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Aspartase-Catalyzed Synthesis of *N*-Hydroxyaspartic Acid*

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Certain microorganisms, such as *B. cadaveris*, can grow in the presence of rather high concentrations of hydroxylamine (10^{-3} M). Hydroxylamine disappears from the medium during growth, and also when incubated with resting cell suspensions of these organisms. This phenomenon is due to the presence of the enzyme aspartase. Partially purified aspartase catalyzes the condensation of hydroxylamine with fumaric acid to yield *N*-hydroxyaspartic acid. That this activity is caused by aspartase was shown by the constant ratio of the activities during purification, and by inhibition studies. Hydroxylamine is a competitive inhibitor of aspartase acting upon aspartic acid. Similarly, aspartic acid competitively inhibits the fumarate-hydroxylamine reaction. K_m and K_i values for aspartic acid and hydroxylamine are reported. The product of the condensation reaction, *N*-hydroxyaspartic acid, is very unstable and could not be crystallized, but was identified by its chemical and physical properties. Enzymatically produced *N*-hydroxyaspartic acid was catalytically reduced to give L-aspartic acid, showing that the enzyme retains its stereospecificity in the condensation of fumaric acid with hydroxylamine.

Within the past decade a number of hydroxamic acids have been isolated from microbial fermentations. In contrast to simple hydroxamic acids, which are *N*-acyl derivatives of hydroxylamine, these naturally occurring hydroxamic acids are *N*-acyl derivatives of *N*-hydroxyamino acids. Although free *N*-hydroxyamino acids are unstable at neutral *p*H and have not been demonstrated in living cells, in several instances these compounds have been isolated from the parent hydroxamic acid after acid hydrolysis. Thus, ϵ-*N*-hydroxylysine has been isolated from mycobactin (Snow, 1954) and δ-*N*-hydroxyornithine from the ferrichrome compounds (Emery and Neilands, 1961) as well as the antibiotic albomycin (Turková *et al.*, 1962). α-*N*-Hydroxyamino acids are also known to occur in hydroxamate linkage: *N*-hydroxyisoleucine in aspergillic acid (Dutcher, 1947), *N*-hydroxyleucine in pulcherrimin (Kluyver *et al.*, 1953), *N*-hydroxytyrosine and alanine in mycelianamide (Birch *et al.*, 1956), and *N*-hydroxyglycine in hadacidin (Kaczka *et al.*, 1962). All these compounds, except hadacidin, are either potent microbial growth factors or have antibiotic activity. Hadacidin has been reported to have antitumor activity.

Although nothing is known about the biogenesis of organically bound hydroxylamine, one interesting possibility is the direct addition of hydroxylamine to a double bond. It will be the purpose of this paper to show that the enzyme aspartase can catalyze the addition of hydroxylamine to fumaric acid to form *N*-hydroxyaspartic acid.

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EXPERIMENTAL

Preparation of Aspartase.—*B. cadaveris* (ATCC 9760) was grown in 10 liters of sterile medium containing 1% Bacto yeast extract, 1% Bactopeptone, and 0.5% monopotassium phosphate. After growth for 48 hours at 32°, with gentle aeration, the cells were harvested by centrifugation and frozen overnight at −17°. All subsequent operations were conducted at 0°. The frozen cells (26.4 g) were ground with about 2 g of Alcoa activated alumina for 15 minutes. To the thick paste was added twice the volume of 0.1 M potassium phosphate buffer, *p*H 6.8, and grinding was continued for 5 minutes. The extract was transferred with additional buffer to centrifuge tubes and the aluminum oxide and cell debris were removed by centrifugation. To the clear supernatant solution (43 ml) was added 3.2 ml of 1% protamine sulfate (Nutritional Biochemicals). After removal of the precipitate by centrifugation, the supernatant solution was brought to 60% saturation with solid ammonium sulfate. The precipitate was redissolved in 10 ml of 0.1 M potassium phosphate buffer, *p*H 6.8, and dialyzed overnight against 0.005 M potassium phosphate buffer, *p*H 6.8. Eight ml of this solution, containing 37.6 mg protein, was fractionated by the addition of solid ammonium sulfate (see Table I).

