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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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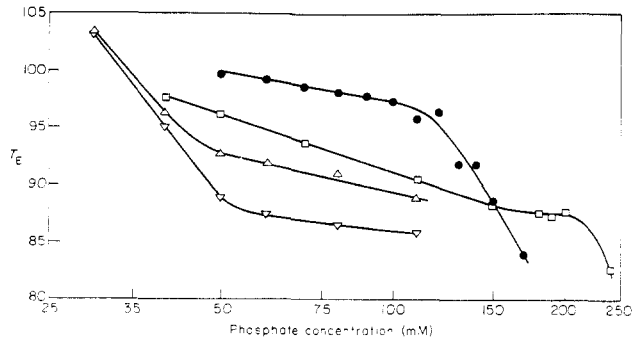

FIGURE 1: Thermal elution chromatography at various phosphate concentrations. (●) Each of 12 columns containing 20 mg of hydroxylapatite was loaded with 5 μg of *M. lysodeikticus* DNA, washed with 0.17 M, and then rinsed with 0.01 M sodium phosphate at 40°. T_E values were obtained for each of the phosphate concentrations indicated. Approximately 4° steps starting at 60° were used. Each step was eluted with 0.4 ml. (□) Each column of 35 mg of hydroxylapatite was loaded with 0.4 μg of T4 [^{14}C]DNA, washed with 0.26 M sodium phosphate at 40°, rinsed with 0.1 M NaCl, and then with incubation buffer. Thermal elution was conducted with 4° steps starting at 75°. The elution volume was 0.5 ml/step. The time elapsed per step was 10 min. (Δ) 35-mg columns of hydroxylapatite were loaded with 0.8 μg of T4 DNA each, washed with 0.27 M sodium phosphate at 40°, and then rinsed with water, 0.11 M phosphate, and incubation buffer in succession. Steps of 4° starting at 75° were used. Two elutions of 0.5 ml each were performed at each step. The hydroxylapatite bed was stirred during the second elution. Elution took about 20 min/step. (▽) As above except that after the rinse with water the columns were eluted with 0.4 M phosphate. The eluate was adjusted to between 0.11 and 0.12 M phosphate and the same columns (after having first been rinsed with water) were then reloaded with the DNA. The preceding procedure was then continued from the point where it had been interrupted.

