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FRACTIONATION OF NUCLEIC ACIDS USING POLYLYSINE KIESELGUHR CHROMATOGRAPHY

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SUMMARY

Polylysine kieselguhr chromatography, which has been used to fractionate bacterial DNA, has now been employed to separate the various components of natural DNA–RNA mixtures. Different genetic markers on the bacterial chromosome are also shown to be partially separated using this method.

INTRODUCTION

Many methods of nucleic acid fractionation have been tried, *viz.* methylated albumin-coated kieselguhr (MAK)[1,2], IRC-50 (a polycarboxylic resin in the Mg^{2+} ion form[3]), hydroxyapatite[4], silk fibrion[5], and ECTEOLA cellulose[6]. Columns of this sort, and several others, have been used to obtain a degree of separation between nucleic acid fractions differing in molecular weight, secondary structure or base composition.

In an attempt to devise a better way of fractionating DNA isolated from *Bacillus subtilis* strain Marburg, AYAD AND BLAMIRE[7] used a column of kieselguhr on which poly-L-lysine (PLK) was absorbed. This procedure was found to resolve the DNA into three components. Investigation showed that the main factor influencing the elution pattern was base composition and that molecular weight and secondary structure played only lesser roles.

The theory behind the method is based on the association of nucleic acids with nucleohistones[8], and the fact that they can be broken down by increasing concentrations of salt[8,9]. Many authors[10,11] have reported that histones are rich in lysine and that polylysine preferentially interacts with specific base sequences in DNA[12]. This was the basis on which the PLK column was developed.

PROCEDURES

Strains of bacteria

Bacillus subtilis strain Marburg served as the wild type, and strains 312 and 234 were used as donors. Strain I^-, an auxotroph requiring indole or tryptophan for

growth, and strain 31, an auxotroph requiring histidine and tryptophan for growth, were also employed. *Escherichia coli* strain B was also used.

Preparation of DNA and nucleic acid mixtures

Bacterial DNA was prepared from the required strain by the method of MARMUR[13], which was modified for the preparation of nucleic acid mixtures. The RNase stage was omitted, and the mixture was precipitated from standard saline citrate with two volumes of 95% ethanol. All the preparations were stored at 4°.

Preparation of polylysine kieselguhr columns

Standard PLK columns were prepared by the method devised by AYAD AND BLAMIRE[7].

Samples of nucleic acids to be fractionated were diluted to a concentration of 100 μg/ml in 0.4 *M* NaCl containing 0.02 *M* KH_2PO_4, adjusted to pH 6.7 with 2 *N* NaOH and loaded onto the column under an air pressure of 3 lb./sq. in. These were then eluted using a linear molarity gradient of 0.4 *M* to 4.0 *M* NaCl (150 ml or 100 ml each) using the method described by AYAD *et al.*[14]. The extinction of the eluate was continuously monitored using a Uvicord recording system at 257 mμ, and 4 ml fractions were collected using an LKB (Stockholm) fraction collector linked to the Uvicord system.

Sedimentation coefficients were determined in a Spinco model E centrifuge fitted with ultraviolet optics. Determination was carried out at 35 000 r.p.m. using DNA solutions in a concentration of 50 μg/ml dissolved in 0.15 *M* NaCl containing 0.015 *M* sodium citrate adjusted to pH 7.0.

Melting temperature of DNA

DNA and samples were examined for temperature dependence of extinction. The samples under investigation were dialysed against 0.15 *M* NaCl containing 0.015 *M* sodium citrate adjusted to pH 7.0, and the extinctions of the resulting solutions were measured at 260 mμ with an Optica recording spectrometer having a heated cell holder. Temperatures were measured with a calibrated thermistor fitted into the top of the cuvette. Solutions of DNA were placed in 1.0 cm cuvettes, degassed under reduced pressure and covered with a layer of liquid paraffin to prevent evaporation.

