PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 166, 167–174 (1981)

Binding of Vitamin E in Mammalian Tumor Cells in Culture (41041)

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Abstract. Radioactive vitamin E (D-α-[5-*methyl*-^{3}H]tocopherol) bound with the cytosol (100,000*g* supernatant), pellet (100,000*g* pellet), crude nuclear, and purified chromatin fractions from mouse neuroblastoma (NBP_2) and rat glioma (C-6) cells in culture. The level and type of vitamin E binding proteins in the cytosol depended upon the cell type. When the cytosol proteins containing radioactive vitamin E were separated by gel filtration (Sepharose 4B gel), there were five protein peaks in neuroblastoma, three peaks in glioma, and one peak in mouse B-16 melanoma cells which contained bound radioactivity. The level of binding in the neuroblastoma cells was higher than that in glioma cells or melanoma cells. Vitamin E remained bound to the proteins from the cytosols of neuroblastoma and glioma even after denaturation and separation by electrophoresis. This suggests that vitamin E is tightly bound with the cytosol proteins. There was only one vitamin E binding protein in the pellet and nuclear fractions of NB, glioma, and melanoma cells. The significance of vitamin E binding proteins in the mechanism of the effect of vitamin E on mammalian cells in culture is unknown.

Vitamin E appears to have a multiple role in cellular functions. Vitamin E has been recognized as a biological lipid antioxidant (1–3). Vitamin E stabilizes membranes probably by participating in enzyme–lipid interactions (4–6). Vitamin E prevents bacterial infection in mice (7), inhibits synthesis of prostaglandins by platelets (8), prevents aggregation (9–12) and release (13) of platelets, and decreases the occurrence of bleomycin-induced lung fibrosis (14). In vitamin E-deficient rats, vitamin E causes a dramatic but transient increase in hepatic nuclear RNA (15). We have recently observed that vitamin E causes irreversible morphological differentiation in mouse neuroblastoma cells in culture (NBP_2 clone) (16), and reversible morphological differentiation in mouse B-16 melanoma cells in culture (17). However, it does not cause morphological differentiation in rat C-6 glioma cells in culture (16). We have also found that vitamin E modifies the effects of several pharmacological agents on mammalian cells in culture (18). The extent of modification of the effect of pharmacological agents depends upon the type of cell and upon the particular agent. In an effort to explain the mechanisms of effect of vitamin E, we have investigated the binding of vitamin E with the subcellular fractions. We now report that radioactive vitamin E binds with the cytosol, pellet, crude nuclear, and purified chromatin fractions. The level and type of vitamin E binding proteins in the cytosol depends upon the cell type.

Materials and Methods. Mouse neuroblastoma clone NBP_2 (19), rat glioma clone C-6 (20) and mouse B-16 melanoma were used in this study. NB cells contain both tyrosine hydroxylase and choline acetyltransferase activities (19). Glioma cells of passages 26–30 were used in this study because they lose certain hormonal responses after passage 50 (21). All cell types were grown in F12 medium containing 10% fetal calf serum containing 100 units penicillin and 100 μg/ml streptomycin. They were maintained at 37° in a humidified atmosphere of 5% CO_2. Cells (NB—0.25×10^6; glioma—1.0×10^6) were plated in Lux tissue culture dishes (100 mm). Fresh growth medium was changed at 2 and 3 days (20 ml of medium) after plating. The subcellular distribution of vitamin E was studied 4 days after plating when the cells were in the confluent phase of growth. Fresh growth medium (5 ml) was changed 30 min prior to addition of D-α-[5-*methyl*-^{3}H]tocopherol (Amersham Corporation; specific activity 17 Ci/mmole or 40 mCi/mg). Radioactive

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vitamin E was added at a concentration of 2 μCi/ml, and incubated at 37° for 5 min. After incubation, the cells were washed two times with phosphate-buffered saline, pH 7.0. Cells were removed from the dish surface by a rubber policeman in ice-cold 0.05 *M* Tris–HCl, 0.25 *M* sucrose, 3 m*M* $MgCl_2$, 4 m*M* 2-mercaptoethanol, and 2 m*M* phenylmethylsulfonylfluoride (PMSF), pH 7.4. The contents of 20 dishes were pooled together in a total volume of 3.7 to 5.6 ml. The contents were homogenized at 5000 rpm for 2 min (5-stroke) using a Potter–Elvehjem tissue grinder. The subcellular fractions were prepared according to the method described previously (22).

