CHAPTER IV

HYDROXYAPATITE BINDING TO DNA

SOME QUESTIONS

4.01 Hydroxyapatite binding to DNA

Hydroxyapatite (HAP) columns was initially developed by Tiselus et al[57] for protein chromatography. Application of HAP to nucleic acids though initiated by Semenza[58] and Main et al[59] owes it present popularity mainly due to the classic work of Giorgio Bernadi[60,61]. Although the major application of HAP lies in the differential binding to double and single stranded DNA, subtle differences in secondary and tertiary structures can also be discriminated by HAP columns. In the present discussion I will first review the present status of the influence of various parameters on the mode of binding DNA to HAP and then discuss the result of experiments in light of the known facts.

Binding of double and single stranded DNA

The most important property of HAP is its differential affinity to double and single stranded DNA.. This property has been gainfully utilised in monitoring the kinetics of DNA reassociation. The nature of binding of DNA to HAP has been extensively studied by Martinson[62-65]. The initial idea put forward by Bernadi[60] based on an earlier work by Ascoli et al[66], that charge density is the prime determinant of adsorption affinity has been thoroughly criticised by Martinson.

Although based on the idea that d.s. DNA has both, more affinity for HAP and a higher charge density than s.s. DNA, it fails to explain why the binding to d.s. RNA and RNA DNA hybrid is less than d.s. DNA though the former has greater charge density than the latter. Also the fact that GC rich DNA having greater charge density than AT rich ones has less binding affinity to HAP makes the charge density hypothesis totally untenable.

Martinson has also discussed the roles of base interaction, phosphate distribution and small entity interaction and have shown that the possibility of explanation through these interactions falls short due to its internal contradictions.

The major - determining factor in binding of DNA to HAP is the availability of charged phosphate group to interact with Calcium ions of the HAP crystals. Conformation analysis of the back bone show that the phosphate group protrude from the helix in case of d.s. DNA whereas are relatively buried in d.s. RNA and DNA-RNA hybrid. Since the interaction of DNA and HAP may be visualised as surface interaction between a cylinder and a plane, the accessibility of the PO_2^- groups when they protrude from the helix is more than when they are buried. Differential binding to GC and AT rich DNA can also be explained on the basis of this model, as in the case of former the charged phosphate group is relatively buried.

One may be tempted to extrapolate the reasons of discrimination among various duplexes into the realm of s.s. DNA, as in case of s.s. DNA the availability of charged phosphate group protruding outwards is even less due to it being in the state of random coil. But as Roe observes[67] from theoretical stand point that co-relation of solution conformation with its adsorption affinity is in-appropriate on the basis that the shape of a flexible polymer is radically altered during the process of adsorption. Thus in principle, as Martinson argues-all the phosphate of a completely denatured molecule in comparison to the calculated one-tenth of a native molecule would be available for adsorption. But this would accompany with a severe loss of conformational degrees of freedom. As is known whether base stacking or base pairing is concerned, increase in structural order corresponding to a loss of configurational entropy always lead to an increase in HAP affinity. Thus it may seem extremely satisfactory that the fractionation of a rigid from a flexible molecule which is a general principle of the HAP system can be explained on the basis of net loss of configurational entropy during adsorption. The more flexible the molecule, the greater is its decrease in configurational entropy. However, there are exceptional cases : where upon melting the binding affinity of 5S. RNA is increased[68]. This is a typical case where an unusually large increase in the number of possible binding sites on denaturation has an overridding influence over the loss of structural

rigidity. Thus we can summarise by saying that - with most nucleic acids, the variable in configurational entropy seems to be the major factor in the discrimination of chemically similar molecules, whereas in case of molecules having more or less similar rigidity (various duplexes) the discriminating factor is the availability of charged phosphates protruding into the outer surface of the helix.

