Biochem. J. (1985) **227**, 629–638
Printed in Great Britain

Separation and characterization of the aldehydic products of lipid peroxidation stimulated by carbon tetrachloride or ADP–iron in isolated rat hepatocytes and rat liver microsomal suspensions

Giuseppe POLI*, Mario U. DIANZANI,* Kevin H. CHEESEMAN,† Trevor F. SLATER,† Johanna LANG‡ and Hermann ESTERBAUER‡

**Istituto di Patologia Generale, Corso Raffaello 30, 10125 Torino, Italy, †Department of Biochemistry, Brunel University, Uxbridge, Middx. UB8 3PH, U.K., and ‡Institute of Biochemistry, University of Graz, Halbarthgasse 5/I, A-8010 Graz, Austria*

(*Received 11 November 1984/Accepted 11 December 1984*)

1. Carbonyl products were separated and identified in suspensions of rat liver microsomal fractions and in isolated hepatocytes, after stimulation of lipid peroxidation by incubation with the pro-oxidants CCl_4 and ADP–iron. 2. The carbonyl products were allowed to react with 2,4-dinitrophenylhydrazine, and the derivatives were extracted and separated by t.l.c. into three zones of non-polar materials, and one fraction of polar derivatives that remained at the origin. Separation of the individual non-polar hydrazones in each zone by h.p.l.c. demonstrated that zone I prepared from microsomal fraction or hepatocytes incubated with CCl_4 or ADP–iron contained mainly 4-hydroxyhex-2-enal, 4-hydroxynon-2-enal and 4-hydroxynona-2,5-dienal. Zone III consisted mainly of the alkanals propanal, pentanal and hexanal, the 2-alkenals propenal, pent-2-enal, hex-2-enal, hept-2-enal, oct-2-enal and non-2-enal, the ketones butanone, pentan-2-one and pentan-3-one, and deca-2,4-dienal. 3. Incubation of a microsomal fraction with ADP–iron was much more effective in producing malonaldehyde and other carbonyl products than an incubation with CCl_4. Despite such quantitative differences, there were no obvious qualitative differences in the h.p.l.c. spectra obtained from zones I and III. However, the stoichiometric evaluation of fatty acid loss and the production of malonaldehyde and other carbonyls suggests that the pathways of lipid peroxidation triggered by CCl_4 and ADP–iron are different. 4. The accumulation of carbonyl products of lipid peroxidation in isolated hepatocytes is strongly affected by their metabolism; in particular, 4-hydroxyalkenals were found to be metabolized very rapidly. Nonetheless, both CCl_4 and ADP–iron produced stimulation in the production of malonaldehyde and non-polar carbonyl production. 5. After incubation of rat hepatocytes with CCl_4 or ADP–iron it was found that approx. 50% of the total amount of non-polar carbonyls produced during incubation escaped into the external medium. This was not leakage from dead cells, as 90–95% of the hepatocytes had retained their integrity at the end of the incubation. Release of carbonyl products from cells stimulated to undergo lipid peroxidation may be a mechanism for spreading an initial intracellular disturbance to affect critical targets outside the parent cell.

The administration of CCl_4 to rats (Cameron & Karunaratne, 1936) and to many other species of animals produces centrilobular necrosis and fatty degeneration of the liver. The hepatotoxic actions of CCl_4 have been extensively studied in the rat (for background references see Slater, 1972; Dianzani, 1978), and it is established that CCl_4 has to undergo a metabolic activation in the endoplasmic reticulum in order to produce its full range of toxic effects (Recknagel, 1967; Slater, 1982). Metabolic activation results in the formation of the trichloromethyl radical, which can be detected by

the e.s.r. spin-trapping technique (Tomasi *et al.*, 1983; Poyer *et al.*, 1980; Albano *et al.*, 1982). The trichloromethyl free radical reacts quickly with molecular O_2 to produce the peroxy radical adduct, which has much greater chemical reactivity (Packer *et al.*, 1978).

The trichloromethylperoxy radical reacts readily with polyunsaturated fatty acids such as arachidonic acid (Forni *et al.*, 1983); one reaction that occurs is hydrogen abstraction, which is the first step in the process whereby CCl_4 stimulates lipid peroxidation. A stimulation of lipid peroxidation by CCl_4 has been demonstrated in liver homogenates (Comporti *et al.*, 1965), in liver microsomal suspensions (Slater, 1967; Slater & Sawyer, 1971), in isolated hepatocytes (Poli *et al.*, 1979) and *in vivo* (Recknagel & Ghoshal, 1966; Jose & Slater, 1972).

It is known that lipid peroxidation results in a wide range of carbonyl products, some of which are extremely reactive towards biomolecules (Schauenstein, 1967; Dianzani, 1982). The aldehydes produced upon stimulation of lipid peroxidation by a mixture of ADP and an iron salt (ADP–iron) in liver microsomal fraction (referred to below simply as 'microsomes') have been separated successfully by h.p.l.c., and most have been identified (Esterbauer, 1982). Although increased amounts of carbonyls have been detected bound to microsomal phospholipid and protein after intoxication of rats with CCl_4 and with $CBrCl_3$ (Benedetti *et al.*, 1982), and a complex mixture of carbonyls is known to be produced upon peroxidation of liver microsomes by CCl_4 (Esterbauer *et al.*, 1982), the identity of these bound and free carbonyls has not been ascertained before the present study.

In this paper we have applied a modification of our previously developed experimental techniques (Esterbauer *et al.*, 1982) to an analysis of the carbonyl products of CCl_4-stimulated lipid peroxidation in rat liver microsomes and in isolated rat hepatocytes. In our view it is important to demonstrate the production of such carbonyls within the cell, to monitor their metabolism and their reaction with cell components, together with their ability to escape into the extracellular fluid, in order to assess the implications of their production in relation to the overall patterns of disturbance produced by CCl_4. Our objectives were to determine if exposure to CCl_4 produces (i) significant amounts of aldehydes other than malonaldehyde, (ii) biologically important concentrations of 4-hydroxylalkenals and (iii) different patterns or amounts of aldehydes compared with lipid peroxidation stimulated by ADP–iron, which is known to be associated with quite distinct features of cytotoxicity (Poli *et al.*, 1983), and to determine (iv) if intact hepatocytes behave similarly to microsomal suspensions with respect to the preceding three points.

