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EFFECT OF HAEMOLYSIS ON THE HEXOSE MONOPHOSPHATE PATHWAY IN NORMAL AND IN GLUCOSE-6-PHOSPHATE DEHYDROGENASE-DEFICIENT ERYTHROCYTES

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Summary

The hexose monophosphate pathway of human glucose-6-phosphate dehydrogenase (EC 1.1.1.49) - deficient erythrocytes is under a severe and unexplained restraint (Gaetani, G.D., Parker, J.C. and Kirkman, H.N. (1974) Proc. Natl. Acad. Sci. U.S. 71, 3584—3587).

In this study the hexose monophosphate pathway activity and the NADPH level of normal and glucose-6-phosphate dehydrogenase-deficient erythrocytes were measured soon after haemolysis. The results indicate a prompt increase in $^{14}CO_2$ evolution and a rise in NADPH levels. Since, in this study, the concentration of the haemolysate is comparable to that of intact erythrocytes, the relief of the restraint on glucose-6-phosphate dehydrogenase through dilution-dependent dissociation from inactivator or inhibitor is excluded. The possibilty that the intracellular restraint may result from compartmentalization of glucose-6-phosphate dehydrogenase and substrates or from properties of the intact membrane of the erythrocytes is suggested.

Introduction

Several mechanisms have been proposed for the regulation of the hexose monophosphate pathway in normal cells as well as in cells carrying the defect for glucose-6-phosphate dehydrogenase (EC 1.1.1.49; D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase). Most of these mechanisms require the concentration of $NADP^+$ to be low or that of NADPH to be high. However, direct measurement of these coenzymes in enzyme-deficient human erythrocytes revealed that most of the nucleotide is in the oxidized form, while normal erythrocytes

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Abbreviation: TES, *N*-tris-(hydroxymethl)methyl-2-aminoethane-sulfonic acid.

have nearly all the nucleotide in the reduced state [1]. Measurement of the hexose monophosphate pathway in intact erythrocytes, in the presence of methylene blue to keep most of the coenzyme in the oxidized form, reveals that the glucose-6-phosphate dehydrogenase activity of both normal and low activity glucose-6-phosphate dehydrogenase variants, seems to be under much greater intracellular restraint than can be accounted for by concentrations of substrates or by properties of the enzyme in cell-free systems [2]. Without such intracellular restraint, the glucose-6-phosphate dehydrogenase-deficient cells should have sufficient enzyme activity to allow them to maintain almost all the pyridine nucleotide in the reduced form, thus avoiding haemolysis by certain drugs [3—5]. Although many metabolites and nucleotides have been tested for their ability to inhibit glucose-6-phosphate dehydrogenase [6], none have been found to be sufficiently inhibitory to account for the observed intracellular impairment. Among the possible explanations for the absence of restraint of glucose-6-phosphate dehydrogenase in diluted haemolysates are prompt destruction of an inhibitor, or rapid activation of the enzyme including release from a concentration-dependent complex.

The present investigation is an effort to evaluate the mechanism that holds the hexose monophosphate pathway under such severe restraint.

Methods

Blood samples were obtained from four normal and eight enzyme-deficient men with the Mediterranean variant. (On the basis of their Sardinian ancestry and enzyme activity, all eight glucose-6-phosohate dehydrogenase-deficient men were regarded as having the Mediterranean variant [7]). Glucose-6-phosphate dehydrogenase deficiency was detected by the brilliant creasyl blue method [8]. Activity of the enzyme was measured spectrophotometrically at 25°C by the rate of formation of NADPH in the presence of the enzyme, glucose-6-phoshate and $NADP^+$. In normal haemolysates, glucose-6-phosphate dehydrogenase activity was estimated by subtracting the values of 6-phosphogluconate dehydrogenase activity from the sum of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities, as suggested by Glock and McLean [9].

