ACIDO BASIC EQUILIBRIA OF NON-TOXIC, DITOPIC AZELAIC AND TEREPHTHALIC ACID DIHYDRAZIDES & AMINO ACIDS PROLINE AND LYSINE IN AQUEOUS MEDIUM

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I. INTRODUCTION

Many biological reactions, including the enzyme catalysis depend on the pH of the biological fluids. This is due to the pH-dependent activity of biomolecules that possess acidic and basic chemical groups. For example, an enzyme must be ready to lose or gain a proton at a particular instant during a biological reaction. It must also revert to the original protonation state to regenerate the active enzyme. This requires a match between the pKa of the corresponding chemical group and the ambient pH, which is one of the fundamental physiological variables. Therefore, determination of the pKa values of various protonation equilibria of a biologically important molecule is vital in understanding its *in vivo* behaviour. Computation of pKa values is challenging if the molecule possesses a number of protonation states with overlapping equilibria. Further, in the case of molecules containing homotopic chemical groups, association or ionization of proton at one centre affects the other. Accurate determination of protonation equilibria therefore requires meticulous data acquisition followed by analysis using efficient algorithms.

The glass electrode probe is used to find the variation of pH for the metal complex formation in acid-base equilibrium occurs in biological systems, for which the determination of acid-base equilibria of proton-ligand is necessary. Therefore, the speciation study of binary and ternary systems could be facilitated by tracking changes in the hydrogen ion concentration of a system containing a metal ion and a ligand using Bjerrum's method1 of potentiometric titration and knowing the protonation constants of the most anionic form of the ligand(s).

II. DATA ACQUISITION

The analysis of the protonation equilibria of the chosen ligands in the aqueous medium was conducted using the potentiometric titration procedure1 of Bjerrum modified by Calvin - Wilson2. It was titrated with approximately 0.2 mol. dm-3 (KOH) potassium hydroxide using the necessary volumes of hydrochloric acid (to maintain the overall concentration of 0.02 -0.05 mol.dm-3), potassium chloride (with an ionic strength of 0.1 mol.dm-3), and water in the presence and absence of a ligand in a cumulative volume of 50.0 cm3. Different studies were conducted with the ligand concentration maintained between 0.004 and 0.015 mol.dm-3. The ionic product of water and the pH correction factor were estimated using Gran's approach 3, 4.

1. **Proton-Ligand Equilibria of Azelaic and Terephthalic Acid Dihydrazides:** Dihydrazides with general formula, R (-CO-NH-NH_2)$_2$ are a group of nitrogenous organic compounds as shown in Figure 1. Depending on 'R', dihydrazides can be classified as aromatic or aliphatic.

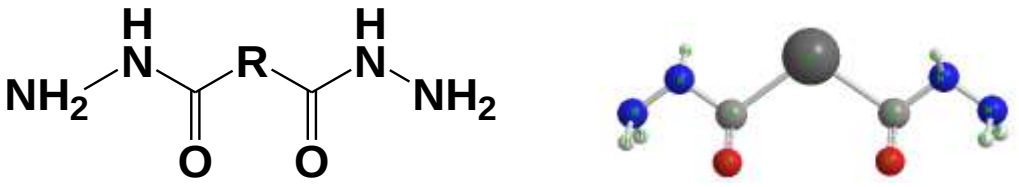


Figure 1: General formula of a dihydrazide

The presence of -NH2 groups in terminal positions in the presence of an acid results in the formation of the mono-protonated (LH+) and biprotonated (LH22+) species. As a result of enolic protons being lost, LH-1- and LH-22-type deprotonated species are created during keto-enol tautomerism of hydrazides (Figure 2).

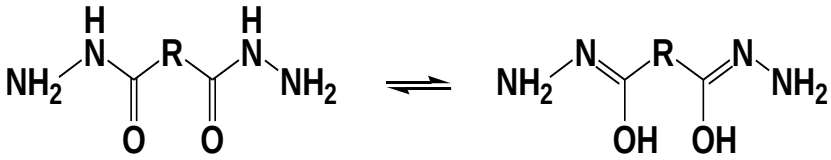
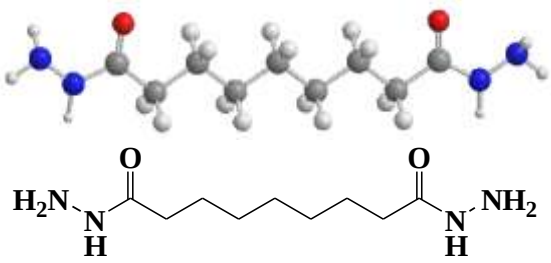


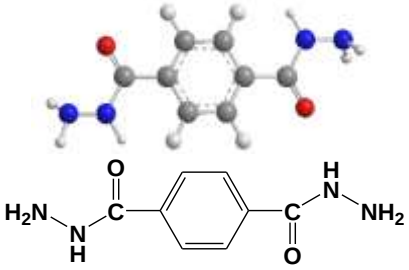
Figure 2: Keto-enol tautomerism of dihydrazides

Thus in the absence of other groups, the possible protonation states of dihydrazides are LH_2^{2+}, LH^+, L, LH_{-1}^- and LH_{-2}^{2-}. Dihydrazides selected for the study were, azelaic acid dihydrazide (AZDH) and terephthalic acid dihydrazide (TPDH). In spite of the catalytic[5-7] and biological importance[8-16] of dihydrazides, there is a paucity of information in the literature on their solution equilibria. There were no literature reports on the acid-base equilibria of the selected dihydrazides.

