Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Nagano, H., and Zalkin, H. (1970), *Arch. Biochem. Biophys. 138*, 58.
Osborne, M. J. (1969), *Annu. Rev. Biochem. 38*, 501.
Peterson, E. A., and Sober, H. A. (1962), *Methods Enzymol 5*, 3.
Strominger, J. L. (1958), *Biochim. Biophys. Acta 30*, 645.

# Biochemical Variability of Human Erythrocyte Membrane Preparations, as Demonstrated by Sodium–Potassium–Magnesium and Calcium Adenosine Triphosphatase Activities†

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ABSTRACT: Membranes have been prepared from human erythrocytes, by hypotonic and isotonic hemolysis. These preparations show considerable variability in (enzymatic) behavior as compared to each other and to the parent cell. The $Na^+$–$K^+$–$Mg^{2+}$ and $Ca^{2+}$ stimulated ATPases have proven satisfactory as monitors for these changes and in illustrating that hemoglobin-free membranes have biochemical properties quite different from those of the parent cell membrane. Hemoglobin-free membranes prepared at pH 7.6 in 20 mosm Tris buffer allow easy access of ATP to the $Na^+$–$K^+$–$Mg^{2+}$ and $Ca^{2+}$ stimulated ATPases. This is contrary to the behavior of intact erythrocytes which show little such activity unless the cells are ruptured, for example, by freezing and thawing. On the other hand, membranes preprared by hypotonic hemolysis at pH 5.8 showed minimal ATPase activities toward extracellular ATP and a significant retention of hemoglobin, Freeze-thaw treatment of the pH 5.8 membranes, however allowed expression of these enzymatic activities. The degree of availability of ATPases was found to be influenced by the composition of the hemolysis buffer, its osmolarity, and pH. No alterations in the availability of acetylcholine esterase in the pH 7.6 or 5.8 membranes were noted. Divalent cations, such as $Ca^{2+}$ and $Mg^{2+}$, had a definitive effect on membranes prepared in hypotonic buffer in that retention of hemoglobin was promoted and the availability of ATPase activities was reduced. A particularly provocative observation was that incubation of human erythrocytes at 44° in isotonic Tris buffer, pH 7.6, led to a time-dependent hemolysis with release of >95% of the hemoglobin in 2 hr. These hemoglobin-depleted membranes developed ATPase sites accessible to extracellular ATP. However, at shorter periods of incubation, significant quantities of hemoglobin could be removed without development or expression of the ATPase activities. It was observed that a 60–75-min incubation at 37° would allow release of nearly 90% of the hemoglobin, with only a slight increase in accessible $Ca^{2+}$-stimulated ATPase. Other buffers such as Hepes, Mes, histidine–imidazole, or $NaHCO_3$, at isotonic levels at pH 7.6, did not cause hemolysis or allow expression of ATPase activities on incubation with human erythrocytes at 37 or 44°. On the other hand, hemolysis of intact erythrocytes in 60 and 100 mosm Tris buffer, pH 7.6 at 4°, yielded membranes with low levels of hemoglobin, *i.e.*, 4 and 20% mean corpuscular hemoglobin, respectively, with low $Na^+$–$K^+$–$Mg^{2+}$ and $Ca^{2+}$ ATPase activites. These experimental findings provide possible routes to further study of some of the molecular changes occurring in the erythrocyte membrane during preparation by hypotonic and isotonic hemolysis. A possible role of hemoglobin in providing structural and functional integrity to the erythrocyte membrane is provocative, yet under these experimental conditions it may serve merely as an indicator of the state of the membrane at any one time.

The biological and biochemical characterization of the plasma membrane of mammalian cells has become the subject of intense interest in many laboratories today. Among the choices of cells available to investigators probably the one most widely used for membrane preparation has been the mammalian erythrocyte. This has not occurred by sheer experimental accident, but has developed due to the fact that this cell has no detectable subcellular organelles and provides, by using a very simple osmotic lysis procedure (Dodge *et al.*, 1963; Weed *et al.*, 1963), a structure considered by many investigators as representative or at least reminiscent of the plasma membrane of the intact cell.

In an earlier study in this laboratory on possible changes in the level of certain enzymes and proteins in membranes obtained by hypotonic hemolysis of human erythrocytes (Mitchell *et al.*, 1965) it was evident that depending on the conditions, *e.g.*, osmolarity and pH, varying quantities of these components could be found in the isolated membranes. Further, as had been suggested by these studies, and more recently emphasized by further experimental evidence (Bramley *et al.*, 1971; Hanahan and Ekholm, 1972), there are very definitive and important biochemical changes occurring during preparation of these membranes. As proposed in the latter study (Hanahan and Ekholm, 1972), the membranes obtained by osmotic lysis must be considered at best as a derivative of the intact erythrocyte membrane. Zwaal *et al.* (1971) and Laster *et al.* (1972) using phospholipase C and Woodward

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and Zwaal (1972) using phospholipase $A_2$ and C as probes of the availability or orientation of membrane phospholipids showed a striking difference in the susceptibility of the isolated membrane to attack by these enzymes as compared to the intact cell. Their results supported quite dramatically the "derivative" concept for the isolated membrane. Even though many investigators (Bodemann and Passow, 1972; Lepke and Passow, 1972; Triplett and Carraway, 1972; Theodore and Robin, 1969; Hoffman, 1962) have shown quite clearly that there are premeability changes in the erythrocyte (membrane) during hemolysis, the fundamental biochemical changes or molecular events underlying these alterations have not been elucidated and unfortunately have not been of concern to many other investigators working on membrane structure.

This present article is concerned primarily with our continuing interest in characterizing the erythrocyte membrane and in establishing those factors most important to maintenance of its structure and function. Particular emphasis is centered on the behavior of the $Na^+$–$K^+$–$Mg^{2+}$ and $Ca^{2+}$ stimulated ATPases and the acetylcholine esterase of human erythrocyte membranes during isolation and handling under a variety of conditions.

## Experimental Section[1]

Human blood samples were obtained, without respect to type, from healthy, adult males and females ranging in age from 21 to 40 years. The blood was collected directly into heparinized Vacutainers (Becton-Dickinson) centrifuged at 4°, washed with desired buffer, and then stored at 4°, if necessary. Hematocrit values for washed cells resuspended in isotonic Tris (0.172 M), pH 7.6, were determined as follows: the blood sample was transferred to a Pre-Cal Heparinized Micro Hematocrit tube (Clay Adams, Inc.) and centrifuged at 6000*g* for 5 min in an Adams Readacrit Micro-Hematocrit centrifuge (Clay Adams, Inc., New York, N. Y.). These latter values are also used in calculation of the packed cell volume.

The ATPase assay was conducted as outlined by Brewer *et al.* (1968), using ouabain as an inhibitor of the sensitive ($Na^+$–$K^+$ stimulated) ATPase activity and an incubation temperature of 44°. The freeze–thaw technique described by Garrahan and Glynn (1967) was employed for expression ATPase activity in intact cells and in membrane preparations where noted. In this procedure, intact cells at a dilution of 4 ml of a 50% hematocrit to 10 ml or membrane suspensions were placed in a polyethylene tube and the tube immersed in a Dry Ice–acetone bath for 3–5 min. The contents were allowed to melt at 37° and refrozen twice more under the same conditions. Activities are expressed mostly as micromoles of $P_i$ released per milliliter of packed cells (or micromoles of phospholipid P) per 2 hr and occasionally on a hemoglobin basis. $Ca^{2+}$-stimulated ATPase was assayed essentially by the method of Brewer *et al.* (1968); $Ca^{2+}$, at 0.5 mM final concentration, was added to give equimolar $Ca^{2+}$–EDTA in the assay mixture.

The activity of the various ATPase was examined at 37, 44, and 51°, at the completion of a 2-hr incubation. Hence, these effects are not necessarily indicative of initial rates in all cases. The results of these experiments showed that the total and ouabain-sensitive ATPase activities were stable up to 51° and in fact increased approximately 1.5-fold over that noted at 37°. On the other hand, the ouabain-insensitive and $Ca^{2+}$-stimulated ATPases tended to plateau at or near 44° and on the basis of these results all ATPase assays were run at 44°. The acetylcholine esterase assay utilized a titrimetric method described by Heller and Hanahan (1972).

Calcium, magnesium, potassium, and sodium were determined by atomic absorption spectroscopy, using a Beckman Model 403 unit. The samples for calcium and magnesium assay were usually dry ashed in platinum crucibles, treated with 12 N HCl, evaporated, and then dissolved in a mixture of HCl, water, and lanthanum chloride (240 mM final concentration). Standard solutions of inorganic cations were used for calibration purposes. Total lipid was isolated and purified, and cholesterol and lipid phosphorus were assayed as described previously (Hanahan and Ekholm, 1972). Hemoglobin was assayed essentially by the cyanmethemoglobin method described by Kachmar (1970). Ouabain octahydrate (strophanthin C), Trizma base (reagent grade), and adenosine triphosphate (disodium salt) were purchased from Sigma Chemical Co., St. Louis, Mo. Acetylcholine was a product of Schwarz BioResearch and was washed repeatedly with diethyl ether and stored in a brown container at 4°. Lanthanum chloride was purchased from A. D. Mackay (New York). All solvents were anhydrous ACS grade analyzed reagents from Matheson Coleman and Bell; Mes,[1] Hepes, and Tris were purchased from Calbiochem (San Diego, Calif.). All inorganic salts were ACS reagent grade products, unless otherwise specified. Glucose, USP, was a Mallinckrodt product.

Cell counting was accomplished through use of a Celloscope unit (Particle Data, Inc., Elmhurst, Ill.).

Membranes were prepared from washed erythrocytes essentially by the procedure of Dodge *et al.* (1963) with 7.5 volumes of 20 imosm[1] Tris buffer at the desired pH used for each volume of cells suspended to a 50% hematocrit in isotonic buffer. First contact of the intact cells with the low ionic strength buffer is called the initial hemolysis. The membranes obtained by centrifugation of this mixture are noted as wash I membranes. Subsequent washes are then numbered in succession. Usually by the third wash at pH 7.6 the membranes (wash III membranes) are a very light pink and most often by the fourth wash (wash IV membrane) nearly colorless. Membranes were prepared at pH 5.8 in a similar manner, but were light red in color at the "wash IV" stage. The latter could contain as much as 3–5% of the original hemoglobin (or *ca.* 25–30% of the membrane by weight).[2] All these operations were conducted at 4°.

