On the Melting Temperature of Nucleic Acid in Solution

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Many natural and synthetic nucleic acids take doubly-stranded helical conformations in aqueous solutions at room temperature.[1] On raising the temperature of such a solution, a strand separation takes place, and the helical molecule turns to two single-stranded random coils. The process can be followed by the viscosity, the optical density (at 260 mμ), or the optical rotation measurement of the solution.[2] It is found that the transition occurs within a narrow temperature range. This transition is considered to be the "melting" of a one-dimensional "crystal." The temperature at the midpoint of the transition is denoted by T_m and may be called "the melting temperature." The melting temperature, T_m, of a nucleic acid in a solution varies according to the base composition of the nucleic acid, the salt concentration of the solution, and the pH of the solution. Some chemicals, such as caffeine,[3] spermine,[4] and actinomycin D,[5] when they are added to the solution, also affect the T_m value. This paper will present some discussion of these variations of T_m on the basis of a simple application of the mass action law to the strand-separation reaction.

Let us assume that, in a solution of a nucleic acid at a temperature in the vicinity of the melting temperature, there are only three kinds of molecules (see Fig. 1): the double-helix molecule (AB), and the two complementary single-stranded molecules (A and B), and that there is no molecule (such as C in Fig. 1) in which a half-way strand-separation takes place. This assumption is somewhat justified by the fact that in the CsCl density-gradient spectrum of partially heat-denatured deoxyribonucleic acid, no bands are observed corresponding to the molecules in which a half-way strand-separation takes place.[6]

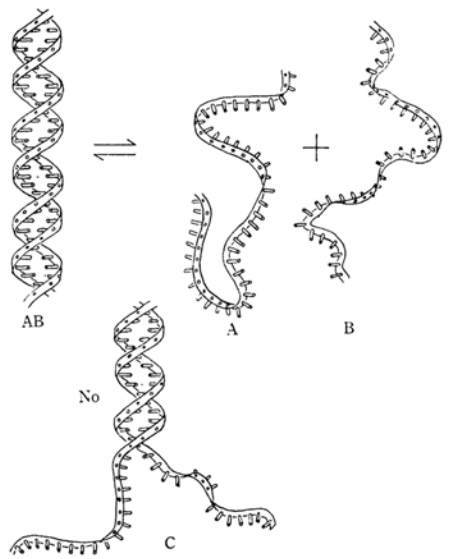

Fig. 1. A schematic representation of a transition of a doubly-stranded helix (AB) of a nucleic acid to two single-stranded random coils (A and B). The form C, where a half-way strand separation takes place, is not considered to exist.

Let us assume also that the melting of nucleic adid is essentially a reversible process, and that the irreversibility observed of deoxyribonucleic acid (DNA) is only an apparent one, an extremely long time being required for the complete "crystallization" (reformation of the perfect double-helix structure). This extremely low reaction rate is probably due to an extremely high activation entropy in the reformation reaction of DNA. As one can easily imagine, the chance is extremely low of any two complementary DNA chains to approach each other in such a way that the original proper base pairings can be reformed. The rate of double-helix reformation is quite high in a system where no such activation entropy is required. Thus, the melting of the double-helix molecule composed of 1:1 polydeoxyribothymidylic acid and polydeoxyriboadenylic acid, poly(dT+dA), was found to be completely reversible.[7] We have also observed

1) See, for example, R. F. Steiner and R. F. Beers, Jr., "Polynucleotides," Elsevier Publishing Company, New York (1961).

2) P. Doty, J. Marmur and N. Sueoka, Brookhaven Symposia in Biology, "Structure and Function of Genetic Elements," No. 12, 1 (1959).

3) P. O. P. Ts'o, G. K. Helmkamp and C. Sander, *Proc. Nat. Acad. Sci.* (*U. S. A.*), **48**, 686 (1962).

4) M. Mandel, *J. Mol. Biol.*, **5**, 435 (1962).

5) M. Tsuboi, S. Higuchi, Y. Kyogoku, K. Matsuo and A. Wada, *Chem. Pharm. Bull.*, **12**, 501 (1964).

6) J. Marmur, C. L. Schildkraut and P. Doty, Fifteenth Annual Symposium on Fundamental Cancer Research, "The Molecular Basis of Neoplasia," Univ. of Texas, Austin, U. S. A. (1961), p. 9.

7) P. Doty, Discussion in Brookhaven Symposium in Biology, No. 12, "Structure and Function of Genetic Elements," 24 (1959).

that, for the 1 : 1 complex of polyriboinosinic acid and polyribocytidylic acid, poly(I+C), the same optical density profile is obtained on heating and cooling.[8]

We can now consider that the "melting" problem is simply the problem of the dissociation equilibrium of the type:

$$AB \rightleftharpoons A + B \tag{1}$$

The equilibrium constant may be expressed as:

$$K=\frac{[A]\,[B]}{[AB]}=\frac{(1-f)^2}{f}a \tag{2}$$

Here, f is the fraction of the double-helix molecules, and a is the total concentration of the nucleic acid in question. At the melting temperature, T_m, f equals[9] 1/2, and the equilibrium constant is given by:

$$K_m=\frac{1}{2}a \tag{3}$$

The K_m is related to the enthalpy (ΔE) and the entropy (ΔS) of melting as:

$$-\boldsymbol{R}T_m \ln K_m=\Delta E-T_m\Delta S \tag{4}$$

1. The Guanine-Cytosine Content and Melting Temperature of DNA

The enthalpy of the melting of a deoxyribonucleic acid (DNA) may be given as:

