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EFFECT OF α-TOCOPHEROL ON THE BINDING OF BENZO(A)PYRENE TO NUCLEAR MACROMOLECULES

Takao MATSUURA, Hisao UEYAMA, Shimpachiro NŌMI, and Kiyoshi UEDA[1]

Department of Medical Biochemistry, Shiga University of Medical Science, Ohtsu, Shiga 520-21, Japan

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Summary The binding of metabolically activated ^{14}C-benzo(a)pyrene (BP) to rat liver nuclei was studied. The nuclear framework structure, termed the nuclear matrix, was found to bind BP preferably. α-Tocopherol inhibited the binding of BP to nuclear macromolecules in the presence of phenobarbital (PB)-induced microsomes. α-Tocopherol decreased the level of BP metabolites in nuclei, though it did not inhibit the activation of BP by PB-induced microsomes.

Keywords tocopherol, antioxidant, benzo(a)pyrene, carcinogen, nuclei, nuclear matrix, nuclear macromolecules, high-pressure liquid chromatography

It is evident from a range of studies (*1–3*) that the administration of synthetic antioxidants to animals inhibits the carcinogenic effect of benzo(a)pyrene (BP). Although the tocopherols are generally considered to be biological antioxidants, there are some conflicting reports (*4, 5*) about the protective effects of α-tocopherol on chemical carcinogenesis.

Metabolic activation of carcinogenic polycyclic aromatic hydrocarbons such as benzo(a)pyrene is necessary for eliciting the carcinogenic effects. However, the relative instability of mutagenic benzo(a)pyrene oxides and dihydrodiol oxides implies that intranuclear distribution of BP metabolites and its interaction with nuclear components may be significant in determining the extent and nature of DNA alteration.

On the other hand, recent studies (*6–8*) have shown the preferential binding of *d*-α-tocopherol to nuclei. Our previous experiments (*8*), in which rat liver nuclei incubated with ^{3}H-*d*-α-tocopherol was fractionated, revealed that a nuclear

[1] 松浦尚雄，上山久雄，能見伸八郎，上田　潔

Abbreviations: BP, benzo(a) pyrene; PB, phenobarbital; MC, 3′-methylcholanthrene; HPLC, high-pressure liquid chromatography.

framework structure, termed the nuclear matrix, was the preferable binding site of *d*-α-tocopherol.

We turned our attention to the intranuclear distribution of BP, and found that BP and its metabolites also preferably associated with the nuclear matrix, and *dl*-α-tocopherol inhibited the covalent binding of BP.

MATERIALS AND METHODS

Chemicals. [7,10-^{14}C]BP with a specific activity of 21.7 mCi/mmole and PCS scintillation cocktail were obtained from Amersham Searle Co. NADP, G-6-P, G-6-PDH, DNase I, and 3′-methylcholanthrene (MC) were obtained from Sigma Chemical Co. PB was obtained from Sankyo Pharmaceutical Co. BP was purchased from Wako Chemical Co. *dl*-α-Tocopherol and other reagents were obtained from Nakarai Chemical Co. *d*-α,β,γ,δ-Tocopherol were generously supplied by Dr. G. Katsui, Eisai Co., Ltd.

Animals. Male Wistar rats, about 250 g in weight, were used as the source of liver nuclei and microsomes. Prior to use, the rats were given *i.p.* injections of BP (50 mg/kg) for 4 days before sacrifice in order to induce the BP-activating system in microsomes, or MC (20 mg/kg, in corn oil) one day before sacrifice to induce the BP-activating system in nuclei.

Isolation of nuclei and microsomes. Nuclei were isolated as follows: The minced livers were homogenized in 2 vol of 0.25 M sucrose–50 mM Tris-HCl, pH 7.5–25 mM KCl–5 mM $MgCl_2$ (TKM). After filtration through nylon cloth, the homogenate was centrifuged at $700 \times g$ for 10 min. The pellet was washed once with 0.25 M sucrose-TKM containing 0.2% Nonidet P-40. The pellet was suspended in 0.25 M sucrose-TKM and mixed with 2 vol of 2.3 M sucrose-TKM. This was underlaid with 2.3 M sucrose-TKM and centrifuged at $55{,}000 \times g$ for 1 hr. The nuclear pellet was washed with 0.25 M sucrose-TKM and suspended in the same solution. Microsomes were isolated by differential centrifugation from the homogenate as a pellet by centrifugation at $78{,}000 \times g$ for 90 min, and frozen at −30°C.

