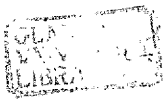
STUDIES ON THE STRUCTURE AND FUNCTION OF *E. COLI* PHENYLALANINE TRANSFER RNA.

MARGARET LOWDON.

A Dissertation submitted to the University of Glasgow for the Degree of Doctor of Philosophy.

Department of Biochemistry. May 1976.



ACKNOWLEDGEMENTS.

I thank Professors R.M.S. Smellie and A.R. Williamson for making available the facilities of the Biochemistry Department for this research.

I am also grateful to the Medical Research Council for the provision of a Research Studentship.

I am deeply indebted to Dr. J.P. Goddard for much helpful advice and encouragement throughout the course of this work.

I should also like to thank Dr. B.E.H. Maden for his advice and the staff and students of A3, particularly Robert Edwards, Ellen Seenan and Tom Carr for their encouragement and support.

Finally I am grateful to Mrs. Pat Brodie for typing this manuscript.

ABBREVIATIONS.

Abbreviations used in this thesis have been defined in 'Instructions to Authors' (Biochem. J. (1976) 153, 1-21), except for the following :-

A	Absorbance.
A_{260} unit	The amount of tRNA which, when dissolved in 1 ml. of 10 mM $MgCl_2$, 10 mM tris-HCl, pH 7·0, has an absorbance, over a pathlength of 1 cm., of one.
R.P.C.-5	Reverse Phase Chromatography Medium 5 (Pearson et al, 1971).
RNase	Ribonuclease.

NOMENCLATURE OF NUCLEOTIDES.

Throughout this thesis, nucleoside -3'- phosphates are represented by single letters (see Sanger et al, 1965) as indicated below. All nucleotides contain ribose as the sugar component. Nucleosides are indicated by the subscript OH, and 3', 5' diphosphates by the letter p preceeding the nucleotide.

A	adenosine -3'- phosphate.
m^1A	1 - methyl adenosine -3'- phosphate.
i^6A	N^6 - isopentenyl adenosine -3'- phosphate.
ms^2i^6A	2 - methylthio -N^6- ispentenyladenosine -3'- phosphate.
C	cytidine -3'- phosphate.
m^5C	5 - methyl cytidine -3'- phosphate.
Cm	2' - O - methyl cytidine -3'- phosphate.
C!	cytidine -2', 3' - phosphate (cyclic).
D	5,6, - dihydrouridine -3'- phosphate.
G	guanosine -3'- phosphate.
m^2G	N^2 - methyl guanosine -3'- phosphate.
m_2^2G	N^7 - dimethyl guanosine -3'- phosphate.
m^7G	N^7 - methyl guanosine -3'- phosphate.
Gm	2' - O - methyl guanosine -3'- phosphate.
'mG'	the nucleotide produced by alkali treatment of m^7G.
G!	guanosine -2', 3' - phosphate (cyclic).
N	The nucleotide produced on bisulphite modification of ms^2i^6A.

R	purine nucleoside -3'- phosphate.
T	thymidine -3'- phosphate.
U	uridine -3'- phosphate.
Ψ	pseudouridine -3'- phosphate.
S^4U	4 - thiouridine -3'- phosphate.
U!	uridine -2', 3' - phosphate.
X	3 - (3 - amino -3 - carboxypropyl) uridine -3'- phosphate.
Y	pyrimidine nucleoside -3'- phosphate.
⊛	nucleotide common to all tRNAs

An asterisk indicates that the nucleotide may be modified.

SUMMARY.

Two different methods have been used for the purification of E. coli $tRNA^{Phe}_2$. Both methods involved initial fractionation of unpurified E. coli tRNA (either unlabelled, or uniformly labelled with ^{32}P) on Benzoylated DEAE-cellulose. The fractions containing $tRNA^{Phe}_2$ (the second phenylalanine isoacceptor tRNA eluted from the Benzoylated DEAE-cellulose column on increasing the NaCl concentration of the eluate) were then fractionated either after charging of the tRNA with phenylalanine on another Benzoylated DEAE-cellulose column, or on an RPC-5 column. After deacylation of purified Phe-$tRNA^{Phe}_2$, the $tRNA^{Phe}_2$ could not be charged with phenylalanine to the original extent. Purified $tRNA^{Phe}_2$ obtained from an RPC-5 column was found to lose phenylalanine accepting activity on storage. Attempts were made to renature both inactivated forms of $tRNA^{Phe}_2$, but they were not successful. Attempts to fully charge the inactivated $tRNA^{Phe}_2$ by increasing the ligase concentration in the assay or by preincubating the $tRNA^{Phe}_2$ with the ligase were equally unsuccessful. However, after investigation of the stability of $tRNA^{Phe}_2$ under various conditions, it was found to be most stable in 10mM $MgCl_2$, 10mM tris-HCl, pH 7·0, in the presence of an equal amount of unpurified E. coli tRNA.

The purified E. coli $tRNA^{Phe}_2$ isolated was found to give identical T_1 and Pancreatic RNase fingerprints to the E. coli $tRNA^{Phe}$ whose sequence was elucidated by Barrel & Sanger (1969).

Treatment of E. coli $tRNA^{Phe}_2$ with sodium bisulphite at 25°C resulted in modification of four nucleoside residues over a period of 48 hours. The cytidine residues of C17, C74 and C75 were converted to uridine residues. Bisulphite modification of ms^2i^6A37 was also observed, the product being an adduct containing one mole of HSO_3^- per mole of 2-methylthio-N^6-isopentenyl adenosine. Cytidine residues of C48 and C56, although in single stranded regions of the cloverleaf structure, were not modified by bisulphite. These residues are probably involved in tertiary structure interactions analogous to those described for Yeast $tRNA^{Phe}$ by Ladner et al (1975b). No conformational change of $tRNA^{Phe}_2$ appeared to occur during bisulphite modification.