$$\Delta E=n[\Delta\varepsilon_1 g+\Delta\varepsilon_2(1-g)] \tag{5}$$

where $\Delta\varepsilon_1$ is the enthalpy of melting assigned to one cytosine-guanine bridge; $\Delta\varepsilon_2$, that assigned to one adenine-thymine bridge; g, the guanine plus cytosine content (GC content) of the DNA in question, and n, the mean number of bridges in the double-helix structure of the DNA in question. From Eqs. 3, 4 and 5, we obtain the relation:

$$g=\frac{\Delta S-\boldsymbol{R}\ln 0.5a}{n(\Delta\varepsilon_1-\Delta\varepsilon_2)}T_m-\frac{\Delta\varepsilon_2}{\Delta\varepsilon_1-\Delta\varepsilon_2} \tag{6}$$

If ΔS, $\Delta\varepsilon_1$, and $\Delta\varepsilon_2$ are assumed to be constants independent of T_m, then Eq. 6 shows that the melting temperature, T_m, should be a linear function of the GC content, g, of the DNA in question. This linear relation is just what was observed by Marmur and Doty.[10]* The straight lines obtained by them are reproduced in Fig. 2. The slope and intercept

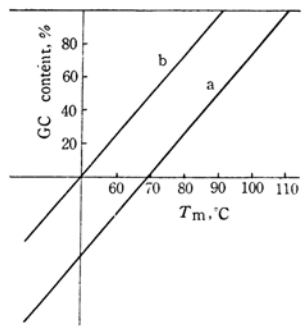

Fig. 2. Dependence of T_m on the guanine plus cytosine content of DNA.[10] The straight line a was obtained from the examination of the solution with 0.15 mol./l. NaCl plus 0.015 mol./l. Na-citrate, and the line b of the solution with 0.01 mol./l. PO_4 plus 0.001 mol./l. EDTA.

of the line for the solutions with 0.15 mol./l. sodium chloride are found to be respectively 1/42 and −8.1. Therefore, from Eq. 6, we obtain

$$\frac{\Delta S-\boldsymbol{R}\ln 0.5\,a}{n(\Delta\varepsilon_1-\Delta\varepsilon_2)}=\frac{1}{42} \tag{7}$$

and

$$\frac{\Delta\varepsilon_2}{\Delta\varepsilon_1-\Delta\varepsilon_2}=8.1 \tag{8}$$

The 7 and 8 relations are given graphically in Figs. 3 and 4 respectively. At present the values of $\Delta\varepsilon_1$, $\Delta\varepsilon_2$, $\Delta\varepsilon_1-\Delta\varepsilon_2$, and $(\Delta S-\boldsymbol{R}\times\ln 0.5\,a)/n$ are all unknown. However, if one

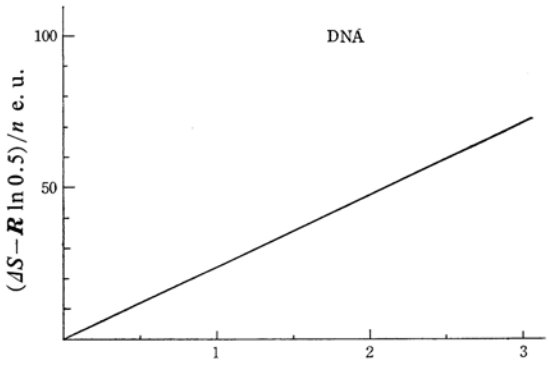

Fig. 3. Relation of $(\Delta S-\boldsymbol{R}\ln 0.5\,a)/n$ and $\Delta\varepsilon_1-\Delta\varepsilon_2$ for DNA in the solution with 0.15 mol./l. NaCl plus 0.015 mol./l. Na-citrate.

8) K. Matsuo and M. Tsuboi, *Chem. Pharm. Bull.*, to be published.

9) It was pointed by J. Applequist (*J. Am. Chem. Soc.*, 83, 3158 (1961)) that the hypochromism of nucleic acid at 260 mμ is not always proportional to f. Therefore, if the transition is followed by the measurement of the optical density at 260 mμ, the apparent midpoint of the transition does not always give the point where f=0.5. However, this does not seriously affect the present discussion, where T_m is required only as an operational quantity.

10) J. Marmur and P. Doty, *J. Mol. Biol.*, 5, 109 (1961).

* Here it should be mentioned that M. Ozawa, M. Tanaka and E. Teramoto (*J. Phys. Soc. Japan*, 18, 551 (1963)) explained the linear relation between the T_m and the GC content of DNA on the basis of essentially the same idea as above but with a somewhat different expression—an experssion of statistical mechanics. The present writer's thermodynamic expression was first described at the 10th Nucleic Acid Symposium, Kanazawa, May, 1962.

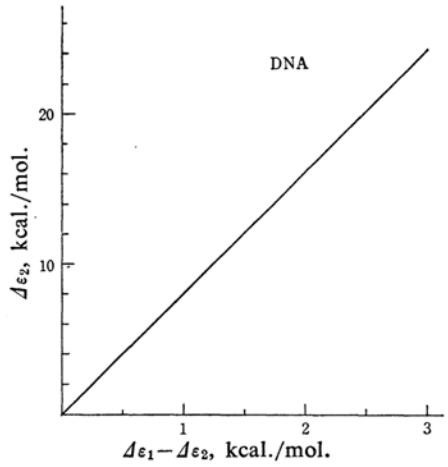

Fig. 4. Relation of $\Delta\varepsilon_2$ and $\Delta\varepsilon_1-\Delta\varepsilon_2$ for DNA in the solution with 0.15 mol./l. NaCl plus 0.015 mol./l. Na-citrate.

