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RNA FRACTIONATION ON MODIFIED CELLULOSES

III. BD-CELLULOSE

R. M. KOTHARI AND MILTON W. TAYLOR
Department of Microbiology, Indiana University, Bloomington, Ind. 47401 (U.S.A.)

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1. INTRODUCTION

Several modified celluloses have been employed[1,2] for the fractionation of RNA into tRNA, rRNA and mRNA; and for further subfractionation of each species into either isoaccepting species or components. None of these celluloses has been completely satisfactory; each has certain operational limitations or inherent drawbacks. Although these celluloses are satisfactory in their own way, and solve the fractionation problem to some extent, in aiming at finer resolution based on a finite criterion, one had still to search for better and alternate methods of RNA fractionation.

In one of such attempts, GILLAM *et al.*[3] discovered that substitution of hydroxyl groups of DEAE-cellulose by aromatic groups increased the non-ionic interactions between polynucleotides and the newly substituted cellulose, benzoylated-diethylaminoethyl-cellulose (B-DEAE-cellulose abbreviated as BD-cellulose). It was also noted that BD-cellulose has more affinity for hydrophobic or lipophilic (*viz.* aromatic) groups than DEAE-cellulose[4]. Secondary forces binding tRNA to DEAE-cellulose were also considerably enhanced by benzoylating or naphthoylating most of the free –OH groups of the cellulose[5]. This was ascertained by the fractionation of yeast glycyl-, alanyl-, and histidyl-tRNAs with 5–30-fold enrichment, using an increasing concentration gradient of sodium chloride. Not only could specific aminoacyl-tRNA be purified, but the column also proved to be useful in the fractionation of isoaccepting species of tRNAs as evidenced by the separation of the glycine acceptor activity into two distinct peaks[5]. It was further noted that the purified isoacceptor species of alanyl-tRNA eluted at the same position on the chromatogram as its uncharged counterparts[5]. It must be emphasized here that the elution profiles of esterified or non-esterified tRNA species remained identical as long as no aromatic group is involved in esterification (as in above case); but once the aromatic amino acid (*viz.* phenylalanine) is involved in esterification, this observation is no longer true[6].

It has been revealed that retardation (strength of binding) of certain species of tRNA on a BD-cellulose column is a function of several environmental factors such as temperature, molarity and pH of the buffer, as well as the concentration of a divalent cation (affecting the secondary structure). Certain species of tRNA were quite strongly held by virtue of their content of special aromatic or hydrophobic groups, now commonly recognised as Y or Yr bases. Incorporation of aromatic amino acids onto specific tRNA yielded a corresponding aminoacyl-tRNA, which had also a higher affinity for the column, thus permitting partial purification from those tRNAs, which were either non-esterified or had no hydrophobic base(s) inherently in their primary structure[7].

Thus, by varying the conditions of secondary interactions, using the above principles, a more general method has been developed that allows purification and fractionation of any tRNA species into isoaccepting species by repeated chromatography on BD-cellulose[4,7]. In achieving this goal, BD-cellulose can be used in two ways: (a) directly for the chromatographic fractionation of aminoacylated tRNA wherein the amino acid is an aromatic one, and (b) when the amino acid is other than an aromatic one, the particular tRNA is aminoacylated with the corresponding amino acid and subsequently derivatized by attaching an aromatic substituent (a phenoxyacetyl- or naphthoxyacetyl-group). The esterification and/or derivatization with an aromatic entity causes a marked shift in the position of elution of that particular tRNA, with no change in the affinities of other tRNA species, ultimately resulting in purification.

Thus, in the present review article, an attempt is made to give an account of the use of BD-cellulose column and the biological significance of the separations achieved from a wide variety of sources. Also, we hope that through personal experience with this column, it may be possible to give a clearcut idea of its operational advantages, limitations and scope.

2. tRNA FRACTIONATION

(a) Yeast

An extensive fractionation of yeast tRNAs has been obtained by elution from a BD-cellulose column using an increasing concentration gradient of sodium chloride[8]. It was observed that 1.0 *M* sodium chloride eluted all the amino acid acceptor activities except for phenylalanine, which was eluted by 10% ethanol in 1.0 *M* sodium chloride yielding 10-fold pure phenylalanyl-tRNA. The authors[3] claimed that such a purification procedure is not only one single step and rapid, but is also superior to counter-current distribution (CCD)[9], and reversed-phase chromatographic (RPC) procedures[10] for purifying phenylalanyl-tRNA. The latter could be further enriched up to 25-fold by chromatography on a BD-cellulose-silicic acid (BDCS) column. Two observations borne out of this study were that the BDCS column has much less capacity than the BD-cellulose column and that BDCS binds tRNAs less strongly than does BD-cellulose as evidenced by the fact that phenylalanyl-tRNA was eluted from the BDCS column by 0.45 *M* sodium chloride. By repeated BD-cellulose chromatography, as high as a 55-fold purification of phenylalanyl-tRNA has been achieved[11]. In further studies, phenylalanyl-tRNA-free effluate of a BD-cellulose column could be utilized for the isolation of 28–32-fold pure tryptophanyl- and tyrosyl-tRNAs by repeated chromatography on BD-cellulose[12]. It was noted that the binding of tyrosyl-tRNA onto the BD-cellulose column was stronger than that of tryptophanyl-tRNA, permitting clear-cut resolution. While in an esterified state, their elution required an ethanol gradient in 1.0 *M* sodium chloride, stripping of their respective amino acid residues resulted in an early elution by a simple salt gradient. The authors[12] claim that their method of chromatographing the esterified tRNA in the first column run (to remove deacylated bulk tRNAs) and then shifting the elution profiles of deacylated species in the second chromatographic run (to space out elution of different species) lead to purification, essentially free of any contaminants.

In another study, by using BD-cellulose and RPC columns, which are operating on a different basis, an attempt has been made to purify isoaccepting species of phenylalanyl-tRNA from yeast, wheat germ and beef liver[13]. The procedure involved the fractionation of total tRNA on BD-cellulose for the removal of bulk tRNAs, giving a partially purified phenylalanyl-tRNA. The latter was then subjected to RPC and again to BD-cellulose chromatography, yielding two 50% pure and active isoaccepting species of phenylalanyl-tRNA at 0.8 and 1.3 *M* sodium chloride concentration.

In the above cases, success of purification largely depended upon interaction of the aromatic group of the amino acid (of acylated tRNA) with the benzoyl group of BD-cellulose. It apparently looked as if the applicability of this method was restricted to tRNAs specific for aromatic amino acids. This problem has been solved and a generally applicable method has been evolved by GILLAM *et al.*[14] as discussed earlier[4,7]. The phenoxyacetyl- or naphthoxyacetyl-group could be easily removed from the charged tRNA after chromatography without disturbing its acceptor activity. Thus, chromatography of a derivatized tRNA (to remove bulk tRNAs) and rechromatography of this partially purified tRNA (to remove residual contaminants) yielded 25–30-fold pure tRNAs as exemplified by the purification of glycyl-, threonyl-, aspartyl-,

arginyl-, and methionyl-tRNAs. In general, rechromatography was highly essential in view of the tendency of a small portion of tRNA to dissociate more slowly from the BD-cellulose column than does the bulk tRNA, causing contamination to some extent.

