STUDIES ON RIBOSOMAL PEPTIDYL TRANSFERASE

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STUDIES ON RIBOSOMAL PEPTIDYL TRANSFERASE

Thesis submitted for the degree of

Doctor of Philosophy by

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from

The Department of Biochemistry,

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STATEMENT

This thesis contains no material previously submitted for any other degree or diploma in any other University. To the best of my knowledge and belief, this thesis does not contain any material previously published or written by another person, except where due reference is made in the text.

D.J. ECKERMANN.

PAPERS PUBLISHED OR IN THE PRESS

1. Eckermann, D.J., Greenwell, P. and Symons, R.H. (1974). Peptide Bond Formation on the Ribosome. A Comparison of the Acceptor-Substrate Specificity of Peptidyl Transferase in Bacterial and Mammalian Ribosomes Using Puromycin Analogues. Eur. J. Biochem., 41, 547.

2. Symons, R.H., Harris, R.J., Greenwell, P., Eckermann, D.J. and Vanin, E.F. (1977). The Use of Puromycin Analogues and Related Compounds to Probe the Active Centre of Peptidyl Transferase on *Escherichia coli* Ribosomes. Bioorg. Chem., in the press.

3. Eckermann, D.J. and Symons, R.H. (1977). Sequence at the Site of Attachment of an Affinity Label Derivative of Puromycin on 23S Ribosomal RNA of *Escherichia coli* Ribosomes. Eur. J. Biochem., in the press.

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DEFINITIONS

The P-site is that site on the ribosome which binds peptidyl-tRNA during peptide bond formation.

The P'-site is that part of the P-site which binds the terminal CpCpA-peptide of peptidyl-tRNA.

The A-site is that site on the ribosome which binds aminoacyl-tRNA during peptide bond formation.

The A'-site is that part of the A-site which binds the terminal CpCpA-amino acid of the aminoacyl-tRNA.

ABBREVIATIONS

A-L-Phe	2'(3')-0-(L-phenylalanyl)-adenosine
Ap-Pan-Gly	adenyl-(3'-5')-Pan-Gly
Ac-L-Phe-tRNA	2'(3')-0-(N-acetyl-L-phenylalanyl)-tRNA
Bap-Pan-Phe	5'-0-(N-bromoacetyl-p-aminophenyl-phosphoryl)-3'-N-(L-phenylalanyl)-Pan
(BrAc-L-Phe)-tRNA	2'(3')-0-(N-bromoacetyl-L-phenylalanyl)-tRNA
BSA	bovine serum albumin
Bzl	benzyl
CNEt-p-Pan-L-Phe	5'-0-(cyanoethyl-phosphoryl)-3'-N-(L-phenylalanyl)-Pan
CpCpA-L-Leu	cytidyl-(3'-5')-cytidyl-(3'-5')-2'(3')-0-L-leucyl-adenosine

All other aminoacyl-oligonucleotides mentioned in the text are similarly abbreviated.

DTE	dithioerythritol
EDTA	ethylene-diamine-tetra-acetic acid
EEDQ	N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline
fMet-tRNA	2'(3')-0-(N-formyl-L-methionyl)-tRNA
Im-benzyl-L-His	imidazole-benzyl-L-histidine
NHS	N-hydroxysuccinimide
Pan	puromycin aminonucleoside
PEI	polyethylenimine
PNPC	p-nitrophenylcarbamyl
POPOP	1,4-bis 2-(5-phenyloxazolyl) - benzene
PPO	2,5 - diphenyloxazole

p-Pan-L-Phe	5'-0-phosphoryl-Pan-L-Phe
TCA	trichloro-acetic acid
t.l.c.	thin layer chromatography
tris	tris(hydroxymethyl)aminomethane

SUMMARY

The work described in this thesis involves several different approaches which aimed to elucidate the composition and mechanism of action of ribosomal peptidyl transferase from E. coli and rat liver, and to study the interactions occurring between the tRNA substrates and the active centre of the enzyme.

Firstly, analogues of the 3'-end of aminoacyl-tRNA were synthesised by coupling various amino acids or their derivatives to the 3'-amino group, and nucleotides to the 5'-hydroxyl group of puromycin aminonucleoside (Pan), itself an analogue of 3'-terminal adenosine of tRNA. These analogues were then tested for their ability to act as acceptor substrates in the fragment reaction of Monro and Marcker, (1967) and the Ac-L-[^{3}H]Phe transfer reaction; i.e. the ribosome (E. coli or rat liver) catalysed transfer of Ac-L-[^{3}H]Phe from Ac-L-[^{3}H]Phe-tRNA to puromycin or its analogue in the presence of poly (U). The major conclusions were:

(a) For both types of ribosome, analogues with a hydrophobic amino acid acted as better acceptors than those with less hydrophobic groups, which indicates the presence of a hydrophobic binding pocket in the A'-site.

(b) The hydrophobic pocket is of restricted size, since analogues with bulky hydrophobic amino acids (e.g. Trp) exhibited lower acceptor activity than those with smaller hydrophobic amino acids (e.g. Phe).

(c) The A'-site is stereo-specific, since Pan-D-Phe had a very low acceptor activity compared to Pan-L-Phe.

(d) Of the four nucleotides tried, only the addition of 3'-CMP to the 5'-hydroxyl group of aminoacyl-Pan allowed retention of maximal acceptor activity, indicating that a binding site for the penultimate CMP of aminoacyl-tRNA is present on the ribosome.

(e) In these simple assay systems, the hydrophobic binding site predominates over the CMP binding site.

(f) Rat liver ribosomes were generally less stringent than those of _E. coli_ in their requirements for high acceptor activity in these assay systems.

(g) Ribosomes from both sources exhibited a greater stringency in their requirements for acceptor substrate activity in the fragment reaction than in the Ac-L-[^{3}H]Phe transfer assay.

Secondly, attempts were made to find evidence of an acyl-ribosome intermediate during peptide bond formation on _E. coli_ ribosomes. Presence of such an intermediate would indicate a double-displacement mechanism for peptidyl transferase in which the peptide is transferred from peptidyl-tRNA to aminoacyl-tRNA via a covalent peptidyl-ribosome intermediate.

Two approaches were used:

(a) Exchange of Ac-L-[^{3}H]Leu from CpApCpCpA-(Ac-L-[^{3}H]Leu) to either tRNA or CpCpA. Formation of Ac-L-[^{3}H]Leu-tRNA or CpCpA-(Ac-L-[^{3}H]Leu) would indicate the formation of the acyl-ribosome intermediate, allowing

CpApCpCpA to diffuse out of the P-site and the second donor molecule to bind and react with the intermediate (assuming all reactions are reversible).

(b) After incubation of CpApCpCpA-(Ac-L-[^{3}H]Leu) with ribosomes, the ribosomes were precipitated under varying conditions and counted to find if any radioactivity had become covalently bound to ribosomal components.

In both cases, no reaction products were found, and thus there was no evidence for the intermediate. During the course of these experiments, the effect of a wide variation in conditions on the binding of CpApCpCpA-(Ac-L-[^{3}H]Leu) to the P'-site of peptidyl transferase was investigated. The results showed that P'-site binding is only slightly affected by a wide range of K^+ and Mg^{++} concentrations and pH values, and that P'-site binding occurs equally as well with ethanol as methanol.

Thirdly, the chemically reactive puromycin analogue Bap-Pan-Phe, which has been shown to be an affinity label of E. coli peptidyl transferase (Harris et al., 1973; Greenwell et al., 1974) was used to affinity label rat liver ribosomal peptidyl transferase. Although covalent binding did occur, it was mainly non-specific, as shown by the following:

(a) Approximately 40 affinity label molecules were bound to each ribosome.

(b) Puromycin only partially protected peptidyl transferase from the inactivation caused by the covalent binding of the affinity label to the active site.

(c) This partial puromycin protection did not reduce the extent of labelling (i.e. 40 molecules) of the total ribosome or the labelling of ribosomal RNA.

(d) The Ki of Bap-Pan-Phe with rat liver ribosomes (4×10^{-3} M) and polysomes (1.0×10^{-4} M) indicated that the affinity label has poor affinity for rat liver ribosomes (c.f. Km = 4.0×10^{-6} M for puromycin with rat liver polysomes). Thus high concentrations of Bap-Pan-Phe were required to obtain significant binding to the active centre of peptidyl transferase, resulting in a very high level of non-specific labelling which made identification of any specific labelling virtually impossible.

These experiments showed that Bap-Pan-Phe is an unsuitable affinity label for rat liver peptidyl transferase, and no specifically labelled component could be identified. However, the results showed that no specific labelling occurred on ribosomal RNA, whereas 23S RNA from *E. coli* ribosomes is specifically labelled by Bap-Pan-Phe (Harris *et al.*, 1973; Greenwell et al., 1974).

Finally, the site of attachment of Bap-Pan-Phe to the 23S RNA of *E. coli* ribosomes was identified as a cytidine residue by affinity labelling ribosomes grown in the presence of each of the [^{3}H]nucleosides, digesting the affinity labelled [^{3}H]RNA to mononucleotides with ribonucleases T_1 plus T_2 and identifying the products by paper electrophoresis.

A pentanucleotide fragment containing the affinity label-CMP was sequenced using the following method:

(a) Ribosomes were affinity labelled and the affinity labelled ribosomal RNA isolated.

(b) The affinity labelled RNA was reacted with Biotin-NHS to form Biotin-affinity label-RNA.

(c) Ribonuclease A or T_1 were used to digest the biotin-affinity label-RNA.

(d) The digests were reacted with avidin to form the very strong avidin:biotin-affinity label-RNA fragment complex.

(e) A phosphocellulose column was used to isolate the complex, which binds because of the basic properties of avidin, while other RNA fragments are eluted.

(f) The complex was dissociated by irreversibly denaturing the avidin with 70% formic acid.

(g) Further purification of the biotin-affinity label-23S RNA fragment was obtained by electrophoresis on DEAE-cellulose paper.

(h) The pure biotin-affinity label-RNA fragment was completely digested by ribonucleases T_1 plus T_2 to mononucleotides which were identified by paper electrophoresis.

The ribonuclease A product was identified as C(affinity label)pCp, which must be preceeded by a pyrimidine nucleotide. The ribonuclease T_1 fragment was identified as UpC(affinity label)pCpGp, which must be proceeded by GMP, thus giving a final pentanucleotide sequence of GpUpC(affinity label)pCpGp. The unusual feature of these experiments was that only one sequence was found, although Greenwell _et_ _al_., (1974) found two specific affinity label molecules were bound per 23S RNA molecule. The reason for this seeming discrepancy is not yet known.

CHAPTER ONE

INTRODUCTION

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INTRODUCTION

The ribosome is a very complex cellular structure which in the case of that of E. coli contains 54 proteins as determined by two-dimensional polyacrylamide gel electrophoresis (Wittmann, 1974) and 3 RNA species (designated 5S, 16S, and 23S according to their sedimentation properties). The ribosome consists of two subunits, with the larger (50S) subunit containing 34 proteins, 5S RNA and 23S RNA species, and the smaller (30S) subunit containing 21 proteins and the 16S RNA species. However, one protein is common to both subunits, thus making the total number of proteins 54 (Wittmann, 1974). Also, protein L_8 has now been shown to be a complex of L_{10} and L_7/L_{12} (Pettersson et al., 1976), and L_7 and L_{12} are identical except for the N-acetylation of the N-terminal serine of L_7. Thus there are really only 52 different proteins in the E. coli ribosome. The smaller (40S) mammalian subunit contains 30 proteins and one (18S) RNA species, while the larger (60S) mammalian subunit contains 40 proteins and two (5S, 28S) RNA species (Perry, 1967; Sherton and Wool, 1972).

The principle function of the ribosome is protein synthesis, which involves a number of reactions, all of which take place on the ribosome. These reactions include:

(a) binding of messenger RNA (mRNA),

(b) binding of fMet-tRNA (prokaryote system) or Met-tRNA (eukaryote),

(c) association of the two subunits,

(d) decoding of mRNA accompanied by the binding of aminoacyl-tRNA,

(e) peptide bond formation,

(f) translocation,

(g) termination,

and finally

(h) dissociation of the ribosomal subunits.

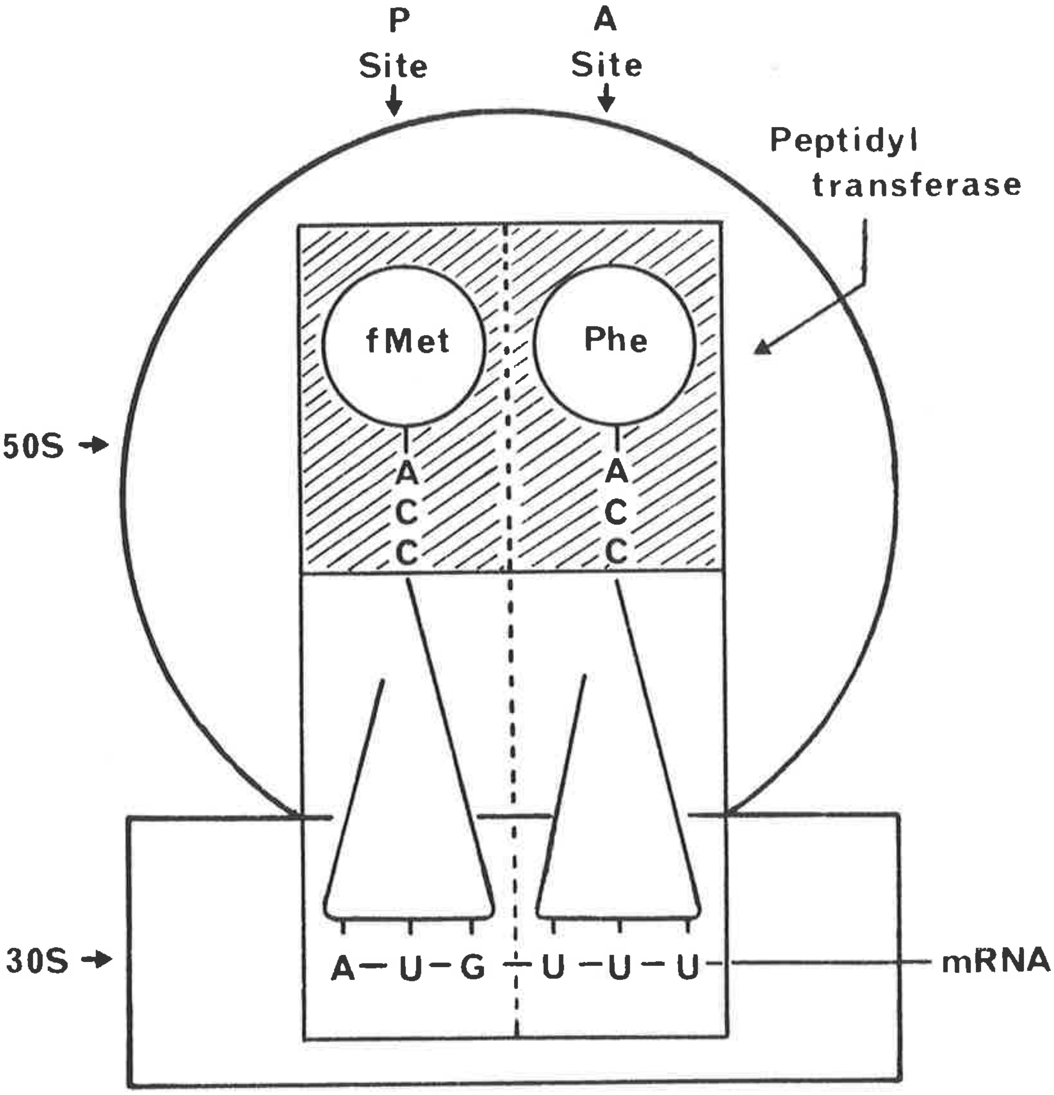
Peptide bond formation (see review by Harris and Pestka, 1977) occurs at a catalytic centre called peptidyl transferase (Monro _et_ _al_., 1969). This enzyme, which is exclusively located on the larger subunit of both prokaryotic (Monro _et_ _al_., 1969) and eukaryotic (Vazquez _et_ _al_., 1969; Thompson and Moldave, 1974) ribosomes, catalyses the transfer of the nascent chain from the peptidyl-tRNA bound to the P-site to the aminoacyl-tRNA bound to the A-site (Fig 1), with the resultant formation of a new peptide bond (Allen and Zamecnik, 1962; Nathans, 1964 a, b).

The work described in this thesis involves a study of the region at, or near, the catalytic centre of peptidyl transferase which aims to:

(a) identify some of the components of the ribosome that make up this catalytic centre by the technique of affinity labelling,

(b) study the interactions occurring between substrates and the enzyme during peptide bond formation through the use of analogues of the 3'-terminus of aminoacyl-tRNA,

FIG. 1: Diagrammatic representation of an *E. coli* ribosome just prior to peptide bond formation.


P Site
A Site
Peptidyl transferase
fMet
Phe
A
C
C
A
C
C
50S
30S
A–U–G
U–U–U
mRNA

(c) study the fine details of the mechanism of peptide bond formation by trying to find evidence for an acyl-enzyme intermediate on the active ribosome, and

(d) Compare the active centres of bacterial (*E. coli*) and mammalian (rat liver) peptidyl transferases.

The ultimate aim of this research was to further the understanding of protein synthesis at the molecular level in both eukaryotes and prokaryotes. The elucidation of the differences between bacterial and mammalian ribosomes may lead to the rational design of selective antibiotics and other therapeutic chemicals in the future.

The following introduction will give a brief review of the literature on *E. coli* peptidyl transferase, with particular emphasis on the substrate specificity of the A'- and P'- sites, and the affinity labelling of the enzyme.

1. Assays for Peptidyl Transferase.

A variety of assays are commonly used to measure peptidyl transferase activity, including

(a) the fragment reaction of Monro and Marcker, (1967), using washed ribosomes, buffer, salts, 30% methanol or ethanol, puromycin (or its analogue) and a low molecular weight fragment of the 3'- end of Ac-L-[^{3}H] aminoacyl-tRNA, $\overset{U}{\text{C}}$ACCA-(Ac-L-[^{3}H]amino acid), with the resultant formation of Ac-L-[^{3}H]aminoacyl - puromycin (or its analogue),

(b) the ribosome catalysed transfer of Ac-L-Phe from Ac-L-Phe-tRNA to acceptor substrate in the presence of ribosomes and poly(U) (Rychlik *et al.*, 1967; Rychlik *et al.*, 1969),

(c) as for (b), but with poly-L-Lys-tRNA and poly (A) (Rychlik *et al.*, 1969; Ringer and Chladek, 1974 a),

(d) the acceptor substrate induced release of peptides from [^{14}C] peptidyl-tRNA formed on ribosomes with natural mRNA (Cannon, 1968; Harris *et al.*, 1971), and (e) the reaction of [^{3}H] puromycin with peptidyl -tRNA on polysomes (Pestka, 1972 a and b; Vanin *et al.*, 1974).

2. Cation and pH Requirements of Peptidyl Transferase.

Peptidyl transferase requires suitable divalent and monovalent cations for activity (Maden and Monro, 1968; Pestka, 1972 a,b; Pestka *et al.*, 1972). The most effective divalent cation used is Mg^{++}, although it can be replaced by Ca^{++} in some assay systems. Either NH_4^+ or K^+ can satisfy the monovalent cation requirements. Response to pH value is similar in most assays, with peptidyl transferase activity progressively inhibited below pH 8.5 (Maden and Monro, 1968; Pestka, 1972 a,b; Pestka *et al.*, 1972). This indicates that a group with a pKa of approximately 7.5 (e.g. histidine) may be necessary for catalysis or the binding of substrates to the ribosome.

3. Structural Requirements for Donor Substrate Activity on Peptidyl Transferase.

The P'-site is responsible for binding the 3'-terminal end of peptidyl-tRNA during protein synthesis. Some of the structural requirements for donor substrate activity have been investigated, and the results have led to the proposal of certain binding pockets.

(a) Hydrophobic site

The donor activity of various N-Ac-aminoacyl-tRNA and dipeptidyl -tRNA molecules was studied by Mao, (1973), who found that aminoacids or dipeptides with high hydrophobicity (e.g. L-Leu and L-Phe) were transferred to puromycin faster than those with low hydrophobicity (e.g. L-Pro). Similarly, donor substrate activity studies by Monro *et al.*, (1968) and Mercer and Symons, (1972) found that the affinity of aminoacyl side-chains for the P'-site decreased in the order L-Met > L-Leu > L-Phe, while Gly was not bound to the site. Thus a binding site for hydrophobic amino acids appears to be present in the P'-site.

(b) Hydrophilic site

This site was suggested by the high donor activity of $(Np)_x$CpCpA-(Ac-L-Arg) when compared to the Ac-Gly-oligo-nucleotides (Monro *et al.*, 1968). Since nucleotide sequences 5'-distal to the terminal CpCpA have little effect on donor activity (Monro *et al.*, 1968), this activity difference must be due to the amino acid moiety. The positively charged amino acid may bind to a carboxyl or phosphate group in the P'-site.

(c) Adenine site

Cerna *et al.*, (1974) and Krayevsky *et al.*, (1975) found that the activity of donor substrates of the type pN-(L-fMet) decreased in the order A > I > G, while the C and U derivatives were inactive. The 3'-terminal adenine of peptidyl-tRNA may be hydrogen-bonded to a uracil in 23S RNA. This RNA species has been implicated in the P'-site of peptidyl transferase since Cerna *et al.*,

(1973) have shown that T_1 ribonuclease treatment inactivates peptidyl transferase and the binding ability of the donor site, but does not affect the acceptor site, while 23S RNA has been labelled by several affinity-labelling derivatives of the type α-N-substituted-L-Phe-tRNA (where the substituent contains an affinity labelling group; see section 6 below).

(d) CpCp site

Since donor activity in the fragment reaction decreases dramatically in the order CpCpA-L-fMet >> CpA-L-fMet >> pA-L-fMet (Monro et al., 1968; Krayevsky et al., 1976), sites must exist for the binding of the two 3'-terminal cytidylic acid residues of tRNA. Nucleotide sequences 5'-distal to the terminal pCpCpA have little effect on donor activity (Monro et al., 1968).

(e) Nascent peptide site

A blocked α-amino group is essential for high donor activity (Monro et al., 1968; Mao, 1973), indicating that peptidyl-tRNA is recognised and preferred by the P'-site. Also, the nascent peptide is protected by the ribosome against protease digestion (Malkin and Rich, 1967), which indicates the presence of a nascent peptide site.

A model of the P'-site has been proposed from much of the above data by Harris and Symons, (1973).

4. Structural Requirements for Acceptor Substrate Activity on Peptidyl Transferase.

The A'-site is responsible for the binding of the 3'-terminal end of aminoacyl-tRNA during peptide bond formation. The substrate specificity of this site has been extensively studied using aminoacyl-nucleoside derivatives or puromycin analogues to define the structural requirements. Puromycin is a structural and functional analogue of the 3'-terminus of aminoacyl-tRNA (Yarmolinsky and de la Haba, 1959) which binds to the A'-site and accepts the peptide from peptidyl-tRNA (Nathans, 1964 a,b). These studies, as with the P'-site, have led to the proposal of binding pockets and recognition sites within the A'-site.

(a) Recognition of the 2'- and 3'-isomers of aminoacyl-tRNA analogues.

The 2'-isomer of puromycin has been shown to be inactive in inhibiting protein synthesis (Nathans and Neidle, 1963; Nathans, 1964 a). Similarly, Chladek *et al.*, (1974) have shown that the non-isomerizable 3'-O-aminoacyl derivatives CpA(2'-O-methyl)-L-Phe, Cp(2'-deoxy)A-L-Phe, and A(2'-O-methyl)-L-Phe acted as acceptor substrates, while the 2'-O-aminoacyl derivatives CpA(3'-O-methyl)-L-Phe, Cp(3'-deoxy)A-L-Phe, and A(3'-O-methyl)-L-Phe were inactive as acceptors of Ac-L-Phe from Ac-L-Phe-tRNA. However, these same 2'-O-aminoacyl derivatives did bind strongly to the A'-site, as shown by their inhibition of CpApCpCpA-L-Phe binding (Ringer *et al.*, 1975). Also, when the complete tRNA molecule was present (e.g. tRNA-CpCp(3'-deoxy)A-L-Phe), the derivative did have acceptor activity, although appreciably less than with the naturally occurring tRNA-CpCpA-L-Phe (Chinali *et al.*, 1974). To explain this activity, Chinali *et al.*, (1974) proposed that the amino group of both the 2'- and 3'- isomers can occupy the same position to act as acceptor molecules in peptide bond formation, a proposal which necessitates some distortion of the normal angles of the aminoacyl groups with the ribose ring (Sundaralingam and Arora, 1972; Yathindra and Sundaralingam, 1973; Mathieson, 1965; Saenger and Suck,1971). Symons et al., (1977) have proposed an equal or more plausible model where no distortion is required, and the amino group of the 2'-isomer can attack from one side of the carboxyl group, and that of the 3'-isomer from the other.

The free 2'-hydroxyl on the terminal adenosine does seem essential for high acceptor activity, since (2'-deoxy) A-L-Phe has negligible activity (Rychlik et al., 1969; Cerna et al., 1970), while the blocking of the 2'-hydroxyl by methylation decreases, but does not completely abolish, the acceptor activity (Chladek et al., 1974). It has been suggested by Chladek et al.,(1974) that the 2'-O-aminoacyl-tRNA isomer is the form in which elongation factor Tu (EF-Tu) presents the aminoacyl-tRNA to the ribosome, and a subsequent transfer of the amino acid from the 2' to the 3'-position occurs while bound to the ribosome. Symons et al., (1977) have proposed that this 2' to 3' -transfer cannot occur with uncoded aminoacyl-tRNA molecules incorrectly bound to the A-site, and thus a fail-safe mechanism is provided to prevent incorrect insertion of uncoded amino acids into proteins.

The above results suggest that, although both the 2' and 3' -isomers of aminoacyl-tRNA can bind to the A-site, the 3' -isomer is the exclusive or strongly preferred one involved in peptide bond formation.

(b) Hydrophobic site.

The presence of a hydrophobic site in the A'-site which binds aromatic and the more hydrophobic amino acids of aminoacyl-tRNA has been suggested by the results of a number of workers using aminoacyl-nucleoside derivatives or puromycin analogues (Rychlik _et al_., 1969; Cerna _et al_., 1970; Rychlik _et al_., 1970; Gottikh _et al_., 1970; Harris _et al_., 1971; Vanin _et al_., 1974; Ringer and Chladek, 1974 a, b; (see also Chapter Two). In general, they found that the more hydrophobic the aminoacyl side-chain (e.g. L-Phe and, to a lesser extent, L-Tyr, S-benzyl-L-Cys, O-benzyl-L-Ser, im-benzyl-L-His, and L-Met), the better the acceptor substrate activity (and hence the binding to the A'-site). The less hydrophobic derivatives showed appreciably less activity with a trend toward decreasing activity with decreasing hydrophobicity (e.g. L-Leu > L-Val > L-Ala > Gly).

The size of the hydrophobic site appears to be limited, since the hydrophobic, but bulky, amino acid derivatives S-benzyl-L-Cys, O-benzyl-L-Ser, im-benzyl-L-His, and the amino acid L-Trp have much lower acceptor activity than the smaller hydrophobic amino acid, L-Phe, indicating that there may be steric hindrance to the larger groups. This specificity for L-Phe is most apparent in the fragment reaction assays (see Chapter Two). Support for the hydrophobic site was also supplied by Pestka _et al_., (1970) and Lessard and Pestka, (1972), who found that CpApCpCpA-L-Phe was bound to ribosomes to a greater extent than the L-Val, L-Met, and L-Leu derivatives, and that the affinity of aminoacyl-trinucleotides for the A'-site decreased in the order CpCpA-L-Phe > -L-Leu > -L-Lys > -L-Ala > -L-Glu.

The reason for the preference of L-Phe derivatives above other hydrophobic groups is not apparent. The suggestion of Krayevsky et al., (1975), that the aminoacyl R group could uniquely affect the conformation of puromycin and related molecules is unlikely in view of the extended nature of the puromycin molecule and the aminoacyl-esters of nucleosides as determined by X-ray analysis (Sundaralingam and Arora, 1972; Yathindra and Sundaralingam, 1973; Mathieson, 1965; Saenger and Suck, 1971). This X-ray data also does not support the proposals of intramolecular stacking of the benzene ring of an amino acid with the adenine ring of the nucleoside (Symons et al., 1969; Raacke, 1971; Ariatti and Hawtrey, 1975).

(c) Hydrophilic site.

The high acceptor activity of A-L-Lys compared to that of related uncharged analogues (e.g. A-L-Val, A-L-Leu, A-L-Ala, and A-Gly) led to the proposal for a hydrophilic site (Rychlik et al., 1970). Support for this site is found in the strong binding of CpCpA-L-Lys when compared to CpCpA-L-Ser, -L-Ala, and -L-Glu (Lessard and Pestka, 1972). Presumably this site binds the aminoacyl side chains of L-Arg- and L-Lys-tRNA, possibly by ionic interactions with a carboxyl or phosphate group in the A'-site.

(d) Adenine site.

The activity of analogues of the type N-L-Phe (where N is a nucleoside) decreased in the order A $\gg$ I $\gg$ C, while the G and U derivatives were inactive (Cerna et al., 1970). This indicates that there is a binding site for the terminal adenosine of aminoacyl-tRNA. Substitutions in the ribose ring of adenosine have shown that the 5'-

hydroxymethyl group and the oxygen of the ring are not required for acceptor substrate activity (Duquette et al., 1974). Cleavage of the ribose ring between the 2' and 3' positions did reduce, but did not completely destroy, acceptor activity (Chaladek et al., 1973).

The role of the C_6-amino group of adenosine in the binding to the A'-site has been investigated (Zemlicka et al., 1975), and all analogues tested had a high acceptor activity. However, a decrease in activity coincident with a decrease in the electron donating properties of the substituent attached to the C_6-carbon was noted ($-NH_2$, $-N(CH_3)_2 > -SCH_3 > -OCH_3 > -H$), which led to the proposal that this decrease in activity may be due to the resultant decrease in electron density of the purine ring, which in turn reduces interactions between the ring and some group on the ribosome. The high activity of all these C_6-substituted analogues does not support the suggestion of Harris et al., (1973) and Greenwell et al., (1974) that the 3'-terminal adenosine of aminoacyl-tRNA base-pairs with a uridine on the 23S or 5S ribosomal RNA.

(e) CpCp site.

5'-substitution of Pan-Gly or A-Gly with Cp leads to a large increase in acceptor activity, but substitution with Gp, Up or Ap gives no increase in activity (Rychlik et al., 1967; Harris et al., 1971; Vanin et al., 1974; see also Chapter Two). Thus a relatively specific binding site for the penultimate nucleotide, Cp, of aminoacyl-tRNA must exist in the A'-site. It also appears likely that a binding site for the third nucleotide (Cp) from the

3'-terminus of aminoacyl-tRNA exists, since CpPan-Gly and CpA-Gly had low acceptor activity in releasing peptides from peptidyl-tRNA, while Takanami,(1964) found that various $(Np)_x$CpCpA-amino acids had high puromycin-like activity. Also, Scolnik et al.,(1970) observed that CpCpA, possibly by binding to the A'-site, allowed nucleophilic attack by ethanol on fMet-tRNA to form fMet-O-ethyl ester, while CpA, ApCpA, GpCpA, and UpCpA were less than 5% as active as CpCpA. Binding assays have provided more direct evidence for a CpCp site. Thus, A-L-Ser was bound very poorly to E.coli ribosomes, whereas CpCpA-L-Ser bound strongly (Pestka et al., 1970). Similarly, the binding of puromycin was just detectable (Fernandez-Munoz and Vazquez, 1973), but CpCpA-L-Phe was bound strongly (Lessard and Pestka, 1972).

A model of the A'-site, drawn from much of the above data, has been proposed by Harris and Symons, (1973b).

5. On the Mechanism of Peptide Bond Formation.

There are two possibilities for the reaction mechanism during peptide bond formation on the ribosome. In the first, a single-displacement mechanism, the peptidyl group is transferred directly from peptidyl-tRNA to aminoacyl-tRNA without covalent interaction with the enzyme; i.e. peptidyl transferase only provides a suitable environment for the reaction to occur efficiently. This reaction can be represented as

$$\text{peptidyl-tRNA} + \text{aminoacyl-tRNA} \xrightleftharpoons{\text{ribosome}} \text{tRNA} + \text{peptidyl-aminoacyl-tRNA.}$$

In the second possibility, a double-displacement mechanism, peptidyl transferase transfers the peptidyl chain to aminoacyl-tRNA via a covalent peptidyl-ribosome intermediate. The two partial reactions can be represented as

$$\text{peptidyl-tRNA} + \text{ribosome} \rightleftharpoons \text{tRNA} + \text{peptidyl-ribosome}$$

$$\text{peptidyl-ribosome} + \text{aminoacyl-tRNA} \rightleftharpoons \text{ribosome} + \text{peptidyl-aminoacyl-tRNA.}$$

Although very little is known about the actual mechanism of peptidyl transfer, a growing body of evidence supports the double-displacement mechanism for the other known transferase reactions (Spector, 1973). Chymotrypsin, which catalyses ester and peptide hydrolysis (i.e. the reverse of peptidyl transferase), has been shown to form a covalently bound acyl-enzyme intermediate which has, in fact, been isolated and the site of attachment identified (McDonald and Balls, 1957). Thus it seems possible that peptidyl transferase may also act via the double-displacement mechanism, although no definitive evidence exists for either mechanism (see also Chapter Three).

