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HEART MITOCHONDRIAL MALIC ENZYMES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By

William Steven Fillers, B.S.

*****

The Ohio State University
1982

Reading Committee:
Dr. J. B. Allred
Dr. K. L. Roehrig
Dr. E. C. Naber

Approved By

Adviser
Department of Food Science and Nutrition

John B. Allred
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VITA

April 1, 1953....... Born - Long Island, New York

1977............... B.S. Agriculture, The Ohio State University, Columbus, Ohio

1977-1980........... Teaching Assistant, Animal Nutrition, The Ohio State University, Columbus, Ohio

1980-1982........... Research Assistant, Food Science and Nutrition, The Ohio State University, Columbus, Ohio

PUBLICATIONS


FIELDS OF STUDY

Major Field: Nutritional Biochemistry

Studies in Enzymology. Dr. K. L. Roehrig
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INTRODUCTION

Malic enzymes oxidatively decarboxylate L-malic acid forming pyruvic acid. The reaction is linked to pyridine nucleotides and requires divalent metal ions. Nomenclature for the isozymes is related to the pyridine specificity and to the ability to catalyze additional reactions. The nicotinamide adenine dinucleotide phosphate (NADP) linked malic enzymes are classified according to the recommendations of the nomenclature committee of the International Union of Biochemistry, Enzyme Nomenclature 1978, as EC 1.1.1.40 whereas EC 1.1.1.38 and EC 1.1.1.39 are linked to nicotinamide adenine dinucleotide (NAD). A general reaction equation for the malic enzymes is as follows:

\[(1) \text{ malate} + \text{NAD(P)} \rightleftharpoons \text{pyruvate} + \text{NAD(P)H} + \text{CO}_2\]

EC 1.1.1.40 and EC 1.1.1.38 are both capable of catalyzing the general reaction in either direction as well as catalyzing the oxidative decarboxylation of oxaloacetate, (OAA), (Ochoa et al. (1948), Korkes et al. (1950)).
EC 1.1.1.39 does not catalyze the carboxylation of pyruvate at a significant rate under physiological conditions and does not decarboxylate oxaloacetate (Saz and Hubbard (1957)). All of the above reactions require the presence of divalent metal ions.

Malic enzymes from a variety of vertebrates have been isolated and studied as noted by the following reports: Bartholome et al. (1972), Lapis and Harrison (1978), Roehrig and Schulz (1978), pigs; Bernstine (1979), Henderson (1966), mice; Davis and Lin (1971), Lin and Davis (1974), rabbits; Caldes et al. (1979), Frenkel (1971, 1972), Saito and Tomita (1972), Simpson and Estabrook (1969), bovine; Li et al. (1975), Mandella and Sauer (1975), Shrago et al. (1963), Swierczynski (1980), rat; Ochoa et al. (1950), Hsu (1970, 1982), Hsu and Lardy (1967), pigeon; Swierczynski et al. (1975), human. Vertebrate tissues in culture have been utilized as a source of malic enzymes: Goodridge and Adelman (1976), rat liver; McKeehan and McKeehan (1982), human fibroblasts; Sauer and Dauchy (1978), mouse ascites tumors; Sauer et al. (1980), Morris hepatoma. Malic enzymes have also been studied in non-vertebrate animals: Hansford and Johnson (1975), Hoek et al. (1976), Imbuga and Pearson (1982),
Lewis and Price (1956), Norden and Matanganyidze (1977), Pearson et al. (1979), Weeda (1981), Weeda et al. (1980), insects; Fodge et al. (1972), Landsperger et al. (1978), Landsperger and Harris (1976), Saz and Hubbard (1957), helminths. Numerous studies in plants have also been conducted: Dalziel and Londesborough (1968), Harary et al. (1953), Hatch and Mau (1977), Hatch et al. (1974), Hatch et al. (1982), Johnson and Hatch (1970), Kraemer et al. (1951), Macrae (1971), Ochoa (1955), Outlaw and Manchester (1980), Slack and Hatch (1967), Tsai et al. (1971), Vennesland et al. (1949), Wedding et al. (1981). Micro-organism malic enzymes have also been investigated: Blanchard et al. (1950), Korkes et al. (1950), Yamaguchi et al. (1973). The distribution and concentration of isozymes varies with the specie, organ, cell type and cell compartment, Brdiczka and Pette (1971), Hatch and Mau (1977), Saz and Hubbard (1957).

The existence of an enzyme that would decarboxylate malate to form pyruvate was suggested by the early work of Thunberg and Weiland. Evidence for the presence of an NADP-linked enzyme that could catalyze this reaction was reported in pigeon liver extracts by Moulder et al. (1945). The isolation of this decarboxylating malic acid dehydrogenase, Ochoa et al. (1948), and a similar enzyme from plants, Vennesland et al. (1949), were described soon
afterward. The name "malic enzyme" was coined by Ochoa and collaborators in order to differentiate the decarboxylating activity of this oxidoreductase from the oxidoreductase activity of the previously described malic dehydrogenase (malate dehydrogenase, EC 1.1.1.37, MDH), reaction (3).

(3) \text{malate} + \text{NAD} \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow 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directed towards the NADP-linked enzyme isolated from pigeon liver.

The cellular location of the pigeon liver enzyme was determined to be almost exclusively cytosolic in the separate laboratories of Ochoa and Lardy in that no appreciable activity of this enzyme could be demonstrated from supernates of isolated particulate fractions. Hülsmann (1962) discussed the existence of malic enzymes in the mitochondria of beef heart and rat liver. Isolation of malic enzyme from acetone powders of guinea pig heart mitochondria was reported by Gertler (1965). The total activity of NADP-malic enzyme from guinea pig heart mitochondria was only 10% of the whole tissue; however, the specific activity of the mitochondrial form was three fold higher. Starch gel electrophoresis of mouse tissue preparations (Henderson (1966)) indicated distinct isozymes of NADP-malic enzyme with respect to specific tissue and cellular compartments. Simpson and Estabrook (1968, 1969) isolated NADP-linked enzyme in the cytosol and the mitochondria of bovine adrenal cortex and postulated a "malate shuttle" for the transport of reducing equivalents from the cytosol into the mitochondria. A study of the distribution of malic enzymes in seven different tissues of eight different mammals and birds was provided by Brdiczka and Pette (1971). The NADP-malic enzyme activities reported
in this study demonstrated specie to specie, organ to organ, cell to cell, and compartment to compartment variation in the enzyme distribution. Of particular interest was the tendency of lipogenic tissues to contain a major portion of total activity in the cytosolic fraction, noted by all of the above mentioned investigators, whereas heart tissue contained the major activity in the mitochondrial fraction. Additionally, Brdiczka and Pette (1971) provided evidence for a malic enzyme form in the mitochondrial matrix of rat liver. This enzyme was electrophoretically different from the cytosolic enzyme. A review of NADP malic enzymes in animal tissues was published by Frenkel (1975). Three NADP malic enzymes were demonstrated with specific staining of electrophoresed polyacrylamide gels containing extract from pig heart mitochondria by Roehrig and Schulz (1978a). Hafez et al. (1978) reported five species of NADP-linked malic enzyme in crude extracts from rat liver and eight species from rat heart when analyzed by thin layer isoelectric focusing.

In a search for an OAA decarboxylase that could operate at physiological pH and have a low enough Michaelis constant (Km) to be active at physiological oxaloacetate concentrations in heart mitochondria, Davis and Lin (1971) reported an NAD-linked malic enzyme that was not readily reversible and did not catalyze OAA decarboxylation. This
activity was similar to enzyme from *Ascaris* described earlier by Saz and Hubbard (1957). An NADP-malic enzyme was simultaneously isolated, by Davis's group, that was reported to have similar kinetic properties to the cytosolic enzyme. Isolated intact mitochondria incubated in NAD or NADP malic enzyme reaction buffers would not catalyze a reaction until the mitochondria were disrupted. Davis et al. (1972) extended these findings to guinea pig and rabbit heart mitochondria and suggested a role for these enzymes in the maintenance of the levels of citric acid cycle intermediates. The strict specificity of the NAD-linked enzyme for pyridine nucleotide was brought into question by Sauer (1973) and Lin and Davis (1974). The activity of the NAD-linked enzyme could not be separated from a lesser activity with NADP. Therefore the term NAD(P) was generally applied to this enzyme form (EC 1.1.1.39). Two well separated NAD malic enzyme activity peaks were reported by Roehrig and Schulz (1978b) in column fractions isolated from pig heart mitochondria. Unsuccessful attempts to isolate NAD-linked malic enzymes from extramitochondrial compartments have been described by these and other investigators. Recently, Swierczynski and Davis (1981) published data indicating the occurrence of an NAD(P) malic enzyme in the cytosol of rat skeletal muscle. At the present time, however, the release of NAD(P) malic enzyme
from disrupted mitochondria during the isolation procedure can not be ruled out as the source of this enzyme.

The juice preparations of parsley root by Vennesland et al. (1949) contained an NADP-linked malic enzyme that supported OAA decarboxylase activity and required Mg++ or Mn++. Isolation of a similar enzyme from wheat germ was described by Ochoa (1955). Malic enzyme (1.1.1.40) association with the chloroplast fraction of C3 and C4 type plants was reported by Slack and Hatch (1967). Johnson and Hatch (1970) determined that some C4 plants contain as much as 45 fold more NADP malic enzyme than C3 plants. NAD-linked malic enzyme isolation from the mitochondria of cauliflower buds was reported by Macrae (1971). This enzyme was active to a lesser extent with NADP and did not catalyze the decarboxylation of OAA (EC 1.1.1.39). Hatch et al. (1974) differentiated two types of NAD-linked malic enzyme activities in C4 plant species. One type of activity was found to reside in the mitochondria of the bundle sheath cells while the other enzyme was localized in the chloroplast. The chloroplast enzyme had a much higher activity with NADP than with NAD while the mitochondrial form appeared to be more specific for NAD. The occurrence of malic enzymes in plant species exhibiting Crassulacean acid metabolism (CAM) has been reviewed by Osmond (1978). A survey of NAD-malic enzyme activities in
three classes of C4 and in C3 plants was recently published by Hatch et al. (1982).

