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ALKYL GLUCOSIDES AS EFFECTIVE SOLUBILIZING AGENTS FOR BOVINE RHODOPSIN

A COMPARISON WITH SEVERAL COMMONLY USED DETERGENTS

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SUMMARY

The suitability of octyl and decyl-β-D-glucoside as solubilizing agents for the bovine retinal rod outer segment disc membrane was investigated and compared to that of hexadecyltrimethylammonium bromide, *N*,*N*-dimethyldodecylamine oxide, Emulphogene BC-720 and digitonin. The properties measured included the thermal stability of rhodopsin, regenerability of bleached rhodopsin by addition of 11-*cis*-retinal, and the rate of denaturation of bleached rhodopsin as measured by changes in the ultraviolet CD spectrum. Denaturing tendencies of the detergents were also evaluated by observing their effects on the absorption and CD spectra of sperm whale metmyoglobin. Our results demonstrate that octyl glucoside is superior to the other detergents, with the possible exception of digitonin, by the above criteria. Unlike digitonin, however, octyl glucoside affords rapid solubilization of the disc membrane and is itself highly soluble. Decyl glucoside has properties equivalent or superior to octyl glucoside, but salts and buffers interfere with its ability to solubilize the disc membrane. The well defined chemical composition, ease of removal by dialysis, and non-denaturing properties of the alkyl glucosides make them attractive detergents for membrane research.

INTRODUCTION

A general approach to the study of complex biological structures is that of disassembly into component parts, characterization of these components, and subsequent reassembly with the aim of understanding the function and structure of the system. In the case of membranous structures, such as the retinal rod outer segment disc, this approach requires the use of an appropriate solubilizing agent. The properties of such a detergent should include the following: (1) the ability to solubilize the membranes thoroughly, allowing isolation of lipid-free rhodopsin in the case of retinal rod outer segment discs; (2) small molecular weight and relatively high critical micelle concentration to permit rapid removal by dialysis; (3) minimal

denaturing tendencies toward the proteins; (4) transparency in the ultraviolet region of the spectrum to permit optical characterization of purified membrane proteins; (5) a well defined chemical composition to insure experimental reproducibility.

The classical extractant for rhodopsin, which has been used in innumerable studies, is digitonin. Unfortunately, its high molecular weight, low solubility and limited ability to solubilize lipids make it unsuitable for reconstitution experiments. Many other detergents have been used in the retinal rod outer segment system, including sodium cholate [1], Tween 80 [2], *N,N*-dimethyldodecylamine oxide [3, 4], Triton X-100 [5–7], dodecyltrimethylammonium bromide [8–10], hexadecyltrimethylammonium bromide [11–15] and Emulphogene [12]. Of these, dodecyltrimethylammonium bromide [8–10] and *N,N*-dimethyldodecylamine oxide [4] have been successfully used for reconstitution experiments.

Baron and Thompson [17] have recently reported the successful use of alkyl glucosides for solubilization of enzymes of a bacterial membrane. Since these detergents possess several of the desirable properties listed above, we decided to compare the efficacy of octyl and decyl glucoside, *N,N*-dimethyldodecylamine oxide, hexadecyltrimethylammonium bromide, Emulphogene BC-720 and digitonin as solubilizing agents for the disc membrane. These detergents fall into three general categories: cationic (hexadecyltrimethylammonium bromide), zwitterionic (*N,N*-dimethyldodecylamine oxide) and non-ionic (alkyl glucosides, Emulphogene BC-720 and digitonin).

The criteria used to compare the detergents include the thermal stability of rhodopsin, the regenerability of bleached rhodopsin upon addition of 11-*cis*-retinal and the rate of light-induced conformation changes in rhodopsin. In addition, we have examined the degree of structural perturbation induced in sperm whale metmyoglobin by the detergents. Our results show octyl glucoside to be the best among the detergents tested, with the possible exception of digitonin, by the above criteria.

MATERIALS AND METHODS

Synthesis of octyl and decyl glucoside. The glucosides were prepared by the method of Noller and Rockwell [16] with modifications described by Baron and Thompson [17]. Acetobromo-α-D-glucose was purchased from Sigma, and octanol and decanol were obtained from Fisher Scientific.

