International Journal of Pharmaceutics, 8 (1981) 101–110

α-TOCOPHEROL RETARDS AUTOXIDATION AND PROLONGS THE SHELF-LIFE OF LIPOSOMES

C. ANTHONY HUNT and SUE TSANG

School of Pharmacy, University of California, San Francisco, Calif. 94143 (U.S.A.)

(Received August 20th, 1980)
(Revised version received January 28th, 1981)
(Accepted January 28th, 1981)

SUMMARY

All liposomes undergo autoxidation that is accelerated by elevated temperature, light, metal ions and some solutes. As a result, there is a dramatic, often abrupt, change in liposome permeability. Incorporation of α-tocopherol into liposomes prolongs the characteristic induction phase of autoxidation. When liposome shelf-life is based on retention of trapped [^{14}C]sucrose, α-tocopherol at 0.1 mol% doubles the shelf-life of multilamellar liposomes containing no cholesterol that are stored in air and light at 22°C. Proportional increases in shelf-life result from increasing amounts of α-tocopherol. Incorporation of cholesterol further improves the shelf-life. A benefit of incorporation of α-tocopherol into liposomes is improved stability in plasma. When it is difficult to avoid exposure of liposomes to oxygen, as for example, when they are to be used as in vitro or in vivo drug carriers, incorporation of α-tocopherol may prove prudent.

INTRODUCTION

Limitations on the use of liposomes as drug carriers have been discussed (Kimelberg and Mayhew, 1978; Papahadjopoulos, 1979). Among these are the difficulty of reproducibly preparing liposomes of defined size and stability of liposomes, particularly with respect to retention of the entrapped solute(s) and their in vivo properties. With the development of extrusion techniques to narrow their size-frequency distribution (Olsen et al., 1979), liosomes of defined size can be prepared reproducibly. Liposome instability remains a problem. In this report attention is focused on oxidation in liposomes.

The various mechanisms involved in phospholipid oxidation are well documented (Holman, 1954). For liposomes the primary concern is autoxidation. Autoxidation of lipids is accelerated by metal ions, light (and other forms of radiation), some organic molecules and high pH. However, autoxidation can be inhibited by metal chelators and antioxidants (Holman, 1954). Konings et al. (1979) have used a spectrophotometric

procedure which provides a measure of oxidation products for studying the sensitivity of small unilamellar (sonicated) and multilamellar liposomes to X-ray-stimulated peroxidation. They find that the primary determinant of liposome sensitivity is the structure of the phospholipid acyl chains, and that α-tocopherol is significantly superior to cysteamine and glutathione as protectors against peroxidation. Using different procedures Dobrestsov et al. (1977) have investigated the effect of $FeSO_4$-induced peroxidation on bilayer fluidity in liposomes composed primarily of egg phosphatidylcholine. They clearly demonstrate that peroxidation increases the rigidity of the liposome membranes. Such decreased bilayer fluidity is typically associated with a reduced permeability of the liposome to entrapped solutes.

During preliminary studies of techniques to improve the shelf-life of liposomes and establish their relative stability in plasma we observed that autoxidation of liposomes results in a rapid and dramatic increase in liposome permeability, contrary to expectations based on the accelerated peroxidation studies of Dobrestsov et al. (1977). This report describes the consequences of liposome autoxidation and results of incorporation α-tocopherol and cholesterol to retard the process.

METHODS AND MATERIALS

Chemicals and supplies

L-α-Phosphatidylcholine (PC), type V-E, L-α-phosphatidic acid (PA), as the sodium salt, cholesterol (CH), analytical standard, D-α-tocopherol, (α-T) type V, and stearylamine were purchased from Sigma (St. Louis, Mo.) and were confirmed pure or purified by thin-layer chromatography (Papahadjopoulos et al., 1973). [^{14}C(U)]-Sucrose, 3.6–4.9 mCi/mmol, was from New England Nuclear (Boston, Mass.). Triton X-100, scintillation grade and PCS scintillation cocktail were from Amersham/Searle (Arlington Heights, Ill.). All other reagents were analytical reagent, USP grade or better. The buffer in all studies was isotonic phosphate-buffered saline (PBS), pH 7.4, without Mg or Ca (Abra et al., 1980). To avoid bacterial growth penicillin (10^5 units/liter) and streptomycin (0.1 g/liter) from the campus cell culture facility were added to all PBS solutions; to avoid catalysis of oxidation by trace metal ions, EDTA, 5 mM, was also included.

