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E. Beutler, E. Guinto

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Research Article

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The Reduction of Glyceraldehyde by Human Erythrocytes

L-HEXONATE DEHYDROGENASE ACTIVITY

E. BEUTLER and E. GUINTO

From the Department of Hematology, City of Hope Medical Center, Duarte, California 91010

ABSTRACT Incubation of red cell suspensions with D-glyceraldehyde resulted in disappearance of glyceraldehyde and appearance of glycerol. Concomitantly, there was an increase of CO_2 formation from glucose. This indicated that the reduction of glyceraldehyde to glycerol occurred through a NADPH-linked system. Studies in hemolysates revealed the presence of an enzyme with the capacity to catalyze the reduction of glyceraldehyde to glycerol by NADPH. This enzyme was partially purified by DEAE chromatography. The elution pattern of the enzyme and its kinetic characteristics indicated that the enzyme was L-hexonate dehydrogenase (L-gulonate: NADP oxidoreductase, EC 1.1.1.19), not aldose reductase (Alditol: NADP oxidoreductase, EC 1.1.1.21), which had previously been thought present in erythrocytes. The reduction of glyceraldehyde to glycerol is one of a number of pathways for the metabolism of glyceraldehyde that have been found in red cells and/or other mammalian tissues.

INTRODUCTION

In the course of studying a patient with hereditary aldolase deficiency, it became necessary to estimate D-glyceraldehyde levels in whole blood. While investigating blood-glyceraldehyde mixtures, we found that glyceraldehyde disappeared from blood during refrigerated storage; to our surprise we discovered that a large proportion of the glyceraldehyde that disappeared could be accounted for as glycerol. In the absence of glyceraldehyde no glycerol was formed. The conversion of glyceraldehyde to glycerol in red cells has not, to our knowledge, been observed previously. We now describe this phenomenon and the enzymatic basis of the reduction process.

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METHODS

Systems for incubating intact red cells were prepared by centrifuging freshly collected heparinized normal adult human blood, removing the plasma and buffy coat, and removing approximately 95% of the remaining leukocytes by filtration through cotton cheesecloth (1). The plasma was freed of formed elements by centrifuging at 5,000*g* for 15 min and the red cell suspension was reconstituted in plasma to give a hematocrit of approximately 40%. Approximately 0.2 μCi of [U-^{14}C]glucose or [1-^{14}C]glucose were added per milliliter of red cell suspension. 2-ml aliquots of the suspension were placed into Warburg flasks. Where indicated, D-glyceraldehyde was added to give a final concentration of 1.5 mM. 1 ml of 10% perchloric acid was placed into the side arm and 0.2 ml hyamine hydroxide (1.0 M *p*-(diisobutyl-cresoxyethoxyethyl) dimethylbenzyl ammonium hydroxide in methanol) into the center well of each flask. The flasks were stoppered and incubated at 37°C. The reaction was stopped in duplicate flasks at the beginning of incubation, and in additional flasks at appropriate time intervals, by tipping perchloric acid into the red cell suspension. The mixture was shaken for an additional 120 min. Then the hyamine was quantitatively transferred into Bray's solution for counting of radioactivity and the perchloric acid extract was analyzed for glycerol and D-glyceraldehyde as described below. An aliquot of the suspension was also deproteinized at the beginning of the experiment for glucose estimations with hexokinase and ATP (2).

The activity of the D-glyceraldehyde-reducing enzyme was measured directly spectrophotometrically in active, hemoglobin-free fractions by measuring at 37°C the rate of decrease of optical density at 340 nm in an assay system containing the following: KH_2PO_4/K_2HPO_4 buffer, 100 mM, pH 7.4; NADPH, 0.2 mM; and D-glyceraldehyde, 2 mM. Kinetic studies were carried out by this procedure, with the enzyme concentrated by ultrafiltration so that 50 μl of enzyme produced an optical density change of approximately 7 mOD U/min in a 1-ml reaction mixture. Direct spectrophotometric measurement of NADPH was not possible at 340 nm in hemoglobin-containing preparations, because glyceraldehyde reacted with hemoglobin, oxidizing NADPH nonenzymatically in the process. The active oxidant appeared to be H_2O_2, since the reaction was inhibited strongly by catalase. To measure the formation

