THE METABOLISM OF VITAMIN E

I. THE ABSORPTION AND EXCRETION OF d-α-TOCOPHERYL-5-METHYL-C14-SUCCINATE

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Vitamin E is widely distributed throughout the tissues of animals and man (2–4), and its deficiency causes a variety of syndromes in laboratory and farm animals (5). Very little information, however, is available concerning either the metabolic fate or the biochemical rôle of this vitamin. We have, therefore, undertaken a study of the metabolism of α-tocopherol.

It has been reported that α-tocopherol and α-tocopherylquinone were not excreted in the urine of animals even after the administration of large doses of vitamin E (6, 7). Some excretion of absorbed tocopherol into the intestinal tract has been postulated (7, 8). In this report we shall present definitive evidence both for the presence of a metabolic product of α-tocopherol in the urine and for the excretion of α-tocopherol and its metabolic products from the blood stream into the intestinal tract.

Materials and Methods

d α Tocopheryl 5 methyl C14-succinate—The crystalline succinate ester of d-α-tocopherol labeled with C14 in the 5-methyl group was used in these studies.1 The material had a specific activity of 0.147 µc. per mg.

β-D-Glucuronidase—Beef spleen and rat liver preparations of β-D-glucuronidase (9) were used.2

Administration and Collection—Young rabbits (1 to 2 kilos) maintained on Diet 11 of Goettch and Pappenheimer (10) supplemented with dl-α-tocopherol were kept in metabolism cages which permitted separation and

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A preliminary report on this work was presented before the 44th meeting of the American Society for Pharmacology and Experimental Therapeutics at Atlantic City, April, 1954 (1).

1 The authors wish to thank Dr. P. L. Harris of Distillation Products Industries for making available the labeled d-α-tocopheryl succinate.

2 The authors are indebted to Dr. M. Levitz of New York University College of Medicine, and Dr. D. Dziewiatkowski of The Rockefeller Institute for Medical Research for generous quantities of β-D-glucuronidase preparations.

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complete collections of all feces and urine eliminated during each 24 hour period. The $d$-$\alpha$-tocopheryl-5-methyl-C$^{14}$-succinate in doses of 10 to 15 mg. (1.5 to 2 $\mu$c.) was administered by various routes. The material was dissolved in 1 ml. of sesame oil for oral and subcutaneous administration. For intravenous injection it was dissolved in 1 ml. of a mixture of 9 parts of 5 per cent aqueous triethanolamine and 1 part of ethanol. Massive doses were administered by mixing 200 mg. of unlabeled $d$-$\alpha$-tocopheryl succinate with the tracer dose in 6 ml. of aqueous triethanolamine-ethanol.

**Extraction of Radioactive Materials**—Urine was acidified to pH 1 to 2 and extracted continuously with ether for 48 hours. About 90 per cent of the radioactivity was recovered in this manner. Longer extraction times did not result in an increased yield of C$^{14}$. Feces were dried in vacuum and ground to a fine powder. Continuous extraction with ethanol in a Soxhlet apparatus for 24 hours yielded 80 to 90 per cent of the radioactivity. The yields were not improved by increasing the time of extraction.

**Methods of Counting**—Whenever levels of activity permitted, samples to be counted were converted to barium carbonate by a modification (11) of the wet combustion method of Van Slyke and Folch (12) and counted at “infinite thickness” in stainless steel planchets in a Tracerlab windowless gas flow counter. The factor relating counts per minute of “infinitely thick” barium carbonate to microcuries was determined by counting as barium carbonate a sample of $d$-$\alpha$-tocopheryl-5-methyl-C$^{14}$-succinate of known weight and specific activity.

When combustion was not feasible, small urine aliquots were dried slowly in tared planchets under an infra-red lamp and counted. A self-absorption curve for these samples was constructed by counting a known quantity of $d$-$\alpha$-tocopheryl-5-methyl-C$^{14}$-succinate with various quantities of urine residues. Samples of dried feces were counted directly at “infinite thickness.” Conversion of counts per minute at “infinite thickness” to microcuries was accomplished by counting fecal samples of sufficiently high specific activity both directly and as barium carbonate. Agreement between counts by direct plating and combustion was reasonably good (±10 per cent).

**Hydrolysis of Urinary Excretion Product; Acid Hydrolysis**—To the residue of an evaporated ether extract of a 24 hour specimen of urine 100 ml. of 3 $N$ HCl were added, and the mixture was refluxed for 2 hours. On cooling to room temperature the hydrolysis mixture was extracted with petroleum ether for 24 hours.

