Biochem. J. (1985) **228**, 363–373
Printed in Great Britain

Metabolism of the lipid peroxidation product 4-hydroxynonenal by isolated hepatocytes and by liver cytosolic fractions

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(*Received 8 October 1984/4 February 1985; accepted 13 February 1985*)

The metabolism of the lipid peroxidation product 4-hydroxynonenal and of several other related aldehydes by isolated hepatocytes and rat liver subcellular fractions has been investigated. Hepatocytes rapidly metabolize 4-hydroxynonenal in an oxygen-independent process with a maximum rate (depending on cell preparation) ranging from 130 to 230 nmol/min per 10^6 cells (average 193 ± 50). The aldehyde is also rapidly utilized by whole rat liver homogenate and the cytosolic fraction (140000*g* supernatant) supplemented with NADH, whereas purified nuclei, mitochondria and microsomes supplemented with NADH show no noteworthy consumption of the aldehyde. In cytosol, the NADH-mediated metabolism of the aldehyde exhibits a 1:1 stoichiometry, i.e. 1 mol of NADH oxidized/mol of hydroxynonenal consumed, and the apparent K_m value for the aldehyde is 0.1 mM. Addition of pyrazole (10 mM) or heat inactivation of the cytosol completely abolishes aldehyde metabolism. The various findings strongly suggest that hepatocytes and rat liver cytosol respectively convert 4-hydroxynonenal enzymically is the corresponding alcohol, non-2-ene-1,4-diol, according to the equation:

$$CH_3-[CH_2]_4-CH(OH)-CH=CH-CHO+NADH+H^+ \rightarrow CH_3-[CH_2]_4-CH(OH)-CH=CH-CH_2OH+NAD^+.$$

The alcohol non-2-ene-1,4-diol has not yet been isolated from incubations with hepatocytes and liver cytosolic fractions, but was isolated in pure form from an incubation mixture containing 4-hydroxynonenal, isolated liver alcohol dehydrogenase and NADH and its chemical structure was confirmed by mass spectroscopy. Compared with liver, all other tissues possess only little ability to metabolize 4-hydroxynonenal, ranging from 0% (fat pads) to a maximal 10% (kidney) of the activity present in liver. The structure of the aldehyde has a strong influence on the rate and extent of its enzymic NADH-dependent reduction to the alcohol. The saturated analogue nonanal is a poor substrate and only a small proportion of it is converted to the alcohol. Similarily, nonenal is much less readily utilized as compared with 4-hydroxynonenal. The effective conversion of the cytotoxic 4-hydroxynonenal and other reactive aldehydes to alcohols, which are probably less toxic, could play a role in the general defence system of the liver against toxic products arising from radical-induced lipid peroxidation.

During the process of lipid peroxidation polyunsaturated fatty acids, especially linoleic acid, arachidonic acid and docosahexaenoic acid, in biomembranes are degraded to a great variety of water-soluble, short-chain carbonyl compounds (Dillard & Tappel, 1979; Benedetti *et al.*, 1979; Esterbauer *et al.*, 1982). Malonaldehyde is most frequently used as a measure for the rate and the extent of lipid peroxidation in biological samples (Slater, 1972) and the metabolic fate of this aldehyde has been extensively studied (Bird & Draper, 1982; Siu & Draper, 1982; Hjelle & Petersen, 1983). Besides malonaldehyde, a great diversity of other aldehydes, such as alkanals, 2-alkenals, hydroxyalkenals (Esterbauer *et al.*, 1982) and phospholipid-bound aldehydes (Tam &

McCay, 1970) are generated in the lipid peroxidation process. Rat liver microsomes stimulated by ADP–Fe^{2+} produce, in addition to 560nmol of malonaldehyde/g of original liver, about 950mmol of other carbonyls/g of original liver (Esterbauer *et al.*, 1982), among which the class of hydroxyalkenals has received increased attention because of their multiple biological reactivities. So far 4-hydroxy-2,3-*trans*-nonenal (Benedetti *et al.*, 1980), 4-hydroxy-2,3-*trans*-hexenal (Heckenast, 1983) and 4,5-dihydroxy-2,3-*trans*-decenal (Benedetti *et al.*, 1984) have been unequivocally identified by mass spectroscopy, 4-hydroxynonenal being by far the major product. It has been shown that this aldehyde is highly cytotoxic to Ehrlich ascites tumour cells (Schauenstein *et al.*, 1977) and *Salmonella typhimurium* (Marnett *et al.*,1985), leads to lysis of erythrocytes (Benedetti *et al.*, 1980) and stimulates chemiluminescence and pentane production in isolated hepatocytes (Cadenas *et al.*, 1983). 4-Hydroxynonenal is highly reactive towards thiol compounds such as glutathione (Esterbauer *et al.*, 1975), cysteine (Esterbauer *et al.*, 1976) and SH-proteins (Esterbauer, 1982) and has an inhibitory action on microsomal glucose-6-phosphatase and cytochrome *P*-450 (Benedetti *et al.*, 1980), aminopyrine demethylase (Ferrali *et al.*, 1980) and adenylate cyclase (Dianzani, 1982). It was also shown that the aldehyde can modify human low-density lipoprotein (Jürgens *et al.*, 1984) in a way similar to malonaldehyde (Fogelman *et al.*, 1980), a reaction which has been implicated in the development of atherosclerotic lesions. Additional biological effects reported for 4-hydroxynonenal or homologous aldehydes include inhibition of DNA and protein synthesis and mitochondrial respiration (for review see Schauenstein *et al.*, 1977), mutagenicity in the *Salmonella* tester strain TA 104 (Marnett *et al.*, 1985) and enhancement of fluorescent chromolipid formation in peroxidizing mitochondria and microsomes (Koster *et al.*, 1985). It has been proposed that 4-hydroxynonenal or similar reactive aldehydes can induce cytopathological effects in liver in the course of lipid peroxidation *in vivo* (Benedetti *et al.*, 1980; Cadenas *et al.*, 1983; Benedetti *et al.*, 1984). The objective of this study was to gain information on the metabolism of 4-hydroxynonenal and related aldehydes by isolated hepatocytes and subcellular fractions. Such knowledge is a necessary requirement to assess the possible implication of hydroxynonenal and related aldehydes for liver injury.

