review

Autoxidation of polyunsaturated esters in water: chemical structure and biological activity of the products

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ABSTRACT When polyunsaturated esters or fatty acids are dispersed for long periods in water in the presence of air, water-soluble substances are formed in great variety. Because these short-chain products are constantly eluted by the aqueous phase and are consequently not available for further reaction in the oil phase, many intermediates of classical autoxidation can be isolated and identified. The identification of several of these compounds is described.

Some of the peroxidic and nonperoxidic autoxidation products show biochemical activity—in particular, inhibition of glycolysis and of respiration during incubation with tumor cells in vitro. Minimal inhibitory concentrations of pure, isolated products have been determined for Ehrlich ascites tumor cells. Synthetic short-chain (C_4–C_{10}) hydroxylated α,β-unsaturated aldehydes have been shown to have this action and also to cause morphological changes in these cells which quickly lead to their death. Normal cells are not affected. Possible therapeutic use of these compounds in the treatment of malignant neoplasms is discussed.

KEY WORDS linoleate · polyunsaturated compounds · autoxidation · lipid peroxides · 4-hydroxyoct-2-en-1-al · glycolysis · respiration · enzyme inhibition · Ehrlich ascites tumor cells · stalagmosis · cancer

THIS REVIEW DEALS WITH the reactions that take place and the products that are formed when polyunsaturated fatty acids or their derivatives are dispersed for extended periods in water in the presence of air, without the addition of any further substances.

The starting point for investigations in our laboratory was the observation that under these conditions water-soluble substances are formed which can be recognized in the filtered water phase by their UV absorption at 222 mμ. These substances are obtained, after evaporation of the water, as a viscous, yellowish oil. Similar results can be obtained not only with linoleic, linolenic, and arachidonic acids and their esters, but also with nonacidic polyenoic compounds such as vitamin A or essential oils.

The reaction is of interest for several reasons. It is, of course, basically an oxidation, but turns out to be one of unusual complexity and chemical interest. Because the oxidation takes place in heterogeneous dispersion, the water-soluble products of oxidation in the oil phase are continuously extracted by the water and become unavailable for further reaction; thus, hitherto unrecognized intermediates of the "classical" autoxidation of polyunsaturated compounds can be isolated.

From the biochemical point of view, the reaction is of interest because many of the water-soluble products exert a number of remarkable effects on the metabolism, structure, and growth of plant, animal, and human cells. An extensive literature exists on the autoxidation, especially peroxidation, of polyunsaturated lipids in living cells and tissues. These oxidation processes are promoted by the presence of highly active biogenic prooxidants but are also kept in close check by the naturally occurring anti-

Abbreviations: DNPH, dinitrophenylhydrazone; GAPDH, glyceraldehydephosphate dehydrogenase, EC 1.2.1.12; HOE, 4-hydroxyoct-2-en-1-al; LDH, lactate dehydrogenase, EC 1.1.1.27; NABC, 4,4′-nitroazobenzene carboxylic; NMR, nuclear magnetic resonance; Prep. LHPO, mixture of oxidation products obtained from methyl linoleate under standard conditions.

oxidants, with the consequence that the concentrations of the oxidation products, when they can be detected at all, are very low. This does not imply that the oxidation products are without meaningful biological action; on the contrary, they seem to be potent and reactive substances whose properties need to be known.

Finally, the oxidative changes which lipids undergo in the presence of water or of moist air are of considerable significance in food chemistry and for the food industry.

CHEMISTRY OF THE REACTION

Historical Survey

As early as 1937, Bloor and Snider pointed out (1) that unsaturated fatty acids become water-soluble upon autoxidation. They inferred from this observation a possible physiological role for the phospholipids as carriers for oxygen. In 1953, Schulte and Schillinger found (2) during a study of the nonenzymatic oxidation of vitamin A acetate that the reaction catalyzed by oleic acid proceeds faster in the two-phase oil–water system than in a homogeneous nonaqueous phase, and that the rate of reaction increases with the amount of water. In contradiction to this, Spetsig found (3) that the autoxidation of methyl linoleate is inhibited by water. Once the chain reaction has started, however, its course is not affected by the presence of water. Variation of the pH between 5.1 and 9.2 is reported to be without influence on the duration of the induction period.

In 1955, papers began to appear on the autoxidation of polyenoic acids in aqueous milieu catalyzed by UV irradiation. It was reported that the water-soluble products of such reactions with linoleate and linolenate inhibit respiration and glycolysis of Ehrlich ascites tumor cells (4), the action of succinate dehydrogenase (5), and the activity of Rous sarcoma virus (6). In 1957 Wilbur et al. reported (7) that the water-soluble oxidation products inhibit cell division and cause morphological changes during spindle formation. Fränz and Cole (8) found inhibition of cell division in yeast and stimulation of endogenous respiration (exogenous respiration was unaffected).

Attempts soon began to be made to correlate the biological activity with a measurable property of the aqueous solution, and for this purpose the thiobarbituric acid test was often used. This test is, unfortunately, very ambiguous. The biological effects can often be brought about by irradiated tissue lipids, and this has led to the conclusion that the effects are due, at least in part, to the formation of lipid peroxides. Thus Lewis and Wills (9) showed that the rate of oxidation of –SH groups in aqueous emulsions of polyenoic acids is proportional to the peroxide content of the emulsion and that the peroxides are consumed during the oxidation of sulfhydryl groups.

The oxidation of lipids in water is strongly influenced, as one would expect, by the degree of contact of the lipid with the oxygenated aqueous phase. The rate of oxidation of carotene in linoleate soap solutions, for example, is affected strongly by the concentration of soap micelles (10); there is a critical middle concentration of linoleate at which the oxidation is minimal. The autoxidation of oil emulsified in water with and without detergents was shown by Jauslin and Leupin (11) to increase with the degree of dispersion of the oil; the effect of the detergents was greater the more double bonds there were in the oil.

Tadros and Leupin (12) have recently established that the oxidation of methyl oleate in water either irradiated with UV light or saturated with O_2 at 50°C proceeds more slowly than when pure methyl oleate is used [cf. Spetsig (3)]. The explanation given by the authors is that oxygen is more soluble in oil than in water. Addition of surfactant to an emulsion, however, always increased the rate of oxidation of the oleate; and increased dispersion in the absence of detergents had a similar effect.

