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Phase Separation of Biomolecules in Polyoxyethylene Glycol Nonionic Detergents

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ABSTRACT: The advantage of aqueous two-phase systems based on polyoxyethylene detergents over other liquid-liquid two-phase systems lies in their capacity to fractionate membrane proteins simply by heating the solution over a biocompatible range of temperatures (20 to 37°C). This permits the peripheral membrane proteins to be effectively separated from the integral membrane proteins, which remain in the detergent-rich phase due to the interaction of their hydrophobic domains with detergent micelles. Since the first reports of this special characteristic of polyoxyethylene glycol detergents in 1981, numerous reports have consolidated this procedure as a fundamental technique in membrane biochemistry and molecular biology. As examples of their use in these two fields, this review summarizes the studies carried out on the topology, diversity, and anomalous behavior of transmembrane proteins on the distribution of glycosyl-phosphatidylinositol-anchored membrane proteins, and on a mechanism to describe the pH-induced translocation of viruses, bacterial endotoxins, and soluble cytoplasmic proteins related to membrane fusion.

In addition, the phase separation capacity of these polyoxyethylene glycol detergents has been used to develop quick fractionation methods with high recoveries, on both a micro- and macroscale, and to speed up or increase the efficiency of bioanalytical assays.

KEY WORDS: aqueous two-phase systems, polyethylene glycol, Triton® X-114, protein purification, membrane proteins, bioanalytical methods, glycosylphosphatidylinositol-anchored membrane proteins, pH-induced conformational changes, detergents.

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I. INTRODUCTION

Phase separation of mixtures of aqueous solutions of two structurally distinct polymers is one of the classic separatory methods in the study of biomaterials.[1] The first observation of this phenomenon was reported as long ago as 1896 by the Dutch microbiologist Beijerinck,[2] who tried to mix aqueous solutions of soluble starch with agar or gelatine. About 60 years later, Albertsson[3] found this tendency to phase partition in certain mixtures of polyethylene glycol (PEG) and potassium phosphate, or PEG and dextran, and recognized the potential of these systems to separate macromolecules,[4] cell organelles,[5] and intact cells[6] because the partition of these polymers occurs under conditions that preserve biological activity.[3] Since then, the study and proposed applications of these aqueous two-phase systems have grown immeasurably to cover new purification procedures, the characterization of new systems, and the development of new biopolymers. An extensive review of this methodology has been published recently.[7–10]

In addition to PEG systems, there are two more liquid-liquid separation systems that can be used for protein purification: reverse micellar systems in organic solvents and aqueous two-phase systems based on polyoxyethylene

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detergents. The first system is based on the extraction of proteins from a buffer into the water pool of a reverse micelle and the subsequent retransfer from the organic phase to another aqueous phase with different pH or ionic strength (for reviews, see References 11 and 12). The second type of system is based on the inverse temperature/solubility behavior of nonionic detergents carrying polyoxyethylene groups as a hydrophilic moiety, which leads to two phases, one rich in detergent and the other depleted.[13,14] These polyoxyethylene-based detergents present an additional feature compared with PEG alone, which is their capacity to disrupt and solubilize biological membranes by interacting with the hydrophobic portion of integral membrane molecules.[15–19] Such a process results in the formation of soluble mixed micelles containing the membrane molecules, integral proteins, and detergent. Water-soluble proteins bind little or no detergent.[20,21]

In 1981 using Triton®* X-114 (TX-114), Bordier[20] combined these two characteristics of PEG detergents to selectively separate integral membrane proteins from peripheral and cytoplasmic proteins. Dilute aqueous solutions of TX-114 (1% w/v) phase separate at the biocompatible low temperature of approximately 20°C into a detergent-rich phase containing integral membrane proteins and a detergent-depleted phase containing water-soluble proteins.[20,21]

The aim of this review is to summarize the data available on the uses of these PEG detergents in protein purification, membrane biochemistry and molecular biology, and bioanalytical techniques. Of the PEG detergents, the review especially concentrates on Triton® X-114 because most published work has been carried out with this detergent. This review is not intended to be comprehensive. Rather, it describes those observations that we felt might contribute to an understanding of the usefulness of these detergents in the aforementioned research fields. In order to accomplish this, the nature and properties of PEG detergents are discussed first so that the solubilizing and precipitating (phase separation) characteristics of these amphiphiles can be understood fully.

II. POLYOXYETHYLENE GLYCOL NONIONIC DETERGENTS

Soluble amphiphiles that effectively solubilize membrane lipids are generally termed detergents.[22] This term is usually synonymous with surfactant, although a surfactant is really an amphiphile that is soluble in aqueous solutions because both polar (or hydrophilic) and nonpolar (or hydrophobic) moieties occur in the same molecule.[18] The hydrophilic groups may be charged (anionic, cationic, or zwitterionic) or uncharged (nonionic) but polar (polyhydroxy residues, polyoxyethylene groups, etc.). The hydrophobic groups are hydrocarbon chains with aliphatic, aromatic, or polycyclic moieties that are barely soluble in water because of the "hydrophobic effect".[23] For more details on detergent properties and basic definitions, several reviews[16,18,22,24–29] and comprehensive synopses on this topic edited by commercial chemical companies[30–32] can be consulted.

One common conclusion in all these reviews and booklets is that the optimum detergent for a particular membrane or membrane protein must be found empirically.[18,24,29]

Among the hundreds of commercially available detergents, nonionic detergents with a polyoxyethylene head group are the most frequently used because they usually do not denature proteins.[24] They are efficient at breaking up lipid-lipid and lipid-protein interactions in membranes but are rather less effective in changing interactions between proteins,[24] thus permitting the study of membrane proteins in their native state. However, the following considerations should be borne in mind before using them to carry out membrane solubilization.

A. Tradenames

Practically identical products can have different tradenames. For instance, Triton® X-100 (Union Carbide, Danbury, CT), Nodinet P-40 (Gallard-Schlesinger Chemical Mfg. Co., Carle Place, NY), and Igepal CA-630 (GAF Corp., NY) are *tert*-octylphenol poly(ethyleneglycolether)$_n$, with n = 9.6, 9.0, and 9.5, respectively. Table 1

* Triton X is a trademark of Union Carbide Corp., Danbury, CT.

TABLE 1
Chemical and Tradenames for Some Commonly Used Heterogeneous Polyoxyethylene Glycol Detergents

Type (chemical name)	General formula	Abbreviation	General trade names
n-alkyl PEG ether [PEG (n) alkyl ether]	$C_n H_{2n+1}(OCH_2CH_2)_xOH$ ***(ART TO COME)*** $[O\text{-}CH_2\text{-}CH_2]_x\text{-}OH$	C_nE_x	Sterox AJ, Thesit, Emulogphene BC, Brij, Lubrol PX, Atlas G, Genapol, Nikkol B
p-tert-octyl phenyl PEG ether [PEG (n) alkyl phenyl ether]	$p\text{-}C_n H_{2n+1}C_6H_4(OCH_2CH_2)_xOH$ ***(ART TO COME)*** $[O\text{-}CH_2\text{-}CH_2]_x\text{-}OH$	*tert*-$C_n\phi E_x$	Triton® X, Igepal CA, Nodinet P, Nikkol OP
n-alkyl phenyl PEG ether [PEG (n) alkyl phenyl ether]	$C_n H_{2n+1}C_6H_4(OCH_2CH_2)_xOH$ ***(ART TO COME)*** $[O\text{-}CH_2\text{-}CH_2]_x\text{-}OH$	$C_n\phi E_x$	Triton N, Nikkol NP, Igepal CO, Tergitol NP, Surffonic N
Acyl PEG sorbitan ester [PEG (n) sorbitan monoacyl-ate]	$H\text{-}[O\text{-}CH_2\text{-}CH_2]_W\text{-}O$ — $O\text{-}[CH_2\text{-}CH_2\text{-}O]_x\text{-}H$; $O\text{-}[CH_2\text{-}CH_2\text{-}O]_y\text{-}H$; ***(ART TO COME)*** $O\text{-}[CH_2\text{-}CH_2\text{-}O]_z\text{-}H$ $W + X + Y + Z = 20$	C_n sorbitan E_x	Tween, Emasol, Nikkol T
Polyoxyethylene-polyoxypropylene alkyl · ether [PEG (n) POP (x) alkyl ether]	$HO\text{-}[CH_2\text{-}CH_2\text{-}O]_X\text{-}[CH(CH_3)\text{-}CH_2\text{-}O]_Y\text{-}[CH_2\text{-}CH_2\text{-}O]_Z\text{-}H$		Pluronic F-, Synperonic F-, Nikkol PBC

Note: Atlas G, Tween, and Brij series are products of Atlas Chemical Industries Inc., Wilmington, DE; Triton® and Tergitol series, Union Carbide Corp., Danbury, CT; Emulgene and Igepal, GAF Corp., NY; Sulfonic series, Jefferson Chemical Co., Houston, TX; Nikkol series, Nikko Chemical Co., Tokyo; Synperonic series, Imperal Chemical Industries PLC; Nodinent, Shell Oil; Sterox AJ, Monsanto; Lubrol PX. ICI American's; Genapol, Hoechst, AG; Thesit, Desitin-Werk Carl Klinke GmbH, Hamburg; Nopalcol, Diamond Chemical; Pluronic, Wyandotte.

shows the chemical and trade names of some polyoxyethylene-derived detergents. Of these, *n*-alkyl PEG ethers and *p-t*-octyl phenyl PEG ethers are the most frequently used nonionic detergents in membrane solubilization, and, for this reason, the considerations below usually refer to these two groups.

B. Impurities

Commercial nonionic detergents are, as a rule, chemically impure.[22] They may contain varying amounts of water and additives, one batch differing from the next, and, after prolonged storage, the composition of the liquid solutions at the bottom of the container may differ from that on the top. In addition, they can undergo autooxidation of their PEG chains catalyzed by trace heavy metal ions.[24,33] Therefore, to obtain reproducible results, it is advisable to purify the detergents whenever possible.[34–37] During the purification process, a free-radical scavenger, such as butylated hydroxytoluene, may be added to remove free radicals from the detergent solution.[22]

C. Heterogeneity

PEG ether detergents are almost always heterogeneous, with a broad distribution of polyoxyethylene chain lengths, the x value given being an average number.[22,32] However, some n-alkyl PEG are available in a pure form (manufactured by Nikko Chemical Co., Tokyo), but, due to their cost, they are generally used in special applications, such as the formation of reverse vesicles.[38,39]

D. Hydrophile-Lipophile Balance

The solubilizing power of nonionic detergents is correlated with the hydrophile-lipophile balance (HLB).[40–43] This parameter is a measure of the balance between the size and strength of the opposing hydrophilic and hydrophobic groups in nonionic detergents.[44,45] This parameter was first introduced by Griffin,[46,47] and the scale (HLB_G) ranges from 0 to 20. Most hydrophobic detergents have a low HLB_G value (1 to 10), and increasing HLB_G values correspond to increasingly hydrophilic characteristics. For detergents whose sole hydrophilic moiety is polyoxyethylene, HLB can be calculated by

$$HLB_G = E/5 \quad (1)$$

where E is the weight percentage of ethylene oxide in the detergent.[46,47] For the first three groups of detergents presented in Table 1, Equation 1 becomes

$$HLB_G = \frac{881x}{44.05 + A} \quad (2)$$

where x is the number of ethylene oxide repeat units per molecule and A is the molecular weight of the lipophilic moiety, namely, 206.3 for the *p-t*-octyl phenyl series.[48] Another HLB scale was proposed by Davies (HLB_D), who considered the HLB as an additive number obtained from the addition of the functional groups of nonionic detergents.[49]

