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ERYTHROCYTE METABOLISM. V. LEVELS OF GLYCOLYTIC ENZYMES AND REGULATION OF GLYCOLYSIS *

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In contrast to many other cell types in which a variety of metabolic pathways may contribute to energy production, the human erythrocyte derives its energy almost exclusively from the breakdown of glucose to lactate via the glycolytic sequence. Lactate can arise also from glucose by an alternate route, namely the hexose monophosphate shunt and the pentose cycle, but this latter pathway is relatively inoperative in the erythrocyte under normal conditions, owing to the unexplained preferential conversion of glucose-6-phosphate to fructose-6-phosphate, rather than to 6-phosphogluconate. The red cell, therefore, offers a unique opportunity to correlate physiological function, or malfunction, with enzymatic activity, since the number of metabolic pathways, fortunately, is somewhat restricted.

In the present investigation a detailed study has been undertaken to define the optimal conditions for the conversion of glucose to lactate in the intact erythrocyte and in hemolysates. In addition, levels of the individual glycolytic enzymes have been determined and this information has been used to discuss regulatory mechanisms of glycolysis in the erythrocyte.

EXPERIMENTAL

Materials. Human blood was collected in acid citrate dextrose (ACD preservative, National Institutes of Health, Formula A). Only fresh blood was used throughout these experiments. Semicarbazide was obtained from Eastman Organic Chemicals; DPN,[1] TPN, DPNH, TPNH, sodium pyruvate, fructose-1,6-diphosphate, fructose-6-phosphate, and glucose-6-phosphate from Sigma Chemical Company; ADP and ATP from Pabst Laboratories; phosphoenolpyruvate, 3-phosphoglycerate, 2-phosphoglycerate, lactic dehydrogenase, pyruvic kinase, 3-phosphoglyceraldehyde dehydrogenase, α-glycerophosphate dehydrogenase, triose isomerase, enolase, aldolase, and glucose-6-phosphate dehydrogenase from C. F. Boehringer and Sons; 2,3-diphosphoglycerate, and dehydrated firefly tails from Schwarz BioResearch, Inc.; glucose oxidase from Worthington Biochemical Corporation; and DL-3-phosphoglyceraldehyde as the diethyl acetal monobarium salt from Nutritional Biochemicals Corporation [conversion to the free aldehyde was accomplished by boiling for 3 minutes in an aqueous suspension of Dowex-50 (H^+)]. Lithium DL-lactate was prepared according to the method of Barker (2) from commercial lactic acid (Mallinckrodt Chemical Co.).

Methods. The pH of the reaction mixtures was measured with a glass, constant temperature, blood electrode (Beckman Co.). Hemoglobin was measured by the procedure of Evelyn and Malloy (3). Lactate and glucose were determined enzymatically with lactic dehydrogenase (4) and glucose oxidase (5), respectively. Phosphate was measured by a method described previously (6). ATP was assayed by the bioluminescent reaction of firefly extracts containing luciferin and luciferase (7, 8) with the use of the G. K. Turner fluorometer. ADP was measured with the coupled reaction, pyruvic kinase-lactic dehydrogenase (9). Fructose was determined by the method of Roe (10), fructose-6-phosphate by a modification (11) of the method of Dische and Borenfreund (12), and glucose-6-phosphate dehydrogenase by the procedure of Kornberg and Horecker (13).

Measurement of glycolytic enzymes. Hemolysates were prepared by freezing and thawing (three times) an aqueous suspension of erythrocytes, previously washed once with 1 vol of 0.15 M KCl. Amounts of hemolysate containing 0.005 to 0.50 mg of hemoglobin were used for measuring the activities of the individual glycolytic enzymes. All enzyme assays were performed in 0.05 M

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[1] The following abbreviations are used in this report: DPN and TPN, di- and triphosphopyridine nucleotide; DPNH, TPNH, reduced DPN and TPN; ADP and ATP, adenosine di- and triphosphate; and P_i, inorganic phosphate.

