Application of HPLC to the Isolation of Molecular Targets in Dosimetry Studies

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High-performance liquid chromatography (HPLC) methods are described which permit the rapid isolation of multiple target macromolecules from the tissues of animals exposed to chemical mutagens. DNA and selected chromosomal proteins are isolated in a simple two step separation scheme. Isolated nuclei are dissolved in 3 M guanidine hydrochloride and the DNA and chromosomal proteins separated on a TSK 3000 SW column. The DNA peak is retained for analysis and the chromatin proteins are dialyzed, lyophylized, and rechromatographed on a PRP-1 column to separate individual histones. Hemoglobin and albumin, two proteins that may prove useful for monitoring mutagen exposure, are isolated from 100 μL of blood by HPLC on a Poly Cat A cation exchange column.

Using this approach, we have monitored the kinetics and dose response of adduct formation (and repair) to DNA, histone, hemoglobin and albumin in mice exposed to 7-bromomethylbenzanthracene and benzo[a]pyrene. The results of these studies are described and briefly discussed. Experiments with other mutagens demonstrate the limits to which DNA adduct quantification may be pushed using radioisotopes. Exposures to very high specific activity (200 Ci/mmole) benzo(a)pyrene have allowed DNA adduct quantification down to a few adducts per cell.

Introduction

Exposure to genotoxic agents may be detected by a variety of somatic and germinal assays in animals and humans, but the biological significance of the observed changes, as measured *in vivo* or *in vitro*, often remains uncertain. One reason for this is that we cannot always define the actual dose of chemical received by specific target molecules. It is essential, therefore, that we develop and routinely apply dosimetric techniques together with these bioassays. This will permit us to define both the extent of damage sustained by specific target molecules and the rate and efficiency of damage repair. By employing dosimetric techniques that can be applied to any tissue, comparisons may be made relating dose to different biological endpoints. Such studies would also provide the common denominator (target dose) required for comparisons among species exposed to similar substances.

One reason that dosimetry measurements are not made an integral part of most exposure/endpoint studies is that target isolations and binding measurements are often labor-intensive. Conventional methods for analyses of DNA adduct formation, for example, require numerous steps to isolate and purify DNA prior to the analysis of bound DNA adducts. Another reason is that few target molecules other than DNA have been extensively characterized with regard to their dose response in exposed animals.

Recent improvements in high-performance liquid chromatography (HPLC) and column packagings make it possible to rapidly separate a variety of macromolecules from exposed animals. We have used HPLC to facilitate the isolation of selected target macromolecules from the tissues of mice exposed to radiolabeled chemical mutagens and quantify the actual dose of chemical that interacts with these molecules. While the work that is described here employs radiolabeled mutagens and scintillation counting to quantify adduct formation, the DNA and proteins provided by these separations may also be analyzed by other techniques that permit direct analyses of adducts and do not require exposures to radioactive compounds.

Isolation of Molecular Targets

The techniques described here emphasize the isolation of macromolecules from two types of targets: molecules that compose or are closely associated with the genome (DNA, histone) and proteins in the blood (hemoglobin, albumin) that can be obtained easily and ana-

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lyzed as dosimeters of recent chemical exposure.

While the ultimate target of mutagens and carcinogens is generally accepted to be DNA, other macromolecules closely associated with DNA may also sustain damage that alters the accessibility or genetic activity of specific chromosomal domains. Chromosomal proteins such as the histones and high mobility group (HMG) proteins are intimately associated with DNA and appear to modulate the packaging of the genome and its accessibility for transcription (1–3). The histones are synthesized coincident with DNA replication and are as stable, biochemically, as DNA itself (4–6). While the HMG proteins, in contrast, do turn over (7,8), two of these proteins appear to maintain genetically active regions of the chromosome in an open, accessible conformation (2,3).

Other targets of potential interest are hemoglobin and serum albumin. Adduct formation to hemoglobin has been considered for use in dosimetry studies as a means of monitoring exposure dose (9–11). Albumin, a natural scavenger and carrier of metals, ions, proteins, fatty acids, antibiotics, sugars, lipids, hormones and a variety of other molecules in serum (12–14) should also be considered. These proteins are excellent nucleophiles, and both may be isolated from blood in reasonable quantities.

