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HUMAN ERYTHROCYTE MEMBRANE BOUND ENZYME ACETYLCHOLINESTERASE

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SUMMARY

1. The activating effects of Na^+ and Ca^{2+} on human erythrocyte membrane-bound acetylcholinesterase are largely independent of acetylcholine concentration. However, the optimal concentration of Mg^{2+} is dependent upon substrate concentration.

2. Solubilization of 10–45 % of erythrocyte membrane protein by hypertonic solutions of NaCl, $CaCl_2$, sucrose or 1 mM EDTA + 50 mM mercaptoethanol was accompanied by loss of only 10–20 % of acetylcholinesterase activity.

3. The nonionic detergent Triton X-100 causes a 50 % decrease in absorbance of turbid membrane suspensions at a detergent concentration $c(\mu M) = 0.76 \pm 0.08$ (mg protein per ml.). This clearing is taken as an indication of membrane solubilization and is not strongly dependent upon pH. The anionic detergent sodium dodecyl sulfate causes a similar decrease at concentration $c(\mu M) = 0.56 \pm 0.06$ (mg protein per ml) at pH 7.5–8. This concentration increases with increasing pH. Acetylcholinesterase is irreversibly inactivated by sodium dodecyl sulfate.

INTRODUCTION

Reports from this laboratory have shown that acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) activity of human or bovine erythrocytes is associated with the membrane[1–3]. There is, however, a difference between the acetylcholinesterase bound to bovine and human membranes by the very fact that upon hemolysis of the erythrocytes with hypotonic salt solutions the former released the enzyme during the process while the latter did not[2,3]. Ca^{2+} or Mg^{2+} at 1–5 mM prevented the release of acetylcholinesterase from bovine erythrocyte only when present during the initial hemolytic event[3].

High concentrations (0.4–1.2 M) of NaCl were shown to release lipoproteins from human erythrocytes ghost or intact bovine erythrocytes[3,4]. Other attemps to solubilize the erythrocyte membrane proteins and concomitantly follow the enzymatic

Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

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activities or other functions of the erythrocytes membrane such as antigenic properties, have also been reported[5-8]. These approaches included treatment of the membranes with mild agents which are known not to cause denaturation, or with compounds which have been shown to cause denaturation (such as high concentrations of urea or certain detergents).

In the present communication we have studied some properties of the acetylcholinesterase of the mammalian erythrocyte membrane, with particular attention to the effects of mono- and divalent cations. We have attempted to detach acetylcholinesterase activity from the membrane by various treatments including high concentrations of NaCl, $CaCl_2$ and sucrose. The effects of an anionic detergent (sodium dodecyl sulfate) and a non-ionic detergent (Triton X-100) on the solubilization of the membrane and on the acetylcholinesterase activity have been studied in some detail.

EXPERIMENTAL

General materials and methods

Fresh human blood type O, Rh^+, obtained from the Community Blood Bank, Tucson, Arizona, was used as source of the erythrocytes. Clotting was prevented by the use of acid–citrate–dextrose U.S.P. solution (0.8 g citric acid, 2.2 g sodium citrate and 2.45 g dextrose per 100 ml solution). The blood was centrifuged at 800–1000 *g* for 20 min, and the plasma and buffy coat were removed. The packed erythrocytes were washed 3 times with equal volumes of either isotonic (0.155 M) Tris–HCl buffer (pH 7.5–8.0), or unbuffered, isotonic NaCl solutions. Following each washing the cell suspension was centrifuged, the supernatant and the remaining leucocytes were removed. Finally, the erythrocytes were suspended in isotonic Tris–HCl (pH 8) or isotonic phosphate buffer (pH 7.5). Membranes were prepared according to DODGE *et al.*[1] by hemolyzing the packed cells with 10–12 vol. of hypotonic (20 imosM) Tris–HCl or phosphate buffers at pH values from 7.27 to 8.0. The membranes were washed 3–6 times with the same hypotonic solution, followed by centrifugation at $30000 \times g$ for 60 min at 4°. The last wash was done with isotonic buffer, which was found to reduce the content of hemoglobin considerably. The milky, sometimes pinkish, membranes were then packed from the isotonic solution by centrifugation, and their shape examined under phase contrast microscope. These were stored for short periods at 4° as concentrated packed preparations in isotonic medium without any preservative, or for longer periods at the same temperature — in the presence of 0.02 % NaN_3, or at −18 to −30°. The azide has no effect on the acetylcholinesterase activity[9].

Protein was determined according to LOWRY *et al.*[10], as modified by HESS AND LEWIN[11], using bovine serum albumin for standard.

Treatment of membranes

Hypertonic solutions. (a) Human erythrocyte membrane containing 4–5 mg protein/ml in a solution of 5 mM Tris–HCl buffer, 1 mM dithiothreitol, 1 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N*, *N*′-tetraacetic acid (EGTA) (pH 8.0), were suspended in 10 vol. of a 1.2 M solution of either NaCl, $CaCl_2$ or sucrose. They were incubated in the presence or absence of 1 mM dithiothreitol, at pH 7.7–8.2 for 15 h at 4°. The suspension was then centrifuged at $35000 \times g$ for 70 min, and the sediment thus obtained suspended in the original solution to the original volume.

(b) Alternatively, the membranes were treated similar to that described in (a), except that the preparations were suspended in 8 vol. each of the hypertonic solutions of 1 M concentration at pH values of 6.2–6.5. The pellets obtained by centrifugation at 35000 × *g* for 60 min were dialyzed against 200 vol. of 5 mM Tris–HCl, 1 mM dithiothreitol, 1 mM EDTA at pH 8.0 for 20 h at 4°. As controls for treatments in Expts. a and b membrane suspensions were treated similarly with solutions in which 1 and 1.2 M salts were omitted.

(c) Membrane suspensions containing 10 mg/ml protein in isotonic saline were treated with 20 vol. of unbuffered 1 M NaCl solution according to MITCHELL *et al.*[2].

Reducing agents. The procedure described in this paper is a combination of the methods of ROSENBERG AND GUIDOTTI[12] and of MITCHELL *et al.*[2]. A membrane suspension (0.6 mg/ml protein) in 10 mM Tris–HCl buffer (pH 7.25), was dialyzed against 100 vol. of 50 mM mercaptoethanol, 1 mM EDTA (pH 7.25) for 36 h at 4°. Following centrifugation at 20000 × *g* for 60 min, the supernatant thus obtained ("Sup 1") was concentrated by passing it through a Diaflo membrane (UM-10) and then dialyzed. The pellet ("Pct 1") was suspended in 10 vol. of 1.2 M NaCl and stirred for 18 h at 4°. It was then centrifuged at 14000 × *g* for 60 min. The supernatant ("Sup 2") was concentrated and dialyzed as described above. The pellet ("Pct 2") was suspended in 10 mM Tris–HCl buffer (pH 7.2). All the fractions were dialyzed overnight at 4° against 100 vol. of 10 mM Tris–HCl buffer (pH 7.25), prior to the determination of the protein concentration or acetylcholinesterase activity.

Sodium dodecyl sulfate. Solubilization of membranes was observed as percentage change in the turbidity of a suspension before and after incubation in detergent solutions at 25° for 30 min. Apparent turbidity was measured as the absorbance at 600 or 530 nm. Subsequent to incubation of the suspensions with 0.1–10 mM dodecyl sulfate, the mixtures were centrifuged for 30–60 min at 105000 × *g* at 4°. Protein content and acetylcholinesterase activity of supernatant and precipitate, as well as of untreated starting materials, were then determined. In several experiments, dodecyl sulfate was added to membrane suspensions maintained at pH values in the range 6–9 in the reaction vessel of a Radiometer Titrigraph/pH-stat. Both acetylcholinesterase activity and decrease in absorbance at 600 nm were determined simultaneously. In another set of experiments, suspensions of membranes at concentrations of 0.11 and 0.22 mg protein per ml, respectively, were maintained at pH 8.7 and 25° in the reaction vessel. The acetylcholinesterase activity was measured as a ml function of time after the addition of dodecyl sulfate. For comparison, membrane suspensions (0.5–1 mg protein per ml) in isotonic saline were subjected to ultrasonic irradiation for 2 min in a cooled vessel (0–4°) using a Branson sonifier with 6 mA output. Also, similar suspensions were frozen at −70° and thawed (37°) 3 times. Subsequent to these treatments the membrane suspensions were diluted with water and observed under phase contrast microscope.

