The Salt-Induced Destacking of Purine in Aqueous NaCl Systems and Its Implications on Life at Elevated Temperatures†

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ABSTRACT: Aqueous purine solutions in the absence and presence of NaCl have been studied by vapor-pressure osmometry and high-resolution proton-magnetic-resonance spectroscopy. The presence of NaCl causes a marked change in the temperature dependence of the molal osmotic coefficient and the chemical shift of the purine resonances when compared with the corresponding quantities in purine solutions containing no added salt. In the absence of salt, the destacking of the purine is gradual as the temperature is increased, whereas there is a sharp decrease in purine stacking at ~42 °C in the presence of NaCl. This salt-induced destacking of purine is consistent with the previously noted salt-induced bulk water destructuring at temperatures above ~42 °C. This effect has been deduced from considerations of species profiles in acid–water systems as a function of temperature (Leifer, L., and Inoue, K., manuscript in preparation). The implications of this phenomenon on biological systems at elevated temperatures will be discussed.

Previous studies of the main ionic species in $HCl-H_2O$ solutions, utilizing the spectroscopically determined Hammett acidity function and the thermodynamically determined activities of HCl and H_2O, have led to the determination of the main ionic species present in solution (Inoue, 1974; Leifer and Inoue, manuscript in preparation). The details of this procedure are given in the Appendix. The analysis of the species profile of these systems indicates the presence of two main species, $Cl(H_2O)_3^-$ and Cl^- (unhydrated). The behavior of the equilibrium constant for the reaction $Cl^- + 3H_2O \rightleftharpoons Cl(H_2O)_3^-$ as a function of temperature indicates a marked change in ΔH and ΔS for this anionic reaction above ~42 °C. These results are summarized in Table I. Two unexpected changes in the thermodynamic parameters are apparent: (1) there is an increase in order for the hydration reaction as the temperature is raised and (2) there is a marked discontinuity in the slope of a plot of $\Delta S°/T$ vs. $1/T$ (this slope is related to the change in heat capacity for the above hydration reaction) at ~40 °C. These results can be explained by postulating that the bulk solvent water is structured at temperatures below ~42 °C, and somewhat destructured at higher temperatures. Thus, for the hydration reaction, the change in entropy at low temperatures between water in the structured solvent and in the hydrated form is small (ca. -1 cal mol^{-1} deg^{-1}) and at high temperatures between water in less structured solvent and the hydrated form is large and negative (cf. Table I).

Previous studies of the hydrophobic interactions of purine in water as a function of temperature utilizing vapor-pressure osmometry and high-resolution proton-magnetic-resonance spectroscopy have shown that the extent of base stacking of the purine decreases with increasing temperature. This behavior of the purine is presumably due to the decrease in water structure with increasing temperature (Ts'o et al., 1963; Ts'o and Chan, 1964; Chan et al., 1964). These studies have shown that purine is an excellent model system for the study of hydrophobic interactions which are a major stabilizing force in biological systems. If indeed the degree of association of purine is sensitive to solvent structure changes, the salt-induced destructuring of the bulk water should show a marked effect on the degree of association of purine in aqueous NaCl solutions.

Experimental Section

Materials. The purine (A grade) was obtained from Sigma Chemical Co. and was sublimed in vacuo before use. The sodium chloride was reagent grade from Matheson Coleman and Bell and was used without further purification. All samples were run in H_2O unless otherwise indicated.

Methods. The vapor-pressure osmometry was performed on a Model 302B Hewlett-Packard vapor-pressure osmometer which was calibrated with known concentrations of NaCl. Osmotic coefficients were calculated from $\Delta R = \nu m K \phi$ where ΔR is the resistance, ν is the number of particles, m is the molality of the solution, K is the instrument constant, and ϕ is the molal osmotic coefficient.

