(*E*)-2-Hexenal-Induced DNA Damage and Formation of Cyclic 1,N^2-(1,3-Propano)-2′-deoxyguanosine Adducts in Mammalian Cells

Petra Gölzer,[†] Christine Janzowski,*[,†] Beatrice L. Pool-Zobel,[‡] and Gerhard Eisenbrand[†]

Department of Chemistry, Division of Food Chemistry and Environmental Toxicology, University of Kaiserslautern, Erwin-Schrödinger-Strasse, Building 52, D-67663 Kaiserslautern, Germany, and Institute of Nutritional Physiology, Federal Research Center for Nutrition, Karlsruhe, Germany

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(*E*)-2-Hexenal (hexenal), a natural flavor compound, acts as directly genotoxic agent and forms cyclic 1,N^2-propano adducts with deoxyguanosine. Formation of this adduct in isolated DNA and in cells was studied with a modified ^{32}P-postlabeling procedure including HPLC separation, nuclease P1 enrichment, two-dimensional TLC of adducted nucleotide bisphosphates on PEI-cellulose, and quantification of adduct spots by liquid scintillation counting. Adduct formation with the more reactive crotonaldehyde was included for comparison. Synthesized adducted dG-3′-phosphates served as external standards for identification and quantification. In calf thymus DNA, hexenal (0.2 mM) shows a time dependent formation of adducts, yielding 1.55 pmol/μmol of DNA at 5 h incubation. With crotonaldehyde (0.2 mM) the adduct rate was about 10-fold higher. Hexenal also generated 1,N^2-propano-dG adducts in the human lymphoblastoid Namalva cell line (0.2 mM, 1 h, 86 fmol/μmol of DNA) and in primary rat colon mucosa cells (0.4 mM, 30 min, 50 fmol/μmol of DNA). In primary colon mucosa cells from rats and humans, hexenal and crotonaldehyde (0.4 mM, 30 min) induced DNA damage, detected by single cell microgel electrophoresis (comet assay). In primary rat gastric mucosa cells, hexenal was only weakly active, inducing detectable DNA damage in 20% of cells at 0.8 mM concentration. In contrast, primary mucosa cells from rat esophagus were as sensitive as colon cells. After single oral application of hexenal to rats (up to 320 mg/kg body wt) DNA damage was not detectable in gastrointestinal mucosa. Analysis of hexenal in selected flavored foods revealed concentrations up to 14 ppm (0.14 mM) that are comparable to its natural occurrence in some fruits and vegetables (up to 30 ppm). Thus, the concentration range selected for the toxicological studies described here clearly is relevant: Hexenal, at concentrations found in food, exerts genotoxic effects in cells from rat and human gastrointestinal tract.

Introduction

(*E*)-2-Hexenal (hexenal)[1] is an important flavoring compound which occurs naturally in many fruits and vegetables (*1*−*5*). Maximum concentrations (up to 76 ppm) were found in bananas (*3*). Predominantly, hexenal and other C_6 aldehydes are formed by enzymatic lipid peroxidation from C_{18} acids after disruption of cells (*6*−*8*). The release of hexenal and other aldehydes by lipoxygenase−hydroperoxide lyase from wounded plant tissue is discussed as possible primary defense mechanism against microbial infection (*9, 10*). In minor amounts, hexenal is also generated by autoxidation processes (*11*). Because of its "green" and fruity flavor, hexenal is used as natural flavoring compound to generate "green notes" (*12, 13*). Hexenal was approved for food use by the US Food and Drug Administration; the Council of Europe included it in the list of artificial flavoring substances (*14, 15*). However, the extent to which hexenal is used as natural food additive in the European Community is not known at present. The C_4 homologue crotonaldehyde ((*E*)-2-butenal) occurs as combustion product and has been detected in tobacco smoke and other environmental media (*16*−*18*).

Both aldehydes are directly acting mutagens in bacteria and mammalian cells (*19*−*22*). They also induce DNA single-strand breaks, chromosomal aberrations, micronuclei, and sister chromatid exchanges in different mammalian cells (*23, 24*). Crotonaldehyde induced liver tumors in F344 rats after chronic administration in drinking water at a dosage of 42 mg/L (*25*). Carcinogenicity experiments with hexenal have not been described yet. Organ toxicity was not observed in rats after feeding hexenal in the diet (4000 ppm) for 13 weeks, which corresponds to a total dose of approximately 250 mg/kg body wt (*26*).

