THE ACTION OF *PSEUDOMONAS FLUORESCENS* EXTRACTS ON ASPARAGINE AND ASPARAGINE DERIVATIVES

N. DE GROOT AND N. LICHTENSTEIN

Department of Biological Chemistry, The Hebrew University, Jerusalem (Israel)

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SUMMARY

Cell-free extracts from *Pseudomonas fluorescens* hydrolyzed L- and D-asparagine, L- and D-β-aspartohydroxamic acid and α-N-alkyl derivatives of DL-asparagine, but failed to attack L- and D-N-(β-aspartyl)-alkylamines. The extracts also catalyze the formation of β-aspartohydroxamic acid from L- or D-asparagine and hydroxylamine and the formation of α-N-alkyl-β-aspartohydroxamic acids from DL-α-N-alkyl derivatives of asparagine and hydroxylamine.

The hydrolysis of D-asparagine, of D-β-aspartohydroxamic acid, of α-N-alkyl derivatives of DL-asparagine as well as the formation of hydroxamic acids from hydroxylamine and D-asparagine or α-N-alkyl derivatives of DL-asparagine were inhibited by α-amino acids. In the cases tested this inhibition proved to be competitive. Heating the extracts at 55° for 8 min caused a complete destruction of all the enzymic activities mentioned above. The presence during this heating of one of the substrates or of one of the amino acids that caused inhibition gave a complete or partial protection of all the above mentioned enzymic activities.

The question whether the enzymic activities mentioned are catalyzed by one or more enzymes is discussed.

INTRODUCTION

In a previous communication the specificity of asparaginase and of β-aspartyl transferase activities of mammalian-liver preparations were studied with the aid of asparagine derivatives bearing various alkyl residues in the amino or in the amide groups[1]. The present paper deals with the specificity of these enzymic activities of bacterial origin (*Pseudomonas fluorescens*), using the same asparagine derivatives.

MATERIALS AND METHODS

Materials

The amino acids were purchased from the Nutritional Biochemicals Corporation, Cleveland, Ohio.

DL-α-N-methyl-, ethyl-, propyl-, butyl-, isopropyl-, ethanolasparagine, N-(β-DL-aspartyl)-methylamine, -ethylamine, -2-aminoethanol, -3-aminopropanol and L- and D-β-aspartohydroxamic acid were prepared as described elsewhere[1-2].

N-(β-DL-aspartyl)-allylamine, N-(β-L-aspartyl)- ethylamine and N-(β-D-aspartyl)-ethylamine were prepared in a way similar to that described for N-(β-DL-aspartyl)-ethylamine. Analyses: N-(β-DL-aspartyl)-allylalamine: Found: N, 16.4; neutral equivalent (LINDERSTRØM-LANG titration), 168. Calculated: N, 16.3; neutral equivalent, 172. N-(β-L-aspartyl)-ethylamine: Found, N, 17.5; neutral equivalent (LINDERSTRØM-LANG titration), 160. Calculated: N, 17.5; neutral equivalent, 160. N-(β-D-aspartyl)-ethylamine: Found: N, 17.4; neutral equivalent (LINDERSTRØM-LANG titration), 158. Calculated: N, 17.5; neutral equivalent, 160.

DL-N-ethylaspartic acid was prepared in the following way: 10 g DL-α-N-ethyl-asparagine were heated for 8 h with 100 ml of hydrochloric acid 1:1. The mixture was evaporated *in vacuo* to dryness with repeated addition of water. The residue was dissolved in 40 ml of water, the pH was adjusted to 3.1 with lithium hydroxyde 2.5 *N* and the *N*-ethylaspartic acid was precipitated by adding 8 volumes of alcohol. The product was crystallized from water–alcohol. Analysis: Found: N, 8.6; neutral equivalent (LINDERSTRØM-LANG titration), 160. Calculated: N, 8.7; neutral equivalent, 161.

Enzyme preparation

The *Pseudomonas fluorescens* used in this study was obtained from the collection of the Department of Bacteriology of the Hebrew University-Hadassah Medical School. The bacteria were grown in Roux bottles at 30° for 40 h containing the following medium: agar, 25 g; peptone, 10 g; sodium chloride, 3 g; potassium dihydrogen phosphate, 3 g; magnesium sulfate (hydrate), 1 g/l of tap-water. The bacteria were harvested, centrifuged, washed twice with sodium chloride 0.9 % and dried *in vacuo* in a desiccator over calcium chloride. The finely powdered dry preparations preserve their activity for several months. Cell-free *Pseudomonas* extracts were prepared in the following way. A suspension of 70 mg of dried bacteria in 20 ml of water was held for 15 min in a 9 kc Raytheon sonic oscillator. After addition of 12 mg of L-asparagine, the mixture was kept for 10 min at 55°, dialyzed overnight against distilled water in the cold, and centrifuged at 15,000 × *g* for 10 min. The clear supernatant was used for enzymic experiments*.

