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# Application of hierarchical thermodynamic interactions to the protonation equilibria of organic polyprotic acids

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**Abstract**

A general method for formulating complex thermodynamic systems in terms of hierarchical interactions has been developed, and has been applied in a previous analyses to the theoretical analysis of cooperativity in a dimeric protein, to the statistical analysis of hemoglobin oxygen binding data, and to the protonation equilibria of inorganic polyprotic acids. Organic polyprotic acids have served as a demonstration system for the development of concepts and methods for treating complex biochemical equilibria. Glutamic acid is the classic test case for understanding proton–proton interactions in organic polyprotic acids, and this system is analyzed using the concept of hierarchical interactions. Second order interactions were apparent between all three possible proton interactions, as has been established previously. The third order interaction between the three protons was found to be insignificant, indicating that protonation of one site on glutamate has no effect on the interaction between the other two protonation sites. This further reinforces the premise that higher order terms, representing more complex interactions, are less likely to be significant than lower order terms. To allow correlation of the interaction values from glutamate with other organic acids, pairwise interaction values between protonation events were then calculated from known $pK_d$ values for a number of diprotic acids and bases. For simple straight chain acids and bases a linear log–log relationship was apparent between the number of intervening atoms between the protons and the $pK_{d,hh}$ ($pK_d$ of interaction). This relationship extended from three atoms (carbonate) up to 11 atoms (azelaic acid) and applied to both dicarboxylic acids and diamine bases. The pairwise interactions in glutamate also followed this simple relationship. 

*Keywords:* Thermodynamics; Hierarchical interactions; Acids; Models/chemical; Allosterism

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## 1. Introduction

We have developed a method for describing complex state systems as a hierarchy of interactions [1–3]. The concept can be applied to the $\Delta G^\circ$ values describing a state system, as well as any other state parameter describing such a system including for example spectroscopic extinction coefficients [4]. The concept of hierarchical interactions is an extension of the well-known concept of pairwise interactions [5–7]. An overview of this method as a prelude to its application to analysis of inorganic polyprotic acid has been presented [8].

Interactions between proton binding events in organic polyprotic acids have been used to study electrostatic, dipolar, and conformational effects between protons in such simple systems [9,10]. More recently these same systems have received attention as models for site-specific thermodynamic interactions in more complex systems such as interactions between thermodynamic events in proteins, where site-specific refers to knowledge of the behavior of a specific site when studied in the context of other sites [11]. These analyses have generally treated all interactions as simple pairwise interactions. However, the possibility for more complex behavior, as revealed by significant third and higher order interactions, exists for polyprotic organic acids as it does for inorganic polyprotic inorganic acids and for protein–ligand binding events. The application of the concept of hierarchical interactions to the analysis of the site-specific thermodynamic properties of organic polyprotic acids would further demonstrate this concept and its utility in analyzing interactions in such systems. This method is therefore used here to analyze the interactions between successive protonation events which occur in the classic case of glutamate. To provide a basis for comparing the interactions in glutamate with the interactions in other polyprotic organic acids, the interactions from a series of symmetrical diprotic organic acids and bases were analyzed. A simple relationship between the number of atoms separating the interacting protons and the degree of interaction was discovered, and the interaction values from glutamate were found to also follow this simple relationship. Deviations from this simple relationship were apparent for conformationally restricted diprotic acids, or in acids where hydrogen bonding was significant.

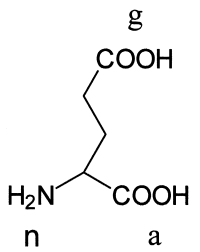



Fig. 1. Glutamate with three protonation sites labeled a, g, and n (for alpha, gamma, and amino).

