

Purification and Properties of NADP⁺:Isocitrate Dehydrogenase from Lactating Bovine Mammary Gland

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NADP⁺:isocitrate dehydrogenase has been purified to homogeneity from lactating bovine mammary gland. Purification was achieved through the use of affinity and DEAE-cellulose chromatography. The isolated enzyme gives one band when stained for protein or enzyme activity on discontinuous alkaline gel electrophoresis. The enzyme has a molecular weight of 55,000 as estimated by sodium dodecyl sulfate-gel electrophoresis and a Stokes radius of 4.1 nm as measured by gel chromatography. The enzyme will not use NAD⁺ in place of NADP⁺ and has an absolute requirement for divalent cations. The apparent K_m values for DL-isocitrate, Mn²⁺, and NADP⁺ were found to be 8, 6, and 11 μ M, respectively. The Mn²⁺-D₃-isocitrate complex is the most likely substrate for the mammary enzyme with a K_m of 3 μ M. The properties of mammary NADP⁺:isocitrate dehydrogenase are compared with those of the homologous enzymes from pig heart and bovine liver, and its characteristics are discussed with respect to the function of the enzyme in lactating mammary gland.

The activity of NADP⁺-dependent isocitrate dehydrogenase [Threo-D₃-isocitrate: NADP⁺ oxidoreductase (decarboxylating) EC 1.1.1.42] rises sharply in bovine mammary gland at the onset of lactation (1, 2). It has been postulated (1) that this enzyme serves as a major source of the NADPH required for the *de novo* synthesis of fatty acids in lactating bovine mammary gland. Purified NADP⁺-dependent isocitrate dehydrogenases have been obtained from heart (3-5) and liver (6); the enzymes isolated from these tissues differ significantly from each other in their chemical, enzymatic, and physical properties. The mammary enzyme, however, has not been studied. Investigation of the properties of the mammary enzyme will lead to a better understanding of the role of NADP⁺-isocitrate dehydrogenase in mammary metabolism. This paper reports the purification and partial characterization of the NADP⁺-dependent isocitrate dehydrogenase of lactating bovine mammary gland.

EXPERIMENTAL PROCEDURES

Materials. All coenzymes, substrates, and biochemicals used in this study were purchased from either Sigma Chemical Company² or Calbiochem. Blue Sepharose CL-6B and Sephaeryl S-200 were products of Pharmacia and DE-32 was obtained from Whatman. All other chemicals were reagent grade.

Small quantities of fresh bovine mammary tissue were obtained at the time of slaughter, through the cooperation of Norristown State Hospital dairy herdsman and a local abattoir. Whole mammary glands from cows of known good health and productivity were obtained through the cooperation of Drs. Wrenn and Bitman of U. S. Department of Agriculture, Beltsville, Maryland. The whole mammary glands were obtained at the time of slaughter, trimmed to remove adipose tissue, and sectioned into pieces approximately 10 × 15 × 5 cm. The tissue was frozen on dry ice and stored at -20°C until used.

Enzyme assays. NADP⁺:isocitrate dehydrogenase activity was measured at 25°C by monitoring the increase in A₃₄₀. The standard reaction mixture

¹ Agricultural Research, Science and Education Administration, U. S. Department of Agriculture.

² Reference to brand or firm name does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

TABLE I
 PROPERTIES OF FRACTIONATIONS OBTAINED IN PURIFICATION OF MAMMARY
 NADP⁺:ISOCITRATE DEHYDROGENASE

Sample	Volume (ml)	Activity (U ^a /ml)	Lowry protein (mg/ml)	Specific activity (U/mg)	Yield (%)	Purification (n-fold)
Whole homog.	800	7.19	23.8	0.302	100	—
1500g supernatant	720	7.85	13.9	0.565	98	2
12,000g supernatant	680	7.85	10.8	0.727	93	2
40,000g supernatant	630	8.50	8.88	0.957	93	3
33–55% (NH ₄) ₂ SO ₄ precipitate	80.0	52.3	20.0	2.62	73	8
G-25 ^b	25	27.9	11.1	2.51	61 ^b	8
Blue Sepharose	7.6	25.3	0.630	40.2	17	130
S-200	10.4	12.2	0.269	45.4	11	150
DE-32	5.6	12.6	0.240	52.5	6.1	170

^a μ mol of NADPH/min at 25°C.