Triton X-100. To ghost suspensions of 0.1–1 mg protein per ml, Triton X-100 to final concentrations 0.05–5 % was added. Assuming a molecular weight of 646, this is approx. 0.8–80 mM detergent. Inasmuch as the detergent possessed a strong phenolic absorption at 280 nm, protein concentration could not be estimated at this wavelength. Further, the detergent interfered with the protein determination as described by LOWRY *et al.*[10]. Therefore the decrease in absorption (turbidity) at 600 nm

was the only measure of solubilization employed. Activity of acetylcholinesterase in the detergent-treated membranes was assayed as described previously.

Combined dodecyl sulfate and Triton X-100. Acetylcholinesterase activity was determined following 2 min incubation of 0.1–0.2 mg ghost membrane protein per ml with variable concentrations of Triton X-100 in the range 1–5 mM (*i.e.* 0.05–0.5 %) with 3.7 or 5.4 mM dodecyl sulfate. The anionic detergent was added to the ghost suspensions at pH 8.5 and 25°, after the addition of Triton X-100.

Density gradient centrifugation of membranes

Suspensions in 3 mM glycine buffer (pH 8.5) of membranes (1–3 mg/ml), or membranes incubated with 5–10 mM dodecyl sulfate at pH 8.5 (1–2 mg/ml) were layered in 0.5-ml aliquots on a 5-ml sucrose density gradient prepared from 15–50 % (w/v) sucrose. The sucrose solutions were prepared in 3 mM glycine (pH 8.5) with and without dodecyl sulfate at the same concentrations used to solubilize the membranes. 0.02 % NaN_3 was added to prevent microbial growth. The density gradients were prepared with a gradient former (Büchler).

The tubes with the membranes were centrifuged at 10, 15, or 24° for 22-24 h at 40000 rev./min (approx. 120000 × *g*) in a SW-65 rotor using a Spinco, Model L2-65B preparative ultracentrifuge. Fractions of 0.2 or 0.5 ml were collected from the bottom of the tubes with a bottom tip collector (Büchler) attached to a peristaltic pump (Büchler) which was connected to a fraction collector (ISCo). The protein concentration of each fraction was determined, and also the density of the corresponding fraction was measured by determining the refractive index by means of an Abbé refractometer (Bausch and Lomb). The refractometer was equipped with a device for circulating water, so that the values were read at the same temperature as that of the centrifugation. The refractive index values were converted to density through use of International Critical Tables. In some cases the fractions thus obtained were extracted with chloroform and methanol according to BLIGH AND DYER[13] and the lipid fraction chromatographed on thin-layer plates with the developing mixture of chloroform–methanol–water (93:35:6, by vol.).

Equal aliquots were used, so that the intensity of the spots, which were made visibly by charring with conc. H_2SO_4, could be compared.

Phase-contrast microscopy

The erythrocytes, membranes and other preparations were observed with a Carl–Zeiss phase-contrast microscope on 100 power oil immersion objective. Using a strobe light, photographs were taken, with a Zeiss camera with iris shutter and 6.3 lens, attached to the microscope. Maximal magnification of the print was × 2500.

Assay of acetylcholinesterase activity

In most cases, acetylcholinesterase activity was assayed using a Radiometer TTT-1c + SBR-2c recording titrigraph operated at pH 8.05 and 25°. The reaction volume was generally 2 ml and 2–10 mM NaOH was used to titrate the acetic acid released. A stream of nitrogen over the reaction mixture reduced background acid production. The acetylcholine chloride concentration used was close to the optimum concentration, 0.4–0.6 mM, except as specified in RESULTS. One unit of acetylcholinesterase activity is defined as that amount of enzyme which hydrolyzes 1 μmole of

TABLE I

EFFECT OF CATIONS ON ACETYLCHOLINESTERASE ACTIVITY IN HUMAN ERYTHROCYTES

Expt. No.	*Preparation* *	*Assay conditions*						*Activity* ***				*Activity ratio* $\frac{+Cation}{-Cation}$
		Medium of incubation	*Substrate (acetylcholine) concn. (mM)*	*pH*	*Temp.*	Ca^{2+} *concn. (mM)*	Na^{+} *concn. (mM)*	*Units/ml packed cells*		*Units/mg membrane protein*		
								−Cation	*+Cation*	*−Cation*	*+Cation*	
1	Whole cell homogenate (3)	Water	10	8.05	31°	0.5–150	—	6.50	9.13	1.28	1.80	1.4
2	Whole cell homogenate (3)	Water	0.6	8.05	25°	—	155	6.21	12.83	—	—	1.9
3	Intact cells (2)	Isotonic NaCl	0.6	8.05	25°	—	155	—	12.38	—	—	—
4	Intact cells (2)	Isotonic sucrose **	0.6	8.05	25°	—	50	4.64	9.70	—	—	2.0
	Whole cell homogenate (2)	Isotonic sucrose	0.6	8.05	25°	—	50	4.40	7.00	—	—	1.5
	Membranes (2)	Water	0.6	8.05	25°	—	50	—	—	0.77	1.44	1.8

* Number of experiments done are given in parentheses.
** Tonicities of (sucrose + NaCl) mixtures remained constant.
*** The values are the mean of the results obtained.

acetylcholine per min; specific activity is expressed in units/ml packed cells or units/mg membrane protein.

The activity of acetylcholinesterase was determined in some cases by the procedure of Mitchel with 10 mM acetylcholine in a barbital–phosphate–KCl buffer of low buffering capacity (pH 8.1)[32]. The activity was followed by measuring the decrease in the pH due to acetic acid formation. Incubation was done at 25° for period of 30–90 min. The pH changes were determined using a Radiometer PHM 22 pH-meter with a scale expander. The sensitivity of this method is much lower than that of the former, and reproducible results can only be obtained with the higher substrate concentrations (*e.g.* at the inhibitory range).

RESULTS

Cation effects on acetylcholinesterase activity

The activity acetylcholinesterase was determined in the intact erythrocytes, in whole cell hemolysates and in the membranes prepared from the erythrocytes. In 155 mM NaCl solutions the acetylcholinesterase activity of intact erythrocytes was considerably higher than in isotonic sucrose (Table I, Expts. 3 and 4). In a series of

TABLE II

EFFECT OF Ca^{2+} ON MEMBRANE ACETYLCHOLINESTERASE ACTIVITY

Ca^{2+} was added to the reaction vessel containing the enzyme, and the reaction was immediately started by the addition of 10 mM acetylcholine at 25° and pH 8.05. Alternatively, the membranes were preincubated with Ca^{2+} for the periods indicated before the addition of the substrate.

Expt. No.	*Ca^{2+} concn. (mM)*	*Preincubation time (min)*	*Specific activity (units/mg protein)*
1	—	—	0.67
	5–15	—	1.32
	27–195	—	1.60
2	—	—	0.64
	0.5	—	0.88
	1.0	—	0.99
	1.5	—	1.09
	2.5	—	1.17
	5–38	—	1.45
3	—	—	0.63
	185	10	1.79
	220	10	1.72
4	0	—	0.30
	12	10–20	0.73
	33	10–20	0.79
	68	10–20	0.87
	130	10–20	0.80
5	0	—	0.53
	530	10	1.29
	970	10	0.59

experiments in which isotonic sucrose was gradually replaced with isotonic NaCl, the acetylcholinesterase activity doubled from 0 to 50 mM NaCl, and did not increase at higher salt concentration. Similar results were obtained when the acetylcholinesterase activity was assayed in the whole cell hemolysates. In addition, Ca^{2+} also activated the hydrolysis of acetylcholine by whole cell hemolysates. Nearly all of the acetylcholinesterase activity was recovered in the membrane prepared from the erythrocytes. Both Na^+ and Ca^{2+} activated the enzymatic hydrolysis of acetylcholine by the membranes.

