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# EFFECTS OF TRITON X-100 TREATMENTS ON THE COMPOSITION AND ACTIVITIES OF MEMBRANE VESICLES FROM PIGEON ERYTHROCYTES *

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## Summary

Membrane vesicles were prepared from pigeon erythrocytes. The effect of various treatments on the ability of these vesicles to trap and transport glycine was measured. Most of the work concerned the effects of the nonionic detergent, Triton X-100 (Triton).

Triton inhibits the capacity of membrane vesicles to trap and transport glycine. The effects of Triton depend on temperature and pH. At neutral pH and 41°C, the half-inactivating dose of Triton is 6 μl/g wet weight of membrane, while at neutral pH and 0°C it is approx. 60 μl/g. At pH 8.5—8.8 and 0°C the half-inactivating dose is 15—20 μl/g. The Triton effect depends on the ratio of Triton to membrane, not the Triton 'concentration' in the aqueous phase.

Protein is released from the membrane by Triton treatment at 0°C. At pH 8.5—8.8, the dose-response curve for protein release is very similar to the dose-response curve for inhibition of the capacities to trap and take up glycine. All three curves have steep and shallow regions, respectively below and above a Triton dose of 15 μl/g. The protein released by Triton treatment at 0°C is largely the lower molecular weight classes and the maximum protein release is 50—60% of the total membrane protein. The percent of this protein class released by a given Triton dose equals the percent inhibition of the capacity to trap glycine.

Triton is bound by the membranes. In the dose range of 0—10 μl Triton/g

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Abbreviations and terms: Glycine uptake, $Na^+$-dependent glycine uptake during a 5 min incubation; trapping, glycine trapped during the annealing step (17 min, 41°C); Triton, Triton X-100, *t*-octylphenoxypolyethoxyethanol ($n$ = 9—10 average), mean molecular weight = 638, density 1.053; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*'-tetracetic acid. The "dipotassium salt" of EGTA was prepared by neutralizing the free acid to pH 6.9 with KOH; TES, *N*-tris(hydroxymethyl)-2-aminoethane sulfonic acid; SDS, sodium dodecyl sulfate; HDL, bovine serum high density lipoprotein [7].

wet membrane, about half of the added Triton is bound. This bound Triton is not readily removed by washing. Like protein release and inhibition of trapping and transport capacities, the binding process has two phases, one above and one below a dose of 15 μl/g. The membrane is saturated with Triton at a dose of 15 μl/g, with 4.5 μl Triton bound/g wet weight, corresponding to 85—90 μl bound/g remaining membrane protein.

Bovine serum high density lipoprotein efficiently removes bound Triton from the membrane. By removing Triton bound at 0°C with lipoprotein, the effects of Triton at 0 and 41°C could be distinguished and the time course of Triton action at 0°C could be measured. Triton action was nearly complete by 10 min at 0°C.

Possible modes of action of Triton on these membranes are discussed. At least part of the action of Triton can be described as a process in which membrane proteins are partitioned between the membrane phase and an extramembranal Triton micelle phase.

## Introduction

Investigations of membrane structure and function frequently begin with the 'solubilization' of the membrane with a detergent. The work to be described began as an attempt to solubilize membranes from pigeon erythrocytes with Triton X-100 (Triton) and to reconstitute transport-active vesicles from solubilized membrane. It quickly became apparent that the effects of Triton on the membrane were complex and needed investigation before reconstitution was attempted.

The membrane preparation used was from pigeon erythrocytes similar to one previously described [1] which has an $Na^+$-dependent glycine transport system [1] as well as $Na^+$-dependent alanine uptake activity [Lee and Vidaver, unpublished] and ATP-dependent $Ca^{2+}$ uptake activity [Ting and Vidaver, unpublished]. These membranes require 'annealing' (exposure to a temperature around 40°C) to cause them to trap low molecular weight solutes [1].

We examined the binding of Triton to these membranes, and the effects of Triton treatment (1) on the ability of membranes to trap glycine upon annealing, (2) on $Na^+$-dependent glycine uptake activity, (3) on the release of protein and (4) on the release of lipid phosphorous. A method was needed for the efficient removal of Triton from membranes and one was found: treatment with bovine high density lipoprotein (HDL).

The abilities of the membranes to trap and take up glycine were abolished by low Triton doses acting at 41°C. Higher doses were required at 0°C. Triton treatment at 0°C resulted in selective release of protein and a proportional inhibition of the ability to trap glycine, but there was little impairment of the glycine uptake activity of those vesicles that were able to seal. The actions of Triton we observed are not explained by the lipid bilayer-to-mixed micelle phase transition mechanism described by Helenius and Simons [2] in their recent review on the action of detergents on membranes. We propose another mode of action for Triton on membranes, a process where membrane components partition between membrane and extramembranal Triton phases.

