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Methods for the isolation and detection of etheno adducts in nucleotide pools in vivo following exposure to ethyl carbamate

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SUMMARY

Cellular extraction and high-performance liquid chromatographic methods were developed for the isolation of etheno adducts from nucleotide pools formed in vivo following exposure to the chemical carcinogen ethyl carbamate. These techniques were employed to detect etheno adduct formation using BDF_1 mice and rainbow trout (*Salmo gairdneri*) as test species following interperitoneal injection of the chemical. Ethenoadenine was detected in spleenocyte nucleotide pools of mice after acute (24 h) exposure and chronic (two weeks) exposure. Several etheno adducts (i.e. ethenoadenine, etheno-AMP, etheno-ADP and etheno-ATP) were also detected in total spleen cell nucleotide pools of trout following acute ethyl carbamate exposure.

INTRODUCTION

Ethyl carbamate (EC), a natural by-product of fermentation processes, is a potent animal and suspect human carcinogen. Low levels of EC have been detected in common foods such as bread, yogurt, beer and wine [1]. Although

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EC has demonstrated carcinogenicity in various tissues of several species [2], its exact mechanism of carcinogenesis is unknown. There is little evidence that the primary DNA adduct, 7-(2-oxoethyl)guanine, is associated with mutations. By contrast, EC produces exocyclic etheno (e) adducts in vivo in hepatic RNA similar to those produced by vinyl chloride [2,3]. Such adducts have been demonstrated to cause genetic miscoding errors in vitro [4–6]. However, e-adducts have not been widely detected in DNA. Perhaps minute quantities of e-adducts formed in DNA are not detectable by current analytical methods or, alternatively, mechanisms other than mutation may be involved in carcinogenesis by this chemical. Etheno adducts of non-DNA cellular targets may be indirectly involved in the mechanism of carcinogenesis by affecting other cellular processes. In vitro studies have demonstrated that DNA damage may arise secondarily as a result of imbalances in deoxyribonucleotide pools [7,8]. In an attempt to further investigate in vivo formation of cellular e-adducts that may participate in neoplastic transformation by EC, methods were developed to detect e-adduct formation in nucleotide pools, following in vivo exposure to EC. Because adenine derivatives are the major constituents of nucleotide pools, e-adducts of these components are expected to be most readily detected. Methods were developed for the isolation and detection of ethenoadenine and its derivatives (i.e. ethenoadenine, ethenoadenosine, e-AMP, e-ADP and e-ATP). These techniques were applied to analysis of e-adduct formation in murine spleen lymphocyte nucleotide pools following acute (24 h) and chronic (two weeks) EC exposure and total spleen nucleotide pools of rainbow trout following acute EC exposure.

EXPERIMENTAL

Chemicals

EC was obtained from Aldrich (Milwaukee, WI, U.S.A.) and stored desiccated at room temperature. Buffered ammonium chloride solution used for lysis of murine spleen red blood cells was prepared according to methods of Mishell and Shiigii [9] using Fisher Scientific chemicals (A.C.S. grade) (Pittsburgh, PA, U.S.A.). Formic acid (FA) and 1-butanol (99%) used in nucleotide pool extractions were from Fisher Scientific (A.C.S. grade). HPLC-grade water, methanol and $NH_4H_2PO_4$ buffer salts were also obtained from Fisher Scientific. Ion-pair buffer was prepared daily by combining 60 ml of 1 *M* $NH_4H_2PO_4$ buffer with 5 ml of 1 *M* tetrabutylammonium dihydrogenphosphate (TBA) (Aldrich, Milwaukee, WI, U.S.A.) and diluting to 1 l, giving a final concentration of 60 m*M* $NH_4H_2PO_4$ and 5 m*M* TBA. The pH was adjusted to 5.0 with 3 *M* NH_4OH. To maintain a constant concentration of TBA, methanol used in chromatography contained 5 m*M* TBA. All biochemical standards were obtained from Sigma (St. Louis, MO, U.S.A.).

High-performance liquid chromatography (HPLC) and detection

Chromatography was performed using a Varian Model 5000 high-performance liquid chromatograph equipped with a column temperature control module. E-adenine derivatives were detected with an Aminco-Bowman spectrophotofluorimeter set at 300 nm excitation and 410 nm emission, which was connected in tandem to a Tracor 970A variable-wavelength UV detector set at 268 nm for detection of unmodified adenine derivatives. To insure linearity of the UV and fluorescent detector response, four-point calibration curves were obtained periodically at the lower limits of detection of the instruments for all normal and e-modified adenine nucleic acid components. Concentrations plotted were at the lower limits of detection for the instruments. Fluorescence emission spectra of the collected HPLC fractions were obtained using the spectrophotofluorimeter set at a 260-nm excitation wavelength. A Perkin-Elmer Lambda 4B UV–VIS spectrophotometer was used for the detection of protein in nucleotide pool samples.

