Biochimica et Biophysica Acta, 382 (1975) 157–171

BBA 76877

ORGANIZATION OF ENZYMES OF GLYCOLYSIS AND OF GLUTATHIONE METABOLISM IN HUMAN RED CELL MEMBRANES

W. TILLMANN, A. CORDUA and W. SCHRÖTER

Department of Pediatrics, University of Göttingen, D-3400 Göttingen (G.F.R.)

(Received August 12th, 1974)

SUMMARY

1) The activities of 16 enzymes of glycolysis and of glutathione metabolism were determined in intact human red cell membranes (ghosts) which were prepared by hypotonic hemolysis.

2) Enzymes and hemoglobin of the ghosts were resolved by two toluene extractions. Only the four enzymes hexokinase, fructose-bisphosphate aldolase, glyceraldehyde-phosphate dehydrogenase and pyruvate kinase could not be released completely from the ghosts.

3) The residual membrane fraction, which was obtained after the toluene extraction of ghosts prepared at 30 imOsM, contained 0.02 % of the original hemoglobin content of the red cell. Between 6.5 and 23 % of the hemolysate activities of glyceraldehyde-phosphate dehydrogenase, phosphoglycerate kinase, pyruvate kinase and fructose-bisphosphate aldolase were detected in this fraction after mechanical disruption.

4) Sonication of intact ghosts increased the activities of fructose-bisphosphate aldolase, pyruvate kinase and phosphoglycerate kinase.

5) In "white" ghosts prepared at 5 imOsM phosphate buffer which contained 0.5 % of the original hemoglobin the activities of fructose-bisphosphate aldolase and glyceraldehyde-phosphate dehydrogenase were detected at high levels. The activities of pyruvate kinase and phosphoglycerate kinase were low in these preparations.

6) The results indicate that one part of all enzymes is loosely attached to the inner surface of the membrane as is hemoglobin. A second part, the "cryptic enzyme activity", is available after resolving by toluene. A residual part of four enzymes is firmly bound to the membrane. Two of them (fructose-bisphosphate aldolase and glyceraldehyde-phosphate dehydrogenase) are oriented toward the inner surface of the membrane, whereas pyruvate kinase and phosphoglycerate kinase are hidden in the lipid core of the membrane.

INTRODUCTION

In recent years the attachment of both hemoglobin and glycolytic enzymes to the erythrocyte membrane has been studied by several authors. Most of the enzymes

are released from the membranes by hypotonic hemolysis to a similar degree as is hemoglobin. They have been classified as enzymes "loosely-bound" to cell membranes by Duchon and Collier [1]. In contrast, fructose-bisphosphate aldolase and glyceraldehyde-phosphate dehydrogenase have been described as "firmly-bound" enzymes. Some years ago, Mitchell at al. [2] arrived at similar conclusions by using different buffer solutions for hypotonic hemolysis. In addition, binding of the enzyme phosphoglycerate kinase to the cell membrane was of intermediate firmness [1]. Parker and Hoffman [3] demonstrated that the activity of this enzyme could also be measured in hemoglobin-free ghosts. They suggested that membrane phosphoglycerate kinase might be a point at which the Na^+-K^+-transport system can influence the metabolic rate of red cells.

The organization of the firmly membrane-bound enzymes was first studied by Schrier et al. [4]. These authors provided evidence that membrane phosphoglycerate kinase was oriented toward the lipid core of the membrane, whereas glyceraldehyde-phosphate dehydrogenase was directed toward the interior of the erythrocyte. Recently Kant and Steck [5] discussed a specific linkage of glyceraldehyde-phosphate dehydrogenase to the inner surface of the membrane in vivo. Furthermore, Duchon and Collier [1] suggested that the glycolytic enzymes were partly hidden in "crypts" of the membrane.

In this paper, the organization of most enzymes of glycolysis and glutathione metabolism has been studied in isolated membranes (ghosts) of human erythrocytes. Using hypotonic hemolysis, toluene extraction and sonication of the ghosts, remarkable differences of enzyme attachment and location within the membrane are demonstrated. Evidence is provided that the firmly bound enzymes fructose-bisphosphate aldolase, glyceraldehyde-phosphate dehydrogenase, pyruvate kinase and phosphoglycerate kinase are partly enclosed in the lipid core of the membrane. Furthermore, enzyme activities in ghosts prepared from "old "and "young" erythrocytes have been determined.

MATERIALS AND METHODS

Chemicals

The biochemical substrates, coenzymes and enzymes were products from Boehringer & Soehne, Mannheim, Germany. All other chemicals were of reagent grade.

Preparation of ghosts

Isolated red cell membranes (ghosts) were prepared by a modification of the method of Dodge et al. [6]. Blood samples from healthy doners were anticoagulated by the addition of 20 I.U. of preservative-free heparin per ml blood. Leukocytes were carefully separated within 2 h of collection by filtration through a cotton wool column according to the method of Busch and Pelz [7]. The final erythrocyte suspensions contained less than 50 leukocytes per 10^6 erythrocytes. After separation, erythrocytes were washed three times in an isotonic 310 imosM phosphate buffer, pH 7.4. With the same buffer solution, a 50 % suspension of erythrocytes was prepared. The cells were lysed by the addition of a hypotonic phosphate buffer (pH 7.4) and were centrifuged for 40 min at 20 000 × *g*. The cells were washed only two more times with the same buffer. The true osmolality of the buffer, ranging from 5–70

imosM, was measured with a half-microosmometer (Knauer, Eppelheim, Germany). As suggested by Jacob et al. [8] 1.0 mM EDTA and 10 mM $NaHCO_3$ were added to all buffer solutions. The cell-buffer ratio used during the washing and lysing of the cells, was 1 : 20 instead of 1 : 30 or 1 : 120 as described by Dodge et al. [6]. All procedures were carried out at 0–4 °C.

