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Prebiotic Origin of Glycolytic Metabolism: Histidine and Cysteine can Produce Acetyl CoA from Glucose via Reactions Homologous to Non-phosphorylated Entner-Doudoroff Pathway

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Conversion of glucose to pyruvate via reactions homologous to the non-phosphorylated Entner–Doudoroff (non-P ED) pathway could be achieved in the presence of two amino acid catalysts, cysteine and histidine: cystine oxidizes glucose to gluconic acid by the reaction homologous to glucose dehydrogenase and histidine changes gluconic acid to 2-keto-3-deoxy gluconic acid, then to pyruvate by the reaction homologous to gluconic acid dehydratase and 2-keto-3-deoxy gluconate aldolase, respectively. Pyruvate can be converted to acetyl CoA by the reaction with CoA, TPP and FAD in the presence of cysteine and histidine, which resembles pyruvate dehydrogenase reaction. It was found that gluconic acid dehydration alone is non-specific, in contrast to other reactions. The non-P ED pathway is used by some extreme thermophiles in bacteria and archaea, usually thought as the oldest among the contemporary organisms. This study suggests the possible contribution of amino acid to the origin of the glycolytic pathway.

Key words: amino acid catalysts, energy acquiring central pathway, origins of life, primitive metabolism, thermophiles.

An enzyme consists of some hundreds of amino acids, most of which are used to construct a pocket for substrates and coenzymes for catalysis. However, only a few amino acids are directly involved in the active center. For instance, in the general acid–base catalysis, only two amino acid residues participate in the reaction, one as a general acid group and another as a general base group. An amino acid may have an acid group as the side chain (not carboxyl group in the peptidyl moiety as discussed later) and a base group (amino group in the peptidyl moiety). The single amino acid could function as a catalyst in the primordial ocean, although very much weaker than a contemporary protein does. Imidazole and thiol groups in histidine and cysteine residues, respectively, are particularly electron mobile groups. Consequently, these two amino acids may play important roles in the primitive metabolic pathway.

It was shown (*1*) that a single amino acid, histidine, (as well as its anticodon GpUpG) has weak but specific enhancing effect on chemical reactions important in the cellular metabolic pathway such as pyruvate carboxylase (ligase), oxalacetate decarboxylase (lyase) and glucose-6-phosphate isomerase. It was demonstrated (*2*) that some other amino acids such as glutamic acid, lysine, cysteine, tyrosine etc. (and their anticodons, too) could catalyze many important reactions in biological metabolism. In particular, cysteine functioned as the oxidoreductase. It was also discussed (*3*) that histidine and cysteine can enhance the metabolic reaction rates in the TCA cycle.

Extreme thermophiles in bacteria and archaea are known as one of the oldest species in the contemporary organisms. Some of them utilize the non-phosphorylated Entner-Doudorff (non-P ED) pathway, in which phosphorylation of hexose is not required in contrast to the Embden-Meyerhof pathway (*4*). At the first step, glucose is converted to gluconic acid (glucose dehydrogenase). Next, H_2O is extracted from gluconic acid to form 2-keto-3-deoxy gluconic acid (gluconic acid dehydratase), which is further cleaved to pyruvate and glyceraldehyde (2-keto-3-deoxy gluconate aldolase). The produced pyruvate may further be converted to reactive acetyl CoA (pyruvate dehydrogenase). The reactions are of ligase, lyase and oxidoreductase type. In this report, the working hypothesis of amino acid catalysts in the first paragraph is checked using visible and ultraviolet spectrophotometry, radioactive thin-layer chromatography (TLC) and mass spectrometry (MS and LC-MS) (*5*, *6*).