6. Affinity Labelling of Peptidyl Transferase.

The technique of affinity labelling (Baker, 1967; Shaw, 1970; Knowles, 1972) has been successfully used to identify the ribosomal components constituting the P'-site, the A'-site and the chloramphenicol binding site (Pellegrini and Cantor, 1977). Chloramphenicol is an inhibitor of protein synthesis which is thought to bind at or near the A'-site (Pestka, 1971). The affinity labelling of peptidyl transferase has been concentrated on the P'-site because of the ease of synthesis of reactive peptidyl-tRNA analogues by blocking the α-amino group of aminoacyl-tRNA with a reactive moiety. The affinity labels which have been used, and the ribosomal components to which they become covalently attached, are listed in Tables 1 and 2.

As can be seen, most of the analogues are derivatives of L-Phe-tRNA, and are directed towards the P'-site because of the blocked α-amino group. Using the numbering system for ribosomal proteins devised by Wittmann, (1974), the components most often labelled by these P'-site affinity labels were L_2, L_{27} and 23 S RNA. Other components labelled by at least one analogue were L_{11}, $L_{14/17}$, L_{15}, L_{16}, L_{18}, L_{20}, L_{24}, $L_{26/27}$, and $L_{32/33}$. Of the affinity labels attached to 23 S RNA, only the site of attachment of (N-BrAc-L-Phe)-tRNA has been sequenced (Yukioka et al., 1977), the sequence being $\overset{*}{A}$pUpUpUpUpApGp (where $\overset{*}{A}$ is the nucleoside to which the affinity label is attached). Although approximately 40% of the 23 S RNA molecule has been sequenced (Branlant et al., 1975; Branlant et al., 1976 a,b), the labelled sequence does not appear in that portion of the 23 S RNA. However, once the complete sequence is known, that part of the 23 S RNA labelled by (N-BrAc-L-Phe)-tRNA will be identified.

Eilat et al., (1974 a) have mapped the peptide groove using affinity labels of the structure (N-BrAc-$[\text{Gly}]_n$-L-Phe)-tRNA (i.e. the distance between the 3' terminus of the tRNA and the reactive bromine increased as n increases). If n = o, the major protein labelled was L_2. As n increased, the labelling of L_2 decreased, while that of L_{27} increased, reaching a maximum at n = 4 or 5. Although the extent of labelling of L_{27} decreased as n increased from 6 to 18, it still remained the major protein labelled, indicating

Table 1: Affinity labelling of peptidyl transferase.

TABLE 1. AFFINITY LABELLING OF PEPTIDYL TRANSFERASE

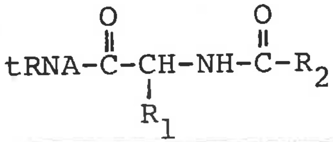
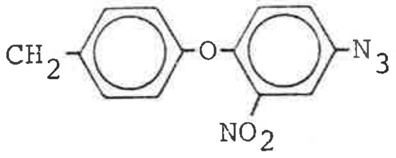
I	Peptidyl-tRNA analogues  $tRNA\text{-}C(=O)\text{-}CH(R_1)\text{-}NH\text{-}C(=O)\text{-}R_2$	Ribosomal components labelled (50S)	Reference
R_1	R_2		
$CH_2Ø$	CH_2Br	L2, L27, L14-17	Oen *et al.*, (1973) Pellegrini *et al.*, (1974)
$CH_2Ø$	CH_2Br	L16, L27, L2 (forced into A site)	Eilat *et al.*, (1974)
$CH_2Ø$	$(Gly)_n\text{-}C(=O)\text{-}CH_2Br$	L2, L26-27, L32-33, L24	Eilat *et al.*, (1974a)
$CH_2Ø$	CH_2Br	23S rRNA	Breitmeyer and Noller (1976)
$CH_2Ø$	CH_2I	23S rRNA	Yukioka *et al.*, (1975) Yukioka *et al.*, (1976)
$CH_2Ø$	CH_2I	L2, L20	Bispink and Matthaei (1973)
$CH_2Ø$		L11, L18, L27	Hsiung et al., (1974)

TABLE 1 (cont'd)

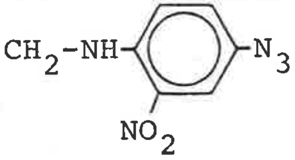
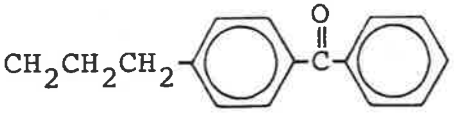
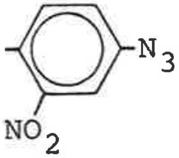
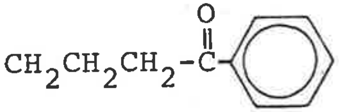
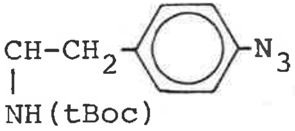
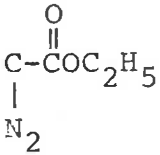
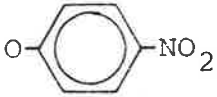
CH_2Ø		L11, L18	Hsiung and Cantor (1974)
CH_2Ø		23S rRNA	Barta et al., (1975)
CH_2Ø		rRNA	Girshovich *et al.*, (1974)
CH_2Ø		23S rRNA	Kuechler *et al.*, (1976)
CH_2Ø		23S rRNA	Sonenberg *et al.*, (1975)
CH_2Ø	 $\underset{N_2}{C}-\overset{O}{C}OC_2H_5$	23S rRNA	Bispink and Matthaei (1973)
CH_2Ø		L27, L15, L2, L16	Bauer *et al.*, (1975) Collatz *et al.*, (1976) Czernilofsky *et al.*, (1974)

TABLE 1 (cont'd).

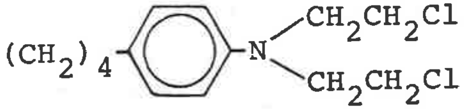
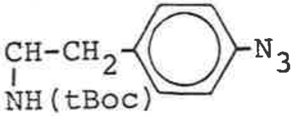
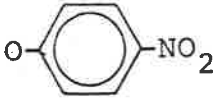
$CH_2Ø$		23S rRNA	Bochkareva et al., (1973) Budker et al., (1972) Knorre (1974)
$CH_2CH_2SCH_3$		23S rRNA	Sonenberg et al., (1976)
$CH_2CH_2SCH_3$		L27, L15	Hauptmann et al., (1974)
$CH_2CH_2SCH_3$	CH_2Br	L2, L27	Sopori et al., (1974)

TABLE 1 (cont'd).

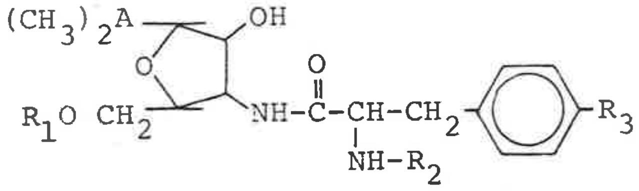
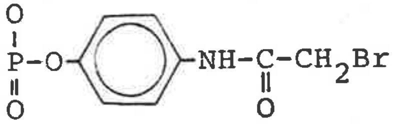
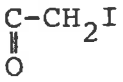
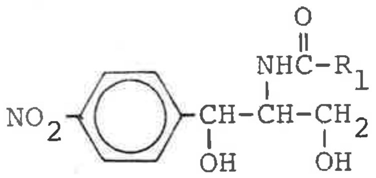
II Puromycin analogues			Ribosomal components labelled (50S)	Reference
				
R$_1$	R$_2$	R$_3$		
	H	H	23S rRNA	Greenwell *et al.*, (1974) Harris *et al.*, (1973)
H	 $\mathrm{C(=O)-CH_2I}$	$O-CH_3$	L6, L2	Pongs (1974)

Table 2: Affinity labelling of the chloramphenicol binding site.

TABLE 2. AFFINITY LABELLING OF THE CHLORAMPHENICOL BINDING SITE

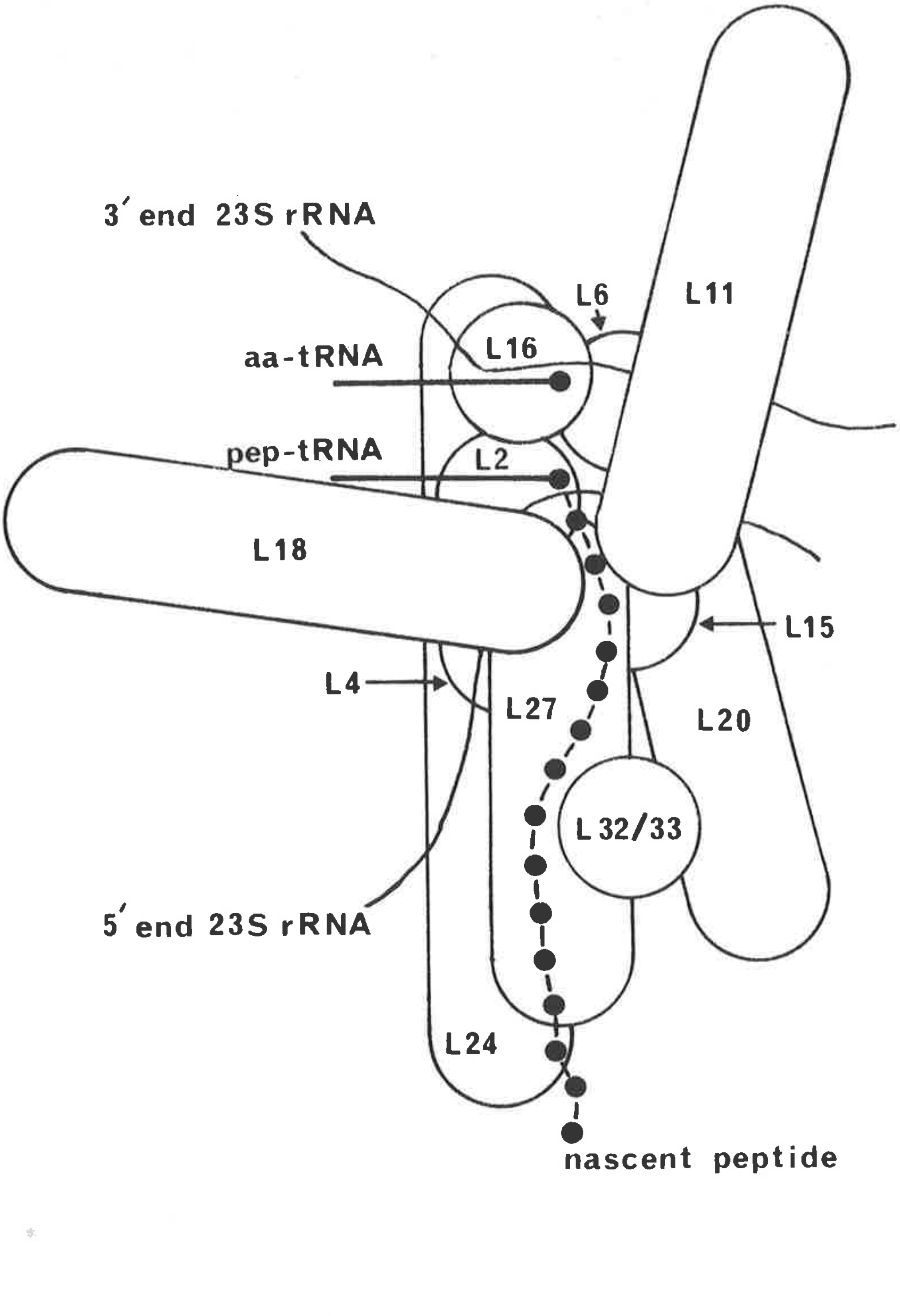
Chloramphenicol analogues	Ribosomal components labelled (50S)	References
		
R_1		
CH_2Br	L2, L27	Sonenberg *et al.*, (1973)
CH_2I	L16, L24	Bald *et al.*, (1972) Pongs *et al.*, (1973) Pongs and Messer, (1976)

that L_{27} is probably an elongated protein. When n > 5, $L_{32/33}$ became labelled, reaching a maximum when n = 8, while further increased n above 8 caused a decrease in labelling of $L_{32/33}$ and an increase in L_{24} labelling which reached a maximum at n = 18. Using these results, Eilat et al., (1974a) were able to give a diagrammatic representation of the peptide groove similar to that described by Vanin,(1977) shown in Fig. 2.

The two photoaffinity labelling analogues of peptidyl -tRNA used by Hsiung and Cantor,(1974) and Hsiung et al., (1974) were found to label proteins L_{11} and L_{18}, which must also be part of, or close to, the P'-site.

Only a small number of affinity labels have so far been successfully used to probe the A'-site. When (N-BrAc-L-Phe)-tRNA was forced into the A -site by the addition of unaminoacylated tRNA, a marked increase in the labelling of protein L_{16} occurred (Eilat et al., 1974a). Reconstitution studies by Moore et al., (1975) found that L_{16} was essential for peptidyl transferase activity. Chloramphenicol binding is also dependent on L_{16} (Nierhaus and Nierhaus, 1973), and the affinity labelling analogue of chloramphenicol, iodamphenicol, labelled L_{16} and L_{24} (Bald et al., 1972; Pongs et al.,1973; Pongs and Messer, 1976). Bauer et al., (1975) have pointed out that non-enzymic binding of (N-PNPC-L-Phe)-tRNA to ribosomes caused significant labelling of L_{16}, while none was labelled during enzymic binding to the P-site. Presumably, the labelling of L_{16} was due to the binding of (N-PNPC-L-Phe)-tRNA to the A-site under non-enzymic conditions. These results

FIG. 2: Model of the active centre of peptidyl transferase (Vanin, 1977).


3′ end 23S rRNA
L6
L11
L16
aa-tRNA
pep-tRNA
L2
L18
L15
L4
L27
L20
L 32/33
5′ end 23S rRNA
L24
nascent peptide

indicate that protein L_{16} is at or near the A'-site of peptidyl transferase.

The reactive puromycin analogue, iodoacetyl -puromycin, labelled L_6 (Pongs, 1974). The presence of this protein near the A'-site is also implicated by the experiments of Dietrich et al., (1974) and Nierhaus et al., (1974), where an interdependence of proteins L_{16}, L_6, and L_{11} for peptidyl transferase and chloramphenicol inhibitory activity was shown. The presence of 23S RNA in or near the A'-site was indicated by its labelling by the puromycin analogue, Bap-Pan-L-Phe. This analogue specifically bound to the A'-site and could still act as an acceptor substrate during peptide bond formation while covalently bound to the 23S RNA (Harris et al., 1973; Greenwell et al., 1974).

Thus the above data indicates that the A'-site of peptidyl transferase is composed of 23S RNA and proteins L_6, L_{16} and possibly L_{24}.

7. Model of the Active Centre of Peptidyl Transferase.

Using similar data to that summarised in Tables 1 and 2, and that described above, Vanin, (1977) has proposed a model, modified from that of Cantor et al., (1974), for the active centre of peptidyl transferase (Fig.2). This was only a schematic representation, and, for simplicity, those proteins for which no evidence for an elongated structure existed were represented as spheres.

The P'-site was shown as being composed of 23S RNA and proteins L_2, L_4, L_{11}, L_{18}, L_{20} and L_{27}, most of which have been labelled by various peptidyl-tRNA analogues (see Table 1). The elongation of proteins L_{11} and L_{18}

was based on the immuno-electron microscopy data of Tischendorf et al., (1975) and of L_{20} on the data of San Jose et al., (1976). The inclusion of L_4 was based on the experiments of Wittmann et al., (1973) who found that mutations in L_4 resulted in a reduction in peptidyl transferase activity. The arrangement of proteins in the peptide groove was based on the data of Eilat et al.,(1974a) as described above (section 6). The large variation in size of the affinity labelling molecules which labelled L_{27} (n = 3 to 18) indicates that this protein is possibly also elongated. Since L_{24} was labelled by both the peptidyl - tRNA analogue, (N-BrAc-$[Gly]_{18}$-L-Phe)-tRNA (Eilat et al., 1974a) and the chloramphenicol analogue, iodamphenicol (Bald et al., 1972; Pongs et al., 1973; Pongs and Messer, 1976), it seems probable that it, too, is elongated. Also, since L_{18} binds to 5S RNA (Gray et al., 1972; Horne and Erdmann, 1972), and L_2 stimulates the binding of the 5S RNA - protein complex to 23S RNA (Gray et al., 1972), it appears that 5S RNA may be near peptidyl transferase. However, due to the elongated nature of many of the ribosomal proteins, it is not possible to localise with certainty any two components by reference to a third.

The A'-site in this model consists of 23S RNA, L_6, L_{16} and L_{24}, as discussed above. The presence of 23S RNA in both the A'- and P'- sites, as shown by its labelling by various affinity labels, is also supported by the fact that the proteins L_2, L_4, L_6, L_{16}, L_{20} and L_{24} (all of which have been affinity labelled - see Table 1) bind to 23S RNA (Garrett et al., 1974). In particular, both the 5'- and 3'- ends of the RNA molecule may be involved, since

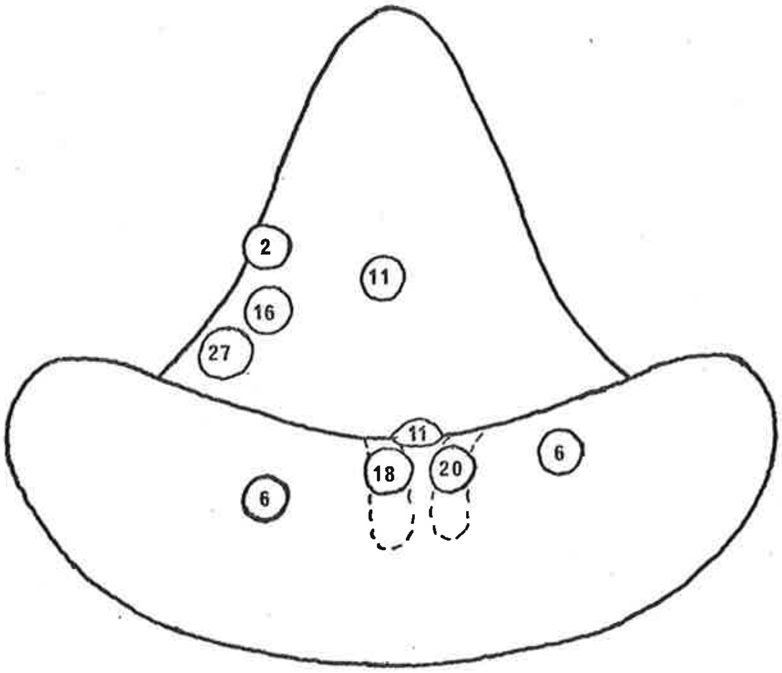
L_2 binds toward the 3'-end (Spierer et al., 1976) and L_{24} binds close to the 5'-end (Branlant et al., 1976c) of 23S RNA.

8. Localisation of Peptidyl Transferase on the 50S Ribosomal Subunit.

It is interesting to note that the proteins L_6, L_{11}, L_{15}, L_{20}, and L_{27} have all been identified as being at, or near, the interface of the two subunits (Morrison et al., 1973; Stoffler, 1974); i.e. on the surface of the 50S subunit to which the 30S subunit becomes associated. This suggests that peptidyl transferase and the aminoacyl- and peptidyl-tRNA molecules may be sandwiched between the two ribosomal subunits during peptide bond formation.

Antibodies to purified ribosomal proteins have been used to localise by immuno-electron microscopy those proteins on the intact ribosome (Stoffler, 1974; Stoffler and Wittmann, 1977). This work has resulted in the localisation of proteins on a three-dimensional model of the 50S subunit (Fig 3.; Stoffler and Wittmann, 1977). Subsequently, the positioning of various active centres (e.g. peptidyl transferase) has been attempted. However, a difficulty in the exact positioning of the active centres exists, since many of the ribosomal proteins are elongated (see above, section 7), and thus the active centre of those proteins with catalytic activity may be some distance from the site to which the antibodies bind. However, it seems from Fig. 3 that there are two possibilities for the site of peptidyl transferase. The first, which is favoured by Stoffler and Wittmann, (1977), is a site on the right-hand side of the "seat" in the region of L_2, L_{16} and L_{27}.

FIG. 3: Frontal view of a model of the 50 S ribosomal subunit, showing the positions of various ribosomal proteins located by immuno-electron microscopy (Stoffler and Wittmann, 1977).



R.J. Harris (personal communication) favours a site on the upper front of the "seat" in the region of L_{11}, L_{18}, and L_{20}, and through which the elongated L_6 may also pass. Further experiments will no doubt differentiate between these two possibilities.

9. Mammalian Peptidyl Transferase.

Information available on peptidyl transferase of mammalian ribosomes is considerably less than that from *E. coli*. Only preliminary physical data (number and size of protein and RNA components) on the ribosome, the production of antibodies to purified proteins and whole subunits, and comparative studies between the ribosomes of different species have been recorded (Wool and Stoffler, 1974; Wool, 1977). Limited attempts at the affinity labelling of peptidyl transferase have been made (Stahl *et al.*, 1974; Minks *et al.*, 1975) using BrAc-puromycin and BrAc-Pan. The 60S subunit was labelled somewhat nonspecifically, although proteins L_{27} and L_{29} were predominantly labelled. Further comparisons between rat liver and *E. coli* ribosomes are discussed in Chapters Two and Four.

CHAPTER TWO

A COMPARISON OF THE ACCEPTOR-SUBSTRATE SPECIFICITY OF PEPTIDYL TRANSFERASE IN BACTERIAL AND MAMMALIAN RIBOSOMES USING PUROMYCIN ANALOGUES

A COMPARISON OF THE ACCEPTOR-SUBSTRATE SPECIFICITY OF PEPTIDYL TRANSFERASE IN BACTERIAL AND MAMMALIAN RIBOSOMES USING PUROMYCIN ANALOGUES

INTRODUCTION

Previous workers in this laboratory have used various 3'-N-aminoacyl and 5'-O-nucleotidyl analogues of puromycin as acceptor substrates for peptidyl transferase in complex bacterial and mammalian cell-free systems (Symons *et al.*, 1969; Harris *et al.*, 1971), and the results have allowed a description of some of the structural requirements for acceptor substrate activity (see Introduction, section 4). The experiments described in this chapter extend this work to a study of the acceptor substrate activity of puromycin analogues in two simpler and more defined cell-free systems; the release of Ac-L-Phe from Ac-L-Phe-tRNA in the presence of ribosomes and poly(U), and the fragment reaction of Monro and Marcker, (1967). The aims of this work are three-fold. Firstly, we wish to extend our understanding of the structural requirements for activity of low-molecular-weight acceptor substrates by using these simpler systems, so that the results can be interpreted more directly in terms of the active centre of peptidyl transferase, since the more complex systems also involve interactions elsewhere on the ribosome. Secondly, by comparing the activity of bacterial and mammalian ribosomes, it is hoped that a molecular explanation of the differential action on peptidyl transferase of such antibiotics as chloramphenicol will eventually be obtained. Thirdly, the data will provide more information for the design of potential affinity labelling compounds of peptidyl transferase.

In order to allow meaningful comparisons of the actual data obtained, the acceptor activity of each analogue has been expressed relative to that of puromycin measured under the same conditions. Further, since changes in the ionic conditions, pH, etc., of the assay medium are likely to cause some variation in the actual results obtained, small differences between the acceptor activity of analogues are not considered significant. Analogues have therefore been grouped depending on their activity into those with high activity (> 50% of that of puromycin), moderate activity (25 - 50% of that of puromycin), low activity (5 - 24% of that of puromycin), and negligible activity (< 5% of that of puromycin).

The work described in this chapter was begun during my B.Sc. (Hons.) and completed during my Ph.D. For completeness, all results obtained have been included in this thesis.

MATERIALS AND METHODS

Materials

Puromycin dihydrochloride (neutralised with Tris base before use) was obtained from Nutritional Biochemicals Corporation. Puromycin aminonucleoside (Pan) was a generous gift from the American Cyanamid Company. All puromycin analogues were prepared by either Dr.R.H.Symons, Mr.E.F.Vanin or myself as described by Harris *et al.*, (1972), and were checked for purity by paper, or thin-layer, chromatography prior to use. Poly (U) was obtained from

Sigma Chemical Co. and E. coli B tRNA, L-[^{3}H]phenylalanine (specific activity 51 Ci/mmole) and L-[^{3}H]leucine (specific activity 54 Ci/mmole) from Schwarz Bioresearch Inc. Scintillation fluid was prepared by dissolving 3.5 g PPO and 0.35 g POPOP in 1.0 l toluene.

Buffer solutions were as follows. Buffer A: 10 mM Tris-HCl, pH 7.4, 25 mM KCl, 0.25 M sucrose, 4 mM $MgCl_2$, 3 mM dithio-erythritol. Buffer B: as for buffer A but with 1.0 M sucrose. Buffer C: 50 mM Tris-HCl, pH 7.4, 0.5 M KCl, 15% (v/v) glycerol, 2 mM $MgCl_2$. Buffer D: 50 mM Tris-HCl, pH 7.2, 0.5 M KCl, 2 mM $MgCl_2$, 1 mM puromycin. Buffer E: 50 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 10 mM KCl.

Preparation of Ribosomes.

Ribosomes from E. coli MRE 600 were prepared essentially as described by Staehelin et al., (1969), except that the ribosomes were washed twice by centrifugation in concentrated salt buffer (0.5 M NH_4 Cl). Ribosomes were stored under liquid nitrogen in a buffer containing 0.1 M NH_4 Cl, 10 mM magnesium acetate, 0.5 mM EDTA, 20 mM Tris-acetate, pH 7.5 (20° C).

Rat liver ribosomes were prepared using an unpublished method kindly provided by S. Pestka and T. Hishizawa. Livers from starved rats were cooled in ice, washed with buffer A, minced with scissors and then homogenised in a Potter-Elvehjem homogeniser using 2 ml of buffer A per g of liver. The homogenate was centrifuged at 6,000 rev./min. for 10 min at 4°C, the supernatant poured through cheesecloth, 0.05 volumes of 10% (w/v) sodium deoxycholate, pH 8.0 added, and the mixture stirred for 30 min at 4°C. Samples (7.5 ml) of the supernatant were layered over 2.0 ml of buffer B in Beckman Ti 50 polycarbonate tubes which were then centrifuged at 40,000 rev/min. for 3 h at 0 - 4°C. The brown pellets were rinsed with buffer A and allowed to resuspend overnight at 0°C in buffer D (1 ml per tube). The solution was centrifuged at 8,000 rev./min. for 10 min and the supernatant was then incubated for 30 min at 30°C to remove nascent peptides. The solution was diluted with 5 volumes of buffer C and the ribosomes centrifuged through buffer B as before. The pellets were resuspended overnight in buffer C (1 ml per tube), the solution clarified by centrifugation and the supernatant diluted 6 times with buffer C. Ribosomes were collected by centrifugation at 50,000 rev./min. for 1 h, resuspended overnight in buffer E (1 ml per tube) at 0°C and the solution clarified by low speed centrifugation before storage in liquid nitrogen.

Ribosome concentrations were determined at 260 nm using $A_{1\ cm}^{1\%}$ = 145 for E. coli ribosomes (Hill et al., 1969) and 140 for rat liver ribosomes (Petermann, 1971).

Preparation of Donor Substrates

CpApCpCpA-(Ac-L-[^{3}H]Leu) (specific activity 20.6 Ci/mmol) and Ac-L-[^{3}H]Phe-tRNA (specific activity 20.2 Ci/mmol) were prepared essentially by the method of Monro, (1971) and stored in 0.1 mM sodium acetate, 0.1 mM EDTA, pH 5.0, under liquid nitrogen or at -15°C. Concentrations of these compounds were estimated by drying aliquots onto Whatman GF/C glass-fibre discs which were counted in toluene scintillation fluid at an efficiency of approximately 13%. The efficiency was determined by firstly counting a known amount of [^{3}H] toluene standard in triton/toluene scintillation fluid to find the efficiency of counting in that medium, and then comparing the counts/min.

obtained when the same amount of CpApCpCpA-(Ac-L-[^{3}H]Leu) was either dissolved in triton/toluene scintillation fluid and counted or dried on a GF/C glass-fibre filter disc which was then placed into toluene scintillation fluid and counted.

Assay of Acceptor Activity of Puromycin Analogues with E. coli Ribosomes.

Assay for acceptor activity of 3'-N-Aminoacyl-puromycin-aminonucleoside Analogues.

(a) Fragment Reaction. The method of Monro and Marker, (1967), as modified by Mercer and Symons, (1972), was used. The reaction mixture contained in 0.17 ml: 0.18 mg ribosomes, 0.67 pmol CpApCpCpA-(Ac-L-[^{3}H]Leu), 300, 100 or 10 µM puromycin or its analogue, 14 mM $MgCl_2$, 0.24 M KCl, 43 mM Tris-HCl, pH 7.2, 18 mM NH_4Cl, 30% (v/v) methanol. The reaction was started by the addition of methanol (0°C) and incubation was at 0°C. The reaction was stopped with 0.10 ml of 0.2 M NaH_2PO_4, pH 5.5, and the product extracted into 1.0 ml ethyl acetate (Leder and Bursztyn, 1966), samples of which (0.5 ml) were mixed with 2.0 ml of scintillation fluid and counted in a Packard liquid scintillation spectrometer. Counting efficiency was 28% as determined with a [^{3}H]toluene internal standard. The quantity of fragment used was sufficient to give approximately 4300 counts/min in the 0.5 ml sample of ethyl acetate if 100% conversion had been achieved. In practice, 1000 counts/min or less were extracted because the incubation times of 10 - 30 min were chosen to represent initial linear rates (see Results). The efficiencies of extraction into ethyl acetate, under peptidyl transferase assay conditions, of the N-acetyl-L-phenylalanine derivatives of puromycin and several puromycin analogues were checked by absorbance measurements: puromycin, 94%; Pan-L-Trp, 97%; Pan-L-Val, 98%; Pan-L-Ala, 87%. The differences are too small to affect the qualitative conclusions derived from the results of the assays.

(b) Ac-L-[^{3}H]Phe Transfer Assay. The reaction mixture was the same as that used for the fragment reaction except that methanol was absent, the donor substrate was 0.86 pmol Ac-L-[^{3}H]Phe-tRNA and 10 μg poly (U) was present. The reaction was started by the addition of ribosomes and the mixture incubated at 37°C. The reaction was stopped and the product assayed as described for the fragment reaction. Complete conversion of the substrate would have given approximately 5300 counts/min; this estimate does not allow for hydrolysis of the substrate under assay conditions.

Assays for Acceptor Activity of 5'-O-Phosphoryl and 5'-O-Nucleotidyl Analogues of Pan-Gly and Pan-L-Phe.

Reaction mixtures and incubations for the fragment reaction and for reaction with Ac-L-[^{3}H]Phe-tRNA were as described above. However, the charged phosphate groups prevent extraction of the product into ethyl acetate so an alternative paper chromatography assay was developed. The ethanol precipitation of unreacted fragment as described by Rychlik *et al.*, (1970) was inefficient under our conditions and resulted in very high levels of radioactivity in control reactions.

The reaction was terminated by the addition of 25 μl of 75% concentrated NH_4OH in ethanol, and the mixture incubated at 37°C for 10 min. to ammonolyse the [^{3}H]acetylamino acid from unreacted fragment or tRNA. Samples of 0.15 ml were dried on Whatman No. 1 chromatography paper and subjected to ascending chromatography with a solvent of acetonitrile - butanone - formic acid- water (10 : 10 : 1 : 1, by vol.). Under these conditions, the labelled reaction products remain at the origin while the [^{3}H]acetyl-amino acid and its amide moved with the solvent front. The origin of the chromatogram was then cut out, dried and counted in 2.5 ml scintillation fluid. The puromycin controls were assayed in the same way, except that the product was extracted into ethyl acetate, 0.5 ml of which was dried on Whatman No. 1 chromatography paper and counted. Less than 5% of the radioactivity in the [^{3}H]acetyl-aminoacyl puromycin was eluted from the paper into the scintillation fluid; the products of the puromycin controls and the test reaction mixtures were therefore counted under the same conditions.

Assays of Acceptor Activity of Puromycin Analogues with Rat Liver Ribosomes.

