Solubilization of Certain Proteins from the Human Erythrocyte Stroma*

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ABSTRACT: It has been shown that treatment of hemoglobin-free human erythrocyte stroma with hypertonic NaCl solutions (0.2–1.4 M) effects a partial solubilization of stromal lipids and acetylcholinesterase plus other protein without complete disruption of the underlying stromal structure. The extent of solubilization is dependent upon the concentration of the NaCl but not directly dependent upon pH. Phase micrographs of treated stroma reveal that the cells are smaller and have undergone distinct peripheral changes; electron micrographs of the NaCl-washed stroma show, in addition, that a number of membrane-bounded fragments have been formed. Ultracentrifugation of the solubilized material in NaBr solutions, density = 1.21 g/ml, yields two protein fractions, one of which floats in this density media with a lipoprotein fraction which contains all of the lipid and almost all of the acetylcholinesterase, and the other of which sediments under these conditions and is free from lipid. The lipid present in the solubilized lipoprotein differs neither in phospholipid distribution nor in fatty acid composition from the lipid of the intact cells. The possible relationship of the solubilized protein and lipoprotein fractions to the stability of the membrane is discussed.

The lipid composition of human erythrocyte stroma[1] has been thoroughly defined (Ways and Hanahan, 1964; van Deenen and de Gier, 1964), but no adequate description has yet been made of the stromal proteins. Efforts to study the composition of these proteins have been hampered by the fact that stromal protein content varies according to the method of stromal preparation, as well as by the difficulties inherent in the separation and study of proteins insoluble in their native state.

Significant solubilization of stromal proteins has been achieved through the use of organic solvents (Moskowitz and Calvin, 1952; Maddy, 1964), cholate and deoxycholate (Richardson *et al.*, 1963), or urea (Azen *et al.*, 1965), but correlation of the solubilized protein fractions with the proteins of the intact stroma has not proved possible, due to alterations produced during the solubilization. A different approach has therefore been taken in this laboratory. Relatively gentle washing procedures have been used to obtain partial removal of proteins from hemoglobin-free stroma. In a previous report (Mitchell *et al.*, 1965) it has been shown that aldolase, glyceraldehyde phosphate dehydrogenase, carbonic anhydrase, and adenosine deaminase can be removed from stroma by hypotonic buffers, without damage to the structural integrity of the fundamental stroma membrane. In the present report, it will be demonstrated that acetylcholinesterase can be solubilized from the stroma with hypertonic NaCl solutions and that removal of this lipoprotein fraction does affect the stability of the basic membrane structure.

Experimental Section

Materials

Hemoglobin-free erythrocyte stroma was prepared from freshly drawn human blood by the procedure described previously (Dodge *et al.*, 1963). Commercial granulated sugar (C & H) was used to concentrate lipoprotein solutions; all other chemicals were reagent grade.

Procedures

Treatment of Stroma. Hemoglobin-free stroma was washed by being suspended in 10–15 volumes of NaCl of the pH and concentration indicated, at 4°. In order to effect solubilization of stromal components, hemoglobin-free erythrocyte stroma was washed with NaCl solutions ranging in concentration from 0.15 M (isotonic) to 1.40 M. In the initial series of experiments, the saline was buffered with 0.005 M Tris, pH 8.2; in the latter series of experiments, unbuffered saline (pH 6.5) was used. The stroma was usually in contact with the saline, without further mixing, for 15 hr at the same temperature. Intermediate extraction periods, ranging from 1 to 8 hr, were tried, and though increasing amounts of soluble material were obtained, the amounts were significantly less than those obtained at 15 hr.

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[1] "Stroma" refers to the post-hemolytic residue or "ghosts," usually considered to be representative of the surface structure of the erythrocyte.