Results on whole cells were more conveniently expressed as units per milliliter of packed cells, using the hematocrit

[1] Abbreviations used are: ATP, adenosine triphosphate; ATPase, adenosine triphosphatase, enzymatic activity catalyzing the hydrolytic cleavage of inorganic phosphate, $P_i$, from ATP; AcChase, acetylcholine esterase; NF, nonfrozen; FT, frozen–thawed; TM, Tris-maleate; NaM, sodium maleate; T, Tris; imosm, ideal milliosmolar; 20 T, 20 imosm Tris; 20 NaM, 20 imosm sodium maleate; 20 TM, 20 imosm Tris-maleate; a subscript number with the abbreviation is the pH value; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

[2] It has been shown on repeated occasions that there are certain distinct differences in the protein pattern of the pH 7.6 membranes as compared to that found in the pH 5.8 membranes. At least as revealed by sodium dodecyl sulfate gel electrophoresis, using the technique of Neville (1971), two proteins, with approximate molecular weights of 17,000 and 25,000, are present in the 20 $TM_{5.8}$ membranes but not in the 20 $TM_{5.8} \rightarrow$ 20 $T_{7.6}$ preparations. A pair of peaks in the 30,000–35,000 molecular weight range, which contain a large quantity of protein, is extensively reversed in their relative magnitudes between the pH 5.8 and 7.6 preparations (G. Hildenbrandt, private communication).

reading for the cell volume. Consequently, many of the assays on membrane or other cell fractions are reported in terms of units per milliliter of cell equivalent, based on the volume of intact cells from which the fraction was derived. Cell counts were done on limited occasions but the hematocrit values obtained under comparable or reproducible conditions were assumed to be related to the number of cells.

*Comments on ATPase Assay.*[3] The assay procedure used in this study derived maximum or at least constant activity from any functioning ATPase site to which ATP was accessible. In our hands, maximum ATPase activity of the intact human erythrocyte, particularly the $Na^+$–$K^+$–$Mg^{2+}$ and $Ca^{2+}$ stimulated types, was achieved by freeze-thaw treatment of the intact cells. Even though sonication of the cells for 5–10 sec at 4° also could express (high) $Na^+$–$K^+$–$Mg^{2+}$ ATPase activity, the $Ca^{2+}$-stimulated ATPase activity was rendered completely inactive by this treatment. However, it should be stressed that the freeze–thaw treatment, though remarkably reproducible on intact cells, must be considered as a nonideal measure of total ATPase activity. This is substantiated by the observations that on occasions in highly washed hemoglobin free membrane preparations, maximum ATPase activity was not expressed by the freeze–thaw treatment. Also, under some conditions the freeze–thaw process actually may be destroying activity by disruption of important protein–lipid interactions near or at the ATPase active sites, resulting in samples with ATPase activities much less than those found for nonfrozen thawed samples. This was particularly true again in highly washed membranes. Notwithstanding these occasional, frustrating problems, the freeze–thaw technique is one of the few procedures available for expressing the ATPase activities in the intact erythrocytes and membranes derived therefrom. If a sufficient number of analyses are performed, the pattern of activity can be established with considerable comfort and confidence.

The increase or change in activity of a particular ATPase cannot be ascribed *a priori* to a change in the number of sites or in the accessibility of the ATP to the same number os sites. It is not clear or established from this study whether all the membranes are slightly leaky so that "all" ATPase sites are operating at very low efficiency due to slow ATP penetration or whether a small fraction of the membranes are completely permeable and exhibit maximum activity. In general, ATPase assays on erythrocytes were conducted within 24 hr after collection. Otherwise, variations in the stability of the different ATPases were noted on longer storage periods and hence caused problems in interpretation of results.

## Results

*Human Erythrocytes.* A. Influence of type of buffer on membrane characteristics. Even though comparable types of membranes have been obtained by lysis of human erythrocytes in low osmolarity $NaHCO_3$ (Bramley *et al.*, 1971) and Tris (Hanahan and Ekholm, 1972) buffers at pH 7.6, it was of considerable import nonetheless to evaluate the ATPase activities in membranes obtained by lysis in other buffers. Consequently, low osmolarity (20 imosm) solutions of the following buffers, Hepes at pH 7.6, Mes at pH 5.8, and Tris-maleate at pH 5.8, were employed in preparation of membranes from human erythrocytes. In addition to the usual assay for total and $Na^+$–$K^+$–$Mg^{2+}$ ATPase activities, the level of $Ca^{2+}$-stimulated ATPase was used as an additional parameter for evaluating the status of the membrane. These data are recorded in Table I.

The results presented here, in general, confirm previous observations from this laboratory (Hanahan and Ekholm, 1972) and from Bramley *et al.* (1971) that membranes prepared at pH 7.6 by osmotic lysis in 20 imosm solutions develop $Na^+$–$K^+$–$Mg^{2+}$ ATPase sites easily accessible to extracellular ATP. An additional finding in these studies was that $Ca^{2+}$-stimulated ATPase activity developed in a comparable fashion to that of the $Na^+$–$K^+$–$Mg^{2+}$ ATPase activity. The latter point is illustrated by examination of the data on intact erythrocytes (Table I) in which there is low $Ca^{2+}$-stimulated ATPase activity in the sample as isolated but high activity in the frozen–thawed samples. On the other hand, $Ca^{2+}$-stimulated ATPase activity became available in wash IV membranes, prepared at pH 7.6 (Table I, expts IB, ID, IIB). However, membranes prepared at pH 5.8 either in 20 imosm Tris-maleate or 20 imosm Mes buffer showed low $Na^+$–$K^+$–$Mg^{2+}$-stimulated ATPase activities. Freeze-thawing of these samples readily produced access of ATP to the ATPase activities.

Significantly, in most instances there was good recovery of ouabain-sensitive ($Na^+$–$K^+$–$Mg^{2+}$) ATPase activity, but a decided loss of the ouabain-insensitive ATPase activity in the membranes prepared at pH 7.6 in 20 imosm buffer. In several experimental runs, the insensitive activity was barely detectable or even missing. Similarly, $Ca^{2+}$-stimulated ATPase activity in these membranes prepared at pH 7.6 was decreased as much as 50% as compared to the intact erythrocytes and this activity was not further expressed by freeze–thaw treatment or gentle sonication. Even though the membranes isolated at pH 5.8 were presumably less "permeable" as judged by inaccessibility of ATPase sites, similar decreases were noted in the activities found in freeze–thawed samples.

B. Effect of varying osmolarity and pH on wash IV membranes. On the basis of previous studies (Mitchell *et al.* 1965) it was expected that osmolarity and pH would play an important role in the expression of ATPase activity in membrane preparations. Particularly interesting was the finding that wash IV membranes, prepared at pH 7.6, could be analyzed for their ATPase activity in an isotonic medium (Brewers assay medium). Under these conditions the membranes exhibited high ATPase activity without the necessity of freeze–thaw treatment. Thus, this type of membrane preparation did not appear to "reseal" on change from a low osmolarity to a high osmolarity medium. Subsequent to this observation a series of experiments was devised to examine the ATPase activities of human erythrocyte membranes under varying conditions of osmolarity and pH. Essentially, these studies centered attention on wash IV membranes prepared at pH 7.6 and 5.8. The results of these experiments are tabulated in Table II.

Under low osmolarity conditions, it was evident that wash IV, pH 7.6 membranes subjected to further washing with 20 imosm Tris-maleate, pH 5.8, buffer (Table II) showed a significant and often dramatic decrease in ATPase activity. Some reduction of $NF^1$ values relative to FT values occurred suggesting that inaccessibility to ATP was partially reestablished. However, wash IV 20 $T_{7.6}$ membranes (expt IA,

[3] The terms "open," overt, and accessible, used at certain points in the article, are meant to convey the same meaning, essentially the availability of membrane-bount ATPase to extracellular ATP. Resealed membranes are meant to connote those membranes which have little or no hydrolyase activity toward extracellular ATP; these membranes at one time would have exhibited significant activity toward added ATP but due to experimental manipulations have been altered, presumably in their permeability.

TABLE I: Total $Na^+$–$K^+$–$Mg^{2+}$ and $Ca^{2+}$ ATPase Activity of Human Erythrocytes and Membranes as Influenced by Type of Buffer and pH.[a]

| Sample and Conditions | | ATPase | | | |
|---|---|---|---|---|---|
| | | Total | Insensitive | Sensitive | $Ca^{2+}$ Stimulated |
| I. Subject, BB | | | | | |
| A. Cells washed in isotonic Tris buffer, pH 7.6 | NF | 4.60 | 3.90 | 0.70 | 4.62 |
| | FT | 15.41 | 9.60 | 5.90 | 45.60 |
| B. Wash IV, 20 imosm Tris, pH 7.6, membranes | NF | 10.00 | 2.20 | 7.80 | 21.70 |
| | FT | 10.40 | 2.50 | 7.90 | 23.60 |
| C. Cells washed in isotonic $NaHCO_3$, pH 7.5 | NF | 5.80 | 4.40 | 1.40 | 5.70 |
| | FT | 16.10 | 10.00 | 6.10 | 38.40 |
| D. Wash IV, 20 imosm $NaHCO_3$, pH 7.5, membranes | NF | 9.00 | 2.10 | 6.90 | 13.10 |
| | FT | 10.80 | 2.80 | 8.00 | 16.20 |
| II. Subject, MH | | | | | |
| A. Cells washed in isotonic Hepes buffer, pH 7.6 | NF | 5.30 | 4.60 | 0.70 | 5.70 |
| | FT | 15.60 | 6.90 | 8.70 | 45.30 |
| B. Wash IV, 20 imosm Hepes, pH 7.6, membranes | NF | 10.10 | 3.20 | 6.90 | 21.10 |
| | FT | 11.40 | 3.20 | 8.20 | 25.30 |
| C. Cells washed in isotonic Mes buffer, pH 5.8 | NF | 5.30 | 4.50 | 0.80 | 5.80 |
| | FT | 14.90 | 6.80 | 8.10 | 44.60 |
| D. Wash IV, 20 imosm Mes, pH 5.8, membranes | NF | 1.80 | 1.20 | 0.60 | 3.60 |
| | FT | 7.60 | 2.70 | 4.90 | 19.50 |
| E. Cells washed in isotonic Tris-maleate, pH 5.8 | NF | 4.40 | 3.80 | 0.60 | Not run |
| | FT | 12.40 | 5.50 | 6.90 | Not run |
| F. Wash IV membranes, 20 imosm Tris-maleate, pH 5.8 | NF | 1.26 | 0.45 | 0.81 | Not run |
| | FT | 8.90 | 3.10 | 5.80 | Not run |
| G. Cells washed in isotonic Tris buffer, pH 7.6 | NF | 4.80 | 4.10 | 0.70 | 5.30 |
| | FT | 16.60 | 7.90 | 8.70 | 48.20 |

[a] Enzyme activity was determined as described in the text, with the sensitive ATPase that component of the complex inhibited by ouabain: NF, nonfrozen; FT, frozen–thawed. ATPase activity is expressed as $\mu$mol of $P_i$ released/ml of packed cells per 2 hr.