## Materials and Methods

*Chemicals.* 2-[$^3$H]Glycine and 1-[$^{14}$C]glycine were obtained from New England Nuclear Corp. Boston, Mass, U.S.A. or Amersham/Searle, Arlington Heights, Ill., U.S.A. [$^3$H]Triton X-100 (ring labelled) was a generous gift from Rohm and Haas, Philadelphia, Pa., U.S.A. Unlabelled Triton (mean $M_r$ 638, density 1.053 g/ml), Dextran sulfate (approx. $M_r$ 500 000) and twice-recrystallized bovine serum albumin were obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A. For scintillation counting the solubilizer used was BBS-3 from Beckman Instruments, Inc., Fullerton, Calif., U.S.A. and the scintillator was Omnifluor from New England Nuclear. Scintillation grade toluene was from Fisher Scientific Co., Chemical Manufacturing Division, Fairlawn, N.J., U.S.A.

*Preparation of bovine high density lipoprotein (HDL).* HDL was prepared by an adaptation of methods [3,4] for the preparation of human high density lipoprotein. 2 to 3 l fresh bovine serum were made 0.1 M in $CaCl_2$ and 0.05% in Dextran sulfate, held 15 min and centrifuged 10 min, 600 × *g*. The supernatant was made 0.2 M in $CaCl_2$ and 1.55% in Dextran sulfate, held 30 min and centrifuged 40 min, 15 000 × *g*. The above steps were at room temperature. The pellet(s) were dissolved in 1 ml 0.39 M trisodium citrate/100 ml original serum, held overnight at 0—4°C, brought to 16% of the original serum volume with 0.02% Tris · HCl (pH 7.3 at 25°C), made 0.2 M in $CaCl_2$ and centrifuged for 10 min, 15 000 × *g* at room temperature. The pellet was dissolved in citrate and precipitated with $CaCl_2$ twice more. The resulting precipitate was dissolved in a minimum volume of 0.39 M trisodium citrate and dialysed 20—24 h at 5—8°C against 2 l of 0.17 M NaCl, 0.02 M Tris (pH 7.3). The dialysed solution was made 0.04 M in $CaCl_2$, centrifuged and the supernatant dialysed 20—24 h against 0.17 M NaCl, 0.02 M Tris (pH 7.3) then dialysed 20—24 h at 5—8°C against 2 l 0.02 M Tris (pH 7.3), 0.041 M $BaCl_2$ and centrifuged. The supernatant was dialysed 20—24 h at 5—8°C against 2 l 0.02 M Tris (pH 7.3), 0.002 M dipotassium salt of EGTA, 0.001 M $CaCl_2$, 0.002 M $KN_3$, 0.129 M KCl. HDL concentration was determined by weighing air-dried aliquots and subtracting the weight of salts. Protein content was 45.5% of the dry weight (range 43—48%, $n$ = 4, method of Lowry et al. [5] using bovine serum albumin as standard) and 24.5% phospholipid (range 24—25%, $n$ = 4, method of Ames [6], assuming a phopholipid molecular weight of 800). This composition corresponds to that of $HDL_3$ ($d$ = 1.12) [7]. SDS gel electrophoresis showed one or two closely spaced main bands and minor amounts of other bands.

*Preparation of membranes.* A simplified version was used of the method previously described [1]. All operations were done in the cold except where otherwise indicated. A 30%, w/v, suspension of pigeon erythrocytes in 143 mM KCl, 5.2 mM $K_2HPO_4$ and 4.8 mM $KH_2PO_4$ was mixed with an equal volume of 134 mM in KCl, 3.6 mM glucose, 4 mM $CaCl_2$ and 8 mM $MgCl_2$, and 60—90 ml portions were sonicated 2.5 min at setting 3.5 with a Branson 20 kHz sonifier, (Model W185D, Heat Systems Ultrasonics, Inc., Plainview, L.I., N.Y., U.S.A.) using a microtip probe. The sonicated suspension was mied with 3/4 vol. ice-cold 0.30 M sucrose and centrifuged 5 min at 5100 × *g* in a Sorvall SS-34 rotor. The supernatants were centrifuged 20 min at 23 500 × *g* and the resulting

pellets were washed twice with 154 mM KCl (15 min, 23 500 × *g*). The washed pellets were suspended in 8 ml phosphate buffered KCl (above) for each 10–15 g original cells and sonicated at setting 5 in 8 ml portions in a 'cold shoulders' cooling cell in an ice bath for three 30 s intervals. The suspension was centrifuged 20 min at 30 900 × *g*. The pellet from one aliquot was weighed to determine membrane concentration in g wet weight/ml. Membrane was stored as pellets at 0°C for use the next day. Membrane preparations had 65.6 ± 5.1 mg protein/g wet weight ($n$ = 18), 54 ± 22 μmol lipid phosphorous/g wet weight ($n$ = 4) and 1 mg DNA/g wet weight.

On the day of use, membrane pellets were first smeared around the bottom of the tubes with stirring rods (pellets were 'smeared' at all steps to facilitate resuspension) and suspended in 1 ml of 2 mM EGTA/1 mM $CaCl_2$/5 mM $K_2HPO_4$/4.8 mM $KH_2PO_4$/137 mM KCl. After standing 10 min the samples were centrifuged for 20 min at 30 900 × *g* and used immediately.

