A General Procedure for the Isolation of Specific Transfer Ribonucleic Acids*

Ian Gillam, Daphne Blew, R. C. Warrington, Margaret von Tigerstrom, and G. M. Tener†

ABSTRACT: A chemical method for the isolation of transfer ribonucleic acids specific for a single amino acid is described. An aminoacyl transfer ribonucleic acid synthetase is used to esterify one amino acid specifically to its acceptors in crude transfer ribonucleic acid. A 2-naphthoxyacetyl or phenoxyacetyl group is introduced onto the amino group of the aminoacyl ester. Such substitution causes a marked shift in the position of elution of the charged transfer ribonucleic acid from benzoylated DEAE-cellulose (BD-cellulose), allowing its separation from uncharged transfer ribonucleic acid. The substituted aminoacyl ester is readily hydrolyzed to liberate transfer ribonucleic acid enriched in acceptor activity for the desired amino acid. Individual species of transfer ribonucleic acid accepting the amino acid used are separated by chromatography of this material on BD-cellulose. The method is likely to be generally applicable. It is illustrated by the isolation of purified transfer ribonucleic acids for aspartic acid, arginine, glycine, methionine, and threonine from crude transfer ribonucleic acid of brewer's yeast (*Saccharomyces cerevisiae*).

Interest in the structure of tRNAs has led to the development of a number of methods for the purification of individual tRNAs, the structures of several of which have now been determined. Most of these methods are based upon differences in the physical properties of the various tRNAs and utilize ion-exchange and adsorption chromatography, liquid–liquid partition, or combinations of these processes. An alternative approach uses the high specificity of aminoacyl-tRNA synthetases to esterify the desired amino acid to its acceptor RNAs in the mixture and then exploits the difference in chemical reactivity between aminoacylated and unesterified tRNAs to separate them. Zamecnik *et al.* (1960) first described such a method using periodate to oxidize the unprotected tRNA and subsequent reaction with a derivative of hydrazine to remove it from that which was aminoacylated. Others have elaborated modifications of this procedure. Related alternative methods involve reaction with the aminoacyl group attached to tRNA, usually to give an insoluble derivative, which is separable from unesterified tRNAs (Brown *et al.*, 1959; Mehler and Bank, 1963; Simon *et al.*, 1964). It appears that such chemical procedures for the isolation of specific tRNAs are not in wide current use. We are aware of only one recent description of such a method for the preparation of a considerable quantity of highly purified tRNA (Grachev *et al.*, 1966).

We have described (Gillam *et al.*, 1967) the benzoylation of the hydroxyl groups in DEAE-cellulose to give an ion exchanger with an affinity for aromatic groups and the use of this material for the chromatographic fractionation of tRNAs. Chromatography on BD-cellulose[1] has been used in a simple procedure to obtain highly purified $tRNA^{Phe}$ (Wimmer *et al.*, 1968). This species binds more strongly than any other tRNA of yeast to BD-cellulose and is readily isolated. The affinity of BD-cellulose for aromatic groups in exposed positions on tRNA has been exploited to obtain purified $tRNA^{Tyr}$ and $tRNA^{Trp}$, also from yeast (Maxwell *et al.*, 1968). This communication presents details of a more general chemical method for the isolation of purified tRNAs using similar procedures.

Experimental Section

Materials

BD-cellulose was prepared as described (Gillam *et al.*, 1967). tRNA of brewer's yeast was a commercial preparation from Boehringer & Soehne, Mannheim, Germany. Amino acids were of the highest purity commercially available. ^{14}C-Labeled amino acids were commercial products of given specific activities and stated to be of greater than 99% purity. *N*-Hydroxysuccinimide was from the Pierce Chemical Co., Rockford, Ill.

* From the Department of Biochemistry, University of British Columbia, Vancouver 8, British Columbia, Canada. *Received June 21, 1968.* Supported by research grants from the National Institutes of Health (GM 14007) and the Medical Research Council, Ottawa.

† Medical Research Associate, Medical Research Council of Canada.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: BD-cellulose, benzoylated DEAE-cellulose; BND-cellulose, benzoylated, naphthoylated DEAE-cellulose; DCC, *N,N'*-dicyclohexylcarbodiimide; A_{260} unit, defined as that quantity of material which dissolved in 1 ml has an absorbance at 260 nm of 1; EF, the ethanol fraction, that fraction of crude tRNA which is eluted from BD-cellulose with 1 M NaCl containing 9.5% ethanol and not by 1 M NaCl; MAK, methylated albumin on kieselguhr.

Methods

Preparation of Phenoxyacetyl Ester of N-Hydroxysuccinimide. Phenoxyacetic acid (625 mg, 4.1 mmoles) and *N*-hydroxysuccinimide (470 mg, 4.1 mmoles) were dissolved in 12 ml of dry dioxane in a stoppered flask. DCC (851 mg, 4.1 mmoles) was added and the flask was shaken to dissolve the crystals. Almost immediately small crystals began to precipitate and reaction was complete in 2 hr at 23°. The crystals were filtered and discarded. The filtrate was concentrated under reduced pressure to a syrup. The product was crystallized from 1-propanol. It sintered at 92° and melted at 102.5–103°. *Anal.* Calcd for $C_{12}H_{11}NO_5$: C, 57.9; H, 4.5; N, 5.6; O, 32.1. Found: C, 57.6; H, 4.6; N, 6.05; O, 32.8. The yield after the first crystallization was 610 mg (60%).

2-Naphthoxyacetyl Ester of N-Hydroxysuccinimide. 2-Naphthoxyacetic acid (2.02 g, 10 mmoles) and *N*-hydroxysuccinimide (1.15 g, 10 mmoles) were dissolved in dioxane (40 ml) to which was added DCC (2.10 g, 10 mmoles) as above, mp 146.5–147°. *Anal.* Calcd for $C_{16}H_{13}NO_5$: C, 64.2; H, 4.4; N, 4.7; O, 26.7. Found: C, 64.1; H, 4.5; N, 4.7; O, 26.6. The yield after the first recrystallization was 2.35 g (79%).

Chromatographic Methods. Columns were packed, washed, loaded, and developed generally as described earlier (Gillam *et al.*, 1967). All operations were performed at room temperature (about 23°). For use in columns to be developed by stepwise elution where high flow rates were used the BD-cellulose was usually of a coarser grade (approximately 30 mesh) than used for elution by a gradient of concentration of salt.

Recovery of tRNA. Pooled fractions were concentrated as described (Gillam *et al.*, 1967), precipitated, washed with ethanol, and freeze dried from water. Solutions with A_{260} about 5 or greater were precipitated directly.

Solutions A–F. Each solution contained 0.01 M magnesium chloride–0.01 M acetic acid (pH 4.5) (NaOH) together with the additions given: solution A, 0.3 M sodium chloride; solution B, 0.8 M sodium chloride; solution C, 1.0 M sodium chloride; solution D, 1.0 M sodium chloride–4.7% (v/v) ethanol; solution E, 1.0 M sodium chloride–9.5% (v/v) ethanol; and solution F, 1.0 M sodium chloride–19% (v/v) ethanol.

