PROTEIN SYNTHESIS

IN *H. CUTIRUBRUM*

ASPECTS OF PROTEIN SYNTHESIS

IN VITRO IN

HALOBACTERIUM CUTIRUBRUM

by

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SCOPE AND CONTENTS:

This thesis proposed to investigate protein synthesis in the extreme halophile, *Halobacterium cutirubrum*, especially in relation to the effect a high salt environment has on codon-anticodon recognitions. Many codon assignments were made on the basis of amino acid incorporation directed by random copolyribonucleotides in an *in vitro* protein synthesizing system. Detailed comparison was made of halophilic and *E.coli* tRNA in high and low salt assay systems. A study was also made of the mechanism of the initiation of protein synthesis in this bacterium.

PREFACE

This thesis describes studies undertaken in the Department of Biology, McMaster University from September 1967 to November 1970. Except where others are specifically mentioned, it consists entirely of my own work. No similar thesis has been submitted at any other university.

My grateful thanks are due to my supervisor Dr. S. T. Bayley and to Dr. K. B. Freeman and Dr. L. Prevec for their help throughout the work. Thanks are also due to the National Research Council of Canada for the award of a scholarship and to McMaster University for the award of a university scholarship. Finally I wish to thank my typist and loyal wife Mrs. Brenda White.

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ABBREVIATIONS

aa	amino acid aminoacyl
ala	alanine
arg	arginine
asp	aspartic acid
asn	asparagine
cys	cysteine
glu	glutamic acid
gln	glutamine
gly	glycine
his	histidine
ileu	isoleucine
leu	leucine
lys	lysine
met	methionine
phe	phenylalanine
pro	proline
ser	serine
thr	threonine
trp	tryptophan
tyr	tyrosine
val	valine

A	adenylic acid
C	cytidylic acid
G	guanylic acid
I	inosinic acid
U	uridylic acid
ATP	adenosine 5'-triphosphate
GTP	guanosine 5'-triphosphate
PEP	2-phosphoenol-pyruvate
RNA	ribonucleic acid
m "	messenger -
r "	ribosomal -
t "	transfer
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
RNase	ribonuclease
DEAE-	diethylaminoethyl-
BD-	benzoylated- DEAE
TCA	trichloracetic acid
THFA	tetrahydrofolic acid

Solutions

D	3.4 M KCl, 0.1 M magnesium acetate, 0.03 M Tris-HCl, pH 7.6
BRS	0.03 M KCl, 0.01 M magnesium acetate, 0.04 M Tris-HCl, pH 7.8

I. INTRODUCTION

1. Introductory Remarks

Since direct, *in vitro* studies on protein synthesis and the genetic code began nine years ago (Nirenberg and Matthaei, 1961), it has become increasingly clear that the same overall mechanism and code are used universally. Although this universality is now generally accepted, it is based in actual fact on the investigations of relatively few species, leaving room for confirmation from the more unusual forms. Good candidates for such a study are the extreme halophiles, which have evolved generally acidic proteins (Brown, 1964; Bayley, 1966) in response to their internal and external high salt environments. Bayley (1966) suggested the acidic nature of these proteins may have evolved through modifications in the recognition of codons by transfer RNA. The work presented below was undertaken to see if such modified responses are found in the halophilic protein synthesizing machinery. As the scope of the work covers too much of molecular biology to be adequately reviewed here, it will only be briefly summarised, and more emphasis placed on the effects that a high salt environment has on macromolecules and their interactions.

2. The Genetic Code

During the 1950's it became apparent that a nucleic acid, template mechanism governed the order of amino acids within a protein. Ideas became crystalized with the publication of Crick's "Adaptor Hypothesis" in 1957. The adaptor was thought of as a small molecule carrying the amino acid, which was capable of specifically hydrogen bonding with a nucleic acid template.The experimental breakthrough for decoding came in 1961 with the discovery of Nirenberg and Matthaei that synthetic polyribouridylic acid acted as a messenger RNA for polyphenylalanine synthesis in an *in vitro* protein synthesizing system. During the next 3 years, extensive work from several laboratories using synthetic random copolyribonucleotides enabled Nirenberg et al., (1963) to assign fifty of the possible 64 codons with arbitrary base sequence. It was not until 1964 with the publication of the ribosomal binding technique by Leder and Nirenberg that it was possible to order the bases within the codons. However it still did not provide complete evidence for all 64 codons. Confirmation and completion of the code came with the use of polyribonucleotides of defined sequence, which were mainly synthesized in Khorana's laboratory. Thus in 1966 the genetic code was broken (Table I). Crick (1966) stated: "The evidence used to produce this table comes mainly from *E.coli*. It is likely that the genetic code in other organisms is either very similar or identical to that shown here".

Table I The Genetic Code

↓ →	U	C	A	G	↓
U	PHE	SER	TYR	CYS	U
	PHE	SER	TYR	CYS	C
	LEU	SER	ochre	?	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

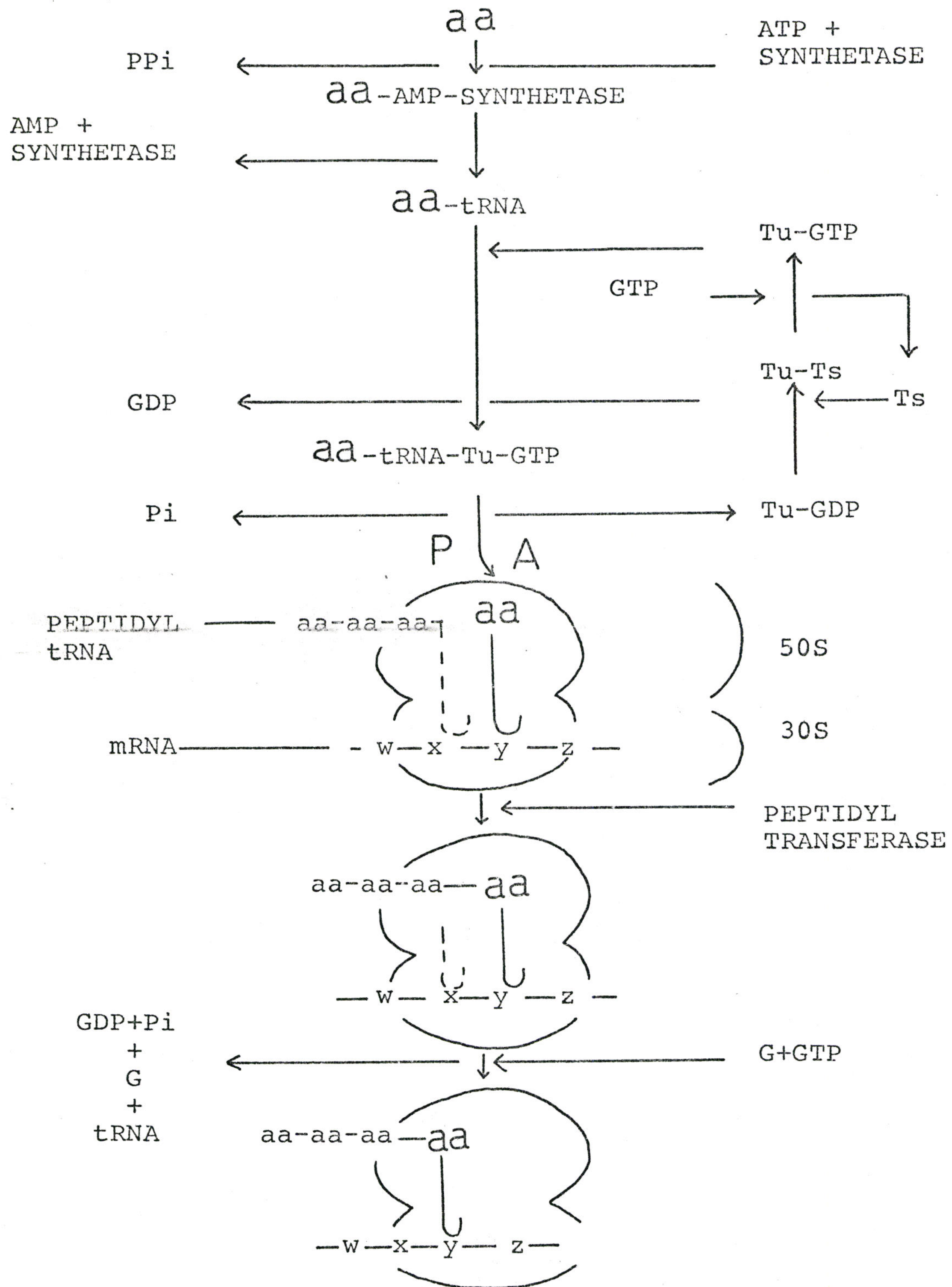
3. Protein Synthesis

In the last decade most of the intricate enzymic steps leading to peptide bond formation have been elucidated. The amount of literature is so immense however that it is difficult to even pick out key papers for each step. The mechanism will be presented below, but it must be remembered that the evidence is not yet complete, and certain parts might be proved incorrect (Cold Spring Harb. Symp. XXXIV 1969).

A. Peptide Chain Elongation

Figure 1 shows a schematic representation of the path of an amino acid to a peptide in E.coli. The process appears to be almost identical in mammalian systems. The first step is the activation of the amino acid and formation of aminoacyl-tRNA by the synthetases. This step involves the specific recognition of the correct tRNA for an amino acid by the synthetase. The code or site on the tRNA, which the synthetase recognises, has yet to be determined. The aminoacyl-tRNA then forms a complex with transferase factor, Tu-GTP. This complex enters the A site of the ribosome with the hydrolysis of GTP and the formation and release of a Tu-GDP complex. Another transferase factor, Ts, displaces GDP, and the Tu-Ts complex interacts with GTP to yield Tu-GTP and Ts. With a peptidyl-tRNA in the P site and an aminoacyl-tRNA in the A site, the peptidyl transferase activity in the 50S subunit forms the peptide bond. At this stage there is a deacylated tRNA in

Figure 1



the P site and the new peptidyl-tRNA in the A site. In a way as yet unclear, the translocase, G, with the hydrolysis of GTP 'kicks out' the deacylated tRNA and moves the peptidyl tRNA to the P site, leaving the A site free for the next aminoacyl-tRNA. In this way the polypeptide is extended one amino acid at a time in an order determined by the codons on the messenger RNA.

B. Initiation

The process outlined above is initiated by a separate sequence of events, which also may be very similar in bacterial and mammalian systems. Three protein factors, which are removed from the 30S subunit of the ribosome, are involved in the process in a way as yet not fully understood. In the presence of the initiator codon ApUpG (or GpUpG) and GTP a unique aminoacyl-tRNA, formyl methionyl-$tRNA^{met}_{F}$, can become bound to the 30S subunit. This species makes up between 60-70% of the $tRNA^{met}$ in E.coli, and when acylated, the methionine is N-formylated by a transformylase. The binding of this F-met-tRNA to the 30S subunit is promoted by one of the protein factors, F_2. The next event is probably the addition of the 50S subunit to form a 70S particle. Factor F_1 appears to enhance considerably the amount of F-met-tRNA bound to the 70S particles. The situation with natural mRNA's appears even more complex. Results indicate that the third factor, F_3, recognises the length of structured RNA, which appears to precede the initiator codon in single stranded viral RNA's.

F_3 thus enhances initiation and polypeptide synthesis with natural mRNA's, but has little effect with synthetic ones preceded by an initiator codon. The actual sequence of events is still unclear, but the net result is the binding of F-met-tRNA to the P site of the 70S ribosomes. The initiation complex is then ready to receive the first aminoacyl-tRNA. The above components and events may prove to be very similar in the cytoplasm of higher cells, except that no transformylase is present, and met-$tRNA^{met}_{F*}$ is the initiator tRNA (F* - formylatable by the E.coli transformylase.)

C. Termination

In this final step of protein synthesis there are again a sequence of events involving several protein factors. Two similar protein molecules, R1 and R2, recognise different sets of the three terminator codons (R1, UAA or UAG; R2, UAA or UGA). Although the mechanism of the recognition has not been elucidated, it is an interaction of extremely high fidelity. A further protein factor, S, increases affinity for the trinucleotide codon in these termination reactions. S may act to increase the rate of formation or stability of the R-terminator codon-ribosome complex. R factor dependent termination yields deacylated tRNA, the identical by-product of the transfer of peptides from peptidyl-tRNA in chain elongation. It is possible that the removal of peptides from the peptidyl tRNA may not be a simple hydrolysis by R factor, but may involve the peptidyl transferase activity in the 50S

ribosomal subunit.

A common feature of these three stages of protein synthesis is the precise interactions and recognitions by the macromolecular components.

4. The Halophilic System

It is clear from the preceding pages that the genetic code and the mechanism for protein synthesis is established, and is probably very similar in both bacterial and mammalian cells. It is pertinent to ask why these mechanisms should be expected to be different in extremely halophilic bacteria. For this reason it is hoped that the short review to follow will explain the rationale behind investigating these processes in _H.cutirubrum_.

Halobacterium cutirubrum is an extreme halophile, requiring 20-30% NaCl in the medium for growth (Larsen 1962). Christian and Waltho (1962) estimated that the major internal ion was potassium, and the total internal ion concentration substantially exceeded that of the medium. Thus all the macromolecules within the cell have to function in an environment saturated with monovalent cations, and the membrane has to contain an efficient pump maintaining Na^+ on the outside and concentrating K^+ on the inside. Accumulating evidence from several sources has indicated specific modifications in certain macromolecules to equip them for the high salt environment.

Brown (1963) compared the mole percentages of NH_2-terminal, acidic and basic amino acids in membranes of a marine pseudomonad and Halobacterium halobium. It was apparent that the different ionic requirements of the two types of membrane were associated with a greater excess of the acidic amino acids in the halophilic membranes. Bayley (1966) made a similar observation with the ribosomal proteins of H.cutirubrum. He found from amino acid analyses that the ratio of the basic amino acids (lysine, histidine and arginine) to the acidic amino acids (glutamic and aspartic acids) was half that of the ribosomal proteins from E.coli. In terms of primary structure then, the proteins of the extreme halophiles are generally acidic in comparison with their non-halophilic counterparts.

The difference in structure of the proteins is reflected by a difference in the activity of the enzymes from extreme halophiles. Baxter and Gibbons (1956) have shown the succinic, malic and isocitric dehydrogenases in crude extracts of H.salinarium to be most active at concentrations of salts ranging from 1 M to 3 M KCl. K^+ is usually the best cation for maximal activity as would be expected in view of the results of Christian and Waltho (1962). Baxter (1959) showed that the lactic dehydrogenase of H.salinarium in crude extracts becomes irreversibly inactivated at low concentrations of salt. From a study of

the inactivation rates Baxter concluded that from 2 M to 3 M NaCl was required to stabilize the enzyme, and from 3 M to 4 M was necessary for activation. There have been several other studies on halophilic enzymes with a variety of responses as far as activity and inactivation in relation to salt concentration is concerned, but in general most need at least 1 M salt for maximal activity, and many require in excess of 3 M.

Halophilic proteins require a high concentration of salt for activity and to prevent their denaturation, and contain a high proportion of the acidic amino acids. The obvious way to correlate the data, as Baxter (1959) does, is to say that the halophilic enzyme is loosely held in its native configuration and removing the salt allows intramolecular electrostatic repulsions to alter the structure sufficiently to denature the protein. Evidence for this comes from the work of Brown (1964), who succinylated the ε-NH_3^+ group of the lysines in the envelope proteins of a marine pseudomonad. These membranes resembled natural halophile membranes in as much as they disaggregated rather than autolyzed at low ionic strength, and they required a higher concentration of salt to stabilize them. Further evidence comes from the results of Bayley and Kushner (1964), who found that at low concentrations of Mg^{2+} and in the absence of K^+ the ribosomes of <u>Halobacterium cutirubrum</u> lost 75% of their protein. The proteins which were lost were acidic, and they concluded that

the stability of the ribonucleoprotein depended on K^+, which shielded the negative groups of the protein. It seems clear then that a major response to the halophilic environment is acidic proteins, the ionised groups of which are neutralised by the high concentration of cations.

In relating the above findings to protein synthesis, two interesting aspects emerge. Does the halophile manufacture these acidic proteins using the same basic code and machinery as the non-halophiles, and if so, what are the modifications to the machinery to allow it to function in saturated KCl? Bayley (1966) suggested that the acidic nature of halophilic proteins may have evolved through modifications in the recognition of codons. An examination of the genetic code (Table I) reveals that many of the codons for acidic and basic residues differ only by the base in the 5'-terminal position. If there was a misreading of the purines G and A in this position aspartic acid would be inserted instead of asparagine and glutamic acid instead of lysine. This hypothesis suggests that the evolution of halophilism may not be through mutations leading to altered codons in the mRNA, but by altered codon recognition because of the high salt concentration.

In preparation for an examination of the above hypothesis Bayley and Griffiths (1968a) developed an *in vitro* protein synthesizing system from *H. cutirubrum*. The basic features of the system were identical to those from non-

halophiles, in that it was dependent on ATP, PEP and GTP for energy, ribosomes, synthetases, transferases and tRNA, and was sensitive to RNase and puromycin. However the system was truly halophilic, requiring nearly saturated salt and specifically 3.8 M KCl, 1 M NaCl and 0.4 M NH_4Cl for maximal activity. Using endogenous mRNA all the amino acids, but ^{14}C-asparagine and ^{14}C-glutamine, were incorporated well into polypeptide. With added polyuridylic acid there was increased incorporation of ^{14}C-phenylalanine, as would be expected from the established code. Bayley and Griffiths (1968b) further investigated the fidelity of translation and the genetic code. Using synthetic random copolyribonucleotides in this system, several codons of known base composition could be assigned. These all agreed with the assignments in the established code. However the codons investigated did not cover many of the ones for basic or acidic amino acids, and therefore only partially ruled out the hypothesis of modification in the recognition of codons in the high salt environment.

Although no clear cut answer to the coding question was achieved, the above work revealed a protein synthesizing machinery essentially the same as in non-halophilic organisms. This machinery was in some form modified to function solely in a high salt environment. One modification was obviously the acidic nature of the protein components of the system, as exemplified by the ribosomes (Bayley, 1966). The other components were the three classes of RNA. The mRNA

appeared essentially the same, as polyuridylic acid and other random copolyribonucleotides seemed to function normally. The ribosomal RNA from recently unpublished data appears only slightly different in its G-C content and sedimentation properties from E.coli. This leaves the tRNA, about which there is little direct evidence. Griffiths and Bayley (1969) found that phenol purified tRNA from H.cutirubrum could be taken to low salt and back to high salt without losing acceptor activity. It could not be charged with ^{14}C-proline by E.coli synthetases, but this is not surprising as many non-halophilic heterologous tRNA and synthetases do not interact. However it was conceivable that the halophilic tRNA's had been modified for their high salt environment, especially in view of the accumulating evidence that these molecules assume conformations more like proteins than nucleic acids.

Although it is not yet clear why the macromolecular components of the halophilic system require saturated salt for the correct conformation and interaction, it is clear that they require besides merely a high concentration, specific cations, indicating specific ionic interactions. For example Bayley and Griffiths (1968a) found that besides saturated KCl there was a requirement for 0.02-0.04 M Mg^{2+}, and the system was greatly enhanced by 1 M NaCl and 0.4 M NH_4Cl. Bayley and Griffiths (1968) suggested that because of the limited solubility of KCl, the NaCl and NH_4Cl may be necessary to lower the water activity to a level at which

the ribosomes and enzymes function properly. Rauser and Bayley (1968) found that NH_4^+ played a specific role in binding the peptidyl tRNA to the ribosome. NH_4^+ also seems to play a specific role in peptide bond formation (Griffiths and Bayley, 1969). Further evidence for specific ionic interactions in the halophilic system comes from a study of the synthetase-tRNA interaction (Griffiths and Bayley, 1969). Kinetic studies on the formation of aminoacyl-tRNA show that replacing K^+ with Na^+ does not affect the apparent Km for the amino acid but causes some reduction in the apparent V max. In contrast replacing K^+ with NH_4^+ does not affect the apparent V max, but considerably increases the apparent Km for the amino acid. From this data the authors suggested that the binding of the amino acid to the aminoacyl-tRNA synthetase involved ionic interaction between the α-amino group of the amino acid and an anionic site on the enzyme. There thus appears at least three roles for the cations in the *in vitro* protein synthesizing system. The first 1 M of the KCl is probably sufficient to neutralise the charges of the acidic proteins (Bayley and Kushner, 1964) while the additional 2.8 M KCl and 1 M NaCl probably binds virtually all the free water in the system. It is difficult to assess the relative importance of neutralisation and reduction in water activity, but in view of the saturated cation concentration required, the latter is probably the most significant effect in the protein synthesizing system. This

leaves other specific roles for NH_4^+ and Mg^{2+} and still allows ionic interactions such as those between the amino acid and the aminoacyl-tRNA synthetase.

The results to follow will shed further light on several aspects of protein synthesis in cell-free systems from H. cutirubrum, especially in answering the following questions.

1. Are tRNA anticodon-codon recognitions modified by the high salt of the system ?
2. Has the primary structure of the tRNA molecule been modified by mutational events during the evolution of the halophile ?
3. Is the initiation process similar to that of non-halophiles ?

II. METHODS

1. Growth of Bacteria

Halobacterium cutirubrum cells were grown at 37°C under continuous aeration in a 14-l New Brunswick Microferm Fermentor. The medium was that described by Sehgal and Gibbons (1960) (Table II), except that 10 ppm of Fe^{2+} (as $FeSO_4$) was added, and the final pH was adjusted to 6.2. The concentrations of the principal inorganic salts in this medium were 4.3 M NaCl, 0.27 M KCl, and 0.08 M $MgSO_4$. Sterilised medium (12-l), which had been filtered to remove the precipitate, was inoculated with a 600 ml, 24 hour culture grown in a New Brunswick Incubator-Shaker. After 18 or 24 hours the cells in early to mid-log phase (Fig. 2) were harvested in the cold by centrifuging in a Sorval, or Sharples continuous centrifuge. The cells were washed at 0°C with centrifuging twice in a solution containing 4.3 M NaCl, 0.03 M KCl, and 0.08 M $MgSO_4$.

For the formation of ^{35}S-methionyl-tRNA and ^{35}S-methionyl-puromycin, the synthetic medium (Table III) of Onishi *et al* (1964) was used. Cells were grown in 100 ml of complex medium in shake culture for 24 hours, and washed as described before. For the formation of ^{35}S-methionyl-tRNA, cells were suspended in the synthetic medium (15 ml), and after 1 hour at 37°C, 50 μC of ^{35}S-methionine added. After a further incubation of

Table II Complex[1] Liquid Medium for Culturing H.cutirubrum - 1 Litre

Component	Amount
Yeast extract (Difco)	10g
Casamino acids (Difco)	7.5g
KCl	2g
$MgSO_4.7H_2O$	20g
NaCl	250g
Sodium citrate	3g
$FeSO_4.7H_2O$	10ppm

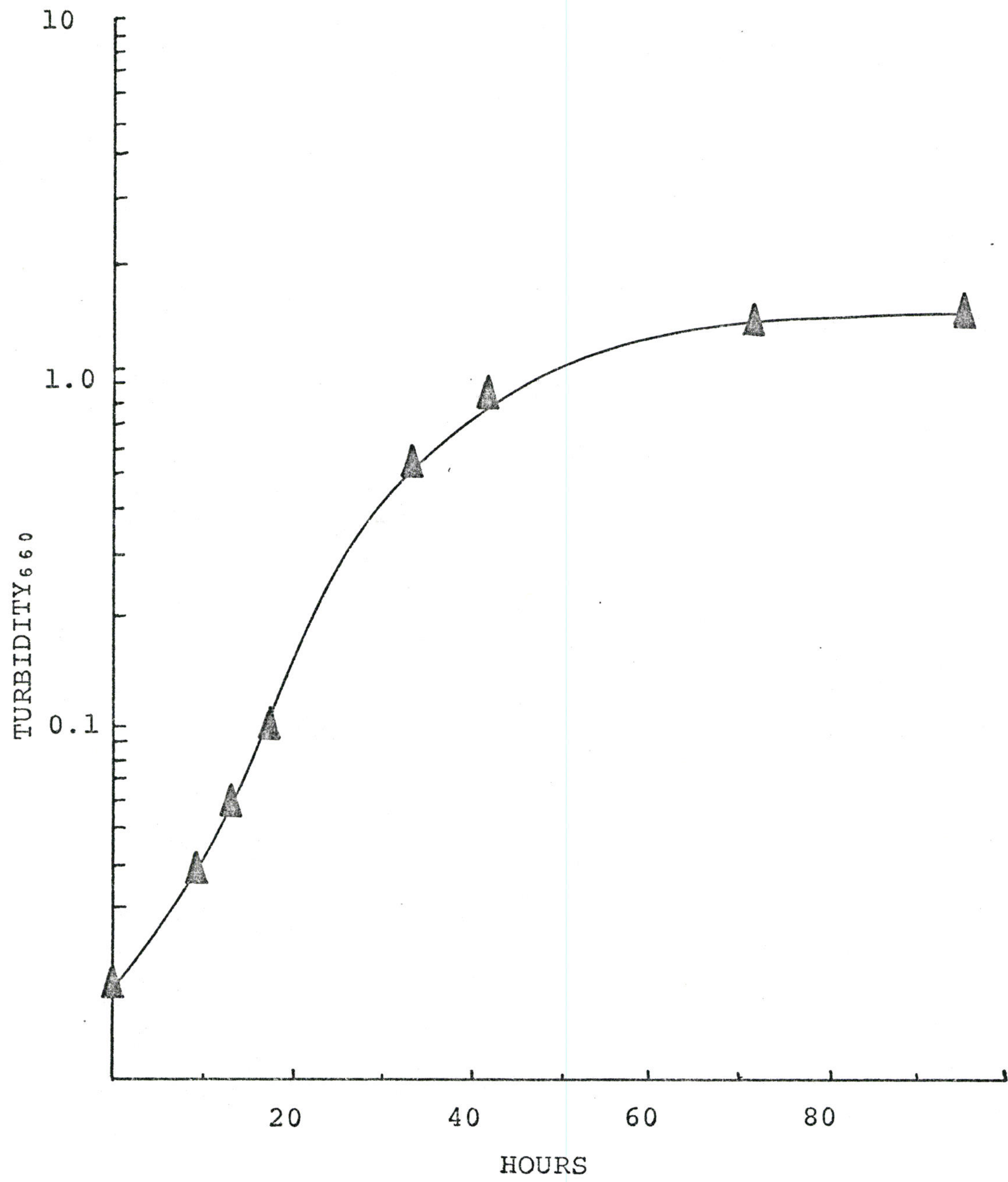
Table III Synthetic[2] Liquid Medium for Culturing H.cutirubrum for ^{35}S-Methionine Incorporation - 1 Litre

Component	Amount	Component	Amount
Uracil	100mg	L-Arginine	400mg
Adenine	100mg	D-L Alanine	430mg
Glycerol	1g	L-Cystine	50mg
NH_4Cl	5g	L-Glutamic Acid	1300mg
NaCl	250g	L-Glycine	60mg
$MgSO_4.7H_2O$	20g	D-L Isoleucine	440mg
KCl	2g	L-Leucine	800mg
KNO_3	100mg	L-Lysine	850mg
K_2HPO_4	100mg	D-L Phenylalanine	260mg
KH_2PO_4	100mg	L-Proline	50mg
Na citrate	500mg	D-L Serine	610mg
$MnSO_4H_2O$	0.3mg	D-L Threonine	500mg
$CaCl_27H_2O$	7mg	L-Tyrosine	200mg
$ZnSO_47H_2O$	0.44mg	D-L Valine	1000mg
$CuSO_45H_2O$	50mg		

[1] Sehgal and Gibbons, 1960. [2] Onishi et al., 1964.

Figure 2   Growth of Halobacterium cutirubrum in a New Brunswick Microferm Fermentor.

12-l of complex medium in a 14-l vessel were inoculated with 600 ml of a 24 hour, shake culture. Optical density readings were taken with a Bausch and Lomb Spectronic 20 at 660 mμ.



10
1.0
0.1
TURBIDITY660
20
40
60
80
HOURS

2 hours, the cells were isolated, and washed. For ^{35}S-methionyl-puromycin formation, puromycin was added to 2 ml cultures, with the ^{35}S-methionine, at a concentration of 5 mM (Bachmayer and Kreil, 1968). Cells were again isolated, and washed as before.

2. Preparation of Biological Extracts.

A. H.cutirubrum

i. S-60 The method used was that described by Bayley and Griffiths (1968a). Harvested cells were homogenised at 0-4°C, in a glass Teflon Potter-Elvenhjem homogeniser with a volume of 3.4 M KCl, 0.1 M magnesium acetate and 0.01 M Tris-HCl, pH 7.6 (modified solution D of Bayley and Kushner, 1964), corresponding to 1.5 times their wet weight, together with 0.015 ml of β-mercaptoethanol and 1 mg of electrophoretically purified DNase per 30 ml of added solution D. A S-60 supernatant extract was obtained from the homogenate by centrifuging once at 40,000g for 20 minutes, and twice at 60,000g for 30 minutes, in each case only the upper, clear part of the supernatant was retained. The S-60 extract was dialysed for 4 hours against 3 changes of solution D and β-mercaptoethanol, and was then frozen in vials in isopentane at liquid nitrogen temperature, and stored under liquid nitrogen.

ii. Polysomes Ribosomes with endogenous mRNA activity were prepared by centrifuging S-60 extracts at 150,000g for 2 hours. The pellet was suspended in solution D and β-mercaptoethanol, and frozen, and stored as described above.

iii. Preincubated, Washed Ribosomes Ribosomes free of endogenous mRNA activity were prepared by preincubating S-60 extracts in bulk at 37°C for 40 minutes in the reaction mixture described in Table IV (Bayley and Griffiths, 1968b), without the ^{14}C-aa or polyribonucleotide. From this mixture ribosomes were isolated by centrifugation at 150,000g for 2.5 hours. The pellet was suspended in solution D by gentle stirring, and again sedimented at 150,000g for 2 hours. Finally the pellet was suspended in solution D, and frozen, and stored in vials as described before.

iv. S-150 S-150 extracts were prepared by centrifuging S-60 supernatant at 150,000g for 2.5 hours (Bayley and Griffiths, 1968b). These were again frozen, and stored in vials under liquid nitrogen.

v. Synthetase Preparation The pH of a S-150 supernatant was adjusted to 5 by adding 1 M acetic acid (Griffiths and Bayley, 1969) at 0-4°C. The precipitate formed was sedimented, resuspended in solution D and β-mercaptoethanol, and dialyzed against this solution overnight. It was then frozen, and stored as before.

vi. Transferase Preparation The S-150 supernatant was dialyzed against 65% saturated ammonium sulfate, pH 7.0, overnight at 0-4°C. The resultant precipitate and the supernatant in the dialysis sac were made up to the original volume with 65% saturated ammonium sulfate and the precipitate sedimented. To the supernatant was added sufficient solid ammonium sulfate to make the resultant solution 80%

saturated (pH maintained at 7.0 with 1 M KOH). The precipitate was sedimented, dissolved in solution D and β-mercaptoethanol, and dialyzed against this solution overnight. The solution was then applied to a Sephadex G-75 column equilibrated with 2.8 M KCl, 0.1 M magnesium acetate and 0.01 M Tris-HCl pH7.6. The first peak at 280 mμ (comparable to fractions 1 and 2 of the S-150 of Fig. 33) was collected, and frozen, or concentrated by a further 80% saturated ammonium sulfate precipitation. The enzyme preparation, which contained both transferase and synthetase activity, was stored as described before.

vii. tRNA tRNA was prepared by phenol extraction as described by Griffiths and Bayley (1969). The pH 5 supernatant from the preparation of synthetases was adjusted to pH 7.6 with 1 M KOH, and the salt concentration reduced by dialyzing overnight against a large volume of a solution containing 0.005 M KCl, 0.005 M magnesium acetate and 0.005 M Tris-HCl, pH 7.6. After dialysis, 0.01 volume of sodium dodecyl sulfate (0.125 g/ml) was added, and the mixture was stirred for 20 minutes. tRNA was then isolated by the usual phenol extraction procedure, and precipitated by acid potassium acetate (pH 5, 20% w/v) and ethanol (Kirby, 1956; von Ehrenstein and Lipmann, 1961; Moldave, 1963). The precipitated tRNA was redissolved in 0.005 M KCl, 0.005 M magnesium acetate and 0.005 M Tris-HCl buffer, pH 7.6, and reprecipitated with potassium acetate and ethanol as before. The final precipitate was washed

with ethanol-H_2O, dissolved in distilled H_2O, and dialyzed against a large excess of distilled H_2O for 48 hours. The tRNA solution was then freeze-dried, and the resultant dry powder stored at -20°C.

viii. <u>^{35}S-Methionyl-tRNA</u> Washed, ^{35}S-methionine labelled cells (see Growth of Bacteria) were suspended in 5 ml of 0.005 M potassium acetate, pH 4.5, and 0.1 ml cutscum (a non-ionic detergent), and homogenised. The cells were phenol extracted, and the nucleic acid precipitated with 2 volumes of ethanol. The precipitate was dissolved in 1 M potassium acetate pH 4.5,and the nucleic acid reprecipitated with 2 volumes of ethanol. To deacylate the tRNA, the nucleic acid was dissolved in 1 ml of 0.1 M NaOH, and incubated at 37°C for 30 minutes. To obtain the ^{35}S-methionine, 2 volumes of ethanol and 0.1 ml 1 M HCl were added to precipitate the nucleic acid. The precipitate was sedimented, and the supernatant dried under vacuum at room temperature. This dried material was later used for identification of the products of ^{35}S-methionine associated with the nucleic acid.

ix. <u>^{35}S-Methionyl-Puromycin</u> Washed, ^{35}S-methionine labelled, puromycin treated cells (see Growth of Bacteria) were suspended in 3 ml H_2O and 0.1 ml cutscum, and homogenised with a Teflon Potter-Elvenhjem homogeniser. This homogenate was extracted with 3 ml of ethyl acetate three times (Leder and Bursztyn, 1966). The ethyl acetate fraction was dried under vacuum at room temperature, and stored for identification

of the ^{35}S products.

B. E.coli

i. S-30 S-30 extracts were prepared as described by Nirenberg (1964). Frozen blocks of *E.coli* W (early log.) were washed in 0.04 M Tris-HCl, pH 7.8 0.01 M magnesium acetate and 0.03 M KCl (BRS) at 3°C, and sedimented at 15,000g for 15 minutes. Packed cells were transferred to a prechilled unglazed porcelain mortar, and an equal weight of neutral alumina added. The cells were ground until the paste became fluid, and an additional equal weight of alumina added slowly. After 15 minutes an equal volume of BRS was added to the mortar, and the paste suspended by stirring. This was centrifuged 20,000g for 20 minutes, and the supernatant fluid decanted. 2µg of pancreatic DNase were added to each ml of extract. After 5 minutes the extract was centrifuged at 30,000g for 30 minutes, and the supernatant of this centrifuged again at 30,000g for 30 minutes. The supernatant, S-30, was then used for preparation of ribosomes and other extracts.

ii. Washed Ribosomes Ammonium chloride washed ribosomes were prepared by the method of Lucas-Lenard and Lipmann (1966). S-30 was centrifuged at 150,000g for 2.5 hours, and the resultant ribosomal pellet resuspended in 0.01 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$. The ribosomes were then sedimented at 150,000g for 2.5 hours, and resuspended in 0.5 M NH_4Cl, 0.001 M $MgCl_2$ and 0.01 M Tris-HCl, pH 7.5.

The ribosomes were resedimented, and resuspended in the same buffer, and recentrifuged. The pellet was then suspended in 0.5 M NH_4Cl, 0.01 M $MgCl_2$ and 0.01 M Tris-HCl, pH 7.5, and centrifuged at 150,000g for 2.5 hours. The ribosomes were then washed by centrifuging 2 times in 0.01 M $MgCl_2$, and 0.01 M Tris-HCl pH 7.5 at 150,000g for 2.5 hours. The ribosomal pellets were finally suspended in BRS, and frozen and stored at liquid nitrogen temperatures.

iii. S-150 S-30 supernatant extracts were centrifuged at 150,000g for 2.5 hours. The supernatant was dialyzed against BRS overnight. The S-150 was stored in vials under liquid nitrogen as described above.

iv. Synthetase Preparation S-150 was adjusted to pH 5 with 1 M acetic acid, and the sedimented precipitate dissolved in BRS, and dialyzed against this buffer overnight. The synthetase preparation was frozen in vials, and stored at liquid nitrogen temperatures as before.

v. Transferase Preparation The procedure adopted was based on that described by Ravel (1967). All operations were conducted at 0-4^{0}C. To the S-150, 22.6 g of solid ammonium sulfate per 100 ml were added slowly with stirring, and the pH maintained at 7.5 with 1 M KOH. After 20 minutes the precipitate was removed by centrifugation for 30 minutes at 30,000g, and discarded. To the supernant, 17.2 g of ammonium sulfate per 100 ml of the original volume were added, and the precipitate collected after standing for at least 1 hour.

The precipitate was resuspended in 0.01 M Tris-HCl, pH 7.5 and 0.006 M β-mercaptoethanol, and dialyzed overnight against this buffer. The transferase preparation was frozen, and stored as before.

3. In Vitro Assay Systems

A. H.cutirubrum

i. Whole Protein Synthesizing System The system was that described by Bayley and Griffiths (1968a). The incubation mixture (with the added polyribonucleotide) and the concentration and amounts of the components are shown in Table IV. The final volumes of the reaction mixtures and consequently the final salt concentrations were uncertain to the extent of the volume of dry KCl added. At the end of the incubation at 37°C for 40 minutes, the sample tube was cooled to 0°C, and to it was added 1000 fold excess of unlabelled amino acid corresponding to the ^{14}C-amino acid used, followed by 2 ml of cold aqueous 10% TCA. The precipitate was washed twice in 5% TCA with centrifuging, heated to 90°C for 15 minutes in 5% TCA (Siekevitz, 1952), cooled at 0°C for 10-20 minutes, collected on a 0.45-μ Millipore filter, and washed with about 3 ml of cold 5% TCA (Nirenberg, 1964). The filters were dried for 30 minutes at 60°C, and counted in a Beckman scintillation counter using omniflour (4 g/l of toluene) as the scintillation solution.

ii. tRNA Charging System This was based on that described by Griffiths and Bayley (1969). The composition of the reaction mixture is shown in Table V. This standard mixture

Table IV Composition of Reaction Mixture for H.cutirubrum Whole Protein Synthesizing System

Component	Amount
3.4 M KCl, 0.12 M magnesium acetate, 0.15 M Tris-HCl, pH 8.0	0.025 ml
1.25 M NH_4Cl, and 3.125 M NaCl	0.040 ml
Solid KCl	24 mg
Polyribonucleotide (5 mg/ml H_2O)	0.010 ml
^{14}C-amino acid (neutralised)	0.005 ml
Cold amino acid mixture less labelled amino acid (each 8×10^{-4} M)	0.010 ml
0.03 M NaATP	0.005 ml
0.012 M NaGTP	0.005 ml
0.12 M NaPEP	0.005 ml
Preincubated and washed ribosomes in solution D	0.005 ml
S-150 in solution D	0.015 ml
Total volume of solutions	0.125 ml

Concentration of Major Salts

Salt	Concentration
Magnesium acetate	0.04 M
KCl	3.8 M
NaCl	1.0 M
NH_4Cl	0.4 M

Amounts of Other Components

Component	Amount
Polyribonucleotide	0.05 mg
19 cold amino acids	0.008 μmole of each
1 ^{14}C-amino acid	0.01-0.0007 μmole
Ribosomes	2-4 A_{260} units

Table V — Composition of Reaction Mixture for H.cutirubrum tRNA Charging System

Component	Amount
5.6 M NaCl	0.055 ml
Solid KCl	41 mg
^{14}C-amino acid (neutralised)	0.010 ml
Cold amino acid mixture less labelled amino acid (each 8 × 10^{-4} M)	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 in solution D	0.020 ml
Water	0.040 ml
Total volume of solutions	0.225 ml

Concentration of Major Salts

Salt	Concentration
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

Component	Amount
NaATP	0.3 μmole
19 cold amino acids	0.008 μmole of each
^{14}C-amino acid	0.02-0.0014 μmole
Synthetases	0.2-0.4 mg protein
tRNA	0.1-0.3 mg

was used for all tRNA charging, except when methionine was the radioactive amino acid, in which case the NaCl was omitted. Again no allowance was made for the volume of the dry KCl, and therefore the final concentrations are slightly lower than those given here. After 20 minutes at 37°C the reaction was terminated, and treated as for the whole protein synthesizing system, except heating at 90°C for 15 minutes was omitted.

iii. Preparation of Aminoacyl-tRNA ^{14}C-aminoacyl-tRNA was prepared by acylating tRNA with one ^{14}C-amino acid and 19 ^{12}C-amino acids in a large scale incubation mixture, increased proportionately from that described above. After an incubation at 37°C for 20 minutes, the reaction was stopped by adding 0.1 volume potassium acetate (20% w/v, pH 4.5), and the mixture dialyzed for 1 hour against 0.005 M potassium acetate, pH 4.5. There followed a normal phenol extraction and precipitation of the charged tRNA with potassium acetate and ethanol. The aminoacyl-tRNA was thoroughly freed of free amino acid by alternately precipitating with 2 volumes of ethanol and dissolving in 1 M potassium acetate, pH 4.5. The final precipitate was washed with 70% ethanol, and dissolved in 0.005 M potassium acetate, pH 4.5. After overnight dialysis against 0.005 M potassium acetate, pH 4.5, it was frozen, and stored at -20°C.

iv. Aminoacyl-tRNA Transfer System This system was based on the whole protein synthesizing system described earlier.

Table VI Composition of Reaction Mixture for H.cutirubrum Aminoacyl-tRNA Transfer System

3.4 M KCl, 0.12 M magnesium acetate, 0.15 M Tris-HCl, pH 8.0	0.025 ml
5.6 M NH_4Cl	0.025 ml
Solid KCl	24 mg
Polyribonucleotide (5 mg/ml H_2O)	0.010 ml
0.012 M NaGTP	0.005 ml
1 ^{14}C and 19 ^{12}C-aminoacyl-tRNA in 0.005 M potassium acetate, pH 4.5	0.020 ml
Ribosomes in solution D	0.010 ml
Transferase preparation in solution D	0.010 ml
Water	0.020 ml
Total volume of solutions	0.125 ml

Concentration of Major Salts

Magnesium acetate	0.04 M
KCl	3.8 M
NH_4Cl	1.12 M
Tris-HCl, pH 8.0	0.03 M

Amounts of Other Components

NaGTP	0.06 μmole
Polyribonucleotide	0.05 mg
^{14}C-aminoacyl-tRNA	5-50 μμmoles
Ribosomes	2 A_{260} units
Transferase preparation	0.25 mg protein

Table VII Composition of Reaction Mixture for H.cutirubrum Ribosomal Binding System

Component	Volume
3.4 M KCl, 0.12 M magnesium acetate, 0.15 M Tris-HCl, pH 8.0	0.020 ml
Solid KCl	24 mg
Polyribonucleotide (5 mg/ml H_2O)	0.010 ml
1 ^{14}C and 19 ^{12}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 volume of solutions	0.125 ml

Concentration of Major Salts

Salt	Concentration
Magnesium acetate	0.088 M
KCl	3.8 M
Tris-HCl, pH 8.0	0.03 M

Amounts of Other Components

Component	Amount
Polyribonucleotide	0.050 mg
^{14}C-aminoacyl-tRNA	5-50 μμmoles
Ribosomes	4-5 A_{260} units

The incubation mixture and the concentration and amounts of components are shown in Table VI, and are similar to mixture I of Bayley and Griffiths (1968a). The incubation for 10 minutes at 37°C was stopped by the addition of 5% TCA-0.25% tungstic acid (Sela and Katchalski, 1959), and the radioactivity assayed as described for the whole protein synthesizing system.

v. Ribosomal Binding System

Nitrocellulose Filter Assay This assay was a combination of the E.coli system described by Leder and Nirenberg (1964) and the protein synthesizing system of Bayley and Griffiths (1968a). The incubation mixture and concentration and amounts of the components are shown in Table VII. Everything was added to the incubation mixture, which was maintained at 0°C, except the 1 ^{14}C + 19 ^{12}C-aminoacyl-tRNA, which was added last. The incubation at 37°C for 30 minutes was stopped by the addition of 4 ml of solution D at 0°C. The millipore filter was washed with 4 ml of solution D at 0°C, and the incubation mixture filtered under gentle suction, and washed with 2 × 4 ml of solution D at 0°C.

RNase-TCA Assay The same basic procedure was followed with the RNase-TCA assay, based on that described by Pestka (1968). After 30 minutes incubation 1.3 μg pancreatic RNase in 0.005 ml solution D was added, and the mixture incubated a further 5 minutes at 37°C. The reaction was then stopped by addition of 4 ml cold 5% TCA, and the precipitate washed

twice in 5% TCA with centrifuging, collected on a 0.45-μ Millipore filter, and further washed with about 3 ml of cold 5% TCA.

vi. Procedure to Stabilize those Polyribonucleotides that Precipitate in High Salt Bayley and Griffiths (1968b) found poly A and AG precipitated in high salt incubation mixtures. This procedure was adopted to give synthetic random copolyribonucleotides, containing significant amounts of A, a protein 'coat' before being exposed to a high ionic environment. Mixtures of S-150 and ribosomes were dialyzed against 40% sucrose, 0.5 M KCl (or 0.1 M KCl), 0.04 M magnesium acetate, 0.01 M Tris-HCl, pH 7.6 and 0.006 M β-mercaptoethanol for 3 hours, the respective polyribonucleotide (dissolved in water) added, and this dialyzed against 40% sucrose, 0.5 M KCl, 0.3 M magnesium acetate and 0.01 M Tris-HCl, pH 7.6 and 0.006 M β-mercaptoethanol for 3 hours. Finally this mixture was dialyzed against solution D for 3 hours, and either used directly or frozen and stored at the temperature of liquid nitrogen.

B. *E.coli*

i. Whole Protein Synthesizing System This system was based on that described by Nirenberg (1964). The incubation mixture and concentration and amounts of components are shown in Table VIII. The incubation for 40 minutes at 37°C was stopped and treated as described for the *H.cutirubrum* system.

Table VIII Composition of Reaction Mixture for E.coli Whole Protein Synthesizing System

Component	Volume
0.4 M Tris-HCl, pH 7.8, 0.1 M magnesium acetate, 0.3 M KCl, 0.06 M β-mercaptoethanol (10 × BRS)	0.008 ml
0.03 M NaATP and 0.012 M NaGTP	0.005 ml
0.12 M NaPEP	0.005 ml
Pyruvate kinase (1 mg/ml H_2O)	0.005 ml
Cold amino acid mixture less labelled amino acid (each 8×10^{-4} M)	0.010 ml
^{14}C-amino acid (neutralised)	0.010 ml
Polyribonucleotide (5 mg/ml H_2O)	0.005 ml
Ribosomes in BRS	0.005 ml
S-150 in BRS	0.015 ml
tRNA (10 mg/ml H_2O)	0.010 ml
Water	0.022 ml
Total volume	0.100 ml

Concentration of Major Salts

Salt	Concentration
Magnesium acetate	0.01 M
KCl	0.03 M

Amounts of Other Components

Component	Amount
Polyribonucleotide	0.025 mg
19 cold amino acids	0.008 μmole of each
^{14}C-amino acid	0.01-0.0007 μmole
tRNA	0.1 mg
Ribosomes	1 A_{260} unit

ii. tRNA Charging System This system was modified from the one described by Marshall et al., (1967). The incubation mixture and concentration and amounts of the components are shown in Table IX. The incubation was for 20 minutes at 37°C, and the ^{14}C-aminoacyl-tRNA assayed in the same way as described for the *H.cutirubrum* system.

iii. Preparation of Aminoacyl-tRNA ^{14}C-aminoacyl-tRNA was prepared by charging tRNA with 1 ^{14}C-amino acid and 19 ^{12}C-amino acids on a large scale incubation mixture, increased proportionately from that described above. Aminoacyl-tRNA was extracted in the same way as that described for the *H.cutirubrum* system, except the dialysis step was not required.

iv. Aminoacyl-tRNA Transfer System This system was adapted from that described by Conway (1964). The incubation mixture and concentration and amounts of the components are shown in Table X. The incubation for 10 minutes at 37°C was stopped by addition of 4 ml of 5% TCA - 0.25% tungstate. The precipitate was treated in the same way as that in the *H.cutirubrum* system.

v. Ribosomal Binding System

Nitrocellulose Filter Assay The system used was identical to that described by Nirenberg and Leder (1964). The incubation mixture and concentration and amounts of the components are shown in Table XI. 1 ^{14}C + 19 ^{12}C-aminoacyl-tRNA was added last to the incubation mixture

Table IX Composition of Reaction Mixture for E.coli tRNA Charging System

Component	Volume
1 M Tris-HCl, pH 7.5	0.005 ml
0.5 M $MgCl_2$	0.005 ml
0.1 M NaATP	0.010 ml
0.05 M NaPEP	0.030 ml
Pyruvate kinase (1 mg/ml H_2O)	0.010 ml
^{14}C-amino acid (neutralised)	0.010 ml
Cold amino acid mixture less labelled amino acid (each 8×10^{-4} M)	0.010 ml
Synthetase or S-150 in BRS	0.020 ml
tRNA in H_2O	0.100 ml
Water	0.050 ml
Total Volume	0.250 ml

Concentration of Major Salts

Salt	Concentration
Magnesium chloride	0.02 M
Tris-HCl, pH 7.5	0.01 M

Amounts of Other Components

Component	Amount
NaATP	1.0 μmole
^{14}C-amino acid	0.02-0.0014 μmole
19 ^{12}C-amino acids	0.008 μmole of each
Pyruvate kinase	0.001 mg
Synthetase or S-150	0.5 mg of protein
tRNA	o.1-0.3 mg

maintained at 0^{0}C. After an incubation of 20 minutes at 24^{0}C, 4 ml of 0.2 M Tris-HCl, pH 7.2, 0.05 M KCl, 0.02M magnesium acetate at 0^{0}C was added. The millipore filter was washed with 5 ml of the same buffer, and the incubation mixture filtered under gentle suction. The filters were washed with 2 times 5 ml of the buffer at 0^{0}C, and the filters dried, and counted as described previously.