Analytical caesium chloride density gradient centrifugation

DNA (1–2 μg/ml) was added to solid CsCl (optical grade, Harshaw Chem.) to give a refractive index of 1.400 and centrifuged at 44 770 r.p.m. in the model E ultracentrifuge[15]. Photographs were taken after 20 h, by which time complete equilibrium had been attained.

RESULTS

Fig. 1 shows the results obtained when a sample of the nucleic acid mixture is fractionated on a 5 g PLK column in which the fractionating layer consists of 5 g kieselguhr and 5 mg polylysine, using a linear salt gradient (0.4–4.0 *M*) of 100 ml of each.

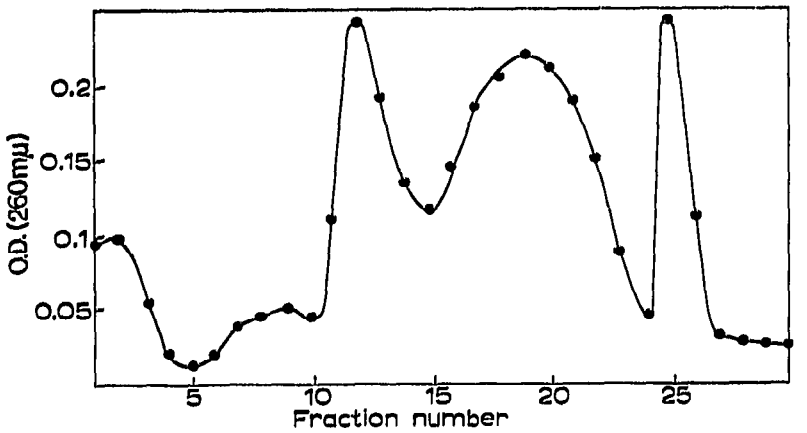

Fig. 1. Elution profile at 260 mμ of a nucleic acid mixture obtained from *B. subtilis* strain Marburg (see text). Fractionation on a 5 g PLK column using a linear salt gradient between 0.4–4.0 *M* NaCl.

Four distinct regions are visible, eluting at the salt molarities of 0.5 *M*, 1.1 *M*, 1.6 *M*, and 2.0 *M*. The first of these, at 0.5 *M* NaCl, consists of nucleotides and oligonucleotides of low molecular weight, as shown by the fact that most of this material can be removed by dialysis. The remaining peaks are all native polynucleotides of high molecular weight and appropriate sedimentation coefficients.

If samples are taken from each of the fractions and assayed for RNA, DNA and protein[16–18], the results show that the 2.0 *M* NaCl peak consists of DNA, with a small amount of protein (see Figs. 2 and 3). The 1.1 *M* NaCl peak consists of RNA, whilst the 0.5 *M* NaCl peak contains both ribonucleotides and deoxyribonucleotides. The material in the 1.6 *M* NaCl peak consists of a complex containing RNA, DNA and protein as ascertained by assay. However, this is not in the form of a simple mixture, since DNase digestion removes the 2.0 *M* NaCl peak but does not remove the material in the 1.6 *M* NaCl peak. RNase digestion acts on both the 1.1 *M* and 1.6 *M* NaCl peaks, removing most of the constituent RNA and increasing the amount of the 0.5 *M* NaCl peak.

