Isoquinoline Alkaloids and their Binding with DNA: Calorimetry and Thermal Analysis Applications

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Abstract: Alkaloids are a group of natural products with unmatched chemical diversity and biological relevance forming potential quality pools in drug screening. The molecular aspects of their interaction with many cellular macromolecules like DNA, RNA and proteins are being currently investigated in order to evolve the structure activity relationship. Isoquinolines constitute an important group of alkaloids. They have extensive utility in cancer therapy and a large volume of data is now emerging in the literature on their mode, mechanism and specificity of binding to DNA. Thermodynamic characterization of the binding of these alkaloids to DNA may offer key insights into the molecular aspects that drive complex formation and these data can provide valuable information about the balance of driving forces. Various thermal techniques have been conveniently used for this purpose and modern calorimetric instrumentation provides direct and quick estimation of thermodynamic parameters. Thermal melting studies and calorimetric techniques like isothermal titration calorimetry and differential scanning calorimetry have further advanced the field by providing authentic, reliable and sensitive data on various aspects of temperature dependent structural analysis of the interaction. In this review we present the application of various thermal techniques, *viz*. isothermal titration calorimetry, differential scanning calorimetry and optical melting studies in the characterization of drug-DNA interactions with particular emphasis on isoquinoline alkaloid-DNA interaction.

Keywords: Thermal techniques, ITC, DSC, thermal melting, isoquinoline alkaloids, DNA binding.

INTRODUCTION

The interaction of deoxyribonucleic acids with small molecule natural products having potential drug value has been under rigorous investigation in several laboratories all over the world since the discovery of the double helical structure of DNA. The interest in understanding the basics of such DNA interactions stems from the need to develop new compounds, particularly natural product based ones, which have the ability to target the genome artificially at specified sequences or structures, for curing many genetic diseases through efficient and less toxic chemotherapy [1, 2]. Most of the research work of the last 50 years on small molecule-DNA interactions was largely confined to elucidating the structural aspects in terms of the mode, mechanism and specificity of binding. Considerable advancement has been achieved and a colossal volume of data is now available in the literature on a variety of small molecules that bind in either of the two dominant modes, *viz*. intercalation and groove binding [3-8]. An important aspect of the binding that has received less attention in the past is the elucidation, analysis and understanding of the energetics of these interactions. Knowledge of the thermodynamics of a reaction enables one to clearly understand the molecular forces that drive the binding, such as entropic effect of the uptake or release of water and ions, enthalpy contribution to the free energy and the burial of water accessible surface areas. With the development of modern calorimetric instruments in recent years that can measure precisely heat changes as small as 0.1μcal/sec, thermodynamic characterization of small molecule-DNA interaction has become possible now, and this can provide a direct and sensitive route to an accurate and complete thermodynamic characterization of these interactions. Understanding the binding thermodynamics has a potential to contribute to an improved rational drug design and optimization process [9-14].

There are mainly three thermal techniques that are essentially useful in the thermodynamic characterization of small molecule-DNA interactions, *viz.* isothermal titration calorimetry (ITC), differential scanning calorimetry (DSC), and optical thermal melting. In this article we will review the utility of these three techniques in characterizing the interaction of biologically active alkaloids with DNA with reference to isoquinoline alkaloids, *viz*. berberine, palmatine, coralyne and sanguinarine, which were studied extensively in the authors' laboratory in the last several years.

BINDING OF ALKALOIDS TO DNA

Alkaloids are small molecules occurring in nature in many plants and other organisms. They constitute one of the most remarkable groups of compounds pharmaceutically useful for human. Among many plant derived alkaloid groups, isoquinolines represent the most abundantly distributed ones that include benzylisoquinolines, protopines, benzo[c]phenanthridines, and protoberberines. Protoberberi-

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nes represent about 25% of the currently known natural alkaloids from all sources. The known DNA binding isoquinoline alkaloids are berberine, palmatine and the synthetic compound coralyne belonging to the protoberberine group, and sanguinarine of the benzo[c]phenanthridine group (Fig. **1**). Structurally, berberine and palmatine are buckled due to partial saturation in the ring B, while coralyne and sanguinarine are planar molecules. Biochemical and pharmacological importance of these alkaloids have been described in detail in several review articles [15-21]. Structural aspects of the DNA binding of these alkaloids like the mode, mechanism and sequence specificity have also been extensively reviewed very recently [6,7,20,22]. Structural data alone, however, provide a partial description of the binding of small molecules to DNA and must be complimented by thermodynamic data that can provide information about the balance of driving forces for a complete understanding of the nature of the complexation. Biophysicists and medicinal chemists engaged in DNA-targeted drug design require accurate and rapid methods to directly obtain reliable thermodynamic parameters. Modern calorimetry techniques can fulfill all these criteria. More recently our laboratory has elucidated the detailed thermodynamics of the interaction of these alkaloids with various natural and synthetic deoxyribonucleic acids [23-27].

ISOTHERMAL TITRATION CALORIMETRY

Isothermal titration calorimetry has recently emerged as a sensitive, precise and highly informative technique that measures the heat of reaction of the solutions of two molecules, a ligand and a macromolecule, when one is titrated against the other [12, 13, 28-33]. This technique is based on the computer aided repetitive addition of one solution *via* automated injection into the calorimeter cell containing the other solution maintained at constant temperature. The heat released (exothermic reaction) or absorbed (endothermic reaction) is directly proportional to the binding that occurs between the two molecules and this enables the thermodynamic parameters to be elucidated precisely. ITC has an unique advantage of allowing accurate determination of the binding constants (K), reaction stoichiometry (n) and thermodynamic profiles of the interaction, *viz*. the calorimetric enthalpy (ΔH), entropy contribution ($T\Delta S$), heat capacity (ΔC_p), and change in free energy (ΔG). Furthermore, compared to other analytical techniques, ITC has the advantage of suitability for characterizing both low affinity and high affinity interactions. The detailed thermodynamic definitions and relationships, theories behind ITC and DSC, instrumentation, experimental protocols *etc*. have been reviewed in details in several recent articles [12, 13, 32-37] and will not be discussed here.

Berberine-DNA Complexation

The energetics of interaction of berberine with various natural DNAs and polynucleotides through ITC studies was first elucidated in the authors' laboratory [24]. The ITC thermograms of the binding of berberine to natural DNAs of varying base composition, *viz*. *Clostridium perfringens* (CP) DNA (27% GC), calf thymus (CT) DNA (42% GC), *Escherichia coli* (EC) DNA (50% GC), and *Micrococcus lysodeikticu*s (ML) DNA (72% GC), and to sequence specific synthetic polynucleotides, *viz*. poly(dA).poly(dT), poly(dA-dT).poly(dA-dT), poly(dG-dC).poly(dG-dC), and poly(dG).poly(dC) were analyzed in details. The representative ITC profiles of berberine binding to CT DNA and poly(dA-dT).poly(dA-dT) are illustrated in Fig. (**2**). Essentially similar amount of heat was found to be liberated in each of the natural DNA systems on complexing with berberine. The binding data obtained from ITC studies revealed the binding affinity to be highest (9.00×10^4 M^{-1}) for complexation of berberine to CP DNA compared to other natural DNAs. The analysis yielded an enthalpy change of -1.80 kcal/mol and an entropy term ($T\Delta S$) of 4.43 kcal/mol. For CT DNA, a K value of 1.20×10^4 M^{-1}, a ΔH value of -2.01 kcal/mol and a $T\Delta S$ value of 3.34 kcal/mol were obtained. The complexation with EC DNA showed a binding affinity of 1.10×10^4 M^{-1}, ΔH = -3.70 kcal/mol and $T\Delta S$ = 1.78 kcal/mol. In case of ML DNA the binding affinity was very weak (9.00×10^3 M^{-1}), while an enthalpy change of - 4.52 kcal/mol and a small entropy term of 0.81 kcal/mol were