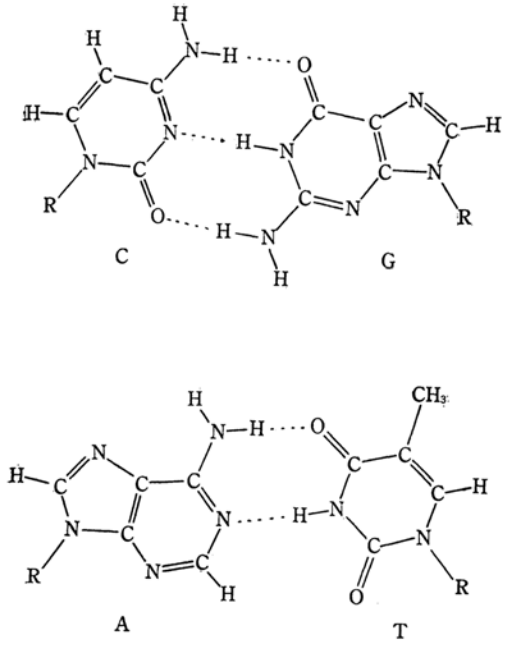

Fig. 5. Hydrogen bonds between cytosine (C) and guanine (G) residues and between adenine (A) and thymine (T) residues.[11]

of these values is fixed, all of these values can be determined by these relations. It has been pointed out[11] that, as is shown in Fig. 5, cytosine and guanine can form three hydrogen bonds, while adenine and thymine can form only two hydrogen bonds. If the stability of the helical conformation came solely from these hydrogen bonds, and if each of these hydrogen bonds made an equal contribution to the stability, $\Delta\varepsilon_2/(\Delta\varepsilon_1-\Delta\varepsilon_2)$ should be equal to 2. The actual value of 8.1 indicates that the third hydrogen bond in the cytosine-guanine bridge makes a relatively small contribution to the stability of the helix.

2. The Degree of Protonation and the Melting Temperature of Polyriboadenylic Acid

Polyriboadenylic acid (poly A) is known to form a two-stranded helical complex in its acidic solution.[12] It has been found that the melting temperature of this complex depends upon the degree of the protonation of the adenine residue. According to the experimental results obtained by Fresco and Klemperer,[12] the melting temperature is 90°C for a 0.88 degree of protonation (at pH 4.25) and 68°C for a 0.76 degree of protonation (at pH 4.86). At pH 4.25, the fraction (g_1) of the AH^+-AH^+ pair is about $0.88\times0.88=0.78$, the fraction (g_2) of the AH^+-A pair is about $0.88\times0.12=0.11$, and the fraction (g_3) of the A−A pair is about $0.12\times0.12=0.01$, where A and AH^+ indicate the adenine residue and protonated adenine residue respectively. At pH 4.86, the fraction (g_1) of the AH^+-AH^+ pair is about $0.76\times0.76=0.58$, the fraction (g_2) of the AH^+-A pair is about $0.76\times0.24=0.18$, and the fraction (g_3) of the A−A pair is about $0.24\times0.24=0.06$. Let the contribution of the AH^+-AH^+ pair to the helix stability be $\Delta\varepsilon_1$, that of the AH^+-A pair, $\Delta\varepsilon_2$, and that of the A−A pair, $\Delta\varepsilon_3$. Now we obtain the relation:

$$\Delta E=n(\Delta\varepsilon_1 g_1+\Delta\varepsilon_2 g_2+\Delta\varepsilon_3 g_3) \tag{9}$$

instead of Eq. 5, and the relation

$$g_1=\frac{\Delta S-\boldsymbol{R}\ln 0.5\,a}{n(\Delta\varepsilon_1-\Delta\varepsilon_2)}T_m-\frac{\Delta\varepsilon_2-(\Delta\varepsilon_2-\Delta\varepsilon_3)g_3}{\Delta\varepsilon_1-\Delta\varepsilon_2} \tag{10}$$

instead of Eq. 6. It is assumed that

$$\Delta\varepsilon_2-\Delta\varepsilon_3=\Delta\varepsilon_1-\Delta\varepsilon_2 \tag{11}$$

then Eq. 10 becomes

$$g_1-g_3=\frac{\Delta S-\boldsymbol{R}\ln 0.5\,a}{n(\Delta\varepsilon_1-\Delta\varepsilon_2)}T_m=\frac{\Delta\varepsilon_2}{\Delta\varepsilon_1-\Delta\varepsilon_2} \tag{12}$$

Thus we obtain

$$\frac{\Delta S-\boldsymbol{R}\ln 0.5\,a}{n(\Delta\varepsilon_1-\Delta\varepsilon_2)}=0.0114\ \text{deg}^{-1} \tag{13}$$

and

$$\Delta\varepsilon_2/(\Delta\varepsilon_1-\Delta\varepsilon_2)=3.4 \tag{14}$$

from the observed relation of g_1-g_3 and T_m (see Fig. 6). Therefore, it may be concluded that, very roughly, the AH^+-AH^+ pair gives $(3.4+1.0)/3.4=1.3$ times the contribution to

11) L. Pauling and R. Corey, *Arch. Biochem. Biophys.*, **65**, 164 (1956).