The high-resolution spectra were obtained on a Varian HA-100 spectrometer. The temperature was controlled to ±1 °C by means of a Varian V-4343 variable-temperature unit and was determined by using an ethylene glycol sample and the calibration curve supplied by Varian. The spectrometer was locked on H_2O and chemical shifts were measured to ±0.2 Hz with reference to an external $CHCl_3$ capillary.

Results

The vapor-pressure osmometry results for a 0.20 m purine solution and a 0.20 m purine plus 0.10 m NaCl solution are summarized in Figure 1, where the molal osmotic coefficients for the solutions are plotted vs. temperature. In calculating ϕ, the value used for ν, the number of particles of purine, was one. Since purine does associate in solution to form aggregates, the value of ϕ will be less than 1 and, with increasing temperature, the value of ϕ for purine should approach 1 as the population of the oligomers decreases. For the 0.20 m purine solution, the value of ν does gradually increase with temperature as would be expected (Ts'o et al., 1963; Ts'o and Chan, 1964). For the 0.20 m purine and 0.10 m NaCl solution, the temperature dependence of ϕ is markedly different. It must be remembered that the ϕ for this solution, which is plotted in Figure 1, represents the deviations from ideality of both the purine and

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TABLE I: Thermodynamic Values for the Hydration Reaction, $Cl^- + 3H_2O \rightleftharpoons Cl(H_2O)_3^-$ between 25 and 80 °C.

T (K)	$1/T$ (K) $\times 10^3$	K	$-\Delta G^\circ$ (cal/mol)	$-\Delta H^\circ$ (cal/mol)	$-\Delta S^\circ$ (cal/deg)	$-\Delta C_p$ (cal/deg)
297.8	3.358	4.162	844	840	−0.01	~70
307.8	3.249	3.914	835	1 500	2.2	
317.7	3.148	3.483	788	3 300	7.9	~260
322.7	3.006	2.383	574	7 100	20	
352.7	2.835	1.012	8.48	12 500	35	

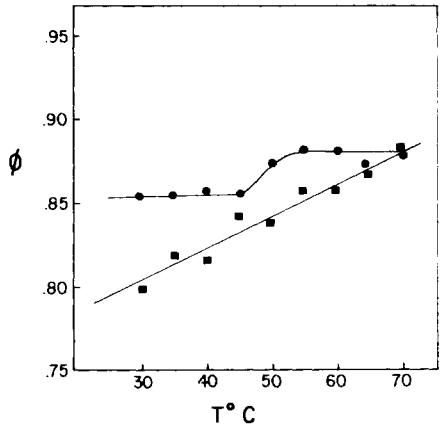

FIGURE 1: The temperature dependence of the molar osmotic coefficients for a 0.20 m purine solution (■) and a 0.20 m purine and 0.10 m NaCl solution (●).

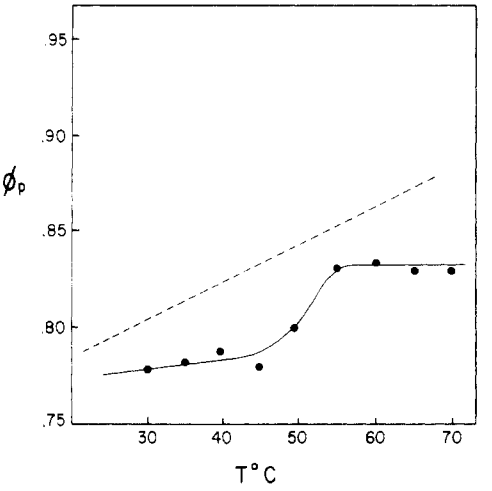

FIGURE 2: The temperature dependence of the calculated molal osmotic coefficient (ϕ_p) of 0.20 m purine in the presence of 0.10 m NaCl. The broken line is that of purine in the absence of salt.

