PROTON-LIGAND EQUILIBRIA OF ALIPHATIC, ATOXIC DIHYDRAZIDES AND WATER-SOLUBLE AMINO ACIDS GLYCINE, ASPARTIC ACID & HISTIDINE

Abstract

This study explores the behavior of amino acids and aliphatic dihydrazides in aqueous solutions at various pH values by examining their proton-ligand equilibria. By investigating the protonation and deprotonation mechanisms, the study reveals the complex molecular interactions affecting the distribution of charge and the overall structure. Understanding the acid-base characteristics is essential for comprehending the compounds' reactivity in a variety of settings, and this is made possible by the determined equilibrium constants. This investigation advances disciplines such as biochemistry and pharmacology, laying the groundwork for future studies on the functions and molecular structure of these substances.

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The equilibrium between protons and ligands, encompassing both dihydrazides and amino acids, is a fundamental prerequisite for determining the formation constants of binary and ternary systems involving metal ions. This prerequisite is indispensable since the formation of a metal complex represents an acid-base equilibrium, wherein a competition arises between the metal ion and the proton for the binding sites of the ligand. Consequently, this competition results in the pH-dependent formation of metal-ligand species, which can be monitored using a glass electrode. Therefore, by tracking changes in the hydrogen ion concentration within a system containing a metal ion and a ligand, employing Bjerrum's potentiometric titration method, and having knowledge of the protonation constants of the anionic forms of the ligands, it becomes possible to facilitate the speciation analysis of binary and ternary systems. This is why the author undertook a study on the proton-ligand equilibria of both dihydrazides and amino acids under similar experimental conditions.

Dihydrazides selected for the study were, succinic acid dihydrazide (SADH) and Adipic acid dihydrazide (AADH). Despite the biological importance of dihydrazides[1-5] there is a paucity of information in the literature on their solution equilibria.

I. DATA ACQUISITION

The Calvin-Wilson titration technique was employed in this study to investigate the protonation equilibria of SADH, AADH, and amino acids in an aqueous medium. The experimental setup involved titrating a total volume of 50 cm^3, which consisted of carefully measured volumes of hydrochloric acid (to achieve an overall concentration of 0.02 - 0.05 $mol.dm^{-3}$), sodium chloride (maintained at an ionic strength of 0.1 $mol.dm^{-3}$), and water. These titrations were conducted both in the presence and absence of the ligand. Sodium hydroxide of approximately 0.2 $mol.dm$[8, 9].

II. PROTON - LIGAND EQUILIBRIA OF SUCCINIC AND ADIPIC ACID DIHYDRAZIDES

Succinic and adipic acid dihydrazides belong to a group of nitrogenous organic compounds with general formula, R $(-CO-NH-NH_2)_2$ as shown in Figure1. In the case of aliphatic dihydrazides R= $(CH_2)_n$, the value of n= 2 for SADH and n=4 for AADH.

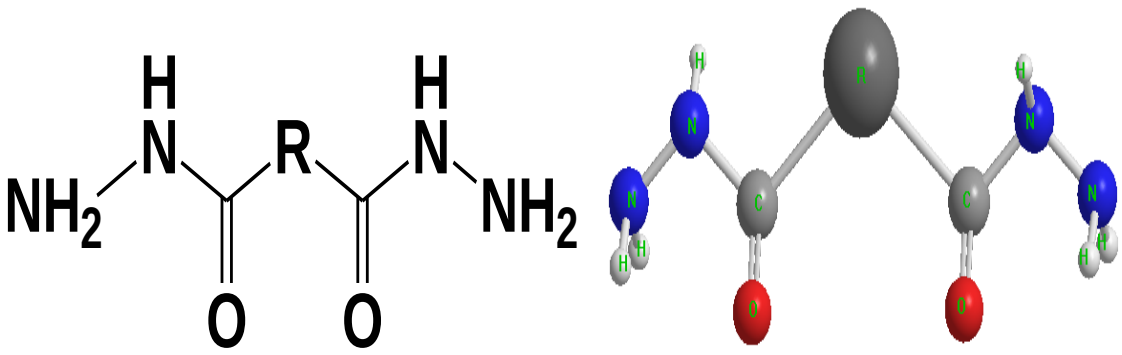


Figure 1: General formula of a dihydrazide

In the existence of an acidic environment, the uncharged state of these ligands (L) can undergo protonation at their terminal -NH2 groups, resulting in the creation of monoprotonated (LH+) and biprotonated (LH22+) species. Hydrazides are recognized for their ability to undergo keto-enol tautomerism and, under basic conditions, they can release enolic protons, leading to the generation of deprotonated species known as LH-1 and LH-2.

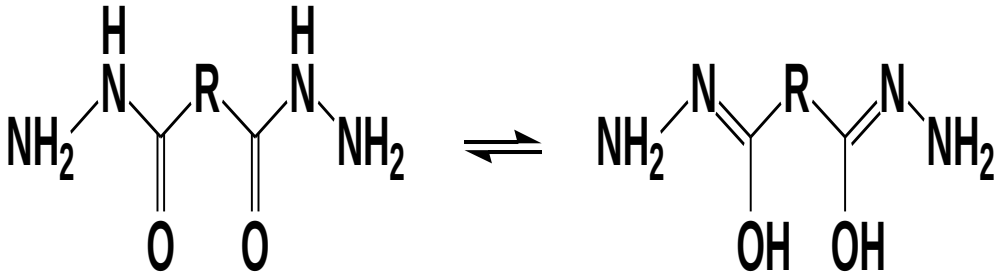
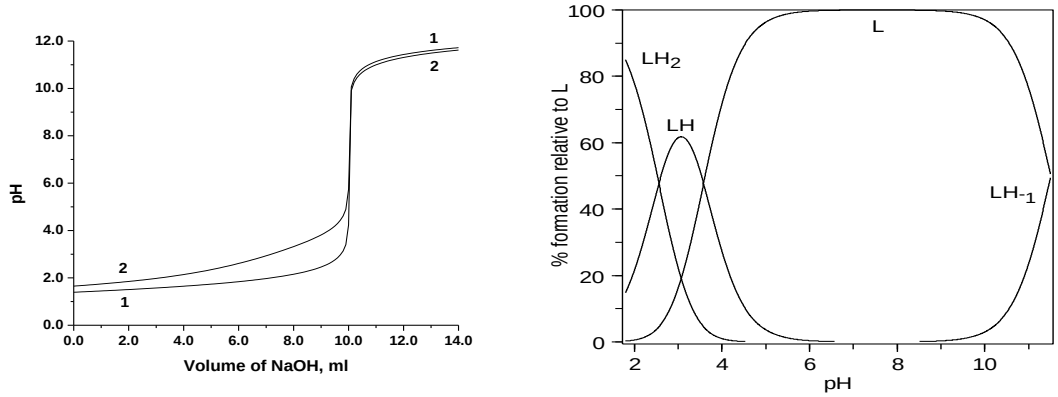


