The Basis of Fractionation of Single-Stranded Nucleic Acids on Hydroxylapatite†

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ABSTRACT: Various single-stranded nucleic acids were chromatographed on hydroxylapatite under a range of conditions. By varying the temperature and cation concentration it was possible to vary the structures of the nucleic acids. It was found that increases in the structural order of single strands, whether resulting from base pairing or base stacking, were always correlated with increases in the affinity of the nucleic acids for hydroxylapatite. This suggests that the conformational entropy of the macromolecules plays an important role during adsorption. An improved method of treating salt gradient elution chromatography data is presented. It is shown that the resolution of two components is often best expressed as a log (M_E ratio) which is the logarithm of the ratio of their elution molarities.

Hydroxylapatite has gained widespread use in the study of nucleic acids (for a review, see Bernardi, 1971). Its most popular analytic application is the separation of ss- and ds-DNA[1] (Kohne and Britten, 1971; Martinson, 1973b). This technique is based on the fact that denatured nucleic acids have less affinity for hydroxylapatite than their double-stranded counterparts. Hydroxylapatite can also be used to distinguish between various types of single-stranded molecules. However there is some disagreement as to whether single-stranded length (Burness and Vizoso, 1961) or the extent of single-stranded structure (Bernardi, 1971) is the basis of fractionation and whether temperature does (McCallum and Walker, 1967) or does not (Bernardi, 1971) affect the degree of fractionation. These uncertainties prompted a series of experiments to reinvestigate the mechanism underlying the fractionation of single-stranded nucleic acids on hydroxylapatite. In the approach chosen, the effect of temperature-mediated structural changes on the chromatography of single strands is studied utilizing a method of data treatment which yields an improved estimate of the true extent of chromatographic displacement on hydroxylapatite. It is found that increases in both length and the degree of structural order in the single strands give rise to greater affinity for hydroxylapatite. The structural effect was studied in some detail and it was determined that both base stacking and base pairing interactions within the strands increase their strength of binding to hydroxylapatite. The mechanism appears to be the same as that involved in fractionation of native from denatured DNA and is apparently related to the conformational entropy of the molecules.

Materials and Methods

Most procedures have been previously described (Martinson, 1973b). *Micrococcus lysodeikticus* DNA was prepared as before (Martinson, 1973a). The TMV and TYMV RNAs (Table I) were gifts of Dr. C. A. Knight and had been prepared at room temperature by the procedure of Gierer and Schramm (1956). One TMV RNA preparation (sTMV) was sonicated in distilled water at 90° for 5 min. Additional information concerning the nucleic acids used is contained in Table I. Bio-Rad "HT" hydroxylapatite and potassium phosphate buffer were used throughout. Phosphate gradient elution was carried out in small steps either as previously described (Martinson, 1973b) or by withdrawing from a buffer reservoir a fixed volume with which to elute the columns and replenishing the reservoir with an equal volume of a more concentrated buffer.

Results

The extent to which single-stranded structure affects the chromatography of polynucleotides on hydroxylapatite was studied by chromatographing various single-stranded nucleic acids (Table I) over a wide range of temperatures. In this way the configurations of the nucleic acids were varied from nearly random at high temperature to much more ordered at reduced temperatures. (For reviews on nucleic acid structure, see Felsenfeld and Miles, 1967, and Yang and Samejima, 1969.)

In the first experiment, a mixture of denatured *B. subtilis* [^{14}C]DNA and denatured *Pseudomonas caryophylli* DNA (Table I) was chromatographed at the highest convenient temperature, 99°. As shown by the pair of overlapping peaks on the left side of Figure 1A, *Bacillus subtilis* and *Ps. caryophylli* DNAs chromatograph quite similarly at 99°, eluting in a very narrow range of phosphate concentrations. To investigate the effect of increased structural order on the chromatographic behaviors of the ss-DNAs, a portion of the peak fraction of the 99° chromatogram was rechromatographed at 45° (Figure 1A, 45° profiles). It can be seen that a large increase in elution molarity (M_E) accompanies the decrease in temperature. Furthermore the (G + C)-rich *Ps. caryophylli* DNA (solid line) experiences a considerably greater increase in M_E than the relatively (A + T)-rich *B. subtilis* DNA (dashed line). This parallels the observation of Eigner and Doty (1965) that as bacterial ss-DNA is cooled from a high temperature, ss-DNA rich in G + C assumes an ordered structure more readily than ss-DNA rich in A + T.

