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Polymerization Reactions Catalyzed by Intracellular Proteinases. IV. Factors Influencing the Polymerization of Dipeptide Amides by Cathepsin C*

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The polymerization of four dipeptide amides by beef spleen cathepsin C was studied as a function of substrate concentration, enzyme concentration, and *p*H. Between *p*H 7 and 8, in the *p*H range of polymerization, L-alanyl-L-phenylalaninamide is polymerized efficiently to form a hexapeptide amide, whereas the polymerization of glycyl-L-phenylalaninamide and glycyl-L-tyrosinamide is accompanied by extensive hydrolysis, and the products formed are largely octapeptide amides. The polymerization of glycyl-L-tryptophanamide also was examined.

The polymerization of dipeptide amides by cathepsin C (Fruton *et al.*, 1953; Würz *et al.*, 1962; Fruton and Knappenberger, 1962) provides a model system for the study of the enzyme-catalyzed elongation of peptide chains under physiological conditions of temperature and *p*H. In previous papers of this series, observations were reported on the polymerization of L-alanyl-L-phenylalaninamide (Ala.Phe[NH_2]), glycyl-L-phenylalaninamide (Gly.Phe[NH_2]), glycyl-L-tyrosinamide (Gly.Tyr[NH_2]), and glycyl-L-tryptophanamide (Gly.Trp[NH_2]). In the work described in the present communication, the polymerization of these substrates was studied as a function of the concentration of substrate, concentration of enzyme, and *p*H.

Experimental

The cathepsin C preparation (De la Haba *et al.*, 1959) had a specific activity $[C.U.]^{GTA}_{mg\ protein} = 62$.[1] The dipeptide amides were prepared as described previously (Fruton *et al.*, 1953; Theodoropoulos and Fruton, 1962). The enzyme experiments were conducted at 37.5°, with 0.01 M β-mercaptoethylamine as the enzyme activator. Ammonia liberation during the course of enzymic action was measured by the microdiffusion method of Seligson and Seligson (1951) and carboxyl liberation was measured by the method of Grassmann and Heyde (1929).

For the determination of the yield of polymer formed from Gly.Tyr(NH_2), the procedure described in Würz *et al.* (1962) was used; the insoluble polymer was filtered with suction (analytical Celite was used as a filter aid), washed thoroughly with water, and dissolved in alkali (final concentration, 0.01 N NaOH), and the tyrosine content of the solution was determined spectrophotometrically at 294 mμ (ε, 2389). For the determination of the amide-N of the polymer from Gly.Tyr(NH_2), a portion of the alkaline solution was heated to 110° for 15 hours in the presence of 6 N HCl in a sealed tube; after removal of HCl, samples of the acid hydrolysate were taken for NH_3 determination by the microdiffusion method.

Since the polymers derived from Gly.Phe(NH_2) and Ala.Phe(NH_2) were insoluble in alkali, the precipitates were filtered, transferred quantitatively into 6 N HCl, and hydrolyzed at 110° for 15 hours in sealed tubes. Samples of the hydrolysates were taken for separate determination of amide-N (by microdiffusion) and of phenylalanine (by spectrophotometry at 258 mμ; ε, 195).

In the case of the polymer derived from Gly.Trp(NH_2), the hydrolysis was conducted with $Ba(OH)_2$ as described in Würz *et al.* (1962). The tryptophan content of the hydrolysate was determined spectrophotometrically at 280 mμ (ε, 5400). Analytical data on the composition of hydrolysates of the polymers were also obtained by means of automatic amino acid analysis by the method of Spackman *et al.* (1958). To facili-

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[1] Cathepsin units per mg protein using glycyl-L-tyrosinamide as substrate.