The assay of acetylcholinesterase was occasionally done with 10 mM, but usually with 0.4–0.6 mM, acetylcholine. Erythrocyte acetylcholinesterase has been shown to be a "true acetylcholinesterase", so that the v_{max} values were obtained only with a very narrow range of substrate concentrations (0.4–0.8 mM). At higher concentrations the substrate inhibited the reaction until at 4 mM acetylcholine a constant inhibition of 50 % was obtained. At 4–20 mM acetylcholine, initial velocity measurements indicated that the reaction had zero order kinetics, although inhibited.

The effect of Ca^{2+} on erythrocyte membrane acetylcholinesterase activity was determined. Table II shows that up to nearly 200 mM Ca^{2+} caused a considerable activation of substrate hydrolysis at 10 mM acetylcholine, irrespective of whether the ion was added immediately after or up to 20 min prior to addition of acetylcholine. Above 200 mM, Ca^{2+} appeared to decrease stimulation. Table III shows the effect of Ca^{2+} on membrane acetylcholinesterase activity at several acetylcholine concentrations. High enzymatic activity was observed at 0.6 mM. As the acetylcholine concentration was increased, the rates of hydrolysis decreased, but the activation by Ca^{2+} was enhanced, as shown by the ratio of activities in the presence and absence of Ca^{2+}.

TABLE III

EFFECT OF Ca^{2+} ON MEMBRANE ACETYLCHOLINESTERASE ACTIVITY AT DIFFERENT ACETYLCHOLINE CONCENTRATIONS

Assay was done with the pH-stat at 25°, pH 8.05 using 6–30 μg membrane protein per assay.

Acetylcholine concn. (mM)	*Ca^{2+} concn. (mM)*	*Specific activity (units/mg protein)*		*Activity ratio $+Ca^{2+}/-Ca^{2+}$*
		$-Ca^{2+}$	*$+Ca^{2+}$*	
0.6	1.7–7	1.295	1.650	1.28
1.5	1.6–7	0.925	1.250	1.35
6–12	5–16	0.645	1.160	1.80

Mg^{2+} in low concentrations also increased membrane acetylcholinesterase activity. As shown in Table IV, optimum activation was obtained at Mg^{2+} concentrations as low as 125 μM and did not appreciably change up to 1 mM when assayed in 0.5 mM acetylcholine. In contrast to brain microsomal acetylcholinesterase[14], we were unable to detect inhibition by ouabain of acetylcholine hydrolysis by the erythrocyte membrane enzyme. Table IV shows that ouabain (0.5 mM) in the absence or presence of Mg^{2+} (0.8 mM), Na^+ (50 mM), Mg^{2+} and Na^+ (1 and 50 mM), did not inhibit the acetylcholinesterase activity. Even using Mg^{2+} and K^+ and Na^+ at con-

TABLE IV

EFFECT OF Mg^{2+}, Na^{+} AND OUABAIN ON HUMAN ERYTHROCYTE MEMBRANE ACETYLCHOLINESTERASE ACTIVITY

Acetylcholinesterase activity was assayed with 20–100 μg membrane protein at 25°, pH 8.05 under N_2.

Additions (mM)			*Acetylcholine concn. (mM)*	*Specific activity (units/mg protein)*
Mg^{2+}	*Na^{+}*	*Ouabain*		
—	—	—	0.5	1.45 (1.17–1.675)
—	—	0.5	0.5	1.67
0.125	—	—	0.5	2.55
0.25	—	—	0.5	2.68
0.8	—	—	0.5	2.79
—	50	—	0.5	3.18
1.0	50	—	0.5	3.45
0.8	—	0.5	0.5	2.84
—	50	0.5	0.5	3.10
1.0	50	0.5	0.5	3.45
—	—	—	0.6	1.15
2.5	—	—	0.6	2.10

TABLE V

EFFECT OF BOILED MEMBRANE EXTRACTS ON HUMAN ERYTHROCYTE MEMBRANE ACETYLCHOLINESTERASE ACTIVITY

0.5 ml of human erythrocyte membrane suspension (4.3 mg/ml protein) in 5 mM Tris–HCl buffer (pH 8) in the presence of 1 mM dithiothreitol and 1 mM EGTA, was maintained under the following conditions: Expt. 1, in boiling water for 5 min, then cooled in an ice bath; Expt. 2, diluted with $CaCl_2$ solution to a final concn. of 0.6 M, warmed to 60° for 2 min then cooled in an ice bath. Aliquots of 0.05 ml and 0.1 ml containing 0.218 mg and 0.436 mg protein, respectively, were taken for assay of the acetylcholinesterase activity with 10 mM substrate at 25° and pH 8.05 in a pH-stat. Control enzyme refers to membranes not heated which were added to the assay mixture in amounts equal to those of the heated membranes.

Expt. No.	*Control enzyme*	*Heated enzyme*	*Ca^{2+} concn. (mM)*	*Specific activity (units/mg protein)*
1	−	+	—	0
	−	+	26	0
	+	−	17	1.09
	+	+	26	1.54
2	−	+	27	0
	+	−	27	1.09
	+	+	27	1.53

centrations of 2.5 and 10 and 100 mM, respectively, (not shown in Table IV) no effect of ouabain on acetylcholinesterase activity was detected.

As shown in Table V, human erythrocyte membranes maintained for 5 min in a boiling water bath lost all acetylcholinesterase activity. At 60° for 2 min, the enzyme was denatured even in the presence of Ca^{2+} (27 mM). However, when heat treated membranes were mixed with control membranes, which were not subjected to heating, and assayed for acetylcholinesterase activity in the presence of Ca^{2+}, an

increase of approx. 40 % in the activity of these control membranes was observed.

Ca^{2+} had similar effects on the acetylcholinesterase of membranes prepared from bovine erythrocytes. The membranes were prepared according to BURGER *et al.*[3] in the absence or presence of divalent cations (5 mM Ca^{2+} or Mg^{2+}) in the hypotonic hemolysis mixture. Comparison of the three preparations shows no difference in the specific activity values (Table VI). Moreover when Ca^{2+} was added to the reaction mixture in the concentration range of 3–12 mM, they increased nearly 2-fold the enzymatic activity of the control membranes, and also of the "cation-prepared" membranes. When calculated on the basis of packed cells, inclusion of Ca^{2+} or Mg^{2+} in the hemolyzing medium assisted in the retention of acetylcholinesterase in the bovine membranes, thus confirming the observations made by BURGER *et al.*[3]. The addition of Ca^{2+} to the assay mixture also caused a considerable activation of the enzyme (Table VI).

Acetylcholinesterase in ghosts treated with hypertonic solutions

Hypertonic solutions of NaCl, $CaCl_2$ or sucrose at 1 M concentrations, caused "solubilization" of 9–43 % of the human erythrocyte membrane protein. The "solubilization" was accomplished at two pH values, 6.5 and 7.9 (Table VII). The activity of acetylcholinesterase was assayed in the sediment, except for Expt. 1c in Table VII, where both the sediment and the soluble fractions were assayed. In this latter case, the total recovery of the enzymatic activity was 78–97 %, most of which (> 75 %) remained insoluble. The fluctuations in the recovery of acetylcholinesterase activity may have resulted from variations in the cation content of the suspensions of the sediments which were obtained following the various treatments.

It was noted earlier that cations activate the acetylcholinesterase activity, although Ca^{2+} at higher concentrations may cause decline in activation. Ca^{2+} was added at increasing concentrations (1–25 mM) to determine their effect on the acetylcholinesterase activity of the sediments which were obtained following the hypertonic treatments. A 2–3-fold increase in the initial rates of acetylcholine hydrolysis were observed with Sediments 1b, 3b and 4b (Table VII) when the Ca^{2+} concentration was about 5 mM. There were some quantitative differences in the maximal values of activity which were obtained in each of the sediments following the addition of Ca^{2+} to the assay mixture, but the shape of the curves was very similar. Furthermore, the enzymatic activities of the sediments, following dialysis to remove the excess salts, were lower than the non-dialyzed samples. On the other hand, the acetylcholinesterase activity of the sediment which was obtained following the hypertonic $CaCl_2$ treatment, was not altered by the addition of increasing concentrations of Ca^{2+} to the assay mixture the activity increased again to 6 units at 5 mM Ca^{2+}. This indicates that prior to the dialysis, the Ca^{2+} content of the enzyme was sufficient to activate fully the hydrolysis of acetylcholine.

