Notes

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Modified Lipophilic Vitamin. III.[1] Distribution and Excretion of Tocopheronolactone[2]

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Tocopheronolactone, the γ-lactone of 2-(3-hydroxy-3-methyl-5-carboxypentyl)-3,5,6-trimethylbenzoquinone (tocopheronic acid), was firstly isolated by Simon, *et al.*[4] from the urine of rabbit and human as a metabolite of α-tocopherol. The formation of tocopheronolactone seems to be essential for the biological actions of tocopherol by the following reasons. (i) Treatment of tocopheronolactone recovers the level of ubiquinone in the uterus of vitamin E-deficient rat more rapidly than tocopherol.[5] (ii) The decline in respiration of necrotic liver caused by vitamin E-deficiency can be prevented by injecting the animal with tocopherol but cannot be arrested by adding it *in vitro*, while tocopheronolactone is completely effective both *in vivo* and *in vitro* for preventing respiratory decline.[6] A more recent view[7] is that vitamin E might function by being degraded to a quinonoid metabolite, which might play a specific role at an enzyme site. We reported[1] previously that tocopheronolactone was effective in inhibiting the formation of lipid-peroxide as well as α-tocopherol.

Abderhalden, *et al.*[8] have reported the content of tocopherol in rat and human organs, and its absorption, accumulation and excretion have already been described by Quaife, *et al.*[9,10] In this paper, we report the incorporation of ^{3}H-tocopheronolactone, in comparison with ^{14}C-α-tocopherol, into organs and subcellular fractions of mouse liver.

Experimental

Materials——Randomly labeled ^{3}H-tocopheronolactone and α-tocopherol-3-$^{14}CH_3$ were obtained from Eisai Co., Ltd. Their specific activities were 7.6 μc/mg and 2.6 μc/mg, respectively.

Animals——Male mice of *dd*-strain, weighing from 18 g to 20 g, were used. ^{3}H-Tocopheronolactone and ^{14}C-α-tocopherol was dissolved in glycerol and cotton seed oil, respectively, and each mouse was injected with 2.5 μc/0.1 ml of the former and 3.5 μc/0.1 ml of the latter intraperitoneally.

Preparation of Subcellular Fractions——The livers were rapidly removed from mice, placed in 10 ml of ice-cold 0.25M sucrose and homogenized. Nuclei and cell debris, mitochondria, microsomes were sedimented at $900\times g$ for 15 min, at $9000\times g$ for 30 min and at $105000\times g$ for 60 min, respectively. Each sediments were resuspended in 10 ml of 0.25M sucrose.

1) Part II.: K. Fukuzawa, Y. Suzuki and M. Uchiyama, *Chem. Pharm. Bull.* (Tokyo), **17**, 1761 (1969).
2) This report will be presented at the 89th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, April 1969.
3) Location: *Aobayama, Sendai.*
4) *a*) E.J. Simon, C.S. Gross and A.T. Milhonat, *J. Biol. Chem.*, **221**, 797 (1956); *b*) E.J. Simon, A. Eisengart, L. Sundheim and A.T. Milhonat, *J. Biol. Chem.*, **221**, 807 (1956).
5) J. Green and J. Bunyan, *Nature*, **190**, 318 (1961).
6) K. Schwarz and E.J. Simon, *Biochim. Biophys. Acta*, **32**, 484 (1959).
7) J. Green and D. Mckale, "*Biochemistry of Quinone*," ed. by R.A. Morton, Academic Press, London & New York, 1965, pp. 261—285.
8) R. Abderhalden, *Z. Vitaminforsch*, **16**, 319 (1945).
9) *a*) M.L. Quaife, W.J. Swanson, M.Y. Dju and P.H. Harris, *Ann. N.Y. Acad. Sci.*, **52**, 300 (1949); *b*) K.M. Brinkhouse and E.D. Warner, *Amer. J. Path.*, **98**, 81 (1941); *c*) G. Klastkin, *J. Lab. Clin. Med.*, **39**, 802 (1952); *d*) H. Schmandke, *Int. Z. Vitaminforsch*, **35**, 346 (1965).
10) K.E. Mason, *J. Nutr.*, **23**, 71 (1942).

