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RAPID ISOLATION OF CARCINOGEN-BOUND DNA AND RNA BY HYDROXYAPATITE CHROMATOGRAPHY

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SUMMARY

Carcinogen-bound DNA and RNA are conveniently isolated by solvent extraction and hydroxyapatite (HAP) chromatography. Tissue is suspended in 8 *M* urea–0.24 *M* sodium phosphate–1% sodium dodecyl sulfate–10 m*M* EDTA, pH 6.8 (MUP–SDS–EDTA) and extracted with chloroform–isoamyl alcohol–phenol (24:1:25; CIP) to remove protein. RNA and DNA are separated by passing the aqueous solution through an HAP column; RNA is eluted with MUP, DNA with 0.48 *M* sodium phosphate, pH 6.8. Examples presented are: (1) calf thymus DNA that has been reacted with N-acetoxy-2-acetylaminofluorene (N-OAc-AAF), (2) isolated intact rat hepatocytes incubated with N-hydroxy-AAF and (3) livers from Sprague-Dawley rats treated with N-hydroxy-AAF.

INTRODUCTION

The covalent binding of chemical carcinogens to cellular macromolecules, especially DNA, seems to be an obligatory step in the initiation of the carcinogenic process[1]. The structural elucidation of these covalent-bound adducts has been a major facet of research in the study of the mechanisms of chemical carcinogenesis[2–16]. As *in vitro* reactions between ultimate carcinogens and nucleic acids generally result in a greater variety of adducts than are found *in vivo*[9,12,17–19], the isolation of DNA from *in vivo* experiments is essential for assessing the importance of particular adducts. To this end, it was necessary to find methods for the isolation of cellular macromolecules. Phenol extraction has been used to separate protein from nucleic acids and then differential precipitation to isolate DNA from RNA[20,21]. The DNA must then be treated with ribonuclease to remove residual RNA contamination and then again be subjected to phenol extractions to remove the ribonuclease. Although this method permits the isolation of reasonable amounts of DNA, RNA and protein, the technique is tedious and time consuming. A method that improves the ease of carcinogen-bound macromolecule isolation, especially DNA, would be a distinct advantage in the understanding of the mechanisms of chemical carcinogenesis.

Britten *et al.*[22] described a very simple method for DNA isolation through

the use of hydroxyapatite (HAP)* chromatography. Their method has proved useful in other studies[23], and further improvements in the technique by Markov and Ivanov[24] and Ivanov *et al.*[25] have permitted the isolation of high yields of pure (<0.1% RNA and protein) DNA. The studies described in this paper extend the use of HAP chromatography for the separation and isolation of carcinogen-bound DNA and RNA.

MATERIALS AND METHODS

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

Hepatocyte isolation and in vitro incubation

Hepatocytes were isolated from male Sprague-Dawley rats (3 months, 250–300 g) by *in situ* collagenase perfusion of the liver as described elsewhere[26–28]. After isolation, intact cells were suspended at approximately $5.0 \cdot 10^6$/ml in Ham's F-12 medium (Microbiological Assoc., Bethesda, Md., U.S.A.) containing 5% fetal bovine serum (Grand Island Biological Co., Grand Island, N.Y., U.S.A.), 90 U/ml of penicillin, 90 μg/ml of streptomycin, 10 mU/ml of insulin (Sigma, St. Louis, Mo., U.S.A.), and 8.5 μg/ml of N-OH-[^{3}H]AAF (137 mCi/mmole). The cell suspensions were placed in sterile erlenmeyer flasks, flushed with oxygen–carbon dioxide (95:5) and incubated for 4 h in a rotary shaker at 37 ± 1°. After 4 h, approximately 97% of the initial cellular inoculum was recovered as intact cells. The incubation was terminated by centrifugation of the entire mixture at 100 *g* for 10 min at 4° and the cell pellets were frozen prior to macromolecular adduct analysis.

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 8 *M* urea–0.24 *M* sodium phosphate–1% sodium dodecyl sulfate–10 m*M* EDTA, pH 6.8 (MUP–SDS–EDTA), using at least 20 ml 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

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* Abbreviations used: HAP = hydroxyapatite; MUP = 8 *M* urea–0.24 *M* 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-OH-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(hydroxymethyl)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.

phenol used in this solution was purified by distillation and stored in sealed containers in a refrigerator to minimize air oxidation. The emulsion resulting from CIP extraction was separated into two phases by centrifugation at 4000 rpm for 15 min in an IEC PR-6000 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 h 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 re-suspended 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 monitored 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 0.014 *M* 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 m*M* Bis-Tris (Sigma), 0.1 m*M* 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[29], orcinol[29] and biuret[30] reactions, respectively. For the diphenylamine assay, the DNA was initially trapped on glass-fiber filters (Reeve Angel 934ah) by precipitation with cetyltrimethylammonium bromide[31].

