The Role of Membrane Phosphoglycerate Kinase in the Control of Glycolytic Rate by Active Cation Transport in Human Red Blood Cells

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ABSTRACT When the internal Na of human red cells is raised, both K influx and lactate production increase and become more sensitive to the inhibitory action of ouabain. This occurs with either glucose or purine nucleoside as substrate. Fresh whole hemolysates enriched with Na and Mg will convert intermediates above the triose phosphate dehydrogenase step to lactate at a rate which is slowed by ouabain. Intermediates beyond the phosphoglycerate kinase step (PGK) are metabolized at a very rapid rate which is not affected by ouabain. No metabolic effects of ouabain were found in ghost-free hemolysates. Hemoglobin-free ghosts were shown to have both triose phosphate dehydrogenase and PGK activity. The rate of this two-enzyme sequence was found to be a function of the ADP concentration, being maximal when ADP $>$ 0.35 mM. Initial addition of ATP to the ghost system rendered the forward rate of the sequence sensitive to the inhibitory action of ouabain. When the sequence was run in reverse, no inhibitory effect of ouabain could be demonstrated. It is concluded that membrane PGK is a point at which the Na-K transport system can influence the metabolic rate and that this action is possibly exerted via a compartmentalized form of ADP which is an immediate substrate for the ghost PGK.

INTRODUCTION

Adenosine triphosphate is a specific, proximate source of energy for the active transport of Na and K by mature red blood cells (Hoffman, 1962), which resynthesize ATP[1] exclusively by the substrate level phosphorylations of the

[1] Abbreviations: ATP, ADP, AMP = adenosine tri-, di-, and monophosphate; ITP inosine triphos-

Embden-Meyerhof pathway. Recently Whittam and Ager (1965) have demonstrated in human red cells that the processes of active cation transport and glucose metabolism are each capable of influencing the other. The rate of Na-K pumping in red cells which are substrate-limited depends on the extent to which ATP levels are maintained by the reactions of the glycolytic sequence; conversely, the rate of glycolysis in cells supplied with ample substrate is a function of their rate of active cation transport. In the present report evidence will be presented that a major rate-determining step in the metabolism of glucose to lactate by human red blood cells is the conversion of 1,3-DPG + ADP to 3PG + ATP catalyzed by phosphoglycerate kinase (PGK). It will be proposed that this enzyme is the point at which the active transport mechanism can influence the glycolytic rate of the cell and that the interaction between the cation pump and PGK occurs on or in the cell membrane. Three systems were studied: (*a*) intact red cells whose cation contents had been altered by storage in media of varying composition, (*b*) hemolysates of red cells in the presence and absence of ghosts, and (*c*) washed, hemoglobin-free ghosts. A brief account of the results obtained has been reported previously (Parker and Hoffman, 1966).

MATERIALS AND METHODS

The following reagents were obtained from commercial sources: (ATP, ADP, AMP, G6P),[2] (FDP, Gly3P, 3PG, PEP, pyruvate, lactate, NAD, NADH, NADP, GSH),[3] adenosine,[2] and ouabain.[2, 3] Hexokinase, glucose-6-phosphate dehydrogenase, aldolase, triose phosphate isomerase, α-glycerophosphate dehydrogenase, pyruvate kinase, and lactic dehydrogenase were all obtained from C. F. Boehringer & Sons, Mannheim, Germany.

Studies with Intact Red Cells

Human blood was drawn into heparinized bags.[4] Following removal of the plasma and buffy coat, the red cells were washed three or four times with the solution in which they were to be stored. Two storage solutions were used: one consisted of NaCl 150 mM, Na phosphate 15 mM, and glucose 10 mM (pH 7.5); the other solution was identical except that K salts were used in place of Na. After the final wash, the cells

phate; G6P = glucose-6-phosphate; FDP = fructose-1,6-diphosphate; Gly3P = glyceraldehyde-3-phosphate; Triose-P = the sum of glyceraldehyde-3-phosphate plus dihydroxyacetone phosphate; 1,3-DPG = 1,3-diphosphoglycerate; 2,3-DPG = 2,3-diphosphoglycerate; 3PG = 3-phosphoglycerate; PEP = 2-phosphoenolpyruvate; NAD, NADH, NADP, NADPH = oxidized and reduced forms of nicotinamide-adenine dinucleotide and nicotinamide-adenine dinucleotide phosphate, respectively; P_i = inorganic phosphate; AsO_4 = arsenate; EDTA = ethylene diamine tetraacetic acid; GSH = reduced glutathione; ATPase = adenosine triphosphatase; PGK = phosphoglycerate kinase.

[2] Sigma Chemical Company, St. Louis, Mo.

[3] Calbiochem, Los Angeles, Calif.

[4] Fenwal Laboratories, Framingham, Mass.

were suspended at a hematocrit of 15 and stored at 4°C with constant, slow agitation for 7–10 days during which they were washed and restored once. On the day of an experiment the stored cells were washed three times with the solution in which they were to be incubated. Unless otherwise noted, the composition of the incubation solution was NaCl 153 mM, KCl 5 mM, glycylglycine 20 mM, Na phosphate 2 mM, plus adenosine 2 mM (pH 7.5). At the start of the incubation period the cell suspensions (Hct 15-30) were placed in a 37°C water bath with constant shaking. At intervals aliquots were removed from each flask and either centrifuged at 20,000 *g* (4°C) for determination of isotopic flux or pipetted immediately into cold, swirling perchloric acid for chemical analysis.

Potassium influx was determined by measuring the rate at which ^{42}K added to the suspension at the beginning of the incubation period entered the cells. Radioactivity was determined by means of a well-type scintillation counter. The Na and K content of the cells and medium were measured by flame photometry. K influx was calculated as the counts entering the cells per unit time divided by the specific activity of the extracellular K. Relative hemoglobin concentration was calculated from absorption at 540 mμ by means of a Beckman Model DU Spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Hematocrit was calculated by dividing the hemoglobin concentration of the lysed, whole suspension by that of the packed red cells.

Measurement of Glycolytic Intermediates

Aliquots of cell suspension were pipetted into three times their volume of cold, swirling, 20% perchloric acid, mixed, allowed to stand for 60 min at 0°C and centrifuged. The clear supernatant was adjusted to pH 7.0–7.5 with K_2CO_3 using phenol red as an indicator, and recentrifuged. Appropriate dilutions of this neutralized extract were prepared for the enzymatic assays. All glycolytic intermediates were assayed by means of enzymatic reactions culminating in the stoichiometric oxidation or reduction of pyridine nucleotide, exactly as described in Bergmeyer (1963): FDP and triose-P were determined with α-glycerol phosphate dehydrogenase, triose phosphate isomerase, aldolase, and NADH; lactate, with lactic dehydrogenase and NAD; ATP, with glucose-6-phosphate dehydrogenase, hexokinase, and NADP; ADP and AMP, with lactic dehydrogenase, pyruvate kinase, myokinase, and NADH. Reduced pyridine nucleotide was measured at 340 mμ on a Beckman Model DU Spectrophotometer in 1 cm quartz cuvettes. Extinction coefficient for both NADH and NADPH at this wavelength was taken as 6.22×10^6 cm²/mole. Recovery of all compounds from the perchloric acid extract was 97–100%, and duplicate samples from the same extract agreed within 2%.