glycylglycine buffer, pH 8.1, and under conditions where activity was proportional to enzyme concentration and time. Enzyme activities are expressed as micromoles of substrate reacting per hour per milliliter of red cells. Except where otherwise noted, assays were carried out spectrophotometrically, using coupled systems linked to the oxidation and reduction of pyridine nucleotides; conditions were arranged whereby the enzyme to be measured was rate limiting. Spectrophotometric measurements were made with a Beckman DU or DK-1 spectrophotometer at 25° C in a 1 ml cuvet of 1 cm optical path. Absorbancy at 340 mμ was followed at convenient intervals (30 seconds or 1 minute) over a 5- to 10-minute period. A millimolar extinction coefficient of 6.22 at 340 mμ was used for DPNH and TPNH.

Specifically, the following assay systems were employed for the determination of enzyme activities. In general, previously described methods were modified and adapted to the measurement of glycolytic enzymes in the hemolysate. The concentrations of the reactants are given for each assay.

Hexokinase was assayed in a system coupled with TPN-dependent glucose-6-phosphate dehydrogenase. The increase in absorbancy at 340 mμ, indicative of TPNH formation, was correlated with the utilization of substrate in the primary reaction catalyzed by hexokinase. The assay mixture contained glucose, 5×10^{-3} M; ATP, 2×10^{-3} M; $MgCl_2$, 7×10^{-3} M; TPN, 1×10^{-4} M; and glucose-6-phosphate dehydrogenase, 0.002 mg. It should be noted that considerably higher values are obtained when hexokinase is assayed by measuring the ADP produced in the primary reaction as a limiting reactant in the pyruvic kinase–lactic dehydrogenase reaction. In this case the "ATP-ase" activity of hexokinase contributes to the measurement.

Phosphoglucose isomerase activity was determined by several methods: *1*) in a system containing glucose-6-phosphate (2×10^{-3} M), $MgCl_2$ (3×10^{-3} M), and ATP (2×10^{-3} M) for the two-step conversion of glucose-6-phosphate to fructose-1,6-diphosphate, utilizing endogenous phosphofructokinase to catalyze the second step; the production of ADP was measured in the pyruvic kinase–lactic dehydrogenase reaction (9); *2*) in a system containing glucose-6-phosphate (2×10^{-3} M) and enzyme source incubated at 25° C for 30 minutes; fructose-6-phosphate was measured chemically (11, 12); and *3*) in a system utilizing the reaction, fructose-6-phosphate → glucose-6-phosphate, coupled with the TPN-dependent glucose-6-phosphate dehydrogenase reaction, according to the method of Srere, Cooper, Tabachnik and Racker (14). The assay mixture contained fructose-6-phosphate, 2×10^{-3} M; $MgCl_2$, 2×10^{-2} M; TPN, 2×10^{-4} M; and glucose-6-phosphate dehydrogenase, 0.005 mg.

Phosphofructokinase was assayed in a system coupled with aldolase, triose isomerase and DPNH-dependent α-glycerophosphate dehydrogenase (15). The assay mixture contained fructose-6-phosphate, 7×10^{-4} M; ATP, 2×10^{-3} M; $MgCl_2$, 1×10^{-3} M; DPNH, 1×10^{-4} M; cysteine, 7×10^{-3} M; aldolase, 0.20 mg; triose isomerase, 0.01 mg; and α-glycerophosphate dehydrogenase, 0.01 mg.

