Stability of the Bovine Erythrocyte Membrane. Release of Enzymes and Lipid Components*

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ABSTRACT: During the process of hemolysis and subsequent washings, bovine erythrocytes release a considerable portion of their acetylcholinesterase (acetylcholine acetylhydrolyase, EC 3.1.1.7) activity as well as membrane lipids in a soluble form. Hemoglobin and certain glycolytic enzymes are solubilized prior to the release of the acetylcholinesterase and lipid. The release of acetylcholinesterase from bovine erythrocytes is almost completely prevented if a divalent cation such as Ca^{2+}, Mg^{2+}, Ba^{2+}, or Sr^{2+}, in 1–5 mM concentration, is added to the hemolyzing mixture. The stroma thus prepared maintain their expected morphological shape and total acetylcholinesterase and lipid levels (of the original cells) even after contact with a hypotonic buffer for several days. The membrane fragment solubilized in the absence of any added divalent cation during hemolysis behaves as a lipoprotein. This latter component is easily sedimented by ultracentrifugation and contains a higher proportion of lipids and acetylcholinesterase of significantly higher specific activity (five- to-sixfold increase) than found in the intact membrane. No glycolytic enzyme activity is detectable in this latter fraction. A similar membrane fragment can also be liberated from intact bovine erythrocytes by a short-term treatment with hypertonic saline. The magnesium (calcium) content of bovine erythrocytes is significantly lower than that of human erythrocytes.

These data suggest a less cohesive structure for the bovine erythrocyte membrane as compared with the human erythrocyte membrane.

Previous reports from this laboratory (Dodge *et al.*, 1963; Mitchell *et al.*, 1965) have shown that hemolysis of human erythrocytes in hypotonic buffer of appropriate osmotic strength, *e.g.*, 25 imOsm, and pH 7.5, followed by repeated washing with the same buffer, yields a ghost or stroma (membrane) preparation that is essentially free of hemoglobin but retains nearly all the lipids and acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) of the original cells. This latter enzyme, which can be solubilized from the stroma by hypertonic (0.8–1.2 M NaCl) saline extraction or treatment, is tightly associated with lipids and has a significantly higher specific activity than that found in the intact stroma. The acetylcholinesterase in erythrocytes has been reported to have the same properties as that in conductive tissue which is called "true" (specific) acetylcholinesterase and which is localized in the surface of the tissue cells (Nachmansohn and Wilson, 1951). Furthermore, evidence is available that suggests the location of this enzyme is on the outer surface of the membrane (Herz *et al.*, 1963; Firkin *et al.*, 1963).

The requirements for larger quantities of membrane enzymes and lipoproteins for detailed chemical and physicochemical investigations led to a study of the bovine erythrocyte as an adequate source for these materials. In contradistinction to the human erythrocyte the integrity of the bovine erythrocyte membrane is highly dependent upon the presence of divalent cations. The present communication presents the results of an investigation into the factors influencing the stability of the bovine erythrocyte membrane.

Experimental Section

Preparation of Bovine Erythrocyte Suspension. Bovine blood was collected at the slaughter house, using as the anticoagulant one part by volume of 3.8% trisodium citrate solution for nine parts of blood. Within 1 hr of collection, the blood was centrifuged, plasma and the "buffy" leucocyte layer were removed by aspiration, and the erythrocytes were washed three times with 0.15 M (isotonic) sodium chloride (unbuffered, pH approximately 6.5). The cells were routinely centrifuged for 20 min at 2000 rpm (1000*g* at the maximum radius) in an International refrigerated centrifuge Model PR-2, with a four-place rotor 276A. All manipulations were carried out at 0–4°. The cells were resuspended in isotonic saline, to a hematocrit of 60–80%, and stored at 4°. Such cell suspensions could be kept for at least 1 week at 4° without appreciable hemolysis or detectable alteration of the various properties examined.

Assay results on whole cells were commonly expressed

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in terms of units per milliliter of packed cells, and assays on stroma or other cell fractions in terms of units per milliliter of packed cell equivalent, based on the volume of intact cells from which the fraction was derived. Cell counts were not done, but the hematocrit values obtained under reproducible conditions were assumed to be simply related to the number of cells.

Preparation of Buffers. Acidic and basic components of the buffer system were made up separately at the desired osmotic strength (ideal)[1] and monovalent or divalent cation content. The basic component was then titrated with the acidic solution to obtain the desired pH as indicated by a Radiometer pH meter, type pHM22p. Buffer pH was commonly adjusted with the solutions at 0–4°, and the pH meter was equilibrated and standardized with standard buffer at the same temperature.

The buffer systems used in this work were Na_2HPO_2–NaH_2PO_4, Tris base (Trizma Base, Sigma) (HCl), and Veronal (sodium barbital, Mallinckrodt) (HCl). All chemicals were reagent grade.

Preparation of Stroma and Stroma Extracts. Stroma were prepared by one-step hemolysis and three to five additional washes in phosphate buffers as described by Dodge *et al.* (1963). Tris-HCl (Trizma Base, Sigma) and Veronal-HCl (sodium barbital, Mallinckrodt) buffers have also been used to prepare bovine stroma, with results identical with those obtained with phosphate buffers under the same conditions of osmotic strength and pH. All procedures were carried out at 0–4°. Optimum conditions for the preparation of hemoglobin-free bovine stroma are described below.

Quantitative recovery of stroma could be achieved by sedimentation of suspensions at 35,000*g* at the maximum radius (17,000 rpm) in a Servall RC-2 for 20–30 min at 4°.

Hypertonic saline extracts of bovine stroma were made, and lipoprotein fractions containing AcChE[2] activity were isolated from these extracts, following the methods described by Mitchell (1965) and Mitchell and Hanahan (1966) for human erythrocyte preparations. These methods, and others that have been found to be applicable to bovine, but not to human erythrocytes, are discussed below.

Phase Microscopy. Erythrocyte, stroma, and lipoprotein fractions were routinely examined under oil immersion with a Zeiss phase microscope, made available to us by Dr. William Rutter of this department. All phase micrographs were kindly made for us by Mr. Roy K. Hayashi, Department of Biological Structure, University of Washington School of Medicine. The photomicrographs were taken on 35-mm Pan X film, using a Zeiss phase microscope fitted with a Zeiss microscopic strobe light.

General Analytical Methods. AcChE ASSAYS. AcChE (acetylcholine acetylhydrolase, EC 3.1.1.7) activity was generally measured by the method of Michel (1949). The sample was incubated at 25° in a barbital–KCl buffer solution containing 0.022 mmole of acetylcholine (Sigma Chemical Co., St. Louis, Mo.) in a total volume of 2.2 ml. Decreasing the substrate concentration by an order of magnitude caused 61% decrease in solubilized bovine erythrocyte AcChE activity, and increasing the substrate concentration tenfold resulted in 75% inhibition, indicating that a "true" acetylcholinesterase rather than a "pseudo" cholinesterase was being measured (Augustinsson, 1950). The sample pH was measured to the nearest 0.005 unit before addition of 0.2 ml of substrate solution and again at the end of the incubation period.

In order to facilitate comparison between various preparations, a unit of activity was defined as that amount of enzyme causing a change of 1 pH unit in 1 hr in the standard buffer system. When it was desired to compare enzyme samples stored in different buffer or salt solutions, the assay was standardized by adding known amounts of acetic acid to the assay mixture or to a solution of identical buffer capacity and measuring the pH change caused by the additions. A separate standard curve was constructed in this way for each different assay mixture. Results were then expressed in terms of micromoles of acetic acid liberated per hour by means of a micromole per ΔpH conversion factor.

In some cases, a pH-Stat assay for AcChE was used to confirm the results of the ΔpH assay. The pH-Stat method was similar to that described by Kremzner and Wilson (1963). A Radiometer automatic titration apparatus with 0.5-ml capacity syringe buret was used, and, generally, a 1-ml volume of reaction mixture was titrated with standard 0.02 N NaOH.

It has recently been reported that Tris is a fairly effective competitive inhibitor of AcChE (Pavlic, 1967). Tris was used as the buffer salt in some of the studies reported here, and was present in some of the assays. However, the concentration in an assay mixture was in no case high enough to cause very serious inhibition, and comparisons of absolute activity values were made only between assays containing identical Tris concentrations.

OTHER ENZYME ASSAYS. Aldolase (fructose-1,6-diphosphate–D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) was assayed through use of a test kit, aldolase (Calbiochem, Los Angeles, Calif.), in which the aldolase reaction is coupled through supplied glycolytic enzymes to the reduction of NAD^+ in the presence of arsenate. Routinely 0.2 ml of sample was incubated with 2.8 ml of the reagent at 30° for 20 min and the increase in the absorbance at 340 mμ was measured on a Beckman DU spectrophotometer. The enzyme unit is defined as the micromoles of fructose diphosphate split per hour.

Glucose 6-phosphate dehydrogenase (D-glucose-6-phosphate–NADP oxidoreductase, EC 1.1.1.49) was also assayed through use of a test kit, glucose

[1] imOsM, ideal milliosmolar. The ideal milliosmolarity was calculated by totaling the concentrations of all ionizable species in the solution, neglecting deviation of the salts from ideal behavior. Thus, 300 imOsM is a close approximation to isotonicity over the pH range studied.

[2] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: WCH, whole cell hemolysate; SFH, stroma-free hemolysate; AcChe, acetylcholinesterase; GDAP, glyceraldehyde phosphate dehydrogenase.

6-phosphate dehydrogenase (Calbiochem). Routinely, 0.2 ml of sample was incubated with 2.8 ml of the reagent at 30° for 10 min and the increase in absorbance at 340 mμ was measured. The enzyme unit is expressed as micromoles of $NADP^+$ reduced per hour.

Glyceraldehyde phosphate dehydrogenase (D-glyceraldehyde-3-phosphate–NAD oxidoreductase, EC 1.2.1.12) was measured essentially according to the method of Warburg *et al.* (1930) in which the rate of reduction of NAD^+, followed spectrophotometrically, is used as a measure of the enzyme activity. The absorbance of the reaction mixture is determined at 340 mμ at the start and at the end of a 10-min incubation at 30°. The enzyme unit is expressed as micromoles of NAD^+ reduced per hour.

PROTEIN DETERMINATION. Protein was determined by the method of Lowry *et al.* (1951). An electrophoretically pure grade of bovine serum albumin (Pentex, Kanakee, Ill.) was used as the standard, and was dissolved in water just before use. Its concentration was accurately determined by absorbance at 280 mμ, using the extinction coefficient ($E^{1\%}_{280\,m\mu}$) of 6.60 reported by Cohn *et al.* (1947) and verified in this laboratory with the albumin sample used.

Small quantities of hemoglobin were determined by the pyridine hemochromogen method, and larger amounts by the cyanmethemoglobin assay using Aculute and Acuglobin (Ortho Pharmaceutical Corp., Raritan, N. J.). These assays were carried out as described by Dodge *et al.* (1963). At very low values of hemoglobin retention in stroma or lipoprotein particles, the usual assays are complicated by turbidity with the large sample aliquots necessary to obtain readings. Qualitative assays for hemoglobin contamination in lipoprotein preparations were frequently made by scanning the spectra of suitably diluted samples from 600 to 240 mμ with a Beckman DB recording spectrophotometer. Fairly large amounts of hemoglobin were determined by means of the 540-mμ absorption band, and trace amounts of heme were detected by the very sensitive Soret band (410-mμ) absorption.

LIPID ASSAYS. Lipids were extracted from stroma or lipoprotein suspensions or from concentrated SFH by the method outlined by Ways and Hanahan (1964). Lipids were extracted from concentrated stroma or lipoprotein suspensions by a similar procedure, modified as outlined below.

