difference between LDH II and III is in the reaction with cytochrome *c*, where two different attachment sites may be operating.

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THE ACTION OF MAMMALIAN LIVER ENZYME PREPARATIONS ON ASPARAGINE AND ASPARAGINE DERIVATIVES

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SUMMARY

α-N-alkyl derivatives of DL-asparagine were not hydrolyzed by rat-liver asparaginase or guinea pig-liver asparaginase. They markedly inhibited the action of these enzymes on L-asparagine. In all cases tested the inhibition was found to be competitive. Rat and guinea pig liver asparaginase did not hydrolyze N-(β-DL-aspartyl)-alkylamines and were inhibited by these compounds only slightly, if at all. The asparagine-α-keto acid transamination-deamidation system did not act on both kinds of asparagine derivatives and was not inhibited by them. Rat-liver preparations which exhibited asparaginase activity also catalyzed β-aspartyl transfer from asparagine to hydroxylamine. The specificity of the transfer reaction towards both kinds of asparagine derivatives was the same as in the case of asparaginase.

INTRODUCTION

Asparaginase is widely distributed in animal and plant tissues, and in microorganisms[1]. A comprehensive study of the specificity of asparaginase activity was carried out by

GRASSMANN AND MAYR[2] with asparaginase prepared from brewer's yeast. They found that this enzyme acted on asparagine only when its amino and amido groups were free. Working with rat-liver asparaginase, GREENSTEIN AND PRICE[3] found that this enzyme did not attack β-L-aspartyl-L-alanine and N-carbobenzoxy-L-asparagine. Similarly, MEISTER *et al.* found that DL-α-N-methylasparagine was not hydrolyzed by rat-liver asparaginase[4].

When rat-liver homogenates are heated at 50° for 10 min, they hydrolyze asparagine only in the presence of α-keto acids such as pyruvate[3,5]. This reaction is due to the presence in the homogenates of an asparagine-α-keto acid transamination-deamidation system. In this system a transamination between asparagine and α-keto acid is followed by the deamidation of the α-ketosuccinamic acid formed[6-8].

The present paper deals with the action of rat-liver and guinea pig asparaginase and of the rat-liver asparagine-α-keto acid transamination-deamidation system on α-N-alkyl derivatives of asparagine and on N-(β-aspartyl)-alkylamines. The effect of both kinds of asparagine derivatives on the above-mentioned enzymes was also investigated. In addition, the specificity of the β-aspartyl transfer reaction catalyzed by rat-liver preparations was studied.

EXPERIMENTAL

Materials and methods

L- and D-aspartic acid and L- and D-asparagine were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

α-N-alkyl-derivatives of DL-*asparagine*

α-DL-chloro-β-succinamide was prepared from DL-asparagine according to the method of HOLMBERG[9]. It was crystallized from hot water. Found: Cl, 23.2; neutral equivalent, 150. Calculated: Cl, 23.4; neutral equivalent, 152. For amination, 1 mole of α-DL-chloro-β-succinamide was treated with 5 moles of alkylamines (33 % aqueous solution) for 3–4 days at 30°. The solutions were evaporated under reduced pressure at a temperature below 50° with repeated addition of water. The residues were treated in one of the following ways: (a) (For the methyl, ethyl, ethanol and isopropyl derivatives.) The residue was rubbed up with alcohol and kept in the cold. The precipitate was filtered, washed with alcohol and dried. The methyl and ethyl derivatives so obtained were twice recrystallized from water–alcohol. The ethanol derivative was crystallized from water–alcohol and then from hot 75 % alcohol. The isopropyl derivative was crystallized from water–acetone and afterwards from hot 90 % alcohol. (b) The propyl and butyl derivatives were obtained by dissolving the residue in a minimum amount of water and precipitating with 5 volumes of acetone. The products so obtained were recrystallized several times from water–acetone.