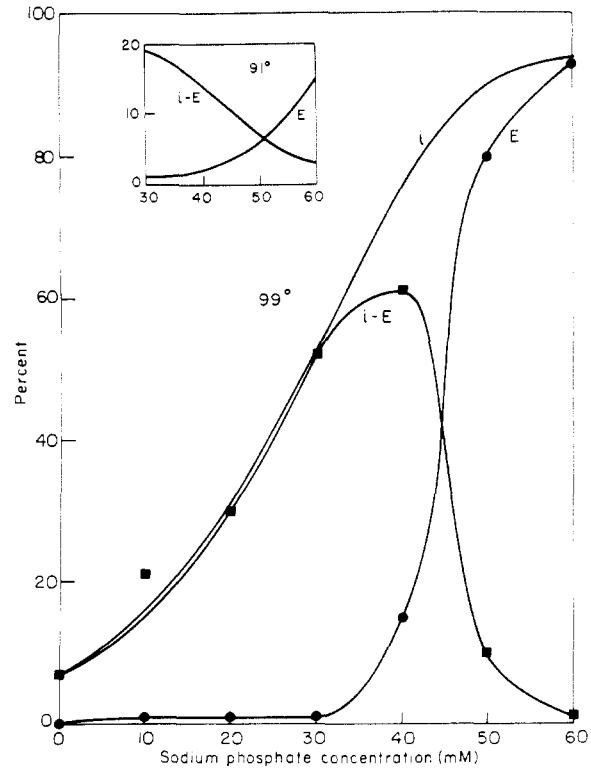

FIGURE 2: Relationship of T_E to (HA)T_{mi} at low phosphate concentrations. 20-mg columns of hydroxylapatite were each loaded with 0.1 μg of *B. subtilis* [^{3}H]DNA. The columns were washed with 0.2 M sodium phosphate at 35° and then with the incubation buffer to be used. One milliliter of the incubation buffer was put on each column and the temperature was raised to 91 or 99°. After 3 min the incubation buffers were passed through the columns and the temperature was returned to 35°. Any ss-DNA remaining on the columns was then eluted with 0.20 M phosphate and undenatured DNA was collected by means of a final high phosphate elution. Curve i represents the total per cent of DNA denatured by the high temperature, some of which was elutable at that temperature by the incubation buffer (curve E) and some not (curve i-E).

For analytical work, four small diameter columns were surrounded by a single water jacket. Eight such water jackets could be connected to a single Haake circulator by means of a distributor made for this purpose. In this way up to 32 separate samples could be analyzed simultaneously at the same temperature (within 0.2°). Thermister wells were included in each water jacket. However, for convenience, a small thermometer was usually used and temperature readings were precise to ±0.1°.

Results

When the thermal stability of DNA is determined in the presence of hydroxylapatite, it is found to increase linearly as the log of the phosphate concentration decreases (Martinson, 1973). The usual method of determining the thermal stability of DNA on hydroxylapatite [(HA)T_{mi}][1] is by means of a temperature gradient at constant phosphate concentration (thermal chromatography) as popularized by Miyazawa and Thomas (1965). However if the thermal elution midpoints (T_E) of DNA are determined over a wide range of phosphate concentrations, striking deviations from linearity are observed. Some representative experiments are illustrated in Figure 1. The pattern which emerges is that T_E is much more strongly dependent on phosphate concentration at high and low than at intermediate buffer molarities. It will be shown below that the nonlinear nature of these curves is the result of three different transitions which occur as the temperature is raised at the various phosphate concentrations. That is, T_E is often not equivalent to (HA)T_{mi}.

Low Phosphate Transition. The transition at low phosphate molarities was investigated by means of one-step thermal elution experiments on a series of samples, each of which was equilibrated with a different concentration of phosphate. The single elution step was at 99°. The percentage of DNA eluted in this step is plotted as a function of the molarity of the incubation buffer in Figure 2, curve E. After elution the columns were cooled and assayed for any ss-DNA present which could not be eluted at 99°. These data are plotted as curve i-E in Figure 2. The sum of curves E and i-E gives curve i which is the total percentage of each DNA sample irreversibly denatured at the various phosphate concentrations by the incubation at 99°. As expected (see Martinson, 1973), more DNA is denatured as the phosphate concentration is increased. However, virtually no ss-DNA is eluted at the lower phosphate concentrations (curve E). Only above 40 mM phosphate is a significant amount of the denatured DNA eluted (curve E) and only above 60 mM phosphate is the amount of DNA eluted equivalent to the amount of DNA denatured (curve i).

[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; T_E and M_E, the temperature and buffer molarity by which 50% of the nucleic acid has eluted during thermal and phosphate gradient chromatography, respectively; ds-DNA, double-stranded DNA; ss-DNA, single-stranded DNA.