(a) Fragment Reaction. The reaction mixture was essentially that of Monro and Marcker, (1967), as modified by S. Pestka (Unpublished), and contained in 0.05 ml: 0.10 mg ribosomes, 0.67 pmol CpApCpCpA-(Ac-L-[^{3}H]Leu), 300, 100, or 10 μM puromycin or its analogue, 40 mM $MgCl_2$, 0.4 M KCl, 50 mM Tris-HCl, pH 7.2, 30% (v/v) methanol. Incubation was at 0°C and products were assayed as described for *E. coli* ribosomes, except that 0.25 ml of 0.2 M NaH_2PO_4, pH 5.5, was added to stop the reaction with aminoacyl analogues and 0.05 ml of the reaction mixture was used for paper chromatography with the nucleotidyl analogues.

(b) Ac-L-[^{3}H]Phe Transfer Assay. The reaction mixture was the same as that for the fragment reaction except that methanol was absent, the donor substrate was 0.86 pmol Ac-L-[^{3}H]Phe-tRNA and 10µg poly (U) and 8 mM dithioerythritol were present. Incubation was at 37°C and products were assayed as above.

RESULTS AND DISCUSSION

Acceptor Substrate Activity of Puromycin Analogues In The Fragment Reaction, With N-Acetyl-L-[^{3}H]Leucine-Pentanucleotide As Donor Substrate.

(a) Activity of 3'-N-Aminoacyl Analogues.

The results obtained with E. coli and rat liver ribosomes are shown in Table 3, and are expressed for each analogue as a percentage of the acceptor activity of puromycin at the same concentration. The time course of reaction with each analogue was checked to ensure that only initial rates were measured. The most noticeable feature is the complete lack of, or negligible, acceptor activity with E. coli ribosomes of all analogues, other than Pan-L-Phe, at the concentrations tested. This contrasts with our previous results with more complex systems (Symons et al., 1969; Harris et al., 1971) where nearly all analogues with a single benzene ring showed high acceptor activity relative to that of puromycin. On the other hand, with rat liver ribosomes, all analogues with a single benzene ring showed low to moderate activity, except for Pan-L-Phe which showed high activity. These rat liver results are qualitatively similar to those reported

Table 3: Acceptor substrate activity of 3'-N-aminoacyl analogues of puromycin in the fragment reaction with *E. coli* and rat liver ribosomes.

All compounds were tested at the concentrations shown as described in Materials and Methods, and the initial rates of reaction for each compound expressed relative to that obtained with puromycin at the same concentration. All values are the average of at least two separate experiments, each of which was done in duplicate.

3'-N-substituted amino acid on Pan.	Initial rate relative to puromycin (%).					
	3.10^{-4}M		10^{-4}M		10^{-5}M	
	E. coli	Rat Liver	*E. coli*	Rat Liver	*E. coli*	Rat Liver
L-Phe	83	92	56	92	50	80
D-Phe	2	20	1	10	4	6
L-Tyr	5	17	7	21	9	21
O-Benzyl-L-Ser	1	39	0	44	2	15
S-Benzyl-L-Cys	2	33	1	36	2	16
Im-Benzyl-L-His	2	12	1	19	1	5
L-Trp	0	8	-	-	1	1
L-Val	1	7	-	-	3	4
L-Ala	0	1	-	-	2	3
L-Leu	0	3	-	-	1	3
L-Met	0	1	-	-	1	1
L-Pro	1	1	-	-	1	1
Gly	0	1	-	-	1	0

by us for more complex rabbit reticulocyte systems (Harris et al., 1971). In the fragment reaction, therefore, the structural requirements for acceptor substrate activity with E. coli ribosomes are much more stringent than in the other systems studied.

It is interesting to note the low but significant activity of Pan-D-Phe with rat liver ribosomes; some activity with this analogue has been noted with other systems (Harris et al., 1971). The less hydrophobic, non-aromatic analogues and the double-ringed Pan-L-Trp showed negligible acceptor activity with both types of ribosomes. In all the work reported here, the pentanucleotide fragment was used as donor substrate. Similar results (not given) were obtained using the chemically prepared trinucleotide donor substrates (Mercer and Symons, 1972) CpCpA-2'(3')-[^{3}H]Ac-L-Leu and the corresponding L-phenylalanyl derivative.

(b) Activity of 5'-0-Substituted Derivatives of Pan-L-Phe and Pan-Gly.

The results obtained with E. coli and rat liver ribosomes are given in Table 4 and clearly show that Cp-Pan-L-Phe was the only analogue which gave significant acceptor activity relative to puromycin and then only with E. coli ribosomes. This analogue had low activity with rat liver ribosomes while all other analogues had much lower or negligible activity with both types of ribosomes. The very high activity of the 5'-0-cytidylyl derivative of puromycin as an inhibitor of peptide bond formation in a rabbit reticulocyte cell-free system has

Table 4: Acceptor substrate activity of 5'-0-phosphoryl and 5'-0-nucleotidyl analogues of Pan-Gly and Pan-L-Phe in the fragment reaction with *E. coli* and rat liver ribosomes.

See Table 3 for experimental details.

	Initial rate relative to puromycin (%).			
	10^{-4}M		10^{-5}M	
Compound	*E. coli*	Rat Liver	*E. coli*	Rat Liver
pPan-Gly	3	1	9	5
ApPan-Gly	0	1	6	1
CpPan-Gly	1	3	10	2
GpPan-Gly	0	0	4	3
UpPan-Gly	3	1	4	2
pPan-L-Phe	6	6	10	12
CpPan-L-Phe	83	14	273	23
Cyanoethyl-p-Pan-L-Phe	2	4	13	11

been reported by Hengesch and Morris, (1971). For *E. coli* ribosomes, the low to negligible acceptor activity of Cp-Pan-Gly, p-Pan-L-Phe, and CNEt-p-Pan-L-Phe indicates that both the 5'-0-cytidylyl and the L-phenylalanyl portions must be in the same molecule for high activity. Further, for both classes of ribosomes, the high activity shown by Pan-L-Phe (Table 3) was substantially reduced by the addition of a 5'-0-phosphoryl or 5'-0-cyanoethyl phosphoryl group (Table 4).

The results of Table 4 contrast also with previous results reported by us (Symons *et al.*, 1969; Harris *et al.*, 1971) for more complex *E. coli* and rabbit reticulocyte systems where Cp-Pan-Gly gave moderate to high activity relative to puromycin. Cp-Pan-L-Phe and CNEt-p-Pan-L-Phe were not tested in our previous work but our preparation of the latter was approximately 50% as effective as puromycin in inhibiting a *Bacillus amyloliquefaciens* cell-free amino acid incorporation system (J.L.McInnes and B.K.May, unpublished results). In addition, 5'-0-cyanoethyl-puromycin gave an inhibitory effect equal to that of puromycin on a poly (U)-directed *E.coli* polyphenylalanine-synthesizing system (Smith *et al.*, 1965). Thus, the results given here for the fragment reaction have shown very stringent structural requirements for acceptor substrate activity, relative to the systems studied earlier.

Acceptor Substrate Activity Of Puromycin Analogues In The Ac-L-[^{3}H]Phe Transfer Assay Using Ac-L-[^{3}H]Phe-tRNA As Donor Substrate In The Presence of Poly (U).

(a) Activity of 3'-N-Aminoacyl Analogues.

The system used here is more complex than the fragment reaction since it depends on the binding of the macromolecule, Ac-L-[^{3}H]Phe-tRNA, to the ribosomes to act as donor substrate under the influence of another macromolecule, poly (U). The results obtained are shown in Table 5. Pan-L-Phe exhibited the highest activity relative to puromycin with both types of ribosomes, especially at the higher concentrations used with E. coli ribosomes where activity similar to that of puromycin was obtained. All other analogues containing a benzene ring (including Pan-D-Phe), as well as Pan-L-Val, showed low to moderate activity, with some exceptions, for several analogues at 10μM with E. coli ribosomes. Pan-L-Trp, with its double ring system, and Pan-L-Met showed low to almost moderate activity only with rat liver ribosomes. The remaining less hydrophobic derivatives showed negligible activity with E. coli ribosomes and low activity with rat liver ribosomes.

Of interest is the low activity of Pan-L-Tyr relative to Pan-L-Phe and puromycin, although the differences are not as marked as those obtained in the fragment reaction (Table 3). Overall, the results of Table 5 indicate that in this Ac-L-[^{3}H]Phe transfer system, the structural requirements for acceptor substrate activity are much less stringent than in the case of the fragment reaction.

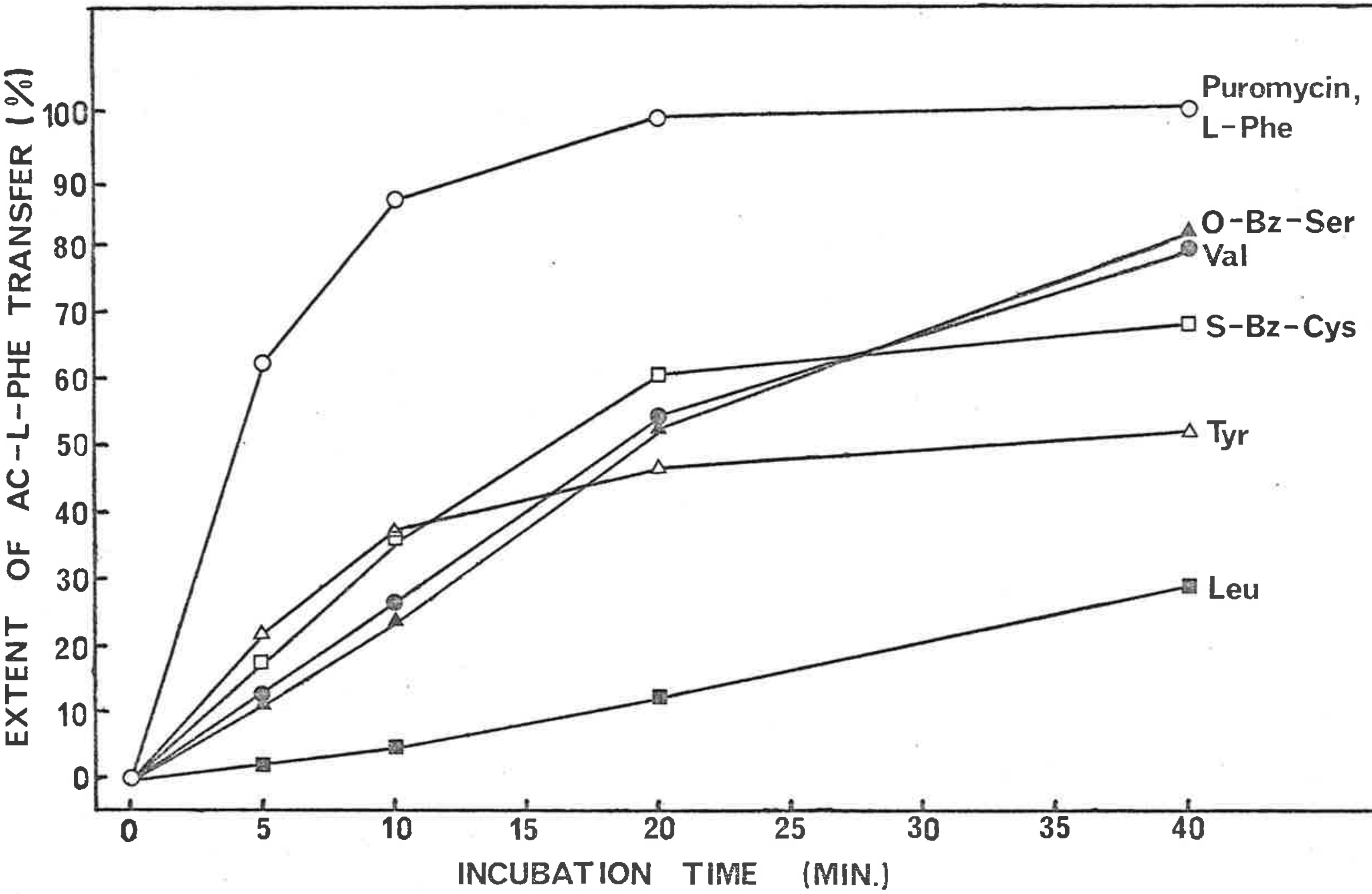
An important, and unusual, feature of this assay system with E. coli ribosomes is demonstrated in Fig. 4, where the extent of reaction for several analogues relative to puromycin (taken as 100% at 40 min) is plotted against time of incubation. The extent of reaction for puromycin,

Table 5: Acceptor substrate activity of 3'-N-aminoacyl analogues of puromycin in the Ac-L-[^{3}H]Phe transfer assay system with *E. coli* and rat liver ribosomes. See Table 3 for experimental details.

3'-N-substitued amino acid on Pan.	Initial rate relative to puromycin (%).					
	$3.10^{-4}M$		$10^{-4}M$		$10^{-5}M$	
	E. coli	Rat Liver	E. coli	Rat Liver	E. coli	Rat Liver
L-Phe	97	57	95	67	68	50
D-Phe	17	13	12	13	2	16
L-Tyr	41	28	29	36	18	33
O-Benzyl-L-Ser	15	36	10	45	2	25
S-Benzyl-L-Cys	25	40	29	50	5	32
Im-Benzyl-L-His	35	32	34	49	8	31
L-Trp	1	21	2	19	0	18
L-Val	19	15	10	14	1	15
L-Ala	2	5	2	7	0	15
L-Leu	4	8	3	8	0	18
L-Met	1	24	1	17	0	15
L-Pro	0	6	2	5	0	13
Gly	2	5	2	4	0	14

FIG. 4: Time course of Ac-L-[^{3}H]Phe transfer from Ac-L-[^{3}H]Phe-tRNA to Pan-amino acids on *E. coli* ribosomes.

All acceptor substrates were tested at 3×10^{-4} M as described in Materials and Methods. For each time point, the counts/min obtained in the product were expressed as a percentage of the counts/min obtained in the product with puromycin after 40 min incubation, which was taken as 100% (about 3000 counts/min).


EXTENT OF AC-L-PHE TRANSFER (%)
0
10
20
30
40
50
60
70
80
90
100
INCUBATION TIME (MIN.)
0
5
10
15
20
25
30
35
40
Puromycin,
L-Phe
O-Bz-Ser
Val
S-Bz-Cys
Tyr
Leu

Pan-L-Phe, Pan-Bzl-L-Cys and Pan-L-Tyr reached, or tended to reach, a plateau after about 20 min, whereas for Pan-Bzl-L-Ser, Pan-L-Val, and Pan-L-Leu, the reaction was more or less linear for 40 min. The plateau with puromycin corresponds to approximately 60% conversion and probably represents exhaustion of the substrate, the remainder having been lost by hydrolysis. In the absence of kinetic parameters of the various analogues, we have not attempted to explain the different time courses. However, the results emphasize the importance of measuring initial rates for comparing the acceptor substrate activity of various analogues. The slower reaction catalysed by rat liver ribosomes was in all cases linear up to 20 min.

(b) Activity of 5'-O-Substituted Derivatives of Pan-L-Phe and Pan-Gly.

As shown in Table 6, only Cp-Pan-L-Phe showed high acceptor activity relative to puromycin, while all other analogues showed either low or negligible activity at both concentrations tested. The results with these analogues are therefore very similar to those obtained with the fragment reaction (Table 4), and emphasize that the only acceptable substitution on the 5'-hydroxyl of Pan-L-Phe for the retention of high acceptor activity is that of the cytidylyl group.

In contrast to a previous report (Siler and Moldave, 1969), the rat liver ribosomes used here with 40 mM $MgCl_2$ catalysed peptide bond formation without the need for added supernatant factors. This confirms the results of Rahamimoff *et al.*, (1972) who showed that reticulocyte

Table 6: Acceptor substrate activity of 5'-O-phosphoryl and 5'-O-nucleotidyl analogues of Pan-Gly and Pan-L-Phe in the Ac-L-[^{3}H]Phe transfer assay system with *E. coli* and rat liver ribosomes. See Table 3 for experimental details.

Compound	Initial rate relative to puromycin (%).			
	10^{-4}M		10^{-5}M	
	E. coli	Rat Liver	*E. coli*	Rat Liver
pPan-Gly	2	5	10	9
ApPan-Gly	1	8	5	10
CpPan-Gly	3	6	4	6
GpPan-Gly	1	5	4	8
UpPan-Gly	0	5	8	8
pPan-L-Phe	14	8	23	8
CpPan-L-Phe	64	76	105	79
Cyanoethyl-p-Pan-L-Phe	16	6	21	3

ribosomes bound Ac-L-Phe-tRNA non-enzymatically and that peptide bond formation occurred with a $MgCl_2$ concentration of 10mM without the need for translocation.

General Conclusions

Various 3'-N-aminoacyl and 5'-O-nucleotidyl derivatives of puromycin were used as acceptor substrates for ribosomal peptidyl transferase in two simple cell-free systems; the release of Ac-L-Phe from Ac-L-Phe-tRNA, and the fragment reaction of Monro and Marcker, (1967). Both *E. coli* and rat liver ribosomes were used in this study to allow comparison of the binding requirements of each type of ribosome under similar conditions. The acceptor activity of each analogue has been expressed relative to that of puromycin measured under the same conditions, and analogues have been grouped, depending on their activity, into high (> 50% of that of puromycin), moderate (25 - 50% of that of puromycin), low (5 - 24% of that of puromycin) and negligible activity (< 5% of that of puromycin).

The results presented here indicate that the conditions of the fragment reaction impose fairly severe limitations on the structural requirements for activity, relative to those of the Ac-L-[^{3}H]Phe-tRNA assay. This effect is more pronounced with *E. coli* ribosomes, where, out of the 21 analogues tested, only Pan-L-Phe and Cp-Pan-L-Phe gave high activity comparable to that of puromycin, while all other analogues gave negligible activity, even at the highest concentration used (3×10^{-4}M). In contrast, in the Ac-L-[^{3}H]Phe-tRNA assay, there was low to moderate activity with a number of the more hydrophobic aminoacyl analogues of puromycin (e.g. Pan-L-Tyr, -O-Benzyl-L-Ser, -S-Benzyl-L-Cys, -Im-Benzyl-L-His), and low activity with some of the 5'-O-derivatives (e.g. pPan-L-Phe, cyanoethyl-pPan-L-Phe). Similarly, with rat liver ribosomes there was a modest enhancement of activity for many of the poorer analogues above that seen in the fragment reaction. Generally, therefore, the specificity pattern of the Ac-L-[^{3}H]Phe-tRNA assay tends towards the broader patterns of the more complex E. coli and rabbit reticulocyte systems used in earlier work (Symons et al., 1969; Harris et al., 1971), where all aminoacyl analogues containing a single benzene ring (and also Cp-Pan-Gly) gave moderate to high activity.

When comparing rat liver with E. coli ribosomes, an obvious feature is the generally higher activity of the hydrophobic puromycin analogues with the eukaryote ribosomes in both assay systems. This suggests that the rat liver ribosomes have a higher intrinsic affinity for the compounds, such that the working concentration of even the less active analogues comes closer to saturating the binding site. With the nucleotidyl analogues, both types of ribosome show quite similar specificity patterns and favour Cp-Pan-L-Phe, the analogue most resembling the 3'-terminus of L-Phe-tRNA, although this compound had low reactivity on rat liver ribosomes in the fragment reaction assay. Both ribosome types in both assay systems differ from the more complex systems referred to above, in that the present work revealed very little activity with Cp-Pan-Gly.

If one considers overall the ability of the amino-acyl-puromycin analogues to undergo peptidyl transfer in the diverse systems described here and earlier (Symons et al., 1969; Harris et al., 1971), it is clear that the more "stripped down" a system is, relative to natural polypeptide synthesis, then the more stringent are its specificity requirements relative to puromycin. This is presumably a measure of the increasingly dominant control by the local interactions at the puromycin binding site of the rate-determining step as the other components and interactions are removed. On the assumption that the rates largely reflect the ability of the analogues to bind to the enzyme, the present measurements with simpler systems provide further support for the two proposed binding sites within the acceptor site of the ribosome (Harris et al., 1971; Harris and Symons, 1973; and references therein); that is, a hydrophobic binding site for aromatic 3'-aminoacyl side chains, and a more specific binding site for the 3'-penultimate CMP residue of aminoacyl-tRNA.

The fragment reaction systems, simplest and most stringent, illustrate that the hydrophobic site is of restricted size, because activity is optimal with puromycin and Pan-L-Phe, and lower with the very hydrophobic but larger Pan-L-Trp, Pan-Bzl-L-Ser and Pan-Bzl-L-Cys. Although Pan-L-Tyr is sterically a close analogue of puromycin, it has low activity which can be explained by the hydrophilic nature of the phenol group.

In both assay systems used here, binding to the proposed CMP site was not sufficient to allow acceptor activity since Cp-Pan-Gly was inactive, whereas Cp-Pan-L-Phe and Pan-L-Phe usually had high activity, results which suggest a dominating effect of the hydrophobic binding site.

In conclusion, the variation of acceptor activity of the puromycin analogues in the assay systems used here further emphasizes the limitation of experiments _in vitro_, particularly with regard to extrapolation of results to the situation _in vivo_. However, surveys of this type are essential to further our understanding of the mechanism of action of peptidyl transferase, and also of those antibiotics and other compounds which affect this reaction (Harris and Symons, 1973).

CHAPTER THREE

ATTEMPTS TO FIND AN ACYL-RIBOSOME INTERMEDIATE DURING PEPTIDE BOND FORMATION ON *E. COLI* RIBOSOMES

ATTEMPTS TO FIND AN ACYL-RIBOSOME INTERMEDIATE DURING PEPTIDE BOND FORMATION ON E. COLI RIBOSOMES

INTRODUCTION

Although the sequence of events occurring during protein synthesis on ribosomes is now well known, the actual fine details of the transfer of the peptide from peptidyl-tRNA to aminoacyl-tRNA is not clear. Thus it is not known if the carboxyl group of the donor molecule reacts directly with the α-amino group of the acceptor molecule, with the ribosome only acting indirectly by providing a suitable environment for the reaction to occur efficiently, or whether the ribosome is directly involved by forming a covalently bound acyl-enzyme intermediate which subsequently reacts with the acceptor substrate. The formation of an acyl-enzyme intermediate would not be surprising, since chymotrypsin, which catalyses the reverse reaction (i.e. peptide hydrolysis), has been shown to form such an intermediate during catalysis (Gutfreund and Sturtevant, 1956; Balls and Woods, 1956; Dixon and Neurath 1957; McDonald and Balls, 1957). The aim of the work described in this chapter was to find evidence for an acyl-ribosome intermediate using two different approaches:

(1) exchange of Ac-L-[^{3}H]Leu from CpApCpCpA-(Ac-L-[^{3}H]Leu) to either tRNA or CpCpA (See Fig. 5 for possible reactions) (J.F.B. Mercer, 1970, unpublished), or

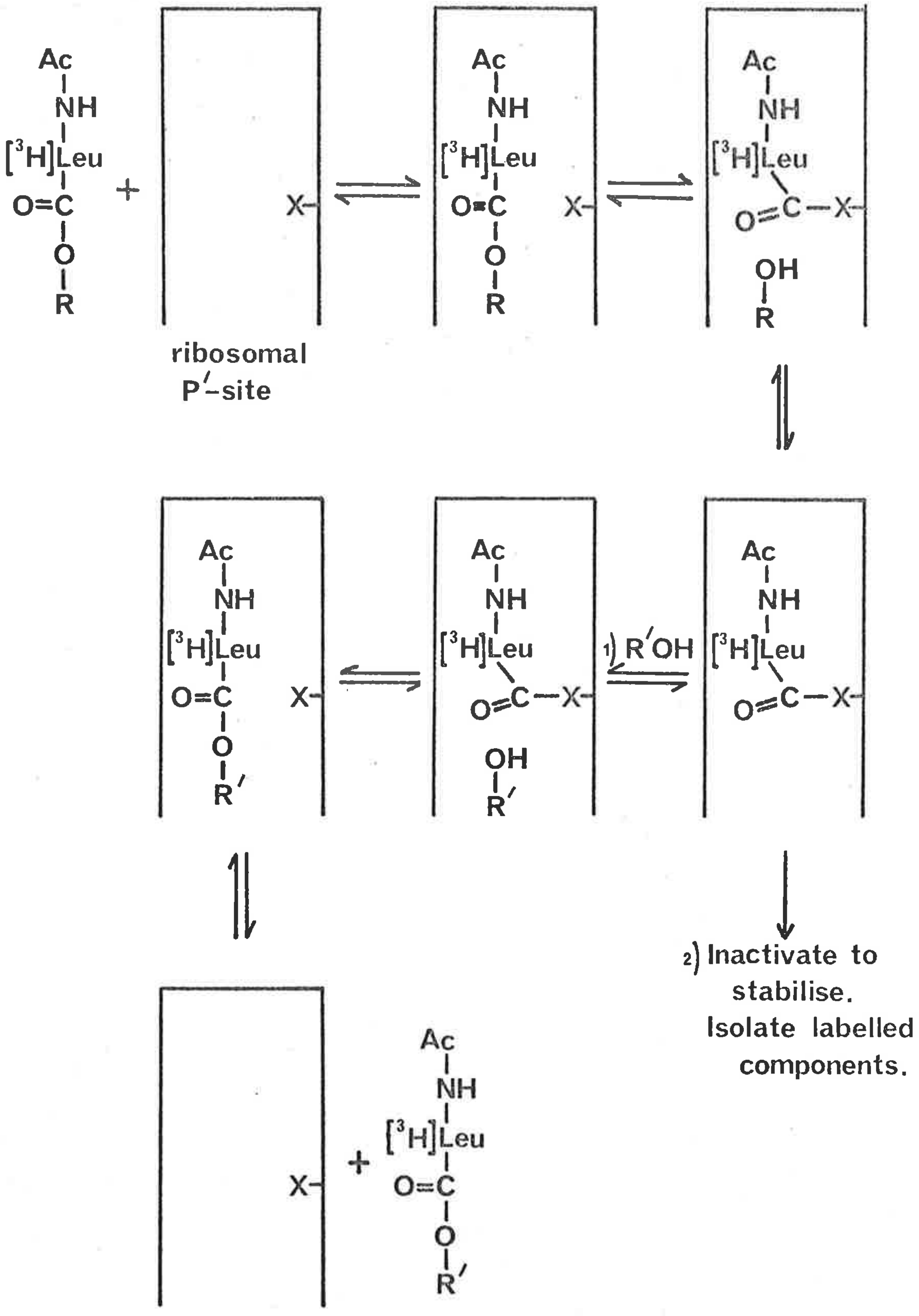
(2) isolation of the Ac-L-[^{3}H]Leu - ribosome intermediate. The rationale for these two approaches is based on the assumption that if the acyl-ribosome intermediate exists,

FIG. 5: Schematic representation of possible reactions occurring in the P'-site (in the absence of A'-site substrates) which would indicate the formation of a peptidyl-ribosome intermediate during peptide bond formation on the ribosome.

X = reactive nucleophile (e.g. serine).

R = CpApCpCpA

R' = tRNA or CpCpA


Ac
NH
[³H]Leu
O=C
O
R
+
X
ribosomal P′-site
C
X
OH
R
1) R′OH
R′
+

2) Inactivate to stabilise.
Isolate labelled components.

then the sequence of events shown in Fig. 5 may occur. If the approaches used here prove unsuccessful, the presence of such an intermediate is still not eliminated, since the intermediate may only exist transiently and be extremely unstable, thus making it impossible to observe under the conditions used.

MATERIALS AND METHODS

Materials.

Puromycin dihydrochloride (neutralised with Tris base before use) and puromycin aminonucleoside (Pan) were obtained from Nutritional Biochemicals Corporation. *E. coli* B tRNA was obtained from Schwarz Bioresearch Inc., EEDQ from Aldrich Chemical Co., and 3-phenyl-propionic acid from Fluka AG Chemische Fabrik. Sigma Chemical Co. supplied ribonuclease T_1, poly (U), BSA, and chloramphenicol. Sparsomycin was obtained from Up John, and PEI chromatography sheets (plastic backed) from Macherey-Nagel Co. Yeast RNA was isolated by Dr. R.H. Symons, and all organic solvents were redistilled prior to use, All t.l.c. was done on Merck Kieselgel F_{254} silica sheets (plastic backed). Toluene scintillation fluid was prepared by dissolving 3.5 g PPO and 0.35 g POPOP in 1.0 l toluene. Triton/toluene scintillation fluid contained triton X-100 / toluene scintillation fluid (1 : 2 by vol.).

Preparation of Ribosomes.

Ribosomes from *E. coli* MRE 600 were prepared as described in Chapter Two.

Preparation of Donor Substrates.

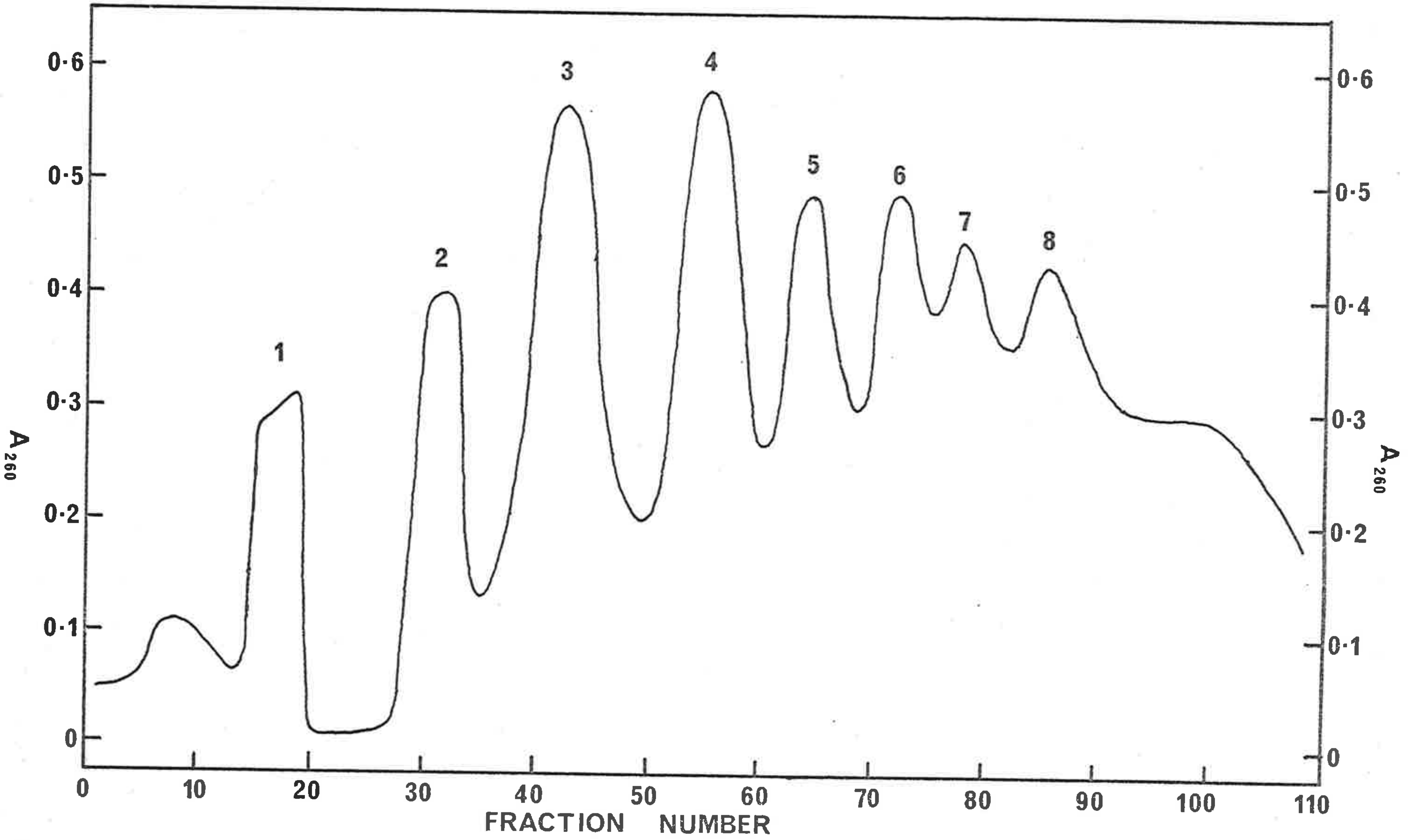
Donor substrates were prepared as described on Chapter Two. Any higher molecular weight material contaminating the pentanucleotide fragment was removed by stepwise chromatography on a small DEAE-Sephadex column prepared in a pasteur pipette. The column was washed with 0.1 M triethyl-ammonium formate, pH 5.0, and the pentanucleotide fragment was then eluted with 0.6 M triethyl-ammonium formate, pH 5.0, and freeze-dried. The purified sample was stored in 0.1 mM sodium acetate, 0.1 mM EDTA, pH 5.0.

Preparation of 3'-Terminal Fragments of *E. coli* B tRNA.