Thawed plasma from various donors was obtained from Moffitt Hospital Blood Bank, pooled and stored frozen (20 ml/ampule) until the day of use when it was thawed and filtered (no. 2 filter) prior to use. Dialysis tubing, 24 Å average pore radius from VWR, was processed by boiling for 1 h in 1% sodium carbonate followed by distilled water rinsing; they were stored at 4°C in 0.02% sodium azide until used.

Preparation of liposomes of defined size

The size range for multilamellar liposomes was controlled by using the extrusion procedures developed in this laboratory (Olsen et al. 1979). Millipore (Bedford, Mass.) 25 mm stirred ultrafiltration cells fitted with Nucleopore (Pleasanton, Calif.) polycarbonate membranes (0.8 μm pore size) were used for all extrusions. Briefly, an organic solution of PA was obtained by extraction of 2 mg/ml PA (Na salt) in 0.5 N HCl with 3 volumes of chloroform/methanol (10/1), followed by two extractions with 2 volumes of chloroform/methanol (9/1); combining these extracts resulted in essentially complete

recovery of PA. Aliquots of PC, CH, α-T, SA (each in chloroform) and the above PA solution, in the desired molar ratios, were combined in a round bottom flask and dried under vacuum. Subsequently, an aliquot (66–100 μl for each mol of lipid) of 5 mM [^{14}C]sucrose in PBS was added to the flask which was gently shaken at 37°C for approximately 2 h. These mechanically dispersed liposomes were then extruded twice at constant flow (approximately 10 ml/min). Untrapped sucrose was removed by dialysis for 12–24 h at 4°C against 500 vols. PBS, which was changed 3 times.

Measurement of liposome stability and oxidation

The stability in buffer of each liposome system was measured based on the percentage of the original trapped [^{14}C]sucrose remaining entrapped at any time. For studies of duration ≤48 h the liposome suspension, 10 mg/ml of phospholipid, was placed in a dialysis tube along with 1 ml PBS, sealed, and then suspended in 500 vols. of stirred PBS at constant temperature (22 or 37°C) in the presence or absence of both light and O_2 (air). Aliquots of the total ^{14}C remaining in the dialysis tube were then measured periodically. For studies of >48 h duration aliquots (1–2 ml) of the liposome suspension were placed along with an equal volume of buffer in 10 ml ampules which were sealed under nitrogen or Ar, and subsequently placed in a water bath at 22 or 37°C. Ampules were removed at various times, their contents were transferred to dialysis tubing, and the sample was dialyzed for 12 h at 4°C at which time an aliquot was measured for total ^{14}C remaining.

Oxidation of liposome components was measured using the thiobarbituric acid (TBA) test as described by Sinnhuber et al. (1958). Limitations of this and other assays of lipid peroxidation were discussed by Gray (1978). In brief, the procedure involved reaction of TBA with malonaldehyde and other peroxidation products under acid conditions to form TBA chromagen, the relative yield of which was quantified by the solution's absorbance at 530 nm. Since the phospholipid concentrations were identical in all liposome systems, a fixed aliquot was used for the TBA test in all cases. The degree of peroxidation was assumed to be proportional to the resulting absorbance at 530 nm, since all other factors were held constant.

Because the in vivo use of liposomes is a principal interest of this laboratory, the effect of addition of α-T on liposome stability in plasma was also evaluated. The procedures were identical to those described above for studies in buffer with the exception that equal volumes of plasma, rather than buffer, were mixed with the liposomes at the beginning of each study.

