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QUANTITATIVE ENZYMATIC HYDROLYSIS OF tRNAs

REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF tRNA NUCLEOSIDES*

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SUMMARY

A rapid quantitative method for enzymatic hydrolysis of microgram amounts of tRNA has been developed, specifically to take full advantage of our precise, accurate, and selective reversed-phase high-performance liquid chromatographic (HPLC) system for separation and measurement of the major and modified nucleosides in tRNA. After study of several enzyme systems, nuclease P1 and bacterial alkaline phosphatase were selected and the hydrolysis parameters were systematically studied. Optimized hydrolysis conditions give quantitative hydrolysis in 2 h and this short incubation time prevents loss of unstable nucleosides. The chromatographic system can tolerate relatively high levels of protein in the sample allowing high enzyme—substrate ratios and direct injection of hydrolysates. This enzymatic hydrolysis—HPLC method is the best described to date for quantitative determination of the nucleoside composition of tRNAs and has already provided important information for investigation of the role of modification in the function of RNAs.

INTRODUCTION

Since the discovery of the wide variety of modified nucleosides present in tRNAs, there has been a great deal of interest in the role of these modified

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nucleosides in the function of tRNA. Current areas of interest include the effect of modifications in, or adjacent to, the anticodon on the fidelity of translation, the importance of modifications in the recognition of tRNA by aminoacyl-tRNA synthetases and the role of modified nucleosides in tRNA–ribosome interactions.

Determination of the modifications present in a tRNA, their position in the sequence, and the extent to which they are modified is essential to the study of modification in tRNA. Sequencing techniques available today are very powerful but often they do not allow identification of the modified nucleoside or exact quantitation of the level of a modification per unit of tRNA. Methodology for accurate identification and quantitation of the complete nucleoside composition of tRNAs is therefore required as a complementary technique to obtain information on the modifications present. Conditions that must be placed on this method are that it be applicable to today's use of microgram amounts of tRNA for sequence determination and allow preparative isolation of unknown modified nucleosides for identification and structure elucidation.

Analysis of the composition of tRNAs at the nucleoside level offers several advantages over analysis at the base or nucleotide level. Nucleosides can be obtained by enzymatic hydrolysis under mild conditions while chemical hydrolysis requiring harsh conditions is needed to obtain the bases and this may alter or destroy structure in sensitive modified nucleosides. Also, chromatographic separation of nucleosides is more efficient than separation of the highly charged nucleotides, and analysis at the base level does not allow the determination of the ribose-methylated nucleosides.

In earlier studies we developed a reversed-phase high-performance liquid chromatographic (HPLC) system for determination of urinary nucleosides [1—3] to use for investigation of tRNA breakdown products as cancer markers [3—5]. Reversed-phase HPLC was found to be ideally suited for separation of ribonucleosides because of their intermediate polarity. We then developed a reversed-phase HPLC method for measurement of the major and modified nucleosides in tRNAs by direct injection of nucleoside mixtures for enzymatic hydrolysates of RNAs [6]. This chromatography has been improved [7] and now allows separation and measurement of more than thirty nucleosides.

Despite a number of investigators reporting the use of various enzymes for hydrolysis of tRNA [8—16], the studies presented in this paper are, to our knowledge, the first systematic investigations on the conditions for enzymatic hydrolysis of tRNA reported and are directed towards developing a hydrolysis procedure that allows us to take full advantage of the chromatographic method. For accurate determination of the nucleoside composition of RNA the hydrolysis procedure must be quantitative, reproducible and capable of hydrolyzing microgram amounts of RNA in microliter volumes without large mechanical loss or chemical breakdown of the resultant nucleosides. Randerath et al. [12] reported some recovery studies with free nucleosides subjected to an enzymatic hydrolysis procedure, which showed conversion of m^1Ado to m^6Ado, and some loss of hUrd, m^3Cyd, and m^7Guo during the 6-h hydrolysis of tRNA with ribonuclease A, snake venom phosphodiesterase, and bacterial alkaline phosphatase. This degradation of nucleosides

was due to the alkaline lability of these particular molecules. We have shown that minimizing the incubation time of the nucleosides at pH 8 eliminated this problem.