Aspartase Assay.—Aspartase was assayed by a modified method of Racker (1950). The 1-cm quartz cuvet

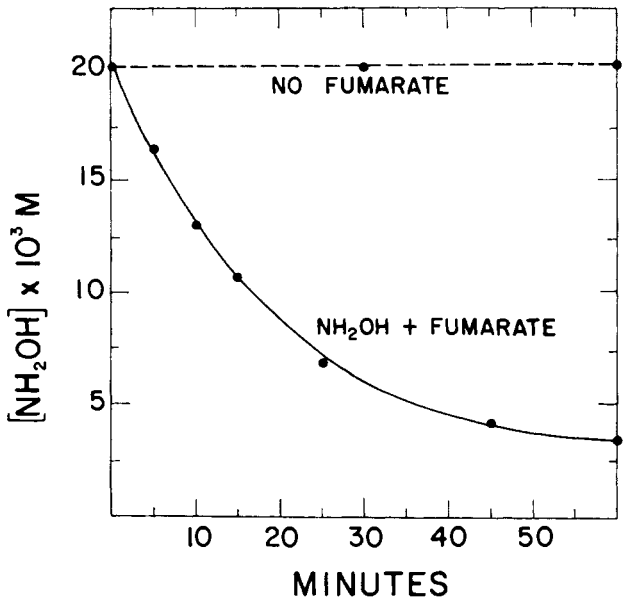

FIG. 1.—The disappearance of hydroxylamine catalyzed by cell-free extracts of *B. cadaveris* or *Proteus vulgaris*. The assay mixture contained 300 μmoles potassium phosphate, *p*H 6.8, 0.3 μmole disodium ethylenediaminetetraacetate, 3.0 μmoles $MgCl_2$, 180 μmoles potassium fumarate, 60 μmoles neutral NH_2OH, and about 0.3 ml extract in a final volume of 3.0 ml. The temperature was 28°. Hydroxylamine was determined in 0.1-ml aliquots by the method of Csáky (1948).

contained 300 μmoles potassium phosphate, *p*H 6.8, 0.3 μmole disodium ethylenediaminetetraacetate, 3.0 μmoles $MgCl_2$, and 160 μmoles neutral potassium L-aspartate (Nutritional Biochemicals) in a final volume of 3.0 ml. After addition of the enzyme and thorough mixing, the optical density at 240 mμ was determined at 30-second intervals using a Beckman DU spectrophotometer equipped with a photomultiplier. The blank contained no aspartate. The temperature was 28°. The increase in optical density was linear with respect to time for several minutes and the rate was proportional to enzyme concentration. Protein was determined by the method of Lowry *et al.* (1951). The molar extinction coefficient of potassium fumarate under identical conditions was determined to be 2.53×10^3 M^{-1} cm^{-1}. Aspartase activity is expressed as micromoles of fumarate formed per milligram protein per hour.

Fumarate-Hydroxylamine Condensation Activity.—This assay was based on the disappearance of hydroxylamine. Into a small test tube were placed 300 μmoles potassium phosphate buffer, *p*H 6.8, 0.3 μmole disodium ethylenediaminetetraacetate, 3.0 μmoles $MgCl_2$, 180 μmoles neutral potassium fumarate (Mann), and 60 μmoles hydroxylamine hydrochloride (Mallinckrodt, A. R.) brought to *p*H 6.8 with potassium hydroxide. The last solution must be prepared immediately before use. The final volume was made to 3.0 ml with distilled water. The enzyme was added and the contents of the tube were mixed well. At zero time and after 15 minutes incubation at 28°, 0.1-ml aliquots were removed and diluted to 15.0 ml with water. The hydroxylamine content of 1.0 ml of this solution was determined by the method of Csáky (1948). The loss of hydroxylamine was proportional to protein concentration over a 20-fold range. The activity is expressed as μmoles hydroxylamine disappearing per milligram protein per hour. There was no detectable nonenzymatic decomposition of hydroxylamine under the conditions of this assay.

Paper Electrophoresis of N-Hydroxyaspartic Acid.—Aliquots (*ca.* 10 μl) of the sample were subjected to paper electrophoresis using Whatman No. 1 filter paper and a potential gradient of 32 volts/cm. The buffer was acetic acid–pyridine–water, 7:5:930, *p*H 5.0. After the paper was air-dried, the spots were detected by spraying with the tetrazolium spray of Snow (1954), periodate-benzidine (Viscontini *et al.*, 1955), or 0.5% ninhydrin in acetone. Hydroxylamine and hydroxamino compounds give a red spot with tetrazolium, and a white spot on a blue background with the periodate spray. α-Hydroxamino acids, but not hydroxylamine, give a brown to purple color with ninhydrin.