Accelerated stabilities

The effects on liposomes of peroxidation potentiation by $FeSO_4$ was evaluated using the procedures of Dobretsov et al. (1977). Oxidation and sucrose leakage were followed for up to 24 h after addition of $FeSO_4$.

RESULTS

Leakage of liposomes following autoxidation

When liposomes are prepared and stored in buffer in the presence of air there is an

autoxidation induction period during which no significant leakage is detected, followed by rapid leakage over an approximately 10-h period. Typical results are shown in Fig. 1 for multilamellar liposomes composed of PC/PA (4/1) and various amounts of cholesterol. Control liposomes treated and processed similarly, but N_2-purged and maintained in a N_2 atmosphere, show no significant leakage. The reproducibility of the induction period duration between studies for liposomes of the same composition and size was poor, yet duplicate studies carried out at the same time showed reproducible (range: ±2 h for the composition PC/PA 4/1) induction periods. Increasing amounts of CH consistently increased the duration of the induction period, but not the duration of the leakage phase. Increasing the fraction of PA consistently decreased the duration of the induction period. Decreasing incubation temperature from 37 to 22°C generally increased the duration of both the induction period and the leakage phase. Careful exclusion of light during incubation consistently gave a 2–10-fold increase in the induction period duration, but not the leakage phase.

Those liposomes most sensitive to autoxidation, PC/PA (4/1) without CH or α-T, showed no significant leakage (<5%) for up to 6 months when stored at 4°C in the absence of light and oxygen.

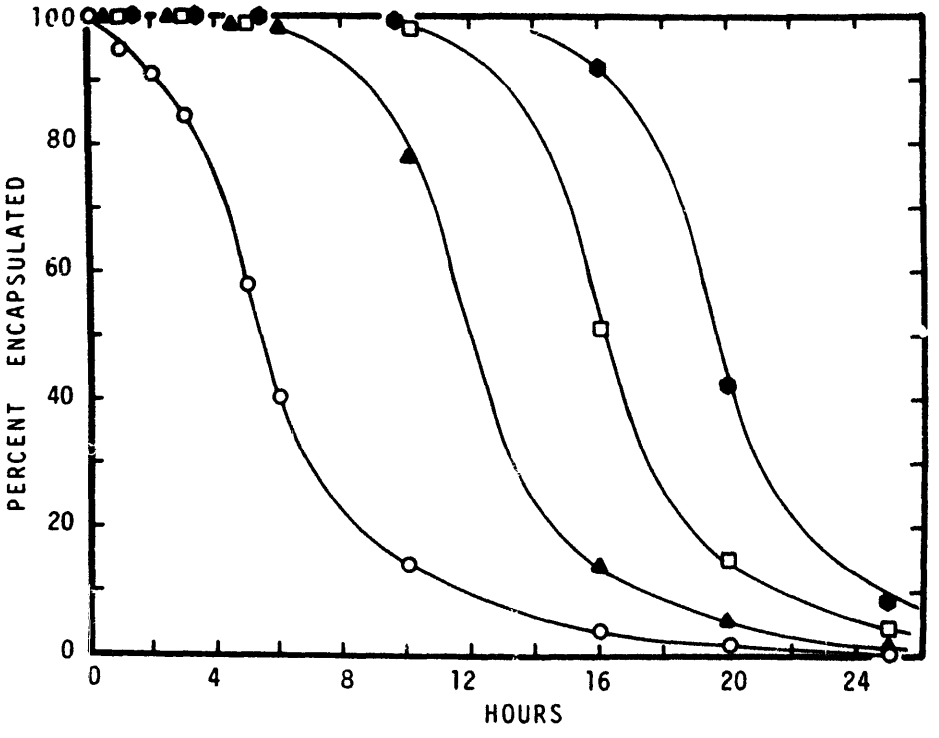

Fig. 1. Per cent [^{14}C]sucrose remaining encapsulated is plotted vs time for multilamellar liposomes without α-T incubated at 37°C in buffer (PBS) while exposed to light and in an air atmosphere. The composition is PC/PA/CH in the following molar ratios: 8/2/0(○), 8/2/2(▲), 8/2/4(□), and 8/2/8(●). Preparation of liposomes was initiated 24 h prior to the t = 0 value. Control liposomes incubated at 37°C in light, but in the absence of O_2, beginning at preparation, show no leakage over the same time period; control liposomes incubated at 4°C in dark, but in the presence of air, also show no leakage. Each point is the mean from duplicate studies.