For the measurement of *aldolase* activity, adaptations of two methods were used: *1*) The procedure of Wu and Racker (16) whereby the reaction is coupled with triose isomerase and DPNH-dependent α-glycerophosphate dehydrogenase, yielding 2 equivalents of DPNH oxidized for each fructose-1,6-diphosphate reacting. The system contained fructose-1,6-diphosphate, 5×10^{-3} M; DPNH, 1.5×10^{-4} M; α-glycerophosphate dehydrogenase, 0.01 mg; and triose isomerase, 0.01 mg. *2*) The procedure of Taylor (17) in which the reaction is coupled with DPN-dependent 3-phosphoglyceraldehyde dehydrogenase; 2 equivalents of DPNH are produced for each fructose-1,6-diphosphate reacting. The assay mixture contained fructose-1,6-diphosphate, 5×10^{-3} M; DPN, 1.5×10^{-4} M; sodium arsenate, 1.7×10^{-2} M; triose isomerase, 0.01 mg; and 3-phosphoglyceraldehyde dehydrogenase, 0.1 mg.

Triose isomerase was measured in the reaction, 3-phosphoglyceraldehyde → dihydroxyacetone-phosphate, by coupling with DPNH-dependent α-glycerophosphate dehydrogenase (18) in a system containing 3-phosphoglyceraldehyde, 3×10^{-3} M (D-isomer); DPNH, 1×10^{-4} M; α-glycerophosphate dehydrogenase, 0.01 mg. After addition of the enzyme to initiate the reaction, 2 minutes were allowed to elapse before absorbancy changes at 340 mμ were measured.

The method for the measurement of *3-phosphoglyceraldehyde dehydrogenase* was that of Velick (19). The assay mixture contained 3-phosphoglyceraldehyde, 2×10^{-3} M (D-isomer); DPN, 1×10^{-3} M; and sodium arsenate, 1.7×10^{-2} M.

The method for the determination of *phosphoglyceric kinase* was that of Bücher (20), utilizing the reaction, 3-phosphoglycerate → 1,3-diphosphoglycerate, and coupling with the reaction of DPNH-dependent 3-phosphoglyceraldehyde dehydrogenase. The assay system contained 3-phosphoglycerate, 5×10^{-3} M; DPNH, 1.5×10^{-4} M; $MgCl_2$, 1×10^{-2} M; cysteine, 2×10^{-2} M; ATP, 3×10^{-3} M; 2,3-diphosphoglycerate, 5×10^{-3} M; and 3-phosphoglyceraldehyde dehydrogenase, 0.1 mg.

3-Phosphoglyceric mutase was determined as the limiting enzyme in the sequence, 3-phosphoglycerate → lactate. The assay system contained 3-phosphoglycerate, 5×10^{-3} M; $MgCl_2$, 3×10^{-3} M; ADP, 2×10^{-3} M; DPNH, 1×10^{-4} M; pyruvic kinase, 0.02 mg; lactic dehydrogenase, 0.05 mg; and enolase, 0.05 mg.

Enolase activity was measured as the limiting enzyme in the reaction, 2-phosphoglycerate → lactate, in a system containing 2-phosphoglycerate, 5×10^{-3} M; $MgCl_2$, 3×10^{-3} M; DPNH, 1.5×10^{-4} M; ADP, 2×10^{-3} M; pyruvic kinase, 0.05 mg; and lactic dehydrogenase, 0.05 mg.

Pyruvic kinase was assayed via the reaction, phosphoenolpyruvate → lactate, in a coupled system (21) containing phosphoenolpyruvate, 5×10^{-3} M; $MgCl_2$, 3×10^{-3} M; ADP, 2×10^{-3} M; DPNH, 1×10^{-4} M; and lactic dehydrogenase, 0.05 mg.

The method for the measurement of *lactic dehydrogenase* was that of Kubowitz and Ott (22). The reaction

TABLE I

Cofactor requirements for glycolysis in the hemolysate *

Component omitted	Lactate production
	μmoles/ hr/g Hb
None	21
P_i	19
DPN	8
ATP	4
Mg^{++}	2
DPN + ATP	1

* Reaction mixtures contained the following components in a total volume of 5 ml: 30 μmoles glucose, 5 μmoles ATP, 1.5 μmoles DPN, 7.5 μmoles $MgCl_2$, 10 μmoles of potassium phosphate (pH 8.1), and 2.0 ml of hemolysate, previously adjusted to pH 8.1 with glycine buffer. The mixtures were incubated for 1 hour at 37° C, and lactate was determined on acid filtrates of the reaction mixtures. Glucose was omitted in the control reaction mixture.

mixture contained pyruvate, 2×10^{-3} M; DPNH, 1×10^{-4} M; and $MgCl_2$, 3×10^{-3} M.

