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FRACTIONATION OF RIBONUCLEIC ACID AND BINDING STUDIES ON COLUMNS OF POLYLYSINE KIESELGUHR

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SUMMARY

Further investigation of RNA fractionation on polylysine kieselguhr (PLK) columns has been carried out, and the elution profiles of the various types of RNA have been determined. Work has also begun on the nature of the binding sites between the nucleic acids and polylysine in the PLK–nucleic acid complex. The preliminary results of this study and those of the first competitive exchange experiments are reported.

INTRODUCTION

Recently reported work from this laboratory[1] on the fractionation of nucleic acid mixtures, extracted from *Bacillus subtilis* strain Marburg, on polylysine kieselguhr (PLK) columns gave rise to speculation about the nature of purified RNA fractionation. When nucleic acid mixtures, prepared by a modification of MARMUR's method[1,2], were fractionated on PLK columns using linear gradients of buffered saline[3] as the eluting agent, a composite peak was obtained with a maximum between 1.6 and 1.8 *M* NaCl. This peak contained RNA when examined by chemical assay[4] but was also contaminated by the presence of small amounts of DNA and two peaks of protein, as described previously[1]. It was thought that this RNA could be of high molecular weight resulting from the breakdown of polyribosomes during preparation, and this led us to investigate the fractionation of various types of RNA and the effect of preparation on the elution profile of these types of RNA.

Concurrently, investigation into the mechanism of PLK fractionation and attempts to demonstrate the nature of the binding sites between nucleic acids and PLK columns have culminated in a new type of experiment (termed competitive exchange), which it is hoped in time will lead to a better understanding of PLK chromatography.

PROCEDURES

Strains of bacteria

Bacillus subtilis strain Marburg and *Escherichia coli* strain MRE 600 (ribonuclease less mutant) were used as sources of the nucleic acids.

Preparations of RNA

(*a*) *Ribosomal RNA*. This type of RNA was prepared by differential centrifugation according to the method of LERMAN *et al.*[5]. Eight grammes (wet weight) of packed *B. subtilis* strain Marburg cells were taken up into 100 ml of RNA medium I (0.01 *M* $MgCl_2$, 0.005 *M* $ZnSO_4$ and 0.05 *M* Tris; pH 7.3) and treated with 80 mg lysozyme at 37° for 30 min. The formation of protoplasts was checked using a microscope, and final lysis of the cells was accomplished by freezing and thawing the suspension using alternately an acetone–dry ice mixture and a water bath at 37°. The cell debris was removed from solution by centrifugation at 12000 r.p.m. for 30 min in the 100-ml head of an MSE High-Speed 18 centrifuge. The supernatant was then recentrifuged at 40000 r.p.m. for 60 min in the 40 head of a Spinco Model L2 centrifuge to collect the ribosomes. The supernatant resulting was decanted off and the ribosomes were taken up into a small volume of RNA medium I by standing at 4° overnight. The protein was dissociated from the ribosomes by dilution of the suspension into several volumes of 0.4 *M* NaCl containing 0.02 *M* KH_2PO_4, pH 6.7 (ref. 6).

(*b*) *Highly polymerised RNA*. One gramme of *E. coli* strain MRE 600 cells (wet weight), harvested in the log phase of growth, were suspended in 10 ml of Tris buffer (0.01 *M* pH 8.1) and 2.5 g of sucrose were added. The solution was made 0.8 m*M* with respect to EDTA and the cell walls were digested using 4 mg lysozyme with shaking at 37° for 10 min. The magnesium ion concentration was then adjusted to 10 m*M* and the cells were lysed by freezing overnight. To ensure complete lysis, sodium dodecyl sulphate (SDS), to a final concentration of 0.5%, was added and deproteinisation was carried out to varying degrees using equal volumes of chloroform–isoamyl alcohol after the method of MARMUR[2]. The RNA was collected by adjusting the potassium acetate concentration to 0.2 *M* and adding two volumes of 95% ethanol. The precipitate that had formed after standing for 3 h at —20° was collected by centrifugation at 3000 r.p.m. for 20 min using an MSE major centrifuge, and dissolved in dilute saline citrate. This was then treated with deoxyribonuclease and 10^{-2} *M* Mg^{2+} for 30 min at 37°. All preparations were stored in concentrated form at 4°.

Preparation of PLK columns

Standard PLK columns were prepared by the method devised by AYAD AND BLAMIRE[7], and samples fractionated and monitored as previously described[1]. Sedimentation coefficients were determined in a Spinco Model E centrifuge fitted with UV optics. Determination was carried out at 35000 r.p.m. using nucleic acid solutions at an approximate concentration of 50 μg/ml dissolved in 0.15 *M* NaCl containing 0.015 *M* sodium citrate pH 7.0 and corrected for zero concentration.

Standards

Calf thymus DNA (Sigma Chemical Co.), ribonucleic acid "core" (BDH, Ltd.), yeast sRNA type XI (Sigma Chemical Co.) and highly polymerised RNA from *E. coli* (BDH, Ltd.) were used as standards and checked against the results previously obtained using DNA extracted from *B. subtilis* strain Marburg by the method of MARMUR[2].