Crude nuclear preparation. The homogenate was centrifuged at 800*g* for 15 min at 4° in a Sorval RC 2-B centrifuge. The supernatant fraction was recentrifuged for an additional 15 min at 800*g*. The nuclear pellets (800*g*) from the two centrifugations were combined, dispersed, and homogenized in 0.5 ml of the above Tris–HCl buffer. An aliquot of nuclear homogenate was dissolved in an equal volume of 1 *N* NaOH. An aliquot of this was taken for protein determination, and another aliquot for determination of radioactivity. The remaining portion of crude nuclear fraction was saved for chromatin preparation.

Cytosol. The 800*g* supernatant fractions obtained after the first centrifugation were centrifuged at 100,000*g* for 90 min at 4° in a Beckman L3-40 ultracentrifuge. The clear supernatant fluid, free of particulate materials, constituted the cytosol fraction. An aliquot of this was taken for protein determination, and another aliquot was taken for determination of radioactivity.

Cytoplasmic particulate. The cytoplasmic particulates (100,000*g* pellet) were washed once by dispersing in 5 vol of the Tris–HCl buffer and centrifuging at 100,000*g* for 90 min at 4°. The pellets were resuspended in 0.2 ml of the Tris–HCl buffer and homogenized in a Dounce tissue grinder. An aliquot was taken for protein determination and another aliquot was taken for counting radioactivity.

Chromatin preparation. The purified nuclei were allowed to swell in 2 vol of distilled water at 0° for 30 min and lysed by homogenization using a Dounce tissue grinder (23, 24). The chromatin materials were mixed with an equal volume of 0.2 m*M* EDTA, 4 m*M* PMSF, and 20 m*M* Tris–HCl, pH 8.0, and centrifuged through 1.7 *M* sucrose in a SW 41-ti Beckman rotor at 115,000*g* for 90 min at 4°. The purified chromatin was resuspended in the same buffer. An aliquot was taken for protein determination, and another aliquot was taken for determining radioactivity.

Protein determination. The protein was determined by the method of Lowry *et al.* (25) using bovine serum albumin (fraction V) as a standard.

Separation of cytosol proteins by gel filtration. Two-milliliter fractions from NB (4.13 mg protein, 36.6×10^4 cpm) and glioma (2.5 mg protein, 17.7×10^4 cpm) cells were passed through a column (55 × 2.5 cm) containing ultragel ACA-34 (LKB 2204-340) in a 20 m*M* Hepes, 120 m*M* KCl, 5 m*M* Mg acetate, 1 m*M* DTT buffer, pH 7.6. Two-milliliter fractions were collected. In another experiment, the cytosols from NB (2.5 mg protein, 22.1×10^4 cpm) and glioma (2.5 mg protein, 15×10^4 cpm), and melanoma (2.5 mg protein, 8.9×10^4 cpm) were filtered through a column (38 × 2.5 cm) of Sepharose 4B gel in a 20 m*M* Hepes, 120 m*M* KCl, 5 m*M* Mg acetate, 1 m*M* DTT buffer, pH 7.6. The pellet and nuclear fractions were solubilized in Hepes buffer containing 0.5% Triton X-100 overnight at 4°. The pellet fraction from NB (1.58 mg protein, 84.2×10^4 cpm), glioma (1.67 mg protein, 46.2×10^4 cpm), and melanoma (1.25 mg protein, 47×10^4 cpm) were filtered through a Sepharose 4B gel in a buffer (20 m*M* Hepes, 120 m*M* KCl, 5 m*M* Mg–acetate, 1 m*M* DTT, pH 7.6) containing 0.5% Triton X-100. The nuclear pellets from NB (2.8 mg protein, 4.6×10^4 cpm), glioma (0.66 mg protein, 2.2×10^4 cpm), and melanoma (2.56 mg protein, 4.4×10^4 cpm) were filtered through a Sepharose 4B gel in the same buffer as described above. An aliquot (0.2 ml) from each fraction was taken for counting radioactivity.

Separation of cytosol by SDS–gel electrophoresis. The procedure for preparing gel and samples for electrophoresis has been described by Laemmli (26). Electro-

phoresis was carried out with a current of 3 mA per gel tube until the bromphenol blue marker reached the bottom of the gel (about 4 hr). After electrophoresis, the gels were divided into 2-mm segments using a Gilson gel fractionator. Each fraction was collected directly into a vial, and the radioactivity was counted in a dioxane solvent system by a liquid scintillation counter.