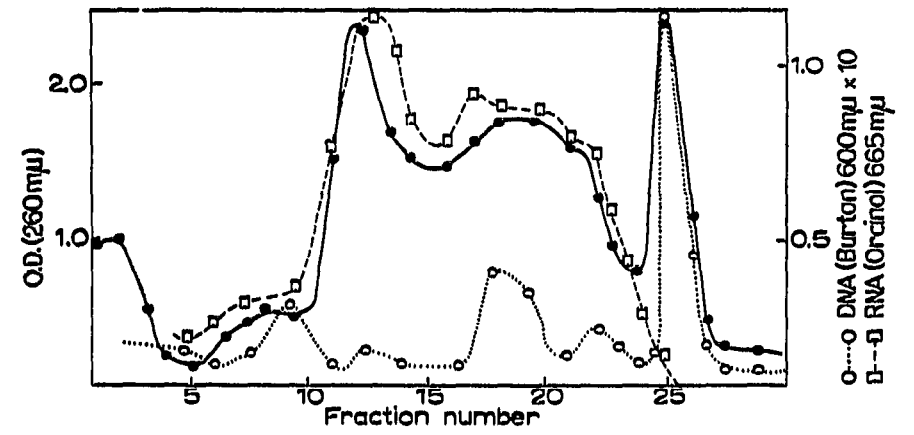

Fig. 2. Results obtained when samples are taken from a fractionation of a nucleic acid mixture (see legend to Fig. 1) and assayed for RNA[16] and DNA[17]. ●—●, O.D. at 260 mμ; ○ · · · · ○, DNA assay; □— — — —□, RNA assay.

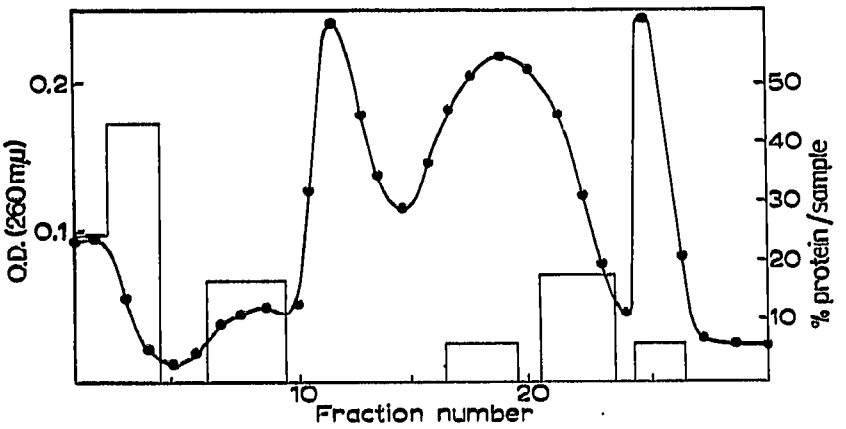

Fig. 3. Result obtained when samples are taken from a fractionation of a nucleic acid mixture (see Fig. 1) and assayed for % protein by the method of LOWRY[18]. ●—●, O.D. at 260 mμ. The histogram represents the % protein of the original unfractionated nucleic acid sample per fraction.

Further confirmation of these findings is provided by the elution profiles in Fig. 4. They are: (1) s-RNA extracted from yeast by the method of HOLLEY *et al.*[19] and supplied commercially by Sigma Chemical Co., and (2) bacterial DNA prepared by the method of MARMUR[13] from *Bacillus subtilis* strain Marburg.

In both these preparations the 0.5 *M* NaCl peak can be seen and in the s-RNA profile the main peak is at 1.1 *M* NaCl. The results obtained with bacterial DNA confirm the findings described in our previous paper[7], where three peaks were also observed, with the main peak appearing at 2.0 *M* NaCl.

The significant difference between the results obtained with whole cell nucleic acid mixtures and the individual DNA and RNA preparations is the 1.6 *M* NaCl peak. The shape of this peak can be altered by increasing the protein concentration. This is done by omitting one or two of the deproteinising steps during the preparation. Elution of this material from a PLK column followed by assay of the fractions for the protein constituent, will give the results shown in Fig. 5. The bulk of the protein