DNA Isolation by Gel Permeation Chromatography

DNA and a number of chromosomal proteins may be isolated from tissue or cultured cells simultaneously. The entire procedure requires approximately an hour to perform and permits the isolation of from a few micrograms to over a milligram of DNA per sample. If both DNA and chromosomal proteins are to be isolated from the same sample, a brief nuclear isolation step must be performed to eliminate contaminating ribosomal and cytoplasmic proteins. Tissues (or cultured cells) are Virtis-homogenized in a medium (grinding medium: 0.25 M sucrose, 10 mM Tris, pH 8, 2.5 mM magnesium chloride) which stabilizes the nuclear membrane, and the nuclei are purified by centrifugation (10 min) through 1 M sucrose containing the detergent Triton X-100. After rinsing in grinding medium, the nuclear pellet is dissolved in 3 M guanidine hydrochloride, sonicated (to reduce DNA viscosity), and centrifuged to remove insoluble material. If only DNA is to be isolated, the nuclear isolation step may be eliminated and the cells or tissues dissolved directly in 3 M guanidine hydrochloride.

DNA and chromosomal protein are then separated by chromatography of the dissociated nuclei on a TSK-3000 SW column (three 7.5 mm × 300 mm columns connected in tandem) in 1 M guanidine hydrochloride, 50 mM phosphate buffer pH 6.5. At a flow rate of 1.0 mL/min, separation of the DNA and chromosomal protein peaks (Fig. 1) is complete in less than 40 min. RNA, a minor contaminant in these preparations (0.35%), elutes midway between the DNA and protein peaks. By judi-
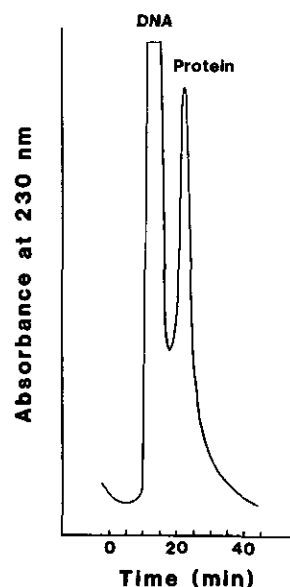

FIGURE 1. Gel-permeation chromatography of dissociated nuclei. HPLC of dissociated mouse liver nuclei was performed on a TSK-3000SW column in 1 M guanidine hydrochloride as described in the text.
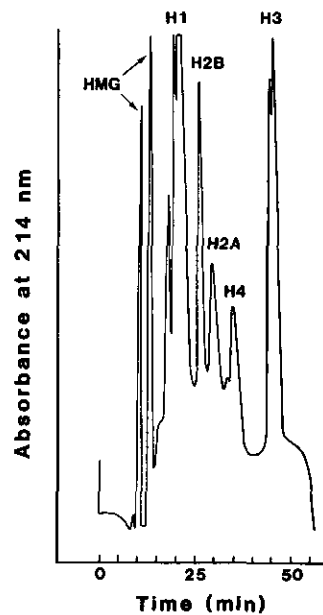

FIGURE 2. Reverse-phase separation of histones and HMG proteins. The lyophylized protein peak obtained following GPC chromatography of dissociated nuclei was dissolved in 0.1% trifluoroacetic acid and rechromatographed on a reverse-phase PRP-1 column. Separation of the HMG proteins and individual histones was accomplished using the following multistep acetonitrile gradient: 35–68% buffer B, 8 min; 68–75% buffer B, 35 min; 75–100% buffer B, 4 min; 100% buffer B, 6 min; 100–35% buffer B, 1 min. Buffer A: 0.1% trifluoroacetic acid. Buffer B: 60% acetonitrile in 0.1% trifluoroacetic acid.

ciously collecting only the early and midpoint (high molecular weight) portion of the DNA peak, RNA contamination of the DNA fraction may be virtually elim-

inated. The DNA peak is processed directly, while the protein peak must be dialyzed against water, lyophylized and rechromatographed to separate specific proteins.

DNA may be isolated from any cell or tissue by this method. Recoveries of DNA and histone from the column typically exceed 95%. Only mammalian sperm require special treatment. The disulfide bridges in the chromosomal proteins associated with the DNA in this cell (protamine) must first be reduced with dithiothreitol or mercaptoethanol and the chromatography performed in 2 M guanidine hydrochloride. The protamines cannot be dissociated from DNA without this reduction and additional guanidine hydrochloride is required to keep the protamine and DNA dissociated and soluble.

