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α-Tocopherol and phylloquinone as non-competitive inhibitors of retinyl ester hydrolysis

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Inhibition of rat intestinal retinyl ester hydrolase by α-tocopherol (vitamin E) and phylloquinone (vitamin K_1) was non-competitive. Maximum inhibition occurred within 10min, and, particularly with α-tocopherol, was substantially reversible. Consequently, increasing tissue concentrations of retinyl esters, which might occur with advancing age or changes in diet, would not diminish the effects of the inhibitors. These data further support the notion that α-tocopherol may, at physiological concentrations, influence the concentration of vitamin A and its ester in tissues.

Vitamin A is essential for vision, reproduction and epithelial differentiation (Moore, 1960; Wolf, 1977; DeLuca, 1978); consequently, knowledge of factors that affect its homoeostasis, such as α-tocopherol, is desirable. Diets deficient in α-tocopherol result in lower amounts of total vitamin A stores in liver (Moore, 1940; Davies & Moore, 1941). The mechanism of this effect had not been elucidated, but the antioxidant properties of α-tocopherol had been excluded as a probable cause (Sondergaard, 1973). A more-recent study has confirmed the effect of low dietary α-tocopherol on the concentration of total vitamin A in liver, and has demonstrated that total vitamin A concentrations in kidney and intestine are only marginally affected (Napoli *et al.*, 1984). As might be expected, the concentrations of retinyl esters in liver were diminished by α-tocopherol depletion, but so were those in kidney and intestine. In contrast, the retinol concentrations were increased in all three tissues. Experiments conducted *in vitro* showed that at least one mechanism was through inhibition of retinyl ester hydrolysis. The same paper also demonstrated that phylloquinone could inhibit retinyl ester hydrolysis *in vitro*.

The present communication reports that inhibition of retinyl ester hydrolysis by α-tocopherol or phylloquinone is non-competitive, and substantially reversible, and that neither seems to require metabolic activation.

Experimental

General

Retinyl [9,10-^{3}H]palmitate (specific radioactivity 807600d.p.m./nmol) was synthesized as described previously (Napoli *et al.*, 1984). 2-ambo-α-Tocopherol (vitamin E), phylloquinone (vitamin K_1) and rat intestinal acetone-extracted powder were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Potassium carbonate/borate buffer, pH 10, was purchased from Fisher Scientific Co., Pittsburgh, PA, U.S.A. Radioactivity was measured in 5ml of Liquiscint (National Diagnostics, Sommerville, NJ, U.S.A.) with an LKB 1217 Rackbeta liquid-scintillation counter.

Assay of retinyl ester hydrolase activity

Assays were conducted by a modification of the procedure of Prystowsky *et al.* (1981). Standard assay conditions consisted of rat intestinal acetone-extracted powder (100ng of protein) in 0.2ml of sodium cholate (2.5%) and 50mM-Tris/maleate, pH8.0 (buffer A). Substrate and/or inhibitors were added in 5μl of ethanol. Incubations were conducted at 37°C. The reaction was quenched by adding methanol/chloroform/heptane (28:25:20, by vol.) (3.25ml) and potassium carbonate/borate buffer (1.0ml) as described by Belfrage & Vaughan (1969). A portion (1ml) of the upper phase, which contained the [^{3}H]palmitic acid, was taken for

measurement of radioactivity. The reaction rate was linear for 90min and from 25 to 500ng of protein. Reactions were conducted at initial-rate conditions (less than 10% of substrate converted into product). Controls were generated by conducting reactions in the absence of protein.