Measurement of glycolysis in the intact cell. Suspensions of erythrocytes were used for the measurement of lactate production from glucose at a constant pH. Adjustment of the pH of a red cell suspension to a desired value is difficult, owing to the necessity of overcoming the formidable buffering capacity of the cells without introducing a high salt concentration which would damage the cells. In the following method, which was found to be satisfactory, all operations were carried out at 4° C, except where noted otherwise, and centrifugations were performed for 5 minutes at 1,800 G in the International refrigerated centrifuge, model PR-2. After a preliminary centrifugation of the blood, the ACD-plasma and buffy coat were discarded, and the erythrocytes were treated by one of the procedures (A or B) described below.

A. pH range 6.4 to 7.0. The cells were washed by centrifugation with 2 vol of 0.15 M KCl and reconstituted to a hematocrit of 50 per cent in 0.08 M potassium phosphate buffer; for cell suspensions having final pH values of 6.4, 6.6, 6.8, and 7.0, the pH of the phosphate buffer must be 6.0, 6.3, 6.6, and 7.0, respectively.

B. pH 7.0 to 9.4. For this pH range, washing of the cells and pH adjustment were carried out in a single operation, namely, by centrifugation of the cells in two volumes of 0.3 M glycine buffer at a pH 0.7 of a unit higher than the desired final pH of the cells. Prior to centrifugation, the cells suspended in the buffer were allowed to stand at room temperature for 10 minutes to insure complete pH equilibration. After the pH had been adjusted by this method, the cells were resuspended in an equal volume of 0.15 M KCl.

Measurement of glycolysis in the hemolysate. Erythrocytes were washed once with 2 vol of 0.15 M KCl, resuspended to a hematocrit of 33 per cent in 0.3 M glycine buffer, 0.9 of a pH unit higher than the desired final pH of the hemolysate, and lysed by rapid freezing and thawing three times. The stromal fraction was not removed. When fortified with cofactors, the hemolysate preparation was used for the measurement of lactate production from glucose at a constant pH.

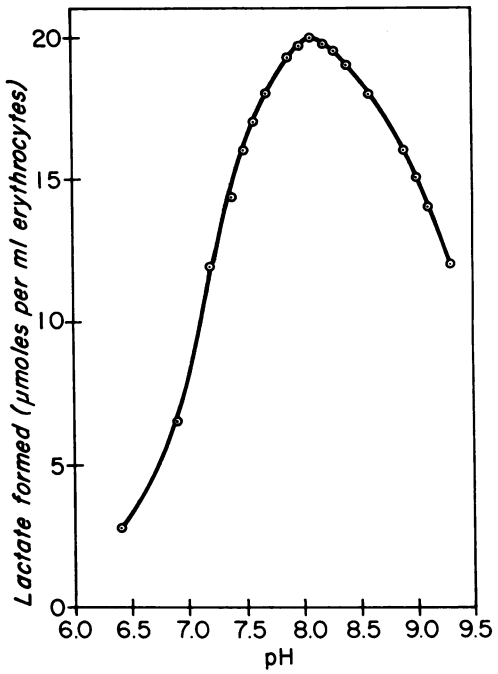


FIG. 1. EFFECT OF PH ON GLYCOLYSIS. Lactate formation was measured after a 3-hour incubation at 37° C in a total volume of 6 ml, containing 70 μmoles glucose, 15 μmoles $MgCl_2$, 180 μmoles phosphate, and 2 ml of red cells, adjusted to the indicated pH values as described in the Experimental section. The reaction was linear over the 3-hour incubation period, and the pH remained constant. Each point represents the average of 8 separate experiments.

