

## Effect of Eu(III) on the Degradation of Malic Acid by *Pseudomonas fluorescens* and the Subsequent Production of Pyruvic Acid

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We studied the effect of Europium, Eu(III), on the degradation of malic acid by the bacterium *Pseudomonas fluorescens*. Its breakdown depended upon the ratio of Eu(III)- to malic acid-concentrations; the higher the ratio, the less malic acid was degraded. The resulting chemical species of Eu(III), determined by calculations using thermodynamic data, indicated that predominant ones were  $\text{Eu}(\text{Mal})_2^-$  and  $\text{EuMal}^+$ ; free Eu(III) constituted less than 1% of the total Eu(III). The degradation of malic acid was independent of  $\text{Eu}(\text{Mal})_2^-$  concentrations. Even though more than 80% of malic acid was present as free malic acid, its degradation was retarded with an increase in the ratio of Eu(III) to malic acid. According to the speciation calculation, the degradation of malic acid was hindered by the presence of  $\text{EuMal}^+$ , as well as by free Eu(III). We also assessed the amount of pyruvic acid generated, which is the major degradation product of malic acid complexed with Eu(III).

### 1. Introduction

Lanthanide elements are widely and increasingly used in superconductors, magnets, fluorescent materials, glass additives, and MRI contrast reagents.<sup>1-4</sup> Accordingly, environmental pollution might well increase as a result of their expanding industrial and medical use. Several authors have noted the toxicity of gadolinium(III) and lutetium(III) to *Escherichia coli* and *Vibrio fischer*.<sup>5-7</sup> In previous investigations of the degradation of malic acid we found that the presence of this acid masked the toxicity of Eu(III).<sup>8</sup> In addition, at higher concentrations of Eu(III), the breakdown of malic acid was retarded. These results suggest that the toxicity of lanthanides depends on the particular chemical species present. However, we did not then identify the chemical species of Eu(III) and malic acid.<sup>8</sup>

The degradation of malic acid is followed by the appearance of at least two kinds of organic acid metabolites.<sup>8</sup> One of them, pyruvic acid, remained in the medium forming complexes with Eu(III) after all the malic acid had disappeared.<sup>8</sup> If pyruvic acid masks the toxicity of Eu(III), then the concentration of pyruvic acid should be nearly the same as, or higher than, the concentration of Eu(III). However, we did not measure its concentration in our previous work.<sup>8</sup>

In this study, we determined the concentrations of the chemical species using the computer codes MEDUSA and HYDRA,<sup>9</sup> we discuss the toxicity of Eu(III) in terms of its chemical species. We also assessed the amount of pyruvic acid generated to identify the type of pyruvate-Eu(III) complexes formed in the medium.

### 2. Experimental

#### 2.1. Culture

*Pseudomonas fluorescens* (ATCC 55241)<sup>8,10</sup> was grown in a mineral-salts solution containing the following: NaCl, 5.6 mg dm<sup>-3</sup>; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 88 mg dm<sup>-3</sup>; KCl, 75 mg dm<sup>-3</sup>;  $\beta$ -glyc-

erophosphoric acid disodium salt, 22 mg dm<sup>-3</sup>; 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 4.76 mg dm<sup>-3</sup>; and trace elements (MgSO<sub>4</sub>·7H<sub>2</sub>O, 7.0 mg dm<sup>-3</sup>; CaCl<sub>2</sub>·2H<sub>2</sub>O, 2.8 mg dm<sup>-3</sup>; MnSO<sub>4</sub>·5H<sub>2</sub>O, 1.2 mg dm<sup>-3</sup>; CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.15 mg dm<sup>-3</sup>; CoCl<sub>2</sub>·2H<sub>2</sub>O, 0.15 mg dm<sup>-3</sup>; FeSO<sub>4</sub>·7H<sub>2</sub>O, 1.5 mg dm<sup>-3</sup>; Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.090 mg dm<sup>-3</sup>; and ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.10 mg dm<sup>-3</sup>). Malic acid and Eu(III)Cl<sub>3</sub> solutions were sterilized separately by passing them through a 0.20- $\mu$ m pore-sized Millipore HA filter (ADVANTEC MFS, Inc., DISMIC-25).

