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Research Article

Extraction in the presence of sodium hydroxide and cysteine allows estimates of NADPH and total NADP in human red cells without the erroneously high values of NADP+ obtained with earlier methods. An application of this technique to G6PD-deficient cells reveals that most of the nucleotide is in the oxidized form. In contrast, normal red cells have nearly all of the nucleotide in the reudced form. In addition to providing information concerning the intracellular regulation of the hexose monophosphate shunt, these findings support the concept that G6PD deficiency is a product-deficiency disorder.

CONCISE

PUBLICATION

Red Cell $NADP^+$ and NADPH in Glucose-6-Phosphate Dehydrogenase Deficiency

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ABSTRACT Extraction in the presence of sodium hydroxide and cysteine allows estimates of NADPH and total NADP in human red cells without the erroneously high values of $NADP^+$ obtained with earlier methods. An application of this technique to G6PD-deficient cells reveals that most of the nucleotide is in the oxidized form. In contrast, normal red cells have nearly all of the nucleotide in the reduced form. In addition to providing information concerning the intracellular regulation of the hexose monophosphate shunt, these findings support the concept that G6PD deficiency is a product-deficiency disorder.

INTRODUCTION

Recent considerations of human red cells (1, 2) indicate that measurements of reduced and oxidized nicotinamide adenine dinucleotide phosphate (NADPH and $NADP^+$) would be of value in understanding not only the dysfunction of glucose-6-phosphate dehydrogenase (G6PD)-deficient red cells but also regulation of the hexose monophosphate shunt (HMS)[1]. G6PD is the first enzyme of the HMS. Together with its tandem enzyme, 6-phosphogluconate dehydrogenase, it accounts for most of the production of NADPH in red cells. Since NADPH is necessary for the regeneration of reduced glutathione (GSH) from oxidized glutathione (GSSG), this pathway serves to protect red cells from agents that place an oxidative drain on GSH. When a genetic impairment of the HMS exists, as with G6PD deficiency, the ingestion of certain drugs or toxic agents results in depletion of GSH and in hemolytic anemia (3). As measured by the response of intact cells to various types of oxidative stress, however, the G6PD of both normal and certain G6PD-deficient red cells seems to be under much greater intracellular restraint than can be accounted for by concentrations of substrate or by properties of the enzyme in cell-free systems (2, 4). If this is true, the concentration of NADPH could be much lower in these G6PD-deficient cells than in normal cells, even in the absence of imposed oxidative stress (2). Without such intracellular restraint, these G6PD-deficient cells should have sufficient activity of G6PD to allow them to maintain nearly all of the nucleotide in the reduced form (2).

The term "total NADP" will be used to mean the sum of NADPH and $NADP^+$. In the present collaborative study, the NADPH and total NADP concentrations were determined in red cells with the two most common types of G6PD deficiency: that seen among white men in the Mediterranean area (in Genoa, Italy) and that among black men (in Chapel Hill, North Carolina). The range of values observed on some of these subjects has been cited in a discussion of HMS regulation (2). We here report the methods used, results on a greater number of subjects, alterations of the total NADP concentration, and statistical analysis of the results.

METHODS

Samples of blood were obtained from 13 healthy, normal men and from 12 G6PD-deficient men with normal hemato-

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[1] *Abbreviation used in this paper:* HMS, hexose monophosphate shunt.

