Enzymatic studies on the muscle of aquatic animals.
5. Purification and properties of malic enzyme from molluscan muscle

By Takeshi Shibata, Tadashi Kitahara and Katsuji Yoshimura

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Takeshi SHIBATA*, Tadashi KITAHARA* and Katsuji YOSHIMURA*

Abstract

1. The existence of malic enzyme (EC 1.1.1.40 l-malate: NADP oxidoreductase), has been discovered in the muscle extract from mollusca. A procedure for the preparation of purified enzyme is presented.

2. After extracting with potassium chloride solution, purification was carried out with fractional precipitation by ammonium sulphate, adsorption with calcium phosphate gel and fractionation by DEAE-cellulose column.

3. In squid muscle (Ommastrephes sloani Pacificus), the final product had a ten-times higher specific activity. Optimum pH is 7.1-7.2. It is stable between pH 6.5 and 7.5 at 30°C, but unstable at temperatures above 35°C. The enzyme is activated by Mg²⁺ and Mn²⁺ ions. Michaelis constant is 1.33 x 10⁻⁵ M for malate and 0.66 x 10⁻⁴ M for NADP.

4. In Scallop (Pecten yessoensis), the enzyme has been purified three-fold. The behaviour of it on a DEAE-cellulose column was shown to differ from that of squid. Other properties were the same as that of squid, except there was less stability.

5. The significance of the malic enzyme in mollusca was discussed.
Enzymatic Studies on the Muscle of Aquatic Animals

5. Purification and properties of malic enzyme from moluscan muscle

Takeshi SHIBATA, Tadashi KITAHARA* and Katsuji YOSHIMURA*

Pyruvic acid will accumulate as the final product of moluscan muscular glycolysis. (1) This pyruvic acid can be utilized in a significant way: i.e. The speed at which pyruvic acid is eliminated from the reacting system will be the restricting factor in the glycolytic process. But pyruvic acid is not directly taken in by the TCA-cycle of the muscles in Omastrephes sloani pacificus (squids) in its original form. (2) To be taken into the TCA-cycle, pyruvic acid must become acetyl Co-A by way of decarboxylation, and then condensed with oxaloacetic acid to become citric acid. The circuit of condensatory enzyme in TCA-cycle will continue to function in cyclical manner until all oxaloacetic acid is consumed, when catalytic amount of oxaloacetic acid or its preceding compound --- C4-Carboxylic acid --- is added to the process. The reaction process, caused by the addition of oxaloacetic acid or C4-carboxylic acid as sparker, can be either of the following:

\[
\text{Phosphoenolpyruvic acid} + \text{CO}_2 + \text{IDP} \ (\text{or GDP}) \Leftrightarrow \text{Oxaloacetic acid} + \text{ITP} \ (\text{or GTP}) \rightarrow (1)
\]

\[
\text{Pyruvic acid} + \text{CO}_2 + \text{NADPH} \Leftrightarrow \text{Malic acid NADP} \rightarrow (2)
\]

The former is phosphopyruvate carboxylase, and the latter is malic enzyme.

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The authors of this paper confirmed the existence of malic enzyme (EC 1.1.1.40, l-malate : NADP Oxidoreductase) when the following experimentation was conducted under the assumption that pyruvic acid may be used for carboxylation, an assumption which was based on the fact that there was a marked Bohr effect (3) in the blood of Ommatrephe locomni pacificus. This existence of malic enzyme had prompted the present authors to conduct a research concerning the characteristics and purifying method of malic enzyme, which is suspected to have a significant role in the combination of TCA-cycle and glycolytic process.

