The Size and Detergent Binding of Membrane Proteins*

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Sucrose density gradient centrifugation has been used to measure the binding of Triton X-100 above its critical micellar concentration to a variety of purified membrane and non-membrane proteins. In addition, binding studies were done on three proteins below the critical micellar concentration of detergent to distinguish between the interaction of proteins with detergent monomers and detergent micelles. A procedure is described for the calculation of the molecular weight of these Triton X-100 protein complexes and measurements were made for opsin, plasma low density lipoprotein, the (Na+ + K+) dependent adenosine triphosphatase, the human red blood cell major sialoglycoprotein (PAS-1) and the human red blood cell minor glycoprotein (band III). These proteins behave as monomers or dimers in detergent and bind between 0.28 and 1.12 g of detergent per g of protein. A general method is also presented for calculating the molecular size and shape of impure membrane proteins in detergent. Finally, Triton X-100 was shown to replace bound Na dodecyl-SO₄ on the minor glycoprotein of the red cell.

Triton X-100 is an effective, non-denaturing detergent which has been used widely to solubilize membrane proteins. Since Triton X-100 does not cause gross changes of protein structure (2), I decided to use this detergent to solubilize and then to determine the size, shape, and subunit composition of several membrane proteins and enzymes which are under extensive investigation at this time. The proteins include the major and minor glycoproteins of human erythrocytes, the (Na+ + K+) dependent adenosine triphosphatase from dog kidney, cattle osin, and human low density lipoprotein.

To obtain these data, I devised a procedure to calculate the molecular weights and the partial specific volumes of detergent-protein complexes by sucrose gradient centrifugation and gel filtration. In addition, I also measured directly the amount of detergent bound to each protein. The results indicate that membrane proteins in Triton X-100 bind up to their own weight in detergent and may have limited subunit contacts. Furthermore, membrane proteins can either bind individual molecules of detergent or they can interact with detergent micelles. Both types of interaction are demonstrated here.

MATERIALS AND METHODS

Detergent Preparations—Triton X-100 (polyoxyethylene octylphenol with an average of 9.6 ethylene oxide units) was a product of Rohm and Haas, Philadelphia, obtained from Sigma (Lot 000C 3130). Ring labeled [H]Triton X-100 (0.276 mCi/g on January 1, 1972) was a gift of Dr. William Lyman of Rohm and Haas. The concentration

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† For a more complete chemical description of Triton X-100 see Ref. 2.
Horse spleen ferritin (five times crystallized), ovalbumin (twice crystallized), and sperm whale myoglobin were Mann Research prepa-
ations; bovine serum albumin (five times crystallized) was obtained from Miles Laboratories.

Human CO hemoglobin was prepared from freshly drawn blood by S.L. White (Harvard University) while rabbit muscle myoglobin was a gift of C. Guidotti (Harvard University). Human carboxy anhydrase B and Aretica hemoglobin were obtained from L. Wexman (Harvard University). A crude preparation of Homarus hemocyanin was obtained from detritinated lobster blood, and apoferritin was prepared by sodium dithionite reduction of ferritin followed by dialysis against sodium acetate, pH 5 (9). Low density lipoprotein was prepared from a 1.06 to 1.063 g/ml density fraction of human plasma, a gift of Dr. S. Goldin (Harvard University), Children’s Hospital Medical Center, Boston. This material was centrifuged through 1.025 g/ml of KBr, and then floated on 1.055 g/ml of KBr to give a preparation consisting of a single polypeptide chain with an approximate molecular weight of 300,000 as determined by polyacrylamide gel electrophoresis in Na dodecyl S0,.

Preparation of Membrane Fractions—Purified Ca*+ adenosine tri-

phosphatase of rabbit muscle was a gift of D.H. MacLennan (Uni-

versity of Toronto). A membrane fraction of the walking leg muscle of Cancer was prepared by K. Swedner (Harvard University). Step IV microsomes from canine renal medulla containing the two peptidases of the (Na* + K*)-dependent adenosine triphosphatase prepared by the method of Kyte (10) were a gift of S. Goldin (Harvard University). The inner membrane/matrix fraction of rat liver mitochondria was prepared by the method of Schmitz and Greenwalt (11), while membranes of sarcoplasmic reticulum were prepared from skeletal muscle using the procedure of MacLennan (12).

Frog rod outer segment membranes were prepared in dim red light from dark-adapted Rana pipiens. Retinas were homogenized in 10% (w/v) sucrose by passage through a 3-inch 17-gauge needle, overlying with 0.5 mM sodium phosphate, pH 7.4, and centrifuged at 20,000 rpm for 30 min. The membranes at the interface were washed twice in 5 mM sodium phosphate, pH 7.4, and collected each time by centrifugation at 38,000 rpm for 60 min. By Na dodecyl-S0, gel electrophoresis, it was estimated that rhodopsin made up 80 to 85% of the protein of this preparation. Cattle retinal rod outer segments were prepared by a similar method except that no care was taken to keep the preparation in the dark. Frozen retinas (Hormel) were homogenized in 40% sucrose, 30 mM Tris-H,S0, pH 7.5, with several strokes of an all glass pestle, overlain with the 10 mM Tris buffer, and centrifuged as with the frog material. The interfacial membranes were resuspended in the 40% sucrose buffer, overlain with sucrose-free buffer and centrifuged before. Membranes were then collected by centrifugation with two washings in this buffer.