The centrifuged ghosts were resuspended in the same buffer, and the number of ghosts was measured in a Coulter Counter, model B using an aperture of 8, an amplification of 2 and a threshold of 17.5. The standard ghost suspension was adjusted to contain about $5 \cdot 10^9$ ghosts $\cdot$ ml^{-1}. The ghosts were viewed under phase contrast microscopy. Ghosts were still intact at osmolalities of 70 to 30 imosM. At 20 imosM only a few small particles, representing destroyed ghosts, were found. At 10 imosM about 10 % of the ghosts and at 5 imosM nearly all of the ghosts were fragmented. The same batch of ghosts was divided into two parts, either for extraction by toluene, or for determining enzyme activities.

Resolving of hemoglobin and non-hemoglobin protein

For resolving of enzymes and the residual hemoglobin of the ghosts, toluene, first employed for solubilization of enzymes by Wallenfels et al. [9] and later on by Ulitzur [10], was used. Two volumes of ghost suspension (about $5 \cdot 10^9$ ghosts $\cdot$ ml^{-1}) were gently swirled for 30 min at 4 °C with one volume of cold toluene to allow three layers to be formed. The upper clear toluene layer was carefully removed and was discarded. No enzyme activity or protein was detected in this phase. The buffer layer underneath the emulsion of the residual membrane fraction was aspirated for measurement of enzyme activities, hemoglobin and protein content. This phase was called "buffer phase I". The remaining membrane fraction was extracted again with toluene under identical conditions. The buffer layer from this step was called "buffer phase II". Using techniques published previously [11], no free fatty acids, triglycerides, phospholipids or cholesterol could be detected in the buffer phase I and II. After the second extraction, the residual membrane fraction was completely white. One volume of this fraction was resuspended in two volumes of deionised water and was disintegrated three times for 10 s in an ultra-turrax (Janke & Kunkel, Staufen, Germany) with two intervals of 30 s each. This suspension was also used for enzyme assays.

The activities of glycolytic enzymes were not appreciably influenced by the presence of different concentrations of toluene in the hemolysates.

Sonication of ghost suspensions

The ghost suspensions were subjected at 0 °C to a sonic vibrator. A Branson sonifier B-12 (Branson, Sonic Power Co., Danbury, Connecticut, U.S.A.) was used. The period of sonication (40 W) was 6×10 s with cooling intervals of 20 s between each sonication. The suspensions containing the destroyed ghosts, called "membrane particles", were used for enzyme assays.

Separation of young and old erythrocytes

Using the method of Oski et al. [12], young erythrocytes were separated from old cells by centrifugation ($30\,000 \times g$ for 30 min). A volume of 10 % of the top and bottom fractions was carefully aspirated from the tube for preparation of the ghosts. Reticulocytes were counted and aspartate amino-transferase activity was measured

in the top and bottom fractions for reference of the mean age of the cell population [12, 13].

Chemical assays

The hemoglobin content of the ghosts was measured by a method described by Dacie and Lewis [14], which is used normally for measuring plasma hemoglobin.

Protein was determined by the method of Lowry et al. [15] using bovine serum albumin as a standard.

Assay of enzyme activities of ghosts and their fractions

Enzyme activities were determined at 37 °C. Enzyme units were defined as the number of micromoles of pyridine nucleotide converted/min/10^{11} ghosts at 37 °C. All assays were carried out in duplicate or triplicate and appropriate blanks were used. Aliquots of the intact and sonicated ghost suspensions, of buffer phase I and II, and of the residual membrane fraction were added for measuring enzyme activities. The following enzyme activities were determined as reported previously [16, 17]: hexokinase, phosphoglucomutase, glucose-phosphate isomerase, 6-phosphofructokinase, fructose-bisphosphate aldolase, glyceraldehyde-phosphate dehydrogenase, triosephosphate isomerase, bisphosphoglyceromutase, phosphoglycerate kinase, phosphoglyceromutase, enolase, glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase (decarboxylating), glutathione reductases and pyruvate kinase. The latter was determined with $2.0 \cdot 10^{-3}$ phosphoenolpyruvate as a substrate.

RESULTS

Resolving of hemoglobin and enzymes by hypotonic hemolysis

The firmness of hemoglobin and enzyme attachment to the membranes of erythrocytes was studied by hypotonic hemolysis. Using phosphate buffer solutions with osmolalities between 5 and 70 imosM, we found a relation between the hemoglobin content of the ghosts and the buffer concentration (Fig. 1). Both, the hemoglobin and the non hemoglobin content of the ghosts declined with decreasing buffer osmolalities. Using a 30 imosM buffer, we found about 1 % of the total hemoglobin

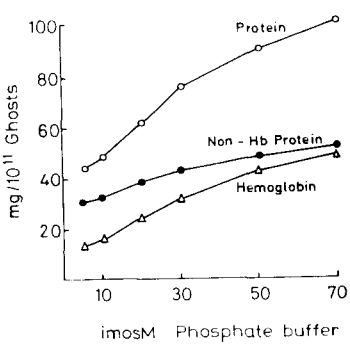


Fig. 1. Protein and hemoglobin content of intact ghosts prepared by hypotonic hemolysis using phosphate buffers of different osmolalities. Non-hemoglobin protein was calculated as the difference of total protein and hemoglobin. The values are the mean of 12 experiments.

of the intact erythrocytes in the ghost suspension. Fig. 1 shows that hemoglobin as well as non-hemoglobin protein was resolved more intensively from the ghosts at lower osmolalities.

