THE ACTIVE CENTRE OF PEPTIDYL TRANSFERASE

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Doctor of Philosophy by

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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 when due reference is made in the text.

E.F. VANIN

PAPERS PUBLISHED OR IN THE PRESS

1. Vanin, E.F., Greenwell, P. and Symons, R.H. (1974). Structure-Activity Relationships of Puromycin Analogues on Escherichia coli Polysomes. FEBS Letters, 40, 124.

2. Vanin, E.F. and Symons, R.H. (1976). The Preparation of High Specific Activity [^{3}H]Chloramphenicol Base and Chloramphenicol Labelled in the Propanediol Side Chain. Anal. Biochem., 76, 259.

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CONTENTS

ABBREVIATIONS

A-L-Phe	2'(3')-O-(L-phenylalanyl)-adenosine
ApPan-Gly	adenyl-(3'-5')-Pan-Gly
Bap-Pan-L-Phe	5'-O-(N-bromoacetyl-p-amino-phenylphosphoryl)-3'-N-(L-phenylalanyl)-Pan
tBoc	t-butyloxycarbonyl
CBZ	carbobenzoxy
CpCpA-L-Leu	cytidyl-(3'-5')-cytidyl-(3'-5')-2'(3')-O-L-leucyl adenosine
CpCpA-L-fMet	cytidyl-(3'-5')-cytidyl-(3'-5')-2'(3')-O-(N-formyl-L-methionyl)adenosine

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

DCC	N,N'-dicyclohexylcarbodiimide
DCU	N,N'-dicyclohexyl urea
EEDQ	N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline
(N-BrAc-L-Phe)-tRNA	2'(3')-O-(N-bromoacetyl-L-phenylalanyl)-tRNA
im-benzyl-L-His	imidazole-benzyl-L-histidine
kPa	kilopascals
NHS	N-hydroxysuccinimide
O-benzyl-L-Ser	O-benzyl-L-serine
Pan	puromycin aminonucleoside
Pan-Gly	3'-N-glycyl-Pan
Pan-(N-α-tBoc-amino acid)	3'-N-(N-α-tBoc-aminoacyl)-Pan

PNPC	p-nitrophenylcarbamyl
PPO	2,5-diphenyloxazole
POPOP	(1,4-bis[2-(5-phenyloxazolyl)]-benzene
SDS	sodium dodecyl sulphate
TFA	trifluoroacetic acid
TLC	thin layer chromatography

DEFINITIONS

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

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

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

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

SUMMARY

Peptidyl transferase, an integral part of the large subunit of the ribosome, catalyses the transfer of the nascent peptide from the 3' terminal end of peptidyl-tRNA, situated in the P site, to the 3' end of aminoacyl-tRNA, situated in the A site. In particular this thesis is concerned with the substrate specificity and affinity labelling of the A' site together with the affinity labelling of chloramphenciol (an acceptor site inhibitor) binding site.

The first section of this thesis describes the synthesis and use of various puromycin analogues, in order to study the substrate specificity of the A' site of peptidyl transferase of E. coli polysomes. Due to the lack of availability of these analogues in radioactive form, the Michaelis-Menten constants could not be determined; instead the analogues were used as competitive inhibitors of the formation of peptidyl - [^{3}H]puromycin. The resultant data was analysed by means of the Dixon plot (Dixon, 1953) to give apparent Ki values, which are a measure of the relative affinities of these analogues for the A' site, and the results obtained are summarised below.

1. The hydrophobic analogues (Pan-Gly, Pan-L-Leu, Pan-L-Tyr and Pan-L-Phe) were found to give decreasing Ki values, that is better binding, with increasing hydro-

phobicity of the aminoacyl side chains.   The higher K_i values and therefore weaker binding of the larger hydrophobic analogues, such as Pan-(im-benzyl-L-His), Pan-(O-benzyl-L-Ser) and Pan-L-Trp can be attributed to the steric hinderance of binding of the very large hydrophobic side chains for the site,

(2)   Pan-D-Phe had a K_i value five times that of Pan-L-Phe.

(3)   of the 5'-O-nucleotidyl derivatives of Pan-Gly only CpPan-Gly gave a low K_i (strong binding) whereas the other nucleotidyl substituted Pan-Gly derivative had K_i values more comparable to Pan-Gly.   Chemical coupling of Cp to Pan-L-Phe also caused a decrease in the K_i value, when compared to Pan-L-Phe.

Therefore these results have indicated the presence of at least two binding regions in the A' site of E. coli polysomal peptidyl transferase.

(1)   a hydrophobic pocket specific for binding the hydrophobic aminoacyl side chains,

(2)   a specific binding site for the penultimate CMP residue of aminoacyl-tRNA.

The enzyme was also shown to exhibit a certain degree of stereospecificity.

The second section deals with attempts to affinity

label the A' site of peptidyl transferase and the chlor-
amphenicol binding site.

Using the data from the previous section, together
with the data supplied by Eckermann et al., (1974) and
Harris and Symons (1973b) two affinity labels were
designed and synthesised for the A' site.   The first
analogue, ε-bromoacetyl-L-lysyl-Pan (Pan-(ε-BrAc-L-Lys))
acted as an acceptor substrate in the fragment reaction
(that is, it was able to bind to the A' site) and follow-
ing overnight incubation with E. coli ribosomes became
covalently attached to the A' site, thereby inactivating
peptidyl transferase.   Attempts, using [^{14}C] affinity
label, to identify the proteins and/or RNA which had the
specific affinity label attached, were unsuccessful
because of the high degree of non-specific reaction (56
affinity labels/ribosome) and the instability of the
ribosomes under the necessarily lengthy incubation con-
ditions used.

The second A' site affinity label used was p-
azido-L-phenylalanyl-Pan (Pan-L-pN$_3$Phe), a photoaffinity
label.   This analogue was also shown to act as an
acceptor substrate in the fragment reaction, but upon
photolysis of [^{3}H]Pan-L-pN$_3$Phe with ribosomes, there was
no inactivation of peptidyl transferase, even though 17
affinity labels were attached per ribosome.

An attempt was also made to affinity label the

chloramphenicol binding site, using p-azido-benzoyl

chloramphenicol base (pN$_3$benzoyl chloramphenicol base),

but, in order to do this, high specific activity radio-

active chloramphenciol base had to be synthesized.

This was achieved by treating chloramphenicol base with

acetic anhydride, in order to protect the amino group,

and subsequently with monomethoxytrityl chloride, to pro-

tect the C$_3$ hydroxyl.   The C$_1$ hydroxyl was then oxidized

to a ketone, using chromium trioxide-pyridine, the mono-

methoxytrityl group removed and the ketone reduced to the

hydroxyl using tritiated potassium borohydride to give

diastereoisomers (both the C$_1$ and C$_2$ positions of the

propanediol side chain are asymmetric centres).   Follow-

ing acetylation of the C$_1$ and C$_3$ hydroxyls the two dia-

stereoisomers were separated by TLC and the correct

isomer isolated.   This was then treated with alkali to

remove the O-acetyl groups, followed by acid to remove

the N-acetyl group to give [^{3}H]chloramphenicol base at

a specific activity of about 2 Ci/mole.   Chloramphenicol

base, prepared using essentially the same procedure as

above, was shown to be identical to a commercial sample

by nuclear magnetic resonance spectroscopy, chromato-

graphic analysis and electrophoretic analysis.   In

addition, chloramphenicol prepared by dichloroacetylation

of the amino group of chloramphenicol base, prepared as

above, had the same Ki value in the fragment reaction as

commercial chloramphenicol.

The photoaffinity label, pN_3benzoyl chloramphen-
icol base, was synthesized and shown to be an inhibitor
of peptidyl transferase, as measured by the fragment
reaction, with a Ki value similar to that of chloram-
phenicol.   It was also shown to inhibit the binding of
[^{3}H]chloramphenicol to ribosomes.   Upon photolysis the
radioactive affinity label became covalently attached to
the ribosome.   However, the affinity labelling was non-
specific because covalently attached affinity label did
not inhibit either the fragment reaction or the binding
of [^{3}H]chloramphenicol and it did not prevent the
inhibition of the fragment reaction by chloramphenicol.

Therefore the results obtained, using these three
affinity labels, emphasises the difficulty in using low
molecular weight compounds as affinity labels.   The
weak binding of these analogues, when compared to the
binding of aminoacyl-tRNA, which may be a reason for the
great success of the peptidyl-tRNA derivatives (for
review see Introduction), necessitates the use of high
concentrations of affinity label, together with long
incubation times.   This, coupled with the complexity of
the ribosome, led to a high degree of non-specific label-
ling, which in one case prevented the identification of
the proteins and/or RNA attached to the specific affinity
label and in the other two cases caused covalent attach-
ment to non-specific sites.

INTRODUCTION

INTRODUCTION

The experimental and theoretical work described in this thesis covers the investigation of three different aspects of protein biosynthesis, namely

    (1)   in vitro specificity of the A' site of peptidyl transferase from E. coli polysomes,

    (2)   the ribosomal components (protein and/or RNA) of the A' site,

and (3)   the ribosomal components which comprise the chloramphenicol binding site.

These studies were aimed at increasing our understanding of the mechanism of peptide bond formation and of the inhibitory action of chloramphenicol on this process.

The ultimate aim of the research described above is to further our understanding of protein biosynthesis at the molecular level, including the mechanism of action of inhibitors (for example, chloramphenicol, lincomycin and the macrolides) on this process. Fulfilment of this aim would most certainly lead to the rational design of selective antibiotics and perhaps advances in other areas (such as transcriptional and stringent control).

Protein biosynthesis involves a number of reactions including binding of mRNA, association of subunits, decoding of mRNA accompanied by the binding of aminoacyl-

FIGURE 1:   Diagrammatic representation of an E. coli
ribosome, just prior to peptide bond formation.
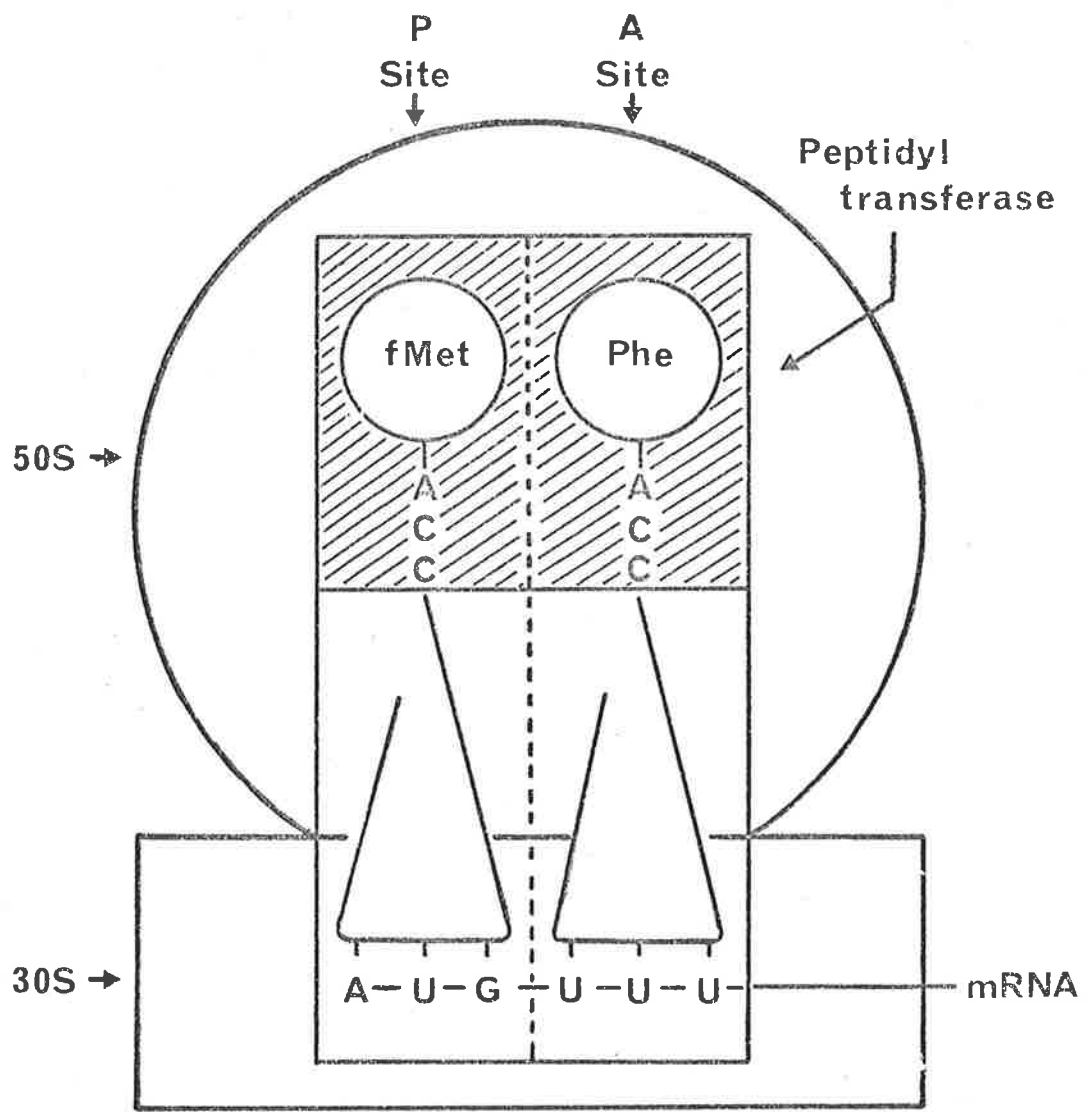
tRNA, peptide bond formation, translocation, termination, release of the newly synthesized peptide and finally dissociation of the ribosomal subunits. One of the most important and central of these reactions is peptide bond formation, which is catalysed by the ribosomal enzyme, peptidyl transferase (Monro et al., 1969). This enzyme, which is exclusively located on the large subunit of both prokaryotic (Monro et al., 1969) and eukaryotic ribosomes (Thompson and Moldave, 1974; Vazquez et al., 1969) catalyses the transfer of the nascent chain from the peptidyl-tRNA bound to the P site, to the amino-acyl-tRNA bound to the A site (Figure 1). This transfer is thought to involve a nucleophilic attack by the α-amino group of aminoacyl-tRNA, on the carboxyl group of the ester link of peptidyl-tRNA (Figure 2). Such an attack affects cleavage of the ester and formation of a new peptide bond (Allen and Zamecnik, 1962; Nathans, 1964a, b).

The following introduction will give a brief review of the literature up to April, 1977, on peptidyl transferase. Particular attention will be paid to the substrate specificity of both the A' and P' sites of the enzyme, affinity labelling of peptidyl transferase and the chloramphenicol binding site and the structure-activity relationships of chloramphenicol analogues. Then using the data available a model for the protein and RNA composition of the A' and P' sites of peptidyl

FIGURE 2:   Proposed nucleophilic attack of the amino

group of puromycin on peptidyl-tRNA.

      R_1 = peptide

      R_2 = aminoacyl side chain

peptidyl-tRNA   puromycin   peptidyl-puromycin
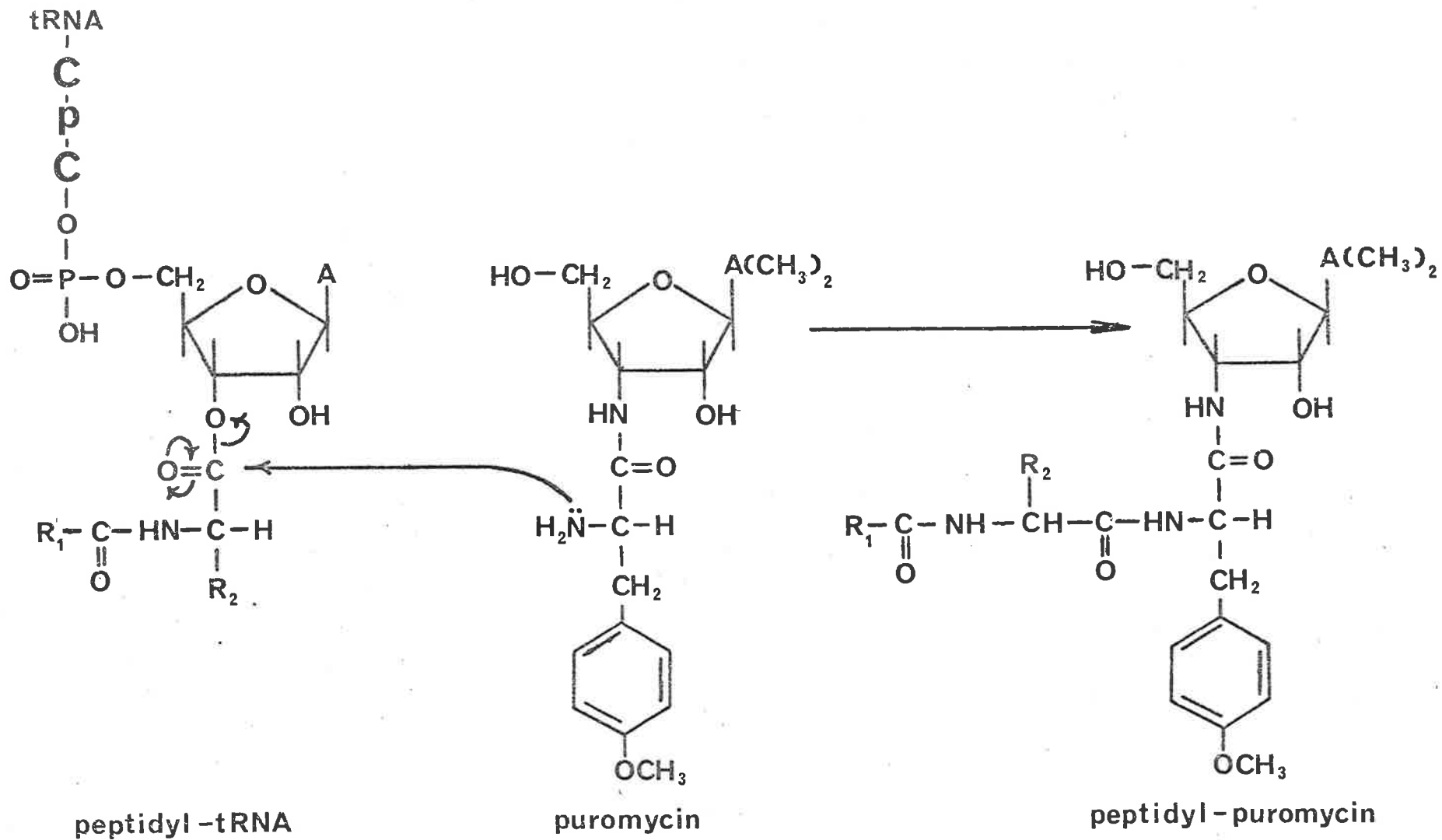
transferase will be presented.   All the studies con-
sidered were carried out using E. coli ribosomes.

1.   Acceptor Site Substrate Specificity of Peptidyl
      Transferase.

The substrate specificity of the A' site, which is
responsible for binding the 3' terminus of aminoacyl-
tRNA, has been studied using a variety of tRNA analogues.
Most of the studies have used either aminoacyl-nucleo-
side derivatives or analogues of puromycin.   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, 1964a, b).   The various aspects
of the specificity investigated are discussed below.

(a)   Activity of 2' and 3' isomers of aminoacyl-
       tRNA analogues at the A' site.

Chladek et al., (1974), using non-isomerizable
2' and 3' aminoacyl-dinucleoside-phosphates, have
shown that the 3'-O-aminoacyl derivatives CpA-
(2'-O-methyl)-L-Phe, Cp(2'-deoxy)A-L-Phe, and
A(2'-O-methyl)L-Phe were acceptor substrates using
(N-Ac-L-Phe)-tRNA as the donor substrate, 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 acceptor substrates.   These results
indicate that the 3'-aminoacyl ester is either

the exclusive or strongly preferred one for peptide bond formation. Consistent with this is the fact that the 2' isomer of puromycin is inactive in inhibiting protein synthesis (Nathans and Neidle, 1963). However, the 2' hydroxyl is essential for high acceptor substrate activity as both Rychlik et al., (1969) and Cerna et al., (1970) have found that (2'-deoxy)A-L-Phe had negligible activity as an acceptor. Chladek et al., (1974) also found that methylation of the 2' hydroxyl of A-L-Phe reduced, but did remove, the acceptor activity. The 2' hydroxyl is also required for elongation factor Tu because it presents the 2' ester of aminoacyl-tRNA to the ribosome (Ringer and Chladek, 1975) after which the aminoacyl moiety is transferred to the 3' position, where it can accept the nascent peptide. Symons et al., (1977) have suggested a possible reason for this transfer may be that the 3' terminal part of an uncoded aminoacyl-tRNA cannot undergo this transfer and so cannot bind correctly to the A' site, therefore, preventing the introduction of erroneous amino acids into nascent polypeptides. This process, if confirmed, exemplifies the remarkable molecular mechanisms that have evolved to ensure that protein synthesis proceeds with high fidelity. Thus, the activity of the 2' and 3' analogues of the 3' terminus of aminoacyl-tRNA has given clues to

the processes occurring before peptide bond form-
ation.

(b)   Hydrophobic site.

Following binding of the 2' isomer of amino-
acyl-tRNA and transfer of the amino acid to the
3' hydroxyl there are several binding interactions
which occur between the acceptor substrate and
specific binding sites of the A' site, one of
which is a hydrophobic site.   Other binding sites
are also discussed (see below).

The presence of a hydrophobic binding pocket
in the A' site of peptidyl transferase has been
postulated by a number of groups (Eckermann et al.,
1974; Harris et al., 1971; Nathans and Neidle,
1963; Rychlik et al., 1970; see also Chapter 2).
In general they have found that the more hydro-
phobic the aminoacyl side chain (natural amino
acids only considered) of the aminoacyl-nucleo-
side derivatives or the puromycin analogues, the
better the binding (as measured by higher acceptor
activity or their ability to inhibit the release of
peptides by puromycin) to the A' site; the less
hydrophobic derivatives showed much weaker binding
(weaker acceptor activity and weaker inhibitory
activity) with decreasing strength of binding
occurring with decreasing hydrophobicity of the

aminoacyl side chain (for example, in order of decreasing activity and hydrophobicity L-Leu, L-Ala and L-Gly analogues). The weak binding, obtained by Eckermann et al., (1974), Harris et al., (1971) and in Chapter 2, of the Pan-(S-benzyl-L-Cys), Pan-(im-benzyl-L-His), Pan-(O-benzyl-L-Ser and Pan-L-Trp can be explained by the fact that the side groups are very bulky and therefore sterically hindered from binding to the hydrophobic site. Support for the hydrophobic site was also supplied by Pestka et al., (1970), who have found that the CpApCpCpA-L-Phe was bound to ribosomes to a greater extent than CpApCpCpA-L-Val, CpApCpCpA-L-Met and CpApCpCpA-L-Leu and also by Lessard and Pestka (1972), who found that the affinity of the amino-acyl-trinucleotides decreased in the order CpCpA-L-Phe, CpCpA-L-Leu, CpCpA-L-Lys, CpCpA-L-Ala and CpCpA-L-Glu.

The increased binding observed by the more hydrophobic analogues could not be due to intra-molecular stacking, as was suggested by Symons et al., (1969), Raacke (1971) and Ariatti and Hawtrey (1975), because of the extended nature of the puromycin molecule, as shown by X-ray analysis (Sundaralingam and Arora, 1972).

(c) Hydrophilic site.

This site was proposed by Rychlik et al.,
(1970) to explain the high activity of A-L-Lys
when compared to A-Gly and related uncharged
analogues (such as A-L-Val, A-L-Leu and A-L-Ala).
This site binds the amino acid side chains of
lysyl and arginyl-tRNA, probably by ionic inter-
action with an α, β or γ carboxyl group or a phos-
phate group. Support for the presence of this
site was also supplied by the strong binding of
CpCpA-L-Lys, when compared to CpCpA-L-Ser, CpCpA-
L-Ala and CpCpA-L-Glu (Lessard and Pestka, 1972).

(d) Adenine site.

The necessity of adenine for high acceptor
activity was shown by the high activity of A-L-Phe
relative to that of I-L-Phe and C-L-Phe, while
G-L-Phe and U-L-Phe were inactive (Cerna et al.,
1970).

The role of the C_6 amino group has been
investigated (Zemlicka et al., 1975) and the
analogues tested (see below) had high acceptor
activity, although activity decreased with a
decrease in the electron donating properties of
the substituent, that is, in decreasing order of
activity, NH_2, $N(CH_3)_2$ > SCH_3 > OCH_3 > H. Two
possible explanations were offered for this by

Zemlicka et al., (1975),

(1)   a decrease in electron density of the nebularine ring (purine ring without C_6 amino group) may decrease hydrogen bond formation between the N_3 nitrogen and some group on the ribosome,

(2)   a decrease in electron density may lead to decreased hydrophobic interactions of the purine ring with some part of the ribosome.

Furthermore, the high activity of the C_6 substituted analogues and of puromycin (which has a C_6 dimethyl amino group) does not support the possibility that the 3' terminal adenosine of aminoacyl-tRNA base pairs with a uridine on the 23S or 5S rRNA (Greenwell et al., 1974; Harris et al., 1973).

(e)   CpCp site.

There is strong evidence for a specific binding site for the penultimate CMP residue of aminoacyl-tRNA.   It was found that 5' substitution of Pan-Gly and A-Gly with 3'CMP resulted in a large increase in acceptor substrate activity, but substitution with GMP, UMP and AMP, in the case of Pan-Gly, and UMP in the case of A-Gly, gave no

increase in acceptor substrate activity (Eckermann et al., 1974; Harris et al., 1971; Rychlik et al., 1967; see also Chapter 2). An additional site for the third nucleotide (CMP) from the 3' end of aminoacyl-tRNA appears likely since CpPan-Gly and CpA-Gly had low activity, when releasing peptides from peptidyl-tRNA, while Takanami (1964) found that $(Np)_x$CpCpA-L-amino acids had high puromycin like activity which was independent of the nature of the amino acid.

More direct data, in support of the proposed CpCp site, was provided by Pestka et al., (1970) who found that A-L-Ser bound very poorly to ribosomes, whereas CpCpA-L-Ser bound strongly. Likewise, the binding of puromycin was just detectable (Fernandez-Munoz and Vazquez, 1973) but CpCpA-L-Phe bound strongly (Lessard and Pestka, 1972).

Alterations in the ribose ring (Duquette et al., 1974) have shown that the 5'-hydroxymethyl group and the oxygen of the ribose ring are not required for acceptor substrate activity. The intactness of the ribose ring had some effect on acceptor activity as was shown by Chladek et al., (1973) who cleaved the ribose ring, using periodate oxidation, reduced the aldehydes and then amino-acylated the alcohols. They found that cleavage of the ribose ring reduced, but did not completely destroy, acceptor substrate activity. Therefore the intactness of the

ribose ring is of importance for acceptor substrate activity presumably due to the positioning and restricted movement of the 2' and 3' hydroxyls.

Therefore, in summary, once the 2' aminoacyl-tRNA is bound to the A' site (via the EF-Tu-GTP complex) each of the nucleotides of the terminal CpCpA interact with the corresponding binding sites in the A' site which correctly positions the 3' terminus of the aminoacyl-tRNA for subsequent peptide transfer. The aminoacyl residue is then transferred from the 2' to the 3' hydroxyl and the aminoacyl side chain, depending on its character, is then able to interact either with the hydrophobic or hydrophilic binding pocket (this transfer presumably does not occur if non-coded aminoacyl-tRNA interacts with the A site (Symons et al., 1977)). This interaction would then correctly position the α-amino group of the amino-acyl-tRNA to accept the peptide from peptidyl-tRNA. Harris and Symons (1973b) have published a diagrammatic representation of the binding pockets of the A' site which is consistent with all of the above data.

2.   Peptide Site Substrate Specificity of Peptidyl Transferase.

The P' site is the site responsible for binding the 3' terminal end of peptidyl-tRNA during peptide bond formation. Although substrate specificity studies are more limited than those of the A' site, enough data has

accumulated to define at least some of the structural requirements for substrate activity.   This data has led to the proposal for the presence of certain binding pockets in the P' site.   One essential feature of the P' substrates is a blocked α-amino group (Mao, 1973; Monro et al., 1968).   The proposed binding pockets of the P' site are as follows,

(a)   <u>Hydrophobic site.</u>

This site is involved in binding the amino-acyl side chains of the hydrophobic amino acids. Mao (1973), using N-acetylated, unacetylated and mono and dipeptidyl-tRNA, studied their donor activity in the fragment reaction.   He found that the amino acids or dipeptides which had high hydro-phobicity (such as L-Leu and L-Phe) were transferred faster than those with low hydrophobicity (such as L-Pro).   Mercer and Symons (1972) and Monro et al., (1968) also found that the affinity of the amino-acyl side chains decreased in the order L-Met > L-Leu > L-Phe, while L-Gly was not bound to the site, in view of the negligible donor activity of the Ac-Gly-oligonucleotides.

(b)   <u>Hydrophilic site.</u>

This was proposed by Harris and Symons (1973b) in view of the high donor activity of (Np)$_x$Cp-CpA-L-Arg-Ac when compared to the Ac-Gly-oligo-

nucleotides (Monro et al., 1968).

(c)   Adenine site.

The activity of donor substrate analogues of
the type pN-L-fMet decreases in the order
A > I > G, while the C and U analogues were inactive
Cerna et al., 1974).

(d)   CpCp site.

Sites exist for the binding of the two ter-
minal CMP residues of peptidyl-tRNA, since donor
activity, in the fragment reaction, decreased
dramatically in the order CpCpA-L-fMet >> CpA-L-
fMet >> A-L-fMet (Krayersky et al., 1976; Monro
et al., 1968).   Nucleotide sequences distal to
the CpCpA terminus had little effect on donor
activity (Monro et al., 1968).   For further evi-
dence on the presence of this site see Symons et al.,
(1977).

(e)   Nascent peptide site.

The presence of this site is based on the
fact that ribosomes are able to protect the nascent
peptides from proteases (Malkin and Rich, 1967).

Therefore, as can be seen from the results pre-
sented above, the binding pockets of the P' site are
similar to those of the A' site.

3.   Affinity Labelling of Peptidyl Transferase.

The technique of affinity labelling (Baker, 1967; Knowles, 1972; Shaw, 1970) has been used successfully, to identify the ribosomal components constituting the P' site, A' site and the chloramphenicol binding site.

Most of the affinity labelling work on peptidyl transferase has concentrated on the P' site, mainly because of the ease of synthesis of these analogues (that is, the blocking of the α-amino group of aminoacyl-tRNA with a reactive group, such as the bromoacetyl group, to give a reactive peptidyl-tRNA analogue, is a relatively simple reaction).   The affinity labels used and the ribosomal components which they label are listed in Table 1.

As can be seen, most of the analogues are deriva-tives of (L-Phe)-tRNA and are directed towards labelling the P' site.   The most often labelled ribosomal com-ponents were proteins L2 and L27 and 23S RNA.   The labelling of the 23S RNA was shown by several groups (Bartō et al., 1975; Bispink and Matthaei, 1973; Breitmeyer and Noller, 1976; Budker et al., 1972; Girshovich et al., 1974; Sonenberg et al., 1975; Sonenberg et al., 1976; Yukioka et al., 1975) and in one case (Barta et al., 1975) the label was located on the 3' two-thirds of the 23S rRNA, but not in the last 480 nucleotides of the rRNA (that is, labelling occurred between nucleotides 1300 to

TABLE 1:   Affinity labelling of peptidyl transferase.

TABLE 1.   AFFINITY LABELLING OF PEPTIDYL TRANSFERASE
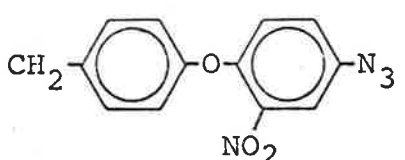
I	Peptidyl-tRNA analogues	Ribosomal components labelled (50S)	Reference

$$\text{tRNA}-\overset{\overset{O}{\|}}{\underset{\underset{R_1}{|}}{C}}-CH-NH-\overset{\overset{O}{\|}}{C}-R_2$$

R_1	R_2	Ribosomal components labelled (50S)	Reference
$CH_2\emptyset$	CH_2Br	L2, L27, L14-17	Oen et al., (1973) Pellegrini et al., (1974)
$CH_2\emptyset$	CH_2Br	L16, L27, L2 (forced into A site)	Eilat et al., (1974)
$CH_2\emptyset$	$(Gly)_n-\overset{\overset{O}{\|}}{C}-CH_2Br$	L2, L26-27, L32-33, L24	Eilat et al., (1974a)
$CH_2\emptyset$	CH_2Br	23S rRNA	Breitmeyer and Noller (1976)
$CH_2\emptyset$	CH_2I	23S rRNA	Yukioka et al., (1975) Yukioka et al., (1976)
$CH_2\emptyset$	CH_2I	L2, L20	Bispink and Matthaei (1973)
$CH_2\emptyset$	(benzene ring—O—benzene ring with CH_2, NO_2, N_3 substituents)	L11, L18, L27	Hsiung et al., (1974)

TABLE 1 (cont'd)
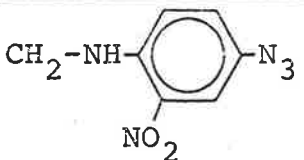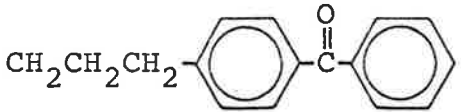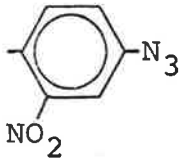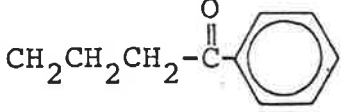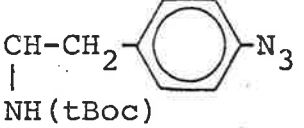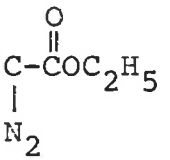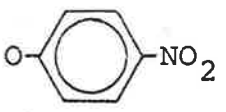
$CH_2\emptyset$	$CH_2-NH-\langle$ ring $\rangle-N_3$ (with NO_2)	L11, L18	Hsiung and Cantor (1974)
$CH_2\emptyset$	$CH_2CH_2CH_2-\langle$ ring $\rangle-\overset{O}{\overset{\|}{C}}-\langle$ ring $\rangle$	23S rRNA	Barta et al., (1975)
$CH_2\emptyset$	$\langle$ ring $\rangle-N_3$ (with NO_2)	rRNA	Girshovich et al., (1974)
$CH_2\emptyset$	$CH_2CH_2CH_2-\overset{O}{\overset{\|}{C}}-\langle$ ring $\rangle$	23S rRNA	Kuechler et al., (1976)
$CH_2\emptyset$	$CH-CH_2-\langle$ ring $\rangle-N_3$; $NH(tBoc)$	23S rRNA	Sonenberg et al., (1975)
$CH_2\emptyset$	$\overset{O}{\overset{\|}{C}}-COC_2H_5$; N_2	23S rRNA	Bispink and Matthaei (1973)
$CH_2\emptyset$	$O-\langle$ ring $\rangle-NO_2$	L27, L15, L2, L16	Bauer et al., (1975) Collatz et al., (1976) Czernilofsky et al., (1974)

TABLE 1 (cont'd).
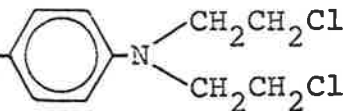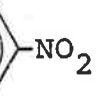
$CH_2\emptyset$	$(CH_2)_4$—⟨benzene ring⟩—N(CH$_2$CH$_2$Cl)(CH$_2$CH$_2$Cl)	23S rRNA	Bochkareva et al., (1973) Budker et al., (1972) Knorre (1974)
$CH_2CH_2SCH_3$	CH(NH(tBoc))—CH$_2$—⟨benzene ring⟩—N$_3$	23S rRNA	Sonenberg et al., (1976)
$CH_2CH_2SCH_3$	O—⟨benzene ring⟩—NO$_2$	L27, L15	Hauptmann et al., (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

$(CH_3)_2A$ — OH ... R_1O CH_2 — NH-C(=O)-CH-CH$_2$— R_3, with NH-R_2

R_1	R_2	R_3			
$O=P-O-$(phenyl)$-NH-C(=O)-CH_2Br$	H	H	23S rRNA	Eckermann and Symons (1977) Greenwell et al., (1974) Harris et al., (1973)	
H	C(=O)-CH$_2$I	O-CH$_3$	L6, L2	Pongs (1974)	

2520 of the 3000 nucleotide long 23S rRNA (Fellner, 1974).

Furthermore, $\overset{*}{A}$pUpUpUpUpApGp (where * is the residue

to which the affinity label is bound (Yukioka et al.,

1976)) has been shown to be the sequence around the site

of attachment of (N-BrAc-L-Phe)-tRNA to 23S rRNA.

Therefore as soon as the primary sequence of 23S rRNA is

known, the part of the 23S rRNA at or near the P' site

can be located. Eilat et al., (1974a) have mapped the

peptide groove using affinity labels of the structure

(N-BrAc-(Gly)$_n$-L-Phe)-tRNA (that is, the distance between

the 3' terminus of the tRNA and the bromine increases as

n increases). They found that if n = 0 the major pro-

tein labelled was L2 while as n was increased the label-

ling of L2 decreased and that of L27 increased (reaching

maximum at n = 4 to 5) after which, even though the

extent of labelling of L27 decreased (n = 7 to 18), it

always remained the major protein labelled. As n was

increased (from n = 5) L32 - 33 became labelled, reaching

a maximum at n = 8; further increase caused a decrease

in labelling of L32 - 33 and an increase in the labelling

of L24 (reaching 20% of label incorporated at n = 18).

Therefore, using this data, Eilat et al., (1974a) were

able to give a diagrammatic representation of the peptide

groove, similar to that shown in Figure 3. Two photo-

affinity labelling analogues of peptidyl-tRNA (having

reactive groups in a similar position to the bromoacetyl

group, when n = 2, of the above discussed affinity labels)

were found to label the proteins L11 and L18 (Hsiung

and Cantor, 1974; Hsiung et al., 1974). Therefore, in terms of ribosomal components the P' site and peptide groove are well characterized.