Human erythrocyte ghosts were prepared by M. K. Ho, P. Royer, and L. Wexman of this laboratory by the method of Dodge et al. (13).

Preparations of Purified Membrane Proteins in Triton X-100—The major sialoglycoprotein of the human red cell membrane was obtained by affinity chromatography on Sepharose-bound concanavalin A. The preparation contained only the PAS-I component described by Fairbanks et al. (15). Purified minor glycoprotein from human erythrocytes (band III) was obtained by affinity chromatography on concanavalin A-Sepharose (14).

Detergent Binding Assay by Sucrose Gradient Centrifugation—Samples were prepared from crystalline proteins in 1 to 2% Triton X-100, 0.1 mM Na SO,, 0.01 M Tris-H,S0, pH 7.5. Membrane preparations were washed in the detergent-free buffer above, collected by centrifugation, and then resuspended in the buffer containing 2 to 5% detergent. The amount of detergent was always in at least a 20-fold weight excess over the protein. Preparations were allowed to equilibrate for 1 hour at 4°. Immediately before sucrose gradient centrifugation, membranes were centrifuged in a Beckman 40 rotor at 4° for 15 min per cm of radial path length in the sample to remove any undissociated membrane fragments. Sucrose gradients 5 to 20% (w/v) were layered on top of the gradients and centrifuged in a SW 50L rotor of a Beckman L3-50 or L2-50 HV ultracentrifuge at a speed of 48,000 rpm at 4°. After the run, 22 to 28 6-drop fractions were collected from the bottom of the tube. Alternatively, 3.8-ml gradients were prepared in polyallomer tubes (10.9 x 54.7 mm) for the SB 405 rotor of a IEC B60 ultracentrifuge. These gradients were run at 90,000 rpm.

Aliquots of the fractions from the sucrose gradients were counted for radioactivity and assayed for protein as described. The radioactivity, over the base-line of the plateau region, for a given fraction was divided by the specific activity of the labeled detergent and the amount of protein in the same sample volume. The values calculated for each fraction of the peak were then averaged to give the amount of Triton X-100 bound per mg of protein.

Enzymatic Assays—Enzyme assays, except for urease and ribonu-
clease, were performed at room temperature on a Cary 15 spectropho-
tometer, generally utilizing the 0 to 1 slide wire. Rates were calculated from the initial portion of the A versus time curve. Unless otherwise noted, substrates were buffered in 40 mM Tris-H,S0, pH 7.5. Catalase was assayed by hydrogen peroxide consumption at 240 nm (11). 10 mM (pH 7.5), containing 0.01% Triton X-100, 0.05% [3H]Triton X-100, 0.1 M Na SO,, 0.01 M Tris-H,S0, pH 7.5. Protein samples (100 to 250 l) were layered on top of the gradients and centrifuged in a SW 50L rotor of a Beckman L3-50 or L2-50 HV ultracentrifuge at a speed of 48,000 rpm at 4°. After the run, 22 to 28 6-drop fractions were collected from the bottom of the tube. Alternatively, 3.8-ml gradients were prepared in polyallomer tubes (10.9 x 54.7 mm) for the SB 405 rotor of a IEC B60 ultracentrifuge. These gradients were run at 90,000 rpm.

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Stark (18). Samples were made up in the borate buffer containing 1% Na dodecyl-S04 and 30 mM β-mercaptoethanol, and were heated for 3 min at 100° to minimize proteolysis (19). The concentration of Na dodecyl-S04 was always kept at least 10 times as high (on a weight basis) as Triton X-100. Gels were stained routinely with Coomassie brilliant blue R (Sigma) and scanned at 560 nm in a Böhr linear transport attachment for a Zeiss PMQ II spectrophotometer (20). The relative amount of stain in each peak was determined by multiplying the width at half-maximum absorbance (peak minus baseline) by the net absorbance. The red cell major sialoglycoprotein, which stains poorly through the use of D2O. This method gives an explicit formulation of both solvents is known.

The complete Svedberg equations for a protein-Triton complex in H2O and D2O are given by:

$$\frac{D_{H}}{D_{D}} = \frac{m_{H}}{m_{D}} (1 + \alpha) \quad [3]$$

$$\frac{\eta_{H}}{\eta_{D}} = \frac{\eta_{P}}{\eta_{D}} (1 + \beta) \quad [4]$$

Where subscripts H and D refer to values measured in H2O and D2O. M is the molecular weight of the complex, s is the measured sedimentation coefficient in solution of density ρ, and viscosity µ, and α and β are the partial specific volume and Stokes radius of the complex, and k is the ratio of the molecular weight of the complex due to deuterium alone. The last item, k, has a maximum value of 1.0155 and in general can be neglected.

The molecular weight and the partial specific volumes of the Triton-protein complexes can be resolved into two terms, one for the protein and one for the bound detergent.

$$\frac{m_{H}}{m_{D}} = \frac{m_{H} - m_{D}}{m_{D}} \quad [5]$$

$$\frac{\eta_{H}}{\eta_{D}} = \frac{\eta_{P} - \eta_{D}}{\eta_{D}} \quad [6]$$

Here α and β denote the grams of detergent bound per g of protein in H2O and D2O, respectively. $m_{H}$ is the molecular weight of the non-detergent portion of the complex and $m_{D}$ is the molecular weight of the detergent portion of the complex. A value of 0.94 cm3/g (24) was used for $\eta_{P}$ of Triton X-100 in these calculations, but other appropriate values may be used for other detergents.

