The Influence of Glutathione and Detoxifying Enzymes on DNA Damage Induced by 2-Alkenals in Primary Rat Hepatocytes and Human Lymphoblastoid Cells

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The reaction of 2-alkenals with GSH to form GSH conjugates by Michael addition is a major detoxification pathway. The reaction proceeds at a much higher rate under catalysis by glutathione *S*-transferase (GST) than the non-enzymatic reaction. Oxidation of 2-alkenals to the corresponding acids by cytosolic and microsomal fraction of rat liver also contributes to detoxification. Primary rat hepatocytes rich in GSH and proficient for GST and other metabolizing enzymes consume much more alkenal than human lymphoblastoid cells (Namalva cells), that are poor in GSH and in metabolic activities. In Namalva cells DNA single strand breaks were induced by much lower concentrations of acrolein, crotonaldehyde and *(E)*-2-hexenal than in primary rat hepatocytes. In both cell systems intracellular GSH depletion by 2-alkenals proceeds in a dose dependent manner, approaching about 20% of pretreatment level before DNA damage becomes detectable. GSH conjugates of *(E)*-2-hexenal and *(2E,6Z)*-2,6-nonadienal induce DNA damage in Namalva cells at high concentrations (1.5 mM). In the absence of GSH these conjugates decompose slowly into aldehyde and GSH. Although the rate of decomposition is only about 10^{-4} times that of Michael adduct formation, such GSH conjugates could potentially function as transport molecules for 2-alkenals, if they reach tissues low in GSH and GST.

Introduction

2-Alkenals occur naturally in a wide variety of foods *(1–3)*. Especially longer chain homologues like *(E)*-2-hexenal and *(2E,6Z)*-2,6-nonadienal play an important role as flavoring substances. Acrolein and crotonaldehyde occur as combustion byproducts and are found in tobacco smoke and automobile exhaust *(4)*. Acrolein is also formed in vivo as a metabolite of the antitumor agent cyclophosphamide.

Acrolein, crotonaldehyde, and hexenal act as bacterial mutagens in *Salmonella typhimurium* TA100 and TA104 *(5, 6)*. 2-Alkenals also show mutagenicity in mammalian V79 cells by induction of 6-thioguanine resistence *(7)*. In a long-term study (2 years) acrolein administered orally at relatively low dosage (0–2.5 mg/kg per day) showed no carcinogenic effects in rats *(8)*. Induction of urinary bladder tumors was observed in rats after ip application of acrolein (2 mg/kg) twice weekly for 6 weeks, followed by uracil treatment *(9)*. Crotonaldehyde, given in drinking water at a dosage of 42 mg/L induced liver tumors in F 344 rats *(10)*.

Because of their potential to form Michael adducts, 2-alkenals are directly acting genotoxic agents. Several types of adducts with DNA bases have been characterized *(11, 12)*. Biotranformations and quenching reactions with noncritical cellular nucleophiles that result in loss of Michael activity should lead to detoxification. Several detoxifying enzymes have been found to be involved in metabolism of 2-alkenals. Aldehyde dehydrogenase (ALDH)[1] oxidizes hexenal to the corresponding acid that is no longer genotoxic *(13)*. Another important pathway results from conjugation to GSH. A mercapturic acid metabolite was found in urine of rats treated with crotonaldehyde and acrolein *(14, 15)*. Conjugation with GSH might occur spontaneously but is also subject to catalysis by glutathione *S*-transferase (GST) *(16, 17)*. In isolated rat hepatocytes acrolein has been found to induce GSH depletion *(18, 19)*.

We here report results of a detailed study on the reactions of 2-alkenals with GSH, the characterization of GSH conjugates, and the influence of GSH interaction on DNA damage. We were interested in clarifying to what extent reaction with noncritical cellular nucleophiles and enzymatic detoxification might contribute to protect DNA from alkenal-induced damage. Induction of DNA single strand breaks (SSB) by alkenals was therefore studied in Namalva cells, a human lymphoblastoid cell line poor in deactivating enzymes and low in GSH and GST activity, in comparison to primary rat hepatocytes. Furthermore, GSH conjugates were tested for their stability under physiological conditions and their propensity to dissociate back into GSH and the alkenal.

Materials and Methods

Chemicals. Acrolein (>97%) was obtained from Janssen (Neuss, Germany), crotonaldehyde (>99.5%) from Fluka (Buchs, Switzerland), *(E)*-2-hexenal (>99%) from Aldrich (Steinheim, Germany), *(2E,6Z)*-2,6-nonadienal from Alfa (Danvers, MA). Collagenase P was obtained from Boehringer (Mannheim, Germany), and equine liver glutathione *S*-transferase was obtained from Sigma (Deisenhofen, Germany). RPMI 1640 essential medium with glutamine and newborn calf serum were obtained from Gibco (Eggenstein, Germany). Hypnorm was

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[1] Abbreviations: ALDH, aldehyde dehydrogenase; GST, glutathione *S*-transferase; SSB, single strand breaks; FAB/MS, fast atom bombardment mass spectrometry; HBSS, Hank's balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; ADH, alcohol dehydrogenase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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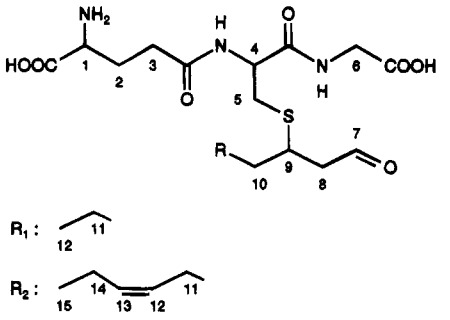
Chart 1. Structures of GSH Conjugates with Hexenal (R_1) and Nonadienal (R_2)



purchased from Janssen (Neuss, Germany). All other chemicals were of analytical grade.