Blood was collected in tubes containing heparin and centrifuged at 1000 × *g* for 10 min at 4°C. Plasma and buffy coat were discarded. The erythrocytes were suspended in 5 vols. of Krebs Ringer buffer that also contained *N*-Tris-(hydroxymethyl)methyl-2-aminoethane sulphonic acid (TES) (sodium salt, pH 7.4) at a final concentration of 20 mM. The suspension was centrifuged again at 1000 × *g* and the supernatant fluid was removed. The resulting erythrocytes were used undiluted or mixed with an equal volume of the buffer. All experiments described in this study were performed in duplicate.

Determination of NADPH and $NADP^+$ + NADPH levels in normal and enzyme-deficient packed erythrocytes, at various times after haemolysis. 1 ml of packed erythrocytes was transferred to a 3 ml plastic centrifuge tube, which was placed in a water bath at 25°C. The packed cell volume was determined each time and varied between 85 and 92%. A micro-tip connected to an ultrasonic probe (model Vibrason 150, Kerry Ultrasonics Ltd., Hitchin, U.K.) was

dipped into the packed cells, and the erythrocytes were haemolysed for 30 s at a frequency of 20 kHz. A slight increase in the temperature was evident at the end of sonication (2° C), whereas the pH remained constant at 7.38.

At various times after haemolysis, 15 μl of the intact packed erythrocytes and of the haemolysates were diluted 1 : 330 in cold 0.04 M NaOH containing 0.5 mM cysteine hydrochloride. The determination of the nucleotides was performed by the enzymatic cyclic method of Lowry and Passonneau [10] as previously described [1]. Three experiments were also carried out on erythrocytes previously incubated under 100% carbon monoxide, an experimental condition which eliminates peroxide production [11].

Kinetic studies in the presence of [1-^{14}C]glucose-6-phosphate and saturating amounts of NADP$^+$ and glucose-6-phosphate. Twice-washed erythrocytes, both normal and glucose-6-phosphate dehydrogenase-deficient, were separately mixed with an equal volume of Krebs Ringer TES buffer (pH 7.4). The reaction mixture consisted of 2.0 ml of erythrocytes in Krebs Ringer TES buffer and 0.3 ml of the buffer solution containing NADP$^+$ at a final concentration of 6.6 mM, glucose-6-phosphate at a final concentration of 6.6 mM, and 0.3 μCi of [1-^{14}C]glucose-6-phosphate (52.5 mCi/mmol). For normal erythrocytes, NADP$^+$ and glucose-6-phosphate concentrations were doubled. Reactions were stopped at intervals of 30 s. Rates were linear up to 1 min after haemolysis. Two experiments with normal and deficient erythrocytes were performed with 1.0 μCi of [U-^{14}C]glucose-6-phosphate (125 mCi/mmol). This allowed an evaluation of other possible sources of $^{14}CO_2$ evolution.

[1-^{14}C]Glucose-6-phosphate was purchased from New England Nuclear Corp., Boston, Mass. and [U-^{14}C]glucose-6-phosphate from the Radiochemical Centre, Amersham, U.K.

The reaction flasks consisted of scintillation vials fitted with a rubber cap and a plastic disposable center well (Kontes, Vineland, N.J.) containing 0.2 ml of 2 M NaOH. Each reaction mixture was preincubated for 5 min at 25° C, then reactions were started by quick haemolysis of the erythrocytes; this was achieved by means of sonication for 30 s at a frequency of 20 kHz, passing the micro-tip, connected to the sonicator, through the rubber cap of each vial. By this method complete haemolysis of the erythrocytes was obtained as demonstrated by the addition of 0.15 M NaCl and determination of the haemoglobin on the supernatant after centrifugation of the saline suspension. The reactions were stopped at various intervals (from 0 to 4 min) by the addition of 0.7 ml of 3.7 M perchloric acid. The perchlorate-reaction mixture was allowed to stand for 30 min for full recovery of $^{14}CO_2$. The disposable center wells were then transferred to new scintillation vials containing 10.0 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) and 1.0 ml of water, and counted in a Packard scintillation counter (Model 3330) with an efficiency of approx 90%.

