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OZONE INDUCED CONDUCTANCE INCREASES ASSAYED WITH LIPID IMPREGNATED FILTER MEMBRANES--EFFECT OF VITAMIN E

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SUMMARY

Lipid impregnated Millipore filter membranes, which model certain permeability characteristics of lipid bilayer, were used to examine the effect of ozone on membrane conductance. It was found that the conductance across a lipid impregnated filter membrane increased progressively upon exposure to a stream of oxygen delivering up to 5 ppm ozone to the aqueous compartment. The extent of this conductance increase depended on duration of ozone treatment (tenfold after 30 minutes), ozone concentration (linear dependence to at least 5 ppm), the bathing solution pH (the lower the pH is, the higher the conductance), lipid composition (unsaturation required), temperature, and the existence of a membrane barrier between ozone and the assay membrane (no penetration of ozone through a membrane). It was also found that the presence of α-tocopherol, or cholesterol as part of the lipid of the filter membrane diminished the conductance increases. Virtually complete protection occurred at about a 1% mole ratio of α-tocopherol/lecithin. Since saturated lipid impregnated filter membranes are relatively unaffected by ozone, it is concluded that reaction products of unsaturated lipids with ozone are responsible for the observed conductance increases.

INTRODUCTION

Ozone damage to cell membranes as a primary event in ozone toxicity is an attractive hypothesis.[2] Biochemical mechanisms of ozone injury have been discussed in a recent review.[7] Evidence for the involvement of membrane damage in ozone effects comes from permeability studies, showing increased solute fluxes for glucose[3] and rubidium[4] in higher plant leaf discs and K^+ leakage in algae.[5] Inhalation of 2 ppm ozone for 8 hr by rats and monkeys has been shown to impair oxidative metabolism of lung mitochondria and to induce increased permeability of mitochondrial membranes to NADH.[14]

Dl-α-tocopherol is known to protect lipids against oxidative chain reactions in vitro,[18] but its role in vivo is not resolved.[6,9,11,19] Both structural and antioxidant functions in biological membranes have been proposed for this vitamin.[8]

In this study the effect of ozone on the impermeability characteristics of biological membranes was investigated by assaying the conductance of model membranes composed of various lipids incorporated into Millipore filters. The effect of the dl-α-tocopherol incorporation into the lipids was also studied.

METHODS

Egg yolk lecithin, dimyristoyl lecithin, dipalmitoyl lecithin, phosphatidylethanolamine (Bovine Brain) and cholesterol were purchased from Sigma Chemical Co. Dl-α-tocopherol was a gift from Hoffman La Roche, Inc. Oxidized cholesterol was prepared by bubbling oxygen through a solution of cholesterol in octane for 6 hours.[3]

The electrical assay of lipid impregnated Millipore filter membrane was previously described;[15,17] a 0.5 cm diameter filter membrane of 0.3 m average pore size was impregnated with lipid in decane solution for 15 hours at 20-22°C. Excess decane was evaporated under a stream of nitrogen. The lipid impregnated filter membrane was glued by its edges onto a 0.5 cm^2 orifice in a 10 ml cylindrical Teflon cup. Then the Teflon cup was placed in a glass cup, both compartments were filled with aqueous medium (10 mM Tris-Cl, 100 mM sucrose, pH 7.4) and the systems were stirred magnetically. For the double membrane experiment two Millipore filter membranes impregnated with egg yolk lecithin were glued over the interior and exterior edges of the aperture in the Teflon cup.

Ozone was generated by passing oxygen through an enclosed UV light source and was bubbled into the aqueous medium of the inner compartment.[4] The flow rate of the incoming oxygen was adjusted by a flowmeter. The ozone influx into 10 ml of the aqueous medium (nmoles/min) was measured by the potassium iodide method[13] in a solution exposed to ozone/oxygen bubbling for 5 minutes. All experiments reported

here were conducted at a flow rate corresponding to the appearance of 47 nmoles of iodine/min in the buffer.*

Membrane resistance was measured by applying an external voltage source in series with the membrane system. The membrane conductance Gm was calculated from the relationship;

$$Gm = \frac{1}{R_m} = \frac{Vm}{(E_i - {}_m)R_i\ A_m}$$

where E_i, V_m, R_i and A_m are applied electrical field, the membrane potential, the external resistance and the membrane area. The membrane conductance has the units mho x cm^{-2}.