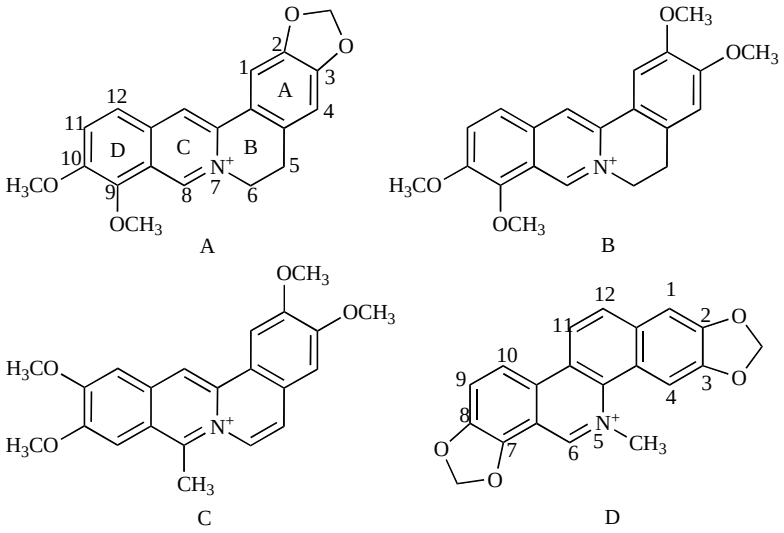

Fig. (1). Chemical structures of some pharmaceutically important isoquinoline alkaloids. **A**. berberine, **B**. palmatine, **C**. coralyne and **D**. sanguinarine.

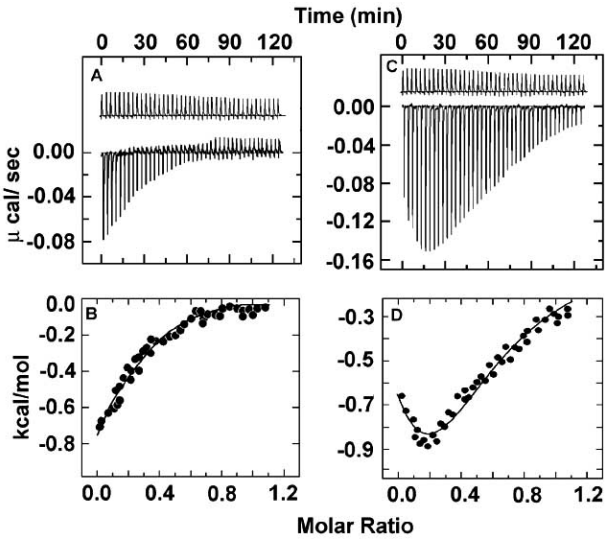

Fig. (2). Representative ITC profiles for the titration of berberine into a solution of (**A**) calf thymus DNA and (**C**) poly(dA-dT).poly(dA-dT). Lower panels (**B**) and (**D**) represent the corresponding normalized heat signals versus molar ratio. (Reprinted in part from Bhadra *et al*., **2008** [24] with permission from Elsevier).

observed. The free energy change (ΔG) in each system was essentially similar, in the range of -5.3 to -6.3 kcal/mol. The values of all the thermodynamic parameters are presented in Table **1**. The thermodynamic results revealed that the binding of berberine to AT rich CP DNA was more favourable compared to that with the GC rich ML DNA in terms of high binding affinity and large entropy change.

Complexation of berberine with the synthetic polynucleotide poly(dA-dT).poly(dA-dT) revealed two distinct exothermic binding events in the thermogram. The first binding equilibrium was assigned to intercalation and the second one to weaker, non-specific interactions. The first binding resulted in an enthalpy value of -1.10 kcal/mol and a K_1 of 1.11×10^5 M^{-1} while the second binding had a large enthalpy value of -32.50 kcal/mol and a relatively weaker K_2 of 8.0×10^3 M^{-1}. The first binding was found to be overwhelmingly entropy driven while the second one was enthalpy driven. The binding data to poly(dA).poly(dT), on the other hand, showed only a single strong endothermic binding event having a binding affinity of 8.0×10^4 M^{-1} and an enthalpy change of 1.56 kcal/mol. The structural differences between the alternating and non-alternating AT sequences have been suggested to account for the observed differences in thermodynamic parameters in the two AT polynucleotides [38]. The ITC profile of the binding of berberine with poly(dG-dC).poly(dG-dC) was shown to yield a relatively lower binding constant (8.09×10^3 M^{-1}), a ΔH of -9.52 kcal/mol, a $T\Delta S$ value of -4.61 kcal/mol and a free energy change of -4.91 kcal/mol. Binding of berberine with poly(dG).poly(dC) was fit to sequential binding site model and was not analyzed in details.

The bindings of berberine with the AT rich DNAs and AT polynucleotides were entropy driven with highest preference of binding to poly(dA-dT).poly(dA-dT) and CP DNA. Thus, the thermodynamic profiles revealed the specificity of berberine towards AT rich DNAs and AT polynucleotides as concluded earlier from spectroscopic observations [39, 40] and gave additional information on the differences in the hydration behavior between the two AT polynucleotides.

Heat Capacity Results for Berberine-DNA Complexation

The authors' laboratory first studied the temperature dependence of the binding enthalpy of berberine complexation to many natural DNAs as well as synthetic polynucleotides and determined the heat capacity changes from the slope of the variation of ΔH with temperature [24]. A representative case of CT DNA - berberine binding showing the variation of ΔG, ΔH and $T\Delta S$ with temperature is presented in Fig. (**3**). The ΔC_p values for berberine binding to CP DNA, CT DNA, poly(dA).poly(dT), and poly(dA-dT).poly(dA-dT) were found to be -120, –110, –98 and –140 cal/mol K, respectively. Since the plots of variation of ΔH and $T\Delta S$ with temperature were parallel to each other in all the above cases, the reaction enthalpy and entropy were suggested to compensate each other to make the reaction free energy ΔG almost independent of temperature. The free energy contribution to the hydrophobic transfer step of berberine to the intercalation site was also calculated from the equations suggested by Record Jr. and coworkers [41]. The calculated values of ΔG_{hyd} for berberine binding to CP DNA, CT DNA, poly(dA).poly(dT), and poly(dA-dT).poly(dA-dT) were found to be -9.60, -8.80, -7.84 and -11.20 kcal/mol, respectively. These values were slightly lower in magnitude than the values observed for classical intercalators and this was suggested to be due to the partial intercalative nature of berberine [24].

Palmatine-DNA Complexation

There were no previous reports of the energetics of palmatine-DNA interaction till we studied the interaction with different DNAs using isothermal titration calorimetry [23, 25]. The ITC profiles for the binding of palmatine to four natural DNAs, *viz.* CP, CT, EC and ML DNA, and to the two polynucleotides poly(dG-dC).poly(dG-dC) and poly(dA).poly(dT) had single binding events. On the other hand, the ITC data on poly(dA-dT).poly(dA-dT) revealed two binding events and hence a two site model was used for the analysis. The ITC data of poly(dG).poly(dC) was complex and could not be fitted to any model. Thus, the profile of palmatine was similar to that of berberine in these cases. The values of the thermodynamic parameters reported from these studies are collated in Table **1**.

