

Phase Separation of Integral Membrane Proteins in Triton X-114 Solution*

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A solution of the nonionic detergent Triton X-114 is homogeneous at 0°C but separates in an aqueous phase and a detergent phase above 20°C. The extent of this detergent phase separation increases with the temperature and is sensitive to the presence of other surfactants.

The partition of proteins during phase separation in solutions of Triton X-114 is investigated. Hydrophilic proteins are found exclusively in the aqueous phase, and integral membrane proteins with an amphiphilic nature are recovered in the detergent phase.

Triton X-114 is used to solubilize membranes and whole cells, and the soluble material is submitted to phase separation. Integral membrane proteins can thus be separated from hydrophilic proteins and identified as such in crude membrane or cellular detergent extracts.

Integral membrane proteins are characterized by a hydrophobic domain which interacts directly with the hydrophobic core of the lipid bilayer (1). Nonionic detergents are widely used for the solubilization and characterization of these proteins (1-3). During solubilization the nonionic detergent replaces most lipid molecules in contact with the hydrophobic domain of the integral membrane protein and leads to the formation of a soluble protein-detergent mixed micelle (1, 2). Several properties of this mixed micelle such as size and hydrophilicity will depend on the properties of the protein solubilized as well as on the properties of the detergent present in the complex. In contrast to integral membrane proteins which have an amphiphilic structure, water-soluble or hydrophilic proteins show little or no hydrophobic interaction with nonionic detergents, and their physicochemical properties are not expected to be influenced by the presence of such a detergent in the solution (2, 23).

The commonly used nonionic detergent Triton X-100¹ forms clear micellar solutions at room temperature. As the temperature is raised the micellar molecular weight increases, and the solution turns suddenly turbid at 64°C (4, 5). At this temperature, called the cloud point, there occurs a microscopic phase separation in the solution. This phase separation could be due to secondary association of small micelles into large micelle aggregates (27). With increased temperature phase separation proceeds until two clear phases, respectively depleted and enriched in detergent, are formed.

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¹ Triton X is a Rohm and Haas Company trade name.

Within the Triton X series the temperature of the cloud point depends strongly on the number of hydrophilic oxyethylene units condensed on the hydrophobic octylphenyl residue (6, 7). The solubility in water of the most hydrophobic members of the Triton X series ($n \leq 6$) barely exceed the critical micelle concentration, and the detergent is found in a separated phase, even at 0°C (7). A diluted solution of Triton X-114 (average $n = 7$ to 8) exhibits a cloud point at about 20°C (6). This temperature is convenient for the reversible condensation and separation of the detergent under conditions compatible with the isolation of native proteins.

Considering that integral membrane proteins, and only those, form mixed micelles with nonionic detergent, I investigated the possibility of isolating them from the hydrophilic proteins by phase separation above the cloud point of the detergent. This article describes the experimental conditions for such a separation in Triton X-114 solution and shows that hydrophilic proteins are recovered in the aqueous phase whereas amphiphilic integral membrane proteins are found in the detergent phase after the separation.

EXPERIMENTAL PROCEDURES²

The protein samples (0.2 to 1.0 mg/ml) were prepared in 200 μ l of 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.5 to 1.0% Triton X-114 at 0°C.

For the separation of the proteins a cushion of 6% (w/v) sucrose, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.06% Triton X-114 (300 μ l) was placed at the bottom of a 1.5-ml conical Eppendorf microfuge tube. The clear sample was then overlaid on this sucrose cushion, and the tube was incubated 3 min at 30°C. Clouding of the solution occurred.

The tube was centrifuged for 3 min at 300 $\times g$ at room temperature or at 30°C in a clinical centrifuge equipped with a swinging bucket rotor. After centrifugation the detergent phase was found as an oily droplet at the bottom of the tube. The separation was then repeated as follows. The upper aqueous phase was removed from the tube and received 0.5% fresh Triton X-114. After dissolution of the surfactant at 0°C the mixture was again overlaid on the sucrose cushion used previously, incubated 3 min at 30°C for condensation, and centrifuged on the previous detergent phase.

