6. THE FATE OF TOCOPHEROLS IN THE ANIMAL BODY

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VERY little is recorded in the literature regarding the fate of the tocopherols in the animal body. The original studies of Evans & Burr [1927] gave strong indications that vitamin E is not stored in tissues to any significant extent and also that such reserves as there may be are gradually reduced as a result of normal metabolic processes when the diet is deficient in that respect. They emphasized that the rate of utilization was not increased by gestation, as might perhaps have been expected.

We have attempted to study the fate of the tocopherols of wheat germ oil by a series of metabolism experiments followed by the examination of the tissues of the animals.

Experiment 1

Two groups, each of 4 adult bucks and 4 does, were compared. One had been fed on a vitamin E-deficient diet for 4 months after weaning. The other had had an adequate diet for the same period. The compositions of the two diets were:

	Vitamin E- deficient	Adequate
Extracted casein (Glaxo)	18	18
Rice starch	45	45
Lard	12	12
Yeast extract (Yeastrel)	8	8
Cane sugar	10	10
Salt mixture	5	5
Cod-liver oil	2	—
Cottonseed oil + 1 % halibut-liver oil	—	2

Each group of rats was kept in a metabolism cage and records were kept of food consumption, body weight etc. After a preliminary period of 3 days, urine and faeces were collected daily over a period of a week; great care was taken to avoid any contamination of either by food particles.

The faeces were ground with anhydrous Na_2SO_4 and the resulting powder was fully extracted with purified ether. The urines were brought to approximately pH 1 and extracted with ether in a continuous-extraction apparatus for 6 hr. The ether was removed from the extracts of urine and faeces and the residues were extracted with light petroleum until no more material went into solution.

Spectroscopic examination of the petroleum-soluble fractions, representing periods of a week for each group, both before and after chromatographic adsorption on aluminium oxide columns failed to reveal any selective absorption which might be taken as indicating the presence of tocopherols. The fractions from the urine extracts showed strong absorption in the region 270–280 $\text{m}\mu$ which was traced to the presence of phenolic substances easily removed by washing with

dilute alkali. Their removal did not unmask any recognizable absorption likely to be due to tocopherols.

Each animal was then given a daily dose of a wheat germ oil concentrate containing approximately 45 % of the natural mixture of α - and β -tocopherols. The concentrate was diluted with 3 parts of olive oil to facilitate dosing and each rat received an amount equivalent to 3.5 mg. of tocopherols daily for a week, the measured dose being given directly into the mouth by means of an Agla syringe.

Spectroscopic examination of extracts of the urines failed to show any trace of tocopherols, even after the extracts had been fractionated by chromatographic adsorption. The fats from the faeces gave clear indications of vitamin E which had presumably escaped absorption from the intestine. Estimates placed this amount at from 3 to 15 % of the total amount administered over the whole week, an interesting point being that the males consistently excreted a larger proportion (10–15 %) than the females (3–6 %).

At the conclusion of the experiment the animals were killed and tissues were removed from them and dehydrated by grinding with anhydrous Na_2SO_4 . Ether extracts, after removal of the solvent and spectroscopic examination in *cyclohexane* solution, did not show selective absorption related to vitamin E in the case of the heart, kidney, adrenals, testes, ovaries, liver or pituitary. Extracts of leg muscles and of body fat showed definite evidence of the presence of tocopherols.

It may be useful to record some of our observations on the spectroscopic examination of extracts of animal tissues for the tocopherols. Small amounts of these substances may easily be missed when they are insufficient (i.e. less than about 0.1 %) to produce an inflexion in the curve representing the ultra-violet absorption of the fat itself. In all such doubtful cases the unsaponifiable fraction, prepared with care to minimize losses by oxidation, should be examined.

Fig. 1. Influence of different amounts of α -tocopherol on the shape of the absorption curves of rat kidney fat.