AZDH is an aliphatic dihydrazide, where R= - $(CH_2)_7$ - and TPDH is an aromatic dihydrazide, R= - (C_6H_4) - (Fig. 3).



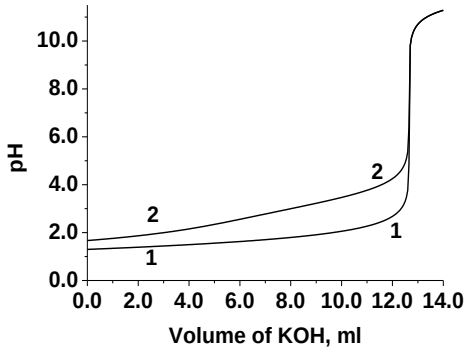
Azelaic or nonanedioic acid dihydrazide

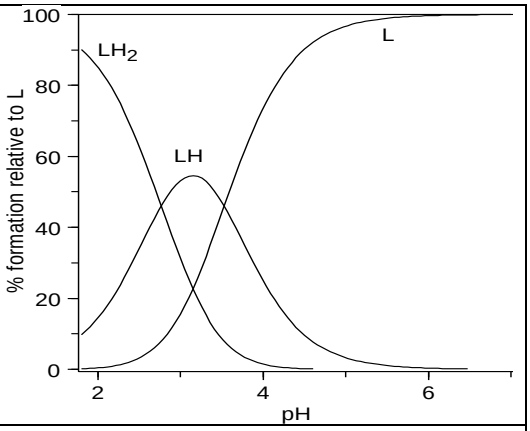


Terephthalic acid dihydrazide

Figure 3: Structures of AZDH and TPDH

The ligands AZDH and TPDH do not possess any ionisable protons below the pH region of 9.0, because the addition of 0.1 mole dm^{-3} alkali about one drop can suddenly raise the pH of both the ligands. Fig 4a and 5a represents the pH-metric titration data for AZDH and TPDH. The titration of hydrochloric acid without a ligand is represented by curve 1, while the titration of acid + dihydrazide using potassium hydroxide is represented by curve 2. Below a pH of about 5.0, the titration curves for both TPDH and AZDH (curve 2 in Figures 4a and 5a) are higher than those for free acid. The existence of proton associable centers is indicated by the difference between the free acid and ligand curves in the lower pH area for both dihydrazides.





a.	1. Titration curve of free mineral acid	**b.** Species distribution diagram
	2. Titration curve for acid + AZDH	
[AZDH] = 0.0150 mol dm^{-3}	[HCl] = 0.05034 mol dm^{-3}	[KOH]= 0.1989 $moldm^{-3}$
Temp..= 30.0 ±0. 1^0C	Total volume= 50.0 cm^3	I = 0.1 mol dm^{-3} KCl

Figure 4: Proton-Ligand System of AZDH

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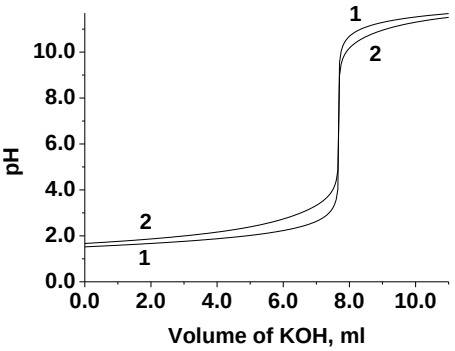
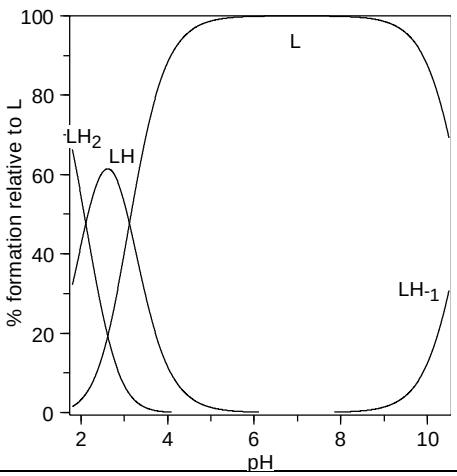
<tr><td colspan="2"> 
</td><td> 
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<tr><td>a.</td><td>1. Titration curve of free mineral acid</td><td>b. Species distribution diagram</td></tr>
<tr><td></td><td>2. Titration curve for acid + TPDH</td><td></td></tr>
<tr><td colspan="2">[TPDH] = 0.00504 mol. dm^{-3}</td><td>[HCl] = 0.03011 mol. dm^{-3}</td><td>[KOH]= 0.1961mol. dm^{-3}</td></tr>
<tr><td colspan="2">Temp. = 30.0 ±0. 1^0C</td><td>Total volume= 50.0 cm^3</td><td>I = 0.1 mol. dm^{-3} KCl</td></tr>
<tr><td colspan="4">Figure 5: Proton-Ligand System of TPDH</td></tr>
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Deprotonation of enolic groups was observed only in the case of TPDH as it is observed that a significant decrease of the titration curve of the ligand relative to the free acid on basic side i.e. above a pH of ~9.0. In the case of AZDH, there is no such a deviation indicating that the pH region of study is not showing any enolic proton's deprotonation. The free acid and ligand curves coincide between these two pH ranges, suggesting that there are no further proton-ligand equilibria.