^b Recoveries from this point on are based on dividing the (NH₄)₂SO₄ precipitate into five equal volumes and processing each separately. The results reported are the average of two trials.

consisted of 100 mM Tris-HCl at pH 7.4, 1.10 mM MnSO₄, 1.50 mM DL-isocitrate, and 110 μ M NADP⁺ in a total volume of 2.70 ml; 10 to 50 μ l of enzyme was added to start the reaction. Reactions were carried out in 1-cm cells and monitored on a Gilford spectrophotometer with a scale setting of 0 to 0.150 absorbance units. The time course of the reaction was linear for the period of the assay. One enzyme unit catalyzes the formation of 1.0 μ mol of NADPH per minute at 25°C. Specific activity is defined as enzyme units per milligram of protein.

Kinetic data were obtained by averaging duplicate assays for each point. The results were analyzed by iterative techniques as recommended by Cleland (7).

Polyacrylamide gel electrophoresis. Discontinuous polyacrylamide gel electrophoresis was carried out on 10% acrylamide gels with a modification of the buffers of Williams and Reisfield (8) and the techniques described by Ornstein (9). All samples contained 30% sucrose and were added directly to the gels. Gels were stained with Coomassie Blue R250 to detect protein or by the nitro blue tetrazolium method to detect enzyme activity as described by Brewer (10).

Electrophoresis in the presence of SDS³ followed the procedures of Laemmli (11). Samples for SDS-gels were prepared by estimating the protein content and adjusting the volume of sample to deliver 30 to 80 μ g of protein per gel. When the total required volume of sample was greater than 200 μ l,

³ Abbreviations used: SDS, sodium dodecyl sulfate; DTT, dithiothreitol; TES, *N*-tris[hydroxymethyl]-methyl-2-aminomethanesulfonic acid; pCMB, *p*-hydroxymercuribenzoate.

the protein solutions were first concentrated in a plastic tube in a Savant Speedvac Concentrator. The monomer molecular weight of the enzyme was calculated by the method of Weber and Osborn (12), with α -lactalbumin, β -lactoglobulin, ovalbumin, and bovine serum albumin used as standards.

Protein determination. Protein concentrations were determined by the method of Lowry *et al.* (13) with appropriate buffer blanks and with bovine serum albumin as the standard.

RESULTS

Isolation of NADP⁺:Isocitrate Dehydrogenase from Lactating Mammary Gland

Frozen mammary gland was minced and weighed. Four volumes (by weight) of cold homogenization medium containing 0.25 M sucrose, 50 mM Tris, pH 7.4, 0.5 mM EDTA, and 100 μ M DTT were added and the mixture was homogenized for 1 min in a Polytron 10 ST at medium speed. The homogenate was then filtered twice through cheesecloth and cooled on ice. All subsequent operations were carried out at 5°C. The homogenate was centrifuged consecutively at 1500g for 15 min, 12,000g for 30 min, and 40,000g for 2.5 h. After each centrifugation, the precipitate was removed, resuspended in homogenization buffer, and tested for enzyme activity.

With frozen mammary gland, more than 93% of the NADP⁺:isocitrate dehydrogenase activity was present in the supernatant (40,000g, 2.5 h). Data are shown in Table I for the fractionation of frozen mammary gland.

Solid (NH₄)₂SO₄ was added to bring the supernatant to 33% saturation. Table I shows that 73% of the total activity of the crude homogenate was recovered in the 33 to 55% precipitate, which represents a convenient stopping point. At this point a preparation was divided into 15-ml portions each of which was processed separately. The resuspended material can be frozen and maintains its activity for over a year.