12) J. R. Fresco and E. Klamperer, *Ann. New York Acad. Sci.*, **81**, 730 (1959).

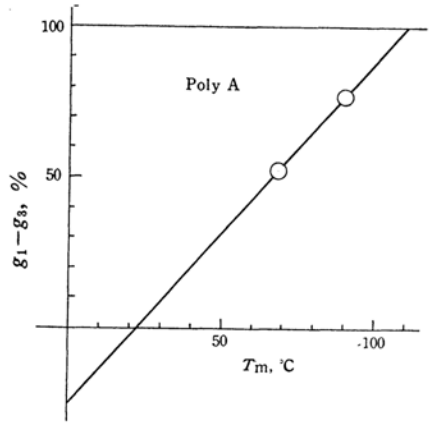

Fig. 6. T_m plotted against g_1-g_3 of poly A in the solution with 0.15 mol./l. NaCl.

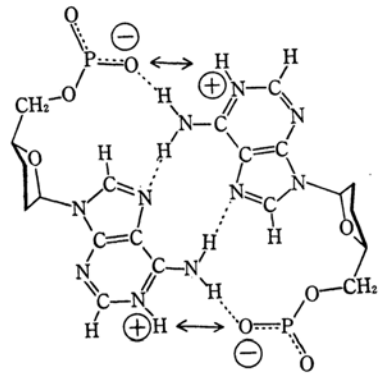

Fig. 7. Hydrogen bonds between two adenine residues.[13]

the helix stability given by the AH^+-A pair.

It has been pointed out[13] that two adenylic acid residues can form the four hydrogen bonds shown in Fig. 7, and that the additional stability of the protonated poly A may be attributed to an electrostatic force between the positively-charged $(N_1H)^+$ group of the protonated adenine residue in one of the two intertwined helices and the negatively-charged phosphate group in the other helix. The amount of this stability may be estimated from the difference between the pK values of poly A and adenosine. From the titration curve (at 25°C) of the poly A solution with 0.15 molal sodium chloride,[12] the pK of poly A may be estimated as about 5.5. On the other hand, Levene and Simms[14] reported the pK for adenosine to be 3.5. This means that protonated poly A needs more free energy for deprotonation than does protonated adenosine. The difference is:

$$(RT/\log_{10} e)(5.5-3.5)=2.8 \text{ kcal./mol.}$$

13) A. Rich, D. R. Davies, F. H. C. Crick and J. D. Watson, *J. Mol. Biol.*, 3, 71 (1961).
14) P. A. Levene and H. S. Simms, *J. Biol. Chem.*, 65, 519 (1925).

where T is the absolute temperature of the solution and e is the base of the natural logarithm. This difference may be attributed mostly to the additional stability of the protonated poly A because of the above-mentioned electrostatic force. If we assume, on this basis, that

$$\Delta\varepsilon_1-\Delta\varepsilon_2=2.8 \text{ kcal./mol.}$$

then the values of $\Delta\varepsilon_1$, $\Delta\varepsilon_2$, $\Delta\varepsilon_3$, and $(\Delta S-R\ln 0.5\,a)/n$ are calculated by Eqs. 11, 13, and 14 as follows:

$$\Delta\varepsilon_1=12.2 \text{ kcal./mol.}$$
$$\Delta\varepsilon_2=9.4 \text{ kcal./mol.}$$
$$\Delta\varepsilon_3=6.6 \text{ kcal./mol.}$$
$$(\Delta S-R\ln 0.5\,a)/n=32 \text{ e.u.}$$

3. The Effect of Salt Concentration

The melting temperature of nucleic acid is greatly influenced by the ionic strength of the solution. Marmur and Doty[10] showed this to be the case for E. coli K-12 DNA heated in various concentrations of potassium chloride. It was found that, when the salt concentration is raised, the T_m goes up in the manner shown in Fig. 8.

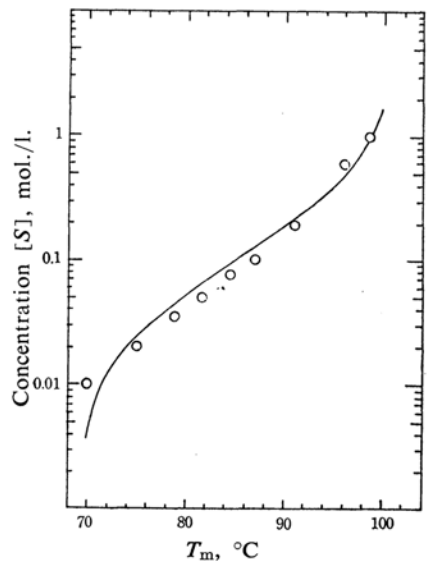

Fig. 8. T_m of E. coli K12 DNA plotted against the KCl concentration (in logarithmic scale) in the solution.

○: From the data given by Marmur and Doty.[10]
—: Theoretical curve on the assumption that $K'=10(\text{mol./l.})^{-1}$, $(\Delta S-R\ln 0.5\,a)/\alpha N=0.0304 \text{ deg}^{-1}$, and $E_0/\alpha N=10.4$.