Temperature effect of binding

As have been already discussed it should be apparent that even among s.s. DNA differences in structural rigidity owing to environmental factors (temperature and ionic concentration) should influence binding and hence the elution molarity. This has experimentally found to be the case. Binding of s.s. DNA to HAP decreases dramatically at high temperature. At room temperature the general elution molarity is about 150 mM of P.b. whereas at 99°C it is 9 mM. The effect of temperature on d.s. DNA is less dramatic. The elution molarity decreases from 250 mM at room to 170 mM just prior to melting. These results are in good agreement with the fact that before the melting starts change in d.s. DNA structure is minimal compared to the changes in s.s. DNA structure in that temperature range.

Stability of DNA on HAP

1) An interesting observation with HAP-DNA binding has been the reverse ion effect on the stability of DNA. While on the one hand increase of sodium ion concentration increases the stability of d.s. DNA in solution, it has been observed that DNA bound to HAP in low molar P.b. (i.e. low sodium ion concentration) has higher melting temperature (as measured by thermal chromatography) than one bound to HAP at high molar P.b. This has been explained as the effect due to stronger binding at low molarity which hinders strand separation of DNA. It is the amount of separated single strands which one measures during the course of thermal chromatography.

2) Kawaski has discussed[69,70] the evolution of binding modes during chromatography. He predicted that under condition which promote strong binding (i.e. low molarity of P.b.) DNA adsorbs in a wide state of configuration many of which are in a relatively high states of free energy. During elution DNA bound in these less stable modes are eluted first and if the HAP bed is not already saturated readsorb down the column in a more stable configuration. That d.s. DNA do have various modes of stability has been beautifully verified by Martinson. Though one is not very certain how the number of binding sites in DNA vary with ionic environment i.e. at low molar P.b. DNA has less binding sites than at high molar P.b. with binding being stronger at low molarity. One cannot conceive of much structural transition of d.s. DNA in this molarity range leading

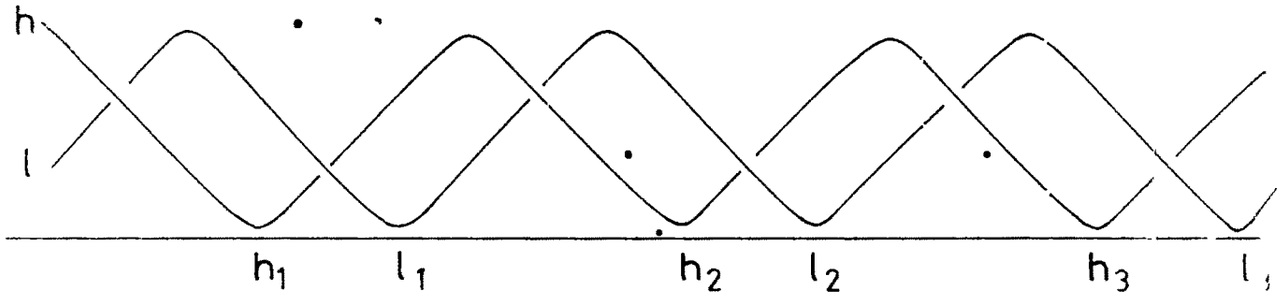
Fig. 15. Schematic diagram of binding pattern for various types of DNA to hydroxyapatite (HAP)

a) The binding of a typical right handed DNA, in which both the strands are equally bound to HAP through the charged phosphate groups at h_1, h_2, h_3 and l_1, l_2, l_3.

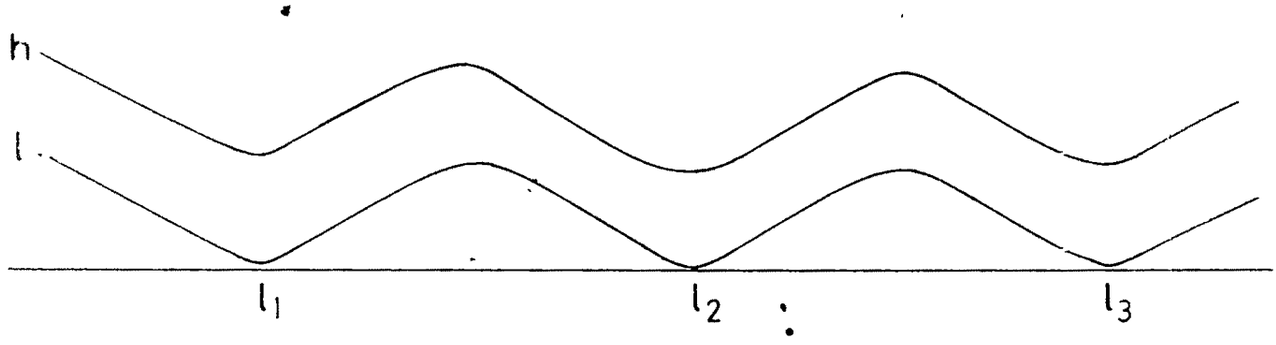
b) The binding for an ideal SBS DNA, here only 'l' strand is bound through l_1, l_2, l_3.