Insect tissue NADP specific malic enzyme was described in blowfly muscle by Lewis and Price (1956). Mitochondrial NAD-linked malic enzyme was demonstrated by Hansford and Johnson (1975) in beetle flight muscle. Hoek et al. (1976) reported an NAD-linked and two distinct NADP-linked malic enzyme activities from the flight muscles of several insects. One of the NADP activities was coincident with the NAD activity (EC 1.1.1.39, NAD(P)). The non-specificity of a mitochondrial malic enzyme for pyridine nucleotides was confirmed by Norden and Matanganyidze (1977) in the flight muscle of the Tse-tse fly.

The distribution of malic enzymes has been investigated in other species. A mitochondrial location for NAD-linked malic enzyme (EC 1.1.1.39) was described by Saz and Lescure (1969) in Ascaris lumbricoides. In the same tissue Fodge et al. (1972) isolated NAD malic enzyme (EC 1.1.1.39) and indicated that this form was specific for NAD. Evidence for an NADP-linked malic enzyme (EC 1.1.1.40) associated with transhydrogenase activity in the mitochondria of Hymenolepis microstoma (cestoda) was recently discussed by Fiorvanti (1982). NAD malic enzyme (EC 1.1.1.38) was demonstrated in Lactobacillus arabinosis.
by Korkes et al. (1950). Yamaguchi et al. (1973) reviewed NAD and NADP malic enzymes in *E. coli*. The occurrence of two isozymes of NAD-linked malic enzyme (1.1.1.39) in the mitochondrial matrix of oocytes of *X. laevis* was suggested by Petrucci et al. (1977). NAD(P) malic enzymes (EC 1.1.1.39) have been reported in the mitochondria of mouse Ascites tumors (Sauer and Dauchy (1978)), Morris hepatomas (Sauer et al. (1980)) and human fibroblasts (McKeehan and McKeehan (1982)).

The occurrence of malic enzymes seems to be ubiquitous across all cells, however, isozyme characterization with respect to molecular weight, net charge, immunochemical cross reactivity, and substrate and cofactor specificity, indicates wide variation in the distribution of enzyme types. In general, the strictly NAD-linked malic enzymes are reportedly found in microorganisms, NAD(P)-linked malic enzymes are located in mitochondrial fractions while NADP specific malic enzymes are primarily located in the cytosolic fractions and are also present in the mitochondria.

Identification and quantitation of malic enzymes may be hampered by the presence of one or more contaminating enzymes. A problem with the quantitation of malic enzyme was noted because of the co-purification of lactate dehydrogenase (LDH, EC 1.1.1.27), reaction (4):
(4) Pyruvate + NADH $\rightleftharpoons$ lactate + NAD

in acetone powder extractions of pigeon liver (Ochoa et al. (1950)). Although this malic enzyme is NADP-linked, the activity of LDH diminished the concentration of pyruvate and interfered with the determination of the equilibrium constant ($K_{eq}$) for the malic enzyme reaction (Veiga Salles and Ochoa (1950)). Contamination with LDH would be of much greater importance in assays of NAD-linked malic enzymes since the substrates of the LDH reaction are produced by malic enzyme, reactions (1)+(4).

$$\text{malate} + \text{NAD} \rightarrow \text{pyruvate} + \text{NADH} \rightarrow \text{lactate} + \text{NAD}$$

LDH has been reported to be a cytosolic enzyme and has been frequently employed as a marker for this compartment; however, substantial contamination of mitochondrial preparations may occur due to the affinity of LDH for the mitochondrial membrane (Roehrig (1982)). The presence of LDH may lead to the underestimation of malic enzyme rates when determined by the production of pyruvate or NADH. The concentration of MDH, while not a problem for NADP-linked enzymes, could be a serious problem in assays for NAD malic enzyme activity. From reactions (1) and (3), it is clear
that identical substrates are required for these enzymes. Reaction rates monitored by the change in NADH concentration of an assay mixture may overestimate the malic enzyme rate if small amounts of MDH are present. The MDH reaction does not require metal ions so that assays run in the strict absence of metal ions reflect the MDH contribution (Appendix A) to the overall rate. Underestimation of malic enzyme may occur in the presence of high concentrations of MDH. The underestimation may be the result of a combination of effects due to the equilibrium of the MDH reaction. Depending on the concentration of substrates and malic enzyme activity in the reaction mixture, the MDH reaction may drop the malate concentration below the concentration required for maximum malic enzyme rate and/or the NADH produced by malic enzyme may cause a shift to the left (reaction (3)) in the equilibrium for NADH/NAD maintained by MDH such that the net production of NADH via malic enzyme is underestimated. The severity of the underestimation is increased with increased malic enzyme concentration in the reaction. Numerous assay systems described in the literature for the determination of NAD malic enzyme in fractions containing MDH contamination include the addition of exogenous MDH before the malic enzyme rate is measured. Additional NADH production after the MDH reaction rapidly reaches
equilibrium has been attributed to malic enzyme (Appendix A).

The production of OAA by MDH activity presents additional difficulties in determination of NAD malic enzyme. OAA is spontaneously decarboxylated to form pyruvate when divalent metal ions are present (Krebs (1942)). The reaction proceeds at physiological pH and is not pyridine linked. This activity is separate from the enzymatic OAA decarboxylase activity attributed to the malic enzymes (reaction (2)) at low pH by Ochoa et al. (1947) and Korkes et al. (1950). The equilibrium of the MDH reaction is constantly shifted towards the production of NADH as OAA concentration drops due to the spontaneous activity. In this case reaction rates determined by the production of NADH, after MDH equilibrium is established, result in the overestimation of NAD malic enzyme when malic enzyme concentration is low. Malic enzyme rates derived from the production of pyruvate in this assay system are also subject to overestimation. Additionally, high levels of OAA (5mM) were demonstrated to completely inhibit the activity of NAD malic enzyme (Norden and Matanganyidze (1977)).

The complications of contaminating enzymes in assays for plant NAD-linked malic enzymes are discussed by Outlaw and Manchester (1980) and Hatch et al. (1982). These
authors suggest mathematical approaches for the determination of apparent malic enzyme rates in the presence of various enzyme contaminants. Regardless of the accuracy of such formulae, the appropriateness of kinetic analysis on such preparations is questionable. Routine additions of MDH to malic enzyme assay systems not only alter the pool sizes of malate and NAD but, more importantly, increase OAA and NADH concentrations far above physiological levels. The difficulty in accurate assessment of malic enzyme activity in the presence of a combination of varying amounts of contamination by MDH and LDH along with the spontaneous decarboxylation of OAA demonstrate the necessity for extensive purification of malic enzymes. Comparison of data using literature values for malic enzymes must consider relative contamination as well as the methods of analysis.

Alternate methods of analysis of malic enzymes in the presence of contaminating MDH include control assays of initial rates of MDH in the absence of metal ions and isotope exchange. Since MDH activity does not require metal ions, initial reaction rates determined in the absence of Mn++ subtracted from initial rates determined with Mn++ reflects the malic enzyme rate. This system avoids the problems of MDH equilibrium provided the MDH concentration is low enough to generate a linear initial rate (Appendix
A). McKeenan and McKeehan (1982) utilized the exchange between $l^{-12}\text{C}\text{ pyruvate}$ and $l^{-14}\text{C}\text{ alanine}$, previously described by Nisonoff et al. (1954), in order to follow the in situ production of pyruvate via malic enzyme in human lung fibroblast cultures. Reactions run in the presence of $l^{-14}\text{C}\text{ alanine}$ involve the glutamate-pyruvate transaminase (GPT, EC 2.6.1.2) reaction catalyzed isotope exchange to pyruvate and subsequent decarboxylation of the $l^{-14}\text{C}\text{ pyruvate}$ forming $^{14}\text{CO}_2$ and acetate. $l^{-14}\text{C}\text{ alanine}$ was resistant to the decarboxylation reaction; thus, the disappearance of label from the alanine pool was proportional to pyruvate production. This procedure involved the subtraction of background activity that occurred in the absence of Mn++ and did not account for possible competition for pyruvate from pyruvate dehydrogenase (PDH). The presence of high endogenous levels of MDH and LDH in this system was demonstrated by the lack of change in the loss of label when additional MDH and LDH were included in the reactions. The loss of compartmentation of competitive enzymes during the isolation treatment may lead to the underestimation of the physiological activity of malic enzyme and ultimately be less useful than purified preparations.

The high activity of NAD malic enzyme found in insect flight muscle has been related to elevated proline
in these tissues (Hoek et al. (1976), (Handsford and Johnson (1975)). A major functional role for malic enzyme in the proline-alanine pathway in this tissue was suggested by Bursell (1963). The conclusion that proline metabolism is a primary energy source for insect flight muscle was supported by the work of Weeda et al. (1980) and Pearson et al. (1979). Bursell and Slack (1976) postulated that malic enzyme was integral to the replenishment of pyruvate for the transamination with glutamate to form alanine and α-ketoglutarate. In this scheme, proline is initially oxidized to glutamate which is then transaminated to α-ketoglutarate. Alanine accumulates in the cell while α-ketoglutarate enters the tricarboxylic acid cycle (TCA) cycle and is metabolized to pyruvate. This function for malic enzyme agrees with the suggestion of Davis et al. (1972) that the role for malic enzyme in mitochondria is to provide net outward flow of carbon from the pool of TCA intermediates.

In contrast to the emphasis on the production of pyruvate, the function of malic enzymes in plants is closely associated with the production and subsequent assimilation of CO₂. The C₄ plants have been subdivided on the basis of the decarboxylation activities of NAD malic enzyme (EC 1.1.1.39), NADP malic enzyme (EC 1.1.1.40) and PEPCK (EC 2.6.1.1) by Hatch et al. (1974). The data of
Hatch indicate that, in at least some C4 plants, NAD malic enzyme is the primary, if not singular, decarboxylating enzyme of four carbon compounds. The role of these enzymes in the photosynthetic process involves decarboxylation of malate or OAA in the bundle sheath cells to form the CO₂ required for hexose synthesis via the Calvin cycle. The presence of NAD malic enzyme in plants exhibiting CAM (Osmond (1978)) is of particular interest in that it may provide a regulated means of CO₂ release from the free malate formed during the dark cycles in these plants (Wedding et al. (1981)). The bundle sheath location of NAD malic enzyme optimizes the transfer of CO₂ to ribulose 1,5 bisphosphate carboxylase (EC 4.1.1.39, R1,5BPcbx) for the production of 3-phosphoglyceric acid (3 PGA). This association in tropical plants is advantageous during the hot daylight hours when atmospheric CO₂ is limited by stomatal closure. If, as suggested by Lehninger (1982), the rate of hexose formation is primarily limited by the supply of CO₂ to R1,5BPcbx then malic enzyme has a major role in photosynthesis. Thus, the anabolic function of NAD malic enzyme in bundle sheath cells of plants is in opposition to the energy linked functions suggested for animal mitochondria.