Griffin's scale has been validated by extensive experiments,[48] and it is the most frequently used parameter in membrane biochemistry to designate membrane solubilization power.[41–43] The most effective nondenaturing membrane protein solubilizers are those PEG nonionics whose HLB_G ranges from 12 to 20. Detergents with an HLB_G at the lower end of the range (12 to 14.5) are best for extracting and solubilizing integral membrane proteins.[22] Detergents with an HLB_G at the upper end of the range (such as Tweens) are frequently used for solubilizing extrinsic proteins.[29] Nonionics with a low HLB_G number are not water soluble and, for this reason, have been little used.[22]

The HLB_G is also useful for predicting whether a detergent can be removed from a solution by methods based on hydrophobic absorption. If the HLB_G is too large, the detergent will not bind very well to the hydrophobic support.[29]

E. Micellation

Each detergent has a characteristic phase diagram that describes the conditions under which

crystalline detergent, micelles, and monomers exist. In general, PEG detergents only exist in an insoluble hydrated crystalline state in water solutions that are below the freezing point of the solvent (<0°C).[50] Above this temperature, known as the critical micelle temperature (cmt), they tend to form micelle aggregates when the critical micelle concentration (cmc) is reached.[22,28]

In general, the cmc values for PEG detergents are very low (2 μ*M* to 1 m*M*) and about 100 times lower than their ionic analogs.[22] These low cmc values correspond to high micellar weights, and there is an inverse relationship between these two parameters.[51] The practical consequence of these low cmcs is that the detergents are very difficult to remove by dialysis, and a chromatographic exchange process is necessary using several high cmc detergents or adsorption to a hydrophobic resin.[52–55] Furthermore, the high micellar molecular weight of PEG detergents, which is due to their low cmc, also interferes with the separation of different proteins by gel filtration according to their size, giving rise to artefactual protein molecular weights.[16,24]

In addition, membrane solubilization generally occurs above the cmc, at which point membrane components are taken into the micelles.[56] Mixed micelles of Triton® X-100 and phosphatidylcholine have been investigated extensively.[18,57–59] At high Triton/phospholipid ratios, the size of the mixed micelles is greater than that of the pure Triton® micelles, the size increasing in proportion to the amount of phospholipid present.[59] Geometrical constraints of the Triton® molecule suggest that an oblate (disk-shaped) ellipsoid is preferred over a prolate (rod-shaped) ellipsoid[57] (Figure 1). These calculations assume that the structure of PEG detergent micelles follows the classic pattern of a hydrophobic core surrounded by randomly coiled polar polyoxyethylene chains in the outer mantle of the micelle. In contrast to the classic picture of micelles, Robson and Denis[57] proposed the presence of spherical micellar shapes for Triton® X-like detergents by allowing oxyethylene units to become incorporated into the hydrophobic core. However, this model seems to be a thermodynamically improbable event.[58]

Any of the above types of arrangement of polyoxyethylene detergent micelles would enable these surfactants to solubilize different substances

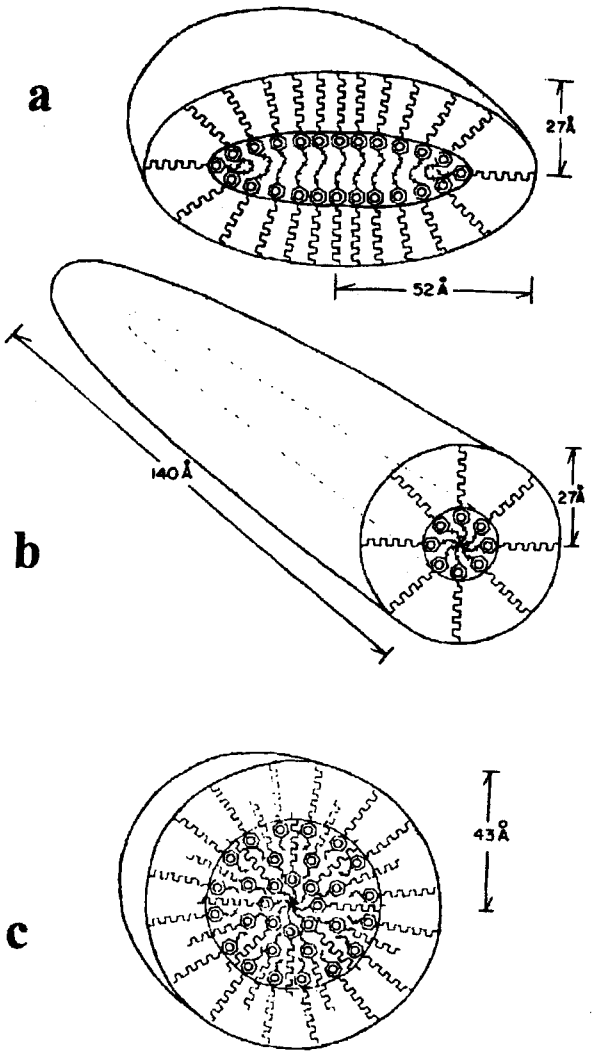

FIGURE 1. Schematic view of the (a) oblate, (b) prolate, and (c) spherical micelle models for Triton® X-100. For the oblate and prolate models, the octylphenyl groups are only packed in a spherical or ellipsoidal core, while, in the spherical micelle model, there are additional oxyethylene groups in the core's vicinity, which extend its radius to 26 Å. Figure c shows Triton® monomers in the center of the hydrophobic core, with their oxyethylene groups extending outward through the core, and the others having less of their oxyethylene chains embedded in the core. (From Robson, R. J. and Dennis, E. A., *J. Phys. Chem.*, 81, 1075, 1977. With permission.)

not only by means of the three classical modes of solubilization (apolar molecules in the liquid-like hydrocarbon micellar core, amphiphilic molecules at the interface, and solubilizates that are insoluble

in hydrocarbon or in water adsorbed onto the surface of the micelle), but also by means of a fourth mode consisting of the inclusion of organic compounds into the polyoxyethylene exterior of the micelle rather than adsorption on the surface (Figure 2).[56] As an example of this last mode, phenol and its homologs have been shown to form complexes with polyoxyethylene groups by hydrogen bonding between the phenolic hydroxy group and the ether oxygen of the polyoxyethylene group.[56] This fourth solubilization mode is of great importance for explaining the removal of phenolic compounds from plant crude extracts, and is discussed in a later section.

F. Cloud Point

The physical state of these detergents is affected at temperatures higher than the cmt. An increase in temperature leads to a decrease in the number of hydrogen bonds between water molecules and ether oxygens of the polyoxyethylene groups.[56] This raises the micellar weight and decreases the cmc.[60] If the temperature continues to increase, the micelle becomes so large and the number of intermicellar interactions increases to such an extent that a sudden onset of turbidity is perceptible even to the naked eye.[14,61] This temperature is called the cloud point. A further rise in temperature causes the solution to begin to separate into two phases, one detergent rich and the other detergent depleted, with few or no micelles present.[56]

This phase separation of PEG detergent has been extensively studied[14,62–65] and there has long been dispute as to whether the phase separation, made manifest by the cloud point, is associated with a rapid increase in micellar size at an increased temperature or with the aggregation of relatively small micelles. Another possibility, suggested by Kjellander,[14] is that this phase behavior is the result of the aggregation of large, flexible, rod-like micelles by an intermicellar force that originates from the increased structuring of water around the polar groups. This force is temperature dependent and can be repulsive at low temperatures and attractive at high ones. The net force is composed of two large, opposing components (enthalpic and entropic) that almost cancel each other out, making the interaction sensitive to changes in surfactant chain lengths, additives, etc. The clouding phenomenon is a consequence of

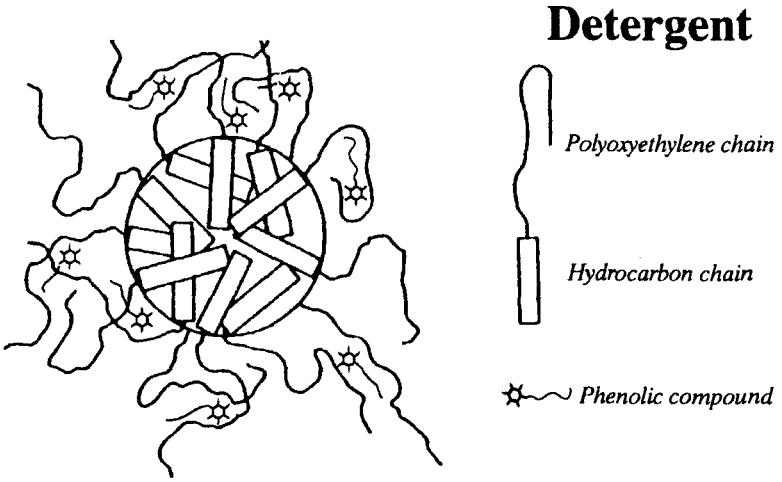

FIGURE 2. Schematic model for a nonclassic mode of solubilization of nonionic surfactants possessing polyoxyethylene glycol groups. The outer shell of hydrated polyoxyethylene probably permits organic compounds with strong affinity for a polyoxyethylene group to be incorporated in this region. This type of solubilization is classified as inclusion into the polyoxyethylene exterior of the micelle rather than as adsorption on the surface of the micelle. Examples of such solubilization are phenols and their homologs.

entropy dominance in the intermicellar interaction and not of micellar growth or dehydration.[14]

The cloud points of several nonionic detergents are listed in Table 2. From this table it can be deduced that the cloud point depends on the lengths of the hydrophilic oxyethylene units and of the hydrocarbon chains. For a given number of oxyethylene units, the cloud point decreases when the alkyl chain increases. The opposite occurs in the case of the oxyethylene units. For example, in the Triton® X-series, the cloud point increases from 22°C for TX-114 (7.5 units) to 65°C for Triton® X-100 (9.6 units). In addition, as shown in Figure 3, the cloud point is also directly related to the HLB within a series of detergents (*n*-alkyl PEG or phenyl-derived PEG): the more hydrophilic the detergent (HLB close to 20) the higher cloud point, and vice versa. In particular, note that Triton® X-114 has an HLB very similar to that of Triton® X-100 and therefore shares the same solubilization capability. However, it is insoluble at room temperature, and because of this, TX-114 was long ignored as a useful detergent for many studies. However, since a protein fractionation method was developed by Bordier,[20] who used its phase partitioning capacity, TX-114 has been at the center of many physicochemical characterizations, especially those affecting its cloud point.

III. FACTORS AFFECTING THE CLOUD POINT OF TRITON® X-114

Triton® X-114, as well as other Triton® X detergents, is prepared by the reaction of octylphenol with ethylene oxide, which renders a Poisson distribution with respect to the length of the polyoxyethylene chain, with an average number of 7.5.[32] Commercial preparations of TX-114, except for protein-grade detergents,[29–31] need to be purified. The normally used purification method consists of a series of phase separations in the aqueous solution (pure water or buffer) that is to be used in the experiments with TX-114. In the first two condensations, butylated hydroxytoluene (16 mg/20 g TX-114) is used to remove the peroxides.[20]

Once TX-114 has been purified, composite phase diagrams can easily be obtained by preparing varying mixtures of detergent and water, and observing the temperature at which turbidity first appears (cloud point) when slowly raising the temperature of the stirred mixture.[66,67] Care must be taken to differentiate between the turbidity caused by precipitation of a new phase and the opalescence occurring near the boundary between the two phases in the phase diagram. To help in this differentiation, amphiphilic dyes such as bromophenol blue can be used.[56,68] Figure 4 shows a typical composite diagram. Note that only solutions below 10% (w/w) have a cloud point in the biological range.