Affinity labelling data for the A' site on the other hand, has not been as plentiful. In fact, only three affinity labels (Eilat et al., 1974; Harris et al., 1973; Pongs, 1974) have been used. Eilat et al., (1974) found that a dramatic increase in labelling of L16 occurred when (N-BrAc-L-Phe)-tRNA was forced into the A' site by using unaminoacylated-tRNA. Therefore the authors concluded that L16 was at or near the A' site. This is in good agreement with the data of Moore et al., (1975) who found, using reconstitution studies, that L16 was essential for peptidyl transferase activity. Bauer et al., (1974) also pointed out that non-enzymic binding of (N-PNPC-L-Phe)-tRNA to ribosomes caused significant labelling of L16 while enzymic binding using (N-PNPC-L-Met)-tRNA did not lead to labelling of L16. Presumably the labelling of L16 was due to (N-PNPC-L-Phe)-tRNA binding to the A site under non-enzymic conditions. Pongs (1974) has also found that L6 was labelled with iodoacetyl-puromycin, thus suggesting that L6 is close to the 3' terminus of aminoacyl-tRNA. This conclusion is supported by reconstitution experiments showing an interdependence of proteins L16, L6 and L11 for peptidyl transferase and chloramphenicol inhibitory activity (Dietrich et al., 1974; Nierhaus et al., 1974), which

led Dietrich et al., (1974) to conclude that L16, L6 and L11 are at the A' site. In addition, Eckermann and Symons (1977), using Bap-Pan-L-Phe, an A' site affinity label (Greenwell et al., 1974; Harris et al., 1973) have shown that the affinity label was bound to the 23S rRNA sequence of GUĆCG (where * indicates the residue to which the affinity label is attached). Therefore, once again, as soon as the sequence of 23S rRNA is available then the part of the 23S rRNA in or near the A' site can be located.

Therefore the A' site of peptidyl transferase is composed of at least 23S rRNA, L16 and L6.

4.   Structure-Activity Relationships of Chloramphenicol.

Chloramphenicol, an inhibitor of protein synthesis (Pestka, 1971), has been shown to bind to the 50S sub-unit (Vazquez, 1964; Vogel et al., 1971) and thereby inhibit peptide bond formation (Monro and Marcker, 1967). Both in vivo (Das et al., 1966; Hurwitz and Braun, 1967) and in vitro binding studies (Fernandez-Munoz et al., 1971; Lessard and Pestka, 1972a; Wolfe and Hahn, 1965) have shown that chloramphenicol binds, with high affinity (Kd = 2.2 x 10^{-6} M (Fernandez-Munoz and Vazquez, 1973)), to only one site on the ribosome.

Structure-activity relationships, for chloramphen-icol, have been studied extensively (Brock, 1961;

Coutsogeorgopoulos, 1966; Contreas et al., 1974; Hahn
et al., 1956; Hansch et al., 1973; Ringrose and Lambert,
1973; Vazquez, 1966) and the conclusions reached from
these studies are discussed below.

Of the four possible isomers of chloramphenicol
(C_1 and C_2 are asymmetric centres) only the D(-)threo
isomer had any antibacterial activity (Brock, 1961;
Hahn et al., 1956) or inhibited [^{14}C]chloramphenicol
binding (Vazquez, 1966). The structure of the propane-
diol side chain was also shown to be important, because
extension of this side chain caused complete loss of
antibacterial activity (Brock, 1961; Hahn et al., 1956).
In addition, both the C_2 hydrogen and the amide hydrogen
were shown to be essential because replacement of these
by a methyl group caused complete loss of antibacterial
activity (Brock, 1961). The two free hydroxyl groups
are also important because either acetylation, or
replacement by a hydrogen completely destroys antibacterial
activity (Brock, 1961; Hahn et al., 1956). These two
hydroxyls may be important in determining the configuration
of chloramphenicol and in fact Sundaralingam (personal
communication to Dr. R.H. Symons) has found, by X-ray
analysis, that the hydrogen of the C_3 hydroxyl is hydrogen
bonded to the oxygen of the C_1 hydroxyl.

The nitro group, on the other hand, can be replaced
by a number of other groups without loss of activity

(Brock, 1961; Contreas et al., 1974; Hahn et al., 1956; Hansch et al., 1973; Ringrose and Lambert, 1973; Vazquez, 1966) although the aromaticity of the ring as well as the para substitution are essential (Hahn et al., 1956). Alterations of the nitro group did affect the activity, and the hydrophobicity of the substituent replacing the nitro group was found to be the most important parameter (Hansch et al., 1973).

The dichloroacetyl side chain could also be altered without drastic effect on the activity of chloramphenicol (Brock, 1961; Coutsogeorgopoulos, 1966; Hahn et al., 1956; Hansch et al., 1973; Ringrose and Lambert, 1973). The most improtant parameter is the high electron withdrawing capacity of the substituent, although very bulky groups are not favourable (Hansch et al., 1973).
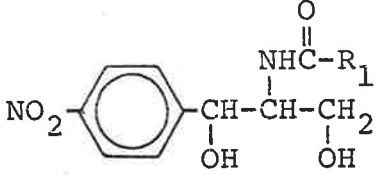
Therefore the parts of the molecule which are essential for activity seem to be the propanediol side chain, the primary amide of C_2, the aromatic ring and the para substitution on the aromatic ring.

5.   Affinity Labelling of the Chloramphenicol Binding Site.

As chloramphenicol is thought to bind at or near the A' site of peptidyl transferase (Pestka, 1971), information as to the protein and/or RNA composition of the chloramphenicol site could be important, in order to

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
NO_2—⟨benzene ring⟩—CH(OH)—CH(NHC(=O)—R_1)—CH_2OH		
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)

understand its mechanism of action (for example, to see if it is near the A' site). Once again the technique of affinity labelling has been used and in fact two similar chloramphenicol affinity labels, iodamphenicol (Bald et al., 1972; Pongs et al., 1973; Pongs and Messer 1976) and bromamphenicol (Sonenberg et al., 1973) have labelled completely different proteins, that is, L16 and L2 and L27, respectively. This result is very surprising because the reactive group of both analogues are in the same position. The labelling of L16 is consistent with the reconstitution studies, demonstrating that L16 was essential for chloramphenicol binding (Nierhaus and Nierhaus, 1973). Sonenberg et al., (1973) also found that covalently bound bromamphenicol did not inhibit peptidyl transferase; therefore the labelling of L2 and L27 by bromamphenicol, was probably a non-specific reaction.

The labelling of L16 suggests that the chloramphenicol binding site is very near, if not at, the A' site of peptidyl transferase, because L16 has been labelled by at least one A' site affinity label (Eilat et al., 1974) and it has also been shown to be essential for peptidyl transferase activity (Moore et al., 1975). However, it should be remembered that most of the ribosomal proteins are highly elongated (Tischendorf et al., 1975) and therefore the chloramphenicol binding site and peptidyl transferase may be widely separated, but

connected by allosteric effects.

6.   Model of the Active Centre of Peptidyl Transferase.

Using the data summarized on Tables 1 and 2, to-
gether with the reconstitution data discussed above, a
model for the active centre of peptidyl transferase is
proposed (Figure 3).   This is only a schematic repre-
sentation and for simplicity all of the proteins for
which there is no evidence for an elongated structure
are represented as spheres.   When designing this model,
particular attention was paid to the definitive studies
involving peptidyl transferase catalysed transfer of
radioactively labelled peptidyl transferase substrates
to cold affinity labels, covalently bound to the A' or
P' sites.   For example, Harris et al., (1973), using
non-radioactive Bap-Pan-L-Phe, inactivated peptidyl
transferase, via A' site labelling and then incubated
these affinity labelled ribosomes with CpApCpCpA(Ac-L-
[^{3}H]Leu), under fragment reaction conditions and found
that radioactivity became associated solely with the
23S rRNA.   Hsiung et al., (1974), Hsiung and Cantor
(1974) and Oen et al., (1973) performed similar experi-
ments, in which the donor substrate was covalently
attached to the ribosome and radioactive acceptor sub-
strates were transferred to the affinity label.   Using
this procedure they were able to identify L11 and L18
(Hsiung and Cantor, 1974; Hsiung et al., 1974) and L2,

FIGURE 3:   Model of the active centre of peptidyl transferase.

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
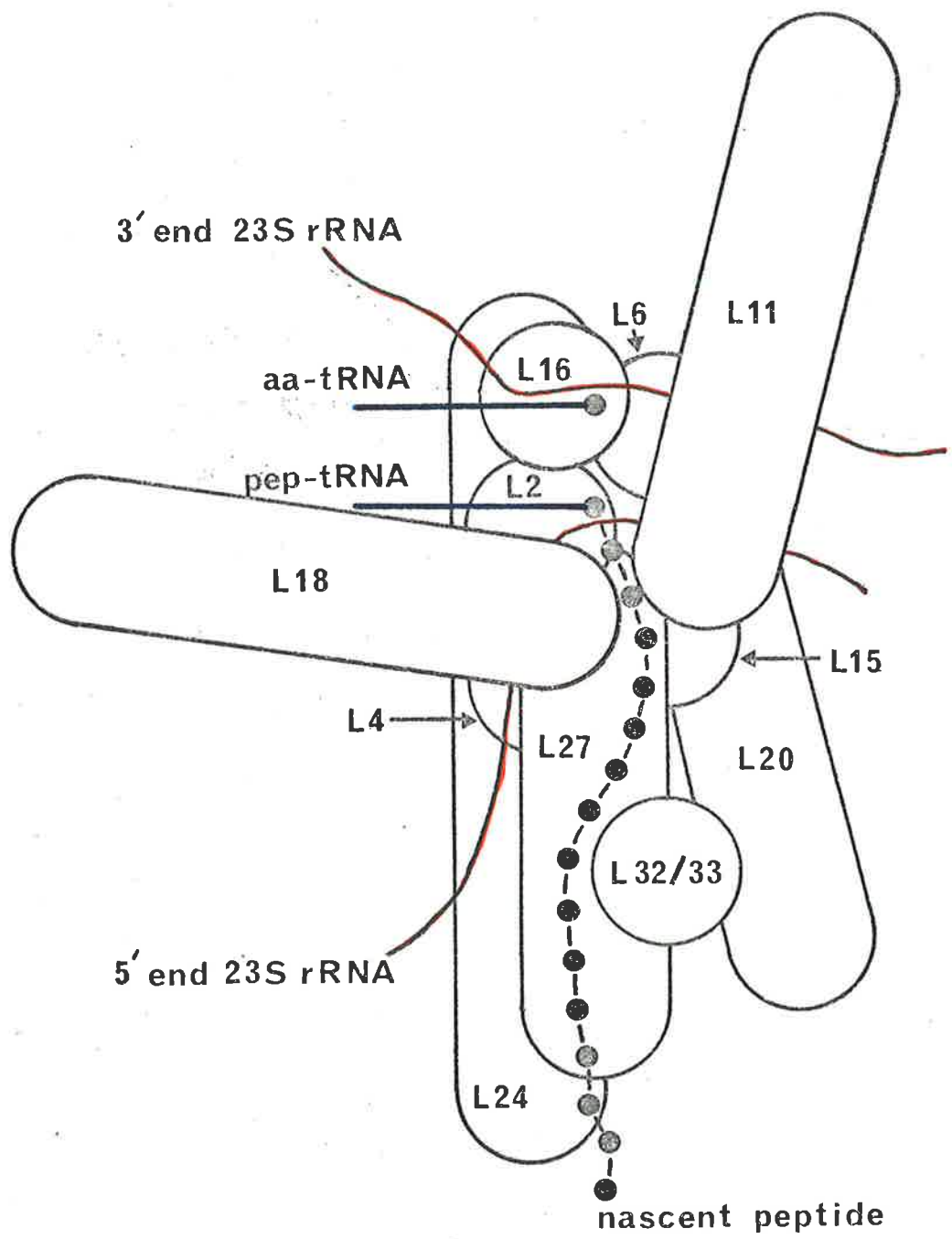
L16 and L27 (Oen et al., 1973) as being in the P' site.

The P' site is shown, composed of L2, L4, L27, L18, L11, L20 and 23S rRNA (Figure 3), most of which have been affinity labelled (Table 1) using different analogues of peptidyl-tRNA. The elongation of proteins L18 and L11 is based on the data of Tischendorf et al., (1975) and of L20 on the data of San Jose et al., (1976). The inclusion of L4 is due to the data obtained by Wittmann et al., (1973) who have found that mutants resistant to erythromycin (an antibiotic which binds to the 50S subunit in the vicinity of peptidyl-tRNA (Olernick and Corcoran, 1969; Mao and Robinshaw, 1972; Pestka, 1974)) have alterations in L4 or L22 and the alteration in L4 caused a reduction in peptidyl trans-ferase activity. Sonenberg et al., (1976) also found that L4 was labelled, using (N-tBOC-L-pN$_3$Phe-L-Met)-tRNA.

The arrangement of the proteins, in the nascent peptide groove, was based on the data of Eilat et al., (1974a), who used affinity labels of the structure (N-BrAc-(Gly)$_n$-L-Phe)-tRNA and found that as the distance between the reactive group and the 3' end of the peptidyl-tRNA was increased different proteins were labelled (see above). The elongation of L27 was indicated from its labelling; as n increased from 3 to 18 (Table 1) L27 was always the major protein labelled. Elongation of

L24 was indicated, because both the peptidyl-tRNA

analogue, (N-BrAc-(Gly)$_{18}$-L-Phe)-tRNA (Eilat et al.,

1974a), and iodamphenicol, which also labelled L16

(Bald et al., 1972; Pongs and Messer, 1976; Pongs et al.,

1973), labelled L24.

The A' site is shown to consist of L24, L16, L6

and 23S rRNA as was discussed earlier. Chloramphenicol

must bind at, or in the close vicinity of the A' site,

as Pongs et al., (1973) and Pongs and Messer (1976)

have shown that iodamphenicol labels L16 as well as L6

and L24. The presence of the 23S rRNA near the active

centre of peptidyl transferase has been shown by affinity

labelling (Table 1). This is not surprising in view of

the fact that L2, L4, L6, L16, L20 and L24 all bind to

23S rRNA (Garrett et al., 1974). Both the 5' and 3'

ends of the 23S rRNA may be involved in or near the

peptidyl transferase centre because L2 is known to bind

towards the 3' end of 23S rRNA (Spierer et al., 1976)

and L24 binds very close to the 5' end of 23S rRNA

(Branlant et al., 1976).

Two interesting features of the model are that

(1)   5S rRNA must be near peptidyl transferase

as L18 (a component of the P' site) is one of the

5S rRNA binding proteins (Gray et al., 1972; Horne

and Erdmann, 1972). Additionally L2 was shown

to stimulate the binding of the 5S rRNA-protein

complex to 23S rRNA (Gray et al., 1972),

(2)   proteins L6, L24, L27 and L15 have all been shown to be interface proteins (Morrison et al., 1973).

This suggests that peptidyl transferase may be sandwiched between the two ribosomal subunits during protein synthesis.

CHAPTER 1

GENERAL METHODS

GENERAL METHODS

INTRODUCTION

This chapter describes the general methods used throughout this work.

MATERIALS

Puromycin, DNase 1 (grade 3), RNase T_1, RNase A, ninhydrin, PPO and POPOP were purchased from Sigma Chemical Co.; tRNA and L-$[^3H]$Leu from Schwarz/Mann and the γ(4-nitrobenzyl)-pyridine from Nutritional Biochemicals Corp. The $[^3H]$ and $[^{14}C]$toluene was supplied by Packard Instrument Company Inc. and the glass scintillation vials from Australian Pharmaglas, Sydney.

SOLVENTS

Pyridine was distilled twice from ninhydrin and stored over CaH_2. Acetonitrile was distilled from CaH_2, then from P_2O_5 and stored over CaH_2. Trifluoroacetic acid was distilled and stored in a well stoppered flask. Acetic anhydride and triethylamine were redistilled and stored in sealed ampoules. Phenol was redistilled and stored at -15° until required. All other solvents were distilled before used.

METHODS

1.   Preparative TLC.

Preparative TLC was carried out on 20 x 20 cm glass plates, coated with Merck Kieselgel HF_{254}. The plates were coated by pouring a slurry of 15 g of Merck Kieselgel HF_{254} in 50 ml of ethanol, on to, and spread evenly over the glass plate. The plates were air dried, after which they were heated at 150° for 2 h to activate the silicic acid and stored at room temperature until required. All compounds were located by viewing under UV light.

2.   Detection of Functional Groups.

(a)   Amino groups.

Detection was achieved by spraying with 0.1% (w/v) ninhydrin in acetone and subsequent heating at 100° for 10 min.

(b)   Alkylating groups.

Detection was the three step procedure used by Santi and Cunnion (1974).

The TLC plate was sprayed with 50 mM potassium biphthalate, dried at 100° for 5 min, sprayed with 2% γ(4-nitrobenzyl)-pyridine (w/v) in acetone, heated again at 100° for 5 - 10 min and finally sprayed with 0.5 M NaOH. Compounds containing

alkylating groups gave an immediate blue colour with the alkaline spray.

(c)   Aldehydes and ketones.

Compounds containing aldehydes or ketones produced an orange or yellow colour upon spraying the TLC plate with 2% 2,4-dinitrophenylhydrazine (w/v) in acidified methanol (Vogel (1957) p.1061) and heating at 100° for 5 min.

(d)   Diazonium salts.

Diazonium salts were detected by the formation of a diazo dye (bright orange-red precipitate) following the addition of a few drops of the solution to β-naphthol in 1 N NaOH (Vogel (1957) p.648).

3.   Estimation of Radioactivity.

(a)   Scintillation fluids.

Scintillation fluid was a solution of 0.35% (w/v) PPO and 0.035% (w/v) POPOP in toluene. Triton scintillation fluid was Triton X-100 mixed with scintillation fluid in a ratio of 1:2 (v/v). Radioactivity was estimated using a Packard Tricarb Liquid Scintillation Spectrophotometer.

(b)   Determination of counting efficiency.

The efficiency was determined as follows.

Known aliquots of $[^3H]$ or $[^{14}C]$toluene were counted under the same conditions as the samples and efficiency (%)

$$= \frac{\text{cpm of } [^3H] \text{ or } [^{14}C]\text{toluene obtained}}{\text{dpm of } [^3H] \text{ or } [^{14}C]\text{toluene added.}} \times 100$$

4.   **E. Coli Ribosomes.**

Ribosomes were prepared according to the modification by Mercer (1971) of the method of Staehelin et al., (1969).

E. coli MRE600 (RNase 1 free strain (Cammock and Wade, 1965)) were grown to late log phase, collected by centrifugation, redissolved in 50 ml of 0.02 M Tris-acetate, pH 7.5, 0.01 M Mg(OAc)$_2$, 0.5 mM EDTA and 0.1 M NH$_4$Cl and ruptured using the French pressure cell (110,000 kPa).   Following incubation of the homogenate with 50 µg of DNase 1 for 40 min at 4° the debris was removed by centrifugation at 38,000 g for 30 min and 4.5 ml of the supernatant layered on to 5 ml of a 1.3 M sucrose cushion containing 0.02 M Tris-acetate, pH 7.5, 0.01 M Mg(OAc)$_2$, 0.5 mM EDTA and 0.5 M NH$_4$Cl.   The clear ribosomal pellet, obtained after centrifugation at 100,000 g for 18.5 h, was resuspended in the above buffer containing 0.1 M NH$_4$Cl and stored at -70° until required. The concentration of ribosomes was determined using an extinction coefficient (mg/ml), at 260 nm, of 14.5 (Hill et al., 1969).

5. $\overset{U}{C}pApCpCpA\,(Ac-L-[^3H]Leu)$.

The pentanucleotide fragment of $(Ac-L-[^3H]Leu)$-
tRNA was prepared according to Monro (1971). Charging
of the tRNA with $L-[^3H]Leu$ was carried out as described by
Nishizuka et al., (1967), the reaction mixture extracted
twice with equal volumes of phenol and the $(L-[^3H]Leu)$-
tRNA precipitated overnight at -15° by the addition of
2 volumes of ethanol to the aqueous phase.

The $(L-[^3H]Leu)$-tRNA, collected by centrifugation,
was redissolved in 0.2 M NaOAc, pH 5.5, (0.5 ml) and
acetylated at 4° by three 30 μl additions of acetic
anhydride at 40 min intervals. The $(Ac-L-[^3H]Leu)$-tRNA
was collected by ethanol precipitation (as above), dis-
solved in 0.15 ml of 5 mM Tris-acetate, pH 7.5, 10 mM
NaOAc, 2 mM EDTA, pH 5.4, and digested with RNase T_1
(400 units) at 37° for 30 min. Electrophoresis of the
reaction mixture at pH 5.1 produced two bands of radio-
activity, both migrating towards the anode; the slower
migrating peak was $CpApCpCpA\,(Ac-L-[^3H]Leu)$ and the
faster peak being $UpApCpCpA\,(Ac-L-[^3H]Leu)$ (Monro, 1971).
Both bands were eluted with 0.1 mM NaOAc, 0.2 mM EDTA,
pH 5.0, and stored at -70° until required

The specific activity of 20 Ci/mmole for the frag-
ment was calculated by assuming that it was identical to
that of the $L-[^3H]Leu$ used for charging the tRNA.

6.   Peptidyl Transferase Assay (Fragment Reaction).

The assay, which measures the transfer of Ac-L-
[^{3}H]Leu from $\overset{U}{C}$pApCpCpA(Ac-L-[^{3}H]Leu) to puromycin, was
that of Greenwell et al., (1974) as modified from Maden
and Monro (1968).

Assays were done in glass scintillation vials
(0.9 x 4.7 cm) and contained in 50 µl : 40 mM Tris-acetate,
pH 8.0, 50 mM Mg(OAc)$_2$, 0.3 M KCl, 30% methanol, approx-
imately 0.43 pmol of $\overset{U}{C}$pApCpCpA(Ac-L-[^{3}H]Leu) of specific
activity 20 Ci/mmole, 1.0 - 2.0 mM puromycin and 6 - 12
pmol of E. coli ribosomes.   Incubation was generally
carried out at 0° for 5 min (unless otherwise stated).
Termination of the reaction was by the addition of 5 µl
of 3 N NaOH, which also ensures that any Ac-L-[^{3}H]Leu
methyl ester formed during the reaction would be hydro-
lysed and therefore not extracted (Miskin et al., 1970).
After 15 min at 37°, 100 µl of 0.2 M NaH$_2$PO$_4$, pH 5.5,
saturated with MgSO$_4$, was added, followed by 2.2 ml of a
mixture of ethylacetate and scintillation fluid (1:4, v/v).
The saturation of the NaH$_2$PO$_4$ solution with MgSO$_4$ creates
a charged aqueous phase which increases the partitioning
of the uncharged product into the organic phase.   The
vials were capped and briefly but vigorously shaken to
selectively extract the product into the organic phase
and the radioactivity estimated.   Due to the lack of
scintillants in the lower aqueous phase any Ac-L-[^{3}H]Leu
or excess substrate in that phase does not contribute to

the radioactivity of the upper organic phase.

7. Sucrose Gradient Analysis of Ribosomal Subunits.

Ribosomes (0.53 mg, 190 pmol) in 0.1 ml of 0.02 M Tris-acetate, pH 7.5, 0.01 M Mg(OAc)$_2$, 0.5 mM EDTA and 0.1 M NH$_4$Cl were precipitated at 4° with 2.0 ml of methanol (containing no salts in order to reduce the magnesium concentration in the ribosomal pellet), the precipitated ribosomes collected by low speed centrifugation and the ribosomal pellet redissolved in 0.15 ml of 20 mM Tris-acetate, pH 7.5 (at 20°), 0.5 mM Mg(OAc)$_2$, 0.025 mM EDTA and 0.5 M NH$_4$Cl, the low concentration of Mg(OAc)$_2$ causes the dissociation of the ribosomes into subunits (Pellegrini et al., 1972). Portions (0.1 ml) were layered on to 11.6 ml 10 - 30 % (w/v) linear sucrose gradients in 20 mM Tris-acetate, pH 7.5 (at 20°), 3.0 mM Mg(OAc)$_2$, 0.15 M EDTA, 0.2 M NH$_4$Cl and centrifuged at 40,000 rpm for 3.5 h using a Beckman SW41 rotor. Fractions (0.5 ml) were collected by upward displacement using 60% sucrose (w/v) and the absorbance at 254 nm monitored using an Isco Model 640 gradient fractionator connected to a W. & W. 1100 recorder.

If radioactivity in the fractions was to be estimated 0.1 ml aliquots of each fraction were added to 0.2 ml of water and 2.0 ml of Triton scintillation fluid.

8.   Separation of Proteins and RNA of E. coli Ribosomes.

(a)   RNase digestion of ribosomes.

A solution of ribosomes (0.5 mg) in 0.3 ml of 4 M urea, 0.15 M EDTA, pH 7.2, was treated with 6 μg of RNase A and 2 μg RNase T_1 for 2 h at 37°. After the addition of 0.5 mg of BSA (carrier protein) the protein was precipitated by the addition of an equal volume of cold 10% (w/v) TCA.   The percentage of affinity labels bound to the RNA was estimated from the radioactivity in the supernatant (counted in Triton scintillation fluid), after low speed centrifugation.   Similarly, the percentage of affinity labels bound to the protein fraction was estimated from the radioactivity in the precipitate, which was determined by redissolving the precipitate in 0.1 M NaOH after which aliquots were counted in Triton scintillation fluid.

(b)   Phenol extraction of ribosomes.

Ribosomes (2.5 mg) in 1 ml of 0.3% SDS (w/v), 0.2 M NaOAc and 2 mM EDTA, pH 6.0, were extracted twice with 1 ml of water saturated phenol.   The lower phenol layer was separated from the aqueous layer by low speed centrifugation and the two phenol layers pooled.   The distribution of radioactive affinity label was then determined by counting the aqueous layer (containing the RNA) and the phenol layer (containing the proteins) in Triton scintillation fluid.

CHAPTER 2

STRUCTURE-ACTIVITY RELATIONSHIPS

OF PUROMYCIN ANALOGUES ON

ESCHERICHIA COLI POLYSOMES

STRUCTURE-ACTIVITY RELATIONSHIPS OF

PUROMYCIN ANALOGUES ON ESCHERICHIA COLI

POLYSOMES

INTRODUCTION

Work by Pestka (1972a) and Pestka and Hintikka (1971), on the effect of inhibitors on the puromycin-dependent release of nascent peptides from E. coli polysomes, has revealed substantial differences in the action of these inhibitors on salt-washed ribosomes compared to polysomes and intact cells. In particular, the peptidyl transferase acceptor site of polysomes has a one hundred-fold greater affinity for puromycin than that of salt-washed ribosomes (Pestka, 1972a,b; Fahnestock et al., 1970.). In view of these important observations and in order to examine the generality of this large increase in affinity, we have extended our acceptor substrate specificity studies using puromycin analogues (Eckermann et al., 1974; Harris et al., 1971; Symons et al., 1969) to include E. coli polysomes. This system should reflect the in vivo situation more closely than the previous studies on the acceptor site of peptidyl transferase, which largely used salt-washed ribosomes and artificial donor substrates (Eckermann et al., 1974; Harris et al., 1971; Rychlik et al., 1969; Rychlik et al., 1970; Symons et al., 1969).

It was hoped that this study, which most closely resembles the in vivo situation, would lead to a greater understanding of the acceptor substrate specificity of peptidyl transferase in the E. coli cell. As will be seen in this chapter, the data provided reinforcement for the previously held theories for the presence of at least two different binding regions in the A' site, the hydrophobic pocket and the binding site for the penultimate CMP residue of aminoacyl-tRNA (Harris and Symons, 1973b).

The work presented in this chapter, which was begun during my B.Sc. (Honours), was completed during my Ph.D.

MATERIALS

Pan, D-Phe, N-α-tBoc-L-amino acids, DNase 1 (noncrystalline) and lysozyme (grade 1) were purchased from Sigma Chemical Co., the EEDQ from Aldrich Chemical Co., Inc., and the Kieselgel 60 F 254 from E. Merck, Darmstadt, West Germany. Brij 58 was supplied by Atlas Chemical Industries, Wilmington, Del., U.S.A. and the [^{3}H] puromycin by New England Nuclear, Boston, Massachusetts, U.S.A. and also by The Radiochemical Centre, Amersham, England.

METHODS

1. Thin Layer Chromatography.

Silicic acid (Kieselgel 60 F 254) was used as the

solid phase for all TLC described and the solvents used were; solvent A, ethanol : chloroform (1:9, v/v) and solvent B, ethanol : chloroform (3:7, v/v).

2.   Synthesis of N-α-tBoc-D-Phe.

The amino group of D-Phe was protected by reaction of the D-Phe with t-butyl azidoformate, using the procedure of Schwyzer et al., (1959) to give N-α-tBoc-D-Phe.   The t-butyl azidoformate, synthesized by the method of Carpino et al., (1959), was kindly suppled by Dr. S. Mander.

3.   Synthesis of 3'-N-aminoacyl derivatives of Pan.

These analogues of puromycin (Figure 4) were prepared by a modification of the method used by Harris et al., (1972).

The Pan-(N-α-tBoc-amino acids) were synthesized by adding Pan (40 μmol) to a solution of EEDQ (80 μmol) and N-α-tBoc-amino acid (80 μmol) in 1.3 ml of ethanol. After 3 h at room temperature the reaction was shown to be complete by analytical TLC (Rf of the product being dependent upon the N-α-tBoc-amino acid used) using solvent A.   The reaction mixture was then evaporated to dryness and the residue dried by coevaporation with acetonitrile on a vacuum line (Greenlees and Symons, 1966).   The tBOC group was then removed by treating the dried residue with anhydrous TFA for 3 min at room

FIGURE 4:   Structures of NpPan-L-amino acid (I),
puromycin (II) and Pan-L-amino acid (III).

    N = adenine, guanine, cytosine or uracil

    R = aminoacyl side chain
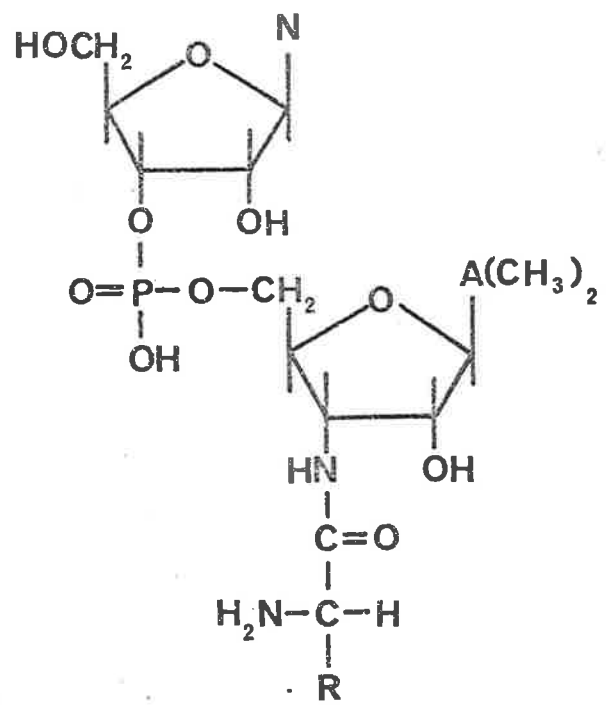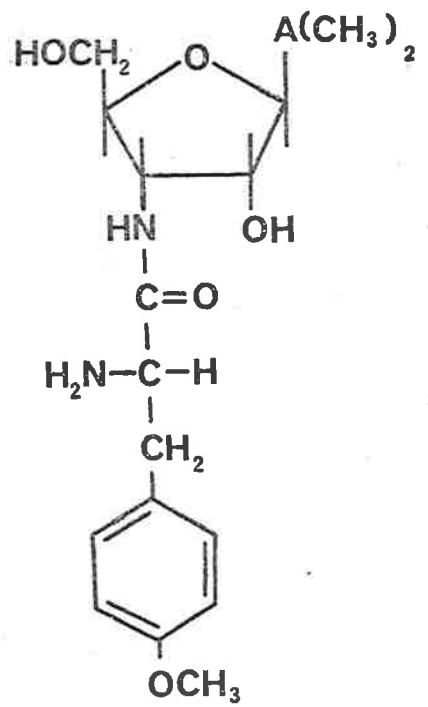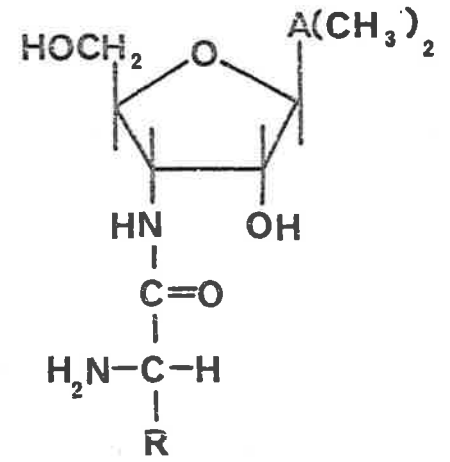
I II III

temperature, after which the TFA was removed on the
vacuum line.   The residue was redissolved in 1 ml of
methanol and the Pan-amino acid purified by preparative
TLC (Chapter 1: section 1) using solvent B (the Rf being
dependent on the Pan-amino acid being purified).   The
appropriate UV absorbing band was scraped off and the
compound eluted from the silicic acid using 3 x 10 ml
of ethanol.   The pooled eluates were filtered, evapor-
ated to dryness and the residue dissolved in 4 ml of
0.1 mM EDTA, pH 8.0.

4.   Synthesis of 5'-O-nucleotidyl derivatives of Pan-
      Gly and Pan-L-Phe.

These derivatives (Figure 4) of Pan-Gly and Pan-L-
Phe were synthesized by Dr. R.H. Symons using the pro-
cedure of Harris et al., (1972).

5.   Preparation of E. coli Polysomes.

The procedure adopted was a modification of the
method used by Pestka and Hintikka (1971), which was
originally developed by Godson and Sinsheimer (1967).
The entire procedure, after growth of the cells (E. coli
MRE 600, a RNase 1 deficient mutant (Cammock and Wade,
1965)) at 37°, was performed at 4°.   The time from
collection of the cells to lysis of the spheroplasts
should take no longer than 20 min, in order to ensure
good polysome preparations.

Cell cultures (500 ml), grown to mid to late log
phase were rapidly cooled to 0-5°, using a dry ice-
ethanol bath, and the cells collected by centrifugation
at 35,000 g for 1 min.   The supernatant was discarded
and the cells resuspended in a total of 2.4 ml of 25%
sucrose (w/v) in 0.01 M Tris-HCl, pH 8.1.   Spheroplasts
were then formed by treating the cells with 0.3 ml of
lysozyme, 2 mg/ml in 0.25 M Tris-HCl, pH 8.1, and the
digestion initiated by the addition of 0.3 ml of 0.01 M
EDTA.   After 1 min the lysozyme treatment was termin-
ated by the addition of 0.15 ml of 1 M $MgSO_4$ and the
spheroplasts pelleted at 35,000 g for 1 min.   Once
again the supernatant was discarded and the spheroplasts
lysed by resuspension in 2.4 ml of 0.5% Brij 58(w/v),
0.01 M Tris-HCl, pH 7.2, 0.005 M $MgSO_4$ and 0.05 M
NH_4Cl, to which 0.06 ml of DNase 1 solution (1.25 mg/ml)
was added.   After 30 min the cell debris was removed
by centrifugation at 12,000 g for 10 min and the super-
natant, containing the polysomes, was stored in aliquots,
in liquid nitrogen, until required.   Yields of 55-90
A_{260} units/ml were generally obtained using this pro-
cedure.   These yields are low when compared to the 121
A_{260} units/ml obtained by Pestka and Hintikka (1971)
but as can be seen from Figure 5 our preparations con-
tained 45% polysomes, whereas Pestka and Hintikka (1971)
obtained only 28%.

FIGURE 5:   Sucrose gradient analysis of E. coli poly-

somes (obtained as in Methods, section 5) and analysed

as in Methods (section 6).
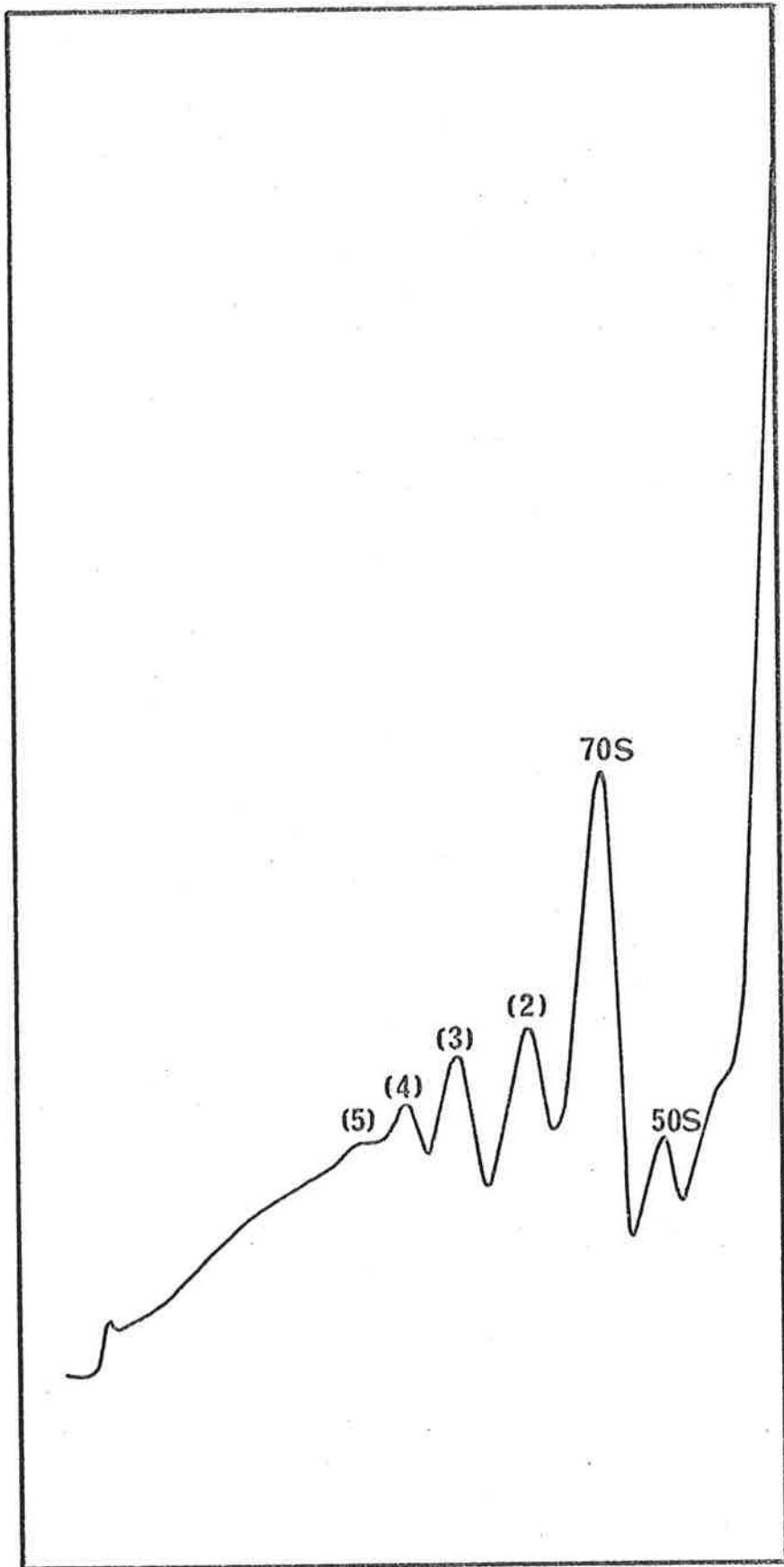
ABSORBANCE 260nm

SEDIMENTATION

6. Sucrose Gradient Analysis of E. coli Polysomes.

Polysomes (5 A_{260} units of the above preparation)
were layered on to an 11.6 ml linear 15 - 30% sucrose
(w/v) density gradient in 0.01 M Tris-HCl, pH 7.5,
0.01 M $MgCl_2$, 0.05 M KCl and centrifuged at 40,000 rpm
(206,000 g (av)) in a Beckman SW41 rotor for 1.5 h.
The A_{254} profile of the gradient was then obtained by
upward displacement, using 60% sucrose (w/v), through
an Isco Model 640 gradient fractionator connected to a
W & W 1100 recorder.

