J. Membrane Biol. 6, 138–157 (1971)

Enzyme Activities of Human Erythrocyte Ghosts: Effects of Various Treatments

GUSZTAV DUCHON* and H. BRUCE COLLIER

Departments of Biochemistry and Pathology, University of Alberta, Edmonton 7, Alberta, Canada

Received 4 May 1971

Summary. Ghosts of human erythrocytes prepared by hypotonic hemolysis were assayed for aldolase, triosephosphate isomerase, glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, pyruvate kinase, lactate dehydrogenase, and glutathione peroxidase and reductase. Cryptic activity of the enzymes was demonstrated by an increase in activity on dilution with water, which caused fragmentation of the ghosts. Aldolase and glyceraldehyde phosphate dehydrogenase were classed as firmly bound; phosphoglycerate kinase was intermediate; the others were loosely bound. Triton X-100 increased the activities of aldolase, glyceraldehyde phosphate dehydrogenase, and phosphoglycerate kinase. The pH of the medium had little effect upon the firmly bound enzymes but it markedly affected the retention of hemoglobin and the activities of the loosely bound enzymes. The presence of Mg or Ca ions enhanced the retention of hemoglobin and the activity of lactate dehydrogenase and pyruvate kinase, with little effect on aldolase and glyceraldehyde phosphate dehydrogenase. Ghosts diluted in water disintegrated into fragments and tubules or vesicles; Mg or Ca at 1 mM afforded protection against this. When ghosts were treated with Triton X-100 and adenosine triphosphate, they contracted to about one-seventh of their volume. The shrunken ghosts had lost a considerable proportion of their cholesterol and protein to the medium.

Great interest has been shown in recent years in cell membrane transport phenomena and membrane structure. Membranes of mammalian erythrocytes (stromata or "ghosts") have been widely used in these investigations, because of the ease of isolation from washed cells. The composition and properties of ghosts may vary enormously, however, according to the method of preparation and subsequent treatment. For example, the hemoglobin content of human ghosts has been variously reported as from 54% of the dry weight (Anderson & Turner, 1960) to 0.2% (Dodge, Mitchell & Hanahan, 1963).

* Present address: Dept. of Pharmacology, University of Alberta.

Bartlett (1958) postulated the presence of three glycolytic enzymes, GAPD[1], LDH, and PGK, in erythrocyte membranes. Schrier (1963, 1966, 1967) demonstrated GAPD and PGK activity. The glycolytic enzymes has been previously thought of as primarily cytoplasmic, but Green, Murer, Hultin, Richardson, Salmon, Brierley and Baum (1965) claimed that the complete glycolytic sequence was operative in membranes from beef erythrocytes. However, modification of the methods used in preparation markedly altered the pattern of activities retained; furthermore, the glycolytic complex could be resolved and reassembled by a cycle of pH changes. Baum, Murer, Slautterback and McConnell (1966), in a continuation of these investigations, concluded that "... the glycolytic complex of enzymes, together possibly with the hemoglobin, may be attached to the interior surface of the erythrocyte stroma as 'headpiece' subunits."

Mitchell, Mitchell and Hanahan (1965) demonstrated that the retention of Hb, ALD, and GAPD by human ghosts was dependent upon pH and ionic strength. These parameters had no effect upon the AChE, which has been shown by many workers to be completely incorporated in the membrane. Lionetti and Fortier (1966) found that ghosts could metabolize deoxyinosine. Parker and Hoffman (1967) found that ghosts possessed both GAPD and PGK activity. Ronquist (1967, 1969) and Nilsson and Ronquist (1969) reported GAPD, PGK, and adenylate kinase activities of the cell membrane; GAPD was firmly bound. Rao, Hara and Askari (1968) described a membrane-bound AMP deaminase. Wins and Schoffeniels (1969) demonstrated a number of enzyme activities in pig erythrocyte stromata, of varying degrees of firmness of attachment; most of them could be solubilized by TX-100. Zamudio, Cellino and Canessa-Fischer (1969) investigated the Na^+, K^+-ATPase and NADH:(acceptor) oxidoreductase activities of human ghosts. The oxidoreductase activity of sonicated fragments was much greater than that of intact ghosts, which led them to the concept of the "crypticity" of such enzymes. Maddy (1970) has recently reviewed the structure and composition of erythrocyte membranes, with emphasis on the proteins.

In the present investigation we describe certain enzyme activities of human erythrocyte ghosts prepared by hypotonic hemolysis. The ionic

1 The following abbreviations are used: AChE, acetylcholinesterase; ALD, aldolase; EDTA, ethylenediamine tetraacetate; GAPD, glyceraldehyde phosphate dehydrogenase; GSH-P, glutathione peroxidase; GSSG-R, glutathione reductase; Hb, hemoglobin; I.U., International Unit; LDH, lactate dehydrogenase; MES, 2-(N-morpholino)ethanesulfonic acid; mOsm, milliosmoles/kg water; MTT, mixture of MES, TES, and TRICINE; PGK, phosphoglycerate kinase; PK, pyruvate kinase; TES, N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; TPI, triosephosphate isomerase; TRICINE, N-tris(hydroxymethyl)methylglycine; TX-100, Triton X-100.

strength, pH, and the presence of Ca or Mg ions have marked effects upon the activity of some of the enzymes, which we have classified as "firmly bound" or "loosely bound" (Collier & Duchon, 1968).

Materials and Methods

The crystalline enzymes used in the coupled assay systems were obtained from Boehringer Mannheim Corp. (New York). Coenzymes and substrates were obtained from Boehringer or Calbiochem (Los Angeles). Triton X-100, a nonionic detergent, was the product of Rohm and Haas (Philadelphia). Other chemicals were of reagent grade. Deionized water was used throughout.

Osmolality Measurements. True osmolalities of the buffers were measured with an Advanced Instruments Osmometer, model 31 L, and were expressed in mOsm (milliosmoles/kg water).

Preparation of Ghosts. Ghosts were prepared from washed human erythrocytes by a modification of the method of Dodge *et al.* (1963) and Weed, Reed and Berg (1963), based upon the gradual osmotic lysis procedure introduced by Danon, Nevo and Marikovsky (1956). The hemolyzing and washing buffers always contained 1 mM Na_2-EDTA, unless otherwise stated.

The washed erythrocytes were made up to a 25% hematocrit and a cell count was done in a Coulter Counter, model B. Volumes of four ml each were hemolyzed in 36 ml of hypotonic buffer, the ionic strength, pH, and composition of which varied with each experiment. The lysates were then centrifuged in a high-speed centrifuge at $9{,}000 \times g$ for 20 min and the ghosts washed three times in the buffer. The washed ghosts were then made up to a known volume in buffer and counted in the Coulter counter. This is referred to as the standard suspension, which was usually adjusted to contain about 5×10^9 ghosts/ml. The ghosts had a mean volume of about 60 μ^3, in the Counter, and were largely intact discs, as viewed by phase-contrast optics in an isotonic medium.