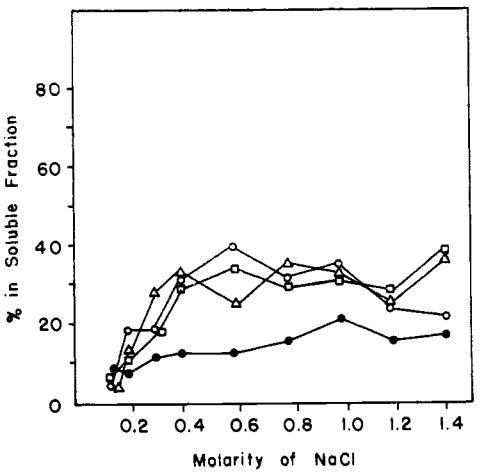

FIGURE 1: Composition of soluble fraction obtained from stroma rewashed with varying concentrations of NaCl + 0.005 M Tris, pH 8.2. Control stroma was washed only with pH 7.4, 20 milliosmolarity phosphate. Protein, ●–●–●; phospholipid, O–O–O; cholesterol, □–□–□; acetylcholinesterase, △—△—△.

After sedimentation in a Servall RC-2 centrifuge at 35,000*g* (No. SS-34 rotor) for 45 min, the stroma was briefly resuspended in the same wash media and re-sedimented twice more. Material which did not sediment under these conditions, *i.e.*, centrifugation at 35,000*g* for 45 min at 4°, was assumed to be soluble. Furthermore, no detectable cellular structures were observed upon examination of the soluble fractions by electron microscopy. Stroma which was washed with potassium phosphate buffer (pH 7.4, 20 ideal milliosmolarity) rather than saline served as a control preparation. Intact erythrocytes were treated with saline similarly in a separate series of experiments.

The clear supernatant solutions obtained from the saline-washed or control stroma were on occasion dialyzed against distilled water. More often, however, these supernatant solutions were first concentrated by dialysis against chilled, concentrated sucrose (as suggested by Squire *et al.*, 1963), then further dialyzed against water, buffer, or salt solutions to remove the sucrose. Aliquots of the concentrated saline extracts were mixed with NaBr to give solutions with a density of 1.21 g/ml for ultracentrifugal separation.[2] These samples were centrifuged for 16–18 hr at 4° at 140,000*g* (No. 50 rotor) in a Spinco Model L preparative ultracentrifuge, which was allowed to decelerate without braking.

Lipid Extractions and Analyses. Lipids were extracted from stromal samples or from lyophilized aliquots of saline extracts by a modification of the procedure of Sloviter and Tanaka (1964). This method consists of extraction of the sample three successive times with chloroform–methanol mixtures, *in vacuo* drying of this extract, re-extraction of the lipid residue with chloroform, and washing of this chloroform extract by the addition of methanol and 0.1 M KCl. Phospholipids were separated by thin layer chromatography on plates spread with silica gel G (E. Merck AG, Darmstadt) and developed in chloroform–methanol–water (95:35:6, v/v). After detection with iodine vapor, the phospholipid zones were scraped into glass-stoppered centrifuge tubes. Each sample of silica gel was extracted with formic acid–chloroform–methanol (2:1:1, v/v)[3]; after 30 min of mixing, the silica gel was sedimented, and an exact volume of the supernatant was taken for determination of phosphorus (by the method of King, 1932). Usually, the recovery of lipid P was 85–90% of the starting amount.

The methyl esters of fatty acids were prepared from the total lipid extracts by the transesterification reaction described by Morgan *et al.* (1963). The methyl esters were dissolved in redistilled hexane and subjected to gas–liquid partition chromatography on columns of 15% ethylene glycol succinate on Anakrom 60/70, at 178°. A Barber-Colman gas chromatograph equipped with an argon ionization detector (Barber-Colman Co., Rockford, Ill.) was used. The methyl esters were identified by comparison of their retention times on the column with those of standard fatty acid esters; the amount of each was approximated by multiplying the height of each peak by the peak width at half-height. Cholesterol was determined by the method described by Mårtensson (1963).

Other Assays. Protein was determined by the method described by Lowry *et al.* (1951). A solution of purified bovine serum albumin (Pentex, Kankakee, Ill.) was used as an arbitrary standard and was itself assayed by measurement of its absorption at 280 mμ, using an extinction coefficient of 6.60 for a 1% solution, as determined by Cohn *et al.* (1947).