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[1] Abbreviations used are: M_E, the buffer molarity by which 50% of the nucleic acid has eluted during chromatography; ss, single stranded; ds, double stranded. For other abbreviations, see Table I.

TABLE 1: Single-Stranded Nucleic Acids Discussed in this Communication.

Abbreviation	Substance[a]	Base Compn (%) A	T-(U)	G	C	Reference	Sonicated?	Source
Bs	*Bacillus subtilis* [^{14}C]DNA			(42–43)		Marmur and Doty (1962)	Yes, 0°	Gift of Dr. R. Burger
CPV	Cytoplasmic polyhedrosis virus RNA			(43)		Miura *et al.* (1968)	Yes, 0°	Gift of Dr. L. Lewandowski
Ml	*Micrococcus lysodeikticus* DNA			(72)		Marmur and Doty (1962)	Yes, 0°	See Materials and Methods
Ps c	*Pseudomonas caryophylli* DNA			(65)		Dr. M. Doudoroff, personal communication	Yes, 0°	Gift of Dr. M. Doudoroff
T2	Bacteriophage T2 DNA			(33–35)		Marmur and Doty (1962)	Yes, 0°	Gift of Dr. K. A. Drlica
TMV	Tobacco mosaic virus RNA (not whole molecules), molecular weight about 300,000	29	27	26	18	Markham (1959)	No	Gift of Dr. C. A. Knight
sTMV	TMV RNA, molecular weight about 60,000	29	27	26	18		Yes, 90°	Gift of Dr. C. A. Knight
TYMV	Turnip yellow mosaic virus RNA	23	22	17	38	Markham (1959)	No	Gift of Dr. C. A. Knight
poly(U)	Poly(uridylic acid), molecular weight about 100,000	0	100	0	0		No	Purchased from Miles Laboratories, Elkhart, Ind.

[a] The approximate molecular weights of the TMV and sTMV preparations were kindly determined by Rosemary Paterson by polyacrylamide gel electrophoresis according to the method of Bishop *et al.* (1967). The molecular weight of poly(U) was given by Miles Laboratories. The molecular weights of the DNA samples have not been determined but were probably in the neighborhood of $1–2 \times 10^5$.

These results strongly suggest that increased order within ss-DNA is responsible for at least a portion of the increase in M_E on going from 99 to 45°. Eigner and Doty (1965) have pointed out in addition that as the temperature reduction is continued, and provided the salt concentration is high enough (they used 0.195 M Na^+), all denatured DNA, regardless of base composition, approaches a maximally structured state of grossly similar conformation. In accord with this observation it was found that rechromatography of another portion of the peak fraction of the 99° chromatogram at 1° yielded still higher M_E values and virtually superimposable profiles (not shown) for the *B. subtilis* and *Ps. caryophylli* DNAs. Taken together all of these results indicate that increased structural order in single-stranded nucleic acids gives rise to increased affinity for hydroxylapatite. However additional experiments are required to determine what aspects of structural variation are the primary factors governing the wide differences in M_E of single-stranded nucleic acids. To lay the groundwork for analyzing the results from such experiments a discussion on the treatment of the data follows.

