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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.

Acetylmalic anhydride and *N*-acetylaspartic anhydride provided reference values for both 0 and 120° to compare with the two *N*-acetyl-3-methylaspartic anhydrides.

Materials

Mesaconic acid was obtained from H. M. Chemical Co., Ltd., Santa Monica, Calif. This material was recrystallized from water, after charcoal treatment, until the melting point was at least 200°. Fumaric and DL-malic acids were obtained from Eastman Organic Chemicals, Rochester 3, N. Y. L-Aspartic acid was obtained from California Corp. for Biochemical Research, Los Angeles 63, Calif. D_2O, 99.8%, was obtained from Bio-Rad Laboratories, Richmond, Calif.

Methods

Each *N*-acetylamino acid anhydride was synthesized from the appropriate amino acid by the method of C. C. Barker (1953) using the suggested molar proportions. Yields were generally from 30 to 50%. The calculated water weights given below for deuteriated compounds are based on one deuterium atom per mole.

N-Acetyl-2-L-3-methylaspartic anhydride (I) was synthesized from the 2-L-3-methylaspartic acid which had been isolated according to the method of Baker and Smyth (1961) after the reaction between ammonia and mesaconate, in the presence of methylaspartate ammonia-lyase, had come to equilibrium. Compound I is therefore the anhydride of the L-3-methylaspartic isomer which is preferred by the enzyme, mp 156.0.

Anal. Calcd for $C_7H_9O_4N$: C, 49.12; H, 5.26; N, 8.10. Found: C, 49.13; H, 5.31; N, 8.11.

N-Acetyl-2-DL-3-methylaspartic anhydride (II) was synthesized from the most soluble fraction obtained in the modified Dakin procedure for the synthesis of 3-methylaspartic acid (Barker *et al.*, 1959). Paper electrophoresis (Barker *et al.*, 1959) and examination of the PMR spectrum of the anhydride showed this fraction to be free of the other, more soluble diastereoisomer. The 2-L component of racemate II is therefore the anhydride of the 2-L-3-methylaspartic isomer which was shown by Barker *et al.* (1959) to be the 100-times-less-active substrate for methylaspartate ammonia-lyase.

Anal. Calcd for $C_7H_9O_4N$: C, 49.12; H, 5.26; N, 8.19. Found: C, 49.06; H, 5.50; N, 8.04.

N-Acetyl-L-aspartic anhydride[2] was synthesized from L-aspartic acid, mp 174.5–176.5°.

Anal. Calcd for $C_7H_9O_4N$: C, 45.86; N, 4.49; H, 8.92. Found: C, 45.93; H, 4.49; N, 8.78.

N-Acetyl-2-L-3-deuterioaspartic anhydride (III). 2-L-3-Deuterioaspartic acid was obtained by allowing methylaspartate ammonia-lyase to add ammonia to fumarate in D_2O. The experimental procedure was identical to that used for the enzymatic synthesis of 3-methylaspartic acid from mesaconate and ammonia in H_2O (Barker and Smyth, 1961) except that all reagents were added as solids. Replacement of exchangeable hydrogens with deuterium, other than those in the fumaric acid and ammonia, was deemed unnecessary. Diammonium fumarate was obtained by neutralizing a saturated solution of fumaric acid in H_2O with concentrated ammonia. The salt was crystallized twice from D_2O before use. The deuteriated L-aspartic acid was crystallized at *p*H 3.1, after the addition of an equal volume of ethanol, and then recrystallized from H_2O. The material was then converted to *N*-acetyl-2-L-3-deuterioaspartic anhydride by the method of C. C. Barker (1953); mp 169–171°.

[2] No racemization occurred, as evidenced by the melting point; cf. Cohen and Khedouri (1961).

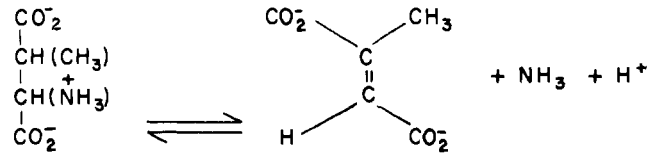

FIG. 1.—The reaction catalyzed by methylaspartate ammonia-lyase.

Anal. Calcd for $C_6H_6DO_4N$: C, 45.56; water wt, 1.85 mg; N, 8.86. Found: C, 45.54; water wt, 1.81 mg; N, 8.76.