Studies with Hemolysates and Hemolysis Mixtures

The term *hemolysis mixture* will be used to apply to a whole lysed suspension of red cells. *Hemolysate* will refer to the supernatant of a hemolysis mixture from which the ghosts have been removed by centrifugation at 20,000 *g*. Red cells from freshly drawn, heparinized blood were washed with a solution which contained NaCl 153 mM and Tris 17 mM (pH 7.5). One volume of packed, washed cells was injected

rapidly into 4 volumes of swirling distilled water at 0°C, and the mixture was stirred for 3 min after which 0.5 volumes of 0.25 M nicotinamide was added. The hemolysis mixture was either promptly frozen at −20°C or centrifuged at 20,000 *g* and the ghost-free supernatant frozen. The frozen preparations were used within 7 days. On the day of an experiment the mixture was thawed and divided into samples to which various substrates and salts were added. All additives were dissolved in a tris-buffered solution (pH 7.5) of which 3 volumes were added to 9 volumes of hemolysis mixture, giving the final concentrations noted in the tables. At the beginning of a two-hour incubation, the mixtures were placed in a 37°C water bath with constant shaking, and samples were removed at 30- or 60-min intervals for the determination of lactate, and in some cases the ATP and ADP content. The enzymatic assays were performed on an extract made by injecting 1 volume of hemolysis mixture into 4 volumes of swirling, cold 10% perchloric acid. Neutralization and assays of the extracts were performed as noted for the studies in intact cells.

Studies with Hemoglobin-Free Ghosts

Fresh red cells were washed in the manner described in the preparation of hemolysis mixtures. Ghosts were prepared by the method of Hoffman and Ryan (see Parker and Hoffman, 1964) in which 1 volume of packed, washed red cells was introduced rapidly into 9 volumes of ice cold, swirling distilled water which contained Na_2EDTA 0.0001 M (pH 7.5). The ghosts were centrifuged at 20,000 *g* and washed in a solution containing Tris 0.0170 M and EDTA 0.0001 M (pH 7.5) until they were cream-white. It was shown by the pyridine hemochromogen method of Dodge et al. (1963) that the ghosts used in the present study contained less than 0.1% of the hemoglobin present in the intact red cells from which they were prepared. Following the final wash the ghosts were frozen at −20°C, thawed, and stored at 4°C for no longer than 7 days. Experiments were done with the ghosts suspended in various media and incubated at 37°C in a water bath with constant shaking. Substrates and cofactors necessary for studying the triose phosphate dehydrogenase and phosphoglycerate kinase reactions were included in the incubation media as noted in the following section. At time intervals, aliquots of the ghost suspension were removed and centrifuged at 0°C and 20,000 *g*. Appropriate dilutions of the clear supernatant were read directly in a spectrophotometer at 340 mμ and were also assayed for adenine nucleotide content. Prior to the enzymatic assays the supernatant was acidified (final HCl concentration 0.1 N), allowed to stand for 1 hr at 0°C and then neutralized with crystalline K_2CO_3 with phenol red as indicator. By this means reduced pyridine nucleotides present prior to the assay were destroyed.

RESULTS

Studies with Intact Red Cells

It is known that the rate of active cation transport in red cells is a function of the intracellular Na concentration (Post and Jolly, 1957). By storing cells at 4°C in media containing all Na or all K salts, cells were prepared which, at the end of the cold storage period, had a wide range of Na concentrations.

Table I shows the effect of ouabain, a specific inhibitor of Na and K transport (Schatzmann, 1953), on K influx and lactate production in cells whose electrolyte concentrations had been modified by cold storage and which were incubated with adenosine as substrate. Both K influx and lactate production were linear over the 2 hr period of the incubation. It is clear that K influx and lactate production are higher in high Na than low Na cells. These same

TABLE I

EFFECTS OF OUABAIN AND VARIATIONS IN INTERNAL Na ON INTACT CELLS

	No. of experiments	High Na cells	Low Na cells
Intracellular Na mM/l cells	9	44–60	6–14
Potassium influx mM/l cells-hr	3	4.8±0.3	1.7±0.2
+ ouabain		0.5±0.1	0.4±0.1
Lactate production mM/l cells-hr	9	3.0±0.4	1.9±0.3
% ouabain inhibition		37±7	9±5
Triose phosphate mM/l cells	6	0.31±0.02	0.66±0.06
Ratio ouabain/control		1.55±0.07	1.05±0.05
ADP mM/l cells	7	0.18±0.05	0.14±0.02
Ratio ouabain/control		1.00±0.07	1.01±0.05
ATP mM/l cells	6	1.19±0.13	1.04±0.06
Ratio ouabain/control		0.98±0.03	0.99±0.03

Cold-stored red cells with high or low Na contents studied with respect to K influx, lactate production, intracellular triose phosphate, ADP, and ATP during a 37°C incubation. The incubation solution contained NaCl 153 mM, KCl 5 mM, glycylglycine 20 mM, Na phosphate 2 mM, (pH 7.5), with adenosine 2 mM as substrate. Ouabain, when present, was at a final concentration of 10^{-4} M. Values for intracellular ion content are given as the range. All other values are given as the mean ± the standard deviation. The incubation period was for 2–3 hr. Samples were taken each hour for lactate determination. The values reported for triose phosphate, ADP, and ATP were obtained after 1–2 hr of incubation.

quantitative effects are observable with inosine as the metabolic substrate for the cell. The effect of ouabain in the high Na cells could be duplicated by the removal of K from the incubation medium, as was also found by Whittam and Ager (1965).

It has been suggested that the basis for the inhibitory effect of ouabain on glycolysis is related to an indirect effect of the glycoside on the activity of the phosphofructokinase reaction (Minakami et al., 1964, 1966). That this is the sole mechanism for the inhibitory action of ouabain on lactate production in cold-stored, high Na cells appears unlikely, since the effect of ouabain is observable with adenosine as substrate. Adenosine and other purine nucleo-

sides, upon entering the red cell, are cleaved by nucleoside phosphorylase to yield a purine moiety plus ribose-1-phosphate. Carbons from the latter compound, through reactions in the pentose shunt pathway, can eventually enter the glycolytic sequence in the form of F6P or triose-P (Dische, 1953; Lowy et al., 1958). Triose-P can be metabolized to lactate without going through the phosphofructokinase step. If the ouabain inhibition of lactate production were mediated via an effect on phosphofructokinase, the effect should be reduced or obliterated with adenosine as substrate. The fact that the metabolic influences of ouabain and internal Na can be demonstrated in

TABLE II
LACTATE PRODUCTION IN GHOST-FREE HEMOLYSATES

Additions (no ghosts)	No. of experiments	Lactate production from FDP	
		Control	Ouabain inhibition
		mM/liter	%
0	5	0.11±0.02	<3
ATP 1 mM	5	0.11±0.01	<3
ADP 1 mM	4	0.34±0.08	<3
P_i 5 mM	4	0.10±0.01	<3
AsO_4 2.5 mM	2	0.96, 0.91	<3

Lactate production from fructose diphosphate in a hemolysate from which the ghosts were removed by centrifugation. To 9 volumes of hemolysate containing nicotinamide 25 mM was added 1 volume of a solution containing FDP 20 mM, NaCl 500 mM, $MgCl_2$ 40 mM, EDTA 30 mM, and Tris 100 mM (pH 7.5). Another volume of tris-buffered solution was added to each flask, and it was via this solution that ATP, ADP, and the Na salts of P_i and arsenate were introduced, so that their final concentrations in the hemolysate were as indicated. Ouabain was added in a final concentration of 10^{-4} M. The increase in lactate concentration in the hemolysate during one hour of incubation is given, together with the standard deviation.

cells utilizing adenosine suggests that alterations in glycolytic rate in these circumstances are mediated by the activity of some step in the pathway subsequent to triose-P formation. This conclusion is supported by the measurements of intracellular triose-P shown in Table I. The levels of triose-P vary inversely with the rates of lactate production under the experimental circumstances shown, being high in low Na cells, low in high Na cells, and increased in the presence of ouabain.

