Solubilization and Purification of Human Erythrocyte Membrane Acetylcholinesterase[1] (37620)

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(Introduced by Elliot Newman)

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In recent years the structure and function of the erythrocyte membrane has attracted considerable attention since: (1) the red cell membrane is an excellent model for studying properties of biological membranes; (2) in several hematologic disorders the basic defect has been shown to be in the red cell membrane; and (3) large quantities are readily available in a relatively homogenous state. Paroxysmal nocturnal hemoglobinuria (PNH)[2] typifies a red cell membrane disorder and is characterized by decreased erythrocyte membrane acetylcholinesterase (AChE) activity (1, 2).

Despite many advances, knowledge concerning the structure and properties of the erythrocyte membrane is far from complete. Disaggregation of the membrane components without destroying biological properties remains a difficult task. Membrane enzymes, such as AChE and ATPase, are usually inactivated during solubilization (3). Ordinarily solubilization of erythrocyte membranes requires the use of high concentration of salts (4, 5), chelating agents, protein dissociating agents, surfactants and organic solvents, separately or in combination. However, none of these procedures has resulted in total disaggregation of components of red cell membrane without some impairment of their biological integrity. In the present investigation, the direct effect of solubilizing agents on AChE activity was studied. Methods for solubilizing membrane protein with recovery of protein and AChE activity were assessed. A relatively simple procedure for isolation and purification of membrane AChE was described.

Methods and Materials. Effect of solubilizing agents on AChE activity. The following solubilizing agents were employed at different concentrations: bile salts, Triton X-100, Tergitol NPX, sodium cholate, sodium deoxycholate, Tweens 20, 40, 60, and 80, saponin, and EDTA. One part of 10% distilled water hemolysates prepared from washed normal human red cells was mixed with one part of solubilizing agent solution of double the final desired strength. The mixtures were stored at 4° for 10 hr prior to analysis for AChE activity.

Solubilization of red cell membrane. Hemoglobin-free stroma was prepared from outdated (21 days old) blood bank blood by the method of Dodge *et al.* (6). Washed stroma was lyophilized, stored at —70°, and used for further processing within 48 hr. The stepwise flow sheet for the solubilization procedure is outlined in Table II.

One hundred milliliters of 1.2 M KCl containing 5 mM EDTA were added to the stroma and the mixture was stirred for 90 min at 4°. The sample was then centrifuged in a refrigerated Sorvall RC-2 centrifuge at 17,000g for 1 hr. The supernatant was diluted to 100 ml with the KCl–EDTA solution (Fraction I). The residue was taken up in 25 ml of 5 mM PO_4 buffer (pH 7.8) and dialyzed overnight against 4 liters of this same buffer. The dialysate was diluted to 100

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[2] Abbreviation used: AChE = acetylcholinesterase, PNH = paroxysmal nocturnal hemoglobinuria, DTNB = dithiobisnitrobenzoic acid.

TABLE I. Effect of Various Membrane Solubilizing Agents on AChE Activity of Intact Erythrocytes.[a]

Solubilizing agent	% of the control activity at concentrations of:			
	0.5 M[b]	1.0 M	2.0 M	5.0 M
Sodium deoxycholate	62	36	33	26
Sodium cholate	99	94		
Bile salts	101	103	100	92
Triton X-100	102	102	101	95
Tergitol NPX	99	101	98	94
Tween 80	98	91	94	
Tween 20	104	100	96	89
EDTA[c]	109	106	103	101
Saponin[d]	105	107	103	109
1.2 M KCl	—	99		
1.2 M KCl + 5 mM EDTA	—	109		
1.2 M KCl + 5 mM EDTA + 5% Triton X-100	—	108		

[a] 5% hemolysates from washed red cells were made in the solubilizing agents at the indicated final concentrations and stored at 4° for 10 hr. The samples were then analyzed for AChE.

[b] Concentrations are expressed as g% except where indicated.

[c] Concentrations expressed in mM.

