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The Nucleic Acid–Hydroxylapatite Interaction. I. Stabilization of Native Double-Stranded Deoxyribonucleic Acid by Hydroxylapatite†

Harold G. Martinson‡

ABSTRACT: The denaturation temperature (T_{mi}) of DNA can be markedly elevated by adsorption of the DNA to hydroxylapatite. This increase is always correlated with factors which stabilize the DNA–hydroxylapatite absorption interaction. For example, the hydroxylapatite T_{mi} *increases* linearly with *decreases* in the logarithm of the phosphate buffer concentration. Other factors which affect T_{mi} are changes of the hydroxylapatite, in the cation of the buffer or in the adsorption mode of the DNA. It is concluded that hydroxylapatite stabilizes the DNA double helix by interfering with the unwinding process required by denaturation.

Hydroxylapatite is widely used in the analysis of nucleic acids (see Kohne and Britten, 1971, and Bernardi, 1971). However, during the course of the present author's work which involved the analysis of RNA–DNA hybridization mixtures, the hydroxylapatite reacted in what at first appeared to be an unpredictable manner as experimental conditions were varied and different nucleic acids were used. This prompted a detailed analysis of the nucleic acid–hydroxylapatite interaction. It was found that the system is considerably more complex than is generally recognized. In this paper, the first of a series, the effect of hydroxylapatite on the thermal stability of ds-DNA is explored.

An increase in the thermal stability of ds-DNA when adsorbed to hydroxylapatite was first observed by Miyazawa and Thomas (1965). They ascribed the effect to an increase in the ionic strength of the buffer near the hydroxylapatite surface. However, the data presented below show that it is the physical immobilization in the adsorbed state which stabilizes the DNA and that electrostatic effects are not significantly involved. Thus as the concentration of phosphate is decreased the DNA becomes more firmly bound to the hydroxylapatite and the thermal stability of the DNA increases despite the reduction in ionic strength of the buffer.

Materials and Methods

Hydroxylapatite. Most of the results were obtained with the dried material "DNA-grade HTP" which was kindly supplied by Bio-Rad, Richmond, Calif. A few experiments were done

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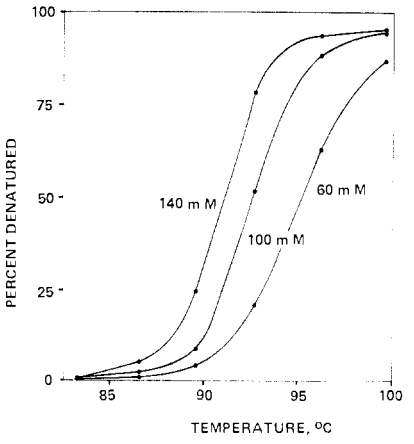

FIGURE 1: The effect of phosphate concentration on the thermal stability of adsorbed DNA. Columns containing 20 mg of hydroxylapatite each were loaded with 0.1 μg of *B. subtilis* [^{3}H]DNA, washed with 0.21 M sodium phosphate, and rinsed with 0.01 M sodium phosphate at 35°. (HA)T_{mi} determinations were then carried out by method B as described in Materials and Methods.

with their original "HTP." The general nature of the results to be described is similar for all types of hydroxylapatite which have been tested.

Buffers. Phosphate buffers were diluted from stocks prepared by dissolving equimolar quantities of the mono- and dihydrogen phosphates in water.

Temperature Control. Water-jacketed columns were used. Water was circulated by a thermostatically controlled Haake circulating pump. By coupling a Polyscience HR30 refrigeration unit to the pump any temperature between 0 and 100° could be maintained.

Column Preparation. Dry "DNA grade HTP" was suspended in water and added to a 5-mm i.d. column partially filled with water. When the bulk of the hydroxylapatite had settled the fines were withdrawn. Slight air pressure was used to compress and stabilize the bed as well as for elution. The columns were usually prepared shortly before using and were not reused.

DNA. For ease of handling, the DNA preparations were degraded by sonic irradiation at 0° in a monovalent cation concentration greater than 0.1 M. The Biosonik Sonifier needle probe was used at a power setting of 60. Sonication times were 1–4 min depending on the volume involved (0.6–6 ml).