RESULTS

Glycolysis in the intact cell. Although the conversion of glucose to lactate in the erythrocyte has been the subject of numerous investigations [e.g. (23–26)], most of the measurements have been carried out at pH 7.4 or below. When a detailed examination is made of the effect of pH upon glycolysis, the pH-activity curve is actually observed to be rather broad and the optimum falls at pH 8.1 (Figure 1). At this pH optimum and with the optimal extracellular phosphate concentration of 0.03 M, the rate of lactate formation was 6.5 μmoles per hour per ml of cells. In the pH range 7.8 to 8.6, the average value for the ratio of lactate production to glucose disappearance was 2.01

TABLE II

Inhibition of glycolysis in the hemolysate *

Ion added	Lactate production
	μmoles/hr/g Hb
None	19
$HPO_4^=$	11
Na^+	12
$Tris^+$	15

* The reaction mixtures contained in a total volume of 5 ml: 30 μmoles glucose, 1.5 μmoles DPN, 5 μmoles ATP, 7.5 μmoles $MgCl_2$, 1,500 μmoles $HPO_4^=$, Na^+, or $Tris^+$ (where indicated), 1,000 μmoles of glycine buffer (pH 8.1), and 1.0 ml of hemolysate. Incubations were carried out for 30 minutes at 37° C, and lactate was determined on acid filtrates of the reaction mixtures.

(range, 1.82 to 2.18 for 8 separate experiments). No requirement for extracellular Mg^{++} was noted. The system was inhibited approximately 23 per cent when the phosphate concentration was raised to 0.085 M.

Glycolysis in the hemolysate. Lysates prepared by repeated freezing and thawing of erythrocytes do not retain the capacity for carrying out the conversion of glucose to lactate. When fortified with 1×10^{-3} M ATP, 3×10^{-4} M DPN, 2×10^{-3} M P_i, and 1.5×10^{-3} M Mg^{++}, however, these preparations are fully reconstituted with respect to the original activity (Table I). The concentrations given above have been determined to be optimal for each cofactor. At levels higher than 10^{-3} M, ATP inhibits glycolysis in the reconstructed system. In addition, Na^+, $HPO_4^=$, or $Tris^+$, each at a concentration of 0.3 M, inhibits lactate production from glucose in this system (Table II). Dialysis of the hemolysate against 0.1 M KCl for periods as short as 6 hours depressed the conversion of glucose to lactate by 20 per cent in the reconstructed system, and after 48 hours of dialysis, essentially all of the activity was lost.

In the reconstructed hemolysate system, the pH optimum for glycolysis was again 8.1, and the expected 1:2 stoichiometry was observed between glucose disappearance and lactate production, When the results in Table I are recalculated on the basis of intact cells—0.33 g of hemoglobin being taken as the equivalent of 1 ml of packed cells (*27*)—6.9 μmoles of lactate are produced per hour per ml of cells; this rate is comparable to that found in the intact cell. In terms of the over-all glycolytic sequence, therefore, the reconstructed hemolysate appears to be metabolically equivalent to the intact cell, and permits more flexibility in the design of experiments.

Measurement of the glycolytic enzymes. The preceding experiments define the over-all requirements for glycolysis in the intact erythrocyte and in the hemolysate. A study of the individual glycolytic enzymes was undertaken in order to examine specific steps in the glycolytic sequence and to obtain information necessary for understanding regulatory mechanisms in the erythrocyte.

The activities of the glycolytic enzymes in the hemolysate were measured by the procedures described in the Experimental section, and the results are summarized in Table III; each value represents the average of at least four separate experiments. Since the over-all glycolytic rate in the red cell is maximal at pH 8.1, the individual activities were measured arbitrarily at that pH.