**Enzyme Hydrolysis**—The residue of an evaporated ether extract of a 24 hour specimen of urine was dissolved in 40 ml. of water, the pH was adjusted to 4.5 with solid sodium bicarbonate, and 4 ml. of acetate buffer (pH 4.5) were added. The mixture was incubated at 37° for 5 days with $\beta$-d-glu-
curonidase in concentration of 400 Fishman units per ml. Penicillin and Streptomycin, in concentrations of 25 units and 25 $\gamma$ per ml., respectively, were added to prevent bacterial growth. The incubation mixture was subsequently extracted with petroleum ether for 24 hours.

Ultraviolet Spectra—Ultraviolet spectra were determined in purified ethanol (distilled over potassium permanganate and potassium hydroxide) and "spectrograde" isooctane (Phillips Petroleum) in silica cells with a 1 cm. light path in a Beckman DU spectrophotometer.

Paper Chromatography—Two reverse phase paper chromatographic systems, developed for the separation of the tocopherols, were employed: the system of F. Brown (13) as modified by Eggitt and Ward (14) in which Whatman No. 1 paper is impregnated with light petroleum, B. P., Nujol, and the chromatogram is developed with 75 per cent ethanol, and the system of J. A. Brown (15) in which Whatman No. 1 paper is treated with silicone stop-cock grease and developed with appropriate mixtures of acetonitrile and water. Spots were identified by scanning under an ultraviolet lamp and, in the case of $\alpha$-tocopherol, by spraying with a ferric chloride-dipyridyl reagent in glacial acetic acid (16).

Isotope Dilution Technique—Known quantities of unlabeled $d$-$\alpha$-tocopherol or $d$-$\alpha$-tocopheryl succinate were added to extracts of feces or urine, and isolated as the crystalline succinate ester. In instances when free $\alpha$-tocopherol had been added, the succinate was prepared by refluxing the dry residue with an excess of succinic anhydride in a few ml. of pyridine for 3 hours. Ether was added to precipitate the excess anhydride, which was removed by filtration. Repeated washings with 5 per cent HCl removed the pyridine. Ether was removed by evaporation. Isolation of tocopheryl succinate was achieved in the following manner: the mixture was dissolved in 83 per cent ethanol and made slightly alkaline. Extraction with three portions of petroleum ether removed impurities. After acidification of the ethanol solution, the tocopheryl succinate was extracted into petroleum ether. The crude tocopheryl succinate was recrystallized to constant specific activity from isooctane or petroleum ether. The total radioactivity of the tocopheryl succinate (specific activity times theoretical yield) divided by the total radioactivity in the extract represents the fraction of isotopic material present in the extract as tocopherol or tocopheryl succinate.

Isolation of Fecal Metabolite—A crude fecal metabolite was isolated by a modification of the series of steps described by F. Brown (13). p-Acetylaminophenol (17) was found to be a better antioxidant than pyrogallol during the saponification. In the chromatographic step $\alpha$-tocopherol was eluted from Florex XXS with benzene; however, ether-ethanol mixtures were necessary to elute the metabolite.
RESULTS AND DISCUSSION

Absorption and Excretion Studies—The oral administration of a sesame oil solution of \(d\)-\(\alpha\)-tocopheryl-5-methyl-\(\text{C}^{14}\)-succinate led to a rapid elimination of radioactivity in the feces, as is shown in Fig. 1. Over 74 per cent of the administered dose appeared in the stool within 3 days after administration. More than 93 per cent of the fecal excretion product was shown by the isotope dilution technique to be either \(\alpha\)-tocopherol or \(\alpha\)-tocopheryl succinate. Only traces of radioactivity were found in the urine.

The subcutaneous administration of labeled \(\alpha\)-tocopheryl succinate in oil was followed by very slow elimination of radioactivity (Fig. 2). During

![Fig. 1. Excretion of radioactivity in feces after oral administration of a tracer dose of \(d\)-\(\alpha\)-tocopheryl-5-methyl-\(\text{C}^{14}\)-succinate.](http://www.jbc.org/)

![Fig. 2. Excretion patterns after subcutaneous and intravenous administration of tracer doses of \(d\)-\(\alpha\)-tocopheryl-5-methyl-\(\text{C}^{14}\)-succinate.](http://www.jbc.org/)

11 days only 10 per cent of the dose appeared in the feces and 4 per cent in the urine. When the animal was sacrificed on the 11th day after injection, 14 per cent of the administered dose was found at the site of injection.

When the isotopic vitamin E was given by the intravenous route, considerable radioactivity was eliminated in both urine and feces, as shown in Fig. 2. The urinary activity was highest on the 1st day and decreased rapidly thereafter, while the fecal output reached its peak on the 3rd or 4th day after injection and fell off more slowly. A period of 15 to 20 days was required for complete elimination of the isotopic material as shown by the absence of detectable radioactivity from both urine and feces after this period. The total recovery of \(\text{C}^{14}\) from the excreta comprised 70 to 75 per cent of the administered dose. About 20 to 30 per cent of the recovered radioactivity appeared in the urine, while 70 to 80 per cent was found in the feces.
When a "massive dose" of tocopheryl succinate was administered by the intravenous route, the total amounts of radioactivity appearing daily in the excreta were roughly the same as when only the tracer dose was given. This would seem to indicate that the fraction of the dose that is eliminated by the body is essentially independent of the size of the dose in the range of 10 to 220 mg.

