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PURIFICATION AND PROPERTIES OF DEOXYRIBOALDOLASE FROM HUMAN ERYTHROCYTES

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SUMMARY

Deoxyriboaldolase (2-deoxy-D-ribose-5-phosphate acetaldehyde lyase, EC 4.1.2.4) from human erythrocytes was purified approx. 2500 times by treatment of hemolysates with $(NH_4)_2SO_4$ (25%) and two successive treatments with calcium phosphate gel. The enzyme was unstable in hemolysates (half life, 2 days) and partially purified preparations, but could be stabilized by 0.01 M $MgCl_2$ (half life, 4 days) or sulfhydryl reagents (dithiothreitol). The latter could also reactivate storage inactivated preparations.

Erythrocyte deoxyriboaldolase was activated by dicarboxylic and tricarboxylic acids of which citrate was most effective and activated 2–3-fold. *Cis*-configuration activators (maleate) were more effective than *trans*-isomers (fumarate), indicating a proximity requirement for adjacent carboxyl groups of the activator. At several citrate concentrations uncompetitive activation kinetics were observed. K_m and v_{max} for the enzyme without citrate was 96 μM and 0.96 mM/h, and with 4 mM citrate was 361 μM and 4.17 mM/h, respectively. Additionally, citrate caused aggregation of the enzyme.

INTRODUCTION

Deoxyriboaldolase (2-deoxy-D-ribose-5-phosphate acetaldehyde lyase, EC 4.1.2.4) catalyzes the reversible reaction:

2-deoxy-D-ribose 5-phosphate $\rightarrow$ acetaldehyde + D-glyceraldehyde 3-phosphate

Originally found by RACKER[1] in extracts of *Escherichia coli*, *Corynebacterium diptheriae* and mouseliver, the enzyme has been described in several mammalian tissues[2–7] and was crystallized from *Lactobacillus plantarum*[8,9], and from *Salmonella typhimurium*[10]. A molecular weight of 60 000 $\pm$ 6000 was reported for the bacterial enzyme[9,10] while that from rat liver was shown to be 253 000 (ref. 11).

We observed that acetaldehyde and triosephosphates (D-glyceraldehyde 3-

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phosphate and dihydroxyacetone phosphate) were synthesized from deoxyinosine in erythrocyte ghosts and from 2-deoxy-D-ribose 5-phosphate in hemolysates[12,13] suggesting the presence of deoxyriboaldolase in human erythrocytes. This paper describes the partial purification of the erythrocyte enzyme and some of its properties. Similar to that from rat liver[11] and *Lactobacillus plantarum*[9], the erythrocyte enzyme was activated by di- and tricarboxylic acids. Citrate, in addition to activation, also effected the reversible aggregation of the enzyme as observed with dextran chromatography.

EXPERIMENTAL

Materials

Deoxyribose 5-phosphate was synthesized from deoxyadenosine monophosphate according to the procedure of LAMPEN[14] or purchased from Calbiochem. Other materials used were obtained commercially. Calcium phosphate gel was prepared according to the method of KEILIN AND HARTREE[15] and was stored in 0.005 M sodium phosphate buffer (pH 7.4). Some was purchased as aqueous suspensions from the BioRad Co.

Protein was determined by the method of Warburg and Christian and the results confirmed with biuret[16]. Hemoglobin was measured from its absorbancy at 418 mμ (ref. 17).

