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SOME CHARACTERISTICS OF THE MITOCHONDRIAL AND SOLUBLE FORMS OF MALATE DEHYDROGENASE IN LEMON FRUITS

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SUMMARY

Studies on malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37) in soluble and mitochondrial fractions from sweet lemon (*Citrus limettioides*, Tanaka) and sour lemon (*Citrus limon*, L.) were undertaken. The first contains low levels of organic acids and the last contains high levels. These studies involved distribution, K_m determinations for 4 substrates (NADH, NAD^+, oxalacetate and malate), oxalacetate substrate inhibition, and effects of 3′-adenylic acid, AMP, ADP and ATP on enzyme activity in two buffers. Product inhibition of NADH oxidation by NAD^+ was also studied.

The studies demonstrated that some differences in enzymatic activity exist between sweet and sour lemon fruits; the most apparent was that intact mitochondria from sour lemon had a much higher K_m value (almost 5 times) for NADH than mitochondria from sweet lemon. Solubilization and partial purification of the mitochondrial enzyme from both lemons resulted in the disappearance of that significant difference.

In both lemons, mitochondrial enzyme accounted for between 23 and 36% of the total enzymatic activity depending on the oxalacetate concentration used in the assay. Oxalacetate inhibition of NADH oxidation by malate dehydrogenase did occur in both soluble and mitochondrial fractions and was found to be of a noncompetitive type. Phosphate buffer alleviated most but not all of the inhibition by oxalacetate. The K_m for oxalacetate was much higher in phosphate buffer than in Tris–HCl buffer. The K_m for NADH was not affected by kind of buffer even though phosphate buffer increased the apparent maximal velocity of the reaction.

Of the 3 adenosine nucleotides, AMP showed the greatest inhibitory effect on NADH oxidation by solubilized mitochondrial enzyme. A 5′-adenosine phosphate moiety was necessary for inhibition. Although the nature of AMP inhibition seemed competitive, high levels of NADH failed to completely overcome it. NAD^+ (simultaneously added with NADH) competitively inhibited NADH oxidation. If, however, the enzyme was preincubated with NAD^+ + oxalacetate with NADH added last, the inhibition was of an uncompetitive nature.

INTRODUCTION

Plant cells as well as animal cells appear to contain all the tricarboxylic cycle acids, at least in catalytic amounts. A peculiarity of higher plant cells, however, is frequently the accumulation of 1 or a combination of the tricarboxylic acid cycle acids at what is, biologically speaking, a high concentration. The 2 main organic acids which citrus fruits accumulate are citric and malic. The sour lemon fruit juice usually contains from 49-67 mg/ml of citric acid and 1.5–4.3 mg/ml of malic acid[1,2].

The absence of conclusive evidence that the site of organic acid synthesis is in leaves and that they are then translocated to young developing fruits, in addition to the existence in young fruit cells of all the enzymes, coenzymes, and all other necessary equipment for the synthesis, interconversion and complete oxidative consumption of citric, isocitric, aconitic, succinic, fumaric, and malic acids, seem to indicate that the site of organic acids synthesis in the fruits is *in situ*[3].

This work was initiated by studying malate dehydrogenase in soluble and mitochondrial fractions of the 2 kinds of lemons to determine if there were any differences in distribution and kinetic properties. Kinetic studies of the mammalian enzyme have revealed some differences between the mitochondrial and soluble forms of this enzyme[4–7]. The mammalian mitochondrial enzyme was inhibited at a high oxalacetate concentration, whereas, the soluble enzyme is not sensitive to high oxalacetate concentrations[4,7]. The mammalian mitochondrial enzyme may function primarily for oxalacetate formation, whereas, the soluble enzyme may primarily result in malate production[4,8]. Furthermore, since adenosine nucleotides have been shown to affect many feedback control mechanisms[9–14], *e.g.*, isocitrate dehydrogenase has been shown to be regulated with adenylic acid[10], it seemed worthwhile to study effects of adenosine nucleotides on malate dehydrogenase from the 2 lemons.

MATERIALS AND METHODS

Materials

Reagents and cofactors. Oxalacetate, NAD^+, NADH, AMP, ADP, ATP, and 3′-adenylic acid were purchased from Calbiochem. Tris was purchased from Sigma Chemicals. DEAE-Sephadex A-25 was purchased from Pharmacia Fine Chemicals.

Fruits. The lemons used for these studies were obtained from the orchard of the University of California, Los Angeles. The sour lemons were of the variety Eureka (*Citrus limon*, Linn). The sweet lemons were of the variety Tunisian sweet lemon (*Citrus limettioides*, Tanaka).

Mitochondria. Mitochondria were obtained from peeled young lemon fruits according to the method described by BOGIN AND WALLACE[1]. Intact mitochondria were suspended in 0.5 M sucrose solution which contained 0.5–1.0 mg/ml of low-fat bovine serum albumin and 0.05 M Tris–HCl buffer (pH 7.5).

