# Effects of Ionic Concentration and pH Upon the State of Aggregation of Neurospora Mitochondrial Malate Dehydrogenase

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Summary:

Five active isoenzymes of Neurospora mitochondrial malate dehydrogenase of 105,000, 91,000, 78,000, 65,000 and 39,000 daltons were observed when the enzyme was extracted from mycelia and centrifuged in a sucrose gradient with 5 mM tris-Cl at pH 9. Only one active species of 65,000 daltons was observed in either 100 mM tris-Cl, pH 9, or 5 or 100 mM sodium citrate at either pH 4 or 6. The addition of 10 mM of either $MgCl_2$ or $CaCl_2$ to the 5 mM tris-Cl pH 9 buffer reversed the aggregation and led to the occurrence of only the 65,000 daltons species. The addition of either 10, 50 or 100 mM KCl to the 5 mM tris-Cl pH 9 buffer yielded 4, 3 and 1 isoenzymes, respectively. The latter's molecular weight was 65,000. Thus, in alkaline solution, monovalent cations at 100 mM and divalent cations at 10 mM prevent the formation of multiple molecular weight species.

Mitochondrial malate dehydrogenase (m-MDH) in Neurospora crassa forms a multiple molecular weight series in vitro (1). The multiplicity of isoenzymes is clearly dependent upon the ionic environment of the enzyme (1). In solutions of high ionic concentration, there exists only one isoenzyme but as the ionic concentration decreases, the number of isoenzymes increases. In solutions of very low ionic concentrations as many as 15 isoenzymes were found. The experiments described here define some ionic and pH conditions which control the aggregation of the isoenzymes.

## Materials and Methods

Enzyme grade sucrose was from Mann Research Lab; L-malic acid, NAD (grade III) and type I bovine blood hemoglobin (twice recrystallized) from Sigma Chemical Co.

Wild-type Neurospora crassa (74A, Fungal Genetics Stock Center No. 936, Humbolt College, Arcata, California) was stored at 4° on silica gel (2). Periodically, new cultures were established on Fries' minimal agar (3).

Cultures of wild-type N. crassa were grown for 16 hours under the conditions previously described (4). Mitochondrial MDH was extracted from the mycelia (4) in the presence of either Na citrate buffer (pH 4.0 or pH 6.0) or tris-Cl buffer (pH 9.0 at 4°).

Linear sucrose (enzyme grade) density gradients from 5 to 20% were formed with a Büchler apparatus. The sucrose solutions were made with either Na citrate (pH 4.0 or 6.0) or tris-Cl (pH 9.0 at 4°) buffer. $MgCl_2$, $CaCl_2$ or KCl were added to the buffer as indicated in the figure legends. An SW 65 Ti rotor in a Spinco L2-65B centrifuge was used to centrifuge the gradients at 178,000 g for 16 hours at 4°. Thirty-eight to 40 fractions, of eight drops each, were collected from the bottom of each gradient. Fractions were assayed for malate dehydrogenase (MDH) activity (1), diluted with 0.8 ml of distilled water, and measured for optical density at 410 nm for location of the hemoglobin marker. Molecular weights were determined by the method of Martin and Ames (5) relative to the marker of 65,000 daltons.

## Results

All of the isoenzymes observed here clearly fit the

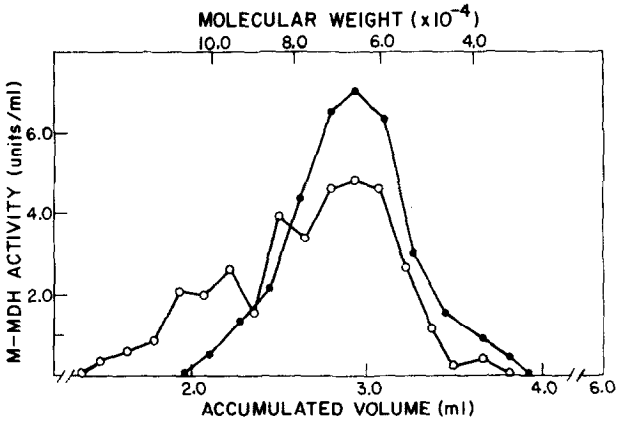


Figure 1. The effect of ionic concentration and pH on the molecular weight distribution of m-MDH.
Linear sucrose density gradients (5-20%) were formed with solutions sodium citrate, pH 4.0 or 6.0 or tris-Cl, pH 9.0 (at 4°). Hemoglobin (50 µg) and 8 units of m-MDH activity were centrifuged at 178,000 g for 16 hours at 4°. Fractions were collected from the bottom of the gradient and assayed for MDH activity. The optical density at 410 nm of the fractions determined the localization of the hemoglobin. Activities and volumes were normalized. Curve (●-●) represents m-MDH activity in gradients formed with one of the following buffers: 100 mM citrate pH 4.0 or 6.0; 5 mM citrate pH 4.0 or 6.0; or 100 mM tris pH 9.0 (at 4°). Curve (○-○) represents m-MDH activity in gradients formed with 5 mM tris pH 9.0 (at 4°) buffer.

molecular weight series that we shall describe in detail elsewhere (1). The weight of each isoenzyme was within 2,000 daltons of a species predicted by the weight series. Therefore, the molecular weights reported here were rounded off to fit the appropriate weight class.