Acetylcholinesterase in membranes treated with mercaptoethanol + EDTA followed by 1.2 M NaCl

Membranes were treated with a solution containing 1 mM EDTA and 50 mM mercaptoethanol according to ROSENBERG AND GUIDOTTI[12]. Approx. 75 % of the ghost membrane proteins were recovered, most of them insoluble (Table VIII, Pct I). All the acetylcholinesterase was recovered in the insoluble fraction, with a 66 %

TABLE VI

ACETYLCHOLINESTERASE ACTIVITY OF BOVINE ERYTHROCYTE MEMBRANES PREPARED IN THE ABSENCE OR PRESENCE OF DIVALENT CATIONS

Membranes prepared according to BURGER *et al.*[3], in 10 mM Tris–HCl in the absence and presence of 5 mM Ca^{2+} or Mg^{2+}. Acetylcholinesterase was assayed with 1.5 mM substrate at 25°, pH 8.05 in the absence and presence of 3–12 mM Ca^{2+}.

Expt. No.	*Membrane prepared in the following*	*Ca^{2+} concn. during assay (mM)*	*Specific activity, units acetylcholinesterase per*				*Activity ratio* $+Ca^{2+}/-Ca^{2+}$*
			ml packed cells		*mg membrane protein*		
			$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	
A	10 mM Tris–HCl buffer (pH 7.4)	3; 6	1.98	4.58	0.33	0.70	2.32
B	10 mM Tris–HCl buffer (pH 7.4) + 5 mM Ca^{2+}	3; 6; 12	3.46	6.35	0.36	0.66	1.84
C	10 mM Tris–HCl buffer (pH 7.4) + 5 mM Mg^{2+}	6	2.85	5.84	0.35	0.71	2.05

* Calculated for the specific activity on ml packed cells.

TABLE VII

PARTIAL SOLUBILIZATION OF HUMAN ERYTHROCYTE MEMBRANES WITH HYPERTONIC SOLUTIONS AND THE EFFECT OF Ca^{2+} ON ACETYLCHOLINESTERASE ACTIVITY

The membranes were treated with the hypertonic solutions as described in MATERIALS AND METHODS. Acetylcholinesterase activity and protein content of control membranes not subjected to these treatments were taken as 100%. In each experiment the assay of acetylcholinesterase activity was done: (a) according to MICHEL[32] with 10 mM substrate; (b) using a pH-stat with 10 mM substrate at pH 8.05, 25° in the absence or presence of 15 mM Ca^{2+}.

Expt. No.	*Solubilization medium*	*Dithiothreitol (1 mM)*	*Sediment dialyzed*	*pH*	*Protein in sediment (%)*	*Acetyl-cholinesterase in sediment (%)*	*Activity ratio $+Ca^{2+}/-Ca^{2+}$*
1a	1.2 M NaCl	−	−	7.82	63	84	—
	1.2 M NaCl	+	−	7.96	71	87	—
1b	1.0 M NaCl	−	−	6.45	—	80	2.35
	1.0 M NaCl	−	+	—	—	58	3.70
1c	1.0 M NaCl*	+	−	6.43	—	73	2.81
2a	1.2 M $CaCl_2$	−	−	7.21	59	59	—
2b	1.0 M $CaCl_2$	−	−	6.44	—	99	1.13
	1.0 M $CaCl_2$	−	−	—	—	60	3.00
	1.0 M $CaCl_2$	+	−	6.40	—	58	2.09
3a	1.2 M sucrose	−	−	8.12	67	54	—
	1.2 M sucrose	−	−	8.17	57	54	—
3b	1.0 M sucrose	−	−	6.52	—	79	2.68
	1.0 M sucrose	−	+	—	—	67	2.94
	1.0 M sucrose	+	−	6.50	—	83	2.41
4a	5 mM Tris, 1 mM EGTA	+	−	7.71	104	91	—
4b	5 mM Tris, 1 mM EGTA	−	−	6.20	—	77	2.70
	5 mM Tris, 1 mM EGTA	−	+	—	—	83	2.00

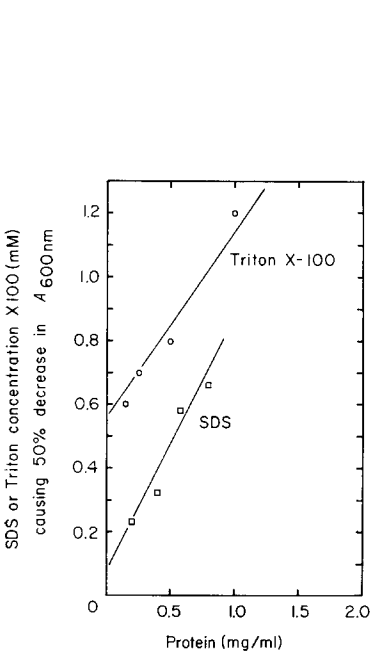
* In one set of experiments membranes were treated with 1.0 M NaCl with 91% protein remaining in sediment and the acetylcholinesterase activity of this residue assayed in the absence or presence of 0.155 M NaCl. Conditions were 0.5–1 mM acetylcholine (pH 8.05), 25° and the following results were obtained: absence of NaCl: 58, 81; presence of NaCl: 94, 127.

TABLE VIII

TREATMENT OF HUMAN ERYTHROCYTE MEMBRANES WITH MERCAPTOETHANOL AND EDTA FOLLOWED BY 1.2 M NaCl

Membranes were treated with mercaptoethanol–EDTA for 36 h at 4°. Following centrifugation at 20000 × *g*, 60 min, the supernatant (Sup 1) was concentrated. The pellet (Pct 1) was suspended in 1.2 M NaCl, stirred for 18 h, then centrifuged at 14000 × *g*, 60 min. The supernatant (Sup 2) was concentrated. All fractions were dialyzed against 100 vol. of 10 mM Tris–HCl buffer (pH 7.25). The assay of acetylcholinesterase was done with 0.6 mM acetylcholine, at pH 8.05, 25°, in the absence and presence of 3–6 mM Ca^{2+}, 51.3 mg of protein and 66.3 units of acetylcholinesterase activity were taken as 100%.

Fraction	*Protein* (%)	*Acetylcholine-sterase activity* (%)	*Activity ratio* $+Ca^{2+}/-Ca^{2+}$
Membranes	100	100	1.29
Mercaptoethanol–EDTA	116	73	1.87
Sup 1	11	3.6	1.58
Pct 1	62	102	1.05
Sup 2	18	—	—
Pct 2	52	62	—



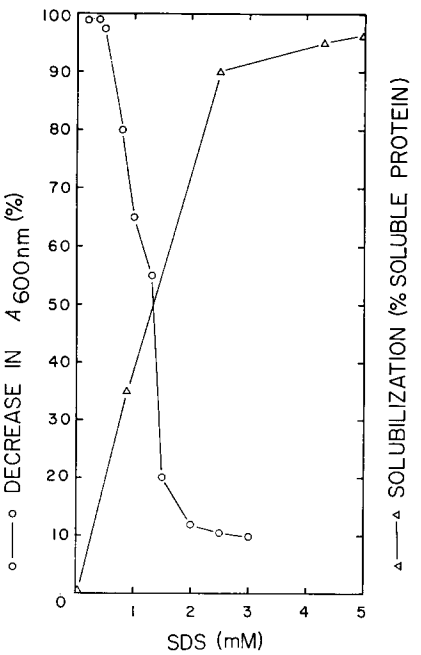


Fig. 1. Detergent requirement for 50% solubilization of membranes. A sample of membrane protein, containing 0.1–1 mg/ml, was mixed with increasing concentrations of sodium dodecyl sulfate (SDS) or Triton X-100 at pH 7.5–8. Solubilization was measured by decrease in absorbance to a constant value at 600 nm. ○—○, with Triton X-100; □—□, with sodium dodecyl sulfate.