It would be expected that raising the salt concentration of the suspending medium would

produce a shielding of the repulsive forces of the charged phosphate groups of the DNA molecule, and that this would produce an additional stability of the helical form and, thus, a higher melting temperature. Let us assume that the shielding occurs through an adsorption of the cations by the DNA molecules at the PO_2^- groups, and that, for this adsorption, Klolz's equation,[15]

$$\frac{1}{r}=\frac{1}{K'N}\frac{1}{[S]}+\frac{1}{N} \tag{15}$$

is valid. In Eq. 15, r is the average number of the cations in question adsorbed by one double-helical DNA molecule AB; N, the maximum number of the cations that can be adsorbed by one double-helical DNA molecule; $[S]$, the concentration of the cations free from the adsorption, and K', the equilibrium constant (at T_m) of each of the reactions:

$$\begin{aligned}&AB+S=AB\cdot S\\&AB\cdot S+S=AB\cdot S_2\\&AB\cdot S_2+S=AB\cdot S_3\end{aligned} \tag{16}$$

and

$$AB\cdot S_{N-1}+S=AB\cdot S_N$$

In the present case, ΔE in Eq. 4 may be given as

$$\Delta E=\Delta E_0+\alpha r \tag{17}$$

where ΔE_0 is the enthalpy of the melting of DNA without cations, and α, the increase of ΔE caused by the adsorption of one cation. From Eqs. 4, 15 and 17, we obtain the relation,

$$\begin{aligned}&(\Delta S-\boldsymbol{R}\ln 0.5\,a)T_m\\&\quad=\Delta E_0+\alpha NK'[S]/(1+K'[S])\end{aligned} \tag{18}$$

The melting temperature of DNA is usually examined at a concentration of 20 μg./ml., i.e., about 6×10^{-5} mol. nucleotide per liter. In a solution with such a low concentration of DNA, the free cation concentration $[S]$ is practically equal to its total concentration ($10^{-2}\sim1$ mol./l.). Therefore, Eq. 18 is considered to give the T_m value as a function of the salt concentration ($\fallingdotseq[S]$). The equilibrium constant K' is a function of T_m, as

$$K'=\exp(\Delta S'/\boldsymbol{R})\exp(-\Delta E'/\boldsymbol{R}T_m) \tag{19}$$

where $\Delta E'$ and $\Delta S'$ are, respectively, the enthalpy and the entropy of each of the reactions 16. If $\Delta E'$ is small, however, K' is practically constant over a small range of T_m (see Fig. 9). As will be mentioned below, this is actually the case for the adsorption of cations by the DNA molecules. It would be convenient to draw curves representing the relations between $[S]$ and $K'[S]/(1+K'[S])$ for various K' values, as in Fig. 10. The abscissa $K'[S]/(1+K'[S])$ should have a linear relation with the T_m in question, since, from Eq. 18,

$$\begin{aligned}&K'[S]/(1+K'[S])\\&\quad=[(\Delta S-\boldsymbol{R}\ln 0.5a)/\alpha N]T_m-\Delta E_0/\alpha N\end{aligned} \tag{19}$$

A comparison of Fig. 8 and Fig. 10 shows that the observed effect of the KCl concentration of the T_m of DNA can be well explained by what has been presented and by assuming that

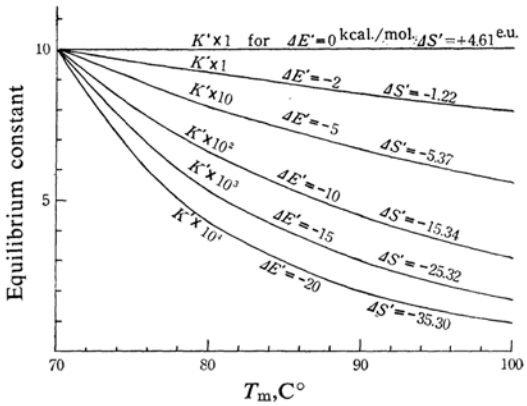

Fig. 9. Equilibrium constant K' plotted against the temperature in question (T_m). $\Delta E'$ and $\Delta S'$ are the enthalpy and entropy of the reaction in question (see Eq. 19 in the text).

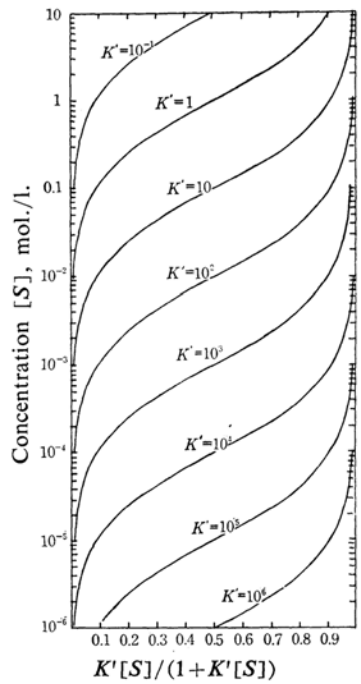

Fig. 10. Relations between $[S]$ (in logalithmic scale) and $K'[S]/(1+K'[S])$ for various K' values.

15) I. M. Klotz, "Protein Interactions" in H. Neurath and K. Bailey (ed.), "The Proteins," Academic Press, New York (1953).