*Treatment with Triton or other agents and assay for trapping ability and glycine uptake activity.* For Triton treatment before annealing, pellets (generally 80 mg wet weight), were suspended in Triton solution (usually 0.50 ml, see Figs. and Tables for details), held for the time and temperature desired (usually 0°C, 30 min) and centrifuged, usually 20 min at 30 900 × *g*. To remove residual Triton, the pellets were suspended in HDL solution, held for 10 min at 0°C, centrifuged and resuspended in annealing solution (4 mM $MgCl_2$/1.7 mM dipotassium salt of EGTA/0.86 mM $CaCl_2$/4.4 mM $K_2HPO_4$/4.1 mM $KH_2PO_4$/131 mM KCl/radioactive trapped space marker, usually 0.33 mM 1-[$^{14}$C]glycine, 7 mCi/mmol).

Annealing was done by incubating suspensions 17 min at 41°C and stopped by chilling for 2 min, addition of 10 ml ice-cold diluent and centrifuging 20 min at 30 900 × *g*. The diluent was a modified Krebs Ringer phosphate solution, 130.6 mM KCl/3 mM $KH_2PO_4$/2.5 mM $CaCl_2$/1.2 mM $MgCl_2$/8.7 mM D-glucose/6 mM $K_2HPO_4$ (cold $K_2HPO_4$ solution must be added last to the cold dilute mixture of the other components). Three quarters of all trapping occurs during annealing (data not shown).

Paired pellets were used. One was suspended in 0.50 ml $Na^+$ incubation medium and the other in 0.50 ml $K^+$ medium, each containing 2-[$^3$H]glycine, usually 0.03 mM, 290 mCi/mmol. The incubation media were the $Na^+$ and $K^+$ versions of the modified Krebs Ringer phosphate solution described above. Samples were incubated 5 min at 40°C, chilled 2 min, diluted with 9.5 ml cold 154 mM KCl and centrifuged 20 min at 30 900 × *g*. The pellets were washed once with 10 ml 154 mM KCl, the tubes drained and the insides wiped dry. Pellets were weighed, extracted with 1.00 ml cold methanol containing 20 μg glycine/ml, 0.40 ml extract was mixed with 15 ml counting cocktail (20 ml BB-S3, 4 g Omnifluor diluted to 1 l with toluene) and counted. Spill corrections were made, quench corrections were unnecessary. Sodium ion-dependent glycine uptake was calculated from the difference between the $^3$H counts of $Na^+$ and $K^+$ samples and trapped space calculated from the $^{14}$C counts of the $K^+$ samples. An untreated control was present in all experiments and the data is presented as 'percent of control' values for uniformity.

*Protein and phospholipid determination.* Protein was determined by the method of Lowry et al. [5] with HDL as the standard. When Triton was

present in unknowns, it was added to the standards, and unknowns and standards were made 1% in SDS to prevent precipitation of Triton.

Lipid phosphorous was determined on organic solvent extracts prepared as described by Kates [8], by ashing and determining inorganic phosphate by the method of Ames [6].

*Determination of Triton binding.* Membrane suspensions were treated with [$^3$H]Triton (0.065—0.26 mCi/ml Triton), centrifuged for 20 min at 30 900 $\times g$, and wet and dry weights of pellets, and dry weights of aliquots of each supernatant were determined. Triton binding to pellets was corrected for Triton dissolved in the pellet water by subtracting pellet water $\times$ (cpm/ml supernatant water) from cpm/pellet. The $^3$H bound was Triton, not a radioactive impurity since a 2-fold dilution of the [$^3$H]Triton used with non-radioactive Triton exactly halved the amount of $^3$H bound.

The centrifugation we used ($10^4 \times g$, 1 h) was 1/10 of the $10^5 \times g$, 1 h usually used [9] to define 'soluble' extracts of membranes. However, $10^5 \times g$, 1 h will begin to sediment substances in the 10—50 s range which could be considered 'soluble'. We found that 65% and 66% (two experiments) of the protein in a 60 $\mu$l Triton/g, $10^4 \times g$, 1 h supernatant remained in the supernatant after a $2.5 \cdot 10^5 \times g$, 1 h centrifugation.

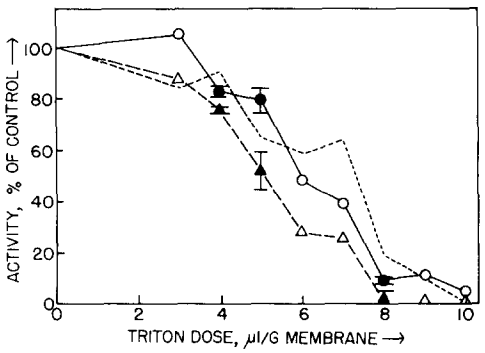
## Results

*Triton effects at 41°C.* When pigeon erythrocyte membranes were exposed to Triton during annealing at 41°C, they lost the abilities to trap glycine during annealing and to take up glycine (Fig. 1). Inhibition of both trapping and transport began at a Triton dose of 3 $\mu$l/g membrane (45 $\mu$l/g membrane protein) and was virtually complete at 8 $\mu$l/g membrane (120 $\mu$l/g protein). Uptake activity was about as sensitive as trapping (uptake/trapping curve vs. trapping curve, Fig. 1). In the dose range of 2—6 $\mu$l Triton/g membrane, about half the added Triton was bound (data not shown). Binding of the first 25 $\mu$l/g membrane protein is innocuous but binding of the next 50 $\mu$l/g protein is accompanied by inactivation.