RNase-TCA Assay   This assay was the same as that described by Pestka (1968). The incubation mixture was identical to that described above, except 0.05 M Tris-acetate, pH 7.2, 0.05M potassium acetate and 0.10 M NH_4Cl was the buffer used. Reactions were incubated at 24^{0}C for 10 minutes, at which time 0.13 µg of pancreatic RNase in a volume of 0.005 ml of 0.05 M Tris-acetate, pH 7.2, 0.05 M potassium acetate, 0.02 M magnesium acetate was added. Incubation at 24^{0}C was continued for an additional 5 minutes, and stopped by addition of 4 ml of cold 5% TCA. The precipitates were treated in an identical way as described for the *H.cutirubrum* system.

vi. Formylation of Methionyl-tRNA   The system used was based on that described by Marcker (1965). The incubation mixture and concentration and amounts of the components are shown in Table XII. All the components were added to the incubation mixture except N^{5} , N^{10} methenyl tetrahydrofolate, which was neutralised immediately prior to addition to give N^{10} formyl tetrahydrofolate. Incubation was for 15 minutes at 37^{0}C, and the reaction stopped by addition of 0.1 vol 20% potassium acetate (pH 4.5). Aminoacyl-tRNA was phenol extracted

Table X Composition of Reaction Mixture for E.coli Aminoacyl-tRNA Transfer System

Component	Volume
0.5 M NH_4Cl, 0.3 M Tris-HCl, pH 7.5 0.06 M β-mercaptoethanol	0.010 ml
0.1 M magnesium acetate	0.010 ml
0.012 M NaGTP	0.005 ml
Polyribonucleotide (5 mg/ml H_2O)	0.005 ml
1 ^{14}C and 19 ^{12}C-aminoacyl-tRNA in 0.005 M potassium acetate pH 4.5	0.015 ml
Ribosomes in BRS	0.005 ml
Transferases in 0.01 M Tris-HCl pH 7.5, 0.006 M β-mercaptoethanol	0.005 ml
Water	0.045 ml
Total volume	0.100 ml

Concentration of Major Salts

Salt	Concentration
Magnesium acetate	0.01 M
NH_4Cl	0.05 M
Tris-HCl, pH 7.5	0.03 M

Amounts of Other Components

Component	Amount
NaGTP	0.06 μmole
Polyribonucleotide	0.025 mg
^{14}C-aminoacyl-tRNA	5-50 μμmoles
Ribosomes	1-1.5 A_{260} units
Transferases	0.05 mg protein

Table XI Composition of Reaction Mixture for E.coli Ribosomal Binding System

0.2 M Tris-HCl, pH 7.2, 0.5 M KCl, 0.2 M magnesium acetate	0.005 ml
Polyribonucleotide (5 mg/ml H_2O)	0.005 ml
1 ^{14}C and ^{12}C-aminoacyl—tRNA in 0.005 M potassium acetate, pH 4.5	0.015 ml
Ribosomes in BRS	0.010 ml
Water	0.015 ml
Total volume	0.050 ml

Concentration of Major Salts

Magnesium acetate	0.02 M
KCl	0.05 M
Tris-HCl, pH 7.2	0.02 M

Amounts of Other Components

Polyribonucleotide	0.025 mg
^{14}C-aminoacyl-tRNA	5-50 μμmoles
Ribosomes	2 A_{260} units

as described before. To remove the amino acids from the tRNA the aminoacyl-tRNA was finally dissolved in 0.1 M NaOH, and incubated at 37^{0}C for 30 minutes. The tRNA was precipitated by addition of 2 volumes of ethanol and 0.1 volume 1 M HCl. The supernatant was then dried under vacuum at room temperature for identification of the products by electrophoresis.

4 Separation of Macromolecules

A. Sucrose Gradients A modified sucrose gradient of Rauser Bayley (1968) was used. Linear 36 ml gradients were prepared in the cold by mixing 18 ml of 5% (w/v) sucrose and 18 ml of 20% (w/v) sucrose, both containing 3.8 M KCl, 0.1 M magnesium acetate, 0.01 M Tris-HCl, pH 7.6. Ribosomes and the other components were in a 3.8 M KCl buffer, from which much of the salt had been crystalised out by cooling in an ice-salt bath. This material was less dense than the top of the gradient, and was easily layered. Gradients were centrifuged in a Spinco SW 27 rotor at a temperature of 4^{0}C, after which they were collected dropwise by puncturing the bottom of the tube. The A_{260} of the tubes was read in a Unicam S.P. 800A, and radioactivity assayed by precipitation with serum albumin and 5% TCA.

B. Column Procedures

i. Diethylaminoethylcellulose (DEAE)-Cellulose (von Ehrenstein, 1967) A 2.5 × 45 cm glass column was packed with DEAE-cellulose and equilibrated with 0.1 M Tris-HCl buffer, pH 7.5. tRNA dissolved in 0.1 M Tris-HCl, pH 7.5

Table XII Composition of Reaction Mixture for Formylation of Methionyl-tRNA

1 M Tris-HCl, pH 7.5	0.250 ml
0.1 M $MgCl_2$	0.375 ml
0.1 M NaATP	0.125 ml
S-150 in BRS	0.100 ml
N^5,N^{10}-methenyl tetrahydrofolate in 0.01 M HCl and 0.05 M β-mercaptoethanol (neutralised)	0.300 ml
^{14}C-methionyl-tRNA OR ^{14}C-methionine and tRNA	1.000 ml
Total volume	2.500 ml

Concentration of Major Salts

$MgCl_2$	0.015 M
Tris-HCl, pH 7.5	0.1 M

Amounts of Other Components

NaATP	15 μmoles
S-150	2.5 mg protein
N^{10}, formyl tetrahydrofolate	0.25 mg
^{14}C-methionyl-tRNA OR tRNA	2.5 mg
^{14}C-methionine	5 μCi

was applied to the column, with a flow rate of 1 ml per minute. The column was washed with approximately 1 litre of the same buffer, and the tRNA eluted with 1 M NaCl in 0.1 M Tris-HCl, pH 7.5, with a flow rate of 0.5 ml per minute. The optical density of the effluent at 260 mμ was monitored, and the peak collected. The tRNA was precipitated with 2 volumes $-20^{0}C$ ethyl alcohol overnight. The tRNA precipitate was removed by centrifugation, dissolved, and dialyzed extensively against distilled water, and freeze dried.

ii Benzoylated Diethylaminoethylcellulose (BD-Cellulose). A glass column was packed with BD-cellulose, by the method described by Gillam et al., (1968), and equilibrated with 0.01 M $MgCl_2$, 0.05 M sodium acetate, 0.45 M NaCl, 0.001 M β-mercaptoethanol. tRNA or aminoacyl-tRNA in the same buffer was applied to the column at $4^{0}C$, with a flow rate of 20 ml per hour. The tRNA was either eluted stepwise by 0.01 M $MgCl_2$, 0.05 M sodium acetate, 0.9 M NaCl, 0.001 M β-mercaptoethanol, followed by the same buffer containing 10% ethanol, or a gradient 0.45 M NaCl-0.9 M NaCl, followed by the ethanol purge. Fractions were, either dialyzed against distilled water and freeze dried, or ethanol precipitated.

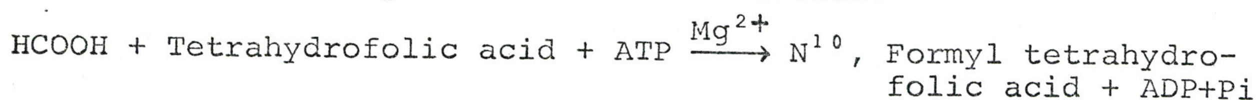
iii. Sephadex Columns Sephadex G-75 was swollen in 2.8 M KCl, 0.1 M magnesium acetate, 0.01 M Tris-HCl, pH 7.6, 0.006 M β-mercaptoethanol by boiling for 3 hours, followed by 24 hours in the same buffer at $4^{0}C$. 45 × 2.5 cm glass columns were packed, and equilibrated with the same buffer,

with a flow rate of 30 ml per hour at 4^0C. tRNA or S-150 in the above buffer was applied to the column, eluted, and 2 ml fractions collected. Protein peaks were usually concentrated by dialysis against 100% ammonium sulfate, and redissolved in solution D with β-mercaptoethanol, while tRNA was isolated by dialysis against distilled water followed by lyophilisation.

Sephadex G-100 was swollen in 0.01 M $MgCl_2$, 0.01 M Tris-HCl, pH 7.5 by boiling for 3 hours and leaving at 4^0C for 24 hours. A 2.5 × 45 cm glass column was packed, and equilibrated in the same buffer. tRNA was applied in, and eluted by the above buffer, and collected from the eluant by direct ethanol precipitation.

5. Preparation of N^5, N^{10} Methenyl Tetrahydrofolate

The method used was that described by Goldwait (1955). Pigeon liver extract catalyses the overall reaction:-



$$\text{HCOOH + Tetrahydrofolic acid + ATP} \xrightarrow{Mg^{2+}} N^{10}\text{, Formyl tetrahydrofolic acid + ADP+Pi}$$

A. Preparation of Extract Pigeon liver was homogenised at 0^0C with 5 volumes of 0.25 M sucrose solution, containing 0.1% EDTA, and centrifuged at 80,000g for 30 minutes. The supernatant was frozen overnight, allowed to stand for 2 hours at room temperature, and the precipitate removed by centrifugation. The fraction that precipitated between 25% and 60% ammonium sulfate saturation was dialyzed against 0.01 M $KHCO_3$ overnight at 4^0C. The extract was frozen, and stored under liquid nitrogen as described before.

Table XIII Composition of Reaction Mixture for the Preparation of N^5,N^{10}-Methenyl Tetrahydrofolate

Component	Volume
1 M $KHCO_3$	0.200 ml
0.1 M $MgCl_2$	0.750 ml
0.1 M NaATP	0.100 ml
0.2 M NaPEP	0.625 ml
Pyruvate kinase (1 mg/ml H_2O)	0.500 ml
Tetrahydrofolate in 0.05 M $KHCO_3$ and β-mercaptoethanol	1.500 ml
Tetrahydrofolate formylase in 0.01 M $KHCO_3$	1.125 ml
0.1 M sodium formate (or ^3H-sodium formate)	1.400 ml
Water	0.300 ml
Total volume	6.500 ml

Amounts of Components

Component	Amount
$KHCO_3$	600 μmoles
$MgCl_2$	75 μmoles
NaATP	10 μmoles
NaPEP	125 μmoles
Pyruvate kinase	0.5 mg
Tetrahydrofolate	125 μmoles
Tetrahydrofolate formylase	25 mg protein
Sodium formate	140 μmoles

B. Reaction Mixture The reaction mixture and concentration and amounts of the components are shown in Table XIII. All the components, except the tetrahydrofolic acid, were added to a tube maintained at 0°C. About 1 cm of petroleum ether was layered over the reaction mixture, followed by the tetrahydrofolic acid. The incubation for 15 minutes at 37°C was terminated by the addition of an equal volume of 4% $HClO_4$. The protein precipitate was centrifuged down and the supernatant allowed to stand under petroleum ether for 30 minutes.

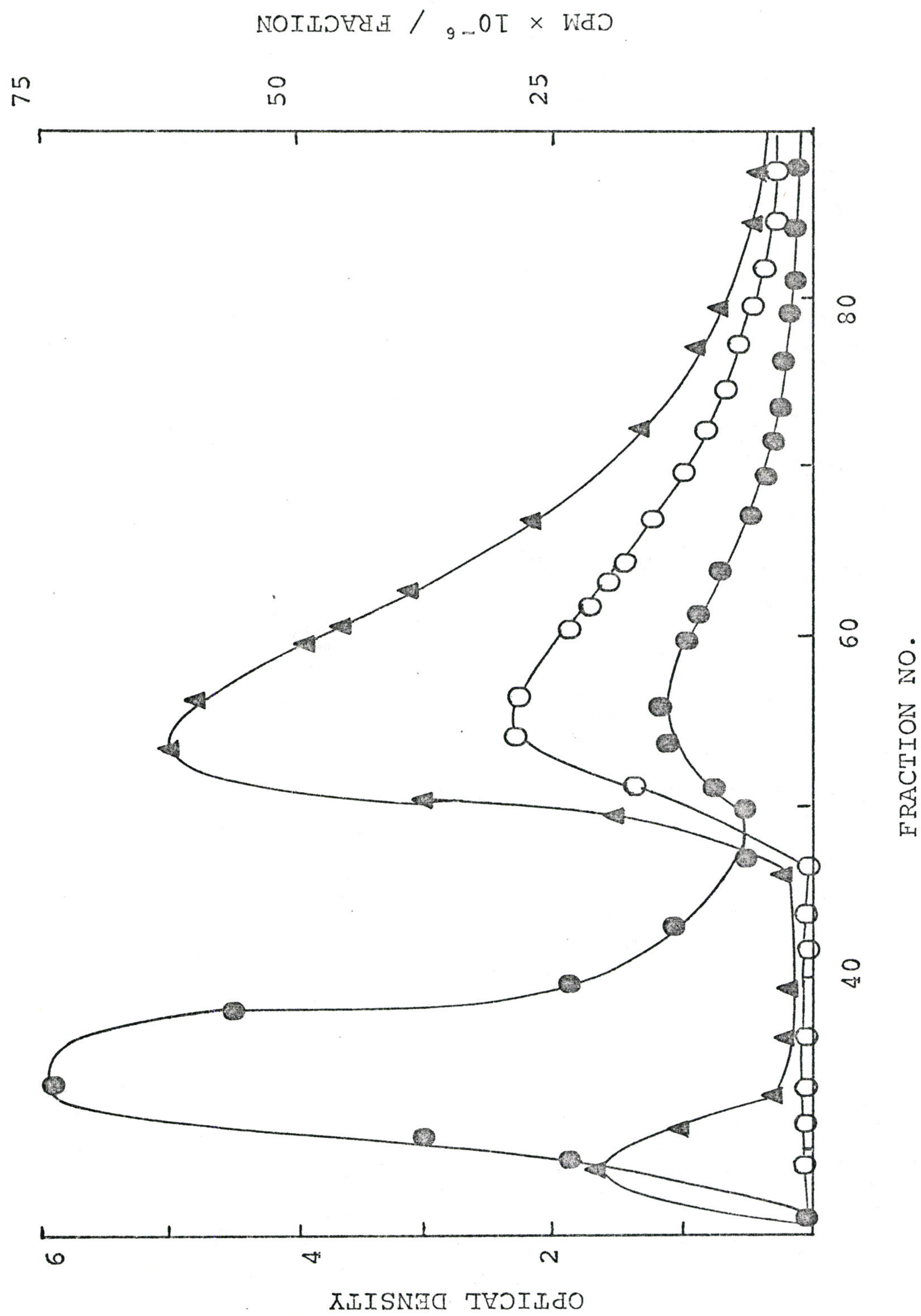
C. Purification of N^5, N^{10}, Methenyl Tetrahydrofolate (Huennekens et al, 1963) A glass column (2.5 × 45 cm) was packed under moderate pressure to a height of 35 cm with Whatman cellulose powder. The column was washed with 500 ml of 0.1 M formic acid 0.01 M β-mercaptoethanol, drained to incipient dryness, and the incubation mixture containing the N^5, N^{10}, methenyl tetrahydrofolate was adsorbed to the column. With a flow rate of 1 ml per minute, elution was carried out with 0.1 M formic acid, 0.01 M β-mercaptoethanol, and the eluant collected in 5 ml fractions. All tubes in the elution profile (Fig. 3) which showed a value greater than 1.6 for the ratio E_{355}/E_{280} were pooled and lyophilised. The powder was dissolved in 0.01 M HCl, 0.05 M β-mercaptoethanol, frozen, and stored at -20°C.

6. Chemical Modifications

A. Partially Hydrolysed Poly U and Poly A The method used

Figure 3 Purification of ^{3}H-N^5,N^{10} Methenyl Tetrahydrofolate

The reaction mixture was applied to a 2.5 × 35 cm column of Whatman cellulose, and eluted with 0.1 M formic acid, 0.01 M β-mercaptoethanol. Fractions (5 ml) were collected immediately the sample was applied with a flow rate of 1 ml/minute. ▲——▲ ^{3}H cpm. ●——● $O.D._{280}^{1cm}$. ○——○ $O.D._{355}^{1cm}$.


CPM × 10⁻⁶ / FRACTION
75
50
25
OPTICAL DENSITY
6
4
2
FRACTION NO.
40
60
80

was that described by Bock (1967). Poly A in 0.1 M NH_4HCO_3, pH 10 was incubated at 100°C for 26 minutes, after which time there should be approximately 10% cleavage of bonds. Poly U in 0.1 M NH_4HCO_3, pH 10 was incubated 7 minutes at 90°C, after which time there should again be approximately 10% cleavage of bonds. After incubation the reaction mixtures were frozen, and freeze dried.

B. Periodate Treatment of tRNA To tRNA or aminoacyl-tRNA, dissolved in 0.075 M potassium acetate pH 5, was added 0.1 volume 0.0075 M sodium periodate (Berg et al., 1962). After incubation at 22°C for 30 minutes in the dark, the tRNA was precipitated with 2 volumes of ethanol. The amount of residual sodium periodate in the supernatant was checked by the addition of 1 drop 1 M ethylene glycol, and the drop in the OD at 232 mμ monitored. The tRNA precipitate was washed free of periodate, and further treated as required.

C. Deacylation of Aminoacyl-tRNA If recovery of acceptor activity from tRNA was required, the aminoacyl-tRNA was dissolved in 0.17 M Tris-HCl, pH 8.8, and incubated at 37°C for 3 hours (Yegian et al., 1966). If acceptor activity was not required, aminoacyl-tRNA was dissolved in 0.1 M NaOH, and incubated at 37°C for 30 minutes. Ethanol precipitation followed either procedure, with the supernatant containing the amino acids or the tRNA pellet being retained.

D. Deformylation of Formyl Methionine N-formyl groups were cleaved by incubation in 0.2 M HCl in methanol at 37°C for 2 hours (Bachmayer and Kreil, 1968).

E. Conversion of Amides to Dicarboxylic Acids Amides were converted to the corresponding dicarboxylic acid by incubation in 2 M HCl for 4 hours at 100^{0}C.

7. Separation of ^{14}C and ^{35}S-Amino Acids and their Derivatives Resolution of formyl methionine from methionine, and the amides from the corresponding dicarboxylic acids, was achieved by high voltage electrophoresis on Whatman 3MM paper. Formyl methionine was separated from methionine in 0.5% pyridine-5% acetic acid, pH 3.5 for 1.5 hours at 3000 V (Marcker and Sanger, 1964). Methionyl-puromycin, methionine, puromycin and related compounds were separated at pH 1.8 in a formic acid-acetic acid buffer (1 M acetic acid adjusted with concentrated formic acid) for 1.5 hours at 2,500 V (Bachmayer and Kreil, 1968). The amides were separated from the dicarboxylic acids in 0.05 M potassium hydrogen phthalate pH 4.0 for 2 hours at 3000 V (Smith, 1960). After drying, the Whatman 3MM paper was cut into 3 × 1 cm pieces, and counted in a scintillation counter.

8. Chemical Analyses

A. Protein Protein concentration was estimated by the technique of Lowry et al., (1951). To the sample in 1 ml of water was added 5 ml of a solution composed of 50 ml of 2% Na_2CO_3 in 0.1 M NaOH and 1 ml of 0.5% $CuSO_4.5H_2O$ in 1% potassium tartrate. After 15 minutes, 0.5 ml of 1 N Folin Reagent was added rapidly, and shaken. After 30 minutes

the OD at 500 mμ was read. Bovine serum albumin was used as the standard.

B. Phosphate Total phosphate was estimated by the technique of Allen (1940). The sample was digested with 1.2 ml $HClO_4$ for 10 minutes, and after it had cooled, 12.5 ml of water, 1 ml 5% ammonium molybdate and 0.5 ml ansa.*, were added. After 15 minutes the sample was read at 680 mμ. The weight of RNA was taken as 11 times the weight of phosphorus.

C. Ribosomes The concentration of halophile ribosomes was calculated from the absorbance at 258 mμ, using $E^{1\%}_{1cm}$ = 158 (Bayley and Kushner, 1964). For both ribosomes and tRNA 1 A_{260} unit was taken as the amount material, which in 1.0 ml would yield a value of 1.0 for the absorbance measured at 260 mμ in a cuvette with a path length of 1.0 cm.

* 1-Amino-2 Naphthol-4 Sulfonic acid

III. MATERIALS

Inorganic salts and TCA were obtained from Fisher Scientific Co., Ltd., and Canlab; ammonium sulfate from Mann Research Labs.; Bovine serum albumin, alumina (neutral 7), and trisodium 2-phosphoenol-pyruvate from Calbiochem Corp.; β-mercaptoethanol from Eastman Organic Chemicals; Tris from Sigma Chemicals Co.; crystalline pancreatic RNase and electrophoretically purified DNase from Worthington Biochemical Corp.; pyruvate kinase (rabbit muscle), E.coli B tRNA (stripped), E.coli W cells (early log.), tetrahydrofolic acid, disodium adenosine 5'-triphosphate and sodium guanosine 5'-triphosphate from P-L Biochemicals Inc.; Antifoam A from Dow Corning Co.; puromycin dihydrochloride from Nutritional Biochemicals Co.; Sephadex G-75 and G-100 from Pharmacia; omniflour from New England Nuclear Corp.; unlabelled L-amino acids from Schwarz Bioresearch, Mann Research Labs., Inc., Fisher Scientific Co., Ltd., and Canlab; poly U, poly A, poly AG (3.6:1), poly AC (1.36:1) and poly GU (3.16:1) from Miles Chemical Corp.; poly CI (4.1:1), poly CG (3.31:1), poly AU (1:5.05), poly CU (3.6:1), poly CU (1:1.44) and poly AC (1:1.59) from Schwarz Bioresearch Inc.

Single or uniform ^{14}C labelled L-amino acids from New England Nuclear Corp., and Amersham/Searle are listed in

Table XIV   Details of ^{14}C-L-Amino Acids

AA	Label	Specific Activity mCi/mmole	Source
ALA	U	112	NENC
ARG	U	234	"
ASP	U	167	"
ASN	U	78.6	"
"	U	207	A/S
CYS	U	218	NENC
"	U	18.3	A/S
GLU	U	197, 186, 195	NENC
GLN	U	94.7	"
"	U	38.8	A/S
GLY	U	116	NENC
HIS	U	267, 239	"
ILEU	U	262, 247	"
LEU	S	31	"
"	U	273, 240	"
LYS	U	241, 247, 257	"
MET	S	60	A/S
"	U	218	NENC
PHE	S	16.5	"
"	U	370, 376, 375	"
PRO	U	201, 209	"
SER	U	131, 118	"
THR	U	164, 163, 167	"
TRP	S	23	"
"	S	38.8	A/S
TYR	U	379	NENC
"	U	475	A/S
VAL	U	219	NENC
"	U	267	A/S

A/S   Amersham/Searle

NENC   New England Nuclear Corp.

U   Uniform label

S   Single   "

Table XIV. ^{3}H-sodium formate (210 mCi/mmole) and ^{35}S-L-methionine (280 mCi/mmole and 12.8 Ci/mmole) were obtained from Amersham/Searle.

IV. RESULTS

1. Codon Assignments from Random Copolyribonucleotides in High Salt

The initial step in determining if there were altered codon-anticodon interactions in the high salt environment was the extension of the assignment of codons using random copolyribonucleotides in the *in vitro* protein synthesizing system (Bayley and Griffiths, 1968b). It was hoped that this technique might especially reveal information on the acidic and basic codons, and also shed light on the low incorporation of asparagine and glutamine with natural messenger RNA (Bayley and Griffiths, 1968a). The incorporation of all 20 ^{14}C-amino acids, each in the presence of 19 ^{12}C-amino acids, was measured with several random copolyribonucleotides in the whole *H.cutirubrum* protein synthesizing system. Results are taken from the net stimulation of incorporation of ^{14}C-amino acids into hot TCA insoluble material by the copolyribonucleotide, above that of the endogenous messenger, and are expressed as μμmoles of ^{14}C-amino acid incorporated per mg ribosomes. The level of endogenous mRNA directed incorporation for the 20 ^{14}C-amino acids, which is similar to that of Bayley and Griffiths (1968b), is shown in Table XV. All the results are the means of at least two separate incubations. The percentage

Table XV Incorporation of 20 ^{14}C-Amino Acids into Hot TCA Insoluble Material Directed by Endogenous mRNA on Preincubated, Washed Ribosomes

Amino Acid	Sp. Act. mCi/mmole	Hot TCA Ppt. cpm
Alanine	112	632
Arginine	234	1783
Asparagine	167	20
Aspartic Acid	207	367
Cysteine	18.3	176
Glutamic Acid	186	98
Glutamine	38.8	58
Glycine	116	333
Histidine	267	146
Isoleucine	262	421
Leucine	251	701
Lysine	241	209
Methionine	218	168
Phenylalanine	16.5	69
Proline	201	824
Serine	131.1	447
Threonine	163	892
Tryptophan	54.5	98
Tyrosine	475	267
Valine	267	297

incorporation of each amino acid in the presence of a polynucleotide is given so as to be directly comparable with the calculated percentage frequency of codons in the random copolyribonucleotide.

Incorporation Directed by Poly CU (3.6:1) - Table XVI

Proline, serine, leucine and phenylalanine incorporations were stimulated in approximately the same proportions as would be expected on the basis of the established code.

Incorporation Directed by Poly CI (4.1:1) - Table XVII

Poly CI was used instead of CG, because of its reported superior messenger RNA activity. Wahbe et al., (1963) have shown inosinic acid in homopolymers and copolymers behaves as guanylic acid, as regards its coding properties in *E. coli* incorporating systems. In the halophilic system proline and arginine were incorporated in approximately the expected proportions, while no significant glycine stimulation could be detected, as would be expected from the percentage of its codons in this polymer. However there was an unexpected, but consistent, stimulation of threonine. Comparing the calculated percentage frequency of codons, to the percentage incorporation, it would appear the triplet ICC was coding for threonine instead of alanine, while ICI perhaps was still coding for alanine. ACU, ACC, ACG and ACA are the established codons for threonine.

Incorporation Directed by Poly CG (3.1:1) - Table XVIII

This polymer was also tried to test if the codon GCC behaved as ICC. It is evident from the results that the

Table XVI Net Incorporation of ^{14}C-Amino Acids into Polypeptide in the Presence of Poly CU (3.6:1)

Amino Acid	Codons	% Calculated No. of Codons		Net Incorporation μumoles/mg Ribosomes		% Incorporation	
				Expt. 1	Expt. 2	Expt. 1	Expt. 2
Proline	CCC	47.25	- 60.75	81	90	44.0	46.0
	CCU	13.50					
Leucine	CUC	13.50	- 17.25	39	43	21.5	22.0
	CUU	3.75					
Serine	UCC	13.50	- 17.25	41	38	22.5	19.5
	UCU	3.75					
Phenylalanine	UUC	3.75	- 4.75	22	24	12.0	12.5
	UUU	1.00					

Each 0.125 ml reaction mixture contained the components and was incubated as described in the "methods" for the whole protein synthesizing system. 0.05 mg of poly CU (3.6:1) and 0.12 mg of ribosomes were present. Results without the random copolyribonucleotide have been subtracted and only those amino acids which show significant net stimulation of incorporation are shown. Results for the other amino acids were the same as shown in Table XV.

Table XVII Net Incorporation of ^{14}C-Amino Acids into Polypeptide in the Presence of Poly CI (4.1:1)

Amino Acid	Codons	% Calculated No. of Codons		Net Incorporation μμmoles/mg Ribosomes		% Incorporation	
				Expt. 1	Expt. 2	Expt. 1	Expt. 2
Proline	CCC	52.00	- 64.75	42.4	23.6	62.00	57.00
	CCG	12.75					
Arginine	CGC	12.75	- 15.75	14.2	10.0	20.75	24.00
	CGG	3.00					
Alanine	GCG	3.00	- 15.75	2.5	2.1	3.75	5.00
	GCC	12.75					
Glycine	GGG	0.75	- 3.75	-	-	-	-
	GGC	3.00					
Threonine	-		-	8.3	5.6	12.00	14.00

Each 0.125 ml reaction mixture contained the components, and was incubated as described in the "methods" for the whole protein synthesizing system except for the designated modifications. 0.05 mg of poly CI (4.1:1) and 0.12 mg of ribosomes were present. For expt. 2 the tubes were preincubated for 15 minutes without the ^{14}C-amino acid in order to further reduce endogenous mRNA directed incorporation.

incorporation in general was much lower than with poly CI. Again proline and arginine were incorporated but this time a significant amount of alanine and no threonine was incorporated. Apparently for the codon ICC, I does not behave as G in the halophilic system.

Incorporation Directed by Poly UA (5.05:1) - Table XIX

This polymer gave a precipitate in 3.8 M KCl, 1.0 M NaCl, 0.4 M NH_4Cl and 0.04 M magnesium acetate, and would not function as a messenger, but it could be stabilized in these ionic conditions by slow dialysis from sucrose to salt in the presence of ribosomes and S-150 supernatant (see "methods"). Phenylalanine, isoleucine, leucine and tyrosine incorporations were stimulated in approximately the same proportions as expected from the established code. No significant incorporation of lysine and asparagine could be detected and UAA, the terminating triplet was not tested.

Poly AG (3.6:1)

This polymer was found to precipitate (Bayley and Griffiths, 1968) in the high salt system, but could be stabilized through sucrose. It did not stimulate the incorporation of amino acids into hot TCA insoluble material after this treatment.

Poly AC (1.6:1)

This polymer behaved as poly AG (3.6:1).

Poly A

This polymer was found to precipitate in the high

Table XVIII Net Incorporation of ^{14}C-Amino Acids into Polypeptide in the Presence of Poly CG (3.1:1)

Amino Acid	Codons	% Calculated No. of Codons		Net Incorporation μμmoles/mg Ribosomes		% Incorporation	
				Expt. 1	Expt. 2	Expt. 1	Expt. 2
Proline	CCC	43.0	- 57.0	13.0	21.9	59.5	66.0
	CCG	14.0					
Arginine	CGC	14.0	- 18.5	4.8	6.6	22.0	19.8
	CGG	4.5					
Alanine	GCG	4.5	- 18.5	4.1	3.7	18.5	11.2
	GCC	14.0					
Glycine	GGG	1.5	- 4.05	-	1.0	-	3.0
	GGC	4.5					
Threonine	-		-	-	-	-	-

Each 0.125 ml reaction mixture contained the components, and was incubated as described in the "methods" for the whole protein synthesizing system. 0.05 mg of poly CG (3.1:1) and 0.12 mg of ribosomes were present for expt. 1 and 0.075 mg of poly CG and 0.20 mg of ribosomes were present for expt. 2.

Table XIX Net Incorporation of ^{14}C-Amino Acids into Polypeptide in the Presence of Poly UA (5.05:1)

Amino Acid	Codons	% Calculated No. of Codons		Net Incorporation μμmoles/mg Ribosomes		% Incorporation	
				Expt. 1	Expt. 2	Expt. 1	Expt. 2
Phenylalanine	UUU	59.00		59.3	43.6	77.25	74.00
Lysine	AAA	0.50		-	-	-	-
Isoleucine	AUA	2.25	- 14.25	5.9	6.1	7.75	10.25
	AUU	12.00					
Leucine	UUA	12.00		4.9	4.5	6.25	7.50
Tyrosine	UAU	12.00		6.1	4.2	8.00	7.25
Asparagine	AAU	2.25		-	-	-	-
ochre	UAA	2.25		-	-	-	-

Each 0.125 ml reaction mixture contained the components, and was incubated as described in the "methods" for the whole protein synthesizing system. Poly UA gave a precipitate and therefore had to be first stabilized in high salt (see "methods"). 0.05 mg of poly UA and 0.12 mg of ribosomes were present.

salt conditions (Bayley and Griffiths, 1968b) and could not be stabilized by dialysis through sucrose.

Incorporation Directed by Poly GU (3.16:1) - Table XX

The only amino acid stimulated by this polymer was cysteine, and this was surprising since only 4.25% of the codons were those allocated to cysteine by the established code. Further experiments, (Table XX) with specific controls to test if the stimulation was due to peptide bond formation, indicated the results were not due to protein synthesis. This was not further investigated, and it was concluded that the polymer was not functioning as a messenger RNA. The reason for this was not clear, because Bayley and Griffiths (1968b) used a poly GU (0.41:1) successfully. Possibly the high proportion of G caused increased secondary structure, or perhaps the polymer was incorrectly manufactured.

Table XXI shows that although many codons have been tested using random copolyribonucleotides, the more interesting ones, namely those for basic and acidic amino acids, have not. From the above results it became obvious that a considerable portion of the code could not be tested in this way, because any synthetic messenger with a significant amount of A precipitated, or would not function as a messenger. There seemed three possible alternative ways of overcoming this problem:-

A. a ribosomal binding system in high salt using triplet codons, or small oligonucleotides, which will not precipitate.

Table XX Incorporation of ^{14}C-Cysteine into Hot TCA Precipitate in the Presence of Poly GU (3.16:1)

Modifications	μμmoles Cysteine/mg Ribosomes in Hot TCA Ppt.
Complete	210
-Poly GU	90
+RNase	212
+Puromycin	190
-Ribosomes	195
-GTP	260

Each 0.125 ml reaction mixture contained the components, and was incubated as described in the "methods" for the whole protein synthesizing system except for the designated modifications. 0.05 mg poly GU, 0.12 mg of ribosomes, 25 μg of puromycin and 20 μg of RNase were used.

Table XXI

[1] Results of Bayley and Griffiths (1968b) included.

[2] Major or only codon for an amino acid in a polymer, or a minor codon with % calculated frequency more than 5%.

[3] Minor codon of 2 in a polymer with % calculated frequency less than 5%.

[4] Codons less than 1%, or stimulating no incorporation.

[5] Random poly UG stimulating methionine incorporation - possible initiator codon.

[6] ICC appears to code for threonine and not alanine.

[7] Not including codons containing I.

Table XXI Codons Tested with Random Copolyribonucleotides in High Salt[1]

A.A	Codons Tested Adequately[2]	Codons Giving Suggestive Results[3]	Codons Not Adequately Tested[4]
Ala	GCC	GCG, ICI	
Arg	CGC, CIC	CGG, CII	
Asp			
Asn			AAU, AAC
Cys	UGU		
Glu			
Gln			
Gly	GGU	GGC	GGG, IIC, III
His	CAC		
Ileu	AUU	AUA	
Leu	UUG, UUA, CUC, CUU		
Lys			AAA
Met		GUG[5]	
Phe	UUU, UUC		
Pro	CCC, CCU, CCG, CCI, CCA		
Ser	UCC, UCU		
Thr	ACC, ICC[6]		ACA
Trp	UGG		
Tyr	UAU		
Val	GUU	GUG	
ochre			UAA
Total[7]	22	5	

B. use non-halophilic ribosomal and protein synthesizing systems with halophilic aminoacyl-tRNA.

C. synthesize mRNA off DNA of known sequence in high salt in the presence of ribosomes, which will prevent precipitation.

Alternative (C.) was difficult because of the availability of DNA of known sequence and it required the development of a halophilic RNA polymerase system. Both (A.) and (B.) were however pursued.

2. H.cutirubrum Ribosomal Binding System: A Comparison of E.coli and H.cutirubrum ^{14}C-Aminoacyl-tRNA

This ribosomal binding system was developed in order to use triplets or oligonucleotides in high salt, and therefore to test codons, which could not be tested in the whole protein synthesizing system, because the relevant random copolyribonucleotides precipitated. As it was first uncertain that H.cutirubrum ribosomes would bind to nitrocellulose filters, the alternate RNase-TCA assay described by Pestka (1968) was developed as well. The final system (see "methods") was essentially the same as that of Leder and Nirenberg (1964), with the high salt modifications. A preliminary comparison was also made of E.coli and H.cutirubrum ^{14}C-aminoacyl-tRNA in this system to determine if non-halophilic tRNA could be recognised by halophilic ribosomes in a high salt environment.

Table XXII shows the general properties of the system, as assayed by the nitrocellulose filter binding technique. The system has an absolute requirement for template mRNA, ribosomes and Mg^{2+} and can use both H.cutirubrum and E.coli ^{14}C-aminoacyl-tRNA, although the former does not apparently bind so well. For the first time the triplet codon AAA was shown to stimulate binding of E.coli and H.cutirubrum ^{14}C-lys-tRNA, indicating a good possibility of testing codons

Table XXII Each 0.125 ml reaction mixture contained the components, and was incubated as described in the "methods" for the H.cutirubrum ribosomal binding system, except for the designated modifications. The amounts of added ^{14}C-aa-tRNA were as follows: 13.26 μμmoles of ^{14}C-lys-tRNA E.coli (0.49 A_{260} unit), 15.52 μμmoles of ^{14}C-lys-tRNA H.cutirubrum (2.50 A_{260} units), 14.10 μμmoles of ^{14}C-phe-tRNA E.coli (0.70 A_{260} unit), 14.28 μμmoles of ^{14}C-phe-tRNA H.cutirubrum (2.75 A_{260} units), 24.10 μμmoles of ^{14}C-leu-tRNA E.coli (0.68 A_{260} unit) and 15.26 μμmoles of ^{14}C-leu-tRNA H.cutirubrum (1.91 A_{260} units). 50 μg of either partially hydrolysed poly U or poly A and 4.54 A_{260} units of ribosomes were present.

Table XXII Characteristics of ^{14}C-Aminoacyl-tRNA Binding to H.cutirubrum Ribosomes

^{14}C-AA-tRNA	Modifications	μμMoles ^{14}C-AA Bound		
		Poly A	Poly U	-Poly
^{14}C-lys-tRNA *E.coli*	-	2.91	0.29	0.31
^{14}C-lys-tRNA *H.cutirubrum*	-	2.45	1.27	1.32
^{14}C-phe-tRNA *E.coli*	-	0.37	2.52	0.34
"	-Ribosomes	-	0.05	-
"	$-Mg^{2+}$		0.07	-
"	+ 1 A_{260} unit *E.coli* tRNA	-	1.01	0.32
"	+ 1 A_{260} unit *H.cutirubrum* tRNA	-	1.72	0.34
^{14}C-phe-tRNA *H.cutirubrum*	-	0.49	1.67	0.61
"	-Ribosomes	-	0.26	-
"	$-Mg^{2+}$	-	0.15	-
"	+ 1 A_{260} unit *E.coli* tRNA	-	0.96	0.55
"	+ 1 A_{260} unit *H.cutirubrum* tRNA	-	1.35	0.61
^{14}C-leu-tRNA *E.coli*	-	0.69	0.81	0.73
^{14}C-leu-tRNA *H.cutirubrum*	-	0.45	0.45	0.47

in polymers which precipitate in high salt systems. Binding of ^{14}C-phe-tRNA was severely reduced by the addition of deacylated tRNA, that of E.coli being more effective on an A_{260} unit basis. The fact that deacylated tRNA did compete for ribosomal binding sites indicated the reason for the poorer binding of H.cutirubrum ^{14}C-aminoacyl-tRNA, as compared with that of E.coli. Table XXIII shows the acceptance of lysine and phenylalanine by E.coli and H.cutirubrum tRNA with two different concentrations of the ^{14}C-amino acid. The lower concentration was that used in preparing ^{14}C-aminoacyl-tRNA. While E.coli tRNA was approximately two thirds fully charged at the low concentration, H.cutirubrum tRNA was only one third charged. The reason for this is probably that the apparent Km of the synthetase for the amino acid is higher in the halophilic system, and the halophilic S-150 contains a considerable amount of endogenous amino acid, which dilutes the label.

The reason for the apparent poorer binding of the H.cutirubrum aminoacyl-tRNA was therefore due to the higher percentage of deacylated tRNA competing for ribosomal binding sites. Fig. 4 shows the different behaviour of the tRNA's very clearly. The amount of binding of ^{14}C-aminoacyl-tRNA's to the corresponding template with a fixed amount of ribosomes was compared, varying the amount of added ^{14}C-aminoacyl-tRNA. It is evident that H.cutirubrum ^{14}C-phe-tRNA or ^{14}C-lys-tRNA was bound 2-3 times less than that of E.coli. The shapes of the curves are in agreement with the suggestion

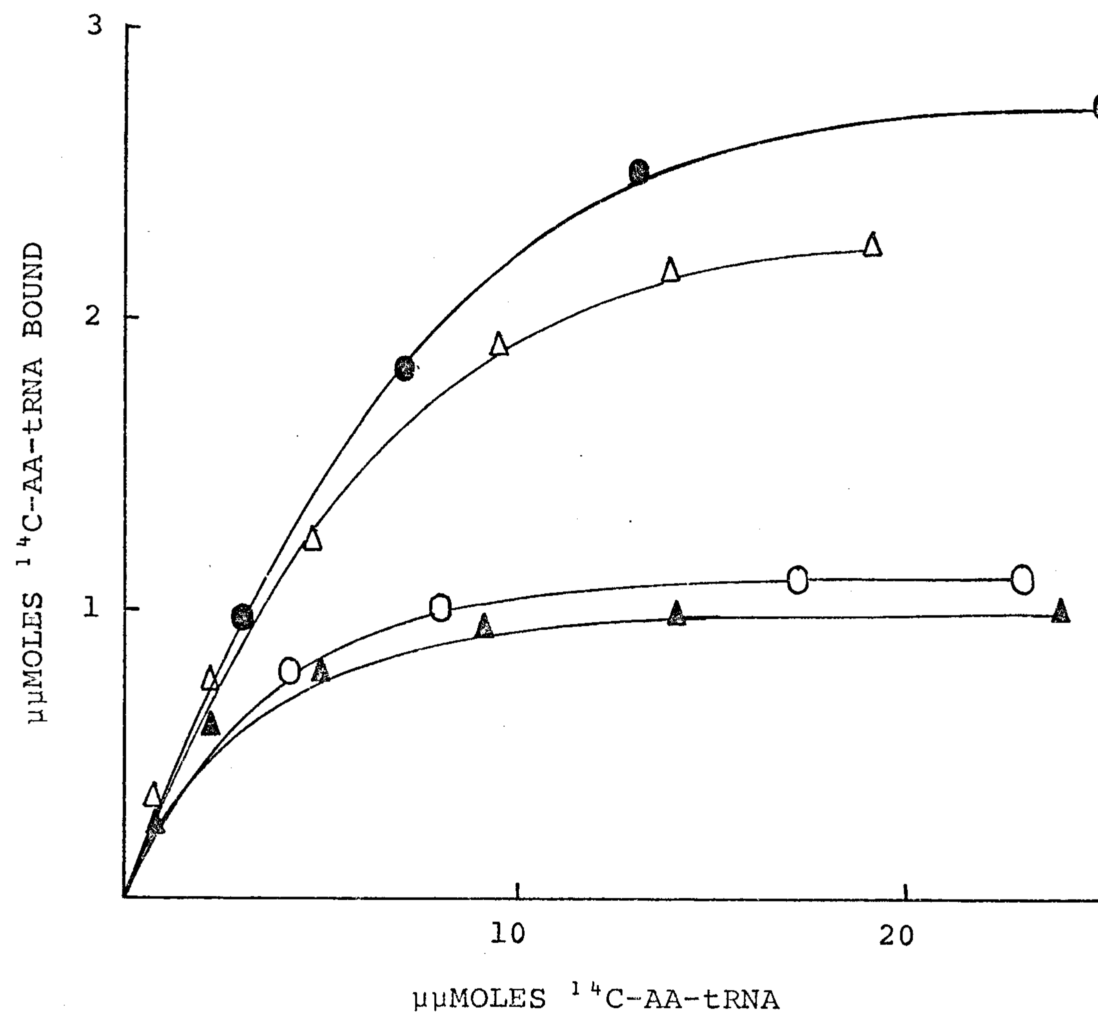
Table XXIII Acceptance of ^{14}C-Phenylalanine and ^{14}C-Lysine by E.coli and H.cutirubrum tRNA

^{14}C-AA	μμmoles ^{14}C-AA per Incubation Mixture	μμmoles ^{14}C-AA accepted per A_{260} Unit of tRNA	
		E.coli	H.cutirubrum
Phenylalanine	0.0013	21.32	6.01
Phenylalanine	0.1300	29.85	16.84
Lysine	0.0020	27.43	6.23
Lysine	0.2000	36.21	16.32

Acceptance of amino acids by the tRNA was measured by the tRNA charging systems described in the "methods". S-150 was used as the source of synthetases in both assay systems, and the 19 cold amino acids were present. The specific activities of phenylalanine were 376 mC/mM and 3.76 mC/mM, and of lysine were 257 mC/mM and 2.57 mC/mM. The results shown are the net incorporation of amino acid into tRNA as the control with added RNase is subtracted.

Figure 4 Ribosomal Binding of ^{14}C-AA-tRNA as a Function of Added µµmoles ^{14}C-AA-tRNA

Each 0.125 ml reaction mixture contained the components and was incubated as described in the "methods", except for the designated modifications. 50 µg partially hydrolysed poly A or poly U and 4.54 A_{260} units of ribosomes were used. ●——● ^{14}C-lys-tRNA *E. coli* (1 A_{260} unit contains 27.3 µµmoles ^{14}C-lys) and poly A. △——△ ^{14}C-phe-tRNA *E. coli* (1 A_{260} unit contains 20.5 µµmoles ^{14}C-phe) and poly U. O——O ^{14}C-lys-tRNA *H. cutirubrum* (1 A_{260} unit contains 6.2 µµmoles ^{14}C-lys) and poly A. ▲——▲ ^{14}C-phe-tRNA *H. cutirubrum* (1 A_{260} unit contains 5.2 µµmoles ^{14}C-phe) and poly U. The results without polymer have been subtracted.


3
2
1
μμMOLES ^{14}C-AA-tRNA BOUND
10
20
μμMOLES ^{14}C-AA-tRNA

that deacylated $tRNA^{phe}$ is saturating the ribosomal binding sites at low imput values of the ^{14}C-aminoacyl-tRNA.

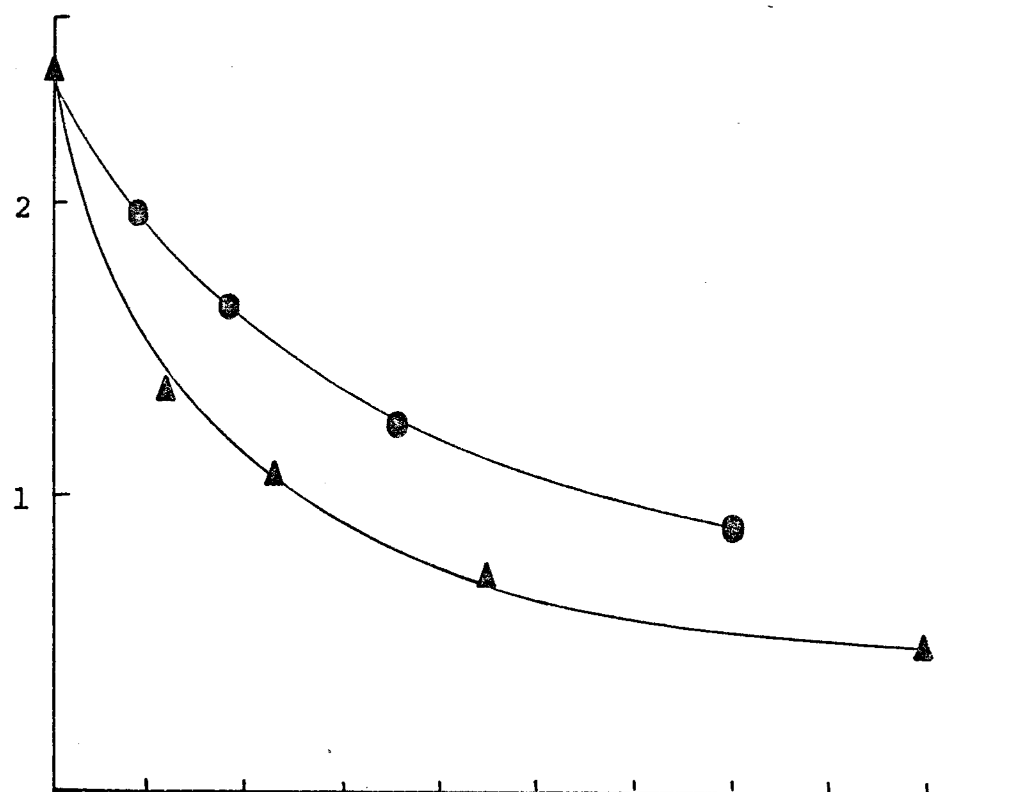
In order to see if the relative proportion of $tRNA^{phe}$ per A_{260} unit was the same in the tRNA preparations from H.cutirubrum and E.coli, a competition experiment was performed. Fig. 5a shows the binding of E.coli ^{14}C-phe-tRNA to ribosomes in the presence of varying proportions of E.coli and H.cutirubrum deacylated tRNA. It is apparent that the E.coli tRNA contained approximately 2 times more $tRNA^{phe}$ than H.cutirubrum tRNA on an A_{260} unit basis, because twice as much H.cutirubrum tRNA was required to reach the same reduction in the binding of E.coli ^{14}C-phe-tRNA to the ribosomes. Further evidence is shown in Fig. 5b, where H.cutirubrum tRNA with enhanced phenylalanine acceptor capacity, and tRNA with reduced phenylalanine acceptor capacity (both gifts from Dr.E.Griffiths) were compared with crude tRNA for the ability to compete with E.coli ^{14}C-phe-tRNA for ribosomal binding sites. The tRNA enriched for $tRNA^{phe}$ behaved more like E.coli crude tRNA, while the tRNA with reduced $tRNA^{phe}$ did not even compete as well as the crude H.cutirubrum tRNA. This is strong indication that crude H.cutirubrum tRNA contains only half as much $tRNA^{phe}$ as E.coli tRNA. The same is true for $tRNA^{lys}$ and it appears that the H.cutirubrum tRNA contains approximately 50% nucleic acid which is not tRNA (see Fig. 28a+b). This and the low charging of H.cutirubrum tRNA explains why 4-5 times more H.cutirubrum tRNA on an A_{260} unit basis is needed to

Figure 5 Ribosomal Binding of *E.coli* ^{14}C-Phe-tRNA as a Function of Added Deacylated tRNA

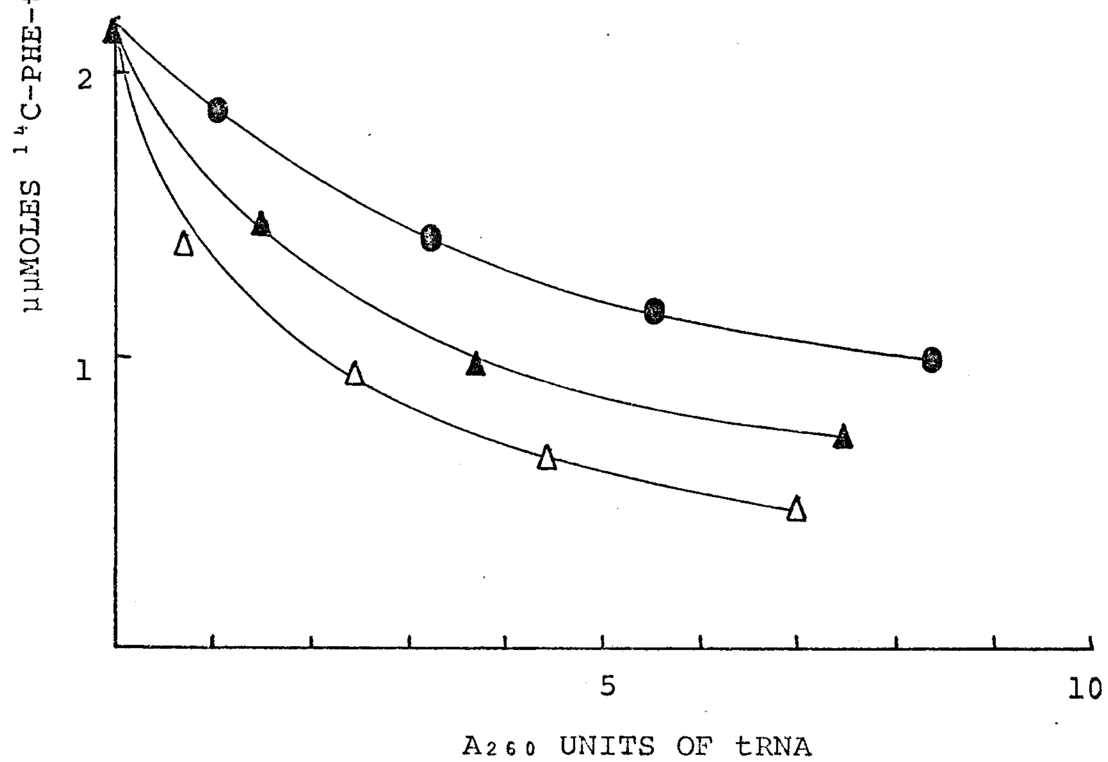
a. Each 0.125 ml reaction mixture contained the components, and was incubated as described in the "methods", except for the designated modifications. 14.10 μμmoles of ^{14}C-phe-tRNA *E.coli* (0.70 A_{260} unit), 50 μg partially hydrolysed poly U and 4.54 A_{260} units of ribosomes were used. ●——● plus *H.cutirubrum* deacylated tRNA. ▲——▲ plus *E.coli* deacylated tRNA.

b. This shows the same experiment as in (a.) ▲——▲ plus original crude *H.cutirubrum* deacylated tRNA. △——△ plus *H.cutirubrum* deacylated tRNA with enhanced phenylalanine acceptor capacity. ●——● plus *H.cutirubrum* deacylated tRNA with reduced phenylalanine acceptor capacity. The tRNA with altered amounts of phenylalanine acceptor capacity were gifts from Dr.E.Griffiths.

a



b



supply the same μμmoles of ^{14}C amino acid as the *E.coli* tRNA (refer to legend of Table XXII). Table XXII shows that the binding of ^{14}C-aminoacyl-tRNA was codon specific, as no binding of *E.coli* or *H.cutirubrum* ^{14}C-leu-tRNA was found with poly A or poly U. This will be dealt with further in Chap.IV,5.