Instrumentation. NMR spectra were obtained on a Bruker spectrometer at 400 MHz. Radioactivity was determined with a Betamatik I 3380 (Kontron Packard Tri-Carb). GC analyses were carried out with a Carlo-Erba 250 with head space sampler. Cell suspensions were frozen in liquid nitrogen and disrupted with a stainless steal ball in a "microdismembrator" (Braun Melsungen, FRG).

Synthesis of *S*-[3-(1-Oxohexyl)]-GSH (Chart 1). Hexenal (2.7 mmol) was dissolved in 15 mL of methanol and added to a solution of 830 mg of GSH (2.7 mmol) in 15 mL of water. After incubation at room temperature for 18 h the solution was extracted with *n*-pentane to remove unreacted hexenal. The mixture was evaporated to dryness, redissolved in 8 mL of water, and separated by HPLC on RP-18 (Lichrosorb, 7 μm, 250 × 20 mm) with methanol/water (40:60) by UV detection at 220 nm. The pure conjugate fraction was collected and lyophilized to form a white hygroscopic powder. Elemental analysis: C 47.6, H 6.71, N 10.36 (calcd C 47.6, H 6.6, N 10.2); ^{1}H-NMR (D_2O): δ 0.91 (m, 3H, H12), 1.43 (m, 2H, H11), 1.58 (m, 2H, H10), 1.75–1.85 (m, 2H, H8), 2.18 (m, 2H, H2), 2.56 (m, 2H, H3), 2.86 (m, 2H, H5), 3.06 (m, 1H, H9), 3.83 (t, 1H, H1), 3.98 (s, 2H, H6), 4.57 (m, 1H, H4), 9.62 (d, 1H, H7); FAB/MS: MH^+ 407, MH^+ − Glu 276.

Derivative formation *(20)*: GSH conjugate (200 μg), dissolved in 700 μL of 0.1 M borate buffer (pH 8), was added to 0.1 mg of fluorescamine, dissolved in 200 μL of acetone. The fluorescamine derivative was submitted to HPLC [RP 18; eluent: acetonitrile/0.1 M citrate buffer (pH 3) (1:1.5), excitation 390 nm, emission 475 nm].

Synthesis of *S*-[3-[1-Oxonon-(*Z*)-6-enyl]]-GSH (Chart 1). Nonadienal (2.9 mmol) was dissolved in 30 mL of methanol and added to a solution of 800 mg of GSH (2.6 mmol) in 7 mL of H_2O. After incubation, the conjugate was isolated as described, using methanol/water (50:50) as mobile phase for HPLC. Elemental analysis: C 51.2, H 6.3, N 9.4 (calcd C 49.9, H 6.6, N 9.1); ^{1}H-NMR (D_2O): δ 0.99 (t, 3H, H15), 1.71 (m, 2H, H10), 1.8 (m, 2H, H8), 2.10 (m, 2H, H2), 2.26 (m, 4H, H14/H11), 2.63 (m, 2H, H3), 2.91 (m, 2H, H5), 3.07 (dd, 1H, H9), 4.05 (s, 2H, H6), 4.07 (m, 1H, H1), 4.61 (m, 1H, H4), 5.44 (m, 1H, H13), 5.52 (m, 1H, H12), 9.7 (s, 1H, H7); FAB/MS: MH^+ 447, MH^+ − Glu − Gly − CO 215. Derivatization of the GSH conjugate with fluram was carried out as described above with the HPLC eluent acetonitrile/0.1 M citrate buffer (pH 3) (1:1).

Primary Hepatocytes. Male Wistar rats (200 g) were anesthesized by Hypnorm (1.2 mL/kg body wt, ip), the peritoneal cavity was opened, and a cannula was introduced into the vena portae. Liver perfusion: Solution A for 2 min at 10 mL/min and for 8 min at 20 mL/min, followed by 5 min solution B at 20 mL/min.The vena jugularis was incised to release the liquid. The liver was removed and disintegrated in solution C, and the hepatocytes were filtered through gauze into 5 mL newborn calf serum. After 10 min, hepatocytes were spun down at 20*g* (300 rpm) for 7 min (2 °C). The pellet was resuspended in RPMI, and viability was determined by trypan blue exclusion. Preparations were only used if viability exceeded 70%.