[d] Concentrations expressed as 50, 100, 200, and 500 mg%.

ml again with the same buffer, and 0.5 ml Triton X-100 was added. The mixture was stirred for 30 min and centrifuged at 17,000g for 1 hr. The supernate was separated and diluted to 100 ml with PO_4 buffer (Fraction II). The residue was then made up to 70 ml with PO_4 buffer, Triton X-100 was added to a final concentration of 1%, and the mixture was stirred and centrifuged as before to obtain the supernatant Fraction III. This process of taking up the residue in PO_4 buffer and collecting the supernatant was continued with increasing addition of Triton X-100 up to 5% concentration. Five soluble fractions were thus collected to this point (Fractions I–V, Table II). The insoluble residue was then stirred with 60 ml of a mixture containing 1.2 M KCl, 5 mM EDTA, and 5% Triton X-100 for 10 min and centrifuged at 17,000g for 1 hr. The supernatant (Fraction VI-A) and a final insoluble residue (Fraction VI-B) were thus obtained. The different fractions were analyzed for protein and AChE.

Purification of AChE from human red cells. Hemoglobin-free stroma was prepared from 200 ml packed red cells as described in the

TABLE II. Stepwise Solubilization of Erythrocyte Stromal AChE.

Fraction no.	Solubilizing agent	% Solubilization	
		AChE	Protein
I	1.2 M KCl containing 5 mM EDTA (90 min, 4°)	12.3	11.5
II	0.5% Triton X-100	13.4	13.4
III	1% Triton X-100	17.2	16.6
IV	2% Triton X-100	25.2	11.0
V	5% Triton X-100	17.8	7.2
VI-A	1.2 M KCl containing 5 mM EDTA + 5% Triton X-100	6.0	21.8
VI-B	None (residue after removing VI-A)	3.0 (insol)	17.5 (insol)
	Total solubilization	91.9	79.5

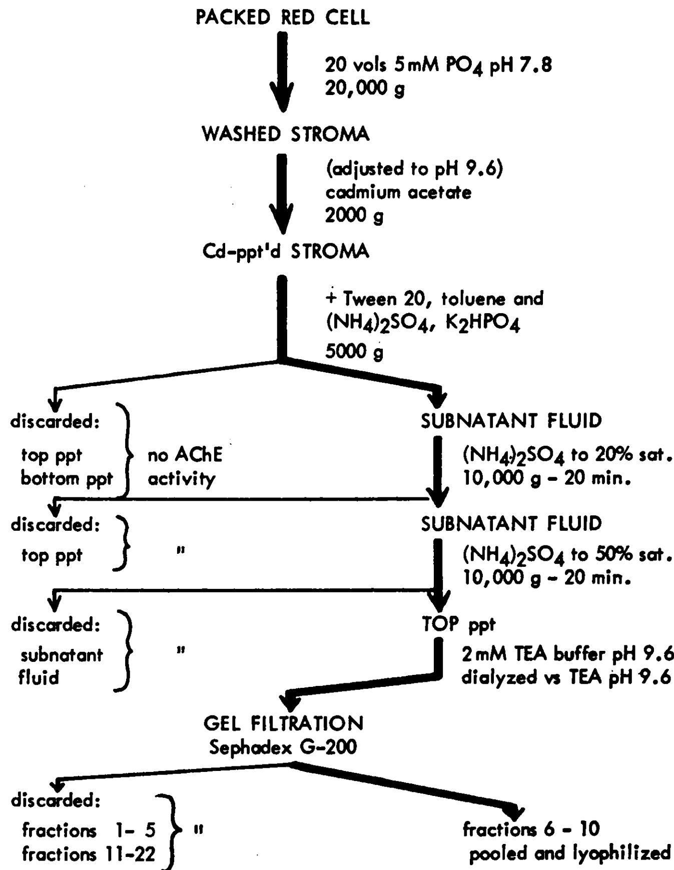

FIG. 1. Solubilization and purification of red cell stromal AChE.

previous paragraph. The outline for subsequent steps is given in Fig. 1. The volume was made up to 200 ml with distilled water, and the pH was adjusted to 9.6 by adding 0.5 *N* NaOH with constant stirring. Twenty ml of 1 *M* Cadmium (Cd) acetate was added with continuous mixing. After stirring for 10 min at 4°, the precipitate was collected by centrifugation at 2000*g* for 10 min at 4° and washed once with an equal volume of cold water.