The spin probes 2-(2-carboxyethyl)-2-tetradecyl-4,4-dimethyl-N-oxazolidinyloxyl, designated as A4NS and 2-(10-carboxydecyl)-2-hexyl-4, 4-dimethyl-N-oxazoldinyloxyl, designated as A12NS[10] were added to asolectin and vitamin E mixtures in decane at a concentration of 1 mole% relative to the asolectin concentration and the solution was equilibrated at 25° for 24 hours. After partial or complete removal of solvent the filters were inserted in a slotted cylindrical TEFLON sample holder for subsequent analysis in a Varian E109-E Electron Paramagnetic Spectrometer operating at x-band. The cylindrical sample holder could be rotated about its long axis to vary the direction of the plane of the filter with respect to the magnetic field of the spectrometer.

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*If it is assumed that all the ozone is absorbed in the aqueous solution, then the ozone concentration in the oxygen stream is calculated as 4.2 ppm. Since it is possible that ozone absorption is incomplete 4.2 ppm should be regarded as a lower limit.

RESULTS

Spin probe studies of filter membrane structure

Asolectin and spin probe impregnated filter membranes were blotted off with tissue paper immediately after their removal from the decane-lipid solution and placed in the slotted sample holder (see Methods of Procedure). The electron spin resonance (ESR) signal of these filter membranes was very similar to that observed for the spin probes in decane solution. Hence it can be inferred that considerable decane remained in the filters after blotting.

When asolectin impregnated filter membranes were dried for five minutes under a stream of nitrogen at 25°C, the spin resonance signals indicated a considerably more immobilized state of motion of the probes. Such decane depleted membranes exhibit high conductances similar to lipid free filter membranes. Both A4NS and A12NS were significantly more immobilized in these millipore filters than in sonicated aqueous asolectin vesicles.

Nitrogen-dried asolectin impregnated filter membranes were hydrated by storing them at 25°C in a 90% humidity chamber for six hours. This treatment led to a slight increase in the mobility of both probes such that spectra were very similar to those observed for these labels in sonicated asolectin vesicles.

In none of the ESR experiments were any effects of vitamin E observed. Hence structural effects of Vitamin E in these filter-supported lipids are too small to lead to alterations in the spin probe mobility or orientation.

For none of the samples examined in the study was there any evidence of any anisotropic character of the signal as the filter membranes were rotated in the magnetic field. This implies that the bulk of the lipid is randomly oriented in the filter membranes.

Ozone causes conductance increases

The effect of ozone concentration and exposure time upon membrane conductance changes--as recorded in Fig. 1, an increase in membrane conductance was observed for egg yolk lecithin impregnated filter membranes exposed to ozone. Conductance increased for 30 minutes of exposure prior to attainment of a steady state conductance (190×10^{-9}mho x cm^{-2}). Such increases in membrane conductance during ozone exposure varied with the lipid composition of the filter. After the ozone treatment was stopped the conductance decreased, reaching a steady state after about 10 minutes. This steady state membrane conductance was ozone concentration dependent. Figure 2 shows a linear relationship between the steady state membrane conductance and the rate of ozone delivery. Rinsing the membrane with fresh bathing solution after the steady state conductance had been reached lowered the membrane conductance induced by ozone by as much as 40% relative to the unrinsed control.

Factors affecting ozone induced membrane conductance changes

Lipid composition--Table I shows changes in steady state membrane conductance with different lipids in the membrane. In general, both the initial conductances, as well as the ozone induced conductance increases are greatest for lipid mixtures containing a high proportion of unsaturated fatty acid residues. Table I shows for cholesterol

membranes that preoxidation leads to a much larger conductance increase than is observed for the unoxidized cholesterol. Cholesterol addition to egg lecithin has two effects: a decrease in the control conductance and a decrease in the effect of ozone. This observation suggests that ozone may be less accessible to the cholestrol containing membrane.