Assay procedures

Hydrolysis of asparagine and its α-N-alkyl derivatives: The reaction mixture contained per ml of 0.045 *M* tris-(hydroxymethyl)-aminomethane buffer, pH 7.5, 6 μmoles of substrate and 0.1 ml of enzyme solution. After incubation for 1 h at 35°, 1 ml of 5 % trichloroacetic acid was added to 3 ml of incubation mixture. After addition of 3 ml of borate buffer pH 10.1[5] and some drops of tributyl phosphate, the ammonia was distilled into 0.1 *N* HCl by aeration at 50° for 10–15 min and estimated colorimetrically with the aid of Nessler's reagent.

Hydrolysis of L- *and* D-*β-aspartohydroxamic acid*: The reaction mixture contained per 1 ml of Tris buffer 0.045 *M*, pH 7.5, 4 μmoles of substrate and 0.1 ml of enzyme solution. After incubation for 1 h at 35°, 2-ml samples were withdrawn. To these samples 1 ml of NH_2OH 2 *M*, pH 6.5 and 3 ml of $FeCl_3$ reagent[6] were added. After

* The heat treatment is necessary to destroy the aspartase activity present in *Pseudomonas* extracts[3,4] which might obscure the results of L-asparagine hydrolysis. The presence of L-asparagine during the heating protects the other enzymic activities described in this paper (see p. 107).

centrifugation the hydroxamic acid was quantitatively measured in a Klett-Summerson colorimeter (filter 54).

β-aspartyl transfer

The reaction mixture contained per 1 ml of Tris buffer 0.030 *M*, pH 7,5, 30 μmoles substrate, 240 μmoles NH_2OH and 0.3 ml of enzyme solution. After incubation for 1 h at 35°, samples of 2 ml were removed and the hydroxamic acid determined as described above.

In several experiments aspartic acid was determined by quantitative paper chromatography. Chromatograms were run on Whatman paper No. 1 with phenol 80 % as solvent. The aspartic acid was eluted from the paper and estimated as described by MEYER[7].

RESULTS

Table I shows that *Pseudomonas* extracts catalyze the hydrolysis of L- and D-asparagine, L- and D-β-aspartohydroxamic acid and DL-α-N-alkyl derivatives of asparagine. They fail to attack N-(β-DL-aspartyl)-methyl- and ethylamine. Methyl and ethylamine added to the reaction mixture in amounts corresponding to those of the substrates gave on aeration recoveries of 75 % and 55 % respectively. Additional evidence for the stability of N-(β-aspartyl)-alkylamines towards the enzyme was obtained by chromatography, which failed to reveal the formation of aspartic acid. When L- or D-asparagine was incubated with the enzyme preparations under the same conditions, aspartic acid could be recovered chromatographically in amounts corresponding fairly well to the amounts of ammonia obtained by aeration.

DL-α-N-methyl-, ethyl-, and ethanolasparagine were hydrolyzed more rapidly than L- or D-asparagine. The rate of hydrolysis of the DL-α-N-alkyl derivatives of

TABLE I

THE ACTION OF *Pseudomonas* EXTRACTS ON ASPARAGINE AND ASPARAGINE DERIVATIVES

Reaction mixtures, as given under *Assay procedures*.

Substrate	*Ammonia formed μmoles*	*Alkylamines liberated μmoles*	*Hydroxamic acid hydrolyzed μmoles*
L-asparagine	2.40		
L-asparagine*	2.40		
D-asparagine	2.25		
DL-asparagine*	2.40		
DL-α-N-methylasparagine	7.30		
DL-α-N-ethylasparagine	3.75		
DL-α-N-propylasparagine	1.45		
DL-α-N-butylasparagine	1.05		
DL-α-N-isopropylasparagine	0.25		
DL-α-N-ethanolasparagine	3.95		
N-(β-DL-aspartyl)-methylamine		0.00	
N-(β-L-aspartyl)-ethylamine		0.00	
N-(β-D-aspartyl)-ethylamine		0.00	
L-aspartohydroxamic acid			1.15
D-aspartohydroxamic acid			1.00

* Concentration, 0.012 *M*.

TABLE II

MAXIMUM HYDROLYSIS OF ASPARAGINE AND ITS α-N-ALKYL DERIVATIVES BY *Pseudomonas* EXTRACTS

Concentration of all substrates 0.0005 *M*. Buffer and enzyme as under *Assay Procedures*.

Substrate	*Ammonia formed μmoles Time (h)*			*Maximum hydrolysis %*
	1	*2*	*3*	
L-asparagine	1.40	1.45	1.45	97
D-asparagine	1.50	1.53	1.53	102
DL-asparagine	1.35	1.42	1.42	95
DL-α-N-methylasparagine	0.72	0.73	0.73	48
DL-α-N-ethylasparagine	0.71	0.70	0.71	47

TABLE III

FORMATION OF HYDROXAMIC ACID FROM ASPARAGINE AND ASPARAGINE DERIVATIVES BY *Pseudomonas* EXTRACTS

Reaction mixtures, as under *Assay procedures*.