Mitochondrial preparations suspended in 0.05 M Tris–HCl buffer adjusted to pH 7.5 and frozen and thawed several times were used as the source of enzyme in the distribution studies. Burst mitochondria were further broken with an H.M.K. cell homogenizer and centrifuged at 20 000 × *g*. The supernatant solution from the 20 000 × *g* was used as the source of solubilized mitochondrial enzyme. Partial

purification of the enyme was achieved by the use of DEAE-Sephadex A-25 column chromatography.

Soluble fraction. The mitochondria-free homogenate was used as the source of soluble fraction malate dehydrogenase enzyme in distribution studies. Partial purification was achieved by passing saturated ammonium sulfate precipitate suspension into DEAE-Sephadex A-25 columns.

Methods

Partial purification of enzyme. DEAE-Sephadex A-25 was equilibrated in 0.1 M Tris-HCl buffer (pH 8.3). Columns 1.5 cm in diameter and 30 cm in length were prepared. The columns were charged with up to 10 ml of saturated ammonium sulfate precipitate suspension or 5-6 ml of the solubilized mitochondrial fraction. The protein was eluted with a linear gradient of NaCl in 0.1 M Tris-HCl (pH 8.3) with a maximum concentration of 0.3 M NaCl. Elutes were collected in fractions of 3 ml.

Protein assay. Protein was determined colorimetrically with the Folin–Ciocalteu reagent[15] or by measuring the optical density of the partially purified solutions at 280 nm.

Enzyme assay. Malic dehydrogenase activity in mitochondrial or soluble fraction preparations was quantitatively measured at 25° essentially as described by OCHOA[16]. Enzymatic activity was measured by following the decrease in optical density of NADH at 340 nm. One enzyme unit is that amount which causes a decrease in optical density at the rate of 0.01 optical density unit per min.

RESULTS

Activity of malate dehydrogenase in soluble and mitochondrial fractions from the 2 lemons

Malate dehydrogenase had an almost 5.5 times higher specific activity in mitochondria from sweet lemon compared with sour lemon mitochondria (Table 1). Total enzyme units were always, however, moderately higher (almost 27%) in preparations from sweet lemon than in preparations from sour lemon.

TABLE 1

DISTRIBUTION OF MALATE DEHYDROGENASE ACTIVITY BETWEEN SOLUBLE AND MITOCHONDRIAL FRACTIONS IN YOUNG LEMON FRUITS

The reaction mixture contained 0.2 μmole NADH, 5 μmoles oxalacetate, and 150 μmoles Tris HCl (pH 7.5). Final volume was 3.0 ml. The enzyme unit is defined as that amount which caused a decrease in absorbance at the rate of 0.01 absorbance unit (at 260 nm) per min under the above specified conditions. The results are means of 6 extractions. Mitochondria were suspended in 0.05 M Tris–HCl (pH 7.5), frozen and thawed several times.

Enzyme source	*Specific activity (enzyme units/mg protein)*	*Enzyme units/ 50 g fresh wt.*	*% of total*
Soluble sour	76	55 300	75
Mitochondria sour	430	18 100	25
Soluble sweet	71	72 700	78
Mitochondria sweet	2361	20 800	22

TABLE II

EFFECT OF 2 OXALACETATE CONCENTRATIONS ON OXIDATION OF NADH BY SOLUBILIZED SOUR AND SWEET MITOCHONDRIAL AND SOLUBLE SOUR AND SWEET MALATE DEHYDROGENASE

Average of 2 extractions. For further explanation see Table I. In addition to what is mentioned in Table I, the mitochondria were further broken by an H.M.K. cell homogenizer and then centrifuged at 20 000 × *g* and the supernatant used as the source of enzyme.

Tissue	*Reaction rate at* $1.67 \cdot 10^{-3}$ *M oxalacetate* ($\Delta A_{340\ nm/min}$)	*Reaction rate at* $1.67 \cdot 10^{-4}$ *M oxalacetate* ($\Delta A_{340\ nm/min}$)	*Activity at* $\frac{1.67 \cdot 10^{-3}\ M}{1.67 \cdot 10^{-4}\ M}$ (%)
Burst sour			
Mitochondria	0.053	0.159	33
Soluble sour	0.084	0.150	56
Burst sweet			
Mitochondria	0.052	0.156	33
Soluble sweet	0.084	0.150	56

Oxalacetate inhibited NADH oxidation in burst mitochondria from both sour and sweet lemons by 67% whereas it inhibited NADH oxidation in the soluble fraction of both lemons by 44% when its concentration was increased by a factor of 10 (Table II). Mitochondrial enzyme accounted for only 22–24% of the total enzyme in both lemons when the assay was carried at the higher oxalacetate concentration. The percentage would be 33–36 if calculations were made at the lower oxalacetate level (Tables I and II).

