Resolving Multiple Overlapping Calorimetric Transitions by Use of a Microcomputer: Studies on Erythrocyte Membranes

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Buffer changes and certain drugs cause temperature shifts, amplitude changes, and transition broadening in the differential scanning calorimetric (DSC) analysis of erythrocyte membranes. However, it has been difficult to interpret and quantitate these shifts and changes because the scans are composed of multiple overlapping transitions and because more than one transition may be simultaneously affected. An empirical approach has been developed by using Gaussian modeling to resolve these calorimetric transitions. Data analysis was carried out on a microcomputer using a nonlinear regression program (PCNONLIN)[1] to fit the data scans. These results show that changes in the calorimetric scans of erythrocyte membranes due to alterations in the buffer environment, such as pH and osmolarity, can be resolved by fitting the data scans with the proposed mathematical model and optimizing the resolution parameters with PCNONLIN. In addition, resolution uncovered hidden characteristics that may not have been readily evident. Under certain conditions, for example, apparent transition shifts were shown to actually be amplitude changes and transition broadening. Determination of the limitations and validity of this method was accomplished with simulation studies. This technique offers a simple means for fitting overlapping DSC transitions by use of a commercially available nonlinear regression program that can be run on a microcomputer.

INTRODUCTION

Differential scanning calorimetry (DSC) has been a useful tool in elucidating the structural properties of biological systems such as proteins,[2-4] nucleic acids,[5,6] and membranes.[7-10] The thermal denaturation of macromolecules such as proteins and nucleic acids involves the disruption of noncovalent interactions such as hydrogen bonds and hydrophobic and electrostatic interactions. In the case of proteins such as chymotrypsinogen[2] and ribonuclease,[3] a simple two-state approximation adequately describes the unfolding process; therefore, useful parameters can be obtained from a simple thermodynamic analysis.

Other proteins may be assembled into two or more domains resulting in several calorimetric transitions. A theoretical model has been developed[11] that describes the reversible unfolding of proteins involving one or more intermediate states. The multiple transitions of troponin C and calmodulin[4] have been deconvoluted from excess heat capacity data by a modification of such a statistical mechanical treatment. An extension of the theory has been developed[12] that applies to thermal transitions of nucleic acids.

The calorimetric transitions produced by suspensions of erythrocyte membranes are known to be due to localized thermal transitions of various membrane proteins.[7,8] Most of these transitions are modulated by membrane phospholipids. In contrast to thermal transitions undergone by various globular proteins and nucleic acids, the erythrocyte membrane transitions are irreversible. For such irreversible transitions there is no thermodynamic model from which to derive a heat capacity function. Therefore, the thermodynamic information that can be obtained from DSC data is mainly limited to the determination of total excess enthalpy of the overlapping transitions and the temperatures at which they occur.

It has been shown that certain additives, including drugs,[13,14] and changes in buffer conditions, such as pH and ionic strength,[8] cause apparent temperature shifts, enthalpy changes, and transition broadening in the calorimetric scans of the erythrocyte membrane. Useful empirical information can be derived from such experiments. DSC studies on RBC membranes have been useful in learning about membrane structural properties[8] and about membrane structure–function relationships.[15] Unfortunately, it is often difficult to quantitate effects due to changes in buffer and solute composition because the scans are composed of multiple overlapping transitions[8] and because more than one transition may be simultaneously affected.

This paper describes a method using a Gaussian model for resolving overlapping calorimetric transitions. The model is then applied to the analysis of experimental DSC curves obtained for RBC membranes under different buffering conditions. Data analysis was carried out on an IBM microcomputer (IBM-PC) using a nonlinear regression program (PCNONLIN) to fit the model.

MATERIALS AND METHODS

The chemicals used for preparing the erythrocyte membranes were obtained from Fisher Scientific Co. Recently outdated, packed human RBC were obtained from the Philadelphia Chapter of the American Red Cross. Citrate phosphate dextrose with adenine (CPDA-1) was used as the anticoagulant.

Erythrocyte membranes were prepared by using the method developed by Dodge et al.,[16] with the exception that during the lysing procedure the membranes were washed a third time with 10 ideal milliosmolar (imOsm) phosphate buffer, pH 7.4, instead of 20 imOsm.