The enzyme activities measured in ghosts prepared by hypotonic hemolysis are shown in Figs 2–6. With lower osmolalities of the buffer solutions the activities of most of the enzymes decreased as did the hemoglobin content. At concentrations lower than 30 imosM phosphate buffer glucose-6-phosphate dehydrogenase, triosephosphate isomerase, phosphoglucomutase, bisphosphoglyceromutase, glutathione reductase 1, glutathione reductase 2, 6-phosphofructokinase, phosphoglycerate dehydrogenase (decarboxylating), phosphoglyceromutase, glucosephosphate isomerase, enolase and phosphoglycerate kinase were resolved more rapidly from the ghosts, whereas in these preparations the activities of hexokinase, fructose-bisphosphate aldolase and glyceraldehyde-phosphate dehydrogenase were measurable larger in amount. As most enzyme activities the activity of pyruvate kinase decreased in proportion to the hemoglobin content of the ghosts, however, at low osmolalities a relatively high activity still remained firmly bound to the ghosts (Fig. 6).

Resolving of hemoglobin and enzymes by toluene

For resolving of hemoglobin and enzymes from the ghosts ghost suspensions

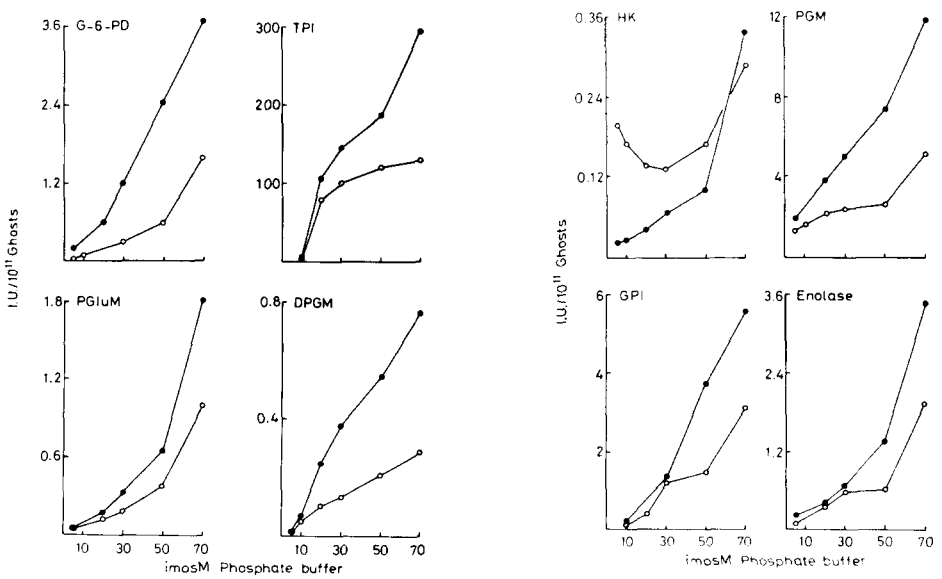
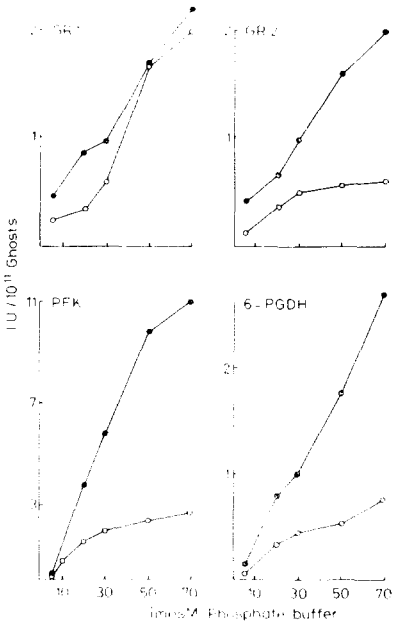


Fig. 2. Activities of glucose-6-phosphate dehydrogenase (G-6-PD), triosephosphate isomerase (TPI), phosphoglucomutase (PGluM) and bisphosphoglyceromutase (DPGM) in ghost suspensions (○ — ○), prepared by hemolysis buffers of different osmolalities, and in buffer phase I (● — ●). The latter was obtained by extraction of one part of the ghost suspensions by toluene. The values are the mean of 24 experiments.

Fig. 3. Activities of hexokinase (HK), phosphoglyceromutase (PGM), glucosephosphate isomerase (GPI) and enolase in ghost suspensions (○ — ○), prepared by hemolysis buffers of different osmolalities, and in buffer phase I (● — ●). The latter was obtained by extraction of one part of the ghost suspensions by toluene. The values are the mean of 24 experiments.