Synthesis of carcinogens

N-Hydroxy-2-acetylaminofluorene (N-OH-[ring-^{3}H]AAF; specific activity 1.7 mCi/mmole) was prepared from 2-nitrofluorene [ring-^{3}H] (NEN, tritium exchange) by M. R. Thissen and W. P. Duncan, Midwest Research Institute, Kansas City, Mo., U.S.A. The former compound was diluted with unlabelled N-OH-AAF and treated with acetic anhydride–pyridine (9:1) overnight at 4°, poured on to ice–water, washed with water and vacuum dried to yield N-acetoxy-2-acetylaminofluorene (N-OAc-[ring-^{3}H]AAF; specific activity 4.3 mCi/mmole; melting point 110–111°, literature

value[32] 109–110°; 88% yield). The purity of these compounds was checked by thin-layer chromatography (TLC) in at least two different solvent systems on a Varian-Berthold Model LB 2723 Radioscanner.

Reaction of N-OAc-AAF with calf thymus DNA

Calf thymus DNA (Sigma, Type I) dissolved (*ca.* 1 mg/ml) in 15 ml of 12.5 m*M* sodium chloride and 5 m*M* sodium acetate, pH 6.0, was purged with argon. To this was added 3 mg of N-OAc-[ring-^{3}H]AAF in 2 ml of methanol and the mixture was incubated for 2 h at 37°. Following the reaction the aqueous solution was washed repeatedly with diethyl ether, made 0.1 *M* in sodium chloride and the DNA was precipitated by the addition of two volumes of cold ethanol.

DNA hydrolysis and adduct chromatography

DNA, dissolved in a convenient volume (*ca* 1 mg/ml) of 5 m*M* Bis-Tris, 0.1 m*M* EDTA, pH 7.1, was made 10 m*M* in magnesium chloride, heated for 3 min in boiling water and rapidly cooled with ice. Deoxyribonuclease I (bovine pancrease, Sigma DN-CI, 0.1 mg/mg DNA) and endonuclease[33] (*N. crassa*, Sigma E4253, 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 P6761, 0.04 units/mg DNA) and alkaline phosphatase (*E. coli*, Type IIIS, Sigma P4377, 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-OAc-AAF with calf thymus DNA were added to serve as UV markers for subsequent column chromatography. For hydrolysis 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 5 g 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 the 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-6000A high-performance liquid chromatograph, equipped with a U6K injector, a 440 UV detector, a 660 solvent programmer and a 10-μm μBondapak C_{18} column (30 cm × 3.9 mm). 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. Fractions (1 ml) were collected and the radioactivity of each was determined with a Packard Tricarb scintillation counter using Scintisol (Isolabs) as the scintillation cocktail.

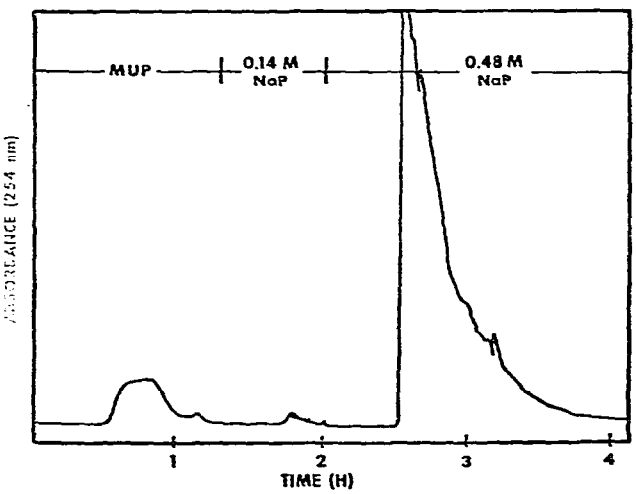
Laboratory lighting

Some of these aromatic amine compounds are highly sensitive to standard fluorescent lighting. Therefore, all experiments were conducted under low-UV fluorescent lights (General Electric, gold, No. 4060).

