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Ubiquinone and Related Compounds. XXVI.[1] The Urinary Metabolites of Phylloquinone and α-Tocopherol

Masazumi Watanabe, Mariko Toyoda, Isuke Imada, and Hiroshi Morimoto

Chemical Research Laboratories, Takeda Chemical Industries, Ltd.[2]

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Urinary metabolites from rabbits dosed with phylloquinone and *dl*-α-tocopheryl acetate were examined. We found that phylloquinone was excreted as conjugate forms of 2-methyl-3-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-naphthoquinone and 2-methyl-3-(3′-carboxybutyl)-1,4-naphthoquinone, and *dl*-α-tocopheryl acetate was excreted mainly as the conjugate form of 2,3,5-trimethyl-6-(5′-carboxy-3′-hydroxy-3′-methylpentyl)-1,4-benzoquinone lactone and in small amounts, as the conjugate form of 2,3,5-trimethyl-6-(3′-carboxybutyl)-1,4-benzoquinone.

Ubiquinone (Q), phylloquinone and tocopherol have a similar multiprenyl side chain. Simon, *et al.*[3] reported that α-tocopherol was excreted in human and rabbit urine as the β-glucuronide of 2,3,5-trimethyl-6-(5′-carboxy-3′-hydroxy-3′-methylpentyl)-1,4-benzoquinone lactone (tocopheronolactone). Gloor, *et al.*[4] and Wiss and Gloor[5] showed by the isotope dilution method that phylloquinone and ubiquinone-9 were metabolized *via* the same route to lactones corresponding to tocopheronolactone. We isolated several new metabolites from the tissues and excrements of rats dosed orally with ubiquinone-7.[6] The metabolite from the adrenals was found to be an ω-*cis*-carboxylic acid which was formed by oxidation of the *cis*-methyl group in the terminal isoprene unit, while the metabolite from the ovaries was the 26,27-dihydro derivative of the adrenal metabolite. In the urine, 2,3-dimethoxy-5-methyl-6-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-benzoquinone (Q Acid-I) and 2,3-dimethoxy-5-methyl-6-(3′-carboxybutyl)-1,4-benzoquinone (Q Acid-II) were found to be excreted as sulfates of the corresponding hydroquinone compounds, and 2,3-dimethoxy-5-methyl-6-(5′-carboxy-3′-hydroxy-3′-methylpentyl)-1,4-benzoquinone lactone (Q Lactone)[4,5] proved to be formed as an artifact from Q Acid-I during isolation. From these facts, we proposed a metabolic pathway of Q, in which the terminal *cis*-methyl in the isoprenoid side chain is oxidized, followed by β-oxidation to give Q Acid-I and Q Acid-II and then the metabolites are excreted into the urine mainly as sulfates of the respective hydroquinone compounds.

Supposing that phylloquinone, menaquinone and tocopherol would be metabolized similarly to Q, we studied their urinary metabolites.

Metabolites of Phylloquinone

Q Acid-I is easily lactonized by acid hydrolysis of its conjugate. To prevent the lactonization of Q Acid-I, lyophilized urine powder was treated with methanolic hydrochloric acid. By this procedure, the conjugates were hydrolyzed to their hydroquinone compounds

1) Part XXV: I. Imada, I. Azuma, S. Kishimoto, Y. Yamamura, and H. Morimoto, *Int. Arch. Allergy Appl. Immunol.*, 43, 898 (1972).
2) Location: *Juso-Nishinocho, Higashiyodogawa-ku, Osaka.*
3) E.J. Simon, A. Eisengart, L. Sundheim, and A.T. Milhorat, *J. Biol. Chem.*, 221, 807 (1956).
4) U. Gloor, J. Würsch, H. Mayer, O. Isler, and O. Wiss, *Helv. Chim. Acta*, 49, 2582 (1966).
5) O. Wiss and U. Gloor, *Vitam. Horm.*, 24, 575 (1966).
6) I. Imada, M. Watanabe, N. Matsumoto, and H. Morimoto, *Biochemistry*, 9, 2870 (1970).