7. Peptidyl-[^{3}H]puromycin Release Assay.

This assay measures the amount of TCA precipitable
peptides released by [^{3}H]puromycin from E. coli poly-
somes.

Each assay, in a total volume of 0.1 ml, contained
0.05 M Tris-HCl, pH 7.2, 0.005 M $MgSO_4$, 0.1 M KCl, 0.25%
Brij 58 (w/v) (contributed by the polysome solution), 0.025 M
NH_4Cl (contributed by the polysome solution) and 2.0
A_{260} units of the polysome preparation. Assays were
initiated by the addition of [^{3}H]puromycin and carried
out at 37° for 1 min (unless otherwise stated).
The reaction was terminated and the [^{3}H]puromycin pep-
tides precipitated by the addition of 2 ml of cold 10%
TCA (w/v). After 15 min at 0°, the precipitates were
collected on glass fibre filters and the filters washed
with 3 x 5 ml of cold 5% TCA (w/v). The filters were

subsequently washed in acidified ethanol (1% HCl (v/v) in ethanol) for 30 min at room temperature, dried and the radioactivity estimated.

The Ki values for the puromycin analogues were measured in the above assay using various concentrations of the analogues (see Table 3) at two concentrations of puromycin (5×10^{-6} M, specific activity 0.55 Ci/mmole and 0.5×10^{-6} M, specific activity 1.1 Ci/mmole).

8.   Determination of Ki Values by Means of Dixon Plots (Dixon, 1953).

The data for the Dixon plots was obtained by measuring the velocity of the reaction at a series of inhibitor (puromycin analogue) concentrations, keeping the substrate ([^{3}H]puromycin) concentrations constant; by using two different [^{3}H]puromycin concentrations for the same inhibitor concentrations and plotting $^1/_v$ against the inhibitor concentration (i) two straight lines were obtained. The point of intersection (being above the x axis and to the left of the y axis) was equivalent to -Ki. The Dixon plots, used to determine the Ki values, were obtained by the method of least squares (computer program by courtesy of Dr. D.B. Keech) with the probable errors of the Ki obtained in this way being ± 10%.

RESULTS AND DISCUSSION

1. Properties of Peptidyl-[^{3}H]puromycin Release Assay.

The time course for the [^{3}H]puromycin dependent release of peptides from these polysomes is shown in Figure 6 and as can be seen the release of peptides by both 5.0 and 0.5 x 10^{-6} M [^{3}H]puromycin was linear for 1 min and greater than 3 min, respectively. Therefore, using an incubation time of 1 min, the Km for puromycin was determined and the results obtained are shown in Figure 7 in the form of a double reciprocal plot. A Km of 3.2 x 10^{-6} M was obtained for puromycin, which is in very good agreement with the value of 3.3 x 10^{-6} M obtained by Pestka (1972b).

2. Inhibition of Peptidyl-[^{3}H]puromycin Release by Puromycin Analogues.

Since the fifteen puromycin analogues used here were not available in radioactive form, the only feasible method for testing them for acceptor site binding in the E. coli polysome system was to use these analogues as competitive inhibitors of [^{3}H]puromycin. Analysis of the velocity inhibition data (in this case the inhibition of peptides released at two different [^{3}H]puromycin concentrations in 1 min) by means of Dixon plots gave apparent Ki values. The two [^{3}H]puromycin concentrations chosen for all our studies were 5 and 0.5 x 10^{-6} M, because at these concentrations the degrees of saturation

FIGURE 6: The rate of $[^3H]$puromycin dependent release of peptides from E. coli polysomes (Methods, section 5) using the assay described in Methods (section 7). The two puromycin concentrations used were 0.5×10^{-6} M (specific activity 2.0 Ci/mmole) and 5×10^{-6} M (specific activity 1.0 Ci/mmole).
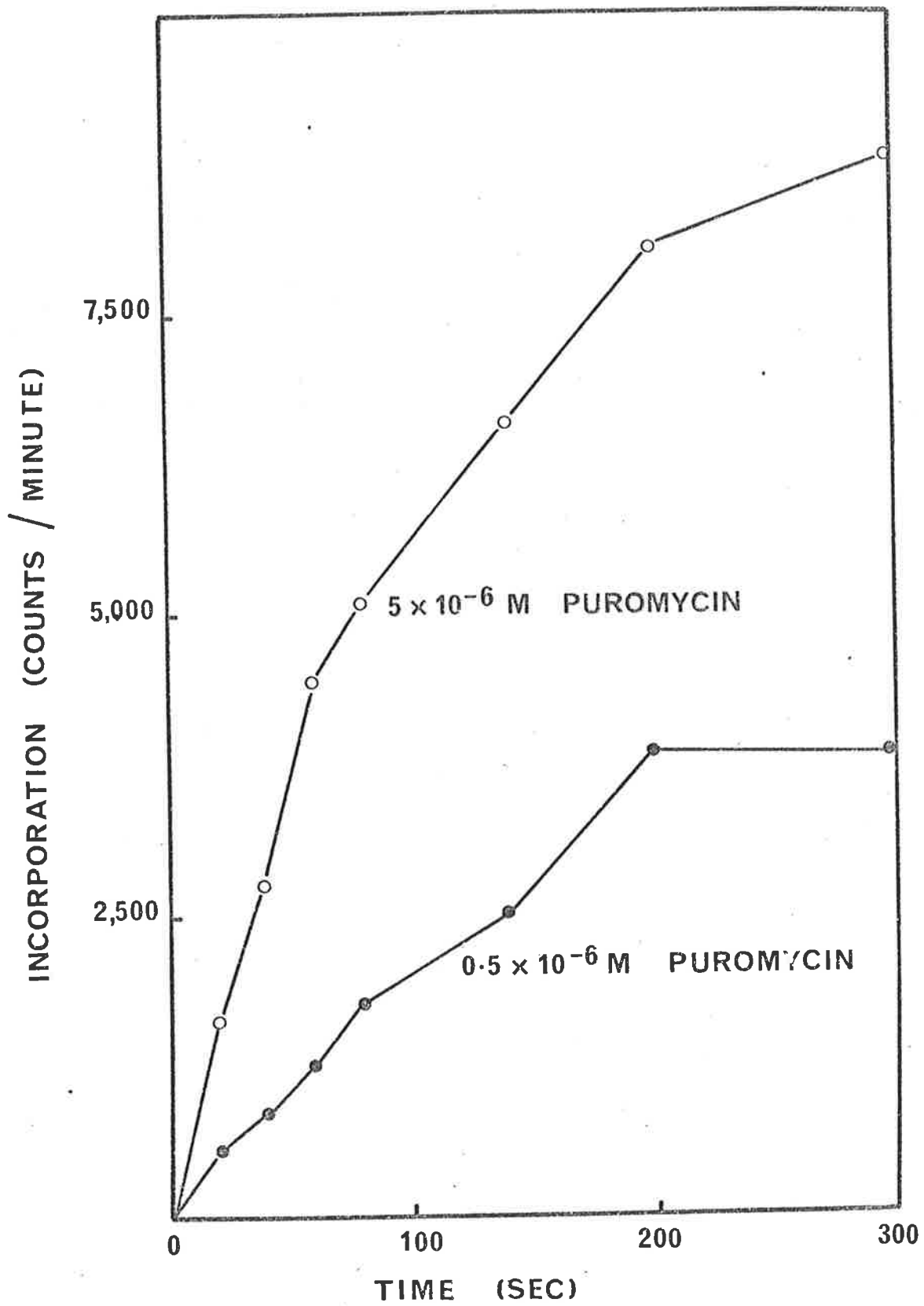
FIGURE 7: Km of E. coli polysomes for puromycin was obtained by varying the [^{3}H]puromycin concentration (specific activity 2.0 Ci/mmole) from 0.25 - 5 x 10^{-6} M in the peptidyl-[^{3}H]puromycin release assay (Methods, section 7). The results obtained were then plotted on a double reciprocal plot.
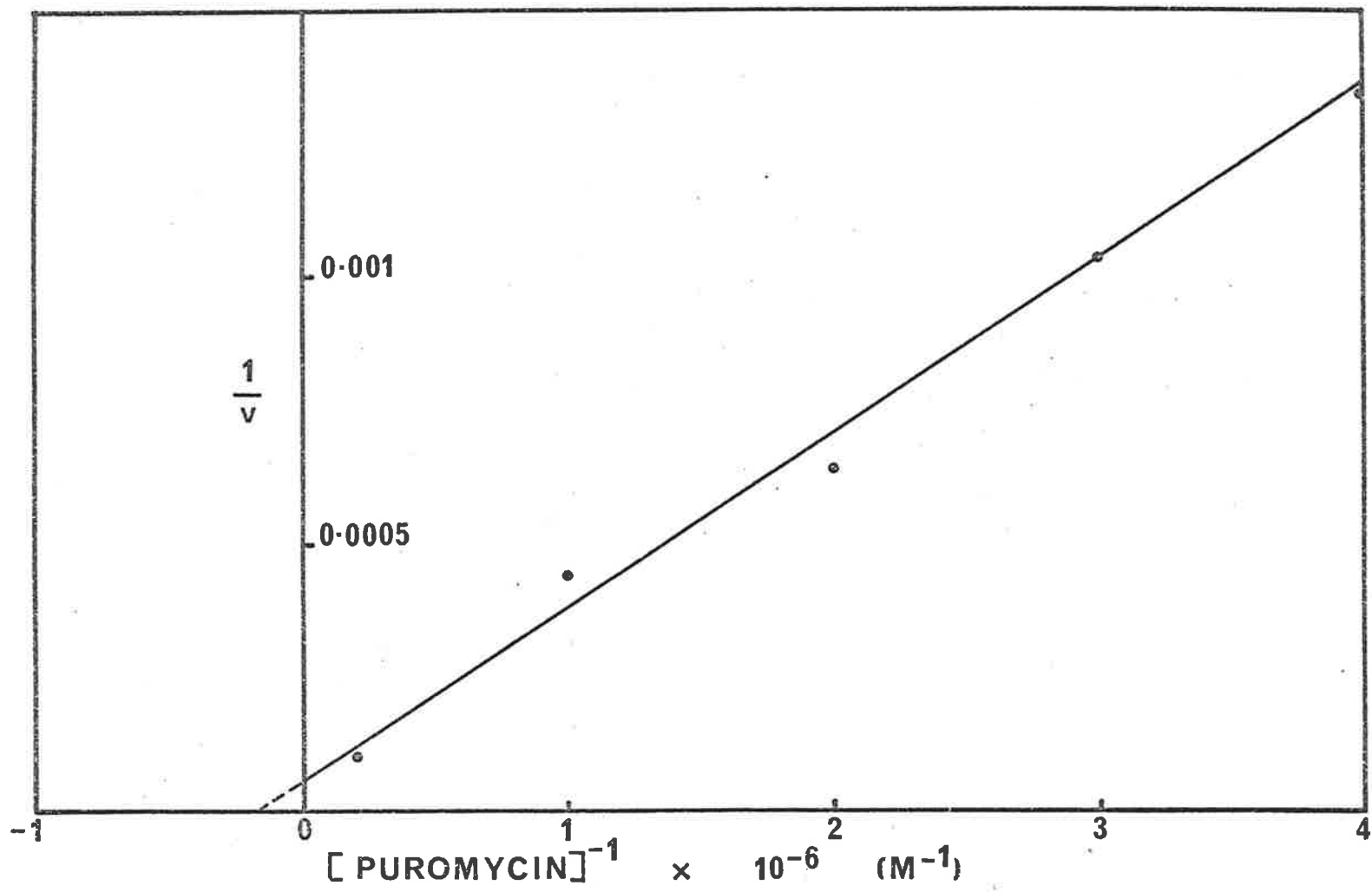
of peptidyl transferase by [3H]puromycin would be quite
different. Therefore, the widely divergent lines,
obtained using the Dixon plot, would give a clear and
unambiguous point of intersection, as can clearly be
seen from the Dixon plot for Pan-(O-benzyl-L-Ser)
(Figure 8). All the Dixon plots obtained gave the
point of intersection above the x axis and to the left
of the y axis, indicative of competitive inhibition.
The apparent Ki values obtained using the above method
would in turn be a measure of the affinities of these
analogues for the A' site.

Theoretically the Dixon plot, which was derived
for an enzyme system obeying Michaelis-Menten kinetics,
cannot be rigorously applied to the present reaction,
since steady-state turnover conditions are not occurring.
However, Fico and Coutsogeorgopoulos (1972), using the
(N-Ac-L-Phe)-tRNA-poly U system and measuring the release
of N-Ac-L-Phe by puromycin, found that the rate for the
reaction was

$$v = \frac{(Eo - Ei).k.S}{Km + S}$$

where Eo is the original concentration of the enzyme
and Ei the fraction that had reacted to form (N-Ac-L-
Phe)-puromycin. However, at t = O, Ei = O and there-
fore the initial rate equation was reduced to

$$v = \frac{Eo.k.S}{Km + S}$$

FIGURE 8: Ki of Pan-(O-benzyl-L-Ser), in the peptidyl-[^{3}H]puromycin release assay (Methods, section 7), was obtained by measuring the inhibition of incorporation of [^{3}H]puromycin (at 0.5 and 5 x 10^{-6} M) at varying concentrations (2.0 - 8.2 x 10^{-5} M) of Pan-(O-benzyl-L-Ser). The results obtained were then plotted on a Dixon plot (Methods, section 8).

$\dfrac{1}{v}$

0·001

0·0005

0·5 × 10⁻⁶ M   PUROMYCIN

5 × 10⁻⁶   PUROMYCIN

-4   -3   -2   -1   1   2   3   4   5   6   7   8

[Pan - (O-benzyl - L - Ser)]   ×   10⁵   (M)
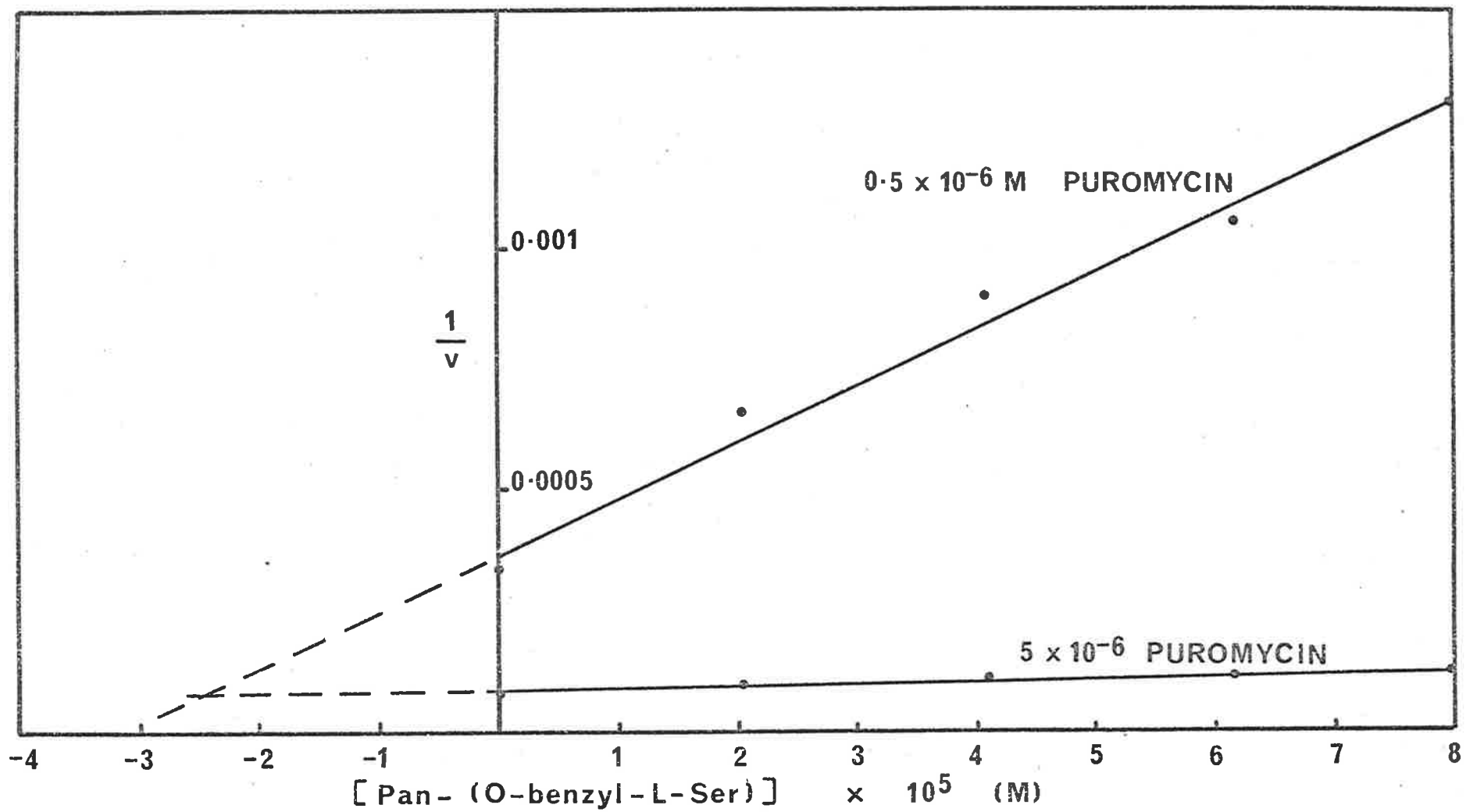
Therefore, for initial rates, Michaelis-Menten kinetics applied. As their assay system is essentially the same as the peptidyl-[^{3}H] puromycin release assay, the same should hold true for the assay used in our studies.

(a)   Inhibition by 3'-N-aminoacyl analogues of puromycin.

The apparent Ki values for the nine different 3'-N-aminoacyl analogues, obtained as in Figure 8, are shown in Table 3.   The Ki values obtained for the hydrophobic aminoacyl analogues decreased in the order Pan-Gly, Pan-L-Leu, Pan-L-Tyr and Pan-L-Phe.   Therefore the data indicates that the more hydrophobic the amino acid side chain, the better the binding (lower Ki) of the analogue.   The weaker binding of the larger hydrophobic analogues, such as Pan-(O-benzyl-L-Ser), Pan-(im-benzyl-L-His) and Pan-L-Trp can be explained by the steric hinderance of the bulky side groups in binding to the A' site.   The data, up to date, is consistent with the presence of a hydrophobic binding pocket in the A' site of peptidyl transferase, as has already been suggested by Eckermann et al., (1974), Harris et al., (1971) and Rychlik et al., (1970).   The high Ki (3.9 x 10^{-5} M) and therefore weaker binding of Pan-D-Phe (20% when compared to Pan-L-Phe) can be explained by the

TABLE 3:   Ki values for puromycin analogues (obtained as in Figure 8) in the peptidyl-[^{3}H]puromycin release assay (Methods, section 7).

1.   Substrate Michaelis constant, Km, for puromycin.

2.   Ratio of Ki of each analogue to the Km of puromycin.

TABLE 3.   INHIBITION CONSTANTS FOR PUROMYCIN ANALOGUES IN THE

RELEASE OF PEPTIDYL-[^{3}H]PUROMYCIN FROM E. COLI POLYSOMES

Analogue	Ki (μM)	Ki/Km^2	Concentration range (M)
Pan-Gly	2200	710	$0.5\text{-}2.3 \times 10^{-3}$
Pan-L-Leu	200	65	$1.0\text{-}4.3 \times 10^{-4}$
Pan-L-Tyr	3.3	1.1	$2.7\text{-}11 \times 10^{-5}$
Pan-L-Phe	2.8	0.9	$1.9\text{-}8.0 \times 10^{-6}$
Pan-D-Phe	15	5	$2.0\text{-}8.0 \times 10^{-5}$
Puromycin	3.1[1]	1.0	$0.2\text{-}5.0 \times 10^{-6}$
Pan-(O-benzyl-L-Ser)	29	9.4	$2.0\text{-}8.2 \times 10^{-5}$
Pan-(im-benzyl-L-His)	540	170	$0.9\text{-}3.9 \times 10^{-4}$
Pan-L-Trp	1400	450	$2.4\text{-}9.8 \times 10^{-4}$
Pan-L-Lys	270	87	$0.3\text{-}1.4 \times 10^{-4}$
Pan-L-Pro	4000	1300	$0.5\text{-}1.9 \times 10^{-4}$

TABLE 3 (cont'd).

Pan-Gly	2200	710	$0.5\text{-}2.3 \times 10^{-3}$
ApPan-Gly	1600	520	$1.4\text{-}5.8 \times 10^{-4}$
GpPan-Gly	1200	390	$2.1\text{-}8.6 \times 10^{-5}$
UpPan-Gly	830	270	$1.4\text{-}5.8 \times 10^{-4}$
CpPan-Gly	61	20	$1.8\text{-}7.4 \times 10^{-5}$
CpPan-L-Phe	1.7	0.55	$1.2\text{-}5.0 \times 10^{-6}$
pPan-L-Phe	11	3.5	$2.7\text{-}11 \times 10^{-5}$
Pan-L-Phe	2.8	0.9	$1.9\text{-}8.0 \times 10^{-6}$

[1]Substrate Michaelis constant, K_m, computed from double reciprocal plot.

[2]The ratio of K_i of each analogue to the K_m of puromycin.

stereochemical requirement of peptidyl transferase for the L-amino acids rather than the D form (Nathans and Neidle, 1963; Calendar and Berg, 1967). The dual hydrophobic-hydrophilic nature of the positively charged lysyl side chain and the rigid conformation of proline may mean that these two analogues do not conform to the simple hydrophobic model. In fact, Rychlik et al., (1970), have also postulated the presence of a hydrophilic binding pocket in the A' site.

(b)   Inhibition by 5'-O-nucleotidyl derivatives of Pan-Gly and Pan-L-Phe.

Of the four Pan-Gly derivatives, substituted on the 5'-hydroxyl (Figure 4), only CpPan-Gly showed a marked reduction in Ki, relative to Pan-Gly. These results are consistent with the previously postulated binding site for the pen-ultimate 3'-CMP residue of aminoacyl-tRNA (Eckermann et al., 1974; Harris et al., 1971; Harris and Symons, 1973b). When the glycyl residue of CpPan-Gly was replaced by L-Phe there was a marked reduction of the Ki value to less than that of puromycin, a result which reflects the importance, for acceptor substrate activity, of both the hydrophobic and the CMP binding sites in the A' site of peptidyl transferase. The

increased binding (lower Ki) of the 5'-O-nucleo-
tidyl substituted analogues was shown to be due
to the base and not the presence of the 5'-O-phos-
phate because pPan-L-Phe had a higher Ki than Pan-
L-Phe.   Therefore the presence of the 5'-O-phos-
phate or the presence of two negative charges on
the phosphate, compared to one negative charge on
the phosphate of CpPan-L-Phe was responsible for
the increased Ki.

3.   <u>Higher Affinity of <u>E. coli</u> Polysomes for Puromycin.</u>

In an attempt to determine whether the increased
affinity of polysomes for puromycin, when compared to
salt-washed ribosomes, was a general phenomenon, the
Km values for puromycin, Pan-L-Phe and CpPan-L-Phe were
determined in the fragment reaction (Chapter 1; section
6) using salt-washed ribosomes; the values obtained were
1.1×10^{-4} M, 1.0×10^{-4} M and 2.5×10^{-5} M, respectively.
Limitations of solubility and availability prevented
the determination of the Km values for the other puro-
mycin analogues in the fragment reaction, but from the
above results it appears that polysomes do have a higher
intrinsic affinity than salt-washed ribosomes for the
puromycin analogues.   The reasons for the difference
between polysomes and salt-washed ribosomes are not
known.   The effect does not appear to be due to the
presence of 30% methanol in the fragment reaction, as

was suggested by Eckermann et al., (1974), because Km

values of 7.5×10^{-6} M and 5.2×10^{-6} M in the presence

and absence of 30% methanol, respectively, were obtained

in the polysome system. However, the catalysis of the

reaction was affected as was shown by a three-fold

reduction in the Vmax in the presence of methanol

(results not shown). Further evidence that methanol

does not reduce the affinity of peptidyl transferase for

puromycin was provided by Fahnestock et al., (1970), who

obtained a Km of 3.1×10^{-4} M for puromycin (which is

similar to that obtained in the fragment reaction) when

using salt-washed ribosomes and (N-Ac-L-Phe)-tRNA as the

donor substrate, in the absence of methanol. A hypo-

thetical explanation for the increased affinity of

E. coli polysomal peptidyl transferase for puromycin has

been forwarded by Pestka (1972a), who has suggested that

the presence of native peptidyl-tRNA in the P site

stabilized a ribosomal conformation which has an increased

affinity for puromycin. Another possibility is that

washing of the ribosomes with high salt may remove a

component(s) which contributes to puromycin binding.

SUMMARY

Peptidyl transferase of E. coli polysomes was

previously shown to have a one hundred-fold higher

affinity for puromycin than salt-washed ribosomes

(Pestka, 1972a,b) and was therefore tested for its

acceptor substrate specificity. Analogues of puromycin
were tested for their inhibitory effect on the [^{3}H]-
puromycin dependent release of peptides from E. coli
polysomes and the data analysed by Dixon plots. The Ki
values obtained from the Dixon plots were then used as
a measure of the affinity of the puromycin analogues
for the A' site.

The results and conclusions from the data were as
follows:

1.   The Ki values decreased in the order of increasing
hydrophobicity of the amino acid side chain, that is
Pan-Gly, Pan-L-Leu, Pan-L-Tyr and Pan-L-Phe. The
values went from 2.2 x 10^{-3} M for Pan-Gly to 2.8 x 10^{-6} M
for Pan-L-Phe, which is indicative of a hydrophobic bind-
ing pocket in the A' site of peptidyl transferase for the
amino acid side chains. The higher Ki values of the
larger hydrophobic residues (Pan-(O-benzyl-L-Ser), Pan-
(im-benzyl-L-His) and Pan-L-Trp) is probably due to the
steric hinderance of binding of the large, bulky side
chains to the hydrophobic pocket.

2.   The Ki value for Pan-D-Phe was five times that of
Pan-L-Phe, indicating that peptidyl transferase has some
degree of stereospecificity.

3.   Of the 5'-O-nucleotidyl substituted derivatives of
Pan-Gly, only CpPan-Gly gave a marked reduction in the

Ki value, when compared to Pan-Gly; this result being indicative of a CMP binding site for the penultimate CMP residue of aminoacyl-tRNA.

The original postulate of Eckermann et al., (1974) for the role of methanol in decreasing the affinity of peptidyl transferase for puromycin was shown to be incorrect, since using E. coli polysomes the Km for puromycin was found to be 7.5×10^{-6} M and 5.2×10^{-6} M in the presence and absence of 30% methanol, respectively. Therefore the higher affinity of puromycin for peptidyl transferase of E. coli polysomes is probably due to either the presence of native peptidyl-tRNA, as suggested by Pestka (1972a), or due to the removal of a ribosomal component, which enhances the binding of puromycin, during high salt-washing.

CHAPTER 3

Pan-(N-ε-BrAc-L-Lys) AS AN AFFINITY LABEL

FOR THE A' SITE OF E. COLI

PEPTIDYL TRANSFERASE

Pan-(N-ε-BrAc-L-Lys) AS AN AFFINITY LABEL

FOR THE A' SITE OF E. COLI

PEPTIDYL TRANSFERASE

INTRODUCTION

Affinity labelling has been successfully used to identify the proteins and/or RNA comprising specific binding sites of the E. coli ribosome (see main Introduction). Most of the affinity labels, used to identify the ribosomal components of peptidyl transferase, have been analogues of peptidyl-tRNA, which have identified the components of the P site. Only two affinity labels, (N-BrAc-L-Phe)-tRNA (Eilat et al., 1974) and Bap-Pan-L-Phe (Eckermann and Symons, 1977; Greenwell et al., 1974; Harris et al., 1973), have been successful in identifying the ribosomal components of the A' site. The (N-BrAc-L-Phe)-tRNA presumably labelled the ribosomal components near the α-amino group of aminoacyl-tRNA, while Bap-Pan-L-Phe presumably labelled the components near the penultimate CMP residue of aminoacyl-tRNA.

The affinity labelling, described in this chapter, was initiated in order to determine the proteins and/or RNA of the aminoacyl side chain binding regions of the A' site (Harris and Symons, 1973b, and references therein), thereby mapping another region of the A' site.

Since puromycin (a low molecular weight analogue of the
3' terminus of aminoacyl-tRNA) was shown to act as an
acceptor substrate for peptidyl transferase (Yarmolinsky
and de la Haba, 1959), it was decided to use a reactive
analogue of puromycin as an affinity label for the A'
site. To date only five analogues of puromycin have
been reported, three of which have been used on bacterial
ribosomes (Cooperman et al., 1975; Eckermann and Symons,
1977; Greenwell et al., 1974; Harris et al., 1973; Pongs
et al., 1973a) and the remaining two on mammalian ribo-
somes (Minks et al., 1975; Stahl et al., 1974). Of
these only Bap-Pan-L-Phe (Eckermann and Symons, 1977;
Greenwell et al., 1974; Harris et al., 1973), which con-
tained a free α-amino group, was shown to be covalently
attached to the acceptor site.

Therefore, using the data of the previous chapter,
Eckermann et al., (1974), Harris and Symons (1973b) and
the criteria of Greenwell et al., (1974), Pan-(N-ε-BrAc-
L-Lys) (Figure 10) was synthesized (Figure 9). It was
hoped that when this analogue of puromycin was bound to
the acceptor site, the lysyl side chain, together with
the bromoacetyl group, would occupy the hydrophilic
pocket of the A' site (Harris and Symons, 1973b and
references therein). This pocket would be expected to
contain hydrophilic residues, such as the ε-amino group
of lysine, the imidazole group of histidine, the γ-
carboxyl group of glutamic acid or the sulphydryl group

FIGURE 9:   Synthetic procedure for the preparation of

Pan-(N-ε-BrAc-L-Lys).

Pan-NH$_2$ = puromycin aminonucleoside

Pan   +   (tBoc)NH—CH—CO$_2$H
|
NH$_2$
                                |
                              (CH$_2$)$_4$
                                |
                             NH(CBZ)

↓ EEDQ

Pan
|
NH
|
C=O
|
CH—NH(tBoc)   —(H$_2$, Pd/BaSO$_4$)→
|
(CH$_2$)$_4$
|
NH(CBZ)

Pan
|
NH
|
C=O
|
CH—NH(tBoc)
|
(CH$_2$)$_4$
|
NH$_2$

↓ (BrCH$_2$CO)$_2$O

Pan
|
NH
|
C=O
|
CH—NH$_2$ ←(TFA)—
|
CH$_2$
|
NH—C—CH$_2$Br
    ||
    O

Pan
|
NH
|
C=O
|
CH—NH(tBoc)
|
(CH$_2$)$_4$
|
NH—C—CH$_2$Br
    ||
    O
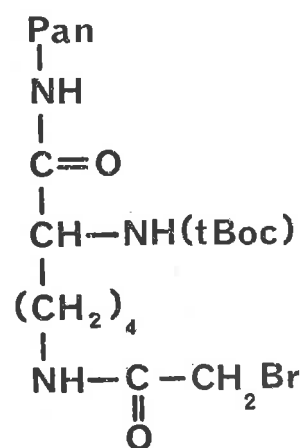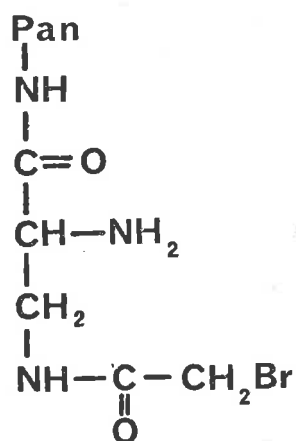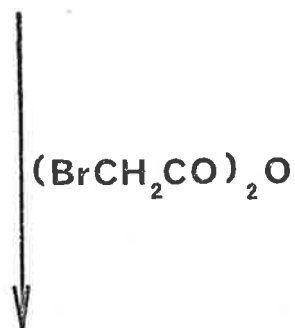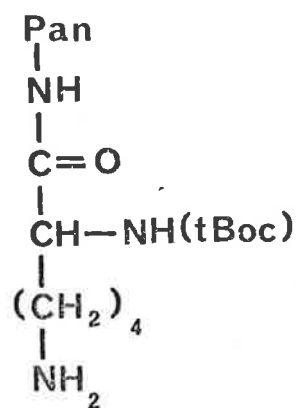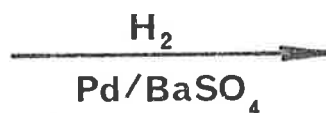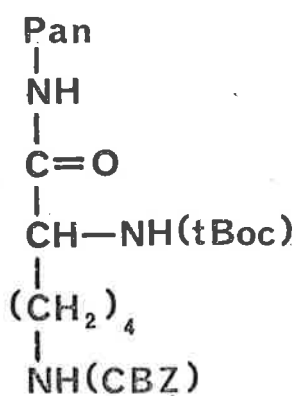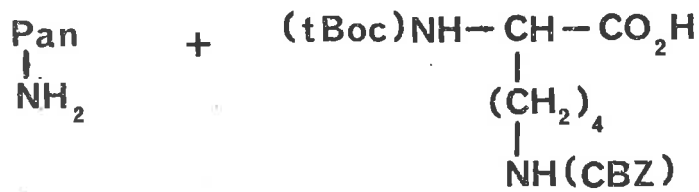
of cysteine. It was hoped that one of these would react with the affinity label, so forming a covalent bond between the affinity label and the proteins and/or RNA of the acceptor site, thus irreversibly inactivating peptidyl transferase. If successful, this approach, using radioactive affinity label, would enable us to identify one or more of the ribosomal components which form part of the A' site of peptidyl transferase.

As will be seen in this chapter, the affinity label did become covalently attached to the acceptor site, but because of the high degree of non-specific labelling the components of the A' site could not be identified.

MATERIALS

Pan,N-α-tBoc,N-ϵ-CBZ-L-Lys, RNase T_1, pancreatic RNase, chloramphenicol and puromycin were supplied by Sigma Chemical Co. and the EEDQ by the Aldrich Chemical Co. Inc. The Eastman 6060 TLC plates were purchased from Eastman-Kodak Co. and the [^{14}C]bromoacetic acid from The Radiochemical Centre, Amersham, England.

METHODS

1. Thin Layer Chromatography.

All analytical TLC described in this chapter was performed using silicic acid (Eastman 6060) as the

solid phase and the solvents used were, solvent A,
ethanol : choloroform (1:19, v/v) and solvent B, ethanol :
chloroform (7:13, v/v).

2.   Synthesis of Pan-(N-ε-BrAc-L-Lys).

All yields stated below were determined spectro-
photometrically using an extinction coefficient, at 275 nm,
of 18,900 $M^{-1}cm^{-1}$ for all of the Pan derivatives.

(a)   Pan-(N-α-tBoc,N-ε-CBZ-L-Lys).

This compound was prepared by a modification
of the method of Harris et al., (1972).   A mixture
of Pan (70 μmol), EEDQ (150 μmol) and N-α-tBoc,N-
ε-CBZ-L-Lys (150 μmol) in 3 ml of methanol was in-
cubated at room temperature for 2 h and the reaction
mixture analysed by TLC using solvent A, which
showed complete conversion of Pan (Rf = 0) to
Pan-(N-α-tBoc,N-ε-CBZ-L-Lys) (Rf = 0.3).   The
reaction mixture was concentrated and the Pan-(N-
α-tBoc,N-ε-CBZ-L-Lys) purified by preparative TLC
(Chapter 1; section 1) in the same solvent.   The
appropriate UV band was scraped off and the com-
pound recovered from the silicic acid by extraction
with 3 x 10 ml of ethanol : chloroform (1:1, v/v)
and the silicic acid pelleted by low speed centri-
fugation.   The pooled supernatants were then
filtered, evaporated to dryness and the residue
dissolved in 3 ml of ethanol to give a 60 - 80%

yield of product, relative to Pan.

(b)   Pan-(N-α-tBoc-L-Lys).

Removal of the CBZ group was achieved by
hydrogenating Pan-(N-α-tBoc,N-ε-CBZ-L-Lys) in
ethanol (5 ml) and 10% acetic acid (0.8 ml) at
20°, using Pd on $BaSO_4$ (20 mg; Vogel, 1957) as
the catalyst.   After 4.5 h removal of the CBZ
group was complete as shown by the total absence
of Pan-(N-α-tBoc,N-ε-CBZ-L-Lys) (Rf = 0.3) on TLC
using solvent A.   The catalyst was then removed
from the mixture by centrifugation, washed with
ethanol (1 ml) and centrifuged.   The pooled
supernatants were then dried by rotary evaporation
and the residue redissolved in methanol (2 ml) to
give Pan-(N-α-tBoc-L-Lys) in 90% yield relative
to Pan-(N-α-tBoc,N-ε-CBZ-L-Lys).

