# AN α-TOCOPHEROL BINDING PROTEIN IN RAT LIVER CYTOPLASM

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Received September 2, 1975

SUMMARY

Gel filtration and sucrose density gradient analysis of rat liver high speed supernatant revealed the presence of a protein capable of binding [$^3$H] α-tocopherol. The protein sedimented with an S value of 3.0. Gel filtration yielded an estimated molecular weight of 31,000. Specificity for α-tocopherol was demonstrated by competition for binding of [$^3$H]α-tocopherol with unlabeled α-tocopherol, but not with α-tocopheryl quinone or α-tocopheryl acetate. Pronase digestion completely abolished binding.

Cytoplasmic binding proteins for the fat soluble vitamins retinol (1,2) retinoic acid (3,4) and 1,25 dihydroxycholecaliciferol (5,6) have been described. Each protein exhibits a high affinity for its respective ligand and specificity can be demonstrated by competition studies. Since no such protein has been described for tocopherol, a study was undertaken to determine if a tissue tocopherol binding protein could be detected. The results demonstrate the existence of a protein in rat liver cytoplasm capable of binding α-tocopherol and exhibiting the above mentioned characteristics of binding proteins. Furthermore the bound α-tocopherol can be separated from the lipoprotein associated tocopherol of rat liver cytoplasm described by Rajaram et al. (7,8).

## Methods and Materials

Experimental animals and tissue fractionation--Male Holtzman rats fed either NIH open formula stock ration or an α-tocopherol free diet (9) for 3-4 months were used. Rats were killed by decapitation, the livers excised and perfused with ice-cold 0.9%NaCl. Livers were homogenized in 4 volumes of 50 mM Tris-HCl buffer, pH 7.5, using 5 strokes of a teflon pestle homogenizer. Homogenates were centrifuged at 10,000 x g for 15 min at 4°C followed by centrifu-

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gation at 105,000 x g for 1 h at 4°C and the high speed supernatant (cytosol) was collected free of floating lipid. Aliquots of the cytosol were incubated for 4 h at 4°C in the presence of 20nM or 90nM d-α-[5 methyl-$^3$H]tocopherol (Amersham Searle-1.4 Ci/mmol) added in 6-8 μl of ethanol per ml of cytosol.

Sephadex gel filtration--A 1.8 ml aliquot of cytosol after incubation with [$^3$H]tocopherol, was applied to a Sephadex G-100 Column (2.6 cm x 50 cm) equilibrated with 50 mM Tris-HCl buffer, 0.1 M KCl, pH 7.5. The flow rate was adjusted to 12 ml per hour and 3 ml fractions were collected. One ml aliquots of each fraction were assayed for radioactivity by adding 10 ml of scintillation solution containing Spectrafluor-(Amersham-Searle) Triton X 100-toluene (50:615:1181). Counting efficiency was about 18%. The remaining 2 ml of each fraction was analyzed for protein at 280 nm.

Sucrose gradient centrifugation--A 0.2 ml aliquot of cytosol, after incubation with [$^3$H]tocopherol, was applied to the top of a 5% to 20% sucrose gradient prepared in 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 10 mM KCl. Gradients were centrifuged in a Spinco 50 L rotor at 45,000 rpm for 18 h at 4°C. The gradient tubes were pierced from the bottom and 15 drop fractions were collected directly into scintillation vials. The radioactivity in each fraction was determined following the addition of 0.5 ml of water and 5 ml of scintillation fluid. Competition for binding was examined by sucrose gradient centrifugation of 0.2 ml aliquots of cytosol incubated with [$^3$H]tocopherol and a 400 fold molar excess of either unlabeled α-tocopherol, α-tocopheryl quinone or α-tocopheryl acetate. Test compounds were added to the incubation in 1 μl of ethanol per ml of cytosol.

Pronase Digestion--Following incubation of cytosol with [$^3$H]tocopherol, 400 μg of Pronase (Calbiochem) in 0.1 ml of 50 mM Tris-HCl buffer, pH 7.5, was added. Buffer alone was added to other aliquots as a control, and the samples were incubated for 2 h at room temperature and applied to gradients.