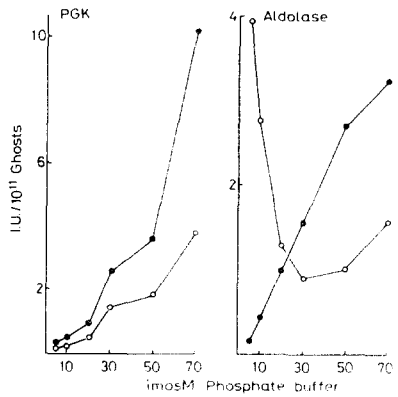


Fig. 4. Activities of glutathione reductase measured with NADH (GR 1), glutathione reductase measured with NADPH (GR 2), 6-phosphofructokinase (PFK) and phosphogluconate dehydrogenase (decarboxylating)(6-PGDH) in ghost suspensions (○ ○), prepared by hemolysis buffers of different osmolalities and in buffer phase I (● ●). The latter was obtained by extraction of one part of the ghost suspensions by toluene. The values are the mean of 24 experiments.

Fig. 5. Activities of phosphoglycerate kinase (PGK) and fructose-bisphosphate aldolase (Aldolase) in ghost suspensions (○ ○), prepared by hemolysis buffers of different osmolalities and in buffer phase I (● ●). The latter was obtained by extraction of one part of the ghost suspensions by toluene. The values are the mean of 24 experiments.

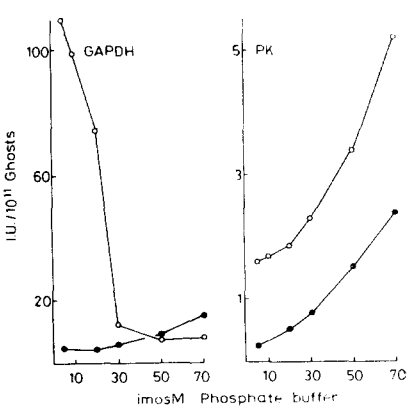


Fig. 6. Activities of glyceraldehyde-phosphate dehydrogenase (GAPDH) and pyruvate kinase (PK) in ghost suspensions (○ ○), prepared by hemolysis buffers of different osmolalities and in buffer phase I (● ●). The latter was obtained by extraction of one part of the ghost suspensions by toluene. The values are the mean of 24 experiments.

were extracted by toluene as described under Methods. Three layers were formed: an upper toluene layer, an intermediate thin layer containing the residual membrane fraction, and the bottom buffer phase containing the resolved hemoglobin and parts of the enzymes. Fig. 7 shows the hemoglobin and non-hemoglobin protein content

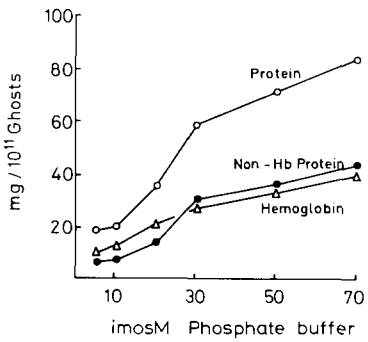


Fig. 7. Protein and hemoglobin content of buffer phase I, obtained by toluene extraction of ghost suspensions. One half of the ghost suspensions used for the experiments shown in Fig. 1 were extracted. The values are the mean of 12 experiments.

of buffer phase I, after extraction of the ghosts, which were prepared at different osmolalities. As expected, enzyme activities were detectable in the buffer phase. In parallel to the decrease of hemoglobin content (Fig. 7), the activities of all enzymes declined with decreasing osmolalities (Figs 2–6), indicating a close relation between the extraction of hemoglobin and of enzymes by toluene. On the other hand, enzymes with high activities in ghosts prepared at osmolalities lower than 30 imosM (hexokinase, fructose-bisphosphate aldolase, glyceraldehyde-phosphate dehydrogenase, pyruvate kinase), were not released into the buffer phase, suggesting that these enzymes are attached more firmly to the cell membranes than hemoglobin and most of the other enzymes. These four enzymes could not be resolved completely even by a second extraction with toluene since activities remained in the residual membrane fraction (Table I).

Table II shows that in contrast to hemoglobin non-hemoglobin protein was not released completely even by two extractions with toluene. Only less than 0.02 % of the original hemoglobin content of the intact erythrocytes was eventually attached to the residual membrane fraction.

Determination of enzyme activities in the residual membrane fraction

Neither in buffer phase I nor in buffer phase II free fatty acids, triglycerides, phospholipids or cholesterol were detected by thin-layer chromatography. Thus, it is assumed that the remaining membrane fraction contained the major part (a minor part, which was not determined, may be transferred into the toluene phase) of their lipids after toluene extraction as well as the residual non-hemoglobin protein. This fraction was desintegrated by an ultra-turrax. Enzyme activities bound to the residual membrane fraction are shown in Table I. When compared with their activities in hemolysates, only the activities of the four enzymes glyceraldehyde-phosphate dehydrogenase, fructose-bisphosphate aldolase, pyruvate kinase and phospho-

TABLE I

ENZYME ACTIVITIES IN DIFFERENT MEMBRANE FRACTIONS

The membrane fractions were obtained by two successive toluene extractions of the ghosts prepared by a 30 imosM phosphate buffer. Values are expressed as I.U./10^{11} ghosts. Enzyme activities given are the mean values ± S.D. of 24 and 8 experiments respectively.