Experimental

Materials

Chemicals were obtained from Boehringer Mannheim ($NADP^+$, ADP, glucose-6-phosphate dehydrogenase) and Sigma Chemical Co. (nicotinamide, glucose 6-phosphate). Solvents, t.l.c. plates and all other chemicals (analytical grade) were purchased from Merck. Male Wistar rats (180–200g body wt.) were used; they were fed on standard laboratory diets (Tagger and Co., Graz, Austria, and Piccioni, Brescia, Italy).

Incubation procedures

Microsomes were prepared from rat livers as described by Slater & Sawyer (1971) after the animals had been deprived of food overnight (16h). Microsomal incubations were carried out in the presence of CCl_4 (8.6mM) or ADP–iron (1.6mM-ADP, 18μM-$FeSO_4$) in a defined medium (Slater & Sawyer, 1971) containing an NADPH-generating system. CCl_4 and ADP–iron were omitted from the control incubations

Hepatocytes were prepared as previously described (Poli *et al.*, 1981*a*) and incubated for 60min in closed 50ml flasks at 37°C in 3ml of a defined balanced salt solution (Poli *et al.*, 1979) at 5×10^6–10×10^6 cells/ml in the presence of ADP–iron (2.5mM-ADP, 100μM-$FeCl_3$) or CCl_4 (final concentration 129μM; see Poli *et al.*, 1979). CCl_4 was added to the centre well and allowed to diffuse into the closed system. Cell viability was checked before and after the incubation by Trypan Blue exclusion and by lactate dehydrogenase release (Poli *et al.*, 1979). The release of carbonyls into the extracellular medium was studied in some experiments with isolated hepatocytes; after incubation for 60min at 37°C the hepatocyte suspension was centrifuged at 50*g* for 10min at room temperature, and both the pellet and supernatant solution so obtained were analysed for carbonyls as described below.

Formation of derivatives and extraction

The following modification of the procedure of Esterbauer *et al.* (1982) was used to obtain improved yields of extracted carbonyl compounds. Microsomal incubation mixtures (14ml) and cell suspensions (adjusted to 14ml) were mixed with 7ml of dinitrophenylhydrazine reagent (0.34mg/ml in 1M-HCl) and allowed to react for 2h (20°C; dark). The complete reaction mixtures were centri-

fuged at 3000*g*, and the dinitrophenylhydrazones present in the supernatant and the pellet were extracted separately. The supernatant was extracted with 10ml of methylene dichloride. The pellet was homogenized in 10ml of chloroform/methanol (2:1, v/v) left to stand at room temperature and, after 30min, filtered (Whatman no. 43 filter paper) into an extraction tube. The filter was washed with 2ml of chloroform/methanol, and then 2.4ml of water was added. After centrifugation, the lower phase was collected, and the upper phase was further extracted with 3ml of chloroform/methanol. The chloroform extracts so obtained were pooled with the methylene dichloride extract of the supernatant, and this mixture was dried down in a rotary evaporator ($\leqslant 35°C$). To remove excess of reagent and to separate the complete mixture of hydrazones into various classes (polar carbonyls, hydroxyalkenals, osazones and alkanals), the residue was separated by t.l.c. (silica gel 60; pre-coated 20cm × 20cm; Merck) with methylene dichloride as developer. The assignment of the various bands into certain carbonyl classes was done by comparison with known standards (Esterbauer *et al.*, 1982). The individual fractions were scraped off the plates and eluted three times with 10ml of methanol, the eluate being collected by centrifugation. The material remaining at the origin was adjusted to 1ml in methanol, whereas the three non-polar fractions (hydroxyalkenals, osazones and alkanals) were further purified by t.l.c and collected as in the first step, then adjusted to 1ml in methanol. The carbonyl content of each fraction was estimated spectrophotometrically (Esterbauer *et al.*, 1982). The individual hydrazones of each fraction were separated by h.p.l.c., with the conditions indicated in the Figure legends. Peak identification and quantification was achieved by separating authentic standards under identical conditions.

Other analytical procedures

Malonaldehyde was measured by the thiobarbituric acid assay (Slater & Sawyer, 1971) and by a direct h.p.l.c. method (Esterbauer & Slater, 1981). Microsomal fatty acids were measured as previously described (Esterbauer *et al.*, 1982). The metabolism by hepatocyte suspensions of externally added hydroxyalkenals was monitored by measuring the decrease in the aldehyde concentration in the suspension as follows: a portion of cell suspension was added to an equal volume of acetonitrile, the mixture was centrifuged and the clear supernatant was separated by h.p.l.c. as described previously (Jurgens *et al.*, 1984). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Effects of CCl_4 on carbonyl formation and fatty acid composition in liver microsomes and comparison with the effects of ADP–iron

In agreement with previous reports (Slater, 1967; Slater & Sawyer, 1971), lipid peroxidation was stimulated in liver microsomes when incubated with CCl_4 and an NADPH-generating system, and when measured by the time-dependent increase of thiobarbituric acid-reacting substances. Since the thiobarbituric acid assay is not necessarily specific for malonaldehyde but could also give a positive reaction with a great variety of other substances (Slater, 1972; Esterbauer *et al.*, 1982), the formation of malonaldehyde during CCl_4-stimulated lipid peroxidation in microsomes was monitored in one kinetic experiment by the more recently developed direct h.p.l.c. method (Esterbauer & Slater, 1981), which measures malonaldehyde in the free, unmodified, form. A complete agreement (correlation coefficient $r = 0.993$) was found between the two methods, demonstrating that only free malonaldehyde is measured by the thiobarbituric acid assay under these conditions (incubation at 37°C for times ranging from 10 to 180min, and with 8.6mM-CCl_4; other conditions as indicated in the Experimental section). Other lipid-peroxidation end products were not significant contributors to the thiobarbituric acid reaction, although they are present at rather high concentrations in the samples studied, as is shown below; for this reason the thiobarbituric acid assay alone is an insufficiently reliable index of the total extent of the peroxidation processes.