Temperature dependence--Filter membranes impregnated with 10 mg/ml egg yolk lecithin, phosphatidyl ethanolamine or cholesterol were treated with 47 n moles ozone/min for more than 50 minutes. Then the value of steady state membrane conductance was measured 10 minutes after ozone removal. In both control and ozone treated systems, a linear decrease in membrane conductance occurred with reciprocal temperature, and the magnitude of the membrane conductance for a membrane treated with ozone was several fold higher than that in the absence of ozone at each temperature studied.

pH--The conductance of untreated membranes decreased as the bathing solution pH increased. The ozone induced conductance increase also decreased with increasing pH (Figure 3). The observation that ozone induced conductance changes, expressed as differences relative to the controls, diminish with increasing pH is consistent with the report that ozone breakdown in water depends on the hydroxyl ion concentration.[1] However, since the conductance of control membranes exhibits a similar pH dependence these data, when expressed as ratios of ozone dependent to control conductances, exhibit a nonlinear dependence on pH.

Exposure of a double membrane to ozone

To gain insight into the extent of ozone penetration through, and the possible release of reaction products from the membrane, the

effect of ozone exposure on a double membrane was studied. Two closely apposed egg yolk lecithin impregnated filter membranes (i.e., separated by less than 1 mm) were placed across an 0.5 cm^2 orifice separating two aqueous compartments. Ozone was bubbled into the aqueous medium of the inner compartment for 40 minutes. The steady state membrane conductance of a single membrane was measured 10 minutes after the end of ozone treatment and after the membrane proximal to the exposure solution had been removed. The conductance of this membrane was measured to be 13×10^{-9} mho/cm^2. This is similar to the conductance of 10×10^{-9} mho/cm^2 observed for an untreated membrane. On the other hand, a single membrane which had been treated with ozone under the same conditions reached a conductance of 147×10^{-9} mho/cm^2.

Conductance measurement of a dl-α-tocopherol enriched membrane during ozone treatment.

Various amounts of dl-α-tocopherol and lecithin were mixed together in decane solvent and then filters were immersed in the membrane forming solution for 15 hours at 24°C. Ten minutes after the lipid and dl-α-tocopherol impregnated filter was inserted into the aqueous medium ozone was bubbled into the inner compartment. Figure 1 shows that in the control system (dl-α-tocopherol absent), membrane conductance was greatly increased within 10 minutes of ozone treatment, began to level off after one hour ozone treatment, and then dropped to a steady state value (140 mho x cm^{-2} x 10^{-9}) 10 minutes after ozone removal. An increase in the molar ratio of dl-α-tocopherol to egg yolk lecithin led to a slower rate of conductance change during ozone

treatment as well as a decrease in the magnitude of the steady state conductance after ozone removal.

There was non-linear decrease in membrane conductance both in egg yolk lecithin and asolectin impregnated filters as the mole ratio of dl-α-tocopherol/lipid increased. Values of 0.7% mole ratio of dl-α-tocopherol/egg yolk lecithin or, 1.7% mole ratio of dl-α-tocopherol/asolectin in the membrane forming solutions could prevent any change in membrane conductance by ozone.

DISCUSSION

Conductance increases: a consequence of ozone action

The Millipore filter membrane assay system used for the ozone studies has been shown to mimic the electrical properties of lipid bilayer membranes and has proven to be a useful model system for the study of simple biological membrane permeability properties.[3] As judged from the stearic acid spin probe spectra appreciable amounts of decane solvent are retained in the filter membrane under the conditions used for the conductance measurements. Hence the membrane can be regarded as a liquid junction separating two bulk aqueous phases where a variety of lipids can be solubilized.