Table II), washed either with 20 $TM_{5.8}$ buffer or 20 $NaM_{5.8}$ buffer, and then subsequently rewashed with 20 $T_{7.5}$ buffer, allowed a partial reexpression of ATPase activity (expt IA,d Table II) and an increase of NF values relative to FT values.

A most interesting effect was noted on treatment of wash IV, pH 7.6, membranes (expts IA, a, c, Table II) with 20 $NaM_{5.8}$ buffer. The presence of $Na^+$ in the latter buffer appears to reseal the membranes with respect to ATPase and in these experiments even treatment with 20 $T_{7.6}$ buffer (expts IA, e, Table II) did not allow full expression of ATPase activities. However, it is entirely possible that the ATPase is lost or irreversibly denatured, but no data are available on this point.

On the other hand, the wash IV 20 $TM_{5.8}$ or $NaM_{5.8}$ membranes (expts IB, IC, Table II) as isolated showed very low ATPase activities but demonstrated high activities upon freeze–thaw treatment. Washing of either the wash IV 20 $NaM_{5.8}$ or the wash IV 20 $TM_{5.8}$ membranes with 20 $T_{7.6}$ buffer led to substantial expression of ATPase activities without freeze–thaw treatment. The further washing of these membranes with low osmolarity, pH 5.8, buffer produced substantial reestablishment of the original wash IV (pH 5.8) membrane condition of low (NF) ATPase values as compared to the freeze–thaw values. It should be noted that in washing membranes prepared at pH 5.8 with 20 $T_{7.6}$ buffer, there is some loss of hemoglobin from the membranes, but it is not a complete removal. Further, as mentioned earlier, at pH 5.8 there was always a significant decrease (loss) in the level of nonsensitive ATPase. This can be seen by examining the ATPase activities presented in Table I, Table II, and in a previously reported study (Hanahan and Ekholm, 1972). Of further interest, the $Ca^{2+}$-stimulated ATPase activity is higher in the pH 5.8 prepared membranes than in those membranes prepared at pH 7.6, but cannot be expressed in the pH 5.8 preparations unless they are freeze–thawed. These data suggest that the $Ca^{2+}$-stimulated ATPase is a different activity than the $Na^+$–$K^+$–$Mg^{2+}$ ATPase, at least in part, and hence its changes in activity or behavior do not necessarily reflect the same membrane changes as indicated by the $Na^+$–$K^+$–$Mg^{2+}$ ATPase. Furthermore, treatment of wash IV 20 $TM_{5.8}$ membranes twice with 20 $T_{7.6}$ buffer (expt B, b, Table II) resulted in a significant decrease in $Ca^{2+}$-stimulated ATPase (FT) activity. However, washing of the wash IV 20 $T_{7.6}$ membrane with 20 NaM, pH 5.8, buffer resulted in a substantial decrease in $Ca^{2+}$ ATPase (NF) activity.

An alternative approach to the study of ATPase expression in these membranes is through graded hypotonic hemolysis, using buffer of osmolarities such that significant amounts of hemoglobin can be retained in the membrane. Human erythrocytes were hemolyzed in 100 imosm Tris buffer, pH 7.6, and the wash IV membranes obtained as described above. These membranes contained 20% mean corpuscular hemoglobin but the ATPase activities were comparable to a control, untreated erythrocyte preparation. On freeze–thaw

TABLE II: Alterations in Total, $Na^+$–$K^+$–$Mg^{2+}$, and $Ca^{2+}$ ATPase Activity of Human Erythrocyte Membranes by Simple Variations in Buffer Washes and pH.[a]

| Pretreatment Conditions | | ATPase Activity | | | |
|---|---|---|---|---|---|
| | | Total | Insensitive | Sensitive | $Ca^{2+}$ Stimulated |
| I. Low Osmolarity | | | | | |
| A. $IV_{20}T_{7.6}$ membranes subjected to following washes | | | | | |
| a. None | NF | 8.50 | 0.30 | 8.20 | 19.60 |
| | FT | 9.60 | 1.40 | 8.20 | 20.10 |
| b. Twice with 20 $TM_{5.8}$ | NF | 4.90 | 0.50 | 4.40 | 11.20 |
| | FT | 6.60 | 1.50 | 5.10 | 14.10 |
| c. Twice with 20 $NaM_{5.8}$ | NF | 2.90 | 1.10 | 1.80 | 4.70 |
| | FT | 5.20 | 0.90 | 4.30 | 7.90 |
| d. Twice with 20 $TM_{5.8}$ plus twice with 20 $T_{7.6}$ | NF | 7.90 | 1.00 | 6.90 | 14.20 |
| | FT | 8.70 | 1.40 | 7.30 | 12.20 |
| e. Twice with 20 $NaM_{5.8}$ plus twice with 20 $T_{7.6}$ | NF | 6.90 | 1.20 | 5.70 | 8.60 |
| | FT | 8.20 | 1.40 | 6.80 | 9.70 |
| B. $IV_{20}TM_{5.8}$ membranes subjected to following washes | | | | | |
| a. None | NF | 3.80 | 1.50 | 2.30 | 6.90 |
| | FT | 8.50 | 0.00 | 8.50 | 31.20 |
| b. Twice with 20 $T_{7.6}$ | NF | 10.40 | 2.30 | 8.10 | 19.00 |
| | FT | 9.60 | 1.80 | 7.80 | 19.00 |
| c. Twice with 20 $T_{7.6}$, twice with $NaM_{5.8}$ | NF | 2.30 | 0.50 | 1.80 | 5.30 |
| | FT | 6.20 | 1.40 | 4.80 | 19.00 |
| C. $IV_{20}NaM_{5.8}$ membranes subjected to following washes | | | | | |
| a. None | NF | 0.80 | 0.00 | 0.80 | 1.50 |
| | FT | 10.40 | 3.10 | 7.30 | 34.00 |
| b. Twice with 20 $T_{7.6}$ | NF | 4.90 | 1.10 | 3.80 | 10.50 |
| | FT | 7.60 | 0.80 | 6.80 | 19.50 |
| c. Twice with 20 $T_{7.6}$, twice with $TM_{5.8}$ | NF | 1.80 | 1.10 | 0.70 | 3.70 |
| | FT | 9.10 | 1.50 | 7.60 | 28.00 |
| II. High Osmolarity | | | | | |
| A. $IV_{20}$ $T_{7.6}$ membranes in Brewer's assay mixture, without ATP or ouabain. Incubated at 44° for 2 hr | NF | 2.04 | 0.57 | 1.47 | 3.03 |
| | FT | 2.72 | 0.67 | 2.05 | 4.38 |
| B. $IV_{20}$ $T_{7.6}$ membranes in isotonic Tris buffer, pH 7.6. Incubated at 44° for 2 hr | NF | 1.37 | 0.84 | 0.53 | 3.20 |
| | FT | 3.00 | 1.70 | 1.30 | 7.40 |

[a] Membranes were prepared at pH 7.6 and 5.8 as described in the text. $IV_{20}T_{7.6}$, for example, refers to wash IV membranes prepared by lysis of erythrocytes in 20 imosm Tris buffer at pH 7.6; $TM_{5.8}$, Tris-maleate buffer, pH 5.8; $NaM_{5.8}$, sodium maleate buffer, pH 5.8; T, Tris buffer; $T_{7.6}$, Tris buffer, pH 7.6. ATPase activity is expressed as μmol of $P_i$ released/ml of packed cells per 2 hr.

treatment of these membranes, high $Na^+$–$K^+$–$Mg^{2+}$- and $Ca^{2+}$-stimulated ATPase activities were elicited. As an example, the $Ca^{2+}$ ATPase activities (based on micromoles of $P_i$ released per micromoles of phospholipid P per 2 hr) were as follows: NF, 0.66; FT, 3.46. On the other hand, hemolysis of human erythrocytes in 60 imosm Tris buffer at pH 7.6 produced membranes containing 4.5% MCH and with obvious accessible ATPase activities. As an example, the $Ca^{2+}$-stimulated ATPase activities were as follows: NF, 1.28; FT, 4.25. Bramley *et al.* (1971) essentially used this type of approach in preparing membranes with different hemoglobin content and different ATPase activities. It is highly possible under these conditions that a number of cells have first become permeable, then have resealed, and do not "open" again with freeze–thaw treatment.

Under high osmolarity conditions as shown in Table II, there were some interesting changes in ATPase activity of the membranes. On incubation of wash IV 20 $T_{7.6}$ membranes in a "Brewer" ATPase assay mixture, without added ATP or ouabain, at 44° for 2 hr with subsequent assay as usual, the ATPase activities decrease significantly (expt IIA, Table II). Even freeze–thaw treatment of this (incubated) membrane did not elicit any significant ATPase activity. A similar type of phenomenon was observed in the wash IV 20 $T_{7.6}$ mem-

