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Phospholipase A_2 of Rat Liver Mitochondria in Vitamin E Deficiency

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1. There is a more than 2-fold increase in phospholipase A_2 activity (EC 3.1.1.4) of liver mitochondria isolated from vitamin E-deficient rats compared with that in normal rats. 2. α-Tocopherol in lipoprotein-bound form is more effective than free α-tocopherol in restoring the enzyme activity to normal.

Alterations in the permeability and the stability of cellular membranes have often been found to be associated with vitamin E deficiency. Electron-microscopic examination revealed that in vitamin E-deficient ducklings the membranes of liver subcellular organelles lost a great deal of positive contrast, which was attributed to a decrease in polyunsaturated fatty acids content of these membranes (Molenaar *et al.*, 1972). It has been proposed that the role of vitamin E in cellular membranes involves the protection of polyunsaturated fatty acids from damage by peroxidation (Witting, 1970; Tappel, 1972; Lucy, 1972), and also from random hydrolysis of phospholipids by membrane-bound phospholipases (Lucy, 1972), which would lead to disorganization of membrane structure and function.

Our interest in membrane-bound phospholipases arose from our observations of significantly increased contents of lysophosphatidylcholine in subcellular membranes of livers of vitamin E-deficient rats (A. S. Pappu, P. Fatterpaker & A. Sreenivasan, unpublished work). Since the major contributing factors to formation of tissue lysophosphatidylcholine are membrane-bound phospholipases it was decided to investigate whether vitamin E influences the activity of phospholipase A_2 (EC 3.1.1.4) in liver mitochondria. This enzyme was chosen for study since it is well characterized (Waite & Sisson, 1971). The results obtained on the effect *in vitro* of free and protein-bound α-tocopherol (Rajaram *et al.*, 1973, 1977) have also been included.

Materials and Methods

Chemicals

α-Tocopherol acetate was from E. Merck, Darmstadt, Germany. Casein was from Amul Products, Anand, Gujarat, India. [^{32}P]P_i (carrier-free) was from Radioisotope Division, Bhabha Atomic Research Centre, Trombay, Bombay, India. Sephadex G-200 was from Pharmacia Fine Chemicals AB, Uppsala, Sweden. 2,5-Diphenyloxazole (PPO) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (dimethyl-POPOP) were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Vitamin E-deficient animals

Male weanling albino rats of Wistar strain weighing 30–40g were raised on either a vitamin E-deficient or vitamin E-supplemented diet (Adhikari *et al.*, 1968). The former contained (per 100g) vitamin-free casein (20g), sucrose (8g), salt mixture prepared according to U.S. Pharmacopia 14, 1950 (4g), groundnut oil (8g), vitamin mixture in sucrose (2g) and starch (58g). The vitamin mixture contained (per 100g), thiamin (10mg), riboflavin (20mg), pyridoxine (20mg), choline (5g), inositol (5g), *p*-aminobenzoic acid (1.5g), nicotinamide (0.5g), calcium pantothenate (50mg), folic acid (12.5mg), cyanocobalamin (0.25mg), menadione (2.5mg) and cholecalciferol (5mg). Retinyl palmitate was included at 25mg/kg of diet. The tocopherol-supplemented diet was identical with the deficient diet except for the addition of 150mg of DL-α-tocopherol/kg of the diet. The animals were fed *ad libitum* for about 5–6 months. The onset of vitamin E deficiency was monitored by determining the susceptibility of erythrocytes to peroxidative haemolysis (Rose & Gyorgy, 1952), from the decrease in the weight of the testes of the animals and from the vitamin E content of the liver. Vitamin E was extracted from the rat livers as described by Rajaram *et al.* (1974) and was measured by a standard procedure (Emmeri & Engel, 1938).

Isolation of mitochondria

Rat liver mitochondria were prepared from a 10% (w/v) liver homogenate in 0.25M-sucrose containing 0.01M-Tris/HCl, pH7.4, and 0.001M-EDTA (Satav *et al.*, 1976). Microsomal and lysosomal contamination of the mitochondrial fraction were determined by monitoring, as marker enzymes, glucose 6-phosphatase (EC 3.1.3.9; Recknagel & Lombardi, 1961) and acid phosphatase (EC 3.1.3.2; Gianetto & de Duve, 1955) respectively.