$K' = 10$ (mol./l.)$^{-1}$

$(\Delta S - \boldsymbol{R} \ln 0.5\, a)/\alpha N = 0.0304$ deg^{-1}

and

$\Delta E_0/\alpha N = 10.4$

It may be pointed out here that straight lines of T_m vs. GC-content observed by Marmur and Doty[10] at different salt concentrations are almost parallel to each other (see Fig. 2). The lines have the same slope but different intercepts. On the basis of Eq. 6, this means that, at different salt concentrations, the values of $(\Delta S - \boldsymbol{R} \ln 0.5\, a)/n(\Delta\varepsilon_1 - \Delta\varepsilon_2)$ are equal to each other, but that the values of $\Delta\varepsilon_2/(\Delta\varepsilon_1 - \Delta\varepsilon_2)$ are different from each other. This suggests that the stability of the helical form of DNA due to the base pairing is not affected (and that thus the $\Delta\varepsilon_1 - \Delta\varepsilon_2$ value is not affected) by the salt concentration.

From the fact that the two straight lines, in question are parallel,[10] another interesting conculsion may be drawn: The $\Delta E'$ is very small for the adsorption of cations by the DNA molecules. The increase in T_m (denoted as ΔT_m) when the salt concentration is raised from "0.01 M PO_4 + 0.001 M versene" to "0.15 M NaCl + 0.015 M Na citrate" should be proportional to the K' value on the basis of Eq. 18. If the $\Delta E'$ in question is -2 kcal./mol. or so, the K' (and therefore ΔT_m) should decrease appreciably when the T_m is raised from 70 to 90°C (see Fig. 9). The experimental results of Marmur and Doty[10] (given in Fig. 2) indicate that this is not actually the case, and that ΔT_m (and therefore K') remains constant at any T_m in the 70~90°C range. Thus, the absolute value of $\Delta E'$ is considered to be much smaller than 2 kcal./mol. If the absolute value of $\Delta E'$ is smaller than 1.6 kcal./mol. ($\Delta E > -1.6$ kcal./mol.) and if $K' = 10$ (mol./l.)$^{-1}$, as has been given above, then $\Delta S'$ should be positive. The positive $\Delta S'$ obtained here may be interpreted as indicating that the adsorption of cations by the DNA molecules takes place through a hydrophobic bond.[16]

4. The Effect of Spermine

Spermine $NH_3^+(CH_2)_3NH_2^+(CH_2)_4NH_2^+ \cdot (CH_2)_3NH_3^+$ is known to cause, when it is added to a DNA solution, an increase in the T_m of DNA.[4,17] This is considered to be due to the formation of complex molecules of spermine plus double-helical DNA, which are more stable than the double-helical DNA molecules free from spermine.

The binding reaction of spermine molecules, S, with double-helical DNA molecules, AB, may be expressed by Eqs. 15 and 16 of the preceding section. The enthalpy of melting the ΔE of such a spermine-DNA complex may be expressed by Eq. 17, and the melting temperature, T_m, may be given by Eq. 18. When T_m is plotted against $\log_{10}[S]$, a curve similar to those given in Fig. 9 is obtained. From a comparison of such a curve and those in Fig. 9, the K' value is estimated to be about 10^5 (mol./l.)$^{-1}$ in solutions with 0.03 mol./l. sodium chloride at 80°C, and about 10^3 (mol./l.)$^{-1}$ in solutions with 0.15 mol./l. sodium chloride at 90°C. Thus, it may be seen that the equilibrium constant, K', for spermine is much higher than that for potassium cation and that it depends greatly upon the salt concentration in the solution.

Mandel[4] examined the effects of spermine on the T_m values of solutions of DNA's obtained from a number of different sources and with different guanine-cytosine contents (GC-content). He observed that the extent of the increase in T_m (ΔT_m) becomes smaller with the increase in GC-content. Thus, $\Delta T_m = 3.1$°C for a DNA with a GC-content of 26% ($T_m = 81.3$°C), while $\Delta T_m = 1$°C for a DNA with a GC-content of 58% ($T_m = 95.8$°C). He concluded that ΔT_m is a function of the adenine-thymine content of the DNA sample. In the present writer's view, however, what he observed is mostly to be attributed to the effect of the temperature on the K' value; it is not to be directly correlated with the base composition of DNA.

As has been mentioned above, the interaction of the spermine molecule with the DNA molecule is very strong, and $\Delta E'$ for the present case may be as high as $-10 \sim -20$ kcal./mol. or so. If it is -20 kcal./mol., for instance, the K' value at 95°C can be only one-third of that at 80°C (see Fig. 9). It is quite possible that such a lowering in K' value causes the lowering in ΔT_m value mentioned above. In addition, we have recently obtained[8] a bit of evidence which shows that the existence of the adenine-thymine base pairing in a nucleic acid is not a necessary condition for the interaction with spermine; the addition of only 0.0005 mol./l. of spermine produces a marked elevation of the T_m (from 58 to 90°C) of 1:1 complex polyriboinosinic acid plus polyribocytidylic acid, poly(I+C), which contains no adenine-thymine base pair.

There it remains much to be done before we can discuss the molecular structure of the spermine-DNA complex in the molecular structure of the spermine-DNA complex in detail. On the basis of what we have so far obtained,

16) See, for example, G. Némethy and H. A. Scheraga, *J. Phys. Chem.*, **66**, 1773 (1962).
17) H. Tabor, *Biochemistry*, **1**, 496 (1962).

however, the writer would like to present a possible model of that structure here.

Quite recently, Iitaka and Huse[18] have determined the crystal structure of spermine phosphate hexahydrate. The results suggest in a very interesting way how the spermine molecule may combine with the DNA molecule in the double-helix form.