Fig. 6 shows the ribosomal requirements for the system. Saturation with 15.6 μμmoles *E.coli* ^{14}C-lys-tRNA (1.30 A_{260} unit), was reached at 6-7 A_{260} units of ribosomes. Fig. 7 illustrates the template requirements of the system. As poly A precipitated in the high salt system it was partially hydrolysed. At the higher concentrations of partially hydrolysed poly A, a precipitate was visible, and this may be the reason for inhibition of *E.coli* ^{14}C-lys-tRNA binding at the higher concentration of this template. Although poly U was soluble in the high salt system, it served as a far more efficient template when it was also partially hydrolysed. This may be due to, either the elimination of the secondary structure in the smaller oligonucleotides of poly U, or the production of more free ends, to which the ribosomes can bind.

Fig. 8 illustrates the time course and response to temperature for binding of *E.coli* ^{14}C-lys-tRNA with partially hydrolysed poly A, and *H.cutirubrum* ^{14}C-phe-tRNA with partially hydrolysed poly U. Unlike the *E.coli* system of Leder and Nirenberg (1964), binding of *E.coli* ^{14}C-lys-tRNA was very slow at 24^{0}C and did not reach maximum even after

Figure 6 Ribosomal Binding of E.coli ^{14}C-Lys-tRNA as a Function of Ribosome Concentration

Each 0.125 ml reaction mixture contained the components, and was incubated as described in the "methods", except for the designated modifications. 15.6 μμmoles of ^{14}C-lys-tRNA (1.30 A_{260} units) and 50 μg of partially hydrolysed poly A were used. ▲——▲ plus poly A. ●——● - poly.

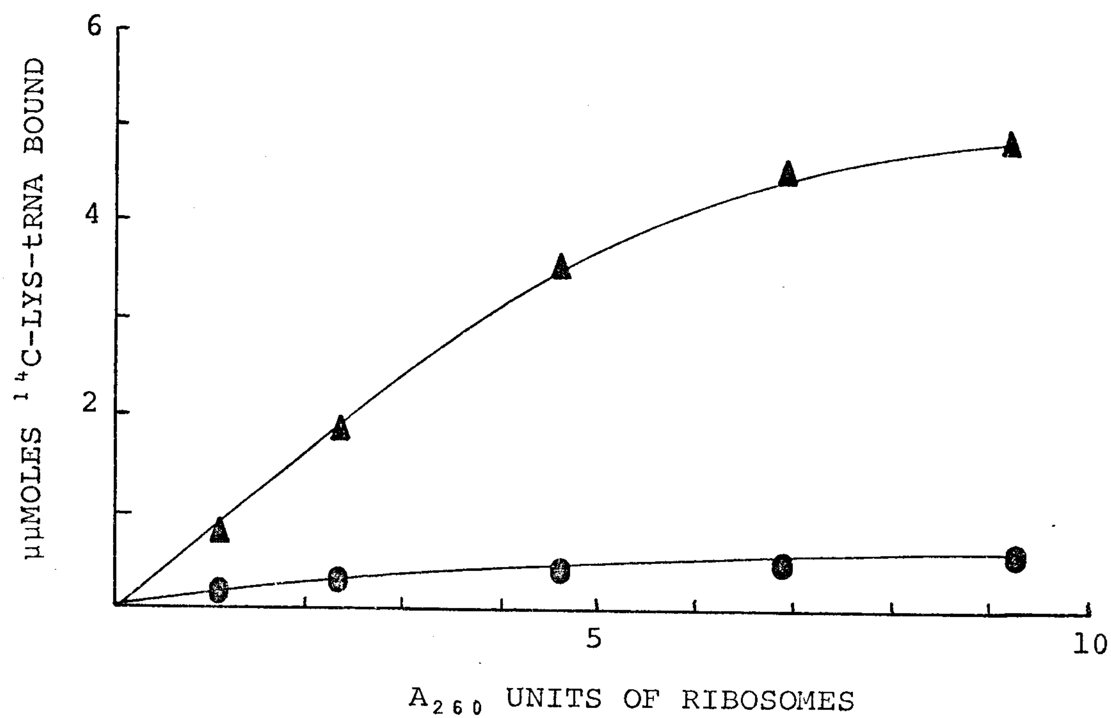
Figure 7 Ribosomal Binding of E.coli ^{14}C-AA-tRNA as a Function of Template Concentration

Each 0.125 ml reaction mixture contained the components as described in the "methods", except for the designated modifications. 14.0 μμmoles ^{14}C-phe-tRNA (0.70 A_{260} unit), 13.26 μμmoles ^{14}C-lys-tRNA (0.49 A_{260} unit) and 3.42 A_{260} units of ribosomes were used.

▲——▲ partially hydrolysed poly A and ^{14}C-lys-tRNA.

○——○ partially hydrolysed poly U and ^{14}C-phe-tRNA.

●——● poly U and ^{14}C-phe-tRNA. Results without polymer have been subtracted.


6
4
2
5
10
μμMOLES ^{14}C-LYS-tRNA BOUND
A_{260} UNITS OF RIBOSOMES

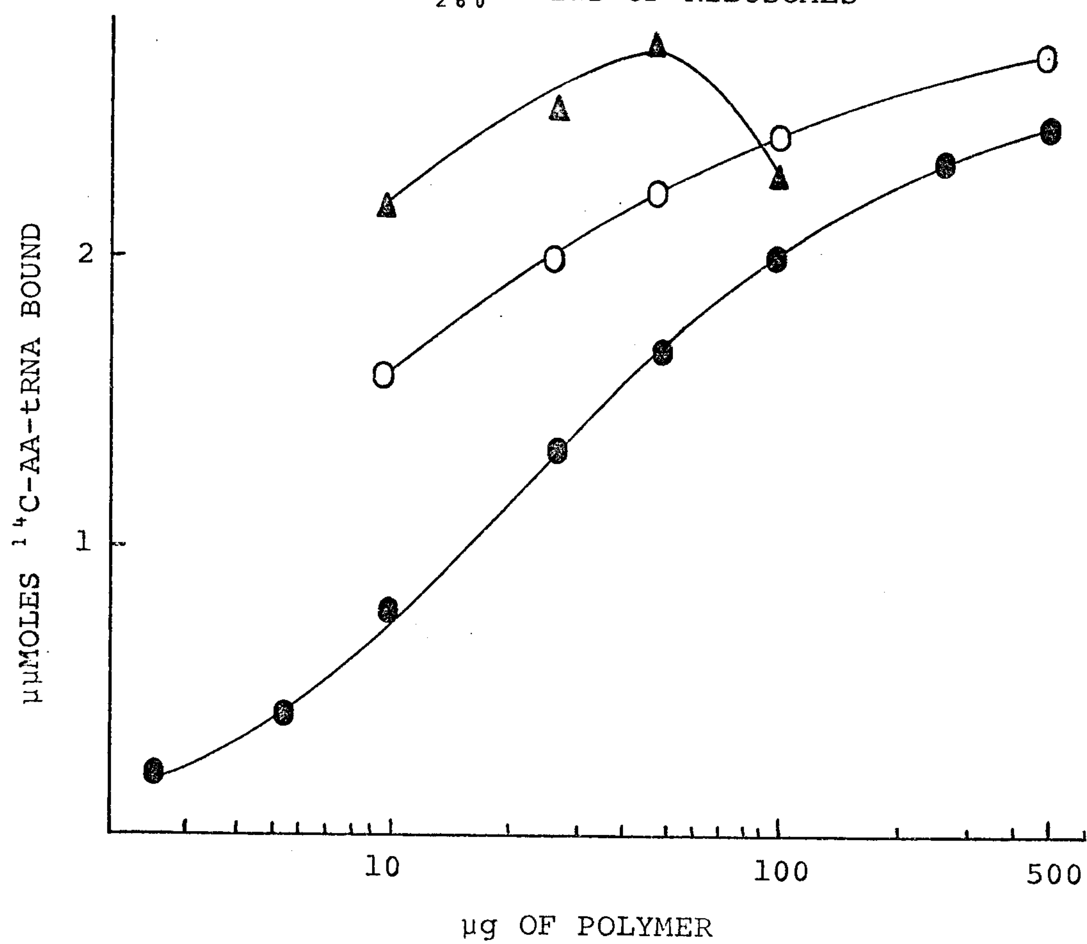
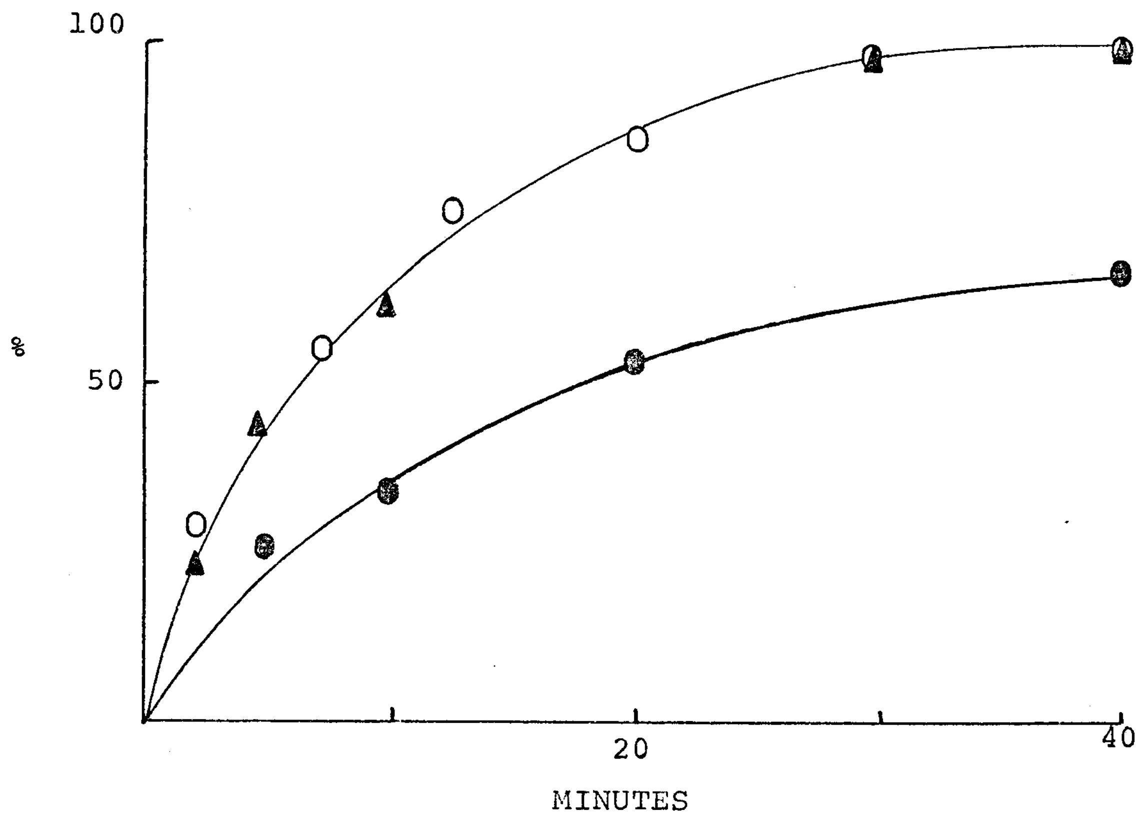

2
1
10
100
500
μμMOLES ^{14}C-AA-tRNA BOUND
μg OF POLYMER

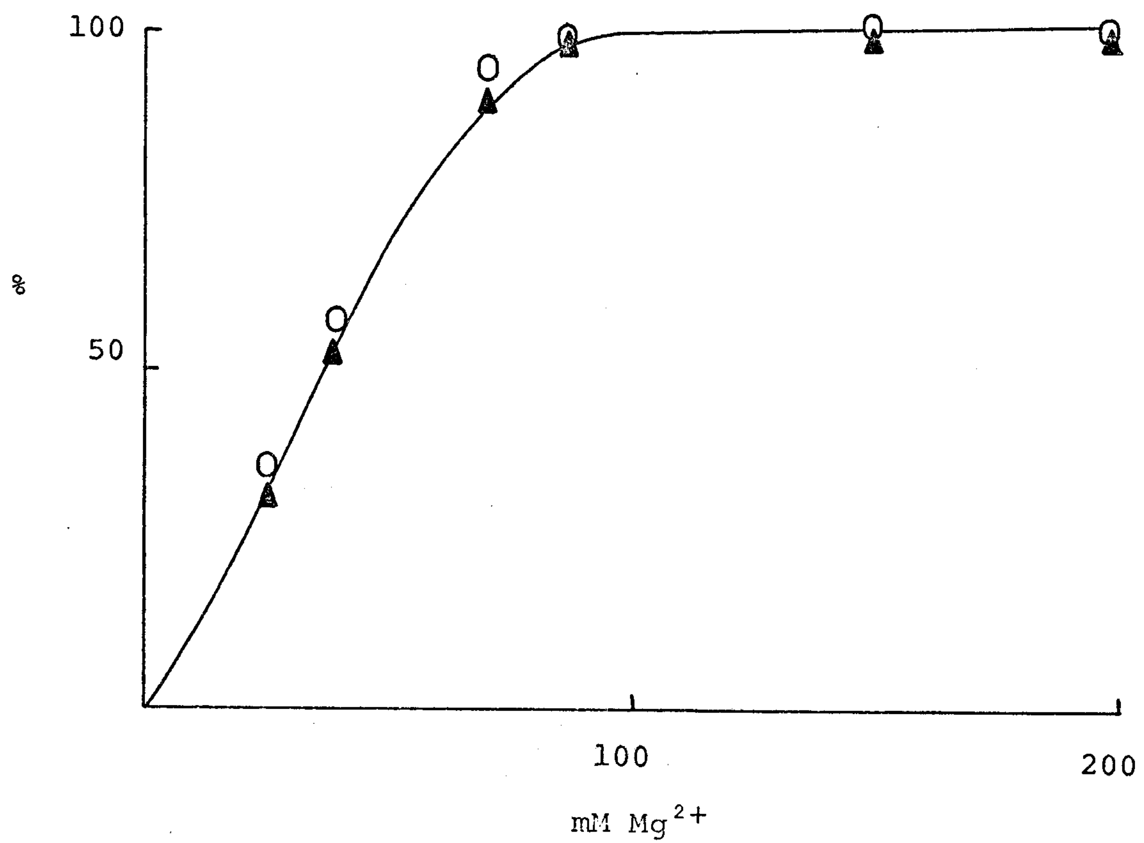
Figure 8 Ribosomal Binding of ^{14}C-AA-tRNA as a Function of Time, at Different Temperatures

Each 0.125 ml reaction mixture contained the components, and was incubated as described in the "methods", except for the designated modifications. 50 µg of partially hydrolysed poly A or poly U, 4.54 A_{260} units of ribosomes, 13.26 µµmoles ^{14}C-lys-tRNA *E.coli* (0.49 A_{260} unit), or 14.28 µµmoles ^{14}C-phe-tRNA *H.cutirubrum* were present. O——O ^{14}C-lys-tRNA *E.coli* and poly A at 37°C (100% = 2.63 µµmoles). ●——● ^{14}C-lys-tRNA *E.coli* and poly A at 24°C (100% = 2.63 µµmoles.) ▲——▲ ^{14}C-phe-tRNA *H.cutirubrum* and poly U at 37°C (100% = 1.09 µµmoles).

Figure 9 Ribosomal Binding of ^{14}C-AA-tRNA as a Function of Magnesium Concentration

Each 0.125 ml reaction mixture contained the components and was incubated as described in the "methods", except for the designated modifications. 50 µg of partially hydrolysed poly A or poly U, 4.54 A_{260} units of ribosomes, 14.10 µµmoles ^{14}C-phe-tRNA *E.coli* (0.70 A_{260} unit) or 14.28 µµmoles ^{14}C-phe-tRNA *H.cutirubrum* (2.75 A_{260} unit) were present. O——O ^{14}C-phe-tRNA *H.cutirubrum* and poly U (100% = 2.16 µµmoles). ▲——▲ ^{14}C-phe-tRNA *E.coli* and poly U (100% = 1.13 µµmoles).


100
50
%
20
40
MINUTES

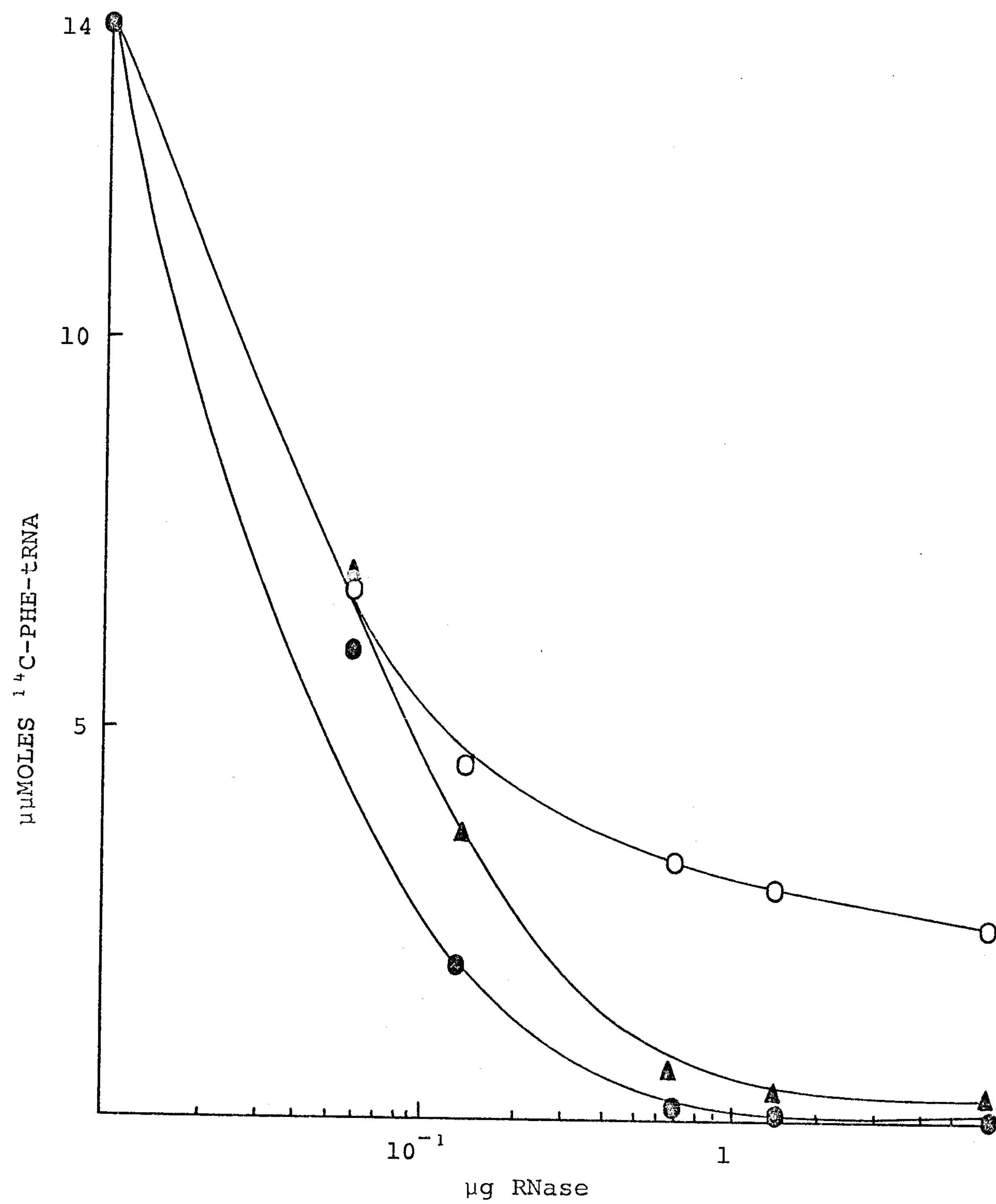

100
50
%
100
200
mM Mg^{2+}

40 minutes. This contrasts to the binding at 37°C which was more rapid and reached maximum after 30 minutes. At 37°C the *E.coli* ribosomal binding system is also far more rapid, but is highly unstable, and after 5 minutes a rapid decrease of binding is apparent. The superior rate and perhaps total extent of binding in the *H.cutirubrum* system at 37°C may be due to a decrease in secondary structure of the polymer template in the high salt. The time course of binding of *H.cutirubrum* ^{14}C-phe-tRNA with partially hydrolysed poly U at 37°C was the same as for *E.coli* ^{14}C-lys-tRNA with partially hydrolysed poly A. The response of *E.coli* and *H.cutirubrum* ^{14}C-phe-tRNA binding with partially hydrolysed poly U to Mg^{2+} concentration is shown in Fig. 9. The optimum for both tRNA's was 88 mM. It was later found that addition of 1.12 M NH_4Cl reduced the Mg^{2+} optimum to 60 mM, but did not significantly affect the total binding at 88 mM.

The RNase-TCA assay was also developed to estimate the binding of ^{14}C-aminoacyl-tRNA to ribosomes. Fig. 10 shows the hydrolysis of *E.coli* ^{14}C-phe-tRNA in the presence and absence of ribosomes and poly U. This system required approximately 100 times more RNase than that of *E.coli*, probably due to high salt inhibition of the RNase activity. Although ribosomes alone afford some protection to the ^{14}C-aminoacyl-tRNA, only when poly U was present also, was there substantial protection at high RNase concentrations. Table XXIV shows a direct comparison of the nitrocellulose

Figure 10 The Hydrolysis of E.coli ^{14}C-Phe-tRNA by Pancreatic RNase as a Function of RNase Concentration in the Presence and Absence of Ribosomes and Poly U

Each 0.125 ml reaction mixture contained the components, and was incubated as described in the "methods" under the RNase-TCA assay, except for the designated modifications. 15.6 μμmoles of ^{14}C-phe-tRNA E.coli (0.76 A_{260} unit), 4.54 A_{260} units of ribosomes and 50 μg partially hydrolysed poly U were used. O——O plus ribosomes, plus poly U. ▲——▲ plus ribosomes, minus poly U. ●——● minus ribosomes, minus poly U.



14
10
5
μμMOLES ^{14}C-PHE-tRNA
10^{-1}
1
μg RNase

Table XXIV Comparison of Nitrocellulose Filter and RNase-TCA Assays for H.cutirubrum Ribosomal Binding System

Assay Method	μμMoles ^{14}C-Phe-tRNA Bound	
	+ Poly U	- Poly
Nitrocellulose filter	2.61	0.37
RNase-TCA	2.75	0.56

Each 0.125 ml reaction mixture contained the components, and was incubated as described in the "methods" for the two assays, except for the designated modifications. 14.10 μμmoles of ^{14}C-phe-tRNA *E.coli* (0.70 A_{260} unit), 50 μg of partially hydrolysed poly U and 4.54 A_{260} units of ribosomes.

filter assay and the RNase-TCA assay. The amount of ^{14}C-aminoacyl-tRNA bound to the ribosomes estimated by either assay was very similar, showing that the bound ^{14}C-aminoacyl-tRNA was protected by the ribosome from RNase as in the *E.coli* system.

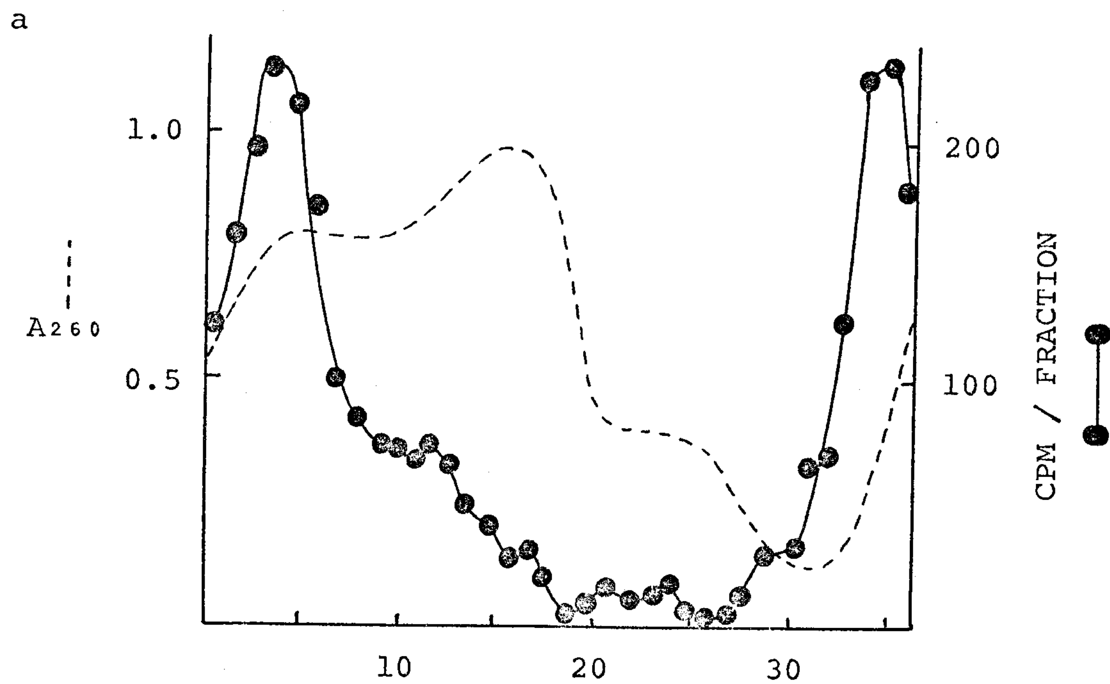
Figs. 11a+b show the results of examining the system on sucrose gradients. *E.coli* ^{14}C-lys-tRNA was bound to *H.cutirubrum* ribosomes with and without partially hydrolysed poly A in expanded incubation mixtures. These were then layered on 5% - 20% sucrose gradients supplemented with 2.8 M KCl and 0.1 M magnesium acetate. Resolution of the various ribosomal components was not very clean. This lack of resolution only appeared with washed and sedimented ribosomes (Dr. Rosa personal communication), and its nature has yet to be determined. However it was evident that radioactivity was only associated with the faster sedimenting 70S particle when partially hydrolysed poly A was present (Fig. 11a). This indicated that the *H.cutirubrum* ribosomal binding system was a real assay for ^{14}C-aminoacyl-tRNA binding to ribosomes in the presence of a specific template.

Table XXV shows the effect of adding different monovalent cations to the binding system. Unlike the whole protein synthesizing system (Bayley and Griffiths, 1968a), or the aminoacyl-tRNA transfer system (to be described) added monovalent cations have no stimulatory effect, and added NH_4Cl gave a very high background. On comparing the halophilic *in vitro* systems it is evident that each has a

Figure 11 Sucrose Gradients of the Binding of E.coli ^{14}C-Lys-tRNA to H.cutirubrum Ribosomes

a. The 0.625 ml (0.125 ml reaction mixture increased five fold) reaction mixture contained the components, and was incubated as described in the "methods", except for the designated modifications. 250 μg of partially hydrolysed poly A, 18 A_{260} units of ribosomes, 40.2 μμmoles ^{14}C-lys-tRNA E.coli (1.46 A_{260} units) were used. The reaction mixture was layered on a 36 ml 5% - 20% sucrose gradient containing 2.8 M KCl, 0.1 M magnesium acetate, 0.01 M Tris-HCl, pH 7.6. The gradient was centrifuged in a Spinco SW 27 rotor at a temperature of 4^0C at 23 K for 15 hours. 1 ml fractions were collected dropwise by puncturing the bottom of the tube. Radioactivity was determined after fractions were precipitated with serum albumin and 5% TCA and filtered.

b. This gradient was identical to that of (a.), except partially hydrolysed poly A was omitted from the reaction mixture.


a
1.0
0.5
A260
200
100
CPM / FRACTION
10
20
30
BOTTOM
TOP

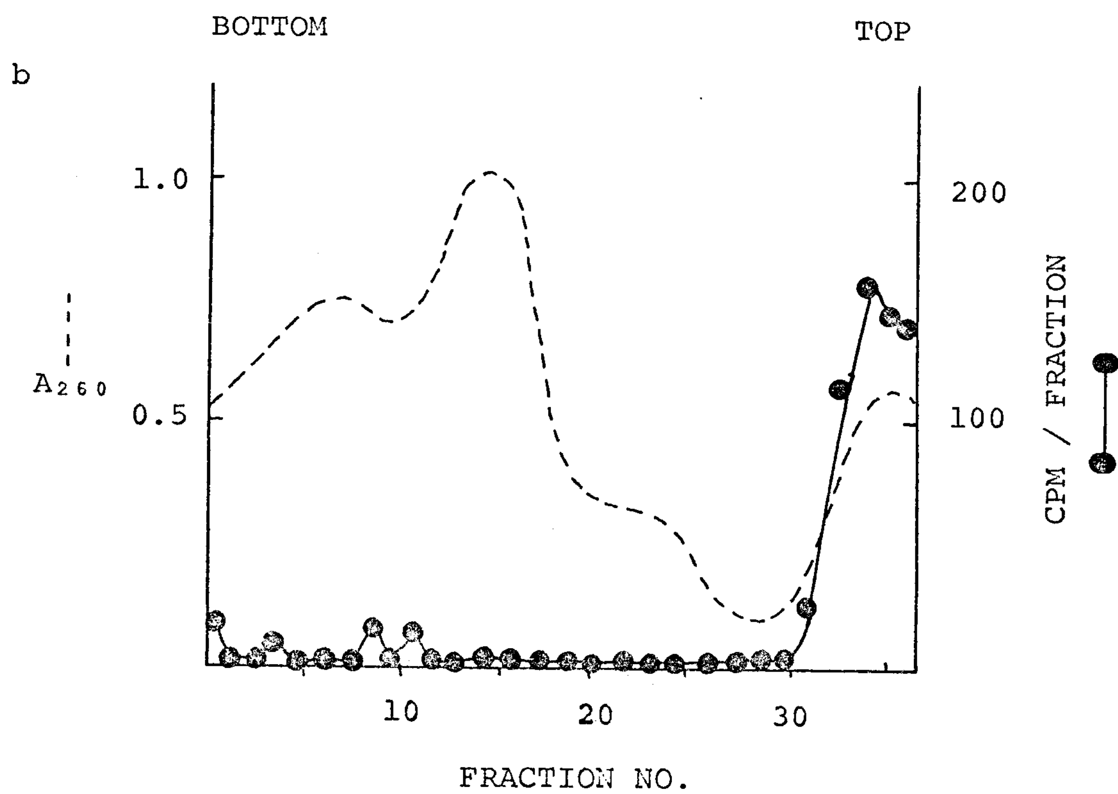

b
1.0
0.5
A260
200
100
CPM / FRACTION
10
20
30
FRACTION NO.

Table XXV Effect of Added NH_4Cl and NaCl on the Binding of E.coli ^{14}C-Phe-tRNA to H.cutirubrum Ribosomes

Monovalent Cations	μμMoles ^{14}C-Phe-tRNA Bound	
	+ Poly U	- Poly
3.8 M KCl	2.73	0.53
3.8 M KCl, 1.0 M NaCl, 0.4 M NH_4Cl	2.72	0.49
3.8 M KCl, 1.12 M NH_4Cl	2.84	0.87

Each 0.125 ml reaction mixture contained the components and was incubated as described in the methods, except for the designated modifications. 14.10μμmoles of ^{14}C-phe-tRNA E.coli (0.70 A_{260} unit), 50 μg partially hydrolysed poly U and 4.54 A_{260} units of ribosomes were used.

slightly different requirement for monovalent cations on top of the 3.8 M KCl and added 1.0 M NaCl and 0.4 M NH_4Cl is the best compromise for the whole protein synthesizing system. It would seem that for binding of aminoacyl-tRNA, added monovalent cations are not as essential as for the whole protein synthesizing system, perhaps reflecting the non-enzymic nature of this binding. NH_4^+ does reduce the Mg^{2+} optimum considerably indicating that these two cations can compensate for each other in some situations. It is also of interest to compare Mg^{2+} concentrations in the two systems. The whole protein synthesizing system has an optimum with poly U around 40 mM Mg^{2+} and a miscoding optimum of about 90 mM, where leucine is incorporated as well as phenylalanine. The ribosomal binding system has an optimum of about 88 mM Mg^{2+}, the concentration at which the ribosomal subunits appear to be forced together.

These results are strong indication that non-halophilic tRNA can bind normally to halophilic ribosomes in a high salt environment. Therefore it would seem that halophilic tRNA has not been grossly modified to function in these saturated cation concentrations. This system does allow codons such as AAA to be tested in the high salt environment and could be used to assign most of the codons, if the relevant triplet templates were available.

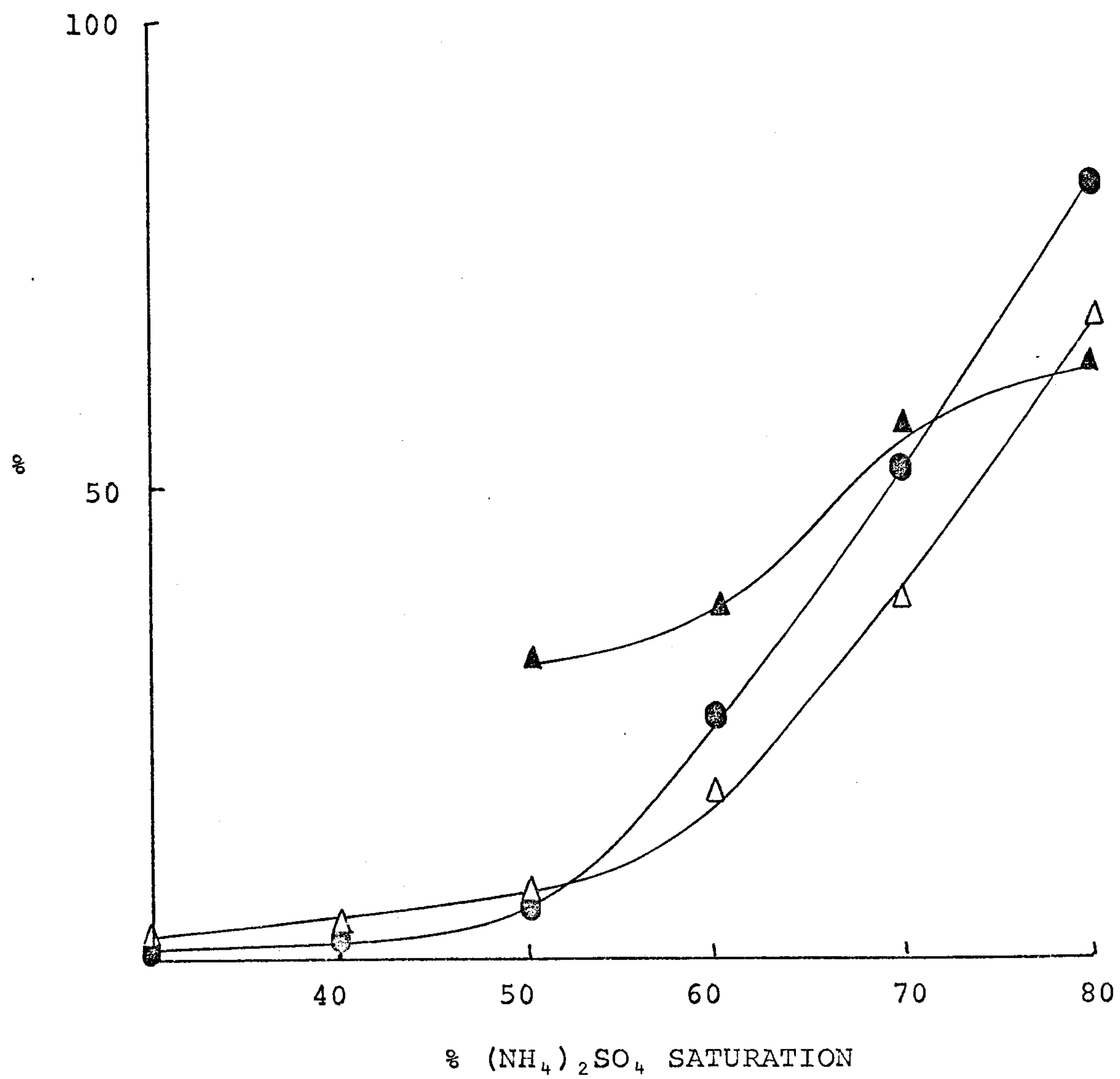
3. H.cutirubrum Aminoacyl-tRNA Transfer System: A Comparison of E.coli and H.cutirubrum ^{14}C-Aminoacyl-tRNA

This system was developed in preparation for the use of E.coli systems, in which the codon responses of halophilic aminoacyl-tRNA could be tested. It was used to thoroughly check if non-halophilic aminoacyl-tRNA could transfer amino acids to polypeptide in a high salt environment. From the previous section it was clear that E.coli aminoacyl-tRNA bound to halophilic ribosomes at very high concentrations of Mg^{2+}, but it was by no means certain that this behaviour could be extended to the recognition of this tRNA by the transferase enzymes, and ultimate transfer of amino acid to polypeptide. The system, unlike the whole protein synthesizing system, utilizes ^{14}C-aminoacyl-tRNA, rather than ^{14}C-amino acid, ATP, synthetases and tRNA. In developing the system greatest priority was given to eliminate as much as possible the tRNA-synthetase interactions. This necessitated, besides separation of transferase from synthetase activity, the elimination of tRNA and ATP from the S-150.

Fig. 12 shows the ammonium sulfate fractionation of the S-150, as regards transferase and phenylalanine synthetase activity, as well as protein content. Fractionation

Figure 12 Ammonium Sulfate Fractionation of *H.cutirubrum* S-150

S-150 was dialyzed against the appropriate ammonium sulfate saturation overnight. The precipitates were removed by centrifugation and dissolved in solution D. Both dissolved precipitates and supernatants were dialyzed back into solution D overnight. Protein was estimated by the Lowry method (100% = 25.4 mg/ml). Transferase activity was measured in the *H.cutirubrum* aminoacyl-tRNA transfer system described in the "methods", using 18.80 μμmoles ^{14}C-phe tRNA *E.coli*, 0.050 mg poly U and 2.5 A_{260} units of ribosomes. (100% = 8.54 μμmoles ^{14}C-phe in hot 5% TCA-tungstate precipitate). Phenylalanine synthetase activity was measured in the *H.cutirubrum* charging system described in the "methods" (100% = 10.4 μμmoles ^{14}C-phe in cold 5% TCA precipitate). Protein concentration and enzyme activities are normalised to that in the S-150 being 100%. ●——● transferase activity in the precipitate. △——△ protein in the precipitate. ▲——▲ phenylalanine synthetase activity if the precipitate.


100
%
50
40
50
60
70
80
% $(NH_4)_2SO_4$ SATURATION

of phenylalanine synthetase between supernatant and precipitate was not clear cut, however transferase activity was, and a small separation was achieved by using the 65-80% precipitate. This preparation still contained tRNA, and to remove this it was passed through a Sephadex G-75 column and the first protein peak was utilized directly, or concentrated by a further ammonium sulfate precipitation. This preparation was used as the source of transferases, although as will be seen later it contained a lot of synthetase activity but little tRNA.

Table XXVI shows the basic features of the system. A small but significant incorporation of ^{14}C-lys from E.coli tRNA could be seen in response to partially hydrolysed poly A, but this was absent when H.cutirubrum tRNA was used as the amino acid donor. E.coli tRNA transferred ^{14}C-phe readily into polypeptide in response to poly U. This was inhibited by RNase and puromycin, and was completely dependent on transfer enzymes and ribosomes, while deacylated tRNA had little effect. Very little incorporation of free ^{14}C-phe was found, showing the systems dependence on ^{14}C-aminoacyl-tRNA. Transfer of ^{14}C-phe from H.cutirubrum tRNA was much lower than from E.coli tRNA, and this will be fully discussed later. The transfer of amino acids was codon specific, little misincorporation of ^{14}C-leu occurring from E.coli tRNA.

Table XXVI Each 0.125 ml reaction mixture contained the components, and was incubated as described in the "methods", except for the designated modifications. The amounts of added ^{14}C-aa-tRNA were as follows: 18.89 μμmoles ^{14}C-lys-tRNA *E.coli* (0.69 A_{260} unit), 15.52 μμmoles ^{14}C-lys-tRNA *H.cutirubrum* (2.50 A_{260} units), 18.80 μμmoles ^{14}C-phe-tRNA *E.coli* (0.92 A_{260} unit), 19.04 μμmoles ^{14}C-phe-tRNA *H.cutirubrum* (3.67 A_{260} units) and 24.10 μμmoles ^{14}C-leu-tRNA *E.coli* (0.68 A_{260} unit) and 15.26 μμmoles ^{14}C-leu-tRNA *H.cutirubrum* (1.91 A_{260} unit). 660 μμmoles of ^{14}C-phe, 25 μg puromycin, 10 μg of RNase, 250 μg of transferases, 2.50 A_{260} units of ribosomes and 50 μg of poly U or partially hydrolysed poly A were used.

Table XXVI Characteristics of the H.cutirubrum Aminoacyl-tRNA Transfer System

^{14}C-AA-tRNA	Modifications	μμMoles ^{14}C-AA in the Hot 5% TCA-Tungstate Ppt.		
		Poly A	Poly U	-Poly
^{14}C-lys-tRNA *E.coli*	-	0.84	0.47	0.50
^{14}C-lys-tRNA *H.cutirubrum*	-	0.73	0.81	0.69
^{14}C-phe-tRNA *E.coli*	-	-	8.91	0.28
"	+ Puromycin	-	0.17	-
"	+ RNase	-	0.34	-
"	- Transferases	-	0.77	-
"	- Ribosomes	-	0.21	-
"	+ 1 A_{260} unit *E.coli* tRNA	-	8.73	-
^{14}C-phe	-	-	0.72	0.31
^{14}C-phe-tRNA *H.cutirubrum*	-	-	2.43	0.39
"	+ Puromycin	-	0.21	-
"	+ RNase	-	0.33	-
"	- Transferases	-	0.61	-
"	- Ribosomes	-	0.15	-
"	+ 1 A_{260} unit *E.coli* tRNA	-	2.53	-
^{14}C-leu-tRNA *E.coli*	-	0.93	1.32	1.14
^{14}C-leu-tRNA *H.cutirubrum*	-	0.67	0.71	0.63

Fig. 13 shows the effect of varying the amount of ^{14}C-phe-tRNA added to the system. Transfer from E.coli tRNA was linear over the range, with about 50% of input aminoacyl-tRNA transferring the amino acid to polypeptide. However, transfer of amino acid from H.cutirubrum tRNA was poor over the whole range. If PEP and ATP were added, the system transferred about twice as much amino acid, indicating one reason for the original poor transfer was a lack of ATP or a generating system. The cause therefore appeared to be a discharge of the H.cutirubrum aa-tRNA by synthetases in the preparation, which were unable to discharge the E.coli ^{14}C-phe-tRNA. In an attempt to overcome this discharge, the system was tested with varying amounts of transfer enzyme preparation, in the hope one concentration would have sufficient transferase activity but minimal synthetase activity. As is evident from Fig. 14 no such concentration was found. The discharge was investigated directly as shown in Table XXVII where E.coli and H.cutirubrum ^{14}C-phe-tRNA were incubated with the transferase preparation. Addition of PEP and ATP severely reduced the amount of discharge of H.cutirubrum ^{14}C-phe-tRNA. Little discharge of amino acid from E.coli ^{14}C-phe-tRNA was observed.

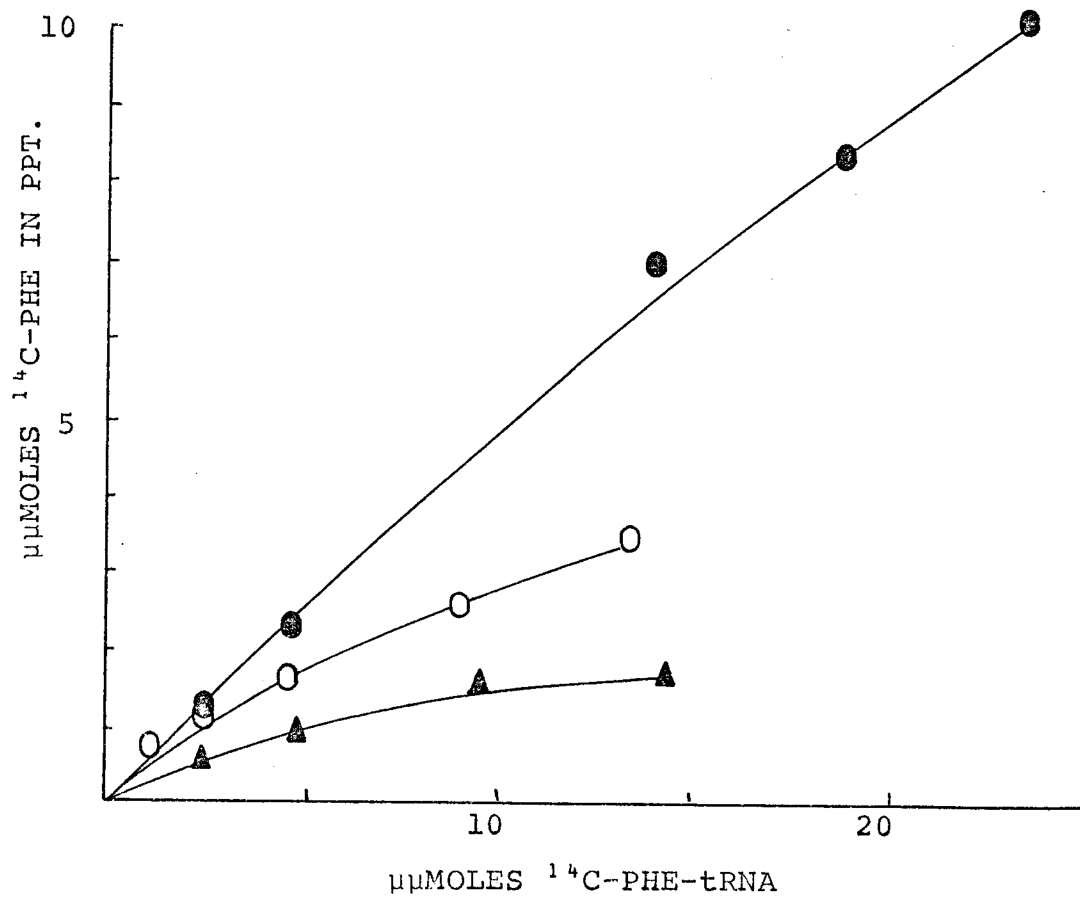
The response of the system to varying Mg^{2+} concentrations is shown in Fig. 15. The optimum for transfer from both E.coli and H.cutirubrum tRNA was 30-40 mM.