E. coli B tRNA (unaminoacylated) (10 mg) was digested with 30 μg ribonuclease T_1 in 0.5 ml of 20 mM Tris-acetate pH 7.5, 1 mM EDTA at 37°C for 3 h. Ribonuclease T_1 digestion results in the range of fragments from internal digestion as well as the 3'-terminal fragments (which vary in size and structure depending on the tRNA molecule from which they originate.) A column of DEAE-Sephadex (27 cm high, 30 c.c. bed volume) was prepared washed with 1 M NaCl, 50 mM ammonium acetate, pH 5.2, 5 M urea, and equilibrated with 0.02 M NaCl, 50 mM ammonium acetate, pH 5.2, 5 M urea. The digested RNA was applied to the column, and the RNA fragments were eluted with 600 ml of a linear gradient of 0.02 - 1.0 M NaCl containing 50 mM ammonium acetate, pH 5.2, 5 M urea. Using a flow rate of 0.42 ml/min, 3 ml fractions were collected. The A_{260} of the eluate was continually recorded on a Uvicord U.V. Absorptionmeter, and 8 distinguishable peaks of A_{260} material were obtained (Fig. 6).

The fractions in each peak were combined, diluted with 10 volumes 0.02 M NH_4HCO_3, and each peak applied to a DEAE-

FIG. 6: Separation of oligonucleotides resulting from ribonuclease T_1 digestion of tRNA on a DEAE-sephadex column at pH 5.2. See Materials and Methods for experimental details.



0·6
0·5
0·4
0·3
0·2
0·1
0
A260
1
2
3
4
5
6
7
8
0
10
20
30
40
50
60
70
80
90
100
110
FRACTION NUMBER

cellulose column (6.5 cm high, 13 cc bed volume) which had been washed with 1.0 M NH_4HCO_3 and equilibrated with 0.02 M NH_4HCO_3. The NaCl and urea were removed with 0.02 M NH_4HCO_3, and the RNA fragments were eluted with 0.5 M NH_4HCO_3. Triethylamine was added to form the triethylammonium salt, and the solution was evaporated to dryness. The residue was redissolved in 0.1 mM EDTA, 0.1 mM sodium acetate, pH 5.0. The purity and constituents of each peak were checked by t.l.c. on PEI paper with 0.5 M NH_4HCO_3 as solvent, and by high voltage paper electrophoresis on Whatman 3 MM paper in 5% acetic acid, 0.6% triethylamine, 0.1 mM EDTA, pH 3.5, at 53 volts/cm for 45 min. The results, shown in Fig. 7, indicate that the first two peaks eluted from the DEAE-sephadex column contained only GMP. Possibly the first peak was cyclic-GMP which hydrolysed during further isolation to form GMP. The third peak from the column contained dinucleotides, the fourth contained trinucleotides, etc. The smallest 3'-terminal fragment, CpCpA, has the same charge as the internal dinucleotides XpGp at pH 5.2 (where the two phosphate groups give each of the molecules a charge of - 2), and thus would co-chromatograph with the dinucleotides.

It was now necessary to separate CpCpA from the dinucleotide contaminants, and here use was made of the lack of a 3'-phosphate on the terminal fragment. At a higher pH, e.g. 7.2, the second hydroxyl on the 3'-phosphate becomes ionized, giving the dinucleotides an extra negative charge. However, the 3'-terminal fragment, with no such phosphate group, retains the same charge as at pH 5.2. Thus the 3'-terminal CpCpA was separated from the contaminating dinucleotides on a second DEAE-Sephadex column as above, except that the pH was kept at 7.2 with 25 mM Tris-HCl instead

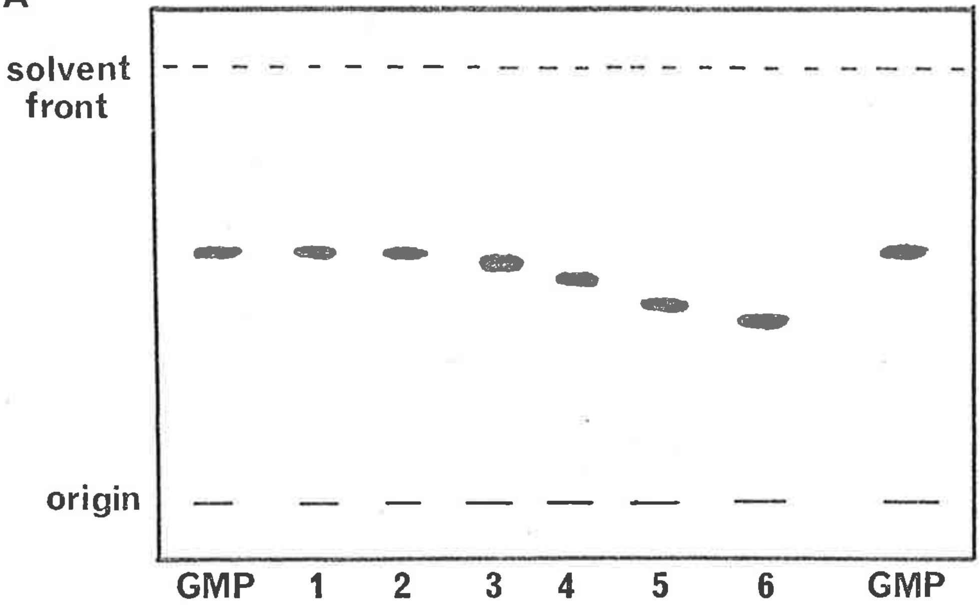
FIG. 7: Characterisation of oligonucleotides obtained from the DEAE-Sephadex column shown in Fig. 6.

A: Thin layer chromatography on PEI paper using 0.6 M NH_4HCO_3 as solvent.

B: Electrophoresis on Whatman 3 MM paper in 5% acetic acid, 0.6% triethylamine, 0.1 mM EDTA, pH 3.5 at 53 volts/cm for 45 min.

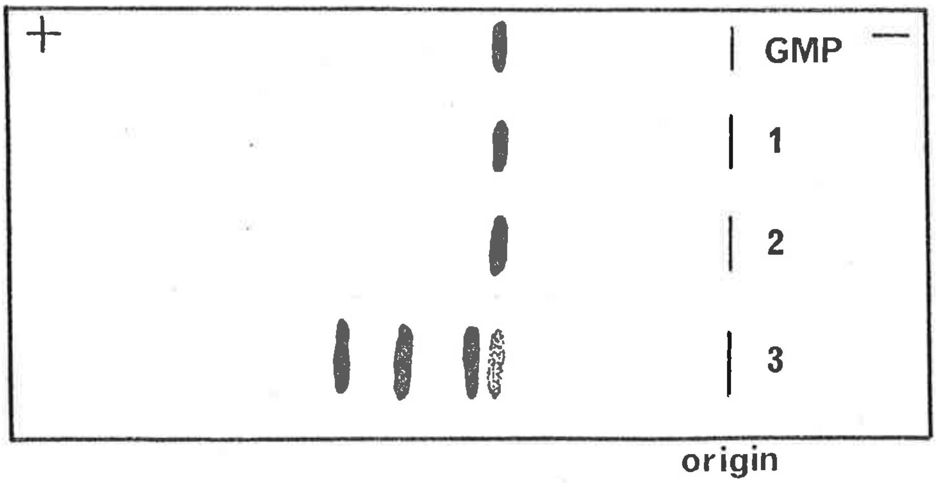
In both experiments, spots were located by viewing under a U.V. light.

A


solvent front
origin
GMP
1
2
3
4
5
6
GMP

T.L.C.

B


+
GMP
1
2
3
origin

ELECTROPHORESIS

of the ammonium acetate, pH 5.2. The A_{260} elution profile is shown in Fig. 8. Ignoring the peaks appearing at the void volume, the CpCpA would be the fastest moving nucleotide material. Thus the leading shoulder of nucleotide material was taken (as shown in Fig. 8), and desalted on DEAE-cellulose and dried as described above. The final residue was redissolved in 0.1 mM sodium acetate, 0.1 mM EDTA, pH 5.0. This solution was taken as the source of CpCpA in subsequent work, although unfortunately it was not in large enough amounts or pure enough to fully characterise and quantitate. However, 5 µl of this crude solution inhibited P site binding of the donor substrate UpApCpCpA-(Ac-L-[^{3}H]Leu) by 29%, indicating that some of the material was entering the P site.

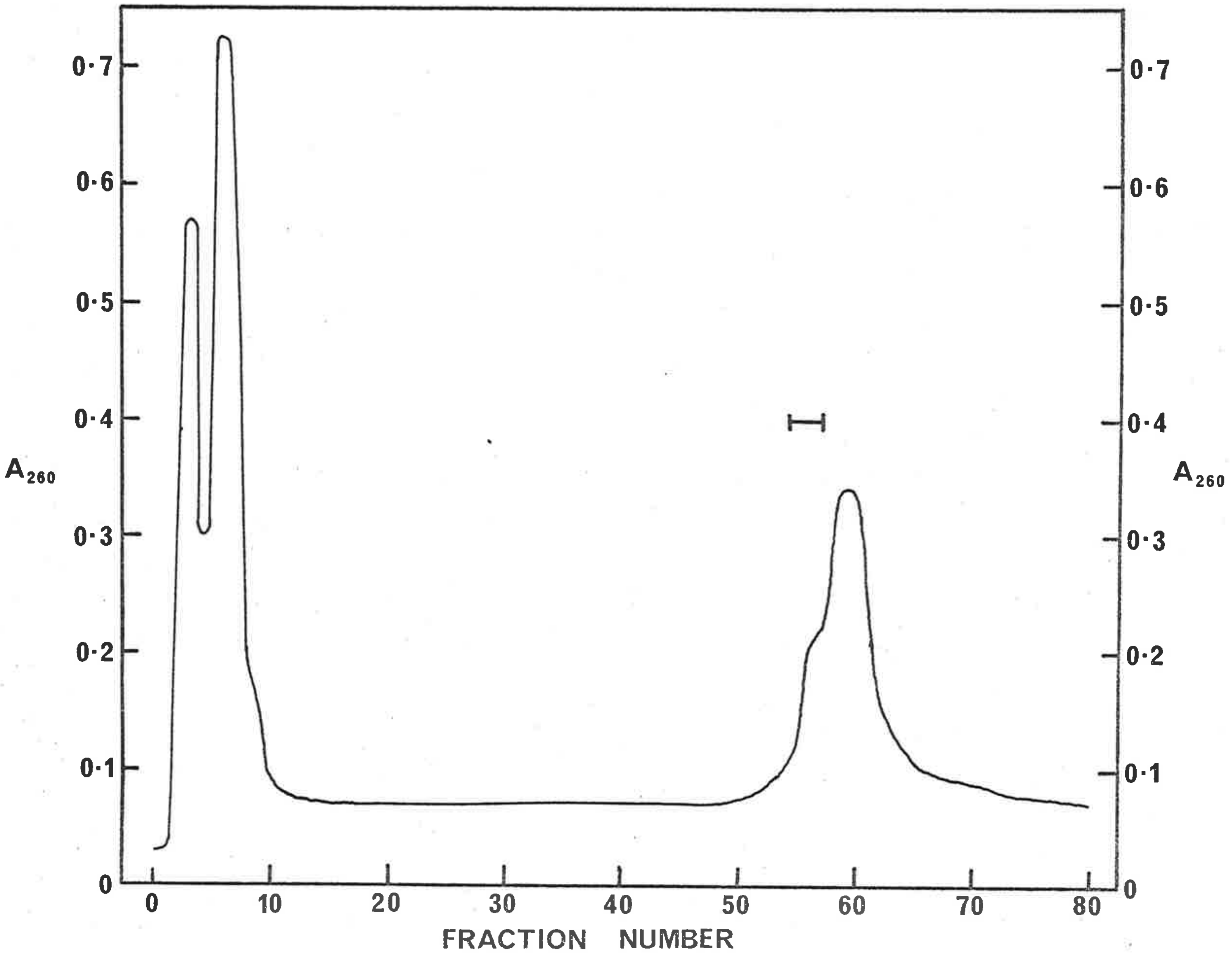
Preparation of 3-Phenyl-propionyl-Pan.

Pan (25 µmole), 50 µmole EEDQ (coupling agent), and 50 µmole 3-phenyl-propionic acid were dissolved together in 0.3 ml ethanol and incubated at 37° C for 1 h. The reaction mixture was then applied to 5 x 8 cm sheets of plastic-backed Merck Kieselgel F_{254} and chromatographed with ethanol: chloroform (1:9, v/v). The lowest band (Rf. = 0.6) was eluted from the silica with ethanol : chloroform (1:1, v/v), and the eluate was filtered to remove remaining silica. The solvent was removed by evaporation, and the solute was dissolved in 2.0 ml ethanol. The yield of chromatographically pure product was 20.7 µmole (83% with respect to Pan), calculated from $E_{mM} = 18.5$ at $\lambda max = 270$ nm.

Inhibition of the Fragment Reaction by 3-Phenyl-propionyl - Pan.

The fragment reaction of Monro and Marcker, (1967) as modified by Mercer and Symons (1972) was used. The reaction mixture contained in 0.17 ml : 14 mM $MgCl_2$, 0.24 M KCl,

FIG. 8: Separation of 3'-terminal fragments from other oligonucleotides on a DEAE-sephadex column at pH 7.2. See Materials and Methods for experimental details. The material used as a crude solution of CpCpA is indicated by ├──┤ .


0·7
0·6
0·5
0·4
A260
0·3
0·2
0·1
0
0
10
20
30
40
50
60
70
80
FRACTION NUMBER
0·7
0·6
0·5
0·4
A260
0·3
0·2
0·1
0

43 mM Tris-HCl, pH 7.2, 18 mM NH_4Cl, 30% (v/v) methanol, 0.12 mM puromycin, 0.18 mg ribosomes, and either 0, 0.37, 1.85, or 3.7 mM 3-phenyl-propionyl-Pan. After incubating the reaction mixture for 30 min at 0°C, the reaction was stopped with the addition of 0.10 ml 0.2 M NaH_2PO_4, pH 5.5, saturated with $MgSO_4$. The product was extracted with 1.0 ml ethyl acetate, and 0.5 ml of the ethyl acetate layer was dissolved in 2.0 ml toluene scintillant for measurement of radioactivity, which is a measurement of the amount of Ac-L-[^3H]Leu-puromycin formed in each reaction mixture.

P'-site Binding Assay.

The standard reaction mixture was modified from that of Celma et al., (1970) and contained in 0.15 ml: 50 mM magnesium acetate, 0.2 M KCl, 50 mM Tris-HCl, pH 7.2, 50% (v/v) methanol, 0.67 pmole CpApCpCpA-(Ac-L-[^3H]Leu), and 0.5 mg ribosomes. After incubation at 0°C for 30 min, the ribosomes (which are precipitated by 50% methanol) were collected by low speed centrifugation at 0°C, and 0.1 ml of the supernatant was dissolved in 0.6 ml triton/toluene scintillation fluid.

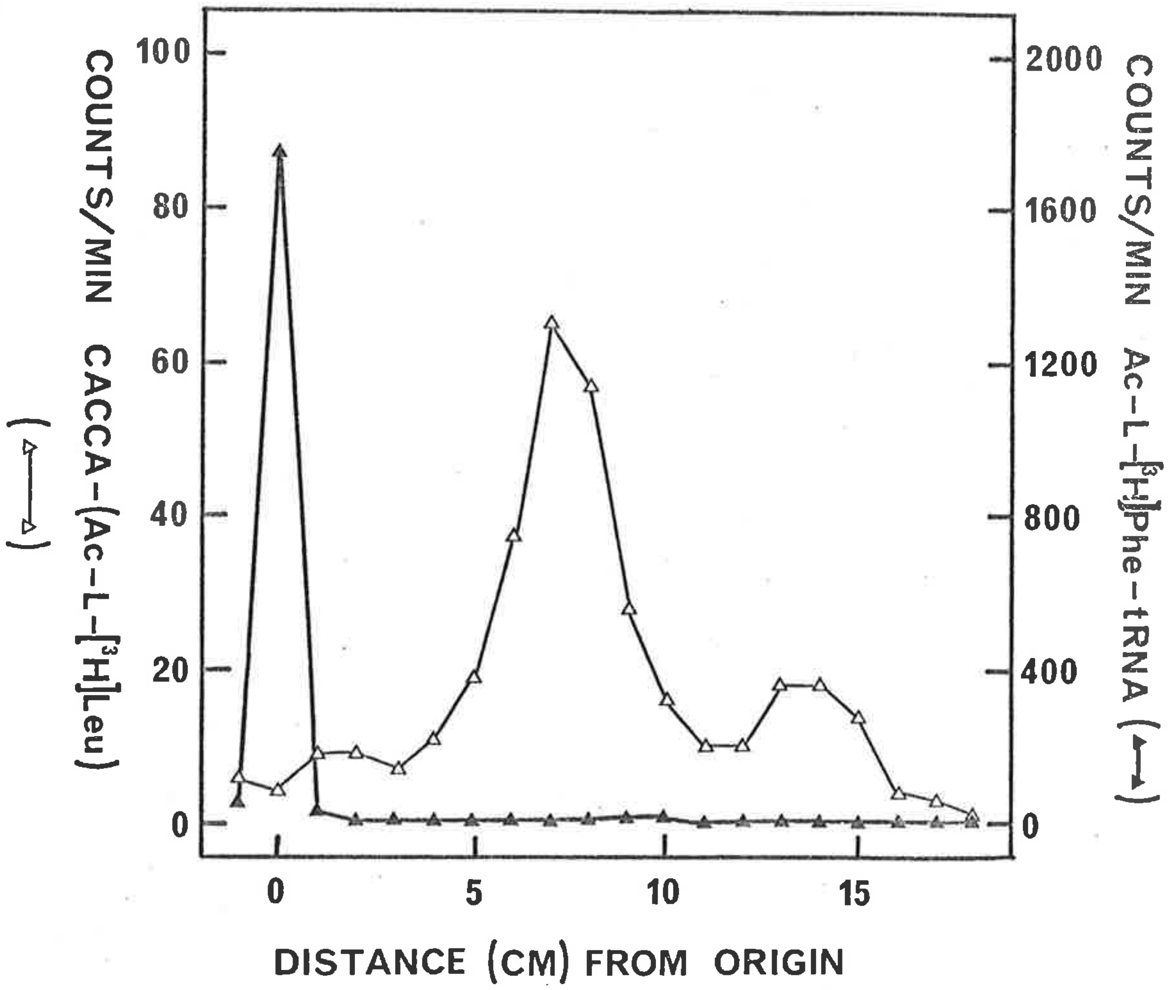
A control minus ribosomes assay was taken as being equivalent to the situation where no donor substrate was bound to the ribosomes. The % reduction in the counts/min present when ribosomes were included was taken as the % bound to the ribosomes.

To optimise conditions for the binding of donor substrate to ribosomes, the donor fragment was incubated with ribosomes under varying conditions. The ranges of conditions used were : 5 - 300 mM magnesium acetate; 0 - 0.5 M KCl; 50 mM Tris - HCl, pH 7.2, 8, 9, or sodium hydrogen maleate, pH 6, or sodium citrate/Na_2HPO_4, pH 3, 4, 5; 50% (v/v) methanol, ethanol, or acetone; 0 - 0.5 mg/ml tRNA; 0 or 0.2 mg/ml poly (U); and 0 or 1.26 mM 3-phenyl-propionyl-Pan. The particular conditions used in a given experiment are described in the legends to Fig. 10.

Assay for Exchange Reaction Between CpApCpCpA-(Ac-L-[^{3}H]Leu) and tRNA.

The standard reaction mixture contained in 0.05 ml : 10 mM magnesium acetate; 0.2 M KCl; 50 mM Tris-HCl, pH 7.2; 18 mM NH_4Cl; 0.67 pmole CpApCpCpA-(Ac-L-[^{3}H]Leu); 2.5 µg E. coli B tRNA; 0.13 mg ribosomes; 50% (v/v) methanol. Control reactions contained no ribosomes. After incubation at room temperature for 0.5 - 2 h, 40 µl of each incubation mix was chromatographed by descending chromatography at 4° C on a strip of DEAE-cellulose paper, using 0.2 M LiCl, 0.02 M triethylammonium acetate, pH 5.0, 5 mM EDTA as solvent, for 3 h. Under these conditions, CpApCpCpA-(Ac-L-[^{3}H]Leu) moved 7 - 8 cm, with little or no material remaining at the origin, while Ac-L-[^{3}H]Leu - tRNA remained at the origin (See Fig. 9). When ribosomes were present in the chromatographed solutions, no change in mobility of either substrate was observed, nor was there any increase in the amount of radioactivity at the origin of the CpApCpCpA-(Ac-L-[^{3}H]Leu) chromatogram. Thus in all assays for exchange, the chromatograms were dried, and the area at the origin was cut out and counted in 2 ml toluene scintillation fluid for the presence of any Ac-L-[^{3}H]Leu-tRNA. In an attempt to observe an exchange, the conditions were varied within the ranges described above in the P'-site binding assays.

FIG. 9: Chromatographic mobility of CpApCpCpA-(Ac-L-[^{3}H]Leu) (△——△) and Ac-L-[^{3}H]Phe-tRNA (▲——▲). Samples were subjected to descending chromatography for 3 h at 4°C on DEAE-cellulose paper using 0.2 M LiCl, 0.02 M triethylammonium acetate, pH 5.0, 5mM EDTA as solvent. The chromatogram was dried, cut into 1 cm strips and counted in 2 ml toluene scintillation fluid.


COUNTS/MIN CACCA-(Ac-L-[³H]Leu)
(△—△)
COUNTS/MIN Ac-L-[³H]Phe-tRNA (▲—▲)
100
80
60
40
20
0
2000
1600
1200
800
400
0
0
5
10
15
DISTANCE (CM) FROM ORIGIN

Assay For Exchange Reaction Between UpApCpCpA-(Ac-L-[^{3}H]Leu) and CpCpA.

The standard incubation mix contained in 0.05 ml: 40 mM Tris-acetate, pH 7.6; 30 mM magnesium acetate; 0.3 M KCl; 50% (v/v) methanol; 0.18 mg ribosomes; 0.67 pmole UpApCpCpA-(Ac-L-[^{3}H]Leu); and 1 µl crude CpCpA prepared as described above. After incubation at 0°C for 1 h, the mixture was electrophoresed for 90 min at 60 volts/cm on Whatman 3 MM chromatography paper in 10% acetic acid, pH 2.4. Under these conditions, UpApCpCpA-(Ac-L-[^{3}H]Leu) would not have any charge, and would remain at the origin, while CpCpA-(Ac-L-[^{3}H]Leu) would have a charge of + 1. Thus the appearance of radioactive material which moved toward the cathode would indicate that exchange had taken place. The electrophoretogram was dried and cut into 1 cm strips which were counted in 2 ml toluene scintillation fluid. In an attempt to find an exchange reaction, conditions were varied over the following ranges: 4 - 300 mM magnesium acetate; 40 mM Tris-acetate, pH 7, 8, 9, or sodium hydrogen maleate, pH 6, or sodium citrate / Na_2HPO_4, pH 3, 4, 5; 50% (v/v) methanol, ethanol, or acetone; 2 - 400 mM KCl; 0 or 1.26 mM 3-Phenyl-propionyl-Pan.

Attempted Isolation of an Ac-L-[^{3}H]Leu -ribosome Intermediate.

The standard incubation mix contained in 0.15 ml : 0.4 M KCl; 20 mM magnesium acetate; 20 mM Tris-acetate, pH 7.6; 33% (v/v) methanol; 0.18 mg *E. coli* ribosomes; 0.67 pmole CpApCpCpA-(Ac-L-[^{3}H]Leu). Control reaction mixes contained no ribosomes. After 20 min incubation at 0° C, the products were assayed by two different methods.

(a) Total precipitation of ribosomes.

After incubation, 50 µl of 10 mg/ml yeast RNA was added to the mixture, and the incubation immediately stopped with 1.0 ml cold 20% TCA. The mixture was left at 0° for 30 min, after which the prescipitated macromolecules (protein and RNA) were collected on Whatman GF/A glass-fibre filter discs. The filter discs were washed with 4 x 5 ml cold 5% TCA and 2 x 2 ml cold ether, dried, and counted in 2.0 ml toluene scintillation fluid. Any radiactivity above that in control experiments would indicate the presence of a covalently bound acyl-enzyme intermediate.

(b) Separate precipitation of RNA and protein from incubated ribosomes.

After incubation, 50 μl of 10 mg/ml yeast RNA was added, and the incubation was stopped by adding 0.4 ml 0.3 M magnesium acetate in glacial acetic acid. After 30 min at 0°C, the RNA precipitate was collected by centrifugation at 17,000 g for 1 h at 4°C. The supernatant (containing ribosomal proteins) was poured off and stored at 0°C. The RNA pellet was resuspended in 0.3 M magnesium acetate in glacial acetic acid at 0°C, and the precipitate was collected on GF/A filter discs under reduced pressure. The discs were washed with 3 x 2 ml cold 0.2 M magnesium acetate in 67% acetic acid, 33% H_2O (by vol.), and then rinsed with cold ether. After drying, the filters were counted in 2 ml toluene scintillation fluid.

The ribosomal proteins in the supernatant of the acetic acid extraction were precipitated, after addition of 20 μl 20 mg/ml BSA as carrier protein, with 2.0 ml 20% TCA. After 30 min at 0°C, the protein precipitate was collected on GF/A glass-fibre filter discs and washed with 5 x 5.0 ml 5% TCA and 2 x 2.0 ml cold ether. The filters were dried and counted in 2.0 ml toluene scintillation fluid.

Variations from the standard incubation mix were: 20 mM glycine -HCl, pH 3, or sodium acetate-acetic acid, pH 4 or 5, or sodium cacodylate-HCl, pH 6 or 7, instead of Tris-acetate, pH 7.6; and addition of either 1.25 mM 3-phenyl-propionyl-Pan, 1.25 mM puromycin, 1.0 mM chloramphenicol, or 0.1 mM sparsomycin (i.e. A'-site specific substrates).

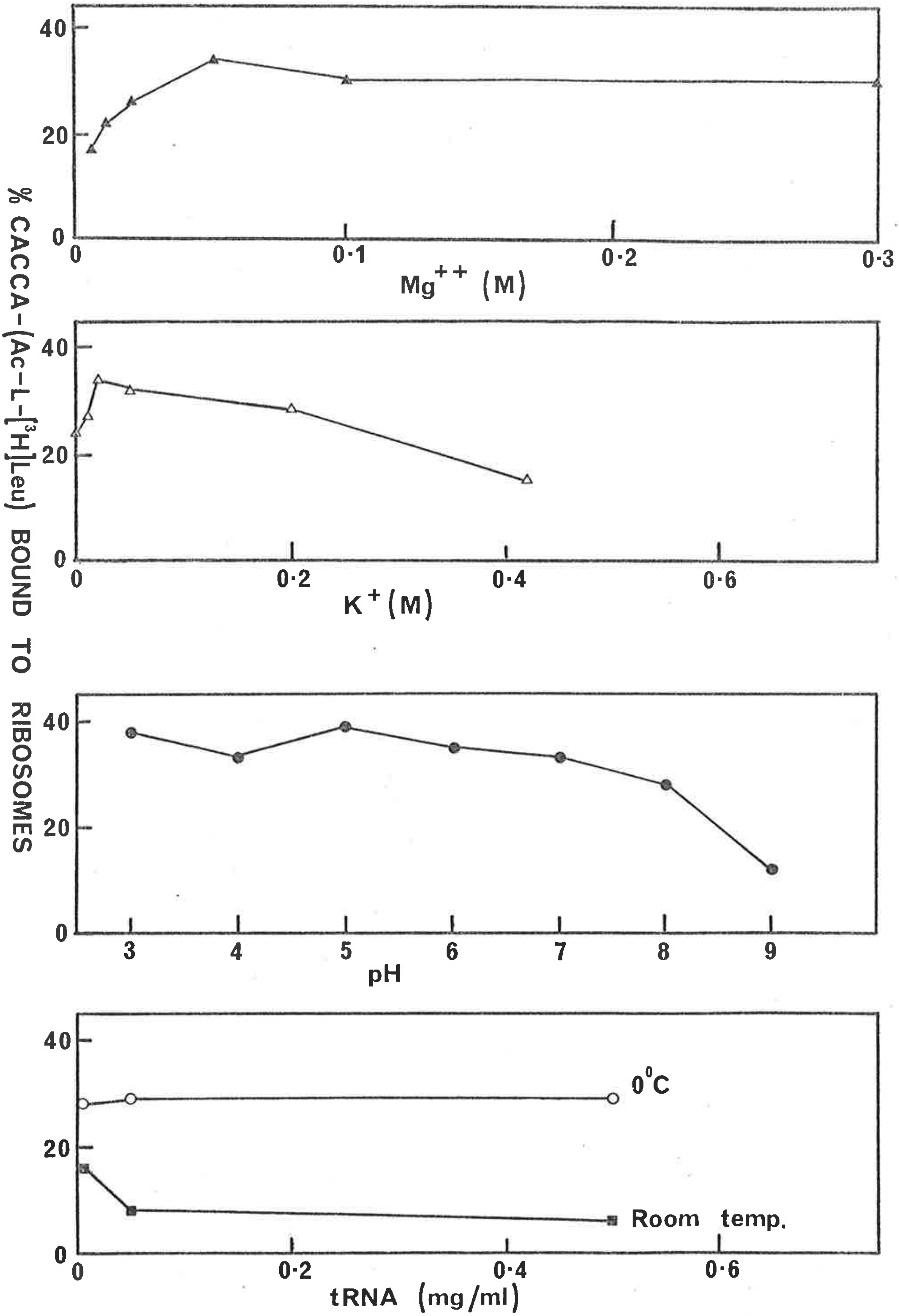
RESULTS AND DISCUSSION

Optimization of P'-site Binding.

The first experiments involved optimization of the P'-site binding of the donor substrate CpApCpCpA-(Ac-L-[^{3}H]Leu) so that the maximum opportunity was given for any formation of the aminoacyl-ribosome intermediate. The P'-site binding assay described in Methods is a difference assay which does represent donor substrate binding, since the addition of 1.0 mM spiromycin, a known inhibitor of donor substrate binding to the P'-site (Oleinick and Corcoran, 1970; Pestka, 1974; Tai et al., 1974), reduced the counts/min present in the ribosome pellet by 70%.

The optimization results are shown in Fig. 10. Increasing the Mg^{++} concentration from 0.005 M to 0.05 M resulted in an increase in fragment binding from 17% to 34%. Further increasing the Mg^{++} concentration to 0.3 M had little effect on the binding. Increasing the K^+ concentration from 0 to 0.02 M increased the binding from 24% to 34%. Further increasing the K^+ concentration resulted in a decrease in the amount of fragment bound to the to the ribosomes : at 0.2 M K^+ it had dropped to 28%, while at 0.42 M it was 15%. However, it appears that a wide range of K^+ concentrations had only

FIG. 10: The effect of varying Mg^{++}, K^{+} and pH and the addition of tRNA on the binding of CpApCpCpA-(Ac-L-[^{3}H]Leu) to the P'-site of peptidyl transferase. The standard reaction mixture described in Materials and Methods was used except for the component under study. The % of CpApCpCpA-(Ac-L-[^{3}H]Leu) bound to the ribosome was determined as described in Materials and Methods.


% CACCA-(Ac-L-[³H]Leu) BOUND TO RIBOSOMES
40
20
0
0
0·1
0·2
0·3
Mg++ (M)
40
20
0
0
0·2
0·4
0·6
K+ (M)
40
20
0
3
4
5
6
7
8
9
pH
40
20
0
0°C
Room temp.
0
0·2
0·4
0·6
tRNA (mg/ml)

a small effect on P'-site binding of the donor fragment. At pH levels between 3 and 7, the amount of binding remained essentially constant at between 33% and 39%. However, at higher pH values, the level of binding decreased until, at pH 9, only 12% remained bound. This decrease at high pH values may also be partly due to hydrolysis of the donor fragment. At 0°C, tRNA had no effect on P'-site binding of CpApCpCpA-(Ac-L-[^{3}H]Leu). However, at 37°C, binding of fragment was reduced by 50% at the higher concentrations of tRNA. The amount bound to ribosomes in the absence of tRNA at 37°C was lower than at 0°C. At 0°C, it appears that tRNA could not enter the P'-site with any efficiency, and thus subsequent experiments with tRNA were done at a higher temperature. Ethanol was found to promote binding of fragment similarly to methanol. However, acetone precipitated the fragment, which prevented its testing in the P'-site binding assay.

In general, a wide variation in conditions had minimal effect on P'-site binding of CpApCpCpA-(Ac-L-[^{3}H]Leu). Variations in concentrations of Mg^{++} from 0.02 M to 0.3 M, in K^+ from 0.01 M to 0.42 M, or varying the pH in the range 3 to 8 only gave a change in the % binding from 26% to 39%. Thus a wide range of conditions can be used to try to find those conditions stabilising the peptidyl-ribosome intermediate with a minimal effect on the level of binding of donor fragment to the P'-site of the ribosome.