Test animals

Male BDF_1 mice (three to four months old) were bred in-house. The parental breeders (C57 B1/6J and DBA/2J) were purchased from The Jackson Laboratory (Bar Harbor, ME, U.S.A.). Mice were maintained on a 12-h light–dark cycle and fed ad libitum. Rainbow trout (*Salmo gairdneri*) was purchased from Bowden Federal Fish Hatchery (Bowden, WV, U.S.A.) and from Angelo's Trout Farm (Normalville, PA, U.S.A.), depending on availability at the time of exposure. Fish were acclimated to laboratory conditions one to two weeks before experimentation and were not fed.

Exposure to EC

Mice. In acute studies, mice were injected with a single interperitoneal (i.p.) high dosage of EC (18.5–19.8 mmol/kg) and killed after 18–24 h. In chronic studies, mice were injected with 3.3 mmol/kg every other day for twelve days (a total dosage of 19.8 mmol/kg). On day 13, mice were killed. Mice were maintained on a 12-h light–dark cycle and fed ad libitum.

Fish. Acute studies were performed by injecting trout with a single i.p. EC dosage of between 25.3 and 28.2 mmol/kg. Trout were killed after 18–24 h. Two to three unexposed fish were placed in a separate tank as a control and killed along with exposed samples.

Cell isolation

Mice. Following sacrifice, spleens were rapidly removed from control and exposed animals and transferred to a centrifuge tube containing 10 ml of phosphate-buffered saline (PBS) and submerged in an ice–alcohol bath. Two or three spleens were placed in each tube and manually pulverized under sterile conditions. Samples were then centrifuged for 15 min at 12 000 *g* at 4 °C. Su-

pernatants were discarded and cells were washed one more time with 5 ml of ice-cold PBS.

After the second washing, cells were brought to room temperature and red blood cells were lysed for 2 min by adding 2 ml of buffered ammonium chloride solution to each tube. The lysing solution was diluted to 6 ml with PBS and samples were centrifuged for 15 min at 12 000 *g* at room temperature. The supernatant was discarded. Samples were washed again with PBS and centrifuged. The supernatant was discarded. Cells were resuspended in 5 ml of PBS, and spleenocytes (i.e. spleen lymphocytes) were counted using a hemocytometer following staining with Trypan Blue, which identified all viable cells by staining dark blue [9]. Cells were then centrifuged at 12 000 *g* and chilled to 0 °C for the remainder of the procedure.

Fish. Preparation of fish spleen cells was performed by the same methodology as described for mice, with the exception that the washing of cells before extraction was performed using ice-cold HPLC water. Since red blood cells were refractory to the lysing procedure, fish samples represent total spleen nucleotide pools rather than spleenocyte nucleotide pools.

Extraction of nucleotide pools and preparation of samples for HPLC analysis

Nucleotide pools from both murine and trout spleen were extracted by modification of the procedures of Martinez-Valdez et al. [10]. After cell counting and centrifugation, supernatant was discarded and tubes were placed on ice. A 5-ml volume of ice-cold 1 *M* formic acid saturated with 1-butanol was added to each sample for 15 min with periodic agitation at which time they were centrifuged for 15 min at 12 000 *g* at 0 °C. Removal of non-precipitated protein and sample clean-up was accomplished by pipetting the supernatant through a C_{18} Sep-Pak cartridge (Waters Assoc.), at a rate of 1 ml/min. The cartridges were washed with 10 ml of cold HPLC water, at a rate of 2 ml/min. Each sample was pooled with one or more similar samples (depending on the number of cells per sample) and lyophilized overnight. Samples were then weighed and stored desiccated in the freezer. HPLC analysis was performed by resuspending a lyophilized, frozen sample in HPLC water to obtain an injection equivalent to 10^6–10^8 cells (i.e. total injection volume was 200 μl). Samples were filtered using a 3cc B-D disposable plastic syringe attached to an Acro-LC13 0.45-μm filter assembly (Gelman Sciences, Ann Arbor, MI, U.S.A.) prior to injection. Two successive 200-μl injections of each sample were made.

Analysis of nucleotide pools by ion-pair reversed-phase HPLC

Ion-pair reversed-phase HPLC was performed using a 250 mm × 4.6 mm I.D. C_{18} Adsorbosphere Nucleotide/Nucleoside column (7 μm particle size) from Alltech Assoc. (Deerfield, IL, U.S.A.) maintained at 27 °C. The column was protected with a prepacked C_{18} guard column obtained from Supelco (Bellefonte, PA, U.S.A.). Samples were analyzed using a linear gradient of 95% ion-

pair buffer–5% methanol to 64% ion-pair buffer–36% methanol over 20 min at a flow-rate of 2.0 ml/min. Chromatographic peaks having retention times similar to any of the e-adenine derivatives were collected for further verification of identity.