At the end of the separation the aqueous phase was rinsed with 2% Triton X-114 in a separate tube without sucrose cushion. The detergent phase of this last condensation was discarded. After separation Triton X-114 and buffer were added, respectively, to the aqueous and detergent phases in order to obtain equal volumes and approximately the same salt and surfactant content for both samples.

Aliquots of the separated phases were analyzed together with the input sample by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on slab gels (14).

² Portions of the paper (including "Materials," "Results," and Figs. 2 to 6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 80M-620, cite author, and include a check or money order for \$5.60 per set of photocopies. Full-size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

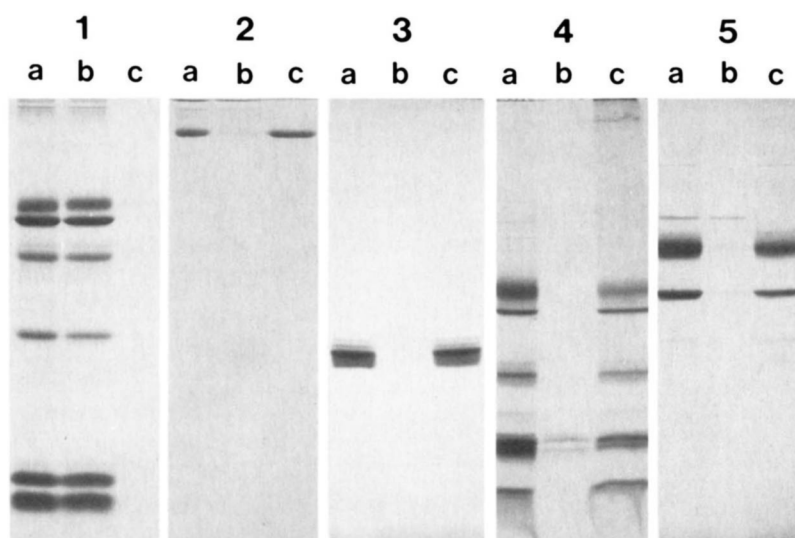


FIG. 1. **Phase separation of hydrophilic and amphiphilic proteins.** Various purified hydrophilic and amphiphilic proteins were mixed with Triton X-114 and submitted to phase separation at 30°C as described under "Experimental Procedures" (samples 4 and 5 were mixed in 1.5% Triton X-114 and incubated at 35°C). Aliquots of the input sample and of the aqueous and detergent phases were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 10 to 15% polyacrylamide gradient gels (sample 2 is unreduced, samples

4 and 5 were warmed up 5 min at 56°C). The samples contained the following proteins: 1, serum albumin, catalase, ovalbumin, concanavalin A, myoglobin, cytochrome c. 2, human erythrocyte acetylcholinesterase. 3, *Halobacterium halobium* bacteriorhodopsin. 4, yeast cytochrome c oxidase. 5, *Paracoccus denitrificans* cytochrome c oxidase. a, input sample before phase separation. b, aqueous phase after separation. c, detergent phase after separation.

RESULTS

Serum albumin, catalase, ovalbumin, concanavalin A, myoglobin, and cytochrome c were used to test the partition of hydrophilic proteins during phase separation in Triton X-114 solution. Fig. 1, part 1, shows the result of such a separation. Lane a is the starting material, lane b is an aliquot of the aqueous phase, and lane c is an aliquot of the detergent phase. It is evident that the hydrophilic proteins were recovered exclusively in the aqueous phase and were even excluded from the volume of the detergent phase after sedimentation through the sucrose cushion. The quality of this separation allows a repeated extraction of the aqueous phase with fresh Triton X-114 without unwanted contamination of the detergent phase with hydrophilic proteins.