2 ml of distilled water were then added to the perchlorate-reaction mixtures, which were incubated at 37° C for 30 min in a Dubnoff metabolic shaker for residual glucose-6-phosphate determination. The contents were then transferred to separate tubes and centrifued at 1500 × *g* for 10 min. The residual glucose-6-phosphate concentration in the neutralized solution was measured according to the method of Lowry and Passonneau [10] in a Farrand fluorometer Model A4 (Farrand Optical Co., Inc. Valhalla, N.Y.).

At the end of each incubation, the specific activity of labelled glucose-6-phosphate was measured as $^{14}CO_2$ evolved from [1-^{14}C]- or [U-^{14}C]glucose-6-phosphate in the presence of reagent glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase [12]. Sawed plastic tubes containing 100 μl of the neutralized solution were inserted into scintillation vials, and 60 μl of a concentrated stock solution having in final concentration 0.25 M imidazole-acetate buffer (pH 7.0), 50 mM $MgCl_2$, 0.15 M ammonium acetate, 5 mM GSSG and 10 mM $NADP^+$, were added to the tubes. The scintillation vials with the plastic tubes were then fitted with rubber stoppers to which were attached disposable center wells containing 0.2 ml of 2 M NaOH. The reaction was started by the addition, with a disposable insulin syringe, of 140 μl of a solution containing, in a final volume of 3.0 ml, the following reagents: 120 μg yeast glucose-6-phosphate dehydrogenase, 480 μg yeast 6-phosphogluconate dehydrogenase, 20 μg yeast glutathione-reductase and 160 μg of bovine serum albumin. GSSG and glutathione-reductase served to maintain most of NADP in the oxidized form and to accelerate the completion of the reaction. The vials were incubated for 5 h at 37° C. At the end of the incubation the wells and the reaction mixtures were transferred to new scintillation vials and counted as described above. The recovery of $^{14}CO_2$ from labelled glucose-6-phosphate present in the reaction mixtures was over 94%. No enzymatically detectable amount of glucose-6-phosphate and 6-phosphogluconate was present at the end of the incubation period. In general, the recovery of total radioactivity was 84—90%.

Reagent glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and glutathione-reductase were purchased from Boehringer Mannheim GmbH, G.F.R..

$^{14}CO_2$ evolution from [1-^{14}C]glucose-6-phosphate without added $NADP^+$. 0.08 μCi (5 μl) of [1-^{14}C]glucose-6-phosphate (52.5 mCi/mmol) were added to 2.0 ml of packed enzyme-deficient erythrocytes. The erythrocytes were haemolyzed as previously described and the reaction was stopped after 2 min by the addition of 1.0 ml of 3.7 M perchloric acid. The procedures for $^{14}CO_2$ counting and for determining the amount of glucose-6-phosphate were as described above. Hexose monophosphate pathway activity was calculated on the basis of the geometric mean of the specific activity between 0 and 2 min.

Results

The measurement of pyridine nucleotides in intact glucose-6-phosphate dehydrogenase-deficient erythrocytes in steady-state conditions confirmed the reduced concentration of NADPH (Table I) [1]. 2 min after haemolysis of packed deficient cells, a significant increase in the concentration of NADPH was noticed ($P < 0.0005$). The NADPH/total NADP ratio passed from 0.33 to 0.89 ($P < 0.0005$), while the amount of total nucleotide did not change significantly ($P > 0.25$). Fig. 1 represents the generation rate of NADPH from steady-state values from one of 5 experiments, without addition of $NADP^+$ and glucose-6-phosphate. Incubations of deficient erythrocytes in the presence of 100% carbon monoxide, did not affect the generation rate of NADPH after haemolysis. No variation in the concentration of NADPH was evident in normal

TABLE I

NADPH AND TOTAL NADP ($NADP^+$ + NADPH) CONCENTRATIONS IN PACKED ERYTHROCYTES (NORMAL AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE-DEFICIENT MEDITERRANEAN VARIANT) BEFORE AND AFTER HAEMOLYSIS

Number of experiments is given in parenthesis. *P* values for the significance of the difference between means are given in the text. NADP and NADPH concentrations were measured 2 min after haemolysis.