In addition, phase volumes are also dependent on the concentration and temperature of TX-114.[69] Lower temperatures with the same detergent concentration result in greater detergent-rich phase volumes.[69] Similar increases in the detergent-rich phase volumes are observed when the concentration is increased at a fixed tempera-

TABLE 2
Cloud Point (°C) of Some Nonionic Surfactants[13,22,28,29,30,32]

	Number of ethylene oxide units[a]									
Alkyl group (1%)	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**12**
n-Hexyl (3%)	+	37	67	75	80	—	—	—	—	—
n-Octyl	+	+	35	55	—	—	—	—	—	—
n-Decyl	+	+	21	45	63	75	85	—	—	—
n-Dodecyl	—	+	7.0	31	51	67	79	88	95	>100
n-Tetradecyl	—	—	—	—	40	58	72	—	—	—
n-Hexadecyl	+	+	+	+	32	53	67	75	—	—
t-Octylphenyl	+	+	+	+	—	22	52	65	78	88

[a] +, insoluble at room temperature; — no results available.

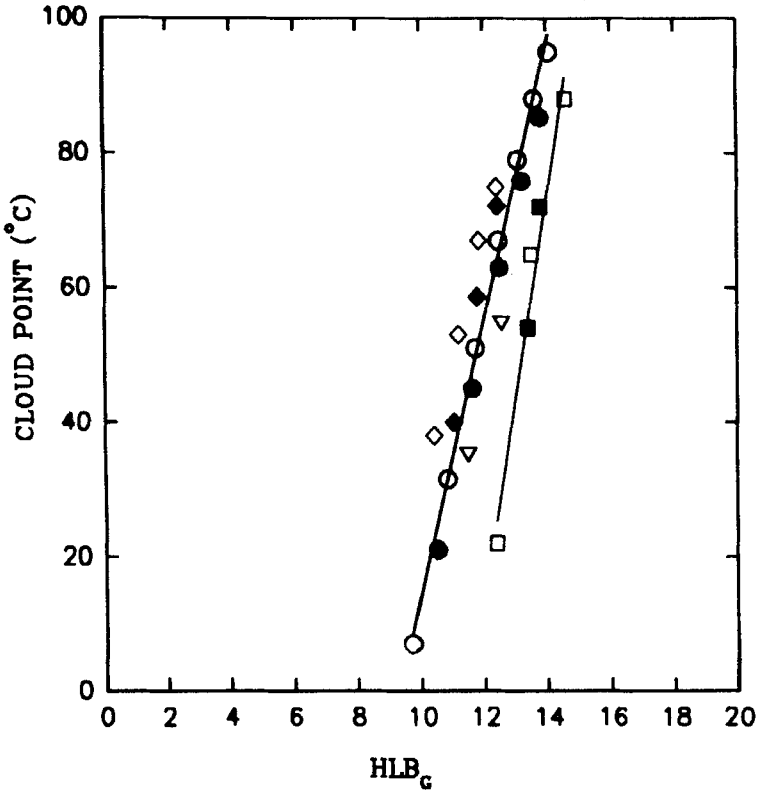

FIGURE 3. Relationship between the degree of hydrophobicity (HLB_G) and the cloud points of nonionic detergents of the *n*-alkyl (▽, $C_8E_{n=4-5}$, ●, $C_{10}E_{n=4-8}$, ○, $C_{12}E_{n=4-10}$, ◊, $C_{14}E_{n=6-8}$, ●, $C_{16}E_{n=6-9}$) and phenyl (□, Triton® X-series; ■, Triton® N-series) polyoxyethylene series. The HLB_G scale ranges from 0 to 20, most hydrophobic detergents being at the lower end of the scale (0 to 10) and most hydrophilic detergents at the upper end (14 to 20).

ture. This increased volume can only represent a greater water content.[69]

The presence of additives in the solution also affects the cloud point. Among them, electrolytes, hydrocarbons, detergents, phospholipids, and protein denaturants are the most studied.

A. Electrolytes

The effect of electrolytes on aqueous solutions of nonionic detergents has been extensively studied.[56,70–73] The cloud point is a sensitive parameter for *salting in* or *out*. Salts that raise the cloud point expand the region in phase diagrams where the isotropic micellar solutions are formed.[74] Therefore, increasing the cloud point is a *salting-in* process. The opposite effect, *salting out*, can also be obtained. Both effects are shown in a TX-114 solution when NaCl and NaSCN are used (Figure 4).[70] Increasing concentrations of NaCl result in lower cloud points, whereas increasing concentrations of NaSCN result in the contrary. These effects are related to the partial molar heat capacity of the additive.[70] Chlorides and sulfates decrease the heat capacity of a nonelectrolyte (detergent) in a water solution, whereas thiocyanate or iodine salts produce an increase in miscibility.[70]

NaCl salts are commonly used in biological buffers containing TX-114 because they decrease the cloud point[20,21] and the time needed for phase separation.[68] However, high salt concentrations should be avoided in order to prevent enzyme inhibition by the salts, particularly Cl^-, and to permit the use of ion-exchange chromatography in subsequent purification steps.[75]

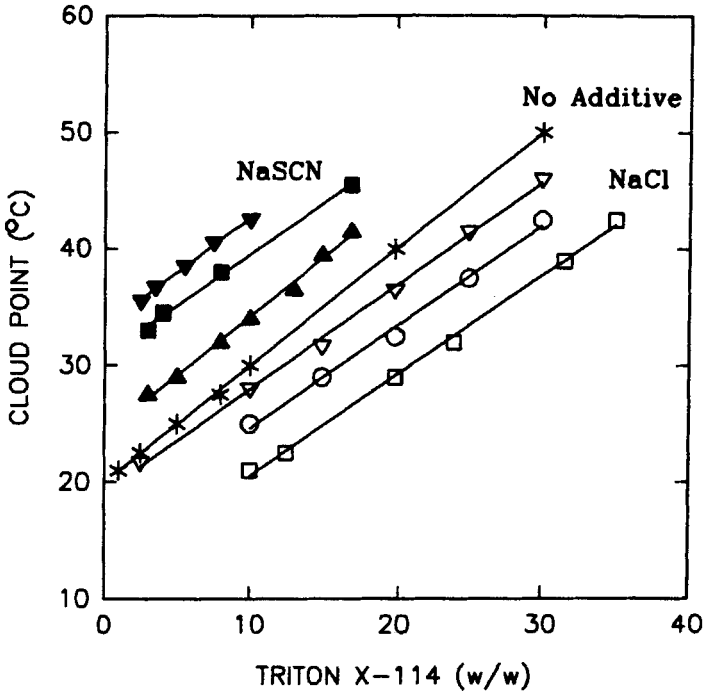

FIGURE 4. Composite phase diagram of Triton® X-114 without added electrolyte (*). Open symbols represent the addition of increasing amounts of NaCl, which decreases the cloud point of the detergent at any given molar fraction: ▽, 0.13 *m*; ○, 0.35 *m*; and □, 0.71 *m*. Filled symbols show the effect of the addition of an electrolyte (NaSCN), which raises the cloud point: (▲, 0.0123 *m*; ■, 0.0619 *m*; and ▼, 0.1245 *m*. (Adapted from Doren, A. and Goldfarb, J., *J. Colloid Interface Sci.*, 32, 67, 1970; Sepúlveda, L. and MacRitchie, F., *J. Colloid Interface Sci.*, 28, 19, 1968. With permission.)

B. Hydrocarbons

Saturated hydrocarbons do not generally depress the cloud point to any great extent and may even raise it, whereas aliphatic alcohols, fatty acids, or phenols decrease the cloud point quite substantially.[56] Among the alcohols, glycerol reduces TX-114 micelle solvation by decreasing the number of hydrogen bonds formed by the detergent's polyoxyethylene groups with water.[75] It has been used with TX-114 to isolate the plant cytochromes P-450 and b_5 by lowering the cloud point to 4°C.[75]

C. Other Detergents

The cloud point of a particular nonionic detergent, such as TX-114, is notably affected by the presence of another surfactant in the solution. In general, highly hydrophilic detergents (with HLB values higher than that of TX-114) increase the cloud point, whereas those with lower HLB values decrease it. The first type of behavior was described by Bordier[20] when using mixtures of TX-114 with Nodinet® P-40, Triton® X-100, *n*-octylglucoside, and sodium deoxycholate. The last is the most effective solubilizer of TX-114 due to its high hydrophilicity (HLB = 16), whereas Nodinet® P-40 (t-C_8 ϕE_9, HLB = 12.4) is the least efficient.

As for the second type of response, the addition of increasing amounts of a more hydrophobic detergent, such as Triton® X-45 (t-C_8 ϕE_5, HLB = 10.4), also lowers the TX-114 cloud point in a linear relationship:[69]

$$CP\ (°C) = 21.4 - 1.25\omega \qquad (3)$$

where ω represents the weight percentage of Triton® X-45. This formula was used to calculate the relative proportions of a TX-114/TX-45 mixture to partition at 9°C, which was used to purify phosphatidylinositol kinase.[69]

D. Membrane Phospholipids

The cloud point of TX-114 is also lowered by phospholipids released from biological membranes due to their high hydrophobicity.[18] The actual decrease depends on the lipidic nature of the membrane. In the case of plant microsomes, the temperature is lowered by 5°C.[75]

E. Protein Denaturants

The clouding phenomenon of nonionic detergents and protein denaturation show some similarities.[76] The former is a reversible precipitation produced by heating and the latter also leads to precipitation, which is caused by heating and may or may not be reversible. In the range of 0.1 to 0.5 *m*, the urea and thiourea derivates, acetamide and guanidinium, increased the cloud point in direct proportion to their concentration.[76] The extent of this effect increases with the degree of alkylation and the number or size of the alkyl substituent. Thiourea derivates raise the cloud point around 60% more than the corresponding urea derivates.[76] Only one urea derivate, phenyl thiourea, has the opposite effect, because it is probably located in the polyoxyethylene shell of the detergent molecules.

This effect of alkylureas and alkylthioureas in raising cloud point can be explained by their behavior as hydrotropes.[76] Such hydrotropes or hydrotropic solubilizing agents, while amphiphilic, are not surface active and do not form micelles because their hydrophilic moiety is not large enough.[77] However, they can increase the water solubility of poorly soluble compounds (such as nonionic surfactants above their cloud points) when added in relatively large amounts.[76,78,79]

IV. FRACTIONATION OF MEMBRANE PROTEINS BY DIFFERENTIAL SOLUBILIZATION AND TEMPERATURE-INDUCED PHASE PARTITIONING IN TRITON® X-114

In the fluid-mosaic model of membrane structures,[80,81] membrane proteins are classified as peripheral or integral, according to their capacity to overcome the thermodynamic constraints that maximize their hydrophobic or hydrophilic interactions.[23] Peripheral[80] (or extrinsic[82] or membrane-associated) proteins are dislodged from the membranes by relatively mild procedures[22] without dissociating the lipid matrix of the membrane. These mild procedures include the addition of chelating agents, lowering or increasing the ionic strength of the medium, or using alkaline pH (200 m*M* of sodium carbonate buffer).[83] Peripheral proteins are usually water soluble after extraction. They include proteins such as cytochrome *c*, which is known to have a tertiary structure similar to that of other water-soluble globular proteins with hydrophilic surfaces and hydrophobic interiors,[84] and with detergent-binding capacities of less than 1 to 3% of their own weight.[85] These proteins are presumably bound by predominantly polar interactions to the proteins or the lipids in the membrane.[22]

Conversely, integral[80] (or intrinsic[82] or membrane-bound) proteins are tightly bound to the membranes and require more drastic methods, such as treatment with detergents, bile acids, protein denaturants, or organic solvents,[86] before they can be solubilized from the membranes. Such membrane proteins are amphipathic molecules and have highly asymmetrical hydrophilic and hydrophobic domains in their structure. The hydrophilic parts of these proteins are exposed to the external aqueous environment, whereas the hydrophobic regions are sequestered in the lipid bilayer due to interactions with hydrophobic sequences of amino acids.[87] The most important factor that determines the amino acid composition of membrane-penetrating segments of integral proteins is the overall hydrophobicity of a particular segment.[88,89] A number of integral membrane proteins have been sequenced and found to contain long sequences of hydrophobic amino

acid residues (19 or more) spanning the bilayer.[23] These proteins can either traverse the bilayer once, as commonly occurs in cell-surface antigens, adhesion molecules, and growth factor receptors, or a number of times, as seems to be generally true in the case of transport proteins.[90]

Another mode of membrane attachment is by means of a lipid covalently linked to protein. This was established long ago for a bacterial coat protein that showed a lipid attached at a NH_2-terminal amino acid.[91] In addition, it was found that eukaryotic plasma membrane proteins with transmembrane sequences could also have covalently attached fatty acids (for a review, see Reference 92). However, the idea that a lipid alone might commonly constitute the hydrophobic group of eukaryotic cell-surface proteins was treated with skepticism until the 1980s. The results of studies concerning membrane-bound enzymes such as alkaline phosphatase, acetylcholinesterase, 5′-nucleotidase, the Thy-1 antigen of rodent thymocytes and neurones, and variant surface glycoproteins (VSG) of protozoa led to a change of mind. Over 60 cell-surface proteins have since been identified as having a glycosyl-phosphatidylinositol (G-PI) moiety covalently attached to C-terminal amino acid by means of ethanolamine (for reviews, see References 93 to 100). They can be solubilized by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC).[101] This class of phospholipid-bound membrane proteins is clearly different from other fatty acid-acylated integral proteins, in which the fatty acids are linked directly via hydroxyester,[102] amide,[102] or thioester bonds,[103] because they are still anchored to the membranes by one or more hydrophobic amino acid sequences (for a review, see Reference 92).