Several functions have been postulated for the NAD linked malic enzyme in heart mitochondria. A well supported
hypothesis (Davis et al. (1972), Hiltunen and Davis (1981), Watford et al. (1980)) involves a pathway of net reduction of carbon from the TCA cycle. The control of fluctuations in TCA intermediates pool was postulated by Kornberg (1966) to be brought about by 'anaplerotic' mechanisms. Some of these enzymatic mechanisms have been demonstrated to operate in heart tissue subjected to ischemia and exercise or work (Davis and Bremmer (1973), Von Korff (1972), Williamson (1972, 1979), Garland and Randle (1964), Nuutinen et al. (1981)). The net influx of carbon to the TCA cycle in heart mitochondria may occur by increases of α-ketoglutarate via transamination reactions, (Safer and Williamson (1973)), or succinyl-CoA via the propionate pathway. Increases in the levels of acetyl-CoA do not lead to net increases in TCA carbon due to the loss of two CO₂ units by the level of α-ketoglutarate dehydrogenase. The tricarboxylate transporter was reported to be of little or no activity in rat heart tissue (Cheema-Dhadli et al., (1975)). This would eliminate increases in citrate and isocitrate due to cytosolic sources. The malate-aspartate shuttle operates in heart tissue but, no net loss or gain of carbon units occurs. A net increase in the malate pool has been described as a result of the operation of a purine nucleotide cycle in skeletal muscle (Lowenstein (1972), Goodman and Lowenstein (1977)), however, the presence of
this cycle in heart tissue has not been supported. Increases in the malate concentration due to the activity of phosphoenolpyruvate carboxykinase (EC 4.1.1.32, PEPCK) were demonstrated to be intramitochondrial and of low activity (Nolte et al. (1972), Nagel et al. (1980)) in heart muscle and would not significantly alter the carbon pool. Pyruvate carboxylase (EC 6.4.1.1) has been reported to be absent in heart tissues (Williamson (1979)). The possibilities for net outward flow of carbon flow include OAA decarboxylase (EC 4.1.1.3), PEPCK, and malic enzymes. PEPCK activity, as previously mentioned, is low in heart mitochondria. OAA decarboxylase has been demonstrated to be absent in rat heart mitochondria (Hiltunen and Davis (1981)). Both NAD and NADP malic enzymes are present in heart mitochondria and have been demonstrated to be essentially non reversible under physiological conditions (Davis et al. (1972), Frenkel (1971)). In heart tissue, increases in the net concentration of carbon due to the influx at $\alpha$-ketoglutarate and succinyl-CoA could be depleted by the activity of malic enzymes.

A number of amino acids feed into the TCA cycle at the levels of $\alpha$-ketoglutarate (glutamine, arginine, histidine and proline) and succinyl-CoA (valine, methionine, and isoleucine) causing a net increase in the TCA intermediates pool (Chang and Goldberg (1978), Cooney
et al. (1981)). The mitochondrial malic enzymes provide a possible mechanism by which the increased carbon pool may be reduced during periods of protein catabolism (Lee and Davis (1979, 1980)).

The previous discussion of insect flight muscle indicated that proline oxidation provided a major contribution to the energy requirement for these tissues. Several investigators suggested a correlation between the level of malic enzyme activity and the ability of a cell type to utilize glutamine as a respiratory fuel (Nagel et al. (1980), McKeegan and McKeegan (1982), Nagel and Sauer (1982)). These reports on malic enzyme activity in highly proliferative mammalian tissues strengthen the hypothesis for a role in depleting carbon from the citric acid cycle.

The NADP-linked mitochondrial malic enzyme from adrenal cortex tissue was suggested (Simpson and Estabrook (1969)) to provide a pathway for mitochondrial steroid hydroxylations via mixed function oxidases. These authors also suggested a "malate shuttle" that was postulated to transport NADPH reducing equivalents from the cytosol into the mitochondria. This system involved the reversible nature of the cytosolic NADP malic enzyme in that cytosolic NADPH was utilized in a reaction with cytosolic pyruvate forming malate. The malate thus formed entered the mitochondria and was oxidized to pyruvate which provided
NADPH to a mixed function oxidase.

A number of effectors have been reported for the mitochondrial NAD malic enzymes. Inhibition by ATP and activation by succinate or fumarate was noted (Sauer (1973), Lin and Davis (1974)). Acetylcarnitine and acetyl CoA were demonstrated to be inhibitory to NAD malic enzyme activity (Spydevold et al. (1976)) along with acetate (Petrucci et al. (1977)). The enzyme has been demonstrated to be inhibited by OAA (Norden and Matanganyidze (1977)) and by hydroxymalonate (Hiltunen and Davis (1981)). A series of possible effectors was reported for the enzyme from Ascaris (Landsperger and Harris (1976)).

Adrenal cortex mitochondrial NADP linked malic enzyme was demonstrated to be activated in the presence of 0.3mM calcium (Pfeiffer and Tchen (1975)). The activation of NAD malic enzyme with calcium has not been reported. The effect on heart mitochondrial malic enzymes subjected to the influx of calcium which occurs during periods of ischemia (Mittnacht et al. (1979), Altschuld et al. (1980, 1981), Denton et al. (1978), Hansford and Castro (1981)) might explain the extensive cell damage in terms of depletion of the citric acid cycle intermediates.

The molecular sequence for the additions of substrates to the mitochondrial malic enzymes and the subsequent release of products have not been elucidated for
the vertebrates. Some preliminary investigations have been published for the NAD malic enzyme from *Ascaris* (Landsperger *et al.* (1978)). The sequence of events may closely resemble those described for the NADP-linked enzyme from pigeon liver as suggested by the separate laboratories of R. Y. Hsu (1967, 1976, 1982) and M. I. Schimerlik (1977).
Materials and Methods

Rabbit hearts were obtained from Pel-Freez Biologicals, Rogers, Arkansas (Code 41218-2 or 41299-0). These hearts were removed from 8-12 week old, 95% albino (New Zealand and Californian), healthy, fasted male and female rabbits. The hearts were individually frozen on dry ice within 30 minutes of death and were either stored frozen at -20 degrees Celsius (C) until shipment or were immediately shipped on dry ice via air freight. Pig heart tissue was procured from the Meat Lab, Dept. Animal Science, The Ohio State University as soon as possible after slaughter and was kept on ice or frozen until use. All reagents not specifically identified were obtained from Sigma Chemical Co. (St. Louis, MO.). The fatty acid synthetase was a generous gift from Dr. P. Cohen, Medical Sciences Institute, University of Dundee, Dundee, Scotland.

Variation in the purity and physical properties of malic enzymes reported in the literature may be due to the source of the enzyme, Brdiczka and Pette (1971), and to the specific isolation procedures employed by the investigator. Malic enzymes from rabbit heart mitochondria were isolated
by the methods of Lin and Davis (1974), Roehrig and Schulz (1978a), Sauer et al. (1980), and Imbuga and Pearson (1982) in order to compare and contrast the procedures with a single enzyme source. The NAD linked malic enzyme from the rabbit heart mitochondria is associated with a less active NADP activity, Lin and Davis (1974). This association has been noted for other tissues and, despite attempts to do so, has not been clearly differentiated into separate NAD and NADP enzymes, Sauer (1973, 1975), Petrucci et al. (1977), Nagel et al. (1980), Nagel and Sauer (1982). Roehrig and Schulz (1978a) reported effective separation of NAD malic enzymes from NADP malic enzyme from pig heart mitochondria. Additionally, these and other investigators report strictly NADP linked malic enzymes from mitochondria, Frenkel (1975), Swierczynski et al. (1980). Therefore, in order to attempt to isolate strictly NAD and NADP malic enzyme, activities were studied in all preparations.

Mitochondria were isolated from the heart tissues by differential centrifugation in 0.25M sucrose, 10mM disodium-dihydro-ethylenediamine tetra acetic acid (EDTA, G. Fredrick Smith Chemical Co., Col., OH), pH 7.4 (isolation buffer). The heart tissues were first thawed on ice, trimmed of excess vessels and fat, sliced into pieces approximately 1 x 1 centimeters (cm), and homogenized in 9
volumes of cold isolation buffer in a Sorvall Omni Mixer, model 17105, (set 7.5) or a Waring blender, model PB 5A, (high) for 30 sec. at 4C. The pH of the homogenate was readjusted to 7.4 as rapidly as possible with 30% potassium hydroxide (KOH). The homogenate was centrifuged (500xg) in an IEC, model B-20A, (870 head) or a Servall, model 2, (SS-34 head) for 5 minutes. The supernatant was filtered through 4 layers of boiled cheesecloth and recentrifuged (10,000xg) for 10 minutes. The resulting mitochondrial pellet was gently resuspended in isolation buffer by hand in a loose fitting Potter Elvejhem homogenizer and recentrifuged (8500xg) for 10 minutes.

The isolated mitochondria were resuspended in a variety of breaking buffers, described below, prior to sonic disruption or a combination of freeze-thaw and sonic disruption. While the temperature was held at 0 - 4C, a Branson-Sonifier, model 185, (microtip, set 6) was activated for alternate 15sec. intervals for 2min. in order to liberate mitochondrial constituents. Additionally, in some experiments, the sonified mitochondria were placed in a large glass beaker, frozen solid, and then slowly thawed on ice.