TABLE III

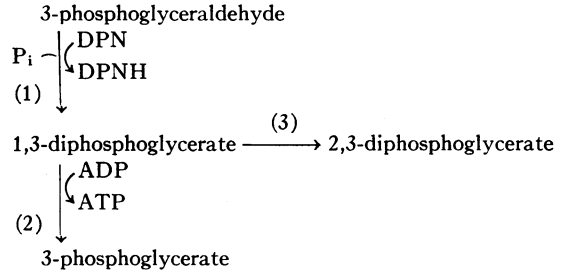
Levels of glycolytic enzymes in the hemolysate

Enzyme	Data from present investigation	Other data	References
	μmoles substrate converted/hr/ml RBC		
Hexokinase	10	6, 6, 21	(26), (28), (29)
Phosphoglucose isomerase	151	150, 630, 990	(30), (31), (32)
Phosphofructokinase	82	33, 97	(33), (29)
Aldolase	31	24, 39	(34), (35)
Triose isomerase	5,100	4, 437	(36)
Phosphoglyceraldehyde dehydrogenase	800	249, 1,083	(36), (29)
Phosphoglyceric kinase	1,910	1,580	(29)
Phosphoglyceric mutase	228	351	(29)
Enolase	95	103	(37), (36)
Pyruvic kinase	158	179	(29)
Lactic dehydrogenase	1,257	2,670, 1,140, 932	(38), (34), (29)

For comparison, other reported values for these activities are shown in the second column of the table. It should be noted, however, that many of the previous studies were carried out under conditions of temperature and pH which differed from those in the present investigation.

A lower value of 249 μmoles per hour for 3-phosphoglyceraldehyde dehydrogenase was reported originally by Löhr and Waller (36), but this has now been superseded (29) by a value of 1,083 μmoles per hour, which agrees more closely with our value of 800. On the other hand, no explanation can be given for the fact that our value of 151 μmoles per hour for phosphoglucose isomerase, which was determined by three different assays (see Experimental section), is low relative to most of the other reported values (31, 32).

Mention should be made of a difficulty encountered in the assay of phosphoglyceric kinase. When this enzyme is assayed by following Reaction 2 in the forward direction (20) (see diagram, below), the substrate, 1,3-diphosphoglycerate, is generated via Reaction 1 by admixing 3-phosphoglyceraldehyde, P_i, and DPN with excess 3-phosphoglyceraldehyde dehydrogenase. After the equilibrium in Reaction 1 is established, as indicated by the cessation of DPNH production, ADP and the preparation to be assayed for phosphoglyceric kinase are added. The production of additional DPNH is then used to follow the rate of Reaction 2.



The occurrence of 2,3-diphosphoglyceric mutase (Reaction 3) interferes with the assay for phosphoglyceric kinase, since it removes 1,3-diphosphoglycerate. The side reaction can be repressed, however, by the addition of excess 2,3-diphosphoglycerate, which has been shown to be an inhibitor of the reaction (39). As indicated in Table IV, the amount of inorganic phosphate converted to various phosphate esters, via Reactions 1, 2, and 3, is reduced to a constant level as the amount of 2,3-diphosphoglycerate is increased. Although erythrocytes contain large quantities of 2,3-diphosphoglycerate (40), the amount of hemolysate added to the assay system is so small that 5 μmoles of exogenous 2,3-diphosphoglycerate must be supplied in order to study the kinase reaction in either direction.

TABLE IV

Effect of added 2,3-diphosphoglycerate in the phosphoglyceric kinase assay *

2,3-Diphosphoglycerate added	P_i converted to phosphate esters
μmoles	*mμmoles*
0	65
5	33
10	34

* Phosphoglyceric kinase was measured in the forward reaction (20) at 25° C, in a cuvet in which the total volume of the assay system was 1.0 ml, containing: 0.4 μmole D-3-phosphoglyceraldehyde; 10 μmoles KH_2PO_4, pH 8.1; 40 μmoles Na_2HPO_4, pH 8.1; 0.1 μmole DPN; 5 μmoles $MgCl_2$; 0.01 mg of 3-phosphoglyceraldehyde dehydrogenase; 2,3-diphosphoglycerate as indicated; and an aliquot of hemolysate equivalent to 0.014 mg of hemoglobin. In the course of an assay, the absorbancy at 340 mμ of a mixture of all the components, except hemolysate and ADP, was measured until it remained constant, at which time the hemolysate and 0.5 μmole of ADP were added. The increase in absorbancy at 340 mμ was then measured over a period of 5 minutes, and the amount of P_i converted to phosphate esters was calculated from the equations given by Bücher (20).