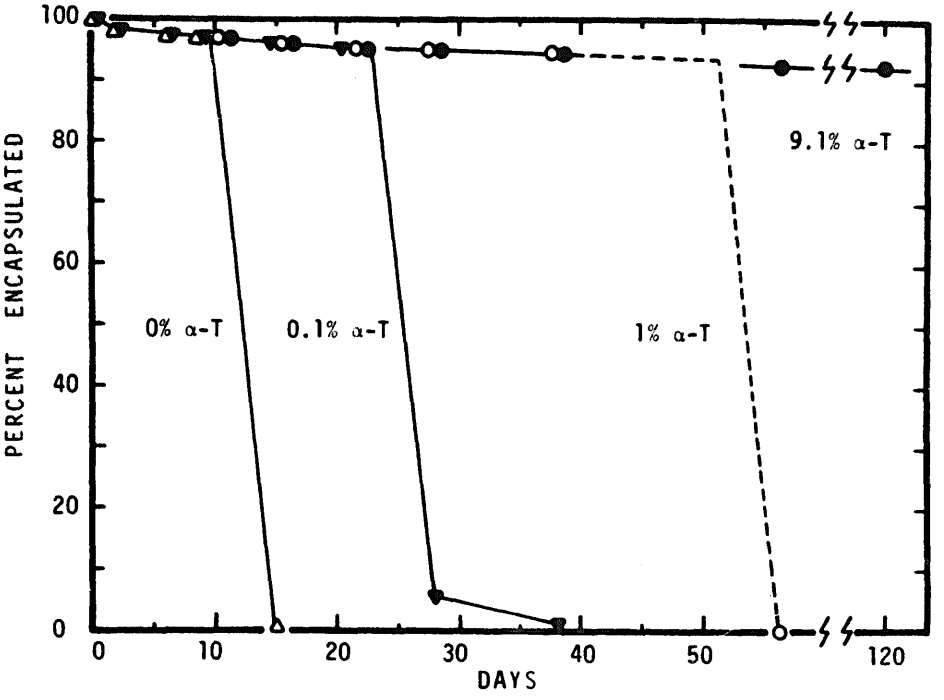

Fig. 2. Per cent [^{14}C]sucrose remaining encapsulated is plotted vs time for multilamellar liposomes composed of PC/PA/CH/α-T: 8/2/0/x. The liposomes are incubated in an air atmosphere at 22°C in buffer (PBS) while exposed to light; x = 0 (△); 0.01 (▼); 0.1 (○) and 1.0 (●). Control liposomes incubated at 22°C in dark, but not in light, in the absence of O_2 beginning at preparation show no leakage over the same time period. Each point is the mean from duplicate studies. Liposomes in Figs. 1 and 2 were prepared at different times; the lipids used were different batches.

