Oxidation of Lipids. I. Quantitative Determination of the Oxidation of Methyl Linoleate and Methyl Linolenate

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The extent of oxidation of methyl linoleate and methyl linolenate has been measured quantitatively by several methods such as oxygen uptake, substrate disappearance, and formation of conjugated diene, TBA-reactive material and of hydrocarbon gas. In the early stage of oxidation of methyl linoleate, oxygen uptake, substrate disappearance, peroxide and conjugated diene formation were all quantitative and their results agreed well each other. Little TBA-reactive material and hydrocarbons were observed. In the oxidation of methyl linolenate, however, no product was formed quantitatively, and the extent of oxidation estimated decreased in the order, oxygen uptake > substrate disappearance > peroxide > conjugated diene > TBA-reactive material.

Oxidation of unsaturated fatty acids has been the subject of research since the onset of studies on autoxidation in 1940's.[1,2] It has recently received renewed attention, especially in relevance to the oxidation of lipids in biological systems as well as the oxidative deterioration of fats and oils.[2]

It is important and crucial to know how much oxidative deterioration has occured, but it is usually quite difficult to evaluate it, especially the oxidation *in vivo*. The oxidation reactions of saturated hydrocarbons are rather simple; they give hydroperoxides as the primary product in a substantially quantitative yield at moderate temperatures and low conversion. Thus, under these conditions, oxygen uptake, substrate disappearance, and hydroperoxide formation are all quantitative and their results agree well each other. The oxidations of olefins are more complicated: The addition to the double bond and abstraction of allylic hydrogen by peroxyl radicals compete. The addition mechanism gives primarily polyperoxides and epoxide, while the abstraction mechanism gives hydroperoxide as a major product.[3]

Oxidations of polyunsaturated fatty acids, major components of lipids, are more complicated, and usually many reactions proceed simultaniously and competitively. Thus, some criteria for accessing the extent of oxidation are required.

The extent of oxidation has been measured by several criteria such as oxygen uptake, peroxide value, conjugated diene formation, TBA-value, ethane and pentane formation, chemiluminescence, and development of fluorescence.[4,5] These methods have been applied by many investigators and are now well accepted. However, only one or two of these methods are usually used for each system, and little work has been done to examine the quantitative interrelationship between these methods.

Moreover, although many works have been done on the oxidation of unsaturated fatty acid, most of them are only qualitative or semi-quantitative; in other words, the oxidation is not measured quantitatively, but only a portion, sometimes a minor portion of these oxidation reactions is followed. Another question is that the ratio of measured oxidation to total oxidation depends on the substrate and experimental variables.

In this study, we intended to measure the oxidation of methyl linoleate and methyl linolenate by various analytical methods in order to find out how quantitative these methods are and what method is suitable for the specific reaction system.

Experimental

Methyl linoleate((*Z*,*Z*)-9,12-octadecadienoate) and methyl linolenate((*Z*,*Z*,*Z*)-9,12,15-octadecatrienoate) were obtained from Sigma Chemical Company and used as received. Prior to the oxidation, it was ascertained that neither conjugated diene nor peroxide was detected in the substrates. The oxidation was carried out in acetonitrile solvent using an ampoule of approximately 30 ml volume, which was immersed in a carefully temperature-controlled oil bath. 2,2'-Azobis(2,4-dimethylvaleronitrile) (AMVN) was used as a radical initiator. The rate of inhibition was measured from the induction period observed in the presence of inhibitor, 2,6-di-*t*-butyl-4-methylphenol (BMP).[6]

The rate of oxygen absorption was measured by following the pressure decrease using a pressure transducer. The oxygen uptake was calculated from the difference between initial and final oxygen amounts measured by Toepler

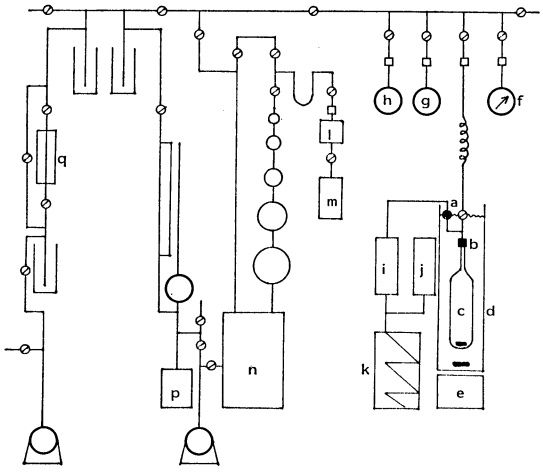

Fig. 1. Oxidation apparatus.
a: Pressure transducer, b: Swagelok teflon fittings, c: ampoule, d: Silicone oil bath, e: magnetic stirrer, f: pressure gauge, g: oxygen, h: nitrogen, i: bridge circuit, j: standard mV supply, k: recorder, l: gas sampler, m: gas chromatography, n: Toepler pump, p: Mcleod gauge (10^{-2}—10^{-6} Torr, 1 Torr≈133.32 Pa), q: mercury diffusion pump.