Stroma suspension (4 ml) (1.31 ml of packed cell equiv) was pipetted with stirring into 20 ml of methanol in a 40-ml glass centrifuge tube and the mixture was allowed to stand 20 min at room temperature. Then 20 ml of chloroform was added with stirring, and after 10 min the protein precipitate was collected by centrifugation and the supernatant was filtered through glass fiber paper into a round-bottom flask. The extraction was repeated twice, as above, on the residue. The combined extracts were evaporated *in vacuo* at 25° with added absolute ethanol and the residue was transferred to a 15-ml glass-stoppered centrifuge tube in a total of 10 ml of chloroform–methanol (2:1, v/v). KCl (2 ml of 0.1 N) was added for the first wash. The tube was cooled to 4°, then rewarmed to near room temperature, and separation of the layers was completed by brief centrifugation. The water phase was removed, and 2 ml of methanol and 2 ml of 0.1 N KCl were added for the second wash. The final lipid extract was evaporated *in vacuo* with added ethanol and the residue was dissolved in a total of 5 ml of chloroform–methanol (2:1, v/v) and filtered through glass fiber paper into a 5-ml volumetric flask.

SFH fractions were concentrated by lyophilization and extracted by the method of Ways and Hanahan (1964). The heme pigment content of the final SFH lipid extract was quite variable and seemed to be influenced by the presence of Mg^{2+} in the sample. Such variable behavior of the heme pigments, together with the fundamental difficulty of quantitative extraction of very small amounts of lipid from very large amounts of protein, subjects the lipid assays on SFH samples to considerable uncertainty. Thus, SFH lipid values obtained by subtracting stroma values from whole cell values are probably more reliable than the direct determinations.

Aliquots of total lipid extracts were digested with 70% perchloric acid by refluxing for 30 min on electric heating coils. Phosphorus content was then assayed by the method of Bartlett (1959).

Cholesterol was determined by the H_2SO_4–$FeCl_3$ procedure as described by Courchaine *et al.* (1959), based on the method developed by Zlatkis *et al.* (1953). It was difficult to obtain reliable cholesterol determinations on samples colored by heme pigment since there was an interference with the colorimetric assay. This interference was negligible for whole cell lipid extracts, which contained relatively little pigment and comparatively high concentrations of cholesterol, but it became quite serious in the case of SFH lipid extracts, which were often darkly colored and required large sample aliquots for the cholesterol assay.

The most effective technique found for cholesterol assay of SFH samples involved application of the sample to a thin-layer plate spread with silica gel G and development of the plate in neutral lipid solvent system (see below). This procedure separated cholesterol from three visible pigments spots. The cholesterol band was visualized by brief exposure to iodine vapor, scraped from the plate, eluted from the gel with chloroform and methanol, then assayed by the usual method.

THIN-LAYER CHROMATOGRAPHY was carried out on silica gel G (Brinkmann Instruments, Inc., Great Neck, Long Island, N. Y.). The coated plates were heated for 2 hr at 100°, then stored in a desiccator until needed. The solvent system for neutral lipids was petroleum ether (bp 30–60°)–diethyl ether–glacial acetic acid (70:30:1, v/v). The solvent system for phospholipids was chloroform–methanol–water (100:40:6, v/v). Visualization in qualitative studies was effected by spraying the plates lightly with concentrated H_2SO_4 and charring at 300° for 5 min in a muffle furnace.

In quantitative phospholipid distribution studies, samples containing 15–20 μg of lipid phosphorus were applied to 4 × 8 in. plates spread with silica gel G that had previously been washed with methanol and ether. After development, the plates were exposed briefly to iodine vapor. The visualized zones were marked im-

mediately with a needle point and later scraped into 15-ml glass-stoppered centrifuge tubes. Eluting solvent (10 ml) (formic acid–methanol–chloroform, 2:1:1, v/v) was added, and the tubes were well shaken and left to stand overnight. The gel was sedimented in a clinical centrifuge, and aliquots were removed for phosphorus assay (2 ml for sphingomyelin zones and 5 ml for the other zones). Very low blank values from elution of a blank zone were subtracted from the readings. Recoveries of phosphorus from the plates were 92–95%.

GEL FILTRATION on Sephadex G-100 or G-200 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) was used to effect partial separation of lipoprotein fractions from smaller molecular weight contaminants such as hemoglobin. Lucite chromatographic columns (50 cm) were used with a bed volume of 140 cm^3 and a height to diameter ratio of 26. In the case of G-200 the hydrostatic pressure head was never allowed to exceed 20 cm during packing and running the columns, and flow rates were usually around 15 ml/hr. With G-100, flow rates of about 30 ml/hr gave good separations. Concentrated lipoprotein suspensions (2–5 ml) were applied to the columns and eluted with buffer of rather high ionic strength to minimize possible adsorption effects; generally 0.01 M Tris–0.05 M NaCl was used. Void volume determinations were made with Blue Dextran 2000 (Pharmacia). Columns were usually run in the 4° cold room.

The lipoprotein peak emerged at the void volume and was easily followed by its turbidity. The remainder of the elution profile was generally monitored by reading the absorbance at 280 mμ for protein or at 410 mμ for hemoglobin. AcChE activity and lipid phosphorus assays determined the position of AcChE–lipoprotein in the eluate, and the lipid distribution was checked qualitatively by thin-layer chromatography of chloroform–methanol extracts from key fractions.

CALCIUM AND MAGNESIUM ASSAYS. Calcium and magnesium were determined by means of a Perkin-Elmer Model 303 atomic absorption spectrophotometer equipped with hollow cathode calcium and magnesiums lamps. The assay conditions were similar to those described by Smuckler (1966). Calcium was determined at the 4227-Å line and magnesium at the 2852-Å line.

All glassware used in these assays were washed with HNO_3 and thoroughly rinsed with distilled water that had passed through a mixed-bed deionizing column. Distilled deionized water was used to prepare all reagents.

Samples of concentrated whole cell suspensions of human or bovine erythrocytes were digested with perchloric acid in 30-ml Kjeldahl flasks (up to 2 ml of packed cells in 3 ml of 70% $HClO_4$) at reflux for 2 hr. This procedure gave a clear or slightly yellowish solution, but on cooling to room temperature, a considerable amount of white solid material crystallized from the digest. This material dissolved on adding water to give a clear solution. Lanthanum chloride solution was added to samples and standards at a 1% final concentration in order to bind phosphates. The final sample volume was 25 ml. NaCl and KCl were also added to the standards in amounts calculated to be present in the most concentrated sample solution, in order to correct for possible monovalent cation interference.

Results

Hypotonic Hemolysis of Bovine Erythrocytes. GENERAL BEHAVIOR. Hemoglobin-free bovine stroma may be conveniently prepared by hypotonic hemolysis of erythrocytes using the osmotic strength and pH conditions reported by Dodge *et al.* (1963). Figure 1 shows the dependence of hemoglobin retention in bovine stroma upon the osmotic strength of the preparative phosphate buffer at pH 7.4 and 4°. Figure 2 shows the dependence of hemoglobin retention in the stroma upon pH at a buffer concentration of 25 imOsM. Hemoglobin removal is optimum in the region of pH 7.4–8.0, which is comparable with that found for the human (Dodge *et al.*, 1963).

Bovine stroma have routinely been prepared in 20–30 imOsM sodium phosphate buffer (pH 7.4–7.6) but identical results have been obtained using Tris or Veronal buffers under the same conditions of pH and osmotic strength. Fresh, intact bovine erythrocytes can be washed repeatedly with isotonic (0.15 M) saline without detectable loss of hemoglobin, AcChE, or lipid. The thoroughly washed cells appear as normal biconcave disks under the phase microscope.

SPECIFIC ALTERATIONS OR CHANGES. Hemoglobin-free bovine stroma, in contrast to human stroma, do not quantitatively retain the intact cell complements of AcChE activity and lipid. In fact, bovine stroma prepared as described above retain only 30–60% of the whole cell AcChE activity. The activity lost from the stroma is quantitatively recovered in the SFH.[3] This solubilized AcChE activity shows inhibition by excess substrate, as expected of a "true" rather than a "pseudo" cholinesterase (Augustinsson, 1950), and is always accompanied by solubilized phospholipid and cholesterol.

The absolute level of AcChE retention in bovine stroma prepared from the same cell suspension is not significantly dependent upon buffer osmotic strength at pH 7.4 between 5 and 50 imOsM, or on the pH of 25 imOsM buffer over the range of pH 5.8–8.0. In an experiment in which bovine stroma were prepared at eight different values of buffer osmotic strength from 5 to 50 imOsM, the average AcChE retention value for the series was 42% of the whole cell activity ±4% standard deviation from the mean. A similar experiment in which eight separate stroma preparations were made, two at each of the following buffer pH values: 5.8, 6.5, 7.4, and 8.0, gave an average value for retention of AcChE activity in stroma of 53 ± 2% standard deviation from the mean.

Even though most of the cells' hemoglobin is lost in the first two hypotonic washes, appreciable AcChE activity does not appear in the SFH until the second or

[3] The term, SFH, is generally used to mean the combined initial hemolysate and subsequent hypotonic buffer washes (35,000*g*, 20-min supernatants). When a specific wash is indicated, the designations SFH 1 (from initial hemolysis step), SFH 2, etc., are used.

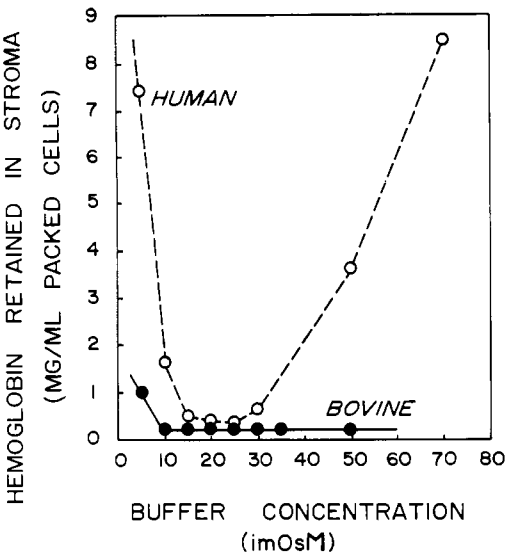

FIGURE 1: Influence of osmotic strength of hemolyzing medium at constant pH on hemoglobin retention in bovine and human erythrocyte stroma. Intact erythrocytes were treated with phosphate buffers of varying ideal osmolarity at pH 7.4, as described in text.

third wash (considering the supernatant from the initial hemolysis step as SFH 1). The time of contact of the cells with hypotonic buffer is important in this regard. For example, if the cells are allowed to stand overnight at 4° in the initial hemolyzing solution before centrifuging, most of the released AcChE activity appears in SFH 1. If the stroma are spun down after 30 min or shorter contact with the hypotonic buffer, a pattern of hemoglobin, enzyme, and lipid release similar to that shown in Figure 3 is obtained. Release of phospholipid

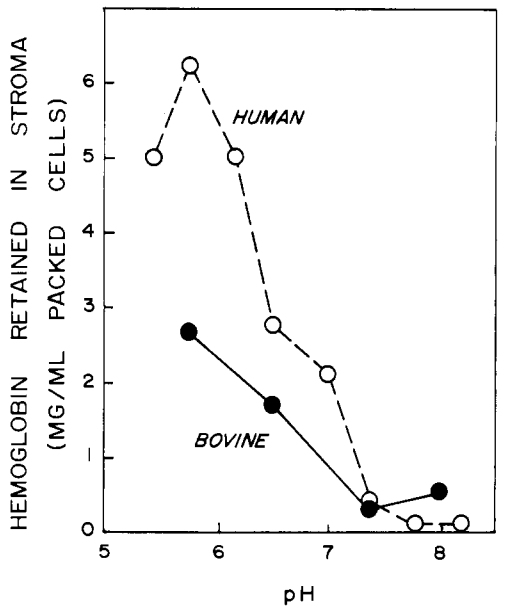

FIGURE 2: Influence of pH of hemolyzing medium at constant osmotic strength on hemoglobin retention in bovine and human erythrocyte stroma. Intact erythrocytes were treated with 25 imOsM phosphate buffer at varying pH values, as described in text.