Calorimetric scans of RBC membranes were conducted on a MicroCal DSC Model MC-1 (MicroCal Inc., Amherst, MA) using matched 1-mL cells[17] and recorded on an X-Y recorder Model 2200G (Bausch & Lomb, Houston Instrument Division, Austin, TX). A heating rate of 1 °C/min was used. Sample masses were determined to ±0.02 mg for the determination of excess specific heat capacities.

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Table I. Parameters of Untreated RBC Membrane Calorimetric Scan (Control Experiments)[a]

parameter	transitions							slope m, mcal g^{-1} $°C^{-2}$
	A_0	A	B_1	B_2	C	H	D	
T, °C	45.3 ± 1.7	51.0 ± 0.1	56.1 ± 0.1	62.3 ± 0.4	66.2 ± 0.3	70.5 ± 0.7	80.1 ± 0.0	1.29 ± 0.28
C_p, mcal g^{-1} $°C^{-1}$	12.5 ± 2.4	97.5 ± 11.7	95.1 ± 10.0	51.4 ± 3.7	128.3 ± 13.2	38.6 ± 2.0	28.4 ± 2.6	
k, $°C^{-2}$	0.022 ± 0.009	0.229 ± 0.040	0.038 ± 0.012	0.219 ± 0.023	0.165 ± 0.019	0.044 ± 0.013	0.069 ± 0.001	
area, mcal g^{-1}	139.6	339.7	810.3	182.9	526.4	306.8	179.5	
% area[b]	5.6	13.7	32.6	7.4	21.2	12.3	7.2	

[a] Mean and standard deviations of three experiments. [b] Percent area of each transition with respect to the area of the entire scan.

DSC scans were carried out under three different buffer conditions in which ionic strength and/or pH were varied: 310 imOsm, pH 7.4 (approximates physiological conditions); 155 imOsm, pH 7.4; 310 imOsm, pH 8.15. Gaussian functions were used to model the endothermic transitions. In addition, a linear base-line correction term was used. The function, C, composed of seven Gaussian transition terms together with a linear base line (correction term) was used to model all three DSC scans of the erythrocyte membranes

$$C = m(T - t_0) + C_1 \exp[-k_1(T - t_1)^2] + \ldots C_7 \exp[-k_7(T - t_7)^2] \quad (1)$$

where C is the calculated heat capacity change, m is the base-line slope (mcal g^{-1} $°C^{-2}$), i = 1, 2, ..., 7 (transition number), C_i is the transition amplitude (mcal g^{-1} $°C^{-1}$), k_i is the coefficient of transition width ($°C^{-2}$), T is the temperature (°C), t_0 is the scan starting temperature (°C), and t_i is the transition midpoint temperature (°C).

The program PCNONLIN was used to fit the experimental spectra with the Gaussian model on an IBM-PC. In general, t_0 was treated as constant, and t_i, C_i, k_i, and m were treated as parameters. Determinations of t_0 and t_i were obtained from the starting temperature and observed transition midpoint temperatures, respectively. Initial estimates for m and C_i were obtained from the base line and the amplitude measured from the base line, respectively. The estimate for k_i was obtained from

$$k_i = \ln 2/[(0.5W_{1/2})^2] \qquad \text{deg}^{-2}$$

where $W_{1/2}$ is the width of the Gaussian transition at half of the transition height.

Each of the DSC scans, as well as subsequent mathematical curve fitting with PCNONLIN, was performed in triplicate. After the initial fitting process, the number of parameters that fitted well (A, C, H, D) was held constant. This resulted in a reduced data set that depended only on those parameters which were not statistically significant in the initial fit (B_1, B_2).

Statistical significance was determined from information furnished by PCNONLIN. As an indicator of "goodness of fit", the program supplied the standard deviation and 95% confidence interval for each fitted parameter in addition to other indicators, such as the sum of squared residuals (SSR) and the correlation coefficient. The standard deviation was the primary indicator used to determine the statistical significance of an individual parameter.

Simulation studies were also performed in which curves were generated by using a set of Gaussian functions with known parameters. In one case, two of the functions were assigned transition midpoint temperatures 1 °C apart (58 and 59 °C), with different amplitudes and widths. In the other case, the transitions midpoint temperatures of two of the functions were assigned identical values, with various amplitudes and widths. Our goal was to test the ability of the model to resolve two closely overlapping transitions.