RESULTS

Grossowicz and Lichtenstein (1961) reported that cell-free preparations of hydroxylamine-resistant strains of an organism similar to *E. coli* could carry out a condensation of fumaric acid with hydroxylamine. They did not identify the product of this reaction, but they state that the reaction was not due to aspartase since this enzyme could not be detected in the extracts ("ammonia did not disappear"). In an attempt to duplicate these findings, a pure culture of an unidentified bacterium that could grow in the presence of 10^{-3} M hydroxylamine was isolated from a soil enrichment culture. Cell-free extracts of this organism could indeed carry out an enzymatic condensation of fumaric acid with hydroxylamine. In contrast to the previously cited results, however, the extracts contained a high aspartase activity, and it was suspected that this enzyme was responsible for the reaction. In support of this belief was the finding that *B. cadaveris*, an organism known to be a rich source of aspartase, could also carry out the fumarate-hydroxylamine condensation. Similar results were obtained with *Proteus vulgaris*, another aspartase-containing organism. The disappearance of hydroxylamine by extracts of these organisms is shown in Figure 1. Under the conditions of these experiments there was no detectable nonenzymatic reaction of hydroxylamine with fumaric acid, nor spontaneous decomposition of hydroxylamine. Since neutral solutions of hydroxylamine are known to be unstable, it is assumed that its unexpected stability here is due to the binding of heavy metals by the protein. Heavy metals are known to catalyze the decomposition of hydroxylamine. Ethylenediaminetetraacetic acid was also found to stabilize hydroxylamine solutions and was consequently added to the reaction mixtures. The disappearance of hydroxylamine was absolutely dependent upon the presence of fumaric acid. Maleic acid could not replace fumaric acid. Even in the presence of excess fumaric acid the concentration of hydroxylamine did not drop to zero. This may indicate the attainment of an equilibrium, but the possibility that the product, *N*-hydroxyaspartic acid, may give a slightly positive Csáky test for hydroxylamine has not been ruled out. In any case, the formation of the condensation product must be highly favored, since even with stoichiometric amounts of fumaric acid over 95% of the hydroxylamine was consistently consumed.

Although crystallization of aspartase has not yet been achieved, evidence was obtained which further indicated that aspartase activity and fumarate-hydroxylamine condensation are catalyzed by the same enzyme. The two activities were followed during ammonium sulfate fractionation of the protamine sulfate–treated extract of *B. cadaveris*. The specific activity of aspartase was highest in the 35–45% fraction, in agreement with the results of Williams and

TABLE I

FUMARATE-HYDROXYLAMINE AND ASPARTASE ACTIVITIES IN AMMONIUM SULFATE FRACTIONS OF *B. cadaveris* EXTRACTS

A protamine sulfate–treated cell-free extract of *B. cadaveris* was precipitated at 60% saturation of ammonium sulfate. The precipitate was dissolved in 10 ml of 0.1 M potassium phosphate, *p*H 6.8, and dialyzed overnight against 0.005 M phosphate, *p*H 6.8. Eight ml of the solution containing 37.6 mg protein was refractionated by the addition of solid ammonium sulfate. The precipitates were dissolved in 2.0 ml of the 0.1 M phosphate buffer and were assayed at 28° (see Experimental). Specific activities are expressed as μmoles/hr/mg protein of fumarate formed (aspartase activity), or μmoles/hr/mg protein of NH_2OH disappearing (fumarate-hydroxylamine activity).

$(NH_4)_2SO_4$ (% saturation)	Fumarate-Hydroxylamine Reaction, Specific Activity	Aspartase Reaction, Specific Activity	Ratio of Specific Activities	Total Protein (mg)
0	98.0	78.0	1.25	37.6
0–25	14.7	11.4	1.29	3.0
25–35	34.8	25.6	1.36	4.6
35–45	153.0	113.0	1.35	12.6
45–60	26.0	17.8	1.46	6.2

McIntyre (1955). The ratio of aspartase activity to fumarate-hydroxylamine condensation activity remained essentially constant in all the fractions. These results are summarized in Table I. All subsequent experiments were carried out using the 35–45% ammonium sulfate fraction.