Materials and methods

Materials

Biochemicals were from Boehringer Mannheim (NADH, NADPH, pyruvate) or from Sigma (pyrazole, sucrose, EGTA). Aldehydes were purchased from Merck (acetaldehyde, pentanal, hexanal), from EGA Chemie (heptanal, octanal, nonanal, undecanal) and from Ventron (heptenal, octenal, nonenal, undecenal). 4-Hydroxyalkenals were synthesized as described (Esterbauer & Weger, 1967) and stored as a chloroform solution (10mg/ml) at −20°C. Other chemicals were of analytical grade and were obtained from Merck.

To prepare aqueous solutions of 4-hydroxynonenal, a sample of the chloroform stock solution containing 0.1mmol (15.6mg) of the aldehyde was evaporated on a rotary evaporator at 20°C and the residue was dissolved in 8ml of distilled water, degassed under vacuum to remove traces of chloroform and filtered. The exact concentration was estimated spectrophotometrically at 223nm ($\varepsilon = 13750 M^{-1} \cdot cm^{-1}$) and the solution was then brought to a final concentration of 10mM by addition of water. Aqueous solutions of the other 4-hydroxyalkenals were prepared similarly. All other aldehydes were used as a 3mM solution in dimethyl sulphoxide.

Animals were male Wistar rats, weighing 200–250g, fed on a standard diet (TACO T 79; Tagger, Graz, Austria).

Incubation of hepatocytes and preparation of subcellular fractions

Rat liver cells were prepared as described by Berry & Friend (1969) as modified by Cadenas *et al.* (1983). To prepare whole liver homogenate and its subcellular fractions, wet liver was homogenized by hand in a Potter–Elvehjem homogenizer in 9vol. of 20mM-Tris/HCl buffer, pH7.4, 250mM-sucrose and 1mM-EGTA. Subcellular fractions were obtained by differential centrifugation at 600*g* (5min), 4600*g* (10min), 12000*g* (7min, pellet discarded) and 140000*g* (40min). The 4600*g* sediment was resuspended in the isolation medium and centrifuged again at 600*g* (5min), the pellet was discarded and the supernatant was centrifuged at 10000*g* (10min) to yield the mitochondria. To obtain purified nuclei the 600*g* pellet was centrifuged in a discontinuous sucrose gradient as described by Maggio *et al.* (1963). Other wet tissues were homogenized in 9vol. of 20mM-Tris/HCl buffer, pH7.4, 250mM-sucrose and 1mM-EGTA with an Ultra Turrax at full speed (3–5min). The homogenate was then centrifuged at 40000*g* (40min) and the supernatant was used for the experiments.

Measurement of 4-hydroxynonenal consumption by hepatocytes

Hepatocytes were incubated in a solution containing 114mM-NaCl, 25mM-$NaHCO_3$, 5.9mM-KCl, 1.18mM-$MgCl_2$, 1.2mM-Na_2SO_4, 12.4mM-

NaH_2PO_4, 1.24mM-$CaCl_2$, 10mM-glucose, 2.1mM-lactate, 0.3mM-pyruvate and 0.5mM-EGTA. 4-Hydroxynonenal was added as a solution in the same medium to give final concentrations of 0.068–1mM, as indicated in the legends to the Figures and Tables. The final incubation volume was 4 or 10ml. Incubations were carried out in Erlenmeyer flasks (25ml or 100ml) in a shaking water bath at 37°C; the flasks were gassed with O_2/CO_2 (19:1) or N_2/CO_2 (19:1). At desired time intervals, samples (1ml) were withdrawn and added to an equal volume of acetonitrile/acetic acid (24:1, v/v) to stop the hydroxynonenal-consuming reaction. The mixture was then centrifuged at 1000*g* for 10min, and the clear supernatant was separated by h.p.l.c. The separation conditions were: Sperisorb S 5 ODS column, with ODS precolumn, methanol/water (13:7, v/v) as mobile phase, flow rate set to 1.0ml/min, detector set to 220nm with 0.64 or 0.08 absorbance units for full recorder scale, injected sample volume was 20μl. Peak identification and quantification of the 4-hydroxynonenal was based on reference chromatograms obtained from standard hydroxynonenal solutions (1μM–1mM) in water/acetonitrile/acetic acid (25:2:1, by vol.).

Measurement of 4-hydroxynonenal consumption by subcellular fractions

Whole liver homogenate and its subfractions were incubated in 10mM-Tris/HCl buffer, pH7.4, containing 150mM-$MgCl_2$ in the presence of 0.1mM-4-hydroxynonenal at 37°C under free access of air. Additional experimental conditions (addition of cofactors, inhibitors, etc.) are given in the legends to Tables 2 and 3 and Fig. 4. Samples were withdrawn at desired time periods and 4-hydroxynonenal was assayed by h.p.l.c. as described above for hepatocyte incubations.

Spectrophotometric assays

Consumption of NADH and NADPH by different subcellular fractions in the presence or absence of 4-hydroxynonenal and other aldehydes was followed spectrophotometrically at 340nm and 25°C in 1cm cuvettes. The medium for the assay contained 90mM-phosphate buffer, pH7.4 and the initial aldehyde concentration was 0.1mM. The rate of NAD(P)H consumption was obtained from the initial linear part of the time curve.

Gas chromatography–mass spectrometry analysis

The product isolated from an incubation mixture of 4-hydroxynonenal, liver alcohol dehydrogenase and NADH was analysed by g.c.–m.s. A Finnigan gas chromatograph 9610 coupled to a Finnigan 4000 mass spectrometer with an Incos Data System was used. The g.c. separation was performed on a 30m fused silica column coated with SE-54. Helium (2ml/min) was carrier gas, with split-less injection and a temperature gradient of 100–300°C at 5°C/min. Chemical ionization was carried out with ammonia as ionization gas with an ion voltage of 120eV and 0.1A; electrical ionization was performed with 70eV; scan time was 1s. The sample was analysed in the underivatized form and as the trimethylsilyl derivative.