Incubation of nuclei with ^{14}C-benzo(a)pyrene. A standard reaction mixture contained rat liver nuclei and microsomes corresponding to 20–40 mg and 7–14 mg protein, respectively, 1 μmole of NADP, 18 μmoles of G-6-P and 3 units of G-6-PDH in a volume of 10 ml of 0.25 M sucrose-TKM. The reaction was initiated by adding 30 nmoles of ^{14}C-BP (0.65 μCi) in 5 μl of acetone and the mixture was incubated at 37°C.

Subfractionation of nuclei labeled with ^{14}C-BP. The procedure of subfractionation of nuclei is composed of four consecutive extractions as follows.

Triton X-100 washing: After incubation for 1 hr with ^{14}C-BP, labeled nuclei were centrifuged at $700 \times g$ for 5 min to remove the added microsomes. The obtained nuclear pellets were suspended in 9 ml of 0.25 M sucrose-TKM, to which 1 ml of a solution of 20% Triton X-100 was added. Incubation of the mixture for

10 min in an ice bath, followed by centrifugation at $700 \times g$ for 5 min, yielded a supernatant (nuclear envelope) and a pellet (unenveloped nuclei).

DNase treatment at pH 8.5: Unenveloped nuclei were suspended in 0.1 mM $MgCl_2$ followed by the addition of 25 μg of DNase I to 500 A_{260} units of nuclei and a solution of 10% sucrose–10 mM triethanolamine-HCl, pH 8.5, –0.1 mM $MgCl_2$. The resulting mixture was incubated for 15 min at 22°C and centrifuged at $20,000 \times g$ for 10 min at 4°C.

DNase treatment at pH 7.5: The pellet obtained from the 1st DNase treatment was suspended in 10% sucrose–10 mM triethanolamine-HCl, pH 7.5, –0.1 mM $MgCl_2$ and digested again in the same way as described above.

Salt washing: The pellet after a 2nd DNase digestion was suspended in 10% sucrose–10 mM triethanolamine-HCl, pH 7.5, –0.1 mM $MgCl_2$, to which an equal volume of 2 M NaCl–100 mM triethanolamine-HCl, pH 7.5, was added. Incubation of the mixture for 10 min in an ice bath, followed by centrifugation at $20,000 \times g$ for 15 min, yielded a pellet containing nuclear matrix.

Binding of ^{14}C-BP metabolites to nuclear macromolecules. After labeling of nuclei with ^{14}C-BP as described above, the obtained nuclear pellets were washed three times with 5 ml of 1% Triton X-100 in buffer A (0.25 M sucrose–20 mM Tris-HCl, pH 7.4, –1 mM EDTA–5 mM $MgCl_2$). Unenveloped nuclei were then subjected to the extraction of BP three times with 5 ml of acetone to remove noncovalently bound BP and its metabolites. The residual pellet was dissolved in formic acid, and aliquots of samples were subjected to radioactivity determination.

Analysis of BP metabolites by high-pressure liquid chromatography (HPLC). Extraction of microsomal metabolic products: After incubation of BP with microsomes and an NADPH-generating system, BP metabolites were extracted from the reaction mixture with 3 vol of ethylacetate–acetone (2 : 1) containing 0.8 mg/ml butylated hydroxy toluene (BHT). Ethylacetate extracts were evaporated to dryness under nitrogen.

HPLC of BP metabolites: Evaporated samples were dissolved in methanol, 40 μl was taken for HPLC on Waters Associates Model 6000A equipped with a μBondapak C_{18} column (3.9 × 300 mm) and the column was eluted stepwise with 60%, 70% and 100% methanol at a constant flow of 1 ml/min. The effluent stream was monitored for absorbance at 254 nm and for fluorescence (Ex 360 nm, Em > 420 nm).

HPLC of intranuclear BP metabolites. After incubation of nuclei with ^{14}C-BP, microsomes and an NADPH-generating system, labeled nuclei were separated from microsomes by low-speed centrifugation at $700 \times g$ for 5 min. The nuclear pellet was washed three times with 1% Triton X-100–Buffer A and extracted three times with acetone. The acetone extract was evaporated to dryness under nitrogen and dissolved in a methanol solution containing the BP metabolites formed by microsomes. HPLC was performed in the same manner as for microsomal metabolites. The effluent stream was monitored for absorbance at 254 nm and collected as fractions (1 ml × 60) for scintillation counting.