A. Solubilization

Nonionic detergents such as those of the Triton® or Brij series have been used extensively to solubilize a number of integral membrane proteins with no loss of biological activity.[22] In fact, Triton® X-114 has been used to solubilize several subcellular membranes, including mitochondria,[104–109] microsomes,[110–112] and vacuoles.[113] Its effectiveness was first demonstrated by Lehninger's group,[104] who used it in a comparative study with other PEG detergents. They found that the most effective detergents for the extraction of mitochondrial proteins were Triton® X-100 (about 70% of the total protein) and Triton® X-114 (65%). In addition, they found TX-114 to be the most effective detergent for phospholipid extraction (19%). These results were later confirmed with bovine heart mitochondrial porin, a pore-forming protein of the mitochondrial outer membrane.[107] Despite these promising results, Triton® X-114 has been used much less frequently than the homologous Triton® X-100 because it renders cloudy solutions at room temperature and has to be used in a cold room. However, this apparent inconvenience of TX-114 makes it a particularly useful detergent for membrane biochemistry in certain circumstances.

B. Differential Fractionation of Proteins

In 1981, Bordier[20] took advantage of the fact that TX-114 binds to integral membrane proteins, and that it forms a detergent-rich phase when warmed to temperatures above its cloud point, to develop a simple and rapid method for the separation of membrane proteins. His idea was to first solubilize a membrane preparation at 0°C, warm it to 30°C, and then recover the detergent-rich phase after low-speed centrifugation. Integral membrane proteins could be recovered from the detergent phase, while hydrophilic proteins remained in the aqueous phase. Bordier tested the efficiency of the phase separation with a mixture of hydrophilic proteins containing serum albumin, catalase, ovalbumin, concanavalin A, myoglobin, and cytochrome *c* (Figure 5). He recovered these proteins exclusively from the aqueous phase, which contained very little residual detergent (0.04% w/v or three to four times the cmc).[20]

On the other hand, well-characterized integral membrane proteins such as acetylcholinesterase from human erythrocyte membrane, bacteriorhodopsin from *Halobacterium halobium* purple membrane, and cytochrome *c* oxidase from yeast and from *Paracoccus denitrificans* showed strong depletion in the aqueous phase and were

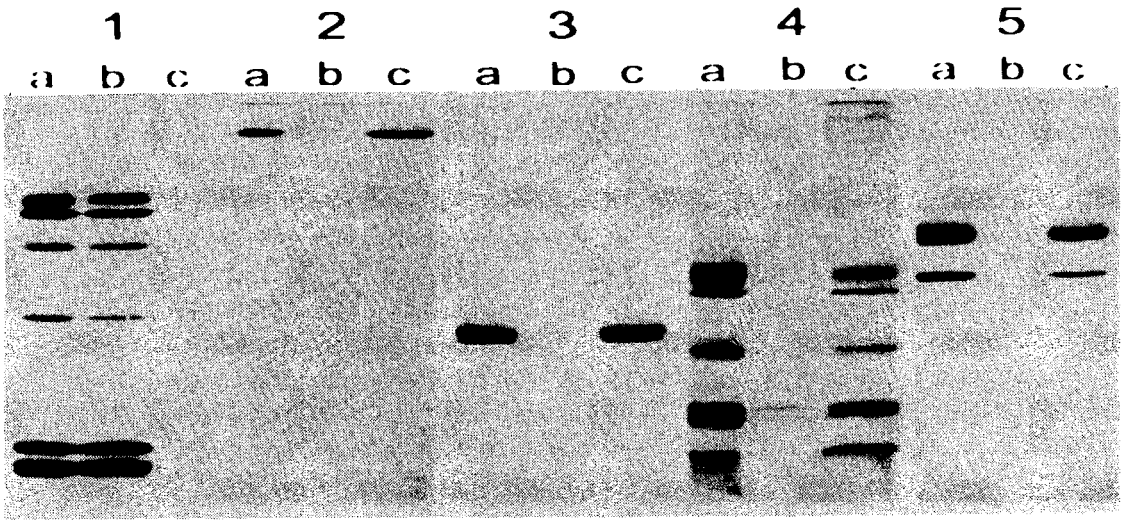

FIGURE 5. Phase separation of hydrophilic and amphiphilic proteins. Various purified hydrophilic and amphiphilic proteins were mixed with Triton® X-114 and submitted to phase separation at 30°C. Aliquots of the input sample and of the aqueous and detergent phases were analyzed by SDS-PAGE on 10 to 15% polyacrylamide gradient gels (sample 2 is unreduced; samples 4 and 5 were warmed up for 5 min at 56°C). The samples contained the following proteins: 1, serum albumin, catalase, ovalbumin, concanavalin A, myoglobin, cytochrome *c*; 2, human erythrocyte AChE; 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. (From Bordier, C., *J. Biol. Chem.*, 256, 1604, 1981. With permission.)

recovered in the detergent-rich phase after phase partitioning (Figure 5). In addition, Bordier extended these experiments to the erythrocyte membranes and demonstrated that following a temperature-induced phase separation, about 75 to 78% of integral membrane glycoproteins, such a Band 3 and PAS 1+2, could be recovered from the detergent phase, while the more hydrophilic proteins associated with the membrane separated into the aqueous phase. Bordier also showed how cellular and secreted proteins of mouse myeloma cells could be separated in Triton® X-114, the latter being recovered in the aqueous phase.

This technique was taken further when more complex membranes were studied, and it was possible to distinguish up to four different types of membrane protein when membrane proteins of adrenal medullary chromaffin granules were subjected to fractionation in TX-114.[21,114] A scheme of the phase separation protocol is presented in Figure 6. The first group of proteins is spontaneously precipitated when a cholesterol- and phospholipid-rich membrane, such as the chromaffin granule membrane, is solubilized with TX-114 at 0°C. The pellet obtained after centrifugation is referred to as the detergent-insoluble or phospholipid-rich phase, which is rich in highly hydrophobic proteins, such as dicyclohexylcarbodiimide-reactive protein, ATPase I, and glycoprotein IV. Warming the resultant solution, layered over a cushion of 0.25 *M* sucrose in buffer containing 0.06% TX-114 (w/v), leads to separation of another hydrophobic phase (the detergent-rich phase) containing the common integral membrane proteins of the chromaffin granules, dopamine β-hydroxylase (DBH), and cytochrome b_{561}. The resulting aqueous phase (0.04% TX-114) contains a mixture of soluble proteins (chromogranin A and soluble DBH) and membrane glycoproteins (glycoproteins III, H, J, K). On removal of the residual detergent after exhaustive dialysis at 4°C in a buffer containing 1% (w/v) Amberlite XAD-2 (Fluka), the glycoproteins precipitate through aggregation. This protocol was also used by the same authors[114] with other subcellular membranes, the Golgi-enriched microsomal frac-

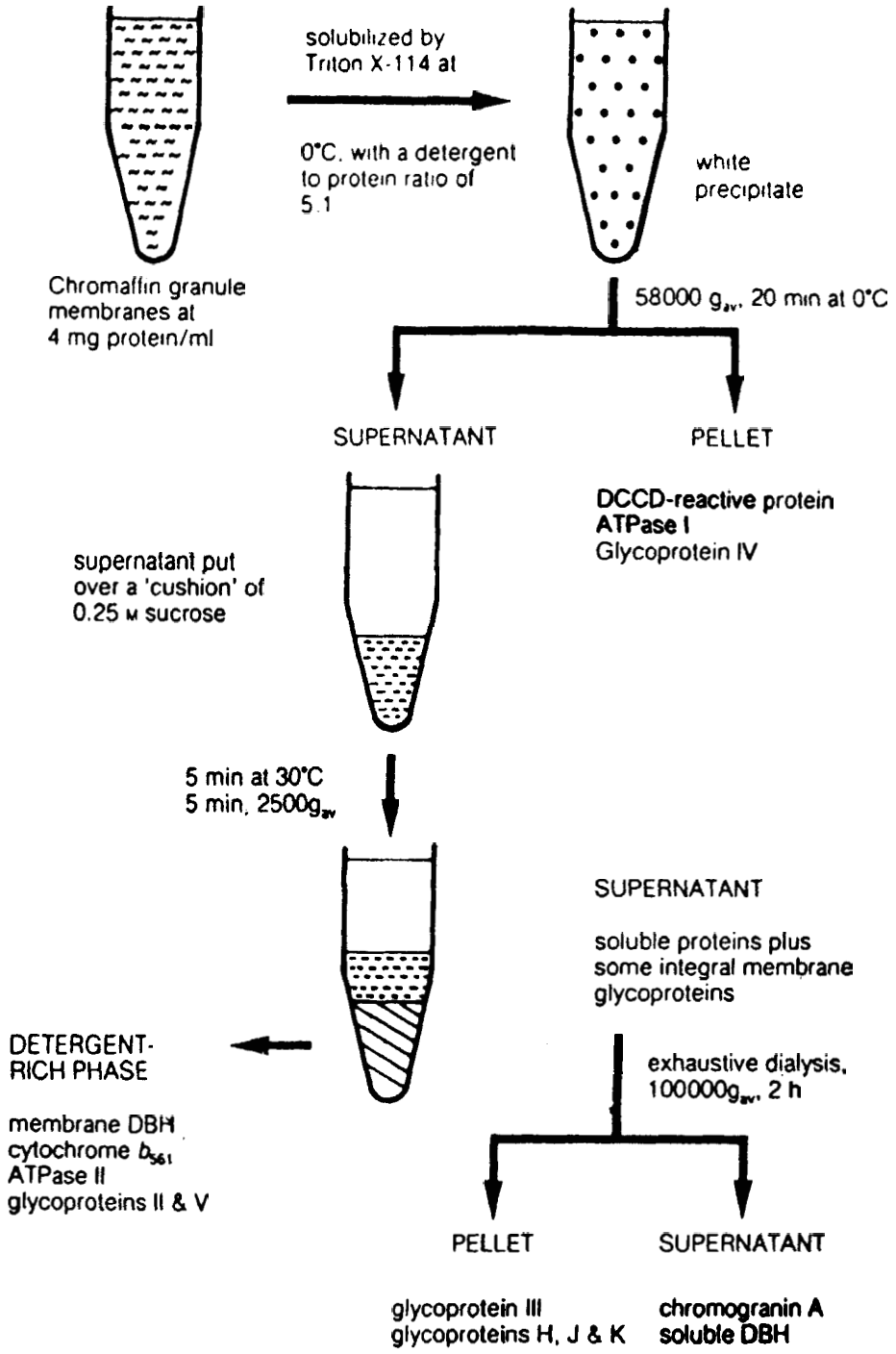

FIGURE 6. Fractionation of chromaffin granule membrane proteins by temperature-induced phase separation in Triton® X-114. DBH, dopamine-β-hydroxylase; DCCD, dicyclohexylcarbodiimide. (From Pryde, J. G., *Trends Biochem. Sic.*, 11, 160, 1986. With permission.)