Ghost suspensions were kept refrigerated and used within 1 to 3 days after preparation, since fragmentation set in after that time. Microscopic examination and cultures failed to demonstrate any bacterial contamination within this period.

Chemical Analysis of Ghosts. For the determination of Hb, the ghosts were solubilized in 1% sodium lauryl sulfate and Hb was read as cyanmethemoglobin at 540 nm. Total protein of the solution in the detergent was determined by the biuret method of Reinhold (1953). Ghost cholesterol was determined by the Liebermann-Burchard method of Sackett (1925).

Assay of Ghost Enzyme Activities

Unless otherwise indicated, all the activities were assayed spectrophotometrically at 28 °C in 10.0 mm cuvettes in a Beckman model DU spectrophotometer coupled to a Gilford model 200 Automatic Cuvette Positioner, a Gilford model 220 Optical Density Converter, and a Sargent model MR Recorder. Conditions were chosen such that the enzyme to be assayed was rate-limiting; in most cases the recorded absorbance changes were linear with time and with ghost concentration.

In the assay of the NADH-coupled enzymes at 340 nm, it was demonstrated that known additions of NADH to a ghost suspension strictly obeyed Beer's Law and that

NADH could be recovered quantitatively. Appropriate blanks were set up for all assays. Enzyme activities are expressed as I. U./ml ghost suspension.

Aldolase (ALD) (ketose 1-phosphate aldehyde lyase, E.C. 4.1.2.7) was assayed by the method of Schrier (1963) or of Wu and Racker (1959). Both are NADH-coupled assays.

Glutathione peroxidase (GSH-P) (Glutathione:H_2O_2 oxidoreductase, E.C. 1.11.1.a) was assayed as outlined by Paglia and Valentine (1967). The GSH concentration was kept constant by addition of NADPH and GSSG-R and the disappearance of NADPH was followed at 340 nm.

Glutathione reductase (GSSG-R) (NADPH:glutathione oxidoreductase, E.C. 1.6.4.2) was followed by determining the rate of liberation of GSH with Ellman's reagent (Collier & Duchon, 1963, *unpublished*). A similar method has since been described by Icén (1967).

Glyceraldehyde 3-phosphate dehydrogenase (GAPD) (D-glyceraldehyde 3-phosphate:NAD oxidoreductase, phosphorylating, E.C. 1.2.1.12) was assayed by combining the methods of Wu and Racker (1959) and Schrier (1963). Since the reaction rates fell off markedly after 1 to 2 min, the initial velocities were determined between 15 and 60 sec from the start of the reaction.

Lactate dehydrogenase (LDH) (L-lactate:NAD oxidoreductase, E.C. 1.1.1.27) was assayed by the method of Bergmeyer, Bernt and Hess (1965).

3-Phosphoglycerate kinase (PGK) (ATP:D-3-phosphoglycerate 1-phosphotransferase, E.C. 2.7.2.3.) was assayed by the NAD-coupled "backward reaction" as described by Schrier (1963).

Pyruvate kinase (PK) (ATP:pyruvate phosphotransferase, E.C. 2.7.1.40) was assayed by the NAD-coupled method of Collier, Ashford and Bell (1966).

Triose phosphate isomerase (TPI) (D-glyceraldehyde-3-phosphate ketol-isomerase, E.C. 5.3.1.1) was assayed by the method of Chapman, Hennessey, Waltersdorph, Huennekens and Gabrio (1962), an NAD-coupled assay.

Each experiment was carried out in duplicate or triplicate on the *same* batch of ghosts, since enzyme activities varied somewhat from preparation to preparation. The replicate experiments gave similar results, and a typical result is reported in each case.

Results

Effects of Ionic Strength

Aliquots of washed erythrocytes were hemolyzed, and the ghosts were subsequently washed in Tris buffers, pH 7.4, of 5 to 80 mOsm and in water. Each batch of ghosts was then made up to the original volume with the buffer used for the hemolysis, and these suspensions were tested for various enzyme activities in two ways: (1) aliquots were further diluted sixfold in the same buffer, or (2) aliquots were diluted sixfold with water.

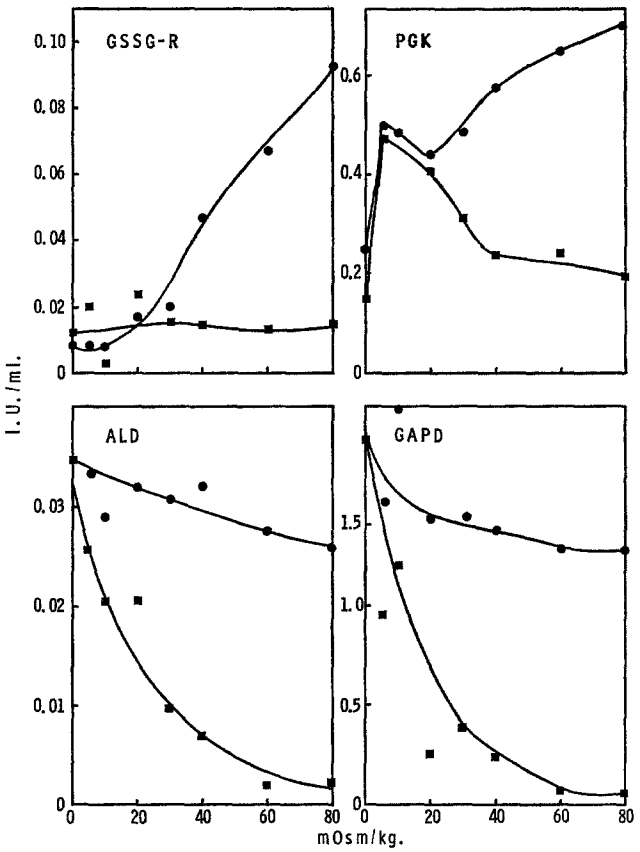

Fig. 1. The effect of ionic strength on enzyme activities of ghost suspensions prepared and washed in Tris buffers, pH 7.4. Aliquots of the suspensions were diluted sixfold in the same buffer (▪—▪) or in water (•—•) before assaying the enzyme activities

Typical results are illustrated in Figs. 1 and 2. The ionic strength of the medium markedly affected the activity of LDH, TPI, PK, GSH-P, GSSG-R, PGK, ALD, and GAPD in the ghost suspensions. In each case, dilution with water increased the measured activity as compared with that in the buffer. The activity on final suspension in buffer will be referred to as the "basic activity", and that in water as the "cryptic activity" (Zamudio *et al.*, 1969).