The breaking buffer of Sauer (1980) was utilized in several experiments designed to isolate malic enzymes. Buffer "A" contained 50mM potassium chloride (KCl), 5mM
N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 0.5mM DL-dithiothreitol (DTT), and 0.1mM EDTA adjusted to pH 7.3 with concentrated hydrogen chloride (HCl). Some experiments also included soybean trypsin inhibitor (5mg/100ml) in this and subsequent buffers. Disrupted mitochondria were centrifuged (105,000xg) in a Beckman Spinco, model L, (type 40 head) for 60 minutes. The supernatant was either treated with enzyme grade solid ammonium sulfate (ICN Pharmaceuticals, Inc., Cleveland, OH.) to saturations between 35% and 70% or was placed, after concentration via an Amicon B-15 Minicon concentrator (molecular weight cut off 15,000, MW CO 15,000), on Pharmacia Sephrose 6B molecular sieve columns pre-equilibrated with the above buffer. Preparations which used ammonium sulfate were gently stirred on ice while the ammonium sulfate was slowly added. The pH was maintained, when necessary, by small additions of Tris(hydroxymethyl)-aminomethane (Trizma base, Tris). Slow stirring on ice was maintained for 20min. at which time the precipitated fraction was isolated by centrifugation (15,000xg, 15min.). Ammonium sulfate pellets were resuspended in a minimum volume of buffer "A" and dialyzed in Spectra/Por 2 (MW CO 12-14,000) cellulose tubing against at least 250 volumes of the same buffer or against 0.05M Tris-Cl, 10mM B-mercaptoethanol, pH 7.4 (buffer "B") for 12
hours at 4C. Sepharose 6B fractions were collected on a drop count basis into glass test tubes using Isco fraction collectors, models 1200 PUP, 2111, or 1850. Protein content of the eluant was qualitatively evaluated with an Isco, Model UA-5, Absorbance/Fluorescence Monitor (type 6 optical unit, 280nm filter). All fractions were collected and stored at 4C.

Batch extraction of calcium phosphate (Ca-P) gels, (Colowick (1955)), with buffer "B" containing KCl in increasing concentrations from 0 to 0.4M, (Roehrig and Schulz (1978a)), was employed to isolate malic enzyme from mitochondria disrupted in buffer "B" or 105,000xg supernatant dialyzed, as previously described, against buffer "B". The 105,000xg supernatant was saturated to 70% with solid ammonium sulfate and centrifuged as previously described. The resulting pellet was resuspended in 5ml of buffer "B" and then combined with a volume of Ca-P gel corresponding to 2mg gel/g heart tissue represented by the pellet. Sequential centrifugation (5,000xg, 5min.), decanting the supernatant, and resuspension of the Ca-P gel in 5ml aliquots of buffer "B" containing 0, 0.05, 0.1, 0.2, and 0.4M KCl, quantitatively eluted malic enzymes from the Ca-P gel. The two fractions containing no KCl were combined and separately stored along with each successive supernatant at 4C until assay or concentration via
ultrafiltration in an Amicon B-15 or PM-10 apparatus.

Malic enzyme isolated for kinetic analysis was liberated from mitochondria disrupted in 0.01M Tris-Cl, 1mM EDTA, (buffer "C"), according to the methods of Imbuga and Pearson (1982) and Hoek (1977). Buffer "C" contained 10mM B-mercaptoethanol in some experiments as noted in Figures and Tables. Disruption of the mitochondria was carried out by sonication as previously described or by three freeze-thaw cycles. Precipitation with ammonium sulfate, as described above, in saturations between 40-50%, 35-55%, and 35-70% followed by overnight dialysis against buffer "C" as previously described, were preparative steps prior to anion exchange chromatography.

Ion exchange chromatography with Whatman DE-52 (diethylaminoethyl, DEAE) or CM-52 (carboxymethyl ,CMC) preswollen, microgranular cellulose was utilized to purify malic enzymes isolated, dialyzed, and concentrated by the above mentioned techniques. Proteins applied to the pre-equilibrated columns were eluted with batch and/or linear gradients beginning with the concentration of the breaking buffer. Anion exchange chromatography (DE-52) on Amicon (2.2x45cm), Pharmacia (0.9x30, 1.6x40, 1.5x90cm) or on (0.84x115cm) custom columns eluted with either Tris-Cl in concentrations from 0.01 to 0.8M or KCl in concentrations from 0 to 0.4M separated NAD(P) and NADP
malic, MDH and LDH enzymes with varying degrees of purity. Enzymes isolated with buffers "A" or "B", that were contained in the peak activity fractions from Sepharose 6B chromatography, were pooled, concentrated, and, in all experiments except one, dialyzed against buffer "B". The dialyzed solutions were applied to the DEAE columns and eluted with linear gradients from 0.05 to 0.8M Tris-Cl containing 10mM B-mercaptoethanol at pH 7.4. The total volumes of the gradients varied with column size but were between 100mls for the 0.9x30cm columns and 400mls for the 2.2x45cm columns. The excepted case mentioned above was initially dialyzed against 0.02M Tris-Cl, 10mM B-mercaptoethanol, pH 7.4; applied to a 0.84x115cm DEAE column, batch eluted with 115mls of the 0.02M buffer and then eluted with a 0.02 to 0.5M Tris-Cl gradient (400mls). Enzymes isolated with buffer "C" were applied to 0.9x30cm DEAE columns pre-equilibrated with buffer "C" at pH 8. Several experiments followed the methods of Imbuga and Pearson (1982) with an additional 0.5M Tris-Cl, 10mM B-mercaptoethanol, pH 7.4 batch elution. Other columns were batch eluted with buffer "C", pH 8 until a protein peak containing the majority of MDH and LDH activity had returned to the base line of the 280nm protein monitor (150-200mls). These columns were then eluted with 0 to 0.4M KCl (in buffer "C") gradients (250 mls) or 0.01 to 0.5M
Tris-Cl (buffer "B") gradients (100 mls). Cation exchange chromatography (CM-52) of pooled activity peak fractions from DEAE (buffer "B") separations which had been concentrated and dialyzed, as previously described, was employed to further purify malic enzymes. The NAD(P) and NADP peak fractions were pooled separately, concentrated and dialyzed against buffer "B" or 0.025M Tris-Cl, 10mM B-mercaptoethanol, 0.05% Triton-X 100, pH 7.4 before application to appropriately equilibrated (0.9x30cm) CMC columns. Columns equilibrated with buffer "B" were eluted with 0.05 to 0.8M Tris-Cl linear gradients whereas those equilibrated with the Triton-X 100 buffer were batch eluted with the equilibration buffer.

Peak activity fractions from DEAE columns eluted with 0 to 0.4M KCl (buffer "C") were pooled, concentrated, and dialyzed with a -p Micro-ProDiCon (Bio-Molecular Dynamics, Beaverton, OR.), negative pressure micro protein dialysis concentrator (MW CO 15,000), against buffer "B" without EDTA. These preparations were applied to Sepharose 6B columns (1.3x85cm) and eluted with 100mls of the "B"-EDTA buffer.

Affinity gel chromatography with an Amicon Dyematrix screen for protein purification of the NAD(P) peak from DEAE fractions (buffer "B", linear gradients) was utilized in an attempt to separate the NAD and NADP
activities of this peak. The dye-ligands included were Blue A, Red A, Orange A, Green A, and Blue B. The columns, including a control gel containing no ligand, were regenerated with 8M urea, equilibrated with 20mM Tris-Cl, pH 7.5 and loaded with pooled and dialyzed (Minicon B-15) enzyme. Each column was eluted with the equilibration buffer and then with the equilibration buffer containing 1.5M KCl. The collected fractions were assayed for NAD and NADP activity and protein concentration as described below.

Qualitative protein elution from all columns was monitored at 280nm as previously described. Quantitative protein content of all samples investigated was determined by the method of Bradford (1976).

Enzyme activities were determined with an Eppendorf spectrofluorimeter (313-366nm primary, 380-3000nm secondary filters) in 1ml quartz cuvettes held at 30C with a Haake, type FE water bath. Numerical data were obtained from linear portions of plots generated by a Leeds and Northrup, Speedomax H, chart recorder (Appendix A). NAD malic enzyme activity was evaluated in preliminary preparations using the following assay buffer (final concentrations): 150mM Tris-Cl, 30mM L-malate, 1mM NAD, 8mM MnCl₂, 3mM B-mercaptoethanol, 10mM oxamate, pH 7.4. Malate dehydrogenase (6.5 units) was added, and the reaction was allowed to reach equilibrium (Figure 16, rate (a)). Rates
resulting from the spontaneous decarboxylation of OAA generated by the MDH equilibrium were subtracted from the malic enzyme rate by means of a control reaction containing MDH only (Figure 16, rate (b)). After achieving a linear rate for several minutes, 40U of LDH was added to the reaction (Figures 15 and 17) to overcome oxamate inhibition of any endogenous LDH (Hakala et al. (1953)) to demonstrate that the product of the reaction was pyruvate, as suggested by Sauer and Dauchy (1978). The NADP linked enzyme was assayed in a similar buffer except that 1mM NADP replaced the NAD and that MDH, LDH, and oxamate were not included. MDH contamination in these fractions was estimated by the difference in initial rate (Figure 16) and the corrected NAD-malic rate (rate a-rate b, Figure 16) or by the reaction rate without MnCl₂ (rate d, Figure 16). Specific assay for MDH contamination was by the method of Ochoa (1955). Contamination due to LDH was assayed according to Singh and Kanungo (1968). Assay for NADH dehydrogenase contained 150mM Tris-Cl, 3mM B-mercaptoethanol, pH 7.4, plus 0.05mM NADH only. Fractions that were free of MDH, LDH, and NADH dehydrogenase contamination were subjected to kinetic analysis with a reaction buffer similar to the NADP assay system.

Slab and disc polyacrylamide gel electrophoresis (PAGE) was utilized to measure the relative purity and the
molecular weights of malic enzymes isolated by the above mentioned procedures. Differing gel concentrations, running buffer pH, denaturing conditions and staining techniques were employed to identify the enzymes. Except for Tris, all reagents required to polymerize and run the gels were obtained from Bio-Rad Laboratories (Richmond, CA.).

