CODING PROPERTIES OF

tRNAs FROM *H. CUTIRUBRUM*

CODING PROPERTIES OF SOME tRNAs FROM *HALOBACTERIUM CUTIRUBRUM*

by

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SCOPE AND CONTENTS:

The assignment of codons in *Halobacterium cutirubrum* for some of the acidic and basic amino acids was undertaken. Although trinucleotides could not be used successfully in the halophile ribosomal binding system, the unfractionated aminoacyl-tRNAs responded to the established codons in the heterologous binding system using *E. coli* ribosomes. This suggested that *H. cutirubrum* uses the established code in full.

Two species of lysine accepting tRNA were purified. Their coding response to Poly(A) and Poly(A,G) was studied. $tRNA_1^{Lys}$ recognized both AAA and AAG codons while $tRNA_2^{Lys}$ recognized AAA preferentially in both the homologous and heterologous ribosomal binding systems.

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I. Introduction

The Genetic Code

The publication of the Adaptor Hypothesis (Crick, 1957) and the discovery of *in vitro* polyphenylalanine synthesis directed by the polyribonucleotide, poly (U) (Nirenberg and Mathaei, 1961) provided the basis for deciphering the genetic code. Crick's adaptor hypothesis (later confirmed) postulated the existence of transfer RNA. The results of Nirenberg and Mathaei established the concept of messenger RNA. With the use of random copolyribonucleotides in a cell-free protein synthesizing system, Nirenberg *et al.*, (1963) were able to assign fifty of the possible sixty four codons. However, the order of the bases in the codons could not be arranged by this method. The problem was resolved with the development of the ribosomal binding method (Leder and Nirenberg, 1964) which permitted the use of one trinucleotide at a time. This approach eliminated the possibility of doubtful assignments using random polyribonucleotides. Khorana and his co-workers chemically synthesized the sixty-four possible codons and used them in the ribosomal binding system to complete the codon assignments. By 1966 only the assignment of the termination codon UGA remained. Table 1 shows the genetic code as it is now accepted. It is clear that it is a triplet code and that it is degenerate - i.e., several different triplets

Table 1: THE GENETIC CODE

1ST LETTER	2ND LETTER U	2ND LETTER C	2ND LETTER A	2ND LETTER G	3RD LETTER
U	PHE	SER	TYR	CYS	U
	PHE	SER	TYR	CYS	C
	LEU	SER	ochre	umber	A
	LEU	SER	amber	TRP	G
C	LEU	PRO	HIS	ARG	U
	LEU	PRO	HIS	ARG	C
	LEU	PRO	GLUN	ARG	A
	LEU	PRO	GLUN	ARG	G
A	ILEU	THR	ASPN	SER	U
	ILEU	THR	ASPN	SER	C
	ILEU	THR	LYS	ARG	A
	MET	THR	LYS	ARG	G
G	VAL	ALA	ASP	GLY	U
	VAL	ALA	ASP	GLY	C
	VAL	ALA	GLU	GLY	A
	VAL	ALA	GLU	GLY	G

can code one amino acid. The latter will be discussed further in the following section.

Transfer RNA

Transfer RNA molecules, the 'adaptors' predicted by Crick (1957) occur in all living organisms. They serve to carry the correct amino acid to the site of protein synthesis on the ribosome. In order to perform this function each transfer RNA molecule is covalently bonded to a specific amino acid. This reaction is catalyzed by the cognate aminoacyl-tRNA synthetase. Three consecutive bases constituting the anticodon on the transfer RNA interact with a specific codon on the messenger RNA bound to ribosomes. The codon and the anticodon are base-paired in an antiparallel fashion. While codon-anticodon interactions use the standard base pairs (A=U and G≡C) in the first two positions from the 5'-end of the codon, Crick (1966) proposed that there may be some 'wobble' in the pairing of the third base. The possible base pairs allowed by the 'wobble' hypothesis are listed in Table . The wobble hypothesis was proposed to explain the degeneracy of the genetic code. Further, it postulates the minimum number of isoaccepting species of tRNA for amino acids. For instance, glycine is specified by four codons. Using the allowed wobble base pairs, it is possible to predict at least two isoaccepting species of tRNA^{Gly}. The possible anticodons may be depicted as :

Table 2: ALLOWED WOBBLE PAIRING

ANTICODON	CODON
U	A, G
C	G
A	U
G	U, C
I	U, C, A

GLY CODONS	POSSIBLE ANTICODONS		
GGU	CCG	OR	CCI
GGC			
GGA	CCU		
GGG			CCC

For an amino acid coded by, say, the triplets $XY\overset{U}{C}$, a tRNA with G in the first position of its anticodon would suffice in translating both codons in protein synthesis. However, examples of isoaccepting tRNAs for amino acids specified by two codons are available. The coding properties of some of these species are not consistent with the response predicted by the wobble hypothesis. Complete and partial nucleotide sequences of some tRNAs show the presence of modified nucleosides in the first position of the anticodon which account for restricted reading of degenerate codons.

In view of these facts, it is pertinent to ask what function(s) tRNAs subserve besides the 'adaptor' role in protein synthesis. tRNA has been implicated in the regulation of gene expression and in translational control. From chromatographic profiles, isoaccepting tRNAs vary in amount and number under different growth conditions, in different tissues, in normal and transformed cells, during differentiation and phage infection (Sueoka and Kano-Sueoka, 1970). Nishimura (1972) has suggested that 2-thiouridine derivatives in the first position of the anticodon prevent mispairing with U or C. His, Tyr, Asn and Asp tRNAs, having

the unknown nucleoside Q in the identical position, recognize codons ending with U more efficiently than those ending with C. Whether Q functions in a manner similar to 2-thiouridine derivatives is not known (Harada and Nishimura, 1972).

Although some modified nucleosides in or adjacent to the anticodon may be involved in ensuring fidelity of translation, this view might be too simplistic. The role of ribosomes in 'anticodon checking' of an aminoacyl-tRNA before insertion at the donor site has been proposed (Swan et al., 1969). The work from Gorini's laboratory on ribosomes from streptomycin resistant mutants strongly supports this concept.

The Halophilic System

It is evident that the genetic code is universal to bacteria, plants, amphibians and mammals (Marshall et al., 1967; Groves and Kempner, 1967; Beaudet and Caskey, 1967). It is known, however, that the fidelity of translation in a cell-free system can be influenced by external factors such as aminoglycoside antibiotics (Davies et al., 1964), genetic suppressors which alter components of the protein synthesizing machinery (Capechi and Gussin, 1965) and high ion concentrations (Szer and Ochoa, 1964; Nishimura et al., 1969). It is therefore pertinent to ask whether the genetic code and the protein synthesizing machinery have been significantly altered in the extremely halophilic bacteria.

Halobacterium cutirubrum, an obligate halophile which was studied here, requires almost saturated salt solutions for growth and has internal concentrations approaching saturation (Christian and Waltho, 1962). The interest in the genetic code in the halophiles stems from the fact that the enzymes and structural proteins studied to date have a higher content of acidic amino acid residues compared to similar proteins from non-halophilic bacteria. Bayley (1966) who studied the ribosomal proteins of H. cutirubrum suggested that the acidic nature of halophilic proteins may be the result of altered codon recognition. It is clear from Table 1 that many of the codons for acidic and basic amino acids differ only by the first base in the codon. Thus, misreading of the purine residues G and A would result in glutamic acid being inserted instead of lysine and aspartic acid instead of asparagine. Bayley and Griffiths (1968a, b) and White and Bayley (1972b) investigated the fidelity of translation and the genetic code in H. cutirubrum using a cell-free protein synthesizing system or the ribosomal binding technique with random copolyribonucleotides. The twenty-eight codons assigned by these authors, all agreed with the established code. Not all the acidic and basic amino acids were investigated and the results only partially ruled out the possibility of altered codon recognition. This work showed, however, that the general features for the protein synthesis system was identical to those from non-halophiles in that it required PEP, ATP, GTP, ribosomes, transferases, aminoacyl-

tRNA synthetases, tRNA and messenger RNA. The halophile system differed in requiring nearly saturated salt (3.8 M KCl, 1 M NaCl and 0.4 M NH_4Cl) for optimal activity. White and Bayley (1972 a, b) examined the tRNA from H. cutirubrum. Some tRNAs were recognized by E. coli aminoacyl tRNA synthetases and many could be used in the E. coli ribosomal binding system. The results indicated that halophile tRNAs were not fundamentally different from tRNAs of other organisms. Ribosomal RNAs from H. cutirubrum are similar in size and composition to those of E. coli (Visentin et al., 1972). It is not possible to demonstrate the presence of isoaccepting species of tRNAs as well as restricted recognition of degenerate codons without fractionating the crude tRNA preparations. Since tRNA has been implicated in modulating several biological processes it was pertinent to ask whether isoaccepting species of halophile tRNA exist beside the two $tRNA^{Met}$ species (White and Bayley, 1972c) and two of $tRNA^{Phe}$ (Griffiths, 1970).

Thus, the objective of this study was to continue the work on codon assignments for the acidic and basic amino acids in order to test the hypothesis proposed by Bayley (1966). The work also entailed the purification of $tRNA^{Lys}$ and an examination of coding properties since purified tRNAs may also be used in examining the genetic code. It was hoped that such a study would provide a better understanding of how the halophilic protein synthesizing machinery was modified in adapting to the extreme ionic environment.

II. Materials and Methods

METHODS

SECTION I. H. cutirubrum

a) Growth of Bacteria

H. cutirubrum strain 9 was grown in the complex medium of Sehgal and Gibbons (1960) with the exception that 10 ppm of Fe^{2+} in the form of ferrous sulfate was added and the final pH of the medium was adjusted to 6.2. For the preparation of tRNA, cells were harvested in late log phase after 36 hours. For all other preparations, the cells were harvested in early log phase after only 16-18 hours (Figure 1). In both cases, cells were harvested and washed as described by Bayley (1972). If necessary, they were stored at -60°C until required.

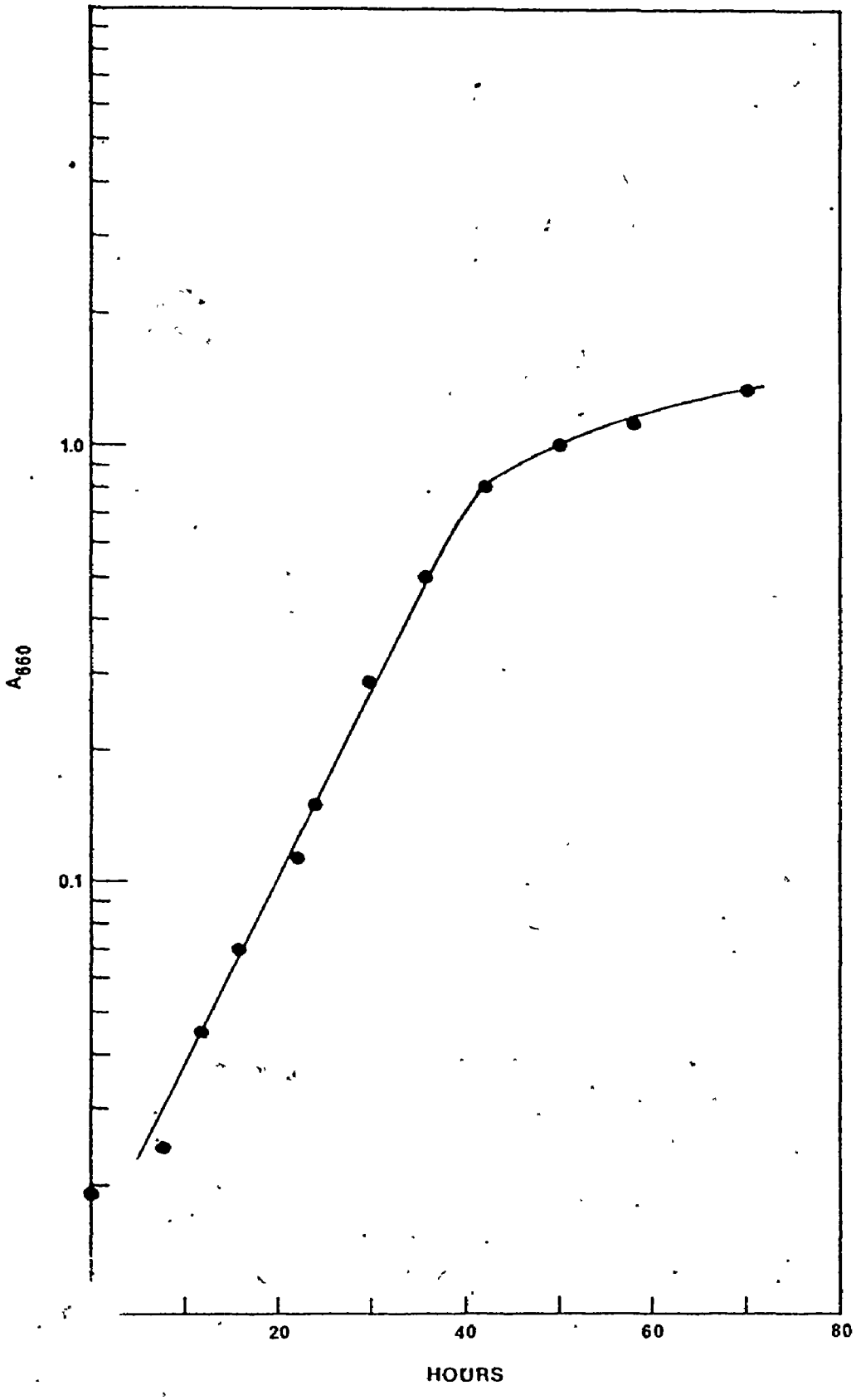
b) Preparation of Biological Extracts (All steps at 0-4°C)

i) Preparation of S-150

Cells were suspended in a volume of solution D' (3.0M potassium chloride, 0.1M magnesium acetate, 0.01M Tris-hydrochloride (pH 7.6) and 0.008M 2-mercaptroethanol) equal to the wet weight of cells together with 1 mg of electrophoretically purified DNase per 30 ml solution D'. The cells were then homogenized, either in a glass Teflon-Potter-Elvenhjem homogenizer or in a Sorval omnimixer at top speed for 5-10 seconds. The homogenate was centrifuged

Figure 1: GROWTH OF H. cutirubrum IN COMPLEX MEDIUM

12 l of medium were inoculated with 600 ml of a 24-hour culture. Turbidity was read in a Bausch and Lomb Spectronic 20 colorimeter at 660 nm.


1.0
0.1
A_{660}
20
40
60
80
HOURS

once at 40,000 x g in a Sorval superspeed centrifuge. The supernatant was retained and centrifuged once at 150,000 x g. The resultant supernatant was dialyzed against a large excess of solution D' for 4-6 hours. This constituted the S-150 supernatant. The S-150 preparation was frozen in liquid nitrogen and stored in liquid nitrogen for up to two months or at -60°C for shorter periods.

ii) "pH5" Synthetases

The pH of an S-150 extract was adjusted to 5 by adding 1 M acetic acid with stirring at 0°C (Griffiths and Bayley, 1969). Stirring was continued for an additional 20 min. The resulting precipitate was collected by centrifugation, resuspended in solution D' and dialyzed against a large excess of the same for at least 4 hours. The preparation was frozen and stored as described above.

iii) Synthetases Free of Endogenous tRNA

S-150 supernatant was passed through a column packed with Sephadex G50 (fine). The gel was swollen in solution D' and elution was with the same buffer. Details of column procedures appear in Section V.

The peak fractions containing synthetase activity were pooled and the synthetases were concentrated by dialyzing against a 100% saturated ammonium sulfate solution. The precipitated proteins were collected by centrifugation, resuspended in a small volume of solution D' and dialyzed

against the same for 4-6 hours. The preparation was frozen and stored as described above.

iv) Preparation of Pre-incubated Ribosomes

The method used was that of Bayley and Griffiths (1968a). Washed early log phase cells were homogenized as described earlier. The homogenate was centrifuged at 40,000 x g for 20 min at 0-4°C. The supernatant was centrifuged at 60,000 x g for 30 min. The resulting supernatant (S-60) was retained and incubated at 37°C for 40 min in the reaction mixture described in Table 3 (Bayley and Griffiths, 1969). After 40 min the reaction mixture was chilled on ice so that excess salt crystallized out. The mixture was then centrifuged at 150,000 x g for 2.5 hours. The ribosomal pellet was suspended in solution D' and centrifuged at 150,000 x g for 2.5 hours. The resulting ribosomal pellet was resuspended in a volume of solution D' corresponding to 1/4 - 1/6 the volume of the S-60 supernatant. Finally the ribosomal suspension was clarified by centrifugation at 2000 rpm for 10 mins. The preparation was frozen and stored as described above.

v) tRNA

The method described below is adapted from von Ehrenstein (1967) and is for processing 100 g of cells. Frozen late log phase cells were thawed and suspended in 1 l of 0.1 M NaCl, 0.01 M $MgCl_2$, 0.01 M Tris-HCl (pH 7.5)

Table 3. REACTION MIXTURE FOR THE BULK INCUBATION OF S-60 EXTRACTS IN THE PREPARATION OF PRE-INCUBATED RIBOSOMES.

0.03 M NaATP	0.25 ml
0.12 M NaPEP	0.25 ml
0.012 M LiGTP	0.375 ml
1.0 M Tris-HCl, pH 8.0	0.125 ml
5.0 M NH_4Cl; 0.6 M $(NH_4)_2 SO_4$	0.75 ml
Solid KCl.	0.61 mg
S-60 Extract in D'	2.00 ml
Total Nominal Volume	3.750 ml

Incubating Temperature: 37°C

Duration of Incubation: 40 min.

NOMINAL FINAL SALT CONCENTRATIONS

KCl	3.8 M
NH_4^+	1.2 M
Mg^{2+}	0.05 M

and 0.001 M 2-mercaptoethanol. An equal volume of liquefied phenol was added and the suspension homogenized in a Waring blendor for 1 min. The mixture was allowed to stand for 5 min and then homogenized for an additional 1 min. The aqueous and phenol phases were separated by centrifugation at 2000 rpm for 20 min in a refrigerated International centrifuge. The upper aqueous phase was retained and the lower phenol phase was re-extracted with 500 ml of the 0.1 M NaCl buffer. The aqueous phases from the first and second extractions were pooled. Nucleic acids were precipitated by adding 0.1 vol. of 20% potassium acetate (pH 5.0) followed by 2 vol. of 95% ethanol. The precipitate was allowed to settle overnight at -20°C.

The precipitated nucleic acids were collected by centrifugation. The supernatant was discarded and the precipitate was drained of ethanol as much as possible and then dissolved in approximately 400 ml of 1 M sodium acetate (pH 7.0). This solution was stirred vigorously in the cold-room and 0.54 vol. of 2-propanol was added dropwise from a separatory funnel. Once the 2-propanol had been added, the temperature of the suspension was raised to 20°C, vigorous stirring being maintained during this operation. The suspension was centrifuged at 20°C for 20 min at 2000 rpm. The supernatant was retained and the precipitate was redissolved in 1 M sodium acetate (pH 7.0) and re-extracted with 2-propanol as described

above. The supernatant fractions of the first and second extractions were combined and tRNA was precipitated from them by adding 2-propanol to a final volume of 0.98. The precipitated tRNA was collected by centrifugation. The pellet was drained, dissolved in a minimal volume of 1 M Tris-HCl (pH 9.0) (Roy, Bloom and Söll, 1971) and incubated at 37°C for 2 hours in order to deacylate any aminoacyl-tRNA which might be present. One-tenth volume 20% potassium acetate (pH 5.0) was then added followed by 2 vol. of 95% ethanol and the preparation left at -20°C for 12 hours.

The tRNA precipitate was collected by centrifugation, washed successively with ice-cold absolute ethanol, ethanol:ether (1:1 v/v) and anhydrous ether and vacuum dried. Finally, the unfractionated tRNA was passed through a DEAE-cellulose column as described in Section V.

SECTION II. *E. coli*

Preparation of Biological Extracts

i) S-30

S-30 extracts were prepared from *E. coli* W (early log phase) cells as described by Nirenberg (1964).

ii) S-150

The S-30 extract was centrifuged at 150,000 x g for 2.5 hours at 0-4°C. The supernatant was dialyzed against a large excess of BRS at 4°C for at least 4 hours.

This supernatant constituted the S-150 extract. It was frozen and stored as described earlier.

The ribosomal pellet resulting from the S-150 preparation was used for preparing high salt washed ribosomes (see below).

iii) High Salt Washed Ribosomes

Ammonium chloride washed ribosomes were prepared as described by Lucas-Lenard and Lipmann (1966).

SECTION III. In Vitro Assay Methods

a) H. cutirubrum

i) tRNA Charging System

The charging system was based on that described by Griffiths and Bayley (1969). The reaction mixture shown in Table 4 was used for charging unfractionated tRNAs. The reaction mixture shown in Table 5 was used for determining the position of tRNA species in the elution profile after reversed phase column chromatography. The reaction mixture shown in Table 6 was used for determining the acceptor activity of the purified tRNA species after they were pooled and precipitated. The main difference between the mixtures in Tables 4 & 5 is in the concentration of lysine.

In all assay systems, the reaction was terminated by adding 2 ml of cold unlabelled lysine (1 mg/ml), 0.1 ml bovine serum albumin (5 mg/ml) and 2 ml of trichloroacetic acid (TCA) at 0°C. The precipitate was washed twice with

centrifugation in 5% TCA and finally filtered onto a Millipore filter. The filter was washed with 4 ml 5% TCA, dried and counted for radioactivity in a Beckman Liquid Scintillation counter using Omnifluor (4 g/l toluene) as the cocktail.

ii) Preparation of Aminoacyl-tRNA

^{14}C-aminoacyl-tRNA for ribosomal binding assays was prepared by charging unfractionated tRNA with one ^{14}C-amino acid and 19 unlabelled amino acids in an incubation mixture scaled up proportionally from the one shown in Table 4. After incubating at 37°C for 20 min, the reaction was terminated by the addition of 0.1 vol potassium acetate (20% w/v, pH 4.5). The mixture was dialyzed for 1 hour against 0.005 M potassium acetate (pH 4.5) and 0.001 M 2-mercaptoethanol. Precipitated proteins were removed by centrifugation. The supernatant was phenol extracted in the normal way followed by precipitation of the aminoacyl-tRNA with potassium acetate (20% w/v, pH 4.5) and 2 vol. of ice-cold ethanol. The aminoacyl-tRNA was freed of amino acids by repeated precipitation with 2 vol. ethanol after dissolution in 1 M potassium acetate (pH 4.5). The final precipitate was dissolved in 0.005 M KAc (pH 4.5) and dialyzed against the same for 2 hours. Finally the aminoacyl-tRNA solution was frozen and stored at -20°C.

Table 4: COMPOSITION OF THE *H. cutirubrum* tRNA CHARGING SYSTEM.

5.0 M NaCl	0.062 ml
Solid KCl	43.6 mg
Cold amino acid mixture less labelled amino acid (each 8×10^{-4} M)	0.010 ml
^{14}C-Amino acid	0.010 ml
0.03 M NaATP	0.010 ml
0.29 M Tris-HCl (pH 8.0)	0.020 ml
tRNA in solution D'	0.070 ml
Aminoacyl-tRNA synthetases	0.020 ml
Water	0.023 ml
Total Nominal Volume	0.225 ml

Incubation Temperature: 37°C

Duration of Incubation: 20 min.

NOMINAL FINAL SALT CONCENTRATIONS

Magnesium acetate	0.04 M
KCl	3.8 M
Tris-HCl (pH 8.0)	0.03 M
NaCl	1.4 M

AMOUNTS OF OTHER COMPONENTS

NaATP	0.3 µmole
19 Cold amino acids	0.008 µmole of each
^{14}C-Amino acid	3.3-3.6 nmoles
Synthetases	0.2-0.4 mg protein
tRNA	1-5 A_{260} units

^{14}C-lysyl-tRNA for BD-cellulose column chromatography was prepared in the same way except that a reaction mixture increased proportionally from that described in Table 6 was used and the final aminoacyl-tRNA precipitate was dissolved in the starting buffer.

Aminoacylation of pure species of $tRNA^{Lys}$ for ribosomal binding assays was achieved by using a reaction mixture similar to the one described in Table 6 except that it was necessary to ensure that the specific activity of the labelled amino acid was 150 mCi/mmole or higher. Purified lysyl-tRNA synthetase was used. The reaction was terminated by addition of 0.1 vol 0.005 M potassium acetate (pH 4.5). The mixture was phenol extracted. The aqueous phase was retained and 2 vol of -20°C ethanol were added and stood at -20°C for 2 hours. If a precipitate was not visible, the ^{14}C-aminoacyl-tRNA was collected onto a washed HA type Millipore filter (0.45 μ) and the aminoacyl-tRNA eluted from the filter in 0.5 ml 0.005 M potassium acetate (pH 4.5) with shaking at 0°C for 30-45 min (Shuggart and Novelli, 1971). If a precipitate was visible, it was collected by centrifugation. The precipitate was drained of ethanol as much as possible and dissolved in a small volume of 0.005 M potassium acetate (pH 4.5). The solution was dialyzed against the same buffer for 2-3 hours at 0°C. In order to minimize losses of the ^{14}C-aminoacyl-tRNA, a piece of

Table 5: COMPOSITION OF REACTION MIXTURE FOR DETERMINING THE ELUTION POSITION OF $tRNA^{Lys}$ AFTER RPC-5 CHROMATOGRAPHY.

