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Formation of Adenosine Triphosphate by a Membrane Fraction from Human Erythrocytes

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The enzyme activities of glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase were determined in various fractions during preparation of a membrane fraction from human erythrocytes. The formation of ATP by the purified membrane fraction was also studied.

The glyceraldehyde-3-phosphate dehydrogenase was found to be firmly attached to the membranous structure, and the concentration of this enzyme per mg nitrogen in the exhaustively washed membrane fraction was four times that in the hemolysate.

The phosphoglycerate kinase activity in the membrane fraction was 1/10 that of the glyceraldehyde-3-phosphate dehydrogenase. Practically no difference was found between the activity of these two enzymes in the hemolysate.

The membrane fraction incorporated labeled orthophosphate into ATP if all phosphorylated substrates and cofactors in the glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase reactions were present in the medium.

The incorporation of labeled orthophosphate into 2,3-diphosphoglyceric acid by the membrane fraction was small in comparison to that into ATP. A considerable amount of ATP was formed in an additional way. It is suggested that it is through the adenylate kinase reaction.

In recent years, interest in membranous structures has intensified. Evidence has accumulated indicating that membranes contain special enzymes participating in both transport of substances and control of cell metabolism. It has, *e.g.*, been established by several authors that ATPase* is an

* *Abbreviations:* $^{32}P_i$ = radioactive orthophosphate, ATP = adenosine triphosphate, ADP = adenosine diphosphate, NAD+ and NADH = nicotinamide adenine dinucleotide, oxidized and reduced form. Tris = tris(hydroxymethyl)aminomethane, 3-GAP = glyceraldehyde-3-phosphoric acid, 3-PGA = 3-phosphoglyceric acid, 2,3 DPGA = 2,3 diphosphoglyceric acid, GAPDH = glyceraldehyde-3-phosphate dehydrogenase, PGK = phosphoglycerate kinase, UV = ultraviolet, Hb = hemoglobin.

anisotropic, stromal-bound enzyme that plays a fundamental role in active transport through membranes. (For reviews, see Hokin and Hokin [1] and Judah and Ahmed.[2]) Much of these studies have been performed on erythrocytes.

Thus Gourley [3] and Bartlett [4] suggested that ATP is formed at the red cell surface when orthophosphate is added to red cells. Schrier [5] found a characteristic pattern of enzyme activities in an erythrocyte membrane preparation that did not parallel the enzyme activities in hemolysate.

Ronquist and Ågren [6] recently reported that ATP was formed by human erythrocyte ghosts, as well as by a soluble membrane fraction, when incubated with substrates and cofactors of the glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase. Moreover, under the conditions used the ATP-synthesizing activity per mg nitrogen was higher in the membranous structures than in the hemolysate.

Green *et al.*[7] found a wide divergence in the association of individual glycolytic enzymes with a preparation of beef erythrocyte membranes. They concluded that, in the intact cell, the complete glycolytic complex of enzymes is associated with the plasma membrane and is not free in solution.

An account is given in this paper of the preparation of an exhaustively washed membrane fraction from human erythrocytes. Glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), E.C. 1.2.1.12) and phosphoglycerate kinase (ATP:D-3-phosphoglycerate 1-phosphotransferase, E.C.2.7.3.3.) activities of this fraction has been compared with those of other erythrocyte fractions. The incorporation of radioactive orthophosphate into ATP by the membrane fraction and by a hemolysate under different conditions is also described.

MATERIAL AND METHODS

Preparation of hemolysate and a membrane fraction from human red cells. Human blood was obtained from the University Hospital, Uppsala, shortly after use in a peristaltic heart-lung machine. For some experiments whole blood was drawn from hematologically normal adult donors with heparin as the anticoagulant.

The blood was centrifuged at 4°C, 2000 *g* for 15 min, the plasma and the "buffy" layer removed. The cells were washed 3 times with 3 volumes of 0.85 % sodium chloride and filtered through cottonwool. All preparative steps were carried out at 4°C. The membranes and the hemolysate were stored at 4°C for short periods (up to 3 days) if not used immediately.