Substrate	*Hydroxamic acid formed, μmoles**
L-asparagine	2.50
D-asparagine	5.05
DL-α-N-methylasparagine	17.30
DL-α-N-ethylasparagine	7.30
DL-α-N-propylasparagine	3.60
DL-α-N-butylasparagine	3.05
DL-α-N-isopropylasparagine	0.65
DL-α-N-ethanolasparagine	7.60
N-(β-DL-aspartyl)-methylamine	0.00
N-(β-L-aspartyl)-ethylamine	0.00
N-(β-D-aspartyl)-ethylamine	0.00

* Synthetic aspartohydroxamic acid was used as standard in all cases.

asparagine markedly decreases from the methyl to the butyl derivatives. There is a striking difference in the susceptibility of DL-α-N-propylasparagine and DL-α-N-isopropylasparagine to the action of the enzyme. While the incubation of small amounts of L-, D- and DL-asparagine with the *Pseudomonas* extracts led to nearly 100% hydrolysis, the hydrolysis of DL-α-N-methyl- and ethylasparagine never exceeded 50% (Table II).

The cell-free extracts of *Pseudomonas* catalyze the transfer of the β-aspartyl group from L- and D-asparagine to hydroxylamine. The extracts are also capable of transferring the DL-α-N-alkyl-β-aspartyl groups from the corresponding DL-α-N-alkyl derivatives of asparagine to hydroxylamine, but fail to bring about the transfer of the β-aspartyl group from N-(β-aspartyl)-alkylamines to hydroxylamine. The results are summarized in Table III. Comparative quantitative evaluation was not possible because of the lack of synthetic α-N-alkyl-β-aspartohydroxamic acids.

On incubation of D-asparagine with cell-free *Pseudomonas* extracts and hydroxylamine the concentration of β-aspartohydroxamic acid at first increases linearly. Then the velocity of hydroxamic acid formation falls until the concentration of hydroxamic acid reaches a maximum value and remains constant. Using L-asparagine as substrate,

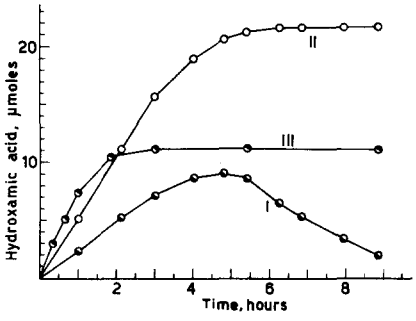

Fig. 1. Time-dependence curves of aspartotransferase activity towards L-asparagine (I), D-asparagine (II) and DL-α-N-ethylasparagine (III). Reaction mixtures as described under *Assay procedures*.

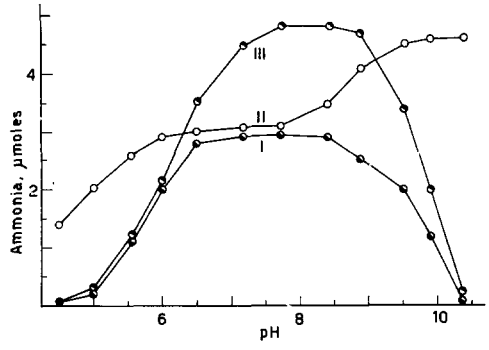

Fig. 2. pH-dependence curves for the hydrolysis of D-asparagine (I), of L-asparagine (II) and of DL-α-N-ethylasparagine (III). The buffers used were citrate–phosphate for pH 4.5 to 7.2 and borate for pH 7.5 o 10.4.

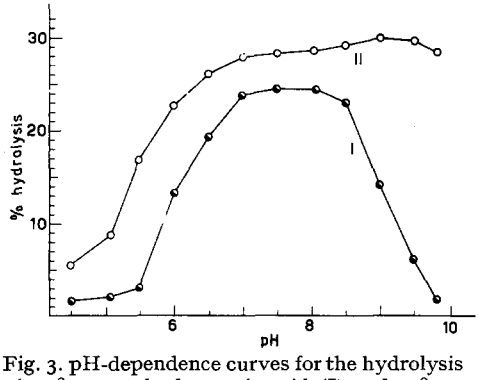

Fig. 3. pH-dependence curves for the hydrolysis of D-β-aspartohydroxamic acid (I) and L-β-aspartohydroxamic acid (II). The buffers used were citrate for pH 4.5 to 6.0, tris–maleate for pH 6.0 to 7.5 and borate for pH 7.5 to 10.0.

