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NADP+-SPECIFIC ISOCITRATE DEHYDROGENASE OF *ESCHERICHIA COLI*

I. PURIFICATION AND CHARACTERIZATION

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SUMMARY

The NADP+-dependent isocitric dehydrogenase of *Escherichia coli* E-26 was purified to electrophoretic homogeneity and partially characterized. The purification was carried out at room temperature and resulted in a 64% recovery of the enzyme from the crude cellular extract.

The enzyme had a molecular weight of 80 000 as determined by gel filtration and sucrose density gradient centrifugation. The K_m values for *threo*-D$_s$-isocitrate, NADP+, Mg^{2+} and Mn^{2+} were $1.56 \cdot 10^{-5}$, $3.7 \cdot 10^{-5}$, $1.27 \cdot 10^{-4}$, and $1.29 \cdot 10^{-5}$ M, respectively. The coenzyme analogs, thionicotinamide–NADP+ and 3-acetylpyridine–NADP+, but not selenonicotinamide–NADP+, were able to replace NADP+ in the reaction. The enzyme exhibited maximum stability at pH 6 and had a broad pH optimum from 7 to 9.

INTRODUCTION

Much of the information pertaining to the physical and chemical properties of NADP+-dependent isocitrate dehydrogenase (*threo*-D$_s$-isocitrate:NADP+: oxidoreductase (decarboxylating), EC 1.1.1.42) has been obtained from the pig heart enzyme[1–7]. However, NADP+-specific isocitrate dehydrogenase is widely distributed in nature and recently several reports have been published concerning the properties of this enzyme from a diverse variety of bacterial species[8–12]. These reports indicate that there are marked differences in the physical and chemical properties of the enzyme, depending upon the source of isolation, and may reflect differences in the metabolic role of the enzyme in a particular biological system. In addition, evidence has been presented for the existence of multiple forms of the NADP+-specific isocitrate dehydrogenase in some organisms[13–16].

Abbreviation: TEMED, *N,N,N',N'*-tetramethylethylenediamine.

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However, many previous characterizations of bacterial $NADP^+$-dependent isocitrate dehydrogenases have not been carried out using homogeneous preparations. Recently, the isolation of electrophoretically homogeneous preparations of $NADP^+$-specific isocitrate dehydrogenase has been reported from a halophile[17] and from a thermophile[18]. These reports, however, indicated a low percentage recovery of the purified enzyme from the crude extract. On the other hand, the enzyme has been purified to electrophoretic homogeneity with a 22–25% recovery from a nitrogen fixing bacteria[19,20]. The latter, however, are unique organisms in many respects and exhibit an unusually high isocitrate dehydrogenase activity even in crude extracts. Thus, the characterization of bacterial $NADP^+$-dependent isocitrate dehydrogenases has been hampered by the extreme lability of the enzyme, the lack of homogeneous preparations and the low yield of the enzyme following purification.

In order to gain a deeper insight into the mechanism of the enzymatic catalysis, as well as the chemical and physical properties of bacterial isocitrate dehydrogenases, an experimental procedure is needed to obtain purified enzyme in greater quantities. In the present report, a procedure is described for the purification of the $NADP^+$-specific isocitrate dehydrogenase from *Escherichia coli*. Since it has previously been reported that the *E. coli* enzyme is cold sensitive[21], a purification procedure is presented which can be conducted at room temperature. The procedure to be described in this report results in an electrophoretic homogeneous enzyme preparation and yields a high percentage recovery. A partial characterization of the *E. coli* $NADP^+$-dependent isocitrate dehydrogenase is also presented.

MATERIALS AND METHODS

Chemicals

The following chemicals were purchased from Sigma Chemicals Co.: $NADP^+$, Sephadex CM-50 and Sephadex G-200, malate dehydrogenase, nitroblue tetrazolium, and phenazine methosulfate. DL-Isocitrate (trisodium salt) and protamine sulfate (salmine) were obtained from Calbiochem. Dehydrated mineral salts medium (R-100 Salt Mixture) was from Grand Island Biol. Co. Enzyme Grade ammonium sulfate and the protein standards used for molecular weight determinations by gel filtration were purchased from Mann Research Laboratory. Acrylamide (electrophoresis grade), *N*,*N*′-methylenebisacrylamide and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine(TEMED) were from Eastman Organic Chemicals. Ribonuclease-free sucrose, used for the density gradients, was from Schwartz Bioresearch ,Inc. The coenzyme analogs were purchased from P. L. Biochemicals. All inorganic salts and other organic reagents were purchased from other commercial sources and were of analytical reagent grade.