Fig. 2. Effect of sodium dodecyl sulfate concentrations on membrane solubilization. A sample of membrane protein, containing 1 mg/ml, in 10 mM sodium dodecyl sulfate. Aliquots were taken for ΔA at 600 nm (○—○) and for centrifugation at 100000 × *g* for 30 min at 4°. Protein was determined in supernatant and sediment (△—△, percent protein in supernatant).

increase in the specific activity. When assayed with 0.6 mM acetylcholine, only a slight activation was observed with 3–7 mM Ca^{2+}.

A combination of the mercaptoethanol–EDTA treatment with hypertonic (1.2 M) NaCl treatment, although "solubilizing" 25 % of the "Pct I" proteins (Table VIII), resulted in the recovery of only 61 % of acetylcholinesterase activity (Table VIII, "Pct 2").

Treatment of membranes with detergents

A turbid suspension of human erythrocyte membranes in 5 mM Tris–HCl buffer (pH 7.5–8), became optically clear following the addition of increasing concentrations of dodecyl sulfate or Triton X-100. The solubilization of the membranes was measured by the decrease in the absorbance of the ghost suspension at a wavelength between 400–600 nm. A plot of the concentration of the detergent causing 50 % solubilization as a function of the ghost protein concentration (at a range of 0.1–1.0 mg/ml) yielded a straight line for both detergents (Fig. 1). The slope of these lines calculated by the least squares method was 0.56 μmole (0.16 mg) and 0.76 μmole (0.49 mg) per mg ghost protein for dodecyl sulfate and Triton X-100, respectively. The membrane suspensions (approx. 1 mg/ml protein) were treated with dodecyl sulfate and centrifuged for 60 min at 100000 $\times$ *g*. The proteins, which did not sediment under these conditions, were considered soluble. A correlation was obtained between the percent decrease in absorbance at 600 nm and the percent solubilization as measured by protein found in the supernatant following high-speed centrifugation (Fig. 2). Sufficient detergent (dodecyl sulfate or Triton X-100) was added to the ghost suspension (Fig. 3) to cause a drop of 50 % in the absorbance at 600 nm and the

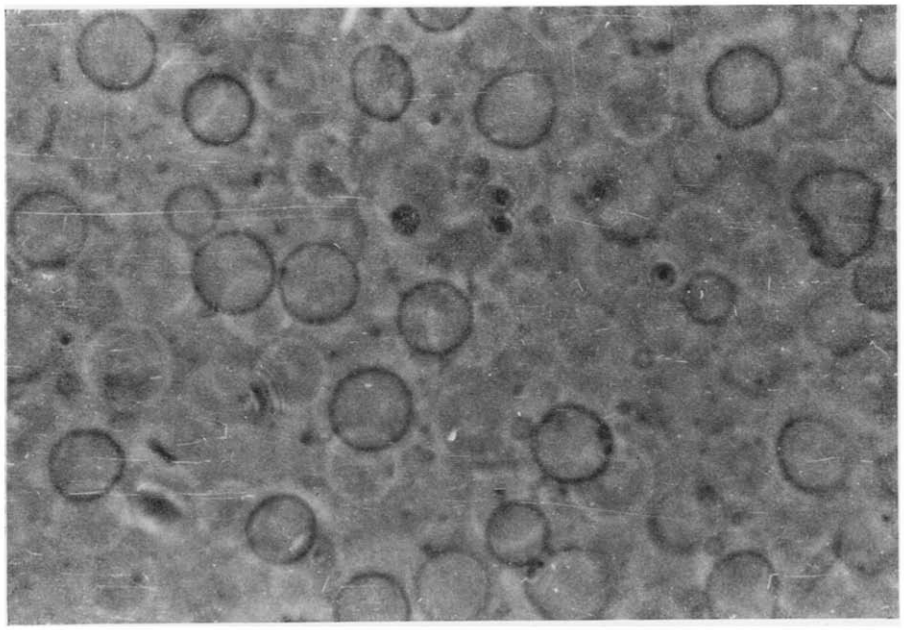


Fig. 3. Phase-contrast micrographs of human erythrocyte ghosts (0.78 mg protein per ml) suspended in 3 mM glycine buffer (pH 8), centrifuged at 35000 rev./min for 60 min. The pellets were suspended in the same buffer prior to photography. Magnification $\times$ 1810.

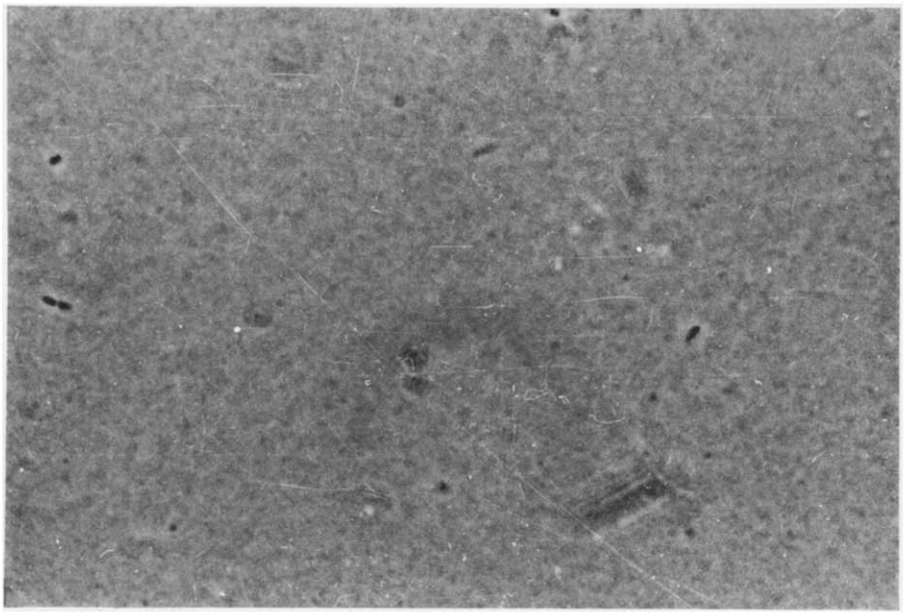


Fig. 4. Phase-contrast micrographs of ghosts (1 mg protein per ml) which were suspended in a solution of 2.5 mM sodium dodecyl sulfate in 3 mM glycine buffer (pH 8.5), centrifuged at 40000 rev./min, 60 min. The pellets were suspended in glycine buffer only prior to photography. Magnification × 1134.

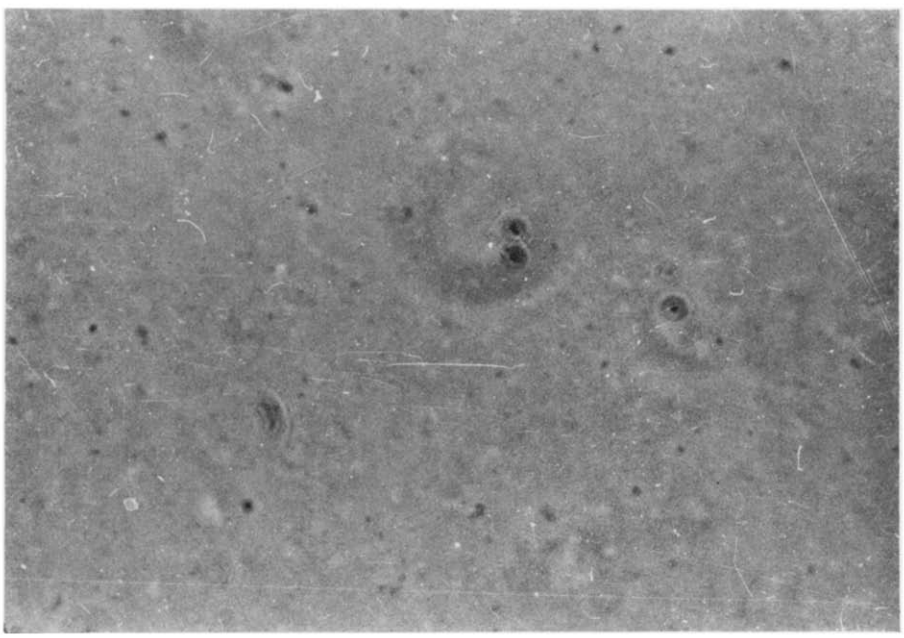


Fig. 5. Phase-contrast micrograph of ghost suspension in isotonic saline which were ultrasonically irradiated for 2 min. Total magnification × 1134.