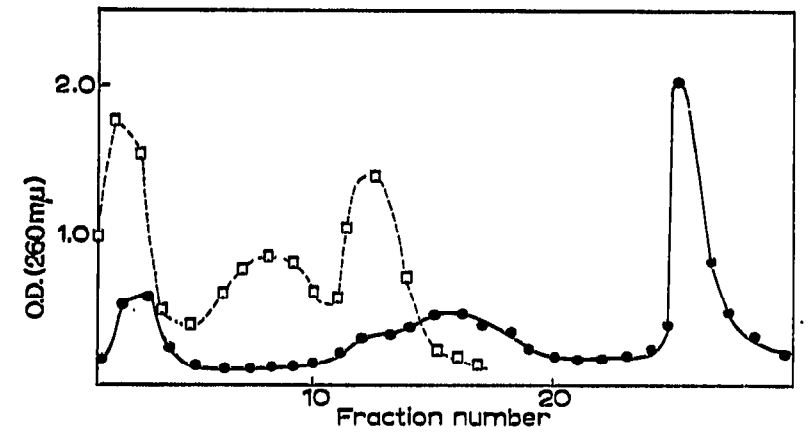

Fig. 4. Elution profiles at 260 mμ of yeast s-RNA (2 mg) and *B. subtilis* DNA (1 mg) from 5 g PLK columns. □— — —□, s-RNA; ●—●, DNA.

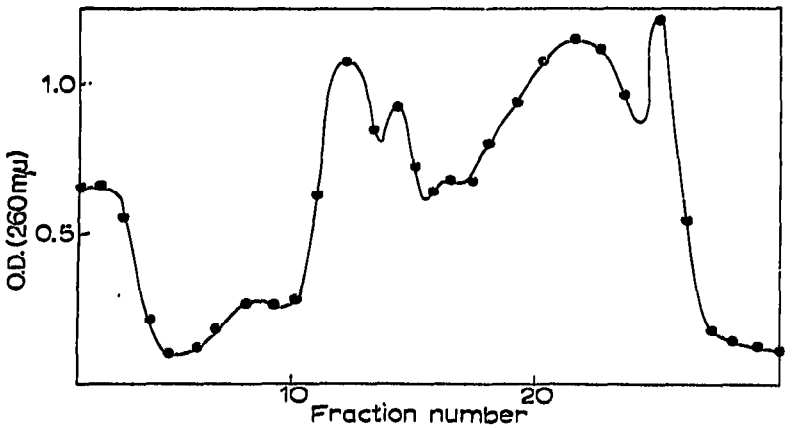

Fig. 5. Fractionation on a 5 g PLK column of a protein-rich nucleic acid mixture (see text), showing the post-RNA peak at 1.2–1.3 *M* NaCl.

does not seem to be held by the column, and is eluted along with the oligonucleotides of low molecular weight. The second largest peak is also eluted early, and precedes the 1.1 *M* NaCl RNA peak by one or two fractions. It is in this region that certain amounts of RNA and DNA are to be found (see Figs. 2–5), so it may be that, at this point, material is eluted which is only weakly adsorbed on the column. This in turn would indicate that it is of a lower molecular weight than the bulk of the polynucleotide material, but of a higher weight than the very low molecular weight oligonucleotides found in the 0.5 *M* NaCl peak.

Two peaks of protein are found within the 1.6 *M* NaCl peak, the main one of which lies very close to the DNA peak and is believed to cause the lopsided appearance of the 1.6 *M* NaCl peak at high protein concentration. It is this protein peak that is progressively removed by successive deproteinisations. The other, smaller, protein peak remains fairly constant, but varies from preparation to preparation, as does the amount of RNA and DNA. The stage of growth of the cells seems to influence the overall nature of the 1.6 *M* NaCl peak significantly.

In certain preparations a small RNA peak can also be observed immediately after the main RNA peak at 1.1 *M* NaCl (Fig. 5). This peak, at 1.2–1.3 *M* NaCl, is not always found in our preparations and varies considerably in size. However, other workers[20,21] have reported RNA peaks at higher salt molarities, which appear when considerable care is taken to ensure that the preparations are not degraded by ribonucleases. This would indicate that our preparations are not entirely free of RNase contamination during the early stages of purification, and that this post-RNA peak, being of a higher molecular weight than the 1.1 *M* NaCl RNA, is more readily attacked and degraded to a lower molecular weight. The RNA in the 1.6 *M* NaCl region may or may not be of high molecular weight, but seems to be in some sort of association with DNA and protein which causes it to be eluted at this salt molarity.