Figure 2: Keto-enol tautomerism of dihydrazides

Thus, the acido-basic equilibria of dihydrazides may contain LH_2^{2+}, LH^+, L, LH_{-1} and LH_{-2} type of species in solution. In literature, there are no reports on the proton-ligand equilibria of SADH. In the case of AADH, there are two reports on the protonation in aqueous-dioxane[10], aqueous-dimethylformamide[10] and aqueous-ethanol[11] media. These authors did not consider the deprotonation of AADH in basic medium. Therefore, the author has taken up a study on the proton-ligand equilibria of these ligands which is an essential pre-requisite for the determination of the formation constants of metal-ligand complexes. According to preliminary experiments, the pH of both the SADH and AADH solutions abruptly increased to around 9.0 with the addition of a drop of 0.1 mole dm-3 alkali. This suggests that in the pH range below 9.0, these ligands do not have any dissociable protons.

In Figures 3a and 4a, respectively, the pH metric titration data for SADH and AADH are displayed visually. Curve 2 shows the titration of acid + ligand using sodium hydroxide, whereas curve 1 shows the titration of hydrochloric acid in the absence of ligand. Under a pH of approximately 5.0, the titration curves of both SADH and AADH (curve 2 in Figures 3a and 4a) are higher than the curve for free acid.The existence of proton associable centers in the ligands is indicated by the difference between the free acid and ligand curves in the lower pH region for both ligands.

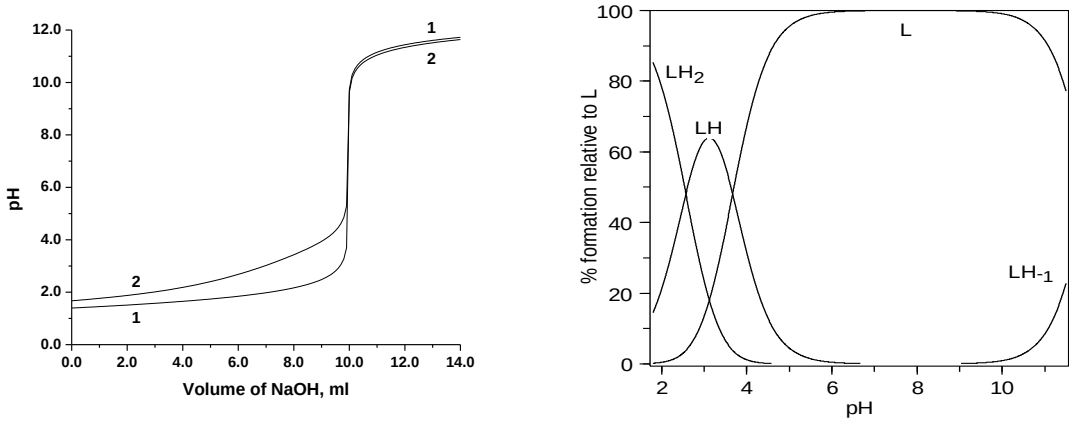


(a) 1.Titration curve of free mineral acid
2. Titration curve for acid + SADH

(b) Species distribution

[SADH] = 0.00960 mol dm^{-3}	[HCl] = 0.04039 mol dm^{-3}	[NaOH]= 0.2016 moldm^{-3}
Temp.: 30.0 ±0. 1^0C	Total volume= 50.0 cm^3	*I* = 0.1 mol dm^{-3} NaCl

Figure 3: Proton-Ligand System of SADH



(a) 1. Titration curve of free mineral acid
2. Titration curve for acid + AADH

(b) Species distribution diagram

[AADH] = 0.0100 mol. dm^{-3}	[HCl] = 0.04016 mol. dm^{-3}	[NaOH]= 0.2016 mol. dm^{-3}
Temp.: 30.0 ±0. 1^0C	Total volume= 50.0 cm^3	*I* = 0.1 mol. dm^{-3} NaCl

Figure 4: Proton-Ligand System of AADH

The ligand titration curves significantly lower than those of the free acid above pH ~9.0, which is indicative of the ligands' deprotonation.Other proton-ligand equilibria are absent because free acid and ligand curves coincide between these two pH zones.

The titration data was first subjected to analysis by ACBA computer program[12], modified by the author to run on a personal computer. The formation constants obtained from ACBA program were taken as initial estimates for refinement by MINIQUAD-75

program[13]. The protonation and deprotonation equilibria of SADH are shown in Figure5. The best-fit model obtained using the Miniquad-75 program (Table1) contained three formation constants β_{011}, β_{012} and $\beta_{01\text{-}1}$ corresponding to the formation of LH, LH_2 and LH_{-1} (charges are omitted for brevity) respectively.

The species distribution diagram of SADH (Figure 3b) indicates that the LH_2^{2+} form exists only below a pH of 4.0. The extent of its formation is~84% (at ~1.8 pH) in the pH region of study. The species LH^+ which has a maximum of 62% around a pH of 3.1 ceases to exist above ~6.0 pH. β_{011} and β_{012} are the formation constants (Figure 5) of mono and biprotonated forms of SADH respectively, the protonation being at the terminal nitrogen atoms of the two hydrazide groups.