RESULTS AND DISCUSSION

The model ultimate carcinogen N-OAc-AAF reacts with DNA to form two adducts, N-(deoxyguanosin-8-yl)-AAF (C-8 adduct) and 3-(deoxyguanosin-N^2-yl)-AAF (N^2-adduct)[8,9,33]. The former adduct, which represents approximately 80% of the bound material, has been shown to cause significant distortion of the DNA helix[34–43] while the latter may reside within the minor groove and cause only slight perturbation[33,44]. In order for HAP to separate DNA from RNA it is necessary for the DNA to maintain a double helical structure. Lavine *et al.*[38], for instance, 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. The fact that N-OAc-AAF reacts with DNA to form multiple adducts, one of which significantly distorts the double helix, makes it an ideal compound to use to test whether Britten *et al.*[22] HAP method may be used for carcinogen-bound DNA isolation.

To investigate this question N-OAc-[ring-^{3}H]AAF was reacted with calf thymus DNA (*cf.*, Materials and methods) to give a product that had *ca.* one carcinogen-bound per 100 nucleotide residues. The precipitated DNA was dissolved in MUP, blended and passed through an HAP column. Two UV-absorbing peaks were observed. The first peak eluted with MUP and contained approximately 5% of the UV-absorbing material and 13% of the radioactivity (Fig. 1). The remainder of the material eluted with 0.48 *M* NaP. Following dialysis, each of these fractions was enzymatically hydrolyzed, purified by Sephadex LH-20 chromatography and the adducts were separated by HPLC. Fig. 2A shows the HPLC profile of AAF–calf thymus DNA that had not passed through an HAP column. Four peaks were observed; peak 4 had a UV spectrum and co-chromatographed with the synthetically prepared dG-AAF C-8 adduct[45]. Peak 3 was established to be the analogous guanosinyl adduct by the above criteria. In addition, this compound gave a positive orcinol reaction and was negative in a diphenylamine assay. The presence of this peak indicated that there was RNA contamination of the calf thymus DNA preparation. This was confirmed



g. 1. HAP chromatography of calf thymus DNA treated with N-OAc-AAF.