Bacillus subtilis strain MY2Y1U5 (a gift of Dr. Yoshikawa), which was auxotrophic for thymidine, leucine, and tryptophan, was grown in a minimal medium supplemented with 50 μg/ml each of leucine and tryptophan and 1 μg/ml of [*methyl*-^{3}H]thymidine (3 Ci/mmol, Schwarz BioResearch) for several generations. The bacteria were collected by centrifugation and then treated with lysozyme (Calbiochem, A grade) at 2 mg/ml in 0.15 M NaCl–0.1 M EDTA (pH 8.0) at 37° for 20 min. Sodium dodecyl sulfate was then added and the DNA was isolated as described by Church and McCarthy (1968) for mouse liver nuclei. The DNA was stored at 100 μg/ml in 20% ethanol at 4° for 10 months. Shortly before use it was sonicated and chromatographed on DNA grade hydroxylapatite at 60° in sodium phosphate buffer. Material eluting between 0.20 and 0.26 M phosphate was pooled, stored at 4°, and used during the following 2 months. The concentration was 8 μg/ml and the specific activity was 17,000 cpm/μg. The bacteriophage T4 and *Escherichia coli* [^{14}C]DNAs were gifts of Dr. R. Burger. As a final purification step the T4 DNA was, after sonication, loaded on to hydroxylapatite and washed extensively with 0.16 M sodium phosphate at 70°. The DNA was then eluted with 0.3 M sodium phosphate and stored at −20°. The specific activity was 3700 cpm/μg. The *E. coli* DNA was similarly purified but at room temperature. The specific activity was 3000 cpm/μg. *Bacillus megaterium* and *Micrococcus lysodeikticus* were grown in nutrient broth and the DNA was isolated similarly to that for *B. subtilis*.

Scintillation Counting. Intertechnique and Beckman scintillation counters were used. In most cases aqueous samples were assayed in a dioxane based scintillation fluid. Where necessary the extent of quenching was carefully controlled and corrected for.

Determination of (HA)T_{mi}.[1] Two methods were used. The first, thermal elution chromatography, was used essentially as described by Miyazawa and Thomas (1965) and is designated method A. It consists of increasing the column temperature stepwise while eluting with a fixed concentration of phosphate buffer. As the DNA becomes thermally denatured it elutes owing to the reduced affinity of the resulting single strands for the hydroxylapatite. Method B was somewhat different as follows. Columns containing hydroxylapatite loaded with DNA were rinsed with several bed volumes of the incubation buffer at 35°. The temperature was raised to the first step, maintained for 3 min and then lowered once again to 35°. The columns were then assayed for the presence of ss-DNA by selectively eluting the single strands with several bed volumes of 0.20 M sodium phosphate at 35°. Control experiments show that no ds-DNA is eluted under these conditions. The cycle was then repeated (rinsing with incubation buffer, etc.) for each in the series of increasing temperatures. At the end of the series any undenatured DNA was removed from the column with a high phosphate elution.

Method A is more rapid than method B because the conditions of assay are the same as the conditions of denaturation. However if denaturation conditions are chosen which are not appropriate for simultaneous assay then method B must be used (see Martinson, 1973). In the range of conditions where both methods are applicable, they yield essentially the same results.

Results

The thermal stability of ds-DNA normally increases as the cation concentration is increased (see, for example, Gruenwedel and Hsu, 1969). However with DNA adsorbed on hydroxylapatite the reverse effect can be observed. Thus as illustrated in Figure 1 the denaturation temperature of *B. subtilis* DNA in the adsorbed state [(HA)T_{mi}] decreases from 95° to less than 91° as the phosphate concentration is increased from 60 to 140 mM. In Figure 2 this anomalous behavior of adsorbed DNA [(HA)T_{mi} line) is contrasted with the usual dependence of T_{mi} on salt concentration exhibited by DNA when in solution (solution T_{mi} line).

In order to determine whether the reverse salt concentration dependence of the (HA)T_{mi} is attributable to a specific buffer ion, (HA)T_{mi} determinations were made in which the

[1] Abbreviations used are: T_{mi} (see Crothers *et al.*, 1965), the temperature by which 50% "irreversible" strand separation has occurred; (HA)T_{mi}, the T_{mi} determined in the presence of hydroxylapatite, the extent of strand separation being assayed under any appropriate conditions; ds-DNA, double-stranded DNA; ss-DNA, single-stranded DNA.