In addition to the enzymes used previously for the hydrolysis of tRNA for nucleoside analysis we report the use of nuclease P1, an endonuclease that quantitatively hydrolyzes both DNA and RNA to 5'-mononucleotides with little specificity requirements for the bases.

The 2-h nuclease P1—bacterial alkaline phosphatase (BAP) hydrolysis procedure presented in this paper, together with our HPLC methods developed earlier, provide the tools for the determination of the complete nucleoside composition of tRNAs needed for research on the modified nucleosides in RNAs.

MATERIALS AND METHODS

The HPLC instrumentation, columns, buffer preparation, chromatographic conditions, standard solutions, and reagents used in these studies were described in detail in earlier publications [2, 3, 6]. Buffers for the step gradient separation of ribonucleoside mixtures were slightly different from those in our earlier tRNA analysis studies [6], but were the same as those used more recently for the separation of deoxyribonucleoside mixtures [17] in research on methylated nucleosides in DNA. Buffer A was composed of 2.5% methanol (v/v), 0.01 *M* $NH_4H_2PO_4$, pH 5.3 and buffer B was 8% methanol (v/v), 0.01 *M* $NH_4H_2PO_4$, pH 5.1.

All enzymes were obtained from commercial sources. Nuclease P1 (EC 3.1.4.-), ribonuclease A (EC 3.1.27.5), snake venom phosphodiesterase (EC 3.1.4.1), and calf intestinal alkaline phosphatase (EC 3.1.3.1) were obtained from Boehringer Biochemicals (Mannheim, G.F.R.); bacterial alkaline phosphatase (EC 3.1.3.1) and ribonuclease T_2 (EC 3.1.4.23) were obtained from Sigma (St. Louis, MO, U.S.A.). Brewer's yeast phenylalanine tRNA accepting 966 pmole phenylalanine per A_{260} unit, was also obtained from Boehringer Biochemicals.

The enzymatic hydrolysis of tRNA with ribonuclease A and T_2 was accomplished according to the following procedure. A tRNA solution (5 μl) containing 25 μg of tRNA was heated at 100°C for 2 min, then quenched in an ice-water bath. Ribonuclease A, 15 μl of a 1 mg/ml solution, and ribonuclease T_2, 25 μl of 1000 unit/ml solution, were added and the mixture was incubated for the desired time (0.25—12 h) at 37°C. The pH of the mixture was adjusted by addition of 6 μl of 0.5 *M* Tris, pH 7.9. BAP, 10 μl containing 2 units, was then added and the incubation was continued for an additional 12 h.

The method developed for the hydrolysis of tRNA with nuclease P1 and BAP is described in the following procedure. A tRNA solution of 5—125 μg of tRNA in 25 μl of water was heated for 2 min at 100°C. The sample was rapidly cooled in an ice-water bath. Then the following reagents were added: 2 μl of 20 m*M* $ZnSO_4$, 10 μl of nuclease P1 (1 mg/ml, 200 units/mg, in 30 m*M* sodium acetate, pH 5.3), and 10 μl of BAP (a commercial suspension containing about 190 units/ml, 30 units/mg, diluted 1:100 with water). This

mixture was incubated at 37°C for 1 h. The pH was adjusted by addition of 15 μl of 0.5 *M* Tris, pH 7.9, and the incubation continued at 37°C for another hour. For hydrolysis of different initial volumes of tRNA solution, the amounts of enzymes and buffers were adjusted to maintain the concentrations at about the same level as those used in the above procedure. The ratio of enzymes to RNA was also kept within or above the indicated range when hydrolyzing greater quantities of RNA. Although no systematic studies were made of the stability of the nucleosides in the hydrolysates, we found that the samples were stable for at least two months when frozen at −20°C.