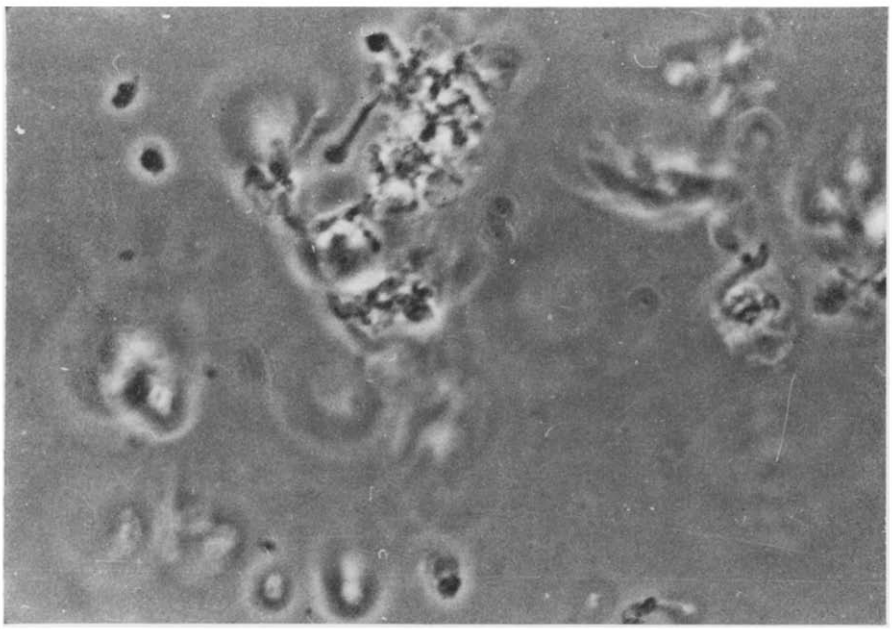


Fig. 6. Phase-contrast micrograph of ghost suspensions which were repeatedly (3 times) frozen at $-70°$ and thawed at 37°, then diluted 1:10 with water prior to photography. Magnification × 1134.

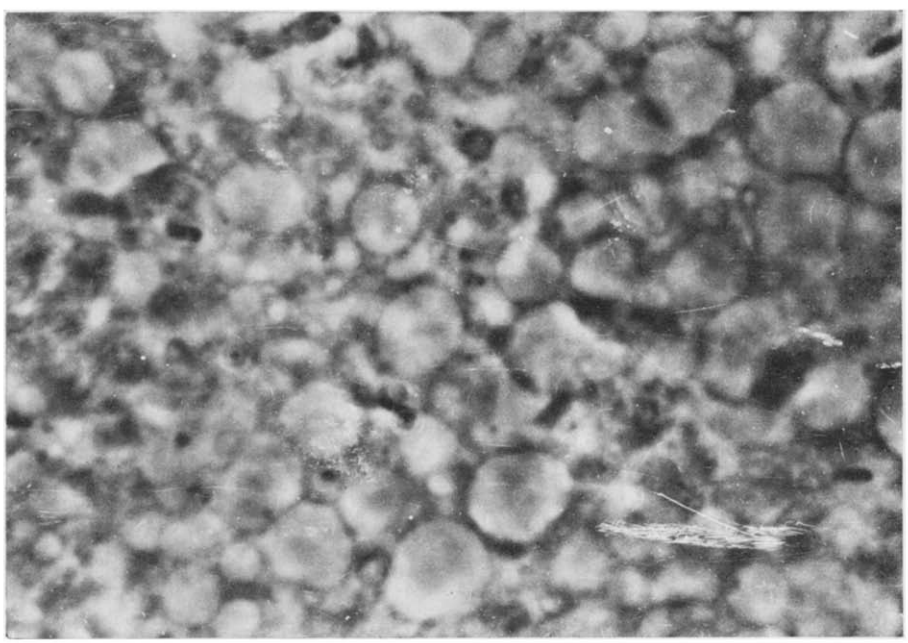


Fig. 7. Phase-contrast micrograph of a human erythrocyte ghost suspension (0.78 mg/ml protein) in 3 mM glycine buffer (pH 8), containing 0.5% Triton X-100, which caused a 50% decrease in the absorbance at 600 nm. Following centrifugation at 35000 rev./min for 60 min the pellets were suspended in 3 mM glycine buffer (pH 8) before the photography. Magnification × 2268.

suspensions were examined with the phase contrast microscope. With dodecyl sulfate no clear structure could be seen even under the highest magnification (Fig. 4). This observation was also confirmed by means of electron microscopic examination following a direct fixation in phosphotungstic acid at a magnification of × 16000. Similar results were obtained with ghost suspensions which were ultrasonically irradiated for 2 min (Fig. 5). On the other hand repeated freezing (—70°) and thawing (37°) of the ghost suspensions yielded membrane fragments of different appearance under the phase contrast microscope (Fig. 6). With Triton X-100, the membranes were detectable with phase microscope, yet the sharp contrast of the membrane rim, which was observed in control preparations disappeared, giving the structure a faded appearance (Fig. 7).

The membrane suspension in the concentration range of 1–3 mg protein per ml was centrifuged on a sucrose density gradient of 15–50 % (w/v). The intact membranes had a density of 1.1303 g/cm³ (range 1.1161–1.1590). The centrifugation was performed at 10, 15 or 24°, and a very slight temperature effect on the banding of the ghost proteins at the above density was observed. The same membrane suspensions (1–2 mg/ml protein) were treated with 4.5–9 mM dodecyl sulfate in the sucrose solutions. The density at the peak of the band was 1.078 g/cm³. The same value was obtained at the different temperatures whether dodecyl sulfate was present or absent. Qualitative lipid analysis (thin-layer chromatography) indicated that the phospholipid and the cholesterol accompanied the protein band in both the dodecyl sulfate treated and untreated ghost. No attempt was made at that time to show separation of the lipids from the proteins.

Effect of detergents on the acetylcholinesterase activity of human erythrocyte membranes

Dodecyl sulfate. A membrane suspension (0.075–0.2 mg/ml protein) was incubated with increasing concentrations of dodecyl sulfate (0.1–3 mM) for periods up

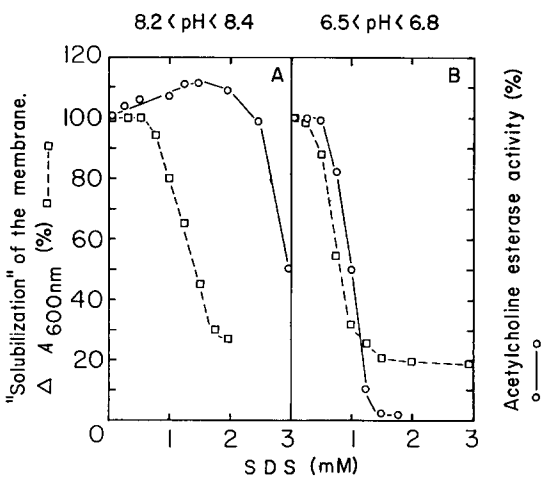


Fig. 8. Effect of sodium dodecyl sulfate (SDS), at acid and basic pH, on membrane acetylcholinesterase activity and absorbance. A sample of membrane protein containing 0.176 mg/ml was incubated with 0–3 mM sodium dodecyl sulfate for 2 min at 25°. Aliquots were taken for ΔA at 600 nm and for assay of acetylcholinesterase activity at pH 8.05 with 0.5 mM acetylcholine substrate. A. $8.2 < pH < 8.4$. B. $6.5 < pH < 6.8$. □---□, decrease in absorbance at 600 nm; ○—○, acetylcholinesterase activity.

to 2 min and the acetylcholinesterase determined. Fig. 8 shows that at pH values 6.5–6.8 the enzymatic activity decreased rapidly at 0.5–1.5 mM dodecyl sulfate, with a simultaneous decrease in the absorbance at 600 nm. At higher pH values (8.2–8.4) more dodecyl sulfate was required to cause the same amount of solubilization (1–2 mM dodecyl sulfate); furthermore, at the higher pH range, an activation of the acetylcholinesterase activity was obtained with 0.5–1.5 mM dodecyl sulfate, but higher concentrations caused inactivation.