*Triton binding at 0°C and removal of bound Triton with HDL.* At 0°C, Triton binding was proportional to added Triton up to 7.5 $\mu$l/g membrane although only approx. half the added Triton was bound (Fig. 2). Surprisingly, the membrane was saturated with Triton at a dose of 15 $\mu$l/g, at which point only 4.5 $\mu$l Triton was bound per g membrane (88 $\mu$l Triton/g membrane protein). The bound Triton was not removed by simple washing (Table I, lines 1—3).

To distinguish between effects of Triton at 0°C and at 41°C, a method was needed for removing bound Triton before warming the membranes to 41°C. Washing with HDL did this (Table I, lines 4—7). After one HDL wash, 1 $\mu$l Triton/g remained bound to the membrane. * This is less than is bound from the Triton dose required to minimally inhibit at 41°C. When HDL and Triton

* This suggested that HDL might be useful for reconstituting transport-active vesicles from Triton-solubilized membrane. Using HDL to remove Triton, we (Yeung and Vidaver, unpublished) have reconstituted vesicles from extracts of pigeon red-cell membranes that show ATP-dependent $^{45}Ca^{2+}$ uptake.



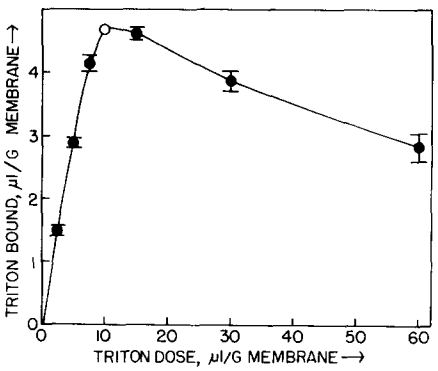




Fig. 1. Effects of Triton treatment at 41°C on $Na^+$-dependent glycine uptake activity and trapping ability. 80 mg membrane pellets were suspended in 0.50 ml of a solution containing 2 mM EGTA, 1 mM $CaCl_2$, 76 mM KCl and 10 mM TES, pH 7.2 and held 70 min at 0°C. Suspensions were centrifuged 20 min at 30 900 × *g*. The pellets were suspended in 0.50 ml annealing solution (Methods) containing [$^3$H]glycine and enough Triton to give the indicated doses. The suspensions were annealed and glycine uptake and trapping determined as described in Methods. ●,○, Trapping of [$^3$H]glycine as % of control sample annealed without Triton; ▲,△, $Na^+$-dependent glycine uptake activity (5 min, 41°C) as % of control; - - - - - -, ratio of uptake to trapping, i.e., relative uptake activity per unit of sealed vesicles. Solid symbols, average ± S.E.M. of two or more experiments; open symbols, single data points.

Fig. 2. Triton binding at 0°C. Unannealed membrane was treated with [$^3$H]Triton for 30 min at 0°C at pH 8.6—8.8 as described for the experiments of Table I. The suspensions were centrifuged and the [$^3$H]-Triton in the (unwashed) pellets determined as in Table I. Triton specific activities were 0.26 mCi/ml Triton, 0.13 mCi/ml, and 0.065 mCi/ml for Triton doses of 2.5—15 μl/g wet weight of membrane, 30 μl/g, and 60 μl/g respectively. In one experiment Triton was unlabelled and Triton in the supernatant was determined chemically [10] and the amount bound calculated by difference. The results were the same as with [$^3$H]Triton. Part of the drop in Triton binding/g original membrane at high Triton doses may be due to release of protein. From a maximum binding of approximately 88 μl/g protein, binding drops to approximately 70 μl/g remaining protein at the highest Triton dose shown. ●, Average ± S.E.M. from two or more experiments; ○, single data point.

were added together, either at 0 or 41°C, Triton inhibition was largely blocked. At 0°C, 6 mg HDL almost completely blocked the action of 4.8 μl Triton (data not shown).

The HDL wash appears to protect the membranes against Triton damage during the subsequent 41°C annealing treatment since (1) bound Triton is removed by HDL, (2) much more Triton is required for inactivation at 0°C than at 41°C (see below) (3) the shape of the 0 and 41°C dose-response curves differ (compare Figs. 1 and 3), and (4) the inhibition of uptake relative to trapping produced by Triton at 41°C (Fig. 1) is absent when the 0°C Triton treatment is followed by an HDL wash (Fig. 3).

*Effects of Triton at 0°C.* When membranes were treated with Triton at pH 7.2—7.6 and 0°C and the Triton removed with HDL before annealing, 50% inhibition of trapping occurred at a dose of 60 μl/g membrane but the uptake/trapping ratio was minimally reduced even at 120 μl/g. At 120 μl Triton/g 20% of both trapping and transport activities remained and only 20% of the membrane protein was released (data not shown).