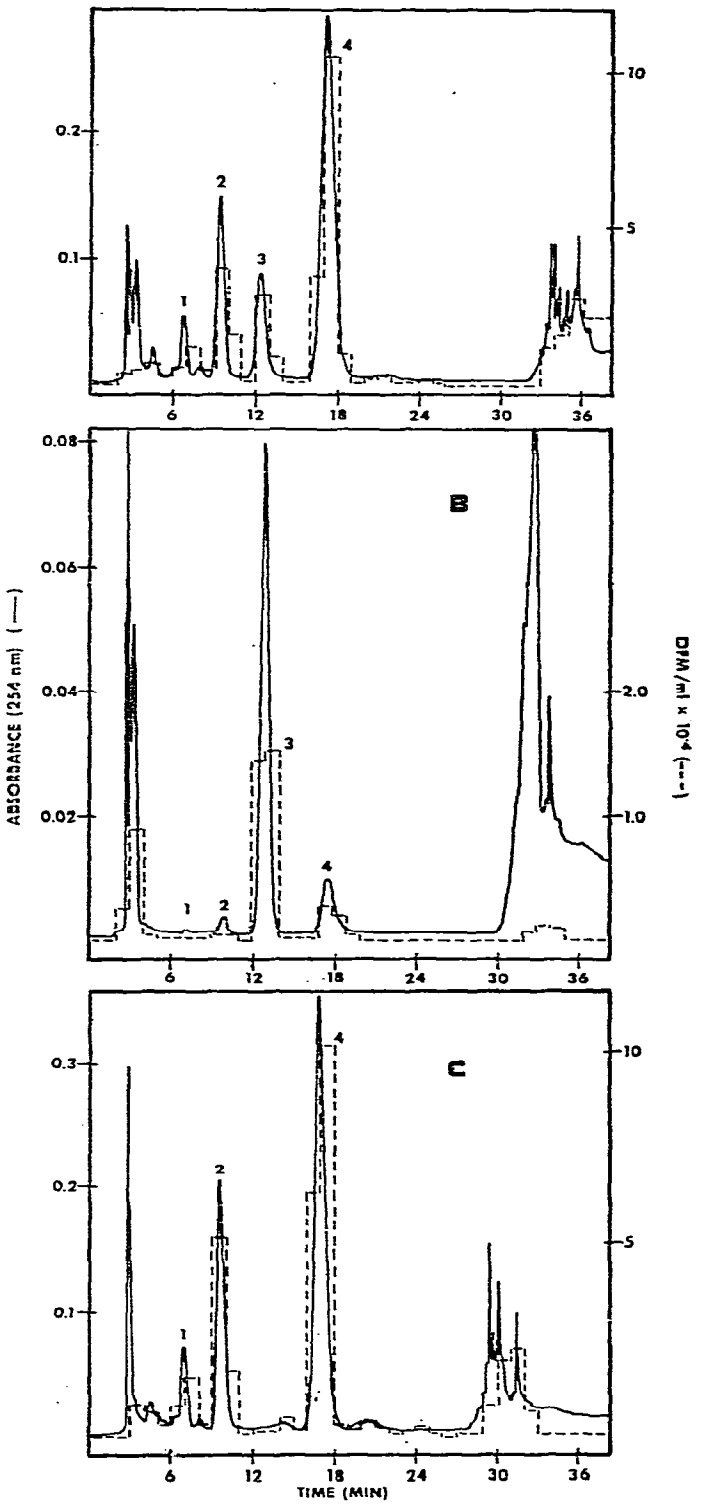


Fig. 2. HPLC profile of enzymatically hydrolyzed AAF bound calf thymus DNA. Conditions: 0-
μm μBondapak C_{18}, 30 cm × 3.9 mm; 56% methanol, for 23–24 min, then 2-min linear program to 100% methanol; flow-rate 1 ml/min. A, AAF–calf thymus DNA that has not passed through the HAP column; B, MUP fraction from the HAP column; C, 0.48 *M* fraction from the HAP column.

by incubating the calf thymus DNA with heat-treated ribonuclease for 30 min. The non-hydrolyzed nucleic acids were precipitated with cetyltrimethylammonium bromide and the supernatant was assayed for RNA with orcinol reagent; this indicated 1% RNA contamination in the DNA. Peak 2 has a UV spectrum consistent with that reported for the N^2-dG adduct[8]. Furthermore, as a peak co-chromatographs with this marker from *in vivo* incubations (see below), it is presumably this adduct. The identity of Peak 1, which was not found *in vivo*, is currently not known.

The HPLC profile of the MUP fraction from the N-OAc-AAF–calf thymus DNA reaction is shown in Fig. 2B. Almost all of the UV-absorbing material and radioactivity is associated with peak 3, which confirms the presence of RNA in this preparation. Small amounts of material in peaks 1, 2 and 4 did elute in this fraction, which indicates that some DNA eluted with MUP. This probably reflects the rather high level of binding in the *in vitro* incubation. *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 HPLC profile for 0.48 *M* NaP fraction adducts is shown in Fig. 2C. Peaks 1, 2 and 4 are evident, as is the lack of peak 3. Thus, even at this high level of carcinogen binding ,an HAP column can separate DNA free of RNA.

The usefulness of this HAP technique for isolating macromolecular adducts formed in cells is demonstrated in Fig. 3, which shows the results obtained from a 4-h incubation of N-OH-[ring-^{3}H]AAF with intact rat hepatocytes. The MUP (RNA) fraction (Fig. 3A) contains only one major radioactive peak, which co-chromatographs with the G-AAF C-8 adduct. The absence of other adducts (*e.g.*, dG-AAF C-8) indicates that there was no DNA contamination, and this was confirmed by diphenylamine assays on this fraction (<1% DNA contamination). Likewise, the 0.48 *M* NaP fraction (Fig. 3B) contains only peaks which co-chromatograph with the DNA adducts. Interestingly, only two DNA–AAF products were observed in this nucleic acid fraction and these co-chromatographed with the C-8 and N^2 adducts.

