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The Effect of Detergent Selection on Retinal Outer Segment $A_{280}/A_{500}$ Ratios*

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$A_{280}/A_{500}$ purity ratios for 5 different retinal rod outer segment (ROS) preparations were determined without prior centrifugation, in 1-cm light path cuvettes, in 1% concentrations of 5 detergents. Mean ratios were 3.66 ± 0.23 for Brij 96, 3.56 ± 0.20 for cetyltrimethylammonium bromide, 3.12 ± 0.14 for Na-cholate, 5.62 ± 0.34 for digitonin, and 1.78 ± 0.07 for Triton X-100. Ratios in Triton were depressed by a large UV absorbance of the detergent, which prevented accurate photodetection of the UV absorbance of the protein. Brij and digitonin also displayed significant UV absorbance. Purity ratios in digitonin were also elevated by UV light-scattering associated with undissolved, sedimentable protein. Differences evidently attributable to interaction of a particular detergent with ROS protein or lipoprotein persisted even when these UV effects were minimized by use of 1-mm light path cuvettes and centrifugation of nonsolubilized material prior to spectrophotometry. Such differences were minimal, however, among the 3 nonionic detergents octyl glucoside, Emulphogene and Ammonyx LO. At 11 μM rhodopsin and detergent concentrations, the 3 nonionic detergents displayed negligible $A_{280}$ and no significant sediment upon centrifugation. In the presence of excess ROS, Emulphogene extracted more protein from the ROS than did Ammonyx LO or octyl glucoside. Despite differences in color of the unextracted, bleached pellets resulting from extraction by these detergents, Emulphogene spectra of the pellets revealed qualitatively similar contents. We have selected Emulphogene for $A_{280}/A_{500}$ ratios because (a) it does not absorb in the 278-nm range; (b) it does not interfere with Lowry protein determinations while all of the other detergents except octyl glucoside do; (c) it apparently extracts more ROS protein than do Ammonyx LO or octyl glucoside when ROS is in excess; and (d) it is much cheaper than octyl glucoside.

The purity of rhodopsin and of retinal rod outer segment preparations from which it is derived have long been estimated in detergent solutions by comparing absorbance at the absorption maximum of rhodopsin (about 500 nm, depending on species) with absorbance at some wavelength which indicates the presence of contaminants, typically 280 nm for protein or 400 nm for hemoglobin, cytochromes, or non-rhodopsin pigments of retinal origin (1-4). Thus an elevated $A_{280}/A_{500}$ ratio would betray the presence of extraneous protein, and an elevated $A_{400}/A_{500}$ ratio would betray a non-rhodopsin pigment. If one is elevated, the other is likely to be also, except in cases where an elevated $A_{400}/A_{500}$ ratio arises from bleaching of rhodopsin rather than contamination. If detergent solutions are to be bleached to determine difference spectra, 0.1 M hydroxylamine is typically present at neutral pH to trap free retinal as its oxime rather than permit it to rebind to protein as adventitious Schiff base pigments.

During the early days of rhodopsin purification, $A_{280}/A_{500}$ ratios almost always were determined in digitonin solutions (4). In recent years, a proliferation of detergents has arisen, each with its own special advantages for extraction, bleaching, regeneration, or reconstitution of rhodopsin or ROS fragments. Rigorous kinetic and thermodynamic analysis of rhodopsin behavior in solution requires as pure rhodopsin as possible. However, in the last decade active soluble enzyme complexes, such as cGMP phosphodiesterase (5-8), and two or more kinases (9-12) have been discovered to co-purify with rhodopsin, at least until washing of the ROS. This has levied even more stringent requirements on preparation purity than did rhodopsin work, because of the possibility of extraneous origin of these mobile proteins. In attempting to reconcile preparative procedures and results from different laboratories, we have often been confused by reported (or absent) purity ratios. Since in recent years, most investigators have used $A_{280}/A_{500}$ ratios rather than $A_{400}/A_{500}$, we focus here on the former. We examined nine papers appearing in the Journal of Biological Chemistry in the last 18 months reporting observations on rhodopsin or ROS preparations. Of the nine, two gave the $A_{280}/A_{500}$ ratio in 1% Ammonyx LO, one gave the ratio in 50 mM CTAB, and one gave the ratio without specifying the detergent, as one of us has also done (7). Five gave no ratio, but referred to earlier reports, of which one gave the $A_{280}/A_{500}$ ratio in 45 mM CTAB, one gave it in 1% Ammonyx LO, one (referred twice) gave it in 3% Ammonyx LO, and one gave no $A_{280}/A_{500}$ ratio but did provide data comparing rhodopsin and protein content. A broader search of other journals revealed a diversity comparable to that found in this journal. Two inferences can be made from this admittedly limited survey: first, that a significant minority of investigators do not use this measure of purity, or report none at all; and second, that a significant number (including ourselves until quite recently) think that the detergent used for determining the $A_{280}/A_{500}$ ratio is unimportant. With respect to the second inference we demonstrate below that the selection of detergent has a marked effect on the ratio.