Malic enzyme was first indentified by Ochoa (4) in the livers of pigeon, and later, also in plants (5) and insects (6). The characteristics of this enzyme was described in detail by Ochoa (7) - (12) and Kun (13). According to their description, Malic enzyme has a double characteristic: firstly, of producing pyruvic acid and CO₂ in a reversible process of decarboxilation - oxidation of l-malate with NADP as its co-factor, and secondly of decarboxylating oxaloacetic acid. However, malic enzyme does not react of NAD, d-malate, fumaric acid, and phosphoenolpyruvic acid. As an exceptional case, the malic enzyme of Ascaris (14) has NAD as its co-factor, but it does not decarboxylate oxaloacetic acid. However, because the attempts to separate these two different kinds of malic enzyme have not proved successful, it is generally recognized that malic enzyme has the double characteristics described above. Also, according to Hiatt (16) malic enzyme is considered as one of the factors that adjust the production process of oxaloacetic acid from malate through the TCA-cycle, with a role in transforming malate into pyruvic acid which is required in the synthesis of liver.
glycogen. In spite of the significance of malic enzyme, its physical and chemical characteristics have not been well established, except Rutter and Lardy's (17) attempt at refining it from pigeon liver.

Experiment

As the material, the present authors used the fresh squids (Ommastrephes sloani pacificus) and scallops fished in the ocean near Hakodate and sold in the market place. For extraction the authors added the solution of 0.15 M potassium chloride containing 10^{-3} M EDTA purified 9 times, homogenized for one minute with a homogenizer manufactured by Nippon Precision Machine Manufacturers, and subjected the solution to centrifugal separation for 10 minutes at 4000 rpm. The upper layer of the solution, which was separated from the sediment, was used as the enzyme solution. The purification operation was always conducted under 4°C temperature, and DEAE-cellulose and CM-cellulose were used in the ion-exchange chromatography. Then it was washed with hydrochloric acid and caustic soda, and then, after being cleansed with water, it was used with respective buffer solutions. For specific enzyme activity, a variation of the Faulkner Method (6) was adopted. The total volume of reaction solution was specified at 3.0 ml, adding the enzyme solution to either Tris-buffer (pH 7.4) or phosphate-buffer (pH 7.2) 200 |mu| moles, MgCl₂, 20 |mu| moles, NADP 0.3 |mu| mole, l-malate 10 |mu| moles. Activity was indicated by the absorbency change of 340 m|mu| of NADP and \( \Delta E_{340 \, m|mu|} = 0.01/\text{min} \) was designated as the enzymatical unit. The reaction temperature was measured at room temperature, and protein content was measured at the absorption of 280 m|mu|. To measure
the absorption of ultraviolet ray, spectrophotometer model Or-50 by Shimazu Manufacturing Co. was used. The L-malate oxaloacetic acid, and other test chemicals, which were used as substrate, were purchased from ordinary market and neutralized to pH 7.0 before use. For auxiliary enzyme, NBO products were used without alteration. For the measurement of the activity of malic dehydrogenase, the method of Delbaëck etc.(18) was adopted. Purification of lactic dehydrogenation enzyme followed the method of Beisenherz et al(19). Pyruvic acid was quantitatively measured by the Friedeman and Haugen method (20) and enzymatical method.(21) The measurement of oxaloacetic acid followed the Friedeman method(22). Finally, the chromatography of keto acid was produced according to Kazuki-Kanayuki method.(23)

Results:

(1) Confirmation of the Existence of malic enzyme:

It was recognized that the reduction of NADP and the emergence of pyruvic acid will occur when L-malate and NADP were added to the solution of extracted squid muscle and 0.15M KCl. As evidence, Table 1 shows the result when L-malate is used as the substrate. From the table, it is clear that while NADP is reduced, NAP is almost entirely non-reduced. Table 1 also shows the results of pyruvic acid measurement by way of producing dinitrophenyl hydrazone compound after deproteinizing the post-reaction solution with trichloroacetic acid. Although oxaloacetic acid can be decomposed into pyruvic acid when treated with nickel sulphate, Table 1 shows that the result does not alter if oxaloacetic acid is treated instead
with or without NiSO₄. Table 1 also shows that whether in the extraction of hydrazone or in the extraction of ethyl acetate or benzene, the results are all the same:

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\[ \text{Table 1. Formation of pyruvate by squid malic enzyme} \]

Reaction mixture contained in a final volume of 3.0 ml: phosphate buffer (pH 7.2) 100 μmoles; MgCl₂ 10; NADP 0.28; L-malate 50; methylene blue 1.6 and enzyme 162 units (ammonium sulphate fraction). Incubation Temp. 30°C