The red cells were suspended in 10 volumes of cold, distilled water, and carbon dioxide was led into the mixture for 15—20 min. To avoid foaming, a few drops of octanol-2 were added. Hemolysis was allowed to continue overnight at 4°C, and the resulting hemolysate was centrifuged after 12—15 h for 60 min at 3000 *g*. The ghosts obtained were washed 6 times with 5 volumes of carbon dioxide-water. They settled easily on centrifugation and were used either as such in some experiments, or were further purified. Two volumes of a 0.016 M Tris-glycylglycine buffer, pH 8.2, were added to the faintly rose-colored ghosts, and the mixture was stirred overnight at 4°C. The homogeneous suspension was centrifuged for 60 min at 40 000 *g* in the Spinco ultracentrifuge.

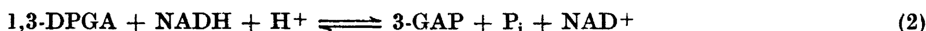
The ghosts, which had disintegrated in the alkaline buffer, were extracted 5 times more after vigorous stirring, each time for 5 min with the same amounts of this buffer and centrifugation for 60 min at 40 000 *g*. The nearly white ghost residue after these washings was called the membrane fraction. A firmly packed red pellet at the bottom of the centrifuge tube was discarded. The pH of the prepared membrane fraction was adjusted to 7.5 with 1 M acetic acid.

Freezing and thawing of membrane fraction. In some experiments, the membrane fraction was frozen by rotating a bottle containing 20 ml of a suspension of the fraction in a freeze-bath at −50°C. The frozen material was stored at −16°C. Thawing was by slow rotation at 37°C.

Concentration of Tris-glycylglycine extract. The pooled, clear 40 000 *g* supernatants were concentrated by ultrafiltration [8] to about 1/15 the original volume. An aspirator bottle of 2 l capacity and 75 cm of Visking dialysis tubing of size 8/32 in. were used, a negative pressure of about 600 mm Hg being applied to the bottle by a vacuum pump.

Determination of glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase activity. The assay method is essentially the same as that given by Bücher [9] and utilizes the two reactions:

$$\text{3-PGA} + \text{ATP} \rightleftharpoons \text{1,3-DPGA} + \text{ADP} \quad (1)$$



$$\text{1,3-DPGA} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{3-GAP} + \text{P}_i + \text{NAD}^+ \quad (2)$$

Reaction 1 is catalyzed by PGK and reaction 2 by GAPDH. The coupled reactions were studied by following the rate of oxidation of NADH at 366 mμ at time intervals up to 15 min. When measuring the GAPDH activity of the sample, PGK was added in excess to the mixture and when the latter enzyme was to be determined GAPDH was added in excess. 0.05 ml of the sample (*e.g.* membrane fraction or hemolysate) was added to a total volume of 3.0 ml to start the reaction at room temperature (about 18°C), and the enzyme activity was determined from the ΔE_{366}/min over the initial linear part of the curve. One unit is defined as that amount of enzyme which will oxidize 0.1 μmole of NADH/min under the aforementioned assay conditions and a cuvette thickness of 1 cm. A check against any possible change in the optical density caused by settling of the ghosts or membrane fraction was provided by shaking the cuvette containing ghosts (or membrane fraction) and reagents and re-reading the optical density at the end of the recording period. Identical readings were obtained in all cases.

Purification of radioactive orthophosphate. Since the commercial $^{32}P_i$* was found to contain small amounts of certain radioactive impurities, it had to be purified by ion exchange chromatography. The carrier-free $^{32}P_i$ was applied to a Dowex 1-X2 (200−400 mesh) formate column and first eluted with 1 N formic acid at a flow rate of about 1 ml/min. After elution for 4 h, the 1 N formic acid was replaced by 4 N formic acid. The migration of $^{32}P_i$ in the column was followed by means of a Panax G M Monitor, and only the eluate with high radioactivity was collected. This was evaporated *in vacuo* at 50°C, dissolved in 1−2 ml of water and deionized by the aid of a Dowex 50 W-X8 (200−400 mesh) column (1 cm² × 2.5 cm). Elution was performed with 3 ml of distilled water under weak positive pressure.