Figure 13 Transfer of ^{14}C-AA from ^{14}C-AA-tRNA to polypeptide as a Function of Added μμmoles ^{14}C-AA-tRNA

Each 0.125 ml reaction mixture contained the components, and was incubated as described in the "methods", except for the designated modifications. 50 μg of poly U and 4.56 A_{260} units of ribosomes were used. ●——● ^{14}C-phe-tRNA E.coli (1 A_{260} unit contains 20.5 μμmoles ^{14}C-phe). ▲——▲ ^{14}C-phe-tRNA H.cutirubrum (1 A_{260} unit contains 5.2 μμmoles ^{14}C-phe). O——O ^{14}C-phe-tRNA H.cutirubrum (1 A_{260} unit contains 5.2 μμmoles ^{14}C-phe) and 0.6 μmole PEP and 0.15 μmole ATP. The results without poly U have been subtracted.

Figure 14 Transfer of ^{14}C-AA from ^{14}C-AA-tRNA to Polypeptide as a Function of Added Transferase Preparation

Each 0.125 ml reaction mixture contained the components, and was incubated as described in the "methods", except for the designated modifications. 50 μg of poly U, 2.50 A_{260} units of ribosomes and 14.10 μμmoles ^{14}C-phe-tRNA E.coli (0.70 A_{260} unit) and 14.22 μμmoles ^{14}C-phe-tRNA H.cutirubrum (2.75 A_{260} units) were used. ●——● ^{14}C-phe-tRNA E.coli ▲——▲ ^{14}C-phe-tRNA H.cutirubrum. The results without poly U have been subtracted.


10
5
10
20
μμMOLES ^{14}C-PHE IN PPT.
μμMOLES ^{14}C-PHE-tRNA

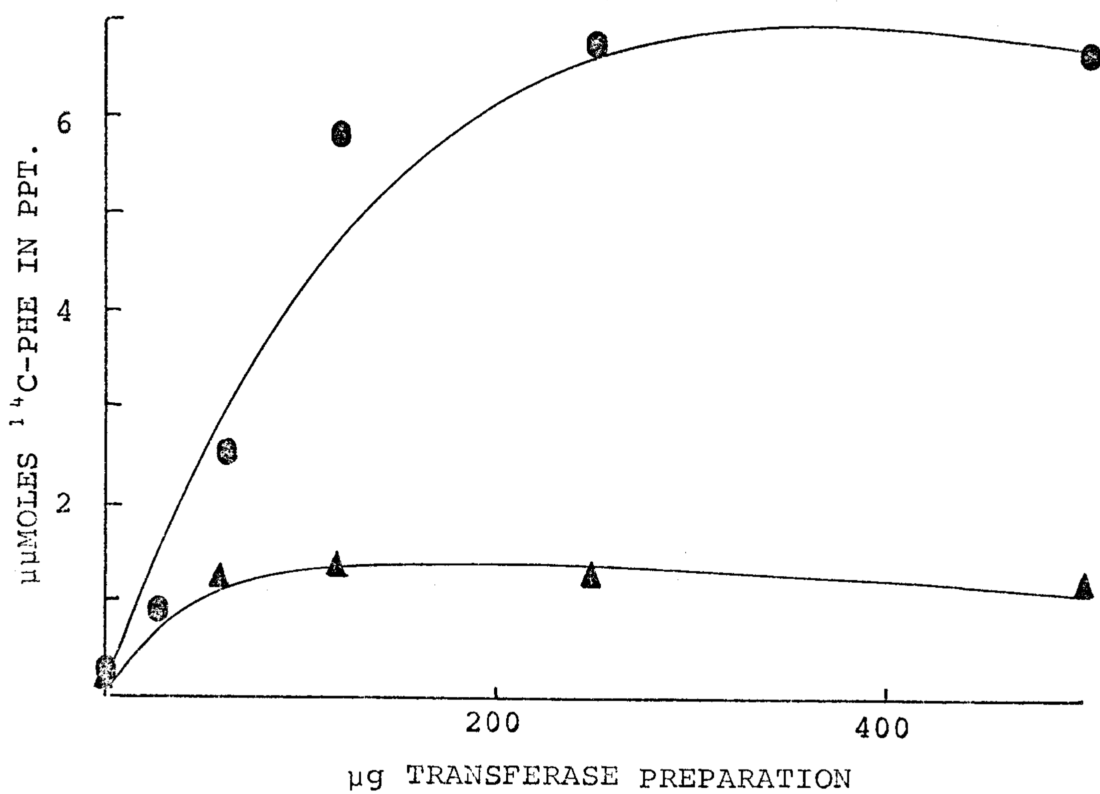

6
4
2
200
400
μμMOLES ^{14}C-PHE IN PPT.
μg TRANSFERASE PREPARATION

Table XXVII Discharge of ^{14}C-Phe from ^{14}C-Phe-tRNA by the Transferase Preparation

Type of ^{14}C-Phe-tRNA	Modifications	μμmoles ^{14}C-Phe in Cold 5% TCA Precipitate
E.coli	-	8.00
"	-transferases	9.03
H.cutirubrum	-	0.52
"	-transferases	4.58
"	+ ATP and PEP	2.23

Each 0.125 ml reaction mixture contained the components (without ribosomes), and was incubated as described in the "methods" for the whole protein synthesizing system except for the designated modifications. 250 μg of transferases, 12.7 μμmoles ^{14}C-phe-tRNA *E.coli*, 7.3 μμmoles ^{14}C-phe-tRNA *H.cutirubrum*, 0.6 μmole PEP and 0.15 μmole ATP were used. ^{14}C-phe-tRNA was precipitated by cold 5% TCA.

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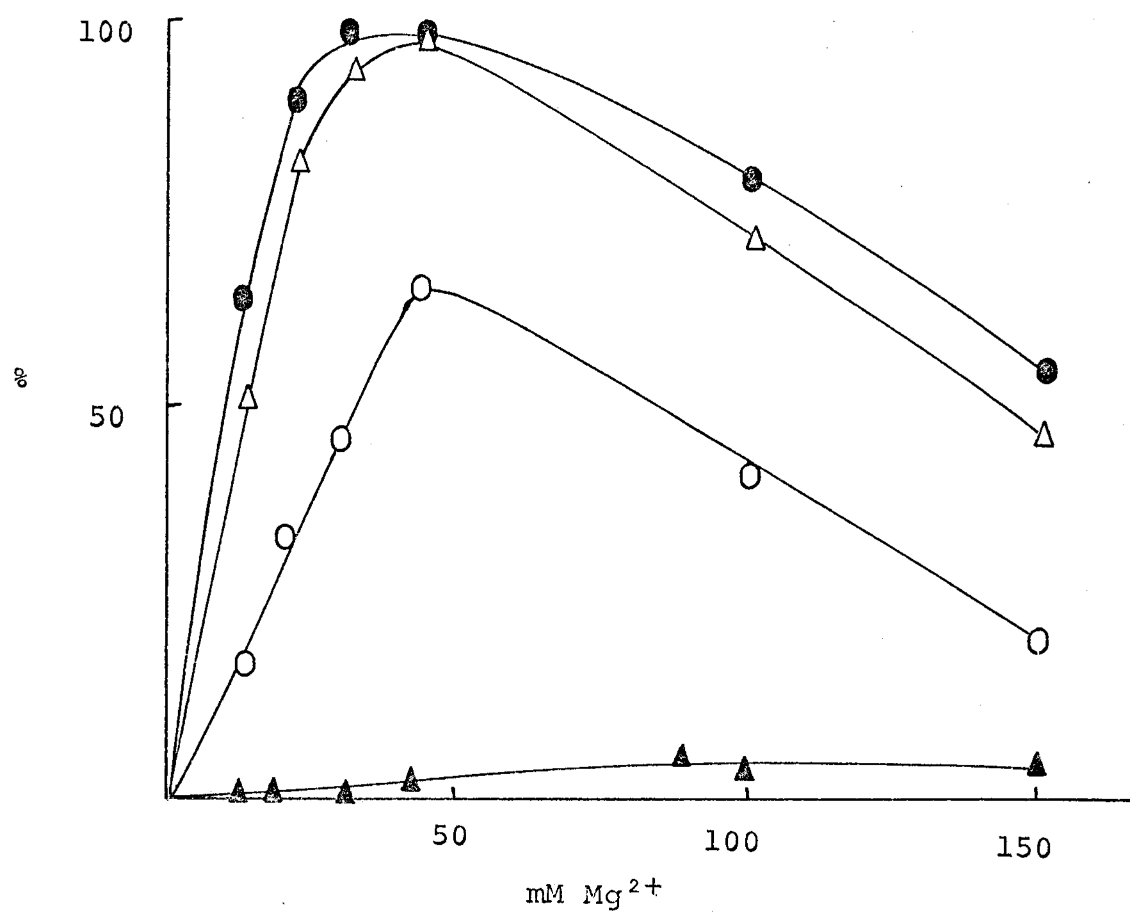
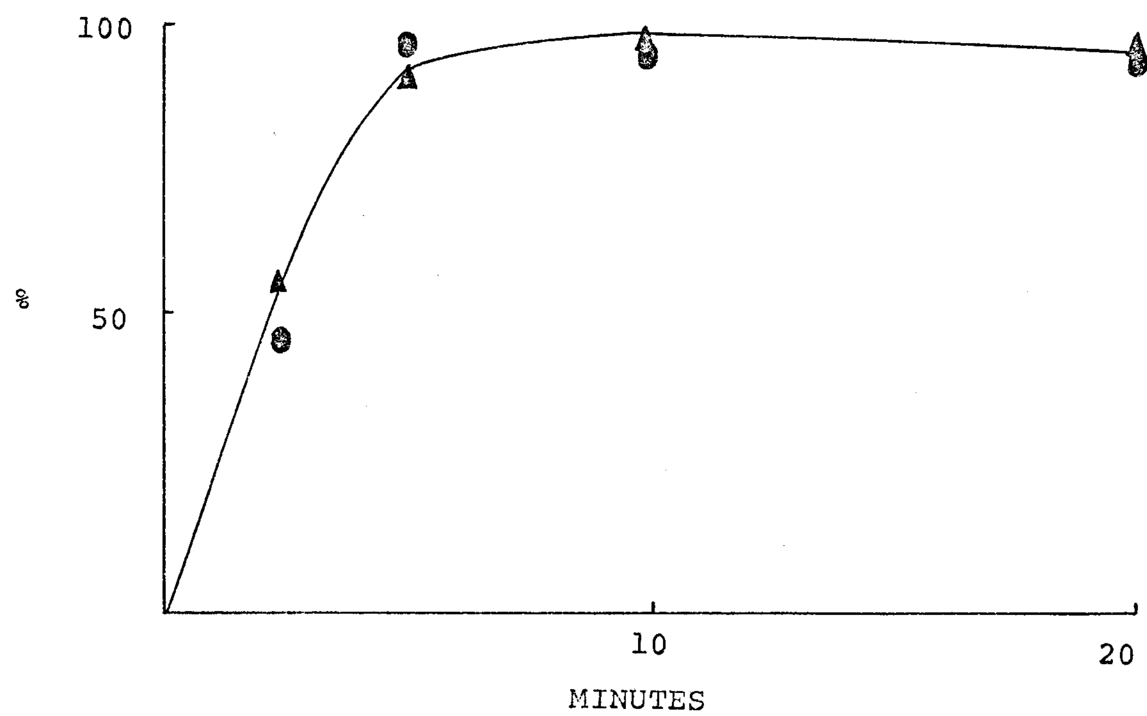
Figure 15 Transfer of ^{14}C-AA from ^{14}C-AA-tRNA to Polypeptide as a Function of Magnesium Concentration

Each 0.125 ml reaction mixture contained the components, and was incubated as described in the "methods", except for the designated modifications. 50 μg of poly U, 250 μg of transferases, 2.50 A_{260} units of ribosomes, 14.10 μμmoles ^{14}C-phe-tRNA E.coli (0.70 A_{260} unit), 14.22 μμmoles ^{14}C-phe-tRNA H.cutirubrum (2.75 A_{260} units) and 24.10 μμmoles A_{260} units of ^{14}C-leu-tRNA E.coli (0.68 A_{260} unit) were used. ●——● ^{14}C-phe-tRNA E.coli (100% = 6.73 μμmoles). O——O ^{14}C-phe-tRNA E.coli - 3.8 M KCl, 1.0 M NaCl, 0.4 M NH_4Cl (100% = 6.73 μμmoles). △——△ ^{14}C-phe-tRNA H.cutirubrum (100% = 1.75 μμmoles). ▲——▲ ^{14}C-leu-tRNA E.coli (100% = 6.73 μμmoles). Results without poly U have been subtracted.

Figure 16 Transfer of ^{14}C-AA from ^{14}C-AA-tRNA to Polypeptide as a Function of Time

Each 0.125 ml reaction mixture contained the components, and was incubated as described in the "methods", except for the designated modifications. 50 μg of poly U, 250 μg of transferases, 2.50 A_{260} units of ribosomes, 4.70 μμmoles ^{14}C-phe-tRNA E.coli (0.23 A_{260} unit) and 14.22 μμmoles ^{14}C-phe-tRNA H.cutirubrum (2.75 A_{260} units) were used. ●——● ^{14}C-phe-tRNA E.coli (100% = 2.49 μμmoles). ▲——▲ ^{14}C-phe-tRNA H.cutirubrum (100% = 1.99 μμmoles). Results without poly U have been subtracted.


100
50
%
50
100
150
mM Mg^{2+}


100
50
%
10
20
MINUTES

This is similar to the Mg^{2+} optimum for the whole system as described by Bayley and Griffiths (1968b). Misincorporation of ^{14}C-leu from E.coli tRNA only began around 100 mM, the point at which the ribosomal subunits were forced together in the binding system (Fig. 9). Fig. 15 also shows the magnesium curve for the system with 1 M NaCl and 0.4 M NH_4Cl (mixture II), instead of 1.12 M NH_4Cl (mixture I). Incorporation was much lower and as is shown in Table XXVIII, NH_4^+ was far the superior additional monovalent cation for the system. This contrasts to the whole protein synthesizing system (Bayley and Griffiths, 1968a) in which mixture II was the optimal cation mixture. The reason for this now seems to be that in the whole protein synthesizing system many synthetases are inhibited by NH_4^+ (Griffiths and Bayley, 1969), thus reducing the incorporation of amino acids into polypeptide in mixture I. In the aminoacyl-tRNA transfer system, synthetase activity is not required, therefore mixture I gives higher activity. This emphasizes that in halophilic protein synthesis each step has a slightly different monovalent cation requirement.

The time course for the transfer of amino acids to polypeptide is shown in Fig. 16. As is evident the reaction with both E.coli and H.cutirubrum ^{14}C-aminoacyl-tRNA was complete in about 5 minutes. This system then can utilize both E.coli and H.cutirubrum aminoacyl-tRNA, although the transfer from H.cutirubrum aminoacyl-tRNA appears less, probably because of discharge of amino acids by synthetases in the

Table XXVIII Effect of Added NH_4Cl and NaCl on the Aminoacyl-tRNA Transfer System

Monovalent Cations	μμMoles ^{14}C-Phe in Hot TCA-Tungstate Ppt.
3.8 M KCl	3.02
3.8 M KCl, 1.0 M NaCl, 0.4 M NH_4Cl	5.61
3.8 M KCl, 1.12 M NH_4Cl	8.54
3.8 M KCl, 1.4 M NaCl	3.61

Each 0.125 ml reaction mixture contained the components and was incubated as described in the "methods" except for the designated modifications. 250 μg of transferases, 2.50 A_{260} units of ribosomes, 50 μg of poly U and 18.8 μμmoles ^{14}C-phe-tRNA E.coli (0.92 A_{260} unit) were used. Results without poly U have been subtracted.

system. These results indicate that *E.coli* aminoacyl-tRNA when placed in the high salt system spontaneously assumes a conformation that is recognised by the *H.cutirubrum* transfer enzymes and ribosomes. Codon-anticodon interactions would also appear to be the same in high and low salt.

In order to utilize polyribonucleotides that precipitate in high salt, similar *E.coli* transfer and binding systems were developed. These would further indicate whether the halophilic tRNA had been modified for its high salt environment.

4. E.coli Ribosomal Binding and Aminoacyl-tRNA Transfer Systems: A Comparison of E.coli and H.cutirubrum ^{14}C-Aminoacyl-tRNA

From the results in the previous two sections, it was evident that E.coli ^{14}C-aminoacyl-tRNA functioned just as well as that from H.cutirubrum, in high salt systems. The E.coli systems were developed to show that this similarity of response extended to halophilic tRNA in a far lower salt environment. These systems would then be used to test codon responses of halophilic aminoacyl-tRNA's, that could not be tested in high salt.

Ribosomal Binding Systems

The system was taken directly from that described by Nirenberg and Leder (1964). Table XXIX shows the general properties of the system. Binding of both H.cutirubrum and E.coli ^{14}C-lys-tRNA occurred specifically in the presence of poly A, with that of H.cutirubrum being lower for the reasons described in Chap.IV,2. The binding was dependent on ribosomes and Mg^{2+} and was reduced by deacylated tRNA. Binding seemed quite codon specific, with little miscoding by ^{14}C-leu-tRNA and poly U. The halophilic aminoacyl-tRNA appears to be accepted by the E.coli ribosomes in low salt, as the E.coli aminoacyl-tRNA was accepted in the halophile systems.

Table XXIX Each 0.050 ml reaction mixture contained the components, and was incubated as described in the "methods" for the E.coli ribosomal binding system, except for the designated modifications. The amounts of added ^{14}C-aa-tRNA were as follows: 13.26 μμmoles ^{14}C-lys-tRNA E.coli (0.49 A_{260} unit), 15.52 μμmoles ^{14}C-lys-tRNA H.cutirubrum (2.50 A_{260} units), 14.10 μμmoles ^{14}C-phe-tRNA E.coli (0.70 A_{260} unit), 14.28 μμmoles ^{14}C-phe-tRNA H.cutirubrum (2.75 A_{260} units), 24.10 μμmoles ^{14}C-leu-tRNA E.coli (0.68 A_{260} unit), and 15.26 μμmoles ^{14}C-leu-tRNA H.cutirubrum (1.91 A_{260} units). 25 μg of poly A or poly U and 2.05 A_{260} units of ribosomes were present.

Table XXIX Characteristics of ^{14}C-Aminoacyl-tRNA Binding to E.coli Ribosomes

^{14}C-AA-tRNA	Modifications	μμMoles ^{14}C-AA Bound		
		Poly A	Poly U	-Poly
^{14}C-lys-tRNA *E. coli*	-	3.22	0.57	0.94
^{14}C-lys-tRNA *H. cutirubrum*	-	1.75	0.49	0.51
^{14}C-phe-tRNA *E. coli*	-	0.21	2.53	0.27
"	-Ribosomes	-	0.11	-
"	$-Mg^{2+}$	-	0.13	-
"	+ 1 A_{260} unit *E. coli* tRNA	-	0.92	0.21
"	+ 1 A_{260} unit *H. cutirubrum* tRNA	-	1.67	0.23
^{14}C-phe-tRNA *H. cutirubrum*	-	0.04	1.48	0.21
"	-Ribosomes	-	0.04	-
"	$-Mg^{2+}$	-	0.36	-
"	+ 1 A_{260} unit *E. coli* tRNA	-	0.87	-
"	+ 1 A_{260} unit *H. cutirubrum* tRNA	-	1.22	-
^{14}C-leu-tRNA *E. coli*	-	0.76	1.05	0.99
^{14}C-leu-tRNA *H. cutirubrum*	-	0.49	0.67	0.65

Fig. 17 illustrates the binding versus ^{14}C-aminoacyl-tRNA concentration. Once again binding of H.cutirubrum ^{14}C-aminoacyl-tRNA appeared poorer because of the lower percentage of $tRNA^{phe}$ and $tRNA^{lys}$ charged with ^{14}C-phe and ^{14}C-lys. Fig. 18 shows that the time course of the binding was the same for aminoacyl-tRNA's from E.coli and H.cutirubrum. The reaction here at 24^0C was over in 20 minutes and can be contrasted with that of the H.cutirubrum binding system, which is not over until 30 minutes at 37^0C. Fig. 19 illustrates that the Mg^{2+} optimum was around 20 mM for both ^{14}C-aminoacyl-tRNA's.

The E.coli binding system confirms the results of the H.cutirubrum systems, in that aminoacyl-tRNA from either bacterium assumes functionally similar conformations in similar environments. To confirm the specificity of the binding, the RNase-TCA assay of Pestka (1968) was used, and the results are shown in Table XXX. With H.cutirubrum ^{14}C-phe-tRNA, there was very little difference in the estimation of that bound to the ribosomes in the presence of poly U by either assay. This shows that the ribosomally bound halophilic aminoacyl-tRNA is insensitive to RNase, and therefore is probably bound in the same way as non-halophilic aminoacyl-tRNA.

Figure 17 Ribosomal Binding of ^{14}C-AA-tRNA as a Function of Added μμMoles ^{14}C-AA-tRNA

Each 0.050 ml reaction mixture contained the components, and was incubated as described in the "methods" for the E.coli ribosomal binding system, with the designated modifications. 25 μg of poly A or poly U, and 2.05 A_{260} units of ribosomes were present. ●——● ^{14}C-lys-tRNA E.coli (1 A_{260} unit contains 27.3 μμmoles ^{14}C-lys) and poly A. △——△ ^{14}C-phe-tRNA E.coli (1 A_{260} unit contains 20.5 μμmoles ^{14}C-phe) and poly U. ○——○ ^{14}C-lys-tRNA H.cutirubrum (1 A_{260} unit contains 6.2 μμmoles ^{14}C-lys) and poly A. ▲——▲ ^{14}C-phe-tRNA H.cutirubrum (1 A_{260} unit contains 5.2 μμmoles ^{14}C-phe) and poly U. The results without the polymer have been subtracted.

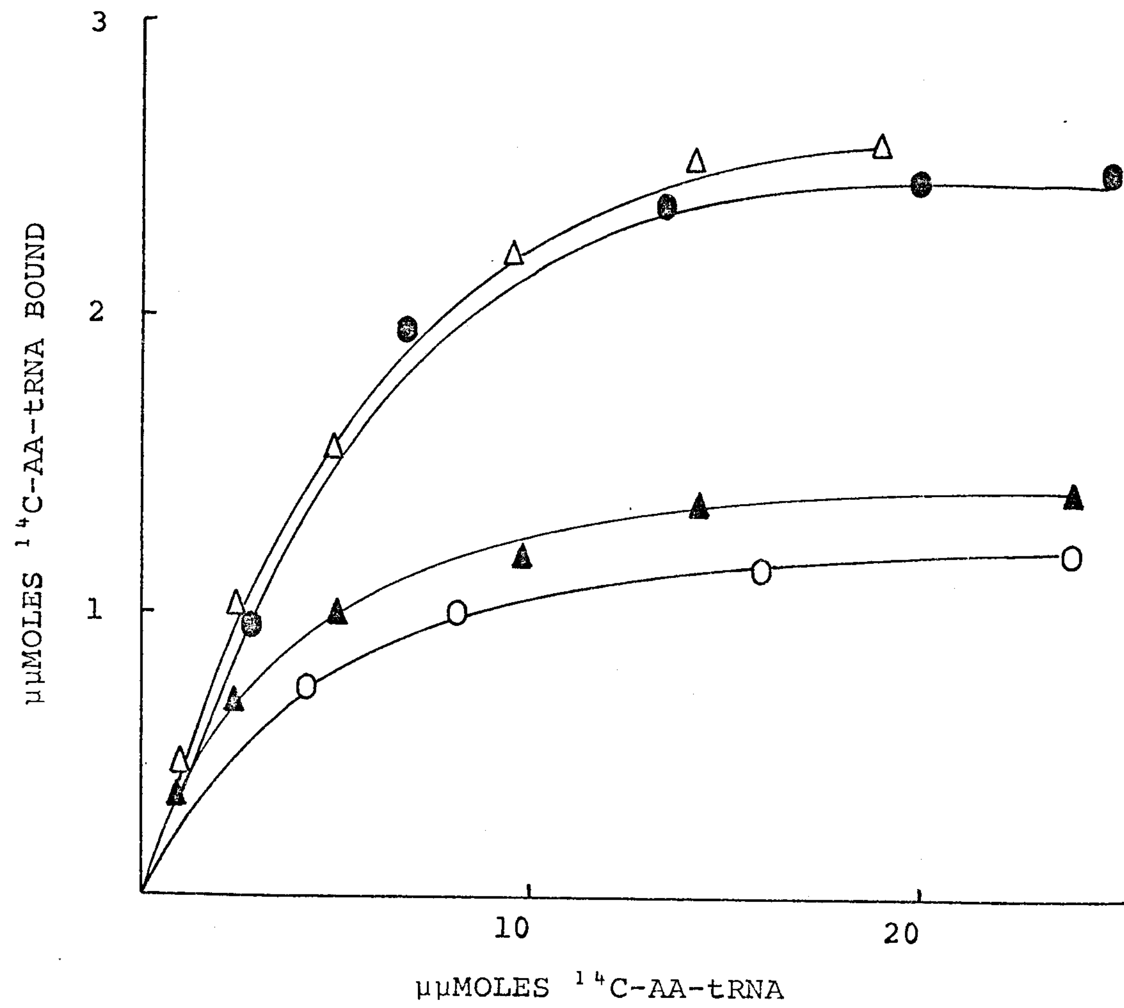
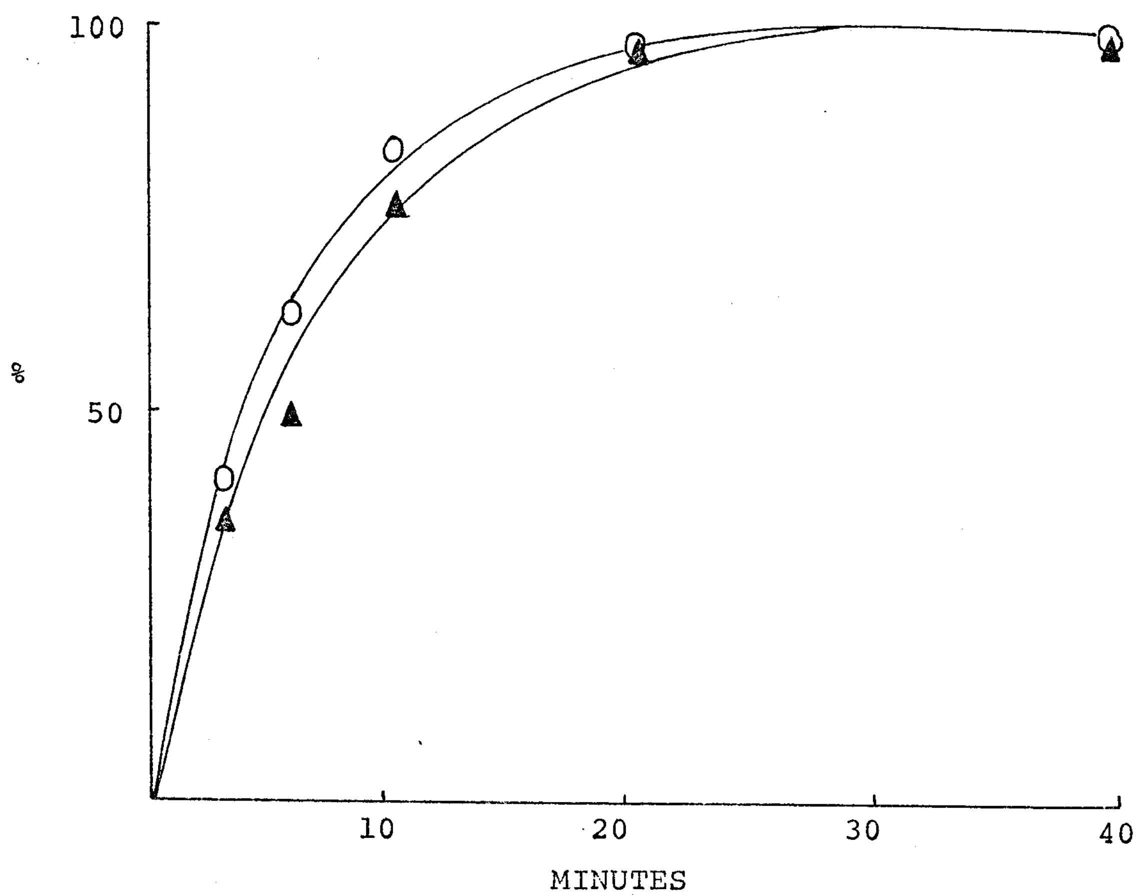

3
2
1
10
20
μμMOLES ¹⁴C-AA-tRNA BOUND
μμMOLES ¹⁴C-AA-tRNA

Figure 18 Ribosomal Binding of ^{14}C-Lys-tRNA with Poly A as a Function of Time

Each 0.050 ml reaction mixture contained the components, and was incubated as described in the "methods" for the E.coli ribosomal binding system, except for the designated modifications. 25 μg of poly A, 2.5 A_{260} units of ribosomes, 13.26 μμmoles ^{14}C-lys-tRNA E.coli (0.49 A_{260} unit) and 23.28 μμmoles ^{14}C-lys-tRNA H.cutirubrum (3.75 A_{260} units) were used. O——O ^{14}C-lys-tRNA E.coli (100% = 2.37 μμmoles). ▲——▲ ^{14}C-lys-tRNA H.cutirubrum (100% = 1.13 μμmoles). Results without poly A have been subtracted.

Figure 19 Ribosomal Binding of ^{14}C-Phe-tRNA with Poly U as a Function of Magnesium Concentration

Each 0.050 ml reaction mixture contained the components, and was incubated as described in the "methods" for the E.coli ribosomal binding system, except for the designated modifications. 25 μg of poly U, 3.1 A_{260} units of ribosomes, 18.8 μμmoles ^{14}C-phe-tRNA E.coli (0.9 A_{260} unit) and 14.28 μμmoles ^{14}C-phe-tRNA H.cutirubrum (2.75 A_{260} units) were used. O——O ^{14}C-phe-tRNA H.cutirubrum (100% = 1.67 μμmoles). ▲——▲ ^{14}C-phe-tRNA E.coli (100% = 3.12 μμmoles). Results without poly U have been subtracted.


100
%
50
10
20
30
40
MINUTES

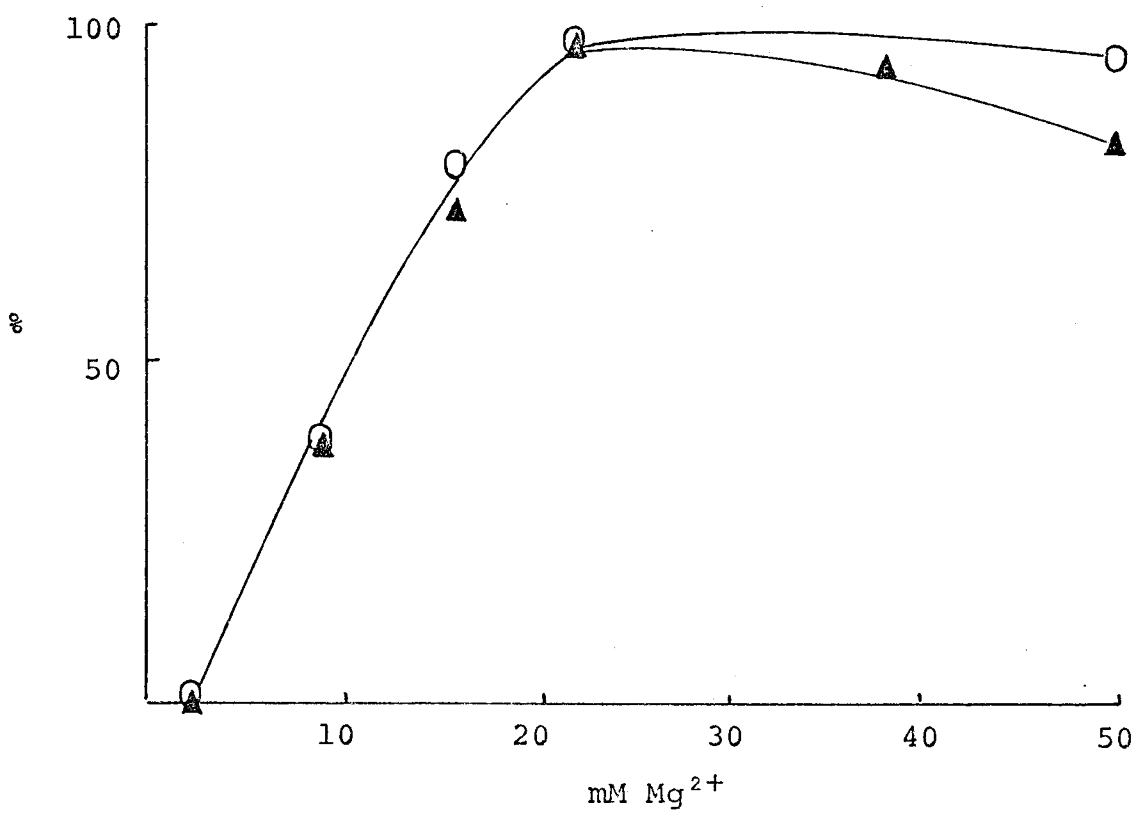

100
%
50
10
20
30
40
50
mM Mg2+

Table XXX Comparison of Nitrocellulose and RNase-TCA Assays

Assay Method	μμMoles ^{14}C-Phe-tRNA Bound	
	+ Poly U	- Poly
Nitrocellulose Filter	1.59	0.19
RNase-TCA	1.63	0.31

Each 0.050 ml reaction mixture contained the components and was incubated as described in the "methods" for the two assay systems, except for the designated modifications. 25 μg of poly U, 2.05 A_{260} units of ribosomes and 14.28 μμmoles ^{14}C-phe-tRNA H.cutirubrum (2.75 A_{260} units) were present.

Aminoacyl-tRNA Transfer System

As in the H.cutirubrum systems transfer of amino acid from aminoacyl-tRNA to polypeptide was the real test of whether the halophilic tRNA could function in a completely foreign ionic environment. Table XXXI illustrates the major features of the system. Both E.coli and H.cutirubrum tRNA transferred ^{14}C-lys into polypeptide in response to poly A. The reason for the lower transfer from H.cutirubrum tRNA is not clear at this time. Transfer of ^{14}C-phe from both E.coli and H.cutirubrum tRNA into polypeptide in response to poly U was equivalent. The transfer was inhibited by puromycin and RNase and was dependent on transfer enzymes and ribosomes. Addition of deacylated tRNA had little effect on the reaction. The transfer was codon specific, in that little miscoding was seen with ^{14}C-leu-tRNA from either bacterium in response to poly U.

Fig. 20 shows that E.coli or H.cutirubrum ^{14}C-phe-tRNA transferred their amino acid with almost the same efficiency into polypeptide with low concentrations of the transferase preparation. At five times the optimal concentration of transferase preparation the transfer from E.coli ^{14}C-phe-tRNA was severely reduced, but this reduction could be partially overcome by the addition of ATP and an ATP generating system. This situation at high transferase enzyme preparation was very similar to that found in the H.cutirubrum transfer

Table XXXI Each 0.100 ml reaction mixture contained the components, and was incubated as described in the "methods", except for the designated modifications. The amounts of added ^{14}C-aa-tRNA were as follows: 18.89 μμmoles ^{14}C-lys-tRNA E.coli (0.69 A_{260} unit) 15.52 μμmoles ^{14}C-lys-tRNA H.cutirubrum (2.5 A_{260} units), 18.80 μμmoles ^{14}C-phe-tRNA E.coli (0.92 A_{260} unit), 19.04 μμmoles ^{14}C-phe-tRNA H.cutirubrum (3.67 A_{260} units), 24.10 μμmoles ^{14}C-leu-tRNA E.coli (0.68 A_{260} unit) and 15.26 μμmoles ^{14}C-leu-tRNA H.cutirubrum (1.91 A_{260} units). 660 μμmoles ^{14}C-phe, 25 μg of poly U or poly A, 1 A_{260} unit of ribosomes, 50 μg transferases, 25 μg puromycin and 10 μg of RNase were used.

Table XXXI Characteristics of E.coli Aminoacyl-tRNA Transfer System

^{14}C-AA	Modifications	μμMoles ^{14}C-AA in the Hot 5% TCA-Tungstate Ppt.		
		Poly A	Poly U	-Poly
^{14}C-lys-tRNA *E.coli*	-	6.63	0.93	1.21
^{14}C-lys-tRNA *H.cutirubrum*	-	2.74	0.63	0.72
^{14}C-phe-tRNA *E.coli*	-	-	9.91	0.34
"	+ Puromycin	-	0.27	-
"	+ RNase	-	0.24	-
"	- Transferases	-	0.31	-
"	- Ribosomes	-	0.17	-
"	+ 1 A_{260} unit *E.coli* tRNA	-	9.63	-
^{14}C-phe	-	-	0.47	0.36
^{14}C-phe-tRNA *H.cutirubrum*	-	-	9.03	0.27
"	+ Puromycin	-	0.23	-
"	+ RNase	-	0.26	-
"	- Transferases	-	0.31	-
"	- Ribosomes	-	0.16	-
"	+ 1 A_{260} unit *E.coli* tRNA	-	8.86	-
^{14}C-leu-tRNA *E.coli*	-	-	0.29	0.27
^{14}C-leu-tRNA *H.cutirubrum*	-	-	0.23	0.19

Figure 20 Transfer of ^{14}C-Phe from ^{14}C-Phe-tRNA to Polypeptide as a Function of Added μμMoles ^{14}C-Phe-tRNA

Each 0.100 ml reaction mixture contained the components, and was incubated as described in the "methods" for the E.coli aminoacyl-tRNA transfer system, except for the designated modifications. 25 μg of poly U and 1 A_{260} unit of ribosomes were used. ●——● ^{14}C-phe-tRNA E.coli (1 A_{260} unit contains 20.5 μμmoles ^{14}C-phe) with 50 μg transferases. ▲——▲ ^{14}C-phe-tRNA E.coli (1 A_{260} unit contains 20.5 μμmoles ^{14}C-phe) with 240 μg transferases. Δ——Δ ^{14}C-phe-tRNA E.coli (1 A_{260} unit contains 20.5 μμmoles ^{14}C-phe) with 240 μg transferases, 0.6 μmole PEP and 0.15 μmole ATP. O——O ^{14}C-phe-tRNA H.cutirubrum (1 A_{260} unit contains 5.2 μμmoles ^{14}C-phe) with 50 μg of transferases. The results without poly U have been subtracted.

Figure 21 Transfer of ^{14}C-Phe from ^{14}C-Phe-tRNA to Polypeptide as a Function of Added Transferase Preparation

Each 0.100 ml reaction mixture contained the components, and was incubated as described in the "methods" for the E.coli aminoacyl-tRNA transfer system, except for the designated modifications. 25 μg of poly U, 1 A_{260} unit ribosomes, 9.40 μμmoles ^{14}C-phe-tRNA E.coli (0.47 A_{260} unit) and 9.48 μμmoles ^{14}C-phe-tRNA H.cutirubrum (1.82 A_{260} units) were used. ●——● ^{14}C-phe-tRNA E.coli. ▲——▲ ^{14}C-phe-tRNA H.cutirubrum. The results without poly U have been subtracted.

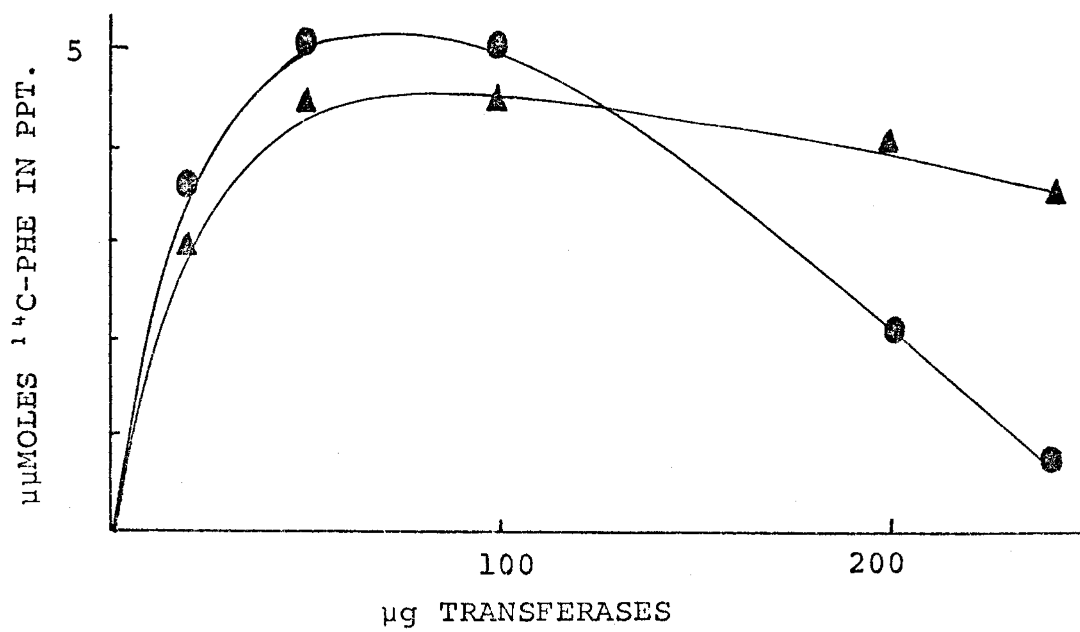
10

5

μμMOLES ^{14}C-PHE IN PPT.

10 20

μμMOLES ^{14}C-PHE-tRNA



system (Fig. 13), and confirms the discharge of only the homologous ^{14}C-phe-tRNA. Fig. 21 illustrates this more dramatically, showing that as the amount of transferase preparation increased past 100 μg there was a rapid decline in transfer from E.coli tRNA, but only a slight decline in that from H.cutirubrum.

The systems response to Mg^{2+} concentration is shown in Fig. 22, where it is evident that transfer from both H.cutirubrum and E.coli ^{14}C-phe-tRNA had an optimum at about 10 mM. This contrasts sharply to the miscoding response of E.coli ^{14}C-leu-tRNA in the presence of poly U. With all other tRNA's charged with the corresponding ^{12}C-amino acids, ^{14}C-leu was transferred slightly with an optimum Mg^{2+} concentration between 20 and 30 mM. However if only ^{14}C-leu was present on the tRNA, a substantial incorporation occurred with increasing magnesium concentration. This observation is explained by the fact that when $tRNA^{leu}$ is the only one acylated it has no competition for codons within the ribosome, and therefore efficiently transfers ^{14}C-leu into polypeptide in response to poly U. Fig. 23 again shows that the time courses using aminoacyl-tRNA from H.cutirubrum and E.coli were very similar.

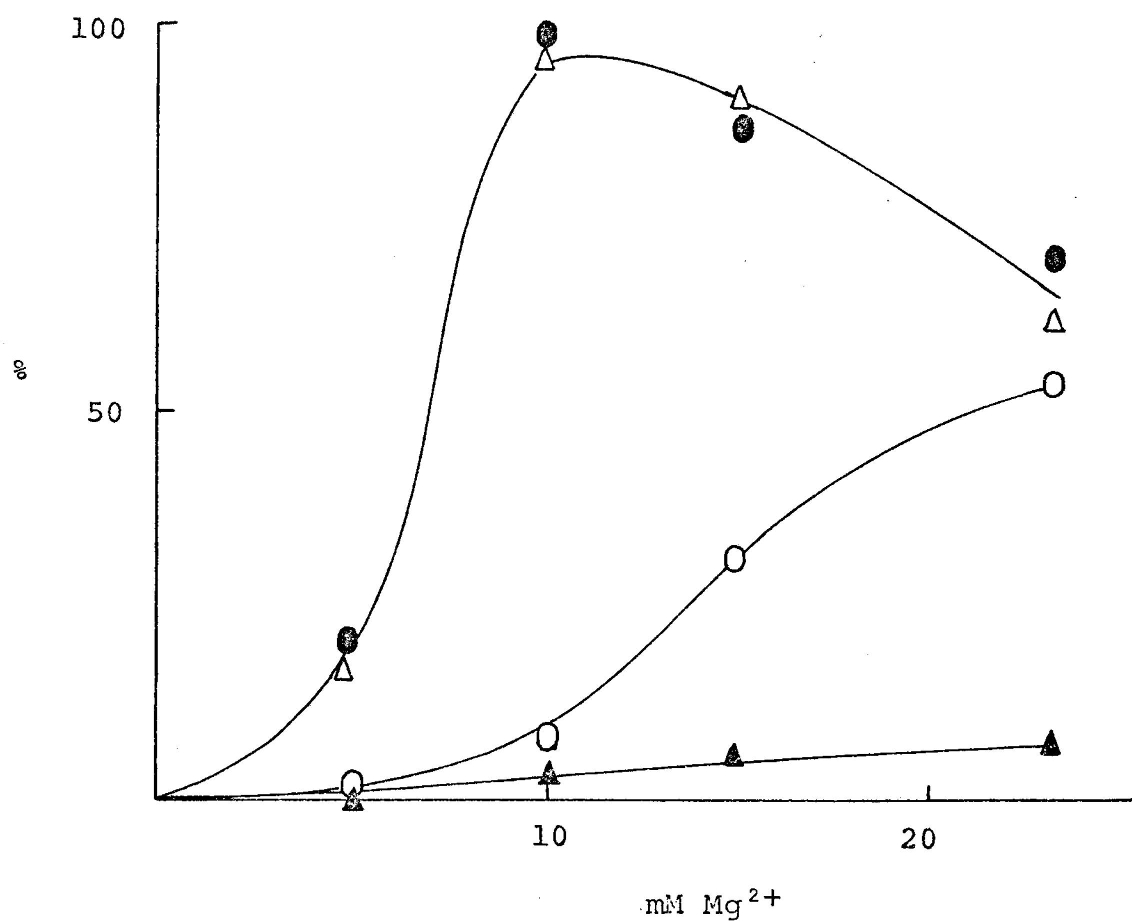
From the results of the E.coli transfer and binding systems, it seemed clear that H.cutirubrum aminoacyl-tRNA behaved almost identically to that of E.coli. This opened the way to trying codons in polymers which precipitate in the

Figure 22 Transfer of ^{14}C-AA from ^{14}C-AA-tRNA to Polypeptide as a Function of Magnesium Concentration

Each 0.100 ml reaction mixture contained the components, and was incubated as described in the "methods", except for the designated modifications. 25 µg of poly U, 50 µg of transferases 2.05 A_{260} units of ribosomes, 14.10 µµmoles ^{14}C-phe-tRNA E.coli (0.70 A_{260} unit), 24.10 µµmoles ^{14}C-leu-tRNA E.coli (0.68 A_{260} unit), 63.2 µµmoles ^{14}C-leu-tRNA E.coli-only $tRNA^{leu}$ charged. (1.72 A_{260} units) and 14.28 µµmoles ^{14}C-phe-tRNA H.cutirubrum (2.75 A_{260} units) were used. ●——● ^{14}C-phe-tRNA E.coli (100% = 6.97 µµmoles). △——△ ^{14}C-phe-tRNA H.cutirubrum (100% = 6.21µµmoles). ▲——▲ ^{14}C-leu-tRNA E.coli (100% = 6.97 µµmoles). ○——○ ^{14}C-leu-tRNA E.coli - only $tRNA^{leu}$ charged (100% = 6.97 µµmoles). The results without poly U have been subtracted.

Figure 23 Transfer of ^{14}C-Phe from ^{14}C-Phe-tRNA to Polypeptide as a Function of Time

Each 0.100 ml reaction mixture contained the components, and was incubated as described in the "methods", except for the designated modifications. 25 µg of poly U, 50 µg of transferases, 2.05 A_{260} units of ribosomes, 9.40µµmoles. ^{14}C-phe-tRNA E.coli (0.47 A_{260} unit) and 9.48 µµmoles ^{14}C-phe-tRNA H.cutirubrum (1.82 A_{260} units) were used. ●——● ^{14}C-phe-tRNA E.coli (100% = 5.13 µµmoles). ▲——▲ ^{14}C-phe-tRNA H.cutirubrum (100% = 4.87 µµmoles). Results without poly U have been subtracted.


100
%
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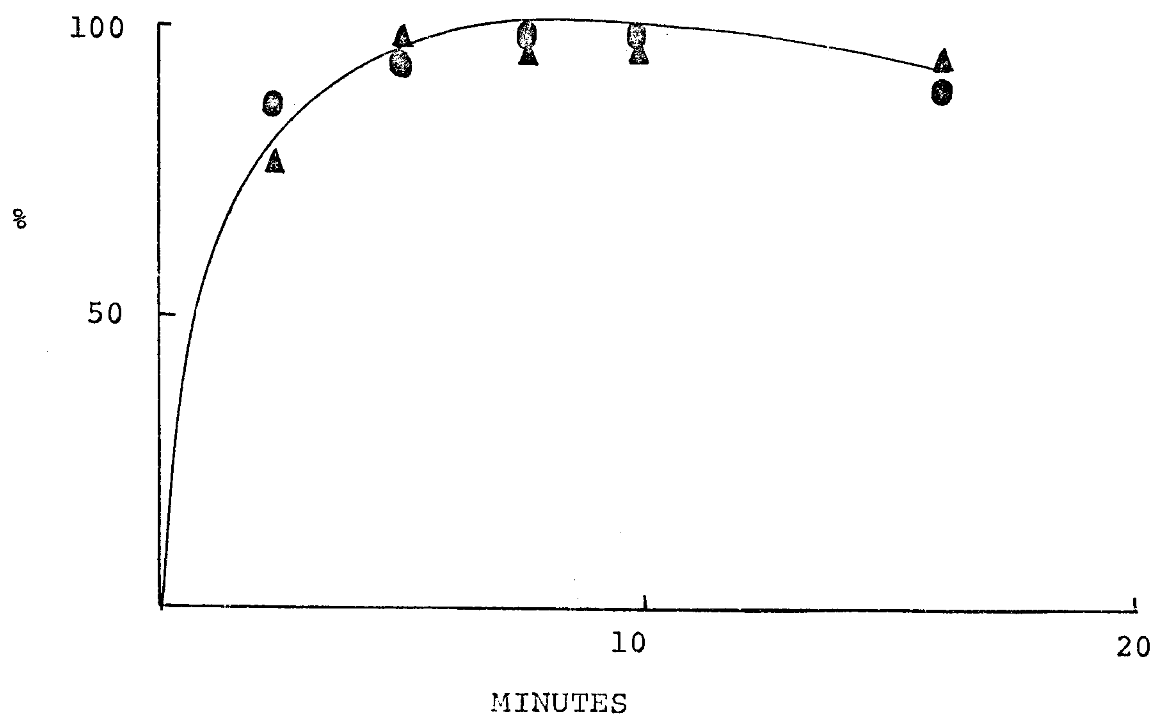
mM Mg^{2+}


100
%
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MINUTES

high salt systems. It further indicated that no gross modification of halophilic tRNA had been made.