The effects of variations in the rate of active Na-K transport on the intracellular concentrations of adenine nucleotides were also studied. In Table I it is shown that under the circumstances of these studies, neither ATP nor ADP levels are affected by ouabain or by variations in internal Na.

Lactate production from glucose was affected by variations in internal

Na and by ouabain in much the same way as when purine nucleosides were the carbon source. With glucose as substrate the ouabain-treated cells had G6P levels and ATP/ADP ratios which were higher than controls. Minakami et al. (1966) have recently reported similar results in red cells metabolizing glucose, as will be discussed.

That a high intracellular Na concentration per se is not responsible for the effects observed was shown in studies with dog red cells, which have an internal Na of 100 mM/liter of cells, and in which no effect of ouabain on lactate production was demonstrable.

TABLE III

LACTATE PRODUCTION IN WHOLE HEMOLYSIS MIXTURES

Additions (+ ghosts)	No. of experiments	Lactate production from FDP	
		Control	Ouabain inhibition
		mM/liter	%
0	5	0.27±0.02	26±10
ATP 1 mM	5	0.36±0.02	34±8
ADP 1 mM	4	0.46±0.07	20±2
P_i 5 mM	4	0.25±0.02	21±2
AsO_4 2.5 mM	4	0.84±0.08	1±2

Lactate production from fructose diphosphate in a whole hemolysis mixture. In some instances the ghosts were removed by centrifugation and then recombined with the hemolysate; in other instances the ghosts were not removed from the hemolysis mixture. The procedure was the same as noted in the legend to Table II, except that ghosts were present.

Studies with Hemolysates and Hemolysis Mixtures

Measurement of the conversion of various substrates to lactate in hemolysis mixtures offers an alternative approach to the study of rate control. Ouabain should inhibit lactate production from intermediates above the step at which the glycoside acts, but this should not be true of compounds further on in the sequence. Hemolysates were prepared as described in Materials and Methods, and it was found that if nicotinamide, NaCl, $MgCl_2$, and EDTA were added in the concentrations given in Table II, lactate was produced from FDP at a linear rate for 2 hr. Linearity was not obtained with lower concentrations of nicotinamide. This was shown in separate experiments to be related to the destruction of NAD by the nicotinamide-sensitive NADase known to exist in red cell membranes (Alivisatos et al., 1956). In Table II it is demonstrated that ouabain has no effect on lactate production from FDP by a hemolysate from which the ghosts were removed. Neither ATP nor P_i have any influence on lactate production by such a hemolysate, but ADP and arsenate both stimulate lactate production severalfold. In Table III it is

shown that if the ghosts are included with the hemolysate, the rate of lactate production is faster, and there is substantial ouabain inhibition. Provided ghosts are present, ATP stimulates lactate production and enhances the effect of ouabain. ADP has the same stimulatory effect noted in the ghost-free hemolysate, and added P_i is without effect. Arsenate causes a marked increase in lactate production, and in the presence of this ion the inhibitory action of ouabain is no longer evident.

TABLE IV

LACTATE PRODUCTION IN WHOLE HEMOLYSIS MIXTURES

Substrates	Additions (+ ghosts)	No. of experiments	Lactate production from various substrates: Control	Lactate production from various substrates: Ouabain inhibition
			mM/liter	%
None	—	2	0.01, 0.03	0, 0.5
None	NADH	2	0.05, 0.06	0.2, 0.5
FDP	—	5	0.36±0.02	34±8
FDP	NAD	5	0.35±0.02	21±7
FDP	NADH	5	0.20±0.03	21±4
FDP	3PG	4	0.49±0.04	12±5
FDP	3PG, NADH	2	0.88, 0.89	0.4, 0
Gly3P	—	2	0.25, 0.26	29, 26
3PG	—	2	0.03, 0.02	0, 0.2
3PG	NADH	4	0.96±0.30	0±2
PEP	NADH	4	0.92±0.24	1±1

Lactate production from various substrates in a whole hemolysis mixture. All mixtures had the following added constituents, expressed in terms of the final concentration in the hemolysate: nicotinamide 25 mM, NaCl 50 mM, $MgCl_2$ 4 mM, EDTA 3 mM, ATP 1 mM, Tris 10 mM (pH 7.5). Substrates and cofactors noted in the table were added in the following final concentrations: FDP 2 mM, Gly3P 2 mM, 3PG 2 mM, PEP 2 mM, NADH 1 mM, NAD 1 mM, ouabain 10^{-4} M. The procedure was the same as noted in Table II, except that ghosts were present in all cases.

The increase in lactate production from FDP observed when ADP was added to the hemolysates suggested that the rate-limiting step might be controlled by the activity of one of the two enzymes in the pathway which utilize ADP as a substrate, either PGK or pyruvate kinase. The accelerating effect of arsenate, which permits the conversion of Gly3P to 3PG without the phosphorylation of ADP by 1,3-DPG (Racker and Krimsky, 1952), suggested that in this system PGK might play an important role in rate regulation.

In Table IV the metabolism of various substrates to lactate by a hemolysis mixture (with ghosts included) is presented. If no substrate is included in the hemolysis mixture, lactate production occurs at a slow rate. Addition of NADH has a stimulatory effect on lactate production in the absence of added

substrate, presumably by allowing the hemolysate to utilize its store of 2,3-DPG. FDP stimulates lactate production, but there is no further stimulation upon the addition of NAD. NADH, a known inhibitor of triose phosphate dehydrogenase, reduces the rate of lactate production from FDP but does not obscure the ouabain effect. The addition of 3PG in the presence of FDP causes a rise in rate of lactate production and some decrease in the inhibitory action of ouabain. 3PG alone is converted to lactate at a slow rate; however, when NADH is added to the hemolysis mixture, the conversion of both 3PG and PEP to lactate is rapid and not sensitive to the action of ouabain.