To increase the effectiveness of Triton at 0°C we tried a higher pH, 8.5—8.8. (Exposure at 0°C to pH 8.5—8.8 does not by itself reduce trapping or glycine uptake activity (data not shown).) at 0°C and pH 8.5—8.8 Triton treatment inhibits trapping ability. The glycine transport activity of vesicles capable

TABLE I

REMOVAL OF TRITON FROM MEMBRANE PELLETS BY SALINE OR HDL WASHES

80 mg unannealed membrane pellets were suspended in 0.50 ml [$^3$H]Triton solution (0.26 mCi/ml Triton) or in 0.50 ml of this solution plus 1.0 ml 154 mM KCl. The [$^3$H]Triton solution also contained 2 mM EGTA, 1 mM $CaCl_2$, 10 mM TES, 24 mM lysine hydrochloride and 57 mM KCl and was brought to pH 9.4 (at 0°C) with KOH. The membrane suspensions were held at 0°C for 30 min, centrifuged 20 min at 30 900 × *g* and the supernatants removed by aspiration. The pH of these supernatants was 8.5—8.8 at 0°C. The pellets were suspended in 0.50 ml of a solution containing 2 mM EGTA, 1 mM $CaCl_2$, 139 mM KCl and 10 mM TES (pH 7.3—7.4 at 0°C) plus an amount of HCl calculated to neutralize the residual alkaline buffer in the pellet. For HDL treatment, the above solution also contained 3 mg HDL/0.50 ml. After 10 min at 0°C the suspensions were centrifuged as above and the supernatants removed. Triton in supernatants and pellets was determined and pellet values corrected for Triton in entrained supernatant as described in Methods. A saline 'wash' treatment consists of suspension in HDL-free solution, holding and centrifuging, while for an HDL 'wash', HDL was present. Each value is the average from two experiments.

| Triton added per g wet weight of membrane (μl) | First wash | Second wash | Triton remaining (μl/g membrane) |
|---|---|---|---|
| 60 | None | None | 3.4 ± 0.1 |
| 60 | Saline | None | 3.8 ± 0.2 |
| 60 | Saline | Saline | 3.0 ± 0.5 |
| 60 | HDL | None | 1.02 ± 0.09 |
| 15 | HDL | None | 0.90 ± 0.05 |
| 60 | Saline | HDL | 0.54 ± 0.06 |
| 60 | HDL | HDL | 0.27 ± 0.04 |

of trapping (uptake/trapping, Fig. 3) was much less affected. Figs. 4 and 5 show the effects of Triton treatment at 0°C and pH 8.5—8.8 on protein release and phospholipid release respectively. Triton binding under these conditions

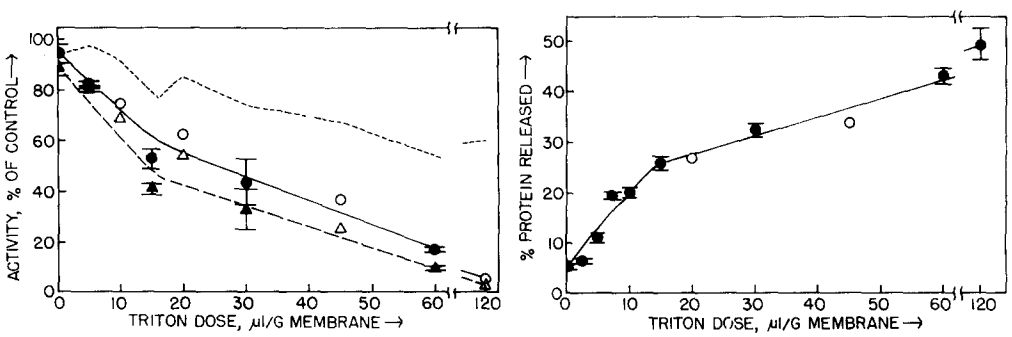



Fig. 3. Effects of Triton dose at 0°C, pH 8.5—8.8, on glycine trapping and uptake. Membrane was treated with the indicated doses of Triton at pH 8.5—8.8 for 30 min at 0°C, the suspensions centrifuged and residual Triton then removed with HDL as described in Table I. For the Triton dose of 120 μl/g (9.6 μl/80 mg), 6 mg HDL was used to remove residual Triton instead of 3 mg. In other experiments (data not shown) we found that with 60 μl Triton/g (4.8 μl/80 mg membrane), washing with 3 mg DHL/80 mg membrane by the procedure of Table I was sufficient to protect the membranes from Triton damage during subsequent annealing. Trapping and uptake were determined as described in Methods. ○,●, Trapping; △,▲, uptake; - - - - - -, uptake/trapping. Solid symbols, average ± S.E.M. from two or more experiments; open symbols, single data points.

Fig. 4. Effect of Triton dose at 0°C, pH 8.5—8.8, on protein release. Membrane was treated with Triton and centrifuged as for Fig. 3. Supernatants were analysed for protein as described in Methods. Data is presented as percent of protein in the original unextracted membrane. ●, Average ± S.E.M. from two or more experiments; ○, single data points.