c) The binding for a mixed DNA with differential binding of the two strands (i.e., h_1, h_2, h_3 for strand h and only l_1 for strand l).

(a)



(b)



(c)

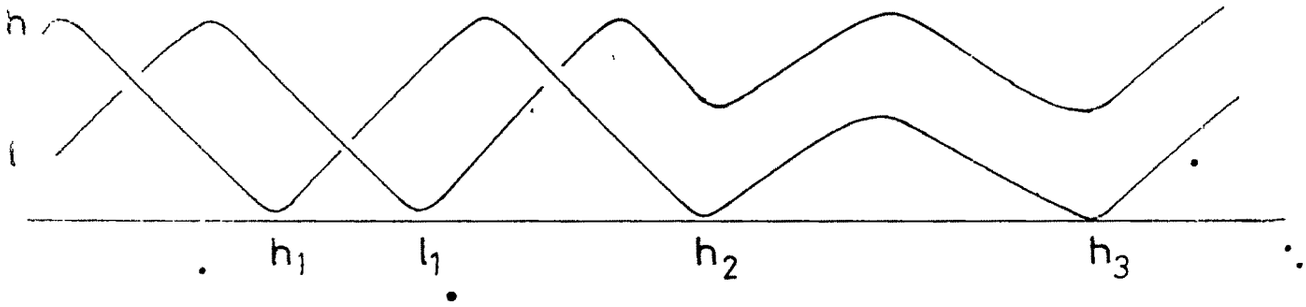


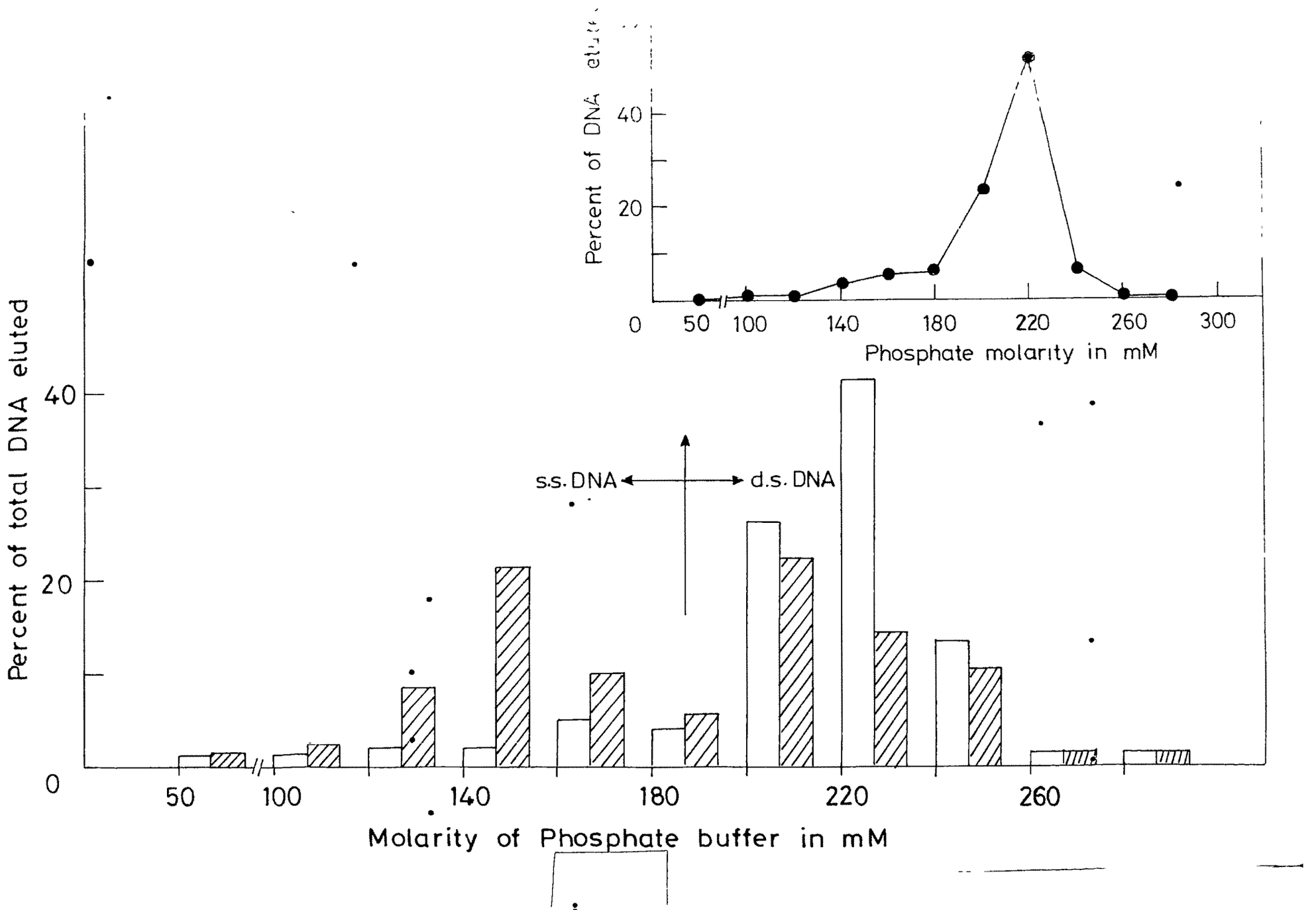
FIG.1

to changing the geometry of the phosphate group in relation to the helix in general. The only solution lies in, Kawaski's model. Though simplified - that one could think of the increase in the number of binding site by assuming that along various direction in the plane of the crystal one has various degrees of binding sites. The most stable mode in the binding of DNA to HAP is in the direction (it being line of contact of the cylinder and the surface) where the number of binding sites is maximum. That mechanism of binding of DNA to HAP actually follow this scheme has yet to be experimentally verified.

4.02 Some questions regarding binding of DNA to hydroxyapatite

One very important question regarding the binding of DNA to HAP has still remained unanswered. Strangely enough it has rarely been asked - how does a d.s. DNA elute and as s.s. DNA at high temperature as it does in thermal chromatography[71]? This question becomes extremely puzzling if one looks at the molecular mechanism of DNA binding to HAP. Binding of DNA to HAP is a surface phenomenon and can be visualised as an interaction between a cylinder and a flat surface. It immediately becomes obvious that on the assumption that DNA is a right handed