(c)   Pan-(N-ε-BrAc-L-Lys).

The Pan-(N-α-tBoc-L-Lys) (30 μmol) was
bromoacetylated by treatment with freshly pre-
pared bromoacetic anhydride (300 μmol) in 1 ml
of ethanol.   The bromoacetic anhydride was pre-
pared by adding bromoacetic acid (600 μmol) to
DCC (300 μmol) in 0.9 ml of dry acetonitrile.
After 1 h at room temperature the precipitated
DCU was removed by low speed centrifugation and

the supernatant used as the anhydride. The bromoacetic anhydride was found to react rapidly with Pan-(N-α-tBoc-L-Lys) to give Pan-(N-α-tBoc, N-ϵ-BrAc-L-Lys). In fact, after 5 min at 20°, the formation of Pan-(N-α-tBoc,N-ϵ-BrAc-L-Lys) (Rf = 0.75) was complete as shown by TLC using solvent A. The reaction mixture was evaporated to dryness and the residue dried by coevaporation with dry aceto-nitrile on the vacuum line (Symons, 1970). Removal of the tBoc group was achieved by treating the dry residue with TFA (0.1 - 0.3 ml) for 5 min at room temperature. Following the removal of the TFA by repeated coevaporations with dry acetonitrile, the residue was dissolved in methanol and purified by preparative TLC using solvent B. The appropriate UV band (Rf = 0.42) was scraped off and the compound recovered by eluting the silicic acid with 3 x 10 ml of ethanol, removing the silicic acid by low speed centrifugation after each elution. The pooled supernatants were filtered, dried by rotary evaporation and the residue dissolved in 2 ml of ethanol to give a 40 - 60% yield relative to Pan-(N-α-tBoc-L-Lys). The overall yield, relative to Pan, was found to be 30 - 40%.

The isolated compound gave a single UV absorbing spot (Rf = 0.63), which was both ninhydrin

FIGURE 10: Structures of puromycin (I) and Pan-(N-ε-BrAc-L-Lys) (II).

HO—CH₂ ... A(CH₃)₂ ... HN ... OH ... C=O ... H₂N—C—H ... CH₂ ... OCH₃

I

HO—CH₂ ... A(CH₃)₂ ... HN ... OH ... C=O ... H₂N—C—H ... CH₂ ... CH₂ ... CH₂ ... CH₂ ... NH ... C=O ... CH₂Br
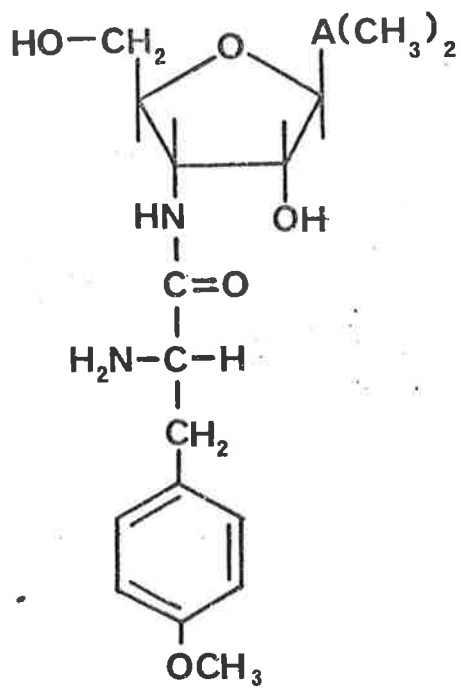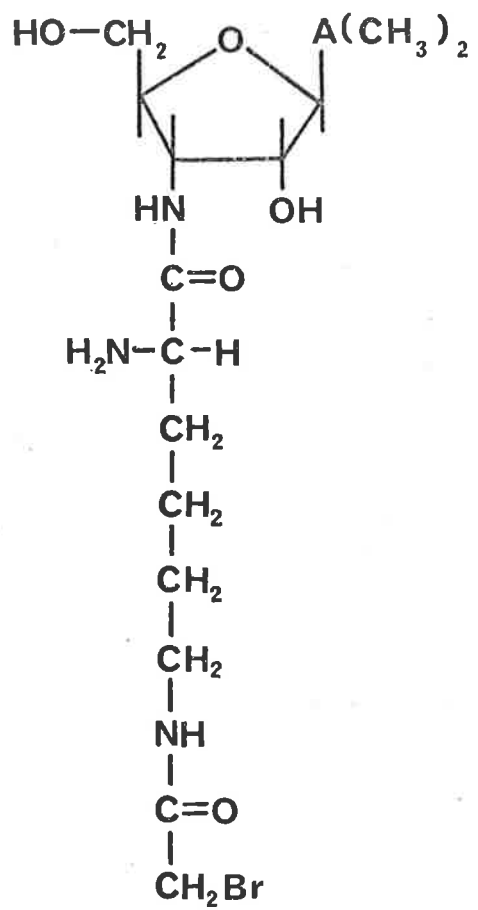
II

positive (Chapter l; section 2(a)) and alkylating positive (Chapter l; section 2(b)), on TLC using solvent B.

3.   Pan-(N-ε-[^{14}C]BrAc-L-Lys).

Pan-(N-α-tBoc-L-Lys) (30 μmol), synthesized as above (section 2(b)), was reacted with EEDQ (90 μmoles) and [^{14}C]bromoacetic acid (27 μmol) in a total volume of 0.5 ml of methanol. After 6 h at 37° the reaction mixture was evaporated to dryness, the residue dried by co-evaporation with dry acetonitrile on the vacuum line and treated with anhydrous TFA for 5 min at room temperature. The TFA was then removed by coevaporation with dry acetonitrile on the vacuum line and the compound purified as above.

The specific activity of the compound was calculated from the radioactivity in a small aliquot of the solution and corrected for the counting efficiency (Chapter l; section 3(b)) to give μCi/ml. Then, by using the concentration of the compound, as obtained from its UV spectrum, the μCi/μmole could be determined.

4.   Affinity Labelling of Ribosomes with Pan-(N-ε-BrAc-L-Lys).

Incubations contained, in a volume of 50 - 100 μl: 0.02 M Tris-acetate, pH 7.5, 0.01 M Mg(OAc)$_2$, 0.5 mM EDTA, 0.1 M NH$_4$Cl, 75 - 150 pmoles of E. coli ribosomes

and Pan-(N-ε-BrAc-L-Lys), puromycin and chloramphenicol as indicated in the legends to the Figures and Tables. After 16 - 18 h (unless otherwise stated) at 37°, ribosomes were precipitated with 2 ml of a methanol-salts solution (98% methanol containing 0.4 mM Tris-acetate, pH 8.0, 0.4 mM $Mg(OAc)_2$ and 4 mM KCl) according to the procedure of Harris et al., (1973). After 10 min at 0° the ribosomes were collected by low speed centrifugation and resuspended in 40 - 80 µl of 0.02 M Tris-acetate, pH 7.5, 0.01 M $Mg(OAc)_2$, 0.5 mM EDTA and 0.1 M NH_4Cl. The precipitation was repeated three or four times (with 95 - 100% recovery of A_{260} each time) to ensure complete removal of non-covalently bound affinity label prior to assaying of the ribosomes for peptidyl transferase activity (Chapter 1; section 6). Generally 5 µl of the final ribosome solution was used for the peptidyl transferase assay and for the determination of the ribosome concentration.

The washing procedure with the methanol-salts solution had little effect on peptidyl transferase activity and the two-dimensional gel electrophoresis, of Howard and Traut (1973), of the isolated ribosomal proteins suggested that almost all of the ribosomal proteins were still present (Greenwell et al., 1974).

5.   Determination of the Stoichiometry of Affinity
      Labelling.

      Following affinity labelling the number of affinity
labels covalently attached per ribosome were determined
as follows.   After washing the ribosomes, by repeated
precipitations, 5 µl of the ribosome solution was added
to 2 ml of Triton scintillation fluid (Chapter 1;
section 3(a)) and the radioactivity estimated.   Using
the efficiency of counting (Chapter 1; section 3(b)) in
Triton scintillation fluid, the dpm/5 µl was determined.
From this value and using the specific activity of the
affinity label, the µmoles of affinity label/5 µl was
calculated.   From the ribosome solution 5 µl was also
taken and added to 1 ml of H_2O, the A_{260} measured and
the µmoles of ribosome/5 µl determined.   Therefore, from
these two values the number of affinity labels per ribo-
some can be calculated,

$$\text{number of affinity labels} = \frac{\text{µmoles of affinity label/5 µl}}{\text{µmoles of ribosome/5 µl}}$$

RESULTS AND DISCUSSION

1.   Synthesis of Pan-(N-ε-BrAc-L-Lys).

      The synthesis of Pan-(N-ε-BrAc-L-Lys), on a 10 - 30
µmole scale, was achieved using a four step procedure
(Figure 9).   Pan was coupled to N-α-tBoc,N-ε-CBZ-L-Lys
using EEDQ as the condensing agent (Harris et al., 1972;

Mercer and Symons, 1971) and the product purified by TLC.
The N-α-tBoc,N-ε-CBZ-L-Lys was chosen because the CBZ
group could be selectively removed (by hydrogenation)
without affecting the tBoc group and also because, after
bromoacetylation, the tBoc group could be removed with-
out affecting the bromoacetyl group.   After removal of
the CBZ group the free ε-amino group was bromoacetylated
in methanol using bromoacetic anhydride.   Methanol was
used to ensure that no O-bromoacetylation of the Pan
moiety of Pan-(N-α-tBoc-L-Lys) would occur.   Coupling
could also be achieved via the NHS ester of bromoacetic
acid (Anderson et al., 1964) or by using EEDQ, but the
use of bromoacetic anhydride proved the more convenient.
After subsequent removal of the tBoc group with anhydrous
TFA, the Pan-(N-ε-BrAc-L-Lys) was purified by TLC.
Yields of 30 - 40% relative to Pan were routinely
obtained and the isolated compound was found to be one
major UV absorbing spot on TLC.   The isolated compound
had a Pan-like spectrum and was both ninhydrin and
"alkylating" positive, which was consistent with the
expected structure of the compound (Figure 10).

2.   Ability of Pan-(N-ε-BrAc-L-Lys) to act as an
       Acceptor Substrate.

Before the compound could be used as an affinity
label, its ability to bind to the A' site was measured.
As can be seen in Figure 11, Pan-(N-ε-BrAc-L-Lys) was

FIGURE 11: Km for Pan-(N-ε-BrAc-L-Lys), in the fragment reaction, was determined by incubating varying concentractions of Pan-(N-ε-BrAc-L-Lys) $(0.4 - 1.8 \times 10^{-3}$ M) for 30 min at 0° (see Chapter 1; section 6) and the amount of N-Ac-[^{3}H]Leu-(Pan-(N-ε-BrAc-L-Lys)) determined, as in Chapter 1; section 6.

$$\frac{1}{v}$$

$$[\text{Pan-(N-}\varepsilon\text{-BrAc-L-Lys)}]^{-1} \times 10^{-3} \quad (M^{-1})$$
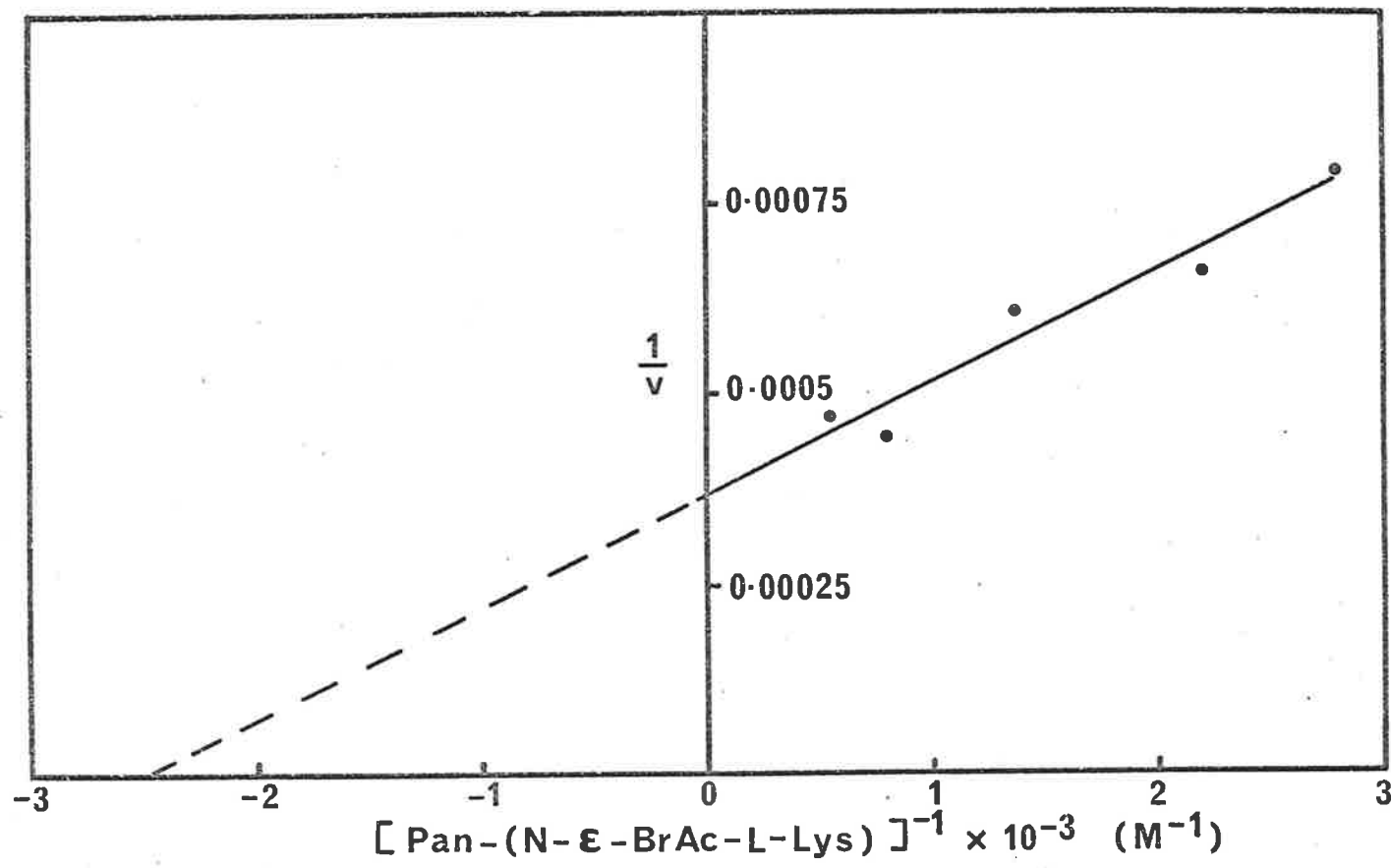
found to act as an acceptor substrate in the fragment reaction (Chapter 1; section 6) with a Km of 3.9×10^{-4} M, which is comparable to the Km of 1.1×10^{-4} M for puromycin (see previous chapter) under the same conditions. Therefore, the Pan-(N-ε-BrAc-L-Lys) has the ability to bind to the A' site, thereby acting as an acceptor substrate.

3. Affinity Labelling of Ribosomes.

When ribosomes were incubated with Pan-(N-ε-BrAc-L-Lys) for 16 h at 37°, washed and then assayed for peptidyl transferase activity (Chapter 1; section 6), it was found that 40% of peptidyl transferase was inactivated when compared to the control (ribosomes incubated without affinity label). In an attempt to maximize the amount of inactivation, both the concentration of affinity label and the time of incubation at 37° were varied.

(a) Effect of concentration of affinity label on inactivation of peptidyl transferase.

Inactivation increased linearly for affinity label concentrations up to 4.0 mM (Figure 12) after which the inactivation plateaued. Therefore 4.0 mM affinity label was chosen for all further work, because any increase above this concentration would give no further inactivation, but would be expected

FIGURE 12: Effect of concentration of Pan-(N-ε-BrAc-L-Lys) an inactivation of peptidyl transferase. Ribosomes were incubated at 37° for 16 h with different concentrations of Pan-(N-ε-BrAc-L-Lys) (0.5 - 5 x 10^{-3} M), washed (see Methods, section 4) and assayed for peptidyl transferase activity (Chapter 1; section 6).
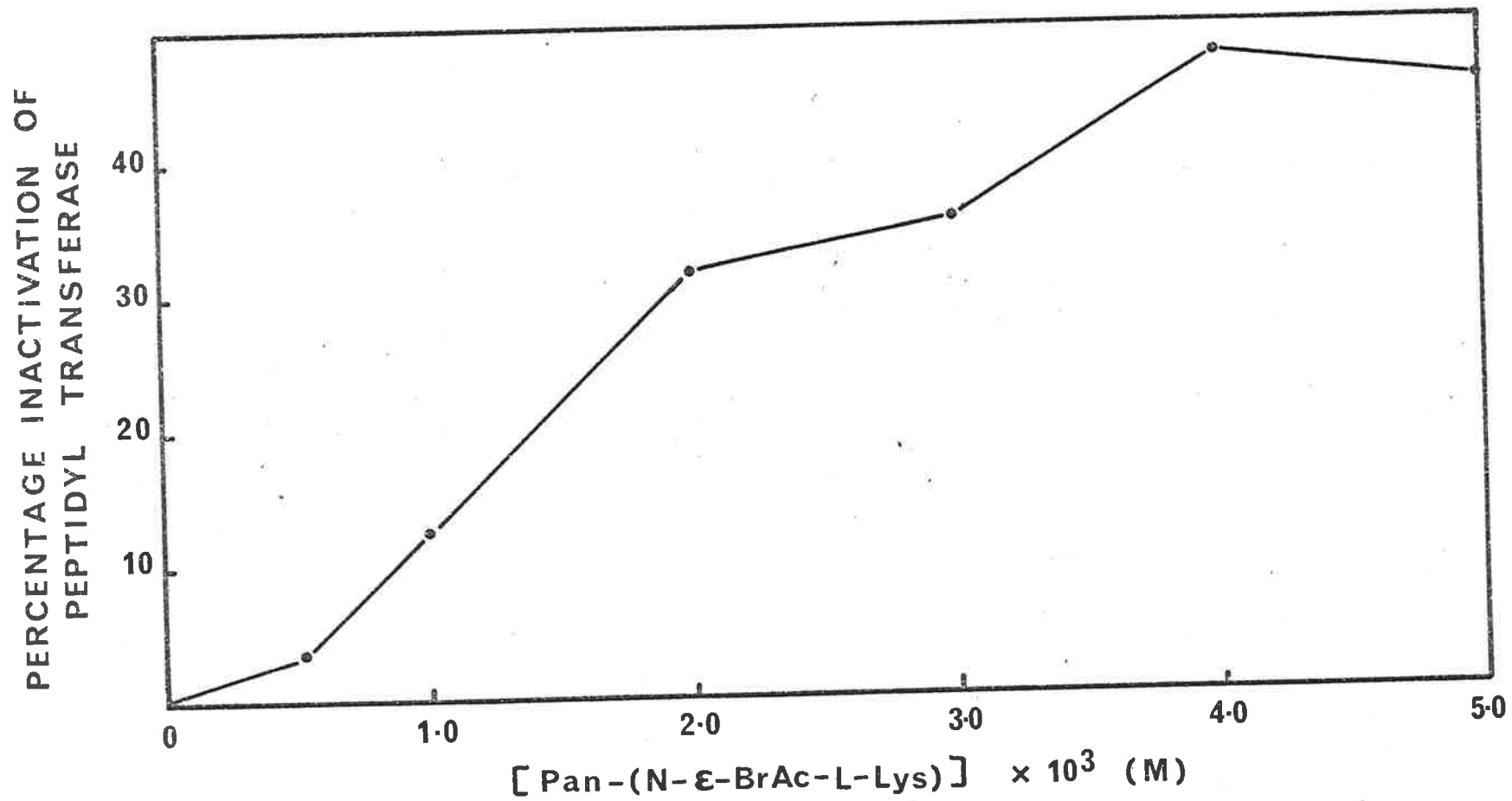
to increase the amount of non-specific reaction.

(b)   Effect of time of incubation on inactivation
        of peptidyl transferase.

Inactivation was also found to increase linearly for up to 24 h at 37° (results now shown); however, for convenience 16 - 20 h was used.

4.   Specificity of Inactivation of Peptidyl Transferase.

In order to demonstrate that inactivation was due to affinity labelling of the A' site, puromycin, chloramphenicol and Pan-Gly were included in the incubation mixture.   Puromycin (a good A' site substrate (Eckermann et al., 1974; Harris et al., 1971; see also Chapter 2)) and perhaps chloramphenicol (an A' site inhibitor (Fernandez-Munoz and Vazquez, 1973; Vazquez, 1974)) were expected to substantially protect against inactivation, while Pan-Gly (a poor A' site substrate (Eckermann et al., 1974; Harris et al., 1971; see also Chapter 2)) was not.   As can be seen from Table 4, puromycin at concentrations of 4.0 mM and 1.0 mM gave complete protection against inactivation, whereas at 0.1 mM there was no protection.   Pan-Gly acted as a control, as reaction of its α-amino group with the bromoacetyl group of the affinity label was not a significant factor during the affinity labelling. Appreciable reaction would have resulted in non-specific

TABLE 4:   Antibiotic protection of peptidyl trans-

ferase against inactivation by Pan-(N-ε-BrAc-L-Lys).

Ribosomes were incubated with 4.0 mM affinity label at

37°, for 16 h in the presence and absence of puromycin,

chloramphenicol and Pan-Gly (concentrations used are

indicated on the table), washed and assayed for pep-

tidyl transferase activity (Chapter 1; section 6).

TABLE 4.   ANTIBIOTIC PROTECTION OF PEPTIDYL TRANSFERASE

AGAINST INACTIVATION BY AFFINITY LABEL

Additions to preincubation mixture		% Peptidyl transferase activity	% Protection by antibiotic
Antibiotic	4.0 mM Affinity Label		
None	−	100	
None	+	58	
4.0 mM Puromycin	+	95	88
1.0 mM Puromycin	+	100	100
0.1 mM Puromycin	+	60	5
4.0 mM Chloramphenicol	+	51	0
1.0 mM Chloramphenicol	+	63	12
0.1 mM Chloramphenicol	+	58	0
1.0 mM Pan-Gly	+	57	0

protection of peptidyl transferase during the overnight
incubation with affinity label. Therefore, by analogy,
puromycin, with an α-amino group of similar reactivity,
did not protect by this non-specific mechanism but
rather by direct competition with the affinity label for
the A' site of peptidyl transferase. As can also be
seen from Table 4, chloramphenicol did not protect
against inactivation. This is very surprising, because
evidence strongly suggests that chloramphenicol inter-
acts at the A' site (Fernandez-Munoz and Vazquez, 1973;
Vazquez, 1974) and in fact, was shown to protect against
inactivation by the puromycin analogue Bap-Pan-L-Phe
(Greenwell et al., 1974; Harris et al., 1973). This
inability of chloramphenicol to prevent inactivation may
be because this affinity label contains a lysyl side
chain, which may bind to the hydrophilic pocket of the
A' site (Harris and Symons, 1973b; Rychlik et al., 1970;
Symons et al., 1977) while chloramphenicol and the
phenylalanyl side chain of Bap-Pan-L-Phe bind to the
neighbouring hydrophobic pocket of the A' site (Harris
and Symons, 1973a).

5.   Specificity of Affinity Labelling.

In order to determine the extent of specific
versus non-specific labelling, the ribosomes were in-
cubated with [^{14}C] affinity label for 16 h at 37°.
Following washing, the ribosomes were found to be heavily

non-specifically labelled to the extent of 56 affinity labels per ribosome, with a concommittant 36% inactivation of peptidyl transferase. To further investigate the distribution of affinity labels, the number of affinity labels per ribosomal subunit were determined (Chapter 1; section 7). Results obtained, given in Figure 13, showed that 38 and 18 affinity labels were attached per 50S and 30S subunit, respectively. It was thought possible that the specific labelling might be confined to the rRNA, as was found by Greenwell et al., (1974) and Harris et al., (1973) for Bap-Pan-L-Phe. Therefore the distribution of affinity labels between RNA and protein was determined, by two techniques to separate the rRNA and protein (Chapter 1; section 8). It was found, by both techniques, that 10.5 affinity labels were on the RNA and the remaining 45.5 affinity labels were on the protein (Table 5). Overall these results show a generalized non-specific labelling and illustrate the difficulty of determining the site of attachment of the peptidyl transferase specific affinity label.

6. Attempts to Locate the Specific Affinity Label.

In approaching the problem of locating the site of specific labelling, three possible methods were considered.

Firstly, if one aliquot of ribosomes were incubated with $[^{14}C]$ affinity label in the presence of, and another

FIGURE 13: Sucrose density gradient analysis of dis-
sociated ribosomes (see Chapter 1; section 7) which
were affinity labelled with 4.0 mM Pan-(N-ε-[^{14}C]BrAc-
L-Lys) for 16 h at 37°, washed (see Methods, section 4)
and dissociated prior to analysis.
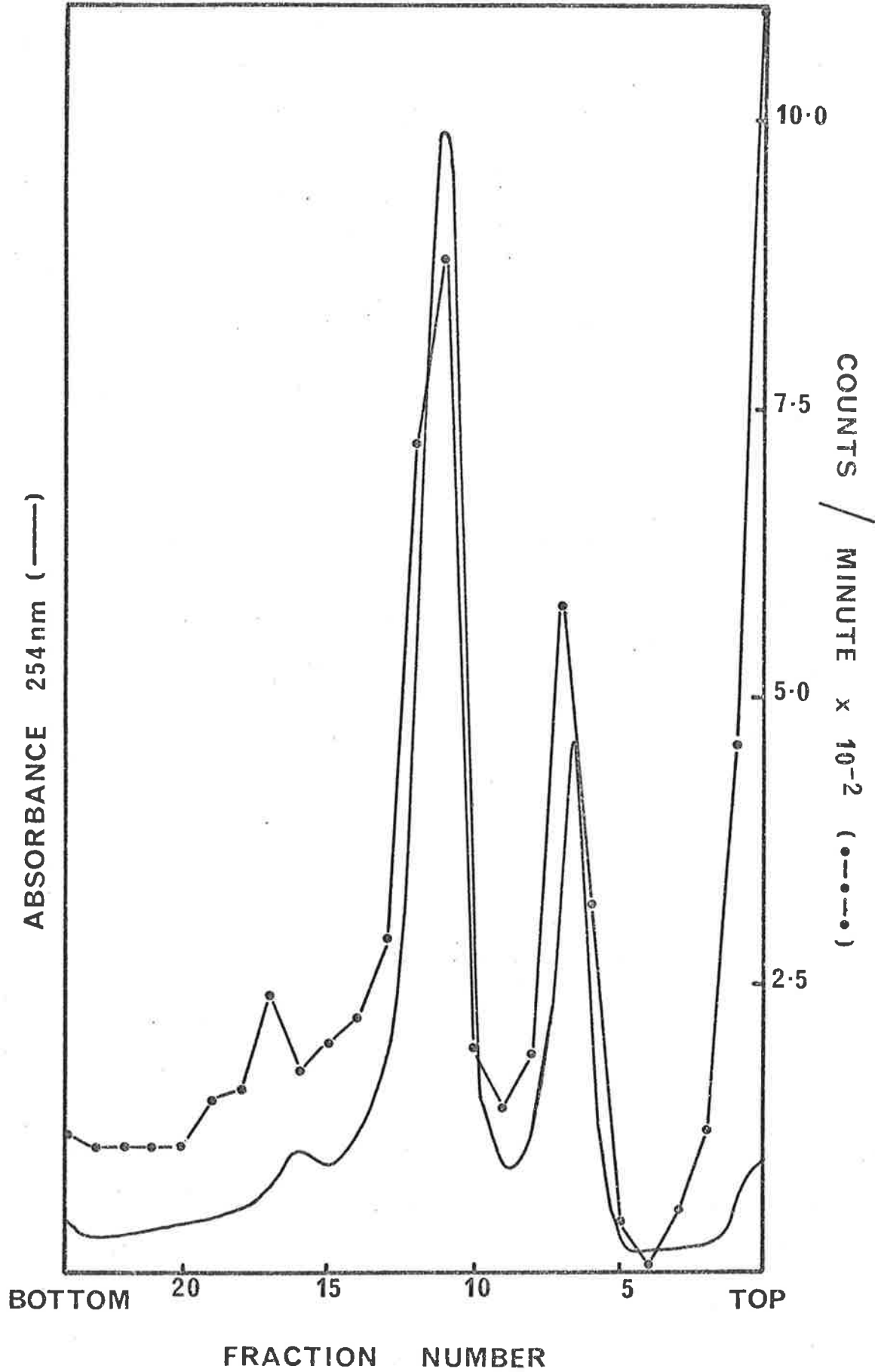
TABLE 5:   Distribution of Pan-(N-ε-[^{14}C]BrAc-L-Lys) between protein and RNA compliments of the ribosomes. The protein and RNA components of ribosomes incubated with 4.0 mM [^{14}C]affinity label (see Methods, section 4) were separated as in Chapter 1; section 8 and the percentage of the total radioactivity in each fraction determined.

TABLE 5. DISTRIBUTION OF [^{14}C]AFFINITY LABEL

BETWEEN RNA AND PROTEIN COMPONENTS

Method of RNA-protein separation	Distribution of radioactivity (%)	
	RNA	protein
1. Ribonuclease digestion	19	81
2. Phenol extraction	19	81

aliquot in the absence of puromycin, then the specific affinity label should be attached in the absence of puromycin but not in the presence of puromycin. The non-specific labelling, on the other hand, should be the same for both samples. Fractionation and comparison of the ribosomal proteins and rRNA, from the two aliquots, may then allow the specific site to be identified. However, this experiment was not attempted due to the small expected difference in labelling, as there are 56 affinity labels per ribosome of which less than one is specific. Therefore if the specific affinity label is wholly confined to one fraction (rRNA or proteins) then there would be a maximal increase of 2% or 10% in the radioactivity associated with the proteins or rRNA fractions, respectively, derived from the ribosomes affinity labelled in the absence of puromycin when compared to the ribosomes affinity labelled in the presence of puromycin. These predicted increases were felt to be too small to be experimentally useful. Two approaches which were attempted are outlined below,

(a) ribosomes were affinity labelled with cold affinity label in the presence of puromycin, all the non-specific sites should react, leaving the specific site unlabelled due to protection by the puromycin. Theoretically, subsequent relabelling of the ribosomes with [^{14}C] affinity label, in the absence of puromycin, would be only at the A' site.

However, in practice, this was not the case; in fact 117 affinity labels were attached per ribosome. Presumably the ribosome was partially unfolded or degraded during the lengthy affinity labelling incubation at 37° and therefore more non-specific sites were exposed.

(b) ribosomes were reacted with cold affinity label, washed free of excess affinity label and then the ribosomes were incubated with the donor substrate, CpApCpCpA(Ac-L-[^{3}H]Leu) under fragment reaction conditions (Chapter 1; section 6) in an attempt to transfer Ac-L-[^{3}H]Leu from the substrate to any affinity label covalently attached to the A' site. The specificity of transfer would be ensured because only peptidyl transferase can catalyse the reaction and therefore only the affinity label bound to the A' site can act as an acceptor. However, no transfer of Ac-L-[^{3}H]Leu was detected.

SUMMARY

In conclusion, it has been shown that Pan-(N-ε-BrAc-L-Lys) was able to bind, non-covalently, to the A' site and act as an acceptor substrate in the fragment reaction. After 16 h at 37° this compound was also able to irreversibly inactivate peptidyl transferase, by covalent attachment to the A' site. This inactivation

was shown to be specific since it was prevented by puromycin and this protection was not due to direct chemical reaction between the affinity label and puromycin. The inability of chloramphenicol to protect against inactivation was unexpected, suggesting, contrary to previous evidence, that either chloramphenicol does not bind to the A' site or both chloramphenicol and Pan-(N-ε-BrAc-L-Lys) can simultaneously bind to the A' site. The localization (protein and/or RNA) of the specific A' site affinity label was not successful, since affinity labelling was largely non-specific (56 affinity labels per ribosome to give 36% inactivation of peptidyl transferase) and the ribosomes proved unstable during the necessarily long incubations.

CHAPTER 4

Pan-L-pN$_3$Phe AS AN AFFINITY LABEL

FOR THE E. COLI PEPTIDYL TRANSFERASE

A' SITE

Pan-L-pN$_3$Phe AS AN AFFINITY LABEL

FOR THE E. COLI PEPTIDYL TRANSFERASE A' SITE

INTRODUCTION

The affinity label described in the previous
chapter was designed to bind to a proposed hydrophilic
binding site in the A' site (Harris and Symons, 1973b
and references therein). The presence of a hydrophobic
pocket in the A' site, which would presumably be formed
by hydrophobic residues, such as hydrophobic aminoacyl
side chains and/or bases from the 23S or 5S RNA, has also
been proposed (references as above). Therefore the
use of a conventional chemical affinity label, which by
its very nature requires a nucleophile in the active
site, would probably have little chance of success in
affinity labelling the hydrophobic pocket of the A' site.
Therefore a photoaffinity label would be best suited to
study the protein and/or RNA environment of this
pocket.

Photoaffinity labels, which are chemically un-
reactive in the dark, form carbenes or nitrenes upon
photolysis; these reactive groups are then able to
react with any bond, including insertion into carbon-
carbon or carbon-hydrogen bonds (Knowles, 1972).
Therefore, photoaffinity labels do not suffer from the
limitations of conventional chemical affinity labels,
in that nucleophiles or any other reactive groups need

not be present in the site to be studied. For "true"
photoaffinity labelling to occur at any particular site,
the rate of covalent attachment of the activated photo-
affinity label to the site must be greater than the rate
of dissociation of that photoactivated species from the
site. If on the other hand the rate of dissociation
is greater than the rate of covalent attachment,
"pseudo" photoaffinity labelling occurs (Ruoho et al.,
1973), which is very similar to conventional chemical
affinity labelling in that the reactive species can
become covalently attached to many sites apart from
its own binding site, thereby increasing the extent of
non-specific labelling. Ideally, photoaffinity labels
are bound to the enzyme and upon photolysis form highly
reactive species in situ which, because of tight bind-
ing to the site, become covalently attached to the site
and by so doing cause inactivation of enzymic activity.

One such photoaffinity label, Pan-L-pN$_3$Phe
(Figure 17), designed for the hydrophobic pocket of the
A' site, has been synthesized. As can be seen this
compound contains a free α-amino group which, as dis-
cussed in the previous chapter, is essential if the
puromycin analogue is to bind to the A' site. It was
hoped that upon photolysis the azide would be converted
to a nitrene (Figure 18), which would then react and so
covalently attach the affinity label to the A' site;
this covalent attachment would then cause inactivation

of peptidyl transferase. Therefore, by using this
photoaffinity label, the proteins and/or RNA of the
hydrophobic pocket of the A' site could be determined.
However, in practice this photoaffinity label proved
unsuccessful.

MATERIALS

Pan, DCC, NHS and L-phenylalanine were all pur-
chased from Sigma Chemical Co. The Whatman No.1
paper was supplied by W. and R. Balston Ltd., the
Kieselgel 60 F 254 TLC plates by E. Merck, Darmstadt,
W. Germany and the [^{3}H]Pan by The Radiochemical Centre,
Amersham, England.

METHODS

1.   Thin Layer Chromatography.

Silicic acid (Kieselgel 60 F 254) was used as the
solid phase for all analytical TLC with the following
solvents: solvent A, ethanol : chloroform : acetic acid
(5:95:3, v/v/v); solvent B, ethanol : chloroform (1:19,
v/v) and solvent C, ethanol : chloroform (7:13, v/v).

2.   Synthesis of N-α-tBoc-L-pN$_3$Phe.

(a)   L-pNO$_2$Phe.

Nitration of L-Phe was achieved as described

by Bergel and Stock (1959).   To a solution of
L-Phe (55 mmol) in concentrated sulphuric acid
(150 ml) at 0°, 1.7 ml of fuming nitric acid was
added, with vigorous stirring, over a period of
20 min, after which the reaction mixture was left
for a further 20 min at 0°.   The reaction mixture
was then poured on to 200 ml of ice-water, the pH
of the solution raised to 8.3, using concentrated
ammonia solution, and the resultant solution left
overnight at 4°, in order to crystallize the pro-
duct.   The crystals formed were collected by
vacuum filtration and dried in a vacuum desicator
overnight.   The overall yield of L-pNO$_2$Phe was
3.7 g (17.6 mmol; 32%).

The UV spectrum of the isolated compound
had a λ_{max} at 273 nm, with a calculated extinction
coefficient at 273 nm of 7,800 M^{-1}cm^{-1}.   The infra-red
spectrum of the product, when compared to that of
L-Phe, showed the appearance of two new peaks at
1550 cm^{-1} and 1350 cm^{-1}, both indicative of a
nitro group (Dyer, 1965; Williams and Fleming,
1966).   The L-pNO$_2$Phe was also analysed, using
a Beckman Model 120C amino acid analyser with
Durrum DC1A resin and Pico buffer system, and gave
a peak eluting at 152 min, corresponding to L-Phe
(constituting 4% of the sample) and also a peak
eluting at 196 min, which was presumably L-pNO$_2$Phe

(constituting 96% of the sample).

(b)   N-α-tBoc-L-pNO$_2$Phe.

The reaction conditions were those of
Schwyzer et al., (1959).

The tBoc group was added to the α-amino
group of L-pNO$_2$Phe by incubating, L-pNO$_2$Phe
(4 mmol) in 14 ml of dioxane, 4 ml of H$_2$O, 1.8 ml
of triethylamine and 2 ml of tBoc-N$_3$ (Chapter 2:
Methods (section 2)) at 37° for 20 h, with stir-
ring. The reaction mixture was then acidified
to pH 3, using citric acid, and the resulting
solution extracted three times with 50 ml of
ethylacetate. The pooled ethylacetate extracts,
dried using MgSO$_4$ (which was removed by filtration),
were evaporated to dryness and the residue re-
dissolved in methanol (10 ml). The isolated
compound was found to be a single UV absorbing
spot (Rf = 0.59) on TLC using solvent A. The UV
spectrum was identical to that of L-pNO$_2$Phe and
using the same extinction coefficient, the yield
of N-α-tBoc-L-pNO$_2$Phe was 2.2 mmoles (55% with
respect to L-pNO$_2$Phe).