Buffer systems

The buffer systems used in the enzyme purification were the following: (a) *Sonicating buffer*: 0.01 M potassium phosphate (pH 7.5) containing 0.5 M NaCl and 2 mM $MgCl_2$. (b) *Buffer A*: 0.0127 M citrate–phosphate (pH 5.5) containing 20% (v/v) glycerol and 2 mM $MgCl_2$. (This buffer was prepared by mixing 33 ml of 0.1 M citric acid, 47 ml of 0.2 M Na_2HPO_4, 200 ml of glycerol and diluted to a final volume of 1 l with distilled water). (c) *Buffer B*: 0.0120 M citrate–phosphate buffer (pH 5.3) containing 20% glycerol. (This buffer was prepared by mixing 20 ml of 0.1 M citric

acid, 40 ml of 0.2 M Na_2HPO_4 and 200 ml of glycerol then diluted to a final volume of 1 l with distilled water). (d) *Buffer C*: 0.0635 M citrate–phosphate buffer (pH 6.0) containing 20% glycerol. (This buffer was prepared by adding 3.47 g of citric acid and 16.85 g of Na_2HPO_4 to 800 ml of distilled water and diluting to one liter with 200 ml of glycerol).

Enzyme assay

Isocitrate dehydrogenase was routinely assayed by following the reduction of $NADP^+$ at 340 nm at 25° using a Cary Model 15 recording spectrophotometer. The reaction mixture contained 150 mM Tris·HCl buffer at pH 7.5, 0.5 mM $MnCl_2$, 0.66 mM $NADP^+$ and 2.5 mM DL-isocitrate (*allo*-free). The reaction was started by the addition of either enzyme or isocitrate. One unit of enzyme activity is the amount of enzyme catalyzing the reduction of 1 μmole of $NADP^+$ per min. Specific activity is expressed as units/mg protein.

Protein determination

Protein was determined by either the spectrophotometric method of WADDELL[22] or by the method described by LOWRY *et al.*[23] using crystalline bovine serum albumin as a primary protein standard. The former method cannot be employed with citrate buffers above 0.1 M.

Analytical polyacrylamide disc gel electrophoresis

A stock mixture of 100 g of acrylamide and 3.67 g of *N,N'*-methylenebisacrylamide was used for all gels. 7% gels (w/v), prepared using this stock mixture, were polymerized in 7.5 cm × 0.5 cm glass tubes under anaerobic conditions using ammonium persulfate (0.85 mg/ml) as catalyst. Electrophoresis was carried out using a Shandon disc electrophoresis cell.

The buffer systems used to determine enzyme homogeneity were the following: (a) *Acetate, pH 5.5*. The gel buffer contained 0.121 M sodium acetate, 20% glycerol and 0.04% TEMED. The electrode buffer was a 1:2 dilution of the gel buffer, but contained neither glycerol nor TEMED. (b) *Citrate–phosphate, pH 6.0*. The gel buffer was Buffer C (see *Buffer systems*) containing 0.04% TEMED. The electrode buffer was a 1:2 dilution of Buffer C but contained no TEMED. (c) *Tris–EDTA–borate, pH 8.0*. The gel buffer consisted of 0.0825 M Tris, 2.7 mM EDTA, 0.04% TEMED and 20% glycerol adjusted to pH 8 with saturated boric acid. The electrode buffer was 0.825 M Tris and 2.7 mM EDTA adjusted to pH 8 with saturated boric acid. Electrophoresis was carried out at constant current of 5 mA per gel using bromophenol blue as the tracking dye.

The gels were subsequently stained for protein for 1 h with a 0.5% solution of amido black in 7% acetic acid and then destained for 18 h by constant stirring in a solution of 7% acetic acid containing 1.2% (w/v) Dowex 1 (Cl^-) of 20–50 mesh.

Gels were stained specifically for $NADP^+$-dependent isocitrate dehydrogenase activity employing a reaction mixture containing: 0.25 mM nitroblue tetrazolium, 0.078 mM phenazine methosulfate, 0.26 mM $NADP^+$, 7.8 mM sodium isocitrate, 4 mM $MgCl_2$ and 150 mM Tris·HCl buffer, pH 8.

Molecular weight determination

Density gradient centrifugation was carried out by the method of MARTIN AND AMES[24] using crystalline malate dehydrogenase as standard. The gradients were prepared using a Buchler universal density gradient sedimentation system (Buchler Instruments). The 5–20% sucrose gradients were centrifuged for 18 h at 117 850 × *g* in a Beckman Model L2-50 ultracentrifuge.

Gel filtration[25,26] was carried out using a 2.5 cm × 45 cm column of Sephadex G-200 equilibrated in Buffer B (see *Buffer systems*) containing 10% glycerol and 0.1 M KCl. Ovalbumin, bovine serum albumin and γ-globulin were used as molecular weight standards.