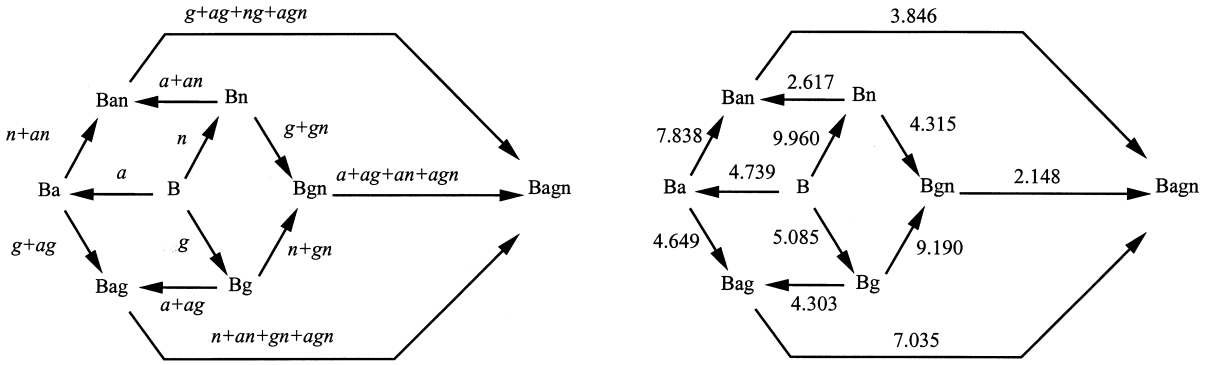
## 2. Materials and methods

### 2.1. $pK_d$ values

$pK_d$ (dissociation) values for glutamate (Fig. 1), its mono and di esters, and derived microscopic values (Fig. 3) were from Neuberger [12] and Hill [10]. $pK_d$ values for all other organic acids were from standard sources [13,14]. Interaction $pK_d$ values for water and carbonate were derived previously [8].

### 2.2. Conversion to hierarchical interaction terms

Systematic rules for formulating complex systems in terms of hierarchical interactions have been developed [1,3], and were summarized for application to inorganic polyprotic acids [8]. Two models are relevant to the following analysis. Glutamic acid has three distinguishable protonation sites (Fig. 1), and the hierarchically formulated model depicted in Fig. 2 applies. Note that




$pK_{d,a} = 4.739$ $pK_{d,ag} = -0.436$ $pK_{d,agn} = -0.033$
$pK_{d,g} = 5.085$ $pK_{d,an} = -2.122$
$pK_{d,n} = 9.960$ $pK_{d,gn} = -0.770$

Fig. 2. Top left; hierarchically reformulated model for protonation of glutamate. B represents the basic form of glutamate, and lower case letters are used to denote protonation of a given site (Ba, mono-protonated on the α-carboxyl group, etc.). Lowercase italicized letters are used to denote hierarchical $\Delta G^\circ$ values. The same pattern applies to hierarchical $pK_d$ values since $pK_d$ values are proportional to $\Delta G^\circ$ values [Eq. (15)]. Top right; microscopic $pK_d$ values for protonation of glutamate from Neuberger [12] and Hill [10]. Bottom; derived hierarchical $pK_d$ values.

this model is essentially the same as the basic model used in the original derivation of the concept of hierarchical interactions [1], except that the three 'ligands' are the same (protons), but that the 'binding sites' are different.

For symmetric diacids and dibases the well-known concept of statistical correction factors must be applied, where the statistical correction factor for a step is equal to the number of ways forward divided by the number of ways back. For a dibase with two macroscopically apparent $pK_d$ values ($pK_{d,1}$ and $pK_{d,2}$) there are several equivalent formulations available (Scheme 1), including using hierarchical interaction $\Delta G^\circ$ values (denoted by lowercase italicized letters), corresponding equilibrium association constants, equilibrium dissociation constants, and hierarchical interaction $pK_d$ values. Conversion between these descriptions is straightforward [8], and the same pattern applies to diacids.

### 2.3. Hierarchical interaction parameter values for glutamate

The hierarchically reformulated model for glu-

$$\begin{array}{ccc} & pK_{d,1} & \qquad pK_{d,2} \\ B + H^+ & <=====> & BH^+ + H^+ <==========> BH_2^{+2} \\ & h - RT\ln 2 & \qquad h + hh - RT\ln(\tfrac{1}{2}) \\ & 2\,K_{a,h} & \qquad \tfrac{1}{2}\,K_{a,h}K_{a,hh} \\ & \tfrac{1}{2}\,K_{d,h} & \qquad 2\,K_{d,h}K_{d,hh} \\ & pK_{d,h} - \log(\tfrac{1}{2}) & \qquad pK_{d,h} + pK_{d,hh} - \log 2 \end{array}$$

Scheme 1.