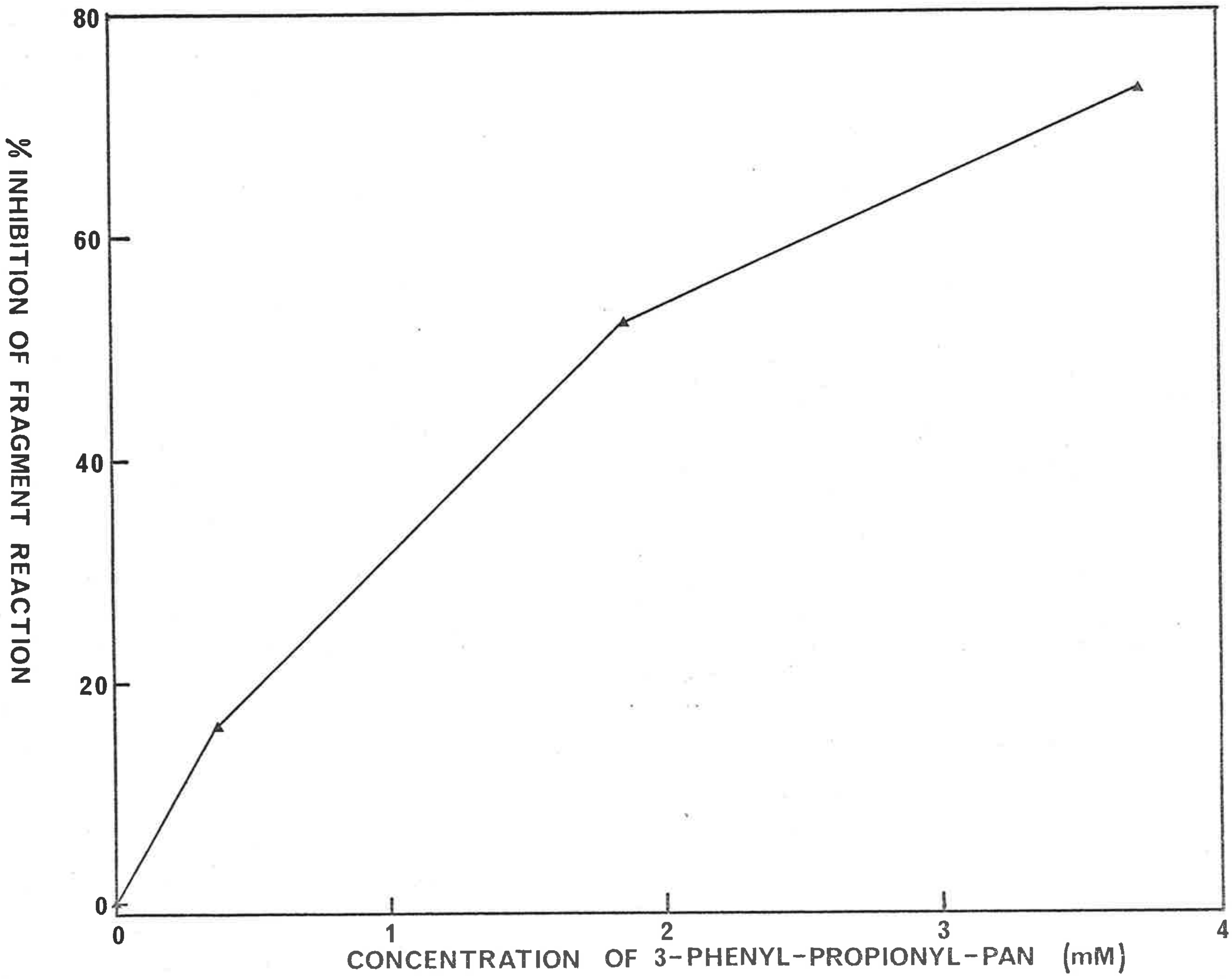
Synthesis and Properties of 3-Phenyl-propionyl-Pan.

It is possible that the A'-site of peptidyl transferase may have to be occupied before any reaction can occur between the donor substrate and the ribosome to form the acyl-enzyme intermediate. To check this possibility, it was desirable to have an analogue of aminoacyl-tRNA which would bind efficiently to the A'-site, yet not accept the Ac-L-[^{3}H]Leu from the donor substrate. One possible analogue was 3-phenyl-propionyl-Pan, which is a structural analogue of the antibiotic puromycin and which does not contain the α-NH_2 group of puromycin that accepts the donor substrate during peptide bond formation. To test its ability to enter the A'-site, 3-phenyl-propionyl-Pan was used as an inhibitor of the fragment reaction of Monro and Marcker, (1967) as described in Methods, and the results are shown in Fig. 11. The graph shows that at 1.75 mM, 3-phenyl-propionyl-Pan caused a 50% inhibition of the fragment reaction using 0.35 mM puromycin. Thus, 3-phenyl-propionyl-Pan effectively binds to the A'-site and inhibits the fragment assay. If puromycin was replaced by 3-phenyl-propionyl-Pan in the fragment reaction, no product at all was obtained; i.e. no peptide bond formation occurred. Thus 3-phenyl-propionyl-Pan enters the A'-site, but, as expected, cannot accept Ac-L-[^{3}H]Leu.

Exchange of Ac-L-[^{3}H]Leu from CpApCpA-(Ac-L-[^{3}H]Leu) to tRNA.

Initial experiments to find evidence for an acyl-enzyme intermediate involved attempts to find exchange of Ac-L-[^{3}H]Leu between CpApCpCpA and tRNA (see Fig. 5). Firstly, it was necessary to devise an assay method which could efficiently separate CpApCpCpA-(Ac-L-[^{3}H]Leu) from any Ac-L-[^{3}H]Leu-tRNA which may be formed, and DEAE-cellulose paper chromatography as described in Methods above successfully did this.

FIG. 11: Inhibition of the fragment reaction by 3-phenyl-propionyl-Pan. The % inhibition of the fragment reaction by various concentrations of 3-phenyl-propionyl-Pan was determined as described in Materials and Methods.


% INHIBITION OF FRAGMENT REACTION
80
60
40
20
0
0
1
2
3
4
CONCENTRATION OF 3-PHENYL-PROPIONYL-PAN (mM)

In all exchange assays under the various conditions described in Methods, a background level of 20 counts/min was found at the origin, which was also present in minus ribosome control assays; i.e. no exchange had taken place.

Exchange of Ac-L-[^{3}H]Leu from CpApCpCpA-(Ac-L-[^{3}H]Leu) to CpCpA.

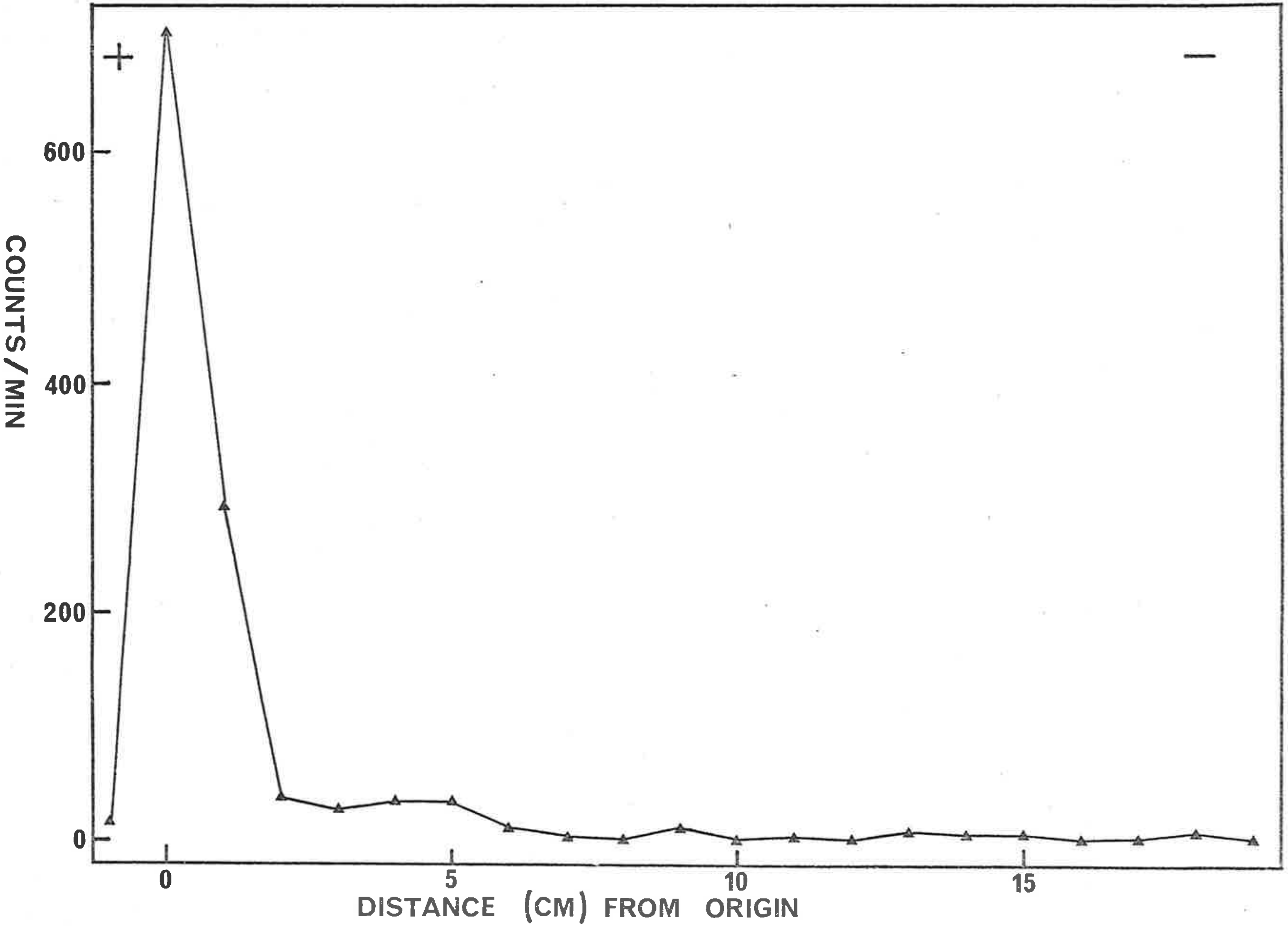
Since unaminoacylated tRNA did not appear to be an efficient inhibitor of the P'-site binding of CpApCpCpA-(Ac-L-[^{3}H]Leu), particularly at lower temperatures (Fig.10), it was thought that a small 3'-terminal fragment of tRNA may be more efficient at binding to the P'-site of the ribosome. Thus CpCpA was partially purified as described in Methods, and used in the exchange assay in place of tRNA.

To assay for any exchange of Ac-L-[^{3}H]Leu which may have occurred between UpApCpCpA and CpCpA, high voltage paper electrophoresis at pH 2.4 was used as described in Methods. Using the various conditions described in Methods, the radioactive profile of the electrophoretogram remained the same in all cases (Fig. 12), including control reactions which did not contain ribosomes. No significant new radioactive peak appeared under any of the conditions used, once more indicating no exchange had occurred.

Attempted Isolation of an Ac-L-[^{3}H]Leu -ribosome Intermediate.

Due to the failure to observe any exchange reaction, a new approach involving an attempt to isolate the peptidyl-ribosome complex was tried as described in Methods. The rationale behind these experiments was firstly to incubate the donor substrate CpApCpCpA-(Ac-L-[^{3}H]Leu) with ribosomes

FIG. 12: Electrophoresis of UpApCpCpA-(Ac-L-[^{3}H]Leu) and reaction mixes used to assay for ribosome catalysed exchange of Ac-L-[^{3}H]Leu from UpApCpCpA-(Ac-L-[^{3}H]Leu) to CpCpA as described in Materials and Methods. Samples were electrophoresed on Whatman 3 MM paper in 10% acetic acid, pH 2.4, for 90 min. at 60 volts/cm. The electrophoretogram was dried, cut into 1 cm strips, and counted in 2 ml toluene scintillation fluid.


+
—
600
400
200
0
COUNTS / MIN
0
5
10
15
DISTANCE (CM) FROM ORIGIN

such that a small proportion of the Ac-L-[^{3}H]Leu might be covalently attached to the ribosome if the intermediate does exist. If the ribosomes are then placed in an environment where they are instantly denatured, the acyl-protein or acyl-RNA complex may remain intact and thus allow its detection. This type of approach was used by Balls and Wood, (1956) to isolate the acyl-enzyme intermediate of chymotrypsin.

Once again, however, under all conditions used, (as described in Methods), no radioactivity became associated with the ribosome. Neither the precipitated total ribosomes nor the separately precipitated ribosomal proteins or RNA were labelled to any significant extent. Various antibiotics (e.g. puromycin, chloramphenicol, and sparsomycin) and 3-phenyl-propionyl-Pan were used in these experiments in the hope that the binding of these molecules to the A'-site may enhance formation of the intermediate. However, all had no effect.

Thus all three approaches used to find evidence for a peptidyl-ribosome intermediate were unsuccessful. However, this in no way proves that the intermediate does not exist. It may be that it only exists transiently, and may be very unstable, such that these experiments could never observe its presence.

General Conclusions

The experiments described above have not provided any evidence for an acyl-ribosome intermediate occurring during peptide bond formation. Despite a wide variation in the conditions used in the experiments (e.g. 5 - 300 mM Mg^{++}, 0 - 0.5 M K^+, pH 3 - 9) and the addition of compounds which could possibly enhance the formation of such an intermediate (e.g. methanol, ethanol, poly (U), and various antibiotics or their analogues which bind to the A'-site), neither the exchange of Ac-L-[^{3}H]Leu between two different P'-site substrates nor the isolation of the acyl-ribosome complex was observed.

CHAPTER FOUR

AFFINITY LABELLING OF RAT LIVER RIBOSOMES BY BAP-PAN-PHE

AFFINITY LABELLING OF RAT LIVER RIBOSOMES BY BAP-PAN-PHE

INTRODUCTION

Previous work by Harris *et al.*, (1973) and Greenwell *et al.*, (1974) in this laboratory showed that the chemically reactive analogue of puromycin, Bap-Pan-Phe, binds covalently to *E. coli* ribosomes in the active site of peptidyl transferase. This covalent attachment was found to be specific for 23S RNA. This chapter describes work done to find if the same analogue was also an affinity label of rat liver ribosomal peptidyl transferase, and if so, to identify the components of the ribosome which were bound to the affinity label.

MATERIALS AND METHODS

Materials.

[^{3}H]puromycin (Specific activity 3.7 Ci/m mol) was obtained from The Radiochemical Centre, Amersham, England. Sigma Chemical Co. supplied DTE and L-Phe.

Buffer solutions were as follows:

Solution A: 0.2 M sucrose, 0.1 M NH_4Cl, 5 mM magnesium acetate, 1 mM DTE, 20 mM Tris-HCl, pH 7.5.

Solution B: as in solution A except with 0.7 M sucrose.

Solution C: as in solution A except with 2 M sucrose.

Solution D: 10 mM Tris-acetate, pH 7.2, 5 mM magnesium acetate, 50 mM KCl.

Preparation of Rat Liver Ribosomes.

Ribosomes were prepared as described in Chapter Two.

Preparation of Donor Substrates.

The donor substrate CpApCpCpA-(Ac-L-[^{3}H]Leu) was prepared as described in Chapter Two.

Activity of Rat Liver Ribosomes.

The reaction mixture was essentially that of Monro and Marcker, (1967) as modified by Greenwell *et al.*, (1974), and contained in 0.05 ml : 0.05 mg ribosomes, 0.67 pmole CpApCpCpA-(Ac-L-[^{3}H]Leu), 2 mM puromycin, 40 mM magnesium acetate, 0.4 M KCl, 50 mM Tris-HCl, pH. 7.2, 30% (v/v) methanol. After incubation at 0°C for 0 - 40 min, reactions were stopped with 5 µl 3N NaOH, and the mixtures incubated at 37° C for 15 min to hydrolyse any Ac-L-[^{3}H]Leu-methyl ester which forms during the assay. Then 0.1 ml 0.2 M NaH_2PO_4, pH 5.5, saturated with Mg SO_4 was added, and the reaction product extracted into 2 ml toluene scintillant / ethyl acetate (4 : 1 by vol.) by vortexing. The samples were then counted in a Packard liquid scintillation spectrometer.

Effect of Ribosome Concentration on the Fragment Reaction.

The reaction mixtures and assay methods were the same as described for the activity measurement above, except that 0, 0.01, 0.02, 0.05, or 0.1 mg ribosomes were used, and samples were incubated at 0° C for 30 min.

Preparation of Affinity Label.

Both [^{32}P] - labelled and non-radioactive Bap-Pan-Phe were prepared by Dr. R.H. Symons essentially as described by Greenwell *et al.*, (1974).

Affinity Labelling of Ribosomes, and Protection by Puromycin.

Incubations contained in 0.05 ml : 0.1 - 2 mM Bap-Pan-Phe, 40 mM magnesium acetate, 0.3 M KCl, 40 mM Na_2CO_3 adjusted to pH 8.8 with $NaHCO_3$, and 0.25 mg rat liver ribosomes. Where indicated, 3 mM puromycin was also added. After 18 h at 27° C, the ribosomes were precipitated at 0°C by a solution containing 40 mM magnesium acetate, 0.4 M KCl, 50 mM Tris-acetate, pH 7.2, and 30% methanol (v/v). The ribosomes were collected by low-speed centrifugation, and resuspended in 0.05 ml 50 mM Tris-HCl, pH 7.4, 2 mM magnesium acetate, 10 mM KCl. The precipitation cycle was repeated a further 3 times, and the ribosomes were then tested for activity in the fragment reaction as described above.

Ki of Bap-Pan-Phe in the Fragment Reaction.

The reaction mixture contained in 0.05 ml : 40 mM magnesium acetate, 0.4 M KCl, 50 mM Tris-HCl, pH 7.2, 30% (v/v) methanol, 0.1 mg ribosomes, either 5×10^{-4} M or 5×10^{-5}M puromycin, and either 2×10^{-5}M, 5×10^{-5}M, 10^{-4}M, 2×10^{-4}M, 5×10^{-4}M, or 10^{-3}M Bap-Pan-Phe. The reactions were assayed as described above for the fragment reaction, and the results expressed in a Dixon plot, (Dixon, 1953). When the graphs of $\frac{1}{v}$ $\left(\frac{1}{cpm}\right)$ against concentration of Bap-Pan-Phe at both puromycin concentrations have been drawn, the Ki of Bap-Pan-Phe can be found from the point of intersection of the two lines corresponding to the two different puromycin concentrations.

Attempted Transfer of Ac-L-[^{3}H]Leu from CpApCpCpA-(Ac-L-[^{3}H]Leu) to Affinity Labelled Ribosomes.

Ribosomes were incubated with Bap-Pan-Phe and washed by precipitation as described above. The transfer reaction mix contained in 0.075 ml : 40 mM magnesium acetate, 0.4 M KCl, 50 mM Tris-acetate, pH 7.2, 60% methanol (v/v), 6 pmole CpApCpCpA-(Ac-L-[^{3}H]Leu), and 0.02 mg affinity-labelled ribosomes. After 3.5h at 37°C, 0.02 ml 20 mg/ml BSA was added, and the macromolecules were precipitated with 2 ml cold 10% (w/v) TCA. After 30 min at 0°C, the precipitate was collected by low speed centrifugation. The pellet was washed by redissolving in 0.15 ml 0.1 N NaOH, and then reprecipitating with 10% TCA. This washing was repeated a further three times, the final pellet was dissolved in 0.2 ml NCS solubiliser in 2 ml toluene scintillation fluid, and the final mixture was counted for bound radioactivity with a counting efficiency of 25% (determined by counting a known amount of [^{3}H]toluene standard under identical conditions).

Identification of Ribosomal Components Labelled with Bap-Pan-Phe.

Ribosomes were affinity labelled with [^{32}P]Bap-Pan-Phe and washed as described above except on a four times larger scale. Aliquots (10 µl) of the affinity labelled ribosomes were taken for determinations of (1) A_{260}, to calculate the ribosome concentration, (2) peptidyl transferase activity (fragment reaction, as described above) to find the extent of affinity labelling, and (3) radioactivity, by dissolving the sample in 2 ml triton/toluene scintillation fluid to calculate the stoichiometry of labelling.

The remaining [^{32}P]affinity labelled ribosomes were fractionated into protein and rRNA as follows to allow determination of the distribution of affinity label. The ribosomes were mixed with 125 pmole untreated ribosomes, and 2 volumes of 0.3 M magnesium acetate in glacial acetic acid was added to precipitate RNA (Kaltschmidt and Wittmann, 1972). After 1 h at 0°C, the precipitate was collected by centrifugation at 17,000 g for 45 min at 4°C. The supernatant, which contained ribosomal proteins, was poured into tubes and stored at 4°C, while the RNA pellet was resuspended in 0.2 ml 50 mM Tris-HCl, pH 7.4, 2 mM magnesium acetate, 10 mM KCl. The RNA was again precipitated and collected as above, and the supernatant discarded. Excess liquid was removed from the pellet with a strip of Whatman 3 MM chromatography paper, the pellet was resuspended in 0.2 ml 0.5 M Tris base (to neutralise excess acetic acid), and the RNA was immediately precipitated with 0.5 ml cold ethanol. After 16 h at -15°C, the RNA was collected by centrifugation at 12,000 g for 30 min at 4°C, the supernatant was discarded, and the pellet dried under vacuum. The residue was dissolved in 0.05 ml 0.1 mM EDTA, pH 6.8, and 5 µl aliquots were taken for either (1) A_{260} readings to compare the amount of RNA in various samples, or (2) measuring the radioactivity bound to RNA following solubilising in 0.5 ml triton/toluene scintillation fluid.

Ribosomal proteins, contained in the supernatant from the first acetic acid precipitation above, were precipitated with 2.0 ml cold 20% TCA. After 30 min at 0°C, the precipitate was collected by low speed centrifugation.

The supernatant was discarded, and the pellet was washed with 1.0 ml ethanol/ether (1 : 1 by vol.). The pellet was recentrifuged at low speed, the supernatant poured off, and excess liquid removed from the pellet by a strip of Whatman 3 MM chromatography paper. The pellet was redissolved in 0.1 mM EDTA, pH 6.8, and 5 µl aliquots were dissolved in 0.5 ml triton/toluene scintillation fluid for measurement of radioactivity bound to the proteins.

Preparation of Polysomes.

Polysomes were prepared from rat liver essentially as described by Pestka et al., (1972). Livers from overnight starved rats were rinsed three times in 0.85% (w/v) NaCl, minced with scissors, and homogenised in a Potter-Elvehjem glass homogeniser using 3.0 ml of solution A per g of liver. The homogenate was centrifuged at 15,000 g for 15 min at 4°C. To the supernatant was added 10% (w/v) sodium deoxycholate in 50 mM Tris-HCl, pH 8.0, to give a final concentration of 1.3% deoxycholate. Aliquots (5.5 ml) of the solution were layered onto 2.0 ml solution B which had been layered onto 2.0 ml solution C in 10 ml Beckmann Ti 50 polycarbonate tubes. The tubes were centrifuged at 90,000 g for 8 h at 4°C. The supernatants were discarded, the polysome pellets were each rinsed with 2.0 ml solution D, the liquid removed, and the pellets then suspended in 0.25 ml solution D. The solutions were combined and cleared of any aggregated material by centrifugation at 12,000 g for 15 min at 4°C. The supernatant, containing polysomes, was stored in liquid nitrogen. Assuming that at 260 nm, the ribosome data of $A^{1\%}_{1\ cm} = 140$ (Petermann, 1971) is also applicable to polysomes (since by far the majority of any A_{260} readings would be due to ribosomal RNA), a yield of 66 mg of polysomes was obtained from 34 g of rat liver.

The polysomes were analysed on 15 - 30% sucrose gradients by layering 0.56 mg polysomes onto the gradients which were then centrifuged at 40,000 revs/min in a SW 41 rotor for 45 min. The gradients were then analysed on an Isco fractionator to give the profiles shown in Fig. 13, which show that polysomes containing up to 14 ribosomes may be present in the sample.

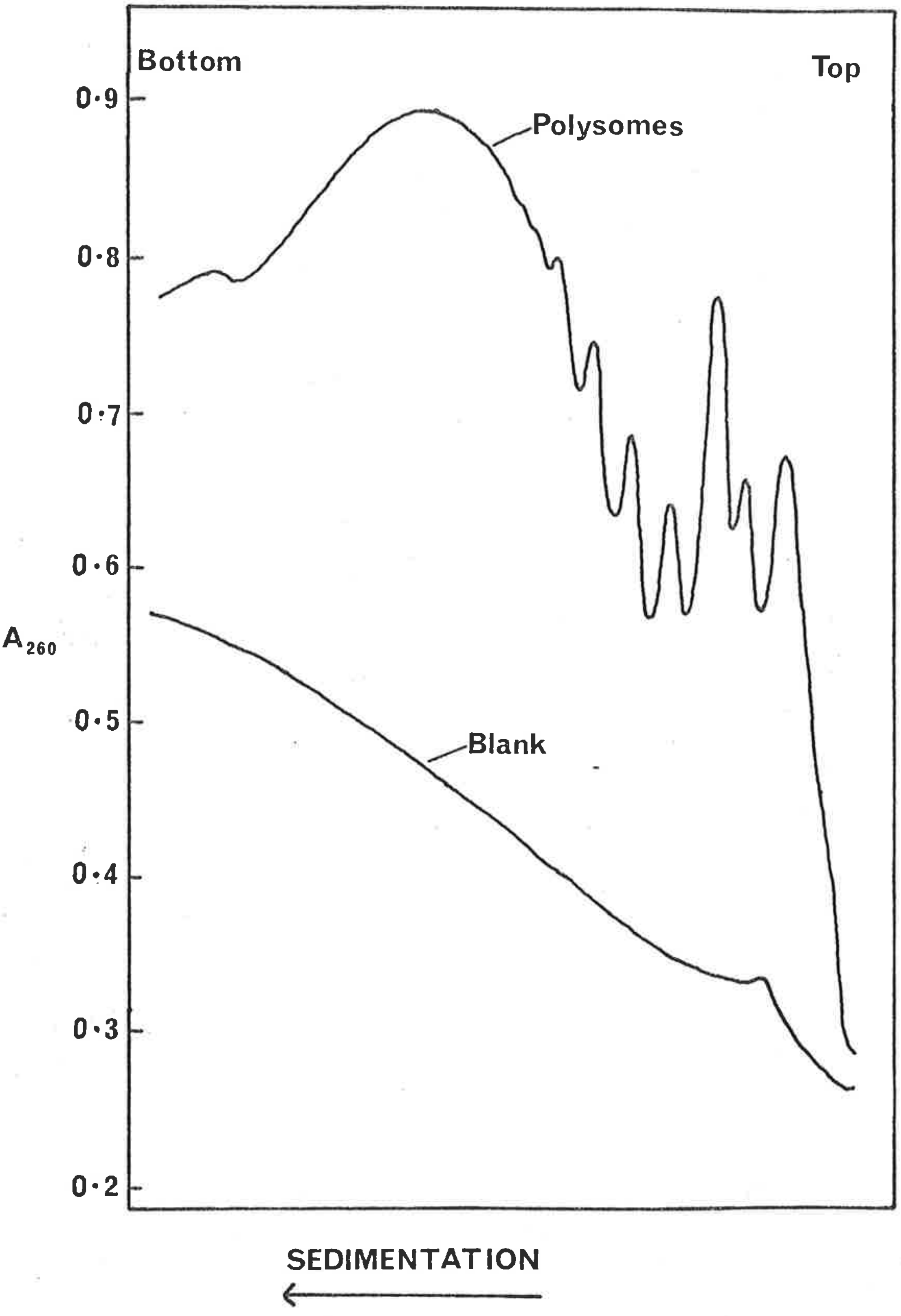
Activity Assay for Polysomes.

The assay, which measures [^{3}H]puromycin-peptide formation, was essentially that of Pestka *et al.*, (1972). The incubation mix contained in 0.1 ml : 5 mM magnesium acetate, 0.5 M KCl, 50 mM Tris-acetate, pH 7.2, 6.5 x 10^{-6} M [^{3}H]-puromycin (spec. act. 595 mCi/ m mol), 0.16 mg polysomes. After incubation at 37°C for 1 min, the reaction was stopped by the addition of 2.0 ml cold 10% (w/v) TCA. The mixture was left at 0°C for 30 min, and the precipitate was collected on Whatman GF/A filter discs, washed with 3 x 5.0 ml cold 5% TCA, and stirred in 1% (v/v) HCl in ethanol. The filters were then dried, and the radioactivity measured by adding 2.0 ml toluene scintillation fluid and counting.

Km For Puromycin On Polysomes.

The reaction mixtures contained in 0.1 ml : 5 mM magnesium acetate, 0.5 M KCl, 50 mM Tris-acetate, pH 7.2, 0.24 mg polysomes, and either 6.25 x 10^{-6} M, 3.125 x 10^{-6} M,

__FIG. 13:__ Sucrose gradiant of rat liver polysomes. Polysomes, prepared as described in Materials and Methods, were centrifuged in a 15 - 30% sucrose gradiant and the gradiant was then fractionated as described in Materials and Methods.


Bottom
Top
Polysomes
Blank
A260
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
SEDIMENTATION

1.25×10^{-6} M, or 6.25×10^{-7} M [^{3}H]-puromycin (spec. act. 595 mCi/mmol.). The reactions were assayed as above, and the results expressed as a double reciprocal plot of $\frac{1}{V}$ vs $\frac{1}{S}$.

Ki of Bap-Pan-Phe On Polysomes.

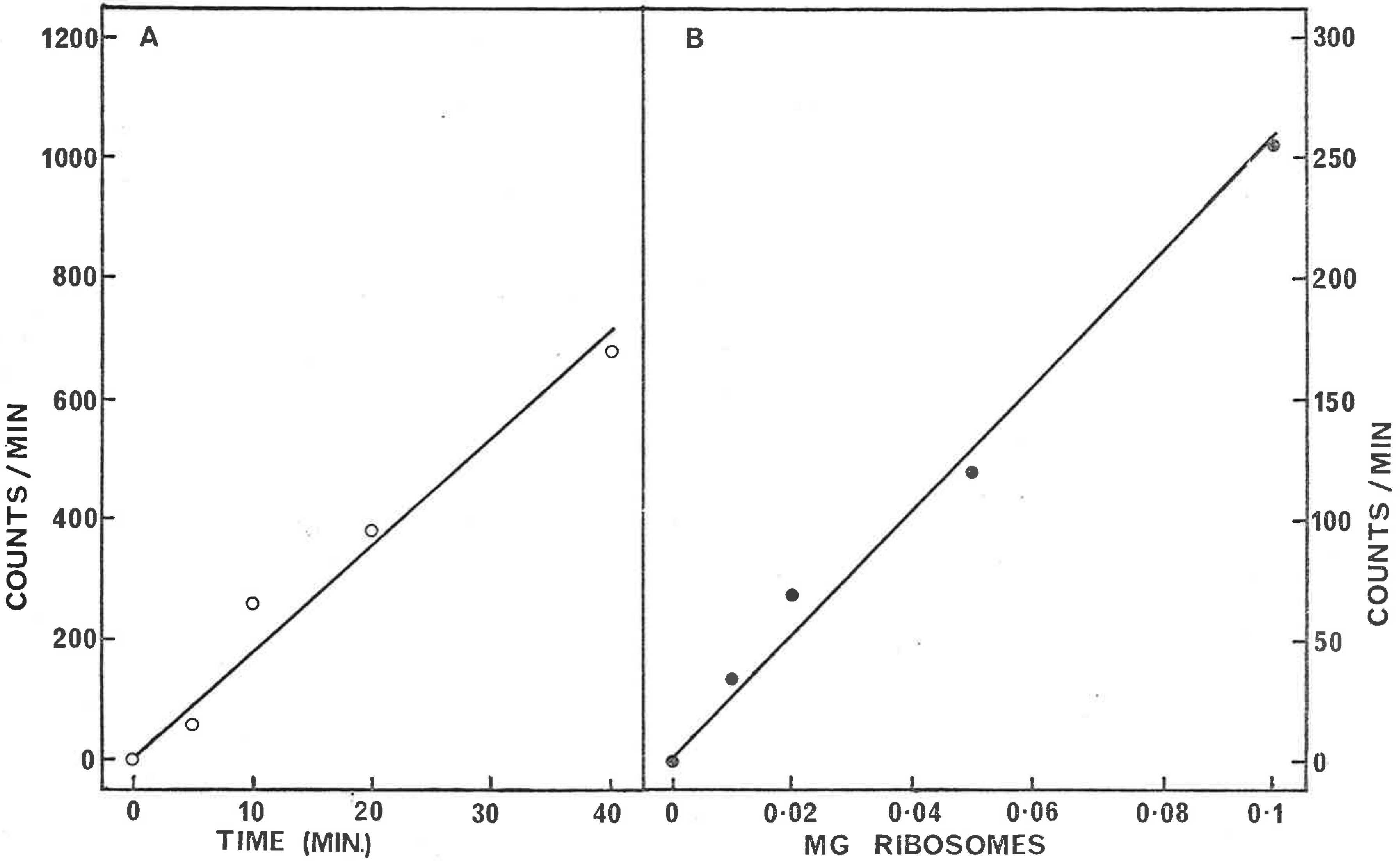
The reaction mixture contained in 0.1 ml : 0.5 M KCl, 5 mM magnesium acetate, 50 mM Tris-acetate, pH 7.2, 0.16 mg polysomes, either 6.25×10^{-6} M or 6.25×10^{-7} M [^{3}H]-puromycin (spec. act. 595 mCi/mmol.), and either 2×10^{-4}M, 10^{-4}M, 5×10^{-5} M, 2×10^{-5} M, or 10^{-5} M Bap-Pan-Phe. Determination of [^{3}H]puromycin-peptide formation was carried out as described above for the polysome assay. The results were expressed in a Dixon plot (Dixon, 1953).