MATERIALS AND METHODS

All non-radioactive biochemicals and enzymes are purchased from Sigma. [^{14}C] radioactive chemicals are from American Radio-labeled Chemicals, Inc. (Nineteen other amino acids as well as the target amino acid and the reference, where the buffer solution was used instead of the amino acid solution, were also checked in each reaction). As discussed earlier (*1*), amino acids and their esters showed the same catalytic activity in the

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metabolic reactions. For leucine, isoleucine, methionine, asparagines, glutamine, phenylalanine, tyrosine and tryptophan, we always used the esters of these amino acids. Some of the amino acids such as lysine, aspartic acid etc. are of salt form.

In the spectrophotometric experiments, absorbance was measured using a DU 65 spectrophotometer (Beckman, NC, USA). The experiments were performed at 25°C, unless stated otherwise. In the following assay, 100 μl of the reaction mixture, whose final concentrations were specified in the figure captions of each reaction, was used.

In the TLC, 0.3 μl of reaction mixture was spotted on a cellulose TLC plate after incubation (the reaction time depended on the reaction) and developed with a *n*-propanol: 2N ammonia aqueous solution (7:3) for 1 h. The radioactive spots were analysed using an imaging plate and an imaging analyser BASS 2000 (Fuji Flm Co., Tokyo, Japan).

For the mass-spectrometric measurement, a LCT TOF Instrument (Micromass, MS Technologies, Manchester, UK) equipped with a 'Z spray' electronspray source was used to analyse the samples. Typical operating parameters were as follows: capillary voltage, 2.5 kV; sample cone voltage, 20 V; source block temperature, 80°C; desolation temperature 150°C. For the analysis of pyruvate dehydrogenation, a Mass Spectrograph, Finnigan LCQ DUO (Thermo Fischer Sci. Inc., MA, USA) connected to a HPLC, Agilent 1100 series (Agilent Tech., CA, USA) with a column Inertsil Diol 3 (GL Sciences Inc., Tokyo, Japan) was used. The elution buffer started from 90% acetonitrile (AN) in 10% of 20 mM ammonium acetate (AA) solution and AN was linearly decreased to 70% by 4 min, with a linear gradient and the 70% AN was used additional 11 min. ESI(−) parameters were: voltage, 4.05 KV, capillary temperature, 250°C and capillary voltage, −9.74 V. The set parameter to detect CoA mode was: parent mass, 808.50, isolation width, 2.0 and helium relative collision, 30.0%. The column temperature was 4°C and the eluent was monitored at 260 nm.

In the above reactions, the concentrations of amino acids are much higher than those of the reaction substrates. Consequently, almost all the substrates are expected to interact with the amino acid catalyst, in contrast to the usual catalytic reaction where catalysts are almost fully occupied with the substrates before the final catalytic reaction proceeds. (We shall use the word catalyst below, since this word would intuitively emphasize a new role of amino acid rate-enhancing function in the origin of metabolism. To be exact, we should show that the amount of amino acid before and after the reaction to use the word catalyst. This is difficult due to the weakness of the activity. However, we could not find any spectra or lines for the amino acid related compounds such as imino acid, keto acid and so on except those of amino acid itself in TLC and HPLC. This is a strong evidence, although indirect.)

RESULTS

Glucose dehydrogenation (DH)—Figure 1A shows the TLC plate of the reaction products of gluconic acid with