Isotope dilution studies showed that in the feces of rabbits given \( \alpha \)-tocopheryl succinate either subcutaneously or intravenously 40 to 50 per cent of the ethanol-extractable excretion products was free \( \alpha \)-tocopherol. Virtually all of the succinate ester had been hydrolyzed in the body.

Previous reports on the absorption of vitamin E are few and are limited largely to studies of absorption from the gastrointestinal tract (18, 19). The results were usually based on the chemical determination of tocopherol and tocopherylquinone in the feces and are somewhat open to question in view of the possibility of excretion of absorbed tocopherol into the intestinal tract. The use of isotopic tocopherol permits the estimation of absorption by comparison of the rate of fecal excretion, the level of urinary radioactivity, and the nature of the fecal excretion products with the corresponding results obtained on intravenous administration of the material. Such a comparison leads to the conclusion that most of the labeled material recovered in the feces after oral administration, particularly during the first 3 days, must have been unabsorbed vitamin E, since after intravenous injection only 12 per cent of the dose had appeared in the stool during the same period, and since only 40 to 50 per cent of this material was \( \alpha \)-tocopherol. A consideration of these results and of the relative levels of urinary radioactivity led us to estimate that the amount of \( \alpha \)-tocopheryl succinate absorbed from the gastrointestinal tract in this experiment did not exceed 10 per cent of the oral dose.

It is of interest that following subcutaneous administration the excretion patterns, including the relative amounts of radioactivity in urine and feces, resemble closely those obtained on intravenous injection, except for smaller absolute amounts of daily excretion and a delay of both maxima by 6 days, presumably due to the very slow rate of absorption from the subcutaneous site. Since the amount of tocopherol absorbed averaged approximately 1 mg. per day, or about the quantity an animal may be expected to absorb from its food, this may be taken as evidence that these excretion patterns would hold under physiological conditions.

A direct investigation of radioactivity present in the respiratory carbon dioxide was not feasible because of the low level of radioactivity of the administered material. It seems justified to conclude, in view of the high recovery of radioactivity in urine and feces over such a long period, that oxidation of the carbon atom of the 5-methyl group and its excretion as carbon dioxide can at best be a very minor pathway of elimination.
The data presented indicate that a significant portion of parenterally administered tocopherol is eliminated in some form in the urine and that excretion from the blood stream into the intestinal tract takes place to a large degree. These results bear considerable similarity to the findings of Siperstein and Chaikoff (20) on the elimination by the rat of intravenously administered C<sup>14</sup>-cholesterol labeled in the steroid nucleus. These authors found that virtually all of the isotopic cholesterol was excreted into the intestinal tract and eliminated in the feces. The period of 15 days required for complete elimination of the administered dose as well as the shape of the fecal excretion curve is strikingly similar to the results reported here for vitamin E. The observation that elimination via the urine is a significant pathway for \( \alpha \)-tocopherol, while it is a negligible one for cholesterol, represents the most important difference between the modes of elimination of these two lipid materials.

**Nature of Excretion Products Following Parenteral Administration: In Feces**—It has already been pointed out that 40 to 50 per cent of the ethanol-extractable fecal excretion products was found to be free \( \alpha \)-tocopherol. Preliminary observations on the balance of the fecal radioactivity indicate that it is composed of materials of much more polar character than \( \alpha \)-tocopherol, as shown by its distribution between aqueous ethanol and petroleum ether and the behavior of a crude metabolite, isolated from the feces, in reversed phase paper chromatography. This material had an \( R_F \) of 0.88 in the system of Eggitt and Ward (14), in which the \( R_F \) of \( \alpha \)-tocopherol is 0.25. The nature of the fecal excretion products is under investigation.

**In Urine**—The reason for the negative findings of previous workers looking for urinary \( \alpha \)-tocopherol became clear when it was shown by isotope dilution as well as by paper chromatographic procedures that no detectable \( \alpha \)-tocopherol or \( \alpha \)-tocopheryl succinate was present in urine. On the assumption that small quantities of tocopherol, if they were to appear in the urine, would most likely be present early after administration, when the blood \( \alpha \)-tocopheryl succinate level is highest, the urine of one rabbit was collected by catheter during the 6 hours immediately following intravenous injection. The first urine sample obtained 2 hours after injection had the highest specific activity, but, even here, metabolic products of \( \alpha \)-tocopherol accounted for 99 per cent of the radioactivity.