<table>
<thead>
<tr>
<th>Reaction time (min)</th>
<th>Colorimetrically²⁸</th>
<th>Enzymatically²¹</th>
<th>NiSO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.73</td>
<td>0.79</td>
<td>0.657</td>
</tr>
<tr>
<td>40</td>
<td>1.04</td>
<td>1.04</td>
<td>0.921</td>
</tr>
<tr>
<td>60</td>
<td>1.23</td>
<td>1.34</td>
<td>1.110</td>
</tr>
</tbody>
</table>

*) These values are expressed as μmoles per ml of reaction mixture

in all cases, the final product is pyruvic acid. In the paperchromatography of hydrazone of Keto acid, no oxaacetic acid was recognized. The results
of enzymatic analysis using the peculiar properties of muscular lactic dehydrogenation enzyme also coincide with the result of colorimetric analysis, confirming that the final product was indeed pyruvic acid. Table 2 shows that pyruvic acid increases in its volume parallel to the acceleration of turn-over in the NADP of methylene blue. In the crude extracted solution, fumaric acid is not linked to this reaction. The malic dehydrogenase of mitochondria related to TCA cycle is not connected to the reaction because of the absence of fumarase, which is necessary in producing malate from fumaric acid. Reversed reaction was recognized in the existence of pyruvic acid, NADPH, and sodium bicarbonate. The result, as shown in Table 2,

Table 2

<table>
<thead>
<tr>
<th>Concentrations of methylene blue (μmole/ml*)</th>
<th>with NiSO₄</th>
<th>without NiSO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.22</td>
<td>0.21</td>
</tr>
<tr>
<td>0.4</td>
<td>0.51</td>
<td>0.49</td>
</tr>
<tr>
<td>0.8</td>
<td>0.80</td>
<td>0.79</td>
</tr>
<tr>
<td>1.2</td>
<td>1.15</td>
<td>1.15</td>
</tr>
<tr>
<td>1.6</td>
<td>1.36</td>
<td>1.35</td>
</tr>
</tbody>
</table>

*) These values are expressed as μmoles per ml of reaction mixture

is the oxidation of NADPH. It is suspected that the existence of malic enzyme was not confirmed because of the advanced stage of reversed reaction. Never-
theless, this reaction requires high enzyme density and long reaction time, and is difficult to take place. Malic enzyme can be decomposed into pyruvic acid and carbon dioxide through decarboxylation of oxaloacetic acid. Table 3 shows that in the muscle of Ommastrephes sloani pacificus the same process takes place: pyruvic acid is created from oxaloacetic acid. In 60 minutes, most of the oxaloacetic acid, which constitutes the substrate, is decarboxylated by enzyme. Under this condition, the autolysis of oxaloacetic acid takes place, and pyruvic acid is formed. What appears as the chemical reaction of malic enzyme may well be the congruent reaction of malic dehydrogenase which is mixed with malic enzyme, and oxaloacetic acid which is undergoing autolysis. This point will be discussed later.

From the chemical reactions described above, it was confirmed that pyruvic acid is formed directly from malic acid, and that malic enzyme does indeed exist.

(2) The purification of malic enzyme:

(A) Method of purification from the muscle of Ommastrephes sloani pacificus:

Incubate the solution of extracted squid muscle and 0.15 M KCl for 10 minutes at 30°C, subject it to centrifugal separation at 6000 rpm for 10 minutes, then

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Autolysis</th>
<th>Decarboxylation by enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>colorimetrically</td>
<td>enzymatically</td>
</tr>
<tr>
<td>20 (min)</td>
<td>μmole/ml</td>
<td>μmole/ml</td>
</tr>
<tr>
<td></td>
<td>1.22</td>
<td>1.42</td>
</tr>
<tr>
<td></td>
<td>1.69</td>
<td>1.74</td>
</tr>
<tr>
<td></td>
<td>1.93</td>
<td>1.91</td>
</tr>
</tbody>
</table>