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Isolation of hepatic cell sap and lipoprotein-bound vitamin E

Hepatic cell sap and lipoprotein-bound α-tocopherol were isolated by using Sephadex G-200 gel filtration by the procedure of Rajaram *et al.* (1974).

Preparation of labelled phospholipids

[^{32}P]Phosphatidylcholine and lyso[^{32}P]phosphatidylcholine were obtained by injecting intraperitoneally 3mCi of [^{32}P]P_i (carrier-free) into Wistar rats weighing 150–250g. The lipids were extracted from the liver by the method of Folch *et al.* (1957) and the labelled phospholipids were separated by t.l.c. (Wagner *et al.*, 1961).

Hepatic mitochondrial phospholipase A_2

Mitochondrial phospholipase A_2 was assayed by the method of Waite & Sisson (1971). The reaction mixture contained 0.1–0.2μCi of [^{32}P]phosphatidylcholine (specific radioactivity, 22.8μCi/mmol), 100mM-glycine/NaOH buffer, pH9.5, 2mM-$CaCl_2$, 500mM-KCl and a mitochondrial suspension equivalent to 2–3mg of protein in 0.25M-sucrose. The total volume was 1ml. The mixture was incubated at 37°C for 30min and the reaction was stopped by the addition of 20vol. of chloroform/methanol (2:1, v/v). The lipids in the reaction mixture were extracted by the procedure of Folch *et al.* (1957). Lyso[^{32}P]phosphatidylcholine was separated from other phospholipids by t.l.c. on silica gel a with chloroform/methanol/water (65:25:4, by vol.) as the developing solvent (Wagner *et al.*, 1961). Lysophosphatidylcholine was identified by co-chromatography with an authentic sample of lysophosphatidylcholine on the same t.l.c. plates. The area corresponding to the lysophosphatidylcholine spot was detected by iodine vapour, scraped off and the scrapings used directly for measurement of radioactivity in counting vials containing 10ml of scintillation fluid [0.3% (w/v) PPO and 0.01% (w/v) dimethyl-POPOP in toluene]. The radioactivity was measured in a Beckman LS-100 liquid-scintillation spectrometer. The counting efficiency for ^{32}P was about 20%.

The effect of either hepatic cell sap or lipoprotein-bound α-tocopherol or α-tocopherol suspension on mitochondrial phospholipase A_2 was assayed with slight modification of the above procedure. Mitochondrial suspension equivalent to 2–3mg of protein in 0.1ml of 0.25M-sucrose was preincubated at 37°C with 0.4ml of cell-sap fraction, which contained 6–7mg of protein, or with 0.4ml of lipoprotein-bound α-tocopherol suspension containing 1.5–2.5μg of vitamin E, or with 0.4ml of a suspension of α-tocopherol (10μg) in 0.9% NaCl containing 1% (w/v) Tween-80 in a total volume of 0.5ml for 15min at 37°C in a shaking water bath. The quantities of cell sap, lipoprotein-bound α-tocopherol and α-tocopherol suspension were fixed arbitrarily with respect to vitamin E concentration, because determination of the α-tocopherol content in cell sap and in the lipoprotein and fixing their concentrations at comparable values before the phospholipase assay was time-consuming and would have led to oxidative losses of the vitamin. Hence the quantities of the cell sap and the lipoprotein were arbitrarily fixed and their α-tocopherol content was determined later. Phospholipase A_2 activity of mitochondria thus incubated was determined by the method described above.

Protein was measured by the procedure of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results and Discussion

Rats exhibiting 95% erythrocyte haemolysis and showing a decrease in testes wet weight from 1.5g (normal) to 0.9g (deficient) were used in these studies. The vitamin E content of these livers was 1.5μg per g wet wt. compared with 200μg in control rats (Table 1).

The mitochondrial fraction used appeared to be fairly pure, since the percentage contamination by microsomal and lysosomal fractions as assessed by the activities of glucose 6-phosphatase and acid phosphatase respectively was less than 5%.