Representative ITC profiles for the interaction of palmatine with CT DNA and poly(dA-dT).poly(dA-dT) are presented in Fig. (**4**). The ITC data of CP DNA complexation yielded a K of 1.0×10^5 M^{-1}, an enthalpy change of -2.70 kcal/mol and an entropy term of 4.04 kcal/mol, indicating that the binding was favored by positive entropy changes. For CT DNA-palmatine complexation, the ITC data yielded a K of 2.00×10^4 M^{-1}, a ΔH of -4.40 kcal/mol and a $T\Delta S$ value of 1.42 kcal/mol, indicating both negative enthalpy and positive entropy changes to be responsible for the binding. The ITC data with EC DNA, on the other hand, yielded a K of 1.14×10^4 M^{-1}, ΔH of -4.75 kcal/mol and a $T\Delta S$ of 0.69 kcal/mol indicating the binding to be favored by

Table 1. Thermodynamic Parameters for the Binding of Berberine, Palmatine, Coralyne, and Sanguinarine to Various DNAs at 20 °C

System	K (x10^5 M^{-1})	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	ΔG (kcal/mol)	ΔC_p (cal/mol K)
Berberine [24, 54]					
CP DNA	0.90 ±0.05	-1.80	4.43	-6.23	-120
CT DNA	0.12±0.02	-2.01	3.34	-5.50	-110
EC DNA	0.11±0.03	-3.70	1.78	-5.47	nd
ML DNA	0.09±0.01	-4.52	0.80	-5.33	nd
Poly dA-dT).poly(dA-dT)[b]	1.11±0.15	-1.10	5.71	-6.81	-140
Poly(dA).poly(dT)	0.80±0.04	+1.56	7.71	-6.16	-98
Poly(dG-dC).poly(dG-dC)	0.08±0.02	-9.52	-4.61	-4.91	nd
Palmatine [25, 54]					
CP DNA	1.00±0.15	-2.70	4.04	-6.74	nd
CT DNA	0.20±0.05	-4.40	1.42	-5.82	-120
EC DNA	0.11±0.02	-4.75	0.69	-5.47	nd
ML DNA	0.09±0.01	-4.92	0.39	-5.33	nd
Poly dA-dT).poly(dA-dT)[b]	1.63±0.06	-1.02	6.02	-7.03	-150
Poly(dA).poly(dT)	2.30±0.15	1.70	9.02	-7.23	-175
Poly(dG-dC).poly(dG-dC)	0.15±0.02	-18.00	-12.48	-5.64	nd
Coralyne [26, 54]					
CP DNA	28.6±0.08	-5.20	3.46	-8.71	nd
CT DNA	71.5±1.75	-7.05	2.30	-9.25	-147
EC DNA	92.0±0.090	-7.55	1.77	-9.40	nd
ML DNA	400.01±5.2	-8.99	1.28	-10.26	-190
Poly dA-dT).poly(dA-dT)	7.13±0.03	-3.09	4.60	-7.89	nd
Poly(dA).poly(dT)	6.35±0.04	-3.10	4.83	-7.83	nd
Poly(dG-dC).poly(dG-dC)	670.0±7.9	-9.08	1.29	-10.56	nd
Poly(dG).poly(dC)	33.40±0.06	-5.07	3.69	-7.89	nd
Sanguinarine[c] [50, 51, 57]					
CT DNA	9.5±0.03	-6.91	1.11	-8.02	-140
Poly dA-dT).poly(dA-dT)	30.1±0.05	-7.16	1.53	-8.69	nd
Poly(dA).poly(dT)	13.1±0.04	5.76	13.94	-8.18	nd
Poly(dG-dC).poly(dG-dC)	60.3±1.54	-9.04	0.06	-9.62	-250
Poly(dG).poly(dC)	4.6±0.02	-4.37	3.19	-7.56	nd

nd: no data, [b]:only the first binding site values are presented. [c]:for sanguinarine no calorimetry data is available with CP, EC and ML DNAs.

negative enthalpy changes. In case of ML DNA, the binding affinity was 9.00×10^3 M^{-1} while an enthalpy change of -4.92 kcal/mol and a small entropy change of 0.39 kcal/mol were observed. The binding data on poly(dA).poly(dT) showed a single strong endothermic binding event as with berberine but the binding affinity with palmatine was quite higher. The binding was therefore enthalpically unfavourable. Since intercalative binding was evidenced from viscometric and fluorescence quenching results [23], the process was clearly entropically driven with the entropy contribution arising from disrupted solvent.

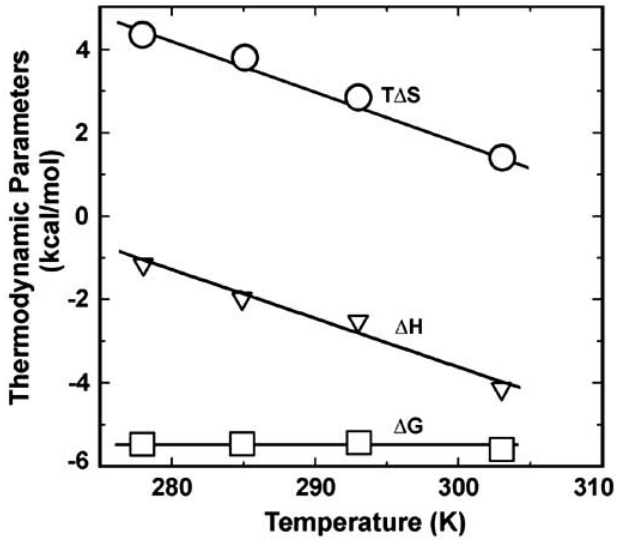

Fig. (3). Variation of the thermodynamic parameters for the binding of berberine to calf thymus DNA as a function of temperature, determined from ITC analysis. (Reprinted in part from Bhadra *et al.*, **2008** [24] with permission from Elsevier).

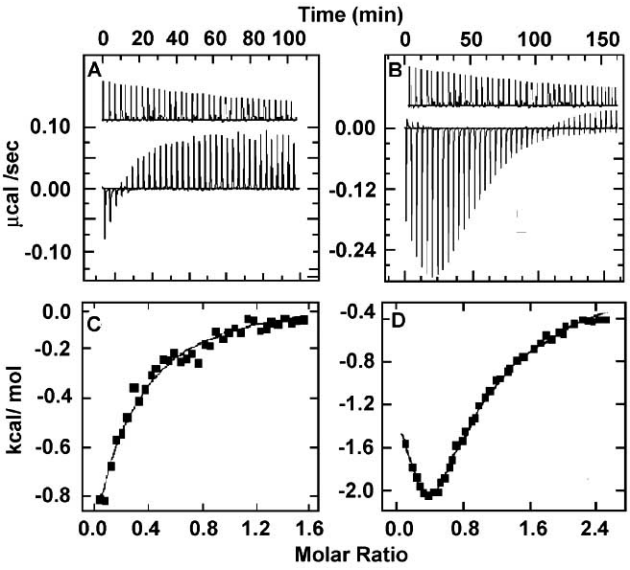

Fig. (4). Representative ITC profiles for the titration of palmatine into a solution of (A) calf thymus DNA and (B) poly(dA).poly(dT). (C) and (D) represent the corresponding normalized heat signals versus molar ratios. (Reprinted in part from Bhadra **2007** [54] and Bhadra *et al.*, **2008** [23, 25] with permission from Elsevier).