Preparative reversed-phase HPLC for collection of e-adenine from murine spleen lymphocyte nucleotide pools

In two separate experiments, a total of 20—25 mice were injected with an acute EC dosage of 19.8 mmol/kg. Exposed and control mice were sacrificed, and spleenocyte nucleotide pools were isolated according to the procedure outlined above. Prior to reversed-phase HPLC analysis, lyophilized extracts were combined, resulting in an exposed sample representing 45 mice and a control sample from a total of 5 mice. HPLC analysis was performed using a 250 mm $\times$ 9.4 mm preparative C_{18} column (5 μm particle size) custom-made by Custom L.C. (Houston, TX, U.S.A.). Optimum separation of e-modified adenine derivatives was achieved using a gradient of 100% of 0.02 *M* $NH_4H_2PO_4$ buffer to 65% buffer–35% methanol over 30 min, at a flow-rate of 3.0 ml/min.

Lyophilized exposed and control samples were each resuspended in 2 ml of HPLC water according to the outlined procedures. Single injections were made of the two samples using a 5-ml sample loop. Fluorescent peaks were identified by comparison of retention times of the adenine derivative standards. Major fluorescent peaks in exposed sample were then collected and the fluorescence spectra were obtained for each peak. To facilitate characterization, peaks were then lyophilized and desalted.

Desalting procedure

Buffer salts (TBA) were removed from collected lyophilized fractions using reversed-phase HPLC. Desalting was achieved with a 250 mm $\times$ 4.6 mm I.D. Zorbax C_{18} analytical HPLC column from Dupont which was protected by a C_{18} prepacked guard column (Supelco). Lyophilized samples were resuspended in a minimum amount of HPLC water and desalted isocratically using 20% methanol–80% water, at a flow-rate of 2.2 ml/min.

Pooling of e-adenine derivatives isolated from exposed fish

Fish fractions collected from exposed samples suspected of containing any e-adenine derivatives (based on comparisons of retention times to standards) were pooled and lyophilized overnight. Samples were desalted and relyophilized to obtain a concentrated material. Dry samples were resuspended in 300 μl of 0.01 *M* HCl and stored at $-20\,^{\circ}C$ before further analysis.

Fluorescence emission spectra

Fluorescence emission spectra of HPLC fractions collected from murine and fish samples were obtained using an excitation wavelength of 260 nm. Emis-

sion spectra of the unknown fractions were obtained under identical conditions. The fluorimetric limit of detection for e-adenine was determined to be approximately 750 ppt. In the case of murine studies, emission spectra of the fraction having the same retention time as the e-adenine standard were obtained without desalting. Peaks collected from trout samples having retention times similar to e-adenosine, e-AMP, e-ADP and e-ATP were pooled and desalted prior to obtaining fluorescence emission spectra.

Protein detection

To detect possible protein contamination in nucleotide pool samples, the Bio-Rad microprotein assay was performed on collected fractions according to the methodology provided by the manufacturer. The detection limit of this assay is approximately 1 μg.

Analysis of data

HPLC peaks were identified by comparison to retention times of standard compounds. Quantitation of unmodified adenosine nucleotides (i.e. ATP, ADP and AMP) was performed in the UV detection mode, by comparison of area integration with standard curves using a Hewlett Packard 3390A recording integrator. Ethenoadenine derivatives were quantitated using fluorescence detection and measurement of peak heights using a Linear recorder. Fluorescence emission spectra of collected fractions were compared to emission spectra of e-adenine standard for further verification of identity.

RESULTS

Separation and detection of nucleotides, nucleosides and bases by ion-pair reversed-phase HPLC

Chromatograms of a standard mixture of the major unmodified and e-modified adenine derivatives are presented in Fig. 1. Fluorescence detection of e-adenine derivatives permitted quantitation in the picomole range. Etheno-ADP is most readily detectable at the excitation and emission wavelengths employed (i.e. 300 nm excitation, 410 nm emission), whereas detection of e-adenine was the least sensitive, with a limit of detection of about 1 ng.

Detection of e-adenine in lymphocyte nucleotide pools of murine spleen cells

Fig. 2 illustrates the detection of e-adenine in murine spleenocytes following acute and chronic exposures to EC. Tryptophan, another fluorescent acid-extractable cellular component, was detected in control and exposed samples in the region of e-adenine elution (Fig. 2a). Ethenoadenine and tryptophan were not fully resolved under the HPLC conditions used (Fig. 2b and c). Each exposure chromatogram represents an injection equivalent to $5 \cdot 10^7$ cells.