For analysis, the compounds were dried *in vacuo* at 100° over phosphorus pentoxide. DL-α-N-methylasparagine: Found: N, 19.0 %; neutral equivalent (LINDERSTRØM-LANG titration), 147. Calculated: N, 19.1; neutral equivalent, 146. DL-α-N-ethylasparagine: Found: N, 17.5; neutral equivalent (LINDERSTRØM-LANG titration), 160. Calculated: N, 17.5; neutral equivalent, 160. DL-α-N-propylasparagine: Found: N, 16.0; neutral equivalent (LINDERSTRØM-LANG titration), 172. Calculated: N, 16.1; neutral equivalent, 174. DL-α-N-isopropylasparagine: Found: N, 16.0; neutral

equivalent (LINDERSTRØM-LANG titration), 174. Calculated: N, 16.1; neutral equivalent, 174. DL-α-N-butylasparagine: Found: N, 14.7; neutral equivalent (LINDERSTRØM-LANG titration), 185. Calculated: N, 14.8; neutral equivalent, 188. DL-α-N-ethanol-asparagine: Found: N, 15.8; neutral equivalent (LINDERSTRØM-LANG titration), 171. Calculated: N, 15.9; neutral equivalent, 175.

N-(β-DL-aspartyl)-alkylamines

β-methyl ester hydrochloride of DL-aspartic acid was prepared according to the method of COLEMAN[10] by treating DL-aspartic acid with methanolic hydrochloric acid and precipitating the ester hydrochloride with ether. The methanolic hydrochloric acid was obtained from acetyl chloride and dry methanol as described by HANBY *et al.*[11]. The ester was converted into N-(β-DL-aspartyl)-alkylamines by treatment with 7 moles of the corresponding amine in 30 % alcoholic solutions at 35° for 5–6 days. The reaction mixtures were evaporated under reduced pressure. The methyl and 2-ethanol derivatives were obtained by dissolving the residues in water and precipitating with alcohol. The products were recrystallized from water–alcohol. The ethyl- and 3-propanol derivatives were obtained by rubbing up the residues with alcohol. The precipitates formed were recrystallized from hot water.

For analysis, the substances were dried *in vacuo* at 100° over phosphorus pentoxide. N-(β-DL-aspartyl)-methylamine: Found: N, 18.9; neutral equivalent (LINDERSTRØM-LANG titration), 144. Calculated: N, 19.1; neutral equivalent, 146. N-(β-DL-aspartyl)-ethylamine: Found: N, 17.5; neutral equivalent (LINDERSTRØM-LANG titration), 156. Calculated: N, 17.5; neutral equivalent, 160. N-(β-DL-aspartyl)-aminoethanol: Found: N, 15.9; neutral equivalent (LINDERSTRØM-LANG titration), 173. Calculated: N, 15.9; neutral equivalent, 175. N-(β-DL-aspartyl)-3-aminopropanol: Found: N, 15.0; neutral equivalent (LINDERSTRØM-LANG titration), 186. Calculated: N, 14.8; neutral equivalent, 189.

β-aspartohydroxamic acid was prepared as described elsewhere[16].

Rat liver enzyme

Adult albino rats (male or females) were killed by decapitation. The liver was rapidly removed, cut in small pieces, and homogenized in an M.S.E. blender (14,000 rev./min) in 4–5 volumes of cold water (0–2°) for 2 min. The pH of the homogenate was adjusted to 7.5 with NaOH 0.1 *N* and the homogenate was centrifuged in the cold for 45 min at 32,000 × *g*. The reddish transparent supernatant was used for enzymic experiments. For the experiments with the asparagine-α-keto acid transamination-deamidation system, the homogenate was kept 10 min at 50° and rapidly cooled before centrifugation. The supernatants so obtained are referred to as "asparaginase-free rat-liver enzyme".

Guinea pig-liver asparaginase

Asparaginase from guinea pig liver was prepared as described by KREBS[12]. Equal amounts of the finely powdered preparations were added to each reaction mixture.

