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Lipid Peroxidation and Biogenic Aldehydes: From the Identification of 4-Hydroxynonenal to Further Achievements in Biopathology

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The formation, reactivity and toxicity of aldehydes originating from lipid peroxidation of cellular membranes are reviewed. Very reactive aldehydes, namely 4-hydroxyalkenals, were first shown to be formed in autoxidizing chemical systems. It was subsequently shown that 4-hydroxyalkenals are formed in biological conditions, i.e. during lipid peroxidation of liver microsomes incubated in the NADPH–Fe systems. Our studies carried out in collaboration with Hermann Esterbauer which led to the identification of 4-hydroxynonenal (4-HNE) are reported. 4-HNE was the most cytotoxic aldehyde and was then assumed as a model molecule of oxidative stress. Many other aldehydes (alkanals, alk-2-enals and dicarbonyl compounds) were then identified in peroxidizing liver microsomes or hepatocytes. The *in vivo* formation of aldehydes in liver of animals intoxicated with agents that promote lipid peroxidation was shown in further studies. In a first study, evidence was forwarded for aldehydes (very likely alkenals) bound to liver microsomal proteins of CCl_4 or $BrCCl_3$-intoxicated rats. In a second study, 4-HNE and a number of other aldehydes (alkanals and alkenals) were identified in the free (non-protein bound) form in liver extracts from bromobenzene or allyl alcohol-poisoned mice. The detection of free 4-HNE in the liver of CCl_4 or $BrCCl_3$-poisoned animals was obtained with the use of an electrochemical detector, which greatly increased the sensitivity of the HPLC method. Furthermore, membrane phospholipids bearing carbonyl groups were demonstrated in both *in vitro* (incubation of microsomes with NADPH–Fe) and *in vivo* (CCl_4 or $BrCCl_3$ intoxication) conditions. Finally, the results concerned with the histochemical detection of lipid peroxidation are reported. The methods used were based on the detection of lipid peroxidation-derived carbonyls. Very good results were obtained with the use of fluorescent reagents for carbonyls, in particular with 3-hydroxy-2-naphtoic acid hydrazide (NAH) and analysis with confocal scanning fluorescence microscopy with image video analysis. The significance of formation of toxic aldehydes in biological membranes is discussed.

Keywords: Lipid peroxidation, cellular membranes, aldehydes, 4-hydroxynonenal, histochemistry for lipid peroxidation

INTRODUCTION

It has been known for a long time that the peroxidation of unsaturated fatty acids gives rise to aldehydes as secondary oxidation products, the primary products being hydroperoxides. The latter are in fact unstable and decompose readily to secondary products which, in turn, may give rise to the formation of a third and a fourth

generation of compounds. Finally, a great diversity of lipid-derived substances accumulate which have various functional groups; such as aldehyde, keto, hydroxy, epoxy and carboxy groups.

The aldehyde products of lipid peroxidation have been of particular interest in food chemistry, because they are mainly responsible for the flavour defects of many foodstuffs and beverages in the well-known process of fat and oil rancidity. Despite this attention, up to 30–40 years ago, relatively few aldehydes, such as malonaldehyde (MDA) and other dicarbonyl compounds, had been definitely identified.

In the 1960s, the group of Prof. Schauenstein, in which Hermann Esterbauer was an active young researcher, showed[1–3] that some aldehydic products of lipid peroxidation exhibit a number of biological effects. Although it was not clear at that time whether such aldehydes could be formed in biological systems, nevertheless the importance of the many biological effects shown by these aldehydes, such as their antitumoral effect, markedly stimulated chemical and biological studies on these products. The Schauenstein–Esterbauer group demonstrated[3,4] the formation from autoxidizing methyl linoleate of 4-hydroxyoctenal, a prototype of a new class of aldehydes, namely 4-hydroxyalkenals. A chemical synthesis was developed by Esterbauer and Weger[5] which allowed the preparation of 4-hydroxyalkenals of almost any desired structure.

One of these synthetic aldehydes, 4-hydroxypentenal (4-HPE), was the most thoroughly investigated[4] since it can be prepared with the highest purity and is water-soluble. As will be examined below, the study of the biological activities of these aldehydes was mostly concerned with the inhibitory effects on cell functions and, in particular, with the inhibition of tumour cell growth.[4]

Although 4-hydroxyalkenals proved to have interesting properties, their significance in a biological sense remained uncertain without unequivocal evidence for their formation in biological systems. The relevance of the subsequent investigations was the recognition that hydroxyalkenals, in particular 4-hydroxynonenal, are formed during lipid peroxidation occurring in a well-known biological condition such as that represented by the NADPH oxidation by liver microsomes in the absence of an electron-accepting terminal substrate.[6,7]