Bisulphite modification was found to destroy the phenylalanine accepting activity of E. coli $tRNA^{Phe}_2$. However, $tRNA^{Phe}_2$ samples in which all of the four reactive residues had been fully modified still had some residual phenylalanine accepting activity. After separation of active and inactive bisulphite modified $tRNA^{Phe}_2$ molecules both fractions were found to contain all four possible bisulphite modifications, although the inactive fraction contained a greater proportion of these than the active fraction. Therefore, no residue could be implicated as a component of the specific ligase recognition site and the loss of phenylalanine acceptor activity was probably due to a conformational change facilitated in bisulphite modified $tRNA^{Phe}_2$.

Bisulphite modification of E. coli Phe-$tRNA^{Phe}_2$ resulted in modification of the same four residues as in uncharged

$tRNA_2^{Phe}$. However, ms^2i^6A37 was modified to a slightly lesser extent in charged $tRNA_2^{Phe}$ than in uncharged $tRNA_2^{Phe}$. No additional residues were modified in Phe-$tRNA_2^{Phe}$.

The pattern of thermal denaturation of E. coli $tRNA_2^{Phe}$ was investigated by bisulphite modification at elevated temperatures. In the absence of Mg^{2+}, quite extensive denaturation of $tRNA_2^{Phe}$ appeared to have occurred at $45^{\circ}C$. At this temperature, in a proportion of molecules C48 and C56 were available for bisulphite modification and therefore some destruction of the tertiary structure interactions involving these residues must have occurred. In some $tRNA_2^{Phe}$ molecules, cytidine residues involved in the secondary structure base pairs of stems b and e were also available implying some melting of these helices. These residues were modified to a greater extent at $55^{\circ}C$, but even at this temperature, no reaction of cytidine residues of stems a and c was observed (except for C72 to some extent) indicating that these helices were still intact.

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TO MY PARENTS .

1.1. GENERAL AND HISTORICAL INTRODUCTION.

Transfer ribonucleic acid (tRNA) is the term, coined by Allen & Schweet (1960), used to describe a group of low molecular weight RNA molecules that play a vital part in protein synthesis, by translating the information carried by messenger RNA into a protein. The existence of this type of molecule had been predicted by Crick (1955), who was unable to envisage DNA or RNA acting as a direct template for amino acids. Crick suggested that "adaptor molecules" were necessary to translate the information carried by nucleic acids into a protein. In its simplest form the hypothesis required that there was one "adaptor molecule" for each amino acid, each molecule having a specific hydrogen bonding surface that would enable it to bind specifically to a nucleic acid template, and one enzyme for each amino acid that would be responsible for the specific attachment of the amino acid to its "adaptor molecule". The role of adaptor molecule was assigned to transfer RNA by Hoagland et al, (1958).

During protein synthesis, each tRNA molecule is recognised by its cognate aminoacyl-tRNA ligase, and charged with the correct amino acid. The aminoacyl-tRNA is carried to the ribosome under the influence of an elongation factor. The aminoacyl-tRNA-elongation factor-GTP complex binds to the ribosome-mRNA complex in response to a codon complementary to the anticodon on the tRNA, and, under the influence of the peptidyl transferase enzyme, the amino acid is transferred to the end of a nascent polypeptide chain. For a review of protein synthesis, see Lengyel (1974).

Transfer RNA is the smallest species of RNA present in the

cell, sedimenting at about 4S. There are a large number of species of tRNAs in each cell, and often there is more than one tRNA specific for the same amino acid. The number of species may vary from about 60 in bacterial cells, to about 100 in mammalian cells (Lengyel, 1974). Transfer RNA molecules may be 72-93 nucleotides long, the molecular weights being in the region 25,000-35,000.

Transfer RNA is the most modified type of RNA found, modifications of each of the four major bases being possible (Nishimura, 1972). The proportion of modified bases in tRNA increases with the evolutionary complexity of the organism. In some cases, e.g. mammalian tRNAs, up to 25% of the bases present in tRNA are modified.

The primary structures of over 60 species of tRNA have been determined (see Barrel & Clark, 1974) and each can be represented in the cloverleaf form, suggested amongst others by Holley et al (1965), which allows the maximum amount of Watson-Crick base pairing (about 60%). The cloverleaf has four loop regions and four helical stem regions.

The structure and functions of tRNA are discussed more fully in the following Sections, 1.2, 1.3, and 1.4.

1.2. THE INVOLVEMENT OF TRANSFER RNA IN CELLULAR PROCESSES.

Transfer RNA has been implicated in a large number of cellular processes, a list of which is given below:-

1.2.1. PROTEIN SYNTHESIS.

(i) Activation of amino acids (see Section 1.2.1.1)

(ii) Recognition of initiator factor by initiator tRNA (Berthelot et al, 1972; Ghosh & Ghosh, 1972).

(iii) Recognition of charged initiator tRNA by transformylase in procaryotic systems (Seno et al, 1970; Berthelot et al, 1972; Ghosh & Ghosh, 1972; Giegé et al, 1973).

(iv) Location of charged initiator tRNA in the initiation site on the small subunit of the ribosome. For reviews of the initiation of protein synthesis, see Revel (1972), Rudland & Clark (1972) and Ochoa & Mazumder (1974).

(v) Recognition of elongation factor by tRNAs involved in peptide chain elongation (Krauskopf et al, 1972; Beres & Lucas-Lenard, 1973; Schulman et al, 1974).

(vi) Location of charged tRNA in the A site of the ribosome (Czernilofsky et al, 1974; Forget & Weissmann, 1967; Erdmann et al, 1973; Dube, 1973a; Richter et al, 1973; Schwarz et al, 1974).

(vii) Decoding mRNA. For reviews of the mechanism of peptide chain elongation, see Moldave (1972), Haselkorn & Rothman-Denes (1973) and Lucas-Lenard & Beres (1974).