Disc gels of 7.5 and 12.5 cm tube lengths were prepared by the method of Davis (1964) at 7.5% acrylamide/bisacrylamide with a 2.5% stacking gel. A Tris-glycine (pH 8.3) running buffer and 0.005% bromophenol blue tracking dye were used. The samples to be examined were saturated with sucrose before application to the gels. A constant current of 1 mA/tube was maintained with a Bio-Rad power supply, model 500, until the tracking dye had passed through the stacking gel at which time the current was increased to 2 mA/tube. Temperature was maintained by circulating ice water through the Buchler electrophoresis unit with a Brinkman, model IC-2, water pump. Gels were reamed and rinsed in deionized water (0-4C) and stained for protein with 0.25% Comassie Brilliant Blue R-250 or for specific enzymes with 150mM Tris-Cl, 30mM L-malate, 1mM NAD or NADP, 0.028% nitro blue tetrazolium (NBT), 0.014% phenazine methosulfate (PMS), +/- 8 mM MnCl₂, pH 7.4. A Bio-Rad, model 172A, diffusion destainer filled with 7% acetic acid in water was used to destain the gels. Slab
PAGE at gel concentrations of 5% and 12.5% were prepared with and without sodium dodecyl sulfate (SDS) according to the methods of Ryrie and Gallagher (1979) in a Markson, model S-12531 (200 mm) dual slab unit with 1.5x18x6mm or 1.5x18x4mm wells. Running, staining and destaining of the slab gels was similar to the procedures described above except that SDS gels were run at room temperature (20°C). The slab gels were dried on filter paper with a Bio-Rad, model 224, gel slab dryer.
RESULTS AND DISCUSSION

Preliminary isolations of malic enzymes from pig heart mitochondria utilizing calcium-phosphate gels eluted with KCl buffers (buffer "B") (Roehrig and Schulz (1978b)) separated several malic enzyme fractions as illustrated in Fig. 1 (graph (A)). Initially these fractions were assayed in the absence of oxamate according to the method outlined in Appendix A for fractions containing high contamination of MDH (Fig. 15). The distribution of NAD malic enzyme activity consistently dropped with each successive elution, however the total activity in each fraction could not be increased with multiple elutions of the same KCl buffer concentration. In agreement with the data of Roehrig and Schulz, no activity of the NAD malic enzyme was found in the 0 KCl fraction in the absence of oxamate and the 0 KCl fraction did contain all of the isolated activity of the NADP malic enzyme (not shown). The effect of LDH contamination and inhibition of LDH with oxamate in fractions assayed for malic enzymes has been discussed (see Methods and Literature Review). Addition of 10mM oxamate to the assay buffer for NAD malic enzyme activity in the KCl
Elution of pig and rabbit heart mitochondrial NAD malic enzyme from calcium phosphate gels. Elution of pig (A) and rabbit (B) heart mitochondrial NAD malic enzyme activity from calcium phosphate gels with batch washes of buffer containing KCl. The clear bar areas represent the percent of total activity found in each fraction when assayed in the absence of oxamate. The hatched bar areas represent the percent of total activity when assayed in the presence of 10mM oxamate. Total activities were 30 nanomoles/min/50g for pig heart and 180 nanomoles/min/50g for rabbit heart.
demonstrated the presence of both LDH and a large proportion of NAD malic enzyme in the 0 KCl fractions (Fig. 1). Fig. 16 demonstrates the effect of oxamate added to the assay of a 0 KCl fraction. A similar distribution pattern of NAD malic enzyme activity from rabbit heart mitochondria was observed (Fig. 1, graph B) despite a six fold difference in yield per gram of heart. The percent of unbound enzyme (0 KCl) was slightly greater in the rabbit than in the pig.

The NAD malic enzyme activities of the 0.05-0.4M KCl fractions from pig and rabbit hearts remained fairly stable for several weeks after isolation except for the 0.05M KCl fraction from rabbit heart which seemed to increase in activity. In previous studies from this laboratory, rabbit heart mitochondrial NAD malic enzyme activities were assayed immediately after elution from the calcium-phosphate gel and again after 12 days storage at 4C (Fig. 2). The initial activity of the 0.05M KCl fraction was very low, but increased to a level similar to that of Fig. 1 after storage. In order to investigate the possible involvement of proteolysis in the activation of this fraction, freshly prepared 0.05M KCl fractions were incubated with or without trypsin inhibitor (Table 1). Fractions assayed immediately after isolation and again after 48 hours of storage at 4C indicated that the
Fig. 2

Storage effects on the activity of NAD malic enzyme from rabbit heart eluted from calcium phosphate gels. Storage effects on the activity of NAD malic enzyme from rabbit hearts isolated with KCl elution of calcium phosphate gels. Open bar areas represent initial activities. Hatched bar areas represent activities after 12 days storage at 4C compared to the initial total activity.
Table 1

EFFECT OF TRYPsin INHIBITOR ON THE ACTIVATION OF THE 0.05M KCL FRACTION OF NAD MALIC ENZYME FROM RABBIT HEART

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>% of control</th>
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<tr>
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<tr>
<td>0</td>
<td>100</td>
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<tr>
<td>48</td>
<td>130 ± 3</td>
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</table>

Activity was assayed as soon as the fraction was isolated and again after 48 hours at 4°C. Data are expressed as % of zero time value in the absence of trypsin inhibitor. Values are the means of triplicate determinations of the 0.05M KCl fraction derived from approximately 60 rabbit hearts ± S.E.M. Trypsin inhibitor was present at 1.25 ug/ml.
proteolytic inhibitor prevented activation of this fraction. In these studies, the competitive inhibition by ATP and allosteric activation by succinate of NAD malic enzyme (Sauer (1973b)) could be demonstrated with fresh preparations or preparations stored in the presence of trypsin inhibitor but not with fractions stored without proteolytic inhibitors.

Disc PAGE of the KCl fractions from rabbit heart failed to differentiate these fractions. If limited proteolysis during storage and isolation created these fractions, then the change in molecular weight was very small.

The differences in the net charge of the enzymes in each of the KCl fractions was demonstrated with the activity patterns from DEAE cellulose columns eluted with linear Tris-Cl gradients (Fig. 3). Four distinctly different activity peaks were collected and, apparently, the 0.4M KCl buffer eluted a combination of the previously eluted NAD malic enzymes from the calcium-phosphate gel. The relatively high activity in the 0 KCl fraction was again evident when assayed in the presence of 10mM oxamate. Additionally, the majority of the MDH and LDH contamination in these fractions eluted with the void volumes. The elution characteristics of NAD malic enzyme activities from calcium-phosphate gels and DEAE cellulose columns was
Elution of rabbit heart NAD malic enzyme KCl fractions from DEAE cellulose columns (1x24cm) with linear Tris-Cl gradients.
indicative of enzymes with different net charges. The *in vivo* and/or *in vitro* genesis of these charge differences was not known, however the function of true isoenzymes in four different polyisozymic complexes within the mitochondria would be a matter of great speculation.

The large quantitity of NAD malic enzyme that did not bind to calcium-phosphate gels and the associated contamination with MDH, LDH, and NADP malic enzyme activities led to the adoption of isolation techniques published by Sauer (1980). The preliminary separation of the 105,000xg mitochondrial supernatant with Sepharose 6B (buffer "A") for pig and rabbit hearts is shown in Fig. 4. The differences in elution volumes may be explained in terms of different column sizes and different flow rates employed in these separate experiments. Graph A depicts NAD malic enzyme activity from 100g of pig heart that was contained in fractions with 100x more activity due to MDH. Total activities were about five times greater for the NADP-linked enzyme than for the NAD-linked malic enzyme. The average molecular size of the NAD malic enzyme was smaller than the NADP malic enzyme and about the same size as MDH. In contrast, the elution of these enzymes from 50g of rabbit heart (graph B) differed significantly in total yields, relative molecular weights, and activity ratios. Total recoveries when expressed as nanomoles/min/gram of
Fig. 4

Elution of pig and rabbit heart malic enzymes and MDH in 105,000xg supernatants from Sepharose 6B columns.

Elution of concentrated 105,000xg pig (A) and rabbit (B) heart mitochondrial supernatants from Sepharose 6B as described in the text. 3.5ml pig supernatant was applied to a 2.5x45cm column and eluted at 170ml/hr. 2.2ml rabbit supernatant was applied to a 2.2x40cm column and eluted at 24ml/hr. 5.5ml fractions were collected and assayed in the presence of 10mM oxamate. NAD (———) and NADP (· · · ·) malic enzymes and malate dehydrogenase (- - - -) activities are shown.
heart were 56, 0.5, and 3.7 for MDH, NAD malic enzyme, and NADP malic enzyme, respectively for pig versus 96, 4.4, and 1.2 for rabbit. Total NAD malic enzyme activity recoveries were 8x greater in rabbit heart than in pig heart while NADP malic enzyme activity was 1/3 less. The elution order of the rabbit heart enzyme activity peaks elution was NAD malic enzyme, NADP malic enzyme, followed by MDH. This indicated that the molecular weight of the NAD enzyme was greater than NADP malic enzyme and MDH. This order of elution from Sepharose 6B was in agreement with that reported by Sauer (1973b) for the enzymes from calf adrenal cortex mitochondria. Of special interest was the multiple peaks for NAD malic enzyme activities from both pig and rabbit sources suggesting the occurrence of isozymes. MDH and LDH contamination was present in all fractions containing malic enzyme activity. The yield of NAD malic enzyme activity from rabbit heart with this separation procedure was 20% greater than the yields represented by the calcium-phosphate isolations (Fig. 1).

