THE ORIGIN AND EVOLUTION OF OPTICALLY ACTIVE COMPOUNDS IN BIOLOGICAL WORLD

- CHIRAL RECOGNITION OF AMINO ACID BY NUCLEIC ACID -

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Contents

ABSTRACT

All the molecules constructing "living matter" which has the characteristics of self-replication and energy metabolism are essentially dissymmetric. Proteins are made only of L-amino acid enantiomers and nucleic acids contain only D-ribose or D-deoxyribose. On the other hand, "non-living matter" which is abiotically synthesized is symmetric without exception. Accordingly, symmetry breaking process is inseparably related to the origin of life, but the origin of dissymmetry is still unsolved regardless of a number of investigations.

Amino acids and nucleic acids are the most important building block molecules for life, which are also typical examples of optically active compounds. However, no explanation of the origin of those chirality has been made. I have proposed a hypothesis that one molecular chirality might complementally have evolved under the influence of the opposite chirality, namely nucleic acids produce amino acid chirality or vice versa. The purpose of my study, therefore, is to elucidate whether nucleic acids recognize amino acid chirality and select their one-handed enantiomers, and to discuss mechanism of chiral recognition and its development process in organisms.

First of all, it must be made clear that abiotically synthesized amino acids are achiral. In Part I, I describe prebiotic syntheses of amino acids (Gly, Ala, Asp and AAnBA) and nucleic acid base and its precursors (adenine, AICA and AICAI)

from HCN in the presence of clay (montmorillonite). It has been known that the synthesized amino acids were racemic. It could be expected that "non-living matters" abiotically synthesized were symmetric because of lack of any chiral aids. In addition, the results suggest that montmorillonite as a prebiotic catalyst could play a role for synthesis of building block molecules.

Since the amino acids abiotically synthesized are all racemic, those symmetry breaking process should have been present during chemical evolution. As indicated, I have hypothesized that nucleic acids break the symmetry of amino acid. In order to prove this, the model system has been constructed. Cellulose was used in place of nucleic acids to know if D-sugar generally recognize amino acid chirality. In **Part II**, optical resolutions of all proteinic amino acids by native cellulose (D-glucose polymer) chromatography are shown. It is concluded that D-glucose is able to recognize the amino acid chirality. It is suggested that the interaction between D-glucose and L-amino acids is stronger than that between D-glucose and D-amino acids. The findings are extremely significant for considering some possibility of chiral amplification process of amino acids upon existing sugars on the primitive earth.

It is indicated that the resolution factors (α) for amino acid racemates on cellulose is dependent on the size of side chain. In order to understand the chiral recognition process by the size of amino acid on cellulose, 2.4-dinitrophenyl(DNP)-DL-

amino amino acids were resolved by a cellulose column (**Part III**). High resolution factors (α) in comparison with underivatized amino acids were obtained. It is suggested that an increase in molecular size and then an amplification of distortion of molecule resulting from the modification of amino group may play an important role on the chiral recognition process by cellulose. It is shown in **Part II** that most L-amino acids interact with cellulose stronger than the opposite. When modified with DNP, then DNP-D-amino acids interact stronger than the opposite (**Part III**). It is also suggested that the form of molecule might be involved in chiral recognition process.

Based on the above experimental results (**Part II** and **III**), a chiral model of amino acid recognized by cellulose was constructed (**Part IV**). The mechanism of chiral recognition process is proposed by using the model. Moreover, the energy difference between DL-amino acids on D-glucose (dissymmetry environment) was calculated basing on the chromatographic results. It was known that the energy difference of enantiomers on cellulose increases by a factor 10^{16} in comparison with that in nature, and it is also influenced by the size of side chain (including DNP-derivatization).

It has been suggested in the previous **Parts** that D-sugar could recognize amino acid chirality and select most L-amino acids to interact, and the recognition process could be influenced by molecular size and form. Next study is to

investigate whether nucleic acids could recognize amino acid chirality (**Part V**). Using ligand exchange chromatography whose mobile phase contained ribonucleic acid and Cu(II), nine proteinic DL-amino acids were resolved on the basis of chiral interaction of ribonucleic acid by mediation of Cu(II). The difference of stability between DL-amino acid - Cu(II) - D-nucleic acid complexes seems to give rise to their resolutions. Such the complex might also be regarded as the model for functional ribozymes which might have let ribonucleic acids recognize amino acid chirality to develop ribonucleoproteins during chemical evolution. By using deoxyribonucleic acids, similar experimental results with those of RNA were obtained. The results suggest that the interaction between nucleic acid and amino acid might have emerged and brought its chiral style to modern biological world.

In the **Part VI**, it is shown that DL-amino acids were resolved using mobile phase containing ADP, NAD or FAD as a chiral additive, and then using chiral stationary phase with cyanocobalamin. These nucleotide cofactors were also able to recognize amino acid chirality. Since NAD, FAD and cyanocobalamin are representative nucleotide cofactors, the above results are very interesting. It is hypothesized that those nucleotide cofactors might be fossils of enzymes in the RNA world and those might have acted for chiral selection.

GENERAL INTRODUCTION

Dissymmetry is essential for life

There are many approaches to the origin of life, for example, abiotic synthesis of essential building block molecules for life (amino acids[1,2], bases[3], sugars[4], nucleosides[5], nucleotides[6], fatty acids[7], cofactors), prebiotic condensation of building blocks (oligopeptides[8,9], oligonucleotides[10-12], lipids) and self-assembly of building blocks (coacervate[13], microsphere[14], marigranule[15]). However, the most important but difficult subject is to solve the origin and evolution of optically active compounds in biological world.

The definition of "living matter" which has characteristics of self-replication and energy metabolism is dissymmetric, in other words, "order" at molecular level. All the modern organisms on the earth are constructed with L-amino acids (proteins) and D-sugars (DNA, RNA and nucleotide cofactor). For example, if a protein is constructed with racemic amino acids, it is never able to be "ordered" (for instance, α-helix and β-sheet) structure. However, "non-living matter" is symmetric ("disorder"), because abiotically synthesized amino acids and sugars are racemic, in other words, D- and L-amino acids (also sugars) are equally synthesized (**Part I**). The symmetry breaking process, therefore, must be inseparably related to the origin of life (Fig. 1).

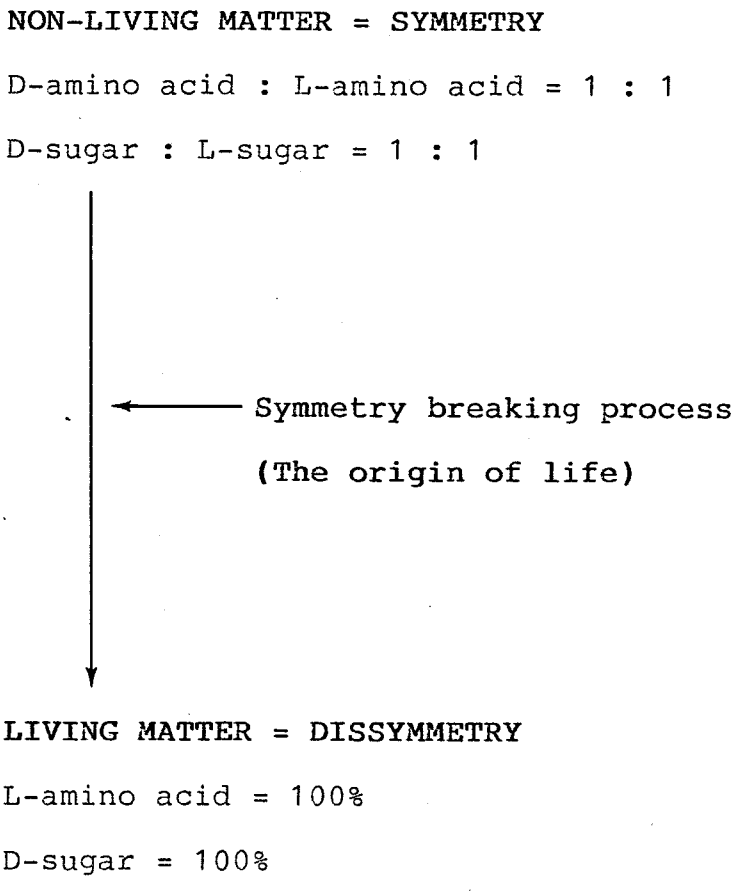

Fig. 1 The symmetry breaking and origin of life

Physical approaches to chiral evolution

In order to know the mechanism of the chiral evolution of biologically interesting molecules, numerous investigations have so far been carried out, which mostly dealt with the physical aids. The methodological basis, however, seems to principally depend upon the theorem by L.Pasteur[16]. Such the physical methodology[17-23] may happen to solve the chiral evolution mechanism, since the above leading approaches have mainly discussed the long term-gradual-gradient amplification of the chiralities by being off from the initially unmeasurable and mimic difference (almost nothing) between enantiomers. For example, Mason[17] recently calculated the energy difference between D- and L- alanine enantiomers in nature to be some 10^{-19} eV. However, an effectively catastrophic machinery for the absolute selection of chirality seems to have naturally occurred during chemical evolution.

It is the fundamental concept in the case of using the physical methodology that the different property between enantiomorphs in the simple system such as DL-molecules vs. energies may become measurable and/or visible at constant condition, under which we may take a long time for the amplification of each difference. On the other hand, it is possible that extreme difference between corresponding enantiomorphs even in the form of racemate may necessarily be produced in the complex system coexisting with other molecules, especially the presence of chiral molecules. The energy difference between D- and L- amino

acid enantiomers in D-glucose environment was calculated to be some 10^{-2} - 10^{-3} eV (**Part II - IV**). In this sense, we must again take up the Pasteur proposition. He has already mentioned such the effectiveness of the complex system. The proposition has once triggered our study on the mechanism of chiral evolution through the complex chemical interactions among different species of molecules. However, my study on chemical interaction does not aim at making a denial of the physical methodology, but at being compatible with it for the present.

Chiral evolution based on complex chemical interaction

A number of plausible chemical interactions can be considered as for chiral evolution (Table 1). Important is to make clear how the chirality of molecules in bionts has evolved before and/or after the appearance of life. Since the present-day situation could be resulted from the historical reflection of the old days', such the chiral world should necessarily have been evolved from sophisticated chemical interactions. First of all, it is hypothesized that all molecules are racemic and therefore none of dissymmetry of molecules is present at the beginning on the earth. It is, accordingly, important to know the mechanism of breaking symmetric balance of molecules. In Table 1, plausible selection environments of chirality are listed.

It has so far been known that quartz could form D- and L-crystal, then some investigators have reported the phenomenon of asymmetric adsorption on the surfaces of D- and L-quartz

Table 1 Plausible environment for selection of chiral compounds

Environment	To be selected	References
sugars	sugars	24-26
amino acids	amino acids	27-29
sugar	amino acids	30-35
amino acids	sugars	
quartz	amino acids	36-39
crystal	amino acids	40

crystals. However, no one knows the existence ratio of D- and L-quartz on the earth. Recently, the interesting finding of enantioselective occlusion into centrosymmetric crystals of glycine has also been reported[40].

Chiral amplification processes of L-amino acids by L-amino acids, and of D-sugar by D-sugar, may easily be understood. A number of experiments have been made to substantiate the hypothesis.

The chiral interaction processes between amino acids and sugars are the most interesting subject for the study of chiral evolution based on complex chemical interaction. Because amino acids and sugars are indispensable and essential for building block molecules, many scientists may be interested in knowing whether D- or L-sugars can be selected by L-amino acids, or D- or L-amino acids can be selected by D-sugars. My concern is to elucidate a possibility the chiral selection of D- or L-amino acids by D-sugars (D-glucose and nucleic acids containing D-ribose and D-deoxyribose). The reason why I have attempted the chiral selection experiments of amino acids by D-sugars is as follows.

The origin of life and RNA world

The discovery of the ribosomal RNA intron excision of Tetrahymena has triggered the development of studies on RNA enzymes (ribozymes) in the modern organisms[41]. It is, accordingly, speculated that the first "living molecule" might

have been RNA on the primitive earth[42,43]. If the RNA world afterwards generates ribonucleoprotein world as more functional molecules, the pre-existed RNAs would in advance select L-amino acids for the proteins from their racemic mixture. It is hypothesized that the symmetry breaking of amino acids abiotically synthesized might have processed after the emergence of ribonucleic acids.

Since the chiral part of nucleic acid is constructed with D-ribose moieties, it is important to know whether such D-sugars recognize the chirality of amino acids. In order to investigate the possibility of chiral interaction between ribonucleic acids and racemic amino acids, an experiment has been carried out using cellulose (D-glucose polymers) and racemic amino acids (**Part II - IV**). It has been known that D-glucose recognizes the chirality of amino acids to give their enantiomeric resolutions[30-35]. It is therefore considered that nucleic acids would also recognize the chirality of amino acids as well. Although a number of the related study which was examined between limited nucleic acids and DL-amino acids has ever been known[44-48], the results discussed in this thesis is the first publication which shows the chiral interaction between various nucleic acids and DL-amino acids (**Part V,VI**).

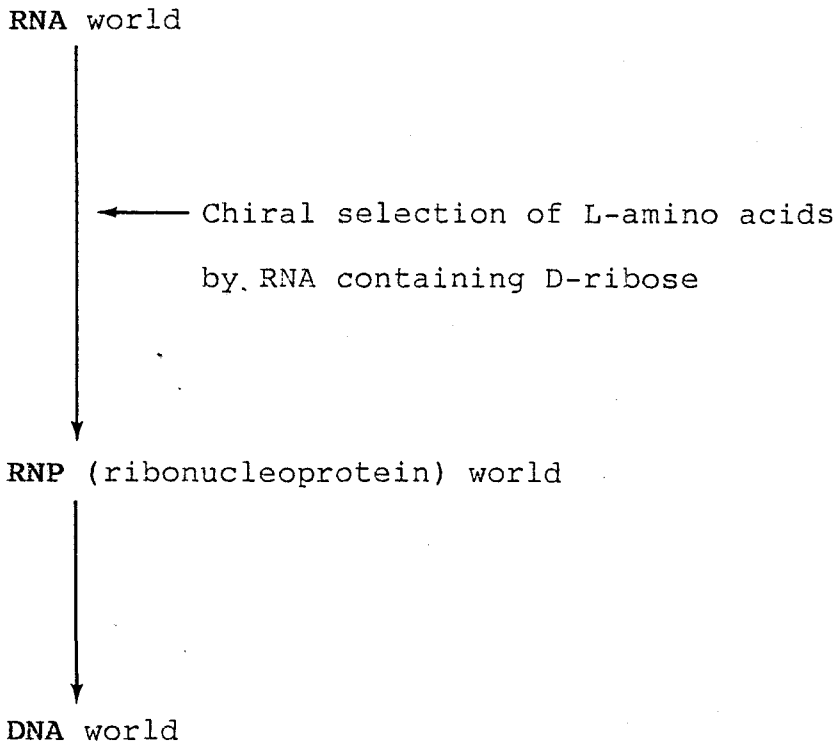

Fig. 2 A scenario of chiral evolution

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Part I

Montmorillonite-catalyzed synthesis of amino acids and heterocyclic compounds from hydrogen cyanide

Abstract

The catalytic action of montmorillonite on hydrogen cyanide (HCN) has been investigated. The acid hydrolysis of the product mixture gave amino acids (yield: 0.4 %) and heterocyclic compounds including adenine (yield: 0.1 %), aminoimidazole carboxyamidine (AICAI) and aminoimidazole carboxyamide (AICA). It became evident that those compounds were formed via diaminomaleonitrile (DAMN). In connection with the study, it has been shown that metal compounds such as Na_2CO_3, $MgCO_3$, $CaCO_3$, MgO, CaO and Al_2O_3, which are related to clay minerals and abundant in earth crust, were substituted for montmorillonite in polycondensation reaction of HCN. The result suggests that clay minerals and metal compounds might have provided an environment for prebiotic synthesis during chemical evolution.

Introduction

It has been proposed that clay minerals would have played important roles on the formation of organic compounds during chemical evolution in terms of selective adsorption and metal-catalyzing reaction[1]. The recent reviews show that a number of investigations on clay have so far been carried out[2-4], among which the following studies would be listed: binding or adsorption of organic compounds[5,6], limited catalytic properties[7-10] and crystal gene theory[11,12].

The experiment for prebiotic synthesis of organic compounds has been initiated by the action of electric discharges on a simulated primordial gas[13,14]. The result showed that HCN is the key molecule for the production of compounds. Accordingly, HCN has been utilized for a number of prebiotic syntheses under ammoniacal condition[15-20]. Although ammonia has been proven to be excellent catalyst for HCN polycondensation, one may throw doubt on the presence of a great quantity of it during chemical evolution, because of its instability, for example, rapid photochemical degradation to nitrogen and hydrogen[21]. Actually, ammonia is not essential for the oligomerization of HCN on the basis of kinetics of DAMN formation where the rate is proportional to the concentration of HCN and CN^- [18]. It has also been known that ammonia is not sole catalyst for HCN polycondensation[22]. An alternative basic catalyst substituted for ammonia would then be clay minerals and metal compounds which are abundant on the earth as has been suggested[23,24]. In this article, the authors report the synthesis of amino acids and adenine from HCN in the presence of montmorillonite, and the synthesis of DAMN by substituting metals such as MgO, CaO, $MgCO_3$, $CaCO_3$, Al_2O_3 for montmorillonite, in order to simulate on the production of organic compounds during chemical evolution.

Materials and Methods

Montmorillonite.

Montmorillonite (lot # M4F4169, extra pure grade, Nakarai Chem. Ltd., Kyoto, Japan) was in advance baked at 100 °C for 24 hr in an oven to eliminate any ammoniacal contaminants. The result of elementary analysis was Si: 64.6%, Al: 11.5%, Ca: 4.2%, Fe: 3.9%, Mg: 1.7 % and Na: 1.0%, respectively.

Chemicals.

HCN was generated by the reaction of H_2SO_4 on NaCN and condensed in a volume-calibrated cold trap. The liquid HCN was used for several reactions after dilution according to the purpose. DAMN (lot #LA9924) purchased from Aldrich Chem. USA was recrystallized in water before measuring IR spectrum (Perkin-Elmer Model 983, USA). Amino acids were obtained from Wako Pure Chem. Osaka, Japan. ^{14}C-adenine (50 µCi/mmole/ml) was purchased from Amersham, UK. Adenine, aminoimidazole carboxyamide (AICA) and aminoimidazole carboxyamidine (AICAI) were obtained from Wako Pure Chem.

Synthesis of DAMN.

A 20 ml of liquid HCN (0.52 moles) was added to 50 ml of glass distilled water (200 ml flask equipped with a condenser),

to which 3 g of montmorillonite was dispersed (pH 7.1 : the pH of slurry of montmorillonite without HCN was 9.7). The reaction mixture was then refluxed at 50 - 55 °C for 30 hr (the final pH was 7.6). After cooling down it to room temperature, the reaction product mixture (dark yellow) was transferred into a new flask (1 liter), to which 300 ml of glass-distilled water was added. DAMN was extracted by adding 500 ml of ether and vigorously shaking. The ether fraction was condensed by an evaporator and then crude DAMN (light yellow) was obtained. The purified DAMN (600 mg) was then prepared by triply recrystallizing it in water (yield: 4.3 %).

In addition, the metal-mediated formation of DAMN was also studied. Naturally occurred forms of metals were employed as catalyst. Ammonium hydroxide was also used as catalyst to compare the above reaction with the leading synthesis[18-20]. Since DAMN shows λ_{max} at 296 nm, the reaction mixture kept at 15 °C was measured at a constant interval.

Synthesis of amino acids.

The reaction mixture (refluxed for 39 hr) shown in the above section of DAMN was further refluxed at 50 - 55 °C for 7 days. The reaction product mixture (dark reddish brown) was filtered with a filter paper to eliminate black solid. The filtrate (reddish yellow) was condensed to 3 ml by an evaporator and then hydrolyzed with 5.7 N HCl at 110 °C for 48 hr in a sealed glass tube. After hydrolysis, the sample was neutralized with NaOH,

passed through a Dowex 50 (H^+) resin column (30 cm x 0.8 cm ID) to eliminate salt, eluted with 2 N NH_4OH, and finally condensed to 1 ml[25]. A 50 - 100 µl of the sample was analyzed by an amino acid analyzer (Irica 500, Kyoto, Japan). The production of amino acids was confirmed by another methods, thin layer chromatography with two different solvent system and high performance liquid chromatography after derivatizing amino acids with 1-fluoro 2,4-dinitrobenzene.

Study of optical activity of amino acids.

The fraction (5 ml) containing an amino acid (Ala, Asp, AAnBA) obtained from an ion exchange column was dried in vacuo and dissolved in 1 ml of water, to which 1 ml of ethanol, 0.1 ml of triethylamine and finally 0.1 ml of 50 mM 1-fluoro 2,4-dinitrobenzene were added. The reaction mixture was then incubated at room temperature in dark box for 10 hr. The derivatized amino acids were purified through a HPLC column (LiChrosorb RP-18, 25 x 0.46 I.D.)[26]. The optical resolutions of DNP-Ala, DNP-Asp and DNP-AAnBA were carried out as follows. The column (stainless, 25 cm x 0.46 cm ID) was packed (150 kg/cm^2) with native cellulose (lot #2330, Merck Co., FRG) and equilibrated with elution mixture [hexane (H): isoamylalcohol (iA): acetonitrile (A): acetic acid (Ac) = 100:50:50:1 (v/v/v/v) for DNP-Ala, H/iA/A/Ac = 200:50:25:1 for -AAnBA and H/iA/A/phosphoric acid = 12000:3000:2000:1 for -Asp, respectively]. The elution was made by using high performance

liquid chromatography (1.0 ml/min, 30 - 40 kg/cm^2) and monitored at 350 nm.

Synthesis of adenine.

The reaction product mixture shown in the section of amino acids was firstly hydrolyzed in 1 N HCl at 80 °C for 3 hr under nitrogen atmosphere and then filtrated with a filter paper. The reddish yellow colored solution (65 ml) was condensed by an evaporator to 2 - 3 ml, to which 1 µl of ^{14}C-adenine was added as a probe. The sample thus prepared was chromatographed by a cellulose column (100 cm x 2.5 cm ID, elution mixture: acetonitrile/water/acetic acid = 8:4:1 (v/v/v) at 120 ml/hr). Every 3 ml was collected by a fraction collector. The fraction (15 ml) showing radioactivity was collected and rechromatographed after condensation. The radioactive fraction collected was condensed, crystallized in water and then provided for measuring IR spectrum.

On the other hand, adenine was also synthesized from DAMN. The mixture containing 100 mg DAMN, 400 mg formamidine, 500 mg montmorillonite and 50 ml water in a 300 ml flask, was refluxed for a week at 55 °C. The reaction product mixture thus obtained was treated as shown above and adenine was finally crystallized.

Results and Discussion

DAMN

In the absence of montmorillonite, none of DAMN was formed (Fig. I-1), indicating that the clay mediates the polycondensation of HCN to DAMN. Fig. I-2 gives its IR spectrum of crystallized DAMN, which coincides with that of the authentic DAMN. It is evident that the key intermediate to polymers from HCN in the presence of montmorillonite is also DAMN, as has previously been shown under ammoniacal condition[18-20]. Since the slurry of montmorillonite under experimental condition gave a basic pH (9.7-10.2), clay seems to play as a weak base for HCN polycondensation as ammonia : 1) a proton of HCN is firstly withdrawn by clay, 2) CN^- attacks to δ^+ carbon of the next HCN, 3) clay then returns H to nitrogen of the above HCN and 4) sequential reaction gives precursors for amino acids and nucleic acid bases.

Amino acids

The chromatogram of amino acid analysis shows that various ninhydrin positive compounds were synthesized (Fig. I-3), among which the listed amino acids are confirmed by three different methods (Table I-1). The yield of amino acids was calculated to be *ca.* 0.4 % based on carbon of input HCN. The total amount of amino acids would also be comparable with the reported[13-15,20].