TABLE I

POLYMERIZATION OF L-ALANYL-L-PHENYLALANINAMIDE, GLYCYL-L-PHENYLALANINAMIDE, AND GLYCYL-L-TYROSINAMIDE BY CATHEPSIN C[a]

Enzyme Concentration (units/ml)	Lag Period (min)			Polymer Yield (μmoles aromatic amino acid/ml)			Aromatic Amino Acid/NH_3		
	Ala.Phe-(NH_2)	Gly.Phe-(NH_2)	Gly.Tyr-(NH_2)	Ala.Phe-(NH_2)	Gly.Phe-(NH_2)	Gly.Tyr-(NH_2)	Ala.Phe-(NH_2)	Gly.Phe-(NH_2)	Gly.Tyr-(NH_2)
0.3	8.5			8					
0.45	5.0			12			3.1		
0.9	3.0	30.0	55.5	23	7	6	3.1		3.6
1.1	2.5	25.5	44.5	26	8	8	3.2		4.2
1.5	2.0	19.0	35.0	31	11	9	3.0		3.6
1.8	1.5		30.0	37		16	3.2		
3.0	<1.0	10.5	20.0	39	14	18	3.2	4.2	4.0
4.5	<1.0	9.0	17.0	42	17	19	3.3[b]	4.3	3.8[c]
9.0	<1.0	4.0	8.5	45	19	14	3.4	4.1	3.9
18.0	<1.0	2.5	5.0	44	21	13	3.4	4.1	

[a] Substrate concentration, 0.05 M; β-mercaptoethylamine, 0.01 M; *p*H adjusted with 0.1 N NaOH to 7.6 for Ala.Phe(NH_2), to 7.8 for Gly.Phe(NH_2), and to 7.5 for Gly.Tyr(NH_2); incubation time, 3 hours; temperature, 37.5°. [b] Automatic chromatographic analysis of the hydrolysate obtained in another experiment under these conditions gave a ratio of Ala/Phe/NH_3 of 3.06:2.96:1. [c] Automatic chromatographic analysis gave a ratio of Gly/Tyr/NH_3 of 4.08:4.06:1.

tate comparison of the data, the amount of polymer is expressed in terms of μmoles of tyrosine, phenylalanine, or tryptophan residues present in the precipitate formed in 1 ml of the enzymic incubation mixture.

RESULTS

Polymerization of Ala.Phe(NH_2).—When the polymerization of 0.05 M Ala.Phe(NH_2) was studied near *p*H 7.6 as a function of enzyme concentration, it was observed that at the higher enzyme levels tested the incorporation of Ala.Phe units into insoluble polymer was essentially complete (80–90%) after prolonged incubation (Table I). Analysis of acid hydrolysates of the polymer (either spectrophotometrically or by automatic chromatographic analysis) indicated that in all samples analyzed the isolated product was largely in the form of a hexapeptide amide, so that the over-all process in the enzyme-catalyzed polymerization may be written:

$$3\ \text{Ala.Phe}(NH_2) \rightarrow \text{Ala.Phe.Ala.Phe.Ala.Phe}(NH_2) + 2\,NH_3$$

As noted previously (Fruton *et al.*, 1953), the appearance of insoluble product in the enzymic polymerization of Gly.Phe(NH_2) is characterized by a lag period. In the present experiments with Ala.Phe(NH_2) it was found that the duration of the lag period (as determined by first appearance of precipitate) was extremely reproducible, and showed an inverse proportionality to enzyme concentration (Table I). Over the range of enzyme concentration tested, and at which accurate time measurement was possible (0.3–1.8 units/ml), the lag period decreased from 8.5 minutes to 1.5 minutes, and the arithmetical product of enzyme concentration times lag period was constant at 2.7 ± 0.2.