On the basis of these activity profiles, the eight enzymes may be divided into two groups. Group I, which includes LDH, TPI, PK, GSH-P, and GSSG-R, show very low activity in the low ionic strength buffers and much higher activity at 80 mOsm. Group II, ALD and GAPD, have maximum activity at low ionic strength, especially the basic activity. PGK is inter-

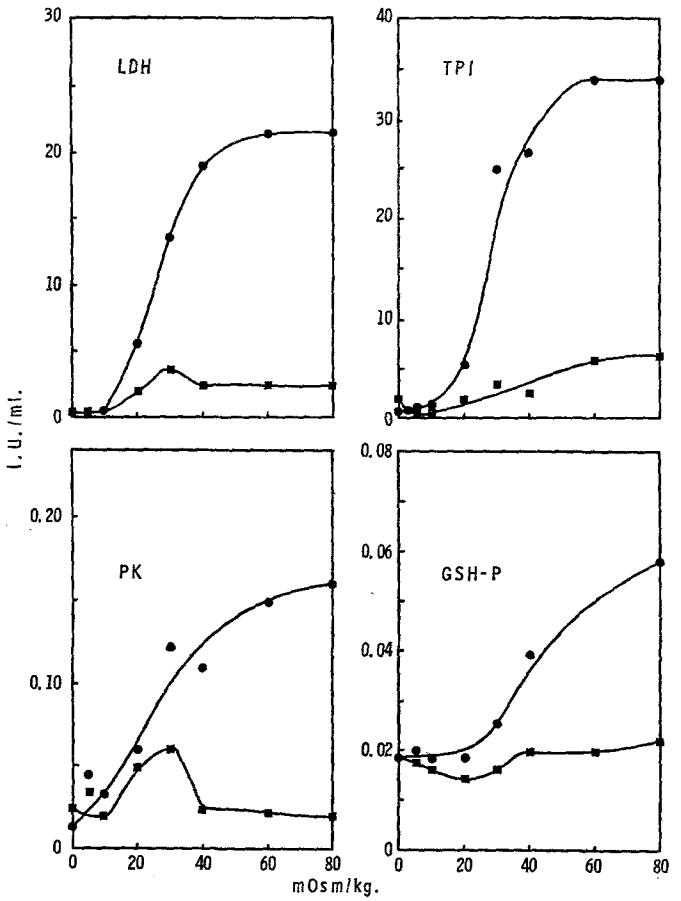

Fig. 2. The effect of ionic strength on enzyme activities of ghost suspensions prepared and washed in Tris buffers, pH 7.4. Aliquots of the suspensions were diluted sixfold in the same buffer (▪—▪) or in water (•—•) before assaying the enzyme activities

mediate. The cryptic activity profiles for the enzymes in group I resemble the curves of Hb retention obtained by Dodge *et al.* (1963).

Examination of the ghosts under phase-contrast optics revealed marked structural differences. At 30 to 80 mOsm the ghosts were intact but contained varying amounts of Hb, as described by Dodge *et al.* (1963). At lower ionic strength, fragmentation took place until, at 10 mOsm or lower, intact ghosts were scarce. This fragmentation was further confirmed by measurements of particle-size distribution in the Coulter Counter.

At intermediate osmolalities, several of the enzymes show marked maxima of activity that we cannot explain. It has been suggested that release of an inhibitor may account for these maxima. The cryptic activity

Table 1. *Effect of osmolality of the wash medium on the distribution of enzyme activities between ghosts and supernatant*

Enzyme	Activity (I.U./ml)							
	(I) Control ghosts [a]		(II) Washed in buffer [b]			(III) Washed in water [c]		
	in buffer	in H_2O	super-natant	in buffer	in H_2O	super-natant	in buffer	in H_2O
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
GSSG-R	0.080	1.15	0.03	0.05	1.19	0.85	0.11	0.23
GSH-P	0.009	0.059	0.012	0.003	0.042	0.056	0.002	0.001
TPI	3.63	11.6	0.63	0.92	10.5	11.9	0.15	0.15
PK	0.004	0.027	0.008	0.002	0.020	0.026	0.002	0.003
LDH	0.31	1.94	0.20	0.16	1.83	1.78	0.04	0.05
PGK	0.10	0.54	0.03	0.09	0.49	0.26	0.28	0.30
GAPD	0.14	0.40	0.02	0.12	0.34	0.03	0.28	0.32
ALD	0.035	0.075	0.00	0.030	0.075	0.003	0.051	0.073

All of the ghost suspensions below were initially aliquots of equal volume, and they were made up to the same final volume for the enzyme assays.

[a] In (I), a stock suspension of ghosts prepared in 80 mOsm buffer, pH 7.4, was diluted with six volumes of the same buffer or of water, and the enzyme activities of the final suspensions were determined.

[b] In (II), ghosts prepared as above were diluted 10-fold with 80 mOsm buffer, then centrifuged down for 30 min at 10,000 × *g*. The supernatant was kept and assayed for enzyme activities. The ghosts were resuspended in either the same buffer or water, and the enzyme activities of the suspensions were determined.

[c] In (III), an aliquot of the stock ghost suspension was diluted 10-fold in water and centrifuged. The supernatant was removed and assayed for enzyme activities. The ghosts were resuspended in either the same buffer or water, and the enzyme activities of the suspensions were determined.

can be explained by the fragmentation at low ionic strength, whereby the ghosts lose their semi-permeability. The enzymes associated with the *inside* then become accessible to substrates and cofactors, or may partially go into solution.

In another type of experiment, a ghost suspension was prepared in 80 mOsm Tris, pH 7.4, and aliquots were treated in one of three ways: (I) an aliquot without further treatment served as a control; (II) an aliquot was diluted 10-fold with the same buffer, then centrifuged at 10,000 × *g* for 30 min; (III) an aliquot was diluted 10-fold in water and centrifuged as in (II). The ghosts from these three treatments were finally suspended in either (a) six volumes of the buffer or (b) six volumes of water. The eight enzyme activities were then determined on the various ghost suspensions

and on the supernatants from (II) and (III). Results of a typical experiment are summarized in Table 1.

It is seen that the control ghosts and those washed in buffer exhibited cryptic activity for each enzyme; i.e., there was a marked increase on dilution with water (column 3 vs. 2 and column 6 vs. 5). The ghosts washed in buffer lost some activity to the supernatant (column 4) except for ALD. On washing in water, there was little further increase in activity on final dilution in water (column 9 vs. 8), as might be expected, except for GSSG-R. On examination of the values in column 7, it is seen that one group of enzymes, GSSG-R, GSH-P, TPI, PK, and LDH, liberated most of the activity into the supernatant; these will be referred to as "loosely bound" enzymes. The second group, ALD and GAPD, liberated very little activity and will be described as firmly bound enzymes. PGK is again intermediate in behavior, having liberated about one-half of its activity.

It may also be noted that the *total* activities in each of treatments (I), (II), and (III) remain constant within the limits of error of the assays; i.e., in each case the values in column 3, columns 4 and 6, and columns 7 and 9 are comparable.