Ring closure in the amino acids was always accompanied by a qualitatively predictable shift of the carbonyl-stretching frequency from 5.97 μ in the free amino acids to bands at 5.33, 5.58, and 5.76 μ in the anhydrides. These are probably due to a coupling of the carbonyl frequencies through the skeleton of the ring.

The *O*-acetylhydroxy acid anhydrides were synthesized from the appropriate free acids by the method of Anschütz (1881) with suitable modifications described. Yields were generally 20–40%.

O-Acetyl-*erythro*-2-L-3-deuteriomalic anhydride was synthesized from the *erythro*-2-L-3-deuteriomalic acid obtained when fumarase was allowed to hydrate fumaric acid in D_2O according to the method of Farrar *et al.* (1957) using a total volume of 100 ml. This malic acid, however, was not isolated. Instead, 25 g of Dowex 50 (acid form) was shaken with the reaction mixture and filtered off. This acid solution was then evaporated to dryness and treated with acetyl chloride as described by Anschütz (1881). The anhydride distilled at 152.5° (6.5 mm). No attempt was made to crystallize the gum so formed and elemental analysis was performed on material which had been twice distilled.

Anal. Calcd for $C_6H_5DO_5$: C, 45.28; water wt, 2.10 mg. Found: C, 45.75; water wt, 2.04 mg.

O-Acetyl-2-L-3-deuteriomalic anhydride (IV) was synthesized from the 2-L-3-deuteriomalic acid which had been obtained from 2-L-3-deuterioaspartic acid (the product of the methylaspartate ammonia-lyase–catalyzed addition of ammonia to fumarate in D_2O), by the method of Whitmore and Langlois (1938). However, the PMR spectrum of this anhydride revealed that about 20% racemization had occurred in the conversion of the 2-L-3-deuterioaspartic acid to 2-L-3-deuteriomalic acid. The amount of compound IV obtained was insufficient for elemental analysis. However, the compound was readily identified by its PMR spectrum (see Results) and infrared spectrum (*vide infra*); bp 152.5° (6.5 mm).

O-Acetyl-DL-malic anhydride was synthesized from DL-malic acid. This anhydride could be crystallized from chloroform; mp 79–81°.

Anal. Calcd. for $C_6H_6O_5$: C, 45.57; H, 3.73. Found: C, 45.51; H, 3.89.

As was the case for the *N*-acetylamino acid anhydrides, ring closure of the hydroxy-acids was always accompanied by a characteristic shift of the carbonyl-stretching frequencies, namely, 5.31, 5.56, and 5.70 μ. These shifts were identical for the following anhydrides: *O*-acetyl-DL-malic, *O*-acetyl-L-malic, *O*-acetyl-*erythro*-2-L-3-deuteriomalic from the fumarase reaction in D_2O, and compound IV. Also, the spectra of *O*-acetyl-*erythro*-2-L-3-deuteriomalic anhydride and compound IV were identical from 2.5 to 15 μ.

Proton Magnetic Resonance.—A Varian high-resolution HR-type spectrometer system operating at 60 Mc was used in this work. The most generally useful solvent for cyclic acetyl anhydrides was found to be deuteriated acetone (99%). When this solvent was

TABLE I
RING COUPLING CONSTANTS IN CYCLIC ANHYDRIDES[a]

Acetyl Anhydride	Interaction (in cps) J_{12}	J_{23}	$J_{23'}$	$J_{33'}$	J_{3D}
I (*threo*-2-L-3-Methylaspartic)	7.4		8.0		
II (*erythro*-2-DL-3-Methylaspartic)	7.3	10.2			
erythro-2-L-3-Deuteriomalic		9.7		(17.6)	2.7
DL-Malic		9.4	5.8	18.8	
III (*erythro*-2-L-3-Deuterioaspartic)	7.6	10.4		(17.6)	2.7
L-Aspartic	7.0	9.8	6.2	18.0	

[a] At 60 Mc in D_6-acetone. For proton nomenclature, see footnote 4. J_{3D} is the coupling constant for the proton and deuteron on the 3-carbon.

