PHENYLALANINE RACEMASE

—— ITS PROPERTIES AND ROLE IN BIOSYNTHESES OF GRAMICIDIN S AND TYROCIDINE ——

by

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ABBREVIATIONS

TEA: Triethanolamine

DTT: Dithiothreitol

K-PO_4: Potassium phosphate

Pi: Inorganic phosphate

PPi: Inorganic pyrophosphate

PALP: Pyridoxal phosphate

PPO: 2, 5-Diphenyloxazole

dimethyl POPOP: 1, 4-Bis (2, (4methyl-5-phenyloxazolyl)) benzene

I. INTRODUCTION

"... if the mysterious influence to which the dissymmetry of natural products is due should come to change in sense or direction, the constituting elements of all living beings would take an inverse dissymmetry. Perhaps a new world would be presented to us. Who could foresee the organization of living beings, if the cellulose, which is right, should become left, if the left alubumen of the blood, should become right? There are here mysteries which prepare immense labors for the future, and from this hour invite the most serious mediations of science." Pasteur (1860),

A. General Considerations

1. Occurrence of D-Amino Acids in Peptide Antibiotics

Many peptide antibiotics are produced by a variety of microorganisms and the structures of these antibiotics have been elucidated by many investigators. Several common features are found (1): one or more D-amino acids are present in the molecules and most of the peptide antibiotics are cyclic peptides. The distribution of D-amino acids in peptide antibiotics are shown in Table I.

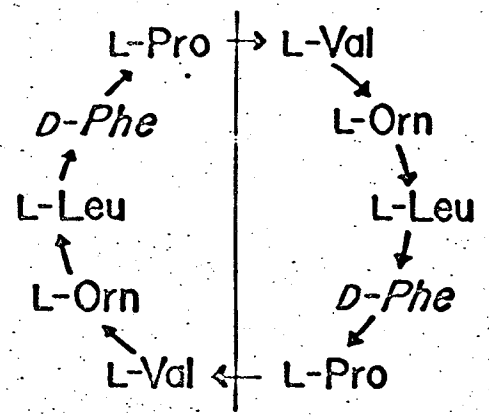
TABLE I

Occurrence of D-amino acid in peptide antibiotics

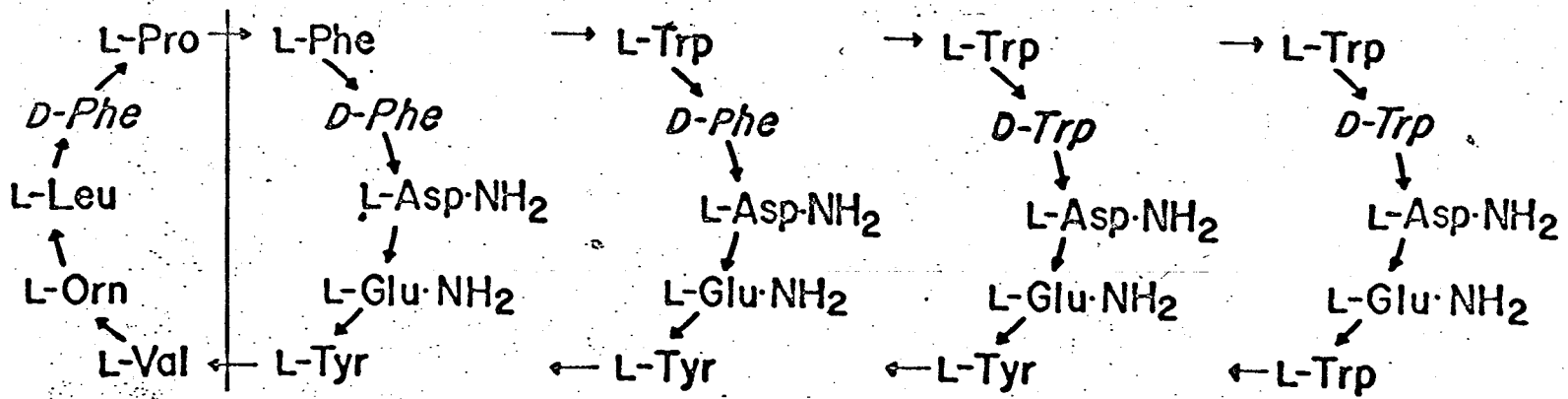
Antibiotics	Antibiotic producing organisms	D-Amino acid
Penicillin	*Penicillium chrysogenum*	valine
Malformin	*Aspergillus niger*	valine, cysteine and leucine
Actinomycin D	*Streptomyces antibiotics*	valine
Polymixin B	*Bacillus polymyxa*	phenylalanine
Polymixin D	*B. polymyxa*	leucine and serine
Circulin	*B. circulans*	leucine
Bacitracin A	*B. licheniformis*	phenylalanine, glutamic acid, aspartic acid, and ornithine
Gramicidin S	*B. brevis* Nagano or ATCC 9999	phenylalanine
Tyrocidines	*B. brevis* ATCC 8185 or 10068	phenylalanine and tryptophan
Gramicidins	*B. brevis* ATCC 8185 or 10068	leucine and valine

Gramicidin S produced by Bacillus brevis Nagano (2) and B. brevis ATCC 9999 (3) is known to be a cyclic decapeptide possessing 2 molecules of D-phenylalanine. The tyrothricin produced by Bacillus brevis ATCC 8185 is a mixture of gramicidins and tyrocidines (4). Gramicidins are linear peptide antibiotics containing D-valine and D-leucine, whereas tyrocidine, A, B, C and D.are cyclic decapeptides containing D-phenylalanine and D-tryptophan. One half of the structure of tyrocidines has the sequence of the five amino acid of gramicidin S (5). The sequences of gramicidin S and tyrocidines are given in the diagram.

AMINO ACID SEQUENCE OF GRAMICIDIN S AND TYROCIDINES



Gramicidin S



Tyrocidine A B C D

2 Biosynthesis of Peptide Antibiotics

The incorporation of amino acids into several antibiotics has been studied with intact cells (6-13) and more recently with cell-free systems (14-18). It is proven from these studies that the mechanism of biosynthesis of antibiotic peptides is different from that responsible for protein biosynthesis.

There is evidence for incorporation of the L-isomers or of both L- and D-amino acids into the D-amino acid moieties of the peptide antibiotics. L-Valine is more rapidly incorporated into penicillin by strains of Penicillum or into actinomycin by Streptomyces antibiotics than is D-valine (19, 7). In addition, the nitrogen atom of L-valine is retained during incorporation in both cases, indicating that there is inversion of the configuration of L-valine during biosynthesis of the antibiotics from this amino acid. (19, 20). Since D-leucine inhibited the formation of polymixin D and L-leucine reversed the inhibition, it was postulated that L-leucine might be the precursor of the D-leucine residue of the polymixin D (9). The formation of bacitracin by B. licheniformis is also inhibited by the addition of D-phenylalanine and the inhibition can be released by L-phenylalanine (11). It was observed by Tomino et al. that in the cell-free system of B. brevis Nagano D-phenylalanine as well as L-phenylalanine

served as the precursor for the D-phenylalanine residue of gramicidin S and that at the simultaneous presence of both forms of phenylalanine the L- form was preferentially incorporated into gramicidin S (17). The cell-free system of B. brevis Nagano enzymatically synthesizes the peptides, such as D-phenylalanyl-L-proline diketopiperazine (DKP) and D-phenylalanyl-L-prolyl-L-valine and D-phenylalanine amide from the L- forms of the constituents amino acids (21, 22, 17). The fact that the configuration of phenylalanine of these peptides and phenylalanine amide is all the D- form suggests that the inversion of the configuration of phenylalanine in gramicidin S may occur prior to the peptidation of the amino acid. The cell-free extract of B. brevis ATCC 8185 also incorporates both D- and L-phenylalanine into D-phenylalanine residue of tyrocidines (23). The similar effect of D- and L-leucine and D- and L-cysteine on the incorporation into the D- moieties of malformin is obtained with an cell-free preparation of Aspergillus niger (14).

It will be concluded that L- rather than D-amino acids is more efficiently utilized in the formation of peptide antibiotics and that the free D-amino acid is not an obligatory precursor. However, the mechanism of the conversion of L-amino acid to the D- enantiomorph has remained obscure in all instances. The phenomena involved in inversion of the configuration suggest some more novel enzymology.

B. Biosynthesis of D-Amino Acids

1 General Considerations

The biosynthesis of D-amino acids has been studied only in microorganisms. D-amino acid can be formed either from the corresponding L-amino acid by racemization or from the corresponding keto acid by D-amino acid specific transamination.

Racemases catalyze the formation of racemic amino acid from either D- or L-amino acid at equal rates. There is evidence for the occurrence of amino acid specific racemases in microorganisms. Alanine racemase was found to be present in Streptococcus faecalis and to involve pyridoxal phosphate (PALP) as a prosthetic group (24). Both PALP and FAD were required for the action of alanine racemase from B. subtilis (25). Similar activity is found in a number of other organisms, including Leuconostoc mesenteroides, Escherichia coli, and Pseudomonas fluorescence (26). Other amino acid racemases, such as glutamic acid racemase, lysine racemase, and proline racemase are known (27-29). A preliminary report of threonine racemase showed that ATP or AMP stimulated its action (30).

An alternative formation of D-glutamic acid from L-glutamic acid was shown by Thorn et al. in B. subtilis and B. anthracis (31, 32). The reaction is catalyzed by a coupled enzymic system of alanine racemase and L- and D-amino acid transaminases:

L-glutamic acid + pyruvate $\rightleftharpoons$ α-ketoglutaric acid + L-alanine

L-Alanine $\rightleftharpoons$ D-alanine

D-Alanine + α-ketoglutaric acid $\rightleftharpoons$ Pyruvate + D-glutamic acid

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Sum: L-glutamic acid $\rightleftharpoons$ D-glutamic acid

Several other D-amino acids such as phenylalanine which are not present in these bacteria was formed in vitro by this enzyme system.

2. Formation of D-Phenylalanine from L-Phenylalanine

It was reported in 1964 by Yamada, Tomino, and Kurahashi that L-phenylalanine is converted to D-phenylalanine by a cell-free extract of B. brevis Nagano and that ATP is essential for the conversion of L-phenylalanine to the D-isomer (33). Absence of D-amino acid transaminase in the cell-free extract of B. brevis Nagano excludes the possibility of the formation of D-phenylalanine through the coupled enzymic reaction of alanine racemase and D- and L-transaminases. The enzyme capable of catalyzing the formation of D- and L-phenylalanine from each one of the isomers was called phenylalanine racemase (34).

The recent study on phenylalanine racemase was described in the attached paper (Yamada and Kurahashi, 1968). The paper dealt with the partial purification and some properties of the enzyme. The enzyme was partially purified by ultracentrifugation, ammonium sulfate precipitation and calcium phosphate gel adsorption and elution. In addition to ATP and magnesium ion, inorganic pyrophosphate was required for the activity. Higher concentration of thiol compound, such as dithiothreitol and 2-mercaptoethanol stimulated the enzyme activity. The rate of the formation of D-phenylalanine from the L-isomer was more rapid than that of L-phenylalanine from the D-isomer under the certain conditions and the equilibrium of the reaction is favorable for the formation of

D-phenylalanine. This ATP-dependent phenylalanine racemase was also found in B. brevis ATCC 8185 producing tyrocidines.

C Nature of P oblem

A novel enzyme of ATP-dependent phenylalanine racemase was shown to be present in B. brevis Nagano. The enzyme gives rise to many problems. The auther has tried to answer the following questions:
(a) How is phenylalanine converted to the D-isomer in dependence on ATP? and (b) Whether or not the racemase is related with the enzyme systems of gramicidin S synthesis and of tyrocidine synthesis.

The requirement of ATP, Mg^{++} and PPi for the racemization of phenylalanine and the coexistence of L- and D-phenylalanine activating activities in the racemase preparation tempted us to formulate a hypothetical reaction mechanism as follows:

$$\text{L-Phe} + \text{ATP} + \text{Enz.} \rightleftharpoons \text{L-Phe}\cdot\text{AMP}\cdot\text{Enz.} + PP_i \quad (1)$$

$$\text{L-Phe}\cdot\text{AMP}\cdot\text{Enz.} \rightleftharpoons \text{D-Phe}\cdot\text{AMP}\cdot\text{Enz.} \quad (2)$$

$$\text{D-Phe}\cdot\text{AMP}\cdot\text{Enz.} + PP_i \rightleftharpoons \text{D-Phe} + \text{ATP} + \text{Enz.} \quad (3)$$

$$\text{Sum :} \quad \text{L-Phe} \rightleftharpoons \text{D-Phe} \quad (4)$$

On this scheme the racemization of phenylalanine occurs at the state of the enzyme bound phenylalanyl adenylate and L-phenylalanine may be incorporated into D-phenylalanine moiety of gramicidin S, not passing through free D-phenylalanine, because D-phenylalanyl adenylate formed from L-phenylalanine would be used for gramicidin S. This idea is compatible with the fact that free D-amino acid is not obligatory precursor of

the D-amino acid moieties of peptide antibiotics.

To verify the above hypothesis, the author attempted successfully to isolate the phenylalanine racemase as a homogeneous protein from B. brevis Nagano and examined its characteristics. The purified sample was shown to exert L- and D-phenylalanine activating fuction which is involved in the phenylalanine racemization. It was also demonstrated with the purified preparation that the phenylalanine racemase participated in both gramicidin S- and tyrocidines-synthesizing systems.

II, MATERIALS AND METHODS

A. Chemicals and Isotopes

The following chemicals were obtained commercially : Sephadex G-50, Sephadex G-150, Sephadex G-200 and DEAE-Sephadex A-50 medium (3.1 meq/g) were from Pharmacia; TEAE-cellulose (0.4 meq/g) was from Serva; nucleoside phosphates, crystalline bovine serum albumin were a product of Sigma Chemical Co; amino acids and the analogues of phenylalanine were from Mann Research Laboratories Inc.; DTT from Calbiochem; tyrocidine-HCl and tyrothricin from Nutritional Biochemicals Corp.; gramicidin S from Meiji Seika Kaisha, Ltd; acid alumina from M. Woelm Eschwege; L-phenylalanine-U-^{14}C (322 mc/mmole) L-leucine-U-^{14}C (214 mc/mmole), L-tyrocine-U-^{14}C (118.8 mc/mmole) from Daiichi Pure Chemicals Co.; L-tryptophan-3-^{14}C (21.4 mc/mmole) and L-valine-U-^{14}C (208.5 mc/mmole) from New England Nuclear Corp.; L-phenylalanine-U-^{14}C was purified by treatment with D-amino acid oxidase followed by column chromatography on Dowex 50 (H^+); L-phenylalanine-3-^{14}C and D-phenylalanine-3-^{14}C were prepared from DL-phenylalanine-3-^{14}C (a product of Section Molécules Marquées Fabriqué par CEA-France, 10 mc/mmole) by treatment with D- and L-amino acid oxidase, respectively. Resulting keto acid was removed by passing through a column of Dowex 50 (H^+); ATP-8-^{14}C (213 mc/mmole) and AMP-U-^{14}C (440 mc/mmole) from Schwarz Bioresearch and New England Nuclear Corp, respectively, were purified by paper chromatography as described below.

Streptomycin sulfate was a gift from Mr. I. Yoshimura of Meiji Seika Kaisha Ltd. ATP-γ-^{32}P was a gift from Dr. T. Horio of Institute for Protein Research, Osaka University. Phenylalanine hydroxamic acid was prepared by the method of Safir and Williams (35) from L-phenylalanine methylester hydrochloride, which was kindly given by Dr. S. Sakakibara of Institute for Protein Research, Osaka University, and the prepared phenylalanine hydroxamic acid represents a single spot by paper chromatography in solvents of sec butanol : formic acid : water (75 : 15 : 10 v/v) (36) and of n-butanol : acetic acid : water (4 : 1 : 5, v/v) (37). Calcium phosphate gel was prepared according to the method of Keilin and Hartree (38). Salt-free hydroxylamine was prepared by the method of Beinert et al. (39) and stored frozen at -20°C.

B. Growth of Bacteria

1. Bacillus brevis Nagano

B. brevis Nagano was supplied by Dr. S. Otani of Osaka City University Medical School. The medium used was nutrient broth which contained 1 % polypeptone (Daigo Eiyo Chemical Co.), 1 % meat extract (Kyokuto pharmaceutical Industrial Co.), 0.25 % NaCl and enough NaOH to bring the pH to 7.2. The organism grown on a nutrient agar slant was washed into 500 ml of the medium in a 2-liter Erlenmeyer flask and the flask was incubated at 37° C for 2 days with reciprocal shaking. One hundred milliliters of the preculture were inoculated to 1 liter of the fresh medium in a 5-liter Erlenmeyer flask and the flask was incubated overnight at 37° C with reciprocal shaking. A total volumes of 5 liters of the inoculum was transferred to a 200-liter fermenter containing 100 liters of the fresh medium. The fermenter was maintained at 37° C and aerated at the rate of 70 liters per minute with stirring at 175 revolutions per minute. The cells were harvested at the late logarithmic phase of growth (4 to 5-hour culture) by a Sharples centrifuge and washed once with 2 liters of 0.01 M TEA-HCl buffer (pH 8.0) containing 0.01 M $MgCl_2$. The packed cells were kept frozen at -20° C.

2. Bacillus brevis ATCC 9999

B. brevis ATCC 9999 cells grown overnight on a nutrient agar slant were washed into the same broth as that described in the growth of B. brevis Nagano. The culture was incubated overnight at 37° C. Then, 30 ml of overnight culture were inoculated into nine 2-liter Erlenmeyer flasks each containing 500 ml of the medium and they were incubated at 37° C with shaking in a New Brunswick gyratory incubator shaker. Cells were harvested by centrifugation at 8,000 x g for 15 minutes and washed with the same buffer as used for B. brevis Nagano cells.

3. Bacillus brevis ATCC 8185

B. brevis ATCC 8185 was grown by the method of K. Fujikawa et al (23). The organism was maintained on milk agar slants and then grown on milk-yeast extract medium. The fully grown culture was inoculated and grown at 37° C on the nutrient medium described in B. brevis Nagano except that the vigorous aeration (90 l/min) and the violent agitation (210 revolutions/min) were needed.

C. Enzymes

1. Preparation of Gramicidin S Synthesizing System

Fraction I and fraction II were prepared from B. brevis Nagano according to the method of H. Itoh et al (40). The procedure condisted of ultracentrifugation, streptomycin treatment, ammonium sulfate fractionation, the adsorption and elution on calcium phosphate gel and the separation of the calcium phosphate gel fraction by Sephadex G-200 into the two complementary fractions, fraction I and fraction II.