Results

Determination of 4-hydroxynonenal in hepatocyte suspensions

The rate of consumption of externally added 4-hydroxynonenal by rat hepatocytes was followed by h.p.l.c. separation (Fig. 1). It was necessary to pretreat the samples prior to h.p.l.c. analysis in order to terminate all 4-hydroxynonenal-consuming reactions. This was accomplished by addition of an equal volume of acetonitrile/acetic acid (24:1, by vol.) to the cell suspension. Acetonitrile was used to precipitate and thereby inactivate 4-hydroxynonenal-degrading enzymes, while the acetic acid component of the mixture decreased the rate of the non-enzymic reaction to negligible levels by lowering the pH to 4. Experimental samples treated in this manner could be stored at 4°C for 24h without any detectable change in their 4-hydroxynonenal content as measured by h.p.l.c. The usual procedure of protein precipitation with $HClO_4$ (3.5% final concn.) is applicable in principle; however, such samples must be neutralized within a few minutes to minimize acid-catalysed 4-hydroxynonenal decomposition reactions. The h.p.l.c. separation of 4-hydroxynonenal did not show any peak interference with other cellular constituents and 4-hydroxynonenal could be quantitatively measured down to levels of 1nmol/ml of cell suspension. A calibration curve obtained from standard 4-hydroxynonenal solutions exhibited a linear relationship between peak height and 4-hydroxynonenal concentration over a range of 1μM–0.5mM.

Consumption of 4-hydroxynonenal by isolated hepatocytes

Incubation of isolated rat liver cells with 4-hydroxynonenal resulted in a rapid loss of the aldehyde from the incubation mixture as measured by h.p.l.c. analysis at various time periods (Fig. 2). Within 1min 92% of 0.1mM- and 50% of 1.0mM-4-hydroxynonenal were consumed by 2×10^6 cells/ml. The concentration of 1mM-4-hydroxynonenal, used by Cadenas *et al.* (1983) to study the effect on chemiluminescence and pentane production by hepatocytes, was highly cytotoxic to our hepatocyte preparations. More than 50% Trypan

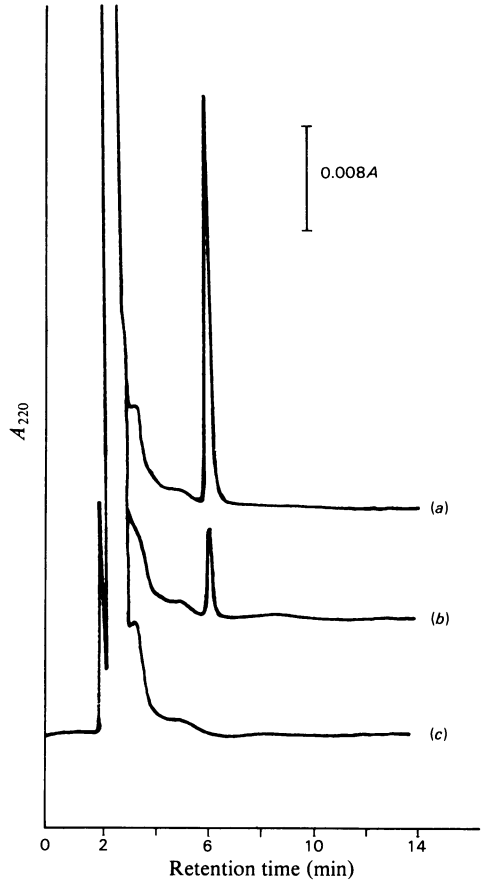

Fig. 1. *H.p.l.c. determination of 4-hydroxynonenal in suspensions of rat hepatocytes*

Hepatocytes (2×10^5 cells/ml) were incubated in the absence and in the presence of 0.1 mM-4-hydroxynonenal. Samples (1 ml) were withdrawn, precipitated with an equal volume of acetonitrile/acetic acid (24:1, v/v), centrifuged and 20 μl of the clear supernatant was injected into the h.p.l.c. Separation conditions were as described in the Materials and methods section. (*a*) 1 min after addition of 4-hydroxynonenal; (*b*) 6 min after addition of 4-hydroxynonenal; (*c*) control, no addition of 4-hydroxynonenal, 30 min incubated.

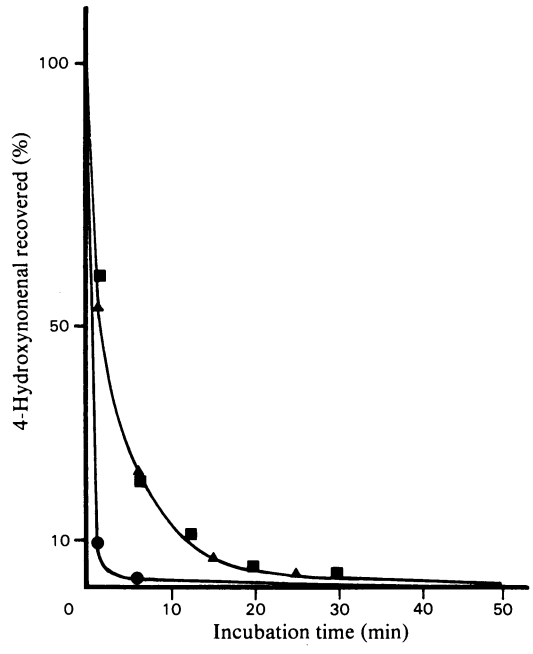

Fig. 2. *Kinetics of 4-hydroxynonenal utilization by rat hepatocytes*

Hepatocytes were incubated aerobically (O_2/CO_2) in the presence of 4-hydroxynonenal. At the indicated time periods, the residual aldehyde was estimated by h.p.l.c. (for separation conditions see Fig. 1). ●, 2×10^6 cells/ml, 0.1 mM-4-hydroxynonenal; ▲, 2×10^6 cells/ml, 1 mM-4-hydroxynonenal; ■, 2×10^5 cells/ml, 0.1 mM-4-hydroxynonenal.

Blue-stainable cells were found after 30 min of incubation with 1 mM-4-hydroxynonenal, whereas no decrease in cell viability was observed over a 60 min incubation period with 0.1 mM-4-hydroxynonenal. Therefore all further experiments with hepatocytes were performed with aldehyde concentrations of 0.1 mM or less. The rate of 4-hydroxynonenal loss increased linearly with the cell number (Fig. 3) over the range 5×10^4–5×10^5 cells/ml. With 2×10^6 cells/ml, the time course could not be accurately followed, since 90% of the added aldehyde was utilized within the first 1 min of addition.