of glycerol from glyceraldehyde in hemolysates or fractions with hemoglobin or in preparations with very low activity we used an assay system in which glucose-6-phosphate, G6PD, and NADP provided a continuous source of NADPH for the reduction of glyceraldehyde to glycerol. The assay system had the following composition: K_2HPO_4/KH_2PO_4 buffer, 200 mM, pH 7.4; NADP 2 mM; G6P, 4 mM; G6PD, 1.5 U/ml; and D-Glyceraldehyde, 4 mM. At appropriate time intervals the reaction was stopped by the addition of perchloric acid, and the amount of glycerol formed was measured as indicated below. The amount of glycerol formed was linear with hemolysate concentration up to a concentration representing 0.1 ml of red cells/ml of reaction system. With this amount of hemolysate, the system was also found to be linear with respect to time for at least 180 min. Routinely, the reaction was stopped at the end of 100 min. The results of assaying the enzyme activity by these two methods in hemoglobin-free enzyme preparations were essentially identical.

D-glyceraldehyde and glycerol determinations were carried out by a modification of the method described by Pinter, Hayashi, and Watson (3). This technique is based upon the fact that glyceraldehyde and dihydroxyacetone are reduced to glycerol by treatment, under appropriate conditions, with potassium borohydride. Perchloric acid extracts were made, a drop of 0.05% methyl orange was added and the extract was neutralized with 3 M potassium carbonate (4). 0.1 ml of 1 M potassium borohydride in 1 N NaOH was added to a 2–2.5-ml aliquot of the neutralized extract. After 10 min at room temperature, 0.05 ml of 12 N HCl were added and the tube was mixed vigorously to destroy excess borohydride. The pH was then adjusted to the methyl orange endpoint with 3 M K_2CO_3. The borohydride-treated and untreated supernates were assayed for glycerol with glycerokinase according to the following three reactions:

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{glycerokinase}} \text{glycerol phosphate} + \text{ADP} \quad (1)$$

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate} \quad (2)$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{lactate dehydrogenase}} \text{NAD} + \text{lactate} \quad (3)$$

The reaction system contained the following: Tris-HCl, 200 mM, pH 8.0; KCl, 100 mM; $MgCl_2$, 5 mM; NADH, 0.2 mM; ATP, 5 mM; phosphoenolpyruvate, 2.5 mM; GSH, 2 mM; pyruvate kinase, 15 U/ml; lactate dehydrogenase, 35 U/ml. The reaction was started by the addition of approximately 0.15 U of glycerokinase/ml. The blank contained water instead of perchloric acid extract. The decrease in optical density at 340 nm after the addition of glycerokinase represents the amount of glycerol in the sample. Assays using α-glycerophosphate dehydrogenase and glycerokinase (2) demonstrated that no appreciable amounts of dihydroxyacetone were present in any of the types of preparation studied. Therefore, the difference between the glycerol content of the borohydride-treated and untreated sample represents the glyceraldehyde content of the sample. Protein estimations were made by the method of Lowry, Rosebrough, Farr, and Randall (5). All biochemical reagents were obtained from Sigma Chemical Co., St. Louis, Mo., with the exception of DE 52 (DEAE cellulose), which was obtained from Whatman Biochemicals Ltd., Springfield Mill, Maidstone, Kent, England.

RESULTS

The metabolism of glyceraldehyde by normal erythrocytes. When D-glyceraldehyde was added to a red cell suspension in plasma at 37°C, it disappeared from the mixture at a rate of approximately 0.1 μmol/ml of red cells/min. Glycerol appeared at a somewhat slower rate and carbon dioxide formation from glucose was stimulated. The time course of these changes is illustrated in Fig. 1. Table I shows the results obtained in eight experiments on the blood of six normal individuals and one person with the A type of G6PD deficiency. In each case, the 15-min point was used to calculate the results. It is apparent that in all cases the amount of glycerol appearing was considerably smaller than the amount of glyceraldehyde consumed. The presence of glyceraldehyde resulted in excess CO_2 formation (i.e., CO_2 formed in the presence of glyceraldehyde minus CO_2 formed in the absence of glyceraldehyde) in normal red cells, but not in the G6PD-deficient cells. In all cases in which both C-1 labeled and uniformly labeled glucose was used in the same experiment, slightly more labeled carbon dioxide was formed from the uniformly labeled sugar, indicating that a slight amount of recycling of glucose was occurring.