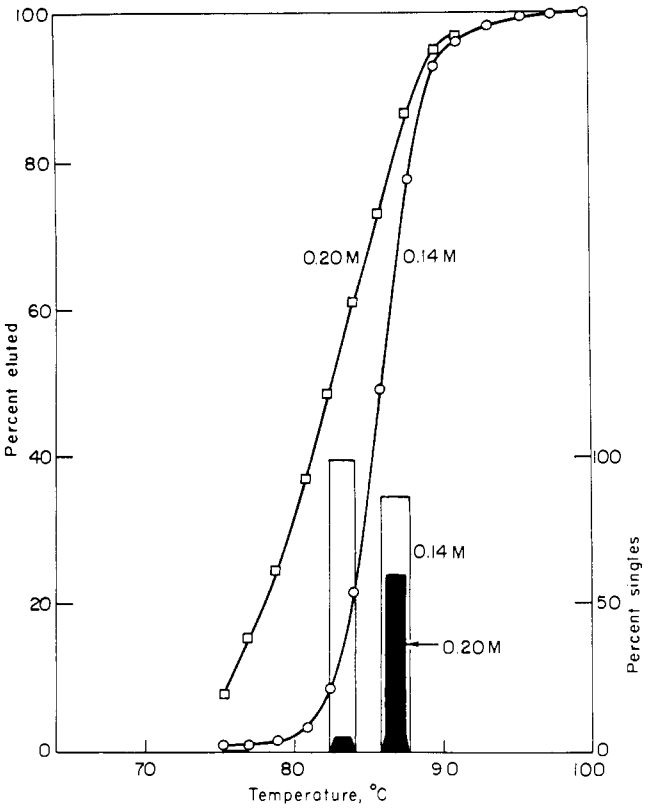

FIGURE 3: Extent of denaturation of DNA by thermal elution chromatography at different phosphate concentrations. T4 [^{14}C]DNA (4 μg) was loaded on columns of 20 mg of hydroxylapatite. Each step was eluted with 0.5 ml of buffer and the eluent was collected in 1 ml of water to rapidly cool and dilute the sample in order to retard renaturation of the DNA. The elution profiles of the DNA in 0.14 and 0.20 M sodium phosphate are shown in the figure. The eluates from selected temperature intervals were assayed for degree of denaturation. The results are displayed as open bars for the 0.14 M chromatograms (two samples) and solid bars for the 0.20 M chromatogram (three samples).

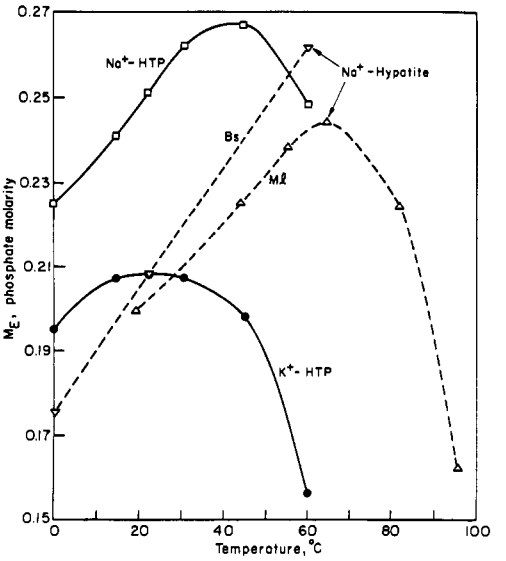

FIGURE 4: Temperature dependence of elution molarity. M_E was determined at various temperatures for different buffer–hydroxylapatite systems. For Na^+–HTP (□), non-DNA grade HTP (35 mg/column) was loaded with 0.5 μg of *B. subtilis* [^{3}H]DNA and eluted with sodium phosphate (0.8 ml/step) at a slope of 0.01 M phosphate/step. For K^+–HTP (●), as above except potassium phosphate was used. For Na^+–Hypatite: Bs (▽), as above with sodium phosphate except Hypatite C was used; M1 (△), a different batch of Hypatite C was loaded with 5 μg of *M. lysodeikticus* DNA and eluted with 0.5 ml/step of sodium phosphate at a slope of 0.01 M phosphate/step. Note the tenfold higher ratio of DNA to hydroxylapatite for this curve which doubtless has served to reduce the M_E values somewhat.