These investigations originate from a completely different line of research; that is the studies carried out by our group and concerned with the pathophysiology of carbon tetrachloride hepatotoxicity. It was, in fact, demonstrated[8-10] that CCl_4, through the haloalkane free radicals originating from its metabolism, promotes lipid peroxidation in the membranes of liver cell, particularly those of the endoplasmic reticulum. This process appeared to be of particular interest in the mechanisms of CCl_4-induced hepatocellular injury. However, even assuming that lipid peroxidation was the key event in the CCl_4-induced liver damage, it remained to be clarified how a process initially restricted to a particular site of the endoplasmic reticulum (for instance, the cytochrome P_{450} site) could produce damaging effects on cellular structures distant from that site. On the basis of theoretical[10] and experimental data, the concept developed according to which toxic products are formed during lipid peroxidation, diffuse from the locus in which lipid peroxidation is set into motion, and act at distant loci in the cell. Supporting evidence for this concept (i.e. action at a distance of diffusible lipid peroxidation products) was found in studies of various laboratories.[11–15] In our studies,[11,12] the effects were shown to occur in revealing or target systems which were separated from the peroxidizing system (liver microsomes, NADPH, Fe^{2+}, etc.) by a dialysis membrane. The cytotoxic effects [lysis of erythrocytes, inhibition of microsomal enzymes, such as glucose-6-phosphatase (G-6-Pase), etc.] indicated that the cytopathological products originating from the peroxidation of liver microsomal lipids were dialysable and therefore of a relatively stable

nature. These cytotoxic products could be recovered in extracts obtained from the dialysate.[16] After separation of the products by thin-layer chromatography (TLC), it was found[16] that the highest inhibitory activity for microsomal G-6-Pase occurred in a band which contained most of the carbonyl functional groups present in the unfractionated dialysate extract.

I communicated these results to Prof. Trevor Slater, who was, besides a friend, the Scientific Director of the National Foundation for Cancer Research (USA), created by the famous Nobel Laureate Albert Szent-Györgyi. Slater and Dianzani (my former Director) were actively engaged in collaborative researches with the Graz group on the biological effects of the above mentioned 4-hydroxypentenal. They immediately promoted an informal meeting with Schauenstein, Esterbauer and myself to prompt investigation aimed to characterize the aldehydes present in the TLC band mentioned above. The meeting was held in Venice on November '78 and it was decided that the products should be identified in Graz. So, this was the first time I met Hermann; we were the youngest fellows of the group and we had a good time to comment on the events of the informal meeting with lot of jokes during the night in the Riviera degli Schiavoni in front of big glasses of beer. The sympathy, the sincerity and the sense of humour of Hermann led us to familiarize ourselves immediately and we became such friends that in all the subsequent years we used to meet very frequently in Siena or in Graz to discuss results of our collaborative studies and to have good time too. Hermann was also Visiting Professor in my Faculty in 1989.

Coming back to science, the aldehydes contained in the TLC band were identified during a 20-day visit of Benedetti (my senior collaborator) and myself in Graz (1979). They were 4-hydroxyalkenals, almost entirely (more than 95%) 4-hydroxy-2,3-trans-nonenal (4-HNE), with minimal amounts of 4-hydroxyoctenal, 4-hydroxydecenal and 4-hydroxyundecenal.[17] The separation was performed by HPLC and the structure was ascertained by infrared and mass spectrometry.

The kinetics of formation of 4-hydroxynonenal (4-HNE) in peroxidizing liver microsomes is

PHOTO An informal meeting in Venice, November 1978. From the left: Hermann Esterbauer, Trevor Slater, Erwin Schauenstein, Mrs. Schauenstein, Mrs. Dianzani, Mario Umberto Dianzani and Mario Comporti.

similar to that of MDA.[18] The ratio between 4-HNE and MDA production is about 1:10 on a molar basis.[18] Another cytotoxic aldehyde, contained in a close TLC band, was identified some years later as 4,5-dihydroxy-2,3-decenal, again in collaboration with Esterbauer.[19] It is formed in lower amounts compared to 4-HNE (ratio of 1.5–10, on a molar base) in the course of microsomal lipid peroxidation. Thus the most important cytotoxic product up to now known to derive from peroxidation of cellular membranes is 4-HNE. This product was also assumed as a model molecule of oxidative stress.

WHOLE PATTERN OF ALDEHYDES ORIGINATING FROM PEROXIDATION OF MEMBRANE LIPIDS. REACTIVITY AND BIOLOGICAL EFFECTS

The whole pattern of carbonyls originating from the ADP–Fe^{2+}-stimulated peroxidation of liver microsomal lipids was studied by Esterbauer *et al.*[20] and Poli *et al.*[21] The products were separated into two fractions (a polar and a non-polar fraction) by TLC. The non-polar fraction contained *n*-alkanals (31%), alk-2-enals (9%), α-dicarbonyl compounds (22%) and 4-hydroxyalkenals (37%). The identified alkanals were propanal, butanal, pentanal, hexanal and nonanal (the latter two being the most represented ones). The alk-2-enals were acrolein, pent-2-enal, hex-2-enal, hept-2-enal, oct-2-enal (the most represented) and 4-hydroxy-2,5-nonadienal. Ketones and osazones were also formed. A similar pattern of aldehydes was also found[21] when isolated hepatocytes were incubated with pro-oxidants such as CCl_4 or ADP–Fe^{2+}. MDA was in any case the most predominant aldehyde. Fifty percent of the total amount of non-polar carbonyls produced by the hepatocytes was released into the incubation medium.