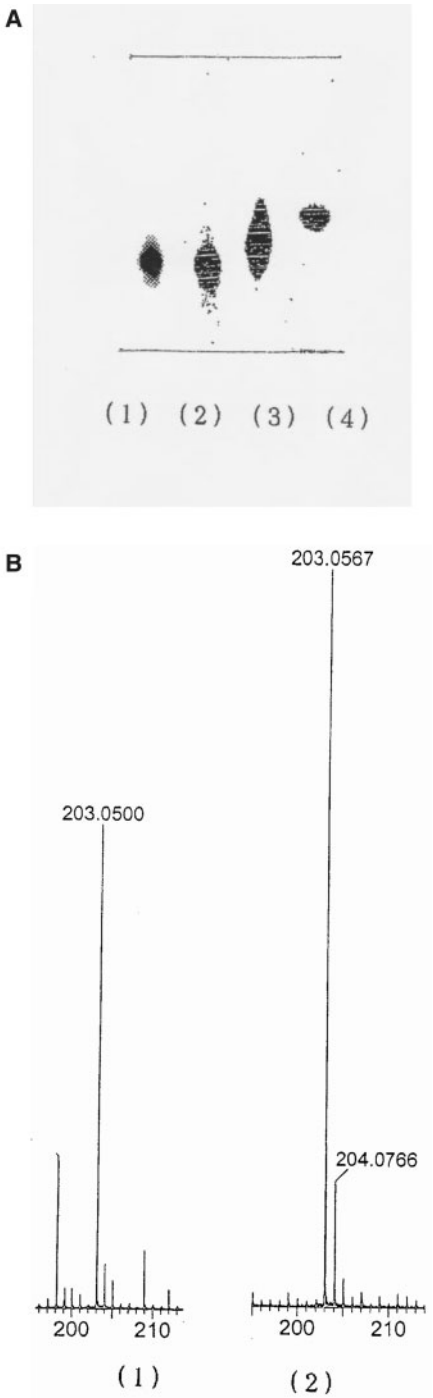

Fig. 1. (**A**) **Radioactive spots detected on TLC plate after the reaction of gluconic acid and cysteine showing the formation of glucose.** The reaction is homologous to the glucose dehydrogenase. The lanes (1) and (2) are for authentic gluconic acid and the reaction mixture at time zero, respectively. The lanes (3) and (4) are for the reaction mixture after 1 h and for authentic glucose, respectively. Reaction mixture: 500 mM sodium phosphate buffer pH 3.7, 500 mM gluconic acid with [1-^{14}C] gluconic acid 3 KBq/100 μl and 500 mM cysteine. B: (1) Mass-spectrum of the reaction mixture after 1 h. *m/z* 203 corresponds glucose plus sodium (plus proton). (2) Mass-spectrum of authentic glucose. Reaction mixture: 100 mM ammonium acetate buffer pH 5.2, 50 mM gluconic acid, 100 mM cysteine or 19 other amino acids (or their esters).

cysteine, which is the reverse reaction of glucose dehydrogenation (DH). The smearing spot extending to glucose was detected on the TLC plate, compatible with the formation of glucose from gluconic acid. To further verify the formation of glucose, mass spectrum of the reaction mixture products was recorded on a Micromass LCT TOF spectrometer (Fig. 1B). The mass corresponding to glucose plus sodium and proton was detected in the reaction mixture.

Gluconic Acid Dehydratation and 2-keto-3-deoxy Gluconate Aldol Cleavage—Both reactions catalyzed by histidine, forming pyruvate as the final product through the two step reactions, were tested. Formation of pyruvate was monitored by coupled enzymatic method in the presence of lactate dehydrogenase, which catalyze the reaction from pyruvate to lactate. The dehydrogenation of NADH is monitored at 340 nm at 25°C (Fig. 2A). The pH dependence for the pyruvate formation was shown in Fig. 2B.

To confirm the formation of 2-keto-3-deoxy gluconic acid and pyruvate, [1-^{14}C] gluconic acid was incubated with histidine and the product analysed by TLC. The reaction mixture was incubated for 10 min to 1 day at 25°C. Week but clear spots were detected at Rf = 0.7, which correspond migrated position of pyruvate (Fig. 2C). The 2- keto-3-deoxy gluconic acid was not commercially available and Rf of its spot is not known. Consequently we developed the reaction mixture without radioactive gluconic acid on a cellulose plate and found the spot at Rf = 0.6.

The spot was scrapped off. After extracting the compounds with water, the mass of the extract was determined by MS technique. Figure 2D depicts the mass spectrum of the compound of Rf = 0.6, the *m/z* being 179, which corresponds to the expected value for 2-keto-3-deoxy gluconic acid.