As shown in Table 1, the stimulation of lipid peroxidation in liver microsomes by CCl_4 leads to the production of 14.2nmol of malonaldehyde/mg of protein (average for five experiments) compared with 5.6nmol/mg of protein in the controls, in addition to 38.2nmol/mg of protein of carbonyls other than malonaldehyde, 52% of which were polar and 48% non-polar. With the same microsomal suspensions, incubations were also performed in the presence of ADP–iron and an NADPH-generating system. Under the experimental conditions used the formation of malonaldehyde and other carbonyls was significantly higher than with CCl_4 treatment: after 30min of incubation 76.8nmol of malonaldehyde/mg of protein and 144nmol of other carbonyls/mg of protein were formed (Table 1). The values obtained here are higher than in our previous report (Esterbauer *et al.*, 1982) as a consequence of the improvement of the extraction procedure leading to a more complete recovery of the aldehyde associated with the microsomal membrane; in consequence, the

Table 1. *Effects of CCl_4 and ADP–iron on the formation of malonaldehyde and other carbonyl compounds by rat liver microsomes*

Microsomes (1 mg of protein/ml) were incubated in the presence of 8.6mM-CCl_4 or ADP–Fe^{2+} (1.6mM, 18μM) at 37°C in the presence of an NADPH-generating system for 30min. All values are means ± S.E.M.; data for the control and CCl_4-treated groups represent five experiments, and for the ADP–Fe^{2+}-treated group three experiments. All values for CCl_4-treated and ADP–Fe^{2+}-treated groups are significantly increased compared with the corresponding control value as evaluated by the Wilcoxon test ($P<0.01$).

	Carbonyl content (nmol/mg of protein)		
	Control	CCl_4-treated	ADP–Fe^{2+}-treated
Total carbonyls, including malonaldehyde	27.4±5.7	52.4±7.3	221.0± 8.5
Malonaldehyde	5.6±1.7	14.2±2.5	76.8± 5.6
Total carbonyls, except malonaldehyde	21.8±4.0	38.2±5.0	144.2±12.5
Total polar carbonyls	11.0±1.1	20.1±1.7	76.9± 8.4
Total non-polar carbonyls	10.8±3.4	18.2±5.0	67.4± 4.9
	Carbonyl composition (% of total carbonyls)		
	Control	CCl_4-treated	ADP–Fe^{2+}-treated
Malonaldehyde	20.4±6.2	27.1±4.8	34.7± 2.5
Polar carbonyls	40.1±4.5	38.3±3.2	34.7± 3.8
Hydroxyalkenals (t.l.c. zone I)	8.1±4.2	8.0±2.4	8.6± 0.3
Osazones (t.l.c. zone II)	3.6±1.5	5.6±1.2	7.5± 0.3
Alkanals, alkenals, alkadienals, ketones (t.l.c. zone III)	27.6±8.8	21.0±6.9	14.2± 2.3

values given are quantitatively more reliable than previously reported (Esterbauer *et al.*, 1982), and can be compared directly with the corresponding values for lipid peroxidation stimulated by CCl_4. Freshly prepared microsomes contained no detectable amounts of malonaldehyde and only trace amounts of other endogenous carbonyls, which were not sufficient for quantitative analysis.

The carbonyls produced during CCl_4 and ADP–iron treatment of liver microsomes were quantitatively analysed after their preseparation by t.l.c. into one class of polar and three classes of non-polar compounds, which are categorized by the three zones that they occupy on the t.l.c. plates: the three classes of non-polar carbonyls representing 4-hydroxyalkenals (zone I), osazones (zone II), and alkanals, 2-alkenals and ketones (zone III). The justification for such categorization can be found in Esterbauer *et al.* (1982).

The distribution within these three classes of non-polar compounds relative to total carbonyl production was 8% zone I, 6% zone II and 21% zone III for the CCl_4 system, whereas the values in the ADP–iron system were respectively 9%, 7% and 14%. The individual carbonyls present in each zone were then qualitatively analysed by h.p.l.c., as illustrated in Figs. 1 and 2 as examples. Zone I consisted mainly of 4-hydroxyalkenals, e.g. 4-hydroxyhex-2-enal, 4-hydroxynon-2-enal and 4-hydroxynona-2,5-dienal. The last-mentioned aldehyde was identified only by means of its mass spectrum, but the others were identified by use of standard reference compounds as well as by mass spectrometry. No significant qualitative difference was found between the patterns of the zone I carbonyls obtained from ADP–iron-treated or CCl_4-treated microsomes, despite a striking quantitative difference, which is also evident from the values reported in Table 1.

Zone III consisted mainly of alkanals (propanal, pentanal, hexanal), 2-alkenals (propenal, pent-2-enal, hex-2-enal, hept-2-enal, oct-2-enal, non-2-enal), ketones (butanone, pentan-2-one, pentan-3-one) and deca-2,4-dienal. Again no significant qualitative differences were observed in the patterns of products resulting from exposure to the two pro-oxidant agents, although quantitative variations occurred. The data in Fig. 2 considerably amplify the information given previously (Esterbauer *et al.*, 1982) for lipid peroxidation stimulated by ADP–iron. Moreover, with the improved extraction procedure used here it is possible to make a direct quantitative comparison between the two pro-oxidant stimuli studied: Figs. 1 and 2 and Table 1 emphasize that incubation with ADP–iron results in a generalized increase in carbonyl production as well as in malonaldehyde when compared with CCl_4. The h.p.l.c. chromatograms of zone II and of the polar carbonyls are not shown here, since the identification of these substances requires additional studies.