RESULTS AND DISCUSSION

Monitoring enzymatic hydrolysis

Determination of the nucleoside composition of tRNA requires both quantitative hydrolysis of the macromolecule and precise and accurate analysis of the resulting ribonucleoside mixture. For the following investigations, the yield of the hydrolysis under each set of the conditions was determined by reversed-phase HPLC [6, 7] separation and quantitation of the nucleosides. This HPLC method allowed direct injection of the enzymatic hydrolysates onto the column without removal of protein or any other sample preparation. The analyses were performed at the rate of one every 2 h and automated instrumentation allowed unattended overnight operation.

A readily available commercial preparation of phenylalanine tRNA from yeast was chosen as the model substrate for these studies. This tRNA has been widely studied and the complete sequence and three-dimensional X-ray crystallographic structure are known.

Selection of enzymes for RNA hydrolysis

The enzymatic hydrolysis of RNA is a two-step process requiring a nuclease to release the mononucleotides and a phosphatase to remove the phosphate groups yielding nucleosides. Three nuclease systems and three phosphatases were investigated prior to selecting the best combination of enzymes for simple, rapid and complete hydrolysis.

The phosphatases considered were acid phosphatase, calf intestinal alkaline phosphatase, and BAP. The use of an acid phosphatase would allow the entire hydrolysis to be carried out at the pH optimum for the nucleases without incubation at high pH. However, the very low activity of these enzymes would require the addition of an excessive amount of protein to the mixture to ensure complete hydrolysis within a reasonable period of time.

The commercial preparation of calf intestinal alkaline phosphatase was contaminated with very high adenosine deaminase activity and gave almost quantitative conversion of Ado to Ino. This prevented determination of Ino residues present in tRNA and the accurate quantitation of Ado and modified Ado. For this reason we chose the BAP with only a trace of adenosine deaminase activity.

Each lot of BAP was checked for adenosine deaminase activity. If the activity was high enough to convert more than 1% of the Ado to Ino under the conditions used for hydrolysis of RNA, the BAP suspension was heated at

100°C for 5 min to denature the adenosine deaminase [18]. The activity of the BAP was checked before and after this heat treatment to assess how much BAP activity was lost. If 50% or more of the activity remained after heating the BAP could still be used effectively.

Three nuclease systems were investigated for hydrolysis of tRNA to nucleotides: nuclease P1; ribonucleases A and T_2; and ribonucleases A and T_2 with snake venom phosphodiesterase. The use of snake venom phosphodiesterase without other nucleases was not studied. This enzyme is an exonuclease and consequently its activity is decreased in the hydrolysis of oligonucleotides having appreciable secondary or tertiary structure.

Hydrolysis of yeast $tRNA^{phe}$ with nuclease P1 and BAP yielded all of the expected chromatographic peaks for major and minor ribonucleosides (Fig. 1), whereas hydrolysis with ribonucleases A and T_2 failed to release 2'-O-methylcytidine (Cm) and 2'-O-methylguanosine (Gm) as seen by the absence of these two peaks from the chromatogram (Fig. 2). This result was expected since hydrolysis with ribonucleases A and T_2 requires a free 2'-hydroxyl group for formation of a 2'—3'-cyclic nucleotide as an intermediate.

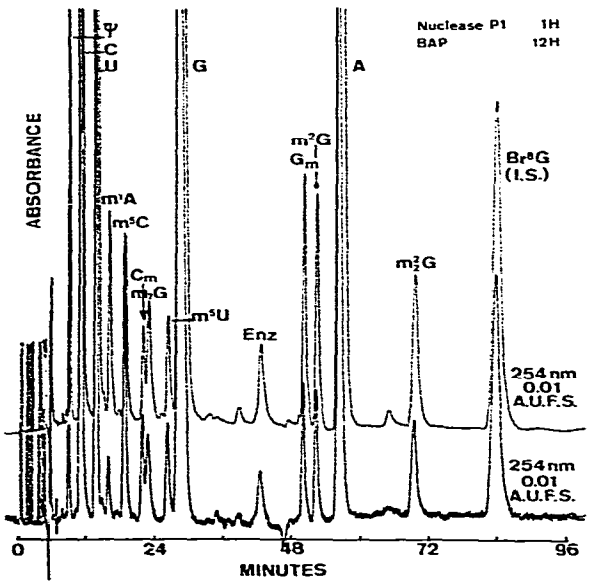

Fig. 1. A representative chromatogram for analysis of yeast $tRNA^{phe}$ hydrolyzed with nuclease P1 and BAP; 5 μg of tRNA were injected.