The Cd-precipitated stroma was suspended in 200 ml of 0.1 *M* triethanolamine (TEA) buffer (pH 9.6). Ten milliliters each of 2.3 *M* K_2HPO_4, toluene and Tween 20, and 54 ml of 4 *M* $(NH_4)_2SO_4$ were added while the sample was stirred at 4° for 10 min and then centrifuged at 5000*g* for 20 min. The solid top layer which contained the bulk of lipids and Tween as well as the precipitate at the bottom which contained no appreciable enzymic activity was discarded. Solid $(NH_4)_2SO_4$ was added to the solution to 20% saturation and centrifuged at 17,000*g* for 20 min. The solid top layer which contained the remainder of the lipids and Tween was discarded. $(NH_4)_2SO_4$ was then added to the solution to 50% saturation, and the mixture was centrifuged at 17,000*g* for 20 min. The solid top layer was dissolved in 20 ml TEA buffer. A clear solution containing most of the AChE activity was obtained. The enzyme was dialyzed for 24 hr against 4 liters of 2 m*M* TEA buffer with four buffer changes at approximately 6-hr intervals. The dialyzed enzyme was then passed through an 18 × 2.5 cm column of Sephadex G-200 at 4° (equilibrated with 2 m*M* TEA buffer). Five-milliliter fractions were collected. Fractions 5–11 into which the enzymic activity was eluted were pooled and lyophilized.

Assay for AChE activity. In the studies

of the effect of solubilizing agents on AChE activity, analysis was carried out by the method of Ellman *et al.* (7) adapted to the Technician Autoanalyzer in the manner described by Levine *et al.* (8). In other studies, the Ellman procedure for AChE was used without modification, employing a Beckman DU spectrophotometer.

Protein. Protein was assayed by Lowry's method (9).

Polyacrylamide gel electrophoresis. A 9.5% polyacrylamide system was used with 10% glacial acetic acid as the buffer. The gel column was 10 cm and electrophoresis was carried out for 15 hr (0.6–1 mA/gel). The gels were then stained with 1% Amido Schwartz.

Results. Effect of membrane solubilizing agents on AChE activity. Preliminary to solubilization attempts, commonly employed membrane solubilizing agents were tested for any possible direct effect on human erythrocyte AChE activity. Only sodium deoxycholate significantly inhibited the enzymic activity in human red cell hemolysate (Table I). Saponin had a slight stimulating effect in agreement with previous observations by Doizaki *et al.* (10). However, saponin alone directly reduced DTNB, which could readily account for the observed stimulating effect. The ten other substances studied had no effect on the assay system. Similar results were obtained employing purified *bovine* red cell AChE (Sigma Chemical Company).

Solubilization of membrane protein and AChE. The effect of KCl, EDTA, and Triton X-100 in solubilizing the red cell membrane proteins and AChE is shown in Table II. Stepwise solubilization procedures demonstrated that only about 80% of the membrane proteins could be solubilized *in toto*, whereas about 92% of the AChE could be brought into solution. Protein electrophoresis on polyacrylamide gel revealed at least

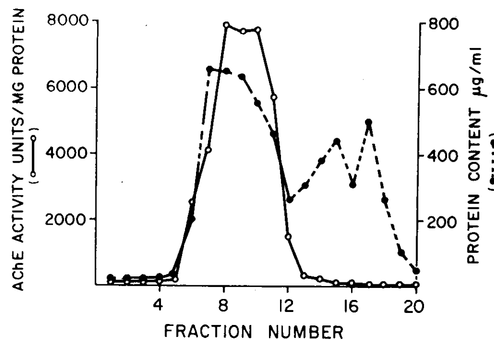

FIG. 2. AChE gel filtration on Sephadex G-200 (18 × 2.5 cm).

11 bands in all the fractions. The starting material showed 17 bands as previously reported by Rosenberg and Guidotti (11).