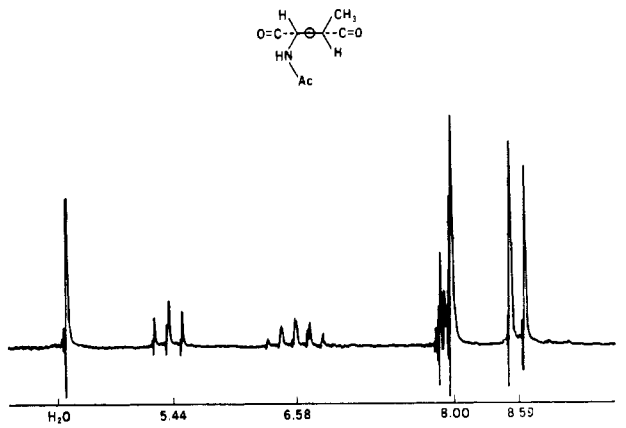

FIG. 2.—PMR spectrum of the *N*-acetyl anhydride I in deuteriated acetone. (Prepared from the 2-L-3-methylaspartic acid isomer preferred by the enzyme.) Included is the configuration of compound I which has been proved in this study (see text).

unavailable or when certain spin-spin couplings had to be observed, trifluoroacetic acid was used. The anhydrides appeared to be stable in this solvent for several hours.

All spectra were internally referenced to 1% tetramethylsilane, and peak positions were determined by interpolation using the standard modulation-sideband technique. Several spectra were averaged for each compound. Shielding values are believed to be accurate to within 0.02 τ while the peak separations on which the coupling constant determinations are based could be reproduced to 0.2 cycle/sec. It is difficult to estimate the additional uncertainty introduced by the ABX calculation[3] in the case of malic and aspartic anhydrides, but it should in no case exceed 0.2 cycle/sec.

RESULTS

Proton Magnetic Resonance Studies

N-Acetyl-3-methylaspartic Anhydrides.—Figure 2 shows the spectrum of the anhydride I (from the isomer preferred by the enzyme).[4] The low-field singlet comes from water in the deuteriated acetone. The multiplet centered at 5.44 τ can be assigned to H(2) on the basis of its field position and because its triplet structure shows that it is roughly equally spin-spin coupled to two protons on adjacent atoms. Likewise, H(3) (see Discussion) can be assigned to the band at 6.58 τ

[3] A spin-coupling pattern. For explanation see J. A. Pople, W. G. Schneider, and H. J. Bernstein, *High-Resolution Nuclear Magnetic Resonance*, New York, McGraw-Hill, 1959; or J. D. Roberts, *An Introduction to Spin-Spin Splitting in High-Resolution Nuclear Magnetic Resonance Spectra*, New York, W. A. Benjamin, 1961.

TABLE II
PROTON-PROTON COUPLING CONSTANTS OF POTASSIUM *threo*-2-L-3-METHYLASPARTATE AND SEVERAL MODEL COMPOUNDS[a]

Compound	Interaction (in cycles/sec) J_{23}	$J_{23'}$	$J_{33'}$
threo-2-L-3-Methylaspartate[b]		4.0	(J_{34} = 7.5)
L-Malic acid[c]	7.5	4.1	15.7
L-Malic acid[d]	7.1	4.4	17.1
L-KH malate[d]	8.5	3.2	16.4
L-K_2 malate[d]	9.7	3.1	15.3
erythro-2-L-3-Deuteriomalic acid[e]	7.1		
erythro-2-L-3-Deuteriomalic-K_2 malate[d]	9.4		

[a] At 60 Mc. See footnote 4 for nomenclature. [b] In D_2O (dianion). Shielding constants H(2), 6.14, and H(3'), 7.14. [c] In D_6-acetone. Shielding constants H(2), 5.46; H(3), 7.13; and H(3'), 7.27. [d] Alberty and Bender (1959) in D_2O. Assignments corrected for presently accepted configuration of the deuteriomalate. [e] In D_6-acetone.

since only it could be equally coupled to four protons on adjacent atoms. (The proton of pentadeuteriated acetone, which is coupled to two deuterons on the same carbon, is responsible for the incompletely resolved quintet just below 8.00 τ.) The sharp singlet at 8.00, typical of acetyl methyl protons, is assigned to H(5). The 7.2 cycles/sec doublet splitting of the band at 8.59 τ shows that there is only a single proton on an adjacent atom, and on this basis as well as from its area and position it can be assigned to H(4). To low field, outside the interval shown in Figure 2, a very broad band without structure, which represents one proton, is found at 1.80 τ. Such a peak is characteristic of protons attached to nitrogen in a sufficiently nonuniform electric field so that quadrupolar relaxation of the nitrogen-14 nucleus partially, but not totally, averages the otherwise quite large (60 cycles/sec) proton-nitrogen-14 spin-spin coupling.

The spectrum of the synthetic 2-DL-anhydride II can be similarly assigned. These and all subsequent assignments are summarized in Tables I, II, and III.