(c)   N-α-tBoc-L-pNH$_2$Phe.

Reduction of N-α-tBoc-L-pNO$_2$Phe was achieved

by hydrogenation using palladium on charcoal (Pd/C) as the catalyst. N-α-tBoc-L-pNO$_2$Phe (1 mmol) in 10 ml of methanol : acetic acid (1:1, v/v) with 100 mg of Pd/C was hydrogenated, with vigorous stirring, for 24 h at room temperature. The catalyst was removed by low speed centrifugation, the pellet washed with 2 ml of methanol, centrifuged again and the pooled supernatants evaporated to dryness. The resultant residue was dissolved in 5 ml of methanol and when analysed by TLC using solvent A was found to contain one major UV absorbing (95%), ninhydrin positive spot (Rf = 0.25), as expected for N-α-tBoc-L-pNH$_2$Phe. The UV spectrum showed a shift in the λ_{max} from 273 nm for the starting compound to 239 nm for the product, which was also consistent with the aromatic nitro group being reduced to an aromatic amine (Dyer, 1965).

(d)   <u>N-α-tBoc-L-pN$_3$Phe.</u>

Due to the light sensitivity of the azide group all reactions involving N-α-tBoc-L-pN$_3$Phe were carried out in the dark or in reaction vessels coated with aluminium foil.

To N-α-tBoc-L-pNH$_2$Phe (1.1 mmol) in 4 ml of 2N HCl at 0°, NaNO$_2$ (1.2 mmol) in 1 ml of H$_2$O was added dropwise over a period of 20 min, with vigorous stirring, after which the reaction

mixture was left for a further 10 min at 0°. As expected a diazonium salt was present at this stage as shown by the formation of a bright red precipitate when reacted with β-naphthol (Chapter 1; section 2(d)).

The N-α-tBoc-L-pN$_3$Phe was then formed by dropwise addition of NaN$_3$ (1.4 mmol) in 0.3 ml of H$_2$O to the above reaction mixture, over a period of 20 min at 0°, with stirring. After a further 10 min at 0° the reaction mixture was extracted with 4 x 4 ml of ethylacetate. The pooled ethylacetate extracts were dried using Na$_2$SO$_4$(anhydrous), which was removed by filtration, and the filtrate evaporated to dryness. The residue, which was dissolved in 5 ml of ethylacetate, was analysed by TLC using solvent A and found to contain one major UV absorbing spot (80%) and two minor UV absorbing spots. Further purification of N-α-tBoc-L-pN$_3$Phe was achieved by preparative TLC (Chapter 1; section 1) using solvent A. The compound was recovered from the silicic acid by extraction with 3 x 10 ml of ethanol : chloroform (1:1, v/v) and the silicic acid pelleted by centrifugation. The pooled supernatants were filtered, evaporated to dryness and the residue redissolved in ethanol (3 ml). N-α-tBoc-L-pN$_3$Phe, purified in this way, was found to be one single UV absorbing spot on

FIGURE 14: Synthetic procedure for the preparation of
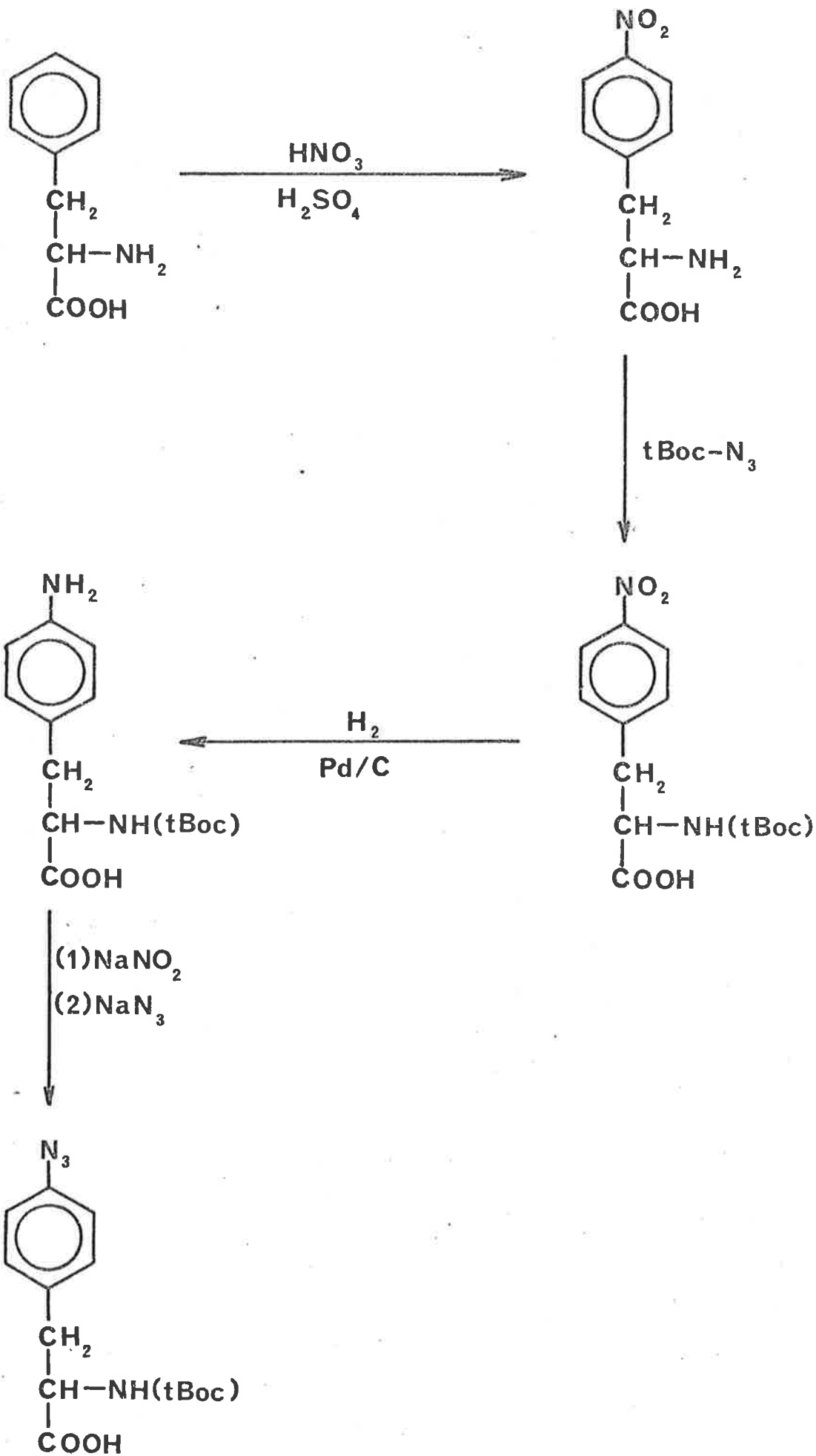N-α-tBoc-L-pN$_3$Phe.

$$HNO_3 \atop H_2SO_4$$

tBoc-N$_3$

$$H_2 \atop Pd/C$$

(1) NaNO$_2$
(2) NaN$_3$

FIGURE 15: The infra-red spectrum of N-α-tBoc-L-pN$_3$Phe, using a nujol mull.

Wavelength (cm^{-1}) x 10^{-2}
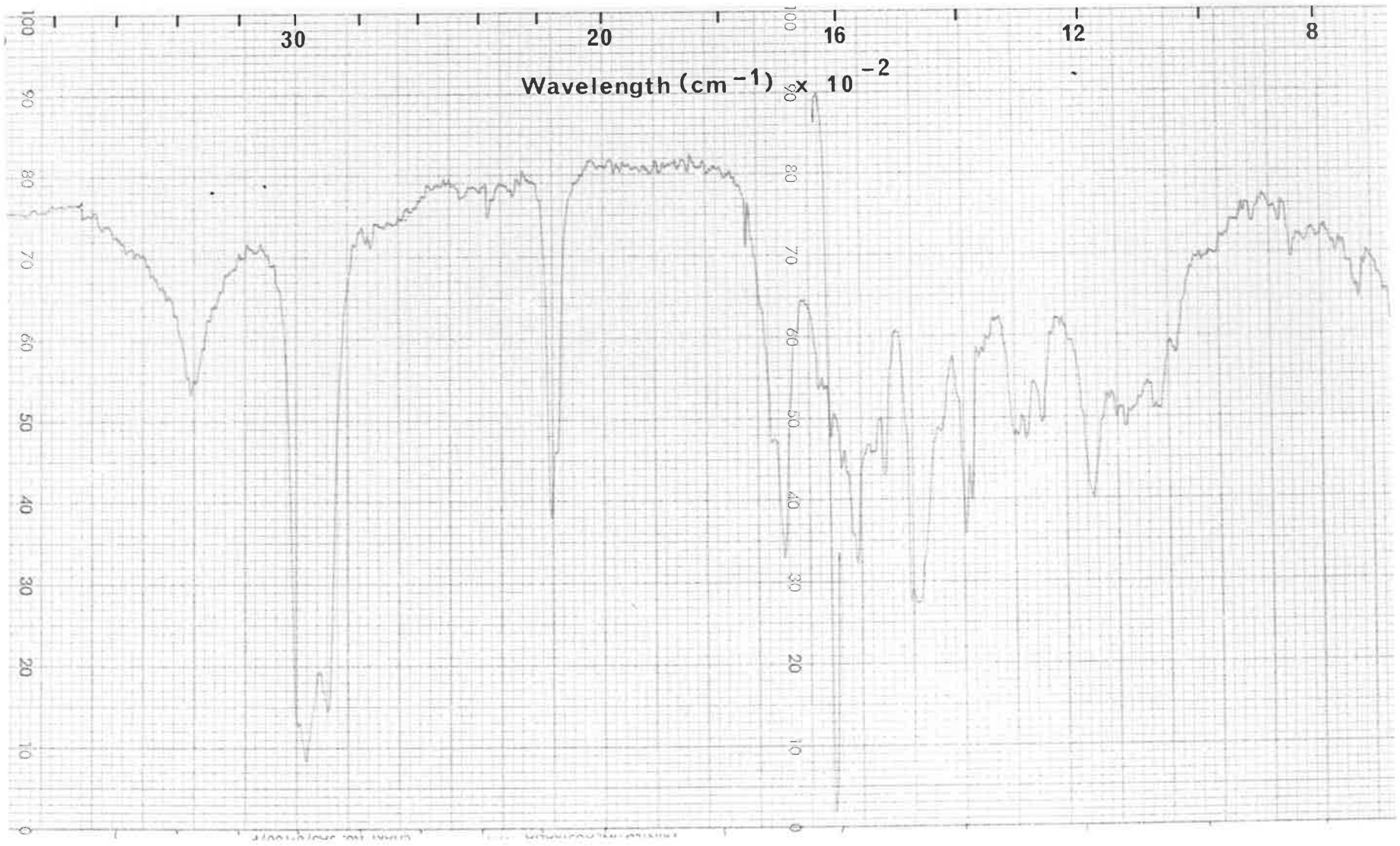
analytical TLC using the above solvent system.
The yield of N-α-tBoc-L-pN$_3$Phe was 510 μmoles (46%),
using the calculated extinction coefficient at
250 nm of 8,200 M^{-1}cm^{-1}.

The UV spectrum showed a shift in the λ_{max}
from 239 nm, for N-α-tBoc-L-pNH$_2$Phe, to 250 nm,
for N-α-tBoc-L-pN$_3$Phe.   The infra-red spectrum
showed peaks at 1683 cm^{-1} and 1691 cm^{-1}, both in-
dicative of carbonyls and another at 2170 cm^{-1},
indicative of the azide group (Dyer, 1965;
Williams and Fleming, 1966).

3.   Synthesis of the NHS ester of N-α-tBoc-L-pN$_3$Phe.

The NHS ester was formed by reacting N-α-tBoc-L-
pN$_3$Phe (50 μmol) in 0.5 ml of dry acetonitrile containing
NHS (55 μmol) and DCC (55 μmol)(Anderson et al., 1964).
After 2 h at room temperature the DCU formed was pelleted
by centrifugation and the supernatant, containing the NHS
ester, used for coupling to the amino group.

4.   Synthesis of Pan-L-pN$_3$Phe.

Coupling of Pan to N-α-tBoc-L-pN$_3$Phe was achieved
by reacting Pan (20 μmol) in 0.5 ml of methanol with the
NHS ester of N-α-tBoc-L-pN$_3$Phe (100 μmol) at room tempera-
ture for 90 min, after which the reaction was complete
as shown by the disappearance of Pan (Rf = 0) on analy-
tical TLC using solvent B.   The Pan-(N-α-tBoc-L-pN$_3$Phe)

(Rf = 0.71) was then purified by preparative TLC (Chapter 1; section 1), using solvent B, and the compound recovered by extraction of the silicic acid with 3 x 10 ml of ethanol : chloroform (1:1, v/v) and the silicic acid removed by centrifugation. The pooled supernatants were filtered, in order to remove any residual silicic acid, the filtrate evaporated to dryness and the residue dried by coevaporation with acetonitrile (Symons, 1970). The tBoc group was then removed by treating the dried compound with anhydrous TFA for 5 min at room temperature, after which the TFA was removed on the vacuum line, by coevaporation with acetonitrile and the residue finally dissolved in 1 ml of methanol. The compound isolated gave one major UV absorbing, ninhydrin positive (Chapter 1; section 2(a)) spot on analytical TLC using solvent C. The UV spectrum of the compound was the sum of the two isolated chromophores, Pan and L-pN_3Phe. Using an extinction coefficient at 265 nm of 19,400 $M^{-1}cm^{-1}$ (see below), the yield was 12.0 μmoles (60% with respect to Pan).

5. Determining the Extinction Coefficient of Pan-L-pN_3Phe.

Pan-L-pN_3Phe (5 A_{265} units) was evaporated to dryness and the residue dissolved in 0.1 ml of 1.0 N HCl and heated at 100° for 1 h in a sealed tube. The solution was then evaporated to dryness, the residue redissolved in 0.1 ml of H_2O, of which 0.03 ml was applied

FIGURE 16:   Synthesis procedure used for the preparation of Pan-L-pN$_3$Phe.

Pan-NH$_2$ = puromycin aminonucleoside

Pan
|
NH₂

Pan
|
NH_2

+

CO—NHS
|
CH—NH(tBoc)
|
CH₂
|
(benzene ring)
|
N₃

Pan
|
NH
|
C=O
|
CH—NH(tBoc)
|
CH₂
|
(benzene ring)
|
N₃

—— TFA ——→

Pan
|
NH
|
C=O
|
CH—NH₂
|
CH₂
|
(benzene ring)
|
N₃
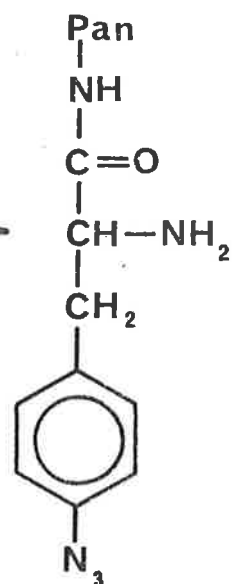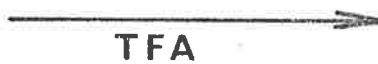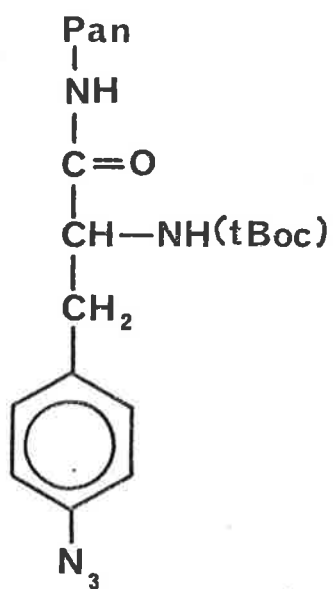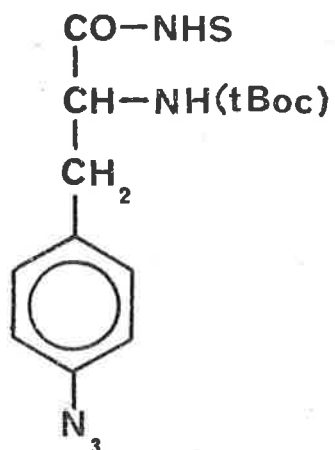
to Whatman No.1 paper and chromatographed (descending)
using propan-2-ol : HCl : H_2O (170:39:41, v/v/v) as the
solvent.   The UV spot corresponding to Pan (Rf = 0.53)
was cut out and the compound eluted from the paper, using
0.1 N HCl (5.0 ml), overnight at room temperature and
the A_{275} values determined.   A correction for the
efficiency of elution was determined by using a known
amount of Pan, as a control, during the entire procedure.
An extinction coefficient at 265 nm of 19,400 $M^{-1}cm^{-1}$
was determined for Pan-L-pN$_3$Phe using the following
equation,

$$\frac{A_{265} \text{ units of compound}}{\text{extinction coefficient at 265 nm of compound}}$$

$$= \frac{A_{275} \text{ units of Pan, obtained on hydrolysis of compound}}{\text{extinction coefficient at 275 nm of Pan}}$$

6.   Synthesis of [^{3}H]Pan-L-pN$_3$Phe.

[^{3}H]Pan-L-pN$_3$Phe was synthesized using the same
basic procedure as for the non-radioactive compound, but
on a smaller scale.

[^{3}H]Pan (1.0 - 1.5 µmol) in 0.1 ml of methanol was
treated with the NHS ester of N-α-tBoc-L-pN$_3$Phe (8 µmol)
at room temperature.   At appropriate times samples were
analysed by TLC in solvent B.   The small TLC plates were
cut into 0.5 cm strips, which were placed into vials
containing scintillation fluid (Chapter 1; section 3(a))
and the radioactivity estimated.   Generally, after 3 h
at room temperature, 60% of the [^{3}H]Pan had been converted

to [^{3}H]Pan-(N-α-tBoc-L-pN$_3$Phe).   Purification was achieved

using Kieselgel 60 F 254 and solvent B.   The appropriate

UV absorbing band (Rf = 0.73) was scraped off and the

compound eluted from the silicic acid with 3 x 3 ml of

ethanol : chloroform (1:1, v/v).   The pooled eluates were

evaporated to dryness, the residue dried by coevaporation

with dry acetonitrile and treated with anhydrous TFA.

After 5 min at room temperature the TFA was removed by

coevaporation with dry acetonitrile and the resultant

residue dissolved in 1 ml of methanol.   The concentration

of the compound was then estimated by using the extinction

coefficient at 265 nm (section 5 of this chapter) and

the specific activity estimated as in Methods (section 4)

of Chapter 3.

7.   Photoaffinity Labelling of Ribosomes with Pan-L-
       pN$_3$Phe.

In a final volume of 50 - 100 μl, ribosomes (90 -

180 pmol) in 0.02 M Tris-acetate, pH 7.5, 0.01 M

Mg(OAc)$_2$, 0.5 mM EDTA and 0.1 M NH$_4$Cl were photolysed,

at room temperature, with Pan-L-pN$_3$Phe (0.9 - 1.0 mM).

Samples were photolysed by irradiation with a Philips

HPK 125W lamp, encased in a water cooled jacket, at a

distance of 3 cm in a pyrex apparatus; the pyrex ensured

that the ribosomes were photolysed with light of wave-

length greater than about 300 nm.   Light of wavelength

less than 300 nm is known to cause extensive photo-

oxidation of biological systems (Knowles, 1972). After photolysis, the ribosomes were precipitated with 2 ml of a methanol-salts solution (98% methanol containing 0.4 mM Tris-acetate, pH 8.0, 0.4 mM $Mg(OAc)_2$ and 4 mM KCl) according to the procedure of Harris et al., (1973). After 10 min at 0° the ribosomes were collected by low speed centrifugation and resuspended in 40 µl of 0.02 M Tris-acetate, pH 7.5, 0.01 M $Mg(OAc)_2$, 0.5 mM EDTA and 0.1 M NH_4Cl. This precipitation procedure was repeated three to four times (with 95 - 100% recovery of A_{260} each time) to ensure complete removal of non-covalently bound affinity label, prior to assaying of the ribosomes for peptidyl transferase activity (Chapter 1; section 6).

The stoichiometry of labelling was determined as in Methods (section 5) of Chapter 3.

RESULTS AND DISCUSSION

1.   Synthesis and Characterization of Pan-L-pN$_3$Phe.

The synthetic procedure, used to synthesize Pan-L-pN$_3$Phe (Figure 17), was as outlined in Figure (16). Pan was coupled to N-α-tBoc-L-pN$_3$Phe using the NHS ester to activate the carboxyl group and the Pan-(N-α-tBoc-L-pN$_3$Phe) formed, purified by preparative TLC. The tBoc group was removed by subsequent treatment with anhydrous TFA. The N-α-tBoc-L-pN$_3$Phe, used above, was synthesized using a four step procedure (Figure 14), which required

FIGURE 17: Structures of puromycin (I) and Pan-L-pN$_3$-

Phe (II).

I

HO—CH₂ O A(CH₃)₂
HN OH
C=O
H₂N—C—H
CH₂

OCH₃

I

HO—CH₂ O A(CH₃)₂
HN OH
C=O
H₂N—C—H
CH₂

N₃
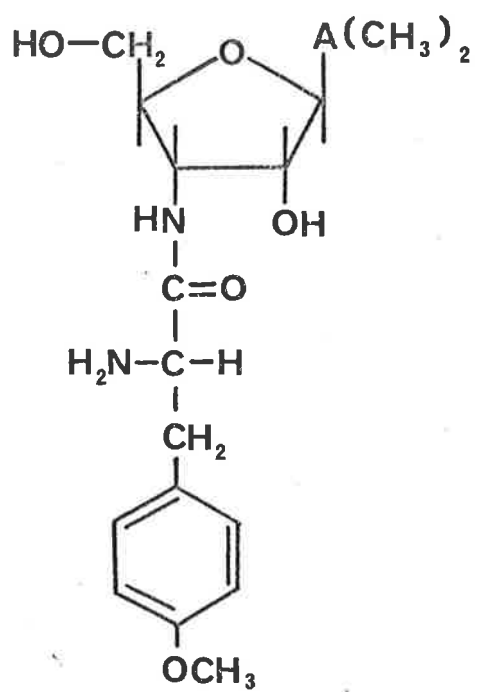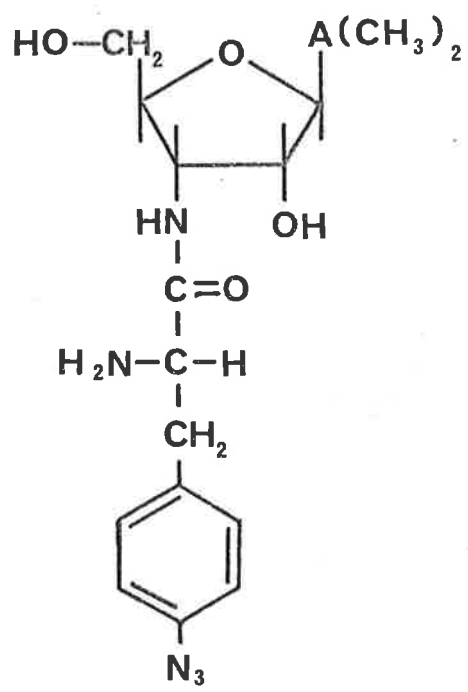
II

nitration of L-phenylalanine, according to the procedure
of Bergel and Stock (1959), as the first step.   The L-pNO$_2$-
Phe formed was purified and reacted with tBoc-N$_3$ in
order to protect the α-amino group; the tBoc group was
chosen as the protecting group because of its inertness
in any of the subsequent steps of the procedure.   The
aromatic nitro group, which was reduced using catalytic
hydrogenation, was converted to the azide by treating
the aromatic amine with NaNO$_2$, to form the diazonium
salt, which was then reacted with NaN$_3$ to form the
aromatic azide.   The N-α-tBoc-L-pN$_3$Phe formed, was
purified by preparative TLC and the infra-red spectrum
of the purified compound taken (Figure 15).   As can be
seen the infra-red spectrum demonstrated the presence
of an azide (peak at 2170 cm^{-1}) and of two carbonyls
(peaks at 1695 cm^{-1}), one corresponding to the carbonyl
of the carboxyl group and the other to the carbonyl of
the tBoc group.

Figure 18 shows the UV spectrum for Pan-L-pN$_3$Phe,
which was found to be the sum of the UV spectra of the
isolated chromophores, Pan and L-pN$_3$Phe.   As can also
be seen from Figure 18, upon photolysis of the compound
in the presence of N-α-Ac-Gly-Gly, using the apparatus
described in section 7 (see Methods), the spectrum of
Pan-L-pN$_3$Phe was altered.   In fact the UV spectrum
was converted to one similar to that of puromycin.
This was due to the reaction of the nitrene, formed upon

FIGURE 18: The effect of photolysis (see Methods, section 7), in the presence of N-α-Ac-Gly-Gly (1 mM) on the UV spectrum of Pan-L-pN$_3$Phe. The spectral lines, in order of decreasing A$_{260}$ are the spectra of Pan-L-pN$_3$Phe after 0, 2, 3, 4, 5, 30 and 60 min of photolysis.
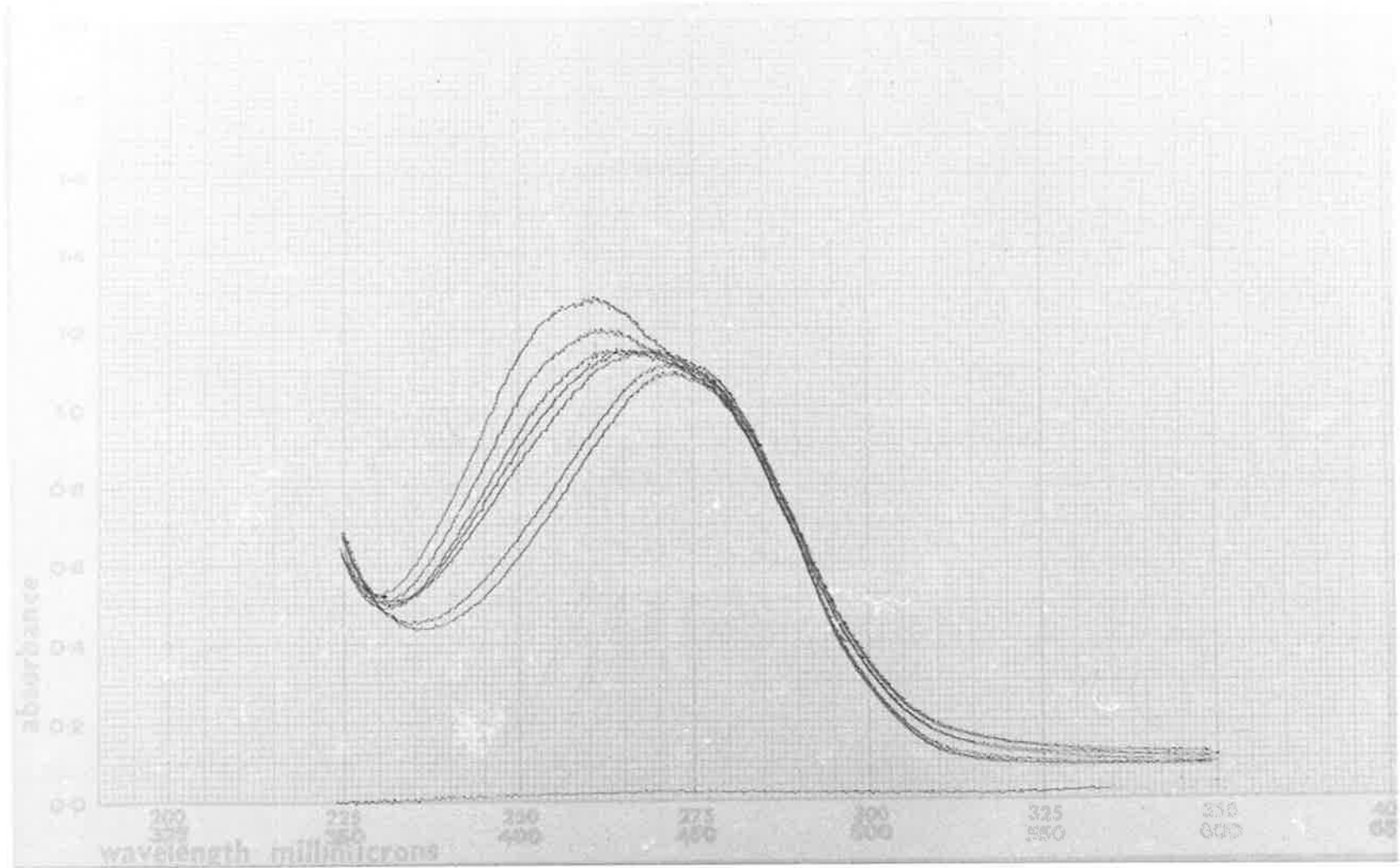
FIGURE 19:   Insertion of nitrene, formed by photolysis
of Pan-L-pN$_3$Phe, into carbon-hydrogen bond.
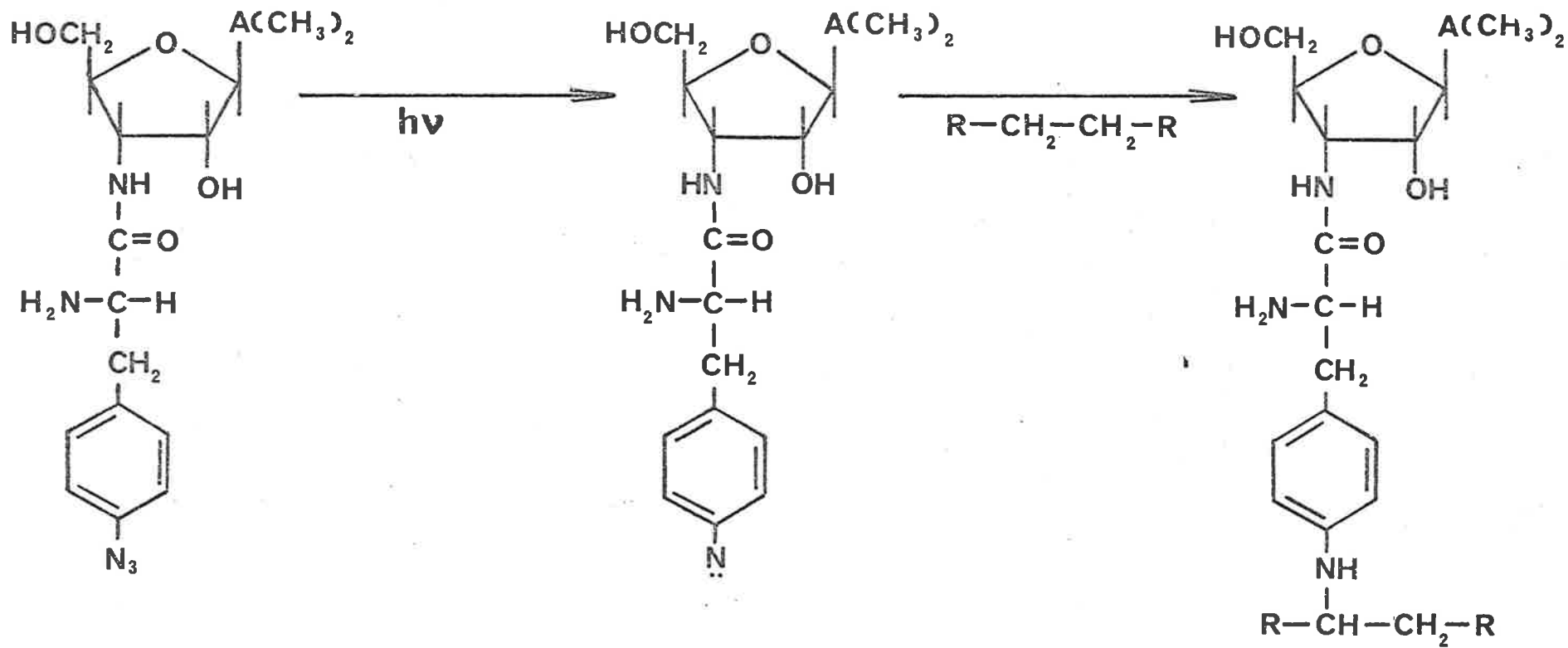
photolysis, with N-α-Ac-Gly-Gly (Figure 19) to produce a secondary amine, which having similar properties to the methoxy group of puromycin, would have the same spectral properties. If the ln (% A_{260} remaining) was plotted against time, a $t_{\frac{1}{2}}$ for the azide group of 5 min was obtained. Therefore the photolysis data on Pan-L-pN_3Phe and the infra-red spectrum of N-α-tBoc-L-pN_3Phe were both consistent with the presence of an azide group in the compound (Figure 17).

2.   <u>Ability of Pan-L-pN_3Phe to act as an Acceptor in the Fragment Reaction.</u>

The ability of Pan-L-pN_3Phe to bind to the A' site of peptidyl transferase had to be determined before this compound could be used as an affinity label. As can be seen from Figure 20, Pan-L-pN_3Phe was able to act as an acceptor in the fragment reaction (Chapter 1; section 6) with a Km of 1.5×10^{-4} M, which was similar to that of puromycin (Km = 1.1×10^{-4} M) under the same conditions (Vanin <u>et al</u>., 1974). Therefore, Pan-L-pN_3-Phe) is able to bind to the A' site.

3.   <u>Photoaffinity Labelling of Ribosomes with Pan-L-pN_3Phe.</u>

(a)   <u>Effect of time on affinity labelling.</u>

Ribosomes were photolysed in the presence of 0.9 mM Pan-L-pN_3Phe for different times (0 - 5 min)

FIGURE 20:   Km for Pan-L-pN$_3$Phe, in the fragment reaction, was determined by incubating varying concentrations of Pan-L-pN$_3$Phe (0.09 - 0.9 x 10^{-3} M) for 5 min at 0° (see Chapter 1; section 6) and the amount of N-Ac-L-[^{3}H]Leu-(Pan-L-pN$_3$Phe) determined as in Chapter 1; section 6.
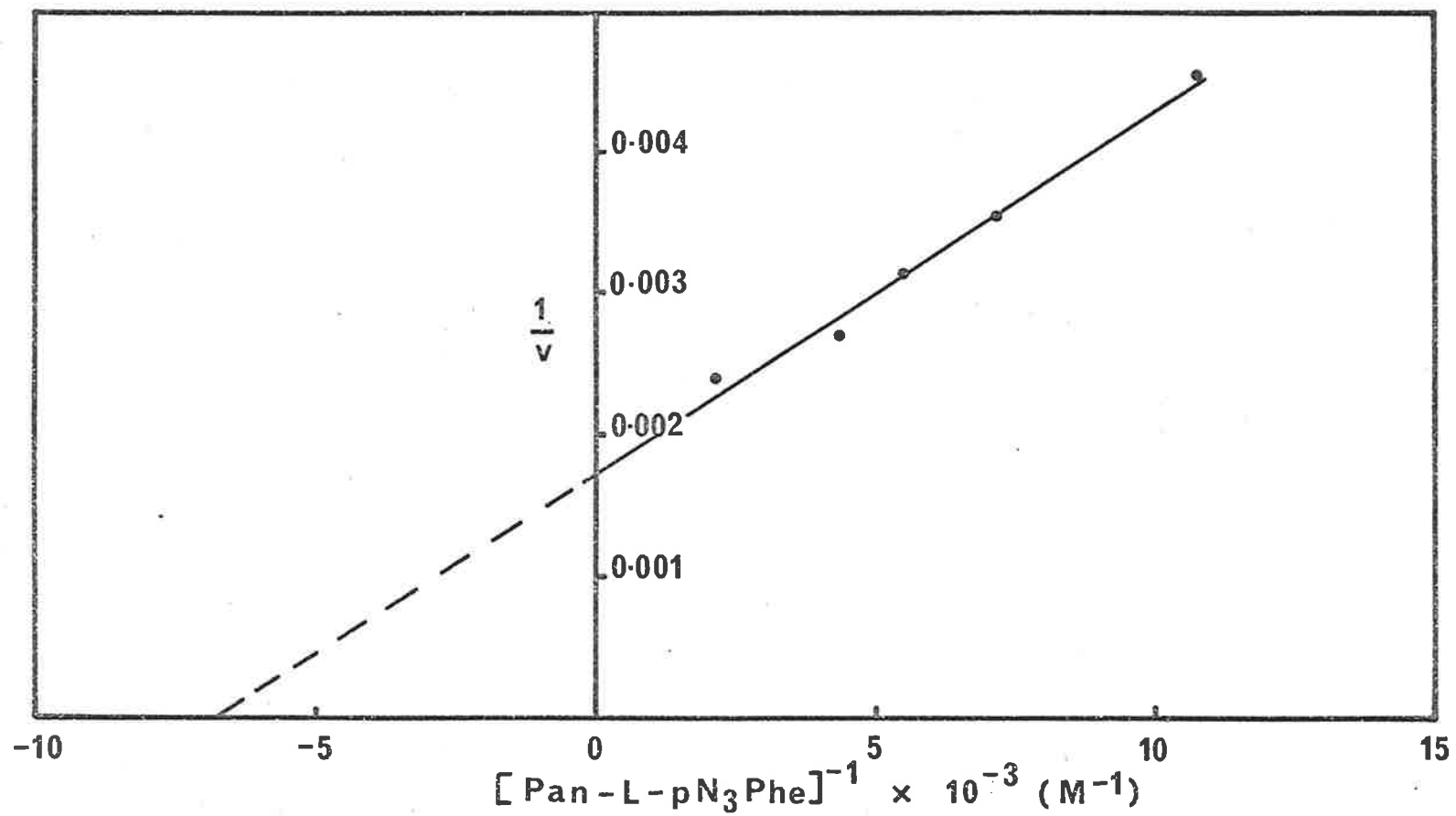
after which the ribosomes were washed.   The
washed ribosomes were then assayed for peptidyl
transferase activity and the stoichiometry of
labelling determined.   The stoichiometry of
labelling increased linearly for 2 - 3 min after
which it began to plateau at 17 affinity labels
per ribosome (Figure 21); however, peptidyl trans-
ferase was unaffected.   The lack of inactivation
of peptidyl transferase can be attributed to the
high Km of the affinity label, because upon photo-
lysis the activated photoaffinity label was able
to easily dissociate from the A' site and thereby
react with other regions of the ribosome.   In
fact, very few, if any, affinity labels were
covalently attached to the A' site.

