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Existence of only a single functional pool of adenosine triphosphate in human erythrocytes

(red cell/metabolism/membrane)

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ABSTRACT **The question of whether separate "membrane" and "soluble" pools of ATP exist in erythrocytes has been examined. Phosphoglycerate kinase (EC 2.7.2.3)-derived ("membrane") ATP was labeled by short-term incubation with inorganic [^{32}P]phosphate. Pyruvate kinase (EC 2.7.1.40)-derived ("soluble") ATP is not labeled under these circumstances. The specific activity of the γ-phosphate of "soluble" ATP was then evaluated by the addition of 2-deoxyglucose and measurement of the specific activity of 2-deoxyglucose-6-[^{32}P]phosphate formed. This specific activity was essentially the same as the overall specific activity of erythrocyte ATP γ-phosphate, indicating that no functional pools of phosphoglycerate kinase-derived and pyruvate kinase-derived ATP exist in erythrocytes.**

Lacking nucleus, mitochondria, and other intracellular organelles, the erythrocyte was long considered to be a simple, single metabolic unit. However, the association of certain glycolytic enzymes with the erythrocyte membrane (1) spawned an interesting hypothesis. It was proposed that two pools of ATP exist in erythrocytes and that a membrane-associated pool of ATP is preferentially involved in the pumping of sodium and potassium ions. The ATP in this pool would be replenished by the action of phosphoglycerate kinase (EC 2.7.2.3) (2–5). A second, soluble pool of ATP, replenished in the pyruvate kinase (EC 2.7.1.40) reaction, presumably provides ATP for phosphorylation of glucose in the initial steps of glycolysis.

Certain features of this model are attractive. The ATP used for sodium and potassium transport would be produced in proximity to the site where it functions. Moreover, the amount of ATP required for glucose phosphorylation very closely approximates the amount of ADP that is phosphorylated to ATP in the pyruvate kinase step. Attractive as the two-compartment model of ATP metabolism in the erythrocyte may be, no direct experimental evidence supports its existence. The purported existence of a specific linkage between ATPase activity and ATP generated in the phosphoglycerate kinase step was initially based upon the failure of ouabain to inhibit lactate production from phospho*enol*pyruvate in crude hemolysates. However, this interpretation failed to take into account the reversibility of the phosphoglycerate kinase step, and reinterpretation of the data shows them to be irrelevant to the question of selective linkage of ATP produced in the phosphoglycerate kinase step to ion pumping (6). Other data that have been presented to support the existence of a membrane-associated ATP pool are quite indirect. For example, the effect of ouabain on crossover plots (7), the lack of effective ion transport in substrate-depleted erythrocytes in which 2,3-diphosphoglycerate was the source of glycolytic energy (5), and the blocking of sodium-magnesium-ATPase activity exerted on ^{32}P-labeled ATP by pretreatment in the cold with nonradioactive ATP (2) have been cited as evidence for the existence of two ATP pools. However, a study of phosphoglycerate kinase-deficient erythrocytes failed to show any decrease in ion pumping (4).

We have now devised a technique that allows us to examine under more physiological circumstances the possible existence of two ATP pools in erythrocytes. This method depends upon the fortunate circumstance that the inorganic phosphate consumed during glycolysis is quickly incorporated into the γ-phosphate of ATP formed in the phosphogylycerate kinase reaction. In contrast, the phosphate transferred from phospho*enol*pyruvate to ADP is derived almost entirely from phosphate esterified to glucose in the early stages of glycolysis (Fig. 1). Thus, it is possible to selectively label ATP formed in the phosphoglycerate kinase reaction with [^{32}P]phosphate. The specific activity of the putative soluble ATP pool may readily be estimated by measuring the labeling of 2-deoxyglucose phosphorylated in the hexokinase reaction to its nonmetabolizable derivative, 2-deoxyglucose-6-phosphate.