Different cell preparations showed some variation in their 4-hydroxynonenal consuming capacity, ranging from 130 to 230 nmol/min per 10^6 cells (mean ± S.D., 183 ± 50). In order to study whether 4-hydroxynonenal consumption by hepatocytes is dependent on oxygen, incubations were also carried out in an O_2-free, N_2/CO_2 (19:1) atmosphere. Essentially the same time course of 4-hydroxynonenal disappearance was found under these anaerobic conditions; after 3, 6, 9 and 16 min of incubation, 10, 22, 27 and 37 nmol of 4-hydroxynonenal (0.068 mM) were consumed by 1×10^5 cells. The respective values for aerobic conditions were 11, 24, 27 and 38 nmol/10^5 cells. This indicates that 4-hydroxynonenal utilization by hepatocytes is not an oxidative process.

The chain length of the hydroxyalkenal has a significant effect on the rate of its consumption by hepatocytes. The initial velocity of aldehyde loss relative to 4-hydroxynonenal (= 1.0) decreased with decreasing chain length of the hydroxyalkenal

in the order 1.00, 0.825, 0.500 for 4-hydroxynonenal, 4-hydroxyoctenal and 4-hydroxypentenal respectively. This chain-length-dependence shows that hepatocytes possess a higher capacity for metabolizing the biogenic aldehyde 4-hydroxynonenal than for the other two homologous non-biogenic 4-hydroxyalkenals.

Utilization of 4-hydroxynonenal by rat liver homogenate and subcellular fractions

Whole rat liver homogenate supplemented with NADH gave a rapid consumption of 4-hydroxynonenal (Table 1). Without NADH supplementation some 4-hydroxynonenal loss occurred, though at a markedly reduced rate, compared with the complete system. Among the subcellular fractions, only the 140000*g* supernatant showed substantial 4-hydroxynonenal-metabolizing activity, whereas essentially no such activity was found in the purified nuclei and mitochondria. A small activity was present in the microsomal fraction. In the cytosolic fraction, 4-hydroxynonenal consumption was also observed in the presence of NADPH, however at a much lower rate as compared with the NADH-supplemented system, indicating that NADPH can only in part substitute for NADH. In whole liver homogenate no difference between the control (without addition) and the NADPH-containing incubation system was observed. Addition of NAD (0.3mM) to cytosol led to production of NADH as shown by the increase in absorption at 340nm. Upon addition of 4-hydroxynonenal (0.3mM) this intrinsic NADH production was strongly decreased. This clearly shows that 4-hydroxynonenal is not metabolized by an aldehyde dehydrogenase.

To compare 4-hydroxynonenal and NADH consumption, rates of NADH consumption in whole homogenate and its subfractions were measured spectrophotometrically. The results summarized in Table 2 confirm that the NADH-dependent 4-hydroxynonenal-metabolizing system is localized nearly exclusively in the cytosolic fraction. In the same preparations as used for measuring 4-hydroxynonenal-mediated NADH consumption, lactate dehydrogenase and alcohol dehydrogenase were also measured as cytosolic marker enzymes. The distribution of both enzymes in the various fractions is in good agreement with the distribution of the 4-hydroxynonenal-mediated NADH consumption. Moreover the presence of low activities of both marker enzymes (5–8% of total activity) in the 600*g* sediment suggests that the 4-hydroxy-

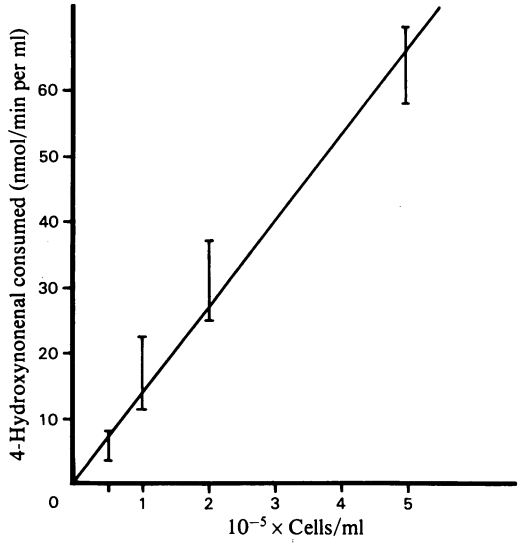

Fig. 3. *Relationship between hepatocyte concentration and rate of 4-hydroxynonenal utilization*
The initial rates were calculated from the loss of 4-hydroxynonenal measured within the first 1min after addition (0.1mM) of the aldehyde. Cells were incubated aerobically as described in the Materials and methods section.

Table 1. *Consumption of 4-hydroxynonenal by whole rat liver homogenate and subfractions in the presence and absence of NAD(P)H*
Incubation systems contained whole homogenate and subcellular fractions equivalent to 0.33mg of liver/ml, 0.3mM-NAD(P)H and an initial 4-hydroxynonenal concentration of 0.1mM. Consumption of 4-hydroxynonenal was followed by h.p.l.c. and the values given are those obtained after 10min of incubation. Values are means ± S.D. from three measurements.

Cell fraction	Consumption of 4-hydroxynonenal (nmol/10min per ml)		
	+NADH	+NADPH	Without addition
Whole homogenate	73±5	16±5	13±6
600*g* sediment (nuclear fraction)	32±3	11±2	10±5
Purified nuclei	0	0	0
12000*g* sediment (mitochondria)	0	0	0
140000*g* sediment (microsomes)	7±5	3±2	0
140000*g* supernatant (cytosol)	68±6	16±5	3±2

Table 2. *Subcellular distribution of the 4-hydroxynonenal (HNE)-metabolizing activity and the alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH) activities*

The assays contained total liver homogenate and subcellular fractions in a dilution equivalent to 0.33 mg of liver/ml. NADH and NADPH were 0.3 mM, and 4-hydroxynonenal, pyruvate and acetaldehyde were 0.1 mM. Activities were calculated from the initial linear decrease of NAD(P)H, monitored at 340 nm. Abbreviation used: n.d., not detectable.