	Buffer phase I (n = 24)	Buffer phase II (n = 24)	Residual membrane fraction (n = 8)	% Activity of hemolysates*
Glyceraldehyde-phosphate dehydrogenase	6.00 ± 1.80	5.00 ± 1.90	42.00 ± 6.00	8.20
Fructose-bisphosphate aldolase	1.58 ± 0.32	0.12 ± 0.03	5.10 ± 0.70	30.70
Pyruvate kinase	0.78 ± 0.10	0.67 ± 0.08	11.00 ± 1.00	21.84
Phosphoglycerate kinase	2.53 ± 0.35	0.10 ± 0.02	48.00 ± 5.60	13.20
Glucose-6-phosphate dehydrogenase	1.20 ± 0.18	0.09 ± 0.01	0.30 ± 0.05	5.19
6-Phosphofructokinase	5.80 ± 0.74	0.06 ± 0.01	1.30 ± 0.17	12.15
Bisphosphoglyceromutase	0.38 ± 0.08	< 0.05	0.40 ± 0.08	6.14
Hexokinase	0.06 ± 0.01	< 0.05	< 0.05	2.57
Phosphogluconate dehydrogenase (decarboxylating)	1.00 ± 0.16	0.13 ± 0.03	< 0.05	4.31
Phosphoglucomutase	0.33 ± 0.05	< 0.05	< 0.05	2.42
Glucosephosphate isomerase	1.35 ± 0.32	0.20 ± 0.03	< 0.05	1.25
Enolase	0.72 ± 0.11	0.06 ± 0.01	0.60 ± 0.12	3.08
Glutathione reductase 1	0.97 ± 0.11	0.42 ± 0.06	0.35 ± 0.07	6.77
Glutathione reductase 2	0.97 ± 0.12	0.15 ± 0.04	0.30 ± 0.05	11.09
Triosephosphate isomerase	144.00 ± 22.80	37.00 ± 5.20	35.00 ± 6.20	3.56
Phosphoglyceromutase	5.10 ± 0.73	1.10 ± 0.20	6.00 ± 1.30	6.47

* Total activity of both, the two buffer phases and the residual membrane fraction, represented in % of the enzyme activities measured in hemolysates. The activities in hemolysates of normal erythrocytes has been published elsewhere [16, 17]. The activities in hemolysates are expressed in I.U./10^{11} erythrocytes.

TABLE II

SOLUBILISATION OF HEMOGLOBIN AND NON-HEMOGLOBIN PROTEIN BY TOLUENE EXTRACTION OF THE GHOSTS

Ghosts were prepared by a 30 imosM phosphate buffer and hemoglobin and protein were measured in the ghosts as described under Methods. Subsequently the ghosts were extracted by toluene and the hemoglobin and protein content was measured in the buffer phases. All values are in mg/10^{11} ghosts and are expressed as the mean ± S.D.

	Untreated ghosts (n = 24)	Buffer phase I (n = 24)	Buffer phase II (n = 12)
Protein	76.3 ± 8.8	58.8 ± 6.7	6.0 ± 0.6
Hemoglobin	32.0 ± 4.1	28.4 ± 3.8	3.1 ± 0.4
Non-hemoglobin protein	44.3 ± 5.6	30.4 ± 4.9	2.9 ± 0.4

glycerate kinase are found in considerable amounts in the residual membrane fraction. From this we conclude that these enzymes are firmly attached to the residual membrane fraction of ghosts after hemolysis by 30 imosM phosphate buffer.

We cannot explain why the hexokinase activity, which was high in ghosts prepared by low osmolality hemolysis, could not be detected in the residual membrane fraction. The addition of $5 \cdot 10^{-4}$ mercaptoethanol to the ghost suspensions had no effect. Since enzymes firmly attached to the cell membrane are expected to be detectable in hemoglobin-free ghosts, the activities of glyceraldehyde-phosphate dehydrogenase, fructose-bisphosphate aldolase, pyruvate kinase and phosphoglycerate kinase were determined in so-called "white ghosts". These white ghosts represent the ghosts prepared by 5 imosM phosphate buffer containing less than 0.5 % of the original hemoglobin of intact cells.

Table III shows that about 17 % of the glyceraldehyde-phosphate dehydrogenase and fructose-bisphosphate aldolase activities of hemolysates are found in white ghosts, but that only 2.9 % of the pyruvate kinase activity and a negligible

TABLE III

COMPARISON OF THE ENZYME ACTIVITIES IN "WHITE" GHOSTS AND IN THE RESIDUAL MEMBRANE FRACTION

"White" ghosts were prepared by a 5 imosM phosphate buffer. The data are mean values of 24 experiments, expressing enzyme activities in % of hemolysate activity, as explained in Table I.

	Activity in "white" ghosts	Activity in the residual membrane fraction[†]
Glyceraldehyde-phosphate-dehydrogenase	17.03	6.50
Fructose-bisphosphate aldolase	17.80	22.97
Pyruvate kinase	2.93	19.30
Phosphoglycerate kinase	0.03	12.57

[†] Mean values of 8 experiments representing the percentage of activity of the respective enzymes in hemolysates. The residual membrane fraction was obtained by toluene extraction of ghosts which were prepared by a 30 imosM phosphate buffer and subsequently destroyed by an ultra-turrax.

activity of phosphoglycerate kinase were detectable in these preparations, although activities of these enzymes were also high in the residual membrane fraction. It is remarkable that the activity of phosphoglycerate kinase could only be detected in completely desintegrated membranes and that only a small part of pyruvate kinase activity was demonstrable in white ghosts. The fact that glyceraldehyde-phosphate dehydrogenase activity was relatively low in the residual membrane fraction may be explained by the lack of Mg^{2+} ions in the hemolysing mixtures [4].

Sonication of the ghosts

In order to determine the activities of the enzymes firmly bound to erythrocyte membranes by two independent methods, ghosts were destroyed by sonication and the enzyme activities were compared with the activities found in the residual mem-

brane fraction. Table IV shows that the activities in membrane particles prepared by sonication are lower than the activities in the residual membrane fraction. Prolonged and more intensive sonication did not increase enzyme activities.