TABLE I

NADPH and Total NADP Concentrations in Red Cells of Normal and G6PD-Mediterranean Men

Subjects	Total NADP	NADPH	NADPH/total NADP
	μM	μM	
Normals			
1	30.3	29.0	0.96
2	30.2	31.8	1.05
3	34.4	33.8	0.98
4	27.1	27.9	1.03
5	35.5	33.7	0.95
6	29.0	27.4	0.94
7	29.4	27.8	0.95
8	29.8	29.8	1.00
Mean ± SD	30.71 ± 2.82	30.15 ± 2.62	0.9825 ± 0.0406
G6PD Mediterranean			
1	57.6	9.2	0.16
2	57.1	12.8	0.22
3	60.9	17.0	0.28
4	60.2	16.8	0.28
5	55.9	16.2	0.29
6	62.8	14.8	0.24
Mean ± SD	59.08 ± 2.63	14.47 ± 3.01	0.2450 ± 0.0497

P values for the significance of the difference between various means are given in the text.

crits and without apparent hematological disease. G6PD Mediterranean has been defined as the prevalent enzymic phenotype among G6PD-deficient men of Sardinian and Sephardic Jewish ancestry (5). No major heterogeneity has been found for the enzymic phenotype among Sardinian, deficient men, although the same techniques of characterization revealed heterogeneity among Greek subjects (6, 7). On the basis of their Sardinian origin and enzyme activity, all six G6PD-deficient men in Genoa were regarded as having the G6PD Mediterranean variant. Furthermore, the enzyme of subject 1 was purified, characterized (5, 7), and found to have the properties of G6PD Mediterranean. His sister's son is also included in Table I. Otherwise, these men are unrelated. Similarly, on the basis of definition of the phenotype, their race, the level of activity in red cells, and electrophoretic migration of their enzyme, the six black, G6PD-deficient men were regarded as having G6PD A-. These six men and five control subjects (Table II) were unrelated, black male employees. The controls had normal activity and electrophoretic migration of red cell G6PD.

Screening for deficiencies in activity of G6PD was by the brilliant cresyl blue method described in a WHO technical report (8). Deficiencies in activity were confirmed by spectrophotometric measurement of the rate at which NADPH is formed in the presence of the enzyme, $NADP^+$, and G6P (8). The electrophoretic phenotype of the G6PD in each of the 12 G6PD-deficient men and of the normal black men was determined on starch gel with Tris-EDTA-borate buffer (8). Blood was collected in tubes containing heparin, mixed, placed at room temperature, and mixed once again before samples were removed for determinations of NADPH and total NADP. Determination of these nucleotides were carried out in duplicate within 2 min of venipuncture. The procedure used for determination of total NADP was the cycling method of Burch, Bradley, and Lowry (9) and Lowry and Passonneau (10). The concentration of NADPH was determined in samples in which $NADP^+$ was destroyed by heat at alkaline pH (9, 10). Reagent G6PD, 6-phosphogluconate dehydrogenase, and glutamate dehydrogenase were purchased from the Boehringer Mannheim Corp., New York. Hemoglobin was isolated and converted to methemoglobin by the method of Hegesh, Calmanovici, and Avron (11). In the following paragraphs, we describe the slightly different techniques used in the two laboratories involved in this study.

In Genoa, determinations of nucleotide in G6PD-deficient red cells were performed simultaneously with a normal control. The blood was diluted 1:200 in cold 0.04 N NaOH containing 0.5 mM cysteine hydrochloride, as suggested by Burch, et al. (9). A portion of the diluted blood was immediately heated at 60°C for 10 min to destroy the $NADP^+$, while blanks, standards, and the remaining samples were kept at 0°C for the measurement of total NADP. $NADP^+$ standards and samples were added to the cycling reagent (10) to give a final concentration between 1 nM and 10 nM. At these concentrations, the reaction rates were proportional to nucleotide concentrations. Fluorometric measurements (10) were performed with a Farrand fluorometer, Model 4A (Farrand Optical Co., Inc., Valhalla, N. Y.), with a primary Corning 5840 filter (Corning Glass Works, Science Products Div., Corning, N. Y.) and with secondary 3387 and 4303 filters. The cycling rate for standards was 1,200–1,500/h.