A quantitative comparison of these two enzyme systems is given in Table I. In addition to the failure of ribonucleases A and T_2 to release the 2'-O-methylated nucleosides, hydrolysis with these enzymes yielded only 80% as much m^7Guo and 50% as much m^2_2Guo as hydrolysis with nuclease P1. These observations reflect a difference in the substrate specificity of the two enzyme systems and not differences in the specific activities for they were compared over a wide range of enzyme incubation times (0.25—12 h).

The addition of snake venom phosphodiesterase as well as ribonucleases

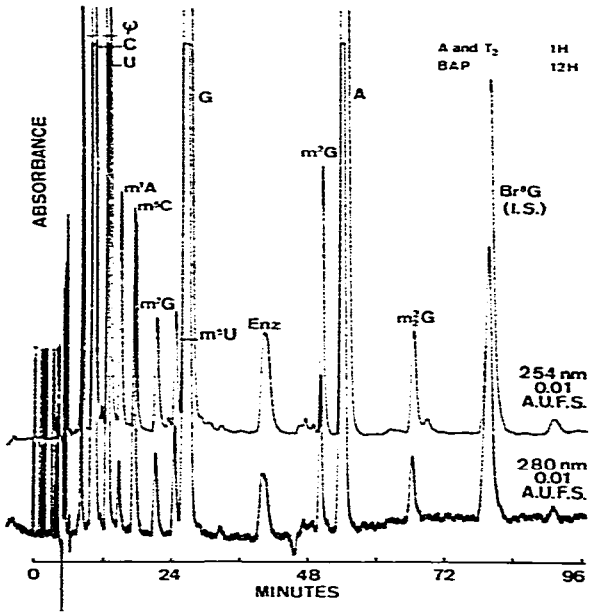

Fig. 2. A representative chromatogram for analysis of yeast $tRNA^{phe}$ hydrolyzed with ribonuclease A, ribonuclease T_2 and BAP; 5 μg of tRNA were injected.

TABLE I

HYDROLYSIS OF $tRNA^{phe}_{yeast}$

Nucleoside	Residues per 73 residues (hydrolyzed with nuclease P1)	Residues per 71 residues (hydrolyzed with nucleases A and T_2)
ψ	2.1	2.1
Cyd	16.2	16.4
Urd	11.6	11.0
m^1Ado	0.88	0.88
m^5Cyd	1.68	1.70
Cm	0.74	—
m^7Guo	0.79	0.64
m^5Urd	0.93	0.92
Guo	18.8	19.0
Gm	0.97	—
m^2Guo	0.87	0.88
Ado	16.5	17.1
m_2^2Guo	0.90	0.49

A and T_2 to the sample gave results essentially identical to those for nuclease P1. Since hydrolysis with a single enzyme is much more desirable than use of a three-enzyme system we chose nuclease P1 for optimization of hydrolysis conditions.

Effect of incubation time on hydrolysis of $tRNA^{phe}$ with nuclease P1 and BAP

A very rapid hydrolysis procedure is desirable to prevent the breakdown of sensitive nucleosides during hydrolysis, and to allow the preparation of hydrolysates within one 8-h working day. Therefore, the time required for complete hydrolysis of tRNA was investigated using high concentrations of nuclease P1 and BAP. HPLC allows injection of high concentrations of protein without interfering with the analysis. This means high enzyme to RNA ratios may be used but column life will be shortened somewhat.