Reverse-Phase Separation of Chromosomal Proteins

Further separation and isolation of specific histones and nonhistone chromosomal proteins requires rechromatography of the protein peak on a reversed-phase column. The lyophylized protein is dissolved in aqueous trifluoroacetic acid (TFA, 0.1%) and injected into a 10 μm PRP-1 column (two 7.5 mm × 300 mm columns connected in tandem; Hamilton Co., Reno, NE). Separation of the individual histones is accomplished by using a multistep acetonitrile gradient (Fig. 2). The HMG proteins elute early, just prior to H1 histone, while the core mouse histones elute in the order H2B, H2A, H4, and H3. Only the histones and HMG proteins are present in sufficient quantities to allow analyses of mutagen binding at exposure levels which result in the formation of less than one DNA adduct per 10^5 nucleotides.

Isolation of Hemoglobin and Albumin

Mouse hemoglobin and albumin are obtained by HPLC of whole blood lysates. Aliquots of mouse blood (100 μL) are added to 1.0 mL sodium citrate (1%) and promptly frozen at -20° C. After thawing and centrifuging, 0.5 mL samples are chromatographed on a 7.5 mm × 300 mm Poly Cat A cation exchange column (Custom LC, Inc, Houston, TX) by using a multistep sodium chloride gradient (Fig. 3). Under these conditions, albumin and hemoglobin separate as individual peaks in less that 20 min. The hemoglobin peak is a composite of at least four peaks; these represent sequence variants of hemoglobin that readily separate on this column.
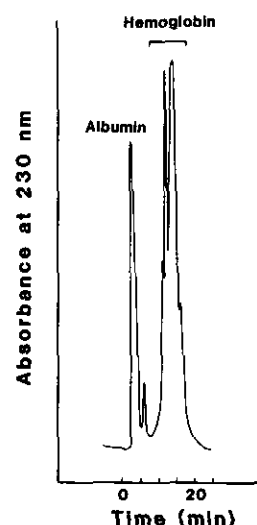

FIGURE 3. Separation of albumin and hemoglobin from lysed mouse blood. Whole mouse blood was added to 1.0 mL 1% sodium citrate and promptly frozen. After thawing, 500 μL was chromatographed on a Poly Cat A cation exchange column by using the following multistep sodium chloride gradient: 0% buffer B, 3 min; 0–40% buffer B, 0.1 min; 40–60% buffer B, 10 min; 60% buffer B, 2 min; 60–0% buffer B, 1 min. Buffer A = 25 mM potassium phosphate, pH 6. Buffer B = 0.3 M sodium chloride in 25 mM potassium phosphate, pH 6.

Sample Processing

Following their isolation, the concentration of chromosomal protein or DNA is determined from the absorbance of the sample at 230 nm or 260 nm, respectively. Albumin and hemoglobin concentrations are determined by using their absorbance at 280 nm. Absorbance measurements are made directly on the DNA fractions or blood proteins, while the chromosomal proteins are lyophylized and redissolved in water. Each sample is then extracted with ethyl acetate or water-saturated butanol to remove noncovalently bound mutagen. This step is essential if adduct formation is to be quantified using radiolabeled compounds and scintillation counting or other techniques that do not differentiate hydrophobic or electrostatic binding and the formation of covalent adducts. Experiments with several different radiolabeled mutagens have revealed that a substantial fraction of the mutagen that coisolates with DNA is not covalently bound to it (Table 1). This extractable mutagen is tightly bound; extensive dialysis does not significantly diminish the level of its binding.

Table 1. Mutagen binding in untreated and water-saturated butanol-extracted DNA and albumin samples.