Partial purification of malate dehydrogenase from soluble fraction and burst mitochondria from sour and sweet lemons

Attempts were made to purify and, if possible, isolate different isozymes from both mitochondrial and soluble fractions by adsorption and elution on DEAE-

TABLE III

PARTIAL PURIFICATION OF MITOCHONDRIAL MALATE DEHYDROGENASE

Fraction	*Total activity (units)*	*Total protein (mg)*	*Specific activity (units/mg protein)*
Sour lemon mitochondria			
(1) Mitochondria suspended in 0.05 M Tris-HCl	18 000	42.6	423
(2) Solubilized mitochondrial malate dehydrogenase	13 100	3.8	3 450
(3) Combined fractions from DEAE-Sephadex A-25	6 600	0.2	32 800
(4) Activity not adsorbed to resin	7 000	1.0	7 000
(5) Fraction 3 + 4	13 600	1.2	11 300
Sweet lemon mitochondria			
(1) Mitochondria suspended in 0.05 M Tris HCl	19 900	9.6	2 070
(2) Solubilized mitochondrial malate dehydrogenase	16 000	1.1	14 500
(3) Combined fractions from DEAE-Sephadex A-25	8 800	<0.05	$>175\ 000$
(4) Activity not adsorbed to resin	7 400	0.20	36 800
(5) Fractions 3 + 4	16 100	0.25	64 500

TABLE IV

PARTIAL PURIFICATION OF SOLUBLE FRACTION MALATE DEHYDROGENASE

Fraction	*Total activity (units)*	*Total protein (mg)*	*Specific activity (units/mg protein)*
Soluble sour			
(1) Mitochondria free supernatant	56 100	765.0	73
(2) 100% $(NH_4)_2SO_4$ precipitate	18 600	103.0	181
(3) Combined fraction from DEAE-Sephadex A-25	10 100	11.2	905
(4) Activity not adsorbed to resin	8 800	30.3	290
(5) Fractions 3 + 4 pooled together	18 900	41.5	456
Soluble Sweet			
(1) Mitochondria free supernatant	73 100	1050.0	70
(2) 100% $(NH_4)_2SO_4$ precipitate	27 100	98.8	274
(3) Combined fraction from DEAE-Sephadex A-25	15 000	10.0	1500
(4) Activity not adsorbed to resin	12 100	19.5	622
(5) Fractions 3 + 4 pooled together	27 100	29.5	920

Sephadex A-25. In both types of enzyme, almost one-half of the activity was not retained on the resin at 0.1 M Tris–HCl (pH 8.3). The remaining adsorbed activity could be eluted with NaCl (0.1–0.3 M) in Tris–HCl buffer (0.1 M, pH 8.3) gradient.

A representative partial purification of solubilized mitochondria and soluble fraction malate dehydrogenase from both lemons is shown in Tables III and IV. No

TABLE V

MICHAELIS CONSTANTS FOR THE 4 SUBSTRATES (NADH, OXALACETATE, NAD^+ AND MALATE) (K_m) IN μM AT 25°

Circumstances: (1) NADH varied from $2.5 \cdot 10^{-5}$ to $1.33 \cdot 10^{-4}$ M. Oxalacetate was kept constant at either $1.67 \cdot 10^{-4}$ M or $1.67 \cdot 10^{-3}$ M. (2) Oxalacetate varied from $1 \cdot 10^{-5}$ to $1.67 \cdot 10^{-3}$ M. NADH constant at $6.7 \cdot 10^{-5}$ M. (3) NAD^+ varied from $1 \cdot 10^{-4}$ to $1 \cdot 10^{-3}$ M. Malate constant at $1 \cdot 10^{-2}$ M. (4) L-Malate varied from $1 \cdot 10^{-4}$ to $1 \cdot 10^{-3}$ M. NAD^+ constant at $1 \cdot 10^{-3}$ M.

Fraction	*NADH (1)*		*Oxalacetate (2)*		*NAD^+ (3)*		*Malate (4)*	
	In Tris HCl (0.05 M, pH 7.5)	*In phosphate (0.05 M, pH 7.5)*	*In Tris HCl (0.05 M, pH 7.5)*	*In phosphate (0.05 M, pH 7.5)*	*In Tris–HCl (0.05 M, pH 8.5)*	*In hydrazine glycine (pH 9.5)*	*In Tris HCl (0.05 M, pH 8.5)*	*In hydrazine-glycine (pH 9.5)*
Solubilized malate dehydrogenase from sour lemon mitochondria	77	83	19.5	67	244	180	909	455
Intact sour lemon mitochondria	67	*	29.0	91	263	180	909	455
Solubilized malate dehydrogenase from sweet lemon mitochondria	53	46	19.5	67	250	180	909	455
Intact sweet lemon mitochondria	14	*	19.5	67	270	180	909	455
Soluble sour	23	*	19.5	67	322	227	770	455
Soluble sweet	22	*	19.5	67	322	227	770	455

* Not determined.

