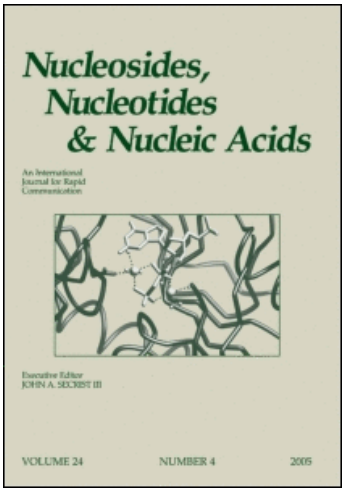


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# STRUCTURAL TRANSITIONS IN POLYCYTIDYLIC ACID: PROTON BUFFER CAPACITY DATA

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# STRUCTURAL TRANSITIONS IN POLYCYTIDYLIC ACID: PROTON BUFFER CAPACITY DATA

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## ABSTRACT

The pH-dependences of proton buffer capacity of poly(C) were computed on the basis of the literature data. In these curves there were observed four peaks: two narrow and two wide ones. The first narrow peak reflects the process of cooperative formation of double helices, which is induced by protonation of the N3 atom of nucleotide bases. The first wide peak is assigned to noncooperative process of poly(C) double helices protonation at the N3 nitrogen atom. It is proposed that the second wide peak corresponds to noncooperative protonation of the neutral cytosine bases at the oxygen atom. This reaction causes cooperative dissociation of the poly(C) double helices. The second narrow peak reflects the dissociation process.

*Key Words:* Poly(C); Proton buffer capacity method; Protonation mechanism; Conformation transitions

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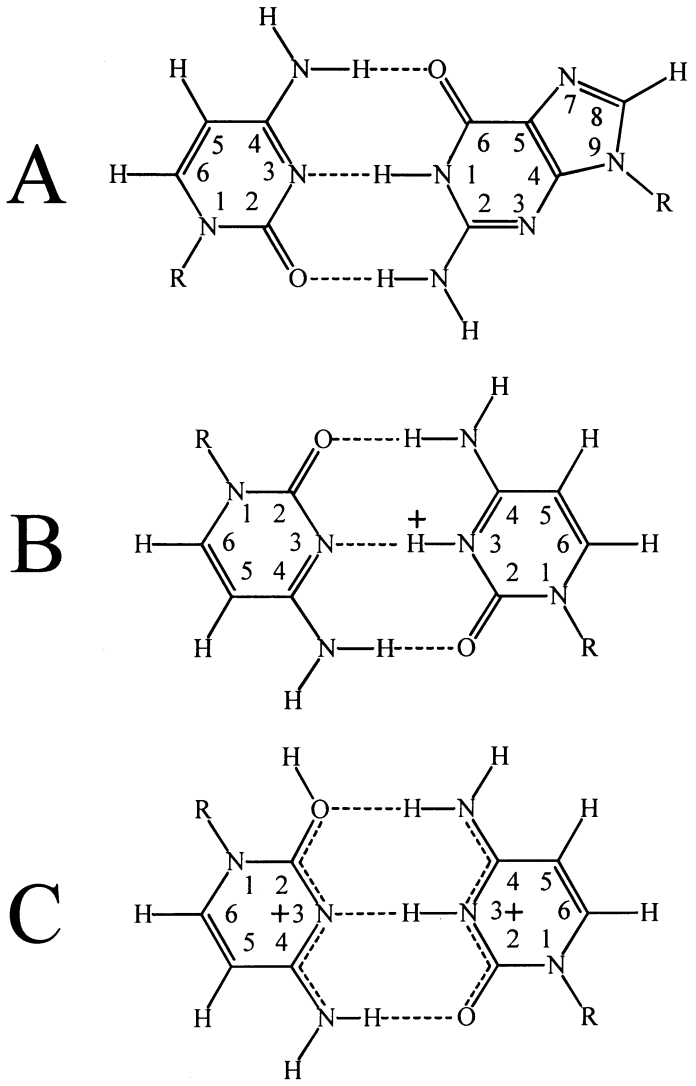
## INTRODUCTION