Solution A: 300 mL of Hank's balanced salt solution (HBSS) [8 g/L NaCl, 0.4 g/L KCl, 0.05 g/L Na_2HPO_4, 0.06 g/L KH_2PO_4, 0.35 g/L $NaHCO_3$,1 g/L glucose, 4.8 g/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4 with NaOH)] mixed with 3 mL of [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) (50 mM). Solution B: 100 mL of HBSS mixed with 0.5 mL of $CaCl_2$ solution (1 M) and 20 mg of collagenase P. Solution C: 300 mL of HBSS mixed with 0.5 mL of $CaCl_2$ solution (1 M).

Namalva Cells. Namalva cells, a human lymphoblastoid cell line, originate from a Burkitt lymphoma. They were cultivated at 37 °C in 5% CO_2 atmosphere using RPMI 1640 essential medium with glutamine, 5% newborn calf serum, and gentamycin (0.1 mg/mL).

Determination of Enzyme Activities. Cytosolic fraction was prepared from Namalva cells and rat hepatocytes as described earlier *(21)*. Briefly, pottered hepatocytes and Namalva cells, disrupted in a dismembrator, were centrifuged stepwise up to 9000*g*. The supernatant was centrifuged at 100000*g* for 75 min to obtain the cytosolic fraction.

Alcohol dehydrogenase (ADH) activity: Ethanol (0.5 mM) and NAD^+ (4 mM) were preincubated in Tris buffer (0.1 M; pH 8.5) at 25 °C. The reaction was started by addition of cytosol (0.1 g of protein/L), and NADH formation was followed spectrometrically at 340 nm for 4 min.

ALDH activity: Propionaldehyde (2.5 mM), NAD^+ (6.25 mM), and pyrazole (0.5 mM) were preincubated in phosphate buffer (0.02 mM; pH 8.5) at 25 °C. After addition of cytosol (0.1 g of protein/L) NADH formation was followed at 340 nm for 7 min *(22)*.

GST activity: GSH (32.2 μM) and 1-chloro-2,4-dinitrobenzene (32.2 μM) were preincubated in potassium phosphate buffer (0.1 M; pH 6.5) at 25 °C. The reaction was started by addition of cytosol (60 mg of protein/L). Extinction was determined at 340 nm *(23)*.

Effects of Alkenals on Cellular GSH. Cells (2×10^7) were incubated in RPMI medium for 1 h at 37 °C with aldehyde dissolved in 10 μL of Me_2SO. Controls were treated with Me_2SO alone. Thereafter, cells were centrifuged (10 min at 280*g*), and the pellet was resuspended in 2.5 mL of phosphate buffer (0.1 M, pH 7.2). Trichloroacetic acid (10%, 0.5 mL) was added, and the suspension was centrifuged for 18 min (4 °C, 100000*g*). GSH was determined photometrically in the supernatant (cytosol) with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). Cytosol (0.4 mL) was mixed with 1.6 mL of sodium phosphate buffer (0.3 M, pH 9), 0.2 mL of EDTA buffer (34.4 mM EDTA in 0.1 M potassium phosphate buffer, pH 7.6), and 0.1 mL of DTNB solution (1.01 mM in 1% sodium citrate). Extinction was measured at 412 nm.

Aldehyde Consumption by Cells. Cells (2×10^6/mL) in RPMI medium were incubated with aldehyde (predissolved in Me_2SO) in gas tight vials (1 h, 37 °C). Aldehyde concentration was measured by head space GC. Calibration runs were carried out with aldehyde incubated in RPMI medium without cells. GC separation: Column: diphenyl (86%)–cyanopropylmethyl (14%)–polysiloxane DB1701 at 100 °C, for nonadienal at 120 °C. Temperature of sample vial: 37 °C; temperature of injection needle: 47 °C; detector: flame ionization.

Labeling of Cellular Components by [^{14}C]Hexenal. Hepatocytes (2×10^7 cells) were incubated with [2-^{14}C]hexenal (1, 3, 5 mM; specific activity 0.6 mCi/mmol) for 1 h at 37 °C. After centrifugation (10 min, 200*g*) the pellet was washed twice with phosphate buffer (25 mM, pH 7.4) and resuspended in 25 mL of Tris buffer (5 mM Tris, 0.5 mM $MgCl_2$, 5 mM NaCl, pH 7.5). Triton X-100 (1%) was added, the sample was centrifuged for 10 min, and the pellet was resuspended in 25 mL of Tris buffer (10 mM Tris, 10 mM $MgCl_2$, pH 7.3).

RNA isolation: Proteins were extracted twice with the same volume of chloroform/isoamyl alcohol (24:1) for 5 min. After centrifugation the water phase was submitted to CsCl gradient centrifugation [CsCl: optical densitiy 1.390 in Tris buffer (10 mM Tris, 1 mM Na_2EDTA, pH 8.0)] for 50 h at 15 °C (100000*g*).

The RNA pellet was resuspended in Tris buffer (10 mM Tris, 10 mM $MgCl_2$, pH 7.3) and quantified with orcinol.

DNA isolation: Proteinase K was added (500 μg/mL) to the resuspended pellet in Tris–$MgCl_2$ buffer and incubated for 90 min at 37 °C in a shaking water bath. The solution was extracted twice with the same volume of chloroform/isoamyl alcohol/phenol (24:1:25) and centrifuged. After addition of twice the volume of ice cold ethanol, DNA was precipitated. DNA was collected on a polycarbonate filter (2 μm) and redissolved in 3 mL of Tris–$MgCl_2$ buffer by sonication (30 min). The amount of DNA was determined with diphenylamine *(24)*.