RESULTS

Calorimetric data for erythrocyte membranes obtained by using different buffer conditions were fit to the mathematical model as illustrated in Figures 1 and 2. Each figure shows the combined fit of the function C to the data, the resolved Gaussian peaks, and the linear base line. The individual transitions at 310 imOsm, pH 7.4, in Figure 1 (control) are labeled A, B_1, B_2, C, and D according to the method of Brandts et al.[8] The small peak near 45 °C, A_0, has not been previously identified, and the peak near 72 °C, H, is presumably due to hemoglobin. Evidence for this latter assignment came from calorimetric studies of the hemoglobin containing supernatant obtained from the initial exposure of RBC to the lysing buffer (20 imOsm). A single transition was observed for the hemoglobin containing supernatant at 72 °C (data not shown). The scan at 155 imOsm, pH 7.4 (Figure 2), shows that the two B transitions, B_1 and B_2, appear to merge into one tran-

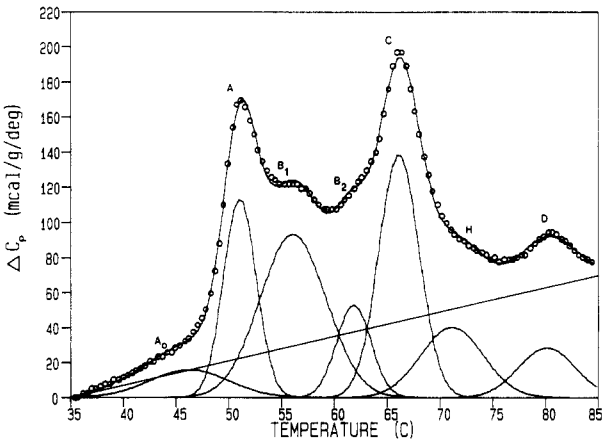

Figure 1. DSC scan of RBC membranes at 310 imOsm sodium phosphate, pH 7.4 (single experiment). The open circles represent the experimental data. The line drawn through the circles is the nonlinear regression fit of the data to the equation. The resolved transitions and linear base line are shown below the experimental and calculated data. The letters identify each transition.

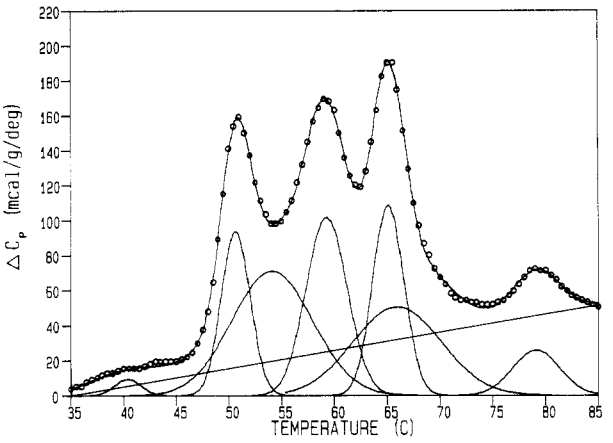

Figure 2. DSC scan of RBC membranes at 155 imOsm sodium phosphate, pH 7.4 (single experiment). The open circles represent the experimental data. The line drawn through the circles is the nonlinear regression fit of the data to the equation. The resolved transitions and linear base line are shown below the experimental and calculated data.

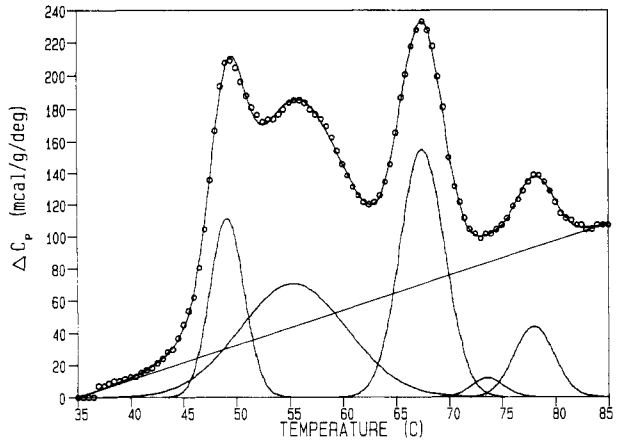

Figure 3. DSC scan of RBC membranes at 310 imOsm sodium phosphate, pH 8.15 (single experiment). The open circles represent the experimental data. The line drawn through the circles is the nonlinear regression fit of the data to the equation. The resolved transitions and linear base line are shown below the experimental and calculated data.