While making comparative studies on yeast and mammalian phenylalanyl-tRNA, it was observed that normal adult rat liver phenylalanyl-tRNA displayed high affinity for BD-cellulose providing a simple chromatographic procedure for its purification[15]. It was revealed that the observed high affinity of phenylalanyl-tRNA towards BD-cellulose was due to the presence of a fluorescent and lipophilic nucleotide, Yr, similar to the nucleotide Y, present in yeast phenylalanyl-tRNA. Phenylalanyl-tRNAs from fetal rat liver, rat liver nuclei and mitochondria, rabbit reticulocytes, and Novikoff hepatoma were screened for their chromatographic characteristics with respect to BD-cellulose. Exhibition of a similar chromatographic behaviour indicated that this nucleotide or a closely related nucleotide was widely distributed in mammalian phenylalanyl-tRNAs. A probe was made to unravel further structural details of the purified yeast phenylalanyl-tRNA by reacting with kethoxal or glyoxal[16]. It was noted that kethoxal or glyoxal attached on the twentieth and thirty-fourth nucleotide residues of phenylalanyl-tRNA, with subsequent loss of 50% and 10% acceptor activity, respectively. However, the charged and uncharged phenylalanyl-tRNAs after such modification could be separated by BD-cellulose chromatography.

An interesting approach to studying tRNA function involved polynucleotide chain scission under controlled conditions, and analysis of the acceptor activity before and after reconstitution of the oligonucleotide halves. Accordingly, a mixture of halves of yeast phenylalanyl-tRNA produced by chemical cleavage of the anticodon loop, was resolved first on Sephadex G-100 and then on a BD-cellulose column at 57° in both cases[17]. The 3'- and 5'-halves, thus separated, showed no acceptor activity before reconstitution, but exhibited almost original acceptor activity after reconstitution. High temperature (57°) was purposefully maintained during the chromatography to avoid base pairing between complementary sequences, since a long column, a shallow gradient, and high pH did not favour the separation of halves.

Yeast alanyl-tRNA has been purified from the bulk tRNAs using BD-cellulose chromatography[18–22]. Gel filtration chromatography and RPC were also employed to ensure high purity and yield[21]. Two peaks of alanyl-tRNA were observed, of which the minor peak, elutable at 0.75 *M* sodium chloride concentration, represented 15% of the total alanyl-tRNA, and was a mixture of alanyl-tRNA monomers and dimers. Alanine acceptor assay of this fraction showed that two molecules of alanine could be esterified to the dimer at a rate of esterification equal to that of the monomer. A double clover-leaf model can explain the structure of this dimer which was relatively more resistant to RNAase T_1 than the monomer, indicating its anticodon region was less exposed than that of the monomer. This study also indicated the probable site of dimerization.

It was surprising to note that the alanyl-tRNA purified by BD-cellulose chromatography[19] differed in its nucleotide sequence as well as in codon response from the alanyl-tRNA which was isolated by CCD[23].

In order to examine more structural features of alanyl-tRNA and to elucidate the chemical basis of recognition of tRNA by its synthetase, inactivation of purified alanyl-tRNA (from yeast, *E. coli* and wheat germ) by UV light was studied. Active

and UV-inactivated alanyl-tRNAs were separated on a BD-cellulose column by stepwise elution and it was found that the inactivation was due to extensive photochemical modification in the stem nucleotides near the acceptor end of tRNA. It was inferred that a localized ordered structure near the acceptor end of tRNA was essential for the exhibition of acceptor activity; a unique native conformation of the entire molecule was not necessary[20].

By repeated chromatography on BD-cellulose and DEAE-Sephadex A-50 columns, glutamyl-tRNA was purified and fractionated into four isoaccepting species, I–IV (refs. 24, 25). Glutamyl-tRNA I and III fractions could bind to *E. coli* ribosomes in the presence of codon GAG and GAA, respectively; while glutamyl-tRNA II and IV fractions did not exhibit any specificity or order of choice, they could bind well in the presence of either GAG or GAA. The specificity of fractions I and III was further tested by the transfer of glutamic acid residue to different positions in rabbit hemoglobin.

Yeast lysyl- and valyl-tRNAs have been purified on a BD-cellulose column[26,27]. It was originally thought that the BD-cellulose column chromatography yielded a tRNA species possessing reduced structure as a contaminant in the ethanol fraction, which required removal by subsequent procedures. That the purified tRNA has neither reduced nor altered its secondary or tertiary structure as a result of passage through the BD-cellulose column was confirmed by the analysis of elution profiles on a methylated albumin-silicic acid (MASA) column.

Total tRNA preparations from *S. lactis* and *S. cerevisiae* were fractionated on DEAE- and BD-cellulose columns[28]. The fractions, in each case, were tested for their cytokinin activity in the tobacco leaf bioassay. It was found that cytokinin activity was associated with those tRNAs whose codons began with uridine. These studies were further extended with *E. coli* tRNAs[29,30].

(b) E. coli

Chromatographic comparisons on the BD-cellulose and the MASA columns of the genetically (Su^+_{36}-tRNA) and chemically (HNO_2-glycyl-tRNA) derived suppressors have shown them to be different molecular species[31]. It was apparent that suppressor-tRNA was present in a relatively small quantity in Su^+_{36}, probably up to the extent of 1.0% of the total glycyl-tRNA. Although the nature of the structural change in these species was not known, BD-cellulose column profiles of glycyl-tRNA before and after mutation were different[32]. Suppressor mutants were found in both glycyl-tRNA I and glycyl-tRNA II. The BD-cellulose column furnished three isoaccepting species of glycyl-tRNA from *E. coli*[33]. These glycine acceptor activities were characterized for their codon specificity and observed that while glycyl-tRNA I recognized predominantly GGG, fraction II recognized both GGG and GGA, and fraction III recognized GGU and GGC[34,35].

ROY AND SÖLL[36,37] obtained highly pure species of seryl-, cysteinyl-, tyrosyl-, phenylalanyl-, and tryptophanyl-tRNAs on a BD-cellulose column. The fractionation was done at 2° in the absence of Mg^{2+}, presumably with less folded secondary structure to facilitate easy elution and to space out distribution of different species of tRNA. Upon rechromatography, seryl-tRNA obtained in the ethanol fraction yielded five isoaccepting species[37]. In further studies[38], three species (I, IIIa and IIIb) of

seryl-tRNA were obtained in a highly pure state by the combined use of DEAE-Sephadex A-50, BD-cellulose, and RPC columns. It was noted that DEAE-Sephadex A-50 could remove seryl-tRNA II contamination from seryl-tRNA I-furnishing sharp resolution. The characterization of these isoaccepting species for their codon specificity and for acylation with homologous and heterologous seryl-tRNA synthetases furnished the following information: (a) seryl-tRNA I predominantly recognized the codons UCA and UCG, and less effectively the codon UCU, and contained uridine-5-oxyacetic acid and methyl-thio-isopentenyl-adenosine as the minor bases; (b) while seryl-tRNA IIIa recognized the codon AGU and contained adenosine derivative of threonine as a minor base, seryl-tRNA IIIb recognized the codon AGC and contained a minor constituent analogous to threonine-containing adenosine derivative; (c) the region participating in the codon–anticodon interaction in tRNA had no relation to the recognition of synthetase since a single seryl-tRNA synthetase from *E. coli* served in charging all the isoaccepting species of seryl-tRNA; and (d) charging of these isoaccepting species by yeast and rat liver synthetases implied that these tRNAs have some common structural features with those from yeast and rat liver.