The above experiment concentrated to the detection of pyruvate in the case of the specific catalyst histidine, which did not explicitly test the possibility that the gluconic acid dehydratase is also specifically performed by histidine alone. To clarify the point, the reaction solution with radioactive gluconic acid (thus without non-radioactive gluconic acid) and various amino-related compounds including ammonia after 2 h were developed on a cellulose plate. After taking the radio image (upper part of the figure), the plate was strained with ninhydrin, resulting in the spots for amino acid catalysts (only ammonia had no trace) in the lower part of the figure. Figure 2E shows that the gluconic acid dehydratation was catalyzed by a variety of amino acids but aldol cleavage of 2-keto-3-deoxy gluconic acid is specifically catalyzed by histidine. The temperature and pH dependences of the catalytic reaction rate of histidine that are analogous to gluconic acid dehydratase and 2-keto-3-deoxy gluconate aldolase were studied. These are the key reactions in the metabolic pathway to cleave C6 sugar to C3 sugars. The temperature dependence of the initial velocity was estimated between 25°C and 45°C. The activation energy of catalysis is calculated from the initial velocities and is 123 kcal/mol. Assuming the constant activation energy extended to 85°C, the reaction rate at 85°C is estimated to be 27 times higher than that at 25°C. The pH dependence is shown in Fig. 2B.

Pyruvate dehydrogenation (7)—The reaction mixture of pyruvate, CoA, TPP (thiamine pyroposphate), FAD and two amino acids, cysteine and histidine, was monitored at 340 nm (Fig. 3A). It is noteworthy that cysteine alone can induce reduction of pyruvate (corresponding to the lactate dehydrogenation activity). The difference between Cys and Cys-His curves gives the contribution from the acetyl CoA formation. To confirm the formation of acetyl CoA, 0.3 μl of a mixture was spotted on a TLC cellulose plate after the reaction time of 2 h. The spot with Rf = 0.15, which is close to Rf of acetyl CoA, was detected (Fig. 3B). A LC-MS was attempted for further confirmation. The HPLC mass chromatogram at *m/z* 808 of authentic acetyl CoA is depicted in (C1) of Fig. 3C, while that of acetyl CoA formed by the reaction analogous to the pyruvate dehydrogenase by cysteine and histidine is shown in (C3). Both acetyl CoAs were destructed by neutral helium bombardment and their fragment spectra are depicted in (C2) and (C4), respectively. These fragmentation spectra are quite similar to each other, supporting the formation of acetyl CoA in the above reaction.

DISCUSSION

The recent geochemical idea on the primitive earth favours its hot early history due to the accretion of planetesimals in a short time (1–100 million years?), which ended about 3.8 billion years ago (*8*, *9*). The carbon isotope data of the oldest rocks also suggested the generation of living system (even having the photosynthetic system) in that period (*10*). The paleotemperature of the oceans at that time appears to be 80°C or so, suggested from the recent silicon isotopic data, which is completely independent of the earlier oxygen isotope data concluding similar high oceanic temperature, since oxygen isotope came from oceanic water but silica isotope from silica dissolved in water (*11*, *12*). The silica curve exhibits the presence of ice ages at aroud 2.5 billion years, in accord with the claim by other geologists (*13*). All archaea and some of ancient bacteria have the lipids with ether bonds, which are more stable at high temperature than the lipids with the ester bonds in other species. It is also noteworthy that amino acids are stable at high temperature in contrast to proteins and RNA. Yamagishi *et al*. (*14*) named the thermophilic common ancestor Commonote.