TABLE VI

RELATIVE APPARENT MAXIMAL VELOCITIES IN DIFFERENT BUFFERS (PERCENTAGES)

For further explanation, see under RESULTS and Table V.

Fraction	*NADH oxidation*				*Oxalacetate reduction*		*NAD⁺ reduction*		*Malate oxidation*	
	Tris HCl Oxalacetate (M)		*Phosphate Oxalacetate (M)*							
	$1.67 \cdot 10^{-4}$	$1.67 \cdot 10^{-3}$	$1.67 \cdot 10^{-4}$	$1.67 \cdot 10^{-3}$	*Tris–HCl*	*Phosphate*	*Tris–HCl*	*Hydrazine–glycine*	*Tris–HCl*	*Hydrazine glycine*
Solubilized mitochondrial malate dehydrogenase from sour lemon	75	25	100	82	54	100	96	100	100	100
Solubilized mitochondrial malate dehydrogenase from sweet lemon	68	22	100	73	55	100	100	100	100	100
Soluble fraction malate dehydrogenase from sour lemon	100	56		—	—	—	89	100	97	100
Soluble fraction malate dehydrogenase from sweet lemon	100	56		—	—		94	100	100	100

significant differences in kinetic properties could be detected between the partially purified enzymes and the corresponding crude fractions, *i.e.* soluble fraction and solubilized mitochondrial enzyme preparations.

Kinetics of NADH oxidation

Tables V and VI contain data obtained by plotting information obtained according to the double reciprocal plot technique of LINEWEAVER AND BURK[17]. Enzyme solubilization from the mitochondria increased the K_m for NADH only slightly in sour lemon but drastically in sweet lemon. It should be noted also that the intact sour lemon mitochondria have a much higher K_m for NADH, almost 5 times higher than that for intact sweet lemon mitochondria.

Soluble enzyme has the same K_m for NADH for the 2 lemons. A high oxalacetate concentration caused a significant inhibition of the apparent maximal velocity, whereas it did not affect the K_m for NADH. Also noted was that under the same experimental conditions, *e.g.* same pH and ionic strength, the solubilized mitochondrial enzyme from sour and sweet lemon had a significantly higher K_m for NADH than did the soluble enzyme from both lemons, whereas the intact sweet mitochondrial enzyme had a lower K_m for NADH which is even lower (35–45%) than that of the soluble enzyme from the 2 lemons.

Effect of phosphate vs. Tris–HCl buffer on the kinetics of NADH oxidation

During the course of the study, it was observed that if the reaction was carried out in 0.05 M phosphate buffer (pH 7.5) instead of 0.05 M Tris HCl (pH 7.5), the velocity was apparently increased. A study was initiated to compare the effect of phosphate *vs.* Tris HCl on NADH oxidation by solubilized sour and sweet lemon mitochondrial enzyme. The results are summarized in Tables V and VI. High oxalacetate concentration caused a sharp decrease in the apparent maximal velocity in enzymes in Tris-HCl buffer from both lemons. On the other hand, phosphate buffer gave a higher maximal velocity at the 2 levels of oxalacetate than Tris HCl buffer gave at the lower level of oxalacetate. Thus, it seems that phosphate not only alleviated oxalacetate inhibition when compared with Tris-HCl buffer, but it also caused an increase in the apparent maximal velocity regardless of the oxalacetate concentration. However, there was still a decrease in the maximal velocity at the higher oxalacetate concentration (about 23%) in the phosphate buffer. It is of importance to note that the K_m remained unaffected regardless of the oxalacetate concentration or the kind of buffer.

Kinetics of oxalacetate reduction in Tris-HCl and phosphate buffers

As shown in Table V, only intact sour lemon mitochondrial enzyme had a slightly increased K_m. This occurred in either Tris-HCl buffer or phosphate buffer. While the K_m for NADH was not affected by the kind of buffer, the K_m for oxalacetate was higher in phosphate buffer than in Tris–HCl buffer. This was true for enzymes from both lemons. Phosphate buffer gave a higher maximal activity and also alleviated oxalacetate inhibition at higher concentrations to a very great extent with solubilized mitochondrial malate dehydrogenase from both lemons (Table VI).

Kinetics of NAD^+ reduction in Tris–HCl buffer (0.05 M, pH 8.5) and hydrazine–glycine buffer (0.4 M hydrazine, 1 M glycine, pH 9.5)

Since the equilibrium constant of the reaction is strongly toward malate formation, it was necessary to use higher substrate concentrations as well as a higher amount of enzyme to get a quantitatively measurable rate. Also, it was necessary to use a buffer of high pH since the pH optimum for the enzyme when catalyzing this direction is rather high. The K_m for NAD^+ was noticeably decreased, whereas the apparent maximal velocity was not significantly changed when the reaction was carried out in hydrazine–glycine buffer (Tables V and VI). The soluble sweet and sour lemon enzymes showed the same K_m for NAD^+ in both buffers (Table V). However, in the case of either intact or solubilized sweet and sour lemon mitochondrial enzyme, the K_m for NAD^+ seemed to be slightly lower in both buffers than the K_m of the soluble enzyme for NAD^+.