Removal of EF from tRNA. A column (3.5 × 22 cm) of BD-cellulose was equilibrated with solution A and loaded with tRNA (1 g) dissolved in solution A (50 ml). The column was washed with solution A until the effluent had A_{260} less than 0.01 and then washed with solution B. Flow through the column was about 2 ml/min and fractions were collected in the usual way. The fractions containing the main peak of absorbancy were pooled and the RNA was recovered. Washing of the column with solution B was continued until the effluent contained less than 0.3 A_{260} unit/ml when elution with solution E was begun. Fractions containing the two peaks of absorbancy were pooled separately and the RNA was recovered. The second peak contained the EF.

Sham Acylation of tRNA. Uncharged tRNA was incubated with the phenoxyacetyl ester of *N*-hydroxysuccinimide, recovered, and passed through BD-cellulose as described below for the preparation and isolation of *N*-phenoxyacetylaminoacyl-tRNA.

Preparation of Aminoacyl-tRNA Synthetases (Millward, 1967). Fleischmann's baker's yeast was used as the source of extracts prepared by method A of Gillam *et al.* (1967). The desalted crude preparation in buffer containing 35% (v/v) glycerol was chromatographed on a column of hydroxylapatite in the presence of 35% (v/v) glycerol at 4° (*cf.* Muench and Berg, 1966). Some aminoacyl-tRNA synthetases were not eluted by buffers containing 0.2 M potassium phosphate and required the addition of ammonium sulfate as a gradient or stepwise to 10% (w/v) in the same buffer without glycerol. Aminoacyl-tRNA synthetase activities were located in fractions obtained from these columns by a qualitative assay dependent upon the formation of acid-insoluble radioactive material when samples were incubated on paper disks with [^{14}C]amino acid, tRNA, ATP, and cacodylate buffer in much the same way as described previously (Maxwell *et al.*, 1968). Fractions containing the required activity were pooled and concentrated by ultrafiltration (Blatt *et al.*, 1965) using the Diaflo apparatus with membrane type UM 1 (Amicon Corp., Cambridge, Mass.). The resultant solutions were desalted by passage through Sephadex G-25 (Pharmacia, Uppsala, Sweden) and stored at −20° in the presence of glycerol (40%, v/v).

Assays for Amino Acid Acceptor Activity. Samples of tRNA were assayed for acceptance of [^{14}C]amino acids as described previously (Gillam *et al.*, 1967).

Charging of tRNA on a Preparative Scale. Aminoacyl-tRNA synthetase partially purified as above was freed of glycerol by passage through a column of Sephadex G-25 in 0.01 M potassium phosphate, 1 mM 2-mercaptoethanol, and 0.1 mM EDTA (pH 7.5). The resultant solution of enzyme was used to study the kinetics of charging of a small sample of crude tRNA with the appropriate ^{14}C-labeled amino acid, in the presence of magnesium chloride, ATP, and cacodylate buffer. Conditions were generally found such that reaction was complete in 10 min at 30°. Actual concentrations of reagents used are given in Table I. Details given below are for a sample containing 500 mg of tRNA. In parallel, a small sample of the same material was charged with [^{14}C]amino acid. Reaction was terminated by cooling in ice and adjusting the pH to 5.0 with formate buffer. Sodium chloride solution (5 M) was added to give a concentration of 0.9 M and the RNA was recovered by precipitation with ethanol (two volumes).

N-Phenoxyacetylaminoacyl-tRNA. The pellets of tRNA prepared above were carefully drained to remove as much ethanol as possible and suspended in 25 ml of a solution of 0.1 M triethanolamine hydrochloride–0.01 M magnesium chloride (pH 4.3). ^{14}C-Labeled and [^{12}C]-aminoacyl-tRNAs were combined, centrifuged to remove insoluble protein, and cooled to 0°. To the stirred solution was added phenoxyacetyl ester of *N*-hydroxysuccinimide (60 mg) in dry tetrahydrofuran (2.5 ml). (Much of the added material precipitated.) The pH of

TABLE I: Concentrations of Reagents Used in the Charging of tRNA with Various Amino Acids.

Amino Acid	RNA Sample[a]	Quantity Used	Total Vol (ml)	Concentration of Reagent (mM)					
				Amino Acid	Cacodylate (pH)	$MgCl_2$	ATP	EDTA	KCl
Arg	A	500 mg	40	8.0	50 (7.4)	5	2.5	0.5	150
Asp	A	1000 mg	80	0.5	100 (7.5)	10	2.5	0.4	
Gly	B	1000 mg	170	1.5	75 (7.4)	10	2.0	0.3	20
Met	A	500 mg	40	2.5	50 (7.4)	10	5.0	0.5	80
Thr	C	9200 A_{260}	38.5	1.3	100 (7.5)	15.5	2.6	0.5	21

[a] A, crude tRNA stripped of EF and of phenoxyacetylated fraction; B, crude tRNA stripped of EF; and C, material recovered from A after removal of $tRNA^{Asp}$.

the mixture was quickly adjusted to 8.0 by addition of 1 N sodium hydroxide and stirring was continued at 0°. After 10 min glacial acetic acid was added to pH 4.5. RNA was recovered by precipitation with two volumes of ethanol. The precipitate was washed twice with 95% ethanol and carefully drained.

N-2-Naphthoxyacetylaminoacyl-tRNA. The 2-naphthoxyacetyl ester of *N*-hydroxysuccinimide was used at the same concentration by weight and in a similar manner except that it was usually added to the reaction mixture in two equal portions, one at the start and the other half-way through the 10-min reaction period.

Separation of N-Phenoxyacetylaminoacyl-tRNA from Uncharged tRNAs. The pelleted tRNA (above) was dissolved in solution A (100 ml). Samples were taken for determination of A_{260} and radioactivity, the latter by scintillation counting in Bray's (1960) solution. The remainder was loaded onto a column (3.5 × 22 cm) of BD-cellulose equilibrated with solution A. The column was washed with solution A until the eluate had negligible A_{260}, when elution with solution B was started. Fractions of about 10 ml were collected each 1.2 min and their A_{260} was recorded. When this dropped below about 0.2 elution with solution E was begun. Fractions were collected as before and examined for A_{260} and radioactivity, determined as above. Individual examples often differed slightly from this protocol in the details of loading and elution; such details are recorded in the legends to the appropriate figures.

Separation of N-2-Naphthoxyacetylaminoacyl-tRNA from Uncharged tRNA. The procedure was as above except that the column of BD-cellulose was eluted with solutions A, C, D, and E in that order. Details are recorded in the legends to the figures.

Stripping of the N-Substituted Aminoacyl Group from tRNA. The recovered RNA was dissolved in 1.0 M Tris-HCl (pH 9.0), incubated for 1 hr at 23°, and recovered as described (Maxwell *et al.*, 1968).