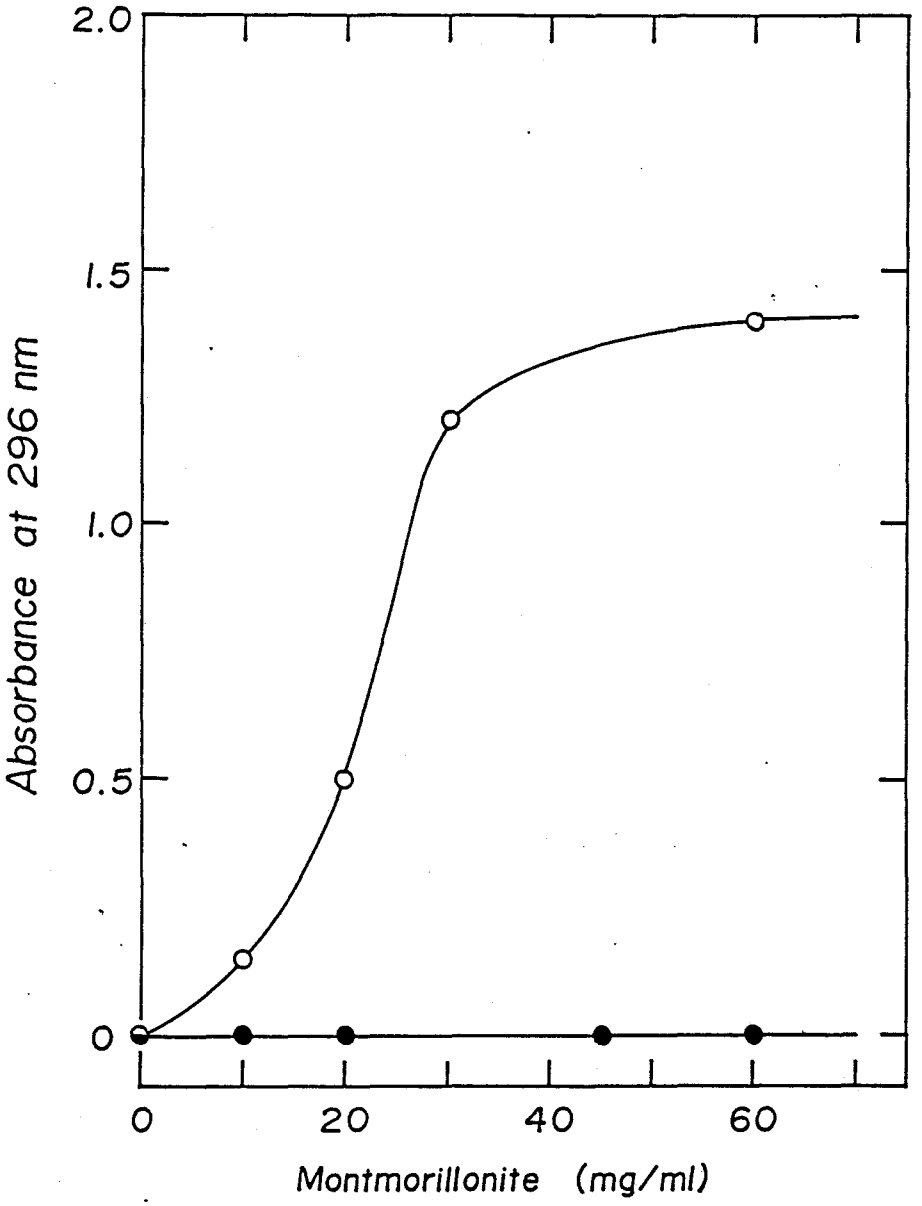

Fig. I-1. Formation of DAMN with concentration of montmorillonite. The indicated amount of clay powder was gently dispersed in a quartz cuvette (light path: 1 cm) containing 3 ml of 0.1 M HCN. The vessels were stood at 15 °C without shaking for 1 week. The absorbance at 296 nm of the reaction mixture was measured. The value of $OD_{296\ nm}$: 1.0 was corresponding to 7 x 10^{-5}M DAMN. --o--: with and --●--: without montmorillonite.

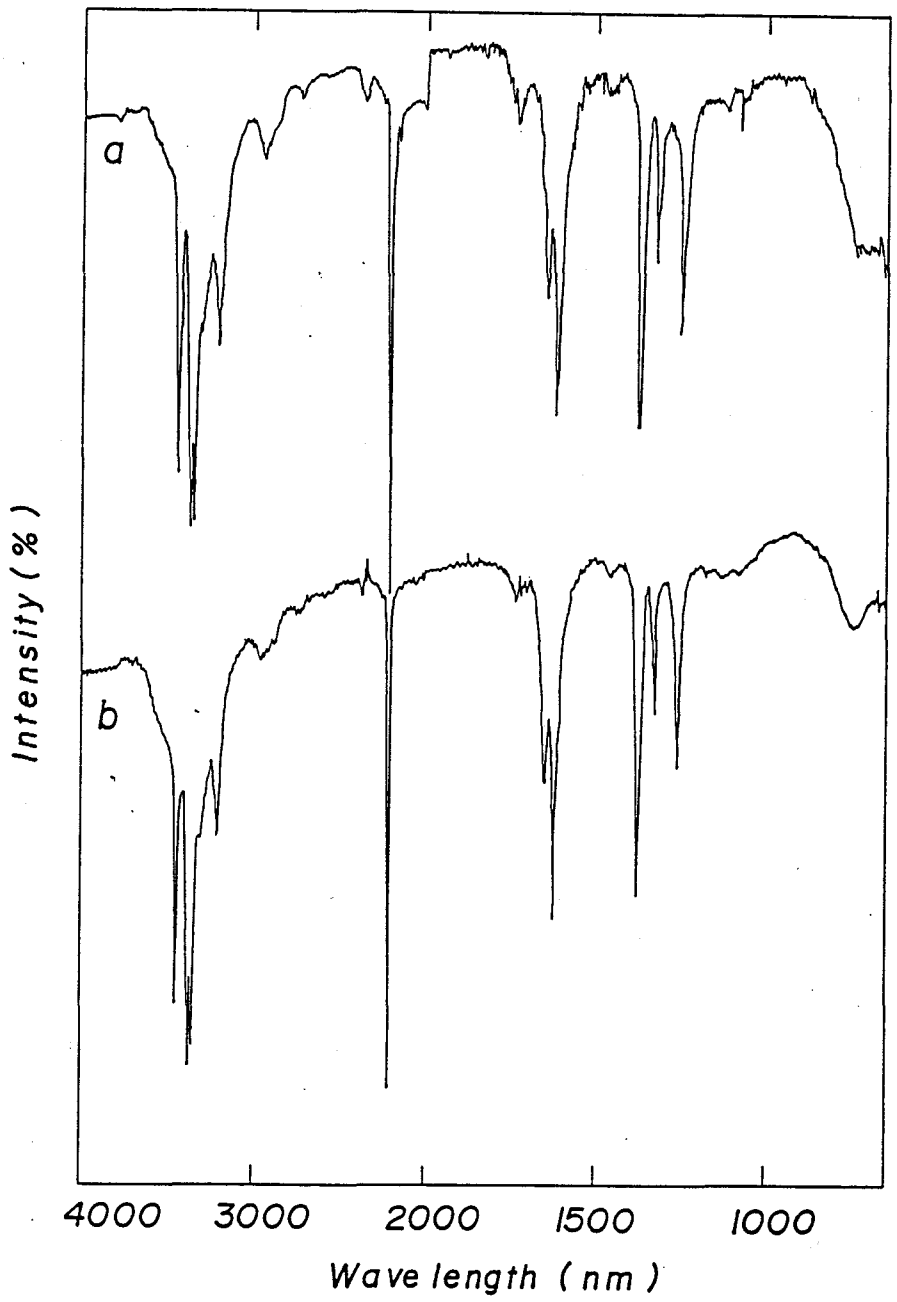

Fig. I-2. IR spectra of synthesized and authentic DAMN. (a) synthesized DAMN and (b) authentic DAMN.

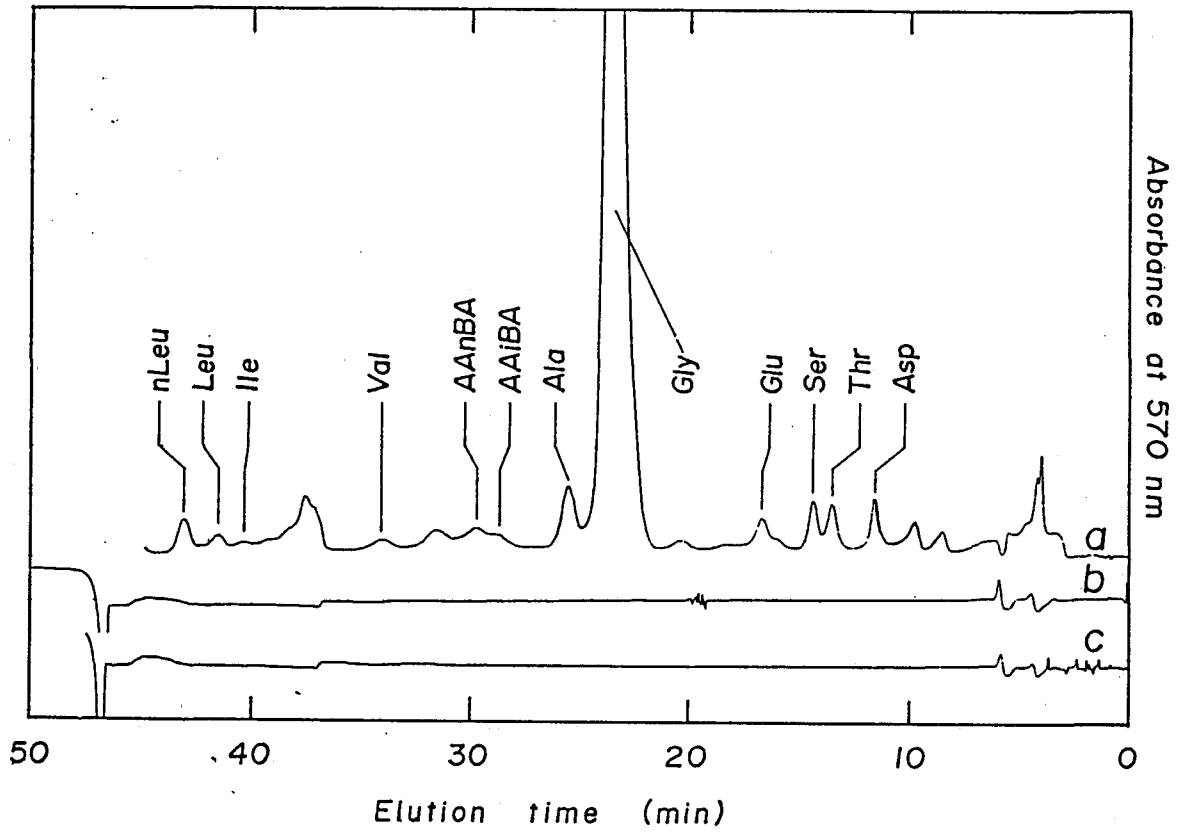

Fig. I-3. Chromatograms of amino acid analysis. Ninhydrin positive compounds from HCN, water and montmorillonite (a). The amino acids indicated show the position where the authentic amino acids are eluted. The confirmed amino acids are then given in Table I-1. Two blank experiments were made as follows. To 50 ml of water, only 3 g of montmorillonite was dispersed (b). 20 ml of liquid HCN was mixed with 50 ml of water (c). The experiment was separately treated as the same as the condition to produce amino acids as shown above.

Table I-1. Amino acids from HCN in the presence of montmorillonite.

amino acids	analytical methods			yield[4] (μmoles)
	TLC[1]	IELC[2]	HPLC[3]	
Gly	+[5]	+	ut[7]	83.9
Ala	+	+	+	6.9
Asp	+	+	+	2.1
AAnBA	-[6]	+	+	0.2

1) Thin layer chromatography by two different solvent system : n-butanol-acetic acid-water (4:1:1, v/v/v) and ethanol-conc NH_4OH-water (90:5:5, v/v/v)

2) Ion exchange liquid chromatography (amino acids analyzer) (Fig. 3)

3) Optical resolution of amino acids derivatized with DNP by high performance liquid chromatography (Fig. 4)

4) Total yield : 0.4 % based on input carbon of 50 mmoles HCN (calculated from the chromatogram of Fig. I-3)

5) Confirmed, 6) Not confirmed, 7) Untested

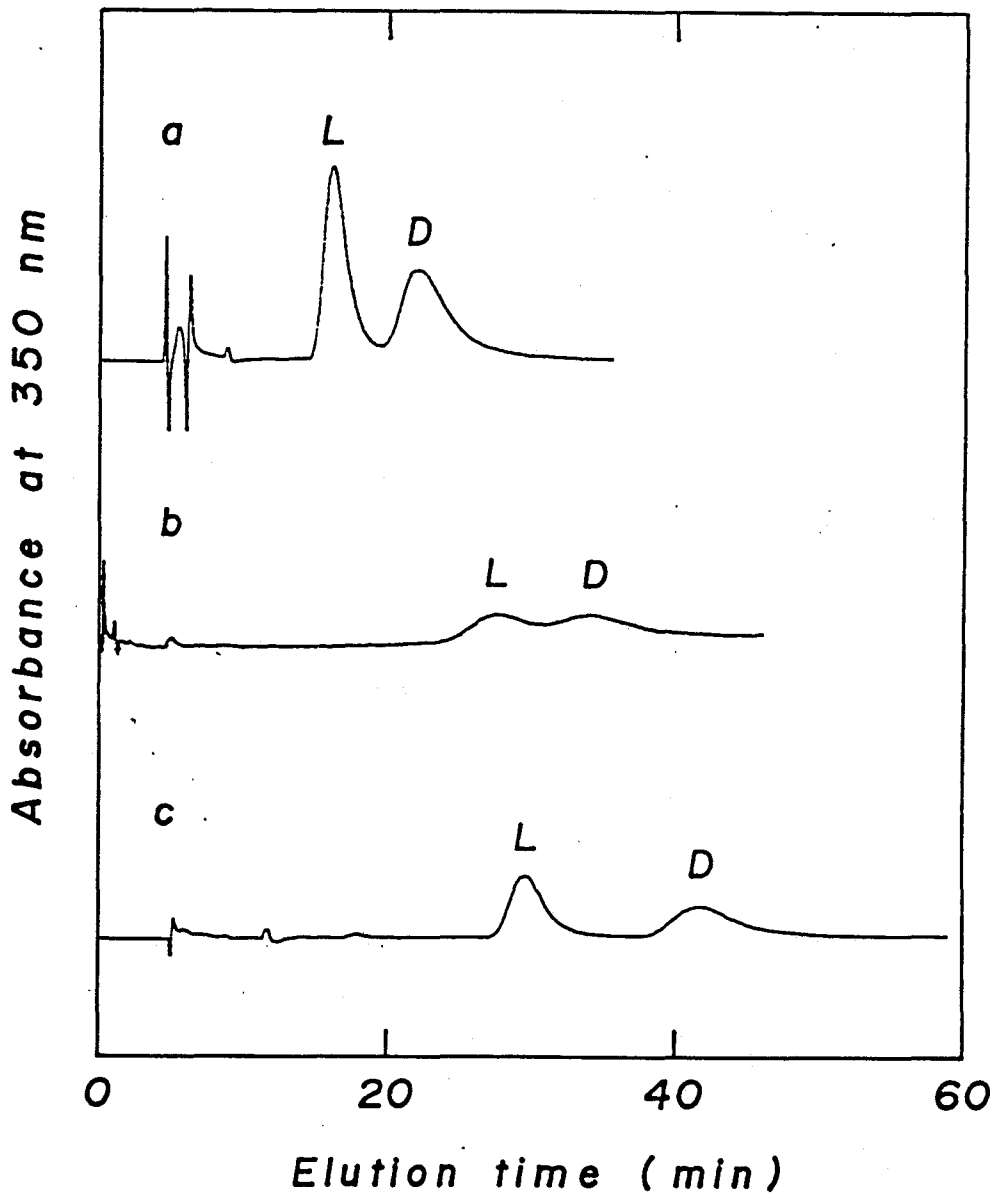

Fig. I-4. Optical resolution of the derivatized amino acids. a: DNP-Ala, b: DNP-ASP and c: DNP-AAnBA.

These amino acids would be produced from various HCN polymers by means of hydrolysis, decarboxylation or deamination, probably as follows : Gly from dimer of HCN, Ala from trimer and Asp and AAnBA from tetramer, respectively. On the other hand, two blank runs gave no peak (b and c in Fig. I-3), indicating that the present amino acids are newly synthesized from HCN in the presence of montmorillonite.

All the amino acids which are abiotically synthesized without aid of microorganisms give racemic mixture. Three amino acids, Ala, Asp and AAnBA separated in the pure form from an ion exchange column, were derivatized to give DNP-Ala, DNP-Asp and DNP-AAnBA and resolved. The result shows that the ratio of D-enantiomer by L-enantiomer is roughly 1 (Fig. I-4). It is concluded that the amino acids and other ninhydrin positive compounds shown in Fig. I-3 are abiotically synthesized.

Adenine

The production of DAMN from HCN was shown in the presence of montmorillonite (Fig. I-1). DAMN once formed is converted to heterocyclic compounds. The authors synthesized adenine (13 mg, 0.1 % yield) from HCN in the presence of montmorillonite (Fig. I-5 a). In the absence of clay, neither adenine nor amino acid (Fig. I-3 c) was found.

For reference, the reaction containing DAMN, formamidine and montmorillonite gave 17 mg of adenine (16 % yield) (Fig. I-5b), but adenine was not formed only from DAMN and formamidine in the

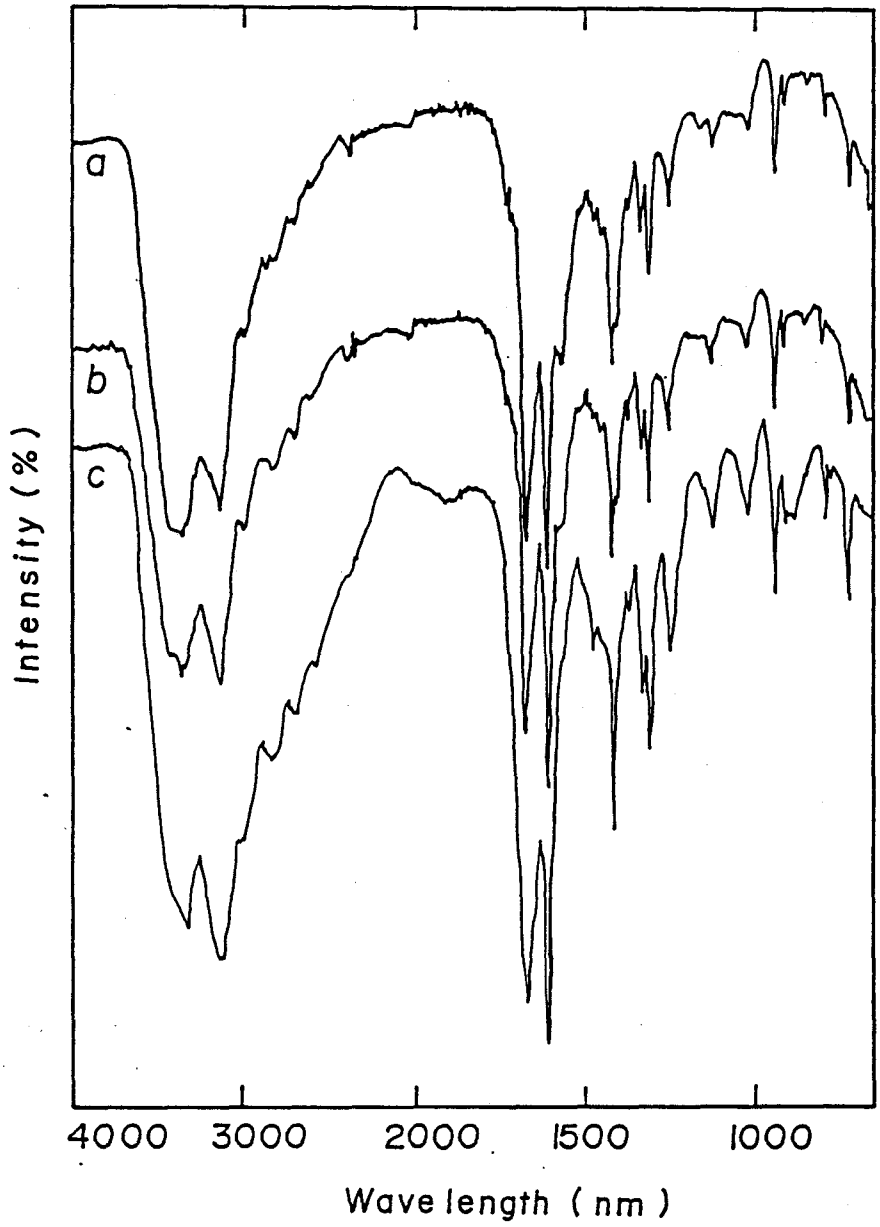

Fig. I-5. IR spectra of synthesized and authentic adenine. (a) HCN and montmorillonite, (b) DAMN, formamidine and and montmorillonite and (c) authentic adenine, respectively.

absence of montmorillonite. The fractions containing AICAI and AICA were also collected through a cellulose column. After condensation, they were determined by TLC and UV absorbance by comparing them with corresponding authentic standard. Their yields were photochemically calculated to be 0.1 mg (0.1 %) for AICAI and 23 mg (13 %) for AICA. A large amount of AICA in comparison with that of AICAI would be due to hydrolysis of AICAI during preparation. The result indicates that in the presence of montmorillonite HCN produces DAMN from which adenine is formed, of which mechanism seems to be the same as those in the presence of ammonia as catalyst[16-19]. Although guanine was not isolated in the present experiment, it could be synthesized because of the presence of a large amount of AICA. Ferris et al[27,28] have shown the clay-mediated decomposition of DAMN. However, the synthesis of adenine and amino acids from HCN in the presence of montmorillonite would be significant for simulating prebiotic synthesis.

Clay and metal compounds

It has been shown that montmorillonite provides weak basic environment (the pH of slurry of montmorillonite was 9.7) under which polycondensation of HCN can be catalyzed. Such the environment can also be produced in the presence of metal compounds which are abundantly available in earth's crust in the form of oxide or carbonate, for example silica (SiO_2), alumina (Al_2O_3), hematite (Fe_2O_3), calcite ($CaCO_3$),brucite ($MgO \cdot H_2O$),

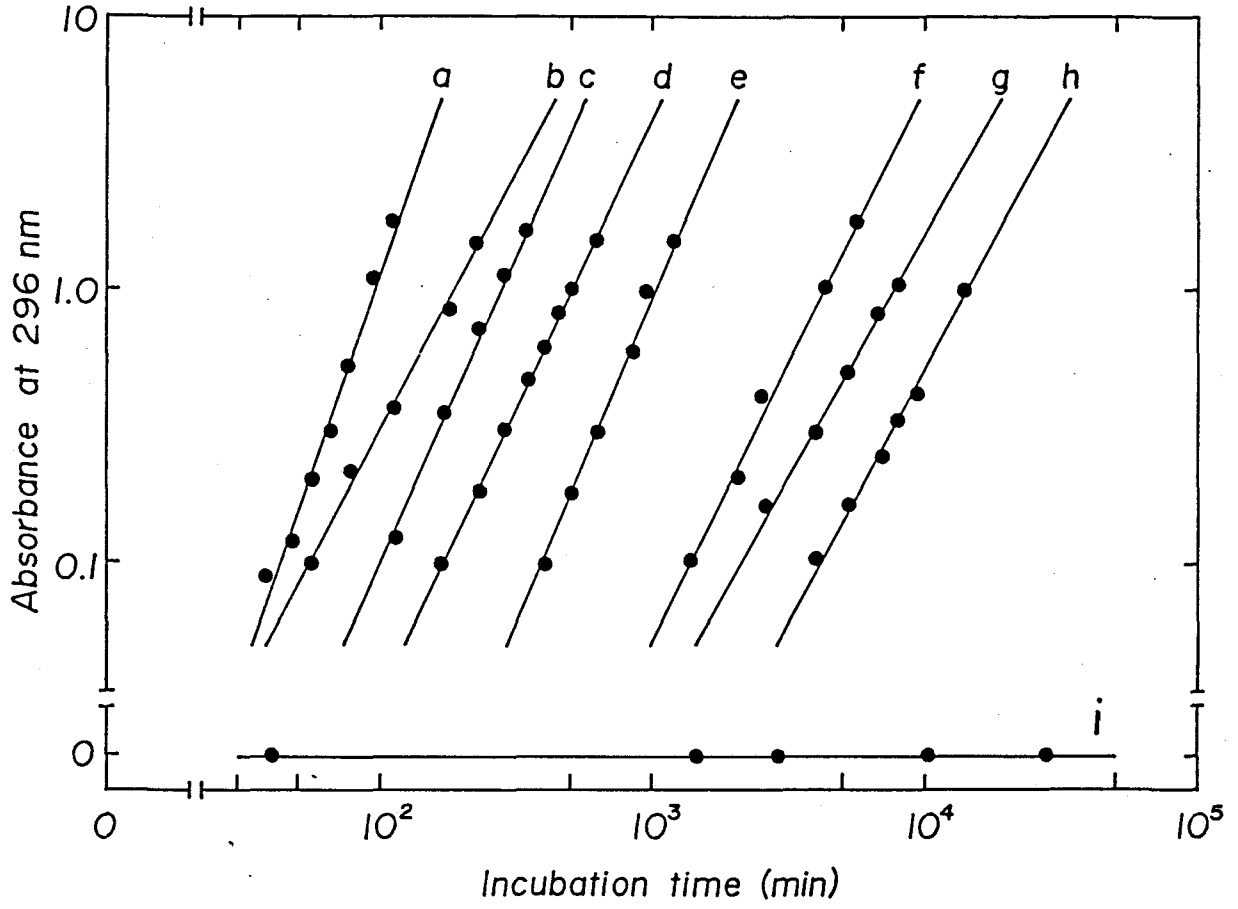

Fig. I-6. Formation of DAMN from HCN in the presence of montmorillonite and metals. Naturally occurred forms of metals were employed. Before use, all the metals were baked in an oven at 100 °C for 24 hr to eliminate ammoniacal contaminations. They were added to 3 ml of 0.11 M HCN in a 5 ml cuvette (light path: 1 cm) at a final concentration of 0.1 M, except montmorillonite (30 mg/ml). Since DAMN has λ_{max} at 296 nm, the reaction mixture stood at 15 °C was measured at the indicated interval. (a) NH_4OH, (b) CaO, (c) MgO, (d) $MgCO_3$, (e) Na_2CO_3, (f) $CaCO_3$, (g) montmorillonite, (h) Al_2O_3, and (i) Fe_2O_3 and SiO_2, respectively. The rates of synthesis per hour were calculated as follows: (a) 6×10^{-5} M, (b) 3×10^{-5} M, (c) 2.5×10^{-5} M, (d) 1.3×10^{-5} M, (e) 7×10^{-6} M, (f) 1.3×10^{-6} M, (g) 5.6×10^{-7} M and (h) 2.5×10^{-7} M, respectively.

periclase (MgO), dolomite ($CaCO_3 \cdot MgCO_3$) and soda (Na_2CO_3). If these metal compounds as well as clay minerals show certain catalytic activity on the polycondensation of HCN, further opportunity would be added to the prebiotic formation of organic compounds. In order to survey this possibility, the formation of DAMN has been investigated in the presence of montmorillonite, and metal oxides and carbonates has been investigated (Fig. I-6). The rate of polycondensation of HCN in the cases of CaO (b), MgO (c), $MgCO_3$ (d) are mostly comparable with that of ammonia (a). The other catalysts also gave a certain level of DAMN production with time of incubation (e-h). However, silicate and hematite showed no catalytic activity during experimental period (i).