Kinetics of malate oxidation in Tris–HCl (0.05 M, pH 8.5) and hydrazine glycine buffer (0.4 M hydrazine, 1 M glycine, pH 9.5)

Under the experimental conditions specified in Table V, all enzyme preparations showed lower K_m for malate in hydrazine-glycine buffer than in Tris–HCl buffer. However, as shown in Table V, in Tris–HCl buffer the K_m of the soluble enzyme for malate was slightly less than that of either solubilized or intact mitochondrial enzyme from both lemons. It is also apparent that the soluble enzyme from both lemons has the same K_m for malate in both buffers.

Effect of AMP on the kinetics of NADH oxidation

The study was carried out at 3 levels of AMP for solubilized and partially purified sweet and sour lemon mitochondrial enzyme. The inhibition of NADH oxidation by AMP appeared to be of a competitive nature at the NADH levels used ($0.3 \cdot 10^{-4}$–$1.33 \cdot 10^{-4}$ M) at a constant oxalacetate level of $1.67 \cdot 10^{-4}$ M (Table VII). No change in apparent maximal velocity was observed. In both lemon enzymes, the inhibition type and degree were almost the same in either phosphate buffer or Tris-HCl buffer at the same pH (7.5). However, AMP (at 3 concentrations) increased the K_m for NADH much more in the case of partially purified sour lemon mitochondrial malate dehydrogenase than in the sweet lemon enzyme (Table VII). Thus, it seems that the sour

TABLE VII

EFFECT OF AMP ON KINETICS OF NADH OXIDATION IN Tris–HCl OR PHOSPHATE BUFFERS (0.05 M, pH 7.5) BY PARTIALLY PURIFIED MITOCHONDRIAL MALATE DEHYDROGENASE

NADH varied from $0.33 \cdot 10^{-4}$ to $1.33 \cdot 10^{-4}$ M. Oxalacetate kept constant at $1.67 \cdot 10^{-4}$ M. Relative apparent maximal velocities were unchanged, *i.e.* the AMP inhibition was competitive.

AMP concn. $\times 10^3$ *(M)*	K_m *(NADH)* $\times 10^6$ *(M)*	
	Sweet lemon enzyme	*Sour lemon enzyme*
0.00	44	83
0.33	67	200
1.67	100	303
3.33	133	400

TABLE VIII

K_i (AMP) VALUES FOR INHIBITION OF NADH OXIDATION BY PARTIALLY PURIFIED MITOCHONDRIAL MALATE DEHYDROGENASE

See under RESULTS.

	K_i (AMP) $\times 10^3$ (M)	K_i (AMP)/K_m (NADH)
Sweet lemon enzyme		
in phosphate	2.3	53
in Tris–HCl	2.3	53
Sour lemon enzyme		
in phosphate	3.0	36
in Tris–HCl	2.3–3.1	27–37

lemon enzyme is consistently much more sensitive to AMP at the 3 concentrations used than is the sweet lemon enzyme. To determine the K_i of the enzyme for the inhibitor, AMP, the data were replotted as $1/v_i$ *vs.* inhibitor concentrations from which the data in Table VIII were obtained. The K_i for AMP in the sweet lemon enzyme was almost essentially the same in both buffers (K_i AMP $= 2.3 \cdot 10^{-3}$ M). On the other hand, the K_i for AMP in the case of the sour lemon enzyme was almost $3 \cdot 10^{-3}$ M in the phosphate buffer, whereas the lines did not intercept at one point in the case of Tris HCl buffer with K_i increasing with NADH concentration (from $2.3 \cdot 10^{-3}$ to $3.1 \cdot 10^{-3}$ M).

The ratio K_i (AMP):K_m (NADH) was lower in both buffers for the sour lemon enzyme than that for the sweet lemon enzyme (it was 53 in both buffers for the sweet lemon enzyme, whereas it was 36 in phosphate buffer and between 27 and 37 in Tris–HCl buffer for the sour lemon enzyme). This indicates more sensitivity for inhibition of NADH oxidation by AMP in the sour lemon enzyme than in the sweet lemon enzyme.

TABLE IX

EFFECT OF HIGH NADH ON AMP INHIBITION OF NADH OXIDATION BY PARTIALLY PURIFIED MITOCHONDRIAL MALATE DEHYDROGENASE FROM SWEET LEMONS

For explanation, see under METHODS.