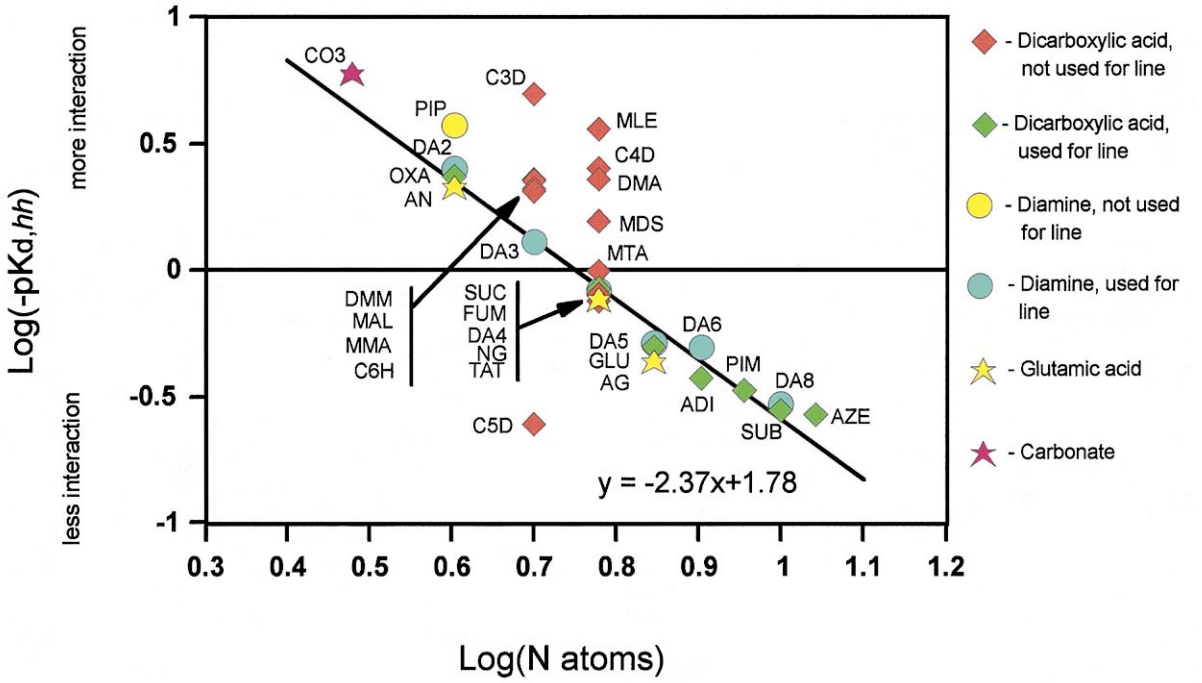



Fig. 3. Plot of $\log(-\mathrm{p}K_{\mathrm{d},hh})$ vs. log(N atoms) from the values listed in Table 1.

tamate and the microscopic $\mathrm{p}K_{\mathrm{d}}$ values [10,12] are summarized in Fig. 2. Derivation of values for the second and third order interaction $\mathrm{p}K_{\mathrm{d}}$ values are straightforward [8] and are summarized in the bottom of Fig. 2 and also included in Table 1.

### 2.4. Pairwise interaction values for a series of symmetrical diprotic acids and bases

Microscopic $\mathrm{p}K_{\mathrm{d}}$ values can be readily calculated from macroscopic $\mathrm{p}K_{\mathrm{d}}$ values for symmetrical diprotic acids and bases. Statistical effects of multiple binding sites (Scheme 1) must be taken into consideration. Macroscopic ($\mathrm{p}K_{\mathrm{d},1}$ and $\mathrm{p}K_{\mathrm{d},2}$) and microscopic ($\mathrm{p}K_{\mathrm{d},h}$ and $\mathrm{p}K_{\mathrm{d},hh}$) constants are related by the following equations.