Disc membrane preparation. All manipulations were carried out under dim red light unless otherwise stated. Spectral measurements were taken on a Cary 15 recording spectrophotometer. Disc membranes were prepared from frozen bovine retinas (Hormel) according to the procedure of Smith et al. [18]. When solubilized this material routinely had an A_{280nm}/A_{500nm} ratio of 2.1.

Rhodopsin purification. Purified rhodopsin with an A_{280nm}/A_{500nm} ratio of 1.7, which contained less than 1 mol of organic phosphate per mol of rhodopsin, was obtained by the concanavalin A-Sepharose (Pharmacia) affinity column procedure of Steinemann and Stryer [14]. Octyl glucoside (0.05 M) was substituted for hexadecyltrimethylammonium bromide, and rhodopsin was removed from the column by addition of 0.1 M *a*-methyl-D-mannoside to the eluting buffer.

Regeneration of rhodopsin. Regeneration with 11-*cis*-retinal (gift from Hoffmann-La Roche Inc.) was measured at 23 °C by the following procedure. Discs were solubilized in 0.1 M KH_2PO_4 buffer (pH 7) containing the detergent of interest.

Aliquots with A_{500nm} of approx. 0.6 and a volume of 0.25 ml were used. Decyl glucoside-solubilized samples were regenerated in water rather than phosphate buffer because of solubility problems encountered with this detergent. The solubilized samples were thoroughly bleached by exposure to a fluorescent light for 45 s. At 1 min from the start of bleaching, 1 μl of 39 mM 11-*cis*-retinal in ethanol was added to each sample and mixed by vortexing. After a 1.5 h dark incubation at 23 °C, the samples were diluted 2-fold to yield a final volume of 0.5 ml by addition of hexadecyltrimethylammonium bromide and neutralized hydroxylamine in phosphate buffer. The final concentrations were 1 % hexadecyltrimethylammonium bromide and 0.1 M hydroxylamine. The percent regeneration was determined by dividing the ΔA_{500nm} of the regenerated samples by the ΔA_{500nm} of an equivalent unbleached sample.

Thermal stability. The thermal stability of rhodopsin was measured by incubating samples of solubilized discs at 42 °C, and periodically measuring their A_{500nm} over a period of 1 h. Samples were either incubated in the thermostated cell holder of the Cary 15 or in a water bath from which they were transferred to the Cary 15. A first-order time constant was calculated based on the initial A_{500nm} and its initial rate of decay.

Circular dichroism measurements. All CD measurements were made on a Cary 60 spectropolarimeter equipped with a 6001 CD attachment. The sample compartment was thermostated at 27 °C. The samples were prepared in 0.1 M potassium phosphate buffer (pH 7) except for those containing decyl glucoside, which were in water. Values quoted are mean residue ellipticities in degree-cm^2 per dmol of amino acid residues. A mean residue molecular weight of 115 was used, and the molar extinction coefficient and molecular weight of rhodopsin were taken to be 40000. All ultraviolet CD measurements were made in a 0.1-cm cell with sample concentrations of 0.1–0.2 mg/ml of rhodopsin. The visible CD spectrum was measured in a 1-cm cell with a sample concentration of 1.14 mg/ml. The dynode voltage was maintained below 500 V in all cases. The helical content of rhodopsin was estimated by the formula, percent helix = $100([\theta]_{222}+2340)/-30300$ [19].

Detergent concentrations. Unless otherwise stated, the detergent solutions used consisted of the following concentrations in 0.1 M potassium phosphate buffer (pH 7): hexadecyltrimethylammonium bromide, 1 % (Eastman); *N*,*N*-dimethyldodecylamine oxide, 1 % (prepared according to Hoh et al. [20]); Emulphogene, 0.5 % (GAF Corp.); digitonin, 2 % (Nutritional Biochemicals); octyl glucoside, 30 mM; decyl glucoside, 3 mM.