TABLE III: Effect of Buffer and pH on ATPase Levels in Human Erythrocytes Incubated at 44°.[a]

| Sample | | ATPase Activity | |
|---|---|---|---|
| | | Nonfrozen | Frozen–Thawed |
| A. Original cells, not incubated, but suspended 0.172 M Tris, pH 7.60 | Total | 4.02 | 15.10 |
| | Insensitive | 3.31 | 6.32 |
| | Sensitive | 0.71 | 9.78 |
| | $Ca^{2+}$ stimulated | 4.30 | 50.30 |
| B. Cells incubated at 44° in | | | |
| 1. 0.155 M $NaHCO_3$, pH 7.50 | Total | 4.82 | 15.80 |
| | Insensitive | 4.10 | 7.40 |
| | Sensitive | 0.72 | 8.40 |
| | $Ca^{2+}$ stimulated | 5.03 | 53.00 |
| 2. 0.155 M Hepes,[b] pH 7.58 | Total | 3.08 | 15.00 |
| | Insensitive | 3.08 | 5.39 |
| | Sensitive | 0.00 | 9.61 |
| | $Ca^{2+}$ stimulated | 3.24 | 53.70 |
| 3. 0.172 M Tris[c] | Total | 11.70 | 12.70 |
| | Insensitive | 3.88 | 4.03 |
| | Sensitive | 7.82 | 8.67 |
| | $Ca^{2+}$ stimulated | 47.80 | 45.80 |
| 4. 0.155 M $KHCO_3$, pH 7.60 | Total | 3.73 | 14.40 |
| | Insensitive | 3.52 | 6.12 |
| | Sensitive | 0.21 | 8.28 |
| | $Ca^{2+}$ stimulated | 4.53 | 48.40 |
| 5. 0.155 M NaCl with 1% Tris, pH 7.60 | Total | 3.66 | 15.00 |
| | Insensitive | 3.30 | 7.20 |
| | Sensitive | 0.36 | 7.80 |
| | $Ca^{2+}$ stimulated | 4.37 | 53.30 |
| 6. 0.155 M KCl with 1% Tris, pH 7.60 | Total | 3.52 | 15.50 |
| | Insensitive | 3.66 | 5.96 |
| | Sensitive | 0.00 | 9.54 |
| | $Ca^{2+}$ stimulated | 3.88 | 48.60 |
| 7. 0.155 M histidine–imidazole buffer,[d] pH 7.6 | Total | 2.80 | 14.80 |
| | Insensitive | 2.37 | 4.82 |
| | Sensitive | 0.43 | 9.98 |
| | $Ca^{2+}$ stimulated | 3.23 | 44.20 |
| C. Cells exposed to 0.155 M $NH_4HCO_3$,[e] pH 7.5, without incubation | Total | 3.66 | 8.94 |
| | Insensitive | 1.55 | 3.70 |
| | Sensitive | 2.11 | 5.24 |
| | $Ca^{2+}$ stimulated | Not run | Not run |
| D. Cells incubated in 0.158 M Tris-maleate buffer,[f] pH 5.8 | Total | 4.95 | 8.65 |
| | Insensitive | 3.08 | 4.70 |
| | Sensitive | 1.87 | 3.95 |
| | $Ca^{2+}$ stimulated | 14.40 | 31.60 |

[a] Four-milliliter aliquots of a hematocrit 50.5% freshly drawn cell suspension were washed well with 1.5 vol of indicated buffer, centrifuged, and supernatant discarded. The cells were suspended to 10 ml with specified buffer and assayed. ATPase activities are given as μmol of $P_i$ released/ml of packed cells per 2 hr; subject, BB. [b] pH of sample adjusted to indicated value with dilute NaOH. [c] Pronounced hemolysis occurred. [d] Buffer is that used in the ATPase assay (Brewer *et al.*, 1968). [e] Cells hemolyzed immediately without incubation at 44°. These cells were washed three times with the same buffer and analyzed. [f] Cells hemolyzed to a small degree on incubation at 44°. These cells were obtained from a different subject, but the original cells, nonincubated, in 0.172 M Tris, pH 7.6 exhibited ATPase activities comparable to footnote *b* above. The values were as follows: nonfrozen, total, 4.96; insensitive, 4.14; sensitive, 0.82; $Ca^{2+}$ stimulated, 4.68; frozen–thawed, total, 16.30; insensitive, 8.90; sensitive, 9.40; $Ca^{2+}$ stimulated, 49.50.

branes incubated in isotonic Tris buffer, pH 7.6, at 44° for 2 hr (exp IIB, Table II). On the other hand, incubation of wash IV 20 $T_{7.6}$ membranes in a complete "Brewer" ATPase assay mixture gave high ATPase activities. Thus, it would appear that under these conditions ATP is required to provide an "open" or permeable membrane.

C. ATPase ACTIVITIES IN VARIOUS ISOTONIC BUFFERS. The possible influence of isotonic Tris and other buffers on the

TABLE IV: Distribution of ATPase Activities in Human Erythrocytes Incubated at 44° in Isotonic Tris Buffer, pH 7.6.[a]

| | | ATPase Activity | |
|---|---|---|---|
| | | Nonfrozen | Frozen–Thawed |
| A. Cells suspended in 0.172 M Tris, pH 7.6, but not incubated[b] | Total | 4.10 | 14.40 |
| | Insensitive | 3.54 | 7.50 |
| | Sensitive | 0.56 | 6.90 |
| | $Ca^{2+}$ stimulated | 4.40 | 40.30 |
| B. Cells incubated at 44° for 2 hr in 0.172 M Tris, pH 7.6, total mixture[b] | Total | 10.60 | 10.90 |
| | Insensitive | 3.68 | 3.80 |
| | Sensitive | 7.00 | 7.10 |
| | $Ca^{2+}$ stimulated | 34.20 | 33.00 |
| C. "Intact" cells recovered[c] from B by centrifugation at 1000*g* (pellet) | Total | 8.21 | 7.76 |
| | Insensitive | 2.80 | 3.71 |
| | Sensitive | 5.41 | 4.05 |
| | $Ca^{2+}$ stimulated | 22.65 | 16.98 |
| D. Membranes recovered[c] from C supernatant, by centrifugation at 20,000*g* | Total | 9.40 | 8.87 |
| | Insensitive | 2.78 | 2.92 |
| | Sensitive | 6.62 | 5.95 |
| | $Ca^{2+}$ stimulated | 29.40 | 20.17 |

[a] The incubated cell suspension, expt B3, Table III, was centrifuged at 20,000*g* for 40 min at 4°, and washed in the 0.172 M Tris, pH 7.6. The pellet was resuspended in the same buffer and the above process repeated twice more. The resulting "membranes" were resuspended in 0.172 M Tris at pH 7.6 and centrifuged at 1000*g*, and the pellet saved. The supernatant was centrifuged in a separate tube at 20,000*g* for 40 min at 4° and the pellet labeled as membranes. ATPase activities are given as μmol of $P_i$ released/ml of packed cells per 2 hr. [b] Recovery of acetylcholine esterase in the nonincubated and incubated preparations was in the range of 85–95%. Typical activity values (expressed as μmol of acetic acid released/ml of packed cells per hr); original cell suspension (nonincubated), 762 ± 30; cells incubated at 44° for 2 hr (pelleted at 20 20,000*g*), 780 ± 40. [c] Recovery based on lipid P ~70% of the activity was recovered in the membrane fraction (D), and ~28% in the "intact" cells (C).

stability of the ATPase system upon incubation at 44° for 2 hr was explored. This temperature was chosen, since it was the one employed in the ATPase assay system. Surprisingly, after approximately 1 hr of incubation in an isotonic Tris buffer, pH 7.6, intact erythrocytes underwent hemolysis and at the end of 2 hr well over 90% of the cells had hemolyzed, as judged by hemoglobin release. This was a highly reproducible phenomenon. Examination of the ATPase activities of these incubated cells showed that they were now available to added ATP, as indicated by the data in Table III. At least at pH 7.6, Tris was the only buffer causing any hemolysis under these conditions. A particularly important fact is that other buffers such as isotonic Hepes, Mes, or $NaHCO_3$ did not cause any hemolysis or other discernible alterations to the cells on incubation at 44°. Isotonic Tris-maleate, at pH 5.8, caused slight hemolysis at 44° in 2 hr. Intact cells stored at 4° in isotonic Tris, pH 6, overnight showed slight hemolysis and a significant increase in cell volume. Several different batches and preparations of Tris buffer exhibited the same behavior. Interestingly, incubation of erythrocytes in isotonic glucose at 44° also led to a time-dependent hemolysis and an expression of ATPase activities. Other facets of the time-dependent hemolysis of intact erythrocytes in isotonic media are described in a later section (see F, below).

In an effort to define more carefully the alterations occurring during incubation of eythrocytes at 44° in isotonic Tris buffer, pH 7.6, the levels of lipid P and cholesterol and enzymatic activities in the various components of the final incubate were explored. As it was evident visually that hemoglobin depleted membranes were produced during incubation, the mixture was subjected to differential centrifugation. Essentially, this involved initial recovery of all cellular components by centrifugation at 20,000*g* for 40 min at 4°, and then recentrifugation of this pellet at 1000*g* to recover the "intact" cells. Further details are given in Table IV. The "intact" cells and membranes were analyzed for lipid P, cholesterol, and ATPase activities. The recovery of lipid P was excellent (nearly 100%), but the recovery of cholesterol was variable. It was not possible to correlate any loss of cholesterol with development of accessible ATPase sites. The data in Table IV show that all the cellular components have ATP accessible sites, even in the cells (expt C, Table IV) which retain a considerable amount of hemoglobin.

Another approach to expression of ATPase activity in intact cells is through the freeze–thaw procedure. On the basis of the above results, it was of some value to examine the frozen–thawed cells in similar manner. The membranes resulting from frozen–thawed cells were centrifuged at 20,000*g* for 40 min at 4°, washed with isotonic Tris buffer, pH 7.6, and then analyzed for lipid P and cholesterol, plus ATPase activities. The results showed that lipid P could be recovered in excellent yield (>95%) in the membranes, whereas cholesterol loss (ranging from 40 to 51%) was a feature of this treatment. The availability of ATPase sites to added ATP was at a level expected from previous experiments (see Table I for example).