Results and Discussion

The basis for this chemical method for the isolation of particular tRNAs has been described in brief (Tener *et al.*, 1966, 1967). When tRNA prepared from yeast is chromatographed on BD-cellulose, 1 M sodium chloride in the presence of 0.01 M magnesium ion elutes most of the material from the column (Gillam *et al.*, 1967). That remaining represents 7–8% of the total A_{260} units applied to the column and can only be eluted in the presence of salt and an organic compound, such as urea or an aliphatic alcohol, which disrupts hydrophobic interactions (as well as hydrogen bonding). In practice ethanol (5–10%, v/v) in 1 M sodium chloride is used to elute this material, which is referred to as the ethanol fraction. This fraction contains all of the acceptor activity for phenylalanine found in the crude tRNA (Gillam *et al.*, 1967) and little acceptor activity for other amino acids, though it does contain RNA devoid of detectable acceptor activity. Wimmer *et al.* (1968) have described the purification of $tRNA^{Phe}$ from the EF.

Columns of BD-cellulose may be used for the chromatography of aminoacyl-tRNA provided the eluent is buffered around pH 4 to stabilize the aminoacyl ester. In this respect these columns resemble those of MAK (Sueoka and Yamane, 1962). This has been illustrated (Gillam *et al.*, 1967) for [^{14}C]Ala-$tRNA^{Ala}$, which elutes from the closely related BND-cellulose around the salt concentration expected for uncharged $tRNA^{Ala}$. This is not so for $tRNA^{Tyr}$ and $tRNA^{Trp}$ of yeast (Maxwell *et al.*, 1968). The uncharged acceptors elute from BD-cellulose with salt alone whereas after aminoacylation with their specific aromatic amino acids they require the presence of ethanol for elution. This increased affinity of tRNAs containing an aromatic group for BD-cellulose is the basis of what is likely to be a general method for the purification of tRNAs specific for the aromatic amino acids. The method to be described extends this convenient means of separating aminoacylated from uncharged tRNA to acceptors for other amino acids by introducing an aromatic substituent onto the reactive amino group of the aminoacyl-tRNA. When this is done the substituted aminoacyl-tRNA shows increased affinity for BD-cellulose which allows it to be separated from the rest of the tRNA.

The general procedure is outlined in a series of steps. First the crude tRNA, stripped of aminoacyl esters, is eluted from BD-cellulose with 1 M sodium chloride to

free it from material already having a strong affinity for BD-cellulose (the EF). The next step is not obligatory but has usually been used. It is termed sham acylation, that is, incubation of the recovered uncharged tRNA with the acylating agent under the conditions to be used later to substitute aminoacyl-tRNA. Passage of the RNA again through BD-cellulose gives material eluted with ethanol and salt which is separated from that eluted by salt alone. This procedure is designed to remove polynucleotides susceptible to acylation under the conditions used. In particular these might be expected to include tRNAs containing the nonformylated forms of the N^6-aminoacyladenines found in yeast by Hall (1964). In fact a small part (usually 2–3%) of the RNA so treated is recovered from the column only after elution with ethanol and salt. This fraction is usually greater than the proportion of material nonspecifically held (described later) which appears as a small peak with ethanol and salt when unacylated, uncharged tRNA already stripped of the EF is passed through BD-cellulose. Material isolated by sham acylation has not been examined further.

The third step in the procedure is the charging of specific acceptors for the amino acid of interest with that amino acid, the process being catalyzed by a preparation of aminoacyl-tRNA synthetase. The objective is to obtain complete charging of the one or more acceptors without esterification of other amino acids to their acceptors. Hence pure amino acid must be supplied, purified aminoacyl-tRNA synthetase is desirable, and the enzyme, which may still contain other activating enzymes, should be free of amino acids. (This may be accomplished by gel filtration immediately before use.) It should also be free of enzymes capable of generating amino acids. As the aminoacyl-tRNA is rapidly hydrolyzed at the pH of the enzymatic incubation mixture the proportion of an acceptor esterified with its amino acid at equilibrium is dependent upon the rates of esterification and of hydrolysis. To obtain the maximum yield of aminoacyl-tRNA it is necessary to perform the incubation under conditions where esterification occurs most rapidly. Optimal concentrations of various reagents and cofactors are found by preliminary experiments.

Step four is the substitution of the amino group of the aminoacyl ester by an aromatic acyl group. The aminoacyl ester linkage in aminoacyl-tRNAs is generally considered to be stable under mildly acidic conditions and to be hydrolyzed quite rapidly at pH 7 or above. Our investigations (unpublished) on the substitution of the amino group of aminoacyl-tRNA by various electrophilic reagents showed conditions could be found such that little hydrolysis occurred in the period required for complete substitution. It is clear that with such reagents only the uncharged amino group of the aminoacyl ester will be reactive; the ionized form will be unreactive. Hence the reaction must be performed at a pH near the pK of that amino group. The reagent in these early studies of particular interest here was phenoxyacetyl chloride. (It is likely that any simple aromatic system substituted into the aminoacyl ester will have the desired effect.) Though this reagent has low solubility in water it was shown to react completely with the amino group of the aminoacyl esters of tRNA within 1 or 2 min at 0° and pH 8.0 in aqueous triethanolamine buffer. The extent of substitution of [^{14}C]aminoacyl groups esterified to the tRNA was readily checked in three ways. Both substituted and unsubstituted esters were rapidly hydrolyzed by exposure to ammonium hydroxide solution as was verified by simple radiochemical methods. Introduction of a large lipophilic group into an amino acid profoundly alters its behavior upon paper chromatography, and substitution of its amino group changes its rate and often its direction of migration upon electrophoresis, depending upon the pH used. Thirdly, phenoxyacetylation of the aminoacyl group in a charged tRNA alters dramatically its affinity for BD-cellulose, as will be seen. All three criteria indicated rapid phenoxyacetylation under these conditions. Ip and Fraser (1965) have used acetic anhydride in glacial acetic acid to prepare *N*-acetylglycyl-tRNA, but the even more reactive phenoxyacetyl chloride presented two problems with regard to use in an aqueous system. First is its quite rapid hydrolysis at pH 8 and second is the possibility that it may react with hydroxyl or amino groups in the tRNA itself. From such positions phenoxyacetyl groups would not be removed by the mild treatment used to strip off the substituted aminoacyl ester and could lead to loss of biological activity. All experiments to be described here made use of esters of *N*-hydroxysuccinimide as acylating agents. These active esters were first used by Anderson *et al.* (1964) as acyl donors in the synthesis of peptides in an aqueous system where they are more stable than acyl chlorides. de Groot *et al.* (1966) have used the acetyl ester to prepare *N*-acetylaminoacyl-tRNAs, though in a system in which the aminoacyl-tRNA was not soluble. The phenoxyacetyl and 2-naphthoxyacetyl esters of *N*-hydroxysuccinimide have rather low solubility in water but give rapid and complete substitution of aminoacyl-tRNA. There appears to be little if any reaction with uncharged tRNA under the conditions specified. Under the rather different conditions used by de Groot *et al.* (1966) and using radioactively labeled acetyl ester of *N*-hydroxysuccinimide they were unable to detect significant reaction with unesterified tRNA when aminoacyl-tRNA was completely substituted. Without recourse to similar technique it is difficult to eliminate the possibility that under the conditions of reaction described here there is some slight degree of acylation of uncharged tRNA. However, the presence of water and triethanolamine is likely to limit attack upon hydroxyl groups in the RNA. The only evidence that can be offered on this point is that a second sham acylation of uncharged tRNA gives little or no more material eluted from BD-cellulose by ethanol than the slight tail found on rechromatography of untreated RNA. Any such acylation is clearly of low frequency of occurrence since tRNA with high biological activity is recovered by the over-all procedure. The reasons for the use of the 2-naphthoxyacetic ester of *N*-hydroxysuccinimide as the preferred reagent for acylation in certain instances will be described later.