Chromatographic characterization of the glyceraldehyde reducing enzyme. Crude dialyzed hemolysate was found to have the capacity to reduce approximately 0.08 μmol/min/g Hb of D-glyceraldehyde to glycerol. Washed erythrocytes were dialyzed against 0.01 M KH_2PO_4/K_2HPO_4 buffer, pH 7.2, with 7 mM β-mercaptoethanol. The pH of the hemolysate was adjusted to 7.2 with 0.01 M K_2HPO_4 or KH_2PO_4, depending on the length of time the red cells had been stored. The dialyzed hemolysate was centrifuged at 30,000*g* for 45 min at 4°C and was passed over a 1.5 × 30-cm DE 52 column, equilibrated with 0.01 M phosphate buffer, pH 7.2, containing 7 mM β-mercaptoethanol. The hemoglobin was washed from the column with the starting buffer. The remaining enzyme activity was eluted with a slightly curvelinear gradient from 0.01 to 0.20 M potassium phosphate in the ratio of 0.604/0.396 K_2HPO_4/KH_2PO_4 containing 7 mM β-mercaptoethanol. The result of the chromatographic separation is shown in Fig. 2. The peak tube showed 386-fold purification of enzyme activity.

Chromatography of the glyceraldehyde-reducing enzyme was also investigated in the system described by Gabbay (6) and Moonsammy and Stewart (7) using the Tris-PO_4-NaCl gradient described by Moore and McGregor (8) since this system is known to discriminate between the two glyceraldehyde reducing enzymes, L-hexonate dehydrogenase and aldose reductase. The enzyme was eluted in a single peak with maximum activity after 135 ml of gradient, corresponding to 50