tion, yielding three phases of different polypeptide composition similar to those obtained in chromaffin-granule membranes. In contrast, the preparations of less lipid-rich mitochondrial membranes and rough endoplasmic reticulum produced very little initial phospholipid-rich phase, although each yielded a distinct detergent and aqueous phase.[114]

A modification of Pryde and Phillips' phase separation method[114] was developed for the identification of two integral membrane proteins of the malaria parasite *Plasmodium falciparium*.[115] The changes made were in the volume of TX-114 used (only 0.5% in the first solubilization step) and in the incubation and mixing times in order to obtain maximal solubilization and purification of these two membrane proteins. However, the main difference from Pryde and Phillips' method[114] was the sequential repetition of the washing and centrifugation steps (generally three times) to obtain more specific detergent-insoluble, detergent-rich, and detergent-poor phases.[115]

Based on these two modifications of Bordier's phase separation method[114,115] and the earlier observation that detergents with low cmcs (e.g., TX-100, TX-114, and Nodinet P-40) are relatively ineffective at solubilizing proteins anchored by a glycosyl-phosphatidylinositol,[116] Hooper and Bashir[117] developed a technique of differential solubilization and temperature-induced phase separation in TX-114 to distinguish between those proteins anchored by a G-PI moiety and those anchored by a simple membrane-spanning polypeptide. When this method, which is depicted Figure 7, was applied to pig kidney microvillar membranes, which are abundant in both G-PI-anchored and polypeptide-anchored ectoenzymes, TX-114 at 0°C effectively solubilized those ectoenzymes possessing a polypeptide anchor, whereas the G-PI-anchored ectoenzymes were not solubilized and were sedimented by low-speed centrifugation and recovered in the detergent-insoluble pellet.[116]

The detergent-solubilized supernatant was then further fractionated by phase separation at 30°C into a detergent-rich phase, which contained the ectoenzymes with a polypeptide anchor, and an aqueous phase. When microvillar membranes were treated first with bacterial PI-PLC, the G-PI-anchored ectoenzymes (alkaline phosphatase and membrane dipeptidase) were recovered predominantly in the aqueous phase, which was consistent with the removal of the hydrophobic glycolipid anchor by PI-PLC. Similarly, after treatment of the membranes with either trypsin or papain, which cleave the hydrophobic polypeptide anchors from transmembrane-polypeptide-anchored ectoenzymes (angiotensin-converting enzyme and aminopeptidase N), these two ectoenzymes were recovered predominantly in the aqueous phase. Thus, Hooper and Bashir's method[117] effectively separates G-PI-anchored proteins without the lipid anchor having to be removed, but is not applicable to purified protein samples because it requires the proteins to be associated with the lipid bilayer for them to be sedimented during the first centrifugation.

All these modifications of Bordier's original method have contributed to the vast diffusion of this technique, consolidating it as a fundamental step in the study or isolation of membrane proteins. Tables 3 to 5 summarize the membranes and their corresponding proteins phase-fractionated in TX-114 in animal[20,68,69,86,117–177] and plant cells,[75,178–186] and in microorganisms,[115,187–218] respectively.

C. Changes in Protein Hydrophobicity

From all of these studies shown in Tables 3 to 5, a common conclusion is reached that the distribution of a polypeptide or polypeptide complex in the phases formed by TX-114 is not simply related to the protein's ability to bind detergent.[68] It appears, rather, that the distribution of a component reflects the overall hydrophilic-hydrophobic properties of its surface.

1. Interactions with Specific Ligands

This was first demonstrated by Alcaraz et al.,[68] who studied the distribution of the receptor for immunoglobulin E (IgE) and its subunits in the two TX-114 phases. The intact receptor, $\alpha\beta\gamma_2$, partitioned preferentially in the detergent-rich phase, whereas the complexation of the receptor with its ligand (IgE) separated in the aqueous upper phase. This change in hydrophobicity during phase separation in TX-114 has also been described in another receptor[126] and membrane-bound enzyme.[201] The receptor, human placenta insulin receptor $(\alpha\beta)_2$, changes from hydrophobic to hydrophilic following insulin binding.[126] These changes imply restricted access of the hydrophobic amino acids of the plasma-membrane domains exposed to Triton® X-114.[126] In the case of the

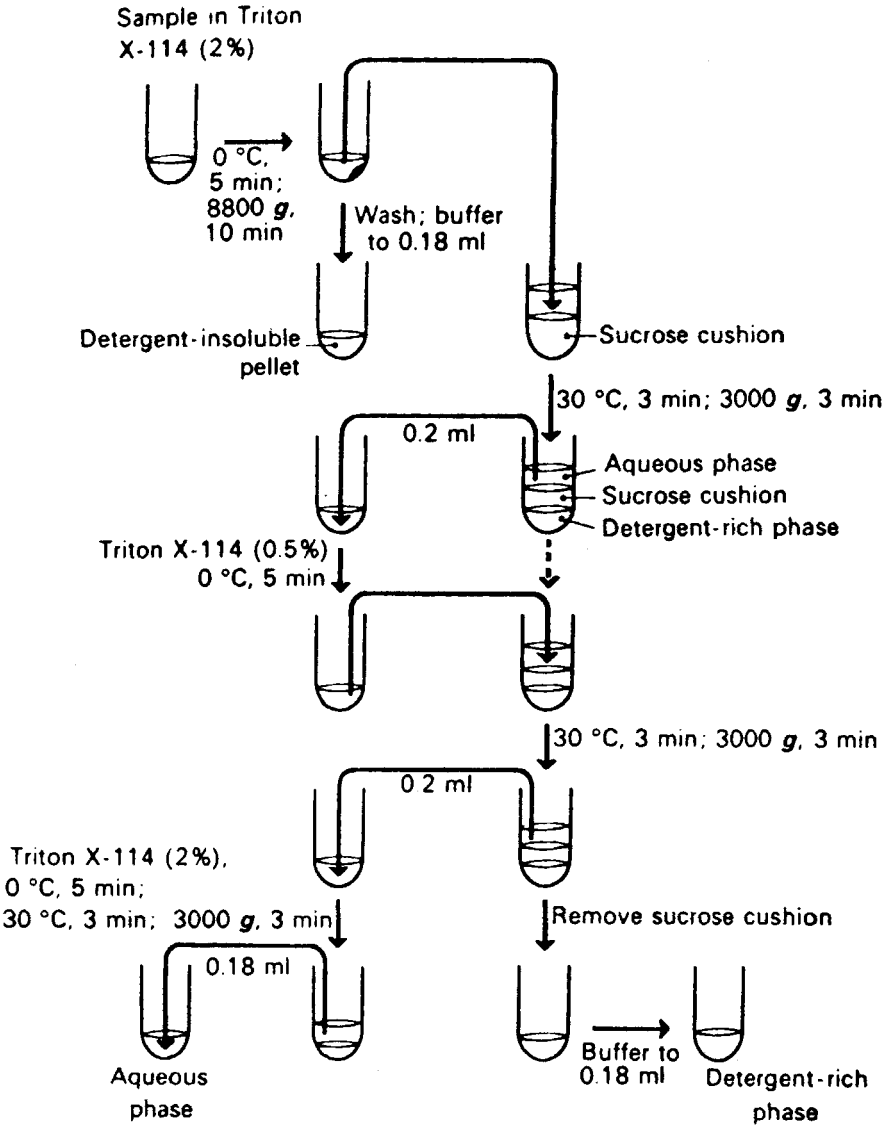

FIGURE 7. Differential solubilization and temperature-induced phase separation in Triton® X-114 of kidney microvillar glycosylphosphatidylinositol-anchored membrane proteins. (From Hooper, N. M. and Bashir, A., *Biochem. J.*, 280, 745, 1991. With permission.)

enzyme pyruvate oxidase from *Escherichia coli*, the wild-type form partitioned into the aqueous phase upon phase separation.[201] However, the presence of the substrate (pyruvate) and cofactor (thiamin pyrophosphate) causes the conformational change that exposes the lipid binding site of the enzyme and induces a substantial partitioning of the complex into the detergent-rich phase.[201]

In addition to interaction between a protein (enzyme or receptor) and its specific ligand, two more factors bring about changes in the partitioning of membrane proteins: the specific conformational changes induced by other proteins (mainly specific enzymes) and changes in the pH. In the first case, the removal of the G-PI anchor by specific phospholipases[93,117,219,220] or the proteolytic removal of the hydrophobic peptide[177] are the most common enzyme-mediated changes in the hydrophobic-hydrophilic balance of membrane-bound proteins. Also, specific phosphorylation of

TABLE 3
Protein Phase-Fractionationated in Triton® X-114 in Animals

Membrane/cell	Protein(s)	Aqueous phase	Detergent phase	Insoluble phase	Ref.
Plasma Membrane					
Epithelial cells					
Epithelial cell lines from different species and tissues	Carcinoembryonic antigen		++++		118
	CD44		++++		119
	Extracellular matrix receptor III		++++		
	150- and 180-kDa TGF-β1 binding proteins		++++		120
	TGF-β1/β2-binding proteins	++++			121
	94-kDa cell surface protein		++++		122
Human HeLa cells	Urokinase-type plasminogen activator, 55–60 kDa		++++		123, 124
	Collagen receptors				
	87 KDa		++++ (93%)		
	102 KDa			++++ (100%)	125
	38 KDa			++++ (92%)	
Human placenta	Insulin receptor $(\alpha\beta)_2$		++++ (95%)		
	$(\alpha\beta)_2$ + insulin	+++ (83%)			
	Insulin receptor (αβ)	++	++ (67%)		126
	αβ + insulin	++	++ (42%)		
Human squamous carcinoma cell line	Palmitate-labeled proteins		++++		127
	Myristate-labeled proteins	++++			
3T3 mouse fibroblast	$pp60^{c\text{-}src}$ (membrane-bound tyrosine kinase)		++++		128
	$pp60^{c\text{-}src}$ + phosphorylation	++++			
Chicken intestinal brush border membranes	Microvillus 110-kDa cytoskeletal protein		++++		129
Rat intestinal brush border membranes	Disaccharidases (glucoamylase, sucrose-isomaltase, trehalase, lactase)	++++			
	Intrinsic factor-cobalamin (IF-cbl)	++++			
	Alkaline phosphatase		++++		130
	Ca^{2+}-Mg^{2+}ATPase		++++		
	Leucine aminopeptidase		++++		
	γ-Glutamyl transpeptidase		++++		