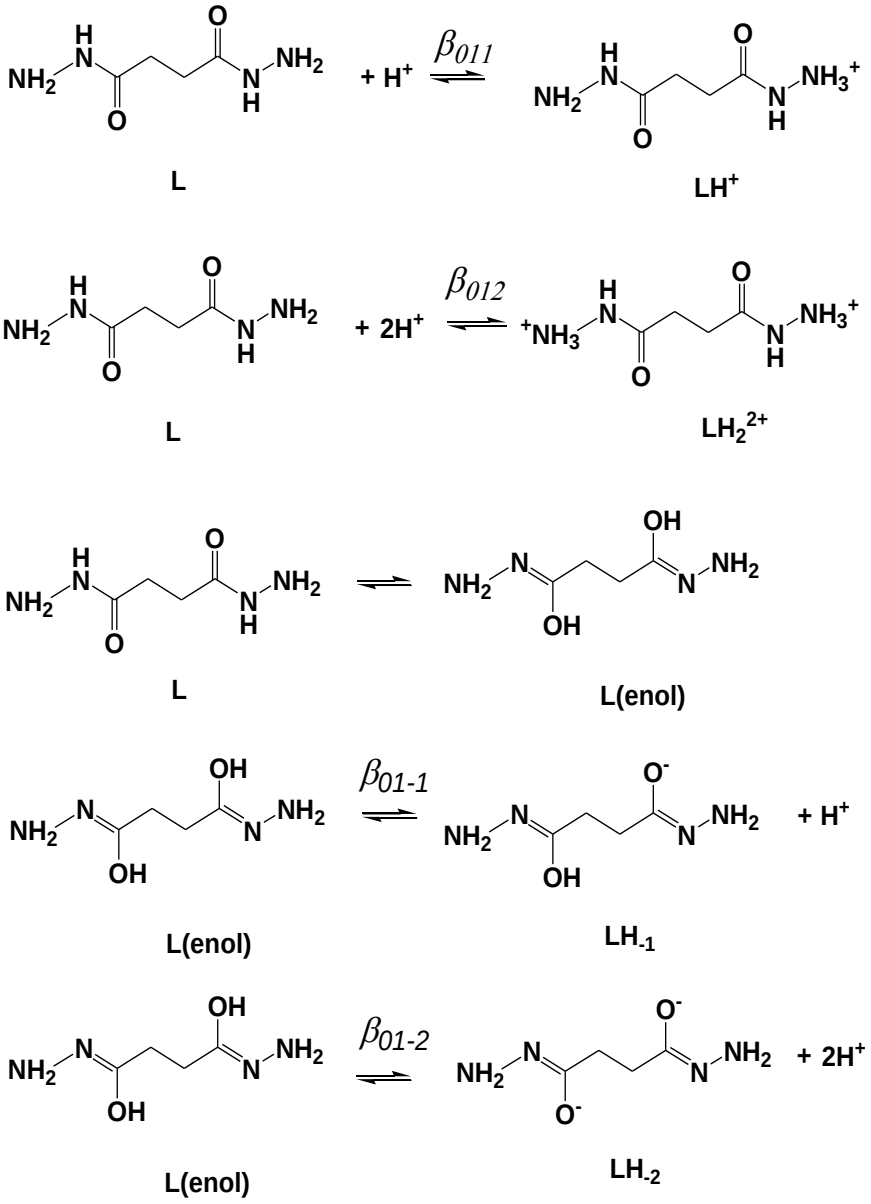


Figure 5: Protonation and Deprotonation Equilibria of SADH

Table1: Best fit chemical model for acido-basic equilibria of succinic acid dihydrazide in aqueous medium. Temp. = 30.0 ± 0.1°C and ionic strength, I = 0.1 mol dm^{-3} (NaCl)

Up to ~99% of the ligand is in neutral form between 6.0 and 9.0 pH. In basic medium

Species *mlh*	L*og* β_{0lh} *(SD)*	Number of experimental points analysed	Sum of the squares of residuals, U	χ^2
011	3.58 (2)	110	1.642 X 10^{-8}	12.34
012	6.14 (2)			
01-1	-11.91 (3)			

hydrazides are prone to lose a proton from the enolic form. As SADH contains two hydrazide groups, there is a probability of losing two enolic protons at higher pH. The formation constant, $\beta_{01\text{-}1}$ in the best-fit model corresponds to the deprotonation of one of the enolic protons leading to the formation of LH_{-1} species. This species appears in the solution above a pH of ~9.0 and represents 49% of the total ligand at a pH of 11.5. The formation constant, $\beta_{01\text{-}2}$ which corresponds to the deprotonation of the second enolic group leading to the formation of LH_{-2}, was not converged. This is because its equilibrium may lie well above the pH range of the study. However, in the presence of a metal ion the ligand may also lose the second enolic proton forming both deprotonated $M_mL_lH_{-1}$ and $M_mL_lH_{-2}$ type of species.

The best-fit model and the corresponding protonation and deprotonation equilibria of AADH are shown in Table2 and Figure6 respectively. The best-fit model for AADH (L) indicates the formation of LH_2^{2+}, LH^+, L and LH_{-1} species in aqueous medium. At lower pH (below ~3.0 pH), the biprotonated form, LH_2^{2+} of AADH dominates and with increase in pH, undergoes successive deprotonation to form the mono-protonated (LH^+) and neutral species (L). β_{011} and β_{012} are the formation constants (Figure 6) of mono and biprotonated forms of AADH from its neutral form.

Table 2: Best fit chemical model for acido-basic equilibria of adipic acid dihydrazide in aqueous medium.Temp.= 30.0 ± 0.1°C and ionic strength, I = 0.1 mol dm^{-3} (NaCl)

Species *mlh*	L*og* β_{0lh} *(SD)*	Number of experimental points analysed	Sum of the squares of residuals, U	χ^2
011	3.67 (3)	75	4.099 X 10^{-8}	11.92
012	6.24 (7)			
01-1	-12.03 (5)			