RESULTS AND DISCUSSION

Peptidyl Transferase Activity of Rat Liver Ribosomes.

The peptidyl transferase activity of rat liver ribosomes was assayed using the fragment assay, which is based on the formation of Ac-L-[^{3}H]Leu-puromycin from the reaction between puromycin and the donor substrate CpApCpCpA-(Ac-L-[^{3}H]Leu) in the presence of ethanol (to promote P-site binding on the ribosome). The activity was essentially linear up to the maximal incubation time (40 min) and ribosome concentration (0.1 mg) tested (Fig. 14). All further work described in this chapter involved incubation times or ribosome concentrations within this linear range.

FIG. 14: The effect of time of incubation (A) and ribosome concentration (B) on the extent of peptidyl transferase activity as measured by the fragment reaction. The formation of Ac-L-[^{3}H]Leu - puromycin was measured as described in Materials and Methods.


A
B
COUNTS / MIN
1200
1000
800
600
400
200
0
0
10
20
30
40
TIME (MIN.)
0
0·02
0·04
0·06
0·08
0·1
MG RIBOSOMES
300
250
200
150
100
50
0
COUNTS / MIN

The precipitation method used for washing Bap-Pan-Phe treated E. coli ribosomes (Harris et al., 1973; Greenwell et al., 1974) caused extensive (almost 100%) inactivation of rat liver peptidyl transferase as measured by the fragment reaction. However, an alternative washing method using fragment reaction conditions (30% [v/v] methanol and appropriate salts as described in Methods) was found to give quantitative precipitation of ribosomes with no loss of activity.

Extent of Affinity Labelling of Peptidyl Transferase by Bap-Pan-Phe, and Protection by Puromycin.

Affinity labelling by Bap-Pan-Phe can occur either specifically at peptidyl transferase following binding to the active site, or non-specifically to any reactive group on the ribosome. The addition of puromycin during affinity labelling would decrease the former (by direct competition for peptidyl transferase) but have little or no effect on the latter. Determination of the extent of affinity labelling of peptidyl transferase can be conveniently followed by the fragment assay. In simple terms, an affinity label covalently bound to the active centre of peptidyl transferase prevents the entry of puromycin and thus lowers Ac-L-[^{3}H]Leu-puromycin formation.

The results of treatment of rat liver ribosomes with Bap-Pan-Phe both in the absence or presence of puromycin are shown in Table 7. Although treatment of the ribosomes with the affinity label caused inhibition of peptidyl transferase activity, protection against this inactivation was only obtained at high concentrations of puromycin;

Table 7: The extent of affinity labelling of rat liver ribosomes (%) at various concentrations of Bap-Pan-Phe in the presence or absence of puromycin, measured by the loss of activity of peptidyl transferase in the fragment reaction as described in Materials and Methods.

	Concentration of Bap-Pan-Phe (mM)			
	0.1	0.5	1	2
No puromycin	22	40	65	78
3 mM puromycin	11	31	55	72
% protection by puromycin	50	22	15	8

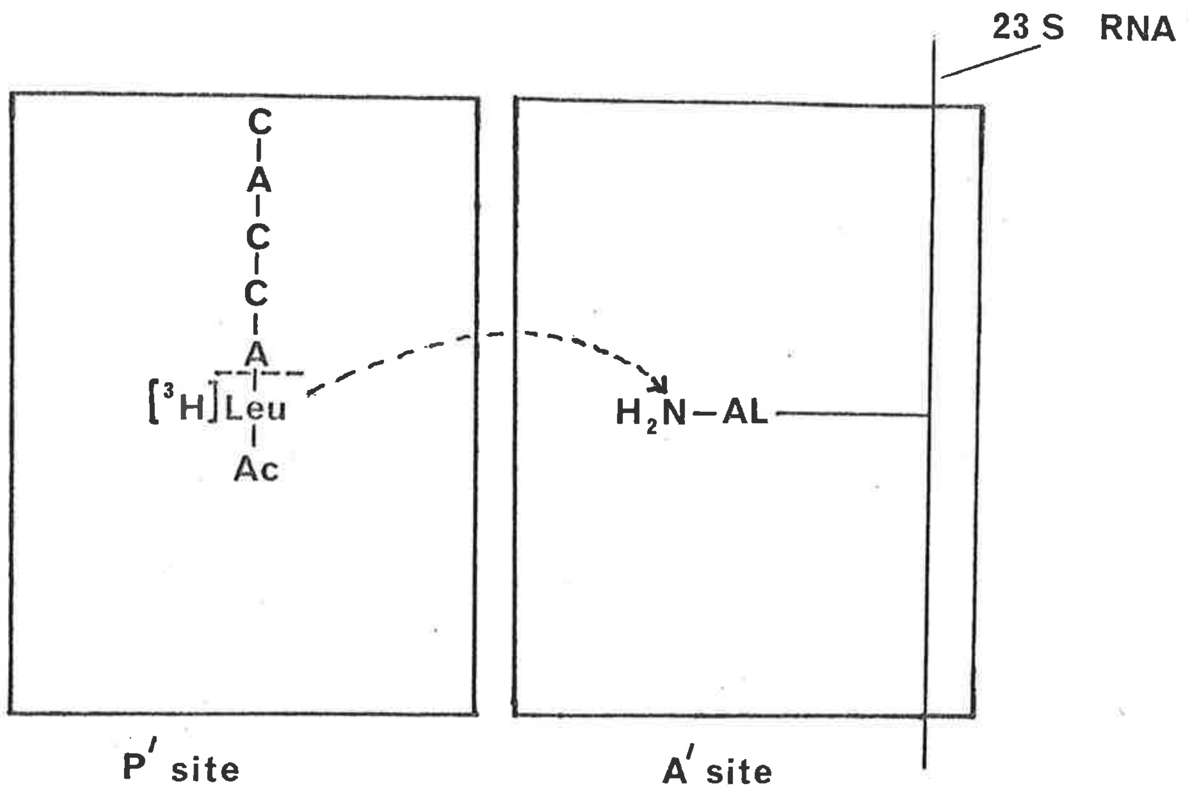
e.g. 3 mM puromycin gave 50% protection from the inactivation (22%) achieved by 0.1 mM Bap-Pan-Phe, but only 8% protection when 2 mM Bap-Pan-Phe was used (78% inactivation). Also, compared to the results of Greenwell *et al.*, (1974) with *E. coli* ribosomes, five times higher concentrations of Bap-Pan-Phe were necessary to produce similar inactivation of peptidyl transferase with rat liver ribosomes. This poorer reaction rate, together with the inability of puromycin to effectively protect against inactivation, suggested that Bap-Pan-Phe may be inhibiting peptidyl transferase non-specifically.

Specificity of Labelling by Bap-Pan-Phe.

To test if non-specific inhibition of peptidyl transferase by Bap-Pan-Phe was occuring, two experiments were done. Any affinity label covalently bound to the A'-site should act as an acceptor substrate (Fig. 15). Indeed, Harris *et al.*, (1973) and Greenwell *et al.*, (1974) have shown that Bap-Pan-Phe, covalently bound to *E. coli* peptidyl transferase, will act as an acceptor substrate. Therefore, affinity labelled rat liver ribosomes were incubated with the donor fragment CpApCpCpA-(Ac-L-[^{3}H]Leu) under fragment reaction conditions. No Ac-L-[^{3}H]Leu was transferred to ribosome - bound affinity label. This indicates that little, if any, Bap-Pan-Phe is correctly bound to the A'-site of rat liver peptidyl transferase.

Secondly, the Ki for Bap-Pan-Phe was determined in the fragment reaction as described in Methods. The extremely poor inhibition by Bap-Pan-Phe made an accurate value for the Ki impossible to obtain. However, an approximate Ki

FIG. 15: Schematic representation of the reaction occurring between CpApCpCpA-(Ac-L-[^{3}H]Leu) in the P'-site and covalently bound Bap-Pan-Phe in the A'-site of peptidyl transferase.


23 S RNA
C
A
C
C
A
[³H]Leu
Ac
H₂N–AL
P' site
A' site

$$\text{RNA–AL–NH}_2 + \text{CACCA} - (\text{Ac}-[{}^3\text{H}]\text{Leu})$$

$$\longrightarrow \text{RNA}-\text{AL}-(\text{Ac}-[{}^3\text{H}]\text{Leu}) + \text{CACCA}$$

of 4 x 10^{-3} M was obtained (data not shown), thus demonstrating a very low affinity of Bap-Pan-Phe for peptidyl transferase. The above results show that Bap-Pan-Phe is a poor affinity label for the A'-site of rat liver peptidyl transferase.

The Ribosomal Components Labelled With $[^{32}P]$Bap-Pan-Phe.

The lack of specificity of Bap-Pan-Phe was further emphasised by the use of $[^{32}P]$affinity label, which gave a stoichiometry of approximately 40 affinity label molecules bound per ribosome. No protective effect of puromycin on this labelling was detected. Of the total bound radioactive label, 93% was attached to ribosomal proteins. The small amount of affinity label attached to RNA was due to non-specific labelling, since affinity labelling in the presence of puromycin gave no reduction in the level of binding of $[^{32}P]$Bap-Pan-Phe to the RNA. This contrasts with the specific (puromycin protectable) labelling of *E. coli* 23S rRNA by this same affinity label (Harris *et al.*, 1973; Greenwell *et al.*, 1974). Due to the low affinity (high Ki) of Bap-Pan-Phe, it was necessary to use high concentrations of the affinity label to ensure a high level of binding to peptidyl transferase. Therefore a high level of non-specific protein labelling was unavoidable.

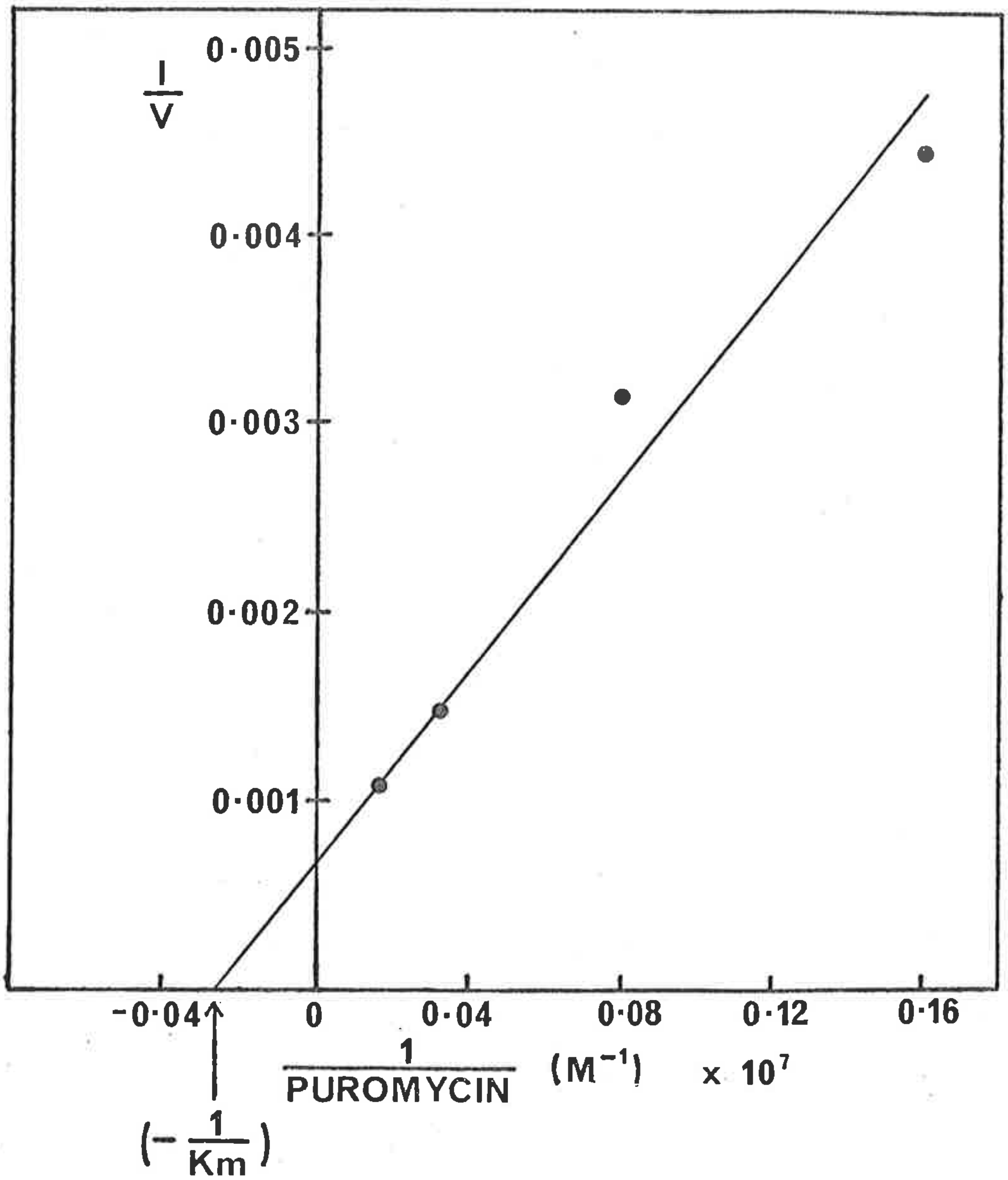
Km of Puromycin and Ki of Bap-Pan-Phe with Rat Liver Polysomes.

Pestka *et al.*, (1972) showed that puromycin had a much higher (approx. 100 times) affinity for mammalian polysomes than for ribosomes. It was anticipated that, if Bap-Pan-Phe had a similar large increase in affinity for rat liver polysomes compared to salt-washed ribosomes, then affinity labelling might be more specific; i.e. it should be possible to use lower concentrations of Bap-Pan-Phe and therefore increase the ratio of specific to non-specific labelling. Subsequent identification of specifically labelled species may then be possible.

The initial characterisation of the $[^3H]$puromycin mediated peptide release assay using rat liver polysomes containing nascent peptidyl-tRNA showed that 13.1 pmol / min of peptidyl-$[^3H]$puromycin was formed per mg of polysomes, which compares favourably with the results of Pestka et al., (1972) for similar levels of polysomes. The Km for puromycin deduced from the double-reciprocal plot as shown in Fig. 16 was 4.0×10^{-6} M, which also compared favourably with the value obtained by Pestka et al., (1972). Thus the polysomes were functionally active. With Bap-Pan-Phe added to the assay system, a Dixon plot (Fig. 17) gave a Ki of 1.0×10^{-4} M, which indicated a 40 fold increased affinity of Bap-Pan-Phe for polysomes compared to salt-washed ribosomes (Ki of 4×10^{-3} M). However, the Ki also indicated that Bap-Pan-Phe has 25 times less affinity for polysomes than does puromycin. Thus the specificity of labelling may still have been a problem in the reaction between Bap-Pan-Phe and polysomes. In addition, during the slow affinity labelling process there should be a rapid formation of a peptide bond between the nascent peptides of the polysomes and the α-amino group of Bap-Pan-Phe. This loss of the nascent peptides may convert the polysomes to a state in which the affinity of peptidyl

FIG. 16: The Km for puromycin of rat liver polysomes. The concentration of [^{3}H]puromycin was varied in the peptide release assay as described in Materials and Methods, and the formation of peptidyl -[^{3}H]puromycin expressed as a double reciprocal plot.

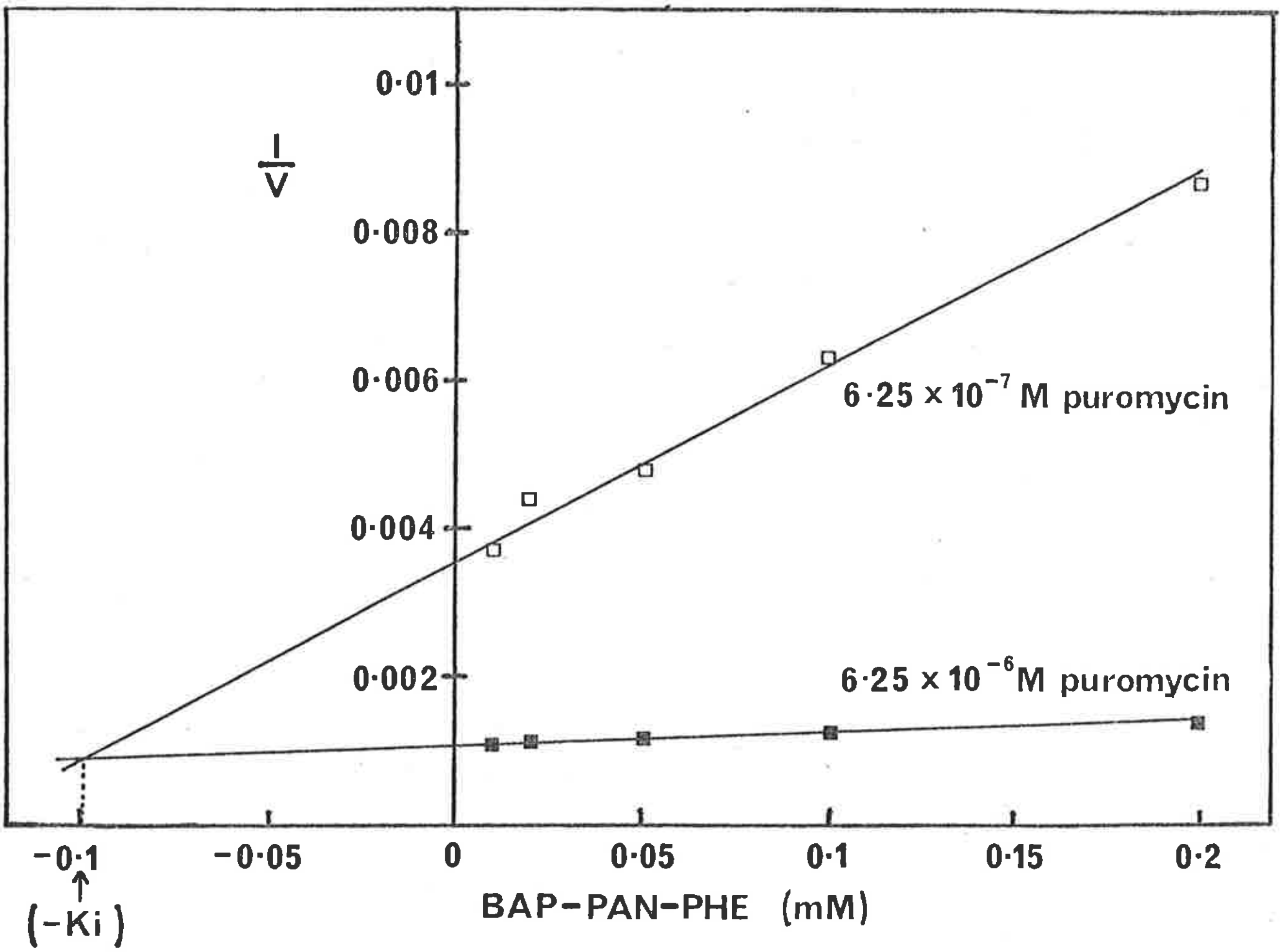
Km = 4·0 x 10^{-6} M



$\frac{1}{V}$
0·005
0·004
0·003
0·002
0·001
−0·04
0
0·04
0·08
0·12
0·16
$\frac{1}{\text{PUROMYCIN}}$ (M^{-1}) x 10^7
$(-\frac{1}{Km})$

FIG. 17: The Ki of Bap-Pan-Phe in the peptide release assay using rat liver polysomes and [^{3}H]puromycin. The extent of inhibition by various concentrations of Bap-Pan-Phe of the formation of peptidyl -[^{3}H]puromycin (using 6.25×10^{-7} M and 6.25×10^{-6} M [^{3}H]puromycin) was measured as described in Materials and Methods, and the results expressed as a Dixon plot.

Ki = $1{\cdot}0 \times 10^{-4}$ M



transferase for Bap-Pan-Phe is reduced to that of isolated ribosomes (as observed in the fragment assay). In view of these potential problems, no further experiments were attempted with rat liver polysomes.

General Conclusions

The results in this chapter indicate that Bap-Pan-Phe has a poor affinity for both rat liver ribosomes (Ki of 4×10^{-3} M) and polysomes (Ki of 1.0×10^{-4} M). The low affinity is possibly the major factor contributing to the poor affinity labelling observed with Bap-Pan-Phe. Although Bap-Pan-Phe at high concentrations does covalently bind to the ribosomes, the binding is extensive (approx. 40 affinity labels per ribosome), and specific (i.e. puromycin protectable) binding could not be detected on either ribosomal RNA or proteins. This is in contrast to the situation with *E. coli* ribosomes, where the reaction between Bap-Pan-Phe and 23 S RNA was highly specific (Harris *et al.*, 1973; Greenwell *et al.*, 1974), thus showing that inherent differences exist between mammalian and bacterial ribosomes.

These results demonstrate the problems associated with affinity labelling when using small molecular weight molecules, as also found by Vanin, (1977). The relatively low affinity of these affinity labels for ribosomes (compared to the high affinity of the tRNA derivatives used as affinity labels by most researchers) results in much higher levels of non-specific labelling because of the high concentrations needed for a significant level of specific affinity labelling. This high non-specific labelling makes identification of the specific site of attachment on the ribosome extremely difficult, unless the specifically labelled component(s) can be easily identified; e.g. when the majority of the specific label is associated with a single ribosomal component while the non-specific label is spread over many components, or when the specific label is associated with rRNA and the non-specific label with proteins (Harris et al., 1973; Greenwell et al., 1974).

CHAPTER FIVE

SEQUENCE AT THE SITE OF ATTACHMENT OF AN AFFINITY LABEL DERIVATIVE OF PUROMYCIN ON 23S RNA OF *E. COLI* RIBOSOMES

SEQUENCE AT THE SITE OF ATTACHMENT OF AN AFFINITY LABEL DERIVATIVE OF PUROMYCIN ON 23S RNA OF *E. COLI* RIBOSOMES

INTRODUCTION

The puromycin analogue, Bap-Pan-Phe, acts as an affinity label in covalently binding to the active centre of *E. coli* ribosomal peptidyl transferase (Harris *et al.*, 1973; Greenwell *et al.*, 1974). Specific labelling of the 23S RNA was found, with two affinity label molecules bound per 23S RNA molecule when 100% inactivation (as measured by the fragment reaction) of peptidyl transferase occurred. In addition, there was extensive non-specific labelling of ribosomal proteins (up to 25 molecules of affinity label per 70S ribosome).

Affinity labelling of 23 S RNA is not unique to Bap-Pan-Phe, as various analogues of L-Phe-tRNA containing reactive moeities attached to the α-amino group of L-Phe also bind to 23S RNA in intact ribosomes (see Table 1). Since these analogues contained a blocked α-amino group, they would most likely enter the P-site of peptidyl transferase, whereas Bap-Pan-Phe enters the A'-site (Harris *et al.*, 1973; Greenwell *et al.*, 1974). Only Yukioka *et al.*, (1977), have sequenced a fragment of 23S RNA to which an affinity label is attached, namely $\overset{*}{A}$pUpUpUpUpApGp (where $\overset{*}{A}$ is the alkylated nucleoside). Puromycin itself can act as a photoaffinity label, with 23S RNA being one of the components labelled (Cooperman *et al.*, 1975). However, Bap-Pan-Phe is the only affinity label reported to date which specifically enters the A'-site of peptidyl transferase and covalently binds to 23S RNA, with part at least of the bound affinity label acting as an acceptor substrate in the fragment reaction (Harris et al., 1973; Greenwell et al., 1974).

The aim of the work described in this chapter was to isolate fragment(s) of 23S RNA to which Bap-Pan-Phe was attached, and to obtain the nucleotide sequence of the fragment(s). When the complete sequence of 23S RNA becomes known, the site of attachment of Bap-Pan-Phe, and hence that portion of 23S RNA at or near the A'-site, could then be accurately identified.

MATERIALS AND METHODS

Materials

[^{3}H] uridine (49 Ci/mmol.) was obtained from Schwarz-Mann, [^{3}H]adenosine (23 Ci/mmol.) from The Radiochemical Centre, Amersham and [^{3}H]guanosine (19.7 Ci/mmol.) and [^{3}H]cytidine (28 Ci/mmol.) from ICN Pharmaceuticals. High specific activity inorganic [^{32}P]phosphate was supplied by the Australian Atomic Energy Commission, New South Wales. Lysozyme (Grade 1), biotin, avidin and ribonucleases A and T_1 were obtained from Sigma Chemical Co. Brij - 58 was obtained from Atlas Chemical Industries.

A partly purified preparation of ribonucleases T_1 plus T_2 was prepared by the method of Hiramaru et al., (1966). The N-hydroxysuccinimide ester of biotin (biotin - NHS) was prepared by E.F.Vanin following the method of Bayer and Wilchek, (1974).

The donor fragment substrates $\overset{U}{C}$pApCpCpA-(Ac-L-[^{3}H]-Leu) were prepared as described inChapter Two.

Ribosomes

Unlabelled ribosomes were prepared from E. coli MRE 600 as described by Greenwell et al., (1974).

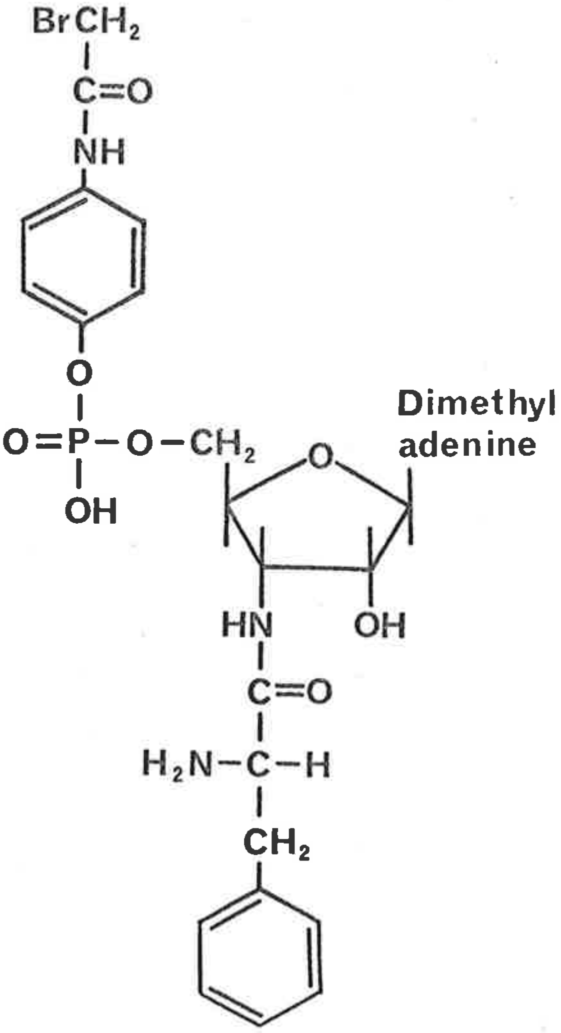
[^{32}P]Ribosomes were prepared by modifying the method of Pestka and Hintikka, (1971) for preparing polysomes. E. coli MRE 600 were grown in 200 ml of growth medium containing 20 mM KCl, 85 mM NaCl, 18.7 mM NH_4Cl, 0.1 M Tris-HCl, pH 7.4, 10 mM Mg SO_4, 0.4% (w/v) sucrose, 0.4 g vitamin - free casamino acids, 0.4 g Bactopeptone, and 5 mCi [^{32}P]phosphate to late log phase (3 h). Approximately 75% of the [^{32}P]phosphate was incorporated into TCA-insoluble material. The bacteria were collected by centrifugation at 26,000 g for 5 min at 4° C and resuspended in 2.4 ml of 25% (w/v) sucrose in 10 mM Tris-HCl, pH 8.0. Spheroplasts were then formed by the addition of 200 µl of 10 mg/ml lysozyme in 125 mM Tris-HCl, 5 mM EDTA, pH 8.0, followed by 400 µl of 10 mM EDTA and incubation at 0° C for 5 min. The reaction was stopped by the addition of 200 µl of 1.0 M magnesium acetate, the spheroplasts were collected by centrifugation at 26,000 g for 5 min and resuspended in 4 ml of 0.5% Brij - 58, 0.05 M NH_4Cl, 10 mM magnesium acetate, 10 mM Tris-acetate, pH 7.2, and lysis allowed to proceed at 0° C for 30 min. After the addition of 14 ml of ribosome buffer (10 mM magnesium acetate, 0.1 M NH_4Cl, 0.5 mM EDTA, 20 mM Tris-acetate, pH 7.5), cell debris was removed by centrifugation at 12,000 g for 10 min and 4.5 ml fractions of the supernatant were layered over 5.0 ml of 1.3 M sucrose, 0.5M NH_4Cl, 10 mM magnesium acetate, 0.5 mM EDTA, 20 mM Tris-acetate, pH 7.5, in Beckman Ti 50 centrifuge tubes. The ribosomes were sedimented by centrifugation at 50,000 rev./min in a Beckman Ti 50 rotor for 16 h at 4° C, resuspended in 400 μl of ribosome buffer and stored at - 80°C. Up to 9 mg of [^{32}P]ribosomes (4 x 10^5 counts/min/μg) were prepared by this method.

Ribosomes labelled with [^3H]nucleosides were isolated in the same way as the [^{32}P]ribosomes except that the E. coli MRE 600 were grown to late log phase (approximately 3 h) in 50 ml of a medium (Lindquist, 1971) of 8 mM NaCl, 0.1 M KCl, 18.7 mM NH_4Cl, 7 mM KH_2PO_4, 7.2 mM sodium pyruvate, 1 mM Mg SO_4, 0.16 mM Na_2 SO_4, 1 mM $CaCl_2$, 0.2 μg/ml Fe SO_4, 0.2% glucose, 0.1 M Tris-HCl, pH 7.4, and 1% vitamin-free casamino acids. After incubation for 1 h at 37° C, 0.5 μmol [^3H]nucleoside (250 μCi) was added and shaking continued for a further 2 h before the ribosomes were isolated as described above. Usually 5 mg of [^3H]ribosomes (1,600 counts/min/μg) were obtained.

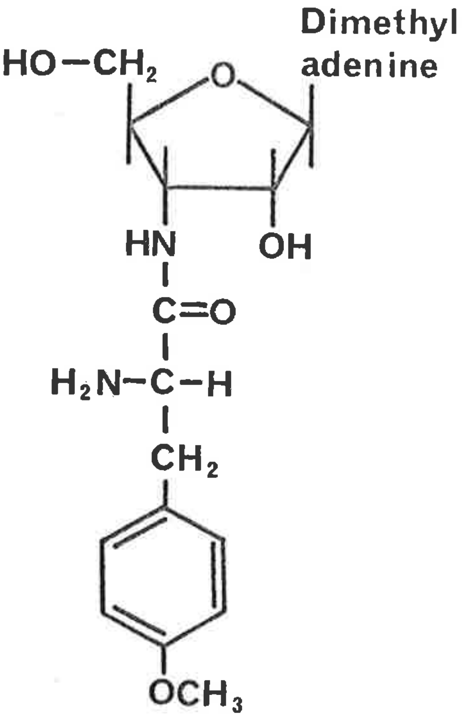
Synthesis of Bap-Pan-Phe and [^{32}P]Bap-Pan-Phe. (Fig. 18).

These compounds were prepared by Dr. R.H. Symons essentially as described by Greenwell et al., (1974) with minor modifications which increased the reliability of the method and the yield of products. The intermediate, 5'-0-(p-aminophenylphosphoryl)-3'-N-(t-butyloxycarbonyl-L-phenylalanyl) puromycin aminonucleoside, was not purified from the reaction mixture. Instead, ammonium ions were removed from the mixture by the addition and evaporation of excess triethylamine in ethanol, and the crude reaction mixture in ethanol was then reacted directly with excess bromoacetic acid anhydride in acetonitrile.

