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The interaction of β-*N*-methylamino-L-alanine with bicarbonate: an ^{1}H-NMR study

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The mode of action of the neurotoxic, non-protein amino acid β-*N*-methylamino-L-alanine (L-BMAA) is unknown. We have shown, using ^{1}H-NMR spectroscopy, that L-BMAA forms a stable adduct with bicarbonate (probably a carbamate). The properties of this adduct may explain the observation that L-BMAA and *N*-methyl-D-aspartic acid appear to act at the same central nervous system receptors.

Methylamino-L-alanine, β-*N*-; Bicarbonate; Carbamate; NMR, ^{1}H-; Methyl-D-aspartate, *N*-

1. INTRODUCTION

The neurotoxicity of the non-protein amino acid β-*N*-methylamino-L-alanine [1] (L-BMAA, α-amino-β-methylaminopropionic acid, L-MeDAP, found in all the species of *Cycas* that have been examined [2,3]), appears to be mediated, in part, by activation of *N*-methyl-D-aspartate (NMDA) receptors. For example, both non-competitive [4] and competitive [5] antagonists protect neurones in organotypic cultures of foetal mouse spinal cord from the effects of L-BMAA in the millimolar range. Similar conclusions may be drawn from electrophysiological studies of cultured rat neurones [6].

An understanding of the mechanism of action of L-BMAA is vital if its deleterious effects upon motor systems in macaques [4] are to be understood. The structure of L-BMAA, which is a neutral amino acid at physiological pH values, is very different from that of the acidic amino acids that activate the NMDA receptor and hence its interaction with this receptor seems unlikely. Weiss and Choi proposed recently [7] that L-BMAA might interact with bicarbonate/carbonate at the β-methylamino function, and thus form a ternary receptor/bicarbonate/L-BMAA complex of a similar overall shape to that formed by glutamate and the NMDA receptor. Although, in the context of explaining the neurotoxicity of L-BMAA this is an attractive proposal, there are problems in substantiating this hypothesis, especially as no clear bonding scheme for the adduct was presented. In this paper, the results of some physical measurements we made recently on L-BMAA [8] and a detailed study of the ^{1}H-NMR spectrum of L-BMAA are used to propose likely interactions between L-BMAA and carbonate/bicarbonate.

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2. MATERIALS AND METHODS

NMR spectra were recorded with a Bruker WM-250 MHz spectrometer, using TSS as an external reference. L-BMAA was prepared as described previously [9]. Solutions of L-BMAA and sodium bicarbonate in D_2O were allowed to equilibrate for 1 h before the spectra were measured. Measurements of pH were made with a conventional glass electrode system and a Pye 9421 pH meter. Values of pH and pD were related by the method of Glasoe and Long [10]. The pD of solutions was altered by the

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addition of small quantitites of NaOD or DCl as appropriate. All measurements were carried out at room temperature (close to 22°C).

3. RESULTS AND DISCUSSION

3.1. *Microscopic and macroscopic pK_a values*

L-BMAA (**I**, fig.2) has three titratable protons. The macroscopic pK_a values may be assigned as follows: pK_1 as the carboxylate, with pK_2 and pK_3 accounted for by the deprotonation of the two amino groups which overlap. The fact that the compound contains two different amino functions, secondary and primary, suggests that there might be some separation of the deprotonations; secondary amines are in general stronger bases than primary amines.

A quantitative calculation by Martin's method [11,12] suggests that the doubly deprotonated form of the amino acid is 86% α-deprotonated and correspondingly 14% β-deprotonated. This result (table 1) is in marked contrast to the related 2,3-diaminopropionic acid (DAP), in which the two amino groups are believed to deprotonate statistically [11,13].

Thus, if specific hydrogen bonding effects, as apparently suggested by Weiss and Choi [7], are considered, hydrogen bonding of HCO_3^- to L-BMAA appears to be more likely at the α- rather than the β-position. However, the results of ^{1}H-NMR studies (vide infra) suggest a very different route for any such hydrogen bonding.

3.2. *^{1}H-NMR studies of L-BMAA*

All solutions of L-BMAA showed a classic $ABMX_3$ ^{1}H-NMR spectrum characteristic of an optically active compound with different couplings to prochiral hydrogens (**II**; fig.2) coupling constants and chemical shifts were derived from a first-order analysis of such spectra (tables 2 and 3 and fig.1). The NMR spectra of solutions of L-BMAA depended on the pH (or more accurately the pD) due to differing populations of the various deprotonated forms of L-BMAA. The effects we see are caused mainly by deprotonation of the α-amino function and this is reflected in the greater changes in chemical shift observed for the proton attached to the α-carbon proximate to this site of deprotonation; the changes in chemical shifts take the order $H_M > H_B \approx H_A > H_X$ (table 2).