The present result provides new perspective for prebiotic synthesis, since clays are of great advantage to selective adsorption and catalytic function. When a large amount of HCN was dissolved into a shallow marsh or a tideland, it might be condensed under slightly basic environment in the presence of clay and metals, in which effective polycondensation and replication of molecules would take place. On the other hand, a number of sugars have been synthesized from formaldehyde in the presence of clay minerals[29,30] and metals[31-34]. The present result might show a possible environment for orderly and co-operative development of nucleic acids and proteins during chemical evolution.

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Part II

Resolution of all proteinic DL-amino acids on native-cellulose chromatography

Abstract

We have studied on the resolution of DL-amino acids on native-cellulose chromatography. Recently, we have accomplished the resolution of all proteinic DL-amino acids without any modification. It has known that the resolution capability of their racemates on cellulose chromatography was mainly affected by 1) bulkiness of the side group attached to α-carbon of amino acids, 2) hydrophobic environment 3) physico-chemical property of cellulose and 4) structural and functional interaction of D- or L-amino acids with cellulose (e.g. the relation such as key and lock). The results so far obtained are reviewed in the present article. In discussion, the resolution mechanism of DL-amino acids on chiral stationary phase of cellulose is proposed. The significance and application for the present study are also presented.

Introduction

More than three decades ago, the limited DL-amino acids on paper chromatography were resolved[1-4]. None of development of the related investigation has so far been made, perhaps because of difficulty of obtaining qualified cellulose, e.g. microcrystaline with uniform microparticles. However, our extensive investigation led us to find such the qualified cellulose. Since then, we have put emphasis on the effectiveness of using native

cellulose on the resolution of racemic amino acids from various points of view[5]. The results obtained by native-cellulose chromatography were mostly comparable with those by the leading techniques which were mostly reviewed by Davankov et al[6] and Souter[7].

The resolutions of DL-amino acids (non-derivatized and derivatized) have been conducted by using native-cellulose column chromatography[5,8-11]. The purpose of this article is to describe the comprehensive study of the resolution of all the proteinic DL-amino acids on cellulose chromatography.

Materials and Methods

Chemicals and reagents

DL-amino acids (Kit # DLAA-24), L-amino acids (Kit # LAA-21), D-amino acids (Kit # DAA-16) were purchased from Sigma Chemical, USA and other amino acids (Wako Pure Chem., Osaka, Japan) were also used. Amino acid abbreviations are given in the legend to Table 1.

Unmodified (native) cellulose (Art. # 2331 and #2330, Merck, Co., Germany) was suspended in 1N-HCl and stirred with a magnetic stirrer for 1 hr to eliminate some contaminants, and then fully washed with glass-distilled water, using the method of suspension and decantation. The cellulose thus prepared was provided for the experiment.

Chemicals and reagents, which were from Wako Pure Chem., were reagent-grade. All aqueous solutions were prepared by using deionized and glass-distilled water.

All glassware was washed by soaking for more than three days in a special detergent (Clean 99L, Terauchi Chem., Osaka, Japan) and then thoroughly rinsed in deionized and glass-distilled water and dried in an electric oven.

Column chromatographic resolution of DL-amino acids and reactivation of cellulose column

The cellulose was packed into a glass column (0.85 x 250 cm, bed height: 240 cm). The methods for analyses were given in legend to the correspondent results.

When we repeatedly used a cellulose column, the resolution capability decreased. Such the cellulose column could easily be reactivated by its original performance with 10 ml of 1N-HCl. After washing with HCl, the column was equilibrated with 100 - 200 ml of the elution mixture. However, it has been known that the resolution capability, in spite of the above reactivation, gradually decreased with the repeated usage.

Computer analysis of chromatograms

It became evident that all proteinic DL-amino acids are resolved on native-cellulose column chromatography. However, some DL-amino acids gave the chromatograms which were poorly

resolved. Such the chromatograms were analyzed by a computer to display the theoretical elution patterns, which provide us basic informations for calculating the resolution factors in Table II-1.

Effect of different source of cellulose on resolution

Three kinds of cellulose were chosen and independently packed in the same column (1.1 cm x 120 cm), to compare respective resolution capability among the source of cellulose. In the present experiment, used were 1) Cellulofine GC-700M (Lot # 1298, Biochem. Ind., Tokyo, Japan), 2) Whatman CF-11 (Lot # 2311110, Whatman, USA) and 3) Merck (Art # 2331, Merck Co, Germany). The column (bed height of 105 cm) was equilibrated only with water. Usually, 0.1 ml of 1mg/ml DL-tryptophan was charged and eluted with water (20 ml/hr) at room temperature. The eluant was monitored at 280 nm (UVICON-540M, Toyo Kagaku Sangyo, Osaka, Japan).

Result

Non-polar side groups.

DL-amino acids only having non-polar side group (Ala, Val, Ile, Leu, Met, Phe, Trp and Pro) were separated (Fig. II-1). Since the resolutions for DL-Ala, -Val, -Ile and -Leu were not

satisfactory, those chromatograms were analyzed by a computer so that the theoretical resolution patterns could be obtained (inset of Fig. II-1). On the other hand, the resolutions of DL-Met, -Phe, -Trp and -Pro were completed. The results indicate that DL-amino acids having large side group can be well-resolved. The detailed data were also summarized in Table II-1. The conformations of D- and L- enantiomers were determined by either measurement of circular dichroism spectra (CD-spectra) for Phe and Trp, or co-chromatography for others.

Polar-uncharged side groups.

Fig. II-2 gives the results of chromatographic resolution of DL-amino acids which have polar but uncharged side group (Ser, Thr, Cys, Tyr, Asn and Gln). Although the complete resolution for DL-Tyr was made, others were not under the present condition. The analyzed patterns by a computer are given in inset of Fig. II-2. The detail explanation is also presented in Table II-1. The identifications of enantiomers were carried out by measuring CD-spectra (Ser, Thr and Tyr), and applying co-chromatography (Cys, Tyr and Asn) and cellulose-thin layer chromatography (CTLC) (Asn and Gln), respectively.

Since DL-Cys is quite labile under the basic condition containing pyridine, the special elution mixture (ethanol and water) for its resolution was employed. The chromatogram for DL-Cys in Fig. II-2 was not resulted from the formation of cystine (Cys-Cys) after dimerizing cysteine (Cys), which was confirmed by

Table II-1. Summary of resolution of all proteinic DL-amino acids

The optical purity of resolved enantiomers (aromatic, heterocyclic etc) was determined by measuring their CD-spectra. Prior to obtaining the spectra, concentrations of each enantiomer were adjusted using the α-value for UV-absorbance and visible absorbance (after reaction with ninhydrin reagent). The CD-spectra of the sample solutions were measured at neutral pH with a magnetic polarimeter (JASCO-MOE 1, Jasco, Tokyo, Japan).

Since aliphatic amino acids absorb extremely short wavelength, none of CD spectra for them can be measured. The resolution was accordingly assigned by co-chromatography, in which the sample solution mixtures containing enantiomers in different ratios (e.g. D:L=1:3 or vice versa) were co-eluted.

In order to identify the enantiomeric conformation of polar DL-amino acids, the CTLC was adopted. D- and L- enantiomers were alternately spotted at the origin of a plate, and ascendingly developed with the solvent that was the same as the elution mixture for column chromatography.

Table II-1 Summary of resolution of all proteinic DL-amino acids

DL-Amino acids		Retention time (min)			Resolution factor[1]	Resolution conditions	Identification[4]
Name	Abbreviations	D(peak)	L(peak)	Trough			
alanine	Ala	892(892)[2]	938(938)[2]	915(915)[2]	0.050(0.050)[2]	P/E/W=5.5:1:1[3]	B
valine	Val	625(625)	663(663)	644(644)	0.059(0.059)	P/E/W=5:1:1	B
leucine	Leu	523(515)	540(542)	531(529)	0.032(0.053)	P/E/W=5:1:1	B
isoleucine	Ile	512(512)	547(547)	529(529)	0.066(0.066)	P/E/W=5:1:1	B
methionine	Met	634	695	664	0.092	P/E/W=5:1:1	B
phenylalanine	Phe	641	711	679	0.103	P/E/W=4:1:1	A
proline	Pro	710	770	740	0.081	P/E/W=3:1:1	B
tryptophan	Trp	380	435	413	0.133	P/E/W=1:1:1	A
serine	Ser	1263(1263)	1337(1348)	1300(1300)	0.057(0.066)	P/E/W=4:1:1	A
threonine*	Thr	1006(1006)	927(927)	966(966)	0.082(0.082)	P/E/W=4:1:1	A
cysteine	Cys	990(989)	1040(1043)	1015(1016)	0.048(0.053)	E/W =6:1	B
tyrosine	Tyr	596	674	635	0.123	P/E/W=4:1:1	A
asparagine*	Asn	286(293)	252(258)	269(276)	0.126(0.127)	A/P/W=2:2:1	B,C
glutamine*	Gln	434(436)	409(408)	422(422)	0.057(0.066)	A/P/W=2:2:1	C
asparatic acid	Asp	1184(1183)	1242(1243)	1213(1213)	0.048(0.049)	A/P/5×10^{-3}N HCl =5:5:2	C
glutamic acid	Glu	844(840)	882(892)	863(866)	0.044(0.060)	A/P/5×10^{-3}N HCl =5:5:2	C
lysine*	Lys	317(321)	302(302)	310(312)	0.048(0.061)	A/P/1×10^{-3}N NaOH =2:2:1	C
arginine*	Arg	304(306)	277(276)	291(291)	0.093(0.103)	A/P/1×10^{-3}N NaOH =1:1:1	C
histidine*	His	925	820	872	0.120	P/E/W=1:1:1	A

1) Resolution factor = [D(peak) - L(peak)] / Trough

2) All the values in parentheses were obtained by a computer.

3) P : pyridine, E : ethanol, W : water and A : acetonitrile, respectively. All the ratio was represented by "volume by volume".

4) The methods of determining the resolved enantiomers were as follows; A: CD spectra, B: Co-chromatography under which the sample solution mixtures containing enantiomers in different ratios (e.g. D:L - 1:3 or vice versa) were eluted, and C: thin layer chromatography on cellulose plates [(Avicel SF, Funakoshi Yakuhin Co. Ltd., Osaka Japan), which was achieved by ascendingly developing D- and L-enantiomers alternately spotted at the origin].

* L-enantiomers were eluted faster than the opposite.

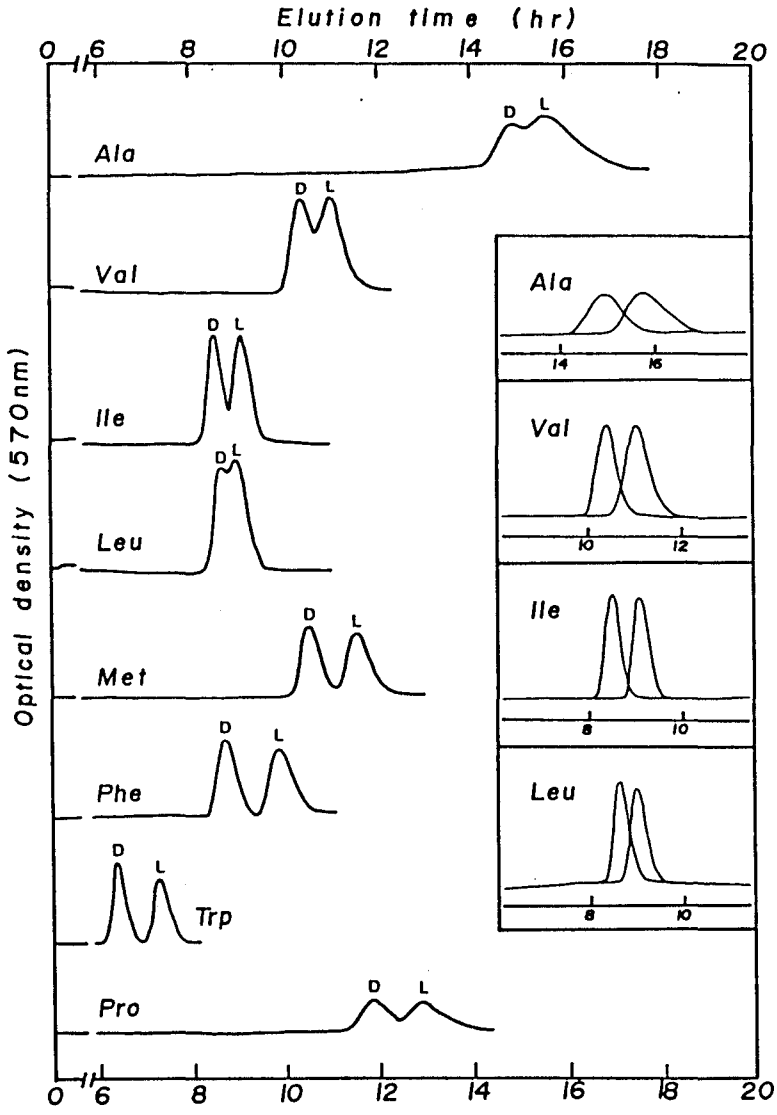

Fig. II-1. Resolution of DL-amino acids having non-polar side group. The packed cellulose (#2331) column (0.85 cm x 250 cm, bed height: 240 cm) was at first washed with 10 ml of 1N-HCl. The column thus washed was equilibrated with the elution mixture containing different ratio of pyridine, ethanol and water as summarized in Table II-1. Using this method, 13 non-polar and polar-uncharged DL-amino acids were resolved. Eluants (30 ml/hr) were monitored at 440, 570 and 660 nm, after mixing with ninhydrin reagent (15 ml/hr), using a UV-Vis effluent monitor (Hitachi, Tokyo, Japan). All resolutions were made at room temperature, except for that of DL-Cys (0-5 °C). Inset shows the computer-analyzed patterns.

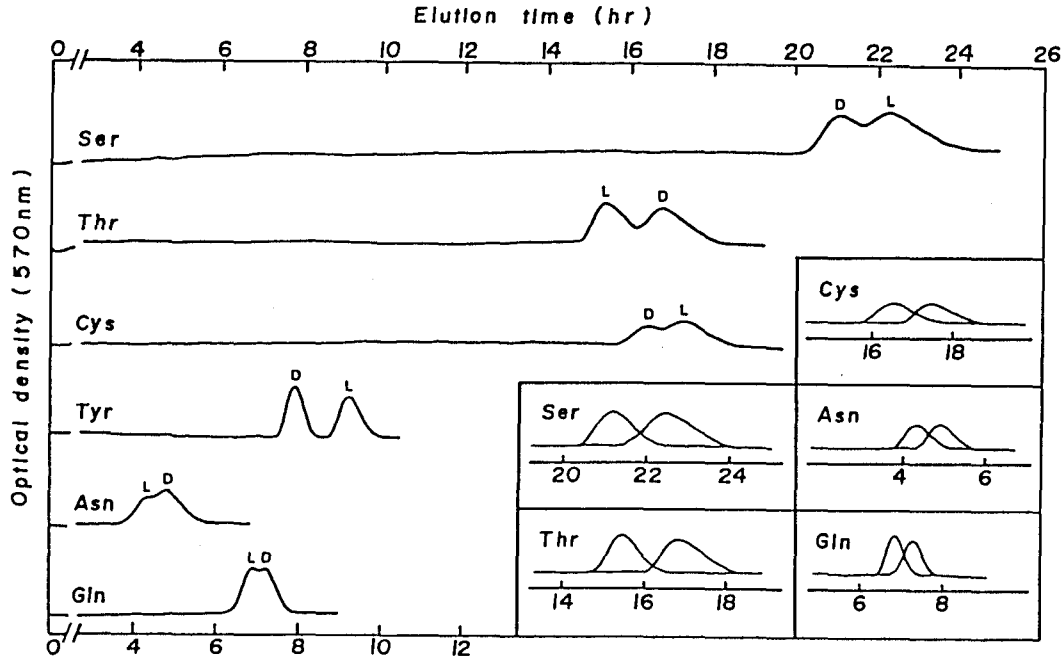

Fig. II-2. Resolution of DL-amino acids having polar-uncharged side group. DL-Ser, -Thr and -Tyr (500 μg each) were resolved with the elution mixture containing pyridine, ethanol and water (4:1:1, v/v/v). DL-Cys (1 mg) was with that containing ethanol and water (6:1). DL-Asn (200 μg) and DL-Gln (300 μg) were with that containing acetonitrile, pyridine and water (2:1:1). The details were in Table II-1. The eluants were monitored at 570 nm after mixing with ninhydrin reagent. The computer analyzed patterns for incomplete resolutions are also given in inset. All resolution were at room temperature, except for that of DL-Cys (0-5 °C).

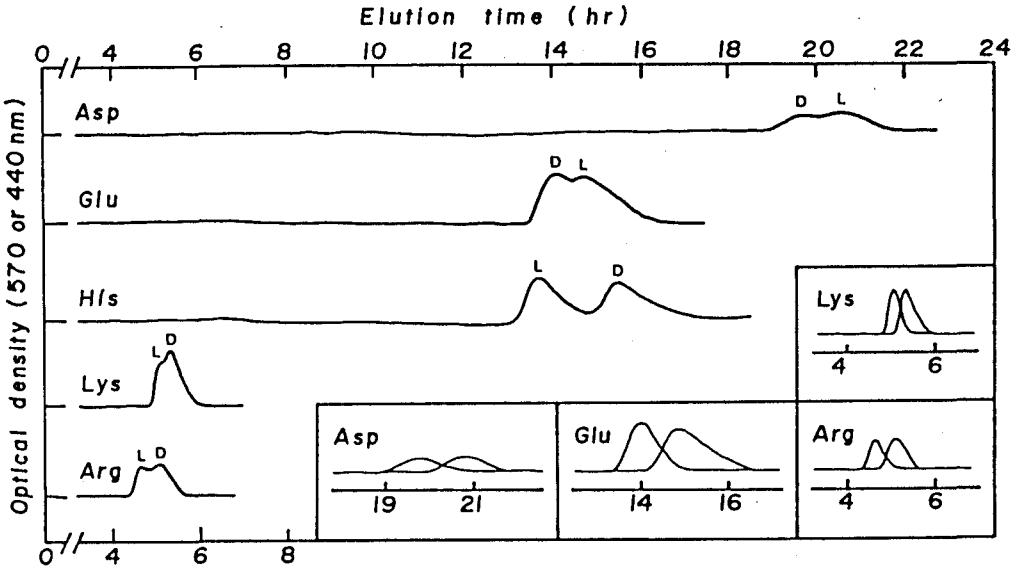

Fig. II-3. Resolution of DL-amino acids having polar-charged side group. The washed cellulose column with HCl was neutralized with 30 ml of 0.01N-NaOH. The column was then equilibrated with the elution mixture (see Table II-1). DL-Asp and -Glu (300 µg each) were resolved with the elution mixture containing acetonitrile, pyridine and $5x10^{-3}$N-HCl (5:5:2). The eluants (60 ml/hr) were monitored at 440 nm. On the other hand, DL-Lys (1mg) and DL-Arg (500 µg) were with that containing acetonitrile, pyridine and $1x10^{-3}$N-NaOH (2:2:1 for DL-Lys or 1:1:1 for DL-Arg). DL-His (500 µg) were with that containing pyridine, ethanol and water (1:1:1). Eluants of these three amino acids were monitored at 570 nm. Inset also shows the computer-analyzed patterns.

the comparison of both elution profile through a column and CTLC plate.

For the resolutions of DL-Asn and -Gln, the different elution mixture (acetonitrile/pyridine/water) from that that consisted of pyridine/ethanol/water, was used. Judging from the computer analyzed patterns (inset of Fig. II-2), it is known that those DL-enantiomers are resolved on chromatography. Moreover, the resolution factors are comparable with other amino acids (Table II-1).

Polar-charged side groups.

DL-His (basic), which is heterocyclic, was resolved by using the elution mixture containing pyridine, ethanol and water (Fig. II-3) and its resolution profile was confirmed by CD-spectra. On the other hand, DL-Asp and -Glu (acidic) and DL-Lys and -Arg (basic) were resolved into their enantiomers with the elution mixture closed to their isoelectric point (Fig. II-3 and Table II-1).

The acidic DL-amino acids (Asp and Glu) were resolved under acidic condition, whose condition was known to be most effective for their resolution among the tested elution mixtures. The identification of D- and L-enantiomers was based on the results obtained from CTLC.

The basic amino acids (Lys and Arg) were resolved under basic condition. This result suggests that the positively charged amino group(s) of these amino acids may strongly interact with

the negatively charged hydroxyl group(s) of cellulose. D- and L-conformations for those amino acids were assigned by CTLC.

Discussion

1. Resolution mechanism

Non-polar and polar-uncharged side groups.

The resolution of DL-amino acids are basically depending upon their bulkiness of side group (see the resolution factors in Table II-1). Also, all aliphatic DL-amino acids are resolved under relatively high content of pyridine in elution mixture (Table II-1), indicating that hydrophobic environment may play an important role on their resolutions as has been suggested[12]. Furthermore, it is interesting to know the role of amino group attached to α-carbon on the resolution, since it may interact with cellulose. For this purpose, the amino groups were modified with 1-fluoro 2,4-dinitrobenzene (DNP-amino acid). The DNP-DL-aliphatic amino acid thus prepared, however, could be completely resolved by using a native-cellulose column[13], suggesting that such the chemical interaction of amino groups with cellulose may not important for resolution of DL-amino acids. Rather, it is considered that their resolution may depend on the way of structural accommodation of D- or L-amino acid on cellulose as has been speculated[12].

Polar-charged side group.

The resolution mechanism for these DL-amino acids may probably be different from the above. The charged side groups are β-carboxylic (Asp), γ-carboxylic (Glu), β-imidazolic (His), ε-amino (Lys) and σ-guanidinic (Arg), respectively. However, it is noteworthy that DL-His was resolved with the elution mixture containing pyridine, ethanol and water (Fig. II-3). This result indicates that the bulkiness of side group may contribute to the resolution rather than its polarity.