TABLE 3 (continued)
Protein Phase-Fractionationated in Triton® X-114 in Animals

Membrane/cell	Protein(s)	Aqueous phase	Detergent phase	Insoluble phase	Ref.
Renal brush border membrane	Trehalase		++++ (>80%)		
	γ-Glutamyl transpeptidase		+++ (>80%)		
	Leucine aminopeptidase		+++ (>80%)		131
	Maltase	++++			
	5′-Nucleotidase	+++	+		
	Alkaline phosphatase	+++	+		
Pig kidney microvillar membranes	Aminoacylase I	++++			
	Alkaline phosphatase		++++		132
	Endopeptidase-24.11		++++		
	Renal dipeptidase		++++		133
	Alkaline phosphatase			++++ (92%)	
	Dipeptidyl peptidase IV		++ (61%)	++ (32%)	117
	Aminopeptidase W		+++		134
Dog kidney glomeruli and proximal tubules, plasma membranes	Neutroendopeptidase 24.11 from				
	Glomeruli tubules		++++ (93%)		135
	Proximal tubules		++++ (94%)		
Madin-Darby canine kidney cells	175-kDa tyrosine-o-sulfate (TyrS)-binding protein	++++			136
Scallop gill cilla plasma membrane	22-kDa protein		++++		137
Immune Cells					
B lymphocytes	Membrane immunoglobulin heavy chain (μm)		++++		138
Human T lymphocyte	Phosphatases				
	type 1_C	++++			
	type 2A	++++			139
	type 2B (calcinearin)	++ (50%)	++ (50%)		
Murine T lymphoma cells	GP200 oncofetal antigen		++++		140
Murine lymphoblasts	Mitogen receptor		++++		141
Human cytotoxic T lymphocytes	Cell surface antigen T8	++++			142
Mast cell	Immunoglobulin E receptor ($\alpha\beta\gamma_2$)				
	($\alpha\beta\gamma_2$) + IgE	+++	+		
	α + IgE	+++	+		68
	$\alpha\beta\gamma_2$	+	+++		
	α	+	+++		
	β,γ		++++		

TABLE 3 (continued)
Protein Phase-Fractionationated in Triton® X-114 in Animals

Membrane/cell	Protein(s)	Aqueous phase	Detergent phase	Insoluble phase	Ref.
Human neutrophils	Myeloperoxidase α-band	++++ (95%)			
	Vitamin B_{12}-binding protein β-band	++++ (90%)			
	FAD β-band	++++ (86%)			
	FAD γ-band	+++ (72%)			143
	FAD S_2 band	++++ (95%)			
	Alkaline phosphatase γ-band		+++ (79%)		
	b-Cytochrome β-band		++++ (92%)		
	NADH-b_5 reductase γ-band		++++ (85%)		
Thymocytes	Thy-1		++++		144, 145
Human platelets	Glycoproteins IIb and IIIa		++++		146
	Ib, $GP_{17}^{5.8-6.5}$	++++			
	IIb, IIIa,IIIb,V		++++		147
	GPIIb, III, IV, VI, VII, VIII, GP IIb β-subunit[38']		++++		
	GPIa, Ib, IIb, III, V, GPIx	++++			148
	Phosphoproteins				
	P20		++++		
	P27	++	++		147
	P47	++++			
Neural Cells					
Rabbit central (CNS) and peripheral (PNS) nervous systems	Wolfgram protein (WP)		++++		
	Proteolipid protein (PLP)		++++		
	Myelin-associated protein (MAG)		++++		149
	P_0		++++		
	P_2	++++			
	Myelin basic protein (MBP)		++++ (>80%)		
Peripheral rat nerve	Major glycoprotein P_0 (29 kDa)	++++			86
Bovine brain	Membrane-bound annexin V isoforms (CaBP33 and CaBP37)		++ (50–60%)		150
Pig brain	22-kDa neuran-specific surface antigen		++++		151, 152
Rat brain	5'-Nucleotidase		++++		153

TABLE 3 (continued)
Protein Phase-Fractionationated in Triton® X-114 in Animals

Membrane/cell	Protein(s)	Aqueous phase	Detergent phase	Insoluble phase	Ref.
Rat brain	Rat brain rolipram-sensitive cAMP-specific type IV phosphodiesterase (RD1)	++ (50–56%)	++ (50–46%)		154
	met[26]-RD1	++++ (97%)			
	Voltage-sensitive sodium chanels				
	α-Subunit	++++			155
	β1, β2 Subunits		++++		
	L-Glutamate binding sites	+ (14%)	+++ (68%)		156
Chicken brain	Neural cell surface protein, 135-kDa		++++		157
Cholinergic NS-204 cell line neuronal plasma membranes	Choline-*o*-acetyl transferase (ChAT)				
	Membrane bound	+	+++		158
	Free hydrophilic form	+++	+		
Pig oligodendrocytes	Myelin basic protein (MBP)	++++			149
Bee head neural membranes	Acetylcholinesterase				
	5S/6S form		++++		159
	7.1 form	+ (5%)			
Musca domestica neural membranes	Endopeptidase 24.11-like protein		++++		160
Drosophila melanogaster plasma membranes	Choline-*o*-acetyl transferase (ChAT)				
	Hydrophilic	+++	+		161
	Membrane bound	+++	+		
Torpedo electric organ	Acetylcholine receptor	++++			162
	Choline-*o*-acetyl transferase (ChAT)				
	Hydrophilic	++++			
	Membrane bound		++ (40%)		163–165
	Detergent-insoluble			++ (40%)	
Olfactory cilia	Glycoprotein gp 95		++++		
	Glycoprotein gp 58	++++			166
	Glycoprotein gp 120	++++			
Erythrocytes					
Human erythrocytes	Band 3	+	+++ (74%)		20
	Acetylcholinesterase		+	+++	
	Human	(4%)	(26%)	(70%)	
	Pig	(5%)	+ (16%)	+++ (78%)	
Hepatocytes					
Fetal liver plasma membrane	42- to 43-kDa non classical, non-HLA A, B soluble class I protein	++++			173
Oocytes					
Xenopus oocytes	5′-Nucleotidase		++++		
	Alkaline phosphodiesterase		++++		174

TABLE 3 (continued)
Protein Phase-Fractionated in Triton® X-114 in Animals

Membrane/cell	Protein(s)	Aqueous phase	Detergent phase	Insoluble phase	Ref.
Spermatocytes					
Human sperm	Ion chanel protein		++++		175
	Ram sperm surface antigen (ESA 152 antigen)		++++		176
	Angiotensin-converting enzyme (ACE)				
	Free form	++++			177
	Membrane bound		++++		
Subcellular Membranes					
Microsomal Membranes					
Microsomal bovine brain membranes	Phosphatidylinositol kinase		+++ (84%)		69
Mitochondrial Membranes					
Rat liver outer mitochondrial membrane	Outer membrane cyt *b* (OM *b*), 23 kDa		++++		167
Secretory Granules					
Bovine adrenal medulla chromaffin granule membranes	Low molecular weight GTP binding protein (Gn)		++++		168
	Phosphatidylinositol kinase		++++		169
	Chromogranin A	++++			
	Soluble DBH	++++			
	Glycoproteins III, H, J, K	++++			
	Membrane bound-DBH		++++		
	Cytochrome b_{561}		++++		114
	ATPase II		++++		
	Glycoprotein II, V		++++		
	DCCD-reactive protein			++++	
	ATPase I			++++	
	Glycoprotein IV			++++	
	p36 (the large subunit of calpactin I)	++++			170
Pancreatic zymogen granule membrane	GP-2 membrane-bound (PI anchored)		++++		171
	free GP-2	++++			172

certain amino acid residues leads to changes in hydrophobicity, as in the case of the pp60$^{c\text{-}src}$ membrane-associated tyrosine kinase,[128] which is translocated from the plasma membrane to the cytosol by phosphorylation in both tyrosine and serine/threonine residues.[128]

2. pH-Induced Changes

Regarding changes in the hydrophobicity induced by pH, Madshus et al.[221] used TX-114 to follow the pH-induced transfer of poliovirus type I into their target cells by following the ability of the virus to enter the detergent phase. The virus remained in the water phase at neutral pH, whereas at pH values below 5, the amount of labeled virus present in the Triton® X-114 phase increased abruptly, and at pH 3 most of the labeled virus was found in the detergent phase. This indicates that the virus exposed its hydrophobic domains at low pH. This dependence of the virus on pH to enter the

TABLE 4
Protein Phase-Fractionated in Triton® X-114 in Plants

Membrane	Protein(s)	Aqueous phase	Detergent phase	Ref.
Jerusalem artichoke tuber microsomal membranes	Cytochrome P-450		++++	
	Cytochrome b_5		++++	75, 178
	NADPH cyt C reductase		++++	
Maize thylakoid membranes	$CF_1\alpha$	++++		
	$CF_1\beta$	++++		
	$CF_1\gamma$	++++		
	32, 25, 24, and 14 kDa	++++		
	Apo CP_1		++++	179
	49- and 45-kDa PSII		++++	
	Cyt *f*		++++	
	Major LHCP		++++	
	Cyt b_6		++++	
Spinach chloroplast membranes	5-kDa protein associated with phosphosystem II	++++		180
	54-kDa polypeptide		++++	181, 182
	Protochlorophyllide oxidoreductase	+	+++	183
	CF_1	+	+++	
Spinach leaf plasma membranes	52, 49, 33, 29, 25, and 17 kDa		++++	184
	41 kDa	++++		
Spinach leaf mitochondria membranes	Glycine decarboxylase	++++		185
	125-kDa protein		++++	
Pea leaf chloroplast	64-kDa marker protein for intramembrane space between the outer and inner chloroplast envelope	++++		186

TX-114 phase was also described for the non-enveloped DNA-containing adenovirus (Ad_2)[222] and influenza virus.[223] Among the three major external proteins of Ad_2[222] (hexon, penton base, and fiber), penton base had the highest association with TX-114 at pH 5.0. In the influenza virus,[223] the large N-terminal domain of the glycopeptide HA2 from the hemagglutinin spike glycoprotein undergoes the pH-induced conformational changes that produce membrane fusion and partition into the TX-114 phase at pH 5.0.

It is interesting to note that other proteins associated with membrane fusion (clathrin),[224] secretory granules (carboxypeptidase E),[225] and toxins (*Pseudomonas aeruginosa* exotoxin A,[226–228] diphtheria toxin,[226] and bactericidal colicin E3)[229] show a pH-induced translocation into the TX-114 phase at acidic pH. Figure 8 shows the partitioning of clathrin in Triton® X-114 at different pHs. This protein was found in the aqueous phase at neutral pH, in both the aqueous and detergent phases at about pH 5 to 6, and solely in the detergent phase below

TABLE 5
Protein Phase-Fractionated in Triton® X-114 in Microorganisms

Membrane/microorganism	Protein(s)	Aqueous phase	Detergent phase	Ref.
Archaebacterium				
Methanococcus voltae	31- and 33-kDa flagelling	++		187
	Wall protein (RS)	++		
Bacteria				
Treponema pallidum outer membrane	190 kDa (4D ordered ring)	++++		190
	47-kDa surface antigen		++++	190-193
	15-kDa major membrane immunogen (lipid anchored)		++++	194, 195
	34-kDa surface antigen (lipid anchored)			196, 197
Borrelia burgdorfen	OspA, OspB membrane antigens		++++	198, 199
Leptospira interrogans	Lipopolysaccharide-like substance		++++	
	41 kDa		++++	200
	44 kDa		++++	
Escherichia coli	Pyruvate oxidase (pox)			
	Pox (wild type)	++		
	Pox (wt) + Pyr (subst.)		++	
	Pox B6 (mutant without the last 24 aa of the C terminal)	++		201
	Pox B6 + Pyr	++		
	Pox B4 + Pyr + TPP-Mg^{2+}		++++	202
Paracoccus denitrificans	Membrane-bound hydrogenase		++++	203
Microplasma hyorhinis	p23 surface antigen		++++	204
Yeast				
Yeast inner mitochondrial membrane	Membrane-bound cAMP-binding protein		++++	205
Protozoa				
Eimeria tenella	Anti-CRD (cross-reacting determinant)-binding protein		++++	206

TABLE 5 (continued)
Protein Phase-Fractionated in Triton® X-114 in Microorganisms

Membrane/microorganism	Protein(s)	Aqueous phase	Detergent phase	Ref.
Plasmodium falciparium	45-kDa antigen		++++	115
	55-kDa antigen		++++	
Leishmania sp. promastigotes	Major surface protein, p63		++++	207–209
Trypanosoma brucei gambiense	Gp 44p		++++	
	Gp 57p		++++	210
	Mf VSG (membrane form of variant surface glycoprotein)		++++	
	Mf VSG		++++	211, 214
T. cruzi	Ssp-4, a stage-specific surface antigen		++++	215
Trypanosoma sp. (M238)	88- and 70-kDa proteins		++++	
T. vespertolionis	96, 77, 60 kDa		++++	216
T. dionissii	84, 72, 60 kDa		++++	
Tetrahymena thermophila ciliary membrane	Dynein	++++		
	Detergent-soluble α- and β-tubulins	++++		217
	58-, 50-, and 49-kDa proteins		++++	
	ATPase		++++	
Virus				
Semiliki Forest virus	Transmembrane glycoprotein p62/E2	++	++	218
	Capside	++++		

pH 5.0.[224] This membrane translocation induced at low pH is probably a general mechanism for the vectorial attachment and release of proteins in biological membranes.[225] TX-114 phase-partitioning is an ideal technique for studying such a translocation.