	Activity [μmol of NAD(P)H/min per g wet wt.]			
Substrate ...	HNE	HNE	Pyruvate	Acetaldehyde
Cosubstrate ...	NADH	NADPH	NADH	NADH
Enzyme ...	-	-	LDH	ADH
Whole homogenate	6.8	0.27	386.2	11.8
600*g* sediment (nuclear fraction)	0.4	n.d.	32.1	0.6
12000*g* sediment (mitochondria)	n.d.	n.d.	5.6	n.d.
140000*g* sediment (microsomes)	n.d.	n.d.	2.3	n.d.
140000*g* supernatant (cytosol)	4.5	0.21	342.5	7.7
Recovery from whole homogenate (%)	72%	78%	99%	70%

nonenal-metabolizing activity present in the 600*g* fraction (6% of total activity) is solely due to cytosolic contamination.

Based on the literature on aldehyde metabolism (Petersen & Hjelle, 1982) the inability of mitochondria to utilize 4-hydroxynonenal as a substrate was rather unexpected. Additional experiments with a 50-fold higher concentration of mitochondrial protein (1 mg/ml) in fact showed that 40% of the added aldehyde disappeared within 10 min of incubation. However, the rate and extent of the disappearance was not altered by the presence of dinitrophenol (8.3×10^{-5} M) as uncoupler and glutamate (3 mM) as NAD(P)H supplier. Therefore the 4-hydroxynonenal consumption by high concentrations of mitochondria is most likely to be due to covalent binding of the aldehyde to mitochondrial protein thiol groups.

Characterization of the cytoplasmic 4-hydroxynonenal-metabolizing system

Fig. 4 shows the time course of 4-hydroxynonenal utilization in the presence and absence of NADH by two different cytosol concentrations. When a concentration equivalent to 0.56 mg of liver/ml was used, 73% of the aldehyde was metabolized within 10 min in the presence of NADH, while only a small loss of 4-hydroxynonenal occurred in the system not supplemented with NADH. With a 10-fold higher cytosol concentration in the NADH-supplemented incubation mixture, the 4-hydroxynonenal was completely consumed within 10 min. Under these conditions, loss of 4-hydroxynonenal also occurred in the absence of externally added NADH. This could be due to some endogenous NADH and to the reaction of 4-hydroxynonenal with glutathione and SH-containing proteins.

The stoichiometry of the NADH-dependent 4-

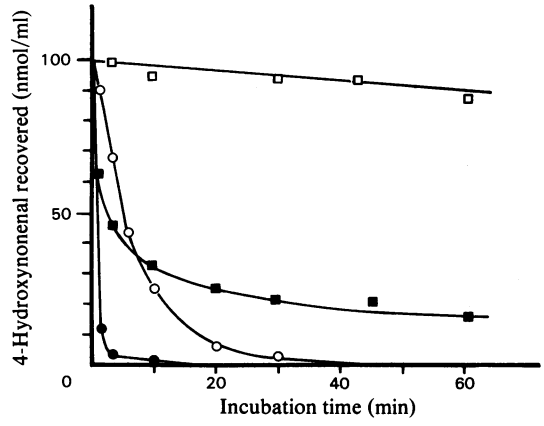

Fig. 4. *Kinetics of 4-hydroxynonenal utilization by rat liver cytosol with and without addition of NADH*

The incubation system contained 0.1 mM-4-hydroxynonenal and cytosol, equivalent to 0.8 mg of liver/ml (□ and ○) and 8.0 mg of liver/ml (■ and ●). □, ■, NADH omitted; ○, ●, 0.3 mM-NADH added. 4-Hydroxynonenal was measured by the h.p.l.c. method.

hydroxynonenal-metabolizing process was studied in incubation systems containing cytosol equivalent to 1.12 mg of liver/ml by monitoring both NADH and 4-hydroxynonenal consumption. The results (Table 3) clearly indicate that under these experimental conditions 1 mol of NADH was oxidized when 1 mol of 4-hydroxynonenal was consumed. The situation could be different in incubation systems with less-diluted cytosol, since in such cases competing NADH-independent side reactions, such as covalent binding to thiol groups, may contribute to the overall 4-hydroxynonenal consumption. Heat inactivation of the cytosol (5 min, 80°C) and addition of 10 mM-pyrazole, an

Table 3. *Stoichiometry of 4-hydroxynonenal and NADH consumption catalysed by rat liver cytoplasm*
The incubated system contained cytoplasm in a dilution equivalent to 0.56mg of liver/ml, 0.08mM-4-hydroxynonenal and 0.1mM-NADH. Loss of hydroxynonenal was measured by h.p.l.c., consumption of NADH was followed spectrophotometrically; the reference cuvette contained the complete system without hydroxynonenal. Hydroxynonenal consumption corrected is experimental (+NADH) minus control (−NADH).

Time (min)	4-Hydroxynonenal consumed (nmol/ml)			NADH consumed (nmol/ml)	Ratio NADH/hydroxynonenal
	+NADH	Control	Corrected		
6.15	20.0	3.0	17.0	18.4	1.08
13.75	36.0	5.5	30.5	32.0	1.05
19.00	48.0	5.5	42.5	41.1	0.97
26.75	57.0	5.5	51.1	48.0	0.93
33.00	61.5	5.5	56.0	51.2	0.91
Average ± S.D.	—	—	—	—	0.99 ± 0.074

inhibitor of alcohol dehydrogenase, reduced 4-hydroxynonenal utilization to 2 ± 1% of the control value observed in the presence of cytosol (equivalent to 0.56mg of liver/ml), 0.1mM-4-hydroxynonenal and 0.1mM-NADH. NADPH gave only 25 ± 5% of the 4-hydroxynonenal consumption observed in the presence of NADH. In the absence of NADH, 4-hydroxynonenal consumption was only 5 ± 2% of the control. Incubation under an atmosphere of N_2 had no influence on reaction rate (102 ± 5% of control). Cytosol separated from the low-M_r compounds by Sephadex G-25 column chromatography was able to metabolize 4-hydroxynonenal in the presence of NADH, indicating that no additional low-M_r cofactors are required.