The Effect of TX-100

To obtain further information on the firmly bound enzymes, disintegration of the membranes was achieved in the presence of TX-100, a nonionic detergent that is known to dissociate lipoprotein complexes (Mazia & Ruby, 1968). At a concentration of 0.05% (v/v), TX-100 decreased the turbidity

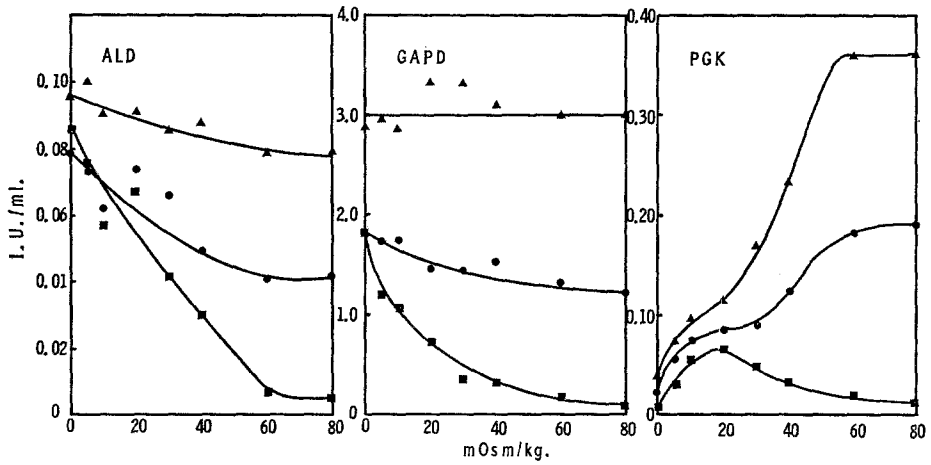

Fig. 3. The effect of ionic strength on some enzyme activities of ghost suspensions prepared and washed in Tris buffers, pH 7.4. Aliquots of the suspensions were diluted sixfold in the same buffer (▪—▪), in water (•—•), or in 0.05% TX-100 (▲—▲)

of a ghost suspension at 660 nm from an absorbance of 0.3 to virtually zero (*see* Miller, 1970). At this concentration, TX-100 had no inactivating effect upon any of the enzymes.

Fig. 3 illustrates the results of experiments in which ghosts were prepared in pH 7.4 Tris buffers of varying osmolalities .The suspensions were then diluted sixfold in one of the following: the corresponding buffer, water, or water containing 0.05% TX-100. In the case of each enzyme tested, TX-100 treatment resulted in further activity beyond that of water treatment alone. The scatter of some of the points is attributed to experimental error.

The Effects of pH

For this study we used a MTT buffer mixture which has a fairly wide range of buffering capacity. This is an equimolar mixture of three of Good's buffers, MES (pK 6.15), TES (pK 7.50), and TRICINE (pK 8.15). Ghosts were prepared in buffers at pH values from 5.5 to 8.5 and at ionic strengths of 5 and 30 mOsm (to test both fragmented and intact ghosts). The enzymes were assayed, however, at the optimum pH for the method in question, after addition of TX-100 to 0.05%. Control experiments showed that the lowest and highest pH values had no detrimental effect upon the enzymes.

Results summarizing the retention of Hb and the activities of six enzymes are illustrated in Figs. 4 and 5. The curves for Hb are similar to those of Mitchell *et al.* (1965), with maximum retention at about pH 6.5. At this pH

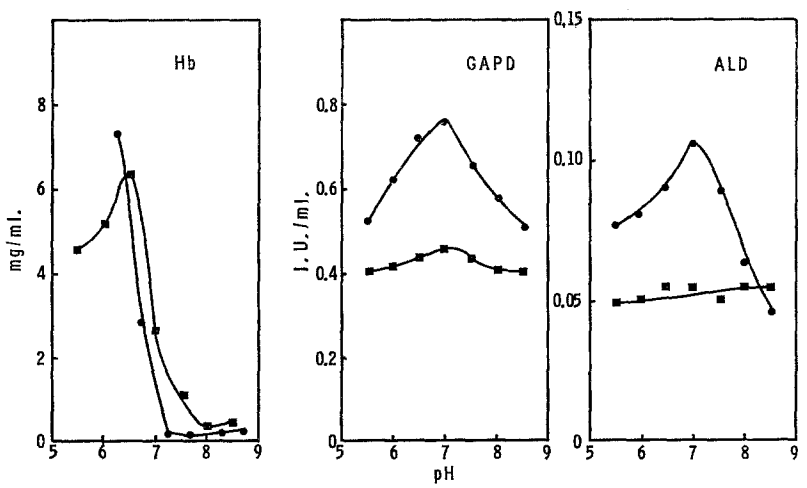

Fig. 4. The effect of pH on the retention of hemoglobin and the activities of GAPD and LDH in ghost suspensions prepared and washed in either 5 mOsm (▪—▪) or 30 mOsm (•—•) MTT buffers. Enzyme activities were assayed after adjustment to the optimum pH for the enzyme, and addition of TX-100 to 0.05%

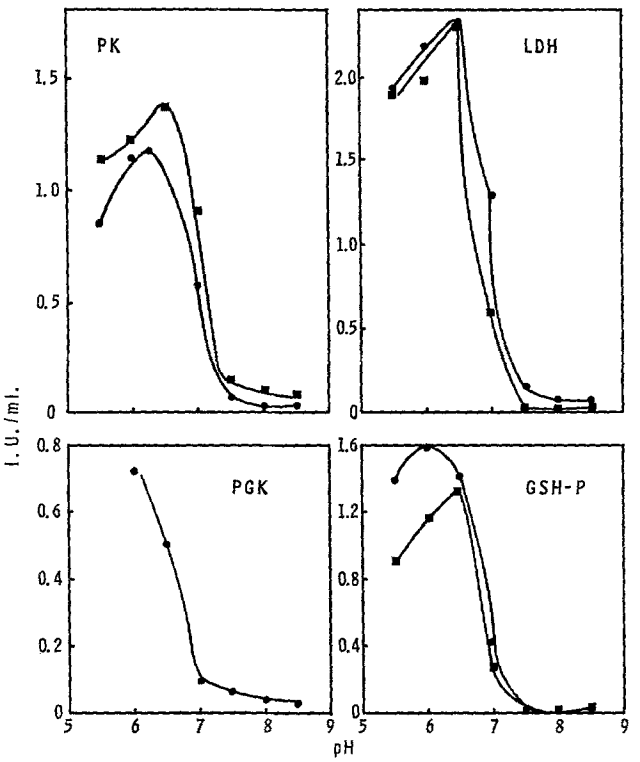

Fig. 5. The effect of pH on the activities of PK, LDH, GSH-P, and PGK in ghost suspensions prepared and washed in either 5 mOsm (▪—▪) or 30 mOsm (•—•) MTT buffers. Enzyme activities were assayed after adjustment to the optimum pH for the enzyme, and addition of TX-100 to 0.05%

the ghosts retained about 4% of the original erythrocyte Hb; at pH 7.5 the retention was 0.05% (5 mOsm buffer). The effect of pH upon the two firmly bound enzymes, ALD and GAPD, was not marked, and in the 5 mOsm buffer there was almost no effect. These results resemble those of Mitchell *et al.* (1965). In contrast, three loosely bound enzymes, PK, LDH, and GSH-P, are markedly susceptible to the effect of pH; PGK must be included here. The osmolality had little effect upon the retention of these.

