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Effect of Vitamin A and E on the Interaction Between 2-Acetylaminofluorene and Rat Liver DNA in Vivo

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Vitamin A와 E 투여가 2-Acetylaminofluorene과 흰쥐 간조직 DNA와 결합에 미치는 영향

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간암물질로 알려진 2-acetylaminofluorene(AAF)가 흰쥐 간조직에서 DNA와 공유결합을 하므로 tumorigenesis에 매우 중요한 과정이라는 것이 보고되어 왔다. 흰쥐에 고농도의 vitamin A와 E를 4주간 투여하고 〔9-^{14}C〕AAF를 일회 주사한 2시간 후에 동물을 희생시켜 간조직에서 DNA를 분리하였고 분리한 DNA는 MUP에 용해시키고 HAP colume을 통과시켰다. 다시 UV-absorbing물질을 모아서 0.48 M Nap로 elute시키고 투석한 후 enzyme으로 가수분해 시켜서 Sephadex LH-20 chromatography로 정제하였다. 이때 분리된 adducts는 HPLC에 의하여 분리하였다.

Vitamin A와 E투여로 간조직의 DNA와 2-AAF의 결합물질인, N(deoxyguanosine-N^2-yl) AAF(C-8-adduct)의 형성이 현저히 억제되었다. 고농도의 vitamin A와 E투여는 억제되는 정도가 더욱 현저하였다.

이상의 결과는 vitamin A와 E가 2-AAF carcinogenesis에 있어서 C-8 deoxyguanosine adduct 형성을 저해함으로써 2-AAF에 의한 carcinogenesis를 억제함을 시사하였다.

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ABSTRACT

Binding of the hepatocarcinogen 2-acetylaminofluorene(AAF) to the DNA of rat liver homogenates was examined in vivo. Animals fed low and high concentrations vitamin A and E for 28 days and DNA isolated 2 hr following a single i.p. injection of 2 [9-^{14}C] acetylaminofluorene. The precipitated DNA was dissolved in MUP, blended and passed through an HAP column. UV-absorbing material eluted with 0.48M Nap. Following dialysis, the fraction was enzymatically hydrolyzed purified by Sephadex LH-20 chromatography and the adducts were separated by HPLC. The ability of vitamin A and E on the binding of AAF to liver DNA in vivo was markedly inhibited the formation of the proximate carcinogen, N-(deoxyguanosine-N^2-yl) AAF(C-8 adduct). Vitamin A at higher concentration(2,000 I.U.) exerted a stronger inhibitory effect on the formation C-8 adducts. A similar inhibition pattern was observed in higher concentration vitamin E administered rats.

The data presented here demonstrated marked inhibits in the pattern of C-8 deoxyguanosine adduct formation during the premaligment phase of 2-AAF carcinogenesis.

INTRODUCTION

Chemical carcinogens have been characterized as electrophilic agents capable of binding covalently to cellular macromolecules. These agents may act directly, but many acquire their electrophilic properties as a result of being oxidized by enzymes associated with microsomal membranes.

The covalent interaction of chemical carcinogens with DNA in widely believed to be a critical step in the initiation of tumorigenesis. A wide variety of chemical adducts have been identified and, depending upon the type of carcinogen and the site of attachment to the nucleic acid bases, these products exhibit a broad spectrum of chemical stability and biological persistence. Substitution at N-7 of deoxyguanosine by alkylating agents, for example, creates chemically labile lesions which undergo spontaneous depurination[1)2)3)]. The potent hepatocarcinogen aflatoxin B_1 also binds to N-7 of deoxyguanosine[4)5)6)], but in addition to causing depurination the imidazole ring may open to form a chemically stable and apparently biologically persistent product[7)8)].

The inhibitory effect of vitamin A on the process of chemical carcinogenesis has recently become a subject of considerable research interest. The ability of vitamin A to protect laboratory animals against the induction of cancer by different classes of carcinogenic chemicals has been demonstrated under a variety of experimental conditions[9)~14)].