Measurement of enzyme activity

Deoxyriboaldolase activity was determined enzymatically by measuring the acetaldehyde produced from deoxyribose 5-phosphate with yeast alcohol dehydrogenase[1]. The reaction mixture contained 80 μmoles sodium phosphate buffer (pH 7.4); 0.1 μmoles NADH; 0.03 ml yeast alcohol dehydrogenase (1:10 dilution); 5 μmoles deoxyribose 5-phosphate; and, 0.1–0.3 ml enzyme in a final volume of 1 ml. Controls containing no substrate oxidized negligible amounts of NADH. Acetaldehyde was also determined by its reaction with buffered semicarbazide at 224 mμ according to the method of BURBRIDGE *et al.*[18]. The assays were carried out in Obrink-modified Conway microdiffusion dishes (44 mm). The reaction compartment contained 100 μmoles sodium phosphate buffer (pH 7.4), 2 μmoles sodium deoxyribose 5-phosphate, in a total volume of 1.5 ml. The reaction was initiated by an aliquot of enzyme 0.05–0.20 ml. The center well contained 0.5 ml of 0.0067 M semicarbazide in 0.2 M phosphate buffer. The covers were sealed in a compartment containing 1.0 ml of 0.2 M phosphate buffer with 0.025% detergent (tergitol). The dishes were incubated for 1 h at 37°. At the end of the incubation, 0.3-ml aliquots of semicarbazide solution were withdrawn from the center well and diluted to 3.0 ml with distilled deionized water. After 20 min standing at room temperature, the solutions were transferred to cuvettes of 1.0 cm path length and the acetaldehyde-semicarbazone content of each solution was estimated from its absorbance at 224 mμ. The concentration of acetaldehyde was estimated by comparison to a standard curve. Under the experimental conditions used, 0.133 absorbance units corresponded to 0.125 μmoles acetaldehyde. A substrate and enzyme control were run simultaneous to each group of test samples. A unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmole of acetaldehyde per h at 37°.

RESULTS

Purification of enzyme

The enzyme purification is summarized in Table I. All isolation steps were carried out at 5°.

Cell preparation

Human blood (500-ml quantities) was either freshly drawn into anticoagulant solution (heparin or acid citrate–dextrose) or obtained as outdated (whole blood in acid citrate–dextrose) from the hospital blood bank. Packed erythrocytes free from white cells were obtained by centrifugation and washed 3 times with 0.15 M NaCl

TABLE I

ISOLATION OF ERYTHROCYTE DEOXYRIBOALDOLASE

Fraction	*Activity (μmoles/h per ml)*	*Protein (mg/ml)*	*Specific activity (μmoles/h per mg protein)*	*Purification (× initial)*	*Yield (%)*
Hemolysate	0.38	236	0.0016	—	—
$(NH_4)_2SO_4$ (25%)	0.72	124	0.0058	4	49
Calcium phosphate gel, I	0.23	0.60	0.383	240	26
Calcium phosphate gel, II	0.17	0.04	4.25	2650	26

or isotonic bicarbonate buffer (pH 7.4)[19]. After each wash, the sample was centrifuged and the supernatant wash solution was removed by aspiration. The top layer of white cells was separated from erythrocytes with a cotton-tipped swab.

Hemolysate preparation

Packed red cells (300–400 ml) were resuspended in an equal volume of 0.15 M bicarbonate buffer (pH 7.4). Hemolysis was initiated by the addition of 2 vol. of distilled deionized water and completed when the sample was twice frozen in a dry ice–alcohol bath and thawed at 37°. The hemolysate was freed of stroma by centrifugation or by filtration through Whatman No. 12 filter paper. No loss of enzyme activity occurred when particulate matter was removed indicating that deoxyribo-aldolase is a soluble intracellular enzyme. The specific activity of the hemolysate preparation (shown in Table I) was low relative to other erythrocyte enzymes[20] being approx. 10% that of hexokinase. No significant differences in specific activity for washed cells was found between hemolysates from heparinized blood or blood collected in acid citrate–dextrose anticoagulant. Values from all sources ranged between 1.1 and 1.6 μmoles/h per mg protein.