Frequent occurrence of homopolynucleotide sequences in native RNAs implies their possessing of specific functions. Polyadenylic acid (mainly as poly(A) tails of mRNA) is the most widespread among such sequences and its various functions are the most studied. Poly(A) tails take part in such processes as mRNA export from the nucleus[1], determination of mRNA lifetime[2,3], and initiation of translation[4]. Some mechanisms of poly(A) functioning were suggested in the work[5]. Guanylic oligonucleotides fairly often can be found in native nucleic acids as clusters of short repeats, which are able to form G-quadruplex structures, like polyguanylic acid[6,7], and are supposed to be involved in such processes as meiotic synapsis and recombination[8], immunoglobulin gene rearrangements[9] etc. Functionally significant stretches of polyuridylic acid occur frequently in cellular RNAs. For example, oligo(U)-tracts more then 12 nucleotides long, localized in the 3′ untranslated region (UTR) of some maternally inheritable mRNAs, are responsible for cytoplasmic polyadenylation of these mRNA on embryogenesis[10]. An oligo(C) segment 14 nucleotides in length was recently proved to play a role of signal of cytoplasmic polyadenylation as well[11]. Poly(C)-stretches of tens and hundreds of nucleotides are located in the 5′ UTR of encephalomyocarditis viruses, mengoviruses and apthoviruses of the picornavirus family[12], but their specific function(s) has not yet been adequately elucidated[13]. Investigations on physico-chemical properties of synthetic polycytidylic acid and, in particular, conformational transitions may cast light on its possible role in vivo.

Structural transitions of poly(C) were investigated in vitro by changing the pH of the medium[14]. But in vivo they can occur as a result of proton transfer from acidic amino acid residues of proteins to the RNA bases. Such transfer has been observed in several experiments with model compounds[15]. Moreover, the protonation of RNA cytosines has been shown by laser Raman spectroscopy in tymoviral virions[16,17] and was proposed[18] to be a result of interactions between bases and acidic amino acid residues of the coat protein.

At alkaline and neutral pH poly(C) is a single-stranded helical structure[14,19]. Protonation of poly(C) at the N3 atom of nucleotide bases results in the formation of a double-stranded helix[14]. Poly(C) transition from the single-stranded state to a double-stranded helix occurs at different pH depending on temperature and ionic strength[20]. Stability of the double-helical poly(C) depends on the degree of polymer protonation ($\alpha$)[21–24] defined as the ratio of the number of protonated bases to their total number. The melting temperature increases up to $\alpha = 0.5$ and decreases at $\alpha > 0.5$. At a high degree of poly(C) protonation the double helix dissociates[22,24–26]. This process occurs at $\alpha \approx 0.7$ at an ionic strength of 0.1 M NaCl and room temperature[22,24].

There are different opinions about the structure of the poly(C) double helix and the reason for its dissociation. The scheme of hydrogen bonding between cytosine residues in the poly(C) double helix proposed by Akinrimisi et al.[21] and Langridge & Rich[27] is presented in Scheme 1B. But this scheme, though presented in many papers devoted to poly(C) transitions, is not supported by X-ray diffraction analysis. As in the case of Watson-Crick




***Scheme 1.*** Base pairs formed with the participation of cytosine. **A**. Watson-Crick G. C base pair; **B**. C. $+C^+$ base pair (27), the degree of poly(C) protonation is equal to 0.5; **C**. $C^+$. $C^+$ base pair, the degree of poly(C) protonation exceeds 0.5. Delocalization of the additional positive charge is showed by dashed line.

Gua:Cyt pair (Scheme 1A), three H-bonds are formed between Cyt and $Cyt(N3H)^+$ (Scheme 1B), but the strands in the poly(C) double helix are parallel. The extra proton is shared by the N3 atoms of two cytosines, thus in half-protonated poly(C) all the bases are paired.

In as much as every base of poly(C) can be protonated by acidification of the medium, the question arises: what is the site of proton binding at $\alpha > 0.5$? According to Hartman and Rich[22], the additional protons can be bound only *via* opening of the base pairs of the double helix, which is accompanied by its destabilization. Considering penetration of an proton inside the poly(C) double helix to be improbable Klump[24] supposed that it may be bound to a position of the pyrimidine ring another than N3. Based on the assumed equilibrium between the protonated imino and amino tautomeric forms of cytosine, he proposed a pairing scheme similar to Scheme 1B, but with localization of the surplus positive charge at the amino group of cytosine, which reduces repulsion between protons. To the contrary Suleimanova et al.[28,29] believed that the completely protonated double helices of poly(C) are conformationally stable, but did not proposed any structure for such helices.