The present studies were conducted to obtain a better understanding of the molecular underlying the anticarcinogenic effect of vitamin A and E, we have undertaken studies into the hepatocarcinogen, AAF. The latter was chosen as a model compound since its metabolism and interaction with tissue nucleophiles has been extensively studied[15)16)]. As in vitro reactions between ultimate carcinogens

and nucleic acid generally result in a greater variety of adducts than are found in vivo, the isolation of DNA from in vivo experiments is essential for assessing the importance of particular adducts. The investigations focused on the effect of low and high concentrations vitamin A and E on the interaction between AAF and liver DNA in vivo.

MATERIALS AND METHODS

Animals: Male wister strain rats weighing 150 to 200g were used throughout this study and were given free access to food pellets and water. The rats, in groups of 3 or 4 animals, were given oral administration by stomach tube of vitamin A(40 I.U. or 2,000 I.U/100g body weight in corn oil) and vitamin E(0.12 I.U. or 12 I.U/100 g body weight in corn oil) for 4 weeks. The rats were each given i.p. injections of AAF containing 40 μCi [9-^{14}C] AAF in 50 μl ethanol. β-Naphthoflavone (80 mg/kg in corn oil) was administered by S.C. 48 and 24hr before death in rats. Rats were killed 2hr after AAF administration, and DNA was isolated from their livers.

Chemicals : [9-^{14}C] AAF (Sp. activity 6.25 mCi/mmol) was obtained from New England Nuclear Corp., Boston, Ma., USA. Vitamin A and E were obtained from Merk Co. & Inc. West Germany. β-Naphthoflavone was obtained from Aldrich, W. Germany.

Unless otherwise stated, all chemicals were of reagent or analytical grade and were used without further purification.

Protein isolation from tissue

Freshly isolated or frozen liver tissue was minced with scissors (except in those experiments using intact hepatocytes) and suspended in filtered 8M urea-0.24 M sodium phosphate-1% sodium dodecyl sulfate-10 mM EDTA, pH 6.8 (MUP-SDS-EDTA), using at least 20ml per gram of tissue. Unless the MUP-SDS-EDTA solution was gravity filtered a green precipitate formed. This suspension was transferred to a Waring blender, cooled with ice for 5 min., blended at high speed for 30 sec and then cooled with ice for 1 min. The blending-cooling sequence was repeated five times and then the homogeneous solution was stirred for 15 min with a magnetic stirrer at room temperature to insure complete lysis. It is essential that air be excluded during the blending process to minimize SDS-induced foam formation. This was accomplished by using an Eberbach 8575 sealed blender vessel. An equal volume of chloroform-isoamyl alcohol-phenol (24:1:25; CIP) saturated with MUP-SDS-EDTA was then added and the mixture was stirred for an additional 15 min. The phenol used in this solution was purified by distillation and stored in sealed containers in a refrigerater to minimize air oxidation. The emulsion resulting from CIP extraction was separated into two phases by centrifugation at 4,000 rpm for 15 min. in an IEC PR-6,000 centrifuge equipped with a No. 284 swinging bucket head. The CIP phase was removed and saved for protein isolation while the MUP-SDS-EDTA layer was extracted at least one additional time with CIP. Following CIP partitioning the aqueous phase was treated twice with diethyl ether to remove trace amounts of

phenol. This consisted of adding an equal volume of diethyl ether, mixing with a magnetic stirrer for 5 min and centrifuging as above. At this point the MUP –SDS–EDTA solution which contains the nucleic acids could be stored for up to 72 hr or applied directly to the HAP column.

Protein was precipitated from the combined CIP extracts by addition of an equal volume of acetone. The solution was stirred for approximately 15 min and the solid isolated by centrifugation. The protein was resuspended in acetone and again isolated by centrifugation. This process was repeated with diethyl ether and ethanol and the protein was then dried in a vacuum desiccator.