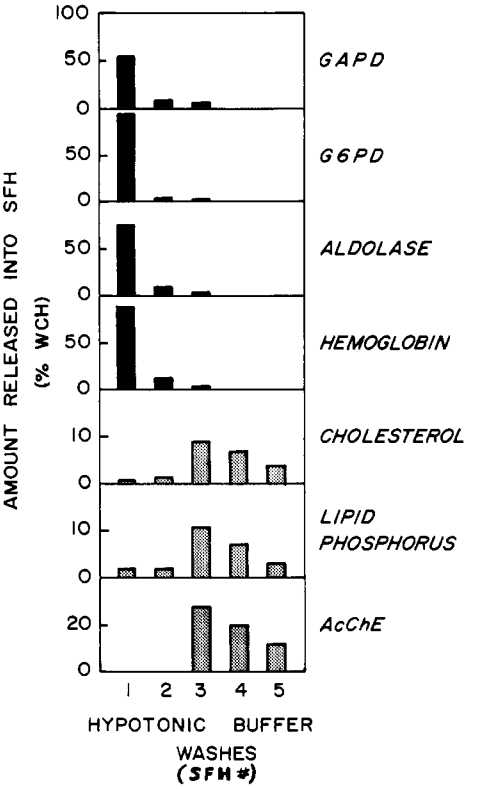

FIGURE 3: Differential solubilization of bovine erythrocyte components by hypotonic hemolysis and washing in absence of added divalent cations. Cells remained in contact with each wash solution 20–30 min before centrifuging. Typical values for whole cell hemolysates were as follows: AcChE, 270–300 μmoles of acetic acid released/hr per ml of cells; aldolase, 50–60 units/ml of cells; G-6-PD, 4–5 units/ml of cells; GAPDH, 150–200 units/ml of cells.

and cholesterol follows the pattern of AcChE release, while G-6-PD, aldolase, and GAPD are released according to the hemoglobin pattern.

The replacement of the hemolyzing buffer with distilled water gives considerable, but incomplete, hemolysis of both human and bovine erythrocytes and causes release of AcChE from the bovine erythrocyte in essentially the same pattern as 25 imOsM buffer. Isotonic (0.15 M) saline will not release AcChE activity from intact, nonhemolyzed bovine erythrocytes; yet, after one hypotonic wash with a 25 imOsM buffer, additional washes with isotonic saline are just as effective as additional hypotonic buffer washes in effecting release of AcChE from the stroma. This observation focuses attention on the primary hemolytic event as an irreversible structural alteration of the membrane, a prerequisite to the release of AcChE from the membrane in hypotonic or isotonic media. Figure 4 shows the morphological appearance of bovine stroma after hypotonic washing followed by resuspension in isotonic saline.

On the basis of this above information, the following procedure has been routinely used to obtain acetylcholinesterase of fairly high specific activity. The washed cell suspension in isotonic saline is added to 10–15 volumes of hypotonic buffer (pH 7.4, 25 imOsM), mixed for 5–10 min, and then centrifuged immediately at 35,000g

TABLE I: AcChE and Hemoglobin Release from Intact Bovine Erythrocytes by Hypotonic and Hypertonic Treatments.[a]

Medium	% AcChE in				% Hemoglobin in	
	1st SFH[b]	2nd SFH[c]	Stroma	Total Recov	1st SFH[b]	2nd SFH[c]
Hypotonic phosphate buffer (pH 7.4, 25 imOsM)	4.4	24.4	66.5	95.3	73	15
Hypertonic saline (1.2 M NaCl, unbuffered)	34	14	51.8	99.8	14	68

[a] Values as per cent of the activity or amount in the WCH. [b] First treatment: 1.5-hr cell solution contact at 4°. [c] Second treatment: 16 hr at 4°.

for 10 min. To the packed stroma is added the same hypotonic buffer, and after mixing for 5–10 min, the mixture is centrifuged as above. The combined supernatants from these two short-term treatments contain almost all the soluble enzymes and hemoglobin but no acetylcholinesterase and only a few per cent of the lipids originally present in intact erythrocytes. The stroma thus obtained are further treated with the same buffer two or three times, each time for 7–14 hr. Through these long-term hypotonic treatments, 80–85% of the acetylcholinesterase activity of the intact cells is released into a stroma-free solution. The latter is then centrifuged at 100,000*g* for 1 hr and the pellet is washed once and centrifuged again. The resulting pellet is the acetylcholinesterase-containing lipoprotein fraction. It has a specific AcChE activity five- to tenfold higher than that of the intact cell, based on nonhemoglobin protein, is largely free of hemoglobin, and has only traces of aldolase and G-6-PD activity.

Effect of Hypertonic Saline on the Intact Bovine Erythrocyte. Hypertonic (1.2 M) saline solubilizes AcChE–lipoprotein from human erythrocyte stroma, but has very little effect on intact human erythrocytes, even after 16-hr exposure at 4° (Mitchell and Hanahan, 1966). On the other hand, 1.2 M NaCl does release AcChE activity from the intact bovine erythrocyte, and this release is considerably more rapid than the release of hemoglobin. Table I compared hypertonic saline and hypotonic buffer treatments on identical samples of bovine erythrocytes under identical conditions of temperature, cell:solution ratio, time of contact, and centrifugation. Thus, hypertonic saline treatment allowed solubilization of 34% of the whole cell AcChE activity in the first 1.5-hr wash whereas only 14% of the hemoglobin was released. In the first wash with a hypotonic solution, on the other hand, only 4% of whole cell AcChE was solubilized in 1.5 hr, whereas 73% of the hemoglobin was released at this stage. The enzyme release on hypertonic treatment was also accompanied by a release of lipid constituents from the intact erythrocytes. In one experiment, it was demonstrated that 10–13% of the total cell lipids and 15% of the AcChE were solubilized by a single hypertonic treatment for 30 min.

Phase microscopy of hypertonic saline-treated bovine erythrocytes resuspended in isotonic saline showed a few intact stroma and a considerable amount of material, apparently consisting of membrane fragments and shrivelled "stromalytic forms." The treatment had obviously caused considerable membrane damage (Figure 5).

The bovine red cell is evidently much less stable to hypertonic media than the human red cell. It is difficult

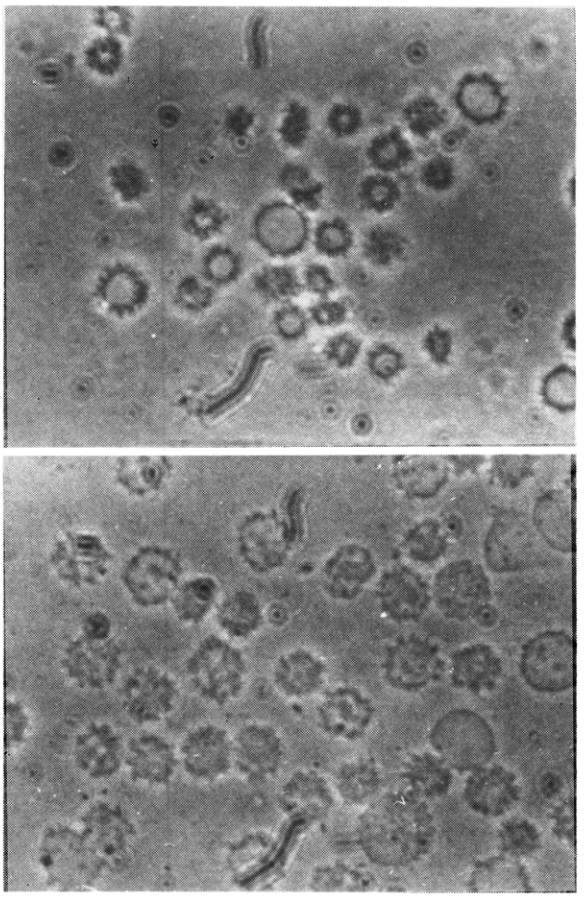

FIGURE 4: Phase micrographs of bovine stroma prepared by hypotonic hemolysis and washing with 25 imOsM sodium phosphate buffer (pH 7.4), then resuspended and allowed to stand 48 hr at 4° in 0.15 M (isotonic) saline. Total magnification on the prints = 2270-fold. Top: Stroma given two 15-min hypotonic washes before resuspension in 0.15 M NaCl; note crenated membranes. Bottom: Stroma given five 15-min hypotonic washes before resuspension in 0.15 M NaCl; note small, spiked membranes.

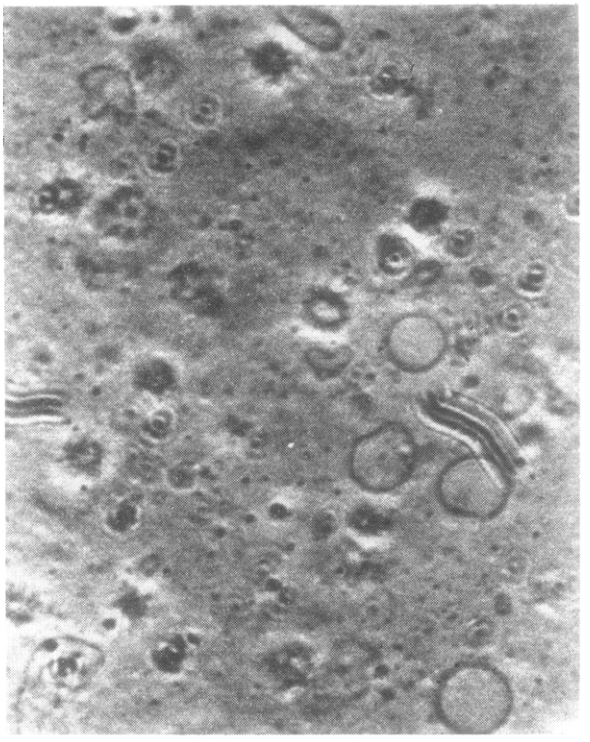

FIGURE 5: Phase micrograph of bovine erythrocytes after two 15-min washes with 1.2 M NaCl and resuspension for 48 hr at 4° in 0.15 M NaCl. Note several stroma-like forms in lower right, and considerable particulate material, too small to be resolved by the phase microscope. A control sample of erythrocytes washed and resuspended in 0.15 M (isotonic) NaCl remained morphologically intact and showed negligible hemolysis. Total magnification on the print = 2500-fold.

TABLE II: Effect of Calcium Ion Added to Hypotonic Buffer at Initial or Later Stages of Hemolysis and Washing on AcChE Retention in Bovine Stroma.[a]

Addition of Ca^{2+} to[b]		AcChE Retention in Stroma (% WCH)	
1st and 2nd Hypotonic Washes[c]	3rd and 4th Hypotonic Washes[d]	Expt 1	Expt 2
−	−	35	18
−	+	78	19
+	−	87	71
+	+	91	96

[a] Bovine erythrocytes were washed with isotonic saline, then twice rapidly with 30 imOsM Veronal buffer, and finally twice more with the same buffer for longer periods of time. [b] Final concentration, 1 mM. [c] First and second washes; for 3 min each; only hemoglobin and loosely bound enzymes are released. [d] Third and fourth washes; for 8 hr each in expt 1, and 20 hr each in expt 2; AcChE release is initiated within 30 min during third treatment.

to interpret the rapid solubilization of AcChE relative to the slower release of hemoglobin from the bovine erythrocyte without assuming the AcChE to be a surface component. Brief treatment of intact cells with hypertonic saline provides a second novel approach to the preparation of AcChE, but the relative advantages of this procedure have not yet been thoroughly investigated.

Effects of Addition of Divalent Cations during Hypotonic Hemolysis of Bovine Erythrocytes. The possibility was considered that the release of AcChE during hypotonic hemolysis might result from the leaching out of some cofactor involved in the binding of lipid–protein components within the membrane. On the basis of observations by Hoffman (1962) and Manery (1966), divalent cations such as Ca^{2+} and Mg^{2+} seemed likely candidates. Consequently a number of bovine stroma preparations (Mg-phosphate, Mg-Tris, or Mg-Veronal-stroma) were made using 5 mM $MgCl_2$, 10 imOsM sodium phosphate, Tris-Cl, or Veronal-Cl (pH 7.4) at 4° (25 imOsM over-all osmotic strength). In addition Ca-Tris or Ca-Veronal-stroma were prepared with 1 mM $CaCl_2$, 25 imOsM buffer salt (pH 7.4) at 4° (28 imOsM over-all osmotic strength). Finally, control stroma[4] were obtained either with 25 imOsM buffer salt (PO_4-, Tris-, or Veronal-stroma) or with 10 imOsM buffer salt, 7 mM NaCl (pH 7.4) at 4°, 24 imOsM over-all (Na-PO_4-, Na-Tris, or Na-Veronal-stroma).