Over 90 per cent of the isotopic material in the urine was strongly acidic and could be removed from acidified urine by continuous ether extraction for 48 hours. Continuous extraction with petroleum ether removed less than 10 per cent of the total radioactivity.

Acid hydrolysis of the evaporated ether extracts increased the amount of petroleum ether-extractable isotopic material to 60 to 70 per cent, while incubation with the enzyme \( \beta \)-d-glucuronidase gave a petroleum ether-
soluble radioactive substance in 70 to 90 per cent yield. These results provided evidence that the major excretion product may be a glucuronide.

Since hydrolysis and petroleum ether extraction gave a considerably purified product, as indicated by its spectrum and specific activity, our attention was directed towards a study of this material. Urines of rabbits injected intravenously with massive doses of \( \alpha \)-tocopheryl succinate were used. The petroleum ether extracts of acid or enzyme hydrolysates from the urines of such rabbits showed the characteristic absorption spectrum depicted in Fig. 3. The proportionality of the optical density at the max-

![Optical Density vs Wavelength](image)

**Fig. 3.** Ultraviolet absorption spectra of urinary metabolite after hydrolysis and petroleum ether extraction. Curve A, after enzyme hydrolysis; Curve B, after acid hydrolysis.

ima to the specific activity of the sample as well as the similarity of the spectrum to that of \( \alpha \)-tocopherylquinone (21), from which it differs only by the presence of end-absorption in the short ultraviolet region, was taken as evidence that the spectrum is really due to the metabolite rather than to impurities. The substance was shown not to be \( \alpha \)-tocopherylquinone by its considerably more polar character. While the latter is quantitatively extracted from 50 per cent ethanol by one extraction with an equal volume of petroleum ether, the metabolite distributes itself between these solvents at a ratio of 3:1 in favor of the aqueous ethanol. In the chromatographic system of Eggitt and Ward (14) the metabolite had an \( R_F \) of 0.88 compared to 0.50 for \( \alpha \)-tocopherylquinone and 0.25 for \( \alpha \)-tocopherol. An even more striking demonstration of the vast difference in polarity is shown in Table I,
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which gives the $R_f$ values for these compounds in the system of J. A. Brown (15).

Whereas tocopherol and its quinone separated only slightly in this system, the metabolite traveled with the solvent front in concentrations of aqueous acetonitrile in which tocopherol and its quinone did not leave the origin.

Paper chromatography of the hydrolyzed material also indicated the complete absence of a conjugated form of $\alpha$-tocopherol.

The increase in polarity and the characteristic spectrum of the urinary metabolite obtained on hydrolysis point to oxidation of the chroman ring to the $p$-quinone state and to drastic reduction and possible alteration of the 16-carbon isoprenoid side chain of $\alpha$-tocopherol. Further purification

<table>
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<th>Compound</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>70</th>
<th>90</th>
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<tr>
<td>$\alpha$-Tocopherol</td>
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<td>0.74</td>
<td>0.83</td>
<td>S. F.*</td>
<td>S. F.</td>
<td>S. F.</td>
</tr>
<tr>
<td>$\alpha$-Tocopherylquinone</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0.35</td>
<td>0.82</td>
</tr>
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</table>

TABLE I

Paper Chromatography of $\alpha$-Tocopherol, $\alpha$-Tocopherylquinone, and Hydrolyzed Urinary Metabolite of Vitamin E

<table>
<thead>
<tr>
<th>RF value</th>
<th>20</th>
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<th>40</th>
<th>50</th>
<th>70</th>
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<td>0.82</td>
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<tr>
<td>S. F.*</td>
<td>S. F.</td>
<td>S. F.</td>
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* Solvent front.

and characterization of this compound had to await the discovery of a more lucrative source which permitted the accumulation of mg. quantities of this material. This work will be discussed in Paper II of this series (22).

SUMMARY

1. $d$-$\alpha$-Tocopheryl-5-methyl-$C^{14}$-succinate was administered to young rabbits by various routes, and its absorption and excretion were studied.

2. Absorption from the gastrointestinal tract and from the subcutaneous site was low when an oily vehicle was used.

3. Complete excretion of radioactivity after intravenous administration required 15 to 20 days. Urine contained 20 to 30 per cent and feces 70 to 80 per cent of the radioactivity recovered from the excreta.

4. The excretion pattern after subcutaneous injection resembled that obtained after intravenous administration, except for a delay of the maxima by 6 days.
5. Free α-tocopherol was found to comprise 40 to 50 per cent of the ethanol-extractable fecal radioactivity after parenteral administration of the tracer dose.

6. The bulk of the urinary radioactivity was shown to be a metabolic product of α-tocopherol. Evidence is presented that it may be conjugated with glucuronic acid. Some of its properties are discussed.

BIBLIOGRAPHY

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