(viii) Regulation of protein synthesis, tRNA may be involved in:-

a) Repression (see Littauer & Inouye, 1973).

b) Feedback inhibition (see Littauer & Inouye, 1973).

c) Supression (see Smith, 1972).

d) Interferon production during viral infection.

e) Control of enzyme activity such as tryptophan pyrrolase activity in Drosophila (Jacobson, 1971).

f) Control of RNA synthesis by the production of magic spot compounds (see Block & Haseltine, 1974).

1.2.1.1. Amino Acid Activation.

The reaction involving tRNA most relevant to this thesis is that of amino acid activation, and this is the only reaction involving tRNA that is discussed in detail.

The aminoacyl-tRNA ligases are a group of enzymes that attach amino acids to the 3'-Termini of their cognate tRNAs, according to the overall equation.

Amino acid + ATP + tRNA $\rightleftharpoons$

Aminoacyl -tRNA + AMP + PPi.

This reaction usually requires the presence of Mg^{2+}. In the absence of tRNA, most aminoacyl-tRNA ligases are able to form an enzyme-bound aminoacyl-adenylate complex. Such compounds have been isolated. For reviews, see Loftfield (1972) and Söll and Schimmel (1974).

Amino acid + ATP + enzyme $\rightleftharpoons$

enzyme. (aminoacyl$\sim$AMP) + PPi.

This reaction has been proposed as thefirst step in the aminoacylation reaction (Hoagland, 1955; Berg, 1956). There is evidence that the order of addition of substrates varies with the aminoacyl-tRNA ligase used. In some cases, the enzyme is thought to form an initial complex with the amino acid, eg. E.coli leucyl-, Seryl-, and valyl-tRNA ligases (Myers et al, 1971), in others it is thought that the formation of an enzyme-ATP complex takes place initially, e.g. Yeast lysyl-tRNA ligase (Berry & Grunberg-Manago, 1970), while in other cases the order of addition of ATP and the amino acid appears to be random, e.g. E. coli methionyl-tRNA ligase (Blanquet et al, 1974) and E.coli phenylalanyl-tRNA ligase (Mulivor & Rappaport, 1973).

There are some reasons for doubt as to whether this proposed mechanism for aminoacylation is the true one. In several cases, the enzyme bound aminoacyl-adenylate is not formed in the absence of tRNA (Loftfield, 1972; Söll & Schimmel, 1974). Some tRNAs may be aminoacylated in the absence of Mg^{2+}, although this cation is required in all cases for the formation of an enzyme bound aminoacyl-adenylate complex (see Loftfield, 1972). These and other reasons discussed fully by Loftfield (1972) have led him to propose a concerted reaction mechanism with simultaneous involvement of all three substrates. However, detailed investigation of the mechanism of aminoacylation, using kinetic data obtained in the presence of all three substrates, is difficult. Therefore it is not easy to prove that such a concerted reaction mechanism really occurs. For the purpose of simplification, it is generally assumed that the reaction does involve the intermediate formation of the enzyme bound aminoacyl-adenylate, and partial reactions are usually studied.

During aminoacyl-tRNA formation, the amino acid is connected by an ester linkage to the 2' or 3' hydroxyl group of the 3' terminal adenosine residue of the tRNA. The initial placing of the amino acid on the 2' or 3' hydroxyl group depends on the aminoacyl-tRNA ligase. Some ligases transfer the amino acid to the 2' hydroxyl group and some to the 3' hydroxyl group (Sprinzl and Cramer, 1973; Cramer, 1975; Rich, 1975). After the initial placement, isomerisation between the 2' and 3' forms takes place rapidly. There is some evidence to suggest that during protein synthesis, aminoacyl-tRNA with the amino acid linked to the 3' position of the terminal adenosine is required (Nathans & Neidle, 1963; Sprinzl & Cramer, 1973).

According to the adaptor hypothesis, it is the nucleotide sequence of the anticodon alone that recognises the codon. Therefore, for faithful translation to occur, the charging of a tRNA with its cognate amino acid must be highly specific, requiring that each tRNA must specifically recognise its cognate aminoacyl-tRNA ligase.

1.2.2. RNA Metabolism.

Transfer RNA is a substrate for:-

(i) Cleavage and maturation enzymes, as precursor tRNA (see Altman, 1975).

(ii) Modification enzymes responsible for the production of modified bases (see Söll, 1971).

(iii) tRNA nucleotidyl-transferase, i.e. the "-CCA_{OH} repair enzyme" (see Deutscher, 1973).

(iv) Peptidyl-tRNA hydrolase (as peptidyl-tRNA (De Groot et al, 1969)).

(v) Nucleases.

Transfer RNA also acts as a primer for reverse transcriptase.

1.2.3. Aminoacyl-tRNA as a Donor of Amino Acids in Reactions not Involving Ribosomes.

(i) Bacterial cell wall biosynthesis (see Littauer and Inouye, 1973).

(ii) Post - translational addition of amino acids to proteins (see Soffer, 1973).

(iii) Biosynthesis of aminoacyl-phosphatidyl glycerol (Nesbit & Lennarz, 1968; Gould et al, 1968).

(iv) Biosynthesis of glycyl-lipopolysaccharide (Gentner & Berg, 1971).

1.2.4. Other activities of tRNA.

(i) tRNA is capable of altering the specificity of E.coli ENDO I restriction endonuclease.

(ii) Some viral RNAs appear to have a tRNA-like structure at the 3' end (Haenni et al, 1973; Cory et al, 1970).

The relationship of structure to function of tRNA is discussed in Section 1.4.

1.3. STRUCTURE OF TRANSFER RNA.

In this Section, a brief account is given of some of the methods that have been used to elucidate the primary, secondary and tertiary structures of tRNA. This is followed by a brief description of how the results obtained from such studies have led to a better understanding of the structure of tRNA.

1.3.1. Primary Structure Determination.

Sequencing of RNA molecules involves digestion of the RNA, followed by fractionation and identification of the constituent oligonucleotides. Two methods have primarily been used for the separation of oligonucleotides, the method of Holley (1968) and that of Sanger et al, (1965). These methods have been described in detail by Brownlee (1972).