When these values are substituted into the Svedberg equations, the ratio of Equation 1 to Equation 2 is:

$$\frac{D_{H}}{D_{D}} \frac{\eta_{H}}{\eta_{D}} = \frac{1 + \beta}{1 + \alpha} \frac{\eta_{P}}{\eta_{D}} \frac{\eta_{H}}{\eta_{D}} \frac{\eta_{P}}{\eta_{D}} \frac{\eta_{H}}{\eta_{D}}$$

If α and β have similar values, then the Stokes radii will vary with the cube root of the difference and αβ can be set equal to αβ. It is now possible to solve Equation 7 for $\eta_{P}$ and then use this value in Equation 4 to solve for the partial specific volume of the protein-Triton complex in H2O.

$$\frac{D_{H}}{D_{D}} \frac{\eta_{H}}{\eta_{D}} = \frac{1 + \beta}{1 + \alpha} \left( \frac{\eta_{P}}{\eta_{D}} \frac{\eta_{H}}{\eta_{D}} \frac{\eta_{P}}{\eta_{D}} \frac{\eta_{H}}{\eta_{D}} \right)$$

The value of $\eta_{P}$ can be obtained from the measured sedimentation coefficients together with knowledge of the densities and viscosities of the two solvents, and the amount of detergent bound by the protein in both solvents. The latter data are obtained by the method described above. Equation 8 can be used directly for sedimentation coefficients determined in the analytical ultracentrifuge with uniform solutions. However, it was determined that the method used for sedimentation coefficients determined in the analytical ultracentrifuge with uniform solutions.

Determination of Partial Specific Volumes of Membrane Protein

Triton X-100 Complexes—The method used here was developed to determine $\phi$ for detergent protein complexes by analyzing sedimentation coefficients in density gradient ultracentrifugation, in solvents made in H2O and D2O after the experimental procedure of Meunier et al. (22). The principle of the method has been described by Edelstein and Schachman (23) and is based on the change in sedimentation coefficient brought about by increasing the density of the solution by the method of Glossmann and Neville (21) and then scanned at 560 nm as above. The molecular weights of the polypeptides were determined by comparison with the heavy chain of myosin (220,000), β-galactosidase (135,000), bovine serum albumin (68,000), catalase (60,000), ovalbumin (43,500), carbonic anhydrase (30,000), and cytochrome c (11,500).

Sedimentation coefficients calculated in this way are used directly in Equation 8. The density of the medium is a linear function of the sucrose concentration and can be determined from a linear interpolation of the measured densities of the 5% and 20% sucrose buffers. Since the sedimentation coefficient is calculated for $r = r_{avg}$, the density of the medium at $r_{avg}$ must be calculated from:

$$\frac{D_{H}}{D_{D}} \frac{\eta_{H}}{\eta_{D}}$$

$\frac{D_{H}}{D_{D}} \frac{\eta_{H}}{\eta_{D}}$ is the molecular weight of the complex, $s$ is the measured sedimentation coefficient of the complex, $s_{20,w}$ is the sedimentation coefficient of the complex, $T$ is the temperature at which the experiment was performed, and $T_{avg}$ is the temperature at which the experiment was performed.

The complete Svedberg equations for a protein-Triton complex in H2O and D2O are given by:

$$s_{20,w} = s_{20,w}(1 - \rho_{s} \eta_{20,w} / \rho_{avg})$$

$\rho_{s}$ is the density of the medium at $r_{avg}$, and $\rho_{20,w}$ is the density of the medium at $r_{avg}$.
that the protein binds the same amount of detergent in H₂O and D₂O. Setting α = β in Equation 5, one obtains:

\[ v = \frac{F_H - 1}{F_D - F_H} - \frac{F_H}{F_D} \]  \hspace{1cm} (14)

This equation is especially useful in calculating \( s_{z0.0} \) and \( s_0 \) for impure proteins, for which only the enzymatic activity can be measured on sucrose gradients, because it does not require knowledge of the amount of detergent bound to the protein.

RESULTS AND DISCUSSION

Triton X-100 Binding to Purified Proteins and Membrane Extracts above cmc—Fig. 2 shows the results of sucrose density gradient centrifugation experiments done to measure the amount of [3H]Triton X-100 bound to three proteins. The principle of the method is the centrifugal separation of protein-detergent complexes from detergent micelles. The top panel illustrates the results obtained with hemoglobin where there is no peak of Triton X-100 corresponding to that of the protein. An upper limit for the amount of Triton bound can be established by assuming that there is a peak of radioactivity of the same magnitude as the scatter of the data. If hemoglobin bound 0.01 mg of Triton X-100 per mg of protein, a peak of 400 cpm (shown by the vertical bar in the figure) would be expected. Since the deviation of the plateau region is less than 400 cpm, the amount of Triton bound by hemoglobin must be less than this amount. Based on an average molecular weight of Triton X-100 of 636 (2), each hemoglobin chain then binds less than 0.25 molecule of detergent.

The center panel in Fig. 2 shows the binding of detergent to the purified minor glycoprotein of the human erythrocyte membrane (component a of Bretschet (35), or band III of Fairbanks et al. (15)) which is thought to be the red cell anion transport protein (36, 37). Here there is a peak of radioactivity coincident with that of the protein, which corresponds to 0.8 mg of Triton X-100 bound per mg of protein.