Long-chain saturated aldehydes show a relatively low reactivity towards molecules of biological interest, i.e. amino acids, proteins, nucleic acids, etc. The only reaction which takes place readily is the reaction with cysteine to give thiazolidine carboxylic acids.[4,22] This reaction could be responsible for the inhibitory effect of some saturated aldehydes on protein biosynthesis in liver slices and hepatoma cells.[23]

Highly hydrated aldehydes such as formaldehyde, acetaldehyde and other short-chain aldehydes are very reactive. They can react with the amino groups of amino acids and primary amines to give carbinolamines and Schiff's bases in readily reversible reactions.[4] Aldehydes which are predominantly present as hydrates have the capacity to produce cross-linkages between two protein molecules.[4] However, the bulk of longer-chain alkanals derived from lipid peroxidation show a low reactivity.

In contrast to alkanals, 2-alkenals and 4-hydroxyalkenals are highly reactive lipid peroxidation products which react readily even at neutral pH and at low concentrations with biomolecules, especially those having sulphydryl groups such as glutathione, cysteine, coenzyme A. Proteins or enzymes containing –SH groups are also attacked.[4,24] Under physiological conditions, the main reaction is the Michael addition, that is, one molecule of the α,β-unsaturated aldehyde reacts with the –SH group in a 1,4-addition reaction (Figure 1). The reaction product initially formed is a saturated aldehyde in which the –SH group is bound to C(3) by a thioether linkage. 4-Hydroxyalkenals react about five times faster than 2-alkenals and the adducts formed are about 500 times more stable.[25] The higher stability of the adduct is due to the formation of cyclic hemiacetals (Figure 1).

It has been shown that under certain conditions (alkaline pH, high concentration of the reactants) 2-alkenals and 4-hydroxyalkenals can also react with the amino groups of amino acids[4] and proteins. However, the reactivity of amino groups with 2-alkenals of 4-hydroxyalkenals is 2–3 orders of magnitude lower than the reactivity with –SH groups. Therefore, the biological effects of the 4-hydroxyalkenals investigated so far can be explained mainly by the reaction with sulphydryl groups and the inactivation of essential –SH

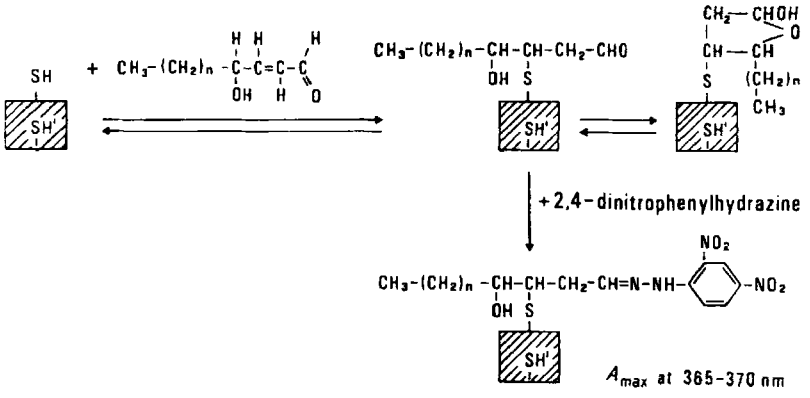


FIGURE 1 Reaction of 4-hydroxyalkenals with the –SH groups of protein and detection of protein-bound aldehydes by 2,4-dinitrophenylhydrazine.

groups. If isolated proteins and enzymes containing –SH groups are incubated with 4-hydroxyalkenals, the number of aldehyde molecules bound to the protein equals the number of –SH groups lost during the incubation.[24] Similar results were obtained in our laboratory[26] when liver microsomes were incubated with 4-HNE. A substantial amount of the aldehyde could be recovered bound to the microsomal protein. Concomitantly with the binding, a decrease of exposed and total –SH groups of the microsomal protein occurred. The decrease in total –SH groups appeared to be accounted for by the amount of aldehyde bound. Coincidentally with the binding, the G-6-Pase activity of the microsomes decreased markedly.[26]

4-Hydroxyalkenals show inhibiting effects on a broad number of cellular functions (see Refs. [27,28], for reviews). They can be summarized as follows:

(a) inhibition of mitochondrial functions (respiration, dinitrophenol-stimulated ATPase, swelling and phosphate transport[28,29]), as observed with isolated liver mitochondria, hepatocytes and Ehrlich Ascites tumour cells (EATC);[3,24,30]
(b) inhibition of anaerobic glycolysis in EATC;[3,24]
(c) inhibition of DNA and RNA synthesis in EATC and fibroblasts;[24,30–32]
(d) inhibition of protein synthesis in cell-free systems,[30,33] isolated hepatocytes[28] and other cellular lines;[32]
(e) inhibition of cell growth and transplantability in cultured EATC;[4,31] inhibition of growth of transplanted solid tumours;
(f) inhibition of G-6-Pase and cytochrome P_{450} in isolated liver microsomes[17,26] and hepatocytes;[34]
(g) inhibition of calcium sequestering activity of liver microsomes;[35]
(h) inhibition of plasma membrane adenylate cyclase and 5-nucleotidase;[36]
(i) loss of viability of isolated hepatocytes[34] and EATC;[31]
(j) loss of triglyceride secretion by isolated hepatocytes;[34]
(k) genotoxic effects;[37,38]
(l) induction of heat shock protein HSP 31;[39]
(m) chemotactic activity towards polymorphonuclear cells.[40]

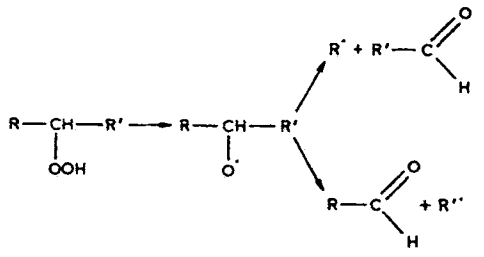
The aldehyde concentrations necessary to produce the various biological effects ranged from 500 to 1 μM. Plasma membrane adenylate cyclase[36] and microsomal calcium pump[35] were affected at the lowest concentrations (4-HNE). In every case, 4-HNE was more effective than 4-HPE.

As mentioned above, our studies on the biological effects of aldehydes were primarily

concerned with the possibility that 4-HNE and other carbonyls originating from the peroxidation of membrane lipids were responsible, at least in part, for some aspects of the liver injury induced by CCl_4 and other pro-oxidants. As already seen, the main alterations of the CCl_4-induced liver damage (inhibition of microsomal activities, G-6-Pase, cytochrome P_{450} and calcium sequestration, inhibition of protein synthesis, inhibition of triglyceride secretion, loss of viability of isolated hepatocytes and consequent release of cellular enzymes, see Refs. [41,42] for reviews), are reproduced, at least in model system *in vitro*, by the addition of 4-HNE to the incubation medium. Furthermore, 4-HNE, while inhibiting those microsomal enzymes (such as G-6-Pase,[17,26,34] cytochrome P_{450} and aminopyrine demethylase[26]) which are affected by either lipid peroxidation *in vitro*[41] or carbon tetrachloride intoxication *in vivo*,[41] does not inhibit microsomal NADPH cytochrome *c* reductase,[26] which is not inhibited by either lipid peroxidation[26,43] or CCl_4 poisoning.[26,41]

POSSIBLE MECHANISM OF ALDEHYDE FORMATION

The chemical pathways leading to the formation of various aldehydes from peroxidized unsaturated fatty acids has been extensively studied in chemical systems.[44] Lipid peroxides, which are the primary products of peroxidation, decompose easily and yield a great variety of products containing oxygen functional groups. Theoretically, aldehydes can be generated by chain-cleavage reactions from the primary monohydroperoxides, or from the other, secondary and/or tertiary, oxygenated products. In the case of cleavage of monohydroperoxide, the most likely reaction is the dismutation of monohydroperoxide which proceeds, most probably, via the formation of an alkoxy radical which then decomposes by a chain-cleavage reaction to an aldehyde and an alkyl radical:[44]



For instance, hexanal (which is the main *n*-alkanal originating from the peroxidation of membrane lipids) can be formed by dismutation of various monohydroperoxides, among which 15-hydroperoxy-arachidonic acid or 13-hydroperoxy-linoleic acid.

As shown in the scheme, the aldehyde group can occur after chain cleavage, on either side of the carbon chain. Therefore, in membrane phospholipids, where most of the polyunsaturated fatty acids are in the β position, β-acyl residues bearing carbonyl functions are formed. This occurrence has been demonstrated[45] in liver microsomes peroxidized *in vitro*. Membrane phospholipids bearing carbonyl functions have also been demonstrated by our group in the liver of both haloalkane-poisoned rats[18] and bromobenzene-poisoned mice.[46] Thus, in addition to the release of cytotoxic carbonyls such as 4-HNE, an alteration of the membrane structure is caused directly by lipid peroxidation, since the formation of polar groups at sites normally containing non-polar hydrocarbon chains could result in alterations of the lipid domain of the membrane.