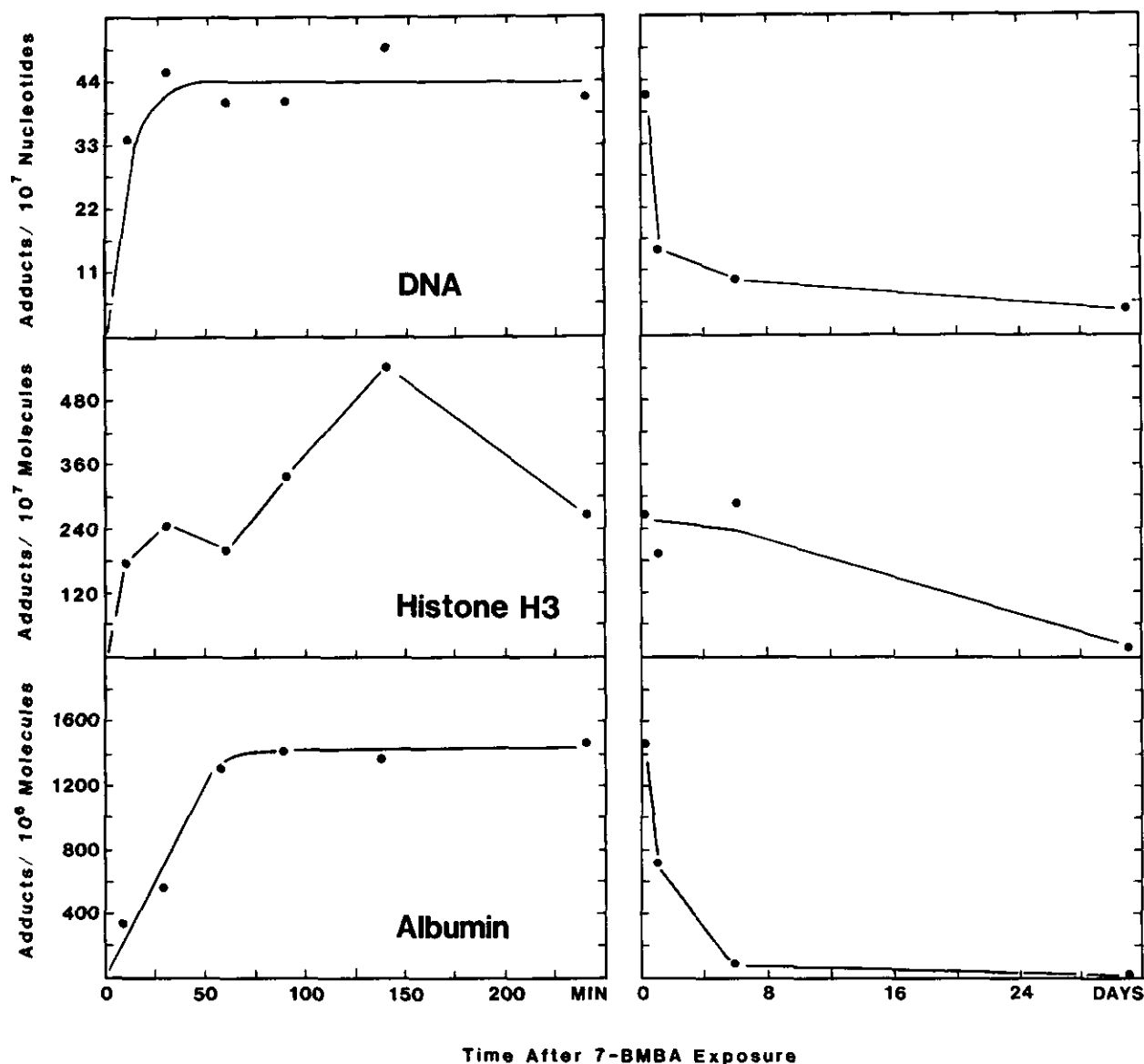|	DNA (adducts/nucleotide)		Albumin (adducts/amino acid)	
	Unextracted	Extracted	Unextracted	Extracted
7-BMBA	$188/10^7$	$92/10^7$	$19.8/10^9$	$13.7/10^9$
Benzo(a)pyrene	$15.6/10^9$	$15.1/10^9$	$25.1/10^9$	$17.5/10^9$
7,12-DMBA	$53.1/10^9$	$34.4/10^9$		
TRP-P-2	$126/10^7$	$17.5/10^7$		

FIGURE 4. Time course of adduct formation in mouse liver DNA, histone, and albumin following exposure to 7-bromomethylbenzanthracene. Male C57B1/6 mice were injected intraperitoneally with 7.7 nmole 3H-7-BMBA (0.8 Ci/mmole) per gram body weight. Mice were sacrificed at various intervals and the liver DNA, histone, and albumin isolated and analyzed as described in the text.