Similar results are obtained at 91° (see Figure 2 inset). The net result is the displacement of the elution curve E (corresponding to T_E) to higher phosphate concentrations than the denaturation curve i (corresponding to (HA)T_{mi}). While the exact phosphate concentrations will vary with the sample and the experiment, the results of Figure 2 afford a clear understanding of the low phosphate region of Figure 1. Thus for the region of shallow slope in Figure 1, T_E is the midpoint of the simultaneous denaturation–desorption transition and is equivalent to (HA)T_{mi}, but for the region of steep slope at low phosphate concentrations T_E corresponds only to the desorption transition of ss-DNA as the temperature is raised and is not equivalent to the (HA)T_{mi} transition which will have already occurred at a lower temperature. In this region of low buffer molarity (HA)T_{mi} must be determined by some other means, either experimentally (see Martinson, 1973) or by extrapolation.

Intermediate Phosphate Transition. The plateau region of Figure 1 corresponds to the range of phosphate concentrations for which thermal elution chromatography yields true (HA)T_{mi} values. That is T_E = (HA)T_{mi} in that range. The factors which affect (HA)T_{mi} values have been discussed previously (Martinson, 1973).

High Phosphate Transition. The transition occurring in the high phosphate region of Figure 1 was investigated by recovering the DNA eluted at various phosphate concentrations during thermal elution chromatography and assaying the DNA for extent of denaturation. The results in the case of two such analyses are presented in Figure 3. The bars in the figure represent the proportion of ss-DNA in the nucleic acid eluted over the designated intervals. It can be seen that the thermal elution conducted in 0.14 M sodium phosphate has produced nearly completely denatured DNA and is therefore a true denaturation profile. On the other hand, the thermal chromatogram in 0.2 M phosphate actually eluted primarily native DNA with the DNA being only partially denatured even at the highest temperatures.

The explanation for this is that temperature has a strong effect on the elution molarity (M_E) of DNA from hydroxylapatite (Figure 4). In particular Figure 4 shows that the M_E decreases sharply as the temperature is raised through the range in which thermal elution studies are generally performed. (The erroneous belief that temperature has little effect on M_E doubtless has arisen from studies in which a temperature on each side of the M_E maximum has been tested.) Consequently, as the thermal elution proceeds, if the M_E value drops below the molarity of the eluting buffer before the denaturation temperature of the DNA is reached, the native DNA elutes and T_E but not (HA)T_{mi} is obtained.

The interplay of temperature and phosphate molarity is depicted in Figure 5 as "phase" diagrams for *B. subtilis* DNA adsorbed to DNA–HTP in the presence of either sodium or cesium phosphate. Each diagram consists of four distinct regions. The boundaries refer specifically to the changes of state which occur when the coordinate values are increased giving rise to the SSH → SS + H, S + S + H or SHS transitions. Because renaturation of ds-DNA depends on system parameters in a complicated way (Wetmur and Davidson, 1968),