Table II. "Composite" Standard Deviations for Each Fitted Transition of DSC Scans of Erythrocyte Membranes

conditions		transition, mcal g^{-1} $°C^{-1}$						
pH	imOsm	A_0	A	B_1	B_2	C	H	D
7.4	310	2.81	0.58	2.43	7.15	6.52	5.87	0.66
7.4	155	0.01	1.36	3.89	6.75	3.90	8.00	0.12
8.15	310	1.03	0.20	w[a]	w[a]	2.97	1.01	0.38

[a] PCNONLIN warned that a fitting error occurred.

sition as a result of decreasing buffer concentration.

The results of a typical curve-fitting analysis are presented in Table I for RBC membranes in 310 imOsm, pH 7.4 (Figure 2). The mean values and standard deviations for these experiments are listed for the parameters corresponding to each calorimetric transition. Curve-fitting analyses of the DSC data for RBC membranes in 155 imOsm, pH 7.4 (Figure 2), and in 310 imOsm, pH 8.15 (Figure 3), were also performed. The composite standard deviations for these conditions are shown in Table II.

The most readily apparent effect of the decrease in ionic strength (Figure 2 compared to Figure 1) from 310 to 155 imOsm is the decrease in the number of distinct peaks between 50 and 70 °C from four to three. This effect appears visually to be due to a merging of the B_1 and B_2 transitions on the temperature axis. The results of the curve-fitting analyses, however, offer a different interpretation. The best fit for the experimental data in Figure 2 indicates that the B_1 and B_2 transitions are still resolved on the temperature axis (at approximately 54 and 59 °C, respectively), even though the composite experimental curve suggests otherwise.

Although we were confident the resolved individual B_1 and B_2 transitions gave rise to the data in Figure 2, there are additional interpretations of the data. Two of the most likely are (1) that distinct B_1 and B_2 transitions occur, but their transition midpoint temperatures are nearly identical, or (2) that the B_1 and B_2 transitions have merged into a single B transition. Simulation studies were undertaken to determine whether or not we could differentiate between these two possibilities with confidence.

Two types of simulation studies were undertaken to generate calorimetric curves with known B_1 and B_2 parameters. In one case, the transition midpoint temperatures were 1 °C apart; in the other case, the transition midpoint temperatures were identical. These studies showed that a single structure B transition arising from a single peak near 56 °C cannot be distinguished from two transitions with identical or nearly identical midpoint temperatures, amplitudes, and widths.

DISCUSSION

Infinitely sharp two-state thermal transitions will occur in a membrane if (1) all of the initial-state molecules have the same energy, (2) all of the final-state molecules have the same energy, and (3) the applied thermal energy is instantaneously homogeneous. Perturbations and deviations from these ideal conditions lead to transition broadening.

Empirically, the Gaussian function appears to adequately describe the transition broadening and the resultant line shape of the RBC thermal transitions we observed. These results are consistent with successful Gaussian modeling of ultraviolet spectroscopy,[18] circular dichroism,[19] gas chromatography,[20] mass spectroscopy,[21] and electrophoresis.[22] However, current theory cannot adequately explain why most irreversible, broadened transitions have a Gaussian symmetric character and why, occasionally, some transitions are asymmetric. Asymmetry has been observed, for example, in the gel–liquid-crystal transition of dipalmitoylphosphatidylcholine (DPPC) multilamellar bilayers.[23] For this situation, asymmetry from the Gaussian distribution may be due to chemical impurities. DPPC thermal transitions at 41 °C have been shown to become more symmetric when the sample is purified.[24]

The assumption was, therefore, made that any symmetries that occurred were the result of overlapping transitions. The work of Orlov et al.[25] supports the assumption. Subsequent Gaussian resolution of asymmetries in this study reinforced this point.