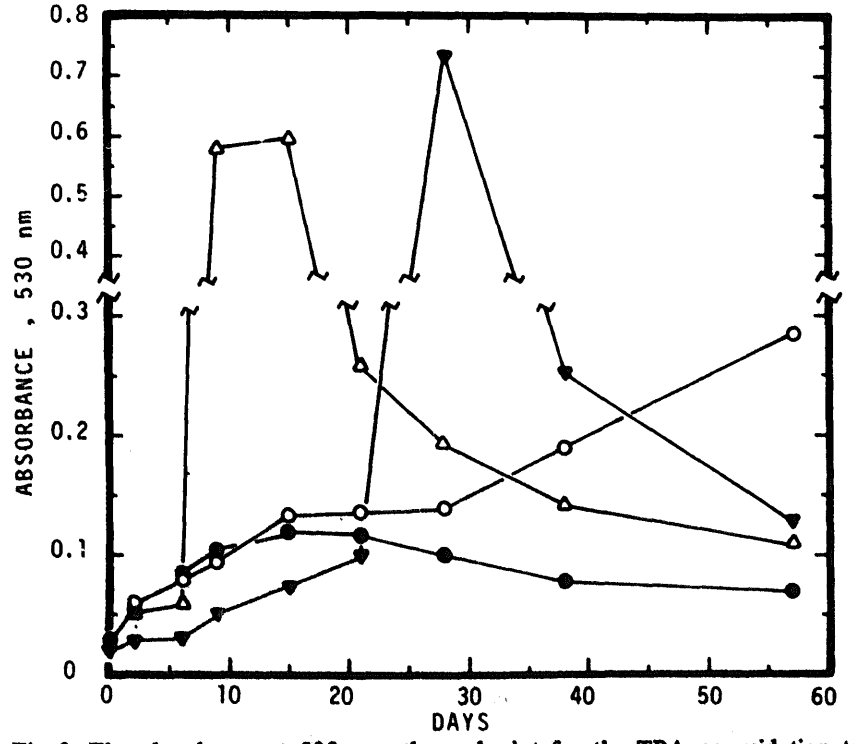

Fig. 3. The absorbance at 530 nm, the endpoint for the TBA peroxidation test, is plotted vs time for the same liposomes and conditions as in Fig. 2. The composition is PC/PA/CH/α-T: 8/2/0/x, where x = 0 (△); 0.01 (▼); 0.1 (○) and 1.0 (●). Each point is the mean from duplicate studies.

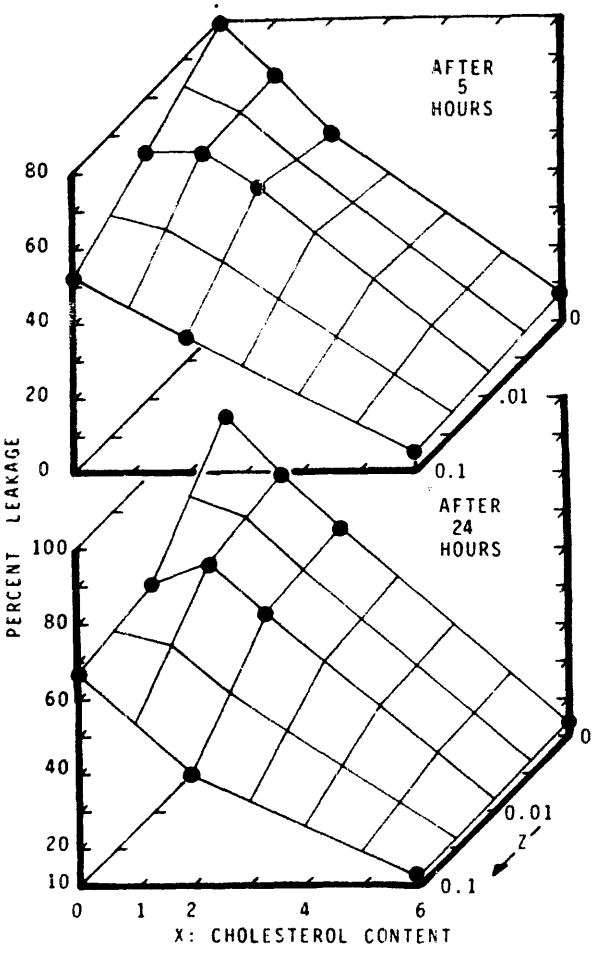

Fig. 4. Per cent leakage after 5 and 24 h for liposomes incubated in plasma at 37°C as a function of α-T (Z) and CH (X). Liposomes were exposed to light in equilibrium with air. The composition is PC/PA/CH/α-T in the molar ratio 8/2/X/Z.