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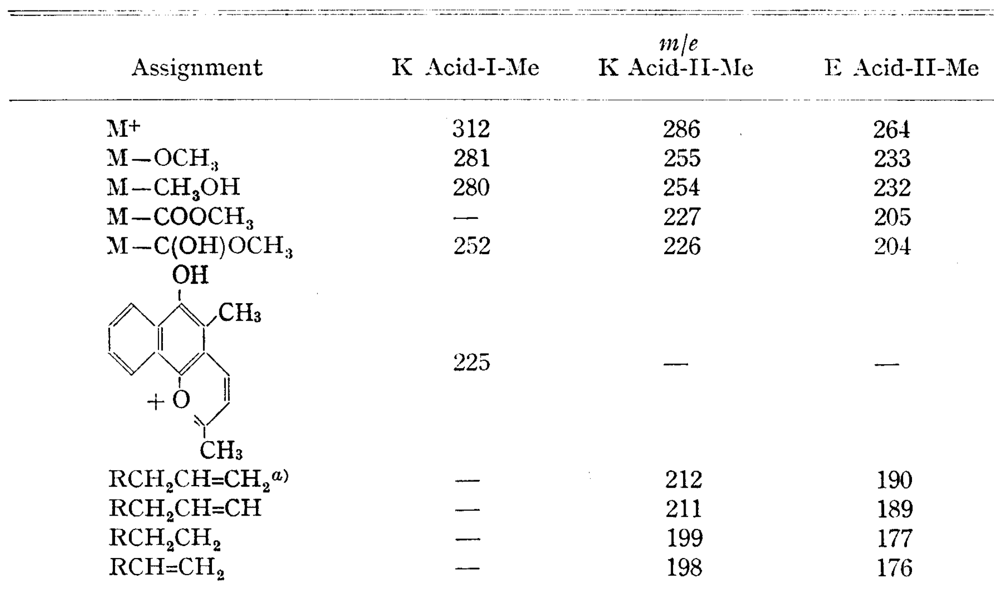
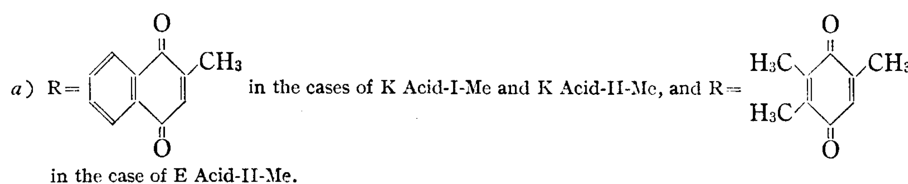
Table I. Mass Spectral Data of Metabolites of Phylloquinone and α-Tocopherol

Assignment	K Acid-I-Me	m/e K Acid-II-Me	E Acid-II-Me
M^+	312	286	264
$M-OCH_3$	281	255	233
$M-CH_3OH$	280	254	232
$M-COOCH_3$	—	227	205
$M-C(OH)OCH_3$	252	226	204
	225	—	—
$RCH_2CH{=}CH_2$[a]	—	212	190
$RCH_2CH{=}CH$	—	211	189
RCH_2CH_2	—	199	177
$RCH{=}CH_2$	—	198	176



a) R= (structure) in the cases of K Acid-I-Me and K Acid-II-Me, and R= (structure) in the case of E Acid-II-Me.

Table II. *Rf* Values of Metabolites of Phylloquinone in Several Solvent Systems

Compound	Solvent system[a]				
	A	B	C	D[b]	E[b]
K Acid-I-Me	0.73	0.65	0.94	0.36	0.71
K Acid-II-Me	0.73	0.67	0.94	0.43	0.69
K Acid-I	0.08	—	0.40	0.83	0.84
K Acid-II	0.08	—	0.42	0.85	0.74
K Lactone	0.39	0.16	0.82	0.57	0.86

a) solvent system: A, benzene–ethyl acetate (4 : 1); B, ether–hexane (3 : 2); C, chloroform–ether–ethanol (14: 5: 1); D, acetone–water (1: 1); E, acetonitrile–water (3: 2)
b) Reversed phase silica gel plate was used.

and simultaneously converted into the methyl esters.[6,7] Following this method, the lyophilized powder of urine from rabbits which had been injected intravenously with phylloquinone, was treated with methanolic hydrochloric acid. The resulting hydroquinone compounds were oxidized to quinone, then extracted with ethyl acetate. The lipid fraction was chromatographed on a column of silicic acid and then purified by thin–layer chromatography (TLC) using markers of synthetic 2-methyl-3-(3′-methyl-5′-methoxycarbonyl-2′-pentenyl)-1,4-naphthoquinone (K Acid-I-Me) and 2-methyl-3-(3′-methoxycarbonylbutyl)-1,4-naphthoquinone (K Acid-II-Me).[8] These quinones were difficult to separate from each other by the