*) These values are expressed as μmoles per ml of reaction mixture.
collect the portion of the separated solution which is to be subjected to fractional precipitation with 30 - 60% saturated ammonium sulphate, dialyse it over night with 0.01M phosphate buffer pH 7.0 in order to eliminate the ammonium sulphate, absorb it with calcium phosphate gel, and dialyse it with 0.2M phosphate buffer at pH 7.0. Dialyse the calcium phosphate gel solution with 0.01 M phosphate buffer (pH 7.0) for one night, then put it through DEAE-cellulose column to fractionate it by Stepwise method. Malic enzyme can be fractionated by 0.2 M phosphate buffer (pH 7.0). Fig. 3 shows the result of the above operations. The relative activity of this part was about 10-11 times that of the test chemicals (the maximum relative
activity is 653.6). One example of the purification method is indicated in Table 4. In the case of calcium phosphate gel, the loss in activity is

**Table 4**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Volume (ml)</th>
<th>Activity (unit/ml)</th>
<th>Total unit</th>
<th>Protein (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Specific activity</th>
<th>Yield (%) Protein</th>
<th>Yield (%) Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial extract</td>
<td>825</td>
<td>252</td>
<td>207900</td>
<td>7.32</td>
<td>6039</td>
<td>34.4</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Incubation at 30°C for 10 min.</td>
<td>820</td>
<td>188</td>
<td>154120</td>
<td>5.31</td>
<td>4255</td>
<td>61.9</td>
<td>72</td>
<td>74</td>
</tr>
<tr>
<td>30-60% ammonium sulphate ppt.</td>
<td>72</td>
<td>1485</td>
<td>106920</td>
<td>29.6</td>
<td>2131</td>
<td>103</td>
<td>35</td>
<td>52</td>
</tr>
<tr>
<td>Treatment with calcium phosphate</td>
<td>48</td>
<td>586</td>
<td>28129</td>
<td>3.33</td>
<td>160</td>
<td>183</td>
<td>2.6</td>
<td>14</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>10</td>
<td>231</td>
<td>2910</td>
<td>0.49</td>
<td>4.9</td>
<td>654</td>
<td>0.08</td>
<td>1.4</td>
</tr>
</tbody>
</table>

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larger: the maximum relative activity in calcium phosphate gel is 200, and the average relative activity is approximately 100. However, in the case of ommastrephes sloani pacificus, the white turbidity caused by protein frequently poses an obstacle to the measurement of activity. This white turbidity can be eliminated with calcium phosphate gel. Later, it was discovered that this white turbidity in the extracted solution can also be eliminated with 10 minutes of super-speed centrifugal separation at 50,000 rpm. The existence of phosphate stabilizes the activity, and the stability is unaffected even when malic enzyme is subjected to dialysis. In the two salting-out operation with ammonium sulphate, no marked increase in relative
activity was recognized. Although activity is maintained when malic enzyme is in ammonium sulphate, the existence of ammonium sulphate makes it more difficult to measure activity.

B. Method of purifying malic enzyme from the muscle of scallop (pecten yessvenesis):

To purify malic enzyme from the muscle of scallops, the present authors applied the method of purification used to obtain malic enzyme from squid muscle. Fig. 4 and Table 5 shows the method of purification on DEAE-cellulose-column and a general method of purification. However, when scallop muscle was used to obtain malic enzyme, the relative activity of the purified product increased only about threefold, and on DEAE-cellulose column the malic enzyme was fractionated with 0.1M phosphate buffer (pH 7.0).

**Fig. 4**

![Chromatography of scallop malic enzyme on DEAE-cellulose column at 4°C, pH 7.0](image)

Experimental condition was the same as in Fig. 3
Table 5

Table 5. Purification of malic enzyme from scallop muscle

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Volume (ml)</th>
<th>Activity (unit/ml)</th>
<th>Total unit (mg)</th>
<th>Protein (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Specific activity</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial extract</td>
<td>770</td>
<td>48</td>
<td>36960</td>
<td>7.09</td>
<td>5465</td>
<td>13.5</td>
<td>100</td>
</tr>
<tr>
<td>Treatment with calcium phosphate</td>
<td>450</td>
<td>24</td>
<td>10800</td>
<td>2.64</td>
<td>1184</td>
<td>36.5</td>
<td>21.7</td>
</tr>
<tr>
<td>30-60% ammonium sulphate ppt.</td>
<td>38</td>
<td>18</td>
<td>684</td>
<td>6.6</td>
<td>246</td>
<td>11.1</td>
<td>4.5</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>10</td>
<td>1.8</td>
<td>180</td>
<td>3.3</td>
<td>33</td>
<td>41.0</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Thus, the result shows considerable difference from the purification of malic enzyme from squid muscle. This difference seems to have arisen from the difference in the protein contents of the two muscles, squid muscle being smooth muscle and scallop muscle being striated muscle. The stability of enzyme also differs in two cases: The malic enzyme purified from scallop muscle was found to be more unstable than the malic enzyme purified from squid muscle.