Inasmuch as freeze–thawed erythrocytes (human) exhibited such high $Na^+$–$K^+$–$Mg^{2+}$ ATPase activities, plus removal of nearly all the hemoglobin, it was thought that this preparation could serve as a starting point for isolation of membranes

TABLE V: Effect of Divalent Cations Added during Hemolysis of Human Erythrocytes on ATPase Activities of Resulting Membranes.[a]

| | | Control[c] (No Added $Ca^{2+}$ or $Mg^{2+}$) | | | | | | $Ca^{2+}$ Treated | | | | $Mg^{2+}$ Treated | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Wash II | | Wash IV | | Wash II | | Wash IV | | Wash II | | Wash IV | |
| | | NF | FT | NF | FT | NF | FT | NF | FT | NF | FT | NF | FT | NF | FT |
| A[b] | | | | | | | | | | | | | | | |
| pH 7.6 | Total | 3.75 | 13.00 | 3.68 | 10.00 | 10.00 | 10.10 | 1.81 | 9.95 | 4.82 | 9.60 | 1.82 | 9.10 | 8.30 | 9.45 |
| | Insensitive | 3.19 | 6.22 | 1.43 | 2.48 | 2.38 | 2.58 | 0.55 | 4.15 | 1.94 | 2.63 | 0.73 | 2.63 | 2.29 | 4.26 |
| | Sensitive | 0.56 | 6.78 | 2.25 | 7.52 | 7.62 | 7.52 | 1.26 | 5.80 | 2.88 | 6.97 | 1.03 | 6.12 | 6.01 | 5.19 |
| | $Ca^{2+}$ stimulated | 3.90 | 41.00 | 4.55 | 43.50 | 27.10 | 29.80 | 2.70 | 36.80 | 10.00 | 29.60 | 3.36 | 38.00 | 24.60 | 30.90 |
| pH 5.8 | Total | 5.05 | 12.80 | 1.21 | 5.78 | 2.94 | 6.84 | 2.04 | 8.30 | 1.25 | 6.12 | 2.16 | 7.2 | 1.14 | 8.40 |
| | Insensitive | 4.45 | 5.90 | 0.71 | 2.72 | 1.92 | 2.84 | 1.09 | 2.99 | 1.06 | 2.58 | 1.74 | 3.4 | 0.60 | 2.96 |
| | Sensitive | 0.60 | 6.90 | 0.50 | 3.06 | 1.02 | 4.00 | 0.95 | 5.31 | 0.19 | 3.54 | 0.42 | 3.8 | 0.54 | 5.44 |
| | $Ca^{2+}$ stimulated | 5.75 | 48.40 | 1.59 | 19.60 | 3.62 | 21.70 | 4.68 | 31.00 | 2.50 | 18.20 | 2.75 | 20.0 | 2.40 | 28.60 |

| | Orig Erythrocyte Suspension[c] | | Control Membranes | | | | $Ca^{2+}$-Treated Membranes | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Wash II | | Wash IV | | Wash II | | Wash IV | |
| | NF | FT | NF | FT | NF | FT | NF | FT | NF | FT |
| B[b] | | | | | | | | | | |
| Total | 4.25 | 11.50 | 3.51 | 8.90 | 8.70 | 8.60 | 2.09 | 14.30 | 5.25 | 9.65 |
| Insensitive | 3.70 | 6.80 | 2.05 | 3.28 | 2.48 | 2.51 | 1.41 | 7.25 | 1.95 | 3.32 |
| Sensitive | 0.55 | 4.68 | 1.46 | 5.62 | 6.22 | 6.09 | 0.68 | 7.05 | 3.30 | 6.33 |
| $Ca^{2+}$ stimulated | 5.09 | 29.30 | 4.60 | 26.70 | 24.30 | 28.20 | 3.65 | 42.00 | 13.30 | 42.60 |

| | Orig Erythrocyte Suspension[c] | Membranes Wash IV | | | | |
|---|---|---|---|---|---|---|
| | | Control | $Ca^{2+}$ Treated | | $Mg^{2+}$ Treated | |
| | FT | NF | NF | FT | NF | FT |
| C[b] | | | | | | |
| Total | 12.29 | 7.26 | 3.08 | 9.97 | 3.31 | 6.28 |
| Insensitive | 7.83 | 2.33 | 1.64 | 6.24 | 1.72 | 3.31 |
| Sensitive | 4.46 | 4.93 | 1.44 | 3.73 | 1.59 | 2.97 |
| $Ca^{2+}$ stimulated | 42.40 | 21.92 | 9.17 | 30.90 | 7.78 | 23.00 |

[a] The preparation and assay of the various membrane samples noted below were accomplished as described in the text and in Table I. The hemolysis was conducted in a 20 imosm Tris buffer, pH 7.6, or 20 imosm Tris-maleate buffer, pH 5.8, with the specific cation included as part of the total osmolarity. ATPase values are expressed as μmol of $P_i$ released/ml of packed cells per 2 hr: NF, nonfrozen; FT, frozen–thawed. See text for definition of wash II and wash IV membranes and other details. [b] A, 2 mM $Ca^{2+}$ or $Mg^{2+}$ added during *initial hemolyzing buffer wash only*, followed by three washes with buffer only; subject, TA; B, 5 mM $Ca^{2+}$ added in initial hemolyzing wash only, followed by three washed with 20 imosm Tris buffer only (pH 7.6); subject, DD; C, 5 mM $Ca^{2+}$ or $Mg^{2+}$ added in initial hemolyzing wash and in the second buffer wash, followed by two washes with buffer only (pH 7.6); subject, SM. [c] Suspended in isotonic Tris buffer, pH 7.6.

with high enzyme activity. However, washing of these freeze–thawed cells with 20 T, pH 7.6, buffer caused complete and irreversible loss of all ATPase activity. No further exploration of the reasons for this type of behavior has been undertaken at present.

D. DIVALENT CATIONS ON MEMBRANE BEHAVIOR. Earlier observation on the protective influence of divalent cations on the cow erythrocyte membrane (Burger *et al.*, 1968) prompted a study of the effect of $Ca^{2+}$ and $Mg^{2+}$ on the behavior of human erythrocyte membranes. Several experimental conditions were selected to test the effect of these divalent cations, as indicated by ATPase activities, on the isolated membrane. In the first protocol, $Ca^{2+}$ or $Mg^{2+}$, at a final level of 2 mM, was included during the initial hemolyzing event only. The second protocol was similar except only $Ca^{2+}$, at a final level of 5 mM, was included in the initial hemolyzing mixture. In the third protocol, $Ca^{2+}$ or $Mg^{2+}$, at a final level of 5 mM, was added to the wash II buffer as well

TABLE VI: Acetylcholine Esterase Levels in Human Erythrocyte Hemolyzate and Membrane Preparations.[a]

| Sample | AcChase Levels[b] | % Recovery |
|---|---|---|
| A. Whole cell hemolyzate | 782 ± 32 | 100 |
| B. Wash IV 20 $T_{7.6}$ membranes | 716 ± 22 | 91 |
| C. Wash IV 20 $T_{5.8}$ membranes | 733 ± 34 | 93 |

[a] AcChase was assayed by the technique of Heller and Hanahan (1972). Activity is expressed as μmol of acetic acid released per ml of packed cells per hr. [b] In each case, these values represent the average of duplicate samples on two separate samples.

as to the initial hemolyzing mixture. It was not technically feasible to include divalent cations in all the washes since under such conditions the level of divalent cations in the final membrane preparation was so high as to distort seriously the true ATPase levels. In all these protocols, the wash IV membranes were obtained and assayed for ATPase activities and the results are given in Table V. These data show very clearly that $Ca^{2+}$, in particular, can exert a decided influence on the ATPase activity of membranes prepared from human erythrocyte. The addition of $Ca^{2+}$ in the initial hemolysis buffer only, at pH 7.6, yielded a membrane preparation with reduced ATPase activity, but of more importance the inclusion of $Ca^{2+}$ in the second wash (wash II) as well as in the initial hemolyzing buffer gave membranes (as isolated) with a very low ATPase activity. Yet, freeze–thaw treatment of these two types of membrane preparation gave a high level of ATPase activity. These results must be interpreted as illustrating the importance of $Ca^{2+}$ in "sealing" the membrane. $Mg^{2+}$ as expected from previous studies on the cow erythrocyte membrane (Burger *et al.*, 1968) proved much less effective in "sealing" the membranes.

Interestingly, wash IV membranes, prepared at pH 5.8 in the absence of added divalent cations, and exhibiting very low activity toward added ATP, on exposure to one wash with 20 T, pH 7.6, buffer containing 5 mM $Ca^{2+}$ showed low (NF) ATPase activities, whereas with a single wash with 20 $T_{7.6}$ buffer containing no added $Ca^{2+}$ the resulting membrane had a high (NF) ATPase activity. Concomitantly, treatment of wash IV 20 $T_{7.6}$ membranes, prepared in the absence of added $Ca^{2+}$, with a 20 $T_{7.6}$ buffer containing 5 mM $Ca^{2+}$ produced a membrane with low activity toward added ATP. Freeze–thaw treatment of this latter preparation allowed expression of a significant but depressed ATPase activity.

It is important to comment on these results as they relate to the characteristics of the final membrane preparations. In the experiments conducted at pH 7.6 (Table V), where $Ca^{2+}$ was added in the hemolyzing (wash I) and second wash (wash II) at pH 7.6, the final membranes (wash IV) contained significant amounts of hemoglobin (~3–5%) and at the same time had a reduced activity toward ATP, at least as expressed by the $Na^+$–$K^+$–$Mg^{2+}$- and $Ca^{2+}$-stimulated ATPases. These membranes behaved like the hemoglobin containing wash IV 20 $T_{5.8}$ membranes in that both preparations express ATPase activity, but retain hemoglobin on treatment with 20 $T_{7.6}$ buffer. On the other hand, membranes prepared at pH 7.6 in the absence of $Ca^{2+}$ yielded essentially hemoglobin free membranes, and had high ATPase activity. This latter ATPase activity is decreased almost completely in the absence of hemoglobin, when the membranes are washed with 5 mM $Ca^{2+}$ in a 20 $T_{7.6}$ buffer. These latter membranes appear less stable than those prepared by inclusion of $Ca^{2+}$ in the initial hemolyzing buffer.

E. ACETYLCHOLINE ESTERASE LEVELS. Acetylcholine esterase is an enzyme characteristic of the erythrocyte membrane and hence it was of some import to examine, in a limited manner, its activity in certain of these membrane preparations. Table VI presents data on the level of AcChase in the whole cell hemolyzate of human erythrocytes and membranes prepared from these cells by lysis in 20 imosm buffer at pH 5.8 and 7.6. These results show that within the limits of the assay method, the retention of AcChase in these membranes is nearly quantitative. Thus, it appears evident that AcChase and the ATPase activities vary greatly in their reactivity toward certain conditions or reagents and hence must be considered as having quite different membrane environments.

F. HEMOLYSIS IN ISOTONIC MEDIA. As noted briefly in section C above, incubation of human erythrocytes in isotonic Tris buffer, pH 7.6, or in isotonic glucose at 44° unexpectedly gave rise to a time-dependent hemolysis. A similar phenomenon, though occurring at a slower rate, was observed at 37°.[4] Release of a significant amount of hemoglobin preceded any observed accessibility of $Na^+$–$K^+$–$Mg^{2+}$ and $Ca^{2+}$ ATPase activities at each temperature. These results are given in Figures 1 and 2.