Purification of AChE from red cell ghosts. The flow sheet for solubilization and purification of AChE from red cell ghosts is shown in Fig. 1. It can be seen from Table III that this procedure yielded a preparation with sp act of 7100 units/mg protein and about 4,200-fold purification.

The 50% $(NH_4)_2SO_4$ fraction was freely soluble in 2 m*M* TEA buffer. Unlike previously described methods for gel filtration of solubilized red cell membrane (11–13), no protein denaturing agent or detergent was necessary to keep the enzyme in solution. Figure 2 shows AChE activity and protein content of the fractions obtained from the Sephadex G-200 column. AChE was eluted in a single peak in the void volume, eliminating other proteins which came off the column in later fractions. The homogeneity of the protein part of this fraction was demonstrated by immunizing rabbits against the enzyme and obtaining a single precipitin band in immunodiffusion on agar plates by the method of Ouchterlony (14). However, the fact that the "precipitate" with 50%

TABLE III. Purification of Erythrocyte AChE.

Sample	AChE activity[a]	Fold purification	Yield (%)
1. Packed, washed red cells	1.7	—	—
2. Red cell stroma	24.2	14	93
3. 50% $(NH_4)_2SO_4$ fraction	1213	714	44
4. Pooled sephadex fractions	7100	4176	26

[a] µmoles of acetylthiocholine iodide hydrolyzed/mg protein/hr.

$(NH_4)_2SO_4$ saturation floated on the top of the solution indicates that the preparation may still be associated with some lipid, perhaps as an integral part of the enzyme.

Discussion. A close association between lipids and proteins is essential for stability and integrity of the erythrocyte membrane. Our experiments with commonly used membrane solubilizing agents were intended to establish whether there was any direct effect of such on AChE as a marker enzyme. Sodium deoxycholate, commonly used for solubilization, was strongly inhibitory toward human erythrocyte AChE as well as toward purified bovine red cell AChE. However, bile salts which contain compounds closely related to sodium deoxycholate (*e.g.,* salts of taurocholic and glycocholic acid) did not affect this enzyme.

From studies on the effect of concentrated salt solutions on the red cell membrane, Mitchell and Hanahan (4) concluded that AChE is located in the outer regions of the membrane which are more accessible to solubilization of AChE. This suggestion has been strengthened by studies showing that exposure of intact red cells to proteases substantially reduces AChE activity (15, 16). In our studies, however, a variety of drastic treatments with salt, chelating agent, and detergent were required to bring most of the AChE into solution from lyophilized red cell stroma, suggesting that the enzyme is an "integral part" of the membrane structure and not just loosely associated therewith. Moreover, enzymic activity closely paralleled the solubilization of membrane proteins (Table II). Furthermore, cytochemical staining of red cells for AChE employs the use of concentrated Na_2SO_4 solution. Yet even with the use of such concentrated salt solution, the reaction products of the staining procedure appear to originate in the red cell membrane (17).

To our knowledge, our procedure for the isolation of AChE from *human* red cell membrane gives a preparation much purer than any previously reported. Isolation of a pure preparation of AChE from human red cells has a variety of practical applications. For example, immunological studies with the antibody for this enzyme could prove useful in elucidating the nature of the reduced red cell AChE activity seen in PNH and related disorders (18). This might help determine whether there is a reduction in enzymic synthesis or a defect in the enzymic protein with poor catalytic activity as a result. Furthermore, similar studies might be extended with preparations of other components of the membrane, so that, using immunoelectrophoresis the basic defect in the various red cell membrane disorders might be pinpointed.

Summary. A variety of commonly employed membrane solubilizing agents were studied for a possible effect on erythrocyte AChE activity. Only sodium deoxycholate had any appreciable inhibitory effect.

Stepwise solubilization of red cell membrane with a variety of agents showed no selective removal of membrane AChE. Rather the release of enzymic activity closely paralleled the solubilization of membrane proteins, suggesting that AChE is an integral part of the membrane.

A relatively simple method was described for the isolation and purification of human red cell AChE which resulted in a 4200-fold purification. The enzyme appears to be associated with lipids and gave a single precipitin band on immunodiffusion.

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