5.0 M NaCl	0.062 ml
Solid KCl	43.6 mg
0.29 M Tris-HCl (pH 8.0)	0.020 ml
0.03 M NaATP	0.023 ml
^{14}C-Lysine	0.005 ml
tRNA in eluting buffer	0.025 ml
Solution D'	0.070 ml
Aminoacyl-tRNA synthetases	0.020 ml
Total Nominal Volume	0.225 ml

Incubation Temperature: 37°C

Duration of Incubation: 12 min.

Table 6: COMPOSITION OF THE REACTION MIXTURE FOR DETERMINING THE SPECIFIC ACTIVITY OF $tRNA^{Lys}$ SPECIES.

5.0 M NaCl	0.062 ml
Solid KCl	43.6 mg
0.29 M Tris-HCl (pH 8.0)	0.020 ml
0.1 M NaATP	0.023 ml
tRNA in D'	0.020 ml
^{14}C-Lysine	0.030 ml
Aminoacyl-tRNA synthetases	0.020 ml
Solution D'	0.050 ml
	0.225 ml

Incubation Mixture: 37°C

Duration of Incubation: 12 min.

NOTE: When partially purified lysyl-tRNA synthetase was used 60 µl of the enzyme in solution D' was added and the reaction was allowed to proceed for 30 min.

NOMINAL FINAL CONCENTRATION OF SALTS

KCl	3.8 M
Magnesium acetate	0.04 M
Tris-HCl (pH 8.0)	0.03 M
NaCl	1.4 M

AMOUNTS OF OTHER COMPONENTS

NaATP	2.3 µmoles
^{14}C-lysine	66-69 nmoles
Synthetases	0.05-0.5 mg
tRNA (purified)	0.01-0.05 A_{260} units
tRNA (unfractionated)	7 A_{260} units

dialysis tubing, previously boiled in 10 mM EDTA, was slipped over a piece of tightly-fitting, siliconized glass tubing. The ends of the glass tubing had been fire-polished. A double knot was tied in the dialysis tubing a short distance from one end of the glass. The other end of the dialysis tubing was sealed around the glass tubing with an elastic band. Through the open end of the apparatus the ^{14}C-lysyl-tRNA species was introduced and the closed end submerged in a beaker of the potassium acetate buffer. By this method more than 90% of the sample was retrieved.

iii) Ribosomal Binding Assay

The assay developed by White and Bayley (1972b) was used. The incubation mixture is described in Table 7. The reaction was terminated by adding 4 ml of cold solution D' and filtering the mixture through a Millipore filter (0.45 μ pore diameter) previously washed with 4 ml solution D'. The filter was washed with another 4 ml of solution D', dried and counted for radioactivity as described earlier. Since the filter was treated with 3.0 M potassium chloride, it was necessary to check routinely for quenching using appropriate standards.

b) E. coli

i) tRNA Charging System

The charging system was modified from the one described by Marshall et al., (1967). The composition

Table 7: COMPOSITON OF THE REACTION MIXTURE FOR THE *H. cutirubrum* RIBOSOMAL BINDING SYSTEM.

Solid KCl	24.6 mg
3.4 M KCl; 0.12 Magnesium acetate 0.15 M Tris-HCl (pH 8.0)	0.025 ml
Ribonucleotide template in H_2O	0.010 ml
^{14}C-Aminoacyl-tRNA in 0.005 M potassium acetate (pH 4.5)	0.015 ml
Ribosomes in solution D'	0.020 ml
1 M Magnesium acetate	0.006 ml
Water	0.049 ml
Total Nominal Volume	0.125 ml

Incubation Temperature: 37°C

Duration of Incubation: 30 min.

NOMINAL FINAL CONCENTRATIONS OF SALTS

Magnesium acetate	0.088 M
KCl	3.8 M
Tris-HCl (pH 8.0)	0.03 M

AMOUNTS OF OTHER COMPONENTS

^{14}C-Aminoacyl-tRNA	8-35 pmoles
Ribosomes	2-4 A_{260} unit
Ribonucleotide template	
a) Polyribonucleotide	25-75 µg
b) Trinucleotides	0.4-1.2 A_{260} units

of the reaction mixture is shown in Table 8. The ^{14}C-aminoacyl-tRNA was determined by the cold TCA method described for the H. cutirubrum system.

ii) Ribosomal Binding Assay

The nitrocellulose filter assay described by Nirenberg and Leder (1964) was used. The composition of the reaction mixture is described in Table 9. The labelled aminoacyl-tRNA was added last to the incubation mixture. The reaction was terminated by addition of 4 ml of 0.05 M KCl, 0.02 M magnesium acetate and 0.02 M Tris-HCl (pH 7.2) at 0°C. The mixture was filtered through a Millipore filter previously washed with 4 ml of the 0.05 M KCl buffer. The filter was then washed with 4 ml of the same buffer, dried and counted as described earlier.

SECTION IV. RIBONUCLEOTIDE TEMPLATES

a) Enzymatic Synthesis of Trinucleotides

Trinucleotides were synthesized enzymatically by the procedure described by Thach and Doty (1965). The reaction mixture described in Table 10 was used. Under these conditions, the products were tri-, tetra- and pentanucleotides which were separated by anion exchange chromatography described in Section V. When guanosine was the 3'-terminal nucleoside, the yield of the trinucleotide

Table 8: COMPOSITION OF THE REACTION MIXTURE FOR THE *E. coli* CHARGING SYSTEM.

1 M Tris-HCl (pH 7.5)	0.005 ml
0.5 M Magnesium chloride	0.005 ml
0.1 M NaATP	0.020 ml
0.05 M NaPEP	0.030 ml
Pyruvate Kinase (1 mg/ml H_2O)	0.010 ml
^{14}C-Aminoacid	0.030 ml
Synthetases in BRS (S-150)	0.020 ml
tRNA in H_2O	0.100 ml
Water	0.030 ml
Total Volume	0.250 ml

Incubation Temperature: 37°C

Duration of Incubation: 20 min.

FINAL CONCENTRATION OF SALTS

Magnesium chloride	0.02 M
Tris-HCl (pH 7.5)	0.01 M

AMOUNTS OF OTHER COMPONENTS

NaATP	2.0 µmole
^{14}C-Amino acid	66-69 nmoles
Pyruvate Kinase	0.001 mg
Synthetases	0.2-0.5 mg protein
tRNA (unfractionated)	1-5 A_{260} units
tRNA (purified)	0.01-0.05 A_{260} units

Table 9: COMPOSITION OF REACTION MIXTURE FOR THE *E. coli* BINDING SYSTEM.

0.2 M Tris-HCl (pH 7.2); 0.5 M KCl	0.005 ml
0.2 M Magnesium acetate	0.005 ml
Ribonucleotide template in H_2O	0.005 ml
^{14}C-Aminoacyl-tRNA in 0.005 M KAc(pH 4.5)	0.015 ml
Ribosomes in BRS	0.010 ml
Water	0.010 ml
Total Volume	0.050 ml

Incubation Temperature: 24°C

Duration of Incubation: 20 min.

FINAL CONCENTRATION OF SALTS

Magnesium acetate	0.02 M
KCl	0.05 M
Tris-HCl (pH 7.2)	0.02 M

AMOUNTS OF OTHER COMPONENTS

Ribosomes	2-3 A_{260} units
Aminoacyl-tRNA	8-30 pmoles
Ribonucleotide template:	
a) Polyribonucleotide	25-75 mg
b) Trinucleotide	0.4-1.2 A_{260} units

Table 10: COMPOSITION OF REACTION MIXTURE FOR SYNTHESIS OF TRINUCLEOTIDES.

2.0 M Tris-HCl (pH 8.8)	0.050 ml
0.1 M Magnesium chloride	0.100 ml
0.1 M Nucleoside-5'-diphosphate (NDP)	0.150 ml
Dinucleoside phosphate (XpY) (20 mg/ml)	0.250 ml
5 M NaCl	0.080 ml
Polynucleotide phosphorylase	0.100 ml
[T_1 RNase] or Water	0.050 ml
Water	0.220 ml
Total Volume	1.0 ml

Incubation Temperature: 37°C

Duration of Incubation: 24 hours

was increased by including 50 units of T_1 RNase in the incubation mixture. T_1 RNase degradation product gave trinucleoside triphosphates and the 3'-terminal phosphate was removed by treating the incubation mixture with 10 units of alkaline phosphatase for 1 hour at the end of the 24-hour incubation period.

b) Chemical Modification of Polyribonucleotides

Partial hydrolysis of poly(U), poly(A) and poly(A:G) was achieved by the method of Bock (1967).

SECTION V. CHROMATOGRAPHIC AND PURIFICATION PROCEDURES

a) Diethylaminoethyl-cellulose (DEAE-Cellulose) Chromatography of Crude tRNA

Crude tRNA, prepared as described in Section I, was purified further by the method of Kelmers et al., (1965). Crude tRNA was dissolved in 0.1 M NaCl, 0.01 M $MgCl_2$, 0.01 M Tris-HCl (pH 7.5) with 0.005 M 2-mercaptoethanol and charged onto a 2.5 x 45 cm glass column packed with DEAE-cellulose equilibrated in the same buffer. The column was washed at a flow rate of 36 ml per hour at 4°C with about 100 ml of the 0.1 M NaCl buffer. Subsequently elution was with a buffer containing 0.3 M NaCl plus the other constituents described. When the absorbance of the effluent was approximately 10% of the peak fraction, the eluting buffer was changed to 1.0 M NaCl plus the other constituents. The material eluting with this buffer was

taken as the tRNA fraction. The tRNA was precipitated with 2 vol. of ethanol and stood overnight at -20°C. The tRNA precipitate was collected by centrifugation and washed successively with ethanol, ethanol:ether (1:1 v/v), anhydrous ether and finally vacuum dried.

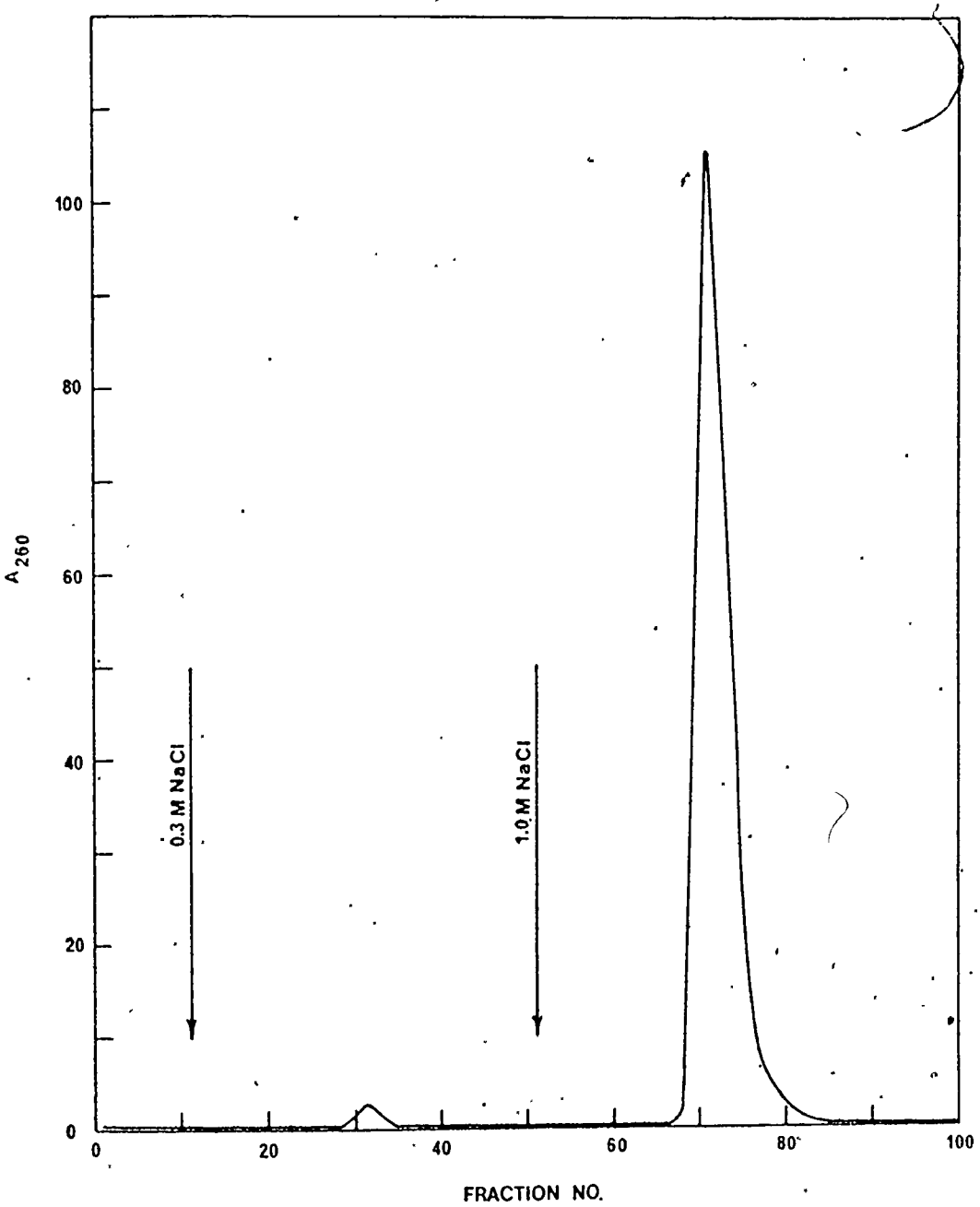
A typical chromatographic profile is shown in Figure 2.

b) Benzoylated Diethylaminoethyl-cellulose (BD-Cellulose) Column Chromatography

The method used for preparing the column was that of Gillam et al., (1968). Tighter packing of the bed was achieved by firmly stroking the sides of the column with a pad of tissue paper moistened with alcohol (Gillam and Tener, 1971). The column was equilibrated in a buffer containing 0.45 M NaCl, 0.01 M $MgCl_2$, 0.45 M sodium acetate (pH 4.5) and 0.005 M 2-mercaptoethanol. ^{14}C-lysyl-tRNA was dissolved in the same buffer and applied to the column at a flow rate of 36 ml per hour at 4°C. Elution was stepwise or with a linear gradient followed by a purge with ethanolic salt. Details of elution conditions are given with the chromatographic profiles. Appropriate fractions were pooled and precipitated with 2 vol. cold ethanol as described earlier. $tRNA^{Lys}$ for reversed phase chromatography was discharged by dissolving the tRNA precipitate in 1 M Tris-HCl (pH 9.0) and incubating at 37°C until no TCA precipitable radioactivity was evident (Roy, Bloom and Söll, 1971).

Figure 2: PARTIAL PURIFICATION OF CRUDE tRNA ON DEAE-CELLULOSE

A 2.5 cm x 45 cm glass column was packed with DEAE-cellulose and equilibrated in 0.1 M NaCl, 0.01 M $MgCl_2$, 0.01 M Tris-HCl (pH 7.5) and 0.005 M 2-mercaptoethanol. 4920 A_{260} units of H. cutirubrum tRNA in 30 ml of the same buffer were applied to the column and washed with 0.1 M NaCl buffer at a flow rate of 36 ml/hour with 10 ml fractions being collected. Elution was stepwise with 0.3 M NaCl buffer and 1.0 M NaCl buffer.


100
80
60
40
20
0
A_{260}
0.3 M NaCl
1.0 M NaCl
0
20
40
60
80
100
FRACTION NO.

Crude uncharged tRNA in the 0.45 M NaCl buffer was chromatographed on a BD-cellulose column in the manner described. Lysine acceptor activity in the salt fractions was detected using the reaction mixture given in Table 5. The presence of lysine tRNA in the ethanol fractions was determined in the same way except that 1 ml fractions were previously dialyzed against the 1.2 M NaCl buffer to remove the ethanol.

c) Reversed Phase Chromatography V (RPC-5)

The preparation of the packing for reversed phase chromatography was as described by Pearson et al., (1971) except that the preparation was scaled down proportionally. 30 grams of Plaskon CFTE 2300 powder was placed in an Erlenmyer flask to which was added 1.2 ml Adogen 464, a trialkylmethylammonium chloride, dissolved in chloroform (total vol., 60 ml). The flask was agitated at top speed in a New Brunswick incubator-shaker at ambient temperature for 2-3 hours. The slurry of plaskon in chloroform was dried in a glass tray with continuous stirring with a ceramic spatula. Just prior to reaching dryness, the mixture was tumbled mechanically for 2 hours. The packing was returned to a glass tray to remove the remaining chloroform.

The dry RPC-5 packing was slurried with starting buffer consisting of 0.45 M NaCl, 0.01 M $MgCl_2$, 0.01 M Tris-HCl (pH 7.0) and 0.001 M 2-mercaptoethanol. The slurry was shaken vigorously for 1 hour in a New Brunswick incubator shaker at ambient temperature (Pearson et al., 1973).

A jacketed glass column (either 1 cm x 100 cm or 0.6 cm x 70 cm) maintained at 37°C was partly filled with the 0.45 M NaCl buffer described above. The slurry was poured into the column and compacted under maximum aqueous flow with a Milton Roy Minipump. Before each chromatographic run, the column was equilibrated with several column volumes of the starting buffer. The tRNA sample dissolved in 5-10 ml of the equilibrating buffer was applied to the column and a linear gradient was generated at a flow rate of 36 ml per hour. The absorbance at 260 nm was monitored and the elution position of $tRNA^{Lys}$ species was determined as described in section IIIa. Appropriate fractions containing the tRNA of interest were pooled and precipitated with 2 vol of ethanol at -20°C. If a precipitate was not visible, the tRNA was recovered on an HA type Millipore filter (Shuggart and Novelli, 1971).

d) DEAE-Cellulose Chromatography of Trinucleotides

A protocol for the isolation of trinucleotides was obtained from Dr. Clelia Ganoza, Banting and Best Institute. Whatman DEAE-Cellulose DE 32 (Microgranular) was suspended in water and defined. The DEAE-Cellulose was slurried in a small volume of water and poured into a 1.5 cm x 30 cm glass column at 4°C. Subsequently, the column was washed with 0.5 M Tris-HCl (pH 8.1) until the pH of the effluent was the same as that of the buffer in

Figure 3: ISOLATION OF CpApU BY DEAE-CELLULOSE (MICROGRANULAR) CHROMATOGRAPHY

A 1.5 cm x 30 cm glass column was packed with DEAE-cellulose in water and washed successively with 0.5 M Tris-HCl (pH 8.1) and water. The sample was applied to the column in 50 ml water or at a flow rate of 12.5 ml per hour. Elution was with a linear gradient consisting of 250 ml water in the mixing chamber and 250 ml 0.5 M ammonium carbonate (pH 8.6) in the reservoir. 2.5 ml fractions were collected.

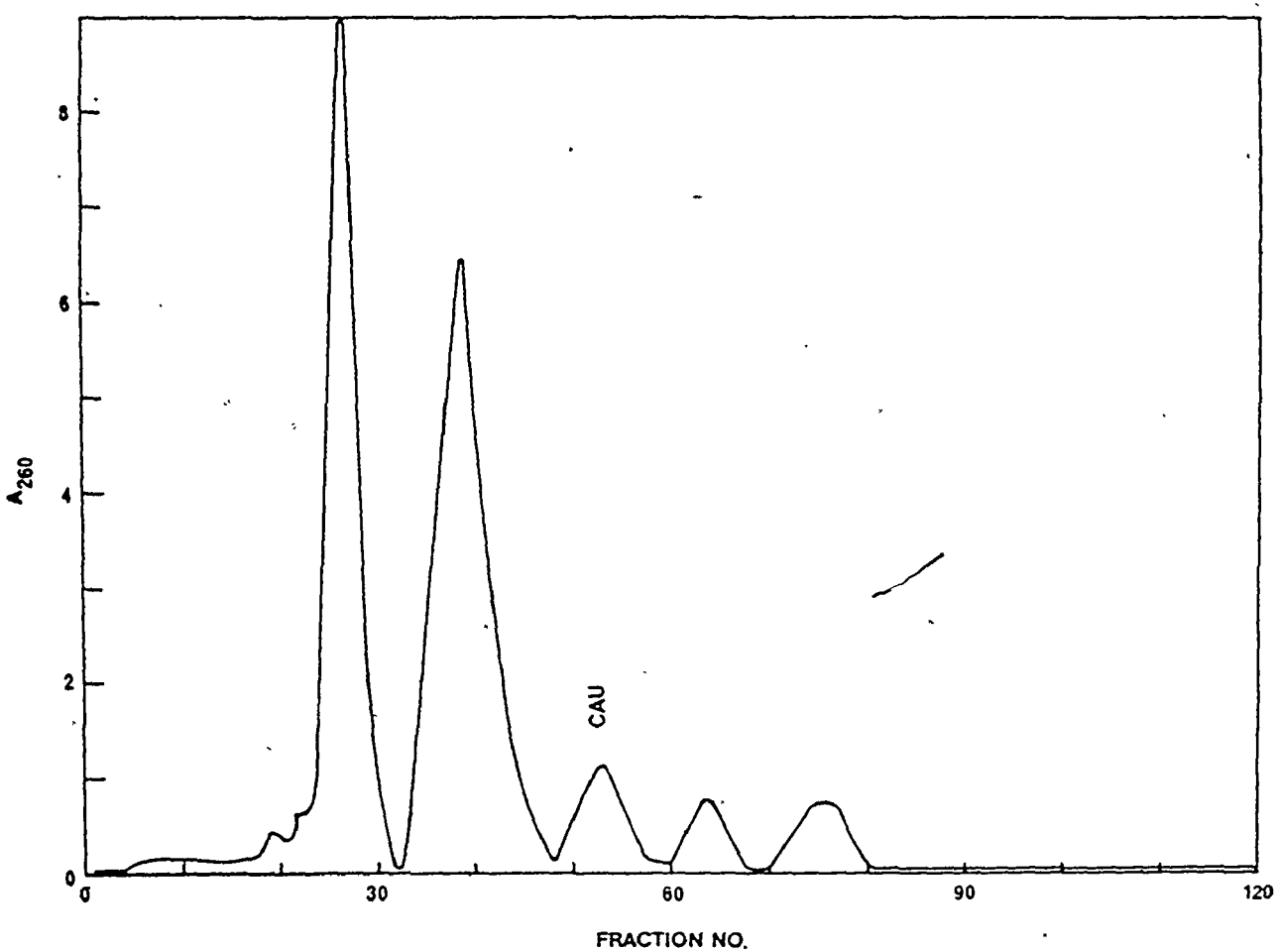

8
6
4
2
0
A_{260}
CAU
0
30
60
90
120
FRACTION NO.

Figure 4: ISOLATION OF ApApU BY DEAE-CELLULOSE (MICROGRANULAR) CHROMATOGRAPHY

The conditions of chromatography were as for Figure 3.

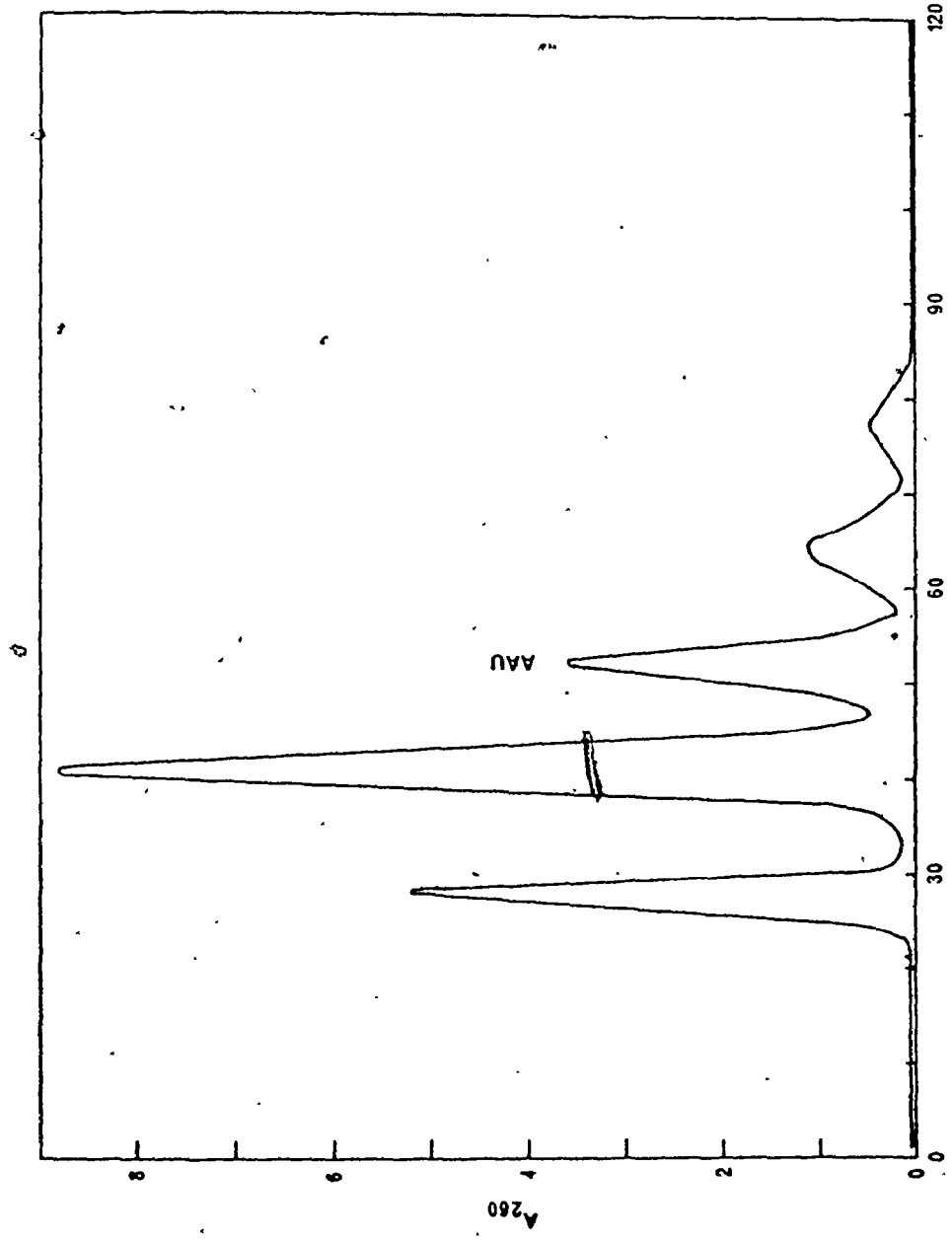

A_{260}
0
2
4
6
8
AAU
30
60
90
120
FRACTION NO.