DISCUSSION

The over-all glycolytic rate in the intact erythrocyte was found to be independent of extracellular Mg^{++}, indicating that a sufficient amount of this ion is available within the cell to meet the needs of the Mg^{++}-requiring enzymes in this pathway. In the hemolysate system, however, a definite requirement emerges for added Mg^{++}. On the other hand, although there is a requirement for extracellular inorganic phosphate (optimal level, 0.03 M) for glycolysis in the intact cell, the addition of phosphate to the hemolysate system does not appreciably affect the glycolytic rate. It is possible that adenosine triphosphatase and other phosphatases, "activated" by the lytic procedure, produce a continuous supply of inorganic phosphate by the breakdown of phosphate esters.

At concentrations higher than 0.03 M, phosphate inhibits glycolysis in the intact cell. This effect can be localized between glucose and 3-phospho-

glyceraldehyde, since the conversion of glucose to lactate and of inosine to lactate involves a common pathway at the triose phosphate level, and since the latter sequence is stimulated rather than inhibited by high (> 0.03 M) concentrations of phosphate (unpublished results, this laboratory). Phosphate inhibition of glycolysis in erythrocytes is likely to occur in cells stored for long periods of time in ACD, since under these conditions inorganic phosphate accumulates due to the breakdown of ATP and 2,3-diphosphoglycerate (41).

The pH optimum (8.1) for erythrocyte glycolysis is approximately the same as that observed for hexokinase in the red cell (42). The experimentally determined ratio of lactate production to glucose disappearance is 2 in the presence of excess glucose, which would follow if the first step in the sequence (i.e., glucose → glucose-6-phosphate) were rate limiting. If hexokinase is located at the cell surface, and if the phosphorylation of glucose is a rate-limiting step, it would not be surprising to find that the pH requirement for hexokinase would largely determine the pH at which the over-all rate is maximal.

In the present investigation the activity of each of the glycolytic enzymes in the erythrocyte has been determined. In general, the enzyme levels in Table III may be grouped into three categories: *1*) the low activities (< 85 μmoles per hour) represented by hexokinase, phosphofructokinase, and aldolase; *2*) the intermediate values (100 to 300 μmoles per hour) of phosphoglucose isomerase, phosphoglyceric mutase, enolase, and pyruvic kinase; and *3*) the high values (> 800 μmoles per hour) of triose isomerase, 3-phosphoglyceraldehyde dehydrogenase, phosphoglyceric kinase, and lactic dehydrogenase. Thus, the enzymes which catalyze the ATP-dependent reactions, hexokinase and phosphofructokinase, are in the lowest category, and hence potentially rate limiting, while the enzymes involved in ATP-regeneration (phosphoglyceric kinase and pyruvic kinase) are both present at much greater levels. Comparable findings have been reported for other predominantly glycolyzing systems, such as ascites tumor cells and HeLa cells (43) and chicken erythrocytes (44).