7) M. Watanabe, M. Toyoda, R. Negishi, I. Imada, and H. Morimoto, *Internat. J. Vit. Nutr. Res.*, **41**, 51 (1971).
8) M. Watanabe, M. Kawada, M. Nishikawa, I. Imada, and H. Morimoto, *Chem. Pharm. Bull.* (Tokyo), 22 (1974), "in press".

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above procedure, but could be separated by reversed-phase TLC and identified with synthetic compounds by physicochemical data.[1] The mass spectral data and *Rf* values of TLC in various solvent systems of metabolites are shown in Table I and II, respectively. When the urine was treated with dilute hydrochloric acid instead of methanolic hydrochloric acid, only 2-methyl-3-(5′-carboxy-3′-hydroxy-3′-methylpentyl)-1,4-naphthoquinone lactone (K Lactone) was detected, and neither K Acid-I-Me nor K Acid-II-Me was detected in the acid fraction from the hydrolysate after treatment with diazomethane, respectively, by gas-liquid chromatography (GLC) (Table III). This showed that K Acid-I converted into K Lactone on acid hydrolysis. One reason for failure to detect K Acid-II is probably its lability to hydrochloric acid. Although the phylloquinone administered in our experiment was a mixture of *cis* and *trans* isomers in a 1: 3 ratio, the K Acid-I-Me isolated from urine was a mixture of *cis* and *trans* isomers in a 1: 2 ratio.[9] This difference of the *cis–trans* ratio is probably due to either interconversion of *cis* and *trans* isomers during isolation or a difference in metabolic behavior. The *cis*-isomers of phylloquinone and menaquinone are known to be isomerized to *trans* by irradiation[10] and have little or no biological activity.[11] The relationship between biological activities and metabolic behavior of these isomers seems interesting.

Table III. Metabolites from Urine[a] under Different Condition of Hydrolysis

Compound	Condition	
	1.5N methanolic HCl at room temperature for 15 hr	1.5N HCl at 75° for 2 hr
K Acid-I-Me	205.8 μg[b]	—
K Acid-II-Me	177.1	—
K Lactone	7.2	273.1

a) One-tenth volume of urine collected for 5 days after beginning of administration.
b) Measured by gas chromatographic analysis.

Table IV. Phylloquinone Metabolites from Urine

Group	K Acid-I-Me (μg/day)[a]	K Acid-II-Me (μg/day)
Control	4.8	31.5
Administered[b]	462	354

a) Measured by gas chromatographic analysis.
b) Phylloquinone (91.5 mg/day) was injected intravenously.

Small amounts of K Acid-I and K Acid-II were also detected as methyl esters in the urine of the control group (Table IV). This would be the metabolite from the phylloquinone contained in the diet and/or menaquinone biosynthesized by the intestinal frola. But the amounts of K Acid-I and K Acid-II detected in the urine of dosed rabbit were higher than those from the control group. As mentioned above, Gloor, *et al.*[4] detected the K Lactone as the metabolite of phylloquinone, using labelled compound. From these results, these two

9) Determined by nuclear magnetic resonance (NMR) analysis. L.M. Jackman, R. Rüegg, G. Ryser, C. von Planta, U. Gloor, H. Mayer, P. Schudel, M. Kofler, and O. Isler, *Helv. Chim. Acta*, **48**, 1332 (1965).
10) S.J. DiMari and H. Rapoport, *Biochemistry*, **7**, 2650 (1968).
11) F. Weber and O. Wiss, *Helv. Chim. Acta*, **42**, 217 (1959); O. Wiss, F. Weber, R. Rüegg, and O. Isler, *Z. Physiol. Chem.*, **314**, 245 (1959); F. Weber and O. Wiss, "The Vitamins," 2nd ed., Vol. III, ed. by W.H. Sebrell, Jr. and R.S. Harris, Academic Press, New York and London, 1971, p. 457; J.T. Matschiner and R.G. Bell, *J. Nutr.*, **102**, 625 (1972).