Excess ammonium sulfate was removed by passing the resuspended precipitate over a Sephadex G-25 column equilibrated with 50 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, and 100 μM DTT containing 10% glycerol (Buffer B). The desalted effluent was applied directly to a Blue Sepharose column (Fig. 1). Most of the protein, along with some enzyme activity, was eluted immediately. After the protein concentration approached the base line (usually 120 ml), the enzyme was eluted by addition of 2 mM NADP⁺, and tubes with the highest specific activity were pooled. Other methods of elution were attempted to increase the yield at this step; these included addition of substrate, metal ion, and KCl. Quantitative recovery of the enzyme activity can be achieved with 2 M KCl, but the enzyme has a lowered specific activity. The high specific activity (Table I) of the enzyme obtained with NADP⁺ elution, coupled with its good purity as determined by gel electrophoresis, prompted the use of this step to obtain purity at the expense of yield. After each cycle, the affinity column was treated with 4 M urea and 2 M KCl. A small degree of purification was obtained by chromatography on Sephacryl S-200 (2.6 × 30 cm, 20 ml/h) as seen in Table I; this step also removed excess NADP⁺.

Examination of the preparation by gel electrophoresis showed the major contaminants to be more acidic than the NADP⁺:isocitrate dehydrogenase, hence DEAE-cellulose equilibrated with Buffer B was employed as the next step in the

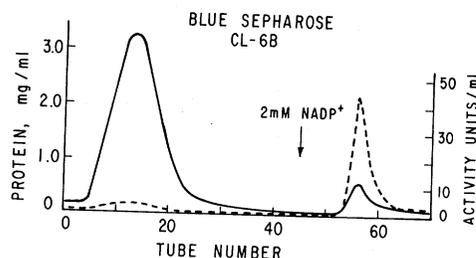


FIG. 1. Affinity chromatography of NADP⁺:isocitrate dehydrogenase on Blue Sepharose CL-6B. Samples of 15 ml each were placed on a column 2.5 × 12 cm equilibrated with (50 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 100 μM DTT, 10% glycerol). The column flow was maintained at 30 ml/h, and fractions of 3 ml each were collected and analyzed for protein (—) and activity (---). After the protein concentration approached the base line, a solution (15 ml) of 2 mM NADP⁺ was pumped onto the column followed by elution with starting buffer.

purification procedure. The enzyme was applied to a 1 × 10-cm column and eluted with a linear gradient of 0 to 0.3 M NaCl. A brownish band representing most of the impurities remained bound to the column. The fractions with the highest specific activity were pooled and stored at -20°C. The purified enzyme represents 6% of starting material with a specific activity of 52.5 units/mg protein. The overall purification is roughly 170-fold, based on specific activity (Table I).

Purity of Preparation and Estimated Molecular Weight

The purified enzyme obtained from the procedure described above was subjected to gel electrophoresis on 10% acrylamide discontinuous gels. The buffers were those of Williams and Reisfeld (8) for a pH 7.5 system except that their STOCK B was adjusted to pH 6.0. This increases the pH of the running gel, but the enzyme, as isolated, was not stable below pH 6.0 for long periods of time; therefore, exposure to the pH 5.5 gel was avoided. The purified mammary NADP⁺:isocitrate dehydrogenase is homogeneous and displays one single band when stained for protein (Fig. 2, P). A companion gel when stained for enzymatic activity (Fig. 2, A) shows a single band in the same position. Electrophoresis

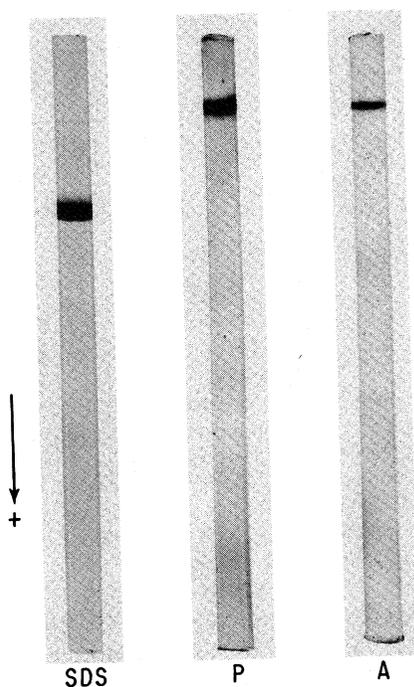


FIG. 2. Acrylamide gel electrophoresis of purified NADP⁺ isocitrate dehydrogenase. Discontinuous gel electrophoresis as described in Experimental Procedure stained for protein (P); companion gel stained for enzyme activity (A). Sodium dodecyl sulfate-gel electrophoresis of purified enzyme (SDS) as described in Experimental Procedures.

of the enzyme in sodium dodecyl sulfate again yields one band (Fig. 2, SDS). By calibration of the dodecyl sulfate gels as described under Methods, the monomer molecular weight was estimated to be 55,000.