The fatty acid compositions of the microsomal

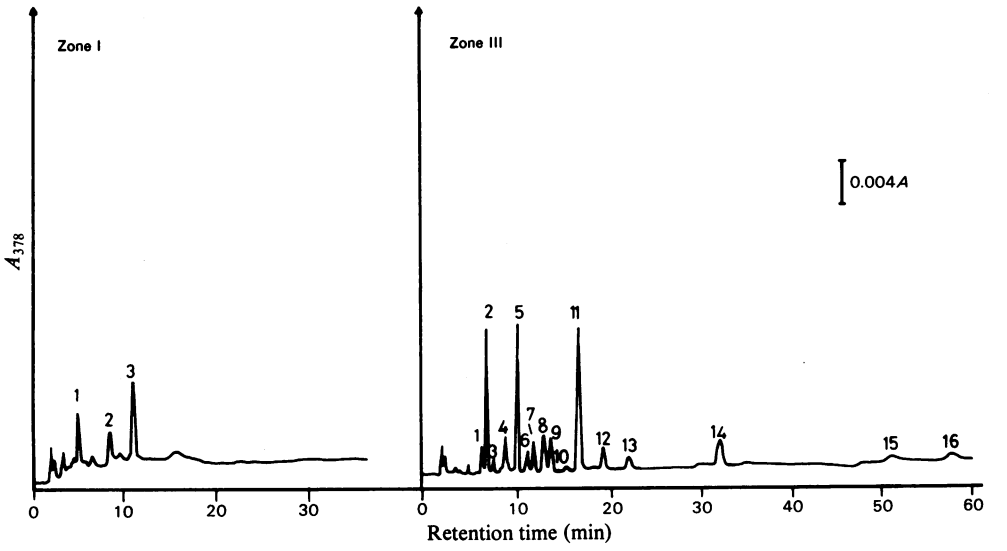

Fig. 1. *Separation by h.p.l.c. of the dinitrophenylhydrazones of the non-polar carbonyl compounds produced by rat liver microsomes incubated in the presence of 8.6mM-CCl_4 for 30min*

Zone I is the class of hydroxyalkenals recovered by t.l.c. separation (see Table 1): 1, 4-hydroxyhex-2-enal; 2, 4-hydroxynona-2,5-dienal; 3, 4-hydroxynon-2-enal. Zone III is the class of alkanals, 2-alkenals, alkadienals and ketones recovered by t.l.c. separation (see Table 1): 1, propenal; 2, propanal; 3, unknown; 4, but-2-enal; 5, butanone; 6, pent-2-enal; 7, pentanal; 8, pentan-2-one; 9, pentan-3-one; 10, hex-2-enal; 11, hexanal; 12, hepta-2,4-dienal; 13, hept-2-enal; 14, oct-2-enal; 15, non-2-enal; 16, deca-2,4-dienal. The dinitrophenyl-hydrazones obtained from 14ml of microsomal incubation medium (14mg of protein) were dissolved in 1ml of methanol, and 20μl was separated on a 5μ-Spherisorb ODS column (4.6mm × 250mm) with methanol/water (31:9, v/v) at a flow rate of 1ml/min, 378nm detector wavelength, 0.04 absorbance unit full scale and ambient temperature.

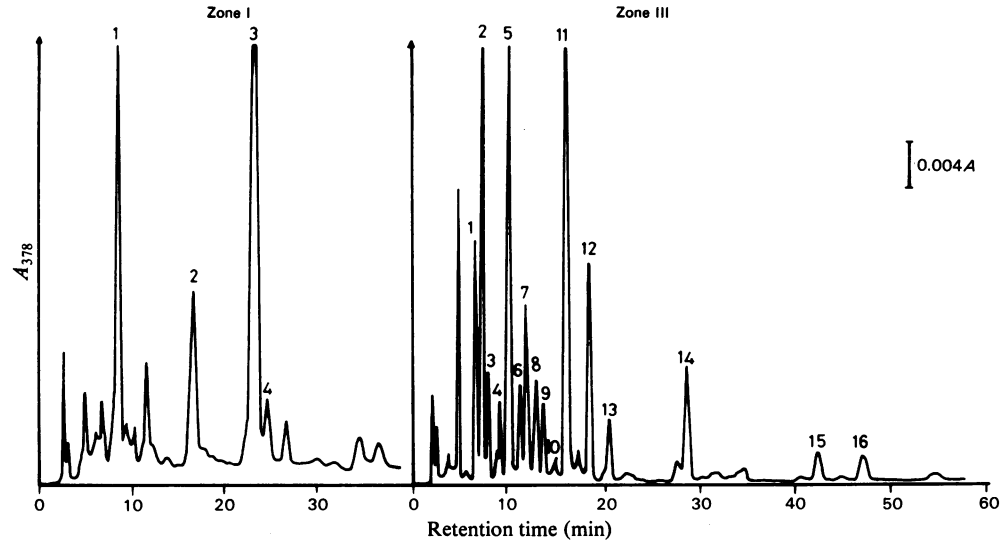

Fig. 2. *Separation by h.p.l.c. of the dinitrophenylhydrazone derivatives of the non-polar carbonyl compounds produced by rat liver microsomes incubated in the presence of 1.6mM-ADP and 18μM-Fe^{2+} for 30min*

Peak identification for zones I and III were made as outlined for Fig. 1. Experimental conditions were as indicated in Fig. 1 legend.

preparations were also investigated. Treatment with CCl_4 led to a significant loss of total polyunsaturated fatty acids: 130nmol/mg of protein, corresponding to 24% of the polyunsaturated fatty acid content of freshly prepared microsomes. After treatment with ADP–iron, the loss of polyunsaturated fatty acids was 235nmol/mg of protein, corresponding to 58% of the original value. In both cases the major decreases were in the $C_{20:4}$ and $C_{22:6}$ fatty acids. Microsomes incubated in the absence of pro-oxidant agents (controls) also showed, on average, a small decrease in their polyunsaturated fatty acid content. Combining the values for the polyunsaturated fatty acid losses with the corresponding value for the production of malonaldehyde and total other carbonyls (polar and non-polar) reported in Table 1, it can be calculated that 10mol of polyunsaturated fatty acids utilized in the presence of CCl_4 or ADP–iron yields, respectively, 1.1mol of malonaldehyde and 2.9mol of other aldehydes or 3.3mol of malonaldehyde and 6.1mol of other aldehydes. From these findings it can be postulated that the lipid-peroxidation pathways triggered by CCl_4 and ADP–iron are different. As well as malonaldehyde and other carbonyls, there are other undefined products, including polyunsaturated fatty acid–free radical adducts resulting from attack by species such as $CCl_3^{\cdot}$ (Reynolds, 1967) and cross-linked lipid materials (Frank & Link, 1984).