If the mixture of polynucleotides, normally stored in standard saline citrate, is first dialysed overnight at 4° against distilled water before fractionating, the resulting elution profile is slightly altered. The greater part of the 0.5 *M* NaCl peak disappears, leaving only a small sharp peak at about 0.59 *M* NaCl. The 1.1 *M* NaCl RNA peak moves, by one fraction, to a lower eluting salt molarity, as does the DNA

peak. Heat denaturation of the sample, however, causes reduction of the DNA peak, and moves it by four fractions to a lower eluting salt molarity.

Whilst studying the parameters of bacterial DNA fractionation it was found that base composition was the most important factor, with secondary structure and molecular weight playing smaller but significant roles[7]. This finding has now been confirmed in a number of ways.

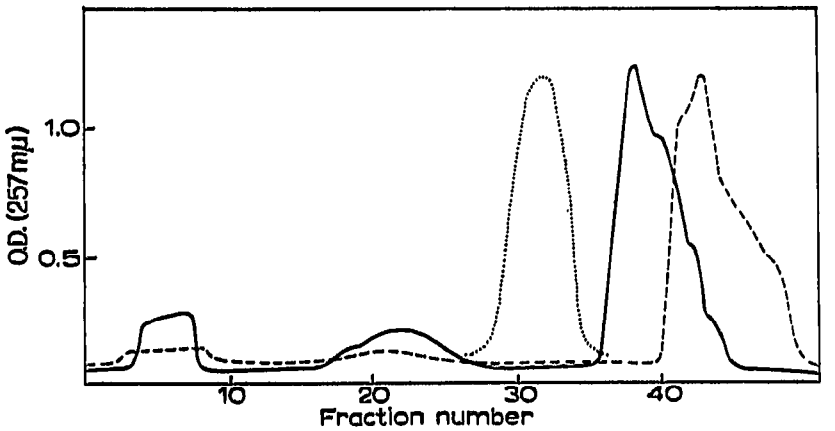

Fig. 6. Continuous elution profile from a 10 g PLK column monitored using an LKB Uvicord U.V. spectrophotometer of DNA (1.5 mg) isolated from calf thymus (· · · · ·), *B. subtilis* (————), and *E. coli* (— — — —).

Elution profiles from PLK columns (10 g of fractionating layer) using calf thymus DNA, *B. subtilis* DNA and *Escherichia coli* DNA can be seen in Fig. 6. Calf thymus DNA is eluted first in a single peak. *B. subtilis* DNA exhibits the usual three peaks, which can also be seen in the *E. coli* DNA fractionation, but with a reduced middle peak. The main *E. coli* peak is also eluted at a higher salt molarity than that of *B. subtilis*, which is consistent with the fact that the average guanine–cytosine content of *E. coli* DNA is higher than that of *B. subtilis*[22].

The bouyant densities of the fractions within the 2.0 *M* NaCl peak have been determined using caesium chloride density gradient centrifugation in a Beckman Model E ultracentrifuge as described above. The sample of bacterial DNA used in fractionation and determinations had been very extensively deproteinised, and the PLK column used had a 10 g fractionating layer. The results obtained confirm our previous findings that the DNA fractions eluted at the higher salt molarities are richer in the bases guanine and cytosine. Similarly the melting temperatures of these fractions in standard saline citrate show an increase across the peak. Using the formula of MANDEL AND MARMUR[23] these melting temperatures, when translated in % guanine–cytosine, indicate a 5.5% increase between adjacent fractions.

All these results show that PLK columns can separate DNA regions which differ in chemical structure. Thus it was decided to investigate whether or not separation of biologically active markers could be achieved using this method.

The assay chosen to test the DNA fractions for transforming ability was that devised by AYAD *et al.*[24]. 1.5 mg of DNA isolated from strain 234 of *B. subtilis* was fractionated on a 10 g PLK column, and samples of DNA were taken from the three peaks obtained. These samples were then used to try and transform the auxotrophic

bacterial strain *B. subtilis* I⁻ to tryptophan independence. It was found that only DNA in the 2.0 *M* peak had this ability, the other two peaks giving consistently negative results.