Amino acid analysis

Samples were exhaustively dialyzed against several changes of deionized water and then lyophylized to dryness. 1 ml of water and 1 ml of 12 M HCl were then added. The tubes were frozen in an acetone–dry ice slurry, evacuated and sealed. The sealed tubes were then placed in an oil-bath at 110° and hydrolyzed for the desired period of time.

After hydrolysis, the tubes were opened, placed in a desiccator over NaOH and evacuated until dry. The hydrolysate was dissolved in 0.2 M citrate buffer, pH 2.2 and a 100-μl aliquot applied per column of a Beckman Model 120 C amino acid analyzer[27]. The cysteine content of the enzyme was determined on a hydrolysate which had been treated with performic acid[28]. The tryptophan content was not determined.

RESULTS

Purification of the enzyme

Step 1. Source of the enzyme. Escherichia coli, strain E-26, was maintained on trypticase soy agar slants at 4°. In order to obtain sufficient cells for an enzyme purification, the growth from two 18-h trypticase soy agar slants was inoculated into two 2-l flasks, each containing 1 l of mineral salts medium with 0.2% glucose as carbon source. The cultures were incubated at 37° for 7 h with constant shaking at 200 rev./min in a New Brunswick controlled environment incubator. The cells were then harvested by centrifugation at 4° for 10 min at 10 400 × *g* using a Sorval RC2-B centrifuge.

The cell pellet was suspended in 60 ml of cold Sonicating buffer (see *Buffer systems*), and subjected to sonic disruption for 15 min employing a sonifier cell disruptor (Model 140 C). The temperature was maintained below 15° during the sonic treatment. Cell debris was removed by centrifugation at 4° for 30 min at 27 000 × *g* and the crude cell-free extract diluted to 100 ml with cold sonicating buffer.

Step 2. Protamine sulfate treatment. The extract was maintained at 0–4° in an ice-water bath while 20 ml of a protamine sulfate solution, prepared in Sonicating buffer to contain 0.3 mg of protamine sulfate per mg of protein in the extract, was added slowly. The mixture was stirred gently for 10 min and the heavy white precipitate which formed was removed by centrifugation at 4° for 10 min at 27 000 × *g*. From this point on, except for the $(NH_4)_2SO_4$ extraction (Step 4), all operations were carried out at room temperature (23°).

Step 3. $(NH_4)_2SO_4$ precipitation. The supernatant solution from the previous step was dialyzed for 4 h at room temperature against an appropriate volume of $(NH_4)_2SO_4$ solution (previously saturated at 4°) to result in a 45% saturated solution at equilibrium. The precipitate was removed by centrifugation for 15 min at 27 000 × *g* and discarded.

Step 4. $(NH_4)_2SO_4$ extraction. The protein remaining in the supernatant fluid was precipitated by dialysis against a saturated $(NH_4)_2SO_4$ solution, recovered by centrifugation and extracted at 4° with 20 ml of cold 75% $(NH_4)_2SO_4$ solution. After centrifugation, the supernatant fluid was discarded. The pellet remaining was resuspended in 20 ml of cold 55% $(NH_4)_2SO_4$ solution and extracted at 4°; the supernatant obtained following centrifugation contained the enzyme. Two additional 10-ml extractions of the precipitate, using cold 55% saturated $(NH_4)_2SO_4$, sufficed to quantitatively recover the enzyme. The extracts were combined, and precipitated by dialysis at room temperature for 2 h against a saturated solution of $(NH_4)_2SO_4$. The precipitate was recovered by centrifugation and the supernatant fluid discarded.

Step 5. Salting out. The precipitate obtained in Step 4 was dissolved in 6 ml of Buffer B and dialyzed against the same buffer for 2 h. The resulting heavy white flocculent precipitate was removed by centrifugation and discarded. Dialysis of the solution for more than 2 h caused precipitation of the enzyme. The supernatant fluid was immediately dialyzed for 18 h against Buffer A containing 0.01 M $(NH_4)_2SO_4$.

Step 6. Sephadex CM-50. 30 ml of a slurry of Sephadex CM-50, previously equilibrated with Buffer A containing 0.01 M $(NH_4)_2SO_4$, was used to pack a small column (1.5 cm × 8.0 cm). The dialyzed enzyme solution from Step 5 was passed through the gel at a flow rate of 16 ml/h using a perstaltic pump (Harvard Apparatus Co., Model 1201). The enzyme did not bind to the gel.