Measurement of the UV absorption of the aqueous phase after these oxidative reactions shows that conjugated double bonds are present. In addition, the occurrence of a tetrahydroxycarboxylic acid has been reported (13). The advent of gas–liquid chromatography has provided more information about several reaction products hitherto detected only by ambiguous color reactions (8). Kaufmann, Garloff, and Deicke (14) found the most polar products of autoxidation of cholesteryl polyenoates to contain several vicinal hydroxy groups (IR spectrum), as the Farmer mechanism of autoxidation (15) would predict. The other product formed a mixture too complex to analyze. Baker and coworkers (16) showed that the only water-soluble product after a very short treatment of polyunsaturated acid in CCl_4 with ozone is hydrogen peroxide. They went on to show that water-soluble products of UV-irradiated linoleic and linolenic acids could be divided into volatile and relatively involatile fractions, each of which reacted with thiobarbituric acid and peroxidase. The volatile fraction contained hydrogen peroxide and probably malonaldehyde; the involatile one at least 12 substances, C_7–C_{13}, containing α,β-unsaturated carbonyl groups.

Work from This Laboratory

It was possible that the original observation (17) of water-soluble products after the dispersion of polyunsaturated compounds in water was due to extraction into the aqueous phase of impurities present in the original material. This possibility was eliminated by the finding (18) that if the highly purified starting material was dispersed in water in several successive small portions, a higher

yield of UV-absorbing water-soluble products could be obtained.

Chemical characterization of the products was aided by the demonstration (19), by IR spectrography of the dry residue from the clear filtered water phase, that hydroxyl groups were present which were, of course, absent from the starting material.[1] Intense CH_2 bands, indicating with certainty the presence of aliphatic chains, were also present, whereas double-bond absorption was either absent or greatly reduced in intensity compared with the starting material. Addition of H and OH groups from the water across the double bond to produce hydrophilic substances seemed a reasonable working hypothesis. (In addition, one or more IR peaks corresponding to C=O groups were observed in the reaction product.)

However, if oxygen was rigorously excluded no transformation took place (20), and the first hypothesis had to be discarded. Since after prolonged dispersion in the presence of air the aqueous phase could be shown to contain "active oxygen" (21), the reaction is clearly an oxidation; this conclusion is supported by the finding that a higher yield results when the same amount of starting material is repeatedly dispersed in small portions (see above) and when the number of double bonds in the substrate is increased.

Standardization of Experimental Conditions

Subsequent experiments employed standardized conditions. The substrate was methyl or ethyl linoleate (9-*cis*, 12-*cis*-octadecadienoate) of high purity, the ester:water ratio was 1:50, the water used had been twice distilled in glass, and the oil was dispersed by means of a glass stirrer in the presence of air at 40°C. At timed intervals samples were removed and filtered to clearness and their contents of hydrogen peroxide (with $TiOSO_4$) and of total peroxide [by the method of Hartman (22)] were determined. The difference between these two values was attributed to lipid peroxides.

Fig. 1 shows typical results for either methyl or ethyl linoleate. After an induction period (in this case about 50 hr) lipid peroxides begin to appear; hydrogen peroxide becomes measurable only later and seems therefore to be a secondary product. The lipid peroxide value increases to a plateau at about 30 μg of active oxygen per ml, while the hydrogen peroxide concentration continues to rise after this time. We have marked the point of intersection of the curves for lipid peroxide and hydrogen peroxide *S* in Fig. 1; it occurs at 90 hr in this example. For other samples of methyl linoleate the point *S* was reached between 70 and 240 hr, varying only with the induction

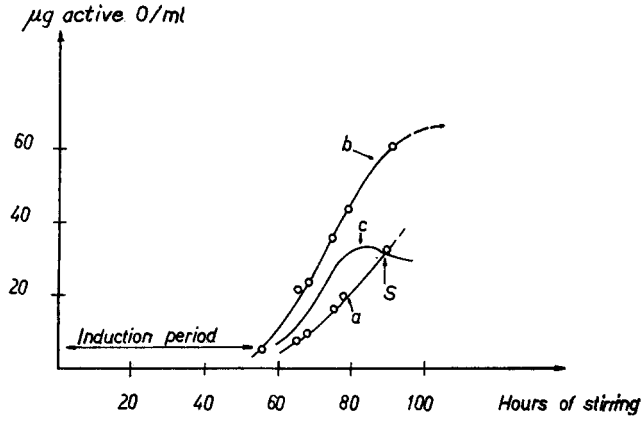

FIG. 1. Increase of peroxide content with stirring time. Methyl linoleate in distilled water (weight ratio 1:50) stirred with a glass stirrer at 40°C for number of hours shown. *a*, hydrogen peroxide content (determined with $TiOSO_4$); *b*, total peroxide (determined as in ref. 22); *c*, lipid peroxide (difference curve); *S*, intersection of curves *a* and *c*. Values are μg of active oxygen per ml of the filtered aqueous solution.

period (which depended presumably on the initial content of oxidation products in the starting material); the height and mutual relation of the curves was constant. In subsequent chemical investigations of the products, stirring was stopped, for consistency's sake, at the time corresponding to the point *S*.

Analysis of Oxidation Products

The aqueous phase was neutralized with bicarbonate and extracted with chloroform (21). The product after removal of solvent ("Prep. LHPO") contained about 20 μg of active oxygen per mg, and gave a remarkably reproducible pattern (23, 24) on thin-layer chromatography (Fig. 2). Nine spots were revealed by phosphomolybdic acid spray, four of which contained peroxides (Table 1).

Column chromatography on silica gel (Merck, 50–200 μ) can be used to separate the components if precautions against the decomposition of the peroxides are taken. Fig. 3 shows densitometric tracings of thin-layer analyses of the whole mixture (top) and of component 6, partially and completely purified by method b described in ref. 24. Further chromatography with different eluents showed components 1, 4, 5, and 7 to be single compounds whereas the others were complex (see Table 2, in which subfractions of each are denoted by two-digit numbers; the second digit rises with increasing polarity). The identification of substances 1, 23, 64, 65, 7, 81, 82, 91, and 93 is described in the following section.