NaCl. It will be shown later that the purine and NaCl act independently in solution and, therefore, their deviations from ideality may be separated as:

$$\nu_{tot} m_{tot} \phi_{tot} = \nu_p m_p \phi_p + \nu_s m_s \phi_s \quad (1)$$

where the subscripts tot, p, and s denote the various parameters due to the total concentration of purine plus salt, the concentration of the purine and of the salt, respectively. Using the appropriate values in eq 1 for a 0.2 m purine and 0.1 m NaCl solution, one obtains:

$$0.4\phi_{tot} = 0.2\phi_p + 0.2\phi_s \quad (2)$$

Rearrangement of eq 2 results in:

$$\phi_p = 2\phi_{tot} - \phi_s \quad (3)$$

Utilizing ϕ_{tot} for the purine plus NaCl solution and the values of ϕ_s for a 0.10 m NaCl solution (Lang, 1967; Gardner et al., 1963), the values for the molal osmotic coefficients of purine in the presence of salt were obtained. These results are summarized in Figure 2. For reference purposes, the behavior of ϕ for purine in the absence of salt is also included. As can be seen in Figure 2, the degree of association of purine is fairly constant over two temperature ranges below ~45 and above ~55 °C with a sharp transition in between.

To confirm the assumption that the purine and NaCl act independently in solution, the dependence of the chemical shifts of the purine resonances as a function of temperature was monitored in the presence and absence of NaCl in H_2O and D_2O solutions. Normal and heavy water solutions were chosen for comparison because the isotopic substitution of deuterium for a proton in water increases the strength of hydrogen bonding, as evidenced by an increase in the melting point from 0 °C for H_2O to 3.8 °C for D_2O. These two solvents are sufficiently different in structure to enable one to distinguish effects of solute–solute interactions and those due to solvent changes. The best samples to compare to VPO[1] results with the NMR results would be ones at the same concentration. This is not possible for the following reason. Utilizing our VPO data, the VPO data of Ts'o et al. (1963), the computed distribution of the population of associated species of purine in aqueous solution (Ts'o and Chan, 1964), and the chemical-shift data for purine systems (Chan et al., 1964), it can be calculated that, for a 0.2 m purine solution in the presence of 0.1 m NaCl, the change in chemical shift of the purine resonances from those in the absence of salt would be ~2 Hz at the maximum change in the molal osmotic coefficient (40 to 50 °C). Although a shift of ~1–2 Hz is observed for the 0.2 m purine and 0.1 m NaCl solution when compared with a 0.2 m purine solution with no NaCl added, a chemical-shift change of this small magnitude cannot be relied upon since the chemical shifts are not corrected for bulk susceptibility difference between the sample and reference capillary. An internal reference would be of equally little use for detection of such a small change in chemical shift since it has recently been shown that the chemical shifts of the commonly used reference compounds (i.e., $(CH_3)_4NCl$) also vary considerably with temperature (Ludemann, 1972). These restrictions dictated that sample conditions be chosen other than those of the VPO data. The chemical-shift difference of the purine resonance in the absence and presence of NaCl became pronounced at salt concentrations of ~3 m. In the absence of salt in H_2O, the downfield shift of the purine resonance is almost linear with temperature indicating a gradual decrease in the degree of association (Figure 3). For an H_2O solution which is 3.0 m NaCl and 0.20 m pu-

[1] Abbreviations used: VPO, vapor pressure osmometry; NMR, nuclear magnetic resonance.

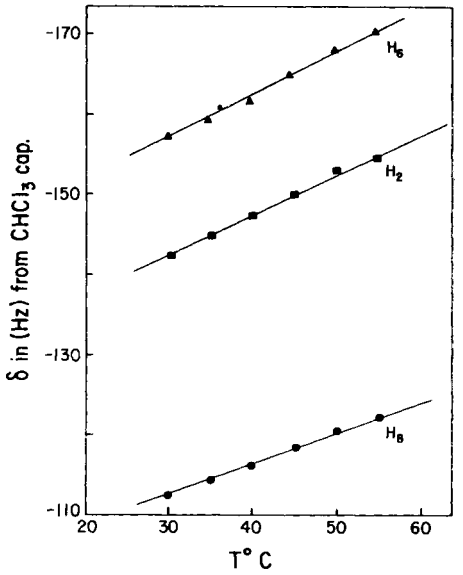

FIGURE 3: The temperature dependence of the chemical shifts of the purine resonances for a 0.20 *m* purine solution.

