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# RNA FRACTIONATION ON HYDROXYAPATITE COLUMNS

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## 1. INTRODUCTION

Various chromatographic methods are employed for the resolution of different types of ribonucleic acids (*viz.*, tRNA, rRNA, viral RNA, dsRNA, mRNA, RNA–DNA hybrid, etc.). These methods include the use of one or a combination of more than one ion exchangers and/or adsorbents: pure cellulose and different modified celluloses[1–3]; Kieselguhr columns impregnated with different naturally available basic proteins and synthetic polyamino acids[4]; different reversed-phase columns[5]; starch, agar and polyacrylamide gels[6]; DEAE-Sephadex and its modified forms[7]; and gels of inorganic origin such as silica gel, alumina gel and hydroxyapatite gel. The main objective of these methods has been to obtain a homogeneous entity of a particular type of RNA possessing a single biological function by sequential chromatography on a variety of these columns. Possibly the degree of heterogeneity will become even more evident as the methods of fractionation improve.

While the chemistry of nucleic acids was still in its infancy, different inorganic gels were utilized in the partial purification of enzymes and serum proteins[8,9]. The applicability of calcium phosphate gel as an adsorbent in the preparative purification of enzymes by both batchwise operation and column chromatography was demonstrated[10]. Since the role of the nucleus as a centre of cell metabolic and physiological activities was established, various workers have shown interest in purifying DNA and DNase depolymerized products using calcium phosphate gel[11,12]. With the background experience on the purification of proteins, it apparently seemed that (a) the preparation of hydroxyapatite columns from readily available materials was relatively easy and (b) DNA and its degraded products could be adsorbed and eluted under mild conditions of pH and ionic strength from such material[12].

With the increasing use of hydroxyapatite columns in the analysis of different molecular species of RNA, we thought it worthwhile to give a consolidated account of the use of this column, the biological significance of the separations achieved,

different modifications and alternatives which improve the resolution, its operational advantages, limitations and scope and the possible mechanism of separation. The fractionation of DNA from a broad range of sources, various environmental conditions that influence the elution patterns and the precautions that must be rigidly followed in order to obtain reproducible results have been reviewed extensively by many workers[6,13–19] and therefore will not be discussed here.

## 2. FRACTIONATION OF RNA

### *A. Preparation of hydroxyapatite*

At least six to eight procedures have been described for the preparation of hydroxyapatite and these have been summarized by Bernardi[13]. Hydroxyapatite, which is calcium phosphate with small crystal dimensions, is also available commercially under two brand names: brushite [$CaHPO_4 \cdot 2H_2O$] and hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] or hypatite C; brushite can be converted into hydroxyapatite by chemical treatment as discussed in detail by Bernardi[13]. De Lorenzo and Ames[20] found that hydroxyapatite prepared by the method of Siegelman *et al.*[21] gave a much better flow-rate than a commercially available preparation obtained by Tiselius' method[13]. Flow-rate is a very important factor, especially when the solution to be loaded is viscous. During the conversion of brushite into hydroxyapatite, if a low rate of stirring and gentle boiling are carefully maintained, the resulting adsorbent is free from aggregates, "fines" and incompletely converted brushite crystals; such a hydroxyapatite preparation has a high capacity, good flow-rate and reproducible chromatographic characteristics. Not only the above precautions must be rigidly observed during the preparation of hydroxyapatite, but sufficient care must also be taken to pack the column slowly, yet continuously, so that the adsorbent settles evenly within the column, with particles of uniform size. This method of preparation of the column ensured the reproducibility of subsequent results[22]. It was found that a freshly prepared hydroxyapatite gel stored at 4° for at least 3 months showed no significant variations in the elution pattern[23]. The details of hydroxyapatite chromatography, such as packing and regeneration of the column, adsorption–elution and recovery of the adsorbate, have been disussed extensively by Barnardi[13].

### *B. Fractionation*

There have been far fewer investigations on the chromatographic separation of RNAs, synthetic polyribonucleotides, etc., than of DNAs using hydroxyapatite. This is particularly striking if one considers the multiplicity of secondary structures that exist among the natural and synthetic polyribonucleotides. However, it has recently been pointed out by Kohne and Britten[16] that (a) the capacity of hydroxyapatite for the retention of RNA is less than that for DNA, (b) excess of RNA prevented binding of RNA–DNA hybrids and (c) in the presence of 8.0 *M* urea, only the hybrids could bind on to the column, whereas non-utilized RNA and single-stranded DNA were just retarded. In addition to these factors, a major obstacle in fractionation of RNA on hydroxyapatite is the apparent helical content of 40–60% of tobacco mosaic virus (TMV) RNA or rRNA[24]. It was suggested that a high helical conformation leads to difficulties in separating different RNAs. By this time, it was already known that

under conditions of DNA resolution, tRNAs were eluted as a single peak[25]. However, after extensive studies on the separation of native and denatured DNA, natural single-stranded DNA (ssDNA), the replicative form (RF) of DNA, synthetic polynucleotides and oligonucleotides, it was thought that by exploiting the secondary and tertiary structures, operational conditions for the optimal separation of RNAs on hydroxyapatite columns could be devised.

*(a) Low-molecular-weight RNA other than tRNA*

A low-molecular-weight RNA preparation from rat liver microsomes (for convenience called membrane RNA) was chromatographed on a hydroxyapatite column using a 0.02–0.2 *M* sodium phosphate buffer (pH 6.8) gradient[26]. There was no separation between membrane RNA and marker tRNA and both species showed partial overlapping and considerable tailing, indicating that they might not be homogeneous species. The partially enriched membrane RNA exhibited unique properties: (i) it was not present in ribosomes prepared from microsomes by deoxycholate treatment; (ii) it comprised 15–20% of the total microsomal RNA; and (iii) like tRNA, it had a high G + C content; (iv) unlike tRNA, it had a low content of 3′-terminal adenosine residues, methylated bases and pseudouridine, and exhibited no acceptor activity. Gardner and Hoagland[26] presented evidence to indicate that membrane RNA was neither 5S RNA nor a degradation product of high-molecular-weight RNAs.

In another study, 5S RNA from wheat germ, shown to be homogeneous by gel filtration and polyacrylamide gel electrophoresis, gave three peaks corresponding to mono-, di- and triphosphate at the 5′-terminal group[27]. This novel ability of hydroxyapatite to fractionate 5S RNA on the basis of degree of phosphorylation at their 5′-terminal group was further exploited in order to investigate the pattern of 5′-phosphorylation of 5S RNA from diverse sources[28]. At the conclusion of these studies, the most striking generalization observed was that 5S RNA from prokaryotic organisms (*Escherichia coli*, *Pseudomonas fluorescens*, *Streptomyces argenteolus*, *Euglena gracilis* and *Saccharomyces cerevisiae*) contained only 5′-monophosphates, while in contrast the 5S RNA from eukaryotic organisms (*Triticum vulgare*, liver of female Wistar rats and reticulocytes of New Zealand white rabbits) contained 5′-mono-, -di- and -triphosphate molecules. However, whether the extent of 5′-terminal phosphorylation contributed to the differences in conformation due to alterations in the charge density was not clear from these studies.

Low-molecular-weight nucleolar RNAs (distinct from tRNA, 5S RNA and 28S associated 7S RNA) obtained from Chinese hamster ovary (CHO) cells could be adsorbed on to and eluted from negatively charged bentonite, provided that they contained residual protein[29]. However, pure RNAs alone were not adsorbed. As a close similarity exists between bentonite and hydroxyapatite, it is probably worth investigating whether such a phenomenon occurs in hydroxyapatite chromatography.

*(b) tRNA*

*(i) Yeast.* The first attempt to fractionate yeast tRNAs met with a limited success, as all the tRNAs were eluted with 0.13 *M* phosphate buffer (pH 6.8)[30]. However, using a phosphate buffer (pH 5.4) gradient, Dirheimer[31] obtained a good resolution of aspartyl-, lysyl-, arginyl- and phenylalanyl-tRNAs. The limited success reported by Bernardi[20] was possibly due to use of conditions that had been optimised

for DNA fractionation rather than for RNA fractionation. This explanation seems reasonable as altered conditions lead to extensive fractionation of RNA[31–33].

As with a methylated serum albumin–Kieselguhr (MAK) column[4], a hydroxyapatite column fractionated 5-fluorouracil-containing tRNA (FU-tRNA) from their normal counterparts[34]. Thus, glycyl-, lysyl- and phenylalanyl-tRNAs from yeast were enriched. It was noted that FU was incorporated more into tRNA than into rRNA and the site of incorporation was largely uracil (U), and to some extent pseudouridine, ribothymidine, etc. The substitution of FU in place of U to the extent of 65% might have altered the conformation of tRNA as judged by melting profiles and possibly reflected in hydroxyapatite chromatographic separation[34].