Step 7. Sephadex G-200. The enzyme solution from Step 6 was passed through a Sephadex G-200 column (2.5 cm × 100 cm) equilibrated with Buffer A containing 0.1 M KCl. The flow rate was adjusted to 0.1 ml/min and 10-ml fractions were collected. The active peak fractions were pooled and concentrated by an 18-h dialysis against a saturated $(NH_4)_2SO_4$ solution. The resulting precipitate was recovered by centrifugation, dissolved in 2 ml of Buffer A and dialyzed for 18 h against the same buffer. The enzyme could be stored in this buffer at 4° for several weeks without any detectable loss in activity.

Step 8. Preparative polyacrylamide gel electrophoresis. The enzyme solution from Step 7 was subjected to electrophoresis employing a "Quickfit" preparative electrophoresis apparatus (Quickflt, Fairlawn, N.J.). 30 ml of 7% acrylamide mixture prepared in Buffer C containing 0.04% TEMED and 0.085% ammonium persulfate was poured into the apparatus, overlaid with 30 ml of the elution buffer (Buffer C) and allowed to undergo complete polymerization at room temperature for 18 h. The electrode buffer employed was a 1:2 dilution of Buffer C containing no glycerol. The initial current was 100 mA and after the enzyme had entered the gel the current was slowly increased and maintained constant at 200 mA. The temperature was maintained at 20° throughout the system by means of a constant temperature circulating water bath Model 2000 (Forma Scientific, Inc.). A peristaltic pump was used to maintain a constant flow (16 ml/min) of the elution buffer and fractions of 3 ml were collected. Table I summarizes the results obtained.

TABLE I

SUMMARY OF PURIFICATION

Step	*Total protein (mg)*	*Total units*	*Specific activity*	*Recovery (%)*	*Purification (-fold)*
Crude	2056	685	0.3	100	–
Protamine sulfate	1090	718	0.7	105	2
$(NH_4)_2SO_4$ precipitation	558	518	0.9	76	3
$(NH_4)_2SO_4$ extraction	246	455	1.9	66	6
Sephadex CM-50	157	495	3.2	72	11
Sephadex G-200	106	465	4.4	68	15
Preparative polyacrylamide gel electrophoresis	37	437	11.8	64	39

Criteria of purity

The enzyme migrated in analytical polyacrylamide disc gels as a single protein band in both an acetate pH 5.5 and a citrate–phosphate pH 6 system (Fig. 1). The protein band coincided with the enzyme activity band when duplicate gels were stained specifically for isocitrate dehydrogenase.

Disc gel electrophoresis in citrate–phosphate buffer at pH 7.3 and in Tris–EDTA–borate buffer at pH 8 resulted in the appearance of a minor protein band which migrated ahead of, and very close to, the major band. When duplicates of these gels were stained specifically for $NADP^+$-dependent isocitrate dehydrogenase activity, only one band, which coincided with the major protein band, exhibited enzymatic activity.

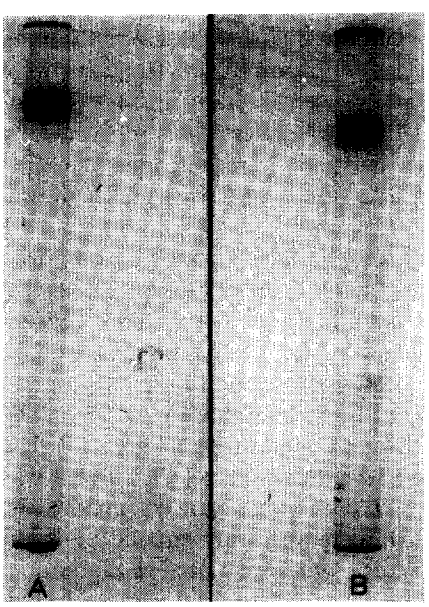


Fig. 1. Polyacrylamide disc gel electrophoresis using 30 μg of isocitrate dehydrogenase in the acetate, pH 5.5, system (A) and the citrate–phosphate, pH 6, system (B). For experimental details see MATERIALS AND METHODS.

TABLE II

EFFECT OF pH ON THE ACTIVITY AND ELECTROPHORETIC BEHAVIOR OF THE ENZYME

System	*% Activity**		*Number of protein bands*
	Before dialysis	*After dialysis*	
Citrate–phosphate, pH 6.0	100	95.7	1
Tris–EDTA–borate, pH 8.0	100	0	2
Acetate, pH 5.5	100	52.2	1
Citrate–phosphate, pH 7.3	100	56.5	2

* The enzyme was assayed before dialysis and the initial activity taken as 100%. In the Tris–EDTA–borate and citrate–phosphate (pH 7.3) systems, only one of the two protein bands exhibited catalytic activity.