DNA and RNA isolation by HAP chromatography

The aqueous extract from the CIP partitioning was applied to an HAP column. This column media was prepared by suspending 1 g of DNA–grade hydroxyapatite (Bio–Rad Labs., Richmond, Calif., U.S.A.) per milligram of DNA in 0.014 M sodium phosphate (NaP), pH 6.8, by gently swirling the slurry and by decanting the fines. This process was repeated with MUP and the suspension was poured into a 2.6×40 cm glass column. A peristaltic pump was used to pump one column volume of MUP at a flow–rate of approximately 1 ml/min. The aqueous nucleic acid solution was then applied and the elution progress minitored with an ISCO UA–5 UV detector (254 nm). MUP was passed through the column until the absorbance returned to zero. This was then followed by 0.014M NaP, pH 6.8, to purge the urea from the system. After re–establishing the initial absorbance, 0.48 M NaP, pH 6.8, was applied to the column and the DNA was eluted.

The MUP, which contained RNA, and the 0.48 M NaP, which contained DNA, were dialyzed against 5 mM Bis–Tris (Sigma), 0.1 mM EDTA, pH 7.1, concentrated in vacuo on a rotary evaporator at 40° and re–dialyzed. In some instance the nucleic acids were precipitated by making the solutions 0.1 M in NaCl followed by the addition of two volumes of cold ethanol. The yields of DNA, RNA and protein were determined by diphenylamine[17], orcinol[17] and biuret[18] reactions, respectively. For the diphenylamine assay, the DNA was initially trapped on glass –fiber filters (Reeve Angel 934 ah) by precipitation with cetyltrimethylammonium bromide[19].

DNA hydrolysis and adduct chromatography

DNA, dissolved in a convenient volume (ca 1 mg/ml) of 5 mM Bis–Tris, 0.1 mM EDTA, pH 7.1, was made 10 mM in magnesium chloride, heated for 3 min in boiling water and rapidly cooled with ice. Deoxyribonuclease I (bovine pancrease, Sigma DN–CI, 0.1mg/mg DNA) and endonuclease[20] (N. crassa, Sigma E 4253, 6 units/mg DNA) were added and the solution was incubated overnight at 37°. The pH was then adjusted to 8.0 by addition of 1 M Tris base (Sigma) and then phosphodiesterase I (Crotalus atrox, Sigma P 6761, 0.04 units/mg DNA) and alkaline phosphatase (E. coli. Type IIIS, Sigma P 4377, 1 unit/mg) were added. The incubation was continued overnight, after which the pH was re–adjusted to 7.0 with 1 N hydrochloric acid and the solution was stored at –20°. In instances when DNA from in vivo incubations was being hydrolyzed, unlabeled deoxynucleoside adducts obtained from the reaction of N–OH–AAF with calf thymus DNA were added to serve as UV markers for subsequent column chromatography. For hy-

drolysis of MUP fractions, ribonuclease-A (Sigma 5500, 0.1 mg/mg DNA) replaced the deoxyribonuclease I.

The aqueous solutions were thawed and passed through a 1.6 × 20 cm glass column packed with 5g of Sephadex LH-20 and eluted with water to remove deoxynucleosides, protein and salts. After the absorbance had returned to the initial baseline, methanol was passed through the column and carcinogen-bound deoxynucleosides were collected. This latter fraction was taken to dryness on a rotary evaporator, dissolved in a small volume of methanol, filtered through a glass-frit filter and injected into a Waters Model M-6000 A high-performance liqued chromatograph, equipped with a U6K injector, a 440 UV detector, a 660 solvent programmer and a 10-μm μBondapak C_{18} column (30 cm × 3.9mm). The adducts were separated by isocratically running 56% methanol for ca. 20 min followed by a linear program to 100% methanol in 2 min at a flow-rate of 1 ml/min.