In this and subsequent studies, the tRNA solutions were heat denatured for 5 min at 100°C and immediately quenched in an ice-bath prior to addition of the enzymes. There was no clear evidence that this heat denaturation was necessary; however, denatured tRNA is more easily attacked by nucleases than native molecules and heat treatment does not damage the nucleosides. Thus, a heat treatment prior to hydrolysis is recommended.

Mixtures of denatured $tRNA^{phe}$ (25 μg), nuclease P1 (2 units, 10 μg) and $ZnSO_4$ (10 nmole) in 50 μl of 30 m*M* sodium acetate buffer, pH 5.3, were incubated for 0.25, 0.5, 1, 2, 3, 8 and 12 h. At the end of the incubation period the sample was heated at 100°C for 5 min to stop the nuclease P1 activity. The pH of each sample was then adjusted with 6 μl of 0.5 *M* Tris, pH 7.9, and incubation was continued for an additional 12 h with 3.8 units (63 μg) of BAP in a final total volume of 66 μl.

Less than 15 min of incubation time were required for complete hydrolysis of $tRNA^{phe}$ by nuclease P1 at the concentration used in this study. The qualitative and quantitative results for analysis of the samples from each incubation time were identical indicating the nucleotides released by nuclease P1 were stable under these conditions for at least 12 h.

The effect of incubation time on the BAP hydrolysis of nucleotides to nucleosides was studied in an analogous manner, except the samples were all incubated with 2 units (10 μg) of nuclease P1 for 1 h prior to incubation with BAP for 0.25, 0.5, 1, 2, 3, 4 and 24 h. A BAP concentration of 3.8 units in 66 μl of sample solution hydrolyzed the nucleotides completely in less than 15 min. The levels of all nucleosides except m^7Guo remained constant regardless of the BAP incubation time. The m^7Guo level in the hydrolysates decreased from 0.84 residues per 73 residues when BAP incubation time was 15 min to 0.61 residues per 73 residues when incubated with BAP for 24 h. This is consistent with the known base lability of m^7Guo [12].

Thus, quantitative hydrolysis of $tRNA^{phe}$ (25 μg) to nucleosides was completed in less than 30 min incubation, 15 min with each enzyme, when the enzyme concentrations were high: 40 units nuclease P1 per ml and 570 units of BAP per ml.

Optimization of nuclease P1 concentration for hydrolysis of $tRNA^{phe}$

To determine the concentration of nuclease P1 required for complete hydrolysis of 25 μg of $tRNA^{phe}$ in 1 h, a series of samples were prepared that were identical except for the nuclease P1 concentration. The use of a very large excess of enzyme is undesirable because injection of too much protein onto the HPLC columns would shorten column life. Obviously, the