RESULTS

Fig. 1 shows the results obtained when a sample of nucleic acid mixture, prepar-

ed as described by AYAD AND BLAMIRE[1] but omitting two of the deproteinising stages, is fractionated on a 2.5-g PLK column in which the fractionating layer consists of 2.5 g kieselguhr and 2.5 mg polylysine, using a linear gradient of buffered saline (0.4–4.0 *M*; 100 ml of each).

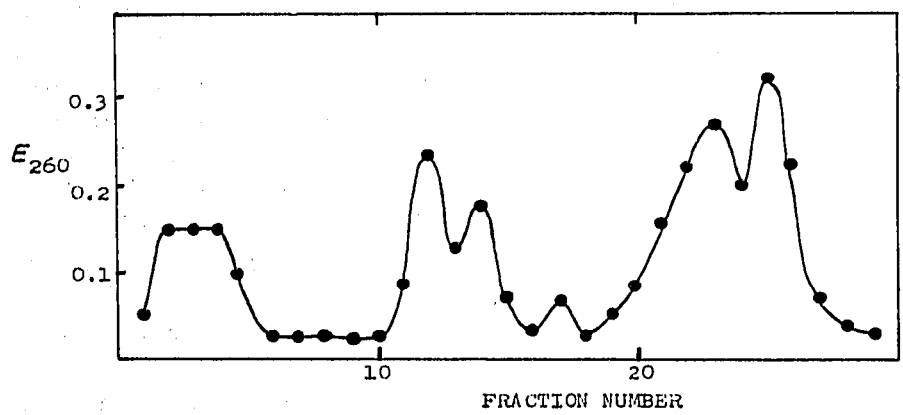

Fig. 1. Fractionation on a 2.5-g PLK column of a nucleic acid mixture extracted from *B. subtilis* strain Marburg.

Similar results have already been reported[1], but it is now possible to reproduce these preparations more consistently than was previously thought. The profile is readily altered if insufficient care is taken during the preparative procedures to avoid

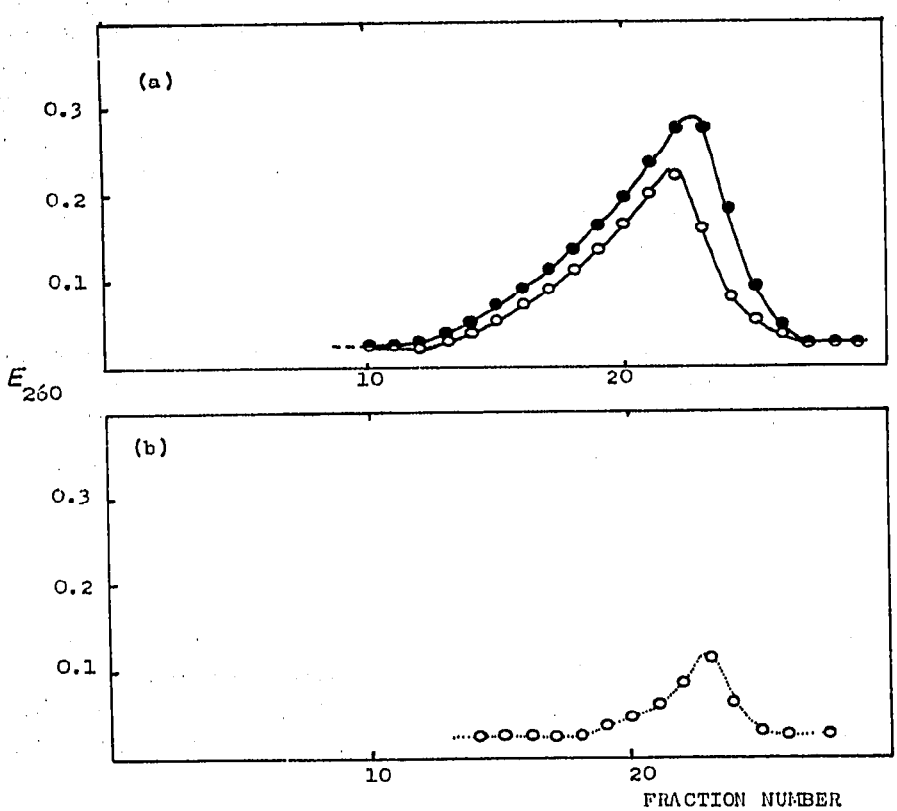

Fig. 2. (a) Fractionation on a 2.5-g PLK column of highly polymerised RNA. ○—○, standard (BDH, Ltd.); ●—●, prepared from *E. coli* strain MRE 600 (see text). (b) R17 phage replicative form RNA.

chemical and mechanical degradation, which can give rise to the loss of the smaller peaks at 1.2–1.3 *M* NaCl. The composition of these paeks is known from previous studies[1]. RNA is found exclusively in the 1.0–1.1 *M* NaCl and the 1.2–1.3 *M* NaCl peaks, and is the major constituent of the 1.6–1.8 *M* NaCl paek. DNA comprises the 2.0–2.2 *M* NaCl peak and small quantities can also be detected by assay in the 1.6–1.8 *M* NaCl (see AYAD AND BLAMIRE[1] for further details). Indications that the RNA in the 1.6–1.8 *M* NaCl peak could be of a high molecular weight were obtained when, upon heating these nucleic acid mixtures to 100° for 10 min in dilute saline citrate and cooling rapidly, it was found that the 1.6–1.8 *M* peak moved from this high salt molarity and the material so formed eluted much closer to the 1.0–1.1 *M* NaCl peak, which seemed unaffected by this treatment. Thus it was determined to investigate the nature of this peak more fully.