To test if the soluble enzymes could recombine with ghost fragments, the latter were prepared in 5 mOsm MTT buffer of pH 7.5, and the ghost-free supernatant of the first hemolysis was saved for later use. The ghosts were then divided into three parts. Aliquot *A* was washed three times in the pH 7.5 buffer. Aliquot *B* was washed once with the hemolysate then twice with buffer, pH 7.5. Aliquot *C* was washed once in the hemolysate, then adjusted to pH 6.5, and this was followed by two more washings in pH 6.5 buffer.

Table 2. *The effect of changes in pH upon binding of PK and LDH by ghost fragments*

	I.U./ml of ghost suspension		
	A. Ghosts prepared and washed at pH 7.5 [a]	*B.* Ghosts washed with lysate at pH 7.5 [b]	*C.* Ghosts plus lysate at pH 7.5, then washed at pH 6.5 [c]
PK	0.110	0.113	0.40
LDH	0.310	0.320	0.83

All suspensions were made up to the original volume for assay of enzyme activities. The assays were performed at the optimum pH values for PK and for LDH.

[a] Ghost fragments were prepared in 5 mOsm MTT, pH 7.5, and washed three times with the same buffer.

[b] Ghost fragments were prepared as above, washed once with the hemolysate and twice with the pH 7.5 buffer.

[c] Ghost fragments were prepared as above, washed once with the hemolysate at pH 7.5. The suspension was then adjusted to pH 6.5, and this was followed by two washings at pH 6.5.

Table 3. *The effect of change in pH on the liberation of PK and LDH activity from ghost fragments*

	I.U./ml of supernatant	
	Supernatant of pH 6.5 ghosts [a]	Supernatant of pH 7.5 ghosts [b]
PK	0.080	0.85
LDH	0.220	2.54

The enzyme assays were performed at the optimum pH for PK and for LDH.

[a] Ghost fragments were prepared by hemolysis in 5 mOsm MTT buffer, pH 6.5. The ghosts were centrifuged down and the supernatant was assayed for enzyme activities.

[b] Ghost fragments were prepared at pH 6.5, as above. The suspension was then adjusted to pH 7.5 and the ghosts were centrifuged down. The supernatant was assayed for enzyme activities.

PK and LDH activities were then determined upon these ghosts. The results of a typical experiment are summarized in Table 2.

The ghosts prepared in pH 7.5 buffer recombined with PK and LDH of the hemolysate when the pH was lowered to 6.5, and subsequent washings failed to remove the bound activities. No recombination took place at pH 7.5. Table 3 shows that when ghosts were prepared at pH 6.5 and subsequently adjusted to pH 7.5, both enzymes were liberated into the supernatant.

The Effects of Calcium and Magnesium Salts

Schrier (1966) noted that ghosts prepared in the absence of Mg, when "dissected" ultrasonically or with lipid-active agents, showed an increased activity of PGK but not of GAPD. In the presence of Mg, an increase in both was noted.

In the present investigation, the effect of Ca and Mg on ghost morphology was first examined under phase-contrast optics. Ghosts were prepared in 10 and 30 mOsm Tris, pH 7.4 (omitting the EDTA), with or without the addition of 0.5 mM $CaCl_2$ or $MgCl_2$. They were then diluted sixfold in either the buffer or water. The ghosts prepared and diluted in 30 mOsm buffer appeared intact, whether the divalent ions were present or not. On dilution in water, the Ca and Mg ghosts were somewhat swollen but intact. Standard ghosts diluted in water were very faint, swollen, fragmented, or showed long ribbon-like or tubular structures.

In 10 mOsm buffer, the untreated ghosts were highly fragmented. The presence of Ca or Mg, on the other hand, exerted a marked protective action, and many intact ghosts were visible, in both the buffer and water dilutions.

Ghosts prepared in the presence of Mg were spherical, without indentation, and had a prickly appearance. Calcium ghosts were smooth, biconcave or cup-shaped, and more closely resembled standard ghosts (*see* Fig. 8 of Burger, Fujii & Hanahan, 1968).

In the absence of added cations, these ghosts retained only 0.06% of the original Hb at 10 mOsm tonicity. The percentages of Hb retained were, for the 10 and 30 mOsm media, respectively: for Mg, 1.20 and 2.50; for Ca, 3.40 and 4.90. Thus, 0.5 mM concentrations of Mg or Ca markedly enhance the retention of Hb, as previously reported by Dodge *et al.* (1963).

Table 4 summarizes the results of an experiment in which 1 mM $MgCl_2$ or $CaCl_2$ was added to the 10 mOsm Tris buffer pH 7.4 (EDTA omitted) used for hemolysis and washing of the ghosts. In the presence of the divalent cations, the ghosts do not fragment at this low ionic strength. For the determination of the activities of PK, LDH, ALD, and GAPD, the ghost suspensions were diluted sixfold: in the 10 mOsm buffer, in water, or in 0.1% TX-100.

There seemed to be no consistent pattern of divalent cation effects on the activities in buffer (basic activity). On dilution in water (cryptic activity), both Mg and Ca provided enhanced activity of PK and LDH, decreased activity of ALD. Dilution in TX-100 brought about very large increases in PK and LDH activity in the ghosts prepared in the presence of the cations, but there were no significant differences in the activity of ALD and GAPD, both firmly bound enzymes.

Table 4. *The effect on some enzyme activities of preparing ghosts in the presence of 1 mM Mg or Ca*

Enzyme	Treatment	Activity (I.U./ml ghost suspension)		
		Diluted in buffer	Diluted in water	Diluted in TX-100
PK	standard	0.61	0.75	1.11
	Mg	0.50	1.25	5.00
	Ca	1.06	1.72	9.7
LDH	standard	0.36	0.83	1.25
	Mg	1.25	2.17	10.8
	Ca	1.03	5.25	17.2
ALD	standard	3.95	3.95	4.59
	Mg	1.30	1.92	4.39
	Ca	1.00	1.61	4.45
GAPD	standard	1.45	1.45	14.5
	Mg	2.78	4.86	14.7
	Ca	0.58	0.58	14.7

The ghosts were prepared, and subsequently washed, in Tris buffer (omitting EDTA), 10 mOsm, pH 7.4, either without addition (standard treatment) or in the presence of 1 mM $MgCl_2$ or $CaCl_2$.

