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EFFECT OF TWEEN 80 ON LIPID VESICLE PERMEABILITY

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As part of an examination of procedures for protein/lipid reconstitution we incubated solubilized cytochrome oxidase at different temperatures with preformed vesicles composed of saturated lipids. We observed that the ^{22}Na permeability of these vesicles was increased following such an incubation step, with the maximal effect occurring following incubations at temperatures in the vicinity of the lipid gel to liquid-crystal phase change. However, these permeability changes could be completely duplicated by incubating similar vesicles with concentrations of Tween 80 used to solubilize the extracted oxidase. Thus detergent molecules used to solubilize extracted membrane proteins can induce significant changes in the reconstituting lipid environment and the possibility of such changes must be taken into account when interpreting the properties of the reconstituted lipid/protein system under study.

In this communication we describe an effect of Tween 80 on the barrier properties of lipid vesicles at detergent concentrations commonly used to solubilize membrane proteins.

Recently we have been examining different procedures for protein-lipid reconstitution using extracted cytochrome oxidase as the protein component. One approach involved directly incubating the solubilized protein at different temperatures with preformed vesicles composed of saturated phospholipids. We anticipated that a temperature dependent incorporation of the protein could occur during the incubation step. Such an interaction if it changed the structure of the lipid bilayer would most probably alter its barrier properties as well. We therefore measured ^{22}Na effluxes from such vesicles following incubation as an indication of whether such a protein induced change in lipid structure had taken place. Indeed we did observe that after incubation of vesicles with solubilized oxidase at temperatures in the vicinity of the lipid phase transition ^{22}Na efflux rates were greatly increased. However, as described below, these effects could be completely duplicated by concentrations of Tween 80 used to solubilize the extracted oxidase, implying the the permeability change was not due to the protein itself but to the detergent associated with the protein.

Cytochrome oxidase was extracted from bovine heart and purified according to the method of Kuboyama et al. [1]. The final precipitate is dissolved in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.25% Tween 80 (Emasol was used in the original method). Thereafter the oxidase was dialyzed for 72 h against 2% sodium cholate/100 mM phosphate/25% ammonium sulphate (pH 8.0). The mixture was centrifuged and the supernatant fraction was dialyzed for a further 24 h against 100 mM phosphate buffer only (pH 7.4), to remove cholate and ammonium sulphate. The final preparation was stored in this buffer at −80°C. The phospholipids 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were obtained

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from Sigma Chemical Company, St. Louis, MO. These lipids showed a single spot by TLC. The preparation of multilamellar liposomes and the measurement of ^{22}Na effluxes were carried out by established procedures as described elsewhere [2].

Fig. 1 illustrates the effects of solubilized oxidase and Tween 80 on vesicle permeability. Liposomes were formed from an equimolar mixture of DMPC and DSPC. Solubilized cytochrome oxidase or an equivalent amount of Tween 80 (see figure legend) was incubated at different temperatures for 15 min with these preformed vesicles. Thereafter the vesicles were cooled to 5°C and dialyzed over-night (at 5°C) to remove untrapped tracer. Following dialysis all ^{22}Na effluxes were measured at 15°C, a temperature at which these vesicles normally display a low efflux rate *. Both cytochrome oxidase and Tween 80 caused comparable temperature dependent increases in sodium permeability with the maximum effects occurring following incubations at 30 to 35°C. Incubations performed at lower or higher temperatures were less effective in altering ^{22}Na effluxes. Although not illustrated, we observed that even concentrations of Tween 80 and oxidase as low as 0.0006% (v/v) still gave equivalent permeability changes. In addition similar results were obtained with vesicles composed of DMPC alone except that with this lipid maximum changes in ^{22}Na efflux occurred following incubations carried out at 20 to 22°C rather than 30 to 35°C.