Measurement of radioactivity. The radioactivity of a sample was determined in a vial containing 10 ml of PCS scintillation cocktail by Packard Model 3255 Tri-Carb scintillation spectrometer. The counting efficiency was over 84% (usually 90%).

Protein assay. After precipitation of nuclei with cold TCA, protein was determined by the biuret method using BSA as a standard.

RESULTS

Intranuclear distribution of ^{14}C-benzo(a)pyrene

In order to compare the intranuclear distribution of ^{14}C-BP with that of ^{3}H-*d*-α-tocopherol, subfractionation of labeled nuclei was carried out as follows. Isolated rat liver nuclei were incubated for 60 min with ^{14}C-BP (2.1 μM, 0.47 μCi) in the presence of a microsomal hydroxylation system, and then the labeled nuclei were separated from the added microsomes by low-speed centrifugation. The nuclear pellet obtained was treated with 1% Triton X-100. This procedure completely removed both the inner and outer nuclear membranes leaving behind envelope-denuded nuclei with about 64% of the total nuclear radioactivity. This result suggests the existence of preferential binding sites for BP in unenveloped nuclei.

The unenveloped nuclei were further subjected to a series of treatments with DNase I at pH 8.5 and DNase I at pH 7.5, followed by washing with 1 M NaCl. An architectural framework of the nuclei, termed a nuclear matrix, was obtained by these treatments with about 50% of the original nuclear radioactivity. Radioactivity per mg protein is highest in the nuclear matrix among those of the subnuclear fractions as shown in Table 1. This high affinity of BP to the nuclear matrix may be significant in determining the extent and nature of covalent binding to nuclear components.

Table 1. Distribution of benzo(a)pyrene and α-tocopherol in rat liver nuclei. Ten-milliliter reaction mixtures containing rat liver nuclei (40 mg protein) plus PB-induced microsomes (8.4 mg protein) were incubated at 37°C. ^{14}C-BP (20.5 nmoles, 0.45 μCi) was added with an NADPH-generating system (1 μmole of NADP, 18 μmoles of G-6-P, 3 units of G-6-PDH). After incubation for 60 min, labeled nuclei were obtained from the reaction mixture, and resolved into five fractions, as described in "MATERIALS AND METHODS."

Fractions	^{14}C-Benzo(a)pyrene		^{3}H-*d*-α-Tocopherol[a]
	per mg protein		
Nuclear envelope	1,045 pmoles	36.1%	89.5%
1st DNase treatment supernatant	157	13.2	7.9
2nd DNase treatment supernatant	189	0.3	0.1
Salt wash supernatant	61	0.8	0.2
Nuclear matrix	1,436	49.6	2.4

[a] Data obtained with the same subfractionation procedure as previously (*8*).

Time course of covalent binding of ^{14}C-benzo(a)pyrene metabolites to nuclear macromolecules

It is known that metabolically activated BP is covalently bound to DNA, RNA, histone and nonhistone proteins of nuclei isolated from liver (*9, 10*). To determine the amount of covalently bound BP metabolites, nuclei labeled with ^{14}C-BP were subjected to a series of washings with 1% Triton X-100 and acetone. The radioactivities recovered in the intranuclear components, which are probably due to covalently bound BP metabolites, increased with time of incubation up to 60 min, as shown in Fig. 1.

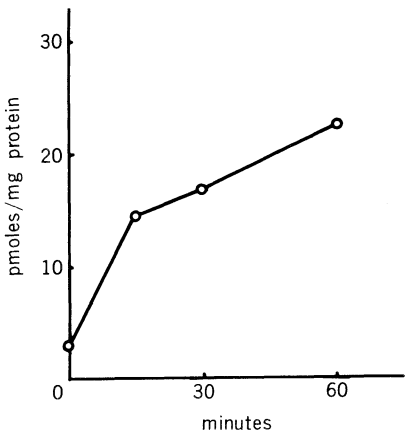

Fig. 1. Binding of ^{14}C-benzo(a)pyrene to nuclear macromolecules. Rat liver nuclei (26.4 mg protein) were incubated with PB-induced microsomes and ^{14}C-BP (26.5 nmoles, 0.58 μCi) in a 10-ml reaction mixture as Table 1. After incubation for various times, labeled nuclei obtained from 2 ml of the reaction mixture were washed with 1% Triton X-100 and acetone. Remaining radioactivities were measured as described in "MATERIALS AND METHODS" and expressed as pmole of BP metabolites bound to one mg nuclear protein.