Proteins were precipitated from the chloroform/isoamyl alcohol extract with the same volume of acetone and quantified according to Lowry *(25)*. Radioactivity was determined by liquid scintillation counting.

Oxidation and Reduction of 2-Alkenals and 2-Alkenols by Rat Liver Cytosol and Microsomes. The cytosolic and microsomal fractions of rat liver were prepared as described earlier *(21)*. Rat liver fractions were incubated with cofactors and alkenal or alkenol at 37 °C. Formation or decrease of NADH was followed photometrically at 340 nm for 5 min. Oxidation of 2-alkenols by rat liver cytosol: The alkenol predissolved in acetonitrile (5 μL) was added to a solution (5 mL) of glycine (10 mM) phosphate (75 mM) buffer (pH 9), cytosol (7 mg of protein/mL), and NAD^+ (2 mM). Reduction of 2-alkenals by rat liver cytosol: The alkenal predissolved in acetonitrile (5 μL) was added to a solution (5 mL) of glycine (10 mM) phosphate (75 mM) buffer (pH 9), cytosol (7 mg of protein/mL), and NADH (2 mM). Oxidation of 2-alkenals by rat liver cytosol: The alkenal predissolved in Me_2SO (10 μL) was added to a solution of Tris-HCl (0.1 mM, pH 8), cytosol (8 mg of protein/mL), and NAD^+ (2 mM). Oxidation of 2-alkenals by rat liver microsomes: The alkenal predissolved in Me_2SO (10 μL) was added to a solution of phosphate buffer (70 mM, pH 7.4), microsomes (2 mg of protein/mL), $MgCl_2$ (20 mM), and $NADP^+$ (2 mM).

The formation of alkenol and alkenal was confirmed by head space GC: Column: DB1701 [diphenyl (86%)–cyanopropylmethyl (14%)–polysiloxane]; water bath: 80 °C; oven temperature: crotonaldehyde/crotyl alcohol 55–80 °C with 20 °C/min, hexenol/hexenal 70 °C, nonadienol/nonadienal 100 °C.

The formation of alkenoic acid was determined by GC. After addition of HCl the incubation mixture was extracted with diethyl ether and centrifuged and the extract submitted to GC. Column: WG 11 polyethylene glycol esterified with nitroterephthalic acid, oven temperature 180 °C.

Reaction of 2-Alkenals with GSH. Reaction in the absence of GST: GSH (500 μM) was incubated with aldehydes (30–100 μM) in phosphate buffer (0.1 M; 0.1 mM EDTA; pH 7.2) at 25 °C. Aldehyde consumption was followed photometrically at 230 nm. For confirmation reaction rates were determined by head space GC (500 μM GSH, 700 μM aldehyde). Reaction in the presence of GST: The activity of GST from equine liver was calibrated with 1-chloro-2,4-dinitrobenzene as substrate. GST (1.5 units) was incubated with GSH (1 mM) for 5 min at 25 °C in phosphate buffer (0.1 M, pH 7.2). The reaction was started by the addition of aldehyde (10–70 μM) and followed photometrically at 230 nm. The conditions were selected to obtain a reaction rate 2–3 times that of the nonenzymatic reaction. The results were corrected for the nonenzymatic reaction rate.

GSH conjugate decomposition: The release of GSH from the GSH conjugates was determined with Ellman's reagent (39.6 mg of DTNB and 15 mg of $NaHCO_3$ in 10 mL of phosphate buffer, 0.1 M, pH 7.2). GSH (180 μM) was incubated with the aldehyde (150 μM) in 2.8 mL of phosphate buffer until the reaction reached equilibrium. Ellman's reagent (100 μL) was added to scavenge residual free GSH. The decomposition of the GSH conjugate was followed at 230 nm (extinction of aldehyde) and 412 nm (extinction of GSH). The rates of decomposition of the hexenal and nonadienal conjugate were independently determined by direct incubation of the conjugates (1 mM) at 25 and 37 °C, following aldehyde liberation by head space GC.

Table 1. Formation and Decomposition of GSH Conjugates with 2-Alkenals at 25 °C (pH 7.2)

alkenal	k_1 ($mol^{-1} \times s^{-1} \times L$)[a]	k_2 (s^{-1})[b]	$t_2(1/2)$ (h)[c]
acrolein	120	–[d]	–
crotonaldehyde	0.72	2.8×10^{-5}	7
hexenal	0.34	2.8×10^{-5}	7
octenal	0.28	3.2×10^{-5}	6.5
nonadienal	0.57	5.5×10^{-5}	3.5

[a] k_1 (rate constant of formation) was determined with excess of GSH. [b] k_2 (rate constant of decomposition) was determined after GSH had been scavenged from the equilibrium with Ellman's reagent. [c] $t_2(1/2)$ was determined by $(\ln 2)/k_2$. [d] Not determined.