2. Preparation of Tyrocidine Synthesizing System

The method employed in the preparation of tyrocidine synthesizing system from B. brevis ATCC 8185 was that described by K. Fujikawa et al. (23): Cells were lysed with the action of muramidase; the lysate was fractionated by ammonium sulfate saturation (33 % - 41 %), the selective precipitation by protamine sulfate, 2nd ammonium sulfate saturation (32 % - 38 %) and resolution of the enzyme system into the component I and II by column chromatography on DEAE-cellulose.

3. Cell-free Extracts of B. brevis ATCC 9999

For the preparation of the crude extract, cells were suspended in 4 volumes of 0.01 M TEA-HCl (pH 8.0) containing 0.01 M $MgCl_2$ and disrupted in 22 ml to 25 ml portions for 5 minutes in a 10-kc Kubota sonic oscillator. The crude extract was obtained on

centrifugation of the sonicate at 18,000 x g for 20 minutes.

4. Inorganic Pyrophosphatase

Inorganic pyrophosphatase was purified from Escherichia coli K-12 through the 2nd DEAE-cellulose chromatography according to the method of Josse (41). One unit of enzyme activity is that amount hydrolizing 10 μmoles PPi in 15 minutes at pH 7.5.

5. D-Amino acid Oxidase

D-Amino acid oxidase was purified from the pig kidney according to the method of Kubo et al. (42). At the end of the purification, the benzoic acid was released by the addition of DL-alanine and removed by gel filtration on Sephadex G-50 equilibrated with 0.02 M $Na_4P_2O_7$-HCl buffer (pH 7.2). The lyophilized powder of the effluent was stored in a desiccator at 4° C without loss of activity at least for 20 months. Some of the preparation used was a kind gift from Dr. H. Watari of Osaka University.

6. Catalase

Crystalline catalase was a kind gift of Dr. T. Ohoka of Tokyo Metroporitan University.

7. Snake Venom

Snake venom, which served as L-amino acid oxidase, was the kind gift from Dr. T. Suzuki of Institute for Protein Research Osaka University.

D. Enzyme Assays

1, Assay of Phenylalanine Racemase

For routine measurement of phenylalanine racemase activity, the amount of D-phenylalanine formed from the L-isomer was determined (34). The reaction mixture contained 50 µmoles of TEA-HCl buffer (pH 8.6), 1 µmole of ATP (adjusted to pH 7.2 with NaOH), 1 µmole of $MgCl_2$, 25 µmoles of DTT, 0.5 µmole of EDTA (adjusted to pH 7.0 with NaOH), 0.5 µmole of potassium or sodium pyrophosphate (adjusted to pH 7.0 with KOH or NaOH), 25 mµmoles of L-phenylalanine-U-^{14}C (5 µc/µmole), and an appropriate amount of enzyme protein in a final volume of 500 µl. The reaction mixture was incubated for 30 minutes at 37° C, and the reaction was terminated by immersing the tube in a boiling water bath for 1.5 minute. The denatured protein was removed by centrifugation. To a 300-µl portion of the supernatant fluid were added 20 µl of D-amino acid oxidase (40 mg of lyophilized powder per ml of 0.1 M NaPPi-HCl buffer, pH 8.0) and 3 µl of catalase (1.7 mg of protein per ml) together with 50 mµmoles of unlabeled D-phenylalanine as carrier. After 30 minutes incubation at 37° C with shaking, 0.1 ml of 0.1 % 2, 4-dinitrophenylhydrazine in 2 N HCl was added to the solution. The solution was allowed to stand for 5 minutes at 37° C, and diluted to 1 ml with 10 % metaphosphoric acid. The

aqueous solution containing 2, 4-dinitrophenylhydrazone of phenylpyruvic acid was shaken with 1 ml of ethyl acetate on a test tube mixer. After brief centrifugation, 50-µl aliquots of the ethyl acetate layer were withdrawn and placed on aluminum planchets. The radioactivity was determined in gas-flow counter. The radioactivity found was corrected by subtraction of the counts found in the control run which was treated similarly as described above except for the omission of D-amino acid oxidase treatment. One milli-unit of the phenylalanine racemase was defined as that amount of enzyme which converted 1 mµmole of L-phenylalanine into the D-isomer per minute under the assay conditions. Specific activity of the enzyme was expressed as milli-units per mg of protein.

The amount of L-phenylalanine formed from the D-isomer was determined by the treatment with L-amino acid oxidase. The procedure was the same as that of the determination of D-phenylalanine except that 20 µl of L-amino acid oxidase (40 mg of the lyophilized snake venom in 0.1 M KCl) and the carrier of L-phenylalanine were used.

In the experiments in which the effect of pH on the racemase reaction was examined, after the termination of the racemase reaction, the pH of the reaction mixture was made up to be optimal (pH 7.2 to pH 7.5) for the action of L-amino acid oxidase by the addition of TEA or HCl.

2. Assay of D- and L-Phenylalanine-activating Activity

The amino acid activation was determined by measuring phenylalanine hydroxamic acid or phenylalanine-dependent exchange reaction between ATP and PPi.

(a) Measurement of Phenylalanine Hydroxamic Acid

The method was similar to that used by Davie (43). Enzyme was incubated with 10 µmoles of either L- or D-phenylalanine at 37° C for 30 minutes, in a final volume of 1 ml of a reaction mixture containing 10 µmoles of ATP, 10 µmoles of $MgCl_2$, 1000 µmoles of salt-free NH_2OH, 5 units of inorganic pyrophosphatase, and 100 µmoles of TEA-HCl, pH 8.0. A control without phenylalanine was run simultaneously. The reaction was stopped by the addition of 2.3 ml of a solution containing 10 % $FeCl_3$, 5 % trichloroacetic acid, and 2/3 N HCl. The precipitate was removed by centrifugation and the phenylalanine hydroxamic acid formed was measured in a Klett-Summerson photoelectric colorimeter with a No.54-filter. One unit of enzyme was defined as that amount of enzyme catalyzing the synthesis of 1 µmole of phenylalanine hydroxamic acid per minute.

(b) Measurement of Phenylalanine dependent ATP-PPi Exchange Reaction

The assay relies on the conversion of γ-P^{32} labeled ATP

to a Norit-nonadsorbable form. The assay mixture included in 0.5 ml: 25 µmoles of TEA-HCl (pH 8.0), 1 µmole of ATP containing γ-P^{32} labeled ATP (23,000 cpm), 1 µmole of $MgCl_2$, 5 µmoles of 2-mercaptoethanol, 1 µmole of $Na_4P_2O_7$, 1 µmole of L- or D-phenylalanine and the appropriate amount of enzyme. The reaction mixture was incubated for 15 minutes at 37° C with and without phenylalanine. The reaction was stopped by the addition of 0.2 ml of 1 N HCl and 0.3 ml of 5 % Norit A suspension. After being mixed for several minutes, Norit was sedimented by centrifugation at a low speed, and the supernatant fluid was obtained. The radioactivity of 50-µl aliquot of the supernatant containing Norit-nonadsorbable ^{32}PPi was determined by counting in a gas-flow counter.

3. Assay of Gramicidin S Synthesizing Activity

The method was that described by Itoh et al. (40). The amount of gramicidin S formed was measured as the amount of L-phenylalanine-U-^{14}C incorporated into gramicidin S. The reaction mixture, 0.11 ml, contained 5 µmoles of TEA-HCl (pH 8.6), 0.5 µmole of ATP, 1 µmole of $MgCl_2$, 1 µmole of DTT, 20 mµmoles of each of the four unlabeled L-amino acid (leucine, valine, ornithine, and proline), 5 mµmoles of L-phenylalanine-U-^{14}C (total counts, 40,000 cpm), and appropriate amounts of enzyme. After incubation for 30 minutes at 37° C, the phenylalanine-U-^{14}C incorporated

into gramicidin S was precipitated together with 0.1 ml of the carrier gramicidin S (10 mg per ml in ethanol) and with 2 ml of 2 % NaCl in 5 % trichloroacetic acid solution. After centrifugation, the residue was dissolved in 1 ml of ethanol, and the solution was passed through a acid alumina column(0.9 x 1.0 cm). The column was washed with 2 ml of ethanol. The radioactivity of an aliquot of the combined effluent was counted in a gas flow counter.

4. Assay of Tyrocidine Synthesizing Activity

The amount of tyrocidine formed was determined by following the method of Fujikawa et al. (23). The amount of L-leucine-U-^{14}C incorporated in the presence of the constituent amino acids, ATP and Mg^{++} into the fraction which was insoluble in NaCl solution and nonadsorbable in the acid alumina column wad determined. The radioactivity was measured in gas-flow counter.

5. Assay of Catalase

Catalase was assayed by following the decrease in the absorbancy at 240 mμ of H_2O_2. The reaction mixture, 1.5 ml, contained 7.5 μmoles of K-PO_4 buffer (pH 7.0), 7.5 μmoles of H_2O_2 and 5 to 10 μl of enzyme fraction. Enzyme activity was expressed as the change of the absorbancy per 20 seconds per 5 μl of enzyme of each fraction. The decrease in the absorbancy was followed in a Cary 14 Recording Spectrophotometer.

6. Assay of Phenylalanine-dependent AMP-ATP Exchange Reaction

The reaction mixture contained 25 µmoles of TEA-HCl (pH 8.0), 1 µmole of ATP, 1 µmole of $MgCl_2$, 5 µmoles of 2-mercaptoethanol, 1 µmole of AMP containing 1.48×10^5 cpm, 1 µmole of L-phenylalanine and enzyme protein in a total volume of 0.5 ml. Reaction was carried out at 37° C. A 25-µl portion of the reaction mixture was removed and applied onto a Toyo filter paper No. 51A. The separation of the adenosine nucleotides and the estimation of the AMP and ATP was carried out as described below.

E. Analytical Methods

1. Separation of Adenosine Nucleotides

An aliquot of the reaction mixture containing the resulting radioactive ATP, ADP and AMP was mixed with 0.05 µmole of each of the unlabeled adenosine nucleotides, placed onto a Toyo filter paper No.51A (2.5 x 50 cm) and developed descendingly with a solvent of isobutyric acid : ammonia water : H_2O (66 : 1 : 33, v/v) at 23-25° C for 12 to 14 hours. After drying the chromatogram, the area of the corresponding nucleotides were located by the ultra-violet absorption, cut in pieces and counted in a liquid scintillation counter.

2. Determination of Molecular Weight by Sucrose Density Gradient Centrifugation

The molecular weight of phenylalanine racemase was determined by sucrose density gradient centrifugation according to the method of Martin and Ames (44).

Sucrose gradient : Each of the two chambers of the gradient forming apparatus was filled with 2.3 ml of sucrose-buffer solution. 20 % of cold sucrose in 0.05 M Tris-HCl (pH 7.5) containing 0.005 M DTT was placed in the mixing chamber and 5 % of cold sucrose in 0.05 M Tris-HCl (pH 7.5) containing 0.005 M DTT was placed in the adjacent chamber. The chamber next to the outflow tube was bubbled with air.

Layering of sample : Sample, 0.2 ml, consisted of 0.18 ml of phenylalanine racemase (about 1.9 mg protein in 0.001 M Tris-HCl, pH 7.5, solution) and of 0.02 ml of catalase (2.9 mg of protein per ml) and the sample was layered on 5 % sucrose.

Centrifugation : The swinging bucket rotor SW-65 was fitted to the model L2 Spinco centrifuge. The rotor was run at 35,200 rpm for 13 hours at 2° C.

Sampling : Each fraction of 7 drops was collected in a total fraction of 36 and used for assay of catalase, L- and D-phenylalanine activating enzyme and phenylalanine racemase.

3. Disc Electrophoresis on Acrylamide Gel

Disc electrophoresis on acrylamide gel was carried out according to the method of Ornstein and Davis (45). The procedure of sample application was simplified as described by Hjertén et al. (46). 100 µg protein in Fraction XIII were applied to a polyacrylamide column (0.7 x 8.5 cm) consisting of 7.5 % pore size. Direct current was applied in the cold at 6 milli- amperes per tube for 60 minutes. Protein was stained with 1 % Amido Black 10 B for 20 minutes at room temperature. Destaining was carried out by applying the direct current at 7 milli- amperes per tube in electrolyte of 7 % acetic acid for 16 hours in the cold.

4. Determination of Protein Concentration

Protein was determined by the biuret method (47) with crystalline bovine serum alubumin as the standard. Protein in 20-µl of each fraction from the sucrose density gradient was precipitated by 5 % trichloroacetic acid and determined by the method of Lowry et al.(48).

5. Measurement of Radioactivity

Radioactivity was determined as follows:

(a) the radioactive material was placed onto an aluminum planchet as an infinite thinness sample and the radioactivity was determined in a Nuclear-Chicago gas-flow counter fitted with a "micromil" window,

(b) the radioactive material on the pieces of chromatogram was put into 5 ml of a toluene-PPO-dimethyl POPOP scintillator solution and the radioactivity was determined in a Packard Liquid scintillation counter. The scintillator solution was composed of 4 g of PPO and 300 mg of dimethyl POPOP in 1 l of toluene.

III RESULTS

A. Preparation of Phenylalanine Racemase from B. brevis Nagano

All operations were carried out at 0-4° C.

Step 1: Preparation of Crude Extract —— The frozen cells (576 g) were thawed and suspended in 4 volumes of 0.01 M TEA-HCl buffer (pH 8.0) containing 0.01 M $MgCl_2$. 2-Mercaptoethanol was added to the cell suspension at a final concentration of 0.01 M. The cell suspension was sonicated in 50-ml portions in a 10-kc Raytheon sonic oscillator for 5 minutes at 1.1 ampere. Unbroken cells and cellular debris were removed by centrifugation at 20,000 x g for 20 minutes.

Step 2: Centrifugation at 78,000 x g —— The supernatant fluid at 20,000 x g was centrifuged at 78,000 x g for 60 minutes. After the addition of a two thousandths volume of 2-mercaptoethanol to the supernatant, the supernatant liquid was stored overnight at 0° C.

Step 3: Streptomycin Sulfate Treatment —— The supernatant fluid was adjusted to contain 14 mg of protein per ml by the addition of 0.01 M TEA-HCl (pH 8.0) containing 0.01 M $MgCl_2$ and 0.01 M 2-mercaptoethanol. A twentieth volume of 20 % streptomycin sulfate solution in 0.01 M TEA-HCl (pH 8.0) and 0.01 M $MgCl_2$ was added to the supernatant fluid. After stirring for 15 minutes, the precipitate was removed by centrifugation at 18,000 x g for 15 minutes.

Step 4: Fractionation on Ammonium Sulfate —— The enzyme solution was brought to 45 % saturation by the slow addition of a saturated solution of ammonium sulfate (pH adjusted to 7.2). After being stirred for 20 minutes, the solution was centrifuged at 20,000 x g for 25 minutes. The precipitate was dissolved in a minimum volume of 0.02 M K-PO_4 buffer (pH 7.0) containing 0.01 M 2-mercaptoethanol and the solution was passed through a Sephadex G-50 column previously equilibrated with 0.02 M K-PO_4 buffer (pH 7.0). To the effluent a sixtieth volume of saturated ammonium sulfate solution (pH 7.2) and a thousandth volume of 2-mercaptoethanol were added and stored overnight at 0° C.

Step 5: Adsorption and Elution on Calcium Phosphate Gel—— The ammonium sulfate fraction was diluted to contain 10 mg of protein per ml with 0.02 M K-PO_4 buffer (pH 7.0) containing 0.01 M 2-mercaptoethanol. Calcium phosphate gel suspension (40 mg dry weight per ml) was added to the enzyme solution at a gel-protein ratio (mg dry weight per mg) of 0.7. The pH of the suspension was brought to 5.5 by the addition of 1 % acetic acid. After stirring for 10 minutes, the gel was collected by centrifugation at 8,000 x g for 7 minutes and washed once with 250 ml of 0.02 M K-PO_4 buffer (pH 7.0). The enzyme was eluted with two 250-ml portions of 0.1 M K-PO_4 buffer (pH 7.0). To the enzyme solution 2-mercaptoethanol was added at a final concentration of 0.01 M.

Step 6: Ultracentrifugation at 269,000 x g —— The protein of calcium phosphate gel fraction was precipitated by 45 % saturation with regard to ammonium sulfate and the precipitate was dissolved in a minium volume of 0.02 M K-PO_4 buffer (pH 7.0) containing 0.1 M KCl, 1 mM EDTA and 1 mM DTT. The enzyme solution was dialyzed against 2 l of the same buffer for 18 hours. The dialyzed enzyme solution was centrifuged at 269,000 x g for 2 hours. The supernatant was pooled and the tubes and the surface of precipitate were washed twice with 0.5 ml of the buffer used for the dialysis. The supernatant and washings were combined and stored overnight at 0° C.

Step 7: Gel-Filtration on Sephadex G-150 —— The column (6 x 98 cm, volume of 2.8 l) of Sephadex G-150 was equilibrated with 0.02 M K-PO_4 buffer (pH 7.0) containing 0.1 M KCl, 1 mM EDTA and 0.1 mM DTT for 4 days. Thirty two ml of the combined enzyme fraction were layered on the top of the gel column and sunk in the gel. Then, the protein was gel-filtrated with 0.02 M K-PO_4 buffer (pH 7.0) containing 0.1 M KCl, 1 mM EDTA, and 0.6 mM DTT. Each fraction, 20 ml, was collected at 12 minutes intervals in a time-regulated fraction collector. Active fractions were pooled and concentrated by ultrafiltration through the cellophan tube mounted in a vacuum chamber. The concentrated fraction was stored at 0° C.