Acetylcholinesterase was measured by the method of Michel (1949). The sample was incubated for 60–90 min at 25° in a buffered solution containing 0.022 mmole of acetylcholine in a total volume of 2.2 ml. The pH of the mixture was determined to the nearest 0.005 unit prior to the addition of the substrate and again at the end of the incubation with a Radiometer pH meter, Type pHM 22p, with an expanded scale. The rate of change of pH over an interval of 60 min was linear with respect to sample concentration for samples containing the equivalent of 0.085–0.200 ml of packed red cells. In the intact-cell studies, the extent of hemolysis was estimated by comparing the absorbancy at 418 mμ of hemoglobin-containing supernatants with the absorbancy of known dilutions of red cells.

Phase and Electron Microscopy. The samples for

[2] This component was considered a lipoprotein on the basis of its flotation on ultracentrifugation in an adjusted density medium and the presence of lipid in the same fraction. Interestingly this lipoprotein fraction sedimented in a 1.104 density salt solution, but floated in a 1.21 density salt solution. This behavior would place this material in the high-density lipoprotein class.

[3] Use of this solvent was kindly suggested to us by Dr. Frank Parker and Mr. Norman Peterson, University of Washington School of Medicine.

TABLE I: Phospholipid Distribution in the Lipids of Control and 1.2 M Saline-Washed Stroma.

Lipid	% in Control[a]	% in Treated[a]	Significance[b] (*P*)
Phosphatidylethanolamine	27.4 ± 2.7	28.9 ± 2.2	0.08
Phosphatidylcholine	31.3 ± 1.6	31.6 ± 1.5	>0.50
Sphingomyelin	27.7 ± 1.3	24.5 ± 1.8	0.002
Phosphatidylinositol + phosphatidylserine	13.4 ± 3.5	14.9 ± 2.2	>0.10

[a] Sample standard deviation = $[\Sigma x^2/(n-1)]^{1/2}$, where x = the difference from the mean. [b] Significance of differences tested by the Student's t distribution.

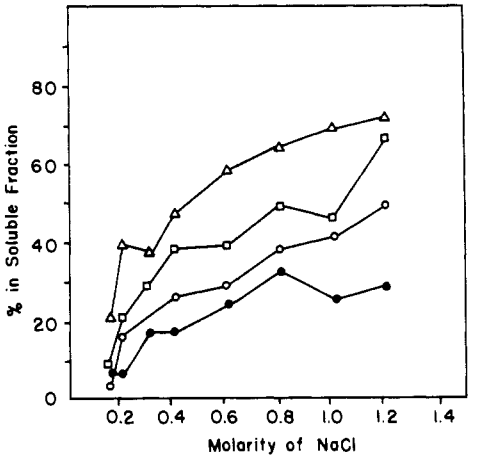

FIGURE 2: Composition of soluble fraction obtained from stroma rewashed with varying concentrations of unbuffered NaCl, pH 6.5. Control stroma was washed only with pH 7.4, 20 milliosmolarity phosphate. Protein, ●–●–●; phospholipids, O–O–O; cholesterol, □–□–□; acetylcholinesterase, △–△–△.

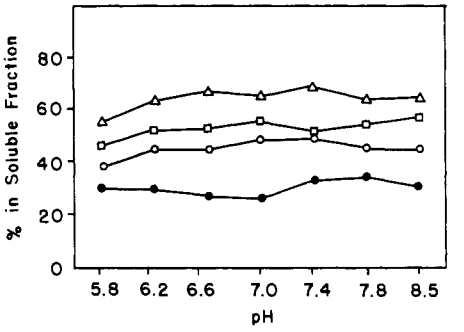

FIGURE 3: Composition of soluble fraction obtained from stroma which have been rewashed with 0.8 or 1.2 M NaCl, at one of several pH values. Control stroma was washed with pH 7.4, 20 milliosmolarity phosphate. Total acetylcholinesterase activity recovered stroma and soluble fraction at each pH was 100%. Protein, ●–●–●; phospholipid, O–O–O; cholesterol, □–□–□; acetylcholinesterase, △–△–△.

phase microscopy were suspended in 0.15 M NaCl and examined with a Zeiss phase microscope equipped with a Zeiss microscopic strobe light. Unfixed stroma was studied by electron microscopy after negative staining with 2% phosphotungstic acid. Similar stroma was fixed in 2% osmium tetraoxide, buffered in *s*-collidine at pH 7.2–7.4, then dehydrated in graded ethanol and propylene oxide mixtures. The fixed stroma was embedded in Epoxy resin according to Luft (1961), sectioned, and double stained with lead tartrate and uranyl acetate.