Derivatized leucyl-tRNA was purified on a BD-cellulose column from the bulk of unesterified non-derivatized tRNAs. Upon rechromatography, leucyl-tRNA yielded five (I–V) isoaccepting species[39] with sharp resolution possibly suggesting that various species possessed large differences in their primary structures. Fractions IV and V contained an isopentenyl-adenosine derivative and were, therefore, relatively retarded on the column. The major leucyl-tRNA fraction represented 50–80% of the total leucine acceptor activity. It was found that the species differed in their coding properties as well as in charging by leucyl-tRNA synthetase either from KB cells or from yeast. While *E. coli* leucyl-tRNA synthetase could charge all the isoaccepting species equally well, synthetase from KB cells and from yeast could esterify only some of the species. The analysis of the primary structure of leucyl-tRNA I and II species from *E. coli* K-12 showed that both possessed a chain-length of eighty-seven nucleotides and differed in twenty-two positions in the complete primary structure[40]. The sequential arrangement of nucleotides of one of these species was identical to that of leucyl-tRNA from *E. coli* B.

In the further course of studies[41], leucyl-*amber* suppressor-tRNA of *E. coli* Su^+_6 strain was purified on a BD-cellulose column. The purified leucyl-tRNA participated in *in vitro* polyleucine synthesis directed by poly (UAG). It was notable that this tRNA possessed the same charging ability as the homologous species from the normal source (*i.e.* without suppressor activity, from Su^-_6 strain). Each of the species accounted for about 5% of the total leucine acceptor activity[42]. While the binding of leucyl-suppressor-tRNA to ribosome was stimulated by *amber* codon UAG and not by leucine codon UUG, the homologous leucyl-tRNA exhibited the opposite codon specificity, indicating a change in anticodon due to suppressor mutation in Su^-_6 gene. It was noticed that another species of leucyl-tRNA not affected by suppressor mutation also bound ribosomes with UUG. Chromatography and rechromatography of leucyl-suppressor-tRNA furnished six peaks of which two were suppressor-specific and were contaminated with seryl-, tyrosyl-, phenylalanyl-, and tryptophanyl-tRNAs. With additional repurification on BD-cellulose, their removal was possible, resulting in up to 60-fold increase in specific suppressor activity. A comparison of leucyl-tRNA profiles from Su^+_6 and Su^-_6 strains on a BD-cellulose column showed the following: (a) whereas in

Su^+_6 strain, only one fraction of UUG-binding-leucyl-tRNA existed, in the Su^-_6 strain, two fractions were present; and (b) in the Su^+_6 strain, one of the UUG-responding tRNAs had been replaced by a UAG-responding tRNA. The most probable mechanism underlying this alteration appears to be a base substitution in the anti-codon region of leucyl-tRNA converting the codon CAA to CUA in the suppressor species[42].

A BD-cellulose column has been employed also to compare leucyl-tRNA profiles before and after phage T_4 infection[43]. It was noted that a particular isoaccepting species of leucyl-tRNA present before the infection was found in the form of two halves 1 min after infection; the halves started disappearing from the chromatogram after 5 min of infection and completely lost after 8 min. The significance of the observed cleavage of leucyl-tRNA species, 1 min after infection, is still not understood; it may be due to a phage-induced nuclease which leads to cessation of host-specific protein synthesis.

A general method has been devised for the separation of isoaccepting species of tRNAs, consisting of chemical derivatization and subsequent chromatography on BD-cellulose, RPC, and DEAE-Sephadex columns[44]. Five species of leucyl-tRNA, having a purity of the order of 80–100%, were purified using this method. Four species of arginyl-tRNA have also been recovered using a similar procedure, but they were less pure due to considerable damage during the chemical derivatization step. The limitation of this method is that, due to large number of steps involved, losses are multiple and therefore overall recovery is low.

Isoleucyl-tRNA has been purified from bulk tRNAs and was rechromatographed on a BD-cellulose column to yield four isoacceptor species[45]. These species were studied for substrate–enzyme interaction to examine which regions of tRNA structure were involved in recognizing the synthetase during aminoacylation.

^{32}P-labeled total tRNA preparation has been fractionated on a BD-cellulose column, and the valine acceptor activity detected on the chromatogram, was rechromatographed after phenoxyacetylation to obtain pure valyl-tRNA[46]. Similar purification has been obtained also by NISHIMURA *et al.*[47]. Further subfractionation on a BD-cellulose column yielded two isoaccepting species of valyl-tRNA with 95% purity (ref. 48). These species were used for complex formation with cognate synthetase from *E. coli*. The complex formed could be detected either by sucrose density gradient centrifugation or by membrane binding assays, both of which indicated that valyl-tRNA, irrespective whether it is acetylated, deacetylated or lacking its terminal adenosine residue, bound to the enzyme with identical affinities[49]. Purification of valyl-tRNA I and II revealed that fraction I represented 80% of the total valine acceptor activity[50]. Being a major fraction, it was subjected to irradiation at 334 nm to examine its structural details. It was noted that the 4-thiouridine entity of tRNA structure was affected (change in fluorescence) due to irradiation resulting in the formation of a covalent linkage between 4-thiouridine (8th residue) and cytidine (13th residue). Still there was no detectable change in size and shape of valyl-tRNA I as revealed by low-angle X-ray scattering pattern[51]. However, thermal denaturation studies did show a local change in the tertiary structure of tRNA without overall change in its shape[47,51]. Irradiated valyl-tRNA I could still be acylated but with acceptor activity drastically reduced. It could also incorporate valine into the polypeptide chain, but at a slower rate than the normal homologue[52]. However, it was

notable that no difference was exhibited by normal and irradiated valyl-tRNA I for ribosome binding in the presence of poly (U,G)

Recently, purified valyl-tRNA was cleaved into halves, each of which alone was insufficient to exhibit acceptor activity indicating that recognition required more structure than was provided by either half individually. The functional complex obtained by simply mixing the halves, however, exhibited acceptor activity, suggesting that the intact anticodon was not necessary[53].

Phenoxyacetylation of methionyl-tRNA and fractionation on a BD-cellulose column was found to be a rapid and convenient method of obtaining highly purified N-formyl-methionyl-tRNA (eluted early) and methionyl-tRNA (eluted later), well separated from each other[54–56]. However, SENO *et al.*[57] have recommended the combination of chromatographic procedures (chromatography and rechromatography on DEAE-Sephadex A-50, then either on BD-cellulose or on RPC) for the large-scale fractionation of isoaccepting species of methionyl-tRNAs. BD-cellulose chromatography was preferred to RPC for two reasons: firstly that resolution was better and secondly, that larger amounts could be handled by a BD-cellulose column than with the RPC column of the same dimensions. It was observed that chromatography at 4° furnished higher degree of purification than at 22°, presumably due to selective stronger binding at low temperature. It is considered that N-acetylation of the methionine residue of tRNA induced differential conformations in these two isoaccepting species of methionyl-tRNA which subsequently appeared at different positions on the chromatogram due to differential affinity towards BD-cellulose[54]. The thermal denaturation profiles, temperature of melting (T_m) and the base composition of N-formyl-methionyl-tRNA (F-met-tRNA) and methionyl-tRNA (met-tRNA) differed significantly, indicating marked differences in their primary structures[57]. In ribosomal binding studies, F-met-tRNA (fraction I) was recognized by the codons AUG, GUG, and UUG, while met-tRNA (fraction II) responded principally to the codon AUG. Both species contained less than 3.0% contamination due to seryl-, glutamyl-, and lysyl-tRNAs and incorporated methionine in a polypeptide synthesis directed by phage f_2 RNA in a cell-free system, but at different positions.

UV-irradiated F-met-tRNA was separated on a BD-cellulose column into two components, an active and an inactive[58]. Inactivation was almost entirely due to modification of cytidine residues in the 3′-terminal-CCA sequence. Selective reduction with sodium borohydride showed that modification of (a) uridine in anticodon and in small loop, and (b) 4-thiouridine and other pyrimidines in double-stranded stem adjacent to the dihydrouridine loop, had no effect on either aminoacylation or transformylation.