Step 8: Chromatography on DEAE-Sephadex A-50 —— The column (2 x 25 cm) of DEAE-Sephadex A-50 was equilibrated overnight with 0.02 M K-PO_4 buffer (pH 6.5) containing 10 % glycerol (v/v) and 0.05 M KCl. 68 ml of Fraction VII containing 221 mg of protein were diluted to 102 ml by the addition of 20 ml of each of glycerol and water. The diluted solution was placed on the top of the DEAE-Sephadex. The column was successively washed with 0.05 M KCl containing 0.02 M K-PO_4 buffer (pH 6.5), 10 % glycerol, 5 mM 2-mercaptoethanol and 1 mM EDTA, and with 0.2 M KCl containing 0.02 M K-PO_4 buffer (pH 6.5), 10 % glycerol, 5 mM 2-mercaptoethanol and 1 mM EDTA. Enzyme protein was eluted with a linear gradient between 400 ml of 0.2 M KCl containing 10 % glycerol, 0.02 M K-PO_4 buffer (pH 6.5), 5 mM 2-mercaptoethanol, and 1 mM EDTA and 400 ml of 0.5 M KCl of the same solvent. Each fraction (13 ml) was collected at 15 minutes intervals. Elution pattern was shown in Fig. 1. Active fractions were pooled and concentrated by ultrafiltration through dialysis membrane. The concentrated enzyme solution was brought to 50 % (v/v) with respect to glycerol and the solution was stored at -20° C without significant loss of activity for several months. The results of the fractionation are shown in Table II. The purified racemase had a specific activity 250-fold higher than the crude extract. The overall yield was 15 %.

Fig. 1. Chromatography of phenylalanine racemase on DEAE-Sephadex A-50.

Protein (221 mg) comprising the active fraction from the Sephadex G-150 column was placed on a DEAE-Sephadex column (2 x 25 cm), and washed with 65 ml of 0.05 M KCl-buffer solution and 130 ml of 0.2 M KCl-buffer solution. The enzyme protein was eluted with a linear gradient of KCl concentration between 0.2 M and 0.5 M. The flow rate was 50-58 ml/hour and 13-14 ml fractions were collected. Each fraction was tested for racemase activity by adding 20-µl samples to tubes of the standard reaction mixture. ○ , absobancy at 280 mµ; ● , racemase activity.

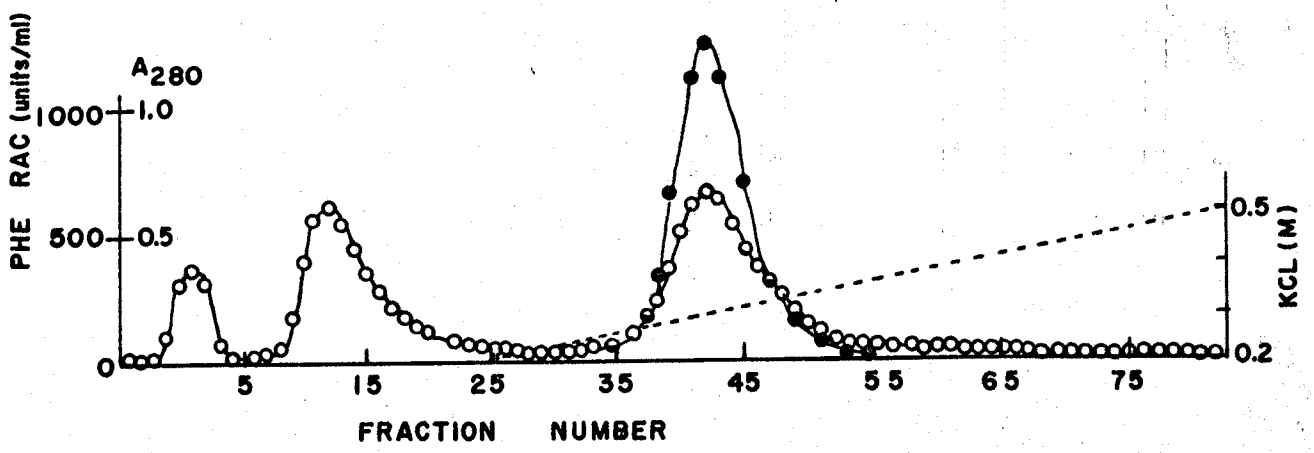


TABLE II

Purification of Phenylalanine racemase

of Bacillus brevis Nagano

Assay conditions were the same as described in the method section except that KF (10 μmoles) was added.

	Fraction	Total protein (mg)	Total activity (milli-units)	Specific activity (milli-units/ mg protein)
I.	Crude extract	92,400	5,544	0.06
II.	78,000 x g supernatant	45,600	5,472	0.12
III.	Streptomycin treatment		5,344	
IV.	$(NH_4)_2SO_4$, 0-45 % saturation	7,500	4,500	0.60
V.	$Ca_3(PO_4)_2$ gel eluate	1,980	3,018	1.57
VI.	269,000 x g supernatant	582	2,014	3.46
VII.	Gel filtration on Sephadex G-150	226	1,418	6.26
VIII.	Chromatography on DEAE-Sephadex	58	879	15.16

B. Characterization of Phenylalanine Racemase

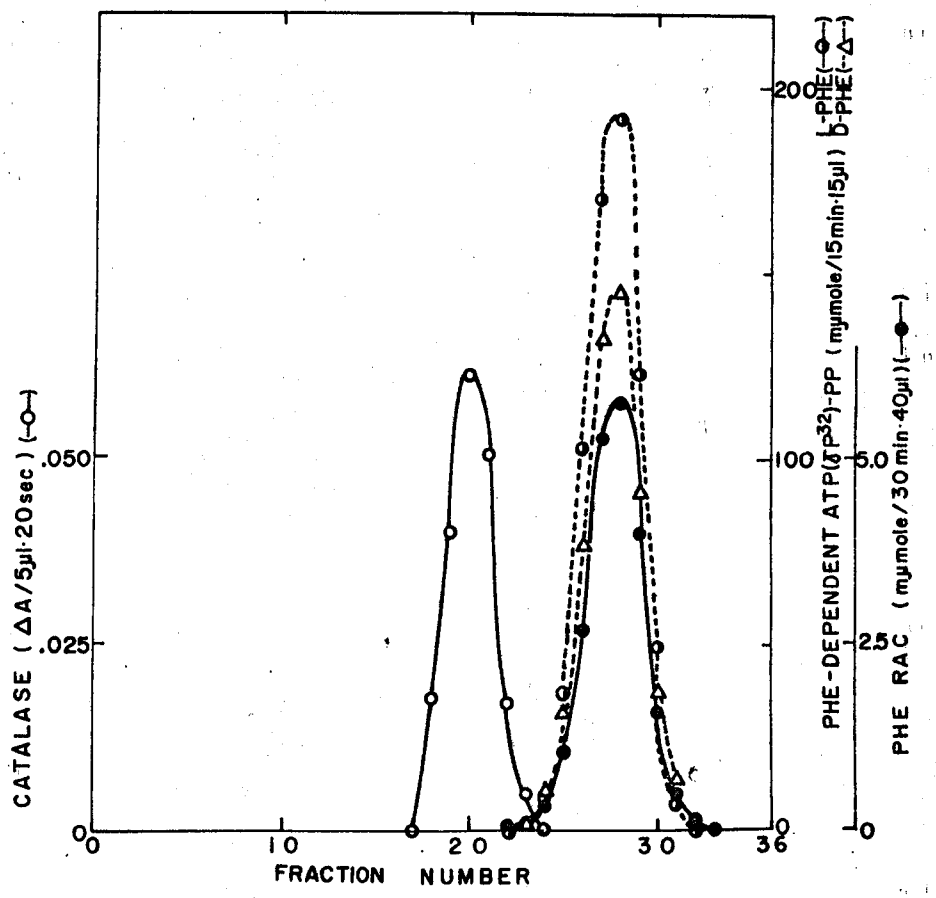
1, Homogeneity of Purified Phenylalanine Racemase

When the phenylalanine racemase was purified by chromatography on DEAE-Sephadex, a single band of protein was eluted with a gradient of 0.2 M to 0.5 M KCl (Fig. 1). The enzyme activity closely paralled the protein, as expected of a pure enzyme.

The purified phenylalanine racemase sedimented as a single homogeneous protein on a sucrose density gradient (Fig. 2). The $S_{20,\,w}$ determined for the racemase was 6.1 S using catalase as a standard with $S^{0.725}_{20,\,w}$ 11.3 S. An apparent molecular weight of the racemase was calculated to be 100,000. Purity of the purified enzyme was also demonstrated electrophoreticaly by disc electrophoresis on acrylamide gel (Fig. 3). There were observed the main band corresponding to the racemase and other faint bands. These findings indicate that the purified enzyme is near homogeneous.

Fig. 2. Sucrose density gradient centrifugation of a mixture of phenylalanine racemase and catalase.

See the method section for the experimental details. Fig. 2a. Phenylalanine racemase, D- and L-phenylalanine activating activity and catalase were determined. Fig. 2b. For the scanty of the amount of each fraction supplied for the measurements of four enzyme activities, the other one of the three tubes was used for the determination of the protein (after Lowry et al.) and the activating activity. It will be shown in the text that the racemase is identical with the protein capable of catalyzing the activation of phenylalanine.



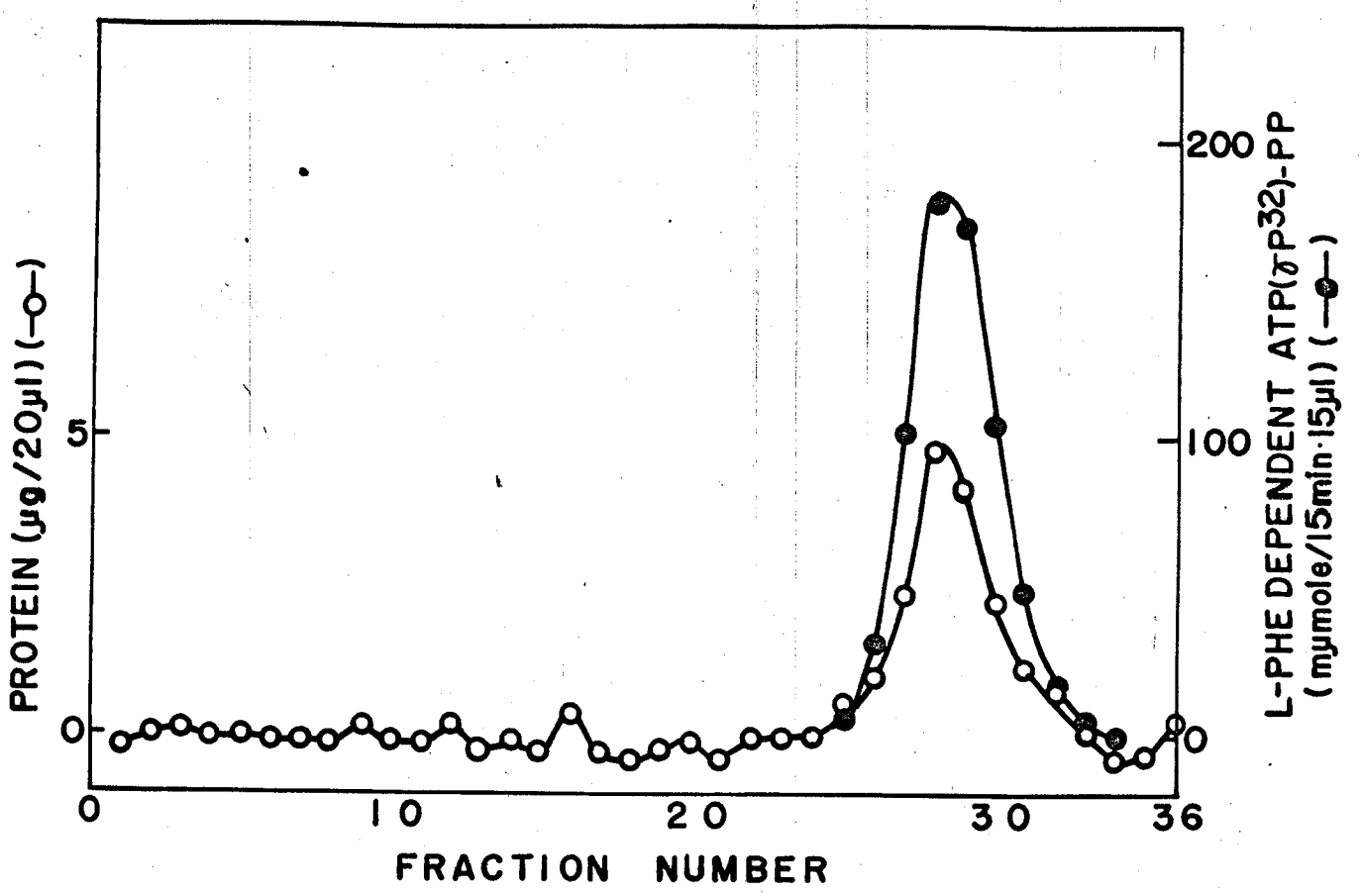
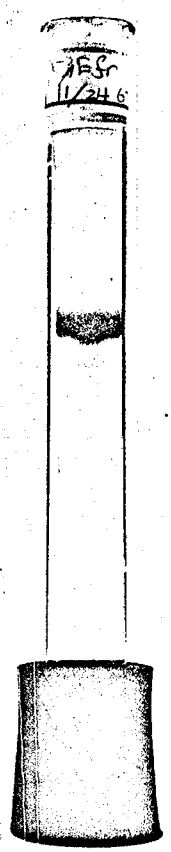

PROTEIN (µg/20µl) (—o—)
5
0
0
10
20
30
36
FRACTION NUMBER
200
100
0
L-PHE DEPENDENT ATP(γP32)-PP
(mµmole/15min·15µl) (—●—)

Fig. 3. Polyacrylamide gel electrophoresis.

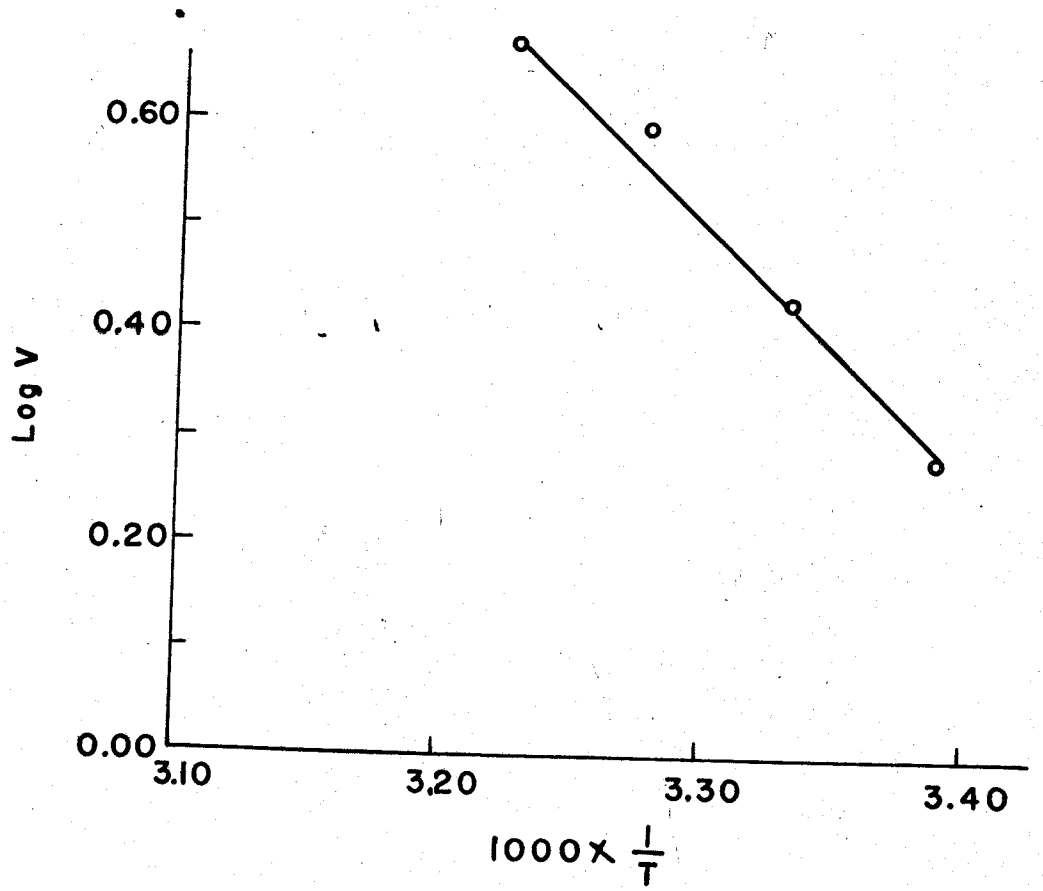
100-μg protein from Fraction VIII was applied to a polyacrylamide column (0.7 x 8.5 cm). Electrophoresis was carried out for 60 minutes at 6 milliamperes per tube. See the text for the experimental details.



2. Effect of Temperature on Reaction Rate

The initial rate was maximum at 37° C and began to fall at 42° C. The activation energy of L-phenylalanine racemization was estimated at temperature between 22° and 37° C. When the logarithm of initial rate (log v) was plotted against the reciprocal of the absolute temperature (1/T), a straight line was obtained. The activation energy was calculated from the slope of the line (Fig. 4). A value of 10.9×10^3 cal/mole was obtained.

Fig. 4. Arrhenius plot of the rate of the racemization reaction of L-phenylalanine as a fuction of reciprocal absolute temperatures, 12.5 µg of Fraction VIII were used.



C. Properties of Phenylalanine Racemase

1. Linearity of the Reaction with Enzyme Concentration

The proportionarity of the reaction with enzyme concentration is shown in Fig. 5. The linear relation persisted up to 0.225 mg of protein and then it leveled off.

2. Time Course of the Racemization Reaction

Fig. 6 represents the relationship between the incubation time and the racemization reaction with L- or D-phenylalanine as substrate. When the conversion of L-phenylalanine to the D-isomer was measured, the reaction was fairly linear with time up to 30 minutes and then it leveled off gradually. When the conversion of D-phenylalanine to the L-isomer was measured, the reaction proceeded at slower rate than the conversion of L-phenylalanine to the D-isomer.

3. Phenylalanine Saturation Curve

When initial velocity was plotted against the increasing concentration of L-phenylalanine, the hyperbolic curve was obtained (Fig. 7). The enzyme was saturated at the concentration of 5×10^{-5} M. The value of Km for L-phenylalanine was calculated to be 2×10^{-5} M from the Lineweaver-Burk plot.

4. Effect of ATP

In Fig. 8, the relation between the enzyme activity and ATP concentration is shown. In the absence of ATP no enzyme activity

Fig. 5. Propotionality of reaction rate to enzyme concentration.

The assay conditions were the same as those in the method section except for 0.05 M TEA-HCl (pH 7.8) used. The amounts of enzyme of the calcium phosphate gel fraction were used as indicated.