Figure 6 shows that the retention of AcChE in bovine stroma depends on the concentration of divalent cation in the hemolyzing buffer. The Mg^{2+} effect is half-maximum near 0.75 mM $MgCl_2$ in phosphate buffer. In Veronal buffer, retention is already near the maximum at 1 mM $CaCl_2$ or $MgCl_2$, and the half-maximum point for both cations is approximately 0.4 mM. Mg-stroma consistently show levels of AcChE retention about twofold higher than comparable control stroma. The absolute retention levels varied with different erythrocyte samples over a range of 30–60% for the control stroma and 60–100% for the Mg-stroma. Ca^{2+} (1 mM) gave somewhat more consistent results, with a range of 75–100% AcChE retention in Ca-stroma and usually greater than 90%.

When 1 mM Ca^{2+} was incorporated only into the first and second hypotonic washes, recovery of AcChE in the final stroma was nearly as complete as when Ca^{2+} was present in all four hypotonic washes (Table II). The inclusion of 1 mM Ca^{2+} in only the isotonic wash also increased retention over the control level, but to a less significant extent, and in fact to about the same degree as Ca^{2+} added in only the third and fourth hypotonic washes. When the cells were washed with 1 mM Ca^{2+} in isotonic saline, followed by a second isotonic wash with 2 mM EDTA, then washed four times with hypotonic buffer in the absence of added Ca^{2+}, there was no increased AcChE retention over the control level in the final stroma. Ba^{2+} or Sr^{2+} at 1 mM concentration gave an effect similar to and perhaps somewhat stronger than Ca^{2+} in preventing release of stromal AcChE.

[4] Control or regular stroma means the stroma prepared with ordinary hypotonic buffer whereas Ca-stroma are those prepared with $CaCl_2$-containing hypotonic buffer, etc.

TABLE III: Attempted Recombination of the Released AcChE with the Residual Stroma.

Expt	Preparation[a]	Fraction	AcChE[b] (μmoles of acetic acid released/ml of packed cells)
1	Stroma in 30 imOsM buffer for 24 hr and centrifuged. No divalent cation added.	Sediment	41.5
			49.5
		Supernatant	194.4
			203.0
2	Stroma in 30 imOsM buffer for 24 hr. Ca^{2+} added and mixture centrifuged.	Sediment	45.0
			54.0
		Supernatant	176.8
			182.2
3	Stroma in 30 imOsM buffer containing Ca^{2+} for 24 hr and then centrifuged.	Sediment	191.5
		Supernatant	25.6
			28.8
Control	Whole cell hemolysate		243.0

[a] Bovine erythrocytes were hemolyzed and washed with ten volumes each of hypotonic buffer of 30 imOsM and pH 7.4 with (in expt 3) or without (in expt 1 and 2) added $CaCl_2$, each for a short period of time (5 min). The stroma thus obtained were suspended in the same buffer for 24 hr at 4° to release AcChE. In expt 2, $CaCl_2$ was then added to such a mixture to give a 1 mM Ca^{2+} final concentration. Each mixture was centrifuged at 35,000*g* for 20 min. [b] Duplicate experiments.

The possibility was considered that divalent cation(s) might not actually protect the red cell membrane from damage in hemolysis but might merely cause the solubilized AcChE-containing membrane fragments to aggregate and thus make them sedimentable at lower centrifugal forces along with the remaining stroma. The following experiments were undertaken to test this hypothesis. Fresh bovine erythrocytes were given two short-contact washes with hypotonic buffer without added divalent cation. The resulting stroma-free hemolysate was discarded. AcChE was then solubilized from the stroma by several additional long-term hypotonic washes, also without addition of divalent cation. Calcium chloride was then added to the AcChE-containing supernatants and they were centrifuged at 35,000*g* for 20 min under conditions generally used to completely sediment stroma. No more than 25% of the enzyme activity was sedimented under these conditions, and essentially the same amount of activity was found in the sedimented fraction when no calcium was added.

The experiment outlined in Table III indicates that recombination of released AcChE with the residual stroma cannot be achieved simply by adding Ca^{2+} (1 mM final concentration) to the mixture following AcChE release in the absence of divalent cations. Bovine stroma prepared by two short-term hypotonic washes, as in the preceding experiment, were allowed to stand in contact with hypotonic buffer in the absence of added Ca^{2+} for 24 hr at 4°. Addition of calcium ion, thorough mixing for 10 min, and subsequent centrifugation at 26,000*g* for 20 min did not bring about any significant increase in AcChE activity in the sedimented fraction over the level obtained with the stroma centrifuged without added calcium. In a separate experiment, the osmolarity of the mixture was restored to the isotonic level prior to the addition of Ca^{2+}, but the result was essentially the same as above. These experiments do not rule out possible recombination at higher concentrations of AcChE–lipoprotein, similar to the reattachment of ATPase with

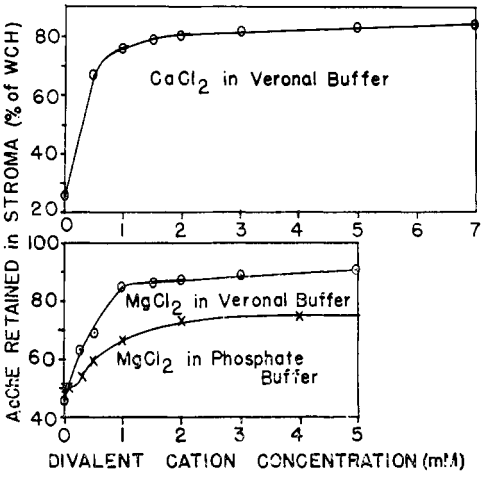

FIGURE 6: The dependence of AcChE retention in bovine erythrocyte stroma upon the concentration of divalent cations in the hemolyzing buffer.

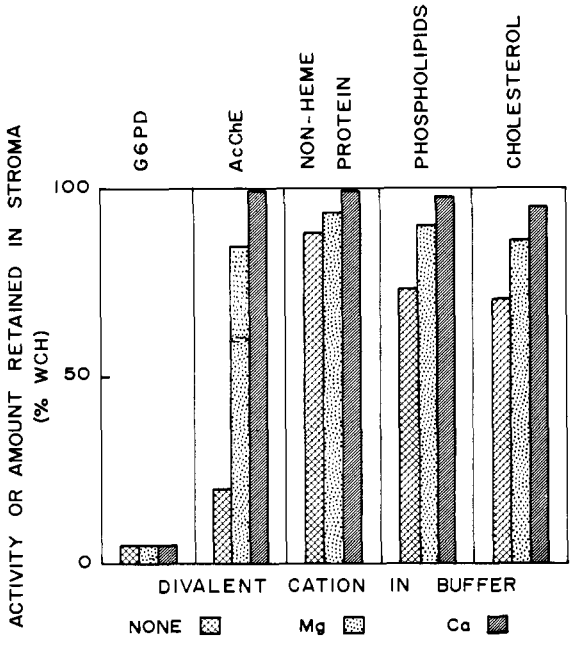

FIGURE 7: The effect of 1 mM Mg^{2+} and 1 mM Ca^{2+} on retention of AcChE activity, nonhemoglobin protein, G-6-PD activity, and lipid in bovine stroma. These results were obtained from long-term (48-hr) treatment of bovine erythrocytes.

streptococcal membranes in the presence of excess ATPase, reported by Abrams and Baron (1968). This possibility remains to be investigated in the case of the bovine erythrocyte AcChE.

Hemoglobin retention in the Ca-stroma or Mg-stroma was somewhat variable. As an example, the Mg-stroma were occasionally quite noticeably pink, even though the final wash was completely colorless. The degree of hemoglobin retention did not seem to correlate with AcChE retention, since some Mg-stroma preparations were unusually pink, but showed rather low retention of AcChE, and the converse situation has also been observed. However, in a series of stroma samples prepared from the same red cell suspension, the color of the pellets definitely increased with increasing Mg^{2+} concentrations from a pearly white control to a strongly pink pellet at 5–8 mM Mg^{2+}. Ca-stroma were more consistent in this effect since they are always quite pink at Ca^{2+} concentrations of 1 mM and above. It should be emphasized, however, that even those stroma preparations which appear unusually pink contain only 0.6% or less of the whole cell hemoglobin.

The decided effect of magnesium and calcium ions on the retention of membrane components such as phospholipids, cholesterol, and certain proteins compared with no influence on the easily solubilized enzyme, G-6-PD, is apparent in Figure 7. In this typical experiment, after a series of hypotonic treatments with an over-all cell–solution exposure of 48 hr, 98% of the G-6-PD, 80% of the AcChE, 12% of the nonheme protein, 26% of the phospholipids, and 28% of the cholesterol were released from the cell to the cell-free hemolysate when the hypotonic medium contained no divalent cation. Addition of Mg^{2+} (final concentration, 1 mM) to the hemolyzing media resulted in a retention of 85–90% of the phospholipid and cholesterol, 90–95% of the nonheme protein, and 60% of the AcChE, but allowed all of the G-6-PD to be released. The effect of Ca^{2+} was still more remarkable in that it almost completely (95–100% retention) prevented the release of all these membrane components, but did not affect the essentially complete release of G-6-PD.

The data shown in Table IV indicate that retention levels of phospholipid and cholesterol, as well as AcChE activity, are higher in Mg stroma than in control stroma. However, the differences in lipid retention are much less striking than differences in retention of AcChE activity. It is evident that the percentage of whole cell lipid released to the SFH in the absence of divalent cations is considerably lower than the percentage released of whole cell AcChE activity. It follows that most of the lipid of the bovine erythrocyte is not intimately related to AcChE. It should be noted, however, that the divalent cation effect on lipid retention has been demonstrated to be significant in several long-term washing experiments such as the one described in the preceding paragraph (also see next section).

The ratio of phospholipid phosphorus to AcChE activity appeared to be fairly constant in the material released to the SFH, at least after rather extensive solubilization of AcChE. The data recorded in Table IV (Na-SFH) and the results of assays on other preparations have consistently shown values for this ratio near 0.11 μg (range 0.09–0.15) of phospholipid phosphorus/unit of AcChE activity (micromoles of acetic acid liberated per hour). Thus, the AcChE that is released to the SFH during hypotonic hemolysis seems to consistently carry with it about one-third the amount of phospholipid per unit of enzyme activity that it possessed in the intact erythrocyte.

However, the Mg-SFH (Table IV), which contains a small percentage of whole cell AcChE, has a much higher ratio of phospholipid to AcChE activity than the intact cell. In other experiments (Figure 3) some release of lipid has been detected in the first two hypotonic washes, before detectable release of AcChE activity. Thus, not all the released lipid would appear to be intimately bound to AcChE protein, but further elucidation of this point must await detailed lipid–AcChE dissociation and binding studies.

It is of interest to note that 1.2 M saline can solubilize AcChE from Ca- or Mg-stroma as well as from the regular stroma (no added divalent cation) of bovine erythrocytes. A typical experiment showed that nearly 50% of the enzyme activity retained in regular and in Ca-stroma was solubilized by three successive, 15-hr 1.2 M NaCl washes. When AcChE is isolated from bovine stroma (Mg-, Ca-, or control stroma) by the hypertonic saline extraction procedure described by Mitchell and Hanahan (1966), the lipid phosphorus to AcChE activity ratio is nearly the same as the ratio found in the intact bovine erythrocyte.