Subsequently successive fractions across the 2.0 *M* peak were tested in a similar way. The results obtained indicated that, despite the fact that the DNA samples were above the saturating concentration in each case, the biological activity for the tryptophan gene was not uniform but had a definite maximum in the third fraction.

B. subtilis strain 31, which requires histidine and tryptophan for growth, was then used as the recipient auxotrophic strain, and the DNA fractions were tested again. The results, shown in Fig. 7, confirmed the findings related to the tryptophan gene (B), where the broken line indicates O.D. 260 mμ, and the histogram % transformation. It can also be seen that the histidine gene (A) has its maximum at fraction 38. Thus some degree of separation between these two genes is achieved. It should be remembered that 4 ml fractions were collected and that it was not always possible to start the 2.0 *M* peak exactly at the beginning of fraction 37. Thus there is an inevitable overlap of results.

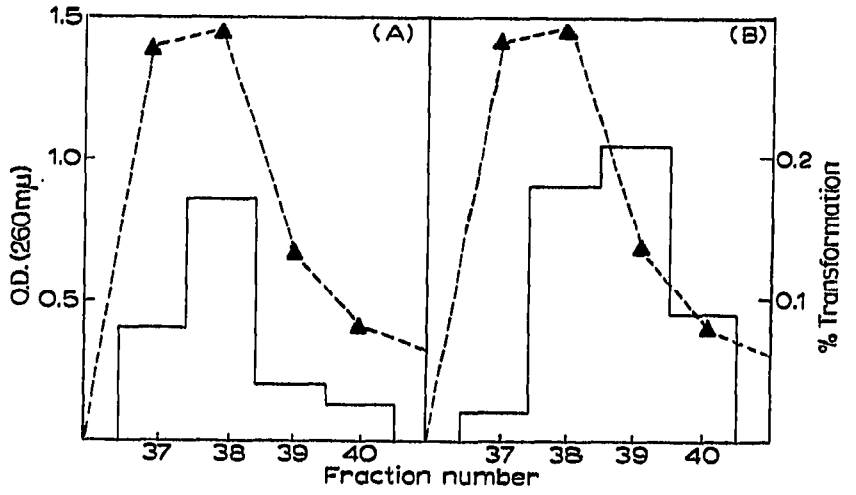

Fig. 7. Results obtained when DNA isolated from the Marburg strain of *B. subtilis* (1.5 mg) is fractionated on a 10 g PLK column[7] and the fractions are assayed for transforming activity using a competent strain of *B. subtilis* (Hist⁻, and Tryp.⁻). ▲— — —▲, O.D. at 260 mμ of the DNA peak eluted at 2.0 *M* NaCl. The histogram represents the % transforming activity for (A) histidine gene, and (B) tryptophan gene.

CONTROL EXPERIMENTS

Provided that the ratio of polylysine to kieselguhr remains constant, the size of the fractionating layer does not affect the results or the elution profile, but more DNA can be loaded onto a larger column.

Linear molarity gradients of buffered sodium chloride were used to elute the nucleic acid samples from the PLK columns in the experiments reported. However, this is not the only type of gradient that can be used. Convex molarity gradients of 0.4–4.0 *M* NaCl were found to be entirely satisfactory and no variation in salt molarity at which the peaks were eluted could be detected. Similarly, by maintaining the concentration of buffered saline at the start at 0.4 *M* NaCl, the molarity of the

concentrated buffered saline was reduced to 3.0 *M* NaCl. Once again the elution profile showed no difference as to the salt molarity at which the peaks were eluted, but, as might be expected, all the peaks except the first moved to a higher fraction number.