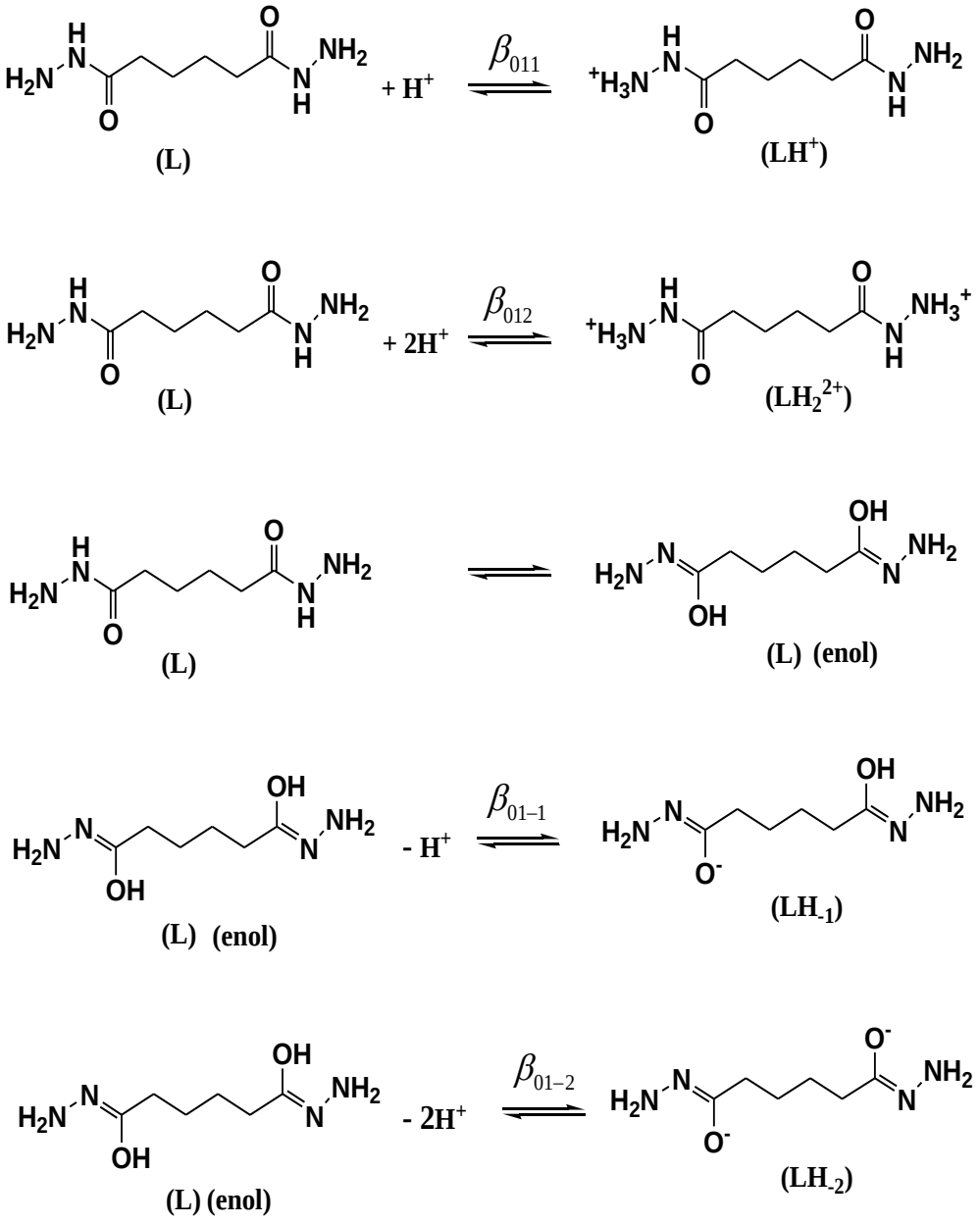


Figure 6: Protonation and Deprotonation Equilibria of AADH

The relatively high values of these formation constants compared to SADH indicate the increase in basicity of the ligand with increase in chain length of "R" group connecting the two hydrazide groups. The formation constant, $\beta_{01\text{-}1}$ in the best-fit model corresponds to the deprotonation of one of the enolic protons leading to the formation of LH_{-1} species. The other, $\beta_{01\text{-}2}$ which corresponds to the formation of LH_{-2}, is not observed as its equilibrium may lie well above the pH range of study. The species distribution diagram (Figure 4b) indicates that AADH exists in the protonated form below pH ~6.5, deprotonated form above pH ~9.0 and in neutral form between pH ~6.5 and ~9.0. The maximum percentage concentrations (with respect to total ligand) of LH_2^{2+}, LH^+ and L^- are 85% (1.8 pH), 64%(3.1pH) and 23%(11.5 pH) respectively in the pH region of study.

III. PROTON-LIGAND EQUILIBRIA OF AMINO ACIDS

Equilibrium constants of acid- base equilibria of amino acids reported in literature were determined using different methods in various solvents and at specific conditions of temperature, ionic strength etc. Several databases are available that give the formation constants of proton-ligand and metal-ligand systems, including,

- The IUPAC Stability Constants Database, SC-Database and Mini-SC Database, 2006 and
- NIST (National Institute of Standards and Technology) -Critically Selected Stability Constants of Metal Complexes- Database Version 8.0 For Windows, R. M. Smith and A. E. Martell, (May 2004)

Along with the formation constants, these databases give the experimental conditions and methods that can be used to adjust the values for different conditions.

Stability constants reported in the literature, mostly for amino acids and some of the other ligands, were critically surveyed and published by Martel et al.[14], Pettit[15], Kiss et al.[16], Sovago et al.[17], Berthon[18]and Yamauchi et al.[19]. Use of the values obtained either from data bases or critical surveys in speciation calculations at different experimental conditions can lead to a significant uncertainty. This may be attributed to the variations in experimental conditions and the use of classical/ graphical methods of formation constant calculations by earlier workers. Most of the classical methods ignore the possibility of formation of protonated and hydroxylated species in addition to the formation of simple mono-nuclear species. The formation constants obtained without considering these aspects are unreliable and associate with some systematic error. Also, the methods used for adjusting the equilibrium constants to the required conditions of temperature and ionic strength introduce some uncertainty in the predicted values as experienced by several workers[20-22].

The protonation constants of glycine, aspartic acid and histidine, although available in literature, were, therefore, redetermined under the same experimental conditions of ionic strength and temperature used to study the binary and the ternary complexes.

IV. PROTON-LIGAND EQUILIBRIA OF GLYCINE

Glycine is the simplest possible proteinogenic non-essential amino acid with the chemical formula NH_2- CH_2- COOH. It is amphoteric in nature and in aqueous solution, depending on the pH, can exist in three different forms (Figure7) *viz.* the cationic **(**XH_2^+**),** the neutral or zwitter ionic (XH) and the anionic (X^-) forms. XH is the zwitterionic form in which the amino group is protonated while the carboxyl group is deprotonated.