Measurement of the dependence of initial rates of NADH consumption (initial [NADH] 0.1mM) on the concentration of the aldehyde over a range from 0.01 to 0.1mM and plotting the data as a Lineweaver–Burk plot (Fig. 5) gave an apparent K_m value for 4-hydroxynonenal of 0.1mM.

When a solution of 50ml of 4-hydroxynonenal (0.1mg/ml) in water was incubated for 2.5h in the presence of 150μl of horse liver alcohol dehydrogenase (27 units/ml) and 30mg of NADH, the aldehyde was completely consumed. The reaction product could be extracted into dichloromethane and the liquid residue remaining after evaporation of the solvent (3mg) was analysed by coupled g.c.–m.s. The NH_3 chemical ionization mass spectrum exhibited ions with *m/z* 176 (*M*+18, 5%), 158 (*M*, 16%), 157 (*M*−1, 22%), 141 (*M*−17, 84%), 124 [*M*−(2×17), 28%], 123 (*M*−17, −18, 84%),100 (31%), 99 (73%), 98 (22%), 95 (31%), 87 (72%), 83 (53%), 81 (90%), 71 (68%), 69 (60%), 67 (100%), 57 (30%) and 51 (34%). The M_r (158) and the loss of OH and H_2O is indicative of a nonenediol. The fragment with *m/z* 71 suggests a CH_3–$[CH_2]_4$ group, a hydroxy group at C-4 is indicated by the fragments of *m/z* 100 ($C_6H_{12}O$) and 57 (*M*-C_3H_5O). An unequivocal proof that the substance

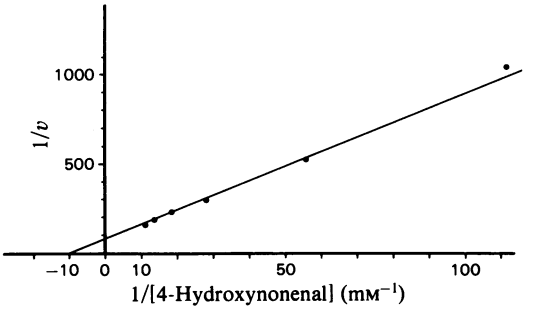

Fig. 5. *Lineweaver–Burk plot of the rate of 4-hydroxynonenal metabolism by rat liver cytoplasm*
The incubation system (3ml) contained cytosol equivalent to 1.12mg of liver/ml and 0.1mM-NADH. The rate of 4-hydroxynonenal metabolism was calculated from the initial rate of NADH consumption, assuming a 1:1 stoichiometry. 4-Hydroxynonenal concentration was varied between 0.1 and 0.01mM. Initial reaction rates were estimated spectrophotometrically at 340nm.

is non-2-ene-1,4-diol was obtained from the mass spectrum of the trimethylsilyl derivative. The fragments at *m/z* 103 and 199 arise from cleavage between C-1 and C-2 and indicate a CH_2OH group. Other typical fragments were at *m/z* 231 (cleavage between C-4 and C-5), 170, 147, 143, 120 and 79.

The effect of the molecular structure of the aldehyde on its NADH-driven metabolism by rat liver cytoplasm was examined with various aldehydes as substrates, differing in structure and chain length (Fig. 6). In the series of 4-hydroxyalkenals the rate of aldehyde metabolism increased nearly linearly with chain length. Different patterns were observed in the n-alkanals and in the 2-alkenals. The length of the linear phase of NADH consumption showed a large variation among different aldehydes; with equal aldehyde concentrations, the total amount of NADH consumed varied. An example of the strong influence of the

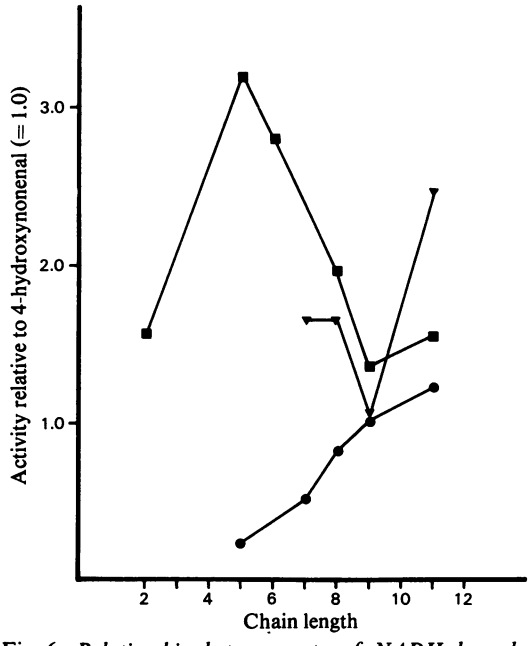

Fig. 6. *Relationship between rate of NADH-dependent reduction by rat liver cytosol and chain length of n-alkanals, 2-alkenals and 4-hydroxyalkenals*
The assay contained cytosol in a dilution equivalent to 0.56mg of liver/ml, 0.14mM-NADH and 0.1mM of the respective aldehyde. The reaction was followed spectrophotometrically at 340nm and the rates were calculated from the initial linear part of the time curve. ■, Alkanals; ▼, alkenals; ●, 4-hydroxyalkenals. All rates are given relative to the rate for 4-hydroxynonenal, which was set to 1.0. The numerical value for 4-hydroxynonenal was 36.2nmol/min per mg of protein.

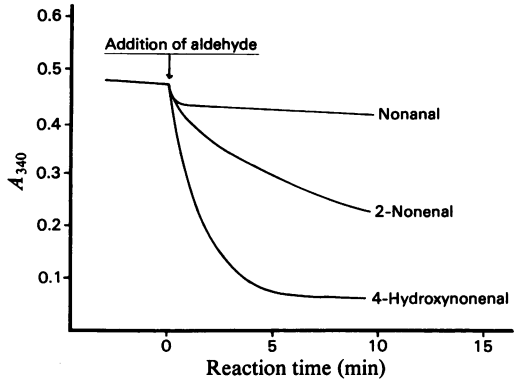

Fig. 7. *Kinetics of NADH-dependent reduction of nonanal, 2-nonenal and 4-hydroxynonenal by rat liver cytosol*
The incubation system (3ml) contained diluted cytosol, equivalent to 0.56mg of liver/ml, 0.14mM-NADH and 0.1mM of the aldehyde. The kinetics were followed by monitoring the decrease of the NADH absorbance at 340nm.

structural elements of the aldehyde on the rate and extent of its reduction by rat liver cytosol is shown in Fig. 7 for three straight chain aldehydes, all with nine carbon atoms, but differing in functional groups adjacent to the aldehyde function. The biogenic aldehyde 4-hydroxynonenal proved to be most susceptible to cytoplasmic reduction, followed by 2-nonenal, which was also readily accepted as a substrate. The saturated analogue nonanal was a poor substrate. The rate of NADH consumption mediated by this aldehyde quickly ceased after a short initial rapid period where about 5% of the added NADH was consumed.