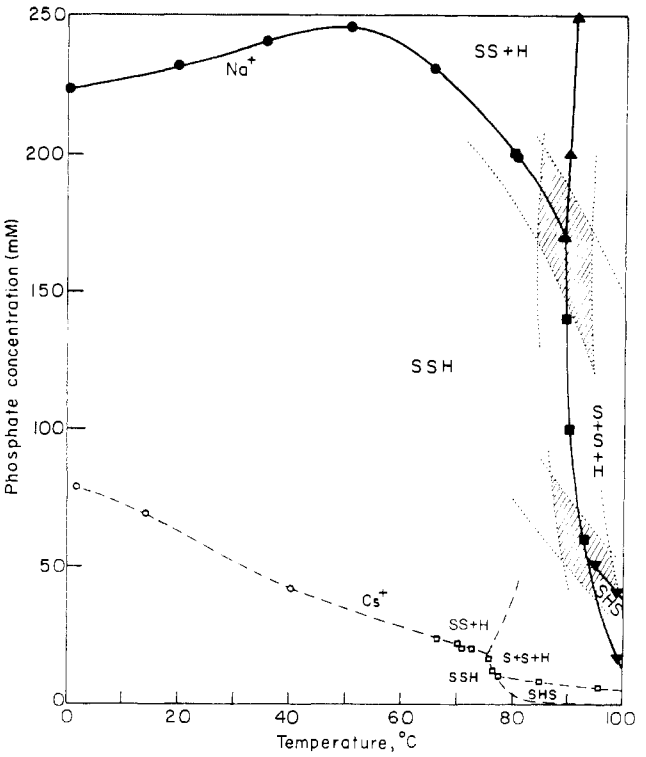

FIGURE 5: Phase transitions in the DNA–hydroxylapatite system. All determinations were made with *B. subtilis* [^{3}H]DNA and DNA grade HTP. Na^+ diagram: (●) temperature dependence of M_E was determined for 0.05 μg of DNA on 20 mg of hydroxylapatite. Elution was with 0.5 ml and at a slope of 0.01 M/step. (■) Thermal elution was performed on 0.3 μg of DNA adsorbed to 20 mg of hydroxylapatite and washed with 0.2 M phosphate. Starting at 70° each step of 2° was incubated 3 min and then eluted with 1 ml of buffer. (▼) Data similar to that of Figure 2 were obtained but longer incubation times were used in order to correspond to the thermal elution data. (▲) The data points were taken from the solution T_{mi} line in Figure 1 of Martinson (1973). For Cs^+ diagram (○), temperature dependence of M_E in cesium phosphate was determined in the same way as that for sodium phosphate above. (□) Thermal elution was performed for 0.05 μg of DNA adsorbed to 20 mg of hydroxylapatite, washed with 30 mM cesium phosphate at 16° and then rinsed with several bed volumes of the incubation buffer. Starting at 45° each step of 5° was incubated about 10 min and then eluted with 0.8 ml of buffer. A plot similar to that of Figure 1 was made and the regions corresponding to the various T_E transitions (*i.e.*, whether elution of doubles, singles, or actual denaturation) were identified by the discontinuities in slope (see text). Appropriate interpolations and extrapolations were then made in order to construct the Cs^+ phase diagram.

this process is considered irreversible in the present context. Thus only the transitions which do not involve DNA strand separation are considered reversible. It can be seen that the SSH and S + S + H regions share a common boundary in but a limited range of temperatures and phosphate concentrations. Only through this "window" can the simultaneous denaturation–desorption transition occur. However, the lines in Figure 5 represent only the midpoints of the transitions. The shaded areas represent, for merging transitions, the region of overlap due to transition breadth. This overlap serves to reduce the effective window in Figure 5 to between 0.08 and 0.14 M phosphate.

A very striking aspect of Figure 5 is the compression of the cesium phosphate phase diagram to low temperatures and phosphate molarities. The effect compared to Na^+ is much greater than that of K^+ (see Figure 4). Furthermore the M_E maximum for cesium phosphate (assuming one exists) is shifted down temperature from that of K^+ which is itself below that of Na^+. In an attempt to shed light on the mechanism for the marked effect of cations on the eluting power of buffers, experiments were done in order to fit other cations into the series. Limited results were obtained which are summarized below.

Lithium and magnesium phosphates are not sufficiently soluble to use as eluting buffers. Therefore *B. subtilis* DNA (13 μg) was eluted from hydroxylapatite (35 mg) with various sulfate salts (35°). The elution molarities for Cs^+, Na^+, and magnesium sulfates were 0.54, 0.74, and 1.30, respectively. Li_2SO_4 gave erratic results perhaps due to aggregation of the DNA. These results show that the Cs^+ salt is more effective than the Na^+ one in the case of both phosphate and sulfate. This suggests that the influence of the cations on relative elution effectiveness is independent of the buffer anion. (Potassium sulfate was not sufficiently soluble to test under the above conditions.) The complete cation sequence in order of decreasing elution power is then: Cs^+, K^+, Na^+, Mg^{2+}.