The lower panel of Fig. 2 shows the binding of detergent to a sample of solubilized (Na⁺ + K⁺)-dependent adenosine triphosphatase containing the same amount of protein as the minor glycoprotein. The binding determined in this experiment is 0.33 mg of Triton per mg of protein, and is a practical lower limit for binding that can be measured with this amount of protein.

Table I is a summary of Triton X-100 binding to various proteins at a free detergent concentration of 0.05%, determined by sucrose density gradient centrifugation as in Fig. 2. The data indicate that there are three classes of proteins: those that do not interact with Triton X-100, those that interact at a few specific sites, and those that interact at a large number of sites. The first category is illustrated by the typical water-soluble proteins, all of which bind less than 0.005 to 0.02 mg of Triton X-100 per mg of protein (corresponding to less than 1 mol of detergent per 30,000 to 120,000 g of protein). The only example of a protein in the second category is serum albumin, for which the measured binding at a few sites (2.5 per molecule) is thought to reflect the known hydrophobic binding sites on this molecule (39). Finally, a significant number of proteins interact with large amounts of Triton X-100. These proteins are all associated with large amounts of lipid in vivo, and all of them but low density lipoprotein are attached tightly to membranes. The amount of binding ranges from that for the Ca²⁺ and the (Na⁺ + K⁺)-dependent adenosine triphosphatases at 0.2 to 0.3 mg of detergent per mg of protein to that for a set of proteins that bind nearly their own weight in detergent. These results are consistent with data indicating that membrane proteins bind Triton X-100 (3, 40–42) and “soluble” proteins do not (2, 40).

Table I also gives data for the binding of detergent to several Triton X-100 extracts of membranes containing more than one protein. The values given reflect the average binding to a number of polypeptides of similar sedimentation coefficients but do not show the binding to components that migrate slowly on the sucrose gradients.³

The data in Table I show that the amount of detergent

³In fact, when an extract of red cell membranes is centrifuged, only binding of detergent to the minor glycoprotein is seen; binding to the major sialoglycoprotein is obscured.
It is of course necessary to exclude detergent protein interactions of the type where a small hydrophobic polypeptide anchor links the protein to a detergent micelle. In the present study, this has been done for three of these proteins (see below) by measuring detergent binding below the critical micelle concentrations, where the interactions are between protein and detergent monomers.

**Triton X-100 Binding below cmc Measurement of Tightness and Type of Binding—**All of the measurements in Table I were made above the critical micelle concentration of Triton X-100 and presumably represent maximum levels of binding. It was of interest to know how tight the interaction of detergent was with the binding sites, and so detergent-protein complexes were sedimented through gradients containing variable levels of Triton X-100 below the cmc of 0.016% (3, 4). It was important in these experiments to use complexes that were free of lipid because reassocation of protein and lipid might occur when the detergent concentration was lowered. Both the red cell major sialoglycoprotein and the minor glycoprotein (band III) were obtained in lipid-free states after purification, while the preparation of low density lipoprotein was delipidated by exchange with a large excess of Triton X-100, followed by sedimentation of the detergent-apoprotein complex away from the lipid detergent mixed micelles in a preparative sucrose gradient containing 0.05% Triton X-100.

Fig. 3 shows the binding curve determined for bovine serum albumin by sucrose density gradient centrifugation. The value of the dissociation constant from these data is 80 \( \mu M \), which is comparable to that of 50 \( \mu M \) obtained by Makino et al. by other methods (2). Fig. 4 shows a similar binding curve for lipid-free low density lipoprotein. Here, the data are best fit by a dissociation constant of 180 \( \mu M \). Although these data do not eliminate the possibility that there may be several classes of binding sites with varied affinities, they indicate that the proteins interact with monomers of Triton X-100, presumably at individual binding sites.

A different picture emerged when the purified red cell major sialoglycoprotein (PAS-1) in 0.1% Triton X-100 was centrifuged through gradients containing from 0.001 to 0.025% Triton X-100 in experiments similar to those described in Figs. 3 and 4. At the highest concentration, the measured binding was identical with that previously measured in 0.05% detergent. However, when the protein was centrifuged through gradients containing submicellar detergent (0.01% and below), it was impossible to measure the binding because all of the protein aggregated and pelleted to the bottom of the centrifuge tube. At detergent levels just below the cmc the protein was in an aggregated state; just above this concentration the protein sedimented as a dimer (see Tables II and III). This evidence suggests that a large part of the interaction of the major sialoglycoprotein with Triton X-100 may be with the micelle and not the individual detergent monomers.

Experiments measuring Triton X-100 binding below the critical micelle concentration may be important in distinguishing between a protein which can insert itself into a membrane or detergent micelle with a hydrophobic “tail” from those proteins which normally interact with a number of individual lipid molecules which can be displaced by Triton X-100. Presumably, the red cell sialoglycoprotein is an example of the

### Table I

**[3H]Triton X-100 binding to proteins above detergent critical micelle concentration**

Number of determinations is in parentheses.