Acetylcholinesterase from the human erythrocyte membrane, bacteriorhodopsin, and the cytochrome c oxidase from yeast and from *Paracoccus denitrificans* are amphiphilic integral membrane proteins or protein complexes. They have been used to test the partition of integral membrane proteins during phase separation in Triton X-114 solution. Fig. 1, parts 2 to 5, show the results of these separations. In contrast to the hydrophilic proteins of Fig. 1, part 1, all four amphiphilic proteins tested show a strong depletion in the aqueous phase and are recovered in the detergent phase (lanes c).

This experiment as well as the separations presented in Figs. 4 to 6 in the miniprint demonstrates that water-soluble proteins and detergent-solubilized integral membrane proteins can be rapidly separated from each other under mild conditions and with an excellent recovery.

DISCUSSION

Integral membrane proteins, bound by hydrophobic interactions to the core of the lipid bilayer, were first operationally defined by the agent required for their solubilization as well as their solubility properties after removal of this agent (18). It became, however, rapidly obvious that these proteins, when solubilized by nonionic detergent were found in solution in the form of protein-detergent mixed micelle and thus could be

characterized by their ability to bind detergent (1, 2, 19, 20). The amount of Triton X-100 bound to solubilized bacteriorhodopsin (21), erythrocyte band 3 and PAS-1 (22) and yeast cytochrome c oxidase (23), all integral membrane proteins used as examples in this article, was found to range from 0.8 g to 4.7 g of detergent per g of protein. In contrast nonionic detergent was not binding to hydrophilic proteins. Catalase, ovalbumin, hemoglobin, and cytochrome c, all hydrophilic proteins used as examples in this article, bind less than 1 to 3% of their own weight of Triton X-100 (22). Serum albumin, an exception among hydrophilic proteins, had four strong binding sites for Triton X-100 (24).

Below the temperature of its cloud point Triton X-114 forms micellar solutions and is an efficient solubilizer of membrane proteins (26). The separation procedure described in this paper is based on the low solubility of Triton X-114 above its cloud point. Under conditions promoting the condensation of Triton X-114 the mixed micelles formed by the amphiphilic proteins and detergent were found to aggregate with the rest of the detergent while the hydrophilic proteins remained in the aqueous phase.

As Triton is a "mild" detergent (1-3), it is anticipated that a number of assays requiring a native protein structure will be feasible after phase separation. The procedure could also be scaled up as a preparative step for the purification of membrane proteins. It is finally suggested that the method could be used as an assay for solubilized amphiphilic membrane receptors of hydrophilic ligands, provided the ligands are available in a labeled form.

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Supplementary material PHASE SEPARATION OF INTEGRAL MEMBRANE PROTEINS IN TRITON X-114 SOLUTION

by Clément BORDIER

MATERIALS

Proteins

Bacteriorhodopsin was prepared from *Halobacterium halobium* purple membrane and solubilized as described by Dencher and Heyn in 0.1% Triton X-100 (8).

Human erythrocyte acetylcholinesterase, a gift of Drs. U. Brodbeck and P. Ott, was prepared by affinity chromatography and freed of surfactant on Bio-Gel HTP column (9). The protein micelles of the enzyme were solubilized in 0.6% Triton X-114.

Saccharomyces cerevisiae and *Paracoccus denitrificans* cytochrome c oxidase, a gift of Dr. B. Ludwig, were prepared respectively according to Mason et al. (10) and Ludwig and Schatz (11). The enzymes were solubilized in 0.1% TX-100.

[³⁵S]methionine labelled mouse myeloma cells MOPC 21 (P3H) were prepared by Dr. B. Schlegel. The cells were suspended at a concentration of 2×10^6 cells/ml in methionine-free Dulbecco modified Eagle's medium supplemented with 0.5 mCi/ml [³⁵S]methionine. After incubation at 37°C for 2 hrs the cells were separated from the culture supernatant by centrifugation and lysed in 1% Triton X-114 at 0°C. Nuclei and debris were discarded by centrifugation at 2500 x g for 5 min at 4°C.