Having successfully introduced an aromatic group

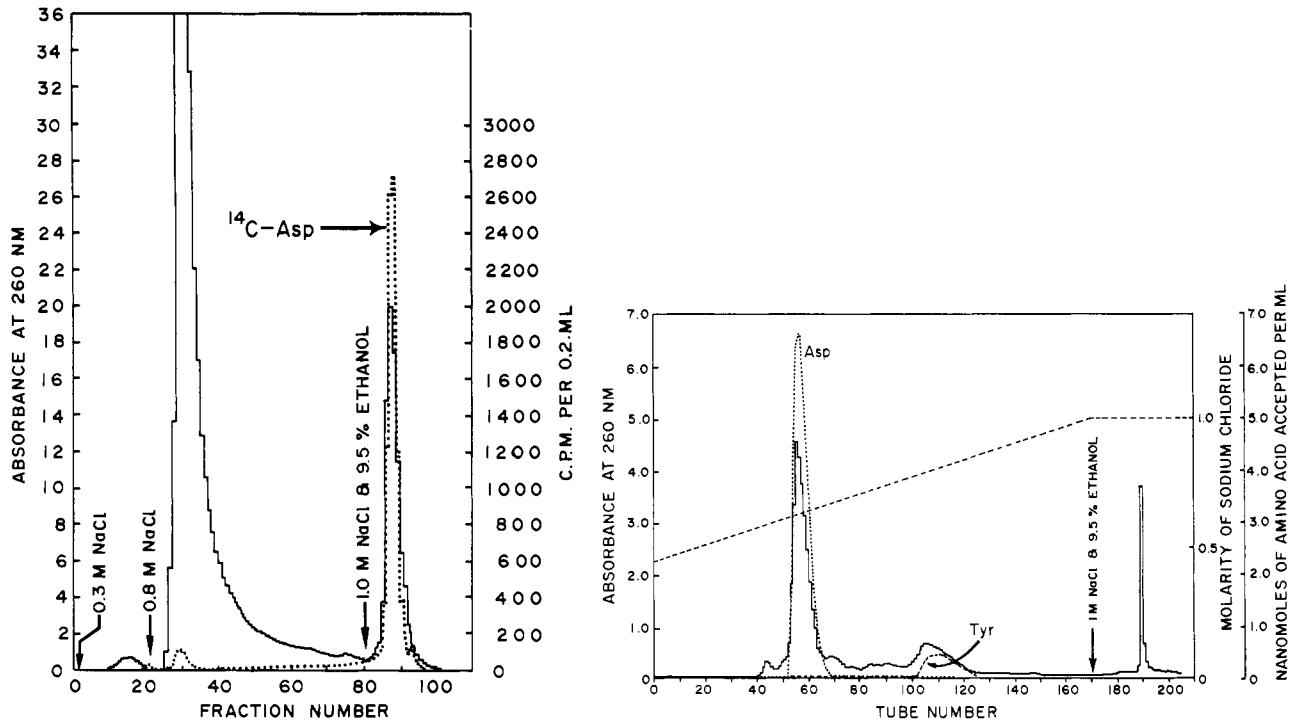


FIGURE 1: Preparation of $tRNA^{Asp}$ by the phenoxyacetylation procedure. (A) Left: Aspartyl-tRNA prepared using purified synthetase with 1 g of tRNA from which the EF and sham-acylated fractions had been removed was phenoxyacetylated after addition of [^{14}C]aspartyl-tRNA prepared similarly from 5 mg of tRNA. The tRNA was applied to a column of BD-cellulose in solution A and subsequently eluted with solutions B and E as indicated by arrows. Solid line: A_{260}; dotted line: radioactivity. (B) Right: The peak eluted by solution E above was stripped of esterified amino acid and chromatographed on a column (1.4 × 110 cm) of BD-cellulose. Elution was with the indicated (dashed line) gradient of concentration of sodium chloride (total of 3 l.), 0.01 M in magnesium chloride. Flow rate was 1.7 ml/min. At fraction 172 elution with 1.0 M sodium chloride–0.01 M magnesium chloride in 9.5% (v/v) ethanol was started. The volumes of fractions decreased after this point. Solid line: A_{260}; dotted line: acceptor activity for aspartic acid; labeled dashed line: acceptor activity for tyrosine.

onto the aminoacyl group of the charged tRNA step five follows. This is the separation of substituted, charged tRNA from the unsubstituted tRNA by use of a column of BD-cellulose. The procedure is similar to the initial removal of the EF in step one. Uncharged tRNA is washed from the column with strong salt solution, eluting together with any charged tRNA which has not been substituted. This fraction may be recovered and used for the isolation of other tRNAs. The substituted aminoacyl-tRNA is then eluted with a solution of salt and ethanol and recovered. This separation depends upon the interaction of aromatic groups. *N*-Acetoacetylalanyl-tRNA is eluted from BD-cellulose by salt alone (unpublished observations), and a naphthoxyacetyl group is more effective than a phenoxyacetyl group in causing the substituted tRNA to adsorb firmly to BD-cellulose (described later). No such marked retardation is seen when *N*-phenoxyacetylalanyl-tRNA is chromatographed on DEAE-cellulose (unpublished).

The sixth step is the recovery of the purified tRNA by removal of the substituted aminoacyl ester. The labile ester is readily removed by incubation in Tris buffer (pH 8 or 9) (Sarin and Zamecnik, 1964), which is not damaging to the majority of species of tRNA. The complete hydrolysis of the ester is readily checked when radioactive amino acid has been attached to the tRNA. If these conditions of hydrolysis are found to cause damage to the tRNA, enzymic hydrolysis at a lower pH may prove useful.

As one amino acid is generally esterified to several specific acceptor RNAs in extracts from any one source, the purified tRNA obtained in this way consists of several species. In the absence of more control over the aminoacyl-tRNA synthetases the method described is thus one for the isolation of a genus of tRNAs specific for a single amino acid. Fortunately the individual species of tRNA in the isolated material are mostly separable by a simple chromatographic procedure using BD-cellulose (Gillam *et al.*, 1967). This has been used to compare the patterns of elution of A_{260} and acceptor activity obtained in the experiments to be described with those expected from earlier work on chromatography of tRNAs from the same source. The next section presents results obtained using the method described above for the isolation of a representative selection of specific tRNAs from brewer's yeast and discusses some of the difficulties encountered.