Subjects	Intact erythrocytes			Lysed erythrocytes		
	NADPH (μM)	Total NADP (μM)	NADPH / Total NADP	NADPH (μM)	Total NADP (μM)	NADPH / Total NADP
Mediterranean variant (7)						
Mean	20.2	62.0	0.33	51.5	58.3	0.89
S.D.	±3.7	±11.1	±0.05	±12.0	±12.0	±0.10
Normal (4)						
Mean	32.2	33.0	0.98	31.8	32.0	0.99
S.D.	±2.1	±2.5	±0.04	±2.5	±3.6	±0.05

erythrocytes after haemolysis (Table I). Two additional series of experiments confirmed that the hexose monophosphate pathway is the prevalent source of NADPH. The first was carried out without added $NADP^+$ and in the presence of labelled glucose-6-phosphate which did not alter the intrinsic concentration of glucose-6-phosphate of enzyme-deficient erythrocytes. By 2 min after haemolysis an increase in the $^{14}CO_2$ evolution was evident (Table II), and since maximum rate of the reaction was obtained within 1 min after haemolysis (Fig. 1), this experiment underestimates the activity of the hexose monophosphate

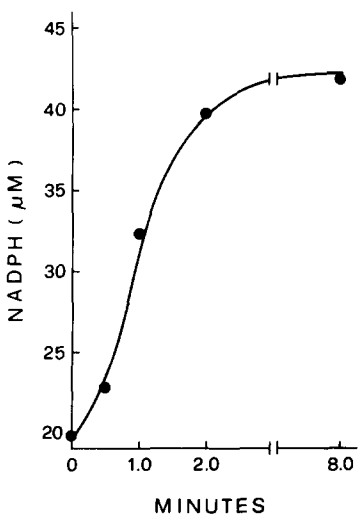

Fig. 1. Spontaneous generation rate of NADPH from steady-state values after haemolysis of glucose-6-phosphate dehydrogenase-deficient packed erythrocytes.

TABLE II

EFFECT OF RAPID HAEMOLYSIS ON HEXOSE MONOPHOSPHATE PATHWAY ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE-DEFICIENT ERYTHROCYTES

Hexose monophosphate pathway activity is expressed as $^{14}CO_2$ evolution 2 min after rapid haemolysis of packed erythrocytes from two enzyme-deficient subjects in the presence of 0.08 μCi of [1-^{14}C]glucose-6-phosphate at 25°C.

Experiment number	Time (min)	Glucose-6-phosphate (μmol/100 ml erythrocytes)	[1-^{14}C]glucose-phosphate specific activity (cpm × 100/μmol)	Hexose monophosphate pathway activity (μmol $^{14}CO_2$/100 ml erythrocytes)
1	0	3.55	25 280	0.00
	2	4.63	5 790	3.54
2	0	2.95	26 500	0.00
	2	4.43	4 890	2.28