SUMMARY

The compound, Pan-L-pN$_3$Phe, which was completely
characterized, was shown to act as an acceptor in the
fragment reaction; that is, it was able to bind to the
A' site.   Upon photolysis of the ribosomes, in the
presence of Pan-L-pN$_3$Phe, there was extensive labelling
(17 affinity labels/ribosome) but no concomittant in-
activation of peptidyl transferase.   Therefore, the
affinity label was not covalently attached to the A'
site.   One possible explanation is that because of the
low affinity of the compound for peptidyl transferase,

FIGURE 21: The effect of time of photolysis on the stoichiometry of labelling and inactivation of peptidyl transferase, using $[^3H]Pan-L-pN_3Phe$. Ribosomes were photolysed for different times (0 - 5 min) in the presence of $[^3H]Pan-L-pN_3Phe$ (0.9 mM), washed and the stoichiometry determined (Methods, section 7). The same ribosomes were assayed for peptidyl transferase activity as in Chapter 1; section 6.
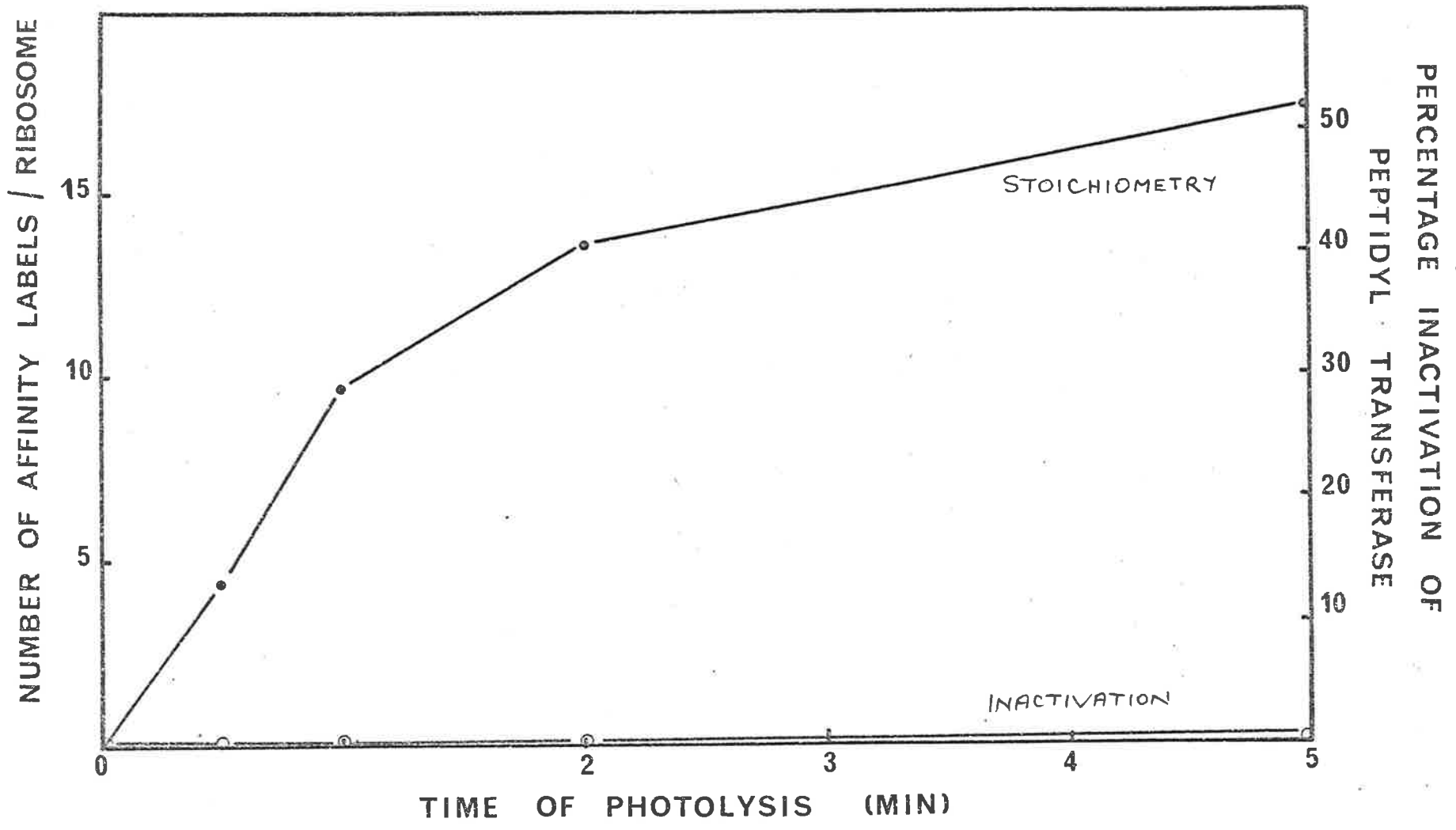
the photolysed compound dissociated from the A' site before the specific affinity labelling could occur.

CHAPTER 5

PREPARATION OF HIGH SPECIFIC ACTIVITY
[^{3}H]CHLORAMPHENICOL BASE AND [^{3}H]

CHLORAMPHENICOL LABELLED IN THE

PROPANEDIOL SIDE CHAIN

PREPARATION OF HIGH SPECIFIC ACTIVITY

[^{3}H]CHLORAMPHENICOL BASE AND [^{3}H]CHLORAMPHENICOL

LABELLED IN THE PROPANEDIOL SIDE CHAIN

INTRODUCTION

The requirement for high specific activity chloramphenicol labelled in the propanediol side chain, for our studies on the mechanism of action of chloramphenicol and its analogues (Harris and Symons, 1973a) and its non-availability from commercial sources led to the development of the synthetic methods described here. The methods allow the preparation of [^{3}H]chloramphenicol and derivatives labelled on carbon one of the propanediol side chain, with a specific activity of greater than 1 Ci/mmole. Since the labelling step involves the reduction of the 1-oxo derivative of chloramphenicol and its analogues by KB[^{3}H]$_4$, the final specific activity was determined by the specific activity of commercially available KB[^{3}H]$_4$.

The reduction step, using KB[^{3}H]$_4$, takes place at an asymmetric carbon atom and therefore produces two diastereoisomers, the biologically active D(-)threo and the inactive D(-)erythro isomers (Brock, 1961; Hahn et al., 1956) which can subsequently be separated. However, if the presence of the inactive isomer is of no consequence, the synthetic steps are considerably simplified and the preparation of [^{3}H]chloramphenicol and its

analogues is a simple, rapid procedure.

The availability of [^{3}H]chloramphenicol base is important, because it now allows the preparation of label-led analogues of chloramphenicol in which the N-dichloro-acetyl group can be replaced by a variety of substituents. If these substituents are chemically reactive, such as 2-bromoacetyl, or photoactivatable, such as p-azido-benzoyl, then these analogues may be used for affinity labelling studies of the chloramphenicol binding site of E. coli ribosomes.

MATERIALS

Chloramphenicol, chloramphenicol base, DCC, NHS and monomethoxytrityl chloride were all purchased from Sigma Chemical Co., and the Kieselgel 60 F 254 TLC plates from E. Merck, Darmstadt, West Germany. The KB[^{3}H]$_4$ (specific activity 4.9 Ci/mmole) was purchased from The Radiochemical Centre, Amersham, England; 100 mCi was dissolved in 0.2 ml of freshly prepared 10 mM KOH and 0.02 ml (10 mCi) samples were dispensed into vials, the contents of which were then dried with an oil pump, sealed and stored at -80° until required (Symons, 1975). The dimethyl formamide used, was distilled under reduced pressure and stored over molecular sieve 4A.

<center>METHODS</center>

1.   <u>Thin Layer Chromatography.</u>

Silicic acid (Kieselgel 60 F 254) was used as the solid phase for all analytical TLC with the following solvents; solvent A, ethanol : chloroform (1:49, v/v); solvent B, ethanol : chloroform (1:19, v/v); solvent C, ethanol : chloroform (1:9, v/v) and solvent D, ethanol : ethylacetate (1:19, v/v).

2.   <u>Synthesis of [^{3}H]chloramphenicol base Labelled at Carbon One of the Propanediol Side Chain.</u>

(a)   <u>3-O-monomethoxytrityl, N-acetyl chloramphenicol base (III).</u>

To a suspension of chloramphenicol base (I, 1.5 mmol) in 5 ml of ethanol was added 1.0 ml of acetic anhydride, and the clear solution was left at room temperature for 30 min. Analytical TLC in solvent C showed complete acetylation to give N-acetyl chloramphenicol base (II). Ethanol was used as a solvent in the above reaction mixture to ensure selective acetylation of the amino group. Excess acetic anhydride and acetic acid were removed by several evaporations with dry acetonitrile on the rotary evaporator and then on the vacuum line. To the dried residue was added monomethoxytrityl chloride (1.6 mmol) and 4 ml of

dry pyridine and the solution left overnight at room temperature. The reaction mixture was then diluted with 50 ml of chloroform and extracted three times with 5% NaHCO$_3$. The chloroform phase was dried with Na$_2$SO$_4$ (anhydrous) and evaporated to dryness. Residual pyridine was removed by several evaporations with acetonitrile and the residue of 3-O-monomethoxytrityl,N-acetyl chloramphenicol base (III) dissolved in 10 ml of chloroform. Analytical TLC in solvent A showed a single UV absorbing, monomethoxytrityl positive spot with an Rf of 0.5. The monomethoxytrityl group was detected by the immediate bright yellow colour when exposed to trifluoroacetic acid. One half of the preparation was then used for the next step.

(b)   1-oxo,3-monomethoxytrityl,N-acetyl chloram-
       phenicol base (IV).

In a conical flask, protected by a drying tube, a mixture of 0.5 g of CrO$_3$, 12.5 ml of dry dichloromethane and 0.81 ml of dry pyridine was stirred for 15 min at room temperature. To the dark burgundy-coloured mixture was added III (0.7 mmol) in 2.0 ml of dry dichloromethane and the mixture was stirred for a further 15 min. The supernatant was decanted and the residue washed with 25 ml of dry dichloromethane. The pooled

supernatants were extracted, twice with saturated NaCl, three times with 5% $NaHCO_3$, dried with Na_2SO_4 (anhydrous), filtered and then taken to dryness. After evaporation with acetonitrile the crude product (IV) was dissolved in 2.0 ml of chloroform. Analytical TLC using solvent A showed a major UV absorbing, ketone positive (Chapter 1; section 2(c)), monomethoxytrityl positive spot, which ran just ahead of the starting material.

(c)   1-oxo,N-acetyl chloramphenicol base (V).

A mixture of about 0.15 mmoles of IV in 2 ml of 80% acetic acid was heated at 100° for 5 min to remove the monomethoxytrityl group.   After removal of the acetic acid, by coevaporation with ethanol, the residue was dissolved in acetone and purified by preparative TLC (Chapter 1; section 1) in solvent B.   The major band (Rf = 0.25) was scraped off, the compound recovered from the silicic acid by elution with 3 x 10 ml of ethanol : chloroform (1:1, v/v) and the pooled eluates evaporated to dryness.   The residue was then dissolved in 2.0 ml of ethanol and the compound isolated, was shown to be one UV absorbing, ketone positive (Chapter 1; section 2(c)) spot (Rf = 0.3) by analytical TLC using solvent B.

The UV spectrum showed an absorption maximum,

FIGURE 22: Synthetic procedure for the preparation of
the l-oxo derivative of N-acetyl chloramphenicol base
(V).

RCl = monomethoxytrityl chloride

$(CH_3CO)_2O$

RCl

CrO_3
pyridine

H^+

I

II

III

IV

V

NO_2

$HO-C-H$

$H-C-NH_2$

CH_2OH

1
2
3

$HO-C-H$

$H-C-NH-C-CH_3$

O

CH_2OH

$HO-C-H$

$H-C-NH-C-CH_3$

O

CH_2OR

$O=C$

$H-C-NH-C-CH_3$

O

CH_2OR

$O=C$

$H-C-NH-C-CH_3$

O

CH_2OH

$R = -C$ —OCH$_3$
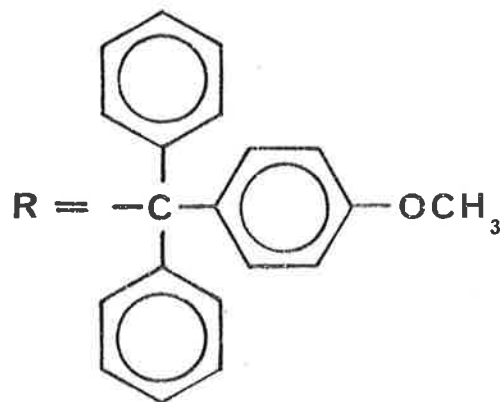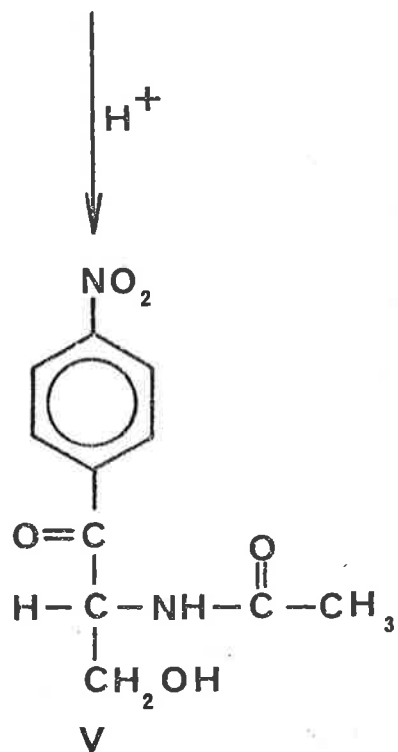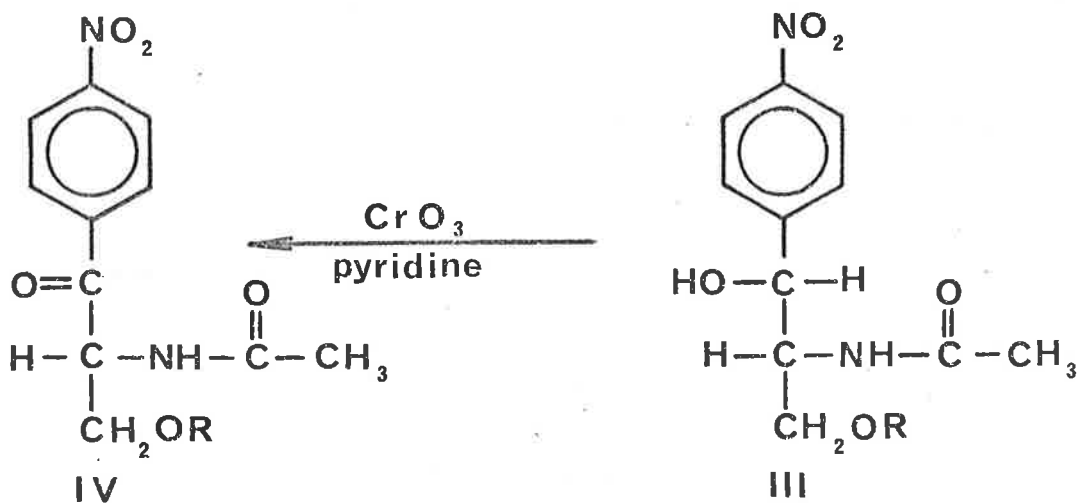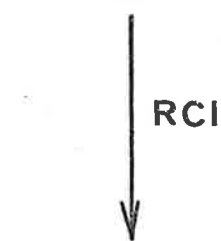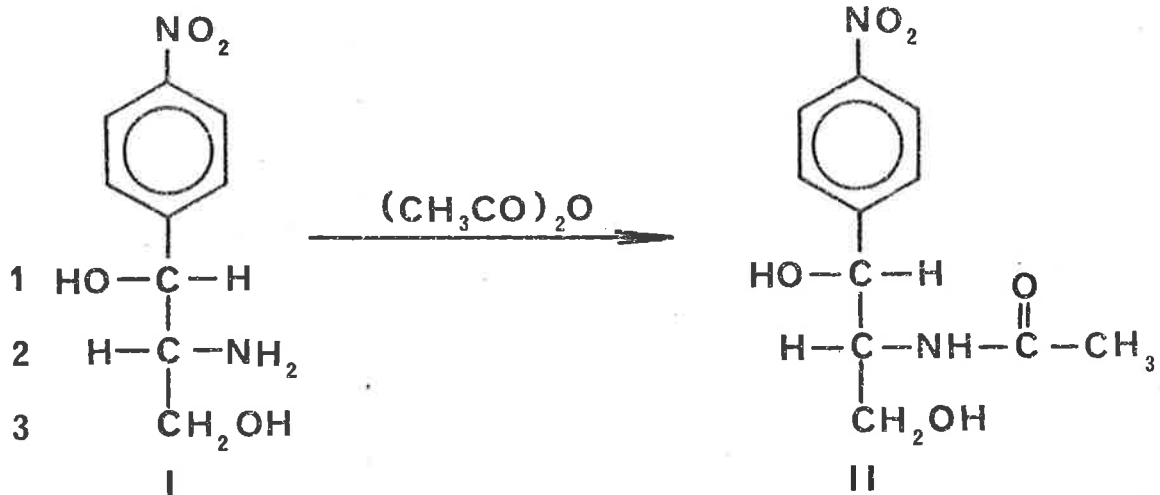
at 264 nm, with an extinction coefficient of 14,000 $M^{-1}cm^{-1}$, as calculated by reduction of the compound with $NaBH_4$ to give N-acetyl chloramphenicol base.

The infra-red spectrum of the compound gave peaks at 1350 cm^{-1} and 1530 cm^{-1} (both indicative of a nitro group), 1650 cm^{-1} (indicative of an amide carbonyl) and 1590 cm^{-1} (indicative of a ketone carbonyl). The NMR spectrum gave peaks at 121 Hz (hydrogens of the acetyl group), 244 Hz (hydrogens of carbon three), 336 Hz (hydrogens of carbon two), 464 Hz (amide hydrogen) and 503 Hz (hydrogens of the aromatic ring).

(d) [^{3}H]chloramphenicol base (VIII).

To a vial containing 2.0 μmoles of KB[^{3}H]$_4$ (10 mCi) and 0.2 μmoles of KOH, 3.14 μmoles of V, in 0.1 ml of ethanol and 0.02 ml of H_2O, was added. After 30 min (reduction of V to N-acetyl chloramphenicol base was shown to be complete in less than 15 min), at room temperature, excess KB[^{3}H]$_4$ was decomposed by the addition of 0.01 ml of 4 M acetic acid, in a well ventillated fume hood. After a further 15 min at room temperature, the acetic acid was removed by coevaporation with ethanol to give a residue containing the diastereoisomers of N-acetyl [^{3}H]chloramphenicol base.

The presence of mixed isomers was shown by synthesizing large amounts of non-radioactive chloramphenicol base (made by deacetylating N-acetyl chloramphenicol base (see below)) and the nuclear magnetic resonance spectrum done in trifluoroacetic anhydride (see Results and Discussion).

The diastereoisomers of N-acetyl [^{3}H]chloramphenicol base, dried by coevaporation with dry acetonitrile on an oil pump, were dissolved in 0.2 ml of pyridine and 0.07 ml of acetic anhydride added. After overnight incubation, at room temperature, volatile solvents were removed by several coevaporations with ethanol (one drop of triethylamine was added at the last coevaporation to ensure removal of all the pyridine). The diastereoisomers of 1,3-di-O-acetyl,N-acetyl [^{3}H]chloramphenicol base (VII) were then separated by TLC in solvent D on two 7 x 10 cm sheets of Kieselgel 60 F 254 (1,3-di-O-acetyl,N-acetyl chloramphenicol base, prepared by acetylation of N-acetyl chloramphenicol base, was applied at each side of the sheet as marker). The two diastereoisomers were clearly separated into two bands, the slower running band (Rf = 0.5) migrating with the authentic marker. Of the total radioactivity in the two bands, 48% was in the slower running, biologically active isomer.

The slower bands were cut out, the silicic acid scraped off and the product eluted with 3 x 3 ml of ethanol : chloroform (1:1, v/v). The eluates were then filtered, taken to dryness and the 1,3-di-O-acetyl groups removed, by treatment of the residue, with 75% concentrated ammonia in ethanol at 37° for 1.5 h. After removal of the ammonia by coevaporation with ethanol, the N-acetyl group was removed by hydrolysis with 1.0 N HCl at 100° for 3 h (Rebstock et al., 1949). The HCl was then removed by coevaporation with water to give [^{3}H]chloramphenicol base (VIII).

3. Synthesis of [^{3}H]chloramphenicol Labelled at Carbon One of the Propanediol Side Chain.

(a) [^{3}H]chloramphenicol (correct isomer, X).

The [^{3}H]chloramphenicol base, synthesized as above, was dried and the residue dissolved in 0.5 ml of dry dimethyl formamide, to which 40 μmoles of the NHS ester of 2,2-dichloroacetic acid (IX) in 0.1 ml of acetonitrile was added and the reaction mixture incubated at 37° for 1 h. The latter compound (IX) was prepared by adding 1.1 equivalents of DCC to 1.1 equivalents of NHS and 1.0 equivalent of 2,2-di-chloroacetic acid in dry acetonitrile. After 30 min at room temperature, the bulky precipitate (DCU) was pelleted by low speed centrifugation and

FIGURE 23: Synthetic procedure for the preparation of the D(-)threo isomer of [^{3}H]chloramphenicol base (VIII). The asterisk represents the tritium atom and the parentheses around HO-C-H* the mixture of D(-)threo and D(-)erythro isomers.

NO$_2$

O=C
H—C—NH—C—CH$_3$
CH$_2$OH
V

KB[^{3}H]$_4$

NO$_2$

(HO—C—H)*
H—C—NH—C—CH$_3$
CH$_2$OH
VI

(CH$_3$CO)$_2$O

NO$_2$

(CH$_3$C—O—C—H)*
H—C—NH—C—CH$_3$
CH$_2$—O—C—CH$_3$
VII

Separation of isomers

(1) OH$^-$
(2) H$^+$

NO$_2$

HO—C—H*
H—C—NH$_2$
CH$_2$OH
VIII
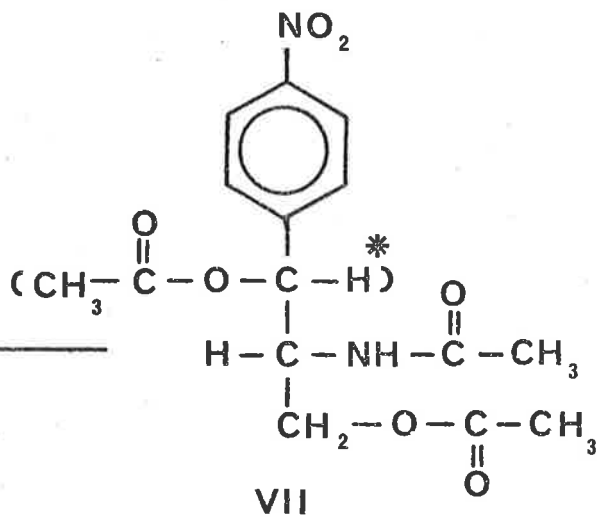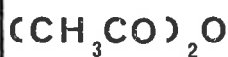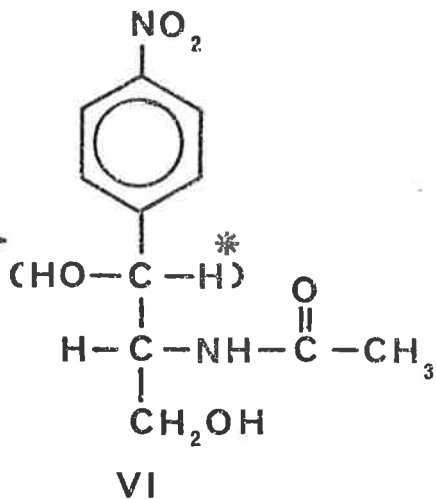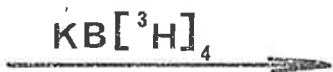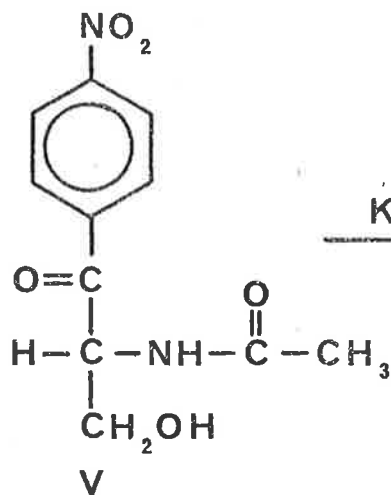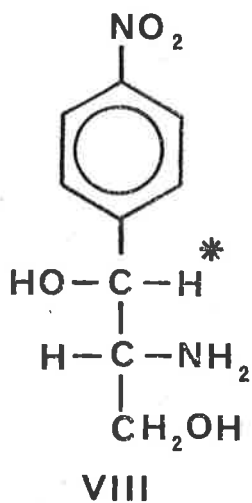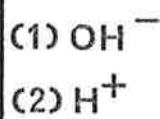
FIGURE 24: Electrophoretic and chromatographic analysis

of mixed isomers of [^{3}H]chloramphenicol base.

├────·──┤ represents where authentic chloramphenicol

base migrated.

(A) Electrophoresis, using Whatman 3 MM paper, was at

pH 3.5, for 20 min using a potential difference of 55 V/cm.

The electrophoretogram was then cut into 1 cm strips and

the radioactivity in the strip estimated (Chapter 1;

section 3(a)).

(B) Chromatography (descending) used Whatman 3 MM

paper and butan-1-ol : acetic acid : water (4:1:1, v/v/v)

as the solvent system. Once developed, the chromato-

gram wad dried, cut into 1 cm strips and the radio-

activity in each strip estimated (as above).

(C) Chromatography was as above but propan-2-ol :

conc. NH$_4$OH : water (8:1:1, v/v/v) was used as the solvent

system and the radioactivity in 1 cm strips estimated.

(D) Thin layer chromatography (Methods, section 1) of

[^{3}H]chloramphenicol base used methanol : ethylacetate :

acetic acid (50:50:3, v/v/v) as the solvent. Once

dried the chromatograph was cut into 0.5 cm slices and

the radioactivity estimated (as above).
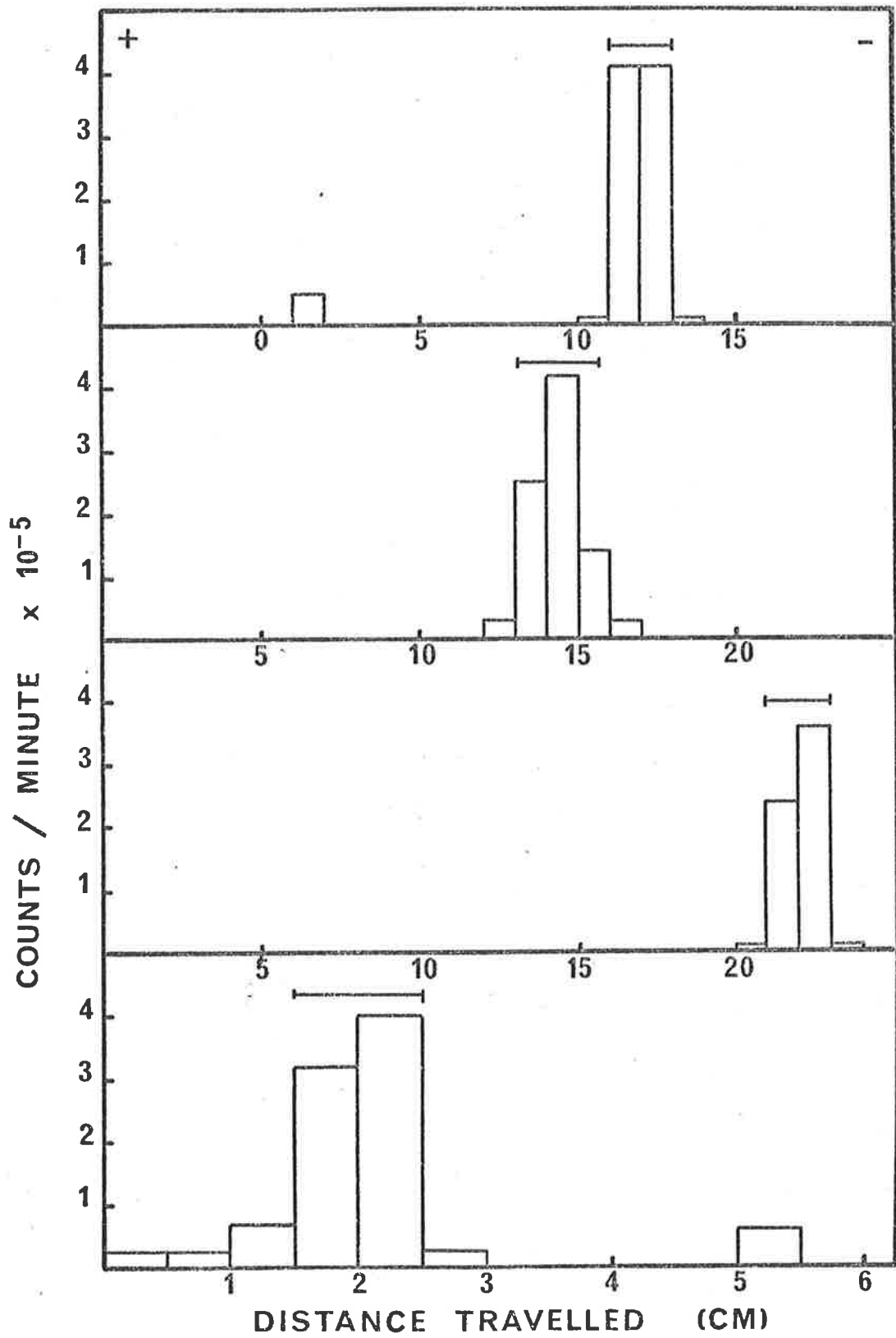
the supernatant used directly. The [^{3}H]chloram-
phenicol formed, was then purified by TLC on a
7 x 10 cm Kieselgel 60 F 254 sheet, using solvent
C. The appropriate band was cut out, the silicic
acid eluted with 3 x 3 ml of ethanol and the
pooled eluates evaporated to dryness. The residue
was redissolved in 1 ml of ethanol and stored at
-15° until required. Based on the extinction
coefficient, at 274 nm, of 9,600 $M^{-1}cm^{-1}$ (Rebstock
et al., 1949), 0.17 µmoles of [^{3}H]chloramphenicol
was obtained, with a specific activity of 2.0 Ci/
mmole.

(b)   [^{3}H]chloramphenicol (mixed isomers, XII).

The l-oxo derivative of chloramphenicol (XI),
was prepared as already described for the l-oxo
derivative of N-acetyl chloramphenicol base (V;
Figure 22), the structure of which was also con-
firmed by infra-red and nuclear magnetic resonance
spectroscopy. A solution of 3.14 µmoles of XI
in 0.1 ml of ethanol and 0.02 ml of H_2O was added
to a vial containing 2.0 µmoles of KB[^{3}H]$_4$ (10 m-
Ci) and 0.2 µmoles of KOH. After 30 min at room
temperature, 0.01 ml of 4 M acetic acid was added
in order to decompose excess KB[^{3}H]$_4$. After a
further 15 min, the solution was dried on to a
9 x 12 cm sheet of Kieselgel 60 F 254, which was
then developed in solvent D. After drying the

FIGURE 25: Conversion of [^{3}H]chloramphenicol base to [^{3}H]chloramphenicol.

NO_2

$HO-\overset{*}{C}-H$

$H-C-NH_2$

CH_2OH

VIII

$+$

$Cl_2CH-\overset{O}{\overset{\|}{C}}-O-N$

IX

NO_2

$HO-\overset{*}{C}-H$

$H-C-NH-\overset{O}{\overset{\|}{C}}-CHCl_2$

CH_2OH

X
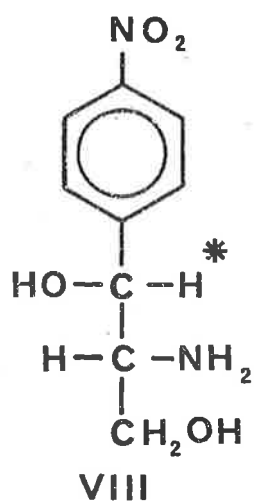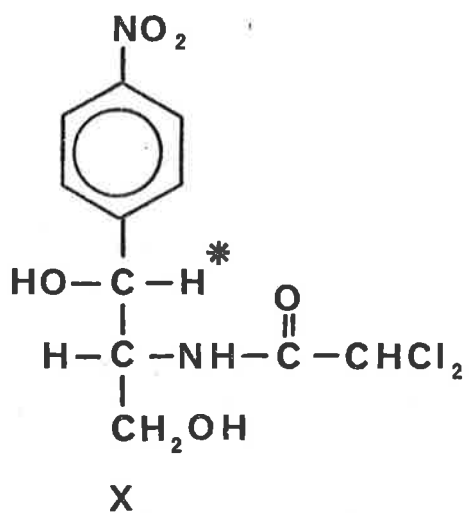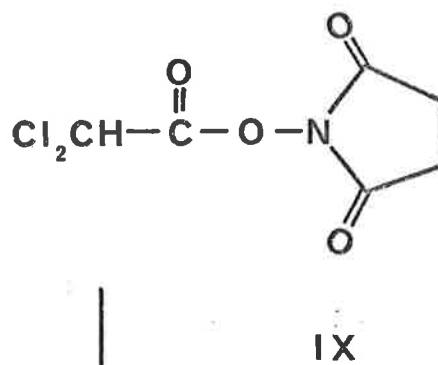
sheet, the strong UV absorbing band, corresponding
to chloramphenicol, was cut out, the silicic acid
scraped off and the product eluted with 3 x 3 ml of
ethanol : chloroform (1:1, v/v).   The pooled eluates
were then evaporated to dryness and the residue
redissolved in 0.5 ml of ethanol to give 1.9 μmoles
of [^{3}H]chloramphenicol (mixed isomers, XII), with
a specific activity of 2.0 Ci/mmole.

The nuclear magnetic resonance spectrum of
non-radioactive material, prepared in this way,
indicated that 42% of the total chloramphenicol was
in the active D(-)threo form and the remainder in
the inactive D(-)erythro form.

RESULTS AND DISCUSSION

1. Preparation of [^{3}H]chloramphenicol base, [^{3}H]-
    chloramphenicol and Analogues Labelled at Carbon
    One of the Propanediol Side Chain.

    (a)   Use of chloramphenicol base as the starting
          material.

    The synthetic route, used for the preparation
of [^{3}H]chloramphenicol base labelled on carbon one
of the propanediol side chain, is given in Figures
22 and 23.   Acetylation of chloramphenicol base (I)
with acetic anhydride, in ethanol, gave rapid and

complete conversion to the N-acetyl chloramphenicol base (II). Selective protection of the primary hydroxyl of carbon three was achieved by reaction of II with a slight excess of monomethoxytrityl chloride in anhydrous pyridine to give III, which was used without further purification, after aqueous extraction of pyridinium chloride and excess pyridine. For the oxidation of the hydroxyl at carbon one, use of the chromium trioxide-pyridine complex, as described by Ratcliffe and Rodehorst (1970), gave complete oxidation of III to IV. After removal of the protecting monomethoxytrityl group, by brief acid treatment, the 1-oxo derivative of N-acetyl chloramphenicol base (V) was purified by preparative TLC. This compound was stable for at least twelve months, when stored in ethanol at -15°.

Introduction of the tritium label, was achieved by the reaction of 1 equivalent of $KB[^3H]_4$ with 1.5 equivalents of the 1-oxo compound (V) (Figure 23); although 1 equivalent of KBH_4 should be able to reduce 4 equivalents of V (Brown et al., 1956). The $[^3H]$chloramphenicol base, prepared by deacetylation of VI, was subjected to chromatographic and electrophoretic analysis (Figure 24). As can be seen, under the four different systems, radioactivity comigrated with commercial chloramphenicol base.

Therefore, under all criteria used, the compound made in this way was, in fact, [^{3}H]chloramphenicol base. Although this analysis has enabled us to identify the compound isolated, it has not been able to detect the presence of diastereoisomers, which would be expected to form on borohydride reduction of V. Therefore, larger amounts of chloramphenicol base were made, as above, and the nuclear magnetic resonance spectrum, in trifluoro-acetic anhydride, recorded. The nuclear magnetic resonance spectrum of commercial chloramphenicol base, in trifluoroacetic anhydride, gave a doublet, for the hydrogen of carbon one (as was expected). The spectrum of the synthetic material produced a quartet in the same position, which was a combin-ation of the doublets produced by each diastereo-isomer; the rest of the spectrum was identical to that of commercial chloramphenicol base. There-fore, the nuclear magnetic resonance data is in agreement with the chromatographic and electro-phoretic data, in that the compound synthesized was chloramphenicol base, but it also showed that the reduction step, using borohydride, produced approximately equal amounts of the D(-)threo and the D(-)erythro isomers. Since the D(-)threo isomer is the active, naturally occuring isomer, whereas the D(-)erythro isomer is inactive as an

inhibitor of protein synthesis (Brock, 1961; Hahn et al., 1956), it was considered necessary to separate the two isomers. (Under conditions where the presence of the inactive isomer of chloramphenicol base or its analogues has no effect on the reactivity of the active isomer, the steps involving the separation of the isomers can be deleted). Since the isomers of VI could not be separated by TLC, VI was acetylated, without prior purification of the reaction mixture from the previous step, to produce VII, which could then be resolved into two well-separated bands on TLC. The slower running of the two bands was shown to be the correct isomer by cochromatography of triacetylated chloramphenicol base. After isolation of the correct isomer (VII), the two O-acetyl groups were removed by alkali treatment and the N-acetyl by acid treatment. The [³H]chloramphenicol base, so formed, was finally purified by TLC.

Overall, the preparative routes of Figures 22 and 23 are relatively simple and straightforward. One preparation of the 1-oxo compound (V) suffices for innumerable labelling steps (Figure 23), each of which requires several hours work over a period of two days.