**2.2. Biodegradation experiments.** Mineral-salt media containing 1 mM malic acid and 0, 0.05, or 0.1 mM Eu(III)Cl<sub>3</sub> were used to study biodegradation. The pH of the medium was adjusted to 6.6. The molar ratios of Eu(III) to malic acid were 0:1, 0.05:1, or 0.1:1 in media containing 0, 0.05, or 0.1 mM Eu(III), respectively.<sup>8</sup>

*P. fluorescens* cells were grown in the mineral-salts medium containing 1 mM malic acid. Cell growth was determined by measuring the optical density (OD) at 600 nm. Cell suspensions (0.5 cm<sup>3</sup>) were withdrawn from the medium at the late exponential growth phase (OD of 0.02) and added to 49.5 cm<sup>3</sup> of the media contained in 100 cm<sup>3</sup> Erlenmeyer flasks. The samples were incubated at 30 °C in the dark while being shaken at 100 rpm. Aliquots were withdrawn from the flasks every 24 h up to 264 h to measure pH, the concentrations of organic materials, and Eu(III). The latter value was obtained after first filtering the media through a 0.20- $\mu$ m pore-size Millipore HA filter (ADVANTEC MFS, Inc., DISMIC-25), and then acidifying 0.5 cm<sup>3</sup> of the filtered solution with 2 cm<sup>3</sup> of 0.2 M HNO<sub>3</sub>.

**2.3. Analytical methods.** The concentration of malic acid was measured by running the filtered solutions through a high-pressure liquid chromatography system (Waters, Alliance 2695) equipped with an organic-acid column (Waters, P/N 023694) with the mobile phase of 0.2 w/v% formic acid. The flow rate was 0.7 cm<sup>3</sup> min<sup>-1</sup>. The column's temperature was 60 °C. The elute was subjected to ESI-Mass spectroscopy (Waters, ZQ 2000) operated in the negative ion mode for  $m/z$  = 50–500 under the following conditions: capillary voltage, –3.07 V; cone voltage, –23 V; desolvation temperature, 150

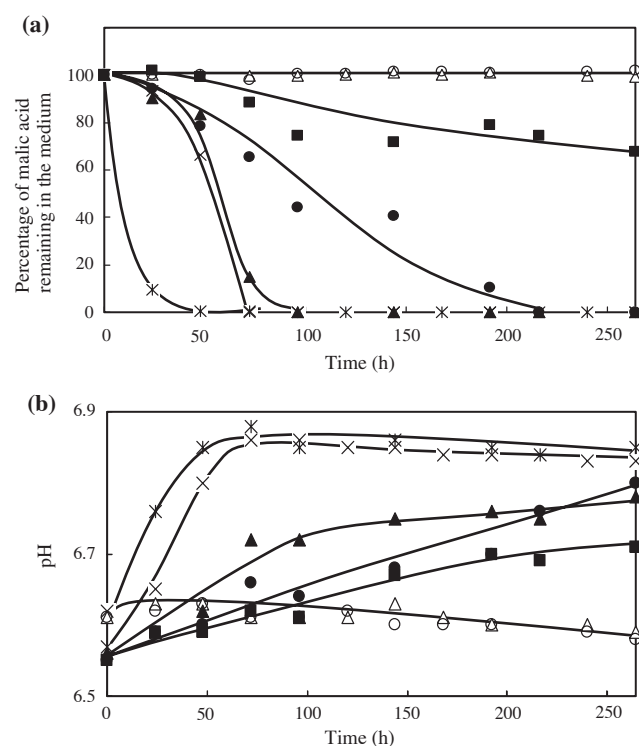
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°C; sheath gas, N<sub>2</sub>; flow rate, 360 dm<sup>3</sup> h<sup>-1</sup>. It also was analyzed using a Photodiode Array Detector (Waters, Alliance 2996) to detect UV absorption of the organic acid coexisting with malic acid. The concentration of malic acid was estimated from the ESI-Mass spectra detected at  $m/z = 133.1$ , and that of the coexisting acid from the absorption intensity detected by Photodiode Array Detector. The Eu(III) concentration was determined by inductively-coupled plasma atomic emission spectroscopy (ICP-AES) (Shimadzu, ICP-7000).

