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Membrane-bound 2,3-Diphosphoglycerate Phosphatase of Human Erythrocytes

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Summary. Gradual osmotic hemolysis of human erythrocytes reduces the cell content of whole protein, hemoglobin, 2,3-diphosphoglycerate and triosephosphate isomerase extensively, but not that of membrane protein and 2,3-diphosphoglycerate phosphatase. After the refilling of the ghosts with 2,3-diphosphoglycerate and reconstitution of the membrane, the 2,3-diphosphoglycerate phosphatase activity equals that of intact red cells. The membrane-bound 2,3-diphosphoglycerate phosphatase can be activated by sodium hyposulfite. The enzyme system of ghosts seems to differ from that of intact red cells with regard to the optima of pH and temperature. It remains to be elucidated if the membrane binding of the 2,3-diphosphoglycerate phosphatase is related to the transfer of inorganic phosphate across the red cell membrane.

In human erythrocytes the concentration of 2,3-DPG[1] is three times higher than that of ATP, and much higher than that in other tissue cells. Its formation is catalyzed by the enzyme 2,3-DPG mutase (1,3-diphospho-D-glyceric acid: 3-phospho-D-glycerate phosphotransferase, E.C.2.7.5.4) in a bypass of the glycolytic pathway (Rapoport & Luebering, 1950, 1952). The low activity of the 2,3-DPG-hydrolyzing enzyme, 2,3-DPG phosphatase (2,3-diphospho-D-glycerate 2-phosphohydrolase, E.C.3.1.3.13), and its kinetic properties are thought to be the rate-limiting factors of the 2,3-DPG cycle (Rapoport & Nieradt, 1955; Rapoport, Dietze & Sauer, 1964; Schröter & von Heyden, 1965; Schröter, 1968). This explains the high concentration of 2,3-DPG in erythrocytes.

Because of its low activity, the determination of the 2,3-DPG phosphatase activity is difficult in hemolysates. Therefore, the measurement of the enzyme activity by hydrolysis of 2,3-DPG shows controversial results (De Verdier, 1964; Schröter & von Heyden, 1965; Schröter & Winter,

1 The abbreviations used are: 2,3-DPG = 2,3-diphosphoglycerate; 1,3-DPG = 1,3-diphosphoglycerate; GAP = glyceraldehyde-3-phosphate; and GAPD = glyceraldehyde-3-phosphate dehydrogenase.

1967; Duhm, Deuticke & Gerlach, 1968; Forstner, Fortier & Lionetti, 1968; Sauer, Jentsch & Rapoport, 1968). In comparison, the determination of the 2,3-DPG phosphatase activity, measured by the breakdown of the intracellular 2,3-DPG, shows a relatively uniform hydrolysis of 6 to 8 μmoles 2,3-DPG/10 ml cells per hr during the incubation of the cells without substrate or in the presence of iodoacetate at 37 °C (Keitt, 1966; Forstner & Fortier, 1968; Schröter, 1968; Schröter & Bodemann, 1968). In both intact erythrocytes and hemolysates, the enzyme can be activated by hyposulfite (Mányai & Várady, 1956; Gárdos, 1966; Forstner *et al.*, 1968; Parker, 1969), by disulfite (Duhm *et al.*, 1968) and by pyrophosphate (Harkness, Ponce & Grayson, 1969). These discrepancies suggest a binding of the 2,3-DPG phosphatase to the erythrocyte membrane, possibly one of the two known isozymes (Sauer & Scholz, 1965; Sauer *et al.*, 1968). The membrane-bound enzyme may be inactivated by the lysis of the cells.

In this study, the erythrocytes of normal human subjects were depleted by gradual osmotic hemolysis in hypotonic saline; thus their intracellular components which were not or only loosely bound to the membrane were washed out, i.e., the metabolites, the cytoplasmatic enzymes and the major part of hemoglobin. Then the ghosts were refilled with 2,3-DPG, and the membrane was reconstituted by the addition of hypertonic saline in an amount which rendered the medium isotonic. In this preparation, the activity of 2,3-DPG phosphatase nearly equals that measured prior to the depletion of the cells from 2,3-DPG. The experiments suggest a relatively strong binding of the enzyme to the erythrocyte membrane.