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1 ROS, retinal rod outer segment; CTAB, cetyltrimethylammonium bromide.
MATERIALS AND METHODS

Bovine ROS fragments were purified on either continuous (density, 1.12–1.14) or discontinuous (densities, 1.12, 1.14, and 1.16) sucrose gradients as previously described (14). All operations were performed in dim red light unless otherwise specified. The top band from the continuous gradient is usually purer, but the top band from the discontinuous gradient has a higher rhodopsin yield with less fragmentation of the ROS. Some of these preparations were made in the presence of 0.15 M KCl, to increase retention by the ROS of cGMP phosphodiesterase activity (15). The ROS preparations, taken directly from the gradient, were stored in liquid N2 prior to use. In the initial experiments, aliquots of 5 to 100 μl (approximately 50 μg–1 mg of Lowry (13) protein) of thawed ROS suspension were dispersed in neutral solutions of 1% of 5 detergents, without sedimentation, to a final volume of 1 ml in a thick-walled quartz cuvette. Final concentration of detergent was 1% w/v. The ROS-detergent complex also contained previously neutralized 0.1 M NH4OH to prevent formation of adventitious visual pigments during rhodopsin bleaching. The matched reference cuvette contained detergent and NH4OH but no ROS (nor buffered sucrose, in which ROS were suspended). Absorption spectra were taken before and after bleaching of the cuvette contents by fluorescent laboratory light. In the second set of experiments, three of the same detergent systems were subjected to a more detailed examination of spectral effects on a single ROS preparation. The third set of experiments compared the purity ratios of the same preparation in cholate, octyl-β-D-glucoside, Emulphogene (polyoxyethylene-1-tri-decyl ether), and Ammonyx L0, a mixture of dodecyl and tetradecyl dimethylamine oxide. The fourth set of experiments compared the effectiveness of 8 detergents in extraction of excess ROS. In all experiments, spectra were performed immediately after ROS dispersion in detergent, unless sedimentation intervened. Additional methods appear in table and figure legends.

Brij 96 (polyoxyethylene 10-oleyl ether), Brij 99 (20-oleyl ether), CTAB, digitonin, octyl-β-D-glucoside, Emulphogene, and sodium cholate were procured from Sigma, and Triton X-100 originated from Rohm and Haas. Ammonyx L0 was a gift from Onyx Chemical Co. of Jersey City, N.J. Digitonin solutions free of turbidity were prepared by the method of Bridges (16).

RESULTS

Fig. 1 shows representative dark and bleached spectra for the same ROS preparation in Triton X-100, digitonin, and CTAB. None of these solutions was centrifuged prior to spectrophotometry. The ratios of A280/A500 determined on the 3 dark spectra for this ROS preparation were 2.0 for Triton, 6.2 for digitonin, and 3.9 for CTAB. Except for digitonin, these ratios were largely independent of ROS concentration within a range of 5–100 μl/1 ml final volume in the cuvette. In the case of digitonin, it was not possible to increase ROS concentration to the same levels as other detergents without also increasing turbidity.