Many aldehydes, in principle those containing two or more oxygen atoms, cannot be originated simply by dismutation of the monohydroperoxide. To explain the origin of these aldehydes, a further oxidation of the molecule should be hypothesized; alternatively, secondary and/or tertiary products and/or rearrangement products should be the precursor. MDA, according to Pryor and Stanley,[47] is formed by cyclic

endoperoxides which are produced by a rearrangement of the parent monohydroperoxide.

As far as the biochemical origin of the 4-HNE in peroxidizing liver microsomes is concerned, we, in collaboration with Esterbauer, have shown[48] that the aldehyde arises mainly from arachidonic acid contained in polar lipids. Neither arachidonic acid of neutral lipids nor linoleic acid of polar or neutral lipids seems to be a substrate for 4-HNE generation. This finding results from the estimation of the specific radioactivity of 4-HNE produced by rat liver microsomes prelabelled *in vivo* with [U-^{14}C] arachidonic acid. Phospholipid-bound 15-hydroperoxy-arachidonic acid would have the structural requirement for 4-HNE. However, microsomes incubated with 15-hydroperoxy-arachidonic acid converted only minimal amounts of the peroxide into 4-HNE. From this study phospholipid-bound 15-hydroperoxy-arachidonic acid did not seem to be an intermediate in the reaction pathway leading to 4-HNE. However, in more recent studies[49] carried out with autoxidizing polyunsaturated fatty acids (PUFAs) two mechanisms for the formation of 4-HNE were proposed. Both mechanisms meet the requirement that 4-HNE can only be formed from omega-6-PUFAs such as 20:4 and 18:2. In one mechanism the precursor is 11-hydroperoxy-arachidonic acid (or 9-hydroperoxy-linoleic acid), while in the other the precursor would be the positional isomers 15-hydroperoxy-arachidonic acid (or 13-hydroperoxy-linoleic acid). The difference in the systems used (microsomes supplemented with NADPH–Fe[48] or autoxidizing PUFAs[49]) may explain the discrepancy.

DETECTION OF ALDEHYDES IN THE LIVER OF ANIMALS INTOXICATED WITH PRO-OXIDANT AGENTS

The *in vivo* formation of aldehydes in the liver of animals intoxicated with pro-oxidants has been shown in several studies from our laboratory.

In a first study,[50] evidence was forwarded for aldehydes (very likely alkenals) bound to the liver microsomal protein of rats intoxicated with CCl_4 and $BrCCl_3$. As previously discussed, the main reaction of alkenals or 4-hydroxyalkenals is the Michael addition to the –SH groups of low molecular weight thiols, proteins and enzymes. This reaction leads to the formation of a modified protein in which the aldehyde moieties are bound covalently by thioether linkages to cysteine residues of the polypeptide chain (Figure 1). Since the aldehyde group remains free after the binding, the reaction of this group with 2,4-dinitrophenylhydrazine (DNPH) results (Figure 1) in the formation of the respective dinitrophenilhydrazone derivative.[24,51] These protein-bound dinitrophenilhydrazones show an absorption spectrum which, under the test conditions used, has a maximum in the 365–370 nm range. Therefore the appearance of such a spectrum in the microsomal protein allowed to react with DNPH is indicative of the aldehyde binding. Furthermore, the evaluation of the spectrum should allow a quantitative estimation of the amount of aldehydes bound to the microsomal protein. Spectrophotometric examination of the DNPH-treated microsomal protein from rats intoxicated with CCl_4 and $BrCCl_3$ showed absorption spectra typical of protein-bound dinitrophenylhydrazones. The amount of protein-bound carbonyls found in the *in vivo* intoxications (0.8–1.1 nmol/mg protein) was not very different from the amount of protein-bound carbonyls (2.5 nmol/mg protein) found in the *in vitro* condition in which lipid peroxidation was induced in liver microsomes by 6 μM Fe^{2+}. It must be noted that in this *in vitro* condition significant membrane damage occurs.[50]

In a second study,[52] we searched for the presence of 4-HNE and other lipid peroxidation products in free forms (that is non-protein-bound) in the liver of bromobenzene-poisoned mice. Bromobenzene hepatotoxicity in fact represents a good model for the study of *in vivo* lipid peroxidation, since in this experimental condition