The results of experiments illustrated in Figs 1–3 show that glucose can be converted to pyruvate, and then, to acetyl CoA in the presence of high concentrations of cysteine and histidine via the reactions homologous to non-P ED pathway. In contrast, the reactions homologous to those of Embden-Meyerhof (EMP) pathway catalyzed by the amino acid was not detected in most cases: the conversion of G6P to fructose-6-phosphate occurred only at a high temperature, 65°C (*1*). The reactions corresponding to the glycel-phosphate isomerase, enolase, pyruvate kinase were not detected at 25°C. This may suggest the earlier evolution of non-P ED pathway than the Embden-Meyerhof (EMP) pathway, since amino acids

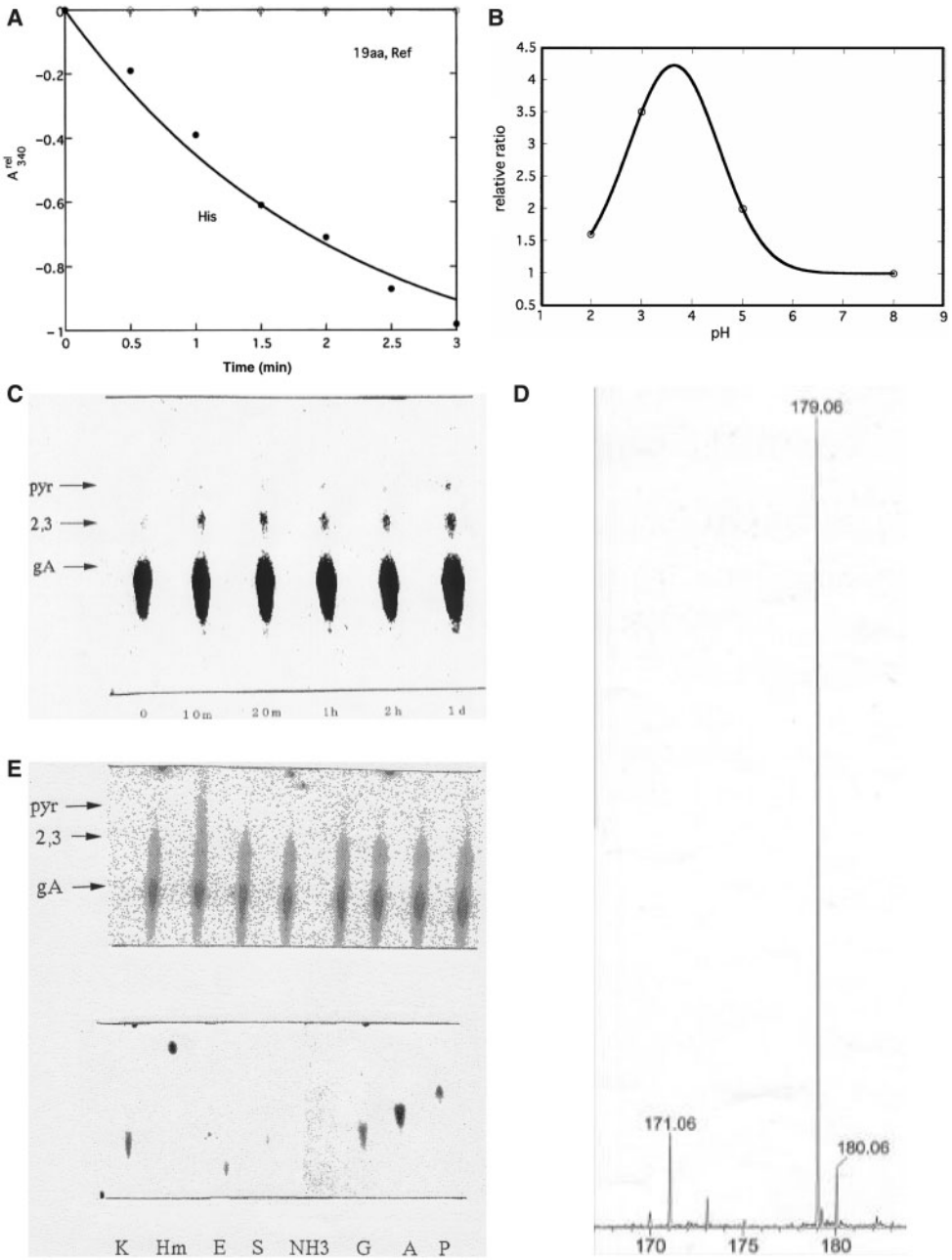

Fig. 2. (**A**) **Time course of the absorption at 340 nm.** NADH was decreased due to its reaction with the formed pyruvate in the presence of histidine (the reactions are homologous to gluconate dehydratase and the 2- keto-3-deoxy gluconate aldolase). The initial velocity is calculated to be 0.068 mM per 1 min. Reaction mixture: 200 mM sodium phosphate buffer pH 7.8, 200 mM gluconic acid, 0.24 mM NADH, lactate dehydrogenase 10 units and 100 mM histidine or each of other amino acids. (B) The pH dependence of the pyruvate formation rates through the gluconic acid dehydratase and 2-keto-3-deoxy gluconic acid aldolase reactions in the presence of histidine. Reaction mixture: 200 mM sodium phosphate buffer of various pH by mixing mono- and di-sodium phosphate solution, 200 mM gluconic acid, 0.24 mM NADH, lactate dehydrogenase 10 units and 100 mM histidine. (C) Radioactive spots detected on TLC plate showing the formation of 2-keto-3-deoxy gluconic acid and pyruvate (and non-visible glyceraldehyde) from gluconic acid. The reaction mixture was analysed after 10 and 20 min, and 1 and 2 h, and 1 day incubation at 25°C. The reactions are homologous to gluconic acid