To elucidate the mechanism of protonation of poly(C) we applied the proton buffer capacity method, developed and adapted for biopolymers by Opanasenko et al.[30]. The method consists in differentiation of the initial potentiometric titration curve and expansion of the resulting curve into basis functions (peaks), the pKs of which correspond to an equilibrium constants of biopolymer between two states characterized by definite conformations and ionization degrees. The position of peaks in the pH scale, their intensity and half-width are determined by quantity and quality of hydrophilic groups of macromolecule and the set of its conformation states. Narrow peaks ($\gamma > 1$, see Methods) correspond to cooperative conformational transitions of the biopolymer and their half-widths in the pH scale determine cooperativity degree of each conformational transition. Earlier this method was successively used in the study of conformational transitions of DNA[31] and poly(A)[32]. In this work we show that there are two different processes of noncooperative protonation of the double-helical poly(C) and propose that it is the protonation of the cytosine O2 atom in poly(C) double helices that leads to their dissociation.

## RESULTS AND DISCUSSION

As to the authors' knowledge there are a few literature data[22,24,28,29] on the curves of poly(C) potentiometric titration. Three of them (at 0.1 M $Na^+$ or $K^+$) were used for calculation of the pH-dependences of proton buffer capacity of poly(C) at room temperature. The results obtained on the basis of the data[22,24] practically coincide. The curve computed on the basis of other

results[29] has the same shape but is essentially shifted to higher pH values. The pK value of the single helix → double helix transition calculated from the latter data does not coincide with the values of others[22,24,25,33–35], taking into account a linear relationship between pK of poly(C) transition and logarithm of the $Na^+$ concentration at constant temperature[33–35]. In Fig. 1 there is presented the proton buffer capacity plot for poly(C) based on the data of Klump[24] as a typical one.

Two narrow peaks in Fig. 1 correspond to regions with a high buffer capacity, which can be seen in curves of poly(C) potentiometric titration[22, 24, 28, 29]. The pH-dependence of proton capacity of poly(C) can be satisfactorily presented as a sum of four basic functions of the type 1 (see Methods), parameters of which are tabulated in Table 1. We assign the narrow peak 1 (Fig. 1) to cooperative formation of the double helices from single-stranded structures. It was exactly in this pH region that poly(C) conformational transition was recorded by UV spectroscopy[22]. The proton buffer capacity plot for poly(C), calculated from reported data[29], is also well



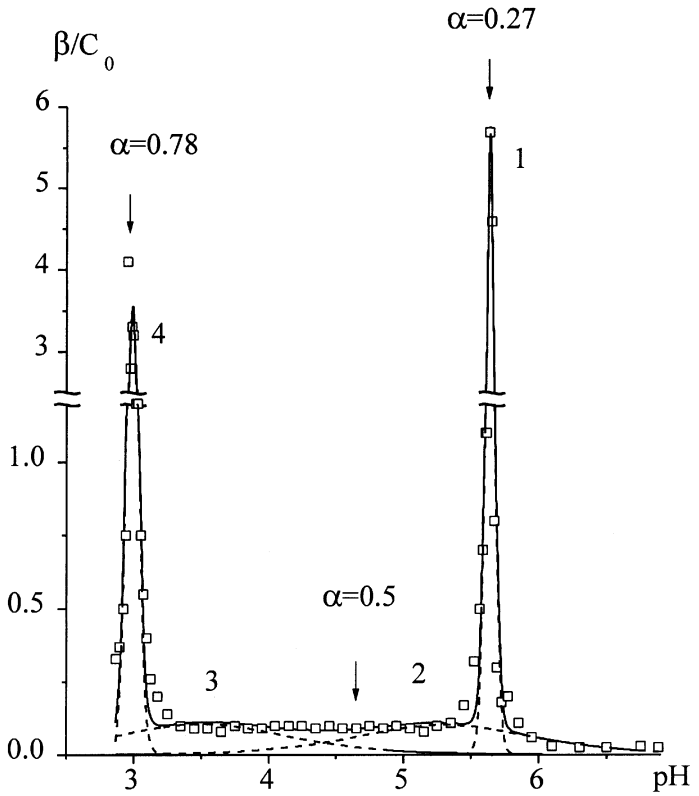



*Figure 1.* Buffer capacity of poly(C) and its expansion. Dashed lines, basis functions; solid line, summary curve.