The bindings of several small molecules, particularly intercalators, to poly(dA).poly(dT) were similarly entropy driven and attributed to the release of bound water molecule from the polynucleotide [42-45]. Calorimetric data yielded an enthalpy change of 1.70 kcal/mol and binding affinity of 2.30×10^5 M^{-1}. On the other hand, titration of palmatine to poly(dA-dT).poly(dA-dT) was shown to reveal two distinct exothermic binding events. The first binding event was significantly steeper than the second one indicating that the affinity for the first binding was much higher than that of the second. The first event resulted in a binding enthalpy of -1.02 kcal/mol and a K_1 of $1.63\text{x}10^5$ M^{-1}, while the second one had a binding enthalpy change of -36.77 kcal/mol and a relatively weaker K_2 of 9.46×10^3 M^{-1}. The ITC profile of poly(dG-dC).poly(dG-dC) was shown to yield a K value of 1.52×10^4 M^{-1}, a ΔH of -18.0 kcal/mol, a $T\Delta S$ value of -12.48 kcal/mol and free energy change of -5.64 kcal/mol. Thus, the reported ITC revealed that palmatine like berberine showed the same trend, i.e. preference for AT base pairs and free energy change between 5-7 kcal/mol. The bindings were reported to be entropically favourable with the AT rich DNAs, but unlike berberine, palmatine showed higher binding affinity with poly(dA). poly(dT).

Heat Capacity Results for Palmatine-DNA Complexes

The temperature dependence of the enthalpy change provides the heat capacity values. Our laboratory studied the temperature dependent behavior of the thermodynamic parameters in the two AT polynucleotides, poly(dA).poly(dT) and poly(dA-dT).poly(dA-dT) [25]. Studies were performed in the range of 5 to 25°C. The association constant for the binding of palmatine to poly(dA).poly(dT) decreased on increasing the temperature and varied from 3.33×10^5 M^{-1} at 5°C to 1.72×10^5 M^{-1} at 25°C . Essentially, the binding affinity decreased only by about half over the temperature range of 5-25°C. The interaction was overwhelmingly entropy driven at all temperatures; both the binding enthalpy and the entropy term (a favorable term in the ΔG) decreased despite increase in temperature. As in the case of berberine-DNA complexation, the free energy exhibited only small changes. The reaction enthalpy and entropy, both of which were strong functions of temperature, compensated to make the reaction free energy almost independent of temperature. On the other hand, the association constants showed a higher trend of decrease in poly(dA-dT).poly(dA-dT) and the K value decreased from $2.58\text{x}10^5$ M^{-1} at 5°C to $0.92\text{x}10^5$ M^{-1} at 25°C. The $T\Delta S$ values were again positive throughout, favoring the reaction. The thermodynamic parameters showed strong dependence of temperature and compensated each other. In both the cases, a negative ΔC_p value for complex formation was obtained. In case of palmatine binding to poly(dA).poly(dT) and poly(dA-dT). poly(dA-dT), the temperature dependence of enthalpy (ΔC_p) was estimated to be -175 and -150 cal/mol K, respectively (Table **1**). In both cases, this was comparatively higher than that evaluated for the interaction of berberine, revealing more hydrophobic contribution from the binding of palmatine with the two AT polymers. Calculation of ΔG_{hyd} revealed a value of -14.0 kcal/mol for palmatine binding to poly(dA).poly(dT) while it was -12.0 kcal/mol for binding to poly(dA-dT).poly(dA-dT). These were higher than the values for the interaction with berberine (*vide supra*) but close to the values observed for intercalators [41].

Coralyne-DNA Complexation

The energetics of coralyne-DNA complexation was first studied using ITC by Bhadra *et al.* [26]. Due to high aggregation tendency and large heat of dilution of coralyne, reverse ITC experiments were carried out by keeping coralyne in the calorimeter cell and titrating DNA from the syringe [46-48]. Fig. **5** shows the ITC profiles of coralyne binding to ML DNA and poly(dG-dC).poly(dG-dC), while the values of the thermodynamic parameters are presented in Table **1**.

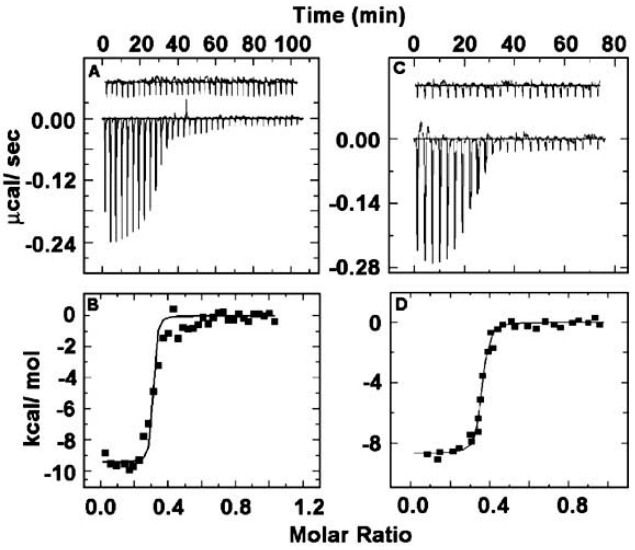

Fig. (5). Representative ITC profiles for the titration of (A) *Micrococcus lysodeilticus* DNA and (C) poly(dG-dC).poly(dG-dC) into a solution of coralyne. (B) and (D) represent the corresponding normalized heat signals versus molar ratios. (Reprinted in part from Bhadra *et al*., **2008** [26] with permission from Elsevier).

There is significant information on the energetics of the interaction from the data. The binding of coralyne to all DNAs studied was exothermic; coralyne released less heat with CP DNA compared to ML DNA. The two GC polynucleotides released more heat compared to the AT polynucleotides. The lower magnitude of heat released with AT rich DNAs and AT polynucleotides was ascribed to weak interactions. Calorimetric data were fit to a single set of identical sites in all cases except for poly(dA-dT). poly(dA-dT). The free energy change in all the eight systems reported varied in the range of -7.82 kcal /mol to -10.56 kcal/mol. ΔG was least with poly(dA).poly(dT) and maximum with poly(dG-dC). poly(dG-dC). The ITC data yielded a K of 2.86×10^6 M^{-1}, an enthalpy change of -5.20 kcal/mol and a $T\Delta S$ value of 3.46 kcal/mol for CP DNA and a K of 7.15×10^6 M^{-1}, a ΔH of -7.05 kcal/mol and a $T\Delta S$ of 2.30 kcal/mol for CT DNA indicating that the binding in both the cases was favored by a negative enthalpy change. In the case of EC DNA, the binding affinity was 9.20×10^6 M^{-1} with an enthalpy change of -7.55 kcal/mol and a small entropy change of 1.77 kcal/mol. The binding data of coralyne to ML DNA revealed a K of 4.00×10^7 M^{-1}, an enthalpy change of -8.99 kcal/mol, and $T\Delta S$ value of 1.28 kcal/mol. Thus, it is very clear that in all the cases the binding of coralyne, unlike that of berberine and palmatine, was favored by a negative enthalpy change, being more negative with increasing GC content in the natural DNAs. The binding affinity was maximum with the GC rich ML DNA. Coralyne showed higher affinity to the GC polynucleotides compared to the AT polynucleotides among the four polynucleotides studied. Between the two GC polymer sequences, the affinity was higher for poly(dG-dC).poly(dG-dC). Calorimetric data of the binding of coralyne to poly(dA).poly(dT) was fit to a model of single set of identical sites yielding a K of 6.35×10^5 M^{-1}, an enthalpy change of -3.10 kcal/mol, and an entropy contribution of 4.83 kcal/mol. On the other hand, binding of coralyne with poly(dA-dT).poly(dA-dT) was found to show two distinct exothermic binding events, a stronger initial one followed by a weaker second one. It is pertinent to note that the behaviors of berberine and palmatine binding to this polymer were similar (*vide supra*). The first binding resulted in an enthalpy of -3.09 kcal/mol and K_1 of 7.13×10^5 M^{-1}, while the enthalpy for the second binding was -46.77 kcal/mol along with a relatively weaker K_2 of 3.07×10^4 M^{-1} (Table **1**). The binding data of coralyne to poly(dG).poly(dC) yielded a K of 3.36×10^6 M^{-1}, an enthalpy change of -5.07 kcal/mol, and $T\Delta S$ value of 3.69 kcal/mol, whereas with poly(dG-dC).poly(dG-dC), it showed a steep sigmoidal curve with a high binding constant of 6.70×10^7 M^{-1}, an enthalpy change of -9.08 kcal/mol and a $T\Delta S$ value of 1.29 kcal/mol. With both the GC polynucleotides the binding was overwhelmingly enthalpy driven with small entropy contributions that favored the binding.