Fig. 2a shows the elution profiles of highly polymerised RNA from *E. coli* (BDH, Ltd.), used as a standard, and RNA extracted by method (b) (see PROCEDURES) from *E. coli* strain MRE 600. In both cases only one peak is observed with its maximum at a salt molarity of 1.85–1.95 *M*. However, because of the method of preparation nothing can be deduced about the physical structure or molecular configuration of RNA molecules, and the heterogeneity of the sample is probably a major contributory factor to the long leading edge of the peak. The sedimentation coefficients of the fractions at the maxima of the peak are high, being in the range of 18.8–24 S, depending on the sample preparation. Thus it would appear that the original belief as to the molecular weight of the RNA found in this region of the profile in nucleic acid mixtures was correct. Unlike the nucleic acid mixture 1.6–1.8 *M* NaCl peak, however, no protein or DNA can be detected by chemical assay [8,9].

R17 phage replicative form RNA (a gift from Dr. T. FRANKLIN), which is known to be double standard[10], elutes as shown in Fig. 2b with a maximum at 1.9 *M* NaCl. This is very close to the maximum observed during highly polymerised RNA fractionation, and would indicate that the secondary structure of the RNA is influencing the elution profile. This is similar to the effect noticed when the secondary structure of DNA is altered[7]. Consideration as to the role that secondary structure and conformation play in the fractionation of RNA, led to the investigation of ribosomal RNA fractionation. The preparative procedures adopted in the isolation of ribosomal RNA, which do not involve chemical deproteinisation or excessive mechanical shear, have been shown to give a closer degree of integrity between molecules[6] and hence less breakdown products which are normally associated with the other methods of preparation.

The results are shown in Fig. 3a. Ribosomal RNA was fractionated at two different concentrations and it was found that at the lower concentration (approx. 500 μg) better resolution was obtained between the two peaks observed at 1.7 and 1.8 *M* NaCl, respectively. Two other peaks, when the higher concentration (approx. 1300 μg) of ribosomal RNA was fractionated, could be observed at 1.1 and 1.25 *M* NaCl. Sedimentation studies were carried out on fractions from all these peaks and the results are shown in Fig. 3b. As can be seen, the peaks at 1.1 and 1.25 *M* NaCl correspond in S value and NaCl molarity to sRNA as observed in the fractionation of nucleic acid mixtures. However the peaks at 1.7 and 1.8 *M* NaCl have S values which correspond to the two units of the bacterial ribosome when they have been stripped of protein. They are also eluted in the region of the highly polymerised and double standard RNA.

This leads us to the conclusion that besides molecular weight and secondary structure being important principles in the fractionation of RNA, tertiary structure or conformation of the molecules is also important.

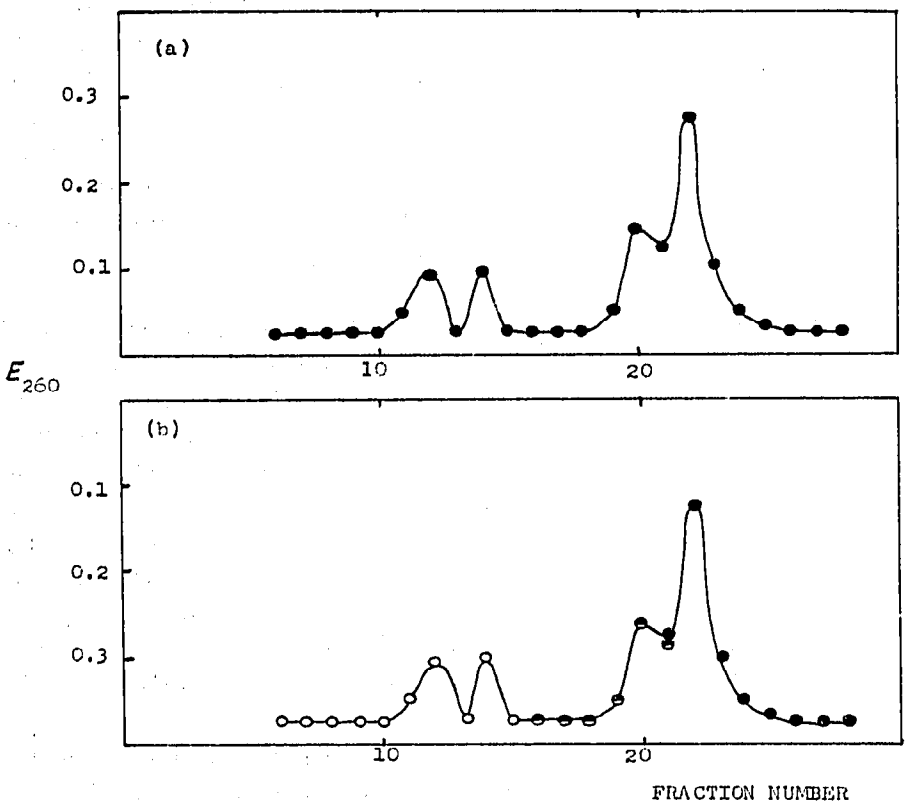

Fig. 3. (a) Fractionation of ribosomal RNA on a 2.5-g PLK column. (b) Study of the sedimentation coefficients of the fractions eluted in (a). O—O, fractions having S values of about 3.5 S; ◒—◒ fractions having S values of 16 S; ●—●, fractions having S values in the range of 23–26 S. (The higher S values are probably due to the incomplete dissociation of protein.)