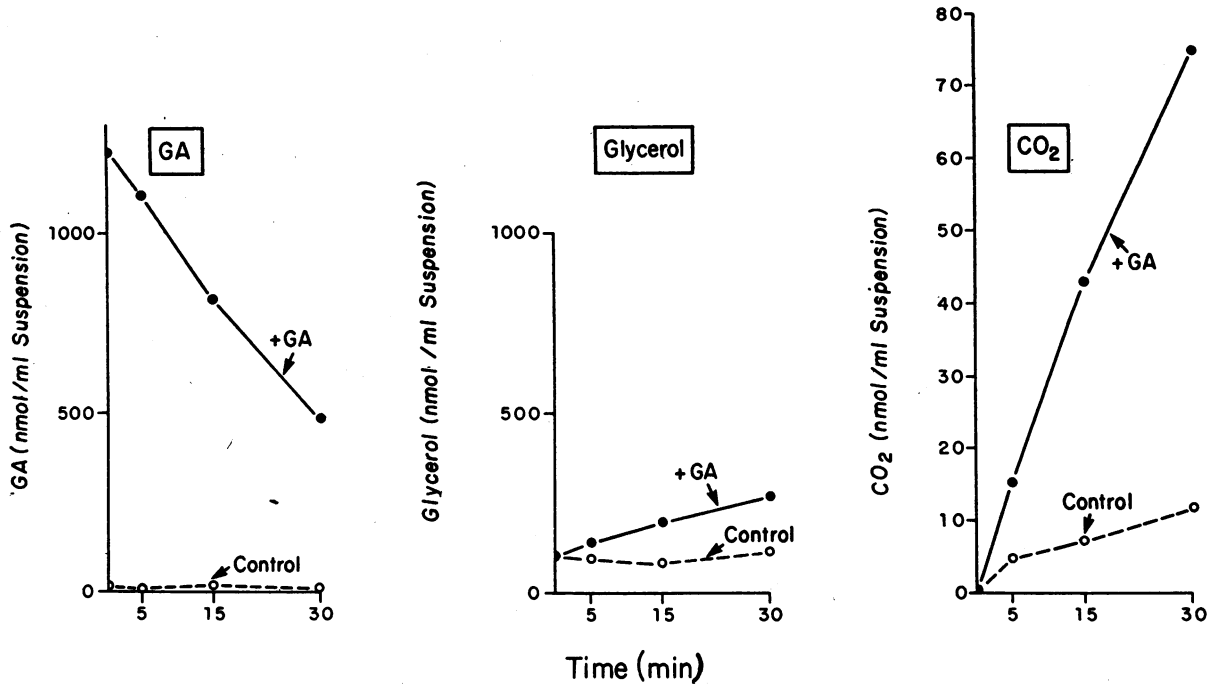

FIGURE 1 The consumption of glyceraldehyde and formation of glycerol and carbon dioxide by leukocyte-free red cell suspensions.

mM NaCl (8), the elution position of L-hexonate dehydrogenase (7).

Kinetic characteristics and substrate specificity of the glyceraldehyde-reducing enzyme. Glyceraldehyde-reducing enzyme was freed of hemoglobin on a DEAE column and was concentrated by ultrafiltration in an Amicon PM 10 ultrafilter (Amicon Corp., Lexington, Mass.). The activity of the enzyme was measured di-

TABLE I

Glycerol Formation and CO_2 Production in Intact Human Red Cells Incubated with D-Glyceraldehyde

Exp no.	Donor	[^{14}C] glucose	D-glyceraldehyde consumed	Excess CO_2 formed*	Excess NADPH oxidized (calculated‡ value)	Glycerol formed
			nmol/min/ml erythrocytes			
1.	B. B.	C-1	108.2	9.11	18.22	26.5
		C-UL	113.5	9.61	19.22	20.0
2.	K. B.	C-1	50.0	6.13	12.26	16.2
3.	B. C.	C-UL	51.1	10.01	20.02	24.9
4.	E. F.	C-1	79.5	5.23	10.46	20.0
5.	M. M.	C-1	95.5	6.79	13.58	18.1
6.	M. M.	C-1	82.5	6.93	13.86	17.8
		C-UL	78.9	8.96	17.92	20.5
7.	J. S.	C-UL	109.9	9.47	18.94	21.2
8.	C. P. G6PD A-	C-UL	89.1	0.95	1.90	6.09

* CO_2 produced in the presence of D-glyceraldehyde minus CO_2 produced in the absence of D-glyceraldehyde.

‡ Excess NADPH oxidized is calculated as two times the amount of excess CO_2 formed (See Fig. 3).

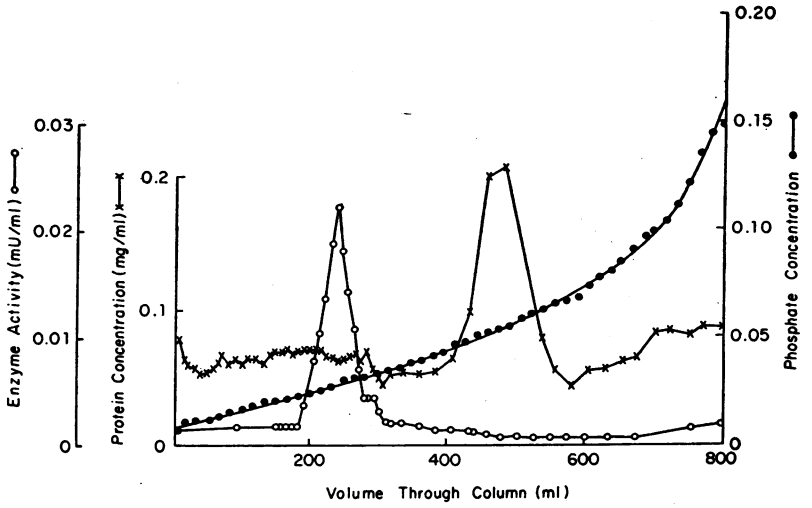

FIGURE 2 Chromatographic purification of glyceraldehyde-reducing enzyme on a DE-52 column with a K_2HPO_4/KH_2PO_4 gradient. The phosphate concentration was estimated by conductivity measurements. Protein determinations were carried out by the method of Lowry, Rosebrough, Farr, and Randall (5). Glyceraldehyde reducing enzyme was measured using the two-step assay. (See text)