The titration data were first subjected to analysis by ACBA computer program[17], modified by the author to run on a personal computer. The formation constants obtained from the ACBA program were taken as initial estimates for refinement by MINIQUAD-75 program[18]. The protonation and deprotonation equilibria of AZDH are shown in Fig. 6. The Miniquad-75 software yielded a best-fit model (Table 1) with two formation constants corresponding to the generation of LH+ and LH22+ species, which correspond to β011 and β012, respectively.

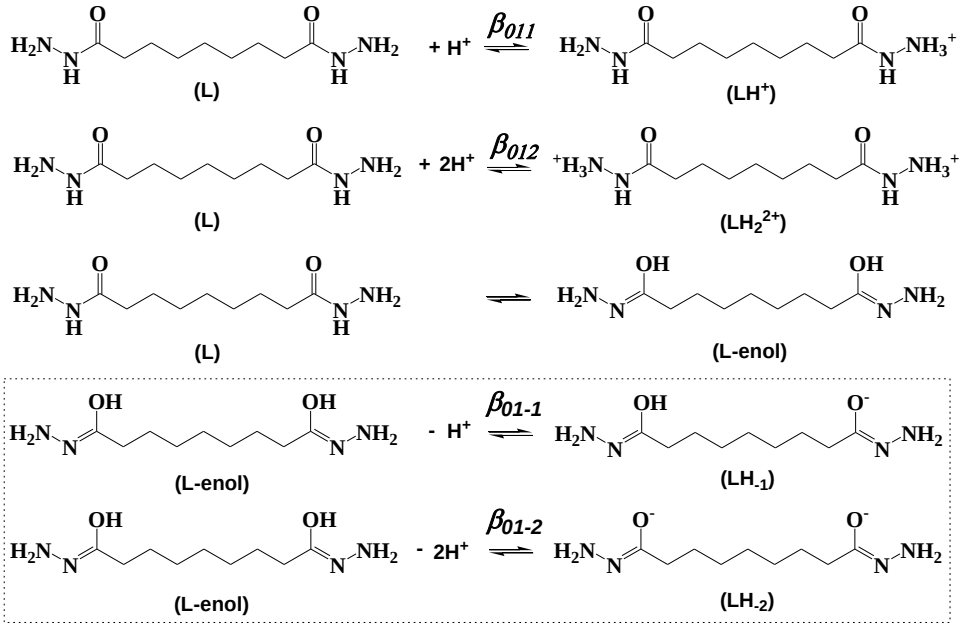


Figure 6: Protonation and deprotonation equilibria of AZDH.

Table 1: Best-fit chemical model for the acid-base equilibria of azelaic acid dihydrazide in the aqueous medium.

Temp. = 30.0 ± 0.1°C and ionic strength, I = 0.1 mol dm^{-3} (KCl)

Species *mlh*	L*og* β_{0lh} *(SD)*	Number of experimental points analysed NP	Sum of the squares of residuals, U/NP	χ^2
011	3.53 (0.01)	190	3.601 e-10	21.14
012	6.30 (0.01)			

The formation constants, $\beta_{01\text{-}1}$ and $\beta_{01\text{-}2}$ related to the deprotonation of enolic groups (Fig. 6) were not converged. The AZDH species distribution diagram (Fig. 4b) shows that the LH22+ form of AZDH is limited to pH values below 4.0. Around 90% of it forms at a pH of 1.8. At pH 3.1, the species LH+ reaches its maximal production rate of 55% and becomes extinct above pH 6.0.

Table 2 and Fig. 7 display the best-fit model together with the associated protonation and deprotonation equilibria of TPDH, respectively. The creation of LH22+, LH+, L, and LH-1 species in the aqueous medium is indicated by the best-fit model. Lower pH (below ~3.0 pH) favors the biprotonated form of TPDH, LH22+, which deprotonates to generate the mono-protonated (LH+) and neutral species (L) as pH rises.

Table 2: Optimal chemical model for terephthalic acid dihydrazide's acid-base equilibria in aqueous solutions.