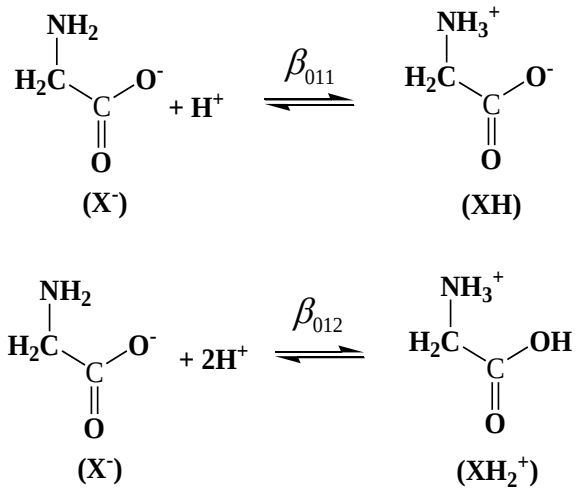
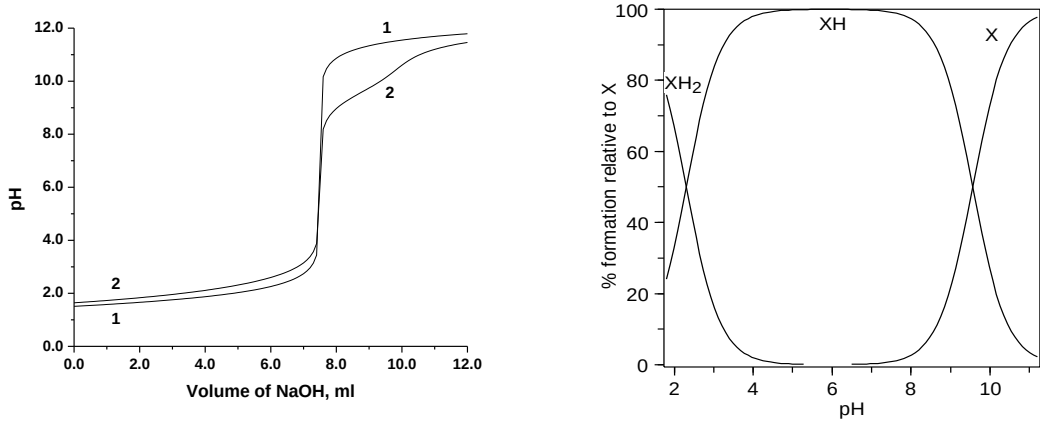


Figure 7: Protonation equilibria of glycine

The Calvin-Wilson[6, 7]potentiometric titration curves obtained for glycine are shown in Figure 8a. Curve 1 represents the titration of free acid in the absence of amino acid. The titration curve for acid + amino acid (curve 2) lies above that of the free acid in the region below a pH of ~ 4.0 indicating association of a proton to the ligand. Above ~7.5 pH, i.e., in the basic region, the titration curve of ligand is well below that of the free acid, indicating the dissociation of proton from the ligand. The difference at lower pH is due to the protonation of the zwitter ionic form of the ligand at the carboxylate group and at higher pH the lowering of the curve relative to that of free acid is due to the ionization of the $–NH_3^+$ group. Therefore, the proton association and dissociation processes of the zwitter ionic form of the ligand (XH) are widely separated.



(a) 1. Titration curve of free mineral acid
2. Titration curve for acid + Glycine

(b) Species distribution diagram

[Glycine] = 0.0100 mol. dm^{-3}	[HCl] =0.03099 mol. dm^{-3}	[NaOH]= 0.2066 mol. dm^{-3}
Temp.: 30.0 ±0. 1^0C	Total volume= 50.0 cm^3	I = 0.1 mol. dm^{-3} NaCl

Figure 8: Proton-Ligand System of Glycine

The protonation equilibria of glycine (XH) can be expressed as,

$$X^- + H^+ \overset{\beta_{011}}{\rightleftharpoons} XH$$

$$X^- + 2H^+ \overset{\beta_{012}}{\rightleftharpoons} XH_2^+$$

The best-fit model obtained using Miniquad-75 program for glycine, is shown in Table-3. The IUPAC recommended[14, 15] values for the protonation constants of glycineare 9.60 and 11.97 for *log* β_{011} and *log* β_{012} respectively at 25.0^{0}C and ionic strength between 0.1 and 0.2M. The observed values are in good agreement with the IUPAC and other literature values[17, 18] after allowing for changes in experimental conditions.

Table 3: Best Fit Chemical Model for Acid-base Equilibria of Glycine in Aqueous Medium Temp. = 30.0 ± 0.1°C and Ionic Strength, *I* = 0.1 Mol Dm^{-3} (NaCl)

Species *Mxh*	**L*og* β_{0xh} *(SD)***	**Literature[14, 15] recommended values At 25.0 ^{0}C**	**Number of experimental points analysed**	**Sum of the squares of residuals, U**	**χ^2**
011	09.56 (1)	09.60	84	3.56 X 10^{-8}	6.86
012	11.86 (2)	11.97			

(SD= Standard Deviation in the least significant digit)

The constant *log* β_{011} is due to the protonation of the amino group of the most basic form of the ligand, to form a zwitter ion. The magnitude of the protonation constant is low, when compared to the corresponding amine and may be explained as due to the electron withdrawing effect of neighbouring deprotonated carboxylate group. The second protonation constant, *log* β_{012} is due to the addition of one more proton to the ligand at the carboxylate group. Again, the relative decrease in magnitude of the constant to that of alkyl carboxylate is due to the strong withdrawing effect of the protonated amino group. The species distribution diagram for glycine is shown in fig. 3.8b. Nearly 75% of the ligand is in biprotonated form at 1.80 pH. The zwitter ion form dominates in the intermediate region i.e., between ~4.0 and ~8.0 pH. The deprotonated form of the ligand (X^-) exists only above 7.5 pH and increases monotonically above this point.

V. PROTON-LIGAND EQUILIBRIA OFL-HISTIDINE

Among the amino acids, L-histidine is one of the strongest metal coordinating ligands which plays an important role in the binding of metal ions by proteins. Histidine has three potential metal-binding sites, namely the carboxylate oxygen, the imidazole imido nitrogen and the amino nitrogen. The imidazole nitrogen of histidine residue often provides the primary means by which the metal ions are bound to proteins.

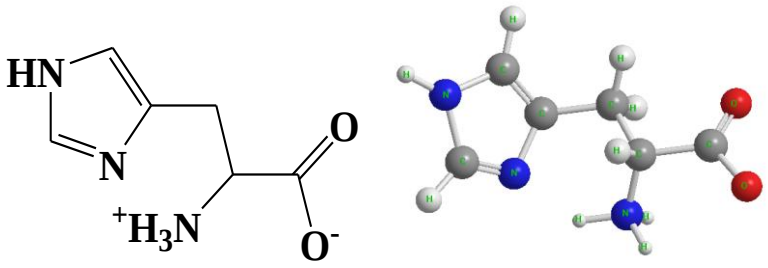


Figure 9: L-Histidine

Histidine belongs to a group of amino acids that contain one or more amino groups in the side chain. Because amine groups can accept protons, they are bases and these amino acids are considered as basic amino acids. In solution they can accept protons from water to become positively charged. In histidine in addition to the amino group, it is the double bonded nitrogen atom that accepts the proton and has a pK_a value in the physiological pH range, hence often is the only amino acid seen in biologically active sites when the donation or abstraction of a proton is needed. Structurally, it is possible that histidine (XH) can exist (Figure10) in solution as XH_3^{2+}, XH_2^{+}, XH and X^{-}.