Incubation of membrane fraction and hemolysate with labeled orthophosphate, substrates and cofactors. 19 ml of membrane fraction or hemolysate was incubated for 1 min at 37°C, under agitation with the following cofactors and substrates: $MgCl_2$, 25 μmoles; nicotinamide, 10 μmoles; glutathione (reduced form), 5 μmoles; 3-GAP, 5.9 μmoles; NAD^+, 3.5 μmoles; ADP 25 μmoles; $^{32}P_i$, 2 μmoles; Tris-acetic acid buffer, pH 7.5, 200 μmoles. The final volume was 20 ml. The reaction was stopped by addition of 3 ml of 3 N perchloric acid, followed by neutralization with about 8.7 ml of 1 M potassium hydroxide to a pH of 6.8 to 7 (indicator paper).

The precipitated and neutralized samples were stored for at least 8 h at 4°C before centrifugation. The precipitate was washed once with ice cold distilled water. The combined supernatants were filtered before application to the ion-exchange columns.

Isolation and identification of $AT^{32}P$. The filtered radioactive fractions were passed through Dowex 1-X2 (200−400 mesh) formate columns of 1.1 cm² × 55 cm. Elution

* Radiochemical Centre, Amersham, Bucks., England.

was performed stepwise by a modification [10] of the method of Hurlbert, Schmitz, Brumm and Potter.[11] The systems were as follows: 4 N formic acid, 0.2 M ammonium formate — 4 N formic acid, 0.4 M ammonium formate — 4 N formic acid, and 1 M ammonium formate — 4 N formic acid. Fractions of 9 ml volume were collected at approximately 20 min intervals and the optical density was read at 260 mμ. The radioactivity of the chromatographic fractions was measured on dried aliquots in aluminum cups, using a Sample Changer (L.K.B. Produkter AB, Stockholm, Sweden) fitted with a TGC-2 end-window Geiger tube, and a Compu Matic II Scaler and Auto Printer (Tracerlab, Waltham, Mass., U.S.A.).

The activity of the radioactive fractions was determined on aliquots plated on aluminum cups, using a Tracerlab SC-18 superscaler with a TGC-2 end-window Geiger tube.

The radioactive material eluted with 0.4 M ammonium formate-4 N formic acid (Fig. 1) was lyophilized and, to remove additional ammonium ions, redissolved in 2—3 ml of distilled water and run through a Dowex 50 column of 1.4 $cm^2 \times 4$ cm, which had been dried by suction. Elution was immediately performed with 3 ml of distilled water under weak positive pressure. The ATP-containing eluate was further purified by high-voltage electrophoresis, on Whatman No. 3 paper at 4°C and 60 V/cm (using a Pherograph Original Frankfurt D.B.G.M. No. 1713858, L. Hormuth) and using a 0.05 M citric acid — sodium citrate buffer, pH 3.6.

Radioautograms were taken by placing the dried chromatograms in contact with Ilford Gold Seal X-Ray film for 2 days. A well-defined spot was observed on the radioautogram, which coincided with the UV-absorbing spot in the electropherogram. The spot was cut out and eluted with water. Samples of the eluate were analysed for ATP by measuring the UV absorption at 260 mμ, the molar absorbancy being assumed to be 14.9×10^3 (see Ref. 12), and by determination of ribose and phosphorus contents. All the remaining material with a UV absorbancy value not exceeding 1.0/cm at 260 mμ was dried and hydrolyzed in 0.5 ml of concentrated formic acid for 30 min at 175°C in sealed tubes.

The hydrolysates were analyzed by thin-layer chromatography, together with adenine, guanine, uracil, cytosine, and methylcytosine as reference substances by a method essentially according to Josefsson.[13] Since the UV spot in the sample coincided with the adenine reference and the molar ratios of the base, ribose, and phosphate was 1:1:3, the labeled substance was identified as ATP.