FIG. 18: Structures of puromycin and the affinity label analogue, 5'-0-(N-bromoacetyl-p-aminophenyl-phosphoryl)-3'-N-L-phenylalanyl puromycin amino-nucleoside (Bap-Pan-Phe).


BrCH2
C=O
NH
O
O=P-O-CH2
OH
O
Dimethyl adenine
HN
OH
C=O
H2N-C-H
CH2

Affinity label


HO-CH2
O
Dimethyl adenine
HN
OH
C=O
H2N-C-H
CH2
OCH3

Puromycin

Work-up of the reaction mixture after the removal of the protecting t-butyloxycarbonyl group by treatment with trifluoroacetic acid involved precipitation of the product out of a methanol solution by the addition of diethyl ether and petroleum ether (Greenwell et al., 1974) followed by purification of the product by t.l.c. as earlier described for $[^{32}P]$Bap-Pan-Phe (Greenwell et al., 1974).

Affinity Labelling of Ribosomes and Reaction of Bap-Pan-Phe with Polynucleotides.

Ribosomes were affinity labelled as described by Greenwell et al.,(1974) except that volumes of 65 - 250 µl containing 0.4 - 4 nmoles of ribosomes and 1.2 mM Bap-Pan-Phe were used. Where indicated, 25 µg of poly(A), poly(C), poly(G) or poly(U) replaced the ribosomes in the reaction mixture. The peptidyl transferase activity of untreated and affinity labelled ribosomes was assayed with the fragment reaction of Monro and Marcker, (1967) as modified by Greenwell et al., (1974).

Isolation of Affinity Labelled RNA.

The RNA was isolated from affinity labelled ribosomes by phenol - SDS extraction (Greenwell et al., 1974).

Ribonuclease Digestion of Affinity Labelled RNA.

Approximately 1.0 mg of affinity labelled RNA (or biotin-affinity labelled RNA; see preparation below) in 0.2 ml of 25 mM Tris-acetate, 0.05 mM EDTA, pH 7.5, was digested with either 30 µg of T_1 ribonuclease, 50 ug of pancreatic ribonuclease A or both at 37° C for 1.5 h.

Similarly, RNA, affinity labelled RNA, or biotin-affinity labelled RNA in 30 mM sodium acetate, pH 4.5 was digested with 10 mg/ml of a partially purified preparation of ribonucleases T_1 plus T_2 at 37° C for 1.0 - 1.5 h.

Benzoylated DEAE-cellulose(BD-cellulose) Column Chromatography.

BD-cellulose columns, 2.5 cm high, were prepared in pasteur pipettes and washed with 3 M NaCl followed by 1 M triethylammonium acetate, pH 5.0. [^{32}P]Bap-Pan-Phe-RNA or Bap-Pan-Phe-[^{32}P]RNA (digested with ribonucleases T_1 plus A) in 1.0 ml of 1 M triethylammonium acetate, pH 5.0, was applied to a column, which was sequentially eluted with 4 ml 1 M triethylammonium acetate, pH 5.0; 20 ml 1 M triethylammonium acetate, pH 5.0, 5% ethanol; and 10 ml 1 M triethylammonium acetate, pH 5.0, 50% ethanol. Fractions of 1.0 ml were collected and counted by Cerenkov radiation. The 1 M triethylammonium acetate, pH 5.0, 50% ethanol fractions containing affinity labelled RNA fragments were pooled and dried on a rotary evaporator.

Preparation of Naphthalene-Acetyl-cellulose (NA-cellulose).

Thionyl chloride was redistilled from quinoline and linseed oil as described by Vogel, (1967a). Naphthalene-acetyl chloride was prepared from 20 g naphthalene acetic acid and 10 ml redistilled thionyl chloride using the method to prepare benzoyl chloride described by Vogel, (1967b), except that the acid chloride was not purified by distillation after the reaction was complete.

Dried cellulose powder (10 g) was suspended in 250 ml anhydrous pyridine in a 500 ml round-bottomed flask, and the crude naphthalene-acetyl chloride was added with shaking. The mixture was slowly brought to the boil, and then boiled under reflux for 1 h with occasional shaking to prevent the cellulose from settling on the bottom of the flask and charring. When cool, the mixture was slowly poured into 3.5 l H_2O with stirring. The precipitated product was collected by filtration, washed twice with 2.0 l H_2O, and then washed sequentially with 2.0 l lots of hot ethanol until little colour was extracted into the solvent. Finally, the product was washed with 2 M NaCl in 50% ethanol, followed by 2 M NaCl, 2 mM EDTA, which was also used as the storage solution.

NA - cellulose Column Chromatography.

NA - cellulose columns, 2.5 cm high, were prepared in pasteur pipettes and washed with 2 M NaCl followed by 10 mM sodium acetate, pH 4.5. [^{32}P]Bap-Pan-Phe-RNA, Bap-Pan-Phe-[^{32}P]RNA (each digested with ribonucleases T_1 plus A), or the semi-purified T_1+A fragments eluted from BD - cellulose columns, in 10 mM sodium acetate, pH 4.5, were applied to the columns. Each column was sequentially eluted with 9 ml 10 mM sodium acetate, pH 4.5; 5 ml 10 mM sodium acetate, pH 4.5, 10% ethanol; 10 ml 10 mM sodium acetate, pH 4.5, 20% ethanol; and finally, 5 ml 10 mM sodium acetate, pH 4.5, 50% ethanol. Fractions of 1.0 ml were collected and counted by Cerenkov radiation. The 10 mM sodium acetate, pH 4.5, 50% ethanol fractions containing affinity labelled RNA were pooled, dried on a rotary evaporator, and re-dissolved in 0.05 ml 0.1 mM EDTA, pH 6.8.

High Voltage Paper Electrophoresis.

High voltage paper electrophoresis on either Whatman 3 MM paper or DEAE-cellulose paper was conducted on a water-cooled plate at 40 - 60 volts/cm using the following buffers:

pH 1.9; 2.5% (by vol.) formic acid, 8.7% acetic acid.
pH 3.5; 5% acetic acid, 0.6% triethylamine, 0.1 mM EDTA.
pH 4.1; 2% acetic acid, 1.07% triethylamine, 0.1 mM EDTA.
pH 5.0; 2% acetic acid, 3.5% triethylamine, 0.1 mM EDTA.

Preparation of Avidin : Biotin - Affinity Label-rRNA and its Ribonuclease Digests.

Approximately 1 mg of affinity labelled rRNA was reacted with 0.3 mg (0.9 μmol) of biotin-NHS in 0.2 ml of 25 mM N-ethylmorpholine acetate, pH 8.0, at room temparature for 30 - 60 min. Unreacted biotin-NHS was removed by the following steps. To the reaction mixture was added 1.0 ml of 0.3 M sodium phosphate, pH 6.6, and then 0.2 ml of 1% cetyltrimethyl-ammonium bromide during agitation of the solution. After 5 min at room temperature and 30 min at 0°C, the RNA precipitate was recovered by centrifugation at 4°C. The pellet was dissolved in 1.0 ml of 20% (w/v) potassium acetate and the RNA again precipitated by the addition of 1.5 ml of acetone (- 15°C) and storage at - 15° C for 15 min. The precipitate was recovered by centrifugation at 4° C, the pellet dissolved in 0.5 ml 0.2 M NaCl, 50 mM sodium acetate, 1 mM EDTA, pH 5.0, and the RNA again precipitated by the addition of 1.5 ml of cold ethanol (- 15° C) and storage overnight at - 15°C. After centrifugation, the final RNA pellet was dissolved in 0.1 ml of 0.1 mM EDTA, pH 6.8, and stored at - 15° C.

Approximately 1 mg of biotin - affinity labelled rRNA was digested with either T_1 or pancreatic ribonuclease as described above. Then 0.1 mg of avidin in 0.1 ml of 10 mM Tris-acetate, 1 mM EDTA, pH 7.5 was added and the mixture incubated at 37° C for 15 min and stored at -15°C.

Phosphocellulose Chromatography of Avidin:Biotin - Affinity Labelled rRNA Digests.

Phosphocellulose columns, 2.5 cm high, were prepared in pasteur pipettes and washed with freshly prepared 1.0 M NH_4HCO_3 followed by 50 mM NH_4HCO_3, 4 M urea. Ribonuclease digests of the avidin:biotin - affinity label- rRNA were diluted in 15 vol. of 50 mM NH_4HCO_3, 4 M urea, and loaded onto the phosphocellulose columns. Each column was then sequentially eluted with 10 ml 50 mM NH_4HCO_3, 4 M urea; 7 ml water; 5 ml 0.2 M NH_4HCO_3; and finally with 5 ml 0.5 M NH_4HCO_3. Fractions of 1.0 ml were collected and counted by Cerenkov radiation. The avidin:biotin - affinity labelled RNA fragments in the 0.5 M NH_4HCO_3 eluate were pooled, triethylamine was added in excess with respect to the NH_4HCO_3 and the two phases were made miscible with ethanol. The solution was then taken almost to dryness on a rotary evaporater, and the concentrated solution transferred to a small tube and lyophilized. To ensure complete removal of the triethylammonium bicarbonate, lyophilization was repeated twice after the addition of 50 µl of water.

Disruption of Avidin:Biotin Complex by Formic Acid.

The lyophilized phosphocellulose eluate was treated with 20 µl of 70% formic acid for 10 min at room temperature (Rylatt et al., 1977) 80 µl of water was then added and the solution immediately used for the next step in the purification.

Electrophoretic Purification of Biotin-Affinity Label - Oligonucleotide.

The biotin - affinity labelled RNA fragments were further purified by electrophoresis on DEAE - cellulose paper for 4 h at 40 volts/cm in pH 1.9 buffer as described above. The electrophoretogram was rinsed in ethanol, dried and cut into 1.0 cm strips which were counted by Cerenkov radiation.

Biotin - affinity labelled RNA fragments were eluted by soaking the strips in 2 M NH_4HCO_3 at 4° C for 1 - 2 h, and the salt was removed from the clarified (by centrifugation) eluate on the rotary evaporator after the addition of excess triethylamine and ethanol. The residue was dissolved in 40 µl of 0.1 mM EDTA, pH 6.8.

Digestion of Biotin-Affinity Label-Oligonucleotide by Ribonucleases T_1 plus T_2, and Paper Electrophoresis.

The purified biotin - affinity labelled RNA fragments were hydrolysed to mononucleotides by incubation with ribonucleases T_1 and T_2 as described above. The reaction mixture was then fractionated by paper electrophoresis on Whatman 3 MM paper for 2 h at 47 volts/cm in pH 4.1 buffer as described above. A marker solution of 2' (3') AMP, CMP, GMP, and UMP was always electrophoresed with each sample.

Assay for Ribosome Binding of UpApCpCpA- (Ac-L-[^3H]Leu).

The standard reaction mixture described in Chapter Three under P-site binding assay was used for P'-site binding assays.

RESULTS AND DISCUSSION

Electrophoretic Characterisation of Ribosome Derived Affinity Labelled Nucleotide.

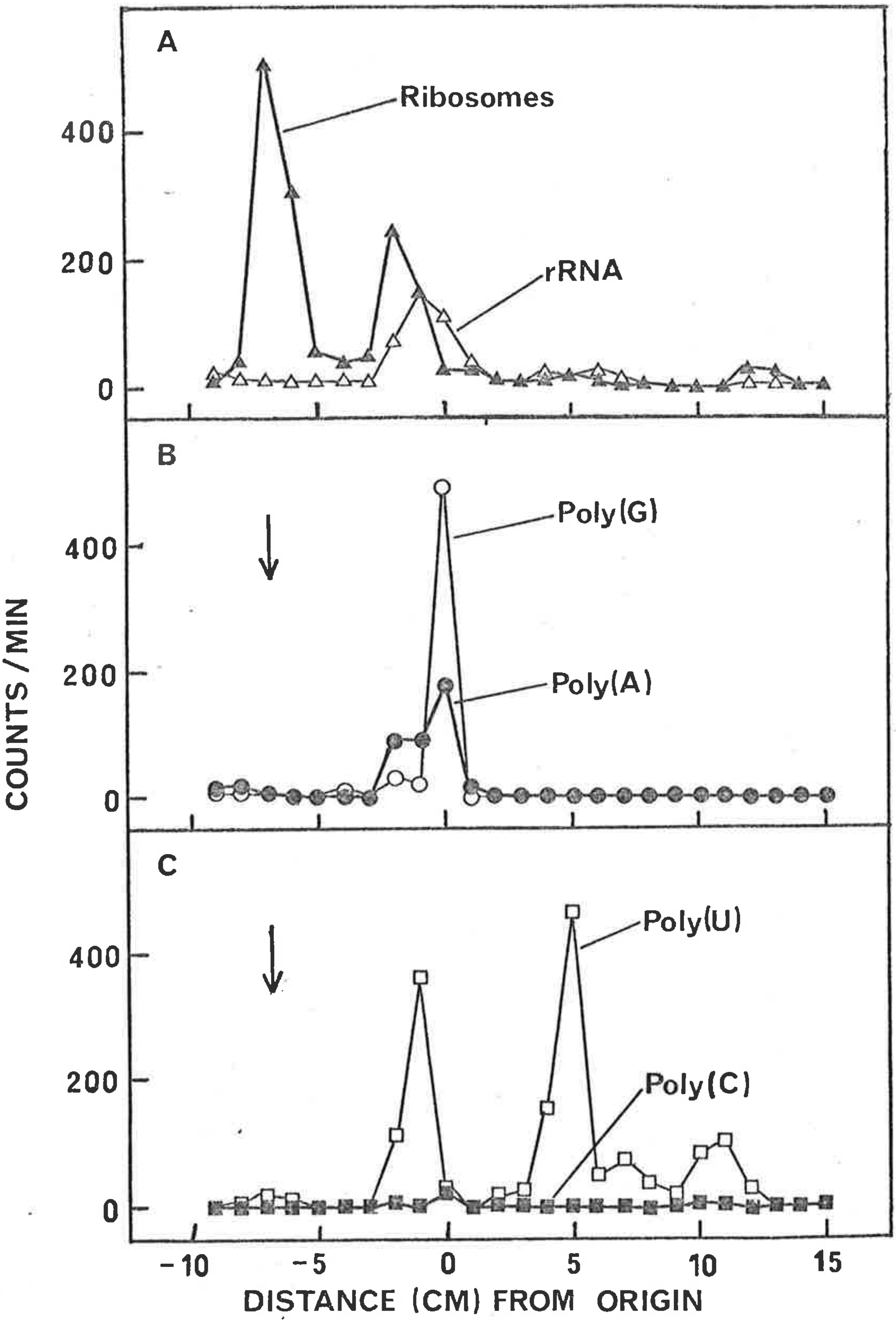
The $[^{32}P]$affinity labelled nucleotide resulting from the ribonuclease T_1 and T_2 digestion (see Materials and Methods) gave two peaks on paper electrophoresis at pH 1.9 (Fig. 19 A). The major peak was identified as the affinity labelled nucleotide since it reacted with either acetic anhydride or the N-hydroxysuccinimide ester of biotin (see below), thus indicating the presence of the free α-amino group of the affinity label (Fig. 18). The minor peak was presumed to be a breakdown product of the affinity labelled nucleotide since it did not have a reactive amino group.

Reaction of $[^{32}P]$Bap-Pan-Phe With Polynucleotides and Isolated Ribosomal RNA.

In an attempt to identify the nucleotide to which the affinity label was specifically bound, $[^{32}P]$Bap-Pan-Phe was reacted with each of poly (A), poly (C), poly (G), poly (U) and purified ribosomal RNA under conditions described in Materials and Methods for affinity labelling of ribosomes. The RNA samples were hydrolysed by ribonucleases T_1 and T_2 and the mononucleotides analysed by paper electrophoresis at pH 1.9 (Fig. 19).

There was little reaction of the affinity label with the polynucleotides and, as previously reported by Greenwell *et al.*, (1974), with isolated ribosomal RNA compared to the reaction of the affinity label with 23S RNA in intact ribosomes. Most reaction occurred with

FIG. 19: Paper electrophoresis at pH 1.9 of $[^{32}P]$- Bap-Pan-Phe labelled ribosomal RNA and polynucleotide species after digestion with ribonucleases T_1 plus T_2. Details are given in Materials and Methods. The percentage of total nucleotide residues in each reaction mixture that reacted with $[^{32}P]$Bap-Pan-Phe were: isolated rRNA, 0.031%; poly (A), 0.038%; poly (C), 0.004%; poly (G), 0.043%; poly (U), 0.114%.

A
Ribosomes
rRNA
B
Poly(G)
Poly(A)
C
Poly(U)
Poly(C)
400
200
0
COUNTS / MIN
- 10
- 5
0
5
10
15
DISTANCE (CM) FROM ORIGIN

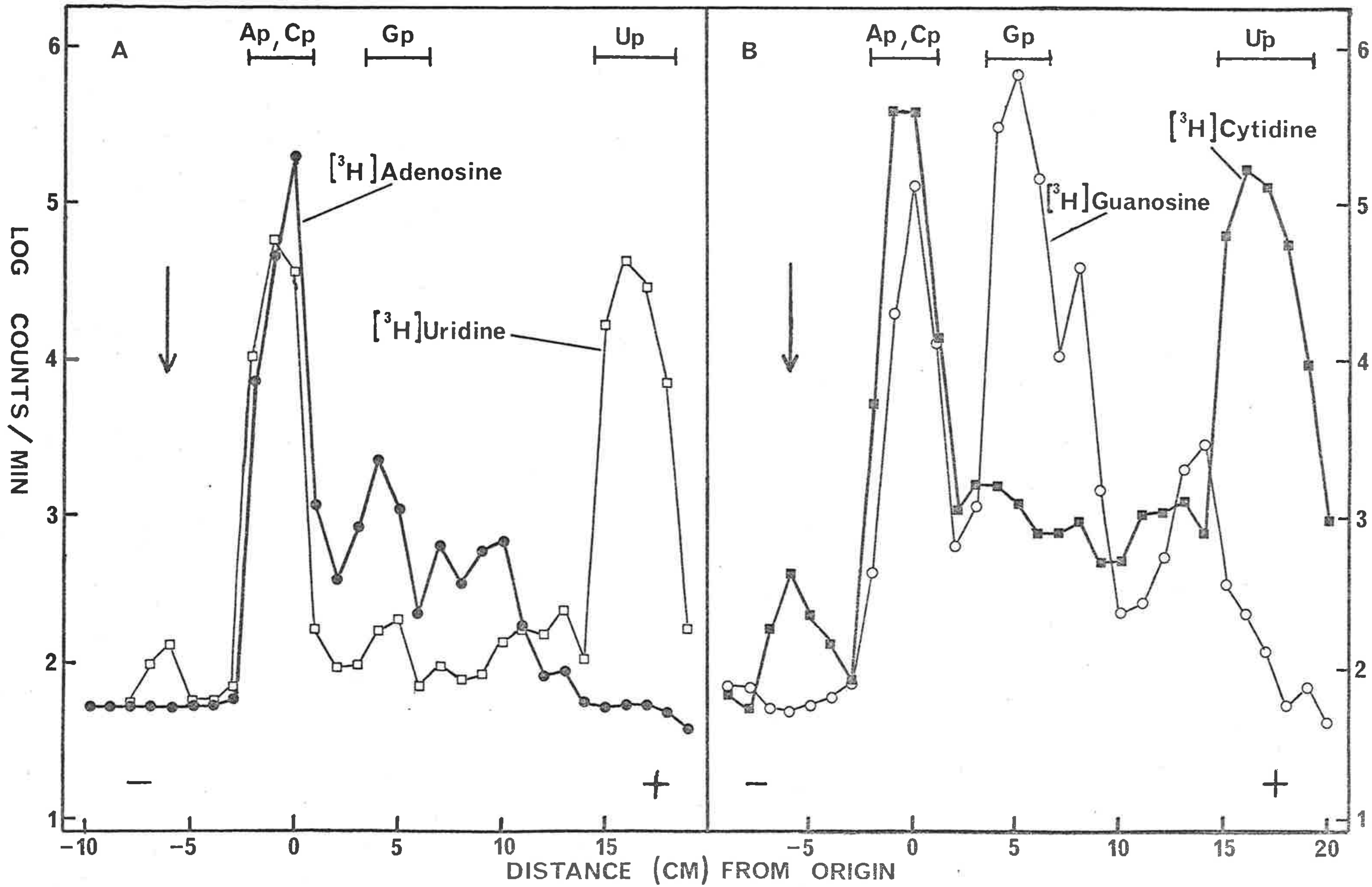
poly (U) and the least with poly (C) (Fig. 19). No significant peak of radioactivity was found coincident with the affinity labelled nucleotide derived from intact ribosomes. Hence, these results further confirm that the functional integrity of the ribosome is necessary for the specific reaction of Bap-Pan-Phe with 23S RNA (Harris *et al*., 1973; Greenwell *et al*., 1974).

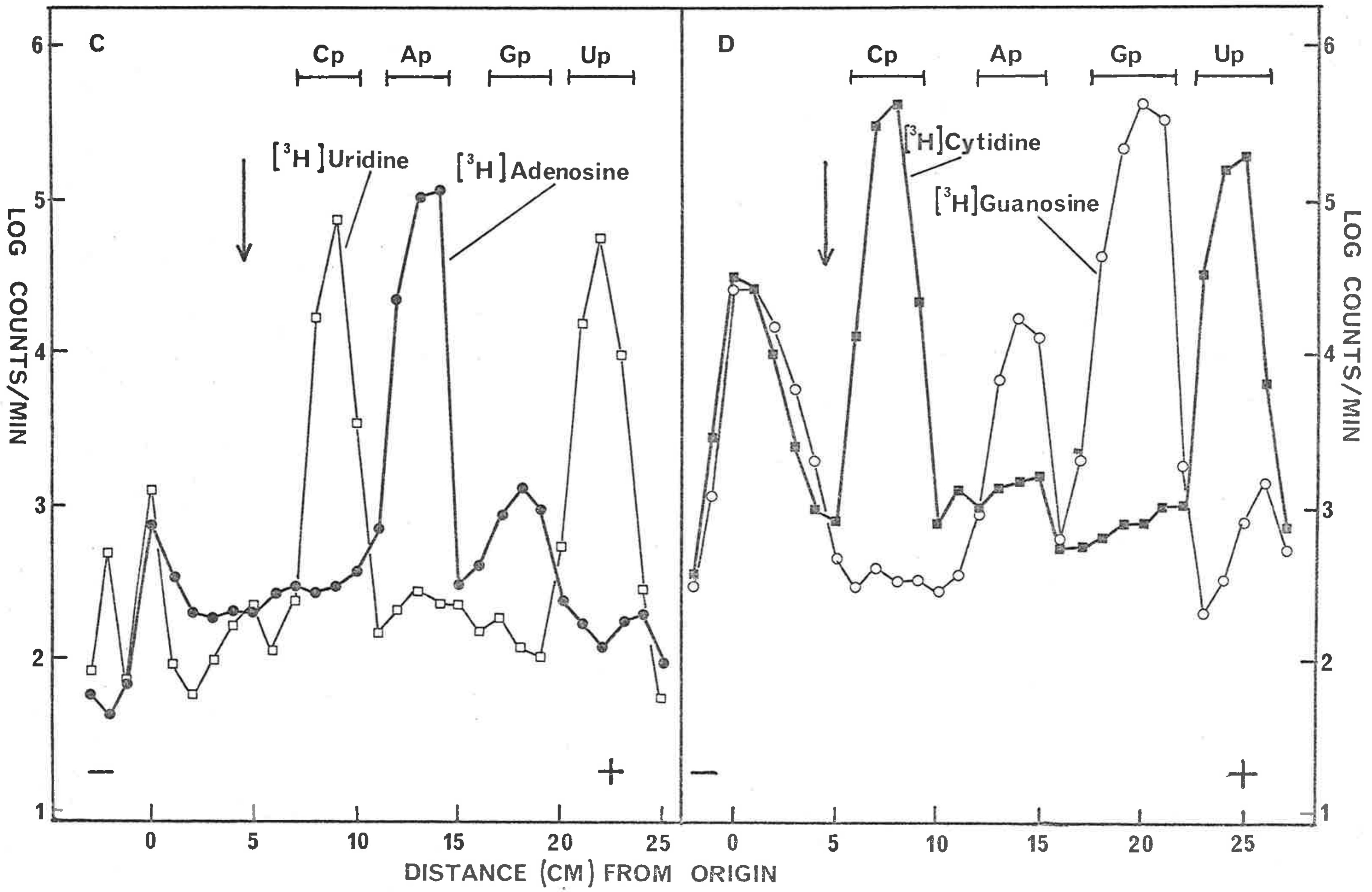
Bap-Pan-Phe is Covalently Coupled to a CMP Residue of 23S RNA.

The approach used to determine which of the four nucleotides of 23S RNA was affinity labelled by Bap-Pan-Phe was as follows. [^{3}H]Adenosine, [^{3}H]guanosine, [^{3}H]cytidine or [^{3}H]uridine labelled ribosomes (see Materials and Methods) were affinity labelled with Bap-Pan-Phe, the RNA was isolated, digested to mononucleotides with ribonucleases T_1 and T_2, and the digests fractionated by paper electrophoresis at pH 1.9 and 4.1 (Fig. 20). Electrophoresis at pH 1.9 allowed separation of the affinity labelled nucleotide (position indicated by vertical arrow, Fig. 20 A and B) but did not separate all four mononucleotides. On the other hand, electrophoresis at pH 4.1 separated the four mononucleotides but the affinity labelled nucleotide was obscured by contaminating material (Fig. 20 C and D).

With [^{3}H]adenosine or [^{3}H]guanosine labelled RNA, no radioactive peak was found coincident with the marker [^{32}P]Bap-Pan-Phe-nucleotide (Fig. 20 A and B); hence, affinity labelling did not occur at an AMP or GMP residue. A small conversion of adenosine to guanosine and vice versa occurred (as shown in Fig. 20 C and D); 1.5% of [^{3}H]-

FIG. 20: Paper electrophoresis at pH 1.9 (A and B) and pH 4.1 (C and D) of Bap-Pan-Phe-[^{3}H]-RNA digested with ribonucleases T$_1$ plus T$_2$. The rRNA reaction products from the incubation of Bap-Pan-Phe with ribosomes labelled with [^{3}H]-adenosine (●——●), [^{3}H] cytidine (■——■), [^{3}H]guanosine (○——○), or [^{3}H]uridine (□——□), were prepared completely with ribonucleases T$_1$ and T$_2$ and electrophoresed at pH 1.9 and pH 4.1 as described in Materials and Methods. The positions of the marker 2' (3') mononucleotides run in each experiment are given. The vertical arrow indicates the position of the affinity labelled mononucleotide.


A
B
Ap, Cp
Gp
Up
[^{3}H]Adenosine
[^{3}H]Uridine
[^{3}H]Cytidine
[^{3}H]Guanosine
LOG COUNTS / MIN
1
2
3
4
5
6
−
+
−10
−5
0
5
10
15
20
DISTANCE (CM) FROM ORIGIN


C
D
Cp
Ap
Gp
Up
[^{3}H]Uridine
[^{3}H]Adenosine
[^{3}H]Cytidine
[^{3}H]Guanosine
LOG COUNTS/MIN
1
2
3
4
5
6
−
+
0
5
10
15
20
25
DISTANCE (CM) FROM ORIGIN

adenosine was converted to [^{3}H]guanosine while 3.5% of [^{3}H]-guanosine was converted to [^{3}H]adenosine.

With both [^{3}H]cytidine and [^{3}H]uridine labelled RNA, a radioactive peak appeared coincident with the marker nucleotide (Fig. 20 A and B). Interpretation of this result was complicated by the extensive inter-conversion of [^{3}H]cytidine and [^{3}H]uridine. Thus when ribosomes were labelled by growing E. coli in the presence of [^{3}H]uridine, 52% of the radioactivity in the RNA was in CMP (Fig. 20 C) and on labelling with [^{3}H]cytidine, 34% of the radioactivity was in UMP (Fig. 20 D). Since only one peak of affinity labelled nucleotide was ever found (by electrophoresis under three different conditions), it was most unlikely that the affinity label was attached to two different nucleotide residues. Hence, the problem was to determine whether the affinity label was attached to CMP or UMP. Attempts to reduce the interconversion of nucleosides by reducing the time of labelling with [^{3}H]nucleoside from 2 h to 15 min had little effect. Additional attempts involving addition of non-radioactive uridine (1 mM) to the growth medium when labelling with [^{3}H]cytidine (initial concentration 0.01 mM) only reduced the conversion of cytidine to uridine from 34% to 25%. Similarly, the addition of non-radioactive cytidine (1 mM) only lowered the conversion of uridine to cytidine from 52% to 35%.

The solution to the problem was found by comparing the radioactivity in the affinity labelled nucleotide peak as a percentage of the radioactivity in CMP and UMP when either [^{3}H]cytidine or [^{3}H]uridine was used to label ribosomes. Since the affinity label was bound to either CMP or UMP but not both, then the radioactivity of the affinity labelled [^{3}H]nucleotide would be a constant percentage of the total of that particular pyrimidine nucleotide in the RNA (either CMP or UMP) but not of the other pyrimidine nucleotide. In the results from three separate experiments (Table 8), the affinity labelled nucleotide remained a reasonably constant percentage of the CMP radioactivity, irrespective of whether [^{3}H]-cytidine or [^{3}H]uridine was used to label the ribosomes, but varied appreciably as a percentage of the UMP radioactivity. Thus CMP was the affinity labelled residue.

Purification of Fragments of 23S RNA Containing the Affinity Label.

The next step was to determine the nucleotide sequence around the affinity labelled CMP residue. The approach used was to digest affinity labelled [^{32}P]RNA with either pancreatic ribonuclease A, ribonuclease T_1 or both, purify the fragment containing the affinity label and determine its sequence. A sample of the appropriate [^{32}P]Bap-Pan-Phe-RNA fragment was always run in parallel with other samples as a marker during column chromatography or paper electrophoresis.

The major problem at this stage was to find a suitable method to purify the small fragment which was only about 0.1% of the total ribosomal RNA. Initial purification attempts involved chromatography of ribonuclease digests of affinity labelled RNA on columns

Table 8: Distribution of radioactivity in affinity label-nucleotide relative to CMP and UMP after digestion by ribonucleases T_1 and T_2 of ribosomal RNA labelled *in vivo* with either $[^3H]$cytidine or $[^3H]$uridine.

All data has been taken from Fig. 20.

Expt. No.	$[^3H]$nucleoside added to label rRNA	% Counts/min in affinity label-nucleotide relative to		% Counts/min in peaks of	
		CMP	UMP	CMP	UMP
1.	Cytidine	0.17	0.34	67	33
	Uridine	0.13	0.14	52	48
2.	Cytidine	0.15	0.38	71	29
	Uridine	0.18	0.25	58	42
3.	Cytidine	0.14	0.35	72	28
	Uridine	0.15	0.21	59	41

containing hydrophobic groups (benzoylated DEAE-cellulose and naphthalene-acetyl-cellulose) which would be expected to preferentially bind the two hydrophobic benzene rings of the affinity label (Fig. 18). In addition, further purification involved electrophoresis on DEAE-cellulose paper at pH 1.9 or 5, or electrophoresis on Whatman 3 MM paper at pH 1.9 or 3.5. However, all the above procedures (described in Materials and Methods) resulted in an impure product being obtained, in which the chief contaminant appeared to be an adenosine containing oligonucleotide (results not shown).

The purification finally used is outlined in Fig. 21 and was based on suggestions by Dr. D. Rylatt; full details are given in Materials and Methods. In the first step, the N-hydroxysuccinimide ester of biotin (biotin-NHS) was reacted at pH 8.0 with the α-amino group of the affinity label attached to 23S RNA. The reaction went to completion, as shown in Fig. 22 by the disappearance of the affinity label - CMP peak and the appearance of a less positively charged compound (which was expected since the α-amino group of Bap-Pan-Phe was acylated during reaction with biotin-NHS). After complete digestion of the biotin-affinity labelled RNA with either ribonuclease A or T_1, avidin was added to produce an avidin:biotin-affinity label - RNA fragment; biotin binds very tightly to avidin with a K_a of 10^{15} M^{-1} (Green, 1963). Since avidin is a very basic protein (Fraenkel-Conrat et al., 1952), it was possible to provide extensive purification of the avidin:biotin - affinity labelled RNA fragments by specific adsorption and elution from phospho-

FIG. 21: Outline of the approach used to purify the affinity labelled oligonucleotide fragments obtained from affinity labelled ribosomal RNA after digestion with either ribonuclease A or T_1. $-AL-NH_2$ represents the affinity label attached to the CMP residue of 23-S RNA.