Extraction of ^{3}H-Tocopheronolactone and ^{14}C-α-Tocopherol——(1) From Tissues and Blood: Mice were decapitated and liver, kidneys, spleen, brain and epididymal adipose tissue were removed. Each tissue was homogenized with 5 ml of water. Blood was collected from the mice by decapitation into a beaker containing 1 mg of sodium citrate. To 0.3 ml of blood was added 5 ml of water. The extraction of ^{3}H-tocopheronolactone was performed as follows; 3 ml of 5% trichloroacetic acid (TCA) and 10 ml of toluene were added to the homogenate or blood, while to the liver homogenate and subcellular fraction of liver were added 6 ml of 5% TCA and 20 ml of toluene. The mixture was shaken vigorously and centrifuged at 3500 rpm for 5 min to separate the water and toluene layer. For the extraction of ^{14}C-α-tocopherol, 1 ml of 5% TCA, 5 ml of MeOH and 10 ml of toluene were added to the tissue homogenate or blood, while to the liver 2 ml of 5% TCA, 10 ml of MeOH and 20 ml of toluene were added. The subsequent procedure was same as ^{3}H-tocopheronolactone.

(2) From Urine: The filter paper inserted under the metabolic cage and infiltrated by urine were chopped into small pieces and allowed to stand for half a day after addition of 10 ml of water. After removing the paper tips by filtration, a portion of filtrate was directly assayed employing Bray's scintillator.

Determination of the Radioactivity of ^{3}H-Tocopheronolactone and ^{14}C-α-Tocopherol——One ml of toluene extracts obtained above was placed in a counting vial containing 10 ml of toluene scintillator fluid (4 g of PPO and 0.2 g of POPOP, were dissolved in toluene to make 1000 ml). For radioactive counting of water soluble materials 0.1 ml of water layer was put into 10 ml of Bray's scintillator fluid (60 g of naphthalene, 4 g of PPO, 0.2 g of POPOP, 60 ml of MeOH, 20 ml of ethylene glycol and dioxane to make 1000 ml). Radioactivity was measured by Packard Tris-Carb liquid scintillation counter 3324.

Identification of ^{3}H-Tocopheronolactone and ^{14}C-α-Tocopherol——The radioactive materials in toluene extracts were identified by thin-layer chromatography (TLC) of silicagel-G. The moving phases were benzene–chloroform–acetone (5:5:1) mixture for ^{3}H-tocopheronolactone, and benzene for ^{14}C-α-tocopherol. The spots were detected under ultraviolet light (365 mμ).

Results and Discussion

The Distribution of Radioactivity after Administration of ^{3}H-Tocopheronolactone and ^{14}C-α-Tocopherol

The radioactivities in toluene extracts from each tissue of mice after the intraperitoneal injection of ^{3}H-tocopheronolactone and ^{14}C-α-tocopherol were shown in Fig. 1, Fig. 2 and

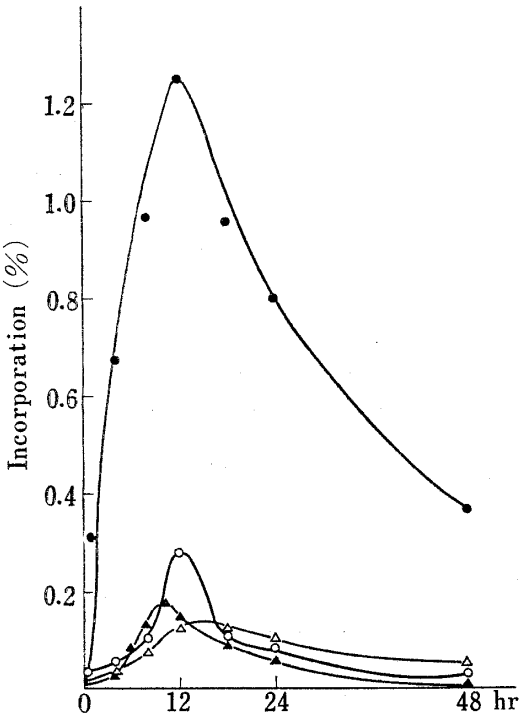

Fig. 1. The Time Course of the Incorporation of ^{14}C-α-Tocopherol into Mouse Tissue