Analytical gel chromatography of the enzyme was carried out on Sepharose CL-6B. A column (1.6 × 40 cm) was calibrated as previously described (14). A 0.5-ml sample of protein containing 0.240 mg/ml was applied to the column, and the elution volume of the protein was detected by enzymatic assay. The activity of the protein eluted as a single peak, and from K_{av} a Stokes radius of 4.1 nm was calculated.

Conditions for Enzymatic Assay

The purified enzyme was used to determine the effects of various buffers on

enzymatic activity at pH 7.4; buffers used were Tris, TES, and *N*-ethyl morpholine. At low buffer concentrations (20 to 50 mM) all buffers produced somewhat lowered activity; addition of KCl at 50 mM raised the activity, indicating an ionic strength rather than a buffer concentration effect. To counteract reduced activity at lower ionic strengths, the fundamental enzyme assay was altered to increase the concentration of Tris to 100 mM.

The effect of pH on enzyme activity was investigated. Precipitation of Mn²⁺ at pH values >7.5 occurred with Tris; use of *N*-ethyl morpholine extended the pH range which could be studied to 9.0 without apparent loss of Mn²⁺ through precipitation. The pH activity curve obtained was quite flat from pH 7.2 to 8.2, but fell off sharply at lower and higher pH values (Fig. 3).

The isolated enzyme exhibited no detectable activity in the absence of added metal ion. With the standard assay conditions, addition of Mn²⁺ up to a total metal ion concentration of 1.5 mM caused activation of the enzyme. At higher concentrations, added Mn²⁺ was inhibitory; under standard assay conditions, additional Mn²⁺ has an I_{50} of 50 mM (added ionic strength due to MnSO₄ = 0.200 M). With 1.5 mM Mn²⁺, KCl produced a lesser degree of inhibition with I_{50} = 0.380 M added ionic strength. Thus the inhibition by added Mn²⁺ may be partially due to increased ionic strength alone.

The purified enzyme has no activity in the absence of added nucleotides; NAD⁺ did not serve as an electron acceptor in the reaction even at concentrations up to 1 mM which is almost 100 times the K_m found for NADP⁺.

Effects of Metal Ions

Mono- and divalent cations were tested for their ability to substitute for Mn²⁺ in the reaction. Table II shows the activity of the enzyme when various metal ions were used in the standard reaction mixture, except that all metals were added at 500 μM. With the exception of Mg²⁺ and Mn²⁺, the binding constants for metal and isocitrate are not accurately known and so the

activities are given in relative terms. Li^+ , Na^+ , K^+ , Cs^+ , Ca^{2+} , Ba^{2+} , Sr^{2+} , and Cu^{2+} were without effect, while Mn^{2+} , Mg^{2+} , Co^{2+} , Zn^{2+} , and Cd^{2+} were partially effective as activators of the enzyme.

Kinetic Constants

The kinetic constants of purified NADP^+ :isocitrate dehydrogenase of lactating mammary gland were determined. Apparent K_m and V values for DL-isocitrate, Mn^{2+} , and NADP^+ were calculated from iterative analysis (Fig. 4), and the results are summarized in Table III. The true substrate for NADP^+ :isocitrate dehydrogenase has been postulated (6, 15, 16) to be the metal-isocitrate complex. Accordingly, the kinetic experiments were repeated with D_s -isocitrate, with the Mn^{2+} and isocitrate concentrations adjusted so that the concentration of free Mn^{2+} remained constant at 30, 100, and 300 μM , while the concentration of the metal isocitrate complex varied. Double reciprocal plots obtained in these experiments were linear and nearly identical, indicating that the metal isocitrate complex is the true substrate and that, over the concentration range studied, variation of the concentration of free metal ion has no effect on V . Table IV summarizes the kinetic constants obtained for Mn-D_s -isocitrate as substrate.

Inhibitor Studies

During the fractionation procedure, it was observed that addition of DTT at 100 μM enhanced the stability of the enzyme. Samples stored without DTT lost 20 to 30% of their activity upon repeated freezing and thawing. The enhancement of stability by DTT could be indicative of the presence of sulfhydryl or other oxidizable groups in or near the active site. A solution of *p*-hydroxymercuribenzoate (pCMB) was prepared (17) and diluted into 0.280 M Tris buffer at pH 7.5 to yield a final concentration of 81.7 μM . The purified enzyme was incubated with pCMB at 25°C for 15 min; pCMB inhibited the enzyme completely.