$(NH_4)_2SO_4$ fractionation

25 g of ground $(NH_4)_2SO_4$ were added slowly with stirring to 100-ml aliquots of hemolysate at 5°. After 15 min of additional stirring, the mixture was allowed to stand at 5° for 1 h. It was then centrifuged at 16 000 × *g* for 45 min. The supernatant was decanted and the precipitate dissolved by dilution 1:5 with 0.10 M sodium phos-

phate buffer (pH 7.4), or with 0.15 M bicarbonate buffer (pH 7.4). This procedure yielded a 4-fold purified enzyme fraction with approx. 50% recovery, and was stable for 1 week at 5°.

Calcium phosphate gel

Step I. The enzyme showed a high affinity for calcium phosphate gel and was readily absorbed from the $(NH_4)_2SO_4$ fraction. It was desorbed from the gel by phosphate buffer (0.2 M). A typical experiment is summarized in Table II. Calcium phosphate gel (275 mg) was added to 22 ml of dissolved $(NH_4)_2SO_4$ precipitate-25 (physiological bicarbonate buffer (pH 7.4) (1:5, v/v)). The sample contained 2.75 g protein and 16.0 units of enzyme assayed as acetaldehyde (μmoles produced per h per 22 ml).

TABLE II

ELUTION OF DEOXYRIBOALDOLASE FROM CALCIUM PHOSPHATE GEL

Experimental conditions are described in the text.

Phosphate (M)	*Cumulative volume (ml)*	*Protein desorbed (mg per 40 ml)*	*Enzyme activity (μmoles/h per 40 ml)*
0.01	40	215	1.8
0.01	80	35	3.3
0.01	120	20	0.4
0.01	160	14	0.4
0.01	200	5	0.8
0.2	240	17	11
0.2	280	10	1.8

A ratio of 10 mg of protein to 1 mg gel (dry wt.) was routinely employed. The suspension was allowed to stand at 5° for 15 min with intermittant stirring and then centrifuged at 4500 × *g* for 15 min. The supernatant was assayed for enzyme activity and protein. Hemoglobin and non-enzyme protein was separated from enzyme by resuspending packed gel in successive steps with 40-ml portions of 0.01 M phosphate buffer (pH 7.4). Table II shows that five successive treatments (200 ml cumulative volume) with 0.01 M phosphate removed most of the protein (90%) and about 30% of the enzyme. The bulk of the enzyme was then desorbed by increasing the phosphate concentration to 0.2 M in two successive washes of 40 ml each (cumulative volume, 280 ml).

Step II. Enzyme was reabsorbed on calcium phosphate gel added in increments to the eluant from the first calcium phosphate gel step. A protein to gel ratio of 20 to 1 mg proved effective for total reabsorption of the enzyme when the eluant was diluted to a final phosphate concentration of 0.005 M. Contaminant proteins were eluted from the gel by two successive washes with 0.02 M sodium phosphate buffer (pH 7.4). The enzyme was eluted from the gel by the addition of 0.2 M sodium phosphate buffer (pH 7.0). This procedure yielded a 10-fold increase in purity (Table I) with essentially no loss of enzyme activity. Deoxyriboaldolase was stable for several months when stored at 5° on calcium phosphate gel from which it could readily be desorbed.

Properties of deoxyriboaldolase

Stability. Enzyme stability depended on storage conditions and the degree of purification. Deoxyriboaldolase was more stable in fresh blood collected into citrate and dextrose at pH 7.0 (half life, 14 days) than in blood collected in heparin (half life, 2 days). The half life of enzyme in hemolysates containing 0.005–0.01 M phosphate was 2 days. In contrast, the half life of enzyme was 4 days when physiological bicarbonate buffer or 0.005 M phosphate buffer was supplemented with 0.01 M $MgCl_2$ and added to hemolysates. $(NH^4)_2SO_4$ (25%) preparations stored in 0.10 M phosphate buffer were the most stable (7 days). Maximum stability was obtained (24–26 days) when the buffers were supplemented with $MgCl_2$ (0.01 M) and the enzyme $(NH_4)_2SO_4$ treated. Mg^{2+} enhanced enzyme stability but was not a cofactor in the reaction as the specific activity of enzyme solutions stored in $MgCl_2$ was not altered by dialysis or by addition of 0.01 M EDTA. Enzyme preparations greater than 240-fold pure (calcium phosphate Gel I) denatured rapidly. Losses of activity in purified enzyme were not preventable by $(NH_4)_2SO_4$ precipitation, concentration of enzyme on Sephadex G-25, or by the addition of bovine serum albumin.