Fractions containing malic enzyme activity from the Sepharose 6B experiment for rabbit and pig hearts were separately pooled, concentrated, applied to DEAE cellulose columns, and eluted with linear gradients of Tris-Cl (Fig. 5). The elution pattern of NAD malic enzyme activity from pig heart (graph A) suggests multiple forms of enzyme as
Fig. 5

Elution of pig and rabbit heart malic enzymes in pooled Sepharose fractions from DEAE cellulose columns with linear gradients. Elution of pooled and concentrated (3.0ml) Sepharose 6B fractions from DEAE cellulose using linear Tris-C1 gradients as described in the text. Pig heart (A) fractions (5.5ml) were collected from a 2.2x40cm column at 30ml/hr. Rabbit heart (B) fractions (5.5ml) were collected from a 1.4x37cm column at 30ml/hr. The fractions were assayed in the presence of 10mM oxamate. NAD (●---●) and NADP (O---O) malic enzyme activities are shown.
compared to a single form for rabbit heart (graph B). The NAD malic enzyme activity from pig hearts that eluted independently of any NADP activity supports the data in Fig. 1 and those of Roehrig and Schulz (1978b) in that NAD malic enzyme occurred in several forms and could be isolated separate from NADP activity. Similarities in the order of elution between the pig heart enzymes isolated in the 0 KCl fractions in Fig. 1 and those contained in the first 30 fractions of Fig. 5 (graph A) were observed. Two distinct peaks of NADP malic enzyme activity were noted for rabbit heart. The first of which (henceforth referred to as peak #1) eluted prior to the NAD malic enzyme and the second (henceforth referred to as peak #2) eluted coincident with the NAD malic enzyme activity. Coincident activities were also noted for the pig heart enzymes, however, the irregular shape of the NADP peak suggested the possibility of more than one enzyme form, and the coincidence of the NAD peak with the NADP peak may be artifactual due to close association with the trailing edge of the void volume. Substantial contamination by MDH may have influenced the assay of NAD malic enzyme in these fractions.

That the activities of NAD and NADP malic enzyme might have represented dual pyridine specificity within the same molecule was of interest. As noted in the literature review and the methods sections, other investigators had
reported NADP activity associated with the NAD malic enzyme obtained from several sources. Lin and Davis (1974) described attempts to separate or alter the activity ratios of a DEAE fraction similar to peak #2 with respect to NAD and NADP malic enzyme activities from rabbit heart mitochondria. If proteolysis contributed to the variation in isolatable malic enzyme forms, then addition of trypsin inhibitor to all stages of isolation might alter the activity ratios and provide evidence that the coincident activities in peak #2 were not attributable to the same catalytic site.

Simultaneous isolations of malic enzymes with and without added trypsin inhibitor (5mg/100ml of buffer) according to the method outlined above for separations with Sepharose 6B and DEAE column chromatography were carried out on 2x50 grams, newly received (Table 2, 11-04-81), rabbit hearts. The elution from identical DEAE cellulose columns (Fig. 6) demonstrated several effects of trypsin inhibitor. The ratio of peak #1 to peak #2 and the ratio of the NAD malic enzyme activity to peak #2 were significantly different between the + and - trypsin inhibitor (TI) treatments. The detection of three NADP malic enzyme activity peaks was demonstrated in the + TI fractions (graph a). Additionally, more total enzyme activity could be isolated in the presence of the proteolytic inhibitor.
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Fig. 6

Elution of rabbit heart malic enzymes and MDH in pooled Sepharose fractions from DEAE cellulose columns with linear gradients ± trypsin inhibitor. Elution of pooled and concentrated (2.0ml) rabbit heart Sepharose 6B fractions from DEAE cellulose using linear Tris-Cl gradients + or - trypsin inhibitor as described in the text. The 2.2x38cm columns had flow rates of 40ml/hr and 5.5ml fractions were collected. The fractions were assayed in the presence of 10mM oxamate. NAD (-----) and NADP (·····) malic enzymes and 10x MDH (-----) activities are shown.
Small losses of protein were observed after concentration in the Minicon apparatus and were thought to be due to nonspecific protein affinity for the membrane. Trypsin inhibitor additions could have lowered the net loss of enzymes during this process by increasing the total protein content of the +TI fractions. Decreases of 78, 54, and 33% for peak #1, peak #2, and NAD malic enzyme activities, respectively were observed. If the average loss of enzyme activities were due to nonspecific binding during concentration, then the difference between the mean loss and the losses of peak #1 and NAD malic enzyme activities was -23% and +22%. This information suggested the possibility that proteolysis might alter the pyridine specificity of the enzyme form in peak #1 to that of the NAD malic enzyme. The differential ratios for peak #2 and NAD malic enzyme supported the contention that these enzyme activities were catalyzed by separate enzymes. The MDH contamination was demonstrated across all fractions containing malic enzymes and occurred as a superimposable peak with the NAD malic enzyme activity.

Pooled and concentrated fractions representing the upper portions of peak #1 and #2 for the + and - TI treatments were subjected to disc PAGE (7.5cm) and were stained for activity and protein as described in the Methods section. Despite five attempts with progressively
increasing sample concentrations (max. = 20ug/50ul), activity stains were not observed in any gel location except an artifact band at the dye fronts. Similar Rf values for protein staining bands were observed in duplicates of three separate gel runs (Plate I) for each of the peak fractions. The peak #1 fractions had bands with Rf values of .28, .31, .39 (heavy), and .92 for the +TI treatment and .28 and .38 for the -TI treatment. Peak #2 fractions had bands with Rf values of .45, .50, and .77 (heavy) for the +TI treatment and .41, .49, and .75 (heavy) for the -TI treatment. Roehrig and Schulz (1978b) demonstrated NADP linked malic enzyme activities from pig heart mitochondria on 7.5% disc gels with Rf values of .25, .29, and .36-.39 when stained for activity. Although the identification of the protein bands was a matter of speculation, the heavy band in the peak #1 +TI gel was most likely MDH due to the high activity in the collected fractions. The similarity between the appearance of the protein bands between the + and -TI treatments indicates that any molecular weight changes due to proteolysis, that could be influenced by TI, were small.

Numerous attempts to replicate the DEAE data from the trypsin inhibitor experiment discussed above were similar in most respects with the notable exception of the inability to demonstrate the relative decrease of peak #1
Plate I

Protein staining of 7.5% disc PAGE (7.5cm) of concentrated peak fractions from DEAE cellulose separations + and - trypsin inhibitor. The peaks represented (left to right) are: peak #1 +TI, peak #2 +TI, peak #1 -TI, and peak #2 -TI as described in the text.
-TI concomitant with a relative increase in the NAD malic enzyme activity (Table 3). The variability in the activity yields and in the peak ratios of these experiments and the fact that several of the orders of rabbit hearts received over this time had been assigned the same lot number gave rise to questions concerning the relationship of storage time to possible proteolysis effects. Upon investigation, the assurance of immediate shipment of freshly isolated tissue offered by a company representative to this laboratory were unfounded (Table 2). Special arrangements with the company were instituted (as of 05-10-82) to insure the receipt of the freshest tissues possible. Data obtained from lot# 2863 (killed 10-14-81) that were received and analyzed at least three different times demonstrated no obvious trend of loss or gain of activities (Tables 2, 3, and 4). Isolation of enzyme activities from fresh hearts (5-13-82) had almost a 4x increase in all three peaks above those previously obtained (Table 4).

Subsequent simultaneous isolations + and - TI (1-07-82 and 5-13-82) indicated that the loss of activity in the -TI treatment was distributed equally across all of the peak fractions. Losses of 44, 36, and 35% (1-07-82) and 12, 13, and 11% (5-13-82) for peak #1, peak #2, and NAD malic enzyme activities, respectively were recorded. That proteolysis shifted NADP malic enzyme activity in peak #1
Table 3

PEAK AREA RATIOS OF NADP AND NAD MALIC ENZYMES FROM DEAE CHROMATOGRAPHY WITH LINEAR GRADIENTS ± TRYPsin INHIBITOR

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Mean ± S.E.M. | +   | 2.05 ± 0.24 | 3.15 ± 0.27 |
              | -   | 1.26 ± 0.27 | 3.54 ± 0.30 |
Table 4

STORAGE TIME AND MALIC ENZYME ACTIVITY FROM DEAE
CHROMATOGRAPHY WITH LINEAR GRADIENTS ± TRYSIN INHIBITOR

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to NAD malic enzyme coincident with peak #2 was not supported by the mean values (Table 3) for the NAD/peak #2 ratios. The parallel decreases in activity of the -TI treatment and the unaltered mean values for the NAD/peak #2 ratios did not support the contention that these were separate enzymes. Sauer (1973b) reported NAD activity at 3/1 to 2/1 the rate of a coincident NADP malic enzyme from rat adrenal cortex mitochondria while Lin and Davis (1974) reported the ratio in similar rabbit heart fractions to be 5/1 to 4/1. The consistent increases in the activities across all enzyme peaks due to the addition of TI in these experiments was in opposition to the indication that trypsin inhibitor tended to increase the peak #1 to peak #2 ratio.

The previously mentioned MDH activity superimposable on the NAD malic enzyme peaks from DEAE separations was a constant feature in all of the these isolations. The possibility that the MDH peak might be an artifact of the activity calculation (Appendix A) was investigated. The fractions corresponding to the NAD malic enzyme activity in one experiment were assayed in the direction of malate formation in the absence of Mn++ (Ochoa (1955), and a coincident MDH activity was present and was approximately 30x more active.
The use of longer column lengths for the Sepharose and DEAE separations, smaller fraction volumes, and shallower gradients did not alter the coincidence of the NAD malic enzyme activity peak with those of MDH and NADP malic enzyme. Two attempts to separate these activities with CMC column chromatography resulted in the elution of all three activities in a single coincident peak in ratios similar to those observed for the DEAE fractions (data not shown). Two attempts to separate these activities with Dyematrex (Amicon) dye ligand affinity chromatography failed to elute detectable levels of NAD and NADP malic enzyme activity. Recoveries of MDH activity were about 60% of the control values and Red A bound the enzyme most strongly while Blue B had the lowest affinity. This DEAE peak fraction was also treated with 0.05% Triton X 100, concentrated against sucrose (12,000 Mkt CO tubing), dialyzed against buffer "A" and placed (1.7ml) on a 0.9x30cm Sepharose 6B column. Assay of the eluted (buffer "A") fractions (2ml) demonstrated coincident activities in a single symmetrical peak. The close association of the NAD and NADP malic enzyme and MDH activities eluted from the CMC and Triton-Sepharose columns did not support the contention for separate enzymes.