Table 2. Formation of GSH Conjugates in the Presence of Equine Liver GSH *S*-Transferase at 25 °C (pH 7.2)[a]

alkenal	V_{max} [μmol × min^{-1} × (mg of protein)$^{-1}$]	K_m (μmol × L^{-1})	V_{max}/K_m	rel reaction rate
crotonaldehyde	0.4	75	5.5	1
hexenal	1.1	162	6.7	1.3
octenal	1.9	79	24.1	4.1
nonadienal	1.3	33	39.2	7.1

[a] Total reaction was determined and corrected for nonenzymatic formation.

Determination of DNA Single Strand Breaks in Cells. Cell suspensions (10 mL, 2×10^6 cells/mL) were incubated with the test compound (dissolved in 10 μL of Me_2SO) in a shaking water bath at 37 °C for 1 h. After incubation cells were washed twice with RPMI medium, centrifuged (10 min, 200*g*, 20 °C), and resuspended to a final concentration of $(2–3) \times 10^6$ cells/mL. Viability was assessed by trypan blue exclusion. Aliquots (2×10^6 cells) were loaded on polycarbonate filters (Nuclepore, Tübingen, Germany) and submitted to alkaline elution as described previously *(26)*. Control experiments were run with Me_2SO. Induction of SSB was expressed as % of DNA retained on filter in fraction 7 of control minus treated sample ($c - t$). Values of $c - t$ exceeding 20% are considered to represent significant effects *(27)*.

Results

Formation and Decomposition of GSH Conjugates. 2-Alkenals react readily with GSH to form GSH conjugates by nucleophilic addition of the thiol group across the C=C double bond (Michael addition). The GSH conjugates of hexenal and nonadienal were synthesized and characterized. Formation of GSH conjugates proceeds at similar rates for crotonaldehyde, hexenal, octenal, and nonadienal (Table 1). Acrolein is exceptionally reactive, displaying a reaction rate about 2 orders of magnitude higher than all other 2-alkenals. Rates of decomposition of the conjugates are lower by several orders of magnitude ($k_1/k_2 \approx 10^4$), resulting in half-lives of 3.5 h for the nonadienal conjugate and about 7 h for the other conjugates at 25 °C. Decomposition rates were determined by removal of GSH with Ellman's reagent from alkenal/GSH reaction mixtures that had reached equilibrium. Similar decomposition rates were determined by head space analysis of alkenal volatilized at 25 °C from the chemically synthesized conjugates in buffer. The initial reaction showed first order kinetics. At 37 °C, half-lives of the synthesized GSH conjugates were considerably shorter (GSH–hexenal: 2.1 h, GSH–nonadienal: 1.2 h), compared to those at 25 °C.

In the presence of GST from horse liver, reaction rates of GSH conjugate formation are strongly increased (Table 2). Conjugation of octenal and nonadienal proceeds at rates 4 and 7 times that of crotonaldehyde, respectively.

Oxidation and Reduction of Alkenals and Alkenols by Rat Liver Cytosolic and Microsomal Frac-

Table 3. Oxidation of Alkenols by Rat Liver Cytosol after Addition of NAD^+

	reaction rate (nmol/h for 2 × 10^6 cells)		
alkenol (mM)	crotyl alcohol	hexenol	nonadienol
0.25	143	190	226
0.5	250	310	440
1	360	360	524
1.5	345	380	536
2	310	415	560

Table 4. Protein, GSH, and Enzymatic Activities in the Cytosolic Fraction of Namalva Cells and Primary Rat Hepatocytes

	Namalva cells	primary hepatocytes (rat)
GSH (nmol for 2 × 10^6 cells)	1.6	80
protein (mg for 2 × 10^6 cells)	0.14	2
alcohol dehydrogenase (nmol of acetaldehyde/min for 2 × 10^6 cells)	3.22	26
aldehyde dehydrogenase (nmol of propionic acid/min for 2 × 10^6 cells)	2.13	31.4
GSH *S*-transferase (nmol of conjugated 1-chloro-2,4-dinitrobenzene/min for 2 × 10^6 cells)	7.6	1754

Table 5. Viability of Namalva Cells (%), Determined as Trypan Blue Excluding Cells[a]

alkenal (mM)	acrolein	crotonaldehyde	hexenal	nonadienal
0.1	72	56	78	60
0.3	60	–	–	–
0.4	–	71	72	0
0.8	–	37	59	0

[a] Viability of control after incubation (1 h) was 75%.

tions. 2-Alkenals are oxidized by cytosolic and microsomal fractions of rat liver to the corresponding alkenoic acids. In both fractions ALDH prefers the longer homologues as substrates, microsomal ALDH contributing about 10 times more to the oxidation of hexenal per cell than cytosolic ALDH. While rates remained essentially unchanged for crotonaldehyde (0.25–2 mM; 96–75 nmol/h for 2 × 10^6 cells), a concentration dependent increase was observed for hexenal (0.25–1.5 mM; 43–150 nmol/h for 2 × 10^6 cells) and nonadienal (0.25–2 mM; 40–325 nmol/h for 2 × 10^6 cells) in rat liver cytosol. In contrast, microsomal ALDH showed a concentration dependent increase in rate for crotonaldehyde (0.5–2 mM; 30–270 nmol/h for 2 × 10^6 cells) but only a slight increase for hexenal (0.25–2 mM; 1170–1884 nmol/h for 2 × 10^6 cells) and no increase for nonadienal (0.5–1.5 mM; 1770 nmol/h for 2 × 10^6 cells). The reaction rate of alkenol oxidation also increases with chain length (Table 3). Reduction of alkenals by cytosolic ADH only occurs at minor rates (2 mM alkenal/2 × 10^6 cells: crotonaldehyde 97 nmol/h, hexenal 122 nmol/h, nonadienal 48 nmol/h), compared to oxidation of alkenols to alkenals.