2-Alkenals react with cellular nucleophiles, predominantly in a Michael reaction where the β-carbon is the prime site of nucleophilic attack. With glutathione, the respective conjugates are formed predominantly under catalysis by glutathione transferases, a process which mainly leads to detoxification (*24*). With proteins, 1,4-addition products and Schiff's bases are formed, leading to cytotoxicity. Both reaction types were observed in vitro

* Author to whom correspondence should be addressed.
† University of Kaiserslautern.
‡ Federal Research Center for Nutrition, Karlsruhe.
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[1] Abbreviations: hexenal, (*E*)-2-hexenal; ATP, adenosine 5′-triphosphate; body wt, body weight; CrotdG, crotonaldehyde derived 1,N^2-(1,3-propano)dG; CrotdG3′p, crotonaldehyde derived 1,N^2-(1,3-propano)-dG-3′-phosphate; CrotdG5′p, crotonaldehyde derived 1,N^2-(1,3-propano)dG-5′-phosphate; dG, 2′-deoxyguanosine; dG3′p, 2′-deoxyguanosine 3′-phosphate; dG5′p, 2′-deoxyguanosine 5′-phosphate; GC-MS, gas chromatography/mass spectrometry; HexdG, hexenal derived 1,N^2-(1,3-propano)dG; HexdG3′p, hexenal derived 1,N^2-(1,3-propano)dG-3′-phosphate; HexdG5′p, hexenal derived 1,N^2-(1,3-propano)dG-5′-phosphate; LMA, low melting agarose; MD, migration distance; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; PEI-cellulose, poly(ethylenimine)−cellulose; SSB, single-strand breaks.

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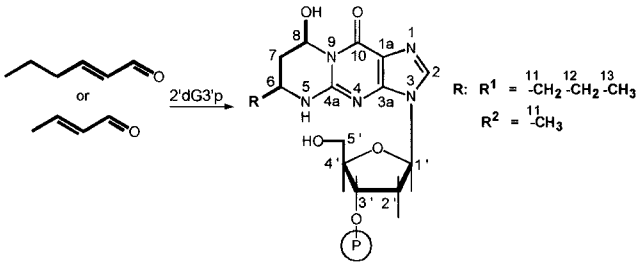
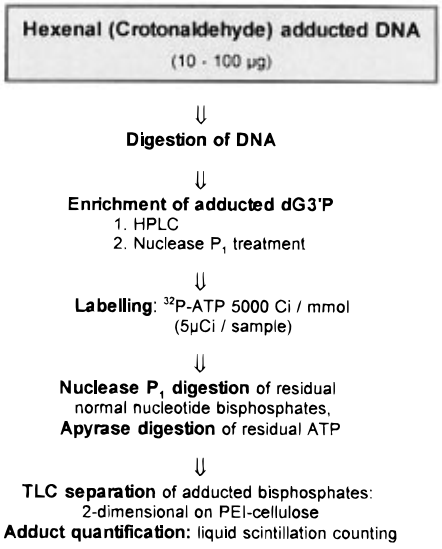
Scheme 1. Formation of 1,N^2-(1,3-Propano)-2′-deoxyguanosine 3′-Phosphate Adducts with Hexenal (R^1) (HexdG3′p) and Crotonaldehyde (R^2) (CrotdG3′p)



Scheme 2. ^{32}P-Postlabeling Assay for 1,N^2-Propanodeoxyguanosine Adducts of Hexenal and Crotonaldehyde (HexdG, CrotdG)



on incubation of hemoglobin with hexenal (*27*). With 2′-deoxyguanosine (dG) and dG-5′-phosphate (dG5′p) hexenal forms cyclic *trans/cis* 1,N^2-(1,3-propano)dG (HexdG) adducts (*28, 29*) by ring closure at the 1 and N^2 positions of guanine from opposite directions as also described for other alkenals (*28, 30, 31*). In general, pairs of diastereomers were found for the respective *trans/cis* adducts, *trans* isomers being preferentially formed. The respective crotonaldehyde 1,N^2-(1,3-propano)dG adducts have been detected in calf thymus DNA (*30*), in chinese hamster ovary cells (*32*), and in vivo in mice skin after topical treatment (*33*). There is some evidence that this adduct is also formed endogenously in the liver of untreated rats (*31*). Cyclic 7,8-dG adducts are also formed from hexenal and crotonaldehyde (*28*).

We here describe the formation of the HexdG adduct (see Scheme 1) in calf thymus DNA and in cells at low hexenal concentrations. A modified ^{32}P-postlabeling method (*34*) was established for sensitive adduct determination (see Scheme 2). The more reactive homologue crotonaldehyde was included for comparison. To further evaluate the genotoxic potential of hexenal, induction of DNA fragmentation was measured with single cell microgel electrophoresis (*35*). Primary gastrointestinal mucosa cells (rat, human) were used in addition to a sensitive human lymphoblastoid cell line. Selected samples of processed foods and flavoring concentrates were analyzed for hexenal in order to obtain more information about the relevant concentration range to be found in various foods.