<table>
<thead>
<tr>
<th>Material</th>
<th>Source</th>
<th>( mg \text{ detergent bound} )</th>
<th>( mg \text{ protein} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol dehydrogenase</td>
<td>Horse liver</td>
<td>0.02 (1)</td>
<td></td>
</tr>
<tr>
<td>Aldolase</td>
<td>Rabbit muscle</td>
<td>0.005 (1)</td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>Bovine liver</td>
<td>0.01 (1)</td>
<td></td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>Horse heart</td>
<td>0.02 (1)</td>
<td></td>
</tr>
<tr>
<td>Glyceraldehyde-3-P-dehydrogenase</td>
<td>Rabbit muscle</td>
<td>0.02 (1)</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>Human</td>
<td>0.01 (1)</td>
<td></td>
</tr>
<tr>
<td>Dyoloyzme</td>
<td>Hen egg white</td>
<td>0.02 (1)</td>
<td></td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>Hen egg white</td>
<td>0.01 (1)</td>
<td></td>
</tr>
<tr>
<td>Serum albumin (N(+\ K)-ATPase)</td>
<td>Bovine</td>
<td>0.025 (4)</td>
<td></td>
</tr>
<tr>
<td>Ca(^{2+}) ATPase</td>
<td>Canine renal medulla</td>
<td>0.28 (3)</td>
<td>0.26 (1)</td>
</tr>
<tr>
<td>Minor glycoprotein (band III)</td>
<td>Rabbit muscle</td>
<td>0.20 (1)</td>
<td></td>
</tr>
<tr>
<td>Major sialoglycoprotein (PAS-1)</td>
<td>Human erythrocyte</td>
<td>0.77 (4)</td>
<td>0.70 (2)</td>
</tr>
<tr>
<td>Rhodopsin</td>
<td>Frog retina</td>
<td>1.10 (1)</td>
<td></td>
</tr>
<tr>
<td>Opisin</td>
<td>Cattle retina</td>
<td>0.70 (1)</td>
<td>0.58 (1)</td>
</tr>
<tr>
<td>Low density lipoprotein</td>
<td>Human plasma</td>
<td>0.92 (3)</td>
<td>0.72 (1)</td>
</tr>
<tr>
<td>Sarcoplasmic reticulum</td>
<td>Rat muscle</td>
<td>0.20 (2)</td>
<td></td>
</tr>
<tr>
<td>Inner membrane/matrix</td>
<td>Rat liver mitochonedia</td>
<td>0.07 (3)</td>
<td></td>
</tr>
<tr>
<td>Low salt extract*</td>
<td>Cancer</td>
<td>0.07 (1)</td>
<td></td>
</tr>
<tr>
<td>High salt extract*</td>
<td></td>
<td>1.00 (1)</td>
<td></td>
</tr>
</tbody>
</table>

*Binding is in a 7 S peak that includes the Ca\(^{2+}\) adenosinetriphosphatase.
*Extracts were made after the procedure of Jacobs et al. (38). The low salt extract contains the matrix proteins and the more loosely attached membrane proteins. The high salt extract is enriched in cytochrome oxidase.
*Binding is to a 7 S component.

# References

1. Rohm and Haas data for an air to water interface.
2. These results depend upon the assumption that Triton X-100 binds to the polypeptide part of these proteins. However, many of these proteins have covalently bound carbohydrate (Table II) and the possibility exists that there is some detergent interaction with the sugar.
3. There is a complete separation of protein from phospholipid on sucrose density gradient centrifugation of these Triton-extracted proteins (43).
solid line indicates the result expected for 1.22 mg of Triton bound per liters of purified delipidated low density lipoprotein (1 mg of protein/protein as a function of detergent concentration. One hundred micro-
ml) in 0.4% Triton X-100 in the buffer given in Fig. 3 were centrifuged
for 8 hours at 60,000 rpm through 4 ml of 5 to 20% sucrose gradients
mg of protein to identical sites with a dissociation constant of 0.18 mM
(114 ~cg of detergent per ml).

plexes Using Detergent-binding Data in H,0 and D,0—The
example of the second case. Preliminary data indicate that the red cell minor
glycoprotein binds Triton below the cmc and is also an
second. The data shown here were used to calculate the values of the s20,w and v
for these proteins in Table II. Also shown in Table II are data
for cattle retinal opsin and low density lipoprotein obtained by
filtration in the presence of 0.05% Triton X-100. A column of
Sepharose 4B (Pharmacia) was calibrated with marker pro-
tin of known diffusion coefficients, and the D20,w of the
Triton-protein complexes were determined by a comparison of the
elution positions (48). Fig. 5 shows the data from this
calculation, plotted in terms of K, and the Stokes radii of the
marker proteins. The Stokes radius, an inverse function of the
diffusion coefficient, is used here for a linear presentation of the data.7 The experimental data used to find K, for the
Triton-protein complexes are shown for the red cell minor
glycoprotein (band III) and the major sialoglycoprotein in Fig.
6 and for the (Na+ + K+) dependent adenosine triphosphatase in Fig. 7. The amount of the major and minor glycoproteins in
each fraction was determined by the integration of A, from the
PAS or Coomassie stain of the appropriate molecular
weight band obtained from Na dodecyl-S0, polyacrylamide gel
electrophoresis. The same procedure was used to quantitate
the small and large chains of the (Na+ + K+) dependent
adenosine triphosphatase (10, 45) and to demonstrate that
they co-elute. The Stokes radius (diffusion coefficient) of
these complexes was interpolated from the standard curve in
Fig. 5 and the results are given in Table II, together with the
values for low density lipoprotein and opsin determined in the
same way. With the exception of opsin, the Triton-protein
complexes had the same width at half-height as the marker
proteins, indicating homogeneous samples. However, the opsin
peak was 50% wider than that of the marker proteins and this
may indicate a range of molecular sizes of these opsin-Triton
complexes.