Materials and Methods

Materials

The following biochemical reagents and enzymes were obtained from Boehringer & Soehne, GmbH, Mannheim, Germany: Ba-diethylacetate glyceraldehyde-3-phosphate; cyclohexylammonium 2,3-diphosphoglycerate; cyclohexylammonium phosphoenolpyruvate; Na_2-ATP; Na_2-NADH; reduced glutathione; enolase; glyceraldehyde-3-phosphate dehydrogenase; glycero-1-phosphate dehydrogenase; 3-phosphoglycerate kinase; and phosphoglycerate mutase.

Gradual Osmotic Hemolysis of Human Erythrocytes, Refilling of the Ghosts with 2,3-DPG and Reconstitution of the Membrane

All the following preparations were carried out at 4 °C. A 70-ml sample of heparinized venous blood of healthy blood donors was washed three times with isotonic saline at 1,500 × *g*. The buffy coat was carefully removed. Then the cells were treated in the following way:

2 ml of the cell sediment was resuspended in 2 ml of 300 mosm saline and stored overnight. → Fraction 1

The remaining cell sediment was resuspended in its fivefold volume of 230 mosm saline. After 5 min, the cells were sedimented at 2,000 × *g* for 10 min.

2 ml of the cell sediment was resuspended in 2 ml of 360 mosm saline and stored overnight. → Fraction 2

The remaining cell sediment was suspended in its fivefold volume of 165 mosm saline. After 15 min, the cells were sedimented at 2,000 × *g* for 10 min.

2 ml of the cell sediment was resuspended in 2 ml of 430 mosm saline and stored overnight. → Fraction 3

The remaining cell sediment was suspended in its 10-fold volume of 130 mosm saline. After 15 min, the cells were sedimented at 2,000 × *g* for 20 min.

2 ml of the cell sediment was resuspended in 2 ml of 460 mosm saline and stored overnight. → Fraction 4

The remaining cell sediment was suspended in its 10-fold volume of 100 mosm saline. After 30 min, the cells were sedimented at 2,000 × *g* for 40 min.

2 ml of the cell sediment was resuspended in 2 ml of 495 mosm saline and stored overnight. → Fraction 5

The remaining cell sediment was suspended in its 10-fold volume of 65 mosm saline. After 30 min, the cells were sedimented at 2,000 × *g* for 20 min.

The first half of the cell sediment was suspended in its own volume of 530 mosm saline and stored overnight. → Fraction 6

The second half of the cell sediment was resuspended in its own volume of 65 mosm saline which contained 4.5 mmoles of 2,3-DPG per liter. After 5 min, the medium was made isotonic by the addition of 530 mosm saline in a quantity corresponding to the whole volume of the suspension. The refilled and reconstituted ghosts were washed three times with isotonic saline, resuspended in 300 mosm saline and stored overnight. → Fraction 6a

Determination of Protein, Hemoglobin and 2,3-DPG Phosphatase Activity in the Fractionated Erythrocytes (Fractions 1 to 6) and in Ghosts Refilled with 2,3-DPG (Fraction 6a)

In the first halves of the sediments, 2,3-DPG, protein, hemoglobin and the activity of triosephosphate isomerase were determined. The second halves of the sediments were incubated at 37 °C in 0.05 M glycylglycine buffer, pH 7.5 containing 10 mM phosphate. After 3 hr of incubation, 2,3-DPG, protein and hemoglobin in the sedimented cells were determined.

The activity of *2,3-DPG phosphatase* was calculated as the difference between the 2,3-DPG concentration prior to incubation and at the end of the incubation.

2,3-DPG was determined enzymatically (Schröter & Winter, 1967). The *triosephosphate isomerase* activity was determined according to Schneider, Valentine, Hattori and Heins (1965) in hemolysates. *Protein* was determined with the method of Lowry, Rosebrough, Farr and Randall (1951), and hemoglobin as cyanomethemoglobin (Betke & Savelsberg, 1950).

Non-hemoglobin protein was calculated as the difference between the whole protein and the hemoglobin protein. The *hemolysates* were prepared with digitonin according to Löhr and Waller (1962). The *hematocrit* of the cell suspensions was determined with the ECCO-Mikrohaematokrit centrifuge (Kollatz & Co., Berlin, Germany).