Cochromatography of [^{75}Se]selenomethionyl- and [^{14}C]methionyl-tRNAs on BD-cellulose by the usual salt gradient yielded two almost overlapping peaks of isoaccepting species in each case[59].

Histidyl- and tyrosyl-tRNA have been purified, and upon fractionation each of the pure tRNAs yielded two isoaccepting species by repeated use of BD-cellulose column chromatography[60,61]. While histidyl-tRNA was subjected to thermal transition studies in the 4-thiouridine region[60], tyrosyl-tRNA was analysed for its primary structure[61].

Tyrosyl-tRNA from *E. coli* infected with transducing phage φ 80 Su^+_{III} was purified first by polyacrylamide gel electrophoresis and subsequently the selected

regions of the electropherogram were purified on a BD-cellulose column[62]. The separation of three discrete species was based upon the presence of a modified isopentenyl base. A similar separation had been achieved on reversed-phase columns. Since these tRNAs have been fully sequenced, it was thought that they would be useful for structural studies using the reaction between methoxyamine and cytosine. Therefore cytosine residues in the Su^{+}_{III} tyrosyl-suppressor-tRNA were allowed to react with methoxyamine. It was already known from the pilot runs that in the pH range 5.0–5.5, approx. 20% cytosine bases in *E. coli* tRNA were reactive towards methoxyamine. Su^{+}_{III} tyrosyl-suppressor-tRNA characterised after such reaction showed that cytosine residues at positions 16, 19, 35, 51, 83 and 84 were sensitive, while the residue at posision 33 (which is at the 5′-terminal of the anticodon loop) was relatively resistant to such a modification.

A combined procedure consisting of three columns (RPC, BD-cellulose and MASA) operating on different approaches was used to purify methyl-deficient phenylalanyl-tRNA and its normal counterpart[63]. Phenylalanyl-tRNA fractionated on a RPC column was pooled, concentrated and freed from salts by ultrafiltration and was chromatographed on BD-cellulose to yield phenylalanyl-tRNA of high purity. The correlation of degree of methylation with amino acid acceptor activity showed that (a) whereas unfractionated methyl-deficient phenylalanyl-tRNA possessed 10–25% higher amino acid acceptor activity than the unfractionated normal preparation, such differences were not observed after fractionation, and (b) while exposure to pH 2.9 reduced the acceptor activity and altered the elution profiles, prolonged exposure to pH 4.5 did not show detectable effect on either acceptor activity or elution profiles. The reduction in acceptor activity and alteration in profiles at pH 2.9 are presumably due to altered conformation, unfavourable for both.

It has been observed that tryptophanyl-tRNA is capable of existing in two frequently interchangeable forms, one of which possesses acceptor activity, while the other is inactive[64]. Taking the advantage of these conformational changes, a quantitative procedure has been developed to purify tryptophanyl-tRNA from the bulk of tRNAs. The procedure consisted of (a) chromatography of the bulk tRNA on BD-cellulose under conditions in which majority of tRNA was eluted except tryptophanyl-tRNA and (b) the enriched tryptophanyl-tRNA was subsequently purified on the methyl albumin on Kieselguhr (MAK) column in the presence of Mg^{2+} which converted it into an active form[65]. Similar procedures using BD-cellulose chromatography for the gross purification of tryptophanyl-tRNA, and using RPC[66] or hydroxyapatite[67] columns in place of the MAK column for final purification are reported. It was found that tryptophanyl-tRNA from Su^{+} strain was more stable and remained in an active form during the chromatography, while the species from Su^{-} strain emerged late in the denatured form as a single peak[66]. Both on hydroxyapatite or BD-cellulose columns, only one major species of tryptophanyl-tRNA was observed, which was in an active form and served as the only substrate for tryptophanyl-tRNA synthetase[67].

The codon UGA has been shown to be a terminating codon in many strains of *E. coli*. Several suppressors of UGA have been isolated and in one of the suppressor strains it has been shown that tryptophan is put in at the UGA-codon site. It was therefore, interesting to purify a particular species of tRNA from the suppressor strain. Total tRNA preparations from Su^{+} and Su^{-} strains of *E. coli* were fractionated using a step-wise elution, and tryptophanyl-tRNA was eluted in the 1 *M* NaCl–19%

ethanol fraction. By repeated chromatography on BD-cellulose columns using a different-concentration-gradient elution, tryptophanyl-tRNA could be purified further. While tryptophanyl-tRNA from Su^+ strain was capable of suppressing a UGA mutation in an *E. coli*–f_2 phage protein synthesizing system, neither the homologous species from the Su^- strain nor other tRNAs purified from Su^+ strain could suppress the UGA codon. The suppressor-tRNA was subjected to further fractionation and the suppressor activity was correlated with tryptophan acceptor activity[68].

When total tRNA preparation from *E. coli* was chromatographed on BD-cellulose, it was observed that partially purified seryl-, leucyl-, tyrosyl-, phenylalanyl,- and tryptophanyl-tRNAs were associated with cytokinin activity[29]. In further studies[30], tRNA preparations from the early, middle and late logarithmic phases of *E. coli* were chromatographed on BD-cellulose; no differences in the elution profiles of tRNAs specific for the above amino acids were found irrespective of the differences in growth phases. Confirmation of these observations revealed that cytokinin activity in these tRNAs was confined to two ribonucleosides, which were subsequently purified and analysed. They are 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosyl purine and its 2-methylthio analogue.

Chromatography of normal tRNA and tRNA treated *in vitro* with a carcinogen N-acetoxy-2-acetyl-aminofluorene (AAF) on BD-cellulose yielded clear and distinctly different profiles[69]. AAF-treated tRNA exhibited less acceptor activity for all amino acids (except for valine), alteration in the codon recognition, ribosomal binding and also profiles on DEAE-Sephadex A-50. It was found that these drastic changes in physico-chemical and biological characteristics were due to a covalent attachment of AAF to the guanosine residues affecting the secondary and tertiary structure. Furthermore, this effect was dependent upon the number of guanosine residues modified. The modification seemed at those sites which were critical for normal functions of tRNA. The observed high acceptor activity for valine may be due to several reasons: AAF-modification must have (a) permitted the mischarging for valine, (b) enhanced the affinity between corresponding tRNA and aminoacyl-synthetase, or (c) renatured some of the denatured species of valyl-tRNA. In our opinion, however, whether the observed marginal differences in the physico-chemical as well as biological characteristics have some significance to carcinogenesis or not will have to be evaluated after a systematic exploration of these effects in an independent system.

A broad separation of *E. coli* tRNAs into three fractions, elutable at low salt concentration, at 0.8 *M* salt concentration, and at 17% methoxyethanol concentration, on a BD-cellulose column has been reported[70].

(c) Salmonella

Total tRNA preparations from the wild strain of *Salmonella typhimurium* and from four classes of mutants constitutive for the histidine operon, were chromatographed on BD-cellulose and RPC columns and the elution profiles were compared for differences; no detectable differences were found in the elution profiles[71]. Although a BD-cellulose column gave no evidence for the multiple species of histidyl-tRNA from the wild strain, a RPC column did indicate a possible presence of a minor histidyl-tRNA species. However, the tRNA preparations from different mutant strains did not reveal any sign of isoacceptor species.

(d) Bacillus

tRNAs from log phase cells of *B. subtilis* were chromatographed on a BD-cellulose column. Elution by an increasing concentration gradient of sodium chloride yielded two peaks of lysyl-tRNA, I and II (ref. 77). The fractions I and II could be recharged after deacylation indicating they were functionally intact. Rechromatography of each fraction separately on BD-cellulose gave sharp single peaks establishing their homogeneity. These fractions could hybridize in an additive fashion with the homologous DNA. Further studies indicated that the primary structure of these two species differed significantly, suggesting that there exist two cistrons, one for each species of lysyl-tRNA.