*Abbreviations used : HAP= hydroxyapatite ; MUP=8M urea-0.24M sodium phosphate, pH 6.8 ; SDS = sodium dodecyl sulfate ; EDTA= ethylenediaminetetraacetic acid, disodium salt ; CIP = chloroform-isoamyl alcohol-phenol (24:1:25), NaP= sodium phosphate ; Bis-Tris = bis-(2-hydroxyethyl) iminotris (hydroxymethyl) methane ; N-AH-AF= N-hydroxy-2-aminofluorene ; N-OH-AAF= N-hydroxy-2-acetylaminofluorene ; N-OAc-AAF = N-acetoxy-2-acetylaminofluorene , ; TLC = thin-layer chromatography ; Tris-HCl = tris (hydroxymethy) aminomethane hydrochloride ; HPLC = high-performance liquid chromatography ; AAF = 2-acetylaminofluorene ; C-8 adduct = N-(deoxyguanosin-8-yl)-AAF ; N^2 adduct = 3-(deoxyguanosin-N^2-yl)-AAF.

RESULTS

Effect of pretreament vitamin A and E with 2-acetylaminofluorene on binding to liver DNA in vivo.

Walker et al.[21] have demonstrated that maximum binding of both AAF and N-OH-AAF occurs 2 to 4 hr after injection. Therefore, 2 hr after injection, liver DNA was isolated.

The precipitated DNA was dissolved in MUP, blended and passed through an HAP column. UV-absorbing material eluted with 0.48M NaP. Following dialysis, the fraction was enzymatically hydrolyzed purified by Sephadex LH-20 chromatography and the adducts were separated by HPLC. Fig. 1 shows the HPLC profile of AAF-rat liver DNA that had not passed through an HAP column. Two peaks were observed; Peak 1 had co-chromatographed with synthetically prepared dG-AAF C-8 adduct[22]. Peak 2 was established to be the analogous guanosinyl adduct by the above criteria.

A comparison was made of the ability of vitamin A and E to inhibit the formation of the proximate carcinogen, N-(deoxyguanosine-N^2-yl) AAF (C-8 adduct). In this study were used concentrations of the 2 vitamin A and E that result