Results

The percentages of the original protein, phospholipid, cholesterol, and acetylcholinesterase solubilized by saline washing of stroma are shown in Figures 1 and 2. In every case, the remainder of the protein, lipid, and enzymatic activity could be quantitatively recovered in the residual stroma.

It is obvious that increasing amounts of protein, lipid, and acetylcholinesterase are solubilized from stroma by increasingly concentrated NaCl solutions. In the presence of 0.005 M Tris, pH 8.2, the maximum release of components was reached at 0.6 M NaCl, with little additional change at higher concentrations. On the other hand, when unbuffered saline is used, additional amounts of protein, lipid, and enzyme are solubilized as the NaCl concentration is raised from 0.6 to 1.2 M, and no apparent maximum is reached. The decrease in solubility of the membrane components in the presence of Tris does not seem to be due to a pH effect alone; as shown in Figure 3, the solubilization of membrane components by NaCl solutions is quite independent of pH over the range pH 6.2–8.2.

Inasmuch as 1.2 M saline treatment had removed as much as 50% of the lipid from the treated stroma (Figure 2), analyses were made to determine if particular classes of lipids had been solubilized. The phospholipid

TABLE II: Fatty Acid Composition of the Total Lipids of Control and 1.2 M Saline-Washed Strom A.[a]

Fatty Acid	% in Control[b]	% in Treated[b]	Significance[c] (*P*)
Palmitic (16:0)	23.2 ± 1.4	22.0 ± 1.9	>0.25
Stearic (18:0)	20.0 ± 1.3	21.8 ± 1.3	>0.06
Oleic (18:1)	21.2 ± 0.8	21.5 ± 0.7	>0.50
Linoleic (18:2)	16.0 ± 1.1	15.4 ± 1.7	>0.20
Arachidonic (20:4)	19.4 ± 2.8	19.1 ± 1.3	>0.50

[a] Treated stroma had been washed with 0.8 or 1.2 M NaCl as described in the text. The sum of the five fatty acids listed was defined as 100%. [b] Sample standard deviation = $[\Sigma x^2/(n-1)]^{1/2}$, where x = the difference from the mean. [c] Significance of differences tested by the Student's t distribution.

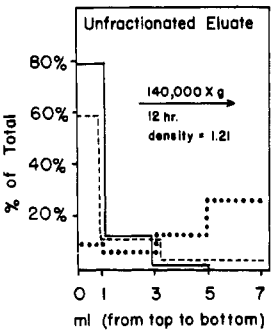

FIGURE 4: Distribution of lipid, protein, and acetylcholinesterase in ultracentrifuged samples of the saline solubilized fraction. Samples were centrifuged for 12 hr, 4°, at 140,000*g* in a medium with final density of 1.21 g/ml. Acetylcholinesterase, ———; phosphorus, – – – –; protein, ●●●●●●.

distribution (Table I) showed no significant differences between the lipids of untreated (control, washed with pH 7.4, 20 milliosmolarity phosphate) and treated (washed with 1.2 M saline, unbuffered) stroma. Likewise, the fatty acid composition of the total lipid extracts showed no significant differences, as demonstrated in Table II. However, as shown in Table III, there was a change in the cholesterol:phospholipid weight ratio in the solubilized material as compared to the stroma. Although the significance of this observation is not readily explainable now, it can be concluded that the solubilization phenomenon was nonselective with respect to phospholipid.

TABLE III: Composition of Human Erythrocyte Stroma and of 1.2 M NaCl Extract from Such Stroma.[a]

Components	Intact Stroma	1.2 M NaCl Extract
Protein	5.40 mg	1.51 mg
Phospholipid[b]	2.75 mg	1.35 mg
Cholesterol	1.65 mg	1.07 mg
Acetylcholinesterase	32.0 units	21.1 units

[a] The quantities given are for the equivalent of 1 ml of packed red cells. [b] Phospholipid was estimated by multiplying the milligrams of lipid phosphorus by 25.

