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# CHARACTERIZATION OF LARGE UNILAMELLAR VESICLES AS MODELS FOR STUDIES OF LIPID PEROXIDATION INITIATED BY AZOCOMPOUNDS

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The aim of this work was to characterize large unilamellar vesicles (LUVETs) prepared by a hand-driven extrusion device in order to use them for studies of lipid peroxidation and antioxidant activity. Vesicle structure and size were examined by electron microscopy. Lipid and antioxidant content was determined before and after the extrusion procedure. Then LUVETs were subjected to autoxidation initiated by both the lipid-soluble 2,2′-azobis(2,4-dimethylvaleronitrile) and the water-soluble 2,2′-azobis(2-amidinopropane hydrochloride) azocompounds. The results demonstrated that: i) LUVETs prepared with lipid concentrations ranging between 25 and 150 mM were essentially unilamellar and reasonably homogeneous, with an average diameter of 90 nm; ii) the phospholipid, cholesterol and antioxidant amounts retained by filters were about 10–15%; iii) LUVETs were suitable for autoxidation studies initiated by the water-soluble azocompound both in the absence and presence of antioxidants. The lipid-soluble azocompound could be used only at low concentrations and its vesicle content had to be determined since part of the initiator was not incorporated into the lipid bilayer. These data suggest that LUVETs seem to be recommended for studies of lipid peroxidation and antioxidant activity.

KEY WORDS: Vesicles, Phospholipids, Lipid Peroxidation, Antioxidants, Azothermal initiator.

Abbreviations: LUVET: large unilamellar vesicle by extrusion technique; PC: egg yolk phosphatidylcholine; $Q_3$: oxidized coenzyme Q-3; $Q_7$: oxidized coenzyme Q-7; $Q_3H_2$: reduced coenzyme Q-3; $Q_7H_2$: reduced coenzyme Q-7; α-T: α-tocopherol; CH: cholesterol; AAPH: 2,2′-azobis(2-amidinopropane hydrochloride); AMVN: 2,2′-azobis(2,4-dimethylvaleronitrile); LH: unsaturated lipid.

## INTRODUCTION

The use of lipid vesicles as model membrane systems is popular in a variety of biochemical studies. A large number of vesicles different in form, size, number of bilayers and trapping efficiency are currently used. Multilamellar vesicles are prepared by simply shaking a dry phospholipid film with an aqueous solution[1] and their trapping efficiency can be increased by techniques involving organic solvents[2] or freeze-thaw procedure,[3] small unilamellar vesicles can be produced by sonication,[4] by a French pressure cell[5] or by ethanol injection;[6] large unilamellar vesicles are obtained *via* dilution from organic solvent,[7] detergent dialysis,[8] or by extrusion of multilamellar vesicles.[9] All of these vesicles have particular advantages and

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drawbacks, depending on the specific application they have been made for. Vesicles are also extensively utilized for the study of lipid peroxidation[10,11] and antioxidant activity,[12,13] however, vesicles suitable for this kind of experiments have to satisfy special requirements such as: i) the absence of organic solvents or detergents, since these compounds can behave as radical scavengers; ii) unilamellar structure with an homogeneous size distribution, in order to achieve a more realistic model of biological membranes; iii) the preservation of the phospholipid integrity, since sonication easily induces phospolipid breakdown.[14,15] Finally, an easy, quick and reproducible procedure for vesicle preparation would be desirable.

It was shown that the repeated extrusion of multilamellar vesicles through polycarbonate filters under moderate pressure resulted in a homogeneous population of large unilamellar vesicles, called LUVETs.[9] The use of these vesicles, having the above cited features, seems to be recommended in studies of lipid peroxidation and antioxidant activity. The aim of this work was: 1) to check, by electron microscopy, the number of bilayer and the size of vesicles formed from different lipid amounts; 2) to measure filter retention of phospholipids, cholesterol and lipophilic antioxidants during the extrusion; 3) to verify whether LUVETs were suitable to undergo peroxidation reactions initiated both by lipophilic or hydrophilic thermolabile azocompounds in the absence or presence of antioxidants.