Effects of CCl_4 and ADP–iron on the production of carbonyls by isolated hepatocytes

Isolated hepatocytes were incubated in the presence of 0.129mM-CCl_4 or ADP–iron (2.5mM-ADP; 0.1mM-$FeCl_3$), and malonaldehyde and other carbonyls formed after 60min of incubation were analysed essentially as described above for the microsomes; Table 2 gives the results obtained. It can be seen that both CCl_4 and ADP–iron stimulate the production of malonaldehyde and non-polar carbonyls. However, CCl_4 does not stimulate the production of polar carbonyls, and, as a consequence, the production of total carbonyls is not significantly different from the controls. Although the results with ADP–iron are few in number, there is a clear tendency for large increases in total carbonyls and total polar carbonyls in addition to the effects already noted above.

The non-polar fraction in CCl_4-treated cells consisted mainly of hydroxyalkenals (21%), osazones (10%) and alkanals with 2-alkenals and ketones (69%). In the cells treated with ADP–iron the corresponding values were 24%, 28% and 48%. These distributions are quite similar to those found with microsomes (Table 1). With the CCl_4 system the formation of non-polar carbonyls is favoured over that of malonaldehyde. In fact, each 1mol of malonaldehyde formed is accompanied by the production of about 5mol of non-polar carbonyls and 9mol of total carbonyls. On the other hand, in the case of the ADP–iron system only 1.5mol of non-polar and 4.5mol of total carbonyls correspond to 1mol of malonaldehyde formed. This difference still exists if all the values are corrected for zero-time values. There are substantial qualitative differences therefore in the relative productions of malonaldehyde and other carbonyls when isolated hepatocytes are incubated with CCl_4 or ADP–iron.

Table 2. *Effects of CCl_4 and ADP–iron on the formation of malonaldehyde and other carbonyl compounds by isolated rat hepatocytes*

For details see the text. All values are means ± S.E.M., with the numbers of experiments in parentheses. *Not significant; **significant at $P<0.01$ (Student's t test).

	Carbonyl content (nmol/10^8 cells)		
	Control	CCl_4-treated	ADP–Fe^{3+}-treated
Total carbonyls, including malonaldehyde	792 ± 204 (3)	923 ± 151* (3)	1730, 1568
Malonaldehyde	32 ± 4 (7)	87 ± 16** (4)	308 ± 27 (4)
Total carbonyls, except malonaldehyde	788 ± 201 (3)	844 ± 157* (3)	1519, 1260
Total polar carbonyls	556 ± 137 (4)	404 ± 112* (4)	1000, 750
Total non-polar carbonyls	229 ± 42 (4)	440 ± 62** (4)	519, 510
	Carbonyl composition (% of total carbonyls)		
	Control	CCl_4-treated	ADP–Fe^{3+}-treated
Malonaldehyde	4.0 ± 0.5 (3)	9.4 ± 1.7 (3)	17.8, 19.6
Polar carbonyls	70.2 ± 17.3 (3)	43.7 ± 12.1 (3)	57.8, 47.8
Hydroxyalkenals (t.l.c. zone I)	5.8 ± 1.9 (4)	9.8 ± 2.1 (4)	6.3, 8.9
Osazones (t.l.c. zone II)	3.5 ± 1.7 (4)	4.9 ± 0.7 (4)	6.5, 10.8
Alkanals, alkenals, alkadienals, ketones (t.l.c. zone III)	19.6 ± 4.3 (4)	32.8 ± 4.0 (4)	16.8, 12.7

The individual carbonyls present in each of the non-polar carbonyl fractions listed in Table 2 were analysed by h.p.l.c. Examples from a typical experiment are shown in Fig. 3 for zone III components. Peak identification was as described above. Major carbonyls present in zone III were alkanals, with chain lengths from 3 to 10 carbon atoms, and butanone. No major qualitative difference existed between CCl_4-treated samples and ADP–iron-treated samples. Zone I consisted mainly of 4-hydroxyhex-2-enal, 4-hydroxynon-2-enal, 4-hydroxynona-2,5-dienal and some other as yet unidentified carbonyls. As in the case of microsomes, zone II and the polar carbonyls gave complex h.p.l.c. chromatograms, and definite peak identifications are not yet completed.

The quantitative distribution in the experimental samples of the major aldehydes mentioned above are given in Table 3. Propanal and hexanal are the major aldehydes found in isolated cells as

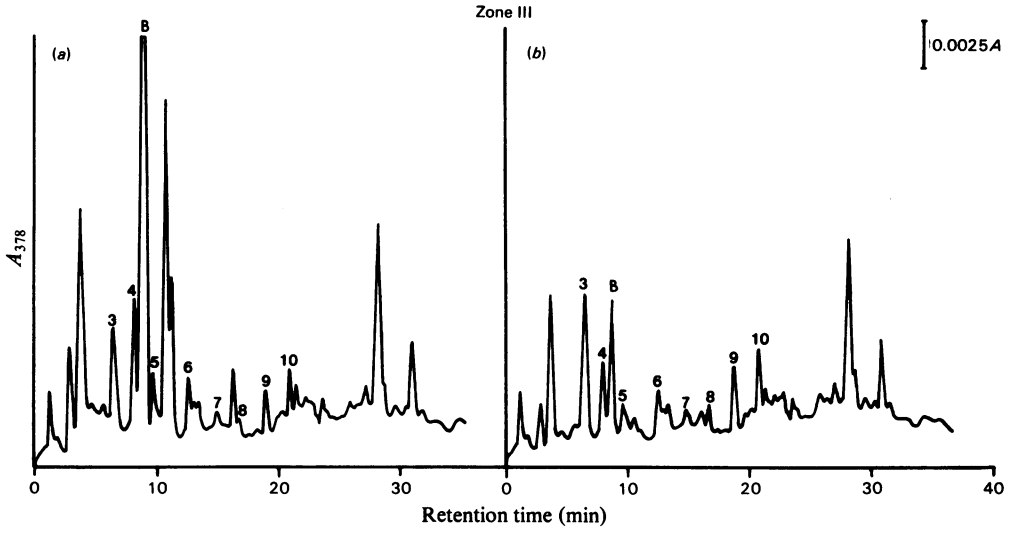

Fig. 3. *Separation by h.p.l.c. of the dinitrophenylhydrazone derivatives obtained from rat liver cells incubated for 60 min in the presence (a) or in the absence (b) of CCl_4*