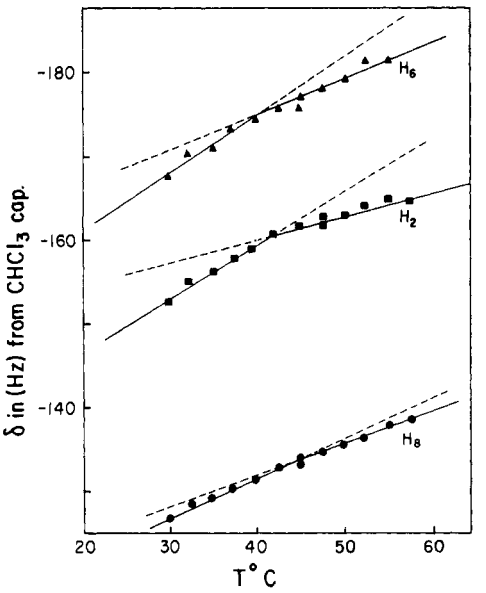

FIGURE 4: The temperature dependence of the chemical shifts of the purine resonances for a 0.20 *m* purine and 3.0 *m* NaCl solution.

rine, the dependence of the purine resonances can be represented by two straight lines with the point of intersection occurring at ~42 °C (Figure 4). The broken lines have been added to show that the slopes of the low- and high-temperature portions of the curve are markedly different. The greater slope of the low-temperature region indicates a greater degree of association than at higher temperatures with a distinct point of transition at ~42 °C, which is in good agreement with the osmometry data. Since the chemical shifts have not been corrected for bulk susceptibility differences, the absolute differences in chemical shift between the two samples cannot be compared. Only the shape of the curves is important. If the change in slope for the sample with NaCl reflected only changes in bulk susceptibility, the deviation from one straight line would be of the same magnitude for all three resonances. This is not the case; the H-8 resonance shows very little deviation from one straight line, whereas the H-2 and H-6 resonances show large deviations. This is to be expected since the H-8 resonance is situated on the less aromatic imidazole ring with a corresponding smaller ring current magnetic anisotropy and thus smaller chemical-shift changes for this proton have been observed as a function of concentration and temperature (Chan et al., 1964). The solution of 0.2 *m* purine and 3.0 *m* NaCl could not be studied by VPO due to instrumental limitations. It is interesting to note that the transition temperature for the change in water structure in the presence of salt is fairly independent of salt concentration. This observation is in total agreement with the species profile analysis which showed that the change in the relative proportions of the two predominant species, Cl^- and $Cl(H_2O)_3^-$, is small over this range from 25 to 80 °C (Högfeldt and Leifer, 1975; Leifer and Inoue, manuscript in preparation). When similar samples of 0.2 *m* purine were run in D_2O, no difference in the shape of the temperature dependence of the chemical shifts of the purine resonances for samples with no salt added and samples with up to 5.0 *m* NaCl was observed. Apparently, the more stable bulk D_2O structure is not as greatly perturbed by the Cl^- ion as is the H_2O structure. Since the only difference between the H_2O and D_2O samples is the increased solvent structure strength of the latter, the absence of any effect of NaCl on the chemical shift of the purine resonances in D_2O confirms the assumption that NaCl does not interact with the purine but the observed behavior in the VPO and NMR experiments is solely due to the effect of the salt on water structure.