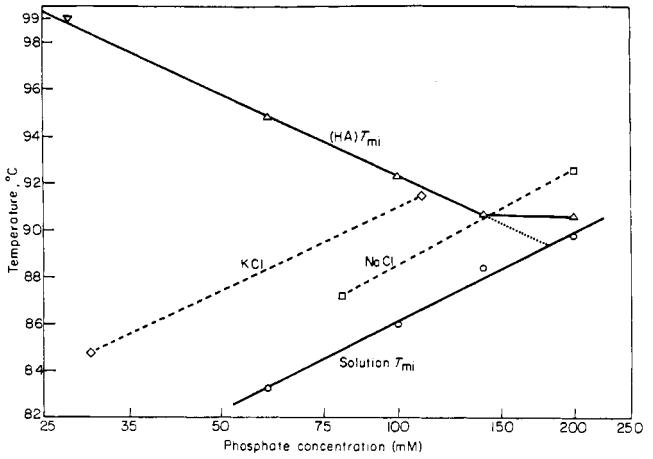

FIGURE 2: The effect of various conditions on the thermal stability of DNA. (HA)T_{mi} (Δ), the 60, 100, and 140 mM points are taken from the data of Figure 1. The 200 mM point was determined at the same time and in the same way. (▽) Three columns were loaded with *B. subtilis* DNA as for Figure 1, washed with 0.20 M sodium phosphate at 35° and then rinsed with several bed volumes of the incubation buffer (0.02 M for one column and 0.03 M for the other two). The (HA)T_{mi} was determined by method B with a single temperature step at 99° and the phosphate molarity at which 99° was the (HA)T_{mi} was found by interpolation. For solution T_{mi} (○), *B. subtilis* [^{3}H]DNA was diluted with various molarities of sodium phosphate to give a DNA concentration of 0.3 μg/ml. One sample at each phosphate concentration was then incubated at each of a series of increasing temperatures (about 4° increments) beginning with 75°. After 3 min at each step 0.1 ml was withdrawn from each sample and diluted to a phosphate concentration of 0.04 M. The extent of denaturation was determined by adsorbing each aliquot to hydroxylapatite (20 mg) at 35° and eluting the denatured and undenatured fractions of DNA with 0.21 and 0.30 M phosphate, respectively. Overlap between the denatured and undenatured fractions was corrected for by reference to the results of parallel assays employing ds- and ss-DNA mixtures of known proportions. For NaCl (□), (HA)T_{mi} values were obtained for bacteriophage T4[^{14}C]DNA by method A in 0.08 M sodium phosphate and also in 0.08 M phosphate supplemented with NaCl to a concentration of 0.18 M. The total sodium ion concentration of the mixture is equivalent to that of 0.20 M phosphate and is plotted as such in the figure. DNA (4 μg) was loaded on 20 mg of hydroxylapatite. Two-degree steps starting at 75° were used. Each step was eluted with 0.5 ml of buffer. For KCl (◇), (HA)T_{mi} values were obtained for a mixture of *B. megaterium* DNA (60%) and *M. lysodeikticus* DNA (40%) by method A in 0.03 M potassium phosphate and also in the same buffer which was supplemented to 0.12 M in KCl. The total potassium ion concentration of the buffer mixture is equivalent to that of 0.11 M phosphate and is plotted as such in the figure. The DNA mixture (85 μg) was loaded on each of two 7-mm diameter columns containing 70 mg of non-DNA grade HTP which had been previously equilibrated with phosphate buffer at 100°. Three degree steps starting at about 60° were used. Each step was eluted with 0.3 ml.

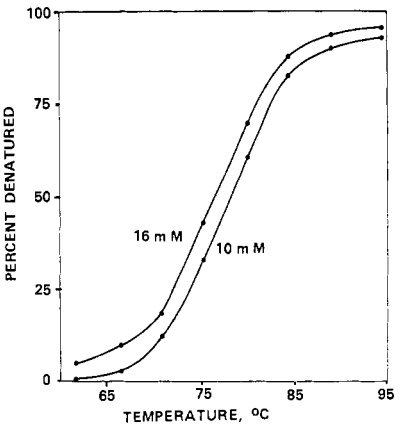

FIGURE 3: Thermal denaturation of adsorbed DNA in cesium phosphate. Columns containing 20 mg of hydroxylapatite each were loaded with 0.05 μg of *B. subtilis* [^{3}H]DNA, washed with 30 mM cesium phosphate, and rinsed with the incubation buffer at 16°. (HA)T_{mi} determinations were then carried out by method A as described in Materials and Methods.

phosphate concentration was kept constant but the cation concentration was increased by the addition of sodium or potassium chloride. The KCl and NaCl lines[2] in Figure 1 show that supplementing the phosphate buffer with these salts causes the (HA)T_{mi} to increase with cation concentration in just the same way as the solution T_{mi}. Thus the thermal stability of both adsorbed DNA and DNA in solution is raised by an increase in cation concentration showing that the increase in (HA)T_{mi} which occurs when the concentration of phosphate buffer is reduced [Figure 2, (HA)T_{mi} line] is attributable to the decreasing concentration of phosphate anion.

Of the ions present in the buffers used (Na^+, K^+, Cl^-, and phosphate) only the phosphate anion has a significant effect on the strength of adsorption of DNA to hydroxylapatite (Bernardi, 1971). Therefore it appears that the (HA)T_{mi} may be sensitive to the strength of the DNA–hydroxylapatite interaction. Specifically it is proposed that it is the increase in adsorption strength accompanying the decrease in phosphate concentration which is responsible for the increase in (HA)T_{mi}. Evidence will now be presented in support of this proposal. It will be shown that three independent and distinct methods of varying the strength of the DNA adsorption to hydroxylapatite are all associated with the appropriate variations in thermal stability of the DNA. Thus (1) the DNA–hydroxylapatite interaction can be weakened and the (HA)T_{mi} decreased by increasing the eluting power of the buffer, (2) the interaction can be weakened and the (HA)T_{mi} decreased by decreasing the adsorbing ability of the hydroxylapatite, and (3) the interaction can be strengthened and the (HA)T_{mi} increased by stabilizing the DNA–hydroxylapatite adsorption mode.