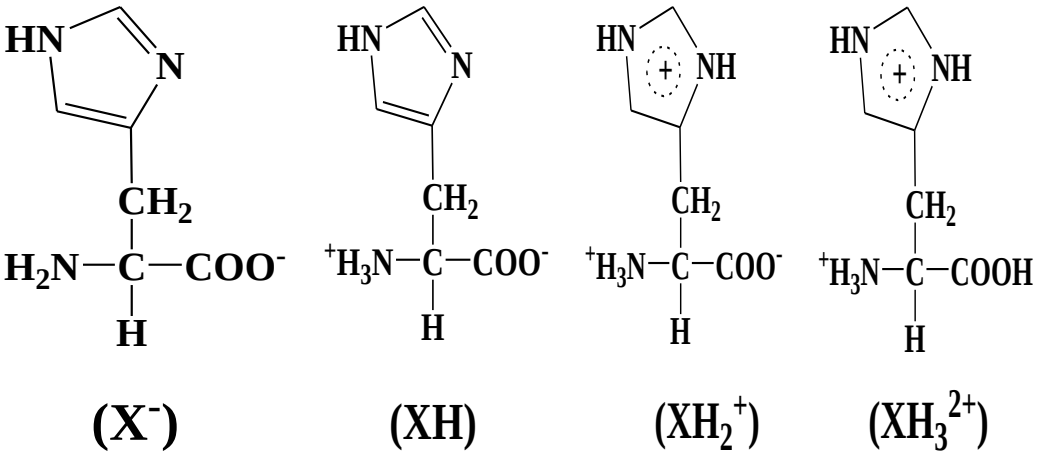


Figure 10: Fischer Projections of successive protonated species of L-Histidine

The first centre to protonate in the fully ionized histidinate anion (X^{-}) is the amino-nitrogen, followed by the pyridine-like imidazole-nitrogen of the side chain and finally the carboxylate group. Since these protonation reactions take place over a widely separated and accessible pH ranges, the formation constants (Fig.11) can be determined comparatively accurately. At higher pH (>14), the pyrrole like proton may also ionize (forming an imidazolate side chain) to give a dianion (X^{2-})[15] which is of course out of the pH region in aqueous solutions.

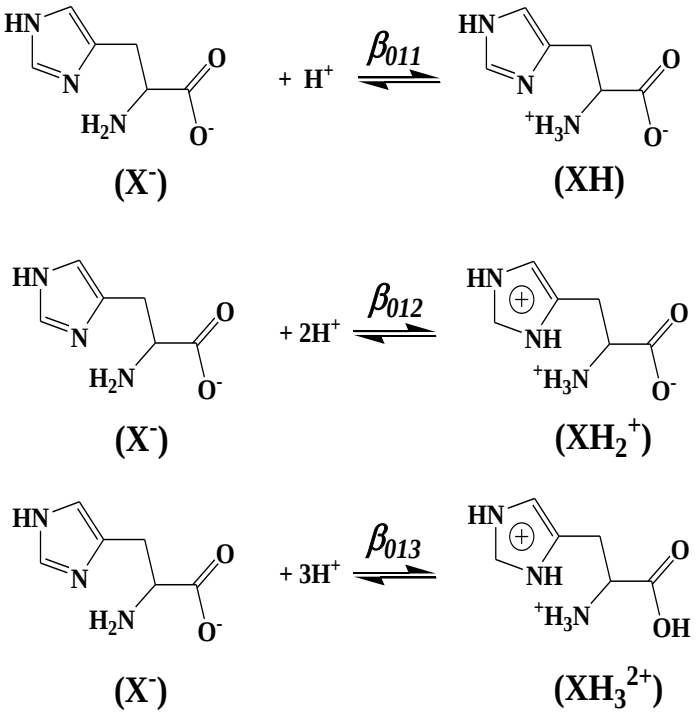
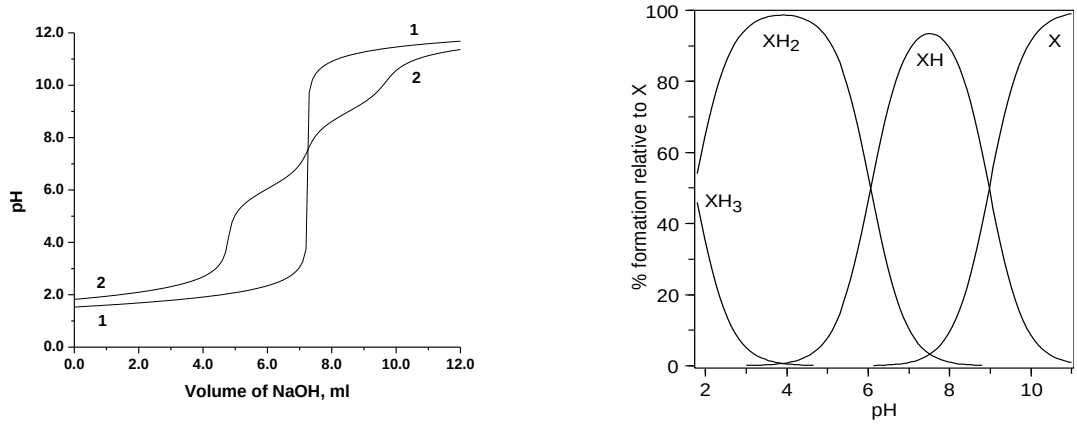


Figure 11: Proton-Ligand Equilibria of L-Histidine

The alkalimetric titration curves obtained, in the presence and absence of histidine are shown in Figure 12a. The titration curve for the acid+ ligand (curve 2) is above that of free acid (curve 1) up to a pH of ~ 7.0 indicating the initial association of the proton to the ligand. The wide difference between the curves indicates proton associations at more than one centre. The two buffer regions below ~7.0 pH correspond to the titrations of carboxylic and ring protons. The buffer region above pH ~7.0 indicates the titration of ammonium proton.