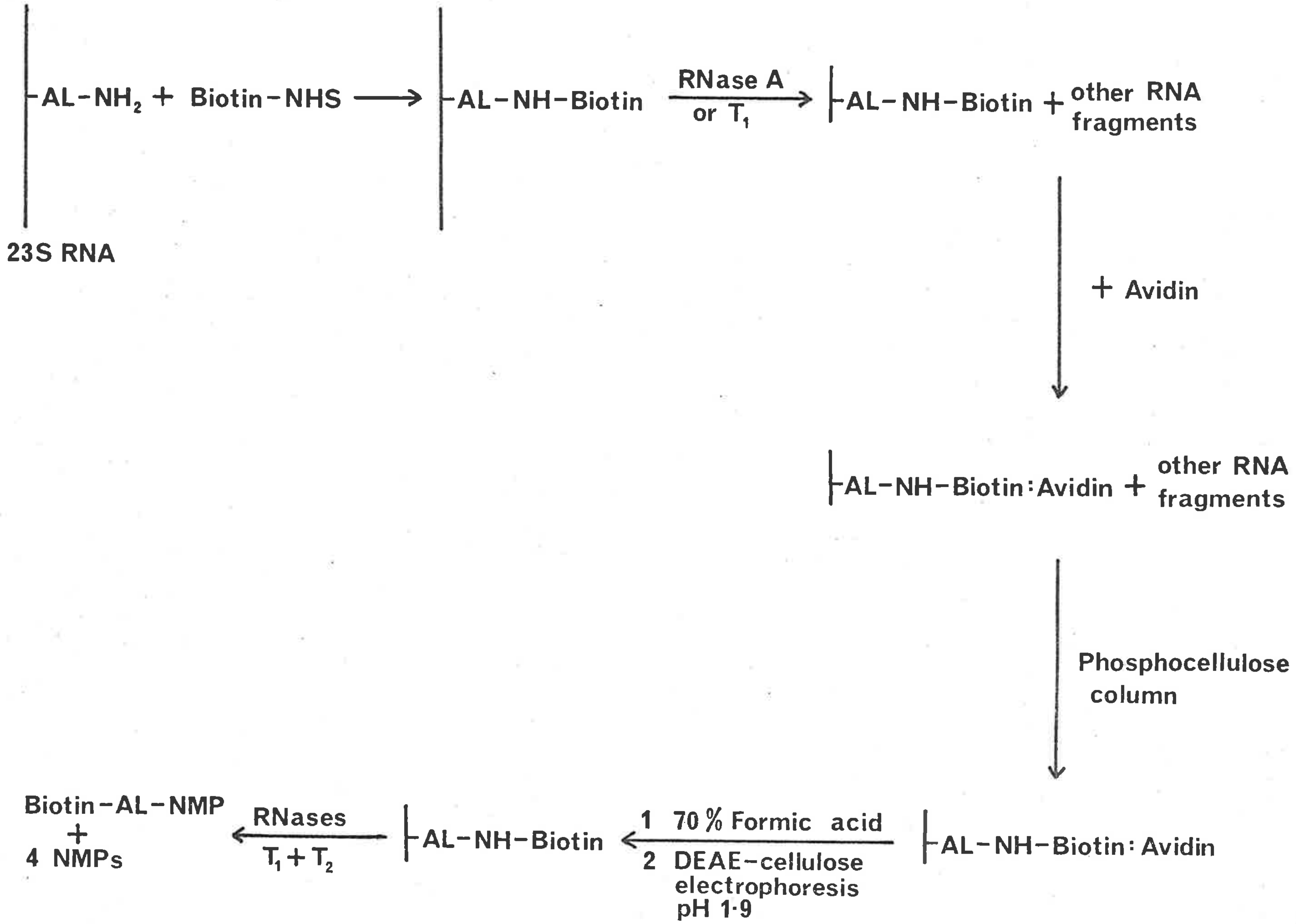
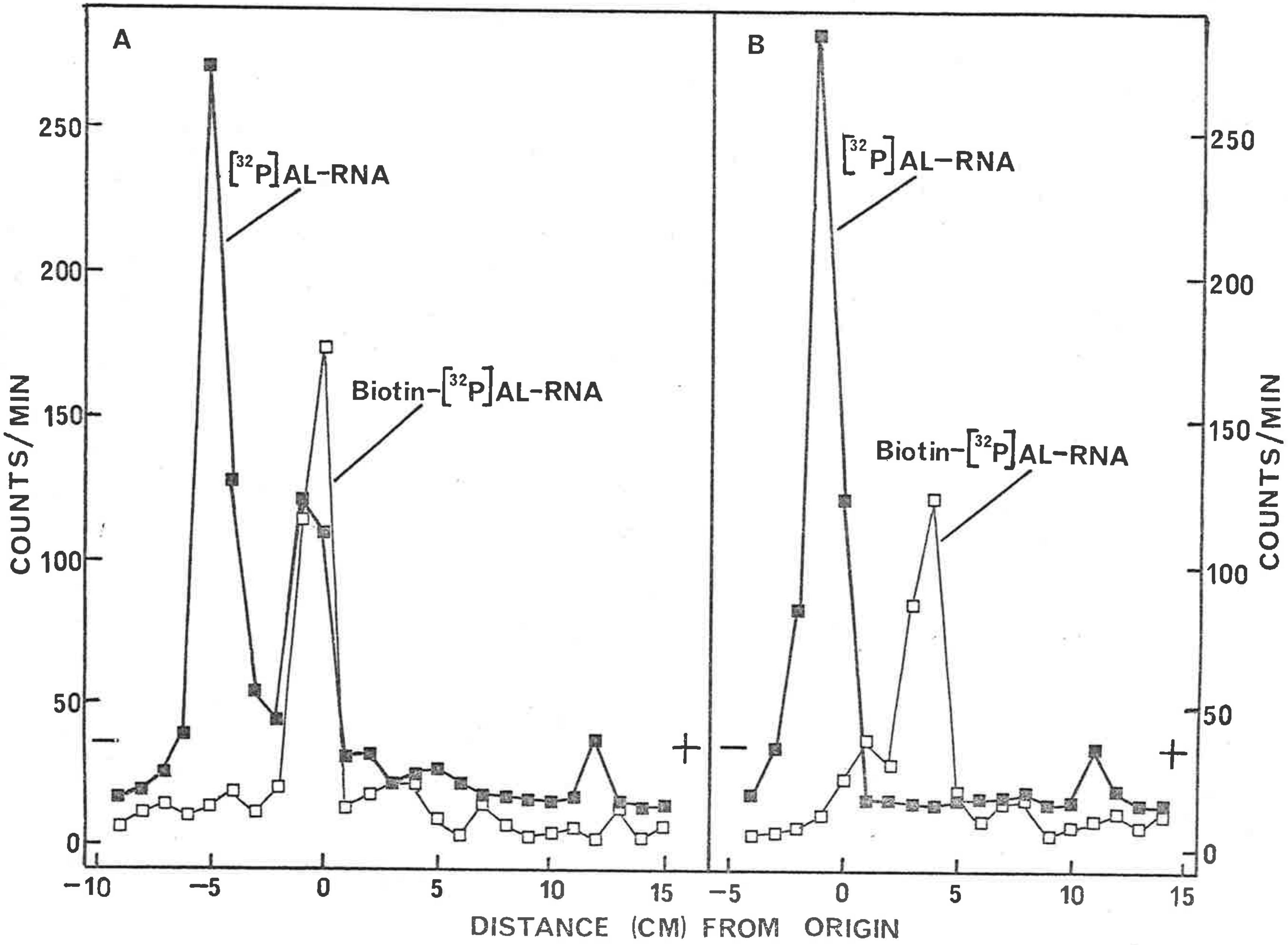

AL-NH2 + Biotin-NHS
23S RNA
AL-NH-Biotin
RNase A or T1
AL-NH-Biotin + other RNA fragments
+ Avidin
AL-NH-Biotin:Avidin + other RNA fragments
Phosphocellulose column
AL-NH-Biotin: Avidin
1 70% Formic acid
2 DEAE-cellulose electrophoresis pH 1·9
AL-NH-Biotin
RNases T1 + T2
Biotin-AL-NMP + 4 NMPs

FIG. 22: Reaction of affinity-labelled RNA with biotin-NHS. [^{32}P] Affinity label-RNA (■——■) and biotin — [^{32}P] Affinity label-RNA (□——□) were prepared and digested to mononucleotides by ribonucleases T_1 plus T_2 as described in Materials and Methods.

A: Digests electrophoresed on Whatman 3 MM paper at pH 1.9 for 1 h at 60 volts/cm.

B: Digests electrophoresed on Whatman 3 MM paper at pH 3.5 for 1 h at 60 volts/cm.


A
B
COUNTS/MIN
COUNTS/MIN
250
200
150
100
50
0
[^{32}P]AL-RNA
Biotin-[^{32}P]AL-RNA
[^{32}P]AL-RNA
Biotin-[^{32}P]AL-RNA
−
+
−
+
−10
−5
0
5
10
15
−5
0
5
10
15
DISTANCE (CM) FROM ORIGIN

The results of step-wise elution of the ribonuclease A digestion products are given in Table 9. Elution with 50 mM NH_4HCO_3, 4 M urea, followed by water and 0.2 M NH_4HCO_3 removed 99.9% of [^{32}P]RNA fragments, but only 37% of the presumed avidin:biotin -[^{32}P]Bap-Pan-Phe-RNA fragments. The required fragments were then eluted with 0.5 M NH_4HCO_3. Similar elution profiles were obtained with ribonuclease T_1 digests. As will be seen below, it was important to include a control of biotin-NHS treated [^{32}P]RNA during purification of the ribonuclease T_1 digests in order to determine the non-specific coupling of biotin to nucleotide residues.

In the next step, the biotin-affinity labelled RNA fragments were obtained from the avidin:biotin fragments by irreversible denaturation of the avidin with 70% formic acid (Rylatt *et al.*, 1977). The details of the final purification involving electrophoresis on DEAE-cellulose paper at pH 1.9 are described below.

The Sequence of the Ribonuclease A Fragment.

Electrophoresis on DEAE-cellulose paper at pH 1.9 of the ribonuclease A fragment after the phosphocellulose column gave the radioactive pattern shown in Fig. 23 A. A single peak of the biotin-affinity label- [^{32}P]RNA fragment co-migrated with the control biotin- [^{32}P]-affinity label-RNA fragment while more than half the total radioactivity represented contamination material which remained at or near the origin. Peak material in the two samples were eluted and digested with ribonucleases T_1 plus T_2 to give the component mononucleotides which were

Table 9:   Stepwise elution of ribonuclease A digests of avidin:biotin-affinity label-RNA from phosphocellulose.

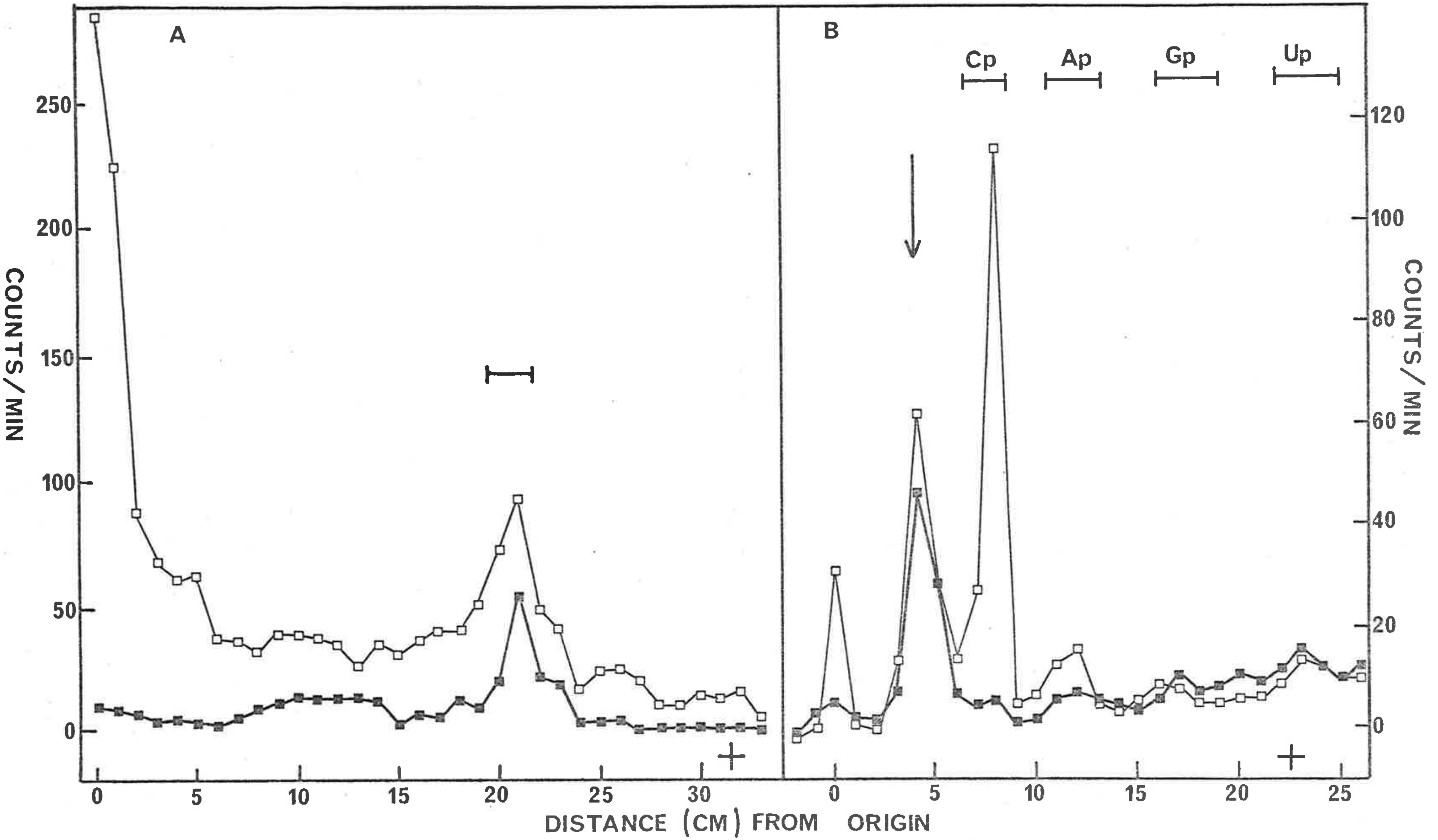
Ribonuclease digests of avidin:biotin-[^{32}P]RNA were prepared and fractionated by stepwise elution from phosphocellulose columns as described in Materials and Methods. Fractions of 1.0 ml were counted by Cerenkov radiations. The total counts/min eluted with each eluting solution are expressed as a % of the total counts/min eluted which usually varied between 70 - 80% of the counts/min applied to each column.

Eluting solution	Counts/min eluted as % of total eluted	
	Avidin:biotin-[^{32}P]-affinity label-RNA	Avidin:biotin-affinity label-[^{32}P] RNA
50 mM NH_4HCO_3, 4 M urea	31.0	99.3
Water	3.3	0.44
0.2 M NH_4HCO_3	2.8	0.13
0.5 M NH_4HCO_3	62.9	0.11

FIG. 23: Determination of the sequence of the affinity labelled oligonucleotide fragment obtained from affinity labelled ribosomal RNA after digestion with ribonuclease A. Biotin-affinity label-$[^{32}P]$RNA and biotin-$[^{32}P]$-affinity label-RNA fragments from ribonuclease A digestion were prepared and partially purified by chromatography on phosphocellulose columns as described in Materials and Methods.

A: Biotin-affinity label-$[^{32}P]$RNA (□——□) and biotin-$[^{32}P]$affinity label-RNA (■——■) fragments were electrophoresed on DEAE-cellulose at pH 1.9. The peak fractions indicated were eluted and digested with ribonuclease T_1 plus T_2 as described in Materials and Methods and the digests fractionated as in B.

B: Paper electrophoresis at pH 4.1 of the component nucleotides obtained from the purified affinity labelled oligonucleotide of A after digestion with ribonucleases T_1 plus T_2. The positions of the marker 2' (3') mononucleotides are given. The vertical arrow indicates the position of the biotin-affinity label-CMP.


A
B
Cp
Ap
Gp
Up
COUNTS/MIN
COUNTS/MIN
250
200
150
100
50
0
120
100
80
60
40
20
0
0
5
10
15
20
25
30
0
5
10
15
20
25
DISTANCE (CM) FROM ORIGIN
+
+

then fractionated by paper electrophoresis at pH 4.1.

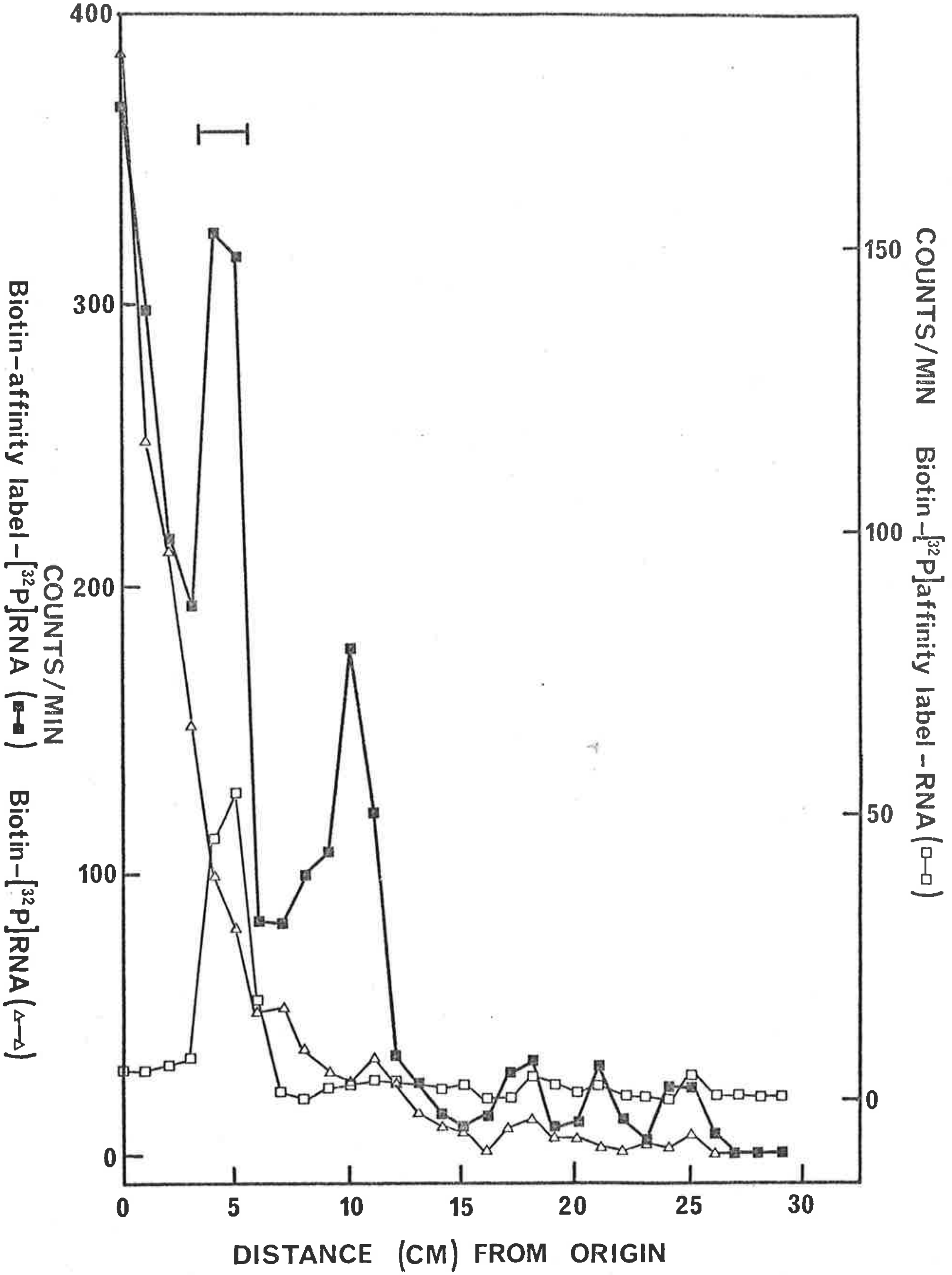
Two main peaks were found (Fig. 23B), the slower of which co-migrated with the single peak obtained from a control biotin-[^{32}P]affinity label-CMP marker while the faster moving peak co-migrated with CMP. Since ribonuclease A hydrolyses RNA on the 3'-side of CMP and UMP, it was concluded that the sequence of the ribonuclease A fragment of biotin-affinity label-RNA must be C(affinity label)pCp and that it was derived from the sequence (Pyr)pC(affinity label)pCp. The molar ratio of biotin-affinity label-CMP : CMP was 0.7 : 1; variation from the expected equimolar ratio was probably due to slow breakdown of the affinity label-CMP which has been observed throughout this work. Further, the attachment of the affinity label to CMP must either sterically prevent attack by ribonuclease A on the 3'-side of this residue or prevent recognition of CMP by the enzyme.

The sequence of the ribonuclease A fragment was confirmed in a second experiment starting with a different preparation of [^{32}P]ribosomes but the same preparation of affinity label.

The Sequence of the Ribonuclease T_1 Fragment.

The radioactive patterns obtained on electrophoresis on DEAE-cellulose paper at pH 1.9 of the ribonuclease T_1 digests of biotin-affinity label-[^{32}P]RNA, biotin-[^{32}P]affinity label-RNA and the control biotin-[^{32}P]RNA after the phosphocellulose column purification are shown in Fig. 24. Since the ribonuclease T_1 fragment of

FIG. 24: Electrophoresis on DEAE-cellulose at pH 1.9 of the ribonuclease T_1 fragments of biotin-affinity label-$[^{32}P]$RNA (■——■), biotin-$[^{32}P]$-affinity label-RNA (□——□), and biotin-$[^{32}P]$-RNA (△——△) after purification by phosphocellulose chromatography as described in Materials and Methods. Peak fractions indicated by the horizontal bar were eluted and digested with ribonucleases T_1 plus T_2 (see Fig. 25).


400
300
200
100
0
COUNTS/MIN
Biotin-affinity label-[32P]RNA (■-■)
Biotin-[32P]RNA (△-△)
150
100
50
0
COUNTS/MIN
Biotin-[32P]affinity label-RNA (□-□)
0
5
10
15
20
25
30
DISTANCE (CM) FROM ORIGIN

biotin-affinity label-RNA did not migrate as far as the ribonuclease A fragment (Fig. 23 A), it was not clearly separated from contaminating material which ran at or near the origin. However, material migrating with the peak of the ribonuclease T_1 fragment of biotin- $[^{32}P]$-affinity label-RNA was eluted, hydrolysed with ribonuclease T_1 plus T_2 and the digests fractionated by paper electrophoresis at pH 4.1 (Fig. 25). In the case of the control biotin-$[^{32}P]$RNA fragment, AMP was the major nucleotide with lesser amounts of CMP, GMP and UMP (Fig. 25B). The four nucleotides were also present in the biotin-affinity label- $[^{32}P]$RNA fragment in addition to the affinity label - CMP nucleotide (Fig. 25 A), although there was a low proporation of AMP present.

The sequence of the T_1 fragment was derived as follows (Table 10). It was assumed that there was only one sequence, which must be terminated by GMP, and that all the minor peak of AMP was derived from contaminating material (since it was the major nucleotide present in the control biotin- $[^{32}P]$RNA fragment). The total counts/min in each of the nucleotide peaks of the biotin-affinity label- $[^{32}P]$RNA fragment were determined (Fig. 25 A, Table 10). Next, from the counts/min in the four nucleotide peaks of the control biotin - $[^{32}P]$RNA fragment (Fig. 25 B), the contamination by CMP, GMP, and UMP relative to AMP (taken as 105 counts/min) was calculated (Table 10). These figures were then subtracted to give the corrected counts/min in each nucleo-

FIG. 25: Determination of the sequence of the purified affinity labelled oligonucleotide obtained from affinity labelled RNA after digestion with ribonuclease T_1. Ribonuclease T_1 fragments purified as in Fig. 23 were digested with ribonucleases T_1 plus T_2 and the mononucleotides separated by paper electrophoresis at pH 4.1. The positions of the marker 2' (3') mononucleotides are given. The vertical arrow indicates the position of the biotin-affinity label-CMP.

A: Biotin-affinity label-[^{32}P]RNA (■——■) and biotin-[^{32}P]affinity label-RNA (□——□).

B: Biotin-[^{32}P]RNA (△——△).

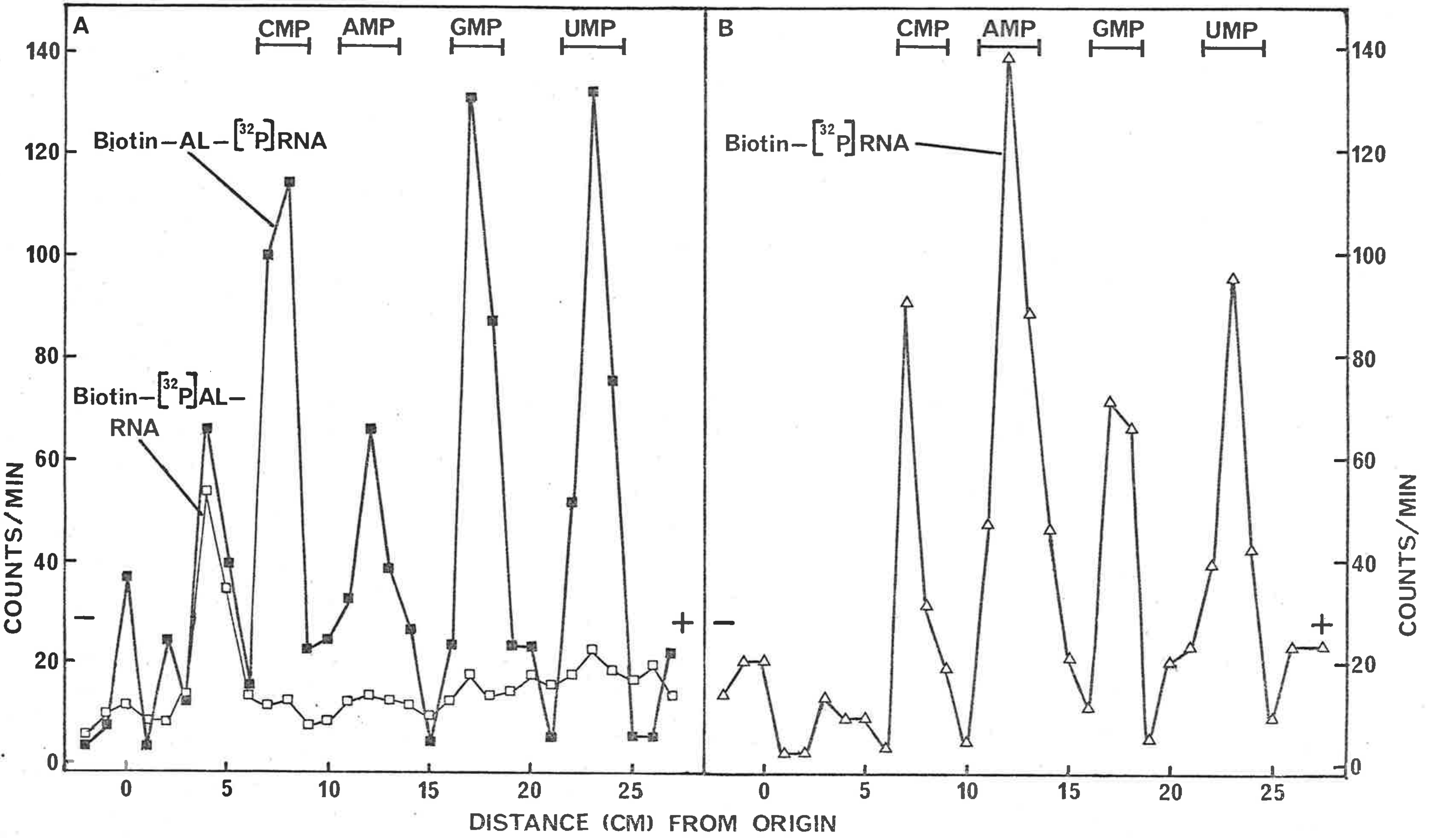
A
B
CMP
AMP
GMP
UMP
Biotin–AL–[^{32}P]RNA
Biotin–[^{32}P]AL–RNA
Biotin–[^{32}P]RNA
COUNTS/MIN
DISTANCE (CM) FROM ORIGIN
0
20
40
60
80
100
120
140
0
5
10
15
20
25
–
+

Table 10: Distribution of radioactivity in nucleotide peaks of Fig. 25 derived from ribonuclease T_1 digestion of purified fragments of biotin→affinity label-[^{32}P]RNA (Fig. 25A) and of biotin→[^{32}P]RNA (Fig. 25B). See Fig. 25 for experimental details.

	Nucleotide peak				
	Affinity label-CMP	Cp	Ap	Gp	Up
Total counts/min in each peak in Fig. 25A	84	197	105	209	227
Total counts/min in each peak in Fig. 25B relative to Ap taken as 105	0	44	105	50	63
Corrected counts/min in each peak in Fig. 25A	84	153	0	159	164
Ratio of corrected counts/min relative to Gp	0.53	0.96	0	1.00	1.03

tide peak of the biotin-affinity label-[^{32}P]RNA fragment and hence the relative molar amounts of the four nucleotides (Table 10). CMP, GMP and UMP were present in equimolar amounts, but the ratio of the affinity label-CMP was again low, presumably because of its instability, as already mentioned. It was concluded, therefore, that GMP, CMP, UMP and affinity label-CMP were present in equimolar amounts in the ribonuclease T_1 fragment of biotin-affinity label-[^{32}P]-RNA.

Since data from the ribonuclease A derived fragment indicated the sequence was (Pyr)pC(affinity label)pCp and ribonuclease T_1 hydrolyses RNA on the 3'-side of GMP, then only one sequence, UpC(affinity label)pCpGp, is possible from this data. Additionally, since this sequence does not appear at the 5'-end of 23S RNA (Branlant _et_ _al_., 1976 a), then it must have been derived from the sequence GpUpC(affinity label)pCpGp. This result was confirmed in three completely separate experiments using two different preparations of affinity label.

General Discussion

The sequence, GpUpCpCpGp, does not occur in any sequences of _E. coli_ 23S rRNA published to date although about 40% of the total sequence is known (Branlant _et_ _al_., 1975; Branlant _et_ _al_., 1976 a, b, c, d). If this sequence only occurs once in 23S RNA, then it would locate that portion of the 23S RNA which is in, or close to, the active centre of peptidyl transferase.

This work has not resolved the site of attachment of the affinity label to the CMP residue in ribosomal RNA. Since the Bap-Pan-Phe reaction with ribosomal RNA is dependent on the integrity of the ribosome (Greenwell et al., 1974; see above also) and since there is no specific reaction of Bap-Pan-Phe with poly (C), it has not been possible to prepare sufficient of the affinity label - CMP for structural analysis.

The results of Greenwell et al., (1974) showed that two molecules of Bap-Pan-Phe were bound to 23S RNA in each ribosome when 100% inactivation of peptidyl transferase was obtained. However, the present work has found only one affinity labelled pentanucleotide sequence. One explanation is that there are two GpUpCpCpGp sequences of 23S RNA in the active centre of peptidyl transferase to which Bap-Pan-Phe is attached. Another possibility is that two affinity label molecules are attached to the same CMP residue, either to different positions on the CMP or as a dimer in which the second affinity label attaches to the first in a reaction between the bromoacetyl group of the second affinity label and the α-amino group of the first. Dimer formation could also explain the very low acceptor activity of the affinity label bound to the A'-site of peptidyl transferase (Harris et al., 1973; Greenwell et al., 1974), since loss of the α-amino group of the first affinity label could prevent acceptance of N-acetyl-amino acid from donor substrates in the fragment reaction. The small amount of acceptor activity found could be due to either a small proportion of ribosomes to which only one affinity label had bound, or even to a low level of acceptor activity of the dimer bound to the ribosome.

In order to explain the binding of two molecules of Bap-Pan-Phe to 23S RNA and the low acceptor activity of bound affinity label, Greenwell et al., (1974) proposed that one affinity label was bound in the A'-site and the other in the P'-site. The low acceptor activity was considered to be due to the small proportion of ribosomes with affinity label bound only in the A'-site. However, binding of the donor substrate, UpApCpCpA-(Ac-L-[^{3}H]Leu) to the P'-site of affinity labelled ribosomes as described in Materials and Methods (82% of ribosomes were affinity labelled according to the fragment reaction) showed only a small reduction in binding (20%) when compared with the binding to untreated ribosomes. This indicates that there was little, if any, Bap-Pan-Phe bound to the P'-site, or that it was bound in such a way that donor substrate binding was not significantly affected.

Concluding Remarks.

A pentanucleotide fragment of 23S RNA containing the bound affinity label, Bap-Pan-Phe, has been isolated and the sequence GpUpC(affinity label)pCpGp obtained. Since two molecules of affinity label become bound to 23S RNA when total inactivation of peptidyl transferase occurred (Greenwell et al., 1974), either two GpUpCpCpGp sequences are present in 23S RNA or the two affinity label molecules are bound to the same CMP residue, either separately or as a dimer.

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