Increasing the Eluting Power of the Buffer. Potassium phosphate has a greater eluting power than sodium phosphate (Bernardi, 1971; Martinson, 1973). This means that adsorbed DNA is bound less tightly to hydroxylapatite in the presence of potassium phosphate than in the presence of sodium phosphate at the same concentration. Furthermore if the strength of adsorption of DNA is correlated with the degree of (HA)T_{mi} increase then lower (HA)T_{mi} values should be obtained when potassium phosphate is used. This is actually found to be the case (H. G. Martinson, unpublished results, and see Figure 2, KCl line and footnote 2). Cesium phosphate is an even more effective eluent than the potassium salt (Martinson, 1973, and unpublished results) and it is found that the (HA)T_{mi} elevation effect is correspondingly even less with the cesium buffer. The denaturation profiles shown in Figure 3 give (HA)T_{mi} values of less than 80° for *B. subtilis* DNA in the cesium buffer compared with greater than 100° predicted for sodium phosphate at the same concentrations (Figure 2, (HA)T_{mi} line). Thus the reduced affinity of DNA for hydroxylapatite in the presence of potassium and cesium phosphate is correlated

[2] In order to allow working with lower more manageable temperatures, a DNA of lower thermal stability than *B. subtilis* DNA was used for the sodium phosphate plus NaCl experiment. The use of K^+ as the cation in the other experiment was for the same reason. The (HA)T_{mi} elevation effect is significantly less for K^+ than for Na^+.

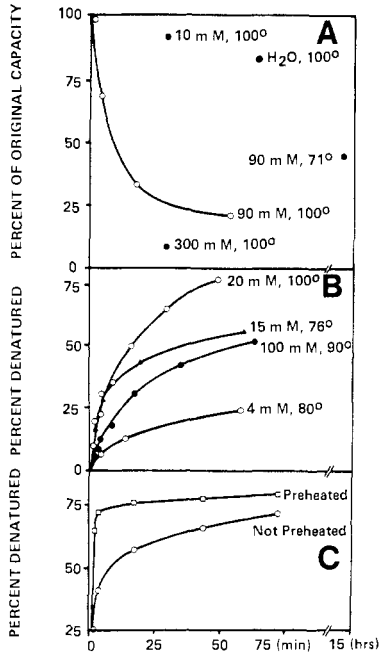

FIGURE 4: Effect of high temperature incubation on the sorptive capacity and DNA stabilizing potential of hydroxylapatite. (A) Several 72-mg portions of hydroxylapatite were incubated at 100° in the sodium phosphate concentrations and for the times indicated. The hydroxylapatite samples were then transferred to columns maintained at 71° and 0.4 μg of T4 [^{14}C]DNA was adsorbed to each sample and washed with 0.4 ml of 0.18 M followed by 0.6 ml of 0.27 M sodium phosphate (which eluted most of the DNA). The amount of DNA which remained bound to the hydroxylapatite was then determined and the DNA binding capacity in 0.27 M phosphate at 71° was calculated for each sample of hydroxylapatite as a percent of that of unincubated hydroxylapatite. (B) For all four curves the basic experiment was to adsorb DNA to hydroxylapatite, wash with an intermediate molarity of phosphate, rinse with the indicated incubation buffer, incubate at the indicated temperature, and assay for extent of denaturation as time progressed. *B. subtilis* [^{3}H]DNA and sodium phosphate (circles) or bacteriophage T4 DNA and cesium phosphate (triangles) were used. The open circle curves represent experiments in which several samples were incubated at the same temperature being withdrawn one by one as time progressed for assay of extent of denaturation in a manner similar to method B of the (HA)T_{mi} determination outlined in Materials and Methods. The solid symbol curves were generated by eluting the single strands with the incubation buffer at the various time points similar to method A of the (HA)T_{mi} determination. Additional details on the individual curves are as follows. For the 20 mM curve, 0.03 μg of DNA was adsorbed to each 20 mg of hydroxylapatite, washed with 0.21 M phosphate, then rinsed with incubation buffer, etc., as described above. For the 15 mM curve, 0.27 μg of DNA was adsorbed to 36 mg of hydroxylapatite and washed with 0.27 M phosphate. For the 100 mM curve, 0.1 μg of DNA was adsorbed to 36 mg of hydroxylapatite and washed with 0.22 M phosphate. For the 4 mM curve, 0.1 μg of DNA was adsorbed to 20 mg of hydroxylapatite, and washed with 0.21 phosphate. (C) Hydroxylapatite (30 mg) was heated in 15 mM cesium phosphate at 100° for 2 hr. T4 DNA was then loaded on this as well as an unpreheated hydroxylapatite sample and was washed with 40 mM cesium phosphate at room temperature. Because of the reduced capacity of "boiled" hydroxylapatite under these conditions less DNA "survived" the wash on the preheated hydroxylapatite, and the final loads were 0.3 and 0.6 μg for the preheated and unpreheated hydroxylapatite, respectively. The loaded hydroxylapatite was heated to 74° in 15 mM cesium phosphate and time points were taken by eluting the denatured DNA with this buffer.