Method of Data Presentation. In comparing the results from various experiments the commonly used linear presentation of data as illustrated in Figure 1A is not convenient because fixed increments in the phosphate molarity become proportionately less effective as the total phosphate concentration is increased. An illustration of this effect is the pronounced peak broadening which occurs at the higher phosphate molarities in Figure 1A. This peak broadening, for the most part, cannot be accounted for by increases in the diversity of molecular conformations because chromatography at 1°, under which conditions the single-stranded configurations of diverse DNAs are similar (Eigner and Doty, 1965), yields profiles as broad as those of 45°. Furthermore, ds-DNA, the conformation of which would not be expected to vary significantly, gives elution profiles of similarly increasing breadth as buffers which give progressively higher M_E values (*e.g.*, cesium phosphate < sodium phosphate < cesium sulfate; Martinson, 1973b) are used (Martinson, 1971). Thus, in order to compare elution profiles over a wide range of phosphate concentrations, a semilogarithmic presentation of the data is more suitable. Accordingly, when the data of Figure 1A are replotted semilogarithmically in Figure 1B the elution profiles at both 99 and 45° become similar.

When several experiments are to be compared, the data are much more easily visualized if the semilogarithmic plot is of M_E *vs.* the temperature of chromatography. The data of Figure 1 and the 1° elutions mentioned above (as well as replicate runs which were averaged in) have been replotted in this way for Figure 2. Also included is an estimate (dashed line) of the conformation independent variation of M_E due to the inherent temperature dependence of adsorption in the hydroxylapatite system (Martinson, 1973b). Unfortunately, based on existing data, this estimate of inherent temperature dependence (probably a minimum) can only be very approximate. Therefore, reliable determinations of the increase in M_E due to conformation factors cannot be made. In light of this uncertainty it appears preferable to examine the *comparative* chromatographic behavior of the single-stranded nucleic acids. A modified presentation scheme which does not imply reference to absolute M_E values would therefore be helpful. Relative values of log M_E could be determined graphically by measuring the vertical distance between the lines in plots such as Figure 2, but since this distance is equal to the logarithm of the ratio of the M_E values of the two DNAs [log (M_E ratio)] it is more easily calculated directly.

Chromatography of Diverse Nucleic Acids. The effect of

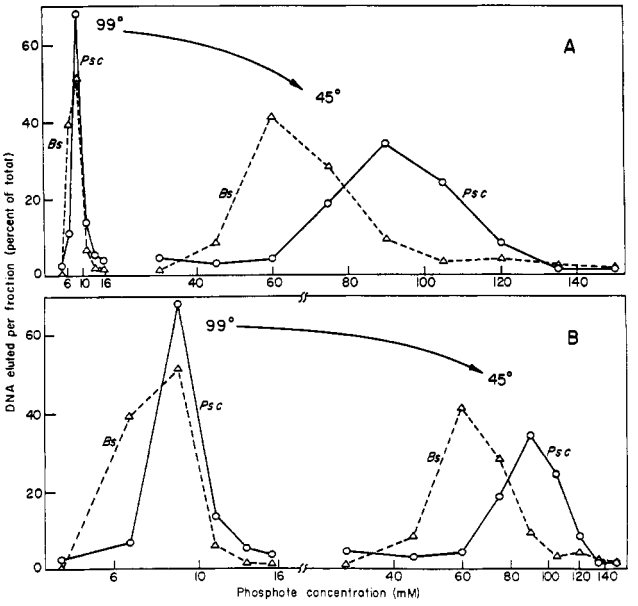

FIGURE 1: Effect of temperature on chromatography of ss-DNA. *Ps. caryophylli* DNA (45 μg) and *B. subtilis* [^{14}C]DNA (1 μg) were mixed, denatured (100°, 5 min, <0.1 M K^+), loaded on a 0.5-ml, 7-mm diameter bed (about 0.3 g dry weight) of hydroxylapatite and chromatographed at 99° with 1.9-ml, 2 mM steps. Two aliquots of the peak fraction were then rechromatographed on 0.05-ml, 5-mm columns at 45° with 0.3-ml steps of 15 mM increments. The 45° profiles shown in the figure are the average of the two determinations. The phosphate concentration is represented linearly in part A and logarithmically in part B. Abbreviations are in Table I. The ordinate represents the amount of DNA eluted per fraction expressed as a percent of the total eluted. Recoveries were about 90%.