Applications

Because numerous macromolecules (DNA and a variety of proteins) may be isolated from the same cell or tissue sample and separated by HPLC, this method is particularly well suited for application in studies comparing the kinetics of adduct formation (and repair) to multiple target molecules. Analyses of 7-bromomethyl-benzanthracene (7-BMBA) binding to mouse DNA, histone and albumin (Fig. 4), for example, show that peak adduct formation is reached earlier in liver DNA than either protein. The majority of the DNA adducts (70%)

are removed within the first 24 hr, and 10% of the adducts persist for more than 30 days. Adduct formation to histone H3 takes longer to peak, repair (or protein replacement) occurs more slowly [other studies (15) have shown that histones containing small adducts are not turned over but that the adducts are removed; the mechanisms for dealing with large adducts (16,17) have not yet been defined], and adduct removal approaches completion. As with DNA, 7-BMBA adducts to serum albumin are rapidly lost. In albumin, however, this removal reflects the normal rapid turnover of the protein (18) in mice ($t_{1/2}$ = 1 day) and does not appear to involve repair.

FIGURE 6. Dose-response of 7-bromomethylbenzanthracene binding to mouse hemoglobin. Hemoglobin was isolated from 50 μL of blood as described for albumin in Fig. 5.
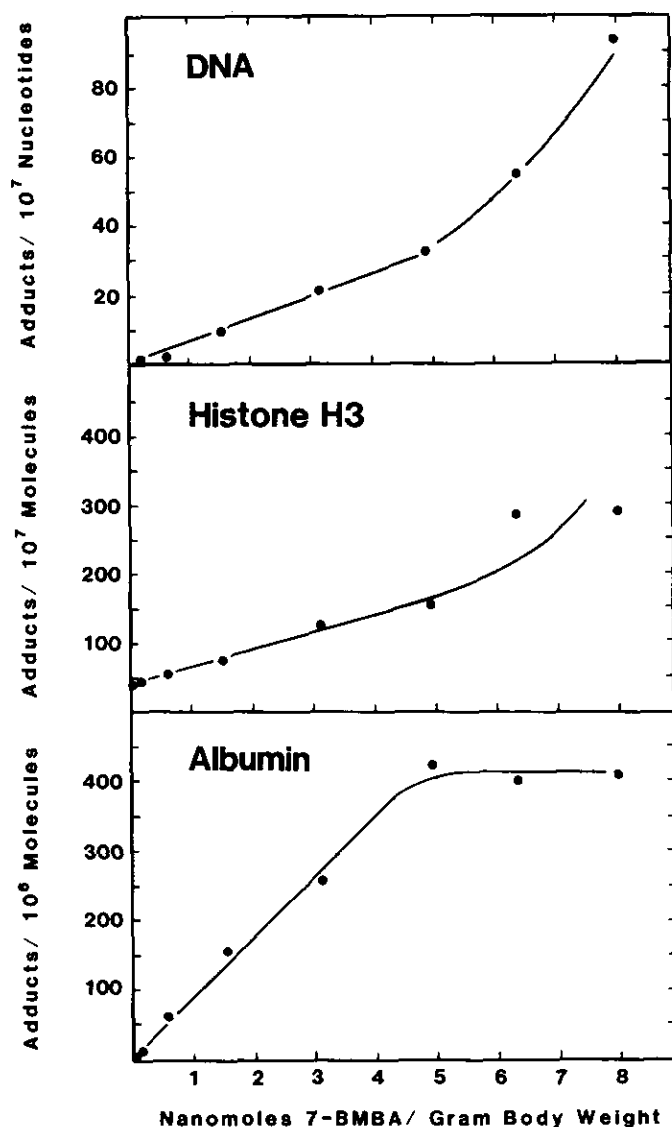

FIGURE 5. Dose-response of 7-bromomethylbenzanthracene adduct formation in mouse liver DNA, histone, and albumin. Male C57BL/6 mice were exposed to 3H-7-BMBA by intraperitoneal injection in dimethyl sulfoxide and sacrificed 2 hr later. DNA was isolated from the livers of sham and exposed animals and processed as described in the text. Albumin was isolated from 50 μL of blood (lysed in 1% sodium citrate) by HPLC on a Poly Cat A cation exchange column.