with decreased thermal stability of the adsorbed DNA in these buffers compared to sodium phosphate.

Decreasing the Adsorbing Ability of the Hydroxylapatite. The affinity of hydroxylapatite for DNA at elevated temperatures can be decreased by preheating the hydroxylapatite in phosphate buffer. The curve in Figure 4A shows that the extent of affinity decrease becomes greater as the heating time increases. This curve describes the progressive decrease in capacity for DNA at high temperature brought about by preheating the hydroxylapatite for increasing lengths of time in 90 mM sodium phosphate at 100° (for special assay conditions see legend to Figure 4A). The solid data points in Figure 4A show that higher preheating temperatures and phosphate concentrations give rise to greater decreases in capacity of the hydroxylapatite for DNA. The effect of this progressive affinity decrease on the thermal stability of adsorbed DNA was observed by means of the following experiment.

DNA was adsorbed to hydroxylapatite, equilibrated with phosphate buffer, and the temperature rapidly raised to a value near the (HA)T_{mi}. The extent of denaturation of the DNA was measured at intervals. Figure 4B shows that denaturation of the DNA occurs over an extended period of time in corollation with the progressive decrease in binding affinity of the hydroxylapatite for DNA at the elevated temperatures.

The progressive denaturation of the DNA at elevated temperatures (Figure 4B) does not appear to be the result of slow DNA degradation. Thermal elution chromatograms of DNA which has been previously incubated on hydroxylapatite at high temperature (but not denatured) are indistinguishable from chromatograms of fresh DNA. Furthermore, preheating of the hydroxylapatite for the most part preempts the progressive melting of DNA (Figure 4C) and most of it denatures at once.

It might be pointed out that the rate of denaturation of DNA in the context of Figure 4 is critically dependent on many experimental factors including temperature, buffer concentration, chemical composition of DNA, native thermal stability of DNA, type of hydroxylapatite, method of column loading, and others. Furthermore, it follows that (HA)T_{mi} determinations are also sensitive to the above factors. For example, the use of preheated hydroxylapatite gives rise to reduced (HA)T_{mi} values. However since thermal chromatography itself constitutes a heat treatment of the hydroxylapatite the effect on (HA)T_{mi} depends, among other things, on the length of the experiment.

Stabilizing the DNA–Hydroxylapatite Adsorption Mode. Kawasaki (1970a,b) has discussed the evolution of binding modes during chromatography. When macromolecules are loaded on an adsorbant column under conditions which promote strong binding interactions at each adsorption site the macromolecules adsorb in a wide range of configurations many of which are in a relatively high state of free energy. During elution chromatography the macromolecules in the less stable adsorption modes are eluted first and, if the column is not already saturated, resorb in a more stable configuration further down the column where they remain until they are eventually reeluted as chromatography proceeds. It follows, therefore, that DNA loaded on hydroxylapatite at a low phosphate concentration will adsorb in a spectrum of less stable configurations than the same DNA loaded at a higher phosphate concentration. Furthermore, the DNA loaded at a high phosphate concentration should have a higher (HA)T_{mi} if increased stability of adsorption causes an increase in the T_{mi} of adsorbed DNA.

Figure 5 shows that adsorption mode does indeed have a dramatic effect on the thermal stability of DNA. The data were obtained by loading phage T4 DNA on several hydroxyl-

apatite columns and then washing them with successively higher molarities of sodium phosphate. The series of columns thus contained DNA adsorbed to hydroxylapatite in a progression of increasingly more stable adsorption modes. The columns were then all equilibrated with 0.07 M sodium phosphate and the temperature raised to 88°. Any denatured DNA produced was eluted with the 0.07 M phosphate at this temperature. Figure 5 shows that the DNA on the hydroxylapatite prewashed with 0.09 M phosphate was 88% denatured whereas the DNA in the 0.27 M prewashed column was virtually completely stable. After the 88° elution the temperature was raised to 93° and the columns were again eluted. It can be deduced from the two temperature steps that the 0.09 and 0.27 M prewashed samples had (HA)T_{mi} values of about 85 and 91.5°, respectively. Even greater differences in (HA)T_{mi} due to adsorption mode have been found in other experiments.