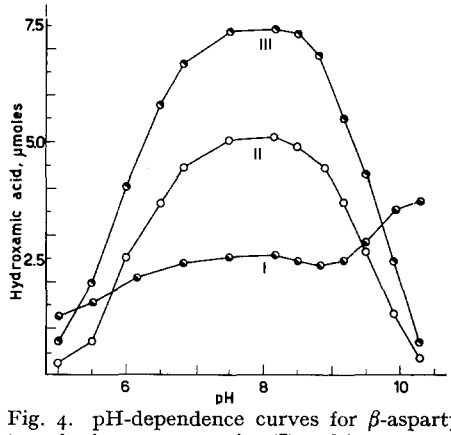

Fig. 4. pH-dependence curves for β-aspartyl transfer from L-asparagine (I) and from D-asparagine (II) to hydroxylamine and α-N-ethyl-β-aspartyl transfer from DL-α-N-ethylasparagine (III) to hydroxylamine. The buffers used were citrate for pH 4.5 to 6.0, Tris–maleate for pH 6.0 to 7.5 and borate for pH 7.5 to 10.0.

the concentration of aspartohydroxamic acid goes through a maximum and then falls to zero (Fig. 1). These results can be explained as follows. Hydroxylamine, in the concentration used in the experiments on β-aspartyl transfer, strongly inhibits the hydrolysis of D-asparagine and D-aspartohydroxamic acid by the extracts, but hardly affects the hydrolysis of L-asparagine and L-aspartohydroxamic acid (Table IV). It follows from Fig. 1, that the hydroxamic acid formed from DL-α-N-ethyl-asparagine was not hydrolyzed under the conditions of the experiments.

Effect of pH

As can be seen from Figs. 2, 3 and 4, the pH-activity curves for the hydrolysis of L-asparagine and L-β-aspartohydroxamic acid and for the L-β-aspartyl transfer are quite similar. There is also a marked similarity between the pH-activity curves for the hydrolysis of D-asparagine and D-aspartohydroxamic acid and for the β-D-aspartyl transfer. There are significant differences between the two groups of pH-activity

TABLE IV

EFFECT OF HYDROXYLAMINE ON HYDROLYSIS OF L- AND D-ASPARAGINE AND OF L- AND D-β-ASPARTOHYDROXAMIC ACID BY *Pseudomonas* extracts

Buffer and substrates, as under *Assay Procedures*. The enzyme concentration was as described under *β-Aspartyl transfer*.

Hydroxylamine concentration	*Hydroxamic acid hydrolyzed, μmoles*		*Aspartic acid formed, μmoles*	
	L-*AHA**	D-*AHA**	L-*asparagine*	D-*asparagine*
0.00 *M*	3.50	3.05	7.20	6.70
0.03 *M*	3.60	1.60	7.10	3.50
0.12 *M*	3.50	0.30	7.00	0.60
0.24 *M*	3.15	0.00	6.60	0.00

* The abbreviation AHA is used for aspartohydroxamic acid.

curves at pH-values below 6 and especially above 9. These differences are not due to the instability of the enzyme at pH-values showing a drop in activity. It was found that enzyme preparations, after being kept at these pH-values under the conditions of the experiment, were again fully active after the pH was adjusted to 7.5.

Figs. 2 and 4 show that the pH-activity curves for the hydrolysis of DL-α-N-ethylasparagine and the α-N-ethyl-β-aspartyl transfer from DL-α-N-ethylasparagine resemble those for the hydrolysis of D-asparagine and D-β-aspartyl transfer.

Effect of substrate concentration on rate of hydrolysis

As can be seen from Fig. 5, the velocity of hydrolysis of D-asparagine and of the DL-α-N-methyl- and ethyl-asparagine is much more dependent on substrate concentration than the velocity of the hydrolysis of L-asparagine. As Fig. 5 shows, the rate of hydrolysis of L-asparagine is virtually independent of substrate concentration over a wide range. K_m values were calculated for D-asparagine, DL-α-N-methyl- and ethyl-asparagine from the slope of the $1/S$ against $1/V$ lines and the points of interception of these lines with the ordinate. The results were: K_m D-asparagine $= 4.8 \cdot 10^{-4}$ mole/l; K_m DL-α-N-methylasparagine $= 7.0 \cdot 10^{-4}$ mole/l; K_m DL-α-N-ethylasparagine =

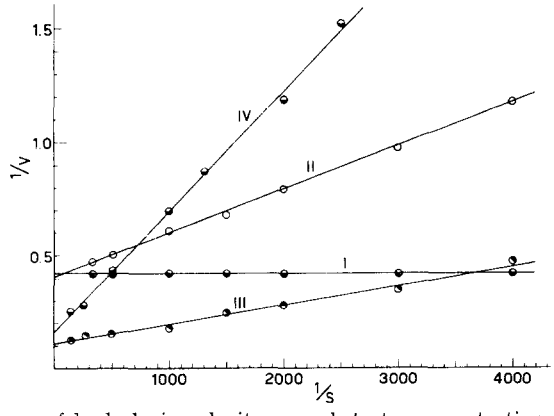

Fig. 5. The dependence of hydrolysis velocity on substrate concentration for L-asparagine (I), D-asparagine (II), DL-α-N-methylasparagine (III) and DL-α-N-ethylasparagine (IV). S is the molar concentration of the substrates. Velocity, V, is expressed in μmoles of ammonia formed/h.