*(ii) Escherichia coli.* Bernardi[25] reported the elution of *E. coli* tRNA from a hydroxyapatite column with 0.13 *M* phosphate buffer (pH 6.8) without resolution into different species or isoacceptor species. In contrast, Hartmann and Coy[35] used a hydroxyapatite column (93 × 3 cm) for the preparative fractionation of tRNA by stepwise elution with potassium phosphate buffer (pH 6.8). Of the several fractions eluted in the concentration range 0.05–0.16 *M*, characterization of threonyl-, valyl- and phenylalanyl-tRNAs showed that valyl-tRNA was purified twelve times. In further studies[36], the reproducibility of results could be verified by re-chromatography. With either stepwise or gradient elution and by modifying the conditions of elution, fractionation of isoacceptor species of threonyl-, valyl- and phenylalanyl-tRNAs was achieved.

Pearson and Kelmers[22] achieved a distinct fractionation and enrichment of several species of *E. coli* tRNA using a linear gradient of 0.1–0.2 *M* sodium phosphate buffer (pH 6.8) and a flow-rate of 12 ml/h. While two peaks of isoleucyl-, tyrosyl- and phenylalanyl-tRNAs were obtained, five peaks of leucyl-tRNA and multiple peaks of alanyl-, seryl- and prolyl-tRNAs were resolved. Pearson and Kelmers[22] claimed that the position of elution of several tRNAs on the chromatogram could be predicted from the shape of the absorbance curve, and this prediction could be subsequently confirmed by examining acceptor activities. Using this procedure, Schofield[37] attempted to separate methionyl- and formylmethionyl-tRNA. Although the presence of at least two species of each of formylmethionyl- and methionyl-tRNA could be demonstrated on the chromatogram, the separation had two major drawbacks: (i) species of methionyl- and formylmethionyl-tRNA overlapped and (ii) the quantitative variations in different species of methionyl-tRNA were so high that sometimes the fourth peak could not be detected.

While examining the optimal conditions for tRNA fractionation, Muench[17,38] observed that aminoacyl-tRNAs have increased stability on hydroxyapatite columns at pH 5.8 rather than at the pH value of 6.8 that was established for the optimal resolution of DNAs. He compared the three peaks of leucyl-tRNA eluted with a potassium phosphate buffer gradient (pH 5.8) at 4° with the five peaks of leucyl-tRNA obtained by partition chromatography. It was decided that leucyl-$tRNA_I$ from hydroxyapatite corresponded to leucyl-$tRNA_I$ from the partition column, leucyl-$tRNA_{II}$ to leucyl-$tRNA_{IV}$ and -$tRNA_V$, and leucyl-$tRNA_{III}$ to leucyl-$tRNA_{II}$ and -$tRNA_{III}$. This result indicated that either the hydroxyapatite column has a lower resolving ability or the optimal conditions of resolution were still to be developed. In further studies[32], it was found that with a prior purification by partition chromatography, alanyl-, leucyl-, valyl- and methionyl-tRNAs could be resolved into multiple peaks

and purified to 80–90% homogeneity on a hydroxyapatite column. Thus, the major species of valyl-tRNA (valyl-$tRNA_{II}$) was further purified by repeated chromatography at pH 5.8 and 6.8 (ref. 39). The chromatographic profiles were similar, irrespective of aminoacylation[32], and the phosphate buffer gradient (pH 5.8) permitted the recovery of tRNAs without undergoing deacylation as observed in certain reversed-phase chromatographic columns[5].

An interesting series of observations was made by Muench[40,41] in an attempt to fractionate tryptophanyl-tRNA: (i) non-aminoacylated tryptophanyl-tRNA was eluted as a single peak from the hydroxyapatite column with an increasing concentration gradient (0.15–0.50 *M*) of potassium phosphate buffer (pH 5.8) and a flow-rate of 30 ml/h; (ii) aminoacylated tryptophanyl-tRNA was eluted as two peaks under experimental conditions similar to those employed for the non-aminoacylated sample; (iii) the two peaks corresponded to an active form (eluted early) and an inactive form (eluted late) of tryptophanyl-tRNA; and (iv) in contrast, partition chromatography yielded three to five peaks of tryptophanyl-tRNA[42,43]. In further studies, both forms of tryptophanyl-tRNA could be purified to homogeneity (specific activity 1.8 nmoles per $A_{260}$) by repeated chromatography on BD-cellulose and final chromatography on hydroxyapatite. The conditions for purification had to be altered in that the elution was carried out by gradient elution of 0.1–0.4 *M* potassium phosphate buffer (pH 5.4) at 4° and a flow-rate of 16 ml/h (ref. 44). As with the MAK column, the hydroxyapatite probably purified these forms on the basis of conformational differences (arising out of aminoacylation) rather than on the basis of repulsive interaction between the tryptophanyl residue and hydroxyapatite. Muench[41] considered that the hydroxyapatite column does not separate tRNAs primarily on the basis of hydrophobic character, as in BD-cellulose or reversed-phase chromatography[3,5]. A possible reason for the failure to resolve two forms of non-aminoacylated tryptophanyl-tRNA might be that they exist in equilibrium at pH 5.8 (ref. 41).

The effects of pH, the shape of the phosphate gradient and the presence of a substance such as urea or a low percentage of alcohol still remain to be studied in order to establish whether they could be exploited to optimize the resolution of various tRNA species.

*(c) rRNA*

By using an increasing concentration gradient of phosphate buffer, Burness and Vizoso[45] fractionated Krebs-2 ascites tumour rRNA at room temperature into two fractions eluting at 0.07 and 0.11 *M*, respectively. The fractions corresponded to peaks in the ultracentrifuge Schlieren pattern which had $S_{20}$ values of 18S and 30S, respectively. However, the position of elution of these species was contradicted in work[46] in which Ehrlich ascites tumour (EAT) high-molecular-weight RNA was eluted into two major peaks at 0.15 and 0.20 *M* phosphate buffer (pH 6.8), corresponding to 19S and 32S, respectively. In our opinion, the elution of rRNA at as low a concentration of phosphate as 0.07 *M* is possibly an artifact of isolation. This view is further supported by the elution profiles of rRNAs from yeast, *E. coli* and other sources[13,47]. In all instances, rRNAs from both prokaryotes and eukaryotes were eluted at a considerably higher phosphate molarity than that observed by Burness and Vizoso[45]. Their observation that fluctuations in temperature or changes in the physical state of RNA did not influence the profiles no longer holds true. The degra-

dation of RNA (decrease in sedimentation coefficient) observed as a result of passage through the column[46] might be due either to disaggregation of the already aggregated species of RNA or to latent RNAse activity associated with rRNA, rather than to inherent drawbacks associated with the hydroxyapatite column. In fact, the S values obtained (19 and 32) do not indicate degradation. Furthermore, Brown *et al.*[48] obtained good recoveries of infectivity of foot and mouth disease viral (FMDV) RNA and other viral RNAs after passage through the column. Finally, no change in the structure of rRNA was detected, even after several passages through the column[49].

In another study, rat liver mitochondrial RNA was separated from DNA by differential elution at room temperature[50], when presumably the rRNA was eluted at 0.18 *M* and DNA at 0.4 *M* phosphate buffer (pH 6.8) concentration. Elution of rRNA and high-molecular-weight viral RNAs as two discrete peaks by stepwise elution and as only one peak by gradient elution has been reported by Bernardi[13]. This discrepancy in the observations as a result of a change in the method of elution indicated that either the gradient elution could not separate viral RNA from rRNA, as noted by Franklin[51], or stepwise elution in this instance afforded extraordinary resolution, as pointed out by Sueoka and Cheng[52] in studies with MAK columns. However, the significance of the elution obtained by stepwise elution has to be treated with caution as it has been a general complaint that stepwise elution generates spurious or false peaks on hydroxyapatite columns.

Total RNA from fresh tobacco leaves yielded three fractions, of which the first eluted showed amino acid acceptor activity, while those eluted at higher molarities corresponded to rRNAs[53]. However, upon alfalfa mosaic virus infection, this elution pattern changed quantitatively and yielded a fraction that was eluted at high molarity (possibly with a different secondary structure) and represented about 0.4% of the total RNA (which was afterwards characterized as RI).