Figure 3 shows on an expanded field scale the H(2) and H(3 and 3') multiplets of the anhydrides I and II.

[4] In this discussion the anhydride protons will be designated as follows: on the nitrogen (when present), H(1); on the 2-carbon, H(2); on the 3-carbon when *cis* to H(2), H(3), and when *trans*, H(3'); the 3-methyl protons, H(4); and the acetyl protons, H(5). Assignment of the 3-carbon protons as 3 and 3' was possible only after inspection of the magnitude of the coupling constant J_{23} obtained from *O*-acetyl-*erythro*-2-L-3-deuteriomalic anhydride (see Discussion).

TABLE III
EFFECT OF CONFIGURATION ON THE PROTON-SHIELDING VALUES OF SOME CYCLIC ANHYDRIDES[a]

Anhydride	Form	Shielding Values (in τ units)					
		H(1)	H(2)	H(3)	H(3′)	H(4)	H(5)
N-Acetyl-3-methyl-aspartic	*threo*-2-L-	1.80	5.44		6.59	8.59	8.00
	erythro-2-DL-	1.76	5.29	6.54		8.83	8.00
O-Acetylmalic	*erythro*-2-L-3-deuterio		4.30	6.54			7.85
	2-DL-3-deuterio		4.24	6.51	6.79		7.86
N-Acetylaspartic	*erythro*-2-L-	1.80	5.24	6.70			8.04
	2-L-	1.80	5.23	6.66	6.96		

[a] At 60 Mc in d_6-acetone. See footnote 4 for nomenclature.

It is obvious from the doublet-of-doublets pattern of the H(2) band and the doublet-split quartet structure of the H(3) band of compound II that the H(2)-H(3) coupling is considerably larger than that of compound I. Also, the H(2) "triplet" of the anhydride of the preferred enzyme substrate is actually also a doublet of doublets.

In trifluoroacetic acid stronger electric-field gradients cause a much enhanced quadrupole relaxation. This results, in many cases, in a complete averaging of the spin-spin coupling between a proton and a nucleus having a quadrupole moment, such as nitrogen-14 (Tiers, 1959), leaving clearly resolved any proton-proton splitting of the signal. When anhydride I was examined in this solvent, the H(1) peak was sufficiently sharpened so that spin-spin splitting caused by H(2) could be observed. In this case J_{12} was 6.7 cps, and $J_{23'}$ and J_{34} were both 7.5 cps (see discussion of deuterioaspartic anhydride spectrum).

O-Acetylmalic Anhydrides.—Figure 4 shows the spectra of the fumarase-derived *erythro*-2-L-3-deuteriomalic and the normal DL-malic anhydrides. The interpretation of the spectrum of the deuteriated compound is similar to that for the 3-methylaspartic anhydrides and entirely straightforward. The width of the components of the H(2) doublet is caused by the unresolved vicinal spin-spin coupling to the *trans* deuteron on the 3-carbon.

In order to obtain reasonably accurate values for the spectral parameters of the normal malic anhydride at least an ABX-type analysis (Bernstein *et al.*, 1957) is required since the chemical shift of H(3) from H(3′) is not large as compared to $J_{33'}$.[5]

The 2-L-3-deuteriomalic anhydride IV obtained from 2-L-3-deuterioaspartate, which was in turn formed by the methylaspartate ammonia-lyase–catalyzed addition of ammonia to fumarate in D_2O (see Methods), gave a spectrum identical to that obtained from the authentic *erythro* form.

N-Acetylaspartic Anhydrides.—Figure 5 shows the spectrum of L-aspartic anhydride and the spectra in both deuteriated acetone and trifluoroacetic acid of the 2-L-3-deuterioaspartic anhydride III obtained from the product of the methylaspartate ammonia-lyase–catalyzed addition of ammonia to fumarate in D_2O. Again the interpretation for the deuteriated form is straightforward so only the results are given. The broad peak on the high-field shoulder of the triplet-split doublet (the triplet splitting is owing to a geminal proton-deuteron coupling) at 6.70 τ is caused by water in the acetone. In trifluoroacetic acid the H(1) peak is "relaxation sharpened" so that the H(1)-H(2) interaction is resolved as discussed previously. For the same reason, however, the H(3) peak is broadened as in this case the relaxation of the deuteron has been increased sufficiently to smear out the geminal H(3)-D interaction, but not sufficiently to completely average to interaction to zero. The peak assignments are obvious from a comparison of the two spectra. In trifluoroacetic acid the spin-spin couplings in cycles/sec are: $J_{12} = 6.7$ and $J_{23} = 9.8$.