Heat Capacity Results for Coralyne-DNA Complexes

ITC studies of coralyne binding with CT and ML DNA in the range 20 to 40°C was performed by Kumar and coworkers [26]. The data evaluated from the titration profiles for CT DNA are presented in Fig. (**6**). The association constants of the binding at 20°C and 40°C varied from 7.15×10^6 M^{-1} to 2.80×10^6 M^{-1} for CT DNA and from 4.0×10^7 M^{-1} to 14×10^6 M^{-1} for ML DNA. The decrease in binding affinity was comparable in the two cases. The binding enthalpy increased and the entropy term which was a favorable term in the ΔG, decreased with increasing temperature, exhibiting only small free energy changes in both cases. ΔC_p values of -147 and -190 cal/mol K, obtained for the complexation of coralyne with CT and ML DNA, respectively were much higher than that obtained for the interaction of berberine and palmatine with these DNAs. Hence the binding coralyne appears to be dominated by more hydrophobic forces. Again,

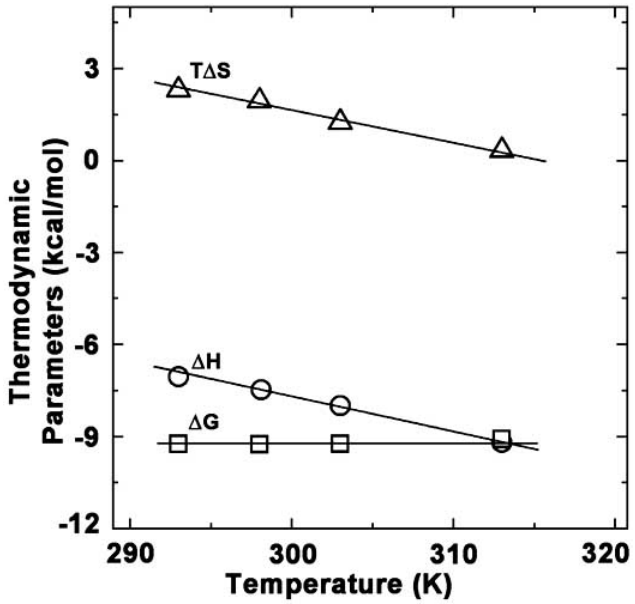

Fig. (6). Variation of the thermodynamic parameters for the binding of coralyne to calf thymus DNA as a function of temperature, determined from ITC analysis. (Reprinted in part from Bhadra *et al.*, **2008** [26] with permission from Elsevier).

the values of ΔG_{hyd} for coralyne binding to CT and ML DNAs were found to be -11.76 and -15.02 kcal/mol, respectively, which were also higher than the values for the interaction of berberine and palmatine. These values are in the range usually observed for classical DNA intercalators [41].

The effect of ionic strength on the binding of coralyne – CT DNA complexation was also studied using ITC at four different [Na^+] concentrations, *viz*. 10, 20, 50 and 100 mM [26]. The binding affinity decreased as the salt concentration increased and a ten-fold increment in [Na^+] concentration resulted in one order of magnitude reduction in the binding constant. The salt dependent binding constants were used to examine the number of counter ions released which was found to be 0.95 [Na^+] ions per bound coralyne with CT DNA at 20°C, very close to the value for a monocationic intercalator as per the polyelectrolytic theories. Compared to berberine and palmatine, the number of released ions was much higher with coralyne. Furthermore the observed free energy change of coralyne-CT DNA complexation, when partitioned into the non-polyelectrolytic (ΔG_t) and polyelelectrolytic (ΔG_{pe}) contributions, revealed that at least 70-75 % of the free energy contribution to the binding arises from the non- polyelectrolytic forces.

Sanguinarine-DNA Complexation

Maiti and coworkers first used vant' Hoff plot to evaluate the thermodynamic parameters of sanguinarine binding to natural and synthetic DNA duplexes at pH 5.2 [49]. Very recently the energetics of sanguinarine binding with natural and synthetic DNAs under various salt and pH conditions was evaluated by isothermal titration calorimetric techniques [50, 51] and later it was reviewed by Maiti and Kumar [52]. The affinity of binding of sanguinarine with CT DNA was found to be in the order of 10^5 M^{-1} (Fig. **7**) and to be dependent on the ionic strength of the medium and temperature. The heat capacity change for CT DNA-sanguinarine complexation obtained from the temperature dependence of enthalpy change gave a value of –140 cal/mol K (Fig. **8**). The salt dependence of the DNA binding data revealed the release of 0.55 units of cations per bound alkaloid to CT DNA [50]. The reason for the low amount of release of ions is not clear. Sanguinarine showed high specificity to alternating purine-pyrimidine sequences with an affinity of the order 10^6 M^{-1} and the affinity to the polynucleotides studied varied in the order poly (dG-dC).poly(dG-dC)>poly(dA-dT).poly(dA-dT)>poly(dA). poly(dT)>poly(dG).poly(dC). ITC analysis further showed that the binding of sanguinarine to both hetero polynucleotides, poly(dG-dC).poly(dG-dC) and poly(dA-dT).poly(dA-dT), was exothermic and enthalpy driven. But to the homo GC polymer poly(dG).poly(dC), the binding was exothermic and favored by both negative enthalpy and positive entropy changes, while that to the homo AT polymer poly(dA).poly (dT) was endothermic and entropy driven. Thus, a complete thermodynamic characterization and specificity of the binding was provided from isothermal titration calorimetry studies.

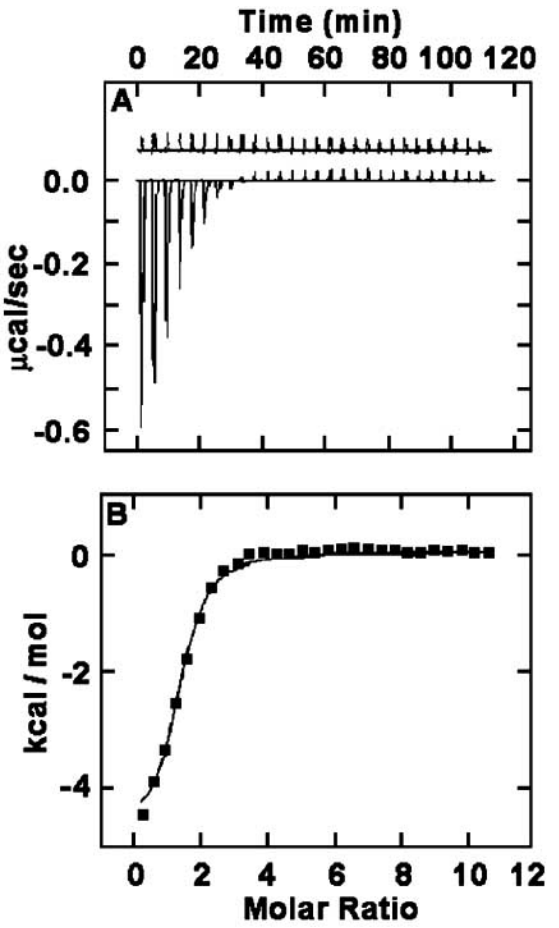

Fig. (7). Representative ITC profiles for the titration of calf thymus DNA into a solution of sanguinarine. The lower panel represents the corresponding normalized heat signals versus molar ratios. (Reprinted in part from Hossain and Kumar **2009** [51] with permission from Elsevier).