Figure 5: ISOLATION OF ApApG BY DEAE-CELLULOSE (MICROGRANULAR) CHROMATOGRAPHY

The conditions of chromatography were as for Figure 3.

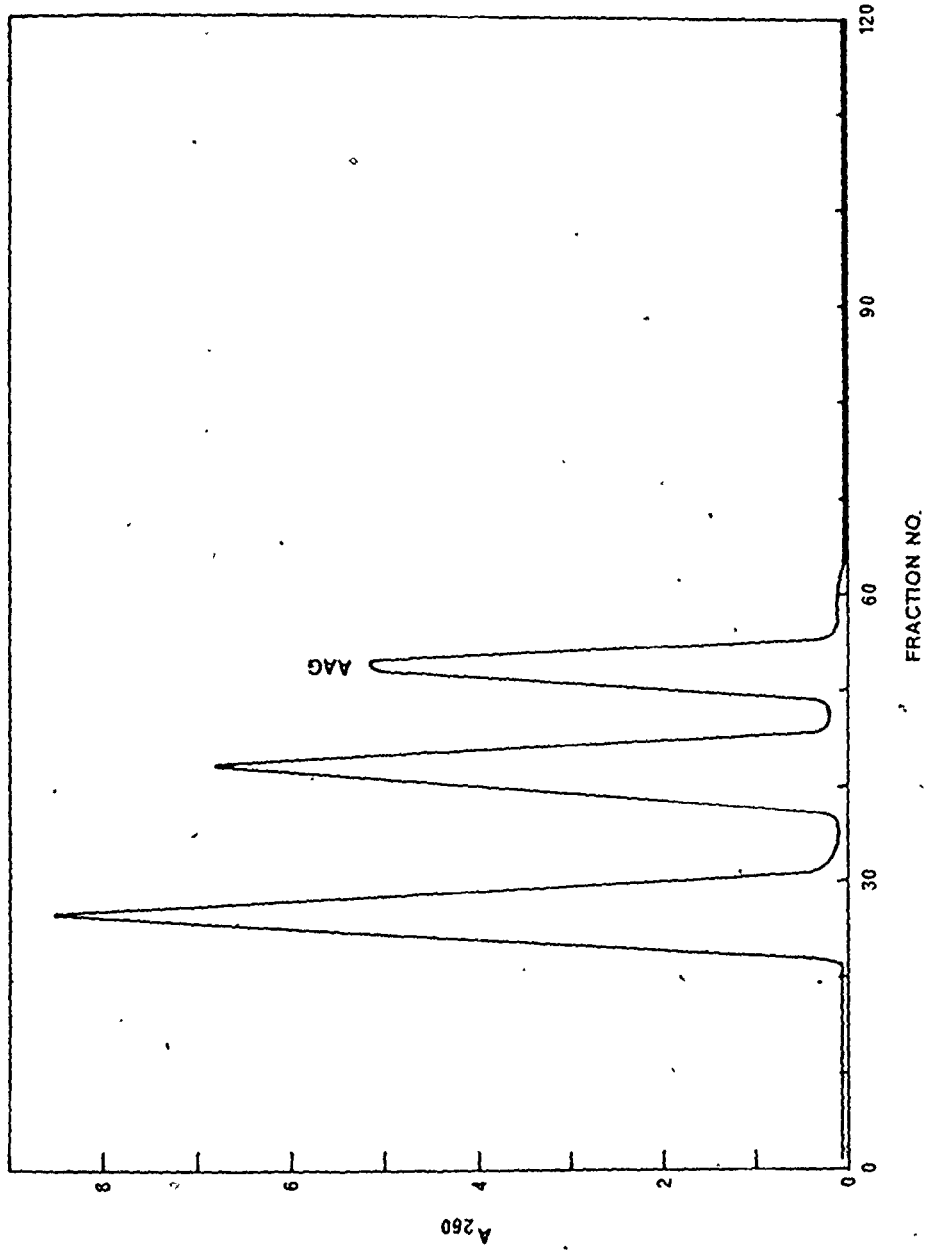

AAG
A_{260}
0
2
4
6
8
0
30
60
90
120
FRACTION NO.

Figure 6: ISOLATION OF GpApU BY DEAE-CELLULOSE (MICROGRANULAR) CHROMATOGRAPHY

The conditions of chromatography were as for Figure 3.

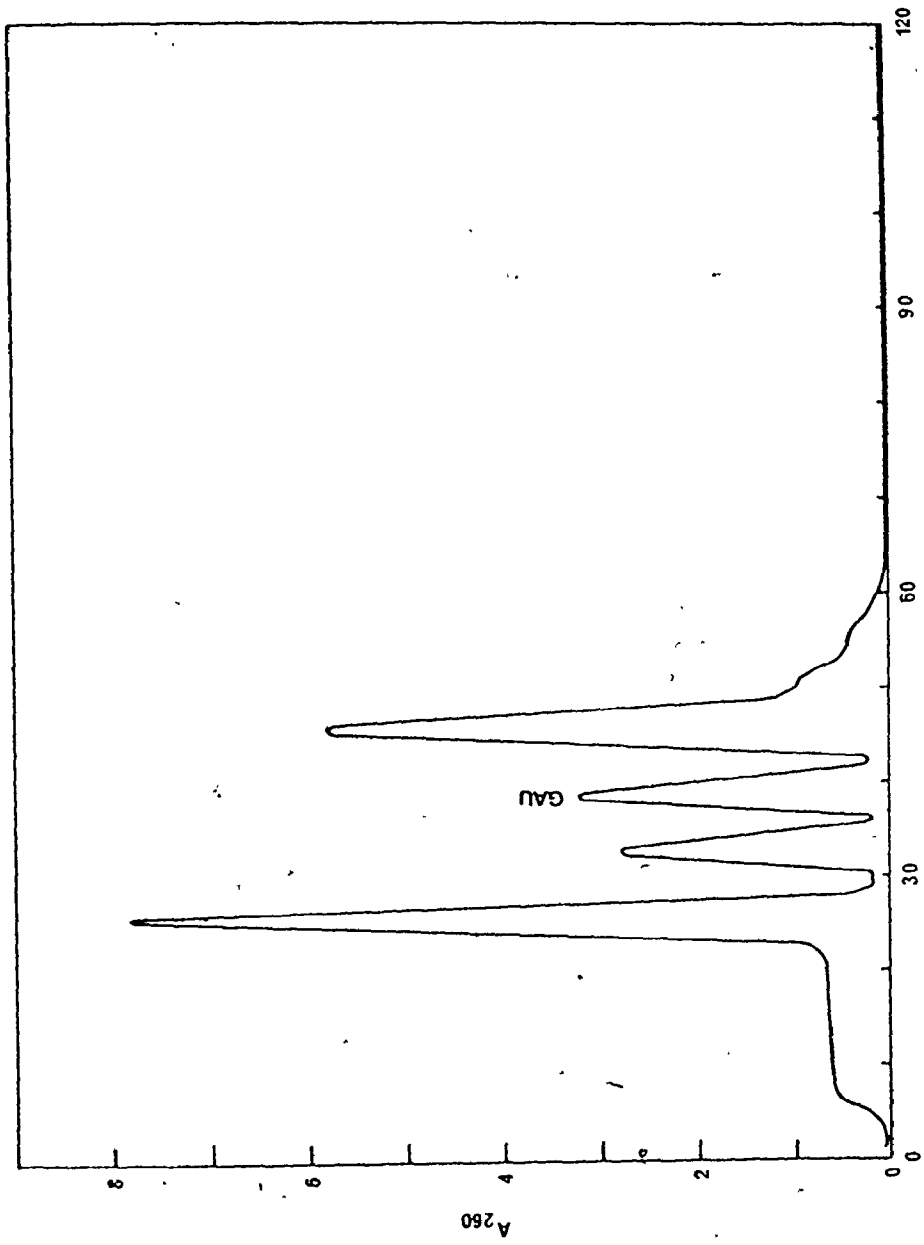

A260
0
2
4
6
8
30
60
90
120
FRACTION NO.
GAU

Figure 7: ISOLATION OF GpApG BY DEAE-CELLULOSE (MICROGRANULAR) CHROMATOGRAPHY

The conditions of chromatography were as in Figure 3.

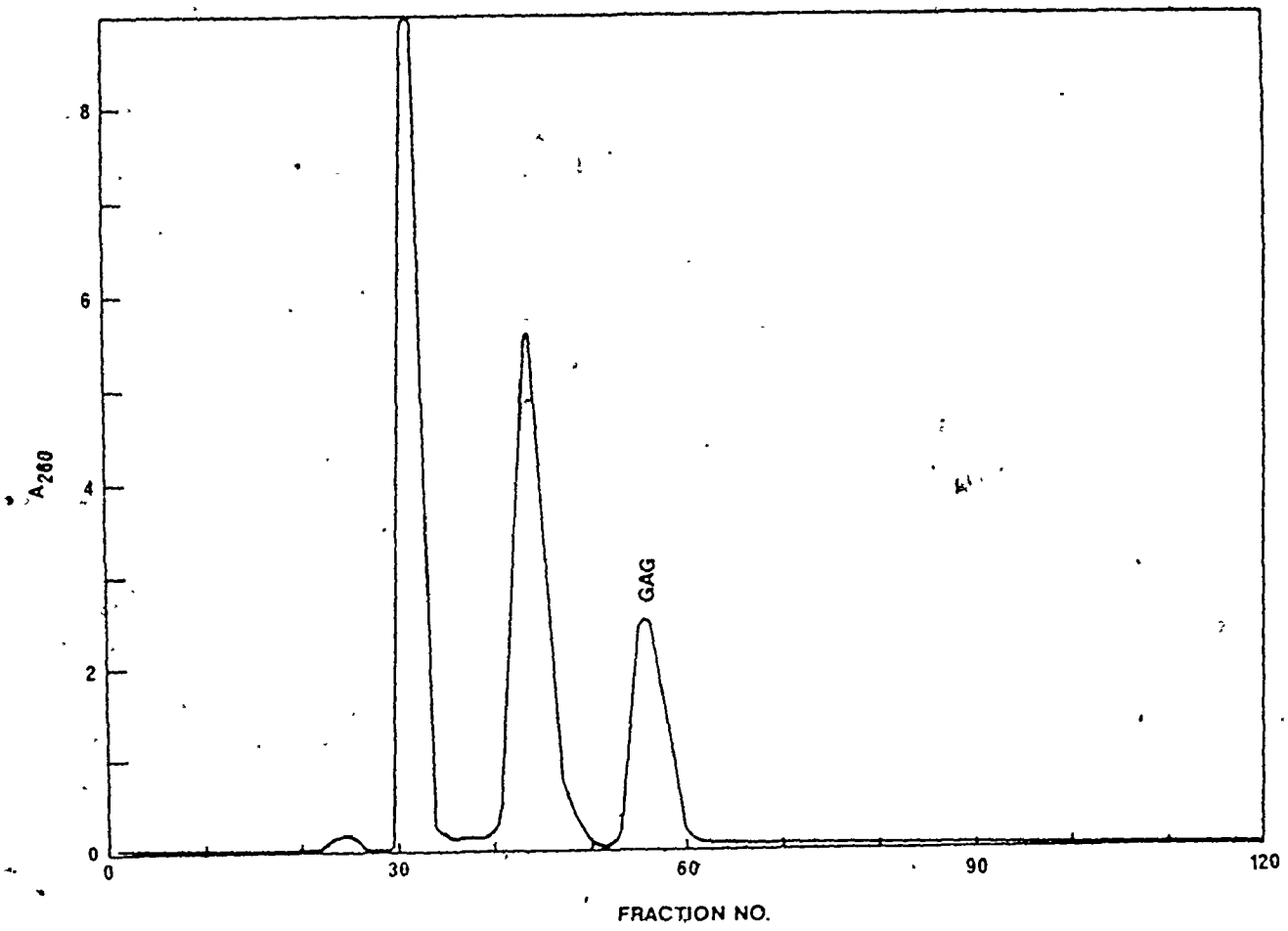
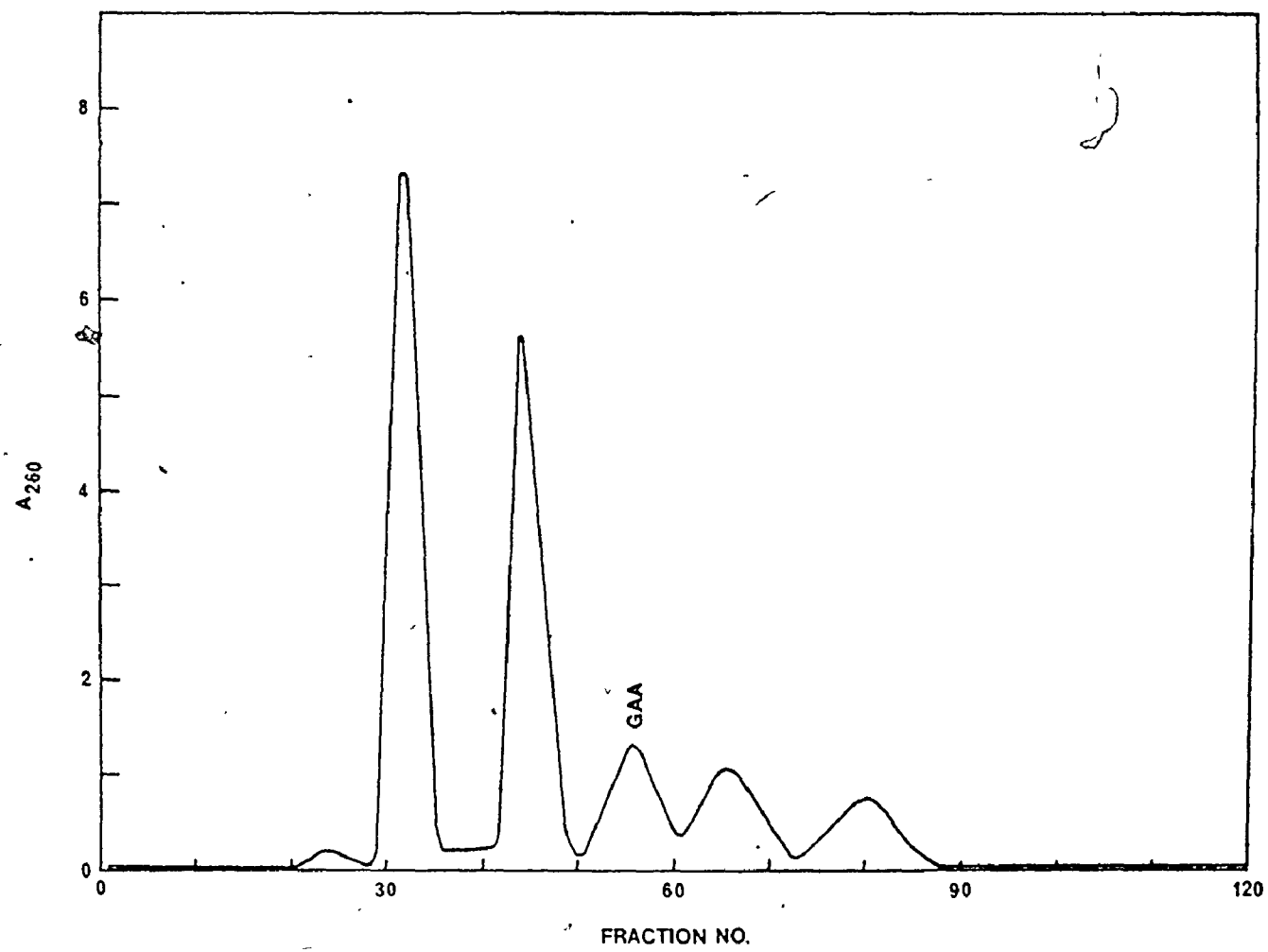

8
6
4
2
0
A_{260}
GAG
0
30
60
90
120
FRACTION NO.

Figure 8: ISOLATION OF GpApA BY DEAE-CELLULOSE (MICROGRANULAR) CHROMATOGRAPHY

The conditions of chromatography were as in Figure 3.


8
6
4
2
0
A_{260}
GAA
0
30
60
90
120
FRACTION NO.

the reservoir. Then the column was equilibrated in glass distilled water and again the pH was monitored. Trinucleotides were isolated from the incubation mixture described in section IV by adjusting the volume of the mixture to 50 ml with water. The solution was charged onto the column at 12.5 ml per hour. Elution was with a linear gradient consisting of 250 ml glass distilled water in the mixing chamber and 250 ml 0.5 M ammonium bicarbonate (pH 8.6) in the reservoir. 2.5 ml fractions were collected. The absorbance at 260 nm was measured. The peak fractions were pooled and lyophilized. The third peak was the ribotrinucleotide in all cases and its identity was confirmed from its chromatographic mobility (see below). Figures 3-8 show the chromatographic profiles of those trinucleotides synthesized and separated by this procedure.

e) Preparation of Synthetases Free of Endogenous tRNAs by Sephadex G50 (fine) Chromatography.

Sephadex G50 (fine) was swelled in solution D' by boiling for 3 hours. After the gel had settled, the supernatant was decanted and fresh solution D' added and left overnight at 4°C. All operations were then carried out at 4°C. The gel was slurried in solution D' and the slurry was poured into a 2.5 cm x 40 cm glass column filled partially with D' and equipped with a packing funnel. The gel was allowed to settle under gravity overnight. Excess gel and equilibrating buffer were removed.

The column was equilibrated with solution D' until the absorbance at 280 nm was zero.

S-150 extract (about 2 ml) was applied to the column at a flow rate of 12 ml per hour. 2 ml fractions were collected and the absorbance at 280 and 260 nm was monitored. The "protein peak" was concentrated by dialysis against 100% saturated ammonium sulfate. The precipitate was collected by centrifugation, resuspended in D' and dialyzed against the same for 4 hours. The synthetase preparation was frozen and stored as described earlier.

f) Affinity Chromatography of Lysyl-tRNA Synthetase

i) Preparation of Hexamethylenediamine-Sepharose

15 ml of Sepharose 4B was activated by cyanogen bromide as described by Axen et al., (1967). The activated sepharose was washed immediately with an excess of cold 0.1 M sodium bicarbonate solution and suspended in 25 ml of the same solution. 25 mmoles of neutralized hexamethylenediamine was added within 2-3 min of activation and washing. The mixture was then stirred at 4°C for 16 hours and washed with 2 l of cold water at the end of the reaction period (Cautrecasas, 1970).

To see whether the diamine had coupled to the sepharose, the qualitative colour test of Inman and Dintzis (1971), using 2, 4, 6-trinitrobenzene sulfonate, was performed.

ii) Preparation of o-Nitrophenylsulfenyl Derivative of L-lysine

L-lysine was derivatized with o-nitrophenylsulfenyl chloride by the method of Zervas et al., (1963) except that the ε-amino group was not blocked with a carboxybenzoyl protecting group. *

iii) Preparation of o-Nitrophenylsulfenyl-derivatized Lysine Succinimide Ester

Equimolar quantities of N-hydroxysuccinimide, dicyclohexylcarbodiimide and the lysine derivative were reacted under the conditions described by Robert-Gero and Waller (1972).

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* It was assumed that by using twice the normal amount of o-nitrophenylsulfenyl chloride the ε-amino group might be protected in addition to the α-amino group. Although this did not give a completely satisfactory column, it did allow purification of some lysyl-tRNA synthetase, enough for the objective desired (see Results).

For subsequent reactions where the molecular weight of the derivative was required, it was assumed that both the α- and ε-amino groups were protected by the o-nitrophenylsulfenyl moiety.

iv) Coupling of Sepharose-diamine with the Succinimide Ester Derivative of Lysine

The coupling reaction was performed as described by the authors cited above except that the reaction was allowed to proceed for 3 hours. The gel assumed a yellow colour indicating that the lysine derivative had coupled.

v) Removal of the o-Nitrophenylsulfenyl Protecting Group

The coupled gel was suspended in 40 ml 200 mM acetate buffer (pH 5.1). To this was added a solution of sodium thiosulfate to a final concentration of 0.4 M. The reaction was allowed to proceed for 2 hours at 30°C. The gel was then washed with water, methanol, water, 50% dimethylformamide and finally water. The gel still assumed a yellow colour, indicating that the blocking group had not been removed entirely. Consequently, the gel was treated for an additional 3 hours followed by washing using the described schedule.

vi) Purification of Lysyl-tRNA Synthetase

A 1 cm x 15 cm glass column was packed with Sepharose-diamine-lysine suspended in solution D'. Equilibration and elution was with solution D' at 4°C. S-150 (1 ml) was applied to the column at a flow rate of 0.5 ml per 3 min. 0.5 ml fractions were collected

and lysyl-tRNA synthetase activity was determined using the incubation mixture shown in Table 5 except that 100 µl aliquots were taken from each fraction and 0.5 A_{260} unit of crude tRNA in 15 µl of solution D' was used. Incubation was for 25 min at 37°C.

g) Paper chromatography of Trinucleotides

The identity of trinucleotides was confirmed by comparing their R_f values with those reported by Lohrmann et al., (1965). The method used was identical to that described by these authors. Paper chromatography, using the descending technique on Whatman No. 1 paper, was performed in the solvent system composed of ethanol-1 M ammonium acetate (pH 7.5) (7:3 v/v).

h) DEAE-Sephadex A-25 Chromatography of 3'-Terminal Oligonucleotides

^{14}C-lysyl-tRNA$_1^{Lys}$ and ^{3}H-lysyl-tRNA$_2^{Lys}$ were mixed with 0.5 mg crude H. cutirubrum tRNA and digested with ribonuclease T_1 (Sen and Ghosh, 1973). The incubation mixture contained 10 mM ammonium formate (pH 5.0), 2 mM EDTA, 100 units of T_1 RNase and the aminoacyl-tRNA^{Lys} species dissolved in 10 mM ammonium formate (pH 5.0). The digest was charged onto a DEAE-Sephadex A-25 column (0.5 cm x 15 cm) equilibrated with 10 mM ammonium formate (pH 7.0) according to the method of Pestka (1971). Elution was with 20 ml 10 mM ammonium formate (pH 5.0).

1 ml fractions were collected. Radioactivity was determined by adding 10 ml Aquasol to the fractions and counting in a Beckman scintillation counter.

SECTION VI. Determination of Protein Concentration

Protein was estimated by the method of Lowry *et al.*, (1951) using bovine serum albumin as a standard.

MATERIALS

Analytical grade inorganic salts and trichloroacetic acid were supplied by Fisher Scientific Co., Canlab and J. T. Baker Chemical Co.; CpA, ApA, GpA, GDP, UDP, ADP and spermidine by Calbiochem; bovine serum albumin and ATP by P-L Biochemical Corp.; Sephadex G100 (fine), G50 (fine), DEAE-Sephadex A25 and Sepharose 4B by Pharmacia; DEAE-cellulose and HEPES by Sigma Chemical Corp.; BD-Cellulose and unlabelled L-lysine by Schwarz/Mann; DEAE-Cellulose DE 32 (microgranular) and number 1 chromatography paper by Whatman; Plaskon CFTE 2300 powder by Allied Chemical Corp.; Adogen 464 by Ashland Chemical Co.; 2-mercaptoethanol, cyanogen bromide, dicyclohexylcarbodidiimide, N-hydroxysuccinimide by Eastman Organic Chemicals; Tris by BDH Chemical Co.; O-nitrophenylsulfenyl chloride by Pierce Chemicals; M. luteus polynucleotide phosphorylase, poly(A), poly(A:G::1.6:1) and poly(A:G::1:1) by Miles Chemical Corp.; E. coli alkaline phosphatase, RNase T_1 and electrophoretically purified DNase by Worthington Chemical Co.; ^{14}C- and ^{3}H-lysine, ^{14}C-histidine, ^{14}C-glutamic acid, ^{14}C-aspartic acid and Omnifluor by New England Nuclear Corp.

III. Results

SECTION I. Ribosomal Binding Assays

a) H. cutirubrum Ribosomal Binding System for Codon Assignments Using Trinucleotides

In the initial phase of this work, it was hoped that the work of Bayley and Griffiths (1968b) and White and Bayley (1972b) on codon assignments in H. cutirubrum might be extended. Specifically, it was proposed that some of the codons for the acidic and basic amino acids might be tested for any altered codon-anticodon interactions in the high salt environment. Since random polyribonucleotides with a high content of adenosine and/or cytosine precipitated out in the high salt (Bayley and Griffiths, 1968b; White and Bayley, 1972b), the trinucleotides CAU, AAU, AAG, GAU, GAG and GAA were synthesized. When these trinucleotides were tested in the high salt environment using the ribosomal technique of White and Bayley (1972b) negligible response was observed to either that aminoacyl-tRNA given in the established code or to those aminoacyl-tRNA's listed in Table 11. Since the H. cutirubrum ribosomal binding assay was developed with polyribonucleotides, it was thought that perhaps the conditions for binding were not optimal for trinucleotides. To this end the following conditions were varied in the hope of improving the binding reaction.