Table I expands the ratios of Fig. 1 to 2 additional detergents and 4 additional ROS preparations. The means of the ratios for each detergent appear in the right-hand column, along with the standard error calculated for the 5 ROS preparations examined in the presence of that detergent. It is quite evident that Triton X-100 produced uniformly lower A280/A500 ratios, and digitonin produced higher ratios than the other 3 detergents (p < 10−4 by Student’s t-test, comparing Triton X-100 with cholic acid, or digitonin with Brij).

Table II presents ΔA280 (dark minus bleached) divided by the size of the aliquot of ROS, for the same spectra on which the purity ratios in Table I were determined. Again, within the limited range of ROS aliquots used (5–100 μl) the size of the ratio did not depend upon aliquot size. However, in digitonin, this normalized ΔA280 was consistently higher than the mean of the 5 detergents together, and the variance of the measure among the 5 ROS preparations in digitonin was also elevated. Thus the elevated A280/A500 ratio in digitonin arose despite a somewhat higher rhodopsin extraction/ml of ROS.

Fig. 2 shows that digitonin has a considerable UV absorbance (A280 in H2O ≈ 0.18 at 1% concentration versus H2O blank), although less than that of Brij 96 (A275 ≈ 0.14 at 0.5%, 0.28 at 1%) or Triton X-100 (A275 ≈ 0.24 at 0.01%, 24 at 1%). Minor differences in effective detergent concentration between the sample and reference cuvettes may thus disproportionately alter A280 of ROS preparations, and in fact neither sample nor reference photodetector probably detects enough light for a valid comparison to be made in the case of Triton X-100.

Studies of the solubilization of ROS disc membranes by octyl glucoside indicate that only the detergent in the micellar form is effective in solubilization. A critical ratio of micellar octyl glucoside to rhodopsin of 270:1 was required for complete solubilization of the disc membrane (17). A rough meas-
Table 1. A280/A600 ratios of five ROS preparations in five detergents. Ratios were determined from absorbances measured directly from a horizontal line extending through the recorded absorbance at 600 nm, which was arbitrarily set equal to 1.

| Detergent | Brij 96 | CTAB | Na Cholate | Digitonin | Triton X-100 |
|-----------|---------|------|------------|-----------|-------------|
| Mean ± S.E. | 3.7 ± 0.2 | 3.9 ± 0.2 | 3.6 ± 0.2 | 6.2 ± 0.2 | 2.0 ± 0.2 |

Table 2. A490/A600 per 456 in each of five ROS dispersions prepared in five detergents.

| Detergent | Brij 96 | CTAB | Na Cholate | Digitonin | Triton X-100 |
|-----------|---------|------|------------|-----------|-------------|
| Mean ± S.E. | 5.0 ± 0.5 | 5.2 ± 0.5 | 4.8 ± 0.5 | 6.0 ± 0.5 | 4.0 ± 0.5 |

Table 3. Ratios of rhodopsin concentrations typically employed in the experiments described, together with estimates of critical micellar concentrations and ratios of rhodopsin to detergent concentrations (mM/mg). The detergent concentration is derived by subtracting the detergent/rhodopsin ratio provided by Becher et al. (6) for complete solubilization of rhodopsin by detergent/CTAB mixture at 2900 nm (x) (d) +2.6. (a) Cholate/Amnonyx (900) (d) +3.0. (b) Cholate/Brij (700) (d) +2.6. (c) Cholate/Cholate (900) (d) +2.6. (d) Cholate/CTAB (900) (d) +2.6. (e) Cholate/Cholate (900) (d) +2.6. (f) Cholate/CTAB (900) (d) +2.6. (g) Cholate/Cholate (900) (d) +2.6. (h) Cholate/CTAB (900) (d) +2.6. (i) Cholate/Cholate (900) (d) +2.6. (j) Cholate/CTAB (900) (d) +2.6. (k) Cholate/Cholate (900) (d) +2.6. (l) Cholate/CTAB (900) (d) +2.6. (m) Cholate/Cholate (900) (d) +2.6. (n) Cholate/CTAB (900) (d) +2.6. (o) Cholate/Cholate (900) (d) +2.6. (p) Cholate/CTAB (900) (d) +2.6. (q) Cholate/Cholate (900) (d) +2.6. (r) Cholate/CTAB (900) (d) +2.6. (s) Cholate/Cholate (900) (d) +2.6. (t) Cholate/CTAB (900) (d) +2.6. (u) Cholate/Cholate (900) (d) +2.6. (v) Cholate/CTAB (900) (d) +2.6. (w) Cholate/Cholate (900) (d) +2.6. (x) Cholate/CTAB (900) (d) +2.6. (y) Cholate/Cholate (900) (d) +2.6. (z) Cholate/CTAB (900) (d) +2.6.
ment (17, 23). Unlike Triton X-100, they have no appreciable absorbance in the 280-nm region. After the initial dispersion in 1% detergent solution, at approximately double the rhodopsin concentration shown in Table IV, centrifugation produced only minimal clearing of these three detergent solutions, while a small pellet and demonstrable clearing resulted in the case of cholate. The difference probably arises from the greater ratios of micellar detergent to rhodopsin estimated for the nonionic detergents as compared to cholate.