Temp. = 30.0 ± 0.1°C and I = 0.1 mol dm–3 (KCl) for the ionic strength

Species *mlh*	L*og* β_{0lh} *(SD)*	Number of experimental points analysed NP	Sum of the squares of residuals, U/NP	χ^2
011	3.12 (0.01)	130	6.230 e-09	37.72
012	5.24 (0.01)			
01-1	-10.85 (0.01)			

Figure 7: Protonation and deprotonation equilibria of TPDH

β011 and β012 represent the formation constants as illustrated in Figure 3.7, denoting the formation of monoprotonated and biprotonated TPDH species from its initially neutral state. In the optimal model, the formation constant β01-1 corresponds to the deprotonation of one of the enolic protons, leading to the creation of the LH-1 species. This particular species emerges in the solution at a pH level of approximately 8.5 and constitutes roughly 30% of the total ligand content at a pH of 10.5. In contrast, the formation constant β01-2, associated with the deprotonation of the second enolic group leading to the formation of LH-2 species, did not reach convergence. This is due to the likelihood that its equilibrium lies well beyond the pH range investigated in this study. Nevertheless, in the presence of a metal ion, the ligand can also undergo the loss of the second enolic proton, resulting in the formation of both monodeprotonated and bideprotonated species, denoted as MmLlH-1 and MmLlH-2, respectively.

III. PROTONATION EQUILIBRIA OF SELECTED AMINO ACIDS

The α-amino acids selected for the study were L-Proline, L-Lysine and L-Aspartic acid. The observed spread in the literature reports[19-24] of the proton complex formation constants of amino acids by several researchers may be due to the use of different experimental techniques and different conditions of temperature, ionic strength, solvent etc. The inconsistency in the proton stability constants even for the systems studied under identical experimental conditions may be due to the use of low precision data acquisition techniques and error-prone graphical methods of analysis. Therefore, the protonation constants of proline, lysine and aspartic acid, although available in the literature, were redetermined using an auto-titrator attached high precision potentiometer for data acquisition followed by the analysis adopting proven reliable computational methods. The formation constants were determined under the same experimental conditions of temperature and ionic strength that were used to study the binary and ternary systems.

1. **Acid-base equilibria of L-Proline:** Proline (2-pyrrolidine carboxylic acid) is a proteinogenic aliphatic α-amino acid (Figure 8). Since the body can produce it by breaking down another amino acid called L-glutamate, it is not necessary. Proline is biologically changed to hydroxyproline in the presence of ascorbic acid, which is a key

constituent of tissue collagen. Proline is a structurally unique amino acid in which the α-amino group is a part of the ring structure making it a secondary amine. Linkage to other amino acids through the secondary amine is responsible for the bends and kinks in the shape of the proteins.

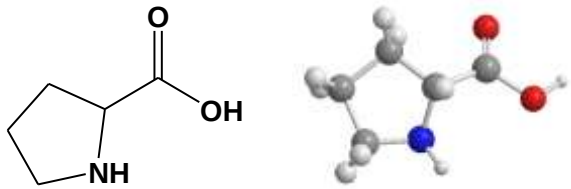


Figure 8: L- Proline

In aqueous solution, proline may exist in anionic (X^-), neutral or zwitterionic (XH) and cationic (XH_2^+) forms. The acid-base equilibria for the formation of zwitterionic and cationic forms from the most anionic form of proline are shown in Figure 9.

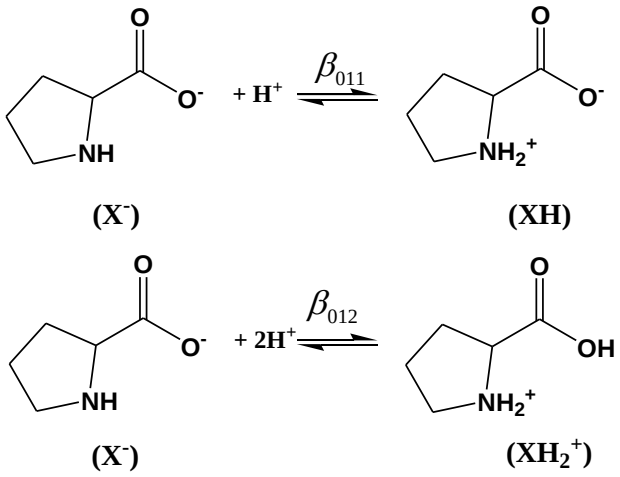
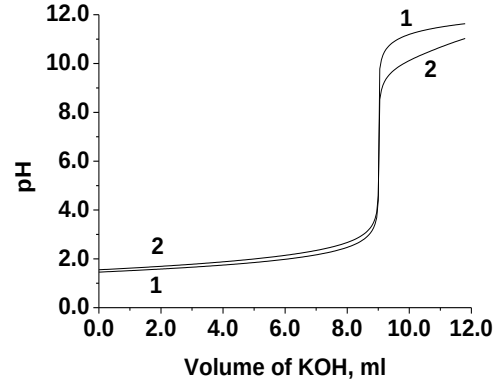
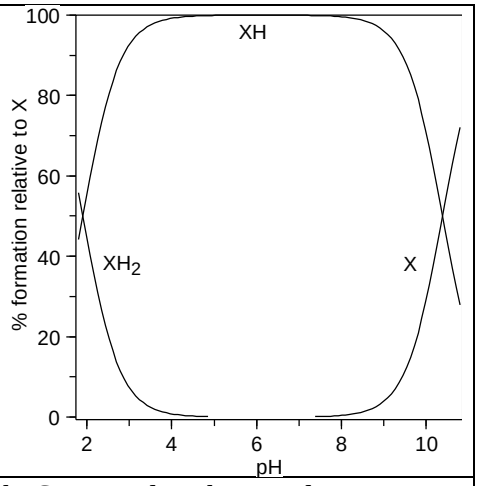