(1) The final Mg^{2+} concentration was varied from 0.04 M to 0.15 M in 0.02 M increments.

Table 11: CODONS TESTED WITH TRINUCLEOTIDES IN HIGH SALT

AMINO ACID	ESTABLISHED CODONS	CODONS TESTED*
HIS	CAU, CAC	CAU
ASN	AAU, AAC	AAU
LYS	AAA, AAG	AAA(polyA)
ASP	GAU, GAC	GAU
GLU	GAA, GAG	GAA GAG AAG

* Each codon was tested with the 5 amino acids listed.

(2) 0.15 M sodium cacodylate (pH 7.5) or 0.15M Tris-acetate (pH 7.2) or 0.15 M HEPES (pH 7.1) was added to the reaction mixture in lieu of 0.15 M Tris-HCl (pH 8.05).

(3) The nominal final potassium chloride concentration was varied from 3.4 M to 4.0 M in 0.02 M increments.

(4) (a) 40% sucrose (w/v) was substituted for potassium chloride in the final reaction mixture. This was achieved by incubating all the components except the triplets at 37°C and then dialyzing the mixture against a 40% sucrose solution containing the other salts at the same concentrations. 0.115 ml of this mixture was then incubated with 0.01 ml trinucleotide in water at 37°C for 30 min.

(b) The trinucleotide in water was added to ribosomes in 40% sucrose, 0.5 M KCl, 0.04 M magnesium acetate, 0.01 M Tris-HCl (pH 7.6) and 0.007 M 2-mercaptoethanol. This mixture was then used in the ribosomal binding assay instead of ribosomes in D' and trinucleotide in water.

(5) The amount of ribosomes added was varied from 2 to 6 A_{260} units.

(6) The amount of ^{14}C-aminoacyl-tRNA added was varied from 14 to 48 pmoles for some of the ^{14}C-aminoacyl-tRNAs used.

(7) The time course of the reaction was monitored from 0 to 60 min.

(8) The amount of trinucleotide added was varied from 0.4 to 1.2 A_{260} units.

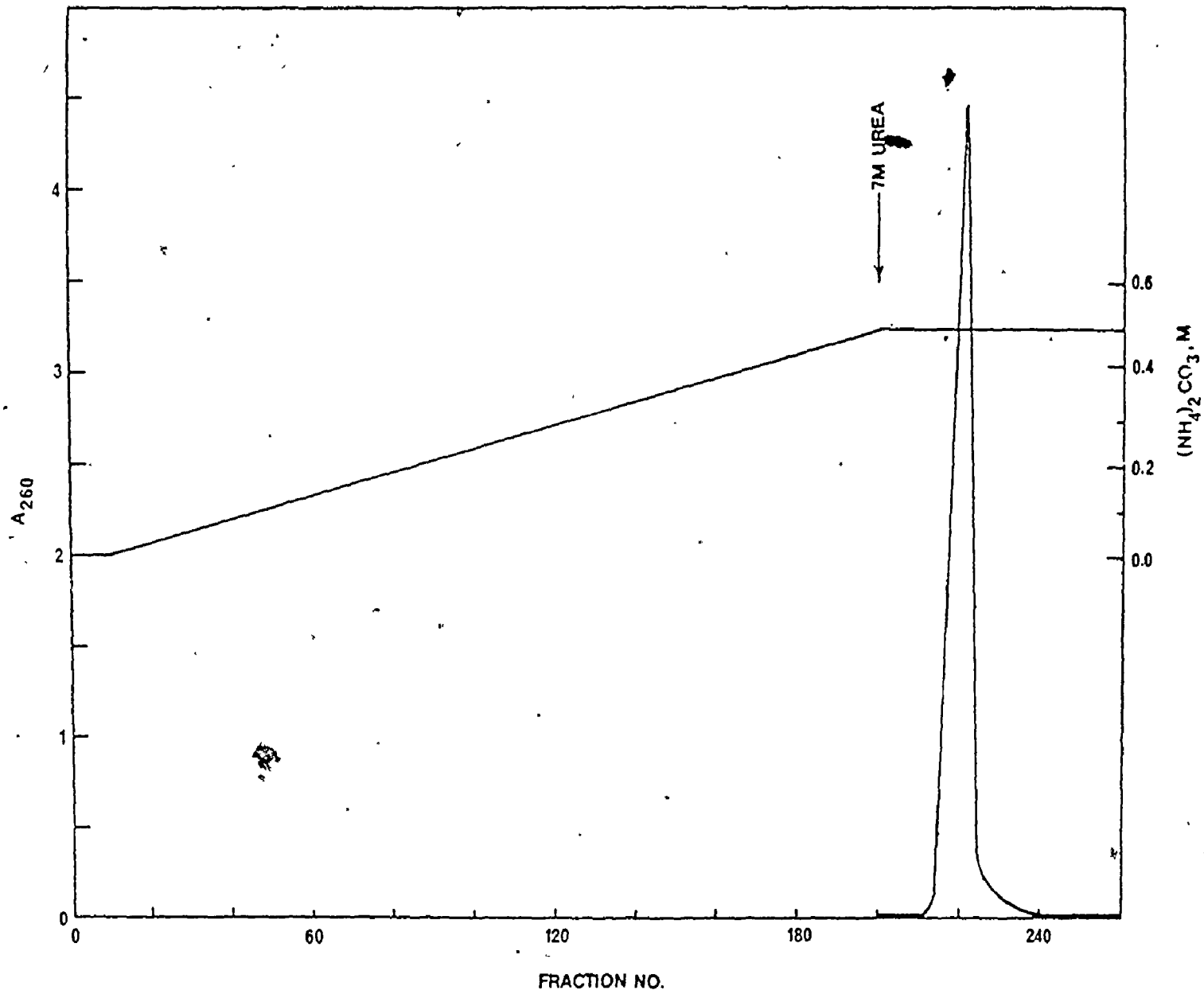
(9) 1.0 M magnesium acetate added to the standard reaction mixture was replaced by 0.2 M to 1.0 M neutralized spermidine.

In all these experiments, the response was negligible usually amounting to no more than 50 counts per min above the control value and this difference was not consistent.

Since the standard binding assay could be used satisfactorily with, for instance, partially hydrolyzed poly(U) and poly(A) it was of interest to examine the proportion of trinucleotides in the hydrolysates. 100 A_{260} units of partially hydrolyzed poly(U) and poly(A) were chromatographed on DEAE-cellulose (microgranular) using the method described in section V of Chapter 2. As shown in figure 9, the amount of trinucleotide present in the hydrolysate of poly(A) was negligible since mono-, di-, tri-, tetra- and pentanucleotides would have eluted between fractions 20 and 100. The chromatographic profile of partially hydrolyzed poly(U) was similar. The homopolymers could be eluted from the column in the presence of 7 M urea. From these results, it was apparent that in order to continue the work on codon assignments in the high salt system, synthetic messenger RNAs of defined sequence would have had to be synthesized and an efficient transfer system developed. The former alone would have entailed a heavy commitment in preparative work and hence, the problem on

Figure 9: CHROMATOGRAPHY OF PARTIALLY HYDROLYZED POLY(A) ON DEAE-CELLULOSE COLUMN

100 A_{260} units of the polymer in 50 ml of water was charged onto the column at a flow rate of 12.5 ml/hr. Elution was with a linear gradient consisting of 250 ml water in the mixing chamber and 250 ml 0.5 M ammonium carbonate (pH 8.6) in the reservoir. The column was purged with 0.5 M ammonium carbonate containing 7 M urea. 2.5 ml fractions were collected.


7M UREA
A_{260}
$(NH_4)_2CO_3$, M
FRACTION NO.
0
1
2
3
4
0.0
0.2
0.4
0.6
0
60
120
180
240

codon assignments in high salt was not pursued further.

b) Heterologous Binding of H. cutirubrum Aminoacyl-tRNA to E. coli Ribosomes

Although the attempt to examine codon-anticodon interactions in high-salt was unsuccessful it was thought that if there were any fundamental alteration in the codon-anticodon response for some of the H. cutirubrum tRNAs, then the difference might be manifest in the heterologous binding system using the E. coli binding system of Nirenberg and Leder (1964). The results in Table 12 show that labelled histidinyl-, asparaginyl-, lysyl-, aspartyl- and glutaminyl-tRNAs responded to the established codons. These results suggest that there is no fundamental difference in codon-anticodon interaction with halophile tRNAs.

SECTION II. Purification of Lysine tRNA

Although the ribosomal binding studies in high salt proved to be unsuccessful, it was hoped that some information on codon-anticodon interactions might be obtained if a tRNA species for either an acidic or a basic amino acid was purified. Lysine tRNA was the species of choice since partially hydrolyzed poly(A) could be used in high salt. Furthermore, if two or more isoaccepting species were present, their response to the codon AAA could be studied.

Table 12: BINDING OF H. cutirubrum ^{14}C-AA-tRNAs TO E. coli RIBOSOMES IN RESPONSE TO TRINUCLEOTIDES

AMINO ACID	HIS	ASN	LYS	ASP	GLU
	pmoles ^{14}C-AA-tRNA bound				
-Trinucleotide	0.15	0.08	0.24	0.39	0.06
+CAU	0.72	0.09	0.24	0.35	0.06
+AAU	0.19	0.20	0.19	0.35	0.07
+AAA*	0.12	0.07	1.10	0.40	0.07
+AAG	0.18	0.07	0.87	0.32	0.05
+GAU	0.21	0.09	0.22	1.08	0.06
+GAA	0.17	0.08	0.27	0.38	0.17
+GAG	0.17	0.09	0.25	0.38	0.14

* As partially hydrolyzed poly(A).

Each 0.05 ml reaction mixture contained the components and was incubated at 24°C for 20 min as described in Section III of Methods except that H. cutirubrum ^{14}C-AA-tRNAs were added. The amounts of ^{14}C-AA-tRNA added were as follows:

^{14}C-His-tRNA (21.1 pmoles)

^{14}C-Asp-tRNA (9.8 pmoles)

^{14}C-Lys-tRNA (27 pmoles)

^{14}C-Asp-tRNA (28.3 pmoles)

^{14}C-Glu-tRNA (8.7 pmoles)

Each reaction mixture contained 0.8 A_{260} unit of template and 2 A_{260} units of E. coli ribosomes. The final Mg^{2+} concentration was 0.02 M.

Before the purification of lysine tRNA could be pursued, it was necessary to optimize the aminoacylation reaction for lysine.

a) Determination of K_m for Crude Lysyl-tRNA-synthetase

The effect of varying the lysine concentration in a reaction mixture containing 3.8 M potassium chloride and .0.04 M magnesium chloride is shown in the form of a Lineweaver-Burk (1934) plot in Figure 10. The K_m for lysyl-tRNA synthetase (pH 5.0 enzyme) was found to be 3.9×10^{-5}M. Thus, in charging reactions for determining the specific acceptor activity of purified $tRNA^{Lys}$, lysine in an amount greater than 5×10^{-5}M was added (see Table 6).

b) Time Course of Lysyl-tRNA Formation

The time course of ^{14}C-lysyl-tRNA formation is shown in Figure 11. The reaction was complete within 15 min. After this period there appeared to be some loss of ^{14}C-aminoacyl-tRNA. This loss occurred consistently with different preparations of pH 5.0 enzyme and is in contrast to the report of Bayley and Griffiths (1969) that there was no apparent loss of ^{14}C-aminoacyl-tRNAs for a period of 40 min after maximal labelling had been reached. As will be seen later the pH 5.0 enzyme could not be used for accurately determining the specific activity of purified tRNA due to degradative activity observed in most preparations.

Figure 10: DETERMINATION OF K_m VALUE FOR LYSINE

The incubation mixture shown in Table 6 was used. 2 A_{260} units of unfractionated tRNA, 2.3 µMoles ATP and 0.2 mg of synthetases (pH 5 enzyme) were present in each reaction mixture. The reaction mixture was incubated at 37°C for 2.5 min. The specific activity of ^{14}C-lysine was 50 mCi/mmole. The reaction velocity, V, is expressed as pmoles ^{14}C-lysine incorporated/A_{260} unit tRNA per 2.5 min reaction time; substrate concentration, S, is expressed as µMoles ^{14}C-lysine added to the incubation mixture.

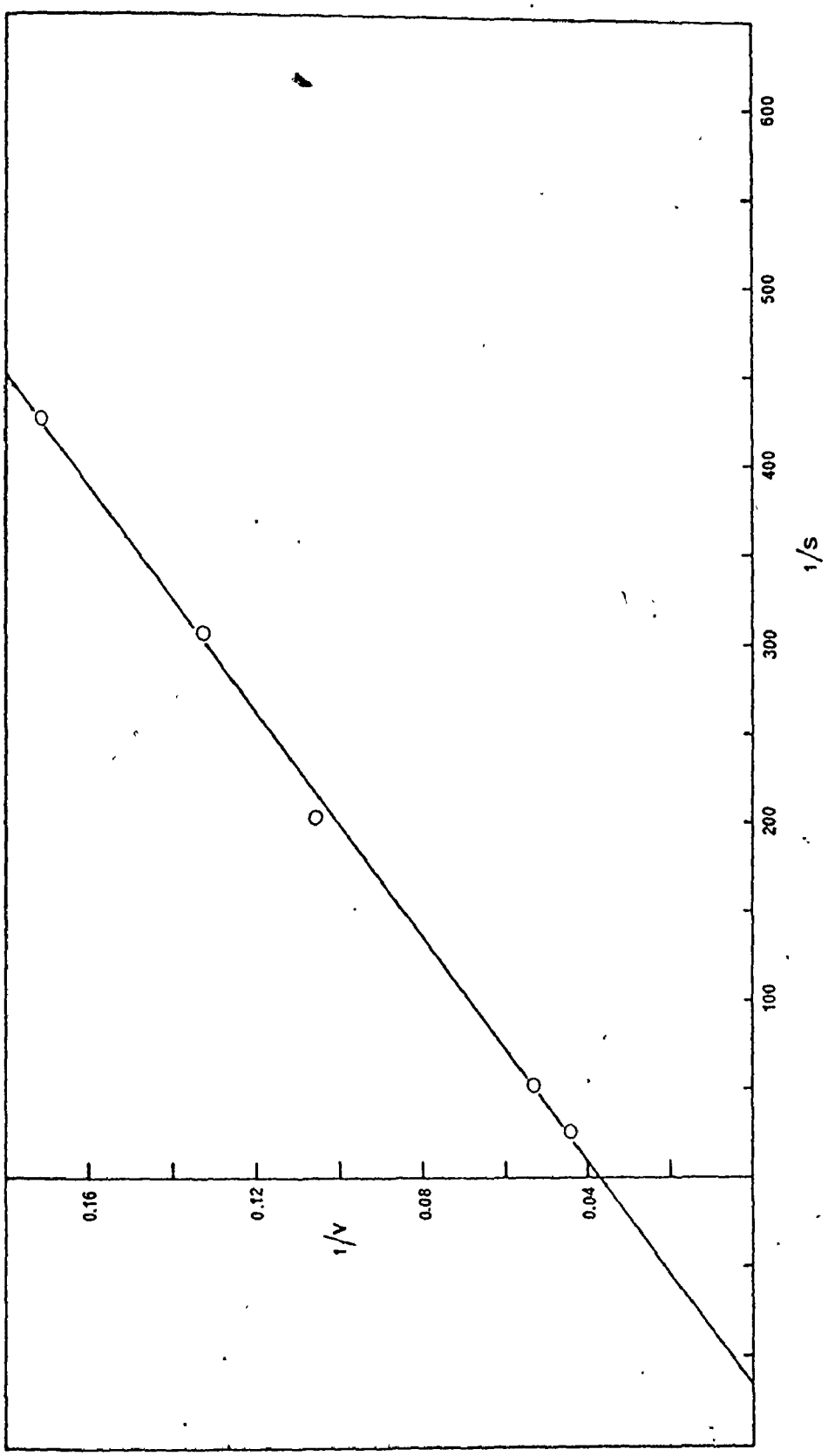

0.16
0.12
0.08
0.04
1/v
100
200
300
400
500
600
1/s

Figure 11: TIME COURSE OF CHARGING OF UNFRACTIONATED tRNA WITH ^{14}C-LYSINE

The incubation mixture shown in Table 6 was used. Each tube contained 2 A_{260} units of tRNA, 0.4 mg S-150 and 2.3 µMoles ATP. The specific activity of ^{14}C-lysine was 50 mCi/mmole. Each point represents the average of a set of duplicates. Background values in the absence of tRNA have been subtracted.

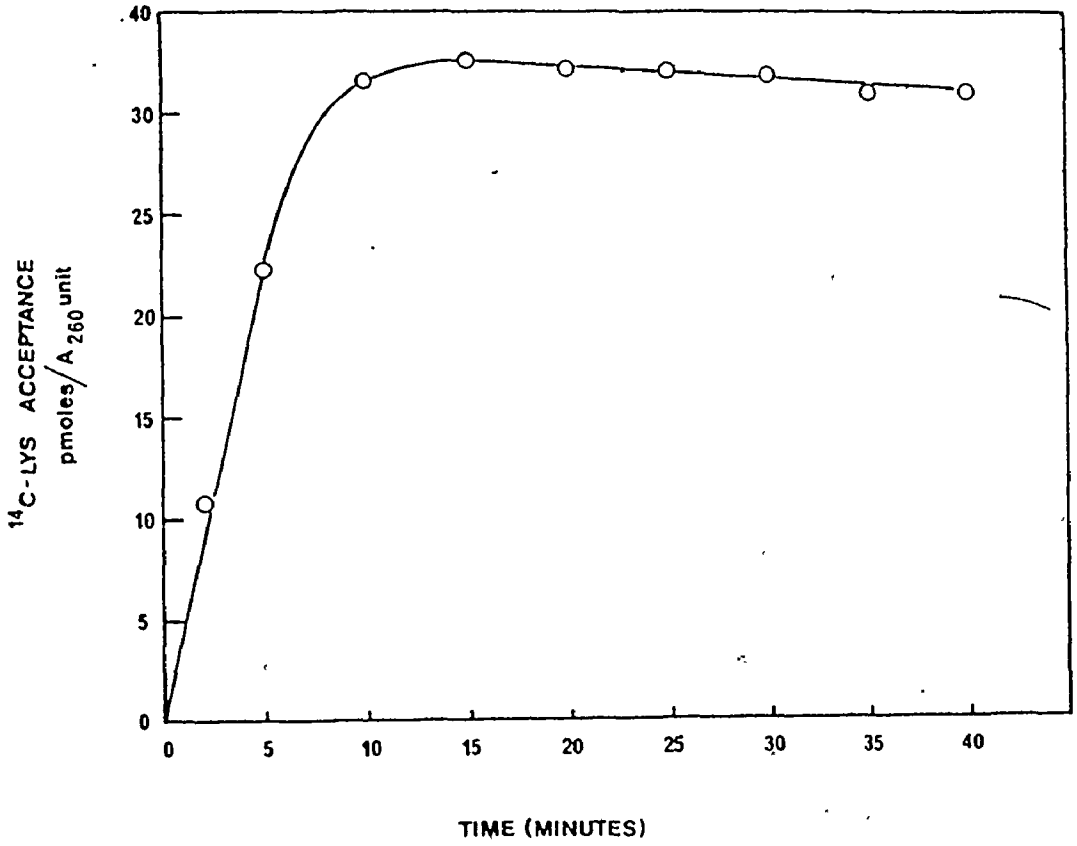
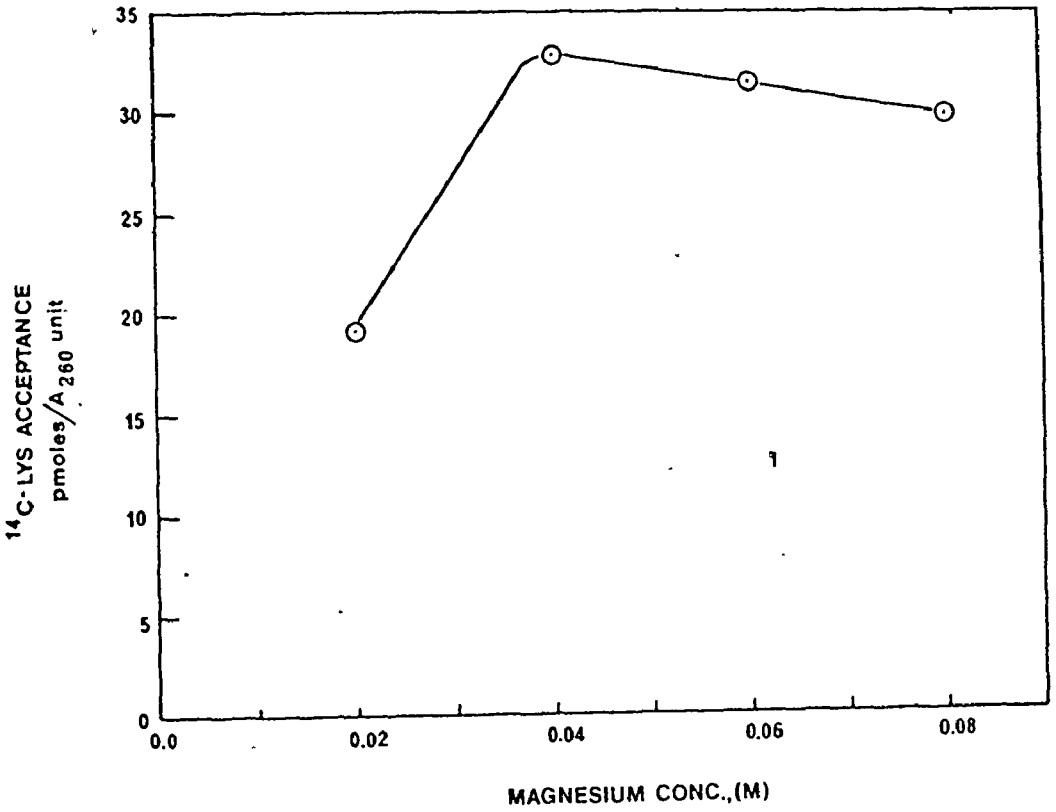

^{14}C-LYS ACCEPTANCE
pmoles/A_{260} unit
0
5
10
15
20
25
30
35
40
TIME (MINUTES)

Figure 12: DETERMINATION OF THE OPTIMAL MAGNESIUM CONCENTRATION FOR ^{14}C-LYSYL-tRNA FORMATION.

The incubation mixture shown in Table 6 was used except that the magnesium concentration was varied. Each tube contained 2 A_{260} units of tRNA, 0.4 mg S-150 and 2.3 µmoles ATP. The reaction was allowed to proceed for 12 min at 37°C. Each point represents the average of a set of duplicates. ^{14}C-lysine incorporation in the absence of tRNA have been subtracted.


35
30
25
20
15
10
5
0
0.0
0.02
0.04
0.06
0.08
^{14}C-LYS ACCEPTANCE
pmoles/A_{260} unit
MAGNESIUM CONC.,(M)

c) Optimization of ATP Concentration

When the amount of ATP added was increased from 0.3 µMole to 2.3 µMoles there was no increase in the amount of ^{14}C-aminoacyl-tRNA formed. Since there was no adverse effect, the higher concentration of ATP was retained in the optimized reaction mixture.

d) Effect of Magnesium Concentration

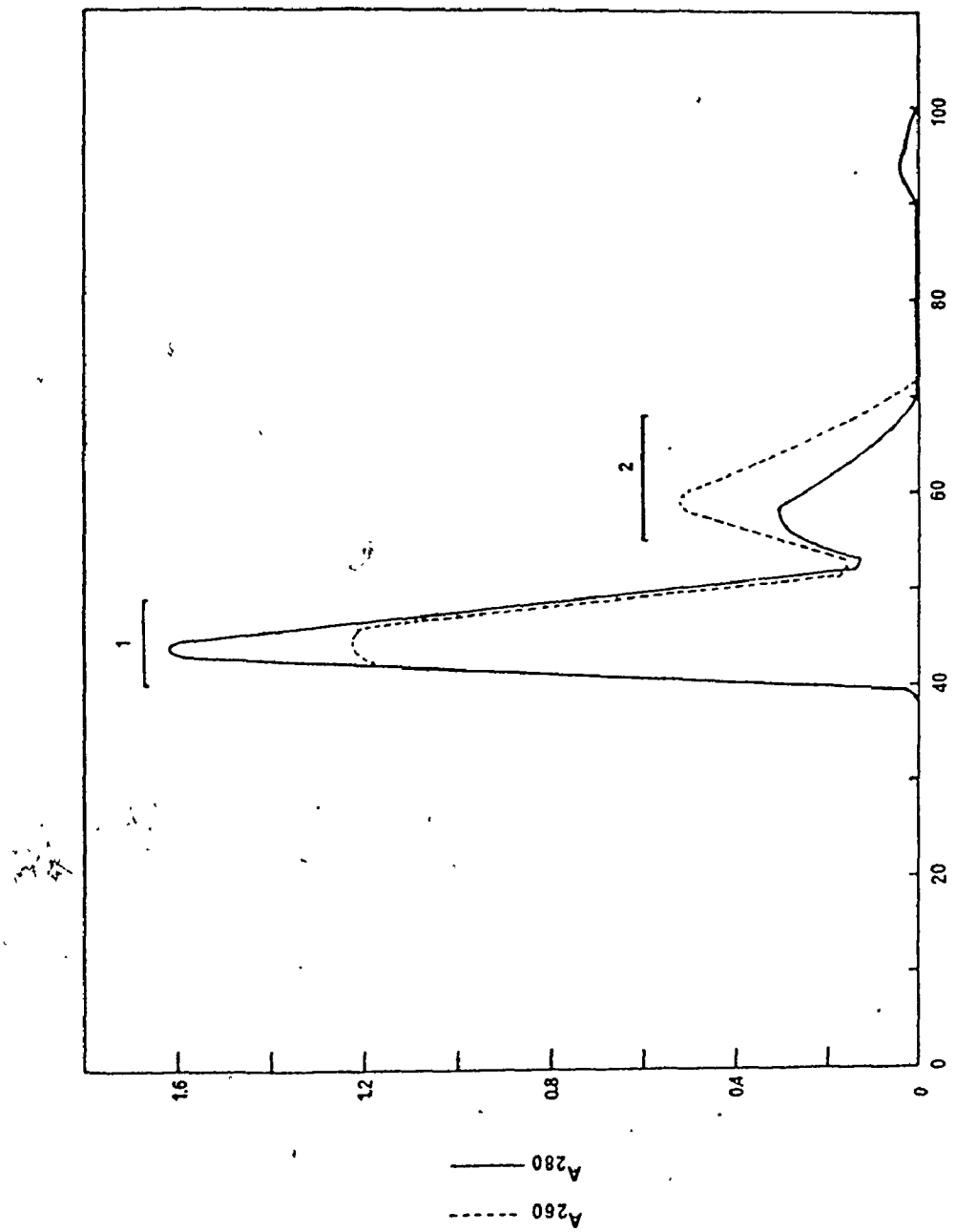
The effect of varying the magnesium concentration on ^{14}C-lysyl-tRNA formation is shown in Figure 12. When the concentration of magnesium was increased from 0.04 M as given in the incubation mixture of Griffiths and Bayley (1969), there was a perceptible decrease in the amount of ^{14}C-aminoacyl-tRNA formed. When the magnesium concentration was lowered to 0.02 M, the formation of ^{14}C-lysyl-tRNA was markedly reduced.

e) Preparation of Synthetases Free of Endogenous tRNA

For the purpose of determining the elution position of $tRNA^{Lys}$ species in the fractions after reverse phase chromatography, the S-150 extract was fractionated on a column packed with Sephadex G50 (fine). Figure 13 shows a typical fractionation of an S-150 extract. The void volume and the higher molecular weight components separated by this column were probably mainly protein as judged by the 280 nm/260 nm ratio. The two peaks were pooled separately and concentrated with 100% ammonium sulfate.