Assay procedures

Asparaginase activity: The reaction mixture contained per ml of 0.08 *M* phosphate buffer, pH 7.4, substrate, 6 μmoles (unless otherwise stated), rat-liver enzyme, 0.2 ml,

or guinea pig-liver preparation, 2.5 mg. Incubations were carried out at 37° for 1 h. At the end of the incubation 3-ml samples were removed and heated for 3 min at 70°. (No loss of NH_3 occurred under these conditions.) To these samples 2 ml of borate buffer (pH 10.1)[13] and ten drops of tributylphosphate were added. The NH_3 was transferred by aeration at 50° for 15–20 min into dilute HCl and measured colorimetrically by Nessler's method. With N-(β-DL-aspartyl)-methyl- or ethylamine as substrate the aeration was carried out at 60° into standard acid, which was then titrated. In these cases, as with N-(β-DL-aspartyl)-2-aminoethanol and N-(β-DL-aspartyl)-3-aminopropanol, a chromatogram was run at the beginning and the end of the reaction (phenol 80 %, Whatman paper No. 1). Aspartic acid was quantitatively estimated by elution and colorimetric determination as described by MEYER[14].

Asparagine-α-keto acid transamination-deamidation system: The reaction mixtures were the same as those given in previous section, but contained, in addition, 12 μmoles sodium pyruvate/ml phosphate buffer. Ammonia and amines were estimated as above. In addition, chromatographic estimations of alanine were made. In these cases, the enzyme solutions were dialyzed in the cold against phosphate buffer for several hours prior to incubation. We found that the asparagine-α-keto acid transamination-deamidation system was not affected by the dialysis.

β-Aspartyl-transfer activity: Each reaction mixture contained per 1 ml of 0.0375 *M* Tris buffer, pH 7.2, substrate, 21 μmoles; rat-liver enzyme, 0.25 ml; NH_2OH, 105 μmoles.

After incubation at 37° for 1 h, 2-ml samples were removed and 1 ml of 2 *M* NH_2OH, pH 6.5, and 3 ml of $FeCl_3$-reagent[15] were added. After centrifugation the hydroxamic acid-ferric complex was estimated in a Klett-Summerson colorimeter (Filter 54) using synthetic β-aspartohydroxamic acid as standard.

RESULTS

Rat-liver and guinea pig-liver asparaginase failed to liberate ammonia or alkylamines from the following derivatives of asparagine: DL-α-N-methylasparagine; DL-α-N-ethylasparagine; DL-α-N-propylasparagine; DL-α-N-isopropylasparagine; DL-α-N-butylasparagine; DL-α-N-ethanolasparagine; N-(β-DL-aspartyl)-methylamine; N-(β-DL-aspartyl)-ethylamine. Under the same conditions, unsubstituted asparagine was readily hydrolyzed. The experiments were carried out at a pH range varying between 6 and 8. Methyl- or ethylamine added to the reaction mixture in amounts corresponding to those of the substrates gave on aeration a recovery of 75 % and 55 % respectively.

In the experiments with N-(β-DL-aspartyl)-2-aminoethanol or 3-aminopropanol at a pH range varying between 6 and 8 no cleavage occurred, since no formation of aspartic acid could be detected by quantitative paper chromatography. Asparagine as substrate in control experiments under the same conditions gave a fair correlation between ammonia as estimated by aeration, and aspartic acid estimated by chromatography.

α-N-alkyl derivatives of DL-asparagine inhibited the action of rat-liver and guinea pig-liver asparaginase, as can be seen from Table I. In all cases the inhibitions obtained with the guinea pig-liver asparaginase were smaller than those obtained with the rat-liver asparaginase under similar conditions. The methyl, ethyl, propyl and butyl derivatives inhibited approximately to the same extent, whereas the inhibitions

shown by the isopropyl or the ethanol derivatives were considerably lower. No inhibitions were obtained with the N-(β-DL-aspartyl)-alkylamines, with the exception of a small inhibition obtained with N-(β-DL-aspartyl)-methylamine in the case of rat liver asparaginase in a molar ratio inhibitor–substrate 10:1.

TABLE I

EFFECT OF ASPARAGINE DERIVATIVES ON RAT-LIVER AND GUINEA PIG-LIVER ASPARAGINASE

Reaction mixtures, as described under *Assay procedures*.