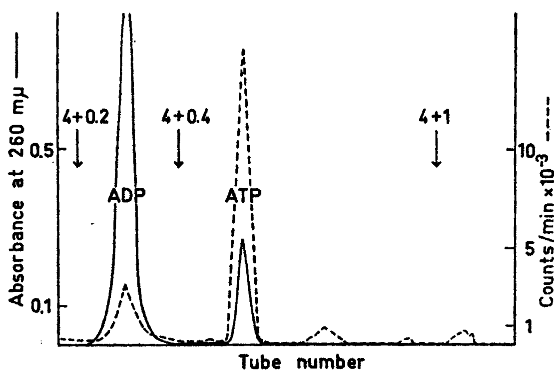


Fig. 1. Dowex 1-X2 (formate) chromatogram of neutralized perchloric acid extract of membrane fraction incubated for 1 min at 37°C with $^{32}P_i$ and all cofactors and substrates in the GAPDH and PGK reactions (see Methods). Only the phosphorus compounds eluted after $^{32}P_i$ are shown in the figure. Elution was performed stepwise as follows: 4 N formic acid; 0.2 M ammonium formate — 4 N formic acid; 0.4 M ammonium formate — 4 N formic acid; 1 M ammonium formate — 4 N formic acid.

Identification of labeled 2,3-DPGA. According to model experiments, 2,3-DPGA could be expected to be eluted by 1 M ammonium formate — 4 N formic acid in the system used. Small amounts of radioactive material were eluted in this region of the chromatogram (Fig. 1). This fraction was concentrated and run through Dowex 50, as described for the ATP fraction. Further identification was performed by ascending paper chromatography on Whatman No. 1 paper at 4°C for 72 h, using solvent A (isopropanol-isoamylalcohol-trichloroacetic acid (5 %, w/v)-lactic acid (72 %, w/w) 15:5:10:0.5) as described by Sekiguchi, Miyamoto, Nakao and Yoshikawa.[14]

Radioautography showed a fairly well-defined spot with R_F 0.42, which did not correspond to any UV-absorbing spot in the chromatogram. In addition, two faint spots were seen, one with R_F 0.62 (corresponding to orthophosphate) and another with R_F 0.25 (unidentified). 2,3-DPGA and orthophosphate were run as reference substances in this solvent system and gave the R_F-values 0.42 and 0.62, respectively. The R_F values for these substances in the same system reported by Sekiguchi *et al.*[14] were 0.44 and 0.65, respectively.

The radioactive compound with R_F 0.42 was eluted with water. The eluate (1.5 ml) was mixed with 0.5 ml of 4 N H_2SO_4 and heated on a boiling water bath for 10 min. During this treatment no phosphate was liberated as orthophosphate. From these results and the behaviour on the Dowex column the substance was identified as 2,3-DPGA.

Analytic methods. Inorganic phosphate was determined by the method of Martin and Doty.[15] Total phosphorus was assayed by the same method after digestion of the samples according to Grunbaum, Schaffer and Kirk.[16] Hemoglobin was determined by the cyanmethemoglobin method.[17]

Nitrogen was determined by a micro-Kjeldahl method. Ribose was estimated according to Mejbaum.[18]

Chemicals. All chemicals were of reagent grade.

RESULTS

Nitrogen and hemoglobin content of various erythrocyte fractions. The presence of significant quantities of hemoglobin in ghosts raises the question of whether an observed property of such ghosts is truly confined to the membrane or represents the activity of some residual intracellular component. The first five carbon dioxide-water extracts of the ghosts were analyzed for Hb and nitrogen content as well as for GAPDH and PGK activity. Fig. 2 shows the values for Hb and nitrogen expressed as g % and mg/ml, respectively. It is obvious that most of the ghost Hb was removed at the first washing. Generally after five washings no measurable Hb could be extracted. It is also seen that