the level of detectable lipid peroxidation is by far greater than that in the case of CCl_4 or $BrCCl_3$ hepatotoxicity.[46] 4-HNE was looked for in liver extracts as either free aldehyde or its dinitrophenylhydrazone derivative. In both cases, by means of TLC and HPLC, a well-resolved peak corresponding to the respective standards of 4-HNE (free aldehyde or dinitrophenylhydrazone derivative) was obtained (Figure 2). Total carbonyls were detected as dinitrophenylhydrazone derivatives. The hydrazones were pre-separated by TLC into three fractions according to different polarity (polar, non-polar fraction I and non-polar fraction II). The amount of carbonyls present in each fraction was determined by UV–VIS spectroscopy. Non-polar carbonyl fraction II was fractionated further by TLC. The fraction containing alkanals and alk-2-enals was analysed by HPLC and several aldehydes were identified. The identified alkanals were butanal, pentanal, hexanal (the highest peak), heptanal and nonanal. The identified alk-2-enals were pent-2-enal, hept-2-enal and oct-2-enal. Therefore the pattern of carbonyls formed in the liver of bromobenzene-poisoned mice is somewhat similar to that produced during the *in vitro* lipid peroxidation of liver microsomes[20] or isolated hepatocytes.[21]

Very similar results were obtained using the same method, for the detection of 4-HNE and other carbonyls in the liver of allyl alcohol-intoxicated mice.[53]

In further studies[54] we detected 4-HNE as free aldehyde in the liver of CCl_4 or $BrCCl_3$-intoxicated mice. Since, as previously mentioned, in these intoxications the level of peroxidation is much lower than in the case of bromobenzene and allyl alcohol, we needed a procedure with higher sensitivity. The electrochemical detection, instead of UV-VIS detection, of the 2,4-dinitrophenylhydrazone derivative of the aldehyde (separated essentially by the same HPLC procedure used previously[20,52]) increased the sensitivity by about 25-fold and allowed the detection of 4-HNE below the pmol level. The hepatic levels of 4-HNE found by this procedure in the *in vivo* intoxications with the pro-oxidants mentioned above are given in Table I.

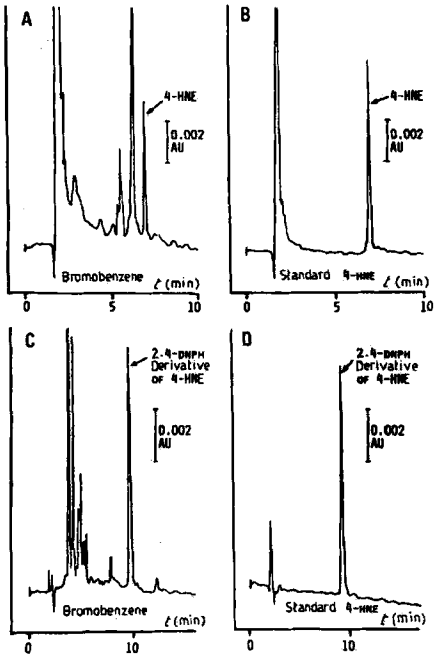


FIGURE 2 Detection by HPLC of 4-hydroxynonenal (4-HNE) as free aldehyde (A) or 2,4-dinitrophenylhydrazone derivative (C) in the livers of bromobenzene-poisoned mice. (B) Standard 4-HNE; (D) 2,4-dinitrophenylhydrazone derivative of standard 4-HNE. Separation conditions: reversed-phase column, Zorbax™ ODS (4.6 mm × 25 cm); mobile phase, acetonitrile/water (1:1, v/v); flow rate 1 cm^3/min; detector wavelength, 222 or 350 nm, for free aldehyde or its 2,4-dinitrophenylhydrazone derivative, respectively. A sample derived from the liver corresponding to 5 mg of protein was injected. (From Benedetti *et al.*,[52] with permission).

TABLE I Hepatic concentrations of 4-HNE and MDA in control and CCl_4, $BrCCl_3$ or bromobenzene intoxicated mice[a]

Treatment	*n*	4-HNE (pmol/mg protein)	MDA (pmol/mg protein)
Control	6	4.5 ± 0.7	18 ± 3
CCl_4	3	11.3 ± 0.7	33 ± 7
$BrCCl_3$	3	26.2 ± 3.8	40 ± 4
Bromobenzene	3	90.6 ± 19.3	1251 ± 420

[a]4-HNE was determined by high-performance liquid chromatography with electrochemical detection. MDA was determined with thiobarbituric acid. The animals were intoxicated and sacrificed at different times as described in Materials and Methods (from Goldring *et al.*[54] with permission). Results are means ± SE.

Various highly sensitive and specific gas chromatography–mass spectrometry methods are now also available for the determination of 4-HNE.[55] The sensitivity of the methods is below 0.01 pmol of 4-HNE.

HISTOCHEMICAL DETECTION OF LIPID PEROXIDATION

As already discussed, sophisticated and sensitive biochemical procedures are nowadays available for the determination of even minimal levels of oxidative stress *in vivo*, besides in experimental models consisting of isolated cells or cellular subfractions. However, such approaches do not generally allow information concerning the distribution of phenomena *in situ*, while this aspect is of great potential importance for the understanding of oxidative mechanisms involved in cell injury, especially in the case of tissues with a heterogeneous cell composition, such as e.g. brain, lung or kidney.