Perhaps the most intriguing and fascinating data were obtained with cells incubated in isotonic Tris buffer. On incubation at 37°, as much as 85% of the human erythrocyte hemoglobin was released in 60 min, yet there was no demonstable increase in the accessibility of the ATPase activities (Figure 1). However, after centrifugation and washing the incubation mixture clearly contained a layer of hemoglobin-depleted membranes above a well-defined hemoglobin rich cell layer. After 75 min at 37°, nearly 94% of the hemoglobin had been released but the ATPase activities had increased only slightly. Freeze–thaw treatment of these samples gave high levels of $Na^+$–$K^+$–$Mg^{2+}$ and $Ca^{2+}$ stimulated ATPase activities. Further, in a 120-min incubation at 37°, over 95% hemolysis occurred and there were significant increases in all of these ATPase activities, especially with the $Ca^{2+}$-stimulated (Figure 1) enzyme without the necessity of any freeze–thaw treatment. However, even in this long incubation, there is a hemoglobin-rich pellet. This suggests that some of the membrane population reseals trapping an amount of hemoglobin equal to (or less than) the concentration of hemoglobin in the supernatant. Thus, there are some cells that hemolyze completely and others that reseal with respect to hemoglobin after initial partial hemolysis.

On the basis of the above observations on a 60-min incubation at 37°, it was apparent that the hemoglobin-rich and the hemoglobin-depleted components could be separated by differential centrifugation. Accordingly, the incubate was centrifuged at 20,000*g* for 40 min at 4° and the supernatant carefully removed by decantation. The residue was washed

[4] A particularly provocative observation concerned the effect of incubation in isotonic Tris on the erythrocytes of the adult cow and the very young calf. Whereas the erythrocytes of the adult cow were stable at 44° over a 120-min period (as determined solely by lack of hemolysis), the cells from the very young calf (approximately 2 weeks old) were nearly completely hemolyzed under similar conditions. Further study is underway on this phenomenon.

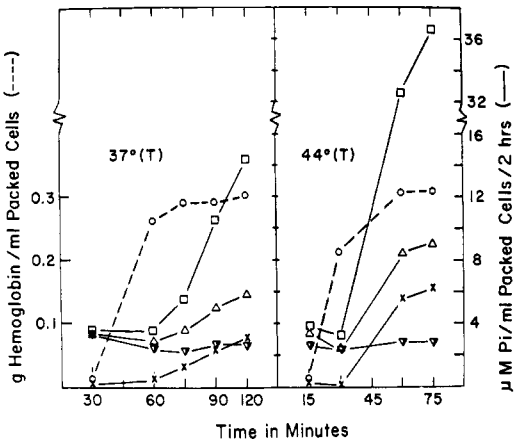


FIGURE 1: Changes in human erythrocytes incubated at 37 and 44° in isotonic Tris buffer, pH 7.6, as monitored by ATPase activities and hemoglobin release. Human erythrocytes, at a hematocrit of 20%, were incubated for the indicated times at 37 and 44°, and then centrifuged. The supernatant was assayed for hemoglobin content (○) and the pellet was assayed (without freeze–thaw treatment) for the following ATPase activities: total, △; ouabain insensitive, ×; and ouabain sensitive, ▼; $Ca^{2+}$ stimulated, □; subject LP.

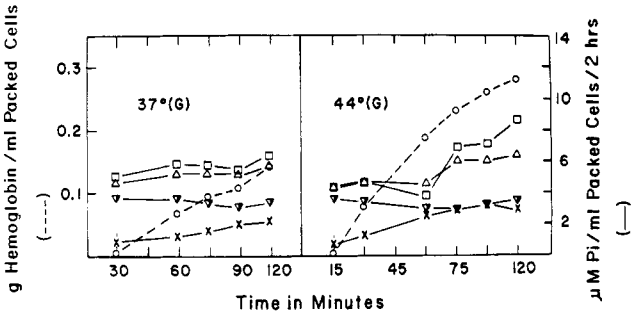


FIGURE 2: Changes in human erythrocytes incubated at 37 and 44° in isotonic glucose, pH 7.6, as monitored by ATPase activities and hemoglobin release. Conditions, types of assays, and symbols are exactly the same as given in the legend for Figure 1.

twice with isotonic Tris buffer, pH 7.6, centrifuged after each wash as above, and the supernatants were discarded. After the second wash, the pellet was suspended again in isotonic Tris, pH 7.6, and spun at 20,000*g* for 40 min at 4°. Two layers of cellular material were clearly visible. The upper, light pink layer loosened easily by gently swirling from the lower, dark red hard packed layer and was recovered by gentle decantation. These two layers, the hemoglobin-rich and the hemoglobin-depleted, were analyzed for ATP activities (E, F, Table VII) and hemoglobin content. In addition it was decided (a) to explore the behavior of these two fractions on further incubation at 44° in isotonic Tris buffer, pH 7.6, *i.e.*, to observe whether there was additional release of hemoglobin and development of ATPase activities, and (b) to investigate the biochemical nature of the membranes prepared from each fraction by hypotonic hemolysis in 20 imosm Tris buffer at pH 7.6.

These results are summarized in Table VII. Reincubation of the hemoglobin-depleted cells at 44° for 2 hr (G, Table VII) showed little or no change in low (NF) ATPase activities, and even freeze–thaw treatment did not give the expected significant increase in these enzyme systems. On the other hand, the reincubation of the hemoglobin-rich cells at 44° for 2 hr (I, Table VII) led to further loss of hemoglobin and a significantly high (NF) ATPase activity, comparable to the FT activities. It is obvious that these two fractions differ significantly in their biochemical behavior. Particularly provocative was the finding that both the hemoglobin-depleted cells and the hemoglobin-rich cells had low ATPase activities as isolated, but on freeze–thaw treatment developed significant ATPase activities. However, this latter expression of ATPase activities was not as pronounced in the hemoglobin-depleted cells as in the hemoglobin-rich cells. Hypotonic washing of the hemoglobin-depleted cells leads to membranes which give high $Na^+$–$K^+$–$Mg^{2+}$ and $Ca^{2+}$ ATPase activities without freeze–thaw treatment (H, Table VII), whereas the hemoglobin-rich cells responded in a much less pronounced manner to this treatment (J, Table VII). As expected incubation of the cells at 44° in isotonic Tris increased the rate of release of hemoglobin, with 74% of the hemoglobin released in 30 min; of interest, though, there was no definitive change in the ATPase accessibility at this time (Figure 1). Further incubation led to increased hemoglobin release and ultimate expression of a significant level of ATPase activities.

The incubation of erythrocytes in isotonic glucose at 37 and 44° provided some interesting data (Figure 2) though much less dramatic as compared to that obtained in the Tris experiments cited above. At 37°, only 20% of the hemoglobin was released from intact cells in 60 min and there was no expression of ATPase activity. At the same temperature, incubation for 120 min allowed release of 50% of the hemoglobin with again only a slight increase in ATPase accessibility (mainly the ouabain-sensitive type). There was a layer of hemoglobin-depleted membranes and also a layer of "intact" erythrocytes in centrifuged samples. At 44° approximately 60% of the hemoglobin was released in a 60-min incubation with a slight increase only in the ouabain-sensitive ATPase. However, after a 120-min incubation at 44° approximately 90% of the hemoglobin was released and there was a significant increase in available ATPase activities. It should be noted, however, that the expression of the ATPase activities in these glucose-treated samples on freeze–thaw treatment appeared to decrease. This behavior has not been examined further at this time.

The above experimental results intimated a relationship of

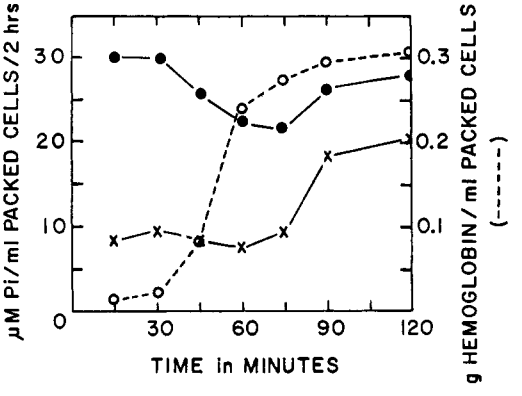


FIGURE 3: $Ca^{2+}$ ATPase accessibility and hemoglobin release in human erythrocytes incubated at 37° isotonic Tris buffer, pH 7.6. Cells were incubated and treated as described in Figure 1, except that only $Ca^{2+}$-stimulated ATPase activity was assayed on NF (×) and FT (●) samples at the times indicated. Hemoglobin release (○) was determined as described under Methods.

TABLE VII: ATPase Levels in Human Erythrocytes Incubated at 37° or 44° in Isotonic Tris Buffer, pH 7.6.[a]

| Sample and Treatment | | ATPase Activity: μmol of $P_i$ Released/ml of Packed Cells per 2 hr | | ATPase Activity: μmol of $P_i$ Released/μmol of Phospholipid P per 2 hr | |
|---|---|---|---|---|---|
| | | NF | FT | NF | FT |
| A. Original cells suspended in 0.172 M Tris, pH 7.6, but not incubated | Total | | 23.20 | | 5.08 |
| | Insensitive | | 16.10 | | 3.52 |
| | Sensitive | | 7.10 | | 1.56 |
| | Ca stimulated | | 38.40 | | 8.39 |
| B. Original cells incubated at 37° for 60 min in 0.172 M Tris, pH 7.6 | Total | 2.30 | 12.50 | 0.50 | 2.70 |
| | Insensitive | 1.70 | 8.50 | 0.40 | 1.90 |
| | Sensitive | 0.60 | 6.00 | 0.10 | 0.80 |
| | Ca stimulated | 2.90 | 23.90 | 0.63 | 5.20 |
| C. Cells incubated at 37° for 60 min in 0.172 M Tris, pH 7.6; reincubated 44° for 120 min | Total | 7.30 | 8.80 | 1.50 | 1.92 |
| | Insensitive | 2.70 | 3.90 | 0.58 | 0.84 |
| | Sensitive | 4.60 | 4.90 | 0.92 | 1.08 |
| | Ca stimulated | 19.50 | 22.70 | 4.30 | 4.97 |
| D. Cells incubated at 37° for 60 min and washed three times with 0.172 M Tris, pH 7.6 | Total | 3.10 | 11.40 | 0.72 | 2.50 |
| | Insensitive | 1.70 | 5.00 | 0.39 | 1.10 |
| | Sensitive | 1.40 | 6.40 | 0.33 | 1.40 |
| | Ca stimulated | 4.70 | 21.80 | 1.10 | 4.96 |
| E. Hb-depleted cells from B | Total | | | 0.44 | 1.25 |
| | Insensitive | | | 0.00 | 0.24 |
| | Sensitive | | | 0.44 | 1.01 |
| | Ca stimulated | | | 1.05 | 2.85 |
| F. Hb-rich cells from B | Total | | | 0.49 | 2.80 |
| | Insensitive | | | 0.31 | 1.74 |
| | Sensitive | | | 0.18 | 1.06 |
| | Ca stimulated | | | 0.80 | 5.06 |
| G. Hb-depleted cells E reincubated at 44° for 120 min | Total | | | 0.24 | 0.83 |
| | Insensitive | | | 0.00 | 0.09 |
| | Sensitive | | | 0.24 | 0.74 |
| | Ca stimulated | | | 0.55 | 1.73 |
| H. Hb-depleted cells (E) washed four times with 20 imosm Tris, pH 7.6 | Total | | | 2.30 | 2.52 |
| | Insensitive | | | 0.56 | 0.77 |
| | Sensitive | | | 1.74 | 1.75 |
| | Ca stimulated | | | 7.31 | 7.60 |
| I. Hb-rich cells (F) reincubated at 44° for 120 min | Total | | | 1.79 | 1.94 |
| | Insensitive | | | 0.58 | 0.60 |
| | Sensitive | | | 1.21 | 1.34 |
| | Ca stimulated | | | 5.50 | 5.15 |
| J. Hb-rich cells (F) washed four times with 20 imosm Tris, pH 7.6 | Total | | | 1.06 | 2.10 |
| | Insensitive | | | 0.27 | 1.20 |
| | Sensitive | | | 0.79 | 0.90 |
| | Ca stimulated | | | 3.10 | 6.30 |