Pure Substances Isolated from Prep. LHPO

Substance 1: oct-2-trans-en-1-al. Identification (25) by IR and UV absorption and polarography, and by UV absorption, paper chromatography, and elementary analysis of the 2,4-DNPH.

[1] Our sincerest thanks are extended to Professor A. Butenandt, who with great kindness expressed his interest in our investigations at this stage and promoted them by personal discussions as well as by experimental investigations in his own institute.

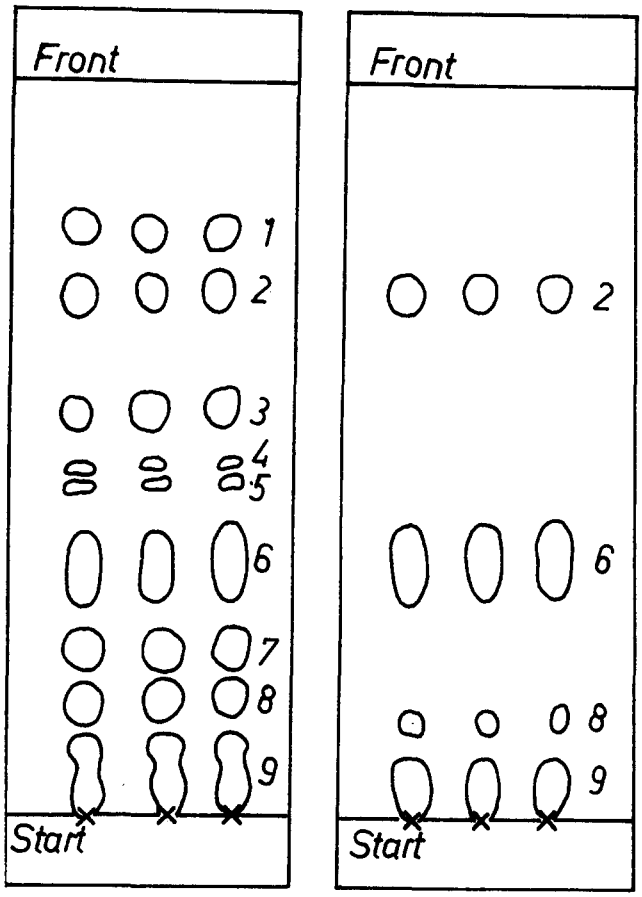

FIG. 2. Tracings of thin-layer chromatograms of Prep. LHPO (on Silica Gel G, Merck, in hexane–ether 3:2), showing the nine components stained by phosphomolybdic acid (left), four of which stain with the peroxide reagent (right).

TABLE 1 THIN-LAYER CHROMATOGRAPHIC SEPARATION OF "PREP. LHPO" INTO NINE MAJOR FRACTIONS

R_f	Staining Reactions: PMO	Staining Reactions: Fe^{++} + Dpi	Component No.
0.83–0.85	+++	—	1
0.71–0.72	+++	+++	2
0.50–0.55	+++	—	3
0.48	+	—	4
0.43	+	—	5
0.35–0.37	+++	++++	6
0.25–0.28	++++	—	7
0.18–0.20	+++++	—	8
0.10–0.15	—	+	
0.08–0.1	+++++	—	9
0.00–0.08	+++++	++	

Abbreviations: PMO, phosphomolybdic acid; Dpi, dichlorophenol–indophenol; +, very weak; ++, weak; +++, medium; ++++, strong; +++++, very strong.

Substance 23: n-amyl hydroperoxide. Identification (25) by IR spectroscopy, polarography, and peroxide value according to Sully (26); reduction to the alcohol with

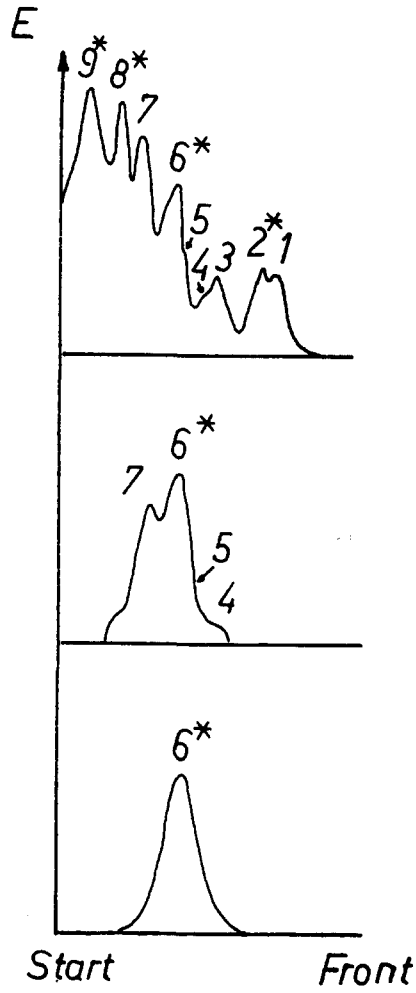

FIG. 3. Purification of component 6 from Prep. LHPO. Densitograms of thin-layer plates for the whole mixture (top), of an intermediate fraction ("Main fraction B") (middle), and the single component (bottom). Stained with phosphomolybdic acid.
* Peroxide-active. E, extinction.

$ZnCl_2$ (27) and formation of the 4,4′-nitroazobenzene carboxylic (NABC) ester.

Substance 64: 8-hydroperoxycaprylic acid methyl ester. Identification (28) as for substance 23: reduction to the corresponding hydroxyl derivative and formation of the NABC ester. From the UV spectrum and elementary analysis of the latter the molecular weight and chain length were deduced. The ester group of the reduced substance 64 was hydrolyzed (pK 4.82). The melting point of the free acid makes it probable that the hydroxyl group (hydroperoxy in substance 64) is at C_8.