*(d) Viral RNA*

Vizoso and Burness[54] were the first to attempt to separate infectious RNA from the bulk of the cellular RNA. There was no difference in the elution profiles of RNA from non-infected (*i.e.*, Krebs-2 ascites tumour) and 6 h-infected (*i.e.*, Krebs-2 ascites tumour infected *in vitro* with encephalomyocarditis virus) cells, indicating that the RNA species responsible for infectivity might be present in relatively low concentration. However, there were marginal differences in the elution profiles of RNA from the non-infected and the 24 h-infected cells. Although there was no clear separation of the infectious species from the cellular RNA, the studies established the usefulness of the hydroxyapatite column in that (a) it has appreciable capacity for RNA (3 mg RNA per millilitre of packed column) and (b) it accounted for 95% recovery. It was found that the optimum $Ni^{2+}$ concentration needed in order to stabilize the infectivity of RNA was one $Ni^{2+}$ ion per nucleotide residue, a value already demonstrated for stabilizing the infectivity of TMV RNA[55]. Passage of FMDV RNA yielded two peaks and their characterization revealed that infectivity was associated with the late-eluting peak[48]. It appears that the early-eluting peak represents degradation products, which probably arise during the isolation procedure. Single-stranded RNA (ssRNA), synthesized *in vitro* by wound tumour virus (WTV)-associated RNA transcriptase, has been purified from viral double-stranded RNA (dsRNA) by differential elution with phosphate buffer at 60° (ref. 56).

*(e) dsRNA*

The first attempt to purify the replicative intermediate (RI) of the bacteriophage $R_{17}$ using the hydroxyapatite procedure developed by Bernardi and Timasheff[46] was unsuccessful, as RI was eluted with rRNA[51]. However, by employing a hydroxyapatite column at high temperature, Bishop *et al.*[57] purified and characterized polio virus specific dsRNA. Bockstahler[58] purified RI from turnip yellow mosaic virus (TYMV)-infected cabbage plants, in milligram amounts. It was found that in conjuntion with the MAK column, a hydroxyapatite column could be used to purify RI to homogeneity by using a gradient elution of 0.05–0.4 *M* phosphate buffer (pH 6.7) at 35°. This procedure gave higher recoveries on the hydroxyapatite column (90–95%) than on the MAK column (50–70%), and hydroxyapatite exhibited a 50-fold greater capacity for RI as determined with ssRNA. In another study, comparison of the chromatographic profiles revealed that the order of elution with potassium phosphate buffer (pH 6.8) was tRNA (0.13 *M*), rRNA (0.25–0.30 *M*) and RI (above 0.30 *M*) from alfalfa mosaic virus-infected tobacco leaves[53]. Further studies led to the purification of RI without prior treatment with RNAse[49].

Bishop *et al.*[59] attempted to purify the polymerase product of Rous sarcoma virus (RSV) with limited success, using conditions (high temperature, stepwise elution) that eliminated the helical content of ssRNA. They considered that analysis on a hydroxyapatite column could be misleading unless the state of template RNA was monitored concomitantly with the DNA analysis. In contrast to these considerations, more recently a procedure consisting in a stepwise increase in phosphate buffer concentration as well as temperature, has been introduced[60] for the separation of ssRNA from dsRNA with 96–100% recovery: at 60° and 0.1 *M* phosphate buffer oligonucleotides and a small amount of ssRNA were eluted, at 90° and 0.1 *M* phosphate buffer the remaining ssRNA was eluted, and finally at 90° and 0.3 *M* phosphate buffer all of the dsRNA was eluted[60]. A combination of a temperature and a salt gradient also facilitated the separation of RNA polymerase product from viral dsRNA of cytoplasmic polyhedrosis virus[61], and from viral dsRNA of wound tumour virus[56].

The stability of dsRNA on the hydroxyapatite bed has been ascribed to the effect of physical immobilization in the adsorbed state[62] rather than to the effect of electrostatic forces[63]. Furthermore, in contrast to the analogy suggested by Bernardi[13] that the separation of ssRNA from dsRNA is equivalent to the separation of denatured DNA from native DNA, Martinson[19] found that both dsRNA of average base composition and RNA–DNA hybrids have a lower affinity than dsDNA (native DNA) for hydroxyapatite. Martinson attributed these differences in affinity towards hydroxyapatite to variations in the backbone conformations of these nucleic acids; the phosphates of dsDNA protrude from the helix, whereas those of dsRNA and RNA–DNA hybrids are relatively hidden and very few loci are available for binding between dsRNA and hydroxyapatite. Further, dsRNA also differed from dsDNA in the temperature dependence of its elution molarity. It is interesting that RNA–DNA hybrids were chromatographed in an essentially similar manner to dsRNA rather than being intermediate between dsRNA and dsDNA. It is not clear why dsRNA and RNA–DNA hybrids have their maximum elution molarities at a higher temperature than dsDNA.

*(f) RNA–DNA hybrid*

Many studies in molecular and developmental biology would be greatly facilitated if it were possible to isolate the desired segments of the genome of higher organisms. Because of the differences in buoyant densities between RNA and DNA, equilibrium density centrifugation has obviously been a method of choice for the enrichment of RNA–DNA hybrids and has been used for this purpose by a number of workers. However, as a routine preparative method, this technique is severely limited in both capacity and cost. According to Brenner *et al.*[64], hydroxyapatite column chromatography should be the method of choice for isolating RNA–DNA hybrids, because (i) it does not require immobilization of unlabelled DNA, as required in other methods[65,66], (ii) it has a reasonable capacity to retain RNA–DNA hybrids and has a reasonable cost of operation, and (iii) the percentage binding of labelled to unlabelled nucleic acid is greater in the free solution on hydroxyapatite (75–95%) than on agar gel (20–40%) or on nitrocellulose (NC) filters (70%). For re-association studies, however, hydroxyapatite column chromatography can be replaced by batchwise procedures, as the former is time consuming and only one or two columns can be operated simultaneously, whereas ten or more samples could be processed by a batchwise procedure or by using NC filters. Hence hydroxyapatite chromatography has been considered to be suitable either for the isolation of a fraction of genome specific for a particular kind of RNA or for the relative enrichment of RNA for the desired genes.

*(i) Mycoplasma.* The partial purification of native rRNA and tRNA cistrons from Mycoplasma sp. (Kid)[67] and *Mycoplasma laidlawii*[68] has been achieved after hybridization and separation on hydroxyapatite columns.

*(ii) Neurospora crassa.* DNA sequences that code for tRNA in *N. crassa* have been isolated in a highly purified state by repeated hybridization[69] between a DNA fragment and tRNA and then by fractionation of DNA–tRNA hybrid from non-hybridized DNA and excess of tRNA by differential elution with a linear gradient of phosphate buffer (pH 6.8) at 60°.

*(iii) Bacillus subtilis.* rRNA genes of *B. subtilis* have been isolated by passage through the column when renatured (self-annealed) DNA formed during hybridization was eluted after the elution of RNA–DNA hybrid[70–72]. The removal of renatured or partially renatured DNA from the hybrid was important, as a substantial amount of renatured DNA banded with RNA–DNA hybrid during caesium sulphate–$Hg^{2+}$ density gradient centrifugation. Denatured ssDNA could be removed either by passage through the column (before or after DNAse treatment to which the RNA–DNA hybrid is resistant) or by the differential density technique. Further experiments revealed that a relatively larger percentage (in comparison with mammalian genome) of the *B. subtilis* genome was transcribed in its exponentially growing stage[73].

*(iv) Escherichia coli.* The annealing product formed between phage $T_2$-infected *E. coli* DNA and complementary RNA was separated with a linear gradient of phosphate buffer. The three fractions, in order of elution, were ssDNA, RNA–DNA hybrid and renatured DNA non-specifically complexed with a negligible amount of RNA. It was also found that RNA from phage $T_2$-infected *E. coli* could be resolved into two fractions: the easily eluted $^{32}P$-labelled RNA probably represented $T_2$ mRNA, while the late-eluting RNA corresponded to cellular high-molecular-weight RNA[74]. In another study, DNA sequences that code for rRNA were purified by

selective elution with 0.14 *M* phosphate buffer at high temperature[75]. Direct comparison of the rRNA cistrons, thus enriched, from *E. coli*, *Proteus mirabilis* and *Salmonella typhimurium* showed that rRNA cistrons of these organisms were very similar to each other and apparently were conserved[75]. These studies revealed that the hydroxyapatite column can be useful (i) to measure the kinetics of re-association of different nucleic acid species, (ii) to isolate repeated nucleic acid sequences and (iii) to determine the extent of complementarity between nucleic acids derived from different species[16].

*(v) Amphibia.* By using differential elution at constant temperature, Davidson and Hough[76] separated amphibian RNA–DNA hybrid formed between the non-repetitive DNA from Xenopus red blood cells and doubly labelled RNA from the lamp-brush stage of the oöcyte of Xenopus.

*(vi) Aves.* McConaughty and McCarthy[77] fractionated RNA cistrons from embryonic chicken red blood cells using a thermal gradient. At 0.12 *M* phosphate buffer concentration and at 90, 95 and 100°, three fractions differing in average G + C content were eluted in the proportions 20:51:29. The segment of chromatin active in RNA synthesis exhibited a low dissociation temperature ($T_m$) and was enriched independent of the RNA–DNA hybridization procedure[77].