The two kinds of malic enzyme purified on DEAE-cellulose column, kept under-20°C freezing storage, both had stable activity for at least several months. But repeated freezing and thawing will cause the loss of stability. The present authors had kept the column-effluence in frozen storage for use of research. CM-cellulose flowed away without being absorbed (pH 7.0).
Properties of Malic Enzyme:

3. Optimum pH value

1. Activity of malic enzyme was measured by using phosphate buffer and tris buffer, results of which are shown in Fig. 5. As is clear from the diagram, activity is greatest in the vicinity of pH 7.1 - 7.2. This is the optimum pH value of very similar to the malic enzyme obtained from pigeon liver. Figure 6 shows the result of oxaloacetate decarboxilation of squid malic enzyme, when oxaloacetic acid was used as the substrate and measured with Warburg vessel. As is shown in Fig. 6, the optimum pH value for decarboxilation is 6.8, as distinct from the optimum the malic enzyme of pigeon liver. In the case of squid, muscle malic enzyme, the optimum pH value found in the process of decarboxilation was quite close to the optimum pH value measured by the absorption of NADPH.

Fig. 5

Fig. 6
2. pH Stability of Squid Malic Enzyme:

Measurement of activity was performed by exposing the enzyme with phosphate buffer, acetate buffer, and Tris buffer, respectively, for two hours at 30°C to the given pH values, and then neutralizing them to pH 7.2 for activity testing. The results are shown on Fig. 7. The pH stability is greatest in the vicinity of pH 6.5 - pH 7.5.

3. Effect of Metal Ions on Scallop Malic Enzyme:

Malic enzyme of scallop (pecten yessoensis) muscle requires metals such as Mn²⁺, Mg²⁺, Co²⁺. Their activities were measured by using the 2-atomic-value ions contained in MnCl₂, MgCl₂, CoSO₄, and CaCl₂. The results of these measurements are shown in Table 6. When the enzyme activity obtained by the standard activity test is given as 100, it is found that the Mn²⁺ ions activate the enzyme most greatly: 110% at 3 x 10⁻⁵M, 148% at 1 x 10⁻⁴M. At 2 x 10⁻¹M the activity is at its maximum of 228%, then the enzyme activity declines as the density is further increased.

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Table 6

<table>
<thead>
<tr>
<th>Metal ion (10⁻¹ M)</th>
<th>Ratio of activity</th>
<th>Metal ion (10⁻³ M)</th>
<th>Ratio of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Assay (Mg²⁺: 6.6)</td>
<td>100</td>
<td>Co²⁺</td>
<td>0.03</td>
</tr>
<tr>
<td>No addition</td>
<td>0</td>
<td>0.10</td>
<td>81</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.16</td>
<td>0.23</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>0.33</td>
<td>0.33</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.6</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>2.3</td>
<td>4.6</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>109</td>
<td>109</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>0.03</td>
<td>0.23</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>0.33</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>3.3</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>136</td>
<td>136</td>
</tr>
</tbody>
</table>
155% at $3 \times 10^{-4}$M, 136% at $1 \times 10^{-3}$M, approaching a plateau. In the case of Mg$^{2+}$ ions, it is 18% when the density is at $2 \times 10^{-4}$M, lower than that of standard activity test condition rising slowly to 64% at $1 \times 10^{-3}$M, to 109% at $1 \times 10^{-2}$M. In case of Co$^{2+}$ ions the maximum activity is obtained at $2 \times 10^{-4}$M (109%) and starts to decline when density exceeds $2 \times 10^{-4}$M.