MATERIALS AND METHODS

Blood from normal human donors was collected in 1 mg of EDTA per ml of erythrocytes and passed through a column of microcrystalline cellulose-α-cellulose (8) to remove the leukocytes and platelets. The erythrocytes were then washed three times at 25° in a buffered saline solution prepared by mixing nine parts of 0.154 M NaCl solution with one part of 0.15 M triethanolamine hydrochloride, pH 7.8 (25°), and containing 0.3 mM glucose. Sixty milliliters of a 45% suspension of the erythrocytes was prepared in the same buffer and 180 μCi of $H_3{}^{32}PO_4$ (ICN, Irvine, CA) was added. After 4 min of incubation at 37°, 20 ml of the erythrocyte suspension was rapidly transferred into 80 ml of ice-cold 4% perchloric acid. 2.1 ml of 2 M 2-deoxyglucose in 0.77 M K_2HPO_4/0.23 M KH_2PO_4 was immediately added to the remaining 40 ml of the erythrocyte suspension; after 2 min this suspension was mixed rapidly with 160 ml of ice-cold 4% perchloric acid. The acid-extracted erythrocytes were centrifuged in the cold and the supernatant was adjusted to pH 7.0 with KOH. Aliquots were removed for measurement of lactate and ATP concentrations (9). The extracts were then diluted with 6 vol of water and applied to Dowex-1 formate (1.5 × 25 cm) columns at a flow rate of 1 ml/min. Chromatography was carried out by modifications of the methods described by Bartlett (10). First each extract was eluted with a 2-liter linear gradient of 0–5 M formic acid/ammonium formate (4:1) adjusted to pH 2.9 with 5 M ammonium formate. The peak containing 2-deoxyglucose-6-phosphate, eluting just before the inorganic phosphate peak, was pooled and lyophilized. After the sample was reconstituted with 3.0 ml of water, 0.03 ml of 0.5 M $MgCl_2$, 0.5 ml of 1 M Tris·HCl

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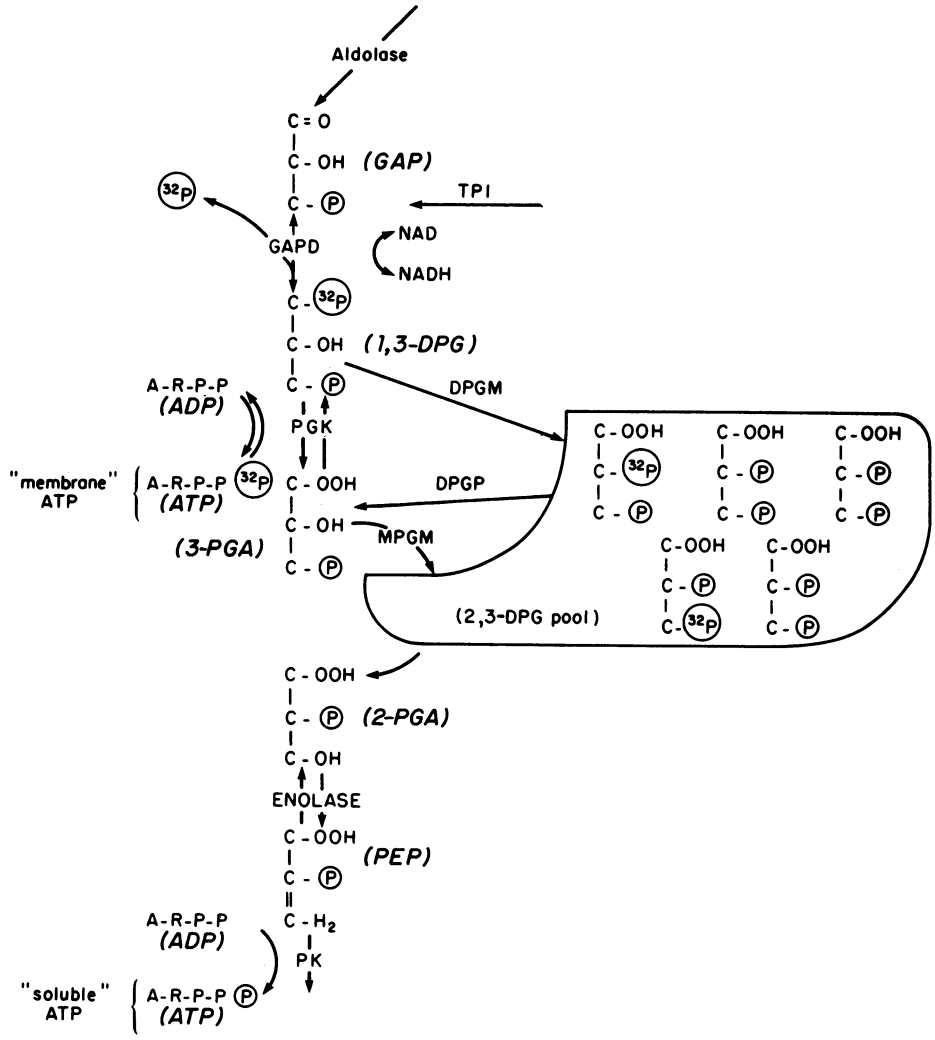