When MDH was extracted and centrifuged in the presence of 100 mM buffer, a single isoenzyme weighing 65,000 daltons was observed with either pH 4.0 citrate or pH 6.0 citrate or pH 9.0 tris buffer (Fig. 1). In contrast, a pH effect was observed when the tris buffer concentration was lowered to 5 mM (Fig. 1). In the acidic citrate buffers MDH contin-

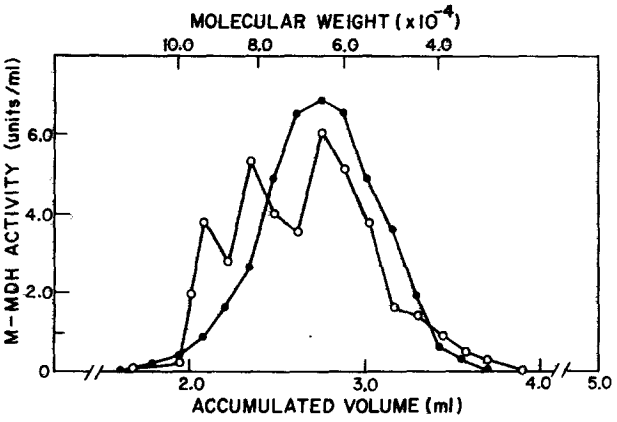


Figure 2. The effect of cations on the molecular weight distribution of m-MDH.
The procedure described in Figure 1 was used to analyze the molecular weight distribution of m-MDH in the presence of a 5 mM tris pH 9.0 (at 4°) buffer containing 10 mM $MgCl_2$ or $CaCl_2$ (●-●) or 10 mM KCl (0-0).

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ued to exist as a single species of 65,000 daltons, but in the basic tris buffer, we observed five isoenzymes with molecular weights of 105,000, 91,000, 78,000, 65,000 and 39,000. If either 10 mM $MgCl_2$ or $CaCl_2$ was added to the 5 mM tris pH 9.0 buffer which was used in the extraction and gradient solutions a single component of 65,000 was observed (Fig. 2). The addition of KCl to the 5 mM tris pH 9 buffer reduced the number of isoenzymes. However, KCl was not as effective as $MgCl_2$ or $CaCl_2$ (Fig. 2,3). There were four isoenzymes in the presence of 10 mM KCl (91,000, 78,000, 65,000 and 52,000); three isoenzymes in 50 mM KCl (78,000, 65,000 and 52,000) and only one isoenzyme in 100 mM KCl (65,000).

## Discussion

The ionic concentration influences the number and size of isoenzymes of MDH of *N. crassa* (1). The present results

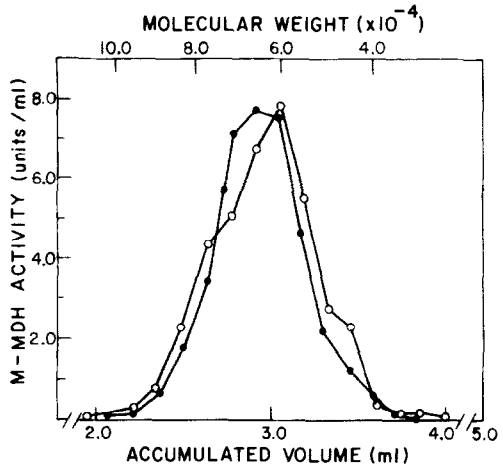


Figure 3. The effect of KCl on the molecular weight distribution of m-MDH.
The procedure described in Figure 1 was used to analyze the molecular weight distribution of m-MDH in the presence of a 5 mM tris pH 9.0 (at 4°) buffer containing 50 mM KCl (0-0) or 100 mM KCl (●-●).

substantiate this observation and demonstrate that the multiplicity of isoenzymes is dependent not only on ionic concentration but also on pH. High molecular weight aggregates existed only when the pH was high and the ionic concentration was low. We have not eliminated the possibility that the failure to observe multiple isoenzymes in 5 mM acidic buffers may have been a direct response to the presence of citrate ion rather than pH.

Divalent cations readily reversed the trend toward high molecular weight aggregates. Higher concentrations of monovalent than divalent cations were required to achieve comparable states of aggregation. The molecular weight of MDH of Lemna minor is also specifically dependent on the cation concentration (6). A low calcium ion concentration favored the occurrence of the smaller isoenzymes and high concentrations favored the occurrence of the larger isoenzymes.

The activity of the larger isoenzymes was maximal in ionic conditions which were optimal for the aggregation. Other divalent cations substituted for calcium but not as effectively. These changes in the state of aggregation may allow the organism to physiologically adapt to various calcium concentrations in the environment.

It is believed that the α and β subunits of m-MDH (7,8) aggregate asymmetrically to form a sheet or filament (1). To test this hypothesis and to ascertain the role of the α and β subunits in the aggregation phenomenon a pure preparation of one or more high molecular weight isoenzymes is needed. Although the molecular weight of N. crassa MDH is clearly dependent upon pH and ionic concentration, no specific combination of these parameters has been found yet which enable the enzyme to exist as a single high molecular weight species greater than 65,000.

Acknowledgments

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