The results of Tables II, III, and IV suggest that in the hemolysate system the principal rate-limiting step in the conversion of FDP to lactate is triose phosphate dehydrogenase. This enzyme is known from studies in other

TABLE V

LACTATE, ATP, AND ADP LEVELS IN WHOLE HEMOLYSIS MIXTURES

Incubation time	Lactate		ADP		ATP	
	Control	Ouabain inhibition	Control	Ratio Ouab./Contr.	Control	Ratio Ouab./Contr.
min	μM/*liter*	%	μM/*liter*		μM/*liter*	
45	351±22	26±4	142±9	0.93±.07	1580±40	1.04±.02
90	589±50	31±8	116±16	0.94±.08	1610±30	1.01±.01
135	831±120	33±10	111±20	0.95±.07	1600±30	1.02±.01

Lactate, ADP, and ATP concentrations in a whole hemolysis mixture incubated at 37°C for 135 min in the presence and absence of Ouabain 10^{-4} M and with the following additions (final concentration): nicotinamide 25 mM, NaCl 50 mM, $MgCl_2$ 4 mM, EDTA 3 mM, ATP 1.8 mM, FDP 2 mM, and Tris 10 mM (pH 7.5). Mean and standard deviations for three experiments are shown.

tissues to be inhibited by its products, NADH and 1,3-DPG (Velick and Furfine, 1963). The removal of NADH depends principally on the oxidation of this substance by pyruvate in the lactic dehydrogenase reaction. The removal of 1,3-DPG is catalyzed by two enzymes: diphosphoglyceromutase, which converts 1,3- to 2,3-DPG, and PGK, which catalyzes the transfer of P from 1,3-DPG to ADP. The stimulation of lactate production by ADP suggests that it is PGK which is the rate-limiting step in the metabolism of 1,3-DPG, since the synthesis of 2,3-DPG in red cells via the diphosphoglyceromutase reaction has been found to be suppressed by ADP (Schroter and Heyden, 1965).

Both 3PG and NADH must be supplied to obtain maximum lactate production and to obliterate the effect of ouabain; neither alone is sufficient. This suggests that the production of lactate from FDP is limited by the activity of triose phosphate dehydrogenase insofar as this step controls the production of both NADH and phosphoglycerate. The rate of the triose

phosphate dehydrogenase reaction is in turn controlled by the activity of phosphoglycerate kinase insofar as this enzyme catalyzes the removal of 1,3-DPG. The rate of the phosphoglycerate kinase reaction might then be expected to be a function of the level of ADP, so long as this compound is present in subsaturation concentrations.

It was postulated initially that the inhibitory action of ouabain was due to the effect of this glycoside on the production of ADP from ATP via the Na, K-ATPase of the ghosts (Post et al., 1960; Dunham and Glynn, 1961). This possibility was tested by measuring lactate production and ADP concentration in hemolysis mixtures to which ATP had been added and into which FDP was introduced as substrate. In Table V it is shown that ouabain inhibits the conversion of FDP to lactate but that the effect of ouabain on the concentrations of ADP and ATP is insignificant. These results are similar to the findings in intact, high Na cells shown in Table I. The metabolism of substances, e.g. adenosine, inosine, and FDP, which can be converted to lactate without passing through the phosphofructokinase step is slowed by ouabain, but there is little or no effect of ouabain on the ADP level.

Studies with Hemoglobin-Free Ghosts

The activity of the ghosts in increasing the rate of lactate production by a hemolysate and in conferring glycoside sensitivity upon the system suggested that the membranes themselves might be able to carry out the rate-limiting reaction. This possibility seemed particularly attractive since Schrier (1963) had described a preparation of ghosts which possessed aldolase, triose phosphate dehydrogenase, and phosphoglycerate kinase activity. Accordingly, a system was devised which included the substrates and cofactors necessary for the sequence of reactions involving these three enzymes. In Table VI the components of the system are presented, and the effects of various omissions and additions are shown. The fact that NAD is converted to NADH in the complete system and that P_i, FDP, and ghosts are necessary is evidence that triose phosphate dehydrogenase is present in the ghosts. The fact that aldolase is not required indicates the presence of this enzyme also in the ghosts. Aldolase added to the system had no stimulatory effect.

The fact that NAD is reduced slowly in the absence of ADP suggests either that the inhibition of triose phosphate dehydrogenase by 1,3-DPG is not complete or that some diphosphoglyceromutase activity is present in the system. The stimulation of NADH production by ADP is evidence of the presence of PGK in the ghosts. If instead of ADP, ATP is added to the system, NAD reduction is stimulated and is ouabain-sensitive. ITP and UTP are less active than ATP in stimulating NADH production, and with neither compound is a ouabain effect seen. Arsenate greatly stimulates NAD reduction even in the absence of ADP. In the presence of arsenate, the product of

the triose phosphate dehydrogenase reaction is 1-arseno-3-phosphoglycerate, a compound which undergoes rapid, nonenzymatic hydrolysis, thus permitting the dehydrogenase reaction to proceed without accumulation of 1,3-DPG.

The stoichiometric relationship between NAD reduction and ATP synthesis is shown in Table VII *a*. When NAD is omitted from the system, there is some synthesis of ATP from ADP, presumbaly via the adenylate kinase reaction which has been shown to occur in or near the cell membrane (Kashket and Denstedt, 1958). With NAD present, ATP synthesis during the first 30 min rises by an amount equal to the amount of NAD reduced. Subsequently the net ATP synthesis lags behind NADH production as would be expected from the ATPase activity known to be associated with ghosts prepared in this manner.

TABLE VI
GENERATION OF NADH BY THE GHOST SYSTEM

Incubation conditions	No. of experiments	NADH production	
		Control	Ouabain inhibition
		μM/liter-min	%
Complete system	5	6.8±.08	4±2
Omit FDP	4	0	—
Omit P_i	4	0	—
Omit ghosts	4	0	—
Omit ADP	8	0.4±0.1	<3
Omit ADP, + ATP	9	2.7±0.2	38±4
Omit ADP, + ITP	4	1.2±0.2	<3
Omit ADP, + UTP	1	0.5	<3
Omit ADP, + AsO_4	3	10.6±1.7	3±2

Rate of change of NADH concentration in the medium bathing hemoglobin-free ghosts. The complete system included: NaCl 40 mM, KCl 20 mM, $MgSO_4$ 2.5 mM, EDTA 1.5 mM, nicotinamide 25 mM, Na_2HPO_4 10 mM, Tris 50 mM, FDP 2 mM, GSH 6 mM, NAD 2 mM, ADP 1 mM, ghosts 0.7 mg dry weight/ml, and when present, ATP, ITP, UTP, all 1 mM, Na_2HAsO_4 2.5 mM, ouabain 10^{-4} M, pH was 7.5. Incubations were performed over a 45-min period with samples taken at 5, 20, and 40 min after placement of the flasks in a 37°C water bath. Values are calculated from the slope of the line drawn between the DPNH concentrations at these time points (see Fig. 1) and are expressed as the mean ± the standard deviation.