Fractionation studies on labeled tRNA from *Lactobacillus acidophilus* on a BD-cellulose column indicated that an unusual nucleotide, N^6-(Δ^2-isopentenyl)-adenosine, was localized in tRNAs specific only for serine, cysteine, leucine, tyrosine, and tryptophan[78]. Further fractionation studies revealed that all the isoaccepting species of tRNA specific for serine and leucine did not contain this unusual nucleotide as seen in case of *E. coli* (ref. 38). The authors[78] feel that the observed distribution of this nucleotide is consistent with the model that a tRNA must have an anticodon whose 3'-end is the base A in order to contain the above nucleotide in the adjacent position[79].

(e) Mycoplasma

tRNAs from *Mycoplasma laidlawii* B were fractionated on a BD-cellulose column. Two peaks of methionine acceptor activity were sharply separated from each other. While the major peak eluting earlier corresponded to methionyl-tRNA, the minor peak eluting later could be formylated by *E. coli* extracts[80]. Two other strains of Mycoplasma also showed the presence of formylable methionyl-tRNA. It may be involved in peptide chain initiation in Mycoplasma just as in other procaryotic organisms. In general, Mycoplasma tRNAs showed close resemblance to *E. coli* tRNAs (*viz.* in sedimentation analysis and melting behaviour).

(f) Other microorganisms

LENARD *et al.*[72,73] resolved phenylalanyl-tRNA and its synthetic acetylated form from *Pseudomonas fluorescens* by chromatography on a BD-cellulose column using an ethanol gradient in 1.0 *M* sodium chloride. Phenylalanyl-tRNA and acetyl-phenylalanyl-tRNA appeared on the chromatogram at 4.7% and 8.4% ethanol concentration, respectively, accounting for 75% recovery.

In another study, N-formyl-methionyl-tRNA and methionyl-tRNA present in the growing cultures of *Pseudomonas aeruginosa* were sharply resolved on a BD-cellulose column[74]. The easily elutable fraction at a low salt concentration apparently corresponded to N-formyl-methionyl-tRNA of *E. coli* in that it was susceptible to *in vitro* formylation by *E. coli* transformylase. This species was hardly detectable with *in vivo* extracts, but an approximately equivalent amount of N-formyl-methionyl-tRNA was eluted at higher salt concentration in an overlapping peak with the second species of methionyl-tRNA, suggesting that methionyl-tRNA was completely formylated *in vivo*. It was concluded that the amount of methionyl-tRNA species (elutable at low

salt concentration) was reduced in proportion to the amount of N-formyl-methionyl-tRNA (elutable at higher salt concentration) formed.

Total tRNA preparation from Neurospora cytoplasm was first fractionated on DEAE-Sephadex gel, and selected fractions of the DEAE-Sephadex chromatography were further resolved on a BD-cellulose column to yield two major species of methionyl-tRNA[75]. One of the species was formylated by *E. coli* transformylase and had more affinity than the other species for ribosome binding in the presence of the codon AUG, indicating that the former species was equivalent to N-formyl-methionyl-tRNA and presumably functioned as an initiator without prior formylation in contrast to the latter species which transferred methionine exclusively in internal positions.

BD-cellulose cochromatography was employed to compare the elution profiles of isoaccepting species of doubly labeled tRNAs from aerobic and anaerobic photosynthetic cultures of *Rhodopseudomonas spheroides*[76]. In methionyl-tRNA, apparent difference existed between the relative amounts of isoaccepting species. Phenylalanyl-tRNA from aerobic culture was separated into two major and several minor species. It was noted that tryptophanyl-tRNA esterified *in vivo* and *in vitro* exhibited closely similar elution patterns. The elution profiles of the isoaccepting aminoacyl-tRNA species from these two sources in most instances showed close correspondence except for prolyl-, phenylalanyl-, and tryptophanyl-tRNAs where profiles differed significantly. These observations were reproducible and were independent of the source of aminoacyl-tRNA synthetase, meaning that the observed changes in each case were intrinsic to tRNA itself. It was suggested that the altered amounts of tRNA species as reflected from the profiles may represent either differential transcription of tRNA genes or an intracellular conversion of one isoacceptor species into another.

(g) Plants

BD-cellulose chromatography has been employed in the fractionation of bulk tRNA isolated from wheat germ[81,82] using an increasing concentration gradient of sodium chloride in the presence of EDTA (Mg^{2+} absent) at pH 4.5. Three fractions (two major and one minor) possessing methionine acceptor activity were detected. The minor species elutable at 0.75 *M* sodium chloride concentration was contaminated with major fraction II, and could be purified to homogeneity by rechromatography on a BD-cellulose column, now in the presence of 10 m*M* magnesium chloride. It was suggested that of the three species, two were formylable and possibly represented cytoplasmic and chloroplastic F-met-tRNA, while the third one was non-formylable. Further studies[83] have confirmed the above hypothesis. It was observed that F-met-tRNA I was located in the cytoplasm of wheat germ cell, was not formylable by wheat extracts, and appeared to function in protein chain initiation in the cytoplasm without prior formylation. The other species (F-met-tRNA II) was located in the chloroplast in formylated form[84]. It was also noted that there were two more species of methionyl-tRNA, one major from the cytoplasm and one minor from the chloroplast, and both functioned to transfer methionine residues exclusively in the internal positions of a growing polypeptide chain. Similar studies have also been reported by YARWOOD *et al.*[85] in the developing seeds of *Vicia faba*. BD-cellulose chromatography has revealed that *Vicia faba* tRNA contained three species of methionyl-tRNA, two major and a minor. Although neither of the species could be formylated by *Vicia faba*

extracts, the major I species could be charged and subsequently formylated by *E. coli* transformylase. Studies on AUG binding, release of methionyl–puromycin complex, N-terminal analysis of endogenous messenger and poly (AUG) directed incorporation indicated, that this species was involved in protein chain initiation, while the minor species possibly acted as an initiator in the cell organelle and major II species acted as methionyl-tRNA for incorporation of methionine exclusively in the internal positions. It is hoped that additional studies will detect the presence of a methionyl-tRNA species, performing the function of exclusive incorporation of methionine into internal positions in protein of organelle origin.

About 10–15-fold purification of wheat germ phenylalanyl-tRNA has been achieved using a BD-cellulose column[86,87]. Partially purified tRNA, obtained as above, could be purified to the extent of 90% using an RPC-5 column[86] and was analysed for its primary structure to provide information on the variability, if any, in the sequential arrangement of nucleotides of phenylalanyl-tRNAs from widely different sources. The analysis showed that (a) wheat germ phenylalanyl-tRNA has a structure very similar to that of yeast phenylalanyl-tRNA, (b) only ten out of the seventy-six nucleotides were different, (c) all but two nucleotide changes were located in the double-stranded region of a clover-leaf model, (d) two changes were found in the single-stranded regions involving a modification of the same nucleotides, and (e) dihydrouridine loop and its supporting stem were identical in structure in both the species. With the complete knowledge of the primary structure, an attempt was made[87] to obtain information on the secondary and tertiary structure. It was observed that when phenylalanyl-tRNA was exposed to pH 9.0, the fluorescent base Y located adjacent to the 3'-end of the anticodon was modified resulting in the formation of phenylalanyl-tRNA II. Although the latter tRNA also exhibited fluorescence, its place on the chromatogram was quite different due to a modification of Y base to a new fluorescent base, which had a free –COOH group in alkaline not present in acidic condition. Except for this change, which resulted in an altered elution profile on the chromatogram, and a reduced rate of poly U-directed polyphenylalanine synthesis, phenylalanyl-tRNA I (original species) and II (after alkali treatment) were identical in all other respects.