In the case of Ca$^{2+}$ ions, no significant activation was recognized. As in the case of squid malic enzyme, scallop malic enzyme's activity is stabilized by use of Mn$^{2+}$, Mg$^{2+}$, and Co$^{2+}$ ions, results of which are shown in Fig. 8.

**Fig. 7**

*Fig. 7. pH stability of squid malic enzyme*

The enzyme was exposed for 2 hours to the pH values given and the activity then tested at pH 7.2. pH 4.4-5.6, acetate buffer; pH 6.0-7.3, phosphate buffer; pH 8.4-9.2, Tris buffer.

**Fig. 8**

*Fig. 8. Effect of metal ions on scallop malic enzyme*

Experimental condition was the same as in Table 6, except that phosphate buffer was used save for Mn$^{2+}$.

---

As shown in the diagram, the effect of Mn$\text{Cl}_2$ on enzyme activity rises sharply.
up to 1 μ mole, then declining slightly after 1 μ mole and reaching a plateau up to 5 μ moles. The effect of CoSO₄ on enzyme activity rises more gently, that of MgCl₂ rises in a straight line, and that of CaCl₂ reaches its maximum at the vicinity of 0.5 μ mole and then shows no marked change afterwards. The activity of malic enzyme from scallop muscle is also stabilized by Mn²⁺ ions, as is the case with the activity of squid muscle malic enzyme.

4. Heat Stability of Malic Enzymes:

Fig. 9 shows the results of activity tests conducted on the sediment which is separated from the enzyme solutions after they are exposed to a given temperature at pH 7.0 for 60 minutes and then immediately followed by cooling-off to 0°C with ice water. Activity tests were conducted at different temperatures. In the case of malic enzyme purified by way of DEAE-cellulose procedure, the coexistence of malic enzyme activity and malic dehydrogenase activity was always recognized. The above-described experiment was conducted as one of the means to tell whether or not malic enzyme and malic dehydrogenase are the same element with identical properties. As a result, it was discovered that although the activity of both malic enzyme and malic dehydrogenase are stable until the temperature reaches 30°C, malic dehydrogenase loses stability rapidly as the temperature approaches 35°C and becomes almost completely inactive at 40°C. On the other hand, malic enzyme's activity is 84% at 35°C, and even at 40°C it still has an enzyme activity of about 50%, showing a greater stability in the enzyme activity then malic dehydrogenase.
The enzyme was exposed for an hour to the temperatures given at pH 7.0 and then cooled to 0°C. Other experimental condition was the same as in the text.

5. Michaelis Constant:

The effect of substrate density on the speed of reactions which lead to the formation of malic enzyme and malic dehydrogenase was measured in the following manner. In the case of malic enzyme, l-malate and NADP, which constitute the substrates, were chosen, and then reaction speeds were measured at different density of these substrates. The same operation was performed on oxaloacetic acid and NADPH, which constitute the substrate of malic dehydrogenase. Table 7 shows the results of this experiments. As shown in that table, the Km (the values of Michaelis constant) of l-malate and NADP in malic enzyme was, respectively, $1.33 \times 10^{-3}M$ and $0.66 \times 10^{-3}M$, and the Km of oxaloacetic acid and NADPH in malic dehydrogenase was, respectively, $0.64 \times 10^{-3}M$ and $0.93 \times 10^{-3}M$. 

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Table 7

Table 7. Michaelis constant of squid malic enzyme and malic dehydrogenase at pH 7.0

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>MgCl₂ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malic enzyme</td>
<td>0.66 x 10⁻⁵ M</td>
</tr>
<tr>
<td>NADP</td>
<td>0.64 x 10⁻⁵ M</td>
</tr>
</tbody>
</table>