Cysteine·HCl, reduced glutathione, betamercaptoethanol and dithiothreitol (0.001 M) partially reactivated stored crude or partially purified enzyme preparations when enzyme was assayed in the presence of citric acid, a known enzyme activator[6]. Dithiothreitol was the most effective of those as demonstrated in Table III. Without

TABLE III

REACTIVATION OF RED CELL DEOXYRIBOALDOLASE BY SULFHYDRYL COMPOUNDS

The reagents were added to 48 h stored (4°) $(NH_4)_2SO_4$ precipitate-25, dissolved in physiological bicarbonate buffer (pH 7.4) (1:5 dilution, v/v). Activity represents μmoles acetaldehyde produced/h per ml assayed in the presence of 4 mM citric acid (alcohol dehydrogenase assay).

	Concn. (M)	*Activity (μmoles/h)*	*% Activation (over control)*
Cysteine	0.001	1.33	84
Reduced glutathione	0.001	0.97	35
B-Mercaptoethanol	0.001	1.29	79
Dithiothreitol	0.001	2.02	180
Control, aged 2 days	—	0.72	—
Control, aged 0 days	—	1.84	—

citric acid little activation was found (approx. 1–8%) for enzyme stored without these compounds. This finding suggests a sulfhydryl dependent shift in enzyme sensitivity to citrate.

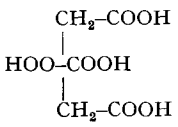
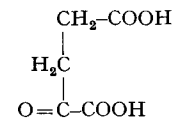
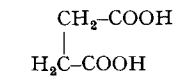
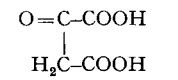
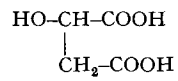
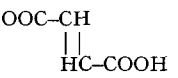
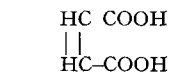
Effect of pH. The enzyme reacted optimally at pH 6.5 as shown in Fig. 1. The pH optimum was the same in the presence of citrate.

Activation by carboxylic acids. Erythrocyte deoxyriboaldolase was activated 3-fold (30%) by carboxylic acid anions as shown in Table IV. The degree of stimulation was greater in the presence of citrate than any other carboxylic acid tested. Evidently, the degree of electronegativity of anion influenced the activation as the tricarboxylic acid anion (citrate) had a greater effect than the dicarboxylic acid anions. The relative proximity of the carboxyl groups was also important in activation as the *cis*-configuration of maleate was a better activator than the *trans*-configuration of fumarate.

TABLE IV

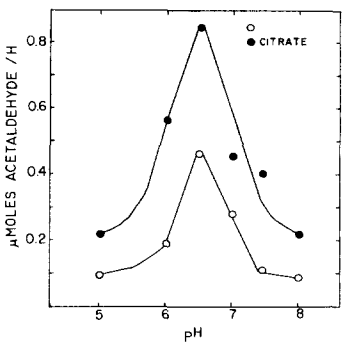
ACTIVATORS OF DEOXYRIBOALDOLASE

Phosphate buffer, 100 μmoles (pH 7.4); deoxyribose 5-phosphate, 1.86 μmoles; enzyme, 0.26 mg (calcium phosphate gel, I); and carboxylate, 3 μmoles were incubated in a volume of 1.5 ml for 1 h at 37°. Acetaldehyde was determined as described in EXPERIMENTAL. Fold stimulation shown in the table represents the ratio of enzyme activity in the presence of activator to that determined in the absence of activator (microdiffusion assay).