Stoll and Neuman (1956) have shown that monovalent cations interact with the hydroxylapatite surface and that Na^+ interacts more strongly than K^+. This suggests the possibility that the cations in the eluting buffers may be exerting their influence *via* direct interactions with the hydroxylapatite. To test this possibility two samples of hydroxylapatite (35 mg each) were incubated in 2 M cesium or sodium phosphate for 3 hr. Each was then loaded with 0.1 μg of *B. subtilis* [^{3}H]DNA and eluted at 25° with a phosphate buffer containing equal amounts of Cs^+ and Na^+. The M_E values were 0.19 and 0.17 for the Na^+ and Cs^+ preincubated hydroxylapatites, respectively. This result shows that Na^+ does indeed induce a change in the hydroxylapatite which is slightly different from that induced by Cs^+. Also illustrated is the fact that in an equimolar Cs^+ and Na^+ eluting buffer Na^+ predominates. This would be expected if Na^+ interacts more strongly than Cs^+ with the hydroxylapatite surface as implied by the results of Stoll and Neuman (1956). At 25° the average of the M_E values for sodium and cesium phosphates is 0.145 M (Figure 5). In contrast the equimolar Cs^+–Na^+ buffer gave an elution molarity significantly higher than this average. In a related experiment a series of 0.147 M phosphate buffers which varied in Cs^+:Na^+ ratio from 0 to ∞ was used to elute *B. subtilis* [^{3}H]DNA. In this case the DNA eluted with a midpoint at 67% Cs^+.

Discussion

The data presented in the previous section are summarized by the "phase" diagrams in Figure 5. Each of the two phase diagrams (Na^+ and Cs^+) consists of four regions. The boundaries refer to the midpoints of the transitions of SSH to each of the other three states (definition of terms given earlier). It can be seen that any of the three transitions can be induced by phosphate increases at appropriate temperatures or temperature increases at appropriate phosphate concentrations. Experimentally the trantition of most frequent analytical use is that from SSH to S + S + H. However, when transition breadth is considered, this transition can occur in a single step only when the temperature is raised in a narrow range of phosphate concentrations. Thus in the case of the sodium phosphate–DNA grade hydroxylapatite–*B. subtilis* DNA system at very low DNA load the effective "window" through which the transition can uniquely occur is only about 0.08–0.14 M phosphate. The upper shaded area in

Figure 5 is an estimate of the overlap of the SSH → SS + H and SS → S + S transitions. Analysis of this region shows that at phosphate concentrations around 0.15 M only the DNA eluted at the beginning of the thermal chromatography is completely denatured. However at still higher phosphate levels the situation is reversed and the DNA eluted at the beginning is not denatured at all. These relationships are illustrated in Figure 3 for the case of T4 DNA.

It must be emphasized that the phase diagrams of Figure 5 are strictly individual examples presented for illustrative purposes. Figure 1 shows that significant variation in the position and extent of the window can occur depending on the type of nucleic acid and the experimental method. For example, the phage T4 DNA curves (open symbols) of Figure 1 show that the slope and temperature level of the window region are both increased by more rapid chromatography (hence less temperature induced change occurs in the hydroxylapatite, see Martinson, 1973) or by washing the adsorbed DNA with higher concentrations of phosphate prior to chromatography (hence DNA is more stably adsorbed, Martinson, 1973). Also the glucosylated T4 DNA, having greater affinity for hydroxylapatite than non-glucosylated DNA (Bernardi, 1971a), has a window which extends to higher phosphate molarities than that of *M. lysodeikticus* DNA (Figure 1, solid symbols). However, many different systems have been investigated along the lines presented in this report and Figure 5 adequately summarizes for any given system and mode of chromatography the various transitions which occur, their relationships to each other and the effects that temperature and salt exert on them.