The general composition of the saline-extractable material is given in Table III. Ultracentrifugation of this solubilized fraction in NaBr solutions, density = 1.21 g/ml, suggested that at least two protein fractions had been removed from the stroma. As shown in Figure 4, a small amount of protein was in lipoprotein form and contained all of the solubilized acetylcholinesterase activity.[4] The bulk of the protein sedimented during this centrifugation. A similar separation into protein and lipoprotein fractions was observed when concentrated saline extracts were dialyzed *vs.* H_2O; a large part of the lipoprotein fraction precipitated irreversibly when the ionic strength was lowered.

Figures 5 and 6 show phase micrographs of stroma before and after treatment with hypertonic NaCl. The treated stroma became reduced in size and contained peripheral, bead-like protuberances. Electron micrographs of negatively stained stroma are shown in Figures 7 and 8, in which the extent of the saline-induced alteration is even more clear. The treated stroma is surrounded by numerous small globules, which in cross section (not shown) appeared to be membrane-bounded spheres. No such globules were seen in the control preparations.

Washing of *intact* erythrocytes with NaCl solutions (0.15, 0.80, and 1.20 M) was carried out under the same conditions as described in the preceding experiments. After 16 hr at 4°, the intact cells had undergone the

[4] The treatment of stroma with 1.2 M NaCl appears to be an excellent route to the preparation of solubilized acetylcholinesterase of high specific activity. As an example, the specific activity of stromal acetylcholinesterase was 0.12 unit/mg of protein, whereas the specific activity of solubilized acetylcholinesterase activity was 22.7 units/mg of protein. Thus, a 190-fold purification of the acetylcholinesterase could be achieved by this rapid, simple procedure (unpublished observations).

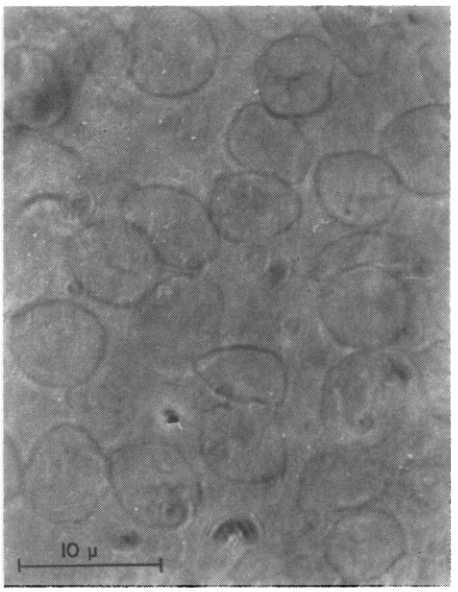

FIGURE 5: Phase micrograph of hemoglobin-free human erythrocyte stroma before NaCl treatment.

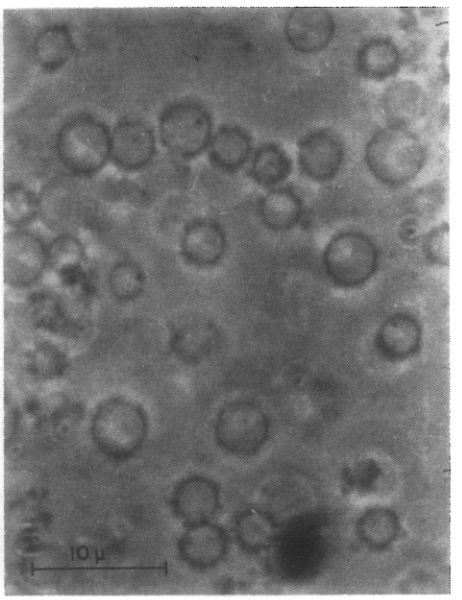

FIGURE 6: Phase micrograph of human erythrocyte stroma which have been washed with 0.8 M NaCl. Stroma washed with 1.2 M NaCl appear similar in every respect.

changes tabulated in Table IV. It is evident from the low level of hemolysis that almost no membrane damage had occurred in his period, and that the effect of NaCl upon acetylcholinesterase in the intact cell was far different from its effect upon this same enzyme in isolated stroma.

TABLE IV: Alterations in Intact Red Cells Suspended for 16 Hr in NaCl Solutions[a] at 4°.