The dinitrophenylhydrazone derivatives were pre-separated by t.l.c. as described in the Experimental section: zone III was dissolved in 1 ml of methanol, and 20 μl was separated on a 5 μ-Spherisorb ODS column with a linear gradient from methanol/water (7:3, v/v) to 100% methanol in 25 min, followed by 100% methanol for 10 min, at a flow rate of 1 ml/min, wavelength 378 nm, 0.025 absorbance unit full scale and ambient temperature. A reference mixture of standards composed of n-alkanals (C_1 to C_{11}) and 2-alkenals (C_6 to C_{10}) was used to identify the peaks in the cell extracts. The peak identifications decided in this way were: 3, propanal; 4, butanal; B, butanone; 5, pentanal; 6, hexanal; 7, heptanal; 8, octanal; 9, nonanal; 10, decanal.

Table 3. *Effects of CCl_4 and ADP–iron on the production of individual carbonyls by rat liver hepatocytes*

Incubation time was 1 h. For other details see the text. All values are means ± S.E.M. for three experiments.

Compound	Carbonyl content (nmol/10^8 cells)		
	Control	CCl_4-treated	ADP–Fe^{3+}-treated
Propanal	16.0 ± 6.6	38.0 ± 4.0	23.5 ± 4.5
Butanal	41.3 ± 29.6	60.6 ± 37.6	26.0 ± 13.0
Pentanal	8.0 ± 8.0	15.0 ± 9.6	5.0 ± 2.0
Hexanal	7.3 ± 2.0	32.0 ± 22.0	31.0 ± 11.0
Heptanal	2.0 ± 1.3	5.0 ± 2.3	3.0 ± 2.0
Octanal	3.6 ± 2.3	8.0 ± 6.0	5.0 ± 4.0
Nonanal	8.8 ± 4.6	4.1 ± 1.0	13.0 ± 12.0
Decanal	4.6 ± 2.3	5.6 ± 1.0	8.5 ± 7.5
Butanone	35.0 ± 7.0	160 ± 93	86.0 ± 83.0
Hydroxyhex-2-enal	0.8 ± 0.6	0.3 ± 0.3	0.8 ± 0.7
Hydroxynon-2-enal	1.3 ± 0.5	2.7 ± 1.8	4.0 ± 1.4

well as in microsomes after ADP–iron or CCl_4 treatment. The longer-chain alkanals (heptanal, octanal, nonanal and decanal) found with intact cells were not found in microsomes and appear to be endogenous metabolic products of the incubated isolated liver cells.

Butanone has been reported as an autoxidation product of cholesterol (Smith, 1981). This ketone was also found in all the experiments performed with isolated liver cells. Both CCl_4 and ADP–iron treatment seemed to increase the cellular content of butanone, but the high variability (e.g. 160 ± 93 nmol/10^8 cells with CCl_4; 86 ± 83 nmol/10^8 cells with ADP–iron) does not permit the conclusion that butanone is a lipid-peroxidation product in these experiments with whole cells. In other experiments, not reported here, we failed to detect butanone in microsomal suspensions.

4-Hydroxyhex-2-enal and 4-hydroxynon-2-enal were also consistently found in the liver cell extracts. In some experiments a peak with the retention time of 4-hydroxyoct-2-enal was also found. Only the amount of 4-hydroxynon-2-enal recovered from the cell suspension appears to be in any way related to the extent of lipid peroxidation, and the 4-hydroxyhex-2-enal content did not show any variation between the peroxidized and non-peroxidized cells.

Metabolism of aldehydes by intact hepatocytes and their distribution between intracellular and extracellular compartments

The amounts of 4-hydroxyalkenals found in isolated hepatocyte suspensions after incubation with CCl_4 or ADP–iron are very much lower than with corresponding incubations of microsomes (compare Tables 1 and 3). These large differences are suggestive of rapid metabolism of the aldehydic materials by intact cells so that the concentration found represents a competition between the rates of formation and metabolism. To confirm the hypothesis that hydroxyalkenals may be metabolized by intact hepatocytes, cells were incubated in the presence of externally added 4-hydroxypent-2-enal, 4-hydroxyoct-2-enal and 4-hydroxynon-2-enal (all present in 0.1 mM concentration). As shown by a typical experiment (Fig. 4), aldehydes were rapidly consumed by liver cells: 0.2×10^6 cells (in 1 ml of incubation medium) metabolized 90% of the added 4-hydroxynon-2-enal in 12 min of incubation at 37°C. Such high activity may explain the low recovery of this type of aldehyde from peroxidized liver cells. Variation in the metabolic rate of different cell preparations might also be in part responsible for the high variability in the experimentally obtained data.

Finally, experiments were performed in order to check the possibility that carbonyls generated in the cell during the stimulation of lipid peroxidation can escape into the incubation medium. Results obtained from three experiments in which cells were incubated in the presence or in the absence of CCl_4 showed that about 50% of the total amount of non-polar carbonyls can actually be found in the incubation medium. When hepatocytes were treated with CCl_4 for 60 min and then centrifuged, 48% of the total non-polar carbonyls were found in the cellular pellet and 52% in the supernatant medium; a practically identical ratio was obtained in the absence of CCl_4. Similar experiments in which only the distribution of malonaldehyde was monitored have led to the same conclusion (Poli *et al.*, 1981*b*). The high amount of extracellular carbonyls cannot be due to leakage from dead cells, as 90–95% of the hepatocytes retained their integrity at the end of the incubation period, as determined by Trypan Blue exclusion and by the measurement of the release of lactate dehydrogenase.