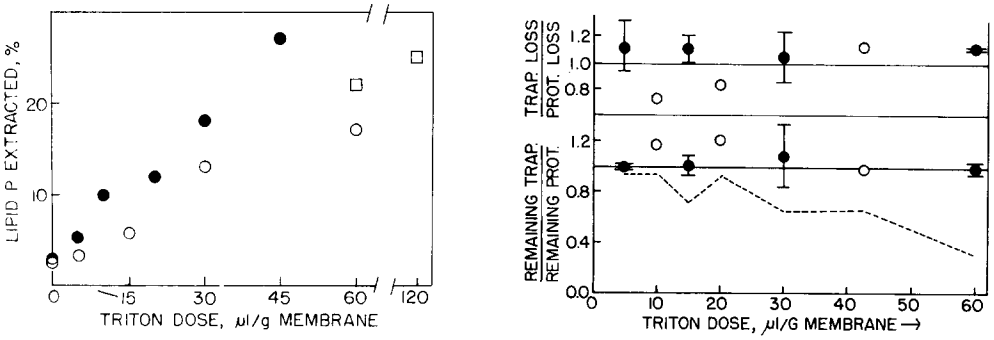



Fig. 5. Effect of Triton dose at 0°C, pH 8.5—8.8, on the release of lipid phosphorous. The procedure was as for Fig. 4 except that lipid phosphorous in supernatants and in the original untreated membrane was determined (Methods). Data is presented as percent of the lipid phosphorous in the untreated membrane. Different symbols show data from different experiments.

Fig. 6. Proportionality between protein released and loss of trapping ability. Membrane was treated with Triton at 0°C, pH 8.5—8.8, as described for Fig. 4. Protein is plotted as fraction of Triton-releaseable protein corrected for the fraction of the protein released in the absence of Triton (0.051). For '●,○' points, the maximum releaseable protein is taken to be 53% of the total membrane protein; the maximum release is taken to be 100% for the dotted curve. Solid symbols are averages ± S.E.M. from two or more experiments, open symbols are single data points. In the upper part of the figure, loss of trapping/loss of protein is plotted, while for the lower part, remaining trapping/remaining protein is plotted. Since the former plot is insensitive at high doses while the latter is intensive at lower doses, both plots are given to show the equivalence over the whole Triton dose range from 5 to 60 µl/g. (The trapping data was not felt to be as accurate at the 120 µl/g dose, so these points were omitted.)

was shown in Fig. 2. Glycine trapping activity, protein release and Triton binding all showed two phase responses to Triton dose; the dose-response curves were steeper below doses of 15 µl/g than at higher doses. In contrast, the release of lipid phosphorous was linear with Triton dose. SDS gel electrophoresis of Triton extracts showed preferential release of the lower molecular weight polypeptides (data not shown).

If we assume (see Discussion) that only 53% of the membrane protein can be released by Triton treatment at pH 8.5—8.8 and 0°C, then the percent loss of trapping ability is equal to the percent loss of this 'releasable' protein (Fig. 6).

The loss of trapping ability appears to be all-or-none. After a 5 min incubation at 40°C, vesicles retained approx. the same percentage of glycine trapped at 0°C (range, 66—89% of unincubated samples, $n = 13$) whether they were untreated or severely inhibited by Triton (e.g. 120 µl/g at 0°C, pH 8.5—8.8 or 8 µl/g at 41°C (data not shown)).

At low Triton doses increasing Triton binding was accompanied by increasing protein release and inhibition of trapping. At higher Triton doses no more Triton was bound, yet the Triton effects still increased with Triton dose. This might occur if membrane proteins partitioned between the membrane and the extramembranal Triton micelles. The data of Tables II and III and Fig. 7 are consistent with this interpretation. First, at a fixed Triton/membrane ratio, protein release, inhibition of trapping and Triton binding were independent of the Triton concentration (Table II). Second, a partition process should eventually reach equilibrium. Triton action on trapping capacity was nearly complete in 10 min at 0°C (Fig. 7). (Triton action was stopped by addition of

TABLE II

TRITON EFFECTS DEPEND ON THE TRITON/MEMBRANE RATIO, NOT THE TRITON 'CONCENTRATION'

80 mg membrane pellets were treated with 60 μl Triton/g membrane at 0°C, pH 8.5—8.8, for 30 min with solutions containing the amounts of TES and lysine buffers present in 0.50 ml of the usual high pH Triton solution (Table I) but 2 mM in EGTA, 1 mM in Ca and with enough KCl to bring the total salt concentration to 180 mosM. Trapping and uptake were determined after an HDL wash as for Fig. 3., protein release and Triton binding were determined as for figs. 4 and 2 respectively. Each value is the average from two experiments except for the last column where the experiments are presented separately (see below).

| Triton concentration (%, v/v) | Extraction volume (ml) | Trapping | Uptake | Uptake / Trapping | Protein release (%) | Triton bound (μl/pellet) | |
|---|---|---|---|---|---|---|---|
| | | | | | | Expt. 92 | 95 * |
| 0.96 | 0.50 | 18.5 ± 3.5 | 10 ± 3.0 | 52.5 ± 3.5 | 43 ± 1 | 0.20 | 0.29 |
| 0.32 | 1.50 | 21.0 ± 4.0 | 25 ± 2.5 | 59.5 ± 0.5 | 38 ± 1 | 0.28 | 0.29 |
| 0.096 | 5.00 | 15.5 ± 0.5 | 10 ± 1.0 | 64.0 ± 6.0 | 40 ± 2 | 0.35 | 0.26 |

* For this experiment, the pellets were, in addition, washed once with saline (saline wash of Table I) since with a high concentration of unbound Triton in the supernatants, the correction of pellet Triton for Triton in the entrapped supernatant is large.