Erythrocytes labelled by treatment with neuraminidase, galactose oxidase and NaB³H₄ (12) were a gift from Dr. J. Finne. Ghosts, prepared according to Dodge et al. (13), were suspended at a concentration of 1.7 mg/ml in 1.7% Triton X-114, 10 mM Tris-HCl pH 7.4, 600 mM KCl. The preparation was incubated 30 min. on ice and centrifuged 30 min. at 10^5 g below 10°C. The supernatant was diluted to a final concentration of 1% Triton X-114 and 350 mM KCl.

Precondensation of Triton X-114

Triton X-114 was purchased from Fluka AG, Buchs, Switzerland. 20 g Triton X-114 containing 16 mg butylated hydroxytoluene were added to 980 ml, 10 mM Tris-HCl pH 7.4, 150 mM NaCl. After dissolution at 0°C the clear solution was incubated at 30°C. Condensation of the detergent occurred and the mixture separated overnight into a large aqueous phase depleted in detergent and a smaller phase enriched in detergent. The aqueous phase was discarded and replaced by the same volume of 10 mM Tris-HCl pH 7.4, 150 mM NaCl. Buffer and detergent phase were mixed at 0°C and condensation was repeated twice at 30°C under the same conditions. The Triton concentration in the third lower phase was measured at 275 nm. This phase was used as Triton X-114 stock for all experiments described here.

[³H]-labelled Triton X-114 was prepared as follows. [³H]-labelled Triton X-100 from New England Nuclear was mixed with [³H] 30,000 x excess of Triton X-114. Condensation of 0.5 ml of [³H]Triton (1% w/v) was performed four times as described above. Phase separation occurred in a centrifuge at 300 x g in 3 min. After these condensations [³H]-labelled Triton showed the same partition as Triton X-114 during phase separation.

RESULTS

Precondensation of Triton X-114

In order to eliminate the most hydrophilic molecules from the commercial Triton X-114 (5,16), a condensation was performed three times as described in Materials and Methods.

This treatment lowered the cloud point by 2°C and the concentration of detergent found in the aqueous phase after condensation decreased from 0.15% to 0.04%. The distribution of the polyoxyethylene chain length of the detergent before and after condensation was analysed by thin layer chromatography (15). No difference could be detected visually. The detergent phase of the third condensation had a concentration of 11.4% (w/v) Triton X-114 and was used as stock of detergent for all the experiments presented in this article.

Cloud point and phase separation of Triton X-114

The cloud point of a 1% solution of Triton X-114 was determined in 10 mM Tris pH 7.4, 150 mM NaCl. The turbidity of the solution, monitored visually, increased sharply above 22°C, the temperature of the cloud point under these conditions (Fig. 2).

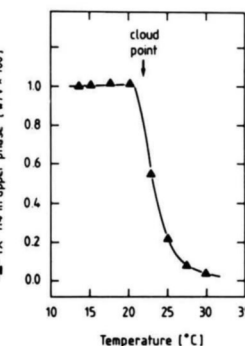


Fig. 2

Cloud point and phase separation of Triton X-114

A 1.0% solution of Triton X-114 in 10 mM Tris-HCl pH 7.4, 150 mM NaCl was prepared on ice. Duplicate tubes containing 1.0 ml of the solution were incubated at the indicated temperatures for 10 min and then sedimented at the same temperature at 300 x g for 3 min. The concentration of Triton X-114 in the upper phase was checked visually and an aliquot (200 µl) was removed from the upper half of the tubes for the determination of the concentration of Triton X-114 remaining in the upper phase.

Macroscopic separation of the solution into two distinct phases was detected after centrifugation above 22°C. This separation is reflected in the decrease in detergent concentration in the upper half of the tube above this temperature (Fig. 2). At 30°C, the standard temperature used for the separation of proteins, the concentration of Triton X-114 in the aqueous upper phase was 0.04% or 3 to 4 times the cmc.

Effect of other detergents on the phase separation of Triton X-114

The cloud point of a mixture of detergents has a temperature between the cloud points of the detergents mixed (5,6). Detergents which are more hydrophilic than Triton X-114 are often used to solubilize membrane proteins. Their presence in the sample will raise the temperature of the cloud point and could prevent the formation of two separated phases at 30°C.