*(vii) Mammals.* Studies on the separation of model hybrids prepared by enzymic transcription on to mouse ssDNA or hybrids of DNA with RNA synthesized on a mouse native DNA primer *in vitro*[78] revealed that (i) elution of RNA–DNA hybrid was dependent upon the relative amounts of RNA and DNA in the hybrid and the degree of double strandedness, (ii) the best separation could be achieved with a hybrid with a high RNA:DNA ratio, (iii) hybrids with a low RNA:DNA ratio were eluted throughout the gradient because DNA strands were too long relative to the RNA molecule and (iv) hybrids with a high RNA:DNA ratio were eluted near to the elution molarity of dsDNA. Further, it was suggested[78] that a high temperature (70°) was useful for the isolation of hybrids of high complementarity, while the recovery of hybrids of low complementarity could be improved by eluting at lower temperatures and by taking precautions to exclude heavy metal ions. It was realized that polyacrylamide gel electrophoresis[79], particularly on a preparative scale[80], has been an asset to hybridization studies as it affords the isolation of RNA species of high purity and in high yields.

RNA cistrons were isolated from the brain, liver, kidney and spleen of mouse by following the procedure used in the enrichment of avian RNA cistrons[77], and the proportion of the genome transcribed in these different organs was estimated by hybridizing purified RNA cistrons with excess of RNA. Saturation hybridization values for liver, kidney and spleen were of the order of 4–5%, but for brain they were as high as 11%. In another study, the degree of hybridization as a function of aldosterone administration in adrenalectomized rats was studied by comparing the profiles of RNA–DNA hybrid before and after the treatment. While there was a specific increase in the hybridization between kidney cortex nuclear RNA and repetitive DNA, no such increase in the hybrid-forming capacity between repetitive DNA and microsomal RNA or tRNA was observed[81].

Chromosomal RNA, which is characterized by its short chain-length (40–60 nucleotide residues), high content of dihydrouridylic acid (8–10 mole%) and its association with the chromatin in higher organisms, could be purified in the form of

RNA–DNA hybrid with homologous or heterologous DNA by differential elution: at a constant buffer composition, unhybridized RNA was eluted between 25° and 40°, denatured DNA was eluted at 80° and the hybrid was eluted at higher temperatures[82]. Two interesting points shown by these studies were that (i) hybridization takes place to equal extents with homologous native and denatured DNA and (ii) chromosomal RNA, unlike mRNA, possesses the ability to interact and bind to native DNA, a property which is not applicable to other RNA molecules.

Recently, a simple and reproducible method, consisting in hybridization with poly-U followed by chromatography on hydroxyapatite, has been devised for the separation of mRNA and heterogeneous nuclear RNA (hnRNA), both of which contain long stretches of polyadenylic acid (poly-A)[83]. By this method, it was found that all mRNAs of mouse L-cells contained poly-A, with the exception of histone mRNA. A similar analysis of hnRNA indicated that only one fifth of these contained poly-A. A similar procedure, using MAK columns in place of hydroxyapatite columns, has been developed in our laboratory[84] for the purification of sea urchin oöcyte mRNA hybridized with [$^3$H]poly-U.

Murine sarcoma viral (MSV) DNA annealed with RNA from MSV-transformed cells was retained at 0.12 *M* and eluted at 0.40 *M* phosphate buffer concentration[85]. Haapala and Fischinger[86] reported that each of six mammalian C-type viruses, including two feline leukaemia viruses, three murine leukaemia viruses and the human "candidate" virus RD-114, could be distinguished from each other on the basis of hybridization of viral RNA with DNA synthesized by viral reverse transcriptase. Characterization of these hybrids on a hydroxyapatite column revealed the diversity in the nucleotide sequences among the viruses, as judged by the percentage of cross-hybridization and by the decreased thermal stability of heterologous hybrids.

*(g) Synthetic polynucleotides*

*(i) Adenylic acid polymers.* Calcium phosphate preparations obtained at different pH values and subsequently modified by heat or treatment with alkali have been investigated for their suitability as adsorbents for the fractionation of polynucleotides. One such preparation of calcium phosphate, precipitated at pH 6.7 and boiled with a saturated solution of calcium hydroxide, was found to possess the desired chromatographic characteristics of an adsorbent and was used to separate poly-A from DNA using gradient elution with phosphate buffer (pH 7.0)[87]. Partial separation of poly-A from TMV RNA and DNA has been reported by Bernardi[13]. He also found that non-alternating poly-(dA–dT) has a higher elution molarity than the alternating poly-(dAT–dAT), which was eluted close to yeast nuclear DNA[13].

In a study[88] with an increasing concentration gradient of phosphate buffer (pH 6.8), yeast RNA, yeast nuclear DNA and poly-(ADP–ribose) [a polymer of 2′-(5″-phosphoribosyl)-5′-AMP] were successively eluted at 0.16, 0.23 and 0.31 *M*. The fact that poly-(ADP–ribose), with a chain length of 18–22 nucleotide residues, was eluted at 0.31 *M* indicated that molecular weight (molecular size) might be of subsidiary importance in determining the sequence of elution. Bernardi[30] also arrived at a similar conclusion by observing that poly-A which possessed a single-stranded helical structure at neutral pH was eluted at the same molarity as double-stranded polynucleotide.

Niyogi and Thomas[89] investigated the chromatographic characteristics of com-

TABLE I

ELUTION PROFILES OF DIFFERENT SYNTHETIC POLYRIBONUCLEOTIDES ON HYDROXYAPATITE COLUMNS

| *Polynucleotide* | *Molarity of elution with phosphate buffer (pH 6.8)* | *Temperature of elution (°C)* | *Structural characteristics at the point of elution* | *Recovery (%)* | *General remarks* | *References* |
|---|---|---|---|---|---|---|
| Poly-U | 0.15 | 4 | Partial helical secondary structure | 100 | Only poly-U eluted | 91, 92 |
| Poly-U | 0.10 | 25 | Completely devoid of secondary structure | 100 | " | 91 |
| Poly-C | 0.12–0.13 | 4 | — | 100 | — | 13 |
| Poly-C | 0.12 | 27 | — | 100 | — | 30 |
| Poly-I | — | 27 | — | Poor | Irregular profiles | 13 |
| Poly-A | 0.22–0.25 | 27 | Random structure with respect to total conformation; ordered structure with respect to short-range interactions[93] | 80 | Elution as broad peak | 13 |
| Poly-U* | 0.05 | 27 | Devoid of secondary structure | 100 | Elution at lower molarity than untreated poly-U | 30 |
| Poly-C* | 0.12 | 27 | Devoid of secondary structure | 100 | Elution molarity constant irrespective of secondary structure | 30 |
| Poly-A* | 0.17 | 27 | Devoid of secondary structure | 100 | Elution at lower molarity than untreated poly-A | 30 |
| Poly-I* | 0.16 | 27 | Devoid of secondary structure | 100 | — | 30 |
| Poly-A–Poly-U | 0.23–0.26 | 27 | Double stranded | 100 | — | 30 |
| Poly-A–2-Poly-U | 0.45–0.50 | 27 | Triple stranded | 95 | — | 25 |
| Poly-I–Poly-C | 0.20 | 27 | Double stranded | 100 | — | 30 |
| Poly-r(G–C)$_n$** | 0.10 | 80 | Partial helical, $T_m$ = 92° in 0.0001 *M* $Na^+$ | 20 | 95% pure; slight contamination due to poly-[$^3$H]-r(I–C)$_n$ | 90 |
| Poly-[$^3$H]-r(I–C)$_n$** | 0.10 | 70–75 | Partial helical, $T_m$ = 49.5° in 0.01 *M* $Na^+$ | 90 | Partially eluted at 60° | 90 |
| Poly-(ADP-ribose) | 0.31 | 27 | — | 100 | Poly-(ADP-ribose) with a long chain length is readily separated from RNA and DNA; with a moderate chain length, separation from RNA and DNA is facilitated if RNase and DNase treatment given prior to chromatography | 88 |

* Additional treatment given to the sample before loading on to the column at 27°: heating at 100° for 5 min in 0.01 *M* potassium phosphate buffer (pH 6.8) containing 1.0% neutral formaldehyde; immediate cooling in ice-bath; elution buffer also contained 1.0% neutral formaldehyde.