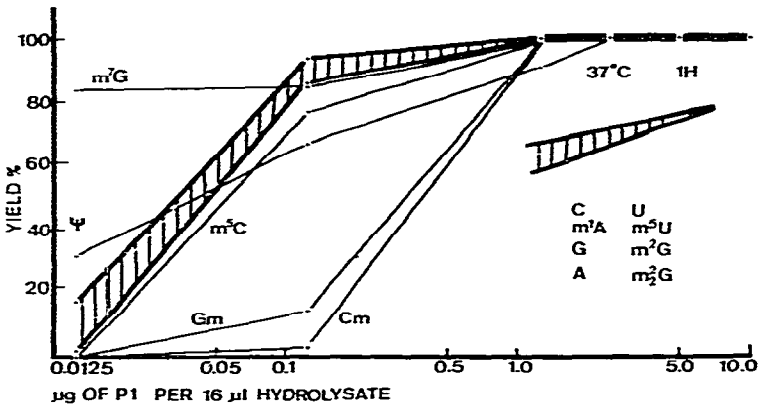

Fig. 3. Effect of nuclease P1 concentration on hydrolysis of yeast $tRNA^{phe}$. Reaction mixtures were prepared containing $tRNA^{phe}$ (25 μg), $ZnSO_4$ (10 nmole) and 0.0125, 0.125, 1.25, 2.5, 5 and 10 μg of nuclease P1 in a final volume of 16 μl (30 m*M* sodium acetate buffer, pH 5.3) and incubated for 1 h at 37°C. Nuclease P1 activity was stopped by heating at 100°C for 5 min. Hydrolysis to nucleosides was then completed by addition of 6 μl of 0.5 *M* Tris, pH 7.9, 10 μl of BAP (3.8 units, 63 μg) and incubation at 37°C for 1 h. The nucleoside levels in each sample were determined by HPLC and expressed as nanomoles per hydrolysate relative to the internal standard added after hydrolysis. The nanomoles per hydrolysate for each nucleoside were converted to per cent yield based on a 100% yield for the hydrolysates having a nuclease P1 concentration of 2.5 μg per 16 μl or greater.

use of too little enzyme would result in incomplete hydrolysis.

The yield of each nucleoside as a function of nuclease concentration is presented in Fig. 3. All nucleosides except ψ were obtained in 100% yields when the nuclease P1 concentration was 1.25 μg per 16 μl or greater. Cm, Gm and ψ exhibited a marked dependence on enzyme concentration while m^7Guo was much less dependent on enzyme concentration.

We suggest that the inhibited release of Cm and Gm was due solely to the nature of the modification, 2'-oxygen-methylation, and its hinderance of phosphate ester cleavage. The yield of these two nucleosides was less than 10% at a nuclease concentration of 0.125 μg per 16 μl and essentially zero below this concentration.

In contrast to the 2'-O-methyl nucleosides the yield of ψ did not drop below 30% for the lowest nuclease concentration, but did not reach 100% until the nuclease concentration was above 1.25 μg per 16 μl. This would suggest that the release of ψ from $tRNA^{phe}$ depends both on the nature of the nucleoside, ψ is the only one with a carbon—carbon ribofuranosyl bond, and on its two different locations within the tRNA structure. One ψ residue is located in the TψCG loop while the other is in the hydrogen-bonded anticodon stem. The high yield of m^7Guo (> 80%) even at the lowest concentration of nuclease P1 could be due to its exposed position in the extra loop.

Based on these studies a nuclease P1 concentration of 2.5 μg per 16 μl is sufficient for complete hydrolysis of $tRNA^{phe}$ in 1 h. Routinely, we use a concentration 2—4-fold higher which gives a margin of safety ensuring complete hydrolysis.

Optimization of BAP concentration for conversion of nucleotides to nucleosides

The study of the concentration of BAP required for complete release of nucleosides from tRNA was analogous to the study of nuclease P1 concentration on extent of enzymatic hydrolysis. Percent yields of the nucleosides from yeast $tRNA^{phe}$ versus phosphatase concentration is presented in Fig. 4.

Only three nucleosides, m^1Ado (45%), m^7Guo (32%) and ψ (not detected) were obtained in yields of less than 70% at the lowest level of BAP studied, 0.0125 μg in 32 μl. All nucleosides were quantitatively released at a BAP concentration of 0.05 μg per 32 μl except m^1Ado and m^7Guo which were obtained in 85% yield. This indicates that phosphatase action on the corresponding nucleotides is influenced by the nature of the modification.