Quantitation of e-adenine content per $1 \cdot 10^6$ murine spleen lymphocytes fol-

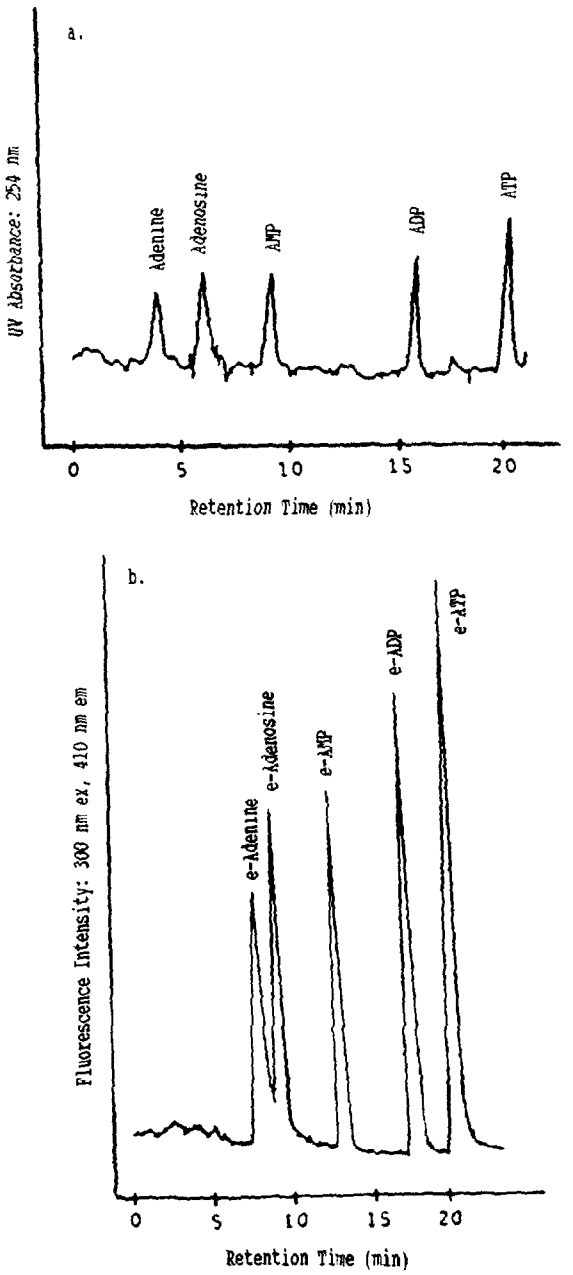

Fig. 1. Ion-pair reversed-phase HPLC separation of unmodified and e-modified adenine derivatives. (a) UV detection of five major adenine derivatives; (b) fluorescence detection of five major e-adenine derivatives.