Tissue distribution of the 4-hydroxynonenal-reducing activity

Various rat tissues were examined for their ability to metabolize 4-hydroxynonenal in a similar NADH-dependent enzymic pathway as shown for rat liver cytosol. For this screening, a 40000*g* supernatant of the whole tissue homogenate supplemented with 0.14mM-NADH was employed and the time course of NADH consumption was monitored in the presence (0.1mM) and absence (control) of 4-hydroxynonenal. Rates of 4-hydroxynonenal-mediated NADH consumption, calculated from the difference of whole system minus control, are given in Table 4 for two separate preparations. Relative to liver (100%) only kidney (7–10%) and heart (1–8%) gave a noteworthy activity.

Table 4. *Tissue distribution of NADH-dependent 4-hydroxynonenal metabolism*
Rat tissue homogenate (10%) was centrifuged at 40000*g* and the supernatant was diluted 1:200. The assay contained 0.5ml of the diluted supernatant, 0.14mM-NADH and 0.1mM-4-hydroxynonenal. The decrease of NADH was monitored at 340nm and the 4-hydroxynonenal reducing activity was calculated from the initial linear part of the time curve, assuming a 1:1 stoichiometry. Values given refer to two separate preparations.

Tissue	4-Hydroxynonenal consumed (nmol/min per g wet wt.)	(% of liver)
Liver	4900, 4974	100
Heart	50, 401	1–8
Muscle	10, 0	0–0.2
Kidney	360, 481	7–10
Fat pads	0, 0	0
Spleen	10, 240	0.2–5
Small intestine	10, 160	0.2–3
Lung	10, 240	0.2–5
Brain	10, 160	0.2–3

All other organs, i.e. spleen, small intestine, lung, brain, muscle and fat pads, possess nearly no detectable 4-hydroxynonenal-metabolizing activity. The accurate measurement of this possible minute activity in these organs was not within the scope of this work.

Discussion

The results of this study show that rat liver cells have a powerful system to utilize the lipid peroxidation product 4-hydroxynonenal. The hydroxynonenal-consuming capacity found for different cell preparations ranged from 130 to 230 nmol/min per 10^6 cells with a mean ± S.D. of 183 ± 50 ($n = 4$). This value is in the upper range of other metabolic activities present in liver cells, i.e. gluconeogenesis from lactate, 10.3 nmol/min per 10^6 cells (Krebs *et al.*, 1976); urea synthesis, 33–39 nmol/min per 10^6 cells (Krebs *et al.*, 1974, 1976); aspartate aminotransferase, 1739 nmol/min per 10^6 cells (Krebs, 1972) and lactate dehydrogenase, 2799 nmol/min per 10^6 cells [calculated from Table 2, whole homogenate, assuming 1.38×10^8 cells/g wet wt. of liver, according to Krebs *et al.* (1974)]. 4-Hydroxynonenal utilization is an oxygen-independent process, as the same rate for aldehyde consumption was observed under aerobic and anaerobic incubation conditions. This clearly indicates that hydroxynonenal metabolism cannot be an oxidative process catalysed by mitochondrial or cytosolic aldehyde dehydrogenases or aldehyde oxidase, enzymes known to catalyse the oxidation of a wide variety of aliphatic aldehydes (Weiner, 1982; Rajagopalan & Handler, 1964). It has been reported that α,β-unsaturated carbonyl compounds (aldehydes, ketones, esters, lactones) rapidly bind to glutathione by the action of a glutathione *S*-transferase (Boyland & Chasseaud, 1968) and in fact 4-hydroxynonenal leads to a rapid loss of glutathione in isolated hepatocytes, as recently reported by Cadenas *et al.* (1983). Moreover, even in a non-enzymic reaction, a 0.1 mM solution of 4-hydroxynonenal was completely consumed by 1 mM-glutathione within 30 min (Esterbauer, 1982). As shown very recently (Alin *et al.*, 1985) this spontaneous conjugation with glutathione can be enhanced by at least two orders of magnitude by rat liver cytosolic glutathione transferases. However, under our experimental conditions (2×10^5 cells/ml or equivalent concentrations of whole homogenate and cytosol, 0.1 mM-4-hydroxynonenal) spontaneous or enzyme-catalysed conjugation with glutathione can only be a minor pathway, since the glutathione available in the incubation systems was only about 8% of the 4-hydroxynonenal metabolized. Detailed further investigation revealed that the main route of 4-hydroxynonenal metabolism in suspensions of rat liver cells *in vitro* is an NADH-dependent reductive process, localized in the cytosol. Experimental evidence for these conclusions is summarized in Tables 1, 2 and 3. Firstly, the subcellular distribution of the hydroxynonenal-metabolizing activity clearly shows that the main activity (92% of total recovered activity) is present in the cytosolic fraction, as its subcellular distribution resembles very closely that of the cytosolic marker enzymes lactate dehydrogenase and alcohol dehydrogenase (Table 2). Secondly, 4-hydroxynonenal metabolism requires NADH as cofactor. Cytosol not supplemented with NADH exhibits only a minute 4-hydroxynonenal-consuming activity. Besides NADH, no other low-M_r factors are required. Thirdly, the experiment where both 4-hydroxynonenal and NADH consumption were monitored simultaneously (Table 3) revealed a stoichiometry of 1 mol of NADH consumed for the disappearance of 1 mol of hydroxynonenal.