FIG. 1. A portion of the glycolytic pathway of erythrocytes showing the manner in which inorganic ^{32}P preferentially enters ATP synthesized in the phosphoglycerate kinase (PGK) reaction. This putative pool of heavily labeled ATP has been designated "membrane" ATP. In contrast, ATP synthesized in the pyruvate kinase (PK) step, designated "soluble" ATP, is labeled only to the extent that ^{32}P has gained access to the large 2,3-diphosphoglycerate (2,3-DPG) pool through the diphosphoglycerate mutase (DPGM) reaction. TPI, triosephosphate isomerase; DPGP, diphosphoglycerate phosphatase; MPGM, monophosphoglycerate mutase; GAP, glyceraldehyde phosphate; GAPD, glyceraldehyde phosphate dehydrogenase; PEP, phospho*enol*pyruvate; 3-PGA, 3-phosphoglyceric acid.

(pH 8, with 5 mM EDTA), 0.6 unit of glucosephosphate isomerase and 0.6 units of yeast glucose-6-phosphate dehydrogenase (Sigma Type VII), and 0.03 ml of 100 mM NADP were added and the mixture was incubated for 30 min to oxidize glucose-6-phosphate and fructose-6-phosphate to 6-phosphogluconic acid. Ammonium ions were then removed by passing the solution through a column of Dowex-50 in the hydrogen form. After the mixture was diluted to 150 ml with distilled water, the 2-deoxyglucose-6-phosphate was applied to a 1 × 8 cm Dowex-1 column and rechromatographed with a 1-liter gradient of 0–1 M formic acid. The peaks containing 2-deoxyglucose-6-phosphate were lyophilized and dissolved in a small quantity of distilled water, and the 2-deoxyglucose-6-phosphate content and radioactivity were determined. 2-Deoxyglucose-6-phosphate was measured by hydrolyzing the phosphate group with alkaline phosphatase and measuring the ADP formed in the hexokinase reaction when the 2-deoxyglucose was rephosphorylated. The pH was adjusted to between 8 and 10 with ammonium hydroxide. Two-tenths milliliter of alkaline phosphatase, 50 mg/ml (Sigma) in 0.2 M glycine buffer, titrated to pH 10.7 with NH_4OH and containing 20 mM magnesium chloride, was added to 0.5 ml of the neutralized fraction and the mixture was incubated at 37° for 2 hr. The pH was then adjusted to between 3 and 4 with 4 M HCl, and the mixture was boiled for 1 min. After centrifugation the extract was neutralized with potassium carbonate, and the 2-deoxyglucose content was measured in a volume of 1 ml containing 0.1 M Tris·HCl (pH 8), 0.5 mM EDTA, 5 mM $MgCl_2$, 2 mM ATP, 1 mM phospho*enol*pyruvate, 0.2 mM NADH, 12 units of pyruvate kinase, and 20 units of lactate dehydrogenase. After the absorbance was recorded, at 340 nm, the reaction was started by the addition of 15 units of yeast hexokinase (Sigma). In this system 1 mole of ADP is formed in the hexokinase reaction for each mole of 2-deoxyglucose phosphorylated. The ADP is measured in the pyruvate kinase reaction linked to the lactate dehydrogenase reaction. One mole of NADH is therefore oxidized for each mole of 2-deoxyglucose present.