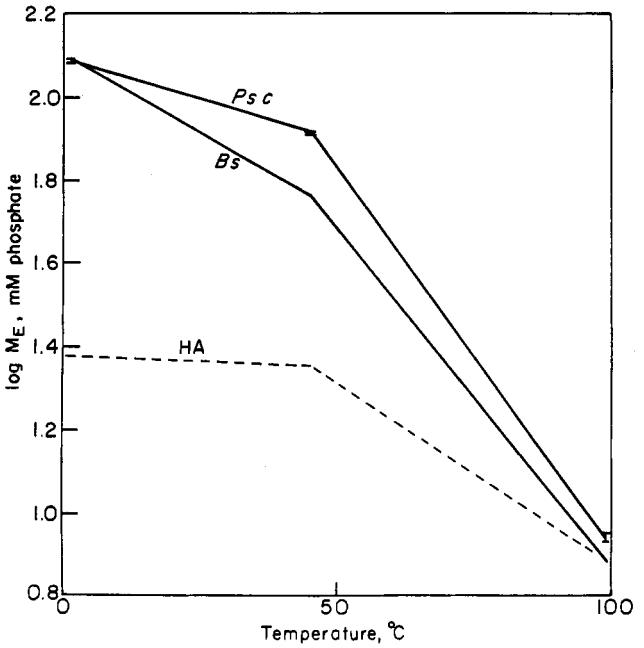

FIGURE 2: Effect of temperature on the M_E of ss-DNA. *Ps. caryophylli* DNA and *B. subtilis* [^{14}C]DNA were chromatographed at 99 and 45° as in Figure 1 except that 1.1-ml steps were used at 99° and one of the 45° chromatograms was made with a previously unchromatographed sample of DNA mixture. In addition, two aliquots of the peak fraction from the 99° elution of Figure 1 were rechromatographed at 1° in the same way as the 45° samples. The M_E values so obtained, as well as those of Figure 1, were averaged for each temperature and DNA species. The range in M_E for the *Ps. caryophylli* DNA at each temperature relative to the marker *B. subtilis* DNA is indicated in the figure by the size of the symbol on the Ps c line. The dashed line (HA) is an estimate of the elution molarity increase which would be expected to occur for *B. subtilis* DNA in the absence of any conformational changes. This estimate is based on measurements (Martinson, 1971, 1973b) of the temperature dependent changes intrinsic to the hydroxylapatite system. Abbreviations (except hydroxylapatite; see above) are in Table I.

temperature on the log (M_E ratio) of a number of single-stranded nucleic acids is illustrated in Figure 3. Each of the various DNA and RNA species (see Table I for abbreviations and base compositions) was chromatographed with *B. subtilis* [^{14}C]DNA as the internal reference. Hydroxylapatite catalyzes the degradation of ss-RNA at high temperature and low buffer concentrations (H. G. Martinson, manuscript in preparation) so 99° points are shown only for the DNA samples. The effect of temperature on the log (M_E ratio) of all of the nucleic acids richer in G + C than *B. subtilis* DNA (Figure 3) is similar to that already seen for *Ps. caryophylli* DNA (Figure 2). Conversely the two nucleic acids tested which had lower levels of G + C [T2 DNA and poly(U)] than *B. subtilis* DNA showed the reverse trend. These results show that as the temperature is increased and single-stranded structural order is reduced, the log M_E of the nucleic acids poor in G + C decreases more readily than that of the (G + C)-rich nucleic acids. This observation can probably be generalized to most natural nucleic acids. However special sequences such as those found in many encaryotic genomes allow the formation of "hairpin" structures by virtue of inverted sequence repetitions (Sueoka and Cheng, 1962; Wilson *et al.*, 1972; R. B. Church, personal communication). Such single-stranded nucleic acids would exhibit considerably more structure than expected on the basis of A + T content alone.

Also illustrated in Figure 3 is the fact that increasing the cation concentration of the buffer (KCl curves in Figure 3A,B) serves to shift the divergence of log M_E values to higher temperatures. Finally it can be seen that reducing the molecular weight (sTMV line, Figure 3) significantly lowers the log M_E of TMV RNA but that the response of the log M_E to variation in temperature is unchanged. The significance of these observations is discussed in the next section.