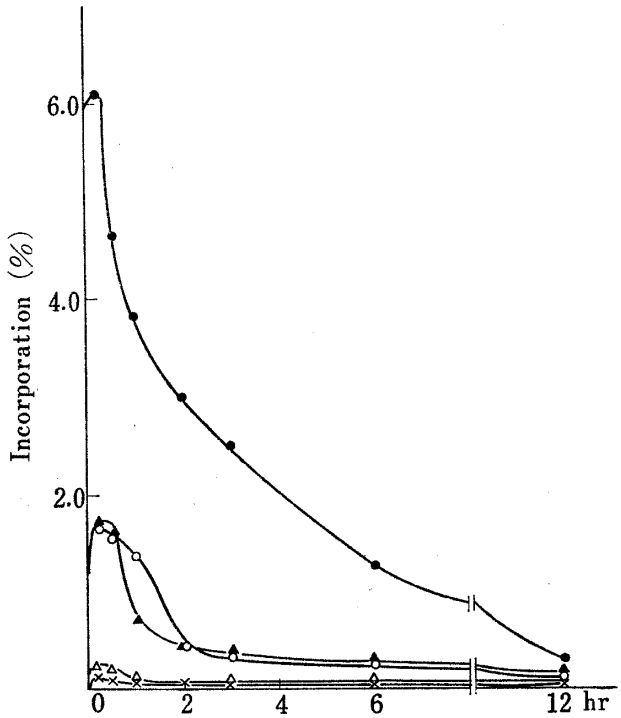

Fig. 2. The Time Course of the Incorporation of ^{3}H-Tocopheronolactone into Mouse Tissue

—●—: liver —△—: spleen
—○—: kidney —▲—: blood
—▲—: blood
—○—: kidney —×—: brain
—△—: spleen

Incorporation is shown by the percentage of the radioactivity incorporated against administrated.

Table I. The distribution of ^{3}H was maximum in liver among organs and the time course of ^{3}H content were approximately the same tendency in all organs tested, namely the peak was appeared at 15 min after administration. On the other hand the incorporation rate of ^{14}C was very slow and the peak was appeared at 12 hr after administration in all organs except the adipose tissue. The absolute amount of incorporated ^{14}C-α-tocopherol was about one fourth of ^{3}H-tocopheronolactone. Turn over rate of ^{3}H-tocopheronolactone was higher than ^{14}C-α-tocopherol as recognized by the faster disappearance of ^{3}H from that of ^{14}C. Even if ^{3}H-tocopheronolactone was dissolved in cotton seed oil instead of glycerol as a injection medium, radioactivity incorporated showed only slight decrease and the time course of distribution differed little.

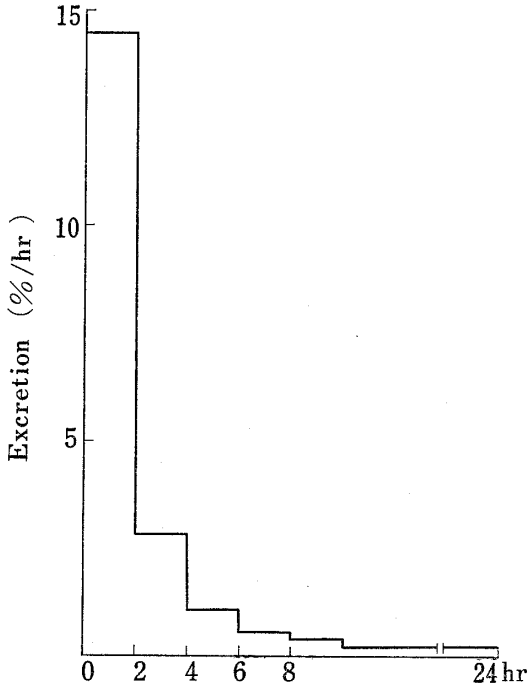

Fig. 3. The Time Course of Excretion of ^{3}H-Tocopheronolactone into Mouse Urine

Excretion is shown by the percentage per hour of the radioactivity excreted against administered.

Mason[10] described that most of vitamin E was accumulated into adipose tissue and muscles. In our experiments, the incorporation of ^{14}C-α-tocopherol in adipose tissue was not identical to other organs. The incorporation of ^{14}C was still increasing at 50 hr after administration, whereas ^{3}H-tocopheronolactone did not show any accumulation.

The Excretion of Radioactivity in Urine after Administration of ^{3}H-Tocopheronolactone and ^{14}C-α-Tocopherol

The time course of the excretion of ^{3}H into mouse urine was shown in Fig. 3. Total radioactivity during 12 hr after administration of ^{3}H-tocopheronolactone was approximately 40% of administered dose, while only 0.7% of ^{14}C was excreted during 50 hr after administration of ^{14}C-α-tocopherol.