**2.4. Determination of Speciation.** We employed the computer programs HYDRA and MEDUSA to determine the speciation of Eu(III) and malic-acid complexes based on their reported stability constants.<sup>9</sup> The acidity ( $pK$ ) of malic acid (Mal) and malic acid mono-anion are 3.24 and 4.67, respectively.<sup>11</sup> The stability constant ( $\log K$ ) of the 1:1 molar ratio Eu(III)-malic acid complex (EuMal<sup>+</sup>) is 4.85, and that of the 1:2 molar ratio complex (Eu(Mal)<sub>2</sub><sup>-</sup>) is 8.11.<sup>12</sup>

### 3. Results and Discussion

**3.1. Degradation of malic acid by *Pseudomonas fluorescens*.** Figures 1a and 1b illustrate the degradation of malic



**Figure 1.** Biodegradation of malic acid by *P. fluorescens* in the presence of Eu(III) with an Eu(III) to malic acid molar ratio of  $\blacktriangle$  0.05:10,  $\bullet$  0.1:10,  $\blacksquare$  0.2:10,  $\triangle$  0.05:1,  $\circ$  0.1:1, and no Eu(III) ( $\times$  1 mM malic acid,  $\times$  10 mM malic acid); (a) Malic-acid concentration vs contact time and (b) pH vs contact time.

acid in media containing 1 mM malic acid and 0, 0.05, and 0.1 mM Eu(III), expressed as the Eu:malic acid molar ratio 0:1, 0.05:1, and 0.1:1 medium, respectively. We also include our previously reported data (those in 0:10, 0.05:10, and 0.1:10 medium).<sup>8</sup> In 0:1 and 0:10 media, the concentrations of malic acid decreased with time (Figure 1a). Malic acid was not degraded in 0.1:1 and 0.05:1 media during the test period (264 h). The acid was broken down in 0.2:10, 0.1:10, and 0.05:10 media. Its degradation occurred as fast in the 0.05:10 medium as in the 0:10 medium. The rate of degradation of malic acid decreased with the increasing ratio of Eu(III) to malic acid.

The initial pH of the medium was 6.55–6.62 (Figure 1b), but it gradually increased with time to almost 6.9 in those media where malic acid was degraded. In all media, the concentration of Eu(III) did not change whatever its initial concentration.

Although the concentration of Eu(III) in the 0.2:10 medium (0.2 mM) is higher than that in the 0.05:1 medium (0.05 mM), malic acid was broken down in the former but not the latter. These results suggest that toxicity of Eu(III) does not depend on its concentration; hence, we calculated its speciation so that we could discuss the toxicity of Eu(III) in terms of individual chemical species.