***Table 1.*** Parameters of Peaks of the Proton Buffer Capacity Plot for Poly(C)

| Peak number | $pK_i$ | $\gamma_i$ | $C_1/C_0$* |
|---|---|---|---|
| 1 | $5.64 \pm 0.01$ | $30 \pm 5$ | $0.34 \pm 0.01$ |
| 2 | $5.3 \pm 0.1$ | 1 | $0.18 \pm 0.1$ |
| 3 | $3.5 \pm 0.1$ | 1 | $0.18 \pm 0.1$ |
| 4 | $2.99 \pm 0.01$ | $20 \pm 5$ | $0.31 \pm 0.01$ |

*$C_0$–concentration of the single-stranded poly(C) at alkaline pH (in moles of residues).

expanded into two narrow and two broad peaks, and the distance between the narrow peaks in the pH scale approximately equals that in Fig. 1.

As to extreme narrowness of the peak 1 (Fig. 1) we believe that high cooperativity of the single helix $\rightarrow$ double helix transition in poly(C) is determined by the interdependence of the Cyt:Cyt(N3H)$^+$ base pair formation (Scheme 1B): a formed base pair facilitates formation of the next one in particular by drawing together two poly(C) strands. High cooperativity of the transition process is apparently determined also by homogeinity of initial and final macromolecules and absence of intermediate conformational substates.

A third of cytosine residues is cooperatively protonated on the poly(C) transition from the single-stranded to double-helical state ($C_1/C_0$ in Table 1). It is worth noting that the deviation of the calculated proton buffer capacity curve for poly(C) from the experimental one served as a single criterion for determining error limits associated with the parameters of peaks (Table 1). *Apropos*, comparison of parameters of the peaks, calculated on the basis of the data [24] (Table 1) and on the data [22] (not shown here) demonstrates rather good agreement between them. For example, the $C_1/C_0$ values are 0.34 (based on [24]) and 0.30 (based on [22]), the peak 1 pKs are 5.64 and 5.58, respectively. The most differences are between the peak 3 parameters (e.g., the pKs 3 are 3.5 and 3.1, accordingly).

We suppose that the first broad peak (peak 2 in Fig. 1) corresponds to the process of noncooperative ($\gamma = 1$) addition of protons to N3 nitrogen atoms of nonprotonated cytosines of the double-stranded poly(C). The protonation in the pH region preceding the first poly(C) cooperative transition (pH $> 5.8$; 0.1 M NaCl) can be explained in accordance with the assumption[29] about existence of a small number of nonprotonated double-helical regions in concentrated solutions of single-stranded poly(C).

The broad peak 3 in the proton buffer capacity plot (Fig. 1) corresponds to another process of noncooperative addition of protons to double-helical poly(C), which evidently paves the way for the second conformational transition (the narraow peak 4). We share the standpoint[24] that addition of protons to the interior of the double helix of poly(C) is rather unlikely. This

follows from the fact of formation of a proton trap by oppositely oriented lone electron pairs of N3 atoms in the Cyt:Cyt(N3H)$^+$ base pair. As a result the proton affinity of N3 atom of a paired nonprotonated Cyt turns out to be significantly lower than that of free one, which is accessible to protonation. Moreover, according to quantum-chemical calculations[36], the energy of the formation of the isolated Cyt:Cyt(N3H)$^+$ pair (41.7 kcal/mole) is 1.7 times higher than in the case of the formation of the Gua:Cyt pair (23.7 kcal/mole). In compliance with quantum-chemical calculations[37, 38] N3 and O2 atoms are the most probable sites of protonation of the isolated Cyt, the oxygen atom being slightly preferable[38]. In aqueous solutions the proton affinity of cytosine N3 atom is somewhat enhanced as compared to the cytosine O2 atom[38]. Therefore we ascribe peak 3 in the proton buffer capacity plot of poly(C) (Fig. 1) to protonation of the double helix at the oxygen O2 of nonprotonated cytidine residues with cis-orientation of the formed ''hydroxyl'' bond relative to the glycosidic bond (Scheme 1C). The appearance in IR spectra of poly(C) at low pH of the $\sim 1440\,cm^{-1}$ band[22,26], which corresponds to stretching vibration of protonated carbonyl group, favours such an interpretation (frequency is determined from the spectrogram in Fig. 2 of work[26]). Such an assignment is supported by ab initio calculation of the vibrational spectrum of isolated cytosine protonated at O2 and by the frequency values of analogous vibration observed in Raman spectra of Cyt protonated at O2[38] both dissolved in water and in the crystal state, at 1418 and $1408\,cm^{-1}$, respectively.