Figure 13: CHROMATOGRAPHY OF S-150 ON SEPHADEX G50 (FINE)

2 ml of an S-150 preparation was layered onto a glass column (2.5 x 45 cm) packed with Sephadex G50 equilibrated in solution D'. The sample was eluted with the same buffer at a flow rate of 12.5 ml/hr. The fraction volume was 2 ml.


1.6
1.2
0.8
0.4
0
0
20
40
60
80
100
FRACTION NO.
1
2
A_{280}
A_{260}

Both fractions were tested for the presence of lysine tRNA synthetase activity and for the presence of $tRNA^{Lys}$ as shown in Table 13. From these results it is evident that most of the $tRNA^{Lys}$ activity was present in fraction 2 while the synthetase activity was in fraction 1.

f) Purification of $tRNA^{Lys}$ on BD-Cellulose

Figure 14 illustrates the chromatographic behaviour of ^{14}C-lysyl-tRNA on BD-cellulose when eluted with a linear gradient from 0.5 M - 1.2 M NaCl. The aminoacyl-tRNA could be eluted from the column only with 1.2 M NaCl plus 10% ethanol. E. Griffiths and S. T. Bayley (personal communication) found that when uncharged H. cutirubrum tRNA was chromatographed on BD-cellulose using stepwise elution with 1.0 M NaCl followed by an ethanol purge, lysine, threonine, glycine, histidine and isoleucine tRNAs were found to elute with the ethanol purge. In an attempt to obtain $tRNA^{Lys}$ separated from the bulk of the A_{260} absorbing material as well as some of the other tRNAs mentioned above, ^{14}C-lysyl-tRNA was chromatographed on BD-cellulose using step elution with 1.5 M NaCl and 1.5 M NaCl + 10% ethanol. Figure 15 shows that under these conditions the ^{14}C-lysyl-tRNA eluted with the 1.5 M NaCl buffer. However, the aminoacyl-tRNA could not be recovered in a precipitable form with 2 volumes of ethanol due to the excessively large volume of eluting buffer. Hence, the tRNA had to be recovered on a Millipore filter

Table 13: LYSINE SYNTHETASE ACTIVITY AND LYSINE ACCEPTANCE CAPACITY OF THE COMPONENTS OF S-150 FRACTIONAED ON SEPHADEX G50 (FINE)

		pmoles ^{14}C-LYS IN THE 5% TCA PPT			
	EXTRACT	A (No Addition)	B (+ tRNA)*	C (+ pH 5 enzyme)	A_{260} UNITS OF EXTRACT ASSAYED
	S-150	9.7	73.8	-	1.94
1	FRACTION 1	0.4	64.1	-	1.20
	FRACTION 2	0.09	0.13	11.4	0.55
	FRACTION 1	0.3	61.1	-	1.42
	FRACTION 2	0.12	0.11	9.7	0.47
3	FRACTION 1	0.8	60.4	-	1.17
	FRACTION 2	0.10	0.13	10.9	0.51

* 3 A_{260} units/incubation mixture.

Each 0.225 ml reaction mixture contained the components described in Table 1 for the H. cutirubrum tRNA charging system. Column A assayed for both $tRNA^{Lys}$ and lysine synthetase activity; column B assayed specifically for lysine synthetase activity; column C assayed for $tRNA^{Lys}$ in fraction 2 with a pH 5.0 enzyme.

Figure 14: COLUMN CHROMATOGRAPHY OF ^{14}C-LYSYL-tRNA ON BD-CELLULOSE: GRADIENT ELUTION

150 A_{260} units of ^{14}C-lysyl-tRNA (649, 740 cpm) dissolved in 0.45 M NaCl, 0.01 M $MgCl_2$, 0.05 M Na acetate (pH 4.5) and 0.005 M 2-mercaptoethanol were applied to a BD-cellulose column (1.5 x 30 cm) previously equilibrated in the same buffer. After step elution of the first 40 fractions with the 0.45 M NaCl buffer, a linear gradient was generated with 180 ml 0.5 M NaCl in the mixing chamber and 180 ml 1.2 M NaCl in the reservoir. The column was purged with 1.2 M NaCl + 10% ethanol. All solutions contained the other constituents of the 0.45 M NaCl buffer. The flow rate was 36 ml/hr; fractions of 3 ml were collected.

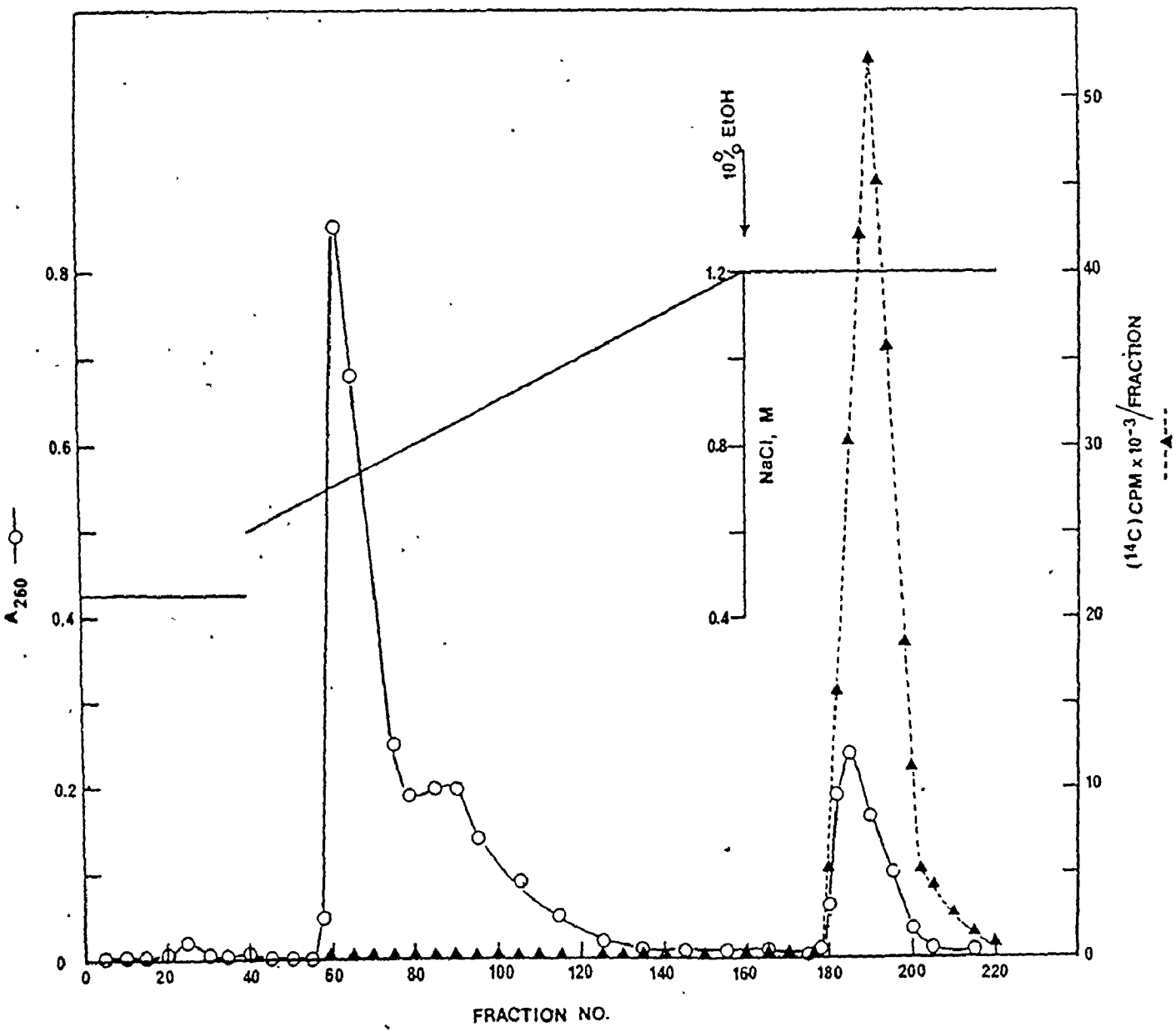
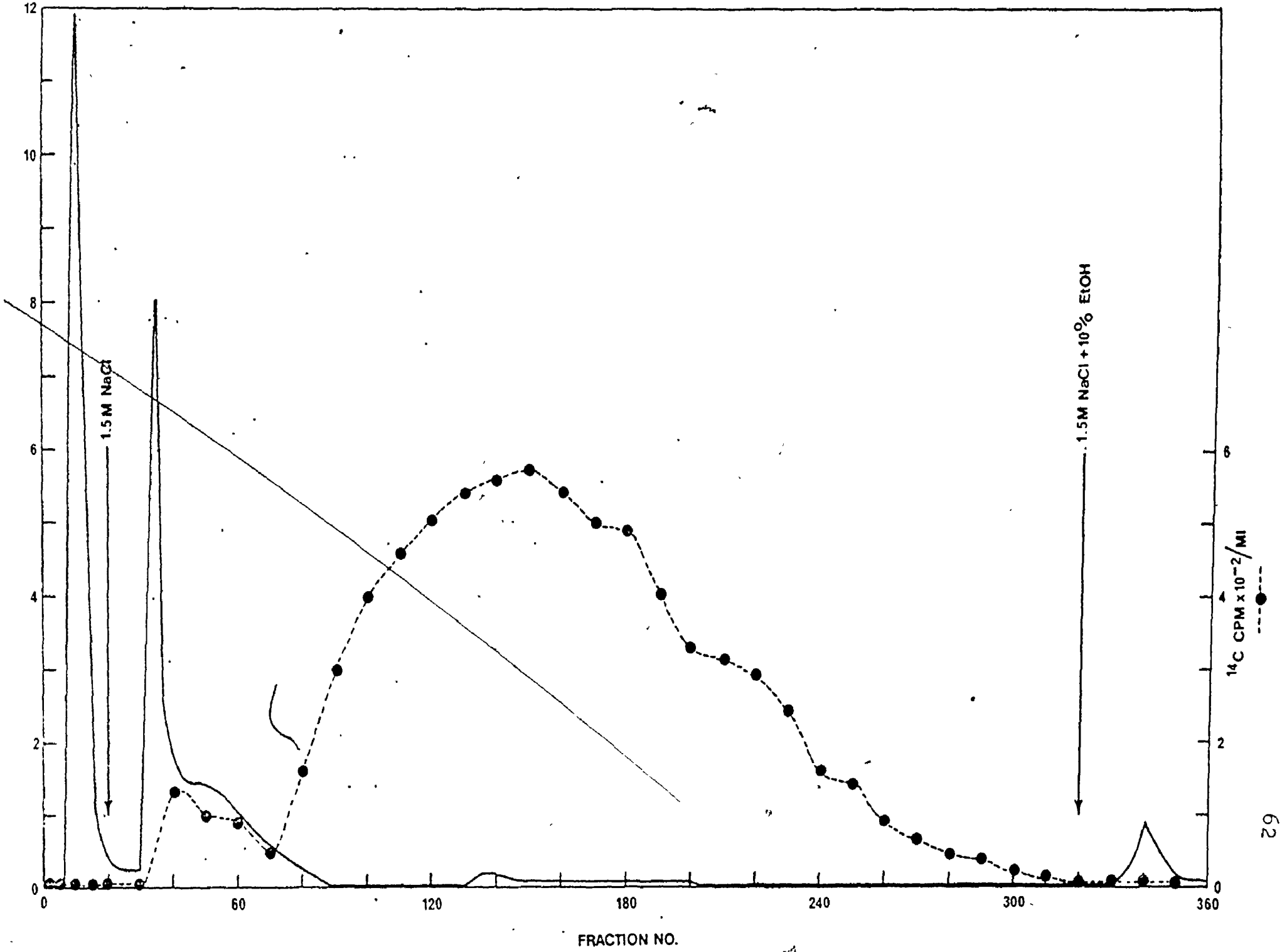

10% EtOH
NaCl, M
A_{260}
(^{14}C) CPM x 10^{-3}/FRACTION
FRACTION NO.
0
0.2
0.4
0.6
0.8
1.2
0.8
0.4
0
10
20
30
40
50
0
20
40
60
80
100
120
140
160
180
200
220

Figure 15: CHROMATOGRAPHY OF ^{14}C-LYSYL-tRNA ON BD-CELLULOSE: STEP ELUTION

900 A_{260} units of ^{14}C-lysyl-tRNA (440, 312 cpm) were charged onto a BD-cellulose column (1.6 x 40 cm) previously equilibrated with the 0.45 M NaCl buffer described in fig. 14. Elution was step-wise with 0.45 M NaCl, 1.5 M NaCl and 1.5 M NaCl + 10% Ethanol in the presence of 0.01 M MgCl , 0.05 M Na acetate (pH 4.5) and 0.005 M 2-mercaptoethanol. The flow rate was 36 ml/hr; 5 ml fractions were collected.


1.5 M NaCl
1.5 M NaCl + 10% EtOH
^{14}C CPM x 10^{-2}/Ml
FRACTION NO.
0
60
120
180
240
300
360
0
2
4
6
8
10
12

and for bulk purifications handling the material in this manner would have been cumbersome. For subsequent purifications on BD-cellulose, stepwise elution with 1.2 M NaCl followed by 1.2 M NaCl + 10% ethanol was carried out. A typical chromatographic profile is shown in Figure 16. From these results there was no indication of multiple isoaccepting species of $tRNA^{Lys}$. [When uncharged tRNA was chromatographed on BD-cellulose, lysine acceptor activity was detected mainly in the ethanol fractions (Figure 17)].

The fractions containing the ^{14}C-lysyl-tRNA were pooled, ethanol precipitated and then discharged as described earlier. The specific activity of the partially purified $tRNA^{Lys}$ was determined, the results of which are presented in Table 14. The $tRNA^{Lys}$ was purified further by RPC-5 chromatography.

g) RPC-5 Chromatography of $tRNA^{Lys}$

The lysine-tRNA-rich fraction from BD-cellulose chromatography was purified further by RPC-5 chromatography at neutral pH. Figure 18 shows a typical profile. From these results it was evident that there were two isoaccepting species of $tRNA^{Lys}$. The faster moving species was designated $tRNA^{Lys}_1$ and the slower moving species, $tRNA^{Lys}_2$. Both $tRNA^{Lys}$ species resolved as sharp peaks and were chromatographically distinct as judged by the lysine acceptor activity. With regard to determining the position

Figure 16: COLUMN CHROMATOGRAPHY OF ^{14}C-LYSYL-tRNA ON BD-CELLULOSE: BULK PREPARATION

The procedure was that described in fig. 15 except that the NaCl concentration was reduced to 1.2 M. 2100 A_{260} units of ^{14}C-lysyl-tRNA (260, 103 cpm) were applied to the column. The flow rate was 36 ml/hr. Fraction volume was 5.5 ml. Column dimensions were 1.6 x 40 cm.

Recovery of A_{260} units: 96%

Recovery of cpm: 93%

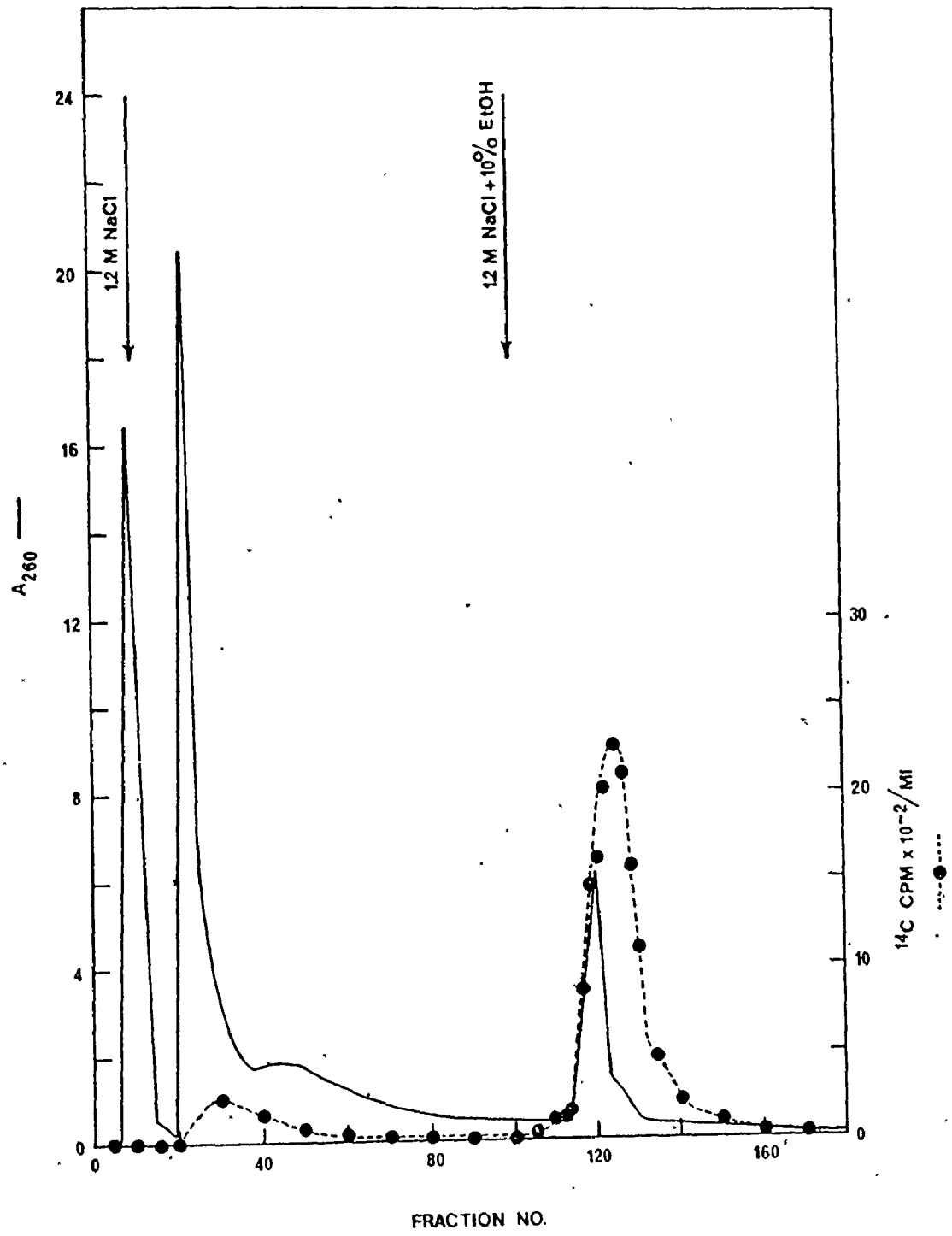

1.2 M NaCl
1.2 M NaCl + 10% EtOH
A_{260}
^{14}C CPM x 10^{-2}/Ml
FRACTION NO.
0
4
8
12
16
20
24
0
40
80
120
160
10
20
30

Figure 17: CHROMATOGRAPHY OF UNCHARGED tRNA ON BD-CELLULOSE

The procedure was the same as that described in fig. 16. 123 A_{260} units were applied to a 1.5 x 30 cm BD-cellulose column. The flow rate was 36 ml/hr; 2.5 ml fractions were collected. The lysine acceptor activity was determined using the incubation mixture described in Table 5. 1 ml aliquots from the ethanol fractions were dialyzed against 1.2 M NaCl buffer (minus the ethanol) before use in the charging reaction.

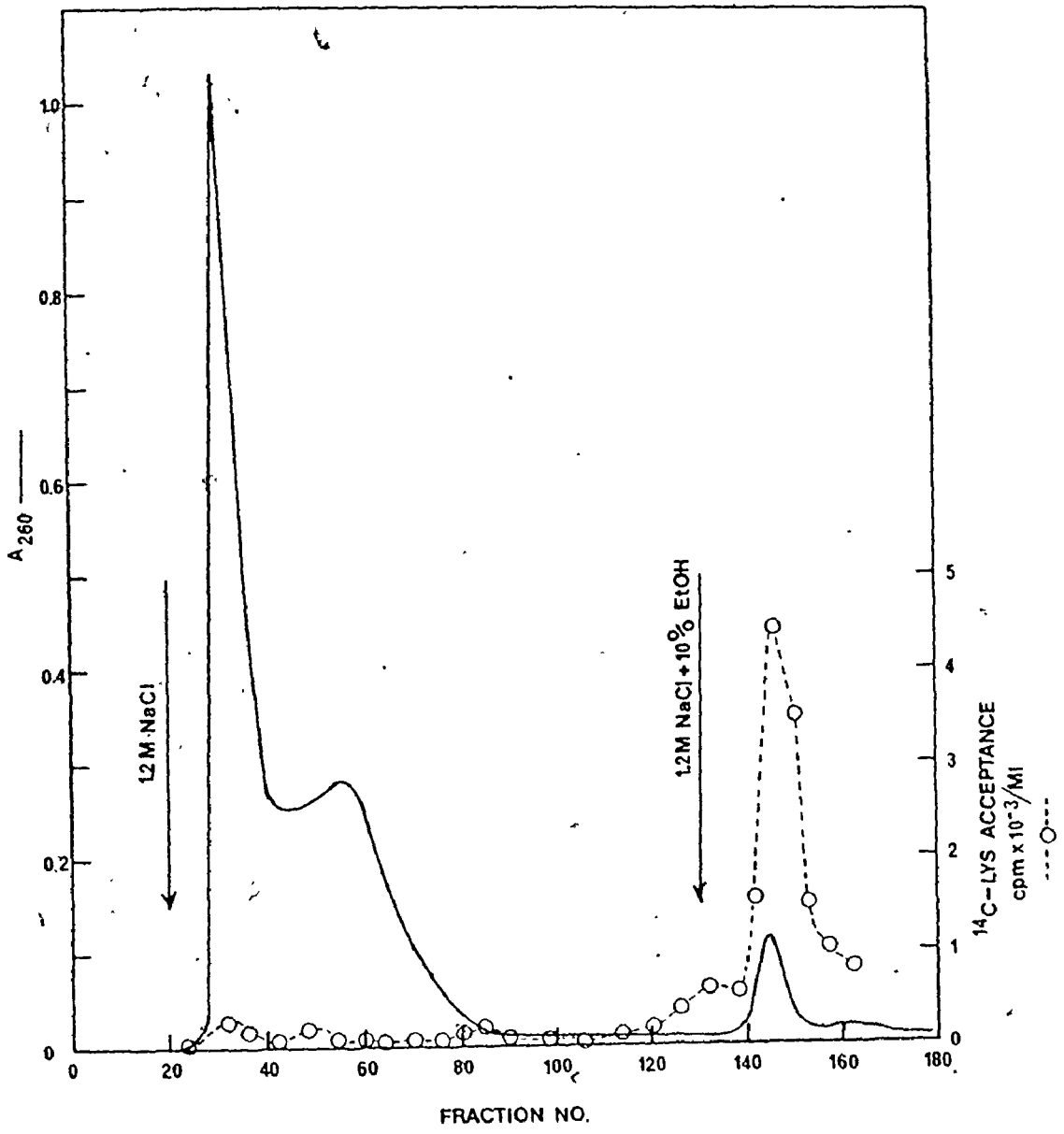
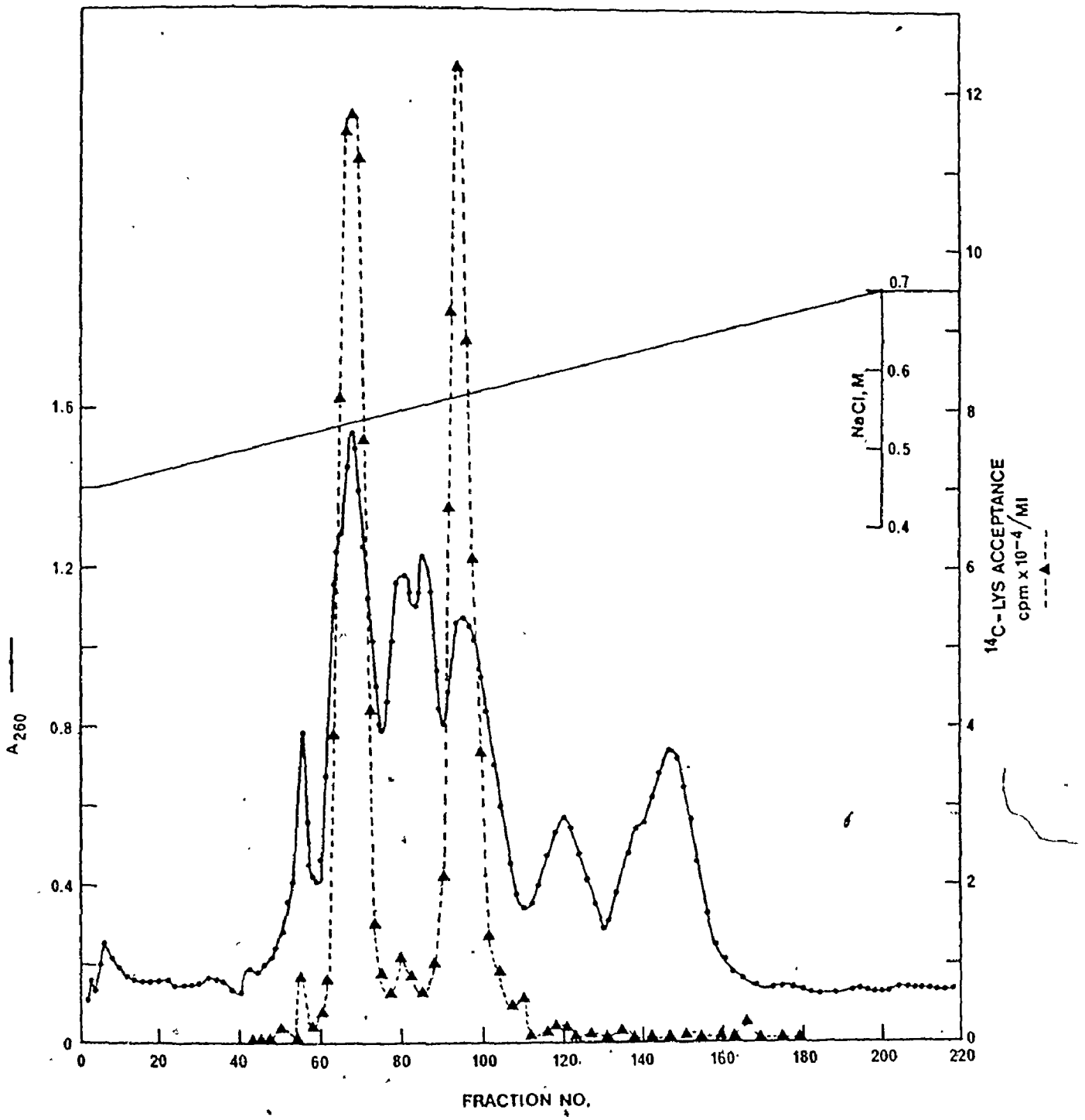