Substance 65: 4-hydroperoxynon-2-en-1-al. Identification (28) similar to that of 64; $ZnCl_2$ reduction gave a hydroxyaldehyde from which the 2,4-DNPH was made. UV absorption in chloroform and in alkali showed the conjugated double bond and gave an estimate of molecular weight. The position of the hydroperoxy group could

TABLE 2 FRACTIONATION OF "PREP. LHPO"

Fractions by Method b (ref. 24)	Component by Method a	Percentage in Prep. LHPO	Contains Substances No.
Main fraction A (7%)	1	2	1
	2*	2.5	21, 22, 23*
	3	2	31, 32, 33
	4	1.5	4
Main fraction B (15%)	5		5
	6*	14	61, 62, 63, 64*, 65*
Main fraction C (11%)	7	2	7
	8*	10	81, 82, 83
Main fraction D (65%)	9*	~60	at least 7 substances

Subfractions are denoted by two-digit numbers; the second digit rises with increasing polarity.
* Peroxide-active.

not be established with certainty because of the small amount of this product.

Substance 7: 1-hydroxyheptan-2-one. Identification by polarography and IR: the latter showed only one CH_3 per molecule and indicated therefore that the other end was CH_2OH. The UV absorption of the NABC ester gave the molecular weight. This ester was then converted to its 2,4-DNPH, the UV spectrum of which indicated a saturated aliphatic ketone. The 2-position of the ketone was proven by conversion of substance 7 to an osazone, the melting point of which gave further information on chain length (29).

Substance 81: 4-hydroxy-2-trans-octen-1-al. Identification (30) by IR and UV spectroscopy and polarography. A 2,4-DNPH was formed. The UV spectrum showed the double bond to be conjugated; elementary analysis gave a hydroxy C_8 aldehyde. The position of the hydroxyl group was established (by Dr. J. Sonnenbichler, Max Planck Institute for Biochemistry, Munich) by NMR spectrometry.

Because of its remarkable biological effects (see below), substance 81 was synthesized by Esterbauer and Weger (31) and its structure thereby confirmed. Among the physicochemical properties of this compound the rapid reaction with SH groups is striking; this reaction was demonstrated with cysteine (29, 32) and recently with glutathione. During this reaction both the SH group and the conjugated chromophore of the aldehyde are lost, so that formation of a thiosemiacetal or addition of the SH across the double bond seem likely. 4-Hydroxyoctenal reacts about 10 times as fast as saturated aldehydes and 2–3 times as fast as unsaturated, nonhydroxylated aldehydes. In the reaction with glutathione (33) the addition of SH across the double bond has been shown to be followed by a cyclization to form a furane-like ring. These reactions with simple SH compounds form models for reactions with SH enzymes in which the enzyme is frequently inactivated (see below).

Substance 82: methyl hydroxycaprylate. Identification (34) by IR spectroscopy and hydrolysis to an acid of pK 5.2 followed by determination of its equivalent weight; determination of molecular weight, elementary analysis, and formation of an NABC ester.

Component 9 represents about 60% of Prep. LHPO. It contains at least seven substances of high polarity. *Substance 91* is, on the grounds of its IR spectrum, the methyl ester of a hydroxyoxocarboxylic acid: it yields an NABC ester and an osazone. Its molecular weight is between 200 and 275. *Substance 93*, on the grounds of its UV and IR spectra, contains a *trans* double bond and aldehyde, ester, and hydroxy groups, and must be a carbomethoxy hydroxyalk-2-en-1-al. It forms a 2,4-DNPH and has a molecular weight above 200 (Schauenstein and H. Esterbauer, unpublished observations).

Mechanism of the Aqueous Oxidation

The observations so far reported allow the following statements about the reaction to be made.

The first major reaction is identical with that which occurs in the autoxidation of linoleic acid, namely the formation of hydroperoxides—$\Delta^{10,12}$-9-hydroperoxide, the $\Delta^{9,11}$-13-hydroperoxide, and the $\Delta^{9,12}$-11-hydroperoxide. This is unequivocally proven by the IR and UV absorption and polarography of the separated primary oxidation products. The values obtained agree well with those reported by other workers.

The "classical" hydroperoxides are known to be unstable and to react readily with further oxygen to yield secondary products—aldehydes, ketones, dimers and polymers, and epoxides—which in turn may be hydrolyzed in the presence of water to tertiary products: hydrogen peroxide, carboxylic acids, and the many components of Prep. LHPO. The formation of substance 1 (2-*trans*-octenal) can readily be envisaged as the result of homolytic cleavage of the linoleic hydroperoxides.

Oxidation of the aldehyde to a carboxyl group may yield either a short-chain carboxylic acid (from the methyl end of the linoleate) or the half-ester of a dicarboxylic acid. The methyl monoesters of suberic, pimelic, and azelaic acids have been isolated (21).

The characteristic feature of the oxidation in *aqueous suspension* is that the large excess of water favors the formation of tertiary products, which will accumulate in the aqueous phase. These products are formed only in minute amounts in the absence of water. Thus despite an extensive literature on the formation of hydroperoxides and secondary and tertiary products such as unsaturated aldehydes in the course of autoxidation, the products described above have not been reported previously, with the exception of substance 1 and possibly substance 82.

BIOLOGICAL EFFECTS OF THE OXIDATION PRODUCTS

Action of Prep. LHPO

As mentioned above, Prep. LHPO exerts several types of action on yeast, plant, and animal cells (20, 21, 35). In early, tentative experiments (21) the active oxygen of Prep. LHPO was found to be resistant to the action of catalase. Of the observed biological actions of Prep. LHPO we describe here those which refer to the metabolism of Ehrlich ascites tumor cells under the influence of Prep. LHPO from methyl 9,12-linoleate (the corresponding preparation from the ethyl ester of the isomeric octadeca-9,11-dienoic acid gives somewhat different results).

Prep. LHPO inhibits anaerobic and aerobic glycolysis by these cells, besides reducing their oxygen consumption. Curves *1*, *2*, and *3* of Fig. 4 (36, 37) relate these inhibitory actions to the concentration of Prep. LHPO (in g/liter) and to the concentration of active oxygen (in μg/ml). If the cells are preincubated in air the inhibitory action is greatly enhanced. Curves *1* and *2* illustrate that anaerobic and aerobic glycolysis are inhibited to about the same extent, respiration less markedly (curve *3*). If the cells are aerobically incubated with Prep. LHPO, but in the presence of glucose, the inhibition is decreased [the "glucose protecting effect" (38)]. The inhibition of anaerobic glycolysis by Prep. LHPO is accompanied by a roughly proportional decrease (up to 85%) of the intracellular level of NAD, which suggests that the NAD content of the cells becomes confined to the intramitochondrial portion.