## MATERIALS AND METHODS

### *Chemicals*

Egg yolk lecithin (PC) was purchased from Lipid Products (Redhill, U.K.) and stored at −20 °C in chloroform/methanol 1: 1 (v/v) under nitrogen. Coenzyme $Q_3$ ($Q_3$) and coenzyme $Q_7$ ($Q_7$) were kindly supplied by Eisai Co. (Tokyo, Japan) and stored as approx. 10 mM solutions in ethanol at −20 °C. Cholesterol (CH) from Boehringer Mannheim (Germany) was used without further purification. The thermolabile azocompounds 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH) and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) were obtained from Polysciences Inc. (Warrington, PA), α-tocopherol (α-T) and MES buffer were from Sigma Chemical Co. (St. Louis, MO). All other chemicals of the highest available purity were from Merck. All aqueous solutions were passed through Chelex-100.

### *Reduction of Coenzymes Q*

The reduction of coenzymes Q was achieved according to Rieske:[16] $Q_3$ or $Q_7$ (10–50 mg in 5 ml ethanol) were added to 15 ml of a solution containing 0.1 M phosphate, pH 7.4, and 0.25 M sucrose. Then a pinch of sodium dithionite was added and the mixture was shaken until it was colorless. The aqueous solution was extracted with cyclohexane and evaporated under vacuum. The residue was dissolved in absolute ethanol and acidified with dilute HCl to ensure a prolonged stability of this solution.

### *LUVET Preparation*

Multilamellar vesicles were prepared by adding in a round-bottom tube PC, CH, lipid-soluble antioxidants and AMVN, when necessary. After each addition the

solvent was carefully removed with a stream of nitrogen to obtain a thin film. Then 0.6 ml of 10 mM MES buffer, pH 6.5, containing 0.14 M NaCl and 1 mM $Na_2EDTA$ was added and the film was vortex-stirred for 7 minutes to achieve a white, milky, multilamellar vesicle suspension. PC, CH and antioxidant concentrations were 25–150 mM, 12–22 mM, 15–400 $\mu$M, respectively. The milky suspension was then transferred into LiposoFast (produced by Avestin, Ottawa, Canada) and extruded 19 times back and forth through two polycarbonate filters (100 nm pore size, Nucleopore Corp., Pleasenton, CA) by simply applying a manual pressure.[17] Oxidation of vesicle preparations before and after extrusion was measured according to Klein.[14] The total volume of LUVETs was then adjusted to give a final PC concentration of about 15 mM. As cited above, when the lipid-soluble azocompound AMVN was used, the initiator was added before the LUVET formation to enable its uniform distribution. In this case vesicles were prepared in a cold room, in order to prevent the start of AMVN decomposition. In the experiments using water-soluble AAPH, the initiator was added to the vesicle suspension at the beginning of the measurement, therefore no special care was taken during LUVET preparation.

### *Freeze-fracture*

LUVET suspensions mounted on gold disks and frozen in Freon 22 and liquid nitrogen, have been fractured in a Balzer BAF 301 device at a vacuum of better than $5 \times 10^{-7}$ Torr and a temperature of $-133$ °C. Immediately after fracture, the specimens were shadowed with platinum-carbon at an angle of 45 °. Replicas reinforced with carbon evaporation were then cleaned in chloroform-methanol bleach and examined by a Philips CM 10 transmission electron microscope at 80 Kv and 46.000 × magnification.

### *Phospholipid and Cholesterol Determination*

The phospholipids were quantitated by phosphate analysis;[18] cholesterol was determined enzymatically by using a Boehringer Mannheim Cholesterol Kit. Both measurements were carried out before and after vesicle extrusion.

### *Antioxidant Determination*

Aliquots of vesicles (0.1 ml) before and after extrusion were added with the same volume of isopropanol to solubilize lipid components. The solutions (20 $\mu$l) were injected in a HPLC apparatus equipped with an Altex ODS 5 $\mu$m column (15 × 0.46 mm). The elution was isocratic at a flow rate of 1.5 ml/min with methanol for the determination of $\alpha$-T and reduced coenzyme $Q_7$ ($Q_7H_2$) and with methanol/water (82.5:17.5) for reduced coenzyme $Q_3$ ($Q_3H_2$). Detection was at 290 nm.[19]

### *Vesicle Autoxidation*

Autoxidation experiments were carried out by monitoring the oxygen concentration with a Clark-type oxygen electrode (Yellow Spring, OH). After thermal equilibration at 40 °C, an appropriate amount of AAPH was added to the LUVET suspension (3 ml) in order to obtain final AAPH concentrations of 10, 20 and 40 mM. When AMVN was used as radical initiator, LUVET suspensions were incubated at

45 °, 50 °, 55 ° and 60 °C. The reaction cell was always protected from room light to avoid initiator photodecomposition. In some experiments vesicle autoxidation was also measured by the formation of thiobarbituric acid reactive compounds according to Gutteridge.[20] Chromogens were measured at 532 against appropriate blanks.