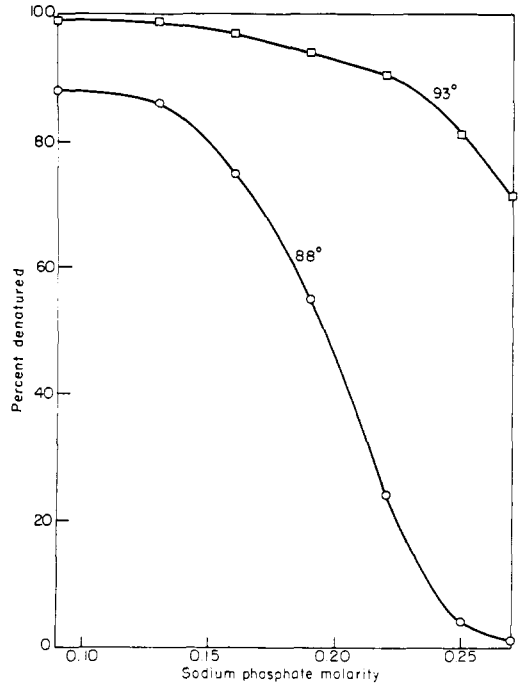

FIGURE 5: Effect of adsorption mode on thermal stability. T4 [^{14}C]-DNA (2 μg) was specially purified on 0.25 g of hydroxylapatite by washing with 0.27 M sodium phosphate. The DNA was then distributed to seven columns and each was washed with a different concentration of sodium phosphate at 40°. All columns were equilibrated with 0.07 M phosphate. A thermal elution of two steps (88 and 93°) and a terminal high phosphate wash were done. The total per cent of DNA denatured at 88 and 93° is plotted *vs.* the molarity of the 40° wash.

Discussion

The data which have been presented in the previous section show that the thermal stability of DNA is increased by adsorption to hydroxylapatite and that the stronger the adsorption, the greater the increase in thermal stability. Thus higher concentrations of phosphate, substitution of K^+ for Na^+ or Cs^+ for K^+, use of heat-pretreated hydroxylapatite and destabilization of the adsorption mode all reduce both the affinity of DNA for hydroxylapatite as well as the thermal stability of the DNA.

Because hydroxylapatite has a greater affinity for double-stranded than single-stranded molecules, it is perhaps not surprising that it has a stabilizing effect on nucleic acid helices (see Li, 1972, and Crothers, 1971). This phenomenon has been known for some time (Miyazawa and Thomas, 1965) but the mechanism has not been clear. The best known mechanism for stabilizing nucleic acid helices is shielding of the electrostatic repulsion of the backbone (see Felsenfeld and Miles, 1967). In the hydroxylapatite system there are three conceivable sources of electrostatic stabilization: (1) increase in salt concentration near the crystal surface, (2) competition of buffer cations with hydroxylapatite for the DNA, and (3) interactions at the adsorption sites themselves.

Localized Increases in Salt Concentration. Elevated salt concentration in the neighborhood of the hydroxylapatite crystals is ruled out as an important contributor to (HA)T_{mi} elevation by the fact that the stability of adsorbed DNA actually increases as the phosphate concentration (and therefore also the cation concentration) of the buffer decreases. The cation concentration in the neighborhood of the crystals is undoubtedly higher than that of the bulk solution (Pak and Skinner, 1968) but it cannot vary in magnitude inversely to solution concentration changes.

Cation Competition. The reverse dependency of the (HA)T_{mi} on sodium phosphate concentration (Figure 2) is at first reminiscent of the data of Dove and Davidson (1962) who showed that in the presence of Mg^{2+}, increases in the Na^+ concentration could lead to a decrease in the stability of DNA over a two log range as sodium competed more and more effectively for the Mg^{2+} binding sites on the DNA. However, an analogous cation competition with hydroxylapatite for the nucleic acid phosphates is ruled out by the fact that the order of effectiveness of the cations in promoting the hydroxylapatite-dependent increase in stability is $Na^+ > K^+ > Cs^+$. Since the affinity of these cations for the nucleic acid phosphates decreases in the same order (see Felsenfeld and Miles, 1967) Na^+, not Cs^+, or K^+, would be expected to compete most effectively with the hydroxylapatite (or calcium ions in its environs) for the DNA. It should also be pointed out that these cations differ little in their effect on DNA stability in solution (Leng and Michelson, 1968; Gruenwedel and Hsu, 1969).