Aspartic Acid. Figure 1 summarizes the purification of $tRNA^{Asp}$ by the phenoxyacetylation procedure. A trace of radioactivity eluted with the major peak of RNA which emerged from the first column with solution B (Figure 1A). This fraction was found to contain 15% of the initial aspartic acid acceptor activity, being $tRNA^{Asp}$ which had not been aminoacylated. The bulk

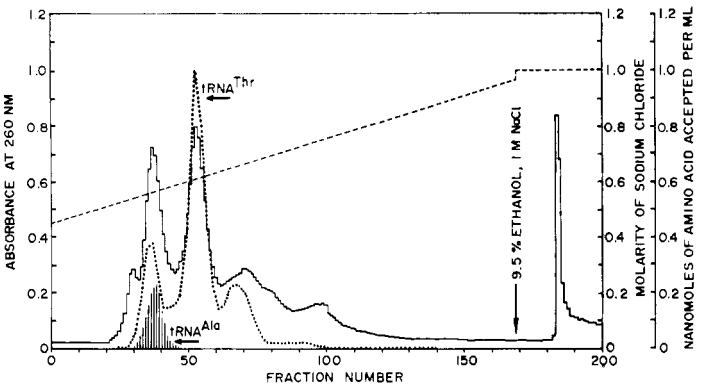


FIGURE 2: Chromatography of $tRNA^{Thr}$ (415 A_{260} units) purified by the phenoxyacetylation procedure. The column (1.4 × 110 cm) of BD-cellulose was eluted with the indicated (dashed line) gradient of concentration of sodium chloride, containing 0.01 M magnesium chloride (a total of 3 l.). Flow rate was 1.7 ml/min. At the end of the gradient (arrow) elution was continued with 1 M sodium chloride–0.01 M magnesium chloride in 9.5% (v/v) ethanol. Dotted line: acceptor activity for threonine; barred area: acceptor activity for alanine.

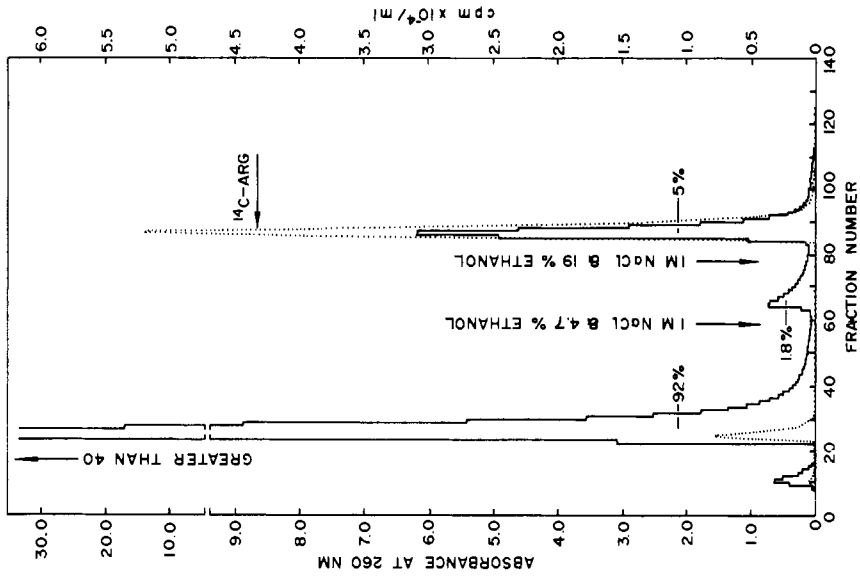
of the radioactivity was eluted only in the presence of ethanol (solution E). This fraction represented 9.4% of the total A_{260} units eluted from the column. After hydrolysis of the substituted amino acid the RNA was found to be enriched about eightfold over the starting material in acceptor activity for aspartic acid. This material was chromatographed on a column of BD-cellulose upon which $tRNA^{Asp}$ gives a single sharp peak eluting around 0.6 M sodium chloride (Gillam *et al.*, 1967). Such a peak about the expected position dominated the pattern of elution (Figure 1B), and this peak had acceptor activity for aspartic acid approaching the anticipated value for pure material (1.85 nmoles of amino acid accepted/A_{260} unit[2]). Several minor peaks of absorbancy were present, the largest eluting around the position expected for $tRNA^{Tyr}$ (Gillam *et al.*, 1967). This latter was shown to have acceptor activity for tyrosine. Figure 1A illustrates the tendency of tRNA eluted with steps of concentration of salt from BD-cellulose to tail after the main peak of absorbancy has eluted and also the occurrence of slight bleeding off of the radioactive *N*-phenoxyacetylaspartyl-tRNA before the application of the ethanolic wash. Solution B, containing 0.8 M sodium chloride, was used here to minimize the problem of bleeding. It is not surprising that it does not elute with complete efficiency the $tRNA^{Tyr}$, the last acceptor to be eluted by salt alone and one which shows particularly marked tailing (Gillam *et al.*, 1967). The material eluted from the second column by a solution containing ethanol represented 8% of the load and was not characterized.

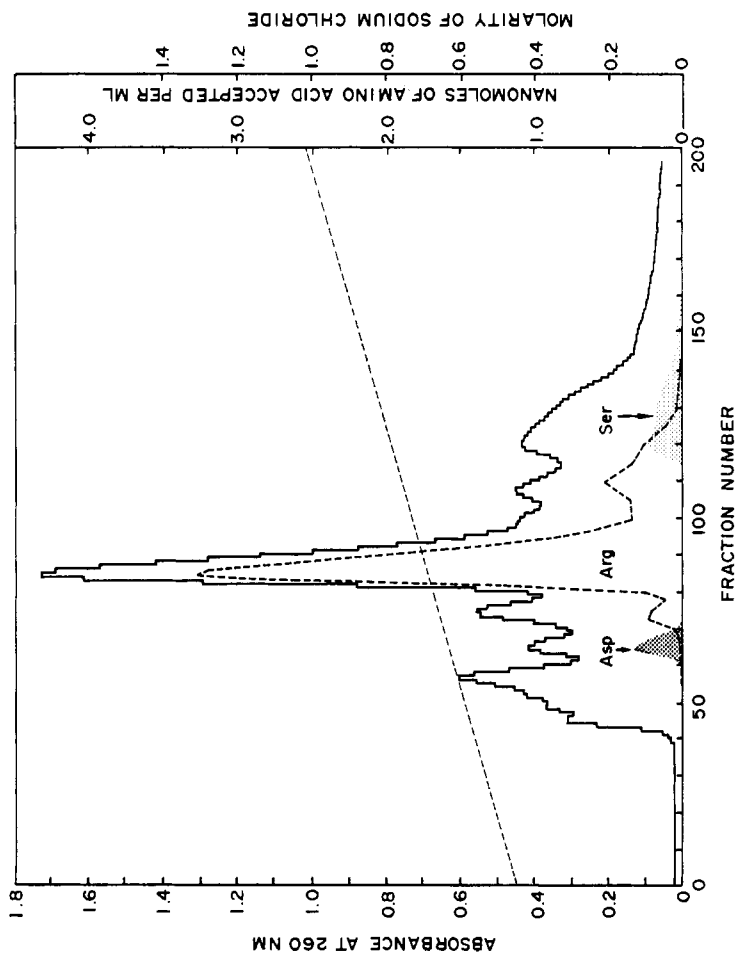
Threonine. Phenoxyacetylation of tRNA charged with threonine gave an unusually low yield of material containing radioactive amino acid which eluted from BD-cellulose with solution E. Only 65% of the radioactivity appeared in this fraction, which represented 6% of the total A_{260} units. The rest of the radioactivity eluted together with the bulk of the RNA (eluted with solution B). The apparent difficulty in acylating the amino group on threonyl-tRNA was not completely overcome when the reaction was performed at pH 9 or for a longer time and remains unexplained. Chromatography of the material recovered from the RNA eluted by solution E is shown in Figure 2. Several peaks of absorbance are seen, more than the three expected for threonine acceptors. The material eluted by solution containing ethanol represented 8% of the total. Only three peaks of threonine acceptor activity were found and their heights relative to absorbance were not uniform. In particular the first was of low activity. This was due to its containing also an acceptor for alanine. The identities of the other smaller peaks were not determined.