Effect of α-tocopherol on liposome stability in buffer

Addition of 0.1–9.1 mol% α-T to liposomes composed of PC/PA (4/1) followed by storage in sealed ampules under air at 22°C in constant laboratory light dramatically increased the duration of the induction phase (Fig. 2). Liposomes composed of 9.1 mol% α-T showed no significant leakage even after 120 days under these suboptimal conditions.

The time course of measurable peroxidation products, based on the TBA test, was also favorably shifted by incorporation of α-T (Fig. 3). An induction and oxidation phase were evident for 0.0 and 0.1 mol% α-T, but the rapid oxidation phase was not apparent when either 1.0 or 9.1 mol% α-T were used. There was an apparent trend of increased oxidation with increasing α-T content during the early phase of the studies (e.g. 6 days in Fig. 3), consistent with the effect of α-T on oxidation of other lipids.

Effect of α-tocopherol on liposome stability in plasma

Plasma destabilizes liposomes; the magnitude of this effect is diminished by addition

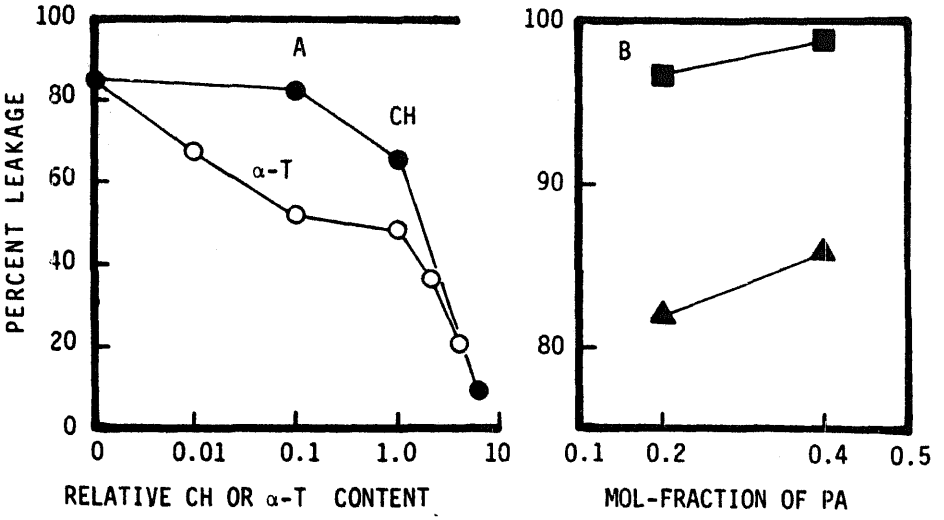

Fig. 5. A: Per cent leakage after 5 h of liposomes incubated in plasma as a function of CH (•) or α-T (○) content at 37°C. Liposomes were exposed to light and in equilibrium with air. The liposome composition is PC/PA/CH (•) in the molar ratio 8/2/X or PC/PA/α-T in the molar ratio 8/2/Z. The additional leakage after 24 h averaged at approximately 5%. B: Per cent leakage after 5 (▲) and 24 (■) h from liposomes as a function of PA (PC + PA) content. The conditions were the same as in A. These liposomes contained no CH or α-T.

of CH (Hunt and Tsang, 1979; Allen and Cleland, 1980). The amount of plasma-induced leakage of sucrose from the various liposomes after 5 and 24 h of incubation in plasma equilibrated with air (37°C) is shown in Fig. 4. Incorporation of 0.1 mol% CH does not improve stability, whereas incorporation of 0.1 mol% α-T does improve stability. Incorporation of 10 or 20 mol% of either CH or α-T into PC/PA liposomes results in essentially identical improvements in stability. However, the beneficial effect of 1 mol% α-T is masked when the CH content of the liposomes exceeds 10 mol%.