(a) 1. Titration curve of free mineral acid (b) Species distribution diagram
2. Titration curve for acid + Histidine

[Histidine] = 0.0100 mol dm^{-3}	[HCl] = 0.02944 mol dm^{-3}	[NaOH]= 0.2030 $moldm^{-3}$
Temp.: 30.0 ±0. 1^0C	Total volume= 50.0 cm^3	I = 0.1 mol dm^{-3} NaCl

Figure 12: Proton-Ligand System of L-Histidine

The titration curve for the acid+ ligand (curve 2) is above that of free acid (curve 1) up to a pH of ~ 7.0 indicating the initial association of the proton to the ligand. The wide difference between the curves indicates proton associations at more than one centre. The two buffer regions below ~7.0 pH correspond to the titrations of carboxylic and ring protons. The buffer region above pH ~7.0 indicates the titration of ammonium proton.

The titration data of all the experiments with different concentrations of the ligand (0.004, 0.01 and 0.015 mol dm^{-3}) were analysed by the ACBA[12] computer program and the constants obtained were used as the initial estimates for the refinement using the MINIQUAD-75 program[13]. The best-fit chemical model thus obtained along with the statistical parameters is shown in Table 4.

Table 4: Best Fit Chemical Model for Acido-Basic Equilibria of L-Histidine in Aqueous Medium. Temp. = 30.0 ± 0.1°C and Ionic Strength, *I* = 0.1 Mol Dm^{-3} (NaCl)

Species *mlh*	L*og* β_{0lh} *(SD)*	Number of experimental points analysed	Sum of the squares of residuals, U	χ^2
011	08.95 (1)	102	4.021 X 10^{-8}	23.12
012	15.02 (2)			
013	16.74 (2)			

(SD= Standard Deviation in the least significant digit)

The results are in good agreement with the literature reports (Table 5) after allowing for the changes in experimental conditions and calculation methods.

Table 5: Some Representative Literature Reports on the Protonation Constants of Histidine

S.No.	Experimental conditions		*Log* β_{011}	*Log* β_{012}	*Log* β_{013}	Reference
	Temp.^{0}C	Ionic strength				
1.	37.0	0.10 M ($NaNO_3$)	8.80	14.55	16.65	23
2.	25.0	0.10 M (KCl)	9.11	15.19	16.96	24
3.	25.0	0.10M (KNO_3)	9.16	15.25	17.31	25
4.	25.0	0.10M (KNO_3)	9.12	15.17	16.60	26
5.	25.0	0.10M (KNO_3)	9.12	15.22	16.93	27
6.	25.0	0.10 M (KNO_3)	9.11	15.15	16.92	28
7.	25.0	0.10 M (KCl)	9.09	15.11	16.81	14,29
8.	25.0	0.10-0.15M (KCl)	9.11	15.16	16.88	15

The species distribution diagram for proton-ligand equilibria of histidine is shown in Figure 3.12b. The fully protonated form XH_3^{2+} exists only below a pH of 4.0. This species loses the carboxylate proton and forms XH_2^+ which is a dominating species between 2 to 6

pH and represents nearly 99% of the ligand at 3.90 pH. Further increase in pH causes neutralization of the side chain proton resulting in the zwitter ionic species XH. The formation of the XH reaches a maximum of 93% of the total ligand around 7.5 pH. Above a pH of 9.0, the major species is the most anionic form of the ligand (X^-) and represents the total ligand concentration around ~11.0 pH.

VI. PROTON-LIGAND EQUILIBRIA OF L-ASPARTIC ACID

Acidic amino acid aspartic acid (2-Aminobutanedioic acid) has two -COOH groups: one on the alpha carbon atom and the other on the side chain. It is a non-essential amino acid that is mostly present in proteins. It functions in the central nervous system as an excitatory neurotransmitter and is involved in the synthesis and release of hormones. It contributes significantly to the citric acid cycle biochemically.

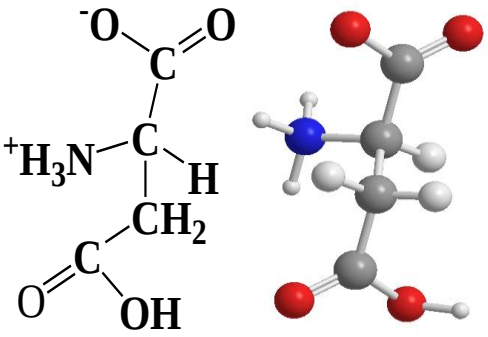


Figure 13: L- Aspartic acid (XH_2)

In aqueous solution depending on the pH, L-aspartic acid may exist (Fig14) in cationic (XH_3^+), neutral zwitter ionic (XH_2) or anionic (XH^- and X^{2-}) forms.

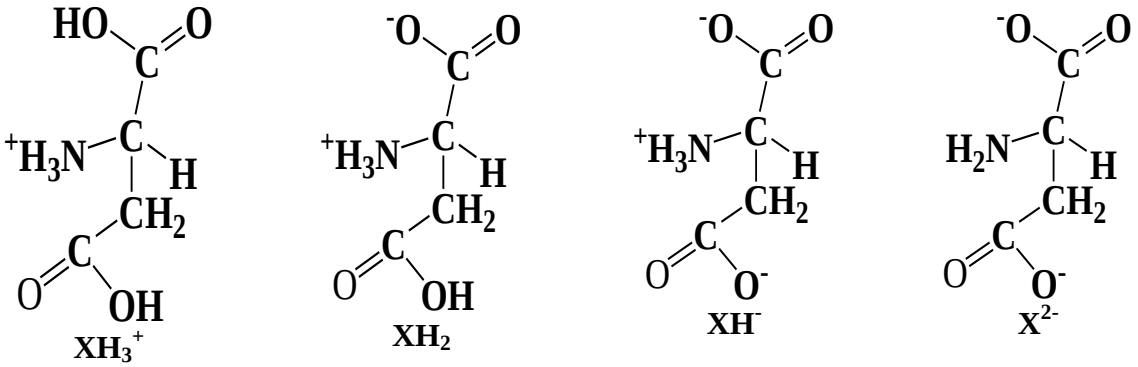
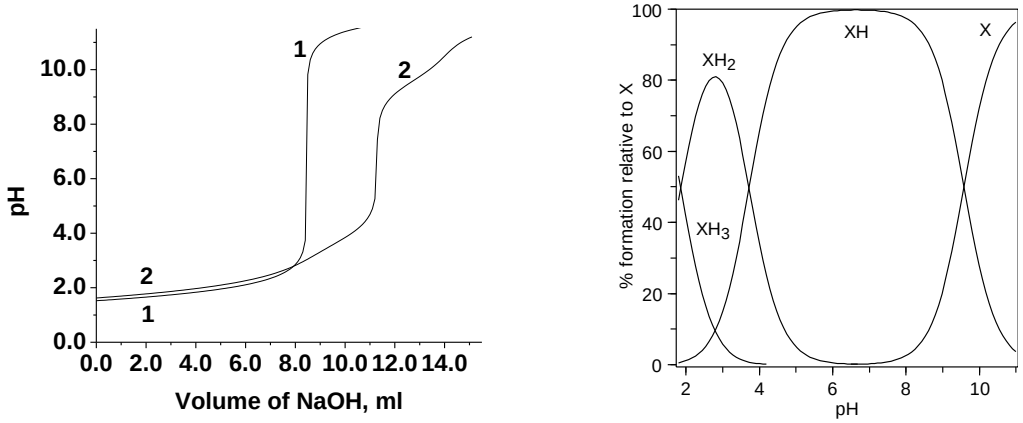