A final example of the isolation of *in vivo* macromolecular adducts by HAP chromatography is given in Fig. 4, which shows an HPLC profile of the liver DNA (0.48 *M* NaP) fraction from a male Sprague-Dawley rat which had been injected three times with N-OH-[ring-^{3}H]AAF at bi-weekly intervals and killed 2 weeks following the last dose. There is no radioactivity co-chromatographing with peak 3, N-(deoxyguanosin-8-yl)-AAF, thus confirming Kriek's observation that this adduct is rapidly excised from rat liver DNA *in vivo*[8]. Two major radioactive peaks were obtained. One was associated with peak 2, the presumed N^2 adduct, and the other (peak 5) co-chromatographed with the synthetic marker, N-(deoxyguanosin-8-yl)-AF[45]. This C-8 AF adduct, however, should distort the DNA helix and be excised in a manner analogous to the C-8 AAF adduct. The identity of the radioactive compound which co-chromatographs with the C-8 AF adduct, therefore, remains to be established. Typical yields from rats treated with N-OH-AAF as described above were: protein (phenol fraction), 112.0 ± 12.7 mg; RNA (MUP fraction), 4.8 ± 0.4 mg; and DNA (0.48 *M* NaP fraction; $A^{260/280} = 1.88 \pm 0.01$), 0.96 ± 0.02 mg per gram of liver (mean ± standard error from four rats).

In conclusion, we have found Britten *et al.*'s HAP method[22] as modified by Markov and Ivanov[24] to be a good method for the rapid isolation and purification of carcinogen-bound DNA, even at high levels of binding (one carcinogen per 100

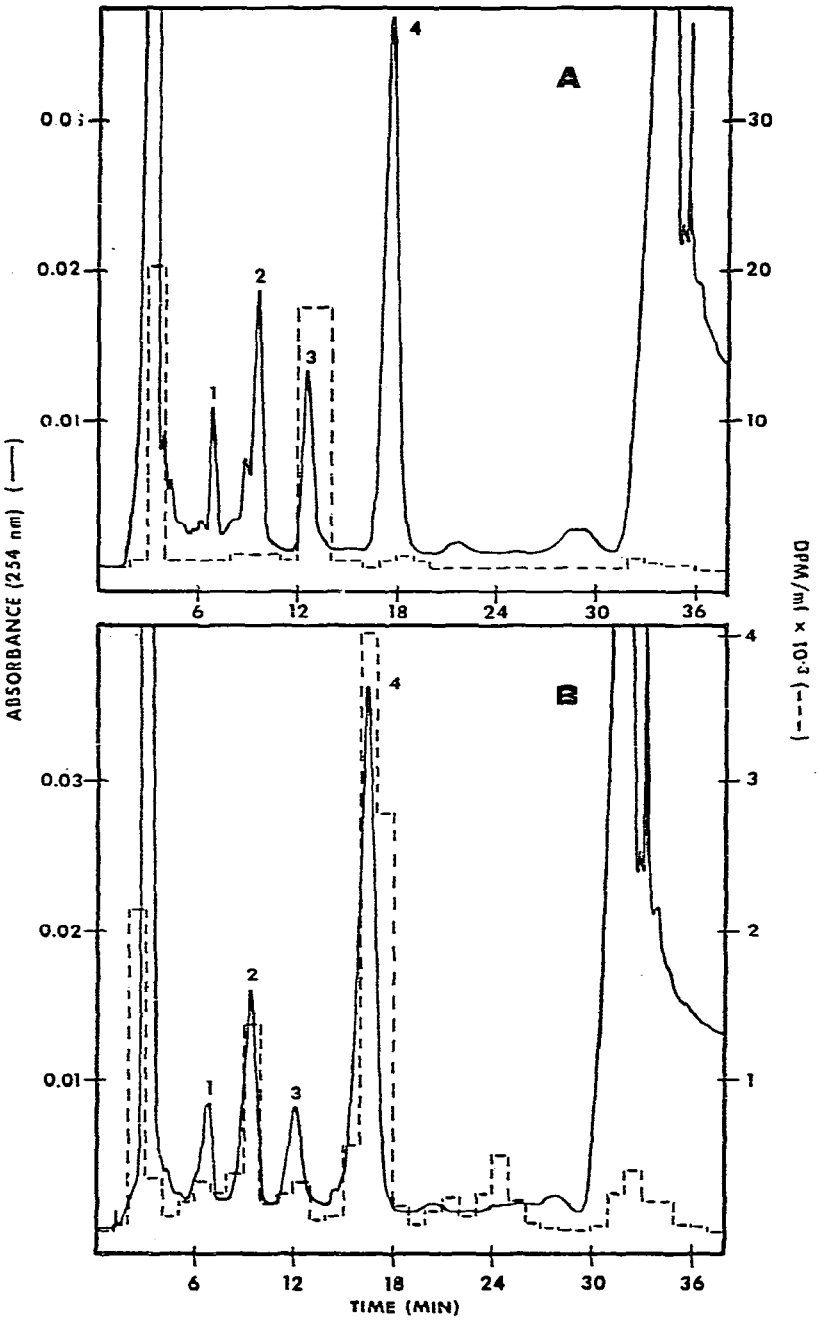


Fig. 3. HPLC profile of adducts isolated from hepatocytes incubated with N-OH-AAF. Following enzymatic hydrolysis, unlabelled adducts were added to serve as UV markers for subsequent chromatography. Conditions: identical with Fig. 2. A, MUP fraction (RNA) from HAP chromatography; B, 0.48 *M* NaP fraction (DNA) from HAP chromatography.