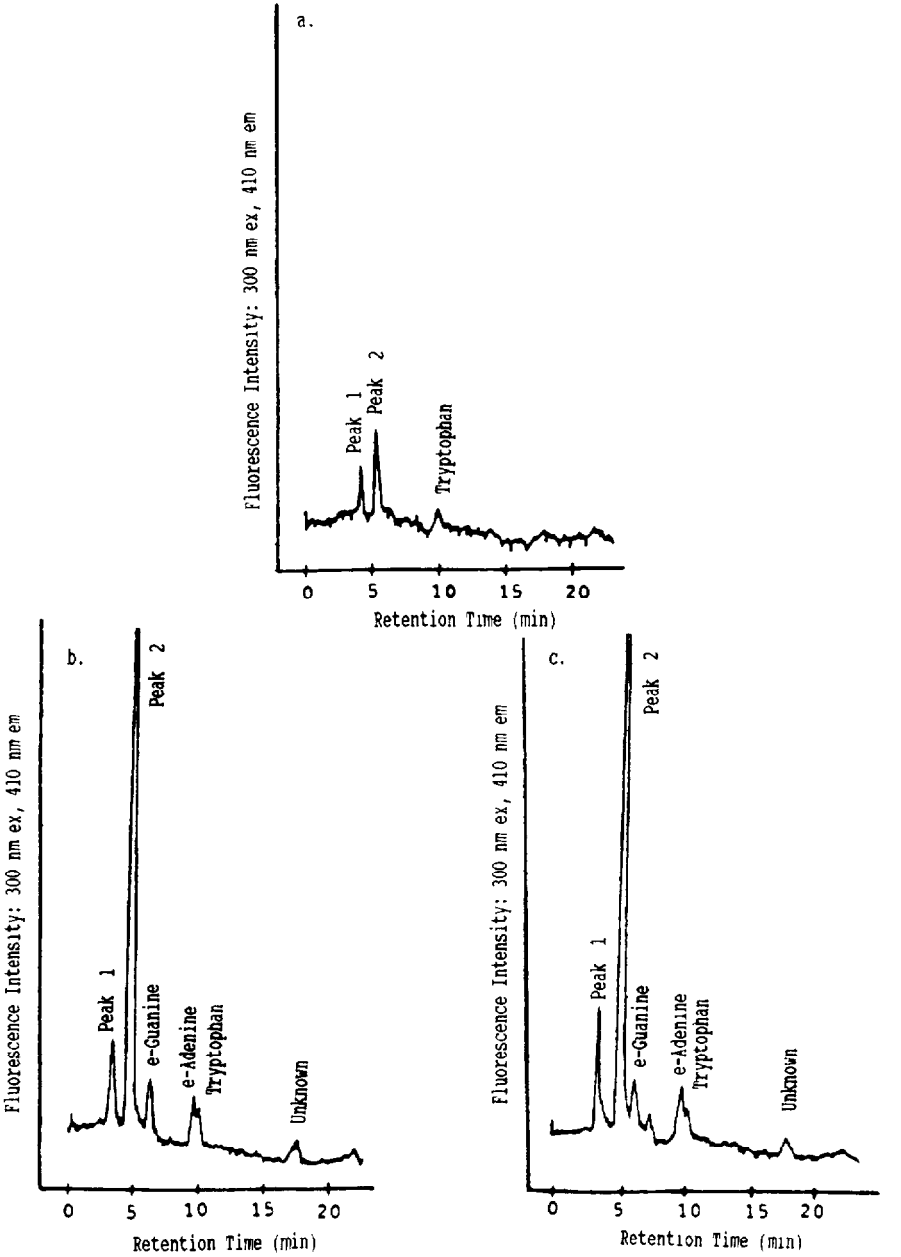

Fig. 2. Isolation of e-adenine from murine spleen lymphocytic nucleotide pools following EC exposure. (a) Control, extract from $8 \cdot 10^7$ cells; (b) acutely exposed, extract from $5 \cdot 10^7$ cells; (c) chronically exposed, extract from $5 \cdot 10^7$ cells.

TABLE I

LEVELS OF e-ADENINE IN NUCLEOTIDE POOLS OF $1 \cdot 10^6$ MURINE SPLEENOCYTES

Sample	e-Adenine (pg)	Adenine derivatives (ng)	eAA/UNMOD[a]
Acute (18.5–19.8 mmol/kg)	103 ± 13[b]	300 ± 198	1 3000
Chronic (19.8 mmol/kg)	194 ± 15[c]	92 ± 15	1 500

[a]Ratio of the amount of e-adenine derivatives detected versus total amount of unmodified adenine derivatives detected.
[b]Standard deviations based on the results of two experiments
[c]Standard deviations based on two HPLC injections of the same sample.

lowing EC exposure is presented in Table I. Mice received approximately an equivalent dosage of EC in both acute and chronic exposures, but in the chronic study this dosage was given over a two-week period every other day (i.e. six i.p. injections). Data from acute exposures represent average values from two separate experiments whereas chronic data are representative of one experiment. The high standard deviation calculated for the quantity of adenine derivatives per $1 \cdot 10^6$ cells in Table I is most likely the result of the high dosage of EC received by the animals in the acute studies (i.e. 18.5–19.8 mmol/kg, 24 h before sacrifice). Experiments employing acute high dosages of carcinogens frequently cause cytotoxic effects, such as variations in the levels of adenine nucleotides [11], as well as in the number of viable cells (i.e. those that stain with Trypan Blue). A comparison of the e-adenine detected in nanograms for each experiment in Table I suggests that there is approximately two times more adduction due to chronic exposure. However, due to the cytotoxic effects which are associated with a high dosage of EC, a portion of the e-adenine detected in the acute sample may be from non-viable cells, whereas all e-adenine detected in the chronic study is most likely from viable cells. Therefore the levels of e-adenine detected in acute versus chronic studies are not directly comparable.

Results of the acute and chronic experiments can be compared by calculating the eAA/UNMOD ratio, which is defined as the amount of e-adenine derivatives detected versus the total amount of unmodified adenine derivatives detected per sample. The eAA/UNMOD ratios for the acute and chronic studies presented in Table I indicate that six times more adduction was detected in the chronic study (i.e. 1:3000 in the acute study versus 1:500 in the chronic study). These results suggest that there is an accumulation of adduction due to chronic EC exposure. It must be noted, however, that these ratios are semiquantitative, due to the possible contribution of e-adenine from non-viable cells in the acute

sample. Future methods' development will include procedures to remove non-viable cells from samples prior to detection of adducts by HPLC.

Evidence of the formation of additional adducts in spleenocyte nucleotide pools is illustrated by the detection of other fluorescent peaks after acute and chronic EC exposures (Fig. 2). The first and second peaks eluting off the column are present in control, acute and chronic samples (i.e. designated as peaks 1 and 2 in Fig. 2). Levels of peak 2 increase following acute and chronic exposures (Fig. 2b and c). This unknown pool component was collected from several samples and concentrated to determine if it could be identified as a protein contaminant using the Bio-Rad microprotein assay. No protein was detected in this fraction.