The effects of using varying concentrations of sodium bicarbonate on the ^{1}H-NMR spectrum of L-BMAA was then investigated. In 5 mM sodium bicarbonate solution, the main species observed is identical to that in the corresponding spectrum in

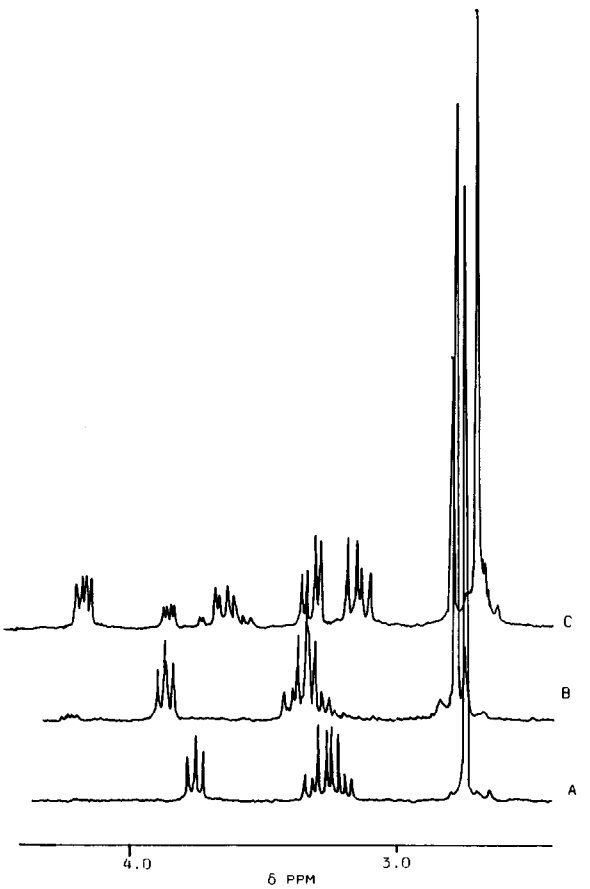

Fig.1. Typical ^{1}H-NMR spectra. (A) L-BMAA, pD = 7.33. (B) 1:0.1 L-BMAA and sodium bicarbonate, pD = 7.29. (C) 1:5 L-BMAA and sodium bicarbonate, pD = 8.09. [L-BMAA] = 50 mM.

Table 1

Results used in calculating microscopic constants

pK_{a2}	pK_{a3}	Separation	Compound
6.63	9.76	3.13	L-BMAA[a] [8]
6.5	9.80	3.30	L-BMAA [9], no ionic strength control
6.77	9.52	2.74	2,3-diaminopropionate [11][a]
6.66	9.39	2.73	2,3-diaminopropionate [13][a]

[a] I = 0.1 mol/dm^3, 25°C

Table 2

Chemical shifts for the various species observed

		pD	H_M	H_B	H_A	H_X
L-BMAA		7.04	3.91	3.40	3.33	2.79
		7.25	3.89	3.39	3.32	2.78
		7.33	3.76	3.31	3.20	2.75
		7.88	3.64	3.23	3.12	2.71
		8.12	3.61	3.21	3.09	2.70
		8.77	3.59	3.19	3.07	2.70
[L-BMAA]:[HCO_3]						
1:0.1	major	7.29	3.86	3.39	3.28	2.77
	minor	7.29	4.22	–	3.20	2.74
1:1.5	major	7.49	3.85	–	–	2.76
	minor	7.49	4.21	–	–	2.74
1:5	minor	8.09	3.86	3.71	3.49	2.80
	major	8.09	4.18	3.32	3.19	2.70

All are at L-BMAA concentrations of 50 mM. The missing entries for the octets due to H_A and H_B are for spectra where the two sets of quartets overlap so severely that assignments are impossible

the absence of bicarbonate. However, a totally new species with the following main features was detected in such solutions: a singlet at δ 2.74 ppm and a quartet at δ 4.22 ppm (table 2 and fig.1).