DISCUSSION

The liposome is inherently a stable system. In the absence of oxygen and free radicals the only chemical reactions which result in a change in liposome properties are those of acid/base-catalyzed ester hydrolysis and acid-catalyzed hydration of double bonds. Because the sites of these reactions are within the lipid bilayer, their rates are necessarily reduced relative to similar processes in aqueous solution. Since the lipid bilayer structure of liposomes is a minimum energy configuration, liposome suspensions would be expected to be quite stable under optimum pH conditions. When stability considerations focus on retention of a trapped drug, the inherent permeability of the selected liposome composition is one basis for determining shelf-life. For the multilamellar liposomes composed primarily of PC which are described in this report, there is no detectable degradation of chemical components and less than 5% leakage of trapped sucrose after 6 months at pH 7.4 and 22°C in the absence of oxygen and light.

In the presence of even trace amounts of oxygen the integrity of the liposome can be 'short lived'. We have demonstrated that when no effort is made to exclude oxygen or

light during or after liposome preparation, autoxidation of the lipids can result in complete loss of trapped solute within 48 h of preparation (Fig. 1). The autoxidation process for liposomes, or for other lipids (Holman, 1954), is characterized by an induction period of unpredictable duration followed by a rapid, autocatalytic oxidation phase which in turn is followed closely by an increasing degree of liposome permeability. Lipid oxidation is detectable prior to loss of trapped solute (Figs. 2 and 3), but detection of peroxidation products (using the TBA test) does not correlate well with the onset of permeability changes because leakage of solute is a terminal event, whereas the measured peroxidation reaction products are kinetic intermediates. To monitor the shelf-life of liposomes it may be necessary to measure both products of peroxidation and the degree of retention of a trapped solute.

Dobretsov et al. (1977) observed an increase in liposome membrane rigidity following $FeSO_4$-stimulated peroxidation. We have successfully reproduced the results of Dobretsov et al. Increased membrane rigidity implies decreased solute permeability. We, however, observe an increase in liposome permeability under conditions of autoxidation. Although these two sets of results appear contradictory, they may, nevertheless, be consistent with the known complexity of lipid oxidation reactions (Holman, 1954). Autoxidation proceeds via a number of parallel reaction pathways each involving a series of reactions; it seems reasonable that $FeSO_4$-stimulated peroxidation may proceed primarily by one of these pathways and does not result in the same decomposition products as does autoxidation. Thus, accelerated stability studies using $FeSO_4$-stimulated peroxidation, and possibly other means, may give invalid estimates of liposome sensitivity to autoxidation.

Results reported here show that α-T successfully retards autoxidation of liposomes similar to its ability to protect against radiation-induced oxidative damage (Konings, 1979). The primary effect of α-T is to prolong the autoxidation induction period, where induction period is defined as the time separating preparation of liposomes and the confirmed leakage of trapped solute or rapid rise in oxidation products (Figs. 2 and 3). α-T does not prevent lipid oxidation (Fig. 3). In fact, as was expected, addition of increasing amounts of α-T to liposomes results in increasing evidence of oxidation at early times. The lack of reproducibility in the induction period between studies, as opposed to within studies where results were reproducible, is to be expected for the experimental conditions used. The list of conditions and agents which accelerate autoxidation or retard it is long (Holman, 1954); and only when all variables are carefully controlled and limited does one observe reproducible induction periods. Under the adverse conditions of this study (22 and 37°C in the presence of light), addition of 0.1 mol% α-T roughly doubles the induction time relative to liposomes containing no α-T; further increases in the induction period by factors of from 5 to 20 are obtained when the sample is stored (37°C) in the absence of light. Addition of CH to liposomes enhances the effect of α-T (Fig. 1), even though CH itself is subject to peroxidation (Suwa et al., 1977), presumably as a result of the decrease in membrane fluidity.