A membrane suspension (0.2 mg protein per ml) was incubated for 2 min with 1.25 and 2.5 mM dodecyl sulfate at pH values from 6–9. The acetylcholinesterase activity was then assayed after the pH was readjusted to 8.05. With 1.25 mM dodecyl sulfate, 50 % inactivation was obtained at pH 7, but with 2.5 mM dodecyl sulfate 50 % inactivation occurred even at higher pH values, *i.e.* 8–8.1 (*cf.* Fig. 9). These data reflect a strong dependence of the acetylcholinesterase inactivation on both pH and dodecyl sulfate concentrations.

The membrane suspension was treated with dodecyl sulfate at a concentration

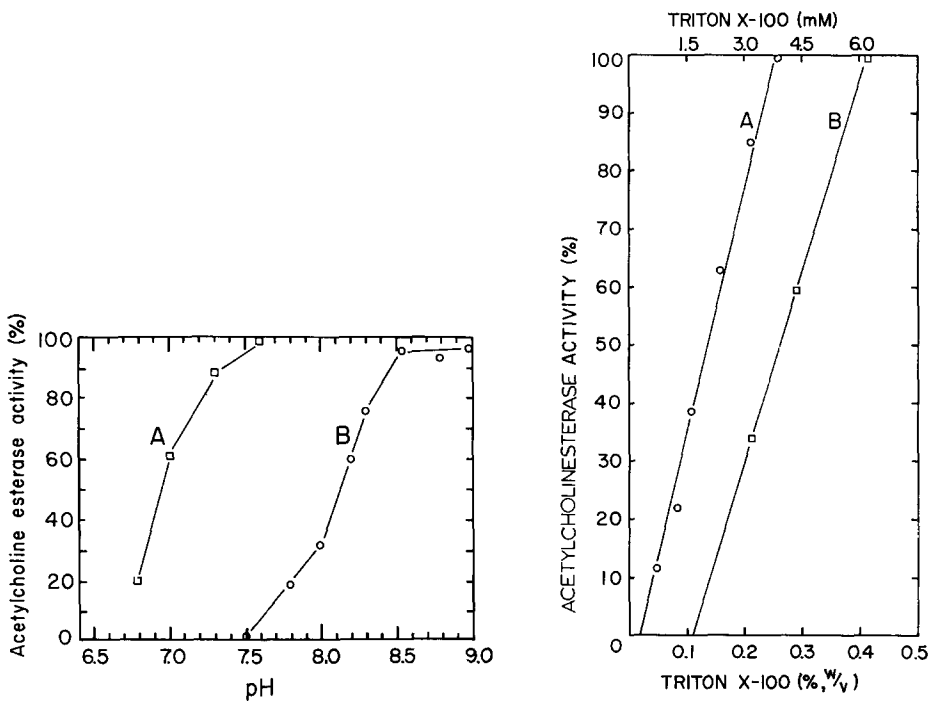


Fig. 9. pH dependence of sodium dodecyl sulfate inactivation of acetylcholinesterase activity. A sample of membrane protein, containing 0.2 mg/ml, was incubated with sodium dodecyl sulfate for 2 min at pH values as indicated adjusted with Radiometer pH-stat at 25°. Assay of acetylcholinesterase as described in the legend to Fig. 8. Curve A, with 1.25 mM sodium dodecyl sulfate; Curve B, with 2.5 mM sodium dodecyl sulfate.

Fig. 10. Effect of Triton X-100 on the sodium dodecyl sulfate inactivation of acetylcholinesterase. A sample of membrane protein, containing 0.08–0.15 mg/ml, incubated with Triton X-100 at 25° for 2 min, pH 8.5, then sodium dodecyl sulfate added. Incubation was continued for 1–2 min, pH readjusted to 8.05 and acetylcholinesterase then assayed. A, with 0.1 % (3.7 mM) sodium dodecyl sulfate; B, with 0.18% (5.4 mM) sodium dodecyl sulfate.

which partially inactivated the acetylcholinesterase and then centrifuged at 100000 × *g* for 60 min. The residual activity was found only in the sediment, indicating a denaturation of the soluble portion by dodecyl sulfate. Subsequently, a membrane suspension (0.11 mg protein/ml) was incubated with sub-inactivating concentrations (< 2 mM) of dodecyl sulfate at pH > 8.7 and 25° for periods up to 45 min. The acetylcholinesterase was not affected during this period and was even slightly increased. Longer periods of incubation caused a rapid loss of the activity (80 % of the initial value).

The inactivation by dodecyl sulfate was irreversible, since dialysis for several days at 4° against a large volume of 3 mM glycine buffer at pH 8.5 in the absence or presence of 5–20 mM Mg^{2+} did not restore the enzymatic activity. The acetylcholinesterase activity of control membranes treated similarly but without added dodecyl sulfate were not affected by the prolonged dialysis. During the dialysis, a heavy precipitate was formed in the dodecyl sulfate-treated samples which, under phase-contrast microscopy showed non-organized aggregates. On examination by electron microscopy, vesicular forms with occasional membrane bilayers were observed.

Triton X-100. Triton X-100 did not exhibit the same effect as dodecyl sulfate. When incubated with erythrocyte membranes (0.075 mg protein per ml) under conditions similar to those used for dodecyl sulfate (*i.e.* at pH 8.5 for 2 min before assaying for acetylcholinesterase activity), Triton X-100 caused a 50 % increase in the enzyme activity at a concentration of 0.05 % (*i.e.* 0.76 mM). The activity remained elevated even at higher concentrations, up to 4.75 % (*i.e.* 73.5 mM). The same membrane preparations (0.75–0.15 mg protein per ml) were preincubated with Triton X-100 at a concentration range of 0.05–0.5 % for 2 min at pH values > 8.5 and dodecyl sulfate was then added at either 3.7 mM (0.1 %) or 5.4 mM (0.18 %). At such concentrations dodecyl sulfate alone causes an irreversible inactivation which was prevented completely when the molar ratio of Triton X-100/dodecyl sulfate was close to 1 (1.07–1.17) (*cf.* Fig. 10). If dodecyl sulfate was added to the membranes prior to the Triton X-100, the damage was irreversible.

DISCUSSION

In the present investigation attempts have been made to solubilize the erythrocyte membrane proteins by several different methods, using the membrane bound enzyme, acetylcholinesterase, as a marker for solubilization.

It has been shown previously[2–4] that membranes prepared by hypotonic hemolysis do not necessarily have the same composition, structure, or stability as the original erythrocyte membrane. These preparations may be considered, however, as a close derivative of that membrane, possessing many of its components and functions, *e.g.* lipids, enzymes, transport of cations, *etc.* In addition to retention of its osmotic properties and lipid content, the human erythrocyte membranes which were prepared by hemolysis with hypotonic salt solutions and rendered hemoglobin-free by repeated washings[1] also retain most of the original acetylcholinesterase activity of the erythrocyte[4].

In contrast, membranes prepared from bovine erythrocytes, using the same procedure, disintegrate and lose acetylcholinesterase during the washing process[3]. The difference between the erythrocytes of the two species was explained, in part, by

the difference in the content of divalent cations (most probably Mg^{2+} but also Ca^{2+}). Actually, both the fragmentation and the simultaneous loss of acetylcholinesterase–lipoprotein was prevented by the addition of Ca^{2+} or Mg^{2+} (1–5 mM) to the buffer used in hemolysis of the bovine erythrocytes[3]. Recent studies have shown that human erythrocytes contain a Ca^{2+}-activated ATPase[15,16]. Furthermore, membranes of human erythrocytes, although probably containing higher concentrations of Ca^{2+} than bovine erythrocytes can still bind more Ca^{2+} and the major binding sites are either the protein fraction[17] or the *N*-acetylneuraminic acid-containing groups[31] rather than the lipid fraction of the membrane. Recent studies[33] indicate that the binding of Ca^{2+} to erythrocyte membrane fragments requires ATP and it is claimed that this process is strongly linked to the Ca^{2+} transport across the membrane.