Amounts of tocopherols sufficient to give rise to distinct inflexions in the absorption curves of the crude extracts from tissues do not always reveal themselves by maxima at 290–294 $\text{m}\mu$. Low concentrations may influence the curve

in the vicinity of 270–280 $m\mu$. With increasing concentration the position of the maximum tends to shift towards the region characteristic of the tocopherols. This shift is illustrated by the curves in Fig. 1. In our experience the direct spectroscopic estimation of tocopherols in crude tissue extracts is unreliable if the proportion is much below 1%, but examination of the unsaponifiable fraction will enable quantities down to about 0.1% to be determined satisfactorily. Actually, however, we have found it advisable, whenever relatively small amounts are likely to be present, to oxidize the unsaponifiable fraction

Fig. 2. Influence of 0.1 and 0.4% of α -tocopherol on absorption curve of body-fat from rats on a vitamin E-deficient diet.

Fig. 3.

Fig. 3. Absorption curves of the unsaponifiable fraction of rat body-fats containing respectively 0, 0.1 and 0.4% of α -tocopherol.

Fig. 4.

Fig. 4. Absorption curves of oxidized unsaponifiable fractions of rat body-fat containing respectively 0, 0.1 and 0.4% α -tocopherol.

with silver nitrate so that the tocopherols are quantitatively converted into the quinones [Moss *et al.* 1938] which show well-marked maxima in the vicinity of 265 $m\mu$ with an intensity about 4 times as great as that of the parent substances.

This modification of the spectroscopic method enables one to detect as little as 0.01 % of a tocopherol in an unsaponifiable fraction and to estimate with reasonable accuracy proportions above 0.03 %. For oxidizing the unsaponifiable fractions we employed a 5 % solution of silver nitrate in 90 % methyl alcohol acting at 65° for only 5 min. This method appears to have advantages over that employed by John [1938] in giving a cleaner material and a better yield. The spectroscopic examination of samples of rat body-fat with added tocopherol and of the corresponding unsaponifiable fractions before and after oxidation are shown in Figs. 2-4.

The detection of traces of tocopherols in extracts of the leg-muscles and of significant amounts in the body-fat of the rats employed in Exp. 1 recall the observations of Evans & Burr [1927].

Experiment 2

In this metabolism experiment a group of 10 rats which had previously been fed for 12 months on a diet unusually rich in natural tocopherols was examined. They received the same diet during the period of 3 weeks that they were under study. The diet was composed of:

Casein	18
Rice starch	48
"Fertilol" (wheat germ oil of tested potency)	12
Olive oil	.8
Dried yeast	10
Salt mixture	4
Cod-liver oil	Bi-weekly dose to provide vitamin A

We again found evidence of incomplete absorption, for the faecal fats accounted for approximately 25 % of the tocopherols contained in the basal diet.

The examination of the urines from this group of rats gave very interesting results. To simplify spectroscopic examination and to facilitate separation from phenolic substances the fats were saponified carefully under conditions giving protection against oxidation.

Material was present which absorbed selectively in the vicinity of 290 $m\mu$ and was recognized as tocopherol. Estimations indicated that the urine passed by 10 adult rats fed on this vitamin E-rich diet for 19 days contained altogether about 6-8 mg. of tocopherols.

Colorimetric estimations carried out on the fractions by use of the ferric chloride- $\alpha\alpha$ -dipyridyl method of Emmerie & Engel [1938; 1939, 1] gave values of the same order. It seems established, therefore, that tocopherols pass into the urines of rats in small quantities when their diet is very rich in that respect.

Spectroscopic examination was made of extracts of tissues removed from these rats. It revealed storage of vitamin E in the body-fat (approximately 0.04 %) and the presence of traces in the blood. Other tissues gave negative results, including the liver.

We were at first in doubt whether it would be possible easily to detect vitamin E by spectroscopic means in extracts, such as those from livers, which might show considerable absorption at or near 328 $m\mu$ as a consequence of the presence of vitamin A. This was put to direct test by examining rat liver fat to which known quantities of vitamin A and α -tocopherol acetate had been added. It was found that quantities of the latter of the order of 0.02 % were detectable.