Materials and Methods

Chemicals. Hexenal (purity 99%) was obtained from Aldrich (Steinheim, FRG), crotonaldehyde (purity > 99.5%) from Fluka (Buchs, Switzerland), and (*E*)-2-octenal from Alfa (Karlsruhe, FRG). **Caution:** *2-Alkenals have been shown to be irritant and genotoxic and therefore should be handled carefully.* 2′-Deoxyguanosine 3′-phosphate and 5′-phosphate (both sodium salts) were purchased from Sigma Chemie (Deisenhofen, FRG), [γ-^{32}P]-ATP triethylammonium salt (specific activity >5000 Ci/mmol) was from Amersham Buchler GmbH (Braunschweig, FRG), TLC plates PEI-cellulose CEL 300 from Macherey & Nagel (Düren, FRG) and scintillation cocktail Aqualuma were from Baker (Gross-Gerau, FRG). Autoradiographic films were X-omat AR (Kodak) and Hyperfilm MP (Amersham). Proteinase K, ribonuclease A type II A, micrococcal nuclease, spleen phosphodiesterase, nuclease P1, and nuclease S1 were provided from Sigma, and cloned T4-polynucleotide kinase was obtained from Amersham. Calf thymus DNA, highly polymerized, was from Sigma, RPMI essential medium with L-glutamine was from Gibco (Eggenstein, FRG), and low and normal melting agarose was from Biozym Diagnostik GmbH (Hess. Oldendorf, FRG). All other chemicals were of analytical grade.

Instrumentation. Single cell electrophoresis was performed with a Biometra electrophoresis unit. GC/FID analysis of hexenal was carried out on a Hewlett Packard HP 5890 Series II with autosampler HP 18596 B.

Synthesis of CrotdG- and HexdG-Phosphates. CrotdG- and HexdG-3′- and -5′-phosphates (CrotG3′p, HexdG3′p, CrotdG5′p, HexdG5′p) were prepared by reacting deoxyguanosine 3′-phosphate and 5′-phosphate (20 mM) with a 5-fold molar excess of alkenal (100 mM) at 80 °C for 24 h in phosphate buffer (0.01 M, pH 7.4) (*36*). Hexenal was predissolved in acetonitrile (15% of incubation mixture). Samples were extracted twice with hexane to eliminate residual aldehyde, evaporated to dryness by vacuum drying, redissolved in water, and submitted to HPLC system 1 (Constametric LDC pump, λ: 254 nm; column: Hibar RP 18, 250 × 10 mm, 7 μm; eluent: 0.02 M ammonium formate, pH 5.4; flow rate: 6 mL/min; sample volume: 1 mL). For separation of HexdGp, acetonitrile (10%) was added to the eluent. Adduct purity was controlled with HPLC system 2 (Hewlett Packard 1050, diode array detection; column: Lichrospher RP-18 select B, 250 × 4 mm, 5 μm; eluent A: 0.02 M ammonium formate, pH 5.4; eluent B: 0.02 M ammonium formate, pH 5.4/acetonitrile (80/20), gradient: 50% B to 80% B in 30 min; flow rate: 0.7 mL/min). Collected adduct fractions were identified by characteristic differential UV absorption (λ_{max}: 259 nm) compared to dG3′p (λ_{max}: 253 nm) and checked by ^{1}H-NMR spectroscopy (*21, 30, 37*). Yields (% of theory) were as follows: CrotdG3′p, 8%; CrotdG5′p, 30%; HexdG3′p, 1%; HexdG5′p, 4%. Identity of nucleotides was confirmed after apyrase induced dephosphorylation: Adduct nucleotides (1 nmol) were incubated in 100 μL of acetate buffer (40 mM, pH 4.5) with 0.2 U of apyrase (grade III, Sigma) at 37 °C for 2 h. Cochromatography of reference nucleosides was performed on HPLC system 2.

Isolation and Digestion of DNA. Cells (Namalva cells, primary rat mucosa cells from colon and stomach) were lysed in EDTA buffer (25 mM EDTA, 75 mM NaCl, pH 8.0) with 1% SDS and 200 μg/mL proteinase K (*38*). After incubating overnight at 55 °C, saturated NaCl solution was added to give a final concentration of 1.5 M. Proteins were extracted by adding 1 volume of chloroform, mixing (60 min), and centrifugation at 10 000 rpm (10 min). DNA was precipitated in the aqueous supernatant by adding 1 volume of 2-propanol and gently mixing (5 min, room temperature). The pellet was washed twice with 70% ethanol and vacuum dried. DNA was dissolved in 250 μL of citrate buffer (150 mM NaCl, 15 mM citric acid, pH 7.4), and residual RNA was digested at 37 °C for 30 min with 40 μg of RNase A type II a (Sigma) (*39*). Saturated NaCl solution was added to a final concentration of 0.6 M. Proteins were extracted by adding 1 volume of phenol/isoamyl alcohol (24/1) and mixing (15 min). DNA was precipitated from the aqueous supernatant with 1 volume of 2-propanol (room temperature), transferred into 70% ethanol, washed twice, and

evaporated to dryness. DNA (10−100 μg) was dissolved in buffer (2.5 mM Tris-HCl, 1.2 mM $CaCl_2$, pH 8.8) (*40*) and digested with micrococcal nuclease (5 U) and spleen phosphodiesterase (0.5 U) in a total volume of 60 μL (9 h at 37 °C).