Fluorescent studies with organelle tRNAs showed that phenylalanyl-tRNA from *Euglena gracilis* chloroplasts or from *N. crassa* mitochondria did not contain a Y base as against its presence in their respective cytoplasmic homologous species[88]. This difference facilitated their easy separation using BD-cellulose chromatography; those containing a Y base (lipophilic group) eluted in the ethanol fraction while those lacking it eluted earlier in the salt gradient. The fluoroscopic examination of a *N. crassa* cytoplasmic tRNA and yeast tRNA was suggestive of the differences in the structure of Y bases although both were closely related. Phenylalanyl-tRNAs, thus purified, were capable or participating in the poly U-directed polyphenylalanine synthesis, irrespective of their origin (cytoplasmic or organelle) or their odd base content.

BD-cellulose chromatography was routinely followed to compare the elution profiles of tRNAs from *Nicotiana tabacum* before and after infection with tobacco mosaic virus (TMV)[89]. No qualitative or quantitative changes were noted in the nature of elution profiles of alanyl-, seryl-, leucyl-, isoleucyl-, methionyl-, and histidyl-tRNAs before and after the TMV infection. However, a shift in the elution profiles of

phenylalanyl-tRNA was detected after the TMV infection if young plant leaves were infected, but was undetectable when old plant leaves were infected. Apparent dependence of the alteration in phenylalanyl-tRNA profiles on the physiological state (*viz.* age) of tobacco leaves may mean that the change in the elution pattern may not be a direct result of TMV infection.

(h) Animals

Rat liver phenylalanyl-tRNA has been purified from a mixture of tRNAs using BD-cellulose and RPC-2 column chromatography[90]. The fluorescent constituent in the tRNA was used to monitor the progress (degree) of purification. Similar studies have also been reported on beef liver phenylalanyl-tRNA and the purified phenylalanyl-tRNA was used to study Mg^{2+}-induced enhancement of fluorescence and conformational changes[91]. By employing DEAE-Sephadex, BD-cellulose and/or RPC column chromatography, a general procedure has been evolved for the excellent resolution and considerable purification of rat liver tRNAs specific for sixteen amino acids[92]. The elution profiles showed the presence of isoaccepting species of tRNAs for almost all the amino acids. The codon recognition studies with the resolved species of tRNA were consistent with the "wobble" hypothesis.

Aminoacylated tRNAs from Vero cells (an established line from African green monkey kidney) were chromatographed after phenoxyacetyl derivation[93]. The tRNAs were subsequently used for homology studies: mouse kidney and *E. coli* DNAs did not hybridize effectively with the monkey kidney tRNAs implying the latter have little, if any, sequence homology with the mouse kidney or *E. coli* DNA. The homology studies concluded that in contrast to *E. coli* and yeast, monkey kidney genome contained several hundred redundant copies of tRNA genes.

A systematic study has been made to examine the changes in the chromatographic profiles of *in vitro* aminoacylated tRNAs during the progress of phosvitin synthesis in rooster liver[94]. It was noted that during the rapid phase of phosvitin synthesis, seryl-tRNA activity in the estrogen-treated roosters was at a higher level than in the control roosters, regardless of the source or concentration of acylating enzyme, concentration of tRNA, period of acylation, etc. Further studies on a BD-cellulose column[95,96] revealed that seryl-tRNA could be fractionated at least into four distinct species from the liver of control as well as estrogen-treated roosters and rats. A marked increase in one major and one minor seryl-tRNA peak was noticed during the rapid phase of phosvitin synthesis. With decreasing rate of synthesis, the level of the seryl-tRNA profiles approached to the control level. Similar chromatographic comparisons of tRNAs specific for other amino acids, before and after administration of estrogen during the rapid phase of phosvitin synthesis did not reveal major noticeable changes like those observed for the seryl-tRNA. However, small changes in minor species of glycyl-, glutamyl-, and histidyl-tRNAs were observed. The analysis of the four species of seryl-tRNA revealed the presence of a species of tRNA specific for O-phosphoserine. While fraction I, II and III were specific for serine, fraction IV contained a mixture of seryl- and O-phosphoseryl-tRNA. This fraction was detected on the chromatogram irrespective of whether the tRNA was charged or uncharged prior to chromatography. In ribosome-binding studies[96], this fraction responded very slightly to poly (U,C), but not to any of the known codons assigned for L-serine, suggesting

that this unique fraction probably arose by the phosphorylation of seryl-tRNA.

AGARWAL and his associates[97–100] have routinely used BD-cellulose chromatography to compare the profiles of tRNAs from normal rat liver before and after either cortisone treatment[97–99] or carcinogen administration[100]. Isoaccepting species of leucyl- and tyrosyl-tRNAs were analysed and no detectable differences were found in their profiles.

The following gross changes were noticed after the administration of the hepatic carcinogen, N-acetoxy-2-acetyl-aminofluorene (AAF) or its hydroxy analogue to rats[100]: (a) shortly after the administration, the tRNA fraction had two to three times higher specific activity (counts of AAF) than the 5S, 18S and 28S rRNA entities; (b) after 12 h, maximal binding of AAF was found in both rRNA and tRNA; (c) before 24 h, tRNA had higher specific activity than rRNA; (d) between 24–48 h, rRNA and tRNA had comparable specific activities, and (e) after 5 days, the activity in tRNA was decreased. Small amounts of AAF-containing tRNAs, due to its affinity for lipophilic groups, were separated from the bulk of unmodified tRNA on BD-cellulose. After extensive purification, these tRNAs were screened for modification in minor thio-bases, which could be potential susceptible sites for attack by various carcinogens.

Distribution of specific species of tRNAs from normal and neoplastic tissues has been compared using BD-cellulose[101–103]. For this purpose, total tRNA preparations from rabbit liver, EAT and L cells were chromatographed with and without aminoacylation and it was noted that the profiles were similar irrespective of aminoacylation, ruling out any possibility of either an aggregation or the degradation of tRNAs. Three species of tyrosyl-, four species of lysyl-, and five species of seryl-tRNA were detected. That the presence of altered isoaccepting species of tRNA may not have any direct functional significance in the regulation of protein synthesis in the neoplastic state, but may possibly reflect minor mutational events at non-functional place(s) in the gene that have become fixed and are translated into tRNA, was considered. In further studies, two isoaccepting species of phenylalanyl-tRNA from normal rat liver have been purified using BD-cellulose and RPC column chromatography[102]. It was noted that the species elutable at a low salt concentration represented a minor fraction of phenylalanyl-tRNA and was 100% pure, while that elutable at higher salt concentration represented the major fraction and was 80% pure, seryl-tRNA being the only contaminant. Similar studies have been extended for the purification of isoaccepting species of phenylalanyl-tRNA from hepatoma[103].

Two species of methionyl-tRNA (normal and formylable) were fractionated from mouse ascites tumour cells, mouse liver and from yeast. Although one of the species was non-formylated, it was responsible for the initiation of protein synthesis and was compared to the N-formyl-methionyl-tRNA from *E. coli*. The authors[104,105] have defined this species as the one, which is formylable *in vitro* by *E. coli* transformylase and can incorporate methionine exclusively at the N-terminal position of a polypeptide chain being synthesized, while the other species which is non-formylable incorporated methionine exclusively into the internal positions.