The time course of the polymerization for a given enzyme concentration (0.45 unit/ml) is given in Table II. It will be noted that the end of the incubation period, when approximately 36% of the substrate had been converted to insoluble polymer, the extent of NH_3 liberation during the course of the incubation amounted to about 65% of the polymer yield (expressed as μmoles of Phe per ml incubation mixture), in agreement with above equation. It may be added that separate experiments to estimate the extent of hydrolytic cleavage, by means of the Grassmann-Heyde method, showed that the extent of liberation of carboxylate groups was negligible. It appears justifiable to conclude, therefore, that the predominant enzyme-catalyzed process

TABLE II

POLYMERIZATION OF L-ALANYL-L-PHENYLALANINAMIDE BY CATHEPSIN C[a]

Time (min)	NH_3 Liberation (μmoles/ml)	Polymer Yield (μmoles Phe/ml)	Phe/NH_3 Ratio in Polymer Hydrolysate
15	3.5		
30	5.3		
60	7.7	7.1	3.4
90		11.2	3.5
120	8.8	11.8	3.1
180		14.5	3.2
240	11.7	18.2	3.1
300	11.7	18.0	3.1

[a] Substrate concentration, 0.05 M; enzyme concentration, 0.45 unit/ml; β-mercaptoethylamine, 0.01 M; *p*H 7.5, adjusted with 0.1 N NaOH; temperature, 37.5°.

under the conditions of these studies is that of transamidation, and that the hydrolytic reaction observed at *p*H values near 5 is essentially absent at *p*H values near 7.5.

It will be noted from Table II that the average chain length of the insoluble product remains essentially unchanged (within the precision of the analytical method employed) during the course of the polymerization. A similar average value of about 3.2 was maintained throughout the course of the reaction in other experiments at higher enzyme concentrations (0.9–1.8 enzyme units). Addition of 0.05 M Ala.Phe to an incubation mixture (under the conditions given in Table II) did not affect significantly the rate of NH_3 liberation, the lag period, the polymer yield, or the average chain length. Similar results were obtained in the case of the polymerization of 0.05 M Gly.Phe(NH_2) and Gly.Tyr-(NH_2) upon the addition of equimolar amounts of the corresponding free dipeptides.

Examination of the effect of *p*H on the yield of polymer showed a broad maximum for Ala.Phe(NH_2) over the *p*H range 7.1–7.6; at all *p*H values in this region the average chain length of the insoluble product was essentially the same (*ca.* 3.3).

The yield of insoluble polymer obtained from Ala-Phe(NH_2) in a 3-hour incubation mixture (*p*H 7.4; 0.9 enzyme unit/ml) is roughly proportional to the substrate concentration over the range 0.01–0.05 M, but the lag period was the same (3 minutes) at all substrate

TABLE III
KINETICS OF POLYMERIZATION OF L-ALANYL-L-PHENYLALANINAMIDE[a]

Substrate Concn (μmoles/ml)	Enzyme Concn (units/ml)	v_D[b]	E/v_D	v_P[c]	v_P/v_D
10	0.9	0.15	6.0	0.11	0.73
12.5	0.9	0.18	5.0	0.12	0.67
16.7	0.9	0.21	4.3	0.12	0.57
25	0.9	0.28	3.2	0.17	0.61
50	0.45	0.22	2.1	0.13	0.59
50	0.9	0.43	2.1	0.27	0.63
50	1.8	0.83	2.2	0.55	0.66

[a] β-Mercaptoethylamine, 0.01 M; pH 7.4, adjusted with 0.1 N NaOH; temperature 37.5°. [b] μmoles of NH_3/ml liberated per minute during first 15 minutes of reaction. [c] Amount of insoluble polymer/ml (expressed as μmoles of Phe) formed per minute during initial period of formation of insoluble polymer (15–60 minutes after start of incubation).

concentrations. In all cases the polymer appeared to be largely a hexapeptide amide.

In Table III are given data on the kinetics of the release of NH_3 and of the formation of insoluble polymer during the initial stages of the reaction. Because of the limitations of the analytical methods employed, these results can be considered only preliminary. From a Lineweaver-Burk plot of the data on the rate of NH_3 release, a K_m value of approximately 0.05 M was calculated.