Results

As can be seen from Table 1, the gradual osmotic hemolysis decreases the hemoglobin content of the erythrocytes from 1,830 mg/100 ml suspension in fraction 1 to 61 mg/100 ml in fraction 6. It was impossible to

Table 1. *Delayed osmotic hemolysis of human erythrocytes and refilling of the ghosts with 2,3-DPG*

Protein or enzymatic activity	Red cell fraction[a]						
	1	2	3	4	5	6	6a
	(300)	(230)	(165)	(130)	(100)	(65)	(65)
Whole protein (mg/100 ml suspension)	2,110	1,827	1,770	1,593	782	338	349
Hemoglobin (mg/100 ml suspension)	1,830	1,530	1,480	1,305	503	61	64
Non-hemoglobin protein (mg/100 ml suspension)	280	293	290	288	279	277	285
Non-hemoglobin protein/ hemoglobin protein	0.15	0.19	0.20	0.23	0.56	4.54	4.46
Triosephosphate isomerase activity (μmoles NADH oxidized/min/g non-hemoglobin protein)	3,530	2,850	2,570	2,080	732	42	21
2,3-DPG (μmoles/g non-hemoglobin protein)	148	120	110	90	24	3	126
2,3-DPG phosphatase activity (μmoles 2,3-DPG hydrolyzed/ 3 hr/g non-hemoglobin protein)	66.0	53.4	52.8	55.0	10.3	2.6	48.0

[a] The red cell fractions 1 to 6 were prepared by gradual osmotic hemolysis with hypotonic saline and resuspension of the cells in a medium, which was made isotonic by the addition of hypertonic saline. Numbers in parentheses indicate osmolarity of saline (mosm). Fraction 6a represents ghosts refilled with 2,3-DPG, the membrane of which was reconstituted. For details *see* Materials and Methods. The results of a typical experiment are demonstrated. Three further identical experiments showed only small deviations.

remove totally the hemoglobin from the membranes without their destruction. Therefore, the methods for the preparation of ghosts or of membrane fragments free of hemoglobin as outlined by Dodge, Mitchell and Hanahan (1963), by Green, Murer, Hultin, Richardson, Salmon, Brierley and Baum (1965), and by Marchesi and Palade (1967) were unsuitable in our experiments, in which the membrane should be reconstituted and the ghosts refilled with 2,3-DPG. The decrease in the whole protein is mainly due to the loss in hemoglobin, because the non-hemoglobin remains relatively constant during the fractionation of the cells. The ratio of non-hemoglobin/hemoglobin protein increases from 0.15 to 4.54 during the preparation. This implies a careful treatment of the membrane, because most of the non-hemoglobin protein may be membrane protein. The decrease of the activity of the cytoplasmic enzyme, triosephosphate isomerase, to one-hundredth of its original activity indicates a predominant loss of most of the intracellular components. This was confirmed by the decrease of the 2,3-DPG concentration. At first glance, the activity of the enzyme 2,3-DPG phosphatase seems to decrease from fraction 4 to fraction 6 (Table 1). But the experiments with ghosts refilled with 2,3-DPG (fraction 6a) indicate that the decrease of the enzyme activity is not due to a loss of enzyme protein. In these preparations, not only is the concentration of 2,3-DPG prior to incubation similar to that measured in the intact cells (fraction 1), but also the activity of 2,3-DPG phosphatase remains nearly the same (Table 1). This indicates that an undersaturation of the enzyme with its substrate 2,3-DPG is responsible for the apparent decrease of the enzyme activity in fractions 5 and 6.

It was impossible to restore the apparent reductions in 2,3-DPG phosphatase activity in fractions 5 and 6 by providing the 2,3-DPG to the outside of the membrane during assay. In other words, it was necessary to present the substrate to the 2,3-DPG phosphatase after trapping it inside the ghosts. In both the refilled ghosts with reconstituted membranes and the hemolysates, hyposulfite activates the 2,3-DPG phosphatase. In hemolysates the effect is much more marked than in ghosts. Fig. 1 demonstrates an activation of about 150% of the enzyme activity in ghosts by 10 mM sodium hyposulfite. This activation correlates well with the activation in intact cells as shown by Mányai and Várady (1956), Gárdos (1966) and Parker (1969). Compared to this, sodium hyposulfite in hemolysates increases the enzyme activity to several times its original level (Table 2).