Adduct Enrichment. Digested DNA was chromatographed on HPLC system 2, and adduct fractions were collected at retention time intervals, known from separate experiments with UV detectable amounts of 5′-nucleotide adducts. Hydrolyzed DNA was quantified using 2′-deoxyadenosine 3′-phosphate as external standard. Adduct fractions were evaporated to dryness and dissolved in 20 μL of water. An aliquot (5 μL) was submitted to digestion by nuclease P1 (10 U in 6 μL of sodium acetate buffer: 100 mM, 55 μM $ZnCl_2$, pH 5.0, 37 °C). After 1 h reaction was stopped with 1 μL of Tris base (0.5 M).

^{32}P-Postlabeling. The sample fraction was incubated (3 h, 37 °C) in 3 μL of labeling buffer (60 mM $MgCl_2$, 60 mM dithiothreitol, 6 mM spermidine, 60 mM Bicine/NaOH, pH 9.5) containing T4 polynucleotide kinase (5 U) and [γ-^{32}P]ATP (specific activity 5000 Ci/mmol; 5 μCi). Subsequently, 4 μL of nuclease P1 mix (200 mM sodium acetate, pH 4.5; 40 mM $MgCl_2$, 5 U nuclease P1) was added. After incubation (1 h, 37 °C) the mixture was reacted with 3 μL of apyrase (5 mU, 0.8 M Tris, pH 8.5; 37 °C, 15 min); an aliquot (10 μL) was submitted to TLC separation on prewashed 10 × 20 cm poly(ethylenimine) (PEI)−cellulose plates (first direction: 0.2 M sodium phosphate buffer, pH 6.5). The upper part of the plate was cut off and the remaining (10 cm) was dried and developed in the second direction (90°) using 2-propanol/water/25% NH_3 (80/58/22). After drying it was autoradiographed with intensifying screen for up to 24 h. Adduct spots were cut out and quantified by liquid scintillation counting. Each ^{32}P-postlabeling assay included one untreated control (untreated DNA, obtained from the same source as the samples and subjected to the whole procedure). Radioactivity of the respective TLC spot area was determined and used to establish the detection limit (2-fold background). Appropriate amounts of synthetic adduct standard, added to untreated controls ($n = 3$) prior to nuclease P1 enrichment, were analyzed to correct for adduct losses.

Incubation of Calf Thymus DNA. Calf thymus DNA (100 μg), dissolved in 200 μL Tris-HCl (0.125 M, pH 7.4), was incubated with hexenal (0.02−2 mM), added in 50 μL DMSO (20 h at 37 °C). The solution was adjusted to 1.5 M NaCl, and DNA was precipitated by addition of 1 volume of 2-propanol and washed twice with 70% ethanol before evaporation to dryness.

Incubation of Namalva Cells. Human lymphoblastoid Namalva cells, originating from a Burkitt lymphoma (*41*), were cultivated at 37 °C in 5% CO_2 atmosphere using RPMI 1640 essential medium with glutamine, supplemented with 5% newborn calf serum and antibiotics. Cell suspensions (10 mL, 2 × 10^6 cells/mL) were incubated with hexenal, predissolved in 100 μL of DMSO, in serum free medium under shaking (37 °C for 1−20 h). Viability was assessed by trypan blue exclusion. After incubation, cells were washed twice with RPMI medium, centrifuged (10 min, 200*g*, 20 °C), and submitted to isolation of DNA.

Isolation and Incubation of Primary Mucosa Cells from Colon, Stomach, and Esophagus. Mucosa cells were isolated according to Burlinson (*42*) with slight modifications (*35*). Colon and stomach were excised, perfused with Ca^{2+}, Mg^{2+} free PBS for 5 min, filled with Ca^{2+} and Mg^{2+} free HBSS containing 50 U/mL protease K, and incubated at 37 °C for 30 (colon) or 45 min (stomach). Then the organs were opened and cells were isolated by gentle agitation. The suspensions were centrifuged at 800 rpm (colon) or 500 rpm (stomach); and the pellet was resuspended in 10 mL of RPMI 1640 and adjusted to 2 × 10^6 cells/mL. Esophagus was directly filled with proteinase K solution and incubated 30 min at 37 °C. Then isolated cells were centrifuged at 800 rpm and resuspended in 1 mL of RPMI. Human colon mucosa biopsy samples were incubated for 30 min at 37 °C with HBSS containing 50 U/mL protease K. Cell suspensions were centrifuged and adjusted to 1 × 10^5 cells/mL in RPMI 1640 (*43*). Viability of cells, as determined by trypan blue exclusion, exceeded 80%.