Counting radioactivity. The homogenate, pellet, nuclear, and chromatin fractions were first dissolved in 1 *N* NaOH, and then an aliquot was counted in a liquid scintillation spectromoter using a Beckman Ready-Solve EP solvent system.

In vitro binding. Neuroblastoma cells were grown to confluency. Cells were washed twice with phosphate-buffered saline (pH 7.0). Cells were removed from the dishes in 50 m*M* Tris–HCl buffer, pH 7.4, with a rubber policeman. The contents of five dishes were pooled together. The cytosol was prepared according to the procedure described in the previous sections. The reaction mixture of 135 ml contained 50 μg protein, 37 m*M* Tris–HCl, pH 7.4, 1.25 IU vitamin E (DL-α-tocopherol acetate) and 101,000 cpm ^{3}H-vitamin E (sp act 17 Ci/mmole). The reaction mixture was preincubated at 4° in the absence of radioactive vitamin E for 10 min, and then radioactive vitamin E was added. The reaction mixture was incubated for an additional 15 min at 4°. The blanks were similarly treated except no protein was added. The reaction was terminated by the addition of 3 ml of cold Tris–HCl buffer. The contents were filtered under reduced pressure on a glass filter (No. 30 glass, 25 mm ZE01, Schleicher and Schuell, Inc., N.H.). The tubes were rinsed once with 3 ml of cold buffer, and the filters were washed twice with 4 ml of ethanol. The filter was placed into a scintillation vial containing 10 ml of aquasol. The specific binding represented total binding in the absense of nonradioactive vitamin E minus total binding in the presence of nonradioactive vitamin E.

Results. Table I shows that the homogenate radioactivity in NB ells was about 45% higher than that in glioma cells, although the total amount of homogenate proteins was similar in both cell types (NB—25.9 mg; glioma—24.5 mg). Except in the chromatin fraction, the proportion of homogenate radioactivity in the cytosol (100,000*g*), pellet (100,000*g*), and crude nuclear fractions of NB and glioma were similar. The reduced amount of radioactivity in the chromatin fraction of NB cells was primarily related to a lesser yield of chromatin (total NB chromosomal proteins—0.29 mg; total glioma chromosomal proteins—0.99 mg). This was further supported by the fact that the specific activity of radioactive vitamin E in the chromatin fractions was similar in both cell types. The specific activity of vitamin E in the pellet and crude nuclear fractions of NB cells was about twofold higher than that in corresponding fractions of glioma cells; however, the specific activity of vitamin E in the cytosol fraction was similar in both cell types.

When the cytosols from NB and glioma cells containing bound radioactive vitamin E were separated by gel filtration (Ultragel ACA-34), only one peak of bound radioactive vitamin E was found (Fig. 1). When the cytosols from both NB and glioma cells were separated by polyacrylamide gel electrophoresis (27), only one area of the gels contained radioactive vitamin E (data not shown). These results are in agreement with those obtained by gel filtration (Ultragel, ACA-34), and support the idea that vitamin E is bound to proteins. The total amount of radioactivity in the material that was excluded from the gel was about threefold higher in NB cells than that in glioma cells. This was in part due to the fact that the total amounts of proteins and radioactivity in NB cells was about twofold higher than those in glioma cells. When the cytosol from glioma cells containing bound radioactive vitamin E was treated with an equal volume of 2.5% trypsin for 2 hr at 37°, then separated by gel filtration (Ultragel ACA-34), two protein peaks were observed (Fig. 2). One peak was at the same location as the initial peak. A second peak was observed in the material that was retarded by the column.