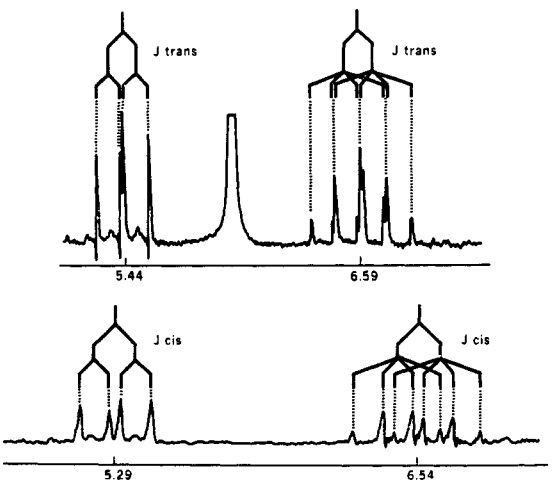

FIG. 3.—Proton splitting in the PMR spectra of the *N*-acetyl anhydrides I (upper spectrum) and II (lower spectrum) in deuteriated acetone. Anhydride I was prepared from the 2-L-3-methylaspartic isomer which is preferred by the enzyme, whereas anhydride II was prepared from a 2-L-3-methylaspartic racemate, the 2-L- component of which is a poor substrate for the enzyme. The basis for the *trans*- and *cis*-coupling-constant assignments is discussed in the text.

As was the case for the undeuteriated malic anhydride, a more detailed analysis of the H(2) and H(3 and 3′) multiplets in the aspartic anhydride spectrum is required. Although H(1), H(2), H(3), and H(3′) form an ABKY system, we have analyzed the H(2) multiplet as the X portion of an ABX system with an additional first-order splitting of each peak by the coupling to H(1). This treatment appears justified on the basis of earlier work (Abraham and Bernstein, 1961; Banwell and Sheppard, 1961) especially since H(1) is coupled only to H(2). (To further check this assignment the anhydride was examined in trifluoroacetic acid. The splitting of the H(1) peak gave J_{12} = 6.8 cycles/sec.) The broad peak at 6.80 τ is again owing to water that was present in the solvent.

2-L-3-Methylaspartate Isomer from Which Compound I Was Prepared (i.e., the preferred substrate).—The potassium salt was examined in D_2O after neutralization with KOH. Because tetramethylsilane is totally insoluble in aqueous solutions, sodium 2,2-dimethyl-2-silapentane-5-sulfonate was used as an internal reference ($\tau' = 10.00$) as recommended for aqueous solutions (Tiers and Coon, 1961).

[5] For the highest accuracy, an ABK analysis would be required, but the requirements of this study did not seem to justify the additional computational effort.

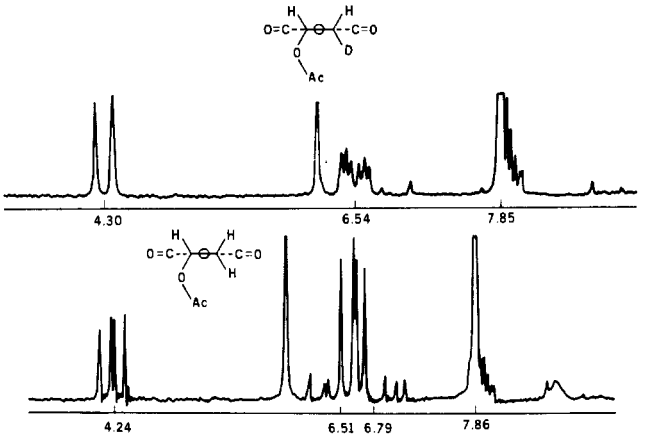

FIG. 4.—The PMR spectra of deuteriated and normal *O*-acetylmalic anhydrides in deuteriated acetone. The upper spectrum is of *O*-acetyl-*erythro*-2-L-3-deuteriomalic anhydride, prepared from deuteriomalic acid which was in turn formed from fumaric acid in deuterium oxide in the presence of fumarase. The lower spectrum is of *O*-acetyl-2-DL-malic anhydride.