Preparative chromatography

To verify the formation of e-adenine in EC-exposed mice, a preparative-scale experiment was performed. Forty-five mice were given a single acute EC dosage of 19.8 mmol/kg. Thus, larger quantities of fluorescent HPLC fractions were collected to enable identification by comparison to fluorescence spectra. Fig. 3a illustrates the fluorescence chromatogram of nucleotide pools isolated from 45 mice (i.e. $6.8 \cdot 10^8$ cells) exposed to 19.8 mmol/kg EC. The peak eluting at approximately 30 min in both control and exposed samples corresponds to the retention time of e-adenine. The quantity of the peak derived from exposed mice was approximately five times greater than that from control mice per $1 \cdot 10^6$ cells. Verification of the identity of e-adenine was made by comparison of the collected fraction's fluorescence emission spectra to standard spectra at 260-nm excitation. Fig. 3b represents a sample of five control mice (i.e. nucleotide pools from $1.82 \cdot 10^8$ cells). The minor peak eluting at approximately 30 min corresponds to the retention time of e-adenine, suggesting that e-adenine may be present in control murine samples. The chromatograms shown in Fig. 3 indicate the presence of other fluorescent compounds in nucleotide pools which have not as yet been identified. Experiments to determine the structure of these components are currently underway.

Detection of e-adducts in fish spleen cells

Fig. 4a illustrates the detection of e-adenine derivatives in nucleotide pools of fish spleen following an HPLC injection equivalent to $9 \cdot 10^7$ cells. External dosages were between 25.3 and 28.2 mmol/kg. At 24 h following acute EC exposure, the prominent adduct in fish samples was e-ATP. An eAA/UNMOD ratio of 4000:1 in Normalville fish and 150:1 in West Virginia fish were calculated for the presence of e-ATP alone. Lower levels of e-adenine derivatives were also detected in exposed samples from both sources, including e-adenosine in three of four samples from Normalville and two of four samples from West Virginia, e-AMP in one of four samples from Normalville and two of four samples from West Virginia and e-ADP in two of four samples from Normal-

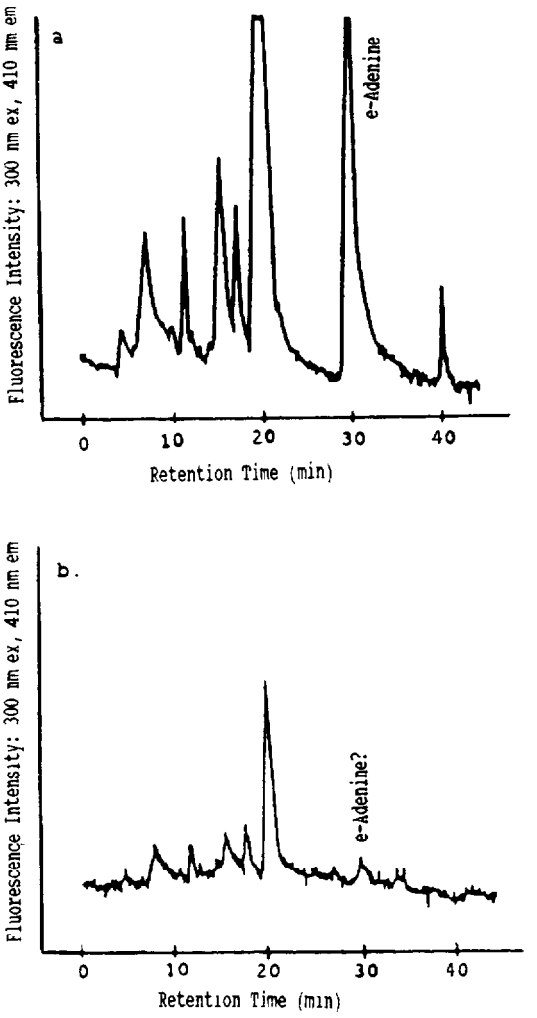

Fig. 3. Results of preparative chromatography study to isolate e-adenine. (a) Fluorescence profile of 45 acutely exposed mice; (b) fluorescence profile of 5 control mice.

ville and one of four samples from West Virginia. The average eAA/UNMOD ratio for all these adducts exclusive of e-ATP was approximately 20 000:1. There was evidence of the presence of e-adenine in all control and exposed samples from both sources (i.e. Normalville and West Virginia University), but quantitation of this adduct was not attempted because of its coelution with tryptophan.

A large number of unknown peaks were detected in both control and exposed samples at retention times prior to that of e-adenine (Fig. 4). Microanalysis of a sample of the pooled unknown peaks did not show the presence of protein.