Discussion

Since the hydrophobic interactions, such as vertical base stacking of purine, are expected to be extremely sensitive to structural changes of the solvent, the temperature dependence of the association of purine in the presence of sodium chloride is in total agreement with the effect of chloride ions on physical properties of bulk water in saline solutions. Previous studies have shown that the chloride ion does have some structuring effect on H_2O (Wen et al., 1966). There are, however, two degrees of structuring. Below 42 °C the bulk water is structured to a greater extent than above this temperature (Inoue, 1974; Leifer and Inoue, manuscript in preparation). Thus, in the low-temperature region, the structuring effect on the bulk water by the chloride ion is reflected by the increase in the extent of base stacking of purine in the presence of NaCl when compared with purine in pure water. As the transition temperature between the structured and less structured forms of the bulk water is exceeded, the degree of association of purine markedly decreases.

Besides the effect of the presence of sodium chloride on the hydrophobic interactions of purine, other drastic changes in the behavior of systems of biological importance in the presence of NaCl have been observed upon reaching the transition temperature of 42 °C. These include a phase transition for dipalmitoyllecithin bilayers in NaCl buffer at 40 °C (Levine et al., 1972), a change in the aggregation properties of sonicated lecithin vesicles in the presence of NaCl at 42 °C (Sheetz and Chan, 1972), the marked increase in the doubling time of *A. hydrophila* at 42 °C (Ringler and Anver, 1975), and others. In light of these results, it would seem more than coincidental that no advanced form of life can exist at temperatures much above 42 °C. Complex biological components, proteins, nucleic acids, and lipids, exist in the living cell in a saline environment. Since a major contribution to the conformational stability of these systems is hydrophobic interactions, even a small change in these forces would denature at least some of these components. Since our results indicate that the strength of hydrophobic forces decreases markedly above 42 °C, death in higher

organisms at elevated temperatures could simply be the result of the destructuring of the bulk water structure in the presence of chloride ions.

Appendix: Determination of the Main Ionic Species in Aqueous Electrolyte Solutions

In relatively concentrated solutions, the main contribution to the excess free energy is considered to arise from ion–solvent interactions

$$H^+ + n_+H_2O = H(H_2O)_{n_+}{}^+ \quad \text{(A1a)}$$

and

$$Cl^- + n_-H_2O = Cl(H_2O)_{n_-}{}^- \quad \text{(A1b)}$$

with the sum of the cationic and anionic hydration number giving the total hydration number n. Techniques for determining the main species in aqueous solution have been developed which involve a combination of the spectroscopically determined Hammett acidity function and the thermodynamically determined activities of HCl and H_2O (Högfeldt and Leifer, 1963; Leifer and Högfeldt, 1964, 1973; Inoue, 1974; Leifer and Inoue, manuscript in preparation). Using uncharged weak base indicators in the determination of the Hammett acidity function, we obtain:

$$BH^+ \rightleftharpoons B + H^+ \quad \text{(A2)}$$

and

$$K_{BH^+} = \frac{(H^+)[B]}{[BH^+]} \frac{y_B}{y_{BH^+}} \quad \text{(A3)}$$

In eq A3 the parentheses denote activity, the brackets denote concentrations of the species enclosed within, and the y's denote activity coefficients on a molar scale. Taking the logarithm of eq A3 and rearranging we obtain

$$\log K_{BH^+} = \log (H^+) + \log [B]/[BH^+] + \log y_B/y_{BH^+} \quad \text{(A4)}$$

Denoting the ratio of the molar activity coefficients of the uncharged weak base indicator to the charged form by the symbol ϕ_0, we obtain the defining equation for the Hammett acidity function H_0

$$-H_0 \equiv \log \phi_0(H^+) = \log K_{BH^+} + \log [BH^+]/[B] \quad \text{(A5)}$$

From eq A5, H_0 can be determined from a knowledge of the equilibrium constant for the protonation reaction of the indicator, K_{BH^+}, and the spectroscopically determined ratio of the concentrations of acid to base forms of the indicators used.