Polymerization of Gly.Phe(NH_2).—In spite of the similarity in structure of Gly.Phe(NH_2) and Ala.Phe(NH_2), there are marked differences in their behavior as substrates for cathepsin C–catalyzed polymerization. At a given enzyme concentration the lag period for the appearance of insoluble polymer is longer and the yield of polymer is much less than in the case of Ala.Phe(NH_2); furthermore, Gly.Phe(NH_2) is converted to a product that is, on the average, an octapeptide amide (Table I). When the enzyme concentration was varied over the range 0.9–18 units/ml, the lag period (at 0.05 M substrate, pH 7.8) for the appearance of insoluble polymer decreased from 30 minutes to 2 minutes; the arithmetical product of enzyme units per ml times lag period (minutes) was 32 ± 4. At a given enzyme concentration the same lag period was observed for 0.025 M, 0.05 M, and 0.075 M substrate. The pH dependence of polymer yield shows a broad optimum over the range pH 7.3–7.9, with no change in the average chain length of the insoluble polymer.

It will be noted from Table IV that the rate of ammonia liberation from Gly.Phe(NH_2) is much less (E/v_D = 6.0 at 50 μmoles/ml) than that with Ala.Phe(NH_2) (cf. Table III). Of special interest is the fact that whereas the enzymic hydrolysis observed with Ala.Phe(NH_2) was negligible at pH 7.5, that found with Gly.Phe(NH_2) for a comparable rate of polymer formation was appreciable (Table IV).

Polymerization of Gly.Tyr(NH_2).—The effect of enzyme concentration on polymer yield from Gly.Tyr(NH_2) is shown in Table I. It will be noted that the polymer yield is much lower than in the case of Ala.Phe(NH_2), and furthermore that, at high enzyme concentrations, a decrease in yield is observed. Since it had been shown that cathepsin C exerts considerable hydrolytic action on Gly.Tyr(NH_2) and Gly.Tyr.Gly.Tyr(NH_2) at pH values near 7.5 (Fruton and Knappenberger, 1962), this decrease in polymer yield may be attributed to such a hydrolytic effect. The inverse proportionality observed between enzyme concentration and lag time of polymer appearance in the case of Ala.Phe(NH_2) does not hold for Gly.Tyr(NH_2) over the entire range of enzyme concentration tested. However for a given concentration (1.8 units/ml) the lag period is essentially independent of substrate concentration over the range 0.025–0.1 M. As in the case of the other two substrates mentioned above, th pH optimum for polymer formation appears to be near pH 7.6.

TABLE IV
POLYMERIZATION OF GLYCYL-L-PHENYLALANINAMIDE BY CATHEPSIN C[a]

Time (min)	NH_3 Liberation (μmoles/ml)	Carboxyl Liberation (μeq/ml)	Polymer Yield[b] (μmoles Phe/ml)	Phe/NH_3 Ratio in Polymer Hydrolysate
30	23	6	10	4.0
60	31	9	13	3.7
120	38	15	19	4.1
180	40	14	18	4.1

[a] Substrate concentration, 0.05 M; enzyme concentration, 4.5 units/ml; β-mercaptoethylamine, 0.01 M; pH 7.6, adjusted with 0.1 N NaOH; temperature 37.5°. [b] It will be noted that the yield of insoluble polymer is much less than that expected from the extent of transamidation (NH_3 liberation–carboxyl liberation). This is similar to the case of the polymerization of Gly.Tyr(NH_2), where soluble polypeptides were demonstrated by electrophoretic analysis (Würz *et al.*, 1962). Such electrophoretic experiments were not done in the present case with Gly.Phe(NH_2).

The data in Table I indicate that the average chain length of the insoluble polymer from Gly.Tyr(NH_2) is that of an octapeptide amide, rather than that of a decapeptide amide as reported previously (Fruton *et al.*, 1953). Because of the concordance of the analytical data obtained by spectrophotometry and by automatic chromatographic analysis, the available evidence favors the conclusion that the insoluble product is largely octapeptide amide.