sRNA fractionation has also been studied, and in Fig. 4a the result of fractionating sRNA type XI from yeast can be seen. The main peak at 1.0–1.1 *M* NaCl is the same as in the previous studies, however there is a notable absence of the 1.2–1.3 *M* NaCl peak, and a significant increase in the region immediately prior to the 1.0 *M* NaCl peak and in the oligonucleotides of low molecular weight found at the beginning of each fractionation. This would suggest in the light of our previous findings a degree of degradation in the preparation. Fig. 4b shows the elution profile obtained from a fractionation of ribonucleic acid "core" (BDH, Ltd.). This is resistant to digestion by ribonuclease, and has been tested in this laboratory. Three peaks can be seen and the major one, although disperse, elutes between 1.0–1.3 *M* NaCl. This could represent double standard portions of RNA which are resistant to ribonuclease[10], but are of lower molecular weight than that found in the R17 phage double standard RNA, and hence elute earlier.

Certain preliminary conclusions can be drawn from this work on RNA fractionation. Firstly, the method of preparation is very important. RNA molecules, particularly those with a high degree of tertiary structure, are very susceptable to degradation. Secondly, the method of PLK chromatography has been able to

separate all the various types of nucleic acids from one another in a quick, convenient manner in high yield (80–90% recovery). Also, the method is a rapid way of ascertaining the purity and homogeneity of any particular preparation which in itself is a useful

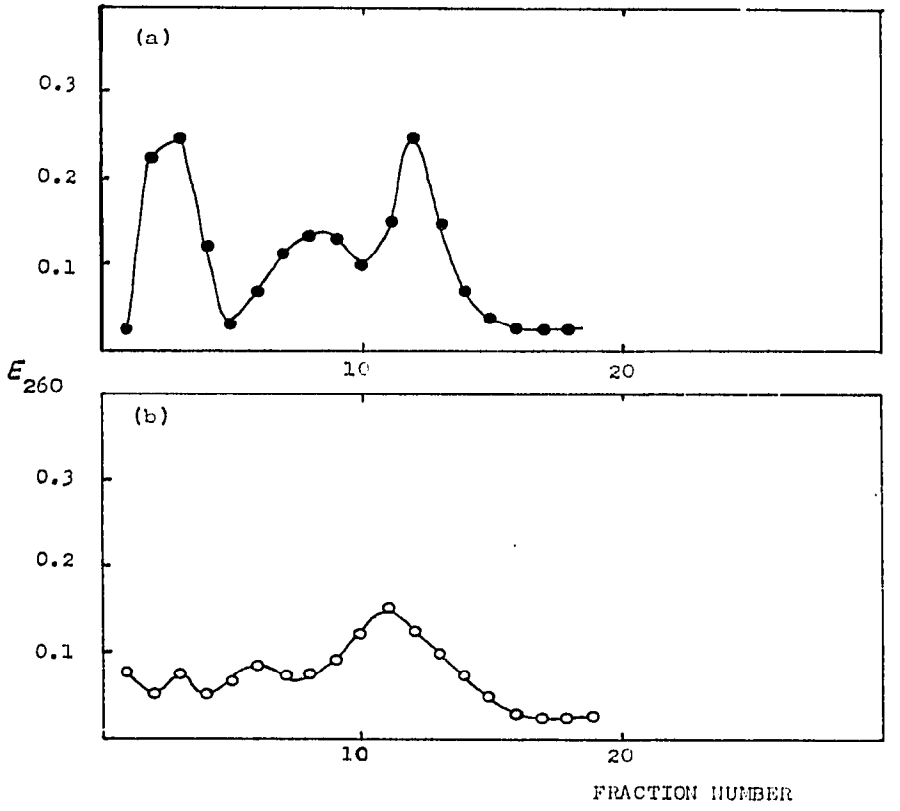

Fig. 4. (a) sRNA type XI (Sigma Chemical Co.) eluted from a standard 2.5-g PLK column. (b) Elution profile of ribonucleic acid "core" (BDH, Ltd.) known to be RNase resistant.

ability. Finally the indications are that definite binding sites, possibly associated with short regions of double standardness, exist on the nucleic acid molecules which interact specifically with the PLK and that it is the manner, number, and distribution of these binding sites on the nucleic acid molecule which determine its salt molarity of elution.