On the other hand, none of other polar DL-amino acids was resolved with the above elution mixture, but resolved with another mixture which had typical pH at around their isoelectric points (Fig. II-3). It suggests that some ionic interaction of those polar groups with hydroxyl group of glucose may be formed, which may affect their resolution of polar DL-amino acids.

Property of cellulose.

We have compared the property of cellulose obtained from different source to know the effect of cellulose conformation on the resolution (Fig. II-4). The cellulose which has been reconstructed (type II) (A) has no resolution capability. The cellulose (B), which is categorized in native cellulose (type I) but is said to be made of pulp, has less capability than C. The third one (C) gives complete resolution, whose cellulose is also categorized in type I but made of linter. The result gave

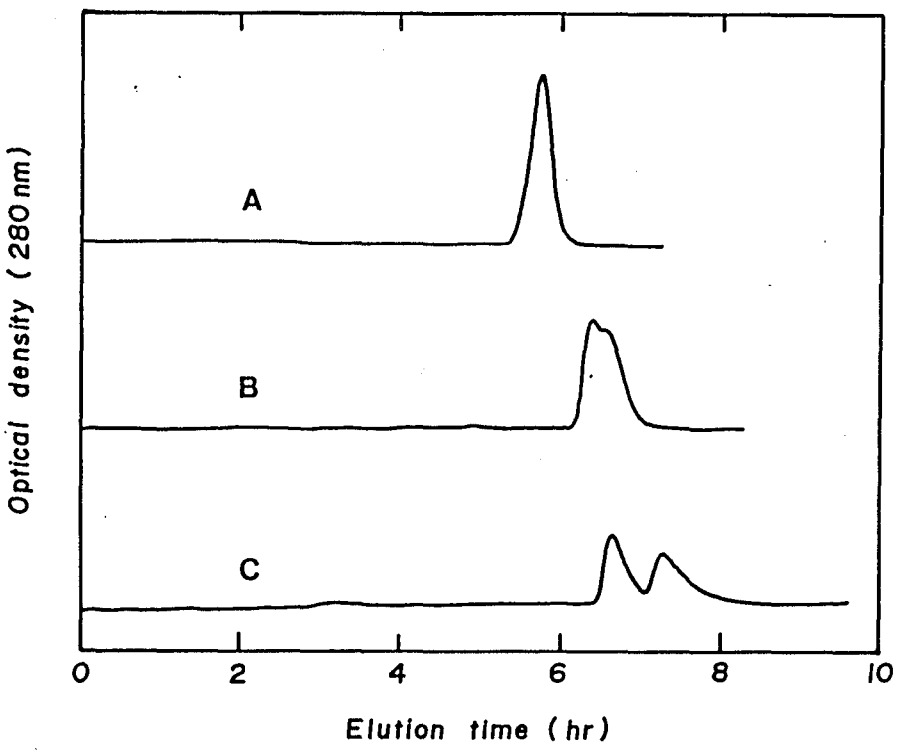

Fig. II-4. Change of resolution capability by using different cellulose. A: Cellulofine, B: Whatman and C: Merck, respectively. The experimental condition was described in Materials and Methods.

us the evidence that the resolution capability can be variable with using different source of cellulose. It is suggested that a certain microcrystalline conformation, which is supposed to be typical for the linter-typed cellulose, may be necessary for the complete resolution.

2. Significance and application of study

Construction of model for resolution.

The resolution mechanism has long been discussed on the basis of chemical interaction between chiral-chiral compounds[5,8-13]. Viewing the interaction between DL-amino acids and cellulose, the following model may be considered. DL-amino acids may accommodate on cellulose surface in the relation such as Key (amino acids) and Lock (cellulose). However, the way of the accommodation may slightly be different between enantiomers. Such the physical interaction may predominantly play an important role on the resolution of DL-amino acids rather than chemical interaction. In this sense, our system may provide valuable information for the construction of model which may explain the resolution mechanism of racemates on chiral phase.

Characteristics of our technique.

The chromatography presently employed has marked characteristics as follows. 1) Commercial native cellulose can

repeatedly utilized without any modification. 2) The cellulose can be reactivated by a certain method with which its resolution capability is kept high (Materials and Methods). 3) Not only non-derivatized but also derivatized DL-amino acids can be resolved into enantiomers. 4) Nearly half proteinic DL-amino acids gave complete resolutions (Figs. II-1-3). 5) Those resolved enantiomers were optically pure. 6) Either water or organic solvents can be used as the liquid phase and eluants other than those mixing with reagent can be recycled. 7) If volatile elution mixtures are used, optically pure samples can be obtained in high yields without any complicated treatments. 8) The separation method can be applied not only in laboratory but also for industrial scale of separations because the resolution depends on the bed volume of the cellulose packed in the column.

On the other hand, the TLC separations of amino acid enantiomers by using chiral thin layers (artificially modified) have recently been demonstrated[14,15]. Since we have used cellulose, it is interesting to compare the data obtained from our CTLC with those from the artificial plates. The result by CTLC is known to be comparable with that by the authentic TLC[14,15]. Therefore, it is concluded that the cellulose column and thin layer chromatography could be useful for the separation of amino acid racemates, although further innovation of the system should be required.

Application of our study.

All the bulky DL-amino acids can be resolved into the mostly pure forms (Met, Phe, Trp, Pro, Thr, Tyr and His, Figs. II-1-3). However , some of other DL-amino acids gave poor resolution on chromatograms which contain 2/3-1/5 impurity based on computer analyses. Those impurities may be excluded by repeated re-chromatography, since the amount of their impurity can be known by a computer.

An application has been made for our biochemical study. Example are bacterial utilizations of D-amino acids[16]. However, since only racemate for the radioactive ^{14}C-Trp was commercially available, ^{14}C-D-Trp was obtained after resolving ^{14}C-DL-Trp on cellulose chromatography. ^{14}C-D-Trp thus prepared can be provided for the above experiment. A number of application will therefore be expected in the future on the basis of the chromatographic characteristics.

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Part III

Rapid resolution of 2,4-dinitrophenyl DL-amino acids on a native-cellulose column

Abstract

Sixteen 2,4-dinitrophenyl (DNP) DL-amino acids were resolved by high performance liquid chromatography (HPLC) using a packed column with native cellulose. The high resolution factor ($\underline{\alpha}$-value) and resolution rate ($\underline{Rs}$-value) obtained from the present experiment were comparable with those obtained from the leading experiment. The mixture of DNP-DL-amino acids were stepwisely separated and the isolated were then satisfactorily resolved, suggesting that the method thus employed may be applicable for not only biochemical and geochemical study but also medical diagnosis.

Introduction

There have been investigated the biochemical racemizations of amino acids in organisms which are occurred with aging[1-4] and the geochemical racemizations of free and/or bounded amino acids with diagenesis[5]. These studies require handy technique for observing those racemization processes. Although the methods for separating DL-amino acids have been reported[6-9], those are inconsistent with our purpose of experiment from the viewpoints of cost-performance, sensitivity and handiness.

In the light of our purpose of study, the limited numbers of resolution of DNP-DL-amino acids on a native-cellulose column have formerly been reported by using conventional liquid chroma-

tography[10]. We described here recent achievement of the high performance liquid chromatographic resolution (HPLCR) of sixteen DNP-DL-amino acids and its application for biochemical and geochemical studies.

Materials and Methods

Cellulose column

A stainless steel column (25cm x 0.46cm ID) which was commercially available was used. Native cellulose (lot. #2330, Merck, FRG) suspended in glass-distilled water was washed with 1N HCl and then with 0.1N NaOH in order to eliminate contaminants and to activate its performance of resolution. After fully washing with water, cellulose thus prepared was suspended in methanol and packed in the above column under the constant pressure (120-200kg/cm^2).

DNP-DL-amino acids

DL-amino acids were purchased from Sigma Chem. U.S.A. and from Wako Pure Chem. Japan. Abbreviation for amino acids are shown in the legend to Table III-1. DL-amino acids were dissolved in 0.3 ml of water (1 mg/ml), to which 0.3 ml of 0.13 M (1.8 %) triethylamine, 0.3 ml of ethanol and 0.3 ml of 50 mM 1-fluoro-2,4-dinitrobenzene (FDNB) in ethanol were added. The

reaction vessels were kept in a dark box at room temperature for 0.5 - 2 hr. To this reaction mixture, 0.5 ml of 0.13 M triethylamine and ca. 2 ml of diethylether were added. After vigorously shaking, the water phase was collected, to which 1 ml of 2 N HCl and 2.5 ml of ethylacetate were added. The phase composed of ethylacetate was collected, evaporated, and finally dissolved in ca. 2 ml of methanol.

Resolution

Usually 1-3 µl (absolute amount ca. 1 n moles, mean 200 ng of DNP-DL-amino acids) was introduced into a cellulose column and resolved. Using the presently employed column, the upper limit of the charged amount is calculated to be around 20 µg. The elution mixtures used were (the ratio refers to the Table III-1); 1) isoamylalcohol (iA) : acetonitrile (A) 10 % triethylammonium acetate in H_2O (TEAA) (pH 4.1), 2) iA : A : TEAA (pH 10.0), 3) iA : A : acetic acid and 4) iA : A : 10 % phosphoric acid, respectively. The eluents were flowed at 0.35 ml/min (40-60 Kg/cm^2) and monitored at 360 nm. The assignment of those resolution was carried out by co-chromatography in difference ratio of DNP-DL-amino acids (for instance, D:L = 3:1 or vice versa).

Separation and resolution of mixture of sixteen of DNP-DL-amino acids

Sixteen of DL-proteinic amino acids (Gly, Trp, Asn and Gln

were omitted from proteinic amino acids) were mixed and pre-labelled according to the above preparation method of DNP-DL-amino acids. The sample thus obtained was firstly chromatographed by Amberlite CG-50-3 (column : 51 x 0.85 cm ID) in order to obtain rough separation. The eluent correspond to the respective peak composed of two or three species of DL-amino acids was collected and condensed. Secondarily, the condensate thus obtained was eluted to give perfect separation for each DL-amino acid by using HPLC (LiChrosorb RP-18, 25 x 0.46 cm ID). Thirdly, a small amount of eluent which is composed of only one species of DNP-DL-amino acid corresponded to the respective single peak was resolved by the method as described in the section for resolution.

Results and Discussion

Resolution of sixteen DNP-DL-amino acids

The result obtained by high performance liquid chromatographic resolution (HPLCR) is summarized in Table III-1. The representative chromatograms for those resolutions of DNP-DL-amino acids, which are nonpolar, polar-uncharged and polar-

All nonpolar DNP-DL-amino acids were perfectly resolved (the upper most in Fig. III-1) which gave high values of α and Rs (Exp. 1 in Table III-1). Among the polar-uncharged, the resolution of DL-Ser, -Thr and -Tyr were perfect as listed in

Table III-1 Summary of HPLCR of DNP-DL-amino acids

DNP-DL-amino acids	Exp. 1			Exp. 2, 3		
	Elution mixture[3]	α[4]	Rs[4]	Elution mixture[3]	α[4]	Rs[4]
Ala (alanine)*	A (80)[5]	1.88	1.14	G (60)	2.11	0.97
Val (valine)	B (50)	2.88	1.50	untested		
Leu (leucine)	C (50)	2.81	1.45	untested		
Ile (isoleucine)*	C (50)	3.00	1.58	untested		
Phe (phenylalanine)	B (100)	3.43	1.98	G (60)	3.12	1.50
Trp (tryptophan)	A (90)	2.42	1.62	G (60)	2.75	1.18
Met (methionine)*	B (100)	2.67	1.60	G (60)	2.43	1.13
Ser (serine)*	D (160)	1.88	1.49	H (80)	2.58	1.09
Thr (threonine)	D (140)	2.73	1.97	H (50)	2.86	1.21
Cys (cysteine)[1]	A (180)	1.59	--	I (150)	2.63	1.41
Tyr (tyrosine)[1]	D (60)	1.94	1.07	I (40)	2.10	0.94
Asn (asparagine)[2]	unresolved			J (120)	1.67	--
Gln (glutamine)*	E (100)	1.21	--	L (80)	2.11	1.33
Lys (lysine)[1]*	A (150)	2.02	0.80	K (120)	1.91	--
Asp (aspartic acid)	F (200)	1.27	--	I (70)	1.48	--
Glu (glutamic acid)*	F (150)	1.34	--	M (120)	2.47	0.82

Table III-1

1) diDNP compounds: Cys (-N and -S), Tyr (-N and -O) and Lys (-N(α) and -N(ε))
2) D-enantiomer eluted faster than the opposite
3) elution mixtures,
   Exp. 1 (isoamylalcohol : acetonitrile : 10 % triethylammonium acetate in water pH 4.1)
   = 8:1:0.1(A), = 8:1:0.05(B), = 8:1:0.025(C),
   = 8:1:0.2(D), = 8:1:0.3(E) and = 8:1:0.25(F),
   respectively.
   Exp. 2 (isoamylalcohol : acetonitrile : acetic acid)
   =8:1:0.05(G), = 8:1:0.3(H), = 8:1:0.2(I),
   =8:1:1.5(J) and = 8:1:0.025(K), respectively.
   Exp. 3 (isoamylalcohol : acetonitrile : 10 % phosphoric acid)
   =8:1:0.2(L) and 20:1:1(M).
4) $\alpha=(t_2-t_0)/(t_1-t_0)$, where t_0: void volume, t_1: for peak of L-enantiomer and t_2: for peak of D-enantiomer. $Rs=2\Delta t/(w_1+w_2)$, where w_1 is for the theoretical base of the eluted L-enantiomer and w_2 is for that of D-enantiomer and $\Delta t=t_1-t_2$.
5) All parentheses indicate the elution time (min).

* Chromatograms are given in Fig. III-1.

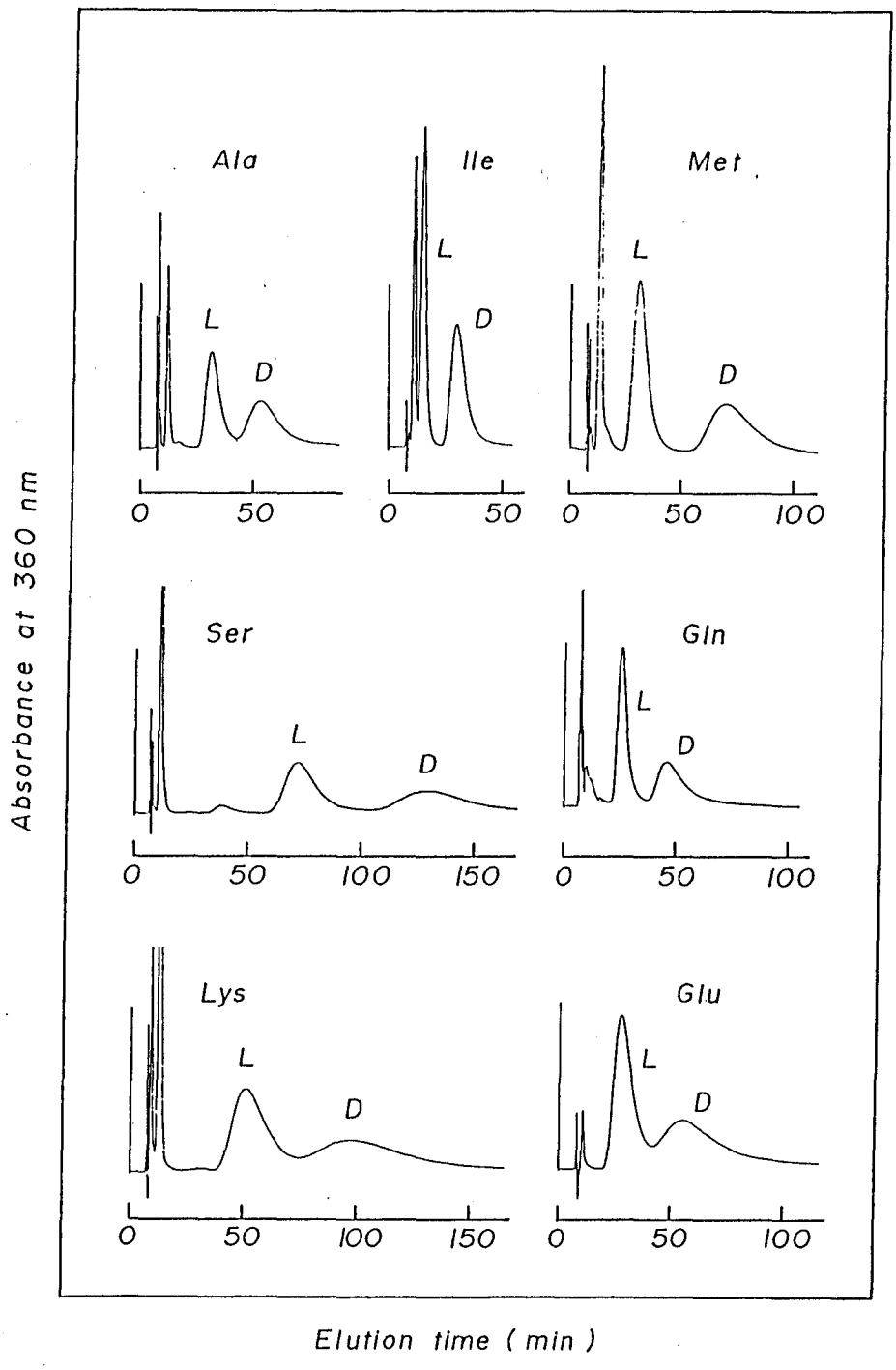

Fig. III-1 Representative chromatograms of HPLCR of DNP-DL-amino acids consisted of three category of side groups, nonpolar, polar-uncharged and polar-charged. The chromatograms shown are obtained from Exp. 1 in Table III-1 except for Gln and Glu (from Exp. 3). The conditions are described in the text.
charged, are given in Fig. III-1.

Exp. 1 and also refers to Fig. III-1 (middle). DL-Cys and -Gln were fairly resolved in Exp. 2 and 3. However, DL-Asn was not fairly resolved even in Exp. 2. On the other hand, it was difficult to resolve the polar-charged DL-amino acids, although the difference of retention time between both enantiomers was clearly seen on chromatogram (Table III-1 and the lower most in Fig. III-1). This is because they have extra charged group(s) after modification with FDNB. Therefore, they may firmly interact with glucose moieties so that the difference between the enantiomers on the cellulose surface is too small to detect on the chromatograms.

Although we have tried to resolve other three DNP-DL-amino acids such as Pro, His and Arg, they were not resolved under the employed conditions. The method for resolving those racemates in addition to the polar-charged amino acids is under investigation.

Separation and resolution of mixed DNP-DL-amino acids

We have investigated the separation of mixed DNP-DL-amino acids and the resolution of the isolated DNP-DL-amino acid (Fig. III-2). Since the present experiment was preliminary, the eluents corresponding to a few peaks were collected and analyzed. At the first step of separation, we collected the eluent from A, B and C, respectively (upper most of Fig. III-2). Then they were subjected to separate by a HPLC column (middle). Finally, the five DNP-DL-amino acids were respectively resolved by a HPLC column enpacked with native cellulose (lower most).

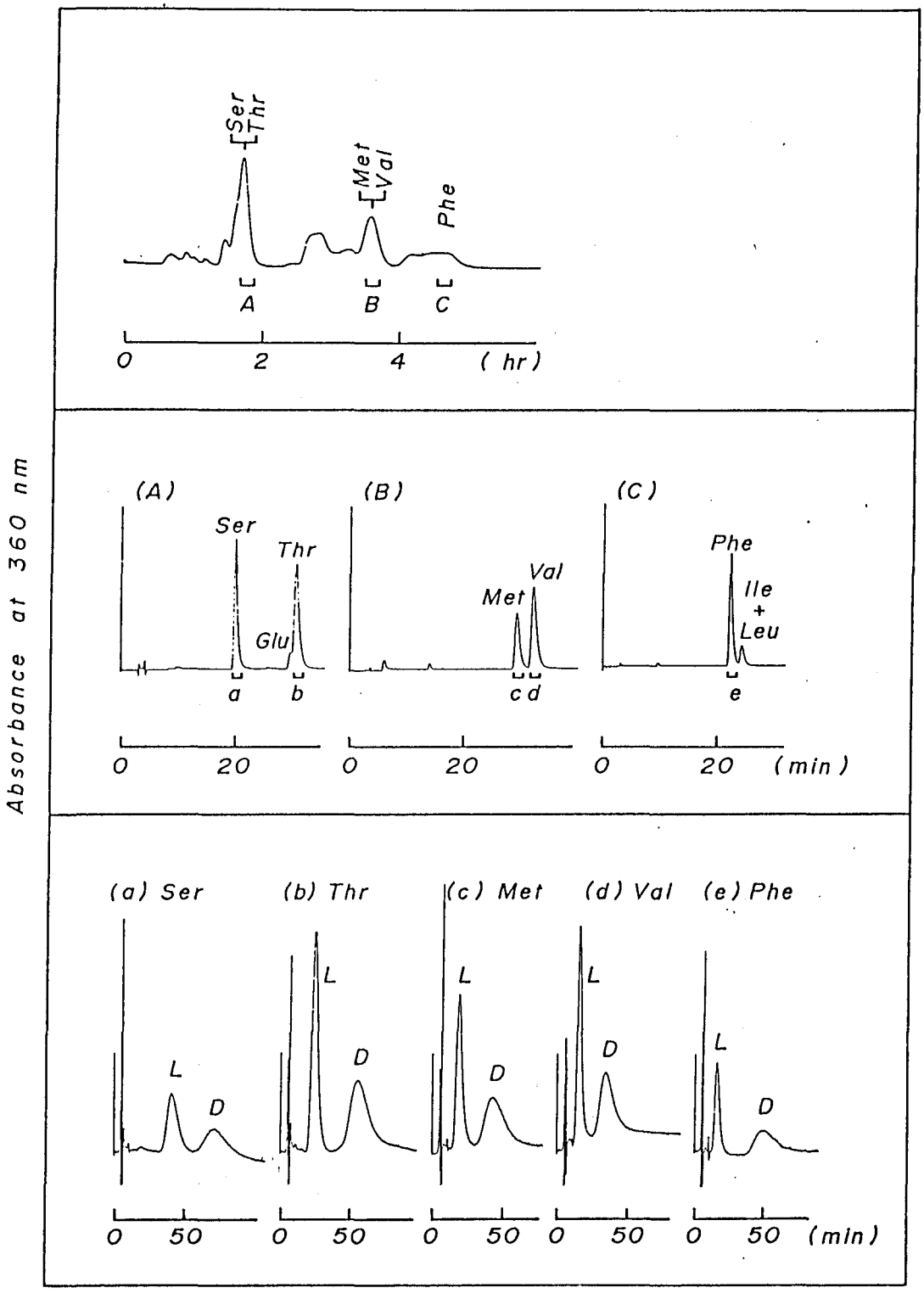
Absorbance at 360 nm
Ser
Thr
Met
Val
Phe
A
B
C
0
2
4
(hr)
(A)
Ser
Thr
Glu
a
b
(B)
Met
Val
c d
(C)
Phe
Ile
+
Leu
e
0
20
(min)
(a) Ser
(b) Thr
(c) Met
(d) Val
(e) Phe
L
D
0
50
(min)
Elution time

Fig. III-2 Separation and resolution of mixture of DNP-DL-amino acids. The first chromatogram was obtained by a Amberlite column to give rough separation (upper most). The elution mixture was methylethylketone : acetone : 0.3N HCl (1:3:5, v/v/v) and flowed at 0.4 ml/min. The eluent corresponded to A, B and C was independently collected and transferred to the next separation by a HPLC column of LiChrosorb RP-18 (middle). The elution mixture was 1 % phosphate in acetonitrile : H_2O [1:3 (v/v) for A, 2:3 for B and 9:11 for C] and flowed 0.75 ml/min. A small amount of eluent corresponded to a, b, c, d and e was respectively collected and finally resolved by a native cellulose column (lower most). The elution mixture was isoamylalcohol : acetonitrile : 10 % triethylammonium acetate in H_2O (pH 10.0) (8:1:0.2 for a and b, 8:1:0.05 for c, d and e) and flowed at 0.5 ml/min. Others are given in the text.

When we charged the mixture of sixteen species of DL-amino acids onto the first column, a few peaks were observed. However, the complete resolution of the isolated DL-amino acid was made after stepwise separation of the mixture as shown in representative chromatogram. Further improvement of technique such as the combined system responsible for separation and resolution of all species is under investigation in order to provide useful application to many fields as described in Introduction.