Discussion

Method of Data Presentation. In the previous section it was shown, on an intuitive and empirical basis, that log M_E is the most appropriate vehicle for comparison of relative elution molarities of nucleic acids from hydroxylapatite. Similarly, it can be deduced from a theoretical treatment of hydroxylapatite chromatography by Kawasaki (1970a,b) that M_E is roughly proportion to $Ce^{cg} - 1$, where C and c are constants (>0) and $-g$ is proportional to the free energy of adsorption. This relationship shows that log M_E is linearly related to the affinity of the nucleic acid for hydroxylapatite when the term Ce^{cg} becomes large enough. However, more data would be required in order to determine if this is a meaningful parallel.

In this paper the log (M_E ratio) has been introduced for characterizing the relative positions of hydroxylapatite chromatographic peaks. In fact in comparing elution profiles of materials of similar heterogeneity the log (M_E ratio) can actually be used as a precise measure of chromatographic resolution. This is illustrated by the broken and solid Ml lines in Figure 3A. The solid line is a plot of the log (M_E ratio) for the cochromatographed *B. subtilis* and *M. lysode-*

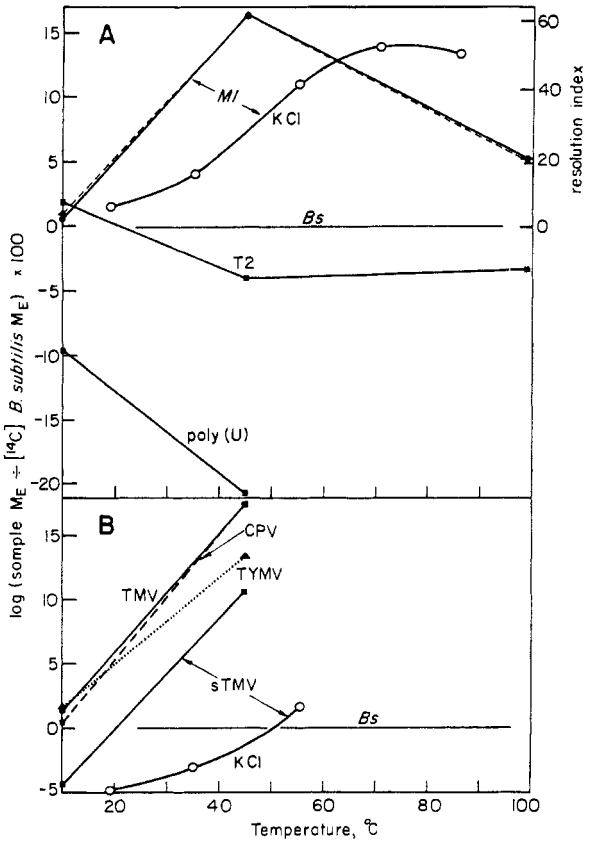

FIGURE 3: Effect of temperature on the chromatographic resolution of various single-stranded nucleic acids. All data except the KCl curves were obtained by chromatography at 10.1, 45.5, or 99° of 4 μg of an unlabeled single-stranded nucleic acid together with 0.1 μg of denatured *B. subtilis* [^{14}C]DNA. Columns (5 mm diameter; 7 mm for the 10.1° elutions) containing 0.05-ml beds of hydroxylapatite were eluted with 0.4 ml/step. This volume elutes at least 90% of the nucleic acid possible at each step. The steps used were 2, 4, 6, 8, 10, 14, 18, 22, 26, 30, 38, 46, 54, 62, 70, 80, 90, etc., to 230 mM phosphate. Abbreviations are listed in Table I. Recoveries were 90–100% at 45.5 and 99° and 70–90% at 10.1°. The dashed line in part A is the "resolution index" for *M. lysodeikticus* DNA and corresponds to the upper right-hand scale. The resolution index as defined here is negatively correlated with peak overlap and was calculated by subtracting from 80 the percentage of *M. lysodeikticus* DNA which eluted in the same fractions (or portion thereof) as the first 80% of the *B. subtilis* DNA. The KCl curves represent elutions performed with a buffer series generated by diluting 0.22 M potassium phosphate with 0.33 M KCl. For part A, 50% of a mixture of 30 μg of *M. lysodeikticus* DNA and 2 μg of *B. subtilis* [^{14}C]DNA was denatured and then recombined with the other portion. The preparation was then distributed to 5-mm diameter columns (except 7 mm at 19°) of hydroxylapatite (0.15-ml bed volume), rinsed with 10^{-3} M phosphate at room temperature, brought to the temperature to be tested and chromatographed with 0.3 ml/step. The steps (26 altogether) extended from 2 to 220 mM phosphate in increments which ranged from 2 mM at the beginning to 15 mM at the end. The elution pattern of the *M. lysodeikticus* ds-DNA was essentially parallel to that of the native *B. subtilis* DNA and it not shown in the figure. The KCl curve in part B was generated as just described except that fragmented TMV RNA (ss-) and native CPV ds-RNA were used instead of the *M. lysodeikticus* DNA. As in part A the elution of the double strands is not shown in the figure.