Carboxylic acid anion (3 mM)	*Formula (acid)*	*% of initial activity*
Citrate		330
α-Ketoglutarate		268
Succinate		230
Oxaloacetate		92
Malate		220
Fumarate		140
Maleate		235

The magnitude of activation of the other dicarboxylic acid anions also reflect the proximity relationship of carboxyl groups. The effect of citrate concentration on enzyme activity is shown in Fig. 2. Enzyme activity was greatly increased by addition of citrate in a linear fashion up to 3 mM citrate, above which no further change in activity was observed to 15 mM. Higher concentrations of citrate caused a reduction in activity.

The mechanism of citrate activation was investigated as to the possibility that citrate activated the enzyme by chelation of an enzyme bound inhibitor. This was tested with EDTA and no activation was found over the range of concentrations at which citrate was stimulatory (3–15 mM). The possibility that an obligatory intermediate was formed between citrate and enzyme was found unlikely when enzyme containing citrate was dialyzed against citrate-free phosphate buffer (0.01 M, pH 7.4). The dialyzed enzyme showed a requirement for citrate similar to unactivated enzyme.



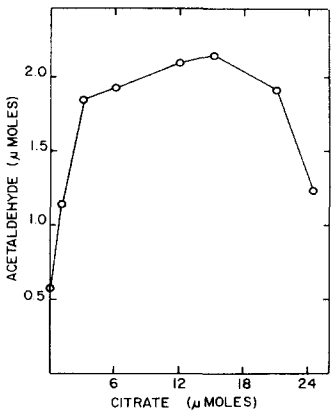


Fig. 1. The pH optimum for deoxyriboaldolase. 1.86 μmoles deoxyribose 5-phosphate, 100 μmoles sodium phosphate buffer (pH indicated in graph), and 0.288 mg of enzyme (calcium phosphate gel, Eluate I) were incubated with (●—●) and without (○--○) 3 μmoles citric acid in a final volume of 1.5 ml. Each point represents enzyme activity (μmoles acetaldehyde produced per h) at the indicated pH (microdiffusion assay).

Fig. 2. Effect of citrate on deoxyriboaldolase activity. Phosphate buffer, 100 μmoles (pH 7.4); deoxyribose 5-phosphate, 1.86 μmoles; enzyme, 11.8 mg $(NH_4)_2SO_4$ precipitate-25); and citrate, 0–24.0 μmoles were incubated in a volume of 1.5 ml for 1 h at 37°. The points represent μmoles acetaldehyde produced per h at each of the amounts of citrate indicated (microdiffusion assay).

Intermediate formation between substrate and citrate also seemed unlikely. Both the forward and reverse reactions catalyzed by the enzyme were stimulated 3–4-fold by citrate and no detectable change occurred in the concentrations of deoxyribose 5-phosphate, glyceraldehyde 3-phosphate or acetaldehyde when each was incubated without enzyme in the presence and absence of citrate.

Substrate specificity. The enzyme (calcium phosphate, Step II) was relatively specific for its substrates in the forward (deoxyribose 5-phosphate) and backward reactions (acetaldehyde and D-glyceraldehyde 3-phosphate). There was no reaction when ribose 5-phosphate and deoxyribose were substituted for deoxyribose 5-phosphate, when dihydroxyacetone phosphate, pyruvate and lactate were tested as triose substrates, or when ethanol or pyruvate were substituted for acetaldehyde.

K_m determination. Fig. 3 illustrates the substrate velocity relationships for a range of citrate from 0 to 4 mM. K_m values for deoxyribose 5-phosphate in the absence of citrate was 96 μM and v_{max} was 0.96 μmoles/ml per h. Lines were obtained parallel to that for unactivated enzyme. These data indicated that citrate caused "uncompetitive" activation of deoxyriboaldolase as each concentration of citrate produced increases in both the v_{max} and the K_m for the reaction. The results are summarized in Table V.