## RESULTS AND DISCUSSION

In this study large unilamellar vesicles were prepared using Liposofast, a hand-driven extrusion apparatus recently constructed.[17] The only disadvantages of this device are the small volume handled, i.e. 0.5–0.6 ml, and a recommended lipid concentration of 6–30 mM. As higher volumes are often required to perform experiments of lipid peroxidation, we used a range of lipid concentration between 25 and 150 mM. However, no information about bilayer number, homogeneity and size of LUVETs obtained at much higher lipid concentration is available. For this reason multilamellar vesicles of 50, 75 and 150 mM PC concentrations were prepared, frozen, fractured and their replicas examined in an electron microscope. The examination showed that the repeated passages through the mini-extruder primarily produced unilamellar vesicles. The electron micrograph of Figure 1 shows that replicas of LUVETs of 150 mM PC do not exhibit a significant number of cross-fractures, indicating the absence of inner lamellae. Thus, even when formed using the highest amount of egg PC, they were essentially all single layered. The average diameter measured from electron micrographs was found approximately 90 nm.

At first we investigated whether the preparation of LUVETs could cause the oxidation of the lecithin. As oxidation of unsaturated fatty acid residues is accompanied by the formation of conjugated dienes, changes in the UV spectrum of

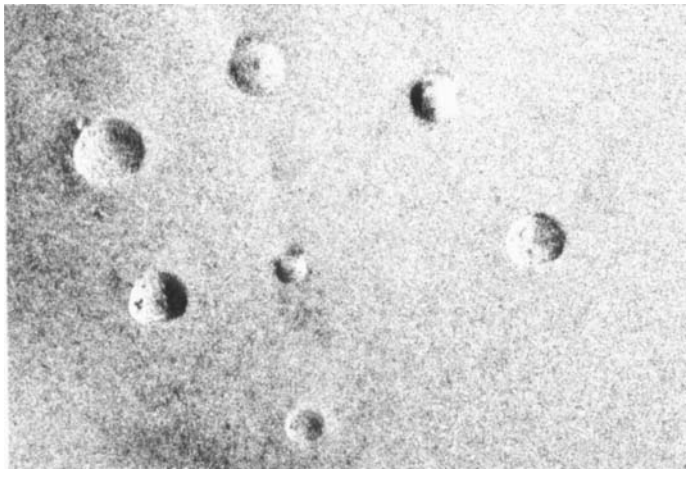

FIGURE 1 Electron transmission micrograph of replicas of freeze fractured vesicles prepared with 150 mM egg lecithin suspended in 10 mM MES buffer, pH 6.5, containing 0.14M NaCl and 1 mM $Na_2EDTA$.

TABLE 1
Phospholipid and cholesterol content of different vesicle preparations before and after extrusion

| MULTILAMELLAR VESICLES | | LUVETs | | LOSS (%) | |
|---|---|---|---|---|---|
| [PC]mM | [CH]mM | [PC]mM | [CH]mM | PC | CH |
| 21.46 ± 0.88 | – | 19.14 ± 1.22 | – | 10.8 | – |
| 45.10 ± 3.21 | – | 38.88 ± 4.11 | – | 13.8 | – |
| 58.67 ± 0.99 | – | 49.75 ± 0.70 | – | 15.2 | – |
| 111.84 ± 3.05 | – | 98.19 ± 1.61 | – | 12.2 | – |
| 38.83 ± 1.78 | 12.12 ± 1.75[a] | 34.20 ± 2.52 | 10.72 ± 0.60 | 11.9 | 11.5 |
| 40.64 ± 1.53 | 22.40 ± 0.52[b] | 35.88 ± 1.32 | 9.00 ± 0.88 | 11.7 | 15.2 |

Data are the means of ten experiments with different vesicle preparations.
[a] PC/CH molar ratio 8:2
[b] PC/CH molar ratio 6:4

vesicles before and after extrusion were recorded and the oxidation index, i.e. $A_{233}/A_{215}$, was calculated. The index was 0.12 ± 0.01 for multilamellar vesicles and 0.13 ± 0.008 for LUVETs, therefore the multiple passages through the filters did not increase diene conjugation.