Given:

$$\mathrm{p}K_{\mathrm{d},1} = \mathrm{p}K_{\mathrm{d},h} - \log(\tfrac{1}{2}) \quad (1)$$

and

$$\mathrm{p}K_{\mathrm{d},2} = \mathrm{p}K_{\mathrm{d},h} + \mathrm{p}K_{\mathrm{d},hh} - \log(2) \quad (2)$$

then:

$$\mathrm{p}K_{\mathrm{d},h} = \mathrm{p}K_{\mathrm{d},1} + \log(\tfrac{1}{2}) \quad (3)$$

$$= \mathrm{p}K_{\mathrm{d},1} - 0.301 \quad (4)$$

and

$$\mathrm{p}K_{\mathrm{d},hh} = \mathrm{p}K_{\mathrm{d},2} - \mathrm{p}K_{\mathrm{d},1} - \log(\tfrac{1}{2}) + \log(2) \quad (5)$$

$$= \mathrm{p}K_{\mathrm{d},2} - \mathrm{p}K_{\mathrm{d},1} + \log(4) \quad (6)$$

$$= \mathrm{p}K_{\mathrm{d},2} - \mathrm{p}K_{\mathrm{d},1} + 0.602 \quad (7)$$

Eqs. (4) and (7) were used to calculate $\mathrm{p}K_{\mathrm{d},h}$ and $\mathrm{p}K_{\mathrm{d},hh}$ values from $\mathrm{p}K_{\mathrm{d},1}$ and $\mathrm{p}K_{\mathrm{d},2}$ values for diprotic acids and bases.

Table 1
Pairwise interactions in organic diprotic acids and bases, glutamate, water, and carbonate[a]