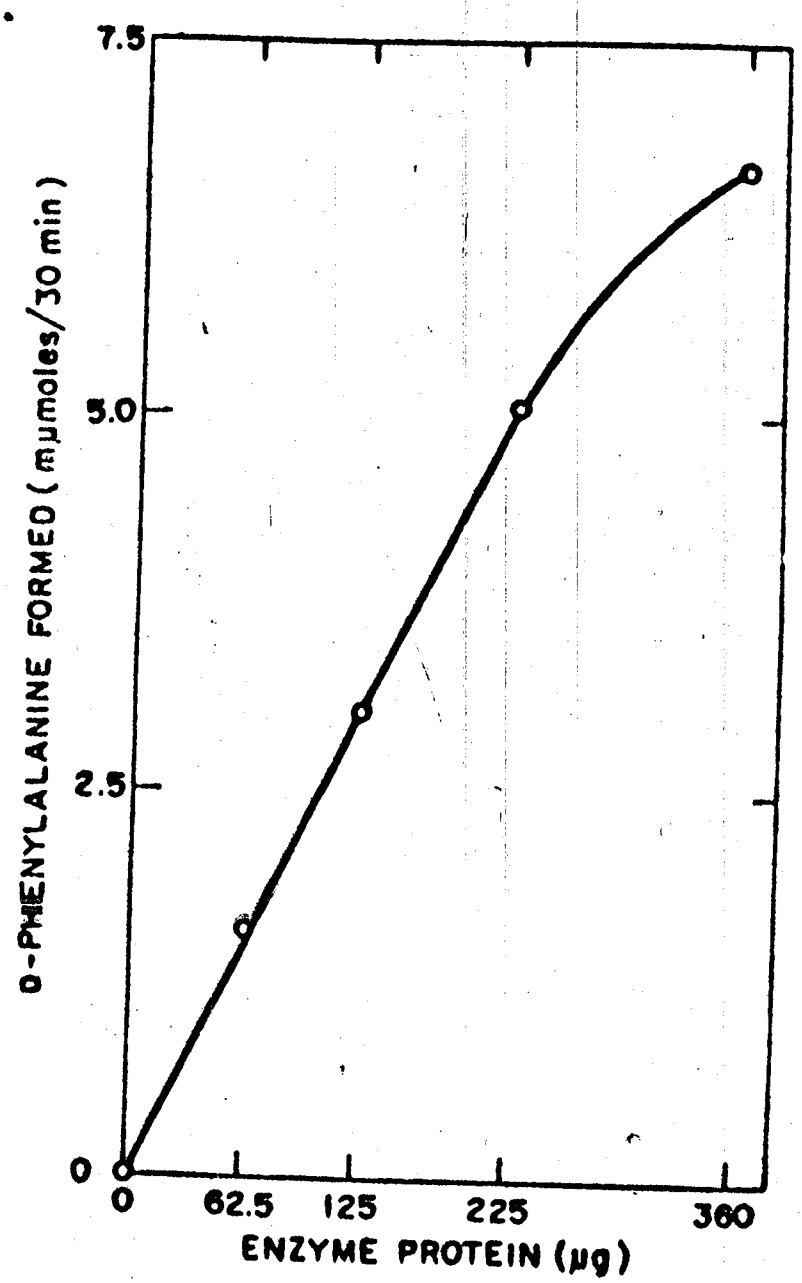


Fig. 6. Dependency of the racemization reaction on incubation time.

The reaction mixture contained 125 µmoles of TEA-HCl (pH 8.0), 5 µmoles of ATP, 5 µmoles of $MgCl_2$, 125 µmoles of DTT, 2.5 µmoles of EDTA, 2.5 µmoles of PPi, 1.125 mg of enzyme protein from the calcium phosphate gel fraction and 125 mµmoles of L-phenylalanine-U-^{14}C for 125 mµmoles of D-phenylalanine-3-^{14}C in a final volume of 2.5 ml. 300-µl portions of the reaction mixture were removed at the times indicated, and the amount of D- and L-phenylalanine formed was determined by D-amino-acid oxidase or L-amino-acid oxidase, respectively.

-●- D-Phenylalanine formed per 0.5 ml of reaction mixture

-○- L-Phenylalanine formed per 0.5 ml of reaction mixture

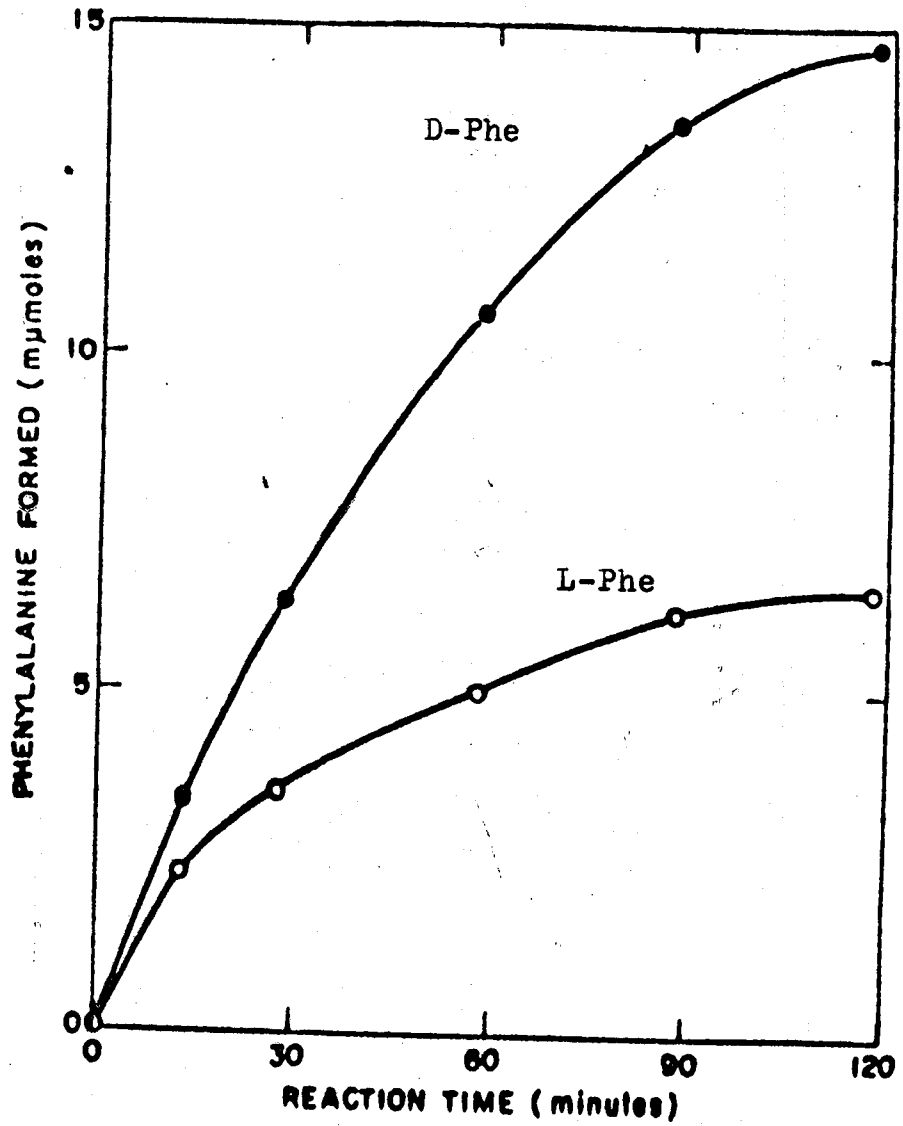

15
10
5
0
0
30
60
90
120
D-Phe
L-Phe
PHENYLALANINE FORMED (mμmoles)
REACTION TIME (minutes)

Fig. 7. L-Phenylalanine saturation curve.

Assay conditions were the same as described in the text except for the L-phenylalanine concentration as indicated. 12.5 µg of Fraction VIII was used.

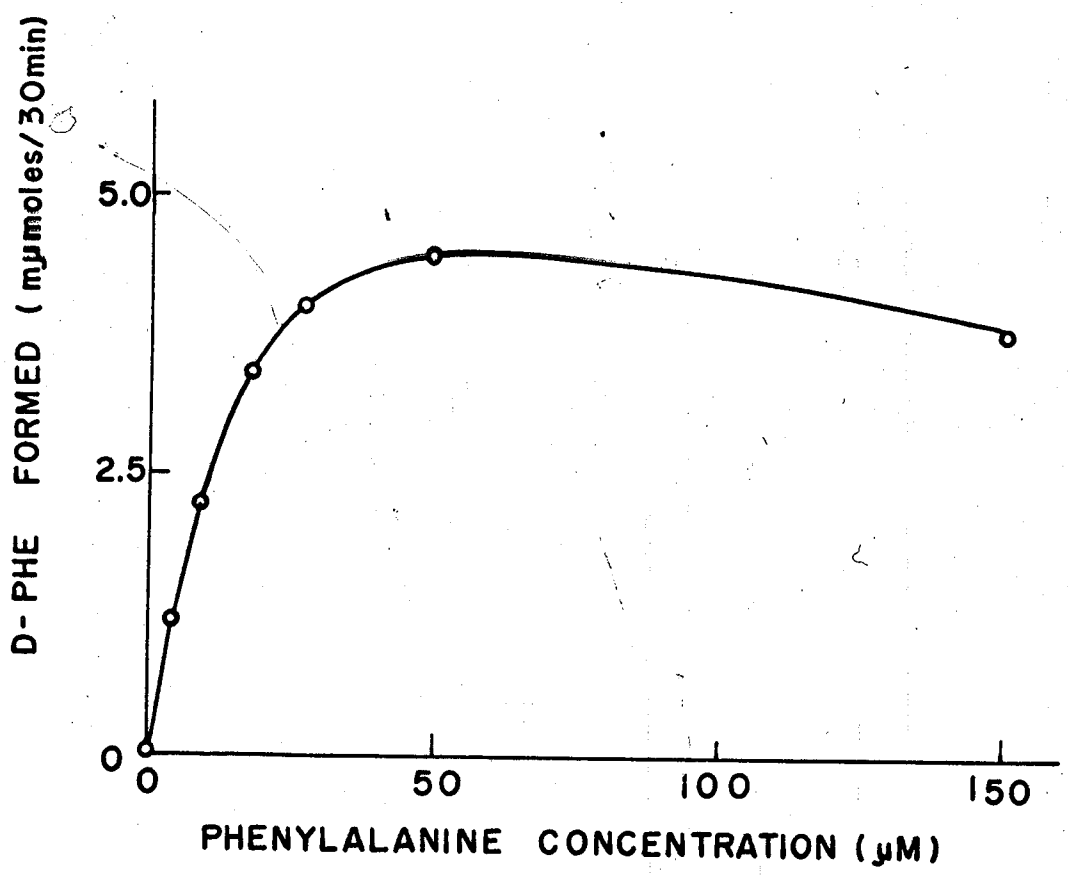
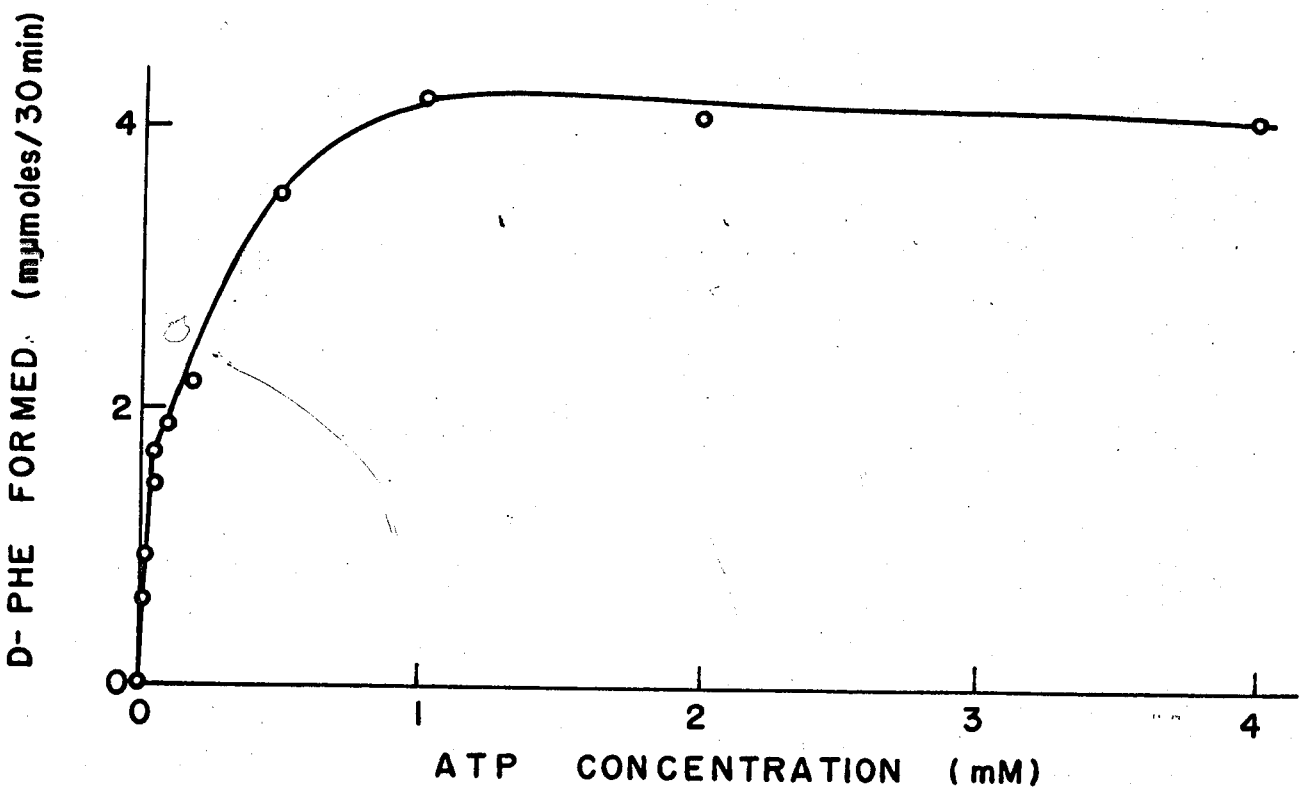


Fig. 8. Effect of ATP concentration on enzyme activity.

Assay conditions were the same as described in the method section except that the ATP concentration was indicated. 12.5 µg of Fraction VIII were used.



was detected and activity increased with the increasing amounts of ATP. Higher concentrations (up to 10 mM) of ATP showed no inhibitory effect. The value of Km for ATP in the racemase reaction was calculated to be 1.5×10^{-4} M from the Lineweaver-Burk plot. Of the nucleoside phosphates tested, ATP was found to be most effective in the reaction (Table III). ADP was effective, but the degree of activation was only 15 % of that of ATP. In the presence of 5 mM KF, which was an inhibitor of adenylate kinase, the effect of ADP decreased to 10 % of that of ATP. Other nucleotides, such as AMP, GTP, CTP, UTP, and TTP, was found to be inactive in this reaction. Since the enzyme preparation (calcium phosphate gel fraction) contained adenylate kinase, it may be concluded that only ATP was the active nucleotide for the reaction.

5. Effect of Magnesium and Other Metals

Fig. 9 shows that the addition of increasing amounts of Mg^{++} up to 0.5 mM to the reaction mixture increased the reaction velocity. The enzyme activity in the absence of added Mg^{++} was less than 5 % of the activity in the presence of Mg^{++}.

Mn^{++} ion had a similar level of activation as Mg^{++}. The addition of metal ions (2 mM), such as Ca^{++}, Ni^{++}, Zn^{++}, Co^{++}, Fe^{++}, Al^{+++}, and Fe^{+++}, to the reaction mixture containing Mg^{++} showed no stimulation of enzyme activity but rather inhibitory effects (Table IV).

TABLE III

Effect of nucleoside phosphates on the rate of phenylalanine racemization

The reaction mixture was that described in the method section except that TEA-HCl (pH 7.8) was used and the indicated nucleotides were added. 0.225 mg of protein of calcium phosphate gel fraction was used.

Additions (μmoles)	D-Phenylalanine formed (mμmoles/30 min)
None	-0.03
ATP, 1	5.10
ADP, 1	0.83
ADP, 1 and KF, 2.5	0.59
AMP, 1	0.00
GTP, 1	0.04
CTP, 1	0.04
UTP, 1	0.16
TTP, 1	0.03

Fig. 9. Effect of Mg^{++} concentration on enzyme activity

Assay conditions were the same as described in legend of Fig. 5 except for $MgCl_2$ concentration as indicated. 0.225 mg of enzyme protein from the calcium phosphate gel fraction was used.

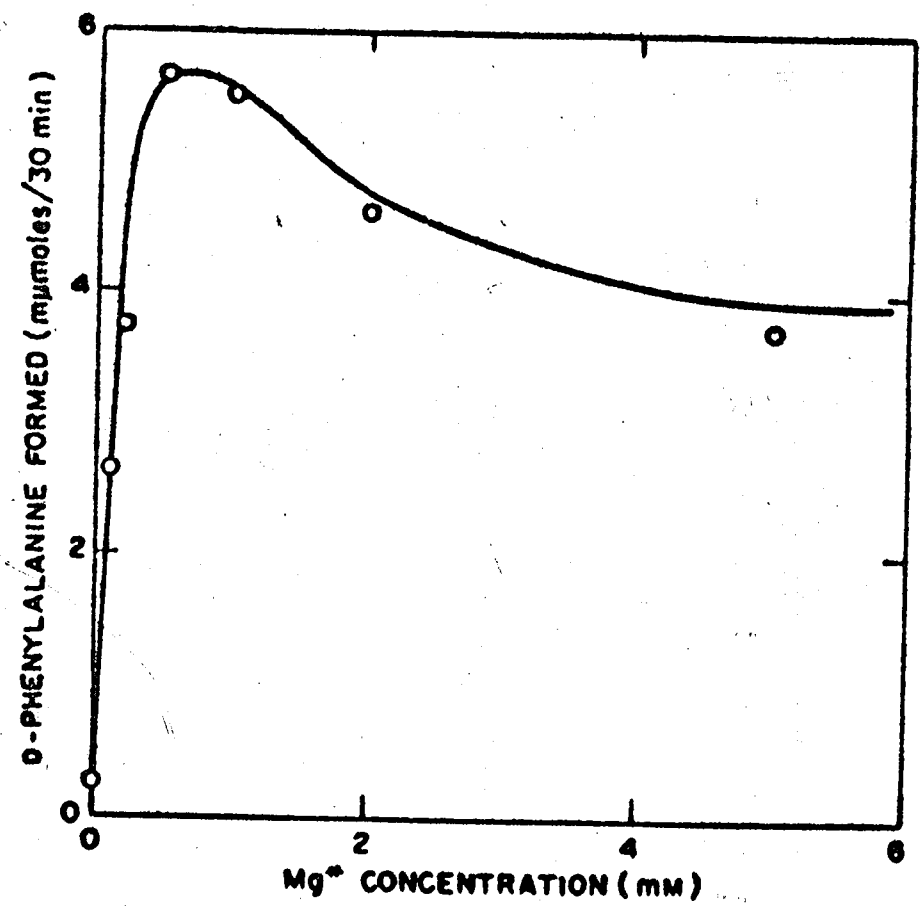


TABLE IV

Effect of various metal ions on racemase reaction

Assay conditions were the same as described in Table III except for the additions of various metal ions. 0.225 mg protein of calcium phosphate gel fraction was used.