If ATP is substituted for ADP in the complete system, and if the production of ADP is measured in the presence and absence of NAD, an alternative stoichiometric calculation can be made: ATP will be hydrolyzed in the presence of the ghosts, with ADP as a product. Since the phosphorylation of 1 mole of ADP via the triose phosphate dehydrogenase-PGK sequence requires

the reduction of 1 mole of NAD, it should be possible, in the presence of NAD, to reduce the net yield of ADP from ATP by an amount approximately equal to the amount of NADH generated. The data in Table VII *b* show that this

TABLE VII *a*
GENERATION OF ATP AND NADH BY THE GHOST SYSTEM

Incubation time	Complete system ATP *a*	Complete system NADH *b*	Complete system minus NAD ATP *c*	Complete system minus NAD NADH *d*	Net ATP synthesis via PGK $\Delta a - \Delta c$	Net NADH production Δb
min	*μM/liter*	*μM/liter*	*μM/liter*	*μM/liter*	*μM/liter*	*μM/liter*
0	98	92	27	0	0	0
15	242	228	37	0	134	136
30	328	309	47	0	210	217
45	363	399	56	0	236	307
60	388	446	64	0	253	354

ATP synthesis in the ghost system in the presence and absence of NAD. The constituents of the system are given in Table VI. The initial concentrations of ATP and NADH were measured after the system had been brought to 37°C. The values of Δa, Δb, and Δc were calculated by subtracting the value at time 0 in each column from the value at each subsequent time point.

TABLE VII *b*
PRODUCTION OF ADP FROM ATP, RELATED TO GENERATION OF NADH BY THE GHOST SYSTEM

Experiment	Incubation time	Complete system ADP *a*	Complete system NADH *b*	Complete system minus NAD ADP *c*	Complete system minus NAD NADH *d*	ADP utilized via PGK $\Delta c - \Delta a$	Net NADH production Δb
	min	*μM/liter*	*μM/liter*	*μM/liter*	*μM/liter*	*μM/liter*	*μM/liter*
1	0	24	45	34	0	0	0
	20	53	76	94	0	31	31
	40	71	123	152	0	71	78
	60	80	168	192	0	102	123
	80	86	210	221	0	125	165
2	0	18	44	26	0	0	0
	20	58	84	101	0	35	40
	60	77	172	193	0	108	128
	80	76	206	232	0	148	162
3	0	10	41	15	0	0	0
	15	33	69	65	0	27	28
	30	50	102	111	0	56	61
	45	65	145	163	0	93	104
	60	68	178	189	0	116	137

ADP generation in the ghost system with ATP as substrate in the presence and absence of NAD. The constituents of the system are as noted in Table VII *a*, except that ADP 1 mM was replaced by ATP 1 mM. The values of Δa, Δb, and Δc were calculated in the same way as in Table VII *a*.

is the case. The fact that NADH production outstrips ADP disappearance via PGK is further evidence that the coupling between the reduction of NAD and the synthesis of ATP is not complete and suggests that the ghosts may possess some diphosphoglyceromutase activity. No lactate or pyruvate were formed under any of the circumstances listed in Table VI. The increase in optical density of the complete system at 340 mμ could be obliterated by adding lactic dehydrogenase plus pyruvate, thus confirming that the compound being measured at this wavelength was indeed NADH.

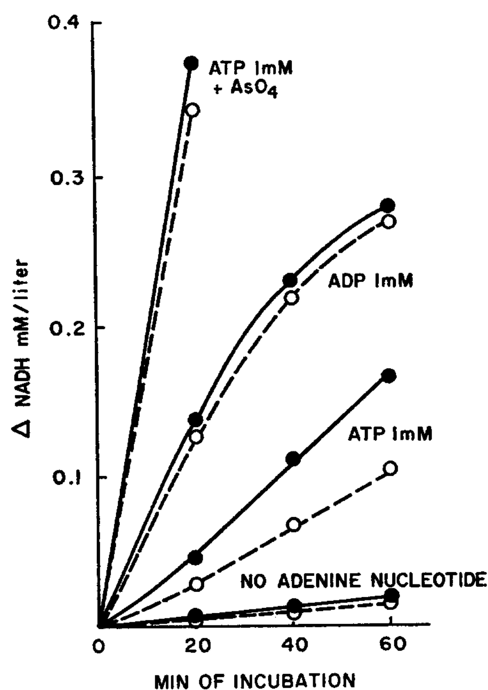


FIGURE 1. NADH production as a function of incubation time at 37°C. The complete ghost system as described in Table VI was used, with and without ADP. In the curves indicated, ADP was replaced by ATP alone or ATP + arsenate 2.5 mM. The dashed lines and open circles denote the presence of ouabain 10^{-4} M. Solid lines and closed circles indicate controls.

The effect of ouabain on the rate of NAD reduction was investigated as shown in Table VI and Fig. 1. It is apparent that with ADP as a substrate there is no effect of ouabain on the reaction rate, but that ouabain does inhibit the production of NADH when ATP is the substrate. That ouabain does not inhibit aldolase or triose phosphate dehydrogenase is shown by the results obtained with arsenate, which bypasses the PGK reaction. In this circumstance ouabain is without effect, even in the presence of ATP.

A series of kinetic studies was performed in the ghost system in order to ascertain the concentration range within which a change in the ADP concentration would affect the rate of NAD reduction. ADP was added to the reaction mixture in varying concentrations, all flasks were brought to 37°C, and the ADP level was measured at the beginning and end of the 20- to 30-min interval over which the rate of formation of NADH was determined.

The resulting curve is shown in Fig. 2, in which it is apparent that the maximum reaction rate is achieved at an ADP concentration of 0.35 mM and that ouabain does not influence the rate of NAD reduction. Hashimoto and Yoshikawa (1964) crystallized phosphoglycerate kinase from hemolysates of human erythrocytes and reported a Michaelis constant for ADP of 0.78 mM, almost an order of magnitude higher than the half-saturation value for ghost PGK shown in Fig. 2.

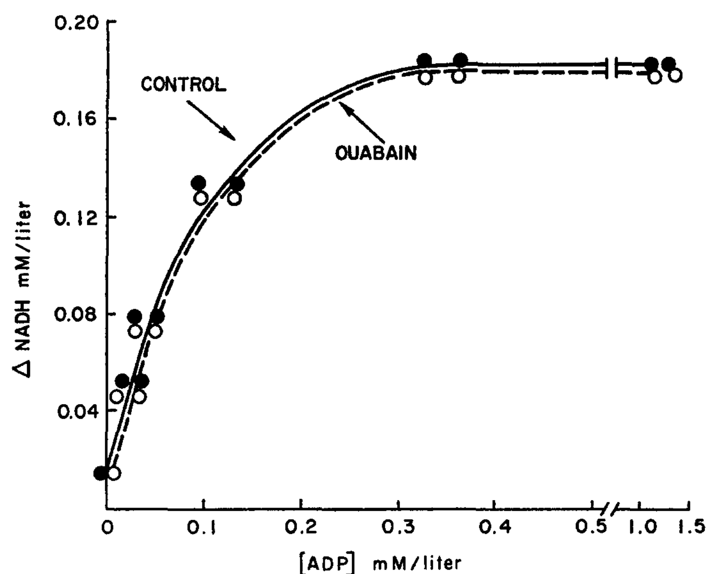


FIGURE 2. NADH production during a 30-min period at 37°C, as a function of the ADP concentration in the complete ghost system described in Table VI. The first samples were taken 5 min after the flasks were brought to 37°C; the second samples were taken 30 min later. NADH production is calculated from the change in optical density at 340 mμ between the supernatants of the first and second samples. ADP was measured in both samples, and the curve is drawn between the initial and final values for ADP. The dashed lines and open circles indicate the presence of ouabain 10^{-4} M. Solid lines and closed circles indicate controls.