These ghost suspensions were then diluted sixfold in the same buffer, water, or 0.1% TX-100.

Table 5. *The effect of Ca or Mg upon retention of Hb and activity of ALD and LDH in ghosts*

Concentration	Hb, mg/ml	ALD, I.U./ml	LDH, I.U./ml
Ca, 0	0.04	0.69	0.04
Ca, 0.01 mM	0.19	0.69	0.59
Ca, 0.02 mM	–	0.69	0.84
Ca, 0.05 mM	0.36	0.69	0.94
Ca, 0.10 mM	0.34	0.70	1.05
Ca, 0.20 mM	0.46	0.73	1.18
Ca, 0.40 mM	0.72	0.74	1.24
Mg, 0	00.5	0.96	00.6
Mg, 0.01 mM	0.05	0.96	0.15
Mg, 0.02 mM	0.05	0.96	0.20
Mg, 0.05 mM	0.05	0.91	0.32
Mg, 0.10 mM	0.08	0.92	0.82
Mg, 0.20 mM	0.15	1.00	1.30
Mg, 0.40 mM	0.35	0.96	1.70

The ghosts were prepared and washed in 10 mOsm MTT buffer (EDTA omitted), pH 7.4, with additions of $CaCl_2$ and $MgCl_2$ as indicated. The determinations of Hb content and enzyme activities were performed after addition of TX-100 to 0.05%.

Since Ca and Mg exert a protective action against hypotonic disintegration, it was of interest to determine what concentrations affected the retention of Hb and activity of LDH and ALD. Table 5 shows the results of an experiment in which ghosts were washed in 10 mOsm buffer (EDTA omitted) containing varying concentrations of $MgCl_2$ or $CaCl_2$. It is clear that these cations had no definite effect on the activity of ALD, but they did have a profound effect on the retention of Hb and the activity of LDH. As little as 0.01 mM Ca markedly increased retention to levels which were achieved only by much higher concentrations of Mg.

On microscopic examination the ghosts prepared in 0.2 to 0.4 mM Ca or Mg were intact. At 0.1 mM there was some fragmentation, and at the lowest concentrations there was almost complete fragmentation and the formation of "vesicles". Under phase-contrast optics it was possible to observe the breakdown of intact ghosts upon dilution of the suspensions, with formation of long ribbon-like tubules or vesicles.

Shrinkage of Ghosts Induced by ATP Plus TX-100

As described under Materials and Methods, we assayed PGK by the backward reaction of Schrier (1963). In this assay, 3-phosphoglycerate and ATP are converted to 1,3-diphosphoglycerate and ADP; this is coupled with NAD-dependent GAPD and the change in absorbance at 340 nm is read. In the experiments on the effect of TX-100 on enzyme retention, somewhat irregular PGK assays were obtained, whereas those on stroma-free hemolysates were quite reproducible. It was noticed that a ghost suspension that had been cleared by TX-100 became opaque again on addition of ATP and this resulted in increased absorbance at 340 nm.

When 0.5% TX-100 and 3 mM ATP were added to a ghost suspension in 30 mOsm Tris, pH 7.4, the ghosts were seen under the microscope to become dense contracted spheres. This effect was specific for ATP among nucleotides tested: it was not induced by ADP, AMP, CTP, GTP, or UTP. TX-100 by itself brought about a "clearing" which made the ghosts practically invisible under the phase microscope. Addition of 1 mM Ca or Mg had no effect on the size of the ghosts, but it did increase the contrast.

Volume changes of the treated ghosts were followed photometrically. Teorell (1952) and Wins and Schoffeniels (1966) have shown that an increase in apparent absorbance (actually light absorption plus scattering) denotes a decrease in cell volume and *vice versa*. Fig. 6 illustrates typical recorded absorbance changes at 500 nm. The reading drops sharply on addition of TX-100, then rises on addition of ATP, presumably indicating shrinkage. The maximum change was exerted at 5 to 6 mM ATP.

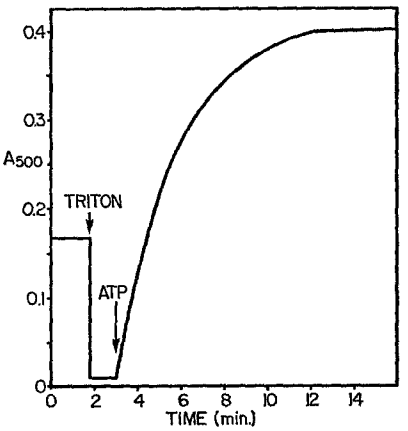

Fig. 6. The effect of the addition of 3 mM ATP on the apparent absorbance at 500 nm of a ghost suspension in 30 mOsm Tris buffer, pH 7.4, containing 0.5% TX-100

Micro-hematocrit readings were also made in an International Hemato-Kit centrifuge head at 5,100 × *g* for 30 min. In one experiment, the control suspension gave a packed volume of 58%, the TX-100- and ATP-treated ghosts 7.3%, representing a volume decrease of about 87%.

Volume distribution curves were also obtained in the Coulter Counter, on suspensions diluted 1:50,000 in 0.5% NaCl. For the untreated suspension, that in 3 mM ATP, and that exposed to 0.5% TX-100 and then ATP, the modal settings corresponded to volumes of 84, 48, and 12 μ^3, respectively. Thus, there is a shrinkage of the TX-100 + ATP-treated ghosts of 86%, a value that agrees well with the hematocrit reading. It would appear that the high dilution in saline had not reversed the volume change.

On analysis, the ghosts exposed to TX-100 plus ATP had lost 40 to 45% of the cholesterol and 35 to 40% of the total protein. Treatment with

Table 6. *LDH and GAPD activities of ghosts contracted in the presence of TX-100 and ATP*

Treatment	Activity (% of the control)	
	LDH	GAPD
Controls	100	100
3 mM ATP	76	61
0.5% TX-100	136	129
ATP + TX-100	5	18

The ghosts were prepared and washed in 30 mOsm Tris, pH 7.4.

TX-100 probably removes surface material, and may expose a contractile protein to the action of ATP.

The contracted ghosts were also tested for activity of one loosely bound enzyme (LDH) and two firmly bound ones (ALD and GAPD). ALD activity remained unchanged. The changes in LDH and GAPD are recorded in Table 6. LDH activity was decreased to about 5%, and GAPD activity was decreased to about 18% of the control activity.

Discussion

The experiments on the effect of ionic strength on ghost enzyme activity showed that all eight enzymes exhibited higher activity when the ghosts were diluted with water as compared with buffer. This appears to confirm the concept of "crypticity" as advanced by Zamudio *et al.* (1969). The intact ghosts are semi-permeable and the enzymes within the membrane, or associated with its inner surface, may be less accessible to substrates and cofactors. Fragmentation of the ghosts could expose these interior enzymes to the reactants, or permit some solubilization. A low concentration of TX-100 results in even greater ghost enzyme activity than hypotonic disruption.