Then the filter retention of lipid components was measured. In fact, vesicle composition can vary depending on the subsequent application: cholesterol is frequently added to obtain a higher bilayer stability[11] and lipid soluble antioxidants are incorporated when their activity as chain-breakers has to be studied. On the other hand, the numerous sample passes back and forth through the two filters could decrease the amount of the different lipid components initially added. Table 1 reports the content of phospholipids and cholesterol of different vesicle preparations before and after the extrusion. It can be seen that the PC loss was about the same for all the concentrations tested, namely 10.8–15.2%. When cholesterol containing vesicles (8:2 or 6:4 lecithin/cholesterol molar ratio) were checked, the cholesterol loss resulted equal to that obtained for lecithin, i.e. 11.5–15.2%. It can be deduced that the amount of these lipid components retained by filters was not affected by their concentration.

Antioxidants incorporated in LUVETs were the main lipophilic physiological antioxidant, i.e. α-tocopherol, and two reduced coenzyme Q homologues, the synthetic short-chain homologue, $Q_3H_2$, and the physiological long-chain one, $Q_7H_2$. Practical reasons can justify the use of homologues of intermediate side-chain length, which have more handy physical properties, when the bioenergetic[21,22] or the antioxidant[23,24] role of $QH_2$ is studied. The antioxidant activity of coenzyme Q is well documented *in vitro* and *in vivo*.[25] Furthermore, previous results[26,27] from our laboratory have shown that the different homologues act as antioxidants with quite similar effectiveness. The losses of $Q_3H_2$, $Q_7H_2$ and α-tocopherol occurring during extrusion in relation both to the antioxidant content originally incorporated and to the PC amount present were determined, as shown in Table 2. Vesicles formed from 25 mM PC and from antioxidant concentrations ranging between 16 and 127 μM exhibited a loss of antioxidants that was about 16% for ubiquinol homologues and 5% for α-tocopherol. When LUVETs were prepared with 50 mM PC and antioxidant concentration changed from 143 to 300 μM, filter retention was very low (3%) for α-tocopherol, while it was very similar to that found in the previous set of experiments for the other two inhibitors of lipid peroxidation. The loss was low for $Q_3H_2$ and $Q_7H_2$ (under 8%) and nearly negligible for α-

TABLE 2
Percent loss of antioxidants during extrusion of vesicles prepared with different amounts of PC and $Q_3H_2$, $Q_7H_2$, α-T

| [PC] mM | [AH] | LOSS (%) |
|---|---|---|
| 25 | $Q_3H_2$ 16–86 μM | 18.9 |
| 25 | $Q_7H_2$ 16–127 μM | 13.2 |
| 25 | α-T 20–89 μM | 5.1 |
| 75 | $Q_3H_2$ 143–271 μM | 18.7 |
| 75 | $Q_7H_2$ 150–300 μM | 12.7 |
| 75 | α-T 170–259 μM | 3.0 |
| 150 | $Q_3H_2$ 400–498 μM | 7.5 |
| 150 | $Q_7H_2$ 402–600 μM | 5.6 |
| 150 | α-T 409–500 μM | 1.6 |

Data are the means of four experiments with different vesicle preparations.

tocopherol in the presence of 150 mM PC and 400–600 μM antioxidants. In our opinion, the very high amount of egg lecithin can saturate all the filter centers able to interact with antioxidants and responsible for their retention. The different behavior of α-tocopherol with respect to $Q_3H_2$ and $Q_7H_2$ can be explained by the susceptibility of ubiquinol homologues to oxidation.[27] In fact, traces of oxidized coenzymes Q were found at the end of the preparation on polycarbonate filters and in LUVETs. Thus the decrease in ubiquinol content within LUVETs can be ascribed both to the filter retention and to the oxidation of the reduced form of coenzymes Q under air.

LUVET suspensions adjusted to give a final PC concentration of about 15 mM were then subjected to autoxidation initiated by azocompounds able to generate peroxyl radicals by thermal decomposition. The use of water or lipid soluble initiators, which generate free radicals at a constant rate and at specific sites, offers the possibility of studying lipid oxidation in aqueous dispersions and the role of chain-breaking antioxidants in lipid peroxidation.[12,13] In the recent years research on antioxidants has received remarkable attention at both basic and applied levels and it has become evident that natural and synthetic antioxidants play an important role in protecting cells and tissues against oxidative damage.[28] Furthermore, antioxidants are of interest to radiation chemistry, food science and polymer chemistry.