Investigation of binding sites

To confirm that one of the determining factors in fractionation was the arrangement of the binding sites on the nucleic acids, experiments were conducted in which the number and concentration of receptor sites on the PLK column were varied in a controlled manner. This was done in two ways. Firstly, the concentration of polylysine in a standard 2.5-g PLK column was varied in a linear manner whilst keeping all other parameters, *e.g.* DNA concentration, eluting buffers, etc. constant. When the ratio of polylysine to kieselguhr fell below 1×10^{-3} (w/w) it could be seen that, in the fractionation of DNA, the major peak at approx. 2.0 *M* NaCl began to broaden with increasing amounts of material eluting at lower salt molarities. This effect was most marked at the lower ratio of 0.2 and 0.4 $\times 10^{-3}$ (w/w). This is interpreted to mean that once a DNA molecule is trapped by one or more binding sites on the column the re-

maining sites of attachment can only be saturated if the polylysine is available in sufficient quantity, and that the point of release of any given nucleic acid molecule from the column is a function of the number of binding sites on that molecule that are attached to the PLK. These results would argue against the idea that it is the strength of the bond between the polylysine and the nucleic acid molecule that determines its point of elution.

Secondly, polylysine was purchased with a mol. wt. of 175 000 (normal polylysine average mol. wt. 50 000) and this was used in the fractionating system. When experiments were carried out in which the polylysine/kieselguhr ratio was maintained at 1×10^{-3} (w/w) no differences could be observed from the standard runs using normal polylysine. Thus the presence of additional receptor sites per molecule of polylysine cannot raise the eluting salt molarity for any of the different types of nucleic acids so far tested. Once again the indication is that the binding sites on the nucleic acid molecules determine the elution patterns.

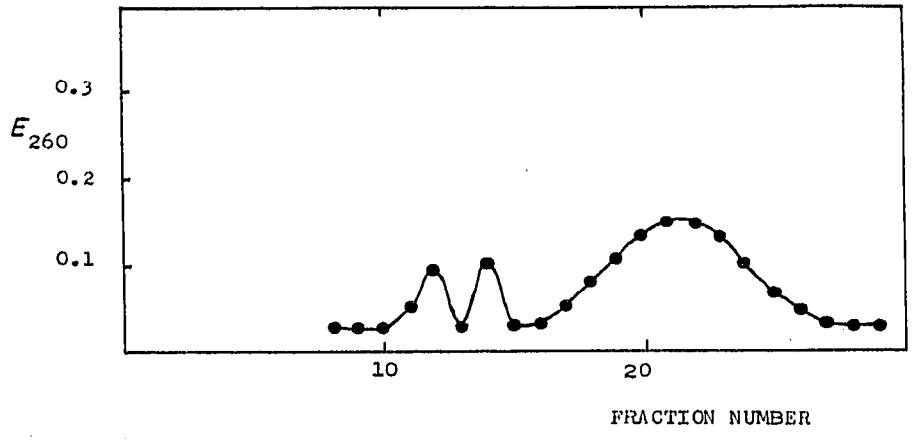

Fig. 5. Elution profile of a ribosomal RNA preparation after heat denaturation, from a 2.5-g PLK column.

Fig. 5 shows the effect of heat denaturation on the elution profile of ribosomes. A ribosomal preparation was heated to 100° in medium I buffer for 10 min and cooled quickly to room temperature. On fractionation a single rounded peak eluted in the region associated with highly polymerised RNA could be seen. Furthermore two smaller peaks at 1.1 and 1.25 *M* NaCl could be observed which were unaffected by the heat treatment. When sRNA was treated similarly no alteration in elution profile could be noted.

Heat treatment is affecting the binding of RNA of high molecular weight to PLK, possibly by destroying or modifying binding sites in some way. This results in a mixture of molecules with a Gaussian distribution of binding sites instead of two distinct species each with a definite number of binding sites. The reason why sRNA remains unaltered by this treatment may lie in the fact that rapid renaturation takes place on cooling[11], and the fact that there can only be a small number of binding sites per sRNA molecule.

In an attempt to isolate the binding sites of RNA and DNA molecules the following procedure was adopted. A standard 2.5-g PLK column was prepared and loaded with highly polymerised RNA in the usual way. 1 ml of an RNase solution in 0.4 *M* NaCl buffer was then run through the PLK–RNA complex at a flow rate of 22 drops per

min followed by 0.4 *M* NaCl buffer. The column was then washed with twice its fluid volume of 0.4 *M* NaCl buffer to remove digestion products not held on the column, and then the remaining material was eluted in the usual way (see Fig. 6a). As the profile shows, no material is eluted in the 1.6–1.9 *M* NaCl region of highly polymerised RNA; instead the non-digested material comes in a diffuse series of peaks in the region of 0.4–1.4 *M* NaCl. Although very little can be deduced from such a profile it is clear that some material was protected from RNase digestion, and that these were probably the binding sites to the polylysine. The fact that they were eluted at lower salt molarities than the parent molecule would indicate that the enzyme had been able to attack regions between binding sites hence giving molecules not only of a lower molecular weight but also with fewer binding sites per molecule.

Fig. 6b shows the elution profile of DNase digestion of a PLK–DNA complex. The method adopted was similar to that used for the PLK–RNA complex except that an environment of 10 *m*M Mg^{2+} was required for the enzyme to act. The profile of the non-digested material had altered but not in the dramatic manner of the highly polymerised RNA. The bulk of the DNA seemed to have been unaffected by the enzyme, but a certain reduction in the peak height coupled with a broad leading edge indicates that some parts of certain molecules were attacked and in consequence the total number of binding sites per molecule was reduced.