** Additional treatment given to the sample before loading on to the column at 60°: heating at 100° for 5 min in 0.1 *M* potassium phosphate buffer (pH 6.8) containing 0.0001 *M* EDTA; immediate cooling in ice-bath; elution buffer also contained 0.0001 *M* EDTA.

plexes formed between defined oligoadenylic acid and polyuridylic acid (poly-U) as a function of temperature. In contrast to the above studies[30,88], they found that the temperature of elution was a function of the chain-length (molecular weight), the presence of $Mg^{2+}$ ions, and the presence or absence of terminal phosphate residues on the oligonucleotides. They also found that oligoadenylic acid alone, with a chain-length of six residues, was not retained on a hydroxyapatite column even at 4°, whereas poly-U was retained at all temperatures of melting, even at 70°, and complexes between oligoadenylic acid and poly-U were retained only up to the melting temperature. As the temperature gradually increased, each oligoadenylic acid–poly-U complex melted, whereupon the labelled oligoadenylic acid was released from the column without the concomitant release of poly-U. Furthermore, the position of elution and width of the peak were independent of the flow-rate in the range 20–120 ml/h. Niyogi and Thomas[89] claimed that thermal chromatography is a sensitive and convenient means of estimating the thermal stability of trace amounts of labelled oligoadenylic acid–poly-U complexes. It seems to us that the present procedure could be applicable to other equivalent polynucleotide systems. These studies revealed that (i) oligonucleotides with a 5′-phosphate terminal group formed the stable complexes with polynucleotides, (ii) oligonucleotides with a 2′- or 3′-phosphate terminal group reduced the stability of the complex formed with polynucleotides and (iii) the stability was reduced still further especially when the oligoadenylic acid had shorter chain-lengths.

*(ii) Guanylic acid polymers.* While exploiting the advantages of hydroxyapatite columns in the fractionation of guanylic acid polymers, it was found that copolymers that contained high molar percentage of G + C had relatively low affinities towards the column. As a result, the molar percentage of G + C decreased in the fractions eluted with increasing molarity of phosphate buffer. It was also found that when large amounts of poly-r (G + C) were used, the recovery was better when several small columns were employed rather than a single large column[90].

For the sake of convenience, the chromatographic behaviour of different polyribonucleotides under various operating conditions on hydroxyapatite columns is summarized in Table 1.

The requirement of a low molarity of elution of poly-U and polycytidylic acid (poly-C) at 27° and the need for an increased molarity of elution at 4° is consistent with the principles of thermal chromatography[63,89].

The fact that polypurinic acids (poly-A, poly-I, etc.) were eluted at higher molarities and with low recoveries indicated their firm binding on hydroxyapatite, in contrast to the polypyrimidinic acids (poly-U, poly-C), which were eluted at lower molarities and with quantitative recoveries. This observation supports the view that if all variables were constant, fractionation into two molecular species could occur on the basis of differences in average base composition. However, such a case of theoretical similarity, as found with synthetic polynucleotides, rarely occurs in nature.

## 3. MODIFICATIONS AND ALTERNATIVES

### *A. Modifications*

The separation of different species of nucleic acids on hydroxyapatite columns

is generally achieved by employing a linear gradient of phosphate buffer at room temperature and it usually results in reproducible separations with reasonable recoveries. However, with this general approach, in certain instances a desired species of nucleic acid could not be purified from other contaminants, and it was proposed that modifications to hydroxyapatite chromatography might lead to better and more extensive resolutions.

Several modifications, such as (a) the change from a column procedure to batchwise operation[64], (b) the employment of stepwise elution instead of gradient elution[13], (c) the change in the cations of the phosphate buffers from $Na^+$ to $K^+$ (ref. 94), (d) the addition of new cations ($Mg^{2+}$, $Cs^+$, etc.) to the phosphate gradient[89,95] and (e) the addition of organic solvents such as formamide, urea and EDTA[30,88,90] to the eluting agents, were introduced. Using a batchwise procedure and centrifugation with a differential molarity of phosphate buffer, bacterial RNA could be separated from DNA[96]. However, some of the procedures were laborious and none of them gave superior or even equal resolutions in comparison with the general procedure.

As hydroxyapatite is an inorganic gel and physico-chemically highly stable at elevated temperatures, a modification was introduced by Miyazawa and Thomas[63] and thermal chromatography (increasing temperature gradient) was developed. The concept underlying this approach was to melt certain fractions of DNAs selectively, depending upon their base composition and to some extent upon their three-dimensional conformation (degree of base stacking), and to elute them. At high temperature, the elution molarity of dsDNA decreased sharply, as anticipated. In spite of a decrease in the elution molarity, DNA became more firmly bound to the column and the thermal stability was further increased despite the reduction in the ionic strength of the buffer[62]. This often resulted in dsDNA being eluted without being denatured (melted) as the thermal chromatography proceeded[95]. Taking this drawback of hydroxyapatite chromatography into consideration, the following modifications were developed.

*(a) Elution at 18° in the presence of organic solvents*

The first modification was introduced by Isaenko and Aksel'rod[97], involving elution of RNAs at 18° using a 0.05–0.20 *M* phosphate buffer gradient containing 0.1% of methanol plus 0.1% of chloroform. Thus, by exploiting another exclusive characteristic of hydroxyapatite (resistance to organic solvents), fractionation of 90% pure valyl-$tRNA_1$ from bakers yeast was possible. In our opinion, several other non-corrosive organic solvents could be used and even at a higher concentration, as high as 10%, in order to achieve better resolution, keeping other variables (particularly the flow-rate) constant.

*(b) Elution at lower temperature in the presence of perchlorate*

Graham[98] observed that the addition of 7.2 *M* sodium perchlorate to a low concentration of phosphate buffer allowed the elution to be carried out at much lower temperatures. Thus, the range of dissociation temperatures ($T_m$) could be reduced by the presence of perchlorate, which in turn facilitated the elution of a melted sequence of nucleic acids at lower phosphate concentration and thus might remove the drawback observed earlier in thermal chromatography[63]. The validity of this modification

stood the test during the enrichment of G + C-rich satellite DNA from Bermuda land crab *(Gecarcinus lateralis)*. However, in our opinion, at such a high concentration of perchlorate, some degree of apurinisation would probably occur.

*(c) Elution at room temperature in the presence of 50% formamide*

Recently, a modification has been suggested in which hydroxyapatite chromatography is carried out at room temperature with an elution buffer containing 50% formamide[99]. This procedure proved useful for (a) separating ssDNA, dsDNA and RNA–DNA hybrid from each other and (b) studying the kinetic analysis of RNA–DNA hybridization at room temperature. The results obtained by this procedure were comparable with those obtained at elevated temperature or those obtained by equilibrium centrifugation in caesium sulphate gradients. Notable features of this procedure are that the use of 50% formamide in the elution buffer obviated the need for elevated temperatures, eliminated non-specific hydrogen binding, abolished the risk of cleavage of phosphodiester bonds in RNA at high temperatures and has retained the original simplicity and rapidity of the technique. Moreover, it is free from the obvious drawbacks of batchwise procedures[61].

*(d) Elution at 37°*

A good compromise between low reaction rates at lower temperatures and the possibility of nucleic acid scission or depurination at higher temperatures is provided by carrying out separations at 37°. This modification[100] has the added advantage of the availability of temperature control at 37° in most laboratories. At this temperature, reaction rates might be augmented by an increase in ionic strength without calling for excessively high concentration of formamide, as used in the earlier method[99].

*B. Possible modification*

Hydroxyapatite chromatography has been used to separate protein sub-units in the presence of sodium dodecyl sulphate (SDS), an idea borrowed from the polyacrylamide gel electrophoresis of proteins[101]. It is claimed that protein–SDS complexes exist as rod-like molecules and the lengths of the complexes are a function of molecular weight. In view of Kawasaki's recent theoretical considerations[102–106], in our opinion it might be worthwhile to attempt to separate dsRNA from other nucleic acid species in the presence of SDS. SDS is expected to act in a similar manner to 7.0 *M* urea or 50% formamide used earlier in nucleic acid chromatography. As with proteins, SDS might facilitate the separation of different entities of nucleic acids by orienting them in a conformation that is repulsive to the hydroxyapatite bed.

*C. Alternatives*

In spite of several modifications of hydroxyapatite chromatography, it was considered necessary to develop alternate procedures that might result in clear, reproducible and extensive fractionation of nucleic acid species. All of the chromatographic procedures in current use[1–4] are based on a single principle. Reversed-phase chromatography is the only technique in which principles of counter-current distribution (CCD) and ion exchange are combined to our advantage. By experience, it has

been realized that reversed-phase chromatography gives superior resolutions that are not possible with the earlier techniques. On similar lines, Kirkegaard and co-workers[107,108] introduced two techniques (see below) that might serve as alternatives to the use of hydroxyapatite columns.