The heat lability of both of these enzyme-catalyzed reactions was found to be essentially identical, as was the sensitivity to a variety of inhibitors, including 1-propanol, a known inhibitor of aspartase (Erkama and Virtanen, 1951), (Table II). The extreme lability of aspartase to heat was not consistently observed and no explanation can be offered for the behavior of this particular preparation. The *p*H-activity profile of the fumarate-hydroxylamine condensation reaction is shown in Figure 2. There is a rather sharp optimum of activity at pH 7.0. Williams and McIntyre (1955) reported a maximum of aspartase activity at *p*H 6.5-6.8 in 0.1 M phosphate buffer.

Final conclusive proof that aspartase is responsible for the fumarate-hydroxylamine condensation was obtained by competitive inhibition studies. If two different substrates are both bound to the same active site of an enzyme, then each substrate should act as a competitive inhibitor of the reaction of the other. In Figure 3 the reciprocal of the initial rate of disappearance of hydroxylamine in the presence of aspartase and excess fumaric acid is plotted against the reciprocal of hydroxylamine concentration. From the graph, the K_m value for hydroxylamine under these conditions was calculated to be 0.03 M. Identical measurements were then carried out in the presence of 0.004 M and 0.008 M L-aspartic acid, respectively. In the presence of L-aspartic acid the rate of disappearance of hydroxylamine was inhibited, and the inhibition was of a competitive nature, as seen in the figure. The inhibition

TABLE II

INHIBITION OF FUMARATE-HYDROXYLAMINE ACTIVITY AND ASPARTASE ACTIVITY[a]

Inhibitor	Per Cent Inhibition[b]	
	Fumarate-NH_2OH Activity	Aspartase Activity
37°, 2 min	36	33
37°, 4 min	52	53
45°, 2 min	97	95
$HgCl_2$, 10^{-5} M	100	100
-Chloromercuribenzoate, 2×10^{-5} M	100	100
KCN, 5×10^{-2} M	100	100
1-Propanol, 10%	56	55

[a] The assays are described under Experimental. [b] Per cent inhibition of partially purified aspartase (35–45% ammonium sulfate fraction, Table I).

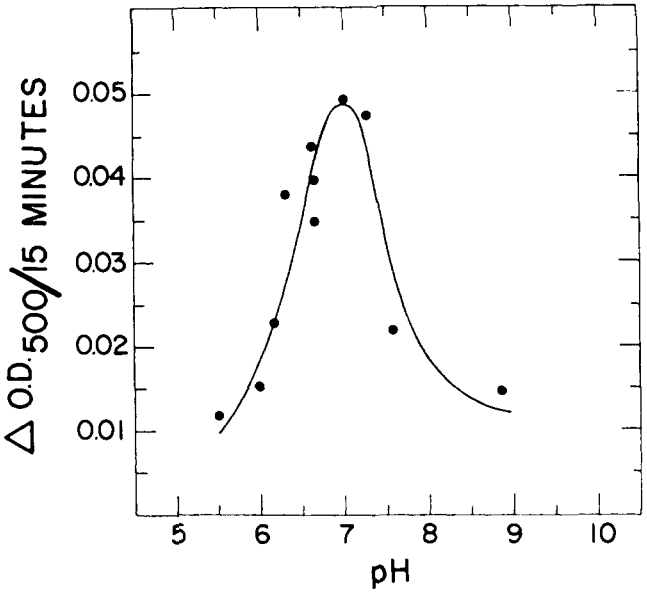

FIG. 2.—Fumarate-hydroxylamine activity of partially purified aspartase (35–45% ammonium sulfate fraction) as a function of *p*H. The assay is identical to that of Figure 1. The loss of hydroxylamine in 0.1-ml aliquots, as determined by the method of Csáky (1948), is expressed by the decrease of optical density at 500 mμ per 15 minutes.