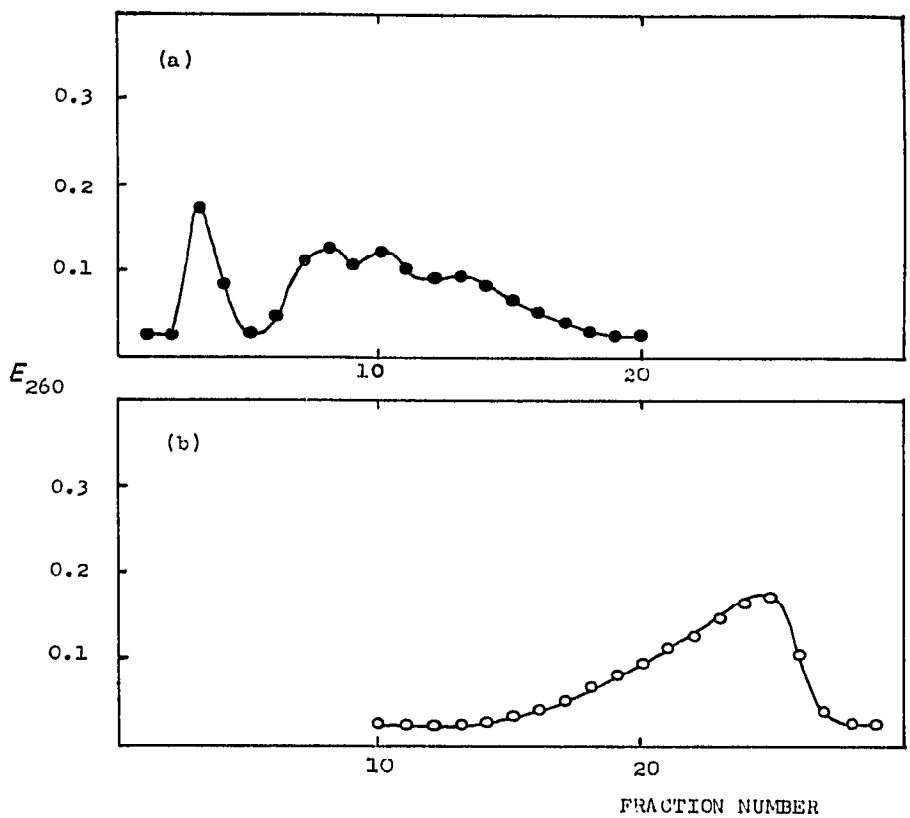

Fig. 6. (a) Resulting elution profile when a highly polymerised RNA–PLK complex is predigested with RNase (see text). (b) Similar experiment involving DNase digestion of a DNA–PLK complex in a Mg^{2+} environment.

Dye-binding studies

OLINS *et al.*[12], working with lysine-rich histone f1 and polylysine, studied the

interaction of ethidium bromide with DNA in the presence and in the absence of the histone and polyamine. From his results he showed that polylysine in solution will prevent completely the binding of the dye, whereas the presence of histone f1 will only reduce the amount of dye bound and not affect at all the binding constant.

Histone f1, it was deduced, is therefore binding to the DNA in a manner which does not materially affect the dye binding, which probably means it is binding in the large grove of the DNA molecule. Polylysine on the other hand, when in solution, will prevent dye binding because it binds in the small grove[13]. It was decided to try and determine which of the above two models PLK–DNA complexes would follow. Acriflavine was chosen as the dye because of its similarity in binding studies to ethidium bromide[14–16]. Two types of experiment were carried out. Firstly, the dye was bound to the DNA in solution before loading onto a PLK column which had been presaturated with the dye at 10^{-4} *M* concentration, and eluted with buffers which also contained 10^{-4} *M* acriflavine. No difference was observed in the elution profile, with the possible exception of a slight rounding off of the peak. However, the top of the fractionating layer of the PLK column became strongly yellow for about 1.5 cm. The majority of this band disappeared when the DNA was eluted. It was clear that the DNA–PLK complex was still being formed in the presence of the dye, and that dye was still bound even after the formation of the PLK–DNA complex.

The second type of study involved first forming the DNA–PLK complex and then passing 0.4 *M* NaCl buffer containing 10^{-4} *M* acriflavine through the column. These experiments showed clearly that the complex would still bind the acriflavine even after it had been formed. On passing the acriflavine-containing buffer over the complex the yellow band appeared again at the top of the fractionating layer, and the dye was removed from the buffer completely. It was not until the top 1.5 cm were completely saturated with dye that the eluate from the column again contained acriflavine. The use of a dilute acriflavine solution in this way demonstrated conclusively that the PLK–DNA complex was still able to bind dye.

Thus, of the two models shown earlier, it would appear that the PLK column behaves more like an artificial lysine-rich histone than a free solution of polylysine.