5. Codon Assignments from Random Copolyribonucleotides in Low Salt

Although halophilic ^{14}C-aminoacyl-tRNA behaved as non-halophilic tRNA in the low salt systems with poly U and poly A, they had not been subjected to the test of a random copolymer, which would direct the incorporation of several amino acids. Before trying a polymer that precipitated in high salt, poly CU was used to examine more precisely codon responses in high and low salt with halophilic and non-halophilic tRNA. Although the responses of *H. cutirubrum* aminoacyl-tRNA in low salt systems was the prime reason for these experiments, it was of interest to see the behaviour of *E. coli* aminoacyl-tRNA in the high salt systems. This would reveal if any alteration of codon response was due to the ionic environment or due to a change in the tRNA itself.

E.coli and H.cutirubrum Whole Protein Synthesizing systems

Poly CU was first tried in the whole protein synthesizing systems from both *E. coli* and *H. cutirubrum*. The *E. coli* system was based on the one described by Nirenberg (1964), and the *H. cutirubrum* system based on that of Bayley and Griffiths (1968a). The results with these systems are shown in Tables XXXII and XXXIII. A comparison of the percentage incorporation of phenylalanine, leucine, proline and serine with the percentage of the codons in the polymer reveals a good similarity. The incorporation of phenylalanine

Table XXXII Incorporation of ^{14}C-AA in Response to Poly CU (1:1.44) and Poly U in E.coli Whole Protein Synthesizing System.

^{14}C-AA	% Codons	^{14}C-AA in the Hot 5% TCA-Tungstate Ppt.			
		+ Poly CU		+ Poly U	- Poly
		Net %	μμ Moles	μμMoles	μμMoles
PHE	35	48	31.77	67.23	3.53
LEU	25	17	15.37	5.29	5.17
SER	25	19	13.58	3.01	2.56
PRO	15	16	9.99	0.73	0.87

Each 0.100 ml reaction mixture contained the components and was incubated as described in the "methods" for the E.coli whole protein synthesizing system, except for the designated modifications. 660 μμmoles ^{14}C-phe (376 mCi/mmole), 950 μμmoles ^{14}C-leu (263 mCi/mmole), 1,900 μμmoles ^{14}C-ser (131 mCi/mmole), 1,000 μμmoles ^{14}C-pro (228 mCi/mmole), 25 μg of poly CU (1:1.44) and poly U and 1.5 A_{260} units of ribosomes were used.

Table XXXIII Incorporation of ^{14}C-AA in Response to Poly CU (1:1.44) and Poly U in H.cutirubrum Whole Protein Synthesizing System

^{14}C-AA	% Codons	^{14}C-AA in the Hot 5% TCA-Tungstate Ppt.			
		+ Poly CU		+ Poly U	- Poly
		Net %	μμMoles	μμMoles	μμMoles
PHE	35	40	12.56	23.44	1.13
LEU	25	23	8.26	1.33	1.28
SER	25	19	8.65	2.49	2.96
PRO	15	18	9.44	4.70	4.28

Each 0.125 ml reaction mixture contained the components, and was incubated as described in the "methods" for the *H.cutirubrum* whole protein synthesizing system, except for the designated modifications. 660 μμmoles ^{14}C-phe (376 mCi/mmole), 950 μμmoles ^{14}C-leu (263 mCi/mmole), 1,000 μμmoles ^{14}C-pro (228 mCi/mmole), 50 μg poly CU (1:1.44) and poly U and 2.70 A_{260} units of ribosomes were used.

was as usual high, because of the high efficiency of poly U as a template. This then showed poly CU was directing incorporation of the 4 amino acids in a ratio similar to that expected from the established code in both systems.

E.coli Aminoacyl-tRNA Transfer System

Using the procedures developed in Chap.IV,4, E.coli and H.cutirubrum ^{14}C + 19 ^{12}C-aminoacyl-tRNA's were compared for their transfer of amino acids in response to random poly CU. Tables XXXIV and XXXV show the results. Again comparison of the percentage incorporation of the 4 amino acids with the percentage of codons for those amino acids in the polymer reveals a reasonably good correlation. This correlation may be fortuitous, because when each ^{14}C-aminoacyl-tRNA was tested it was limiting compared to the 19 ^{12}C-amino acids. This was because in the charging of the tRNA, the ^{12}C-amino acids were in excess while the ^{14}C-amino acid was limiting, resulting in completely charged tRNA's with ^{12}C amino acids, but incompletely charged tRNA's with ^{14}C amino acids (Table XXIII). However incorporation does appear codon specific, with very little incorporation of leucine, serine or proline with poly U. Table XXXV also shows incorporation with random poly CU when one of the four required amino acids were absent from the tRNA. Line 5 shows transfer of ^{14}C-leu in the presence of ^{12}C-pro and ^{12}C-ser-tRNA but in the absence of ^{12}C-phe-tRNA. Comparison of lines 2 and 5 reveals better incorporation of ^{14}C-leu in the absence of ^{12}C-phe than with

Table XXXIV Transfer of ^{14}C-AA from E.coli ^{14}C-AA-tRNA in Response to Poly CU (1:1.44) and Poly U in the E.coli Aminoacyl-tRNA Transfer System

AA-tRNA	% Codons	^{14}C-AA in the Hot 5% TCA-Tungstate Ppt.			
		+ Poly CU		+ Poly U	- Poly
		Net %	μμMoles	μμMoles	μμMoles
^{14}C-phe + 19 ^{12}C-AA	35	55	1.80	4.13	0.15
^{14}C-leu + 19 ^{12}C-AA	25	21	0.92	0.33	0.29
^{14}C-ser + 19 ^{12}C-AA	25	17	0.72	0.27	0.23
^{14}C-pro + 19 ^{12}C-AA	15	7	0.35	0.12	0.15

Each 0.100 ml reaction mixture contained the components, and was incubated as described in the "methods" for the E.coli aminoacyl-tRNA transfer system, except for the designated modifications. 10.12 μμmoles ^{14}C-phe + 19 ^{12}C-aa-tRNA E.coli (0.76 A_{260} unit), 38.0 μμmoles ^{14}C-leu + 19 ^{12}C-aa-tRNA E.coli (1.32 A_{260} units), 70.2 μμmoles ^{14}C-ser + 19 ^{12}C-aa-tRNA E.coli (2.48 A_{260} units) and 21.4 μμmoles ^{14}C-pro + 19 ^{12}C-aa-tRNA E.coli (1.44 A_{260} units), 25 μg of poly CU (1:1.44) or poly U and 1.5 A_{260} units of ribosomes were used.

Table XXXV Transfer of ^{14}C-AA from H.cutirubrum ^{14}C-AA-tRNA in response to Poly CU (1:1.44) and Poly U in the E.coli Aminoacyl-tRNA Transfer System

AA-tRNA	% Codons	^{14}C-AA in the Hot 5% TCA-Tungstate Ppt.			
		+ Poly CU		+ Poly U	- Poly
		Net %	μμMoles	μμMoles	μμMoles
1. ^{14}C-phe + 19 ^{12}C-aa	35	42	4.35	9.77	0.25
2. ^{14}C-leu + 19 ^{12}C-aa	25	26	2.92	0.67	0.42
3. ^{14}C-ser + 19 ^{12}C-aa	25	12	1.43	0.41	0.23
4. ^{14}C-pro + 19 ^{12}C-aa	15	20	2.18	0.18	0.18
5. ^{14}C-leu, ^{12}C-pro,ser.	-	-	3.67	1.66	0.41
6. ^{14}C-leu, ^{12}C-pro,ser. + ^{12}C-phe	-	-	3.10	0.69	0.47

Each 0.100 ml reaction mixture contained the components and was incubated as described in the "methods" for the E.coli aminoacyl-tRNA transfer system, except for the designated modifications. 16.6 μμmoles ^{14}C-phe + 19 ^{12}C-aa-tRNA H.cutirubrum (2.4 A_{260} units), 26.9 μμmoles ^{14}C-leu + 19 ^{12}C-aa-tRNA H.cutirubrum (2.3 A_{260} units), 14.0 μμmoles ^{14}C-pro + 19 ^{12}C-aa-tRNA H.cutirubrum (2.3 A_{260} units), 23.0 μμmoles ^{14}C-ser + 19 ^{12}C-aa-tRNA H.cutirubrum (2.3 A_{260} units), 25.4 μμmoles ^{14}C-leu + ^{12}C-pro + ^{12}C-ser-tRNA H.cutirubrum (2.3 A_{260} units), ^{12}C-phe-tRNA H.cutirubrum (1.9 A_{260} units), 25 μg of poly CU (1:1.44)or poly U and 1.5 A_{260} units of ribosomes were used.

it. This can easily be explained by reference to the incorporation of ^{14}C-leu directed by poly U. The increase is then due to misreading of phenylalanine codons by the ^{14}C-leu-tRNA, because of the lack of competition for these codons from ^{12}C-phe-tRNA (Fig. 22). This misreading was greatly reduced when ^{12}C-phe-tRNA was added as shown in line 6. The purpose of leaving an amino acid out of the tRNA was in preparation for the use of poly AC which has codons for asparagine and glutamine. These amino acids are only very poorly accepted by *H.cutirubrum* tRNA (Griffiths and Bayley, 1969) and therefore use of this polymer with *H.cutirubrum* tRNA seemed to depend on the addition of *E.coli* asparagine and glutamine-tRNA. Table XXXVI shows a similar experiment to that in Table XXXV. Line 4 shows the transfer of ^{14}C-phe in the presence of ^{12}C-pro and ^{12}C-ser-tRNA but in the absence of ^{12}C-leu-tRNA. Incorporation of ^{14}C-phe in response to poly CU was only 50% of that when all 19 amino acids were present in the tRNA (line 1) or when the 3 essential amino acids were present (line 6). When ^{12}C-leu was added (line 5), incorporation of ^{14}C-phe was as high as lines 1 or 6 where all essential amino acids were present during acylation of the tRNA, showing that if the absence of an aminoacyl-tRNA is reducing incorporation, extra aminoacyl-tRNA can be added to allow full translation.

H.cutirubrum Aminoacyl-tRNA Transfer System

To test the codon response of *E.coli* ^{14}C-aminoacyl-tRNA in high salt, poly CU was used as a messenger in this

Table XXXVI Transfer of ^{14}C-AA from H.cutirubrum ^{14}C-AA-tRNA in Response to Poly CU (1:1.44) and Poly U in the E.coli Aminoacyl-tRNA Transfer System

AA-tRNA	^{14}C-AA in the Hot TCA-Tungstate Ppt. μμMoles		
	+ Poly CU	+ Poly U	- Poly
1. ^{14}C-phe + 19 ^{12}C-aa	2.14	4.61	0.08
2. ^{14}C-leu + 19 ^{12}C-aa	0.83	0.09	0.06
3. ^{14}C-pro + 19 ^{12}C-aa	0.92	0.13	0.11
4. ^{14}C-phe, ^{12}C-pro, ser	1.10	4.92	0.07
5. ^{14}C-phe, ^{12}C-pro, ser + ^{12}C-leu	2.72	4.76	0.08
6. ^{14}C-phe, ^{12}C-pro, ser, leu	2.25	5.01	0.08

Each 0.100 ml reaction mixture contained the components, and was incubated as described in the "methods" for the E.coli aminoacyl-tRNA transfer system, except for the designated modifications. 9.32 μμmoles ^{14}C-phe + 19 ^{12}C-aa-tRNA H.cutirubrum (1.70 A_{260} units), 12.73 μμmoles ^{14}C-leu + 19 ^{12}C-aa-tRNA H.cutirubrum (1.65 A_{260} units), 8.52 μμmoles ^{14}C-pro + 19 ^{12}C-aa-tRNA H.cutirubrum (1.65 A_{260} units), 10.13 μμmoles ^{14}C-phe, ^{12}C-pro + ^{12}C-ser-tRNA H.cutirubrum (1.6 A_{260} units), ^{12}C-leu-tRNA H.cutirubrum (1.3 A_{260} units), 10.34 μμmoles ^{14}C-phe, ^{12}C-pro, ^{12}C-ser + ^{12}C-leu-tRNA (1.7 A_{260} units), 25 μg of poly CU (1:1.44) or poly U and 1.5 A_{260} units of ribosomes were used.

Table XXXVII Transfer of ^{14}C-AA from E.coli ^{14}C-AA-tRNA in Response to Poly CU (1:1.44) and Poly U in the H.cutirubrum Aminoacyl-tRNA Transfer System

AA-tRNA	% Codons	^{14}C-AA in the Hot 5% TCA-Tungstate Ppt.			
		+ Poly CU		+ Poly U	- Poly
		Net %	µµMoles	µµMoles	µµMoles
^{14}C-phe + 19 ^{12}C-aa	35	48	10.56	10.87	0.46
^{14}C-leu + 19 ^{12}C-aa	25	36	8.12	1.12	0.81
^{14}C-ser + 19 ^{12}C-aa	25	8	1.83	0.58	0.52
^{14}C-pro + 19 ^{12}C-aa	15	8	1.72	0.31	0.45

Each 0.100 ml reaction mixture contained the components and was incubated as described in the "methods" for the H.cutirubrum aminoacyl-tRNA transfer system, except for the designated modifications. 20.2 µµmoles ^{14}C-phe + 19 ^{12}C-aa-tRNA E.coli (1.52 A_{260} units), 38.0 µµmoles ^{14}C-leu + 19 ^{12}C-aa-tRNA E.coli (1.32 A_{260} units), 70.2 µµmoles ^{14}C-ser + 19 ^{12}C-aa-tRNA E.coli (2.48 A_{260} units) and 21.4 µµmoles ^{14}C-pro + 19 ^{12}C-aa-tRNA E.coli (1.44 A_{260} units), 50 µg of poly CU (1:1.44) or poly U and 2.5 A_{260} units of ribosomes were used.

system. Table XXXVII shows the results. Comparison of the percentage of codons and the percentage incorporation of the 4 amino acids reveals poor correlation, especially for serine. This is not particularly surprising for the reasons stated before. However one possible reason for poor serine incorporation becomes apparent by reference to Table L, where it is evident that H.cutirubrum synthetases charged E.coli tRNA with serine. This indicated that the low serine incorporation could be due to discharge of E.coli ^{14}C-ser-tRNA. Proline, leucine and phenylalanine were not accepted by E.coli tRNA from H.cutirubrum synthetases. Incorporation using H.cutirubrum aminoacyl-tRNA was again very poor as with the poly U system, indicating again rapid discharge of the amino acid. However Table XXXIII expresses the results of the H.cutirubrum tRNA codon responses to poly CU in high salt. Again the results point to complete interchangeability of the halophilic and non-halophilic aminoacyl-tRNA's between ionic environments.

E.coli Ribosomal Binding system

In this system percent binding is virtually meaningless, because of the competition by deacylated tRNA with ^{14}C-aminoacyl-tRNA for ribosomal binding sites. Percent binding is therefore a reflection of not only the percentage of codons in a polymer, but also the percentage of a particular tRNA that is acylated. Another factor influencing quantitative results (Marshall et al., 1967) is the different affinities of codons for their respective tRNA's

Table XXXVIII Binding of E.coli ^{14}C-AA-tRNA to E.coli Ribosomes in Response to Poly CU (1:1.44) and Poly U

^{14}C-AA-tRNA	% Codons	^{14}C-AA-tRNA Bound to Filter			
		+ Poly CU		+ Poly U	-Poly
		Net %	μμMoles	μμMoles	μμMoles
^{14}C-phe	35	31	2.05	3.44	0.44
^{14}C-leu	25	13	1.64	0.68	0.98
^{14}C-ser	25	44	3.82	1.69	1.49
^{14}C-pro	15	12	1.04	0.56	0.47

Each 0.050 ml reaction mixture contained the components, and was incubated as described in the "methods" for the _E.coli_ ribosomal binding system, except for the designated modifications. 57.0 μμmoles ^{14}C-leu-tRNA _E.coli_ (1.98 A_{260} units), 32.1 μμmoles ^{14}C-pro-tRNA _E.coli_ (2.16 A_{260} units), 15.2 μμmoles ^{14}C-phe-tRNA _E.coli_ (0.90 A_{260} units), 105.3 μμmoles ^{14}C-ser-tRNA _E.coli_ (3.73 A_{260} units), 25 μg of poly CU (1.44:1) or poly U and 2.8 A_{260} units of ribosomes were used.

Table XXXIX Binding of H.cutirubrum ^{14}C-AA-tRNA to E.coli Ribosomes in Response to Poly CU (1:1.44) and Poly U

^{14}C-AA-tRNA	% Codons	^{14}C-AA-tRNA Bound to Filter			
			+ Poly CU	+ Poly U	- Poly
		Net %	μμMoles	μμMoles	μμMoles
^{14}C-phe	35	37	1.32	2.22	0.32
^{14}C-leu	25	16	0.97	0.40	0.54
^{14}C-ser	25	24	1.81	0.81	1.15
^{14}C-pro	15	23	0.88	0.27	0.24

Each 0.050 ml reaction mixture contained the components, and was incubated as described in the "methods" for the E.coli ribosomal binding system, except for the designated modifications. 16.6 μμmoles ^{14}C-phe-tRNA H.cutirubrum (2.4 A_{260} units), 26.9 μμmoles ^{14}C-leu-tRNA H.cutirubrum (2.3 A_{260} units), 14.00 μμmoles ^{14}C-pro-tRNA H.cutirubrum (2.3 A_{260} units), 23.0 μμmoles ^{14}C-ser-tRNA H.cutirubrum (2.3 A_{260} units) 25 μg of poly CU (1.44:1) or poly U and 2.8 A_{260} units of ribosomes were used.

Table XXXVIII shows the results with E.coli aminoacyl-tRNA. There was reasonably good positive binding with poly CU for all 4 ^{14}C-aa-tRNA's, with only ^{14}C-phe-tRNA binding significantly in response to poly U. The results with H.cutirubrum aminoacyl-tRNA are shown in Table XXXIX. There was again significant positive binding of the 4 ^{14}C-aa-tRNA's with poly CU while only ^{14}C-phe-tRNA was bound in response to poly U. The amount of ^{14}C-aa-tRNA bound was on the borderline of the point Nirenberg and Leder (1964) call significant. They suggest binding should be 3 times as much with the polymer as without before it is deemed significant. However the results are highly suggestive of correct codon responses, and triplets or mRNA's of defined sequence would give far more clear cut results.

H.cutirubrum Ribosomal Binding System

This system again has the same comments as for the E.coli ribosomal binding system. It tests whether ribosomal binding for either E.coli or H.cutirubrum aa-tRNA remains codon specific in the high salt system. Table XXXX shows the results with E.coli aminoacyl-tRNA. Binding of the 4 aminoacyl-tRNA's was observed in response to poly CU while only ^{14}C-phe-tRNA responded to poly U. Table XXXXI shows similar results for H.cutirubrum aminoacyl-tRNA.

Poly CU has thus been tested in 6 separate *in vitro* systems for the response of seryl, prolyl, leucyl and phenylalyl-tRNA's. Four of the systems were used to compare

Table XXXX Binding of E.coli ^{14}C-AA-tRNA to H.cutirubrum Ribosomes in Response to Poly CU (1:1.44) and Poly U

^{14}C-AA-tRNA	% Codons	^{14}C-AA-tRNA Bound to Filter			
		+ Poly CU		+ Poly	- Poly
		Net %	μμMoles	μμMoles	μμMoles
^{14}C-phe	35	34	1.52	2.72	0.36
^{14}C-leu	25	18	1.24	0.54	0.64
^{14}C-ser	25	32	2.79	1.65	1.71
^{14}C-pro	15	16	0.98	0.27	0.45

Each 0.125 ml reaction mixture contained the components, and was incubated as described in the "methods" for the H.cutirubrum ribosomal binding system, except for the designated modifications. 47.5 μμmoles ^{14}C-leu-tRNA E.coli (1.73 A_{260} units), 26.8 μμmoles ^{14}C-pro-tRNA E.coli (1.80 A_{260} units), 12.68 μμmoles ^{14}C-phe-tRNA E.coli (0.75 A_{260} unit), 87.8 μμmoles ^{14}C-ser-tRNA E.coli (2.58 A_{260} units), 50 μg of poly CU (1:1.44) or poly U and 6.60 A_{260} units of ribosomes were used.

Table XXXXI Binding of H.cutirubrum ^{14}C-AA-tRNA to H.cutirubrum Ribosomes in Response to Poly CU (1:1.44) and Poly U

^{14}C-AA-tRNA	% Codons	^{14}C-AA-tRNA Bound to Filter			
		Net %	+ Poly CU μμMoles	+ Poly U μμMoles	- Poly μμMoles
^{14}C-phe	35	24	1.19	1.77	0.50
^{14}C-leu	25	33	1.45	0.49	0.47
^{14}C-ser	25	27	1.75	0.54	0.95
^{14}C-pro	15	16	0.75	0.26	0.20

Each 0.125 ml reaction mixture contained the components, and was incubated as described in the "methods" for the H.cutirubrum ribosomal binding system, except for the designated modifications. 13.83 μμmoles ^{14}C-phe-tRNA H.cutirubrum (2.0 A_{260} units), 21.93 μμmoles ^{14}C-leu-tRNA H.cutirubrum (1.92 A_{260} units), 11.68 μμmoles ^{14}C-pro-tRNA H.cutirubrum (1.92 A_{260} units), 19.12 μμmoles ^{14}C-ser-tRNA H.cutirubrum (1.92 A_{260} units), 50 μg of poly CU (1:1.44) or poly U and 6.60 A_{260} units of ribosomes were used.

E. coli and H. cutirubrum aminoacyl-tRNA. All the evidence presented strongly indicates correct codon recognition of both aminoacyl-tRNA's in either high or low salt systems. This then was sufficient ground to try H. cutirubrum aminoacyl-tRNA with a polymer (poly AC), which could not be tested in a high salt system.

Codon Assignments from Poly AC in a Low Salt System

Initially poly AC was tested in the E. coli whole protein synthesizing system. The results in Table XXXXII show good incorporation of proline, threonine, histidine and lysine in a qualitative way, but quantitatively correlation between the percentage of codons and amount incorporated is poor. However although the amount of incorporation may not be meaningful, the incorporation of an amino acid would indicate if the codon response was as expected by the established code. Table XXXXIII shows the transfer of ^{14}C-amino acids from E. coli aminoacyl-tRNA directed by poly AC. Good incorporation of threonine, histidine, glutamine and proline was found with poly AC and virtually none with poly U. Table XXXXIV shows the same experiment using H. cutirubrum aminoacyl-tRNA. As is evident incorporation of the amino acids was very poor, except for proline. This poor incorporation was perhaps due to the failure to acylate H. cutirubrum glutamine and asparagine tRNA, leaving 18% of the codons unable to be translated. Table XXXXV shows the same experiment with the addition of E. coli ^{12}C-glutamine and asparagine-tRNA. Unfortunately there was little stimulation

Table XXXXII Incorporation of ^{14}C-AA in Response to Poly AC (1:1.59) and Poly U in the E.coli Whole Protein Synthesizing System

^{14}C-AA	% Codons	μμMoles of ^{14}C-AA in the Hot 5% TCA-Tungstate Precipitate.		
		+ Poly AC	+ Poly U	- Poly
PRO	38	5.37	0.73	0.87
THR	23	4.70	0.82	0.94
HIS	15	10.53	2.18	2.47
GLUN	9	-	-	
ASPN	9	-	-	
LYS	6	5.36	0.87	0.94

Each 0.100 ml reaction mixture contained the components, and was incubated as described in the "methods" for the E.coli whole protein synthesizing system, except for the designated modifications. 1,000 μμmoles ^{14}C-pro (228 mCi/mmole), 1,500 μμmoles ^{14}C-thr (167 mCi/mmole), 1,000 μμmoles ^{14}C-his (267 mCi/mmole), 1,000 μμmoles ^{14}C-lys (247 mCi/mmole), 25 μg of poly AC (1:1.59) or poly U and 1.5 A_{260} units of ribosomes were used.

Table XXXXIII Transfer of ^{14}C-AA from E.coli ^{14}C-AA-tRNA in Response to Poly AC (1:1.59) and Poly U in the E.coli Aminoacyl-tRNA Transfer System

AA-tRNA	μμMoles of ^{14}C-AA in the Hot TCA - Tungstate Ppt.		
	+ Poly AC	+ Poly U	- Poly
^{14}C-thr + 19 ^{12}C-aa	6.10	0.47	0.53
^{14}C-his + 19 ^{12}C-aa	4.94	0.75	0.92
^{14}C-gln + 19 ^{12}C-aa	4.27	0.41	0.38
^{14}C-pro + 19 ^{12}C-aa	3.01	0.30	0.27

Each 0.100 ml reaction mixture contained the components, and was incubated as described in the "methods" for the E.coli aminoacyl-tRNA transfer system, except for the designated modifications. 59.0 μμmoles ^{14}C-thr + 19 ^{12}C-aa-tRNA E.coli (2.12 A_{260} units), 17.8 μμmoles ^{14}C-his + 19 ^{12}C-aa-tRNA E.coli (2.15 A_{260} units), 32.3 μμmoles ^{14}C-gln + 19 ^{12}C-aa-tRNA E.coli (2.60 A_{260} units), 26.85 μμmoles ^{14}C-pro + 19 ^{12}C-aa-tRNA E.coli (1.80 A_{260} units), 25 μg of poly U and 1.5 A_{260} units of ribosomes were used.

Table XXXXIV Transfer of ^{14}C-AA from H.cutirubrum ^{14}C-AA-tRNA in Response to Poly AC (1:1.59) and Poly A in the E.coli Aminoacyl-tRNA Transfer System

AA-tRNA	μμMoles of ^{14}C-AA in the Hot 5% TCA - Tungstate Ppt.		
	+ Poly AC	+ Poly A	- Poly
^{14}C-lys + 19 ^{12}C-aa	0.65	2.32	0.36
^{14}C-his + 19 ^{12}C-aa	1.02	0.71	0.69
^{14}C-thr + 19 ^{12}C-aa	1.21	0.42	0.40
^{14}C-asp + 19 ^{12}C-aa	0.26	0.27	0.27
^{14}C-pro + 19 ^{12}C-aa	1.63	0.12	0.14

Each 0.100 ml reaction mixture contained the components, and was incubated as described in the "methods" for the E.coli aminoacyl-tRNA transfer system, except for the designated modifications. 12.5 μμmoles ^{14}C-lys + 19 ^{12}C-aa-tRNA H.cutirubrum (2.73 A_{260} units), 15.3 μμmoles ^{14}C-his + 19 ^{12}C-aa-tRNA H.cutirubrum (2.28 A_{260} units), 15.3 μμmoles ^{14}C-thr + 19 ^{12}C-aa-tRNA H.cutirubrum (1.55 A_{260} units), 22.3 μμmoles ^{14}C-asp + 19 ^{12}C-aa-tRNA H.cutirubrum (2.15 A_{260} units), 11.68 μμmoles ^{14}C-pro + 19 ^{12}C-aa-tRNA H.cutirubrum (1.92 A_{260} units), 25 μg of poly AC (1:1.59) or poly A and 1.5 A_{260} units of ribosomes were used.

Table XXXXV — Transfer of ^{14}C-AA from H.cutirubrum ^{14}C-AA-tRNA with Added E.coli ^{12}C-Gln + ^{12}C-Asn-tRNA in Response to Poly AC (1:1.59) in the E.coli Aminoacyl-tRNA Transfer System

AA-tRNA	μμMoles of ^{14}C-AA in the Hot 5% TCA-Tungstate Ppt.		
	+ Poly AC	+ Poly A	- Poly
^{14}C-lys + 19 ^{12}C-aa	0.46	2.21	0.13
^{14}C-his + 19 ^{12}C-aa	0.49	0.24	0.22
^{14}C-thr + 19 ^{12}C-aa	0.86	0.11	0.11
^{14}C-asp + 19 ^{12}C-aa	0.12	0.09	0.10
^{14}C-pro + 19 ^{12}C-aa	1.41	0.05	0.04

Each 0.100 ml reaction mixture contained the components, and was incubated as described in the "methods" for the E.coli aminoacyl-tRNA transfer system, except for the designated modifications. 12.5 μμmoles ^{14}C-lys + 19 ^{12}C-aa-tRNA H.cutirubrum (2.73 A_{260} units), 15.3 μμmoles ^{14}C-his + 19 ^{12}C-aa-tRNA H.cutirubrum (2.28 A_{260} units), 15.3 μμmoles ^{14}C-thr + 19 ^{12}C-aa-tRNA H.cutirubrum (1.55 A_{260} units), 22.3 μμmoles ^{14}C-asp + 19 ^{12}C-aa-tRNA H.cutirubrum (2.15 A_{260}units), 11.68 μμmoles ^{14}C-pro + 19 ^{12}C-aa-tRNA H.cutirubrum (1.92 A_{260} units), ^{12}C-gln and ^{12}C-asn-tRNA E.coli (1.6 A_{260} units), 25 μg of poly AC (1:1.59) or poly A and 1.5 A_{260} units of ribosomes were used.

of incorporation.

It became apparent that random polymers would give minimal direct evidence of the code. They enabled systems to be set up to compare E.coli and H.cutirubrum tRNA's in high and low salt systems, but did not allow fine resolution of codon assignments. For this reason polymers of defined sequence will have to be used to check the interesting codons. While preparing some of the ^{14}C-aminoacyl-tRNA's used in this section some difficulty was encountered in the charging of tRNA's for several of the amino acids. The synthetase-tRNA interaction was investigated, as more fully charged tRNA's would allow better resolution in ribosomal binding and aminoacyl-tRNA transfer systems.

6. Homologous and Heterologous Synthetase - tRNA Interactions

While charging H.cutirubrum tRNA's with various ^{14}C-amino acids, it became apparent that they did not all behave as the leucine and isoleucine systems (Griffiths and Bayley, 1969). The amount of acceptance of many amino acids was much lower than that described by Griffiths and Bayley (1969). It soon became clear that a major cause for the discrepancy was the use of high specific activity amino acids instead of the previously used low specific activity ones. The result of the change was that in some cases only 0.01 of the amount of amino acid was now being added to the incubation mixtures. Economy prevented the addition of more high specific activity ^{14}C-amino acid, so ways had to be found to lower the apparent Km of the synthetase for the amino acid.

Another problem that was evident was the failure of H.cutirubrum tRNA to accept ^{14}C-gln and asn. In view of the reports that ^{14}C-gln-tRNA^{gln} was made via the formation of ^{14}C-glu-tRNA^{gln} (Wilcox and Nirenberg, 1968; Wilcox, 1969) it was pertinent to see if this system was present in H.cutirubrum.

As E.coli and H.cutirubrum aminoacyl-tRNA behaved similarly in different ionic environments, it became of

interest to know if *E.coli* or *H.cutirubrum* synthetases could recognise the heterologous tRNA. These heterologous interactions had been reported between bacterial and mammalian synthetase-tRNA systems with a variety of responses. Results of Griffiths and Bayley (1969) indicated little interaction for the proline components in the *E.coli* - *H.cutirubrum* heterologous reactions. The synthetase-tRNA interaction is much more precise than the transferase-aminoacyl-tRNA-ribosome interactions and therefore offers a better chance of examining if there has been significant adaptation of *H.cutirubrum* tRNA to its environment.

Homologous tRNA-Synthetase Interactions

With the charging system of Griffiths and Bayley (1969), amino acids such as phe, leu, ileu and lys were accepted by the tRNA as expected, but poorer acceptance of proline, serine, threonine, aspartic acid and glutamic acid was found. It was evident that a major reason was the decrease of the amount of amino acid in the system. In order to increase the efficiency of utilization of the amino acids, the ionic requirements for the individual aminoacyl-tRNA synthetases were examined. Table XXXXVI shows the acceptance of several amino acids by tRNA with additional monovalent cations to the 3.8 M KCl. It is evident the response is varied between the amino acids. The addition of 1.4 M NaCl markedly increased the acceptance of aspartic acid, glutamic acid, serine, proline and threonine but had little effect on leucine and severely reduced that of methionine. The results for methionine at 45°C

Table XXXXVI Effect of Different Monovalent Cations on the H.cutirubrum tRNA Charging System

Monovalent Cations	Temp. $0^{0}C$	^{14}C-AA accepted per A_{260} unit tRNA expressed as % accepted in 3.8 M KCl at $37^{0}C$						
		Asp	Met	Glu	Ser	Pro	Thr	Leu
3.8 M KCl	37	100	100	100	100	100	100	100
3.8 M KCL 1.4 M NH_4Cl	"	64	91	531	54	-	42	60
3.8 M KCl 1.4 M NaCl	"	391	62	350	180	210	324	105
3.8 M KCl 1.0 M NaCl 0.4 M NH_4Cl	"	-	83	423	-	-	-	95
3.8 M KCl	45	-	31	-	-	-	-	-
3.8 M KCl 1.4 M NaCl	"	-	26	-	-	-	-	-
4.9 M KCl	"	-	90	-	-	-	-	-

Each 0.225 ml reaction mixture contained the components and was incubated as described in the "methods" for the H. cutirubrum tRNA charging system, except for the designated modifications. The specific activities (mCi/mmole) of the amino acids were:- aspartic acid 167, methionine 60, glutamic acid 186, serine 118, proline 209, threonine 164, and leucine 278. 0.5 μCi of each ^{14}C-amino acid and 0.008 μmole of each of the 19 ^{12}C-amino acids were used.

indicated that perhaps at 3.8 M KCl the water activity was too high for maximal activity, while the addition of further KCl at this elevated temperature reduced it to a level comparable with that at 37^{0}C with 3.8 M KCl. The addition of 1.4 M NH_4Cl reduced acceptance in all cases except for glutamic acid which showed a marked increase. This five fold stimulation was found whether the source of the synthetases was a pH 5 precipitate or a S-150. In view of these results 1.4 M NaCl was added routinely to all charging systems except when ^{14}C-methionine was being charged by itself. This modification to the charging system allowed the large scale production of 1 ^{14}C and 19 ^{12}C-aminoacyl-tRNA's (Table XXXXVII) that were used in determining codon responses in low salt systems. Only 18 such preparations could be made because of the difficulty of acylating glutamine and asparagine tRNA.

Glutamine and Asparagine tRNA Formation

Incorporation of asparagine and glutamine into polypeptide by *H. cutirubrum* S-60 extracts (Bayley and Griffiths, 1968a) or into tRNA by synthetase preparations (Griffiths and Bayley, 1969) was very low. Several reasons for this were possible, including the complete absence of these amino acids from *H. cutirubrum* proteins. Amino acid analyses of halophilic proteins (Bayley, 1966) had not estimated these amides, because acid hydrolysis was used. Bayley (unpublished results) has found the amides in whole cell and ribosomal protein by

Table XXXXVII Preparations of H.cutirubrum 1 ^{14}C + 19 ^{12}C-Aminoacyl-tRNA's

^{14}C-AA	Sp. Act. of AA mCi/mmole	Acceptance of ^{14}C-AA by tRNA μμmoles/A_{260} unit
Alanine	112	11.1
Arginine	234	12.7
Aspartic acid	167	10.3
Cysteine	18	4.2
Cystine	218	1.6
Glutamic acid	186	3.3
Glycine	116	8.3
Histidine	239	4.7
Isoleucine	247	11.2
Leucine	278	9.5
Lysine	257	5.0
Phenylalanine	376	5.9
Methionine	60	5.6
Proline	209	6.3
Serine	118	12.1
Threonine	164	5.0
Tryptophan	23	4.5
Tyrosine	379	6.0
Valine	219	12.0

means of enzymic digestion. They constitute about 10% of the total amino acids in the protein, and therefore a mechanism for their incorporation into protein must exist *in vivo*. The possibility that the synthetases or tRNA's were inactivated in the extraction was investigated by using very crude homogenates. High backgrounds in these experiments prevented meaningful interpretation, but nevertheless little incorporation was seen.

The development of the use of *E.coli* aminoacyl-tRNA in the *H.cutirubrum* aminoacyl-tRNA transfer system allowed the investigation of the presence of glutamine codons in the halophilic mRNA. Table XXXXVIII shows the transfer of ^{14}C-gln, ^{14}C-lys and ^{14}C-leu from *E.coli* tRNA to polypeptide in response to endogenous mRNA present on *H.cutirubrum* polysomes. A high incorporation of glutamine besides the other 2 amino acids was found indicating the presence of glutamine codons in the mRNA. The block in the incorporation of glutamine into polypeptide in the complete halophilic system seemed at the synthetase-tRNA level.

Wilcox and Nirenberg (1968) found no glutamine synthetase in Gram positive bacteria, but a transamidation pathway, which converted the intermediate glutamic acid-$tRNA^{gln}$ into glutamine -$tRNA^{gln}$. As a preliminary to investigating this pathway, the effect of the addition of a large excess of certain ^{12}C-amino acids on the incorporation of ^{14}C-gln into polypeptide was investigated. Table XXXXIX

Table XXXXVIII Transfer of ^{14}C-AA from E.coli ^{14}C-AA -tRNA to Polypeptide in Response to H.cutirubrum Polysomes

E.coli ^{14}C-AA-tRNA	μμMoles ^{14}C-AA in the Hot 5% TCA-Tungstate Ppt.	
	-	+ Puromycin
^{14}C-gln + 19 ^{12}C-aa-tRNA	5.77	0.09
^{14}C-leu + 19 ^{12}C-aa-tRNA	12.22	0.94
^{14}C-lys + 19 ^{12}C-aa-tRNA	4.72	1.50

Each 0.125 ml reaction mixture contained the components and was incubated as described in the "methods" for the H.cutirubrum aminoacyl-tRNA transfer system, except for the designated modifications. 32.3μμmoles ^{14}C-gln-tRNA E.coli (2.00 A_{260} units), 38.0 μμmoles ^{14}C-leu-tRNA E.coli (1.32 A_{260} units), 26.52 μμmoles ^{14}C-lys-tRNA E.coli (0.98 A_{260} unit), and 5.6 A_{260} units of polysomes (replacing the ribosomes) were used.

Table XXXXIX Incorporation of ^{14}C-Gln into Polypeptide by H.cutirubrum S-60 Extract

Additions	^{14}C-Gln in Hot 5% TCA Ppt. CPM	
	-	+ Puromycin
-	463	225
0.008 μmoles of all 19 ^{12}C-aa	160	126
0.008 μmoles of ^{12}C-gln	153	81
0.008 μmoles of ^{12}C-glu	186	148
0.008 μmoles of ^{12}C-asp	315	130

Each 0.125 ml reaction mixture contained the components and was incubated as described in the "methods" for the H.cutirubrum whole protein synthesizing system, except for the designated modifications. 0.020 ml of S-60 (replacing the ribosomes and S-150) and ^{14}C-gln (94.7 mCi/m mole) were used.

shows that the presence of glutamic acid inhibited the incorporation of glutamine while aspartic acid did not. This is the result to be expected, if the apparent incorporation of ^{14}C-gln was due to a prior conversion to glutamic acid before entering the tRNA.

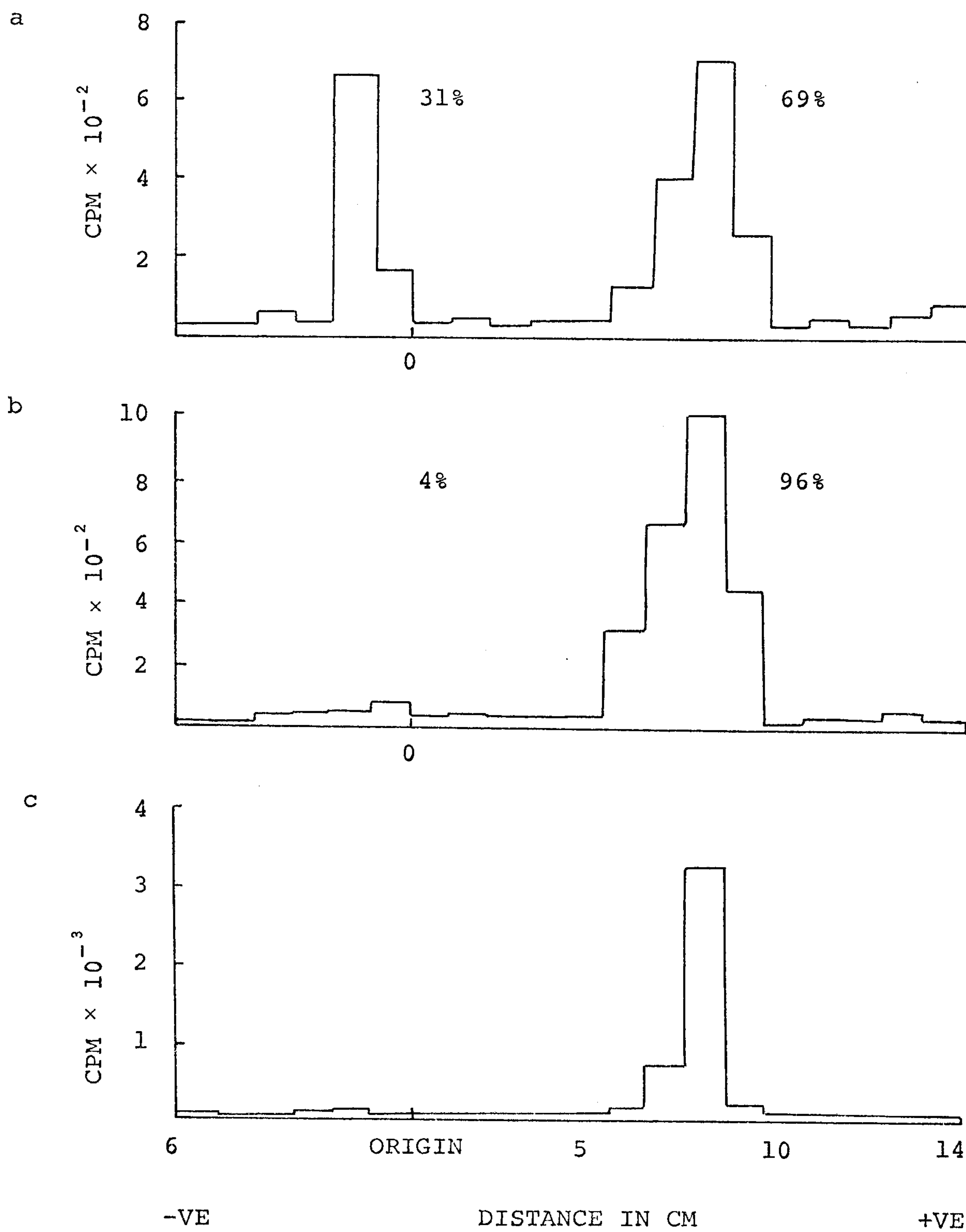
To investigate this process further, tRNA was charged with ^{14}C-glutamic acid in the presence of all 19 cold amino acids. The amino acids were discharged from the tRNA by alkali and their nature investigated by electrophoresis at pH 4.0. Fig. 24 shows the result obtained; 31% of the label moved towards the cathode, and 69% moved to the anode, as glutamic acid (Fig. 24a). The material moving towards the cathode had the same mobility as glutamine. A control with the E.coli charging system revealed no label except that as glutamic acid (Fig. 24c). The nature of the non-glutamic acid material from the halophilic system was further investigated by treating the discharged amino acids with 2 M HCl at 100^{0}C for 4 hours, which converts glutamine to glutamic acid. After this treatment (Fig. 24b) 96% of the label was in the position of glutamic acid, strongly indicating the original material moving towards the cathode to be glutamine. This then appears to be the same sort of system described by Wilcox and Nirenberg (1968). They state however this pathway is confined to Gram positive bacteria which means that either the Gram stain is of little meaning in halophilic bacteria or their generalisation is incorrect.

Figure 24 Electrophoresis of ^{14}C-Gln and ^{14}C-Glu Discharged from E.coli and H.cutirubrum tRNA

a. 5 mg of *H.cutirubrum* tRNA were charged with ^{14}C-glu (186 mCi/mmole) by S-150 in 3.8 M KCl, 1.0 M NaCl 0.4 M NH_4Cl. All 19 ^{12}C-amino acids were also present. The aminoacyl-tRNA was phenol extracted and the amino acids discharged by alkali and separated by electrophoresis, on Whatman 3 MM paper in 0.05 M potassium hydrogen phthalate, pH 4.0 at 3,000 V for 1.5 hours. The 3 MM paper was cut into 3 ×1 cm pieces and counted in a scintallation counter.

b. As in (a) except the products were treated with 2 M HCl at 100^{0}C for 4 hours prior to electrophoresis.

c. 5 mg of *E.coli* tRNA were charged with ^{14}C-glu (186 mCi/mmole) by S-150 in the *E.coli* tRNA charging system. The aminoacyl-tRNA was phenol extracted and the amino acids discharged by alkali and separated by electrophoresis.


a
8
6
4
2
CPM × 10⁻²
31%
69%
0
b
10
8
6
4
2
CPM × 10⁻²
4%
96%
0
c
4
3
2
1
CPM × 10⁻³
6
ORIGIN
5
10
14
-VE
DISTANCE IN CM
+VE

The same technique was used to investigate the aspartic acid - asparagine situation but no asparagine was formed from aspartic acid on tRNA and the problem of asparagine entering protein has yet to be resolved.

Heterologous Synthetase-tRNA Interactions

Aminoacyl-tRNA from either H.cutirubrum or E.coli assumes conformations recognisable by heterologous transferase enzymes and ribosomes in foreign ionic environments. It appears that during halophilic evolution little modification has been made to the tRNA to demarcate it from non-halophilic tRNA. To investigate this further the recognition of halophilic tRNA by non-halophilic synthetases was pursued.

The normal E.coli and H.cutirubrum charging systems were used, except that the heterologous tRNA was added. Halophilic synthetases were only used in high salt systems and E.coli synthetases in low salt systems. Table L shows the results of these experiments in terms of μμmoles of amino acid accepted per A_{260} unit of tRNA. It is evident that E.coli tRNA significantly accepted five amino acids from H.cutirubrum synthetases while H.cutirubrum tRNA accepted approximately 10 amino acids from E.coli synthetases. The reason why the heterologous system for a particular amino acid was positive, while the reverse was negative is not clear, but is consistent with results from heterologous systems between other organisms.

Table L Acceptance of ^{14}C-AA by H.cutirubrum and E.coli tRNA from Heterologous and Homologous Synthetases

^{14}C-AA	μμMoles of ^{14}C-AA accepted per A_{260} unit of tRNA			
	E.coli tRNA		H.cutirubrum tRNA	
	E.coli Synthetases	H.cutirubrum Synthetases	H.cutirubrum Synthetases	E.coli Synthetases
Alanine	14.0	0.1	14.6	5.3
Arginine	9.8	21.1	12.5	2.5
Asparagine	11.0	0.0	0.6	0.6
Aspartic acid	12.3	0.8	8.6	1.3
Cysteine	10.7	0.4	2.8	0.0
Glutamic acid	6.5	0.0	2.9	0.1
Glutamine	21.7	0.0	0.1	0.0
Glycine	1.8	0.0	10.3	2.6
Histidine	10.6	0.0	4.1	1.9
Isoleucine	8.8	8.4	9.6	1.2
Leucine	30.5	0.0	9.4	0.3
Lysine	27.5	0.0	4.8	3.8
Methionine	30.2	6.3	4.9	4.6
Phenylalanine	15.5	0.4	5.6	0.2
Proline	14.1	0.0	5.7	0.1
Serine	28.8	11.8	9.8	0.6
Threonine	24.8	0.2	4.6	13.8
Tryptophan	3.8	0.0	3.8	0.2
Tyrosine	8.9	0.0	5.4	0.1
Valine	15.7	4.5	10.8	7.5

To test whether the acceptance of amino acids by tRNA from the heterologous synthetase was specific, or due to spurious attachment of amino acids to tRNA, the experiment of Fig. 25 was performed. *E.coli* tRNA was charged with arginine by *H.cutirubrum* synthetases. The superscript X indicates that the species of tRNA which accepts arginine is unknown. The tRNA was extracted and treated with periodate, which destroys the acceptor activity of all species of tRNA except those that are protected by arginine. The arginine was discharged from the tRNA and the acceptor activity of the tRNA for the 20 amino acids was tested with *E.coli* synthetases. Table LI shows that only arginine acceptor activity remained, showing the halophile synthetase had charged the correct *E.coli* tRNA with arginine. Table LI also shows the results from the reverse experiment, in which *H.cutirubrum* tRNA was charged with methionine by *E.coli* synthetases. In this case only methionine acceptor activity was protected. For the 2 cases investigated the heterologous synthetase could recognise the correct species of tRNA, although the tRNA was in a completely foreign ionic environment.

