for different rats were so small as to be of doubtful significance, and certainly did not reflect the widely varied tocopherol intakes. With the rats receiving wheat germ the tocopherol content given by the colour reaction was about half the maximum value found by the spectroscopic method. The absence of selective absorption at 294 $m\mu$ indicates that tocopherol, if present, must contribute only a fraction of the absorption at this position. There is, therefore, no inherent inconsistency between the spectroscopic and colorimetric data for these animals. For the same reason the data by the two methods for the rats on the diet deficient in vitamin E, with and without tocopherol, which are in good numerical agreement, are difficult to reconcile with the specificity of both the methods. Unidentified substances may contribute to the colour reaction.

Fig. 1. Spectroscopic absorption of the unsaponifiable fraction of the intraperitoneal fat of a rat (Table 1, No. 12) given wheat germ for 2 years (upper curve). The maximum is at 294 $m\mu$, the position characteristic of vitamin E. The lower curve is for the corresponding fraction from a rat (Table 1, No. 2) which had been restricted to a diet deficient in vitamin E.

Table 2. *Spectroscopic and colorimetric data on the unsaponifiable fractions of the liver fats of rats given diets with and without vitamin E*

No. of rat	Treatment	$E^{100\%}_{1\text{ cm.}}$		Nature of spectroscopic absorption	Max. tocopherol mg. per 10 g. by spectroscopic method	Tocopherol mg. per 10 g. by Emmerie's method
		294 m μ	270 m μ			
Diet deficient in vitamin E						
14	Deficient diet for 2 months	0.49	0.92	Inflexion 280 m μ	—	—
2	Deficient diet for 4 months	0.6	1.5	No inflexion	0.78	0.64
Diet deficient in vitamin E with doses of tocopherol						
4	Tocopherol acetate 25 mg. over 5 days	0.74	0.98	Slight inflexion 275 m μ	0.97	0.95
5	Killed 48 hr. after single dose 64 mg. tocopherol	1.1	1.5	Slight inflexion 285 m μ	—	—
Diet with 60% of wheat germ						
6	Wheat germ diet for 2 months	0.98	1.3	No inflexion	1.3	0.73
7-11 mixed	Wheat germ diet for 3 months	1.1	1.6	No inflexion	1.4	0.69
12	Wheat germ diet for 2 years	1.4	1.8	Inflexion 285 m μ	—	—

Table 3. *Spectroscopic and colorimetric data on untreated liver fats of rats given diets with and without vitamin E*

No. of rat	Treatment	$E^{100\%}_{1\text{cm.}}$			Nature of spectroscopic absorption	Intensity of purple colour on saponification	Max. tocopherol mg. per 10 g. by spectroscopic method	Tocopherol mg. per 10 g. by Emmerie's method
		294 mμ	280 mμ	250 mμ				
Diet deficient in vitamin E								
14	Deficient diet for 2 months	—	—	—	—	Very faint	—	—
2	Deficient diet for 4 months	3.1	6.9	5.4	Band 275 mμ	Strong	16	0.59
Diet deficient in vitamin E with doses of tocopherol								
4*	Tocopherol acetate 25 mg. over 5 days	2.3	4.9	6.1	Shallow band 275 mμ. High absorption in far u.v.	Faint	12	1.2
5	Killed 48 hr. after single dose 64 mg. tocopherol	—	—	—	—	Very faint	—	—
Diet with 60% of wheat germ								
6	Wheat germ diet for 2 months	1.8	5.8	3.2	Band 275 mμ	Strong	14	0.68
7-11 mixed	Wheat germ diet for 3 months	2.0	6.5	3.0	Band 275 mμ	Intense	15	1.1
13	Wheat germ diet for 2 years	—	—	—	—	Intense	—	—

* Fat obtained by Soxhlet extraction.

When the liver fats were examined without saponification (Table 3) a band at about 275 $m\mu$ was always observed. This position is between the maximum at 285 $m\mu$ for tocopherol acetate and the main maximum at 271 $m\mu$ for the highly absorbing substances obtained by Evans *et al.* [1936] from tocopherol by treatment with $AgNO_3$. The band at 275 $m\mu$ varied in sharpness in different extracts, but remained nearly constant in intensity, being slightly more intense for the rat which had received the deficient diet than for the others which had been given wheat germ or tocopherol. The assumption that this band is actually due to an ester of tocopherol seems untenable, since it would imply the presence of about 12 mg. of tocopherol in the liver of the animal which had received the diet deficient in vitamin E. The band at 275 $m\mu$ did not appear in the unsaponifiable fraction after saponification.

It may be mentioned that the saponification of the liver fats was always accompanied by the development of a transient bright purple colour of varying intensity. This colour reaction suggests the presence of vitamin K. No evidence of the complex absorption spectrum with maxima at 248, 261, 270 and 328 $m\mu$ as found by Dam *et al.* [1939] for this vitamin could be distinguished in the fat before saponification. Preliminary tests with chicks in collaboration with Miss E. M. Cruickshank, however, suggested that the liver fat from rats given wheat germ possessed some activity in promoting the clotting of blood. The possibility of the association of the disappearance of the 275 $m\mu$ absorption band with the development of the purple coloration may be kept in mind. The comparatively constant intensity of the absorption band must, however, be contrasted with the great variations in intensity of the purple coloration.

With rat No. 6 grinding of the liver tissues with anhydrous Na_2SO_4 followed by Soxhlet extraction gave an extract which absorbed nearly twice as strongly as the extract obtained by digestion with alkali. The sharpness of the absorption band at 275 $m\mu$ was much reduced, suggesting that the increased absorption was due to the inclusion of additional substances with non-selective absorption rather than to increased stability of the substance absorbing at 275 $m\mu$.