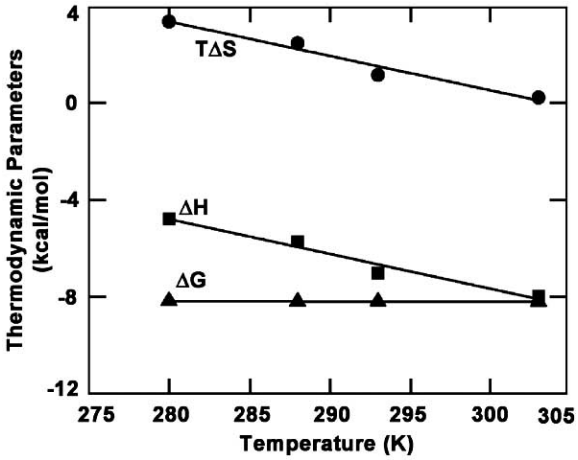

Fig. (8). Variation of the thermodynamic parameters for the binding of sanguinarine to Calf thymus DNA as a function of temperature, determined from ITC analysis. (Reprinted in part from Hossain and Kumar **2009** [51] with permission from Elsevier).

DIFFERENTIAL SCANNING CALORIMETRIC ANALYSIS

DSC is a powerful and informative tool in understanding the molecular aspects of thermal transitions particularly the folded and unfolded conditions of a protein, double and single stranded states of DNA and energetics of small molecule–DNA interactions. DSC has the capability to measure

affinity constants for ultra tight binding reactions. Furthermore, DSC melting curves can give a wealth of information like the stability of the biomolecule aggregation states, cooperativity of the melting transition, and melting temperatures. DSC measures the excess heat capacity of the molecule under investigation as a function of temperature. The transition for DNA appears as a sharp endothermic peak centered around the melting temperature, T_m, and the maximum heat capacity occurs at the T_m value. The integration of the curve of the excess heat capacity versus temperature provides the transition enthalpy (ΔH) and the shift in base line provides the excess heat capacity, C_p. For a truly reversible cooperative transition the ratio of the calorimetric and vant' Hoff enthalpy must be unity (ΔH_{cal} /ΔH_v=1). DSC is the only technique that allows the direct determination of enthalpy and heat capacity changes.

DSC Studies of Berberine-DNA Complexation

The DSC thermogram of CT DNA has a strong transition with maximum at 65±0.1°C (Fig. **9**) and three small satellite peaks thereafter that are attributed to the melting of short repetitive stretches of GC sequences [53]. The DSC thermogram of CT DNA was observed to be only partially reversible probably due to heterogenous base pair composition, restricting the application of equilibrium thermodynamics to evaluate the ratio of vant' Hoff to calorimetric enthalpy. The binding of berberine enhanced the helix stability as revealed by the transitions being concomitantly pushed to higher values [54]. A stabilization of 6.6°C was reported at saturating D/P (drug/nucleotide phosphate molar ratio) of 1.0. The temperature dependencies of the excess heat capacities (C_p, kcal/mol/°C) of CT DNA at saturating D/P values are presented in Fig. (**9A**). The calorimetric enthalpy of helix denaturation of the DNA in the absence of berberine was 8.27 kcal/mol, increasing to 15.4 kcal/mol for the complex at a D/P of 1.0. The satellite peaks of the native DNA remained unaffected in the thermogram of the complex also, revealing that the bound alkaloid had no influence on the repetitive stretches of GC sequences. vant' Hoff enthalpy values of 50.0 and 17.2 kcal/mol were observed for free CT DNA and for its complex with berberine, respectively. DSC of berberine-polynucleotide complexes was also studied by Bhadra [54]. The DSC thermograms of the poly(dA-dT).poly(dA-dT) (T_m = 40.05±0.5 °C) and of its complex with berberine are presented in Fig. (**9B**). There was a helix stabilization of 10.6°C in this polynucleotide. The enthalpy of helix denaturation here was 5.99 kcal/mol, increasing to 7.48 kcal/mol at saturating D/P. vant' Hoff enthalpy values of 5.93 and 11.26 kcal/mol were observed for this AT polynucleotide and for its complex with berberine, respectively. The DSC thermogram of the free poly(dA-dT).poly(dA-dT) was found to be completely cooperative and reversible ($\Delta H_{cal}/\Delta H_v$ =1).

DSC Studies of Palmatine-DNA Complexation

The binding of palmatine was also reported to enhance the helix stability of DNA [54]. A stabilization of about 11°C was reported at the saturating D/P of 1.0 with CT DNA. The temperature dependence of the excess heat capacities of CT DNA in presence of saturated concentrations of the alkaloid is presented in Fig. (**9C**). The enthalpy of helix denaturation of the DNA in the absence of the alkaloid

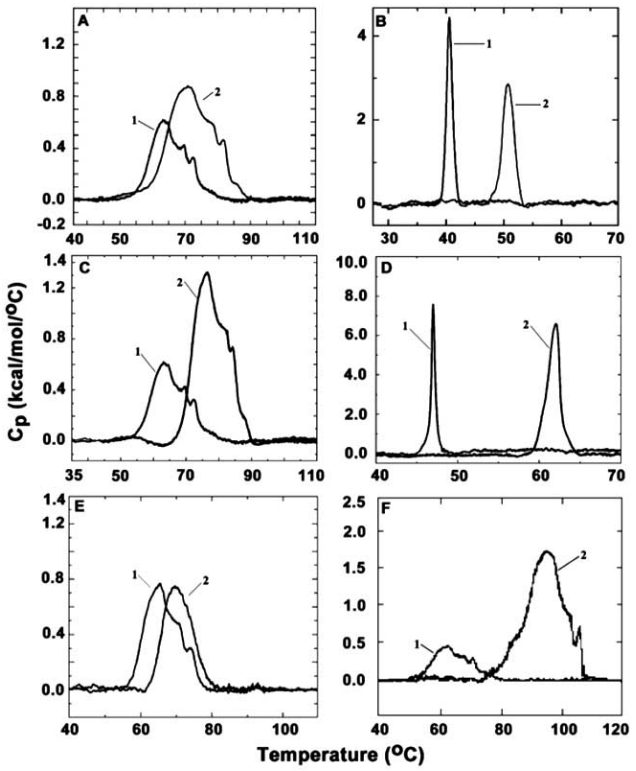

Fig. (9). Representative DSC thermograms of (**A**) calf thymus DNA (curve 1) and its complexation with berberine (curve 2), (**B**) poly(dA-dT).poly(dA-dT)(curve1) and its complexation with berberine (curve 2), (**C**) calf thymus DNA (curve 1) and its complexation with palmatine (curve 2), (**D**) poly(dA).poly(dT) (curve1) and its complexation with palmatine (curve 2), (**E**) calf thymus DNA (curve 1) and its complexation with coralyne (curve 2) and (**F**) calf thymus DNA (curve 1) and its complexation with sanguinarine (curve 2).

was 8.27 kcal/mol, which increased to 16.9 kcal/mol for the complex at a D/P of 1.0. Compared to berberine, palmatine was shown to stabilize the DNA to a greater extent showing larger affinity. DSC thermograms of poly(dA).poly(dT) (T_m = 47±0.5 °C) and its complex with palmatine are presented in Fig. (**9D**). There was a stabilization of 15 °C, in good agreement with the thermal melting study. The enthalpy of helix denaturation of the free polymer was 4.63×10^4 kcal/mol that increased to 6.52×10^4 kcal/mol at the saturating D/P of 1.0. As with poly(dA-dT).poly(dA-dT), the DSC thermogram of free poly(dA).poly(dT) was also found to be completely reversible ($\Delta H_{cal}/\Delta H_v$ =1).