In Chapel Hill, blood samples were collected and assayed singly. The blood was diluted 1:300 in cold 0.04 N NaOH containing 0.5 mM cysteine hydrochloride (9). $NADP^+$ and NADPH were added to separate portions of NaOH-cysteine solutions, to a final concentration of 80 nM. Portions of each dilution, and a blank of NaOH-cysteine, were heated 10 min at 60°C to destroy $NADP^+$. Both heated and unheated portions of blanks, standards, and samples were made less alkaline by the addition of 1/10 part (vol/vol) of 0.38 N HCl. 50 μl of each solution were added to 50 μl of cycling solution. The cycling solution was twice as concentrated, in all components, as that of Lowry and Passonneau (10). The 100 μl portions were mixed and incubated at 38°C for 1 h. Fluorometric measurements were in 5-ml cuvettes in a Turner 111 fluorometer with high-sensitivity

TABLE II

NADPH and Total NADP Concentrations in Red Cells of Normal Black (G6PD B) and G6PD A- Men

Subjects	Total NADP	NADPH	NADPH/total NADP
	μM	μM	
Normals			
1	31.1	32.6	1.05
2	33.9	32.4	0.96
3	35.2	40.5	1.15
4	29.8	29.8	1.00
5	34.7	38.0	1.10
Mean ± SD	32.9 ± 2.36	34.66 ± 4.31	1.0500 ± 0.0759
G6PD A-			
1	43.1	18.0	0.42
2	52.6	16.9	0.32
3	45.7	18.9	0.41
4	47.0	22.1	0.47
5	55.9	16.9	0.30
6	57.2	24.8	0.43
Mean ± SD	50.25 ± 5.80	19.60 ± 3.19	0.3917 ± 0.0667

P values for the significance of the difference between various means are given in the text.

sample holder. A Turner 7-60 filter and a 10% neutral plate served as primary filters, and a Turner 2A filter was the secondary filter (G. K. Turner Associates, Palo Alto, Calif.). The cycling rate with standards was 4,000–19,000/h. The rate was proportional to nucleotide concentration over the range examined.

RESULTS

In the normal red cells, essentially all NADP was in the reduced form, the ratio of NADPH to total NADP being insignificantly different from 1.00 ($P > 0.2$) in both Tables I and II. Similarly, the mean concentration of total NADP of normal samples in Genoa did not differ significantly from that in Chapel Hill ($P > 0.1$). For the Mediterranean, G6PD-deficient samples, the mean ratio of NADPH to total NADP, was 0.24, a value very significantly less ($P < 0.001$) than that for the normal samples (Table I). The mean ratio, NADPH/total NADP, for G6PD A− cells was 0.39, a value also very significantly less ($P < 0.001$) than that for normal cells (Table II), but greater ($P < 0.005$) than that of G6PD Mediterranean cells. The concentration of total NADP was significantly higher ($P < 0.001$) in both variant red cells than in normal cells, but greater in the G6PD Mediterranean cells than in G6PD A− cells ($P < 0.01$). These deviations of total NADP concentration and NADPH/total NADP ratios from normal were related to the degree of G6PD deficiency, since the G6PD Mediterranean cells had less than 5% of the G6PD activity of normal cells, whereas the G6PD A− cells had 10–20% of normal activity.

Table III provides the results of recovery experiments, in which NADPH or total NADP was determined after hemoglobin, methemoglobin, GSSG, normal blood, or G6PD-deficient blood were added to 3-ml portions of NaOH-cysteine solutions containing $NADP^+$ or NADPH.