The reaction product of oxalacetate and glyoxylate, which is known to inhibit the

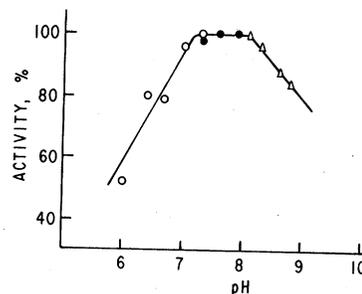


FIG. 3. pH Activity curve obtained for NADP^+ :isocitrate dehydrogenase of bovine mammary gland. Standard assay conditions were used except for the buffers which were (○) TES, (●) Tris, and (△) *N*-ethyl morpholine, all at 100 mM.

NADP^+ :isocitrate dehydrogenase of pig heart (18), also inhibits the mammary gland enzyme (Table V). Neither metabolite significantly affected the enzyme, but after the mixture of the two compounds stood for 1 min, the resulting product was inhibitory.

DISCUSSION

NADP^+ :isocitrate dehydrogenase of bovine mammary gland has previously been shown to occur predominately in the soluble or cytosolic fraction (1). In this study, the enzyme was purified from frozen tissue in which lysis of the mitochondria occurs. However, based on the preponderance of the enzyme in the cytoplasm, and comparisons drawn with enzymes from other tissues, the isolated protein is most likely the cytoplasmic form of the enzyme. An advantage to the isolation procedure presented here is that only one buffer is used throughout, and eluates from one column may be transferred directly to the next without precipitation, concentration, or dialysis, each of which usually results in loss of enzymatic activity. Glycerol at 10% is employed to maintain stability, as is DTT at 100 μM . In this fractionation scheme, affinity chromatography is the chief method employed for purification of the enzyme. This chromatographic method has been used successfully for the preparation of human heart NADP^+ :isocitrate dehydrogenase (4). Experiments aimed at improving the efficiency of the affinity column

TABLE II
EFFECTS OF VARIOUS CATIONS ON THE ACTIVITY
OF NADP⁺:ISOCITRATE DEHYDROGENASE

Metal ion	Oxidation number	Charge:radius ratio	Typical coordination No.	Activity (%)
Li	I	1.5	4	0
Na	I	1.0		0
K	I	0.75	6,8	0
Cs	I	0.60	8	0
Mg	II	3.0	4,6	45
Ca	II	2.0	6	0
Sr	II	1.8	6	0
Ba	II	1.5	6,8	0
Mn	II	2.5	4,6	100
Co	II	2.8	4,6	41
Ni	II	2.9	4,6	6
Cu	II	2.8	4,6	0
Zn	II	2.7	4,6	1
Cd	II	2.1	4,6	32

Note. Standard assay conditions except that the total added metal ion in each case (including Mn²⁺) was 500 μM.

were fruitless; apparently some nonspecific binding of impurities occurs, and when the yield of enzyme is increased the degree of purity decreases.

Carrier and Pantaloni (6) used DEAE-Sephadex in their isolation of the soluble liver isocitrate dehydrogenase. In the isolation reported here DEAE-cellulose is the final step, and significant binding occurs. This is not the case with the heart enzymes (19, 20), which bind to CM- but not to DEAE-celluloses under quite similar buffer conditions. Thus, with respect to column chromatography, the mammary enzyme resembles the liver enzyme and not the heart enzyme. Finally with respect to sub-cellular location, the liver (6) and mammary enzymes (1) are soluble, in contrast to the heart enzymes, which are reported to be mitochondrial in origin (5).