RESULTS AND DISCUSSION

Detergent-induced spectral perturbations of metmyoglobin

In order to evaluate the general denaturing properties of the various detergents employed in this study, the effects of these detergents on the absorption and CD spectra of sperm whale metmyoglobin were measured. Detergent levels normally employed for membrane solubilization were used (see Materials and Methods).

Although it is not a membrane protein, metmyoglobin shares with rhodopsin the common feature that the native protein structure is stabilized through the interaction of the polypeptide chain with an extrinsic hydrophobic moiety (the heme group in metmyoglobin and retinal in the case of rhodopsin). Also, the high degree

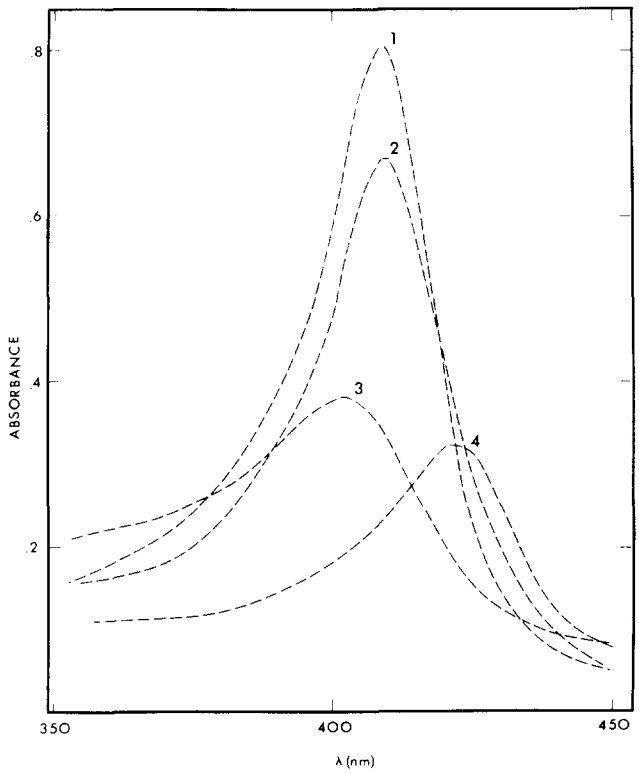

Fig. 1. Effects of detergents on the absorption spectrum of metmyoglobin. The solutions were in a 1-cm path length cell and contained 0.1 mg/ml metmyoglobin, 0.1 M potassium phosphate buffer (pH 7) and the individual detergent of interest at the concentration listed under Materials and Methods. The detergents present are as follows: (1) no detergent, or octyl glucoside or decyl glucoside; (2) Emulphogene or digitonin; (3) hexadecyltrimethylammonium bromide; (4) *N,N*-dimethyldodecylamine oxide.

of helicity of metmyoglobin allows a clear interpretation of the detergent-induced changes in the CD spectrum.

As shown in Fig. 1, no change in the Soret band of metmyoglobin was detected in the presence of either of the alkyl glucosides, while Emulphogene and digitonin produced moderate spectral alterations including a 16 % reduction of the 408 nm absorbance and a broadening of the band toward the red. Hexadecyltrimethylammonium bromide and *N,N*-dimethyldodecylamine oxide produced major spectral perturbations indicative of the denatured [21] and low spin states [22] of metmyoglobin, respectively.

Similar ordering of the detergents was observed in the metmyoglobin CD spectrum (Fig. 2). The glucosides produced no alteration of the native CD spectrum in contrast to the other detergents, which caused the following reductions in the ellipticity at 222 nm: digitonin, 6 %; Emulphogene, 12 %; *N,N*-dimethyldodecylamine oxide, 32 %; and hexadecyltrimethylammonium bromide, 36 %. The existence of a double trough in the CD spectrum of proteins in the wavelength region of 200–250 nm is indicative of helical structure [23, 19], and the observed reduction in