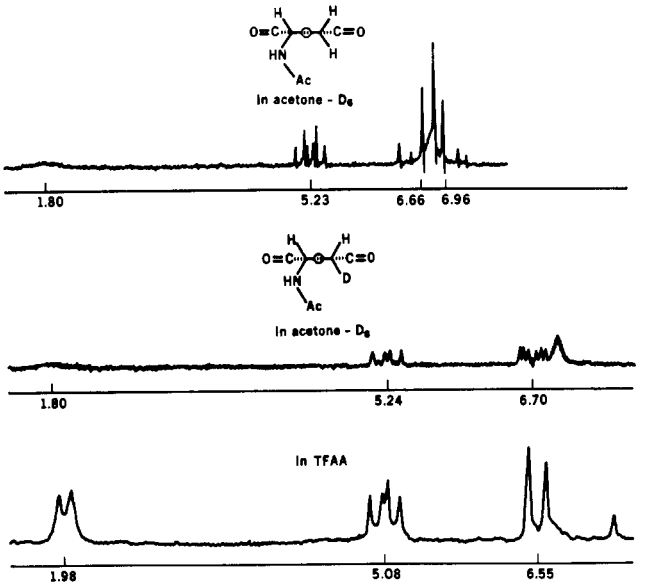

FIG. 5.—The PMR spectra of the *N*-acetyl-2-L-aspartic anhydrides in deuteriated acetone and in trifluoroacetic acid. The top spectrum is that of *N*-acetyl-2-L-aspartic anhydride while the center and lower traces are the spectra of *N*-acetyl-2-L-3-deuterioaspartic anhydride (III) prepared from the product of the methylaspartate ammonia-lyase–catalyzed addition of ammonia to fumarate in D_2O. The basis for the assigned configuration of compound III is discussed in the text.

L-*Malic Acid.*—Malic acid was run in deuterioacetone both in order to compare the coupling constants with those reported by Alberty and Bender (1959) and Krasna (1958) for malate in D_2O solutions and for comparison with the value for J_{23} obtained from the 2-L-3-methylaspartate isomer which is the preferred substrate.

DISCUSSION

Conclusion from PMR.—The H(2)-H(3) or -H(3′) coupling constants for all the anhydrides together with their known or assigned dihedral angles are given in Table I. The 9.7-cycles/sec coupling of H(2) and H(3) in the *O*-acetyl-*erythro*-2-L-3-deuteriomalic anhydride, where these protons are known to have an eclipsed configuration, agrees closely with the 9.4-cycles/sec coupling obtained from the undeuteriated malic anhydride, the 10.4-cycles/sec coupling from the deuteriated aspartic anhydride, the 9.8-cycles/sec value obtained from undeuteriated aspartic anhydride, and, most importantly, the 10.4 cycles/sec from the anhydride II prepared from the 2-L-3-methylaspartic acid isomer which is a poor substrate for methylaspartate ammonia-lyase. The chemically synthesized 2-DL-3-methylaspartic acid and the enzymatically produced form of deuterioaspartic acid on this basis can be assigned the *erythro* configuration. We conclude, therefore, that the preferred substrate form of 2-L-3-methylaspartic acid must be *threo*. This assignment is further confirmed by the finding that the 2-L-3-deuteriomalic acid prepared from deuterioaspartic acid, which was in turn formed by the methylaspartate ammonia-lyase–catalyzed addition of ammonia to fumarate in D_2O, is *erythro*. Our results, therefore, confirm the tentative *threo*- and *erythro*-3-methylaspartate assignments made by Barker *et al.* (1958).

The 8.0-cycle/sec coupling between H(2) and H(3′) on the *threo*-3-methylaspartic anhydride does not agree nearly so closely with the corresponding values of 6.2 cycles/sec for the undeuteriated aspartic anhydride or 5.8 cycles/sec for the undeuteriated malic anhydride. Its value of 7.5 cycles/sec in trifluoroacetic acid corresponds somewhat better but the reason for the discrepancy is not readily apparent. A consistent interpretation of the H(2), H(3′) and H(4) multiplets of the *threo*-3-methylaspartic anhydride spectrum is possible only if the 8.0-cycle/sec value is assigned to J_{23}'. The J_{12} values of both forms agree and are in reasonably good agreement with the 6.7-cycle/sec coupling for the *threo* in trifluoroacetic acid which can be determined directly.