Asparagine derivative added, μmoles/ml		*Rat-liver asparaginase*		*Guinea pig-liver asparaginase*	
		NH_3 liberated μmoles*	*Inhibition %*	*NH_3 liberated* μmoles*	*Inhibition %*
None		6.05		4.20	
DL-α-N-methylasparagine	6	4.15	30		
DL-α-N-methylasparagine	12	3.05	50		
DL-α-N-ethylasparagine	6	4.05	33		
DL-α-N-ethylasparagine	12	3.00	52		
DL-α-N-ethylasparagine	18	2.05	65	3.25	23
DL-α-N-ethylasparagine	60	1.35	78	2.30	45
DL-α-N-propylasparagine	6	4.15	30		
DL-α-N-propylasparagine	18	2.40	60	3.20	25
DL-α-N-propylasparagine	60			2.10	50
DL-α-N-butylasparagine	6	4.00	35		
DL-α-N-butylasparagine	18	2.00	67	3.00	28
DL-α-N-butylasparagine	60			2.05	51
DL-α-N-isopropylasparagine	6	5.15	15		
DL-α-N-isopropylasparagine	18	4.10	32	3.70	12
DL-α-N-isopropylasparagine	60			2.85	32
DL-α-N-ethanolasparagine	6	4.85	20		
DL-α-N-ethanolasparagine	18	3.75	38	3.50	17
N-(β-DL-aspartyl)-methylamine	30	5.40	10	4.25	0
N-(β-DL-aspartyl)-methylamine	60	5.05	17		
N-(β-DL-aspartyl)-ethylamine	60	5.85	< 5	4.20	0
N-(β-DL-aspartyl)-2-aminoethanol	60	5.95	0		

* All blanks substracted.

Experiments carried out with DL-α-N-methyl- and ethylasparagine proved that the inhibition shown by these compounds is competitive (Figs. 1 and 2).

Asparaginase-free rat-liver enzyme, which in the presence of pyruvate was very active towards DL-asparagine, failed to liberate NH_3 from the α-N-alkyl derivatives of DL-asparagine. On incubation of rat-liver enzyme (free of asparaginase) in the presence of pyruvate with N-(β-DL-aspartyl)-methyl- or ethylamine, no liberation of methyl- or ethylamine could be detected. Similarly no formation of alanine could be observed by paper chromatography. In control experiments, asparaginase-free rat-liver enzyme was incubated in the presence of pyruvate with alanine in concentration as small as 5 % of those of the derivatives used in the experiments. The recovery of alanine in these control experiments was nearly quantitative. After incubations of the asparaginase-free rat-liver enzyme with DL-asparagine under similar conditions, a formation of alanine was observed in amount of about 80 % of the amount of NH_3 liberated.

Rat-liver enzyme preparations also hydrolyzed α-asparagine (isoasparagine), but this activity was only slightly affected on heating at 50° for 10 min, which led to the

complete abolition of the action on asparagine. The hydrolysis of α-asparagine was not inhibited by the α-N-alkyl derivatives of DL-asparagine in a molar ratio of derivative to α-asparagine up to 10:1.

Rat-liver enzyme catalyses the transfer of the β-aspartyl group from asparagine

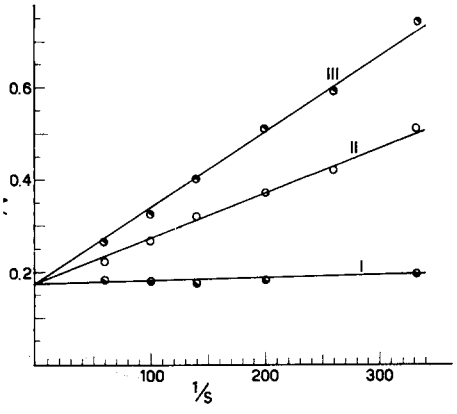

Fig. 1. Competitive inhibition of rat-liver asparaginase by DL-α-N-methyl- and DL-α-N-ethylasparagine. I: DL-asparagine; II: DL-asparagine and DL-α-N-methylasparagine (0.009 *M*); III: DL-asparagine and DL-α-N-ethylasparagine (0.018 *M*). *S* is the molar concentration of DL-asparagine. Velocity, *V*, is expressed in μmoles ammonia/h.