The activities of mitochondrial phospholipase A_2 in vitamin E-deficient rat liver and that of the corresponding controls are given in Table 2. Interference by lysophospholipase (EC 3.1.1.5) localized in the matrix was assessed separately by using lyso-[^{32}P]phosphatidylcholine as a substrate and keeping other conditions of the assay system the same. The

Table 1. *Criteria for vitamin E deficiency in rats*
Male rats of Wistar strain were maintained on vitamin E-deficient diet for a period of 4–5 months. The onset of vitamin E deficiency was monitored by the erythrocyte-haemolysis test, and determining the weight of the testes and the α-tocopherol content of the liver. The results are given as means±S.E.M. for 12 independent observations. The erythrocyte haemolysis test was carried out using dilauric acid. No haemolysis was observed in the control rats. Similar observations are reported by Rose & Gyorgy (1952) and Bunyan *et al.* (1960). *Significantly different from the corresponding control values at $P<0.001$, calculated by Student's t test.

Group	Erythrocyte haemolysis (%)	Testis wet weight (g)	α-Tocopherol content of the liver (μg/g wet wt.)
Control	Nil	1.50±0.17	200 ±25
Vitamin E-deficient	95±4*	0.90±0.08*	1.50± 0.19*

amount of lysophosphatidylcholine hydrolysed was computed from the radioactivity remaining in the lysophosphatidylcholine fraction after incubation. With the assay conditions used for phospholipase A_2 only about 10% of the lysophosphatidylcholine added was hydrolysed. The interference by lysophospholipases was therefore considered to be negligible. The activity of phospholipase A_2 of the mitochondria for vitamin E-deficient rats was twice that of the control (Table 2). However, the activity was decreased to the normal values when these mitochondria were preincubated with cell sap from control animals and not with cell sap from vitamin E-deficient animals. Incubation of control mitochondria with cell sap from either control or deficient animals had no effect on the enzyme activity. These results imply that the control cell sap contains a factor that inhibits enzyme activity and that this factor is probably absent from the cell sap of deficient rats. It is possible that the lysophospholipases present in the control cell sap may hydrolyse lysophosphatidylcholine formed. This would give an impression of inhibition of phospholipase A_2 of the deficient mitochondria. The contribution of lysophospholipase in apparently decreasing the enzyme activity seems to be minimal since the cell-sap lysophospholipase has a pH optimum of 7, and at the experimental pH (9.4) its activity decreases by about 84% (van Den Bosch & de Jong, 1975). Failure of the cell sap from the deficient rats to decrease the enzyme activity also cannot be explained on this basis. In this context the observations (Rajaram *et al.*, 1973) on the presence of a significant amount of α-tocopherol in cell sap, about 80% of which exists in the lipoprotein-bound form and that decreases to negligible values in vitamin E deficiency, were of relevance. The vitamin present in the cell sap from control animals is probably responsible for the inhibition of the enzyme activity in mitochondria from the deficient rats that lack vitamin E. The control mitochondria have sufficient vitamin to control the phospholipase A_2 activity and any further addition of the vitamin from control cell sap has no effect. To verify these assumptions, experiments were carried out to assess the effect of free and bound α-tocopherol on the enzyme activity. It is evident from Table 3 that addition of α-tocopherol *in vitro* in both the free and the bound form inhibits the phospholipase A_2 activity in mitochondria from deficient-rat liver. Since Tween-80 was used to emulsify α-tocopherol, its effect on the enzyme activity was assessed separately. Tween-80 at the concentration used for emulsifying α-tocopherol when added to mitochondrial suspension from control and deficient rats under appropriate conditions activated the phospholipase A_2 activity, expressed as a percentage of the radioactivity (d.p.m.)

Table 2. *Phospholipase A_2 activity in vitamin E deficiency*

The enzyme activity is expressed as percentage hydrolysis of the added phosphatidylcholine to lysophosphatidylcholine. Results are given as means ± S.E.M. of five independent experiments. *Significantly higher than control values at $P<0.001$.