NaCl (M)	% Hemolysis	% of Acetylcholinesterase Released
0.15	0.5	0.4
0.80	0.4	1.1
1.20	0.9	6.1

[a] All solutions were 0.005 M in phosphate, pH 7.4, to minimize the extent of spontaneous hemolysis.

Discussion

It seems clear that no method of hemolysis can produce stroma which accurately represent the "true" cell membrane as it is constituted in the intact cell. The very process of hemolysis would seem to change the environment in an irreversible manner, and thus the properties, of that membrane.[5] The object of these studies has been, therefore, to elucidate the fundamental, structural membrane which may be considered to be the framework of the more complex membrane structure *in situ*.

In this study, an initial attempt has been made to define the enzymes and proteins which are integral components of the basic, structural cell membrane of the human erythrocyte. Some success in removing loosely bound proteins from this basic membrane has already been reported (Mitchell *et al.*, 1965). The possibility is implicit that such stroma, which demonstrate minimal levels of certain components, may have been depleted not only of all these loosely bound proteins but of all physiological functions as well. There is, after all, no reason at present to expect that the fundamental structural membrane necessarily will be a functional one, although this latter possibility cannot be excluded. Nonetheless, it is structural integrity that is a fundamental parameter by which stromal preparations can be compared and judged. Moreover, the structurally intact membrane, whether or not it has physiological function, does represent the basic framework of the cell membrane and therefore is worthy of investigation in its own right.

The morphological effects of concentrated salt solutions upon erythrocyte stroma and intact erythrocytes have been noted by a number of investigators. Furchgott (1940), who published a detailed description of the "stromatolytic forms" and globules which arose from saline-treated stroma, cited references to studies made as early as 1914. Lovelock (1953) found that short ex-

[5] There can be little dissent from the conclusion that the isolated erythrocyte stroma is an artifact, or essentially a derivative of the original membrane. It must not be assumed, however, that careful study of this artifact is meaningless. In actual fact, it provides a convenient and useful entity for an approach to an understanding of the biochemical nature of this membrane.

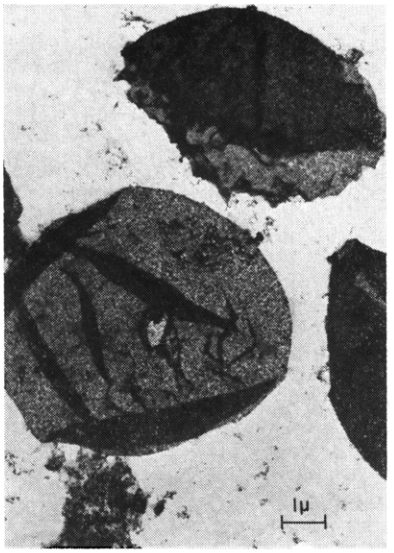

FIGURE 7: Electron micrograph of untreated human erythrocyte stroma, negatively stained with 2% phosphotungstic acid.

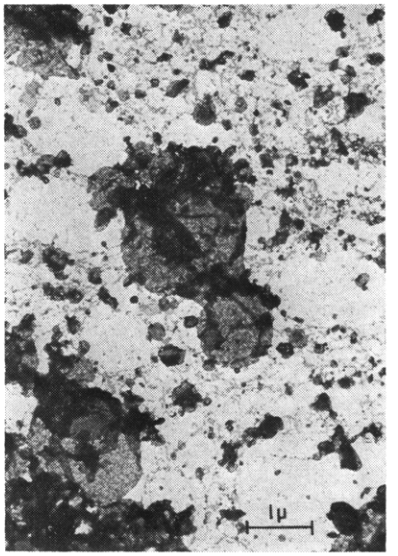

FIGURE 8: Electron micrograph of 1.2 M NaCl washed human erythrocyte stroma, negatively stained with 2% phosphotungstic acid.

posure of intact cells to 3.0 M NaCl produced similar forms; lower salt concentrations did not. Ponder and Barreto (1954) observed that both intact cells and stroma shrink spontaneously in isotonic solutions, then give rise to myelin figures. Tomcsik and Scherrer-Gervai (1961) reported that stroma, when suspended in 0.5–4.0% NaCl, developed bud-like protuberances and then disintegrated into small spheres if this stroma had first been treated with trypsin. These latter investigators did not observe similar behavior in non-trypsinized controls. Finally, Baker (1964) had described the appearance of tube-like "stromalytic" figures formed when intact cells are placed in hypotonic saline solutions.