In the crystal structure of spermin phosphate hexahydrate, there is a sheet which consists of HPO_4^{2-} ions and water molecules formed by the O–H···H hydrogen bonds. The sheet contains parallel chains of phosphate ions running along the a axis. The phosphate-phosphate distance along the a axis is 7.96 Å, while the chain-chain distance along the b axis (in the sheet) is 11.6 Å. As Iitaka and Huse[18] pointed out, there is a certain similarity between the arrangement of phosphate groups found in spermine phosphate and that found in crystalline DNA. Langridge et al.[19] have shown the arrangement of phosphate groups along the polynucleotide helices in the B-form of LiDNA. In this structure, two oxygen atoms of the phosphate group, which share one negative formal charge, are turned outwards and so are readily available for interaction with proteins, water, etc. (see Fig. 11).

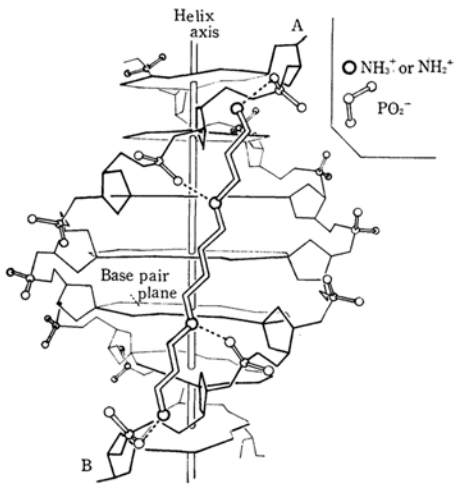

Fig. 11. A proposed model of spermine plus double-helical DNA.

The distance between successive phosphate groups along the helix is about 7.3 Å, which may be compared with the phosphate-phosphate distance, 7.96 Å, along the a axis in the phosphate chain in the spermine phosphate crystal. The perpendicular distance between two polynucleotide chains (chain A and B of the double-helix) across the shallow groove in LiDNA is about 13 Å, while in the spermine phosphate crystal the separation of the phosphate chains within the same sheet is found to be 11.6 Å, as has been given above. The spermine molecule in the spermine phosphate crystal is in the form of the fully-extended zigzag chain, and it is placed parallel to the phosphate-water sheet, forming an oblique bridge across the adjacent two phosphates chains in the same sheet. One of the two NH_3^+ groups makes a strong hydrogen bond with a phosphate group in the one chain, and the other NH_3^+ group makes a similar hydrogen bond with a phosphate group in the other chain. One of the two NH_2^+ groups makes a strong hydrogen bond with another phosphate group in the former chain, and the other NH_2^+ group makes a similar hydrogen bond with another phosphate group in the latter chain.

On the basis of what has been described above, it may be proposed that the spermine molecule combines with the double-helical NDA molecule, so that it forms an oblique bridge across the shallow groove between the two polynucleotide helices, A and B, as is shown in Fig. 11. A molecular model building shows that each of the NH_3^+ and the NH_2^+ groups on one end of a spermine molecule can form a strong hydrogen bond with one of the two adjacent phosphate groups in the DNA chain A, and that at the same time both the NH_3^+ and the NH_2^+ groups on the other end of the spermine molecule can form a similar hydrogen bond with one of the two adjacent phosphate groups in the DNA chain B. In this way, the spermine molecule may function as a clamp for binding two chains (A and B) together, and thus for stabilizing the double-helical structure of DNA.[20]

5. The Effect of Actinomycin D

The effect of actinomycin D on the T_m of DNA has recently been examined by Tsuboi et al.[5] Of what has been observed, the following two facts are to be pointed out here: First, only a very small amount (2.5×10^{-5}

18) Y. Iitaka and Y. Huse, This Bulletin, 37, 437 (1964); *Acta Cryst.*, in press.

19) R. Langridge, D. A. Marvin, W. E. Seeds, H. R. Wilson, C. W. Hooper, M. H. F. Wilkins and L. D. Hamilton, *J. Mol. Biol.*, 2, 38 (1960).

20) Iitaka and Huse (Ref. 18) showed that, in tne spermine phosphate hexahydrate crystal, the phosphate chains of the adjoining sheets as well as of the same sheet are bound together strongly by the spermine molecules. Likewise, a spermine molecule may function not only as a clamp for binding two polynucleotide helices together but also as an "adhesive" for binding two double-helical DNA molecules. It was found (Ref. 4) that, at concentrations of spermine in excess of 0.005 mol./l., visible aggregation of the DNA strands occurs. This fact may be interpreted as due to the above-mentioned "adhesive" function of the spermine molecule.

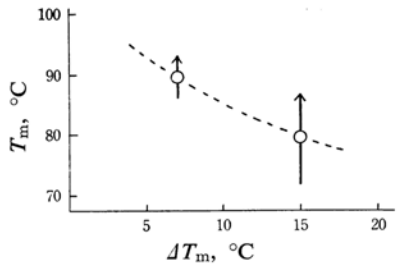

Fig. 12. Amount of elevation of $T_m(\Delta T_m)$ of calf-thymus DNA caused by addition of actinomycin D, plotted against the T_m of the solution.

mol./l.) is necessary to cause a marked elevation of T_m (72°C to 87°C). Secondly, the amount of elevation, ΔT_m, depends upon the original T_m value; ΔT_m is lower in a DNA solution with high a T_m (see Fig. 12).

On the basis of what has been discussed in this paper, the first fact may be interpreted as indicating that actinomycine D molecules are bound by the helical DNA molecule with a very high K' value and/or a very high α value. The second fact may be interpreted as indicating that the K' value depends greatly upon the temperature of the solution, because there is a high $\Delta E'$ value in each of the binding reactions, Eqs. 16.