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acidic compounds were considered to be the metabolites of phylloquinone by a physiological pathway. Since these quinones were not detected directly in the urine until it was treated with methanolic hydrochloric acid at room temperature, they must be excreted as sulfates of their hydroquinone compounds which can be hydrolyzed almost completely under such a mild condition as seen with the metabolites of Q. To confirm the conversion of K Acid-I into K Lactone during the hydrolysis step with hydrochloric acid, the hydroquinone derivative of synthetic K Acid-I was treated with dilute hydrochloric acid, and K Lactone was obtained in a yield of about 70%, no unchanged K Acid-I was recovered. On the other hand, only 13% K Lactone was obtained with similar treatment of the quinone form of K Acid-I. This supports the possibility that K Acid-I is excreted in urine as a conjugated form of the corresponding hydroquinone and phylloquinone is metabolized similarly to Q.

Metabolites of α-Tocopherol

Urine of rabbits given *dl-α*-tocopheryl acetate was treated similarly to the case of phylloquinone, with methanolic hydrochloric acid and extracted with ethyl acetate. The lipid fraction was examined by TLC, and a less intense spot corresponding to synthetic E Acid-I-Me and E Acid-II-Me[8] and an intense spot corresponding to tocopheronolactone were found by leucomethylene blue reagent[12] (Table V). These quinones were purified by repeated column and thin-layer chromatographic procedures. Tocopheronolactone was identified with the authentic material.[8] The two esters were then separated from each other by reversed-phase TLC. One of them was identified with synthetic E Acid-II-Me by physicochemical data.[1] Another quinone had a UV spectrum and *Rf* value in TLC identical with synthetic E Acid-I-Me. Although the latter was found to be contaminated with impurity by GLC, we did not attempt further purification because the quantity was too small.

Under this condition the hydrolysis of the conjugates was incomplete, unlike those of the conjugates of phylloquinone metabolites (Table III) and Q metabolites.[6] Therefore the aqueous layer resulted from the above hydrolysis was heated with hydrochloric acid and the same quinones were obtained again. The relationship between the amounts of a main products and the condition of hydrolysis is shown in Table VI.

TABLE V. *Rf* Values of Metabolites of α-Tocopherol in Several Solvent Systems

Compound	Solvent system[a]				
	A	B	C	D[b]	E[b]
E Acid-I-Me	0.44	0.60	0.71	0.48	0.76
E Acid-II-Me	0.42	0.62	0.73	0.53	0.83
E Acid-I	0.02	0.05	0.03	0.72	0.85
E Acid-II	0.03	0.05	0.06	0.74	0.72
E Lactone	0.15	0.12	0.42	0.64	0.68

a) solvent system: A, benzene–ethyl acetate (19:1); B, petroleum ether–ether (3:2); C, chloroform–ether–methanol (100:1:0.3); D, acetone–water (3:2); E, acetonitrile–water (3:2)
b) Reversed phase silica gel plate was used.

A hypothetical scheme for the metabolic pathway of α-tocopherol was proposed by Simon, *et al.*[3] In their scheme, the chroman ring is hydrolyzed to α-tocopherylquinone (step A), and the terminal methyl group in the side chain is oxidized, followed by degradation of the resulting carboxylate by β-oxidation (step B). Next, a nucleophilic attack by the hydroxy group in the side chain on the carbonyl carbon gives tocopheronolactone, pushing out coenzyme

12) B.O. Linn, A.C. Page, Jr., E.L. Wong, P.H. Gale, C.H. Shunk, and K. Folkers, *J. Amer. Chem. Soc.*, **81**, 4007 (1959).

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TABLE VI. Tocopheronolactone from Urine[a] under Different Condition of Hydrolysis

Condition			Tocopheronolactone formed	
reagent	Temp. (°C)	time (hr)	mg[b]	%
3N HCl	75	2	76.7	100
3N Methanolic HCl	75	2	38.2	49.8
3N Methanolic HCl	20	15	43.5	52.6

a) One-third volume of urine collected 10th to 30th day after beginning of administration.
b) Measured by gas chromatographic analysis.