Test compounds, dissolved in DMSO, were added to the cell suspension to give a final DMSO concentration of 1%. The positive control *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) was dissolved in 0.7% NaCl. Colon and esophagus cells were incubated at 37 °C for 30 min, gastric mucosa cells for 45 min. Aliquots were checked for viability by trypan blue exclusion. For postlabeling, cells were washed twice with RPMI and submitted to isolation of DNA. For microgel electrophoresis, cells were centrifuged and resuspended to 2 × 10^5 cells in 10 μL.

In Vivo Experiments. Male Sprague-Dawley rats (6−8 weeks old) were gavaged with hexenal (40−320 mg/kg body wt), dissolved in corn oil. MNNG (5 mg/kg body wt in 0.9% NaCl solution) was used as positive control, corn oil as solvent control. Rats were sacrificed 1 and 16 h after treatment for preparation of stomach and colon, respectively. Mucosa cells were isolated as described above and submitted to ^{32}P-postlabeling and to microgel electrophoresis.

Microgel Electrophoresis. The assay was performed basically according to Singh et al. (*44*). Cell suspensions (10 μL) were mixed with 65 μL of 0.7% low melting agarose (LMA) and distributed on slides covered with 0.5% normal melting agarose (*45*). After addition of a third layer of LMA, cells were lysed at pH 10 and subjected to electrophoresis at 25 V/300 mA under alkaline conditions. Then DNA was stained with ethidium bromide, and migration distance (MD) of DNA (comet) was determined by image analysis. Cells were classified according to their image length as undamaged (<40 μm), moderately damaged (40−80 μm), and severely damaged (>80 μm) (*35*). A total of 100 cells/slide (2−3 slides/experimental point) was evaluated. The proportion of damaged to undamaged cells was expressed in % of total.

Determination of Hexenal in Food and Flavoring Concentrates. Samples (food: 20 g; flavor concentrates: 20 mg) were homogenized/diluted with varying amounts of water, and hexenal was extracted into hexane. After centrifugation, the organic layer was directly submitted to GC/FID (capillary column: DB Wax: 50 m × 0.32 mm × 1 μm; Macherey & Nagel) using (*E*)-2-octenal as internal standard. Recovery ranged about 90%, and the detection limit varied from 0.2 to 0.5 ppm. Positive results were confirmed by GC-MS.

Results

To investigate the formation of hexenal adducts in DNA, a modified ^{32}P-postlabeling method was developed. Generation of the respective crotonaldehyde adduct was included for comparison. Reference nucleotides obtained from reaction of aldehyde with dG-3′- and -5′p (CrotdG3′p; CrotdG5′p; HexdG3′p; HexdG5′p) showed the expected pairs of signals indicative for mixtures of diastereomers for each adduct. dG3′p adducts were used as external standard to control the ^{32}P-postlabeling procedure (see Scheme 2): After enzymatic digestion of DNA into 3′-nucleotides, the sample was submitted to adduct enrichment. HPLC separation of adducted 3′-nucleotides was achieved on RP-18, using an ammonium formate/acetonitrile gradient and the respective 5′p adducts as UV markers. The collected adduct fraction containing both isomers was incubated with nuclease P1 to further reduce the amount of normal nucleotides (*34*). This two-step adduct enrichment allowed subsequent ^{32}P-postlabeling under ATP excess without addition of nonlabeled ATP. Control experiments showed a maximal adduct-labeling efficiency of about 60%. After labeling, samples were treated again with nuclease P1 to dephosphorylate traces of unmodified nucleotides, while the adducted bisphosphates remain unchanged. This results in a better adduct separation on PEI-cellulose; adduct isomers, however, are not separated under the described conditions. Adduct spots were identified by autoradiography, cut out, and quantified by liquid scintillation counting in comparison to untreated and adduct-spiked control samples. The detection limit for hexdG3′p was approximately 1 adduct/5 × 10^7 nucleotides. For CrotdG3′p

Table 1. Formation of 1,N^2-Propanodeoxyguanosine Adducts in Calf Thymus DNA by Hexenal (HexdG) and Crotonaldehyde (CrotdG)[a]

aldehyde (mM)	incubation time (h)	HexdG (pmol/μmol of DNA)	CrotdG (pmol/μmol of DNA)
0.02	20	0.36*	n/a
0.2	2	0.30 ± 0.16	n/a
	5	1.55 ± 0.89	13.9*
	20	6.9 ± 2.6	n/a
2	2	8.9 ± 0.7	n/a
	5	9.2 ± 3.0	72.6 ± 6.6
	20	16.8*	n/a

[a] n/a = not analyzed. Means and ranges were obtained from 2 experiments except for (*): $n = 1$. After incubation (37 °C; 100 μg of DNA/250 μL of incubation mixture) and digestion of DNA an aliquot ≅20 μg of DNA was submitted to ^{32}P-postlabeling.