Molecular weight. The molecular weight of deoxyriboaldolase was estimated by the use of the molecular seive technique with Sephadex G-100 and G-150 as described by WHITAKER[21]. Proteins of known molecular weight (hemoglobin, bovine serum albumin and trypsin) were used to relate elution volumes to molecular weights of purified protein. Blue dextran was used to determine the void volume. By interpolation the molecular weight of deoxyriboaldolase was estimated to be 70 000 ± 4000. In contrast to the bulk of enzyme, a small percentage of enzyme protein with a molec-

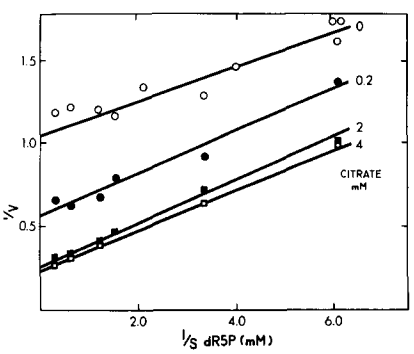
TABLE V

V_{max} AND K_m OF CITRATE-ACTIVATED DEOXYRIBOALDOLASE
Conditions were those given in Fig. 3.

Citrate (mM)	*1/v*	*v_{max} (μmoles/ml per h)*	*K_m (μM)*
0.0	1.04	0.96	96
0.2	0.62	1.61	134
0.4	0.56	1.79	153
2.0	0.26	3.85	304
4.0	0.24	4.17	361

ular weight greater than 100 000 was observed when the enzyme was eluted in the presence of citrate.

Aggregation of deoxyriboaldolase by citrate. Enzyme preincubated with citrate and passed over columns of dextran gels revealed that citrate induced protein aggregation. The elution of untreated and citrate-pretreated enzyme from dextran gel (Sephadex G-100) is shown in Fig. 4. All samples were assayed in the presence of 4 mM citrate (approx. maximal activation). Only one peak appeared in the elution profile of enzyme without citrate. In contrast, when enzyme was pretreated with 4 mM citrate for 1 h two peaks to enzyme activity appeared in the elution profile (closed



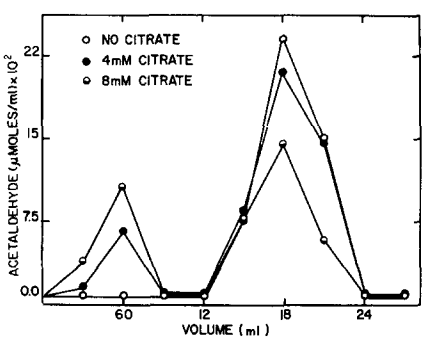


Fig. 3. Lineweaver Burk plots of deoxyriboaldolase activity as a function of deoxyribose 5-phosphate (dR5P) concentration. The reaction mixture contained 80 μmoles sodium phosphate buffer (pH 7.4); 0.31 mg enzyme; 0.03 ml NADH (2 mg/ml); 0.03 ml yeast alcohol dehydrogenase (1:10 dilution); and, 0.082–3.28 μmoles deoxyribose 5-phosphate in a final volume of 1.0 ml. The points represent the initial velocity (v), (μmoles acetaldehyde produced per h) measured in the absence of citrate (○—○), and in the presence of 0.2 mM citrate (●—●), 2 mM citrate (■—■), and 4 mM citrate (□—□).