*(a) Ion-filtration chromatography*

In this technique, the principles of ion exchange and gel filtration are combined to form a rapid and convenient technique for preparative purposes. The striking feature of this technique is that it affords simultaneous purification and concentration so that the resulting fraction could be used directly for further chromatographic purification without prior manipulations[107]. Although the technique is presently applied for the purification of enzymes, it might be suitable for the fractionation of nucleic acids if the operating conditions used in modified cellulose chromatography[2,3] and DEAE-Sephadex A-50 chromatography[109] are adopted.

*(b) Intervant dilution chromatography*

In this technique, the principles of adsorption and molecular sieve separation are combined and it is useful for the selective or complete dissociation and separation of biological complexes such as RNA–protein, DNA–histones and RNA–DNA hybrids without destroying any of the conjugate (associated molecule). In this technique, the macromolecules that are usually polyfunctional entities (containing numerous ionic and non-ionic groups) are separated by adding low-molecular-weight substances that reduce the intermolecular binding strength and space out the species to be separated. Such substances are called "intervants" and hence the technique is called "intervant dilution chromatography"[108].

While developing this technique, Kirkegaard and Agee[108] considered that current chromatographic procedures had limited applicability in the resolution of macromolecules that interact strongly with each other. They further noted that it was not always possible to alter the chromatographic environment that permitted the molecules to behave independently while retaining sufficient interaction with the stationary phase (adsorbent); ultimately, a point was reached where resolution was not feasible with these chromatographic procedures. However, it should be pointed out that this is not entirely so: there are at least two well documented cases where a biological complex (DNA–protein) has been separated either by stepwise elution[110] or by increasing the salt concentration gradient[13]. As the histones or the associated acidic proteins are less strongly adsorbed by the adsorbent than DNA in the presence of high salt concentrations, the fractionation of DNA and protein has been achieved simultaneously in the case of buffalo liver deoxyribonucleoprotein[110] and calf thymus nucleohistone[13].

## 4. THEORETICAL CONSIDERATIONS

As pointed out by Bernardi[13], most of the chromatographic separations on hydroxyapatite columns have been carried out under arbitrary experimental conditions, and a systematic examination of the parameters that affect the chromatographic behaviour of nucleic acids and a methodical study of the optimal conditions of resolution are lacking. Kawasaki and Bernardi[111] studied the optimal conditions for the

resolution of macromolecules of different sizes, and also studied the environmental factors that influence the chromatographic behaviour, such as dependence of the elution molarity on (a) length of the column, (b) slope of the gradient, (c) load of the adsorbate, (d) presence of other components on the column and (e) width of the peak.

Based on these studies and in the light of existing data, Kawasaki[112] proposed a theory for the chromatographic separations of rigid macromolecules on hydroxyapatite columns, with two assumptions: (a) instantaneous thermodynamic equilibrium between the adsorbed phase and solution could be brought about and (b) negligible longitudinal diffusion of macromolecules takes place. Under normal circumstances, at room temperature, perhaps both assumptions may be close to reality, but with an increasing thermal gradient neither the instantaneous attainment of thermodynamic equilibrium nor the avoidance of diffusion seems possible. As an extension of the above theory, the adsorption and elution of macromolecules were studied as a function of (a) phosphate concentration and (b) their position on the column[113].

The applicability of the above theory has been considered for its validity under different theoretical conditions:

(1) It is assumed that there are no mutual interactions among the adsorbed macromolecules[102]. With this assumption, the present theory may not be completely applicable as the molecular species undergoing separation may not be rod-like or elongated structures and might exert considerable interactions among themselves, although all species behave like a single molecule. The ideal example could be different isoacceptor species of the same tRNA from a single source. Furthermore, this theory has the greatest limitation that no experimental data are available.

(2) It is assumed that the molecular species in the mixture to be separated have (a) the same chain length, but adsorption sites are distributed differently on the molecular surface and (b) different chain lengths, but the same adsorption energy per unit length. A common feature in both assumptions is that all of the macromolecules on the column have more or less the same diameter[103]. This situation is suitable for the separation of tRNAs specific for different amino acids.

(3) Mathematical relationships that express the behaviour of tropocollagen molecules may not be applicable to the separation of different types of either RNAs or DNAs, as the well defined tertiary structure assumed for a micro-heterogeneous model of tropocollagen might not be valid for the tertiary structure of nucleic acids[104,105,114].

(4) The theory developed in (2) above is extended to a very general case in which both the chain length and the adsorption energy per unit length need not be constant. With minimal assumptions, the order of preferential adsorption of different macromolecules on to the hydroxyapatite surface is considered[106]. This theory may possibly be applicable to the separation of different nucleic acids for which a broad range of structural differences exists under one set of experimental conditions, for example, the separation of ssDNA from renatured DNA, RNA–DNA hybrid, excess RNA, etc.

## 5. ADVANTAGES AND LIMITATIONS

### *A. Advantages*

Hydroxyapatite can be used for both analytical and preparative purposes, as

it is claimed[16] that it has the potential for handling widely varying amounts of nucleic acids in the range 0.001–2000 $\mu$g. It has an even higher capacity for tRNA per gram and therefore could be employed for preparative purposes[17]. In fact, preparative hydroxyapatite chromatography has been developed for the characterization of RNA synthesized by isolated nuclei from bovine thymus[115]. It separates viral RNA without loss of infectivity and tRNA independent of aminoacylation.

When the separation is incomplete on smaller columns or when large amounts of samples have to be handled (especially during preparative work), the use of longer columns (constant diameter and constant experimental variables) and shallower gradients of phosphate buffer is useful in overcoming the difficulty[11,23,116]. Shallow and linear concentration gradients gave good resolutions of tRNAs and ten void volumes of the buffer were sufficient to elute all tRNAs present on the column[33].

It affords distinct and quantitative separations of ssDNA from dsDNA with a consistent elution pattern which is relatively insensitive to the size of the DNA molecules[15]. Our present knowledge of hybrid separation could be derived from the separation of dsDNA.

A useful operational asset of this column is that it neither entraps air nor gives rise to channelling, even if by oversight the column happened to remain dry for several hours[117].

As it is an inorganic and crystalline adsorbent, it possesses intrinsic physicochemical stability and inertness towards (a) alterations in temperature up to slightly above 100° and (b) a wide range of organic solvents. Because of these two characteristics, several modifications have been possible with hydroxyapatite chromatography.

It is claimed that comparison of experimental results obtained with columns of different sizes is possible only with hydroxyapatite chromatography[33]. For this, one simply has to express the volumes of eluting buffers as multiples of the void volume of the columns: the void volume (*i.e.*, the volume of the mobile phase in the column) is equal to 70% of the geometrical volume of the column[33].

The elution profiles obtained with TMV RNA hydrolyzed with RNAse $T_1$ demonstrated that hydroxyapatite was superior to DEAE-Sephadex chromatography for the distinct resolution of larger oligomers using 0.001–0.2 *M* phosphate buffer (pH 6.8) in 7.0 *M* urea[118].

Since the basis of resolution on hydroxyapatite column seems to depend upon properties other than those which determine partition coefficients, this technique is useful as an adjunct to counter-current distribution and partition chromatography[32].

Finally, hydroxyapatite chromatography also serves as a complementary technique to density gradient centrifugation with a positive advantage over it that like the latter, it is neither a time consuming technique nor requires any sophisticated expensive equipment or costly reagents.

### *B. Limitations*

Some of the limitations of this technique have already been discussed by Walker[119].

Although hydroxyapatite chromatography seems to be a simple and easy method of fractionation and does not require expensive equipment or reagents, it is less frequently used than it should be. This is largely because hydroxyapatite is pre-

pared under empirical conditions, which are often critical, and the whole procedure needs considerable familiarity. The preparation itself is laborious and not completely standardized and therefore it may not be possible to prepare the adsorbent repeatedly with comparable chromatographic characteristics[6,17,120]. However, it is claimed that by introducing certain modifications during the preparation, the difficulties in preparing hydroxyapatite with the desired properties have been partially overcome[17]. Also, the attainment of equilibrium on hydroxyapatite is a very slow process and the column requires prolonged washing with phosphate buffer of low molarity, and the regeneration procedure is therefore time consuming[117].

Although chromatography and rechromatography are claimed to be successful[121], sometimes variations in individual batches of hydroxyapatite require adjustment of the experimental conditions, as different batches have different capacities for the retention of nucleic acids[16]. Therefore, the initial and final concentrations of the phosphate buffers to be used in the gradient must be pre-determined for each batch of the hydroxyapatite in order to obtain reproducibility in the elution patterns[23,50,122].