Chemical reduction of Prep. LHPO greatly diminished its inhibitory effect (curves *4–6*), in agreement with the demonstration by others that peroxides of unsaturated fatty acids have inhibitory properties (39–42). Some inhibitory activity remained after reduction, however, so that nonperoxidic inhibitors are also apparently present.

The mechanism of action of Prep. LHPO may be related to the following observation. When the enzymes GAPDH (glyceraldehydephosphate dehydrogenase, EC 1.2.1.12) and LDH (lactate dehydrogenase, EC 1.1.1.27) were isolated from tumor cells that had been incubated with Prep. LHPO they were found (curve *7* of Fig. 4) to be completely inactive if the concentration of active oxygen had been as little as 2 μg/ml (at this concentration the anaerobic and aerobic glycolysis is blocked to the extent of 50%). If Prep. LHPO is chemically reduced with $ZnCl_2$ before the incubation the enzymes are still inhibited, but to a very limited extent. Addition of nicotinamide during the incubation of the cells with Prep. LHPO raises the NAD level (although not to initial values), but the inhibition of glycolysis is not affected (21). From this we may infer that the inhibition is caused mainly by inactivation of the glycolytic enzymes GAPDH and LDH via the oxidation of their SH groups by hydroperoxides. The NAD system is affected, but not essentially. The selectivity of inhibitory action is remarkable: other SH enzymes such as fructosediphosphate aldolase (EC 4.1.2.13), hexokinase (EC 2.7.1.1), and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) are not measurably affected (43).

Prep. LHPO acts dramatically on the transplantability of tumor cells when incubated with them at high concentrations (about 50 μg of active oxygen per ml). The incubation was performed (44) under various conditions, with and without ascites serum. When the incubated cells were injected into healthy white mice, no tumors were formed for up to 150 days, whereas all control animals (injected with untreated tumor cells) died after a mean of 13 days.

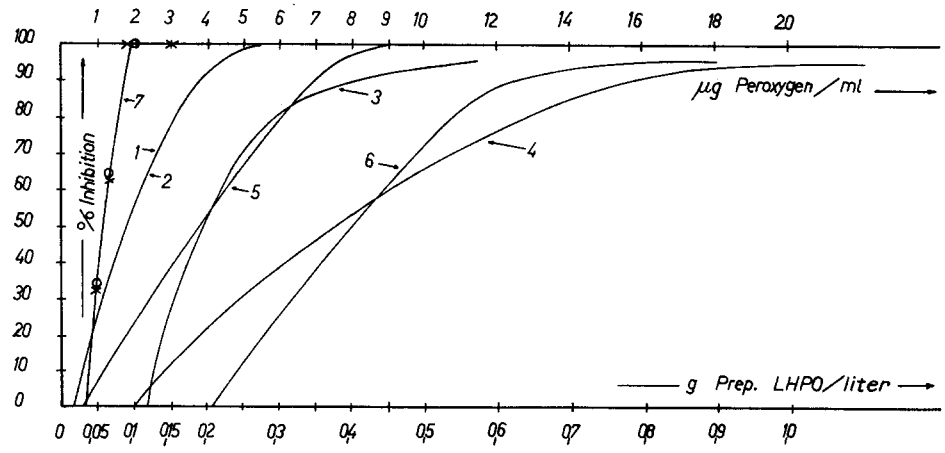

FIG. 4. Dose–response curves for the inhibitory effects of Prep. LHPO. Curves *1–3*, inhibition of aerobic glycolysis, anaerobic glycolysis, and respiration in Ehrlich ascites tumor cells, respectively, after aerobic preincubation. Curves *4–6*, corresponding curves for the $ZnCl_2$-reduced Prep. LHPO (only the bottom scale, grams of total preparation per liter, applies to these curves). Curve *7*, inhibition of the glycolysis enzymes GAPDH (x) and LDH (o). All reactions tested after 30 min incubation of the cells with Prep. LHPO.

Chemically reduced Prep. LHPO gave a retarded tumor growth, but no prolongation of the survival time of test animals.

These properties of the mixture Prep. LHPO made it desirable to test the biological properties of the individual components isolated from it.

Inhibitory Activity of Hydroperoxides 23, 64, and 65

All three of the isolated hydroperoxides 23, 64, and 65 inhibit the metabolism of tumor cells. Table 3 shows the minimum concentrations necessary for inhibition of glycolysis and of respiration (45, 46). As with complete Prep. LHPO, inhibition of the anaerobic glycolysis occurs mainly via an oxidative inactivation of the enzymes GAPDH and LDH, the NAD system being essentially unaffected. Consideration of the center column in Table 3 and the percentages of the components in Prep. LHPO leads to the conclusion (46) that the activity of Prep. LHPO is largely attributable to its content of hydroperoxide 64, although it should not be forgotten that Prep. LHPO retains some activity even after all hydroperoxide groups have been destroyed by reduction. In the inhibition of respiration the contribution of nonperoxidic compounds must be even more important, since even if the effects of the three hydroperoxides were additive they would not be sufficient to account for the action of Prep. LHPO.

Since after reduction of Prep. LHPO with $ZnCl_2$ a significant residual inhibition remains (Fig. 4), and since the content of carbonyl compounds remains unchanged after this treatment, it seemed probable that the nonperoxidic inhibitors of respiration would be carbonyl compounds. If this were true, these compounds would exhibit special features of chemical constitution since simple saturated aldehydes and ketones do not inhibit cell respiration (47). We therefore isolated a representative inhibitory compound from Prep. LHPO and examined its biological activity in detail. Although Prep. LHPO contains a large number of these substances their preparative isolation is extremely difficult, because each of them occurs in vanishingly small percentage and most of them are present in main fraction D (see Table 2), the most polar fraction and the one most difficult to fractionate. However, substance 81 (4-hydroxyoct-2-en-1-al) constitutes 3–4% of Prep. LHPO and is relatively easy to separate from main fraction C.