We believe that protonation of poly(C) double helix at the O2 atom of electroneutral cytidine residues inevitably leads to dissociation of the helix. The arguments are following. Firstly, even if this reaction does not lead to dissociation of Cyt(O2Hcis$^+$):Cyt(N3H)$^+$ pair, it results in its significant destabilization. According to quantum-chemical calculations[39], the protonation of the cytosine O2 atom of Watson-Crick Gua : Cyt pair (the analogy with which is quite pertinent) results in its predissociation. Besides, protonation of O2 atoms disturbs the equilibrium conformation of the sugar phosphate backbone due to Coulomb repulsion between extra protons and neighbouring H1′ atoms of ribose [40]. Secondly, the above-mentioned protonation leads also to destabilization of stacking interactions because of significant electrostatic repulsion between the protons H-bonded with N3 and O2 atoms of adjacent cytidine residues. In the halfprotonated double helical poly(C), neutral and protonated cytosines are evidently distributed in alternating sequence along the strands that minimizes electrostatic repulsion between the protonated residues[26].

Klump's conception of the double-helical poly(C) protonation[24] is not substantiated by quantum-chemical calculations. The point is that the relative concentration of the rare iminoform of Cyt in water solution is $10^{-5}$, according to Sowers et al.[41], and the energy of the Cyt:Cyt(imino) pair formation[36] is 22 kcal/mole less than for the Cyt:Cyt(NH3)$^+$ pair. Therefore

poly(C) double helix formation via Cyt:Cyt(imino) pairs and its subsequent protonation at amino nitrogen atom N4 of the rare Cyt tautomeric form do not seem to be real events.

The narrow peak 4 in the proton buffer capacity plot (Fig. 1) corresponds to the protonation of the single-stranded regions arisen as a result of the dissociation of the double-helical poly(C). This process has a pseudocooperative character reflecting a cooperative process of double helix dissociation. Nucleotide residues of single-stranded polynucleotides are considered to be protonated approximately in the same pH region as free monomers (see refs in the brief review of work[28]). Since pK of cytidine-5′-phosphate equals 4.42 at 20°C in 0.1 M $Na^{+}$[42], the cytidine residues of single-stranded poly(C) have to be almost completely protonated in the pH interval 2.8–3.2 in which the dissociation of the double helices occurs[22,24]. Therefore nonprotonated cytosine residues of poly(C) become protonated (at nitrogen N3 atom) immediately as single-stranded chains form as a result of the dissociation of the double helices. And simultaneously in the protonated cytidine residues proton transfer from O2 to N3 atom could occur along the intramolecular H-bond $O2H^{+}\cdots N3$, which is preceded by transition of the $O2\cdots H^{+}$ hydrogen bond from *cis* to *trans* position.

Another interpretation of the results of poly(C) potentiometric titration was suggested by Suleimanova et al.[28,29]. The authors consider that at $\alpha = 0.5$ fully protonated double-stranded and nonprotonated single-stranded poly(C) coexist in equal amounts (0.1 M KCl). Under further acidification the single-stranded macromolecules become completely protonated, double helices remain stable. We consider that such a picture is not supported by the other experimental data. So, in the pH range between two transitions the CD[25] and ORD[43] spectra of poly(C) do not demonstrate the presence of any single-stranded structures. On the other hand, CD spectrum of fully protonated poly(C) significantly differs from those of double-helical form and corresponds to disordered state of polymer[25]. Besides, Suleimanova et al. scheme of protonation[28, 29] does not explain the bell-shaped dependence of $T_m$ of double-stranded poly(C) on pH.