One must maintain the ratio of adsorbate loaded to the amount of adsorbent constant, as the molarity of elution decreasses as the load ratio increases[95]. Because of this variation, experimental complications could arise simply because of the availability of only a limited amount of sample for analysis. The importance of the ratio of the adsorbate loaded to the amount of adsorbent was also emphasized by Bernardi[17]. He observed peaks of DNA whose relative heights could be altered by varying the amount of adsorbent, and concluded that fractionation might have been guided by the ratio of load to hydroxyapatite bed.

Aggregated DNA samples showed elution patterns different from the normal pattern. Furthermore, individual fractions thus obtained could not be eluted at the same location with the same height on the chromatogram[123], which could create confusion and lead to erroneous conclusions. Discrepancies in the elution pattern during chromatography and rechromatography on hydroxyapatite column are not unusual, because the individual species might become disaggregated owing to passage through the column (during chromatography) and hence behave in a different manner to the originally aggregated species (during rechromatography).

Mattson *et al.*[124], as quoted by Larsen[125], found that hydroxyapatite behaved like an amphoteric colloid and its isoelectric point varied from 6.5–10.2, which might sometimes adversely affect the reproducibility of the elution patterns.

Pearson and Kelmers[22] found that hydroxyapatite prepared by Levin's method[117] and obtained from Bio-Rad Labs. (Richmond, Calif., U.S.A.) yielded a satisfactory resolution, whereas Hypatite C (Clarkson Chemical Co., Williamsport, Pa., U.S.A. and hydroxyapatite–cellulose (Gallard-Schlesinger Chemicals, Carle Place, N.Y., U.S.A.) gave little or no resolution of tRNAs. In their opinion, differences in ability to resolve tRNA could be attributed to differences in the surface area of the hydroxyapatite crystals. The differences in elution patterns, due either to a change in the batch of the adsorbent or to a change in the manufacturing agency, could perhaps be partially explained as above. However, it is still not understood why, when analyzing the same preparation of tRNA using the same batch of the adsorbent, one should obtain irreproducible results. Schofield[37] found that the chromatography of purified tRNA on hydroxyapatite sometimes generated spurious multiple peaks of amino acid acceptor activity, for unknown reasons.

The use of tightly packed or old columns resulted in poor recoveries and very slow flow-rates. Furthermore, with recoveries ranging between 70 and 95%, it is doubtful if the column could separate as little as 0.5 $\mu$g of DNA, as claimed[14]. Any attempt to improve the flow-rate by increasing the pressure caused a further decrease in flow-rate and even lower recoveries. It is suggested that the poor recovery is due to aggregation upon denaturation, owing to non-specific intermolecular base pairing or to interactions between the phosphate of nucleic acid and amino groups of the residual protein associate with nucleic acid[126]. In early work, the Celite used to enhance the flow-rate of hydroxyapatite caused undesirable adsorption[117]. We recommend that "fines" should be removed liberally and repeatedly and that Whatman CF-11 cellulose should be added to "fine-free" hydroxyapatite in the ratio of 1:2 (w/w) in order to obtain satisfactory flow-rates. Such precautionary measures give appreciably higher flow-rates without the application of pressure and without impairment in the resolving ability of hydroxyapatite. During the regeneration of the column, if hydroxyapatite is carefully suspended in the desired buffer and if vigorous agitation is avoided, the breakdown of hydroxyapatite crystals can be prevented, which enables the same flow-rate to be maintained in further experiments. Incidentally, it should be noted that hydroxyapatite gives a higher flow-rate than brushite.

Variations in the pH of the buffer altered the requirements of the volume and concentration of the buffer necessary for complete elution[33], *e.g.*, at pH 5.4, as tRNAs are more firmly bound, larger volumes of buffer of higher concentration are required for complete elution. However, the reverse is the case at pH 7.8, where tRNAs are moderately bound. Moreover, although firm adsorption was facilitated at pH 5.8, it did not enhance the resolution.

The elution profiles obtained with TMV RNA hydrolyzed with RNAse $T_1$ demonstrated that the technique was inferior to DEAE-Sephadex chromatography for the resolution of short oligomers using 0.001–0.20 *M* phosphate buffer (pH 6.8) in 7.0 *M* urea[118].

## 6. BASIS OF ADSORPTION AND RESOLUTION

Most of our knowledge as regards the mechanism of adsorption on and elution from the hydroxyapatite column is derived from the separation of DNAs.

Most of the separations performed on hydroxyapatite columns were carried out under empirically chosen conditions until Martinson[19,62,95,127] attempted to establish certain principles that had previously been only partly appreciated. The analysis of RNA–DNA hybridization mixture revealed that the mode of separation on hydroxyapatite columns is considerably more complex than had generally been recognized[62].

The chromatographic behaviour of different nucleic acid molecules has provided useful information on the relationship between macromolecular conformation and elution molarities and has also been useful in the elucidation of the possible mechanism of adsorption–elution on hydroxyapatite columns.

### *A. Adsorption*

#### *(a) Basis of adsorption*

The non-retainability of purine and pyrimidine bases and nucleosides of ribose

and deoxyribose series[30], the slight retardation of mononucleotides of both series[30], the moderate retention of nucleoside polyphosphates[30], the easy elution of oligonucleotides and partial digests of RNA by pancreatic RNAse[25,30], the limited digest of DNA by DNAse[13,128], the relatively lower affinity of sonicated or high-speed-sheared nucleic acids towards hydroxyapatite[25,120], the elutability of heat-denatured or formaldehyde-denatured DNA at comparatively low molarities of phosphate buffer[13] and the firm retention of tRNA[44], rRNA[53], viral RNA[54], dsRNA[60], RNA–DNA hybrid[72], dsDNA and partially annealed DNA[47] indicate that although molecular size may not be a primary factor in adsorption, a finite three-dimensional macromolecular conformation is a prerequisite for adsorption on the hydroxyapatite column.

*(b) Mechanism of adsorption*

The studies of the adsorption–elution response of different nucleic acids and their degradation products as a function of structure showed that the main factor involved in the adsorption of nucleic acids on hydroxyapatite columns was the interaction between the negatively charged phosphate groups of the nucleic acids and the positively charged $Ca^{2+}$ ions from the surface of the hydroxyapatite crystals with no direct involvement of either the sugar moiety or bases[13]. This conclusion is supported further by the following observations:

(1) phosphoproteins and nucleotides have a higher affinity for hydroxyapatite than non-phosphorylated proteins and nucleosides[30];

(2) the presence of chelating agents decreases considerably the capacity of hydroxyapatite for retaining nucleic acids, which indicates competition between the chelating agent and nucleic acids for $Ca^{2+}$ ions[23]; and

(3) the elution of nucleoside mono-, di- and triphosphate occurs in the order of increasing number of phosphate groups in the molecule.

*(c) Relationship between conformation and strength of adsorption*

It has been found that an increase in both the length and three-dimensional structure in single-stranded nucleic acids gave rise to a greater affinity for hydroxyapatite; also, the base pairing and base stacking interactions within the strands increased their strength of binding on hydroxyapatite[127]. In general, hydroxyapatite showed a greater affinity for adsorption towards rigid and ordered structures than towards flexible and disordered structures[25,120,129,130]. However, Soave *et al.*[28] recently reported that a flexible molecule is more firmly adsorbed because of its ability to conform to the adsorbing surface. They have further proposed that the separation of rigid from flexible molecular species takes place on the basis of a net loss in the conformational entropy of the macromolecular species during adsorption. Martinson also arrived at the same conclusion[127].

*(d) Factors affecting adsorption*

The presence of some cations severely depressed the adsorption affinity of DNA towards hydroxyapatite[95], and their presence in the elution buffer increased the power of elution in the order $Mg^{2+} < Na^{+} < K^{+} < Cs^{+}$. This observation is in contrast to a general finding that an increase in $Mg^{2+}$ concentration facilitated the retention of nucleic acid species on other chromatographic systems[131,132].

The degree of ionization of phosphate ions seemed to control the adsorption

of nucleic acids on hydroxyapatite[128]. Chromatography of a number of water-soluble synthetic homo- and heteropolypeptides on hydroxyapatite columns showed that only those polypeptides which contained accessible carboxyl groups (such as in poly-L-aspartate, poly-L-glutamate and their copolymers) were adsorbed[120]. It seemed most likely that carboxyl groups had taken the role of phosphate groups, which was found to be necessary for the firm retention of phosphoproteins[129] and nucleic acids[130]. Denaturation of these synthetic polypeptides or nucleic acids reduced their affinity for adsorption, probably owing to a reduction in the number of carboxyl and phosphate groups available on the surface for the interaction with the adsorbent.

*B. Elution*

*(a) Stepwise versus gradient elution*

Both stepwise and gradient elution have been used for the resolution of nucleic acid species on hydroxyapatite columns. However, stepwise elution is not preferred as it gives false peaks at the point of change in molarity and results in inferior resolution in comparison with that obtained by gradient elution.