When the [³H]chloramphenicol base was required

for the synthesis of [^{3}H]chloramphenicol or its
analogues, the synthetic step was carried out
prior to purification of [^{3}H]chloramphenicol base
by TLC. The procedure, used for the synthesis
of [^{3}H]chloramphenicol, is outlined in Figure 25
and involves the reaction of [^{3}H]chloramphenicol
base with the NHS ester of 2,2-dichloroacetic acid
in dimethyl formamide-acetonitrile, as the solvent.
The [^{3}H]chloramphenicol (X) was then purified by
TLC. In a standard preparation, as described in
Methods (section 3(a)), 0.17 µmoles of [^{3}H]chlor-
amphenicol was obtained, with a specific activity
of about 2 Ci/mmole. In order to test the bio-
logical activity of chloramphenicol, unlabelled
material, prepared by this route, was tested as
an inhibitor of the fragment reaction (Chapter 1;
section 6). As can be seen from Figure 26, the
synthetic chloramphenicol had a Ki of 1.2×10^{-6} M,
whereas the commercial chloramphenicol had a Ki of
1.3×10^{-6} M. Therefore, the chloramphenicol,
synthesized in this way, was found to have identical
biological activity, when compared to the commer-
cial chloramphenicol.

(b)   Use of chloramphenicol as the starting material.

The synthetic route, described above, started
with chloramphenicol base and is the method of

FIGURE 26: The Ki for commercial chloramphenicol (A) and synthetic chloramphenicol (B) was determined by measuring the inhibition of the fragment reaction (Chapter 1; section 6) by varying concentrations of chloramphenicol (0 - 1.1 x 10^{-5} M) at two puromycin concentrations (0.05 and 0.5 x 10^{-3} M).
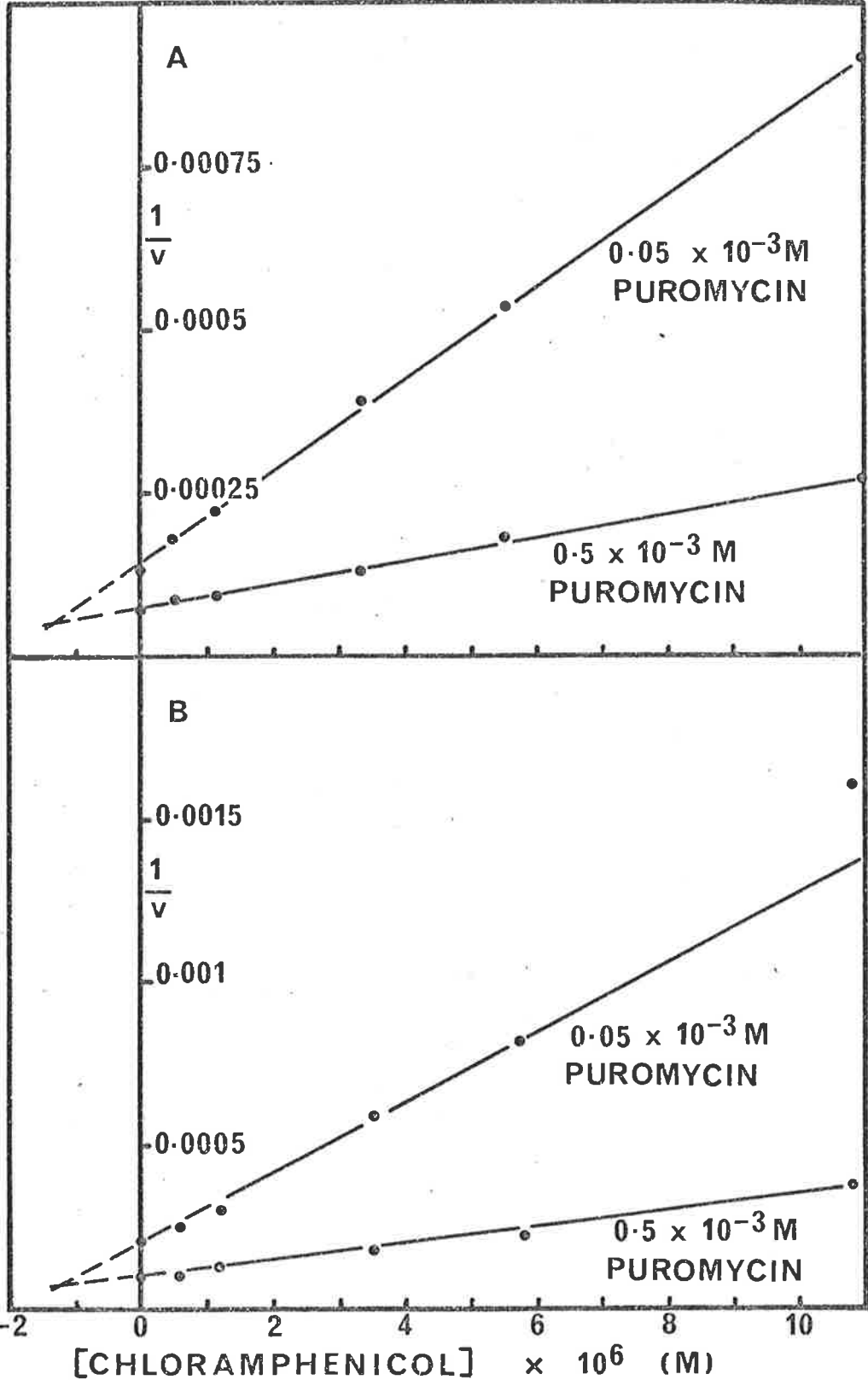
choice for the preparation of the correct isomer of [^{3}H]chloramphenicol and its analogues. However, chloramphenicol can be used instead of N-acetyl chloramphenicol base (II) for the reactions of Figures 22 and 23, but is less satisfactory since the two diastereoisomers formed, in the reduction step, do not separate readily, in contrast to the N-acetyl chloramphenicol base diastereoisomers.

On the other hand, if [^{3}H]chloramphenicol is required and the presence of the inactive D(-)erythro isomer is not important, a rapid, single procedure is to reduce the 1-oxo derivative of chloramphenicol (XI, Figure 27) with KB[^{3}H]$_4$ and purify the [^{3}H]chloramphenicol by TLC. In this way, 3.8 mCi of [^{3}H]chloramphenicol (mixed isomers, XII) was prepared with a specific activity of about 2 Ci/mmole. The proportion of D(-)threo isomer was 42%, as determined by nuclear magnetic resonance spectroscopy on larger amounts of non-radioactive chloramphenicol, prepared in the same way.

(c) Procedure for the preparation of chloramphenicol analogues from chloramphenicol base.

In the methods described, chloramphenicol base was converted to chloramphenicol by reaction with the NHS ester of 2,2-dichloroacetic acid in anhydrous dimethylformamide-acetonitrile as the

FIGURE 27: Reduction of the 1-oxo derivative of chlor-amphenicol, using $KB[^3H]_4$, to give a mixture of the D(-)threo and D(-)erythro isomers of chloramphenicol. The asterisk represents the tritium atom and the parenthesis around HO-C-H* the mixture of D(-)threo and D(-)erythro isomers.

NO$_2$

O=C

H—C—NH—C—CHCl$_2$

$\overset{\text{O}}{\|}$

CH$_2$OH

XI

KB[^{3}H]$_4$

NO$_2$

(HO—C—H) *

$\overset{\text{O}}{\|}$

H—C—NH—C—CHCl$_2$

CH$_2$OH

XII
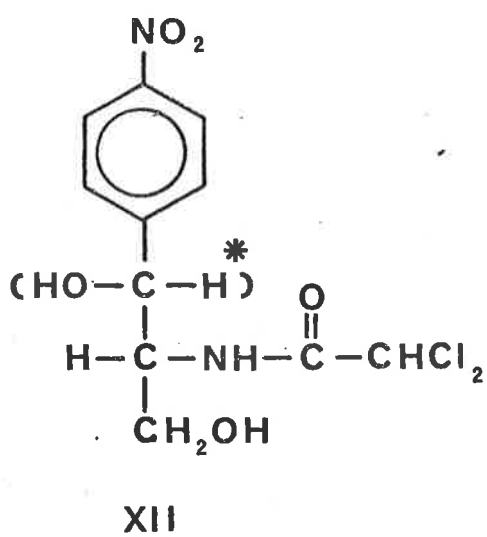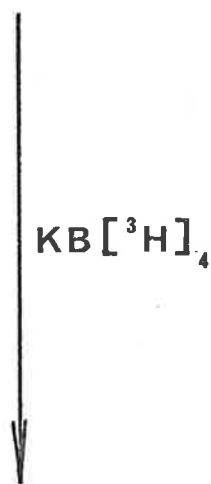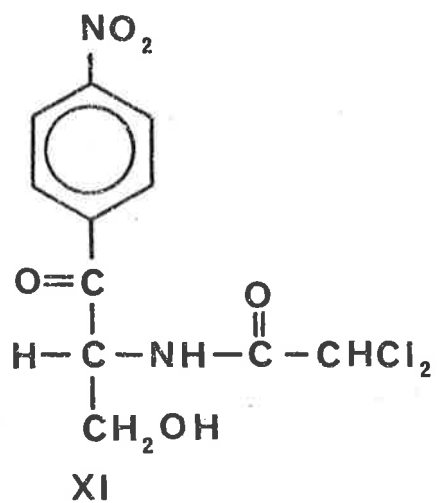
solvent (Figure 25).   The use of NHS esters of
other organic acids represents a general method
for the preparation of a variety of chloramphenicol
analogues, both on a small and large scale.   Where
the NHS esters are reasonably stable, in the
presence of water, ethanol or aqueous pyridine
(Harris et al., 1972) can be substituted for the
anhydrous medium.   A simpler approach is to use
acid anhydrides in ethanol or methanol (both pre-
vent O-acetylation) as solvents, if these anhydrides
are commercially available (acetic anhydride) or
can be readily prepared, such as bromoacetic
anhydride (Greenwell et al., 1974).

A third approach, which has proven to be very
convenient for the preparation of chloramphenicol
analogues on a large scale, is the reaction of 1
equivalent of chloramphenicol base with 2 equiva-
lents of the appropriate carboxylic acid and of
EEDQ in ethanol as the solvent (unpublished data;
see also Greenwell et al., 1974; Harris et al.,
1972).   However, on a small scale, as required
here for the preparation of [^{3}H]chloramphenicol,
the first two approaches are preferred.

2.   Attempted Preparation of [^{3}H]chloramphenicol base
Labelled at Carbon Three of the Propanediol Side Chain.

As was shown above, a practical problem in the

reduction of the 1-oxo derivatives (V and XI) by $KB[^3H]_4$, was that roughly equal amounts of the two diastereoisomers were produced. This problem would be overcome if the reduction step could be carried out on a suitable chloramphenicol derivative, with an aldehyde at carbon three; no isomers would be produced, on reduction of the aldehyde, since carbon three is not an asymmetric carbon atom. However, attempts in this direction proved unsuccessful.

The approach taken, is outlined in Figure 28. The 1-O-acetyl chloramphenicol (XV) was prepared by acetylation, in pyridine, of the 3-O-monomethoxytrityl derivative of chloramphenicol (XIV), followed by acid hydrolysis of the monomethoxytrityl group. Compound XV was not stable on storage for long periods, in ethanol at -15°, as there was appreciable acetyl migration from the hydroxyl of carbon one to that of carbon three. Oxidation of XV by the dimethylsulphoxide-DCC-phosphonic acid procedure (Pfitzner and Moffatt, 1963) gave a number of products, the major one being XVI. Ultravoilet, infra-red and nuclear magnetic resonance spectroscopy and mass spectrometry of all the compounds (XIII to XVI) were done and found to be consistent with the reaction sequence, shown in Figure 28. When the ultravoilet spectrum of XVI was compared to XV we found a shift in the absorption maximum from 274 nm for XV to 311 nm for XVI, which was consistent with an increase in conjugation, as can easily be seen in XVI, when compared to XV. The infra-red spectrums

FIGURE 28: Attempted synthetic procedure for the pre-
paration of carbon three aldehyde derivative of chlor-
amphenicol.

XIII

(1) RCl

(2) (CH₃CO)₂O

XIV

H⁺

XV

DCC, Pi

DMSO
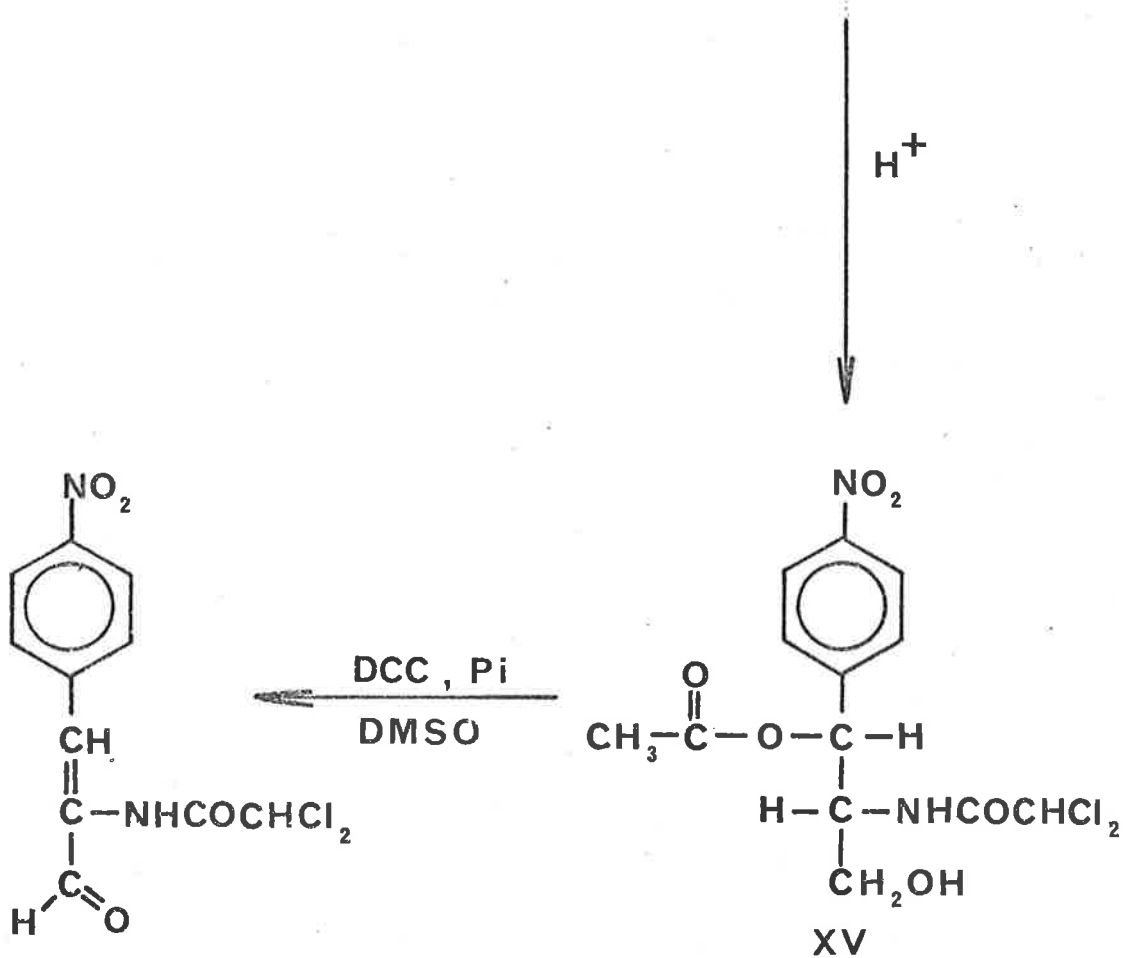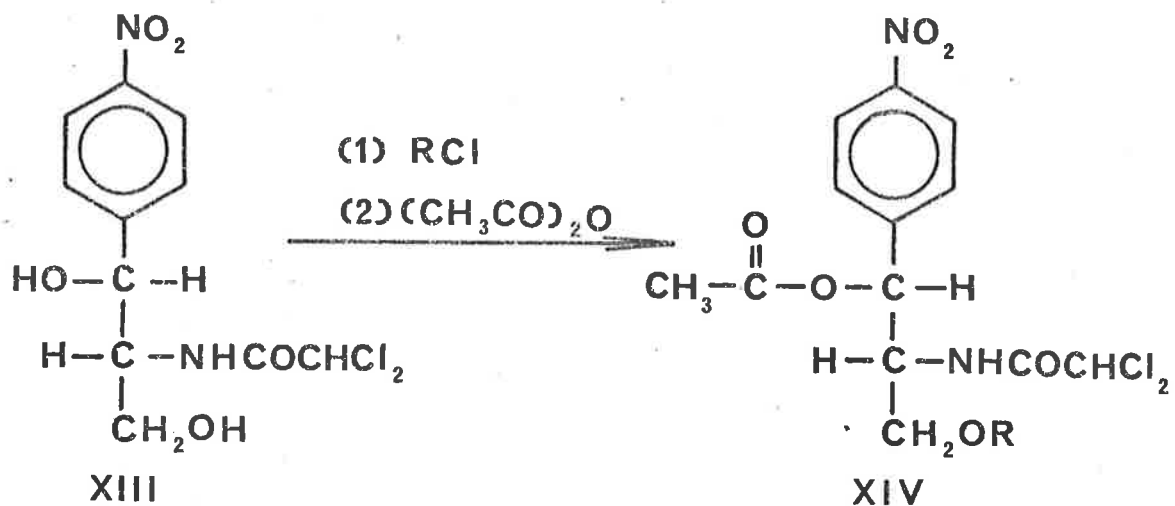
XVI

R as in Figure 22

of XV gave peaks at 1680 cm^{-1} (indicative of an amide carbonyl) and 1740 cm^{-1} (indicative of an ester carbonyl), but upon oxidation the peak at 1680 cm^{-1} remained, but the peak at 1740 cm^{-1} disappeared. Instead two new peaks appeared, one at 1710 cm^{-1} (indicative of an alde-hyde carbonyl) and one at 1635 cm^{-1} (indicative of a carbon-carbon double bond), both of which were consistent with the structure of XVI. The nuclear magnetic reson-ance spectral data was also consistent with the proposed reaction product (XVI), one major change being the loss of a peak at 134 Hz (indicative of the loss of the ace-tyl group). The molecular ion data, obtained from mass spectroscopy, was also consistent with the struc-ture of XVI. Therefore, by using four different spectro-scopic criteria, the reaction sequence was determined.

Other oxidation methods, using chromium trioxide-pyridine (Ratcliffe and Rodehorst, 1970) or chromyl chloride (Sharpless and Akashi, 1975) gave the same major product, as the above oxidation method. Hence, this approach will not be successful until a suitable protecting group, for the carbon one hydroxyl is found, which is stable to the oxidation method used.

SUMMARY

Methods are described, in this chapter, for the synthesis of [^{3}H]chloramphenicol and [^{3}H]chloramphenicol

base, labelled on carbon one of the propanediol side chain. The use of [^{3}H]chloramphenicol base, prepared as above, in the preparation of [^{3}H]chloramphenicol analogues is also described. Attempts were made to synthesize [^{3}H]chloramphenicol, labelled on carbon three of the propanediol side chain, but these proved unsuccessful.

CHAPTER 6

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pN$_3$BENZOYL CHLORAMPHENICOL BASE AS

A PHOTOAFFINITY LABEL FOR THE

CHLORAMPHENICOL BINDING SITE OF

THE E. COLI RIBOSOME

pN_3BENZOYL CHLORAMPHENICOL BASE AS A PHOTOAFFINITY LABEL FOR THE CHLORAMPHENICOL BINDING SITE OF THE E. COLI RIBOSOME

INTRODUCTION

Chloramphenicol, an inhibitor of prokaryote protein synthesis (Pestka, 1971; Vazquez, 1974) has been found to reversibly bind with high affinity (Kd = 2.2 x 10^{-6} M (Fernandez-Munoz and Vazquez, 1973)) to a single site on the ribosome, both in vivo (Das et al., 1966; Hurwitz and Braun, 1967) and in vitro (Wolfe and Hahn, 1965; Fernandez-Munoz et al., 1971a). The binding site is located specifically on the large subunit of the E. coli ribosome (Vazquez, 1964; Vogel et al., 1971) and appears to be at, or in the vicinity of peptidyl transferase. Evidence for the latter proposal includes studies on protein depleted ribosomal cores, which have shown that the large subunit protein L16 is essential for both peptidyl transferase activity (Moore et al., 1975) and chloramphenicol binding (Nierhaus and Nierhaus, 1973). Additionally, affinity labelling studies have demonstrated that L16 is at or near the A' site of peptidyl transferase (Eilat et al., 1974) and also at or near the chloramphenicol binding site (Bald et al., 1972; Pongs et al., 1973; Pongs and Messer, 1976). Clearly, identification of the ribosomal constituents and determination of the structure of the chloramphenicol binding site will aid in under-

standing the molecular mechanism of action of chloram-
phenicol.

To date, two affinity labelling chloramphenicol analogues
have been applied successfully to the ribosome.   The
first, iodamphenicol (Bald et al., 1972; Pongs et al.,
1973; Pongs and Messer, 1976), was found to be covalently
attached to the ribosomal protein L16; the second, brom-
amphenicol (Sonenberg et al., 1973) was covalently
attached to the ribosomal proteins, L2 and L27.   As
both the reactive groups of these two analogues are in
the same position on the analogue, these results seem
in conflict.   Therefore, in order to independently
determine which proteins are part of the chloramphenicol
binding site the photoaffinity label, p-azidobenzoyl
chloramphenicol base (pN$_3$benzoyl chloramphenicol base;
Figure 31) was designed, using the data of Contreas
et al., (1974) and Hahn et al., (1956) on the structural
requirements for inhibition of peptidyl transferase.

In the initial planning of the possible effective-
ness of the photoaffinity label, it was hoped that the
one hundred-fold greater affinity of chloramphenicol
compared to puromycin for E. coli salt-washed ribosomes
(see Chapter 2 and Chapter 5) would eliminate "pseudo"
photoaffinity labelling (Ruoho et al., 1973; see also
Chapter 4).

As will be shown in this chapter, the photoaffinity

label was able to reversibly bind to the chloramphenicol

binding site and so inhibit peptidyl transferase, and

upon photolysis it did become covalently attached to

the ribosome, but, it seems, not at the chloramphenicol

site.

MATERIALS

The p-aminobenzoic acid, chloramphenicol, chlor-

amphenicol base, and DCC were all purchased from Sigma

Chemical Co. and the EEDQ from Aldrich Chemical Co.

The Whatman 3 MM paper was supplied by W. and R. Balston,

England, the cellulose nitrate filters (0.45 μ pore

size (SM11306)) by Sartorius, Gottingen, West Germany

and the Kieselgel 60 F 254 TLC plates by E. Merck,

Darmstadt, West Germany.

METHODS

1.   Thin Layer Chromatography.

All analytical TLC used silicic acid (Kieselgel

60 F 254) as the solid phase and the solvent system used

was solvent A, ethanol : chloroform : acetic acid

(10:90:3, v/v/v).

2.   Synthesis of p-azidobenzoic acid (pN$_3$benzoic acid).

Because of the light sensitivity of the azide group,

all reactions involving the formation and use of the

pN_3benzoic acid were carried out in the dark or with reaction vessels coated with aluminium foil.

Formation of the pN_3benzoic acid involved a two step procedure, the first of which was conversion of the amino group of p-aminobenzoic acid (pNH_2benzoic acid) to a diazonium salt as follows. To pNH_2benzoic acid (5 mmol), in 100 ml of 1.5 N HCl at -10°, $NaNO_2$ (5.5 mmol) in 0.75 ml of H_2O was added dropwise, over a period of 15 min, with vigorous stirring. The reaction mixture, which was left for a further 15 min at -10°, was shown to contain a diazonium salt (Chapter 1; section 2(d)) at this stage. The pN_3benzoic acid was then formed by adding NaN_3 (5.5 mmol) in 0.75 ml of H_2O to the above reaction mixture at -10°, over a period of 15 min, with stirring. After leaving the reaction mixture at 4° over-night, it was extracted with 3 x 100 ml of ethylacetate. The pooled ethylacetate extracts were then dried using Na_2SO_4 (anhydrous), the Na_2SO_4 removed by filtration and the filtrate evaporated to dryness. The residue, pN_3-benzoic acid, was then stored at 4°, until required. The overall yield of pN_3benzoic acid was 4.5 mmoles (90% relative to pNH_2benzoic acid).

The compound, isolated in this way, was shown to be one single UV absorbing spot (Rf = 0.5) on analytical TLC using solvent A and had a melting point of 181 - 183° (with decomposition) which is identical to that obtained

by Gallardy et al., (1974). The infra-red spectrum of
pN_3benzoic acid (Figure 30) had a peak at 2140 cm^{-1}, which
is characteristic for an azide group.

3.   Synthesis of pN_3benzoyl chloramphenicol base.

Coupling of the chloramphenicol base with pN_3benzoic
acid was achieved by incubating chloramphenicol base (25
µmol) with EEDQ (50 µmol) and pN_3benzoic acid (50 µmol) in
1 ml of methanol. After 4 h at room temperature the
conversion of chloramphenicol base (Rf = 0) to pN_3benzoyl
chloramphenicol base (Rf = 0.62) was shown to be complete
by analytical TLC, using solvent A. The reaction mix-
ture was then concentrated and the compound purified by
preparative TLC (Chapter 1; section 1). The appro-
priate UV band was scraped off and the compound re-
covered, from the silicic acid, by 3 x 10 ml elutions
with ethanol : chloroform (1:1, v/v). After removal of
the silicic acid by low speed centrifugation, the pooled
eluates were filtered and the filtrate evaporated to
dryness. The residue was then dissolved in 5 ml of
methanol to give a yield of 22.6 µmoles (90% relative to
chloramphenicol base). The extinction coefficient,
used to determine the yield of pN_3benzoyl chlorampheni-
col base, was 28,700 $M^{-1}cm^{-1}$, at 265 nm (see below).

4.   Determination of the Extinction Coefficient of
      pN_3benzoyl chloramphenicol base.

pN_3benzoyl chloramphenicol base (26 A_{265} units)

FIGURE 29: Synthetic route for the preparation of pN$_3$benzoic acid and subsequent coupling to chloramphenicol base to give pN$_3$benzoyl chloramphenicol base.

NH₂ ... (1) NaNO₂ / (2) NaN₃ ... N₃

NH_2

(1) $NaNO_2$

(2) NaN_3

N_3

$COOH$ ... $COOH$

NO_2

$HO-C-H$
$H-C-NH_2$
CH_2OH

$+$

N_3

$COOH$

EEDQ

$NH-CO$ ... N_3

$CH-CH-CH_2$
OH ... OH

NO_2
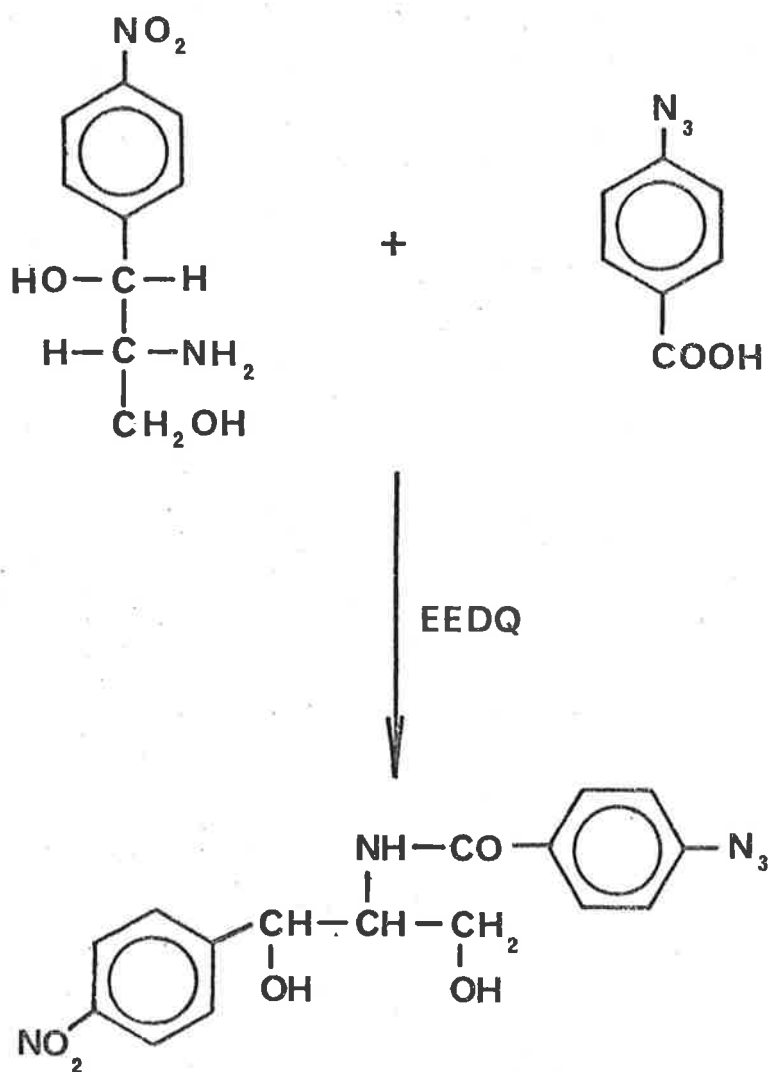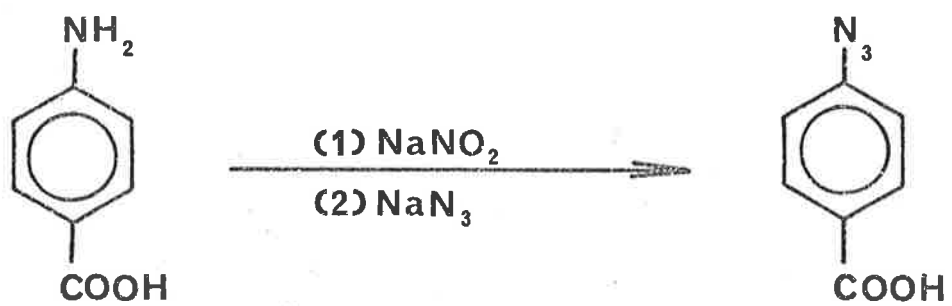
FIGURE 30: The infra-red spectrum of pN_3benzoic acid using a nujol mull.

Wavelength $(cm^{-1}) \times 10^{-2}$
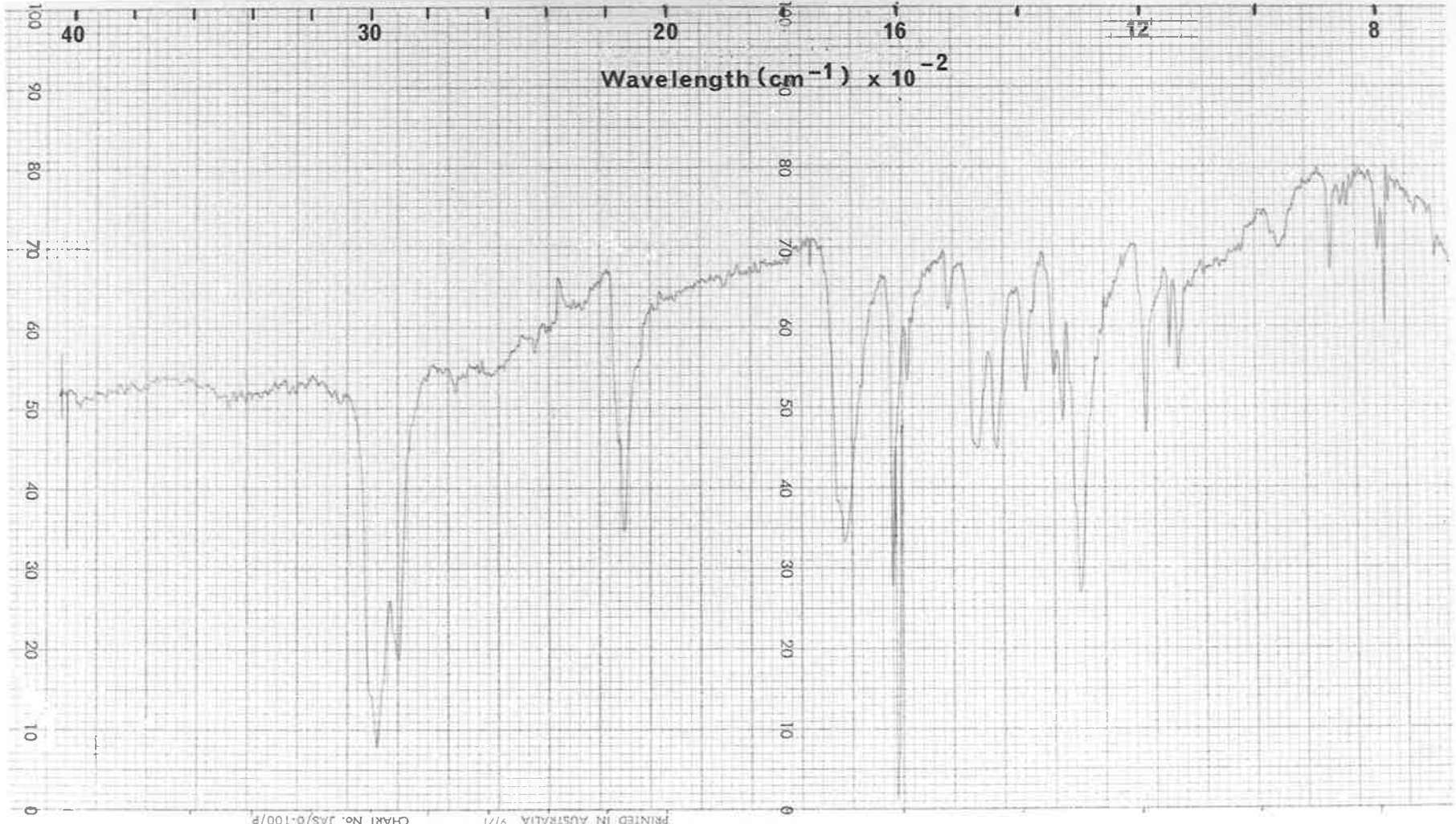
was dissolved in 0.5 ml of 1.0 N HCl, heated at 105° for 8.5 h, evaporated to dryness and the residue redissolved in 0.2 ml of H_2O. An aliquot (0.07 ml) of this solution was then applied to Whatman 3 MM paper and chromatographed (descending) using freshly prepared butan-1-ol : acetic acid : H_2O (4:1:1, v/v/v). The UV spot corresponding to chloramphenicol base (Rf = 0.58) was cut out, the compound eluted from the paper using 0.1 N HCl and the A_{270} values of the eluate determined (following correction for blanks). A known amount of chloramphenicol base was treated as above in order to determine the efficiency of the procedure. The extinction coefficient at 265 nm of 28,700 $M^{-1}cm^{-1}$ was then determined as follows.

$$\frac{A_{265} \text{ units of compound}}{\text{extinction coefficient at 265 nm of the compound}}$$

$$= \frac{A_{274} \text{ units of chloramphenicol base, obtained by hydrolysis of compound}}{\text{extinction coefficient at 274 nm, for chloramphenicol base}}$$

The extinction coefficient at 274 nm, for chloramphenicol base was assumed to be identical to that of chloramphenicol at that wavelength, which is 9,600 $M^{-1}cm^{-1}$ (Rebstock et al., 1949).

5.   <u>Synthesis of pN_3benzoyl [3H]chloramphenicol base.</u>

[3H]chloramphenicol base (1.5 μmol), prepared as in Methods (section 2) of the previous chapter, in

0.05 ml of ethanol, was reacted with 10 μmoles of pN$_3$-benzoic acid anhydride in 0.1 ml of dry acetonitrile. The pN$_3$benzoic acid anhydride was prepared by reacting two equivalents of pN$_3$benzoic acid with an equivalent of DCC for 3 h at room temperature in dry acetonitrile. The DCU crystals formed were then pelleted by low speed centrifugation and the supernatant used as the anhydride solution.

The reaction between [^{3}H]chloramphenicol base and pN$_3$benzoic acid anhydride was allowed to proceed for 5 h at room temperature at which time only 25% of the [^{3}H]-chloramphenicol base was converted to the product, as shown by analytical TLC using solvent A. However, addition of more anhydride, or incubation at a higher temperature, did not increase the conversion. The pN$_3$benzoyl [^{3}H]chloramphenicol base formed was purified by TLC, using a 14 x 10 cm sheet of Kieselgel 60 F 254 and solvent A. The appropriate UV band was scraped off and the compound eluted from the silicic acid with 4 x 2 ml of ethanol : chloroform (1:1, v/v). The pooled eluates were filtered, to remove any silicic acid, and the filtrate was evaporated to dryness. The residue was dissolved in 0.5 ml of ethanol to give a yield of 0.33 μmoles (23%, relative to [^{3}H]chloramphenicol base) using the extinction coefficient of 28,700 M^{-1}cm^{-1} at 265 nm (see above).

The specific activity of the pN$_3$benzoyl [^{3}H]chloramphenicol base was calculated as in Methods (section 3)

of Chapter 3.

6.   [^{3}H]Chloramphenicol Binding Assay.

This assay, which measures the reversible ribosomal binding of [^{3}H]chloramphenicol (prepared as in Methods (section 3(a)) of Chapter 5) was that of Vogel et al., (1971).

The assay, which was carried out in a total volume of 0.05 ml, contained 0.01 M Tris-acetate, pH 7.4, 0.01 M Mg(OAc)$_2$, 0.1 M NH$_4$Cl and 100 pmoles of E. coli ribosomes (unless otherwise stated), prepared as in section 4 of Chapter 1.   The reaction, at 0°, was initiated by the addition of [^{3}H]chloramphenicol to give a final concentration of 2.3 x 10^{-5} M (unless otherwise stated). After 30 min (unless otherwise stated), the reaction was terminated by the addition of 3 ml of cold 0.01 M Tris-acetate, pH 7.4, 0.01 M Mg(OAc)$_2$ and 0.1 M NH$_4$Cl and the ribosomes immediately collected on to cellulose nitrate filter discs (0.45 µ pore size), which were then rapidly washed with 10 ml of the above buffer.   The discs were then dried and the radioactivity estimated.