When an azothermal initiator is present, the kinetics of PC autoxidation in bilayers follow the same classic rate law well known for autoxidation of organic substrates in homogeneous solution.[13,29] The rate of oxidation is given by equation 1:

$$\frac{-d[O_2]}{dt} = k_p(R_i/2k_t)^{1/2}[LH] + R_i \qquad (1)$$

where $R_i$ is the rate of free radical chain initiation, $k_p$ and $k_t$, are the rate constants of chain propagation and termination, respectively. Polyunsaturated fatty acyl residues of the lecithin are represented by LH.

In the presence of a chain-breaking antioxidant (AH), such as α-T or $QH_2$ in the membrane or ascorbate in the water environment, the rate of oxidation is given by equation 2:

$$\frac{-d[O_2]}{dt} = \frac{k_p R_i[LH]}{nk_{inh}[AH]} + R_i \qquad (2)$$

where $n$ represents the stoichiometric factor, i.e. the number of radicals trapped by each molecule of the antioxidant, and $k_{inh}$ the inhibition rate constant for the antioxidant. It is apparent that equations 1 and 2 contain $R_i$, the rate at which new chains are started in the system, i.e. the rate of oxygen consumption in the absence of peroxidation. $R_i$ can be represented by equation 3:

$$R_i = 2ek_i[In] \tag{3}$$

where $e$ is the efficiency of chain initiation and $k_i$ the rate constant for decomposition of the initiator, In. $R_i$ can be determined by measuring the length of the inhibition period, $\tau$, in the presence of $\alpha$-T, for which a stoichiometric factor of 2 is assumed:

$$R_i = \frac{2[\alpha - T]}{\tau} \tag{4}$$

When AAPH was added to the aqueous suspensions of LUVETs in the absence of any antioxidant the autoxidation of these vesicles proceeded by a free radical chain mechanism. In these conditions a constant rate of oxygen uptake was expected to be observed without any appreciable lag period. Figure 2 shows some typical oxygen uptake traces of LUVETs for reactions initiated by different amounts of AAPH in the aqueous phase at 40 °C both in the presence and absence of $\alpha$-tocopherol. When AAPH concentration was 10 mM (see trace 4) a clear lag period of about 23 min was observed, whose length increased by decreasing azocompound concentration (not shown). Vesicles were oxidized at a constant rate only in the presence of 20 and 40 mM AAPH (traces 2 and 1). This behavior might be explained according to considerations recently suggested by Ingold *et al.*[30] in a study of

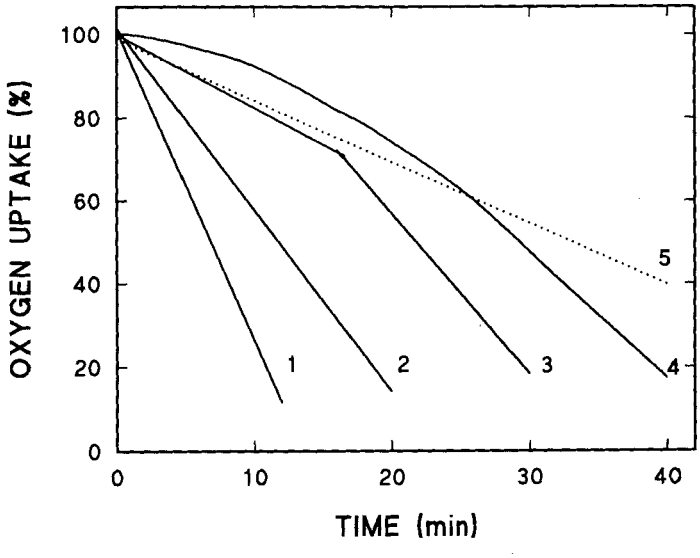


FIGURE 2 Oxygen uptake during LUVET peroxidation initiated by different amounts of AAPH at 40 °C: (1) [AAPH] = 40 mM; (2) [AAPH] = 20 mM; (3) [AAPH] = 20 mM and [$\alpha$-T] = 7.7 $\mu$M; (4) [AAPH] = 10 mM; (5) [AAPH] = 20 mM in the absence of LUVETs. Egg lecithin concentration was 15 mM.