Table 2. Formation of 1,N^2-Propanodeoxyguanosine Adducts (HexdG) in Namalva Cells by Hexenal[a]

hexenal (mM)	incubation time (h)	viability (%)	HexdG (fmol/μmol of DNA)
0		86 ± 4	
0.2	1	88	86 ± 3
	3	85	211 ± 122
	5	81	135 ± 33
	20	44	72 ± 26
0.4	1	80	171 ± 43
	3	65	297 ± 50
	5	30	n/a

[a] n/a = not analyzed. Means and ranges were obtained from 2−4 experiments. Incubation: 37 °C, 2 × 10^7 cells/10 mL of incubation mixture.

it was approximately 1 adduct/10^6 nucleotides because of higher background of untreated control in the respective TLC region.

Results of adduct formation experiments in calf thymus DNA (Table 1) show that adduct levels increased with incubation time at 0.2 mM hexenal, but not at 2 mM. Crotonaldehyde induced about 1 order of magnitude more DNA adducts than hexenal. At high aldehyde exposure incomplete hydrolysis due to strongly modified DNA might lead to underestimation of adduct level.

Incubations of RNA with hexenal had shown that adducted nucleotides are not separated from the respective DNA adducts during enrichment, labeling, and TLC separation. Thus, in experiments with cells, RNase treatment is required before hydrolysis of DNA. Incubation of Namalva cells with 0.2 and 0.4 mM hexenal for 1 h resulted in mean adduct levels of 86 and 171 fmol/μmol of DNA, respectively (Table 2). Under these conditions, viability of cells was not significantly reduced, compared to the control. Higher concentrations or prolonged incubation at 0.4 mM hexenal, however, exerted cytotoxic effects with relative viabilities below 70%. Incubation with 50 μM hexenal (5 h) indicated minute adduct formation, below the determination limit.

Incubation of colon mucosa cells for 30 min with 0.4 and 0.8 mM hexenal induced detectable adducts (see Table 3). As an example, the autoradiogram resulting from 0.8 mM incubation is shown in Figure 1C, in comparison to untreated control (Figure 1A) and control spiked with adducted standard (Figure 1B). In gastric mucosa cells incubated for 1 h with hexenal (up to 1.2 mM) adducts were not detectable, however. Autoradiograms from these cells showed a background spot in the adduct area, which compromised the detection limit. The structure of this background adduct cannot be identified. The spot might, however, well originate from endogenous sources. 1,N^2-Propano-dG adducts from other alkenals (acrolein, crotonaldehyde) have been detected in DNA from untreated rodents and humans. Induction of DNA damage, assayed by single cell electrophoresis, was investigated in parallel. Results are expressed as ratio of undamaged cells in % of total (Figure 2). Rat colon mucosa cells (Figure 2A) were sensitive toward hexenal and crotonaldehyde. Incubation with both aldehydes (0.4 mM for 30 min) clearly induced genotoxic effects (DNA

Table 3. Formation of 1,N^2-Propanodeoxyguanosine Adducts (HexdG) in Primary Rat Colon Cells by Hexenal

hexenal (mM)	viability (%)	HexdG (fmol/μmol of DNA)
0.2	83 ± 5	n/d
0.4	87 ± 2	50 ± 13
0.8*	80 ± 10	162 ± 33

[a] n/d = not detectable (<20 fmol/μmol of DNA, corresponding to 1 adduct/5 × 10^7 nucleotides); (*): autoradiogram shown in Figure 1. Means and ranges were obtained from two independent experiments. Incubation: 30 min, 37 °C, 2 × 10^6 cells/mL.

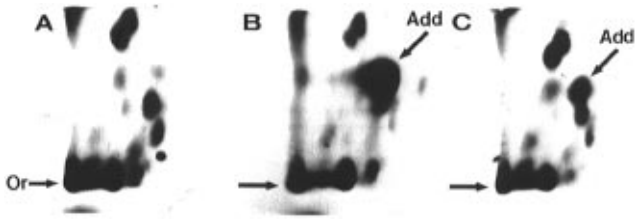


Figure 1. Detection of HexdG in primary rat colon mucosa cells. Autoradiograms of two-dimensional TLC separations. (A) Untreated control. (B) Untreated control, fortified with adduct standard (34 fmol of HexdG3′p) prior to ^{32}P-postlabeling. (C) Sample: 2 × 10^6 cells/mL incubated with hexenal (0.8 mM) for 30 min at 37 °C (see Table 3). The whole sample was submitted to adduct determination by ^{32}P-postlabeling. Or: origin. Add: HexdG-3′,5′-bisphosphate. Autoradiography: 20 h at 22 °C, Amersham Hyperfilm MP, with intensifying screens.