Substituent electronegativity is known to have a pronounced influence on vicinal couplings (Williamson, 1963). The couplings for 120° reported by Williamson for a series of substituted bicycloheptenes vary from 4.6 to 2.5 cycles/sec and decrease approximately linearly with increasing electronegativity. For 0° his values, which agree reasonably well with ours, range from 9.3 to 7.6 cycles/sec. However, since the rate of change of coupling constant with substituent electronegativity was essentially the same for 0° and 120°, this study does not provide an explanation for the anomalous 120° value found for *threo*-3-methylaspartic anhydride. Perhaps it is significant that all the systems studied by Williamson were of the ABX type and our ABX values are reasonably close to his. However, in a recent exhaustive review of vicinal-coupling constants in substituted cyclohexanes (Huitric *et al.*, 1963) no such simple dependence on electronegativity was observed. The variations in *gauche* and *trans* couplings could not be satisfactorily explained either on the basis of changes in bond angle or substituent electronegativity.

A similar variation to that reported here has been observed in the 0° and 120° vicinal couplings of cyclopropane derivatives (Patel *et al.*, 1963). *Trans* couplings vary from 5.4 to 8.0 cycles/sec while *cis* couplings are found in the interval from 8.0 to 11.2 cycles/sec.

As is shown in Table II, Alberty and Bender observed a marked dependence of their 180° coupling and to a lesser extent of the 60° coupling in malic acid on the degree of neutralization of the acid. While this factor cannot be operative in acetone, it does indicate the sensitivity of coupling constants to slight changes of environment.

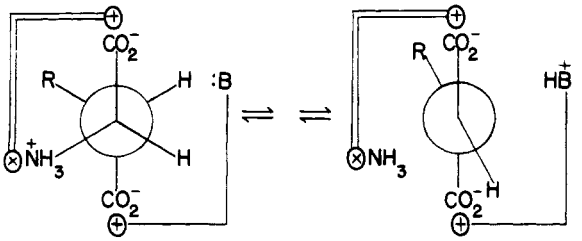

FIG. 6.—The preferred overall stereochemistry of the methylaspartate ammonia-lyase reaction. For *threo*-2-L-3-methylaspartate R is methyl, and for L-aspartate R is hydrogen.

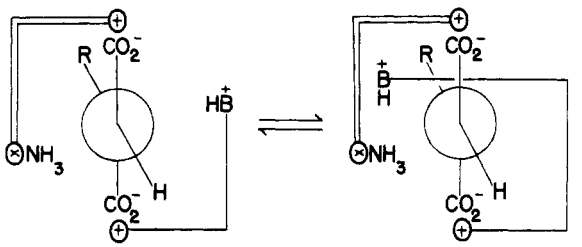

FIG. 7.—Possible conformational change in methylaspartate ammonia-lyase. Conformer A would give rise to *threo*-2-L-3-methylaspartate, while conformer B would give rise to *erythro*-2-L-3-methylaspartate.

The equation relating vicinal-coupling constants to dihedral angle which was developed by Karplus (1959) using a valence-bond approach was apparently moderately successful in predicting a wide variety of constants (Conroy, 1960), but recent studies (Huitric *et al.*, 1963; Williamson, 1963) have emphasized its limitations. Our results are a further indication of the caution which should be exercised in its use since there appears to be no reason for assuming here that the bond angles in the *threo* compound are greatly altered.

A further confirmation of the *threo* configuration assigned to the enzymically more reactive 2-L-3-methylaspartate isomer is provided by a comparison of its coupling constants with those of malate. The malate values agree quite well with those obtained by Alberty and Bender (1959) and Krasna (1958). If the carboxyls are *trans* in solution as all evidence now indicates, a *threo* configuration requires the protons to be *gauche* as is clearly shown by the data of Table II. A further check on the ABX analysis can be obtained by converting the proton-deuteron geminal coupling to the equivalent proton-proton coupling by the factor $\gamma H/\gamma D = 6.514$ (Shapiro *et al.*, 1963). These values for the anhydrides are shown in parentheses in Table I for the two mono-deuterio anhydrides. The agreement is excellent for the aspartic but less good for the malic anhydride.