## Results and Discussion

The *in vitro* binding of [$^3$H]α-tocopherol to rat liver cytosol components

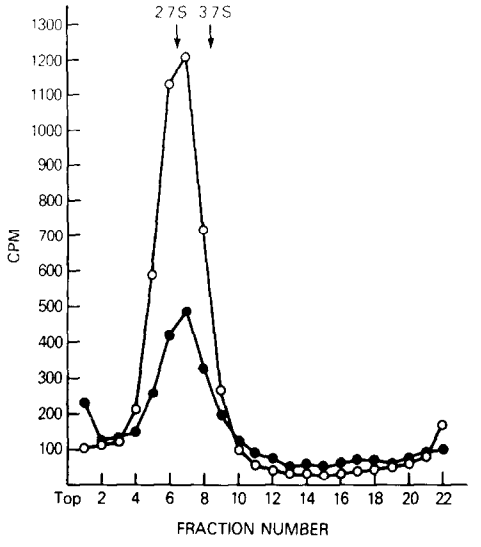


Figure 1. Sucrose density gradient centrifugation of rat liver cytosol incubated in the presence of 90nM[$^3$H]α-tocopherol as described in the text. Chymotrypsinogen and ovalbumin were used as markers. Cytosol was prepared from rats fed a tocopherol free ration (○—○), or a stock ration (●—●).

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is illustrated in Figure 1. A single macromolecular-tocopherol complex was observed following sucrose density gradient centrifugation. The sedimentation coefficient of the bound tocopherol is approximately 3S as calculated by the sedimentation of the markers chymotrypsinogen and ovalbumin (10).

The amounts of bound tocopherol varied somewhat with preparations from different rats. This was most likely due to the presence of varying quantities of endogenous tocopherol since, as illustrated in Figure 1, considerably less binding is observed in the cytosol of rats fed stock ration as compared to the rats fed a tocopherol-free diet. Subsequent experiments were therefore carried out using liver cytosol from rats fed the tocopherol-free ration.

The specificity of the binding component was examined by incubating rat liver cytosol with 90 nM[$^3$H]tocopherol in the presence of a 400-fold excess of unlabeled α-tocopherol. As shown in Figure 2, the unlabeled compound competed for the binding of [$^3$H]tocopherol and reduced the amount of bound radioactivity by greater than 90%. The additions of a 400 fold excess of either α-tocopheryl

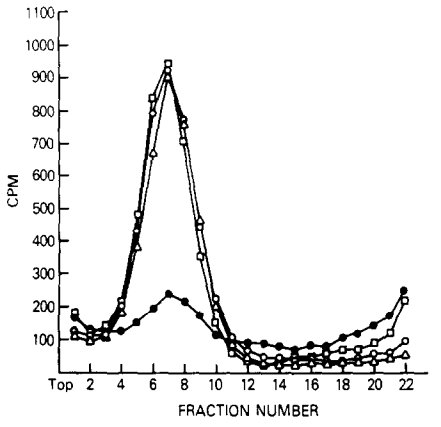


Figure 2. Sucrose density gradient centrifugation of rat liver cytosol incubated in the presence of 90nM[$^3$H]α-tocopherol (○—○) or 90nM[$^3$H]α-tocopherol plus a 400 fold excess of α-tocopherol (●—●), α-tocopheryl quinone (△—△) or α-tocopheryl acetate (□—□). See text for details.

quinone or α-tocopheryl acetate had no effect on the binding of [$^3$H]α-tocopherol (Fig. 2). These results indicate that an intact chromanol ring structure and a free hydroxyl group are specific requirements for binding.

To assess the chemical nature of the binding component, the [$^3$H]tocopherol labeled cytosol was subjected to proteolytic digestion. Treatment with Pronase resulted in the complete loss of bound tocopherol, demonstrating that the binding component is a protein.

The α-tocopherol binding protein observed in sucrose gradients was further characterized by Sephadex G-100 chromatography. The elution pattern (radioactivity and protein) of cytosol incubated in the presence of [$^3$H]tocopherol is shown in Figure 3.

At a concentration of 20 nM[$^3$H]tocopherol most of the radioactivity is associated with a protein fraction that is retained on G-100. The elution volume of the binding protein indicates that the molecular weight is approximately 31,000 as judged by the elution of the molecular weight markers ovalbumin (45,000) and chymotrypsinogen (25,000). This value is in agreement with the sedimentation value of 3.0 obtained from sucrose density gradients. A high affinity of the binding protein for α-tocopherol can be inferred from the per-