1.0
0.8
0.6
0.4
0.2
0
A_{260}
1.2 M NaCl
1.2M NaCl + 10% EtOH
0
20
40
60
80
100
120
140
160
180
FRACTION NO.
5
4
3
2
1
0
^{14}C-LYS ACCEPTANCE
cpm x 10^{-3}/Ml

Figure 18: PURIFICATION OF $tRNA^{Lys}$ BY RPC-5 CHROMATOGRAPHY

528 A_{260} units of partially purified $tRNA^{Lys}$ obtained by BD-cellulose chromatography was charged onto a reversed phase chromatography V column (1 x 100 cm) previously equilibrated with a buffer containing 0.01 M Tris-HCl (pH 7.0), 0.01 M $MgCl_2$, 0.001 M 2-mercapto-ethanol and 0.45 M NaCl. Elution was with a linear gradient of NaCl (0.45 M to 0.7 M) in 2 l of the above buffer. Flow rate was 60 ml/hr; fraction volume was 10 ml. The operating temperature was 37°C.


A260
14C-LYS ACCEPTANCE
cpm x 10-4/Ml
NaCl, M
0.7
0.6
0.5
0.4
1.6
1.2
0.8
0.4
0
12
10
8
6
4
2
0
0
20
40
60
80
100
120
140
160
180
200
220
FRACTION NO.

of the two species of $tRNA^{Lys}$ it is perhaps well to point out that the reaction mixture shown in Table 5 was used. Although the condition described in Table 5 did not give complete aminoacylation of tRNA, they were retained since, for optimal aminoacylation, the amount of L-lysine in the reaction mixture would have had to be increased significantly with cold amino acid concomitantly reducing the specific activity of the label and hence, having to deal with smaller values of radioactivity above background. The method used proved to be satisfactory and avoided the tedium of removing aliquots of the fractions to be assayed for dialysis against solution D'. Furthermore, it was essential to pool and process the appropriate fractions as quickly as possible in order to avoid undue loss of activity of the purified tRNA species. Although, potentially, the problem of using low specific activity ^{14}C-label could have been circumvented by using high specific activity ^{3}H-labelled lysine, it was found that under the high salt conditions quenching became a problem with ^{3}H.

$tRNA_1^{Lys}$ eluted with approximately 0.52 M NaCl and $tRNA_2^{Lys}$ with 0.57 M NaCl. The fractions containing the two $tRNA^{Lys}$s were pooled separately and precipitated. The specific acceptor activities of the two species were determined using the optimized charging conditions. The relative proportions of the two species was estimated from their specific activities and the amount of A_{260} absorbing material having lysine acceptance. $tRNA_1^{Lys}$ and $tRNA_2^{Lys}$

were in the approximate ratio of 3:2 and this ratio was consistent in four different preparations. $tRNA_1^{Lys}$ and $tRNA_2^{Lys}$ were further purified in the reverse phase chromatography system using shallower gradients as illustrated in Figures 19 and 20, respectively.

h) Acceptor Activity of tRNA Samples

The activity of the purified $tRNA^{Lys}$ was defined as the acceptance of ^{14}C-lysine per A_{260} unit of tRNA; 1800 pmoles/A_{260} unit was assumed to be 100% pure (Ghosh et al., 1971). The results in Table 14 show that when a preparation of lysine tRNA was assayed for amino acid acceptance with different enzyme preparations, the values varied considerably. The two $tRNA^{Lys}$ species, chromatographed twice on the RPC-5 system, did not give acceptance values greater than 1200 pmoles/A_{260} unit. When two different $tRNA_1^{Lys}$ pools were checked for glycine, isoleucine, threonine and histidine acceptance, only 12 pmoles and 16 pmoles ^{14}C-histidine/A_{260} unit and 5 and 12 pmoles of ^{14}C-threonine/A_{260} unit were accepted. The $tRNA_2^{Lys}$ pools accepted only 9 and 6 pmoles ^{14}C-histidine/A_{260} unit. One possible explanation for the erratic results was that there was some sort of degradative activity in the enzyme preparations. If this was indeed the case, then in a time course experiment a stable plateau of cold TCA precipitable radioactivity would not be observed. Figure 21 shows the result of such

Figure 19: FURTHER PURIFICATION OF $tRNA_1^{Lys}$ BY RPC-5 CHROMATOGRAPHY

55 A_{260} units of $tRNA_1^{Lys}$ obtained by fractionation on an RPC-5 column were charged onto a 0.7 x 70 cm column equilibrated with the buffer described in figure 18. Elution was with a linear gradient of NaCl (0.45 M to 0.55 M) in 700 ml of buffer. The operating temperature was 37°C and 3.5 ml fractions were collected.

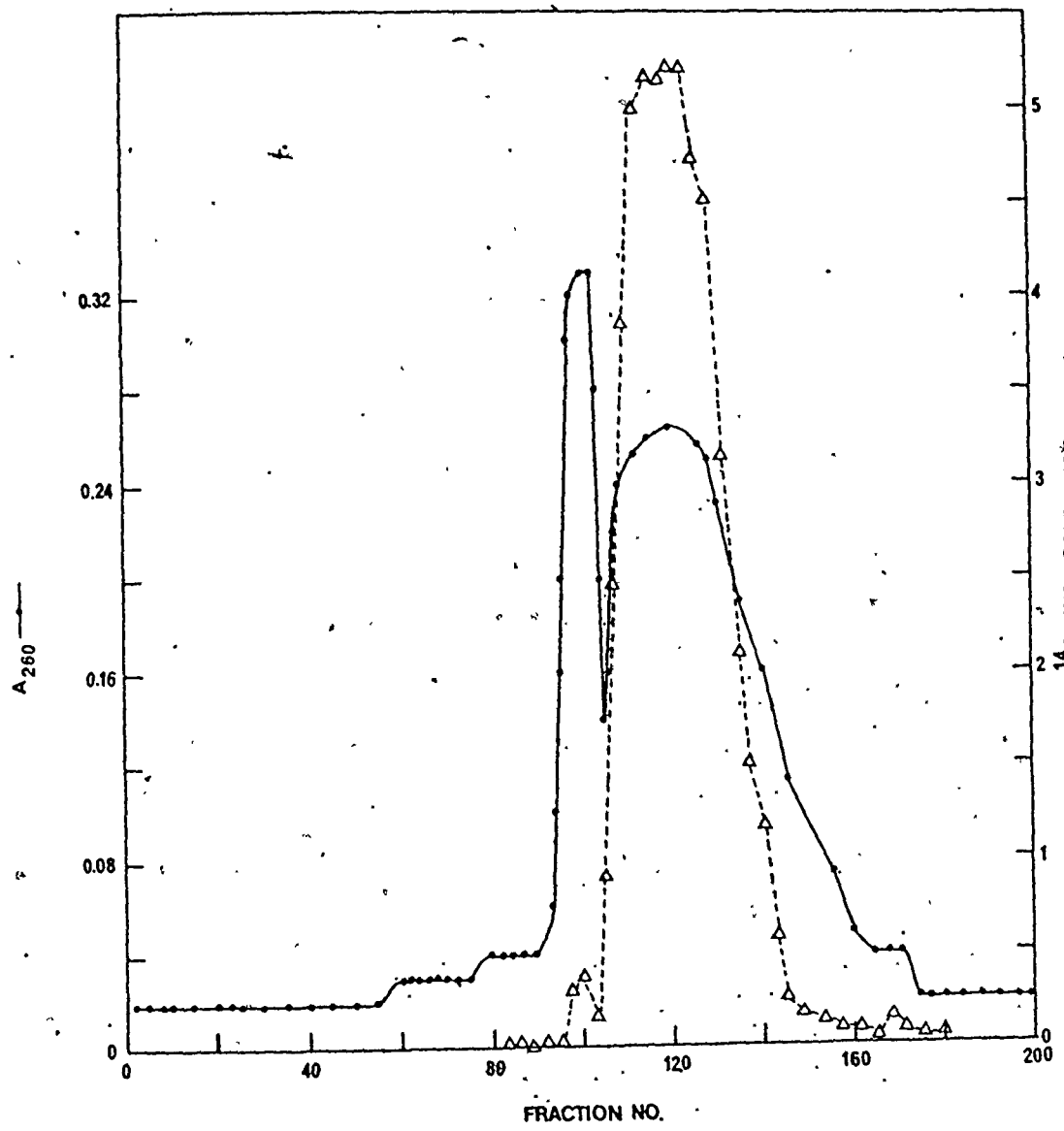
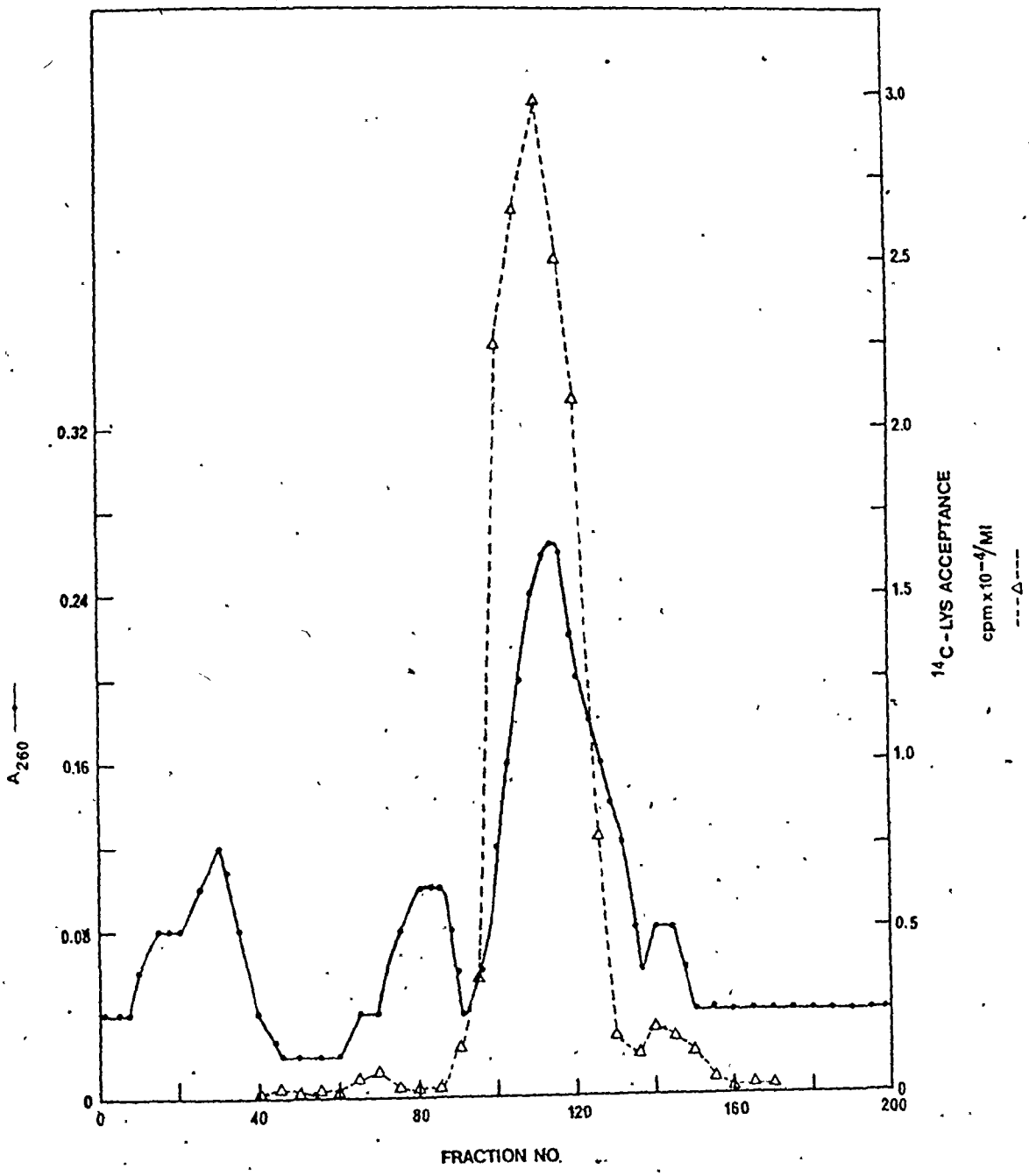

A260
0.32
0.24
0.16
0.08
0
0
40
80
120
160
200
FRACTION NO.
14C-LYS ACCEPTANCE
cpm x10-4/MI
5
4
3
2
1
0

Figure 20: FURTHER PURIFICATION OF $tRNA_2^{Lys}$ BY RPC-5 CHROMATOGRAPHY

36 A_{260} units of $tRNA_2^{Lys}$ were charged onto a 0.7 x 70 cm column. The conditions were as described in figure 19 except that elution was with a NaCl gradient (0.45 M to 0.65 M) in 500 ml of the buffer. Fraction volume was 2.5 ml.


0.32
0.24
0.16
0.08
0
A_{260}
3.0
2.5
2.0
1.5
1.0
0.5
0
^{14}C-LYS ACCEPTANCE
cpm x10^{-4}/Ml
0
40
80
120
160
200
FRACTION NO.

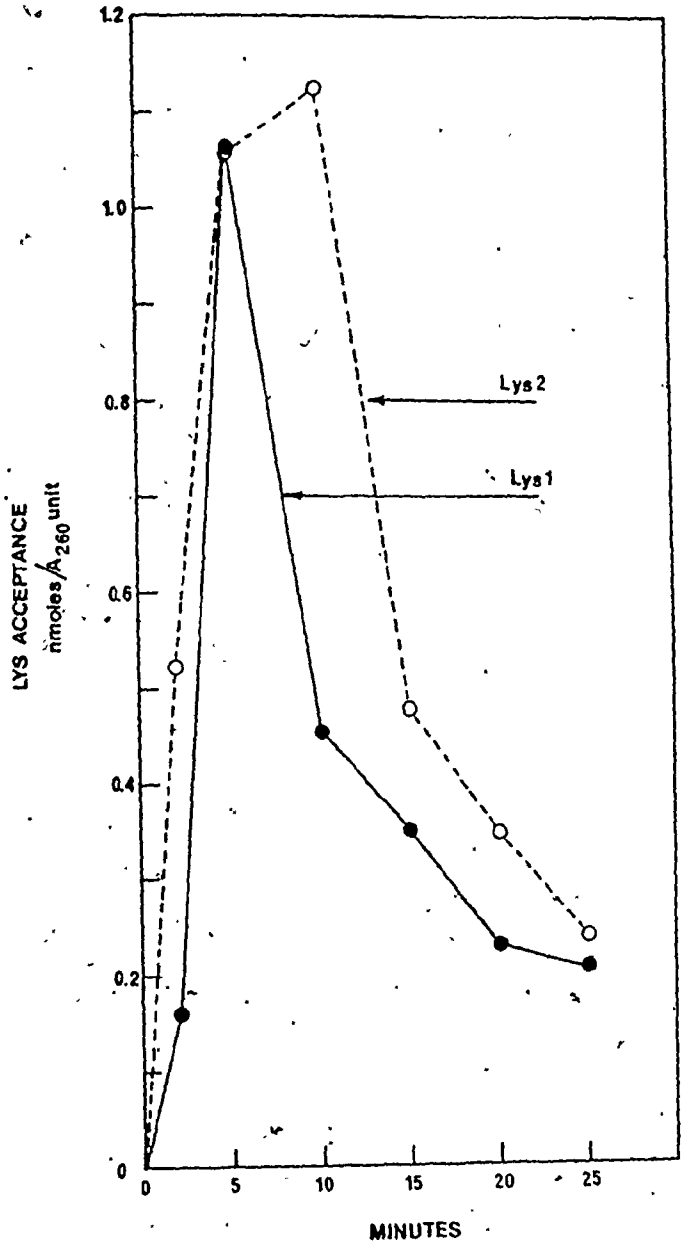


Figure 21: TIME COURSE OF CHARGING: $tRNA_1^{Lys}$ AND $tRNA_2^{Lys}$

The incubation mixture described in Table 6 was used with pH 5 enzyme as a source of synthetases. 0.04 A_{260} unit of $tRNA_1^{Lys}$ and 0.05 A_{260} unit of $tRNA_2^{Lys}$ were used.. Each point is the average of a set of duplicates. Specific activity

an experiment which clearly demonstrated loss of the aminoacyl-tRNAs. When charging was in the presence of 60 µg ribosomal RNA per incubation mixture, the loss of aminacyl-tRNA was reduced but not eliminated. Similar results have been obtained for purified methionine tRNA when crude synthetases were used for obtaining acceptance values (Patel and Bayley, unpublished results). In order to obtain reliable amino acid acceptance values, an attempt was made to purify lysyl-tRNA synthetase by affinity chromatography. Lysyl-tRNA synthetase was obtained from an S-150 extract as shown in figure 22. From the profile it is evident that only some of the lysyl-tRNA synthetase was retarded by the column and eluted later than the bulk of the A_{280} absorbing material. When a larger column was employed, the result was similar. No attempt was made to optimize the chromatographic conditions for better recovery of the synthetase since the material from these columns was satisfactory as shown by the results in Table 14. Amino acid acceptance values were consistent and higher with this preparation. After BD-cellulose and reversed phase chromatography, almost a 50-fold purification of the two lysine tRNA species was achieved.

i) Sephadex G-100 Chromatography and Aminoacylation of Native and Renatured Lysine tRNAs

Since S-150 preparations were used for charging tRNA for BD-cellulose chromatography; it was necessary to show that the presence of two species of $tRNA^{Lys}$ was not the

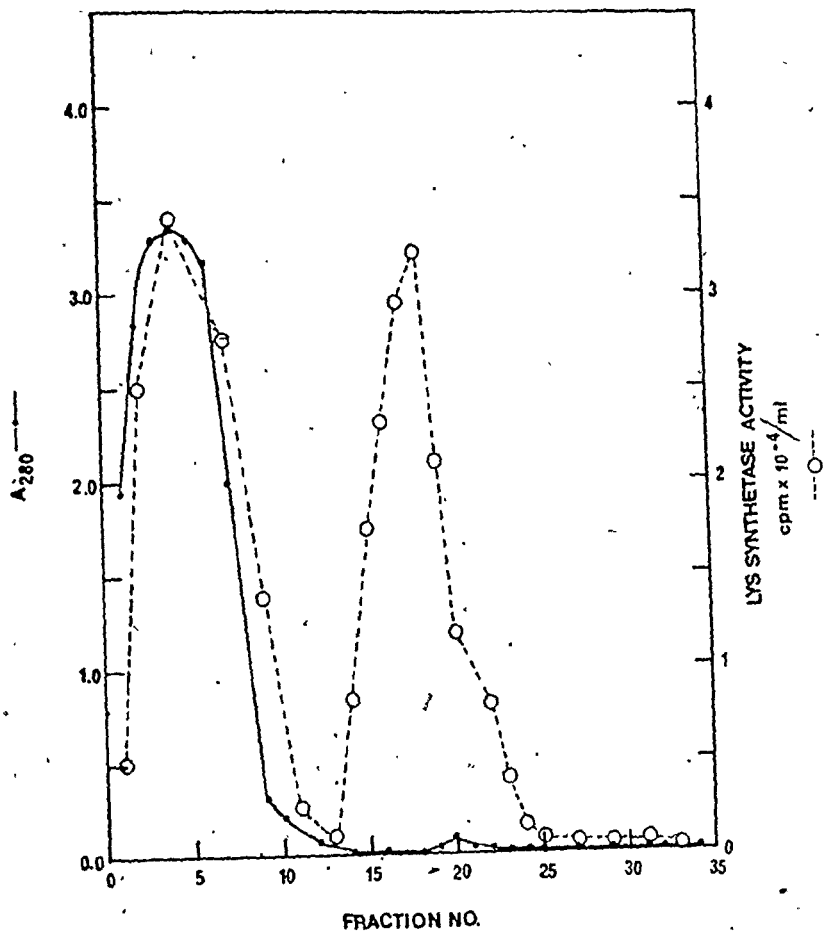


Figure 22: PURIFICATION OF LYSYL-tRNA SYNTHETASE BY AFFINITY CHROMATOGRAPHY

1 ml of a S-150 preparation was loaded onto a 0.5 x 15 cm affinity column, previously equilibrated in solution D'. Elution was with solution D' at a flow rate of 12 ml/hr. Fraction volume was 1 ml. 100 µl of each fraction were assayed. 0.5 A_{260} unit of crude tRNA was used. Specific activity of ^{14}C-lysine was 312 mCi/mMole.

Table 14

Each 0.225 ml reaction mixture contained the components described in Table 6. With the G50, pH 5.0 and S-150 enzymes, 0.05 A_{260} units of $tRNA_1^{Lys}$ and 0.03 A_{260} units $tRNA_2^{Lys}$ were used. With the purified lys-synthetase, 0.02 A_{260} unit of each species was added. The conditions for incubation were as described in "Methods".

Table 14: AMINO ACID ACCEPTANCE OF PURIFIED tRNAs WITH DIFFERENT SYNTHETASE PREPARATIONS

SYNTHETASE PREP	tRNA SAMPLE	^{14}C-LYS ACCEPTANCE pmoles/A_{260} unit	PURIFICATION
S-150	UNFRACTIONATED	32.7	1
G50	BD-CELLULOSE	331.4	
pH5		326.1	
S-150		329.8	
	RPC-5 (POOL 1)	LYS 1 / LYS 2	
G50(2)		926 / 1012	
pH5(1)		1093 / 1213	
pH5(2)		757 / 932	
G50(1)		312 / 446	
	RPC-5 (POOL 2)		
G50(1)		1062 / 1125	
G50(2)		491 / 503	
pH5(1)		1179 / 1250	
pH5(2)		841 / 1087	
LYS SYNTHETASE (affinity column)	BD-CELLULOSE	372 368	11
		LYS 1 / LYS 2	
	RPC-5 (POOL 1)	1224 / 1361	40
		1257 / 1390	
	RPC-5 (POOL 2)	1547 / 1594	49
		1596 / 1601	

result of nicked tRNA. One convenient method to show that the two species were intact was to denature the tRNAs in the presence of 0.5% (v/v) formaldehyde which hydroxymethylated cytosine residues on heating. This treatment prevented reannealing when the samples were cooled (Boedtker, 1967). Chromatography of the samples on Sephadex G-100 (fine) showed that the elution pattern of the native and denatured tRNAs was not much different (Figure 23). These results indicated that the tRNA species were intact. The results in Table 15 showed that there was a loss of hypochromicity on heating in the absence of formaldehyde but there was reannealing after 24 hours. The heating of tRNA samples with formaldehyde, however, resulted in the permanent loss of hypochromicity. The charging data (Table 16) indicated that most of the reannealed tRNA could be aminoacylated. Optimum conditions for reannealing were not worked out and would be a likely explanation for not obtaining complete charging (Fresco et al., 1966).