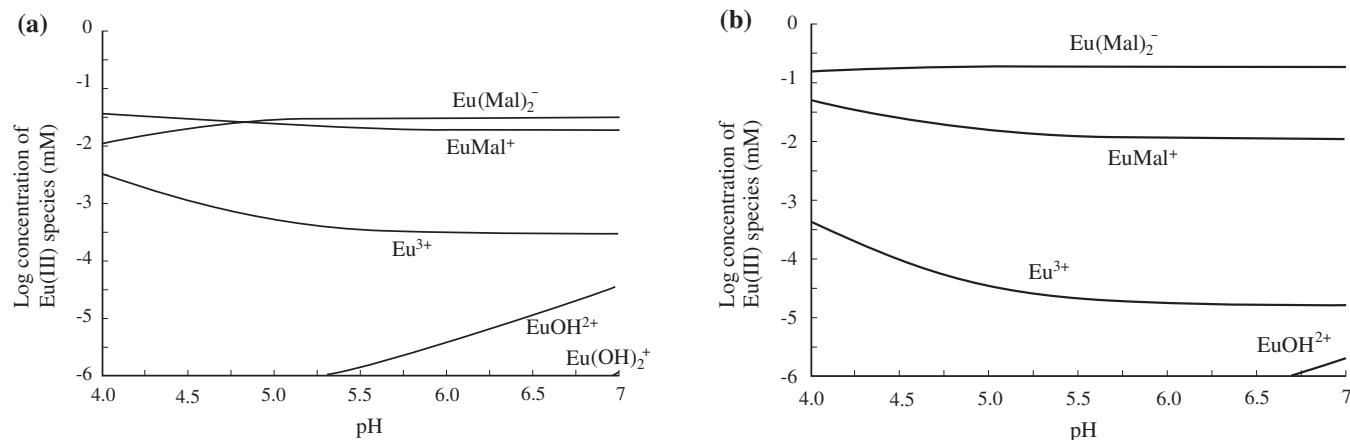
**3.2. Speciation of Eu(III).** Table 1 summarises the initial concentrations of Eu(III) species in solution in each medium. In the 0.1:1 and 0.05:1 ratio media where malic acid was not degraded, more than 80% of it was present as the free acid, indicating that Eu(III) retarded the degradation of free malic acid. In the 0.05:1 medium at pH 6.61 (the initial pH), approximately 61% of Eu(III) (0.031 mM) formed Eu(Mal)<sub>2</sub><sup>-</sup>, and approximately 37% (0.019 mM) formed EuMal<sup>+</sup>. In addition, the concentration of free Eu(III) was  $3.0 \times 10^{-4}$  mM and that of monohydrated Eu(III) was  $1.5 \times 10^{-5}$  mM. The concentrations of EuMal<sup>+</sup>, free Eu(III), and monohydrated Eu(III) decreased with the decreasing ratio of Eu(III) to malic acid. In contrast, the amount of Eu(Mal)<sub>2</sub><sup>-</sup> fell from 0.059 to 0.031 mM as the ratio of Eu(III) to malic acid dropped from 0.1:1 to 0.05:1; it then increased to 0.19 mM at the ratio of 0.2:10, while the concentration of malic acid rose from 1.0 to 10 mM. Because malic acid was broken down in the 0.2:10 medium but not in 0.05:1 medium, this result suggests that Eu(Mal)<sub>2</sub><sup>-</sup> does not hinder the degradation of malic acid by *P. fluorescens*.

The chemical species of Eu(III) in solutions at pH 4.0–7.0 (Figures 2a and 2b) showed that predominant ones, Eu(Mal)<sub>2</sub><sup>-</sup> and EuMal<sup>+</sup>, remained unchanged during cell growth, even though the pH in the medium rose from 6.5 to 6.9.

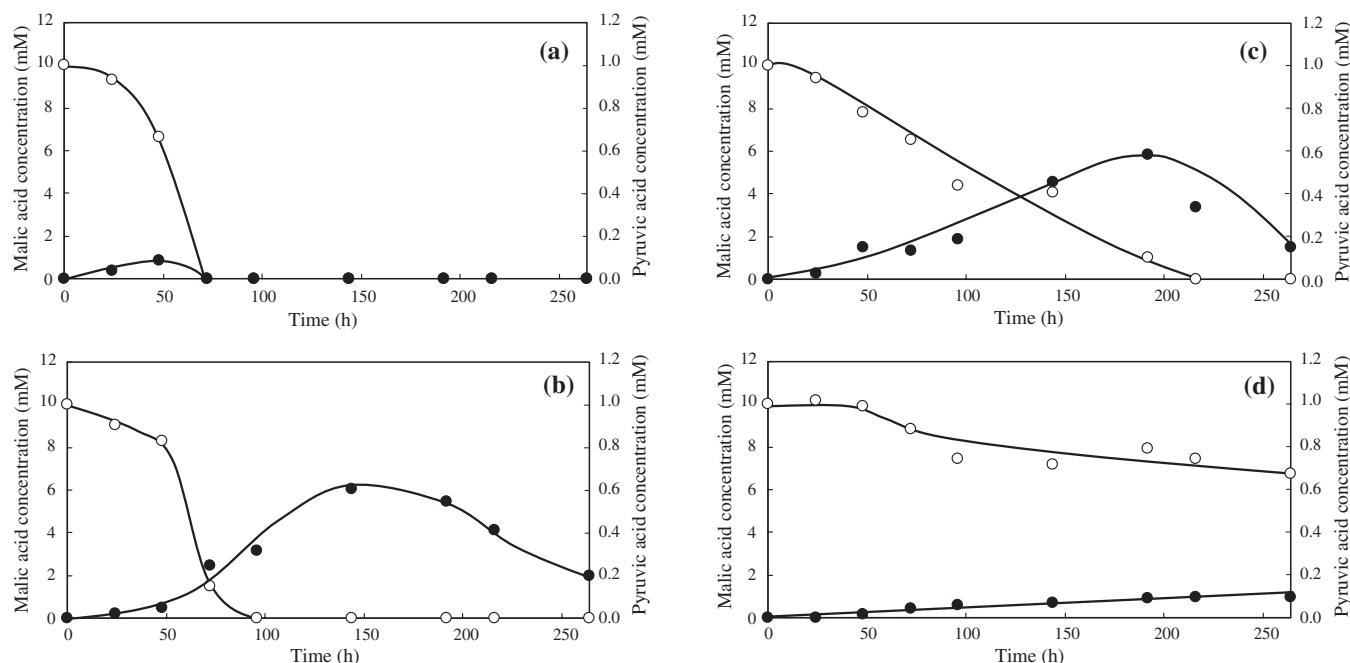
Fuma et al. reported that the toxicity of Gd(III) inhibited the growth of *E. coli* in media containing more than 0.3 mM, although growth was observed in those media containing less than 0.1 mM Gd(III).<sup>5</sup> Weltje and colleagues studied the effect of Lu(III) on the metabolic rate of the Gram-negative *Vibrio fischeri* in the presence of organic acids, such as EDTA, NTA, citrate, oxalate, and malate.<sup>7</sup> They found that metabolic rate