Effect of Temperature on M_E. Perhaps the most significant trend illustrated in Figure 5 is the sharp decrease in M_E at high temperatures. This could conceivably be due to temperature-dependent physical changes either in the DNA or the hydroxylapatite but these possibilities seem unlikely. In the first place Figures 4 and 5 show that the variation in M_E is considerable. However the rather extensive literature which has been published on DNA structure shows that one would expect the variations produced in solution by changes in alkali cation or temperature in the ranges used here to be small (see, for example, Bram, 1971; Arnott, 1970; Tunis and Hearst, 1968; Upholt *et al.*, 1971; Bode and MacHattie, 1968). Furthermore Figures 4 and 5 show that pronounced M_E maxima occur of which the positions are strongly dependent on the type of cation used. There is no evidence of similar trends in ds-DNA structure (Luzzati *et al.*, 1964; Studdert *et al.*, 1972; Gennis and Cantor, 1972). Finally, ds-RNA, the M_E of which also varies strongly with temperature (H. G. Martinson, unpublished data), has not been found to exhibit any temperature-dependent structural variation (Samejima *et al.*, 1968).

It appears equally unlikely that structural changes in the hydroxylapatite could be responsible for the variation of M_E with temperature. The M_E values depicted in Figures 4 and 5 can be varied at will along the various curves by merely adjusting the temperature, no equilibration time being necessary. In contrast, changes in the hydroxylapatite known to be induced by heating occur slowly (Martinson, 1973) and persist at least for days (H. G. Martinson, unpublished data).[2] The variations of M_E with temperature cannot, therefore, be adequately accounted for by changes in either the hydroxylapatite or the DNA.

Most likely the influence of temperature on M_E reflects the type of variation in binding equilibrium which is characteristic of ion association reactions in general. The occurrence of maxima and minima in such reactions is not at all uncommon (see Nancollas, 1966, for example). In the case of the DNA–hydroxylapatite interaction, many simultaneous association and dissociation reactions occur. However, an obvious one, ionization of the buffer ($H_2PO_4^- \rightleftharpoons HPO_4^{2-} + H^+$), is not strongly temperature dependent (Harvey and Porter, 1963) and, having a maximum, actually varies inversely to that necessary to explain the observed M_E maxima. Another possibly enlightening reaction is the binding of Mg^{2+} to double-helical poly(adenylate)–poly(uridylate) which has a minimum between 10 and 40° (Krakauer, 1971) but evaluation of this depends on whether the interaction is considered as a model for association of the counterions or the hydroxylapatite with the DNA. Interestingly, the component of the hydroxylapatite system which appears to have the greatest effect on the position of the M_E maximum is the buffer cation which is often not recognized as even being involved during nucleic acid adsorption. However, since the cation itself can interact with most of the other components of the system its contribution is no better understood than the rest. Consequently at this time it is not clear which association reactions contribute most significantly to the temperaure dependence of M_E.

The complexity of the situation is underscored by the fact that different species of double-stranded nucleic acid, different preparations of hydroxylapatite, or just heat pretreatment of the hydroxylapatite all affect the temperature dependence of M_E (Figure 4 and unpublished data). Furthermore, using hydroxylapatite which has been pretreated at about 100° in phosphate buffer shifts the M_E maximum to lower temperatures compared to untreated hydroxylapatite no matter what preparation of hydroxylapatite or what buffer is used (H. G. Martinson, unpublished data). This explains the paradoxical practices of boiling hydroxylapatite to *reduce* its affinity for ss-DNA, on the one hand (Kohne and Britten, 1971), and to *increase* its capacity for DNA in preparative procedures, on the other (R. Wood, Bio-Rad, personal communication). Since boiling shifts the M_E maximum to lower temperatures, the affinity for DNA at low temperatures is increased while the affinity at elevated temperatures is decreased. Because Kohne and Britten (1971) were involved in high-temperature experiments whereas preparative procedures are usually done at room temperature, the effects of boiling were originally observed from opposite sides of the M_E maximum.