HDL.) The release of protein by Triton and Triton binding were complete in less than 30 min at 0°C (Table III). (In the experiments of Table III the action of Triton was stopped by a 20 min centrifugation. Hence a '5 min' exposure time means 5 min plus the time required for the vesicles to actually pellet.) Finally, a partition mechanism implies that the Triton in a Triton extract should be relatively ineffective in inactivating fresh membrane. This was tested using membrane extracts made with 60 and 120 μl Triton/g membrane. The '60 T' extract was only as effective as 20—30 μl of fresh Triton (trapping 56%; uptake, 34% ($n$ = 2)) although it delivered 56 μl of Triton/g fresh membrane (corresponding trapping, 21%; uptake, 12% (Fig. 3)). The '120 T' extract, delivering 110 μl/g, had the effect of 50—55 μl fresh Triton ($n$ = 2).

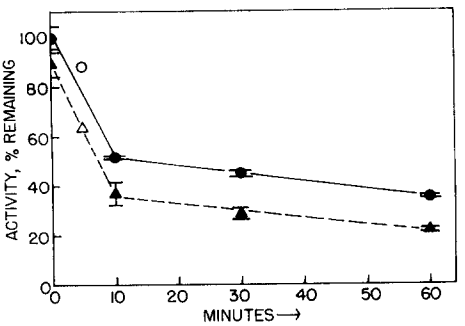



Fig. 7. Time dependence of Triton action at 0°C, pH 8.5—8.8, on glycine uptake and trapping. Membrane was treated for the indicated time with 60 μl Triton/g wet weight as described in Table I. At the end of the treatment period 6 mg HDL (open symbols) or 12 mg HDL (solid symbols) were added. The samples were centrifuged and given an additional wash with 3 mg HDL, as described in Table I, and annealed, and uptake and trapping measured as described in Methods. ●,○, Trapping; ▲,△, uptake. Solid symbols, average ± S.E.M.; open symbols, single data points.

TABLE III

TRITON BINDING TO MEMBRANE, AND PROTEIN RELEASE AS A FUNCTION OF TRITON TREATMENT TIME AT 0°C, pH 8.5—8.8

80 mg membrane pellets were suspended in 0.50 ml Triton-containing solutions as described in Table I. After holding for the indicated time the samples were centrifuged 20 min at 30 900 × *g* and the supernatants and pellets analysed for protein and [$^3$H]Triton as described in Methods.

| Triton dose (μl/g membrane) | Triton bound (μl/g membrane) | | | Protein release (% of total) | | |
|---|---|---|---|---|---|---|
| | After 5 min (*n*) | 30 min (*n*) | 60 min (*n*) | After 5 min (*n*) | 30 min (*n*) | 60 min (*n*) |
| 2.5 | 1.5 ± 0.1 (2) | 1.5 ± 0.1 (2) | 1.5 ± 0.1 (2) | 5.9 ± 0.6 (2) | 6.8 ± 0.3 (2) | 6.7 ± 0.6 (2) |
| 5.0 | | 2.9 ± 0.2 (3) | | | 10.9 ± 2.0 (5) | 10.4 (1) |
| 15.0 | | 4.6 ± 0.2 (3) | 4.9 (1) | | 26.3 ± 2.8 (4) | 23.6 (1) |
| 30.0 | | 3.9 ± 0.2 (2) | 4.2 (1) | | 32.6 ± 2.9 (4) | 33.1 (1) |
| 60.0 | 2.6 ± 0.5 (2) | 2.8 ± 0.4 (3) | 3.0 (1) | 30.8 ± 4.0 (2) | 41.7 ± 2.7 (4) | 40.4 ± 3.4 (2) |

## Discussion

In their recent review, Helenius and Simons [2] expressed the view that detergents (especially nonionic ones) first dissolve in the membrane and then "as a result of their wedge shape which causes a pressure for increasing curvature of the bilayer . . ." (ref. 2, p. 46) cause a phase transition from lipid bilayer saturated with detergent to mixed micelles of detergent containing lipid and protein. "The extent of solubilization depends mainly on the amount of detergent bound and can therefore best be correlated with the ratio of bound detergent to membrane" (ref. 2, p. 59). The ratio of added detergent to membrane governs the action of the detergent because ". . . the free unbound detergent will constitute just a small portion of the total detergent in the sample" (ref. 2, p. 60). "This suggests that it is the ability to disperse the membrane lipid that determines the effectiveness of nonionic detergents in membrane solubilization", (ref. 2, p. 61).

Our observations do not conform to such a mechanism. First, the pigeon erythrocyte membrane is saturated with Triton at a Triton/protein ratio of 0.09. With such a low Triton/protein ratio, the bilayer-to-micelle phase transition seems implausible. Helenius and Soderlund [12] report that breakdown of Semliki Forest virus membrane first begins at a Triton/total protein ratio of 0.17 (only about half of the protein is in the membrane) and membraneous structures are still seen at a Triton/membrane protein ratio of 0.9. Second, we found no correlation between Triton bound and protein released in the high dose range. Third, micellar Triton and membrane phases coexist and at least some of the extramembranal Triton is active. Fourth, protein release is selective and limited (see below), and shows a dose-response pattern quite different from that expected of a phase transition, where a small increase in dose above a critical level should cause a large conversion from one phase to the other. Fifth, protein release can be fitted, as a first approximation to a model where protein distributes between the membrane phase and extramembranal Triton micells (see below).