The monomer molecular weight of the purified mammary NADP⁺:isocitrate dehydrogenase was estimated to be 55,000 by gel electrophoresis in the presence of sodium dodecyl sulfate. This is in agreement

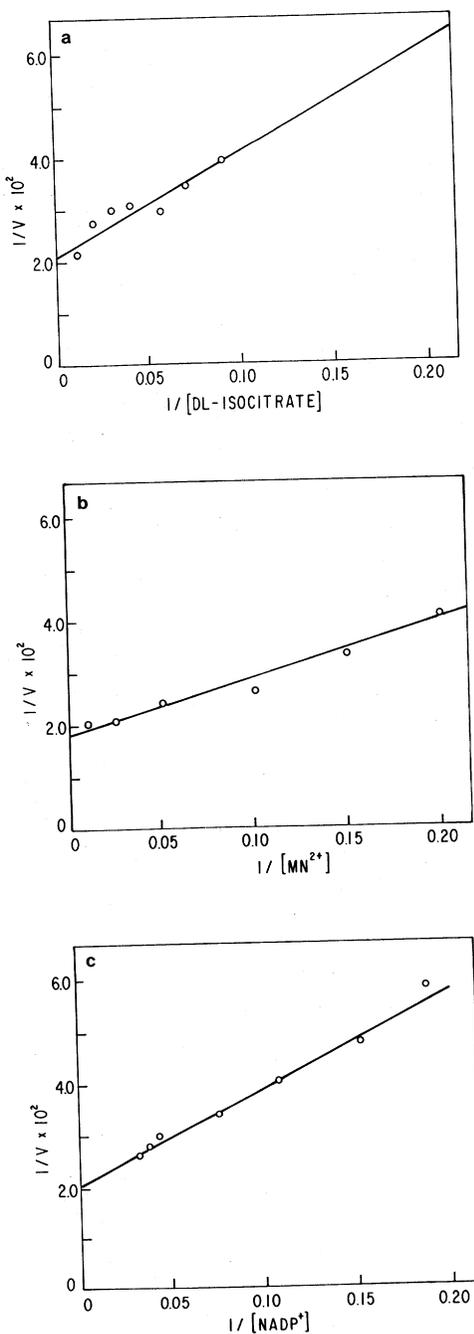


FIG. 4. Double reciprocal plots for the variation of the observed velocity (v is expressed as $\mu\text{mol NADPH}^+ \text{min}^{-1} \text{mg}^{-1}$) with substrate concentration (μM): (a) DL-isocitrate, (b) Mn²⁺, (c) NADP⁺; in each case the standard reaction conditions were used for the unvaried substrates. The lines shown were obtained by the iterative computer analyses described by Cleland (9).

with the values obtained for human heart (19) but differs from the 48,000 obtained for the liver enzyme (6). A Stokes radius of 4.1 nm obtained at low protein concentration (0.25 mg/ml) is close to the 3.9-nm value obtained for the pig heart enzyme and 3.7 for human heart (19) under similar conditions.

Carlier and Pantaloni (21) reported activity in the liver enzyme in the absence of added metal ion, even when the preparation was treated with Chelex resin. In the present study no activity was obtained in the absence of added metal ions. While the mammary enzyme is isolated in the presence of EDTA (0.5 mM), enzyme assays are routinely carried out on 10- μ l samples diluted up to 1:10 into Tris buffer. Thus, the EDTA carryover is always less than 2 μ M and usually 0.2 μ M. Carlier and Pantaloni (21) found I_{50} for EDTA to be 30 μ M for enzyme activity expressed in the absence of added metals. Therefore, the mammary enzyme is probably devoid of activity in the absence of added metal and in this respect is similar to pig heart enzyme. Addition of Mn^{2+} beyond 1.5 mM is inhibitory; however, because KCl will also produce a similar effect, this may be more related to increased ionic strength than to a specific metal ion effect.

With regard to the cations which may be utilized in the reaction, Mg^{2+} , Mn^{2+} , Co^{2+} , and Cd^{2+} have been reported to stimulate the heart enzyme (22). In addition, Zn^{2+} has been reported to be an effective catalyst in the reaction of the pig heart enzyme. In the present study, little or no reactivity was found for either zinc acetate or zinc chloride

TABLE III

SUMMARY OF KINETIC PARAMETERS FOR BOVINE MAMMARY NADP⁺:ISOCITRATE DEHYDROGENASE

	K_m apparent ^a (μ M)	V ^a (μ mol min ⁻¹ mg ⁻¹)
DL-isocitrate	8.0 \pm 1.4	47.7 \pm 1.55
NADP ⁺	10.8 \pm 1.6	49.9 \pm 2.6
Mn ²⁺	6.4 \pm 1.4	54.6 \pm 3.2

^a Average of three trials with three preparations \pm σ .