Thus we can construct ion activity functions in the following manner. We use HCl as an illustrative example.

$$(HCl) = \phi_0(H^+)\phi_0{}^{-1}(Cl^-) \quad \text{(A6)}$$

and

$$\log \phi_0{}^{-1}(Cl^-) = -\log \phi_0(H^+) + \log (HCl) \quad \text{(A7a)}$$

or

$$\log \phi_0{}^{-1}(Cl^-) = H_0 + \log (HCl) \quad \text{(A7b)}$$

Further ion activity coefficient functions may be constructed and the average degree of hydration may be obtained using methods well known in the field of complex ion chemistry.

$$\log \phi_0{}^{-1}(Cl^-) - \log [Cl^-] = \log \phi_0{}^{-1}y_{Cl^-} \quad \text{(A8)}$$

where the term on the right side of eq A8 is the chloride ion activity coefficient function. It is related to the average hydration of the chloride ion by the equation below:

$$-\text{d} \log \phi_0{}^{-1}y_{Cl^-}/\text{d} \log (H_2O) = \bar{n}_{Cl^-} \quad \text{(A9)}$$

where $\bar{n}_{Cl^-}$ is the average degree of hydration of the chloride ion. By plotting the logarithm of the ion activity coefficient function against the negative logarithm of the water activity, the slope at any given water activity (or solute concentration) is the average hydration of the chloride ion. This technique allows a splitting of the hydration between anion and cation as well as giving the hydration as a function of the concentration. Utilizing the assumption that $\phi_0{}^{-1}(Cl^-)$ is the same in both acid and salt solutions when compared at the same water activity, we are able to establish the main ionic hydrates in salt solutions for which there is no known measurement analogous to H_0.

This technique may be illustrated by considering the NaCl–H_2O system. We begin with the generalized expression for anionic hydration:

$$Cl^- + mH_2O = Cl(H_2O)_m{}^- \quad \text{(A10)}$$

We write the following equilibrium expression in which the molar activity coefficients of the hydrated species are absorbed into the constant K_m.

$$\frac{[Cl(H_2O)_m{}^-]}{[Cl^-]y_{Cl^-}} = K_m(H_2O)^m \quad \text{(A11)}$$

Taking the logarithm of eq A11 we have

$$\log \frac{[Cl(H_2O)_m{}^-]}{(Cl^-)} = \log K_m + m \log (H_2O) \quad \text{(A12)}$$

It has been shown (Leifer and Högfeldt, 1973) that

$$\frac{-\text{d} \log \sum[Cl(H_2O)_m{}^-]/(Cl^-)}{\text{d} \log a_{H_2O}} = \bar{m}_{Cl^-} \quad \text{(A13)}$$

in which $\bar{m}_{Cl^-}$ is the average degree of hydration of the chloride ion.

We have already seen that

$$\frac{-\text{d} \log \phi_0{}^{-1}y_{Cl^-}}{\text{d} \log a_{H_2O}} = \bar{m}_{Cl^-} \quad \text{(A14)}$$

We thus identify the ion activity coefficient function, $\phi_0{}^{-1}y_{Cl^-}$, with the numerator on the left side of eq A13 and if over a substantial region of water activity $\log \phi_0{}^{-1}y_{Cl^-}$ is linear when plotted against $-\log a_{H_2O}$, this is taken as evidence that only one term of the series is important.

Analysis of plots of $\log \phi_0{}^{-1}y_{Cl^-}$ vs. $-\log a_{H_2O}$ gave curves which exhibited two linear regions with slopes of 0 and 3 in both HCl–H_2O solutions and NaCl–H_2O solutions. This analysis indicates the ions are acting independently and the main ionic species of the chloride ion in aqueous solution are the Cl^- ion and $Cl(H_2O)_3{}^-$ ion.