ATP was located in the original chromatogram by use of the hexokinase reaction (9). The fractions from this peak were pooled and lyophilized to remove most of the formic acid/ammonium formate, and redissolved in water. The γ-phosphorus was then determined by transferring it to glucose in the

Table 1. Specific activity of phosphorylated intermediates and concentrations of lactate, ATP, and 2-deoxyglucose-6-phosphate in erythrocytes

	4 min	6 min
Specific activity*		
γ-PO_4 of ATP	188, 195, 165	130, 136, 139
2,3-Diphosphoglycerate	1.4, 1.2, 1.1	3.3, 3.4, 2.9
2-Deoxyglucose-6-*P*	—	140, 148, 145
Lactate, mM	1.015	1.034
ATP, mM	0.80	0.75
2-Deoxyglucose-6-*P*, mM	—	0.12

Each set of three specific activity figures represents the three peak tubes of the chromatogram. The values designated "4 min" represent the labeling after 4 min of incubation with $^{32}PO_4^{3-}$. At this time inorganic phosphate and 2-deoxyglucose were added. The values designated "6 min" were obtained after 2 min of further incubation.
* μCi/mmol.

hexokinase reaction and determining the specific activity of the glucose-6-phosphate formed. The solution was made 90 mM with respect to glucose and 9 mM with respect to magnesium chloride; the pH was adjusted to 8.2 by addition of 2 M Tris, and hexokinase was added to a concentration of 10 units/ml. After incubation for 0.5 hr the mixture was applied to a 1 × 8 cm Dowex-1 column and eluted with a 1-liter linear gradient of 0–5 M formic acid/ammonium formate, pH 2.9. The fraction containing glucose-6-phosphate was passed through Dowex-50 in the hydrogen form and was rechromatographed on Dowex-1 with a 0–1 M formic acid gradient to separate it clearly from inorganic phosphate. The concentration of glucose-6-phosphate was estimated by using glucose-6-phosphate dehydrogenase and NADP (9). ^{32}P radioactivity was measured in Scintisol (Isolab) in a liquid scintillation counter.

RESULTS

The results of a typical experiment are summarized in Table 1. As expected, the specific activity of the ATP was much higher than that of 2,3-diphosphoglycerate. The radioactivity of ATP declined slightly, presumably because of the dilution of the radioactive phosphate with a large amount of inorganic phosphate added at 4 min. The radioactivity of 2,3-diphosphoglycerate, however, showed a slight rise but remained much lower than the specific activity of ATP. The specific activity of the 2-deoxyglucose-6-phosphate was intermediate between the specific activity of the γ-phosphate of ATP at the time of addition of 2-deoxyglucose and that at the end of the experiment.