Since the first tRNA was sequenced (Holley et al, 1965), the primary structures of over 70 tRNAs have been determined, (compiled by Barrel & Clark, 1974).

1.3.2. Secondary Structure : The Cloverleaf Model.

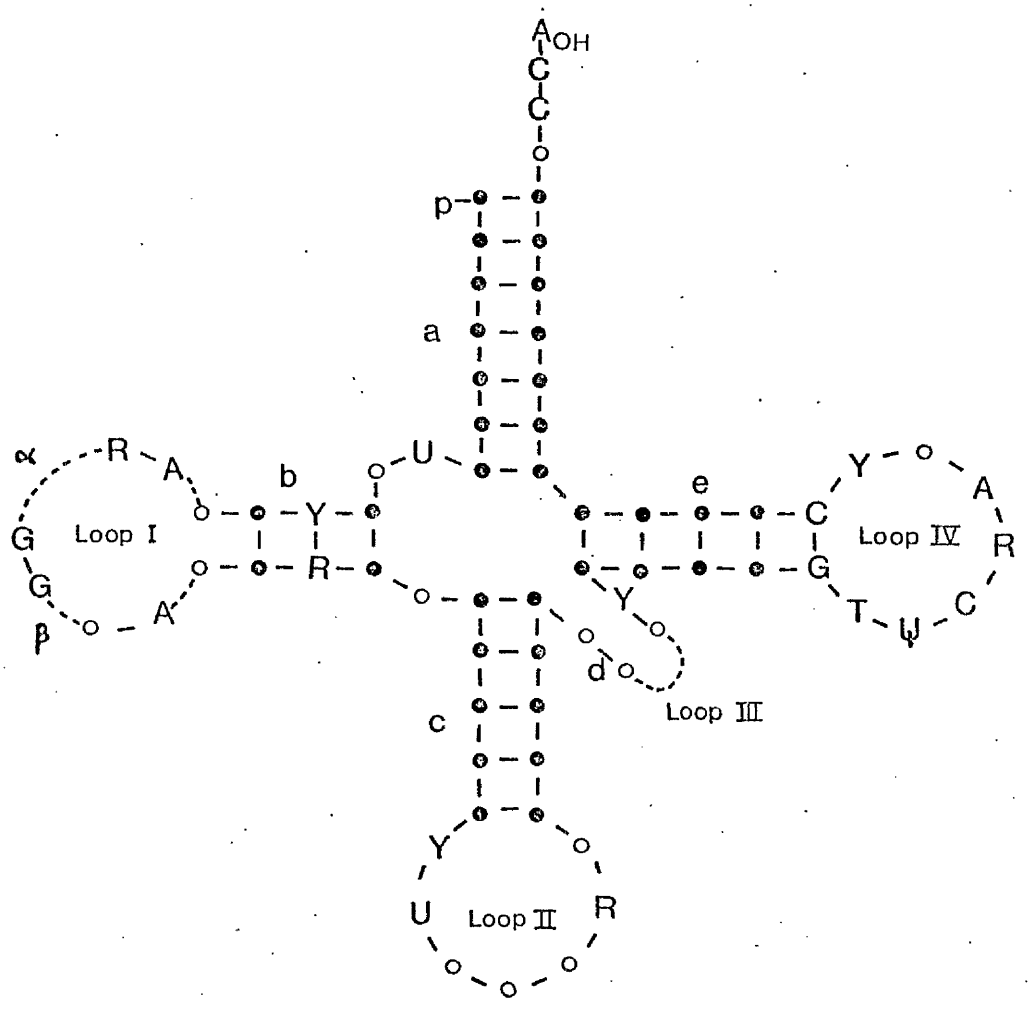
The secondary structure of tRNA is defined as helical elements, formed by Watson-Crick base pairing between the heterocyclic bases

(Cramer, 1971). Evidence for the existence of helical regions has come from the X-ray diffraction patterns of oriented fibres of tRNA (Fuller et al, 1967; Arnott et al, 1967; Doctor et al, 1969) and also from the reversible "melting" curves obtainable for tRNA which resemble those of other double-helical nucleic acids.

Holley (1965) proposed a number of models for the secondary structure of Yeast $tRNA^{Ala}$, involving Watson-Crick base pairing. Of these, the "cloverleaf" model allows the maximum amount of base pairing. The primary structure of all tRNAs sequenced so far can be arranged in this cloverleaf form. This strong circumstantial evidence for a common cloverleaf secondary structure is supported by all results of the structural studies of tRNA tertiary structure.

FIG 1 shows the features that are common to all tRNAs. The constant sequence T Ψ C in Loop IV is not present in a few tRNAs. The exceptions (described by Barrel & Clark, 1974) include eucaryotic initiator tRNAs and several glycine tRNAs from some species of Staphylococcus that are not involved in ribosome-mediated protein synthesis. The extra arm, d varies in length in different tRNAs. Two types of tRNA can be distinguished, those with a short extra arm (3-5 nucleotides long with no helical content), and those with a long extra arm (13-15 nucleotides long with some base pairing).

Loop I varies in size in different species of tRNA, containing between 8 and 10 nucleotides. Kim et al, (1974,a) have defined two regions of Loop I, the α and β regions. The α region is that between the two constant sequences AR and GG, and the β



Generalised tRNA Cloverleaf Structure showing

Constant Features.

after Barrell & Clark (1974).

FIG. 1.

region is that between GG and the constant A residue. The α and β regions each contain from one to three nucleotides. When dihydrouridine residues are present, they occur only in these regions.

Of the other minor nucleosides that occur in tRNA, most are restricted to the loop regions of the cloverleaf model. Pseudouridine is sometimes found in the last base pair of stem c.