Enzyme Activities of Namalva Cells and Rat Hepatocytes. Namalva cells only contain small concentrations of GSH and low GST activity, compared to primary rat hepatocytes (Table 4). They also have only low activities for a wide spectrum of metabolizing enzymes, including cytochrome P450, ADH, and ALDH.

Consumption of Hexenal by Cells. Concentrations of 2-alkenals were selected to allow for maximum tolerated nontoxic concentrations (Tables 5 and 6). Namalva cells were significantly more sensitive toward the cytotoxic effects of alkenals than primary rat hepatocytes, as measured by trypan blue exclusion. Nonadienal was

Table 6. Viability of Primary Rat Hepatocytes (%), Determined as Trypan Blue Excluding Cells[a]

alkenal (mM)	crotonaldehyde	hexenal
0.5	67	63
1	64	59
1.5	60	51

[a] Viability of control after incubation (1 h) was 69%.

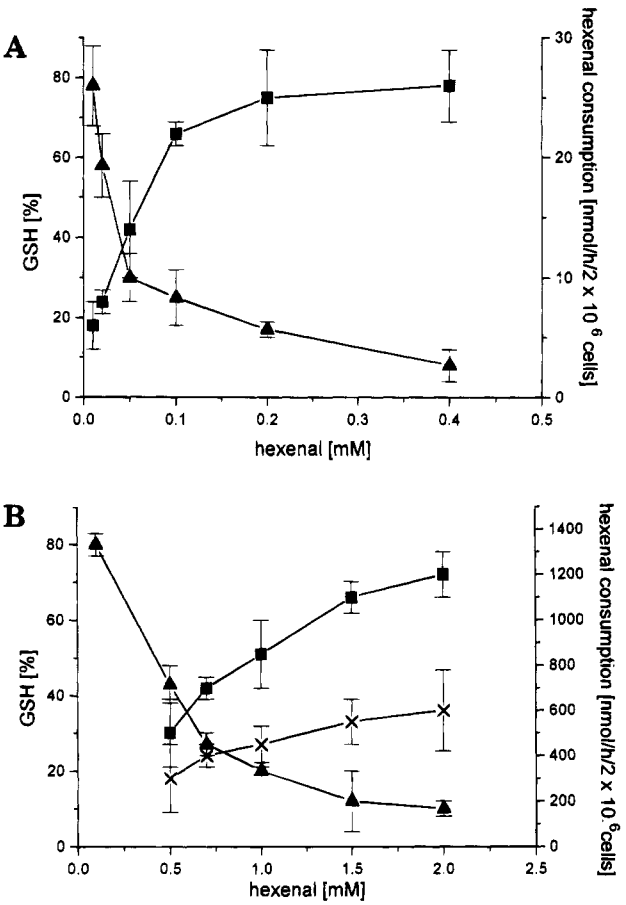


Figure 1. Consumption of hexenal (■) and reduction of GSH level (▲) (A) in Namalva cells and (B) in primary rat hepatocytes (heated cells: ×). Cells were incubated with hexenal for 1 h at 37 °C in gas-tight vials. Consumption was measured as reduction of hexenal by head space GC. GSH level, determined in the cytosol with DTNB [5,5′-dithiobis(2-nitrobenzoic acid)], is given as the percentage of GSH of untreated control. Hepatocytes were denaturated by heating cells in hot water. Values are means ± SD from three individual experiments.

the most toxic alkenal, inducing trypan blue uptake even at 0.1 mM. Hexenal consumption by Namalva cells approached saturation (25 nmol/h for 2 × 10^6 cells) at concentrations of 0.1 mM and more (1 h incubation) (Figure 1A). In contrast, hexenal consumption in hepatocytes does not level out at noncytotoxic concentrations of up to 1200 nmol/h for 2 × 10^6 cells at 2.0 mM (Figure 1B). Heat inactivation of hepatocytes reduced the consumption to about half the value of viable cells.