TABLE V

EFFECT OF AMINO ACIDS ON THE RATE OF HYDROLYSIS OF D-ASPARAGINE, D-β-ASPARTOHYDROXAMIC ACID AND DL-α-N-METHYLASPARAGINE BY *Pseudomonas* EXTRACTS

Concentration of all substrates 0.005 *M*. Buffer and enzyme, as under *Assay Procedures*.

Inhibitor	*Concentration*	D-*asparagine hydrolysis,* % *inhibition*	D-*β-aspartohydroxamic acid hydrolysis.* % *inhibition*	*Hydrolysis of* DL-*α-N-methylasparagine,* % *inhibition*
Glycine	0.005	45	55	50
Glycine	0.015	80	85	
L-alanine	0.005	54	50	61
L-alanine	0.015	88	90	
D-alanine	0.005	23	25	30
D-alanine	0.015	50	55	
L-serine	0.005	48	56	55
L-serine	0.015	80	90	
D-serine	0.015	44	52	50
D-aspartic acid	0.005	32	36	35
L-aspartic acid	0.005	78	85	85
D-phenylalanine	0.015	10		
L-phenylalanine	0.015	20	25	25
D-leucine	0.015	12		
L-leucine	0.015	25		
D-glutamic acid	0.015	23	23	25
L-glutamic acid	0.015	53	60	55
β-alanine	0.020	20		
Glycineamide	0.025	0	0	0
Sarcosine	0.025	0	0	0
N-ethyl-DL-aspartic acid	0.025	0	0	0
Proline	0.015	0	0	0
Acetyl-DL-alanine	0.025	0	0	0
N-(β-DL-aspartyl)-methylamine	0.00125	52		
N-(β-DL-aspartyl)-ethylamine	0.00125	52		
N-(β-D-aspartyl)-2-aminoethanol	0.00125	51		
N-(β-DL-aspartyl)-3-aminopropanol	0.00125	50		
N-(β-DL-aspartyl)-allylamine	0.00125	53		
N-(β-L-aspartyl)-ethylamine	0.010	90	90	90
N-(β-D-aspartyl)-ethylamine	0.010	12	15	15

TABLE VI

EFFECT OF AMINO ACIDS ON THE FORMATION OF HYDROXAMIC ACID FROM D-ASPARAGINE AND DL-α-N-ETHYLASPARAGINE BY *Pseudomonas* EXTRACTS

Reaction mixtures, as given under *Assay procedures*.

Inhibitor	*Concentration (M)*	*Formation of hydroxamic acid*	
		From D-*asparagine,* % *inhibition*	*From* DL-*α-N-ethylasparagine,* % *inhibition*
L-alanine	0.045	65	74
L-alanine	0.050	90	90
D-alanine	0.045	30	35
L-serine	0.045	68	78
D-serine	0.045	35	35
L-aspartic acid	0.030	80	90
D-aspartic acid	0.030	40	45
N-(β-L-aspartyl)-ethylamine	0.030	75	80
N-(β-D-aspartyl)-ethylamine	0.030	15	25

$3.2 \cdot 10^{-3}$ mole/l. The K_m value for L-asparagine could not be calculated in this way, but it is obviously much smaller than the K_m values for the other compounds.

Inhibition experiments

A number of α-amino acids tested inhibited the hydrolysis of D-asparagine, D-aspartohydroxamic acid, and the α-N-alkyl derivatives of DL-asparagine, and also inhibited the transfer reaction between D-asparagine or the α-N-alkyl derivatives of DL-asparagine and hydroxylamine. The inhibitory action of the L- and D-forms of several amino acids was compared and it was found in all cases that the L-form acted as a stronger inhibitor than the D-form. The inhibitions obtained with various amino acids are given in the Tables V and VI. These Tables show that α-N- or α-carboxyl-substituted amino acids as well as proline have no inhibitory action whatever. β-alanine inhibited to a markedly lesser degree than α-alanine. N-(β-DL-aspartyl)-alkylamines are powerful inhibitors of the hydrolysis of D-asparagine and D-aspartohydroxamic acid and of β-aspartyl transfer from D-asparagine to hydroxylamine. As can be seen from Table V, all the N-(β-DL-aspartyl)-alkylamines tested inhibited the hydrolysis of D-asparagine to the same extent. Table VII shows that the hydrolysis of L-asparagine and of L-β-aspartohydroxamic acid is comparatively slightly or not at all inhibited by the same amino acids that strongly inhibited the hydrolysis of the D-forms of these substrates.