double helix, both the strands should be equally clamped to HAP. This is schematically represented in Figure 15a. Since we know that DNA elutes at high temperature as s.s. DNA we are confounded with the puzzle - how the intertwined immobile duplex DNA bound to HAP unwounds to form s.s. DNA. The only way to

Fig. 16. The elution pattern of DNA bound to HAP. DNA ($\sim$2 mg/ml) in 10 mM phosphate buffer pH 6.8 (P.b.) flushed with nitrogen, was sonicated in a MSE sonicator (at setting 3, amplitude high) with 40 pulses each of 30 second duration. This results in molecules of an average length of 200 bases as measured by gel electrophores with markers. The sonicated DNA (200 µg DNA/ml of HAP) was loaded onto a 2.5 ml HAP bed (HAP was prepared according to ref. (71). It had a capacity of binding 300 ug DNA/ml of HAP) and washed with several volumes of 5 mM P.b. The resulting HAP with some added buffer was quantitatively transferred to sealed tubes which were heated in a 95°C water bath for 3-5 minutes. The HAP was transferred back to the column and the DNA was eluted in steps with 5 ml of the mentioned buffer. The O.D. of the eluant was measured, after necessary correction due to UV absorbing material from the bed. The elution of untreated DNA was simply with 5 ml steps of the corresponding buffer. The shaded region shows the heat treated DNA and the unshaded untreated DNA. The inset shows the elution pattern of native (mildly sonicated) heat treated DNA.