Biological Properties of 4-Hydroxyoct-2-en-1-al (HOE)

Effects on Isolated SH Enzymes

As mentioned above, the reactivity of this hydroxylated α,β-unsaturated aldehyde toward enzymes with functional SH groups is extremely high. Its inhibitory action on two such enzymes is shown in Table 4, which also gives an idea of its specificity (32 and unpublished work by M. Taufer). The concentration of 8.5 mM is the solubility of HOE in water. The enzyme inhibition can be completely abolished by the addition of a 100-fold molar amount of cysteine.

TABLE 3 Inhibition of Glycolysis and Respiration in Ehrlich Ascites Tumor Cells by Substances 23, 64, and 65

Substance	Minimum Concn for 100% Inhibition of Anaerobic Glycolysis	Minimum Concn for 95% Inhibition of Oxygen Uptake
	mM	
23	0.30	1.5
64	0.315	2.0
65	0.16	2.7

TABLE 4 Effects of HOE on Different SH Enzymes

Enzyme	Final Concn of HOE	Inhibition
	mM	%
GAPDH	0.03	50
	0.10	100
LDH	8	50
	8.5	64
ALD	8.5	0
HK	8.5	0
G-6-PDH	8.5	0

Enzymes were supplied by Boehringer (Mannheim) and were tested for activity under routine conditions before and after 15 min of incubation with the hydroxyoctenal at 0°C. GAPDH, glyceraldehydephosphate dehydrogenase; LDH, lactate dehydrogenase; ALD, fructosediphosphate aldolase; HK, hexokinase; G-6-PDH, glucose-6-phosphate dehydrogenase.

The selective reaction of HOE with the SH groups of the enzymes is also suggested by the following. Rabbit muscle GAPDH was incubated in HOE solution (2.7 mM) at 2°C for 30 min and the solution was subsequently dialyzed to equilibrium (29, 32, and unpublished work of M. Taufer). Determination of HOE by UV absorption in the dialysate led to an estimate of protein-bound aldehyde corresponding to 18 moles/mole of protein, equivalent to all SH groups in the enzyme molecule. We wish to emphasize that the determination was approximate. More recent experiments (48) showed that actually only 4 moles of aldehyde are bound per mole of enzyme, which corresponds to all of the *functional* SH groups of the enzyme.

The aldehyde-saturated enzyme shows 100% loss of activity compared with a control sample without aldehyde, but full activity is restored by the addition of an excess of cysteine. Thus the inactivation is caused by reversible addition of the aldehyde to the protein; there is no nonspecific denaturation.

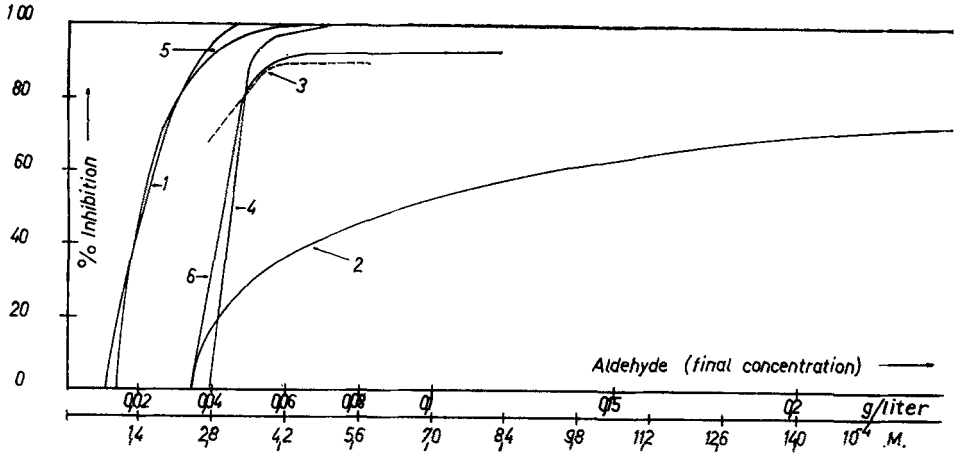

FIG. 5. Dose–response curves for the inhibitory effects of 4-hydroxyoct-2-en-1-al (HOE). Curves *1*, *3*, and *4*, inhibition of anaerobic glycolysis, aerobic glycolysis, and respiration in Ehrlich ascites tumor cells respectively, after aerobic preincubation of the cells. Curve *2*, anaerobic glycolysis after anaerobic preincubation of the cells. Curves *5*, *6*, inhibition of GAPDH and LDH respectively, extracted from the cells after the cells had been incubated with HOE.

Effects on Cell Metabolism

Fig. 5 shows the inhibition by HOE of aerobic and anaerobic glycolysis and of respiration in Ehrlich ascites tumor cells (30). Respiration is inhibited (curve *4*) only by concentrations above those that will completely block anaerobic glycolysis (curve *1*), a result characteristic of true glycolysis poisons such as H_2O_2, X-rays, monoiodoacetate, menadione, and ethyleniminoquinones. HOE differs from a true glycolysis poison, however, since nicotinamide added during the preincubation of the cells with HOE does not abolish or even reduce the inhibition of glycolysis (46, 49).

This suggests that HOE does not inhibit anaerobic glycolysis via inactivation of the NAD system as the cited poisons do. The suggestion is supported by the curves (Fig. 6) relating NAD level to percentage inhibition (49). Although HOE is like the other agents mentioned in lowering NAD levels to about 15% (this presumably corresponds to the intramitochondrial fraction), the shape of the curve (curve *1*) is quite different from that obtained with H_2O_2 or Trenimon (2,3,5-trisethyleniminobenzoquinone) or after X-ray irradiation in glucose-free medium [values obtained by Hilz, Hubmann, Oldekop, Scholz, and Gessler (38), not shown in Fig. 6, lie on curve *2*]. Fig. 6 also shows that although the addition of nicotinamide raises the level of NAD, either by stimulation of synthesis or inhibition of degradation, as it does with true glycolysis poisons, the inhibition of glycolysis shows no measurable alteration.