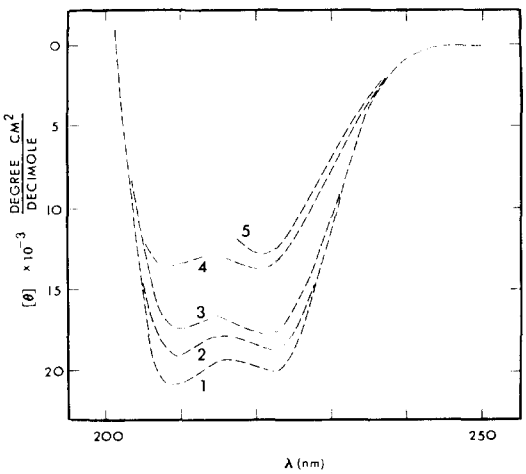

Fig. 2. Effects of detergents on the CD spectrum of metmyoglobin. The samples were the same as those shown in Fig. 1, except that a path length of 0.1 cm was employed. The individual detergents present are as follows: (1) no detergent, or octyl glucoside or decyl glucoside; (2) digitonin; (3) Emulphogene; (4) *N,N*-dimethyldodecylamine oxide; (5) hexadecyltrimethylammonium bromide.

the depth of the trough may be interpreted as disruption of the ordered segments of the metmyoglobin molecule. Therefore, it is clear that while the alkyl glucosides are non-perturbing reagents, the other detergents tested generate varying degrees of unfolding of the native metmyoglobin structure.

It should be pointed out that the glucosides possess excellent optical transparency in the ultraviolet, allowing CD measurements of membrane proteins to be made to 193 nm. This is slightly better than Emulphogene and digitonin, and markedly superior to *N,N*-dimethyldodecylamine oxide and hexadecyltrimethylammonium bromide which cut off in the vicinity of 220 nm.

Properties of solubilized disc membranes

Octyl glucoside is characterized by good water solubility and a very high critical micelle concentration of 25 mM [24]. By contrast, that of the non-ionic detergent Triton X-100 is 0.24 mM [25]. Solubilization* of discs begins at about 21 mM octyl glucoside and requires approx. 2.5 g of detergent per g of rhodopsin solubilized. We have found 30 mM to be a useful detergent concentration for most purposes. Solubilization occurs immediately upon addition of discs to the detergent solution.

The solubility properties of decyl glucoside differ significantly from those of octyl glucoside. The maximum concentration which can exist as a single phase in aqueous solution is about 3 mM at room temperature. Upon refrigeration decyl glucoside crystallizes out of solution. Disc membranes added to a decyl glucoside suspension in water rapidly dissolve forming a clear, non-sedimentable solution. However, in the presence of 0.1 M phosphate buffer, complete solubilization could

* A sample was considered to be solubilized if it failed to sediment when subjected to 100000 × *g* for 30 min.

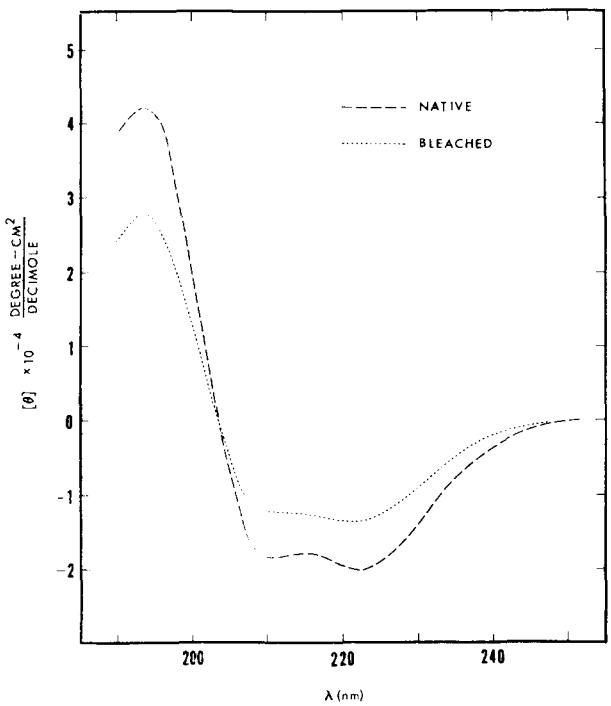

Fig. 3. Ultraviolet CD spectrum of purified rhodopsin in 0.05 M octyl glucoside, 0.1 M potassium phosphate buffer (pH 7).

not be achieved. Other salts and buffers which were tried also interfered with solubilization to varying degrees.