Additions (μmole)	D-Phenylalanine formed (mμmoles/30 min)
None	1.77
EDTA, 0.5	0.26
$MgCl_2$, 0.1	5.45
1.0	4.35
$MnCl_2$, 0.1	5.55
1.0	5.30
$MgCl_2$, 1.0	
plus $CaCl_2$, 1.0	4.19
" $CuSO_4$, 1.0	2.75
" $NiSO_4$, 1.0	2.90
" $ZnSO_4$, 1.0	0.33
" $CoCl_2$, 1.0	2.44
" $FeSO_4(NH_4)_2SO_4$, 1.0	1.27
" $Al_2(SO_4)_3(NH_4)_2SO_4$, 1.0	0.03
" $FeCl_3$, 1.0	1.84

6. Effect of pH

In Fig. 10, the reaction velocities of D- and L-phenylalanine formation from each of the isomers in TEA buffer are plotted against the pH of the reaction mixture. The pH of the reaction mixture was determined by a Beckman pH meter with a one-drop electrode. The reaction of the formation of D-phenylalanine from the L-isomer had a pH optimum between pH 8.2 and pH 8.5 and the velocity increased with pH upto 8.4, while on the formation of L-phenylalanine from the D-isomer the velocity was not so much affected by pH changes and the reaction had a pH optimum between pH 7.6 and pH 8.0.

7. Activation by PPi and AMP

In the assay in which the reaction was carried out without the addition of PPi and KF (33), the enzyme activity was very weak in the crude extract and varied from preparation to preparation. As shown in Table V, the addition of PPi together with KF increased the enzyme activity 8-fold, indicating that inorganic pyrophosphatase in the crude extract might be interfering with the reaction. In another experiment with the use of the purified enzyme (Table V), it was observed that the addition of PPi increased the enzyme activity appreciably, and that the simultaneous presence of PPi and inorganic pyrophosphatase diminished the activity to 30 % of that in the absence of inorganic pyrophosphatase.

Fig. 10. Effect of pH on the rate of the D-phenylalanine formation and of the L-phenylalanine formation.

The assay conditions were the same as described in the text except for the TEA-HCl buffer used and L-phenylalanine-U-^{14}C or D-phenylalanine-3-^{14}C used. 18.8 µg of Fraction VIII were used.

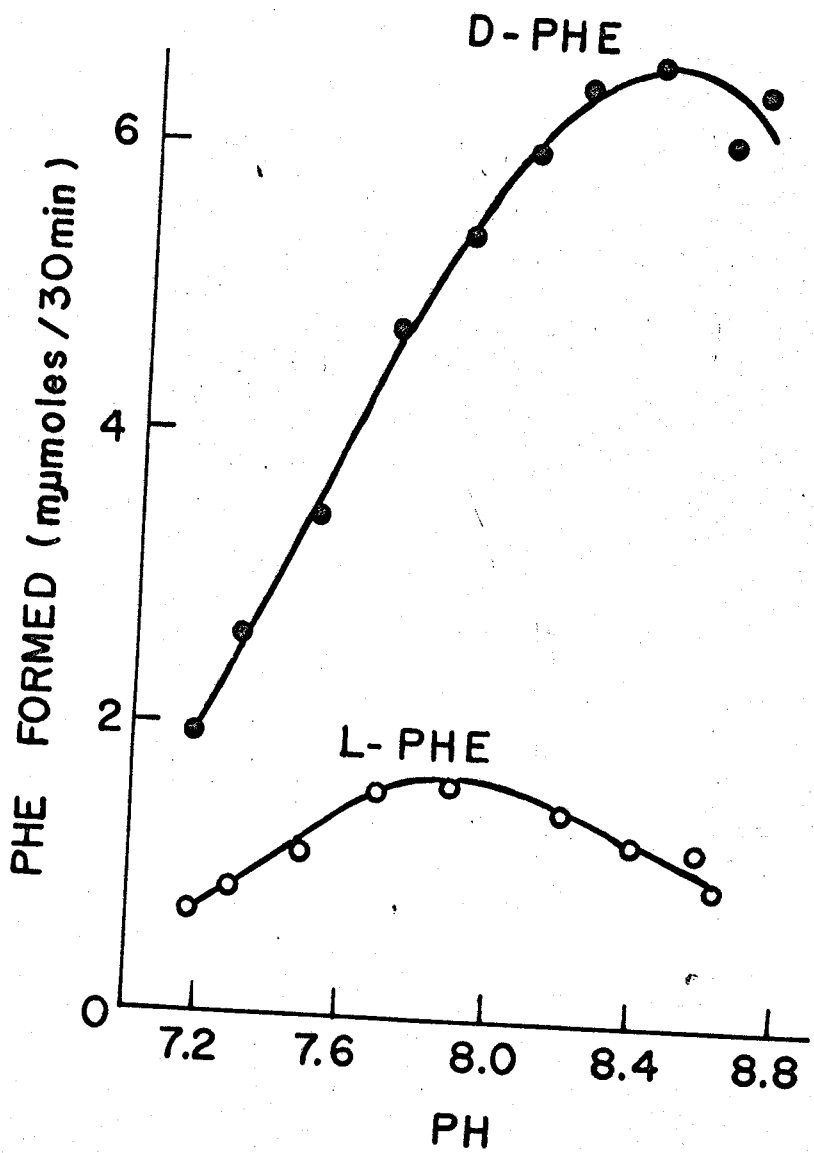


TABLE V

Requirement of inorganic pyrophosphate for phenylalanine racemization

Assay conditions were the same as described in the text except for the additions indicated.

Exp.	Enzyme prep.	Additions (μmoles)	D-Phenylalanine formed (mμmoles/30 min)
I	Crude extract 2.1 mg of protein	PPi, 0.5	0.53
		PPi, 0.5 and KF, 2.5	3.62
		PPi, 0.5 and KF, 10.0	3.95
		PPi, 0.5 and KF, 20.0	2.52
		PPi, 0.5 and KF, 30.0	0.23
		KF, 10.0	2.36
II	DEAE-Sephadex fraction 18.8 μg of protein	none	4.11
		PPi, 0.5	5.98
		PPi, 0.5 and boiled PPase[1], 0.2 unit	6.13
		PPi, 0.5 and PPase 0.2 unit	3.21
		2.0 unit	1.73
		PPi, 0.5 and KF 10	6.89
		PPi, 0.5, KF, 10 and PPase, 2.0 units	7.39

1) The tube containing PPase was immersed for a boiling water bath for 30 minutes. Bovine serum albumin (100 μg of protein) did not inhibit the racemase in another series of experiments.

Addition of KF released the inhibition by inorganic pyrophosphatase. The boiled inorganic pyrophosphatase and bovine serum albumin did not replace inorganic pyrophosphatase. These results indicate that the enzyme action was activated by PPi. The optimum concentration of PPi was found to be 0.2 mM to 2 mM (Fig. 11). The excess amount of PPi was found rather inhibitory.

It is seen from Fig. 12 that AMP as well as PPi had the effect on the racemase activity, but that ADP or Pi did not.

When the racemase activity was determined at the lower concentration of DTT and at the lower pH, the enzyme activity is negligible. The addition of PPi or AMP to this reaction mixture caused the several fold enhancement in the enzyme activity. In the simultaneous presence of AMP and PPi, the enzyme activity was enhanced remarkably and became near to or higher than the activity under the usual assay conditions where the higher concentration of DTT (0.05 M) and the higher pH (pH 8.6) were used. The stimulation by AMP in the presence of PPi shifted to the low concentrations of AMP. This finding showed that there occurred the co-operative interaction between AMP and PPi.

Fig. 11. Effect of potassium pyrophosphate concentration on enzyme activity.

Assay conditions were the same as described in the legend of Fig. 5 except for the PPi concentration as indicated. 0.225 mg of enzyme protein from the calcium phosphate gel fraction was used.

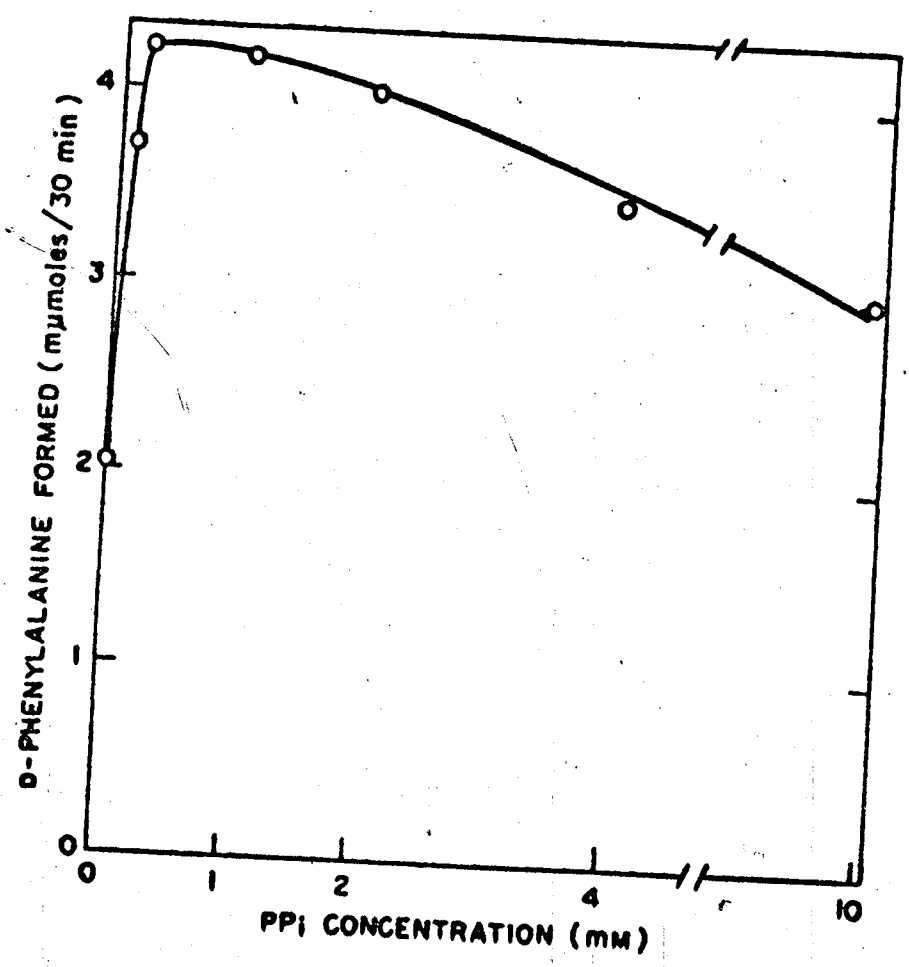
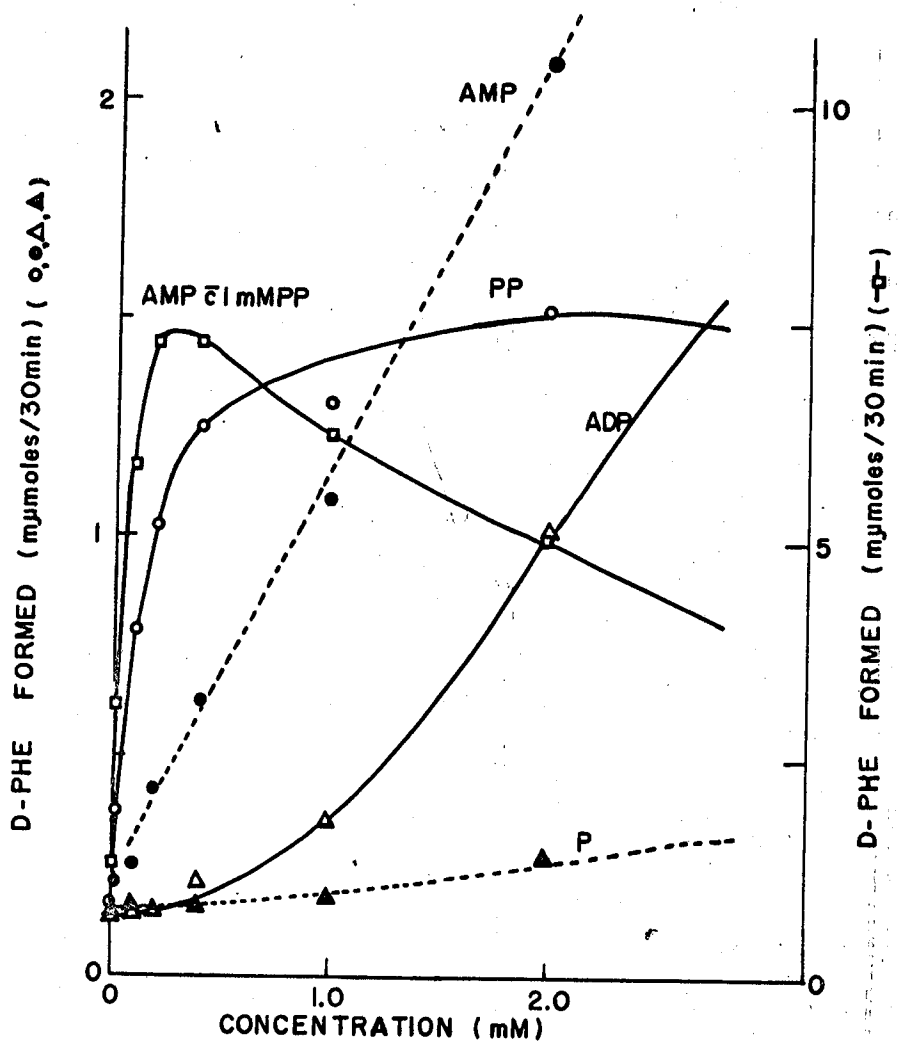


Fig. 12. Effect of ADP, AMP, PPi, and Pi concentrations on the reaction rate.

Assay conditions were the same as described in the text except that 0.1 M TEA-HCl (pH 7.8) and 0.005 M DTT were used with increasing concentrations of ADP, AMP, PPi or Pi or AMP in the presence of the constant amount of 1 mM PPi.



8. Effect of Thiol Compounds

The effect of thiol compounds on enzyme activity is shown in Fig. 13. As DTT was added in increasing amounts to the reaction mixture, a 5.8-fold activation of the velocity of D-phenylalanine formation from the L-isomer attained at 0.1 M DTT. When the formation of L-phenylalanine from the D-isomer was measured in varying amounts of DTT, the velocity was maximum at the similar concentration of DTT to that of the D-phenylalanine formation, whereas the stimulation by DTT is not so profound as that in D-phenylalanine formation. Higher concentrations of DTT had an inhibitory effect on the reaction. Of DTT and 2-mercaptoethanol tested, DTT was more effective (34).

9. Influence of pH and Thiol Compound on the Ratio of D- to L-Phenylalanine at Equilibrium of the Reaction

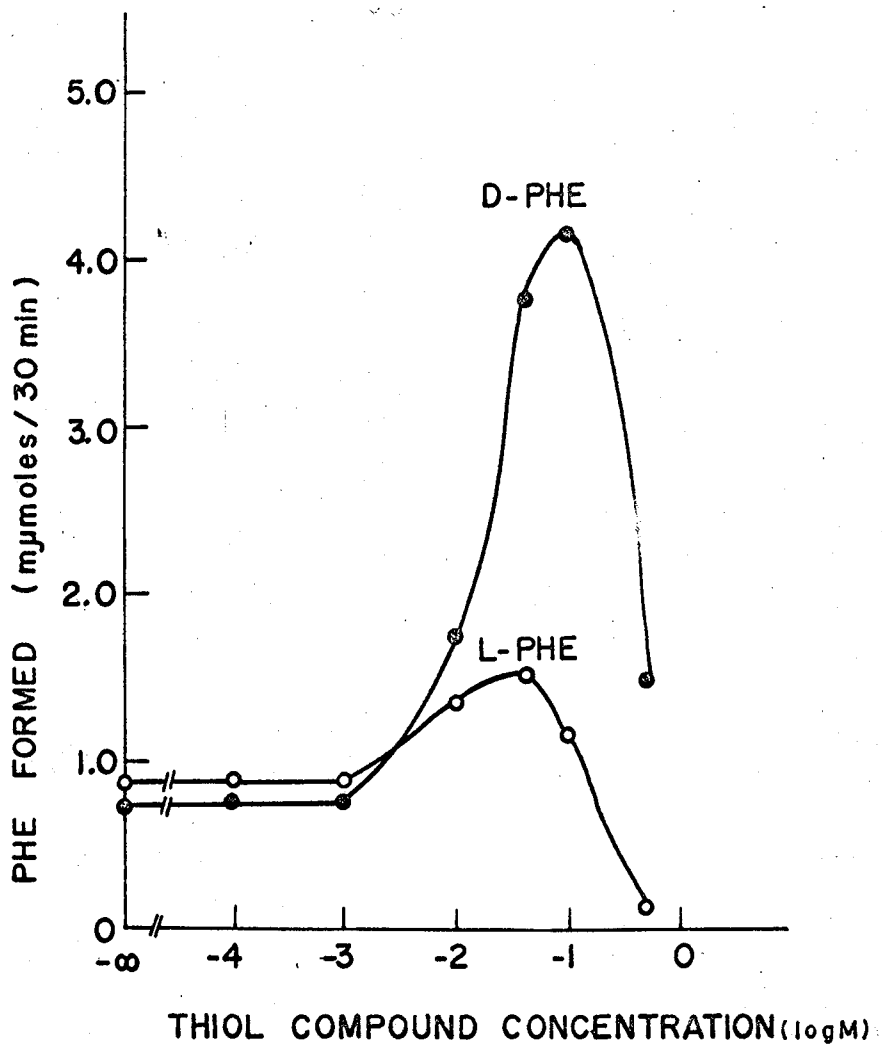
It was described above that the initial velocities of D-phenylalanine formation from the L-isomer were considerably affected by the concentrations of DTT and pH changes (Fig. 10, 13), but that the velocities of L-phenylalanine formation from the D-isomer were not affected by these variables. These findings indicate that D/L-phenylalanine ratio will vary according to the assay conditions and this led us to determine D/L-

Fig. 13. Effect of DTT concentration on the rates of D-phenylalanine formation and of L-phenylalanine formation.