Induction of DNA SSB by 2-Alkenals. Acrolein showed a much higher potential to induce DNA SSB in Namalva cells than crotonaldehyde and hexenal (Figure 2A). Namalva cells were much more sensitive to DNA damage induced by alkenals than primary hepatocytes (Figure 2A,B). In hepatocytes about 3–5 times higher concentrations of aldehyde were necessary to induce significant effects compared to Namalva cells. As a consequence of its high cytotoxicity, nonadienal could only be tested up to 0.2 mM in Namalva cells. No

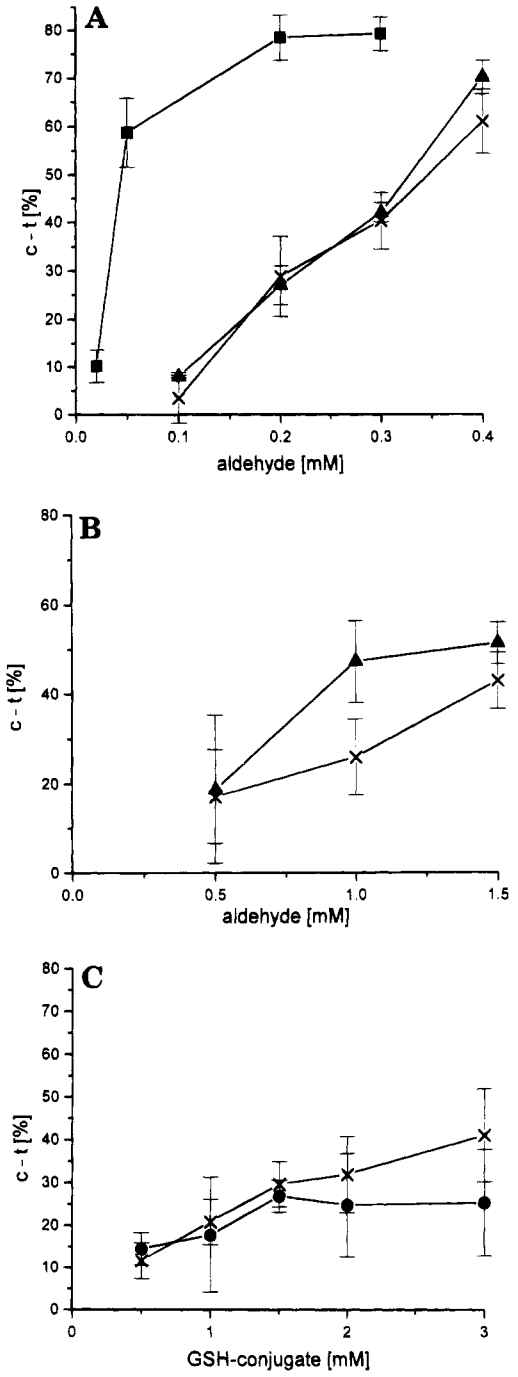


Figure 2. Induction of DNA SSB (A) in Namalva cells by acrolein (■), crotonaldehyde (▲), and hexenal (×), (B) in primary rat hepatocytes by crotonaldehyde (▲) and hexenal (×), and (C) in Namalva cells by GSH–hexenal conjugate (×) and GSH–nonadienal conjugate (●). Cells were incubated for 1 h at 37 °C. Results of the alkaline filter elution are given as the control value minus the test value ($c - t$) of percentage of DNA in the seventh fraction. Values are means ± SD from three elutions.

significant induction of DNA SSB was observed, in contrast to hexenal that already induced DNA damage at this concentration.

Induction of DNA SSB by GSH Conjugates. The GSH conjugates of hexenal and nonadienal display strongly reduced cytotoxicity. The hexenal conjugate is about 1 order of magnitude less effective in inducing DNA damage than hexenal. The corresponding nonadienal conjugate shows similarly low effectiveness (Figure 2C).

Binding of [^{14}C]-2-Hexenal to Biopolymers in Rat Hepatocytes. Radioactive binding to DNA, RNA, and proteins increased in a concentration dependent manner. Binding to DNA was slightly higher than to RNA and protein (Table 7).

Table 7. Binding of [2-^{14}C]Hexenal to Biopolymers in Rat Liver Hepatocytes (μg of Hexenal Equivalents/mg)

hexenal (mM)	DNA	RNA	protein
1	0.3	0.3	0.1
3	1.2	0.8	0.6
5	2.1	1.7	0.8

Discussion

Conjugation to GSH has been described as a major reaction in the biotransformation of acrolein and crotonaldehyde *(14, 18)*. The resulting GSH conjugate is further metabolized to the corresponding mercapturic acid. For acrolein, *S*-(3-hydroxypropyl)-*N*-acetyl-L-cysteine and *S*-(3-carboxypropyl)-*N*-acetyl-L-cysteine were found as urinary metabolites *(15, 28)*. This reaction proceeds by direct Michael addition of GSH across the α,β-double bond, but also is subject to catalysis by GST. For the present investigation GST from equine liver, which resembles the main GST in human liver (GSTα-ε), was used *(29)*. In agreement with results reported earlier by Esterbauer et al. (16), the direct Michael reactivity of acrolein was found to be very much higher than that of the other alkenals tested, its high spontaneous reactivity preventing determination of the GST-mediated reaction rate. For the other alkenals, the GST-mediated conjugate formation proceeds much more rapidly than the spontaneous reaction and increases with increasing alkenal chain length. This is consistent with results obtained for the conjugation of 4-hydroxyalkenals by GST from rat, mouse, and man *(30)*. Based on the assumption that in hepatocytes millimolar GSH levels are present and that GST activity is similar to the rate observed in vitro with equine GST, GST-mediated conjugate formation will be about 3 orders of magnitude more rapid for hexenal than the spontaneous reaction. Therefore, nonenzymatic GSH conjugate formation appears to be of minor importance in hepatocytes.