[a] Freshly drawn erythrocytes were washed to remove plasma and buffy coat and then suspended in 0.172 M Tris, pH 7.6. Samples were incubated as indicated and then assayed: NF, nonfrozen; FT, frozen–thawed; subject, BB.

hemoglobin to membrane stability. A particularly facile route to study of this possibility was afforded by an examination of hemoglobin release (or retention) and development of "overt" $Ca^{2+}$-stimulated ATPase activity in human erythrocytes incubated for varying periods of time at 37° in isotonic Tris buffer, pH 7.6. The results presented in Figure 3 show that there is a sharp rise in accessible $Ca^{2+}$-stimulated ATPase activity only at a time when at least 90–92% of the hemoglobin had been released. Freeze–thaw treatment of the cells at various stages of the incubation revealed an unexplained decrease followed by an increase in this ATPase activity. It would appear that certain cells may become less permeable at the early stages of incubation and may represent a reorientation of the membrane components prior to hemolysis. Subsequent to the onset of hemolysis, the membrane undergoes an additional reorientation that allows increased access of ATP to the $Ca^{2+}$-stimulated ATPase. It may be indicative of a general response of all cells in the sample or may represent differences in behavior of cells of different age groups. On the basis of these results, it is entirely possible to prepare mem-

TABLE VIII: Distribution of $Ca^{2+}$ Stimulated ATPase, Phospholipid Phosphorus (PLP), and Hemoglobin in Human Erythrocytes Incubated at 37 or 44° in Isotonic Tris Buffer, pH 7.6.[a]

| Sample and Treatment | $Ca^{2+}$-Stimulated ATPase in $\mu$mol of $P_i$ per 2 hr | | | | PLP | Hemoglobin | | |
|---|---|---|---|---|---|---|---|---|
| | PCE | | $\mu$mol of PLP | | $\mu$g/ml | mg/ml | mg/$\mu$g | % |
| | NF | FT | NF | FT | of PCE | of PCE | of PLP | MCH |
| A. Cells suspended in 0.172 M Tris, pH 7.6, but not incubated | 4.40 | 40.30 | 0.88 | 8.06 | 155.0 | 346[b] | 2.23 | 100 |
| B. Cells incubated at 44° for 75 min | | | | | | | | |
| a. Total, unwashed | 31.30 | 33.60 | | | | 305[c] | | |
| b. Hb rich | 1.40 | 6.90 | 1.12 | 5.58 | 38.5 | 70 | 1.81 | 81 |
| c. Hb poor | 13.50 | 10.50 | 4.37 | 3.38 | 96.4 | | | |
| C. Cells incubated at 44° for 2 hr, unwashed | 29.40 | 30.70 | | | | 317[c] | | |
| D. Cells incubated at 37° for 70 min | | | | | | | | |
| a. Unwashed | 6.60 | 21.80 | | | | | | |
| b. Washed 3× with 0.172 M Tris, pH 7.6 | 7.70 | 23.80 | 1.55 | 4.81 | 153.7 | | | |
| c. Hb rich, incubated a further 30 min at 37° | 1.20 | 9.20 | 0.78 | 5.79 | 49.3 | 94 | 1.92 | 86 |
| d. Hb poor, incubated a further 30 min at 37° | 4.60 | 6.90 | 1.52 | 2.29 | 93.3 | 10 | 0.11 | 5 |

[a] Samples treated essentially as described in Table VII. Assays for $Ca^{2+}$-stimulated ATPase, phospholipid P, and hemoglobin are described in the text; subject, MH; PCE, packed cell equivalent; MCH, mean corpuscular hemoglobin. [b] Obtained by hypotonic hemolysis. [c] Present in supernatant.

branes with a major amount of the hemoglobin removed and with certain characteristics, *e.g.*, impermeability to extracellular ATP, of the original erythrocyte membrane retained.

Perhaps the most striking observation was that the $Ca^{2+}$ ATPase accessibility developed only subsequent to release of at least 90% of the cells' hemoglobin. The results of another similar study are presented in Table VIII. In these experiments, the protocol was quite similar to that described above in which hemoglobin-rich and hemoglobin-depleted cells from a 37 or 44° incubation in isotonic Tris, pH 7.6, were separated and then assayed. These data are expressed on a phospholipid P as well as packed cell equivalent basis and show in particular that there is a loss of $Ca^{2+}$-stimulated ATPase from the original cell during this procedure and that the ATPase levels (or specific activity) of the hemoglobin-depleted and hemoglobin-rich cells are decidedly different, in both the 37 and 44° incubation. This is a repeatable observation and may reflect in part a difference in the levels of ATPase activities of cells of varying ages.

## Discussion

The results of this investigation confirm and extend an earlier communication from this laboratory (Hanahan and Ekholm, 1972) and from that of Bramley *et al.* (1971) that membranes prepared by hypotonic lysis of mammalian erythrocytes have an altered structure and behavior as compared to the intact cell. This is particularly well illustrated by examination of ATPase activities, specifically the $Na^+$–$K^+$–$Mg^{2+}$ and $Ca^{2+}$ stimulated types, in intact erythrocytes and membranes. As judged by the hydrolysis of added ATP (extracellular ATP) these two enzyme systems are unavailable in the intact erythrocytes and can be expressed only by disruption of the cell by techniques such as freeze–thaw and, to a limited extent, sonication. Yet, hemoglobin-free (wash IV, pH 7.6) membranes derived from intact erythrocytes allow easy access of added ATP to these ATPase systems. The nature of the low osmolarity hemolyzing buffer, whether bicarbonate, Hepes, or Tris, for example, had no effect on the changes noted in the membranes during preparation at pH 7.6. Zwaal *et al.* (1971) and Laster *et al.* (1972) have reported on the pronounced differences in the reactivity of phospholipase C toward the intact erythrocyte as compared to the membrane (ghost) obtained by hypotonic hemolysis. No hydrolysis of the phospholipids of the intact erythrocyte by this enzyme was noted, yet over 70% of the membrane (ghost) phospholipids was hydrolyzed under similar conditions. Many other reports support the basic premise that the hemoglobin-depleted or hemoglobin-free membrane and the intact erythrocyte membrane are substantially different in their biochemical characteristics (Woodward and Zwall, 1972; Triplett and Carraway, 1972; Steck *et al.*, 1971; Lepke and Passow, 1972; Bodemann and Passow, 1972; Duchow and Collier, 1971; Theodore and Robin, 1965). These observations coupled with those reported by Bramley *et al.* (1971) and Hanahan and Ekholm (1972) and in the present report dramatically and convincingly emphasize the point that (hemoglobin-free) membranes obtained through hypotonic hemolysis of intact erythrocytes have been altered in the process and must not be considered as representative of the intact erythrocyte membrane.

The increased accessibility of ATPase sites during hemolysis

is not a phenomenon immediately observed on mixing the intact erythrocyte in the (first) hemolyzing buffer, since the wash I membranes, which contain a significant level of hemoglobin, have low ATPase activities and are considered sealed. Only through subsequent washes with hypotonic buffer solution at pH 7.6, during which the remaining hemoglobin is removed, do the membranes develop an apparent permeability to added ATP. However, preparation of the membranes at pH 5.8 in low osmolarity buffer under wash and volume conditions comparable to those used at pH 7.6 yields a membrane with low (NF) ATPase activity to added ATP and still containing a significant level of hemoglobin.[5] The accessibility to ATPase sites in the pH 5.8 membranes can be expressed by freeze–thaw treatment or by washing the membranes with 20 imosm buffer, pH 7.6. Under these latter conditions, only a part of the hemoglobin is lost. In is of import to note, though, that these latter membranes on rewashing with pH 5.8 hypotonic buffer can undero partial or total reseáling as indicated by a decrease in ATPase activities. Membranes prepared at pH 7.6 in low osmolarity buffer and exhibiting high $Na^+$–$K^+$–$Mg^{2+}$ and $Ca^{2+}$ ATPase activities can be resealed partially by washing with 20 imosm Tris-maleate buffer at pH 5.8. An even more effective resealing could often be achieved by washing the pH 7.6 membranes with 20 imosm Na maleate buffer, pH 5.8. The ability of wash IV 20, pH 5.8, membranes (TM or NaM) to go from a condition of latent ATPase activity to one of overt expression by washing with 20 imosm buffer, pH 7.6, and then back to a condition of latency on washing with 20 imosm, pH 5.8, buffer is suggestive of a pH effect. Such a reversible pH phenomena is most clearly seen with the pH 7.6 membranes, where ATPase activity is lost on washing with pH 5.8 20 imosm buffer. A further point of interest is the observation that this phenomenon does not occur as completely if $Na^+$ is absent both in the initial pH 5.8 buffer and the readjustment pH 5.8 buffer.