1.3.3. Tertiary Structure.

Evidence for the existence of tRNA molecules in a more compact form than the simple cloverleaf model has come from a variety of sources. The R_G of tRNA molecules, as determined by low angle X-ray scattering is smaller than would be expected for the cloverleaf form (Pilz et al, 1970; Connors et al, 1969; Ninio et al, 1969). Changes in conformation, as measured by changes in sedimentation properties and viscosity (Henley et al, 1966), stability to degrading enzymes (Thang et al, 1967) and mobility of the $-CCA_{OH}$ end (Hoffman et al, 1969), have been observed when very little loss of secondary structure, as measured by U.V. absorbtion, occurs.

The maintenance of a defined tertiary structure appears to be important for the functioning of tRNA. Several species of tRNA may exist in two different conformations, only one of which is functional (Lindahl et al, 1966; Fresco et al, 1966; Gartland & Sueoka, 1966; Ishida et al, 1971; Streeck & Zachau, 1971). Divalent cations, particularly Mg^{2+} have been implicated in the maintenance of the correct functional tertiary structure (Fresco et al, 1966; Sueoka et al, 1966; Adams et al, 1967; Chapeville et al, 1969; Reeves et al, 1970). Conversion of the inactive

denatured form to the active native form can often be acheived by heating the inactive form in the presence of low concentrations of Mg^{2+} (about 10mM). Conversely, heating solutions of tRNA in the presence of chelating agents for divalent cations, ie. citrate or EDTA, usually cause conversion of the tRNA to the inactive form.

Alteration of the conformation of tRNA has been postulated, on aminoacylation (Sarin & Zamecnik, 1965; Schofield, 1970; Woese, 1970), and on enzymic (elongation factor Tu-dependent) binding of aminoacyl-tRNA to the ribosome-mRNA complex (Schwartz et al, 1974). There is evidence from low angle X-ray scattering (Connors et al, 1969), Raman spectroscopy (Thomas et al, 1973a), and the fact that several species of tRNA can co-crystallise (Fresco et al, 1968; Blake et al, 1970) that the native tertiary structures of all tRNAs are similar.

Several techniques have been used to examine the tertiary structure of tRNA :-

(i) A critical examination of tRNA primary structure data.

(ii) Chemical modification.

(iii) Enzyme dissection.

(iv) Oligonucleotide binding to tRNA.

(v) A variety of physical methods.

1.3.3.1. Critical Examination of tRNA Primary Structure Data.

Since the tertiary structures of all tRNAs are thought to be similar, it is probable that interactions responsible for the maintenance of tertiary structure involve features common to all tRNAs. These have been discussed (Section 1.3.1. and FIG 1.) In addition, Levitt (1969) noticed that when the constant purine residue

R in Loop I is G, then the constant pyrimidine residue Y in the extra arm, arm d is C and, when the constant purine residue is A, the constant pyrimidine residue is U. On the basis of this observation, Levitt proposed a tertiary structure Watson-Crick base pair involving these two residues.

1.3.3.2. Chemical Modification.

The rationale behind chemical modification as a probe of tRNA conformation is that only bases in exposed single stranded regions of the tRNA are available for modification. A base will not react with a modifying agent if parts of the molecule that would normally react with the reagent are involved in base pairing interactions, or sterically hindered from reacting with the reagent because of the conformation of the base in the tRNA molecule. It is conceivable that a base could be available for modification although it is involved in base pairing interactions, if the reagent attacks part of the molecule not involved in hydrogen bonding. Bases in helical regions, however, are unlikely to be available for modification because of steric hindrance.

For a reagent to be an effective probe of tRNA conformation, the following criteria must be observed (Cramer, 1971) :-

(i) The reagent must show a high selectivity towards single stranded regions.

(ii) Ideally it should be highly selective for one base, so that the pattern of modification can be easily elucidated.

(iii) The reaction should be able to be easily followed analytically.

(iv) There should be no chain breaks, unzipping of secondary structure or disruption of tertiary structure during the reaction.

TABLE 1.

CHEMICAL MODIFICATION OF tRNA .