In Table V data are given on the initial rates of de-

TABLE V
KINETICS OF POLYMERIZATION OF GLYCYL-L-TYROSINAMIDE[a]

Substrate Concn (μmoles/ml)	v_D[b]	v_P[c]	v_P/v_D
25	0.33	0.030	0.091
50	0.66	0.057	0.086
75	0.80	0.073	0.091
100	0.93	0.080	0.086

[a] Enzyme concentration, 1.8 units/ml; β-mercaptoethylamine, 0.01 M; pH 7.7, adjusted with 0.1 N NaOH; temperature 37.5°. In all cases the lag period before appearance of polymer was 32 ± 3 min. [b] μMoles of NH_3/ml liberated per minute during first 30 minutes of reaction. [c] Amount of insoluble polymer/ml (expressed as μmoles of Tyr) formed per minute during initial period of formation of insoluble polymer (30–60 minutes after start of incubation).

amidation and polymer formation, and it is evident that the amount of NH_3 liberated per unit time is far greater than theory for the amount of octapeptide amide formed, although there appears to be a proportionality between the two rates. Comparison of the v_P/v_D ratios given in Table V and in Table III shows that the efficiency of cathepsin C is much lower for the polymerization of Gly.Tyr(NH_2) than for Ala.Phe(NH_2), and may be attributed to the greater extent of hydrolysis observed with Gly.Tyr(NH_2) and to the formation of

appreciable amounts of soluble intermediates, of which the tetrapeptide amide Gly.Tyr.Gly.Tyr(NH_2) predominates (Würz *et al.*, 1962).

Polymerization of Gly.Trp(NH_2).—Earlier studies (Würz *et al.*, 1962) had shown that the polymerization of Gly.Trp(NH_2) proceeds with considerable efficiency with the formation of a product that is, on the average, an octapeptide amide. In the experiments performed by Würz *et al.* (1962) the DL-form of the substrate was employed; in the present studies the L form (in the form of its *p*-toluenesulfonate) was used, after it became available (Theodoropoulos and Fruton, 1962). Over the enzyme concentration range studied, there is inverse proportionality between enzyme concentration (in units/ml) and duration of the lag period (in minutes), the arithmetical product being about 6.3. This value may be compared with 2.7, 32, and *ca.* 55 for Ala.Phe(NH_2), Gly.Phe(NH_2), and Gly.Tyr(NH_2), respectively. The fact that the efficiency of the polymerization of Gly.Trp(NH_2) is comparable to that of Ala.Phe(NH_2) is also indicated by the data in Table VI; from the data on the rate of deamidation, an approximate value of 0.04 M was calculated for K_m under the conditions of the study.

TABLE VI
KINETICS OF POLYMERIZATION OF GLYCYL-L-TRYPTOPHANAMIDE[a]

Substrate Concn (μmoles/ml)	Enzyme Concn (units/ml)	v_D[b]	E/v_D	v_P[c]	v_P/v_D
25	0.9	0.23	3.9	0.13	0.57
50	0.9	0.45	2.0	0.27	0.60
50	1.8	1.0	1.8	0.42	0.42
75	0.9	0.62	1.45	0.32	0.52
100	0.9	0.63	1.4	0.47	0.75

[a] β-Mercaptoethylamine, 0.01 M; *p*H 7.8, adjusted with 0.1 N NaOH; temperature, 37.5°. [b] μMoles of NH_3/ml liberated per minute during first 30 minutes of incubation. [c] Amount of insoluble polymer/ml (expressed as μmoles of Trp) formed per minute during initial period of formation of insoluble polymer (60 minutes after start of reaction).