When the cytosols from neuroblastoma, glioma and melanoma cells containing bound radioactive vitamin E, were sepa-

TABLE I. SUBCELLULAR DISTRIBUTION OF D-α-[5-*methyl*-^{3}H]TOCOPHEROL (VITAMIN E) IN NEUROBLASTOMA AND GLIOMA CELLS IN CULTURE

Fractions	Percentage of homogenate radioactivity		Specific activity cpm/mg protein × 10^4	
	NB	Glioma	NB	Glioma
Cytosol (100,000*g*)	28	33	8.8	7.1
Particulate (100,000*g*)	23	19	53.5	27.7
Crude nuclear	14	12	14.6	8.2
Chromatin	0.88	4.4	11.7	11.8

Note. The procedure for isolating the subcellular fraction has been described in the section on Materials and Methods. The total homogenate radioactivities in NB and glioma cells were 3.86 × 10^6 cpm and 2.64 × 10^6 cpm, respectively. The specific activity of homogenate radioactivities in NB and glioma cells were 14.9 × 10^4 cpm/mg protein and 10.8 × 10^4 cpm/mg protein, respectively. The duplicate samples were taken for the determination of proteins and radioactivity. Each value represents an average of two samples. Each experiment was repeated twice, and similar results were obtained.

rated by gel filtration (Sepharose gel 4B), there were five protein peaks containing radioactive vitamin E in neuroblastoma cells (Fig. 3; molecular weights—7 × 10^6, 7.5 × 10^6, 3 × 10^6, 3.5 × 10^4, and 2 × 10^4), three protein peaks in glioma cells (Fig. 3: molecular weights—9 × 10^6, 2.5 × 10^6, and 2.1 × 10^4), and only one peak in melanoma cells (Fig. 4: molecular weight—2 × 10^4). The amounts of radioactivity of peaks in NB cells were higher than those in glioma cells. This may in part be due to the fact that the total radioactivity of NB cytosol added was about 47% higher than that of glioma cells. The total amount of proteins added to the column was similar for both cell types.

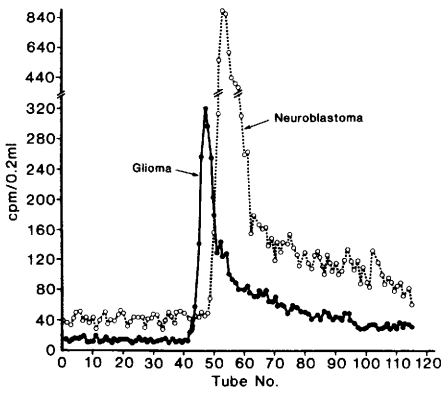

FIG. 1. The soluble proteins from neuroblastoma cells (4.13 mg protein; 36.4 × 10^4 cpm) and glioma cells (2.5 mg protein; 17.7 × 10^4 cpm) containing radioactive vitamin E were separated by gel filtration (Ultragel ACA-34), and the radioactivity in each fraction was counted.

When the cytosols from neuroblastoma and glioma were denatured by boiling in SDS for 1.5 min and then were separated on SDS–gel, the proteins retained their binding activity (Fig. 5). This suggests that vitamin E is tightly bound with the cytosol proteins.

When the pellet fractions from neuroblastoma and glioma were separated by gel filtration (Sepharose gel 4B), there was only one major peak containing radioactivity (Fig. 6; mol wt—2 × 10^4). The extent of bound radioactivity in the pellet fractions of glioma was higher than that of neuroblastoma cells, although the total amounts of proteins added to the gel were similar, and the total amount of radioactivity of the glioma pellet was twofold less than that of the NB pellet. The pellet of melanoma cells also exhibited only one peak at the same location. The peak radioactivity was 3100 cpm/0.2 ml, which was higher than NB cells, but lower than glioma cells.

When the crude nuclear fractions from neuroblastoma, glioma and melanoma were separated by gel filtration (Sepharose gel 4B), there was only one major peak containing radioactivity in all three cell types (Fig. 7; mol wt—2 × 10^4). The extent of binding was similar in both NB and melanoma cells. This was in part due to the fact that the total amounts of proteins and

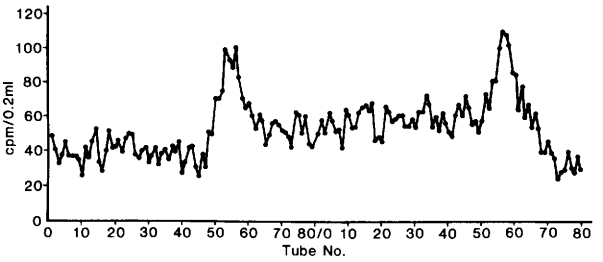

FIG. 2. The soluble proteins from glioma cells containing radioactive vitamin E were treated with an equal volume of 2.5% trypsin for 2 hr at 37°, then separated by gel filtration (Ultragel ACA-34), and the radioactivity in each fraction was counted.

radioactivity added to the gel were similar in both cell types.