The levels of the glycolytic enzymes can be correlated, in part, with the observed steady-state levels of various phosphorylated intermediates of glycolysis. Column chromatography by Bartlett (45) has shown that, with the exception of 2,3-diphosphoglycerate (which is present in abnormally high quantities in the red cell), glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, and monophosphoglycerate are the only glycolytic intermediates which accumulate in measurable quantities. Since the accumulation of an intermediate, B, can result from conditions where $A \xrightarrow{fast} B \xrightarrow{slow} C$, it would follow that only those phosphate esters will accumulate which are substrates for the enzymatic reactions having a low activity. This correlation is seen from the data in Table III, since phosphoglucose isomerase, phosphofructokinase, aldolase, and enolase are, in fact, low in activity. It should be noted, however, that the rate of hexokinase is lower than that of either phosphoglucose isomerase or glucose-6-phosphate dehydrogenase and, according to the above argument, glucose-6-phosphate would not be expected to accumulate if only these factors were involved. There may be another route leading to the formation of glucose-6-phosphate, perhaps from glycogen or a pentose phosphate, which contributes to the observed steady-state level of glucose-6-phosphate.

Even when tested under the favorable conditions of a single-step assay, the hexokinase-controlled reaction (10 μmoles of glucose converted per hour per ml of cells) is rate limiting in the sequence. This value may be compared to the over-all glycolytic rate in the intact cell, or in a suitably reconstructed hemolysate, in which 6.5 μmoles of lactate are produced per hour per ml of cells; this corresponds to an over-all metabolic rate of 3.25 μmoles with respect to glucose utilization. No explanation can be offered for the observation that certain other enzymes in the sequence are present at levels several orders of magnitude higher than that of the apparent pacemaker enzyme, hexokinase.

Regulation of erythrocyte glycolysis may be examined in terms of the steady-state levels of ATP (1 μmole per ml of cells), ADP (0.2 μmole), and inorganic phosphate (0.4 μmole) (45). Since glycolysis involves the net disappearance of both ADP and inorganic phosphate, with the concomitant production of ATP, it is evident that the availability of ADP may be a rate-limiting factor. In

order to sustain an over-all rate of about 3 μmoles of glucose utilized per hour, a minimal rate of ATP breakdown to ADP and inorganic phosphate of about 6 μmoles per hour would be necessary. There are two ways in which this could be accomplished: *1*) by the action of adenosine triphosphatase; and *2*) as a consequence of ATP-dependent synthetic reactions in which ADP and inorganic phosphate are products. It is difficult to assess the magnitude of the latter contribution, but previous studies from this laboratory (46) have shown that the level of adenosine triphosphatase in hemolysates is about 4 μmoles of ATP broken down per hour. This effect, by itself, would almost maintain glycolysis, provided that it occurs at that rate within the intact cell.

The conversion of glucose to lactate in the erythrocyte normally proceeds almost entirely via the glycolytic scheme rather than by the hexose monophosphate shunt and pentose cycle (23). Comparison of the levels of phosphoglucose isomerase (151 μmoles of substrate converted per hour) and glucose-6-phosphate dehydrogenase (74 μmoles per hour) does not reveal why glycolysis should predominate, although the higher level of phosphoglucose isomerase plus the sluggishness of the erythrocyte in reoxidizing TPNH may result in the diversion of most of the glucose-6-phosphate toward fructose-6-phosphate rather than to 6-phosphogluconate.

SUMMARY

1. A method is described for the preparation of erythrocyte suspensions in which the inherent pH of the cells may be adjusted to any desired value in the region of 6.4 to 9.4; the pH is maintained during glycolysis for a period of 3 hours at 37° C. In the intact cell, maximal production of lactate (6.5 μmoles per hour per ml of red cells) from glucose was achieved at pH 8.1, with an extracellular concentration of inorganic phosphate of 0.03 M.

2. When glycolysis is reconstructed in a hemolysate system fortified with ATP, DPN, inorganic phosphate, and Mg^{++}, the pH optimum and the rate of lactate production are the same as those observed for the intact erythrocyte. Lactate production in the hemolysate is inhibited by Na^+, $HPO_4^=$, and $Tris^+$.

3. The levels of the individual glycolytic enzymes have been determined in the hemolysate at pH 8.1. These findings are discussed with reference to the over-all glycolytic rate and are correlated with the steady-state levels in the cell of ADP, ATP, inorganic phosphate, and phosphorylated intermediates of glycolysis.

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