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Part IV

A chiral model of amino acid recognized by cellulose

Abstract

Based on the above experimental results (**Part II** and **III**), a chiral model of amino acid recognized by cellulose was constructed (**Part IV**). The mechanism of chiral recognition process is proposed by using the model. Moreover, the energy difference between DL-amino acids on D-glucose (dissymmetry environment) was calculated basing on the chromatographic results. It was known that the energy difference of enantiomers on cellulose increases by a factor 10^{16} in comparison with that in nature, and it is also influenced by the size of side chain (including DNP-derivatization).

Introduction

Recently, Mason calculated the energy difference between D- and L- amino acid enantiomers in nature to be some 10^{-19} eV[1]. With such small difference, the molecular chirality of amino acids would hardly be recognized by any molecules. The energy difference is realized only when racemic amino acid comes to contact with certain chiral molecules (e.g. enzyme). The great energy difference would arise in enantiomers, after they are recognized in respectively different manner by chiral molecules. this case has been realized in different chromatography which contained racemic amino acids and chiral column or eluent[2-4]. However, the mechanism for their racemic resolutions is yet

unclear. The resolution of racemic amino acids on native cellulose have been achieved[5-7]. The aim of the study was to construct a chiral model of amino acid which is physically recognized by chiral molecules and then to understand the relation between chiral conformation of enantiomers and their energy difference. Recently, we have obtained the new evidence to be able to construct the model. We firstly describe the resolution factors (α value) for twelve hydrophobic DL-amino acids (native or derivatized) on cellulose, showing that the factors depends upon the size of amino acid side chain. We then calculate the difference in energy between enantiomers basing on α values, giving some 10^{-2} - 10^{-3} eV. The evidence that the elution order of enantiomers through a cellulose column is completely inverted before and after derivatizing amino acids with dinitrofluorobenzene is also presented. From these studies, we propose a chiral model (submarine model) of amino acid recognized by cellulose, by which the mechanism of resolution of DL-amino acids can be explained.

Results and Discussion

The size of resolution factors (α) for native amino acid racemates on cellulose is dependent on of side chain (Table IV-1, Experiment I). For instance, the factor for DL-Ala is the smallest among the aliphatic group, but the factor becomes large in accordance with adding carbon to the side chain (Ala < AABA <

nVal < nLeu). The same tendency is observed in the case for aromatics, with adding hydroxyl residue to Phe (Phe < Tyr < Dopa). The factor for Trp is similar with that for Dopa. Accordingly, the amino acids having large side chains give good separation of enantiomers on a cellulose chromatography. This result suggests that cellulose molecule would recognize the conformation around α carbons common to all the α-amino acids by the first groove of cellulose, but it would be fixed by side chain which would be trapped in the second groove of cellulose. D- or L-enantiomer would then be recognized in different fashion. D-enantiomers are eluted faster than the opposite (Experiment I). If the side chain of L-enantiomer is well-accommodated in the second groove of host cellulose so that the conformation around α carbon may be held, the opposite are not necessarily done as such the way. In the latter case, if the conformation around α carbon is held, the side chain would not get between, while if the side chain is fixed in the second groove, the conformation around α carbon is hardly held. In consequence, performing cellulose chromatography of DL-amino acids, D-enantiomers move faster than the opposite.

The size of side chain seems to amplify the asymmetric conformation around α carbon so that it may play a definite role on resolution. Resolving the derivatized DNP-DL-amino acids which give the extremely distorted conformation by binding imino residue of amino acids with DNP, much larger resolution factors than those of the non-derivatized would be expected. The data represent that all the factors show no dependency on the

Table IV-1. Resolution factor and energy difference between enantiomers

1) Experiment I (resolution of native DL-amino acids). Cellulose (lot #2331, Merck, FRG) was prepared as has previously described[6]. The column (250 cm x 0.85 cm ID) was packed with washed cellulose (bed height of 240 cm) and equilibrated with elution buffer [pyridine:ethanol:water = 1:1:1 (v/v/v) for Trp; 4:1:1 for aromatics; 5:1:1 for others]. Usually 500 µg of DL-amino acids were analyzed. Eluants (30 ml/h) were monitored at 570 nm, after mixing with ninhydrin reagent (flow rate, 15 ml/h) by using a UV-Vis Effluent Monitor. The optical purity of the resolved enantiomers was determined by measuring their circular dichroism (CD) spectra using a magnetic polarimeter. The resolution of aliphatic DL-amino acids was determined by co-chromatography of mixtures with the enantiomers in different ratios (e.g. D:L = 1:3 or vice versa).

2) Experiment II (resolution of DNP-DL-amino acids). A stainless steel column (25cm x 0.46cm ID) which was commercially available was used. Native cellulose (lot. #2330, Merck, FRG) was prepared as has previously described[8] and packed in the above column under the constant pressure (140 kg/cm^2). The method of derivatizing DL-amino acids has also been described (8). Usually 1-3 µl (absolute amount ca. 1 n moles, mean 200 ng of DNP-DL-amino acids) was introduced into a cellulose column and

resolved. The elution mixture was isoamylalcohol (iA) : acetonitrile (A) : 10 % triethylammonium acetate in H_2O (TEAA) (pH 4.1) =8:1:0.1 (v/v/v). The eluents were flowed at 0.35 ml/min (40-60 Kg/cm^2) and monitored at 350 nm. The assignment of those resolution was carried out by co-chromatography in different ratios of DNP-DL-amino acids (for instance, D:L = 3:1 or vice versa). DNP-Tyr and DNP-Dopa were not tested, since all hydroxyl groups are derivatized by DNP to give their complex combinations.

$\alpha = (t_2 - t_0)/t_1 - t_0)$, where t_0 = time for void volume, and t_1 is for the peak of L-enantiomer and t_2 is for that of D-enantiomer (elution order was inverted in experiment II, t_1 : D-enantiomer and t_2 : L-enantiomer). t_0 for experiment I was 210 min and that for experiment II was 8 min, respectively. The retention times in experiment II were less than 1/20 of those of experiment I. Considering the value more than 0.03 in experiment I significant from the repeated experiments, those in experiment II would roughly be estimated to be more than 0.6 because of fluctuation of data. Davankov equation (9): $\Delta(\Delta G^0) = RT \ln \alpha$, where $\alpha = (t_2 - t_0)/(t_1 = t_0)$.

Amino acid abbreviations are Ala (alanine), AABA (α-aminobutyric acid), nVal (norvaline), nLeu (norleucine), Val (valine), Ile (isoleucine), Leu (leucine), Met (methionine), Phe (phenylalanine), Tyr (tyrosine), Dopa (2,4-dihydroxy phenylalanine) and Trp (tryptophan), respectively.

Table IV-1

Amino acids	Experiment I[1]					Experiment II[2]				
	Retention time (min)		α	Δ(ΔG°)		Retention time (min)		α	Δ(ΔG°)	
	D	L		cal/mol	eV(x10⁻³)	D	L		cal/mol	eV(x10⁻³)
Ala	1126	1167	1.05	26	1.13	52	31	1.91	382	16.6
AABA	759	827	1.12	69	2.99	34	22.5	1.79	344	14.9
nVal	648	718	1.16	88	3.82	25.5	16.5	2.06	427	18.5
nLeu	558	625	1.19	105	4.56	20.5	14.5	1.92	386	16.8
Val	625	663	1.09	52	2.26	30	17	2.44	527	22.9
Ile	512	547	1.11	65	2.82	24	15	2.29	490	21.3
Leu	523	540	1.05	31	1.35	24	16	2.00	410	17.8
Met	634	695	1.14	80	3.47	42	24	2.13	447	19.4
Phe	641	711	1.16	106	4.46	39.5	20.5	2.52	546	23.7
Tyr	596	674	1.20	129	5.60	--	--	--	--	--
Dopa	503	592	1.31	189	8.20	--	--	--	--	--
Trp	380	435	1.32	219	9.50	68	32	2.50	542	23.5

complexity of side chain (Table IV-1, Experiment II). The result, therefore, suggests that DNP would be substituted for side chain in the second groove. The limit of greatness of molecule capable of entering into the second groove of host cellulose molecule would roughly be the size of DNP. It is also suggested that since the second groove would be large enough for each side chain, its tightness would depend upon the size of amino acids. With native amino acids, the most fitted form in the second groove seems to have obtained by using Trp. The larger amino acids than DNP-amino acids, for example, PTH-DL-amino acids (derivatized by phenylisothiocyanate) gave no resolution (data not shown), indicating that such the large structure would not be accepted by both grooves.

The energy differences between enantiomers were calculated according to Dovankov's equation[9] [$\Delta(\Delta G^0)$ in Table IV-1]. All the data correspond to the α values. DL-amino acids giving extremely large energy difference on cellulose were well-resolved. In organisms, on the other hand, the value of energy difference between two compounds to competitively bind to protein was calculated to be about 200 cal/mol on the basis of calorimetry of the ligand binding proteins[10]. Therefore, the energy values in Table IV-1 would be large enough for the enzymatic recognition of molecular chirality in organisms.

It is interesting evidence that the inverted interaction of amino acid enantiomers with cellulose was observed before and after the derivatization with FDNB. In resolution of DL-amino acids by several chromatographic procedures, the inversion of

elution order of enantiomers through a column has often been reported[4,9]. However, its mechanism is yet unclear. In the present study, resolving native DL-amino acids, all the D enantiomers were eluted faster than the L-enantiomers (Table IV-1, Experiment I). The result suggests that L enantiomers might strongly interact with cellulose, namely L enantiomers might go smoothly into groove of cellulose to accommodate in comparison with the opposite. On the other hand, all the DNP-amino acids gave the inverted elution order of enantiomers (Table IV-1, Experiment II). The DNP-L-amino acids were eluted faster than the D-isomers, suggesting that DNP-D-amino acids are well-accommodated on cellulose rather than the opposite. The reason for the inverted elution of enantiomers is considered as follows. The conformation around α carbon of amino acids is recognized by the first groove of cellulose. This conformation would be fixed by side chain or its equivalent part (e.g. DNP) which goes into the second groove of cellulose. The determinant that fixes the chiral-based distorted conformation would be the side chain for native amino acids, but would be of substituted by DNP after the derivatization. DNP would be more effective determinant rather than side chain, since it would be the size to be accepted by the second groove. The second groove capable of accepting side chain would instead accommodate DNP so that the inversion of the recognition would occur.

On the basis of the above results, we now propose a chiral model of amino acid recognized by cellulose molecule. Plate IV-1 shows the CPK model for L-Phe and DNP-D-Ala (submarine model).

The model gives the information that the both have common parts for recognition. The cellulose molecule would recognize at the first the protruding part (carboxylic residue) using the first groove. Such the part would become fixed with complexity of a lobe consisting of side chain or of DNP in the second groove of cellulose. According to the model, L-Phe and DNP-D-Ala should give the same chromatographic behavior, both of which are eluted slower than the opposite (Table IV-1). L-Phe and DNP-D-Ala would go smoothly into groove of cellulose, but the opposite enantiomers would not. Such a situation results in energy difference between enantiomers and then give good separation of enantiomers on chromatography.

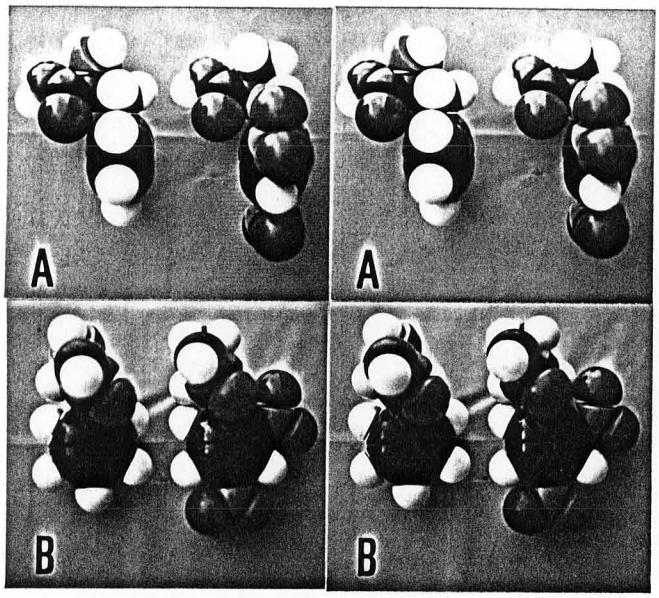

Plate IV-1. Stereograph of CPK model for L-Phe (left hand side) and DNP-D-Ala (right hand side).

A : horizontal view to phenyl residue

B : vertical view

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Part V

Nucleic acids recognize amino acid chirality

Abstract

Using ligand exchange chromatography whose mobile phase contained ribonucleic acid and Cu(II), nine proteinic DL-amino acids were resolved on the basis of chiral interaction of ribonucleic acid by mediation of Cu(II). Firstly, we have attempted to resolve DL-tryptophan (Trp) by using various ribonucleosides and ribonucleotides, among which purine 5'-nucleotides most strongly recognize the chirality of Trp to give the largest α value. Then, we have achieved the resolutions of DL-amino acids by using either 5'-AMP, 5'-GMP, and a mixture of the four 5'-nucleoside monophosphate (4XMP), or enzymatically digested RNA oligomer. The difference of stability between DL-amino acid - Cu(II) - D-ribonucleic acid complexes seems to give rise to their resolutions. Such the complex might also be regarded as the model for functional ribozymes which might have let ribonucleic acids recognize amino acid chirality to develop ribonucleoproteins during chemical evolution.

Furthermore, using deoxyribonucleic acids, the similar experimental results were also obtained. It is suggested that the chiral interaction of nucleic acids and amino acids by mediation of metal ions might have successively occurred through chemical and biological evolution process, and continued to modern biological world.

Introduction

The discovery of the ribosomal RNA intron excision of Tetrahymena has triggered the studies on RNA enzymes (ribozymes) in modern organisms[1]. It is speculated that the first "living molecule" on the primitive earth might have been RNA[2,3]. If the RNA world afterwards generates ribonucleoprotein world as more functional molecules, the pre-existed RNAs might select L-amino acids for the proteins from their racemic mixtures. It is here hypothesized that the symmetry breaking for DL-amino acids abiotically synthesized might have processed after the emergence of such the ribonucleic acids. The chiral part of ribonucleic acids is constructed with D-sugar (ribose moiety). Does D-sugar generally recognize the chirality of amino acids? It has been known that D-glucose polymers recognize the chirality of amino acids to give their enantiomeric resolutions[4-9]. It is therefore possible that ribonucleic acids might also recognize the chirality of amino acids as well, although only a few related studies have so far been known[10-14]. The method[15.16] for ligand exchange chromatography was adopted for the present study, in which nucleic acids were used as a mobile phase additive. This is the first attempt as for optical resolution by ligand exchange chromatography.

Materials and Methods

Apparatus and chemicals

The chromatographic system contained a HPLC Pump [Model 880-PU (Jasco : Japan Spectroscopic Co., Ltd. Tokyo, Japan)], an injector [Model 7125 (Rheodyne, California, USA)] equipped with a 20 µl loop, a detector [Model 875-UV (Jasco)] and a reaction bath [Model TC-100 (Jasco)], respectively.

Amino acids, nucleic acids and other reagents were purchased from Sigma Chem. Co. (St.Louis, Mo, USA) and Wako Pure Chem. Co., Ltd. (Osaka, Japan). 5'-AMP, -ADP and -ATP were from Oriental Yeast Co., Ltd. (Tokyo, Japan), and 3'-AMP, 3'-GMP and 5'-GMP was from Yamasa Co., Ltd. (Chiba, Japan). All abbreviations for chemicals are given in the legend to Table V-1-3.

Preparation of RNA oligomer

A 250 mg of RNA (lot #R7125, Sigma Chem.) was suspended in 12.5 ml of 50 mM Tris·HCl (pH 7.6) to which 5 mg of ribonuclease A (lot #R4875, Sigma Chem.) was added and then incubated for 16 hr at 37 °C. The solution was triply treated with phenol. After removing phenol, the RNA solution was fractionated through Sephadex G-25 (bed volume : 60 cm x 1.6 cm I.D.). The oligonucleotide fraction was collected and its concentration was estimated with the absorbance at 260 nm. A 0.5 mM Cu(II) was then added to 0.5 mM RNA oligomer which was calculated on the

basis of mononucleotide.

Preparation of DNA oligomer

A 300 mg of DNA (lot #D1626, Sigma Chem.) was suspended in 10 ml of 20 mM Tris·HCl (pH 7.6) and 10 mM $MgCl_2$ to which 2 mg of deoxyribonuclease I (lot #D0876, Sigmas Chem.) was added and then incubated for 16 hr at 37°C. Other method was the same as the preparation of RNA oligomer.

Preparation of octyl 5'-AMP coated column

A 1.0 g of 5'-AMP, 0.64 ml of epoxyoctane and 0.18 ml of triethylamine was suspended in 4.9 ml of ethyl alcohol and 2.6 ml of water and then stirred with magnetic stirrer and incubated for 10 hr at 60 °C. The solution was evaporated in vacuo, then suspended in ca. 250 ml of water, and purified by passing through Dowex I x 8 ion exchange resin (bed volume : 20 cm x 1.6 cm I.D.) with 0.01 M formic acid. The octyl 5'-AMP fraction collected was directly passed through Lichrosorb RP-18 column.

Resolution by using chiral additive

LiChrosorb RP-18, 5μm (Merck Co., Darmstadt, FRG) was packed (400 kg/cm^2) in a stainless steel column (250 mm x 4.6 mm I.D.) in our laboratory by a conventional slurry packing technique. The resolution of DL-Trp and it of the other DL-amino acids were

carried out by using a different conditions.

Trp : One or 2 μg of DL-Trp introduced into a column bed was eluted at ca. 100 kg/cm^2 with 50 mM sodium acetate buffer (pH 4.5) containing 1 mM cupric acetate [Cu(II)], either 1 mM nucleoside or nucleotide, and 3 % acetonitrile. Chromatography was carried out after thoroughly equilibrating the packed column with the eluant. The eluant (flow rate : 1 ml/min) was monitored at 300 - 310 nm.

The other amino acids : The eluant consisted of 50 mM sodium acetate buffer (pH 4.5) containing 1.0 mM various nucleic acids and 1.0 mM Cu(II). The flow rate was 0.5 ml/min (ca. 40 kg/cm^2) and the eluant was monitored at either 440 nm (Pro) or 570 nm (others) after mixing with ninhydrin reagent (flow rate : 0.5 ml/min).

The enantiomeric assignments for D- and L-amino acid were made by co-chromatography of different ratio of amino acid enantiomer (eg. D:L = 1:3 or vice versa).

Result

Resolution of DL-Trp by various ribonucleotides and ribonucleosides

Fig. V-1 represents the resolution chromatograms of DL-Trp in the presence of adenosine (a) or adenine nucleotides (b-e). The assignment for D-Trp and L-Trp on chromatogram was made by

co-chromatography with a different ratio of enantiomer (f-h). The results of DL-Trp resolutions by using various ribonucleosides and ribonucleotides are summarized in Table V-1, showing that only purine nucleotides and guanosine as a chiral additive were effective for resolution. The addition of 5'-GMP gave larger α value than that with guanosine, and 5'-AMP showed better separation of DL-Trp in comparison with that of 3'-AMP. It is also interesting that D-Trp was eluted faster than the opposite in the presence of 5'-GMP and 5'-IMP but the elution order was inverted by using adenine nucleotides. The elution profile seems to show the difference of stability for each enantiomer in the complex. The results indicated that purine 5'-ribonucleotides most strongly recognize the chirality of Trp. Then, the resolutions of DL-amino acids by using various 5'-nucleotides have been achieved.

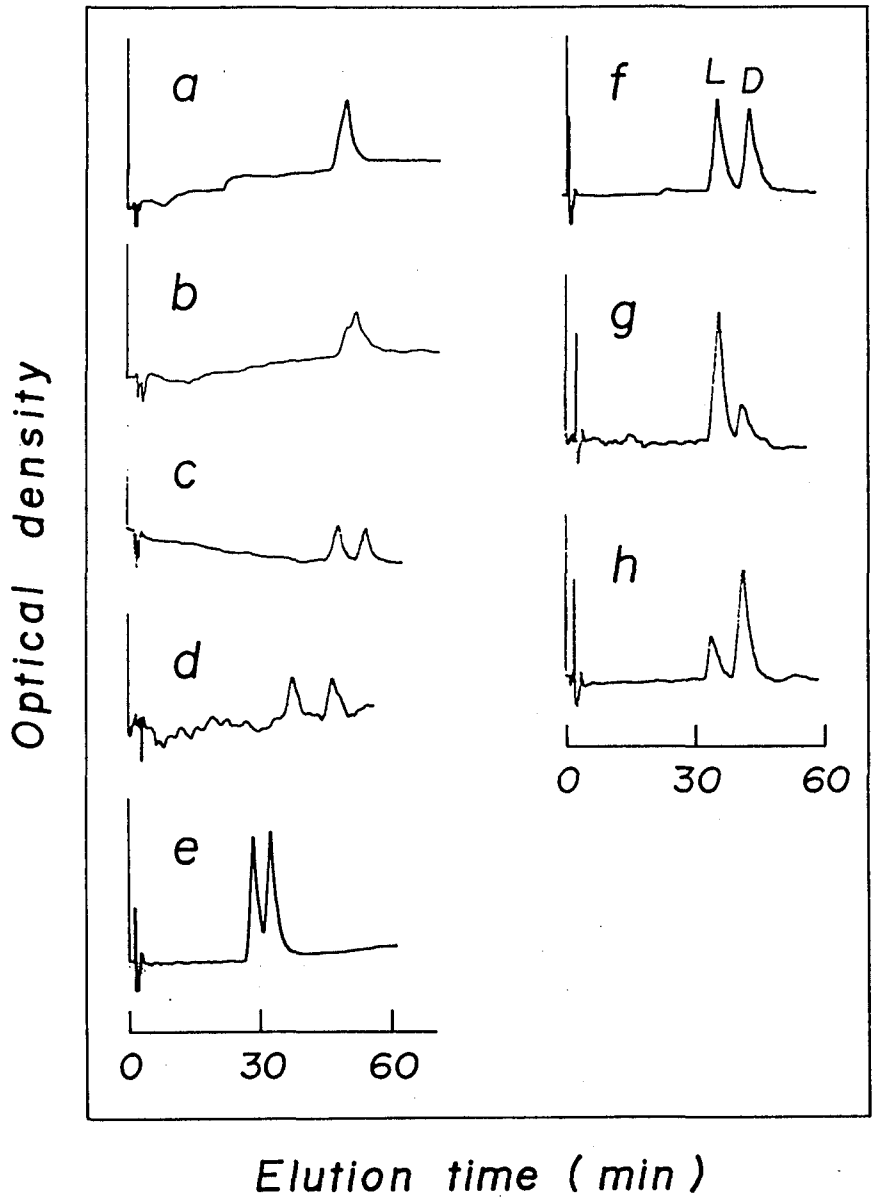

Fig. V-1. Chromatographic resolution of DL-Trp by using eluant containing adenosine - or adenine nucleotide - Cu(II) complex as a chiral additive.

The following chiral additives were used : adenosine (a), 3'-AMP (b), 5'-AMP (C), 5'-ADP (d) and 5'-ATP (e), respectively. On the other hand, the enantiomeric assignments for D-Trp and L-Trp on a chromatogram were made in the presence of ATP - Cu(II) complex (inset, f - h). Chromatographic conditions were the same as a - e, but the eluant contained 1 mM ATP and 2 % acetonitrile. f: 1 μg of DL-Trp, g: 0.25 μg of D- and 0.75 μg of L-Trp and h: 0.75 μg of D- and 0.25 μg of L-Trp, respectively. Other conditions are described in Materials and Methods.

Table V-1. Resolutions of DL-Trp using various chiral additives.

Conditions for chromatography are described in Materials and Methods. Employing guanosine and 5'-CMP, however, the elution mixture contained 0.5 mM ribonucleoside or 0.5 mM ribonucleotide and 0.5 mM Cu(II).

The separation factor (α) was calculated as follows. $\alpha = (t_D-t_0)/(t_L-t_0)$ or $= (t_L-t_0)/(t_D-t_0)$, where t_0 = retention time for void volume (2 min), t_D = that for the peak of the D-enantiomer and t_L = that for the peak of the L-enantiomer.

Chiral additives	Retention time (min)		α
	D	L	
Ado	50.0	50.0	1.00
3'-AMP	52.5	50.0	1.05
5'-AMP	54.5	48.3	1.13
5'-ADP	46.5	37.5	1.25
5'-ATP	32.5	28.5	1.15
Guo	48.0	45.0	1.07
5'-GMP	46.7	54.5	1.17
Ino	47.0	47.0	1.00
5'-IMP	50.8	54.5	1.08
5'-CMP	39.0	39.0	1.00
5'-UMP	45.0	45.0	1.00

Abbreviations : Ado (adenosine), Guo (guanosine) and Ino (inosine), respectively.