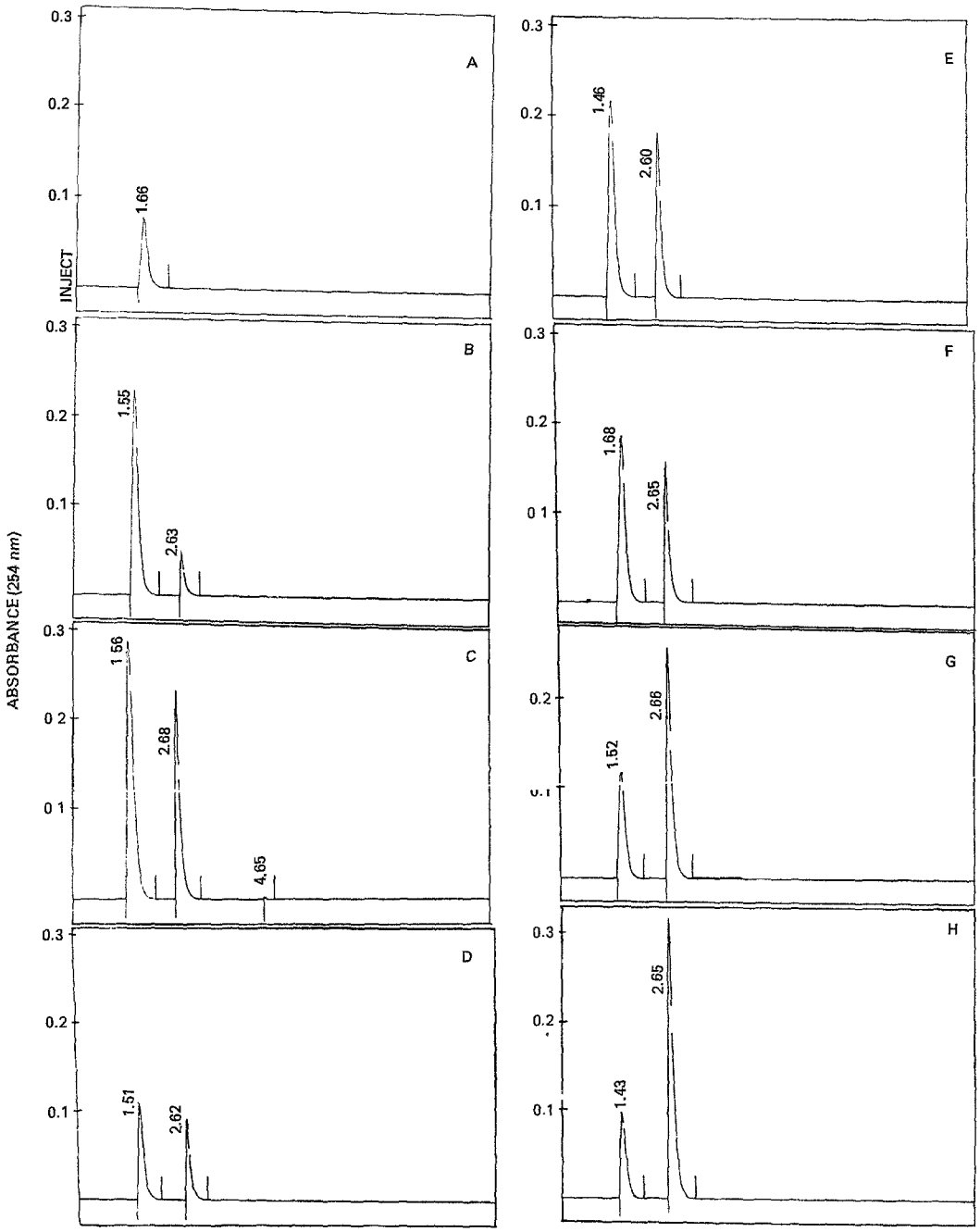

Fig. 1. HPLC profile of AAF–*rat* liver DNA that has not passed through the HAP column. Conditions: 10 μm μBondapak C_{18}, 30 cm×3.9 mm; 56% methanol; flow rate 1 ml/min. A; N–OH AAF–calf thymus DNA, B; Control, C; 40 I.U. Vitamin A+AAF, D; 2000 I.U. Vitamin A+AAF, E; 0.12 I.U. Vitamin E+AAF, F; 12 I.U. Vitamin E+AAF, G; β–naphthoflavone+AAF, H, β–naphthoflavone +AAF.

in the formation of a difference C-8 adducts. The effect of vitamin A and E on the binding of AAF to liver DNA in vivo was markedly inhibited (Fig. 1 or 2). The degree of the inhibition of the binding of N-OH-AAF was dependent on the amount of vitamin A and E administered. The amount of binding in rats administered 40 I.U. vitamin A was different to that in rats administered 2,000 I.U. vitamin A, so the higher dose appeared to have effect on the quantitative formation of C-8 adducts. Vitamin E at low and high concentrations (0.12 and 12 I.U.) exerted a little inhibitory effect on the formation of C-8 adduct than on that of vitamin A. As seen from Fig. 1 D, vitamin A at higher concentration (2,000 I.U.) exerted a stronger inhibitory effect on the formation C-8 adducts. A similar inhibition pattern was observed into higher concentration vitamin E administered rats (Fig. 1. F).

The isolation of macromolecular adducts formed in cells is demonstrated, which shows the results obtained from a incubation of N-OH-AAF with rat liver homogenates. The HPLC profile of 0.48 M Nap fraction from the N-OH-AAF - calf thymus DNA reaction in vitro is shown in Fig. 2. One major peak was co-chromatographed with the synthetic marker, N-(deoxyguanosine-8-yl)-AAF[23]. There was clearly reduction of the formation of C-8 adduct with administered higher concentration vitamin A and E. The 0.48 M NaP fraction contains only major peak which co-chromatograph with the DNA adducts. Interestingly, only one DNA-AAF product was observed in this nucleic acid fraction and this co-chromatographed with the C-8 adduct. The isolation of in vivo macromolecular adducts by HAP chromatography is given in Fig. 3, which shows an HPLC profile of the liver DNA (0.48 M NaP) fraction from a male wister rat liver. The highest concentration of vitamin A and E (2,000 I.U. and 12 I.U.) did result in markedly decrease in the N-(deoxyguanosine-8-yl) AAF adduct.