By thus measuring the rate of NADH formation as a function of ADP concentration, it was possible to study the mechanism of the inhibitory effect of ouabain in the ghost system when ATP was substituted for ADP. For if the rate of the PGK reaction in the ghost system is a function of the concentration of ADP, then the rate of NAD reduction in the presence of ATP should be directly related to the activity of the total ATPase in producing ADP. Ouabain, by inhibiting the Na, K-ATPase, should reduce the concentration of ADP, and the resultant degree of slowing of the PGK reaction should be predictable on the basis of the data in Fig. 2.

Accordingly, the rate of NAD reduction was measured at a range of concentrations of ADP in the presence and absence of added ATP (1 mM). It is shown in Fig. 3 that the rate of NADH production for any given ADP level is less when ATP is added than when ATP is omitted.

Figs. 4 *a* and 4 *b* show that with ATP 1 mM added to the system the rate of NADH production is slowed by ouabain. Furthermore, the slowing is greater than would be predicted from the effect of ouabain on ADP. For if the effect of ouabain on the PGK reaction in the presence of added ATP

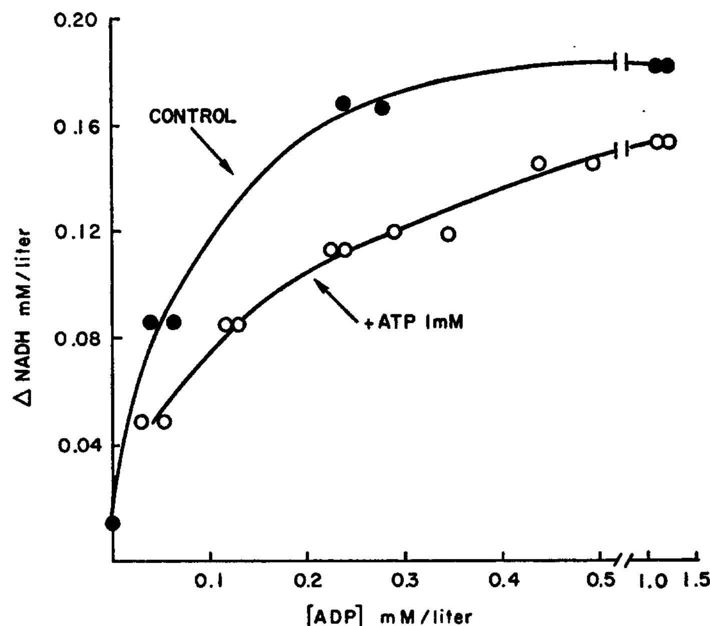


FIGURE 3. NADH production during a 30-min period at 37°C, as a function of the ADP concentration at the beginning and end of the incubation. The effect of adding ATP 1 mM to the system as described in Fig. 2 is shown.

were solely due to a change in the ADP level, the relation between NADH production and ADP concentration should be the same in the presence of the glycoside as in its absence. However, for any given level of ADP in the range from 0 to 0.5 mM in Figs. 4 *a* and 4 *b*, the rate of NADH production is less in the presence of ouabain than in its absence. Thus, in the presence of ATP, ouabain inhibits the rate of NAD reduction in the ghost system by a mechanism which is only indirectly related to the action of the glycoside on the ghost Na, K-ATPase.

It is shown in Fig. 3 that ATP inhibits the rate of NAD reduction for any given level of ADP. Ouabain, which inhibits the ghost Na, K-ATPase, prevents the breakdown of ATP. In order to evaluate the possibility that the

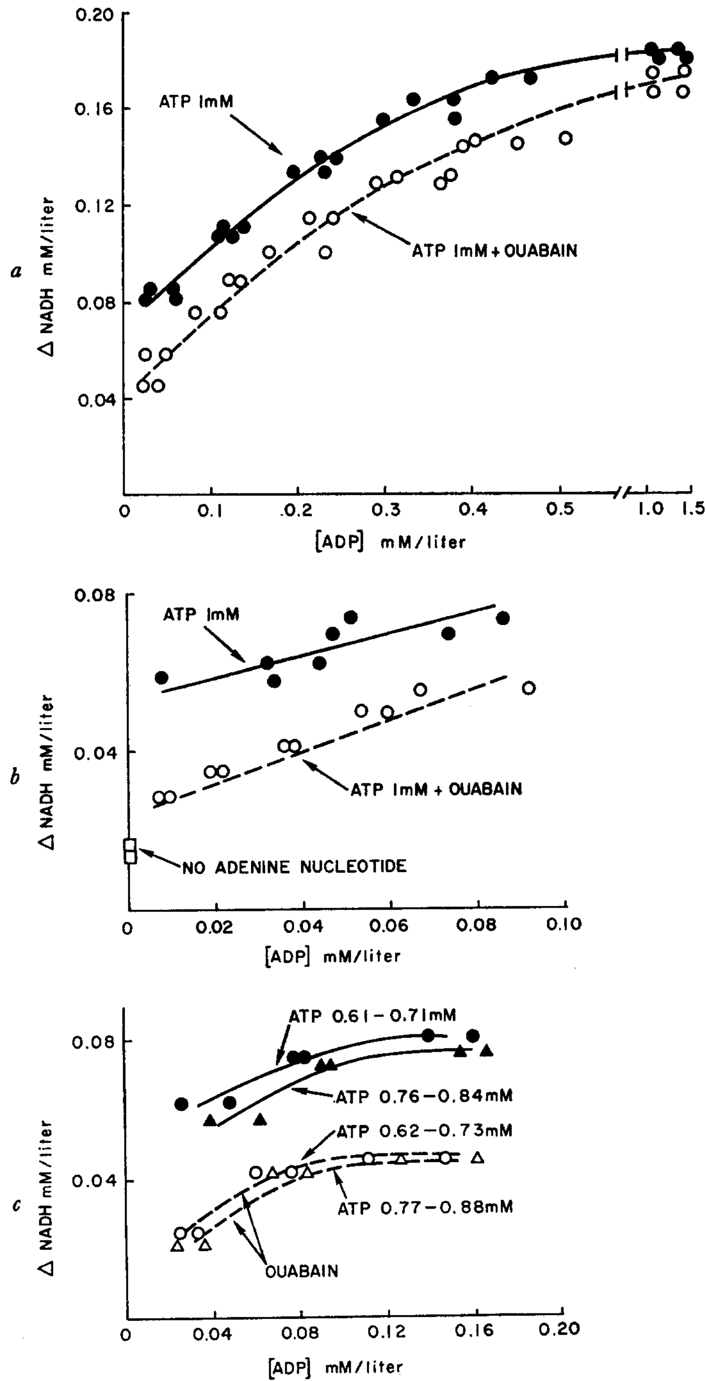


FIGURE 4. NADH production during a 30-min period at 37°C, as a function of the ADP concentration at the beginning and end of the incubation. ATP 1 mM was added to the system as described in Fig. 2, and the effect of ouabain 10^{-4} M, denoted by the open circles and dashed lines, is shown in (*a*) over a concentration range of ADP from 0 to 1.5 mM and in (*b*) from 0 to 0.1 mM. In (*c*) the effect of ouabain at two initial concentrations of ATP was studied. ATP was measured along with ADP at the beginning and end of the time period over which NADH was measured. The maximum and minimum ATP values for all the points on each curve are given.