NADH conen. $\times 10^3$ *(M)*	*Reaction rate No AMP* ($\Delta A_{340\ nm/min}$)	*Reaction rate* $3.33 \cdot 10^{-3}$ *M AMP* ($\Delta A_{340\ nm/min}$)	*Activity in presence of AMP / Activity in absence of AMP (%)*
0.033	0.125	0.055	44
0.067	0.170	0.094	55
0.100	0.200	0.120	60
0.133	0.217	0.143	66
0.200	0.230	0.224	97
0.333	0.240	0.202	84
0.500	0.230	0.221	96
0.667	0.151	0.154	103

Even though the inhibition of NADH oxidation by AMP appeared to be a competitive one as mentioned earlier, when higher concentrations of NADH were used, the inhibition was never completely overcome (Table IX). High NADH levels caused inhibition whether or not AMP was present.

Effect of 3′-adenylic acid, ADP and ATP on the kinetics of NADH oxidation as compared to the effect of AMP

The study was carried out with solubilized and partially purified mitochondrial enzyme from both lemons. Table X shows that 3′-adenylic acid had no effect, whereas ADP and ATP had some inhibitory effect similar to AMP inhibition. ADP was less inhibitory than AMP, whereas it was more inhibitory than ATP. At the same concentration of $3.33 \cdot 10^{-3}$ M, ATP increased the K_m for NADH; from $83 \cdot 10^{-6}$ to $118 \cdot 10^{-6}$ M

TABLE X

EFFECT OF 3′-ADENYLIC ADP AND ATP AS COMPARED TO AMP ON THE KINETICS OF NADH ADENYLIC ACID OXIDATION BY PARTIALLY PURIFIED MITOCHONDRIAL MALATE DEHYDROGENASE

Relative apparent maximal velocities were unchanged, *i.e.* the adenosine nucleotides inhibition was competitive.

Nucleotides	K_m *(NADH)* $\times 10^6$ *(M)*	
	Sweet lemon enzyme	*Sour lemon enzyme*
0	44	83
$3.33 \cdot 10^{-3}$ M 3′-adenylic acid	44	83
$3.33 \cdot 10^{-3}$ M ATP	57	118
$3.33 \cdot 10^{-3}$ M ADP	67	139
$3.33 \cdot 10^{-3}$ M AMP	133	400

(1.4 times), whereas ADP increased it to $139 \cdot 10^{-6}$ M (1.7 times) in comparison to $400 \cdot 10^{-6}$ M (4.8 times) in case of AMP with the partially purified sour lemon mitochondria enzyme. With sweet lemon enzyme the increase in K_m for NADH was from $44 \cdot 10^{-6}$ to $57 \cdot 10^{-6}$ M (1.3 times), $66.7 \cdot 10^{-6}$ M (1.5 times) and $133 \cdot 10^{-6}$ M (3.1 times) for ATP, ADP, and AMP, respectively. Thus, it seems that both lemon enzymes are more sensitive to AMP than to either ATP or ADP at the same concentration of the adenylate nucleotides. It is important to note that 3′-adenylic acid had no inhibitory effect thus indicating the necessity of 5′-adenosine phosphate moiety requirement for the inhibition to occur.

Effects of AMP, ADP, and ATP on NAD^+ reduction

Since adenosine nucleotides appeared to inhibit NADH oxidation, a study was undertaken to see if they affect NAD^+ reduction. As shown in Table XI, AMP increased NAD^+ reduction with partially purified mitochondrial enzyme from both lemon fruits. The stimulation at the highest levels of AMP used ($3.33 \cdot 10^{-3}$ M) was almost 50%. ADP and ATP showed little or no effect. It should be noted that since the equilibrium of the reaction is strongly toward malate formation, it was necessary to use high substrate levels and a buffer of high pH value.

TABLE XI

EFFECT OF AMP, ADP, AND ATP ON REDUCTION OF NAD^+ BY MITOCHONDRIAL MALATE DEHYDROGENASE FROM SOUR AND SWEET LEMON FRUITS

To a cuvette containing 2.0 ml of Tris HCl (0.05 M, pH 8.5), the following reagents were added: (1) 3 μmoles of NAD^+ (0.3 ml of 0.01 M), (2) 3 μmoles of L-malate (0.3 ml of 0.01 M), (3) a suitable amount of 0.01 M or 0.1 M adenylate nucleotide solutions to give the desired concentration (1–10 μmoles), and (4) enough buffer to bring the volume to 2.9 ml. To start the reaction 0.1 ml of suitably diluted enzyme was added.