The reversibility of the inhibition of hydrolysis of D-asparagine, D-aspartohydroxamic acid and of the β-D-aspartyl transfer reactions was tested in experiments with L-alanine and N-(β-L-aspartyl)-ethylamine. Figs. 6, 7 and 8 show that the inhibitions in all these cases were competitive.

K_i values for L-alanine and N-(β-L-aspartyl)-ethylamine were calculated from the data represented in the figures, using the equation: slope of the $1/V$ against $1/S$ line (in the presence of inhibitor) $= K_m/V$ max. $(1 + i/K_i)$ and the K_m value for D-asparagine obtained from Fig. 5. The K_m values for D-β-aspartohydroxamic acid and for D-asparagine in the β-aspartyl transfer reaction were established in separate experi-

TABLE VII

EFFECT OF AMINO ACIDS ON THE RATE OF HYDROLYSIS OF L-ASPARAGINE AND OF L-ASPARTOHYDROXAMIC ACID BY *Pseudomonas* EXTRACTS

Reaction mixtures, as described under *Assay procedures*. The concentration of L-asparagine and L-aspartohydroxamic acid was 0.005 *M*.

Amino acids	*Concentration*	*Hydrolysis of L-asparagine % inhibition*	*Hydrolysis of L-aspartohydroxamic acid % inhibition*
L-aspartic acid	0.005	0	20
L-aspartic acid	0.050	15	68
D-aspartic acid	0.005	0	..
D-aspartic acid	0.050	0	32
L-alanine	0.050	0	27
D-alanine	0.050	0	13
L-serine	0.050	0	26
D-serine	0.050	0	12
N-(β-L-aspartyl)-ethylamine	0.010	5	23
N-(β-D-aspartyl)-ethylamine	0.010	0	0

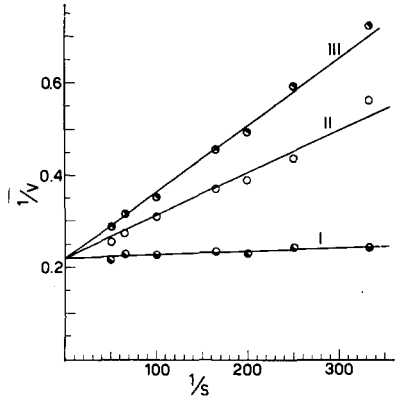

Fig. 6. Competitive inhibition of the hydrolysis of D-asparagine by L-alanine and N-(β-L-aspartyl)-ethylamine. I: D-asparagine; II: D-asparagine and N-(β-L-aspartyl)-ethylamine (0.0005 *M*); III: D-asparagine and L-alanine (0.006 *M*). *S* is the molar concentration of DL-asparagine. Velocity, *V*, is expressed in μmoles ammonia/h.

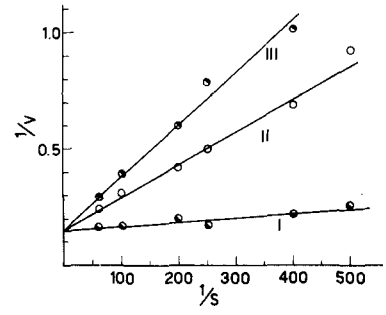

Fig. 7. Competitive inhibition of the hydrolysis of D-β-aspartohydroxamic acid by L-alanine and N-(β-L-aspartyl)-ethylamine. I: D-β-aspartohydroxamic acid; II: D-β-aspartohydroxamic acid and N-(β-L-aspartyl)-ethylamine (0.0005 *M*); III: D-β-aspartohydroxamic acid and L-alanine (0.008 *M*). *S* is the molar concentration of D-β-aspartohydroxamic acid. Velocity, *V*, is expressed in μmoles of hydroxamic acid hydrolyzed/h.

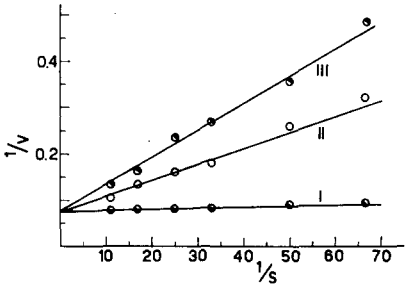

Fig. 8. Competitive inhibition of β-aspartyl transfer from D-asparagine to hydroxylamine by L-alanine and N-(β-L-aspartyl) ethylamine. I: D-asparagine; II: D-asparagine and N-(β-L-aspartyl)-ethylamine (0.0008 *M*); III: D-asparagine and L-alanine (0.015 *M*). *S* is the molar concentration of D-asparagine. Velocity, *V*, is expressed in μmoles of hydroxamic acid formed/h.

ments. The K_i values for L-alanine obtained from Figs. 6, 7 and 8 respectively were $5.0 \cdot 10^{-4}$; $7.0 \cdot 10^{-4}$ and $6.1 \cdot 10^{-4}$. For N-(β-L-aspartyl)-ethylamine the following K_i values were obtained: $6.8 \cdot 10^{-5}$; $7.9 \cdot 10^{-5}$ and $5.5 \cdot 10^{-5}$.