Table 1. Oxidation of methyl linoleate(LH) at 50 °C in acetonitrile, concentrations in μmol

Run No.	1	2	3	4	5
$(LH)_0$(μmol)	2096	2313	2039	2085	2082
$(LH)_0$(mM)	368	402	360	367	367
$(AMVN)_0$[a](mM)	12.6	11.9	12.1	12.4	12.5
Time/min	10	20	30	60	90
Initial O_2(μmol)	724.1	678.0	1166.7	1157.7	1067.6
Initial O_2(Torr)	591	555	951	975	871
Final O_2(μmol)	656.8	587.0	998.3	719.3	592.4
Final O_2(Torr)	536	480	806	612	483
O_2 uptake(μmol)	67.3	91.0	178.4	438.4	475.2
$(-dO_2/dt\times10^6)$/M s^{-1}			17.0	18.3	19.1
k.c.l.[b]			62.6	66.0	68.3
$[k_p/(2k_t)^{1/2}\times10^3]$/$(M\,s)^{-1/2}$			90.9	95.1	98.7
ΔLH(μmol)	68	87	238	402	483
$\Delta LH/(LH)_0\times10^2$(%)	3.2	3.8	11.7	19.3	31.3
Peroxide(μmol)	70	90	165	320	360
$(C=C)_2$(μmol)	74	98	139	243	328
TBA(μmol)	trace	trace	trace	trace	trace
$\Delta LH/\Delta O_2$	1.01	0.96	1.33	1.13	1.37
Peroxides/ΔO_2	1.04	0.99	0.92	0.90	0.76
$(C=C)_2/\Delta O_2$	1.10	1.08	0.78	0.68	0.69
TBA/ΔO_2	0	0	0	0	0

a) 2,2′-Azobis(2,4-dimethylvaleronitrile). b) Kinetic chain length.

pump.[7] A schematic diagram of oxidation apparatus is shown in Fig. 1.

The consumption of the substrate was measured by GLC. Peroxide formation was measured by iodometric titration and/or by formation of triphenylphosphine oxide by the reduction of peroxide with triphenylphosphine. Conjugated diene was measured by the absorbance at 230—236 nm, $\varepsilon=28000\ M^{-1}\,cm^{-1}$ (1 M = 1 mol dm^{-3}). TBA value was estimated by the conventional photometric method, $\lambda_{max}=$ 531 nm and $\varepsilon=1.17\times10^5\ M^{-1}\,cm^{-1}$. Hydrocarbon gas formation was analyzed by GLC.

Results and Discussion

Tables 1 and 2 show some pertinent data for the AMVN-initiated oxidation of methyl linoleate and methyl linolenate, respectively in acetonitrile at 50 °C. The oxidation of methyl linoleate proceeded at 50 °C even in the absence of a radical initiator, but the rate was very slow. In the presence of AMVN, it was oxidized faster and the plot of oxygen uptake as a function of time gave a good straight line at the initial stage.

Under such mild conditions that the primary products are stable and their secondary oxidation is negligible, the oxidation proceeds virtually by the following scheme.

Initiation: $IN_2I \longrightarrow 2I\cdot + N_2$

$I\cdot \xrightarrow{O_2,\,LH} IOOH + LO_2\cdot$

Propagation: $LO_2\cdot + LH \xrightarrow{k_p} LOOH + L\cdot$

$L\cdot + O_2 \longrightarrow LO_2\cdot$

Termination: $2LO_2\cdot \xrightarrow{k_t}$ non-radical products

IN_2I and LH are azo initiator and substrate, respectively. The chain carrying peroxyl radicals abstract doubly allylic hydrogen exclusively from methyl linoleate or methyl linolenate, whereas abstraction of other hydrogen and addition to a double bond are quite

Table 2. Oxidation of methyl linolenate(LH) at 50 °C in acetonitrile, concentrations in μmol