Inhibition of glycolysis by HOE seems to depend, therefore, on the effect of the substance on SH enzymes. Fig. 5, curve *5*, shows 100% inhibition of GAPDH with the same concentration of HOE that prevents glycolysis, and this inhibition of the enzyme seems therefore capable

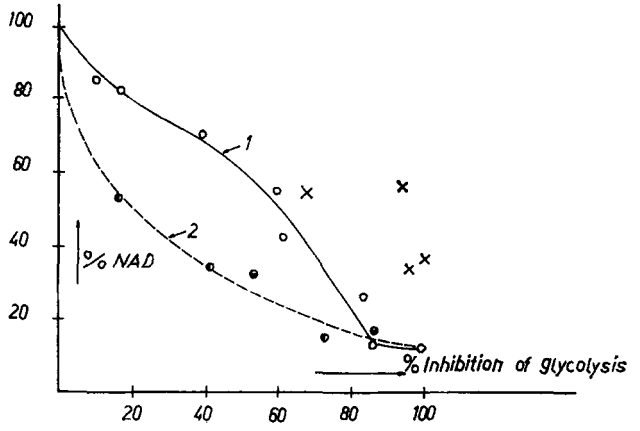

FIG. 6. NAD content of Ehrlich ascites tumor cells compared with degree of inhibition of anaerobic glycolysis. All values after aerobic incubation. O, HOE in buffer without glucose; ◐, H_2O_2; ◒, Trenimon without glucose; x, HOE without glucose but with added nicotinamide.

of accounting for the whole phenomenon. Blockage of LDH requires (curve *6*) higher concentrations of HOE.

No indications of the mechanism whereby respiration is blocked are at present available, other than the observed effect on NAD levels.

Cytomorphologic Studies In Vitro

Incubation of Ehrlich ascites tumor cells in vitro also gives rise to characteristic cytomorphological effects similar to those described by King, Paulson, Puckett, and Krebs (50) after such cells had been exposed to high doses of X-rays or treated with H_2O_2 or alkylating (but not nonalkylating) cytostatics. Schauenstein, Zangger, and Ratzenhofer (51, 52) described two stages of a cytopathological effect of incubation of the cells with HOE. After a short incubation time small extrusions of the membrane

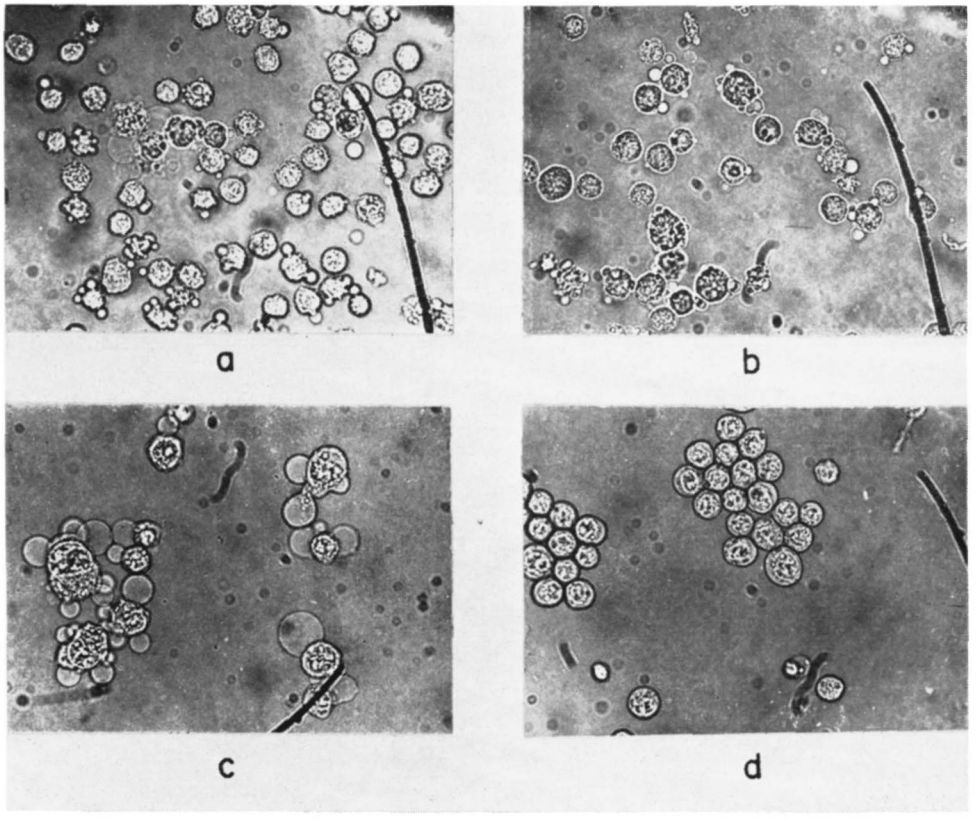

FIG. 7. Cytomorphological changes in Ehrlich ascites tumor cells (8 days after transplantation) induced by incubation with HOE for 30 min at 37 °C at final concentrations of (*a*) 0.5 mM, (*b*) 1 mM, and (*c*) 3 mM. (*d*) Control cells incubated in saline.

appear (Fig. 7*a*); they remain attached to the membrane by a small base and give the cell the appearance of a thorn apple (53). As incubation proceeds the bulges become spherical, although still small ("microstalagmosis," Fig. 7*b*), then increase in size to at least half that of the cell ("stalagmosis," Fig. 7*c*), and finally break off from the cell entirely ("stalagmoptysis"), leaving the cell membrane still intact. The stages up to microstalagmosis are reversible if the HOE is removed; stalagmosis—and of course stalagmoptysis—are irreversible. Stalagmosis should not be confused with the formation of "blebs" (54) or blisters (55) in hypotonic media or with lytic effects of detergents (56); the phenomenon develops extremely rapidly in the presence of the aldehyde.

Table 5 shows the percentages of affected cells after 20 million of them have been shaken with 0.5 ml of an isotonic solution of synthetic HOE in various concentrations. It is remarkable that even with the highest concentrations of the HOE (20 mM; the synthetic material is somewhat more soluble than that from Prep. LHPO) and prolonged incubation about 2% of the cells never show stalagmosis. Since the same results are obtained under anaerobic conditions of incubation, oxidative processes in the cells are not responsible for stalagmosis.