The specific binding of radioactive vitamin E with the cytosol was 1020 ± 149 cpm/50 μg protein (20,400 ± 2480 cpm/mg protein). This represented about 1% of added radioactivity. The binding activity was completely abolished in the presence of a saturated concentration of nonradioactive vitamin E. The blank radioactivities in the absence and in the presence of nonradioactive vitamin E were 210 and 102 cpm, respectively.

To demonstrate the presence of vitamin E binding proteins in peripheral blood, human serum was used. The serum (50 mg protein) was incubated in the presence of radioactive vitamin E (10 μCi) at 4° for 1 hr, and then separated by gel filtration (Sepharose 4B gel). Only one peak (mol wt = ~20,000) with bound radioactivity was obtained. This binding protein may act as a carrier protein for vitamin E (data not shown).

Discussion. The present results show that vitamin E binds with cellular proteins. The number of vitamin E binding proteins and extent of binding depends upon the cell type. The cytosol from neuroblastoma cells contains five proteins, whereas the cytosols from glioma cells and melanoma cells contain only three and one protein peaks, respectively. Significant amounts of radioactivity were also associated with the pellet, crude nuclear and purified chromatin fractions. It was further observed that the pellet fractions of both NB and glioma have only one major radioactive bound peak of similar

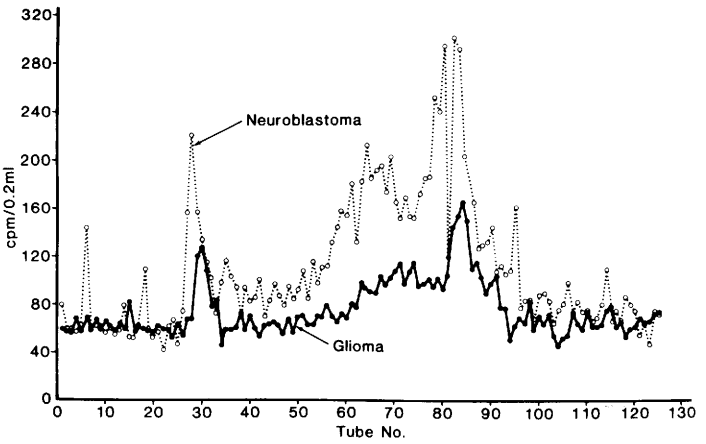

FIG. 3. The soluble proteins from neuroblastoma cells (2.5 mg protein; 22.1 × 10^4 cpm) and glioma cells (2.5 mg protein; 15 × 10^4 cpm) containing radioactive vitamin E were separated by gel filtration (Sepharose gel 4B), and the radioactivity in each fraction was counted.

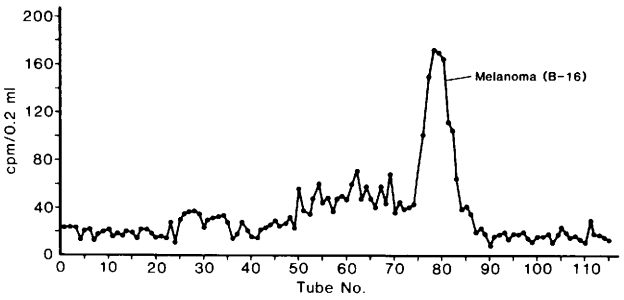

FIG. 4. The soluble proteins from melanoma cells (2.5 mg protein; 8.9 × 10^4 cpm) containing radioactive vitamin E were separated by gel filtration (Sepharose gel 4B), and the radioactivity in each fraction was counted.

molecular weights; however, the extent of binding in glioma cells was much higher than that in NB cells. The total amounts of pellet proteins added to the gel were similar, but the total amount of radioactivity of the glioma pellet was twofold less than that of NB cells. This suggests that either the binding affinity for vitamin E is much higher with the glioma pellet proteins than with the NB pellet proteins, or the amount of vitamin E binding proteins in the glioma pellet is more than that in the NB pellet. There was only one protein peak with bound radioactive vitamin E in the nuclear fraction of all cell types studied. Previous studies have shown that three distinct binding proteins to vitamin E exist in mammalian cells. Two of them are extranuclear components present in the cytosol of rat liver homogenate, while the third one is associated with the nuclear fraction (29–34). However, our studies show that the number of vitamin E binding proteins in cytosols depends upon the cell type.