The tendency of a small portion of the tRNA to dissociate more slowly from the BD-cellulose than does the bulk of the material is well illustrated in comparing Figures 1 and 2. In both these columns eluted by gradients of salt concentration about 8% of the RNA appeared as a peak upon elution with ethanolic salt solution after the gradient had finished. About this proportion is usually found when columns are eluted in this way, regardless of prior treatment of the RNA. (The proportion retained depends in part upon the relative proportions between the load of RNA applied and the quantity of BD-cellulose in the column.) Hence, part of the material in this peak is not retained by virtue of its containing phenoxyacetyl groups. The acceptor for aspartic acid is one of those present in highest concentration in the crude tRNA (Gillam *et al.*, 1967) and chromatographs as a single peak. The total threonine acceptor activity in the crude material is much lower, is divided between three peaks, and was not recovered efficiently in the experiments described. Thus the small amount of nonspecifically adsorbed RNA eluted together with the *N*-phenoxyacetylaminoacyl-tRNA becomes a more important contaminant in the case of $tRNA^{Thr}$ than for $tRNA^{Asp}$. The cause for the occurrence of $tRNA^{Tyr}$ in the partially purified $tRNA^{Asp}$ was discussed above. It was initially thought that the contaminating tRNAs were isolated with the desired species because the incompletely purified enzyme used for aminoacylation also generated and activated certain amino acids to their tRNAs. Though it was not possible to demonstrate generation of sufficient free amino acids to account for the contamination found, the hypothesis was accepted until it was discounted by the experiment to be described next.

Arginine. The proportion of crude tRNA which accepts arginine is almost as great as that which accepts aspartic acid but is composed of three chromatographically distinct species. *N*-Phenoxyacetylarginyl-tRNA was isolated from mixed tRNAs by elution from an unusually large column of BD-cellulose with solution E. This fraction contained all the radioactivity from [^{14}C]arginine and about 15% of the A_{260} units applied. Chromatography of the regenerated $tRNA^{Arg}$ upon a

[2] This figure is based upon an assumed extinction coefficient for a typical tRNA. The actual figure will probably be found to vary from species to species and will be dependent upon the ionic strength, etc., of the solution.





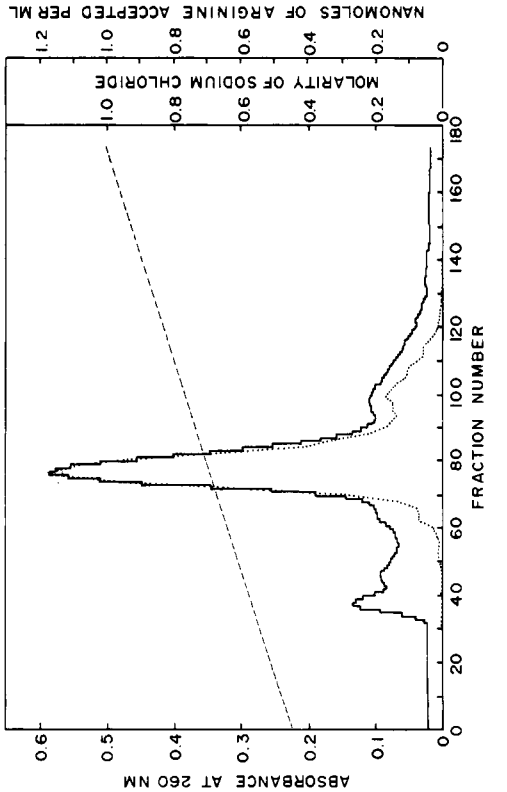


FIGURE 3: Chromatography of $tRNA^{Arg}$. (A) Top left: Material purified by the phenoxyacetylation procedure (1000 A_{260} units) on a column (3.5 × 45 cm) of BD-cellulose followed by hydrolysis was eluted from a column (1.4 × 110 cm) of BD-cellulose by the indicated (dashed line) gradient of concentration of sodium chloride containing 0.01 M magnesium chloride (total of 3 l.). Flow rate, 1.5 ml/min. Solid line: A_{260}; dashed line: acceptor activity for arginine; shaded areas: acceptor activities for the indicated amino acids. (B) Top right: *N*-2-Naphthoxyacetylarginyl-tRNA (2700 A_{260} units) was applied to a column (3.5 × 12 cm) of BD-cellulose in solution A and washed with the same. At fraction 16 elution with solution C was started. At the indicated points (arrows) elution with solutions D and F was begun. Solid line: A_{260}; dotted line: radioactivity from [^{14}C]arginine. (C) Left: Rechromatography of the last fraction from (B) after hydrolysis of the substituted aminoacyl ester. The tRNA (80 A_{260} units) was eluted from a column (0.9 × 100 cm) of BD-cellulose with the indicated gradient (dashed line) of concentration of sodium chloride in 0.01 M magnesium chloride (total of 1100 g). Flow rate was 0.8 ml/min. Solid line: A_{260}; dotted line: acceptor activity for arginine.