The assay conditions were the same as described in the text except for the concentration of DTT as indicated and 0.1 M TEA-HCl (pH 7.8) used.

, D-phenylalanine formed from L-phenylalanine;

, L-phenylalanine formed from D-phenylalanine.



phenylalanine ratio at equilbria of the reaction under the various assay conditions. When the enzyme catalyzed the formation of L- and D-phenylalanine from each of the isomers in the experiment in which the reaction mixture consisted of 0.05 M TEA-HCl (pH 8.0), 2 mM ATP, 2 mM $MgCl_2$, 0.01 M 2-mercaptoethanol, the enzyme and 0.05 mM of L- or D-phenylalanine, the racemic mixture of L- and D-phenylalanine was formed at the equal rate from both isomers (Table VI). Thus the value of the D/L ratio was 1 under theses conditions. When the enzyme was incubated with 0.05 M TEA-HCl buffer (pH 7.8), 2 mM ATP, 2 mM $MgCl_2$, 0.05 M DTT, 1 mM EDTA (pH 7.0), 1 mM PPi (pH 7.0) and 0.05 mM of L-phenylalanine, the initial velocity of D-phenylalanine formation was faster than that of L-phenylalanine formation and the D/L-phenylalanine ratio was determined to be 7/3, at equilibrium of the reaction (Table VII). When the reaction reached equilibrium in the experiment in which the reaction mixture contained 0.1 M TEA-HCl buffer (pH 8.6), 2 mM ATP, 2 mM $MgCl_2$ 0.05 M DTT, 1 mM EDTA (pH 7.0), 1 mM $Na_4P_2O_7$ (pH 7.0), D- or L-phenylalanine and an appropriate amount of enzyme, the initial velocity of D-phenylalanine formation was faster than that of L-phenylalanine formation and at equilibrium the resulting mixture of D- and L-phenylalanine was composed of the D/L-phenylalanine ratio of 8/2 (Table VIII).

TABLE VI

Racemic equilibrium of phenylalanine racemization

Reaction mixture was 5 times larger than the one as described in the text. 2.92 mg protein of 2nd calcium phosphate gel fraction which was obtained by purifying the calcium phosphate gel fraction with alumina gel Cr and 2nd calcium phosphate gel treatments.

Time (min)	D-Phenylalanine formed[1] (mμmoles)[3]	(%)	L-Phenylalanine formed[2] (mμmoles)[3]	(%)
0	0.2	0.8	0.2	0.8
15	4.0	16.0	6.1	24.4
30	8.3	32.2	8.4	33.6
60	12.5	50.0	9.7	38.8
90	12.9	51.5	12.5	50.0
120	13.7	54.7	11.6	46.4

1) L-Phenylalanine-U-^{14}C (5 μc/μmole) served as substrate.

2) D-Phenylalanine-3-^{14}C (10 μc/μmole) was used as substrate

3) The value was expressed as the amount of phenylalanine per 0.5 ml of the reaction mixture.

TABLE VII

Racemic equilibrium of phenylalanine racemization

Reaction mixture was 10 times larger than the one as described in the text. 4.5 mg protein of calcium phosphate gel fraction were used. 300 µl portions were withdrawn at times indicated and L-phenylalanine remaining and D-phenylalanine formed were determined as described in the method section.

Time (min)	L-Phenylalanine remaining (mµmoles)[1)]	(%)	D-Phenylalanine formed (mµmoles)[1)]	(%)
0	23.6	94	0.3	1
15	16.5	66	8.1	32
30	11.5	46	12.8	51
60	8.2	33	16.6	66
120	7.1	28	17.4	70
180			17.4	70
240	7.5	30	18.2	73

1) The value was expressed as the amount of phenylalanine per 0.5 ml of the reaction mixture.

TABLE VIII-I

Racemic equilibrium of phenylalanine racemization

Reaction mixture was 10 times larger than the one as described in the text. 0.5 mg of enzyme of Fraction VIII was used. At 63 minutes, 0.125 mg of enzyme protein was futher added. 300-µl portions of the reaction mixture were withdrawn at the indicated times. L-Phenylalanine-U-^{14}C (5 µc/µmole) was used.

Time (min)	L-Phenylalanine remaining (mµmoles)[1)]	(%)	D-Phenylalanine formed (mµmoles)[1)]	(%)
0	26.7	91.0	0.35	1.4
15	15.3	61.4	8.4	33.7
30	8.4	33.5	15.6	62.3
60	4.8	19.4	22.0	88.2
120	4.9	19.5	20.8	83.1
180	4.1	16.4	21.1	84.6
240	5.0	20.2	21.4	85.5

1) The value was expressed as the amount of phenylalanine per 0.5 ml of the reaction mixture.

TABLE VIII-II

Racemic equilibrium of phenylalanine racemization

The assay condition was the same as described in TABLE VIII-I except that D-phenylalanine-3-^{14}C (10 μc/μmole) was used as substrate.

Time (min)	D-Phenylalanine remaining (mμmoles)[1)]	(%)	L-Phenylalanine formed (mμmoles)[1)]	(%)
0	24.7	97.4	0.65	2.6
15	23.9	89.9	2.7	10.1
30	22.8	88.2	3.0	11.8
60	20.2	83.5	4.0	16.5
120	19.2	78.5	5.3	21.5
180	19.9	82.0	4.4	18.0
240	18.8	77.6	5.5	22.5

1) The value was expressed as the amount of phenylalanine per 0.5 ml of the reaction mixture.

These results indicate that the ratio of D/L-phenylalanine at equilibrium of the reaction changes with the assay conditions, that is, the initial velocities of D- and L-phenylalanine formation which are dependent mainly on the concentrations of thiol compound and the pH of the buffer.

10. Substrate Specificity of Phenylalanine Racemase

The phenylalanine racemase catalyzed only the conversion of L-phenylalanine to the D-isomer and vice versa. L-Tryptophan, L-tyrosine, L-valine, and L-leucine were not converted to the D-isomers by the enzyme (Table IX). The enzyme seems to be specific for phenylalanine.

11. Fate of ATP during the Reaction of the Racemization

The hypothetical reaction mechanism of ATP-dependent phenylalanine racemase described in "INTRODUCTION" suggests that the amount of ATP will not decrease with the reaction of phenylalanine racemization. To elucidate the reaction mechanism, it was examined whether ATP would be consumed during the reaction of phenylalanine racemization or not . Table X presents evidence that 1 molecule of AMP was formed with 1 molecule of D-phenylalanine formation. The data are consistent with the assumption that ATP is used to activate the phenylalanine to the activated phenylalanine such as phenylalanyl adenylate which is to undergo the racemization.

TABLE IX

Substrate specificity for the racemization reaction

The reaction mixture was the same as described in the text except for 25 mμmoles of each of the following amino acid used as a substrate; L-phenylalanine-U-^{14}C (5 μc/μmole), L-tryptophan-3-^{14}C (10 μc/μmole), L-tyrosine-U-^{14}C (11.8 μc/μmole), L-valine-U-^{14}C (5 μc/μmole) and L-leucine-U-^{14}C (5 μc/μmole). 18.8 μg of Fraction VIII were used.

Substrate	D-Isomer formed (mμmoles/30 min)
L-Phenylalanine	6.48
L-Tryptophan	0.00
L-Tyrosine	0.00
L-Valine	0.00
L-Leucine	0.00

TABLE X

Fate of ATP during the racemase reaction

Reaction mixture contained in a total volume of 0.5 ml the followings: 25 µmoles of TEA-HCl buffer (pH 8.6), 0.572 µmoles of ATP (517,920 cpm), 1 µmole of $MgCl_2$, 0.5 µmole of EDTA (pH 7.0), 25 µmoles DTT, 0.5 µmole of PPi, with and without 225 mµmoles of L-phenylalanine-U-^{14}C (103,000 cpm) , 2.5 µmoles of KF, and 250 µg of enzyme protein of Fraction VIII. Reaction time was 30 minutes. D-Phenylalanine formed and the amount of ATP, ADP and AMP were determined as described in the method section.

Conditions	Input ATP (mµmoles)	Adenine nucleotides formed ATP (mµmoles)	ADP (mµmoles)	AMP (mµmoles)	Recovery of nucleotide (%)	D-Phe formed (mµmoles)
Without Phe	572	480.8	24.9	1.8	88.8	
Without Enzyme	572	486.2	26.4	6.6	90.8	
mean:	572	483.5	25.7	4.2		
Complete	572	433.9	31.3	52.2	91.9	46.6
Sum:	572	49.6	5.6	48.0		46.6
Ratio:		ATP / D-Phe	ADP / D-Phe	AMP / D-Phe		
		1.06	~~0.01~~ 0.12	1.03		

12. Effect of Inhibitors of Pyridoxal Phosphate

As described previously, phenylalanine racemase requires ATP for its action, whereas other racemases are known to require pyridoxal phosphate, or FAD, or both for their action. Thus the effect on phenylalanine racemase of various inhibitors of pyridoxal phosphate-containing enzymes was studied. As shown in Table XI, the presence of the inhibitors did not have any inhibitory effect on this reaction. Therefore, it seems unlikely that pyridoxal phosphate is involved in the phenylalanine racemase reaction.

TABLE XI

Effect of inhibitors of pyridoxal phosphate-enzyme on phenylalanine racemase reaction.

Incubation time was 30 minutes at 37° C. Before the reaction was started by the addition of L-phenylalanine, preincubation was carried out for 15 minutes at 37° C with the various inhibitors. 0.225 mg of enzyme protein of the calcium phosphate gel fraction was used.

Additions (M)	D-Phenylalanine formed (mμmoles)
None	4.54
Phenylhydrazine 2×10^{-3}	3.20
Isonicotinylhydrazide 2×10^{-3}	4.22
Hydroxylamine 2×10^{-3}	4.70
Cysteine 2×10^{-3}	3.57
KCN 2×10^{-3}	4.27

D. Properties of Phenylalanine Activation Reaction

In the previous section III C 11 it was shown that phenylalanine was activated by ATP to undergo the racemization. Thus I examined some properties of the activation reaction of phenylalanine as the first step responsible for the racemization of phenylalanine. It is described here that some of the properties of activation reaction is separated from that of the overall racemization reaction of phenylalanine.

Activation reaction was measured by phenylalanine-dependent ATP-PPi exchange reaction.

1. Time Course

The ATP-PPi exchange reaction proceeded linearly up to 20 minutes with L- or D-phenylalanine (Fig. 14). The velocity of the exchange reaction dependent on D-phenylalanine was 60 to 70 % that dependent on L-phenylalanine. When both the D- and L-phenylalanine were simultaneously present in the reaction mixture, the enzyme catalalyzed the exchange reaction at a rate slightly lower than that dependent on L-phenylalanine alone. This result indicates that a single protein is responsible for both L- and D-phenylalanine activations.

Phenylalanine-dependent AMP-ATP exchange reaction was not detected under the same conditions (Fig. 15).

Fig. 14. Influence of incubation time on the ATP-PPi exchange reaction.

The experimental conditions were described in the method section, 0.5 ml of the reaction mixture was incubated and 100-µl aliquots of the reaction mixture were withdrawn at the indicated times. Non adsorbable P^{32} on the charcol was determined as described in the text. The value of the exchange reaction was expressed as the amount of PPi^{32} formed per 0.5 ml reaction mixture. 6.28 µg of Fraction VIII were used.

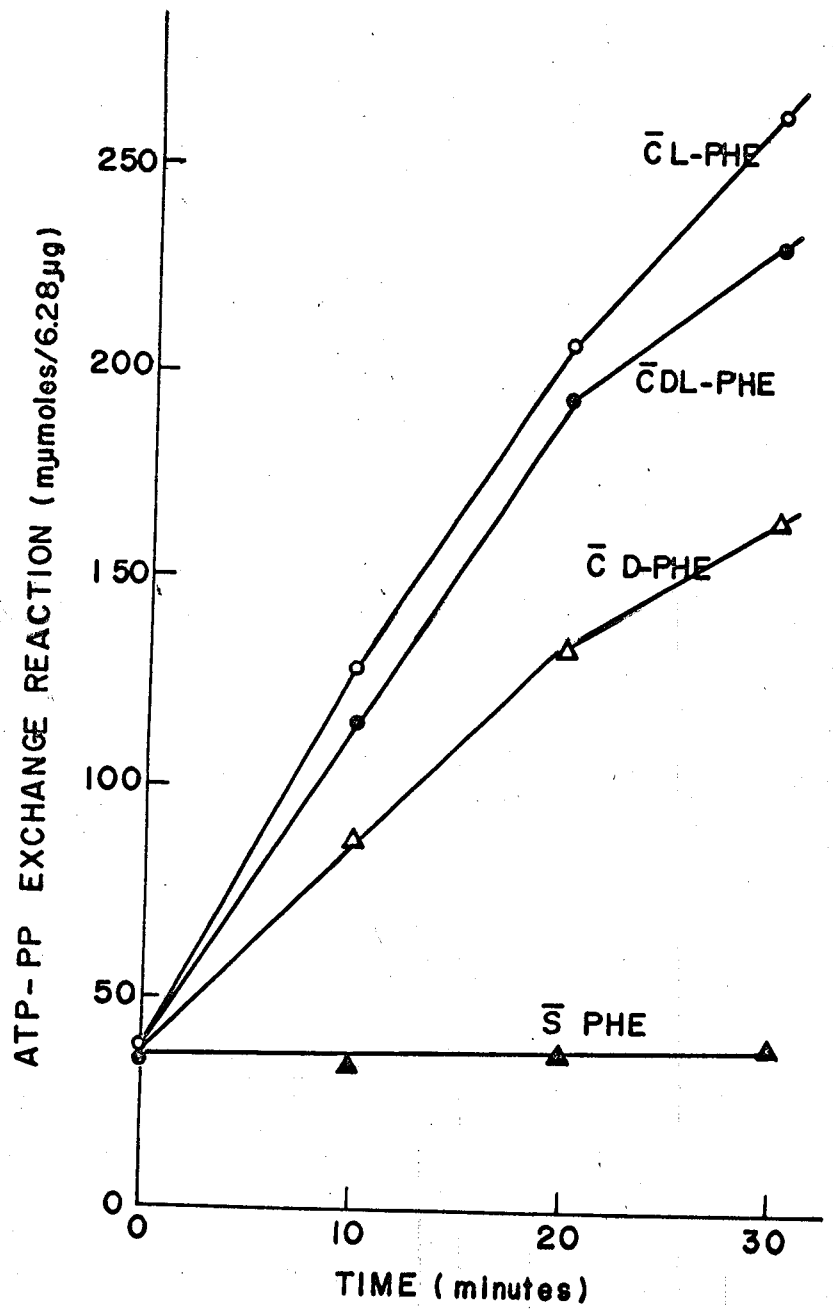
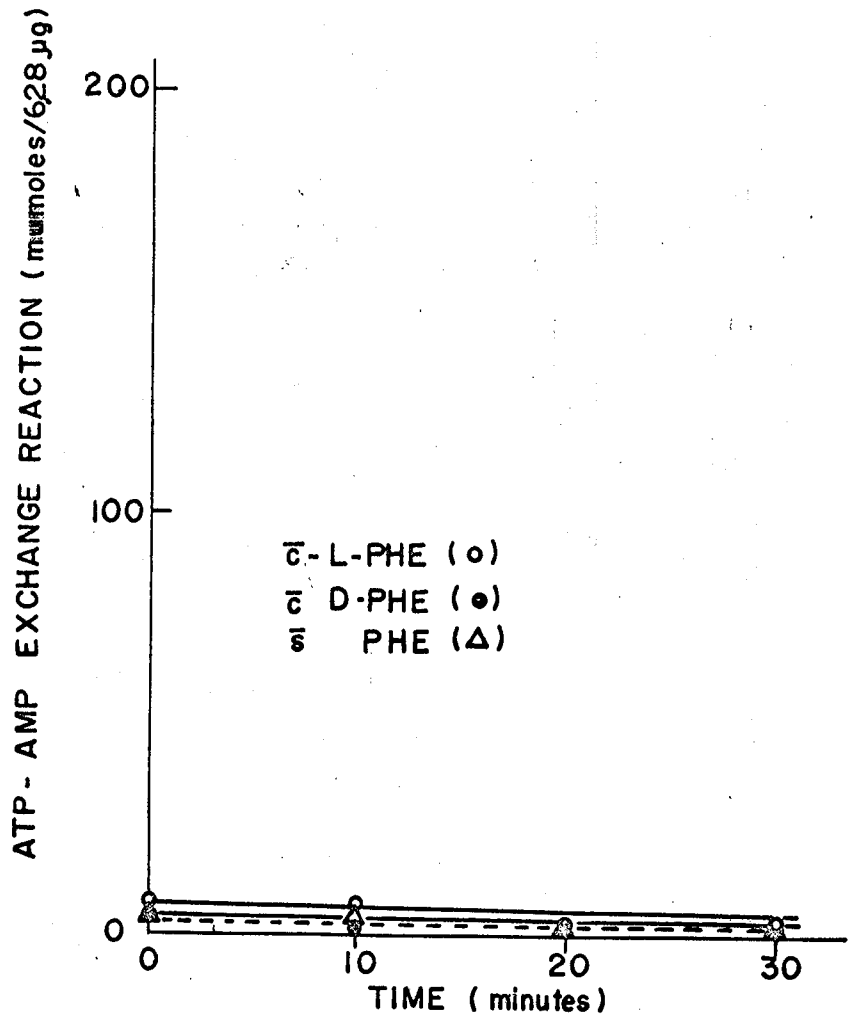

ATP-PP EXCHANGE REACTION (mμmoles/6.28μg)
250
200
150
100
50
0
0
10
20
30
TIME (minutes)
C̄ L-PHE
C̄DL-PHE
C̄ D-PHE
S̄ PHE

Fig. 15. Absence of AMP-ATP exchange reaction.

See the method section for the experimental details. The value of the exchange reaction was expressed as the amount of ATP-^{14}C formed per 0.5 ml reaction mixture. Fraction VIII (6.28 µg) was used.