In principle conductance changes observed after ozone treatment could be due to an increase in the permeability of the filter membrane due to structural alteration or to the release of factors from the membrane which increase conductance. The experiment with the double membrane shows that the increase in conductance is specific to the membrane which has been treated with ozone, and hence there is little or no release of permeability enhancing factors from the untreated side of the membrane into the environment.

The observation that the conductivity increases again after rinsing the membrane with fresh medium implies that some water soluble permeability enhancing factors are produced during ozone treatment. These may be small organic molecules, e.g., aldehydes or carboxylic acids which result from the breakdown of unsaturated lipids by ozone and which are released into the aqueous phase being treated with ozone. However,

experiments with the double membrane suggest that these compounds do not cross the filter membrane to a significant extent. The experiment also shows that ozone does not cross a single filter membrane to a significant extent.

The observation that the membrane conductance decreases again after ozone treatment ceases (Figure 1) implies that some of the reactions of ozone and lipids continue over a period of several minutes and that unstable intermediates of ozone reaction with lipids are responsible for a substantial fraction of the increased conductance. Repeated washing of the membrane with buffer restored the control conductance of the membrane that had been protected with vitamin E but only partially restored the conductance of the unprotected membrane.

Reaction of ozone with lipids

The reaction sites of ozone with lipids are thought to be the double bonds of the fatty acid chains. The failure to observe conductance increases with the saturated lipids dimyristoyl lecithin or dipalmitoyl lecithin show that the measured conductance changes, indeed result from the interaction of ozone with unsaturated lipids.

Vitamin E Protection against Ozone Damage

Dl-α-tocopherol a well known antioxidant, shows a significant dose dependent inhibition of ozone induced conductance increases. The interaction of ozone with lipids is assumed to lead to the accumulation of ozonides and to initiate free radical reactions.[12] In the presence of oxygen the free radical reactions are propagated as chain reactions leading to the production of lipid hydroperoxides and endoperoxides from unsaturated lipids.[16] Vitamin E is very

effective in quenching such chain reactions.[12] Concentrations of this vitamin of the order of 0.1% relative to the bulk lipid are sufficient to quench oxidative chain reactions. The results presented in figure 1 indicate that considerably larger concentrations of vitamin E are required to protect against the ozone induced conductance increases.

Moreover, whereas the usual pattern of the time dependence of autoxidation of nonaqueous lipids with tocopherol is characterized by a dose dependent induction period where lipid peroxidation is minimal, the data (Figure 1) show that the ozone induced conductance increase occurs at a significant rate throughout the exposure period at three of the tested tocopherol concentrations albeit at different rates and to different extents.

Most likely these effects are a consequence of the structure of the lipid impregnated Millipore filter. The spin label data suggest that much of the lipid is not arranged as bilayers so that chain reactions in this system are less likely to occur than in pure lipid system or in biological membranes. In addition it may be that most of the vitamin E is dispersed in the decane phase rather than among the lipids at the aqueous interface of the filter membrane where its free radical scavenging action would be most effective.

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REFERENCES

1. M. G. Alder and G. R. Hill (1950) J. Am. Chem. Soc. 72, 1884-1886.
2. P. Chimiklis and R. L. Heath, Plant Physiol, 49 (1972) suppl:3.
3. P. Shieh (1975) Ph.D. Thesis, Michigan State University.
4. C. Coulson and R. L. Heath (1974) Plant Physiol. 53, 32-38.
5. W. M. Dugger and R. L. Palmer, Internat. Proc. Citrus Sympos, 2, (1969) 711-714.
6. J. Green, Ibid., 29-44.
7. R. L. Heath (1975) Ozone, in Response of Plants to Air Pollution, J. B. Mudd and T. T. Kozlowski, eds., Academic Press, New York, San Francisco and London, 23-55.
8. J. A. Lucy, Ibid., 4-11.
9. J. F. Mead (1976) in Free Radicals in Biology, Vol. 1, W. A. Pryor, ed., 51-68.
10. Mehlorn, R. J. and Keith, A. D., Spin Labeling of Biological Membranes, in Membrane Molecular Biology, C. F. Fox and A. D. Keith, edgs. Sinauer Associates, Stamford, Connecticut.
11. Y. D. Menzel (in press) in Vitamins and Hormones, L. J. Machlin, ed., Marcel Dekker, Inc.
12. D. B. Menzel (1976) in Free Radicals in Biology, Vol. 2, W. A. Pryor, ed., 181-202.
13. J. B. Mudd, R. Leavitt, A. Ongun and T. T. McManus (1969) Atmos. Environ. 3, 669-682.
14. M. G. Mustafa and C. E. Cross (1974), Arch. Biochem. Biophys. 162, 585-594.