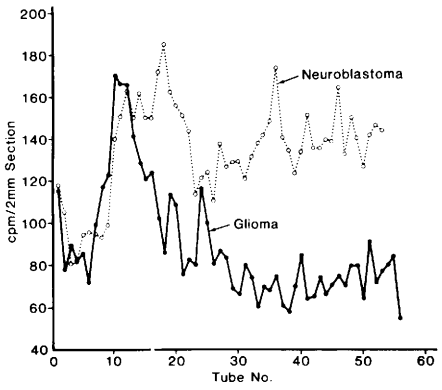

FIG. 5. The soluble proteins from neuroblastoma cells (2.5 mg protein; 22.1 × 10^4 cpm) and glioma cells (2.5 mg protein; 15 × 10^4 cpm) containing radioactive vitamin E were first inactivated, then separated on SDS–gel. After separation, the gel was cut into 2-mm pieces. The radioactivity in each fraction was counted.

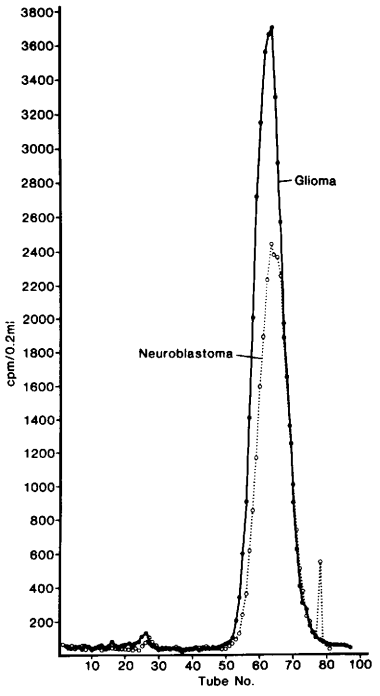

FIG. 6. The solubilized pellet proteins from neuroblastoma cells (1.58 mg protein; 46.2 × 10^4 cpm) and glioma cells (1.67 mg protein; 46.2 × 10^4 cpm) containing radioactive vitamin E were separated by gel filtration (Sepharose gel 4B), and the radioactivity in each fraction was counted.

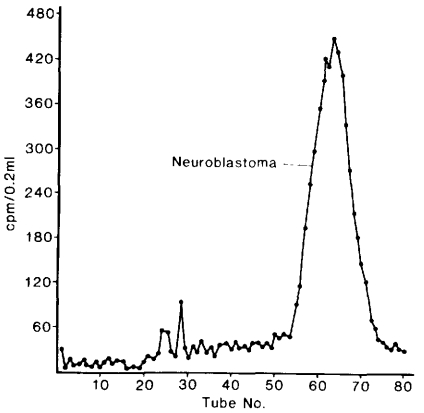

FIG. 7. The solubilized nuclear proteins from neuroblastoma cells (2.8 mg protein; 4.6×10^4 cpm) containing radioactive vitamin E were separated by gel filtration (Sepharose gel 4B), and the radioactivity in each fraction was counted.

It is not fully known whether all the radioactivity bound to subcellular fractions represents radioactive vitamin E. However, radioactive vitamin E was in the form of analogs and the incubation period was only 5 min; therefore, it is unlikely that the bound radioactivity represents the metabolites of vitamin E. In addition, the binding of radioactive vitamin E with NB cytosol is abolished in the presence of a saturated concentration of nonradioactive vitamin E. It should be pointed out that the binding of radioactive vitamin E with various subcellular fractions may involve some uncertainties. For example, it is not fully certain if all the radioactivities of various fractions were incorporated during the incubation of intact cells or if portions or all of the radioactivites could be attributed to redistribution of radioactive vitamin E among the subcellular fractions during homogenization and subsequent fractionation.

The significance of vitamin E binding proteins in the regulation of growth, differentiation, and malignancy, or in the modification of the effects of pharmacological agents is unknown. The fact that a significant amount of radioactive vitamin E was associated with the purified chromatin fraction suggests that vitamin E may modulate the gene expression in mammalian cells.

We thank Marianne Gaschler for her technical help. This work was supported by NIEHS—01576.

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Received June 11, 1980. P.S.E.B.M. 1980, Vol. 166.