That stalagmosis is accompanied by the death of the cells was shown (57) by trypan blue staining (58) and by testing for transplantability (Table 5). Tumor cells are stained by trypan blue only after they have been subjected to heat or cytotoxic agents, and the staining is regarded as a reliable criterion for cell death. Table 5 shows a close correlation between percentages of morphological change and of staining, but even after prolonged incubations with very high concentrations of HOE trypan blue

TABLE 5 CYTOMORPHOLOGICAL AND TOXIC EFFECTS OF HOE ON EHRLICH ASCITES TUMOR CELLS

Final Concn	*Cytologic* Observation	Cells Stained by Trypan Blue	Inhibition of Transplantability
mM		%	
0.3	sporadic formation	5	—
0.5	of thorn-apple	5	—
0.7	shape	5	—
1	50% stal + ptysis	63	—
2	70% "	82	partial
3	94% "	94	100%
4	94% "	96	100%
5	96% "	96	100%

Stal, stalagmosis; ptysis, stalagmoptysis.

failed to stain 2–4% of the cells, which are therefore possibly still viable.

Effect on Oncogenesis

As the test with trypan blue would lead one to expect, HOE reduces the virulence of ascites tumor cells injected into living mice. At a concentration of 3 mM or higher and incubation at 37°C for 30 min, HOE completely inactivated the cells: animals injected with them remained free from tumors at 90 days whereas control animals all died between the 11th and the 18th day after injection. A concentration of 2 mM HOE in the incubation medium caused only partial loss of virulence (57, and unpublished work together with M. Taufer).

Although trypan blue and stalagmoptysis counts indicated that 5% of the cells remain alive after HOE treatment, it is clear that these cells can no longer survive transplantation or induce tumors. Whether the cells have been damaged to such an extent that they are incapable of reproducing or whether their chance of reproduction is too small in the presence of an excess of dead cells remains an open question.

Maximal inhibition of glycolysis and respiration occur at concentrations of HOE (0.3 and 0.4 mM, respectively) which are one-tenth those needed to kill the cells or prevent their reproduction. Thus we have the puzzling result that complete inhibition of glycolysis and 95% inhibition of respiration do not necessarily make the cell nonviable. The explanation may lie in the fact (57) that the inhibition of respiration by HOE is reversible at 0.41 mM but completely irreversible if the concentration of HOE is 2 mM. This concentration causes partial devitalization (Table 5). Thus, whereas measurement of anaerobic glycolysis in vitro is the most sensitive of the tests mentioned for determining an agent's activity, with regard to the life of the cell trypan blue staining and the observation of stalagmosis and stalagmoptysis are much more informative.

Penetration of HOE into Tumor Cells

In cells that had been aerobically incubated with shaking for 30 min at 37°C in the presence of HOE and subsequently washed with 0.9% NaCl solution, a positive reaction with Schiff's reagent could be obtained (59). There was no reaction with control cells. A definite difference from controls was also obtained when the cells were stained with 2,4-dinitrophenylhydrazine, but for both stains results were unequivocal only when the concentration of HOE in the incubation medium was 3 mM or higher. This is the concentration necessary to devitalize the cells completely; it is probable that the aldehyde penetrates at lower concentrations too, but this cannot be proven by these histochemical methods.

That the free aldehyde can be detected within the cell is explainable in one or both of the following ways: (*a*) the aldehyde group does not participate in the metabolic inhibition or in the binding to the substrate; (*b*) the aldehyde group takes part in both these processes but is subsequently liberated under the conditions of histochemical staining. The model experiments with glutathione mentioned above (33) support the latter possibility, since if the aldehyde is bound to cell components by the addition of SH to the C=C of the aldehyde a lactone ring will be formed which can be reopened by the acidic Schiff or hydrazine reagent and give rise to the positive aldehyde reaction.

Action of HOE on Nonmalignant Cells

One of the most remarkable properties of HOE is the selectivity of its damaging action against tumor cells. In normal monkey liver and kidney cells even the highest HOE concentrations tested (10 mM) gave no detectable inhibition of respiration (30, 51). With normal human leukocytes the inhibition of glycolysis and respiration did not exceed 30%. The cytomorphological changes, so marked with tumor cells, could not be detected with liver or kidney cells, leukocytes, or erythrocytes at any concentration of HOE (51, 57).

One possible explanation of the difference is that the membrane of the healthy cells excludes HOE or reduces its rate or degree of entry to a marked extent. Histochemical staining as in the previous section showed aldehyde within the nonmalignant cells after incubation, so that this explanation must be discarded.

A second possibility is that the aldehyde that has entered the cell is rendered ineffective by protective agents that are present in the healthy cell but absent from the abnormal one—a protective action similar to that of catalase in yeast cells, which protects them from the inhibitory effect of H_2O_2 on fermentation. This possibility is under investigation at present.

Therapeutic Aspects

The above observations lead of course to speculation about the possible use of HOE to destroy tumors in vivo without affecting healthy tissue. B. Wünschmann and I (60) have actually been able to confirm this possibility with solid tumors. 10 million virulent Ehrlich ascites tumor cells were implanted subcutaneously on the back of white mice. 1 mg of HOE in 1 ml of 0.9% NaCl solution was injected simultaneously at the same site. After 3 wk about 17% of the animals showed complete, 56% considerable, and 28% no inhibition of tumor growth compared with that in controls. Further experiments were carried out with the C_5-homologue, hydroxypenten-1-al. This gave under the same conditions 50% full, 48% partial, and 2% no inhibition.

Local therapy of the *ascites* tumor is, however, not pos-

sible, since HOE is cleared with remarkable rapidity from the peritoneal cavity: at an injection concentration of 5 mM, only one-third of the injected aldehyde is detectable intraperitoneally after 5 min and only 12% after 10 min. Even though HOE acts quickly in killing tumor cells in direct contact with it, such short survival times do not allow the destruction of a sufficient number of the cells for therapeutic results.

Synthetic Homologues

Gram quantities of synthetic HOE and of the homologous hydroxyenals of chain length C_4 to C_{10} are now available through the work of H. Esterbauer and later of Esterbauer and W. Weger (61). All these substances are qualitatively similar to the C_8 compound in their extraordinary affinity for SH groups, blocking action on enzymes and metabolism, damage to the structure of tumor cells, and inhibition of their growth, but in the living animal considerable quantitative differences with chain-length can be observed.

In general, the 4-hydroxy-2-en-1-als constitute a new class of very reactive aldehydes which exhibit a wide spectrum of biological effects.

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