SECTION III. Properties of the two $tRNA^{Lys}$ species

a) Difference in the 3'-terminal Oligonucleotide Sequences

The structural differences between the two lysine tRNAs were investigated by analyzing the 3'-terminal oligonucleotide fragments obtained by hydrolyzing $tRNA^{Lys}$ charged with radioactive lysine, with T_1 ribonuclease. A mixture of ^{14}C-lysyl-$tRNA_1^{Lys}$ and ^{3}H-lysyl-$tRNA_2^{Lys}$ was

Figure 23: CHROMATOGRAPHY OF NATIVE AND DENATURED $tRNA^{Lys}$ ON SEPHADEX G-100

a) 5.5 A_{260} units of native $tRNA_1^{Lys}$ were loaded onto a 0.9 x 30 cm Sephadex G-100 column equilibrated in 0.5 M NaCl and 0.01 M Tris-HCl (pH 7.2). The sample volume was 0.5 ml. Flow rate was 8 ml/hr, 1 ml fractions being collected.

b) 7.5 A_{260} units of denatured $tRNA_1^{Lys}$ in 0.5 ml were charged onto the column. The procedure was identical to that described above.

c) 6.1 A_{260} units of $tRNA_2^{Lys}$ (denatured) in 0,5 ml were charged onto the column. The procedure was as described above.

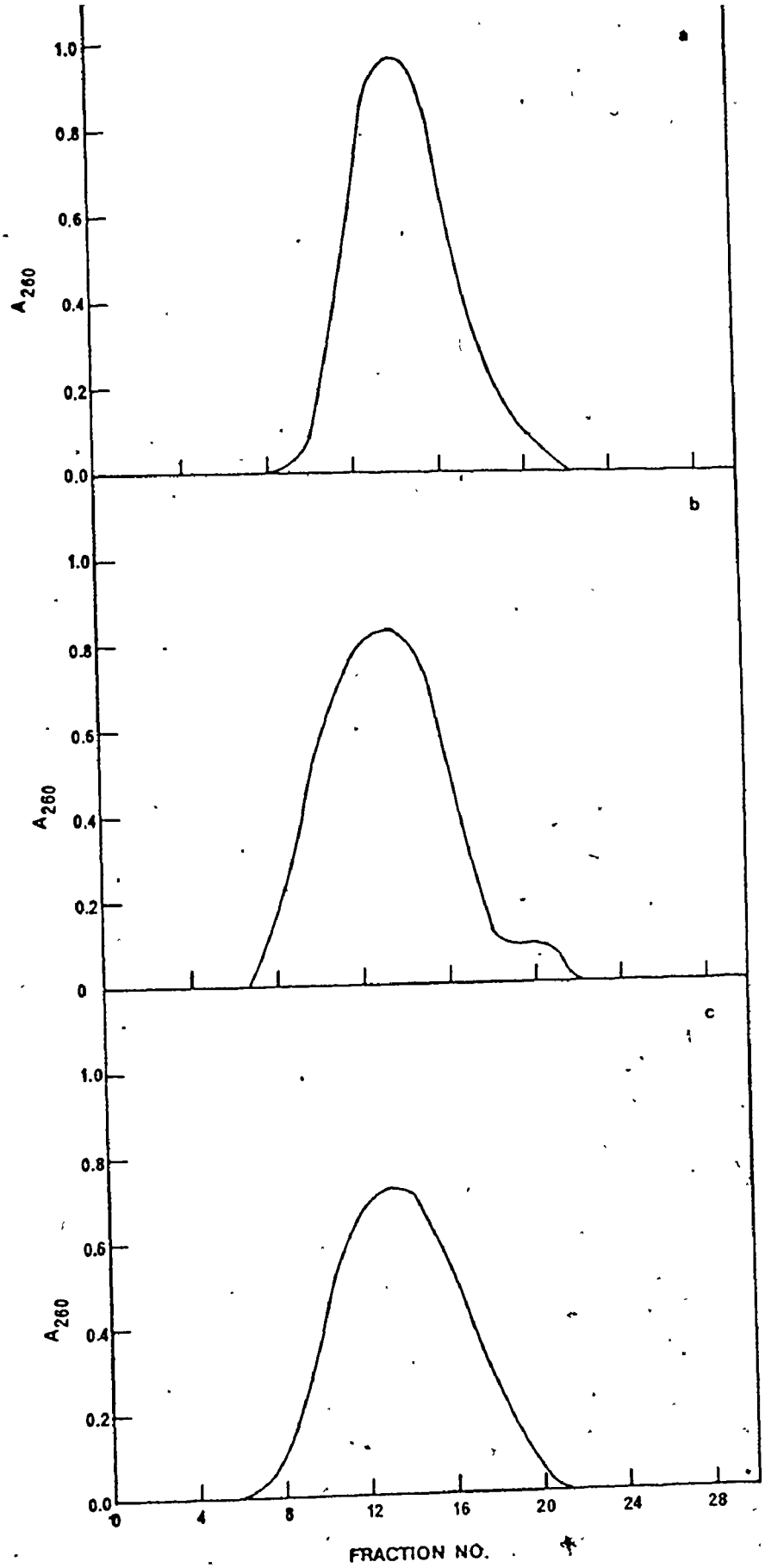

a
b
c
A_{260}
1.0
0.8
0.6
0.4
0.2
0.0
0
4
8
12
16
20
24
28
FRACTION NO.

Table 15: ULTRAVIOLET ABSORBANCE OF NATIVE AND FORMALDEHYDE DENATURED LYSINE tRNAs

	ABSORBANCE			
$tRNA_1^{Lys}$ (918 pmoles/A_{260})	250	260	270	280
NATIVE	0.96	1.09	0.94	0.62
RENATURED	0.96	1.11	0.97	0.62
DENATURED in 0.5% formaldehyde	1.16	1.35	1.21	0.81
NATIVE in 0.5% formaldehyde	0.94	1.07	0.91	0.51
* NATIVE (80°C)	1.09	1.28	1.15	0.83
$tRNA_2^{Lys}$ (1012 pmoles/A_{260})				
NATIVE	0.72	0.83	0.71	0.56
RENATURED	0.73	0.86	0.73	0.56
DENATURED in 0.5% formaldehyde	0.91	1.08	0.99	0.76
NATIVE in 0.5% formaldehyde	0.72	0.82	0.69	0.40
* NATIVE (80°C)	0.83	0.98	0.91	0.72

All samples were in 1/5 x D'. Denaturation in the presence of formaldehyde was carried out at 100°C for 3 min. followed by rapid cooling on ice for 30 min. Renatured tRNA was prepared by heating the sample in the absence of formaldehyde for 3 min. and allowing it to cool at room temperature for 24 hr. Native tRNA in 0.5% formaldehyde was treated in the same manner except heating at 100°C was omitted.

* tRNA in 1/5 x D' was heated for 3 min at 100°C. When the temperature of the sample was approximately 80°C these measurements were made.

Table 16: ACCEPTOR ACTIVITY OF NATIVE AND REANNEALED LYSINE tRNAs

	NATIVE	REANNEALED
$tRNA_1^{Lys}$	918 pmoles/A_{260} unit (100%)	703 pmoles/A_{260} unit (76.5%)
$tRNA_2^{Lys}$	1012 pmoles/A_{260} unit (100%)	867 pmoles/A_{260} unit (80.5%)

tRNA samples in 1/5 x D' were heated to 100°C for 2 min. in the absence of formaldehyde. The samples were left at room temperature for 24 hours before use in the charging reaction.

digested with T_1 RNase and the oligonucleotides formed were chromatographed on a DEAE-sephadex column as described in Methods. The results are shown in Figure 24. Both the ^{14}C- and ^{3}H-radioactivities emerged at the 0.01 M ammonium formate wash. This result showed that the T_1 oligonucleotides from the 3'-terminus of the two $tRNA^{Lys}$ were the same both in their size and in the location of the first G-residue from the 3'-end of the tRNA. In support of this result, unfractionated tRNA was charged with ^{14}C-lysine and treated in the same way as described above. Again, all the ^{14}C-radioactivity emerged at the 0.01 M ammonium formate wash.

b) Recognition of $tRNA_1^{Lys}$ and $tRNA_2^{Lys}$ by E. coli Synthetases

The two lysine tRNAs were charged by H. cutirubrum and E. coli aminoacyl-tRNA synthetases. The results in Table 17 show that both tRNA species were recognized by the heterologous enzyme. Although a quantitative comparison of the level of charging of the tRNAs in the homologous and heterologous system is not valid since the heterologous system was not optimized, the results do indicate that $tRNA_1^{Lys}$ was charged to a greater extent by the E. coli synthetase than $tRNA_2^{Lys}$, under identical conditions. This difference in the recognition of the two lysine tRNAs by the heterologous synthetase could reflect structural differences.

Figure 24: CO-CHROMATOGRAPHY OF RNASE T_1 DIGEST OF ^{14}C-LYS-$tRNA_1^{Lys}$ AND ^{3}H-LYS-$tRNA_2^{Lys}$

66, 704 cpm of ^{14}C-lys-$tRNA_1^{Lys}$ (237 Ci/mole) and 33, 138 cpm of ^{3}H-lys-$tRNA_2^{Lys}$ (4380 Ci/mole) were mixed with 0.5 mg crude tRNA in the incubation mixture containing RNase T_1 described in 'Methods'. The digested tRNA was charged onto a DEAE-Sephadex A25 column (0.5 x 15 cm) equilibrated with 0.01 M ammonium formate (pH 5.0), eluted with 2.0 ml of 0.01 M ammonium formate (pH 5.0); the column was then eluted with a linear gradient of ammonium formate from 0.01 M (50 ml) to 1.0 M (50 ml). 1 ml fractions were colleoted and counted for ^{14}C-lysine and ^{3}H-lysine by adding the fractions to 10 ml Aquasol.

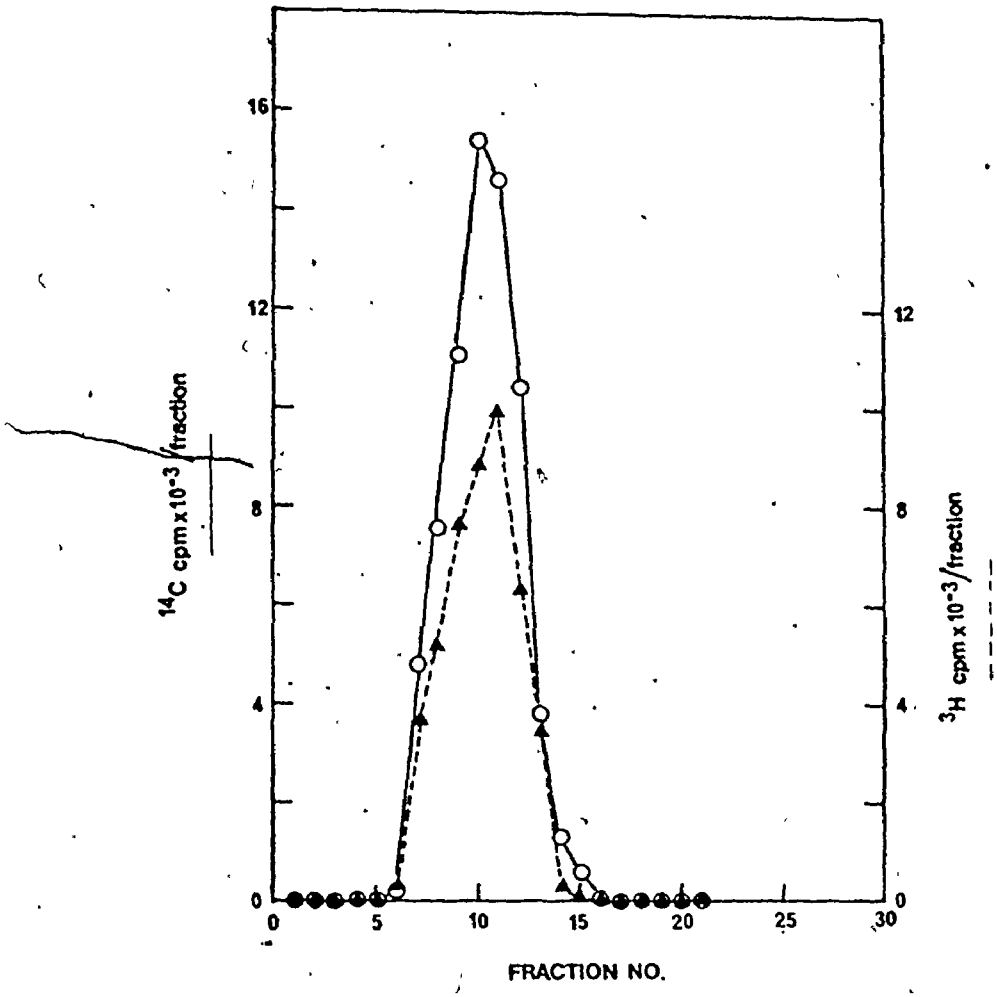

16
12
8
4
0
0
5
10
15
20
25
30
FRACTION NO.
^{14}C cpm x 10^{-3}/fraction
^{3}H cpm x 10^{-3}/fraction

Table 17: RECOGNITION OF $tRNA_1^{Lys}$ AND $tRNA_2^{Lys}$ BY *E. coli* SYNTHETASES

	^{14}C-LYSINE INCORPORATED pmoles/A_{260} unit tRNA	
	E. coli Enzyme	*H. cutirubrum* Enzyme
$tRNA_1^{Lys}$	705	1486
$tRNA_2^{Lys}$	169	1591

The reaction mixtures described in Methods were used. In the heterologous charging mixture 0.35 A_{260} units of $tRNA_1^{Lys}$ and 0.30 A_{260} units of $tRNA_2^{Lys}$ were added. The mixture was incubated at 37°C for 20 min with *E. coli* S-150. In the homologous system 0.02 A_{260} units of the two lysine tRNAs were added and incubated at 37°C for 30 min with lysyl-tRNA synthetase purified by affinity chromatography.

c) Coding Properties of $tRNA_1^{Lys}$ and $tRNA_2^{Lys}$ in High Salt

The coding properties of the two lysine tRNAs were investigated by *in vitro* ribosomal binding experiments using the partially hydrolyzed polyribonucleotides poly(A), poly(A:G :: 1.6:1) and poly(A:G :: 1:1). From the results in Table 18, it is evident that both $tRNA^{Lys}$ species responded almost equally well to poly(A), and hence to the codon AAA. Figure 25a shows the effect of an increasing concentration of partially hydrolyzed poly(A) on the amount of the two ^{14}C-lys-tRNAs bound to ribosomes. This result confirmed that in Table 18. In Figure 25a the slight decrease in binding observed with greater amounts of poly(A) was probably due to some precipitation of the polymer in the high salt. Table 18 and Figures 25 b and c show the binding results with poly(A:G), $tRNA_1^{Lys}$ responded better than $tRNA_2^{Lys}$ to poly(A:G). The frequency of the two lysine codons, AAA and AAG, in poly(A:G :: 1.6:1) was calculated to be 38%. The codon AAA consituted 60% and the codon AAG, 40% of the total lysine codons in this polymer. The efficiency of binding of $tRNA_2^{Lys}$ to this polymer was only 50-67% of the efficiency of $tRNA_1^{Lys}$ as seen in Table 18 and Figure 25b. With poly(A:G :: 1:1) (Figure 25c), $tRNA_2^{Lys}$ responded with 47-52% of the efficiency of $tRNA_1^{Lys}$. In this polymer, the codons AAA and AAG would be equally frequent. Since the binding of the two $tRNA^{Lys}$ species reflects the frequencies of the two codons in the copolymers, the

Table 18: RIBOSOMAL BINDING OF ^{14}C-LYS-tRNA$_1^{Lys}$ AND ^{14}C-LYS-tRNA$_2^{Lys}$ AS STIMULATED BY POLY(A) AND POLY (A:G) IN THE HOMOLOGOUS SYSTEM

pmoles ^{14}C-lys-tRNA^{Lys} bound to ribosomes

	NONE†	+POLY(A)	NET	
^{14}C-LYS-tRNA$_1^{Lys}$	0.16	0.76	0.60	
^{14}C-LYS-tRNA$_2^{Lys}$	0.18	0.68	0.50	
	NONE†	+POLY(A:G::1.6:1)	NET	% LYS CODONS
^{14}C-LYS-tRNA$_1^{Lys}$	0.16	0.54	0.38	38% [AAA=60% AAG=40%]
^{14}C-LYS-tRNA$_2^{Lys}$	0.18	0.37	0.19	
	NONE†	+POLY(A:G::1:1)	NET	% LYS CODONS
^{14}C-LYS-tRNA$_1^{Lys}$	0.16	0.37	0.21	25% [AAA=50% AAG=50%]
^{14}C-LYS-tRNA$_2^{Lys}$	0.18	0.27	0.09	

Each 0.125 ml reaction mixture had the components described. 8.3 pmoles of ^{14}C-lys-tRNA$_1^{Lys}$ and 8.1 pmoles of ^{14}C-lys-tRNA$_2^{Lys}$ were added and 2.3 A_{260} units of H. cutirubrum ribosomes. 25 µg of the different partially hydrolyzed polymers were used. The values are the average of two experiments performed in duplicate.

† -POLY(A)

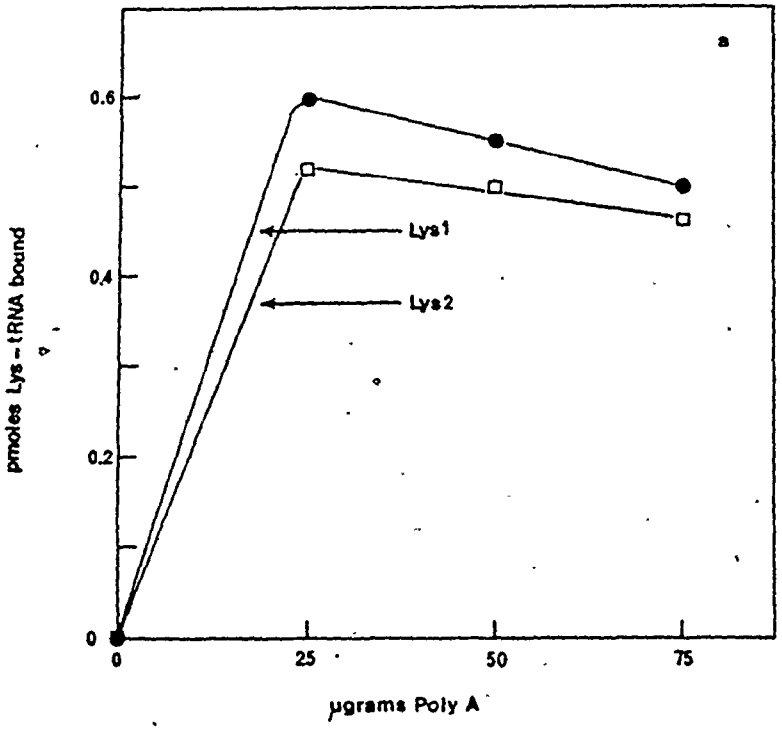
Figure 25: THE EFFECT OF VARYING CONCENTRATION OF POLYRIBONUCLEOTIDE ON THE BINDING ^{14}C-LYS-tRNA$_1^{Lys}$ AND ^{14}C-LYS-tRNA$_2^{Lys}$ TO *H. cutirubrum* RIBOSOMES

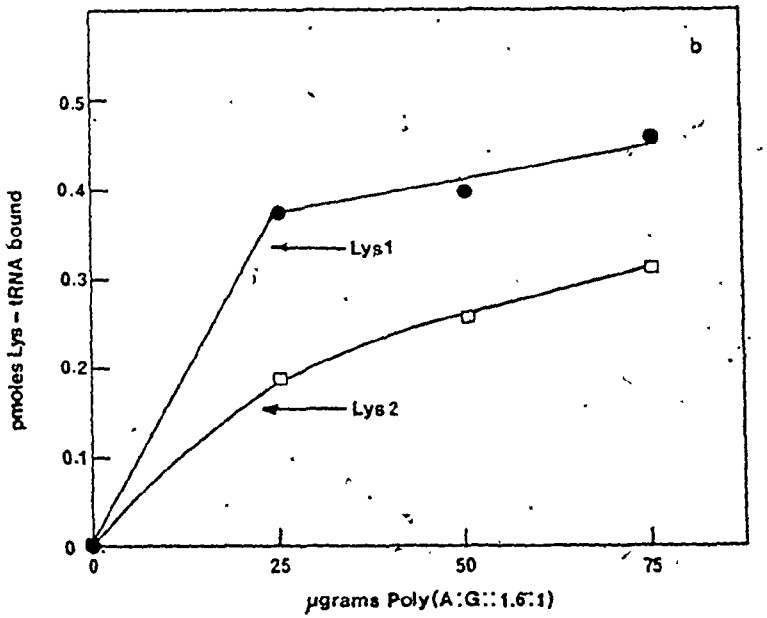
8.3 pmoles of ^{14}C-lys-tRNA$_1^{Lys}$ and 8.1 pmoles of ^{14}C-lys-tRNA$_2^{Lys}$ were added to each reaction mixture and 2.3 A_{260} units of ribosomes. Incubation was at 37°C for 30 min. Each point represents the average of a set of duplicates. Binding of the ^{14}C-lys-tRNAs^{Lys} in the absence of polynucleotide has been subtracted (0.17 pmoles ^{14}C-lys-tRNA$_1^{Lys}$ and 0.19 pmoles ^{14}C-lys-tRNA$_2^{Lys}$ bound to ribosomes in the absence of template).

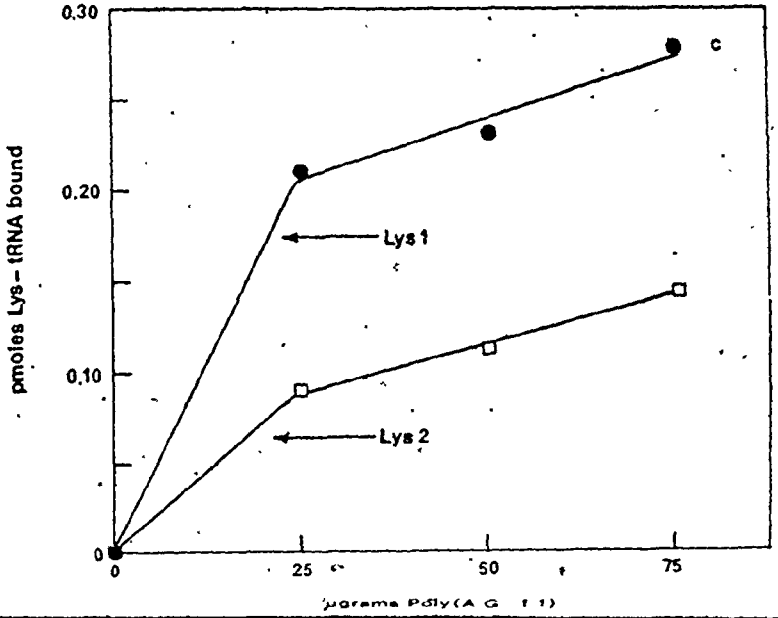
a) Partially hydrolyzed Poly(A)

b) Partially hydrolyzed Poly(A:G::1.6:1)

c) Partially hydrolyzed Poly(A:G::1:1)


a
pmoles Lys – tRNA bound
0.6
0.4
0.2
0
Lys1
Lys2
0
25
50
75
µgrams Poly A


b
pmoles Lys – tRNA bound
0.5
0.4
0.3
0.2
0.1
0
Lys1
Lys 2
0
25
50
75
µgrams Poly(A:G::1.6:1)


c
pmoles Lys – tRNA bound
0.30
0.20
0.10
0
Lys 1
Lys 2
0
25
50
75

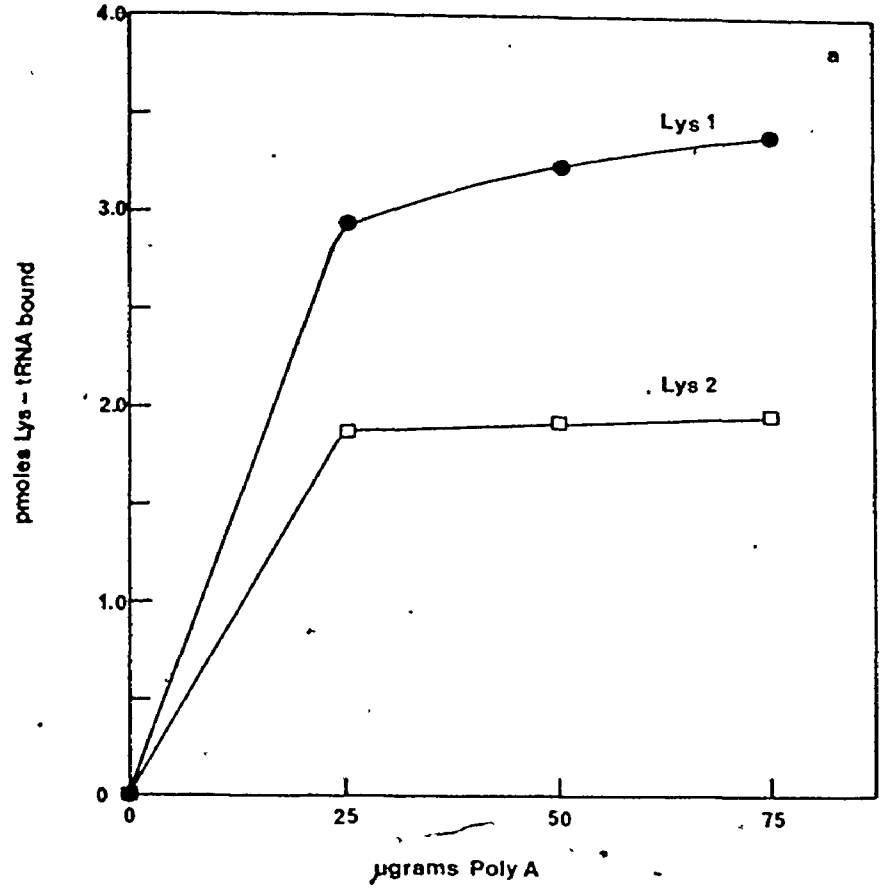
results suggested that while $tRNA_1^{Lys}$ responds to both AAA and AAG, $tRNA_2^{Lys}$ responds only to AAA.