Table V summarizes qualitative and quantitative comparison of the extractive efficiencies of 8 detergents for an excess of ROS. Emulphogene and CTAB demonstrated the highest quantitative extraction of ROS protein, but the unextracted residue from CTAB evidently contained little or no rhodopsin. The first three nonionic detergents in Table V left an unextracted pellet which upon bleaching exhibited a 310-nm peak of unidentified origin. Differences in color of the bleached pellets were also obvious to the naked eye.

**DISCUSSION**

Since the purity of each ROS preparation was a constant, the large differences observed in purity ratio when the preparation was dispersed in the different detergents of the initial experiments (Tables I and II; Fig. 1) have a clear implication.

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3 D. G. Mc Connell, unpublished observations.
for purity estimates. If differences exist not only in preparative procedures but in the detergent used to determine the ratio, the ratio becomes essentially meaningless. To dramatize this, we point out that the ratio of 1.78 ± 0.07 we demonstrated above for Triton-dispersed ROS membranes is precisely what Heitzmann reported (29) for very pure rhodopsin (1.7–1.8) in Emulphogene solutions of ROS prepared by our method and subsequent chromatography.

Differences among purity ratios in various detergents arise from 3 origins. First is the degree of solubilization of the ROS in the detergents, without respect to selectivity among different protein moieties. At 1%, digitonin obviously is the poorest agent among those examined. If it is to be used, centrifugation of preparations is imperative. This is not always feasible when very small aliquots of widely different ROS preparations are stored in large numbers in the freezer for subsequent determinations of rhodopsin and of purity ratios. In contrast, Triton, Emulphogene, and CTAB are very efficient solubilizers, even at ROS concentrations clearly in excess of the capacity of other detergents (Table V).

The second origin of differences is the intrinsic UV absorbance of the detergents. This is manifestly intolerable in the case of Triton, undoubtedly a problem with Brij, and possibly with digitonin. It does not appear to present a difficulty with cholate, although its small UV absorbance may contribute to a slightly higher A360/A500 ratio (Table IV) than was found in three nonionic detergents with even less UV absorbance. To minimize contributions to the purity ratio by intrinsic UV absorbance of Triton, Brij, digitonin, or other detergents, use of 1-mm light paths is not an attractive alternative as a routine laboratory procedure because of required changes in cuvette racks and slide-wires, nosiness of recordings at low concentrations and the fact that even at 1 mm or even 0.5 mm, UV absorbance of the detergent may still significantly exceed that of the protein, as was the case in Fig. 3, E and F. Thus the recorded UV absorbance in those spectra is still not necessarily accurate.

A third origin of differences is apparently attributable to the kinds and amounts of proteins solubilized by each detergent. After minimizing UV absorbance contributions from turbidity or the detergent itself, substantial differences among the ratios in digitonin (3.18, average of Fig. 3, B and C), Triton (3.91, average of Fig. 3, E and F) and cholate (2.63, average of Fig. 3, H, I, and J) still remain. Their interpretation is uncertain, but some differences in solubilizing properties are known. For example, digitonin has been shown by analysis of the behavior of an electron spin probe attached to rhodopsin to exert a smaller effect on rhodopsin conformation than Triton X-100, CTAB, and other detergents (30). This observation was reinforced by determinations that digitonin altered thermal stability of rhodopsin markedly less than Triton X-100, CTAB, or Ammonyx LO, and also increased the rate of metarhodopsin II production less than the other detergents (23).