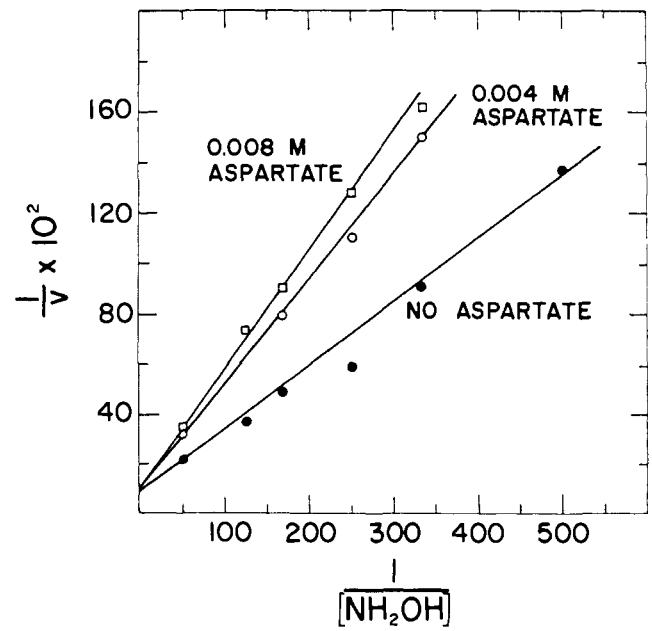

FIG. 3.—Reciprocal of initial rate of disappearance of NH_2OH, *v*, versus the reciprocal of NH_2OH concentration. Assay conditions were identical to those of Figure 1, except the crude extract was replaced by about 0.1 mg of the partially purified aspartase (35–45% ammonium sulfate fraction). The units of $1/v$ are hr μmole^{-1}.

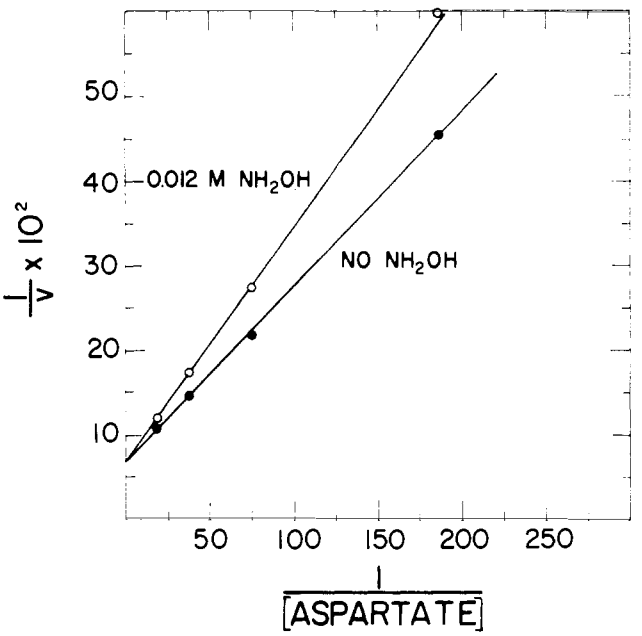

FIG. 4.—Reciprocal of aspartase activity (formation of fumarate) v versus the reciprocal of aspartate concentration. The cuvet contained 300 μmoles of potassium phosphate, pH 6.8, 0.3 μmole disodium ethylenediaminetetraacetate, 3.0 μmoles $MgCl_2$, 160 μmoles potassium aspartate, and about 0.1 mg aspartase (35–45% ammonium sulfate fraction). The rate of fumarate formation was determined by the increase of optical density at 240 mμ against a blank containing no aspartate. Units of $1/v$ are hr μmole^{-1}.

constant K_i for L-aspartic acid was calculated from these data to be 0.008 M.

A Lineweaver-Burk plot of the deamination of L-aspartic acid (formation of fumarate) for the same enzyme preparation is shown in Figure 4. From this graph the apparent K_m for L-aspartic acid was calculated to be 0.03 M. Williams and McIntyre (1955) reported a K_m of approximately 2×10^{-2} M. The competitive inhibition of the deamination of L-aspartic acid by 0.012 M hydroxylamine is also shown in the same figure. K_i for hydroxylamine was calculated to be 0.03 M, identical to its K_m.