Results and discussion

The nature of inhibition of retinyl ester hydrolase by α-tocopherol (vitamin E) and phylloquinone (vitamin K_1) was examined with acetone-extracted powders prepared from rat intestine. Acetone-extracted powders were used because extraction of tissue homogenates with organic solvent removes lipids, thus providing protein depleted of endogenous effectors and/or substrates. Inhibition by either α-tocopherol or phylloquinone was non-competitive, as shown by Lineweaver–Burk plots of the relationships between reaction rate and substrate concentration in the presence and in the absence of the inhibitors (Fig. 1). The apparent K_m, determined from the graph, was 44μM. The apparent $V_{max.}$ (nmol/min per mg of protein) was 167 in the absence of inhibitors. α-Tocopherol (200μM) lowered the apparent $V_{max.}$ to 63nmol/min per mg (62% inhibition). Phylloquinone (50μM) lowered the apparent $V_{max.}$ to 43nmol/min per mg (74% inhibition). These data indicate that the inhibitors act by inactivating the hydrolase, or the hydrolase–retinyl ester complex, and not by competing with retinyl ester for the enzyme's active site. Thus the degree of inhibition depends on the concentrations of the enzyme and the inhibitor, and is independent of the retinyl ester concentration.

The degree of inhibition as a function of time was examined. Within a 10min incubation there was maximum inhibition (Table 1). The potency of inhibition by either α-tocopherol or phylloquinone was unaffected by increasing the incubation time 3-fold. These results show that interaction between the hydrolase and the inhibitors is rapid, and, since there were no added cofactors, suggest that metabolic activation is not required for this function of α-tocopherol or phylloquinone.

To determine whether inhibition was reversible, the following experiments were conducted. Retinyl ester hydrolase (50μg of protein from the acetone-extracted powder) was pre-incubated in buffer A with either 1μM- or 300μM-α-tocopherol for 10min (Expt. 1) or for 30min (Expts. 2 and 3). At the end of the pre-incubation, the homogenates were diluted with buffer A to final protein concentrations of 100ng/0.2ml, i.e. standard assay conditions. The α-tocopherol concentrations after dilution were 1μM and 3μM respectively. α-Tocopherol was added to a portion of the homogenate that had been pre-incubated with 300μM-α-tocopherol to re-establish a concentration of 300μM. The retinyl

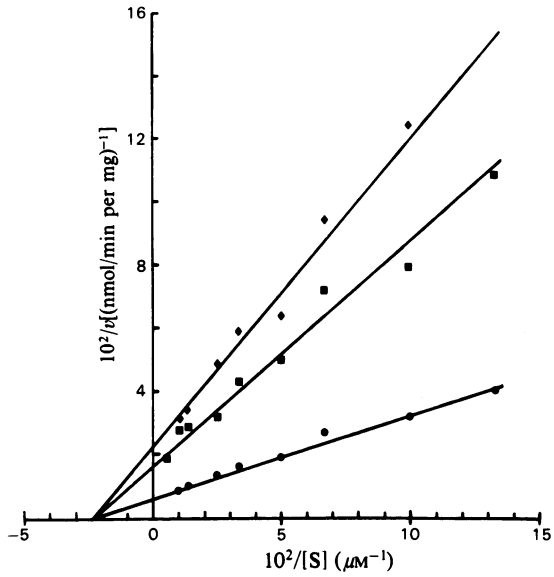

Fig. 1. *Double-reciprocal plot of the rate of retinyl palmitate hydrolysis activity versus substrate concentration*
The rates of hydrolysis of retinyl palmitate in the presence of either 200μM-α-tocopherol (♦) or 50μM-phylloquinone (■) were compared with the rates in the absence of added inhibitors (●). Incubations were conducted for 45min in duplicate; controls were done at each substrate concentration.

Table 1. *Effect of incubation time on inhibition of retinyl palmitate hydrolysis by α-tocopherol or phylloquinone*
For experimental details see the text. Values are the means±S.D. for triplicate determinations.