Inhibitory effect of dl-α-tocopherol on BP binding to nuclear macromolecules

An addition of *dl*-α-tocopherol to the incubation mixture reduces the amount of radioactivity remaining in nuclear macromolecules as shown in Table 2. *dl*-α-Tocopherol at 11 μM, which saturates the intranuclear binding sites in rat liver nuclei, inhibits the covalent binding of BP metabolites by 33%, but at a 10-fold higher concentration it does not further inhibit BP binding.

Effect of dl-α-tocopherol on the metabolism of BP by microsomes

Several mechanisms for the inhibitory effect of *dl*-α-tocopherol have been considered, because both microsomes and the nuclear envelope are potentially important in the activation of BP. It is necessary to establish whether or not added *dl*-α-tocopherol affects the activation of BP by microsomes.

Table 2. Effect of α-tocopherol on binding of benzo(a)pyrene to nuclear macromolecules. *dl*-α-Tocopherol in 5 μl of ethanol were added to 10-ml incubation mixtures containing nuclei (20.8 mg protein). After a 5 min incubation, ^{14}C-BP (34 μmoles, 0.74 μCi) in 5 μl of acetone, PB-induced microsomes (14.8 mg protein) and an NADPH-generating system were added to the mixtures. Formation of bound BP metabolites was measured after 1 hr of incubation as described in "MATERIALS AND METHODS."

Addition	Bound ^{14}C-benzo(a)pyrene metabolites	
Control	81.5 pmoles	100 %
dl-α-Tocopherol		
1.1 μM	62.6	76.8
11. μM	54.1	66.4
110. μM	55.0	67.5

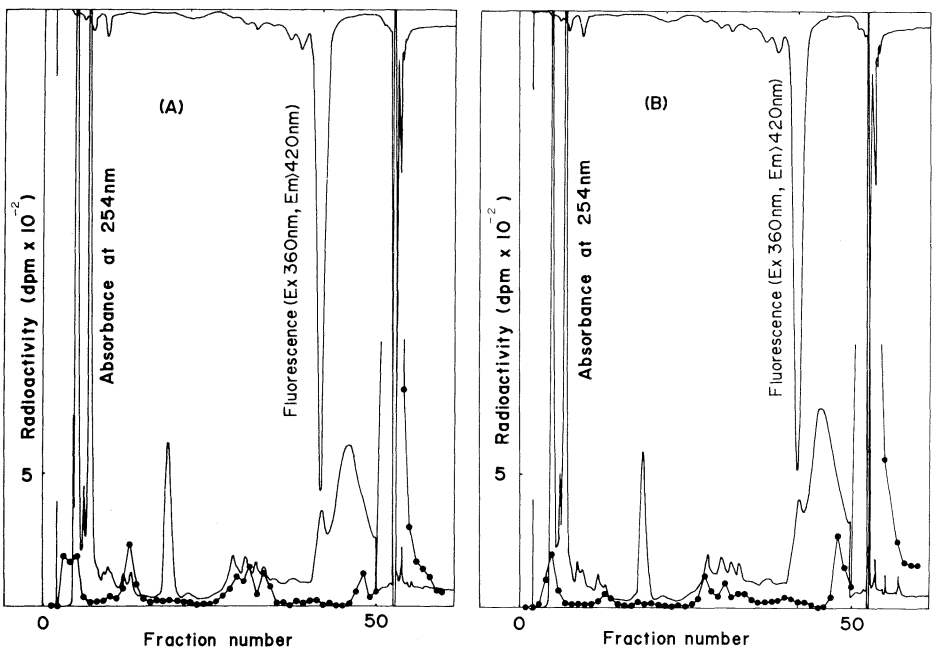

Fig. 2. High-pressure liquid chromatographic analysis of BP metabolites formed by PB-induced microsomes with or without *dl*-α-tocopherol. Incubation mixtures contained PB-induced microsomes (6.7 mg protein), benzo(a)pyrene (30 nmoles) and an NADPH-generating system. After incubation for 20 min at 37°C with (B) or without (A) *dl*-α-tocopherol (110 μM), formed metabolites were subjected to HPLC as described in "MATERIALS AND METHODS."