It is evident that in the halophilic system each interaction and subsequent acylation responds differently to the ionic environment. No one incubation mixture can be optimal for every synthetase (Novelli, 1967). There does not appear to be a specific modification of tRNA to equip it for

Figure 25 Flow Sheet of Experiment to Test Specificity of Heterologous tRNA Charging

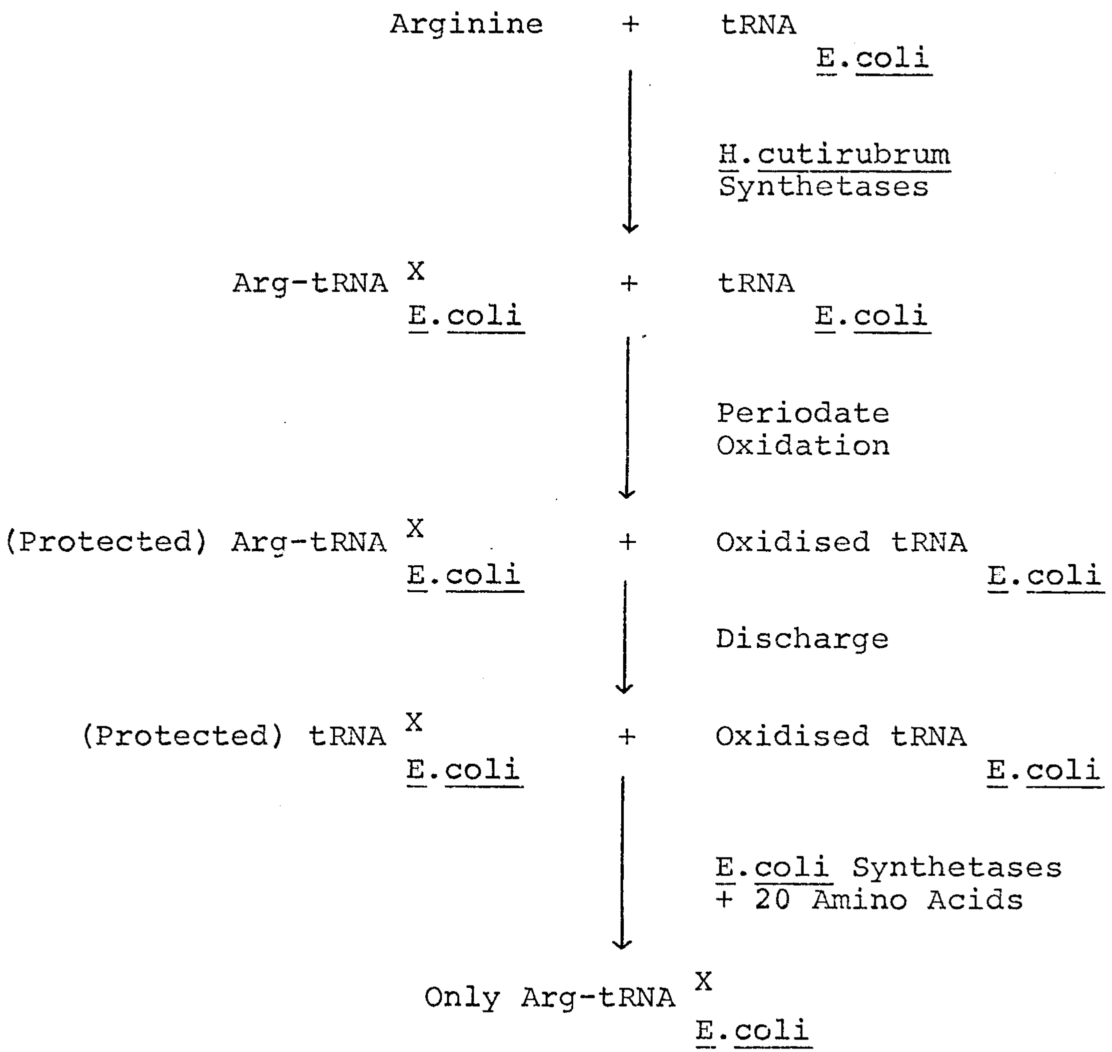


Table LI The experiment was that outlined in Fig. 25. 10 mg of E.coli tRNA were charged with ^{12}C-arginine (1×10^{-4} M) by H.cutirubrum synthetases in the H.cutirubrum charging system . The arg-tRNA E.coli was then treated with periodate and then the arginine discharged from the tRNA. The E.coli tRNA was then assayed for acceptance of all 20 ^{14}C-amino acids from E.coli synthetase . 10 mg of H.cutirubrum tRNA were charged with methionine (1×10^{-4} M) by E.coli synthetases in the E.coli charging system. The met-tRNA H.cutirubrum was then treated with periodate, and the methionine discharged from the tRNA. The H.cutirubrum tRNA was then assayed for acceptance of all 20 ^{14}C-amino acids from H.cutirubrum synthetases. The results of both experiments are expressed as a percentage of the μμmoles of ^{14}C-amino acid acceptor per A_{260} unit of the control tRNA, which was initially charged with all 20 ^{12}C-amino acids (each 1×10^{-4} M). Acceptance of control tRNA was between 60-90% that of untreated tRNA shown on Table L.

Table LI Examination of tRNA Acylated by the Heterologous Synthetase

^{14}C-AA	Arg-Protected *E.coli* tRNA	Met-Protected *H.cutirubrum* tRNA
	% Acceptance of Control	
Alanine	0.0	0.0
Arginine	97.4	0.0
Asparagine	1.3	2.1
Aspartic acid	0.0	1.6
Cysteine	0.0	0.0
Glutamic acid	4.5	0.0
Glutamine	0.0	0.0
Glycine	0.0	2.4
Histidine	0.0	0.0
Isoleucine	1.2	1.3
Leucine	0.0	2.1
Lysine	0.0	4.1
Methionine	0.6	77.8
Phenylalanine	0.6	0.0
Proline	0.9	0.0
Serine	0.0	4.6
Threonine	0.3	0.0
Tryptophan	4.0	0.0
Tyrosine	0.0	3.5
Valine	0.9	0.0

for a high salt environment. The glutaminyl synthetase is absent from *H. cutirubrum* extracts and its activity replaced by a combination of a glutamyl synthetase and an amido transferase. Although this information on the synthetase -tRNA interactions would be useful in investigating codons, several other problems existed in the whole protein synthesizing system. In view of the difficulty in obtaining the relevant template mRNA's for assigning codons, this system was studied more carefully.

7. Purification and Reconstitution of the Halophilic in vitro Protein Synthesizing System

During the work described in Chap.IV,1, it became clear that the system was too crude to do some of the experiments required in investigating the initiation mechanism. These experiments required a system, which could utilize isolated species of tRNA. The use of aminoacyl-tRNA in the system was also difficult, because of the rapid discharge of amino acids by synthetases in the transferase preparation. Another problem was the low efficiency of the system compared to that of E.coli. It required far more template and it polymerised only 1-10% of the amino acids that an E.coli system did with similar amounts of ribosomes and S-150. This low efficiency prevented use of mRNA transcribed from DNA of defined sequence, as prepared in Khorana's laboratory. The normal transcription stage from this DNA synthesizes less than 1 μg of mRNA, while the H.cutirubrum system requires about 50 μg (Bayley and Griffiths, 1968b).

Earlier attempts to reduce endogenous mRNA activity from the ribosomes (Table XV) had met with little success. Centrifuging ribosomes through solution D with sucrose or through solutions with some of the K^+ replaced with NH_4^+ and lower Mg^{2+} concentrations relieved the ribosomes of

significant amounts of protein and some RNA, but also drastically reduced their activity in the protein synthesizing system. The method of ribosome preparation described by Bayley and Griffiths, (1968) proved to be best for final activity. Removal of the residual mRNA activity from these ribosomes by washing results in a loss of activity suggesting a far more fragile structure than the ribosomes of E.coli.

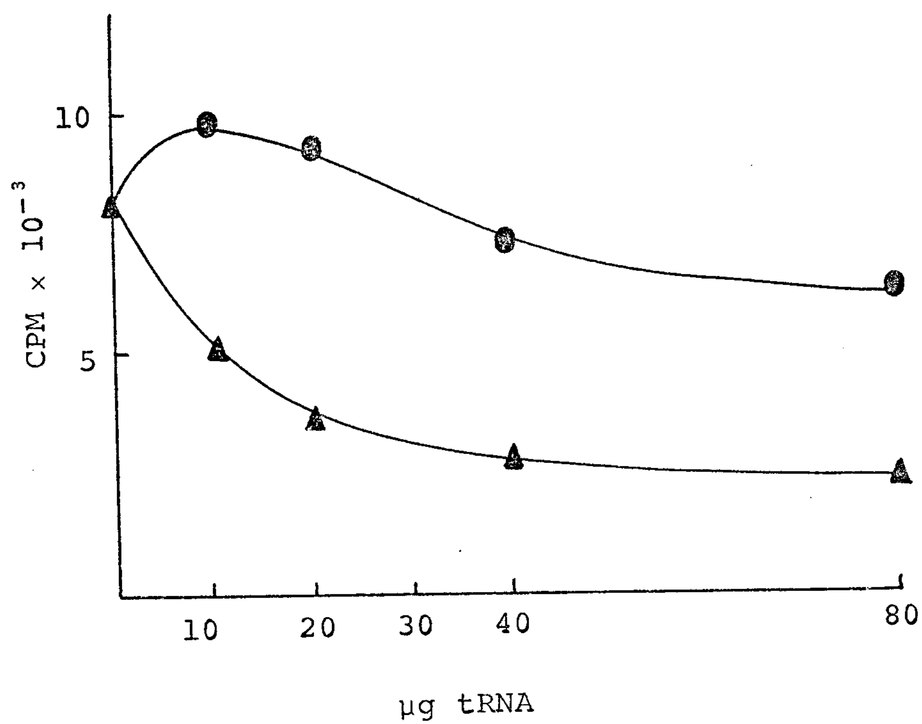
The S-150 had been previously partially purified into synthetases and tRNA (Griffiths and Bayley, 1969) and also transferases (Chap.IV,3). As a preliminary to seeing if the low efficiency in the whole protein synthesizing system was due to a limited amount of tRNA in the S-150, phenol purified tRNA was added to a system programmed with poly U. The results are shown in Fig. 26. As the amount of phenol purified, H.cutirubrum tRNA was increased there was a rapid inhibition in the amount of ^{14}C-phe in the hot TCA precipitate. This effect was not seen with E.coli tRNA and in fact there was an unexplained stimulation at low concentrations. These results contrast sharply with those for charging in Fig. 27, which show that added tRNA made increasing amounts of ^{14}C-phe-tRNA available to the system. There was thus an inhibitor of protein synthesis in the H.cutirubrum tRNA, which did not affect the charging reaction. In an attempt to remove this inhibitor, the tRNA was further purified by column chromatography.

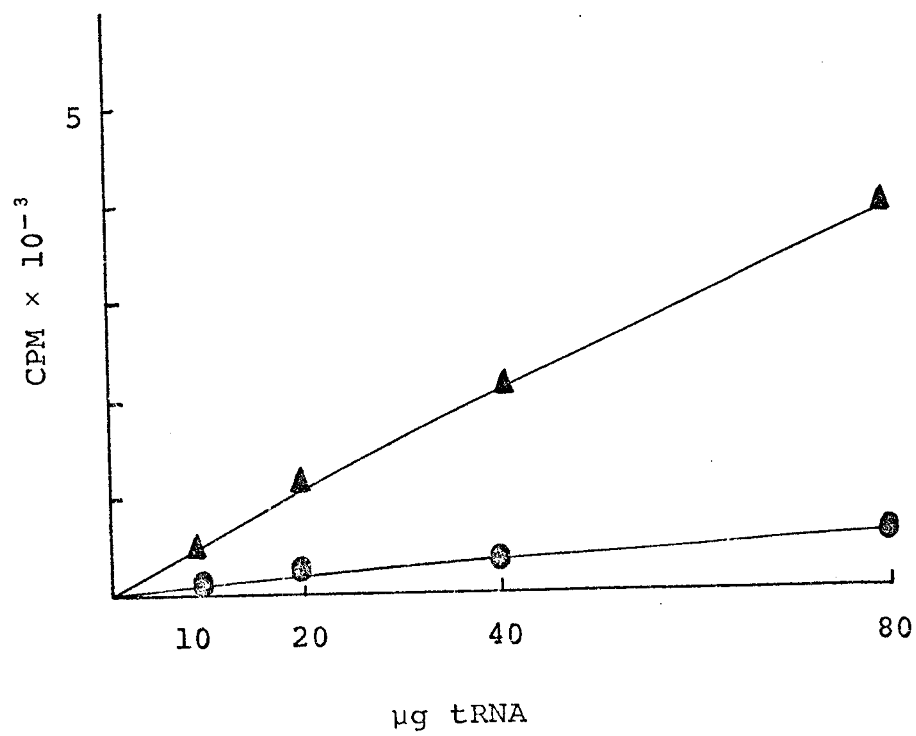
Figure 26 Effect of Adding H.cutirubrum and E.coli tRNA to the H.cutirubrum Whole Protein Synthesizing System

Each 0.100 ml reaction mixture (reduced from the 0.125 ml reaction mixture) contained the components, and was incubated as described in the "methods" for the *H.cutirubrum* whole protein synthesizing system, except for the designated modifications. 0.05 mg of poly U, 2.5 A_{260} units of ribosomes and 0.35 mg of protein in the S-150 were used. Results without poly U for each concentration of added tRNA have been subtracted. ●——● added *E.coli* tRNA. ▲——▲ added *H.cutirubrum* tRNA.

Figure 27 Effect of Adding H.cutirubrum and E.coli tRNA to the H.cutirubrum Charging System

Each 0.100 ml reaction mixture contained the same components as in Fig. 26 except the ribosomes were not present. The reaction was allowed to proceed for 40 minutes and treated normally for estimating amino acid acceptance by tRNA. The control without added tRNA has been subtracted. ●——● added *E.coli* tRNA. ▲——▲ added *H.cutirubrum* tRNA.


10
5
CPM × 10⁻³
10
20
30
40
80
µg tRNA


5
CPM × 10⁻³
10
20
40
80
µg tRNA

Figs. 28 a+b show the profiles of two different preparations of phenol purified tRNA on Sephadex G-75 in 2.8 M KCl, 0.1 M magnesium acetate, 0.01 M Tris-hydrochloride pH 7.6. It was apparent that there were at least three components, the proportions of which varied between the preparations. To see if this was an artefact because of the Sephadex being in high salt, E.coli tRNA was also run (Fig. 28c) and gave one major peak corresponding to the middle one of the tRNA from H.cutirubrum. In order to further rule out high salt artefacts and to purify the peaks on a large scale, the tRNA was run on Sephadex G-100 in 0.01 M Tris-hydrochloride pH 7.6, 0.01 M magnesium acetate. Fig. 29a shows that three peaks were again resolved. The middle peak was pooled and rerun (Fig. 29b) and behaved very similarly to E.coli tRNA on the same column (Fig. 29c).

It was possible to separate the tRNA preparation into three components using Sephadex, but this was not a very satisfactory large scale technique. Purification by means of DEAE-cellulose (von Ehrenstein, 1967) was attempted. The elution pattern is shown on Fig. 30. Very little material was removed by the 0.1 M Tris-HCl wash and the pattern of the material eluting with 1 M NaCl when rerun on the high salt Sephadex G-75 column was similar to the crude tRNA in Fig. 28b. BD-cellulose was then tried and the chromatographic behaviour of the tRNA on this column developed

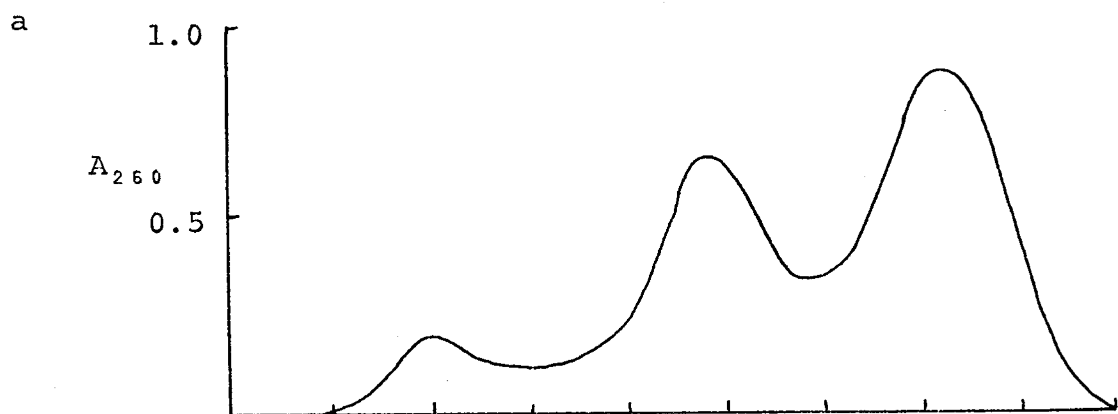
Figure 28 Chromatographic Behaviour of tRNA on Sephadex G-75 in High Salt

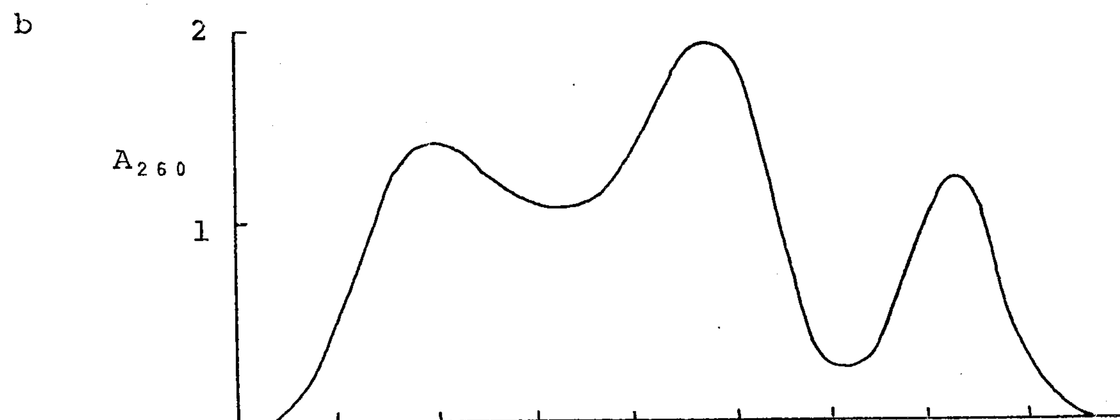
Sephadex G-75 was swollen and equilibrated in 2.8 M KCl, 0.1 M magnesium acetate, 0.01 M Tris-HCl, pH 7.6, and packed into a 2.5 × 45 cm glass column. 2 ml samples were applied in the high salt buffer, and 2 ml fractions collected at a flow rate of 30 ml per hour.

a. 4 mg of *H.cutirubrum* tRNA (preparation A)

b. 10 mg of *H.cutirubrum* tRNA (preparation B)

c. 6 mg of *E.coli* tRNA


a
1.0
A_{260}
0.5


b
2
A_{260}
1

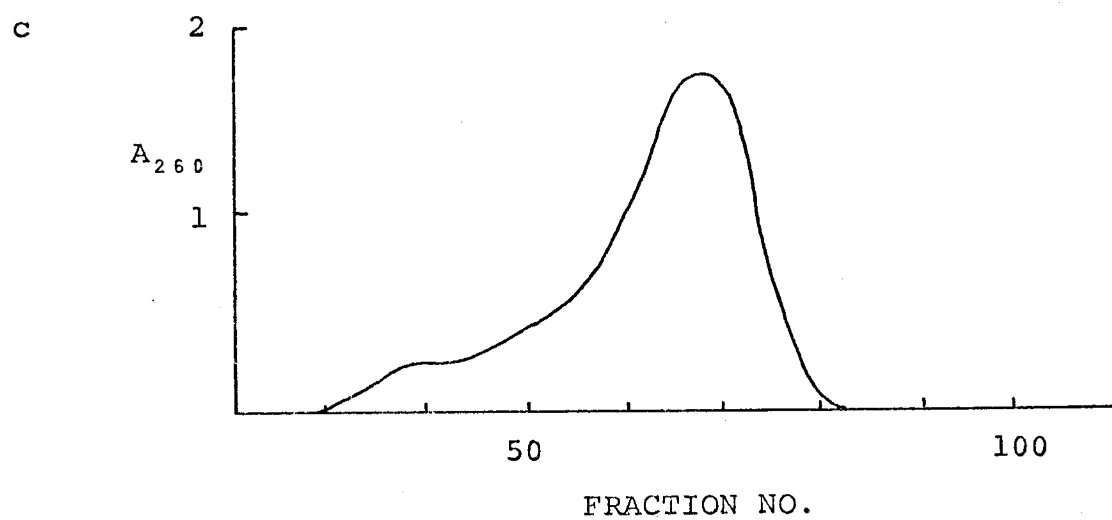

c
2
A_{260}
1
50
100
FRACTION NO.

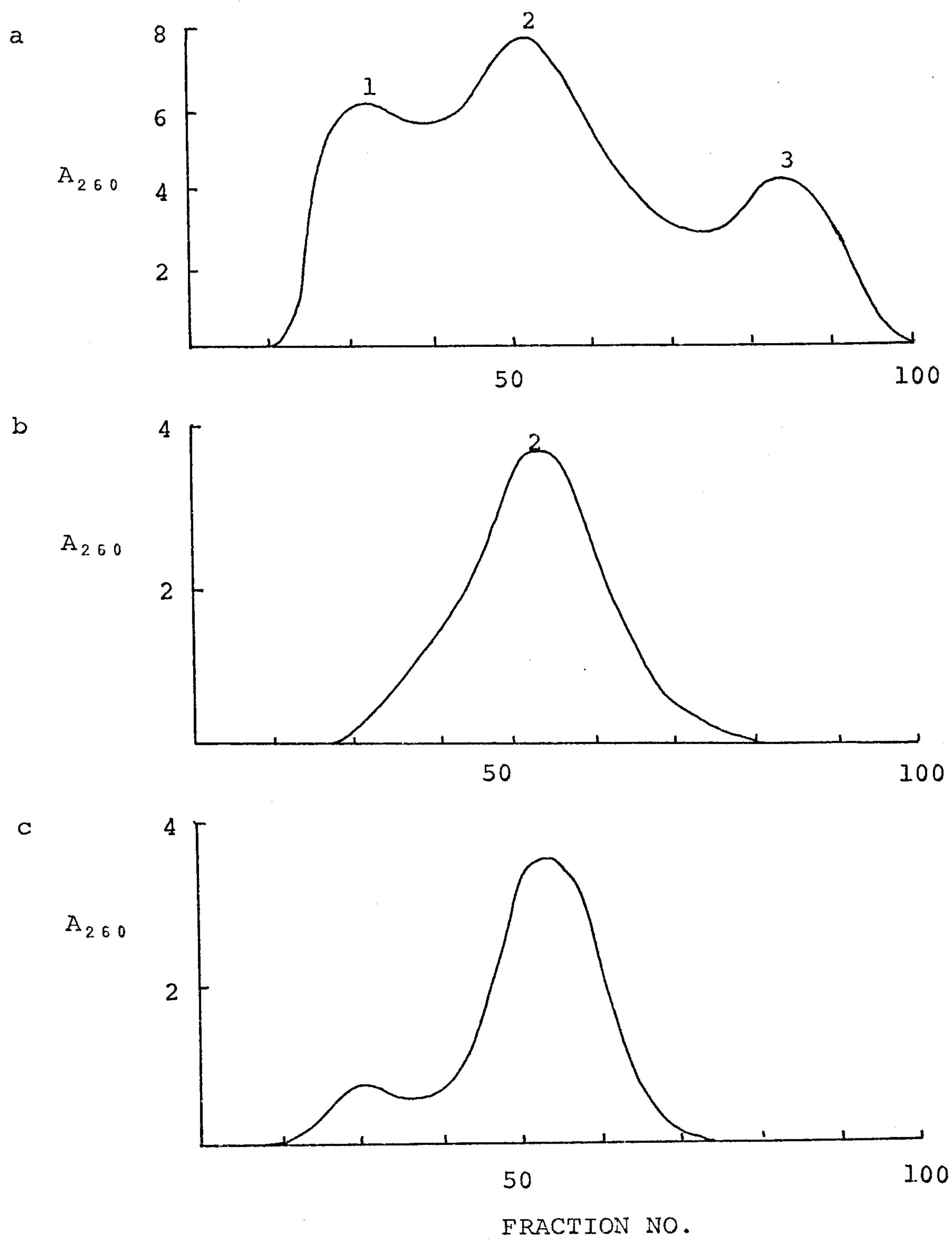
Figure 29 Chromatographic Behaviour of tRNA on Sephadex G-100 in Low Salt

Sephadex G-100 was swollen, and equilibrated in 0.01 M Tris-HCl, pH 7.6, 0.01 M magnesium acetate, and packed into a glass column. Samples were applied in 2 ml of the same buffer, and 2 ml fractions were collected with a flow rate of 30 ml per hour.

a. 40 mg of *H. cutirubrum* tRNA (preparation B)

b. The middle peak of (a) was isolated, ethanol precipitated, and rerun

c. 10 mg of *E. coli* tRNA


a
8
6
4
2
A_{260}
1
2
3
50
100
b
4
2
A_{260}
2
50
100
c
4
2
A_{260}
50
100
FRACTION NO.

<u>Figure 30</u> <u>Chromatographic Behaviour of H.cutirubrum tRNA on DEAE-Cellulose</u>

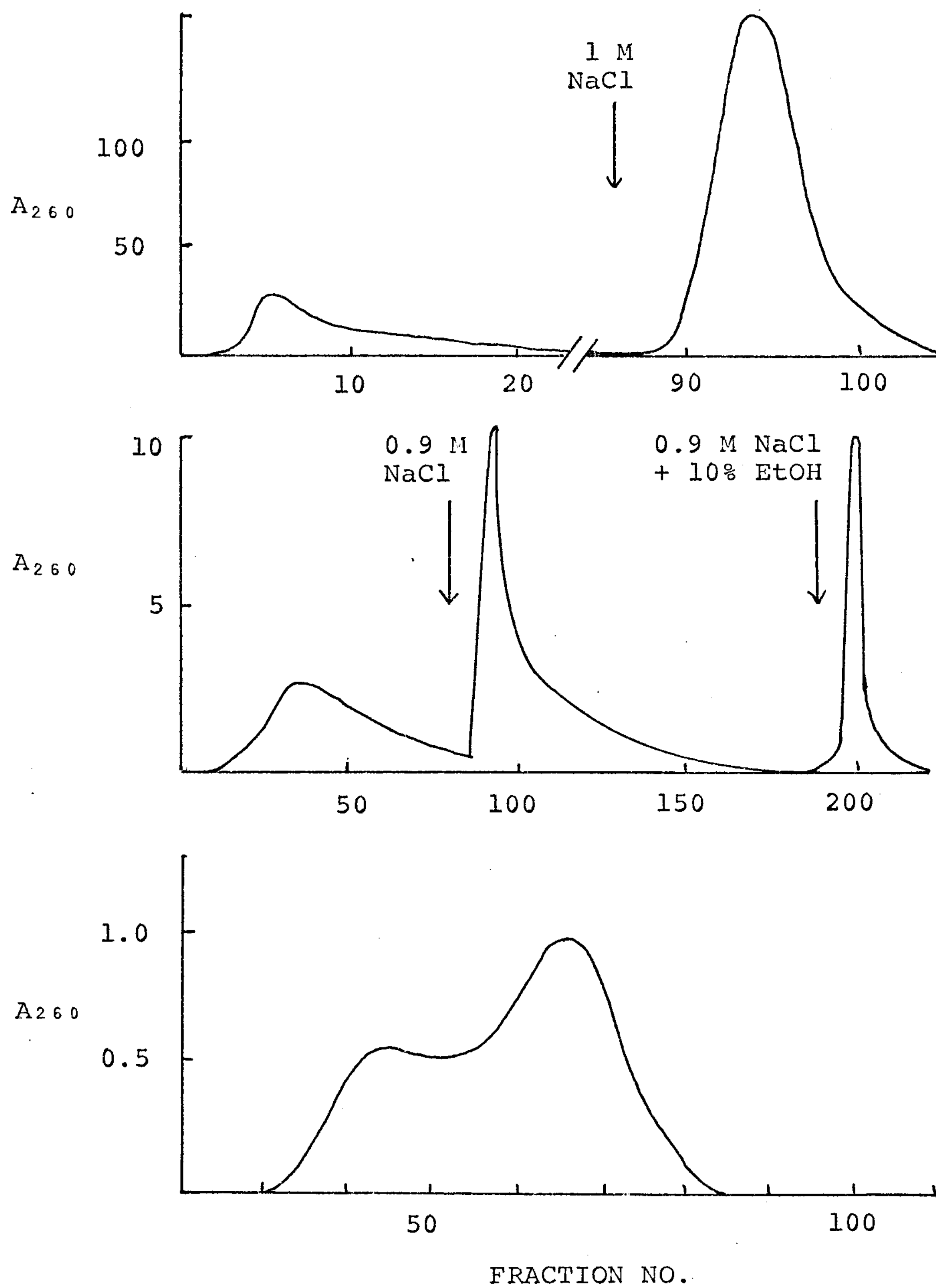
A 2.5 × 45 cm glass column was packed with DEAE-cellulose and washed with 1 litre of 0.1 Tris-HCl pH 7.6. 250 mg of <u>H.cutirubrum</u> (preparation B) in 25 ml of the same buffer were applied to the column and washed with 0.1 M Tris-HCl pH 7.6 at a flow rate of 40 ml per hour with 10 ml fractions being collected. After washing with about 900 ml of this buffer the tRNA was eluted with 1 M NaCl, 0.1 M Tris-HCl, pH 7.6.

<u>Figure 31</u> <u>Chromatographic Behaviour of H.cutirubrum tRNA (DEAE-Cellulose Purified) on BD-Cellulose</u>

A 2.5 × 45 cm glass column was packed with BD-cellulose and equilibrated with 0.45 M NaCl, 0.05 M sodium acetate, pH 4.5, 0.01 M magnesium chloride. 100 mg of <u>H.cutirubrum</u> tRNA (from the DEAE-cellulose column) in 10 ml of the same buffer were applied to the column. Elution was stepwise with 10 ml fractions being collected at a flow rate of 40 ml per hour.

<u>Figure 32</u> <u>Chromatographic Behaviour of H.cutirubrum tRNA (0.9 M NaCl Peak of BD-Cellulose) on Sephadex G-75 in High Salt</u>

A 2.5 × 45 cm glass column was packed with Sephadex G-75 which was swollen and equilibrated in 2.8 M KCl, 0.01 M magnesium acetate, 0.01 M Tris-HCl, pH 7.6. 5 mg of <u>H.cutirubrum</u> tRNA (0.9 M NaCl peak of BD-cellulose) in 2 ml of the above buffer were applied and the column eluted with 2 ml fractions being collected at a flow rate of 20 ml per hour.


1 M
NaCl
100
A_{260}
50
10
20
90
100
10
0.9 M
NaCl
0.9 M NaCl
+ 10% EtOH
A_{260}
5
50
100
150
200
1.0
A_{260}
0.5
50
100
FRACTION NO.

by stepwise elution is shown in Fig. 31. Significant amounts of material came off with each step, the bulk coming off with 0.9 M NaCl. This 0.9 M NaCl material when pooled and rerun on Sephadex G-75 in high salt gave the elution pattern shown in Fig. 32. This material was missing the third peak of Fig. 28b and as will be evident later, the 0.45 M NaCl peak from the BD-cellulose is equivalent to the third peak from the Sephadex columns. From these column profiles it was apparent that although Sephadex columns gave the best separation of the components, BD-cellulose would possibly provide a better larger scale partial purification especially with tRNA similar to preparation A, which contained very little of the first peak material.

The peaks from these columns were collected, ethanol precipitated and/or freeze dried. Table LII shows the acceptor activities of the peaks from the columns. The middle peak from the Sephadex columns and the 1 M NaCl peak from the BD-cellulose column were the only ones with significant ^{14}C-phe acceptor activity. Similar results were found using ^{14}C-methionine.

Table LIII illustrates the chromatographic behaviour of the inhibitory fraction on the columns. It was clear that this fraction was contained in the third peak of the Sephadex columns and the 0.45 M NaCl peak from the BD-cellulose. From the above results it was clear that using the middle peak from the Sephadex columns or the 0.9 M NaCl peak from BD-cellulose was satisfactory, at least for

Table LII Purification of H.cutirubrum tRNA (Preparation B) by Column Procedures

Purification Step			μμMoles ^{14}C-Phe Accepted/A_{260} Unit of tRNA
1.	Crude		3.8
2.	DEAE-Cellulose		4.1
3.	Sephadex G-100	Peak 1	0.4
		" 2	7.4
		" 3	0.0
4.	Sephadex G-100	Peak 2 (Rerun)	7.9
5.	BD-Cellulose	Peak 0.45 M NaCl	0.0
		" 0.9 M NaCl	6.3
		" 0.9 M NaCl + 10% EtOH	0.1

tRNA from the columns shown in Figs. 29, 30 and 31 were isolated, ethanol precipitated, and freeze dried. ^{14}C-phe acceptance was assayed by the H.cutirubrum tRNA charging system described in the "methods" using ^{14}C-phe (376 mCi/mmole).

Table LIII Assay of Peaks from BD-Cellulose and Sephadex G-100 for the "Inhibitor" to Protein Synthesis

Added tRNA (2 A_{260} Units)			µµMoles ^{14}C-Phe in the Hot 5% TCA Ppt.
-			13.7
Crude (Preparation B)			6.7
DEAE-Cellulose			6.9
Sephadex G-100	Peak	1	11.9
	"	2	13.9
	"	3	3.1
BD-Cellulose	Peak	0.45 M NaCl	3.3
	"	0.9 M NaCl	11.3
	"	0.9 M NaCl + 10% EtOH	8.4

tRNA from the columns shown in Figs. 29, 30 and 31 were isolated, ethanol precipitated and freeze dried. Each 0.100 ml reaction mixture (reduced from the 0.125 ml reaction mixture) contained the components, and was incubated as described in the "methods" for the whole protein synthesizing system, except for designated modifications. 0.05 mg of poly U, 0.1 mg of ribosomes and 0.35 mg of protein in S-150 were used. Results without poly U for each concentration of added tRNA have been subtracted.

$tRNA^{phe}$ which was to be used in the protein synthesizing system.

The remaining purification of the system concerned the synthetases and transferase enzymes (T and G). An attempt to purify the transferases had been made in Chap.IV,3 with ammonium sulfate fractionation and Sephadex G-75 in 2.8 M KCl. As the ammonium sulfate fractionation made only a slight improvement over the S-150 as far as tRNA and synthetase content was concerned, fractionation on Sephadex G-75 in 2.8 M KCl was used. Fig. 33 shows the fractionation of the components in the S-150. The void volume and the larger molecular weight components fractionated by the column were probably mainly protein as indicated from the 280 mμ/260 mμ ratio. The 260 mμ peak in fractions 60-75 was in the same position as the phenol purified tRNA was eluting in Fig. 28b. The final peak which proved to be non-precipitable by 100% ammonium sulfate was probably low molecular weight material such as amino acids and oligonucleotides produced from the DNase treatment of the original homogenate. The three regions indicated in Fig. 33 were pooled and precipitated by dialyzing against 100% ammonium sulfate. These fractions were then tested for synthetase activity, and ^{14}C-phe accepting capacity. The results are shown in Table LIV. Phenylalanine synthetase activity was present in all three fractions as the added tRNA was charged with ^{14}C-phe to a similar extent (column B). The amount of ^{14}C-phe accepted

Figure 33 Chromatographic Behaviour of the Components of S-150 on Sephadex G-75 in High Salt

Sephadex G-75 was swollen, and equilibrated in 2.8 M KCl, 0.1 M magnesium acetate, 0.01 M Tri-HCl pH 7.6, and packed into a 2.5 × 45 cm glass column. 2 ml of S-150 were applied, and 2 ml fractions collected at a flow rate of 30 ml per hour

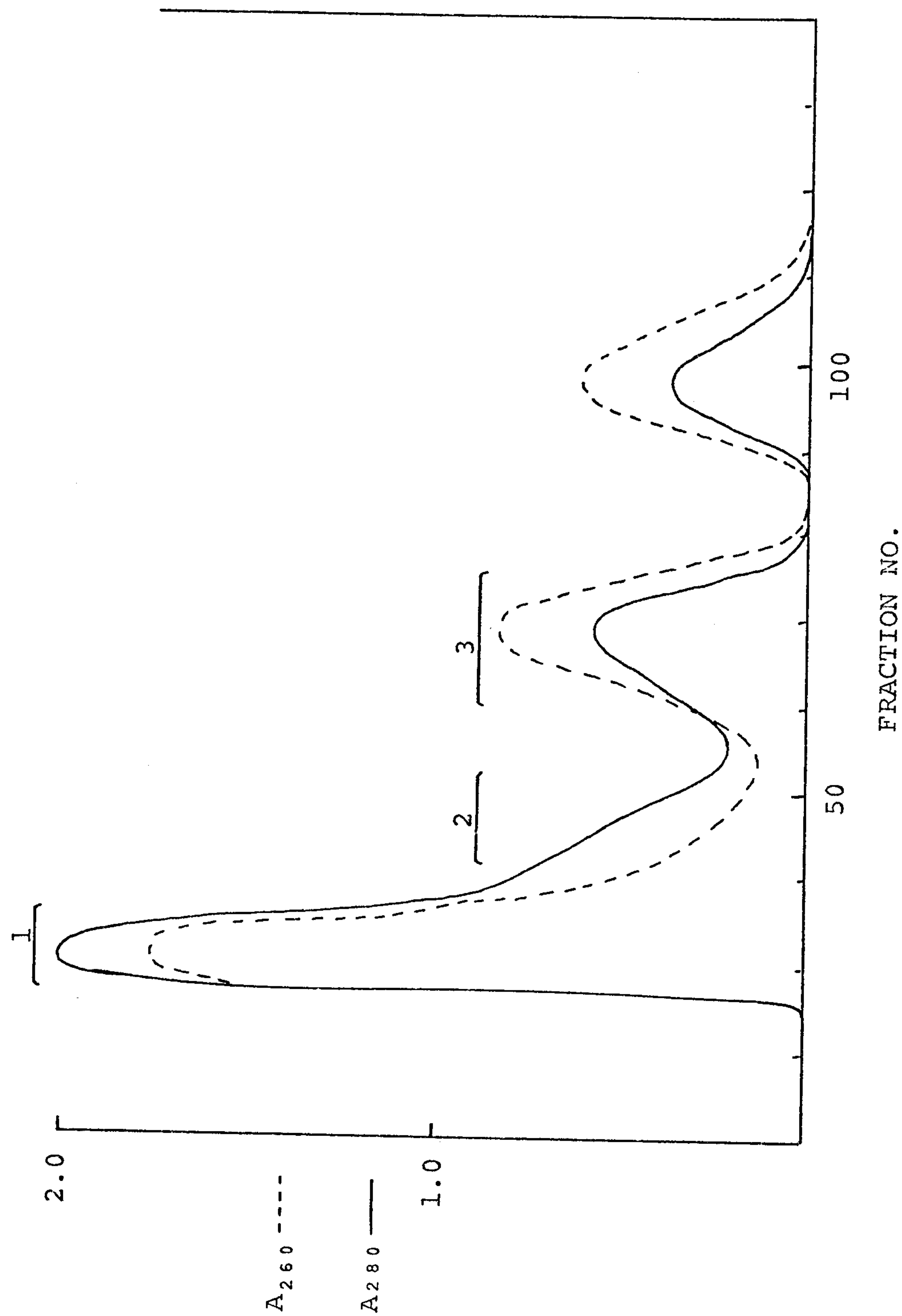

2.0
1.0
A_{260} ----
A_{280} ——
1
2
3
50
100
FRACTION NO.

Table LIV Phenylalanine Synthetase Activity and ^{14}C-Phe Acceptance Capacity of the Components of S-150 Fractionated on Sephadex G-75

Extract		μμMoles of ^{14}C-Phe in the Cold 5% TCA Ppt.		
		A	B	C
		–	+ tRNA	+ RNase
S-150	Complete	2.5	9.8	0.2
"	Fraction 1	0.5	18.4	0.2
"	" 2	0.2	17.9	0.1
"	" 3	10.3	26.3	0.1

Each 0.225 ml reaction mixture contained the components and was incubated as described in the "methods" for the H.cutirubrum tRNA charging system except for the designated modifications. 2.8 A_{260} units of crude tRNA, 1.5 A_{260} units of S-150, 0.36 A_{280} units of fraction 1, 0.20 A_{280} units of fraction 2, and 0.55 A_{260} unit of fraction 3 were used. Column A assayed the presence of both $tRNA^{phe}$ and phe synthetase while B specifically assayed for the presence of phe synthetase.

using S-150 appeared low probably because the ^{12}C-phe, that was not removed from the S-150 by dialysis, lowered the specific activity of the added ^{14}C-phe. The high acceptor activity using peak 3 as the source of synthetases was because this peak contained the tRNA of the S-150. This is evident from the acceptance of ^{14}C-phe without added tRNA (column A). The transferase activity appeared concentrated (Table LV) in fraction 2 although some was in fraction 1.

The fractionation of S-150 on Sephadex in 2.8 M KCl confirmed that the untreated tRNA eluted in the same position as the middle peak of phenol purified tRNA on this column. The transferases T and G seemed to be concentrated in the protein eluting just after the void volume. Phenylalanine synthetase appeared in all three fractions confirming the difficulty in purifying this enzyme by ammonium sulfate fractionation (Fig.12). Although the constituents of the system were far from pure, they were sufficiently so to obtain meaningful results in reconstitution. Table LVI shows the reconstituted system and its absolute dependence on all the components required for protein synthesis.

A protein synthesizing system which could use the phenol purified tRNA was thus obtained. This system, like the crude one, was still inefficient as far as total amount of ^{14}C-phe polymerised was concerned. However no attempt to find optimal amounts of each component was made and if this

Table LV Transferase Activity of the Components of S-150 Fractionated on Sephadex G-75

Extract			μμMoles of ^{14}C-Phe in the Hot 5% TCA-Tungstate Ppt. + Poly U	- Poly
S-150	Complete		7.8	0.3
"	Fraction	1	2.4	0.2
"	"	2	8.3	0.2
"	"	3	0.7	0.1

Each 0.100 ml reaction mixture (reduced from the 0.125 ml reaction mixture)contained the components and was incubated as described in the "methods" for the H.cutirubrum aminoacyl-tRNA transfer system except for the designated modifications. 0.1 mg of ribosomes, 0.05 mg of poly U, 0.75 A_{260} unit of S-150, 0.18 A_{280} unit of fraction 1, 0.10 A_{280} unit of fraction 2, 0.27 A_{260} unit of fraction 3, 17.64 μμmoles ^{14}C-phe-tRNA E.coli (0.87 A_{260} unit) were used.

Table LVI The Reconstituted Protein Synthesizing System

Deletions	^{14}C-Phe μμMoles in the Hot 5% TCA Ppt.	
	+ Poly U	- Poly
-	12.73	0.91
- tRNA	0.52	0.32
- S-150 Fraction 2	0.83	0.64
- Ribosomes	0.12	0.12
- GTP	0.67	0.52
- PEP, ATP	0.81	0.73

Each 0.100 ml reaction mixture contained the components and was incubated as described in the "methods" for the _H. cutirubrum_ whole protein synthesizing system except for the designated modifications. S-150 was replaced by 0.1 A_{280} unit of fraction 2 and 1.3 A_{260} units of tRNA (peak 2 of Sephadex G-100). 0.16 mg of ribosomes and 0.050 mg of poly U were used.

was done it is possible a considerable improvement in incorporation could be made. The system would be sufficient for the purposes of testing for the initiator tRNA.

8. Initiation of Protein Synthesis

Accumulating evidence (Clark and Marcker, 1966; Smith and Marcker, 1969) suggests that initiation on all 70S ribosomes is through the universal initiator N-formyl-methionyl-$tRNA_{F}^{met}$ and initiation on 80S ribosomes is through methionyl-$tRNA_{F*}^{met}$ (F*-formylatable by the *E.coli* transformylase), (Smith and Marcker, 1970; Wigle and Dixon, 1970; Jackson and Hunter, 1970; Rajbhandary and Kumar, 1970; Bhaduri, 1970). To determine whether F-met-$tRNA_{F}^{met}$ is the initiator in *H.cutirubrum*, which has 70S ribosomes (Bayley, 1966) the formylation of met-tRNA was investigated both *in vivo* and *in vitro*. To examine the species of $tRNA^{met}$ more closely, they were separated by BD-cellulose chromatography.

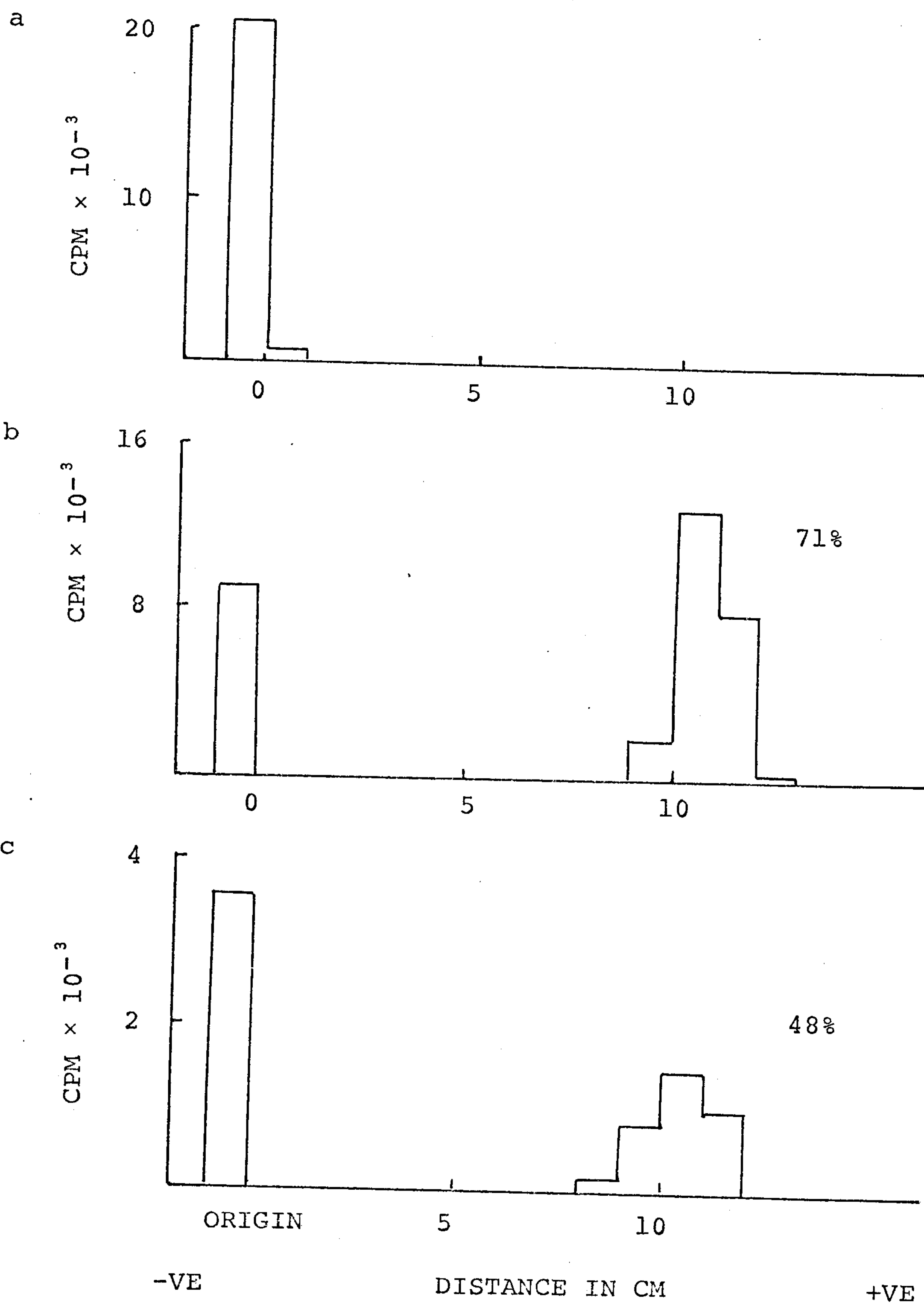
Initially formylation of *H.cutirubrum* met-tRNA was investigated *in vitro*. *H.cutirubrum* tRNA was charged with ^{14}C-methionine in a standard *H.cutirubrum* incubation mixture with the addition of N^{10}-formyl tetrahydrofolate. The tRNA was phenol extracted, and thoroughly freed of ^{14}C-methionine by repeated precipitation with ethanol. The amino acids were discharged from the tRNA by alkali, and analysed by electrophoresis at pH 3.5. Fig. 34a shows the results of such an experiment. All the material from the tRNA had the mobility of ^{14}C-methionine, and further experiments using a variety

Figure 34 Electrophoresis of ^{14}C-Methionine and ^{14}C-Formyl Methionine Discharged from E.coli and H.cutirubrum tRNA

a. 5 mg of H.cutirubrum tRNA were charged with ^{14}C-methionine (0.09 μmole of 60 mCi/mmole per 4.5 ml reaction mixture) in the presence of N^{10}, formyl tetrahydrofolate in a standard preparative charging system using S-150 as the source of the synthetases. The aminoacyl-tRNA was phenol extracted, and thoroughly freed of amino acid by repeated precipitation with ethanol. Amino acids were discharged by alkali, and 3.3×10^4 cpm applied to Whatman 3 MM paper, and electrophoresed at pH 3.5 for 1.5 hours at 3000 V. The paper was cut into 3 × 1 cm pieces, and radioactivity assayed in a scintillation counter.

b. 5 mg of E.coli tRNA were charged with ^{14}C-methionine (0.09 μmole of 60 mCi/mmole per 5 ml reaction mixture) in the presence of N^{10}, formyl tetrahydrofolate in the E.coli formylating system. The aminoacyl-tRNA was extracted, and treated as for (a) 4.7×10^4 cpm were applied to the Whatman paper.

c. 5 mg of H.cutirubrum ^{14}C-met-tRNA (6.4×10^4 cpm) were formylated in the E.coli system. The aminoacyl-tRNA was extracted, and the products examined as above. 8.7×10^3 cpm were applied to the Whatman paper.


a
20
10
CPM × 10⁻³
0
5
10
b
16
8
CPM × 10⁻³
71%
0
5
10
c
4
2
CPM × 10⁻³
48%
ORIGIN
5
10
-VE
DISTANCE IN CM
+VE

of added monovalent cations to the system gave the same result. This indicated that there was no transformylase activity in the S-150 comparable to that found in E.coli.

To show that this result was not due to deformylation during the manipulation of the aminoacyl-tRNA, the E.coli system was used as a control. Fig. 34b shows the results using E.coli tRNA in an E.coli formylating system. This time 71% of the label moved to the anode as formyl methionine, as expected. As the technique was not causing deformylation, it was possible that, either there was no transformylase system in H.cutirubrum, or there was no formylatable met-tRNA. To explore this latter possibility H.cutirubrum ^{14}C-met-tRNA was incubated with E.coli S-150 and N^{10}-formyl tetrahydrofolate in the E.coli formylating system. Cold ^{12}C-met was added to dilute the ^{14}C-met discharged from the H.cutirubrum ^{14}C-met-tRNA and to rule out charging of the E.coli $tRNA^{met}$ in the system, although the amount of $tRNA^{met}$ in the E.coli S-150 was negligible. Incubating at 37^{0}C for 20 minutes gave 32% of the material from the H.cutirubrum tRNA as formyl-methionine. To see if this low amount of formylatable met-tRNA was due to a conformation of the H.cutirubrum tRNA, which was not easily recognisable by the E.coli transformylase, the system was run at 45^{0}C. Fig. 34c shows that now 48% of the material was formyl methionine. It is probable that this apparent increase in formylation at 45^{0}C was due to preferential discharge of the non-formylated met-$tRNA_M$, the aminoacyl bond of which is more alkali labile than that of

F-met-$tRNA^{met}_{F}$.