In an attempt to characterize more intensively the enzymes of the hemolysate and ghosts, the effects of pH and of temperature on the 2,3-DPG phosphatase activity were studied. Fig. 2 demonstrates an optimum

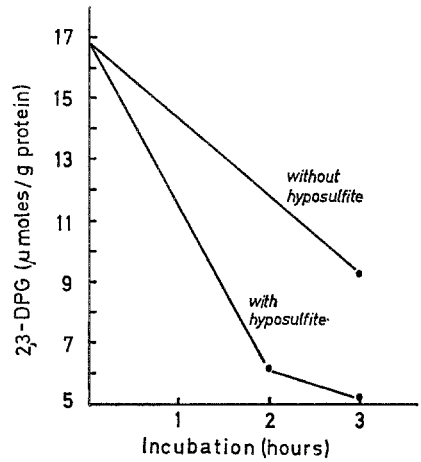

Fig. 1. The effect of hyposulfite on the 2,3-DPG phosphatase activity of ghosts refilled with 2,3-DPG, the membrane of which was reconstituted. The final concentration of hyposulfite was 10^{-2} M

Table 2. *The effect of sodium hyposulfite on the activity of 2,3-DPG phosphatase in hemolysates* [a]

Additions	Time (hr)	2,3-DPG (µmoles/10^9 erythrocytes)	2,3-DPG phosphatase activity (µmoles 2,3-DPG breakdown/4 hr/10^9 erythrocytes)
None	0	0.44	
	4	0.43	0.01
2,3-DPG 6 mM	0	38.2	
	4	38.0	0.2
2,3-DPG 6 mM	0	41.2	
$NaHSO_3$ 10 mM	4	34.8	6.4
2,3-DPG 6 mM	0	40.6	
$NaHSO_3$ 10 mM	4	35.0	5.6
Iodoacetate 1 mM			
$NaHSO_3$ 10 mM	0	0.43	
Iodoacetate 1 mM	4	0.37	0.06
2,3-DPG 6 mM	0	39.8	
Iodoacetate 1 mM	4	39.4	0.4

[a] The hemolysates were prepared with digitonin (Löhr & Waller, 1962). A 0.6-ml sample of the hemolysate was incubated with 0.9 ml of 0.5 M glycylglycine buffer, pH 7.5, at 37 °C for 4 hr. The indicated substances were added to the buffer prior to incubation. After 4 hr the reaction was stopped by boiling in a water bath of 100 °C for 1.5 min. At the beginning and at the end of the incubation, samples were removed for the determination of 2,3-DPG (Schröter & Winter, 1967).

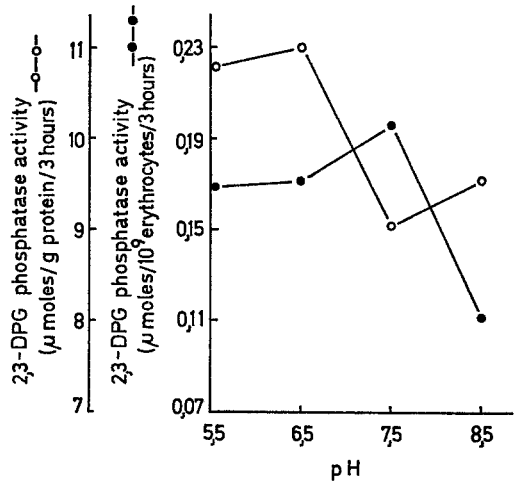

Fig. 2. The effect of pH on the activity of 2,3-DPG phosphatase in intact erythrocytes (•——•) and in ghosts, which were refilled with 2,3-DPG (○——○)