An attempt has been made in this present investigation to combine morphological observations with biochemical analyses. It has been shown in the previous report (Mitchell *et al.*, 1965) that acetylcholinesterase, unlike the glycolytic enzymes, could not be removed from the stroma even by numerous washes with hypotonic solutions. The present communication shows that it is possible to solubilize the bound acetylcholinesterase to a considerable extent by washing hemoglobin-free stroma with hypertonic NaCl solutions. This removal of acetylcholinesterase activity is accompanied by the removal of other stromal components as well, not only protein but also cholesterol and phospholipid. The solubilized lipids seem identical both in phospholipid distribution and in fatty acid composition with those lipids which are in the residual stroma. The morphological changes which accompany this partial solubilization of stromal material are similar to those reported by previous workers.

The deleterious effects of freezing and thawing upon living cells have been ascribed in part to membrane damage caused by concentrated salt solutions (Lovelock, 1954); no correlation between lipid loss and NaCl concentration was made, nor was the composition of the solubilized lipid fraction defined. In the present study, isolated erythrocyte stroma has been used in place of the intact cell. The results reported here substantiate the hypothesis that membrane damage occurs secondarily to the solubilization of proteins and lipoproteins from the cell membrane.[6] In addition, a complete analysis of the solubilized lipids has revealed that the extent of loss is dependent upon the concentration of the surrounding saline and that no selective loss of a particular phospholipid class or classes has occurred. It must be noted, however, that the effect of NaCl upon acetylcholinesterase in the isolated stroma was far greater than the NaCl effect on the intact cell; whether this difference is qualitative as well as quantitative has not yet been determined.

The data presented here may be evaluated with respect to a possible model of membrane ultrastructure. It may be hypothesized that the partial fragmentation of stroma observed in these experiments occurs secondarily, as a result of the solubilization of surface proteins and lipoproteins from the stroma. If this is true, then it might be possible that the residual stroma

[6] A reviewer has raised the interesting point that the acetylcholinesterase might have indeed been present as a soluble component in the cytoplasm of the intact erythrocyte, where a high cation concentration predominates, and on hypotonic hemolysis it became insoluble and bound to the membrane. Then, on subsequent treatment with hypertonic saline this component is solubilized again. Although this proposal is provocative, the very low and slow solubilization of the acetylcholinesterase from stroma treated with isotonic saline and the irreversible precipitation of this lipoprotein (solubilized by hypertonic saline treatment) by dialysis against water or by direct dilution would argue against the above proposal.

still represents a minimal, structural framework of the cell membrane, made up of reduced numbers of lipid and protein molecules in comparison to the untreated stroma. It must be recalled, however, that the residual stroma resulting from treatment with hypertonic saline has lost its original stability. Thus, the structural integrity of the cell membrane might be dependent not only upon a minimal lipid–protein framework but also upon the presence of additional proteins and/or lipoproteins to stabilize the minimal layer.

Acknowledgment

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Valinomycin and Mitochondrial Ion Transport*

Etsuro Ogata and Howard Rasmussen

ABSTRACT: The effects of valinomycin upon K^+ and Ca^{2+} transport in the rat liver mitochondria have been studied. The results indicate that valinomycin acts by altering the permeability of the membrane to K^+, and that under these conditions K^+ and Ca^{2+} compete for the same carrier. It is concluded that the mitochondrial swelling associated with K^+ uptake is an osmotic phenomenon.

Although mitochondrial potassium uptake and binding have been studied for a number of years (Stanbury and Mudge, 1953; Gamble, 1957; Amoore, 1960), only recently have agents been discovered which dramatically alter this mitochondrial activity. Moore and Pressman found that the antibiotic, valinomycin, stimulated K^+ uptake and H^+ ejection in an energy-dependent reaction (Moore and Pressman, 1964; Pressman, 1964). This was followed by the demonstration that parathyroid hormone stimulates K^+ uptake under appropriate circumstances (Rasmussen *et al.*, 1964).

Moore and Pressman (1964) have proposed that valinomycin acts to promote K^+ uptake by increasing

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