Anomalous phase partitioning of proteins in TX-114 has been reported since this technique began to be used,[162] as some well-known integral proteins partition in the aqueous phase instead of in the detergent-rich phase.[121,136,142,155,162] The reason for this anomalous behavior is frequently dependent on the presence of a high content of sugars in the integral glycoproteins,[21] which confers an overall hydrophilic nature on the protein in the presence of small amounts of TX-114 (0.06%). When these trace amounts of TX-114 are removed, these glycoproteins become insoluble after centrifugation.[21,136]

A special example of anomalous behavior in TX-114 is that displayed by pore-forming ion-conducting proteins, such as the nicotinic acetylcholine receptor (AcChoR), the large α-subunit of the sodium channel from the rat brain (α-ssc), and the α-chain of the Na^+,K^+-ATPase of kidney microsome membranes and Ca^{2+}-ATPase of sarcoplasmic reticulum membranes.[155,162] The explanation for this anomalous behavior is still not clear. In the case of AcChoR,[162] it seems to be related to the fact that the irregularly shaped hydrophobic surfaces of the receptor find it difficult to intercalate in the TX-114 lamellar struc-

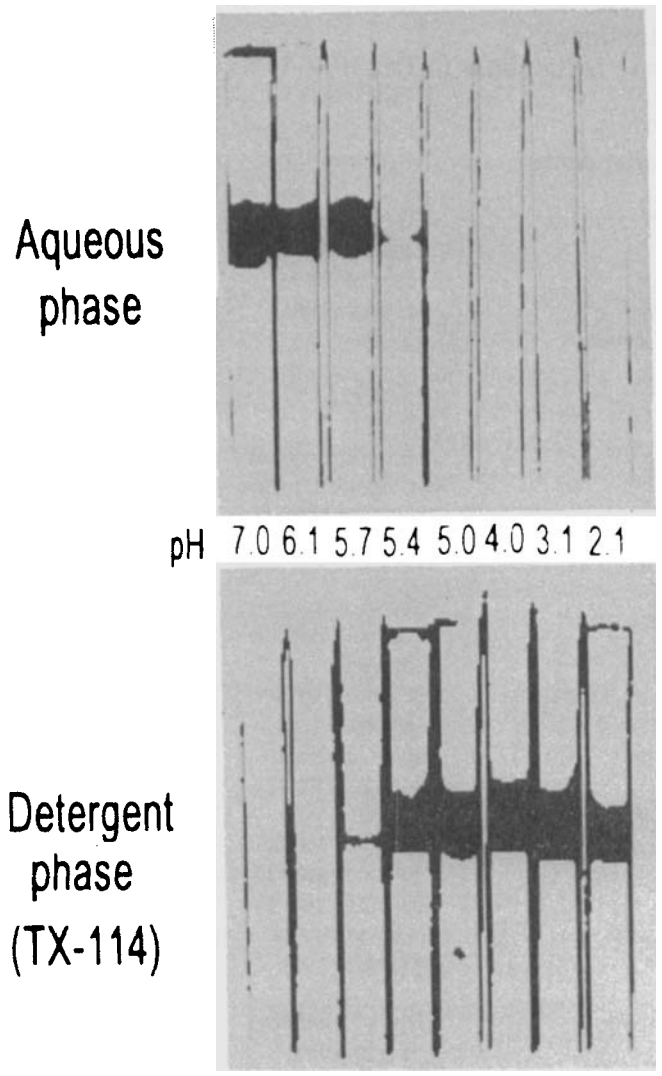

FIGURE 8. Partitioning of clathrin in Triton® X-114 solution. Clathrin (100 μg) was incubated at 0°C for 30 min in buffer solutions of various pH values, containing 1% (w/v) Triton® X-114, and then at 30°C for 10 min. The mixtures were centrifuged at low speed, and the resulting aqueous and detergent phases were analyzed by SDS-PAGE. (From Maezawa, S. et al., *Biochemistry,* 28, 1422, 1989. With permission.)

tures formed in the detergent phase, unless the Triton-organized structures are made more flexible by the inclusion of linoleic acid. Under these conditions of mixed micelles of Triton® X-114 and linoleic acid, the receptor can partition in the detergent-rich phase. However, although this explanation may be valid for AcChoR, it cannot be applied generally because α-ssc does not partition in the TX-114 phase, even in the presence of linoleic acid.[155]

E. Unresolved Proteins

Finally in this section, mention should be made of two interesting membrane protein fractionations carried out with other PEG detergents when TX-114 failed to resolve the wanted proteins. The first example was the fractionation of different lymphocyte surface antigens by ammonium sulfate-induced phase separation in Triton® X-100.[230] These surface antigens failed to parti-

tion into the TX-114 detergent-rich phase at 30°C, although they could be selectively brought into the detergent-rich phase of TX-100 by increasing the amount of ammonium sulfate in the solution. All six cell-surface molecules appeared in the detergent-rich phase when 50% saturated ammonium sulfate was used, whereas with 33% saturation, Ly-2/3 and Ly-24.2 remained in the aqueous phase, Ly-9.1 and Ly-15.2 partially partitioned, and L3T4 and the heparin lectin totally partitioned into the detergent-rich phase.[230]

The second example was the resolution of *Rodocyclus gelatinosus* photoreceptor unit components.[231] Phase separation in TX-114 occurred only at 28 to 30°C, inducing a pigment degradation and a partial loss of activity in the reaction center. To solve this problem, Agalidis and Reiss-Husson[231] used a mixture of $C_{10}E_4$ and octyl-β-D-thioglucopyranoside (OTG) to obtain phase separation at 20°C. The system separated into three phases: a pellet composed of pure B875 antenna, a detergent-rich layer containing cytochrome *c* and other proteins, and an aqueous phase containing crude reaction centers. The partition of these reaction centers in the aqueous phase was due to the $C_{10}E_4$ detergent being mixed with OTG because they partitioned exclusively (as is to be expected from their known hydrophobicity) in the lower, detergent-rich phase of pure $C_{10}E_4$.[231]

V. BULK FRACTIONATION METHODS USING TRITON® X-114

In this section we describe the important contributions of the TX-114 phase separation technique to obtain all or most of the protein from a tissue extract or homogenate, or from a bacterial extract or growth medium, in a bulk form that can serve as the starting material for fine chromatographic fractionation.

A. Animal Tissues

The first report of a bulk fractionation method using TX-114 was by Bjerrum et al.[232] in 1985 using human erythrocyte acetylcholinesterase (AChE). This membrane enzyme was extracted directly from the whole blood so as to eliminate the time-consuming procedure of isolating the membranes. However, due to the high density of lysed blood, it was impossible to pellet the TX-114 phase unless 5% (w/v) sucrose was added. After centrifugation, this rendered a floating TX-114-rich phase. Subsequent washes with buffer and three reextractions of the first infranatant gave a 55-fold purification and 11% yield. The yields are low because losses are sustained in the repeated phase condensations to remove as much hemoglobin as possible.[232] After TX-114 reextractions and phase separation, Cu^{2+} chelating chromatography, dialysis, and concentration to a small volume (3 ml compared with the initial blood volume of 1000 ml), a 650-fold purification can be achieved. Thus, 0.2 mg of human erythrocyte membrane AChE was isolated per 1 l of blood, with a specific activity of 135 U/mg, the major contaminants being glycophorin and hemoglobin.

B. Microorganisms

The above results were the basis for the development of bulk fractionation methods with microorganisms. In 1986, Payne and Trumpower[233,234] developed a simple one-step method for purifying cytochrome *b* from the bc_1 complex (cyt c_1 + cyt *b* + Fe-S protein) of the bacteria *Paracoccus denitrificans* and *Rhodopseudomonas sphaeroides*, using a protocol identical to that described by Bordier.[20] After phase separation with TX-114, cytochrome c_1 and Fe-S protein partition into the aqueous phase, and cytochrome *b* partitions in a pure form in the detergent phase. In a similar way, the peripheral membrane flavoprotein pyruvate oxidase from *E. coli* was purified with TX-114 after preliminary heat fractionation.[235] The only difference from Bordier's method[20] was the 10 n*M* sodium phosphate buffer (pH 6.0) used instead of 10 m*M* Tris-HCl (pH 7.4) and the fact that pyruvate oxidase was converted to its reduced form before partitioning. The lower detergent phase was finally centrifuged at 100,000 g (4°C) for 7 h, rendering a light-yellow pellet. This method renders a 13.6-fold purification and a yield of 52%. The purification results were similar to those obtained with the previously developed DEAE-Sephadex pro-

cedure, but with great saving of time (only 1 d as opposed to 3 weeks).[235]

Other examples of membrane enzymes bulk fractionated in TX-114 are the membrane-bound hydrogenase of *Alcaligenes eutrophus* H16[236] and cholesterol oxidase.[237] In addition, extracellular lipases from a cell-free culture broth of *Pseudomonas cepacia* (DSM 50181)[238] were directly purified 24-fold in only one step into the detergent-rich phase of $C_{14}E_6$ at 35°C. All the above detergent-based aqueous two-phase systems used with microorganisms are very simple, and a scale-up of processes should not be problematic.[238]

C. Plants

TX-114 has became an important alternative to the classic purification procedures, such as ammonium sulfate fractionation, and especially to the drastic use of acetone powders.[239] These classic procedures are used with plant extracts because of the large variety, and frequently large quantity, of secondary products, particularly (poly)phenols and tannins,[240] that bind tightly to the enzymes and change their characteristics. A second problem in plant enzyme purification arises when the enzyme is bound to the chloroplast membrane, because the release of the chlorophylls after detergent treatment precludes the use of colorimetric methods to monitor enzyme activity. Moreover, the extraction method should be very mild when the enzyme is present in a latent state,[241] to prevent its activation or modification, as occurs when acetone powders and ammonium sulfate fractionation are used.[242]

TX-114 was first used to avoid all the above problems in the purification of grape berry polyphenoloxidase (GB-PPO)[243] based on the previous work carried out by Bordier[20] and the fact that TX-114 increases the solubility of banana PPO together with PEG 20000.[244] When TX-114 was used to solubilize the thylakoid-bound GB-PPO, it solubilized the proteins and chlorophylls with the same efficiency as TX-100. However, unlike TX-100, TX-114 failed to maintain all of the proteins and chlorophylls in solution at 4°C. This was used to advantage because after a few minutes, a dark precipitate was formed due to the aggregation of large mixed micelles of TX-114, which contained membrane proteins, phospholipids, phenols, and chlorophylls. After high-speed centrifugation, the supernatant was slightly green, clearly indicating that chlorophylls and phenols had been eliminated from the original extract.[243]

This removal of chlorophylls and phenols by TX-114 at 4°C had never before been described. The total removal of unwanted compounds was achieved by subjecting the clear green supernatant to a classic temperature-induced phase partitioning[20] and adding an additional 4% TX-114 to the mixture and warming it to 37°C for 15 min.[243] After this phase partition, GB-PPO remained in the aqueous phase and as an inactive (latent) form similar to that previously described for other leaf thylakoid-bound PPO (spinach, broad bean).[241]

Table 6 shows the results obtained when the TX-114 method was compared with the well-established methods for purifying GB-PPO involving ammonium sulfate fractionation.[243] The degree of purification was the same in both methods, although the recovery was greater in the TX-114 method. The enzyme purified by TX-114 was latent, and was activated 64 times with trypsin compared with the two times it could be activated with the ammonium sulfate fractionation method. As much as 75 to 80% of the phenols and chlorophylls were removed by TX-114 by ultracentrifugation without the need of any synthetic resin such as Amberlite XAD-2 or organic solvents. Complete removal was attained in the next step by temperature-induced phase partitioning in 4% TX-114.