| | Abbr | Ref | N atoms | $pK_{d,1}$ | $pK_{d,2}$ | $pK_{d,h}$ | $pK_{d,hh}$ | log(N) | $\log(-pK_{d,hh})$ |
|---|---|---|---|---|---|---|---|---|---|
| Acids | | | | | | | | | |
| Oxalic | OSA | 13 | 4 | 4.19 | 1.23 | 3.89 | −2.36 | 0.60 | 0.37 |
| Cyclopropane-1,1-DC | C3D | 13 | 5 | 7.43 | 1.82 | 7.13 | −5.01 | 0.70 | 0.70 |
| Dimethylmalonic acid | DMM | 14 | 5 | 6.06 | 3.17 | 5.76 | −2.29 | 0.70 | 0.36 |
| Malonic | MAL | 13 | 5 | 5.69 | 2.83 | 5.39 | −2.26 | 0.70 | 0.35 |
| Methylmalonic | MMA | 14 | 5 | 5.76 | 3.05 | 5.46 | −2.11 | 0.70 | 0.32 |
| Cyclohexane-1,1-DC | C6H | 13 | 5 | 6.11 | 3.45 | 5.81 | −2.06 | 0.70 | 0.31 |
| Cyclopentane-1,1-DC | C5D | 14 | 5 | 4.08 | 3.23 | 3.78 | −0.25 | 0.70 | −0.61 |
| Maleic | MLE | 13 | 6 | 6.07 | 1.83 | 5.77 | −3.64 | 0.78 | 0.56 |
| Cyclopropane-1,2-DC | C4D | 14 | 6 | 6.47 | 3.33 | 6.17 | −2.54 | 0.78 | 0.40 |
| Dimethylmalic | DMA | 13 | 6 | 6.06 | 3.17 | 5.76 | −2.29 | 0.78 | 0.36 |
| meso-2,3-Dimethylsuccinic | MSD | 14 | 6 | 5.94 | 3.77 | 5.64 | −1.57 | 0.78 | 0.20 |
| Meso-Tataric | MTA | 13 | 6 | 4.82 | 3.22 | 4.52 | −1.00 | 0.78 | −0.00 |
| Succinic | SUC | 13 | 6 | 5.61 | 4.16 | 5.31 | −0.85 | 0.78 | −0.07 |
| Fumaric | FUM | 13 | 6 | 4.44 | 3.03 | 4.14 | −0.81 | 0.78 | −0.09 |
| Tataric | TAT | 13 | 6 | 4.34 | 2.98 | 4.04 | −0.76 | 0.78 | −0.12 |
| Glutaric | Glu | 13 | 7 | 5.41 | 4.31 | 5.11 | −0.50 | 0.85 | −0.30 |
| Adipic | ADI | 13 | 8 | 5.41 | 4.43 | 5.11 | −0.38 | 0.90 | −0.42 |
| Pimelic | PIM | 14 | 9 | 5.42 | 4.48 | 5.12 | −0.34 | 0.95 | −0.47 |
| Suberic | SUB | 14 | 10 | 5.40 | 4.52 | 5.10 | −0.28 | 1.00 | −0.56 |
| Azelaic | AZE | 14 | 11 | 5.40 | 4.53 | 5.10 | −0.27 | 1.04 | −0.57 |
| Bases | | | | | | | | | |
| Piperazine | PIP | 13 | 4 | 9.93 | 5.56 | 9.63 | −3.77 | 0.60 | 0.58 |
| 1,2-Diaminoethane | DA2 | 13 | 4 | 10.71 | 7.56 | 10.41 | −2.55 | 0.60 | 0.41 |
| 1,3-Diaminopropane | DA3 | 13 | 5 | 10.94 | 9.03 | 10.64 | −1.31 | 0.70 | 0.12 |
| 1,4-Diaminobutane | DA4 | 13 | 6 | 11.15 | 9.71 | 10.85 | −0.84 | 0.78 | −0.08 |
| 1,5-Diaminopentane | DA5 | 14 | 7 | 10.25 | 9.13 | 9.95 | −0.52 | 0.85 | −0.29 |
| 1,6-Diaminohexane | DA6 | 14 | 8 | 10.93 | 9.83 | 10.63 | −0.50 | 0.90 | −0.30 |
| 1,8-Diaminooctane | DA8 | 14 | 10 | 11.00 | 10.10 | 10.70 | −0.30 | 1.00 | −0.53 |
| Glutamate | | | | | | | | | |
| *ag* interaction | AG | | 7 | | | | −0.44 | 0.85 | −0.36 |
| *an* interaction | AN | | 4 | | | | −2.12 | 0.60 | 0.33 |
| *ng* interaction | NG | | 6 | | | | −0.77 | 0.78 | −0.11 |
| Misc | | | | | | | | | |
| Water | | | 8 | 1 | | | −17.12 | 0.00 | 1.23 |
| Carbonate | CO3 | | 8 | 3 | | | −5.97 | 0.48 | 0.78 |

[a]Abbr = abbreviation, Ref = reference, DC = dicarboxylic. Other abbreviations defined in table. $pK_{d,h}$ and $pK_{d,hh}$ are calculated as described in the text.

## 3. Results and discussion

### 3.1. Glutamate

Analysis of the hierarchical interactions in glutamate (Fig. 1) reveals significant pairwise interactions between all three protonation events ($pK_{d,ag} = -0.44$, $pK_{d,an} = -2.12$, $pK_{d,ng} = -0.77$) but essentially no significant third order interaction (Fig. 3) ($pK_{d,agn} = -0.033$). In order to compare the results obtained here with the results obtained previously using the concept of aggregate interaction constants [11] it is necessary to define the relationship between hierarchical interaction constants and aggregate interaction constants. Aggregate interaction constants are the

difference between the fundamental constant for a single event and actual observed constant for an event. There is no difference between aggregate pairwise interactions and second order hierarchical interaction constants, but there is a difference between third order constants. The relationships between aggregate interaction constants [11] and hierarchical interaction constants are

$$c_{12} = an \tag{8}$$

$$c_{13} = ag \tag{9}$$

$$c_{23} = ng \tag{10}$$

$$c_{123} = ag + an + gn + agn \tag{11}$$

The pairwise interactions derived in this analysis are essentially identical to those derived by Di Cera [11] ($pK_d$ values; $c_{12} = -2.122$, $c_{13} = -0.452$, and $c_{23} = -0.770$) with the slight differences due to the differences in deriving values of the fundamental constants between Hill [10] and Di Cera [11]. The aggregate third order constant as used in previous analyses represents the sum of second and third order hierarchical interaction constants used here. The hierarchical third order interaction constant has a value of essentially zero. Both Hill [10] and Di Cera [11] have noted the slight discrepancy in $pK_d$ values between the aggregate third order term and the sum of second order terms. The hierarchical third order term represents this discrepancy.