TABLE IV

ENZYME ACTIVITIES IN GHOSTS, IN THE RESIDUAL MEMBRANE FRACTION AND IN MEMBRANE PARTICLES PREPARED BY SONICATION OF GHOSTS

Ghosts prepared by a 30 imosM phosphate buffer were subjected to sonication as described under methods. Values are expressed as I.U./10^{11} ghosts. Enzyme activities are expressed as mean values ± S.D.

	Ghosts (n = 24)	Residual membrane fraction* (n = 8)	Membrane particles* (n = 5)
Fructose-bisphosphate aldolase	0.88 ± 0.21	5.10 ± 0.70	2.53
Pyruvate kinase	2.30 ± 0.42	11.00 ± 1.00	4.78
Phosphoglycerate kinase	1.40 ± 0.34	48.00 ± 5.60	5.39

* The mean of 8 and 5 experiments respectively.

Enzyme activities in ghosts of "young" and "old" erythrocytes

Activities of most glycolytic and hexose monophosphate-shunt enzymes are higher in young erythrocytes than in older cells [18, 19]. On the other hand, the hemoglobin concentration is higher in old than in young erythrocytes [20]. For a better understanding of the mechanism of aging and of erythrocyte destruction, it is important to know the enzyme levels and the protein distribution in erythrocyte membranes of different age groups. Therefore, we separated old and young red cells by centrifugation and prepared ghosts by hypotonic hemolysis using 30 imosM phosphate buffer as described above. Table V shows that the hemoglobin content of older ghosts is increased by about 30 % when compared with the fraction of younger cells. With

TABLE V

SEPARATION OF YOUNG AND OLD ERYTHROCYTES

Ghosts were prepared by a 30 imosM phosphate buffer from young and old erythrocytes obtained by centrifugation (30 000 × g for 30 min). Ten per cent of the top and bottom fractions of the sediment were used in the experiments. All figures are mean values ± S.D. of 8 experiments.

	Top fraction	Bottom fraction
Reticulocytes (%)	4.50 ± 0.80	0.10 ± 0.05
Aspartate aminotransferase activity in hemolysates (units/g hemoglobin)	5.98 ± 0.67	1.71 ± 0.48
Hemoglobin in ghosts (mg/10^{11} ghosts)	2.72 ± 0.32	3.79 ± 0.31
Protein in ghosts (mg/10^{11} ghosts)	7.10 ± 0.90	8.41 ± 0.85
Non-hemoglobin protein in ghosts (mg/10^{11} ghosts)	4.38 ± 0.51	4.62 ± 0.61

TABLE VI

ENZYME ACTIVITIES OF GHOSTS PREPARED FROM YOUNG AND OLD ERYTHROCYTES

Ghosts were prepared by a 30 imosM phsophate buffer from young and old erythrocytes separated by centrifugation.

	Ghosts of normal cell populations ($n = 24$)		Ghosts of young cells** ($n = 8$)	Ghosts of old cells** ($n = 8$)	Ratio of enzyme activities in young: old erythrocytes
	Activity in I.U./10^{11} Ghosts	% Activity of hemolysates*			
Glyceraldehyde-phosphate dehydrogenase	12.00±3.30	1.85	13.20±1.55	9.00±1.19	1.46
Fructose-bisphosphate aldolase	0.88±0.21	3.96	1.11±0.06	0.85±0.07	1.30
Pyruvate kinase	2.30±0.42	4.03	3.40±0.68	2.30±0.47	1.47
Phosphoglycerate kinase	1.40±0.34	0.36	1.62±0.08	1.32±0.08	1.22
Glucose-6-phosphate dehydrogenase	0.30±0.08	0.98	0.43±0.11	0.25±0.08	1.72
6-Phosphofructokinase	1.98±0.41	3.39	1.83±0.40	2.26±0.27	0.81
Bisphosphoglyceromutase	0.13±0.05	1.02	0.14±0.02	0.11±0.02	1.27
Hexokinase	0.13±0.04	5.57	0.15±0.01	0.10±0.03	1.50
Phosphogluconate dehydrogenase (decarboxylating)	0.46±0.09	1.75	0.56±0.03	0.43±0.04	1.30
Phosphoglucomutase	0.18±0.04	1.32	0.28±0.04	0.15±0.05	1.86
Glucosephosphate isomerase	1.24±0.31	1.00	1.77±0.08	1.00±0.12	1.77
Enolase	0.60±0.22	1.34	0.77±0.05	0.50±0.06	1.54
Glutathione reductase 1	0.58±0.12	2.25	0.75±0.08	0.55±0.09	1.36
Glutathione reductase 2	0.48±0.14	3.75	0.53±0.06	0.42±0.07	1.26
Triosephosphate isomerase	98.00±22.60	1.61	110.00±8.27	95.00±9.08	1.15
Phosphoglyceromutase	2.40±0.62	1.27	2.67±0.15	2.17±0.09	1.23

* Activity of normal ghosts expressed in per cent of activity in hemolysates of normal red cells.

** Expressed as I.U./10^{11} ghosts, mean values ± S.D.

the exception of 6-phosphofructokinase, the activities of all enzymes are higher in the younger ghosts than in the older ones (Table VI). It is noteworthy that the enzyme activities are lower in older than in younger ghosts though the hemoglobin content is higher in the latter cell group.