Distribution of ^{3}H-Tocopheronolactone and ^{14}C-α-Tocopherol in the Subcellular Fractions of Mouse Liver

The subcellular distribution of ^{3}H at 20 min and 7 hr after intraperitoneal administration of ^{3}H-tocopheronolactone is shown in Table II. The radioactivity was found predominantly in microsomes and supernatant fraction. This result agrees with the cytoplasmic distribution of tocopheronolactone–reducing enzymes which was reported previously.[1] However, ^{14}C-α-tocopherol showed more affinity to particulate fractions than to supernatant. These

Table I. Incorporation of ^{3}H-Tocopheronolactone and ^{14}C-α-Tocopherol into Mouse Adipose Tissue

	Incorporation (%)		
	1 hr	12 hr	50 hr
^{14}C-α-Tocopherol	2.69	3.24	11.4
	20 min	7 hr	24 hr
^{3}H-Tocopheronolactone	1.92	0.84	0.54

Incorporation is shown by the percentage of the radioactivity incorporated against administrated 200 mg of adipose tissue was used.

Table II. The Distribution of ^{3}H-Tocopheronolactone and ^{14}C-α-Tocopherol into Subcellular Fractions of Mouse Liver

Fractions		Distribution ^{3}H-Tocopheronolactone Time after injection 20 min	7 hr	^{14}C-α-Tocopherol 1 hr	12 hr
Nuclei+Cell Debris	(%)	8.9	21.7	47.6	49.4
	(mμg/mg protein)	8.0	4.5	20.7	60.1
Mitochondria	(%)	16.4	12.3	9.2	21.1
	(mμg/mg protein)	58.3	8.3	17.0	86.9
Microsomes	(%)	19.9	33.1	24.3	18.2
	(mμg/mg protein)	114.4	27.9	56.2	103.9
Supernatant	(%)	54.8	32.9	18.9	11.3
	(mμg/mg protein)	117.1	11.5	17.5	26.3

Total incorporation; ^{3}H-tocopheronolactone 17.8 μg/liver at 20 min, 3.21 μg/liver at 7 hr, ^{14}C-α-tocopherol 7.4μg/liver at 1 hr, 17.7 μg/liver at 12 hr

results seem to be consistent with the facts that vitamin E increased the weight of regenerated liver[11] and that vitamin E is related to the metaboilsm of nucleic acid.[12]

Radioactive Substances in Toluene and Water Extracts

Radioactive substances in the toluene extracts obtained from the experiments showed in Fig. 1 and Fig. 2 were identified by thin-layer chromatography. 62% of total radioactive substances in toluene extracts from the liver was found at tocopheronolactone fraction (*Rf* 0.52) (Fig. 4-(A)). 65% of ^{14}C in toluene extracts in the case of ^{14}C-α-tocopherol was found at α-tocopherol fraction (*Rf* 0.44) (Fig. 4-(C)). The radioactive components recovered from elswhere were not identified.

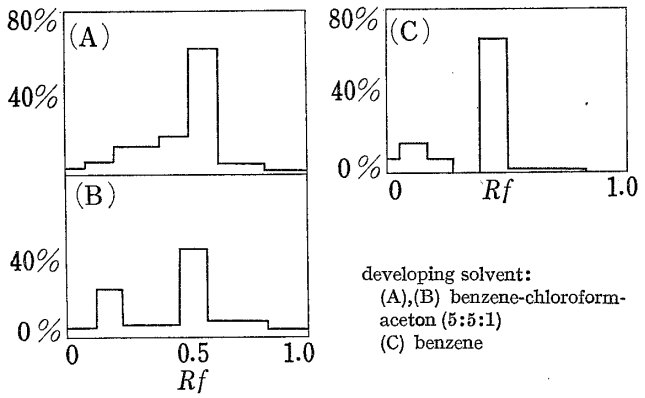

Fig. 4. Identification of ^{3}H-tocopheronolactone and ^{14}C-α-tocopherol by Thin-Layer Chromatography

(A) Radioactive substances in toluene extracts of the liver derived from ^{3}H-tocopheronolactone.
(B) Radioactive substances in toluene extracts of the urine derived from ^{3}H-tocopheronolactone. Extraction from the filter paper infiltrated by urine was carried out with 30 ml of 8% HCl and 20 ml of toluene.
(C) Radioactive substances in toluene extracts of the liver derived from ^{14}C-α-tocopherol.

Simon, *et al.*[1] have reported that tocopheronolactone was excreted in urine as conjugated form. ^{3}H in urine obtained from the experiment showed in Fig. 3 was scarcely extracted with toluene, however, after the water extracts made to 8% HCl solution and warmed at 80° 2 hr, about 70% of radioactivity in water layer was removed to toluene. In this toluene extracts of urine, 43% of ^{3}H was detected at tocopheronolactone fraction and 22% was tocopheronic acid fraction (*Rf* 0.17) (Fig. 4-(B)).

Acknowledgement We wish to express our appreciation to Eisai Co., Ltd. for a generous gift of ^{14}C-α-tocopherol and ^{3}H-tocopheronolactone.

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