At higher concentrations of bicarbonate, these new peaks intensified. With a 5-fold excess of bicarbonate, the new species predominates, and is clearly an $ABMX_3$ spin system; coupling constants and chemical shift information are summarized in

Table 3

Coupling constants and chemical shifts

	L-BMAA (pD = 8.10)	L-BMAA/$NaHCO_3$ (1:5) (pD = 8.09)	
		Minor	Major
CH_2			
δ (ppm) H_A	3.09	3.49	3.19
δ (ppm) H_B	3.21	3.71	3.32
J_{AB}	12.7	15.3	12.7
J_{AM}	6.7	3.3	4.9
J_{BM}	7.1	7.0	8.6
J_{av}	6.9	5.2	6.7
CH			
δ (ppm) H_M	3.61	3.86	4.18
J_{AM}	–	3.3	4.9
J_{BM}	–	7.0	8.6
J_{av}	6.9	5.1	6.7

Coupling constants are given in Hz

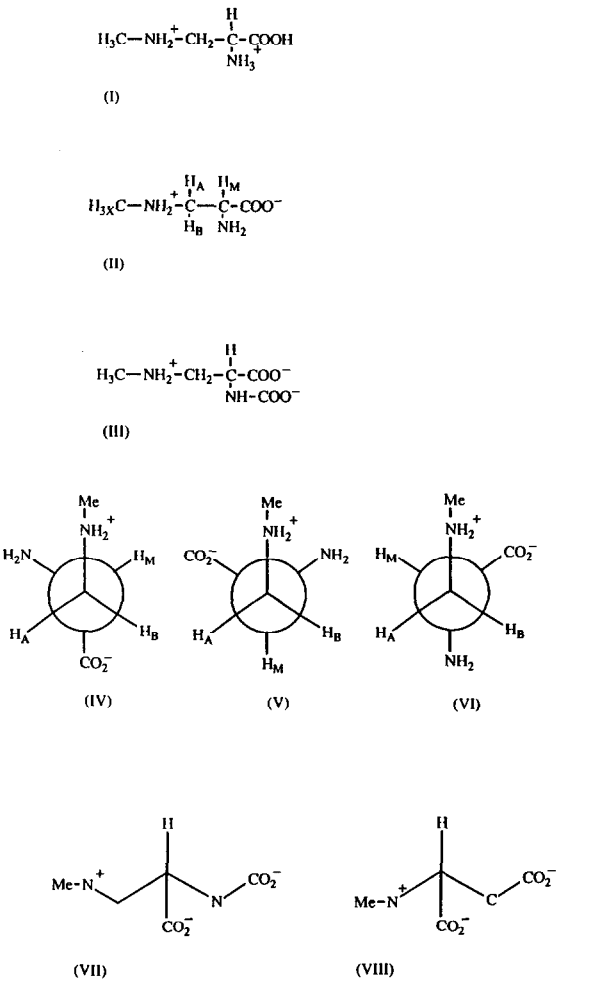

Fig.2. Structures of **I–VIII**. **(I)** Fully protonated L-BMAA. **(II)** L-BMAA dominant form at neutral pH with labelling scheme for NMR spectra. **(III)** Carbamate of L-BMAA. **(IV–VI)** Rotamers of L-BMAA as used in the discussion of NMR spectra. **(VII)** Carbamate of L-BMAA. **(VIII)** *N*-Methyl-D-aspartic acid.

tables 2 and 3 and fig.1. The positions of the peaks for the new species are, unlike those of L-BMAA, relatively independent of both pH and bicarbonate concentration. The coupling constants enable an unequivocal correlation between each quartet and octet. The spectrum in the presence of bicarbonate rapidly reverts to that of L-BMAA on acidification. These results strongly suggest the formation of a new, acid-labile species, involving bicarbonate/carbonate and L-BMAA, which is inert on the NMR time scale.

The most plausible explanation for these results is that we are observing the formation of the carbamate of the amino acid (**III**, fig.2) by the addition of a bicarbonate anion to the α-amino group of L-BMAA.