HPLC was used for a detailed analysis of the BP metabolites. The metabolites are separated from BP into diol, quinone, and phenol fractions on a reverse-phase

column (μBondapak C_{18}) as shown in Fig. 2(a), 2(b). Since structures of the metabolites cannot be assigned with certainty, the quantitative data obtained in the chromatograms are expressed only as diols 1, 2 and 3, quinone 1 and 2, and phenol 1 and 2. There is no remarkable difference between the elution pattern of microsomal metabolites and that of metabolites formed by microsomes with *dl*-α-tocopherol, though a slight increase of diol 2 was observed with *dl*-α-tocopherol.

Inhibitory effect of dl-α-tocopherol on nuclear BP metabolite levels

From the above results, *dl*-α-tocopherol seems not to affect the microsomal activation of BP; it might directly inhibit nuclear activation. After 60 min of incubation of nuclei with ^{14}C-BP (35 nmoles, 0.76 μCi) under the standard reaction conditions, labeled nuclei were separated from used microsomes, and then extracted three times with acetone. After evaporation the extract was dissolved in 150 μl of methanol containing microsomal BP metabolites as markers. A 40-μl aliquot was taken for HPLC, and fractions of 1.0 ml were collected for measurement of radioactivity. As shown in Fig. 3(a), 3(b), diol 1 predominated and

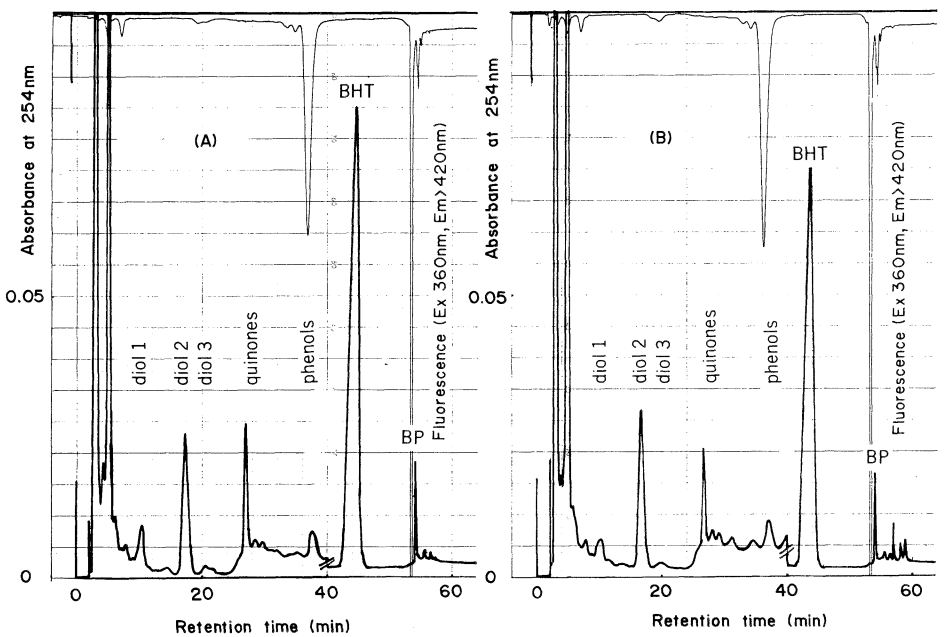

Fig. 3. The effect of α-tocopherol on BP metabolite levels in nuclei. Incubation mixtures containing rat liver nuclei (19.2 mg protein) and PB-induced microsomes (6.7 mg protein) were incubated with ^{14}C-BP (35 nmoles, 0.76 μCi) at 37°C for 1 hr with (B) or without (A) *dl*-α-tocopherol (110 μM). ^{14}C-BP metabolites were resolved by HPLC with microsomal metabolites as markers. UV absorbance was recorded and ^{14}C radioactivity was measured in 1-min fractions.

diol 2 is virtually absent, the latter being a matabolic fraction from microsomes. Three quinones emerge from the column as a single tailing peak. Phenol 2 and unknown polar metabolites were also observed, but a majority of the radioactivity is due to unreacted BP. It is important that a noticeable decrease in the proportion of diol 1 was observed in the nuclei with *dl*-α-tocopherol.