ikticus DNAs (left-hand scale) whereas the broken line is a plot of the actual resolution of the two components as measured by the degree of peak overlap (right-hand scale; see the legend to Figure 3 for the operational definition of the "resolution index"). In additional similar comparisons it has been verified that the log of the M_E ratio, but not the M_E ratio itself nor the M_E difference, is an actual measure of peak resolution. Besides being usually more convenient to compute than the degree of peak overlap, the log (M_E ratio) is particularly useful when the degree of separation of completely resolved peaks is of interest since in such a situation no peak overlap exists by which to compare adjacent or widely separated peaks. Nevertheless it should be recognized that log M_E is a measure only of the average elution behavior of a population of molecules and indicates nothing about the breadth of an elution profile. Therefore the log (M_E ratio) is insensitive to peak width and hence to changes in peak overlap which result mainly from changes in the shapes of the peaks. This consideration is immaterial if peaks are completely separated or if, as in the present study, similar elution profiles are being compared; indeed it is an asset if, again as in the present study, only the net behaviors of populations of molecules independent of heterogeneity, are of interest. However as shown in Figure 1, even the breadth of an elution profile can be meaningfully assessed only in terms of the logarithm of the salt concentration and it would seem advisable to plot all salt elution data in similar semilogarithmic fashion whether or not the log (M_E ratio) is eventually used.

Molecular Weight vs. Structure. The data of Figure 3 show that both molecular weight and structure affect the log M_E of single-stranded nucleic acids. Thermal chain scission can be assumed to be negligible during chromatography at 1 and 45° (Bernardi, 1971) and rechromatography of DNA eluted at 99° showed that it behaved identically with previously unchromatographed DNA. Therefore, because molecular weight does not change significantly with temperature, its contribution to the log (M_E ratio) is essentially constant and the TMV and sTMV lines in Figure 3B are parallel. Structure, on the other hand, does vary with temperature. Thus TMV RNA fragments (sTMV) elute before *B. subtilis* DNA (Bs) at 10° because of their small size but nevertheless elute later than *B. subtilis* DNA at 45° because of the greater importance of structural differences at the higher temperature. In the discussion to follow it will be shown that all of the log M_E variations can be understood in terms of known structural transitions which occur in single-stranded nucleic acids.