There seems to be a critical point at about 30 mOsm (approximately one-tenth of blood plasma osmolality) below which membrane disruption begins. The mechanism of disruption is not clear, but it seems to involve the removal of ions from the medium. Mazia and Ruby (1968) reported that ghosts lysed in TX-100, and with complete removal of all ions by means of a mixed-bed ion-exchange resin, could be dissolved in water adjusted to pH 9.3 to 9.5 with ammonia. Harris (1968) found that ghosts produced cylindrical structures when dialyzed against water.

It must be made clear that the disintegration of the ghosts results in an increase in *measurable* enzyme activity. This involves the assumption that the enzymes were in or on the membrane, but were not able to exert full activity in the intact ghost. The mechanism of the increase in activity is not clear. It could be because of fragmentation, whereby enzymes on the interior of the membrane become more accessible to substrates or cofactors. It might be related to increased solubilization of membrane enzymes, from fragmented surfaces or the vesicles that are formed.

Our results give clear evidence of a distinction between loosely bound and firmly bound enzymes in the ghosts. The former are readily removed from the membrane as ionic strength is lowered. Also, their association with the membrane is also markedly affected by changes in pH, whereas that of the firmly bound enzymes is not. Mitchell *et al.* (1965) found that

the retention of ALD and GAPD was dependent on pH and ionic strength, but they failed to recognize the phenomenon of crypticity.

At pH 7.4, the Hb and loosely bound enzymes are probably bound by readily disruptible ionic bonds. Early methods for the preparation of ghosts involved precipitation at about pH 6, and thus large amounts of Hb were bound. The pH effect on the binding of Hb and of the loosely bound enzymes evidently does not involve permeability, since intact and fragmented ghosts gave similar results. The activities of the firmly bound enzymes were completely unaffected by the presence of Ca or Mg, in contradistinction with the loosely bound activities. The effects of decreasing the concentrations of Ca and Mg in the washing water confirmed this distinction.

The role of the firmly bound enzymes in the erythrocyte membrane requires further investigation. Bartlett in 1958 proposed a role for these enzymes in the transfer of inorganic phosphate from the exterior to the interior of the cell as organic phosphates. Parker and Hoffman (1967) have discussed in considerable detail a role of PGK in membrane phosphorylation and the pumping of cations. How these enzymes are incorporated into the membrane is of course unknown. In considering both firmly and loosely bound enzymes, measurements on hemolysates or on isolated ghost fragments cannot exactly reproduce conditions within the intact cell in its physiological state.

Our experiments showed that low concentrations of Ca and Mg ions were associated with a marked increase in activity of the loosely bound enzymes, LDH and PK, on dilution with TX-100; there was a smaller increase on dilution with water. Ca and Mg had no effect on the activities of the firmly bound enzymes, ALD and GAPD. Schrier (1966), on the other hand, reported that membranes disrupted by ultrasonication or lipid-active agents showed relatively large increases in GAPD and PGK activity when prepared in the presence of 0.25 mM Mg. Burger *et al.* (1968) found that the release of AChE from bovine ghosts was almost completely prevented if an alkaline-earth cation was added to the hemolyzing solution. They suggested that the bovine ghost had a less cohesive structure than the human ghost.

The significance of the increased activity of the loosely bound enzymes in the presence of Ca and Mg is not clear. The lower latent activity of Ca and Mg ghosts in the 30 mOsm preparations, as compared with the standard ghosts, suggests that these cations may have an effect on permeability. Actually, as demonstrated in Table 4, a clear-cut effect of the cations on LDH and PK would not have been evident if only the dilutions in buffer and in water had been compared. The dilution in TX-100 permitted a large

increase in activity; possibly the detergent exposes cation-binding centers that also play a role in the binding or the activity of the enzymes.

Our results on the effect of Ca and Mg ions in maintaining the integrity of the ghosts may be related to the erythrocyte deformability studies of Weed, LaCelle and Merrill (1969), reviewed by Weed (1970). They suggested that membrane deformability is related to ATP, Ca-dependent sol-gel changes in the membrane. The presence of Mg minimized the effect of Ca in increasing rigidity of the membrane. Forstner and Manery (1970) have recently reported that when Hb-free human ghosts were incubated with ^{45}Ca, 80% of the bound Ca was associated with protein, 15% was associated with lipid. All the evidence seems to indicate that Ca and Mg ions can have a profound effect upon the physical properties of the erythrocyte membrane.

The contraction of TX-100-treated ghosts on addition of ATP suggests the presence of a contractile protein. The smaller mean volume of the ghosts in TX-100 is probably due to the loss of surface material, lipid and protein. Nakao, Nakao and Yamazoe (1960) suggested the presence of an ATP-dependent contractile system, and Ohnishi (1962) claimed to have isolated actin- and myosin-like proteins. Wins and Schoffeniels (1966) discussed a similar phenomenon. Penniston and Green (1968), and Penniston (1969) have described an ATP-energized pinocytosis in erythrocytes from several species; GTP and ITP were ineffective. Palek, Curby and Lionetti (1969) found a relationship between a Ca-dependent ATPase and contraction of human ghosts; Mg had a similar but weaker action. The inner Ca-ATPase may be related to the contractile protein.

We observed formation of tubes and vesicles during hypotonic disintegration of the ghosts. Baker (1967) reviewed the ultrastructure of the erythrocyte and published electron micrographs of similar tubes on the surface of hypotonic ghosts which he described as stromatolytic forms. Rosenthal, Kregenow and Moses (1970) have recently reported the presence of fibrils in the membrane and discussed "a possible role for the Ca^{++}-activated, Mg^{++}-inhibited ATPase and the associated fibrils in the maintenance of erythrocyte deformability".

This work was supported by a grant from the Medical Research Council of Canada.

References

Anderson, H. M., Turner, J. C. 1960. Relation of hemoglobin to the red cell membrane. *J. Clin. Invest.* **39**:1.

Baker, R. F. 1967. Ultrastructure of the red blood cell. *Fed. Proc.* **26**:1785.

Bartlett, G. R. 1958. Organization of red cell glycolytic enzymes: Cell coat phosphorus transfer. *Ann. N. Y. Acad. Sci.* **75**:110.

Baum, H., Murer, E., Slautterback, D. B., McConnell, D. G. 1966. Association of integrated metabolic pathways with membranes. II. Electron micrographic studies on glycolytically active preparations from erythrocytes. *Arch. Biochem. Biophys.* **113**:487.

Bergmeyer, H.-U., Bernt, E., Hess, B. 1965. Lactic dehydrogenase. *In*: Methods of Enzymatic Analysis. H.-U. Bergmeyer, editor. p. 736. Verlag Chemie, Weinheim.