The spectrum of the new species formed has several features that support the above formulation:

(i) The peak most shifted is the quartet associated with the H_M; this is the proton most proximate to the α-amino function.
(ii) The new H_M resonance is exactly in the position expected (~4.2 ppm) and shifted by the amount (0.38 ppm) normally associated with the acylation of the α-amino function of an α-amino acid. The comparison of the chemical shift with that observed for a wide range of related compounds [14] supports the suggestion that the new species contains the H_M-C-NH-CO grouping.
(iii) The intensity of this new peak depends on the concentration of bicarbonate in the system.
(iv) The positions of the peaks due to this compound are relatively insensitive to both pD and the bicarbonate concentration in the region studied (tables 2,3). In the carbamate, it seems likely that the species is stabilized by intramolecular hydrogen bonding between the $-CO_2^-$ group (α-amino carbamate) and the protonated β-methylamino group. This conformation could be quite rigid and consequently relatively insensitive to pD and bicarbonate ion effects (see **IV–VI**, fig.2).
(v) Such intramolecular hydrogen bonding would lead to an enhanced stabilization of the rotamers **IV** and **V** (fig.2) consistent with the asymmetry in the coupling constants J_{AM} and J_{BM} experimentally observed for this species (table 3).
(vi) In similar experiments using L-alanine, for which the α-amino group is protonated at physiological pH, no bicarbonate adducts were observed.

At the highest concentrations of bicarbonate, the spectrum of L-BMAA was also shifted and the coupling constants were altered (table 3). The explanation for this observation may also lie in the stabilization of rotamers **IV** and **V** (fig.2), but this time by hydrogen bonding from hydrogen carbonate ions between the two amino functions (table 3).

The results for both the carbamate and L-BMAA in the more concentrated bicarbonate solutions may be contrasted with those for L-BMAA at the similar values of pD in the absence of bicarbonate (table 3 and fig.1). Under these conditions, the coupling constants J_{AM} and J_{BM} are very similar, which may be explained if all three rotamers (**IV–VI**) contribute equally to the observed spectrum.

The equilibrium position for the formation of the adduct between bicarbonate and L-BMAA may be estimated from the NMR spectra. A conditional equilibrium constant of ~14 has been calculated for the formation of the adduct (pD 7.3–8.1, [amino acid] = 50 mM, 1 h after mixing). Extrapolated to a physiological value for the concentration of bicarbonate (~25 mM), this equilibrium constant suggests that about 26% of L-BMAA in tissue culture media would be present as the new species. This equilibrium constant must be treated with some caution as the reactions in these solutions may be under kinetic control.

4. CONCLUSIONS

Clearly the reactions of this amino acid in bicarbonate buffer are complicated and require still further investigation to be understood fully. However, our work has shown that specific interactions, leading to species inert on the NMR time scale, occur between L-BMAA and bicarbonate. At high concentrations of bicarbonate, the ^{1}H-NMR spectrum of L-BMAA itself is affected, the NMR results indicating that the species formed may have more restricted rotation about the C–C bond, suggesting bridging of the amino functions. At low concentrations of bicarbonate, the NMR spectrum of L-BMAA is unaffected.

At bicarbonate concentrations lower than those found in vivo (5 mM), there is evidence for the rapid formation of the new species, which is assigned as the α-carbamate of the amino acid. The resemblance between the α-carbamate of L-BMAA and NMDA (**VII** and **VIII**) may be important. The similar spatial orientation of the charged groups is a consequence of the opposite chiralities of the two molecules. There may be other specific interactions with receptor sites or metal ions in

receptors that lead to the potent effects of L-BMAA; for example, we have shown recently [8] that L-BMAA binds very strongly to zinc, a metal which is known to have important functions in the central nervous system [15].

These results may explain the much higher concentrations of L-BMAA (1–3 mM) [1] required to produce neuronal effects in vitro, as compared to NMDA (21–100 μM) [16]. Weiss and Choi [7] noted that neuronal damage by millimolar concentrations of L-BMAA is produced only in the presence of 10 mM sodium bicarbonate and after 1 h incubation. Assuming that the equilibria set up in their assay solutions are similar to those that we have discussed above, the concentrations of the α-carbamate in the medium would be approx. 120–360 μM, comparable to the effective concentrations of NMDA. However, these results refer only to the speciation of the amino acid in aqueous solution and cannot exclude the possibility of different interactions at the receptor surface.

If the structural resemblance of **VII** and **VIII** has any significance in vivo, it may be relevant that an enzyme exists in human blood which accelerates the formation of carbamates from bicarbonates/CO_2 and compounds possessing amino functions with pK_a values in the same range as that of the α-amino group of L-BMAA [17]. There is also precedence for the suggestion that carbamates are important in mediating the neuroactivity of compounds with such amino groups. A similar mechanism has been proposed [18] to explain the effect of 1,2-diaminoethane in activating γ-aminobutyric acid receptors in the presence of bicarbonate.

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