Adsorption Site Effects. It has been shown in the previous section that increases in (HA)T_{mi} are in all cases correlated with increases in the strength of the DNA–hydroxylapatite interaction. It is reasonable to assume that the tighter the binding, the greater the extent of charge neutralization attributable directly to the adsorption interactions (either qualitatively from more effective interactions or quantitatively from a greater number of them). However owing to the geometry of the double helix (see Arnott, 1970), no more than 10% of the helix phosphates can be bound to hydroxylapatite at once and under normal conditions it is probably somewhat less than this. It therefore seems unlikely that this charge neutralization, no matter how effective on a per charge basis, can account for the remarkable stability of DNA adsorbed to hydroxylapatite. This intuitive argument is borne out experimentally. Some salts, such as chlorides, can be added to phosphate buffers in moderate amounts with little effect on the affinity of ds-DNA for hydroxylapatite (Bernardi, 1971). It is therefore possible to vary the cation concentrations of phosphate buffers with very little effect on the binding strength of DNA for hydroxylapatite. When this is done (Figure 2, KCl and NaCl lines, see footnote 2) it can be seen that the (HA)T_{mi} responds to changes in cation concentration with the same slope as solution T_{mi}. However if adsorption interactions themselves were contributing significantly to electrostatic stabilization of the helix, then increases in the cation

concentration of the solution would be a less effective elevator of (HA)T_{mi} than of solution T_{mi}. This conclusion follows from the well known semilog relationship (see Figure 2 in this paper or Schildkraut and Lifson, 1965, for example) which shows that increases in cation concentration become proportionately less effective as the double helix is electrostatically stabilized.[3] Thus the fact that adsorption of DNA to hydroxylapatite does not detectably modify the reaction of the DNA to changes in solution salt concentration demonstrates that the adsorption interactions do not themselves contribute significant additional electrostatic stabilization to DNA.

It is thus evident that the stabilization of DNA by hydroxylapatite is not primarily electrostatic in origin. An obvious alternative is that hydroxylapatite stabilizes DNA by physically preventing unwinding of the helix. This explanation is consistent with all of the data which have been presented. An increase in the affinity of DNA for hydroxylapatite is always associated with an increase in thermal stability of the DNA and the stronger the DNA–hydroxylapatite adsorption interaction, the more difficult unwinding of the double helix would be expected to become.

The kind of helix stabilization just proposed is quite different from the type of unwinding restriction which occurs with intact circular DNA (Vinograd *et al.*, 1968). The enhanced stability of circular DNA compared to linear DNA is due to an entropy effect resulting from restrictions on the independent movement of the entwined single strands of circular DNA after denaturation. However enhanced stability is observed on hydroxylapatite even under conditions which allow complete desorption of the denatured DNA (for example, Figure 5). Thus the stabilization by hydroxylapatite of double-stranded DNA is due to an effect exerted on the native double-stranded molecule and not to some hindrance of single-strand migration over the hydroxylapatite. Probably the form of helix stabilization which comes closest to the type produced by hydroxylapatite is that of DNA within formaldehyde-fixed chromatin (Li, 1972) but the latter stabilization is far less dramatic.

Although the stabilizing effect of hydroxylapatite is adequately visualized as being the result of hindered unwinding of the double helix, the physical–chemical nature of the phenomenon is not clear. Are the high (HA)T_{mi} values the result of true increases in thermodynamic stability or are they rather evidence of a potent kinetic barrier to denaturation? In the former case the visualization of hindered unwinding may not be precisely appropriate. In the latter case (HA)T_{mi} increases would represent not increases in actual stability but in the activation energy of unwinding.

It is difficult to distinguish between these two possibilities. For example, experiments of the type illustrated in Figure 4C all show residual progressive denaturation with time even after preheating of the hydroxylapatite up to 2 days. But while this is consistent with the kinetic as opposed to the thermodynamic explanation for the elevation of (HA)T_{mi}, it cannot be considered as strong evidence at this point for too little is known about what ongoing changes there may be in the hydroxylapatite surface at high temperature. Furthermore, evidence that the hydroxylapatite surface changes progressively with washing (Deitz *et al.*, 1964) suggests that the residual time-dependent denaturation which occurs despite preheating of the hydroxylapatite may be partly the result of surface changes in the hydroxylapatite caused by the repeated elution steps.

Another uncertainty which is not resolved by the data presented in this paper is whether variations in the number or variations in the strength of binding of sites determine the relative affinities of DNA under various conditions. It is difficult to predict *a priori* whether heat-treated hydroxylapatite has fewer adsorption sites or merely less effective ones. A similar question arises with regard to DNA in adsorption modes of varying stability. On the other hand, increases in phosphate concentration can be assumed to decrease the binding constants at all of the adsorption sites. Very likely both the strength and number of adsorption sites vary as conditions are changed.