AMP			*ADP*			*ATP*		
Concn. $\times 10^3$ (M)	*Reaction rate ($\Delta A_{340\ nm/min}$)*	*Activity (%)*	*Concn. $\times 10^3$ (M)*	*Reaction rate ($\Delta A_{340\ nm/min}$)*	*Activity (%)*	*Concn. $\times 10^3$ (M)*	*Reaction rate ($\Delta A_{340\ nm/min}$)*	*Activity (%)*
Sour lemon solubilized mitochondrial malate dehydrogenase								
0.00	0.110	100.0	0.00	0.110	100.0	0.00	0.110	100.0
0.33	0.115	104.5	0.33	0.109	99.1	0.33	0.112	101.8
1.67	0.161	146.4	1.67	0.111	100.9	1.67	0.112	101.8
3.33	0.167	151.8	3.33	0.108	98.2	3.33	0.114	103.6
Sweet lemon solubilized mitochondrial malate dehydrogenase								
0.00	0.082	100.0	0.00	0.082	100.0	0.00	0.082	100.0
0.33	0.087	106.1	0.33	0.083	101.2	0.33	0.083	101.2
1.67	0.110	134.1	1.67	0.085	103.7	1.67	0.084	102.4
3.33	0.117	142.7	3.33	0.084	102.4	3.33	0.084	102.4

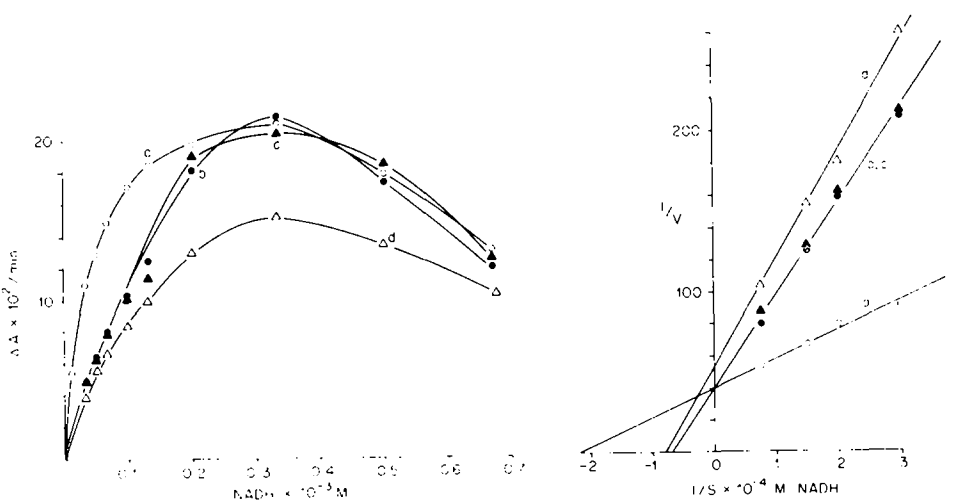

Fig. 1. Effect of NAD^+ on NADH oxidation by partially purified mitochondrial malate dehydrogenase from sweet lemon in Tris–HCl (0.05 M, pH 7.5): a, no NAD^+; b, the enzyme was incubated for 10 min with NADH and buffer. The reactions were initiated by adding NAD^+ and oxalacetate simultaneously to the reaction mixture. c, the enzyme was incubated for 10 min with NAD^+ + NADH simultaneously. The reaction was initiated by oxalacetate addition. d, the enzyme was incubated for 10 min with NAD^+ and oxalacetate and buffer. To initiate the reaction NADH was added. Oxalacetate was used constant at $1.67 \cdot 10^{-4}$ M. NAD^+ was used constant at $10 \cdot 10^{-4}$ M. NADH concentration ranged from $3.3 \cdot 10^{-5}$ to $6.67 \cdot 10^{-4}$ M.

Fig. 2. The data of the low concentration portion of Fig. 1 are represented as a double reciprocal plot; a, b, c, d are the same as in Fig. 1.

Effect of NAD^+ on NADH oxidation by solubilized and partially purified mitochondrial enzyme from sweet lemon

As anticipated in case of product inhibition, NAD^+ inhibition appeared to be competitive with respect to NADH. However, by incubating the enzyme with NAD^+ prior to the addition of NADH, uncompetitive inhibition was obtained. This phenomenon is reported in Figs. 1 and 2 and the studies have been repeated 3 times. It is interesting to note, as seen in Figs. 1 and 2, that the incubation of the enzyme either with NADH alone, with NAD^+ added later with oxalacetate or with NAD^+ + NADH simultaneously with the reaction initiated later by addition of oxalacetate, resulted in competitive inhibition. However, the incubation of the enzyme with NAD^+ and oxalacetate with the reaction initiation with NADH later caused uncompetitive inhibition.

Thus, it seems that both NAD^+ and oxalacetate are responsible for the change in kinetic pattern of the enzyme from competitive to uncompetitive.