Heating the extracts for 8 min at 55° completely destroys the ability of the extracts to hydrolyze L- and D-asparagine, L- and D-β-aspartohydroxamic acid and the DL-α-N-ethyl derivatives of the asparagine, and to transfer the β-aspartyl group from L- and D-asparagine to hydroxylamine. On heating the extracts for 1 or 2 min at 55° the rate of hydrolysis of L-asparagine, D-asparagine, of L- or D-β-aspartohydroxamic acid decreases almost to the same extent (Table VIII). The presence during the heating (55° for 8 min) of varying amounts of one of the substrates, or of one of the compounds capable of inhibiting the hydrolysis of D-asparagine (see Table V) protects, either partially or completely, all the above-mentioned enzymic activities from inactivation. Heating at 55° for 8 min in the presence of D-asparagine or D-alanine in amounts insufficient to preserve the enzymic activities entirely caused a decrease in the rate of hydrolysis of L-asparagine, D-asparagine, L- and D-β-aspartohydroxamic acid to nearly the same extent (Table VIII).

The influence of several cations on the rate of hydrolysis of L- and D-asparagine was also investigated. It was found that Co^{++} ions, in a final concentration of 10^{-2} *M*, accelerate D-asparagine hydrolysis without affecting the hydrolysis of L-asparagine

TABLE VIII

EFFECT OF HEATING ON THE HYDROLYZING ACTIVITIES OF *Pseudomonas* EXTRACTS

Reaction mixtures, as under *Assay Procedures*.

Treatment of extract	*Ammonia formed µmoles*		*Hydroxamic acid hydrolyzed µmoles*	
	L-*asparagine*	D-*asparagine*	L-*AHA**	D-*AHA**
None	4.80	4.50	2.30	2.00
Heating, 55°; 8 min	0.00	0.00	0.00	0.00
Heating, 55°; 1 min	2.50	2.10	1.20	1.00
Heating, 55°; 2 min	1.35	1.10	0.60	0.50
Heating, 55°; 8 min in the presence of D-alanine (20 µmoles/ml)**	3.50	3.30	1.70	1.40
Heating, 55°; 8 min in the presence of D-alanine 6 µmoles/ml)**	1.80	1.80	0.90	0.85
Heating, 55°; 8 min in the presence of D-asparagine (5 µmoles/ml)**	2.95	2.80	1.40	1.25
Heating, 55°; 8 min in the presence of D-asparagine (3,5 µmoles/ml)**	2.25	2.05	1.10	0.95

* The abbreviation AHA is used for aspartohydroxamic acid.

** After heating in the presence of D-alanine or D-asparagine, the extracts were dialyzed in the cold against distilled water.

TABLE IX

EFFECT OF Co^{++} ON THE HYDROLYSIS OF L- AND D-ASPARAGINE

Reaction mixtures, as described under *Assay procedures*.

Concentration of Co^{++} *(M)*	*Ammonia formed µmoles*	
	L-*asparagine*	D-*asparagine*
0.000	2.60	2.45
0.001	2.65	2.70
0.005	2.55	3.45
0.01	2.60	3.75
0.05	2.50	2.60

(Table IX). This accelerating effect depends on the concentration of the Co^{++} ions. Other cations tested, namely Mn^{++}, Mg^{++}, Zn^{++}, Fe^{++} and Cu^{++}, did not influence the rate of hydrolysis of L- and D-asparagine.

From experiments conducted at different incubation temperatures it can be concluded that the hydrolysis of D-asparagine is much more temperature-dependent than the L-asparagine hydrolysis. At 38° the rates of L- and D-asparagine hydrolysis were practically the same, but at 45° D-asparagine hydrolysis velocity exceeded that of L-asparagine. At 25° L-asparagine is hydrolyzed at a velocity twice as great and at 12° 5 times as great as that of D-asparagine.

DISCUSSION

ALTENBERN AND HOUSEWRIGHT working with extracts from *Brucella abortus* observed a hydrolysis of L-asparagine as well as of D-asparagine[8]. A slight but distinct hydrolysis of D-asparagine by guinea pig serum asparaginase was described by MEISTER *et al.*[9].

In addition to the hydrolysis by *Pseudomonas* extracts as described in the present paper, the hydrolysis of D-asparagine by extracts of a strain of the yeast *Torulopsis utilis* was recently found in our laboratory[10]. It may be recalled that GROSSOWICZ AND HALPERN found that D-asparagine which was not hydrolyzed by extracts from *Mycobacterium phlei* competitively inhibited the hydrolysis of L-asparagine by these extracts[11]. Similarly it was found by KOTT that D-asparagine competitively inhibited the hydrolysis of L-asparagine by certain strains of *Salmonella*[12].