In a few laboratories, including ours, the issue has been dealt with from a histochemical point of view. Thus, the possibility has been evaluated to develop procedures provided with high sensitivity and specificity in order to directly reveal the effects of oxidative stress on sections of tissue or cellular smears, making feasible in this way to discriminate areas, cellular types and – possibly – subcellular sites being involved in these processes.

Our attention was originally directed to lipid peroxidation, by developing methods aimed at revealing aldehyde and carbonyl products. In fact, as previously mentioned, in the case of alkenals, the binding to –SH groups of proteins leaves the aldehyde grouping free for the reaction with aldehyde reactants (Figure 1). The same reaction could also involve the carbonyl functions present, as previously shown[18] in acyl residues of membrane phospholipids and formed as a consequence of the peroxidative breakdown of unsaturated fatty acids. The direct Schiff's reaction was first used by us for the visualization of small foci with decreased sensitivity to induction of lipid peroxidation *in vitro*, in cryostat sections obtained from the liver of rats to which a carcinogenic treatment had been administered.[56] Subsequently, the same procedure was applied to the detection of hepatic lipid peroxidation *in vivo* in the model of bromobenzene intoxication.[57] At low, initial levels of lipid peroxidation – as assessed by biochemical methods on adjacent tissue specimens – histochemistry of lipid-derived aldehydes revealed in these animals a mediolobular distribution, which then spreads to involve the remaining parenchyma.

Direct Schiff's reaction was subsequently employed with success in other experimental conditions, allowing the demonstration of the selective involvement of the *substantia nigra* during *in vitro* iron-induced lipid peroxidation,[58] and of rat tubular proximal epithelium during kidney lipid peroxidation induced *in vivo* by the nefrocarcinogen iron nitrilotriacetate.[59]

Our subsequent efforts were focused on the development of procedures provided with sensitivity and reproducibility higher than those of Schiff's reaction. Good results were obtained by using a reaction based on 3-hydroxy-2-naphthoic acid hyrazide (NAH) followed by coupling with a tetrazolium salt.[60] As well as in the case of direct Schiff's reaction,[57] the reliability of the NAH reaction was assessed by means of microspectrophotometrical analysis of tissue sections, with a comparison with data provided by biochemical determination of lipid peroxidation in the same specimens.[60] The employment of the NAH reaction allowed to visualize the regions first involved by lipid peroxidation *in vivo*, following the intoxication with haloalkanes; such lipid peroxidation had proved lower than the detection limits offered by direct Schiff's reaction.[60] NAH reaction also avoids the lack of specificity of Schiff's reaction in tissues other than the liver, thus allowing selective visualization of cells involved by lipid peroxidation in lung or kidney tissue.[60]

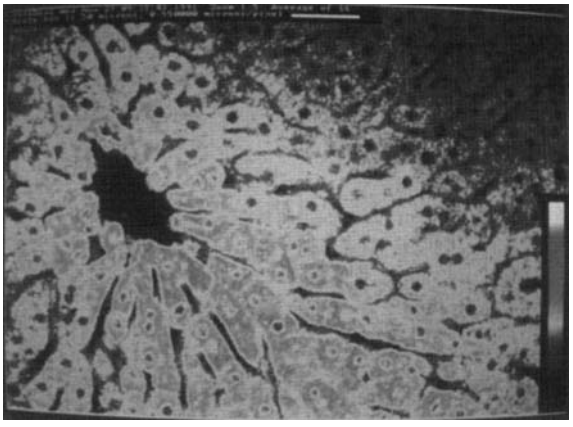


FIGURE 3 Confocal, pseudo-colour image of fluorescence distribution in a liver section obtained from a rat intoxicated with carbon tetrachloride (250 μmol/100 g body wt, 1 h). False colours represent lower to higher fluorescence intensities (see scale bar). Hydroxy-naphthoic hydrazide reaction of tissue carbonyls deriving from lipid peroxidation. An involvement of the first rows of hepatocytes around the central vein can be appreciated. (Reproduced from Pompella and Comporti,[61] with permission). (See Color plate I at the end of this issue.)