SDS slab PAGE of pooled and concentrated peak #2 fractions (+0.05% Triton X 100) that were combined with
marker proteins and then eluted from a 0.9x26cm Sepharose 6B column in 2ml collection volumes were run on 12.5 and 5.0% gels. The marker proteins were catalase (subunit MWT=58,000), trypsin inhibitor (MWT=22,500), and B-lactaglobulin (subunit MWT=18,400). The fractions eluted from the Sepharose 6B in a single symmetrical protein peak and were collected in 10 fractions. A portion of each of these fractions was prepared as described in the methods section and placed on a slab gel along with controls containing the marker proteins and fatty acid synthetase (subunit MWT= 250,000). Preliminary gel experiments indicated that the test fractions were overloaded with marker proteins and that the marker proteins contained many bands which obscured the detection of bands representing the peak #2 activity. One band, however, clearly did not correspond to any of the marker protein bands. The Rf of this band was 0.68 and when plotted against the log MWT standard, had an estimated MWT of 31,800. In two replicate 12.5% gel experiments that included peak #2 fractions that did not contain added marker proteins, three additional bands were identified. The average Rf values were 0.28, 0.42, and 0.53 which corresponded to MWts of approximately 123,000, 76,000, and 53,000. Lin and Davis (1974) reported the MWT for the NAD malic enzyme to be between 260,000 and 280,000 and 165,000 and 195,000 for the NADP malic enzyme.
If the mitochondrial malic enzymes are tetramers as suggested by Lapis and Harrison (1978) then the subunit MWts would be near 67,000 and 46,000, respectively. Lin and Davis (1974) did not include proteolytic inhibitors in their isolation buffers so that the MWt values obtained from their preparation might have reflected clipped enzyme forms. All of the test fractions in this experiment were overloaded with trypsin inhibitor due to its addition to the media used during the isolation procedures. This high protein load appeared to impede the migration of all proteins within those wells which could also have accounted for the higher molecular weights observed. The subunit MWt for pig heart mitochondrial MDH as reported by Place and Beynon (1981) was 35,000. Slab gels (5.0%) that were specifically stained for NAD and NADP malic enzymes and MDH did not react for the peak #1 and peak #2 fractions. Since an equal quantity of either fraction would react in the buffer when spotted onto filter paper, the lack of activity on the slab gel was thought to be due to inactivation of the enzymes during the relatively lengthy procedure (8-10hrs).

Disc gels (12.5cm, 7.5%) were run in less than 4 hours on the same fractions with the same activity staining. NADP malic enzyme staining resulted in Rf values (0.19, 0.46-0.49) for peak #1 and (0.25) for peak #2. The
data indicated that peak #1 could contain two separate NADP enzymes and that the observed bands from peak #1 did not correspond to the previously mentioned Rf values reported by Roehrig and Schulz (1978b). However, the Rf=0.25 for peak #2 is very similar to the data from pig heart mitochondria (Roehrig and Schulz (1978b)). NAD specific staining of these gels detected singular bands at Rf=0.29 and Rf=0.24 for the respective activity peaks. The Rf values obtained were the average of duplicate gel determinations. In a separate experiment, similar gels were run and incubated with the NAD activity stain in the absence of MnCl₂. Singular bands (Rf=0.26) developed for the peak #1 + and -TI while an Rf value for peak #2 + and -TI was 0.33. In the presence of MnCl₂, the identical amount of protein developed much darker and broader bands in the same relative locations for the peak #2 fractions while peak #1 fractions were not darker. These results indicated that the NAD malic enzyme and the associated MDH activities had similar molecular weights and net charges and that the molecular weights were minimally effected by TI.

The contamination of the NAD malic enzyme fractions with MDH and the difficulty in demonstrating activity staining led to the adoption of procedures described for the isolation of NAD malic enzymes from insect flight
A preliminary isolation (2g rabbit heart) demonstrated the elution of several protein peaks (Abs. 280) in each of the 0, 0.04, and 0.09 KCl buffer washes of the DEAE column (0.9x25.5cm). Several peaks of MDH activity were present in each of the KCl washes, however, NAD malic enzyme activity was not detected in any of the fractions. Since the ionic strength for the isolation buffer (0.01M Tris-Cl) was lower than those previously employed, a 0.5M Tris, 10mM B-mercaptoethanol wash was added to the procedure. NAD malic enzyme activity eluted almost entirely in one fraction (2ml) with a total yield of approximately 38 nanomoles/min/g heart. Nagel and Sauer (1982) recently reported a very high specific activity of 40.6 micromoles/min/mg protein in a highly purified preparation of canine intestinal mucosa. The value reported herein represented all cellular compartments and the possibility of activation of the enzyme via proteolysis could not be discounted. The MDH contamination in this fraction amounted to less than 3.3% of the NAD malic enzyme activity when assayed + and - MnCl₂ (Appendix A, Fig. 15) and might represent small rates of NAD malic enzyme activity occurring due to residual Mn++ present in the cuvettes. Coincident NADP malic enzyme activity amounted to 26% of the NAD activity. The homogenization of the entire heart tissue in this procedure resulted in contamination by
high levels of LDH (90 nanomoles/min/g heart). The LDH peak was coincident with the NAD malic enzyme activity but could be overcome with oxamate addition (10mM) to the assay buffer. Subsequent preparations included the isolation of the mitochondria and changes in the elution of the DEAE column as noted in the Methods section. Small amounts of contamination were present in the DEAE elution fractions but were eliminated after concentration (Pro-Di-Con) and elution of the pooled activity fractions from a 1.3x85cm Sepharose 6B column. One set of DEAE fractions demonstrated low initial activities in the presence of MnCl₂ and oxamate which reached a lower than normal equilibrium within 3min incubation time. Addition of MDH resulted in the usual rapid shift towards NADH production but, was immediately followed by a rapid rate in the opposite direction. When 0.4ml of the most active fraction for this activity was assayed in a buffer that contained 150mM Tris-Cl, pH 7.4, with 50ul (0.8mg/ml) NADH, a rate of 7.82 nanomoles of NADH oxidation/min/ml of enzyme was observed. All other isolations were screened for this activity and were found to contain minimal activity of this NADH dehydrogenase. The activity was not observed in the Sepharose fractions. The NAD and NADP malic enzyme activities coincided with the peak protein elution (280nm) and did not have activity in the absence of MnCl₂. Oxamate addition to the assay buffer
for the fractions did not increase the reaction rate. NAD activity staining of 7.5% disc PAGE (7.5cm) of these fractions had positive reaction bands with Rf values (duplicates) of 0.34, 0.35 and 0.51, 0.52. When the Rf vs log MWt was plotted with marker proteins; ferritin (Rf=0.11, MWt 440,000), catalase (Rf=0.20, MWt 232,000), and trypsin inhibitor (Rf=0.69, MWt 22,460) the NAD malic enzyme activity bands corresponded to molecular weights of 126,000 and 54,000. Nagel and Sauer (1981) reported nonidentical subunits (62,000 and 53,000 daltons) for the NAD(P) malic enzyme from canine intestinal mucosa. Recently, these investigators (Nagel and Sauer (1982)) reported the formation of a dimer (MWt 141,000) from the same source during electrophoresis at pH 9.0. The activity gels above represented molecular weights that corresponded to active dimer and active subunits for malic enzymes. The fact that no activity staining was ever observed at Rf values equivalent to the tetrameric weight suggested that the isolation procedures or the gels dissociated the enzyme.

The malic enzyme activity isolated as described above was free of contamination that would influence the determination of NADH production by NAD malic enzyme and was utilized for kinetic analysis. A typical Lineweaver-Burk plot of reaction velocity of NADH
production versus the concentration of L-malate is shown in Fig. 7. The $K_m = 2.95 \text{mM}$ was somewhat lower than the $3.2\text{mM}$ value (Lin and Davis (1974)) previously reported for rabbit heart and might reflect the elimination of low levels of MDH contamination present in these fractions. Replications of the isolation method published by Lin and Davis (1974), in this laboratory, resulted in considerable contamination of the DEAE fractions with MDH. The much higher $K_m$ value for malate in the presence of NADP is demonstrated in Fig. 8. The $K_m = 5.73 \text{mM}$ was significantly lower than the $(9.8\text{mM})$ value reported earlier by Lin and Davis (1974). Fig. 9 demonstrates the competitive inhibition of NADH production from malate oxidation by the presence of 10mM oxamate. The $K_m$ for malate was increased 300% in the presence of oxamate while the change in the maximum reaction velocity was approximately 10%. Thus, additions of oxamate to reaction buffers containing 30mM malate had a minimal effect on reaction velocity.

The rate of the reduction of NAD compared to the concentration of NAD is shown for two separate experiments in Fig. 10 and Fig. 11. The average NAD concentration required to attain one half maximal velocity was $0.16\text{mM}$ which was higher than the $(0.10\text{mM})$ value obtained by Lin and Davis (1974). Fig. 12 depicts the $K_m$ for NADP $(0.81\text{mM})$ was lower than the $1.32\text{mM}$ previously reported.
NAD malic enzyme activity vs the concentration of malate. Double reciprocal plot of NAD malic enzyme activity as a function of malate concentration for a typical experiment. The reaction was carried out in the presence of 150 mM Tris-Cl, 8 mM MnCl₂, 1 mM NAD, at pH 7.4, 30°C. Reaction velocity was expressed as nanomoles of NADH produced per min. Malate concentrations were varied from 0.5 to 30.0 mM. For this experiment, the Km for malate was 2.95 mM, and the Vmax = 0.073. The line and its slope was determined using the regression formula built in the Texas Instruments SR-51-II (Appendix B). The correlation coefficient, r, was 0.99.
Fig. 8

NAD malic enzyme activity in the presence of NADP vs the concentration of malate. Double reciprocal plot of NAD malic enzyme activity as a function of malate concentration for a typical experiment. The reaction was carried out in the presence of 150mM Tris-Cl, 8mM MnCl₂, 1mM NADP, at pH 7.4, 30C. Reaction velocity was expressed as nanomoles of NADPH produced per min. Malate concentrations were varied from 0.3 to 30.0mM. For this experiment, the Km for malate was 5.73mM, and the Vmax=0.134. The line and its slope was determined using the regression formula built in the Texas Instruments SR-51-II (Appendix B). The correlation coefficient, \( r \), was 0.99.
NAD malic enzyme activity vs the concentration of malate ± oxamate. Double reciprocal plot of NAD malic enzyme activity as a function of malate concentration and the presence or absence of oxamate (Ox). The reaction was carried out in the presence of 150mM Tris-Cl, 8mM MnCl₂, 1mM NAD, + or - 10mM oxamate, at pH 7.4, 30°C. Reaction velocity was expressed as nanomoles of NADH produced per min. Malate concentrations were varied from 0.5 to 30.0mM. For this experiment, the Kₘ for malate -Ox was 2.93mM, and the Vₘₐₓ=1.216; in the presence of 10mM oxamate the Kₘ was 8.68mM, and the Vₘₐₓ=1.340. The line and its slope was determined using the regression formula built in the Texas Instruments SR-51-II (Appendix B). The correlation coefficients, r, were 0.98 -Ox and 0.99 +Ox.
Fig. 10