Extracts of the livers of the rats of Exp. 2 showed no sign of absorption at or near $263\text{ m}\mu$ after oxidation of the unsaponifiable fraction with silver nitrate under the conditions we have described. We support, therefore, the view of Moore *et al.* [1939] that storage of vitamin E in the liver does not occur when rats are fed on a diet rich in tocopherols. This method enabled us to detect traces of tocopherols in the blood of these rats, but the amounts were too small to be estimated with any reliability by the spectroscope. They were probably of the same order as the concentrations ($0.03\text{--}0.09\text{ mg./100 ml.}$) found by Emmerie & Engel [1939, 2] in the serum of rats after oral administration of doses of $1\text{--}5\text{ mg.}$ of tocopherol.

Our detection of what appeared to be vitamin E in the urine of rats which had been living for a long time on a diet unusually rich in that substance had some bearing on a curious observation made by Jones [1936; 1937; 1938] in certain studies on the excretion of oestrogens by pregnant women. Following up the discovery that in certain cases there were marked discrepancies between the colorimetric estimation of these compounds and the values given by spectroscopic assays, he reached the conclusion that in a normal pregnancy there is a substance in the urine which shows absorption in the same region of the ultra-violet as oestriol and oestrone and which may be responsible for the higher values which the spectroscopic method sometimes gives. He attempted to isolate this substance and at length obtained from the toluene residues yielded by Marrian's well-known process for the separation of urinary oestrogens a few mg. of a crystalline material, m.p. $225\text{--}230^\circ$, possessing some oestrogenic activity and showing marked selective absorption at $294\text{ m}\mu$. He suspected that it might be vitamin E or a closely related compound, not only because of its spectroscopic properties but because it appeared to be absent from urines in cases in which intra-uterine death had occurred.

We have been unable to confirm his more important claims. Quantities of the order of 15 l. of urine from women in the latter part of pregnancy were evaporated down to 1 l. and hydrolysed with HCl at pH 1 by heating in the autoclave for 2 hr. at 15 lb. pressure. The residue was extracted with ether and the extract was washed, first with several quantities of 10% Na_2CO_3 and secondly with several changes of $N/10\text{ NaOH}$. The washed ether layer was freed from solvent, taken up in toluene and extracted several times with $N\text{ NaOH}$. The toluene-soluble residue was a bright red, viscous oil which on recrystallization from ethanol yielded a few mg. of a crystalline substance with a low melting point, but which was spectroscopically transparent. The residual solution on evaporating down and cooling yielded a further crop of crystalline material. This proved, after several recrystallizations from alcohol, to be a compound similar to that isolated by Jones. It melted at $228\text{--}229^\circ$ and, although not purified fully, was almost certainly impure pregnandiol. It was spectroscopically transparent.

Examination of the mother liquors showed distinct signs of selective absorption in the vicinity of $280\text{ m}\mu$. The thick, red, oily material was, therefore, dissolved in benzene and subjected to chromatographic adsorption on aluminium oxide. Several fractions showed on spectroscopic examination pronounced bands between 280 and $290\text{ m}\mu$ which seemed to indicate the presence of tocopherols or closely related substances. Estimations were then made on all the fractions by the colorimetric method of Emmerie & Engel [1938; 1939, 1]. The reducing powers of the extracts corresponded to amounts of tocopherols approximately half those indicated by the spectroscopic data. This was probably due to interference caused by other substances absorbing in the vicinity of $280\text{ m}\mu$.

The whole of the fractions representing 15 l. of urine from late pregnancy cases contained about 10 mg. of material estimated as tocopherols by the reduction method.

It appears reasonably certain, therefore, that the material isolated by Jones was impure pregnandiol, contaminated with traces of oestrogens and vitamin E. McArthur & Watson [1939] have recently reported their failure to detect vitamin E in fractions separated from the ether extract of 40 gallons of non-pregnant cows' urine.

It seems clear from these results, taken as a whole, that the tocopherol molecule is readily broken down in the body of the rat and that storage occurs in the adipose tissues only when the diet provides an unusually high intake of vitamin E. The results of the experiments do not give any indication of the function of the vitamin in the tissues.

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