**TABLE 1: Speciation of Eu(III) in Media Containing Various Ratios of Eu(III) to Malic Acid**

Eu(III) to Malic acid ratio in the medium	Mal (mM)	Eu(III) (mM)	Eu(Mal) <sub>2</sub> <sup>-</sup> (mM)	EuMal <sup>+</sup> (mM)	Eu <sup>3+</sup> (mM)	EuOH <sup>2+</sup> (mM)	Mal <sup>2-</sup> (mM)	Percentage of malic acid remaining in the medium at 72 after inoculation (%)
0 : 1	1	0	0	0	0	0	0.98	0
0 : 10	10	0	0	0	0	0	9.8	0
0.1 : 1	1.0	0.1	0.059	0.039	$6.8 \times 10^{-4}$	$3.9 \times 10^{-5}$	0.83	100
0.05 : 1	1.0	0.05	0.031	0.019	$3.0 \times 10^{-4}$	$1.5 \times 10^{-5}$	0.89	100
0.2 : 10	10	0.2	0.19	0.011	$1.6 \times 10^{-5}$	$7.2 \times 10^{-7}$	9.5	88
0.1 : 10	10	0.1	0.095	0.0054	$7.8 \times 10^{-6}$	$3.5 \times 10^{-7}$	9.7	65
0.05 : 10	10	0.05	0.047	0.0026	$3.8 \times 10^{-6}$	$1.8 \times 10^{-7}$	9.8	15



**Figure 2.** Calculations of the speciation of Eu(III)-malate complexes in media containing (a) 0.05 mM Eu(III) and 1 mM malic acid (the 0.05:1 medium), and (b) 0.2 mM Eu(III) and 10 mM malic acid (the 0.2:10 medium). The concentration of each species is expressed on a logarithmic scale.



**Figure 3.** Time course of changes in the concentration of  $\circ$  malic acid and  $\bullet$  pyruvic acid in the medium after exposure to *P. fluorescens*; (a) without Eu(III), and with Eu(III) with a malic acid to Eu(III) molar ratio of (b) 0.05:10, (c) 0.1:10, and, (d) 0.2:10.

abruptly decreased with increasing free  $\text{Lu}^{3+}$  concentration from  $10^{-3}$  to  $10^{-2}$  mM and fell almost to zero when the concentration was above  $10^{-2}$  mM. In our study, a concentration of free Eu(III) in the 0.05:1 medium of  $3.0 \times 10^{-7}$  mM retarded the metabolism of malic acid by *P. fluorescens*; this value is much less than those reported for *E. coli* and *V. fischer*.

Trivalent lanthanide ions bind to the surface of human erythrocytes by electrostatic interaction, forming pores on the membrane.<sup>13</sup> Further, this effect is caused by positively charged species, especially free  $\text{Eu}^{3+}$ . From our study, we suggest that  $\text{EuMal}^+$  may affect the degradation of malic acid because the concentration of free Eu(III) is very low.