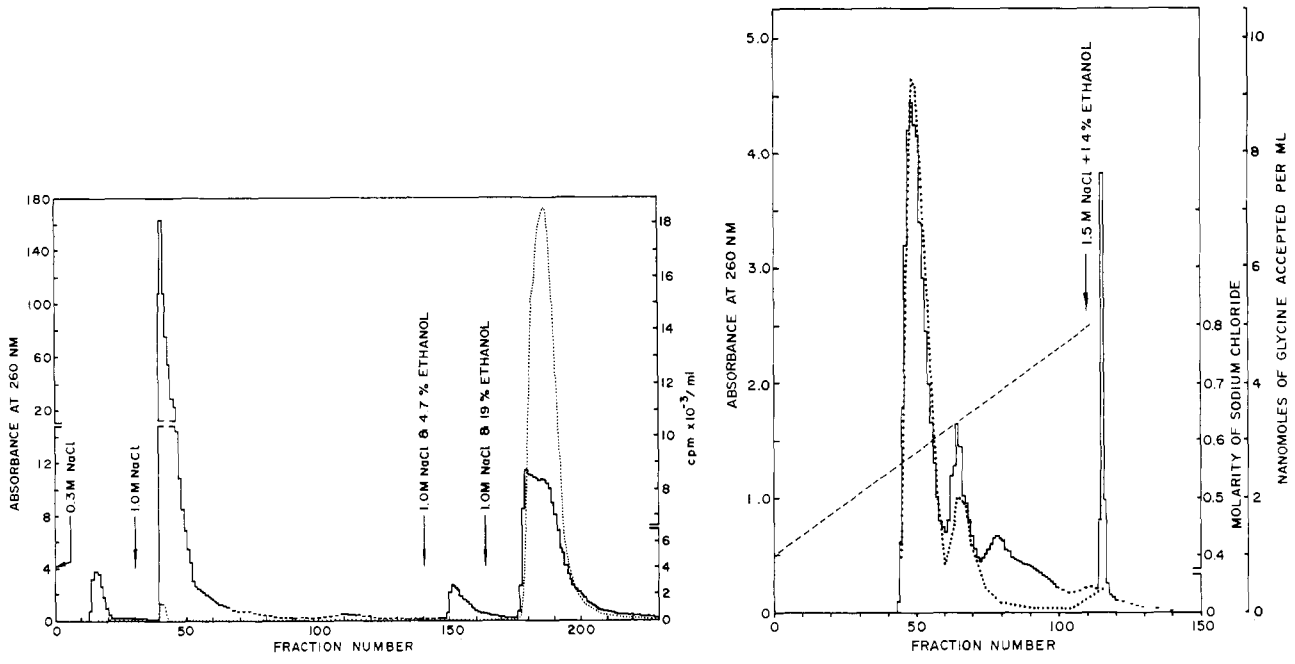


FIGURE 4: Isolation and subsequent chromatography of $tRNA^{Gly}$. (A) Left: *N*-2-Naphthoxyacetylglycyl-tRNA prepared from 1 g of mixed tRNAs was applied to a column (2.5 × 37 cm) of BD-cellulose in solution A. The column was washed with the same solution. At the indicated points elution with solutions C, D, and F was started. Solid line: A_{260}; dotted line: radioactivity from *N*-2-naphthoxyacetyl-[^{14}C]glycyl-tRNA. (B) Right: Chromatography of $tRNA^{Gly}$ obtained by hydrolysis of *N*-2-naphthoxyacetylglycyl-tRNA. The tRNA (1400 A_{260} units) was chromatographed on a column (1.2 × 98 cm) of BD-cellulose eluted with the indicated (dashed line) gradient of concentration of sodium chloride containing 0.01 M magnesium chloride (total of 2 l.). Flow rate, 1.0 ml/min. At the indicated point elution was continued with 1.5 M sodium chloride–0.01 M magnesium chloride in 14% (v/v) ethanol. Solid line: A_{260}; dotted line: acceptor activity for glycine.

column of BD-cellulose (Figure 3A) showed a complex pattern of elution. The expected distribution of peaks of acceptor activity for arginine was found and areas of activity for aspartic acid and serine were identified.

In the purification of aminoacyl-tRNA synthetases by chromatography on hydroxylapatite used here (Millward, 1967) aspartyl-tRNA synthetase is strongly bound to the column and well separated from arginyl-tRNA synthetase. The enzyme used in this experiment was completely free of aspartate-activating enzyme. Thus the contaminating $tRNA^{Asp}$ found could not have arisen from the formation of aspartyl-$tRNA^{Asp}$ during the synthesis of arginyl-$tRNA^{Arg}$. Attention was focussed upon the dilemma examined in the case of aspartic acid above, namely the tendency of some of the uncharged tRNA to tail behind the main peak eluted with solution B or C while the phenoxyacetylated aminoacyl-tRNA tended to begin to bleed off with these eluents before the addition of ethanol. It was hoped that a new derivative could be found which would cause the substituted aminoacyl-tRNA to bind even more firmly to the BD-cellulose and sharpen the separation between the two classes. Of those tried, the 2-naphthoxyacetyl group proved most promising and its use is described. Reaction with the *N*-hydroxysuccinimide ester of this acid was performed as usual. Figure 3B shows the elution of ^{14}C-labeled *N*-2-naphthoxyacetylarginyl-tRNA from BD-cellulose. Together with the bulk of the RNA a small amount of radioactive material was eluted by solution C. This was not characterized but probably represented [^{14}C]arginyl-tRNA which was not substituted. The tRNA eluting with salt tailed as usual, and material still bound to the column when little absorbance was detectable in the eluate could be eluted as a small peak by salt containing 4.5% ethanol (solution D). This small peak contained no detectable radioactivity, the *N*-naphthoxyacetylaminoacyl-tRNA still being held by the column. The desired tRNA was released upon washing with salt containing 19% ethanol (solution F). The material so recovered represented only 5% of the whole and was likely to be substantially pure. The rechromatography of stripped tRNA prepared from this fraction is shown in Figure 3C. The distribution of absorbance showed the principal peak expected for $tRNA^{Arg}$. Since this had a specific acceptor activity higher than the value estimated above for a pure sample it was considered to be close to pure $tRNA^{Arg}$. The presence of small amounts of other tRNAs shown by the elution pattern may be accounted for by a small degree of nonspecific reaction between the 2-naphthoxyacetyl ester and free 2′-hydroxyl groups of tRNAs.

Glycine. In crude tRNA the acceptor activity for glycine is the highest for any amino acid tested and separates on BD-cellulose in the unbuffered system into two major peaks (Gillam *et al.*, 1967). Purification of this group of acceptors by the 2-naphthoxyacetylation procedure is illustrated in Figure 4. The preparation of activating enzyme used in this experiment was not fractionated by passage through hydroxylapatite. Thus the experiment was a direct test of the necessity to use purified enzymes in the isolation of tRNAs. In this case the added protein was removed from the incubation

mixture by extraction with phenol. Figure 4A shows the isolation of *N*-2-naphthoxyacetylglycyl-tRNA labeled with [^{14}C]glycine. The bulk of the tRNA was eluted together with a trace of radioactivity by buffer containing 1 M sodium chloride. Extensive washing of the BD-cellulose with this buffer caused no loss of radioactivity though it failed to remove all of the trailing RNA which was eluted as a small peak by solution containing salt and 4.5% ethanol. This material contained no radioactivity, demonstrating again the effectiveness of the naphthoxyacetyl group in reducing the bleeding of derivatized RNA from the column. *N*-2-Naphthoxyacetylglycyl-tRNA was recovered by elution with buffer solution containing 19% ethanol. This material represented 8.4% of the whole, about the value expected for the proportion of glycyl acceptor.

Chromatography of the tRNA recovered from this (Figure 4B) showed the anticipated two principal peaks of tRNA^{Gly}, though not in the proportions seen previously (Gillam *et al.*, 1967). The first peak had a specific acceptor activity for glycine higher than the average value for other tRNAs (2.1 *vs.* 1.85 nmoles per A_{260} unit). The second peak was of lower specific activity. A third peak of material was not identified and a trace was eluted by a solution containing ethanol. Thus use of a crude preparation of enzyme appears to be satisfactory in this case. However, crude preparations contain enzymes which rapidly destroy arginine and proline, and partially purified synthetases are needed for the esterification of these amino acids.

Methionine. Methionine acceptor tRNA was isolated by the phenoxyacetylation procedure. It is described because of interest in the role of one species of this tRNA in the initiation of protein synthesis (Marcker and Sanger, 1964). Separation of *N*-phenoxyacetylmethionyl-tRNA was routine with 4% of the absorbance eluted from BD-cellulose with solution E. This was hydrolyzed and the recovered RNA chromatographed on BD-cellulose (Figure 5). The expected three peaks of optical density appeared and each contained acceptor activity for methionine.