Table III shows the effect of configuration on the shielding values for the anhydrides studied. In aspartic and malic anhydrides the resonance of H(3′), which is *cis* to the *N*- or *O*-acetyl, is shifted upfield from H(3) (*trans* to substituent), respectively, by 0.30 and 0.28 τ. In 3-methylaspartic anhydride the 3-methyl protons are likewise shifted upfield by a slightly lesser amount, 0.24 τ, as the methyl is shifted from a *trans* to a *cis* configuration. That the ring protons experience the same shift as the methyl protons would seem to indicate that the source of the *cis* shielding must be some distance removed from the 3-carbon. It seems reasonable, therefore, to ascribe this effect to the magnetic anisotropy of the acetyl carbonyl group since it is fairly far removed and is common to all the anhydrides, unlike the oxygen or nitrogen ring substituent. In order for the ring proton and methyl protons to fall within the "shielding cone" of the carbonyl (Jackman, 1960) the plane of the acetyl carbonyl would have to be, at least on the average, approximately perpendicular to the plane of the ring. Steric considerations would appear to favor such a configuration. Because of the constancy of the shielding, it seems to us less likely that it could be a through-space-induced electronic polarizability shift resulting from the negative-charge character of the nitrogen or oxygen.

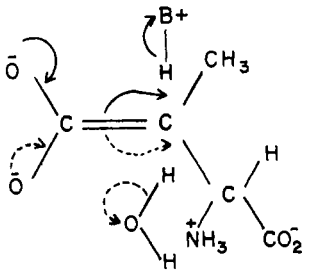

FIG. 8.—Partitioning of the intermediate enzyme-bound carbanion between an enzyme-donated proton and a solvent proton.

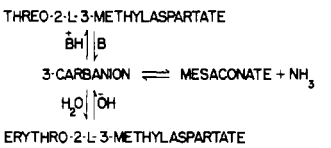

FIG. 9.—Proposed interconversions on enzyme involving partitioning of intermediate carbanion between the conjugate acid of the enzyme and a solvent proton.

The shielding of H(2) in 3-methylaspartic anhydride likewise increases when it is *cis* to the 3-methyl in the *threo* form, although in this case the effect is only one-half as large. This upfield shift can clearly be ascribed to the greater anisotropic shielding induced by the carbon-carbon bond.

The PMR data unequivocally demonstrate that (1) the isomer of 2-L-3-methylaspartate, which is more reactive with methylaspartate ammonia-lyase by a factor of 100, has the *threo* configuration; and (2) the 3-deuteriated isomer of L-aspartate, which is produced by methylaspartate ammonia-lyase from fumarate and ammonia in deuterium oxide, has the *erythro* configuration. The preferred overall stereochemistry of the methylaspartate ammonia-lyase reaction is therefore *trans* as depicted in Figure 6. An effort has also been made in Figure 6 to suggest the types and spatial arrangement of groups at the active center of the enzyme which could control the stereochemistry of the reaction. These groups consist of a base, :B, which would extract the 3-proton, and a positive group, ⊕, to bind the 3-carboxylate group of the substrate. These groups at the active center have been tentatively assigned as the sulfhydryl anion and the divalent metal activator, respectively (Bright and Silverman, 1964; Bright, 1964). In addition it is postulated that a positive group ⊕ must bind the 2-carboxylate and that another group ⊗ at the active center binds the 2-ammonium group.

The finding that the preferred overall stereochemistry of the reaction is *trans* can be used to argue against the possibility that the mechanism of the reaction involves initial displacement of ammonia from substrate by a nucleophile at the active center followed by concerted elimination of the nucleophile and the 3-proton. Such a mechanism, with *threo*-2-L-3-methylaspartate as sub-

strate, would involve concerted *cis* elimination. Since concerted eliminations are predominantly *trans* (Gould, 1959), this possibility is unlikely.

The ability of the enzyme to form both *erythro*-2-L-3-methylaspartate and *threo*-2-L-3-methylaspartate from mesaconate could be owing to (1) a change in conformation of the enzyme such that the base is moved in space relative to the other three groups as depicted in Figure 7 (the relative rates of formation of the two amino acid isomers from mesaconate would therefore depend in part on the fraction of each enzyme conformer present); or (2) racemization of a 3-carbanion intermediate in the reaction as depicted in Figure 8.

Considerable evidence has been adduced to show that the mechanism of the methylaspartate ammonia-lyase reaction involves a carbanion intermediate (Bright, 1961; Bright *et al.*, 1964; Bright, 1964). A partitioning of this intermediate carbanion between the conjugate acid of the enzyme base and a proton source in the solvent (a water molecule or a buffer component) can be pictured as in Figure 8.

The partitioning of the intermediate carbanion could occur at a rigid active center and the overall mechanism shown in Figure 9 would hold. The *p*H dependence of the formation of the *erythro* isomer according to this mechanism would probably be quite different from the *p*H dependence of the formation of the *threo* isomer. It appears, therefore, that racemization of a carbanion intermediate may be distinguished experimentally from the mechanism involving a conformational change in the enzyme.

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