Inhibition of the formation of covalently bound ^{14}C-BP in MC-induced nuclei by tocopherols

To establish further the mode of action of α-tocopherol, an alternative incubation system containing MC-induced nuclei alone was used. Addition of tocopherols to the incubation mixture without microsomes markedly reduced the formation of covalently bound ^{14}C-BP in nuclei. However, the inhibitory potency of tocopherols does not correlate with their power as antioxidants under the experimental conditions (Table 3).

Table 3. Inhibition of the formation of covalently bound ^{14}C-BP in MC-induced nuclei by tocopherols. *d*-α-, β-, γ-, or δ-Tocopherol in 5 μl of ethanol was added to 10-ml incubation mixtures containing 3-methylcholanthrene-induced nuclei (9.6 mg protein). After 5 min of incubation, ^{14}C-BP (23 nmoles, 0.50 μCi) in 5 μl of acetone and an NADPH-generating system were added to the mixtures. Bound BP metabolites were measured after 1 hr of incubation as described in "MATERIALS AND METHODS."

Addition of tocopherols (55 μM)	Bound ^{14}C-BP metabolites pmoles/mg pr.	Percentage control formation
Control	53.0	100 %
α-Tocopherol	32.3	60.9
β-Tocopherol	19.7	37.2
γ-Tocopherol	21.5	40.6
δ-Tocopherol	39.8	75.1

DISCUSSION

A nuclear framework structure, termed the nuclear matrix, was obtained from rat liver nuclei by a series of treatments with Triton X-100, DNase and 1 M NaCl. The matrix consists largely of protein with smaller amounts of RNA, DNA and lipids. The details of isolation and characterization of the nuclear matrix obtained by our method will be presented elsewhere. It has been found, as shown in Table 1, that ^{14}C-BP and its metabolites preferentially associate with the nuclear matrix, suggesting that this matrix is the locale of important biological alternation of DNA and nuclear macromolecules by covalent binding of BP metabolites.

Unreacted BP accounts for a large portion of the intranuclear BP associated

with the matrix, but some considerable amount of bound BP metabolites remained with nuclear macromolecules after washing with 1% Triton X-100 and acetone. It is known that metabolically activated BP binds to DNA, RNA, histone and the nonhistone proteins of nuclei (*9, 10*). Characterization of BP covalently bound to the nuclear matrix will be presented elsewhere. The amount of bound BP increased with time of incubation (Fig. 1).

α-Tocopherol, which also preferably bound to the nuclear matrix amongst possible intranuclear sites, caused a noticeable decrease in the amount of covalently bound BP. It should be noted that a minimum amount of *dl*-α-tocopherol (11 μM), which saturates the intranuclear binding sites of rat liver nuclei, caused the same degree of inhibition as an excess amount of *dl*-α-tocopherol, suggesting the existence of specific inhibition.

This inhibitory effect of *dl*-α-tocopherol is considered to be due to its antioxidant action. Recent evidence suggests that nuclear and microsomal cytochrome P-450 containing mixed-function oxidase in conjunction with epoxide hydrase results in the formation of a diol-epoxide of BP that is probably the ultimate carcinogen (*11–14*). Whether the nuclear and microsomal activation are related to the effect of *dl*-α-tocopherol, therefore, has to be established. Addition of a large amount of tocopherol to the microsomal activation system of BP caused a slight increase in the amount of BP metabolites, being consistent with the stimulatory effect of α-tocopherol on the microsomal hydroxylation of drugs in liver (*15*). However, this observation may not rule out the possibility that the effect of added α-tocopherol on microsomal activation of BP resulted in a decrease of nuclear binding of BP metabolites, because the extent and nature of the metabolites varied with the incubation conditions of BP.

On the other hand, addition of α-tocopherol to the nuclear activation system containing PB-induced microsomes caused a noticeable decrease of BP metabolites in nuclei. This result suggests that α-tocopherol directly inhibits the nuclear activation of BP. This mode of action is also consistent with the fact that α-tocopherol inhibits the formation of covalently bound ^{14}C-BP in MC-induced nuclei in the absence of microsomes (Table 3). However, α-tocopherol might prevent the nuclear matrix from covalent binding of BP metabolites, because both BP metabolites and α-tocopherol have a high affinity for the nuclear matrix.

The effect of α-tocopherol of preventing covalent binding of BP to nuclear macromolecules in MC-induced nuclei or nuclei with BP-induced microsomes was observed *in vitro* in our experiment. However, evaluation of the significance of these findings with regard to the protective effects of tocopherols against chemical carcinogenesis will not be possible until suitable *in vivo* studies are effected.

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