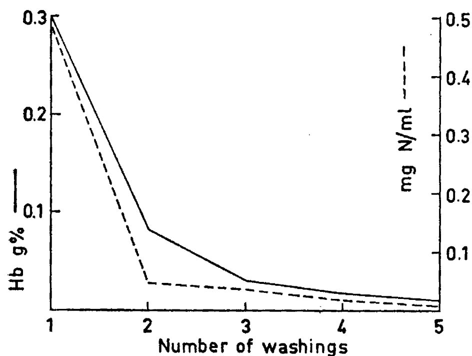


Fig. 2. Hemoglobin (Hb) and nitrogen (N) content of the first 5 washings with carbon dioxide-water.

the nitrogen curve runs parallel to that of the Hb. After washing with carbon dioxide-water the ghosts were further purified by treatment with an alkaline Tris-glycylglycine buffer. This brought about a disintegration of the ghosts and a slight release of Hb from the broken structures. Alkaline Tris-glycyl-

Table 1. Total Hb and nitrogen content as well as total GAPDH and PGK activities of different erythrocyte fractions.

	Total Hb (g)	Total N (g)	Ratio Hb/N	Total GAPDH and PGK activities (in units)	
				GAPDH	PGK
Hemolysate	43.5	8.736	5.0	17450	18065
Carbon dioxide-water washings	11.7	1.934	6.0	7500	7950
Ghosts (washed 6 times with carbon dioxide-water)	not measurable	0.151		1393	328
Tris-glycylglycine extract	0.2	0.060	3.3	108	138
Membrane fraction	not measurable	0.095		625	57

200 ml red cells were hemolyzed as described in Methods. The ghosts obtained after centrifugation were washed first 6 times with carbon dioxide-water and then 6 times more with 0.016 M Tris-glycylglycine, pH 8.2. The ghost residue after these washings was the membrane fraction.

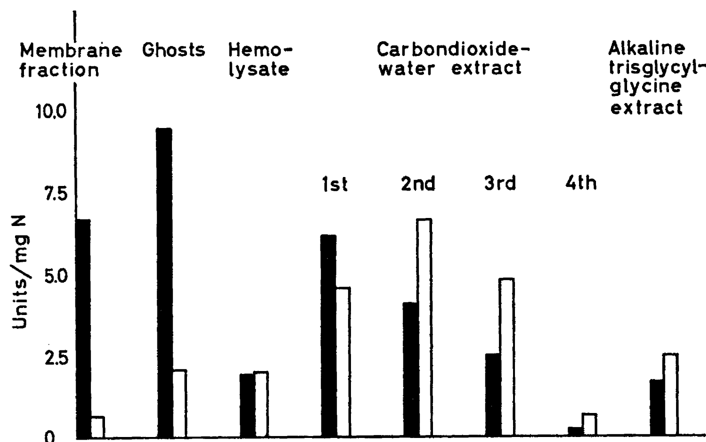


Fig. 3. Specific activity (units/mg nitrogen) of GAPDH (black columns) and PGK (white columns) in the membrane fraction and other erythrocyte fractions obtained during preparation. (For definition of a unit, see Methods). The ghosts were washed 6 times with carbon dioxide-water. The alkaline Tris-glycylglycine extract was concentrated *ca.* 15 times before enzyme determinations.

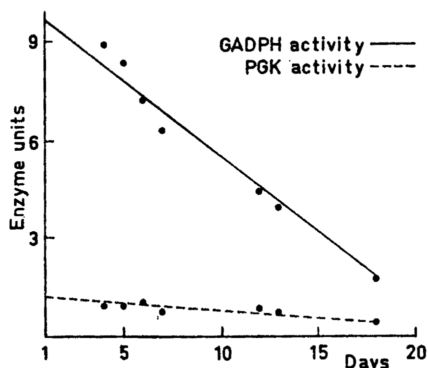


Fig. 4. Stability of GAPDH and PGK activity in 20 ml of prepared membrane fraction during 18 days' storage at 4°C. The enzyme activities are expressed in units/ml membrane fraction. (For definition of a unit, see Methods).

glycine extraction was also tried at a higher buffer concentration (0.1 M) in some experiments but less Hb was found to be extracted by this buffer than by the weaker one (0.016 M). Table 1 gives the total Hb and nitrogen content of various fractions obtained during preparation of the membrane fraction.

Glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase activity in various erythrocyte fractions. Fig. 3 shows the specific enzyme activities (units/mg nitrogen) in the various fractions. The specific GAPDH activity was about four times higher in the ghosts and the membrane fraction than in the hemolysate. This also applied to the first carbon dioxide-water extract. However, the total GAPDH activity of the hemolysate was 28 times higher than that of the corresponding membrane fraction (Table 1).

In the membrane fraction, the specific activity of PGK was 10 times less than that of GAPDH. In the hemolysate, practically the same value was noted for both enzyme activities.

It was also observed that the GAPDH activity in the membrane fraction decreased with time when stored at 4°C. This change in activity was enhanced if the membrane fraction was warmed several times to 20°C. The enzyme activities were followed during a period of 18 days. Fig. 4 illustrates this decrease in enzyme activity with time. It was found, however, that the activity could be kept intact if the membrane fraction was stored at —16°C for only a few days. After 60 days' storage at —16°C, the GAPDH activity had decreased by nearly 50 %, whereas the loss of PGK activity was only about 10 %. Some of the lost activity of GAPDH could be restored if a sulfhydryl-containing reagent (*e.g.* mercaptoethanol, dithiothreitol) was added to the membrane fraction before enzyme assay.

To find out if any ATP or 3-PGA was present in the prepared membrane fraction, the same assay was performed with both GAPDH and PGK present in excess but in the absence of 3-PGA. In another experiment ATP was omitted instead of 3-PGA. In neither case there was any oxidation of NADH.

$AT^{32}P$ formation by the membrane fraction and hemolysate. The membrane fraction and hemolysate were incubated with labeled orthophosphate and the substrates and cofactors of the glyceraldehydephosphate dehydrogenase and phosphoglycerate kinase as described under Material and Methods.

Fig. 1 shows a Dowex 1 chromatogram of the acid extract of an incubated membrane fraction. Only the phosphorus compounds eluted after the $^{32}P_i$ peak are shown in the figure.

In some experiments ADP was omitted from the incubation medium and, as was to be expected, little ATP was formed, but the 2,3-DPGA peak increased if 3-PGA, which serves as a catalyst in the mutase reaction,[19] was present in the incubation system. The hemolysate was used without further treatment as mentioned in Methods. It is therefore not surprising that the ADP present in the hemolysate could react with the other cofactors to form ATP independent of any addition of ADP to the medium (Table 2). When the amount of ATP

Table 2. Incorporation of ^{32}Pi into ADP, ATP, and 2,3-DPGA by the membrane fraction and hemolysate.

Membrane fraction:	µmoles P_i incorporated into ADP/ml red cells × 10^5	µmoles P_i incorporated into ATP/ml red cells × 10^5	µmoles P_i incorporated into 2,3-DPGA/ml red cells × 10^5
1. Ordinary system plus 3-PGA	9.2 (0.180)	37.2 (0.025)	1.1
2. Incubation as 1: ADP omitted	0.04 (0)	0.36 (0)	7.0
3. Control (ordinary system)	3.4 (0.178)	22.0 (0.022)	0.24
Hemolysate:			
1. Ordinary system plus 3-PGA	1081.0 (2.85)	2083.0 (2.8)	36.0
2. Incubation as 1: ADP omitted	721.0 (0.12)	1950.0 (0.2)	330.0
3. Control (ordinary system)	655.0 (3.0)	1192.0 (2.7)	24.0

Membrane fraction and hemolysate incubated for 1 min at 37°C. Besides the substrates and cofactors in the GAPDH and PGK reactions as described in Methods, 5 µmoles of 3-PGA was added to 1 and 2. Incubation volume: 20 ml. Figures in brackets indicate the total amount of ADP and ATP in µmoles per ml red cells calculated on UV extinction basis (see text).