The observation of an essentially zero third order interaction is analogous to what was found for the inorganic polyprotic acids phosphate and arsenate [8]. A third order interaction term represents physically how one event affects the interaction between two other events [1,8], and in the case of glutamate demonstrates that the protonation state of one site in glutamate has no effect on the interaction between the other two sites. Such a result was expected for conformationally rigid inorganic polyprotic acids such as phosphate and arsenate, but was uncertain for a conformationally flexible system such as glutamate.

### 3.2. Diprotic acids and bases

A significant effort has been made to determine the effect of structure on the $pK_d$ values of organic carboxylic acids (reviewed by Kine [15]), and several studies are available on the relationship between structure and proton–proton interaction in diprotic acids and bases, and glutamate [9,10]. It was of interest to correlate the results obtained for pairwise interactions in glutamate with the interactions in simple diprotic acids and bases to reveal any relationships. Only for symmetrical diprotic acids and bases can the two observable $pK_d$ values ($pK_{d1}$ and $pK_{d2}$) be used to derive values for the two intrinsic parameters ($pK_{d,h}$ and $pK_{d,hh}$) without the use of chemical derivatives as has been done for glutamate. One way of explaining this is that in an unsymmetrical diprotic system there are two intrinsic protonation constant and one pairwise interaction constant, for a total of three constants necessary to describe the system. It is not possible to determine values for the three fundamental constants from the two observable constants. In the case of glutamate the use of suitable derivatives allowed a solution for all the microscopic $pK_d$ values [12]. A series of simple diprotic acids and bases were included in this analysis as listed in Table 1. A number of symmetrical acids and bases were excluded from this analysis, such as aromatic and halogenated acids and bases, since a focus on the basic features of interaction was desired. The $pK_{d,h}$ and $pK_{d,hh}$ for the included acids and bases are also summarized in Table 1. Among the diprotic acids and bases listed in Table 1 are a number of simple unbranched hydrocarbon dicarboxylic acids and diamine bases. A graphical examination of the relationship between the number of atoms between the interacting protons revealed a linear relationship between $\log(-pK_{d,hh})$ and log(N atoms) (Fig. 3), with malonic acid being an exception and deviating significantly from the line.

Linear regression (excluding the value for malonic acid) gave the relationship

$$\log(-\mathrm{p}K_{\mathrm{d},hh}) = -2.37 \times \log(\mathrm{N\ atoms}) + 1.78 \quad (12)$$

Note that a $\mathrm{p}K_{\mathrm{d}}$ is proportional to a corresponding $\Delta G^{\circ}$ (of association)

$$\Delta G^{\circ} = -RT\ln(K_a) = -2.303RT\log(K_a) \quad (a \text{ for association}) \quad (13)$$

$$p\mathrm{K}_d = -\log(K_d) = \log(K_a) \quad (d \text{ for dissociation}) \quad (14)$$

Therefore

$$\Delta G^{\circ} = -RT\ln(K_a) = -2.303RT\,\mathrm{p}K_{\mathrm{d}} \quad (15)$$

The interaction values from carbonate and water [8] were also included in Table 1, and the value for carbonate was include in Fig. 3.