Inhibitor	Activity [nmol/min per mg of protein (% inhibition)]	
Incubation time ...	10min	30min
None	96±12 (0)	76±5 (0)
200μM-α-Tocopherol	33±2 (66)	26±3 (66)
50μM-Phylloquinone	63±3 (34)	48±0.5 (37)

Table 2. *Effect of pre-incubation with α-tocopherol or phylloquinone on inhibition of retinyl palmitate hydrolysis*
The pre-incubation time was 10min in Expt. 1, and 30min in Expts. 2 and 3. The incubation time was 30min. For full experimental details see the text. Values are the means ± S.D. for triplicate determinations.

Pre-incubation	Incubation	Activity [nmol/min per mg of protein (% inhibition)] Expt. 1	Expt. 2	Expt. 3
α-Tocopherol (μM)				
1	1	92±15 (0)	71±15 (0)	66±1 (0)
300	3	40±6 (56)	30±2 (48)	46±11 (30)
300	300	9±1 (90)	9±0.3 (87)	5±0.2 (92)
Phylloquinone (μM)				
1	1	92±11 (0)	82±3 (0)	104±17 (0)
100	1	52±5 (43)	46±3 (44)	57±6 (45)
100	100	30±1 (67)	29±1 (65)	25±3 (76)

ester hydrolase activities of the three homogenates were compared. A similar experiment was performed with phylloquinone (Table 2). Pre-incubation with 1μM-α-tocopherol or -phylloquinone did not affect the activity of the retinyl ester hydrolase (control). Pre-incubation and incubation with 300μM-α-tocopherol or 100μM-phylloquinone produced for the three experiments an average 9.5-fold or 3.3-fold decrease in the rate of hydrolysis respectively. In contrast, pre-incubation with the higher concentrations of α-tocopherol, and subsequent dilution of the inhibitor before the incubation, restored hydrolase activity to a large extent, such that the rate was only 2-fold lower than the control rate. Qualitatively similar results were obtained with phylloquinone. Lack of complete recovery may be attributable to the hydrophobic nature of the inhibitors and the hydrolase (Harrison *et al.*, 1979), which would tend to arrest dissociation in aqueous media.

Increasing the pre-incubation time did not affect the results. Thus these results are consistent with those of Table 1, and reinforce the conclusion that metabolism of the inhibitors is not essential for their action. The degree of restoration of hydrolase activity, and the inability of increased pre-incubation time to prevent restoration, suggests that no irreversible covalent interaction occurs between inhibitor and hydrolase.

We demonstrated recently that low dietary α-tocopherol not only decreases the steady-state concentration of total vitamin A in liver, but also decreases retinyl ester and increases retinol concentrations in liver, kidney and intestine. These studies performed *in vivo* were extended by studies done *in vitro*, which showed that α-tocopherol inhibits retinyl ester hydrolase in liver, kidney and intestine, and that inhibition is unlikely to be a non-specific effect (Napoli *et al.*, 1984). For example, the synthetic antioxidant *NN'*-diphenyl-*p*-phenylenediamine, the most effective vitamin E substitute known (Draper, 1980), did not inhibit retinyl ester hydrolase *in vitro* (Napoli *et al.*, 1984), a result expected from its failure to reproduce the liver vitamin A-sparing effects of α-tocopherol *in vivo* (Sondergaard, 1973).

The present paper provides additional evidence that α-tocopherol influences tissue vitamin A stores through modulating the rate of retinyl ester hydrolysis. Since α-tocopherol is a non-competitive inhibitor, its potency depends only on its concentration and the concentration of the hydrolases, and would be unaffected by the vitamin A concentration. Thus increased accumulation of vitamin A, with age or as a result of diet, would not affect the degree of interaction between α-tocopherol and the hydrolase. The influence of α-tocopherol on the tissue concentrations of retinol and retinyl esters would be independent of the total vitamin A. Non-competitive inhibition by phylloquinone is not unexpected in view of its structural similarities to α-tocopherol. Phylloquinone might affect the relative amounts of retinol and retinyl esters *in vivo*, if localization with respect to the hydrolase is important, rather than the total tissue concentration. The demonstration of such a role for phylloquinone, however, will require further work.

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