The phospholipid distributions in Mg-stroma, control stroma, and whole bovine erythrocytes were determined by quantitative thin-layer chromatography, and

TABLE IV: The Effect of Mg^{2+} on the Distribution of Lipid and AcChE Activity between Hemoglobin-Free Stroma and SFH after Short-Contact Hypotonic Hemolysis and Washing of Bovine Erythrocytes.[a]

Sample	AcChE, % of WCH Act.	Lipid–P		Cholesterol		Cholesterol (wt)/ Lipid–P (wt)	Lipid–P/ AcChE Act.[c]
		μg/ml of Cells	% of WCH	mg/ml of cells	% of WCH		
Whole cells	100	122 ± 3	100	1.48 ± 0.06	100	12.0	0.36
Mg-stroma	92	104 ± 3	85	1.32 ± 0.03	89	12.8	0.33
Mg-SFH							
Calculated[b]	8		15		11	9	0.66
Found	4	10 ± 3	8	0.05 ± 0.02	2–5	(2.5–9.5)	0.72
Na-stroma	48	95 ± 4	78	1.21 ± 0.05	82	12.8	0.58
Na-SFH							
Calculated[b]	52		22		18	10	0.16
Found	50	19 ± 2	16	0.18 ± 0.04	9–16	(6.75–12.5)	0.11
						12.5)	

[a] Cells were washed five times with hypotonic buffer, with a total contact time of about 6 hr. The following buffers were used: Mg-stroma, 5 mM $MgCl_2$, 10 imOsM Tris-Cl (pH 7.4) at 4°, 25 imOsM over-all. Na-stroma, 7 mM NaCl, 10 imOsM Tris-Cl (pH 7.4) at 4°, 24 imOsM over-all. [b] Calculated by subtraction: WCH − stroma = SFH. [c] Micromoles of acetic acid liberated per hour; average of four determinations on two separate stroma preparations. Uncertainty values given as standard deviation from the mean = $\sqrt{\Sigma d^2/(n-1)}$, where d = deviation of an individual determination from the mean, and n = number of determinations. Lowry protein concentration for all stroma samples = *4.80 mg ± 0.24* per ml of packed cell equiv; average of 14 determinations on 7 separate stroma preparations from the same bovine erythrocyte suspension. Stroma lipid values = average of eight assays on four separate chloroform–methanol extractions from two separate stroma preparations; whole cell lipid values = average of four assays on duplicate $CHCl_3$–MeOH extracts; SFH lipid values = average of four assays on duplicate $CHCl_3$–MeOH extracts from lyophilized SFH samples.

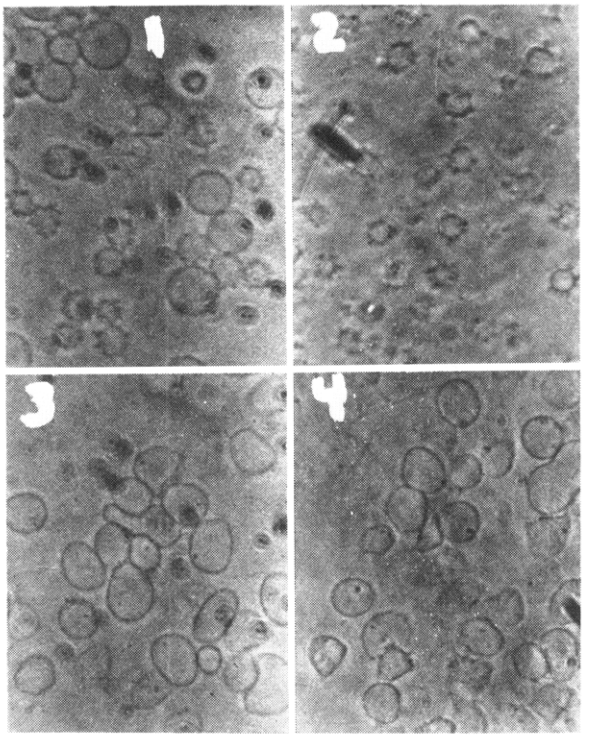

FIGURE 8: Phase micrographs of bovine stroma prepared by hypotonic hemolysis and repeated washing in the presence and absence of Ca^{2+}. All buffers were 30 imOsM over-all osmotic strength. Total magnification on each of the prints = 1730-fold. (1) Control stroma (Na-Veronal) after short-term treatment in the absence of added divalent cations; total time of buffer cell contact = 6 hr; (2) control stroma (Na-Veronal) after long-term treatment (48 hr) in the absence of added divalent cations; (3) Ca-stroma (1 mM Ca^{2+} in Veronal buffer) after short-term treatment (6 hr); (4) Ca-stroma after long-term treatment (48 hr).

no significant differences were found. The phospholipid distribution common to each of the samples examined was as follows (based on recovered lipid–phosphorus): sphingomyelin, 60%; phosphatidylethanolamine, 26%; phosphatidylserine and phosphatidylinositol, 12%; and other, 2%. Recoveries of lipid-P from the plates were 92–95%. In addition, analysis of Ca-stroma and the AcChE–lipoprotein isolated from hypotonic SFH revealed no significant differences in phospholipid distribution patterns.

Thus, the release of lipid from the bovine erythrocyte is not selective for any particular phospholipid class. This is also the case for hypertonic solubilization of lipoprotein from human stroma (Mitchell and Hanahan, 1966). Loss of lipid from erythrocyte stroma probably involves the release of lipoprotein particles or free lipid micelles.

The data in Table IV indicate that the hypotonic solubilization process may be selective for material with a cholesterol to lipid phosphorus ratio different from that of the intact bovine erythrocyte; however, this aspect of the data has been rather variable in separate experiments, so no definite statement concerning relative lipid phosphorus and cholesterol solubilization may be made at this time.

Comments on the Divalent Cation Effect. Lowry protein assays on Mg-stroma and control stroma revealed no significant differences in total protein content of the stroma samples, in spite of the very large difference in AcChE retention and small, but probably significant, differences in lipid phosphorus and chlolesterol retention (Table IV). This indicates that the effect of the divalent cation on loss of protein to the SFH during hypotonic washing does not apply generally to all stromal protein, but is at least partially specific for AcChE. That fraction of the stroma protein, including AcChE, whose retention in stroma is enhanced by the divalent cation represents only a small proportion of the total stroma membrane protein. It has been found that Mg^{2+} and Ca^{2+} have no demonstrable effect on the retention of aldolase and G-6-PD but can alter the "apparent" level of GAPD in the stroma. Although variability was noted in the levels of GAPD and aldolase activity in bovine stromal preparations, there was no doubt that these two enzymes could be retained (ranging from 20 to 40%) under conditions wherein AcChE activity was largely removed through solubilization. Preliminary results to date indicate, though indirectly, that the GAPD activity in particular is dependent both upon the true retention in the stroma and the permeability of the stroma to the substrate of this enzyme.

Short-term hypotonic washes, with about 15–30-min contact time before centrifugation, were used for the preparation described in Table IV. In experiments in which long-term washes (10–16-hr contact) have been employed, small differences in nonhemoglobin protein retention have been detected (see Figure 7). These differences are quite small, however, compared with differences in AcChE activity and lipid retention. Such long-term experiments show definite differences in phospholipid and cholesterol release in the presence and absence of divalent cation. Compared with data from the short-term treatment (Table IV), these results indicate a roughly parallel increase in solubilization of AcChE activity and lipid from the stroma with time of exposure to hypotonic medium in the absence of added divalent cation.

Morphological Appearance of Stroma Prepared under Varying Conditions. It has been possible to correlate differences in the morphological appearance of the bovine erythrocyte membrane with the loss of AcChE and lipid. Several experiments have been carried out in which duplicate stroma preparations have been used for assay of AcChE and lipid release into successive SFH's, and for examination of the stroma by phase microscopy after each hypotonic wash. The results of a typical experiment are described below.

Control (PO_4) and Mg-stroma (5 mM Mg^{2+}) were prepared from fresh, isotonic saline-washed bovine erythrocytes which appeared normal and intact under the phase microscope. Essentially no hemolysis and no detectable loss of AcChE activity occurred during isotonic washing. After the initial hemolysis (first hypotonic wash, 20-min contact before centrifuging) no morphological difference between control and Mg-stroma samples was noted. The stroma membranes were sharp and intact. After the second hypotonic wash, there was still no detectable loss of AcChE activity, although about

TABLE V: The Extent of Long-Term Protection of Mg^{2+} on AcChE Loss from Stroma.[a]

No. of Hypotonic Washes	Total Time of Buffer–Cell Contact	Total AcChE Released to Combined SFH (% of WCH)	
		Mg-PO_4-SFH	Na-PO_4-SFH
5	5 hr	15	52
6	6 days	26	66
7	7 days	34	72
8	4 weeks	53	77

[a] Bovine stroma were prepared in the usual manner in the presence or absence of Mg^{2+} (5 mM) and stored at 4° in hypotonic media.

5% of the whole cell cholesterol and phospholipid was found in the first two combined SFH's. After the third and subsequent hypotonic washes (all short contact), the control and Mg-stroma samples were morphologically different. The Mg-stroma appeared mainly intact, with sharp, distinct membranes, although some apparently damaged cells with crenated membranes could be found. The control stroma sample contained a much higher proportion of apparently damaged cell membranes. Such damaged stroma are reduced in size, and their peripheries are crenated or spiny (see Figures 4 and 8). They appear rather similar to hypertonic saline-treated human erythrocyte stroma residues (Mitchell and Hanahan, 1966). After five short-contact hypotonic washes, the hemoglobin-free control stroma sample contained roughly half-damaged and half-intact membranes and retained 60% of the whole cell AcChE activity. Most of the Mg-stroma were intact, and this sample retained 86% of the whole cell activity.

Ca-stroma remain morphologically intact after extensive hypotonic washing. As mentioned earlier, they also show essentially quantitative retention of AcChE and lipid. Figure 8 shows the morphological differences consistently observed between stroma prepared in the presence and absence of added divalent cation.

The effect of 1 mM $CaCl_2$ on morphology of the stroma is quite long-lasting when the stroma are kept suspended at 4° in the preparative buffer, while the effect of 1 mM $MgCl_2$ is only temporary. The morphological distinction between control and Mg-stroma preparations described above was still evident 24 hr after preparation, but after 5 or 6 days, it was no longer apparent. The long-term change in morphology of Mg-stroma was accompanied by gradual loss of AcChE activity into three additional long-term washes at 4° with 5 mM $MgCl_2$ in 10 imOsM phosphate buffer (pH 7.4).

Control stroma and Mg-stroma prepared as usual by five short-contact hypotonic washes were given three additional washes in their respective preparative buffers over a period of 4 weeks with storage at 4°. Total release of AcChE into the SFH (35,000*g*, 20-min supernatant) after the additional washes is given in Table V. The loss of AcChE activity by denaturation during this period of prolonged storage was negligible, as shown by assay of the final stroma residue. Total recovery of whole cell activity in Mg-stroma and SFH's was 103%, and 90% from control stroma and SFH's.

Thus, 5 mM Mg^{2+} appears to offer bovine stroma some, but rather incomplete, protection from loss of AcChE activity during extended washing and storage in hypotonic buffer. In contrast, a bovine stroma sample prepared and stored in 1 mM $CaCl_2$–25 imOsM Tris-Cl appeared essentially intact under the phase microscope 1 week after preparation, and concomitantly there was no appreciable release of AcChE activity from these stroma.

TABLE VI: Magnesium Content of Human and Bovine Erythrocytes.[a]

Erythrocyte Species[b]	No. of Isotonic Saline Washes	Mg Content (μmoles/ml of packed cells)	% SD[c]
Human (4)	3	1.87 ± 0.06[b]	±3.2
Human (2)	1	2.14 ± 0.09	±4.2
Bovine (3)	3	0.52 ± 0.03	±5.2

[a] Erythrocytes were isolated from citrated blood, washed, and resuspended in 0.15 M NaCl. Samples were washed as described in the text and levels of magnesium determined by atomic absorption spectrophotometry. [b] Numbers in parentheses represent the number of samples assayed. [c] Standard deviation from the mean = $\sqrt{\Sigma d^2/(n-1)}$, where d = deviation of individual determination from the mean and n = number of determinations. % SD = % standard deviation from the mean.