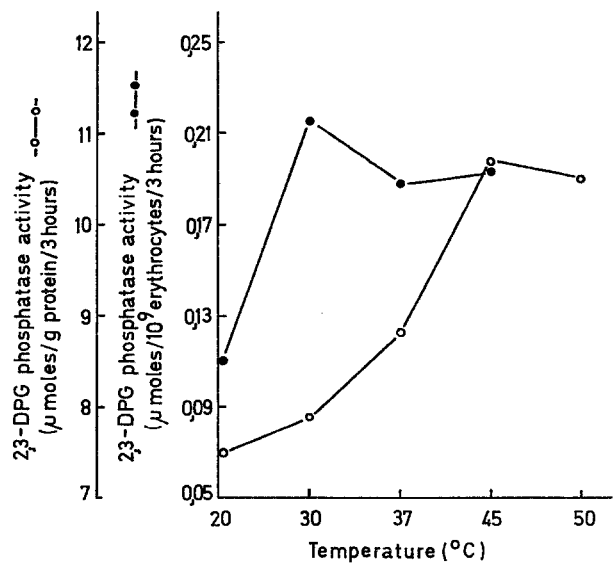

Fig. 3. The effect of temperature on the activity of 2,3-DPG phosphatase in intact erythrocytes (•——•) and in ghosts (○——○) refilled with 2,3-DPG, the membrane of which was reconstituted

of the membrane-bound enzyme of ghosts at pH 6.5, whereas the 2,3-DPG breakdown in intact erythrocytes has its optimum at pH 7.5. The former corresponds with the pH optimum of the mixture of the partly purified two isozymes of the 2,3-DPG phosphatase, as has been shown by Sauer and Scholz (1965). The optimum of the enzyme of ghosts at pH 6.5

suggests either an enzyme which differs from the enzyme of intact erythrocytes or an alteration of the active center, caused by the fixation to the membrane. But the possibility cannot be excluded that the shift in pH optimum may also be explained by a change in the Donnan ratio consequent to the loss of intracellular hemoglobin and ATP.

The effect of temperature on the 2,3-DPG phosphatase activity produces much more marked differences between the two enzyme activities than does the change of the pH (Fig. 3). The membrane-bound enzyme of ghosts exhibits its highest activity at 45 °C. Sauer *et al.* (1968) found the optimum of the partly purified phosphatase at 55 °C. Fig. 3 demonstrates that the highest rate of the 2,3-DPG breakdown in intact erythrocytes has been measured at 30 °C. It was impossible to determine the activity at 50 °C, because too many of the cells hemolyzed at this temperature.

Discussion

The controversial results obtained in measuring the 2,3-DPG phosphatase activity in the hemolysates of human erythrocytes may be partly explained by the experiments presented in this study. As has been shown with 2,3-DPG-refilled ghosts, the membrane of which was reconstituted, the main portion of the 2,3-DPG phosphatase may be located in the membrane or it may be more strongly bound to the membrane than other enzymes of the cytoplasm, i.e., in this study, triosephosphate isomerase. There is only little or negligible 2,3-DPG phosphatase activity in hemolysates, but, after addition of sodium hyposulfite to the incubated hemolysates, 2,3-DPG phosphatase activity can be measured. To determine if there are different enzymes in ghosts and hemolysates, requires further investigation. Probably the process of hemolysis used for the preparation of hemolysates destroys the membrane and releases the enzyme into the hemolysate. The amount of the enzyme released may vary with the method used for hemolysis. In intact erythrocytes, some characteristics of the enzyme differ from those found in refilled ghosts, especially the optima of the enzyme activity for pH and temperature. Other parameters, such as the affinity to 2,3-DPG and the inhibition by 3-phosphoglycerate, cannot be studied in both intact cells and ghosts. The mentioned differences do not prove that the membrane-bound enzyme of ghosts and the enzyme measured in intact erythrocytes are different enzymes or isozymes, because the alteration of the cell milieu by gradual osmotic hemolysis may vary the active center of the membrane-bound enzyme. But the possibility previously proved that there are two or more enzymes in the erythrocyte

hydrolyzing 2,3-DPG cannot be excluded (Sauer & Scholz, 1965; Sauer *et al.*, 1968).

Concerning the general metabolism of the red cell, the significance of the location of the 2,3-DPG phosphatase in or at the cell membrane is not yet clearly understood. The enzyme may be partially adsorbed to the inner aspect of the membrane, as are some other enzymes, such as glyceraldehyde-3-phosphate dehydrogenase (Green *et al.*, 1965; Nilsson & Ronquist, 1969). It remains to be elucidated if the 2,3-DPG phosphatase is connected with the transfer of inorganic phosphate across the red cell membrane according to the hypothesis of Gomperts (1968).

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