TOTAL SOLUBILIZATION OF ERYTHROCYTE MEMBRANES BY NONIONIC DETERGENTS

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The solubilization of the erythrocyte membrane by nonionic detergents was measured under various conditions. Optimum results were obtained using Triton X-100 at a total salt concentration of 5 ideal mosmolar and a pH of 7.4. Under these conditions virtually total solubilization is possible with no apparent loss of acetylcholine esterase activity and only partial loss of sodium-potassium sensitive adenosine triphosphatase activity.

Extensive studies have been made recently on the effectiveness of various detergents in solubilizing the cytoplasmic membrane. Sodium dodecyl-sulfate (SDS) in particular has been found to be most efficient at disaggregating the membranes of mycoplasmas (1-5) and of Micrococcus lysodeikticus (6) converting them to a form which is non-sedimentable at 100,000 G for one hour. One of the more interesting aspects of this work was the discovery that this process is reversible; that is, if the detergent is removed from the solubilized material by dialysis in the presence of magnesium ions, a precipitate is formed which resembles the original membrane under the electron microscope. One unfortunate failing of this detergent, however, is its tendency to denature proteins, as indicated by the irreversible destruction of the activity of some of the more sensitive enzymes present in the membrane (7,8). Certain nonionic detergents, on the other hand, produce less effect on these enzymes but are inefficient as solubilizers under the conditions in which they are generally employed (7,9). The present work demonstrates, however, that conditions do exist under which some of these detergents will produce almost total solubilization of the membrane with relatively little effect on such sensitive enzymes as acetylcholine esterase and sodium-potassium dependent ATPase.

EXPERIMENTAL SDS was obtained from Fisher Scientific Co. Both Triton X-100 and ^{14}C-Triton X-100 (22.8 μc/gm labelled in the polyethoxy chain) were gifts of Rohm and Haas (Philadelphia, Pa.), Lubrol WX was kindly donated by ICI America Inc. (Stamford, Conn.) while other detergents were supplied by Atlas Powder Company (Brantford, Ontario) and Union Carbide (New York, N. Y.). Oubain and the Tris salt of ATP were obtained from the Sigma Chemical Company. Membrane preparations were derived from outdated human blood obtained from the local Red Cross blood bank.

Membrane preparation. Red cell membranes were prepared by the method of Dodge, Mitchell and Hanahan (10).

Determination of the degree of solubilization. For the present purposes, any material still in suspension following centrifugation at 100,000 G for one hour is considered to be "solubilized". The degree of solubilization resulting from a particular treatment was determined by measuring the protein content of the suspension both before and after centrifugation, and is expressed as a percent ratio of the two.

Buffers. Two buffers were used in this work. Tris buffer was made up to a known concentration and then titrated to the required pH with HCl. At pH 7.4, the ideal milliosmolarity (imosM) of Tris buffer prepared in this way was taken as 1.8 times its millimolar concentration. Phosphate buffers were prepared by mixing 5 imosM NaH_2PO_4 with 5 imosM Na_2HPO_4 in such proportions as to give the required pH at a constant osmolarity.

Protein analysis. Proteins were determined by the method of Lowry et al. (11).

Phospholipid analysis. The sample was made up to one ml with water, extracted twice with two ml of a chloroform-methanol (2:1) mixture and the combined extracts evaporated to dryness. The residue was oxidized by the addition of 0.2 ml 70% perchloric acid followed by heating at 180° on a fluidized sand bath (12). The inorganic phosphate content of the remaining material was determined by the method of Fiske and SubbaRow (13) and phospholipid content of

the original sample taken to be 25 times this value.

Cholesterol analysis. Cholesterol analyses were performed as specified by Clark et al (14).

Enzyme activities were determined as follows:

Acetylcholine esterase (EC 3.1.1.7) (ACHase). Samples of known protein content were added to 10 ml of a solution of 0.1 M NaCl, 0.04 M $MgCl_2$ and 2 mM acetylcholine bromide. The acetic acid released was titrated with 0.02 M NaOH using a recording titrator which maintained the pH at 7.0. A straight line plot of alkali consumption against time resulted, and from this the enzyme activity could be determined as mmoles acetylcholine split per min per mgm protein.