The effect of β-naphthoflavone on the binding of liver DNA was also studied. There was no inhibition of DNA-AAF products pretreatment with β-naphthoflavone. In contrast, a significant increase N^2-AAF adduct from administered β-NF rat in vitro and in vivo were observed at 2 hr after treatment with the carcinogen.

DISSCUSSION

The studies dercribed in this report were undertaken the anticarcinogenic effect of vitamin A and E. The investigations focused on the effect of vitamin A and E on the interaction between AAF and liver DNA in vivo.

The covalent binding of carcinogens to DNA in target tissues is generally considered to be a crucial event in the induction of cancer[24]. Furthermore, studies conducted over the past several years have demonstrated a positive association between the ability of various agents to protect animals against the induction of cancer and their ability to inhibit the binding of carcinogens to target tissue DNA[25]. For these reasons, it was deemed important to investigate the effect of vitamin A and E on the binding of AAF to liver DNA. Accordingly, rats from each group were given a single i.p. injection of [9-^{14}C] AAF and sacrified 2 hr thereafter.

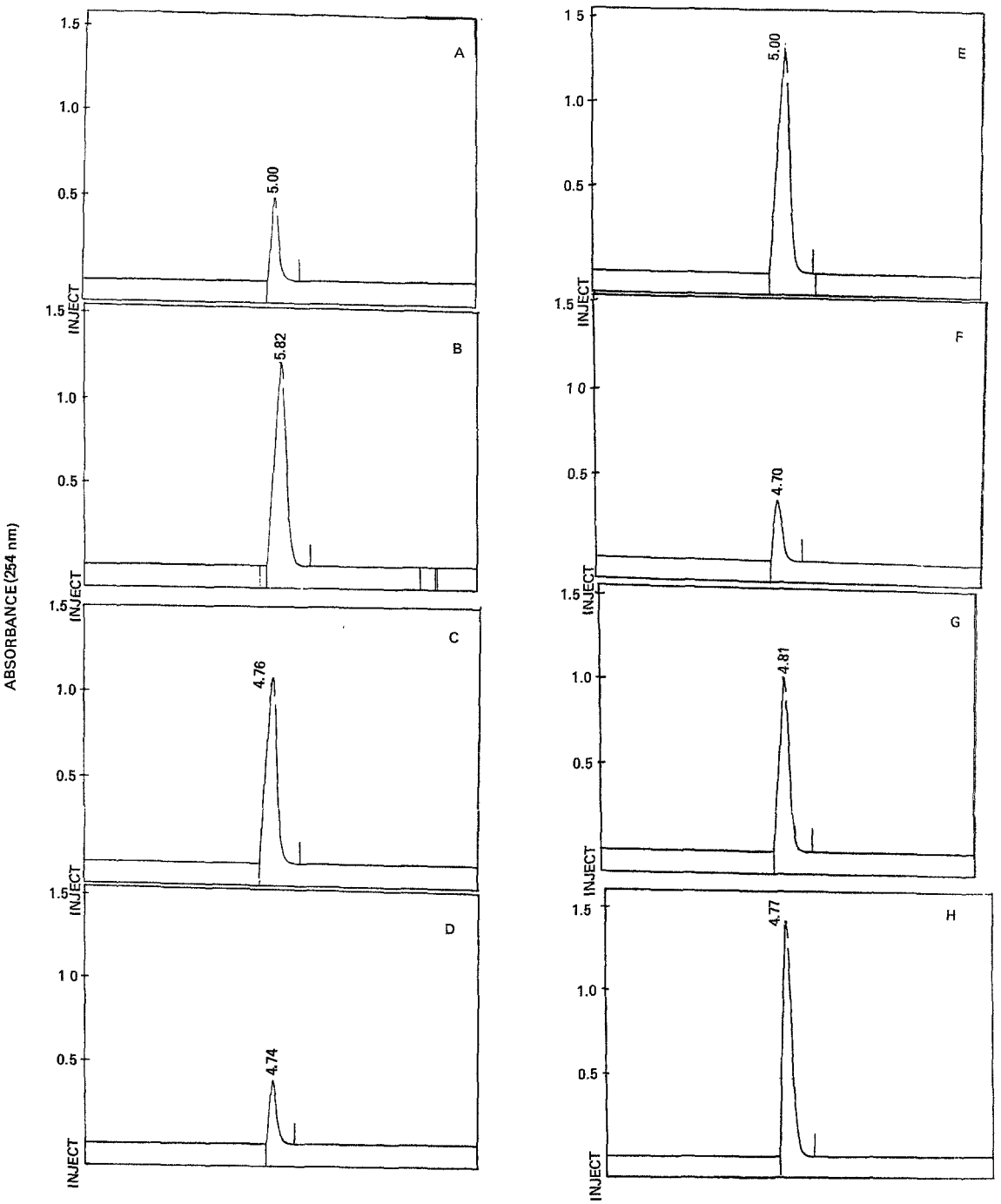

Fig. 2. HPLC profile of enzymatically hydrolyzed AAF bound calf thymus DNA. 0.48 M Nap fraction from the HAP column. Conditions : 10 μm μBondapak C_{18}, 30 cm × 3.9mm ; 100% methanol, flow rate 1 ml/min. A ; N–OH AAF–calf thymus DNA, B ; Control, C ; 40 I.U. Vitamin A+AAF, D ; 2000 I.U. Vitamin A+AAF, D ; 0.12 I.U. Vitamin E+AAF, F ; 12 I.U. Vitamin E+AAF, G ; β–naphthoflavone, H ; β–naphthoflavone + AAF.