15. L. Packer, P. K. Shieh, J. K. Lanyi and R. S. Criddle, Bioenergetics of Membranes. L. Packer et al., ed. (Elsevier/North-Holland Biomedical Press), (1977) 149-159.

16. W. A. Pryor, J. P. Stanley, and E. Blair (1976) Lipids 11, 370-379.

17. P. Shieh, L. Packer and J. Lanyi, "Biomembranes Part D", in Method in Enzymology, eds. Fleischer, S and Packer, L. (Academic Press, New York), in press.

18. A. L. Tappel (1972) Ann. N.Y. Acad. Sci. 203, 12-28.

19. J. Walton and L. Packer (in press) in Vitamin E, L. J. Machlin, ed., Marcell Dekker, Inc.

TABLE LEGENDS

Table I. Dependence of ozone induced conductance increase on lipid composition

Table II. Effect of dl-α-tocopherol addition on ozone induced filter membrane conductance changes as a function of lipid composition.

TABLE I

Lipid in Membrane Forming Solution	mg/ml	Membrane Conductance (mho x cm^{-2} x 10^{-9}) Control	Ozone	Ratio
Asolectin	20	40.0	1200	30.0
Oxidized cholesterol	20	1.4	243	173.6
Egg yolk lecithin	10	12.0	147	12.3
Egg yolk lecithin + dimyristoyl lecithin	5	11.7	123	10.5
Phosphatidyl ethanolamine + Egg yolk lecithin + Dimyristoyl lecithin + Cholesterol	0.5 2.5 6.5 0.5	2.8	68.5	24.4
Phosphatidyl ethanolamine	10	20.0	46.5	2.3
Egg yolk lecithin + Cholesterol	8 2	2.7	16.8	6.2
Dimyristoyl lecithin	10	0.3	0.3	1.0
dl-α-tocopherol	30	2.0	2.0	1.0

TABLE II

Membrane Forming Solution	mg/ml	Membrane Conductance (mho x cm^{-2} x 10^{-9}) Control	+ Ozone	Ratio
Asolectin	15.0			
dl-α-tocopherol	0.25	20.0	200	10.0
Egg yolk lecithin	10.0			
dl-α-tocopherol	0.17	1.6	2.0	1.3
Egg yolk lecithin	5.0			
+ dimyristoyl lecithin	4.3			
dl-α-tocopherol	0.16	28.5	28.5	1.0
Egg yolk lecithin	6.5			
+ Dipalmitoyl lecithin	2.5			
+ Cholesterol	2.5			
+ Phosphatidyl ethanolamine	0.5			
dl-α-tocopherol	0.17	5.0	11.0	2.2
Egg yolk lecithin	8.0			
+ Cholesterol	2.0			
dl-α-tocopherol	0.17	1.5	3.8	2.5

FIGURE LEGENDS

Figure 1. Effect of dl-α-tocopherol on the time, course and extent of ozone induced membrane conductance changes.

Figure 2. Effect of ozone concentration on filter membrane conductance. Ozone exposure time was 30 minutes.

Figure 3. Effect of pH on ozone induced membrane conductance changes.