According to Table 1, lower levels of Eu(III) generated lower concentrations of  $\text{EuMal}^+$ ,  $\text{Eu}^{3+}$ , and  $\text{EuOH}^{2+}$  with the same ratio of malic acid to Eu(III). On the other hand, from comparing media containing the same amount of Eu(III), we see that if the malic-acid concentration increases ten-fold the concentration of  $\text{EuMal}^+$  decreases 1/8 fold, and those of  $\text{Eu}^{3+}$  and  $\text{EuOH}^{2+}$  drop 1/100 fold because two atoms of malic acid can coordinate to one Eu(III).

### 3.3. Pyruvic acid concentration in the malic-acid medium.

In our previous work, we found that two types of organic metabolites were produced following the degradation of 10 mM malic acid or in medium containing 0.1:10 and 0.05:10

Eu:malic acid.<sup>8</sup> These organic metabolites were eluted from LC at 8.85 and 12.03 min. To characterize the organic acids produced by *P. fluorescens* during the inoculation period, we measured the elution time and ESI-Mass spectra of standard acid solutions and compared them to those of these organic-acid metabolites. The standards used were organic acids in the TCA cycle; fumaric-, maleic-, succinic-, oxaloacetic-, citric-, isocitric-, glyceric-, glyoxilic-, and pyruvic acid. Because the ESI-Mass spectrum and elution time of pyruvic acid is similar to that of the organic acid eluted at 8.85 min, the most probable designation for it is pyruvic acid. Pyruvic acid is an intracellular metabolite of L-malic acid that reacts with the cellular enzyme malate dehydrogenase; the pyruvic acid so generated is excreted into the medium.<sup>14</sup>

The  $m/z$ s of the other unidentified organic acid detected at 12.03 min were 115 and 161. We could not plausibly assign them to any organic acid in the TCA cycle. We are pursuing their identification.

The pyruvic acid generated remained in solution in the medium and formed complexes with Eu(III). Figures 3a-d show the concentrations of malic acid and pyruvic acid. In the medium containing only 10 mM malic acid, the concentration of pyruvic acid reached its maximum (0.08 mM) at about 48 h and fell to zero at 72 h after inoculation. This finding demon-

strated that *P. fluorescens* metabolized pyruvic acid.

In the 0.05:10 medium (Figure 3b), pyruvic-acid concentration was 0.60 mM at 144 h after inoculation and decreased to approximately 0.2 mM at 264 h. In the 0.1:10 medium (Figure 3c), its concentration was 0.58 mM at 192 h after inoculation, and had reached to 0.15 mM at 264 h. In the 0.2:10 medium (Figure 3d), the amount of pyruvic acid still was increasing at 264 h because malic acid still was present in the solution.

For media with ratios 0.05:10 and 0.1:10 Eu(III) to malic acid, the maximum concentrations of pyruvic acid were 0.60 and 0.58 mM, respectively, compared with 0.08 mM in the solution without Eu(III). These results suggest that the amounts of pyruvic acid produced are not directly correlated with the concentration of Eu(III). The concentrations of pyruvic acid remaining in these media after malic acid was completely degraded was higher than that of Eu(III), suggesting that they were enough to mask the toxicity of Eu(III), and further, that the pyruvic acid generated was metabolically degraded by *P. fluorescens*.

Appanna et al. studied the biodegradation of trivalent cation-citrate complexes by *P. fluorescens*.<sup>15, 16</sup> They showed that Al(III)-citrate was metabolized intracellularly, while organic acids, such as oxalic acid and glyoxalic acid, were excreted into the medium. With Ga(III)-citrate, its  $\beta$ -hydroxyaspartate derivative was released into the solution, while Ga(III) predominated in the solution after citric acid was broken down.<sup>16</sup>

The stability constant of the 1:1, 1:2, and 1:3 Eu-pyruvic acid complexes are 1.88, 3.3, and 3.8, respectively.<sup>17</sup> Although the stability constants of the 1:1 and 1:2 Eu(III)-malic acid complex are greater than those of their respective pyruvic acids, more malic acid was degraded in the presence of Eu(III).

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