Figure 9: Protonation equilibria of most anionic form of L-Proline

The potentiometric titration curves obtained for acid-base systems of L-Proline are shown in Fig. 3.10a. The titration of mineral acid without proline is represented by Curve 1. The titration curve for mineral acid + proline i.e. curve 2 is above that of free acid in the pH region below a pH of ~4.0. This indicates an association of proton to the ligand. Above a pH of ~8.0, the titration curve of proline is below that of free acid indicating dissociation of a proton from the ligand. Therefore, the two buffer regions corresponding to proton association and dissociation equilibria are well separated.

Miniquad-75 program18 was used to analyze the titration data, and Table 3 displays the best-fit model that was found.





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

[Proline] = 0.0100 mol dm^{-3}	[HCl] =0.03495 mol dm^{-3}	[KOH]= 0.1939 moldm^{-3}
Temp..= 30.0 ±0. 1^0C	Total volume= 50.0 cm^3	I = 0.1 mol dm^{-3} KCl

Figure 10: Proton-Ligand System of L-Proline

Table 3: Best-fit chemical model for acid-base equilibria of L-Proline in the aqueous medium.

Temp. = 30.0 ± 0.1°C and ionic strength, I = 0.1 mol dm^{-3} (KCl)

Species *mlh*	$log\ \beta_{0lh}\ (SD)$	Number of experimental points analysed, NP	Sum of the squares of residuals, U/NP	χ^2
011	10.39 (0.01)	196	4.606 e-10	8.69
012	12.29 (0.01)			

The protonation of the amino group in the ligand's most basic form results in the formation constant, β011, which is a zwitterion. 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 the neighbouring deprotonated carboxylate group. The second protonation constant, $log\ \beta_{012}$ is due to the addition of one more proton to the ligand at the carboxylate group. Again, the relative decrease in the magnitude of the constant to that of alkyl carboxylate is due to the strong withdrawing effect of the protonated amino group. The observed values of the protonation constants are in good agreement (Table 4) with the published values of the critical stability constants[23] and other literature values[24-30] after allowing for changes in the experimental conditions.

Table 4: Some literature reported protonation constants of L-Proline

S.No.	*log* β_{011}	*log* β_{012}	*I*, Temp.	Method	Ref.
1.	10.38	12.28	0.1M, 25^0C	Pot.	25
2.	10.34	12.34	0.15M, 37^0C	Pot.	26
3.	11.20	13.80	0.1M, 15^0C	Pot.	27
4.	10.54	-	0.02 M, 25^0C	Pot.	28
5.	10.48	-	0.1M, 20^0C	Spec, Pot.	29, 30
6.	11.40	13.41	0.1M, 15^0C	Pot.	31
7.	10.65	-	0.1M, 30^0C	Pot	32

Fig. 10b displays the species distribution diagram for L-proline. Nearly 55% of the ligand is in biprotonated form at 1.80 pH. The zwitterion 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 8.0 pH and increases monotonically above this point.

2. Acid-base equilibria of L-Lysine: An important aliphatic α-amino acid is lysine (2, 6-diminohexanoic acid). With an extra amino group bonded to the ω-carbon atom in the side chain, it is a polar amino acid (Fig. 3.11). Since the ω-NH2 group is protonated in aqueous solution over a broad pH range, including the physiological pH, lysine is categorized as a positively charged basic amino acid. L-lysine is necessary for healthy growth and is a key component in the synthesis of carnitine, a nutrient that helps decrease cholesterol and converts fatty acids into energy.

The ω-NH2 group of the side chain is the first center to protonate in the fully ionized lysine anion (X-), followed by the α-NH2 group and the carboxylate group. Structurally, it is therefore, possible that lysine (XH) can exist in solution as XH_3^{2+}, XH_2^+, XH and X^-.