Phospholipase A_2 activity $\left[\left(\frac{\text{d.p.m. in lysophosphatidylcholine}}{\text{d.p.m. in added phosphatidylcholine}} \times 100\right) \Big/ \text{mg of protein}\right]$

Group	Mitochondria	Mitochondria + control cell sap	Mitochondria + deficient cell sap
Control	7.50 ± 1.12	8.00 ± 1.14	6.80 ± 0.74
Vitamin E-deficient	16.5 ± 0.8*	6.00 ± 0.75	17.0 ± 0.7*

Table 3. *Effect of vitamin E addition on phospholipase A_2 activity in vitamin E deficiency*

Lipoprotein-bound vitamin E was obtained from cell sap by the procedure of Rajaram *et al.* (1974). Other details are as described in the Materials and Methods section. Results are given as a mean ± S.E.M. for five independent experiments. * Significantly different from the value for vitamin E-deficient mitochondria at $P<0.001$. † Significantly different from the value for control mitochondria at $P<0.001$.

Phospholipase A_2 activity $\left[\left(\frac{\text{d.p.m. in lysophosphatidylcholine}}{\text{d.p.m. in added phosphatidylcholine}} \times 100\right) \Big/ \text{mg of protein}\right]$

Group	Mitochondria	Mitochondria + lipoprotein-bound vitamin E from control cell sap	Mitochondria + vitamin E
Control	7.50 ± 1.12	6.50 ± 0.51	5.50 ± 0.67
Vitamin E-deficient	16.50 ± 0.83†	6.00 ± 0.72*	9.60 ± 0.64*

of the added [^{32}P]phosphatidylcholine recovered in lyso[^{32}P]phosphatidylcholine, from 7.50±1.12 to 12.60±2.10. The enzyme activity of the mitochondria from the deficient rats in the presence of Tween-80, however, was 14.20±1.66, which was not significantly different from the value (16.50±0.83) for the mitochondria from deficient rats alone. This is understandable in view of the solubilizing effect of the detergent on cellular membranes in bringing about alterations in bound-enzyme activities. The phospholipase A_2 activity of the mitochondrial membrane from deficient rats must have been maximally activated, with the result that any further disruption of the membrane lipids has no effect. It is noteworthy that α-tocopherol in the presence of Tween-80 is more efficient in decreasing the enzyme activity in normal mitochondria than with the mitochondria from deficient rats. Lipoprotein-bound α-tocopherol restores the enzyme activity of the mitochondria in deficient rats more effectively than does free α-tocopherol: 10μg of unbound α-tocopherol brought about 42% inhibition, whereas 1.5–2.5μg of the lipoprotein-bound α-tocopherol caused 63% inhibition. The inhibitory effect of the cell sap from control rats could possibly be attributed to the presence of α-tocopherol in the bound form. The binding of α-tocopherol to proteins or lipoproteins may be expected to facilitate its uptake by membranes where it is functional (Rajaram *et al.*, 1973, 1977). The bound vitamin has no effect on the phospholipase A_2 activity of the control mitochondria.

Vitamin E may be inhibiting the enzyme by masking the active site by either direct physical association or affecting enzyme conformation. As Lucy (1972) has suggested, it is likely that the vitamin may decrease the availability of substrate by binding to the arachidonoyl residue, thus rendering them more resistant to hydrolysis. An oxidation product of α-tocopherol has been shown to form stable complexes with fatty acid residues of phosphatidylcholine (Porter *et al.*, 1971). Alternately, vitamin E may be involved in maintenance of the fluidity of membrane bilayer that determines membrane conformation that, in turn, may keep the enzyme in an inactive form (Fourcans & Jain, 1974). These problems, admittedly, need to be worked out in greater detail.

Increase of phospholipase A_2 activity can account for the enhanced content of arachidonic acid observed in tissues of vitamin E-deficient animals (Witting & Horwitt, 1967; Carpenter, 1971), as these moieties are usually associated with the C-2 position of phospholipids from which they can be readily hydrolysed by phospholipase A_2. It remains to be seen whether the function of vitamin E in relation to the activation of phospholipase A is specific and limited to mitochondria alone or is common to other cellular membranes.

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