TABLE IV

KINETIC PARAMETERS CALCULATED FOR Mn^{2+} -D₃-ISOCITRATE AS SUBSTRATE

$[Mn^{2+}]_f$ (μ M)	K_m (μ M)	V (μ mol min ⁻¹ mg ⁻¹)
300	3.7	47.9
100	2.3	47.4
30	2.9	55.2
$\bar{x} \pm \sigma$	3.0 \pm 0.8	50.2 \pm 4.4

at concentrations of 5, 50, and 500 μ M; higher concentrations of Zn^{2+} might, however, be active. With this exception the relative activities given for divalent cations are similar to those previously reported (22). A charge to radius ratio of 2.5, coupled with a "typical coordination number" of 4 or 6 appears to be optimal (Table II), although other factors may play a role.

The apparent Michaelis constants calculated for mammary NADP⁺:isocitrate dehydrogenase are compared (Table VI) with those obtained for pig heart and bovine liver enzymes. The K_m values of the mammary enzyme are twofold greater than those obtained for the pig heart enzyme for DL-isocitrate and NADP⁺, while " K_m apparent" for Mn^{2+} differs. The observed K_m for the mammary enzyme with Mn-D₃-isocitrate is different than the pig heart enzyme but close to the uncorrected K_m of 2 μ M obtained for the human heart enzyme (19). The kinetic constants of the liver enzyme, however, are quite different from those of the mammary enzyme. Inhibition by pCMB and the glyoxalate-oxalacetate complex (18) apparently is common to all NADP⁺:isocitrate dehydrogenases studied thus far.

Purification of the mammary NADP⁺:isocitrate dehydrogenase allows for the comparison of its properties with previously studied homologous enzymes. With respect to its subcellular location and its affinity for DEAE-cellulose, the mammary enzyme resembles the liver enzyme. However, preliminary molecular weight measurements place the enzyme closer to pig heart enzyme. The apparent K_m values are, again, closer to the heart enzymes, though

TABLE V
EFFECT OF THE GLYOXYLATE-OXALOACETATE
COMPLEX ON MAMMARY NADP⁺:ISOCITRATE
DEHYDROGENASE

	Relative activity ^a
No additions	100
+0.17 mM glyoxylate	100
+0.17 mM oxaloacetate	95
+0.17 mM both ^b	70
+1.0 mM both ^b	31

^a Standard assay except for additions given.

^b Metabolites mixed and held for 1 min prior to assay.

not identical. Further characterization of the chemical properties of the mammary enzyme will be necessary to determine its structural relationship to the liver and heart enzymes.

With respect to its function in mammary tissue, the enzyme has a V of 50 $\mu\text{mol min}^{-1} \text{mg}^{-1}$. It has recently been reported (23) that *in vitro* the fatty acid synthetase complex of bovine mammary gland could consume 1.60 μmol of NADPH $\text{min}^{-1} \text{mg}^{-1}$ when acting on model substrates. If the proteins occurred on an equal weight basis and the reported V values approximate the rates of reaction in mammary tissue, then, at least as a first approximation,

TABLE VI
COMPARISON OF APPARENT MICHAELIS CONSTANTS
FOR NADP⁺:ISOCITRATE DEHYDROGENASE FROM
DIFFERENT TISSUES GIVEN AS
 μM CONCENTRATIONS

Substrate	Mammary gland	Bovine liver	Pig heart
DL-isocitrate	8.0	—	5.7 ^a
Mn ²⁺	6.4	—	0.3 ^b
NADP ⁺	10.8	2.8 ^c	4.6 ^a
Mn-D ₅ -isocitrate	3.0	0.7 ^c	0.02 ^b

^a Ref. (3).

^b Values calculated for comparison from graphs (16) at concentration of substrates used in this study.

^c Ref. (6).

the NADPH required for *de novo* fat synthesis could easily be supplied by isocitrate dehydrogenase. This supports the hypothesis of Bauman *et al.* (1) that the latter enzyme could be the primary source of NADPH for fat synthesis. However, confirmation of this will await determination of the precise molecular weights and of the mammary concentration of the enzymes in question.

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