DSC Studies of Coralyne-CT DNA Complexation

Bhadra studied the helix-coil transition of coralyne-CT DNA complexation up to a D/P of 0.2 (Fig. **9E**), where a helix stabilization of 5°C was observed [54]. Due to self-aggregation of coralyne, coralyne-DNA complexation beyond this D/P value was not performed. Hence the binding constants were not reported from the DSC data.

DSC Studies of Sanguinarine-DNA Complexation

Similar to berberine and palmatine, the binding of sanguinarine to CT DNA was found to enhance the DNA helix stability (Fig. **9F**). Stabilization of about 28°C was reported

at a saturating D/P of 1.0 revealing strong binding with the DNA.

OPTICAL THERMAL MELTING STUDIES

DNA has a double helical structure, the two strands of which are being held together by Watson-Crick H-bonding of the base pairs. The base pairs also stack on each other on the double helical structure, the absorbance of the bases buried inside the helical structure being 30-40 % lesser compared to that in the free state. On thermal activation, the DNA structure undergoes dramatic changes. Thermal denaturation manifested by breakage of the H-bonds and concomitant unstacking of the base pairs, results in the enhancement of the absorbance. The absorbance can be easily monitored at the wavelength maximum, *ie* around 260 nm. The midpoint of the melting curve is T_m, the melting temperature, which is characteristic of each DNA. The melting temperature of DNA depends on several factors like the base composition, counter ion concentration *etc*. DNA samples with higher GC content melt at higher temperature compared to those with lower GC content. T_m is also dependent on the nature and concentration of counter ions of the DNA. Nearly fifty years ago Marmur and Doty studied the correlation of T_m of DNA with GC content and salt concentration [55]. By determining the T_m of a DNA at one particular salt concentration, it is also possible to determine the GC content of the DNA. Melting temperature of DNAs increased on interaction with organic molecules and the data can also be used to ascertain the strength of the interaction. Higher stabilization temperature can be correlated to a stronger binding. Thermal melting experiments are generally done by increasing the temperature of degassed samples of DNA and drug-DNA complexes at varying D/P ratios, monitoring the absorbance at 260 nm, the UV absorbance maximum of DNA.

Berberine-DNA Complexation

Berberine enhanced the thermal melting temperature of all the DNAs studied. The difference in the melting temperature of free and complexed DNA (ΔT_m) was maximum (Table **2**) for poly(dA-dT).poly(dA-dT) among the synthetic polymers and for CP DNA among natural DNAs. The least T_m enhancement was observed in GC polymers, *viz*. poly(dG-dC).poly(dG-dC) and poly(dG).poly(dC). The ΔT_m variation was in the order poly(dA).poly(dT) > CP DNA > CT DNA > EC DNA > ML DNA > poly(dG).poly(dC) [54]. Representative melting profiles of poly(dA).poly(dT) and poly(dA). poly(dT) at saturation D/P of 1.0 have been presented in Fig. **10 A** and **B**, respectively.

Palmatine-DNA Complexation

Palmatine was found to enhance the thermal melting temperature of all the DNAs studied. The variation of ΔT_m at saturation D/P values with various DNAs are presented in Table **2**. Increase in ΔT_m was maximum for poly(dA).poly (dT) (Fig. **10 A**,**B**), followed in the order poly(dA-dT). poly(dA-dT)>CP DNA>CT DNA>EC DNA>ML DNA> poly(dG).poly(dC) [54]. Thus, the preference of palmatine was to homo sequences of AT compared to the preference of berberine for alternating sequences of AT. Cooperativity of the thermal melting pattern in all the cases was unaffected in the presence of the alkaloid.

Coralyne-DNA Complexation

Thermal melting studies of the coralyne-DNA complexes were performed in 8% methanol to prevent aggregation of coralyne [46, 47]. The ΔT_m values at the saturation D/P values for coralyne complexes with several DNAs and synthetic polynucleotides are presented in Table **2**. The stabilization varied in the order ML DNA > EC DNA > CT DNA > poly(dG).poly(dC) > CP DNA > poly(dA-dT).poly(dA-dT)> poly(dA).poly(dT), showing the preference of coralyne to GC base pairs [26].

McGhee derived equations correlating optical thermal melting data and binding constants of small molecules to DNA in combination with calorimetric techniques [56]. Thus,

$$1/T^o_m - 1/T_m = (R/n\Delta H_{wc})\ln(1+K_{Tm}L)^{1/n} \quad (1)$$

where T^o_m is the optical melting temperature of the DNAs in absence of the drug, T_m is the melting temperature in presence of saturating amounts of the drug, ΔH_{wc} is the enthalpy of DNA melting calculated from DSC, R is the universal gas constant, K_{Tm} is the drug binding constant at the T_m, L is the free drug activity that may be estimated by one half of the total drug concentration, and n is the site size of the drug binding. The calculated apparent binding constant at the melting temperature may be extrapolated to a reference temperature, say 20°C, using the standard relationships

$$\ln(K/K_{Tm}) = -(\Delta H_b/R)(1/T - 1/T_m) \quad (2)$$

where K_{Tm} is the drug binding constant at the reference temperature T (in Kelvin) and ΔH_b the binding enthalpy directly determined from the isothermal titration calorimetry experiment. Using these equations the binding constant data for alkaloids to all the DNAs and polynucleotides have been calculated. These values depicted in Table **2** are in reasonable agreement with those evaluated directly from ITC data, validating the use of this approach.

CONCLUSIONS

This review provides detailed insights into the use of thermal techniques for the characterization of small molecule-DNA interaction taking the example of isoquinoline alkaloids. Thermal techniques like calorimetry and melting profile evaluation have been found to be versatile tools that can provide reliable and significant information on the nature, specificity and energetic aspects of the interactions in solution. Additionally, they provide thermodynamic parameters that may be dissected to have a clear idea of the nature of the forces involved in the complexation. The overall variations of the thermodynamic parameters of the four alkaloids with many DNAs have been presented for comparison (Fig. **11**). Comparison of the data on four isoquinoline alkaloids revealed that the planar molecule coralyne showed maximum binding affinity and stabilization with the DNAs, followed by sanguinarine, palmatine and berberine in that order. Interactions with berberine and palmatine were entropically driven with AT rich DNAs. Sanguinarine and coralyne, on the other hand, showed enthalpy driven

Table 2. Thermal Melting, DSC and Binding Constant Data of Berberine, Palmatine, Coralyne and Sanguinarine Binding to Various DNAs