TX-114 also avoids one of the problems normally encountered when plant PPO extracts are subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), namely, the multiplicity of activity bands arising from the formation of artefactual bands caused by the covalent interaction of *o*-quinones and phenols with the enzyme.[241]

The TX-114 method was also used to purify the aforementioned latent leaf thylakoid-bound PPO.[241] In the case of spinach[245] and broad bean leaves,[246] 1.5% TX-114 partially removed the undesirable phenol and chlorophyll components by centrifugation at 4°C, when osmotically shocked chloroplast membranes were digested by the detergent. The remaining chlorophylls and phenols were removed after phase partitioning at 37°C in 8% TX-114.

TABLE 6
Partial Purification of Grape Berry Polyphenoloxidase Using Both Ammonium Sulfate and Triton® X-114 Methods

	Total protein (mg)	Total activity[a] (units) – Trypsin	Total activity[a] (units) + Trypsin	Specific activity (units/mg)	Purification (-fold)	Recovery (%)	Activation (%)	Chl (μg/ml)	Phenolic compounds (mg/ml)
Ammonium Sulfate Method									
Crude extract	15	500	500	33	1	100	100	11	42.5
TX-100 extract	7.8	30	370	47	1.5	74	1233	16	14.5
Supernatant of TX-100 extract	5.7	5.7	324	57	1.7	65	5684	13	12.1
45–95% ammonium sulfate	0.6	50	101	168	5.1	20	200	ND[b]	0.5
Triton® X-114 Method									
Crude extract	15	500	500	33	1	100	100	11	42.2
TX-114 extract	7.8	10	270	47	1.4	74	3700	19	14.5
Supernatant of TX-114 extract	4.3	5.5	322	75	2.3	65	5854	4	3.5
Supernatant 4% of TX-114	1.9	5.0	320	168	5.1	64	6400	ND	0.6

[a] Assayed with 4-methyl catechol as substrate.
[b] ND, not detected.

From Sánchez-Ferrer, A., Bru, R., and García-Carmona, F., *Plant Physiol.*, 91, 1481, 1989. With permission.

Latent PPO remained in the aqueous phase, in both cases with fivefold purification and a recovery of 86 and 43%, for spinach and broad bean PPO, respectively.[245,246] The activation found in both latent enzymes was five to ten times higher than that obtained in previously published methods (acetone powders and ammonium sulfate fractionation),[247] clearly demonstrating the "gentle" purification of the TX-114 method compared with the classic methods.

The final example of a leaf thylakoid-bound enzyme being purified by the TX-114 method is that of potato leaf PPO, which previously had never been purified. The method used differs from the above TX-114 methods in that the second phase partitioning step must be repeated because the level of phenols is higher than in the other leaves studied. This additional step is necessary because at high phenol concentrations, the phenol-binding sites in the TX-114 micelles are saturated.[248]

Recently, TX-114 has been used with nonaerial tissues such as tubers and seeds. The purification of potato tuber PPO is completely different from that of the leaves because the level of phenols, most as chlorogenic acid, is so high that the extract becomes black only seconds after homogenization. To avoid this, ascorbic acid, metabisulfite, and insoluble phenol scavengers were used at high concentrations in previously published methods.[249,250] However, all of these are inhibitors of potato PPO, and so the new strategy was to keep phenols away from the enzyme by adding 6% TX-114 to the homogenization buffer without any other phenol scavenger and to insolubilize the starch by keeping the homogenate at 4°C for 90 min.[251,252] The extract remained light yellow during this time due to the presence of TX-114. After centrifugation, the starch was removed and the supernatant subjected to phase partitioning by increasing the TX-114 concentration by another 4% and warming it for 15 min. The PPO remaining in the supernatant was not latent and was further purified by ammonium sulfate fractionation, leading to a fivefold purification and an almost complete removal of phenols, only 3% remaining.[251] This prevented browning of the enzyme solution and enabled enzyme stability to be preserved for months at –20°C. This removal of phenols also avoids the multiplicity of enzyme bands found in PAGE for potato PPO (up to 18 bands) when it is purified with ammonium sulfate alone, without TX-114.[253]

In addition, another tuber plant enzyme, cinnamic acid 4-hydrolase from Jerusalem artichoke tuber microsomes, was purified in the presence of 2% (w/v) TX-114 and 30% glycerol from the detergent-rich phase.[178] Two phases formed spon-

taneously during centrifugation at 4°C; the upper was the detergent-rich phase and contained 80 to 90% of the spectrophotometrically detectable cytochrome P450.[178]

Finally, a 128-kDa basic cystine-rich seed protein involved in the defense mechanism of plants against predators was isolated from wheat endosperm by TX-114 phase partitioning in 4% (w/v) solution.[254] The protein was recovered from the detergent-rich phase.

D. Patents

Finally, two patented applications of the TX-114 bulk fractionation method must be mentioned. The first corresponds to a 170-kDa membrane-bound protease purified from malignant melanoma cells lines and which is used as a diagnostic marker. The enzyme is absent from the corresponding normal cell types.[255] The second is the obtention of 5-lipoxygenase from potato tuber, which is found in the upper aqueous phase after phase separation and which can be fully purified to homogeneity in only 1 d compared with the 3 d used in the standard purification method. The interest in this enzyme resides in its analogy with human 5-LOX involved in leukotriene synthesis for pharmaceutical and medical uses.[256]

VI. TRITON® X-114 IN BIOCHEMICAL METHODS

The phase separation of low-molecular-weight biomolecules in certain research areas such as analytical biotechnology, public health, or the study of environmental pollutants is an obvious use of TX-114 phase extraction prior to high-performance liquid chromatography (HPLC) analysis, although there are two important disadvantages:[257] a high background absorbance during ultraviolet light (UV) detection and the lengthy operating time (up to several hours) required for the total elution of surfactant. A simple way to overcome these drawbacks is to use electrochemical detection[257] because TX-114 does not contain any functional group that is susceptible to electrodic reactions. When UV is used, probably for economic reasons, mobile phases with high methanol or acetonitrile concentrations should be used for complete elution of the detergent. In fact, with vitamin E, either UV or electrochemical detection can be used in a methanol/water mobile phase (99:1 v/v) because its peak is eluted after more than 10 min (the time required for the elution of surfactant and baseline recovery). Prior condensation of samples by TX-114 phase partitioning enhances the detection of vitamin E by a factor of 30, with 100% recovery.[257]

Nonspecific turbidity of pork meat was also effectively removed by TX-114 phase partitioning.[258] In this method, TX-114 acted both as a solubilizing and a clarifying agent. After phase separation, a clear supernatant containing all the meat pigments was found. Meat pigments were then determined by their extinction coefficient at 490 nm, as in the commonly used methods. The TX-114 method also reduces by one half the time necessary for the assay and minimizes the equipment needed because it is suitable for routine analysis in nonscanning photometers.[258]

In protein analysis, TX-114 has been used for the removal of endotoxins,[259] for quantitative receptor assays,[260] and for the clinical determination of tumor markers.[148,255,261] The endotoxin contamination of protein solutions was reduced 1000-fold by only one cycle of phase partitioning in contaminated preparations of cytochrome *c*, catalase, and albumin.[259] The endotoxins could be completely removed by further cycles of phase separation, each cycle resulting in only a 2% loss of protein and being completed in less than 15 min. The major disadvantage of the procedure was the small amount of detergent (0.018%) present in the aqueous phase, because it could prove harmful to living cells and difficult to remove without loss or dilution of the desired proteins.[259]

In addition, TX-114 can be used to assay cell surface-binding proteins for tissue-type and urokinase-type plasminogen activators (t-PA and u-PA).[260] After incubation of integral membrane proteins with radiolabeled ligand, a solution of TX-114 was added at 4°C and phase separated at 37°C. The radiolabeled ligand bound to membrane proteins was recovered in the detergent-rich phase.[260] The amount of radiolabeled t-PA or

u-PA recovered in the detergent-rich phase was proportional to the protein concentration of the solubilized membranes. This procedure was also used to estimate the dissociation constant of the binding of ligand to solubilized binding proteins, giving results similar to those reported previously.[260] For the clinical determination of normal and pathological cells, several methods have been developed that depend on determining the presence or absence of certain specific proteins in the detergent-rich or -poor phases.[148,255,261]

TX-114 was also used to improve two previously described methods. Nitrocellulose was made transparent by simply impregnating the filters used with concentrated TX-114.[262,263] Within a few seconds, protein blots became transparent and could be photographed by transillumination or scanned on a densitometer. TX-114 is more effective than xylene in bringing about the complete transparency of the membranes, it produces a fast destaining of Amido Black-stained blots without significant loss of proteins, and retains the immunoreactivity of protein blots even after several cycles.[262]

The other method improved by using TX-114 is the visualization of membrane proteins in the detergent-rich phase by electron microscopy.[264–268] Insulin receptor[264] and Band-3 protein[265] erythrocyte membrane have been visualized by freeze fracturing after purified samples were reinserted into TX-114 lamellar membranes in a situation similar to that found in biological membranes. The size of both protein particles is comparable with their size in biological membranes.[265]

VII. CONCLUSIONS

Detergent-based aqueous two-phase systems induced by temperature have become an obligatory step in the isolation and fractionation of membrane proteins since the first report on the differential phase distribution of integral and peripheral membrane proteins by Bordier in 1981. Although the method is widely applicable, some exceptions have been described that throw light on the overall transfer mechanism. Taken as a whole, the available data confirm that it is the overall surface hydrophobicity and the globular or irregular shape of the protein that are the determining factors in the transfer of the protein to a detergent-depleted phase or a detergent-rich phase rather than the simple binding of the detergent to the protein. Bearing this in mind, the partition of macromolecules can be manipulated to enable the protein to partition in the desired phase by changing the environment of the protein with salts, alcohols, other detergents, and even by introducing specific hydrophobic ligands to concentrate the protein in the detergent phase. In addition, many alternatives presumably remain to be found.

One possible strategy might be the design of new biological detergents. These new detergents should fulfill the following requirements: they should be as transparent as possible in the UV region and easily removed by dialysis, they should possess some functional groups to which specific substrate analogs or ligands can be attached, and it should be possible to select an appropriate cloud point. Interdisciplinary collaboration between biochemists and synthetic organic chemists to develop these new detergents will be necessary.

In conclusion, TX-114 temperature-induced phase partitioning is a powerful, simple, rapid, and reproducible technique with which to undertake studies on membrane biochemistry. In addition, it is easily scalable from micro- to macropurification processes and from research project to practical student classes.[269–271] Hopefully, the examples and suggestions described in this review will stimulate and help other biochemists and molecular biologists to take full advantage of the potential of these detergent-based aqueous two-phase systems.

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