dehydratase and 2-keto-3-deoxy gluconic acid aldolase). pyr for pyruvate, 2,3 for 2- keto-3-deoxy gluconic acid, and gA for gluconic acid. Reaction mixture: 400 mM acetate buffer pH 5.2, 100 mM non-radioactive gluconic acid with [1-^{14}C] gluconic acid 185 KBq/100 μl and 200 mM histidine methyl ester. (D) The mass-spectrum of the spot at Rf = 0.6 of the reaction mixture of Fig. 2C after 1 day reaction. *m*/*z* 179 correspond to 2-keto-3-deoxy gluconic acid (plus proton). (E) Radioactive spots (upper figure) and amino acid spots steined with ninhydrine (lower figure). TLC diagrams for the gluconic acid dehydratase and 2-keto-3-deoxy gluconic acid aldolase reactions without non-radioactive 100 mM gluconic acid for the reaction mixture of Fig. 2C in the presence of various amino acids in place of histidine methyl ester. K, for lysine; Hm, histidine methyl ester; E, glutamic acid; S, serine; G, glycine; A, alanine; P, proline. In the upper figure, the formation of pyruvate was observed only for histidine methylester, while the formation of 2-keto-3-deoxy-gluconic acid was observed for all amino acids and even for ammonia. The spots in the lower figure exhibit the presence of these amino acids explicitly.

might have been produced abiotically in the primitive ocean in a great amount and the pathway could have been functioned in the earliest history of hot earth (prebiotic origin of glycolic metabolism).

A reactive molecule, acetyl CoA, was formed from pyruvate in the above non-P ED pathway. It was already shown (*1*) that oxalacetate was catalytically produced from pyruvate and carbonate by histidine. Oxalacetate and acetyl CoA was catalytically reacted in the presence of histidine to form citrate (*3*), which is the starting molecule of the TCA cycle. It was also demonstrated that the TCA cycle was circulated possibly forming ATP (*3*) in the presence of histidine and cysteine as catalysts.