Competitive exchange experiments

These experiments were designed to show the degree to which a nucleic acid molecule, once it was bound to PLK, was free to dissociate itself from its binding site and exchange with other bound molecules on other receptor sites. A 1-g PLK column was prepared as described previously[7] and connected to a Uvicord recording system. In this way the eluate from the column can be continuously monitored for UV absorbance. A solution of sRNA at a concentration of 100 μg/ml in 0.4 *M* buffered saline was first passed through the Uvicord and the position of maximum absorbance noted. The column was then connected to the Uvicord and the solution of sRNA passed through the column at a steady rate of 33 drops/min. The profile recorded on the chart recorder can be seen in Fig. 7a. Once the plateau value reached was the same as for sRNA alone the flow was halted and the column washed with 0.4 *M* buffered saline until the absorbance returned to zero. In order to check that all the available binding sites on the 1-g column were saturated with sRNA the 100-μg sRNA solution was reconnected to the top of the column and the flow recommenced. The resulting profile (shown in Fig. 7b) indicates that no more sRNA was bound, as the UV absorbance

returned immediately to 100% (with reference to the sRNA solution) once the fluid volume of the column had been passed. The column was once again washed with 0.4 *M* buffered saline until the UV absorbance again returned to zero.

Eight millilitres of a solution of calf thymus DNA (100 μg/ml in 0.4 *M* buffer) were then passed through the column at a flow rate of 19 drops/min and followed by

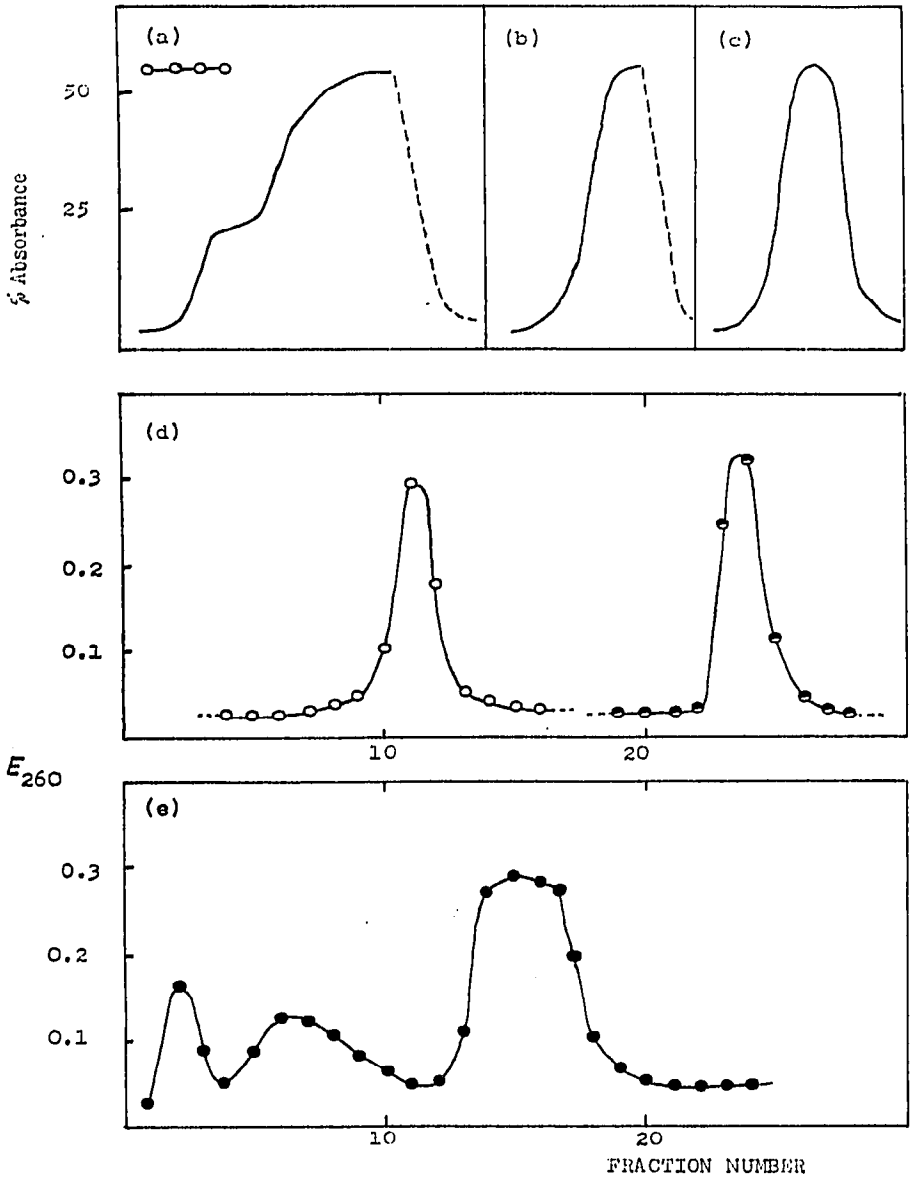

Fig. 7. (a) Uvicord trace obtained during the saturation of receptor sites on a 1-g PLK column using sRNA at 100 μg/ml in 0.4 *M* NaCl buffer. O—O—O—O, maximum UV absorbance of the solution before loading; — — — —, washing of column with 0.4 *M* NaCl buffer back to zero absorbance. (b) Uvicord trace obtained on re-running the sRNA solution through the charged column to test that all receptor sites were occupied. (c) Material eluted from the 1-g PLK sRNA charged column during the loading of DNA (100 μg/ml) at 19 drops/min. (d) O—O—O, standard PLK fractionation of the material collected in (c); ●—●—●, standard PLK fractionation of material eluted between fractions 14–20 in run of Fig. 7e. (e) Elution profile of the 1-g PLK column after the loading of the DNA solution, showing the imperfectly bound DNA peak which re-runs on standard PLK columns as normal DNA (see Fig. 7d).