was measured spectrophotometrically (E^{260}) or chemically (total phosphate determination) more ATP was found to have been formed than could be expected from the amount of added ^{32}Pi. This additional ATP formation suggests the presence of adenylate kinase (ATP:AMP phosphotransferase, E.C. 2.7.4.3) in the membrane fraction as well as in the hemolysate. The observation of

the presence of adenylate kinase in the erythrocyte membrane is in accordance with Ascari and Fratantoni [20] as well as with Sen and Post.[21]

ADP was also labeled during the incubation probably as a consequence to the adenylate kinase activity in the membrane fraction. The labeling of this fraction was found to be about 1/6 of that of ATP in several experiments.

In another series of experiments the membrane fractions were incubated in the ordinary medium but with different orthophosphate concentrations. It appears (Table 3) that the additional formation of ATP is not affected by

Table 3. Membrane fraction incubated with different orthophosphate concentrations.

	ADP μmoles P_i incorporated into ADP/ml red cells $\times 10^5$	ATP μmoles P_i incorporated into ATP/ml red cells $\times 10^5$
Ordinary system	3.2	20.6
10^{-4} M P_i (control)	(0.189)	(0.025)
Ordinary system	14.6	71.4
10^{-3} M P_i	(0.185)	(0.024)
Ordinary system	2.6	15.3
10^{-2} M P_i	(0.184)	(0.024)
Ordinary system	0.6	5.3
10^{-1} M P_i	(0.184)	(0.027)

Membrane fraction incubated for 1 min at 37°C in the ordinary medium (see Methods) together with increasing concentrations of orthophosphate. Incubation volume: 20 ml. Figures in brackets indicate the total amount of ADP and ATP in μmoles per ml red cells calculated on UV extinction basis (see text).

varying the orthophosphate concentration (range: 10^{-4} M—10^{-1} M) while there is an inhibition of the orthophosphate incorporation into ATP in the GAPDH and PGK steps at the concentrations 10^{-2} M and 10^{-1} M. Maximal incorporation into the ATP fraction was obtained at 10^{-3} M orthophosphate concentration.

DISCUSSION

Previous work in this laboratory [6] showed that formation of $AT^{32}P$ (calculated per mg nitrogen) by erythrocyte membranous structures incubated with $^{32}P_i$ and the substrates and cofactors of GAPDH and PGK was, in fact, higher than that by the hemolysate under corresponding conditions. In the present paper the activities of GAPDH and PGK have been measured and the specific activity (in units/mg nitrogen) of GAPDH was four times higher in the thoroughly washed membrane fraction than in the hemolysate. The specific activity of PGK in the membrane fraction was, on the other hand, 1/3 of that in the hemolysate. It is thus concluded that GAPDH in contrast to PGK, is firmly bound to the membrane (Fig. 3). This is in agreement with

the findings of Schrier [5] and Green *et al.*[7] However, the membrane-hemolysate ratios of the two enzymes given by these authors are different from those reported in this paper. This can probably be explained to a certain extent by the fact that the membranes were prepared under different conditions, *e.g.* different pH and osmotic strength in the buffers. Schrier prepared the membranes by dialysis of whole erythrocytes against increasingly hypotonic salt solutions in which the erythrocytes retained their bioconcave shape. Green *et al.* had a membrane fraction which had been obtained by washing beef erythrocytes up to only three times. It has been claimed that even slight changes in pH and osmotic strength will grossly alter the final stromal composition.[22] In the present study, the membrane fraction prepared had been exhaustively washed 12 times in all. The alkaline Tris-glycylglycine treatment brought about a disintegration of the membranes. It is difficult to say whether the activities measured are from enzymes originally bound to the membrane or from intracellular enzymes associated to the membrane during preparation. It has been claimed that the properties of enzymes may vary according to whether they are in solution or bound to particles.[23] Maybe, a part of the erythrocyte enzymes is localized in the membranous structure appearing in close vicinity to each other to facilitate coupled reactions.

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