2. Effect of D- and L-Phenylalanine Concentration

As the concentration of L- and D-phenylalanine was increased, the initial velocity of the exchange reaction was increased and the maximum reaction velocity was attained at 5×10^{-4} M phenylalanine (Fig. 16). The Km value for phenylalanine was determined from the Lineweaver-Burk plot. The values of Km for L-phenylalanine and D-phenylalanine were 6.3×10^{-5} M, and 2.2×10^{-4} M, respectively. The value of Km for the D-isomer was 3.5 times as large as that for the L-isomer. In addition, the value of Km for the L-isomer in the exchange reaction is the same magnitude as that for the L-isomer in the racemase reaction.

3. Effect of pH

The velocity of the reaction dependent on L-phenylalanine was plotted against the pH of the buffers used. It is seen from Fig. 17 that the reaction had a optimum pH between pH 7.4 and pH 7.8 with TEA-HCl buffer, wherease the enzyme catalyzed optimally the reaction between pH 6.2 and 7.2 with K-PO_4 buffer.

It is noticed that an optimum pH of the activation reaction is different from that of the overall conversion of L-phenylalanine to the D-isomer under routine assay conditions (pH 8.6).

Fig. 16. Effect of D- or L-phenylalanine concentration on the ATP-PPi exchange reaction.

The assay conditions were those described in the method section except for D or L-phenylalanine concentration as indicated. 6.28 µg of Fraction VIII were used.

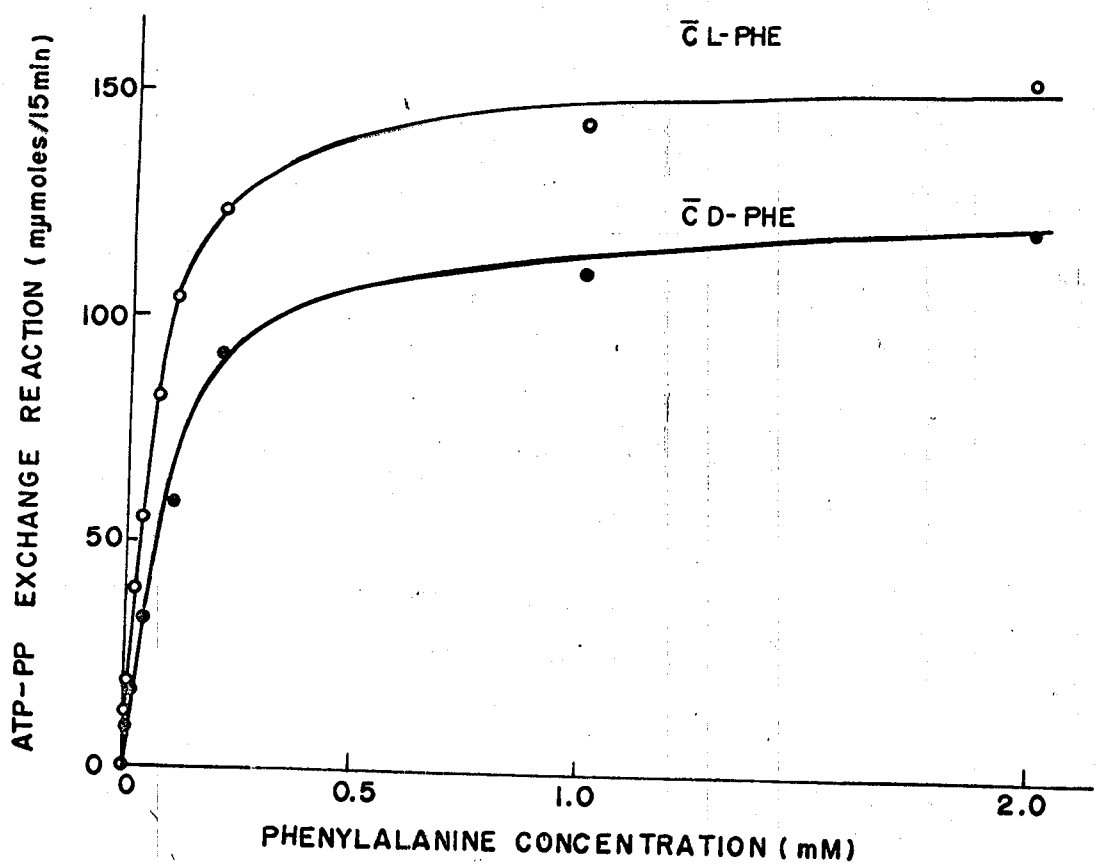
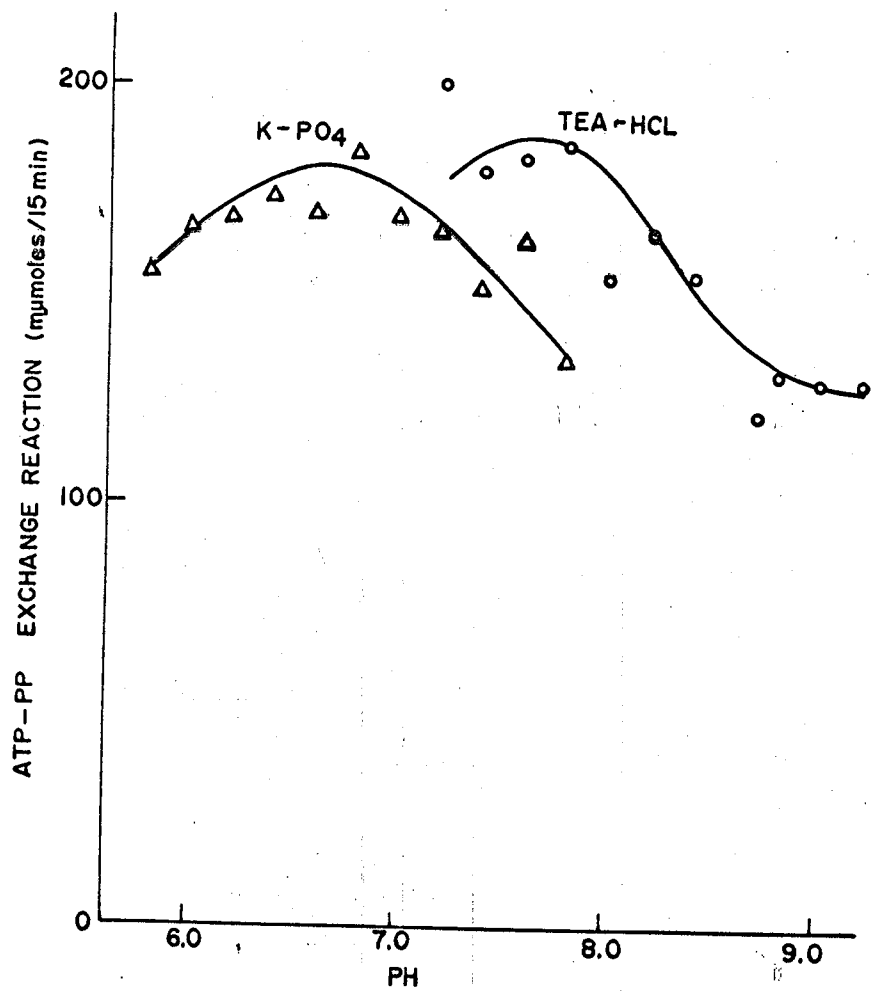


Fig. 17. Effect of pH on the L-phenylalanine dependent ATP-PPi exchange reaction.

The assay conditions were the same as those described in the method section except for TEA-HCl buffer or $K\text{-}PO_4$ buffer as indicated. 6.28 µg of Fraction VIII were incubated.



4. Effect of DTT Concentration

The addition of increasing amounts of DTT did not enhance significantly the exchange reaction (data not presented).

5. Substrate Specificity

Berg et al. reported that DL-p-fluorophenylalanine replaced completely phenylalanine in the synthesis of gramicidin S and that β-thienylserine inhibited the gramicidin S formation as the phenylalanine antagonist.

Fujikawa et al. reported that, of four possitions of the phenylalanine in tyrocidine E, three phenylalanine could replace the other aromatic amino acids, tryptophan and tyrosine. The finding indicates that the activation of three phenylalanine of four is not specific for phenylalanine and that substrate specificity of activating enzyme(s) may be ambiguous with respect to aromatic amino acids, phenylalanine, tyrosine and tryptophan. Table XII shows that the purified enzyme is highly specific for L- and D-phenylalanine and their analogues: DL-p-fluorophenylalanine, β-2-thienylalanine, and DL-phenylserine. But the enzyme did not catalyze the exchange reaction dependent on benzoic acid, phenylacetic acid, L-tryptophan, L-tyrosine, L-proline, L-valine, L-ornithine and L-leucine.

TABLE XII

Substrate specificity of amino acid-dependent ATP-PPi exchange reaction

The assay conditions were that described in the method section except for the substrate indicated. 6.25 µg of protein at Fraction VIII step were used.

Substrate	(µmoles)	PPi formed (mµmoles/15 min)
L-Phenylalanine	1	153
D-Phenylalanine	1	133
DL-Phenylalanine	2	106
DL-P-Fluorophenylalanine	2	55
β-2-Thienylalanine	2	59
DL-β-Phenylserine	2	42
Benzoic acid	1	-3
Phenylacetic acid	1	-3
L-Tryptophan	1	-3
L-Tyrosine	0.4	3
L-Proline	1	-2
L-Valine	1	-3
L-Ornithine	1	-3
L-Leucine	1	0

E. Evidence for the Identical Entity of two Activities: the Activation Reaction and the Racemization Reaction

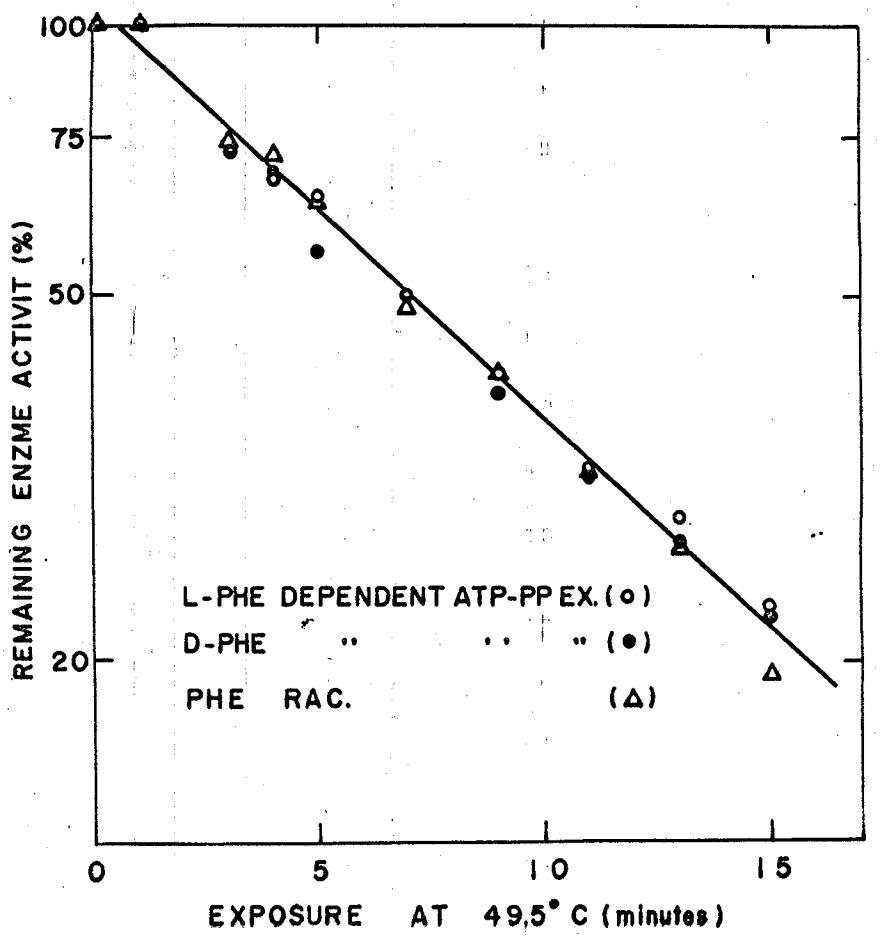
In the preceding sections, it was noted that activation reaction of phenylalanine was involved in the racemase reaction. It is helpful to the understanding of the reaction mechanism to know whether or not the activation step and the overall racemization reaction are catalyzed by a single enzyme protein. The following several lines of evidence indicate that the activation reaction of D- and L-phenylalanine is a constituent step in the series of reaction leading to racemization, which are catalyzed by a single enzyme

The enzyme, which was proven to be homogenous as judged by ultracentrifugation, disc electrophoresis and chromatography, catalalyzes both phenylalanine-dependent exchange reaction and racemization reaction.

Fig. 2 showed that three enzyme activities sedimented with a superimposable single peak on a sucrose density gradient and that the ratio of three enzyme activities was constant throughout the fractions.

When the enzyme was exposed to 49.5° C, the three enzyme activities decrease with time (Fig. 18). The curve followed typical first order kinetics. The inactivation kinetics of the three activities are undistinguishable.

Fig. 18. Inactivation kinetics of phenylalanine racemase and ATP-PPi exchange enzyme. 0.22ml of Fraction VIII consisting of 550 µg of protein, 50 % (v/v) glycerol, 0.02 M K-PO_4 buffer (pH 7.0), 0.2 M KCl and 1 mM DTT was diluted to 2.2 ml by the addition of 1.98 ml of the solution containing 0.1 M KCl, 0.02 M K-PO_4 buffer (pH 7.0) and 1 mM EDTA (7.0). The test tube containing the diluted enzyme solution was immersed into a 49.5° C water bath. After equilibration for 30 seconds, the sampling started. Every 200-µl aliquot was withdrawn at times indicated and quickly chilled at 0° C. The heated aliquots were then assayed for the three activities of racemase and D- and L-phenylalanine-dependent ATP-PPi exchange reaction as described in the method section.

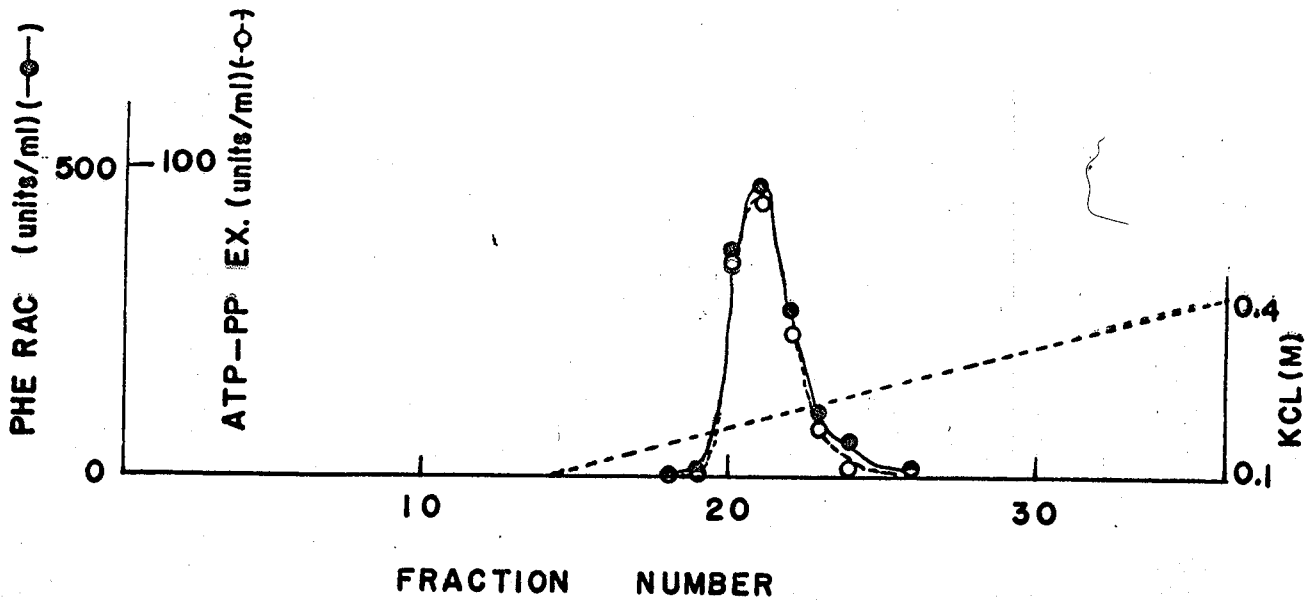


The enzyme was subjected to TEAE-cellulose chromatography and L-phenylalanine-dependent ATP-PPi exchange and the racemase activities were determined on the eluates. It is noted from the elution profiles in two activities that there was virtual coincidence of two activities (Fig. 19).

It follows from these findings that phenylalanine racemase activity and L- and D-phenylalanine activating abilities reside in a single enzyme entity.

Fig. 19. Cochromatography of phenylalanine racemase and L-phenylalanine-dependent ATP-PPi exchange activities on TEAE-cellulose.

23 mg protein of Fraction VIII were placed on the column (1.3 x 10.5 cm) of TEAE-cellulose which was previously equilibrated with 0.05 M KCl containing 0.02 M Tris-HCl buffer (pH 7.4), 20 % (v/v) glycerol, 1 mM EDTA and 5 mM 2-mercaptoethanol. The column was then washed with 24 ml of the starting solution and with 42 ml of 0.1 M KCl in the same buffer system. The protein was eluted with a linear gradient between 75 ml of 0.1 M KCl in the above buffer system and 75 ml of 0.4 M KCl in the above buffer solvent system. A flow rate was 24 ml/ hour, and 6.1 ml fraction, each, was collected in a time-regulated fraction collector. Fractions were assayed for the respective activities by the usual methods. The elution profile of protein agrees with both of the enzyme activities.


PHE RAC (units/ml)(—●—)
ATP—PP EX. (units/ml)(-○-)
500
100
0
10
20
30
0.4
0.1
KCL (M)
FRACTION NUMBER

F. Formation of Phenylalanine Racemase during the Growth of B. brevis cells

When the activity of phenylalanine racemase was followed during the growth, the racemase began to appear at the middle of the logarithmic phase of growth and increased towards the end of the logarithmic phase. The activity was maintained at its maximum for 1 to 1.5-hour period and disappeared rapidly when the cells entered the stationary phase as shown in Fig. 20. The changes in D- and L-phenylalanine-activating activities during the growth paralleled to that in phenylalanine racemase. The results of measurement of gramicidin S synthesizing activity and gramicidin S production during the growth are also included in the figure.