It was subsequently found that dialysis of the purified enzyme against citrate–phosphate buffer at pH 7.3 or Tris–EDTA–borate buffer at pH 8 resulted in a loss of enzymatic activity and the concomitant appearance of the second minor protein band upon subsequent electrophoresis at pH 6. The data in Table II summarizes these results.

pH optimum

The activity of the enzyme was determined as a function of pH by buffering the reaction mixture with 100 mM acetate buffer from pH 4 to 6, 100 mM phosphate buffer from pH 5.5 to 7.5 and 100 mM Tris·HCl buffer from pH 7 to 10. The activity of the enzyme increased up to pH 7, remained constant through pH 9, and subsequently decreased. The pH optimum was quite broad and was considered to be from pH 7 to 9.

Enzyme stability

The data presented in Fig. 2 illustrates the activity of isocitrate dehydrogenase

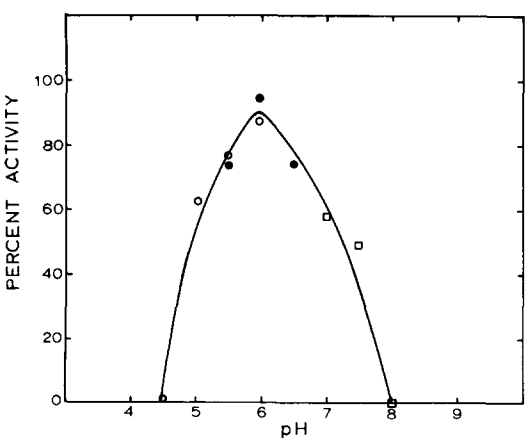


Fig. 2. Stability of isocitrate dehydrogenase. The enzyme (8 μg) was incubated at 23° for 18 h in 0.25 M acetate (○) phosphate (●) or Tris·HCl (□) buffers ranging from pH 4 through 9. The percent activity was determined by assaying the enzyme initally and after the incubation.

TABLE III

BUFFER EFFECT ON ISOCITRATE DEHYDROGENASE STABILITY

Buffer	*% Activity**
Citric acid–sodium citrate	52.7
Sodium citrate–potassium phosphate	42.4
Sodium phosphate	17.7
Potassium phosphate	18.0
Tris–maleic acid–sodium hydroxide	16.9
Phthalic acid–sodium hydroxide	13.8
Succinic acid–sodium hydroxide	3.5
Glycylglycine–Imidazole	0.0

* % of the initial activity remaining after 20 h dialysis at 23° against the indicated buffers. All buffers were pH 6.0 and 0.1 M.

following incubation for 18 h at 23° in buffers from pH 4.0 to 9.0. It can be seen that the enzyme exhibited a rather narrow pH optimum range for stability with a maximum at pH 6.0.

In order to determine whether the stability was a result of pH *per se*, or whether the nature of the buffer employed affected enzyme stability, further studies were undertaken. The enzyme was dialyzed for 20 h at 23° against a 1000-fold volume of several different buffers, each at pH 6.0 and each at a final concentration of 0.10 M. The data presented in Table III illustrates the results obtained. The enzyme was found to be most stable in a citric acid–sodium citrate buffer and exhibited extreme lability in glycyglycine–imidazole buffer.

To investigate further the apparent stabilizing effect of citrate, the enzyme was dialyzed for 20 h at 23° against a 1000-fold volume of 0.05 M potassium phosphate buffers at pH 6.0 containing final citrate concentrations ranging from 0 to 100 mM. The data presented in Table IV clearly illustrate that the stability of the enzyme increases with increasing concentrations of citrate. Similar experiments in which citrate was replaced by EDTA indicated that the protection afforded by citrate could not be explained in terms of the chelation of heavy metal ions. Since it has been previously reported[17] that isocitrate stabilized the enzyme, it does not seem unreasonable to suggest that citrate may bind to the isocitrate site on the enzyme and thereby

TABLE IV

EFFECT OF CITRATE ON ISOCITRATE DEHYDROGENASE STABILITY AT pH 6.0

Citrate concentration (mM)	*% Activity**
0.1	68.8
0.05	63.1
0.025	56.3
0.01	46.8
0.005	39.3
0.0025	32.1
0.001	32.4
0	12.4

* % of the initial activity remaining after 20 h dialysis at 23° against 0.05 M phosphate buffer, pH 6.0, containing the indicated concentrations of sodium citrate.