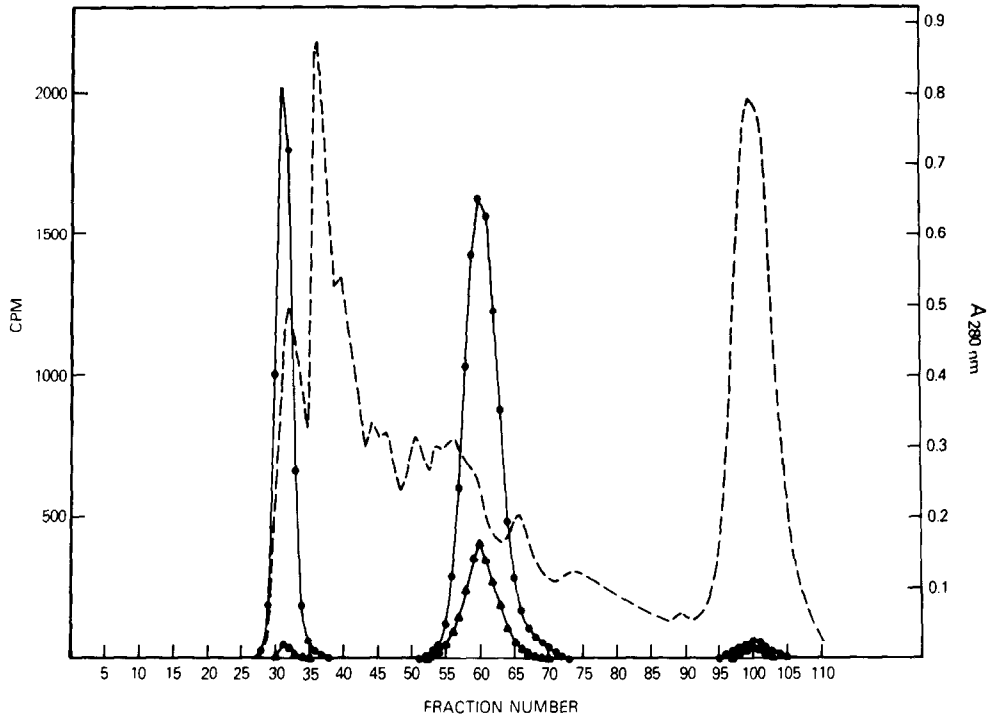


Figure 3. Sephadex G-100 gel filtration of rat liver cytosol incubated in the presence of 20nM(▲—▲) or 90nM(●—●) [$^3$H]α-tocopherol. Fractions were analyzed for protein at 280 nm(----) and radioactivity (●—●, ▲—▲) as described in the text.

sistence of bound radioactivity following sucrose density gradient centrifugation and gel filtration.

The association of tocopherol with a lipoprotein fraction of liver cytosol from rats fed a tocopherol adequate ration has been reported (7, 8). Following in vivo administration of [$^3$H]tocopherol, rat liver cytosol was subjected to gel filtration over Sephadex G-200. The results showed that the tocopherol was eluted just after the void volume as a single peak and the binding component was therefore judged to have a high molecular weight, substantiated by acrylamide gel electrophoresis. It should be noted from the present study that at a concentration of 20nM[$^3$H]tocopherol little radioactivity is eluted in the void volume. When the concentration is raised to 90nM, however, a much greater proportion of the radioactive tocopherol is eluted in this fraction (Fig. 3). These data

demonstrate that the α-tocopherol binding protein exhibits a greater affinity for tocopherol than the lipoprotein described by Rajaram et al. (7, 8). The failure of these authors to detect the tocopherol binding protein is probably the result of several factors. The animals used in their study received a diet adequate in tocopherol. As shown in Figure 1 considerably less binding is observed in cytosol of animals ingesting adequate tocopherol. Furthermore it can be estimated from the amount of radioactivity present in the supernatants of the animals used by Rajaram et al. (7) that a substantially greater quantity of tocopherol was present than the amounts added in vitro in this study. Since the competition experiment described here (Fig. 2) suggests that the α-tocopherol binding protein contains a limited number of binding sites, it would seem likely that the large excess of [$^3$H]tocopherol bound to the lipoprotein fraction in the experiments of Rajaram et al. (7) could have made the relatively small amounts of tocopherol bound to the α-tocopherol binding protein go unnoticed.

The molecular events mediated by α-tocopherol are presently unknown, but one frequently suggested hypothesis on its mode of action is that it serves some specific but thus far undefined regulatory role. Future work will hopefully determine whether the α-tocopherol binding protein mediates cellular events in a manner similar to other binding (receptor) proteins, and thus provide a new and useful approach toward efforts to define the mechanism of action of α-tocopherol.

ACKNOWLEDGEMENT

I wish to thank Dr. John G. Bieri for his helpful suggestions during the course of this investigation and the preparation of this manuscript.

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