The parallel appearance and disappearance of the racemase and the gramicidin S synthesizing system during the growth resulted in one of the indications that the racemase might be closely related with the biosynthesis of the antibiotic.

When the calcium phosphate gel fraction purified from the crude extract of the cells at the late logarithmic phase was mixed with the crude extract of the cells at the stationary phase, the enzyme activity of the gel fraction was not inhibited by the addition of the latter extracts (Table XIII). This finding indicated the absence of inhibitors in the extracts of

latter cells or activators in the former cells for the reaction of the phenylalanine racemase. The decrease in the enzyme activities at the stationary phase of the cell growth may be attributed to the degradation or inactivation of the enzyme. Fujikawa *et al.* also reported in *B. brevis* ATCC 8185 the appearance of the tyrocidine synthesizing system at the late logarithmic phase and the abrupt disappearance of the system on entering the stationary phase of the growth (23). It seems to be general phenomena that the formation and breakdown of the antibiotic synthesizing enzymes coordinate with the production of antibiotics.

Fig. 20 Change of activities of phenylalanine racemase, D- and L-phenylalanine activating enzyme, gramicidin S synthesizing enzyme activity, and of gramicidin S production during the growth of B. brevis cells.

B. brevis Nagano was grown on a nutrient broth at 37° C with shaking in a New Brunswick gyratory incubator shaker. The details of the cultivation condition of the cells were described in the method section. At the indicated times, an aliquot of the culture was removed and centrifuged at 8000 x g for 10 minutes. For the preparation of crude extract, cells were suspended in 4 volumes of 0.01 M TEA-HCl buffer, (pH 8.0), containing 0.01 M $MgCl_2$ and disrupted in 22-ml portions for 5 minutes in a 10-kc Kubota sonic oscillator. After sonication, the extract was centrifuged at 105,000 x g for 60 minutes to remove the intact cells, cell debris, and the particulate fraction. The supernatant at 105,000 x g was used as an enzyme source for the assay of the various enzyme activities. For the determination of the amount of gramicidin S in the cells gramicidin S was extracted from the cells with ethanol and bioassayed. The growth of bacteria was followed by determining the increase of the turbidity in a Klett-Summerson photoelectric colorimeter with a No. 66 filter. The asaay

conditions of phenylalanine racemase were the same as those described in Table III. D- and L-Phenylalanine activating activity was determined by measuring the amount of the hydroxamic acid formed. The gramicidin S-synthesizing activity was assayed according to Tomino et al. (17).

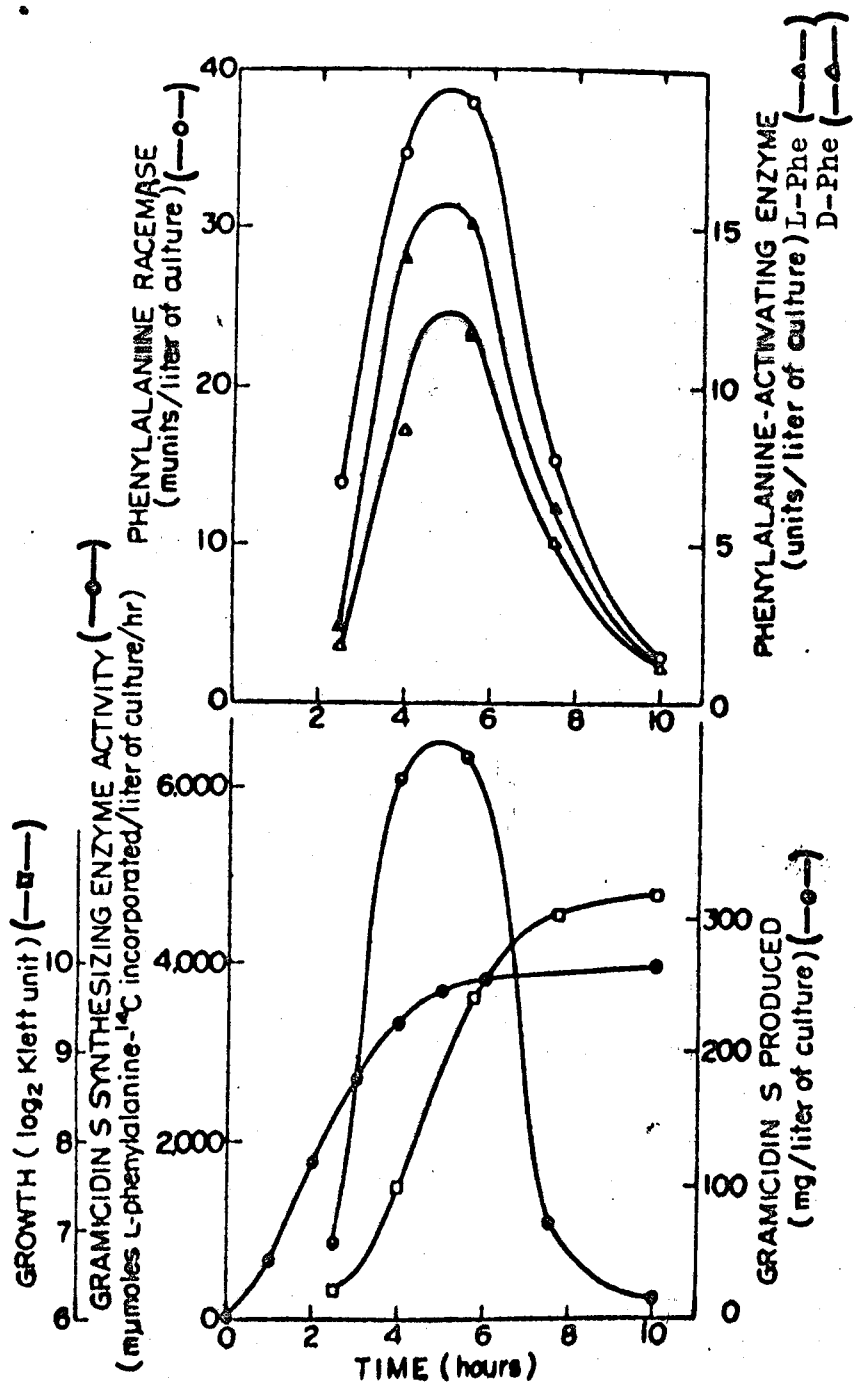


TABLE XIII

Mixing of extract of 4-hr old culture with crude extract of 10-hr old culture

The reaction mixture was the same as described in the Table III except that indicated fractions were added and KF (2.5 µmoles) was used. Reaction time was 30 minutes.

Enzyme fraction	D-Phenylalanine formed (mµmoles/30 min)
Crude extract of 10-hr old culture (1.6 mg of protein)	0.11
Extract[1] of 4-hr old culture (0.225 mg)	4.77
Boiled crude extract of 10-hr old culture plus extract[1] of 4-hr old culture	4.55
Crude extract of 10-hr old culture plus extract[1] of 4-hr old culture	4.60

1) Calcium phosphate gel fraction purified from the crude extract of 4-hr old culture

G. Occurrence of Phenylalanine Racemase in Other Strains Producing Gramicidin S and Tyrocidines

As described in INTRODUCTION, gramicidin S and tyrocidine contain D-phenylalanine residues in their molecules. The survey study on the distribution of ATP-dependent phenylalanine racemase was undertaken by routine measurement in the presence and absence of ATP.

It is seen from Table XIV that B. brevis ATCC 9999 producing gramicidin S and B. brevis ATCC 8185 producing tyrocidine possessed the ATP-dependent phenylalanine racemase. Each of enzyme preparation did not catalyze any formation of D-phenylalanine from the L-isomer in the absence of ATP and $MgCl_2$. In both cases, inorganic pyrophosphate stimulated appreciably the reaction. The amounts of phenylalanine racemase activity catalyzed by two kinds of preparations are comperable to the amount of gramicidin S forming activity, and tyrocidine forming activity, respectively.

The data are compatible with the fact that the phenylalanine racemase is involved in gramicidin S-and tyrocidine-synthesizing systems.

TABLE XIV

Occurrence of phenylalanine racemase in B. brevis ATCC 9999 producing gramicidin S and B. brevis 8185 producing tyrocidine

The reaction mixture was the same as described in the text except that 25 µmoles of TEA-HCl (pH 7.8), 10 µmoles of KF and the indicated enzyme fraction were used. Incubation time was 30 minutes.

Exp.	Enzyme source	Omissions and additions	D-Phenylalanine formed (mµmoles/30 min)
I	Crude extract of B. brevis 9999 (1.5 mg of protein)	none	5.70
		minus PPi	2.90
		" ATP	0.00
		" $MgCl_2$	0.00
II	1 st Ammonium sulfate fraction of B. brevis 8185 (0.63 mg of protein)[1]	none[2]	4.85
		minus ATP	0.07
		minus $MgCl_2$	0.31
		plus PPi	
		, 0.01 µmole	5.37
		, 0.05 "	4.21
		, 0.50 "	1.26
		none[3]	3.13
		plus PPase, 2.4 units[3]	0.48

1) Equivalent to the amount of enzyme synthesizing 2.17 mµmole of tyrocidine in 30 minutes at 30° C

2) Essentially the same as in "Exp. I" except for the omissions of KF and PPi

3) Enzyme was preincubated with and without inorganic pyrophosphatase (PPase) for 15 minutes at 37° C. Then, the reaction was started by the addition of other ingredients.

H. Role of the Phenylalanine Racemase in Gramicidin S- and Tyrocidine-Syntheses

The following lines of evidence suggested the functional association of phenylalanine racemase with gramicidin S biosynthesis: (1) ATP-dependent phenylalanine racemase is present in B. brevis Nagano, 9999 and 8185; (2) phenylalanine racemase formation is closely related with the formation of gramicidin S-synthesizing system. (3) Gramicidin S-synthesizing system is separated into the two complementary fractions, I and II. The fraction II contains only L- and D-phenylalanine activating enzyme activity and the other is responsible for that activation of the rest of the constituent amino acids (40).

However, the direct involvement of phenylalanine racemase in gramicidin S-synthesizing system should come to be proven.

The purified racemase preparation, the separation of gramicidin S-forming system into the fractions, I and II and the resolution of tyrocidine-synthesizing system into the two components, I and II promoted to reconsitute the antibiotic synthesizing system from the two complementary fractions or components and the racemase.

It is seen from Table XV and XVI that the racemase purified from the gramicidin S producing bacteria could replace completely

the fraction II for the gramicidin S synthesis and the component I responsible for the biosynthesis of tyrocidine. Phenylalanine racemase and each of the two fractions for gramicidin S synthesis and for tyrocidine synthesis alone did not catalyze anything of gramicidin S and tyrocidine. The combined system consisting of the racemase from B. brevis Nagano and of the component II from B. brevis ATCC 8185 did catalyze tyrocidine formation, but not gramicidin S. The product was tyrocidine which was verified by paper electrophoresis of the product with gramicidin S and tyrocidine as markers. In the abscence of glutamine in the reaction mixture, no incorporation of L-leucine-^{14}C was observed, indicating also that the product is tyrocidine.

TABLE XV

Replacement of fraction II by the phenylalanine racemase for gramicidin S synthesis

The assay conditions were that described in the text except for the enzyme fraction indicated; fraction I (31.1 μg), fraction II (30.3 μg) and racemase (Fraction VIII 6.3 μg).

Enzymes	L-Phenylalanine-U-^{14}C incorporated into gramicidin S (μμmoles/30 min)
Fraction I and fraction II	229
Fraction I and racemase	220
Fraction II and racemase	24
Fraction I	5
Fraction II	10
Racemase	7.5

TABLE XVI

Replacement of component I by the phenylalanine racemase for tyrocidine synthesis

The assay conditions were the same as described in the text except for the enzyme indicated; component I (DEAE-fraction), 450 µg of component II (hydroxylapitite) and 25 µg of phenylalanine racemase (Fraction VIII) purified from B. brevis Nagano. Incubation time was 30 minutes at 30° C.

Enzymes	L-Leucine incorporated into tyrocidine (mµmoles/30 min)
Component II	0.06
Component I and component II	4.26
Component II and racemase	4.77
Component II and racemase without glutamine	0.26

IV. DISCUSSION

A. Participation of Phenylalanine Racemase in Gramicidin S Synthesis and Tyrocidine Synthesis

Many studies were undertaken in an effort to understand the formation of D-amino acids contained in antibiotics. Any information, however, have not been obtained about the biochemical formations of D-amino acids coupled with the biosynthesis of antibiotics.

The discovery of the existence of the ATP-dependent phenylalanine racemase in the gramicidin S producing bacteria B. brevis Nagano and ATCC 9999 and the tyrocidine producing B. brevis ATCC 8185 have shed light on the understanding of the biochemical aspect of the D-amino acids formation in the antibiotics. Conclusive evidence for the participation of the racemase fraction in the syntheses of gramicidin S and tyrocidine was obtained by the recombination of the racemase with each one of the two complementary fractions of the gramicidin S-and tyrocidine-synthesizing systems.

Tyrocidine A and B contain 2 moles of D-phenylalanine in their molecules (See diagram): one of D-phenylalanine is adjacent to L-leucine and the other follows L-phenylalanine. The former is not replaced by other aromatic amino acid, whereas the latter

could be replaced by D-tryptophan as seen in the structure of of tyrocidine C and D. The substitution D-tryptophan for D-phenylalanine and *vice versa* is determined by the relative concentrations of phenylalanine and tryptophan available on the tyrocidine synthesis. As it is known that the racemase from *B. brevis* Nagano have the strict specificity for phenylalanine, but not for tryptophan, the ATP-dependent phenylalanine racemase maybe take part in only the formation of the D-phenylalanine adjacent to L-leucine on the tyrocidine synthesis. An alternative pathway may exist for the formation of the other D-phenylalanine or D-tryptophan.

The amount of the production of gramicidin S is coordinated with the formation of the racemase during the growth of the cells. It is not known whether the amount of both two fractions of the antibiotic forming system is regulated by the biosynthesis and breakdown of the enzyme system or not. The absence of activator of the racemase in the crude extract suggest that there may occur the *de nove* synthesis of the racemase with the increase of the enzyme activity. The absence of the inhibitor of the racemase in bacterial cells at the stationary phase of the growth rises the problem on the mechanism of the abrupt disapperance of the racemase on entering the stationary phase of the growth. The mechanism on the regulation of the biosynthesis

and destruction of gramicidin S synthesizing system remains to be elucidated.

B. Mechanism of ATP-Dependent Phenylalanine Racemase Reaction

For each mole of ATP and L-phenylalanine utilized, a mole of each of AMP, PPi and D-phenylalanine is formed during the racemase reaction. The equation of the overall reaction will be described as follows:

$$\text{L-Phe} + \text{ATP} \xrightarrow[Mg^{++}]{} \text{D-Phe} + \text{AMP} + \text{PPi}$$

The presence of D- and L-phenylalanine-dependent exchange reaction and the absence of D- and L-phenylalanine dependent AMP-ATP exchange reaction indicate that L-phenylalanine is activated to L-phenylalanyl adenylate enzyme complex, and that the activated complex is not a pyrophosphorylated compound. The activation reaction in the racemase reaction is specific for both the optical isomers of phenylalanine, which is probably catalyzed by the same site on the racemase.

It is assumed that the racemase reaction proceeds as follows:

the 1st reaction; L-phenylalanine is activated to L-Phe•AMP•Enz complex.

the 2nd reaction; L-phe AMP Enz complex is converted to D-Phe•AMP•Enz complex.

the 3rd reaction; D-Phe•AMP•Enz complex is broken down

to free D-phenylalanine, AMP and enzyme.

The 2nd or 3rd reaction will be enhanced by DTT at higher concentration (0.05 M to 0.1 M) and of the alkaline range of pH (pH 8.2 to pH 8.5) or by the effects of the co-operative interactions of PPi and AMP on the state of Phe•AMP•Enz. complex. In the presence of PPi and AMP, in which the formation of gramicidin S is inhibited, the racemase activity is markedly enhanced, while in the absence of PPi and AMP, in which the formation of gramicidin S proceeds optimally, the racemase reaction does not proceed. The data are compatible with the assumption that the activated D-Phe, AMP, Enz.• complex is non-enzymatically released with the formation of D-phenylalanine and AMP by the artificial factors described above. If the other one of the two complementary fractions is accessible to the utilization of the D-phenylalanyl•AMP•Enz. complex in the presence of the other constituent amino acids and ATP, D-phenylalanine of the activated complex is incorporated directly into gramicidin S of tyrocidines.

V. SUMMARY

In "INTRODUCTION" the developement of the biosynthesis of D-amino acid in conjuction with the biosynthesis of the antibiotics was described.

Recent developement concerning the ATP-dependent phenylalanine racemase, the mechanism of the enzyme reaction and its relation to the antibiotic biosynthesis was also discussed.

The phenylalanine racemase was purified to a near homogeneous state from B. brevis Nagano. The study on the properties of the enzyme has revealed that the racemase reaction consists of the activation reaction of phenylalanine and the subsequent racemization of the activated phenylalanine complex and its breakdown to free phenylalanine. The racemase reaction of D-phenylalanine fromation from the L-isomer is activated by the higher concentration of DTT and the alkaline buffer or by the simultaneous presence of PPi and AMP, and D-phenylalanine formation is not affected, while the activation reaction responsible for the racemization of phenylalanine is not affected appreciably by DTT and the alkaline buffer.

The D/L-phenylalanine ratio at equilibrium of the reaction is dependent on the initial velocity of D-phenylalanine formation which is markedly affected by the variable of the DTT concentration

and pH of buffer or by the variable of AMP, PPi and the both.

The racemase is specific for both optical isomers of phenylalanine and the phenylalanine analogues. L-Tryptophan, L-tyrosine, L-leucine, L-valine, and L-ornithine do not serve as substrate.

In the study of the distribution of the ATP-dependent phenylalanine racemase, it was found that the gramicidin S producing bacteria, B. brevis Nagano and B. brevis ATCC 9999 and the tyrocidine producing bacterium, B. brevis ATCC 8185 possessed the enzyme activity.

The racemase has the direct relations with the biosynthesis of gramicidin S and tyrocidine. The racemase could completely replace the one of the two complementary fractions or components responsible for gramicidin S or tyrocidine biosyntheses, respectively.

The mechanism of action of this enzyme and the substrate specificity were discussed with its relation to the biosyntheses of gramicidin S and tyrocidines.

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