Substituted celluloses in general have been extensively used for studying the differences in profiles as a function of *in vivo* or *in vitro* modifications (*viz.* methylation, thiolation, rearrangement in pseudouridine formation, etc.) of tRNA. HANKINS AND FARKAS[106] have discovered a new mode of modification of tRNA, *viz.* guanylation (addition of a guanosine mononucleotide residue into a polynucleotide chain after the

completion of tRNA synthesis). The phenoxyacetyl-derivative of a guanylated rabbit reticulocyte tRNA was chromatographed on BD-cellulose and the purified guanylated tRNA species was examined for its amino acid acceptor activity. It was noted that two species of tRNA were guanylated, of which one possessed histidine acceptor activity. Significance of guanylation is not understood; however, it has been suggested[106] that (a) guanylation may be a necessary step in modification of tRNAs, which were synthesized at earlier state of maturation of rabbit reticulocytes, or (b) it may be a cellular mechanism of converting tRNA to a function other than that required in protein synthesizing machinery.

(i) Viral RNA

BD-cellulose chromatography has been employed for the separation of pancreatic RNAase-depolymerized fragments of TMV-RNA[107]. The elution yielded a single homogeneous peak indicating that the fragments eluted were physico-chemically identical, a fact which was subsequently ascertained by analytical ultracentrifugation and polyacrylamide gel electrophoresis. By calculation of the sedimentation coefficient, it was estimated that the fraction was of the order of fifty-five nucleotide residues.

A BD-cellulose column was useful in resolving the different forms of RNA of the replicative cycle of a group A arbovirus, Semliki Forest Virus (SFV). RNA synthesized in SFV-infected chicken cells (pretreated with actinomycin D), and chromatographed on a BD-cellulose column furnished the following elution pattern with a linear gradient of sodium chloride (a) An RNAase-resistant 16S fraction, which coeluted with double-stranded SFV-RNA and with a homopolymer duplex, poly I:poly C, and which probably corresponds to the base-paired replicative form (RF). (b) A partially RNAase-resistant form of SFV-RNA representing the replicative intermediate (RI) was also eluted by the sodium chloride gradient. (c) However, RNAase-sensitive single-stranded viral RNAs required either urea or dimethyl sulfoxide for elution suggesting the presence of a minimal secondary structure. The elution pattern was independent of the molecular weight and appeared to be related to the degree of a secondary structure of RNA[108]. Presumptive double-stranded RNA of host origin eluted as a single peak on BD-cellulose. This material was synthesized in the presence of actinomycin D and has been detected in a number of cell types[109]. Its biological significance is still not clear[110].

(j) Phage

A low-molecular-weight RNA synthesized very early after induction of prophage λ has been purified on a BD-cellulose column and the different fractions eluted were studied for their hybrid-forming ability with the light strand of prophage λ DNA (ref. 111). It was noted that this low-molecular-weight RNA (a) could form a complex with λ DNA, (b) was electrophoretically homogeneous, (c) had an S value between 4 and 5, and (d) was more unstable than tRNA. As yet, its biological function is not known; it is suggested that (a) it may be a small piece of a large mRNA molecule coding short peptides, (b) it may have a regulatory function at DNA level, or (c) it may be a waste product of phage evolution.

3. rRNA AND mRNA FRACTIONATION

(a) Yeast

Yeast rRNA was so strongly bound on a BD-cellulose column that 30% of rRNA (possibly 16S component) could only be eluted with 1.0 *M* sodium chloride in 4% methoxyethanol[3]. The observed strong binding of rRNA with the BD-cellulose column could be due to either a high molecular weight or an open structure, since either heat denatured or Mg^{2+} deprived (in both the cases, high degree of the secondary structure has been obtained) tRNA molecules bound more strongly on BD- as well as benzoylated-naphthoylated-DEAE-celluloses (BND-celluloses) and a higher concentration of salt (than required for native molecules) was necessary for elution[3].

(b) Bacillus

A foolproof procedure has been worked out for the purification of 5S rRNA[112]. Total RNA preparation from *B. megaterium* was adsorbed onto the BD-cellulose column and eluted by a step-wise elution with sodium chloride. 5S RNA and most of the tRNAs were eluted at 0.6 *M* sodium chloride concentration. This step thoroughly removed potential contaminants like 16S and 23S rRNAs. The mixed fraction of 5S RNA and tRNA was then subjected to 8% polyacrylamide gel electrophoresis. 5S RNA eluted from the gel slices was rechromatographed on BD-cellulose and this time eluted with an increasing concentration gradient of sodium chloride as against the step-wise elution. This step eliminated all minor contamination due to tRNA as confirmed by subsequent studies.

(c) E. coli

An excellent procedure has been introduced for the simultaneous purification of differently metabolically (tRNA, rRNA and mRNA) active species, using a BD-cellulose column and two sets of gradients[113]. The first set consisted of a gradient of an increasing concentration of sodium chloride and dimethyl sulfoxide in 0.02 *M* Tris–hydrochloride buffer (pH 7.5), while the other set consisted of a gradient of an increasing concentration of ammonium chloride in 0.1 *M* acetate buffer (pH 3.5) along with 8.0 *M* urea. It was noted that bulk of the tRNA was eluted by the sodium chloride gradient without dimethyl sulfoxide, while bulk of the rRNA appeared at 0.5 *M* sodium chloride in 13% dimethyl sulfoxide. This fraction (eluted at pH 7.5) upon rechromatography at pH 3.5, separated the mRNA species from rRNA. However, the basis of this separation is not clear. By this method, phage-T_4 specific mRNA could also be purified. The recoveries ranged from 60–100% and the authors[113] have suggested that absolute purification may be possible with repeated chromatography. 15-Fold purification of a rapidly labeled fraction of *E. coli* RNA (metabolically unstable and likely to be mRNA) has been accomplished using BD- and BND-cellulose columns[114]. The yield was 4.5% of the total RNA.

(d) Animals

Total RNA from whole and anucleated eggs of sea urchin were chromatographed on BD-cellulose columns using a combined gradient of an increasing concentration of sodium chloride (0.3–1.2 *M*), and of urea (0.0–4.0 *M*) and then using a gradient of 0.0–1.0 *M* ammonium chloride in 8.0 *M* urea[115]. The nature of elution profiles resembled those obtained by SEDAT *et al.*[114] and suggested that very little rRNA is synthesized in the anucleated eggs of sea urchin.

4. BASIS OF RESOLUTION ON BD-CELLULOSE

Systematic studies on the resolution of synthetic polyribonucleotides have been helpful in understanding the mechanism underlying adsorption and fractionation on the BD-cellulose column[116]. It was observed that when the base composition of different RNA species remained constant (as in the case of synthetic polymers), elution was a function of molecular size. If the base composition was not constant (as in the case of natural polymers), the order of elution was solely a function of the secondary structure of RNA and was independent of the molecular size. It was further noted[116] that the greater was the proportion of base-pairing in RNA, the fewer were the bases available for the interaction with BD-cellulose, subsequently the easier was the elution. That a finite three-dimensional macromolecular conformation plays a major role in determining the chromatographic characteristics of RNA on BD-cellulose was evidenced also by earlier studies[3,109]. The presence of EDTA either on the column or in the eluting medium required higher salt concentration to elute tRNA than when Mg^{2+} was present, indicating that exposition of a secondary structure is of a prime importance during adsorption and elution processes. Such structures basically decide the non-ionic interaction between BD-cellulose and incoming RNA molecules. Some of the common variables such as pH, temperature, concentration of Mg^{2+}, concentration of urea, 2-methoxyethanol, ethanol, etc., also facilitate easy elution presumably attributing a particular conformation[3,110,114,115].

A systematic exploration of pH and/or temperature gradient, as in the case of a DEAE-cellulose column, may throw more light upon the basis of resolution of a BD-cellulose column.