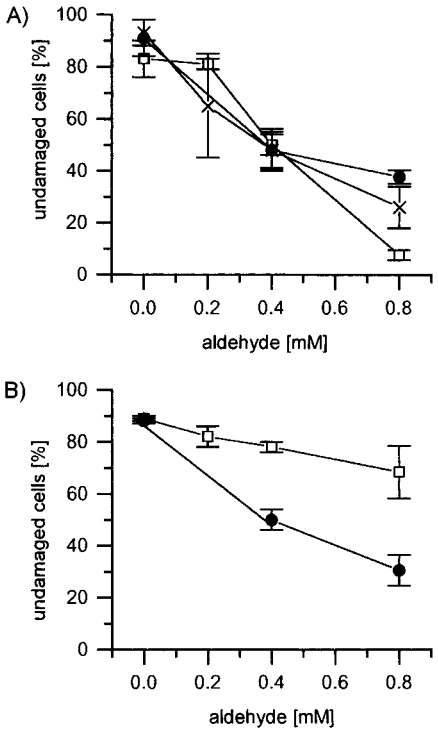


Figure 2. Concentration dependent induction of DNA damage with hexenal and crotonaldehyde in primary rat and human cells. (A) Primary rat colon mucosa cells: hexenal (□) and crotonaldehyde (●); human colon biopsy samples: hexenal (×). (B) Primary rat stomach mucosa cells: hexenal (□) and crotonaldehyde (●). Means and SD were obtained from 600 cells of 2 independent experiments, each experiment performed in triplicate (100 cells/slide; 3 slides/experiment). Viability of cells >80%; undamaged cells: MD <40 μm.

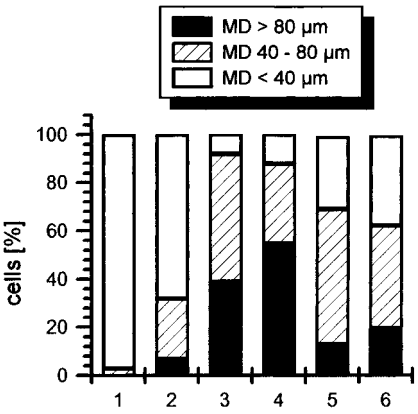


Figure 3. DNA damage with hexenal and crotonaldehyde (both 0.8 mM) in primary rat mucosa cells of different tissues. Ratios of undamaged cells (MD <40 μm), moderately damaged cells (MD 40−80 μm), and severely damaged cells (MD >80 μm) are given in percent of total. Bars: (1) untreated control, esophagus, $n = 1$*; (2) stomach: hexenal, $n = 6$; (3) colon: hexenal, $n = 6$; (4) esophagus: hexenal, $n = 1$*; (5) stomach: crotonaldehyde, $n = 6$; (6) colon: crotonaldehyde, $n = 6$; n = number of pooled slides from repetitive experiments. (*) Preliminary pilot experiment. Viability of cells >80%.

damage in more than 40% of cells). In primary human colon mucosa cells hexenal was as potent as in rat colon cells. In rat stomach mucosa cells (Figure 2B) hexenal was only weakly effective (DNA damage in 20% of cells at 0.8 mM) whereas crotonaldehyde was equally active in both cell types. Effects of hexenal and crotonaldehyde at 0.8 mM in rat tissues are presented in detail in Figure 3. The percentage of undamaged cells (MD <40 μm) after crotonaldehyde treatment (bars 5 and 6) is significantly different from that obtained with hexenal (bars 2 and 3) in the corresponding rat tissues (Student's T test, $p < 0.01$). A pilot experiment with primary mucosa cells from rat esophagus, included for comparison, suggests comparable sensitivity toward hexenal.

After oral application of hexenal to rats, DNA damage was not detectable in mucosa cells of stomach (160 mg/kg body wt) and colon (320 mg/kg body wt) whereas the positive control MNNG (5 mg/kg body wt) was clearly effective in both tissues.