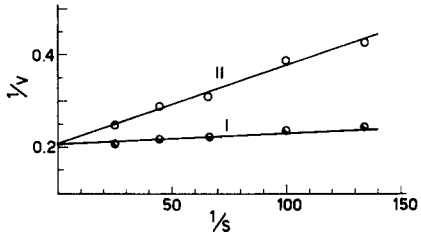

Fig. 2. Competitive inhibition of guinea pig-liver asparaginase by DL-α-N-ethylasparagine. I: DL-asparagine; II: DL-asparagine and DL-α-N-ethylasparagine (0.09 *M*). *S* is the molar concentration of DL-asparagine. Velocity, *V*, is expressed in μmoles ammonia/h.

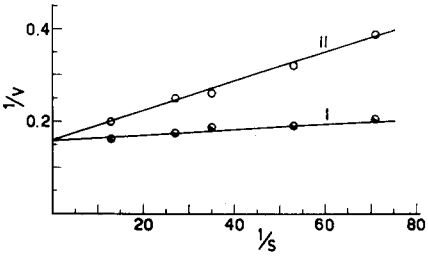

Fig. 3. Competitive inhibition of rat liver β-aspartyl transferase by DL-α-N-methylasparagine. I: DL-asparagine; II: DL-asparagine and DL-α-N-methylasparagine (0.021 *M*). *S* is the molar concentration of DL-asparagine. Velocity, *V*, is expressed in μmoles hydroxamic acid/h.

TABLE II

EFFECT OF ASPARAGINE DERIVATIVES ON THE β-ASPARTYL TRANSFER ACTIVITY OF RAT-LIVER ENZYME

Reaction mixtures, as described under *Assay procedures*.

Inhibitor, μmoles/ml		*Hydroxamic acid formed μmoles**	*Inhibition %*
None		5.40	
DL-α-N-methylasparagine	36	2.90	46
DL-α-N-methylasparagine	63	1.35	75
DL-α-N-ethylasparagine	36	3.15	42
DL-α-N-ethylasparagine	63	1.60	70
DL-α-N-propylasparagine	36	2.85	47
DL-α-N-propylasparagine	63	1.20	78
DL-α-N-butylasparagine	36	2.80	48
DL-α-N-butylasparagine	63	1.35	75
DL-α-N-isopropylasparagine	36	4.30	20
DL-α-N-isopropylasparagine	63	3.35	38
DL-α-N-ethanolasparagine	36	4.10	24
DL-α-ethanolasparagine	63	3.15	42
N-(β-DL-aspartyl)-methylamine	105	5.10	5
N-(β-DL-aspartyl)-ethylamine	105	5.50	0

* All blanks subtracted.

to hydroxylamine. Homogenates heated for 10 min at 50° completely lost this aspartyl transfer activity. No hydroxamic acid was formed by the rat-liver enzyme from α-N-alkyl derivatives of DL-asparagine or from N-(β-DL-aspartyl)-alkylamines. Only the L-form of asparagine reacted with hydroxylamine. Table II shows that α-N-alkyl derivatives of DL-asparagine markedly inhibited the β-aspartyl transfer reaction, whereas no inhibition was obtained by N-(β-DL-aspartyl)-alkylamines in molar ratio derivatives to substrate up to 6:1.

Experiments carried out on the inhibition of the β-aspartyl transfer reaction by DL-α-N-methylasparagine showed that this inhibition was competitive (Fig. 3).

DISCUSSION

Experiments with rat-liver and guinea pig asparaginase demonstrate that the substitution of one hydrogen atom of the α-amino group of asparagine by an alkyl group prevents the hydrolysis of the amide group. The α-N-alkyl derivatives of asparagine can, however, combine with the enzyme as can be concluded from their inhibitory action on asparagine hydrolysis.

The substitution of one of the hydrogen atoms of the amide group of asparagine by an alkyl group prevents hydrolysis and also prevents, or at least strongly diminishes, the binding to the enzyme.

α-N-alkyl derivatives of asparagine gave no ammonia with the asparagine-α-keto acid transamination-deamidation system. This proved that no transamination reaction took place, since otherwise the keto-succinamic acid formed would be deamidated by the system. Similarly, the fact that with the N-(β-aspartyl)-alkylamines as substrates no formation of alanine could be detected in this system, indicated that such compounds are not affected by asparagine-α-keto acid transaminase.

The similarity of the specificities of the asparaginase and the β-aspartyl transferase activities of rat-liver preparations seems to indicate that both reactions are catalyzed by the same enzyme.

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