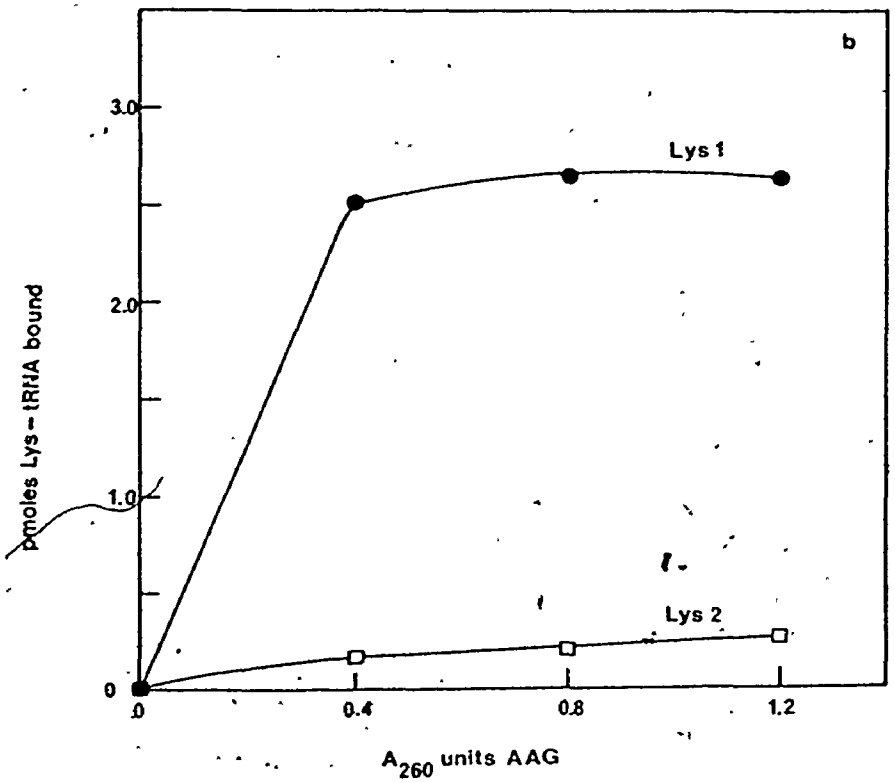
d) Binding of $tRNA_1^{Lys}$ and $tRNA_2^{Lys}$ to E. coli Ribosomes in Low Salt

The coding properties of the two lysine tRNAs were investigated in the E. coli system using partially hydrolyzed poly(A) and the trinucleotide AAG. With partially hydrolyzed poly(A) (Figure 26a), $tRNA_2^{Lys}$ responded with approximately 55% of the efficiency of $tRNA_1^{Lys}$. This result was in contrast to that observed in the high salt system. With AAG (Figure 26b), $tRNA_2^{Lys}$ responded with only 10% (approximately) of the efficiency of $tRNA_1^{Lys}$, again suggesting that $tRNA_2^{Lys}$ does not respond to AAG. Neither $tRNA_1^{Lys}$ nor $tRNA_2^{Lys}$ responded to the codon ApApU (results not shown).

Figure 26: BINDING OF $tRNA_1^{Lys}$ AND $tRNA_2^{Lys}$ TO E. coli RIBOSOMES IN LOW SALT IN RESPONE TO PARTIALLY HYDROLYZED POLY(A) AND AAG.

8.3 pmoles of ^{14}C-lys-$tRNA_1^{Lys}$ and 8.1 pmoles of ^{14}C-lys-$tRNA_2^{Lys}$, and 2.68 A_{260} units of E. coli ribosomes were added to the reaction mixture described in Methods. Incubation was at 24°C for 20 min. Binding of ^{14}C-lys-tRNAs in the absence of template has been subtacted (0.41 pmoles ^{14}C-lys-$tRNA_1^{Lys}$ and 0.37 pmoles ^{14}C-lys-$tRNA_2^{Lys}$ bound to ribosomes in the absence of template). Each point represents the average of a set of duplicates.


a
Lys 1
Lys 2
pmoles Lys – tRNA bound
4.0
3.0
2.0
1.0
0
0
25
50
75
μgrams Poly A


b
Lys 1
Lys 2
pmoles Lys – tRNA bound
3.0
2.0
1.0
0
0.4
0.8
1.2
A260 units AAG

IV. Discussion

The intent of this thesis was to extend the work on codon assignments in H. cutirubrum for some of the acidic and basic amino acids for the reasons discussed in the introduction. $tRNA^{Lys}$ species were also purified with the aim of studying codon-anticodon response with partially hydrolyzed poly(A) and poly(A,G).

The experiments on codon assignments were unsuccessful because of the difficulties encountered with the H. cutirubrum ribosomal binding system using trinucleotides as templates. The response of unfractionated aspartyl-, asparaginyl-, glutamyl-, histidinyl- and lysyl-tRNA to the codons GAU, GAC, AAU, AAC, GAA, GAG, CAU, CAC and AAG was negligible in the high salt system. Of these only lysyl-tRNA responded to partially hydrolyzed poly(A) confirming the result of White and Bayley (1972b). The use of other copolymers in this work was precluded since those containing adenine and cytosine precipitated out in the high salt (Bayley and Griffiths, 1968b). Attempts at improving the binding reaction by varying the conditions of the incubation mixture were unsuccessful. Two of these are of interest. It was thought that under mild ionic conditions which preserved the integrity of the ribosomes, the likelihood of forming ribosome-aminoacyl-tRNA-template complexes might be increased. Hence, potassium chloride was replaced by 40% sucrose in the incubation mixture. White and Bayley

(1972b) showed that a mixture of ribosomes and poly(A,U) in a 40% buffered sucrose solution formed stable complexes. After dialysis against a solution containg 3.4M KCl, such preparations could be used in cell-free protein synthesis. A mixture of ribosomes and a trinucleotide in sucrose added to a modified reaction mixture failed to improve the response. In this experiment the ribosomal-trinucleotide mixture could not be dialyzed against high salt before use as the template would have been lost. When spermidine was used in place of magnesium acetate, again no response was observed. This result is in contrast to that of Takeda (1969a, b) who showed that spermidine could replace magnesium *in vitro* in both the ribosomal binding assay and the cell-free protein synthesis system of *E. coli*.

It was of interest to study the response of the halophile tRNAs under investigation in the heterologous system using *E. coli* ribosomes. The results clearly showed that these aminoacyl-tRNAs responded to the established codons. These results, however, leave unanswered the question of the effect of ions on the fidelity of translation but they do support the idea that in the course of evolution of the halophilic bacteria the tRNA molecule and the mechanism of codon recognition were essentially nonvariant. It is probable that the ancilliary factors in protein synthesis may have been modified in adapting to the high salt environment (Bayley and Griffiths, 1968b). Bayley and co-workers assigned 28 codons for *H. cutirubrum* using either

the cell free protein synthesis system or ribosomal binding system. Their data supported the concept of the universality of the genetic code. Moreover, it was shown that both E. coli and H. cutirubrum tRNA recognized the same codons in both the homologous and heterologous systems. On the basis of these findings and the data presented here, it is likely that H. cutirubrum uses the established code in full. The negative results obtained for the halophile system may be due to an inherent inefficiency in using trinucleotides in the in vitro ribosomal binding assay. Rottman and Nirenberg (1966) and Thach and Sundarajan (1965) showed that the template activity of homooligonucleotides depends on their chain length. Recently, Gassen et al., (1972) studied the binding of oligonucleotides to ribosomes. These authors suggested that the binding of a short-chain oligonucleotide strictly depended on the presence of a tRNA coded by it. However, their results clearly show that pentanucleotides bind ribosomes more readily than tri- and tetra-nucleotides in the absence of tRNA. Similar studies in the halophile system could clarify the results presented here.

Purification of $tRNA^{Lys}$ on BD-cellulose columns did not indicate the presence of two isoaccepting species. From the elution pattern, it was infered that either the lysine tRNAs were hydrophobic or that under the chromatographic conditions, the two species assumed a less compact conformation than normally, thus resulting in tighter binding

to BD-cellulose. Uncharged tRNA chromatographed on these columns also showed lysine acceptor activity in the ethanol fractions. Thus, charging the tRNAs does not appear to alter the chromatographic behaviour of lysine tRNAs. These two species appear to be peculiar in this respect since $tRNA^{Lys}$ from E. coli (Gillam et al., 1967), baker's yeast (Sen and Ghosh, 1973), Bacillus subtilis (Chuang et al., 1971) and rabbit reticulocytes (Rudloff and Hilse, 1971) eluted with sodium chloride buffer alone and at concentrations gnerally lower than those used here. The other chromatographic conditions were comparable.

The two isoaccepting species, $tRNA_1^{Lys}$ and $tRNA_2^{Lys}$, resolved by the RPC-5 system were in the approximate proportion of 60% and 40% respectively. The purity of the two lysine tRNAs could not be judged by acceptance of ^{14}C-lysine using the crude synthetase since this preparation degraded the tRNAs. This problem was eliminated by purifying the lysyl-tRNA synthetase by affinity chromatography. However, the problem was only serious with highly purified preparations of tRNA where the amount of tRNA used in the incubation mixture was small. Because of the procedure used in purifying lysine tRNA on BD-cellulose, it was possible that one of the two species from RPC-5 system was an artifact. Gel filtration of native and denatured forms of the two species on Sephadex G-100 yielded peaks not differently retarded. Reannealed tRNA samples (heat denatured in the absence of formaldehyde) showed ^{14}C-lysine acceptance

greater than 75% of the native forms. These results confirmed the presence of two $tRNA^{Lys}$ species.

The 3'-terminal fragments of the lysine tRNAs obtained by ribonuclease T_1 digestion were of the same size and composition as shown by the elution pattern from a DEAE-Sephadex column. Both $tRNA_1^{Lys}$, and $tRNA_2^{Lys}$ were recognized by E. coli synthetase. Although the heterologous aminoacylation reaction was not optimized, it was clear that $tRNA_1^{Lys}$ was more readily recognized than $tRNA_2^{Lys}$ under identical conditions. Both lysine tRNA species were recognized equally well by the H. cutirubrum enzyme. The results of heterologous charging indicated that the two species were not only chromatographically distinct but may have structural differences not apparent in the study of 3'-terminal fragments. Furthermore, the results of the heterologous charging of the purified tRNAs are in agreement with those of White and Bayley (1972a) who showed that unfractionated H. cutirubrum tRNA was recognized for lysine by the E. coli enzyme.

Lysine is coded by the triplets AAA and AAG in the established code. The 'wobble' hypothesis (Crick, 1966) predicts that a lysine tRNA species with the anticodon CUU will recognize only AAG while a lysine tRNA species with the anticodon UUU can recognize both AAA and AAG. Thus, U in the first position of the anticodon will not only form the standard base pair U-A but also the non-standard base pair U-G. The results of the coding response of the two

$tRNA^{Lys}$ species from H. cutirubrum strongly suggest that $tRNA_1^{Lys}$ recognized both AAA and AAG codons while $tRNA_2^{Lys}$ recognized primarily AAA. The results of the heterologous binding assays were consistent with those obtained in the homologous system. The lower response of $tRNA_2^{Lys}$ compared with that of $tRNA_1^{Lys}$ to AAA in the heterologous binding system may reflect structural or conformational differences in the anticodon loop of the two species not apparent in the homologous system. Species of $tRNA^{Lys}$ which primarily recognize AAA but respond to AAG less efficiently have been purified from baker's yeast (Sen and Ghosh, 1973), B. subtilis (Chuang and Doi, 1972), rat liver (Nishimura and Weinstein, 1969; Liu and Ortwerth, 1972) and from rabbit reticulocytes (Woodward and Herbert, 1972). The specificity of recognizing AAA by a lysine tRNA species of baker's yeast has been correlated to the presence of 2-thio-5-carboxymethyluridine methyl ester (U*) at the first position of the anticodon. Indeed, preferential reading of adenosine in the third letter of the codon has been shown to be due to uridine derivatives in the first letter of the anticodon of other species of tRNA. Yeast $tRNA_{II}^{Glu}$ recognizes only GAA and this was shown to be due to the presence of U* in an identical position. Similarly, $tRNA^{Glu}$ species which responded only to GAA from rat liver (Folk and Yaniv, 1972) and E. coli (Ohashi et al., 1972) had 5-methyl-2-thiouridine and 5-methylamino-methyl-2-thiouridine respectively in the first position

of the anticodon. Furthermore, E. coli $tRNA^{Gln}$ which specifically recognizes CAA also has a thiouridine derivative in the first position of the anticodon. Thus, the apparently low recognition of AAG by H. cutirubrum $tRNA_2^{Lys}$ may be due to the presence of a 2-thiouridine derivative in the first position of the anticodon. However, another explanation for this behaviour of $tRNA_2^{Lys}$ may be tenable. The result could be due to a unique conformation of the anticodon loop of this species. Ghosh and Ghosh (1972) reported that yeast and wheat germ phenylalanine tRNA species showed restricted recognition of uridine in the third position of the codon after removal of the 'Y' base normally located adjacent to the 3'-end of the anticodon. These authors attributed their results to a conformational change in the anticodon. Similar structural studies remain to be done for the halophile $tRNA_2^{Lys}$ to account for its behaviour.

Since $tRNA_1^{Lys}$ responds to both AAA and AAG, it is reasonable to postulate UUU as its anticodon. This species did not respond to the triplet AAU in the heterologous system suggesting that uridin-5-oxyacetic acid is not present in the first position of its anticodon.

The existence of isoaccepting species of tRNAs have been demonstrated in bacterial and mammalian systems. Their possible involvement in modulating several biological processes or in ensuring the fidelity of translation has been discussed. The latter suggestion is particularly appealing in the halophile system because of the extreme ionic conditions under which protein synthesis occurs.

Nishimura et al., (1969) showed that increased salt concentration in the cell-free protein synthesis system of E. coli resulted in ambiguity in translation. Further, chromatographically distinct H. cutirubrum isoaccepting tRNA species for phenylalanine (2) (Griffiths, 1970), glutamic acid (2) (Dingle, 1973) glycine (3) (Dingle and Bayley, personal communication), cysteine (2) and histidine (2) (see appendix) have been demonstrated. These isoaccepting species are in relatively high proportions. Thus, it is tempting to suggest that the fidelity of translation in halophilic bacteria may be partly achieved by discriminative reading of the code by some species of tRNA. Studies in this direction would be valuable in understanding how miscoding is avoided under such extreme ionic conditions.

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APPENDIX

Figure 27: CHROMATOGRAPHY OF ^{14}C-CYSTEINYL-tRNA ON BD-CELLULOSE

94 A_{260} units of crude tRNA charged with ^{14}C-cysteine (130, 682 cpm) were charged onto a 1.6 x 27 cm BD-cellulose column previously equilibrated in 0.01 M magnesium chloride, 0.05 M sodium acetate (pH 4.5), 0.005 M 2-mercaptoethanol and 0.45 M sodium chloride. Elution was with a linear gradient of sodium chloride (0.45 M to 0.85 M) in 560 ml of the above buffer. 2 ml fractions were collected at a flow rate of 36 ml/hr.

The first peak of radioactivity constituted approximately 35% and the second peak 65% of the total radioactivity in the salt fractions. In another experiment, the two peaks were pooled separately, discharged and recharged with ^{14}C-cysteine. Chromatography of the two fractions under the conditions described above, showed that the tRNAs eluted in identical positions.

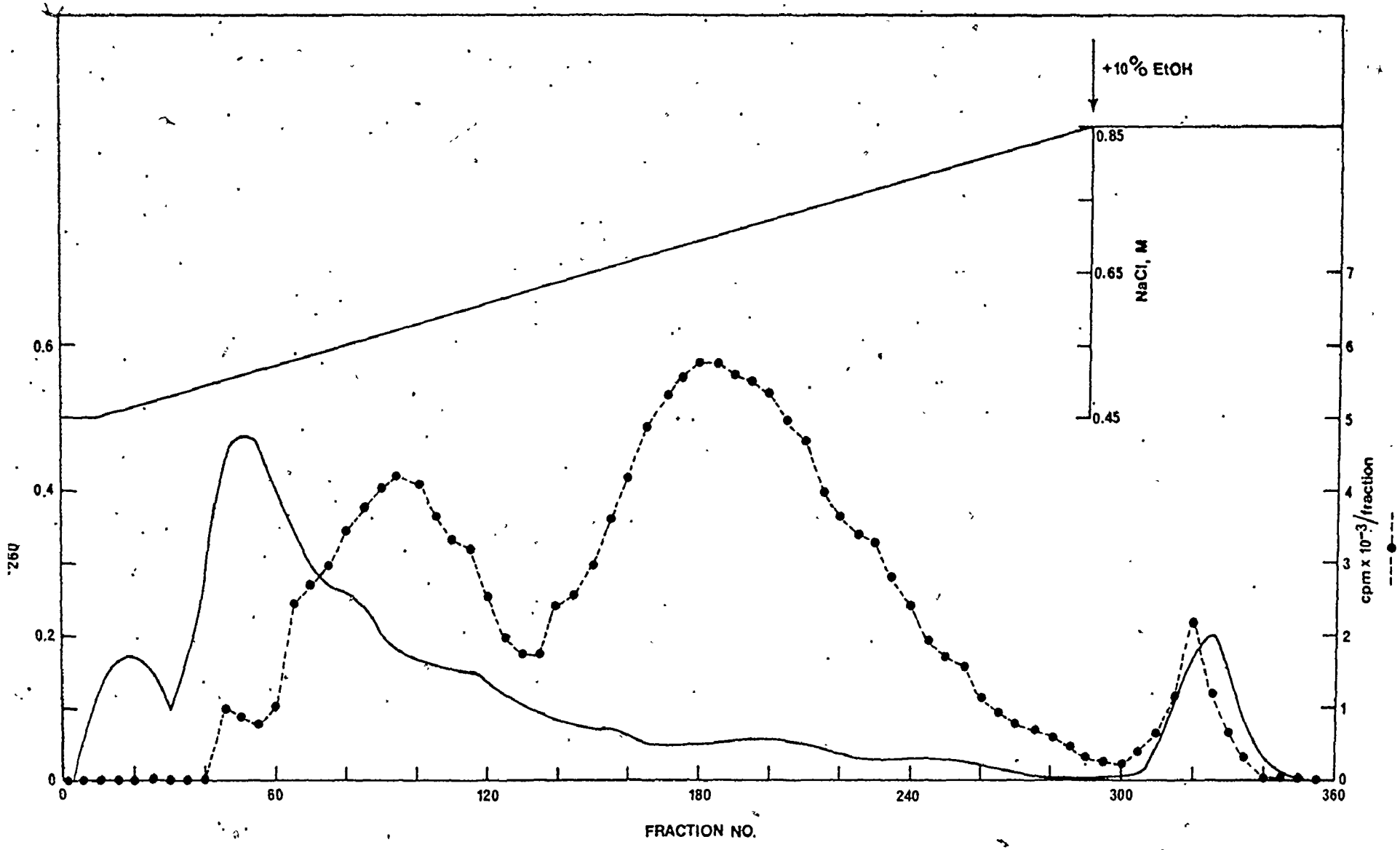
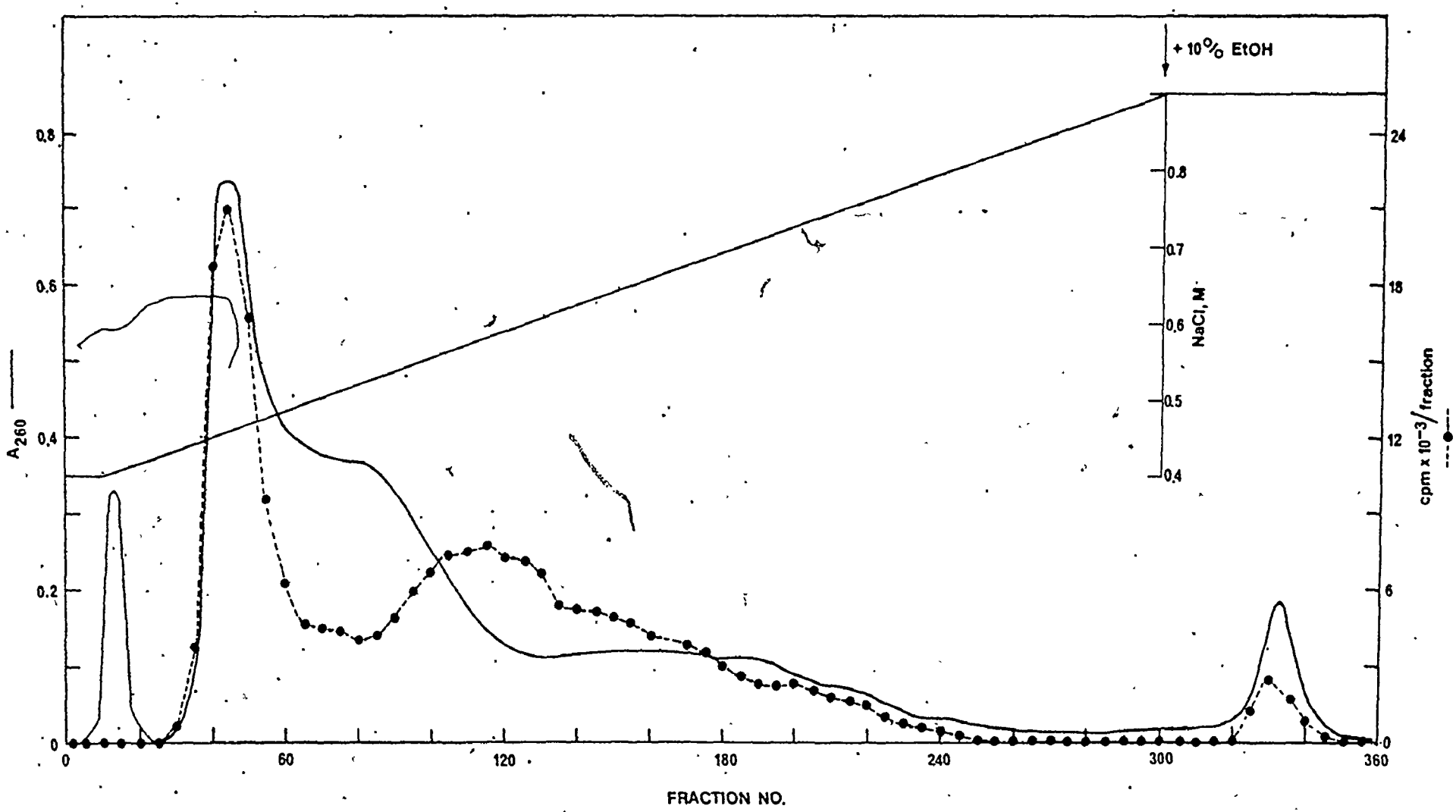

+10% EtOH
0.85
0.65
0.45
NaCl, M
0.6
0.4
0.2
0
0
60
120
180
240
300
360
FRACTION NO.
cpm x 10⁻³/fraction
7
6
5
4
3
2
1
0

Figure 28: CHROMATOGRAPHY OF ^{14}C-HISTIDINYL-tRNA ON BD-CELLULOSE

108 A_{260} units of crude tRNA charged with ^{14}C-histidine (1.1 x 10^6 cpm) were applied to a 1.6 x 30 cm BD-cellulose column equilibrated in the starting buffer described in fig. 27. Elution was with a linear gradient of sodium chloride (0.45 M to 0.9 M) in 580 ml of the sodium acetate buffer (pH 4.5) described previously. 2 ml fractions were collected at a flow rate of 36 ml/hr.

The first peak of radioactivity contained approximately 40% and the second peak 60% of the total radioactivity in the salt fractions. The two peaks eluted in the same positions when pooled and re-chromatographed separately in another experiment under the conditions described above.


+ 10% EtOH
0.8
0.7
0.6
0.5
0.4
NaCl, M
A_{260}
cpm x 10^{-3}/fraction
0
0.2
0.4
0.6
0.8
6
12
18
24
0
60
120
180
240
300
360
FRACTION NO.