In conclusion the following points can be made. The denaturation temperature of DNA is increased by adsorption to hydroxylapatite and the stronger the adsorption the higher the denaturation temperature. Electrostatic stabilization of the DNA is not significantly involved. Neither is the effect due to restricted migration of the single strands. Rather the hydroxylapatite apparently stabilizes the double helix by interfering with the unwinding process which necessarily accompanies denaturation. The increased denaturation temperatures could be the result either of increases in the actual thermodynamic stability of the helix or of increases in the activation energy required to simultaneously desorb and unwind the DNA.

Acknowledgments

The author is indebted to Professor E. B. Wagenaar in Lethbridge and to Professor C. A. Knight in Berkeley for support and encouragement. The author also thanks Dr. C. A. Knight and Dr. K. A. Drlica for criticism of the manuscript. Special appreciation is due to Dr. K. A. Drlica for bringing much of the early literature to the author's attention and for many critical discussions.

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[3] McConaughy and McCarthy (1972) have found that chromatin thermal stability is less sensitive to changes in salt concentration than is that of naked DNA. This is consistent with the above argument and the belief that histones stabilize the DNA, at least in part, by virtue of their electrostatic interactions. However, the reduced dependency of T_{mi} on salt concentration could be due instead to a progressive weakening of the histone–DNA bond as salt concentration increases.

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The Nucleic Acid–Hydroxylapatite Interaction. II. Phase Transitions in the Deoxyribonucleic Acid–Hydroxylapatite System†

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ABSTRACT: The technique of thermal elution chromatography on hydroxylapatite has been studied. It was found that at high temperatures the elution molarity of double-stranded DNA decreases sharply as the temperature is raised. This often results in double-stranded DNA being eluted without being denatured as thermal chromatography proceeds. In order to avoid this problem the eluting power of the buffer employed must be decreased. However, too large a decrease gives rise to the situation where the DNA denatures but does not elute. The cation of the buffer was found to increase the eluting power in the order $Mg^{2+} < Na^+ < K^+ < Cs^+$.

Hydroxylapatite is widely used in the preparation and analysis of proteins and nucleic acids (see Kohne and Britten, 1971, and Bernardi, 1971a,b). However relatively little has been published about the effects of temperature and type of cation on the nucleic acid–hydroxylapatite complex. Consequently it is generally assumed that the system is relatively insensitive to these experimental parameters.

Given the complementary strands of a nucleic acid and a quantity of hydroxylapatite, several combinations are possible. The native double-stranded nucleic acid will be designated SS, implying that each strand (S) is joined to its complement. The denatured nucleic acid is then S + S. Let hydroxylapatite be H. Then denatured nucleic acid in the presence of hydroxylapatite but not adsorbed can be designated S + S + H, or in the adsorbed state SHS. Native nucleic acid in the presence of hydroxylapatite but not adsorbed is then SS + H and in the adsorbed state SSH.

It is commonly believed that the SSH → SS + H transition is only brought about by changes in the buffer whereas the SSH → S + S + H transition occurs only upon raising the temperature. In the present communication the influence of buffer and temperature changes on the transition from SSH to each of the other three states (S + S + H, SHS and SS + H) is explored. It is shown that under appropriate conditions each of the three transitions can be brought about by increases in the eluting power of the buffer at constant temperature as well as by increases in the temperature using the same buffer. Some aspects of the mechanism involved are discussed.

Materials and Methods

DNA. All DNA preparations were those previously described (Martinson, 1973) except that the *Micrococcus lysodeikticus* DNA was further purified by collecting only material adsorbed to hydroxylapatite in 0.16 M sodium phosphate at 70°.

Hydroxylapatite. Unless otherwise indicated, "DNA-grade HTP" (kindly provided by Bio-Rad, Richmond, Calif.) was used. However the original Bio-Rad product "HTP" and the Clarkson Chemical Co. (Williamsport, Pa.) "Hypatite C" were occasionally used where indicated.

The scintillation counting, phosphate buffer preparation, temperature control, and column preparation have been described (Martinson, 1973). Thermal elution chromatography was conducted essentially as described by Miyazawa and Thomas (1965).

Phosphate Gradient Elution Chromatography. The sample was loaded on the column at a phosphate (and sulfate, citrate, EDTA, etc.) concentration that was sufficiently low for adsorption of the species desired. Unadsorbed material was washed through with low molarity phosphate or a Tris-Cl^- buffer near neutral pH. When many columns were being eluted simultaneously, it was most convenient to generate a gradient in small steps using a series of buffers prepared for this purpose. A volume of buffer equal to several times the bed volume of hydroxylapatite was applied to the column and the temperature allowed to equilibrate for at least a minute if other than room temperature was being used. The buffer was then pushed through the columns with air pressure at a rate which varied from column to column between 2 and 10 ml per min per cm^2. Flow rate in this range has only a minor effect on the results of a step elution.

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