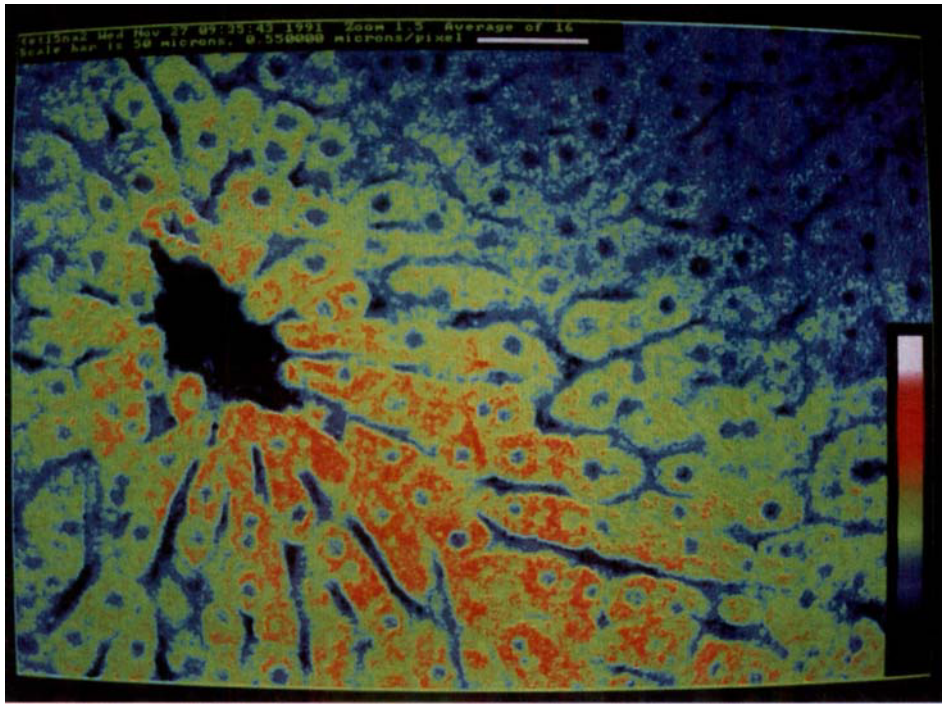
A further improvement in histochemistry of lipid peroxidation is offered by the employment of fluorescent reagents for lipid peroxidation-derived carbonyls in tissues and isolated cells, with an appreciable increase in the sensitivity of detection, and the possibility of analysis by means of confocal laser scanning fluorescence microscopy with image video analysis. Interesting results with this procedure were obtained by us[61] by exploiting the fluorescence of the NAH reagent itself. Figure 3 shows the selective involvement of the first rows of pericentral hepatocytes by lipid peroxidation induced *in vivo* in the rat by intoxication with carbon tetrachloride. With the same method we showed increasing levels of lipid peroxidation in isolated rat hepatocytes incubated *in vitro* with carbon tetrachloride.

An elegant improvement of the approach employing fluorescent derivativization of cellular carbonyls followed by confocal laser scanning microscopy has been recently published by others, using a biotin-labelled hydrazide coupled with fluorescent-conjugated streptavidin.[62]

Several recent studies dedicated to the visualization of lipid peroxidation in tissues have used an immuno-histochemical approach, basing on the fact that some important lipid peroxidation-derived products – MDA and 4-hydroxyalkenals in the first place – can easily react with cellular macromolecules, thus leading to the appearance of recognizable epitopes in proteins. This approach has produced important results such as the revelation of MDA-modified proteins in collagen-producing fibroblasts[63,64] and in the liver of human alcoholics[65] as well as the localization of 4-HNE-modified proteins in arterial wall during experimental atherosclerosis[66] and in renal carcinogenesis.[67]

FINAL CONSIDERATION

Thus I have summarized how I met Hermann, how we became very strict friends, how we worked in collaboration and how many researches arose thereafter. We were together at the 1985 Gordon Conference in St. Barbara, where the whole story of 4-HNE and other lipid peroxidation aldehydes was reported. After that our scientific interests somewhat diverged, he became interested mainly in oxidative modifications of LDL and the consequent molecular mechanisms of atherosclerosis.[68] I was mainly interested with the mechanisms of cell damage by GSH depletion and then by the intracellular release of iron in a reactive form. However, the reciprocal interest in our studies was continuously rekindled as well as our friendship, which remained very firm. We met each other frequently to discuss future plan and to enjoy our company and the beauty of our towns. When he was in Siena, he always wanted to sit in the Palio Square and notwithstanding my efforts to show him other places of the town, he used to say "There is no other place you could show me like this". Some years ago, when I was leaving for a heart surgical operation, he called me very apprehensively and wanted to know in detail



Colour Plate I (see page 632, figure 3) Confocal, pseudo-colour image of fluorescence distribution in a liver section obtained from a rat intoxicated with carbon tetrachloride (250 μmol/100 g body wt, 1 h). False colours represent lower to higher fluorescence intensities (see scale bar). Hydroxy-naphthoic hydrazide reaction of tissue carbonyls deriving from lipid peroxidation. An involvement of the first rows of hepatocytes around the central vein can be appreciated. (Reproduced from Pompella and Comporti,[61] with permission).

what was my problem. My problem was very simple, but when I heard about his problem I was really shocked. I called him several times just to encourage him, but I had understood that I was losing a great, unforgettable friend and that the scientific community was losing a very strong, honest, profound, outstanding scientist.

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