DISCUSSION

Previous work in this laboratory had shown that the action of cathepsin C near *p*H 7.5 on dipeptide amides such as Gly.Phe(NH_2), Gly.Tyr(NH_2), Gly.Trp(NH_2), and Ala.Phe(NH_2) leads, in each case, to the formation of a sparingly soluble polymeric peptide apparently having as a repeating unit the dipeptide residue present in the original substrate. It was suggested (Würz *et al.*, 1962) that the enzyme catalyzes the polymerization of its substrates through successive transamidation reactions with the intermediate formation of acyl-enzyme intermediates. The results presented in the present communication are in accord with this working hypothesis.

In the polymerization of Ala.Phe(NH_2), both the extent of ammonia liberation and the amount of insoluble polymer formed are in satisfactory agreement with the over-all equation for the conversion of the dipeptide amide to a hexapeptide amide by two successive transamidation reactions. At relatively high enzyme concentrations, the yield of insoluble polymer approached 100% of theory. These results indicate that little, if any, of the soluble intermediate Ala.Phe.Ala.Phe(NH_2) should accumulate during the polymerization reaction. Furthermore, since Ala.Phe(NH_2) does not undergo measurable hydrolytic cleavage at *p*H values near 7.5, there should not be much Ala.Phe either. Electrophoretic analysis of the supernatant fluids obtained from incubation mixtures, according to the procedure of Würz *et al.* (1962), showed only trace amounts (below the level of accurate quantitative estimation) of the tetrapeptide amide and the free dipeptide.

A convenient measure of the efficiency of the polymerization reaction is the ratio of the rates of polymer formation and of ammonia liberation (v_P/v_D), and the data presented above indicate that Gly.Trp(NH_2) approaches Ala.Phe(NH_2) in the efficiency with which it is polymerized by cathepsin C. On the other hand, the polymerization of Gly.Phe(NH_2) or Gly.Tyr(NH_2) proceeds at a markedly lower efficiency, largely because these two substrates (or intermediates derived from them) are subjected to measurable hydrolysis at *p*H values near 7.5. On the basis of the hypothesis that the action of cathepsin C involves the intermediate formation of a dipeptidyl-enzyme, the yield of polymer will depend on the extent to which the acyl-enzyme compounds are cleaved by water instead of an amine acceptor in a transamidation reaction. The efficiency of the polymerization may also be expected to depend on the tightness with which the growing chain is held in the catalytic region of the enzyme. If, as in the case of the polymerization of Gly.Tyr(NH_2), the intermediate tetrapeptide amide is readily released, it must be cleaved to active dipeptidyl units for reincorporation into the growing chain (Fruton and Knappenberger, 1962).

The earlier conclusions that the polymeric products contain the dipeptidyl group as a repeating unit were based on analytical determinations, in hydrolysates of the polymers, of the ratio of aromatic amino acids to ammonia, and by analysis of partial hydrolysates by paper chromatography. The present study provides more definitive data on this question, since quantitative automatic amino acid analysis shows that the polymers contain equivalent amounts of the constituent amino acids, and simultaneous determination of the ammonia in the hydrolysate permits an estimate to be made of the chain length. Whereas Ala.Phe(NH_2) yields a hexapeptide amide, the predominant product from Gly.Tyr(NH_2) or Gly.Phe(NH_2) is an octapeptide amide. The chain length of the insoluble polymers is relatively constant throughout the incubation period, and it would appear that dipeptidyl units are not added to the peptide chain after the product has separated from the solution.

In this study it was found that the lag period which precedes the initial appearance of polymer under a given set of conditions is quite reproducible. Within the ranges studied, this lag period is inversely proportional to the enzyme concentration and independent of the substrate concentration. This behavior may be interpreted as indicating the gradual synthesis of the polymer until a concentration is reached that exceeds its solubility. At the initial stages of the reaction, where the substrate concentration is high, the rate of this process may be expected to be proportional only to the concentration of enzyme, other factors being held constant.