Therefore the basic equation for the 4-hydroxynonenal-metabolizing process can be written as:

$$CH_3-[CH_2]_4-CH(OH)-CH=CH-CHO + NADH + H^+ \rightarrow CH_3-[CH_2]_4-CH(OH)-CH=CH-CH_2OH + NAD^+$$

The reaction product non-2-ene-1,4-diol has not yet been isolated from hepatocytes and liver cytosolic fractions and up to now no analytical method exists for direct measurement of the alcohol in tissue extracts. Several years ago a few milligrams of a structural analogue, pent-2-ene-1,4-diol, was isolated from incubation mixtures of rat liver slices with 4-hydroxypentenal (Binder, 1969, cited also in Schauenstein *et al.*, 1977).

Several findings strongly suggest that the enzyme catalysing the metabolism of the aldehyde in hepatocytes and liver cytosolic fractions is alcohol dehydrogenase. (*a*) In incubation mixtures containing 4-hydroxynonenal the aldehyde is converted to non-2-ene-1,4-diol by isolated liver alcohol dehydrogenase in the presence of NADH. From such an incubation mixture, the alcohol could be isolated in pure form and its structure was confirmed by mass spectroscopy. (*b*) Pyrazole, a potent inhibitor of liver alcohol dehydrogenase (Theorell & Yonetani, 1963) abolishes the NADH-driven 4-hydroxynonenal metabolism to the alcohol. (*c*) The distribution of the hydroxynonenal-reducing activity in liver subcellular fractions (Table 1) resembles that of alcohol dehydrogenase, as measured with acetaldehyde/NADH as substrates. (*d*) It is known from the literature (Sund &

Theorell, 1963) that the liver alcohol dehydrogenase has broad substrate specificity and accepts a wide range of aliphatic and aromatic aldehydes. The apparent K_m value of the enzyme for 4-hydroxynonenal is 0.1 mM, a value which is very close to that of liver alcohol dehydrogenase for acetaldehyde (0.21 mM; Sund & Theorell, 1963). NADH can in part be substituted by NADPH and it seems reasonable to assume that the NADPH-dependent enzyme reaction is due to aldehyde reductases, which are liver cytosolic enzymes possessing a broad substrate specificity and requiring NADPH as a cofactor (Flynn, 1982). In intact hepatocytes this route is most likely of minor importance, because cytosolic NADH concentration by far exceeds that of NADPH.

The finding that *in vitro* the conversion of 4-hydroxynonenal to the alcohol is the main process in liver cells does not exclude the possibility that a small fraction of the aldehyde also reacts with glutathione or other thiol compounds. In fact it has been shown that incubation of hepatocytes (2×10^6 cells/ml) with 4-hydroxynonenal (1 mM) results in complete loss of the cellular glutathione (Cadenas *et al.*, 1983). The proportion of the aldehyde reacting by this alternative route depends on several factors, in particular on the concentration and the amount of available glutathione and aldehyde and the activity of glutathione transferase (Alin *et al.*, 1985). Under conditions *in vivo* the situation may be more complex and with present knowledge it is not possible to predict quantitatively the extent of 4-hydroxynonenal metabolism by either pathway.

The present findings have some important biological implications regarding cytotoxicity of aldehyde lipid peroxidation products, in particular 4-hydroxyalkenals. It has been repeatedly suggested (Benedetti *et al.*, 1979, 1980, 1984; Dianzani, 1982) that tissue damage associated with lipid peroxidation might at least in part be induced by diffusible cytotoxic products such as hydroxyalkenals, which are much more stable than the free radicals and therefore could diffuse from the site of their origin to other cellular targets. It seems clear from our investigations that hydroxynonenal generated in a lipid peroxidation process and released from the lipid membrane compartment into the cytosol would effectively be converted by the alcohol dehydrogenase to the corresponding alcohol, which itself is most likely to be unable to induce cytopathological effects because it does not possess the reactive α,β-unsaturated aldehyde function. In our hepatocyte system *in vitro*, initial aldehyde concentrations of 1 mM or 0.1 mM were reduced to less than 3 μM within 30 min or 6 min, respectively (Fig. 2). In close agreement with this value, the intracellular steady state concentration of 4-hydroxynonenal in ADP–Fe-intoxicated hepatocytes was found to be about 7 μM (Poli *et al.*, 1985). It can therefore be concluded that 4-hydroxynonenal produced endogenously in liver or liver cells as a consequence of lipid peroxidation cannot accumulate above a low level of approx. 3–7 μM, the exact concentration depending on the dynamic state of production and removal. The concept of cytotoxic effects induced by hydroxyalkenals is therefore only applicable to cell functions which are influenced at such low concentrations. Up to now, effects of 4-hydroxynonenal in the micromolar range were only reported for adenylate cyclase (Dianzani, 1982) and for the chemotactic response of polymorphonuclear leukocytes (Curzio *et al.*, 1982). It can be definitely excluded that 4-hydroxynonenal released during microsomal lipid peroxidation into the cytoplasm could have effects on mitochondrial respiration or microsomal glucose-6-phosphatase, because such effects need aldehyde concentrations of 0.1 mM. Since hydroxynonenal is a lipophilic compound, the main proportion formed in a lipid peroxidation process would remain in the membrane and not immediately be exposed to the alcohol dehydrogenase. Therefore the possibility exists that 4-hydroxynonenal accumulates in the lipid membrane and directly affects membrane-bound enzymes without encountering the cytosolic barrier. In principle, the possibility exists that the alcohol is toxified again in liver or other tissues by the reverse reaction, if targets with high affinity for the aldehyde exist.

The 4-hydroxynonenal-induced low level chemiluminescence and alkane evolution of isolated hepatocytes showed maximal rates approx. 30 min after addition of the aldehyde (Cadenas *et al.*, 1983), at a time period where most of the added aldehyde in fact has been converted to the alcohol. It seems therefore reasonable to assume that both chemiluminescence and pentane evolution are not related to the aldehyde, but rather to its metabolic product, the alcohol. The fact that the liver alcohol dehydrogenase shows high activity not only towards hydroxynonenal but also to all other aldehydic lipid peroxidation products so far identified (i.e. alkanals, 2-alkenals) suggests, but of course does not prove, that the enzyme plays a role in the cellular defence system against radical-induced lipid peroxidation by effectively removing cytotoxic aldehydes.

These studies were conducted pursuant to a contract with the National Foundation for Cancer Research, Bethesda, MD, U.S.A.

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