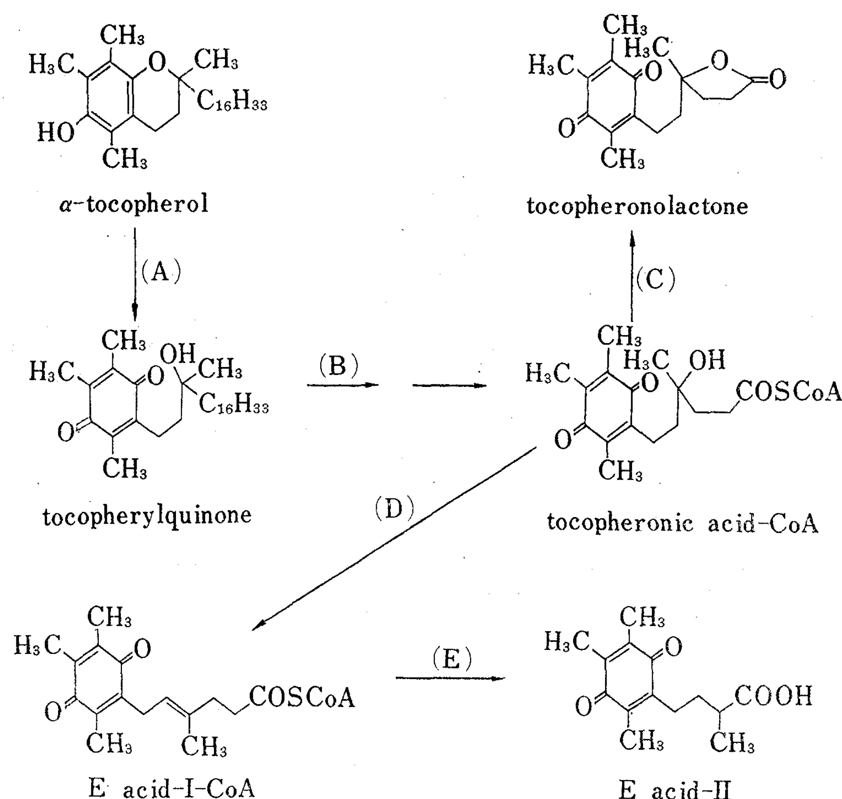

Chart 1. Metabolic Pathway of α-Tocopherol

A (step C) as shown in Chart 1.[13] This scheme is supported by the facts that tocopherylquinone is formed from α-tocopherol in animal bodies,[14] and the administration of tocopherylquinone leads to excretion of more tocopheronolactone in urine than in the case with α-tocopherol.[5,15] In the present study, E Acid-I and -II proved to be new urinary metabolites, after conversion into the corresponding ester as described above. From our results, the metabolic pathway was considered to proceed further as follows: Tocopheronic acid formed by β-oxidation is mainly converted into tocopheronolactone, and a part of this acid is dehydrated to E Acid-I (step D), followed by reduction and β-oxidation to give E Acid-II (step E), and then these metabolites are excreted as conjugates of their hydroquinone compounds.

The conjugates of α-tocopherol metabolites could be hydrolyzed almost completely by heating with dilute hydrochloric acid but only partially with methanolic hydrochloric acid,

13) In the Simon's scheme metabolites were shown in hydroquinone forms or those conjugates but in this chart, those were shown in quinone forms.
14) A.S. Csallany, H.H. Draper, and S.N. Shah, *Arch. Biochem. Biophys.*, **98**, 142 (1962).
15) U. Gloor and O. Wiss, *Helv. Chim. Acta*, **49**, 2590 (1966).

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suggesting that the conjugating group was more difficult to remove than that of Q Acid-I and K Acid-I. It is known that glucuronides of phenolic substances resist hydrolysis more than sulfates,[16] and some metabolites are excreted as a mixture of conjugate such as glucuronide and sulfate. The metabolites of α-tocopheryl acetate in rabbit also may be excreted as mixtures of such conjugates. The hydroxy group of hydroquinone monoconjugates shows considerable acidity and is not conjugated further.[17] There was no definite proof whether one or both of the two phenolic hydroxy groups was conjugated in this study on tocopherol metabolites.

Experimental[18]

Animals——Adult buck albino rabbits (initial body weight, 2.95 to 3.42 kg) were kept in metabolism cages on a standard diet of rabbit pellets. Phylloquinone (40 mg/kg/day) and *dl*-α-tocopheryl acetate (150 mg/kg/day) were injected intravenously in the form of an aqueous solution containing a detergent, Nikkol HCO-50 (Hydrogenated caster oil, Nikko Chemical Ltd.), for five weeks, respectively. Urine was collected in a vessel containing toluene.