autoxidation of lipids and antioxidation by α-tocopherol and reduced coenzymes Q in LDL. The authors claim that probably the efficiency of reaction between water-soluble peroxyls of AAPH and oxidizable particles is not 100%, i.e. each effective hit may be preceded by many unreactive encounters. For this reason a lag period was observed at 10 mM AAPH, while a constant rate of oxygen uptake occurred only when autoxidation started simultaneously in every vesicle, i.e. at 20–40 mM AAPH. In fact, effective hits, able to abstract hydrogen from a polyunsaturated fatty acid molecule, became more probable by increasing AAPH concentration. In the same figure oxygen uptake during 20 mM AAPH initiated autoxidation of LUVETs containing 7.7 μM α-T is also reported (see trace 3). A clear induction period of 16 min occurred and, as expected, after all the antioxidant had been depleted, the reaction proceeded at the same rate observed in the absence of the inhibitor (see trace 2). These experimental conditions were used in our recent paper[27] that aimed to compare, on a quantitative basis, the antioxidant activity of α-tocopherol with that of ubiquinol homologues in LUVETs. Figure 2 also reports the rate of oxygen uptake due only to the decomposition of 20 mM AAPH at 40 °C (see trace 5), which was nearly superimposable to that obtained in the presence of α-T. The rate of oxygen consumption is an overall index of the progress of peroxidation; however, each technique for measuring the rate of peroxidation of lipids measures something different and no one method by itself can be said to be an accurate measure of lipid peroxidation.[31] For this reason some experiments were also repeated by determining the peroxidation of fatty acids with the TBA test. As shown in Figure 3 the course of TBARS formation in LUVETs in the presence of 10, 20, 40 mM AAPH matched the rate of oxygen consumption determined by the oxygen electrode.

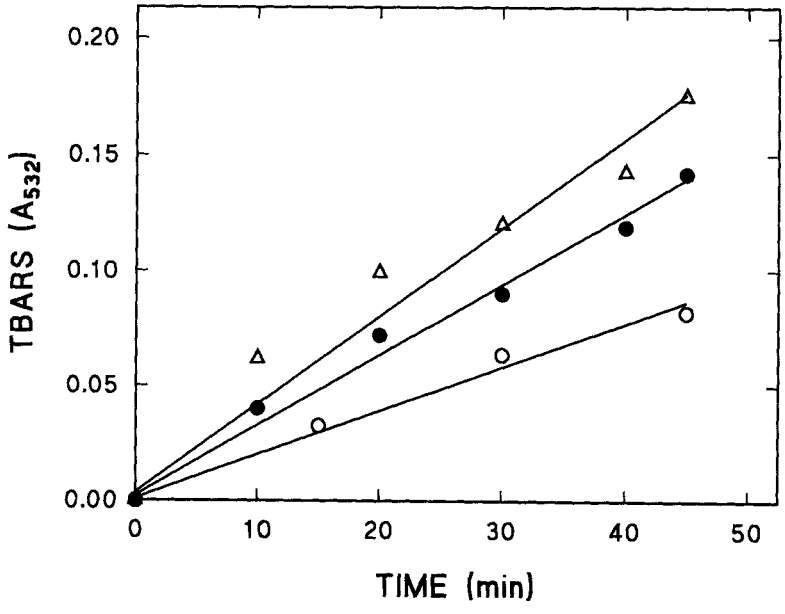


FIGURE 3 TBARS formation during LUVET peroxidation initiated by different amounts of AAPH at 40 °C. (○) [AAPH] = 10 mM; (●) [AAPH] = 20 mM; (Δ) [AAPH] = 40 mM. Assays were performed using 3 μmol of PC.