Concanavalin A-Sepharose affinity column chromatography was used to prepare essentially lipid-free rhodopsin from discs dissolved in 50 mM octyl glucoside. This material was used to obtain the absorption and CD spectra shown in Figs. 3 and 4. The visible CD spectrum shown in Fig. 4 for purified rhodopsin has a $[\theta]_{335nm}/[\theta]_{485nm}$ ratio of 2.67. In contrast, discs solubilized in 30 mM octyl glucoside displayed a somewhat higher α band yielding a $[\theta]_{335nm}/[\theta]_{485nm}$ ratio of 2.0. Due to the transparency of the detergent, we were able to resolve the 194 nm peak in the rhodopsin CD spectrum. The spectra are in general agreement with other reports [26–28], and indicate a helical content of 58 %. Bleaching the purified rhodopsin in 50 mM octyl glucoside produced a 33 % reduction in ellipticity at 194 and 222 nm, and abolished the visible CD spectrum.

Since the ultraviolet CD spectrum of rhodopsin remains unchanged upon bleaching of sonicated disc membranes [29], it has been concluded that bleaching does not greatly alter the structure of rhodopsin in these preparations. The same is true for digitonin-solubilized discs [8]. The light-induced changes seen here for purified rhodopsin and disc membranes dissolved in octyl glucoside are typical of those seen in all other detergents for which the data have been reported. Although octyl glucoside does not perturb the native metmyoglobin structure, and yields rhodopsin solutions in which the visual pigment is stable for weeks at room tempera-

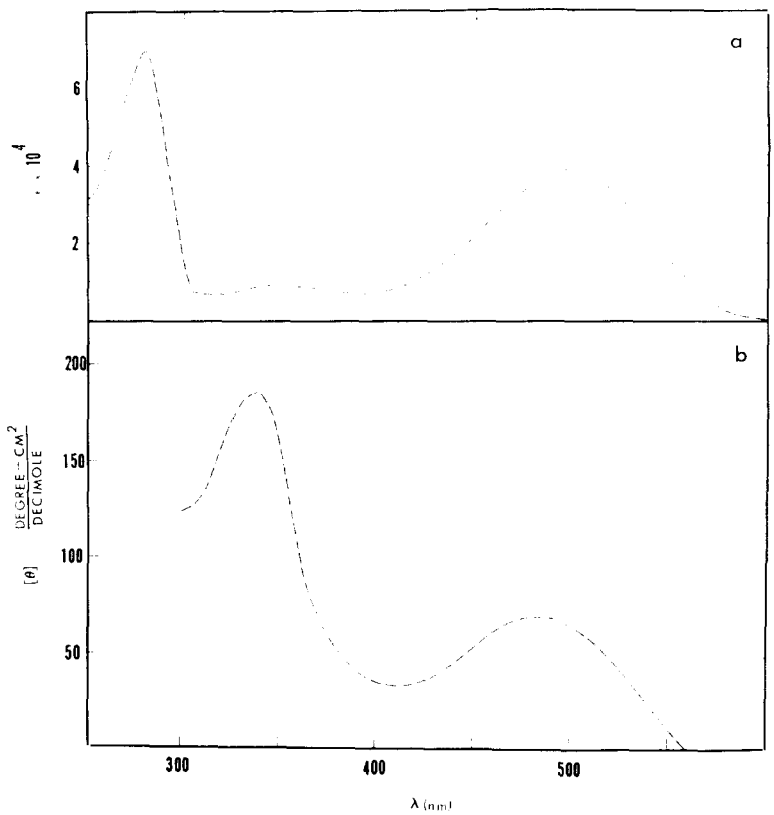

Fig. 4. (a) Absorption spectrum of purified rhodopsin in 0.05 M octyl glucoside, 0.1 M potassium phosphate buffer (pH 7). (b) Visible CD spectrum of the same material (A_{500nm} = 1.14, 1-cm path length).