This technique has also been used to monitor the relationship between exposure dose and the formation of 7-BMBA adducts to several different target macromolecules in the mouse (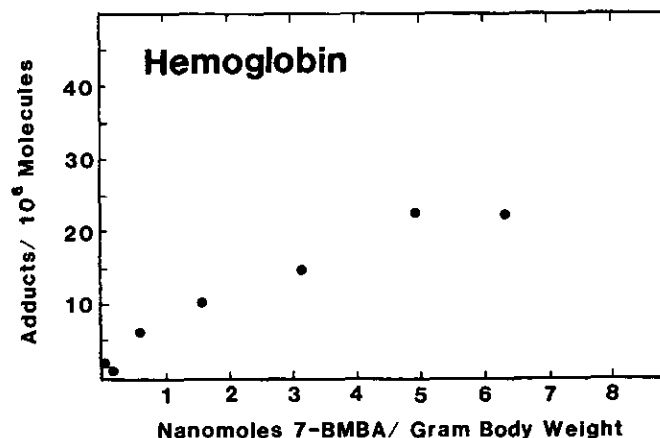Fig. 5). 7-BMBA is clearly more reactive toward DNA than protein (chromosomal or blood). Adduct binding to DNA (per nucleotide) is at least 20-fold higher than to histone H3 (per amino acid) and 4-fold higher than albumin. 7-BMBA binding to hemoglobin is also observed (Fig. 6), but at a level approximately 20-fold lower than albumin. At low doses of mutagen (below 5 nmole/g body weight), dose and adduct formation in DNA, histone, albumin, and hemoglobin are linearly related. At higher doses this binding plateaus in both albumin and hemoglobin and increases nonlinearly in DNA and histone. The plateau observed in albumin and hemoglobin binding appears to represent the saturation of 7-BMBA binding sites in blood. The concomitant nonlinear increase in binding to chromosomal targets may reflect the proportionately larger dose of 7-BMBA received by these targets once the bulk of the binding sites in hemoglobin and albumin become saturated.

Mice exposed to benzo(a)pyrene at doses between 1 and 100 pmole/g body weight exhibit a linear relation between exposure dose and albumin adducts (Fig. 7). The observation that 7-BMBA adducts to albumin are present in at least an order of magnitude greater concentration than hemoglobin adducts was also observed with benzo[a]pyrene; hemoglobin binding was nearly three orders of magnitude lower than albumin. The results with these two compounds suggest that albumin binding may provide an extremely sensitive method for monitoring mutagen exposure in short term experiments. While albumin cannot, obviously, be considered for monitoring exposures occurring over long periods

Table 2. Characteristic levels of DNA-adduct formation in mouse liver DNA following exposures to tritiated mutagens.

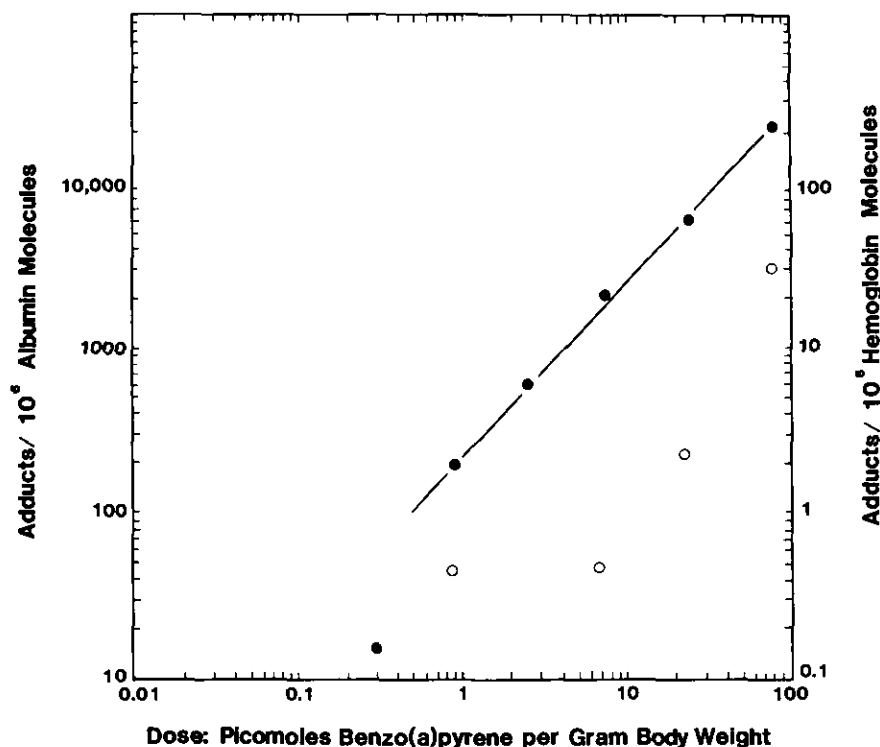| 3H-Mutagen | Specific activity, Ci/mmole | Amount injected/mouse | | Adducts/nucleotide (liver DNA) |
		μmole	cpm	
7-BMBA	0.55	0.18	2.2×10^9	6.9 per 10^6
TRP-P-2	1.5	0.15	3.0×10^5	4.0 per 10^7
Benzo(a)pyrene	25	2.9×10^{-3}	0.8×10^5	1.4 per 10^8
7,12-DMBA	47	7.8×10^{-3}	4.9×10^8	1.7 per 10^8
Benzo(a)pyrene	200	2.5×10^{-6}	2.4×10^7	9.2 per 10^{11}