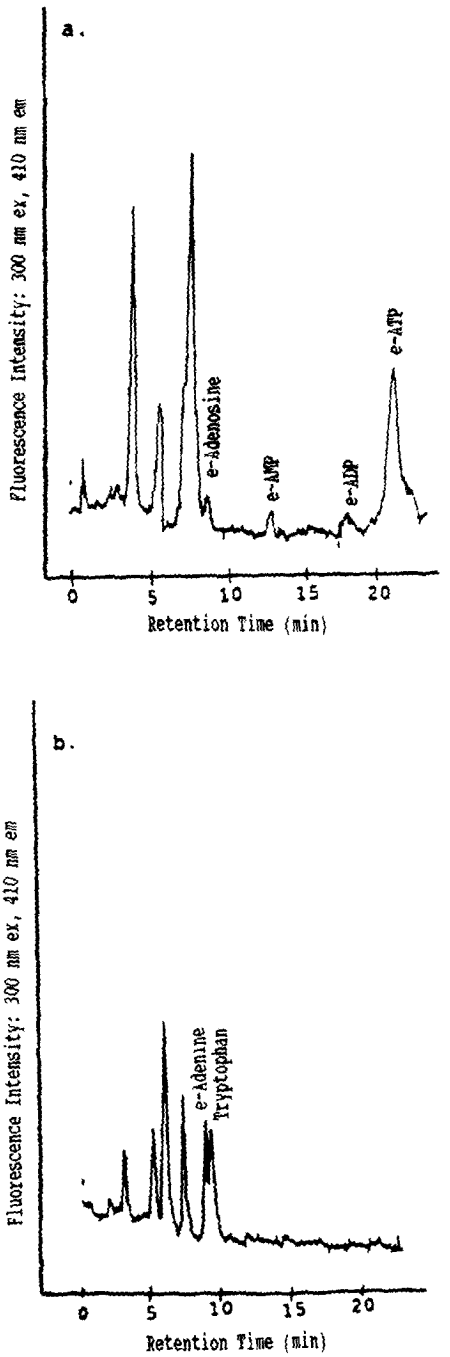

Fig. 4. Detection of e-adenine derivatives in nucleotide pools of fish. (a) Acutely exposed, extr: from $9 \cdot 10^7$ cells; (b) control, extract from $1 \cdot 10^7$ cells.

Thus, if unknown peaks are protein contamination, the total concentration is less than 1 μg per total cell extract.

Identification of e-adenine derivatives in fish

In order to obtain the maximum amount of sample for analysis, all peaks suspected of being e-adenine derivatives in trout nucleotide pools were combined into a single sample for verification of identity (i.e. with exception of the e-adenine base, due to its partial coelution with tryptophan). Following lyophilization and desalting, the fluorescence emission spectra of the pooled fish fraction containing peaks having retention times similar to the e-adenine derivatives were compared to the emission spectra of an e-adenine standard. The spectra of the relyophilized fish sample at 260-nm excitation demonstrate maximum emission at 410 nm which corresponds to the maximum emission wavelength of the e-adenine standard. Detection of e-adducts by fluorescence will not differentiate between the various adenine derivatives [12].

DISCUSSION

We have established analytical methods for extraction and analysis of unmodified and e-modified adenine derivatives in nucleotide pools. Ion-pair reversed-phase HPLC successfully separates adenine nucleotides, nucleosides and bases and their respective e-modified derivatives within a single chromatographic run of short duration (i.e. less than 25 min), yielding sharp, well resolved peaks necessary for quantitation of components. Fluorescence detection permits selective detection of trace quantities (i.e. in the picomole range for e-adenine derivatives), while UV monitoring provides the ability to quantitate levels of unmodified adenine derivatives. These values have been used to estimate the total amount of adducts produced by exposure to EC. Fluorescence detection was not affected by the use of a methanol gradient in ion-pair reversed-phase HPLC. However, increasing the concentration of the ion-pairing reagent to 0.01 *M* decreases the fluorescence intensity of e-adenine derivatives.

Preliminary studies demonstrated that formic acid–butanol yielded optimal quantities of unmodified adenosine nucleotides [13] as well as nucleoside and base etheno derivatives. Perchloric acid, the most widely used nucleotide pool extractant, was not effective in extraction of e-adenine derivatives from spiked spleenocyte samples. Formic acid alone resulted in 50% recovery, whereas formic acid–butanol produced 70–90% recovery of e-adenine derivatives. Subsequent extraction of spiked samples with 100% butanol or chloroform provided no additional recovery of e-adenine derivatives.

The purpose of this investigation was not to determine in vivo levels of specific nucleotides, but rather to detect e-modification of adenine components in general. Therefore, butanol–formic acid was selected as the extractant in spite

of its extensive hydrolysis of unmodified nucleotides as reported by Perrett [14] and of e-adenosine nucleotides observed in the present study. The hydrolytic action of the formic acid–butanol extractant most likely accounts for the fact that e-adenine was the only adduct consistently detected in murine samples following exposure. Alternatively, purine catabolic enzymes may contribute to degradation of nucleotides and nucleosides.

Acute and chronic exposure studies performed in mice provided the same total dosage of 19.8 mmol/kg EC. Comparing the eAA/UNMOD ratios generated from these experiments, EC given in multiple injections every other day for six days (i.e. total dose of 19.8 mmol/kg) results in an accumulation of e-adducts in spleenocyte nucleotide pools, as compared to the same dosage given as a single injection (Table I). Six injections of EC resulted in a six-fold increase in the quantity of adducts detected, suggesting that nucleotide pool damage accumulated with each subsequent injection. A similar pattern of DNA damage, as measured by the induction of sister chromatid exchanges, has also been observed in BDF_1 mice following multiple dosages of EC [15]. Thus, removal and repair of e-adenine derivatives from the cell appears to be limited. In this respect, it is of interest to note that e-AMP is not a substrate for adenine deaminase [16] and consequently is expected to escape the primary route of purine catabolism (i.e. deamination).