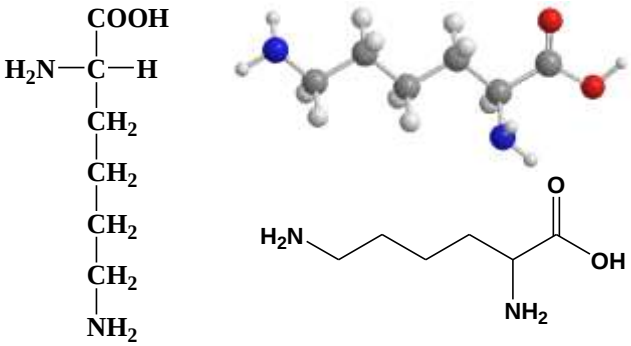


Figure 3.11: L-Lysine

The equilibria for the formation of the protonated species from the most anionic form of lysine (X^-) are shown in Fig. 12. β_{011}, β_{012} and β_{013} are the overall formation constants for the formation of XH, XH_2^+ and XH_3^{2+} respectively from X^-.

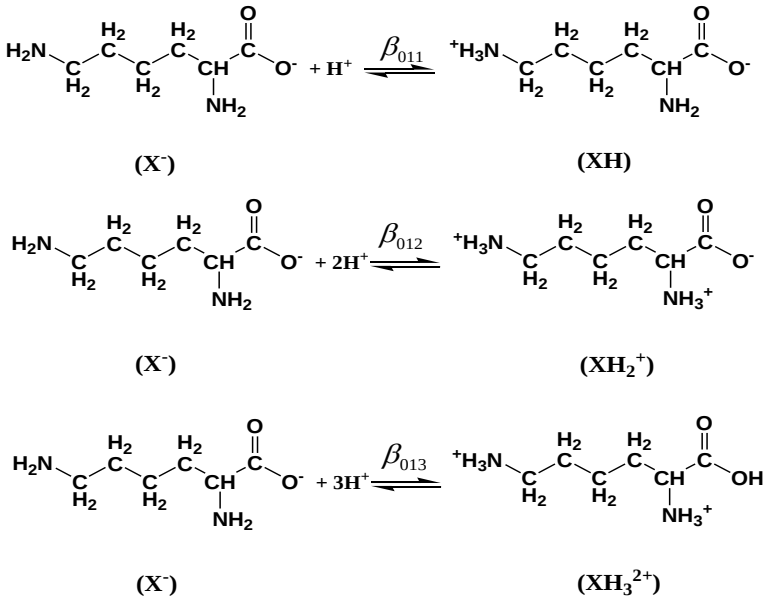
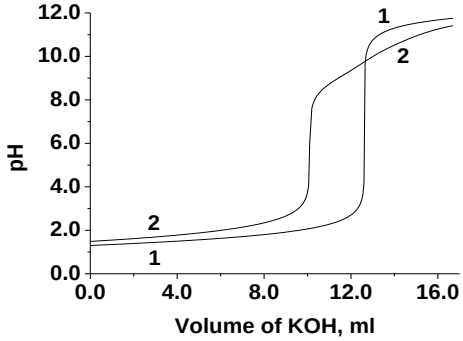
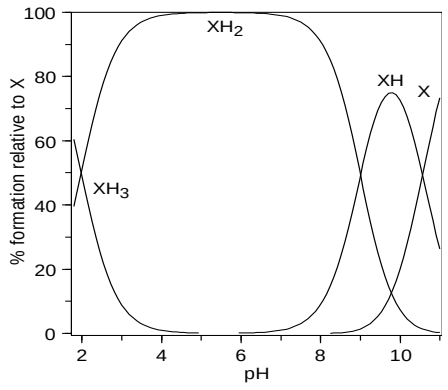


Figure 12: Protonation equilibria of the most anionic form of L-Lysine.

The pH-metric titration curves obtained, for acid-base systems of lysine are shown in Fig. 13a. The titration curve for the acid+ lysine (curve 2) is above that of free acid (curve 1) over a wide range of pH indicating the initial association of protons to the ligand. There are two well-separated buffer regions. The buffer region below ~3.0 pH corresponds to the titration of a carboxylic proton. The buffer region above pH ~7.0 indicates the titration of both α- and ω-ammonium protons. The protonation equilibria of the two $–NH_2$ groups are overlapping.





a.	1. Titration curve of free mineral acid	b. Species distribution diagram
	2. Titration curve for acid + Lysine	
[Lysine] = 0.0100 mol dm^{-3}	[HCl] = 0.05005 mol dm^{-3}	[KOH]= 0.1983 moldm^{-3}
Temp.: 30.0 ±0. 1^0C	Total volume= 50.0 cm^3	I = 0.1 mol dm^{-3} KCl

Figure 3.13: Proton-Ligand System of L-Lysine

The titration data for all the experiments with different concentrations of the ligand (0.004, 0.01 and 0.015 mol dm^{-3}) were analysed using the MINIQUAD-75 program[18]. The initial estimates for the protonation constants were taken from the literature reports. The best-fit chemical model thus obtained along with the statistical parameters is shown in Table 3.5.

Table 5: Best-fit chemical model for acid-base equilibria of L-Lysine in the aqueous medium.