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Effects of Podophyllotoxin and VP-16-213 on Microtubule Assembly in Vitro and Nucleoside Transport in HeLa Cells†

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ABSTRACT: VP-16-213, a semisynthetic derivative of podophyllotoxin, is an active antitumor agent. In this paper, the effects of VP-16-213 and podophyllotoxin on microtubule assembly in vitro and nucleoside transport in HeLa cells are compared. At 100 μM, VP-16-213 does not inhibit microtubule assembly in vitro, while 5 μM podophyllotoxin completely prevents the formation of microtubules. The presence of the glucoside moiety in VP-16-213 is responsible for the inactivity of VP-16-213 in this system because 4′-demethylepipodophyllotoxin, the nonglucoside congener of VP-16-213, inhibits microtubule assembly. In HeLa cells, VP-16-213 and podophyllotoxin share a common biological property; both agents inhibit the uptake of thymidine and uridine into cells by inhibiting the facilitated diffusional component of nucleoside transport. The concentrations of drug necessary to inhibit thymidine and uridine uptake into HeLa cells by 50% are 10 and 5 μM, respectively, for podophyllotoxin, and 25 and 20 μM for VP-16-213. The action of podophyllotoxin on nucleoside transport appears unrelated to its effect on microtubule assembly, since VP-16-213, which does not inhibit microtubule assembly, inhibits nucleoside transport.

Podophyllum resin is a crude extract isolated from the roots and rhizomes of the plants of the podophyllum species. It has been used in man as a cathartic, anthelminthic agent and a remedy for condyloma acuminatum (Kelly and Hartwell, 1954; Hartwell and Schrecker, 1958). The active constituent of this resin is podophyllotoxin, a cytotoxic compound whose mechanism of action involves at least two cellular processes, cell mitosis and nucleoside transport. During cell mitosis, spindle fibers composed of microtubules separate the duplicated chromosomes to poles located at opposite ends of the dividing cell. At low concentrations (1 μM), podophyllotoxin binds to the microtubule subunit, tubulin, inhibiting its polymerization into microtubules and arresting cell division in mitosis (Wilson et al., 1974). At higher concentrations (10–100 μM), Mizel and Wilson (1972) have shown that podophyllotoxin inhibits nucleoside transport.

Over the last decade many investigators have synthesized or isolated compounds related to podophyllotoxin in search of clinically effective antitumor agents. Recently, a semisynthetic podophyllotoxin derivative, 4′-demethylepipodophyllotoxin ethylidene-β-D-glucoside, VP-16-213, was synthesized by Stähelin (1973) and has activity against certain solid tumors and leukemias (Dombernowsky et al., 1972; Nissen et al., 1972; Creaven et al., 1975). VP-16-213 differs from podophyllotoxin at three positions: (1) VP-16-213 contains a glucoside moiety at the C-4 carbon, (2) it has enantiomeric configuration of podophyllotoxin at C-4 carbon atom, and (3) VP-16-213 contains a hydroxyl group at the C-4′ position (Figure 1). In contrast to podophyllotoxin, which blocks cells in mitosis, VP-16-213 has been reported to block L-1210 cells in a premitotic stage of the cell cycle (Grieder et al., 1974). VP-16-213 has also been shown to cause a high incidence of chromosomal aberrations in human hematopoietic cell lines (Huang et al., 1973).

In the present paper, we have examined the effects of podophyllotoxin and VP-16-213 on microtubule assembly in vitro and report that, in contrast to podophyllotoxin, VP-16-213 does not inhibit the in vitro polymerization of tubulin. We have also compared the effects of VP-16-213 and podophyllotoxin on macromolecular synthesis and nucleoside transport in HeLa cells, and found that both drugs are effective inhibitors of nucleoside transport. VP-16-213 also induces single-stranded breaks in HeLa cell DNA, an effect not seen in podophyllotoxin-treated cells. The characterization of this effect on DNA is presented in the following paper (Loike and Horwitz, 1976).

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