Results by the colorimetric method on the unsaponified liver fats were usually in fair agreement with those obtained after saponification.

DISCUSSION

Interpretation of the above results is complicated by the difficulties which have already been mentioned. The anomalous behaviour of tocopherol towards saponification under different conditions undoubtedly presents the most serious problem. Saponification, both cold and hot, was employed at various stages in the original concentration of vitamin E by Evans & Burr [1927]. In the present work sharp absorption bands at the position characteristic of vitamin E were shown by concentrates from storage fat which had been submitted to saponification with hot alcoholic KOH. On the other hand the work of Emmerie & Engel [1939] has shown that pure tocopherol is much less stable to alkali than previously supposed. We have ourselves found that both with synthetic *dl*- α -tocopherol and a concentrate prepared from wheat germ a brief hot saponification may cause 50 % deterioration as measured spectroscopically. Probably the stability of tocopherol towards KOH is influenced by such factors as the excess of KOH used, access to O_2 and the presence of other substances.

It would, therefore, seem permissible to accept the positive results obtained as qualitatively correct, while regarding negative results with caution. The band at 294 $m\mu$ shown by the unsaponifiable matter of the storage fat of rats given wheat germ indicates that significant amounts of vitamin E may be

accumulated. The quantity of about 5 mg. found in rat No. 12 is large when compared with the dose (about 2 mg.) necessary for fertility in the female, but represents only about the amount ingested daily in the wheat germ. Since the latter was given for about 2 years only about $\frac{1}{700}$ part of the tocopherol ingested was stored. It may also be noted that the spectroscopic absorption of the fat was much greater in rats which had been given wheat germ for 2 years than in those which had received the wheat germ for 2-3 months. The storage of vitamin E, at least as measured by the 294 $m\mu$ absorption band, therefore seems inefficient and slow.

With regard to the other tissues of the rats given wheat germ, and to all tissues of rats given the diet deficient in vitamin E with and without supplements of tocopherol, it must be assumed either that vitamin E was present in amounts too small to survive the processes of extraction, or that for some reason it was present in less stable form. The first explanation is in accordance with the early biological tests of Evans & Burr [1927], who failed to find any animal tissue that was a particularly rich source of vitamin E.

Our inability to trace large doses of tocopherol after ingestion by the rat is in agreement with the experience of Drummond [1939] who suggests that tocopherol may be changed in the tissues, probably with opening of the oxygen ring. Evans *et al.* [1936] have also shown that tocopherol may undergo changes in its chemical structure and absorption spectrum *in vitro* without complete loss of biological activity. The possibility that tocopherol, except in the storage fat, is distributed in the tissues in changed form cannot, therefore, be excluded. We have, however, been unable to associate the intensity of any of the unfamiliar absorption bands encountered in our experiments with the level of the tocopherol intake. The contrast between the storage of vitamin E by rats receiving the diet containing wheat germ and the failure to store by rats given tocopherol might suggest that the vitamin is present in wheat germ in more available form, but there is probably no need to seek an explanation beyond the quantitative superiority of the amounts of vitamin E given as wheat germ.

The purple colour given by liver fat on saponification is of interest in the present work in view of the points of similarity between members of the E and K vitamin groups, which differ chiefly in being derived from duroquinone and naphthoquinone nuclei respectively. If the purple chromogen in liver is in fact a vitamin K, its concentration in liver and absence from storage fat presents a striking contrast with the distribution of the chemically similar vitamin E.

In our limited experience with the colorimetric method of Emmerie & Engel the results for storage fats were qualitatively consistent with the spectroscopic data. With liver extracts our results suggest that the colour reaction may be given by substances other than tocopherol. It was stated by the originators of the method that reactions are also given by carotene and vitamin A, but these were not present in amounts sufficient for detection by yellow colour or the $SbCl_3$ method.

SUMMARY

1. Rats were kept on diets low in vitamin A to prevent the accumulation of reserves of vitamin A, and their tissues were then examined for vitamin E by the spectroscopic method.

2. The investigation was complicated by the possible effects of the processes of extraction, and particularly the use of alkali for digestion and saponification, on the stability of the vitamin. Treatment designed to conserve the vitamin led to the inclusion in the extract of other absorbing substances.

3. Even when alkali was used for digestion and saponification the absorption band at 294 $m\mu$ which is characteristic of vitamin E was shown by the depot fat of rats which had been given much wheat germ over long periods. The vitamin was not detected spectroscopically in other tissues.

4. When large doses of *dl*- α -tocopherol, or its acetate, were given to rats over short periods no clear spectroscopic evidence of its storage in the tissues was obtained.

5. In some tissues, even from rats not receiving vitamin E, unidentified substances were present which had absorption near the maximum for esterified tocopherol. Thus, liver fat showed before saponification a relatively intense band at 275 $m\mu$. The intestines also showed a band at about 280 $m\mu$. This band also seemed to be uninfluenced by the dietary history, but differed from the band in liver in resisting saponification.

6. In a few experiments the spectroscopic data were compared with results by the colorimetric method of Emmerie & Engel. In the fat deposits, in qualitative agreement with the spectroscopic results, the vitamin was only detected in significant amounts in rats which had received wheat germ. In liver the amounts of vitamin E present according to the colour test showed little variation with dietary history. Comparison with the spectroscopic data suggested that in liver substances other than tocopherol may have contributed to the colour reaction.

7. On saponification rat-liver fats gave a transient bright purple colour reminiscent of vitamin K.

Our thanks are due to Dr L. J. Harris for his valuable criticism and to Messrs Roche Products, Ltd., for generous gifts of tocopherol.

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