Review of Single-Strand Structure in Solution. At high temperature and low salt concentration single-stranded nucleic acids are relatively devoid of order (for reviews, see Felsenfeld and Miles, 1967, and Yang and Samejima, 1969). Conversely at low temperatures and high salt concentrations they are highly ordered. RNA is more easily converted from the disordered to the ordered form than DNA is (Yang and Samejima, 1969; also compare Eigner and Doty, 1965, and Sinsheimer, 1959, with Doty *et al.*, 1959, Boedtker, 1960, and Billeter *et al.*, 1966, for examples of data). Similarly single-stranded nucleic acids rich in G + C (Eigner and Doty, 1965; Doty *et al.*, 1959) and/or purine bases (see Felsenfeld and Miles, 1967; Yang and Samejima, 1969) become ordered more readily than nucleic acids poor in these substituents. It has been shown for both DNA (Eigner and Doty, 1955) and RNA (Loening, 1969) that the degree of ordering in single strands containing high and low proportions of G + C converges and reaches a maximum (Studier, 1969; Boedtker, 1960, and others herein) as the salt concentration is raised and the temperature reduced.

The studies which have been cited above were done by measuring a variety of structure related properties of the nucleic acids such as optical activity and absorbance, sedimentation velocity and polyacrylamide gel electrophoresis. It will now be shown that studies using hydroxylapatite chromatography are consistent with all of those summarized

above if it is assumed that increased structural order gives rise to an increased log M_E.

Effect of Structure on M_E. Figure 2 shows that log M_E decreases dramatically as the structural order of the single strands is reduced by raising the temperature. In fact if native and denatured DNAs are cochromatographed at increasing temperatures there is a considerably greater decrease of log M_E for the single strands than for the double strands[2] (H. G. Martinson, unpublished data) consistent with the gradual disordering of the former with temperature. Similarly Figure 3 shows that those single-stranded nucleic acids which would be expected to become disordered more easily than *B. subtilis* ss-DNA because of their lower G + C content show a greater decrease than *B. subtilis* DNA in log M_E [*i.e.*, give a negative slope in a plot of log (M_E ratio) *vs.* temperature] between 10 and 45°. Conversely the nucleic acids which have a higher G + C content than *B. subtilis* ss-DNA show a smaller decrease than *B. subtilis* DNA in log M_E (*i.e.*, they give a positive slope between 10 and 45° in Figure 3). Furthermore, ss-RNA, which is more stably structured than ss-DNA, in each case gives a considerably greater slope than DNA of the same base composition would be expected to give. Indeed CPV RNA and TMV RNA which have virtually the same base composition as *B. subtilis* DNA nevertheless give the greatest slopes tested. Finally, the addition of salt (KCl curves) which shifts the thermal transitions of most single-stranded nucleic acids to higher temperatures, has the same effect on the log (M_E ratio) in the hydroxylapatite system. All of these correlations leave little doubt that the structure of single-stranded nucleic acids exerts an important influence on nucleic acid chromatographic behavior on hydroxylapatite.

Base Stacking vs. Base Pairing. It is of interest to resolve the chromatographic effects of the two types of structure which single-stranded nucleic acids normally contain, namely base stacking and base pairing. Both types of interaction are sensitive to changes in temperature (Felsenfeld and Miles, 1967; Yang and Samejima, 1969; Boedtker, 1967; Studier, 1969) but, because only base pairing is significantly stabilized by alkali cations (Record, 1967), the two types of structure can be distinguished to some extent by determining if the temperature of the thermally induced order → disorder transition is raised by the addition of salt. In the case of adsorption to hydroxylapatite, the KCl curves in Figure 3 show that when the base pairing interactions of single-stranded nucleic acids are selectively stabilized by the addition of a little salt, the log (M_E ratio) transitions (as well as the log M_E transitions themselves) are shifted to significantly higher temperatures. This shows, not unexpectedly, that base paired folds increase the affinity of single-stranded nucleic acids for hydroxylapatite.

Two lines of evidence suggest that base stacking is also important. First, the steep negative slope of the poly(U) line in Figure 3A indicates that the log M_E for poly(U) is very sensitive to temperature between 10 and 45° suggesting that poly(U) becomes disordered more readily than *B. subtilis* DNA in that range. At 10°, elution of poly(U) takes place before the K^+ concentration of the phosphate gradient reaches 0.2 M and under these conditions base stacking is thought to be the only form of structure in poly(U) (Thrierr *et al.*, 1971; Michelson and Monny, 1966). Note also that poly(U), being relatively disordered even at 10°, has a much lower log (M_E ratio) than sTMV which is about the same size.