We do not suppose the interpretation and generalizations presented by Helenius and Simons are wrong, but rather that there is an additional mode of action of detergents. The chemical potential of the monomeric detergent is limited by the critical micelle concentration [13]. If this chemical potential is too low to cause the bilayer-to-mixed micelle phase transition, then additional detergent will exist as a separate phase (as we observe) and partition of membrane components can occur between membrane and detergent micelle phases. Taking $P_0$ to be the fraction of the total protein which can be released by Triton, $P_t$ the fraction of releasable protein released at a given Triton dose, and $P_m$ the fraction of releasable protein remaining in the membrane, $P_0 = P_t + P_m$ and $P_t/P_m = K_d T/m$. $K_d$ is the distribution coefficient for releasable protein, $T$ is unbound Triton and $m$ is membrane phase. Combining and rearranging gives $1/P_t = m/K_d P_0 T + 1/P_0$. Fig. 8 shows data plotted according to this equation. Lines connect data points from the same experiment. From $1/T = 0.1$ to $1/T = 0.5$ (corresponding to Triton doses of 120 to 30 $\mu$l/g, the 'high dose' range) the slopes of these lines are approximately the same. From the intercept, $P_0$ is 0.5. Using a value for $P_0$ of 0.53 produced a 1 : 1 relation-

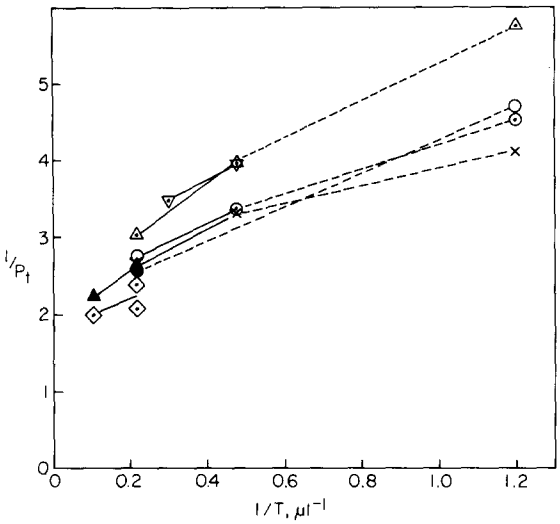



Fig. 8. Double reciprocal plot of protein released vs. unbound Triton. Protein is expressed as the fraction of the total membrane protein. $P_t$, the protein released, and $P_0$, the maximum total protein that can be released were both corrected for protein release in the absence of Triton (0.051). $T$ is unbound Triton. The equation plotted in $1/P_t = m/K_d P_0(T) + 1/P_0$ (Discussion). Triton treatment was for 30 min at pH 8.5—8.8 and 0°C as for Fig. 4. Different symbols represent different experiments. Data points obtained in the same experiment are connected.

ship between protein release and loss of trapping ability (Fig. 7). This equivalence is lost if $P_0 = 1$. That $P_0 < 1$ is also indicated by SDS gel electrophoresis of Triton extracts, which showed selective release of low $M_r$ proteins. Thus, the relationship between protein released and the amount of extramembranal Triton is consistent with a phase distribution mechanism for Triton action in the high dose range, and the $P_0$ value obtained with this model is consistent with the $P_0$ value relating protein release to loss of trapping.

There is precedent from simple lipid-Triton systems for such a phase distribution process. Dennis and Owens [14] interpreted their NMR data on the states of Triton and phosphatidylcholine protons, below the limiting Triton/lipid mole ratio of 2, in terms of distribution of components between separate phospholipid-Triton and Triton-phospholipid phases. Yedgar et al. [15] also noted several coexisting sphingomyelin-Triton phases below a critical Triton/lipid mole ratio of 0.32.

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## References

1 Lee, J.W., Beygu-Farber, S. and Vidaver, G.A. (1973) Biochim. Biophys.Acta 298, 446—459
2 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29—80
3 Burstein, M. and Scholnick, H.R. (1973) in Advances in Lipid Research (Paoletti, R. and Kritchevsky, D., eds.), Vol. 11, 68—108

4 Burstein, M. (1962) Life Sci. 12, 739—744
5 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
6 Ames, B.N. (1966) Methods Enzymol. 8, 115—117
7 Scanu, A.M. (1972) Biochim. Biophys. Acta 265, 471—508
8 Kates, M. (1972) Techniques of Lipidology, p. 352, North-Holland/American Elsevier
9 Razin, S. (1972) Biochim. Biophys. Acta 265, 241—296
10 Garewal, H.S. (1973) Anal. Biochem. 54, 319—324
11 Yu, J., Fischman, D.A. and Steck, T.L. (1973) J. Supramol. Struct. 1, 233—248
12 Helenius, A. and Soderlund, H. (1973) Biochim. Biophys. Acta 307, 287—300
13 Tanford, C. (1973) The Hydrophobic Effect: Formation of Micelles and Biological Membranes, p. 48, John Wiley and Sons, New York
14 Dennis, E.A. and Owens, J.M. (1973) J. Supramol. Struct. 1, 165—176
15 Yedgar, S., Barenholz, Y. and Cooper, V.G. (1974) Biochim. Biophys. Acta 363, 86—97