Observations

In this enzyme system, malic dehydrogenase is stronger than malic enzyme. The presumption that the formation of pyruvic acid is not due to the existence of malic enzyme, but is due to the existence of malic dehydrogenase, a presumption based on the fact that malic dehydrogenase forms oxaloacetate and oxaloacetate in turn directly decomposes into CO₂ and pyruvic acid, shall be denied in the experiment which is due for publication in the next issue of this Bulletin. In that experiment, it will be shown that malic enzyme can definitely be distinguished from malic dehydrogenase; that malic enzyme has a higher specific activity as a cofactor of NAD rather than as the cofactor of NADP; and that malic dehydrogenase has high affinity for NADH. These findings all repudiate the conclusion that pyruvic acid is formed by the reaction process involving malic dehydrogenase. As the reduction speed of NADP shown in Fig. 1 and the value of Km both show that malic dehydrogenase must have abnormally high affinity to NADP, it is therefore, not possible to conceive of the existence of malic dehydrogenase.
having NADP as its auxiliary enzyme system. The working of malic enzyme can be distinctly recognized even when malic dehydrogenase is mixed up with malic enzyme in the purified enzyme which was used in the experiment. Unless the existence of oxaloacetate oxaloacetic carboxylase is confirmed, it is probable that malic enzyme has a dual characteristic.

Certain species of intestinal worms (ascaris) have malic enzyme in their muscles, although Ochoa(4) denies the existence of malic enzyme in ascaris muscles. Squid muscle also contains malic enzyme, but as opposed to ascaris muscle, squid muscle has the capability to decarboxylate oxaloacetic acid. To find out if the existence of malic enzyme in moluscan muscle such as squid muscle is a unique phenomenon comparison was made of the distribution of malic enzyme in squid scallop, and carp muscles. The results of this comparison is shown in Table 8, which seems to suggest that squid muscle does indeed have high content of malic enzyme. One may presume that the existence of malic enzyme is a unique phenomenon in moluscan muscles.

Table 8

<table>
<thead>
<tr>
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<th>Squid units/g. tissue</th>
<th>Scallop units/g. tissue</th>
<th>Carp units/g. tissue</th>
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</table>
| As can be seen from the examples of ascaris and first worm(25), the content of malic enzyme is higher in the more primitive animals. The biological function of malic enzyme is conceivably in the regulation
and adjustment of the speed of TCA-cycle. But its relationship with the accumulation of pyruvic acid in the glycolytic process of squid muscle cannot be more than hypothetical unless the process of carboxylic-acid-fixation is established. It may be more fruitful to relate malic enzyme to phosphopyruvic carboxylase than to follow Hiatt etc (16) in relating malic enzyme to glycogenesis, because in the former process it is not necessary to undergo the process of pyruvate kinase. That malic enzyme is not related to glycogenesis is implied in the report by Shrago et al (26)(27) that malic enzyme and gluconeogenesis are not connected. However, this question remains unsettled since the carboxylase process is not yet confirmed.

In liver and adipose tissue, it was suggested by Lardy etc (28) that malic enzyme is the supply source of NADP required for the synthesis of fat. Also, Lardy etc had conjectured that malic enzyme has a role in lipogenesis by indirectly causing NADH to become NADPH, or by directly forming NADPH, since they recognized the possibility of hydrogen-transfer between NADP and NADPH through the covalence of malic enzyme and --- cytoplasmic malic dehydrogenase (27). Another possible explanation of the above phenomenon is that the high content of fat in squid entrails may have some influence over squid muscle's chemical characteristics.

Summary

The existence of malic enzyme was confirmed by analysing extracted solution of moluscan muscle. The enzymatic properties of purified malic enzyme was studied, using 1-malate as the substrate. After extraction with 0.15M potassium chloride, malic enzyme was purified by fractional precipitation
with 30% - 60% ammonium sulphate, then absorbed by calcium phosphate gel, and fractionation by DEAE-cellulose. The purified squid muscle malic enzyme had a 10 - 11 times higher specific activity. The optimum pH value for squid muscle malic enzyme was 7.1 - 7.2, and activity was most stable in the vicinity of pH 6.5 - 7.5. Its heat stability was found to be greatest in the vicinity of 35°C. The enzyme was activated by metal ions such as Mg²⁺, Mn²⁺, and Co²⁺. The values of Michaelis constant (Km) for 1-malate was 1.33 x 10⁻⁵M, and that for NADP was 0.66 x 10⁻³M. Since malic enzyme was also found to exist in the muscle of scallops (though activity was lower), it can be inferred that malic enzyme has a unique and specific function in moluscan muscles.
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