A linear relationship was observed for the simple acids and bases (Fig. 3). The deviation of malonic acid is not surprising since in its mono-protonated state it forms a cyclic hydrogen bonded structure, which would tend to favor formation of the mono-protonated form and disfavor formation of the di-protonated form, increasing the apparent interaction between protonation events. A number of other than unbranched hydrocarbons acids are observed to fall on or close to this line, including carbonate with three atoms, and fumarate, tatarate, and meso-tatarate with six atoms. Malonic acid, methly malonic acid, and dimethylmalonic acid are all closely grouped together above the line. Maleic acid, with a *cis* double bond lies well above the line, in contrast to fumarate with a *trans* bond which lies on the line, an observation which can be rationalized as due to the closer carboxyl groups in maleic acid. Dimethylsuccinate also lies off the line presumable due to conformational effects. The cyclic 1,1-diacids shown in Fig. 3 are significantly above the line except for cyclopentane-1,1-dicarboxylic acid C5D), and the $\mathrm{p}K_{\mathrm{d}}$ values for C5D may be in error.

The pairwise interaction values from glutamate are also plotted in Fig. 3, and are found to also lie close to the line. It seems reasonable to expect that the simple linear relationship observed in Fig. 3 will hold in the absence of conformational effects and hydrogen bonding interactions. The $\log(-\mathrm{p}K_{\mathrm{d},hh})$ value for water (1.12) is significantly below the linear relation projected value of 1.78 ($\log(1\ \mathrm{atom}) = 0$), so the linear relationship does not accurately describe this case. The slope of $-2.37$ in the log–log plot is equivalent to a power of 2.37 relationship between number of bonds and $\mathrm{p}K_{\mathrm{d}}$, the $\mathrm{p}K_{\mathrm{d}}$ in turn being proportional to the $\Delta G^{\circ}$for the interaction [Eq. (15)].

### 3.3. Summary

The method of hierarchical interactions is used to demonstrate that the third order interaction, physically associated with how one proton affected the interaction between two other protons [1], is insignificant in the case of the organic polyprotic acid glutamate. Although such a result was expected for the rigid inorganic polyprotic acids treated previously [8], it was not so certain for the conformationally flexible glutamate. This result can be interpreted as evidence that protonation of a given site in glutamate does not have a significant effect on the overall solution conformation of glutamate. The degree of pairwise interaction between the three protons in glutamate (Fig. 3) was correlated with the number of bonds separating the protons.

Pairwise interactions for a series of diprotic acids were calculated and examined to see if any simple relationships emerged between structure and degree of pairwise interaction. A simple linear relationship was discovered between log(N atoms) and $\log(-\mathrm{p}K_{\mathrm{d},hh})$. Interestingly the pairwise interactions in glutamate also fall on this line. The thermodynamic interactions between sites in glutamate are: (1) simple in the sense that no third order interaction between the three protons exists; and (2) simple in the sense that the pairwise interactions in glutamate are predictable

from the number of intervening atoms between protons and the simple log–log relationship discovered from simple symmetrical diacids and diamines.

To date all of the systems which have been treated quantitatively using the method of hierarchical interactions have demonstrated no evidence of third or higher order interactions, including hemoglobin [4], inorganic polyprotic acids [8], and the organic polyprotic acids described above. This observation does support the premise that higher order interactions will be less significant than lower order interactions, but it is also of interest to consider the question: do third order interactions exist in any known systems? One quantitative example is available where third order interactions do exist, in the λ cI repressor protein binding to the operator DNA sites OR1, OR2, and OR3 [16]. The measured association constants and aggregate interaction constants for this system (taken from [11]) was $K_1 = 1.3 \times 10^{10}$ $M^{-1}$, $K_2 = 1.1 \times 10^8$ $M^{-1}$, $K_3 = 1.7 \times 10^7$ $M^{-1}$, $c_{12} = 241$, $c_{13} = 1$, $c_{23} = 801$, $c_{123} = 950$. These correspond to hierarchical association constants of $k_1 = 1.3 \times 10^{10}$ $M^{-1}$, $k_2 = 1.1 \times 10^8$ $M^{-1}$, $k_3 = 1.7 \times 10^7$ $M^{-1}$, $k_{12} = 241$, $k_{13} = 1$, $k_{23} = 801$, and $k_{123} = 0.005$. This $k_{123}$ value represents a significant third order interaction in this system and demonstrates that higher order interactions can exist, at least in complex biochemical equilibria.

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