The sedimentation coefficients and the partial specific
volumes of the complexes were determined by the procedures
described under “Materials and Methods,” which involve
measuring the apparent sedimentation coefficients by sucrose
density gradient centrifugation in solvents made with H,0 and
D,0. Figs. 8 and 9 show the data obtained for the sedimenta-
tion of the red cell glycoproteins and the (Na+ + K+) dependent
adenosine triphosphatase with marker enzymes on sucrose
density gradients in H,0 and D,0. The change in the relative
positions of these proteins in comparison to that of the marker
proteins on the H,0 and D,0 gradients indicates that the
partial specific volumes of the protein-Triton complexes are
indeed different from those of the marker enzymes. The data
7The conversion is given by D20,w = kT/6p, where k is Boltz-
mann’s constant, T is 293° K, and g is the viscosity of water at this
temperature.
TABLE II
Molecular size and shape of Triton X-100 protein complexes in H₂O

<table>
<thead>
<tr>
<th>Material</th>
<th>sₑ,oₙ</th>
<th>0</th>
<th>Dₑ,oₙ</th>
<th>Mₑ, complex</th>
<th>fₐ,e</th>
<th>g Triton / g protein</th>
<th>g carbohydrate / g protein</th>
<th>Mₛ, protein portion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minor glycoprotein red blood cells (band III)</td>
<td>6.9</td>
<td>0.81</td>
<td>2.7</td>
<td>320,000</td>
<td>1.7</td>
<td>0.77 (36)</td>
<td>0.08 (36)</td>
<td>175,000</td>
</tr>
<tr>
<td>Major sialoglycoprotein red blood cells (PAS-1)</td>
<td>3.6</td>
<td>0.75</td>
<td>3.3</td>
<td>110,000</td>
<td>2.0</td>
<td>1.12 (7)</td>
<td>1.81 (7)</td>
<td>28,000</td>
</tr>
<tr>
<td>Low density lipoprotein</td>
<td>8.7</td>
<td>0.84</td>
<td>1.9</td>
<td>690,000</td>
<td>1.8</td>
<td>0.92 (36)</td>
<td>0.05 (44)</td>
<td>360,000</td>
</tr>
<tr>
<td>Opsin, cattle</td>
<td>4.3</td>
<td>0.80</td>
<td>4.0</td>
<td>130,000</td>
<td>1.6</td>
<td>0.70 (36)</td>
<td>0.05 (45)</td>
<td>75,000</td>
</tr>
<tr>
<td>(Na⁺ + K⁺)-ATPase</td>
<td>6.1</td>
<td>0.76</td>
<td>3.35</td>
<td>185,000</td>
<td>1.7</td>
<td>0.28 (36)</td>
<td>0.05 (45)</td>
<td>140,000</td>
</tr>
</tbody>
</table>

* The values of the molecular weight in parentheses were calculated assuming that the protein binds the same amount of detergent in H₂O and D₂O according to Equation 14. The values of fₐ,e were essentially the same when these values of molecular weight were used in the calculation.

* References in parentheses.

Fig. 5. Determination of Stokes radii of protein-detergent complexes by gel filtration. A column (1.85 x 47.5 cm) of Sepharose 4B was equilibrated and eluted with 0.1 M Na₂SO₄, 0.01 M Tris·H₂SO₄, pH 7.5, 10% glycerol, and 0.05% Triton X-100 at 4°C. The void volume was determined in each case by the elution position of the high molecular weight aggregate of β-galactosidase activity or blue dextran 2000 (Pharmacia). The total volume was determined by DTNB assay for β-mercaptoethanol. The elution positions of each of the Triton-protein complexes was determined by ninhydrin assay and the quantitation of the appropriate molecular weight band on stained Na dodecyl-SO₄ polyacrylamide gels. Kₑ values of both the marker and unknown proteins was determined by the relation (Vₑapex - Vₑvoid)/(Vₑtotal - Vₑvoid). Standard proteins, the method for determining the elution position, and Dₑ,oₙ were: 1, Arenicola hemoglobin, Aₑ,oₙ 1.77 x 10⁻¹ cm²/s (49); 2, Homarus hemocyanin, Aₑ,oₙ 2.46 (50); 3, β-galactosidase, enzymatic activity, 3.12 (30); 4, urease, enzymatic assay, 3.46 (51); 5, apoferritin, ninhydrin, 3.61 (30); 6, catalase, enzymatic assay, 4.10 (34); 7, glyceraldehyde-3-phosphate dehydrogenase, enzymatic assay, 5.46 (31); 8, malate dehydrogenase, enzymatic assay, 6.10 (27, 28); and 9, myoglobin, Aₑ,oₙ 11.3 (30). To ensure that data from separate experiments could be compared, β-galactosidase and catalase were chromatographed with each sample. The measured Kₑ values were 0.595 ± S.D. 0.009 (ten determinations) and 0.711 ± S.D. 0.006 (nine determinations), respectively.