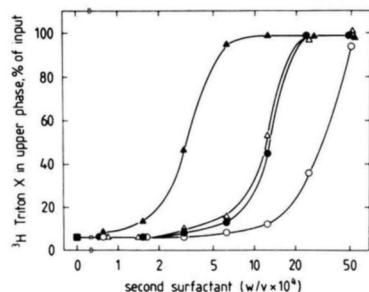


Fig. 3

Effect of other surfactants on the phase separation of Triton X-114

[³H]-labelled Triton X-114 (3250 cpm/mg) was mixed at different ratios with a number of hydrophilic surfactants in 10 mM Tris-HCl pH 7.4, 150 mM NaCl. In each series of tubes the weights of Triton X-114 and of the second surfactant were varied but the volume (500 µl) and the total surfactant concentration (1% w/v) were kept constant.

After standing 3 hr. at 4°C the tubes were incubated 10 min. at 30°C and centrifuged 3 min. at 300 x g at 30°C. 200 µl were removed from the upper half of the tube and counted.

Second surfactant: ■, none; ○, Nonidet P-40; ●, Triton X-100; ▲, n-octyl glucoside; △, Na-deoxycholate.

In the experiment presented in Fig. 3, [³H]Triton X-114 was mixed at different ratios with detergents commonly used for membrane solubilization. The condensation of the detergent mixtures was tested at 30°C in the presence of 150 mM NaCl. When mixed in a 1:1 ratio (50:50 w/v second surfactant) with Triton X-114 all detergents tested prevented the formation of two separate phases. At lower weight ratios the extent of solubilization depended on the nature of the second surfactant used. The ionic bile salt deoxycholate was the most efficient solubilizer of Triton X-114, whereas Nonidet P-40, a compound closely related to Triton X-114, was the least efficient. Essentially the same result was obtained when the concentration of Triton in the upper phase was determined spectroscopically.

Comments on Triton X-114

Triton X-114 was chosen as the most hydrophobic commercially available Triton X which forms micellar solutions above 0°C. More hydrophobic surfactants are insoluble in water and more hydrophilic have a cloud point at a higher temperature (6,7). With an average difference of two oxyethylene units the length distribution of the polar moiety of Triton X-114 and Triton X-100 overlap by more than 70%. Triton X-114 can be used to solubilize membranes and whole cells (26).

The protein separations described here were performed in 10 mM Tris HCl pH 7.4, 150 mM NaCl in two condensations at 30°C with 0.5 to 1% Triton X-114. These conditions can be modified. In particular at a higher ionic strength the cloud point of the Triton solution is lowered and phase separation can also be performed at a lower temperature (17). Alternatively, phase separation is more complete and less sensitive to the presence of contaminating detergents when performed at higher temperature. A higher Triton X-114 concentration increases also the yield of amphiphilic proteins in the detergent phase but interferes later with the SDS-PAGE of the samples.

Phase separation of bacteriorhodopsin and hemoglobin

A 1% solution of Triton X-114 separates at 30°C into a large upper phase depleted in detergent and a small lower phase enriched in detergent. Fig. 4 shows the relative volumes of the two phases as well as the Triton X-114 content of the separation. 94% of the detergent was recovered in the lower phase at a concentration of 10% (w/v).

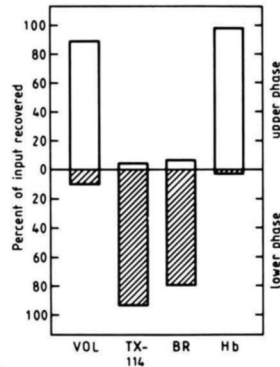


Fig. 4

Phase separation of bacteriorhodopsin and hemoglobin

Bacteriorhodopsin or hemoglobin were added to two tubes containing 500 µl of 1% Triton X-114, 10 mM Tris HCl pH 7.4, 150 mM NaCl at 0°C.