Resolution of DL-amino acids by RNA and its building blocks

Fig. V-2 and Table V-2 shows the comparison of resolution by using four 5'-ribonucleoside monophosphate (5'-AMP, -GMP, -CMP and -UMP). The results show that 5'-AMP and 5'-GMP recognize the chirality of the listed amino acids in addition to Trp to give enantiomeric separations. All the L-amino acids were eluted faster than the opposite in the presence of 5'-AMP, while the elution order of enantiomers was completely inverted in the presence of 5'-GMP. Furthermore, the GMP system gave better resolution than the AMP system (see α values). 5'-pyrimidine nucleotides showed no detectable recognition for amino acid chirality as far as this experimental system. It is concluded that the order of capability of recognizing amino acid chirality is 5'-GMP > -AMP > -CMP = -UMP, which is the same as the results of the resolution of DL-Trp.

Therefore, I focus on 5'-purine mononucleotide, and then on 4XMP and RNA oligomer. Fig. V-3 gives a representative chromatogram for resolution of DL-amino acids in the presence of 5'-GMP - Cu(II) complex. The results of nine proteinic DL-amino acid resolutions by using either 5'-AMP, 5'-GMP, 4XMP, or RNA oligomer are also summarized in Table V-3. The third experiment (4XMP) reflects the result of the GMP system, namely the ability of recognizing amino acid chirality by 5'-GMP is also superior to that by 5'-AMP in the mixed system of 4 ribonucleotides. The same tendency was also seen in the last experiment (RNA oligomer).

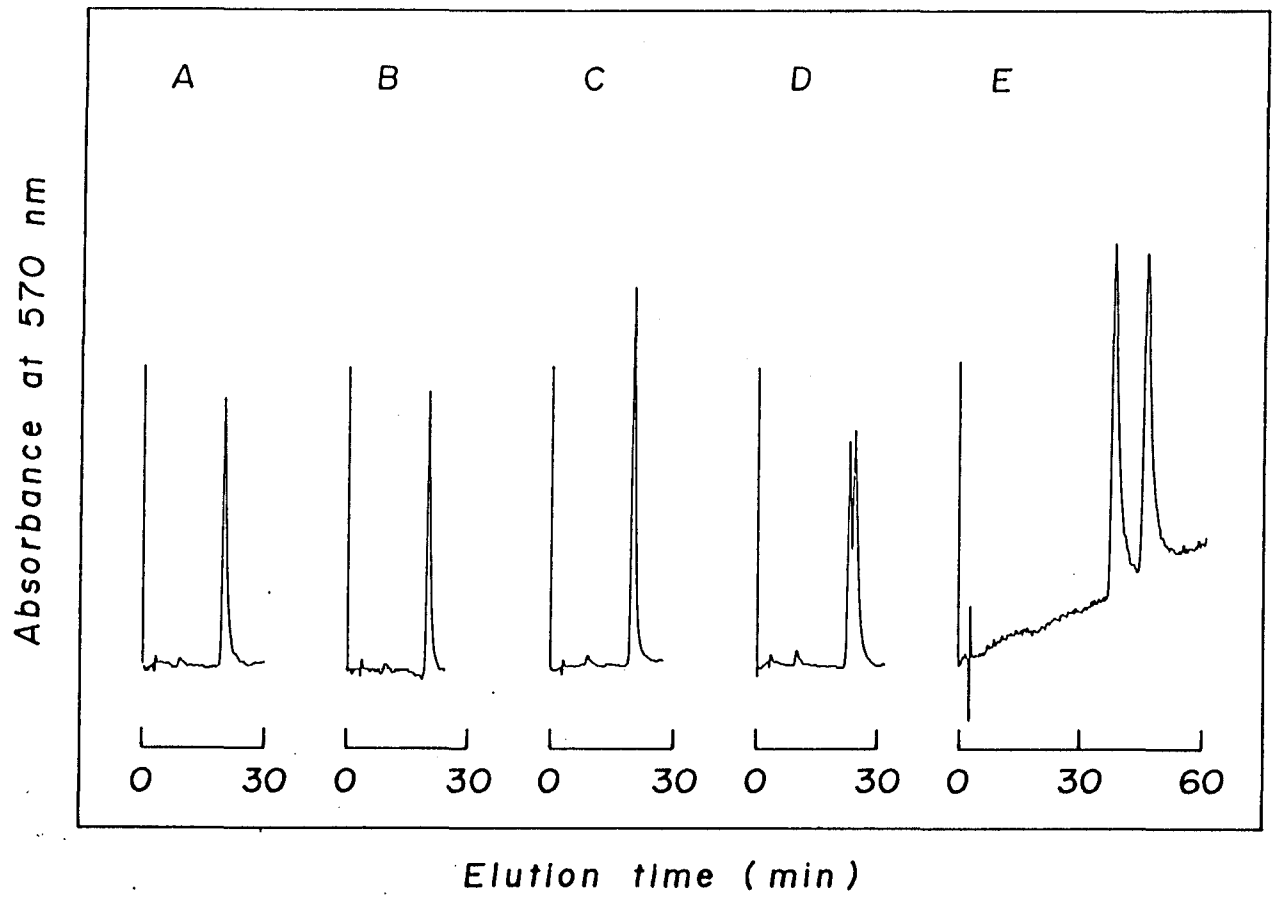

Fig. V-2 Resolution of DL-Leu by using various ribonucleoside 5'-monophosphate. Condition were shown in Table V-2. A (no ribonucleotide), B (5'-CMP), C (5'-UMP), D (5'-AMP) and E (5'-GMP), respectively.

Table V-2. Resolution of seven DL-amino acids using ribonucleoside 5'-monophosphate.

A 50 mM sodium acetate buffer (pH 4.5) containing 1.0 mM Cu(II) and 1.0 mM 5'-ribonucleotide was used as eluant. The separation factor (α) was calculated as described in Table 1 (t_0 = 8 min). Other chromatographic conditions are described in Materials and Methods.

Chiral additives	Control	5'-CMP			5'-UMP			5'-AMP			5'-GMP		
DL-amino acids	retention time* D (L)	retention time* D	L	α	retention time* D	L	α	retention time* D	L	α	retention time* D	L	α
Tyr	26.2	26.0	26.0	1.0	26.7	26.7	1.0	23.0	21.4	1.12	42.0	59.5	1.51
Met	15.5	14.4	14.4	1.0	15.5	15.5	1.0	18.4	17.2	1.13	27.0	31.0	1.21
Val	11.5	11.5	11.5	1.0	11.7	11.7	1.0	12.2	11.7	1.14	17.2	21.0	1.41
Leu	19.6	20.0	20.0	1.0	20.0	20.0	1.0	24.3	22.7	1.11	38.0	45.5	1.25
Ile	18.7	18.8	18.8	1.0	19.1	19.1	1.0	22.4	20.5	1.15	35.0	44.5	1.35
Pro	9.6	9.4	9.4	1.0	9.7	9.7	1.0	11.5	11.5	1.0	14.5	18.5	1.62
Arg	9.3	10.5	10.5	1.0	10.0	10.0	1.0	12.0	12.0	1.0	19.0	20.5	1.14

Amino acid abbreviations : Tyr (tyrosine), Met (methionine), Val(valine), Leu (leucine), Ile (isoleucine), Pro (proline) and Arg (arginine), respectively.

* : minute

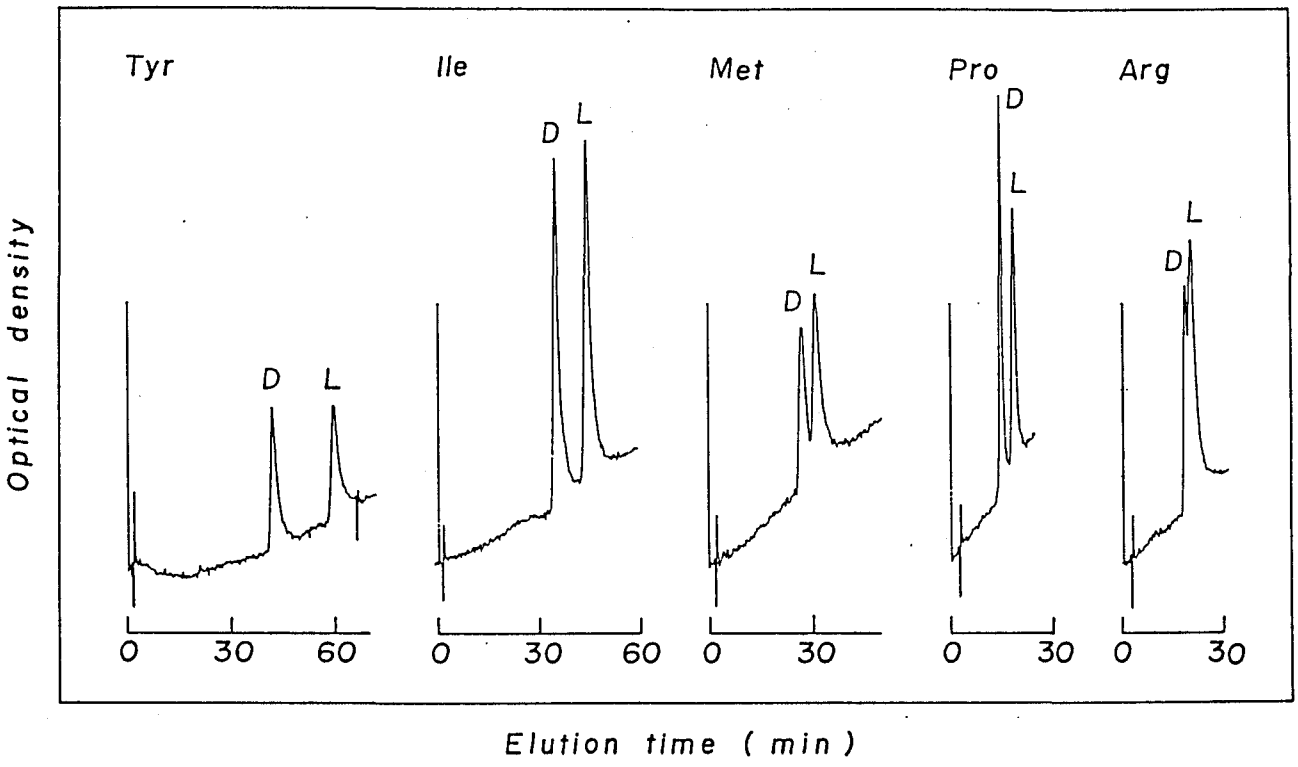

Fig. V-3. Example for chromatographic resolutions of DL-amino acids by using eluant containing 5'-GMP - Cu(II) complex as a chiral additive. Representative chromatograms are DL-Tyr (aromatic), DL-Ile (aliphatic), DL-Met (sulfur containing), DL-Pro (imino) and DL-Arg (charged), respectively. Other chromatographic conditions were the same as that in Table V-3. Amino acids abbreviations are given in the legend to Table V-2.

Table V-3. Resolutions of nine proteinic DL-amino acids by using eluant containing either 5'-AMP, 5'-GMP, 4XMP, or RNA oligomer as a chiral additive and Cu(II) complex.

DL-amino acids (0.5 μg) were analyzed with the same elution mixture as that in Fig. V-1. A 1 mM 5'-AMP or 5'-GMP was used. 4XMP contained 0.25 mM each of 5'-AMP, -GMP, -CMP and -UMP. Preparation of RNA oligomer and other chromatographic conditions are described in Materials and Methods.

Chiral additives	5'-AMP			5'-GMP			4XMP			RNA oligomer		
DL-amino acids	retention time*		α	retention time*		α	retention time*		α	retention time*		α
	D	L		D	L		D	L		D	L	
Trp	(54.5)	(48.3)	(1.13)	(46.7)	(54.5)	(1.17)	(50.5)	(50.5)	(1.0)	ut	ut	
Phe	82.0	72.0	1.15	95.0	129.0	1.39	80.5	85.5	1.07	95.0	101.0	1.07
Tyr	23.0	21.4	1.12	42.0	59.5	1.51	37.0	40.5	1.12	41.5	45.0	1.10
Met	18.4	17.2	1.13	27.0	31.0	1.21	24.8	24.8	1.0	23.5	23.5	1.0
Val	12.2	11.7	1.14	17.2	21.0	1.41	16.6	16.6	1.0	21.6	22.8	1.09
Leu	24.3	22.7	1.11	38.0	45.5	1.25	34.0	36.0	1.08	45.0	47.0	1.05
Ile	22.4	20.5	1.15	35.0	44.5	1.35	32.7	35.0	1.09	33.0	36.0	1.12
Pro	11.5	11.5	1.0	14.5	18.5	1.62	13.0	14.0	1.20	17.3	19.2	1.20
Arg	12.0	12.0	1.0	19.0	20.5	1.14	20.2	20.2	1.0	54.0	54.0	1.0

Amino acid abbreviations : Trp (tryptophan) and Phe (phenylalanine).

* : minute. ut : untested

Resolution of DL-amino acids by DNA and its building blocks

The similar experiments by using deoxyribonucleic acids instead of ribonucleic acids have been carried out. The results of seven proteinic DL-amino acid resolutions by using either 5'-dAMP, 5'-dGMP, 4dXMP, or DNA oligomer are summarized in Table V-4. furthermore, the comparison of 5'-dAMP with 5'-AMP, and that of 5'-dGMP with 5'-GMP are shown in Table V-5 and Fig. V-4. The results indicate that the degree of chiral recognition of amino acid by deoxyribonucleic acid is similar with that by ribonucleic acids (see α value and elution order in Table V-5).

Table V-4. Resolutions of nine proteinic DL-amino acids by using eluant containing either 5'-dAMP, 5'-dGMP, 4dXMP, or DNA oligomer as a chiral additive and Cu(II) complex.

A 1.0 mM 5'-dAMP and 5'-dGMP was used. 4dXMP contained 0.25 mM each of 5'-dAMP, -dGMP, -dCMP and -dTMP. Preparation of DNA oligomer and other chromatographic condition are described in Materials and Methods.

Chiral additives	5'-dAMP			5'-dGMP			4dXMP			DNA oligomer		
DL-amino acids	retention time*		α	retention time*		α	retention time*		α	retention time*		α
	D	L		D	L		D	L		D	L	
Tyr	26.8	25.5	1.07	41.8	56.9	1.45	28.8	33.2	1.21	46.0	51.4	1.14
Met	16.8	16.8	1.0	24.7	28.4	1.22	18.4	18.4	1.0	37.8	37.8	1.0
Val	11.5	11.5	1.0	15.0	18.0	1.43	12.3	13.1	1.19	18.3	19.0	1.07
Leu	21.3	21.3	1.0	34.5	41.5	1.26	23.4	26.6	1.21	54.6	54.6	1.0
Ile	20.9	20.0	1.08	34.5	47.7	1.50	22.6	25.5	1.20	67.5	70.7	1.05
Pro	10.1	10.1	1.0	13.8	17.5	1.64	10.7	11.3	1.22	15.5	18.1	1.35
Arg	13.0	13.0	1.0	16.0	16.0	1.0	13.8	13.8	1.0	47.8	47.8	1.0

* : minute

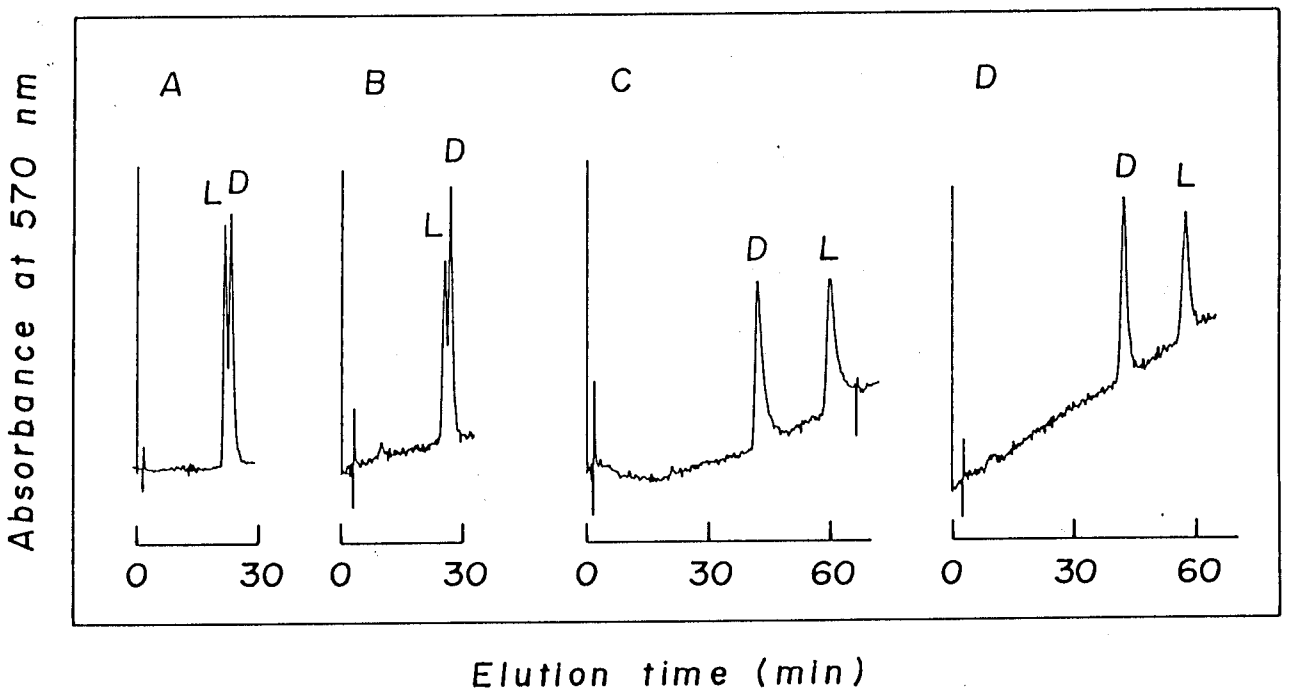

Fig. V-4. Comparison of the resolution of DL-Tyr by using 5'-AMP (A), 5'-dAMP (B), 5'-GMP (C) and 5'-dGMP (D). A 50 mM sodium acetate buffer (pH 4.5) containing 1.0 mM Cu(II) and 1.0 mM 5'-nucleic acid was used as eluant. Other chromatographic condition was the same as that in Table V-5.

Table V-5. Comparison of the resolution of DL-amino acid by using ribonucleotide and deoxynucleotide.

A 1.0 mM 5'-AMP, 5'-dAMP, 5'-GMP or 5'-dGMP was used. Other conditions are described in Materials and Methods.

Chiral additives	5'-AMP			5'-dAMP			5'-GMP			5'-dGMP		
DL-amino acids	retention time*		α	retention time*		α	retention time*		α	retention time*		α
	D	L		D	L		D	L		D	L	
Tyr	23.0	21.4	1.12	26.8	25.5	1.07	42.0	59.5	1.51	41.8	56.9	1.45
Met	18.4	17.2	1.13	16.8	16.8	1.0	27.0	31.0	1.21	24.7	28.4	1.22
Val	12.2	11.7	1.14	11.5	11.5	1.0	17.2	21.0	1.41	15.0	18.0	1.43
Leu	24.3	22.7	1.11	21.3	21.3	1.0	38.0	45.5	1.25	34.5	41.5	1.26
Ile	22.4	20.5	1.15	20.9	20.0	1.08	35.0	44.5	1.35	34.5	47.7	1.50
Pro	11.5	11.5	1.0	10.1	10.1	1.0	14.5	18.5	1.62	13.8	17.5	1.64
Arg	12.0	12.0	1.0	13.0	13.0	1.0	19.0	20.5	1.14	16.0	16.0	1.0

* : minute

Discussion

The structure of the mixed ligand complex

Table V-1 shows that purine 5'-ribonucleotides most strongly recognize the chirality of Trp. The chiral recognition process of DL-Trp by ribonucleotides may be explained as follows : 5'-phosphate may act for the complex formation that is stabilized by stacking purine and indole, as has previously been shown[17]. Moreover, the inversion of elution order by using adenine nucleotides and GMP or IMP suggest that $NH_2(6)$ of adenine nucleotides and O(6) of GMP or IMP considerably affect the complex formation.

Further experiments have been carried out in order to further understand the chiral recognition process of amino acids by nucleic acids. Table V-6 shows the comparison of chiral recognizing ability by 5'-AMP, 3'-AMP and 5'-dAMP, and Table V-7 shows that of 5'-GMP, 3'-GMP and 5'-dGMP. The results are 5'-A(G)MP = 5'-dA(G)MP > 3'-A(G)MP. Namely, the oxygen atom of 5'-phosphate is the important role for the complex formation, but that of 2'-hydroxyl group is not necessarily involved in the complex formation.

Table V-6. Copmparison of the resolution of DL-amino acid using various adenine nucleotides.

A 1.0 mM 3'-AMP, 5'-AMP or 5'-dAMP was used. Other condition are described in Naterials and Methods.

Chiral additives	3'-AMP			5'-AMP			5'-dAMP		
DL-amino acids	retention time*		α	retention time*		α	retention time*		α
	D	L		D	L		D	L	
Tyr	30.2	30.2	1.0	23.0	21.4	1.12	26.8	25.5	1.07
Met	17.2	17.2	1.0	18.4	17.2	1.13	16.8	16.8	1.0
Val	11.8	11.8	1.0	12.2	11.7	1.14	11.5	11.5	1.0
Leu	21.3	21.3	1.0	24.3	22.7	1.11	21.3	21.3	1.0
Ile	21.2	21.2	1.0	22.4	20.5	1.15	20.9	20.0	1.08
Pro	10.5	10.5	1.0	11.5	11.5	1.0	10.1	10.1	1.0
Arg	16.0	16.0	1.0	12.0	12.0	1.0	13.0	13.0	1.0

* : minute

Table V-7. Comparison of the resolution of DL-amino acid using various guanine nucleotides.

A 1.0 mM 3'-GMP, 5'-GMP or 5'-dGMP was used. Other conditions are described in Materials and Methods.

Chiral additives	3'-GMP			5'-GMP			5'-dGMP		
DL-amino acids	retention time*		α	retention time*		α	retention time*		α
	D	L		D	L		D	L	
Tyr	33.4	39.1	1.22	42.0	59.5	1.51	41.8	56.9	1.45
Met	20.7	21.8	1.09	27.0	31.0	1.21	24.7	28.4	1.22
Val	13.6	14.2	1.11	17.2	21.0	1.41	15.0	18.0	1.43
Leu	26.5	28.1	1.09	38.0	45.5	1.25	34.5	41.5	1.26
Ile	27.5	30.1	1.13	35.0	44.5	1.35	34.5	47.7	1.50
Pro	11.8	11.8	1.0	14.5	18.5	1.62	13.8	17.5	1.64
Arg	13.2	13.2	1.0	19.0	20.5	1.14	16.0	16.0	1.0

* : minute

Which enantiomer of amino acid is selected by nucleic acid?

Although DL-amino acids were synthesized under simulated primitive earth conditions[18], those symmetry breaking processes have never been demonstrated. Showing the resolution of all proteinic DL-amino acids by using cellulose (Part II), it is concluded that L-amino acids (13 of 19 DL-amino acids) were adsorbed on cellulose surface more strongly than the opposite enantiomers. It is suggested that the interaction between D-glucose and L-amino acids is stronger than that between D-glucose and D-amino acids. In the present experiment using nucleic acid, the discussion is rather complicated, because chiral nucleic acids are contained in the eluant. The following two results give a solution to me.

(1) When DL-Trp, -Phe and -Tyr were resolved by a Lichrosorb RP-18 column coated with octyl-5'-AMP by using achiral eluant, L-Trp, L-Phe and L-Tyr were eluted faster than the opposite enantiomers, which is the same result as those in Table V-3. The slow elution, therefore, suggests the formation of more stable complex in a column.