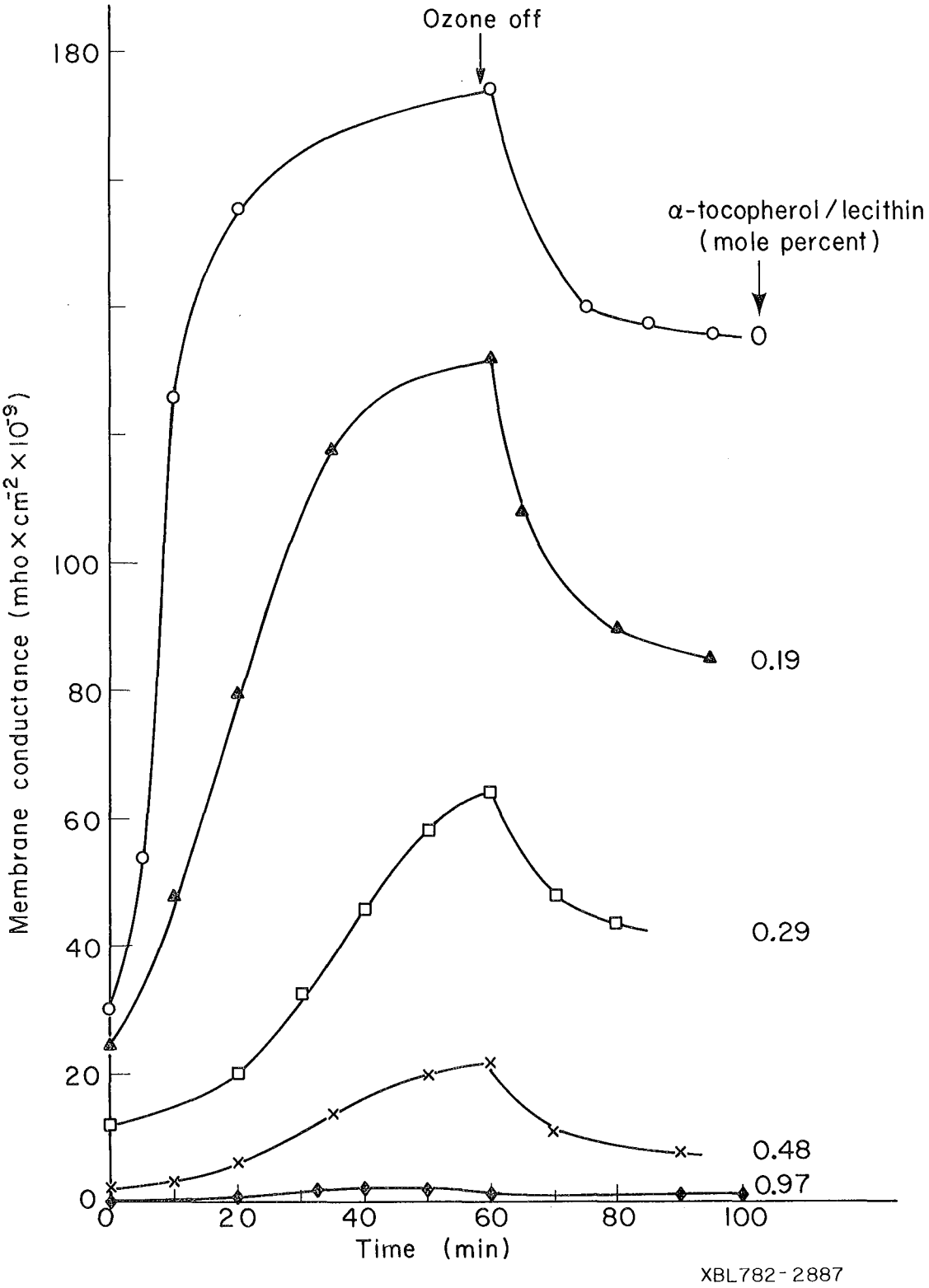