NAD malic enzyme activity vs the concentration of NAD. Double reciprocal plot of NAD malic enzyme activity as a function of NAD concentration for a typical experiment. The reaction was carried out in the presence of 150mM Tris-Cl, 8mM MnCl₂, 30mM malate, at pH 7.4, 30°C. Reaction velocity was expressed as nanomoles of NADH produced per min. NAD concentrations were varied from 0.05 to 5.0mM. For this experiment, the Km for NAD was 0.17mM, and the Vmax=0.135. The line and its slope was determined using the regression formula built in the Texas Instruments SR-51-II (Appendix B). The correlation coefficient, r, was 0.98.
NAD malic enzyme activity vs the concentration of NAD. Double reciprocal plot of NAD malic enzyme activity as a function of NAD concentration for a typical experiment. The reaction was carried out in the presence of 150mM Tris-Cl, 8mM MnCl₂, 30mM malate, at pH 7.4, 30°C. Reaction velocity was expressed as nanomoles of NADH produced per min. NAD concentrations were varied from 0.05 to 5.0mM. For this experiment, the Km for NAD was 0.14mM, and the Vmax=1.29. The line and its slope was determined using the regression formula built in the Texas Instruments SR-51-II (Appendix B). The correlation coefficient, r, was 0.99.
NAD malic enzyme activity vs the concentration of NADP. Double reciprocal plot of NAD malic enzyme activity as a function of NADP concentration for a typical experiment. The reaction was carried out in the presence of 150mM Tris-Cl, 8mM MnCl₂, 30mM malate, at pH 7.4, 30°C. Reaction velocity was expressed as nanomoles of NADPH produced per min. NADP concentrations were varied from 0.06 to 5.0mM. For this experiment, the Km for NADP was 0.81mM, and the Vmax=0.363. The line and its slope was determined using the regression formula built in the Texas Instruments SR-51-II (Appendix 'B'). The correlation coefficient, r, was 0.99.
The average value for the $K_m$ for $Mn^{++}$ shown in Fig. 13 and Fig. 14 in the absence of oxamate was 0.22mM for NAD reduction. Oxamate significantly altered the $K_m$ and the $V_{max}$ of the reaction and appeared to inhibit the reaction uncompetitively with respect to $Mn^{++}$ concentration. The $K_m$ value for the reaction without the inhibitor was slightly higher than the 0.14mM value obtained by Lin and Davis (1974).
NAD malic enzyme activity vs the concentration of Mn++. Double reciprocal plot of NAD malic enzyme activity as a function of Mn++ concentration for a typical experiment. The reaction was carried out in the presence of 150mM Tris-Cl, 30mM malate, 1mM NAD, at pH 7.4, 30C. Reaction velocity was expressed as nanomoles of NADH produced per min. Mn++ concentrations were varied from 0.08 to 8.0mM. For this experiment, the Km for Mn++ was 0.20mM, and the Vmax=0.105. The line and its slope was determined using the regression formula built in the Texas Instruments SR-51-II (Appendix B). The correlation coefficient, r, was 0.99.
NAD malic enzyme activity vs the concentration of Mn++ in the presence and absence of oxamate. Double reciprocal plot of NAD malic enzyme activity as a function of Mn++ concentration and the presence or absence of oxamate (Ox). The reaction was carried out in the presence of 150mM Tris-Cl, 30mM malate, 1mM NAD, + or - 10mM oxamate, at pH 7.4, 30C. Reaction velocity was expressed as nanomoles of NADH produced per min. Mn++ concentrations were varied from 0.08 to 8.00mM. For this experiment, the Km for Mn++ was 0.24mM -Ox, and the Vmax=0.578; in the presence of 10mM oxamate the Km was 0.17mM, and the Vmax=1.032. The line and its slope was determined using the regression formula built in the Texas Instruments SR-51-II (Appendix B). The correlation coefficients, r, were 0.99 for + and - Ox.
SUMMARY AND CONCLUSIONS

Malic enzyme activity that was specific for the reduction of NAD but not NADP could be isolated from both pig and rabbit heart mitochondria. The extraction of calcium-phosphate gels with KCl buffers produced at least 4 different forms of the NAD malic enzyme. Three peaks of NADP malic enzyme activity were eluted from DEAE columns. The PAGE with NADP activity staining indicated that the DEAE fractions contained in peak #1 represented two different enzyme forms. The addition of oxamate to assay buffers for NAD malic enzyme inhibited LDH contamination effects and was integral to the discovery of hitherto unknown large quantities of NAD malic enzyme in the 0 KCl fraction. The 0.05M KCl fraction from rabbit heart became activated in a time dependent manner, and the activation could be slowed by additions of proteolytic inhibitor. The loss of influence by known effectors on the activated fraction suggested the proteolytic loss of regulatory sites on the enzyme molecule. Quantitative and molecular weight differences in the distribution of NAD and NADP malic enzyme activities were present in the same tissues of...
different mammals.

The yield of malic enzymes varied with the isolation procedure employed and with the time from death. The Sauer method of isolation produced more enzyme activity than the calcium-phosphate extraction, however, NAD specific malic enzyme could not be isolated from fractions obtained with this procedure. Additionally, the NAD/NADP activity that was evident in these fractions was constant despite treatments designed to separate or alter the activities. The highest amount of NAD malic enzyme activity/g heart was extracted with the low ionic strength buffers on the freshest available rabbit hearts.

The possibility of large quantities of NAD malic enzyme existing in compartments other than the mitochondria were suggested by the large yields obtained from a whole cell preparation assayed in the presence of oxamate. The possibility of the activation of mitochondrial NAD malic enzymes by one or more extramitochondrial factors was not eliminated by these experiments, but, would have provided an additional avenue of investigation.

All of the published methods duplicated in the above mentioned experiments for the isolation of NAD malic enzyme contained some level of contamination by MDH and/or LDH. The validity of the kinetic data generated from these isolations were questionable in terms of the effects of
contamination. A method for the isolation of NAD malic enzyme from small quantities of tissue was developed and was free of the effects of MDH and LDH contamination.

The high yield of mitochondrial NAD malic enzyme from rabbit heart tissue in concert with the demonstration of small scale preparation has suggested the feasibility of studies on myocytes. The ability to manipulate substrates, effectors, and ischemic conditions in viable heart cells and to quantitate the resultant alterations in the mitochondrial malic enzymes would hasten the elucidation of the role for these enzymes in the functioning heart.
Appendix A

DETERMINATIONS OF ENZYME ACTIVITY

A linear portion of the chart recorder plot, Figure 15, was extended with a straight edge to cover at least 5 min of rate. The number of chart units in 5 min of rate per amount of enzyme added to the 1 ml cuvette was multiplied by the sensitivity of the Eppendorf and expressed as nanomoles of NADH produced/min/ml of enzyme (see calculation).

\[
15 \text{ chart units/5 min/0.2 ml enzyme} = 15 \text{ chart units/min/ml}
\]
\[
15 \text{ chart units/min/ml} \times 0.161 \text{ nanomoles of NADH/chart unit} = 2.415 \text{ nanomoles of NADH produced/min/ml of enzyme}
\]

The sensitivity of the Eppendorf (0.161 nanomoles of NADH/chart unit) was calibrated periodically against a Gilford, model 252, (Beckman DU, model 2400) spectrophotometer (340 nm) using NADH solutions in quartz cuvettes to generate a standard curve.
Sample chart recorder plot for the assay of NAD malic enzyme.
Sample chart recorder plots for the assay of NAD malic enzyme and MDH. Two methods for determining NAD malic enzyme activities in fractions containing MDH contamination. Fractions containing high endogenous levels of MDH were brought to rapid equilibrium with the addition of 6.5 units of MDH from a commercial source. The resulting rate (a) was indicative of the NAD malic enzyme activity plus a small contribution attributed to the spontaneous decarboxylation of OAA formed from the MDH equilibrium (see text). NAD malic enzyme activities were calculated by subtracting a control rate (b), resulting from the added MDH, from rate (a). Fractions containing low MDH contamination were assayed in the absence of MnCl₂ (d). MnCl₂ was added to the reaction after several minutes of rate (d). MDH activity does not require metal ions so that rate (d) represents MDH rate and rate (c) represents MDH plus NAD malic enzyme rates. NAD malic enzyme activities were calculated by subtracting rate (d) from rate (c).
Fig. 16

Malic enzyme rate = a - b

Malic enzyme rate = c - d
Fig. 17

Sample chart recorder plot demonstrating the effect of oxamate in the assay buffer for NAD malic enzyme.
Factors effecting the determination of NADH production from NAD malic enzyme in the presence of contaminating enzymes.
Appendix B

CALCULATION OF X AND Y INTERCEPTS AND CORRELATION COEFFICIENTS FOR LINEWEAVER-BURK PLOTS

The reaction velocities (nanomoles of NADH produced/min/ml of enzyme) and substrate concentrations for kinetic analysis were expressed as inverse functions and were graphically represented as Lineweaver-Burk plots. The best fit line was drawn through the data points using the x and y intercepts calculated with a Texas Instruments SR-51-II linear regression program. The formulas utilized for the calculation of the intercepts and for the correlation coefficients are as follows:

\[
b = \frac{\frac{\sum x_i \sum y_i}{N} - \frac{\sum x_i y_i}{N} \left( \frac{\sum x_i}{N} \right)}{\frac{\left( \sum x_i^2 \right)^2}{N} - \sum x_i^2}
\]

\[
r^2 = \frac{\frac{\sum x_i \sum y_i}{N} - \frac{\sum x_i y_i}{N} \left( \frac{\sum x_i}{N} \right)}{\left( \frac{\sum x_i^2}{N} \right)^2 - \frac{\sum x_i^2}{N^2} \left( \frac{\sum x_i^2}{N} \right)^2}
\]
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