Figure 14: Successive protonated species of L-Aspartic acid

The entire molecule has a negative charge when the pH is neutral or higher. Proteins nearly entirely include it because of the negatively charged carboxyl group that it possesses. An essential idea in the solubility of amino acids in water is that the charged group can generate dipole interactions with water and ionic bonds with different metal ions.

The pH-metric titration curves of free acid (curve 1) and acid + ligand (curve 2) is shown in Figure 15a.



(a) 1. Titration curve of free mineral acid (b) Species distribution diagram
2. Titration curve for acid + Aspartic acid

[ASP]= 0.0100 mol dm^{-3}	[HCl] = 0.0300 mol dm^{-3}	[NaOH]= 0.1773 moldm^{-3}
Temp.: 30.0 ±0. 1^0C	Total volume= 50.0 cm^3	I = 0.1 mol dm^{-3} NaCl

Figure 15: Proton-Ligand System of L-Aspartic Acid

The titration curve of the ligand possesses three buffer regions corresponding to the titration of the three protons associated with the cationic (XH_3^+) form of the ligand. The ligand curve is above that of the free acid in the lower pH region indicating proton association to the ligand. The experimental data were subjected to analysis by the Miniquad-75 program[13]and the best-fit chemical model obtained along with statistical parameters is shown in Table 6.

Taking into account the variations in experimental conditions, the reported values are in close agreement with the IUPAC suggested values. The formation constants obtained represent the equilibrium.

Table 6: Best Fit Chemical Model for Acid-base Equilibria of L-Aspartic Acid in Aqueous Medium. Temp. = 30.0 ± 0.1°C And Ionic Strength, I = 0.1 Mol Dm^{-3} (NaCl)

Species *Mlh*	***Log β_{0lh} (SD)***	**Literature[14] recommended values. At 25.0 ^{0}C**	**Number of experimental points analysed**	**Sum of the squares of residuals, U**	**χ^2**
011	09.58 (2)	9.62	104	4.28 X 10^{-9}	6.37
012	13.30 (2)	13.32			
013	15.16 (2)	15.26			

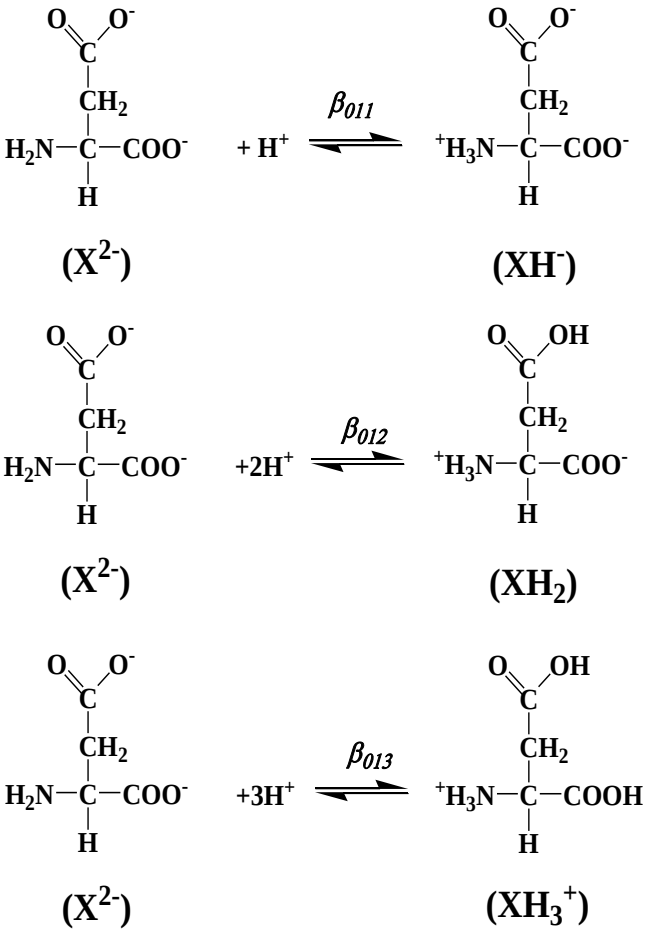


Figure 16: Proton-Ligand Equilibria of L-Aspartic Acid

The most anionic form of the ligand's amino group is protonated, which results in the formation constant β011. The protonation of the carboxylate and amino groups in the molecule's side chain is the cause of β012. The total formation constants for fully protonating the ligand's most anionic form are represented by β013 in this case.

The species distribution diagram indicating the percentage of formation of each species against pH is shown in Figure 15 b. The fully protonated form XH_3^+ exists up to a pH of ~4.0. With increase in pH, it loses the 'α- carboxylic' proton leading to the formation of g neutral zwitter ion. The maximum extent of formation of XH_2 form is 80.6% at 2.83 pH. This species exists up to ~6.0 pH. The neutral form of the ligand, with increase in pH, loses carboxylic proton of the side chain forming anionic XH^- form of aspartic acid. Nearly 99% of the ligand is in this form between 5.8 to 7.8 pH. Further increase in pH leads to the formation of the most anionic X^{2-} form by the loss of the proton associated to amino group.

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