The other correlation of a higher log (M_E ratio) with a higher degree of stacking can be seen at 99° in Figures 2 and 3A. [The log (M_E ratio) is the distance between the lines in Figure 2.] At 99° all of the ss-DNAs elute before the K^+ concentration in the phosphate gradient reaches 50 mM. Under these conditions even *M. lysodeikticus* ds-DNA becomes fully denatured (Marmur and Doty, 1962; Owen *et al.*, 1969, or Gruenwedel and Hsu, 1969). Base pairing is therefore absent. However even at high temperatures stacking interactions appear to persist (Davis and Tinoco, 1968; Gulik *et al.*, 1970) and are stronger for nucleic acids rich in G + C (Owen *et al.*, 1969) and low in T or U (Yang and Samejima, 1969). Thus at 99° where the only structural order present is limited base stacking, the high G + C DNAs in Figures 2 and 3 (*M. lysodeikticus* and *Ps. caryophylli*) have higher log (M_E ratios) than the low G + C DNAs (*B. subtilis* and phage T2). Unequal sizes of the DNA molecules cannot account for these differences because at low temperature the high G + C DNAs do not have higher log (M_E ratios) than other DNAs (Figures 2 and 3).

Bernardi (1971) also has suggested that base stacking affects elution molarity because polypurines, which are highly stacked, are eluted at a higher molarity of phosphate at room temperature than polypyrimidines, which have comparatively little structure.

Mechanisms. The many fundamental parallels which have been found between known structural transitions of single-stranded nucleic acids and their chromatographic behavior leave little doubt that increases in base pairing and base stacking interactions lead to increases in affinity for hydroxylapatite. However, this deduction still leaves open the question of *why* increased order leads to increased affinity. Bernardi (1971) has suggested that the reduced affinity due to denaturation is related to the reduced number of phosphates on the "outer surface" of a flexible, coiled molecule. However, any such attempt to correlate the solution conformation of flexible macromolecules with their adsorption affinities is inappropriate since the shape of a flexible polymer is radically altered during the adsorption process (Roe, 1965). In fact one might expect a flexible molecule to be *more* firmly adsorbed because of its ability to conform to the adsorbing surface, a phenomenon recently reported for 5S RNA by Soave *et al.* (1973). Thus, in principle, all phosphates of a completely denatured molecule, but only about one in ten of a completely helical molecule (and even less for native 5S RNA), would be available for adsorption. Indeed the severe loss of conformational degrees of freedom which must attend the adsorption of flexible molecules may explain their reduced affinity. We have seen that, whether base stacking or base pairing is involved, increases in structural order lead to increases in adsorption affinity for hydroxylapatite. A basic common denominator of single-stranded stacking and folding as well as of native double-stranded pairing is the loss of configurational entropy involved. It therefore seems possible that the fractionation of rigid from flexible molecules, which is a general principle in the hydroxylapatite system, can be explained on the basis of the net loss in configurational entropy of the macromolecules which occurs during adsorption. Thus, more flexible molecules which must suffer greater decreases in configurational entropy than more rigid mole-

[2] It is particularly important that changes in log M_E be compared in this case because changes in M_E itself are actually greater for native DNA owing to its higher elution molarity. The temperature dependence of log M_E for native DNA is unrelated to structural changes in the DNA (Martinson, 1973b) and has been used to estimate the dashed line in Figure 2.

cules as a result of adsorption are usually less stably adsorbed to hydroxylapatite. Certainly other factors, such as the number or individual strengths of the interactions, also influence the affinity for hydroxylapatite. The increase in affinity on denaturation of the compact 5S RNA (Soave *et al.*, 1973) is a good example of a case in which an unusually large increase in the number of possible adsorption interactions on denaturation appears to be the overriding influence. However, with most nucleic acids, the variable decrease in configurational entropy attending adsorption seems to be the overriding factor in the discrimination between chemically similar molecules.

Acknowledgments

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