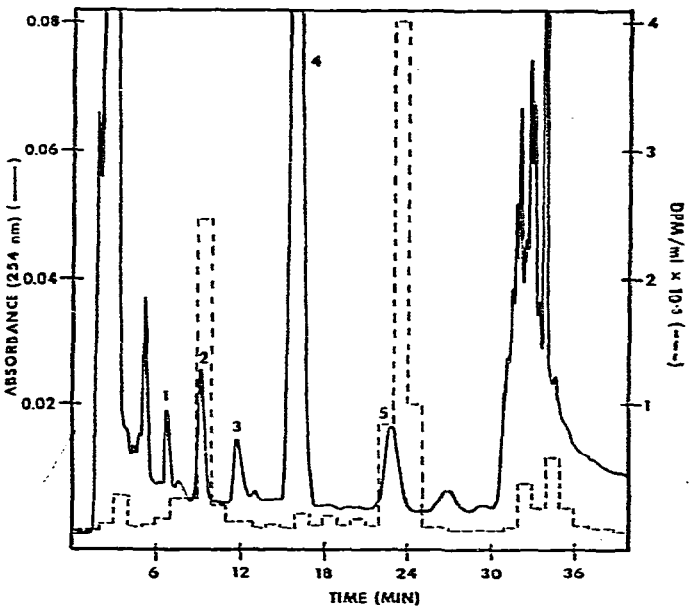


Fig. 4. DNA isolated by HAP chromatography from male Sprague-Dawley rat treated with N-OH-AAF. Following enzymatic hydrolysis, unlabelled adducts were added to serve as UV markers for subsequent chromatography. Conditions identical with Fig. 2.

nucleotides). Furthermore, protein and RNA could be conveniently obtained from the phenol and MUP fractions, respectively. Finally, in addition to the results presented here, by using this method we have also isolated carcinogen-bound macromolecules from (1) hepatocyte primary cultures incubated with benzo(*a*)pyrene, the *anti*diol epoxide of benzo(*a*)pyrene, and N-benzoyloxy-N-methylaminoazobenzene; (2) rats dosed with N-methyl-4-aminoazobenzene; and (3) dog and rat bladder incubated with N-hydroxy-1-naphthylamine.

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REFERENCES

1 E. C. Miller, *Cancer Res.*, 38 (1978) 1479.
2 A. Loveless, *Nature (London)*, 223 (1969) 206.
3 P. D. Lawley, in R. Montesano, H. Bartsch and L. Tomatis (Editors), *Screening Tests in Chemical Carcinogenesis*, IARC Scientific Publications, Lyon, 1976, No. 12, p. 181.
4 P. Sims and P. L. Grover, *Advan. Cancer Res.*, 20 (1974) 165.
5 P. Brookes, *Mutat. Res.*, 39 (1977) 257.
6 A. M. Jeffrey, I. B. Weinstein, K. W. Jennette, K. Grzeskowiak, K. Nakanishi, R. G. Harvey, H. Autrup and C. Harris, *Nature (London)*, 269 (1977) 348.
R. C. Moschel, W. M. Baird, A. Dipple, *Biochem. Biophys. Res. Commun.*, 76 (1977) 1092.