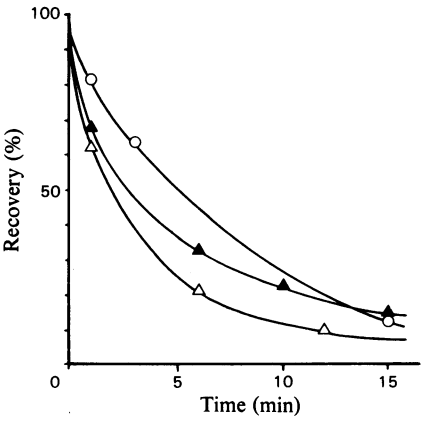

Fig. 4. *Kinetics of the metabolism of 4-hydroxyalkenals by isolated rat hepatocytes*

○, 4-Hydroxypent-2-enal; ▲, 4-hydroxyoct-2-enal; △, 4-hydroxynon-2-enal. Hepatocytes (0.2×10^6 cells/ml) were incubated in the presence of each aldehyde (0.1 mM), and the disappearance of the aldehyde was monitored by h.p.l.c.

Discussion

It has now been found that an almost complete recovery of the non-polar aldehyde products of lipid peroxidation in liver microsomes and cells is possible by employing a Folch-type extraction; subsequent use of h.p.l.c. permits the separation of all the constituents in the complex mixture (see Figs. 1 and 2).

CCl_4 is a rather weak stimulator of microsomal lipid peroxidation when compared with the effect of ADP–iron; moreover, CCl_4-stimulated lipid peroxidation results in a smaller ratio of carbonyl

compounds produced/polyunsaturated fatty acids lost (52:130 and 221:235 respectively). Thus peroxidations of liver microsomes stimulated by ADP–iron or CCl_4 have different stoichiometries. This may be due to the two pro-oxidant stimuli acting on the polyunsaturated fatty acids in different ways (perhaps by the initiating free-radical species having some considerable degree of selectivity in their loci of interaction with the fatty acid chains), or through a different pathway of degradation of the lipid hydroperoxides in the presence of relatively high iron concentration in the case of the ADP–iron stimulus. Another contribution to the difference in stoichiometry results from the covalent binding of the trichloromethyl group to polyunsaturated fatty acids; this can result in dimeric and polymeric fatty acid structures by inter-chain reactions, and will lead to an increased loss of polyunsaturated fatty acid relative to the production of carbonyl compounds.

Despite the large quantitative differences in the carbonyl content between control, CCl_4-treated and ADP–iron-treated groups, no significant qualitative differences could be detected between the three peroxidizing systems (Table 2 and Figs. 1 and 2). In each case free malonaldehyde was present as the major individual aldehydic substance, comprising 20.4% (control), 27.1% (CCl_4) and 34.7% (ADP-iron) of the total carbonyls formed in the peroxidation process. Our previously developed h.p.l.c. technique (Esterbauer & Slater, 1981; Esterbauer *et al.*, 1984) permits the measurement of free malonaldehyde present in biological samples without interferences by other malonaldehyde-like material that would give a positive reaction in the thiobarbituric acid assay. In this investigation we have found that CCl_4 stimulation produces only free malonaldehyde and no detectable precursors that would otherwise give a positive thiobarbituric acid reaction. In previous studies, similar agreement between the h.p.l.c. assay and the thiobarbituric acid reaction has also been found in the ADP–iron-stimulated liver microsomal lipid peroxidation (Esterbauer & Slater, 1981) and in various tissue fractions exposed to different pro-oxidant stimuli (Esterbauer *et al.*, 1984). It can therefore be concluded that in biological systems possible precursors for free malonaldehyde such as the endoperoxides immediately break down and release the malonaldehyde into the aqueous incubation medium.

Isolated microsomes are a good model system for studying the basic chemistry of stimulated lipid peroxidation in a biological situation, and to analyse the chemical nature of the immediate, secondary and tertiary peroxidation products. Such basic knowledge is a prerequisite for discussion of the effect and significance of lipid peroxidation in more-complex systems such as isolated hepatocytes or the living animal. The background knowledge gained from studies on microsomes facilitated the investigation of the question as to whether isolated hepatocytes respond to pro-oxidant stimuli with the formation of lipogenic carbonyls. It is well established that isolated hepatocytes peroxidize when exposed to CCl_4 or ADP–iron (Poli & Gravela, 1982; Poli *et al.*, 1983), as demonstrated by an increased production of malonaldehyde. Increases also occur in fluorescent by-products, in chemiluminescent emission and in alkane formation (Tomasi *et al.*, 1983; Smith *et al.*, 1982); similar findings have been reported for microsomes incubated with CCl_4 (Beswick *et al.*, 1981). As with isolated microsomes, complexed iron is a more powerful pro-oxidant stimulus in isolated hepatocytes than is CCl_4 (308 compared with 87 nmol of malonaldehyde/10^8 cells). The ratio of the malonaldehyde produced upon iron stimulation to malonaldehyde produced upon CCl_4 stimulation is 5.4:1 for microsomes and 3.5:1 for hepatocytes. In addition to malonaldehyde, large quantities of other carbonyls are present in cells exposed to ADP–iron or CCl_4. The respective values were 1650 nmol/10^8 cells (ADP–iron) and 923 nmol/10^8 cells (CCl_4). Moreover, from the similarities of the various classes of carbonyl compounds, and the aldehyde patterns obtained by h.p.l.c. separation, it appears that no qualitative difference occurs between the products formed in intact liver cells after stimulation of peroxidation by ADP–iron or CCl_4.

Liver cells have powerful systems to metabolize various types of aldehydes (Weiner & Wermuth, 1982). 4-Hydroxyalkenals are very rapidly consumed by isolated hepatocytes (Fig. 4), and therefore the aldehyde concentrations actually measured are steady-state concentrations. Assuming a liver cell volume of 3.6×10^{-12} litre, the following approximate intracellular concentrations of carbonyls in CCl_4-intoxicated cells can be calculated from the values in Tables 2 and 3: total carbonyls 2–5 mM, hexanal 90 μM, hydroxynon-2-enal 7 μM and butanone 0.45 mM. These concentrations are at the lower end of the range of concentrations that have effects on enzyme functions *in vitro* (for review see Dianzani, 1982). However, the lipophilic nature of some aldehydes (e.g. 4-hydroxynon-2-enal) may lead to their accumulation in the biomembranes, resulting in much higher concentrations than in free solution.