Specificity.—That the strongly nucleophilic hydroxylamine can substitute for ammonia in the aspartase-catalyzed condensation with fumaric acid is perhaps not too surprising at first glance. Hydroxylamine is known to replace ammonia in transamidation reactions (Jones *et al.*, 1952), and esterases can catalyze hydroxamate formation (Lipmann and Tuttle, 1950). If one accepts the unprotonated form of the base as the reactive species, the greater reactivity of hydroxylamine follows from a consideration of the acid-dissociation constants involved. Thus, ammonia ($pK_a' = 9.3$) is almost completely in the unreactive protonated form at *p*H values near neutrality, whereas hydroxylamine ($pK_a' = 6.0$) is largely in the reactive unprotonated form. However, the specificity of the enzyme must also be taken into account. It was found that neither bacterial nor pig heart fumarase showed any reactivity towards hydroxylamine; that is, the highly reactive hydroxylamine cannot replace water in the fumarase reaction. An enzyme exactly analogous to aspartase is β-methylaspartase, which catalyzes the reversible condensation of ammonia with α-methylfumaric acid (mesaconic acid). It was found that hydroxylamine could not replace ammonia in this reaction. The substitution of hydroxylamine for ammonia in the aspartase reaction is thus indicative of more than a high reactivity of hydroxylamine, and other factors must play a role in allowing this substitution. Finally, hydrazine is only about one-tenth as active as hydroxylamine in the aspartase-catalyzed reaction with fumaric acid.

Nature of the Product.—Attack of an α-carbon of fumaric acid by hydroxylamine would be expected to lead to formation of *N*-hydroxyaspartic acid. *N*-Hydroxyamino acids, like hydroxylamine itself, are very unstable compounds at neutral *p*H. It was not too surprising, therefore, that paper chromatography of the reaction mixture did not lead to the identification of any of the expected product. Spenser and Ahmad (1961) showed that paper chromatography of α-*N*-hydroxyamino acids leads to extensive decomposition. Paper electrophoresis at *p*H 5 in pyridine-acetate buffer proved the only practicable method of detecting *N*-hydroxyaspartic acid. At *p*H 5, *N*-hydroxyaspartic acid should have a net negative charge and move as an anion, while hydroxylamine itself should be cationic. At *p*H 3 or 7 extensive decomposition took place. The reactivity of hydroxamino acids with periodate, tetrazolium, and ninhydrin offered convenient and sensitive methods for detection of the spots.

After incubation of fumaric acid and hydroxylamine in the presence of aspartase until about 95% of the hydroxylamine had disappeared, 10-μl aliquots of the reaction mixture were subjected to paper electrophoresis (experimental). Deproteinization was unnecessary and, in fact, was avoided due to the previously mentioned instability of the product. After drying and spraying the paper, a spot moving with a mobility of 7.5 cm/hr was detected with all three sprays. The enzyme solution alone was completely negative. Solutions of hydroxylamine, or hydroxylamine and fumaric acid, yielded only a spot of hydroxylamine moving as a cation (no reaction with ninhydrin). Aspartic acid moved with electrophoretic mobility identical to that of the reaction product, but gave no reaction with the periodate or tetrazolium spray.

The above observations led to the tentative conclusion that the reaction product was indeed *N*-hydroxyaspartic acid. Extensive efforts were made to crystallize this compound, or prepare crystalline derivatives thereof. The compound, purified by column chromatography on Dowex 50 resin, yielded a white, apparently crystalline material when taken to dryness in a vacuum dessicator. Upon exposure to air, however, there was almost immediate formation of an intractable gum which darkened upon standing, making elemental analysis impossible. Attempts to crystallize the sodium, potassium, barium, or cupric salts yielded similar results, the latter cation causing extensive decomposition. *N*-Acylation with acetic anhydride, acetyl chloride, or benzoyl chloride, in order to form the more stable hydroxamic acid, was unsuccessful. Others have reported difficulty in the *N*-acylation of α-hydroxamino acids (Neelakantan and Hartung, 1958).