The experiments presented above serve to demonstrate that the proposed method of isolation of specific aminoacyl-tRNAs based upon a shift in their position of elution from BD-cellulose after substitution with an aromatic group is effective for a variety of amino acids. It has also been used in purification of acceptors for alanine and histidine (unpublished). Kuo and Keller (1968) have described isolation of alanyl-tRNA^{Ala} and Reeves *et al.* (1968) have separated charged from uncharged tRNA using this procedure. The procedure is expected to be widely applicable and is likely to offer the best means of isolating those species of tRNA which occur only as traces in crude tRNA. However, it is subject to some limitations. In the form described it clearly cannot be used for purification of a tRNA which binds strongly enough to BD-cellulose in the uncharged state to require the presence of ethanol for elution. In the yeast used here only one tRNA, that for phenylalanine, has this property and its purification has been described (Wimmer *et al.*, 1968). Where several tRNAs occur in this fraction it may well be feasible

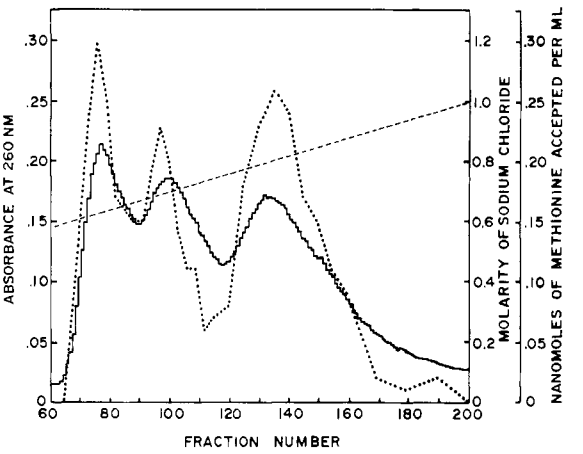


FIGURE 5: Chromatography on BD-cellulose of tRNA^{Met} prepared by the phenoxyacetylation procedure. The RNA (90 A_{260} units) was chromatographed on a column (0.9 × 90 cm) of BD-cellulose by elution with the indicated (dashed line) gradient of concentration of sodium chloride containing 0.01 M magnesium chloride (total of 1100 g of solution). Flow rate, 0.7 ml/min. Solid line: A_{260}; dotted line: acceptor activity for methionine.

to separate one by the method described here except that the eluents would all contain ethanol. tRNAs charged with aromatic amino acids bind firmly to BD-cellulose without further substitution. This property has been used for the purification of tRNA^{Trp} and tRNA^{Tyr} of yeast (Maxwell *et al.*, 1968). However, introduction of another aromatic group to these tRNAs by the procedure used here might be useful in helping to eliminate the problems of tailing and bleeding discussed earlier. The imino group in proline is more basic than that of other amino acids and prolyl-tRNA is hydrolyzed at pH values between 7 and 11 considerably more rapidly than typical aminoacyl-tRNAs (Gatica *et al.*, 1966). This combination of properties may result in difficulties in obtaining complete naphthoxyacetylation of this particular aminoacyl-tRNA. Since different species of acceptor RNA for a given amino acid may be charged at different rates or even by different enzymes (Vescia, 1967) it is clearly unwise to use any method dependent upon enzymatic aminoacylation to make estimates of the relative concentrations of such species without carefully defining the conditions of reaction (Heyman *et al.*, 1967). In view of the considerable change in properties of aminoacyl-tRNAs upon substitution with an aromatic acyl group it may be that a similar scheme can be applied to the purification of tRNAs in the countercurrent distribution system (Apgar *et al.*, 1962; Karau and Zachau, 1964) or that using partition chromatography (Weiss and Kelmers, 1967).

References

Anderson, G. W., Zimmerman, J. E., and Callahan, F. M. (1964), *J. Am. Chem. Soc. 86*, 1839.

Apgar, J., Holley, R. W., and Merrill, S. H. (1962), *J. Biol. Chem. 237*, 796.

Blatt, W. F., Feinberg, M. P., Hopfenberg, H. B., and Saravis, C. A. (1965), *Science 150*, 224.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.
Brown, G. L., Brown, A. V. W., and Gordon, J. (1959), *Brookhaven Symp. Biol. 12*, 47.
de Groot, N., Lapidot, Y., Panet, A., and Wolman, Y. (1966), *Biochem. Biophys. Res. Commun. 25*, 17.
Gatica, M., Allende, C. C., Mora, G., Allende, J. E., and Medina, J. (1966), *Biochim. Biophys. Acta 129*, 201.
Gillam, I., Millward, S., Blew, D., von Tigerstrom, M., Wimmer, E., and Tener, G. M. (1967), *Biochemistry 6*, 3043.
Grachev, M. A., Menzorova, N. I., Sandakhchiev, L. S., Budovskii, E. I., and Knorre, D. G. (1966), *Biokhimiya 31*, 840.
Hall, R. H. (1964), *Biochemistry 3*, 769.
Heyman, T., Seror, S., Desseaux, B., and Legault-Demare, J. (1967), *Biochim. Biophys. Acta 145*, 596.
Ip, P. M. K., and Fraser, M. J. (1965), *Can. J. Biochem. 43*, 1071.
Karau, W., and Zachau, H. G. (1964), *Biochim. Biophys. Acta 91*, 549.
Kuo, T. H., and Keller, E. B. (1968), *Federation Proc. 27*, 341.
Marcker, K., and Sanger, F. (1964), *J. Mol. Biol. 8*, 835.
Maxwell, I. H., Wimmer, E., and Tener, G. M. (1968), *Biochemistry 7*, 2629.
Mehler, A. H., and Bank, A. (1963), *J. Biol. Chem. 238*, 2888.
Millward, S. (1967), Ph.D. Thesis, University of British Columbia, Vancouver, Can.
Muench, K. H., and Berg, P. (1966), *in* Procedures in Nucleic Acid Research, Cantoni, G. L., and Davies, D. R., Ed., New York, N. Y., Harper & Row.
Reeves, R. H., Imura, N., Schwam, H., Weiss, G. B., Schulman, L. H., and Chambers, R. W. (1968), *Proc. Natl. Acad. Sci. U. S.* (in press).
Sarin, P. S., and Zamecnik, P. C. (1964), *Biochim. Biophys. Acta 91*, 653.
Simon, S., Littauer, U. Z., and Katchalski, E. (1964), *Biochim. Biophys. Acta 80*, 169.
Sueoka, N., and Yamane, T. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1454.
Tener, G. M., Gillam, I., von Tigerstrom, M., Millward, S., and Wimmer, E. (1966), *Federation Proc. 25*, 519.
Tener, G. M., Gillam, I. C., von Tigerstrom, M., Wimmer, E., Millward, S., Blew, D., and Maxwell, I. (1967), *7th Intern. Congr. Biochem., Tokyo*, p 643.
Vescia, A. (1967), *Biochem. Biophys. Res. Commun. 29*, 496.
Weiss, J. F., and Kelmers, A. D. (1967), *Biochemistry 6*, 2507.
Wimmer, E., Maxwell, I. H., and Tener, G. M. (1968), *Biochemistry 7*, 2623.
Zamecnik, P. C., Stephenson, M. L., and Scott, J. F. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 811.