(2) Table V-8 shows the effects of the chiral ligand concentration on the resolution. Using the eluant containing only 0.5 mM 5'-GMP, the retention time was very small and the resolution did not occur. Using other eluant containing 0.2 mM 5'-GMP and Cu(II), however, the retention time increased and then three DL-amino acids were resolved. It is suggested that Cu(II) ion, which acts as the mediator between nucleic acid and amino

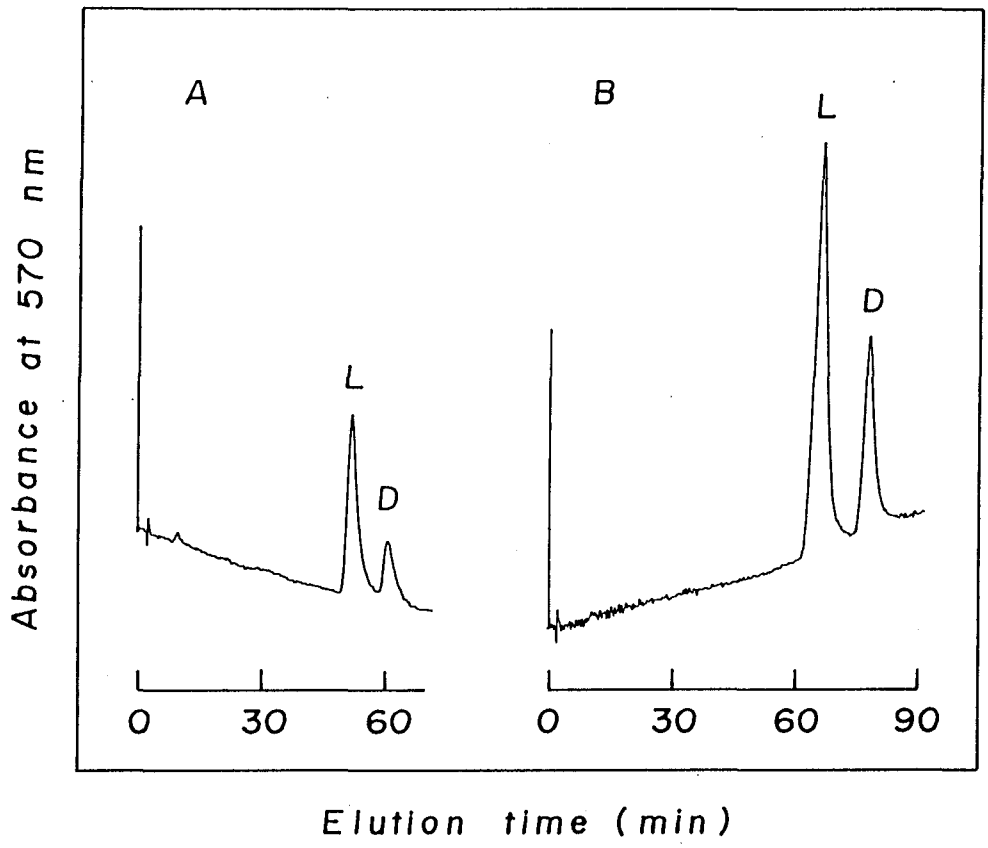

Fig. V-5 Resolution of DL-Phe By using a octyl-5'-AMP coated column (A) and eluant containing 1.0 mM 5'-AMP - Cu(II) complex (B). Preparation of octyl-5'-AMP coated column and other chromatographic condition are described in Materials and Methods.

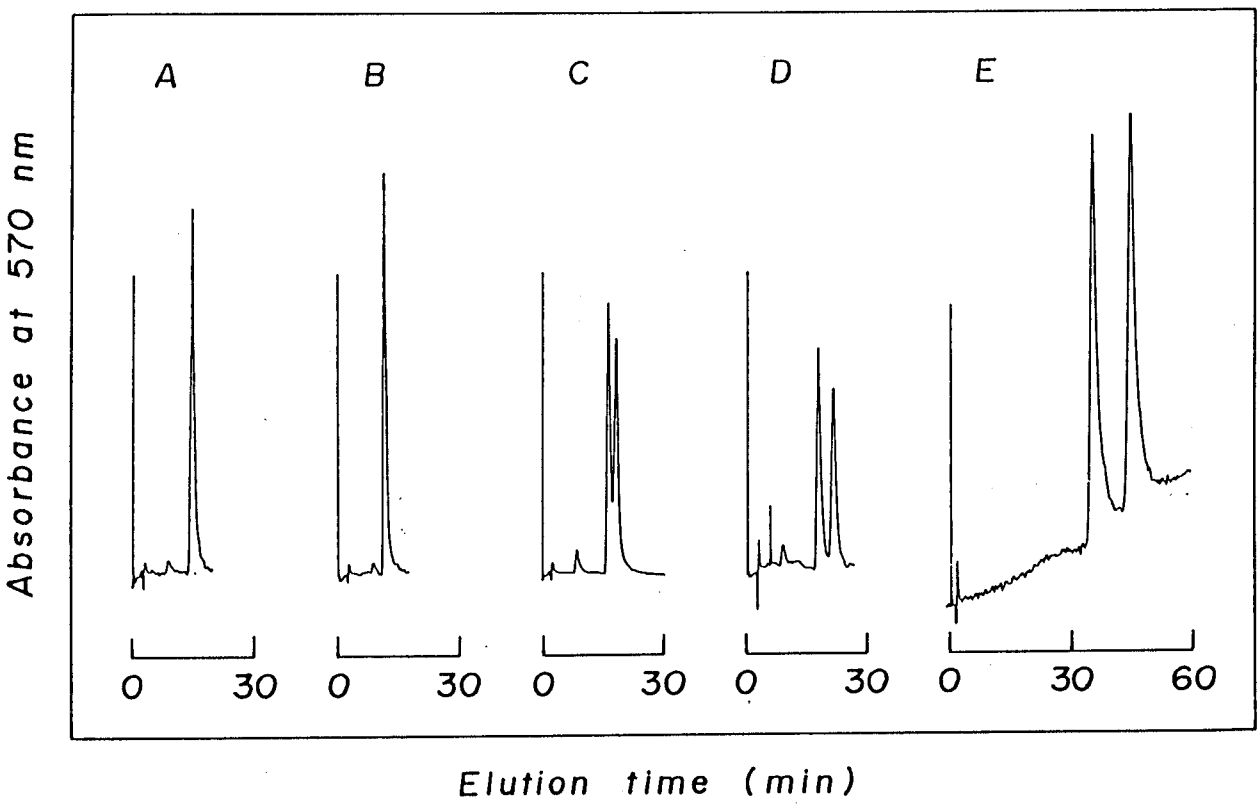

Fig. V-6 Effect of concentration of 5'-GMP and Cu(II) on resolution of DL-Ile. A [0.5 mM Cu(II)], B [0.5 mM 5'-GMP], C [0.2 mM Cu(II) + 5'-GMP], D [0.5 mM Cu(II) + 5'-GMP] and E [1.0 mM Cu(II) + 5'-GMP], respectively.

Table V-8. Effect of concentrations of 5'-GMP and Cu(II) on resolution of DL-amino acids.

Eluant contained either 0.5 mM Cu(II), 0.5 mM 5'-GMP, 0.2 mM 5'-GMP and Cu(II), 0.5 mM 5'-GMP and Cu(II) or 1.0 mM 5'-GMP and Cu(II). Other conditions are described in Materials and Methods.

Chiral additives	0.5 mM Cu(II)	0.5 mM 5'-GMP	0.2 mM 5'-GMP 0.2 mM Cu(II)			0.5 mM 5'-GMP 0.5 mM Cu(II)			1.0 mM 5'-GMP 1.0 mM Cu(II)		
DL-amino acids	retention time*	retention time*	retention time*		α	retention time*		α	retention time*		α
	D (L)	D (L)	D	L		D	L		D	L	
Tyr	19.5	14.1	23.4	27.3	1.25	22.1	30.0	1.56	42.0	59.5	1.51
Met	11.6	10.1	14.1	14.1	1.0	14.8	16.8	1.29	27.0	31.0	1.21
Val	9.6	8.3	10.8	10.8	1.0	11.2	12.3	1.34	17.2	21.0	1.41
Leu	15.4	11.6	18.0	19.6	1.16	18.4	21.3	1.29	38.0	45.5	1.25
Ile	14.5	11.2	16.1	18.0	1.23	17.7	21.4	1.38	35.0	44.5	1.35

* : minute

acid by coordinate bonds, is necessary for the resolution. Furthermore, Using 0.5 mM and 1.0 mM 5'-GMP and Cu(II), the retention time further increased and all five DL-amino acids were resolved. Therefore, the chance of the mixed ligand complex formation of amino acid and nucleic acid was increased by increasing the concentration of chiral ligand, resulting in the retention time was increased. Namely, the amino acid retained in a column by the mixed ligand complex formation of the nucleic acid with mediation of Cu(II), well-resolved. This result is compatible with the suggestion that slow elution is formed more stable complex in a column.

On the other hand, the elution order of amino acids by using 5'-AMP or 5'-GMP is perfectly opposite, suggesting that the complex of D-amino acids - Cu(II) - 5'-AMP and that of L-amino acids - Cu(II) - 5'-GMP would be more stable than those for the opposite enantiomeric complex. Using GMP, 4XMP and RNA oligomer, D-enantiomers were eluted faster than the opposite, suggesting that the complexes composed of L-amino acid are more stable than those composed of D-enantiomer. Such sterically significant complex, which can be detected by this experimental system, would not be constructed with pyrimidine nucleotides.

In deoxynucleic system, the similar experimental results with those in RNA system were obtained, which reaches to the above conclusion. The results of the DNA system suggested that the chiral amplification process described above might have continued to DNA world, that is, modern biological world.

Metal ions and the origin of life

In the present experiment, I have tested Cu(II), Ni(II), Co(II), Mn(II) and Mg(II), respectively, [Pb(II) and Zn(II) were not examined because of heavy precipitation]. Table V-8 shows the resolution of five DL-amino acids by using only 0.5 mM 5'-GMP, and 0.5 mM 5'-GMP and 0.5 mM metal ion [Cu(II), Ni(II) and Co(II), data of Mn(II) and Mg(II) are not shown]. The chromatograms of DL-Leu using various metal ions are shown in Fig. V-7. The retention time of amino acids by using 0.5 mM Cu(II) were larger than that by using other metal ions, and DL-amino acids were resolved only by using Cu(II). Using either Ni(II) or Co(II), amino acids were retained in a column longer than using a eluant containing only 5'-GMP, which gave no resolutions. It is reasonable, because it has previously been known that Cu(II) ion facilitates to form a complex with amino acids and nucleic acids rather than Ni(II) and Co(II). Considering the result of Table V-8 more detail, the retention time of amino acids using Ni(II) is slightly larger than that using Co(II). It is compatible with knowledge of inorganic chemistry which tells that Ni(II) facilitates to form a complex with nucleic acids and amino acids rather than Co(II).

Since Cu(II) ion has existed abundantly on the earth[19], it might have played as metal ion agent for chiral interaction between amino acids and nucleic acids. The role of metal ions on the prebiotic synthesis of oligonucleotides has been reported[20-22]. It has recently been reported that the yeast $tRNA^{Phe}$ was

Table V-9. Resolution of DL-amino acids by using 5'-GMP and various metal ions.

Eluant contained 0.5 mM 5'-GMP and either 0.5 mM Co(II), Ni(II) or Cu(II). Control experiment was made without metal ion. Other conditions are described in Materials and Methods.

Chiral additives	No metal ions 0.5 mM 5'-GMP			0.5 mM Co(II) 0.5 mM 5'-GMP			0.5 mM Ni(II) 0.5 mM 5'-GMP			0.5 mM Cu(II) 0.5 mM 5'-GMP		
DL-amino acids	retention time*		α	retention time*		α	retention time*		α	retention time*		α
	D	L		D	L		D	L		D	L	
Tyr	14.1	14.1	1.0	18.7	18.7	1.0	19.8	19.8	1.0	22.1	30.0	1.56
Met	10.1	10.1	1.0	11.4	11.4	1.0	12.1	12.1	1.0	14.8	16.8	1.29
Val	8.3	8.3	1.0	9.3	9.3	1.0	9.5	9.5	1.0	11.2	12.3	1.34
Leu	11.6	11.6	1.0	13.6	13.6	1.0	14.6	14.6	1.0	18.4	21.3	1.29
Ile	11.2	11.2	1.0	12.8	12.8	1.0	13.2	13.2	1.0	17.7	21.4	1.38

* : minute

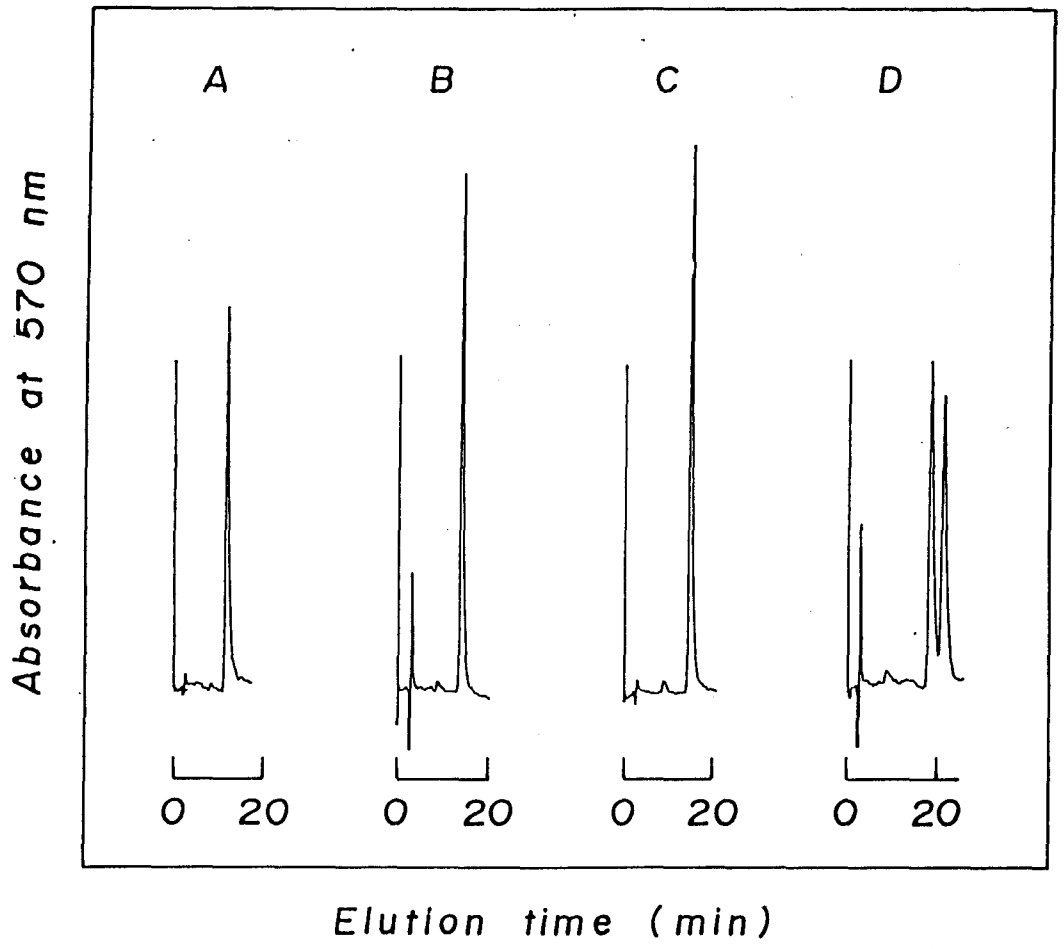

Fig. V-7 Resolution of DL-Leu by using 0.5 mM 5'-GMP and (A) : without metal ion, (B) : 0.5 mM Co(II), (C) : 0.5 mM Ni(II) and (D) : 0.5 mM Cu(II).

site-specifically cleaved by Pb(II) ion and its mechanism could be an intramolecular version of a metallo-enzyme-catalyzed reaction[23]. Even during chemical evolution, a primitive version of such metallo-enzyme reaction system might have existed. If mononucleotides are synthesized on the primitive earth[18], they might form metal complexes which could give shortening and/or elongation of nucleotide molecules and furthermore recognize chirality of amino acids.

The chiral mixed ligand complex and evolution

If small RNA or its derivatives had been present on the primitive earth, L-amino acids might have necessarily been selected to give the emergence of the interaction of D-ribonucleotides and L-amino acids. Above all, the role of 5'-GMP even in the above RNA would be emphasized to select L-amino acids. On the other hands, more generally, if D-ribonucleotides are formed after DL-amino acids, the ribozyme - like construction composed of ribonucleotide - Cu(II) complex might recognize the existed amino acid chirality and select corresponding enantiomers. Such the system might develop the primitive ribozymes to ribonucleoproteins as more functional molecules.

Furthermore, it has been known that D-deoxyribonucleic acids as well as D-ribonucleic acids also select L-amino acids. It is suggested that the chiral amplification process described above might have continued to DNA world. Going a step forward, the chiral interaction of nucleic acid and amino acid must be found

in modern organisms, because many surprising findings related to nucleic acids (eg. RNA editing[24,25]) have recently been reported, and it may be concerned with a very important reaction of life.

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Part VI

Novel ligand exchange chromatographic resolution of DL-amino acids by using 5'-ADP and coenzyme

Abstract

DL-amino acids were resolved using mobile phase containing ADP, NAD or FAD as a chiral additive, and chiral stationary phase with cyanocobalamin (ligand exchange chromatographic resolution). Separation factor (α) and resolution rate (Rs) in mobile phase was known to be dependent upon the concentration of chiral additives. A chiral stationary sorbent was obtained by letting cyanocobalamin flow through a ODS-silica gel column, which was repeatedly utilized for amino acid resolutions. The results are compared with those obtained by the leading ligand exchange chromatography using amino acids or their derivatives for chiral mobile and stationary phase.

On the other hand, one attractive hypothesis that nucleotide cofactors are metabolic fossils of the enzymes in the RNA world is supposed. NAD, FAD and cyanocobalamin are representative nucleotide cofactors, so the present experimental results are very interesting.

Introduction

Numerous publications of the chromatographic resolutions of DL-amino acids have been accumulated (1). For the last decade, the ligand exchange chromatography which includes two types of chiral modifications (chiral stationary and mobile phase) has been adopted to the optical resolution of DL-amino acids (2).

The reported chiral ligands, however, are limited only to amino acids and their derivatives. Utilizations of nucleic acid and its derivatives for ligand exchange chromatography to resolve DL-amino acids have never been demonstrated. In this article, novel ligand exchange chromatographic resolutions of sixteen DL-amino acids using chiral mobile phase with either ADP, NAD or FAD and stationary phase with cyanocobalamin are described.

Materials and Methods

Apparatus and chemicals

The chromatographic system contained a HPLC Pump [Model 880-PU (Jasco : Japan Spectroscopic Co., Ltd. Tokyo, Japan)], an injector [Model 7125 (Rheodyne, California, USA)] equipped with a 20 µl loop, a detector [Model 875-UV (Jasco)] and a reaction bath [Model TC-100 (Jasco)], respectively.

Amino acids, cyanocobalamin and other reagents were purchased from Sigma Chem. Co. (St.Louis, Mo, USA) and Wako Pure Chem. Co., Ltd. (Osaka, Japan). ADP and NAD were from Oriental Yeast Co., Ltd. (Tokyo, Japan), and FAD was from Yamasa Co., Ltd. (Chiba, Japan). All abbreviations for chemicals are given in the legend to Table VI-1.

Resolution by using chiral additive (mobile phase)

LiChrosorb RP-18, 5μm (Merck Co., Darmstadt, FRG) was packed (400 kg/cm^2) in a stainless steel column (250 mm x 4.6 mm I.D.) in our laboratory by a conventional slurry packing technique. The eluant consisted of 50 mM sodium acetate buffer (pH 4.5) containing either ADP, NAD or FAD at the same concentration (0.2 mM) and 0.2 mM cupric acetate [Cu(II)]. Chromatography was carried out after thoroughly equilibrating the packed column with the eluant. About 200 ml was let flow to reach a state of equilibrium for the case of ADP and NAD, and ca. 600 ml was for FAD. Usually, 0.2 - 1.5 μg of DL-amino acids were analyzed. The flow rate was 0.5 ml/min (ca. 40 kg/cm^2) and the eluant was monitored at either 440 nm (Pro) or 570 nm (others) after mixing with ninhydrin reagent (flow rate : 0.5 ml/min).

Resolution by column coated with cyanocobalamin (stationary phase)

LiChrosorb RP-18 (ca. 3 g) was coated with cyanocobalamin using hydrophobic interaction. Namely about 3 liter of 0.1 mM cyanocobalamin and 0.1 mM Cu(II) in 50 mM sodium acetate buffer (pH 4.5) was passed through a column to saturate the chiral agent in a column. The column thus prepared was repeatedly utilized and it resolution capability was maintained for at least fifty cycles of analysis. Eluant consisted basically of sodium acetate buffer and Cu(II) (details are given in the legend to Table VI-

1). Other conditions are the same as those for the resolutions using mobile phase.

Results

The chromatographic results are summarized in Table VI-1. The resolved chromatograms of DL-Leu using four kinds of chiral reagents are shown in Fig. VI-1. The representative co-chromatographic resolution patterns of four DL-amino acids using a cyanocobalamin-coated column are also demonstrated in Fig.VI-2.

Table VI-1 shows that seven or six DL-amino acids were resolved by using either ADP or NAD as a chiral additive in mobile phase, and 14 DL-amino acids were resolved using FAD. The results suggest that FAD may be superior agent to either ADP or NAD for the resolution of DL-amino acids. The reason may be considered as follows : (1) FAD may have suitable structure than others in order for recognizing the chirality of amino acid, or (2) since FAD are more strongly retained in a reverse phase sorbent matrix than others, FAD-Cu(II)-amino acid complexes are then more gently eluted from the reverse phase column than other complexes. The former possibility is doubtful considering that NAD having mostly similar structure to FAD showed no comparable ability of resolving DL-amino acids, although detail still remains unknown. The latter would be rather favorable. The capacity factors (k') obtained using ADP and NAD were extremely small. Under the condition, amino acid enantiomers were eluted

Table VI-1. Resolution of DL-amino acids by using mobile phase containing either ADP, NAD or FAD, and stationary phase with cyanocobalamin.

Mobile phase : DL-amino acids (0.2 - 1.5 µg) were analyzed using a 50 mM sodium acetate buffer (pH 4.5) containing 0.2 mM Cu(II) and 0.2 mM respective chiral ligand (ADP, NAD or FAD). However, slight modifications of the condition were made for respective resolution as follows : (a) contained 2 % acetonitrile, (b) 5 µM FAD and (c) 5 µM FAD and 2 % acetonitrile. All the eluant (0.5 ml/min) through a column was monitored at 570 nm except for Pro (440 nm), after mixing with ninhydrin reagent (0.5 ml/min).

Stationary phase : Using a column coated with cyanocobalamin, 0.5 - 1.5 µg of DL-amino acids were resolved. The eluants used were as follows : (d) 10 mM sodium acetate buffer (pH 5.0) containing 0.1 mM Cu(II), (e) 10 mM buffer (pH 5.5) containing 0.1 mM Cu(II), (f) 10 mM buffer (pH 5.0) containing 0.5 mM Cu(II), (g) 10 mM buffer (pH 4.5) containing 0.5 mM Cu(II) and (h) 5 mM buffer (pH 4.5) containing 0.1 mM Cu(II). The flow rate (Phe and Trp) was 1.0 ml/min and the eluants were directly monitored at 254nm (Phe) and 282 nm (Trp) without mixing with ninhydrin reagent. Other chromatographic condition was the same as that for the study of mobile phase.

Capacity factor (k'), separation factor (α) and resolution rate (Rs) were calculated as follows. $k' = [t_D \text{ (or } t_L) - (t_0 - 2.99)]/t_0$, where t_0 is the time for void volume (5 min at the flow rate of 0.5 ml/min and 2.5 min at 1.0 ml/min) which was corrected by calculating a volume of a ninhydrin-reaction coil (2.99 ml), t_D is the retention time for the peak of D-enantiomer and t_L is the that for L-enantiomer. $\alpha = k'_D/k'_L$ or k'_L/k'_D. $Rs = 2[t_D - t_L \text{ or } (t_L - t_D)]/(w_D + w_L)$, where w_D is the theoretical base for eluted D-enantiomer and w_L is that for L-enantiomer. In the case of incomplete resolution, Rs was not calculated (i). The enantiomeric assignments for DL-amino acids were made by co-chromatography of different ratio of amino acid enantiomer (eg. D:L = 1:3 or vice versa).

Abbreviations for nucleic acid and coenzymes were as follows : ADP (adenosine 5'-diphosphate), NAD (nicotinamide adenine dinucleotide), FAD (flavin adenine dinucleotide) and cyanocobalamin (Co α - [α-(5,6-dimethylbenzimidazolyl)] - Co β -cyanocobamide), respectively. Amino acid abbreviations : Ala (alanine), AAnBA (α-amino normal-butyric acid), nVal (normal-valine), Val (valine), nLeu (normal-leucine), Leu (leucine), Ile (isoleucine), Pro (proline), Met (methionine), Phe (phenylalanine), Tyr (tyrosine), Trp (tryptophan), His (histidine), Lys (lysine), Arg (arginine) and Asp (aspartic acid), respectively.