It is not clear what changes in the hydroxylapatite surface lead to the shifts in M_E maximum. McConaughy and McCarthy (1972) have observed that the (HA)T_{mi} values of both DNA and chromatin are increased by the substitution of Clarkson Hypatite for Bio-Rad HTP. In the case of DNA this can be ascribed to the shift in M_E maximum to higher temperatures for Hypatite (Figure 4) with the result that Hypatite has greater affinity than HTP for the DNA at thermal elution temperatures (see Martinson, 1973). In the case of proteins the primary adsorbing groups are the carboxyls (Bernardi, 1971b) and these must be distributed over chromatin in quite a different way than the phosphates are on DNA. The fact that both DNA and chromatin respond similarly to the change in hydroxylapatite therefore implies that the crystal surface variatons are relatively nonspecific and are not related in a definite way to the geometry of DNA.

[2] Unless preheated hydroxylapatite is used, small long-lasting changes in the hydroxylapatite do occur during chromatography at the higher temperatures. However these effects are distinct from and usually very much smaller than those exhibited in Figures 4 and 5.

Effect of Cation on M_E. The importance of the cations in determining the position of the M_E maximum has already been pointed out. However even more important is the effect the cations have on the average M_E itself. The fact that the order of effectiveness of the cations in eluting DNA is independent of the anion again shows that the cations play a significant role in the DNA–hydroxylapatite interaction. It seems unlikely that the cations exert their effect by depressing the ionization of the buffer anions as suggested by Bernardi *et al.* (1968) because the strongest association reaction in solution is the opposing one of the cations with the DNA, which would more than compensate for any effect on the buffer. The strength of interaction with the DNA phosphates increases in the order $Cs^+ < K^+ < Na^+ < Mg^{2+}$ (Felsenfeld and Miles, 1967) which is the reverse of the order of their effectiveness as eluents for DNA from hydroxylapatite. This is in contrast to chromatography on methylated albumin kieselguhr in which case increased shielding of the DNA phosphates by the cations *is* correlated with a decrease in the affinity of the DNA for the methylated albumin (Lichtenstein and Shapot, 1971). This suggests that the difference lies with the hydroxylapatite itself. However the data of Stoll and Neuman (1956) and Pak and Bartter (1967) on the interactions of various cations with the hydroxylapatite surface apparently lead to the sequence Ca^{2+}, Cs^+, K^+, Na^+, which does not correspond to that found for elution of DNA. Other sequences involving cation properties such as extent of hydration *are* correlated with the elution sequence for hydroxylapatite but are difficult to interpret in view of the reverse elution sequence found for methylated albumin kieselguhr. Therefore, because the interaction of the hydroxylapatite surface with the solvent is extremely complex (see also Rootare *et al.*, 1962, and Deitz *et al.*, 1964) it seems likely that the role of the cations must eventually be understood in relation to their direct involvement with the hydroxylapatite. This involvement, as with temperature, can apparently be of two types. Since pretreatment of the hydroxylapatite with different cations can influence a subsequent M_E determination it appears that cation mediated structural changes in the hydroxylapatite surface have some influence. However, as with temperature, the major effect of cations is apparently their involvement directly in some aspect of the adsorption interaction itself.

A detailed discussion of the experimental ramifications of the findings which have been reported above will be deferred to a later publication. However, some salient points bear emphasis. Chromatography on hydroxylapatite is considerably more complex than is generally recognized. Changes in both cation and hydroxylapatite can severely depress the adsorption affinity of DNA. These factors take on greatest significance when thermal elution studies are performed. Because the affinity of ds-DNA for hydroxylapatite decreases as the temperature is raised, the introduction of any element which further decreases the M_E of a nucleic acid increases the risk, already inherent in the system, that thermal chromatography may not produce true denaturation transitions. For example, the DNA:hydroxylapatite load ratio is important in this regard because the M_E decreases as the load ratio increases. The result is to give a phase diagram with a considerably narrower effective "window." In addition imperfect duplexes, DNA–RNA hybrids, ds-RNA and, to a lesser extent, high G + C DNA all have relatively low affinities for hydroxylapatite and therefore also give phase diagrams with narrow windows. Because of these factors and others it is often advisable to determine the extent of the window for each type of nucleic acid and set of conditions to be used when thermal stabilities are to be determined by thermal elution chromatography.

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