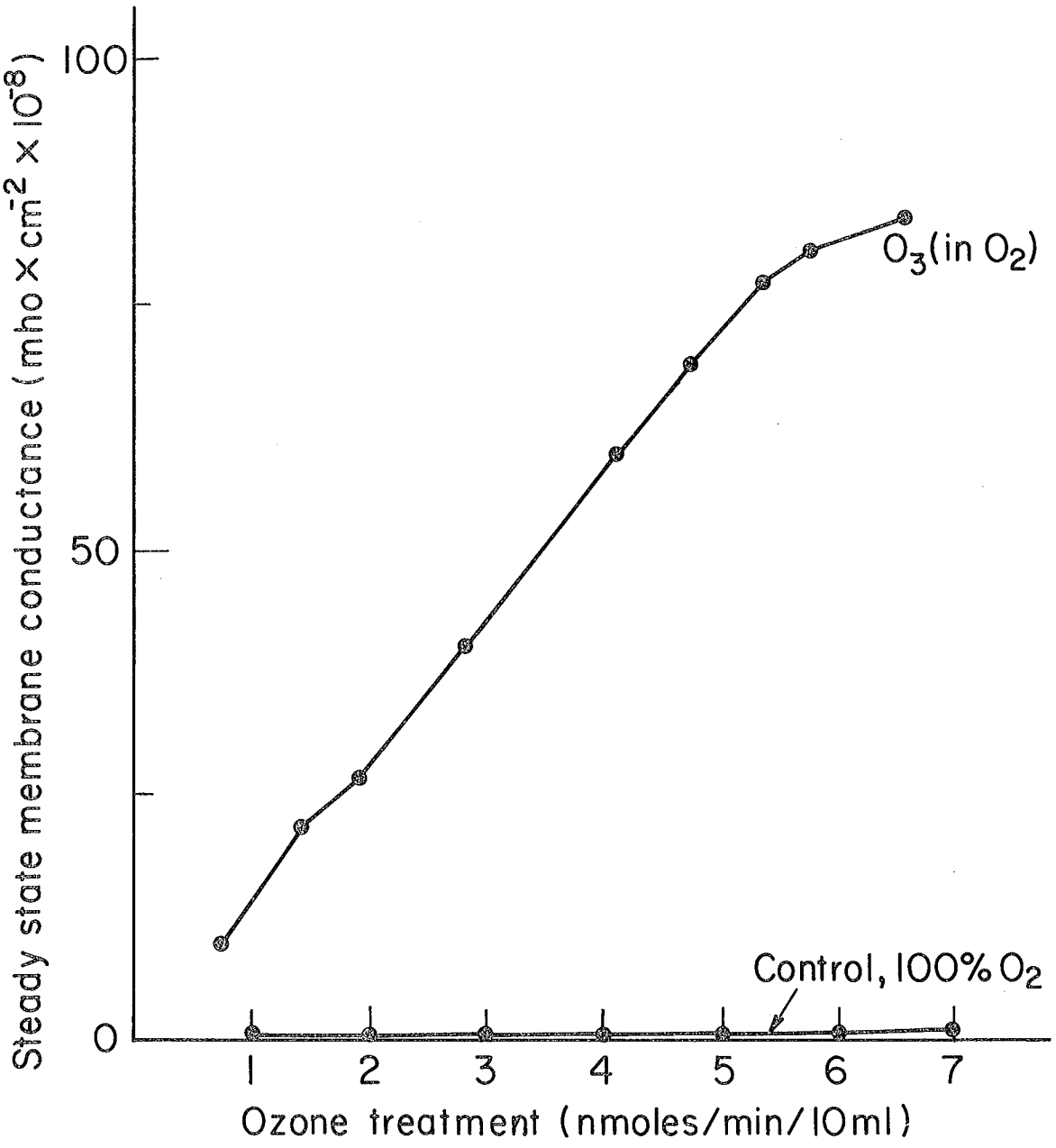