GC analysis of 26 selected foods and flavoring concentrates showed hexenal in substantial amounts in banana (42 ppm), endive (27 ppm), kiwi−apple fruit preparation (7 ppm), alcoholic beverage with apple taste (14 and 1.9 ppm), and apple flavor concentrate (11 400 ppm). Thus, the hexenal concentrations in apple−kiwi fruit preparation and in the alcoholic beverages with apple taste were comparable to the natural occurrence in some fruits or vegetables such as banana or endive. In other food samples such as baby foods, milk products, and alcoholic and nonalcoholic drinks hexenal was not detectable. One apple flavor concentrate contained more than 1% hexenal, while a sample of banana flavor concentrate tested in parallel was negative.

Discussion

To evaluate the potency of hexenal to interact with DNA, we focused on cyclic 1,N^2-propano-dG adducts as it was known that this adduct type, formed in vivo from acrolein and crotonaldehyde (*33, 40*), induces mutations. With the model adduct, primarily 1,N^2-propano-dG G → T transversions were observed in *Escherichia coli* and simian kidney cells (COS) (*46−48*). In certain sequences 1,N^2-propano-dG also induces frame shift mutations (*49*). Since hexenal is less reactive than the shorter chain homologues, a sensitive detection method was required. The ^{32}P-postlabeling procedure was therefore modified accordingly. A two-step adduct enrichment by reversed-phase HPLC and nuclease P1 treatment was found appropriate; adducted bisphosphates were resistant to dephosphorylation (*34*). TLC separation, a critical step for small adducts, was facilitated by a second nuclease P1 treatment after the ^{32}P-postlabeling step. A similar approach has recently been published for cyclic adducts of acrolein and crotonaldehyde (*31*).

Adduct formation by hexenal was observed in Namalva cells, a human lymphoblastoid cell line poor in glutathione, glutathione *S*-transferase, and detoxifying enzymes (*24, 50*). Hexenal has been shown earlier to induce DNA single-strand breaks (SSB) in Namalva cells (*24*). As hexenal is mainly ingested with food, primary gastric, colon, and esophagus mucosa cells were examined for DNA adduct formation and DNA damage (comet assay). The comet assay has been found more appropriate for mucosa cells than alkaline elution, since a smaller number of cells is required and secretion of mucous material causes fewer interferences. Adduct levels in primary rat colon cells, extrapolated to an incubation time of 1 h, were about 50% those found for Namalva cells. This might be due to a higher capacity of colon cells for detoxification by quenching reactions with noncritical cellular nucleophiles or by deactivating biotransformation, as described earlier for primary hepatocytes (*24*). It is not clear why primary gastric mucosa cells were only weakly sensitive toward hexenal but highly sensitive to crotonaldehyde. Substrate specific differences in uptake or detoxification might play a role.

Depending on the respective aldehyde and also on cell type, the in vitro results show distinctly differential sensitivity with regard to DNA interactions. Similar differences have also been described for mutagenicity of acrolein in mammalian cells (*32*). Significant induction of DNA damage was only observed at alkenal concentrations rather close to the cytotoxic concentration limit, as evidenced by a reduction in the percentage of surviving cells. This is in line with in vitro mutagenicity experiments in chinese hamster lung V79 cells with homologous alkenals (*22*). Hexenal and crotonaldehyde (5−250 μM) have shown concentration dependent induction of micronuclei, sister chromatid exchanges, and numerical aberrations in Namalva cells and also in human blood lymphocytes (*23*). Hexenal has been found to be more potent than crotonaldehyde in induction of numerical chromosomal aberrations and inhibition of tubulin polymerization (*23, 51*).

The negative results of the in vivo experiments with hexenal in rats need further investigation. It is not clear whether the time interval between application and isolation of cells (gastric mucosa: 1 h; colon: 16 h) was appropriate. These time points had been shown to be suitable for MNNG in gastric and colon, respectively (*35*), but hexenal may require different exposure lengths. Moreover, a single dose experiment might not reflect effects of chronic exposure. Organs such as the liver, the target organ of crotonaldehyde carcinogenicity, might be more relevant. In volunteers, exposure to hexenal (mouth washing) indicated cytogenetic effects in human oral mucosa cells.[2]

[2] Dittberner et al., unpublished results.

Human exogenous exposure to hexenal mainly results from food. In addition to its natural occurrence in a variety of fruits and vegetables, hexenal is widely used as a flavoring agent. Potential adduct formation by hexenal in vivo needs further elucidation, also with respect to the endogenous adduct burden resulting from potential in vivo formation of hexenal during lipid peroxidation. The respective crotonaldehyde adducts have been detected in liver DNA of untreated rodents and humans (*52*). Hexenal has been identified as lipid peroxidation product in the liver of bromobenzene-poisoned mice (*53*). Our in vitro results show that hexenal forms DNA adducts and induces DNA damage in human lymphoblastoid cells and in colon mucosa cells from rats and humans. Appropriate in vivo studies are required to evaluate the risk of chronic hexenal exposure in food.

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