Klump[24] divides the course of poly(C) protonation into four consecutive steps. According to his standpoint, the third step is protonation of the double helices and the fourth one is the formation of protonated coiled poly(C), which is consistent with our opinion. But we do not agree with his interpretation of the initial steps of poly(C) protonation. Klump considers that at $\alpha \leq 0.4$ the poly(C) is protonated as single-stranded form (step 1) and then the coil-to-helix transition occurs (step 2). In the accordance with the above-mentioned notion that the nucleotide residues of single-stranded polynucleotides are ionized at the approximately the same pH as free monomers[20, 28], the notable protonation of single-stranded poly(C) can take place only in the pH range (3.4–5.4)[22, 42] (at room temperature; 0.1 M $Na^{+}$)

i.e., at $\alpha > 0.4$[22]. Thus, poly(C) double helices are not formed from the protonated single strands as it is proposed in Klump's work[24].

Though single-stranded structures of poly(C) are not detected at room temperature, as noted above, in the pH region between the two cooperative transitions they naturally exist in this region at high temperatures when melting of poly(C) double helices occurs. Authors of some works[23,44] came to the conclusion of pH-dependent deprotonation on melting of poly(C) double helices. This phenomenon can be easily explained exactly from the point of view that nucleotide residues of single-stranded poly(C) are protonated almost in the same pH region as monomers. Actually, calculated values of degree of CMP protonation (taking into account the dependence pK of CMP on conditions[42]) in the pH region 4.0–5.0 at the temperatures, just above those at which the melting process of the poly(C) double helix is practically completed, is lower than the degree of protonation of poly(C) at room temperature at the same pH values. The difference is greater at higher pH. So, on melting single-stranded poly(C) is overprotonated in comparison with its equilibrium degree of protonation and additional protons dissociate.

It is necessary to note that at low pH poly(C) may exist not only as double-stranded helix. For example, possibility of its existence in the form of a four-stranded structure so-called i-motif, known for poly(dC)[45, 46], can not be excluded. C-rich RNA sequences can form i-motif structures but they are much less stable than their DNA equivalents[47–49]. It is worth noting that the base-pairing in the duplexes of i-motif structure is the same as that in double-helical poly(C). Therefore, the mechanism of destabilization of i-motif poly(dC) and poly(C) at high degree of protonation can be supposed to be analogous to the mechanism proposed in present work for the case of double-helical poly(C). The decrease of $T_m$ of intercalated deoxyribonucleotides with pH decrease is demonstrated in the work[47].



## CONCLUSION

The proton buffer capacity method has been for the first time applied to analysis of potentiometric titration curves of poly(C). On the basis of the results obtained and consideration of literature data, new mechanisms of the protonation of half-protonated poly(C) and dissociation of double-helical poly(C) were suggested as well as explanation of the reason of the pH-dependent proton release under thermal dissociation of double-stranded poly(C). As a whole, the following processes take place under the acidification of poly(C): cooperative formation of double helices from single-stranded structures induced by protonation of the N3 nitrogen atom of cytosine bases; noncooperative protonation of the double helices at the N3 nitrogen atom of cytosine bases not involved into the proton trap formation; external noncooperative protonation of the double helices at the O2 oxygen atom of

electroneutral cytidine residues which results in destabilization of the double helices; cooperative dissociation of the double helices followed by the complete protonation of the formed single-stranded structures at the N3 nitrogen atom.

## METHODS

The proton buffer capacity $\beta$ was determined as $\Delta b/\Delta pH$[50], where $\Delta b$ is the titrant concentration change upon addition of its small portion, and $\Delta pH$ is the small pH change caused by $\Delta b$. The b and pH values were taken from the experimentally obtained curve of potentiometric titration [24]. The following $\Delta pH$ intervals were chosen: 0.01 in the regions of high buffer capacity and $\leq 0.1$ in the other regions. The basis function, describing *i*-peak, was calculated according formula[31,50].

$$\beta_i = \frac{C_i \gamma_i \ln 10}{10^{\gamma_i(pH-pK_i)} + 10^{\gamma_i(pK_i-pH)} + 2} \quad , \tag{1}$$

where $C_i$ is the concentraiton of the ionized groups determining the *i*-peak on the buffer capacity curve; $pK_i$ is the thermodynamic parameter of ionization of the groups involved; and $\gamma_i$ is the cooperativity parameter, a factor by which the pH range of the proton addition or removal is narrowed as compared with the noncooperative process for the same number of the proton-accepting groups of the biopolymer.



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