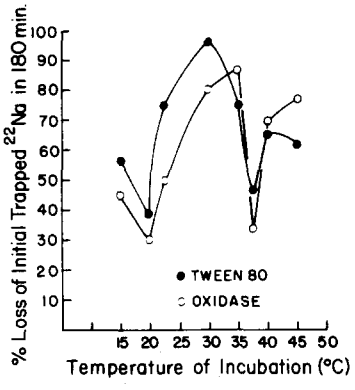

Fig. 1. Effects of Tween 80 and solubilized cytochrome oxidase on vesicle permeability. Liposomes composed of an equimolar mixture of DMPC/DSPC were swollen in 50 mM NaCl/5 mM Tris-HCl/$^{22}NaCl$ (pH 7.5). The cytochrome oxidase preparation used in these experiments had an cytochrome aa_3 concentration of 138 μM, a protein concentration of 27 mg·ml^{-1} and a heme to protein ratio of 10.3 (nmol heme a/mg protein). As indicated in the text, the oxidase was solubilized in 0.25% Tween 80. In the experiments illustrated in the figure 10 μl of Tween 80 (0.25%) or 10 μl of solubilized oxidase was added to 1 ml of lipid dispersion (detergent/lipid molar ratio 1:250) and then the mixture was incubated at a given temperature for 15 min. Thereafter the incubated mixtures were cooled to 5°C and dialyzed overnight to remove excess tracer. Following dialysis ^{22}Na effluxes were measured at 15°C. Each incubation represents a separate experiment performed in triplicate. The results illustrated are the means. The ordinate refers to the percentage of initial trapped isotope lost over 180 min. Although not illustrated we have examined concentrations of oxidase and Tween 80 over the range 2.5 μl to 50 μl per ml lipid dispersion (final detergent concentration 0.0006 to 0.013% v/v). For all concentrations, Tween 80 and solubilized oxidase gave comparable permeability effects. Although error bars have not been included for the data points, the range of values for any given experiment was never larger than 20%.

Cytochrome oxidase when reconstituted into lipid vesicles can generate a transmembrane hydrogen ion electrochemical gradient under certain conditions. To test for this possibility we examined the rate of oxygen consumption, both with and without an uncoupler, by DMPC/DSPC vesicles that had been incubated with cytochrome oxidase at 30°C as described above. Rates of oxygen uptake were measured with a Yellow Springs Instruments oxygen electrode as described in Ref. 3. The assay mixture contained cytochrome c (75 μM), sodium ascorbate (5 mM) and N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD) 0.18 mM. An enzyme turnover number (TN) was calculated by dividing oxygen consumption in nmol·ml^{-1}·s^{-1} by the oxidase concentration (nmol cytochrome aa_3/ml). The TN (s^{-1}) measured at 20°C and 30°C was 7 and 19, respectively, values comparable to those previously reported [3]. Addition of the uncoupler trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP) did not increase the rate of oxygen consumption at either temperature indicating that these oxidase/lipid vesicles are incapable of generating a transmembrane hydrogen ion electrochemical gradient. The existence of such a gradient could have influenced sodium movement.

* At 15°C, vesicles formed from an equimolar mxiture of DMPC/DSPC display a ^{22}Na efflux rate of 10–15% loss of initial trapped isotope in 180 min [2].