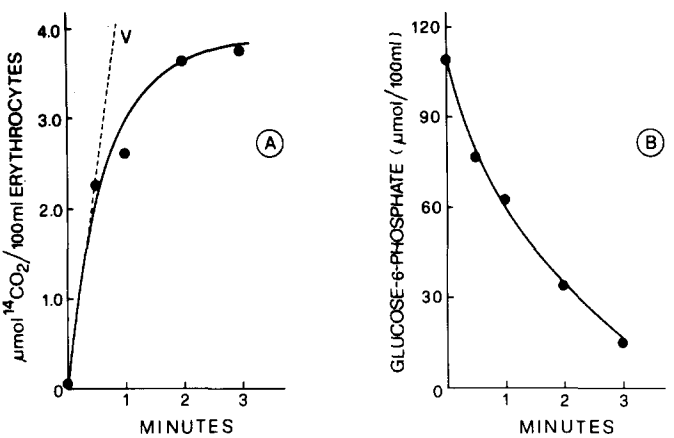

Fig. 2. A. Kinetic representation of the hexose monphosphate pathway activity as $^{14}CO_2$ evolution after haemolysis of glucose-6-phosphate dehydrogenase-deficient erythrocytes in the presence of [1-^{14}C]glucose-6-phosphate and saturating amounts of $NADP^+$ and glucose-6-phosphate. B. Glucose-6-phosphate concentration at various times.

TABLE III

EFFECT OF SONICATION ON THE HEXOSE MONOPHOSPHATE PATHWAY ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE-DEFICIENT ERYTHROCYTES

Erythrocytes were suspended in Krebs Ringer TES solution (packed cell volume 40%) in the presence of 0.9 mM glucose-6-phosphate, 0.9 mM $NADP^+$, and [1-^{14}C]glucose-6-phosphate.

Time (min)	Residual [1-^{14}C]glucose-6-phosphate (cpm/ml)	Glucose-6-phosphate concentration (μmol/100 mol)	[1-^{14}C]glucose-6-phosphate specific activity (cpm/μmol)	$^{14}CO_2$ evolution (cpm)	Hexose monophosphate pathway activity (μmol $^{14}CO_2$/100 ml erythrocytes)
0	162 445	94.01	172 813	278	0.05
1	104 624	52.23	200 429	1867	1.16
2	83 844	43.14	194 533	2792	1.78
3	48 299	24.45	197 946	3288	2.06
4	28 475	16.10	176 863	3416	2.40

TABLE IV

HEXOSE MONOPHOSPHATE PATHWAY ACTIVITY AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY OF NORMAL AND DEFICIENT ERYTHROCYTES UNDER VARIOUS CONDITIONS

Values are means of different experiments and are expressed as μmol/min per 100 ml of erythrocytes. Number of experiments is given in parenthesis. Data of intact erythrocytes are drawn from a previous paper [2].

Hexose monophosphate pathway activity				Glucose-6-phosphate dehydrogenase activity
Intact erythrocytes		Concentrated haemolysates		
Steady-state	Methylene Blue (100 μM)	A *	B **	
Normal erythrocytes				
0.07 (1)	2.92 (1)	109.10 (3)	—	121.0 (3)
Glucose-6-phosphate dehydrogenase-deficient erythrocytes				
0.04 (3)	0.08 (3)	5.76 (6)	1.46 (2)	6.0 (6)

* In the presence of [1-^{14}C]- or [U-^{14}C]glucose-6-phosphate and saturating amount of $NADP^+$ and glucose-6-phosphate (packed cell volume 40—43%).

** In the presence of [1-^{14}C]glucose-6-phosphate only (packed cell volume 85—92%).

pathway. Despite that, the activity in the haemolysate was about 40-fold higher than the steady-state values of intact erythrocytes (Table IV).

The second series of experiments was carried out in order to evaluate the maximum rate of the hexose monophosphate pathway activity of concentrated haemolysates, in the presence of labelled glucose-6-phosphate and with concentrations of substrate and coenzyme well above the Michaelis constants (Table III, Fig. 2, A and B). In normal and deficient erythrocytes the rate was comparable to the spectrophotometrically determined V of glucose-6-phosphate dehydrogenase, (Table IV), and the shape of the curve suggests no apparent lag in achieving this activity. A similar result with a separate experiment appears in Table III. No differences were noticed using [1-^{14}C]- or [U-^{14}C]glucose-6-phosphate.