One approach employed in this current study to effect solubilization of erythrocyte membrane components was the use of high salt (NaCl) solutions. MITCHELL *et al.*[2] have shown that intact (human) erythrocytes were not affected, but their membranes and also intact bovine erythrocytes lost acetylcholinesterase–lipoprotein fragment following prolonged contact with the hypertonic (0.4–1.2 M NaCl) solutions. These observations emphasize the "sideness", polarity or asymmetry of the membrane with respect to the location of enzymes in general and acetylcholinesterase in particular. It was believed that with appropriate precautions diisopropyl phosphofluoridate, which is a potent and irreversible inhibitor of acetylcholinesterase, could bind specifically to this enzyme and also serve as a tag of the outer surface of the erythrocyte membrane[18,19]. This approach was challenged recently by SEARS AND WEED[20] who showed that diisopropyl [^{32}P]phosphofluoridate crossed the membrane of human intact erythrocytes and labeled the intracellular components to an even greater extent than the membrane.

Proteolytic enzymes reduce or even abolish the membrane-bound acetylcholinesterase activity. These enzymes have been reported to act on the outer surface of intact erythrocytes only since neither significant hemolysis or changes in the activities of the intracellular lactate dehydrogenase or glucose-6-phosphate dehydrogenase[21] or in choline transport[22] were detected. Other enzymes (lipase, neuraminidase or lysozyme) had no effect on the acetylcholinesterase activity[21]. Therefore, acetylcholinesterase appears to be accessible to those proteases which do not cross the cell membrane suggesting that acetylcholinesterase is on the outer surface of the erythrocyte membrane. On the other hand, recent studies by MARCHESI *et al.*[8] have shown that ($Na^+ + K^+$)-activated ATPase is located on the inner surface of the guinea-pig erythrocyte membrane. Trypsin digestion, however, caused ATPase inactivation and structural breakdown of the ghost membrane, both effects being preventable by ATP + Mg^{2+} (ref. 23).

The present study demonstrates the activating effects of cations on the activity of the membrane bound acetylcholinesterase. At the substrate concentrations tested, maximal activation by Na^+ was at 50 mM and by Ca^{2+} at 5 mM. With Mg^{2+}, the maximum activation with 10 mM acetylcholine was obtained at 2.5 mM Mg^{2+} while with 0.5 mM substrate, only 0.1 mM Mg^{2+} was required. In spite of the considerable activation by the cations, which was of the order of 3-fold, the addition of these cations does not seem to be essential to activity. It seems possible that the membrane-bound concentrations of these cations, and in particular Ca^{2+} or Mg^{2+}, are sufficient to maintain the measured basal activity. BURGER *et al.*[3] have shown that the levels

of Mg^{2+} in bovine erythrocytes are much lower than those of human. It would therefore be expected that the amount of acetylcholinesterase in bovine erythrocyte membranes isolated after hemolysis in the presence of Ca^{2+} or Mg^{2+} should be higher and the effect of added Ca^{2+} or Mg^{2+} to the assay mixture should be smaller compared with the control membranes. The results shown in Table VI indicate that though the "Ca^{2+} (Mg^{2+})-membranes" have a higher acetylcholinesterase activity compared with the control membranes, the Ca^{2+} added to the assay caused the same effect on both preparations. Therefore, higher apparent basal acetylcholinesterase in the bovine "Ca^{2+} (Mg^{2+})-membranes" could mean only increased retention of the enzyme.

Prolonged dialysis of human erythrocyte membranes against EGTA reduced the basal acetylcholinesterase activity, indicating that part of the bound divalent cations can be removed, thus emphasizing their role in the activity of acetylcholinesterase and integrity of the structure. However, high concentrations of Ca^{2+} (approx. 0.2 M) inhibited the enzyme (Table II) and furthermore, prolonged contact with 1 M $CaCl_2$ yielded membranes having acetylcholinesterase which could no longer be activated by the addition of Ca^{2+}. The lower activity of acetylcholinesterase was however, increased by dialysis against Ca^{2+}-free medium. MYERS[24] concluded that the salts, NaCl and KCl, decrease the affinity of true acetylcholinesterase for acetylcholine as well as for the inhibitors eserine and prostigmine[25,26]. Divalent cations caused marked potentiation of the hydrolytic activity and the possibility of altering the charge of the enzyme was considered as an explanation, although MYERS[24] favors the idea of structural alteration of the active center. It is possible that acetylcholine and Ca^{2+} compete for similar or adjacent sites. Excess substrate inhibits the reaction (Table III) and therefore the activation by Ca^{2+} was maximal with high (10 mM) inhibitory concentrations of substrate while with the low concentrations (0.5 mM) the effect was minimal.

The effect of Ca^{2+} on the acetylcholinesterase activity, may, however, be secondary to its effect on the membrane conformation. Several observations point to the lipoprotein nature of acetylcholinesterase although no evidence has been presented to indicate the requirement of lipids for the enzymatic activity (*cf.* footnote 7 in ref. 3). It may be fortuitous that the treatment which removes acetylcholinesterase from the membrane also removes lipids and other proteins[3,4]. Detergents have been used by many investigators to study the organization of proteins and lipids in membrane structure[27]. Dodecyl sulfate in particular but also other detergents have been found to be efficient at disaggregating the membranes of Mycoplasma[27] and other microorganisms[28]. Dodecyl sulfate was used in the present study as a solubilization agent by following both solubility of proteins and alterations in the activity of acetylcholinesterase. The loss of activity of acetylcholinesterase with dodecyl sulfate was correlated under one set of conditions (acid pH) with the destruction of the membrane. Using model systems of pure protein, it has been shown that dodecyl sulfate binds to a variety of proteins, causing denaturation at very low concentrations (0.5–1 mM)[29]. It may be argued that dodecyl sulfate does not denature all proteins because some enzymes (NADH-oxidase, ribonuclease, and deoxyribonuclease) present in membranes of *Mycoplasma laidlawii* resisted denaturation by 10 mM dodecyl sulfate[30]. However, 10 mM dodecyl sulfate inactivated ATPase and *p*-nitrophenyl phosphatase[30] at pH 7.4, and 1.5 mM dodecyl sulfate inactivated the ($Na^+ + K^+$)-activated ATPase of guinea-pig erythrocyte membrane[8] at pH 7.0.

The acetylcholinesterase of human erythrocyte is also inactivated irreversibly by dodecylsulfate, a process which is pH dependent in that at lower pH values (< 7) lower concentrations of dodecyl sulfate caused a damage which required higher concentrations of dodecyl sulfate at pH > 8. At this point the available data do not allow one to decide whether the inactivation by dodecyl sulfate is due to denaturation of the enzyme or removal of lipids. The dodecyl sulfate was shown to shift the density of the ghost proteins from 1.1303 g/cm^3 of intact ghost to 1.078 g/cm^3 after solubilization. The amount of dodecyl sulfate required to cause 50 % solubilization of the ghost membrane as obtained from Fig. 1 was 0.56 μmole/mg protein while that of Triton X-100 was 0.76 μmole/mg protein above a basal level of both detergents. Triton X-100 causes solubilization of the membrane without destroying the acetylcholinesterase activity which agrees with the recent data of MILLER[6]. The protective effect of Triton X-100 against inactivation caused by dodecyl sulfate with a molar ratio of approx. 1 may be explained by interaction of the Triton X-100 with docedyl sulfate to form a 1:1 complex. This might indicate that Triton X-100 disrupts the membrane by a different mechanism, and does not cause denaturation of the sensitive enzymes as does dodecyl sulfate. Compared to the attempts to solubilize the ghost membrane by milder agents, the detergents seem to be much more efficient in total solubilization rather than in use for selective removal of components from the erythrocyte membrane.

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