MODIFYING AGENT	NUCLEOTIDES MODIFIED	tRNA	REFERENCE
Sodium Borohydride	D, ac^4C, s^4U, Y, m^7G, m^1A	Yeast $tRNA^{Phe}$, $tRNA^{Ser}$	Igo-Kemenes & Zachau (1969)
		E. coli $tRNA^{Phe}$	Shugart & Stulberg (1969)
		Yeast $tRNA^{Phe}$	Igo-Kemenes & Zachau (1971)
Sodium Bisulphite	C, i^6A, ms^2i^6A, s^4U, U, Cm	Yeast $tRNA^{Tyr}$	Furuichi et al (1970)
		Yeast $tRNA^{Tyr}$	Kucan et al (1971)
		E. coli $tRNA^{Met}_f$	Goddard & Schulman (1972)
		Yeast $tRNA^{Val}_2$	Chambers et al (1973)
		E. coli $tRNA^{Glu}_2$	Singhal (1974)
		E. coli $tRNA^{Trp}$	Seno (1975)
$TICl_3$ / NaI	C	Yeast $tRNA^{Met}_f$	Schmidt et al (1973)
Methoxyamine	C	Yeast $tRNA^{Val}$	Jilyaeva & Kisselev (1970)
		E. coli $tRNA^{Tyr}_{su+3}$	Cashmore (1970)
		Mutant E. coli $tRNA^{Tyr}_{su+3}$	Cashmore (1971)
		E. coli $tRNA^{Tyr}_{su+3}$	Cashmore et al (1971)
		Mouse myeloma $tRNA^{Met}_f$	Piper & Clark (1974)
		E. coli $tRNA^{Met}_f$	Chang (1973)
		E. coli $tRNA^{Leu}_1$	Chang & Ish-Horowicz (1974)
^{14}C Methylamine/bisulphite	C, Cm.	E. coli $tRNA^{Met}_f$	Schulman et al (1974)
Kethoxal	G	Yeast $tRNA^{Phe}$	Litt (1969)
		E. coli $tRNA^{Phe}$, $tRNA^{Val}_1$	Litt & Greenspan (1972)
		Yeast $tRNA^{Leu}$	Hawkins & Chang (1974)
N-Acetoxy-2-acetylamino fluorene	G	E. coli $tRNA^{Met}_f$	Fujimura et al (1972)
		Yeast $tRNA^{Tyr}$	Pulkrabek et al (1974)
Carbodiimide reagents :	U, G, rT, , D	Yeast $tRNA^{Ala}$	Brostoff & Ingram (1967)
		E. coli $tRNA^{Tyr}_{su+3}$	Chang et al (1972)
		Yeast $tRNA^{Val}_1$	Vlasov et al (1972)
		E. coli $tRNA^{Met}_f$	Chang (1973)
		E. coli $tRNA^{Leu}$	Chang & Ish-Horowicz (1974)
		Yeast $tRNA^{Phe}$	Rhodes (1975)
^3H - labelling	Purine nucleotides	Yeast $tRNA^{Phe}$	Gamble & Schimmel (1974)
Monoperphthalic acid	A	Yeast $tRNA^{Phe}$	Cramer et al (1968)
		Yeast $tRNA^{Phe}$, $tRNA^{Ser}$	Cramer (1971)
Nitrous acid	G, A, C	Yeast $tRNA^{Ala}$	May & Holley (1970)
		Yeast $tRNA^{Ala}$	Nelson et al (1967)
Photohydration	C, U	Yeast $tRNA^{Ala}$	Schulman & Chambers (1968)
Irradiation at 335 n.m.	s^4U	E. coli $tRNA^{Val}_1$	Favre et al (1969)
		E. coli $tRNA^{Met}_f$	Berthelot et al (1972)
		E. coli tRNAs	Carre et al (1974)
Cyanogen Bromide Parachloromercuribenzoate	s^4U	E. coli $tRNA^{Phe}$	Pal et al (1972)
		E. coli $tRNA^{Tyr}$, $tRNA^{Met}_f$	Walker & Rajbhandary (1972)
Iodine	s^4U, ms^2i^6A.	E. coli $tRNA^{Tyr}$, $tRNA^{Phe}$, $tRNA^{Val}$	Lipsett & Doctor (1967)
		E. coli $tRNA^{Phe}$	Faulkner & Uziel (1971)
		E. coli $tRNA^{Tyr}$	Gross & Czerny (1973)

A number of chemical reagents meet these requirements and have been used for the chemical modification of tRNA. For a review see Brown (1974). In addition to being specific for single stranded regions some reagents are inhibited by base stacking. TABLE 1 lists studies on the chemical modification of tRNA, relevant to the tertiary structure. Some general points emerge on reviewing these data:-

(i) There are no reports of chemical modification of bases that are involved in helical regions of the cloverleaf model.

(ii) In almost all of the cases listed, the basesin Loop IV have been found to be resistant to chemical modification. An exception is the modification of E. coli tRNA $^{Tyr}_{Su_3}$+ with a carbodiimide reagent (Chang et al, 1972), both the guanosine and uridine residues in this loop being partially modified, and the modification of E. coli tRNA$^{Glu}_{2}$ with bisulphite (Singhal, 1974), the C residue of Loop IV being modified.

(iii) All attempts to modify bases in the anticodon have been successful, indicating that the anticodon is exposed in the tertiary structure. Other bases in the anticodon loop have been found to be at least partially reactive. (Schulman & Chambers, 1968; Cashmore et al, 1971; Chang et al, 1972; Chang, 1973; Chang & Ish-Horowicz, 1974).

(iv) The two constant guanosine residues in Loop I appear in most cases to be unavailable for modification (Litt, 1969; Litt & Greenspan, 1972; Brostoff & Ingram, 1967; Chang et al, 1972; Chang, 1973; Chang & Ish-Horowicz, 1974), although modification of one or both of these guanosine residues has

been observed (Fujimura et al, 1972; Pulkrabek et al, 1974; Vlasov et al, 1972; Rhodes, 1975). Where the purine nucleotide R in the constant sequence AR is G, this is usually not available for modification (Litt, 1969; Litt & Greenspan, 1972; Fujimura et al, 1972; Pulkrabek et al, 1974; Brostoff & Ingram, 1967; Vlasov et al, 1972; Chang, 1973; Chang & Ish-Horowicz, 1974; Rhodes, 1975). This guanosine residue reacts to a small extent on modification of E. coli $tRNA^{Tyr}_{su_3^+}$ with a carbodiimide reagent (Chang et al, 1972), but in the case of yeast $tRNA^{Tyr}$, only becomes available for modification by N-acetyl-2-acetylaminofluorene at elevated temperatures, (Vlasov et al, 1972).

Nucleosides in the α and β regions of Loop I appear to be available for modification in all of the cases listed, but the constant adenosine residues in Loop I do not seem to be available for modification (Cramer, 1971).

(v) The bases of Loop III, in tRNAs with a small extra loop, appear on the whole to be unavailable for modification. Exceptions are the modification of U48 in E. coli $tRNA^{Met}_f$ (Chang, 1973) and U47 in yeast $tRNA^{Phe}$ (Rhodes, 1975) with a carbodiimide reagent, and the photohydration of U48 and C49 in yeast $tRNA^{Ala}$ (Schulman & Chambers, 1968). In tRNAs with a large extra loop, modification of the bases at the end of the loop does seem to occur (Chang & Ish-Horowicz, 1974; Chang et al, 1972; Cashmore, 1970). Partial modification of bases presumed to be involved in base pairing interactions in Loop III of E. coli $tRNA^{Tyr}_{su_3^+}$ have been reported (Cashmore, 1970; Chang

et al, 1972). Modification of the constant pyrimidine nucleotide Y in the extra loop has not been reported.