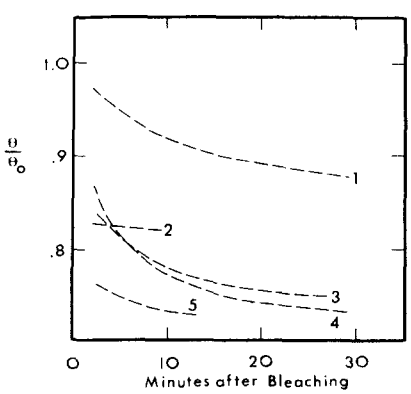

Fig. 5. Kinetics of light-induced denaturation of discs solubilized in various detergents at 27 °C. The ellipticities at 222 nm divided by the ellipticities observed prior to bleaching are plotted as a function of time after bleaching. Detergent concentrations are as indicated in Materials and Methods. The decyl glucoside sample was prepared in water rather than phosphate buffer. The detergents present are as follows: (1) decyl glucoside; (2) hexadecyltrimethylammonium bromide; (3) Emulphogene; (4) octyl glucoside: (5) *N,N*-dimethyldodecylamine oxide.

ture, stabilization of the native structure is apparently not strong enough to prevent a conformation change upon bleaching. The native structure of rhodopsin appears to be stabilized by both its interaction with phospholipid and the binding of 11-*cis*-retinal. Most detergents seem to be capable of substituting for the lipid interactions as long as the chromophore is intact, but only phospholipid bilayers or digitonin provide adequate stability to maintain the structure of the bleached pigment. This unique property of digitonin is still not fully explained, but may be related to the relative rigidity of the molecule [8] or to its tendency to solubilize rhodopsin as a lipoprotein complex [30].

While the light-induced denaturation of rhodopsin in solubilized discs is common to almost all detergents, we found that the rate of denaturation varied greatly from one detergent to another. This variation in rate provides another means of ordering the detergents as to their strength as denaturants. Fig. 5 shows the kinetics of the light-induced change in ellipticity at 222 nm at 27 °C. In both hexadecyltrimethylammonium bromide and *N,N*-dimethyldodecylamine oxide the denaturation was substantially complete within the 2 min required to bleach the samples and replace them in the spectropolarimeter. In octyl glucoside and Emulphogene (as first observed by Shichi [28]) the transition occurred over a period of 20–30 min, while the decyl glucoside-solubilized sample denatured slower and to a lesser extent. In agreement with Hong and Hubbell [8] we observed little or no change in the ellipticity of digitonin-solubilized discs.

The two additional measurements designed to assess the structural integrity of rhodopsin in solubilized disc solutions are summarized in Table I. These measurements are the rate of thermal denaturation of rhodopsin, and the extent to which light-bleached rhodopsin could be regenerated by addition of 11-*cis*-retinal.

The thermal stability measurement was carried out at 42 °C over a period of 1 h as described in Materials and Methods. At this temperature insignificant loss of A_{500nm} occurred for unsolubilized discs (determined by solubilization following the 42 °C incubation) or for discs incubated in the presence of 4 mM decyl glucoside. Octyl glucoside and digitonin produced a slow but detectable loss with a first-order time constant of about 20 h, Emulphogene displayed an intermediate rate of decay with a time constant of 10 h, while the *N,N*-dimethyldodecylamine oxide and hexa-

TABLE I

THERMAL STABILITY AND REGENERABILITY OF RHODOPSIN IN VARIOUS DETERGENTS

Detergent	Thermal stability τ (min) at 42 °C	Regenerability (%)
Unsolubilized discs	∞	81
Decyl glucoside (4 mM)	2400	90
Digitonin (saturated)	1200	75
Octyl glucoside	1100	86
Emulphogene	600	18
N,N-Dimethyldodecylamine oxide	35	0
Hexadecyltrimethylammonium bromide	2.8	0

decyltrimethylammonium bromide solutions underwent rapid thermal bleaching.