Rogers and Neilands (1963) have reported the synthesis of ω-hydroxamino acids, and methods are available for the synthesis of α-hydroxamino acids. Unfortunately, none of these methods is applicable to the synthesis of *N*-hydroxyaspartic acid, either because of the unavailability of starting materials or the inherent instability of intermediates. Refluxing of bromosuccinic acid with neutral solutions of hydroxylamine in methanol yielded a product with electrophoretic mobility and reactivity to periodate, tetrazolium, and ninhydrin identical to that of the enzymatic product, but the yields were almost negligible. Much better yields of an apparently identical material were obtained by the addition of a methanolic solution of fumaric acid to a refluxing methanolic solution of salt-free

hydroxylamine. The material could be purified on a Dowex 50 column, but the same difficulties of crystallization and instability were encountered as with the enzymatically synthesized product. The electrophoretic mobility, the reactions with periodate, tetrazolium, and ninhydrin, and the mode of synthesis, both chemical and enzymatic, provided convincing evidence that the compound in question was *N*-hydroxyaspartic acid. Additional evidence was the fact that the compound could be catalytically hydrogenated at 1 atm using platinum oxide catalyst to yield L-aspartic acid. The reduction was apparently quantitative. The aspartic acid was isolated as the copper salt. The decomposition point (220°) and infrared spectrum were identical to an authentic sample of the copper salt of L-aspartic acid prepared under identical conditions. Hydrogen sulfide decomposition of the copper salt yielded aspartic acid which was fully active in the aspartase system, which is known to be specific for the L-isomer of aspartic acid. It can be concluded that the configuration of the enzymatically produced *N*-hydroxyaspartic acid is of the L-configuration.

Discussion

That hydroxylamine can replace ammonia in the aspartase reaction may be of no more significance than a lack of specificity of the enzyme. However, there are two considerations that should not be overlooked. In the first place, this reaction represents the first demonstration of an enzymatic synthesis of a hydroxamino acid. Although it is not known if free hydroxamino acids are intermediates in the biosynthesis of the naturally occurring hydroxamic acids, this possibility exists. In two cases, with aspergillic acid (MacDonald, 1961) and with mycelianamide (Birch and Smith, 1958), it has been shown that the carbon skeletons of the hydroxamino acids in the hydroxamate compounds are derived from the corresponding α-amino acids. It has not been demonstrated, however, that the α-amino group is also incorporated. It is possible that the amino group is first lost in a reductive deamination to give the α-β-unsaturated carboxylic acid. Hydroxylamine addition to the double bond by an aspartase-type reaction, followed by *N*-acylation, would result in the formation of the hydroxamic acid. Such a mechanism would obviously not be possible in the biosynthesis of *N*-hydroxylglycine, a constituent of hadacidin (Kaczka *et al.*, 1962). Other routes of biosynthesis of the *N*-hydroxy group are also possible. Cramer *et al.* (1960) have presented evidence for the *in vivo* *N*-hydroxylation of an amide bond.

A second consideration of the results reported in this paper is in relation to inorganic nitrogen metabolism. Hydroxylamine is generally accepted as a logical intermediate in nitrogen fixation, nitrification, and denitrification reactions. Its toxicity to most cells has prevented the gathering of any definitive evidence concerning its participation in these pathways of metabolism, and it has been speculated that hydroxylamine occurs organically bound rather than free. Virtanen (1947) postulated that it condenses with α-keto acids to form oximes, which are subsequently reduced to amino acids. It would be expected on theoretical grounds that hydroxamino acids be intermediates in reductions of oximes to amines. The results presented in this paper indicate an even more direct formation of organically bound hydroxylamine. Specific aspartase-catalyzed condensation of hydroxylamine with fumaric acid to form *N*-hydroxyaspartic acid has been demonstrated. That this reaction can occur *in vivo* is known, since resting cell suspensions as well as actively growing cells were shown to carry out this reaction. It is quite probable that the hydroxylamine resistance of these organisms is due to their high aspartase activity. This does not rule out the possibility that other cells containing aspartase, an enzyme widely distributed in plants and microorganisms, may carry out a similar reaction in the presence of the smaller concentrations of hydroxylamine encountered under physiological conditions. This reaction could provide a pathway for entry of hydroxylamine into general nitrogen metabolism if the necessary enzyme were present for reduction of the *N*-hydroxyaspartate to aspartate. Preliminary experiments in this laboratory suggest that *N*-hydroxyaspartate may not be an inert end product of cellular metabolism. Resting cell suspensions of *B. cadaveris* can bring about a disappearance of *N*-hydroxyaspartic acid from the suspending medium, although it has not yet been demonstrated that the immediate product is in fact aspartic acid.

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