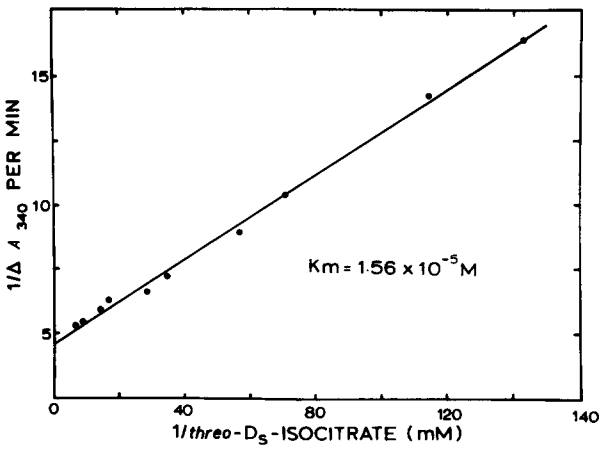


Fig. 3. Lineweaver–Burk plot for the determination of the K_m for *threo*-D_s-isocitrate. The reaction mixture contained 150 mM Tris·HCl buffer (pH 7.5), 0.5 mM $MnCl_2$, 0.66 mM $NADP^+$, and 2.5 μg of enzyme. The isocitrate concentration was varied as indicated.

exert a protective effect. This suggestion is strengthened by the observation that at high concentrations (5–10 mM) citrate is a competitive inhibitor of isocitrate in the enzymatic reaction.

Enzyme kinetics

The K_m values calculated from double reciprocal plots of enzyme activity with respect to substrate, coenzyme and metals are shown in Figs. 3–6.

Although the NADP analogs, 3-acetylpyridine–$NADP^+$ and thionicotinamide–$NADP^+$, are both able to serve as cofactors in the reaction catalyzed by the *E. coli* enzyme, the activity with these analogs was 37.9 and 2.0%, respectively, of that observed with the natural cofactor. Selenonicotinamide–$NADP^+$ did not serve as a cofactor in the reaction.

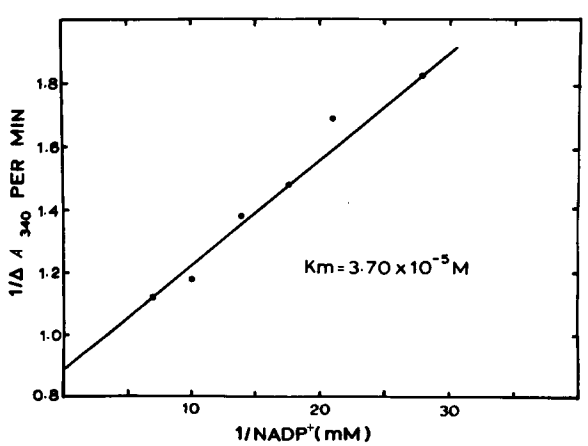


Fig. 4. Lineweaver–Burk plot for the determination of the K_m for $NADP^+$. The reaction mixture contained 150 mM Tris·HCl buffer (pH 7.5), 0.5 mM $MnCl_2$, 2.5 mM DL-isocitrate and 2.5 μg of enzyme. The $NADP^+$ concentration was varied as indicated.

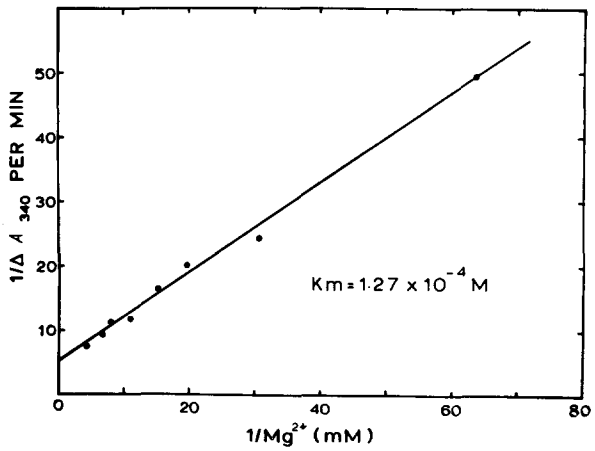


Fig. 5. Lineweaver–Burk plot for the determination of the K_m value for Mn^{2+}. The reaction mixture contained 150 mM Tris·HCl buffer (pH 7.5), 2.5 mM DL-isocitrate, 0.66 mM $NADP^+$ and 2.5 μg of enzyme. The Mn^{2+} concentration was varied as indicated.

Molecular weight

The molecular weight of isocitrate dehydrogenase, obtained by gel filtration on Sephadex G-200, was 79 000 (Fig. 7). As determined by density gradient centrifugation, a molecular weight of 82 500 was obtained (Fig. 8). On the basis of these data, the molecular weight of the *E. coli* isocitrate dehydrogenase was considered to be 80 000.