Regeneration of light-bleached rhodopsin with 11-*cis*-retinal followed roughly the same ordering of the detergents as the other measurements described above. The *N*,*N*-dimethyldodecylamine oxide and hexadecyltrimethylammonium bromide solutions yielded no significant recovery of A_{500nm}, Emulphogene showed an intermediate regenerability of 18 %, whereas in the digitonin and alkyl glucoside solutions 75–90 % of the rhodopsin was regenerated.

Reconstitution experiments

A major advantage of the alkyl glucosides over other detergents is the rapidity with which they can be removed by dialysis. This is particularly true of octyl glucoside with its high critical micelle concentration. Permeability coefficients of octyl and decyl glucoside were determined to be 0.18 and 0.16 cm/h, respectively, through a Union Carbide 27/100 dialysis membrane. This corresponds to a half-time of about 2 h from a 2×5 cm dialysis bag.

Several preliminary reconstitution experiments were performed in octyl glucoside. Purified rhodopsin from the concanavalin A-Sepharose affinity column was mixed with octyl glucoside-solubilized egg phosphatidylcholine in molar ratios from 1:100 to 1:200. Dialysis of the mixture against water at 4 °C in the dark for 24 h produced vesicles which frequently resembled native discs in the phase contrast microscope. Negatively stained samples appeared by electron microscopy to contain predominently sealed vesicles with a wide variety of sizes and no multilamellar structures. Centrifugation on sucrose density gradients established that the recombined vesicles contained both lipid and protein in a ratio approximately equal to that which was loaded into the dialysis bag. The amount of unbleached rhodopsin recovered ranged from 75 to 96 %.

CONCLUSION

The use of cationic and anionic detergents for the solubilization of membrane proteins is generally unsatisfactory, since the concentration of detergent required for solubilization usually falls into the region of high, non-specific binding that produces denaturation of most proteins [25, 31]. Non-ionic detergents do not exhibit this non-specific, cooperative binding, and are, therefore, preferable for solubilization of membrane proteins when denaturation is to be avoided.

The commerically available non-ionic detergents such as Emulphogene and Triton X-100 consist of mixtures of chemical species, and are subject to variation from batch to batch [6, 25]. Elimination of this possible source of experimental variability was one of our motives for investigating the alkyl glucosides, since they are prepared as pure, well defined compounds.

We have demonstrated that octyl and decyl glucoside are capable of rapidly solubilizing the disc membrane. Essentially, complete removal of lipid from rhodopsin was achieved in octyl glucoside by affinity column chromatography. This purification was not attempted in decyl glucoside because of the technical problems encountered with its use in the presence of salts and buffers. However, decyl glucoside provided significantly greater stabilization of the rhodopsin structure than did octyl glucoside according to our CD, thermal bleaching, and regeneration experiments.

This observation suggests that nonyl glucoside may provide an optimal compromise in properties between the octyl and decyl glucosides.

Their optical transparency makes the alkyl glucosides suitable for characterization of purified membrane proteins, and their rapid rates of dialysis allow detergent removal for reconstitution experiments.

The bulk of the experiments reported here was intended to evaluate the denaturing and stabilizing effects of the various detergents toward membrane proteins, specifically rhodopsin. In all cases the cationic hexadecyltrimethylammonium bromide and zwitterionic *N,N*-dimethyldodecylamine oxide were observed to be inferior. The non-ionic detergent used for comparison, Emulphogene BC-720, was significantly better than hexadecyltrimethylammonium bromide and *N,N*-dimethyldodecylamine oxide, but was still inferior to the alkyl glucosides, particularly with regard to the thermal stability and regenerability of rhodopsin.

In their study employing alkyl glucosides, Baron and Thompson [17] have demonstrated that octyl glucoside is capable of solubilizing the membrane-bound ATPase and NADH dehydrogenase of *Streptococcus faecalis* without loss of enzymatic activity. Their observations, and our own work presented here, show that the alkyl glucosides have the ability to solubilize membrane proteins while maintaining their structure and function, thus offering a significant improvement over the detergents presently being used in membrane research.

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