Besides GSH conjugation other enzymes are also contributing to alkenal metabolism. Oxidation by ALDH is a major deactivating reaction for hexenal and nonadienal and, to a lesser extent, also for crotonaldehyde, as shown by incubation with rat liver microsomal fraction. In view of the comparatively poor activities in these deactivating enzymes (Table 4), Namalva cell fractions were not tested in parallel. In agreement with our results, Mitchell et al. have reported reaction rates for rat liver microsomal ALDH to increase from pentenal to octenal while acrolein was not found to be a substrate *(13, 31)*. Detailed studies with mitochondrial and cytosolic rat liver ALDH have revealed that acrolein irreversibly inhibits the enzyme in the absence of GSH or other thiol groups while crotonaldehyde causes a reversible and noncompetitive inhibition of the high affinity cytosolic ALDH *(32, 33)*. In contrast to acrolein itself, the acrolein–GSH conjugate has, however, been found to be oxidized by rat liver ALDH *(34)*.

GSH conjugates are not stable and undergo reversible decomposition into GSH and 2-alkenal. Half-lives (37 °C) of about 2 h for the hexenal and about 1 h for the nonadienal conjugate may permit such conjugates to act as transport forms for alkenals. After systemic distribution they may reach tissues low in GSH and GST and

might liberate the alkenal by dissociation, thus generating potentially genotoxic activity *(35)*. GSH conjugates induce SSB in Namalva cells obviously due to alkenal release. Their potential to act as transport forms for alkenals therefore has to be further elucidated.

Primary rat hepatocytes and Namalva cells were chosen by us as cell systems because of their significantly different enzymatic status. Primary rat hepatocytes are proficient in metabolizing activity and are able therefore to metabolize much higher quantities of alkenals than Namalva cells. Moreover, microsomal activities like those depending on cytochrome P450, cytochrome b_5, UDP-glucuronyltransferase, and aryl hydrocarbon hydroxylase are not detectable in Namalva cells *(21)*. In addition to these differences in enzymatic activity, the higher overall protein content and their much higher GSH level obviously also protects hepatocytes against cytotoxic effects by alkenals. This is consistent with our observation that Namalva cells are much more sensitive to cytotoxic effects of 2-alkenals than primary rat hepatocytes. Toxic damage determined by trypan blue exclusion became detectable in both cell types at hexenal concentrations that caused a reduction of GSH level to about 10% of the control. The marked differences in cellular protein, GSH, and metabolizing activity account for the much higher rate of hexenal consumption in hepatocytes, as compared to Namalva cells. In both cell types, hexenal consumption directly correlates with intracellular GSH depletion. Heat inactivation of the enzymes in hepatocytes results in 50% reduction of hexenal consumption. However, the value after heat inactivation of the hepatocytes is still much higher than that in Namalva cells. This phenomenon obviously reflects direct reaction of the alkenals with cytosolic proteins in hepatocytes, because their protein content is more than 10 times greater than that of Namalva cells. The substantial contribution of the direct reaction with proteins is also evident from the binding studies of [^{14}C]2-hexenal in hepatocytes. Although the relative binding of alkenals to proteins is somewhat lower than that to DNA or RNA, the high protein content per cell ensures that most of the hexenal will be bound to protein.

Namalva cells are much more sensitive toward the DNA damaging activity of alkenals than rat hepatocytes.This can be reconciled with the marked differences in GSH, GST, metabolizing enzymes, and cellular protein contents of both cell types. Although cellular GSH concentrations were quite different in both cell systems, GSH levels had to be depleted to less than 20% of the control levels before DNA single strand breaks became detectable. In Namalva cells, acrolein showed much greater DNA damaging potential than crotonaldehyde and hexenal. The latter are about equipotent in both Namalva cells and hepatocytes. Nonadienal was not found to be genotoxic in Namalva cells at a concentration where hexenal already induced DNA damage. The high cytotoxic potential of nonadienal prevented its testing at higher concentrations. Since however, in all further aspects investigated, nonadienal behaved quite similarly to hexenal, its DNA damaging potential in hepatocytes was not expected to be significantly more potent than that of hexenal and therefore was not investigated.

We assume from the results of our experiments, that metabolically proficient cells rich in GSH and GST are efficiently protected against genotoxic effects of alkenals. However, alkenals may induce DNA damage in tissues low in protein, GSH, and GST activity after reaching them directly or after being formed from the decomposition of the GSH conjugate. Moreover, GST activity also has been observed to be subject to significant oscillations during the cell cycle, with a decrease in activity during G1 phase, rendering cells in G1 more sensitive toward directly DNA damaging agents *(36)*.

Alkenals ingested in foods obviously have the greatest potential for damaging tissues which they directly contact: the oral mucosa and other epithelial cells of the gastrointestinal tract. Studies in exfoliated cells of human oral mucosa have shown that mouth rinsing with hexenal solutions at concentrations reflecting those in foods can induce chromosomal damage.[2] Studies are underway to determine the extent of DNA damage and adduct formation in mucosa cells from the upper gastrointestinal tract of rats.

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