rectly by estimating the rate of NADPH oxidation at various concentrations of D-glyceraldehyde, DL-glyceraldehyde, D-xylose, D-glucuronic acid, D-glucose, and D-galactose. All sugar solutions were allowed to stand for at least 18 h to permit equilibration between α- and β-forms. The K_m values and relative V_{max} values are presented in Table II. Tetramethylene glutaric acid,[1] a potent inhibitor of aldose reductase at a concentration of 0.1 mM (9), produced only 9% inhibition of the glyceraldehyde-reducing enzyme when added to a system containing 2 mM D-glyceraldehyde as substrate. Under the same conditions 3 mM barbital produced 87% inhibition and lithium sulfate 51% activation. The relationship between pH and activity of the enzyme was investigated in a Tris-glycine-phosphate buffer (10). As shown in Fig. 3, maximum activity was observed at pH 7. The partially purified enzyme also exhibited some activity with NADH as a substrate. When 0.2 mM NADH was substituted for NADPH in the assay system, 12–17% of the activity with NADPH was observed with 30 mM D-glucuronate, 150 mM D-xylose, and 2 mM D-glyceraldehyde as substrate.

DISCUSSION

Incubation of human erythrocytes with D-glyceraldehyde clearly demonstrated that red cells have the capacity to reduce glyceraldehyde to glycerol. The production of CO_2 from glucose by red cells provides a means of measuring the rate at which NADPH is oxidized within red cells, since the availability of NADP is the limiting step in the hexose monophosphate pathway of red cells: each mole of carbon dioxide formed from glucose results from the reduction of 2 mol of NADP to NADPH. In the steady state, it is necessary, of course, that an equivalent amount of NADPH has been oxidized in reducing another substance, and the total amount of NADP + NADPH in red cells is sufficiently small that significant accumulation of NADP or NADPH is not possible. If the formation of glycerol were all due to the reduction of glyceraldehyde by NADPH, one-half as much carbon dioxide should have been formed as

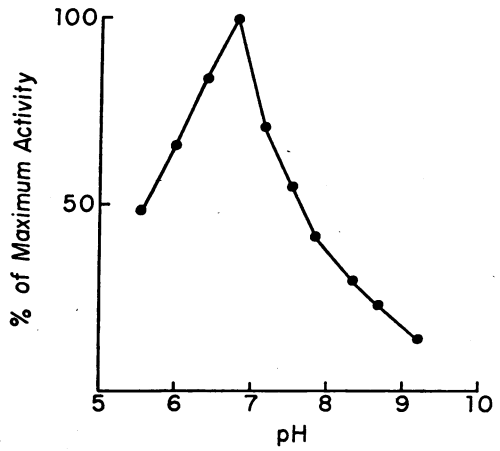

FIGURE 3 The pH-activity curve of hemoglobin-free red cell L-hexonate dehydrogenase. A Tris-glycine-phosphate buffer (10) was used. The pH given at the dilution (0.05 M) used and at the temperature of assay, 37°C.

[1] Obtained through courtesy of Dr. Jin Kinoshita.

TABLE II

Comparison of Properties of Human Red Cell Glyceraldehyde Reducing Enzyme with Those of Aldose Reductase and L-Hexonate Dehydrogenase

	Aldose reductase	L-Hexonate dehydrogenase	Human red cell enzyme
Elution position on DEAE column			
KH_2PO_4/K_2HPO_4, pH 7.2	75 mM*		30 mM
Tris-phosphate-NaCl (pH 7.3–6.2)	300 mM‡; 180 mM§	70 mM‡; 50 mM§	50 mM
Kinetic properties K_m			
DL-Glyceraldehyde			0.68 mM
D-Glyceraldehyde	0.03 mM*; 0.11 mM‡; 0.46 mM§	2.2 mM§	0.63 mM
D-Xylose	5 mM*; 15.7 mM‡; 48 mM§	192 mM‡; 910 mM§	470 mM and 9 mM¶
D-Glucuronate	4 mM*; 17.5 mM‡; 7.6 mM§	1.7 mM‡; 0.45 mM§	1.9 mM
D-Glucose	37.0 mM‡	175 mM‡	396 mM
D-Galactose	20 mM*; 12.0 mM‡	159 mM‡	91 mM
Relative V_{max} (percent of glucuronate)			
D-Glyceraldehyde			60.2
DL-Glyceraldehyde	384‡; 167‖		42.3
D-Xylose	238‡	38‡	44.4
D-Glucuronate	100‡; 100‖	100‡	100
D-Glucose	96‡; 150‖	8.8‡	13.2
D-Galactose	146‡; 87‖	7.0‡	15.7
Glucuronate (30 mM): D-xylose (150 mM) activity ratio	1:2‡	5.4:1‡	5.3:1
Inhibition by 0.1 mM 3,3'tetramethylene glutaric acid	68%‖		9%