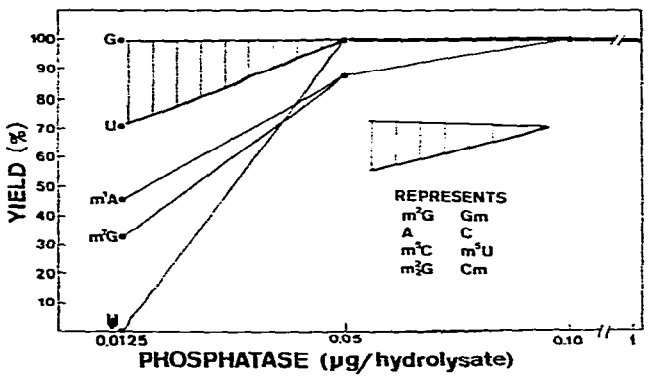

Fig. 4. Effect of phosphatase concentration on hydrolysis of yeast $tRNA^{phe}$. Samples containing 25 μg of $tRNA^{phe}$ were subjected to nuclease P1 (2 units, 10 μg) hydrolysis for 1 h. The resulting nucleotides were then incubated for 1 h with BAP (30 units/mg) at concentrations ranging from 0.0125 μg per 32 μl to 10 μg per 32 μl. Calculations of yields were made in the same manner as those in Fig. 3.

We have concluded that under these conditions, 0.1 μg per 32 μl is the minimum concentration of BAP required for quantitative dephosphorylation of the nucleotides resulting from nuclease P1 hydrolysis of 25 μg of tRNA. In subsequent experiments the concentration of phosphatase was increased 5-fold to ensure complete dephosphorylation.

Since BAP is appreciably active at a pH of 5.3 and was not irreversibly inactivated at this pH there was a decided advantage in adding the phosphatase enzyme at the same time as the nuclease P1. Dephosphorylation of nucleotides would then start at the pH optimum for nuclease P1 and would continue at a higher rate after adjustment of the pH to the optimum (pH 7.9) for BAP. When BAP was added to the samples with nuclease P1, the analytical results were identical to those obtained when BAP was added after completion of hydrolysis with nuclease P1. The combined addition of the enzymes has the added advantage of simplifying sample handling.

Maximum capacity of the enzymatic hydrolysis method

With establishment of the optimum enzyme concentrations and incubation times to assure quantitative hydrolysis of 25 μg of tRNA, the capacity

of the system for hydrolysis of larger quantities of tRNA was studied. Samples were prepared containing 25, 50, 125 and 200 μg of $tRNA^{phe}$ with 10 μg of nuclease P1 and 0.63 μg of BAP. The 200-μg tRNA sample was in an initial volume of 50 μl and the others were in an initial volume of 25 μl. The samples were hydrolyzed for 1 h and then 15 μl of 0.5 *M* Tris, pH 7.9, were added prior to incubation for another hour. Complete hydrolysis of the tRNA was achieved under the above conditions. Normally only a limited amount (1—50 μg) of pure tRNA is available for nucleoside composition analysis so the enzymatic capacity demonstrated here is more than adequate. We have used the HPLC method for the determination of the nucleoside composition of 1 μg of tRNA, so the high capacity is required only for preparative scale isolation of modified nucleosides.

Application of the enzymatic hydrolysis to nucleoside analysis of various tRNAs

The enzymatic procedures reported here (see Materials and methods) have been used to hydrolyze a wide variety of tRNA samples for nucleoside analysis by HPLC. Excellent precision was obtained for replicate analyses of 5 μg of tRNAs with relative standard deviations (R.S.D.) ranging from 0.5 to 1.0% for the major nucleosides and from 2—4% for the modified nucleosides. A representative chromatogram from the analysis of a yeast $tRNA^{ser}$ isoacceptor (Fig. 5) illustrates the HPLC separation of the nucleosides from a tRNA with a greater variety of modification than is found in yeast $tRNA^{phe}$. This sample is a single pure species; however, quantitative comparison of these results with other serine tRNAs cannot be made since the sequence for this species