To try to obtain a more quantitative estimation of the amount of formylatable met-tRNA in H.cutirubrum, use was made of the fact the E.coli synthetases charge H.cutirubrum $tRNA^{met}$. In this system there would be an equilibrium between charged and uncharged $tRNA^{met}_{M}$, so the proportions of $tRNA^{met}_{M}$ and $tRNA^{met}_{F}$ would be more accurately estimated. Results of such an experiment are shown in Fig. 35a, in which 30% of the label from the tRNA was formyl methionine. Another result indicating that in Fig. 34c the proportion of the formylatable species may be exagerated is shown in Figs. 35 b+c. In this experiment there was approximately 90 times less methionine than in Fig. 35a. Fig. 35b shows the result of charging and formylating E.coli tRNA with this reduced amount of methionine. 94% of the methionine was now formylated compared to 71% in Fig. 34b. The results with H.cutirubrum tRNA were similar (Fig. 35c) in that 56% of the methionine was now formylated compared to 30% in Fig. 35a. With this concentration of methionine the equilibrium was more favourable for discharge of the met-$tRNA_{M}$, while the met-$tRNA^{met}_{F}$ once formylated could not be deacylated as it was no longer recognised by the synthetase and the bond was more alkali stable.

As a confirmation of the above results, use was made of the ^{3}H-N^{5}, N^{10} methenyl tetrahydrofolate, prepared as described in the "methods". Table LVII shows that when added to an H.cutirubrum system in the presence of all 20 amino acids,

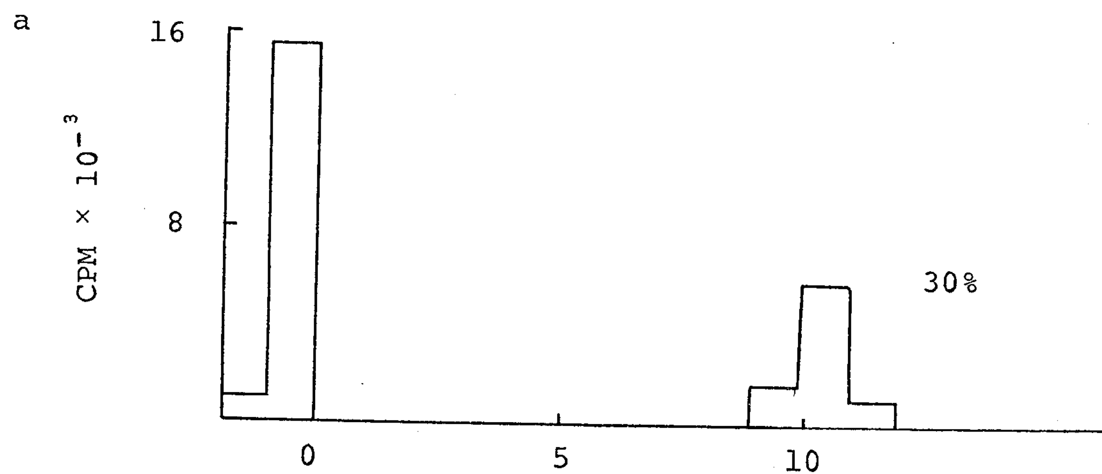
Figure 35 Electrophoresis of ^{14}C-Methionine and ^{14}C-Formyl Methionine Discharged from E.coli and H.cutirubrum tRNA

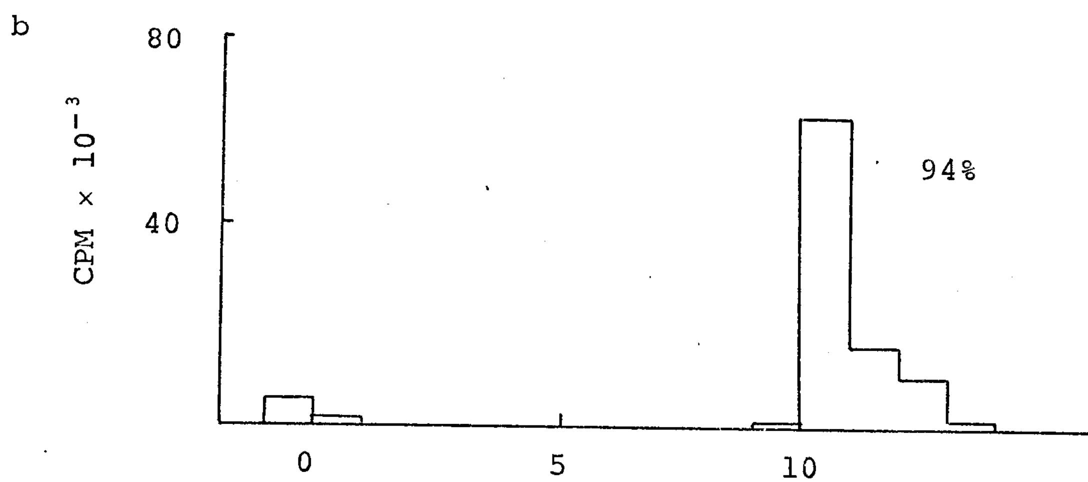
a. 5 mg of H.cutirubrum tRNA were charged with ^{14}C-met (0.09 μmoles of 60 mCi/mmole per 5 ml reaction mixture) in the presence of N^{10}, formyl tetrahydrofolate in the E.coli formylating system using both the E.coli transformylase and methionine synthetase. The aminoacyl-tRNA was extracted and the products examined as for Fig. 34. 3.3×10^4 cpm were applied to the Whatman 3 MM paper, and electrophoresed at pH 3.5, for 1.5 hours, at 3000 V.

b. The conditions were the same as in Fig. 34b except ^{35}S methionine (12.8* Ci/mmole) was used. There was 0.001 μmole of ^{35}S methionine in the 5 ml incubation mixture. The aminoacyl-tRNA was extracted and the products examined as before. 1.2×10^5 cpm were applied to the Whatman 3 MM paper.

c. The conditions were the same as in Fig.35a except ^{35}S methionine (12.8* Ci/mmole) was used. There was 0.001 μmole ^{35}S methionine in the 5 ml incubation mixture. The aminoacyl-tRNA was extracted, and the products examined as before. 1.2×10^5 cpm were applied to the Whatman 3 MM paper.

* Approximate specific activity as this ^{35}S methionine contained 20% sulfone (Dr. Ghosh - personal communication).


a
16
8
CPM × 10⁻³
30%
0
5
10


b
80
40
CPM × 10⁻³
94%
0
5
10

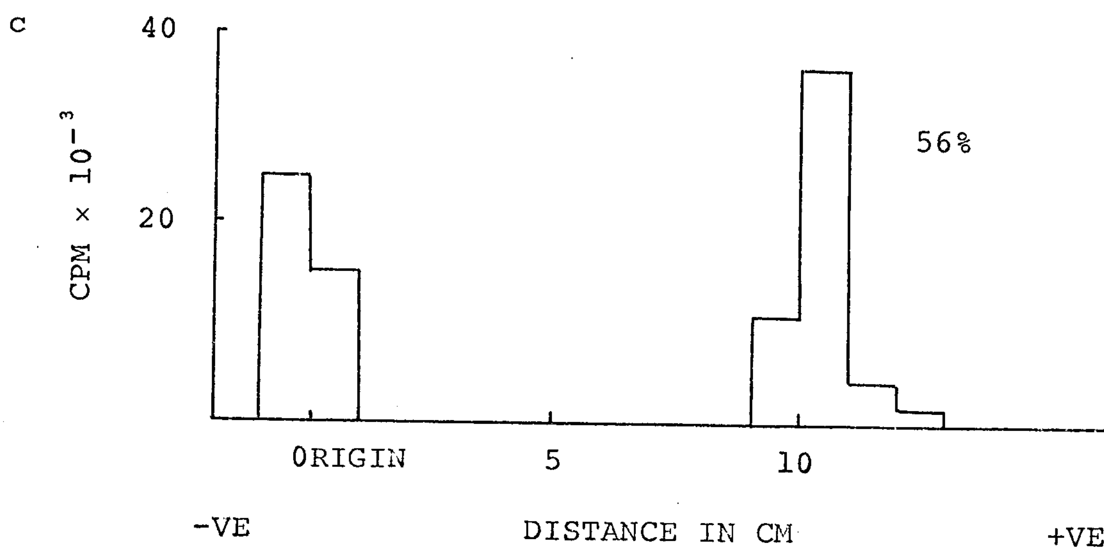

c
40
20
CPM × 10⁻³
56%
ORIGIN
5
10
-VE
DISTANCE IN CM
+VE

Table LVII Acceptance of the ^{3}H-Formyl Group by H.cutirubrum Aminoacyl-tRNA's in the H.cutirubrum tRNA Charging System

Deletions	^{3}H-Formyl Group in the Cold 5% TCA Ppt. cpm	
	-	+ RNase
-	88	69
- 20 ^{12}C-AA	63	54

Each 0.225 ml reaction mixture contained the components and was incubated as described in the "methods" for the H.cutirubrum charging system except for the designated modifications. 0.0025 mg of ^{3}H-N^{10}, formyl terahydrofolate and 0.008 μmole of each of the 20 amino acids were used. S-150 was used as the source of synthetases and possible transformylase system.

the ^{3}H-formyl group was not accepted by the aminoacyl-tRNA. A similar experiment in the E.coli system (Table LVIII) revealed significant acceptance of ^{3}H-formyl group by both E.coli and H.cutirubrum aminoacyl-tRNA. All the above results indicated the absence of a transformylase system in H.cutirubrum S-150, but that a portion of the met-tRNA was formylatable by the E.coli transformylase system.

A possible reason for the absence of formyl methionine after incubation with H.cutirubrum S-150 was the presence of a strong deformylase. To test this, ^{14}C-formyl methionyl-tRNA (both E.coli and H.cutirubrum) was incubated with H.cutirubrum S-150. The results for the E.coli tRNA are shown in Figs. 36 a+b. There was little difference in the ratio of methionine to formyl methionine in the aminoacyl-tRNA incubated either with or without the S-150. There was therefore no indication of a deformylase.

The above in vitro experiments could not rule out the possibility that the transformylase system in the H.cutirubrum S-150 was, either inactivated in extraction, or could not use the N^{10}, formyl tetrahydrofolate. To examine this, an attempt was made to find formyl-methionyl-tRNA in vivo. 24 hour cells were washed, and suspended in a synthetic medium devoid of methionine. After one hour ^{35}S-methionine was added, and following incubation for varying lengths of time, the cells were isolated and washed. The aminoacyl-tRNA was isolated by phenol extraction at pH 4.5, and the amino acids discharged by alkali. Fig. 36c

Table LVIII Acceptance of ^{3}H-Formyl Group by H.cutirubrum and E.coli Aminoacyl-tRNA's in the E.coli Formylating System

tRNA	Deletions	^{3}H-Formyl Group in the Cold 5% TCA Ppt. cpm –	+ RNase
E.coli	-	6450	124
"	- 20 ^{12}C-AA	570	98
H.cutirubrum	-	950	103
"	- 20 ^{12}C-AA	192	77
-	-	123	112

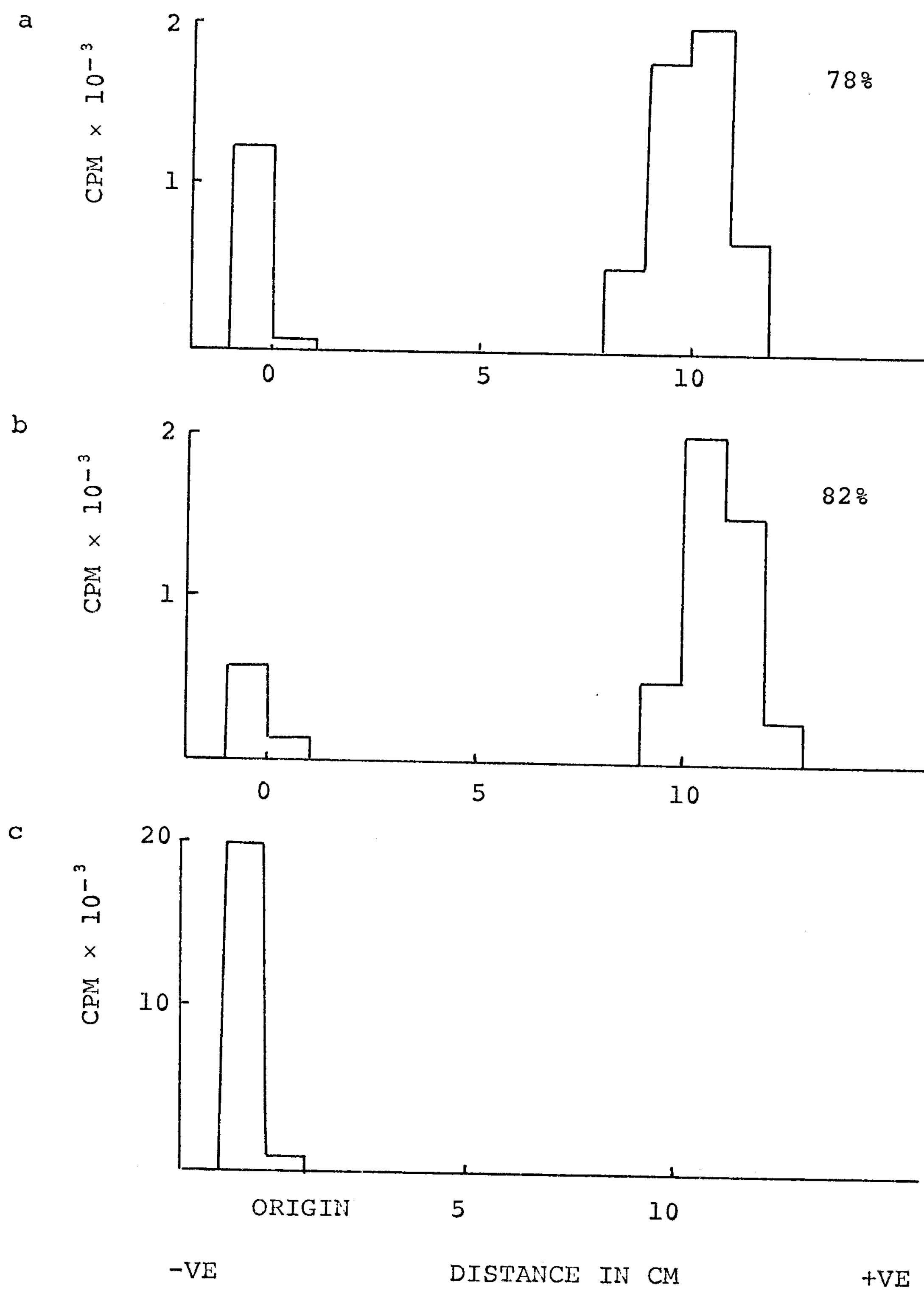
Each 0.250 ml reaction mixture contained the components and was incubated as described in the "methods" for the E.coli formylating system. 0.025 mg of ^{3}H-N^{10}, formyl tetrahydrofolate and 0.008 μmole of each of the 20 amino acids were used. S-150 was used as the source of synthetases and transformylase.

Figure 36 Electrophoresis of ^{14}C and ^{35}S-Methionine and Formyl Methionine Discharged from E.coli and H.cutirubrum tRNA

a. E.coli ^{14}C-methionyl and ^{14}C-formyl methionyl-tRNA prepared as in Fig. 34b were incubated in a standard H.cutirubrum charging system. S-150 was the source of the synthetases. The aminoacyl-tRNA was extracted and the discharged amino acids separated as for Fig. 34. 9.8×10^3 cpm were applied to the Whatman 3 MM paper.

b. As for (a) except no S-150 was present in the charging system. 8.3×10^3 cpm were applied to the Whatman 3 MM paper.

c. 24 hour H.cutirubrum cells were suspended in 15 ml of synthetic medium and after one hour at 37^0C, 50 μC of ^{35}S methionine (280 mCi/mmole) were added. After a further hour incubation the cells were washed, and lysed by homogenisation in 0.005 M KAc pH 4.5. Nucleic acids were phenol extracted, and the ethanol precipitate thoroughly washed by repeatedly dissolving in 1 M potassium acetate, pH 4.5, and precipitating with 2 volumes of ethanol. Amino acids were discharged by alkali, and electrophoresed as for Fig. 34. 3.1×10^4 cpm were applied to the Whatman 3 MM paper.


a
2
1
CPM × 10⁻³
78%
0
5
10
b
2
1
CPM × 10⁻³
82%
0
5
10
c
20
10
CPM × 10⁻³
ORIGIN
5
10
-VE
DISTANCE IN CM
+VE

shows the results of such an experiment. All the ^{35}S-methionine discharged from the tRNA had the mobility of methionine. This experiment, based on that described by Smith and Marcker (1969), should have revealed formyl methionyl-tRNA, if any was present *in vivo*. ^{35}S-methionine was found on the tRNA and therefore the absence of formylation was not due to the lack of ^{35}S-methionyl-tRNA.

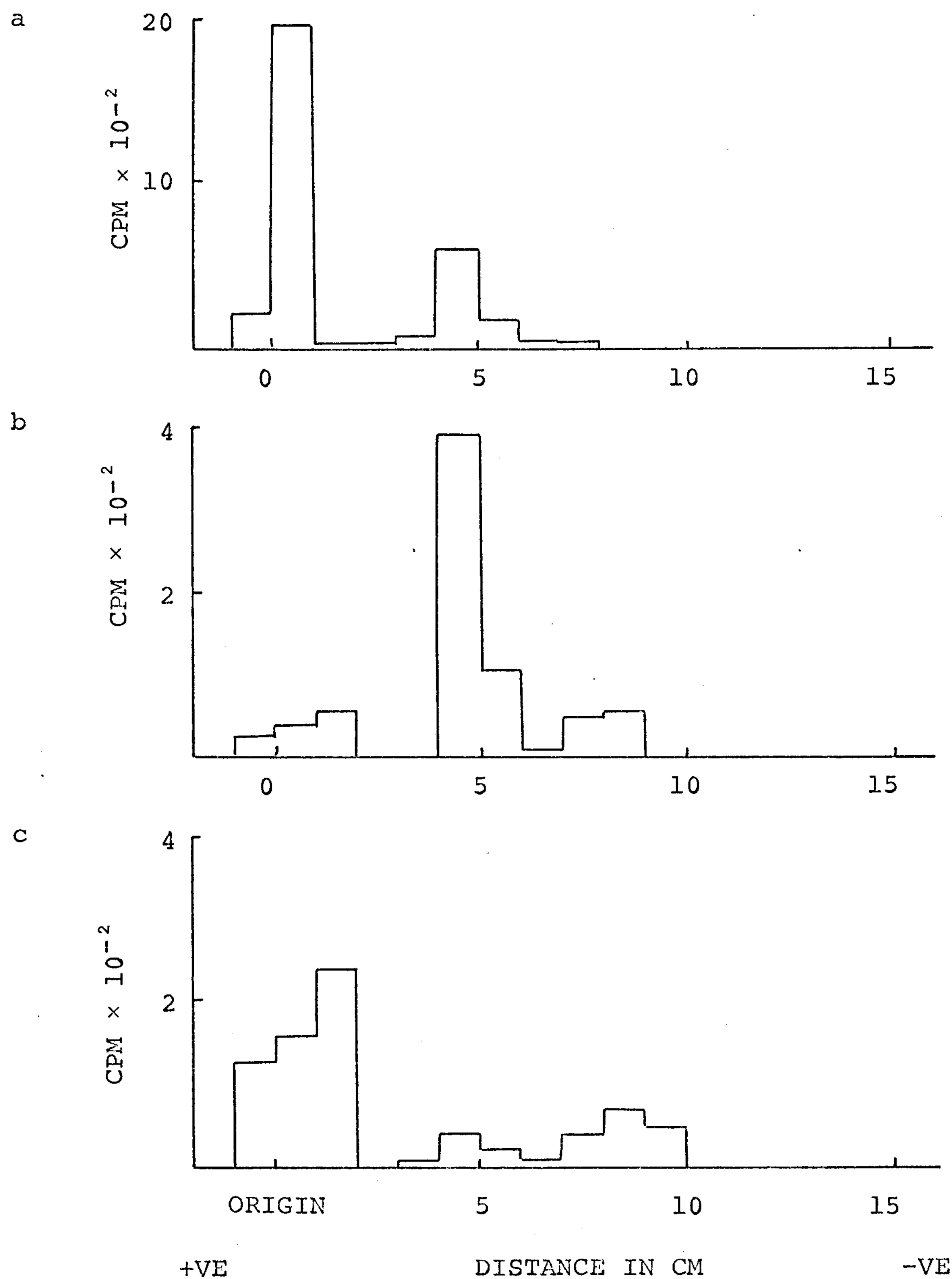
An alternative way of investigating the initiation mechanism was by use of a technique described by Bachmayer and Kreil (1968). In this experiment cells were incubated with ^{35}S-methionine in the presence of puromycin. Puromycin should cause the release of all ribosomes from the mRNA, followed by reinitiation at the beginning of the messenger RNA. Only the initiator tRNA would then be able to enter the P site, and puromycin would compete with aminoacyl-tRNA for the A site. The end result in other bacterial systems is the formation of initiator amino acid - puromycin, which is selectively extracted from cells with ethyl acetate. The results of such an experiment with *H.cutirubrum* cells are shown in Fig. 37a. Apart from the material at the origin, the only peak was in the position Bachmayer and Kriel (1968) show formyl methionyl-puromycin and formyl methionyl-aminoacyl-puromycin to migrate to. If puromycin was not present during the labelling of the cells, little radioactivity entered the ethyl acetate, and all this remained at the origin. This peak was a direct result of the presence of puromycin, and to determine its nature the peaks were eluted and a part

Figure 37 Electrophoresis of ^{35}S-Methionine Products Extracted from Cells Incubated in the Presence of Puromycin

a. 24 hour cells were washed free of complex medium, and suspended in 2 ml of synthetic medium. After 1 hour ^{35}S-methionine (280 mCi/mmole) and puromycin to a final concentration of 5 mM were added. The cells were incubated for 2 hours, and the cell pellet after centrifugation was suspended in 3 ml H_2O and 0.1 ml cutscum, and homogenised. This homogenate was extracted with ethyl acetate, and the ^{35}S-products identified by electrophoresis at pH 1.8 for 1.5 hours, at 2500 V. 4.1×10^3 cpm were applied to the Whatman 3 MM paper. The ^{35}S-methionine marker migrated 9 cm and the puromycin marker 12-14 cm towards the cathode.

b. The peaks migrating 5 cm towards the cathode from several runs like (a) were pooled, eluted, and rerun. 1.1×10^3 cpm were applied to the Whatman 3 MM paper.

c. The peaks migrating 5 cm towards the cathode from several runs like (a) were pooled, eluted, and treated with methanolic HCl. 1.3×10^3 cpm were applied to the Whatman 3 MM paper.

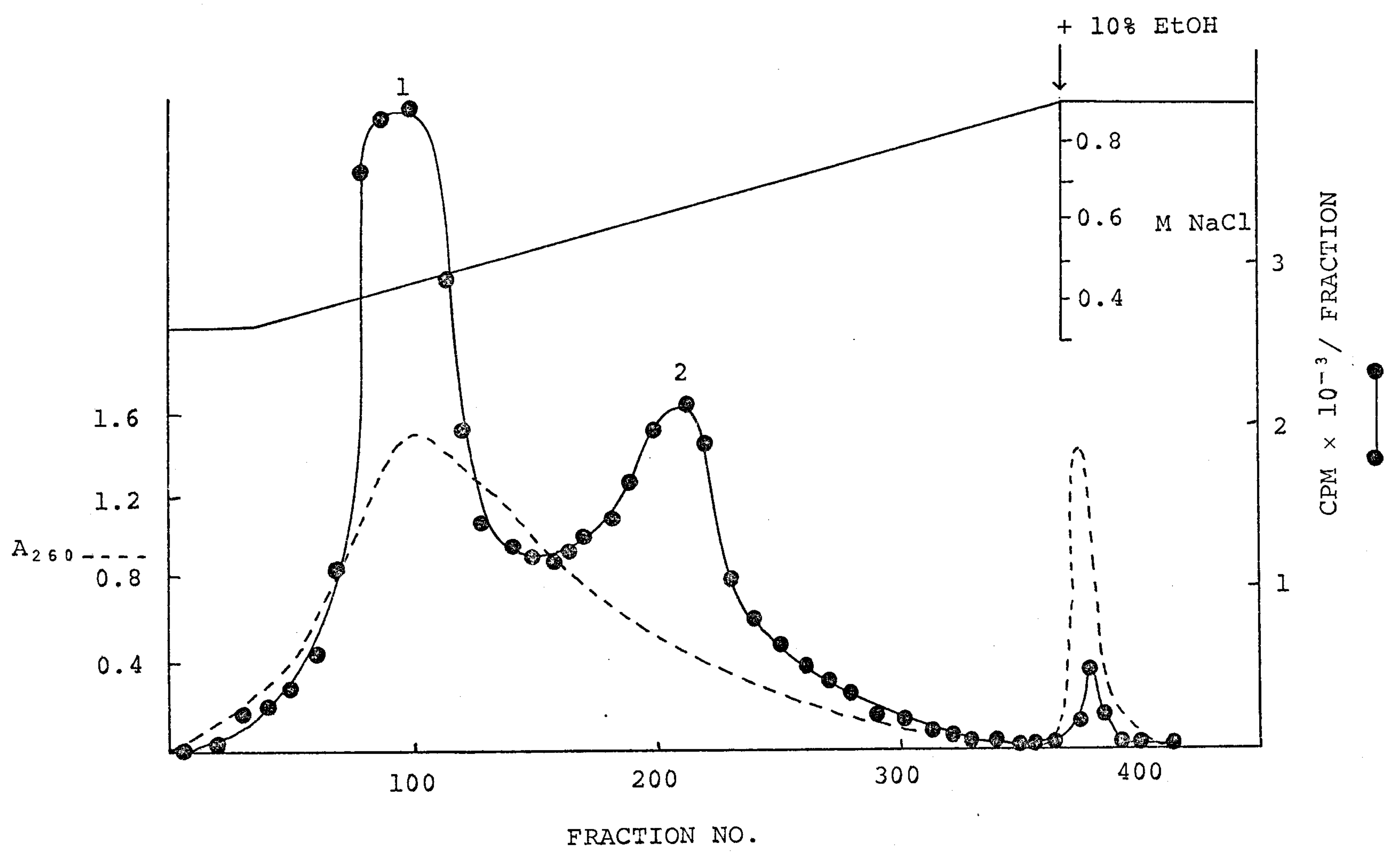

a
20
10
CPM × 10^{-2}
0
5
10
15
b
4
2
CPM × 10^{-2}
0
5
10
15
c
4
2
CPM × 10^{-2}
ORIGIN
5
10
15
+VE
DISTANCE IN CM
-VE

rerun (Fig. 37b). The other part was treated with methanolic HCl, which selectively removes the formyl group from formyl methionine. Fig. 37c shows that this treatment caused most of the material to remain at the origin. If the material was formyl methionyl-puromycin, it should have migrated to the position of methionyl-puromycin between the methionine and puromycin markers (10-11 cm towards the cathode). The peak did not appear to contain formyl methionyl-puromycin, and its nature remains to be determined. This experiment would appear to rule out methionine itself as the initiator, because of the absence of methionyl-puromycin in Fig. 37a. However because the prime aim of the experiment was to detect formyl methionyl-puromycin, no attention was paid to the pH of the solution after lysis of the bacteria. Lysis (see "methods") was achieved by addition of distilled water to the bacterial pellet, and it was likely the resultant lysate was acidic. In this case although formyl methionyl-puromycin would enter the ethyl acetate readily, methionyl-puromycin would not (Leder and Bursztyn, 1966).

Even though the above experiment was far from conclusive, it again indicated that formyl methionine was not the initiator amino acid. The earlier experiments however had shown that 30% of the methionyl-tRNA from H.cutirubrum was formylatable by the E.coli transformylase. For this reason an attempt was made to resolve the species of $tRNA^{met}$. Fig. 38 illustrates the chromatographic

Figure 38 Separation of Methionine Acceptor tRNA's by Chromatography on a BD-Cellulose Column

37 mg of *H.cutirubrum* tRNA in 0.45 M NaCl, 0.01 M $MgCl_2$ 0.05 M sodium acetate, pH 4.5, 0.001 M β-mercaptoethanol were applied to a 1.5 × 26 cm column, equilibrated with the same buffer. Elution was by a gradient of 300 ml of 0.45 M NaCl buffer and 300 ml of 0.9 M NaCl buffer, followed by the ethanol purge. 2 ml fractions were collected at a flow rate of 20 ml per hour. Selected fractions were dialyzed against distilled water, and freeze dried. To the dry tRNA was added the normal *H.cutirubrum* charging system, and the fractions assayed for ^{14}C-methionine (60 mCi/mmole) acceptor activity.



+ 10% EtOH
1
2
0.8
0.6
0.4
M NaCl
3
2
1
CPM × 10^{-3}/ FRACTION
1.6
1.2
A_{260}
0.8
0.4
100
200
300
400
FRACTION NO.

behaviour of *H.cutirubrum* tRNA on BD-cellulose when eluted with a 0.45 - 0.9 M NaCl gradient. The methionine acceptor activity of the fractions was resolved into two peaks, the one eluting with the bulk of the A_{260} units being about twice as large as the second peak. Although this gave a reasonably good separation of two chromatographically distinct species, the separation of charged ^{14}C-met-tRNA was also examined on the column. Fig. 39 shows that when charged, the $tRNA^{met}$ species elute differently. One peak elutes at about 0.66 M NaCl while the other is eluted with the ethanol fraction. The proportion of the ethanol peak varied from 30% - 40% of the total ^{14}C-met-tRNA with different preparations. Fig. 40 shows the results of a similar experiment except that step elution was used instead of the salt gradient. The separation of the two peaks was very clean and again the percentage of the ethanol peak was about 30. It was possible that the ethanol peak represented the same material as the salt peak which non-specifically adhered to the column and was merely concentrated by the ethanol purge. To rule this out peaks from Fig. 40 were isolated and rerun. Fig. 41 shows the salt peak rerun with step elution. Almost all the material eluted with the salt eluant and very little with the ethanol purge. The same experiment with the ethanol fraction from Fig. 40 is shown in Fig. 42. In this case while some material did elute with the salt fraction over 80% was eluted by the ethanol.

Figure 39 Separation of ^{14}C-Methionine-tRNA Species by Chromatography on a BD-Cellulose Column

10 mg of *H.cutirubrum* tRNA were charged with ^{14}C-methionine (60 mCi/mmole) in the standard preparative *H.cutirubrum* charging system. The ^{14}C-met-tRNA was phenol extracted, and freed of ^{14}C-methionine by dissolving in potassium acetate, pH 4.5, and precipitating with 2 volumes of ethanol 3 times. The ^{14}C-met-tRNA was dissolved in 0.45 M NaCl, 0.05 M sodium acetate, pH 4.5, 0.01 M $MgCl_2$ 0.001 M β-mercaptoethanol, and applied to a 1.5 × 26 cm column of BD-cellulose, equilibrated in the same buffer. Elution was by a linear gradient of 200 ml of 0.45 M NaCl buffer and 200 ml of 0.9 M NaCl buffer, followed by the ethanol purge. 2 ml fractions were collected at a flow rate of 20 ml per hour. Radioactivity was assayed by precipitating selected fractions with serum albumin and 5% TCA.

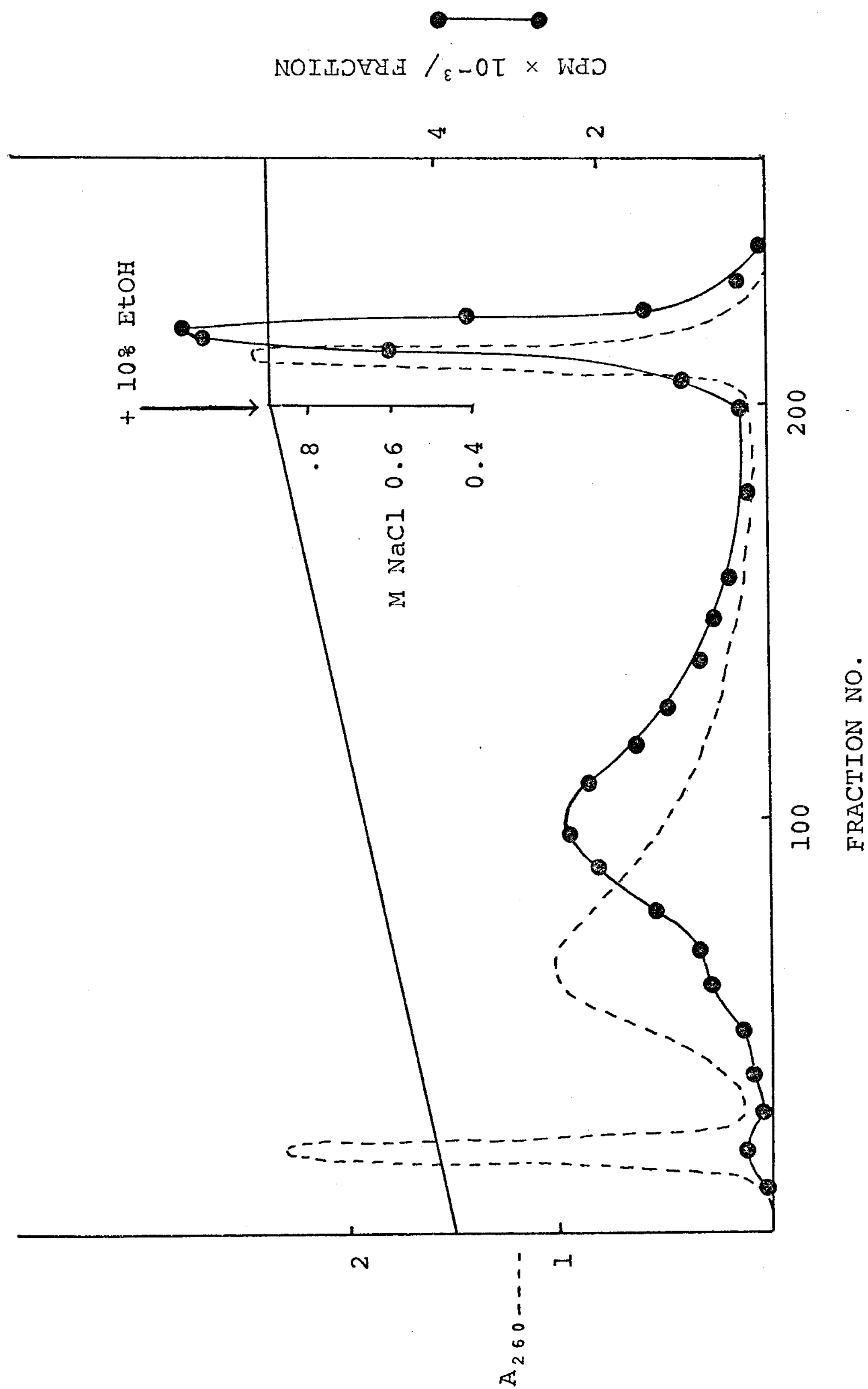
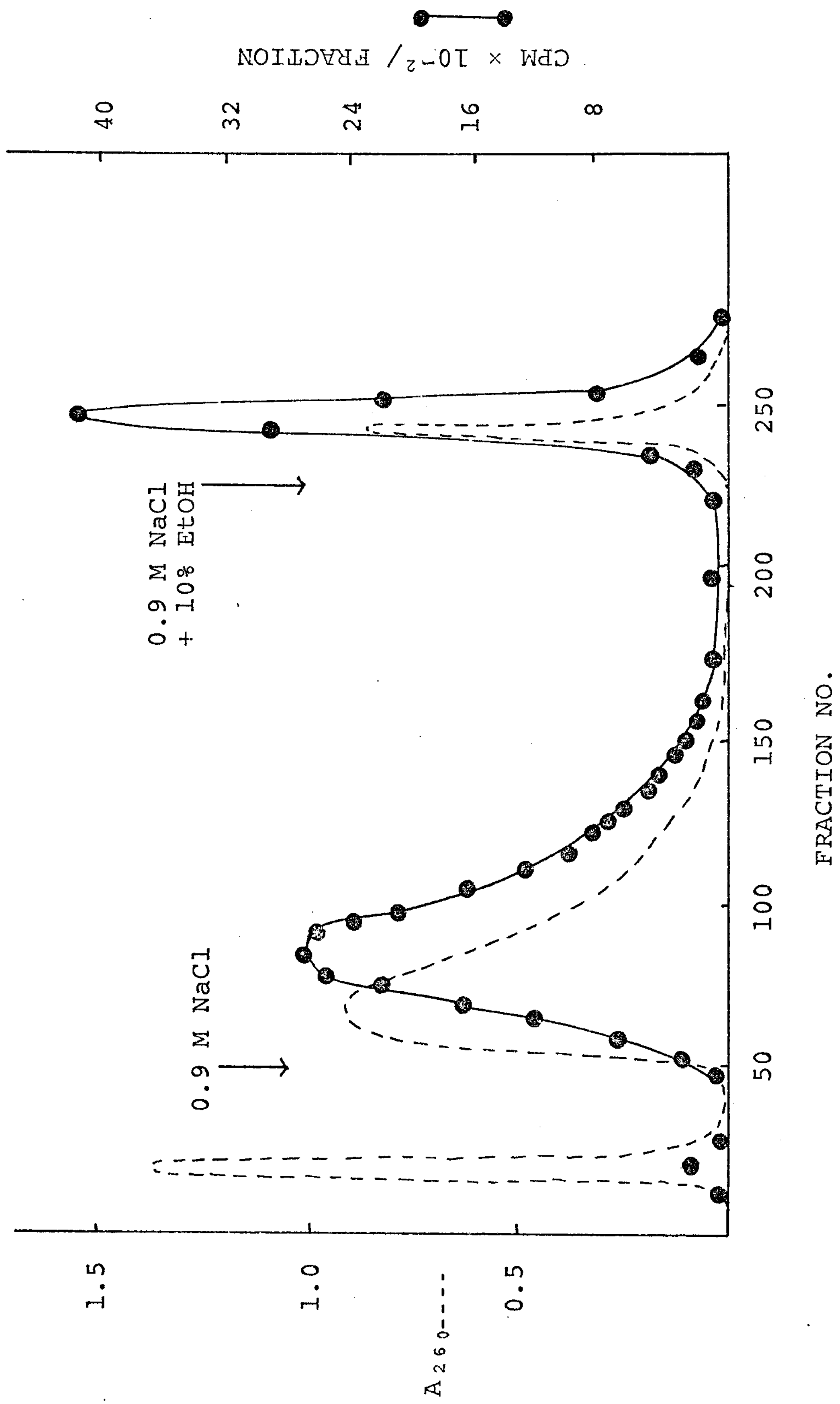

CPM × 10⁻³/ FRACTION
4
2
+ 10% EtOH
.8
M NaCl 0.6
0.4
200
100
FRACTION NO.
2
1
A260 ----

Figure 40 Separation of ^{14}C-Methionine-tRNA Species by Chromatography on a BD-Cellulose Column

6 mg of *H. cutirubrum* tRNA were charged with ^{14}C-methionine (60 mCi/mmole) and purified as for Fig. 39. Elution in this case was stepwise. 2 ml fractions were collected at a flow rate of 20 ml per hour. Radioactivity was assayed by precipitating selected fractions with serum albumin and 5% TCA.



CPM × 10^{-2}/ FRACTION
40
32
24
16
8
0.9 M NaCl
0.9 M NaCl
+ 10% EtOH
FRACTION NO.
50
100
150
200
250
1.5
1.0
0.5
A_{260}-----

Figure 41 Rechromatography of the Salt Eluting ^{14}C-Met-tRNA of Fig. 40 on BD-Cellulose

3.2×10^3 cpm of the salt eluting ^{14}C-met-tRNA peak of Fig. 40 were applied to a 1.5×26 cm BD-cellulose column and eluted stepwise. Radioactivity was assayed by precipitating selected fractions with serum albumin and 5% TCA.

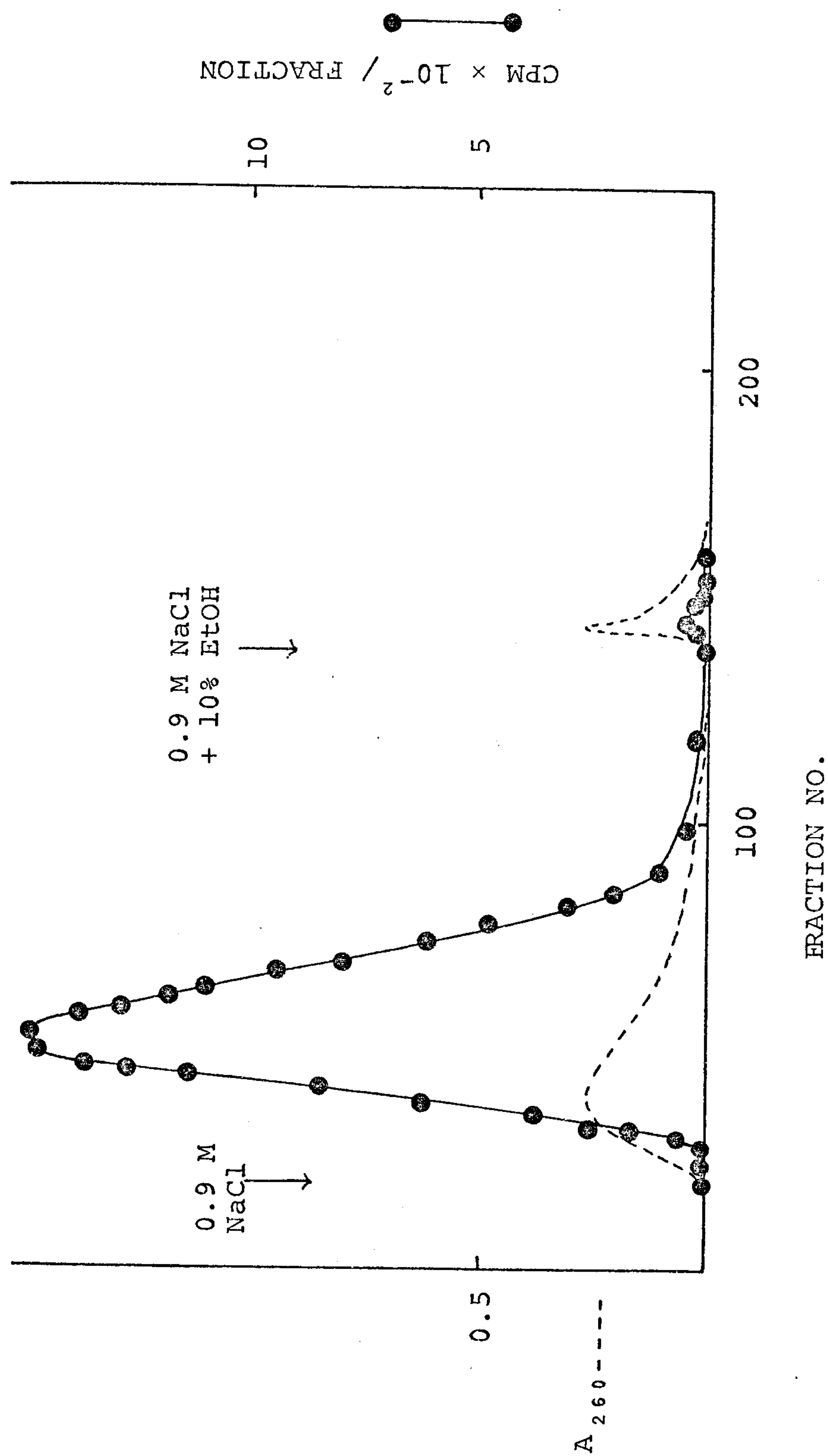
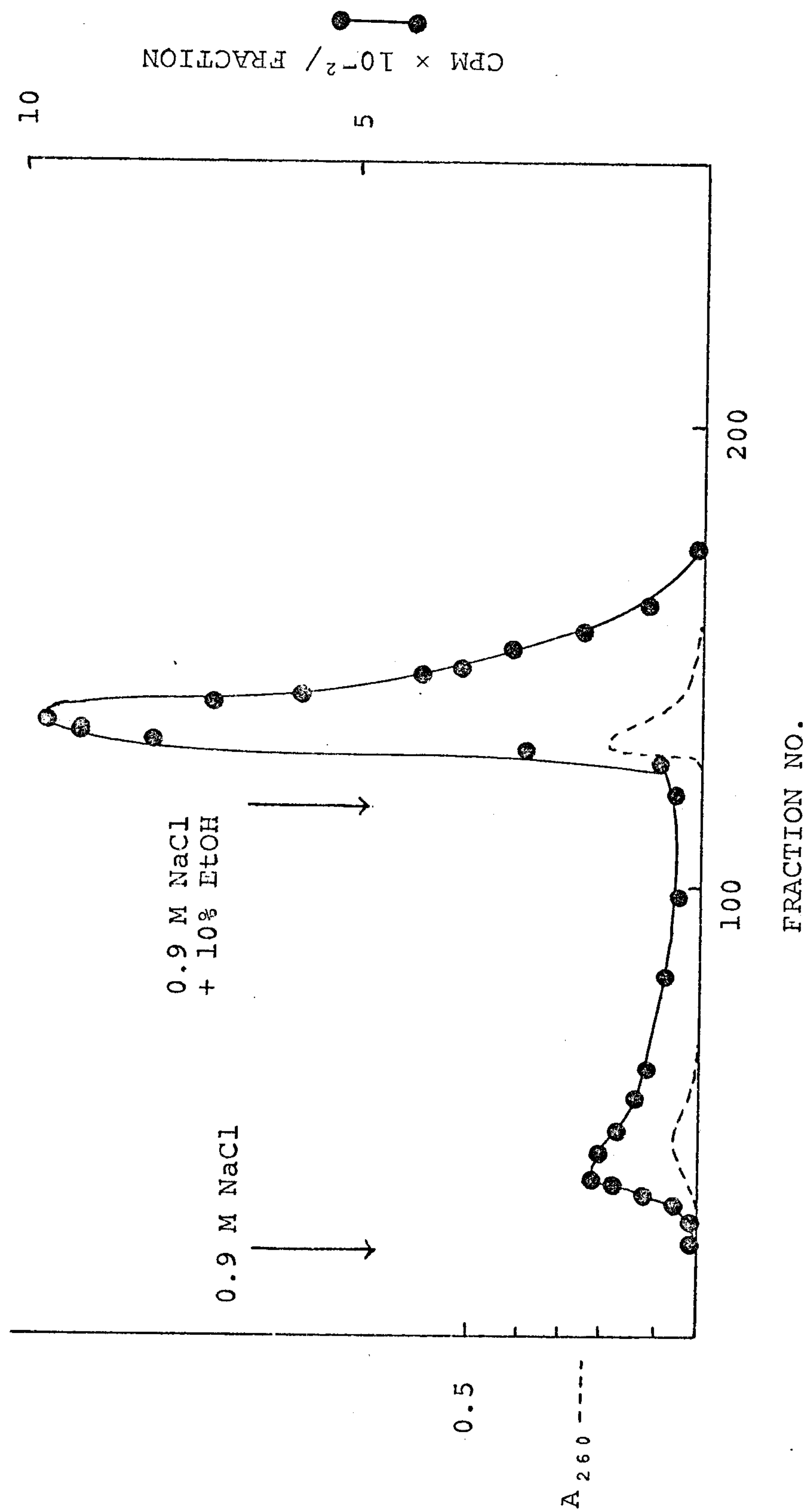

CPM × 10^{-2}/ FRACTION
10
5
0.5
A_{260}
0.9 M NaCl
0.9 M NaCl + 10% EtOH
100
200
FRACTION NO.

Figure 42 Rechromatography of the Ethanol Eluting ^{14}C-Met-tRNA of Fig. 40 on BD-Cellulose

9.6×10^3 cpm of the ethanol eluting peak were applied to a 1.5×26 cm BD-cellulose column and eluted stepwise. Radioactivity was assayed by precipitating selected fractions with serum albumin and 5% TCA.


CPM × 10⁻²/ FRACTION
10
5
0.9 M NaCl
0.9 M NaCl
+ 10% EtOH
200
100
FRACTION NO.
0.5
A260 ----

This indicates that the fractionation of the ^{14}C-met-tRNA in Fig. 40 was a real separation of tRNA^{met} species.

It was of interest to see the relationship of the ^{14}C-met acceptor activity peaks in Fig. 38 and the ^{14}C-met-tRNA peaks of Fig. 40. Peaks from Fig. 38 were pooled, precipitated, freeze-dried and charged with ^{35}S-met. Fig. 43 shows the results with the larger first peak,which when acylated was displaced from the A_{260} peak but was still eluted with the salt fraction. In contrast a large proportion of the smaller second peak, when charged with ^{35}S-met (Fig. 44) eluted with the ethanol peak. These results indicated that the smaller second peak of acceptor activity of Fig. 38 was equivalent to the ethanol eluting ^{14}C-met-tRNA of Fig. 40.

From the earlier results using the E.coli transformylase system it was apparent that approximately 30% of the H.cutirubrum ^{14}C-met-tRNA was formylatable. To determine which of the peaks in Fig. 40 was the formylatable species, tRNA was charged with ^{35}S-met and the species separated by step elution on BD-cellulose. The isolated peaks were then placed in an E.coli transformylase system at 45^{0}C for 20 minutes. Fig. 45a illustrates that the ^{35}S-met-tRNA prior to separation gave 49% formylatable (cf Fig. 34c). In sharp contrast the salt peak of ^{14}C-met-tRNA was almost entirely non-formylatable (Fig. 45b). The ethanol peak gave 80% formylatable in this experiment (Fig. 45c). The amount of this peak that was formylatable

Figure 43 Chromatography of ^{35}S-Met-tRNA from Peak 1 of Fig. 38 on BD-Cellulose

10 mg of tRNA isolated from the peak 1 region of Fig. 38 were charged with ^{35}S-met (128* mCi/mmole) in the normal *H. cutirubrum* charging system. ^{35}S-met-tRNA was phenol extracted, freed of ^{35}S-methionine, and applied to the 1.5 × 26 cm BD-cellulose column. Elution was stepwise and radioactivity assayed by precipitation of fractions with serum albumin and 5% TCA.