Divalent Cation Concentrations in Human and Bovine Erythrocytes. The Mg^{2+} content of suspensions of human and bovine erythrocytes have been determined by atomic absorption spectrophotometry. Bovine blood was collected in citrate and processed as described in the Experimental Section. Human erythrocytes, type O, Rh^+, were obtained from the King County Hospital Blood Bank, Seattle, Wash. The human blood had been drawn into acid–citrate–dextrose (ACD) anticoagulant, stored 1 week in the blood bank, then centrifuged to obtain plasma. The cells were washed with isotonic saline in our laboratory and showed negligible hemolysis.

Sample aliquots containing from 0.9 to 2.2 ml of packed cells were digested with perchloric acid and assayed as described in the Experimental Section. The data obtained for Mg^{2+} concentration in terms of packed cell volumes are summarized in Table VI. The values obtained for Mg^{2+} concentration in human erythrocytes are in excellent agreement with the value of

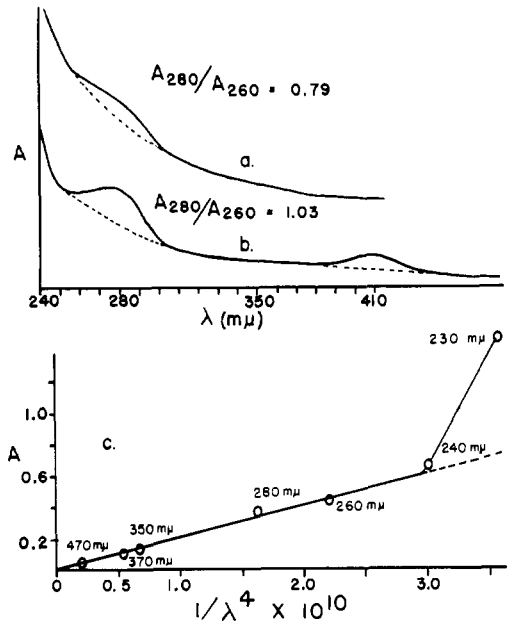

FIGURE 9: Ultraviolet-visible absorption spectra of AcChE–lipoprotein preparations from bovine erythrocytes. (a) Beckman DB scan on 35,000*g* pellet (AcChE–lipoprotein) from hypertonic saline extract of bovine stroma. Sample was suspended in 0.08 M sodium phosphate–0.5 N NaCl (pH 7.4) at 4°. (b) Beckman DB scan on 35,000*g* supernatant corresponding to the 35,000*g* pellet for a in the same buffer. (c) Absorbance readings for sample a taken on a Beckman DU and plotted according to the light-scattering relationship: Absorbance due to scattering, $A_s = k/\lambda^4$, where k is a constant.

2 mM given by Pennell (1964). The slightly higher Mg content after only one 0.15 M NaCl wash than after three isotonic washes suggests a gradual displacement of Mg^{2+} by Na^+, but the difference caused by the usual amount of NaCl washing would not appear to be particularly significant.

The observation that the Mg^{2+} content of the bovine red cell is nearly fourfold lower than that of the human erythrocyte is of considerable interest, and lends some support to the idea that relative divalent cation concentrations or binding affinities in the membranes of the two erythrocyte species may provide a partial explanation for their strikingly different stability characteristics.

It should be pointed out that the human and bovine cells were not treated in exactly the same way and comparison of the absolute values presented in Table VI may be somewhat incautious. The human blood sample had been kept for 1 week in ACD preservative solution before isolation and washing of the erythrocytes, while the bovine cells were isolated and washed within 2 hr after collection of the blood. Hence, if some Mg^{2+} was leached out of the human cells during the 1-week aging period, the differences indicated in Table VI between human and bovine cells might, in fact, represent a minimum value. Nonetheless, there is no doubt a significant difference in Mg^{2+} content in the two types of isolated erythrocytes.

In both the human and bovine erythrocytes, the Ca^{2+} content was too low to be detected. The Ca assay is only about one-tenth as sensitive as the Mg assay, so at present it can only be stated that the Ca^{2+} concentration of both human and bovine stroma lies somewhere in the range of 0.0–0.5 mM. According to Pennell (1964), the Ca^{2+} concentration in human erythrocytes is about 0.5 mM.

Mg^{2+} would appear to be the physiologically important divalent cation which might play the role of an "internal membrane cohesive," as Hoffman (1962) has suggested. It is well known, however, that Ca^{2+} is a significant ionic component of plasma, and thus is constantly present in the red cell's external environment. Gent *et al.* (1964) have shown that Ca^{2+} does bind reversibly to the human erythrocyte membrane. Since the primary effect of the anticoagulant (citrate, oxalate, EDTA, etc.) is to complex Ca^{2+}, it is not surprising that the isolated erythrocytes are deficient in Ca^{2+}, particularly if this cation is mainly restricted to the outer surface of the membrane.

Some Characteristics of Solubilized AcChE. METHODS OF PREPARATION. It is possible to prepare AcChE–lipoprotein material from bovine erythrocytes by at least three different methods: (a) hypertonic saline extraction of Ca- or Mg-stroma; (b) short hypertonic saline treatment of intact bovine erythrocytes; and (c) extended hypotonic washing of bovine erythrocytes in the absence of added divalent cations. Procedure a is essentially the method described by Mitchell and Hanahan (1966) for human erythrocytes, requiring only the addition of divalent cation to the hypotonic hemolysis buffer to apply equally well to the bovine erythrocyte. Procedures b and c are applicable to the bovine, but not to the human erythrocyte, since extended treatment with either hypertonic or hypotonic solutions results in negligible release of AcChE from the human erythrocyte (Mitchell and Hanahan, 1966). The AcChE–lipoprotein prepared from bovine erythrocytes by any of the three methods listed appears to be quite analogous to the human AcChE–lipoprotein described by Mitchell and Hanahan (1966) on the basis of the admittedly superficial data presently available.

STABILITY. Bovine erythrocyte AcChE activity is quite stable for at least 8 days in a whole cell suspension stored in 0.15 N NaCl at 4°. Activity released from the membrane by hypertonic saline extraction appears to be quite stable when stored as a concentrated suspension in phosphate, Tris-NaCl, or Veronal buffer (pH 7.4) at 4°. One preparation lost only 2% of its activity in 25 days, and another lost 8% of its original activity over 36 days. Such preparations may be conveniently concentrated without loss in activity by dialysis against sucrose, followed by dialysis against the storage buffer.

AcChE–lipoprotein released from stroma by hypotonic washing was concentrated by 100,000*g* centrifugation and stored as a concentrated suspension in hypotonic buffer (pH 7.4–7.6) at 4°. Such preparations are stable for several days but lose considerable AcChE activity over a period of 2 weeks. The presence of salt appears to stabilize the AcChE activity of these lipoprotein preparations. For example, a concentrated AcChE–lipoprotein suspension in 25 imOsM Tris buffer

(pH 7.5) lost 50% of its original activity in 2 weeks at 4°; however, part of the same preparation, after passage through a Sephadex G-200 column in 0.5 N NaCl–0.01 M Tris (pH 7.5) followed by concentration by 100,000*g* centrifugation and storage at 4° as a concentrated suspension in the high salt buffer, lost no appreciable AcChE activity over a 2-week period. Bovine AcChE–lipoprotein preparations cannot be stored for long periods in distilled water or dialyzed extensively against distilled water without loss of AcChE activity.

BEHAVIOR OF LIPOPROTEIN SUSPENSIONS. Bovine AcChE–lipoprotein will not sediment appreciably at 35,000*g* from the hypertonic saline extract. However, after concentration by dialysis against sucrose, followed by dialysis against water or buffer, 50–80% of the AcChE activity sediments in 1 hr at 35,000*g*, similar to the result reported by Mitchell (1965) and Mitchell and Hanahan (1966) for human cells. Lipid is present in both 35,000*g* pellet and supernatant fractions, and the distribution of individual lipid classes is qualitatively similar to that of the whole bovine erythrocyte.

Sedimentation of the 35,000*g* supernatant fraction at 100,000*g* for 11 hr at 4° resulted in complete recovery of AcChE activity and lipid in the pellet fractions although protein was present in the 100,000*g* supernatant fractions as indicated by absorbance readings at 280 and 260 mμ. Additional experiments have shown that a 1-hr centrifugation at 100,000*g* is sufficient to sediment all the AcChE activity and lipid from hypertonic saline extracts of bovine stroma, but only about 50% of the total protein is sedimented under these conditions.

Bovine AcChE–lipoprotein prepared by procedure c can also be concentrated by 1–2-hr centrifugation at 100,000*g*, with essentially complete recovery of AcChE activity and lipid in the pellet. These pellets can be easily resuspended in water or buffer solutions to give homogeneous, turbid suspensions which do not settle out appreciably on standing. Such suspensions can be reproducibly sampled using micropipets or ordinary glass pipets for larger volumes, and are suitable for column chromatography on Sephadex.

ULTRAVIOLET–VISIBLE SPECTRA; TURBIDITY OF LIPOPROTEIN SUSPENSIONS. All the lipoprotein suspensions were visibly turbid, and when the spectrum of a typical AcChE–lipoprotein$_A$ (35,000*g* pellet) preparation was scanned on a Beckman DB recording spectrophotometer from 400 to 220 mμ, it became apparent that the observed low A_{280}/A_{260} ratio (0.79–0.85) could be accounted for simply on the basis of Rayleigh scattering. The 280-mμ protein peak appears as a very small peak on a base line which rises continuously toward lower wavelengths (Figure 9a). A plot of absorbance (read on a Beckman DU spectrophotometer) against the reciprocal of the fourth power of the wavelength, according to a simplified form of the Rayleigh scattering equation (absorbance due to scattering of light = k/λ^4, where k is a constant), gives a quite reasonable straight line (Figure 9c). Drawing this line through the 260-mμ point and computing the scattering constant k give an A_s value at 280 mμ which accounts for 88% of the total absorbance of the sample. Assuming that the other 12% of the 280-mμ absorbance is attributable to protein and comparing this value with the protein content of the sample as determined by the Lowry method, give $E^{1\%}_{280\,m\mu}$ 6.4, in close agreement with the extinction coefficient for electrophoretically pure bovine serum albumin ($E^{1\%}_{280\,m\mu}$ 6.6). Thus, it seems unlikely that the AcChE–lipoprotein contains any appreciable amount of nucleotide or other 260-mμ-absorbing material.

The spectrum of the corresponding 35,000*g* supernatant fraction, scanned on the Beckman DB from 660 to 220 mμ, showed a more obvious 280-mμ peak and an A_{280}/A_{260} ratio of 1.03 (Figure 9b). A small peak at about 410 mμ (Soret band) indicated that some hemoglobin was present.

GEL FILTRATION OF ACCHE–LIPOPROTEIN. Gel filtration on columns of Sephadex G-100 or G-200 (or an bead-form Agarose gel) provided an effective method for freeing AcChE–lipoprotein from contamination by smaller molecules, and particularly for removing residual amounts of hemoglobin. AcChE activity, lipid phosphorus, and all but possible traces of cholesterol are completely eluted in the void volume of Sephadex G-100 or G-200 columns, while hemoglobin and perhaps other smaller molecular weight contaminants are retarded. Figure 10 shows a representative elution profile of the AcChE from Sephadex G-100. In this instance, the sample was a concentrated,[5] but unfractionated, hypertonic saline eluate from bovine Mg-stroma. The lipoprotein peak was conveniently located by its turbidity. A_{280} values corrected for scattering as described in the preceding section correspond fairly well to protein values in this peak, as determined by Lowry protein assays. The specific activity of AcChE remains essentially constant through the main part of the peak, but drops off in the trailing edge. The ratio of lipid phosphorus to Lowry protein, constant through the leading edge and peak fractions at 34 μg of P/mg of protein, also decreases in the trailing edge. Thus, it would appear that non-AcChE, nonlipophilic protein is present in the saline eluate and is incompletely resolved from AcChE–lipoprotein on Sephadex G-100.