5. ADVANTAGES OF BD-CELLULOSE

BD-cellulose is an anion exchanger with a higher capacity than the other known adsorbents, except benzoylated-DEAE-Sephadex (BD-Sephadex), and has the technical convenience of DEAE-cellulose with a greater selectivity for more hydrophobic groups or molecules. Its preparation has always yielded an adsorbent of reproducible properties, a factor lacking in many other adsorbents. It furnishes quite extensive (as in case of leucyl-tRNA, it affords as much as six isoacceptor species) and reproducible separations with a recovery up to 90% within wide limits of column size, which could be varied to any extent keeping a length to diameter ratio of the column from 5:1 to 150:1 (ref. 117). The resolutions on BD-cellulose are quite satisfactory and almost

complete in the temperature range of 4–22° and pH range of 4.0–7.5. This has greatly avoided the risk of either chemical or enzymatic degradation, which are accelerated in extreme temperature and/or pH ranges. The greatest advantage of a BD-cellulose column is that all the isoaccepting species of tRNA are simultaneously isolated and purified without loss of their biological expression.

6. LIMITATIONS OF BD-CELLULOSE

The major drawback of BD-cellulose chromatography has been the considerable damage during the derivatization step[44], which is a necessary prerequisite for the purification of tRNAs specific for all non-aromatic amino acids.

Just like the DEAE-Sephadex has a 10-fold higher capacity than the DEAE-cellulose, it has been observed that BD-Sephadex has a 6–7-fold higher capacity than the BD-cellulose[118]. It may act as a substitute for BD-cellulose in the fractionation of tRNA under suitable experimental conditions. However, preliminary observations in this regard from this laboratory are not encouraging[119].

7. OTHER MODIFIED DEAE-CELLULOSES

A BD-cellulose column has been preferred to most of the modified celluloses due to its high capacity, efficiency in extensive fractionation and reproducibility. The reproducibility of BD-cellulose properties has largely been a function of DEAE-cellulose, which is used as a starting material for modification. These qualities, however, do not hold true for many other modified celluloses. For instance, DEAE-cellulose substituted with *p*-phenyl-, azobenzoyl-, anisoyl-, nicotinoyl-, acetyl-, octadecyl-, and nonanoyl-groups as modifiers gave newly modified DEAE-celluloses (substituted with respective groups), which either had poor physical properties of an adsorbent or were inferior to BD-cellulose in their resolving ability.

(a) Naphthoylated DEAE-cellulose

This adsorbent was explored[3] for the separation of RNAs and it was noticed that fully benzoylated-DEAE-cellulose gave superior separations to naphthoylated-DEAE-cellulose indicating that the degree and the nature of the substituting group were both important. Although naphthoylated-DEAE-cellulose gave a product of good physical properties suitable for an adsorbent, the tRNA binding on this adsorbent was so firm that it could not be eluted by maximum sodium chloride concentration; 10 % methoxyethanol only could facilitate its elution. Bearing this difficulty in mind, it was concluded that naphthoylated-DEAE-cellulose may not be of any use in the fractionation of rRNAs, which are still difficult to elute from BD-cellulose. However, it may be useful for the separation of either small or less strongly bound molecules.

(b) Benzoylated-naphthoylated-DEAE-cellulose

With the experience on BD-cellulose and naphthoylated-DEAE-cellulose, it

was thought that a mixed polymer, by virtue of benzoyl- and naphthoyl-groups substituted in it may have better adsorbent properties and perhaps higher capacity than BD-cellulose. The preparation of benzoyl-naphthoyl-cellulose by the introduction of 9% naphthoyl- and 91% benzoyl-groups on the molar basis yielded an adsorbent possessing mixed properties of BD-cellulose and naphthoylated-DEAE-cellulose[3]. Like BD-cellulose, it has also a strong affinity towards tRNAs by comparison of the starting material—DEAE-cellulose.

Benzoylated-naphthoylated-DEAE-cellulose (BND-cellulose) was first prepared by TENER *et al.*[5] and was used in the fractionation of yeast tRNA (ref. 3). It was noted that on BND-cellulose at pH 5.0, ester bond between an amino acid and the corresponding tRNA was labile to some extent. Therefore, use of solutions having lower pH was advised. It was also noted that on BND-cellulose, unlike DEAE-cellulose, 7.0 *M* urea in the eluting medium did not improve upon the elution profiles. In further studies[114], it was observed that double-stranded DNA and RNA from *E. coli* infected with phage MS_2 eluted at nearly the same salt concentration, while infectious MS_2 RNA eluted after them, but before 16S rRNA. Introduction of a dimethyl sulfoxide gradient superimposing upon the sodium chloride gradient sharpened the resolution of these species. Although mRNA could be eluted by cesium trichloroacetate in caffeine (pH 4.0) and rRNA could be successfully rechromatographed into two peaks, poly A and poly U could not be eluted by 1.2 *M* sodium chloride (pH 7.2). This suggested a very strong binding between BND-cellulose and stacked or unstacked synthetic polynucleotides. Likewise, phage φ X 174 DNA, a covalent circular single-stranded polynucleotide, was non-elutable even at 4.0 *M* sodium chloride concentration. However, it could be eluted at 0.25 *M* sodium chloride concentration with the aid of 20 mg caffeine/ml (pH 7.4). In contrast, φX174 RF could be eluted quite easily in 0.3–1.2 *M* sodium chloride gradient. Further studies[120] revealed that the RI of phage MS_2 was eluted from a BND-cellulose column after DNA, but before single-stranded MS_2 RNA. RI consists of a double-stranded core to which are attached single-stranded tails of variable lengths and fractionation seems to occur with respect to fail length. It was indicated that RF existed into two forms, RF_I (two closed DNA strands) and RF_{II} (one closed and one open DNA strand). RF_I could be purified in a single chromatographic run[121] as follows: a mixture of *E. coli* DNA, RF_I and single-stranded phage φ X 174 DNA were kept at pH 11.8 for 3 min at 22°, rapidly neutralized, cooled, and then chromatographed on a BND-cellulose column; *E. coli*-denatured DNA and single-stranded phage φ X 174 DNA were firmly bound onto the column, while RF_I was eluted at 1.0 *M* sodium chloride concentration with 75–85% recovery.

BND-cellulose has appreciably high capacity; a 5 × 5-cm column retained about 40 mg of nucleic acids. Application of a sample on the column at 0.3 *M* (than at 0.1 *M*) sodium chloride resulted in high recovery, but with inferior subfractionation. For reproducible results on BND-cellulose, careful control of moisture content was required.

8. SUMMARY

Substitution of the benzoyl groups in DEAE-cellulose has appreciably increased the capacity of BD-cellulose for retention of tRNAs, maintaining all the technical

conveniences of DEAE-cellulose. BD-cellulose has been found to be the most suitable adsorbent for the column chromatographic fractionation of individual tRNA from a mixture of tRNAs from a wide variety of sources. Furthermore, it has successfully afforded reproducible and extensive subfractionation of isoaccepting species of specific tRNA. The notable feature of the BD-cellulose column by comparison of the other adsorbents used for RNA fractionation[1,2,122] is its exceptionally high flow-rate without affecting the resolution. The basis of resolution on a BD-cellulose column appears to be a function of the secondary structure of RNA and is independent of the molecular size. Its role in the fractionation of rRNAs or ribosomes keeping their biological expression intact has yet to be explored. With the meagre data available on the performance of naphthoylated-DEAE- and benzoylated-naphthoylated-DEAE-celluloses, it may be premature to evaluate their potential in RNA fractionation at this stage.

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