The use of PCNONLIN for curve-fitting analysis of Gaussian functions to DSC data enabled us to make important observations not readily apparent upon inspection of the raw data. It was found that seven Gaussian terms were required to fit the calorimetric data to the mathematical model. If fewer terms were used, the resulting goodness of fit was significantly reduced. For example, in Figure 1 there are only five clearly resolved transitions present. However, upon closer inspection, the A_0 and H transitions were found to be necessary to fit the data. Although the A_0 peak has not been previously identified, it was retained in the model even though a low-temperature transition is not readily apparent in the experimental curves. Certain scans required an additional low-temperature peak for a good fit whose existence Brandts has postulated (unpublished data). Orlov et al.[25] observed an irreversible calorimetric transition in the region of 41 °C, which they also called the A_0 transition. The transition between the C and D transitions is due to hemoglobin.

Our studies showed that apparent shifts in transitions due to changing buffer osmolarity conditions may actually be due to broadening of the individual transitions. An example of this is illustrated in Figure 2, which shows the DSC scan of erythrocyte membranes at 155 imOsm, pH 7.4. Brandts et al.[8] previously performed calorimetric studies on erythrocyte membranes at these buffer conditions. Their interpretation of the effects at these buffer conditions on the B_1 and B_2 transitions is that the B transitions appear to have merged into a single transition near 59 °C.

Our data suggest an alternative interpretation. Reduction of buffer concentration from 310 to 155 imOsm, at pH 7.4, results in minimal shifts in the transition temperatures, whereas the predominant effect is the change in the amplitude of the resolved transitions. These effects are not readily apparent in the experimental heat capacity curve; however, closer inspection of this curve reveals an asymmetry in the B peak. This asymmetry suggests the presence of an additional transition, since a single transition should be (nearly) symmetrical. This transition is the B_1 (Figure 2) transition that occurs at a temperature corresponding to a valley in the experimental

curve. If this transition is moved, restricted in size, or removed completely, the resulting fit is poor. We conclude that what appears to be a single B transition may in fact still arise from the two separate B_1 and B_2 transitions.

The curve-fitting results for RBC membranes at 310 imOsm, pH 8.15 (Figure 3), give another interpretation. Here also the B_1 and B_2 transitions appear to have merged on the temperature axis, resulting in a single B transition. Brandts et al.[8] demonstrated that the B_2 transition is very sensitive to pH, shifting to lower temperature as the pH is increased. According to their study, at 310 imOsm, pH 8.15, the B_2 transition nearly coincides on the temperature axis with the B_1 transition. This results in a single apparent transition near 57 °C, which our data also suggest in view of the simulation studies.

In conclusion, this technique offers a simple and rapid method to aid in fitting and resolving overlapping DSC transitions by use of a nonlinear regression program and Gaussian curves for the transitions. This ability offers the possibility of extracting more information from DSC scans which otherwise may have been overlooked.