The lipid-soluble AMVN has been frequently used in autoxidation studies of multilamellar vesicles both in the presence and absence of antioxidants.[12,13] This azocompound, due to its lipophilicity, has to be incorporated into vesicles when they are formed. Multilamellar vesicles of 50 and 100 mM PC containing 16 and 33 mM AMVN, respectively, were prepared and extruded. In these conditions, i.e. with a phospholipid/initiator molar ratio of 3, the flow of vesicles was hindered and precipitation of the azocompound on polycarbonate filters was observed. Then large vesicles were formed with a higher phospholipid/initiator molar ratio, i.e. 6; in this case the extrusion was easier and the filter retention of the initiator was negligible. Therefore, provided that AMVN concentration into the bilayer was determined (since AMVN solutions exhibited an absorbance spectrum with a maximum at 350 nm) useful conditions for peroxidation studies could be found by varying the assay temperature. Figure 4 shows some typical oxygen uptake traces of LUVETs for reactions initiated by 2 mM AMVN within the lipid bilayer at different temperatures and the oxygen consumption due only to the decomposition of AMVN at 50 °C (trace 5). Vesicles were oxidized at a constant rate without any noticeable lag period. From the traces of Figure 4 it is also possible to observe that, as expected, the rates of oxygen consumption increased by increasing assay temperature. As far as the oxygen uptake in the absence of oxidizable substrate is concerned, the same considerations reported above for AAPH are also tenable for AMVN.

The aim of this work was to characterize large unilamellar vesicles to be used particularly in quantitative kinetic studies of autoxidation both in the presence and absence of antioxidants. To this purpose the rate of chain initiation must be known and controlled. This was achieved by using thermal initiators and by working under

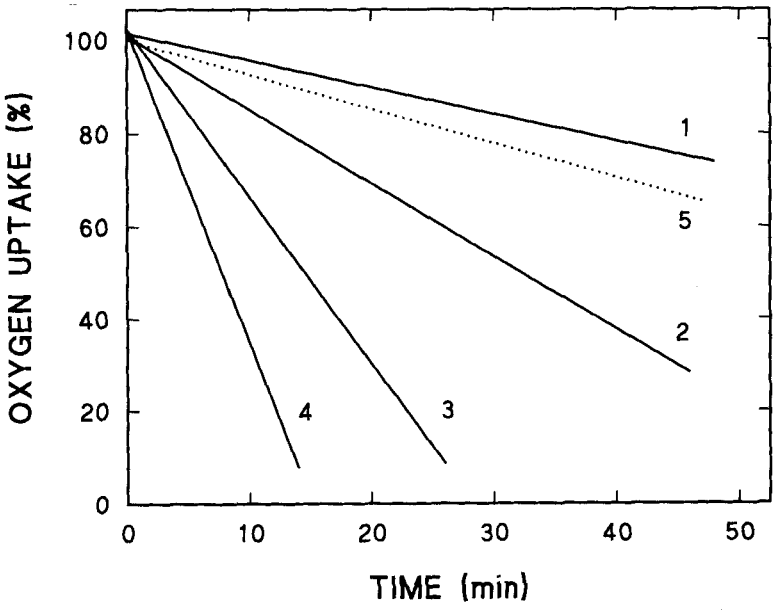


FIGURE 4 Oxygen uptake during LUVET peroxidation initiated by 2 mM AMVN at different temperatures: (1) T = 45 °C; (2) T = 50 °C; (3) T = 55 °C; (4) T = 60 °C; (5) T = 50 °C in the absence of LUVETs. Egg lecithin concentration was 15 mM.

conditions where new chains started only by the decomposition of azocompounds, therefore, the addition of EDTA during vesicle preparation was necessary to chelate adventitious iron ions present in water and reagents. However, LUVETs can also be used when lipid peroxidation is initiated by superoxide-dependent Fenton chemistry or stimulated by iron by a peroxide-decomposition reaction. In this case EDTA has not to be added and Good type buffers, such as MOPS, MES or HEPES should be used, since they have low affinity for[32] $Fe^{2+}$ and do not interfere with its rate of autoxidation.[33]

In conclusion the results here reported demonstrate that: i) LUVETs formed from high lipid amounts by the hand-driven extrusion apparatus are essentially unilamellar and their diameters are distributed around 90 nm, therefore phospholipid concentration can be increased at least five times with respect to that recommended (6–30 mM); ii) extrusion does not cause oxidation of lipids; iii) the amount of lecithin and cholesterol retained by filters is always in the order of 10–15%, independent of the concentration initially added and the loss of lipophilic antioxidants is low, particularly at high lipid concentrations; iiii) LUVETs can be profitably used in autoxidation studies initiated by water and lipid soluble initiators both in the absence and presence of lipophilic antioxidants. As for the use of LUVETs containing lipophilic azocompounds, the molar ratio between phospholipid and AMVN must be considered in order to incorporate the added initiator, otherwise the precipitation of the lipid-soluble azocompound on filters becomes a serious drawback.



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