Adenosine triphosphatase (EC 3.6.1.3) (ATPase). The ATP hydrolyzed by various membrane preparations was determined by standard methods following the addition of 0.3 gm sugar charcoal per ml solution to remove proteins, lipids and detergent. The Na-K-ATPase activity was taken as the difference between the amounts of ATP hydrolyzed in the presence and absence of 1.5×10^{-5} M oubain.

RESULTS The basic finding in this work is illustrated in Fig. 1A where the importance of salt concentation to the process of solubilization by Triton X-100 is illustrated. Here it can be seen that the reduction of tris concentration brings about an increased efficiency which reaches a maximum at about 5 imosM and then declines rapidly at lower salt concentrations. An optimum pH near 7.5 is indicated in Fig. 1B and since 5 imosM phosphate buffers were used in these experiments, one may conclude, tentatively at least, that it is the total salt concentrations rather than the effect of a particular buffer which is important in determining solubilization efficiency. That this effect is not osmotic in nature was demonstrated by a further experiment in which membranes suspended in 5T buffer (5 imosM tris, pH 7.4) were solubilized both in the presence and absence of 300 mM sucrose with no discernable difference being noted.

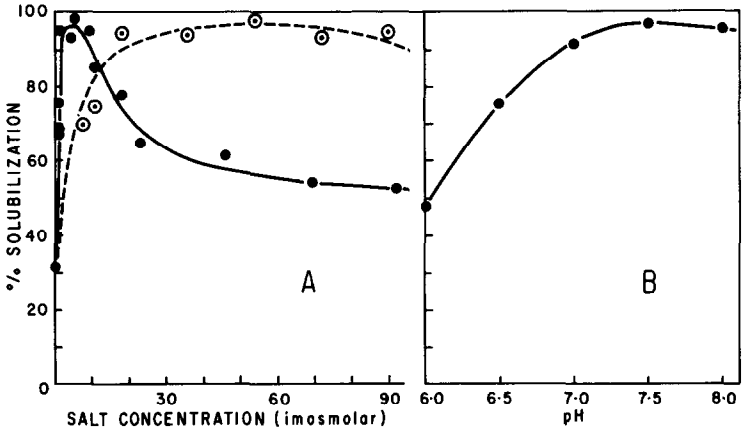

Fig. 1

Optimum conditions for membrane solubilization

A Erythrocyte membrane (1.2 mgm protein/ml) were washed several times with water or tris buffer (pH 7.4) of the indicated concentration, and titrated with Triton X-100 until a constant OD_{500} was obtained. The percent solubilization was then determined and plotted here against buffer concentration. For the solid line and points, buffer concentrations are as shown; for the broken line and open circles, concentrations are 1/10 those given in the abscissa.

B Percent solubilization of erythrocyte membranes by Triton X-100 at various pH's. Five imosM phosphate buffers were used; procedure otherwise as above.

As a result of the above demonstration of its ability to promote solubilization, 5T buffer was used exclusively in the following experiments.

The effect of Triton X-100 concentration on optical density and solubilization. A suspension of membranes tends to be cloudy, but with the addition of detergent visual clearing results. This phenomenon is shown to correspond with solubilization in Fig. 2 where the optical density and percent solubilization are plotted as a function of detergent concentration. The amount of Triton X-100 required to produce 50% solubilization was measured as a function of the amount of membrane protein present and this data, when plotted, was found to correspond to a straight line with a least squares calculated slope of 0.40 $\pm$ 0.006 mgm X-100/mgm protein (highest measurement being made at 8 mgm protein/ml).