DISCUSSION

It has been proposed that two pools of ATP exist, a membrane-associated pool and a soluble pool. It has been suggested that the putative membrane pool of ATP represents that ATP which is formed in the phosphoglycerate kinase reaction. It is a happy circumstance that the γ-phosphorus of ATP formed in this reaction is derived entirely from inorganic phosphate, while ATP formed in the pyruvate kinase reaction is derived almost entirely from phosphate transferred to hexose in the early steps of glycolysis (see Fig. 1). A very small amount of labeled inorganic phosphate may be incorporated into the phosphate of phospho*enol*pyruvate through the randomization of the phosphate of 2,3-diphosphoglycerate in the monophosphoglycerate mutase reaction. If 1,3-diphosphoglycerate is converted to 2,3-diphosphoglycerate in the diphosphoglycerophosphate mutase reaction, the 2-phosphate of 2,3-diphosphoglycerate becomes labeled. This phosphate equilibrates with the phosphate at the 3-position in the monophosphoglycerate mutase reaction (Fig. 2). In this reaction, the 3-phosphate of 2,3-diphosphoglycerate is transferred to the 2-position of 3-phosphoglyceric acid, and through this reaction the phosphate at the 2- and 3-positions of 2,3-diphosphoglycerate quickly become randomized (11). Thus the labeling of phospho*enol*pyruvate serving as substrate for the pyruvate kinase reaction may be estimated from the specific activity of phosphate in the 2,3-diphosphoglycerate pool. However, the specific activity of the pyruvate kinase-derived ATP pool is in reality much lower than the specific activity of phosphate in the 2,3-diphosphoglycerate pool, since only a small amount of ATP is formed in this reaction during the short time periods involved in our labeling experiments. The precise quantity of the ATP synthesized per min in the pyruvate kinase reaction can be determined by measuring the amount of lactate formed per min since no mechanisms exist for the further metabolism of lactate and pyruvate in erythrocytes. The greatest portion of erythrocyte ATP must of necessity be in the "soluble" pool, and we assume that in our experiment the concentration of ATP in this pool might be of the order of 0.7 mM. Since lactate production during the period of labeling was only 0.019 mM or 42 μmol/ml of erythrocytes, only approximately 5.4% of the soluble pool would have been turned over during the experiment. The specific activity of this "soluble" pool at approximately 0.12 μCi/mmol stands in sharp contrast to the specific activity of about 160 μCi/mmol of the γ-phosphorus of the combined membrane and soluble pools. If the 2-deoxyglucose-6-phosphate had been formed from a very slightly labeled pyruvate kinase-derived pool of ATP, its specific activity would have been equal to that of this pool. If, on the other hand, only a single pool of ATP actually existed in the erythrocyte, the specific activity of the 2-deoxyglucose-6-phosphate should be the same as the average specific activity of ATP during the time of its formation. Experimentally, we found the specific activity of 2-deoxyglucose-6-phosphate formed to be essentially identical to the latter specific activity.

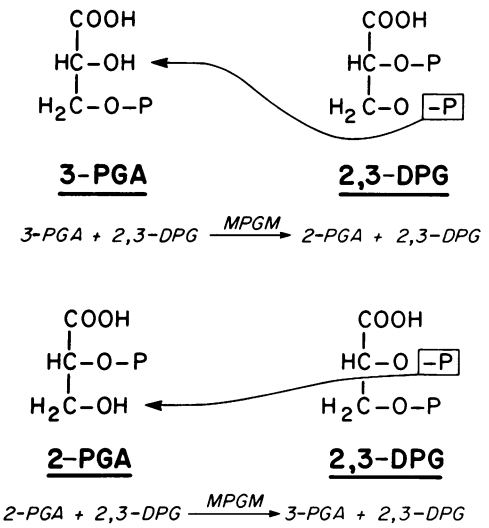

FIG. 2. Monophosphoglyceromutase (MPGM) reaction. The conversion of 3-phosphoglyceric acid (3-PGA) to 2-phosphoglyceric acid (2-PGA) results in randomization of the phosphate of 2,3-diphosphoglycerate (2,3-DPG).

Our studies clearly show that all erythrocyte ATP is equally available for sugar phosphorylation by hexokinase; there is no division of ATP into functional pools. While one might argue

that two pools exist but are in rapid communication with one another, this concept loses credibility in view of the time period of but 2 min encompassed by our experiment. Pools that equilibrate completely in this period of time can hardly be considered to be pools at all. Moreover, the indirect data cited to support the existence of ATP pools were obtained from studies conducted over much greater time periods and could therefore not have reflected the effects of functional pool systems of the erythrocyte. We propose that the data that have been interpreted as indicating that separate "membrane" and "soluble" pools of ATP exist do not reflect the state of ATP in the living erythrocyte, and require reinterpretation.

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