*(b) Choice of eluting agents*

The main criterion that any eluent has to satisfy is that it should not bring about significant changes in the physico-chemical and biological characteristics of the molecule during elution. This criterion rules out the use of strong acids and alkalis, oxidizing and corrosive agents, and complex-forming (chelating) agents such as EDTA, fluoride, citrate and tartrate. Chelating agents have a greater affinity for $Ca^{2+}$ ions of the hydroxyapatite and therefore their use would indiscriminately elute anything present on the column. It was found that 30 $\mu$mole of sodium citrate drastically reduced the affinity of even bacteriophage DNAs towards hydroxyapatite[23].

The eluents used should have an appreciable buffering capacity, should facilitate solubilization of nucleic acids and yet remain harmless. Phosphate buffers satisfy all of the criteria required for an ideal eluent. Of the widely used phosphate buffers, potassium phosphate buffer has been reported to be more effective than its sodium counterpart as an eluent, possibly owing to the higher degree of ionization of $K^+$ ions. Therefore, $K^+$ buffers have a greater eluting power than $Na^+$ buffers at comparable molarities. The elution molarity, therefore, varied if $K^+$ or $Na^+$ buffers were used[94].

*(c) Choice of pH*

As hydroxyapatite is more stable at pH levels above 5.0, the buffers used could be in the pH range 5.4–7.8. However, as tRNAs are stable and firmly retained on the hydroxyapatite column between pH 5.4 and 5.8, this pH range would seem to be ideal for the fractionation of tRNA. The arbitrary choice of pH 6.8 for the fractionation of different types of DNAs seems to have resulted in partial resolution.

*(d) Means of elution*

The application of a temperature gradient with or without a simultaneous gradient of pH or salt concentration yields a resolution superior to that when room temperature is used. An increase in temperature decreases the strength of adsorption by affecting the secondary structure of the adsorbate and by accelerating the degree

of ionization of the eluting phosphate buffer. These factors, either alone or together, reduce the affinity of nucleic acids for hydroxyapatite to the point of selective elution. In thermal chromatography, separations can be achieved on any scale (analytical or preparative) provided that the width of the column is kept within limits that permit rapid thermal equilibrium[133].

*(e) Mechanism of elution*

The decrease in the adsorbing forces appears to be due to specific competition between phosphate ions in the eluent and the phosphate groups of the polynucleotides for $Ca^{2+}$ sites on hydroxyapatite, and not simply to an increase in ionic strength. Thus, increasing the molarity of the eluting phosphate buffer progressively reduces the forces responsible for retaining the binding strength between the adsorbent and an adsorbate. Eventually, at a particular concentration of phosphate buffer, the already unstable and ever weakening forces collapse, at which point a gradual shift in the $R_F$ value from zero to unity is completed, resulting in elution.

Strong indirect evidences which indicate that slight changes in the secondary and tertiary structures underly the basis of separation of RNAs and DNAs on hydroxyapatite columns are summarized below.

*(i) Effect of partial depolymerization.* Oligouridylic acid with an average chain-length of 25 residues and a partial digest of different strains of TMV RNA by RNAse $T_1$ in the presence or absence of 7.0 *M* urea exhibited a substantial loss of affinity towards the column[134] in comparison with the intact molecule[53].

The early elution of the limited digest of DNA, sonicated fragments and high-speed-sheared DNA support the view that conformation plays a definite role in determining the sequence of elution, as all of the above treatments cause significant modification(s) in the secondary structure.

Only a moderate degree of fractionation is obtained with an artificial mixture of polymerized and partially depolymerized DNA[128]. In this instance it would appear as if fractionation is a function of the displacement of shorter fragments by the longer fragments by virtue of their hydrodynamic volume (which, in turn, is a reflection of overall conformation). The partial separation of bacteriophage $T_2$ DNA (glucosylated) from *E. coli* DNA (non-glucosylated) suggested that the hydrodynamic volume possibly contributes to the sequence of elution. Whether the hydroxymethylcytosine (HMC) residues play a role in separation is still not known.

*(ii) Effect of differences in base composition.* The fractionation of DNAs and sonicated fragments from phage $T_2$, $T_5$, $\lambda$, *E. coli* and *Haemophilus influenzae*, using a temperature gradient showed that fractionation took place on the basis of differences in average base composition; fractions eluted at high temperature (high $T_m$) exhibited a proportionately higher percentage mole fraction of G + C (ref. 63). DNA rich in G + C showed a lower affinity at room temperature than DNA poor in G + C (ref. 19). These observations indicated that hydroxyapatite is sensitive to differences in base composition if it also reflects differences in secondary structure. However, differences in the percentage of G + C alone do not distinguish the molecular species of nucleic acids[14].

*(iii) Effect of molecular size.* The separation of phage T even DNA (55–58S) from *E. coli* DNA (40S) and the elution of sheared and unsheared DNA from phage $T_4$ at almost the same point indicated that molecular size (molecular weight) might

not be a major factor controlling the separation pattern[14]. This is further substantiated by the finding that active and inactive forms of tryptophanyl-tRNA could be distinguished on the hydroxyapatite column[42,135].

In the case of single-stranded nucleic acids, however, both molecular size and overall conformation affect the molarity of elution[127]. This is supported by the observation that TMV RNA was eluted before DNA from *B. subtilis* at 10° because of its small size, but nevertheless was eluted later than *B. subtilis* DNA at 45°, because of the greater importance of structural differences at higher temperatures. From the above finding, one may conclude that at higher temperatures and low salt concentrations, single-stranded nucleic acids are devoid of ordered structure: conversely, single-stranded nucleic acids exhibit a highly ordered structure at low temperatures and high salt concentrations.

*(f) Physical characteristics related to elution pattern*

The twisted circular DNA from polyoma virus could be eluted at a lower molarity than the linear open or circular forms of the same DNA, which were eluted at the same molarity[136,127]. The mitochondrial DNA from wild strains and cytoplasmic "petite" mutants of yeast were eluted at higher concentration than their nuclear counterparts[138]. Single-stranded DNA preparations from phage $\Phi$X174, phage $T_2$ and denatured DNA were eluted at lower molarities than native dsDNA and completely annealed DNA, indicating that limited fractionation might occur on the basis of molecular weight. At the same time, it provided evidence that hydroxyapatite distinguished DNAs by virtue of differences in their secondary structures[47,126,139]. This was further supported by two independent studies: (a) rapidly renaturing and slowly renaturing DNAs of rat liver could be separated by differential adsorption at 70 (ref. 140) and (b) a triple-stranded structure (2-poly-U·poly-A) was eluted at a molarity higher than that required for dsDNA.

Finally, according to Martinson[19], variations in the interaction of nucleic acid with hydroxyapatite, the charge densities of nucleic acids per unit length, the distribution of phosphate on nucleic acids and the redistribution of ions and water molecules during the adsorption of different nucleic acids are of minor importance when one considers the mechanism of adsorption and elution.

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## 8. SUMMARY

Hydroxyapatite column chromatography has provided a sensitive and powerful technique for the fractionation of nucleic acid species. Because of the widely differing molarities of elution, dsDNA, RNA–DNA hybrids and dsRNA could be separated from single-stranded RNAs, deliberately denatured DNAs and native ssDNA by either stepwise or gradient elution, in the presence or absence of formaldehyde, at room temperature or with a temperature gradient, at constant or different molarities, or by a combination of more than one of these factors. As hydroxyapatite is a

physico-chemically stable inorganic adsorbent, it has afforded many modifications for improving resolutions by the use of increased temperatures or by permitting the use of organic solvents in the eluents; modifications of this type are unique to hydroxyapatite alone.

Because of the high capacity (24 mg of tRNA/per square centimetre of the column bed) and the high recoveries (90%), hydroxyapatite columns offer a suitable analytical and also a preparative means for the fractionation of labelled aminoacyl-tRNAs at room temperature by using a gradient of potassium phosphate buffer (pH 5.4). Because of its increasing use in the separation of DNAs, RNA–DNA hybrids and tRNAs, it has provided ample experimental data for theoretical aspects to be considered, from which it will be possible to predict the chromatographic characteristics of certain molecules under fixed experimental conditions. Although it has several operational advantages, the inherent drawbacks introduced during its preparation often make it difficult to obtain good flow-rates, reproducible resolution patterns, etc. Randomly coiled polynucleotides are eluted at lower molarities than rigid, helical polynucleotides; moreover, factors that are capable of altering the conformation (such as changes in temperature or ionic strength or the presence of certain cations and organic solvents) determine the sequence of elution on hydroxyapatite columns. If conformational differences are negligible and other variables are constant, a hydroxyapatite column distinguishes nucleic acid molecules to a limited extent by virtue of differences either in their molecular size or in the base composition.

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