* Calf lens (11) Kinetic studies in phosphate buffer, pH 6.2.
‡ Ox brain (7) Kinetic studies in phosphate buffer, pH 7.0.
§ Bovine retina (6), conditions not described.
‖ Calf lens (9), phosphate buffer, pH 6.8.
¶ D-xylose consistently gives a two-component Lineweaver-Burk plot.

glycerol produced in the incubation experiments. Slightly less carbon dioxide was found, even when uniformly labeled glucose was used as substrate. Thus, a small fraction of the gycerol produced must be accounted for through a hydrogen donor other than NADPH. The fact that some glyceraldehyde reduction may occur independently of NADPH generation was supported by the study of G6PD-deficient erythrocytes. As expected, no stimulation of CO_2 production was observed in these cells. Nonetheless, a modest amount of glycerol was formed. One must presume that a small portion of glycerol formation occurs through a NADH linked pathway.

Two mammalian enzymes are known to catalyze the reduction of D-glyceraldehyde to glycerol, aldose reductase (alditol: NADP oxidoreductase, EC 1.1.1.21) (6, 7, 11), and L-hexonate dehydrogenase (L-gulonate: NADP oxidoreductase, EC 1.1.1.19) (6, 7, 12). Both enzymes preferentially utilize NADPH as a hydrogen donor. Travis, Morrison, Clements, Winegrad, and Oski (13) demonstrated that human erythrocytes accumulate sorbitol in the presence of high concentrations of glucose. They suggested that aldose reductase activity was present in erythrocytes; because of the high K_m which L-hexonate dehydrogenase had been reported to have for glucose, they surmised that the enzymatic activity present in erythrocytes was not this enzyme. Gabbay and Cathcart (14, 15), on the other hand, reported that only L-hexonate dehydrogenase was present in red cells. No direct measurement of this enzymatic activity in erythrocytes or studies of the characteristics of the red cell enzyme are known to us, however.

Although aldose reductase and L-hexonate dehydrogenase have many similarities, the two enzymes are most easily distinguished from one another because of their marked difference in the ability to catalyze the reduction of xylose, and the differences in their relative capacity to catalyze reduction of DL-glyceraldehyde and glucuronic acid (6, 7). These enzymes have also been separated chromatographically from bovine retina (6), ox brain (7) and rabbit aorta (16). Table II compares the properties of the glyceraldehyde-reducing enzyme of erythrocytes with aldose reductase and L-hexonate dehydrogenase characterized from various mammalian sources. It is apparent that the properties of the red cell enzyme more closely resemble those of L-hexonate