System	GC (mol %)	T^o_m °C	T_m °C	ΔT_m °C	ΔH_{wc} (kcal/mol)	K (x10^5 M^{-1})
Berberine [24, 54]						
CP DNA	30	51.2	59.4	8.2	6.78	0.86±0.15
CT DNA	42	65.0	70.0	5.0	8.27	0.13±0.06
EC DNA	50	67.5	70.5	3.0	8.18	0.13±0.01
ML DNA	72	79.0	81.0	2.0	9.20	0.09±0.01
Poly dA-dT).poly(dA-dT)	0	39.7	49.7	10.0	5.99	1.70±0.40
Poly(dA).poly(dT)	0	47.0	54.6	7.6	4.63	1.15±0.16
Poly(dG-dC).poly(dG-dC)	100	94.0	nd	nd	46.40	nd
Poly(dG).poly(dC)	100	84.0	87.0	3.0	10.40	0.02±0.01
Palmatine [25, 54]						
CP DNA	30	51.2	64.2	13	6.78	1.07±0.04
CT DNA	42	65.0	76.0	11	8.27	0.32±0.01
EC DNA	50	67.5	77.5	10	8.18	0.12±0.04
ML DNA	72	79.0	85.0	6	9.20	0.09±0.01
Poly (dA-dT).poly(dA-dT)	0	39.7	51.7	12	5.99	2.09±0.05
Poly(dA).poly(dT)	0	47.0	62.0	15	4.63	1.50±0.20
Poly(dG-dC).poly(dG-dC)	100	94	nd	nd	46.40	nd
Poly(dG).poly(dC)	100	84.0	88.0	4	10.40	0.02±0.01
Coralyne [26,54]						
CP DNA	30	51.2	60.2	9.0	6.78	11.3±0.30
CT DNA	42	65.0	77.0	12.0	8.27	35.0±0.40
EC DNA	50	67.5	81.3	13.8	8.18	63.7±0.75
ML DNA	72	79.0	96.0	17.0	9.20	173.0±0.40
Poly dA-dT).poly(dA-dT)	0	39.7	47.7	8.0	5.99	2.5±0.02
Poly(dA).poly(dT)	0	47.0	53.0	6.0	4.63	1.8±0.35
Poly(dG-dC).poly(dG-dC)	100	94.0	>110	nd	46.40	nd
Poly(dG).poly(dC)	100	84.0	94.5	10.5	10.40	16.4±0.26
Sanguinarine[a] [51,57]						
CT DNA	42	65.0	86.3	21.3	8.27	17.6±0.02
Poly (dA-dT).poly(dA-dT)	0	39.7	80.43	40.73	5.99	78.6±0.05
Poly(dA).poly(dT)	0	47.0	79.76	32.76	4.63	6.4±0.04
Poly(dG-dC).poly(dG-dC)	100	94.0	>110	nd	46.40	nd
Poly(dG).poly(dC)	100	84.0	nd	nd	10.40	nd

[a]: No data available for CP, EC and ML DNAs.

reactions exhibiting GC specificity in their DNA binding. Thus, the specificity aspects and binding affinity in addition to the thermodynamic profiles also were clearly exemplified in the calorimetric data of the DNA binding of these alka-

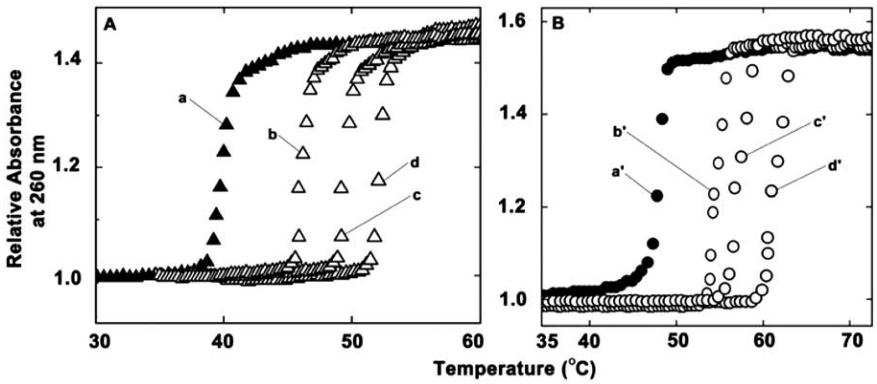

Fig. (10). Representative thermal melting profiles of (**A**) poly(dA-dT).poly(dA-dT) (curve a) and its complexation with berberine (curve b), palmatine (curve c) and coralyne (curve d), (**B**) poly(dA).poly(dT) (curve a') and its complexation with berberine (curve b'), palmatine (curve c') and coralyne (curve d').

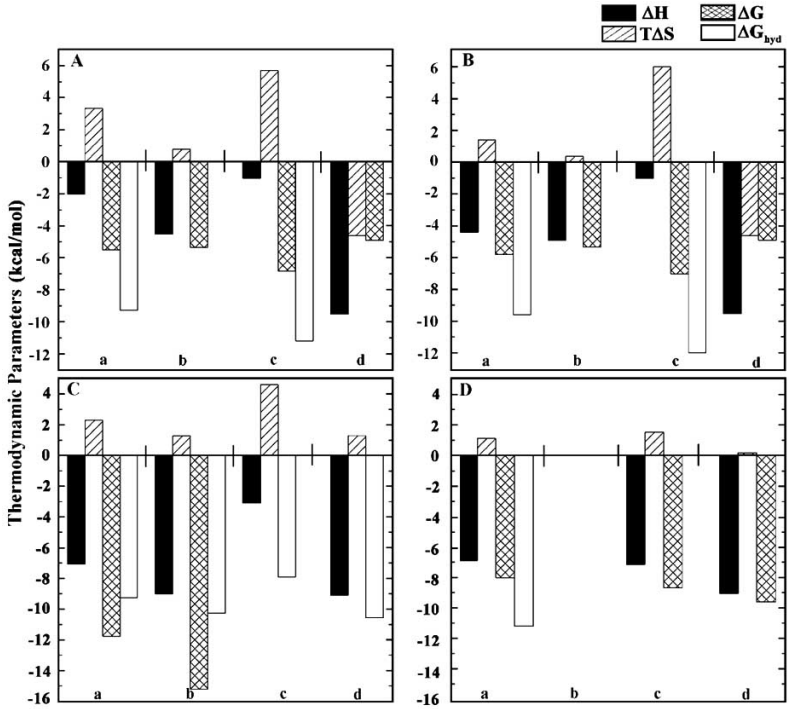

Fig. (11). Overall thermodynamic profiles for the binding of (A) berberine (B) palmatine (C) coralyne and (D) sanguinarine to (a) calf thymus DNA, (b) *Micrococcus lysodeikticus* DNA (c) poly(dA-dT).poly(dA-dT) and (d) poly(dG-dC).poly(dG-dC) determined from ITC analysis. (Refer Table **1**). In case of B (b) and D(c) and (d) ΔG_{hyd} data are not available in the literature.

loids. Furthermore, data on the change in heat capacity suggested the involvement of significant hydrophobic contribution to the binding process, probably to a greater extent with coralyne and sanguinarine compared to others. Overall, it is clear that the thermodynamic data is highly informative, and taken in conjunction with the structural information, may be helpful for the development of pharmaceutically useful small molecule based drugs for therapeutic application.

ACKNOWLEDGEMENTS

Dr. Kakali Bhadra was supported by the Research Associateship of the Council of Scientific and Industrial Research (CSIR), Government of India. The authors thank Dr. Basudev Achari, CSIR Emeritus Scientist of IICB for the critical reading of the manuscript and Prof. Siddhartha Roy, Director, for his patronage. The contribution of the erstwhile and current colleagues of Biophysical Chemistry Laboratory towards the understanding of isoquinoline alkaloid-nucleic acid interactions is also acknowledged. We are thankful to the two anonymous reviewers for their comments and suggestions that enabled us to improve upon the manuscript.

ABBREVIATIONS

DNA	=	Deoxyribonucleic acid
RNA	=	Ribonucleic acid
AT	=	Adenine-thymine
GC	=	Guanine-cytosine
CP	=	*Clostridium perfringens*
CT	=	Calf thymus

EC	=	*Escherichia coli*
ML	=	*Micrococcus lysodeikticus*
CD	=	Circular dichroism
T_m	=	Thermal melting
ITC	=	Isothermal titration calorimetry
DSC	=	Differential scanning calorimetry
D/P	=	Alkaloid or drug/DNA molar ratio
C_p	=	Molar heat capacity at constant pressure

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Received: May 25, 2010 Revised: July 29, 2010 Accepted: August 04, 2010