Ozone off
α-tocopherol / lecithin
(mole percent)
0
0.19
0.29
0.48
0.97
Membrane conductance (mho x cm⁻² x 10⁻⁹)
180
100
80
60
40
20
0
0
20
40
60
80
100
Time (min)
XBL782-2887

Fig. 1



XBL782-2888

Fig. 2

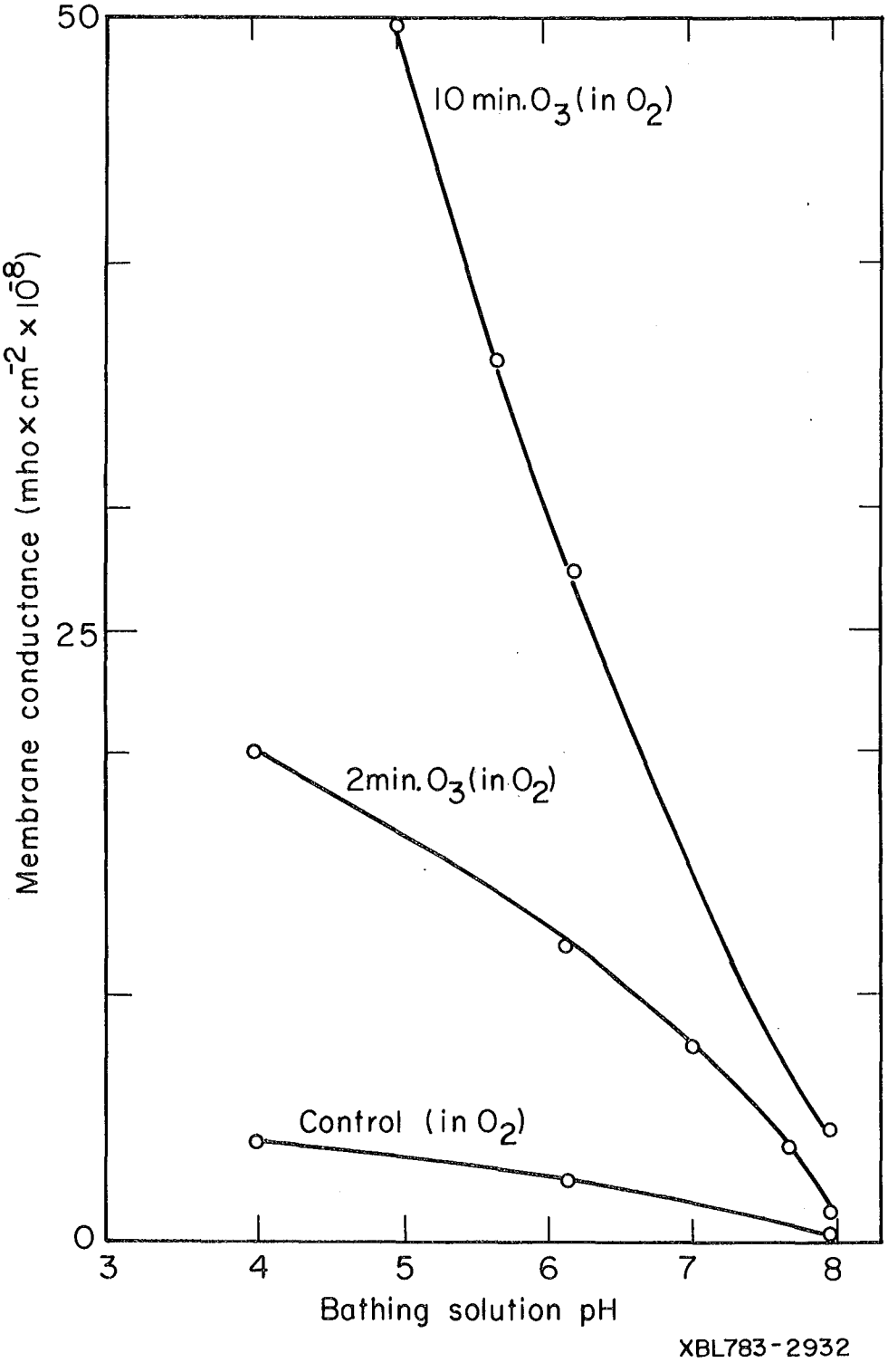


Fig. 3

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