In addition to the $Na^+$–$K^+$–$Mg^{2+}$ ATPase system, another excellent indicator of erythrocyte membrane behavior was the $Ca^+$ stimulated ATPase. This enzymatic activity was inaccessible to added ATP in the human (intact) erythrocyte but was readily available in the hemoglobin-free membranes prepared at pH 7.6 in 20 imosm buffer. In those instances where membranes were apparently resealed as judged by lack of $Na^+$–$K^+$–$Mg^{2+}$ ATPase activity toward added ATP, the $Ca^{2+}$-stimulated ATPase activity was low. Contrariwise, in those instances where there was high $Na^+$–$K^+$–$Mg^{2+}$ ATPase activity toward added ATP, the $Ca^{2+}$-stimulated ATPase activity of the hemoglobin-free membrane was high. Of further interest, sufficient evidence has now accumulated to support the conclusion that the $Ca^{2+}$-stimulated ATPase is different, either in location or in structure, than the $Na^+$–$K^+$–$Mg^{2+}$ ATPase. The finding of higher $Ca^{2+}$ ATPase activity in pH 5.8 (low osmolarity) prepared membranes than in the pH 7.6 (low osmolarity) membranes, and that washing with 20 $T_{7.6}$ buffer reduces the NF value of $Ca^{2+}$ ATPase in wash IV 20 $TM_{5.8}$ or IV 20 $NaM_{5.8}$ membranes while not similarly affecting the $Na^+$–$K^+$–$Mg^{2+}$ ATPase, are added support for differences in these two ATPase systems.

A study of the influence of $Ca^{2+}$ (and $Mg^{2+}$) on the stability and activity of the erythrocyte membrane, as reflected by AcChase and ATPase activities, provided an important insight into membrane behavior. These two enzyme systems are particularly good choices, since AcChase is considered a surface or "outside" enzyme, and the $Na^+$–$K^+$–$Mg^{2+}$ and $Ca^{2+}$ ATPases are considered distributed through the membrane and act as a double-sided system, exposed to the inside as well as outside of the membrane. In an earlier study, Burger *et al.* (1968) reported that divalent cations added during hemolysis were particularly effective in preventing fragmentation (and concomitant "release" of AcChase) of the membrane of the cow erythrocyte. Fujii *et al.* (1971) has noted a similar fragmentation with pig and cow erythrocytes but not with human erythrocyte. In the case of the AcChase it was very evident that these divalent cations had little or no influence on the level of this enzyme in the human erythrocyte membrane. Consistently good recoveries of AcChase were noted in membranes prepared at pH 7.6 and 5.8, in the absence of any $Ca^{2+}$ added during hemolysis. On the other hand, it was evident that $Ca^{2+}$ could exert a significant effect on the availability of the ATPase in membranes prepared in hypotonic hemolysis at pH 7.6. Depending on the wash at which $Ca^{2+}$ was added during the hemolysis procedure, a significant reduction in the accessibility to ATPase activities was noted. At pH 7.6, $Mg^{2+}$ exerted little effect when added in the hemolyzing washes, but at pH 5.8 it had a very positive action, similar to $Ca^{2+}$ in reducing even further the availability of any ATPases to added ATP. However, high ATPase activity could be expressed through freeze–thaw treatment of these membranes. The possibility that a protein factor, similar to the "nectin" described by Baron and Abrams (1971) as important to the retention of ATPase activity in the membranes of *S. faecalis*, has not been overlooked in this study. Our limited efforts in this direction (Dr. M. Luthra, personal communication) have been provocative but definitive proof for existence of such a factor in the human erythrocyte membrane is lacking at this time. Though little insight into the mechanism by which these divalent cations exert their effect can be offered here, it is evident that divalent cations must be considered as another factor of potential contributory import to the behavior and structure of the membrane.

It is apparent from the data presented in this investigation that acetylcholine esterase is not affected by various treatments or conditions which dramatically affect the ATPase activities, at least in the human erythrocyte membrane. Thus, the acetylcholine esterase system certainly reflects one portion of the erythrocyte membrane behavior but by no means does it reflect alterations or changes in the behavior of other enzyme systems, such as the ATPase, in the same membrane.

The rapid hemolysis of human erythrocyte on incubation at 37 or 44° in isotonic Tris buffer, pH 7.6, was unexpected, but could have been predicted from the report of Omachi *et al.* (1961). These investigators reported that isotonic Tris (as well as other amine) buffers penetrated the human erythrocyte at room temperature and led to a significant increase in cell volume but not hemolysis in 60–90 min. This rise in cell volume was pH dependent, with the undissociated species of Tris considered to be the penetrant. Interestingly, on osmotic reversal with hypertonic saline, cells swollen in Tris buffer reverted to near their original volume. Thus, according to these authors, Tris unlike the saponins did not appear to damage the membranes *per se*. Of interest, though, Omachi *et al.* (1961) noted that Tris-treated cells allowed to stand overnight did exhibit some hemolysis. This same phenomenon has been observed in this laboratory. Thus, the

[5] It is of course entirely possible that the pH 5.8 membranes have opened and resealed during preparation, or that these membranes are actually permeable to ATP, for example, but the ATPase system may be in an inhibited state under these conditions of preparation. These possibilities are under active study.

elevated temperatures used here, *i.e.*, 37 and 44°, only accelerated the process of hemolysis, though the exact mechanism is still unclear. Omachi *et al.* (1961) proposed that Tris changes the cell pH and probably affects sodium transport. Obviously the mechanism of the Tris effect is complex and must have pH and concentration as two important components in this process. Another route to hemolysis of the cells was through use of isotonic glucose solutions. Even though the results obtained on incubation of erythrocytes in isotonic glucose at 37 and 44° were not as dramatic as with isotonic Tris, this approach does have some merit. It allows one to prepare membranes with very low concentrations of hemoglobin, but with no overt $Na^+$–$K^+$–$Mg^{2+}$ and $Ca^{2+}$ ATPase activities prior to freeze–thaw treatment. Yet it was obvious that the cell membranes from these incubations had reduced ATPase activities even after freeze–thaw exposure. Whether this is an inhibitory action of the glucose on the membrane ATPase remains to be elucidated.

On the basis of the evidence presented in this article, it is possible to prepare membranes of varying characteristics, through the use of hypotonic and isotonic hemolyses, respectively, in different media and through variations in conditions of the pH and temperature. This approach should be helpful in evaluating the relationship of hemoglobin level to cell membrane behavior, for example, and in evaluating the importance of certain cellular components to the stability of membranes. The question of the importance of hemoglobin to the membrane is still a moot, though important, one. Thus, whether it is intimately associated with the membrane as a structural component or whether it is simply a good indicator of the state of the membrane at any one time remains to be established. Another important facet of any study on the mammalian erythrocyte demands recognition of the population of age groups present in a typical cell sample. There is sufficient evidence now available to suggest a decided difference in the biochemical behavior and stability of various age groups of cells and to warrant an exploration of the separation of cells based on a difference in density and a detailed examination of the characteristics of the separated cells.

The field of membrane biochemistry has exhibited explosive growth over the past several years with a considerable attention directed toward use of the mammalian erythrocyte, usually the human or cow, as a model system. Membranes were prepared from the cells under conditions of hypotonic hemolysis in which complete removal of the hemoglobin was the major criterion for purity. In many instances, these hemoglobin-free membranes, without any particular characterization, were used in a series of quite sophisticated experiments, some of which led to provocative and "definitive" models of membrane structure. Notwithstanding the importance of a variety of approaches to membrane structure proof, it is evident from the findings presented here that hemoglobin-free erythrocyte membranes possess certain characteristics quite different than those found in the membrane associated with the parent cells. These differences are not minor and must represent a considerable change in the conformation and configuration of the membrane during isolation. The mammalian erythrocyte is still an excellent cell type for membrane studies and its structure should be forthcoming in due time if one is cognizant of the alterations that can occur in preparations of its membranes and if steps are taken to minimize these changes.

## References


Baron, C., and Abrams, A. (1971), *J. Biol. Chem. 246*, 1542.

Bodemann, H., and Passow, H. (1972), *J. Membrane Biol. 8*, 1.

Bramley, T. A., Coleman, R., and Finean, J. B. (1971), *Biochim. Biophys. Acta 241*, 752.

Brewer, G. J., Eaton, J. W., Beck, C. C., Feither, L., and Schreffler, D. C. (1968), *J. Lab. Clin. Wed. 71*, 744.

Burger, S. P., Fujii, T., and Hanahan, D. J. (1968), *Biochemistry 7*, 3682.

Dodge, J. T., Mitchell, C., and Hanahan, D. J. (1963), *Arch. Biochem. Biophys. 100*, 119.

Duchow, G., and Collier, H. B. (1971), *J. Membrane Biol. 6*, 138.

Fujii, T., Komatsu, Y., and Murofushi, M. (1971), *Chem. Pharm. Bull. 19*, 2325.

Garrahan, P. J., and Glynn, I. M. (1967), *J. Physiol. (London) 192*, 189.

Hanahan, D. J., and Ekholm, J. (1972), *Biochim. Biophys. Acta 255*, 413.

Heller, M., and Hanahan, D. J. (1972), *Biochim. Biophys. Acta 255*, 251.

Hoffman, J. F. (1962), *J. Gen. Physiol. 45*, 837.

Kachmar, J. F. (1970), *in* Fundamentals of Clinical Chemistry, Tietz, N., Ed., Philadelphia, Pa., W. B. Saunders, pp 268–269.

Laster, Y., Sabban, E., and Loyter, A. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 20*, 307.

Lepke, S., and Passow, H. (1972), *Biochim. Biophys. Acta 255*, 696.

Mitchell, C. D., Mitchell, W. B., and Hanahan, D. J. (1965), *Biochim. Biophys. Acta 104*, 348.

Neville, D. M. (1971), *J. Biol. Chem. 246*, 6328.

Omachi, A., Macey, R. I., and Waldeck, J. G. (1961), *Ann. N. Y. Acad. Sci. 92*, 478.

Steck, T. L., Fairbanks, G., and Wallach, D. F. H. (1971), *Biochemistry 10*, 2617.

Theodore, J., and Robin, E. D. (1965), *Clin. Res. 13*, 283.

Triplett, R. B., and Carraway, K. L. (1972), *Biochemistry 11*, 2897.

Weed, R. I., Reed, C. F., and Berg, G. (1963), *J. Clin. Invest. 42*, 581.

Woodward, C. B., and Zwaal, R. F. A. (1972), *Biochim. Biophys. Acta 274*, 272.

Zwaal, R. F. A., Roelofsen, B., Confurios, P., and van Deenen, L. L. M. (1971), *Biochim. Biophys. Acta 233*, 474.