Isolation and Purification of K Acid-I-Me and K Acid-II-Me——Lyophilized powder (360 g) of urine (6.7 liters) which had been collected 2 to 5 weeks after the beginning of additive administration of phylloquinone was stirred with 1.5N methanolic HCl (1.3 liters) for 15 hr at room temperature, then oxidized with 5% $FeCl_3$ (0.65 liter). The hydrolysis mixture was extracted with four 2 liter portions of EtOAc and washed with water. The combined extracts were dried over Na_2SO_4 and evaporated *in vacuo*, yielding 22.8 g of brown lipid material. This lipid was dissolved in $CHCl_3$ and chromatographed on a column of silicic acid (250 g, 7.5×10 cm). The first fraction (6.9 g), eluted with $CHCl_3$ (2 liters), was further purified by preparative TLC using benzene–EtOAc (4: 1). A quinone band corresponding to the synthetic K Acid-I-Me and K Acid-II-Me, which could not be separated in this developing solvent system, was scraped off and extracted with ether. The extract was then rechromatographed using hexane–ether (4: 1) as the developing solvent system. These quinones were separated into two bands by reversed phase chromatography developed in acetone–water (1: 1). They were further purified by successive chromatography developed in $CHCl_3$–ether–EtOH (14: 5: 1), benzene–MeOH (97: 3) and by reversed phase chromatography developed in acetonitrile–water (3: 2), to yield K Acid-I-Me (3 mg) and K Acid-II-Me (2 mg) as yellow oils.

Analysis of Phylloquinone Metabolites——Hydrolysis with methanolic HCl: To 4 g of powder obtained from 82 ml of urine, 1.5N methanolic HCl (30 ml) was added and the mixture was stirred at room temperature for 15 hr. The hydrolysis mixture was stirred with 5% $FeCl_3$ (15 ml) for 1 hr and extracted with four 50 ml portions of EtOAc. The combined extracts were washed with three 30 ml portions of water and dried over Na_2SO_4. The residue from the extracts was subjected to TLC using benzene–EtOAc (4: 1). The two quinone bands corresponding to synthetic K Lactone and K Acid-I-Me and K Acid-II-Me (the latter two not having been separated by this solvent system), were extracted with ether. After further purification by TLC using hexane–ether (2: 3), they were dissolved in an aliquot volume of acetone and analyzed by GLC (Table III).

Hydrolysis with dilute HCl: Urine powder (4 g) was hydrolyzed with 1.5N HCl (30 ml) in a stream of N_2 at 75° for 2 hr and treated as mentioned above. The residue from the EtOAc extract was subjected to TLC using benzene–EtOAc (4: 1). The bands corresponding to synthetic K Lactone and a mixture of K Acid-I and K Acid-II were scraped off and extracted with ether. K Lactone was further purified by TLC using hexane–ether (2: 3) and analyzed by GLC (Table III). The fraction corresponding to synthetic K Acid-I and K Acid-II was further purified by TLC using $CHCl_3$–ether–EtOH (14: 5: 1), and then methylated with diazomethane. After purification by TLC using benzene–EtOAc (4: 1), K Acid-I-Me and K Acid-II-Me were not detected in the resulting oil by GLC.

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16) S. Lieberman and K. Dobriner, *Recent Progr. Horm. Res.*, 3, 71 (1948); S. Burstein and S. Lieberman, *J. Biol. Chem.*, 233, 331 (1958).
17) D.V. Parke, "The Biochemistry of Foreign Compounds," Pergamon Press, London, 1968, p. 147.
18) Phylloquinone and *dl*-α-tocopheryl acetate were obtained from Nisshin Chemicals Co., Ltd. TLC was carried out on Silica gel GF_{254} (Merck A.G.), and reversed phase chromatography on Silica gel GF_{254} impregnated with 5% (v/v) liquid paraffin in ether. Visualization was performed by spraying with H_2SO_4 for phylloquinone metabolites and with leucomethylene blue reagent for α-tocopherol metabolites, and by ultraviolet light examination (253 mμ) for both. The silicic acid (mesh 100) used in column chromatography was purchased from Mallinckrodt Co. Mass spectra were recorded on either a Hitachi RMU-6D double focusing or a Hitachi RMS-4 mass spectrometer at an ionization potential of 70 eV. The sample was vaporized at the ion source with a heated direct inlet system operating at 200°.