* Approximate specific activity as original 12.8 Ci/mmole ^{35}S-met contained 20% sulfone.

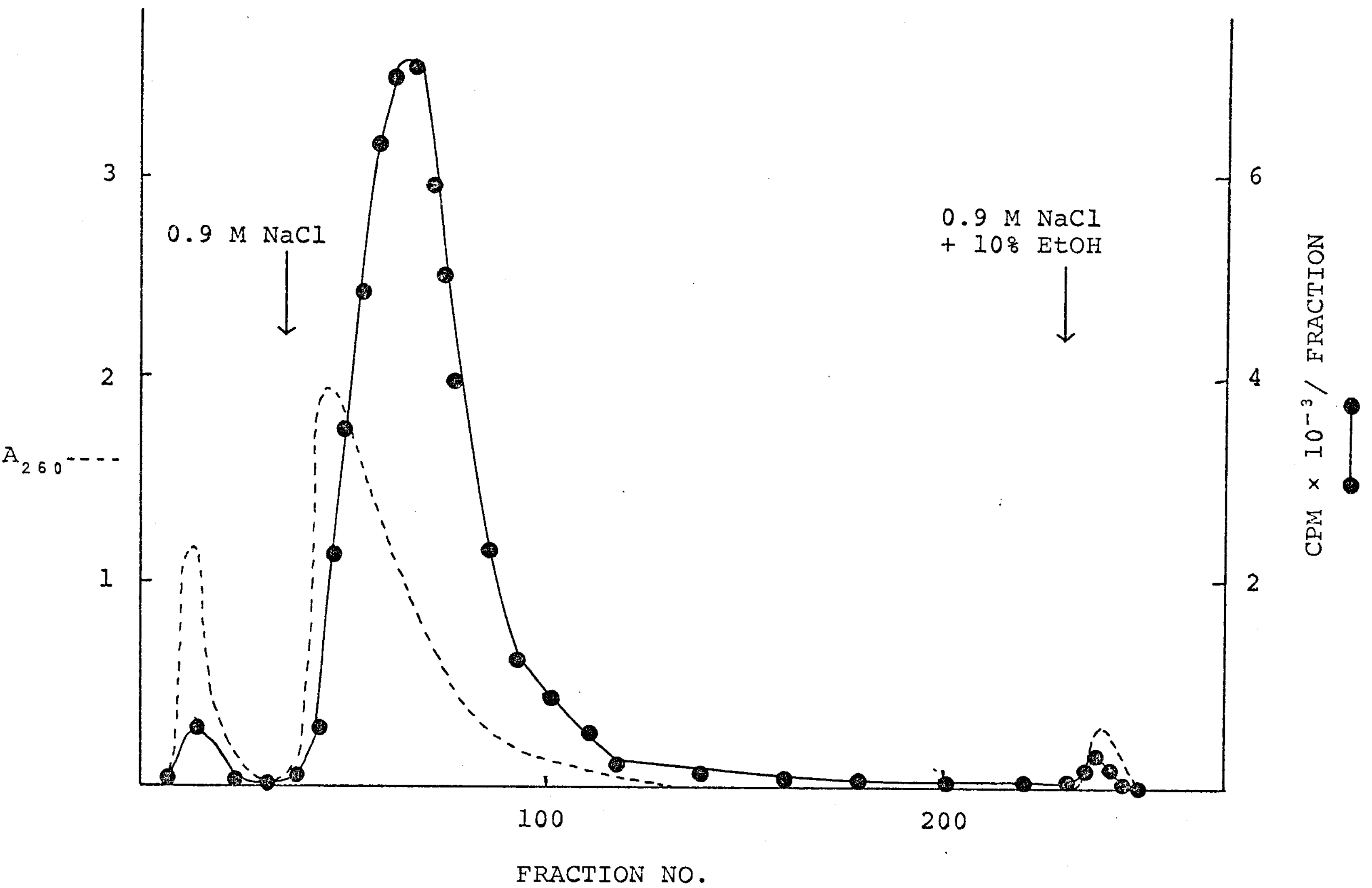

3
2
1
A260
0.9 M NaCl
0.9 M NaCl
+ 10% EtOH
6
4
2
CPM × 10-3/ FRACTION
100
200
FRACTION NO.

Figure 44 Chromatography of ^{35}S-Met-tRNA from Peak 2 of Fig. 38 on BD-Cellulose

3 mg of tRNA isolated from the peak 2 region of Fig. 38 were charged with ^{35}S-met (128* mCi/mmole) in the normal H.cutirubrum charging system. ^{35}S-met-tRNA was phenol extracted, freed of ^{35}S-methionine and applied to a 1.5 × 26 cm BD-cellulose column. Elution was stepwise and the radioactivity assayed by precipitation of fractions with serum albumin and 5% TCA.

* Approximate specific activity as original 12.8 Ci/mmole ^{35}S-met contained 20% sulfone.

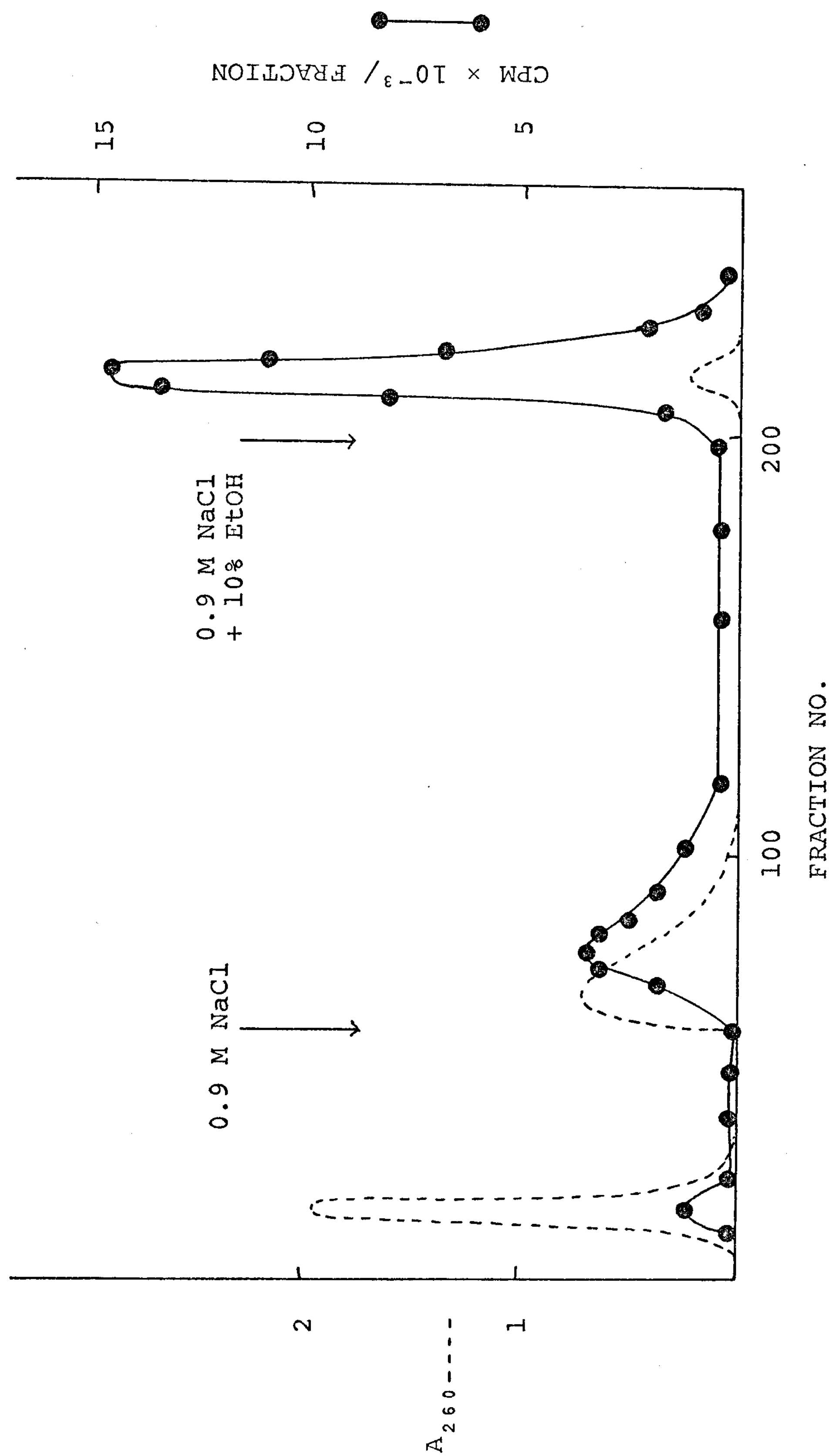
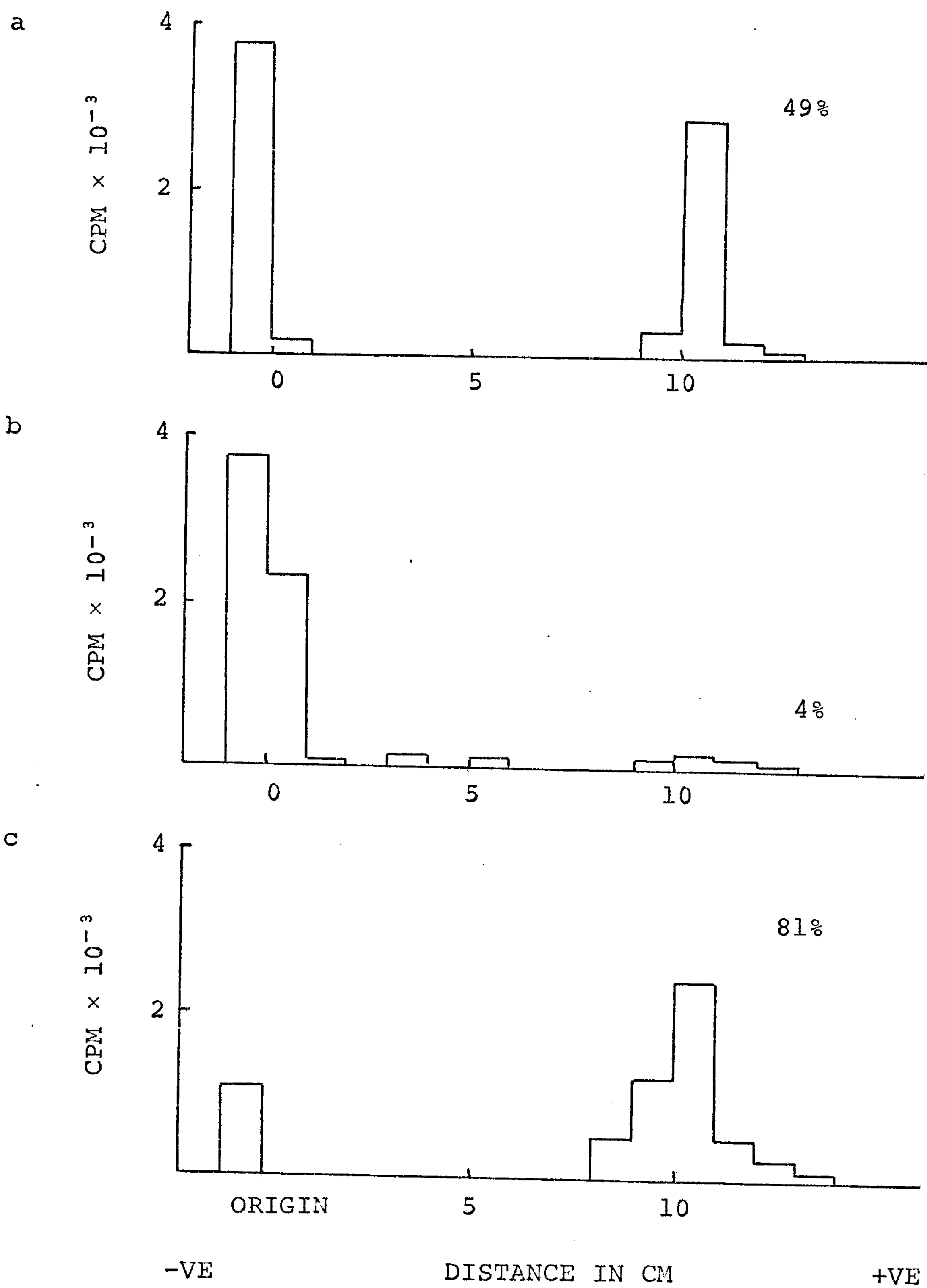

CPM × 10^{-3}/ FRACTION
15
10
5
0.9 M NaCl
+ 10% EtOH
0.9 M NaCl
200
100
FRACTION NO.
2
1
A_{260} -----

Figure 45 Electrophoresis of ^{35}S-Methionine and ^{35}S-Formyl Methionine Discharged from H.cutirubrum tRNA

a. 4.3×10^4 cpm of *H.cutirubrum* ^{35}S-met-tRNA were formylated in the *E.coli* formylating system for 20 minutes at 45°C. 1.1×10^4 cpm discharged from this tRNA after formylation were applied to the Whatman 3 MM paper.

b. 6.2×10^4 cpm of the salt peak (Fig. 40) *H.cutirubrum* ^{35}S-met-tRNA were formylated as in (a). 8.1×10^4 cpm were applied to the Whatman 3 MM paper.

c. 2.8×10^4 cpm of the ethanol peak (Fig. 40) *H.cutirubrum* ^{35}S-met-tRNA were formylated as in (a). 8.6×10^4 cpm were applied to the Whatman 3 MM paper.


a
4
2
CPM × 10⁻³
49%
0
5
10
b
4
2
CPM × 10⁻³
4%
0
5
10
c
4
2
CPM × 10⁻³
81%
ORIGIN
5
10
-VE
DISTANCE IN CM
+VE

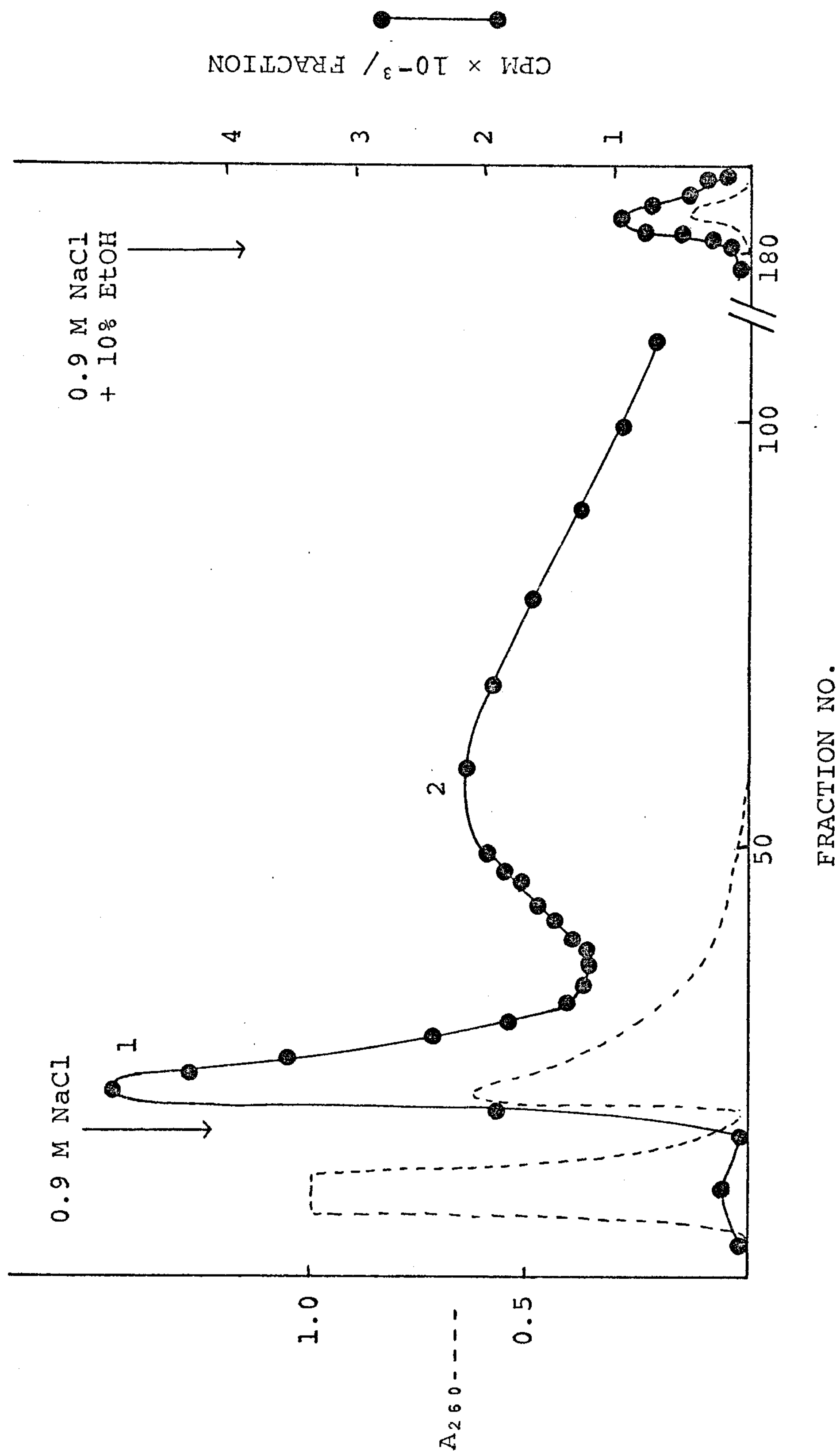
varied between 55-80% depending on the preparation. The reason why this peak was not 100% formylatable, if it indeed was a single species of tRNA, was not clear, especially in view of the fact that the conditions for formylation were in favour of excess formylation. However it seemed clear that the peak eluting with the ethanol was the formylatable species.

In order to overcome the problems of acylating in the H.cutirubrum system and formylating in the E.coli system an attempt was made to purify the $tRNA^{met}$ species after acylation by the E.coli synthetases. From the results in Chap. IV,7 it seemed clear that the E.coli methionine synthetase recognised a large proportion H.cutirubrum $tRNA^{met}$. Fig. 46 shows the results of separating H.cutirubrum ^{35}S-met-tRNA charged by E.coli synthetases on BD-cellulose. The profile was completely different to that of ^{14}C-met-tRNA charged by H.cutirubrum synthetases (Fig. 40). There were several possibilities to explain this. Perhaps the E.coli methionine synthetase did not recognise H.cutirubrum $tRNA^{met}$ species. This seemed unlikely in view of the result in Chap. IV,6. It was possible that acylation by the different synthetases in the different ionic environments induced chromatographically different conformations. To investigate this, H.cutirubrum ^{35}S-methionine-tRNA preparations charged by both E.coli and H.cutirubrum synthetases were heated in 0.45 M NaCl, 0.05 M

Figure 46 Separation of H.cutirubrum ^{35}S-Met-tRNA Species After Acylation with E.coli Synthetases by Chromatography on BD-Cellulose

4 mg of H.cutirubrum tRNA were charged with ^{35}S-methionine (128* mCi/mmole) by E.coli synthetases. The ^{35}S-met-tRNA was phenol extracted, freed of ^{35}S-methionine and applied to a 1.5 × 26 cm BD-cellulose column. Elution was stepwise and radioactivity assayed by precipitation of fractions with serum albumin and 5% TCA.

* Approximate specific activity as original 12.8 Ci/mmole ^{35}S-met contained 20% sulfone.


0.9 M NaCl
0.9 M NaCl
+ 10% EtOH
1
2
$CPM \times 10^{-3}$/ FRACTION
4
3
2
1
A_{260}- - - -
1.0
0.5
50
100
180
FRACTION NO.

sodium acetate, pH 4.5, 0.01 M $MgCl_2$, 0.001 M β-mercaptoethanol at 60°C for 1 hour. After this treatment the chromatographic behaviour of both preparations was altered somewhat but they were still far from similar. The meaning of the different chromatographic behaviours of H.cutirubrum ^{35}S-met-tRNA charged with E.coli and H.cutirubrum synthetases was not further investigated. However to determine which of the peaks was formylatable, the peaks from Fig. 46 were isolated and formylated in the E.coli formylating system at 45°C for 20 minutes. Fig. 47a shows the results of formylating the ^{35}S-met-tRNA before separation on the BD-cellulose column. As with the ^{14}C-met-tRNA charged in the H.cutirubrum system the amount of formylatable ^{35}S-met-tRNA under these conditions was between 40-50%. The absolute amount of the two peaks from the BD-cellulose that was formylatable varied but as is shown in Fig. 47 b+c, peak 1 was always only half as formylatable as peak 2. It seemed apparent that this 0.9 M NaCl elution which resolved these two peaks was not achieving a clean separation. The whole nature of this E.coli synthetase H.cutirubrum tRNA heterologous system was not resolved.

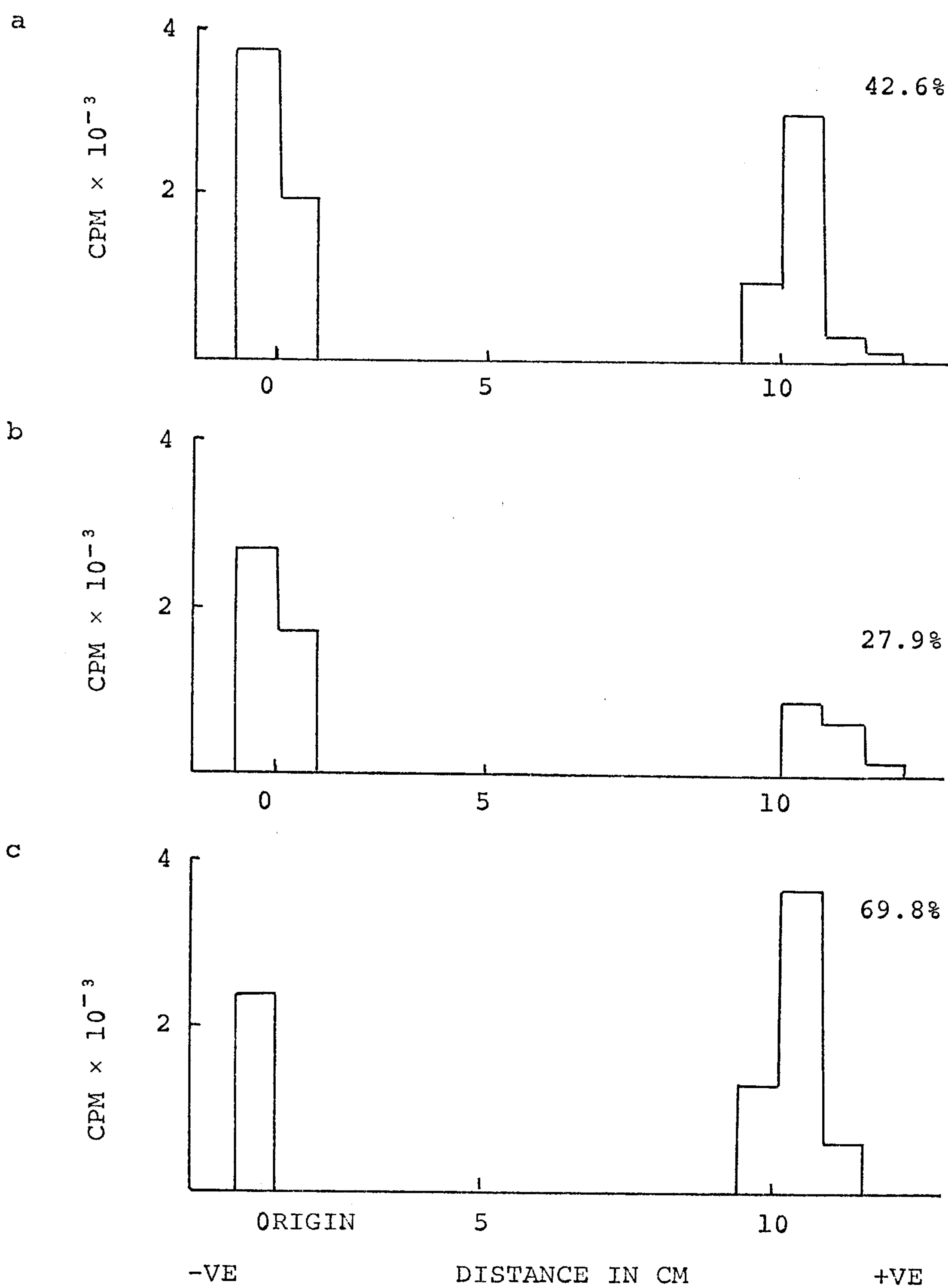
The above results strongly indicate the absence of a transformylase system in H.cutirubrum and therefore it is likely that F-met-$tRNA^{met}_{F}$ is not the initiator aminoacyl-tRNA in this bacterium. Furthermore in view of the results with yeast and mammalian systems and the resolution of the two species of $tRNA^{met}$ in H.cutirubrum it is possible that

Figure 47 Electrophoresis of ^{35}S-Methionine and ^{35}S-Formyl Methionine Discharged from H.cutirubrum tRNA Charged with E.coli Synthetases

a. 6.2×10^4 cpm of H.cutirubrum ^{35}S-met-tRNA charged with E.coli synthetases were formylated in the E.coli formylating system for 20 minutes at 45^0C. 1.3×10^4 cpm discharged from this tRNA were applied to the Whatman 3 MM paper.

b. 4.2×10^4 cpm of H.cutirubrum ^{35}S-met-tRNA from peak 1 of Fig. 46 were formylated as in (a). 8.2×10^3 cpm were applied to the Whatman 3 MM paper.

c. 8.6×10^4 cpm of H.cutirubrum ^{35}S-met-tRNA from peak 2 of Fig. 46 were formylated as in (a). 1.0×10^4 cpm were applied to the Whatman 3 MM paper.


a
4
2
CPM × 10^{-3}
42.6%
0
5
10
b
4
2
CPM × 10^{-3}
27.9%
0
5
10
c
4
2
CPM × 10^{-3}
69.8%
ORIGIN
5
10
-VE
DISTANCE IN CM
+VE

an unformylated met-$tRNA^{met}$ is the initiator. The met-tRNA eluting with the ethanol fraction which is formylated by the *E. coli* transformylase may be the initiator but this has yet to be demonstrated.

V. DISCUSSION

It was the intent of this thesis to examine three main areas of halophilic protein synthesis. These were the effect a salt environment has on codon-anticodon recognition, the modifications made to halophilic tRNA, and the mechanism of initiation in this bacterium. The results just described do not provide unequivocal answers to these questions, but do give strong indications concerning the mechanisms involved.

1. <u>The Effect a High Salt Environment has on Codon-Anticodon Recognition</u>

As was indicated in the Introduction, this was investigated because of the acidic nature of halophilic proteins and the possibility that the high salt environment induced misreading of codons to insert acidic amino acids instead of basic ones. It has been known for some time that the fidelity of translation in a cell-free system can be influenced by external factors such as aminoglycoside antibiotics and high ion concentrations as well as suppressors, which alter components of the protein synthesizing machinery (Davies, 1966; Szer and Ochoa, 1964; Capecchi and Gussin, 1965). Nishimura et al., (1969) reported that at 20 mM Mg^{2+} tyrosine was specifically inserted instead of cysteine

with an alternating poly UG template in an *E. coli* protein synthesizing system *in vitro*. Similar specific replacements of amino acids were found by Davies et al., (1966) using streptomycin and neomycin with several ribonucleotide templates containing 2 nucleotides in alternating sequence. What effect does 3.8 M KCl, 1 M NaCl, 0.4 M NH_4Cl and 0.04 M magnesium acetate have on these interactions?

Bayley and Griffiths (1968b) showed that codon responses of several amino acids to random copolyribonucleotides in the *H.cutirubrum* protein synthesizing system *in vitro* were in agreement with the established code. The results presented in Chap. IV,2 extended the number of codons reasonably tested for base composition to 27 (Table XXI). The stimulations of amino acid incorporation agreed well with the initial work on codon assignments in the *E.coli* system (Wahba et al., 1963; Speyer et al., 1963; Nirenberg et al., 1963) except that total incorporation was only 1-10% for the same amount of ribosomes per incubation mixture. The only odd result was the stimulation of threonine incorporation by the codon ICC. Wahba et al., (1963) using a random copolyribonucleotide of CI (5:1) found that inosinic acid behaved exactly as guanylic acid as far as coding properties were concerned. As proline and arginine incorporation were stimulated by poly CI in the halophilic system as expected, it is possible that this odd result with threonine represents a modified base in the 3' position of the anticodon of a

threonine tRNA, which recognises adenylic acid and inosinic acid in the high salt system. This thr-tRNA may be a much more effective competitor for ICC than the ala-tRNA, which normally recognises the codon GCC. This could be a possible explanation for this apparent modified recognition. The normal codon GCC does stimulate incorporation of alanine as expected (Table XVIII).

Of the 27 codons tested by the random copolyribonucleotides only 2 were for the basic amino acids and none were for the acidic amino acids or the amides. The major reason for this was that most of the interesting codons were contained in polymers with significant amounts of adenylic acid which precipitated in the high salt system. The H.cutirubrum ribosomal binding system, which was developed to overcome this problem, allowed the triplet AAA to be assigned to lysine. However the system did not contribute much evidence toward codon assignments because the relevant triplet templates were not available. This ribosomal binding system, like the one developed by Leder and Nirenberg (1964), could establish most of the codon assignments when triplets become available. Twenty eight codons had thus been tested in high salt environments and all the assignments agreed with the established code shown in Table I. This indicated that the genetic code used by halophilic bacteria was probably essentially the same as that used by the non-halophiles. If organisms such as halophiles use the same genetic code as E.coli, yeast and guinea pig (Marshall et al., 1976) it is

obviously very stable to evolutionary change. Although the codons for amino acids were the same, this did not prove that the codon-anticodon recognition was the same. Halophilic tRNA could have been modified to compensate for a high salt effect and therefore still respond to the same codons.

2. Halophilic tRNA

Griffiths and Bayley (1969) demonstrated that the halophilic tRNA could be placed into low salt and transferred back into high salt without loss of acceptor activity. This indicated that if conformational changes of the tRNA occur in the transition between high and low salt environments they are reversible. Smith (1969) concluded that, although NaCl in an *E.coli* charging system inhibited aminoacyl-tRNA synthetases, it also reduced the final level of aminoacylation by inducing changes in the *E.coli* tRNA. In the high salt environment the enzymes which interact with the tRNA could either have been modified to recognise a different structure or possibly the tRNA could have been modified to compensate for high salt induced conformational changes or a combination of such modifications could have occurred. The first indication that halophilic tRNA had not been modified was the fact that *E.coli* aminoacyl-tRNA would bind to *H.cutirubrum* ribosomes in response to the specific codon in high salt. This implied that at least for this artificial binding the halophilic ribosome accepted *E.coli* aminoacyl-tRNA and that codon-anticodon recognition was the same in high and

low salt. This kind of test was fairly crude because of the high Mg^{2+} concentration required for the binding system.

The results in Chap. IV,3 provide evidence for the non-modification of halophilic tRNA. The fact that ^{14}C-phe could be transferred from E.coli aminoacyl-tRNA to polypeptide, at low Mg^{2+} in the halophilic system, implied that the T factor could recognise and complex with non-halophilic aminoacyl-tRNA. The conformation of E.coli aminoacyl-tRNA in high salt was therefore sufficiently similar to that of H.cutirubrum to replace it in the aminoacyl-tRNA transfer system. The results in sections 3 and 4 of Chap. IV show that the reverse was also true. Aminoacyl-tRNA from H.cutirubrum could bind to E.coli ribosomes and transfer their amino acids to polypeptide in a completely foreign ionic environment. In view of the fact that many other systems can use heterologous aminoacyl-tRNA (Marshall et al., 1967), it is reasonable to ask whether such results are surprising. While the macromolecules of most other organisms function in similar low ionic environments very few, if any, halophilic enzymes can function normally in the absence of relatively high concentrations of monovalent cations. The response to a high salt environment was therefore the evolution of specialised protein molecules. It could be argued that because there are 20 amino acids to vary in the synthesis of proteins while there are only 4 bases to vary in nucleic acids, proteins are obviously the only class of macromolecule which will be modified. While this argument may be true for DNA, mRNA and

rRNA it may not be so applicable to tRNA. It is now well known that tRNA contains besides the four major bases of RNA numerous modified ones. This means that it can have many more than just 1 of 4 bases in any position and therefore allows the formation of a far more complex structure than the other classes of RNA. The recent evidence on the tertiary structure of tRNA suggests it has a conformation resembling a small protein more than DNA. It is quite conceivable that modifications of bases in the primary structure of the tRNA during the evolution of halophiles could have produced a uniquely halophilic tRNA. The evidence presented is quite contrary to this.

Further evidence in support of an evolutionary stable tRNA molecule during the development of halophiles comes from the study of heterologous tRNA-synthetase interactions presented in Chap. IV,6. How a synthetase recognises the correct species of tRNA from the population present in a cell is not clear. For this reason the nature of heterologous synthetase-tRNA interactions is even less clear. However the synthetase-tRNA recognition is very precise and heterologous interactions indicate that a certain structure of the tRNA and the corresponding site on the synthetase have been conserved. A number of investigators have studied the formation of aminoacyl-tRNA's in heterologous systems using aminoacyl-tRNA synthetases from one source and tRNA from a different source (Novelli, 1967). Doctor and Mudd

(1963) studied the cross-reaction between rat liver, yeast and *E.coli* synthetases and tRNA's for 12 amino acids and concluded that the extent of incorporation of an amino acid into tRNA is dependent on the source of the enzyme, the tRNA and the particular amino acid used. They observed some striking species differences but also noted a number of homologies. Lagerkvist and Waldenström (1964) used purified valyl-, phenylalanyl-, and leucyl-tRNA synthetases from yeast and the corresponding enzymes from *E.coli* to study the esterification of amino acids with homologous and heterologous tRNA's. All of the yeast enzymes could esterify tRNA from *E.coli* while only valyl synthetase from *E.coli* could esterify tRNA from yeast.

The heterologous interactions between the corresponding *E.coli* and *H.cutirubrum* tRNA and synthetases are shown in Table L. *E.coli* synthetases charged *H.cutirubrum* tRNA with about 10 amino acids to different degrees while the *H.cutirubrum* synthetases charged *E.coli* tRNA with only 5. The only point to be made from this is that the synthetase-tRNA recognition is far more precise than that between the aminoacyl-tRNA and the T factor. Both *E.coli* and *H.cutirubrum* synthetases recognise some of the heterologous tRNA's which are in a completely foreign ionic environment. This indicates that there has not been a drastic change to halophilic tRNA's to make them completely unrecognisable by heterologous synthetases. Table LI further shows that for at least 2 of

these heterologous interactions the correct amino acid was put on the correct tRNA. The results presented in Chap. VI,8 indicate that the H.cutirubrum $tRNA^{met}$ - E.coli synthetase interaction may not be as simple as shown in Table LI and this will be discussed later.

There seems little doubt that during the evolution of halophiles the genetic code and the basic structure of the tRNA molecule were conserved. This places the full responsibility for salt dependence of the protein synthesizing system on the protein moiety. This agrees with the results of Malcolm (1969) who concluded that the temperature-sensitive physiology of a psychrophile was caused by the rapid inactivation of glutamyl- and prolyl-tRNA synthetases when exposed to temperatures above 25°C. He states that any change that occurs in the stereochemical arrangement of the tRNA at elevated temperatures does not preclude their recognition by enzymes derived from other sources, which normally function at these temperatures. Nomura et al., (1968) came to similar conclusions concerning thermophile rRNA. Although the rRNA of B.stearothermophilus had a higher GC content and a higher Tm than that of E.coli, it appeared that a major factor in the heat stability of the thermophile ribosomes was the special property of the ribosomal proteins. Szer (1970) removed a protein factor, P, from the ribosomes of a psychrophilic pseudomonad which seemed essential for their activity at 0°C. Addition of P to E.coli ribosomes enabled them to translate poly U at 0°C. Proteins therefore

seem to be the class of macromolecule most modified in the adaptation of an organism to a specialised environment.

3. The Protein Synthesizing System

The protein synthesizing system of *H. cutirubrum* functions optimally in 3.8 M KCl, 1 M NaCl, 0.4 M NH_4Cl, 0.02-04 M magnesium acetate (Bayley and Griffiths, 1968a). The tRNA charging system however, at least for leucine, is not greatly influenced by added monovalent cations above the 3.8 M KCl (Griffiths and Bayley, 1969). At low amino acid concentrations however, NH_4^+ does act as a competitive inhibitor for the amino acid site on the synthetase. During the course of examining codon-anticodon recognitions, the whole system was further analysed for its ionic requirements at each step.

From the results in Chap. IV,6 it was clear that all the synthetases did not have the same ionic requirements as the leucyl and isoleucyl enzymes. The role the high salt environment may play in enzyme activity was discussed in the Introduction. For the leucyl-tRNA synthetase there is a requirement of 3.8 M KCl to neutralise the negative charges on the enzyme and probably more importantly to lower the water activity in the system. There seems to exist a variable tolerance to water activity between the synthetases. While leucyl and isoleucyl-tRNA synthetases function optimally at 3.8 M KCl, prolyl, threonyl and other aminoacyl-tRNA synthetases require added monovalent cations in the form of NaCl to reduce this activity still further. The methionyl-tRNA synthetase

appears inhibited by the addition of NaCl and the reason for this is not clear. One experiment with methionyl-tRNA synthetase however further indicated that reduction of water activity was a primary function of the monovalent cations for enzymic activity. In 3.8 M KCl at 45^{0}C the extent of acylation of met-tRNA was only 31% of that at 37^{0}C. However by increasing the KCl concentration to 4.9 M (approx.), the acylation at 45^{0}C was 90% of that at 37^{0}C in 3.8 M KCl. The synthetases therefore seem to have different requirements for the absolute amount of monovalent cations in the system, but they all prefer K^+ over Na^+ or NH_4^+ as the major ion (Griffiths and Bayley, 1969). There is an indication that NH_4^+ would be better as a substitute for K^+ than Na^+ in the tRNA charging system (Griffiths and Bayley, 1969) if it was not a competitive inhibitor for the amino acid site on the synthetase.

The special cases of glutaminyl and asparaginyl-tRNA synthetases were also investigated (Chap. IV,6). Glutamyl-tRNA synthetase activity was the only one which was greatly enhanced by addition of NH_4^+ to the reaction mixture and this may be related to the apparent lack of a glutaminyl-tRNA synthetase in this bacterium. Wilcox (1969) reported the absence of a glutaminyl-tRNA synthetase in five Gram positive bacteria, while it was present in E.coli and two other Gram negative bacteria, as well as yeast, rat, pig, chicken and rabbit livers. In the Gram positive bacteria gln-tRNA^{gln} is made via glu-tRNA^{gln} using the glutamic acid aminoacyl-tRNA

synthetase and an amidotransferase. The results in Fig. 24 strongly indicate that this system is present in H. cutirubrum. H. cutirubrum tRNA when charged with ^{14}C-glu by S-150 contained 30% ^{14}C-gln. Wilcox (1969) concluded only Gram positive bacteria had this system. As H. cutirubrum is Gram negative, this generalisation is either incorrect or the Gram stain is not meaningful in relation to extremely halophilic bacteria. The acceptance of asparagine by H. cutirubrum tRNA was also investigated but this proved not to be via asp-$tRNA^{asn}$ and the mechanism by which asparagine is incorporated into H. cutirubrum protein has yet to be resolved.

The results presented in Chap. IV,2 further indicated the ionic requirements of the specific steps of protein synthesis. The binding of ^{14}C-aminoacyl-tRNA to H. cutirubrum ribosomes required only 3.8 M KCl and 0.088 M magnesium acetate. Addition of extra monovalent cations at this Mg^{2+} concentration did not increase the binding, indicating this non-enzymic process could tolerate a higher water activity than the whole system. Addition of NH_4^+ did reduce the Mg^{2+} optimum to 0.06 M however, which may mean that Mg^{2+} and NH_4^+ can perform similar roles in the ribosome. This is in agreement with the results of Rauser and Bayley, (1968) who found that release of peptidyl-tRNA from the ribosome requires a suitably low level of both Mg^{2+} and NH_4^+.

The roles of the different cations in the whole protein

synthesizing system were further clarified by the aminoacyl-tRNA transfer system (Chap. IV,3). Bayley and Griffiths (1968a) found that 1 M NaCl with 0.4 M NH_4Cl gave better activity in the whole system than either 1.4 M NaCl or 1.4 M NH_4Cl. The reason for this now seems clear. Griffiths and Bayley (1969) found NH_4^+ was a competitive inhibitor with the amino acid for the synthetase. Therefore a high amount of NH_4^+ in the whole system would reduce the amount of aminoacyl-tRNA available for protein synthesis. In the aminoacyl-tRNA transfer system, no charging was required and it is apparent from Table XXVIII that NH_4^+ was by far the preferred extra monovalent cation. NH_4^+ is probably playing a dual role here by lowering water activity and performing specific functions during peptide bond formation (Conway, 1964). The reaction mixture, 3.8 M KCl, 1.0 M NaCl, and 0.4 M NH_4Cl, (Bayley and Griffiths, 1968a) is obviously the best compromise between the requirements of the aminoacyl-tRNA transfer system and the tRNA charging system.

An attempt was made to dissociate and reconstitute this protein synthesizing system (Chap. IV,6). Manipulation of the ribosomes by washing resulted in a drastic loss of activity in the protein synthesizing system and a minor decrease in their capacity to bind aminoacyl-tRNA. The activity of the ribosomes may be a factor in the low level of incorporation in this system, but the results of the aminoacyl-tRNA transfer system would argue against this. More than 50% of the amino acid on the *E.coli* aminoacyl-tRNA

was transferred to polypeptide. The problem did not appear to be particularly located in the charging system. A certain reduction of incorporation would naturally be expected in view of the compromise salt conditions of the system, which are slightly inhibitory to both tRNA charging and aminoacyl-tRNA transfer steps.

The isolation of an inhibitory nucleic acid fraction (Table LII) in the phenol purified tRNA may indicate another cause for the low activity. Such an inhibitor was found by Bichowsky-Slomnicki (1969) in crude yeast tRNA preparations. The nature of the inhibitor was not determined, but it varied in proportion depending on the growth conditions. The inhibitor in *H.cutirubrum* may be present in the S-150 or it may be formed during the phenol extraction of the tRNA. It however was not the cause for the relatively low activity of the reconstituted system shown in Table LV. The finding of this inhibitor indicates that care must be taken to remove this material from tRNA which is to be used in the protein synthesizing system.

The low activity may be due to insufficient amounts of the transferase factors. Increasing amounts of the 65-80% precipitated protein from the S-150 added to the protein synthesizing system did increase incorporation by over 100%. More careful experiments using purified tRNA and fraction 2 protein from the Sephadex G-75 separation of S-150 components shown in Fig. 33 could elucidate this. Further purification

of the transfer factors may prove very difficult as they are irreversibly denatured at lower salt concentrations. The lower activity of the H. cutirubrum protein synthesizing system compared to that of E. coli may be an inherent feature or it could possibly be markedly improved by further purification of components and reconstitution.

4. Initiation of Protein Synthesis

A study of the initiation mechanism in this bacterium was made to determine if F-met-$tRNA^{met}_{F}$ was as a universal initiator of 70S ribosomes as was indicated in the literature. This unique aminoacyl-tRNA has been found in several species of bacteria as well as mitochondria and chloroplasts (Schwartz, et al., 1967; Horikoshi and Doi, 1967; Seligman and Finch, 1970; Bachmayer and Kreil, 1968; Burkard et al., 1969; Smith and Marcker, 1969). It has been found to be conspicuously absent from the cytoplasm of eukaryotic cells (Marcker and Sanger, 1964). Recent evidence suggests that initiation in higher cells is by met-$tRNA^{met}_{F*}$ which is formylatable by the E. coli transformylase (Smith and Marcker, 1970; Migita and Doi, 1970; Rajbhandary and Kumar, 1970; Leiss and Keller, 1970; Jackson and Hunter, 1970; Wigle and Dixon, 1970).

Bayley and Griffiths (1968b) found that ^{14}C-methionine incorporation was stimulated by the presence of a random poly GU in the H. cutirubrum whole protein synthesizing system. This was an indication the F-met-$tRNA^{met}_{F}$, which responds to

GUG in other bacterial systems, was the initiator aminoacyl-tRNA. Attempts to demonstrate the presence of this F-met-tRNA$^{met}_{F}$ in H.cutirubrum using the tRNA charging system supplemented with N^{10}, formyl tetrahydrofolate proved unsuccessful (Fig. 34). This did not appear to be due to a deformylase or compounds which might cause deformylation, present in the H.cutirubrum S-150 (Fig. 36 a+b). It further did not appear to be because of inactivation of the trans-formylase in the S-150 as F-met-tRNA could not be demonstrated in vivo (Fig. 36c) by an experiment based on that described by Smith and Marcker (1969).

Using an E.coli transformylase system it was demonstrated that a portion of H.cutirubrum met-tRNA could be formylated (Fig. 34c). It was shown in Chap. IV,6 that E.coli synthetases could charge H.cutirubrum tRNA^{met}. In the presence of a formyl donor and a transformylase, approximately 30% of the met-tRNA charged with the heterologous synthetase was formylatable (Fig. 35a). Therefore it appeared that there was a formylatable met-tRNA$^{met}_{F*}$ in H.cutirubrum but no transformylase system. Resolution of methionine acceptor tRNA species and met-tRNA species on BD-cellulose columns yielded two peaks. The smaller second acceptor activity peak from BD-cellulose (Fig. 38) appeared to be the same tRNA species as the ethanol eluting met-tRNA (Figs. 39 and 40) which was shown to be the formylatable species (Fig. 45). It seems clear that the ethanol eluting met-tRNA is comparable to the met-tRNA$^{met}_{F*}$ found in the

cytoplasm of higher cells but whether this is the initiator aminoacyl-tRNA has yet to be demonstrated.

Fig. 46 shows the elution profile of H.cutirubrum met-tRNA, charged by E.coli synthetases, on a BD-cellulose column. This is completely different to the profile of met-tRNA charged by H.cutirubrum synthetases (Fig. 39). There are several possible explanations for this difference. One is that the different synthetases induce a conformational change in the tRNA during charging in the different ionic environments. If this is so it would tend to support the argument that charged tRNA assumes a different conformation to uncharged tRNA (Danchin and Grunberg-Monago, 1970). Other possibilities include the modification of the primary structure of the tRNA by modifying enzymes in the E.coli S-150. It is also possible that contrary to the results in Chap. IV,6 other tRNA's besides $tRNA^{met}$ are charged with methionine by the E.coli synthetases.

VI. CONCLUSION

Codon-anticodon recognition is unaffected by the saturated cation concentration present in the *H.cutirubrum* protein synthesizing system. *E.coli* and *H.cutirubrum* aminoacyl-tRNA's not only recognise the same codons in both high and low salt but are also fully recognised by the heterologous transferase enzymes and ribosomes. The tRNA molecule does not seem to have been especially modified during the evolution of the halophile. Several *E.coli* synthetases recognise *H.cutirubrum* tRNA's and several *H.cutirubrum* synthetases recognise *E.coli* tRNA's. The adaptation of the macromolecules of the protein synthesizing system to the high salt environment seems to be entirely located in the protein moiety. The salt requirements of the proteins of the whole protein synthesizing system are a compromise between the requirements of the tRNA charging and aminoacyl-tRNA transfer steps.

There is no glutaminyl-tRNA synthetase in *H.cutirubrum* extracts. Glutaminyl-$tRNA^{gln}$ is made via glutamyl-$tRNA^{gln}$ *in vitro* by means of an amidotransferase system. There is no indication of the presence of F-met-$tRNA^{met}_{F}$ either *in vivo* or *in vitro*. There is a met-$tRNA^{met}$, which comprises 30% of the met-$tRNA^{met}$ in phenol extracted tRNA, that is formylatable

by the E.coli transformylase system. Methionine incorporation is stimulated by random poly GU in the protein synthesizing system. These facts strongly indicate that a met-tRNA may be the initiator aminoacyl-tRNA in H.cutirubrum and that F-met-$tRNA^{met}_{F}$ may not be a universal initiator of 70S ribosomes.

Several aspects of this study were not completed mainly because of the lack of suitable template RNA's. All the codon assignments in high salt especially those for the basic and acidic amino acids could be made by use of the H.cutirubrum ribosomal binding system and triplet codons. Further evidence for codon assignments could be obtained by use of H.cutirubrum aminoacyl-tRNA in E.coli protein synthesizing systems programmed with synthetic mRNA's of defined sequence. Use of synthetic mRNA's of defined sequence in the H.cutirubrum system would seem to depend on making this system far more efficient. An attempt was made to dissociate and reconstitute this system and this seems to be the best approach to improve the system.

The techniques have been established for determining which one of the met-tRNA species is the initiator aminoacyl-tRNA. These include the H.cutirubrum and E.coli aminoacyl-tRNA transfer systems for testing if the transferase factors fail to interact with one of the species as they do with E.coli F-met-$tRNA^{met}_{F}$. By combining the H.cutirubrum ribosomal binding system and the met-puromycin assay described by Leder and Bursztyn (1966), it can be determined which of the met-tRNA

species enters the P site in the ribosome preferentially. The formation of met-puromycin from either species of met-tRNA can be determined over Mg^{2+} concentrations and with added transferase enzymes as well as initiation factors, if any are present on H. cutirubrum ribosomes. These experiments could elucidate which species is the initiator and whether formylation is really not required for the initiation process.

Although the extension of codon assignments to the basic and acidic amino acids and the definition of the initiator aminoacyl-tRNA would make the work more complete, there are sufficient results to strongly indicate that the genetic code and the protein synthesizing machinery are basically the same as in non-halophiles. The evidence presented points to the proteins being the macromolecules of the protein synthesizing system which have been modified for their high salt environment. It is clear that to understand the adaptation to a high salt environment, attention must be concentrated on the modified proteins of these bacteria. Many of the pertinent questions would be answered by directly comparing the amino acid sequence and structure of the same protein from a halophilic and non-halophilic bacterium.

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