The molecular weights and the apparent frictional ratio (fₐ,e) were calculated from the data in Table II as described by Edsall (52). The data indicate that, unless the protein-detergent complexes are hydrated to a much greater extent than are soluble proteins (and this possibility may not be unlikely, especially if water molecules can order themselves around the polyoxyethylene tails of the detergent*), all of the protein-detergent complexes deviate significantly from globular shapes. Whether or not this is due to a partial unfolding of globular membrane components is not known. It is clear that the activity of the (Na⁺ + K⁺)-dependent adenosine triphosphatase is inhibited by detergent, but this effect may simply be the result of the replacement of an essential lipid cofactor with a molecule of Triton X-100 (54).

The novel feature of the procedure used here to calculate the hydrodynamic results on Triton X-100 micelles (53) can either be interpreted as showing a large amount of hydration (1.4 g/g) for a spherical micelle, or a lower hydration for a nonspherical micelle (zero hydration with an axial ratio of 6).
were solubilized as in Fig. 2 and 30 μg of protein applied to the column for K+-dependent adenosine triphosphatase. Step IV microsomes of Kyte gels on Coomassie brilliant blue-stained Na dodecyl-SO₄ polyacrylamide amounts of the large (O—O) and small (O—O) polypeptide chains were determined by the integrated A₄₅₀ of the respective bands on Coomassie brilliant blue-stained Na dodecyl-SO₄ polyacrylamide gels.

The method described here for calculating molecular weights is dependent on knowing the amount of detergent bound in both H₂O and D₂O and is thus limited to purified protein preparations for which such binding determinations can be made.

**Determination of Molecular Weight of Triton-Protein Complexes for Impure Proteins**—Many proteins cannot be analyzed by the method described above because they are contaminated with other proteins or are present in concentrations too low to allow the measurement of detergent binding. However, with the assumption that the binding of detergent in H₂O is the same as that in D₂O, such data would not be necessary and a molecular size and shape could be calculated from sucrose density gradient centrifugation and gel filtration data alone, where only an enzymatic activity need be measured (Equation 14 under “Materials and Methods”).

This abridged method of calculating the partial specific volume and s₂₀,w (Equation 14) can also be applied to non-membrane molecular weights was the method used to determine  and  from the sedimentation data. Although it is possible to estimate  from the relative contribution of protein and detergent (3, 42), the presence of other components and the uncertainty of the values of  for the various components may make this estimate inaccurate. If the partial specific volume of the complex is known or can be estimated, one can correct the apparent sedimentation coefficient obtained only in H₂O by comparison with marker proteins on sucrose gradients utilizing the tables prepared by McEwen (55). This procedure was adapted by Simons et al. when they measured the molecular weight of a membrane component of Semliki Forest Virus in Triton X-100 (3). One reason for their choice of this approach, which involved estimating  rather than measuring  by the methods described here, was that they found lower amounts of Triton bound in the presence of D₂O than H₂O. They stated that this difference would preclude the direct determination of  in sucrose gradients in H₂O and D₂O. However, the method used here explicitly takes into account the difference in the binding of detergent to the protein in H₂O and D₂O in the determination of the partial specific volume.

The method described here for calculating molecular weights is dependent on knowing the amount of detergent bound in both H₂O and D₂O and is thus limited to purified protein preparations for which such binding determinations can be made.

![Fig. 7. Gel filtration of the Triton X-100-solubilized (Na⁺ + K⁺)-dependent adenosine triphosphatase.](image)

![Fig. 9. Centrifugation of Triton X-100-solubilized (Na⁺ + K⁺)-dependent adenosine triphosphatase in 5 to 20% sucrose gradients, in H₂O and D₂O to determine s₂₀,w and .](image)
enzyme does not bind more than 0.2 mg of Triton X-100 per mg proteins in detergent are all oligomers, while low density lipoprotein is either a monomer or a mixture of monomers and carbohydrate. The data indicate that the four membrane glycoprotein (band III, 36)
dimers resulting from disaggregation of the two polypeptide chains in the intact molecule (46).

While it is possible that the subunit structure of the protein-Triton X-100 complexes is different from that of the protein-lipid complexes in the membrane, a simple explanation of the data is that the membrane proteins described in Table III are indeed oligomers even in the membrane. The data for cattle opsin indicate that two polypeptides make up the Triton complex. This result can be contrasted to that of Osborne et al. (42) who measured the size of the unbleached Triton complex of rhodopsin and found it contained only one polypeptide chain. They also measured higher amounts of bound detergent. These differences may reflect either the solubilization procedures or a genuine difference in the protein in the bleached and unbleached states. The subunit structure of the Triton-solubilized (Na⁺ + K⁺)-dependent adenosine triphosphatase is probably an αβ dimer consisting of one large chain and one small chain, which would have a molecular weight of 141,000 (based on Na dodecyl-SO₄ gel electrophoresis) to 174,000 (based on gel filtration) (45). While the existence of an αβ structure of the native enzyme is supported by cross-linking experiments (45), larger molecular weights (and presumably higher orders of quaternary structure, i.e. α₁β₂) have been obtained by radiation inactivation analysis (57) and by the osmometry of cardiac glycoside and ATP binding sites (58). The latter results may indicate that Triton X-100 causes a partial disaggregation of this membrane protein.