Temp. = 30.0 ± 0.1°C and ionic strength, I = 0.1 mol dm^{-3} (KCl)

Species *mlh*	L*og* β_{0lh} *(SD)*	Number of experimental points analysed NP	Sum of the squares of residuals, U /NP	χ^2
011	10.55 (0.01)	214	1.507 e-09	5.05
012	19.55 (0.01)			
013	21.54 (0.01)			

After accounting for the modifications to the experimental setup and computation techniques, the outcomes are in good agreement with the reports from the literature (Table 6). The stepwise protonation constants of the most anionic form of lysine are log K1, log K2, and log K3.

Table 6: Some representative literature reports on the step-wise protonation constants of Lysine

S.No.	*log* K_1	*log* K_2	*log* K_3	*Ionic str.*, Temp.	Method	Ref.
1.	10.71	9.19	2.16	0.1-0.2 M, 25^0C	Pot	19
2.	10.69	9.08	2.04	0.1M, 25^0C	Pot.	33
3.	10.67	9.14	2.20	0.1M, 25^0C	Pot.	34
4.	10.65	9.14	2.18	0.1M, 25^0C	Pot.	35
5.	10.66	9.20	2.15	0.2M, 25^0C	Pot.	36
6.	10.79	-	-	0.1M, 25^0C	Pot.	37
7.	10.53	8.95	2.18	0.1M, 25^0C	Pot.	38
8.	10.63	9.10	2.09	0.1M, 25^0C	Pot.	39
9.	10.55	9.00	1.99	0.1M, 30^0C	Pot,	Present Work

Fig. 13b displays the species distribution diagram for lysine proton-ligand equilibria. The fully protonated form XH_3^{2+} exists only below a pH of 4.0. This species loses the carboxylic proton and forms XH_2^+ which is a dominating species between 3 and 7pH. It represents nearly 99% of the total ligand over a wide pH region. Further increase in pH causes neutralization of the α-ammonium proton resulting in the zwitterionic species $XH^{\pm}$. The formation of the XH reaches a maximum of 75% of the total ligand around 9.8 pH. Further increase in pH leads to the deprotonation of ω-ammonium proton

of the side chain. The carboxylate group's ability to extract electrons from the α-NH2 group more effectively than the ω-NH2 group may be the reason for the α-NH2 group's lower basicity.

3. **Protonation states of** L-**Aspartic Acid:** Two carboxylic acid groups, one on the alpha carbon atom and the other in the side chain, characterize aspartic acid (2-aminobutanedioic acid), an acidic amino acid (Fig. 14). This unneeded α-amino acid is typically present in proteins and functions as an excitatory neurotransmitter in the central nervous system, as well as in the synthesis and release of hormones. It contributes significantly to the citric acid cycle biochemically.

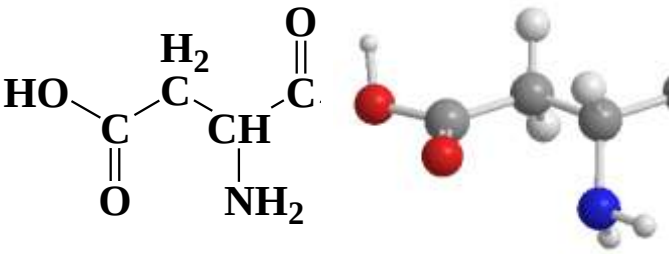


Figure 3.14: L- Aspartic acid (XH_2)

In aqueous solution depending on the pH, L-aspartic acid may exist (Fig. 15) in cationic (XH_3^+), neutral zwitterionic ($XH_2^{\pm}$) or anionic (XH^- and X^{2-}) forms.

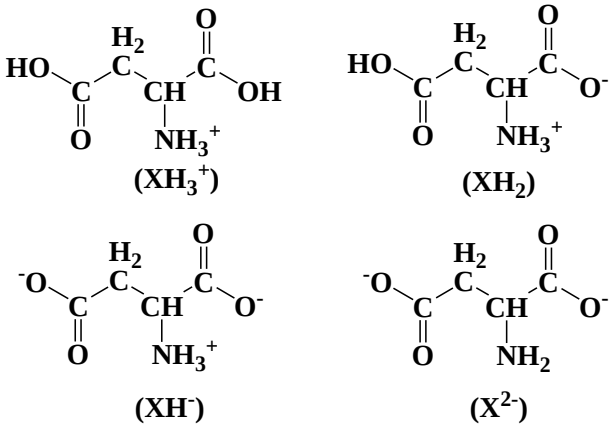
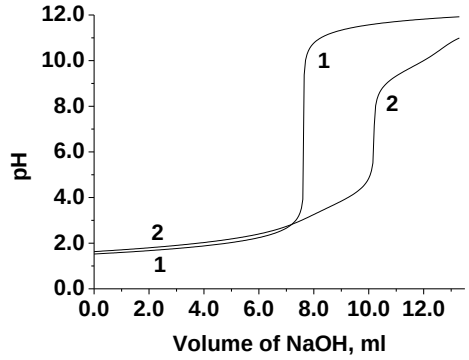
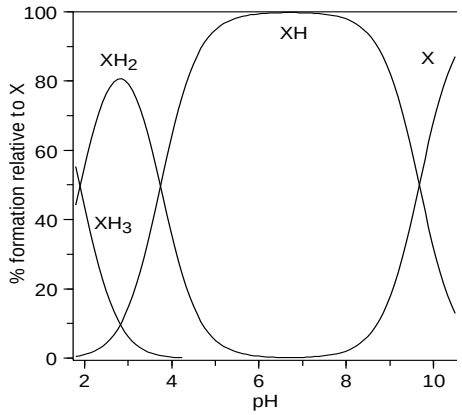