In separate experiments, gel filtration of AcChE–lipoprotein has been carried out on Sephadex G-200

[5] Concentrated by ammonium sulfate flotation. Attempts to fractionate hypertonic saline extracts by ammonium sulfate precipitation were unsuccessful. No precipitate was found at any stage of saturation up to 80%. However, on centrifugation of 80–100% saturated solutions at 4° (pH adjusted to about 7.4 with ammonium hydroxide) a floating layer formed containing all the AcChE activity and lipid of the saline extract. When a hard, floating pellet was formed by centrifugation at 35,000*g* for about 10 min, the underlying solution could be removed almost quantitatively by means of a long-needled syringe, as the pellet generally adhered to the wall of the tube as liquid was being withdrawn. This convenient method has been used frequently to concentrate AcChE–lipoprotein preparations from large volumes of hypertonic saline extract or to rapidly concentrate preparations before and after column chromatography. It has not been used, however, in the preparation of AcChE–lipoprotein by procedure c in order to avoid exposing those preparations to media of high salt concentration. In contrast to the 35,000*g* or 100,000*g* sedimentation methods, ammonium sulfate flotation has not been found to give any significant increase in AcChE specific activity.

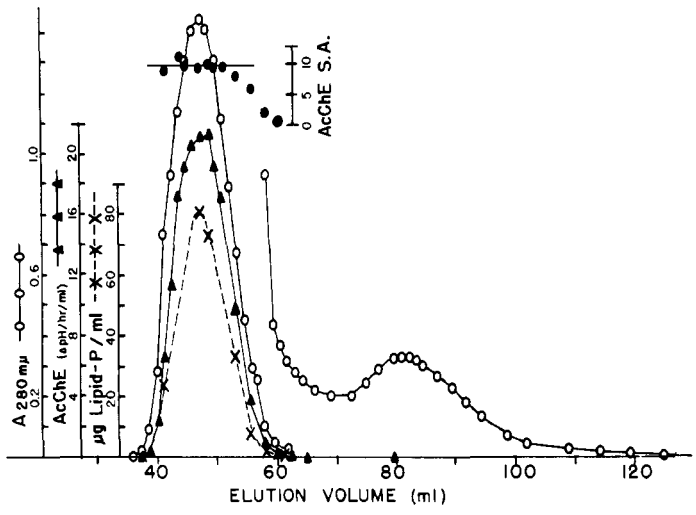

FIGURE 10: Sephadex G-100 column chromatography of AcChE isolated from bovine erythrocyte stroma. Void volume by Blue Dextran = 41 ml; sample volume = 5 ml; eluting buffer = 0.001 M Tris–0.5 M NaCl (pH 7.8) at 4°; flow rate = 30 ml/hr. For column characteristics see Experimental Section. AcChE specific activity units = ΔpH/hr per mg of Lowry protein. Initial lipoprotein peak was visibly turbid. Open circles in this peak represent 12% of actual $A_{280\,m\mu}$ reading and give approximate absorbance due to protein. $A_{280\,m\mu}$ values beyond the initial peak are not corrected for light scattering. The second $A_{280\,m\mu}$ peak also absorbed strongly at 410 mμ, indicating that it contained hemoglobin.

and 3.5% bead-form Agarose. In each case, the AcChE activity and lipid phosphorus peaks have been eluted together in the void volume. The preliminary data obtained from Agarose chromatography indicated that the apparent molecular weight of the lipoprotein lies somewhere above the two million mark.

As mentioned earlier, Ca-stroma are always visibly pink and may retain as much as 0.6% of the whole cell hemoglobin content. Hypertonic saline extraction of Ca-stroma removes some of this residual hemoglobin and the concentrated saline extracts are also obviously pink. Hemoglobin has been located in column elution profiles by following either the 540-mμ heme absorption band when considerable hemoglobin is present, or the very strong 410-mμ absorption to detect trace contamination by heme-containing material. Considerable amounts of 410-mμ-absorbing material were removed in both the Sephadex G-200 and Agarose chromatography experiments, and the heme peak fractions in both cases were very well separated from the lipoprotein peak fractions. Nevertheless, the broad heme absorption peaks did overlap somewhat with the lipoprotein turbidity peaks, and small 410-mμ absorption peaks could still be detected on scanning even the leading edge fractions of the AcChE–lipoprotein peaks. Thus, a small amount of hemoglobin (or some material possessing the Soret band absorption characteristic of heme) appears to be very tightly bound to the AcChE–lipoprotein particles. However, in lipoprotein preparations from essentially hemoglobin-free stroma, no such heme absorption has been detected, so it does not follow that hemoglobin or heme-containing material is an essential component of bovine erythrocyte AcChE–lipoprotein. Presumably, additional gel filtration steps or the use of longer columns with hemoglobin-containing samples could eventually remove all detectable heme contamination without appreciable separation of the phospholipid and AcChE components.

Discussion

The present investigation has shown that bovine erythrocytes behave quite differently from human erythrocytes on hypotonic hemolysis. In the case of human cells, hypotonic hemolysis and subsequent washing with hypotonic buffer produces morphologically intact, hemoglobin-free stroma with quantitative retention of AcChE activity and lipid (Mitchell and Hanahan, 1966). When stroma are prepared in the same way from fresh bovine erythrocytes, the final hemoglobin-free preparation has lost most of its AcChE activity and a significant amount of the lipid of the intact cell by solubilization. In addition, the morphological integrity of the bovine stromal membrane is lost during hemolysis. The incorporation of 1 mM Ca^{2+} into the hemolysing buffer leads to essentially complete retention of AcChE activity and lipid in bovine erythrocyte stroma and preserves the morphological integrity of the membrane. Mg^{2+} exerts a similar but somewhat variable and less permanent effect; Sr^{2+} and Ba^{2+} are also very effective in this process.

Disruption of the bovine erythrocyte membrane by hypotonic hemolysis is a stepwise process. Subsequent to the initial hemolytic event, the bovine stroma appear round and intact under phase contrast microscope and exhibit no loss of AcChE and only trace losses of lipid. Additional hypotonic buffer washes cause severe alterations in the membrane with loss of components such as AcChE and lipids. Under similar conditions human stroma do not undergo these changes (Mitchell and Hanahan, 1966). It is possible that progressive lowering of the concentration of divalent cations, which act as membrane stabilizers, may play a critical role in the disruptive process, since addition of certain divalent cations such as Ca^{2+} or Mg^{2+} to the hemolyzing buffer and wash solution actually protect the membrane against such damage.

The lipoprotein released from fresh bovine erythrocytes, either by hypotonic hemolysis in the absence of divalent cations or by hypertonic saline extraction of bovine stroma, appears to be quite analogous to the AcChE–lipoprotein preparation obtained from human stroma by hypertonic saline extraction (Mitchell, 1965; Mitchell and Hanahan, 1966). The differential release of AcChE, as compared with hemoglobin and glycolytic enzymes, allows a very mild procedure for isolation of this enzyme with a concomitant 200-fold purification and in good yields (80–85%).

The data presented here tend to support the hypothesis that AcChE may exist in the erythrocyte membrane as a lipoprotein complex, since AcChE released from the bovine red cell membrane by three different procedures is always accompanied by and appears to be tightly bound to lipid in a fairly constant amount rel-

ative to the enzyme activity.[6] However, definitive experimental evidence for such a conclusion would require studies on the binding of lipid to a lipid-free preparation of AcChE and delineation of the effects of lipid on the properties of the enzyme. Preliminary delipidation studies[7] have indicated that at least 92% of the phospholipid complement of AcChE–lipoprotein may be removed without altering the specific activity of the enzyme, but possible effects of lipid on other important properties of AcChE such as its peculiar kinetic parameters or multiple aggregate forms have not been ruled out. It should be emphasized that the physiological role of erythrocyte AcChE is still a matter open to speculation.

A soluble lipoprotein fraction has previously been isolated from human erythrocyte ghosts by ultrasonic irradiation in 10% 1-butanol solution (Morgan and Hanahan, 1966). This low-density lipoprotein fraction contained 68–80% of the original stroma lipid, but only about 15% of the total stroma protein. Furthermore, the amino acid compositions of the lipophilic and nonlipophilic stroma protein fractions were shown to be different. These experiments indicated that fractionation of the erythrocyte membrane protein is possible, but the rather harsh butanol ultrasonic treatment proved unsuitable for enzyme fractionation studies. AcChE, for example, is totally and rapidly inactivated by this procedure (C. D. Mitchell, unpublished observations).

Maddy (1966) has described the solubilization of 90–95% of the total protein of ox erythrocyte stroma by butanol extraction of aqueous ghost suspension, with separation of the bulk of the lipid into the butanol phase. Maddy's procedure has been tried in this laboratory on bovine Ca-stroma and on bovine AcChE–lipoprotein, but no AcChE activity could be recovered in the aqueous phase. This result is not surprising in view of the finding of Doizaki *et al.* (1958) that 1-butanol inactivates bovine erythrocyte AcChE. Maddy has recently described some properties of the protein of the ox erythrocyte plasma membrane, as isolated by his butanol extraction procedure (Maddy, 1966; Maddy and Malcolm, 1965). As Maddy himself has stressed, the stroma preparation involves extensive washing, and thus the final ghost preparation might be depleted of some important proteins of the intact, functional membrane. The data presented here show that this is indeed the case with the bovine erythrocyte, and provided that Scottish oxen are similar in this regard to American cattle,[8] our findings presumably apply to the stroma prepared by Maddy. Of course, the amount of protein lost from the bovine erythrocyte membrane during hypotonic hemolysis and washing in the absence of divalent cations represents a very small percentage of the total protein that can be retained in Ca^{2+}- or Mg^{2+}-treated, morphologically intact stroma. Therefore, our findings do not constitute a serious objection to Maddy's work, which presumably applies to the bulk of the protein of the fundamental structural membrane.

Green *et al.* (1965) have suggested that the entire sequence of glycolytic enzymes is associated with and organized on the red cell membrane *in vivo*. This is an extremely attractive hypothesis from a teleological point of view, particularly in the case of the erythrocyte with its viscous, hemoglobin-filled interior, in which efficient diffusion of substrate and intermediates from one soluble, randomly distributed enzyme of the glycolytic pathway to the next is rather difficult to imagine. Also, as pointed out by Schrier (1963), certain glycolytic enzymes localized in or on the erythrocyte membrane could provide ATP to drive active transport processes and might also serve as carrier molecules. Rigorous experimental support for this hypothesis, however, has been difficult to obtain. Contributions from this laboratory have shown that glycolytic enzymes can be completely removed from human stroma, as can hemoglobin, with essentially complete retention of AcChE activity, lipid, and the intact morphological appearance of the membrane (Dodge *et al.*, 1963; Mitchell *et al.*, 1965). Treatments leading to loss of AcChE activity, however, are always accompanied by loss of lipid and the morphological integrity of the membrane (Mitchell and Hanahan, 1966).

Thus there is some justification for calling AcChE a membrane enzyme, but the case for association of glycolytic enzymes with the membrane is considerably more tenuous. Green *et al.* (1965), who used the bovine erythrocyte, have shown only that these enzymes are centrifuged together with membranous entities to various extents under specific conditions. On the basis of data previously obtained on human erythrocyte stroma (Schrier, 1963; Dodge *et al.*, 1963; Mitchell *et al.*, 1965; Weed *et al.*, 1963) it seems reasonable to conclude that neither hemoglobin nor any of the glycolytic enzymes are components of the basic structural membrane. The data presented here indicate that the same conclusion is valid for the bovine erythrocyte. Baum *et al.* (1966) examined bovine erythrocyte membrane fragments by electron microscopy and also concluded that the glycolytic enzymes are probably not essential structural features of the membrane continuum; instead, they visualized glycolytic enzyme complexes which would be attached to the membrane *in vivo*, but would not disrupt the basic structural membrane when detached.