The subunit structure calculated for the red cell minor glycoprotein (band III) is dependent upon the particular choice of the polypeptide chain molecular weight. In spite of the apparent value of 150,000 obtained by gel filtration in 6 M guanidinium chloride (36), the most likely value for the polypeptide molecular weight is 80,000 to 100,000 and the Triton complex would consist of a dimer of this polypeptide. A dimeric structure for the minor glycoprotein (band III) in Triton was also obtained by Yu and Steck, who observed that

preparations. Here, there is no complication of bound detergent and the equation is explicit. This procedure is useful because it allows a direct measurement of \( \bar{d} \) for proteins present in crude mixtures or in low amounts and can indicate the presence of large amounts of carbohydrate, lipid, or nucleic acid components bound to the polypeptide.

Some caution is necessary when bound detergent is estimated by the partial specific volume instead of by direct measurement. For example, the major sialoglycoprotein (PAS-1) has a measured \( \bar{d} \) of a typical soluble protein in spite of the fact that it binds its own weight in detergent. Presumably, in this case the low partial specific volume of the carbohydrate compensates for the higher \( \bar{d} \) of the detergent.

Triton X-100-protein complexes do not contain significant amounts of phospholipid. This has been shown here for low density lipoprotein, as well as for the erythrocyte proteins (43) and rhodopsin (42).
the 7.5 S sedimentation coefficient on sucrose gradients was not altered when the isolated polypeptide was cross-linked to a dimer (59). Evidence that this protein also exists as a dimer in the membrane has been reviewed by Wang and Richards (60).

The best estimate of the polypeptide chain molecular weight of the major sialoglycoprotein (PAS-I) comes from the results of sedimentation equilibrium experiments in Na dodecyl-SO₄ which give a value of 13,000 for the protein portion (61). This value is supported by the value of 11,000 obtained by subtracting the contribution of carbohydrate to the mass of the native molecule (31,400) determined by hydrodynamic measurements (7). The polypeptide molecular weight determined by Na dodecyl-SO₄ gel electrophoresis is unreliable here because the proteins used for calibration have no bound carbohydrate and bind Na dodecyl-SO₄ in a uniform way (62), while 65% of the mass of the glycoprotein is carbohydrate and this protein binds an abnormal amount of Na dodecyl-SO₄ (61). These results indicate that the Triton X-100 complex of the major sialoglycoprotein contains two polypeptide chains. In contrast to the situation with the minor glycoprotein, there is no evidence for the existence of dimers of this protein in the membrane (63).

Replacement of Sodium Dodecyl Sulfate by Triton X-100 on Red Cell Minor Glycoprotein—The ability of Triton X-100 to compete with Na dodecyl-SO₄ for binding sites on the minor glycoprotein (band III) was determined by mixing an aliquot of the protein in Na dodecyl-SO₄ with a 10-fold weight excess of Triton, and then centrifuging the sample into a sucrose molecule (31,400) determined by hydrodynamic measurements (7). The polypeptide molecular weight determined by Na dodecyl-SO₄ gel electrophoresis is unreliable here because the proteins used for calibration have no bound carbohydrate and bind Na dodecyl-SO₄ in a uniform way (62), while 65% of the mass of the glycoprotein is carbohydrate and this protein binds an abnormal amount of Na dodecyl-SO₄ (61). These results indicate that the Triton X-100 complex of the major sialoglycoprotein contains two polypeptide chains. In contrast to the situation with the minor glycoprotein, there is no evidence for the existence of dimers of this protein in the membrane (63).

Replacement of Sodium Dodecyl Sulfate by Triton X-100 on Red Cell Minor Glycoprotein—The ability of Triton X-100 to compete with Na dodecyl-SO₄ for binding sites on the minor glycoprotein (band III) was determined by mixing an aliquot of the protein in Na dodecyl-SO₄ with a 10-fold weight excess of Triton, and then centrifuging the sample into a sucrose gradient containing only [H]Triton X-100. A sample of this protein, which had not been exposed to Triton, was purified by gel filtration in 0.2% Na dodecyl-SO₄, 40 mM Tris H₂SO₄, pH 8, on a column of Sepharose 4B by M. K. Ho (Harvard University). 35S-labeled Na dodecyl-SO₄ (New England Nuclear) was added to the concentrated and dialyzed sample so that the protein concentration was 10 mg/ml and the free Na dodecyl-SO₄ concentration was between 2 and 10 mg/ml. Equal volumes of 10% Triton X-100 and this sample were incubated 1 hour at room temperature, and then applied to a 5 to 20% sucrose gradient in 0.05% [H]Triton X-100, as in Fig. 2. Fig. 10 shows the complete separation of protein and Na dodecyl-SO₄ that was achieved after centrifugation. The "renatured" protein bound 0.4 mg of Triton per mg of protein and had a sedimentation coefficient of 4 S, which indicated that the polypeptide had either not refolded or had not dimerized to the structure of the "native" Triton X-100-protein complex. Presumably the Na dodecyl-SO₄ was removed from the binding sites on the protein by incorporation into the micelles of Triton X-100.

The ability of Triton X-100 to replace Na dodecyl-SO₄ on the red cell minor glycoprotein (and perhaps on soluble proteins as well) may be useful from the standpoint of the purification of proteins by ion exchange chromatography after initial fractionation in Na dodecyl-SO₄ and may supplement existing methods for removing Na dodecyl-SO₄ from proteins, such as the method of Weber and Kuter (64).

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REFERENCES


The size and detergent binding of membrane proteins.
S Clarke


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