0.4 *M* buffered saline. The recorded profile is shown in Fig. 7c. The material so eluted was collected and found to be 16 ml in volume. This was then loaded and eluted in the usual way from a standard 2.5-g PLK column. Similarly, the material now held on the 1-g PLK column was also eluted with a linear gradient of phosphate-buffered saline in the usual way. The results can be seen in Figs. 7d and 7e.

The material, which was seen to elute from the 1-g column during the loading of the DNA, when analysed by PLK chromatography and sedimentation studies is seen to be the major peak of sRNA, whilst the 1-g column retains a certain amount of sRNA but all the DNA loaded.

Thus, under the conditions of the experiment, despite the fact that all the receptor sites on the PLK column were saturated with sRNA, DNA was also held, and sRNA was exchanged in a competitive manner. It will be seen from the profile of the 1-g column, however, that the DNA peak is not sharp but is characteristic of DNA which has not been able to bind all its binding sites to the column. If this material is collected, adjusted to 0.4 *M* NaCl and rerun on another 2.5-g PLK column under standard conditions, then the elution profile so obtained is undistinguishable from a normal DNA profile (Fig. 7d).

This type of experiment was also carried out using highly polymerised RNA instead of DNA and a similar pattern of exchange was observed. This time the highly polymerised RNA held on the 1-g column gave a completely normal elution profile. This contrasted with the pattern of DNA elution at this stage, and would indicate that most if not all the RNA binding sites are occupied after the exchange.

DISCUSSION

PLK chromatography has now been extended to RNA fractionation and the findings obtained so far show that it is possible to resolve different species of RNA into separate peaks. We have also shown that RNA fractionation depends in great part on the care that is taken during preparation to avoid degradation, as loss of secondary or tertiary structure will affect the binding of the RNA to PLK, and hence its salt molarity of elution. In this context PLK chromatography is a quick reliable method of ascertaining not only what type of nucleic acids are in any given sample, but it is also a way of determining to what degree each type of nucleic acid is homogeneous.

The binding of nucleic acids to PLK has now been studied in several ways, and from the results a tentative model is proposed whereby certain regions on the nucleic acids (termed binding sites), because of their secondary structure and base composition, have a particular affinity for short lengths of polylysine. The PLK column acts in the manner of a synthetic lysine-rich histone and traps the nucleic acids in an immobile complex near the top of the fractionating layer. This complex is not a static entity, but in 0.4 *M* buffer it is in a continuous state of exchange with molecules of a similar nature. When equilibrium is reached each nucleic acid molecule has a certain number of its binding sites fully occupied and it is a function of a number of these binding sites per molecule which determined the salt molarity of elution.

Because of this ready exchange, which is probably more dynamic in the smaller sRNA molecules, competition for given receptor sites on the PLK column means that DNA, because of its larger number of binding sites and more ordered structure, has a natural advantage. However, it is not able to complex all its binding sites. Highly

polymerised RNA on the other hand, having fewer binding sites and a less ordered structure, will have a greater possibility of saturating all its binding sites under the conditions of exchange. These preliminary experiments open an interesting avenue of research into the size and distribution of the binding sites per molecule, and, coupled with further dye-binding studies, could reveal more about the mechanism of PLK chromatography.

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REFERENCES

1 S. R. AYAD AND J. BLAMIRE, *J. Chromatog.*, 42 (1969) 248.
2 J. MARMUR, *J. Mol. Biol.*, 3 (1961) 208.
3 S. R. AYAD, R. W. BONSALL AND S. HUNT, *Anal. Biochem.*, 22 (1968) 533.
4 H. G. ALBAUM AND H. W. UMBREIT, *Arch. Biochem.*, 19 (1948) 455.
5 M. I. LERMAN, A. S. SPIRIN, L. P. GAVRELOVA AND V. F. GOLOV, *J. Mol. Biol.*, 15 (1966) 268.
6 P. SPITNIK-ELSON AND A. ATSMON, *J. Mol. Biol.*, 45 (1969) 113.
7 S. R. AYAD AND J. BLAMIRE, *Biochem. Biophys. Res. Commun.*, 30 (1968) 207.
8 O. H. LOWRY, N. J. ROESBROUGH, A. LEWIS FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
9 K. BURTON, *Biochem. J.*, 62 (1956) 315.
10 R. LANGRIDGE AND P. J. GOMATOS, *Science*, 141 (1963) 694.
11 I. GILLAM, S. MILLWARD, D. BLEW, E. I. VON TIGERSTROM, E. WIMMER AND G. M. TENER, *Biochemistry*, 6 (1967) 3043.
12 D. E. OLINS, *J. Mol. Biol.*, 43 (1969) 439.
13 D. E. OLINS, A. L. OLINS AND P. H. VON HIPPEL, *J. Mol. Biol.*, 24 (1967) 151.
14 L. S. LERMAN, *J. Mol. Biol.*, 3 (1961) 18.
15 L. S. LERMAN, *J. Cellular Comp. Physiol.*, 64 (1964) 1.
16 D. M. NEVILLE AND D. R. DAVIES, *J. Mol. Biol.*, 17 (1966) 57.