All the various permutations and combinations of gradients, linear, convex, 150 ml of each buffer, 100 ml of each buffer, 0.4 *M* NaCl to 3.0 *M* NaCl, 0.4 *M* NaCl to 4.0 *M* NaCl have been tired but in each case only the fraction number of each peak varied, never the eluting salt molarity.

A 10 g PLK column was prepared and eluted, but without prior loading of DNA. No peaks were observed. A column was prepared in which the centre layer of kieselguhr was not pretreated with polylysine. This was loaded with 1.5 mg of Marburg strain DNA and eluted using a linear gradient of buffered saline in the usual way. A single large peak was observed with its maximum at fraction number 6. This means that the DNA had not been retained by the column at all, but eluted at the fluid volume of the column.

The 2.0 *M* NaCl peak from a previous run was isolated, dialysed against the starting buffer, and re-run on a 10 g PLK column. Only one peak was observed at a salt molarity of 1.9–2.2 *M* NaCl.

Once a column has been used for a fractionation, provided it has not been allowed to run dry, it can be regenerated by passing the 0.4 *M* buffered saline through it until the ionic strength of the column falls to loading concentration. This column can then be re-used in the usual way.

The amount of polylysine being eluted from the column was tested. A 10 g column was prepared and eluted without loading DNA. Fractions of 4 ml were collected, and 1 ml of each fraction was tested using the protein assay method of LOWRY[18]. This was then compared with a standard curve of polylysine. The amount of polylysine eluted and the position at which it was eluted was noted. The results indicate that 15% of the amount loaded is eluted at 1½ times the fluid volume of the column.

The sedimentation coefficients (see PROCEDURES) of the 1.0 *M* and 2.0 *M* NaCl peaks were determined and found to be 2S and 25S, respectively.

DISCUSSION

From the manner in which the bouyant density and the melting temperature increased when successive fractions were taken from the 2.0 *M* NaCl DNA peak, it was concluded that the DNA within this peak was gradually increasing in guanine and cytosine since higher salt molarities were needed to elute it. This dependence on the base composition for eluting salt molarity is in agreement with the findings we reported previously[7].

Investigation into the fractionation of nucleic acid mixtures, extracted by ethanol precipitation from bacterial cell lysates has shown that s-RNA can be separated from DNA of high molecular weight. PLK chromatography, however, does more than that, for these nucleic acid mixtures contain other components as well. This method has shown that the bulk of the protein left in the preparation is not held by the column but eluted immediately. Other protein peaks can also be observed within the elution profile.

Immediately prior to the 1.0 *M* NaCl s-RNA peak there is a second large protein peak which also contains RNA and DNA. The exact nature of the material found here is not known, but it is likely that most of it consists of degradation products. Within the 1.5 *M* NaCl peak a complex situation is found. There are two regions of protein. The first is not removed by deproteinisation, and is in conjunction with the peak of DNA in this region, which is not removed by DNase. The second protein peak varies from preparation to preparation and can be removed almost completely by successive deproteinisations (see Fig. 5). There is also some protein in association with the DNA of high molecular weight.

RNA is found in three main regions. The bulk of the material is eluted at 1.0 *M* NaCl and consists of s-RNA as shown by orcinol assay[16] and measurements of the sedimentation coefficient. Prior to this peak, as has been mentioned above, there is a small peak in which RNA can be found, and immediately after the 1.0 *M* NaCl peak, smaller peaks of RNA can be seen in some preparations. These peaks are very labile and sometimes are not observed at all. It is believed that the RNA within these peaks is of a higher molecular weight than 2S, and therefore is sometimes degraded to a lower molecular weight, eluted earlier and thus not observed. Other workers[20,21] working on the fractionation of RNA using this method have reported the existence of RNA of higher molecular weight eluted at higher salt molarities.

RNA is also shown to be the bulk of the material in the 1.5 *M* NaCl peak, and can be eliminated using RNase. Within this peak some sort of complex may exist between RNA, DNA and protein and, as KEMP[20] suggests, it is possible that this complex is a naturally occurring hybrid of RNA and DNA, protected in some way by a protein constituent.