Discussion

Several studies relate the regulation of the hexose monophosphate pathway to inhibition or reversible inactivation by NADPH [6,13,14]. These mechanisms fail to account for the inhibition of glucose-6-phosphate dehydrogenase in deficient erythrocytes, where the NADPH/$NADP^+$ ratio is low [1,2,15]. Our results indicate that the intracellular restraint on glucose-6-phosphate dehydrogenase is completely lifted within 1 min from haemolysis, even when the haemolysates are very highly concentrated. Furthermore, the generation rate of NADPH (Fig. 1) suggests a discrete and discernible lag in resumption of full activity by the Mediterranean mutant hemolysate.

The lag is not discernible when erythrocytes, both normal and glucose-6-

phosphate dehydrogenase deficient, are lysed in the presence of saturating amounts of $NADP^+$ and glucose-6-phosphate (Fig. 2A). This discrepancy could be due to an apparent initial low concentration of glucose-6-phosphate (Table II), below the K_m values of glucose-6-phosphate dehydrogenase, despite the fact that the Mediterranean variant had a more favourable Michaelis constant. Soon after haemolysis occurs, the specific activity of [1-^{14}C]glucose-6-phosphate rapidly falls and the intracellular concentration of glucose-6-phosphate slightly increases (Table II). This is presumed to result from a more active recycling of glucose-6-phosphate through the shunt, via transketolase and transaldolase [16], as well as cross flow-exchange between glucose-6-phosphate and the reservoir of other intermediates of the Embden-Meyerhof pathway. An alternative explanation could be that, at the concentrations of substrate and coenzyme present in the deficient erythrocytes, the reaction is not too fast to follow and the possibility of destruction of an intracellular inhibitor within seconds after haemolysis could be better demonstrated than in the presence of larger amounts of $NADP^+$ or glucose-6-phosphate. This possibility, at least for ATP and 2,3-diphosphoglycerate, two of the most active inhibitors of the hexose monophosphate pathway [6], is ruled out by the fact that no significant variations in the concentration of these metabolites have been observed in packed erythrocytes up to 1 h after haemolysis by sonication [17]. The lag in glucose-6-phosphate dehydrogenase-deficient erythrocytes being due to a modification of the $NADP^+$-dependent monomer-dimer equilibrium [14] is unlikely, since in enzyme-deficient erythrocytes, due to the high $NADP^+$/NADPH ratio, the equilibrium should already be shifted toward the dimeric form. Activation of glucose-6-phosphate dehydrogenase through dilution-dependent dissociation from an inactivator is also excluded, since the concentration of the haemolysates remained comparable to that of intact erythrocytes. If the reduced intracellular activity of glucose-6-phosphate dehydrogenase [2] results from binding of substrate by an intracellular component, this binding could be abolished by sonication. These results therefore do not exclude the possibility that the intracellular restraint results from compartmentalization of glucose-6-phosphate dehydrogenase and substrates or from properties of the intact membrane of the erythrocytes. Both membranes and microstructures are disrupted by haemolysis.

The possibility that stimulation of the hexose monphosphate pathway could be related to an increased production of peroxides through sonication is also ruled out by experiments performed in the presence of carbon monoxide.

It can be argued that the rise in the NADPH values could be ascribed to other sources, such as the enzymes isocitrate or $NADP^+$-malate dehydrogenase. However, the activities reported for these enzymes in human haemolysates [18], are too low to account for this observation. Moreover, this possibility is made unlikely by the finding that the hexose monophosphate pathway activity, as measured by $^{14}CO_2$ recovery, also corresponds to the activity of glucose-6-phosphate dehydrogenase, and this activity is more than sufficient to account for the observed rise in NADPH (Tables I and IV).

The results of the present investigation include several possibilities as the causes of the intracellular restraint [2] and provide information for further investigations on the role of intact membrane in the regulation of the pathway and on

the influence of haemolysis on the intracellular kinetics.

The results also point out the advantages of using glucose-6-phosphate dehydrogenase-deficient erythrocytes in studies related to the regulation of the hexose monophosphate pathway. These allow the use of highly concentrated haemolysates without having glucose-6-phosphate dehydrogenase and hexose monophosphate pathway reactions too fast to follow.

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