Use of α-T to retard auto oxidation has the unexpected but beneficial effect of improving liposome stability in plasma[1]. In the absence of plasma, peroxidation, or other liposome

[1] Liposome stability in plasma depends on composition (Hunt and Tsang, 1979). The PC/PA ratios used for these studies were selected because of the 'relative' sensitivity of the resulting liposomes to the destabilizing effects of plasma.

destabilizing processes, incorporation of α-T into liposomes at levels above 2 mol% can reduce solute permeability (Diplock et al., 1977) when the liposome lipids contain a relatively high proportion of arachidonyl residues. Such decreased permeability is presumed to be similar to that produced by incorporation of CH into the lipid bilayers. Our observation that incorporation of 10 or 20 mol% of either α=T or CH results in a similar decrease in the plasma-induced liposome leakage is consistent with the above report. However, the mechanism by which α-T, at levels of 0.1 mol%, decreases plasma-induced liposome leakage is unknown; we have no evidence that this stabilizing effect of α-T is a result of its acting as an antioxidant.

Because of the demonstrated antioxidant effect of α-T in liposomes, all liposome preparations in this laboratory, whether intended for in vitro or in vivo use, now incorporate at least 0.1 mol% α-T, added either at the time of preparation or after the final lipid purification phase. As a consequence, there has been a significant decrease in the variance of data from all studies.

ACKNOWLEDGEMENTS

This research was supported in part by PHS Grant GM-24612.

The authors wish to thank Mark Bosworth for his technical assistance and Elma P. Belenson for help in preparing the manuscript.

REFERENCES

Abra, R.M., Bosworth, M.E. and Hunt, C.A., Liposome disposition in vivo: effects of pre-dosing with liposomes. Res. Comm. Chem. Path. Pharmacol., 27 (1980).

Allen, T.M. and Cleland, L.G., Serum-induced leakage of liposome contents. Biochem. Biophys. Acta, 597 (1980) 418–427.

Diplock, A.T., Lucy, J.A., Verrinder, M. and Zeileniewski, A., α-Tocopherol and the permeability to glucose and chromate of unsaturated liposomes. FEBS Lett., 82 (1977) 341–344.

Dobretsov, G.E., Borschevshaya, T.A., Petrov, V.A. and Vladimirov, Y.A., The increase of phospholipid bilayer rigidity after lipid peroxidation. FEBS Lett., 84 (1977) 125–128.

Gray, J.I., Measurement of Lipid Oxidation: A review, J. Am. Oil Chem. Soc., 55 (1978) 539–546.

Holman, R.T., Autoxidation of fats and related substances. In Holman, R.T., Ludberg, W.O. and Molkin, T. (Eds.), Progress in the Chemistry of Fats and Other lipids, Vol. 2, Pergamon Press, London, 1954, pp. 51–98.

Hunt, C.A. and Tsang, S., The effect of size, charge and composition on the stability of liposomes in plasma, Abstracts A.A.A.S., 145th Meeting, Houston, 1979 p. 128.

Kimelberg, H.K. and Mayhew, E.G., Properties and biological effects of liposomes and their uses in pharmacology and toxicology. Crit. Rev. Toxicol., 6 (1978) 25–79.

Konings, A.W.T., Damen, J. and Triebing, W.B., Protection of liposomal lipids against radiation induced oxidative damage. Int. J. Radiat. Biol., 35 (1979) 343–350.

Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J. and Papahadjopoulos, D., Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. Biochem. Biophys. Acta, 557 (1979) 9–23.

Papahadjopoulos, D., Liposomes as drug carriers. Ann. Rep. Med. Chem., 14 (1979) 250–260.
Sinnhuber, R.O., Yu, T.C. and Yu Te, C., Characterization of the red pigment formed in the 2-thiobarbituric acid determination of oxidative rancidity. Food Res., 23 (1958) 626–638.
Suwa, K., Kimura, T. and Shaap, P., Reactivity of singlet molecular oxygen with cholesterol in a phospholipid membrane matrix. A model for oxidative damage of membranes. Biochem. Biophys. Res. Commun., 75 (1977) 785–792.