The DNA within the 2.0 *M* NaCl peak has been shown to vary in base composition, but the molecular weight remains high and the secondary structure intact as judged from the melting behaviour. This would indicate that the method does not degrade the sample very much. A more conclusive test for this is the fact that the biological activity of the DNA and its ability to transform auxotrophic bacteria to forms without specific growth requirements remains intact even after fractionation. Not only is the biological activity unimpaired, but separation can also be achieved between different genes on the bacterial chromosome. In Fig. 7A a histogram of the biological activity of the DNA for the histidine gene is shown, and a maximum can be observed at fraction number 38. When this is compared with the tryptophan gene (shown in Fig. 7B), the activity is seen in fraction number 38, but maximal activity is found in fraction number 39 where at least a partial separation is achieved.

This is one of the most interesting aspects of the fractionation technique investigated so far. If smaller fractions were collected, the overlap between them could be reduced and a better separation obtained. Also stepwise elution of this peak could be tried, and the exact salt molarity for a particular gene could be established.

PLK chromatography is thus proving a useful tool in the investigation into the nature of nucleic acids from various sources.

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REFERENCES

1 L. S. LERMAN, *Biochim. Biophys. Acta*, 18 (1955) 132.
2 J. D. MANDELL AND A. D. HERSLEY, *Anal. Biochem.*, 1 (1960) 66.
3 F. R. FRANKEL AND C. F. CROMPTON, *J. Biol. Chem.*, 237 (1962) 3200.
4 Y. MIYAZAWA AND C. A. THOMAS, *J. Mol. Biol.*, 11 (1965) 223.
5 T. Y. HUH AND C. W. HELLEINER, *Anal. Biochem.*, 19 (1967) 150.
6 H. S. ROSENKRANZ AND A. BENDICH, *J. Am. Chem. Soc.*, 81 (1959) 902.
7 S. R. AYAD AND J. BLAMIRE, *Biochem. Biophys. Res. Commun.*, 30 (1968) 207.
8 C. F. CROMPTON, R. LIPSHITZ AND E. CHARGAFF, *J. Biol. Chem.*, 206 (1954) 499.
9 J. PALOU, J. F. PARDON AND B. M. RICHARDS, *Biochim. Biophys. Acta*, 138 (1967) 633.
10 P. F. DAVISON AND J. A. V. BUTLER, *Biochim. Biophys. Acta*, 15 (1954) 439.
11 E. W. JOHNS, *European J. Biochem.*, 4 (1968) 437.
12 M. LENG AND G. FELSENFIELD, *Proc. Natl. Acad. Sci.*, 56 (1966) 1325.
13 J. MARMUR, *J. Mol. Biol.*, 3 (1961) 208.
14 S. R. AYAD, R. W. BONSALL AND S. HUNT, *Anal. Biochem.*, 22 (1968) 533.
15 H. MESELSON, F. W. STAHAL AND J. VINOGRAD, *Proc. Natl. Acad. Sci.*, 43 (1957) 581.
16 H. G. ALBAUM AND H. W. UMBREIT, *Arch. Biochem.*, 19 (1948) 455.
17 K. BURTON, *Biochem. J.*, 62 (1956) 315.
18 O. H. LOWRY, N. J. ROESBROUGH, A. LEWIS FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
19 R. W. HOLLEY, J. APGAR, J. FARROW, M. A. MARINI AND S. H. MERRILL, *J. Biol. Chem.*, 236 (1961) 200.
20 C. J. KEMP, *Ph. D. Thesis*, Manchester, 1968.
21 F. BROMLEY, personal communication.
22 L. R. HILL, *J. Gen. Microbiol.*, 44 (1966) 419.
23 M. MANDEL AND J. MARMUR, *Methods in Enzymology*, Vol. XII, Academic Press, New York and London, 1967, p. 195.
24 S. R. AYAD, G. R. BARKER AND J. WEIGOLD, *Biochem. J.*, 107 (1968) 387.