DISCUSSION

Measurements of $NADP^+$ and NADPH present several difficulties in red cells. A major problem is the tendency of NADPH, in the presence of hemoglobin, to undergo oxidation to $NADP^+$ at acid pH or when heated at alkaline pH. The consequence is underestimation of NADPH and overestimation of $NADP^+$. The method of Burch et al. (9) and of Lowry and Passanneau (10) avoids this effect by using cysteine to protect NADPH from oxidation at alkaline pH. With this precaution, the estimates of NADPH and total NADP concentration were free of the error observed (9) with earlier methods, caused by oxidation of NADPH to $NADP^+$ during preparation and heating of the hemolysates. The absence of this error was indicated by the consistently high ratios of NADPH to total NADP observed with normal red cells (Tables I and II). With estimates of

TABLE III

Recovery of NADPH and $NADP^+$ Added, before Sample, to 3 ml of NaOH-Cysteine Solution

Sample	Nucleotide: No addition	Amount added	Expected	Found	Found/Expected
	pmol				%
Assay for NADPH, +/− addition of NADPH					
Hemoglobin, 20 nmol	0	160	160	162	101
Methemoglobin, 20 nmol	0	160	160	150	94
Methemoglobin, 20 nmol, and 3.7 nmol GSSG	0	160	160	141	88
Blood samples, 5 or 10 μl					
Normal (I-3)	180	160	340	316	93
A- (II-1)	89	160	249	226	91
A- (II-4)	65	120	185	180	97
A- (II-6)	51	120	171	161	93
Assay for NADPH, +/− addition of $NADP^+$					
Normal (I-3)	180	160	180	183	102
A- (II-1)	89	160	89	88	99
Assay for total NADP, +/− addition of $NADP^+$					
Normal (I-3)	165	160	325	316	97
A- (II-1)	209	160	369	342	93

NADPH and total NADP, comparable, high degrees of recovery were obtained for NADPH and $NADP^+$ in the presence of either normal or G6PD-deficient blood (Table III). Accordingly, very little difference was noticed in the ratios of NADPH to total NADP with several samples of G6PD-deficient and normal blood, whether internal or external standards of nucleotide were used. For both deficient and normal samples, values of total NADP were 4–17% lower with external standards than with standards added with the samples (internal standards). Internal standards, however, exhibited less agreement among replicates, necessitating the use of more incubation tubes and making difficult the disposing of samples within 2 min of venipuncture. Values in Tables I and II were obtained with external standards.

Both methemoglobin and GSSG can be present in G6PD-deficient red cells in greater than normal amounts, especially during oxidative stress. It is unlikely, however, that these caused erroneously low estimates of NADPH in G6PD-deficient red cells. The NADPH and total NADP concentrations were found to be 32.2 μM and 35.4 μM, respectively, in a boy with congenital methemoglobin reductase deficiency. Both values were 33 μM in a similarly affected brother. Methemoglobin represented 11–14% of their hemoglobin. Similarly, little effect on estimates of NADPH is seen when methemoglobin and GSSG are present (Table III). These substances were added in amounts comparable to those of red cells having all hemoglobin

as methemoglobin and twice the usual amount of glutathione, all oxidized.

Concentrations of $NADP^+$ can be calculated from the difference in concentrations between total NADP and NADPH. Heating samples in acid, followed by the cycling technique, has served as a means of direct estimation of $NADP^+$ in red cells of rats (9). With this technique, we found a large difference in $NADP^+$ concentration between G6PD-deficient and normal cells (39–55 μM in G6PD A− subject 1 versus 0, 6, and 11 μM in three determinations on two normal men), but this method sporadically resulted in lack of agreement among replicates and higher values for $NADP^+$ when NADPH was added to human blood. The problem was less at higher dilution of samples, but at the cost of high blank to signal ratios and lower accuracy. It was encountered in both laboratories and was not eliminated in over 15 efforts with six different concentrations of ascorbic acid and hemolysate. Values in Tables I, II, and III were obtained without this technique.

Omachi, Scott, and Hegarty (12), using the extraction procedure of Burch et al (9), demonstrated that almost all of the nucleotide in fresh, normal red cells is in the reduced form. This method of extraction has not been used to estimate the concentrations of $NADP^+$ and NADPH in G6PD-deficient red cells. Our results confirm the earlier observations on normal red cells but show lower concentrations of NADPH, and higher concentrations of total NADP, in G6PD-deficient red cells.

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