In the present study, the differences in extraction of excess ROS by 8 detergents (Table V) also appear to imply important differences among the detergents with respect to selectivity for protein or lipoprotein moieties in the ROS or its inevitable contaminant membrane fractions. CTAB, for example, apparently leaves behind unextracted protein of non-rhodopsin origin and would consequently appear to be the detergent of choice for purification of rhodopsin, although not for determining ROS preparation purity. Why it would produce a higher A360/A500 ratio than cholate (Table I) is unexplained. In contrast with CTAB, the 3 nonionic detergents Emulphogene, Ammonyx LO, and octyl glucoside left behind an unextracted protein residue clearly containing rhodopsin, although differences were observed in pellet color to the naked eye as well as in extractive efficiency. We make no pretense in the present study of rigorous interpretation of such differences. In other detergents, they may very well contribute to observed differences in A360/A500 ratios even when UV absorption and turbidity have been minimized.

To resolve confusion among purity ratios in different detergents, we propose the following criteria. Select detergents with low UV absorbances, good extraction efficiencies for excess ROS, and A360/A500 ratios in the middle range (2.5–3.5) for ROS purified on sucrose density gradients. Further purification to rhodopsin will bring the ratio down to less than 2.0. The nonionic detergents octyl glucoside, Emulphogene, or Ammonyx LO would seem acceptable on the basis of Table IV ratios and the differences observed in Table V. Of these three, Emulphogene may be most convenient. Octyl glucoside is quite expensive, and Ammonyx LO can be bought at present only in 50-gallon drums, although the Onyx Chemical Co. has thus far been generous in providing pint volumes of it. Other detergents may also meet these criteria. For our own use we have selected Emulphogene because (a) it does not absorb in the 278-nm range; (b) it does not interfere with Lowry protein determinations while all of the other detergents except octyl glucoside do (The Biuret method of Gornall et al. (31) was also unsuccessful with the same detergents); (c) it apparently extracts more ROS protein than Ammonyx LO or octyl glucoside when ROS is in excess, and (d) it is much cheaper than octyl glucoside.

Since visible difference spectra do not appear to be significantly affected by differences in intrinsic UV absorbance of the detergents (Fig. 3), one might also use detergents only to determine rhodopsin concentration by difference spectrum and determine protein by the Lowry method, expressing rhodopsin purity directly as nanomoles/mg of Lowry protein. However, Lowry estimates may render high values in the presence of elevated lipid content of membranes (32), a caution which is especially applicable to the ROS. Thus we do not recommend this alternative.

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Regulation of insulin receptor metabolism. Differentiation-induced alteration of receptor synthesis and degradation.

Brent C. Reed, Gabriele V. Ronnett, Peter R. Clements, and M. Daniel Lane

Page 3924, Table I, line 1:

Under $k_d$, 0.5 should be 0.05 h$^{-1}$

Vol. 256 (1981) 4843–4846

On the mechanism of ribonucleoside triphosphate reductase from Lactobacillus leichmannii. Evidence for 3'-C–H bond cleavage.

JoAnne Stubbe, Deborah Ackles, Raj Sehgal, and Raymond L. Blakley

The third author’s name should be Raj K. Sehgal

The effect of detergent selection on retinal outer segment $A_{280}/A_{900}$ ratios.

David G. McConnell, Charles A. Dangler, Deborah M. Eadie, and Burton J. Litman

Page 4913, Summary, line 22:

Due to a printer error "1%" was omitted before detergent. The correct sentence should read:

At 11 $\mu$M rhodopsin and 1% detergent concentrations, the 3 nonionic detergents displayed negligible $A_{280}$ and no significant sediment upon centrifugation.

Vol. 256 (1981) 5144–5152

The yeast his4 multifunctional protein. Immunochemistry of the wild type protein and altered forms.

Ramunas Bigelis, Joseph K. Keesey, and Gerald R. Fink

Page 5150, Fig. 10:

Fig. 10 is reprinted here from a new original print supplied by the author:

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