(vi) In all cases listed where tRNAs have been modified with cytidine - specific reagents, the two cytidine residues at the CCA_{OH} end have been found to be available for modification, indicating that these residues are exposed in the 3-dimensional structure. The terminal adenosine residue has also been found to be modified by monoperpthalic acid in yeast $tRNA^{Ser}$ and yeast $tRNA^{Phe}$ (Cramer et al, 1968; Cramer, 1971). There is very little evidence about the reactivity of the nucleotide fourth from the 3' end of tRNA, but it does not seen to be reactive in yeast $tRNA^{Ser}$ and yeast $tRNA^{Phe}$ (Cramer et al, 1968; Cramer, 1971).

(vii) There is no evidence to suggest that the two nucleotides between stems a & b and the nucleotide between stems b & c are reactive except in the case of the -SH group of s^4U which appears in position 8 in many bacterial tRNAs (Carré et al, 1974).

1.3.3.3. Enzyme Dissection.

This technique is similar in theory to that of chemical modification, and relies on the fact that the initial sites of cleavage of a tRNA molecule by a nuclease are likely to be in exposed regions of the 3-dimensional structure. Limiting conditions of enzyme digestion are employed, i.e. usually in the presence of Mg^{2+} at low temperatures.

Table 2 shows some of the results obtained using different nucleases. Loops I and II have been found to be cleaved the most

TABLE 2.

ENZYME DISSECTION OF tRNA.

ENZYME	tRNA	PRIMARY SITES OF CLEAVAGE	REFERENCE
T_1	E. coli $tRNA^{Met}_f$	Loop I	Seno et al (1969)
T_1	Yeast $tRNA^{Phe}$	Loop I then much more slowly Loop IV	Schmidt et al (1970)
T_1	Yeast $tRNA^{Phe}$	Loop I	Streeck & Zachau (1971)
T_1	Yeast $tRNA^{Phe}$	Loop I then more slowly Loop IV	Samuelson & Keller (1972)
Panc	Yeast $tRNA^{Phe}$	Loop I, removed of CCA_{OH} end	Harbers et al (1972)
T_1	Yeast $tRNA^{Tyr}$	Loop II	Hashimoto et al (1969)
T_1	Yeast $tRNA^{Ala}$	Loop II	Penswick & Holley (1965)
T_1	Yeast $tRNA^{Ala}_2$	Loop II	Imura et al (1969)
T_1	Yeast $tRNA^{Val}_1$	Loop II	Bayev et al (1967)
Panc	Yeast $tRNA^{Val}_1$	Loop I	Mirzabekov et al (1969)
T_1	E. Coli $tRNA^{Tyr}_2$	Loop III and more slowly Loop IV	Seno & Nishimura (1971)
T_1	Yeast $tRNA^{Ser}$	Loop II, then removal of CCA_{OH}	Streeck & Zachau (1971)

readily. Although Loop IV almost always contains a guanosine residue, it is cleaved much more slowly than Loops I and II. Cleavage of Loop III has only been reported in the case of E. coli $tRNA^{Tyr}_2$ (Seno & Nishimura, 1971).

1.3.3.4. Oligonucleotide Binding as a Probe of the 3-D Structure of tRNA.

This technique pinpoints regions of tRNA that are able to form a double helix with a complementary oligonucleotide and therefore must be exposed in the tertiary structure. Regions of tRNA involved in base pairing and strained single stranded regions will not be available for binding to complementary oligonucleotides.

Oligonucleotide binding has been used to study the conformation of several tRNAs. These include yeast $tRNA^{Leu}_3$ (Uhlenbeck et al, 1974) and $tRNA^{Phe}$ (Cameron & Uhlenbeck, 1973; Eisinger & Spahr, 1973; Pongs et al, 1973) and E. coli $tRNA^{Met}_f$ (Högenauer, 1970; Uhlenbeck et al, 1970; Uhlenbeck, 1972), $tRNA^{Tyr}$ (Uhlenbeck, 1972) and $tRNA^{Ileu}$ (Schimmel et al, 1972). In all of these cases, the anticodon and the base adjacent to its 5' end have been found to be available for duplex formation. The base adjacent to the 3' end of the anticodon seems to be unavailable for binding. Other regions that appear to be available for binding of complementary oligonucleotides are the CCA_{OH} end and non-hydrogen bonded regions of Loops I and III. Lower values have been obtained for the association constants of complementary tetranucleotides to Loop I than would be expected. This may imply that Loop I is in a strained conformation and may be involved in tertiary structure interactions. However, in the cases of E. coli $tRNA^{Ileu}$ and yeast $tRNA^{Phe}$ it may be due to the presence of D

which is unable to fit into an RNA double helix.

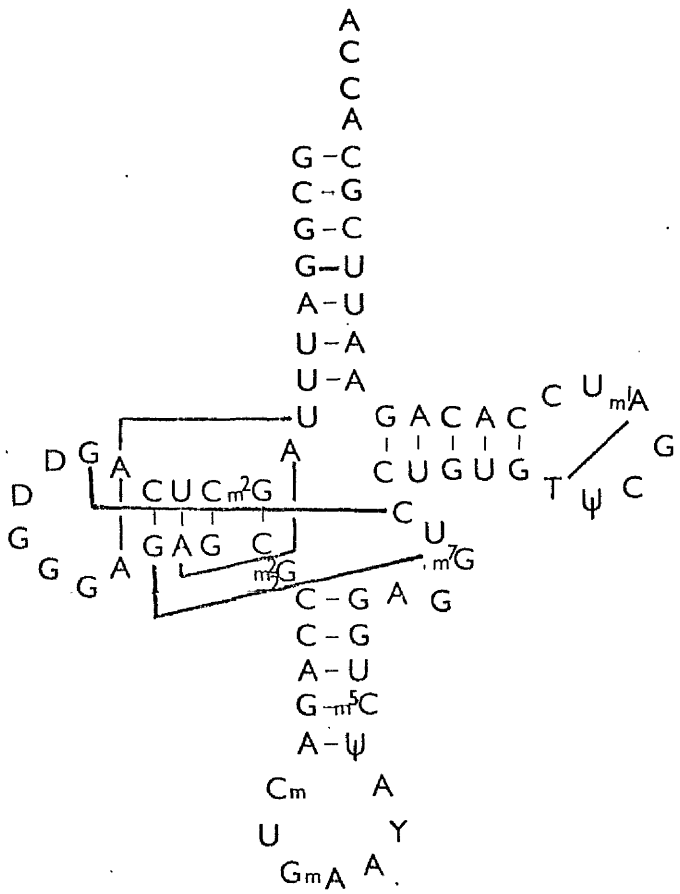
The majority of such studies indicate that Loop IV of most tRNAs is buried in the tertiary structure, i.e. not available for the binding of complementary oligonucleotides. However, Pongs et al, (1973) has observed the binding of complementary oligonucleotides to this loop, although this conflicts with the results of Cameron & Uhlenbeck (1973).