Amino acid composition

The amino acid composition of the purified enzyme was determined and the results are presented in Table V. The protein was hydrolyzed for the times indicated and the number of amino acid residues per mole of enzyme were extrapolated to zero time. These values, to the nearest integer, are expressed relative to alanine.

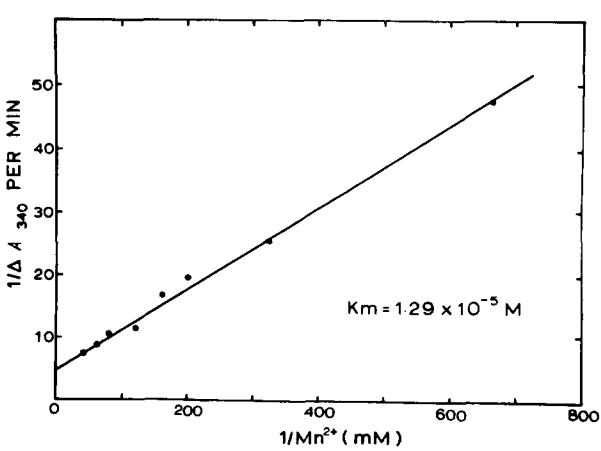
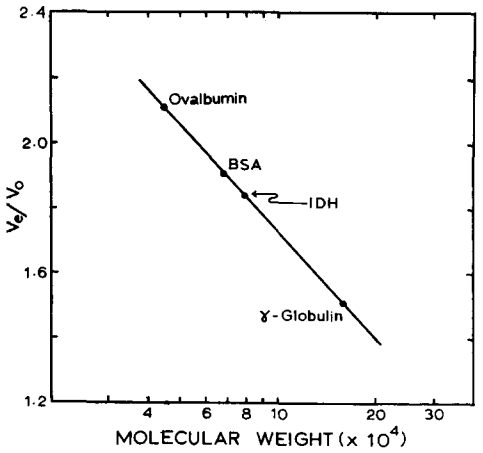


Fig. 6. Lineweaver–Burk plot for the determination of the K_m value for Mg^{2+}. See Fig. 5 for details.



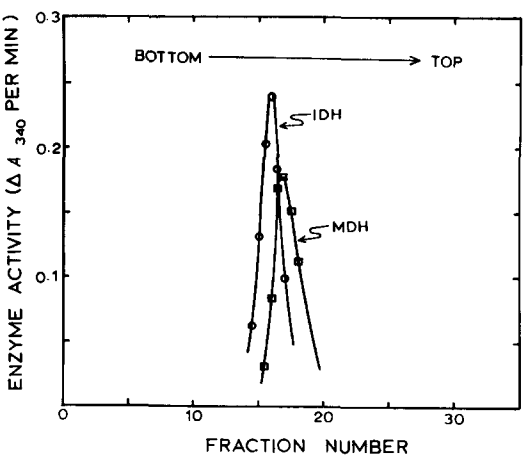


Fig. 7. Molecular weight determination of isocitrate dehydrogenase by gel filtration. The V_e/V_0 values were plotted against the $\log_{10}$ molecular weight of standards. V_e, elution volume; V_0, void volume. BSA, bovine serum albumin; IDH, isocitrate dehydrogenase.

Fig. 8. Molecular weight determination of isocitrate dehydrogenase (IDH) using sucrose density gradient centrifugation. Malate dehydrogenase (MDH) was assayed by following the oxidation of NADH as described by DAVIS[30].

TABLE V

AMINO ACID COMPOSITION OF *E. coli* ISOCITRATE DEHYDROGENASE

Amino acid	*Time of hydrolysis**			*Molar ratio*	*Residues per molecule****
	20 h	*44 h*	*68 h*		
Lysine	0.824	0.729	0.764	0.772	57
Histidine	0.200	0.182	0.176	0.186	14
Arginine	0.382	0.373	0.378	0.378	28
Aspartic acid	1.041	0.987	1.027	1.018	75
Threonine**	0.471	0.449	0.466	0.471	36
Serine**	0.424	0.378	0.338	0.495	34
Glutamic acid	1.124	1.138	1.162	1.141	84
Proline**	0.459	0.440	0.453	0.451	31
Glycine	0.829	0.831	0.865	0.842	62
Alanine	1.000	1.000	1.000	1.000	75
Half cystine†	–	0.071	0.068	0.104	8
Valine	0.659	0.724	0.750	0.786	58
Methionine	–	0.124	0.169	0.147	10
Isoleucine	0.588	0.644	0.655	0.687	51
Leucine	0.847	0.867	0.885	0.897	66
Tyrosine	0.300	0.329	0.351	0.327	24
Phenylalanine	0.306	0.311	0.318	0.312	23

* Data is based on the average of three determinations.