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Apparatus and Conditions: A nitrogen gas chromatograph apparatus equipped with a hydrogen ionization detector (Ohkura gas chromatograph, Model 2100) and a pyrex glass column (0.35×120 cm) packed with 6% QF-1 on Gas Chrom Q (60—100 mesh) were used. Gas flow rates of nitrogen, hydrogen and air were adjusted to 60,50 and 500 ml/min, respectively. Column temperature was adjusted to 190° for analyses of K Acid-I-Me and K Acid-II-Me, which had retention times of 8.4 and 3.7 min, respectively, and to 236° for K Lactone which had a retention time of 6.4 min. The temperature of evaporation was kept at 200°.

Isolation and Purification of α-Tocopherol Metabolites——The lyophilized powder (80 g) of urine collected for 6 days after the beginning of additive administration of α-tocopheryl acetate was stirred with 3N methanolic HCl (160 ml) in a stream of N_2 at room temperature for 20 hr. The hydrolysis mixture was diluted with 200 ml of water and extracted with five 300 ml portions of EtOAc. The combined extracts were washed with four 200 ml portions of water and evaporated *in vacuo*. The resulting residue was stirred with 5% $FeCl_3$ solution in MeOH–water (1: 1) (200 ml) for 1.5 hr and extracted with four 300 ml portions of EtOAc. The combined extracts were washed with four 200 ml portions of water and dried over Na_2SO_4. The residue from the extracts was chromatographed on a column of silicic acid (55 g, 3.5×10 cm).

Fraction	Solvent	Volume (ml)	Contents (g)
1	$CHCl_3$	200	0.84
2	$CHCl_3$	300	0.50
3	$CHCl_3$	400	0.88
4	EtOH	500	2.71

The first fraction was subjected to TLC using petroleum ether–ether (3: 2). Quinone bands corresponding to synthetic E Acid-I-Me and E Acid-II-Me, which had not been separated in this solvent system, were scraped off and extracted with ether. The extract was rechromatographed using benzene–EtOAc (19: 1). These quinones were separated by reversed phase TLC using acetone–water (3: 2). Each fraction was further purified by TLC using benzene–EtOAc (4: 1) to yield E Acid-I-Me (below 1 mg) and E Acid-II-Me (below 1 mg) as yellow oil. The second fraction of the column chromatograph was subjected to TLC using $CHCl_3$–ether–EtOH (14: 5: 1) and a quinone band corresponding to the synthetic tocopheronolactone was extracted with ether and further purified by TLC using hexane–ether (1: 4) to yield tocopheronolactone (15 mg).

The aqueous layer resulted from the above hydrolysis was neutralized with $NaHCO_3$ and lyophilized. The resulting powder was stirred with 3N HCl (160 ml) in a stream of N_2 at 75° for 1.5 hr to remove the conjugating group. The hydrolysis mixture was worked up as described above. After purification by TLC, tocopheronolactone (7.8 mg) and quinones which exhibited the same *Rf* values as synthetic E Acid-I-Me and E Acid-II-Me were obtained.

Analysis of Tocopheronolactone——Urine powder was hydrolyzed under the three different conditions as shown in Table VI. The residue obtained on evaporation of the EtOAc extract from the hydrolyzate was subjected to TLC using benzene–EtOAc (4: 1). A quinone band corresponding to synthetic tocopheronolactone was extracted with ether. After further purification by TLC using $CHCl_3$–ether–EtOH (14: 5: 1), it was dissolved in an aliquot volume of acetone and analyzed by GLC (Table VI).

Apparatus and Conditions: A helium gas chromatograph apparatus equipped with a hydrogen ionization detector (Shimadzu gas chromatograph, GC-5A) and a pyrex glass column (0.2×100 cm) packed with 5% OV-17 on Gas Chrom Z (60—80 mesh) were used. Gas flow rates of helium, hydrogen and air were adjusted to 90, 30 and 900 ml/min, respectively. Temperatures of column and evaporator were adjusted to 209° and 250°, respectively. The retention time of tocopheronolactone was 8.6 min.

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