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A Proton Magnetic Resonance Study of the Stereochemistry of the Methylaspartate Ammonia-Lyase Reaction*

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The *threo* and *erythro* diastereoisomers of 3-methylaspartic acid were converted to the two corresponding *N*-acetyl anhydrides and the structures of the anhydrides were determined by comparing their proton magnetic resonance spectra with that of *O*-acetyl-2-L-3-deuteriomalic anhydride of known (*erythro*) configuration. The latter compound was prepared from 2-L-3-deuteriomalate which was in turn formed from fumarate in deuterium oxide in the presence of fumarase. The structure of the 3-deuteriated L-aspartate formed by methylaspartate ammonia-lyase from fumarate and ammonia in deuterium oxide was determined in a similar manner. The 2-L-3-methylaspartate isomer which is 100 times more reactive with methylaspartate ammonia-lyase has the *threo* configuration and the 2-L-3-deuterioaspartate formed by methylaspartate ammonia-lyase from fumarate and ammonia in deuterium oxide has the *erythro* configuration. Therefore the preferred overall stereochemistry of the elimination and addition of ammonia catalyzed by methylaspartate ammonia-lyase is *trans*.

Methylaspartate ammonia-lyase catalyzes the reversible conversion of 3-methylaspartate to mesaconate and ammonia (Barker *et al.*, 1959) as shown in Figure 1. These authors showed that L-aspartate can also serve as a substrate. The enzyme, which was later crystallized (Bright and Ingraham, 1960), is active with two of the four optical isomers of 3-methylaspartate and it was shown that both reactive isomers having the 2-L configuration (Barker *et al.*, 1958; Benoiton *et al.*, 1959). However, the evidence which was used to assign the *threo* configuration to the 2-L-diastereoisomer, which is the more reactive in terms of V_{max} in the methylaspartate ammonia-lyase–catalyzed reaction by a factor of 100, was derived from an application of the principle of optical superposition of model compounds and was not conclusive (Barker *et al.*, 1958). Because a knowledge of the preferred stereochemistry of an elimination or addition reaction has a certain usefulness in the discussion of mechanism, we wish to report the determination by proton magnetic resonance (PMR)[1] of the configuration of the internal anhydrides of the 2-L-3-methylaspartic acids and of the internal anhydride of the 3-deuteriated L-aspartic acid which is obtained when methylaspartate ammonia-lyase catalyzes the addition of ammonia to fumarate in deuterium oxide.

Cyclic anhydrides were used so that the stereochemistry could be determined unambiguously from the relative configuration of the protons on the 2- and 3-carbons. In a previous high-resolution PMR study of the product of the fumarase-catalyzed addition of D_2O to fumarate, 2-L-3-deuteriomalic acid (Alberty and Bender, 1959), the relative displacement of the carboxyl groups was assumed to be *trans*, and it seemed to us that this assumption might explain the surprising spin-spin coupling constants that were reported. However, the subsequent stereospecific synthesis of this compound (Gawron and Fondy 1959; Gawron *et al.*, 1961; Anet, 1960) showed that the stereochemistry of the deuteriomalate which had been determined in an earlier solid-state PMR study (Farrar *et al.*, 1957) was incorrect, probably as the result of the assignment of *trans* configuration to the carboxyls in the solid. With the revised overall configuration, the high resolution data became consistent with other PMR results on the basis of the assumed carboxyl configuration.

Since the internal anhydride of 3-methylaspartic acid is a presumably planar five-membered ring, its stereochemistry can be established by determining whether the dihedral angle subtended by the protons on the 2- and 3-carbons is approximately 0 or 120 degrees. In this study the magnitude of the spin-spin coupling between these protons, which is known to be very sensitive to configuration (Conroy, 1960), has been used to define this angle. Because the configuration of the 3-deuteriomalate obtained via the fumarase reaction has been conclusively proved to have the *erythro*-L- configuration, its anhydride was used as a model compound for a zero-degree dihedral angle. *O*-

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‡ Reference to a company or product does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

[1] Abbreviations used in this work: PMR, proton magnetic resonance; D_6-acetone, 99% deuteriated acetone.