8 E. Kriek, *Cancer Res.*, 32 (1972) 2042.
9 J. G. Westra, E. Kriek and H. Hittenhausen, *Chem.-Biol. Interact.*, 15 (1976) 149.
10 J. D. Scribner and N. K. Naimy, *Cancer Res.*, 35 (1975) 1416.
11 J.-K. Lin, B. Schmall, I. D. Sharpe, I. Miura, J. A. Miller and E. C. Miller, *Cancer Res.*, 35 (1975) 832.
12 J.-K. Lin, J. A. Miller and E. C. Miller, *Cancer Res.*, 35 (1975) 844.
13 F. F. Kadlubar, J. A. Miller and E. C. Miller, *Cancer Res.*, 38 (1978) 3628.
14 J. M. Essigmann, R. G. Croy, A. M. Nadzan, W. F. Busby, V. N. Reinhold, G. Buchi and G. N. Wogan, *Proc. Nat. Acad. Sci. U.S.*, 74 (1977) 1870.
15 J.-K. Lin, J. A. Miller and E. C. Miller, *Cancer Res.*, 37 (1977) 4430.
16 Y. Kawazoe, M. Araki, G.-F. Huang, T. Okamoto, M. Tada and M. Tada, *Chem. Pharm. Bull.*, 23 (1975) 3041.
17 P. D. Lawley, in C. E. Searle (Editor), *Chemical Carcinogens*, American Chemical Society, Washington, D.C., 1976, No. 173, p. 83.
18 K. W. Jennette, A. M. Jeffrey, S. H. Blobstein, F. A. Beland, R. G. Harvey and I. B. Weinstein, *Biochemistry*, 16 (1977) 932,
19 M. Tada and M. Tada, *Biochim. Biophys. Acta*, 454 (1976) 558.
20 C. C. Irving and R. A. Veazey, *Biochim. Biophys. Acta*, 166 (1968) 246.
21 C. C. Irving and R. A. Veazey, *Cancer Res.*, 29 (1969) 1799.
22 R. J. Britten, M. Pavich and J. Smith, *Carnegie Inst. Wash. Yearb.*, 68 (1970) 400.
23 W. Meinke, D. A. Goldstein and M. R. Hall, *Anal. Biochem.*, 58 (1974) 82.
24 G. G. Markov and I. G. Ivanov, *Anal. Biochem.*, 59 (1974) 555.
25 I. G. Ivanov, P. V. Venkov and G. G. Markov, *Prep. Biochem.*, 5 (1975) 219.
26 R. J. Bonney, *In Vitro*, 10 (1974) 130.
27 E. D. Helton, D. A. Casciano, Z. R. Althaus and H. D. Plant, *J. Toxicol. Environ. Health*, 3 (1977) 953.
28 D. A. Casciano, J. A. Farr, J. W. Oldham and M. D. Cave, *Cancer Lett.*, 5 (1978) 173.
29 A. J. Shatkin, in K. Habel and N. P. Salzman (Editors), *Fundamental Techniques in Virology*, Academic Press, New York, 1969, p. 231.
30 A. G. Gornall, C. J. Bardawill and M. M. David, *J. Biol. Chem.*, 177 (1949) 751.
31 A. Sibatani, *Anal. Biochem.*, 33 (1970) 279.
32 P. D. Lotlikar, J. D. Scribner, J. A. Miller and E. C. Miller, *Life Sci.*, 5 (1966) 1263.
33 H. Yamasaki, P. Pulkrabek, D. Grunberger and I. B. Weinstein, *Cancer Res.*, 37 (1977) 3756.
34 A. M. Kapuler and A. M. Michelson, *Biochim. Biophys. Acta*, 232 (1971) 436.
35 R. Fuchs and M. Daune, *FEBS Lett.*, 14 (1971) 206.
36 R. Fuchs and M. Daune, *Biochemistry*, 11 (1972) 2659.
37 R. Fuchs and M. Daune, *FEBS Lett.*, 34 (1973) 295.
38 A. F. Lavine, L. M. Fink, I. B. Weinstein and D. Grunberger, *Cancer Res.*, 34 (1974) 319.
39 R. P. P. Fuchs and M. P. Daune, *Biochemistry*, 13 (1974) 4435.
40 R. P. P. Fuchs, J.-F. Lefevre, J. Pouyet and M. P. Daune, *Biochemistry*, 15 (1976) 3347.
41 G. Metzger and M. P. Daune, *Cancer Res.*, 35 (1975) 2738.
42 I. B. Weinstein and D. Grunberger, in P. Tso and J. Di Paolo (Editors), *Chemical Carcinogenesis, Part A*, Marcel Dekker, New York, 1974, p. 217.
43 D. Grunberger and I. B. Weinstein, in J. M. Yuhas, R. W. Tennant and J. D. Regan (Editors), *Biology of Radiation Carcinogenesis*, Raven Press, New York, 1976, p. 175.
44 F. A. Beland, *Chem.-Biol. Interact.*, 22 (1978) 329.
45 E. Kriek, J. A. Miller, U. Juhl and E. C. Miller, *Biochemistry*, 6, (1967) 177.