Table VI-1 Resolution of DL-amino acids by using mobile phase containing either ADP, NAD or FAD, and stationary phase with cyanocobalamin.

Chiral additives	ADP				NAD				FAD				Cyanocobalamin			
DL-amino acids	k'_D	k'_L	α	Rs	k'_D	k'_L	α	Rs	k'_D	k'_L	α	Rs	k'_D	k'_L	α	Rs
Ala	0.10	0.10	1.0	-	0.10	0.10	1.0	-	0.30	0.30	1.0	-	[d]0.46	0.46	1.0	-
AAnBA	0.36	0.36	1.0	-	0.34	0.34	1.0	-	1.30	0.90	1.44	[i]-	[e]1.94	2.37	1.22	[i]-
nVal	0.74	0.74	1.0	-	0.76	0.76	1.0	-	4.32	2.64	1.64	1.58	[e]5.44	7.76	1.43	2.05
Val	0.90	0.90	1.0	-	0.66	0.66	1.0	-	5.22	2.86	1.83	2.41	[f]3.60	6.10	1.69	2.03
nLeu	2.90	2.56	1.13	[i]-	2.80	2.80	1.0	-	15.7	9.02	1.74	3.41	[d]8.24	12.3	1.49	3.44
Leu	2.10	1.90	1.11	[i]-	2.80	2.56	1.09	[i]-	7.98	5.58	1.43	3.58	[g]5.34	8.62	1.61	2.85
Ile	2.00	1.60	1.25	[i]-	2.46	2.14	1.15	[i]-	11.0	6.10	1.80	6.45	[d]7.25	11.0	1.52	3.25
Pro	0.14	0.14	1.0	-	0.30	0.30	1.0	-	2.21	1.36	1.62	1.65	[d]1.22	3.44	2.82	2.39
Met	1.20	1.02	1.18	[i]-	1.60	1.41	1.14	[i]-	4.61	3.46	1.33	2.53	[g]3.84	6.14	1.60	1.73
Phe	11.2	8.40	1.33	2.43	14.3	11.8	1.21	1.77	[b]13.0	9.22	1.41	3.53	[h]18.4	25.9	1.41	3.82
Tyr	2.64	2.21	1.20	1.29	4.08	3.49	1.17	1.45	8.90	5.50	1.62	5.00	[d]8.54	11.2	1.31	3.08
Trp	[a]8.96	7.60	1.16	1.50	[a]14.8	13.9	1.06	[i]-	[c]12.1	10.7	1.13	[i]-	[h]44.0	52.8	1.20	2.62
His	0.70	0.70	1.0	-	0.90	0.90	1.0	-	31.3	29.2	1.07	[i]-	[g]1.94	1.94	1.0	-
Lys	0.30	0.30	1.0	-	0.50	0.50	1.0	-	8.24	7.70	1.07	[i]-	[g]0.90	0.90	1.0	-
Arg	0.50	0.50	1.0	-	0.70	0.70	1.0	-	18.8	16.5	1.14	1.71	[g]1.70	1.70	1.0	-
Asp	0.06	0.06	1.0	-	0.05	0.05	1.0	-	0.40	0.40	1.0	-	[g]0.18	0.18	1.0	-

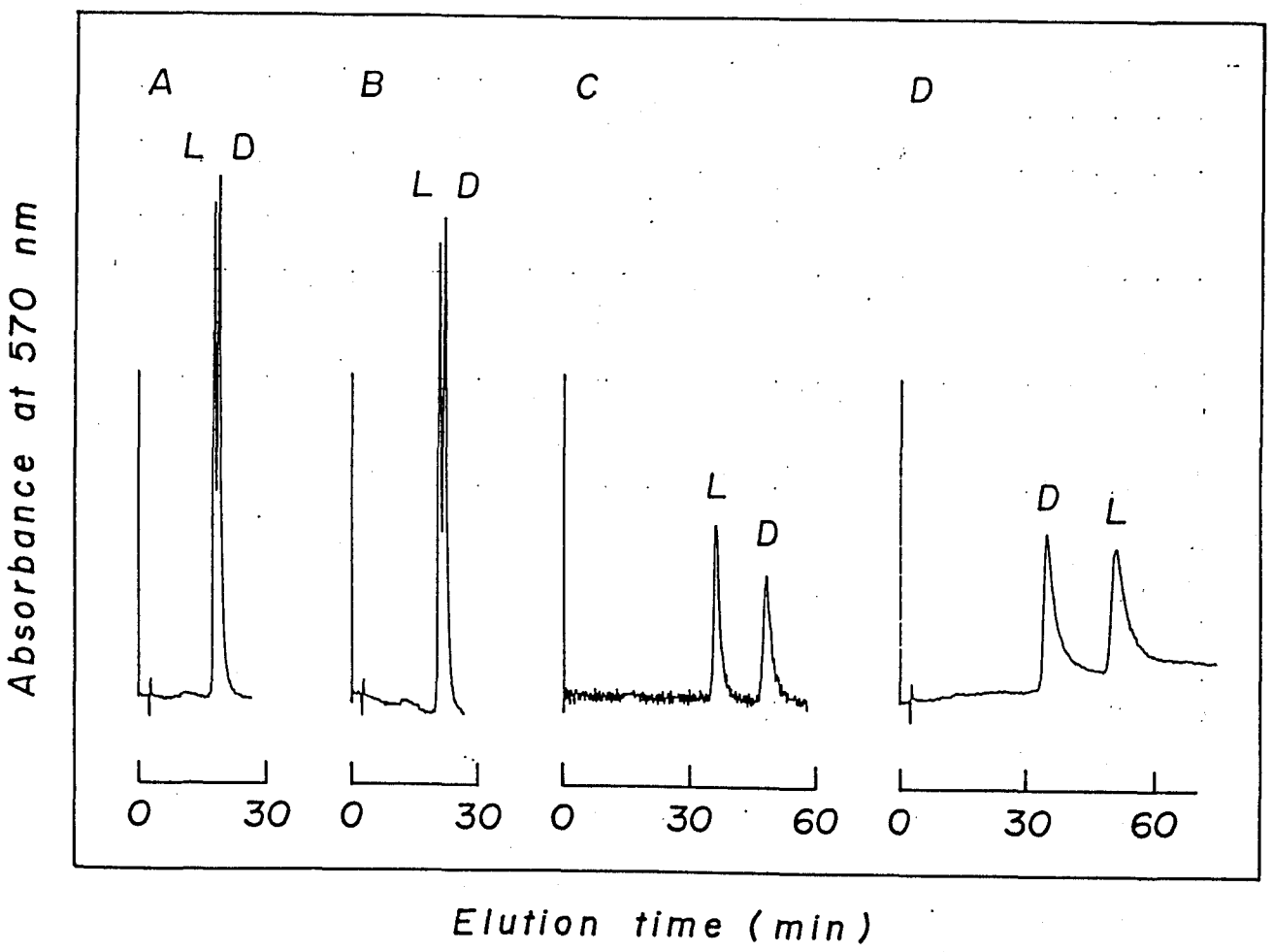

Fig. VI-1. Resolution of DL-Leu by using either mobile or stationary phase. Conditions were shown in Table VI-1. A (ADP), B (NAD), C (FAD) and D (cyanocobalamin), respectively.

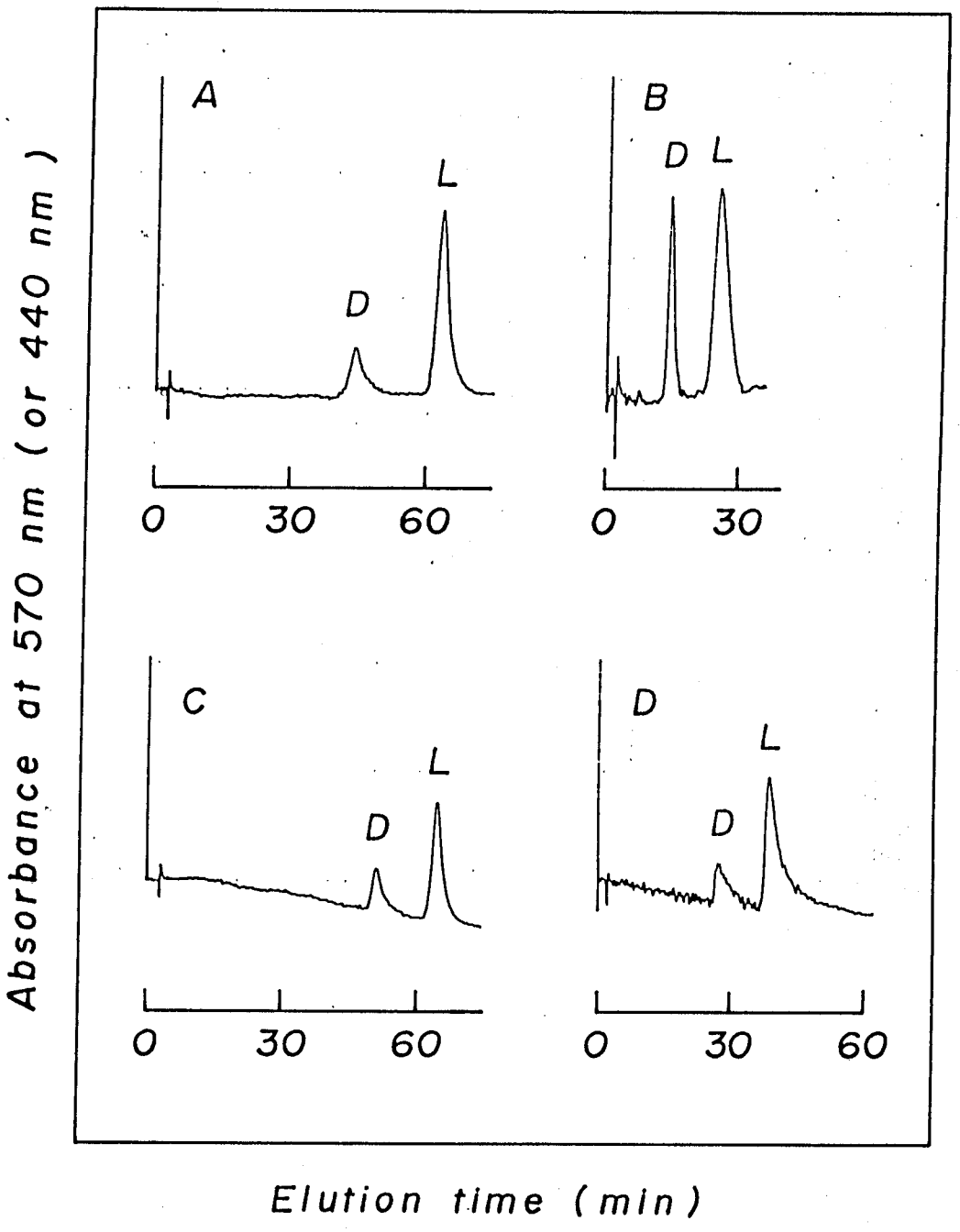

Fig. VI-2. Example of co-chromatography of DL-amino acids by using a cyanocobalamin-coated column. Co-chromatography (D:L = 1:3) was made under the same condition as shown in Table VI-1. A (Ile), B (Pro), C (Tyr) and D (Met), respectively.

much faster than those in the presence of FAD in mobile phase(cf. Fig. VI-1). Therefore, it might be necessary for the resolution that DL-amino acids are retained on the sorbent with a constant interaction between ligand complex and sorbent. Since flavin moiety of FAD is much more hydrophobic than nicotine moiety of NAD, FAD would be more strongly retained than NAD on sorbent so that FAD-mobile phase might give larger k'. In addition to such complex stability, however, the direct hydrophobic interactions of some amino acids with sorbent were shown, giving relatively large k' in comparison with others (nLeu, Leu, Ile, Phe Tyr and Trp). All the results suggest that those integrated interactions might give well-separation of DL-amino acids.

It is stressed that the stationary phase with cyanocobalamin gives good separations (Table VI-1, Figs. VI-1 and -2). Cyanocobalamin was strongly retained on sorbent and was not easily eluted from a reverse phase column by the presently employed eluant. Using the cyanocobalamin-coated column for resolution, further addition of cyanocobalamin to eluant was not necessary. Upon usage of achiral eluant, well-resolutions of amino acid racemates were obtained, whose data were comparable with those using the mobile phase containing FAD (α and Rs in Table VI-1). The result shows that one may conveniently construct a chiral stationary column using hydrophobic resin to provide for amino acid resolutions.

As shown in Table VI-1 and Fig. VI-1, the elution orders of amino acid enantiomers resolved are inverted using stationary phase or mobile phase. Since cyanocobalamin has complicated

structure in comparison with other three chiral ligands, the mechanism of recognizing amino acid chirality by cyanocobalamin seems to be different from that by others. However, detail mechanism remains unresolved.

Table VI-2 and Fig. VI-3 show the effects of concentration of chiral additives on the resolution of DL-amino acids. Using four kinds of DL-amino acid (Val, Leu, Met and Tyr), the resolution capability using 0.2 mM and 1.0 mM chiral additives (ADP and NAD) was compared each other. The concentrated chiral additive (1.0 mM) gave improvement of resolution in comparison with 0.2 mM, suggesting that the resolutions of DL-amino acids would depend upon the concentration of chiral additives in mobile phase.

Table VI-2 Effect of concentrations of chiral resolving agent on resolution of DL-amino acids.

Eluant contained either 0.2 mM or 1.0 mM chiral ligands. The other chromatographic condition was the same as that in Table 1. Control experiment was made without chiral additive.

Chiral additives	None (Control)	ADP								NAD							
		0.2 mM				1.0 mM				0.2 mM				1.0 mM			
DL-amino acids	$k'_D(k'_L)$	k'_D	k'_L	α	Rs	k'_D	k'_L	α	Rs	k'_D	k'_L	α	Rs	k'_D	k'_L	α	Rs
Val	0.20	0.90	0.90	1.0	-	1.86	1.26	1.48	1.02	0.66	0.66	1.0	-	2.12	1.34	1.22	1.56
Leu	1.08	2.10	1.90	1.11	-	4.90	3.70	1.32	1.25	2.80	2.56	1.09	-	6.88	5.04	1.37	2.27
Met	0.54	1.20	1.02	1.18	-	3.32	2.46	1.35	1.18	1.60	1.41	1.14	-	2.26	1.70	1.33	0.95
Tyr	1.44	2.64	2.21	1.20	1.29	6.34	4.24	1.50	3.04	4.08	3.49	1.17	1.45	5.01	3.44	1.45	1.84

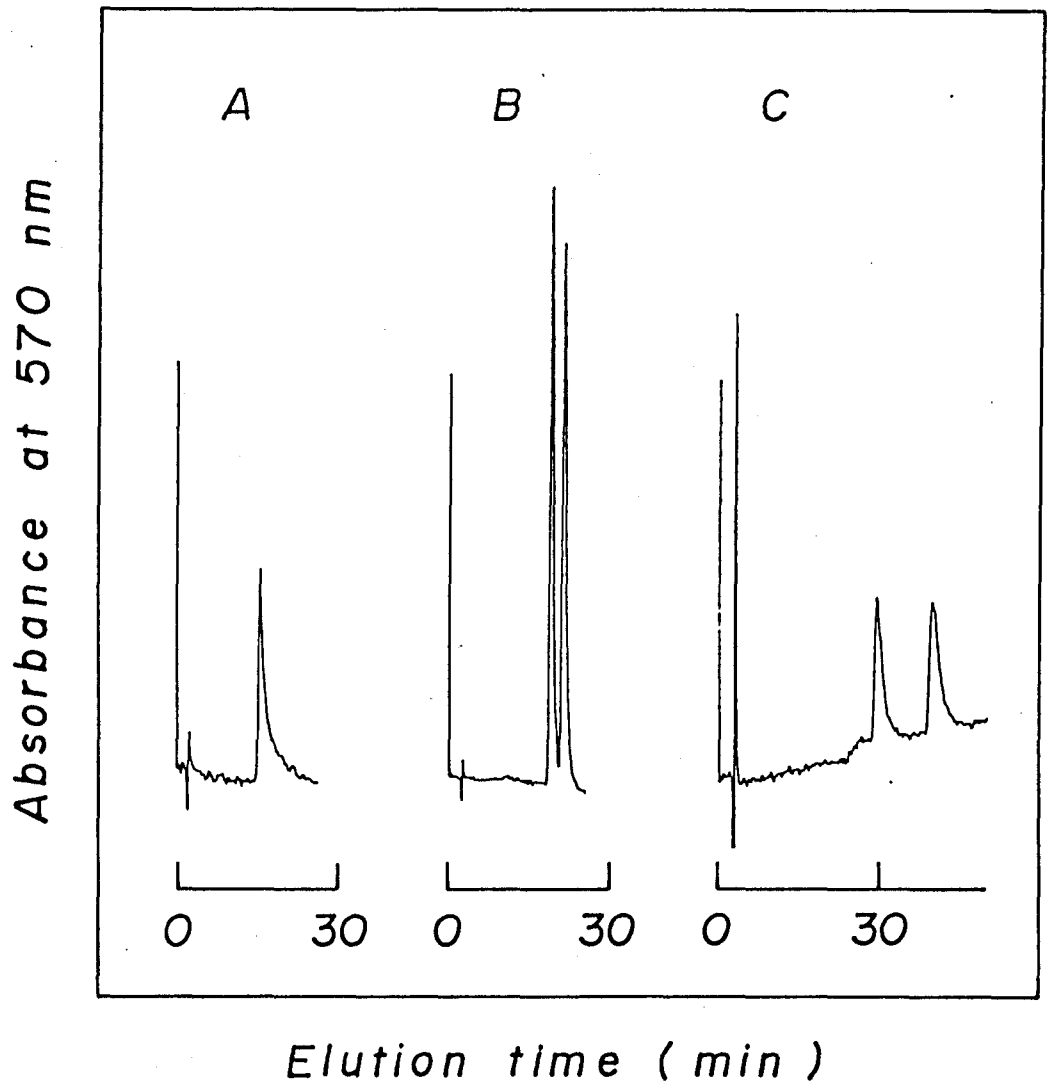

Fig. VI-3. Effect of chiral concentration on resolution of DL-Tyr. A (0 mM ADP), B (0.2 mM ADP) and C (1.0 mM ADP), respectively. Other condition was the same as that in Table VI-1.

Discussion

The present study would be significant, since no one has ever attempted to use nucleic acid and coenzyme for ligand exchange chromatography to resolve DL-amino acids, and the resolution system contains adenine nucleotide or coenzyme which is biologically important.

For example, FAD has been known to play as co-factor for D-amino acid oxidase activity[3,4]. It might be speculated from the present results that FAD in holoenzyme may recognize amino acid chirality.

Since the self-splicing RNA has been discovered[5], many catalitically active RNA molecules has been reported[6]. It is speculated that the first "living molecule" on the primitive earth might have been RNA (RNA world)[7,8]. One attractive hypothesis that nucleotide cofactors are metabolic fossils of the enzymes in the RNA world is proposed[8,9]. Since NAD, FAD and cyanocobalamin are representative nucleotide cofactors, the present experimental results are significant.

The present study also suggests further development for optical resolutions by modifying sorbent matrix using nucleic acids and their derivatives.

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CONCLUSION

[**Part I**] Amino acids (Gly, Ala, Asp and AAnBA) and nucleic acids base and its precursors (Adenine, AICAI and AICA) were abiotically synthesized from HCN in the presence of clay (montmorillonite). Synthesized amino acids (Ala, Asp and AAnBA), as expected, were racemic. The results suggest that montmorillonite as a prebiotic catalyst could play a role for the synthesis of building block molecules.

[**Part II**] All proteinic DL-amino acids were resolved by native cellulose (D-glucose polymer) chromatography. It is concluded that D-glucose is able to recognize the amino acid chirality. It is indicated that most L-amino acids (13 of 19) are selected by D-glucose.

[**Part III**] DNP-DL-amino amino acids were resolved by a cellulose column. High resolution factors (α) in comparison with underivatized amino acids were obtained. It is suggested that an increase in molecular size and then an amplification of distortion of molecules resulting from the modification of amino group may play an important role on the chiral recognition process by cellulose. In **Parts II** and **III**, D-amino acids were eluted faster than the opposite, while DNP-L-amino acids were faster than DNP-D-amino acids, namely the inversion of elution order by derivatization was observed.

[**Part IV**] Based on the above experimental results (**Part III** and **VI**), a chiral model of amino acid recognized by cellulose was constructed. The inversion of elution order phenomena could also be interpreted by the model. Moreover, the energy difference between DL-amino acid on the D-glucose (dissymmetry environment) was calculated to be some 10^{-2} - 10^{-3} eV.

[**Part V**] Using ligand exchange chromatography whose mobile phase contained nucleic acid and Cu(II), nine proteinic DL-amino acids were resolved on the basis of chiral interaction of ribonucleic acid by mediation of Cu(II). The difference of stability between DL-amino acid - Cu(II) - D-nucleic acid complexes seems to give rise to their resolutions. Nucleic acids are also able to recognize amino acid chirality.

[**Part VI**] DL-amino acids were resolved using mobile phase containing ADP, NAD or FAD as a chiral additive, and chiral stationary phase with cyanocobalamin. The result are significant because NAD, FAD and cyanocobalamin are representative nucleotide cofactors, and those might be considered as fossils of the enzymes in the RNA world. Those might have acted for chiral selection.

GENERAL DISCUSSION

Evaluation of the experiment

The present findings are extremely significant. The result of the experiment clearly shows the selection L-amino acids by D-sugars (D-glucose and D-nucleic acids) and gives novel discussion for chiral interactions (L-amino acids and D-sugars).

On the primitive earth, any possible superiority of D-sugars (D-nucleic acids) rather than L-sugars (L-nucleic acids) by means of somewhat specific interaction to, for instance, clay or metal ions have been supposed. It has previously been shown that they could be adsorbed on a clay surface[1,2], oligomerized by metal ions[3-5], and simultaneously amplified chirality[6-8]. If such a D-RNA world existed, as far as the present results are concerned, it is possible that L-amino acids might have been selected to give the emergence of the interaction of D-ribonucleotides and L-amino acids. Above all, the role of D-5'-GMP even in the above D-RNA might have been emphasized to select L-amino acids (Part V).

On the other hand, more generally, if D-ribonucleotides had been formed after DL-amino acids, the ribozyme - like construction composed of ribonucleotide - Cu(II) complex might have recognized the existing amino acid chirality and select corresponding enantiomers. Such the system might have developed the primitive ribozymes to ribonucleoproteins as more functional molecules.

Furthermore, in the experiment using deoxyribonucleic acids, D-deoxyribonucleic acids also selected L-amino acids. It is suggested that chiral amplification process described above might have continued to DNA world as we presently know. Going a step forward, these chiral interaction of nucleic acid and amino acid must be found in modern organisms. It is my belief that the chirality of sugars would necessarily play an important role on selecting amino acid chirality in primitive environment as well as contemporary world.

Model for chiral recognition

When an enzyme catalyzes a substrate, a substrate must have a matching shape to fit into the active site of an enzyme. An enzyme, a protein which is constructed with L-amino acids, can not recognize only the molecular weight and the shape of the substrate, but also its chirality, if it is optically active (e.g. D- or L-amino acids and D- or L-sugar). The Emil Fisher's metaphor[9] of the key and lock has been proven to be essentially correct and highly fruitful, looking at the stereospecificity of catalysis.

On the basis of the present experimental results that cellulose (D-glucose polymer) recognizes DL-amino acids, the key and lock interaction is proposed. The value of energy difference between DL-amino acids on cellulose (D-glucose polymer) is comparable to that when two ligands competitively bind to protein (L-amino acids polymer). This chiral recognition model may be

considered to be fruitful for recognizing optically active compounds in biological as well as non-biological world.

Model for nucleic acid reactions

It is well-known that Mg(II) ion is essential for reactions of all ribozymes[10]. It has recently been reported that the yeast $tRNA^{Phe}$ was site-specifically cleaved by Pb(II) ion[11,12], and single-stranded DNAs were site-specifically cleaved by a copper-dependent redox reaction[13]. Moreover, Some metal ions, especially Mg(II) ion, usually participate in reactions of nucleic acids (e.g. replication, transcription and translation) by forming complexes[14]. Consequently, such the nucleic acid - Cu(II) complex as described in **Part V** might be regarded as the model for reactions of ribozymes as well as for more familiar reactions of nucleic acids in modern organisms.

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