DISCUSSION

There was no significant difference observed between the sour and sweet lemon fruits in the enzyme distribution between soluble and mitochondrial fractions. The mitochondrial enzyme accounted for only 22–36% of the total depending on oxalacetate level. PIERPOINT[18], studying the distribution of malate dehydrogenase among components of tobacco leaf extracts, also found that over half (55–70%) of the enzyme was recovered in the supernatant fraction from which the enzyme could not be sedimented by additional centrifugation at 25 000 × *g* for 2 h. However, the results obtained here showed that the ratio of the soluble enzyme to the mitochondrial one is dependent on the oxalacetate concentration at which the activity was assayed since in contrast to reports that only mitochondrial enzyme from animal tissues is inhibited at high oxalacetate concentrations[4–7] it was found that both the soluble and mitochondrial enzymes from the 2 lemon fruits were inhibited by oxalacetate, the difference being only in the magnitude. CASSMAN AND ENGLARD[19] reported that oxalacetate inhibition of beef heart supernatant malate dehydrogenase took place at pH values below 7.8. Also, it was recently reported that the ionic strength of the reaction mixture can modify the inhibition at high oxalacetate levels[6]. In this study, there was also some evidence that the kind of ionic environment did affect the inhibition at high oxalacetate concentrations.

Kinetic properties showed definite differences between the intact mitochondrial fractions of the 2 lemons. Intact mitochondria from sweet lemon showed the lowest K_m for NADH, even lower K_m than that of the soluble fraction from either sour or sweet lemon. Intact mitochondria from sour lemon had a K_m value for NADH about 5 times greater than that of the intact sweet mitochondria. It has been suggested that soluble enzyme in mammalian tissues is for malate formation, whereas the mitochondrial enzyme is for oxalacetate production[4]. However, the results of the present study indicate that not only the soluble enzyme from both fruits, but the intact mitochondrial enzyme from sweet lemon favors NADH oxidation (low K_m) and thus malate formation. On the other hand, the intact mitochondrial enzyme from sour lemon is less favorable for NADH oxidation which could lead to more oxalacetate (a substrate to form citrate). Solubilization and partial purification of mitochondrial malate

dehydrogenase almost caused the alleviation of this difference. These observations could be speculatively connected to citric acid accumulation in sour lemons.

Three significant phenomena have been observed. First, the affinity of the enzyme for NADH was not changed either at the 2 oxalacetate concentrations which were used or with the type of buffer. The inhibition at the higher level of oxalacetate was also observed in the soluble enzyme as well as in the mitochondrial one, and the difference was in degree only rather than in type.

Second, the phosphate buffer seemed to alleviate the higher oxalacetate level inhibition for NADH oxidation, and even increased the apparent maximal velocity at the higher oxalacetate level more than that at the lower oxalacetate level in Tris–HCl. These 2 observations are self-explanatory since it seems that oxalacetate inhibition for NADH oxidation at the higher concentration used in this study is strictly of a noncompetitive type and phosphate buffer seemed to alleviate most but not all of the inhibition.

Third, in the case of oxalacetate reduction, it was noticed that phosphate buffer increased the apparent maximal velocity and decreased the affinity of the enzyme for oxalacetate when compared with Tris–HCl buffer. The explanation of this observation is rather complicated, however, it is very similar to the effect reported for NaCl (0.02 M or more) on oxalacetate reduction by pea seed soluble malate dehydrogenase[20]. To explain the effect of NaCl several hypotheses were developed. In agreement with WEIMBERG[20], the results reported here rule out any connection between the oxalacetate substrate inhibition and effect of phosphate buffer on oxalacetate reduction since neither phosphate buffer nor oxalacetate altered the affinity of the enzyme for NADH. A possible explanation is that certain ionic species might have some effect on the tertiary and/or quarternary structure of the enzyme, thus producing such kinetic behavior. In support of this view is the recent finding reported by SIEGEL[21] that under certain conditions the mitochondrial malate dehydrogenase of bovine heart muscle was reversibly dissociated to subunits of almost 25% of the molecular weight of the native enzyme.

Studies on the effect of adenosine nucleotides (AMP, ADP, and ATP) showed that AMP had the greatest inhibitory effect on NADH oxidation by solubilized mitochondrial enzyme from the 2 lemon fruits. The results of the present study showed that within the range of NADH levels where the reaction is of a first order rate, the inhibition seems strongly competitive. However, as the levels of NADH favor zero order rate, the inhibition by AMP was never completely overcome as was anticipated. The observation that 3′-adenylic acid was of little or no effect indicates that a 5′-adenosine phosphate moiety was essential for the manifestation of adenosine nucleotide inhibition and this might be of some value in assessing the mechanism of adenosine nucleotides effects.

Studies on the effect of NAD^+ on NADH oxidation by solubilized mitochondrial malate dehydrogenase revealed an interesting observation, *i.e.* if the enzyme was preincubated either with NADH (with NAD^+ added later with oxalacetate), or with NAD^+ + NADH simultaneously (with the reaction initiated later by oxalacetate), a competitive inhibition was observed. However, the incubation of the enzyme with NAD^+ and oxalacetate (with the reaction initiated later with NADH) resulted in an uncompetitive inhibition. It can only be concluded that some conformational change in the enzyme protein might have been initiated by NAD^+ in the presence of oxal-

acetate or possibly NAD^+ slowly became attached to a second site on the enzyme, thus resulting in the alteration in the kinetic behavior from competitive to uncompetitive.

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