6. The Effect of Caffeine, etc.

Ts'o, Helmkamp, and Sander[3] observed that the T_m's of polyriboadenylic acid (poly A) and DNA are lowered when caffeine, nucleosides, and related compounds are added to the solutions. Ts'o and Lu[21] found, on the other hand, that caffeine, for example, is bound by the random coil poly A with $K'=5.8$ (mol./l.)$^{-1}$ at 5°C, while it is much less strongly bound by the helical poly A ($K'<(1$ mol./l.)$^{-1}$ at 5°C). As they mentioned, the lowering of T_m may be related to the fact that caffeine (for example) interacts more strongly with the coil form of nucleic acids than with the helical form.

Also, for the present problem, Eqs. 17 and 18 are considered to be valid provided that the meanings are somewhat different of α, r, N, K' and $[S]$. α is now a negative value and represents the decrease in ΔE caused by the binding of caffeine to the coil form poly A. K' is the equilibrium constant (at T_m) of each of the reactions:

$$\begin{aligned} A+S&=AS \\ AS+S&=AS_2 \\ AS_2+S&=AS_2 \end{aligned} \qquad (20)$$

and

$$AS_{N-1}+S=AS_N$$

r is the average number of caffeine molecules bound by one random-coil poly A molecule (A); N, the maximum number of caffeine molecules that can be bound by one random-coil poly A molecule (A), and $[S]$, the concentration of the caffeine molecules free from poly A (which is practically equal to the total concentration of caffeine in the present case). From Eq. 18, the amount of the lowering of T_m ($-\Delta T_m$) due to the addition of $[S]$ of caffeine, etc., to the solution is expressed as:

$$\Delta T_m=\frac{\alpha K'[S]/(1+K'[S])}{(\Delta S-\boldsymbol{R}\ln 0.5\,a)/N} \qquad (21)$$

By the use of this expression, and by the use of the data obtained by Ts'o et al.[3,21] (plotted in Fig. 13 by circles) an attempt has

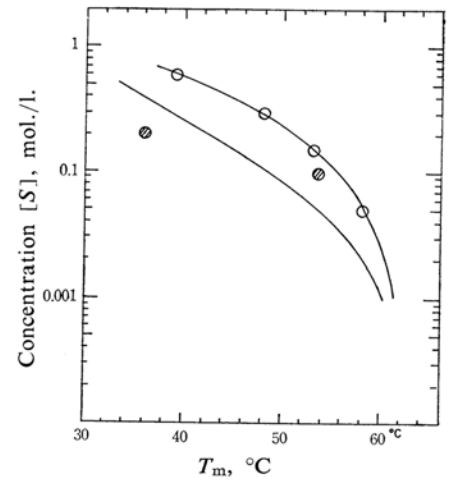

Fig. 13. T_m of poly A at pH 4.85 plotted against the concentration of caffeine (black circles) and purine (white circles) added to the solution.[3] Curves were obtained by a calculation based upon Eq. 21, and by assuming that $\alpha=1.2$ kcal./mol. and $K'=5.8$ (mol./l.)$^{-1}$ for purine and that $\alpha=1.3$ kcal./mol. and $K'=2$ (mol./l.)$^{-1}$ for caffeine. $(\Delta S-\boldsymbol{R}\ln 0.5\,a)/N$ was assumed to be 32 e.u. for both cases.

been made to estimate the α value for the case of caffeine plus poly A and also for the case of purine plus poly A. In both of these cases, $(\Delta S-\boldsymbol{R}\ln 0.5\,a)/N$ for poly A is assumed to be 32 e.u., which is equal to the value of $(\Delta S-\boldsymbol{R}\ln 0.5\,a)/n$ of poly A given in the last paragraph of section 2. Because the hydrophobic forces are considered to play a predominant role in the interaction of caffeine (or purine) with random-coil poly A,[3] the enthalpy of binding $\Delta E'$ for the present cases would be small. Therefore, K' value for caffeine plus

21) P. O. P. Ts'o and P. Lu, *Proc. Nat. Acad. Sci. (U. S. A.)*, **51**, 17 (1964). Their nK corresponds to K' in this paper.

poly A is assumed to be 5.8 $(\text{mol./l.})^{-1}$ not only at 5°C but also in the vicinity of T_m (40～60°C). The data given in Fig. 13 suggest that the K' value is slightly lower for the case of purine plus poly A than that for the case of caffeine plus poly A; it is assumed to be 2 $(\text{mol./l.})^{-1}$ for the former case. Therefore, the α value is calculated to be −1.2 kcal./mol. for the case of caffeine plus poly A and −1.3 kcal./mol. for the case of purine plus poly A. By these values of α, the date given in Fig. 13 can be well explained (see the theoretical curves in the figure). Thus, it may be concluded that the binding of one caffeine or purine molecule to the random-coil poly A casuses a lowering of the ΔE (enthalpy of melting) by 1.2～1.3 kcal./mol.

Summary

The mass action law has been applied to the "melting" process of nucleic acids and also to the binding process of small ions or molecules. On the basis of previously-obtained data on the variation of the melting temperature of nucleic acids caused by interaction with small ions and molecules, some of the enthalpy, entropy, and free energy changes involved in the processes have been estimated. A model has been proposed of how the spermine molecule may interact with the deoxyribonucleic acid molecule to stabilize the double-helical conformation of the latter molecule.

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