Figure 15: Protonation states of L-Aspartic acid

It has an isoelectric point at roughly 2.8 pH. Since the aqueous medium is basic and neutral, the entire molecule has a negative charge. Proteins nearly entirely include it because of the negatively charged carboxylate group that it possesses. The charged group's solubility in water is mostly due to its ability to establish ionic bonds with different metal ions and engage in dipole interactions with water molecules.

The pH-metric titration curves of free acid (curve 1) and acid + ligand (curve 2) are shown in Fig. 16a.





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

[ASP] = 0.01002 mol. dm^{-3}	[HCl] = 0.02990 mol. dm^{-3}	[KOH]= 0.1958 mol. dm^{-3}
Temp.: 30.0 ±0. 1^0C	Total volume= 50.0 cm^3	I = 0.1 mol. dm^{-3} KCl

Figure 16: 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. In the lower pH range, the ligand titration curve is above the free acid's, showing proton interaction with the ligand. The Miniquad-75 program18 analyzed the experimental data, and Table 7 displays the best-fit chemical model and statistical parameters that were found.

Table 7: Best-fit chemical model for acid-base equilibria of L-Aspartic acid in the aqueous medium.

Temp. = 30.0 ± 0.1°C and ionic strength, I = 0.1 mol dm^{-3} (KCl)

Species *Mlh*	L*og* β_{0lh} *(SD)*	Number of experimental points analysed NP	Sum of the squares of residuals, (U/NP)	χ^2
011	9.67 (0.01)	187	2.508 e-09	9.96
012	13.42 (0.01)			
013	15.32 (0.01)			

After taking into account the variations in the experimental settings, the observed values are in close agreement with the "Critical Stability Constants" published by Martell et al.40 and other researchers41-44. Fig. 3.17 displayed the proton-aspartate equilibria corresponding to the formation constants in the best-fit model.

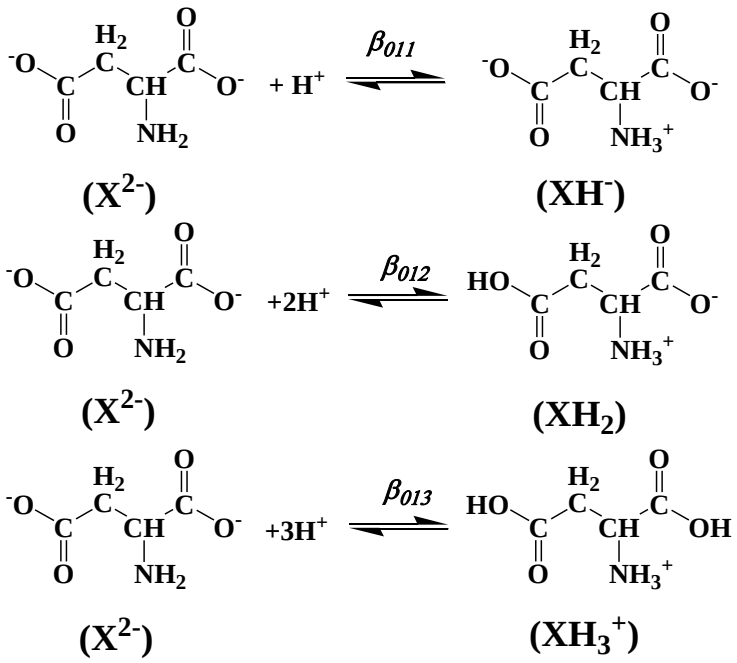


Figure 17: Protonation equilibria of the most anionic form of L-Aspartic acid

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. β013 represents the total formation constant of the fully protonated version of the ligand that is the most anionic.. The species distribution diagram indicating the percentage of formation of each species against pH is shown in Fig. 16 b. The fully protonated form XH_3^+ exists up to a pH of ~4.0. With the increase in pH, XH_3^+ loses 'α- carboxylic' proton leading to the formation of neutral zwitterion. The maximum extent of formation of XH_2 form is 80% at 2.8 pH. This species exists up to ~6.0 pH. The neutral form of the ligand, with the increase in pH, loses carboxylic proton of the side chain forming anionic XH^- form of aspartic acid. Nearly 99-100% of the ligand is in this form between 5.8 and 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 the amino group.

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