Verification of e-adenine formation was accomplished by first exposing 45 mice to a single EC dose and then separating nucleotide pool components on a preparative scale using reversed-phase chromatography. This experiment provided sufficient sample to obtain, without desalting, a fluorescence spectrum indistinguishable from that of an e-adenine standard. The preparative nucleotide pool sample contained several unknown fluorescent components which corresponded to the retention times of unknown peaks in the control sample (Fig. 3a and b). Ongoing studies are aimed at further characterization of these unknown samples.

Another significant finding was the detection of a trace component that eluted at the retention time of e-adenine. The scarcity of this component impeded its detection by ion-pair reversed-phase HPLC due to the interference of tryptophan. This observation could suggest that e-adenine is formed endogenously in murine spleenocytes. This possibility is not unreasonable, in light of a recent investigation which demonstrated that similar mutagenic exocyclic adducts are formed in vitro by the reaction of malondialdehyde (i.e. the major end-product of lipid peroxidation in plants and animals), with guanine and guanine nucleosides [17]. Further support for endogenous occurrence of e-adducts is the existence in mammalian cells of an N-glycosylase specific for excision repair of e-modified deoxyadenosine and deoxyguanosine nucleotides in DNA [18]. Continuing studies in this laboratory will focus on elucidation of the mechanism of endogenous e-adduct formation.

Finally, the existence of unknown fluorescent components in murine spleen nucleotide pools must be addressed. In control pools, one or two unknown peaks

(the peaks can coelute depending on condition of column) eluting at a retention time around 3 min using ion-pair reversed-phase HPLC were not detected as protein (Fig. 2a). Other acid-extractable cellular components such as nucleotide precursors (i.e. inosine and xanthine), ribose sugars, coenzymes, amino acids and ascorbic, uric and orotic acids, which may potentially appear in chromatograms, are not fluorescent at the excitation and emission wavelengths employed in this study (i.e. 300-nm excitation and 410-nm emission). These unknown peaks may represent endogenous nucleotide pool e-adducts not previously detected. Furthermore, since the concentration of the compound giving rise to peak 2 increases during either chronic or acute EC exposure (Fig. 2b and c), it is possible that monitoring levels of this nucleotide pool component may be a useful indicator of EC exposure.

Ethenoguanine was tentatively identified as one peak appearing after exposure (Fig. 2b and c), based on comparison of its retention time with that of a standard synthesized according to a method described by Sattsangi et al. [19]. Further purification of the standard must be performed before a more positive identification can be reported. Verification of in vivo formation of this adduct is of particular importance, in light of the recent suggestion that e-guanine may be implicated in genetic mutation induced by vinyl chloride [20].

The major purpose of extracting nucleotide pools from fish spleen cells was to determine whether the procedures developed for murine cells are applicable for detection of adducts in aquatic biological samples. Because of its known susceptibility to carcinogenic adduct formation, rainbow trout was chosen as the test species [21]. To our knowledge, the present investigation is the first report of detection of adducts in nucleotide pools of fish.

Detection of several e-adducts in fish following acute EC exposure compared to the detection of only e-adenine in mice is most likely due to the presence of red blood cells that were refractory to lysis by methods used for muring spleen samples. As in mammals, erythrocytes of primitive vertebrates contain higher levels of ATP than do lymphocytes [22,23], and it is likely that this contributes to the detection of higher levels of e-adenine derivatives following chemical exposure. Furthermore, hydrolysis of nucleotides in fish following dissection may occur at a lower rate because of inherently less active enzyme degradation systems, as compared to higher vertebrates.

More reproducible adduct formation in exposure studies with fish from the Normalville hatchery may be due to the fact that the extraction technique was more refined when these experiments were performed. Hydrolysis of the adduct due to improper sample handling would lead to a decrease in the level of e-adenosine nucleotides and an increase in the e-nucleoside. Less perfected techniques may have enhanced hydrolysis of the nucleotides and produced relatively higher levels of e-nucleosides.

As in control mice, e-adenine was detected in spleen nucleotide pools of control fish from both Normalville and West Virginia hatcheries (Fig. 4b). Unlike

in mice, however, the level of this adduct was measurable in pools extracted from a single fish spleen and suggests endogenous formation of e-adenine in fish as well as in mice.

Although EC exposure via the aquatic environment is not of current concern, this investigation provides evidence of e-adduct formation in nucleotide pools of fish following chemical exposure. Moreover, methods described in the present study are adaptable for investigation of in vivo adduct formation in nucleotide pools of fish following exposure to other relevant carcinogenic pollutants of the aquatic environment.

Future studies are required to enhance methodology, to characterize unknown adducts and to verify e-adduct formation. Likewise, the significance and role in genotoxic processes of EC-induced as well as endogenously formed e-adducts remain to be elucidated.

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