Percent of DNA eluted
40
20
0
50
100
140
180
220
260
300
Phosphate molarity in mM
Percent of total DNA eluted
40
20
0
s.s. DNA
d.s. DNA
50
100
140
180
220
260
Molarity of Phosphate buffer in mM

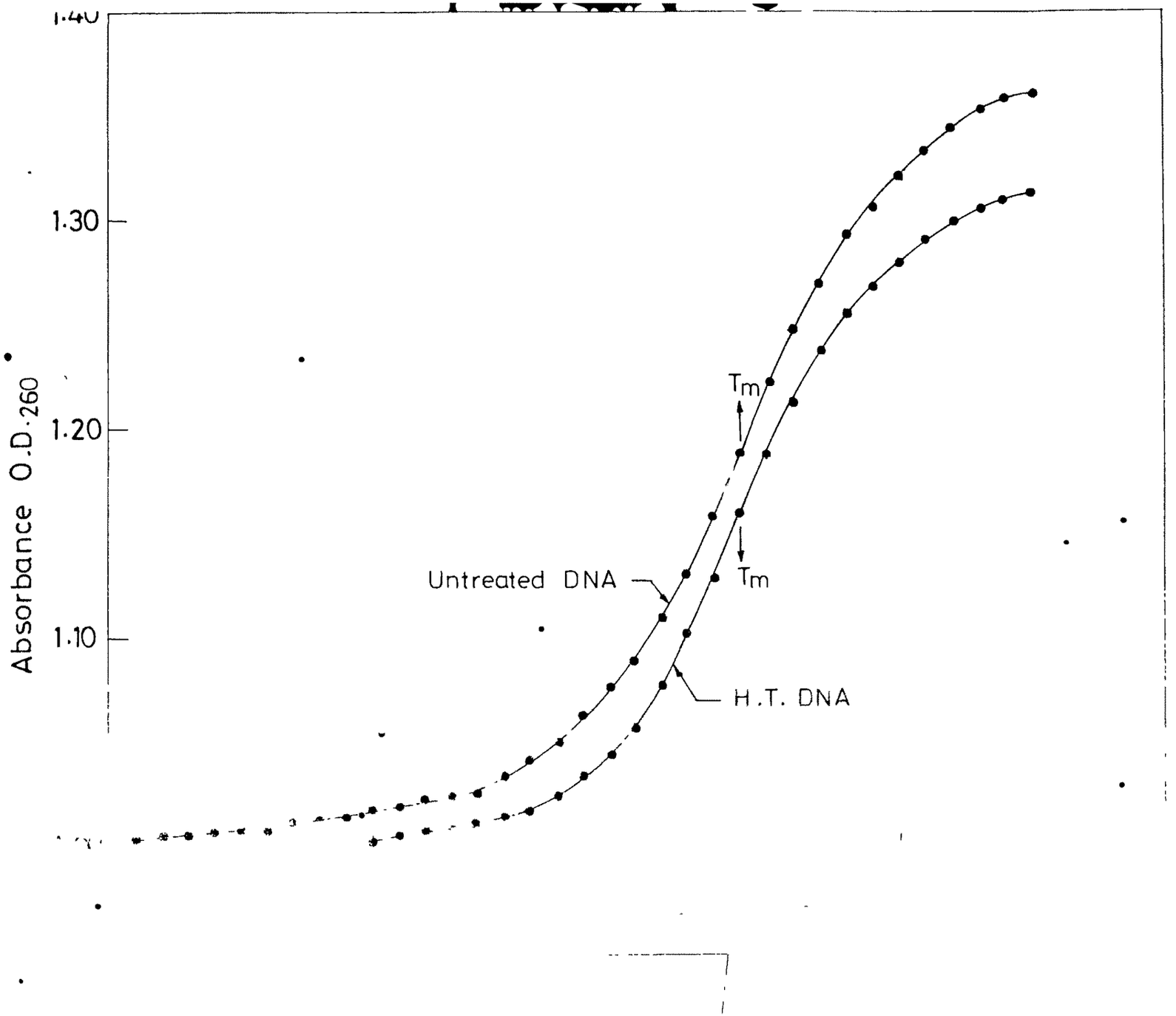
explain this phenomenon would be to think that before uncoiling the duplex DNA is released from HAP and the uncoiling now occurs in solution and not while bound to HAP.