The tubes were incubated 10 min at 30°C and centrifuged 3 min at 300 x g at the same temperature. The relative volumes of the resulting two phases were measured. Aliquots of the two phases were analysed by SDS-PAGE and the relative concentration of bacteriorhodopsin and hemoglobin was determined by quantitative estimation of the Coomassie blue stain bound to the protein bands in the gel. The relative concentration of Triton in the two phases of a third tube was measured photo-metrically at 275 nm.

VOL: Volume, TX-114: Triton X-114, BR: bacteriorhodopsin, Hb: hemoglobin.

The distribution in the two phases of the amphiphilic bacteriorhodopsin matches closely the distribution of Triton X-114 (Fig. 4). This result shows that the mixed micelles formed by Triton X-114 and this protein aggregate above the cloud point almost as efficiently as pure detergent micelles.

On the other hand 98% of the hydrophilic hemoglobin was recovered in the aqueous upper phase (Fig. 4). The concentration of hemoglobin in the detergent phase was lower than the concentration before phase separation.

Solubilization and phase separation of plasma membrane proteins and glycoproteins

The membranes of human erythrocytes were externally labelled with tritium in the glycoproteins and solubilized under hypertonic conditions with Triton X-114. The soluble material was analysed by the phase separation method. Fig. 5.1 shows the stained protein pattern and Fig. 5.2 is a fluorogram of the same gel. With a few exceptions most proteins are recovered either in the aqueous or in the detergent phase. In particular 74% of band 3 and 78% of PAS 1 + 2, both integral membrane proteins, are recovered in the detergent phase (lanes c).

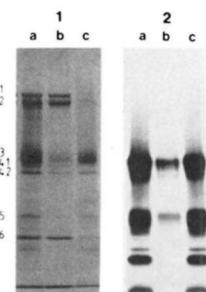


Fig. 5

Extraction and phase separation of human erythrocyte membrane proteins

Human erythrocytes were radioactively labelled in the glycoproteins and lipids (12). Membranes were prepared and extracted with 1% Triton X-114 as described in Materials. The soluble extract was analysed by phase separation as described in Experimental Procedures and in the legend to Fig. 1. 1, Coomassie blue protein stain. 2, Fluorogram of 1 (25). a, Triton X-114 solubilized proteins before phase separation (36 µg protein, 420'000 cpm). b, aqueous phase after separation. c, detergent phase after separation.

Some proteins are recovered in both phases and may be peripheral membrane proteins which are incompletely separated from integral membrane proteins during solubilization.

Phase separation of cellular and secreted proteins

[³⁵S]-Mouse myeloma cells were metabolically labelled with [³⁵S]methionine and after separation of the culture supernatant lysed in Triton X-114. The soluble material was analysed by the phase separation method. Fig. 6.1 shows the separation of the cellular proteins. The vast majority of the label was incorporated into hydrophilic proteins. In particular the intracellular precursor of the secreted immunoglobulin G (arrow) is found in the aqueous phase. A few proteins, however, are selectively recovered in the detergent phase.

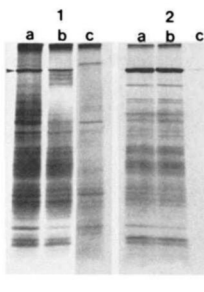


Fig. 6

Phase separation of cellular and secreted proteins

Mouse myeloma cells were labelled *in vivo* with [³⁵S]methionine and a Triton X-114 soluble extract was prepared as described in Materials. This extract as well as the proteins secreted in the culture medium were analysed by phase separation as described in Experimental Procedures and in the legend to Fig. 1. Samples were prepared without reducing agent. The lanes 1a and 1b were exposed 2.8 x less than the other lanes of the figure.

1, Triton X-114 solubilized cellular material. 2, culture supernatant. a, samples before phase separation, b, aqueous phase after separation, c, detergent phase after separation.

Fig. 6.2 shows the separation of the secreted proteins found in the culture medium. As expected from their soluble nature, these proteins are recovered in the aqueous phase.