sparing of ATP by ouabain was the cause of the drop in NADH production, the study shown in Fig. 4 *c* was done. In this experiment two concentrations of ATP were added to the complete system, and the effect of ouabain on NADH production at three different ADP levels was studied. ATP was measured along with ADP in each flask at the beginning and end of the incubation period. The lowest and highest ATP concentrations for each curve

TABLE VIII

GENERATION OF NADH BY THE GHOST SYSTEM AT VARIOUS ATP/ADP RATIOS

	Initial added concentrations		NADH production	
Experiment No.	ATP	ADP	Control	Ouabain inhibition
	μM/liter	*μM/liter*	*μM/liter-min*	%
1	1000	0	2.6	37
	500	0	2.3	38
	200	0	1.7	32
	100	0	1.4	15
	0	0	0.5	0
2	1000	100	3.3	30
	500	100	3.3	30
	200	100	2.7	19
	100	100	2.5	20
	0	100	1.9	5
3	1000	200	3.8	25
	500	200	4.2	29
	200	200	3.7	19
	100	200	3.4	14
	0	200	3.0	8

Correlation between the initial ATP/ADP ratio and NADH production in the presence and absence of Ouabain 10^{-4} M in the complete system as described in Table VI with initial added concentrations of ATP and ADP as indicated.

are given in the graph. It can be seen that the difference in added ATP had much less of an effect on the curve than did the addition of ouabain, which in turn had a negligible effect on the range of ATP concentrations over the relatively brief period of the incubation.

Table VIII shows the results of studies designed to ascertain the initial concentration of ATP necessary to demonstrate an effect of ouabain on NADH production in the ghost system. The inhibitory effect of ouabain at the three initial ADP concentrations studied was maximal when the initial ATP was between 0.2 and 0.5 mM.

If ouabain were acting directly as an inhibitor of PGK in the presence of

ATP, it might be expected that the rate of the reverse reaction would be decreased in the presence of ouabain. However, in Table IX it is shown that when ATP 2 mM, 3PG, and NADH are included as substrates in the ghost

TABLE IX

OXIDATION OF NADH BY THE GHOST SYSTEM

Incubation conditions	No. of experiments	NADH disappearance	
		Control	Ouabain inhibition
		μM/liter-min	%
Complete system	5	2.0±0.2	2±1
Omit ATP	2	0	—
Omit 3PG	2	0	—
Omit ghosts	2	0	—

Measurement of the rate of disappearance of NADH in the presence and absence of Ouabain 10^{-4} M, and with various omissions from the complete system, which included NaCl, KCl, $MgSO_4$, Tris, and ghosts in the concentrations listed in Table VI. Also present were GSH 10 mM, 3PG 3 mM, ATP 2 mM, and NADH 0.12 mM (pH 7.5). Rates were calculated from the slope of the NADH disappearance curve, which was linear over a 45-min incubation at 37°C. They are expressed as the mean ± the standard deviation.

TABLE X

NET CHANGE IN ADP IN THE GHOST SYSTEM

Experiment	Initial ADP	Change in ADP in 30 min
	μM/liter	*μM/liter*
1	14	+44
	118	+2
	218	−23
	345	−56
2	32	+25
	129	−12
	238	−38
	394	−88
3	9	+47
	42	+35
	86	+16
	122	+11
	181	−10
	411	−35

The net change in ADP concentration as a function of the initial ATP/ADP ratio in the complete system as described in Table VI. The initial added concentration of ATP was 1 mM in all cases. ADP was added to the system in various amounts. The initial measured concentration of ADP and the net change in ADP concentration over a 30-min period are given.

system, there is no effect of ouabain on the rate of disappearance of NADH. At low initial ATP concentrations in this system (0.1–0.5 mM), the oxidation of NADH was shown to proceed more rapidly in the presence than in the absence of ouabain.

The relative rates of the total ATPase and the total ATP synthetic apparatus in the ghosts were measured as a function of the initial ATP/ADP ratio. If, during the course of an incubation, the rate of the total ATPase is faster, there should be a net accumulation in the system of ADP; conversely, if the ATP synthetic mechanisms are faster, ADP should disappear. Table X shows that the net change in ADP is minimal when the initial ATP/ADP ratio is between 1000/122 and 1000/129, or close to the ATP/ADP ratio in intact red cells (Table I).

TABLE XI

GENERATION OF NADH BY THE GHOST SYSTEM AT VARIOUS Na/K RATIOS

Na	K	NADH production	
		Control	Ouabain
mM/liter	*mM/liter*	*μM/liter-min*	*μM/liter-min*
50	10	2.6±0.3	1.6±0.1
50	0	1.7±0.2	1.7±0.2
0	10	1.8±0.3	1.8±0.2
0	0	1.8±0.2	1.8±0.2

NADH production in the presence and absence of Ouabain 10^{-4} M with Na and K deletions from the system as noted. The complete system as described in Table VI was used with the following modifications: phosphate was added as H_3PO_4; ATP 1 mM was substituted for ADP 1 mM. The Tris or cyclohexylammonium salts of the adenine nucleotides and FDP were used. Values from four experiments are expressed as the mean ± the standard deviation.

In Table XI the requirement of the ghost system for Na and K is shown. It is clear that the effect of ouabain is dependent upon the simultaneous presence of Na and K.

DISCUSSION

Two central conclusions from these studies are that PGK plays an important role in the regulation of red cell metabolic rate and that the activity of the Na-K pump can influence lactate production at this step. These conclusions are based on studies with intact cells and hemolysis mixtures. Red cells with a raised internal Na concentration produce lactate faster than cells with a low internal Na. This increment in metabolic rate is abolished when Na-K transport is inhibited by ouabain or by the removal of extracellular K. With the cells metabolizing purine nucleoside, lactate production can occur without the participation of hexokinase and phosphofructokinase, although sub-

strate for the latter reaction is formed. Under these circumstances, changes in the rate of active Na-K transport result in levels of triose-P which vary inversely with the rate of lactate production, indicating that the rate-controlling step is subsequent to triose-P formation. Furthermore, since ouabain has no effect on the ATP/ADP ratio with adenosine as substrate, the net rate of resynthesis of ATP in the presence of ouabain must decrease by an amount equal to the decrease in rate of ATP utilization by the Na-K transport mechanism.