DISCUSSION

The life-span of red cells may be partly determined by the stability and function of the erythrocyte membrane. Therefore, enzyme activity as an indicator of membrane behaviour and membrane organization seems to be important. Studies of membranes of intact erythrocytes are of limited value only. Of course, studies on isolated ghosts do not necessarily represent the intact cell membrane in its physiological state. In order to study isolated membranes under conditions as physiological as possible, ghosts were prepared in the presence of 1 mM EDTA by hypotonic hemolysis using a 30 imosM phosphate buffer. Ghosts prepared in such a way do not lose lipids [6]. They are morphologically intact by phase and electron microscopy, contain nearly all of their non-hemoglobin protein (Fig. 1) and, finally, they are highly permeable for molecules in the size of the substrates used [21]. These ghosts contained about 1 % of the hemoglobin content of intact erythrocytes (Table II). Such a hemoglobin content is higher than the values found by Dodge et al. [6] in ghosts prepared by 30 imosM phosphate buffer. The difference may be explained by the fact that hemolysis was followed by two washings only, and that the cell : buffer ratio during hemolysis was 1 : 20 instead of 1 : 30 or 1 : 120, as used by Dodge et al. [6]. With these modifications a linear decrease in the hemoglobin content of the ghosts was found, paralleling the decreasing osmolalities of the lysing buffer solutions (Fig. 1). In ghost suspensions prepared with 30 imosM buffer, all the 16 enzymes were found to be of intermediate strength, whereas the activities in ghosts prepared at lower osmolalities were either decreased (glucose-6-phosphate dehydrogenase, triosephosphate isomerase, phosphoglucomutase, bisphosphoglyceromutase, glutathione reductase 1, glutathione reductase 2, 6-phosphofructokinase, phosphoglycerate dehydrogenase (decarboxylating), phosphoglyceromutase, glucosephosphate isomerase, enolase, phosphoglycerate kinase, pyruvate kinase) or increased (hexokinase, fructose-bisphosphate aldolase, glyceraldehyde-phosphate dehydrogenase). The first group of enzymes is released from the ghosts in the same manner as hemoglobin is. These enzymes are therefore being termed "loosely bound", as proposed by Duchon and Collier [1]. Consequently, the remaining three enzymes, hexokinase, fructose-bisphosphate aldolase and glyceraldehyde-phosphate dehydrogenase, are being called "firmly bound".

Ghosts of normal erythrocytes prepared at 30 imosM buffer solutions still contain about 1 % of the original hemoglobin and between 0.98 and 5.57 % of the enzyme activities measured in hemolysates when calculated on the basis activity per cell, or per ghost respectively (Table VI).

Though the hemoglobin content of ghosts prepared from younger erythrocytes is lower than in older erythrocytes (Table V), the activities of all enzymes except for 6-phosphofructokinase are higher (Table VI). This result shows that the binding of hemoglobin and of enzymes seems to be independent of each other during aging. The increased binding of hemoglobin to the erythrocyte membrane during aging

possibly is the consequence of a reduced production of ATP, as suggested by Weed and La Celle [22]. We suggest that the production of ATP in the compartment near the membrane may be impaired because of diminished activities of the glycolytic enzymes in old erythrocytes.

Duchon and Collier [1] argued that a part of both the loosely bound and the firmly bound enzyme is masked. They called this fraction "cryptic enzyme activity". These authors demonstrated the cryptic activities by the dilution of the ghosts with water. In our experiments toluene extraction was used for further solubilization of membrane-attached enzymes. Figs 2–4 show that the activities of the enzymes known as loosely bound, are higher in the buffer phase obtained after toluene extraction, than in intact ghost suspensions. This part of enzyme activity may represent the cryptic activities as described by Duchon and Collier [1]. Nevertheless, it cannot be excluded that enzymes at the inside of the membrane became more accessible to substrates and cofactors after disruptive treatment resulting in an elevation of the measured enzyme activities, as suggested by Schwoch and Passow [23]. The firmly bound enzymes fructose-bisphosphate aldolase and glyceraldehyde-phosphate dehydrogenase were not solubilized by toluene from ghost suspensions prepared by low osmolality buffers, though these ghosts exhibited high activities as demonstrated in Figs 5 and 6. The remaining activity of pyruvate kinase could not be resolved by toluene (Fig. 6).

This behavior of the firmly bound enzymes leads to the assumption that a substantial amount of enzymes must be hidden in the residual membrane fraction. Table II shows that this fraction contains non-hemoglobin protein and less than 0.02 % of hemoglobin of the original content of erythrocytes. After desintegration of this fraction by an ultra-turrax, the activities of the four enzymes glyceraldehyde-phosphate dehydrogenase, fructose-bisphosphate aldolase, pyruvate kinase and phosphoglycerate kinase could be demasked at high levels of activity (Tables I and III). From these results it is concluded that all four enzymes are firmly bound to the membrane. Sonication, on the other hand, demasked these enzymes to a lower degree only (Table IV).

Since high levels of activity in white ghosts prepared at 5 imosM, were only detected in the enzymes glyceraldehyde-phosphate dehydrogenase and fructose-bisphosphate aldolase, it is suggested that pyruvate kinase and phosphoglycerate kinase are more deeply hidden in the membranes than glyceraldehyde-phosphate dehydrogenase and fructose-bisphosphate aldolase are. Thus, pyruvate kinase may play an important role for the supply of ATP within the membrane, in addition to phosphoglycerate kinase, as already suggested by Parker and Hoffman [3] and by Schrier [4].