The α-N-alkyl derivatives of DL-asparagine which were found to inhibit the hydrolysis of L-asparagine by mammalian liver asparaginase[1] were readily hydrolyzed by *Pseudomonas* extracts. The observation that the α-N-methyl-, ethyl-, and ethanol DL-asparagine were hydrolyzed by the *Pseudomonas* extracts more rapidly than L- and D-asparagine is of interest. We found that in the non-enzymic hydrolysis carried out at 37° with either N-hydrochloric acid or N-sodium hydroxyde asparagine was hydrolyzed at a more rapid rate than its α-N-alkyl derivatives. α-amino acids inhibited the hydrolysis of D-asparagine and of β-D-aspartohydroxamic acid as well as the β-D-aspartyl transfer from D-asparagine to hydroxylamine. The K_i values obtained from the data given in any one of the Figs. 6, 7 and 8 for L-alanine and N-(β-L-aspartyl)-ethylamine were approximately the same. This, in addition to the similarity of the pH-activity curves for the hydrolysis of D-asparagine, D-β-aspartohydroxamic acid and for the β-D-aspartyl transfer from D-asparagine to hydroxylamine, seems to support the conclusion that the three reactions are catalyzed by the same enzyme[13]. The 3 pH-dependence curves for the 3 corresponding activities towards the L-form are quite similar to each other but are markedly different from those of the D-forms.

It is difficult to decide whether the hydrolysis and transfer reaction of the L-forms and the D-forms are catalyzed by the same enzyme. The possibility that the cell-free *Pseudomonas extracts* contain an isomerase that catalyzes the reaction L-asparagine $\rightleftharpoons$ D-asparagine and one stereospecific asparaginase hydrolyzing either L- or D-asparagine, can be excluded. The fact that in some conditions one can obtain a much more rapid hydrolysis of D-asparagine than of L-asparagine (see Table IX) and that in other conditions the L-asparagine hydrolysis rate greatly exceeds that of D-asparagine is incompatible with this assumption. Moreover, on incubating L- or D-asparagine with *Pseudomonas* extracts containing aspartase, a maximum amount of 1 mole of NH_3 can be obtained from 1 mole D-asparagine, whereas from 1 mole of L-asparagine at least 1.5 moles of NH_3 are liberated. ALTENBERN AND HOUSEWRIGHT observed additive values of ammonia on incubating D- and L-asparagine together with enzyme preparations from *Brucella abortus*[8]. This was regarded as a strong indication that the isomers were hydrolyzed by different enzymes. In our experiments, the addition of D-asparagine to L-asparagine at saturation level did not cause an increase in the amount of ammonia formed. However, these results cannot be regarded as a proof that the hydrolysis of both asparagine isomers is catalyzed by the same enzyme, since it is possible that the hydrolysis of D-asparagine by a separate enzyme is completely inhibited by L-asparagine. The fact that heating the extracts at 55° for 1 or 2 min or heating them for a more prolonged time (8 min) in the presence of small amounts of D-alanine or D-asparagine causes a decrease in the rate of hydrolysis of L-asparagine, D-asparagine and L- and D-aspartohydroxamic acid to the same extent, suggests that all these activities are catalyzed by one and the same enzyme[13]. The difference in the pH-activity curves of L- and D-asparagine hydrolysis and the changes

in the ratio between the hydrolysis velocities of the two isomers obtained under the influence of Co^{++}, or by altering the incubation temperature do not necessarily presuppose the existence of different enzymes. The points of attachment of L- and D-substrates to the same enzyme may be only in part identical and in part not. Changes in pH may affect in a different way the non-identical points of attachment, causing a difference in pH-activity curves. Similarly, the difference in effect of Co^{++} on the hydrolysis of L- and D-asparagine may be explained by difference in its way of reaction with the non-identical points of attachment.

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STUDIES ON THE MECHANISM OF FATTY ACID SYNTHESIS

VII. BIOSYNTHESIS OF FATTY ACIDS FROM MALONYL CoA

J. GANGULY*

Institute for Enzyme Research, University of Wisconsin, Madison, Wisc. (U.S.A.)

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SUMMARY

Malonyl CoA can serve as the starting point for the synthesis of long-chain fatty acids in animal tissues. For any given tissue the rate of formation of fatty acids from malonyl CoA is many times faster than that from acetyl CoA. TPNH and one enzyme fraction (R_2) are required for the synthesis of fatty acids from malonyl CoA. DPNH is much less effective than TPNH in some tissues.

The fatty acids formed from malonyl CoA by the isolated enzyme system of cow mammary gland are similar in chain length distribution to those found in milk.

The following abbreviations are used: ATP, adenosine triphosphate; CoA, coenzyme A; DPNH, reduced diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; R_1, enzyme fraction that carboxylates acetyl CoA to malonyl CoA; R_2, enzyme fraction that catalyzes the synthesis of fatty acids from malonyl CoA.

* Postdoctoral trainee of the University of Wisconsin, Institute for Enzyme Research. Present address: Indian Institute of Science, Bangalore, India.