** Values taken after extrapolation to zero time of hydrolysis.

*** A molecular weight of 80 000 was used to determine the nearest integer relative to alanine. This value was obtained by gel filtration and sucrose gradient centrifugation as described in the text.

† Half-cystine was measured as cysteic acid after performic acid oxidation[28].

DISCUSSION

The procedure described for the purification of the $NADP^+$-specific isocitrate dehydrogenase of *E. coli* results in an electrophoretically homogeneous enzyme, can be conducted at room temperatures and provides a method for obtaining highly purified enzyme in high yields. The final step of the purification, preparative polyacrylamide gel electrophoresis, takes advantage of the fact that the enzyme has a low isoelectric point and migrates towards the anode at pH 6.0. This has also been found to occur with the isocitrate dehydrogenases of several other bacterial species (unpublished observations) and may be of general use in the purification of the enzyme from other organisms.

The stability of the enzyme is influenced by both the pH and the nature of the buffer; further, the incorporation of 20% glycerol in all buffers is necessary for the protection of the enzyme[29]. Citrate buffers, particularly, tend to stabilize the enzyme while succinate and glycylglycine buffers are extremely deleterious.

Below pH 5, the enzyme is rapidly inactivated but does not undergo dissociation. Above pH 6.5, however, the enzyme also loses catalytic activity with concomitant irreversible dissociation. Thus, polyacrylamide gel electrophoresis above pH 6.0 reveals the presence of a second, but minor protein band, which does not exhibit catalytic activity. This behavior is in contrast to the NAD^+-specific enzyme described by LEJOHN *et al.*[31] which undergoes aggregation to an inactive form at acid pH levels and exists as a catalytically active monomer at alkaline pH values. These workers report that the difference of one pH unit (6.5 to 7.5) changes the association–dissociation equilibrium in favor of monomer formation from the polymeric form.

The *E. coli* $NADP^+$–isocitrate dehydrogenase has an absolute requirement for a divalent metal ion. Whereas both Mg^{2+} and Mn^{2+} will serve as cofactors, the latter is 10-fold more effective than the former at the same concentration. This finding is in agreement with recent reports concerning several other $NADP^+$–isocitrate dehydrogenases from bacterial sources[8,9,17–19] but is in contrast to the report by CHUNG AND FRANZEN[20] in which they report that the enzyme from *Azotobacter vinelandii* (ATC 9104) is maximally stimulated by Mg^{2+}. SIEBERT *et al.*[32], as well as COLMAN[3], report that the $NADP^+$–enzyme from mammalian sources is more effectively activated by Mn^{2+} than Mg^{2+}.

The $NADP^+$ analogs, 3-acetylpyridine–$NADP^+$ and thionicotinamide–$NADP^+$, were able to serve as coenzymes in the reaction catalyzed by the *E. coli* enzyme but were less active (38 and 2%, respectively) than the natural coenzyme. Selenonicotinamide–$NADP^+$ exhibited no activity. This observation corroborates the earlier report by CHRIST *et al.*[33] that those dehydrogenases which were active with selenonicotinamide–$NADP^+$ involve a transfer of hydrogen with β-sterospecificity for the nicotinamide ring of $NADP^+$ (refs. 34–36). In contrast, the heart muscle isocitrate dehydrogenase and horse liver alcohol dehydrogenase which do not reduce selenonicotinamide–$NADP^+$ have an α-stereospecificity for the nicotinamide ring[34,37]. NAD^+ exhibited activity which was less than 1% of that observed with $NADP^+$.

The molecular weight of 80 000, obtained by gel filtration and sucrose gradient centrifugation, is similar to that reported for the $NADP^+$–enzyme from Azotobacter[19,20] but somewhat smaller than the 92 500 molecular weight reported for the enzyme obtained from *Bacillus stearothermophilus*[18]. No evidence was obtained which

indicated that the *E. coli* enzyme could be dissociated into catalytically active subunits.

Amino acid analysis of the *E. coli* enzyme indicates that it contains a high glutamic acid content as do the enzymes isolated from other bacterial species[18–20]. The most striking observation, however, was the unusually high sulfhydryl content of the *E. coli* isocitrate dehydrogenase. Whereas the enzyme isolated from several other bacterial species[18–20], and the pig heart enzyme[3], contain three or less half cystine residues, the *E. coli* enzyme contains eight. Neither the stability nor the catalytic activity of the enzyme, however, appear to be markedly affected by such groups since they remain unchanged in the presence or absence of reducing agents such as dithiothreitol and mercaptoethanol.

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