The results presented in this paper allow a preliminary hypothesis concerning the organization of glycolytic enzymes within the red cell membrane. It is schematically shown in Fig. 8. The bulk of glycolytic enzymes is loosely associated with the inner surface of the membrane, together with hemoglobin. This fraction can be measured in ghosts prepared by hemolysis at 30 imosM buffer. A second fraction of enzymes, probably more firmly bound in "crypts", is extracted by toluene and by more hypotonic hemolysis, i.e. by 5 imosM buffer solution. The firmly bound enzymes glyceraldehyde-phosphate dehydrogenase, fructose-bisphosphate aldolase, pyruvate kinase and phosphoglycerate kinase are present in large quantities in the residual membrane

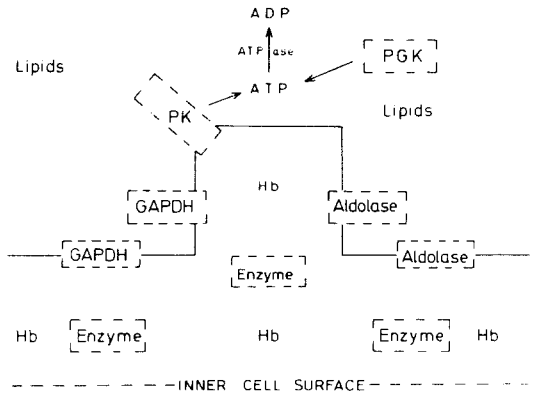


Fig. 8. Preliminary scheme for the organization of enzymes in the human erythrocyte membrane For further details refer to the section of discussion.

fraction after mechanical desintegration. The observation that glyceraldehyde-phosphate dehydrogenase and fructose-bisphosphate aldolase are detectable also in white ghosts, the membranes of which have not been disrupted, suggests that these two enzymes are directed more toward the inner cell surface than pyruvate kinase and phosphoglycerate kinase. Pyruvate kinase has an intermediate position: a small part of activity is detected in white ghosts, whereas the majority is hidden and only detectable after destruction of the membrane.

Membranes contain activities of firmly bound enzymes corresponding in quantity to 6.5 to 23 % of the activities found in hemolysates (Table III). Whether the enzyme activities normally hidden in the cell membrane are necessary for optimal membrane function and stability and eventually for a normal life-span of the erythrocytes remains a question open to further experimental effort. Possibly, the study of ghosts prepared from erythrocytes of patients suffering from hereditary enzymopenic hemolytic anemia may contribute to clarify these points.

ACKNOWLEDGEMENT

This work was supported by the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg, G.F.R. (Schr 86/10 and Schr 86/11).

REFERENCES

1 Duchon, G. and Collier, H. B. (1971) J. Membrane Biol. 6, 138–157
2 Mitchell, C. D., Mitchell, W. B. and Hanahan, D. I. (1965) Biochim. Biophys. Acta 104, 348–358
3 Parker, I. C. and Hoffman, F. F. (1967) J. Gen. Physiol. 50, 893–916
4 Schrier, S. L. (1966) Am. J. Physiol. 210, 139–145
5 Kant, A. J. and Steck, T. L. (1973) J. Biol. Chem. 248, 8457–8464
6 Dodge, J. T., Mitchell, C. and Hanahan, D. I. (1963) Arch. Biochem. 100, 119–130
7 Busch, D. and Pelz, K. (1966) Klin. Wochenschr. 44, 983–984
8 Jacob, H. S., Brain, C. M. and Dacie, J. V. (1968) J. Clin. Invest. 47, 2664–2677
9 Wallenfels, K., Zarnitz, M. L., Laule, G., Bender, H. and Keser, M. (1959) Biochem. Z. 331, 459–485
10 Ulitzur, S. (1970) Biochim. Biophys. Acta 211, 542–549
11 Hürter, P., Schröter, W., Schedel, I. and Gercken, G. (1970) Pediatrics 46, 259–266

12 Oski, F. A., Naiman, J. L., Blum, S. F., Zarkowsky, H. S., Whaun, J., Shohet, S. B., Green, A. and Nathan, D. G. (1969) New Engl. J. Med. 280, 909–916
13 Sass, M. D., Vorsanger, E. and Spear, P. W. (1964) Clin. Chim. Acta 10, 21–26
14 Dacie, J. V. and Lewis, S. M. (1970) In Practical Haematology, 4th edn, pp. 481–482, J. and A. Churchill, London
15 Lowry, O. H., Rosebrough, H. I., Farr, A. L. and Rendall, R. I. (1951) J. Biol. Chem. 193, 265–275
16 Schröter, W. (1970) Klin. Wochenschr. 48, 1407–1414
17 Schröter, W. and Kalinowsky, W. (1969) Clin. Chim. Acta 25, 283–285
18 Marks, P. A., Johnson, A. B., Hirschberg, E. (1958) Proc. Natl. Acad. Sci. U.S. 44, 529–536
19 Löhr, G. W. and Waller, H. D. (1961) Folia Haematol. 78, 384–395
20 Kleihauer, E. and Steenblock, U. (1967) Blut 14, 193–198
21 Bramley, T. A. and Coleman, R. (1972) Biochim. Biophys. Acta 290, 219–228
22 Weed, R. I., La Celle, P. L. and Merrill, E. W. (1969) J. Clin. Invest. 48, 795–809
23 Schwoch, G. and Passow, H. (1973) Mol. Cell. Biochem. 2, 197–218