Burger, S. P., Fujii, T., Hanahan, D. J. 1968. Stability of the bovine erythrocyte membrane. Release of enzymes and lipid components. *Biochemistry* **7**:3682.

Chapman, R. G., Hennessey, M. A., Waltersdorph, A. M., Huennekens, F. M., Gabrio, B. W. 1962. Erythrocyte metabolism. V. Levels of glycolytic enzymes and regulation of glycolysis. *J. Clin. Invest.* **41**:1249.

Collier, H. B., Ashford, D. R., Bell, R. E. 1966. Three cases of hemolytic anemia with erythrocyte pyruvate kinase deficiency in Alberta. *Can. Med. Assoc. J.* **95**:1188.

– Duchon, G. 1968. Retention of enzyme activity by human erythrocyte "ghosts". *Abstr. XII Congr. Int. Soc. Hematol.*, NewYork. No. 0–1.

Danon, D., Nevo, A., Marikovsky, Y. 1956. Preparation of erythrocyte ghosts by gradual haemolysis in hypotonic aqueous solution. *Bull. Res. Coun. Israel* E **6**:36.

Dodge, J. T., Mitchell, C., Hanahan, D. J. 1963. Preparation and chemical characterization of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100**:119.

Forstner, J. F., Manery, J. F. 1970. Calcium-binding sites in human erythrocyte ghosts. *Fed. Proc.* **29**:664.

Green, D. E., Murer, E., Hultin, H. O., Richardson, S. H., Salmon, B., Brierley, G. P., Baum, H. 1965. Association of integrated metabolic pathways with membranes. I. Glycolytic enzymes of the red blood corpuscle and yeast. *Arch. Biochem. Biophys.* **112**:635.

Harris, J. R. 1968. Release of a macromolecular protein component from human erythrocyte ghosts. *Biochim. Biophys. Acta* **150**:534.

Icén, A. 1967. Glutathione reductase of human erythrocytes. *Scand. J. Clin. Lab. Invest.* **20**, Suppl. 96, 67 pp.

Lionetti, F. J., Fortier, N. L. 1966. Metabolism of deoxyinosine by human erythrocyte ghosts. *Biochim. Biophys. Acta* **119**:462.

Maddy, A. H. 1970. Erythrocyte membrane proteins. *Seminars in Hematol.* **7**:275.

Mazia, D., Ruby, A. 1968. Dissolution of erythrocyte membranes in water and comparison of the membrane protein with other structural proteins. *Proc. Nat. Acad. Sci.* **61**:1005.

Miller, D. M. 1970. Total solubilization of erythrocyte membranes by nonionic detergents. *Biochem. Biophys. Res. Commun.* **40**:716.

Mitchell, C. D., Mitchell, W. B., Hanahan, D. J. 1965. Enzyme and hemoglobin retention in human erythrocyte stroma. *Biochim. Biophys. Acta* **104**:348.

Nakao, M., Nakao, T., Yamazoe, S. 1960. Adenosine triphosphate and maintenance of shape of the human red cells. *Nature* **187**:945.

Nilsson, O., Ronquist, G. 1969. Enzyme activities and ultrastructure of a membrane fraction from human erythrocytes. *Biochim. Biophys. Acta* **183**:1.

Ohnishi, T. 1962. Extraction of actin- and myosin-like proteins from erythrocyte membrane. *J. Biochem. Tokyo* **52**:307.

Paglia, D. E., Valentine, W. N. 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med.* **70**:158.

Palek, J., Curby, W. A., Lionetti, F. J. 1969. Relationships between calcium dependent ATPase and contraction of human erythrocyte ghosts. *Fed. Proc.* **28**:339.

Parker, J. C., Hoffman, J. F. 1967. The role of membrane phosphoglycerate kinase in the control of glycolytic rate by active cation transport in human red blood cells. *J. Gen. Physiol.* **50**:893.

Penniston, J. T. 1969. ATP energized pinocytosis in erythrocyte ghosts. *Fed. Proc.* **28**:404.

— Green, D. E. 1968. The conformational basis of energy transformations in membrane systems. IV. Energized states of pinocytosis in erythrocyte ghosts. *Arch. Biochem. Biophys.* **128**:339.

Rao, S. N., Hara, L., Askari, A. 1968. Alkali cation-activated AMP deaminase of erythrocytes: some properties of the membrane-bound enzyme. *Biochim. Biophys. Acta* **151**:651.

Reinhold, J. G. 1953. Total protein, albumin, and globulin. *In*: Standard Methods in Clinical Chemistry, Vol. 1. M. Reiner, editor. p. 88. Academic Press, New York.

Ronquist, G. 1967. Formation of adenosine triphosphate by a membrane fraction from human erythrocytes. *Acta Chem. Scand.* **21**:1484.

— 1969. Enzyme activities at the surface of intact human erythrocytes. *Acta Physiol. Scand.* **76**:312.

Rosenthal, A. S., Kregenow, F. M., Moses, H. L. 1970. Some characteristics of a Ca^{2+}-dependent ATPase activity associated with a group of erythrocyte membrane proteins which form fibrils. *Biochim. Biophys. Acta* **196**:254.

Sackett, G. E. 1925. Modification of Bloor's method for the determination of cholesterol in whole blood or blood serum. *J. Biol. Chem.* **64**:203.

Schrier, S. L. 1963. Studies of the metabolism of human erythrocyte membranes. *J. Clin. Invest.* **42**:756.

— 1966. Organization of enzymes in human erythrocyte membranes. *Amer. J. Physiol.* **210**:139.

— 1967. ATP synthesis in human erythrocyte membranes. *Biochim. Biophys. Acta* **135**:591.

Teorell, T. 1952. Permeability properties of erythrocyte ghosts. *J. Gen. Physiol.* **35**:669.

Weed, R. I. 1970. The importance of erythrocyte deformability. *Amer. J. Med.* **49**:147.

— LaCelle, P. L., Merrill, E. W. 1969. Metabolic dependence of red cell deformability. *J. Clin. Invest.* **48**:795.

— Reed, C. F., Berg, G. 1963. Is hemoglobin an essential structural component of human erythrocyte membranes? *J. Clin. Invest.* **42**:581.

Wins, P. 1969. The association of some oxidoreductases with the red cell membrane. *Biochim. Biophys. Acta* **185**:287.

— Schoffeniels, E. 1966. ATP + Ca^{++}-linked contraction of red cell ghosts. *Arch. Int. Physiol. Biochim.* **74**:812.

Wu, R., Racker, E. 1959. Regulatory mechanisms in carbohydrate metabolism. III. Limiting factors in glycolysis of ascites tumor cells. *J. Biol. Chem.* **234**:1029.

Zamudio, I., Cellino, M., Canessa-Fischer, M. 1969. The relation between membrane structure and NADH:(acceptor) oxidoreductase activity of erythrocyte ghosts. *Arch. Biochem. Biophys.* **129**:336.