Run No.	6	7	8	9
$(LH)_0$(μmol)	2056	2068	2181	1020
$(LH)_0$(mM)	360	365	382	191
$(AMVN)_0$[a](mM)	12.0	13.6	11.8	12.2
Time/min	20	30	40	60
Initial O_2(μmol)	793.2	989.3	1235.2	1190.7
Initial O_2(Torr)	631	807	1009	956
Final O_2(μmol)	567.3	627.1	769.9	654.0
Final O_2(Torr)	451	511	629	606
O_2 uptake(μmol)	225.9	362.2	465.3	436.7
$(-dO_2/dt\times10^6)$/M s^{-1}	34.8	34.3	36.8	23.3
k.c.l.[b]	130	113	140	85.4
$[k_p/(2k_t)^{1/2}\times10^3]$/$(M\,s)^{-1/2}$	188	171	188	234
ΔLH(μmol)	180	260	299	261
$\Delta LH/(LH)_0\times10^2$(%)	8.8	12.6	13.7	25.6
Peroxide(μmol)	129	176	208	
$(C=C)_2$(μmol)	33	46	58	27
TBA(μmol)	33	46	61	60
$\Delta LH/\Delta O_2$	0.80	0.72	0.64	0.60
Peroxide/ΔO_2	0.57	0.49	0.45	
$(C=C)_2/\Delta O_2$	0.15	0.13	0.12	0.06
TBA/ΔO_2	0.15	0.13	0.13	0.14

a) 2,2′-Azobis(2,4-dimethylvaleronitrile). b) Kinetic chain length.

scarce.[8]

Under these conditions, the rate of oxidation is expressed by

$$\text{rate of oxidation} = R_o = k_p(R_i/2k_t)^{1/2}[LH],$$

where k_p, k_t, and R_i are the rate constants for propagation and termination, and the rate of initiation, respectively. The ratio of the rate constants $k_p/(2k_t)^{1/2}$ gives the relative reactivity of the substrate. Tables 1 and 2 show that $k_p/(2k_t)^{1/2}$ for methyl linoleate and methyl linolenate is 0.0949 and 0.182 $(M\ s)^{-1/2}$, respectively, indicating that methyl linolenate is approximately twice as reactive as methyl linoleate.

It should be noted that this value does not always indicate the relative reactivities of the substrates, or more strictly of C–H bonds, toward peroxyl radicals. The rate and oxidizability are determined not only by k_p but also by k_t. Howard and Ingold[8] found that k_p (per reactive C–H bond) and k_t for methyl linolenate are about two and four times, respectively, larger than those for methyl linoleate at 30 °C.

Methyl linoleate was subjected to oxidation for different reaction times and conversions. As shown in Table 1, kinetic chain length was long, and oxygen uptake and reaction products increased as the oxidation proceeded. A small increase with reaction time in the ratio of rate constants, $k_p/(2k_t)^{1/2}$, was observed, indicating that this oxidation is slightly autocatalytic.

Peroxides were analyzed by high pressure liquid chromatography after reduction with triphenylphosphine, and as reported previously[9,10] four isomeric methyl hydroxylinoleates were found: (9*Z*,11*E*)-13-hydroxy-, (9*E*,11*E*)-13-hydroxy-, (10*E*,12*Z*)-9-hydroxy-, and (10*E*, 12*E*)-9-hydroxyoctadecadienoic acid methyl esters.

Table 1 shows that, at early stages where conversion is low, the values of substrate disappearance, peroxide and conjugated diene formation agreed well. As the oxidation proceeded, however, the amount of the conjugated diene formed became smaller than oxygen uptake. On the other hand, only a trace amount of TBA-reactive material was found and neither pentane nor ethane was observed. It has been pointed out that the TBA-reactive materials are formed from olefins with three or more double bonds.[11]

The oxidation of methyl linolenate exhibited a somewhat different pattern from that of methyl linoleate. Methyl linolenate was oxidized faster than methyl linoleate and $k_p/(2k_t)^{1/2}$ increased with increasing conversion, again suggesting autocatalytic oxidation. The most interesting point is that the amount of substrate reacted, and peroxides and conjugated diene formed were considerably smaller than oxygen uptake, whereas some TBA-reactive materials were observed, although they were 15% of oxygen uptake. Pryor[12] also observed in the oxidation of methyl linolenate by air containing 1.5 ppm ozone that the formation of peroxide was larger than that of conjugated diene which was still larger than TBA-reactive material.

The results given above demonstrate that none of the presently accepted tests is quantitative and that the validity of each method for measuring the extent of oxidation of lipids and tissues depend on the profile of unsaturated fatty acids existing in each sample. Care should be taken in the application of methods suitable for the specific system. For example, the oxidation may have proceeded considerably even if the TBA value is small. The oxygen uptakde must be the most useful, versatile and quantitative measure, but it should be emphasized that the oxidation should be followed by as many approaches as possible. Which the best criterion relevant to pathological effect is, remains, of course, as another question.

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