Aldehydes generated within the hepatocytes can be released to a significant extent into the surrounding medium (Table 4). In the incubation systems with 5×10^6–10×10^6 cells/ml the aldehyde concentration in the medium is extremely low as a result of a dilution effect. From the values in Table

2 and 4 the concentration of total non-polar carbonyls in the medium can be calculated to be about 50 μM. In the situation *in vivo* the concentration in the extracellular space in the immediate neighbourhood of the cells might be orders of magnitude higher. Such aldehydes can therefore spread out from the site of their origin, reach critical targets far away and induce so-called 'long-range' effects (Slater, 1976).

We are grateful to the National Foundation for Cancer Research and the Cancer Research Campaign for financial assistance.

References

Albano, E., Lott, K. A. K., Slater, T. F., Stier, A., Symons, M. C. R. & Tomasi, A. (1982) *Biochem. J.* **204**, 593–603

Benedetti, A., Fulcieri, R., Ferrali, M., Ciccoli, L., Esterbauer, H. & Comporti, M. (1982) *Biochim. Biophys. Acta* **712**, 628–638

Beswick, P. H., Cheeseman, K. H., Poli, G. & Slater, T. F. (1981) in *Recent Advances in Lipid Peroxidation and Tissue Injury* (Slater, T. F. & Garner, A., eds.), pp. 156–176, Brunel University Printing Services. Uxbridge

Cameron, G. R. & Karunaratne, W. A. E. (1936) *J. Pathol. Bacteriol.* **52**, 7–21

Comporti, M., Saccocci, C. & Dianzani, M. U. (1965) *Enzymologia* **29**, 185–204

Dianzani, M. U. (1978) in *Biochemical Mechanisms of Liver Injury* (Slater, T. F., ed.), pp. 45–95, Academic Press, London

Dianzani, M. U. (1982) in *Free Radicals, Lipid Peroxidation and Cancer* (McBrien, D. C. H. & Slater, T. F., eds.), pp. 129–158, Academic Press, London

Esterbauer, H. (1982) in *Free Radicals, Lipid Peroxidation and Cancer* (McBrien, D. C. H. & Slater, T. F., eds.), pp. 101–128, Academic Press, London

Esterbauer, H. & Slater, T. F. (1981) *IRCS Med. Sci. Libr. Compend.* **9**, 749–750

Esterbauer, H., Cheeseman, K. H., Dianzani, M. U., Poli, G. & Slater, T. F. (1982) *Biochem. J.* **208**, 129–140

Esterbauer, H., Lang, J., Zadravec, S. & Slater, T. F. (1984) *Methods Enzymol.* **105**, 319–328

Forni, L. G., Packer, J. E., Slater, T. F. & Willson, R. L. (1983) *Chem.-Biol. Interact.* **45**, 171–177

Frank, H. & Link, B. (1984) *Biochem. Pharmacol.* **33**, 1127–1130

Jose, P. J. & Slater, T. F. (1972) *Biochem. J.* **128**, 141P

Jurgens, G., Lang, J., Esterbauer, H. & Holasek, H. (1984) *IRCS Med. Sci. Libr. Compend.* **12**, 252–253

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Packer, J. E., Slater, T. F. & Willson, R. L. (1978) *Life Sci.* **23**, 2617–2620

Poli, G. & Gravela, E. (1982) in *Free Radicals, Lipid Peroxidation and Cancer* (McBrien, D. C. H. & Slater, T. F., eds.), pp. 215–242, Academic Press, London

Poli, G., Gravela, E., Albano, E. & Dianzani, M. U. (1979) *Exp. Mol. Pathol.* **30**, 116–127

Poli, G., Cheeseman, K. H., Slater, T. F. & Dianzani, M. U. (1981*a*) *Chem.-Biol. Interact.* **37**, 13–24

Poli, G., Gravela, E., Albano, E., Chiarpotto, E., Miglietta, A. & Dianzani, M. U. (1981*b*) in *Recent Advances in Lipid Peroxidation and Tissue Injury* (Slater, T. F. & Garner, A., eds.), pp. 118–132, Brunel University Printing Services, Uxbridge

Poli, G., Chiarpotto, E., Albano, E., Biasi, F., Cecchini, G. & Gravela, E. (1983) *Chem.-Biol. Interact.* **43**, 253–261

Poyer, J. L., McCay, P. B., Lai, E. K., Janzen, E. G. & Davis, E. R. (1980) *Biochem. Biophys. Res. Commun.* **94**, 1154–1160

Recknagel, R. O. (1967) *Pharmacol. Rev.* **19**, 145–208

Recknagel, R. O. & Ghoshal, A. K. (1966) *Lab. Invest.* **15**, 132–147

Reynolds, E. S. (1967) *J. Pharmacol. Exp. Ther.* **155**, 117–126

Schauenstein, E. (1967) *J. Lipid Res.* **8**, 417–428

Slater, T. F. (1967) *Proc. FEBS Meet. 4th (Oslo)* Abstr. no. 216

Slater, T. F. (1972) *Free Radical Mechanisms in Tissue Injury*, pp. 1–283, Pion, London

Slater, T. F. (1976) *Panminerva Med.* **18**, 381–390

Slater, T. F. (1982) in *Free Radicals, Lipid Peroxidation and Cancer* (McBrien, D. C. H. & Slater, T. F., eds.), pp. 243–274, Academic Press, London

Slater, T. F. & Sawyer, B. C. (1971) *Biochem. J.* **123**, 805–814

Smith, L. L. (1981) *Cholesterol Autoxidation*, pp. 1–692, Plenum Press, New York

Smith, M. T., Thor, H., Hartzell, P. & Orrenius, S. (1982) *Biochem. Pharmacol.* **31**, 19–26

Tomasi, A., Billing, S., Garner, A., Slater, T. F. & Albano, E. (1983) *Chem.-Biol. Interact.* **46**, 353–368

Weiner, H. & Wermuth, B. (1982) *Enzymology of Carbonyl Metabolism: Aldehyde Dehydrogenase and Aldol-keto Reductase*, pp. 1–428, Alan R. Liss, New York