Recently Minakami and Yoshikawa (1966) have demonstrated inhibition of glycolysis by ouabain in intact red cells metabolizing glucose. When compared with control cells, the glycoside-treated cells had a higher ATP/ADP ratio and increased levels of hexose monophosphates. Our observations in high Na cells metabolizing glucose (not shown) confirm these findings. In addition, these authors report that in the ouabain-treated cells the level of triose-P is higher and that of 3PG lower than in control cells. They explain their observations by postulating that the ATP/ADP ratio is higher in the ouabain-treated cells because of decreased utilization of ATP by the cation pump. They hypothesize that the increased ATP/ADP ratio results in a decrease in the activity of phosphofructokinase and a change in the mass action ratio for the triose phosphate dehydrogenase–PGK sequence, resulting in decreased lactate production and an increase in the ratio of triose-P to 3PG. An alternative interpretation would be that the glycoside is affecting the activity of some step subsequent to triose-P formation and that the increase in ATP/ADP ratio is due to a decrease in hexokinase activity as a result of the increased levels of G6P (Rose and O'Connell, 1964; De Verdier and Garby, 1965).

In whole hemolysis mixtures enriched with Na and Mg, the rate of lactate production from substrates proximal to the triose phosphate dehydrogenase reaction is slowed by ouabain. When arsenate is added to the hemolysis mixture, or when substrates beyond the PGK reaction are metabolized, the rate of lactate production is very rapid and not subject to ouabain inhibition. Removal of ghosts from the hemolysis mixture results in a decrease in the rate of lactate production from FDP and a disappearance of the ouabain effect. NADH, a known inhibitor of the triose phosphate dehydrogenase reaction, slows lactate production from FDP. These studies were interpreted as showing that the rate of lactate production from FDP in a whole hemolysis mixture is limited by the rate of the triose phosphate dehydrogenase step, which in turn is controlled by the activity of PGK in removing 1,3-DPG. Ouabain was thought to influence the metabolic rate via an effect on the activity of PGK in ghost membranes.

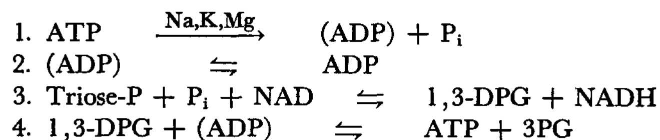
The mechanism of the interaction between the cation pump and the membrane PGK was not clear, however, from the studies with intact cells and

hemolysis mixtures. ADP seemed a likely intermediary compound, since it is both a product of the ouabain-sensitive Na, K-ATPase and a substrate for PGK. However, the retarding effect of ouabain on metabolism in high-Na cells and hemolysis mixtures could not be closely correlated with a change in the ADP concentration (Tables I and V). Eckel et al. (1966) concluded from experiments with cold-stored intact red cells that the influence of K transport on metabolic rate was mediated via the triose phosphate dehydrogenase–PGK sequence. Although they postulated an intermediary role for ADP, they were unable to demonstrate an effect of K transport on the intracellular concentration of this compound in one-third of their experiments.

Hemoglobin-free ghosts are known to possess both triose phosphate dehydrogenase and PGK activity (Ronquist and Agren, 1966; Schrier, 1966). Since in the present study ghosts both accelerated the rate of metabolism in hemolysates and rendered them sensitive to the action of ouabain, it seemed likely that the interaction between the cation pump and the glycolytic pathway might take place on or in the membranes themselves and might therefore not be mediated by changes in intracellular ADP. The initial rate of the triose phosphate dehydrogenase–PGK sequence in the ghosts was shown to depend on the ADP concentration between 0 and 0.35 mM and to be insensitive to ouabain unless ATP was added to the system initially. In the presence of a sufficiently high initial concentration of ATP (Table VIII), the effect of ouabain on the rate of NADH production was greater than one would have predicted from its effect on the Na, K-ATPase in reducing the concentration of ADP. At high ATP, no effect of ouabain on the reverse reaction of ghost PGK was demonstrable.

Jones et al. (1963) have demonstrated the presence of both PGK and Na, K-ATPase activity in a preparation of rat kidney microsomes which possesses the ability to stimulate glycolysis in a soluble fraction of the kidney homogenate. Although these authors do not report studies of the metabolic effects of ouabain or variations in cation content, it is likely that their system bears many resemblances to the one presented here.

A model which might account for the observations in the ghost system is shown in the following series of reactions, all of which are envisaged as being catalyzed by hemoglobin-free ghost membranes:



1. $\text{ATP} \xrightarrow{\text{Na,K,Mg}} (\text{ADP}) + \text{P}_i$
2. $(\text{ADP}) \leftrightharpoons \text{ADP}$
3. $\text{Triose-P} + \text{P}_i + \text{NAD} \leftrightharpoons \text{1,3-DPG} + \text{NADH}$
4. $\text{1,3-DPG} + (\text{ADP}) \leftrightharpoons \text{ATP} + \text{3PG}$

Reaction 1 is the Na, K, Mg–activated ATPase. It is visualized that the ADP produced by this reaction is localized or compartmentalized, and this is shown by the use of parentheses. The compartmentalized form of ADP is

exchangeable with the ADP in the medium, as shown in reaction 2. Triose phosphate dehydrogenase, reaction 3, is coupled with phosphoglycerate kinase, reaction 4, in the sense that the production of NADH is linked to the conversion of ADP to ATP (Table VII, Fig. 2). It is hypothesized that the immediate substrate for the ghost PGK (reaction 4) is the compartmentalized form of ADP, or (ADP). In the presence of a high concentration of ATP, the initial rate of NADH production is slowed (Fig. 3), because the net forward rate of reaction 4 is diminished, owing to the large amount of product and the reversibility of this reaction. Ouabain is presumed to act only on the Na + K–activated portion of the ATPase (reaction 1) in limiting the supply of (ADP), and thus slowing the rate of NAD reduction (Fig. 4). Thus, in the absence of a high concentration of ATP (Fig. 2, Table VIII), there is no effect of ouabain or NADH production, since the formation of (ADP) from ADP is not affected by the glycoside. In this model, ouabain should have no inhibitory effect on the rate of reaction 4 in the reverse direction (Table IX).

Evidence that a significant portion of PGK is intimately associated with the red cell ghost has been recently presented by Schrier (1966), who proposed that ATP was transferred from PGK to the cation pump within the microenvironment of the membrane. The results of the present study suggest that there is indeed a species of interaction between PGK and Na, K-ATPase in the sense that the activity of the ouabain-sensitive ATP–utilizing system affects the activity of the ATP-generating system.

It is of interest to consider the significance of the interaction between cation transport and metabolism for the red blood cell under physiological conditions since in order to demonstrate the relationship in normal red cells, the Na concentration of the cells must be raised by experimental manipulation. Inhibition of cation transport has little or no effect on lactate production in freshly drawn, normal red cells with an internal Na concentration of 10 mM (Jacob and Jandl, 1964). The experiments reported here, however, permit some speculation concerning the arrangement of the glycolytic enzymes in the cell and their relationship to the cation pump on the membrane. It is possible to infer, for instance, that when the rate of lactate production is enhanced in rapidly transporting cells, the flow of metabolic substrates is increasingly channeled through that fraction of the PGK in the cell which is capable of interaction with the pump mechanism on the membrane. Alternatively, increased pump activity might cause the PGK in the cell to come into a closer relationship to the transport apparatus. In either event, the increased energy demands of Na-K transport might be served by a tighter coupling between the utilization of ATP by the pump and the regeneration of ATP by phosphoglycerate kinase.

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