A considerable body of evidence has accumulated in the literature suggesting that divalent cations have physiologically important effects on the properties of biological membranes. Recently, Manery (1966) has reviewed the role of Ca^{2+} in membrane-related phenomena, such as cell adhesiveness, membrane hardening, and cell permeability. The ability of divalent cations to produce significant changes in membrane structure and

[6] Preliminary experiments on the sedimentation behavior of solubilized AcChE–lipoprotein in linear sucrose gradients revealed a heterogeneous distribution of AcChE activity in the Spinco Model L (SW 39 L rotor). Consistent evidence was obtained for two major forms of AcChE, one a light fraction in the density range of 1.07–1.13 g/cc and a heavy fraction, $d \geq 1.15$ g/cc. The different density components strongly suggest the possibility of enzymatically active species with different lipid protein ratios, and this facet of the problem is under investigation.

[7] S. P. Burger, M.S. thesis, University of Washington, 1967.

[8] Webster's New World Dictionary defines an ox as "loosely any animal of the bovine family."

properties is presumably related to the capacity of these cations to form chelation complexes and to bridge adjacent negative charges, thereby stiffening the membrane, decreasing its net negative charge, and displacing water of hydration.

On the basis of monovalent cation permeability studies, Hoffman (1962) has suggested that Mg^{2+} may act as an internal membrane cohesive in the human erythrocyte. Gent *et al.* (1964) have presented evidence for binding of Ca^{2+} by the human erythrocyte membrane. This binding is not dependent upon metabolic activity and was interpreted as ion-exchange adsorption–desorption at relatively nonspecific negative sites on the membrane. Rojas and Tobias (1965) have performed monolayer studies which implicate the phosphate and carboxyl groups of phosphatidylserine and the phosphate group of phosphatidylethanolamine as possibly important ion-exchange sites at cell surfaces. Cerbón (1965) has shown that Mg^{2+} and Ca^{2+} can cause changes in the nuclear magnetic resonance spectra of bacterial membranes in that part of the spectrum associated with the aliphatic CH_2 chains of the lipid components. These data were interpreted as evidence for alteration of the physical state of membrane lipids by the divalent cations. Such an effect on the lipids could presumably induce conformational changes in other membrane components and bring about significant rearrangement of the entire membrane structure.

Abrams (1965) found that Mg^{2+} and some other di- and trivalent cations influenced the binding of Mg-ATPase in membrane ghosts of *Streptococcus faecalis*, produced by osmotic lysis of protoplasts. After several buffer washes in the absence of Mg^{2+}, this enzyme activity was released abruptly from the bacterial membranes. The amount of ATPase released was found to be dependent upon the concentration of Mg^{2+} in the membrane suspension.

The data presented in this current study indicate a requirement for divalent cations in the case of AcChE–lipoprotein association with the bovine erythrocyte membrane. Such an effect has not been demonstrated with the human erythrocyte, but neither has the possibility of a role for divalent cations been eliminated in this system. For example, divalent cations may be found to antagonize the slow release of AcChE–lipoprotein from human stroma in isotonic saline and the more rapid release in hypertonic saline. In this regard, the relative concentrations of divalent cations in human and bovine erythrocytes and the relative binding affinities of their membranes for the various cations may be of importance. The concentration of Mg^{2+} in human red cells has been found to be about 2 mM in terms of packed cell volume, and about 0.5 mM in bovine erythrocytes; the Ca^{2+} concentration is reported to be about 0.5 mM in human red cells (Pennell, 1964).

Soo-Hoo and Brown (1967) have reported that 0.02 M $MgCl_2$ completely prevents the disaggregation of *Halobacterium halobium* membrane into subunits, which occurs at NaCl concentrations less than 1 M in the absence of divalent cations. However, Ca^{2+} or Mg^{2+} concentrations less than 0.5 M are not sufficient to protect the whole cell from lysis during a transient increase in osmotic pressure. By analogy, very low concentrations of Ca^{2+} or Mg^{2+} (1 mM) prevent loss of AcChE–lipoprotein units from the bovine erythrocyte membrane, but do not protect the cell from hemolysis in solutions of low ionic strength. Of course, this analogy breaks down immediately when the effect of high salt concentration is considered. Disaggregation of the *H. halobium* membrane is probably caused by an excess of negative charges on the membrane subunits, which can be effectively shielded only at high concentrations of monovalent cations (Brown, 1963). The bovine erythrocyte membrane is partially degraded in hypertonic NaCl solutions, with release of some membrane surface components (including AcChE and lipid) occurring at a more rapid rate than hemolysis or loss of internal cell constituents.

When erythrocytes are placed in hypotonic medium they swell due to uptake of water. They rapidly lose their biconcave disk shape and become spherical. Additional swelling causes stretching of the membrane and opening of holes through which hemoglobin can pass, resulting in hemolysis. Seeman (1967) has recently provided convincing electron microscopic evidence for transient hole formation in the human erythrocyte membrane during hypotonic hemolysis. The membrane was found to be permeable to ferritin and colloidal gold only between 10 and 20 sec after the lowering of medium tonicity, and this period coincided with the maximum rate of hemoglobin release. Holes in the membrane (200–500-Å diameter) could be fixed in the open state with glutaraldehyde and observed by electron microscopy only during the period of permeability to ferritin and colloidal gold.

It is of interest that immediately after the initial hemolysis event, both human and bovine stromal membranes appear round and intact by phase microscopy; they show no loss of AcChE activity and a very slight reduction in lipid. Additional hypotonic washes and centrifugations probably cause some further osmotic changes, as well as removal of membrane-bound hemoglobin and other loosely bound enzymes. Human stroma stand up quite well to these additional washing steps, but bovine cells are disrupted with loss of membrane components. The partial disruption of bovine cells may result from their fragility to the mechanical stress of centrifugation. Progressive lowering of the concentration of divalent cations or other membrane stabilizers may also be important, since divalent cations in the hemolyzing and washing buffers can protect against this damage.

It seems significant that the divalent cations are considerably less effective in preventing membrane damage to bovine erythrocytes when added only after the initial hemolysis step, although no detectable damage occurs during this initial stage of the procedure. This observation, along with the differential effects of 0.15 M saline on the membrane before and after hemolysis, indicates that hemolysis may cause certain irreversible alterations in the membrane structure, even though some of the morphological and permeability characteristics of the intact cell membrane may be retained or recovered. Therefore, it must be recognized that the

erythrocyte stroma represents a derivative of the cell membrane; accordingly, projection of stroma characteristics to the membrane of the intact erythrocyte must be done with some reservations.

Events occurring during hypertonic (1.2 M NaCl) hemolysis seem to reverse the sequence observed during hypotonic hemolysis. The cells shrink in hypertonic media as water flows out. In the case of bovine erythrocytes, release of membrane components, such as AcChE, follows fairly rapidly and proceeds until lesions are extensive enough to allow release of hemoglobin. These facts may supply additional evidence for the localization of AcChE on the outer surface of the erythrocyte membrane. The disruptive effect of hypertonic saline on the membrane may involve the breaking of ionic bonds, dehydration of membrane components, or replacement of divalent cations that were part of the normal membrane structure. Lovelock (1953) noted that human erythrocytes in 0.8–3.0 M NaCl solutions developed sensitivity to mechanical shock (for example, centrifugation), and he attributed the general membrane disruption in >3 M salt solutions to the lyotropic effect of NaCl.

Finally, the results described here have revealed some striking differences between human and bovine erythrocytes as regards membrane-related phenomena in the two species. In this regard, it is of interest to consider briefly other reported differences in these two species. The choline-containing phospholipids of human and cow erythrocytes comprise 55–60% of the total phospholipid. However, in the bovine erythrocyte this fraction contains nearly 100% sphingomyelin, while human choline phospholipids contain slightly more phosphatidylcholine than sphingomyelin. Significant levels of plasmalogens (vinyl ethers) are found in human erythrocyte phospholipids, particularly in the ethanolamine phosphoglyceride fraction, but none was demonstrated in bovine red cell phospholipids (Hanahan *et al.*, 1960; Ways and Hanahan, 1964). On the other hand, in bovine erythrocyte ethanolamine phosphoglycerides, glyceryl ethers appear to substitute for plasmalogens (vinyl ethers) in the corresponding human phospholipid fraction (Hanahan and Watts, 1961; Hallgren and Larsson, 1962). Certain differences in permeability behavior and lipid composition in erythrocytes from a series of mammalian species, including human and bovine, have been summarized by van Deenen and de Gier (1964).

The data presented here indicate that the bovine erythrocyte membrane is a considerably less cohesive structure than the human erythrocyte membrane. While it is intriguing to speculate on possible effects of varying lipid composition on membrane characteristics such as flexibility, cohesiveness, and permeability, there is at present too little understanding of the role of lipids in membrane and lipoprotein structure to allow meaningful conclusions to be drawn. Probable species differences in membrane protein, carbohydrate, and metal ion components remain to be defined. At present it seems adequate to stress the fact that species differences in erythrocyte membrane composition and properties certainly exist, and to caution against a strictly universal outlook in the general field of membrane structure.

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The Metabolism of Glyceride Glycolipids. II. Biosynthesis of Monogalactosyl Diglyceride from Uridine Diphosphate Galactose and Diglyceride in Brain*

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ABSTRACT: Microsomal enzyme preparations of brain from rats 13 to 20 days of age catalyze the synthesis of 1,2-di-*O*-acyl-3-*O*-(β-D-galactopyranosyl)-*sn*-glycerol from uridine diphosphate galactose and 1,2-diglyceride. The enzyme requires the 1,2 isomer of the diglyceride substrate and prefers diglycerides with long-chain-saturated fatty acid constituents. Consistent and relatively uniform stimulation of the biosynthesis by exogenous diglyceride was best achieved by adsorbing the diglyceride substrate directly to a lyophilized enzyme preparation. The activity of the enzyme is highest in preparations made from brains of rats 14–18 days old. The age of highest enzymatic activity corresponds to the age during which the concentration of monogalactosyl diglyceride in brain increases most rapidly (Wells, M. A., and Dittmer, J. C. (1967), *Biochemistry 6*, 3169), and to the age during which myelination occurs at a maximal rate (McIlwain, H. (1966), Biochemistry and the Central Nervous System, 3rd ed, London, Churchill). This coincidence suggests that the enzyme responsible for the biosynthesis of monogalactosyl diglyceride in brain may function significantly in myelination.

The presence of monogalactosyl diglyceride in brain tissue was first reported by Steim and Benson (1963), Norton and Brotz (1963), and Rouser *et al.* (1967). The initial findings of these workers have been confirmed and extended by studies appearing in more recent publications (Rumsby and Gray, 1965; Pelick *et al.*, 1965; Rumsby, 1967; Steim, 1967). The monogalactosyl diglyceride of animal origin, which has been convincingly demonstrated thus far to occur only in tissues of the central nervous system, has been characterized as 1,2-di-*O*-acyl-3-*O*-(β-galactopyranosyl)-*sn*-glycerol.

The interest of this laboratory in monogalactosyl diglyceride of brain has been in the enzymatic processes responsible for the synthesis of this glycolipid. The results to be reported in this paper demonstrate that microsomal preparations of brains of rats of age greater than 13 days catalyzed the formation of 1,2-di-*O*-acyl-3-*O*-(β-D-galactopyranosyl)-*sn*-glycerol from UDP-galactose[1] and 1,2-diglyceride in the presence of magnesium ion. The enzymatic synthesis of monogalactosyl diglyceride is greatest during the period of most active myelination. A portion of this work has been presented in a preliminary report (Wenger *et al.*, 1967).

Experimental Procedure

Enzyme Source and Preparation. Fractionation of whole homogenates of brain from 13- to 18-day-old rats (bred and generously given by The Fels Research Institute, Temple University School of Medicine) was carried out according to the method of DeRobertis and coworkers (1962). All fractions were suspended in 0.32 M sucrose and were lyophilized overnight in small round-bottom flasks. The residues obtained after lyophilization were then extracted four times with 15 ml of acetone at 0° for 10 min with stirring. Acetone was completely removed from the residues by evaporation at reduced pressure. Microsomal fractions of brain were

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[1] See *Biochemistry 5*, 1445 (1966), for a list of abbreviations.