dehydrogenase than those of aldose reductase. No peak of enzymatic activity was found in the elution position corresponding to that of aldose reductase of various mammalian species. While we could not co-chromatograph the red cell enzyme with authentic human aldose reductase since the latter enzyme was not available to use, our findings lead us to suggest, tentatively, that aldose reductase is not present in human erythrocytes. Although the activity of L-hexonate dehydrogenase in red cells is relatively low, and its K_m for the substrates investigated is much higher than physiologic levels, it may play an important role in red cell metabolism under certain circumstances. It is no doubt responsible for the reported accumulation of sorbitol in erythrocytes in the presence of high concentrations of glucose (13, 17). Under these circumstances an increase in the lactate/pyruvate ratio occurred, presumably because of the oxidation of sorbitol to fructose by NAD; other changes of red cell intermediates, including a fall in 2,3-diphosphoglycerate levels were also documented (13). Since red cell sorbitol levels have been found to be increased in red cells incubated with plasma containing up to 30 mM glucose, it was suggested that alterations in red cell 2,3-diphosphoglycerate might be induced by diabetics with hyperglycemia (13). Travis and Oski (18) also found that sorbitol did not accumulate in G6PD-deficient erythrocytes, a finding quite analogous to the lack of glycerol accumulation in G6PD-deficient erythrocytes incubated with glyceraldehyde. The presence of L-hexonate dehydrogenase may also account for the "high K_m pathway" of galactose metabolism of erythrocytes (19). This pathway results in the reduction of methemoglobin, even in the absence of galactose-1-phosphate uridyl transferase or galactokinase (20 and unpublished observations). This could occur through the mediation of L-hexonate dehydrogenase if the dulcitol formed by the reduction of galactose served as a substrate for sorbitol dehydrogenase, reducing NAD to NADH. According to Smith (21) sorbitol dehydrogenase from sheep liver catalyzes the reduction of dulcitol at only 3% of the rate found with sorbitol. Our understanding of the reactivity of sorbitol dehydrogenase from human erythrocytes (22) with dulcitol awaits partial purification of the red cell enzyme. The characterization of this possible pathway of galactose metabolism in red cells is currently underway.

Only between half and one-fifth of the D-glyceraldehyde consumed during incubation with red cells was converted to glycerol. The various reported potential metabolic fates of glyceraldehyde are summarized in Fig. 4. They include not only the reduction of glyceraldehyde to glycerol, as reported in the current study, but also the phosphorylation of glyceraldehyde to glyceraldehyde-3-phosphate by triokinase (23), the nonenzymatic conversion of glyceraldehyde to methylglyoxal (24), which in red cells is rapidly metabolized to D-lactic acid (25), the reported oxidation of glyceraldehyde to glyceric acid by aldehyde dehydrogenase (26), and the oxidation and phosphorylation of glyceraldehyde to 1-phosphoglyceric acid by glyceraldehyde phosphate dehydrogenase, which has been reported to occur in mammalian tissues (27) and which we have observed in human red cell lysates. Finally, the active aldehyde group of glyceraldehyde may form a Schiff base with protein amino groups. It is not certain what role, if any, each of these potential pathways of D-glyceraldehyde metabolism plays in the disappearance of glyceraldehyde from red cell suspensions.

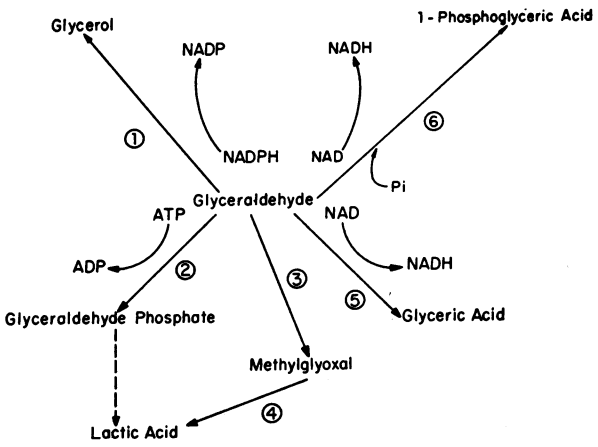

FIGURE 4 Possible pathways of glyceraldehyde metabolism in erythrocytes: 1. L-hexonate dehydrogenase (this paper); 2. Triokinase (23); 3. Nonenzymatic decomposition of glyceraldehyde to methylglyoxal (24); 4. The conversion of methylglyoxal to lactic acid through the mediation of glyoxalase I and glyoxalase II (25); 5. The reported oxidation of glyceraldehyde to glyceric acid by a red cell aldehyde dehydrogenase (26); 6. The oxidation and phosphorylation of glyceraldehyde to 1-phosphoglyceric acid by glyceraldehyde phosphate dehydrogenase (27).

Quantitative investigations of the fate of glyceraldehyde consumed during incubation with erythrocytes is rendered very difficult, both by the lack of commercial availability of isotopic glyceraldehyde, and by the rapid equilibration of some of the potential products of glyceraldehyde metabolism.

ACKNOWLEDGMENTS

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