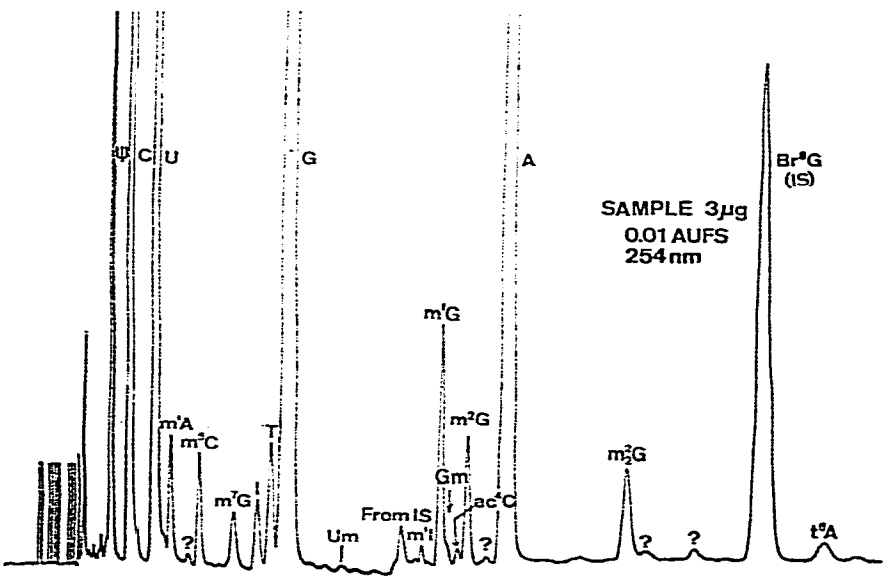

Fig. 5. Reversed-phase HPLC of a 3-μg hydrolysate of a purified species of *S. pombe* $tRNA^{ser}$. The tRNA was hydrolyzed with nuclease P1 and BAP according to the procedure in the Materials and Methods section.

TABLE II

NUCLEOSIDE COMPOSITION ANALYSIS OF tRNAs BY HPLC

The amount of each nucleoside found is expressed as the number of residues per molecule. The calculation was made using the equation:

$$R_N/\text{molecule} = \left[\frac{\text{nmols } N}{\text{total nmols}}\right] \times [\text{total number of residues } (R) \text{ in the tRNA}]$$

where R_N/molecule is the number of residues of the same nucleoside N per tRNA molecule; nmols N is the measured nanomoles of the nucleoside in the total sample; total nmols is the total nanomoles of all nucleosides in the sample (determined from the analysis); and the total number of residues per molecule is based on the sequence. Dihydrouridine and i^{6}Ado residues in the sequence were not counted. Dihydrouridine has a very low absorptivity at 254 and 280 nm preventing its detection by UV absorption at these wavelengths and i^{6}Ado was not quantitated because different chromatographic conditions are required for elution of this strongly retained nucleoside.

Nucleoside	Residues per molecule			
	$tRNA_f^{met}$ (*E. coli*)		$tRNA^{trp}$ (yeast)	
	Analysis	Sequence***	Analysis	Sequence***
ψ	1.01	1	5.8	6
Cyd	25.6	25	15.9	16
Urd	8.2	8	9.6	10
m^{1}Ado	—	—	0.81	1
Cm	0.91	1	1.81	2
m^{7}Guo	0.51	1/0*	0.91	1
m^{5}Urd	1.02	1	1.05	1
Guo	23.9	24	16.1	16
S^{4}Urd	0.8	1	—	—
m^{1}Guo	—	—	0.88	1
Gm	—	—	1.09	1
m^{2}Guo	—	—	0.96	1
Ado	14.1	14/15*	16.4	16
hUrd	NA**	1	NA	3

*The numbers on the right represent the sequence for a minor suhspecies.
**This nucleoside was not analyzed.
***Ref. 19.

has not been determined. The presence of all of the expected modified nucleosides, by comparison to all published yeast $tRNA^{ser}$ sequences, indicates nuclease P1 was capable of releasing all of the wide variety of modified nucleosides present in this tRNA. Enzymatic hydrolysis for analysis of many different tRNA samples has shown that nuclease P1 is capable of releasing all of the expected nucleosides regardless of the extent of modification.

The nucleoside compositions determined for $tRNA^{met}$ (from *E. coli*) and $tRNA^{trp}$ (from *S. cerevisiae*) are presented in Table II along with the predicted results based on the published sequences from these tRNAs [19]. The agreement of the values from HPLC analysis with those predicted from the sequence is excellent.

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