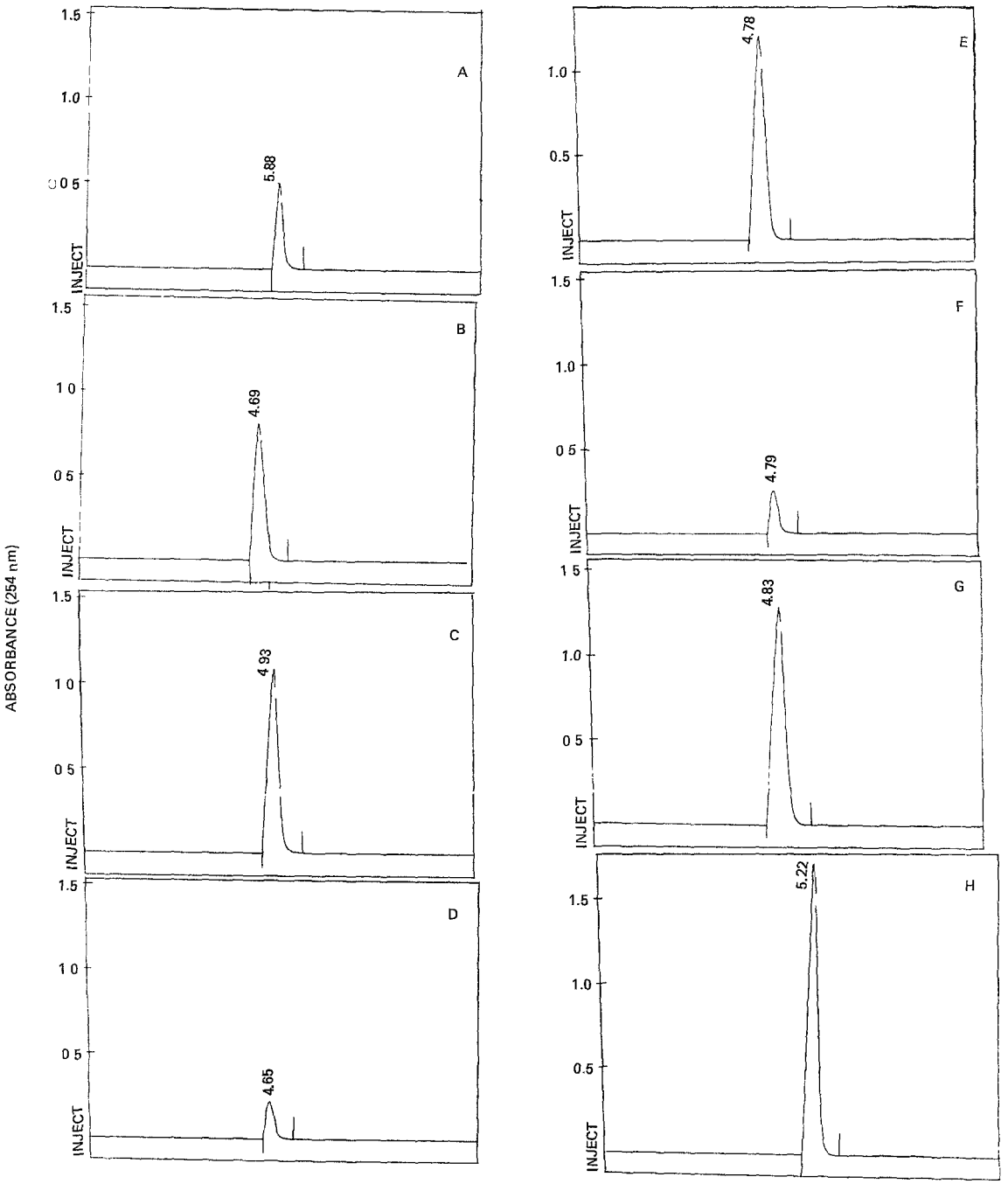

Fig. 3. HPLC profile of enzymatically hydrolyzed AAF bound rat liver DNA. 0.48 M Nap fraction from the HAP column. Conditions : 10 μm μBondapak C_{18}, 30 cm × 3.9 mm ; 100% methanol ; Flow rate 1 ml/min. A ; N–OH AAF–calf thymus DNA, B ; Control, C ; 40 I.U. Vitamin A+AAF, D ; 2000 I.U. Vitamin A+AAF, E ; 0.12 I.U. Vitamin E+AAF, F ; 12 I.U. Vitamin E+AAF, G ; β–naphthoflavone, H ; β–naphthoflavone + AAF.

The model ultimate carcinogen N-OH-AAF reacts with DNA to form two adducts, N-(deoxyguanosine-8-yl)-AAF (C-8 adduct) and 3-(deoxyguanosine-N^2-yl)-AAF (N^2-adduct)[20][26]. In order to for HAP to separate DNA from RNA it is necessary for the DNA to maintain a double helical structure. Lavine et al[27] demonstrated that at high levels of bound carcinogen there was a decreased affinity of AAF-DNA on HAP, presumably owing to a loss in integrity of the double helix. HAP method may be used for carcinogen-bound DNA isolation[28]. In vivo binding is typically two orders of magnitude lower, and therefore there should be significantly less perturbation of the DNA helix and little loss of the DNA in the MUP fraction of in vivo experiments. The usefulness of HAP column for isolating macromolecular adducts formed in cell is demonstrated in Fig. 2, which shows the results obtained from a incubation of N-OH-AAF with rat liver homogenates.

A final example of the isolation of in vivo marcromolecular adducts by HAP chromatography is given in Fig. 3, which shows an HPLC profile of the liver DNA (0.48 M Nap) fraction from a male wister strain rat which had been administered vitamin A and E for 28 days and single injected with [9-^{14}C] AAF killed 2 hr thereafter. The effect of vitamin A and E on the binding of AAF to hepatic DNA in vivo is shown in Fig. 1, 3. The data show that vitamin A and E had effect on either binding of the carcinogen to DNA. The confirming Kriek's observation that this adduct is rapidly excised from rat liver DNA in vivo[26].

In our studies, when male wister strain rats received 40 I.U. or 2000 I.U. vitamin A and 0.12 I.U. or 12 I.U. vitamin E oral administered for 28 days there was decrease in the levels of C-8 deoxyguanosine adduct in liver DNA. The data presented here demonstrated marked inhibits in the pattern of C-8 deoxyguanosine adduct formation during the premalignant phase of 2-AAF carcinogenesis.

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