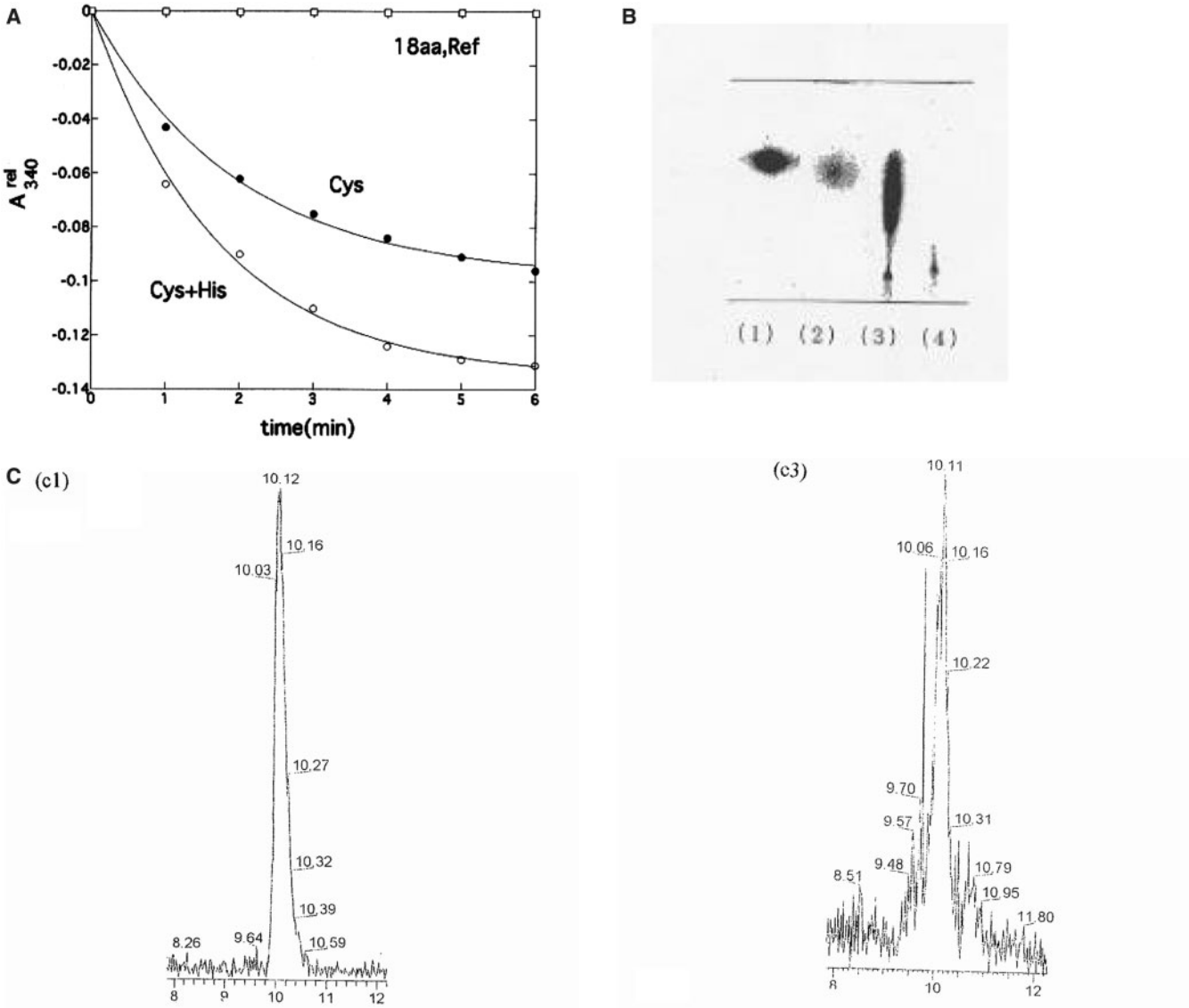

Fig. 3. (**A**) **Time course in the photometric measurements for the reaction homologous to pyruvate dehydrogenase to detect the absorption decrease of pyruvate at 340 nm in the presence of cysteine and histidine.** The Cys curve is for the reaction of pyruvate with cysteine alone (the reaction homologous to lactate DH between these two compounds). The Cys + His curve includes all compounds with cysteine and histidine. Omission of either TPP or CoA or FAD in the Cys + His reaction resulted in the same relative absorption curve for the Cys curve. The initial velocity is computed to be 0.12 mM/1 min. Reaction mixture: 500 mM sodium citrate buffer pH 5.3, 10 mM pyruvate, 10 mM CoA, 50 mM thiamine pyrophosphate(TPP), 0.2 mM FAD and 50 mM cysteine plus 50 mM histidine or 100 mM 18 other amino acids. (B) Radioactive spots detected on TLC plate. The formation of acetyl CoA from pyruvate and CoA in the presence of histidine and cysteine (pyruvate DH) was tested. The lanes (1) and (2) shows the spots for authentic pyruvate and the reaction mixture before the reaction, respectively. The lanes (3) and (4) are those for the mixture 1 day after the reaction and the authentic acetyl CoA, respectively. Reaction mixture: 500 mM sodium citrate buffer pH 5.3, 30 mM pyruvate with [1-^{14}C] pyruvate 3 KBq/100 μl, 