Removal of detergent. Most of the detergent may be removed from the

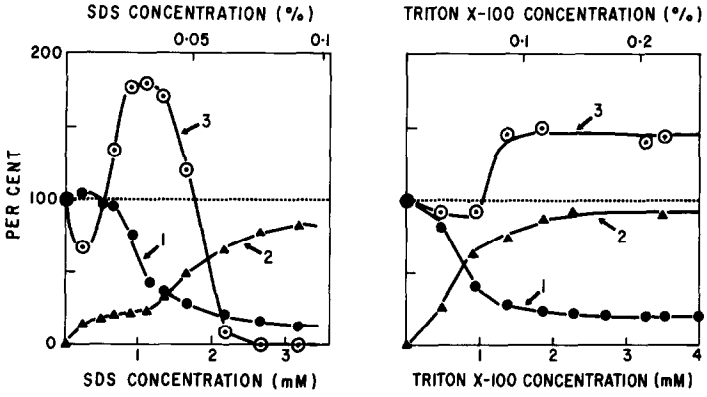

Fig. 2

Effect of detergent concentration on OD, solubilization and ACHase

Membranes were suspended on 5T buffer at a protein concentration of 1.1 mgm/ml. OD_{600}, percent solubilization and ACHase activity were measured for various detergent concentrations.

Curve 1 - OD_{600} as a percentage of initial value.

Curve 2 - Percent solubilization.

Curve 3 - ACHase activity as a percentage of initial value.

solubilized membrane without causing it to precipitate, either by dialysis or by gel filtration. To test for the effectiveness of these procedures, membranes solubilized by radioactive Triton X-100 were either passed through a Sephadex G-50 column (90 x 2.5 cm) or dialysed against 5T buffer for four days at $0^{\circ}C$ and the residual radioactivity determined. About 7% of the detergent was found to be retained by the membrane material so that 3-4% of the weight of dialysed membrane was detergent.

Reconstitution of the membranes. Dialysis of solubilized membranes at $0^{\circ}C$ against either Ca^{++} or Mg^{++} according to the procedure of Razin et al (1), resulted in the formation of a precipitate containing both protein and lipids in the same proportions as found in the dissolved membrane which when embedded and examined under the electron microscope revealed structures similar in appearance to those of the original membrane. This procedure seems to be analogous to the reconstitution of solubilized mycoplasma membranes.

Effect of divalent ions on solubilization. If either Ca^{++} or Mg^{++} is

present during detergent addition, solubilization is inhibited. This inhibition reaches 50% at a Ca^{++} concentration of 3.6 mM or a Mg^{++} concentration of 4.4 mM. One might infer from this that the addition of a sequestering agent would assist solubilization by assuring that any divalent ions were removed from solution. To the contrary, however, it was found that concentrations of EDTA below one mM were ineffective, while higher concentrations simply raised the total salt concentration and resulted in reduction of solubilization.

ATPase activity. SDS was found to destroy all the ATPase activity of the membrane at concentrations sufficient to give complete solubilization, while Triton X-100 under the same conditions reduced the activity of this enzyme to about 30% of the original. Removal of the detergent restored the activity to about 60%, whereas reconstitution reduced it further to about 20%.

ACHase activity. Fig. 2 shows the effect of solubilization on the cholinesterase activity of the membrane. Both SDS and Triton X-100 produced an increase in enzyme activity at low concentrations, but only with X-100 is this activity sustained at higher concentrations. In fact, such activity was found to be undiminished even in the presence of 2% X-100.

The increase in enzyme activity resulting from detergent addition varied from sample to sample, in some cases reaching as high as three times that of the original membrane. Either reconstitution or removal of the detergent reduced this activity to varying amounts, but still left it higher than that of the original membrane.

Other detergents. A number of other detergents structurally related to X-100 were tested for their ability to reduce the optical density of a membrane suspension. Only Lubrol WX (I.C.I.) and Tergitol TMN (Union Carbide) proved to be as effective as Triton X-100 in this respect, but membranes solubilized by these two detergents possessed only about two thirds of the ATPase activity of the X-100 solubilized material. As a consequence, these two compounds have not been used as extensively as X-100 in the present work.

CONCLUSION Suspension in 5 imosM tris buffer at pH 7 in the presence

of Triton X-100 appears to be an effective means of solubilizing the membranes of stored erythrocytes. Much more work will be required, however, to determine the nature of the solubilized material. For example, it would be of interest to know whether this method separates the lipid and protein, as appears to be the case with SDS solubilization, or whether these two components are released from the membrane as lipoprotein subunits. Studies on this problem are in progress.

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