7.   Photoaffinity Labelling of Ribosomes with pN$_3$- benzoyl chloramphenicol base.

Ribosomes (90 µmol), in 0.05 ml of 0.02 M Tris-acetate, pH 7.5, 0.01 M Mg(OAc)$_2$, 0.5 mM EDTA and 0.1 M NH$_4$Cl, were photolysed at room temperature, with pN$_3$-

benzoyl chloramphenicol base (45 - 150 µM) in the presence
and absence of 1.0 mM chloramphenicol, for 5 min (unless
otherwise stated) using the apparatus described in Methods
(section 7) of Chapter 4.   After photolysis the ribosomes
were precipitated as described in Methods (section 4) of
Chapter 3 and the ribosomes resuspended in 0.04 ml of
0.02 M Tris-acetate, pH 7.5, 0.01 M Mg(OAc)$_2$, 0.5 mM
EDTA and 0.1 M NH$_4$Cl.   The precipitation procedure was
repeated four times to ensure complete removal of non-
covalently attached affinity label, prior to assaying
the ribosomes for peptidyl transferase activity (Chapter
1; section 6) or [^{3}H]chloramphenicol binding activity
(Methods (section 6) of this chapter).

The stoichiometry of labelling was determined as
in Methods (section 5) of Chapter 3.

RESULTS AND DISCUSSION

1.   Synthesis and Characterization of pN$_3$benzoyl
chloramphenicol base.

The pN$_3$benzoyl chloramphenicol base (Figure 31) was
synthesized by coupling chloramphenicol base with pN$_3$-
benzoic acid, using either EEDQ (for a large scale
reaction) or the anhydride of pN$_3$benzoic acid (for a
small scale reaction) (Figure 29).   The pN$_3$benzoic acid
used was synthesized by converting the pNH$_2$benzoic acid
to a diazonium salt, using NaNO$_2$, and then to pN$_3$benzoic

FIGURE 31: Structures of chloramphenicol (I) and

pN$_3$benzoyl chloramphenicol base (II).
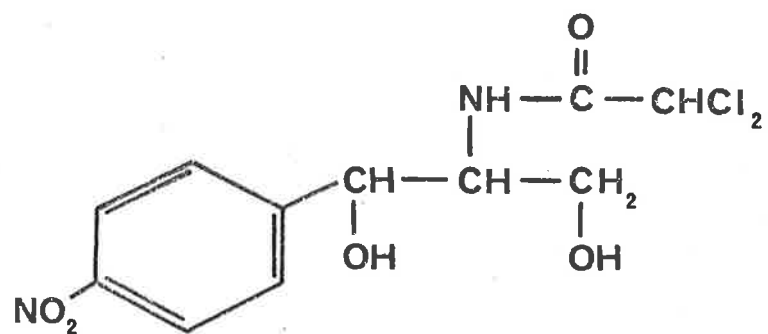
I
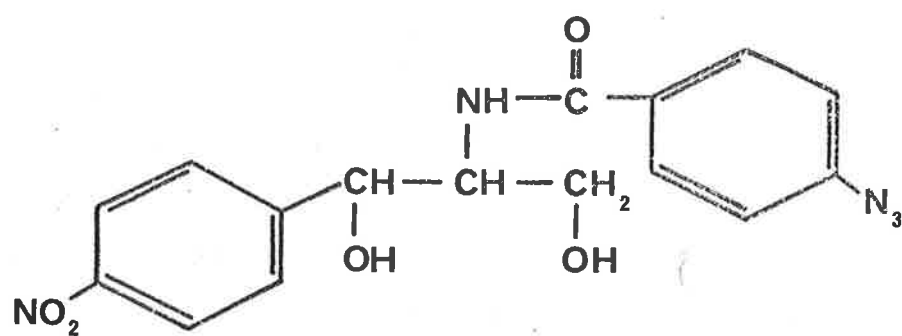
II

acid by reacting the diazonium salt with NaN_3 (Figure 29).
The resultant pN_3benzoic acid had a melting point iden-
tical to that reported by Gallardy et al., (1974) and
the infra-red spectrum of pN_3benzoic acid gave a charac-
teristic azide peak at 2140 cm^{-1} (Figure 30). Further
evidence for the presence of an azide group was the
observation that the spectrum of pN_3benzoyl chloramphenicol
base was destroyed upon photolysis of the compound in
the presence of N-α-Ac-Gly-Gly (scavenger) with a t½ of
5.3 min (Figure 32), thus indicating the presence of a
photolysable group. Therefore, the infra-red spectrum
and melting point of pN_3benzoic acid and the photolysis
of pN_3benzoyl chloramphenicol base are all consistent
with the presence of an azide group in the compound
(Figure 31).

2. Binding of pN₃benzoyl chloramphenicol base to the E.
coli ribosome.

Before this compound could be used as a photo-
affinity label, its ability to bind to the chloramphen-
icol site of the ribosome was determined. This was
achieved by measuring the compoundsability to inhibit
both the fragment reaction and the binding of [³H]chloramphenicol
to the ribosome.

(a) Inhibition of the fragment reaction by pN₃-
benzoyl chloramphenicol base.

Chloramphenicol, an inhibitor of peptidyl

FIGURE 32: The half-life of pN$_3$benzoyl chloramphenicol base. Photolysis of pN$_3$benzoyl chloramphenicol base (40 μM) in the presence of N-α-Ac-Gly-Gly (1 mM) was performed for varying times and the UV spectrum recorded at each time point. The results were then plotted as ln (% A$_{270}$ remaining) against time, using A$_{270}$ after 60 min of photolysis as complete photolysis.
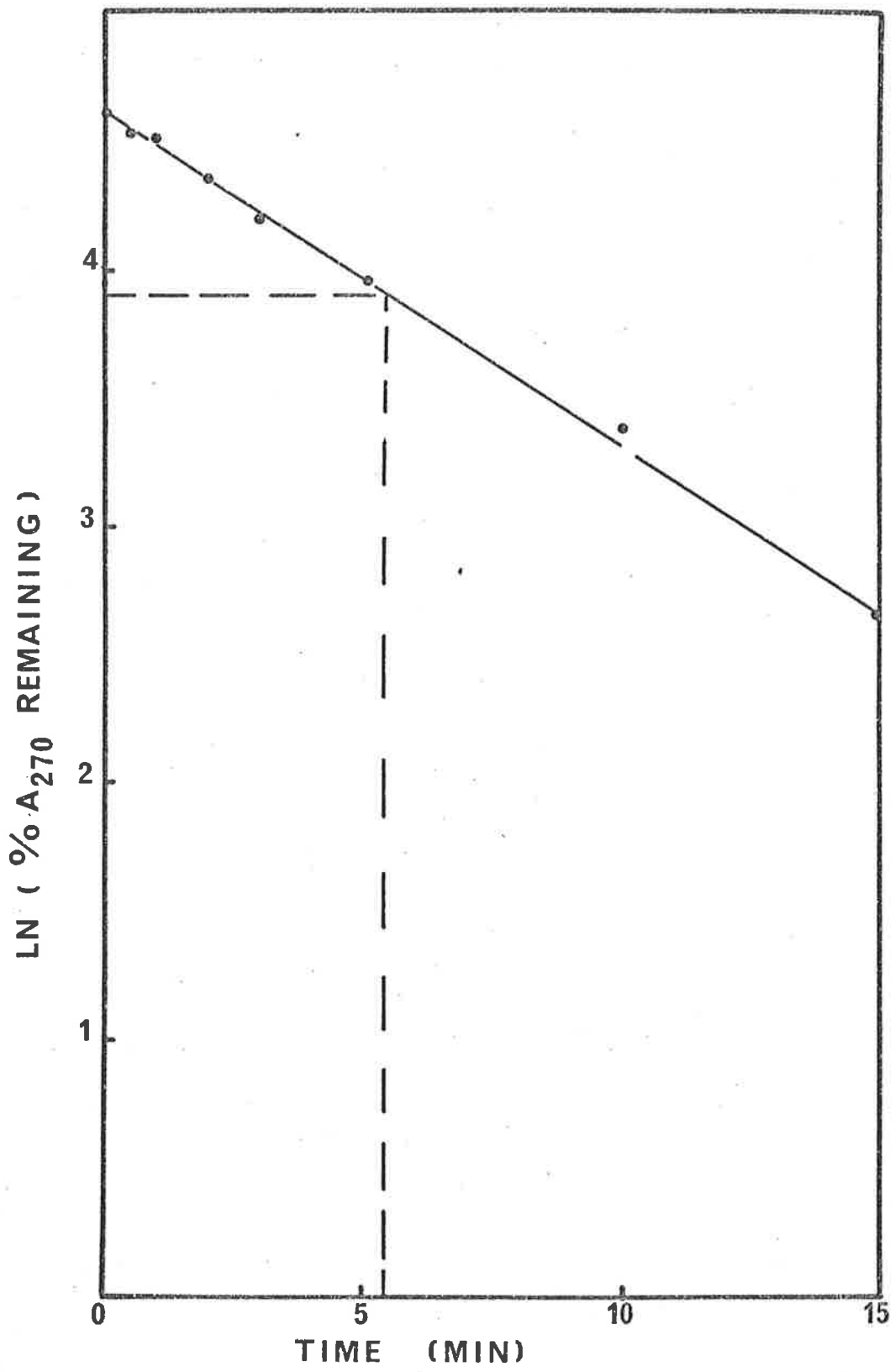
transferase (Vazquez, 1974) was shown to have a
Ki of 1.3×10^{-6} M in the fragment reaction (see
Chapter 5), as measured by the Dixon plot (Dixon,
1953).   Similarly, pN_3benzoyl chloramphenicol
base gave a Ki of 3.6×10^{-6} M (Figure 33).   It is
therefore apparent that this compound binds to the
chloramphenicol site and thereby inhibits peptidyl
transferase in a similar manner to chloramphenicol.

(b)   Inhibition of $[^3H]$chloramphenicol binding
       by pN_3benzoyl chloramphenicol base.

The binding assay used, as described in
Methods, is that of Vogel et al., (1971), in which
the $[^3H]$chloramphenicol is reversibly bound to the
ribosomes and the ribosomes collected on cellulose
nitrate filters.   Initial characterization of this
assay indicated that the $[^3H]$chloramphenicol bind-
ing was linear up to 200 pmoles of ribosomes per
binding assay (Figure 34A) and at equilibrium
(maximum binding) in 15 min (Figure 34B).   There-
fore all subsequent assays used 100 pmoles of
ribosomes per binding assay, which is well within
the linear range, while a convenient assay time
of 30 min was chosen.   Using these assay conditions
pN_3benzoyl chloramphenicol base was demonstrated
to inhibit the binding of $[^3H]$chloramphenicol to
the ribosomes (Figure 35).

FIGURE 33: The Ki of pN_3benzoyl chloramphenicol base, in the fragment reaction (Chapter 1; section 6) was determined by measuring the inhibition of the fragment reaction by varying concentrations of pN_3benzoyl chloramphenicol base ($0 - 7 \times 10^{-6}$ M) at two fixed concentrations of puromycin (0.06 and 0.36×10^{-3} M).

$\dfrac{1}{v}$

0.0008

0.0006

0.06 x 10^{-3} M
PUROMYCIN

0.0004

0.0002

0.36 x 10^{-3} M
PUROMYCIN

-6   -4   -2   0   2   4   6

[pN$_3$ benzoyl chloramphenicol base] × 10^6 (M)
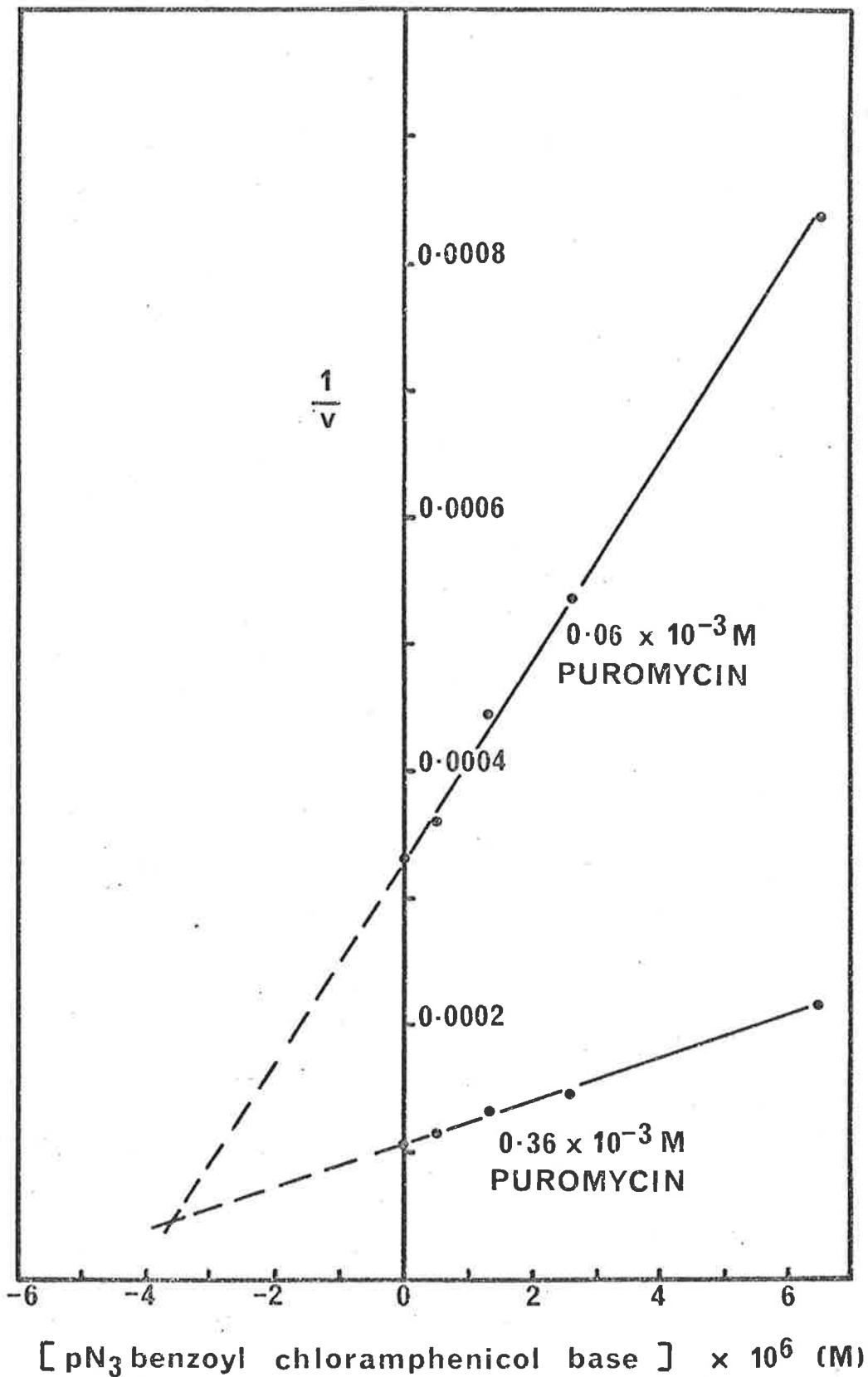
Therefore, both the inhibition of peptidyl transferase and the inhibition of [3H]chloramphenicol binding are indicative of the fact that pN_3-benzoyl chloramphenicol base is able to bind to the same site as chloramphenicol on the ribosome.

3. <u>Photoaffinity Labelling of Ribosomes with pN_3-benzoyl chloramphenicol base.</u>

Photoaffinity labelling of the ribosomes was found to be variable, with respect to the concentration needed to give a particular stoichiometry of labelling, and the variability seemed to be due to the preparation of pN_3-benzoyl [3H]chloramphenicol base used.   In all cases a concentration of pN_3benzoyl [3H]chloramphenicol base which gave a stoichiometry of one affinity label per ribosome was chosen (unless otherwise stated).

In order to determine whether the affinity label could become covalently attached to the ribosome and whether this covalent attachment was to the chloramphenicol site, ribosomes were affinity labelled with varying concentrations of affinity label, in the presence and absence of chloramphenicol (1 mM).   The results obtained (Figure 36) indicate covalent attachment, with increasing stoichiometry as the affinity label concentration is increased.   Chloramphenicol was also shown to protect against this covalent attachment.   Experiments to determine whether this protection, effected by chloramphenicol,

FIGURE 34:   Ribosome and time dependency of the $[^3H]$-
chloramphenicol binding assay (Methods, section 6).

A.   Ribosomes (0 - 400 pmoles) were incubated at 0°
for 30 min, collected on to cellulose nitrate filters
and the radioactivity on the filters estimated (Chapter
1; section 3(a)).

B.   Ribosomes were incubated for varying times
(0 - 30 min) at 0° and at each time indicated the ribo-
somes were collected on to cellulose nitrate filters and
the radioactivity on the filter estimated.
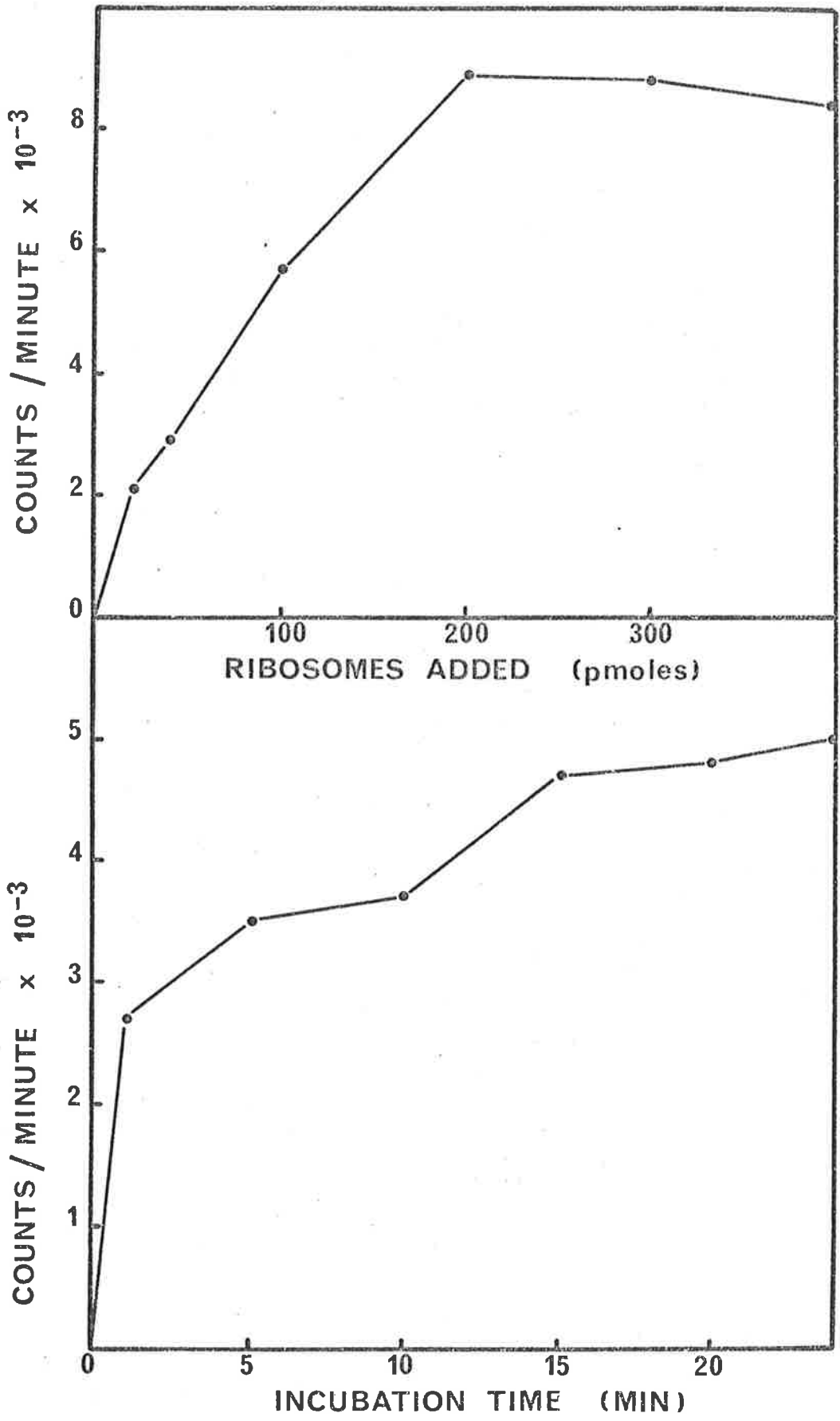
FIGURE 35: Inhibition of [^{3}H]chloramphenicol binding (Methods, section 6) by pN$_3$benzoyl chloramphenicol base.

Ribosomes were incubated in the presence of [^{3}H]chloramphenicol (10 μM) and varying concentrations (1.1 x 10^{-7} - 5.5 x 10^{-3} M) of pN$_3$benzoyl chloramphenicol base. After 30 min at 0°, the ribosomes were collected on to cellulose nitrate filters and the radioactivity estimated.

PERCENTAGE [³H]CHLORAMPHENICOL BOUND

[pN₃ benzoyl chloramphenicol base] (M)
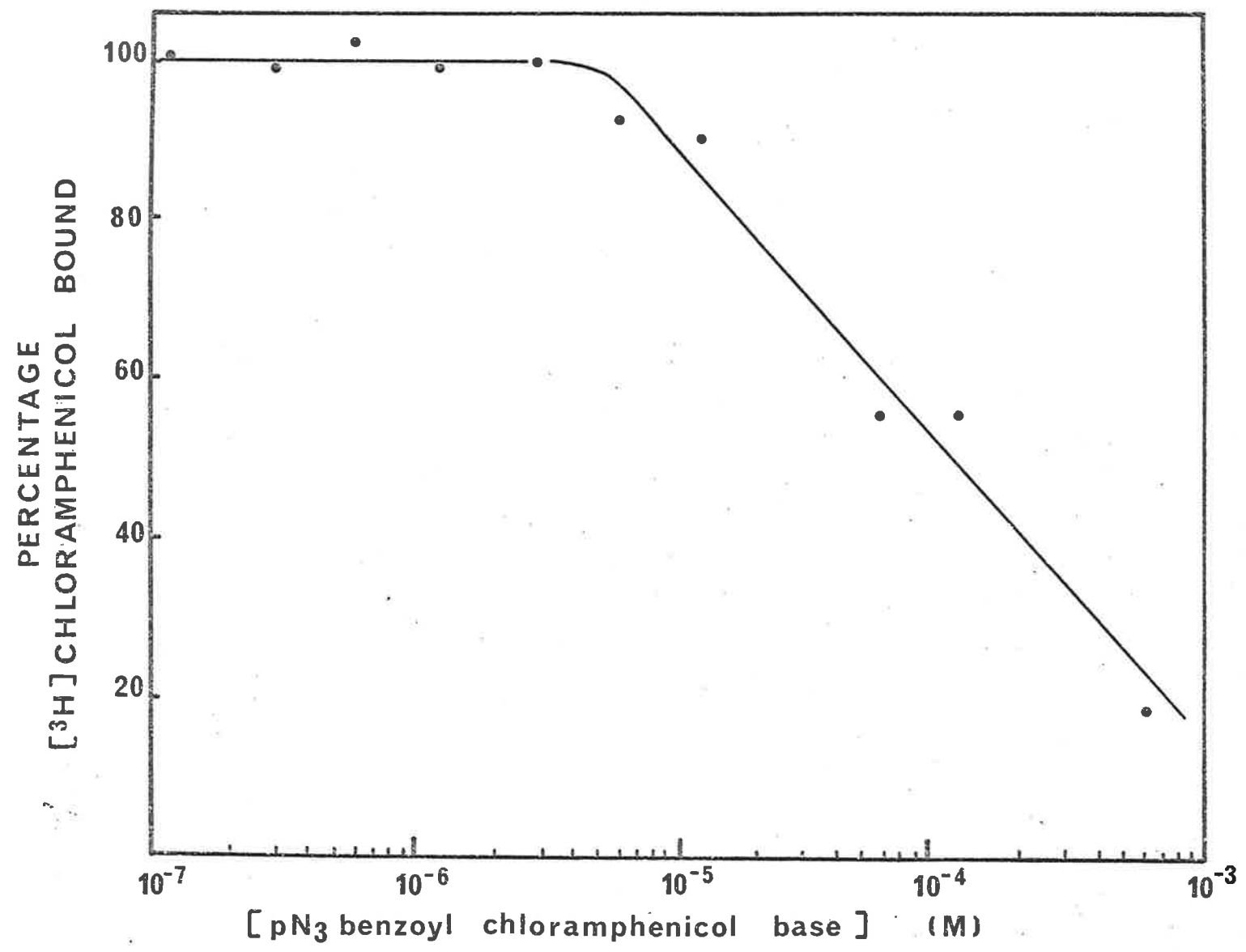
was specific were carried out as follows.   Ribosomes were photolysed with pN_3benzoyl chloramphenicol base (44 μM) in the presence of varying concentrations of chloramphenicol.   If the protection against covalent attachment, offered by chloramphenicol, was non-specific (that is, the protection was only due to chloramphenicol absorbing UV light and thereby preventing UV light from photolysing the affinity label), then a plot of the number of affinity labels per ribosome against the chloramphenicol concentration would give a straight line (that is the stoichiometry would decrease linearly with increasing chloramphenicol concentration).   If, on the other hand, the protection is specific (that is, chloramphenicol is binding to the chloramphenicol binding site, and so preventing the affinity label from binding), then the stoichiometry would be expected to decrease hyperbolically with respect to chloramphenicol concentration, with the result that at saturation of the binding site by chloramphenicol the stoichiometry would plateau at a value of one or less affinity labels per ribosomes.   This, indeed, was obtained and indicated that 0.4 affinity labels per ribosome were specifically attached to the chloramphenicol site, under the conditions used (Figure 37).   Therefore, the protection offered by chloramphenicol does, in fact, seem to be specific.   Hence, it was completely unexpected and pussling that the photoaffinity labelled ribosomes had full peptidyl transferase activity (relative to controls

FIGURE 36: Affinity labelling of ribosomes with pN_3-
benzoyl chloramphenicol base in the presence of chlor-
amphenicol.

Ribosomes were photolysed with varying concentrations
of pN_3benzoyl [^{3}H]chloramphenicol base ($4 - 110 \times 10^{-6}$ M)
in the presence of chloramphenicol (1 mM), washed and
the stoichiometry of labelling determined (see Methods,
section 7).

NUMBER OF AFFINITY LABELS / RIBOSOME

$[pN_3$ benzoyl chloramphenicol $] \times 10^6$ (M)

−CHLORAMPHENICOL

+ CHLORAMPHENICOL

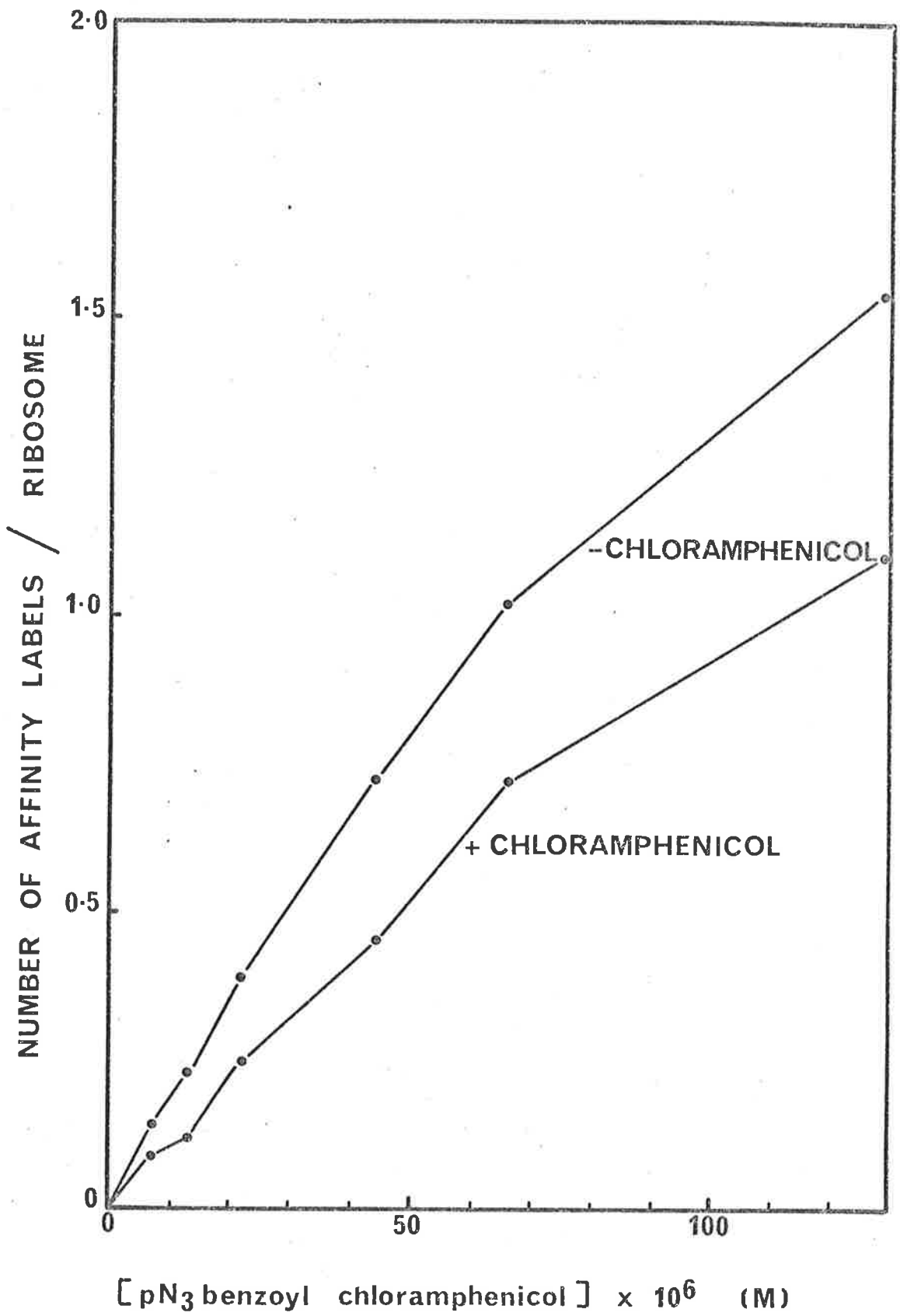FIGURE 37: Affinity labelling of ribosomes with pN$_3$-
benzoyl chloramphenicol base in the presence of varying
concentrations of chloramphenicol.

Ribosomes were photolysed with pN$_3$benzoyl [^{3}H]chloram-
phenicol base (44 μM) in the presence of varying con-
centrations of chloramphenicol (1.1 x 10^{-5} - 2.3 x 10^{-3} M),
washed and the stoichiometry determined (see Methods,
section 7).
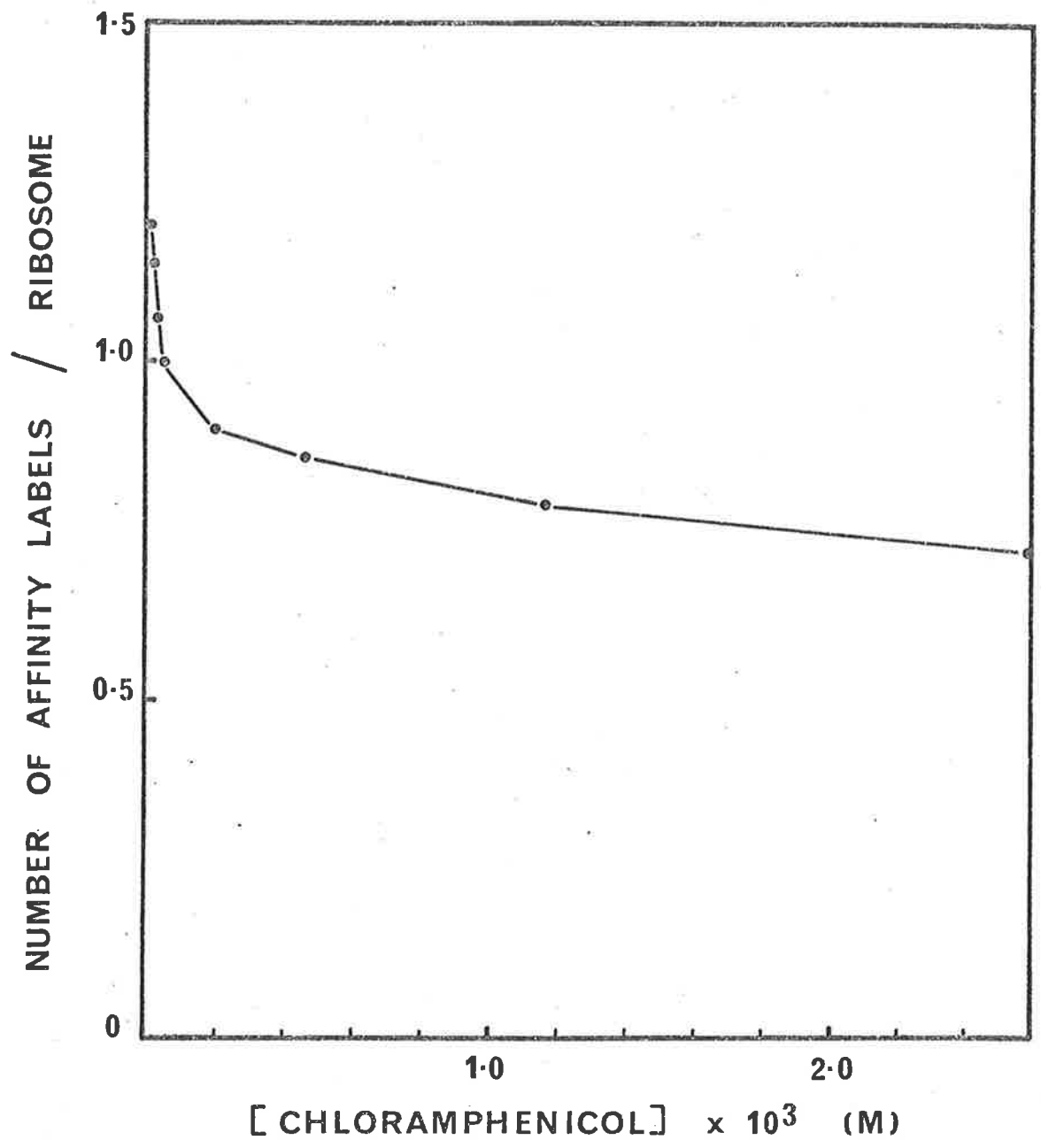
irradiated without affinity label present (Table 6))

These results could be explained by the fact that the affinity label was being bound to the correct site, but that it was bound in the incorrect spacial configuration and therefore could not inhibit peptidyl transferase. If this were the case, then the bound affinity label might

(1) inhibit the binding of [3H]chloramphenicol

and (2) prevent the inhibition by chloramphenicol of the fragment reaction.

Therefore, using the same ribosomes as above, these two possibilities were examined and the results are also shown on Table 6. The binding of [3H]chloramphenicol was not affected by irradiation and when the ribosomes were photolysed with affinity label there was little or no decrease in the subsequent binding of [3H]chloramphenicol to the ribosomes. The inclusion of chloramphenicol during the irradiation had little or no effect on the binding of [3H]chloramphenicol. The inhibition of the fragment reaction by chloramphenicol should be prevented if the affinity label was covalently attached to the chloramphenicol site, because it should prevent the entry of chloramphenicol. However, this was not so, as chloramphenicol was able to inhibit the fragment reaction to the same extent if ribosomes were photolysed in the presence or absence of affinity label (Table 6). The

TABLE 6:   Ribosomes were photolysed with pN_3benzoyl chloramphenicol base (150 μM) in the presence and absence of chloramphenicol (1 mM).   After washing (see Methods, section 7), the ribosomes were assayed for peptidyl transferase activity (Chapter 1; section 6) in the presence and absence of chloramphenicol (3 μM) at a puromycin concentration of 0.45 mM.   The same ribosomes were also assayed for [^{3}H]chloramphenicol binding activity (Methods, section 6).

TABLE 6. **EFFECT OF COVALENTLY ATTACHED pN_3 BENZOYL CHLORAMPHENICOL BASE ON RIBOSOMAL ACTIVITIES**

Samples	Irradiation	Peptidyl transferase activity (cpm/pmoles of ribosomes)			$[^3H]$-chloramphenicol binding activity (cpm/pmoles of ribosomes)
		-Chloramphenicol	+Chloramphenicol	% Inhibition by chloramphenicol	
Ribosomes	-	300	155	48	100
Ribosomes	+	300	160	47	110
Ribosomes + affinity label	+	260	140	46	105
Ribosomes + affinity label + chloramphenicol	+	265	140	47	120

inhibition was the same if chloramphenicol was included during the photolysis.

Clearly the chloramphenicol protected affinity label must be either covalently attached to a hydrophobic site other than the chloramphenicol site (to which chloramphenicol also binds non-specifically due to it's hydrophobic character), or alternatively the compound was removed from the chloramphenicol site by the process of forming a covalent bond between itself and the ribosome. Furthermore, the covalent bond, so formed, was such that the compound was unable to rebind to the chloramphenicol site (or indeed unable to even restrict entry of other compounds to the site). As far as we can see it is not possible to devise simple experiments to eliminate the above. Support for the former idea was offered by the fact that chloramphenicol base was able to protect against labelling to the same extent as chloramphenicol.

Therefore, the conclusion reached was that the affinity label was covalently attached to a hydrophobic site, which could also bind chloramphenicol, but that the site labelled was not the chloramphenicol site. In fact, Lessard and Pestka (1972a) have suggested the presence of two chloramphenicol binding sites on the E. coli ribosome, of which only the high affinity site is thought to be specific.

SUMMARY

The chloramphenicol analogue, pN$_3$benzoyl chloramphenicol base, has been synthesized and shown to bind to the chloramphenicol site of the E. coli ribosome and so inhibit both peptidyl transferase and [^{3}H]chloramphenicol binding. Photolysis of the ribosomes with the affinity label demonstrated covalent attachment of the affinity label to the ribosome (1.0 - 1.5 affinity labels per ribosome). The attachment of 0.4 affinity labels per ribosome was prevented by the addition of excess chloramphenicol and this protection was shown not to be due to non-specific light absorption by chloramphenicol. Paradoxically covalently bound affinity label did not inhibit peptidyl transferase or the binding of [^{3}H]chloramphenicol or prevent the inhibition of the fragment reaction by chloramphenicol. Therefore, the most likely explanation, for the data obtained, is that the affinity label was covalently attached to a hydrophobic site on the ribosome, which also has the ability to bind chloramphenicol non-specifically (because of its hydrophobic nature). An alternative explanation is that the affinity label was removed from the site by the process of formation of the covalent bond between itself and the ribosome, which in turn prevented the bound affinity label from rebinding to the chloramphenicol site.

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