But some serious objections to this explanation may be raised. Firstly the bases have only minor role to play in the binding which is mediated through the charged phosphate of the backbone. Thus temperature which breaks hydrogen bonds between the bases in a sequential manner (i.e. AT's are preferred to GC's) should not identically destabilise the binding of HAP to DNA. But that is what is actually observed - Thermal chromatography is said to follow the optical melting profile of DNA[71].

In order to get an idea as to what actually happens during the uncoiling i.e. a) Is the duplex DNA released from the HAP before uncoiling or b) The uncoiling occurs while DNA is bound to HAP. Some simple experiments were performed.(Fig.16)

The idea behind the choice of molarity i.e. 5 mM during the heating of DNA is that at this low molar even s.s. DNA is bound to HAP at 95°C[62]. Thus it was expected that DNA should not become single stranded. It was thought that at this molarity the duplex DNA should remain bound to HAP and since it is intertwined, both the strand should be clamped equally to HAP. Uncoiling in this immobile phase thus could not occur. The experiment was carried with DNA fragmented too 200 base pairs because it is known that around this length DNA bind to HAP as a stiff rod[72].

Fig. 17. The melting profile of DNA fractions eluted at 200 mM P.b. (Fig. 16) was carried out in a Shimadzu Spectrophotometer with temperature attachment. The temperature of the solution was measured at 1°C intervals by inserting a thermistor probe inside the cuvette and connected externally to a calibrated Wheatstones bridge. For the melting to be in a suitable temperature range the melting was done after half dilution of the samples (i.e. at 100 mM P.b.). H.T. DNA denotes heat treated DNA.


1.40
1.30
1.20
1.10
Absorbance O.D.260
Tm
Tm
Untreated DNA
H.T. DNA

But the result seemed extremely puzzling. It immediately indicated as shown in Figure 16 that half the DNA eluted as single stranded DNA. That the DNA eluted at the second peak was really a duplex DNA was confirmed from its melting profile (Fig. 17) which was more or less identical to its native profile. Though its hyperchromocity was slightly less. The first peak gave a typical melting of single stranded DNA with about 5% hyperchromocity. The experiment was repeated under various conditions such as lowering the phosphate molarity to 1 mM and even less, with different batches of HAP.

It was found that the elution profile remained qualitatively same with two distinct peaks of more or less equal intensity.

This result may be of far reaching consequence. One is immediately tempted to think of DNA possessing some other structures in addition to its classical one. With some recent experimental evidences on the possibility of left handed structures[73,74] backed up by its theoretical prediction[75-78], one could think streches of DNA segments having alternative left and right handed structures. It could be possible that in such structures its binding to HAP would not be as in Figure 15a with both the strand equally clamped. Here both the strands being unequally bound would lead to differential flexibility of both the strands, so far its binding to HAP (fig 15c) is concerned. Random fragmentation of DNA (as it is sonicated) would thus lead to a broad spectrum of flexibilities.

The fragments which possess monotonous handedness (either left or right) would thus be bound to HAP and not elute as single strand DNA, while fragments possessing mixed handedness (alternate left and right) may elute as single strand DNA. It is obvious that if DNA were a true SBS molecule[75,76] (its binding is shown in Fig. 15b) all of them would elute as single stranded DNA, which is found not to be the case. However, this is only a preliminary study. But it points to the fact that hydroxyapatite can be gainfully utilised as a suitable probe to investigate into the structure of DNA.