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REFERENCES AND NOTES

(1) Metzler, C. M.; Weiner, D. L. *PCNONLIN User's Guide*; Statistical Consultants, Inc.: Lexington, KY, 1985; Vol. A.
(2) Jackson, W. M.; Brandts, J. E. *Biochemistry* **1970**, *9*, 2294-2301.
(3) Tsong, T. Y.; Hearn, R. P.; Wrathall, D. P.; Sturtevant, J. M. *Biochemistry* **1970**, *9*, 2666-2677.
(4) Tsalkova, T. N.; Privalov, P. L. *J. Mol. Biol.* **1985**, *181*, 533-544.
(5) Brandts, J. F.; Jackson, W. M.; Ting, T. Y. *Biochemistry* **1974**, *13*, 3595-3600.
(6) Elson, E.; Rewd, T. *Biopolymers* **1974**, *13*, 797-824.
(7) Brandts, J. F.; Erickson, L.; Lysko, K.; Schwartz, A. T.; Taverna, R. D. *Biochemistry*, **1977**, *16*, 3450-3454.
(8) Brandts, J. F.; Taverna, R. D.; Sadasivan, E.; Lysko, K. A. *Biochim. Biophys. Acta* **1978**, *512*, 566-578.
(9) Farach, M. C.; Martinez-Carrion, M. *J. Biol. Chem.* **1983**, *258*, 4166-4170.
(10) Stein, J. M. in *Molecular Association in Biological and Related Systems*; Gould, R. F., Ed.; American Chemical Society: Washington, DC, 1968; p 259.
(11) Freire, E.; Biltonen, R. L. *Biopolymers* **1978**, *17*, 463-479.
(12) Freire, E.; Biltonen, R. L. *Biopolymers* **1978**, *17*, 497-510.
(13) Snow, J. W.; Brandts, J. F.; Low, P. S. *Biochim. Biophys. Acta* **1978**, *512*, 579-591.
(14) Marwaha, L. K.; Tirrell, D. A. in *Biological Activities of Polymers*; Carraher, C. E., Gerelein, C. G., Eds.; American Chemical Society: Washington, DC, 1982; pp 163-175.
(15) Snow, J. W.; Vincentelli, J.; Brandts, J. F. *Biochim. Biophys. Acta* **1981**, *642*, 418-428.
(16) Dodge, J. T.; Mitchell, C.; Hanahan, D. J. *Arch. Biochem. Biophys.* **1963**, *100*, 119-130.
(17) Davio, S. R.; Low, P. S. *Biochemistry* **1982**, *21*, 3585-3593.
(18) Hook, T. M., Jr.; Silbellman, J. A. *Biopolymers* **1970**, *9*, 1319-1348.
(19) Fletcher, R.; Powell, M. J. D. *Comput. J.* **1963**, *6*, 163-168.
(20) Knorr, F. J.; Thorshein, H. R.; Harris, J. M. *Anal. Chem.* **1981**, *53*, 821-825.
(21) Rosenbaum, M.; Hancil, V.; Kromers, R. *J. Chromatogr.* **1980**, *191*, 157-167.
(22) Agard, D. A.; Steinberg, R. A.; Stroud, R. N. *Anal. Biochem.* **1981**, *111*, 257-268.
(23) Mastrangelo, S. V. R.; Dornte, R. W. *J. Am. Chem. Soc.* **1955**, *77*, 6200-6201.
(24) Albon, N.; Sturtevant, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 2258-2260.
(25) Orlov, S. N.; Shnyrov, V. L.; Zhadan, G. G.; Litvinov, I. S.; Gulak, P. V.; Pokudin, N. I.; Postnov, Y. V. *Bull. Exp. Biol. Med.* **1983**, *96*, 55-56.

Search for Concealed Non-Kekulêan Benzenoids and Coronoids

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Benzenoid and coronoid systems consist of congruent regular hexagons. A concealed non-Kekulêan benzenoid or coronoid is a system that does not possess any Kekulé structure ($K = 0$) and yet has $\Delta = 0$, where Δ is the color excess. The systematic search for concealed non-Kekulêan benzenoids is reviewed and supplemented by some of the smallest such systems with trigonal symmetry. An original systematic search for the smallest concealed non-Kekulêan coronoids is reported. This search resulted in 23 such systems with $h = 15$, where h is the number of hexagons. The theory of segmentation, which has been developed for benzenoids, is applied to coronoids for the first time. Finally, the smallest concealed non-Kekulêan double coronoid (two corona holes) is identified.

INTRODUCTION

The perfect matchings of benzenoid graphs correspond to the Kekulé structures of benzenoid (polycyclic aromatic) hydrocarbons. It is recognized that these notions have great chemical importance and mathematical interest.[1] Benzenoid systems[1-3] (or simply benzenoids), which have chemical counterparts in benzenoid hydrocarbons, are planar geometrical constructions consisting of congruent regular hexagons. Here "planar" is taken in the geometrical sense, and it is implied that helicenic systems are excluded. For the sake of simplicity one also speaks about Kekulé structures for benzenoid systems. Benzenoids that do not possess any Kekulé structure are called non-Kekulêan, otherwise, Kekulêan.

Sometimes in the following we shall designate a benzenoid system B. The Kekulé structure count (number of Kekulé structures) of B is denoted K.

It is well-known that benzenoid graphs are bipartite; all vertices can be colored by two colors (say, black and white) so that two adjacent vertices never have the same color. Black and white vertices correspond to starred and unstarred[4,5] (or marked and unmarked[6]) carbon atoms.

The color excess (or Δ value) is an important invariant for benzenoid systems. The quantity Δ is defined as the absolute magnitude of the difference between the numbers of black and white vertices, say $n^{(b)}$ and $n^{(w)}$, respectively. In other words

$$\Delta = |n^{(b)} - n^{(w)}| \quad (1)$$

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