Fig. 4. Effect of citrate on elution of deoxyriboaldolase from Sephadex G-100. Three 5-ml aliquots of enzyme (calcium phosphate gel, I) were diluted with 0.005 M sodium phosphate buffer (pH 7.4) to contain 9 mg protein/ml and were preincubated at 5° without citrate and with 4 and 8 mM citrate. The samples were then added to three columns of Sephadex G-100 (2.4 cm × 36.5 cm) equilibrated with 0.005 M sodium phosphate buffer (pH 7.4) containing no citrate, 4 and 8 mM citrate, respectively. Each column was eluted with phosphate buffer containing the same molarity of citrate. 3-ml aliquots were collected at flow rates of approx. 30 ml/h. The points in the graph represent enzyme activity as acetaldehyde produced (μmoles/h per ml × 10^2) from the sample containing no citrate (○—○), 4 mmoles/ml citrate (●—●) and 8 mmoles/ml citrate (◒—◒). Samples from the experiments without citrate were assayed in 4 mM citrate microdiffusion assay).

circles). The size and position of the peak at 6 ml relative to the second peak at 18 ml demonstrated that a significant amount of enzyme was converted to an active aggregate form by citrate. This aggregate accounted for 15% of the original enzyme activity. The position (elution volume) of the second peak (18 ml) obtained when the enzyme was pretreated with citrate coincided with the peak obtained when no citrate was added to the enzyme. However, this peak contained less activity (85%) when compared to the control enzyme. This suggested that the two components obtained after citrate treatment were related as subunit and aggregate. When enzyme was pretreated with 8 mM citrate for 1 h at 5° (Fig. 4, half circles), approx. 33% of total enzyme appeared in the first peak while the remainder was present in the second peak. Pooled fractions containing enzyme from the unaggregated fraction (4 mM citrate) eluted as one peak in the same position when rechromatographed without further preincubation with citrate on a Sephadex column equilibrated with 5 mM phosphate buffer (pH 7.4). If, however, the rechromatography was performed identically in 8 mM citrate, an additional 17% of enzyme aggregated as observed by the appearance of a small peak at 6 ml with a corresponding diminution of the larger unaggregated fraction (not shown). In one chromatography experiment at 4 mM citrate, when the unaggregated fraction was rechromatographed the total enzyme aggregated was 34%. This was similar to 33% obtained in a single step pass over a similar column pre-equilibrated and chromatographed at 8 mM. In all instances two peaks of enzyme were observed on Sephadex chromatography in the presence of citrate; but, the amount present in the first (aggregated) peak varied from a few percent to a third of the total activity. Similar properties have been observed with ribonuclease[22], glyceraldehyde phosphate dehydrogenase[23], acetylcholinesterase[24] and cytochrome *c* (ref. 25). They may represent anomalous behavior, but probably represent differences in the equilibrium of two enzyme forms at various stages of purification.

DISCUSSION

We have described a method for the purification of human erythrocyte deoxyriboaldolase which yields an enzyme with a specific activity approx. 2500 times greater than the specific activity present in hemolysates. Its presence provides an understanding of the chemical pathway for the production of triose phosphate from deoxypentose phosphate and thus accounts for its synthesis during the catabolism of deoxynucleosides by hemolysates[26] and ghosts[12]. The formation of acetaldehyde with a high reducing potential from the deoxypentose constituent of deoxynucleosides may provide explanation for enhanced methemoglobin reduction observed in intact erythrocytes[27].

In previous studies, it was reported that deoxyriboaldolase is activated by carboxylic acids and different molecular weights for the enzyme from different species were described[9–11]. In the current study, several properties of erythrocyte deoxyriboaldolase indicate that enzyme aggregation also occurs in the presence of citrate. One is the molecular sieve characteristic in which a change in enzyme mobility on Sephadex G-100 columns occurred. Although other explanations are possible for the hyperbolic uncompetitive activation kinetics observed, changing K_m values in the presence of citrate may imply also that aggregation has occurred[28]. Increased enzyme stability in the presence of $(NH_4)_2SO_4$ and $MgCl_2$ further suggests that heterogeneous

forms of the aldolase occur. It has been suggested[29] that metals influenced activity by changing the equilibrium between two forms of an enzyme. The enhanced stability of deoxyriboaldolase that we found in the presence of each salt may thus result from a shift in the equilibrium between a lower molecular weight and higher molecular weight form of the enzyme.

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