FIGURE 7.   Dose–response of benzo(a)pyrene binding to hemoglobin and albumin. Male C57B1/6 mice were exposed to 3H-benzo(a)pyrene
(200 Ci/mmole) by intraperitoneal injection of the mutagen in dimethyl sulfoxide. Hemoglobin and albumin were isolated from 50 μL of
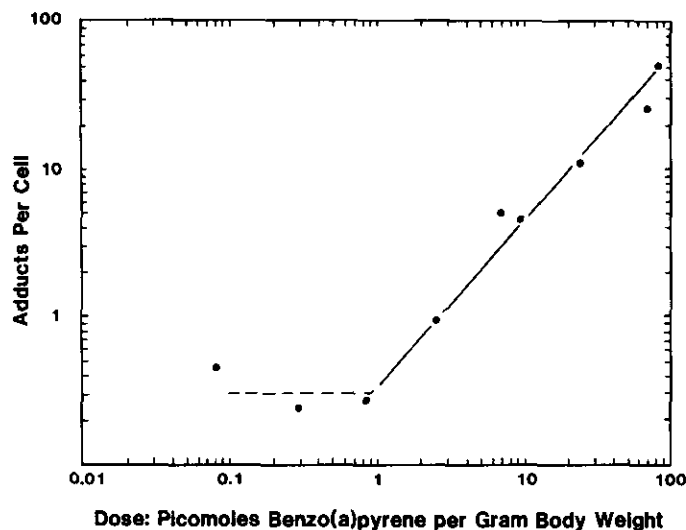blood as described in the text: (●) albumin; (○) hemoglobin.

FIGURE 8.   Dose–response of DNA-adduct formation in the liver of
mice exposed to 3H-benzo(a)pyrene. Mice were exposed as de-
scribed in Fig. 7. Liver DNA was isolated as described in the text
and the number of DNA adducts determined by scintillation count-
ing after extraction with equal volumes (three times) of water-
saturated butanol.

of time [the half-life of the albumin molecule in mice is
approximately 1 day (18) and 20 days in humans (19)],
its sensitivity does offer advantages for monitoring do-
simetry immediately after exposure.

The data shown in Table 2 illustrate how the quan-
tification of DNA-adduct formation in in vivo studies is
limited by the specific activity of the radiolabeled mu-
tagen. In experiments with mutagens containing 1-25
Ci tritium/mmole (the normal specific activity for most
tritium-labeled mutagens), we routinely detect the for-
mation of several DNA adducts per 10^7 or 10^8 nucleo-
tides. By increasing the specific activity of the mutagen
8-fold, as with benzo(a)pyrene (specific activity of 200
Ci/mmole), we have been able to increase the detection
limit to a few adducts per cell (Fig. 8).

Conclusions

The HPLC techniques that we have described offer
a number of advantages over currently used methods
for target DNA and protein isolation. Using this ap-
proach, one can easily monitor and quantitate the bind-
ing of mutagens or carcinogens in vivo to multiple target
molecules (DNA, histones, HMG proteins, albumin,
hemoglobin) within the same individual. The ease and
speed of sample processing allow the analysis of more
samples than can be handled by cesium chloride gradient
centrifugation or gel electrophoresis. The mild condi-
tions employed to dissociate and separate DNA and
chromosomal protein also minimize the loss of chemi-
cally unstable DNA adducts.

With this approach, analyses of DNA adduct forma-
tion require only a few tenths of a gram of tissue. If

only DNA is to be isolated, the entire cell may be dissolved in guanidine hydrochloride and the DNA separated from total protein by GPC. Using radiolabeled mutagens, the sensitivity of the assay permits adduct detection at the level of several adducts per cell. This technique may also be coupled with monoclonal antibody or mass spectrometry methods for quantifying adduct formation *in vivo* without the use of radioisotopes.

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