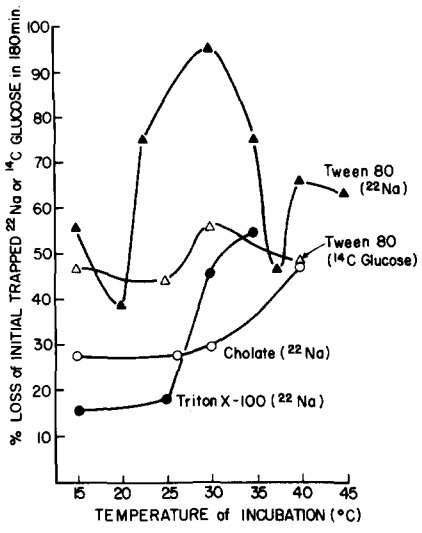

Fig. 2. Effects of Tween 80, cholate and Triton X-100 on vesicle permeability. Liposomes composed of an equimolar mixture of DMPC/DSPC were swollen in either 50 mM NaCl/5 mM Tris-HCl/^{22}NaCl (pH 7.5) or 50 mM NaCl/5 mM Tris-HCl/5 mM glucose/[^{14}C]glucose (pH 7.5). The 'permeability curve' for Tween 80 (^{22}Na) is taken from Fig. 1 and included for comparison. A given detergent was added to 1 ml of lipid dispersion to yield detergent/lipid molar ratios of 1:250 for Triton X-100 and Tween 80 and 1:10 for cholate (sodium salt). The molar ratio for Triton X-100 and Tween 80 is the same as the detergent/lipid ratio used in the experiments illustrated in Fig. 1. For cholate a detergent/lipid molar ratio of 1:10 has been reported to give a significant change in lipid phase properties [11] but this ratio is still less than the ratio used in most reconstitution studies. The detergent/lipid mixtures were incubated at the given temperatures for 15 min. Thereafter the incubated mixtures were cooled to 5°C and dialyzed over-night to remove excess tracer. Following dialysis, ^{22}Na or [^{14}C]glucose effluxes were measured at 15°C. Each incubation represents a separate experiment performed in triplicate. The results illustrated are the means. The ordinate refers to the percentage of initial trapped isotope lost over 180 min. Although error bars have not been included for the data points, the range of values for any given experiment was never larger than 20%.

Fig. 2 illustrates the effects of two other detergents (cholate and Triton X-100) commonly used in reconstitution studies, on vesicle permeability. The experimental design was identical to that described for the experiments using Tween 80 or solubilized oxidase (Fig. 1). Triton X-100 appears to be less potent (on a molar basis) than Tween 80 in altering lipid barrier properties although like Tween 80 a large effect occurs after incubation with the vesicles in the temperature region of the main phase change. Cholate also increases the ^{22}Na efflux rate but demonstrates a different temperature dependence with no obvious maximum following incubation at 30 to 35°C. Tween 80 also causes a temperature dependent increase in glucose permeability * although the maximum effect after incubation at 30°C is much less pronounced than observed for sodium.

Although the permeability effects described above are themselves interesting we believe the real importance of these observations relates to the implications that can be drawn with regard to protein-detergent complexes. Non-ionic detergents, as for example Tween 80, are commonly used to solubilize extracted membrane proteins such as cytochrome oxidase [4,5]. Such solubilized protein preparations are often used to reconstitute protein lipid systems [6,7]. The results illustrated in Figs. 1 and 2 indicate that the detergent associated ** with the protein may have significant effects upon the reconstituting lipid environment and that such effects can not necessarily be ignored when interpreting the properties of the protein/lipid system under study.

This conclusion is supported by several studies published in the literature. Kagawa [8] reported that detergents such as Triton X-100 are capable of inhibiting membrane-dependent functions at concentrations several to a few hundred fold lower than those needed to solubilize proteins. For example, Triton X-100 inhibited mitochondrial respiratory control at a concentration of 0.05 mM, a value about 20-fold below its critical micelle concentration. Van Zutphen et al. [9] demonstrated that sublytic concentrations of non-ionic detergents, including Triton X-100, induced cation selective leaks in planar lipid membranes prepared from either mitochondrial lipids or egg phosphatidylcholine.

Although the nature of the mechanism underlying the detergent induced permeablity change is

* At 15°C, DMPC/DSPC vesicles display a [^{14}C]glucose permeability of 15–20% loss of initial trapped isotope in 180 min (not illustrated).

** We have used the word 'associated' since we do not know how much of the Tween 80 is actually bound to the protein. Certainly it is not removed by dialysis.

not directly addressed in our experiments it is interesting that the alteration in ^{22}Na efflux is maximal when Tween 80 or Triton X-100 is incubated with the lipid vesicles in the temperature vicinity of the main gel to liquid-crystal phase change (30 to 35°C for DMPC-DSPC 1 : 1 and 20 to 22°C for DMPC). There is evidence that at the main phase change defects develop at domain boundaries [2]. The shape of the curves in Fig. 2 for Tween 80 and Triton X-100 imply that the presence of such defects may be an important condition for the detergent induced alteration of ^{22}Na permeability in these vesicles formed from saturated phospholipids.

Finally, as indicated by Lichtenberg et al. [10] detergent/phospholipid mixtures are actually ternary systems composed of detergent/lipid/water and complete phase diagrams for such systems are not generally available. Such data will be needed to characterize in detail the mechanisms involved in detergent-mediated permeability alterations. Detergent/lipid/water/protein systems will be even more complex.

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