10 mM CoA, 50 mM TPP, 10 mM FAD and 50 mM each cysteine and histidine. (C) HPLC mass chromatograms at *m/z* 808 of (C1) authentic acetyl CoA and (C3) the solution of the Fig. 3B, using the LC-MS technique. Figures (C2) and (C4) illustrate the fragment mass distribution by an neutral helium impact of the molecules of peak at 10.12 in C1 or 10.11 min in C3, respectively. The spectra of (C2) and (C4) resemble with each other, showing that the peak of 10.11 min in (C3) is due to acetyl CoA.

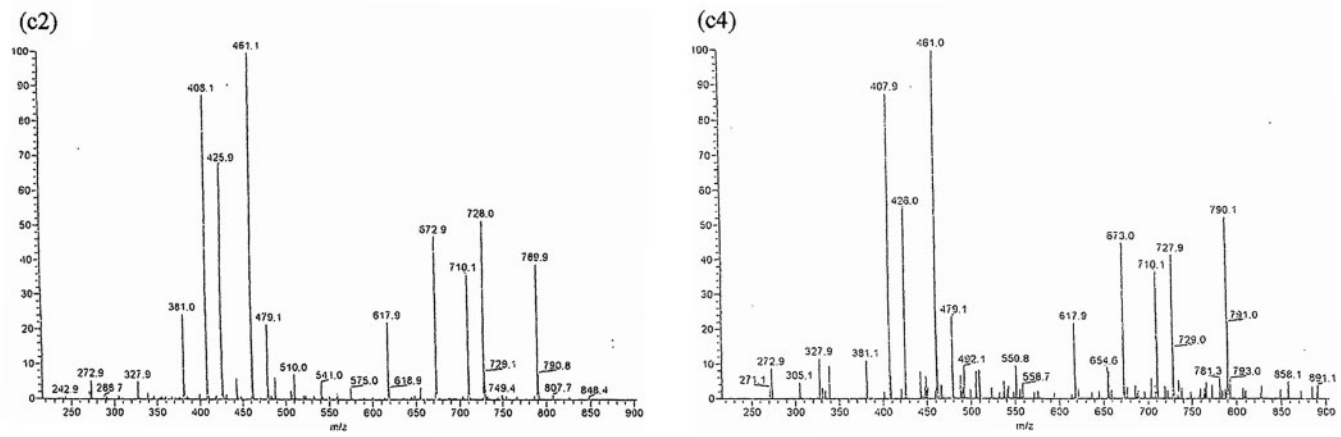

Fig. 3. Continued.

The oxidative decarboxylation of pyruvate and the formation of acetyl CoA in the presence of amino acid rate-enhancers, cysteine and histidine, was observed and this results is in accord with the thioester world as advocated by De Duve (*15*) before the formation of system employing various complicated molecules including coenzymes such CoA, NAD, FAD etc. NADH formed in this way may be oxidized by various oxidants such as iron oxides in the environments and NAD might be recruited: for instance, Egami (*16*) advocated the existence of nitrate fermentation before nitrate respiration, with which the formation of ATP would also be occurred. The existence of the complex ATPase-membrane quinone system was unlikely in early history of the earth.

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