1.3.3.5. Physical Techniques.

Several physical techniques have been used to investigate the conformation of tRNA. Some of these provide relatively little direct evidence about the conformation, but they may be useful in comparing conformations of tRNA molecules, e.g. in deciding whether the conformations of all tRNAs are similar (Thomas et al, 1973a), and whether the conformation changes on aminoacylation (Thomas et al, 1973b) and denaturation (Webb & Fresco, 1973). Circular dichroism and optical rotary dispersion (Willick et al, 1973), spin labelling (Schofield et al, 1970) and fluorescent dye binding (Urbanke et al, 1973)are also principally useful in such comparisons.

Other techniques provide information about the overall size and shape of the molecule, e.g. Low angle X-ray scattering and sedimentation studies, (Lake & Beeman, 1967, 1968; Connors et al, 1969; Henley et al, 1966). More direct information about the conformation can be obtained from N.M.R. and fluorescence studies. Using such techniques, it is possible to investigate the environment of particular bases in tRNA e.g. of methylated bases (Kan et al, 1974; Koehler & Schmidt, 1973; Smith et al, 1969), of the Y nucleoside (W) in yeast $tRNA^{Phe}$ (Beardsley et al, 1970) and of the CCA_{OH}end (Ward et al, 1969; Maelicke et al, 1974). However,

FIG. 2.



—— tertiary structure interactions

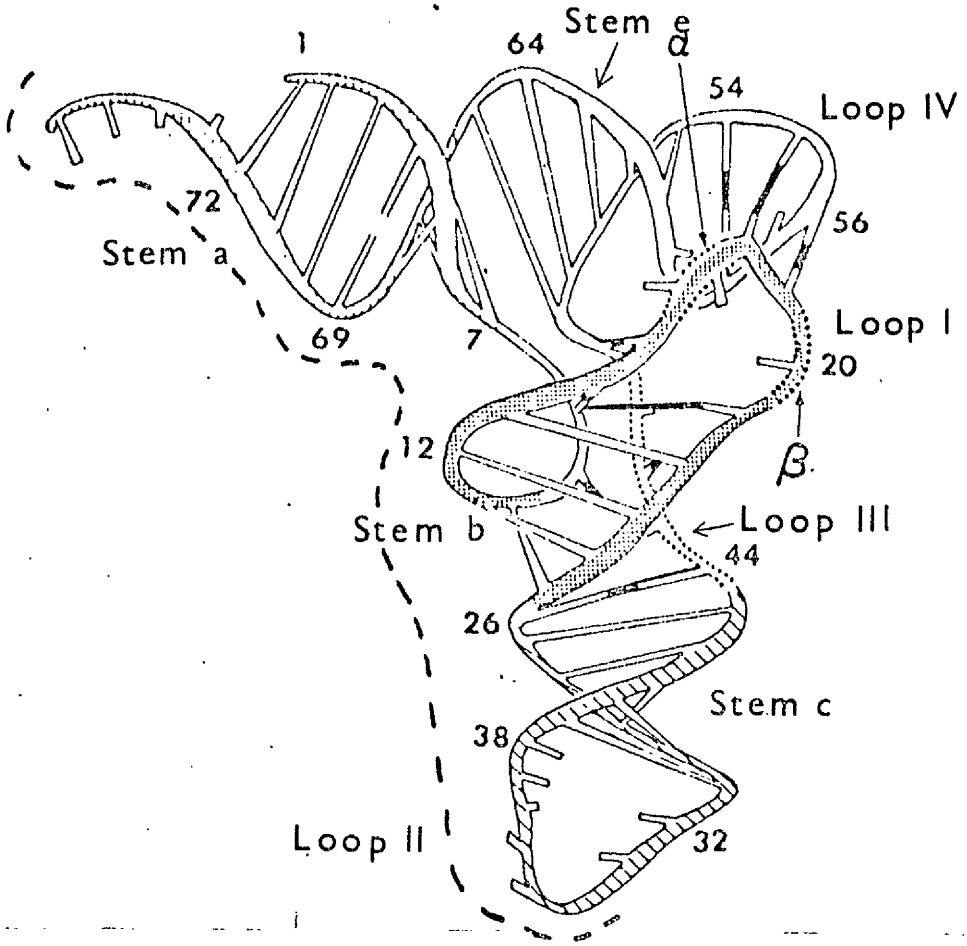
Tertiary structure interactions proposed for Yeast $tRNA^{Phe}$ by Kim et al (1974b) and Robertus et al (1974a).

the big breakthrough in the study of tRNA tertiary structure has come from X-ray diffraction studies. The 3-dimensional structure of yeast $tRNA^{Phe}$ determined to a resolution of 3Å has been reported (Kim et al, 1974b; Robertus et al, 1974a), and recently to a resolution of 2·5Å (Ladner et al, 1975a). This should enable the remaining ambiguities of tertiary structure to be resolved.

The models proposed by the two groups have much in common (FIG.2.). FIG.3 is a schematic diagram illustrating the folding of the yeast $tRNA^{Phe}$ molecule (Kim et al, 1974a). The models involve hydrogen bonding interactions implied by the "cloverleaf" structure plus extra "tertiary structure" hydrogen bonding interactions many not of the standard Watson-Crick type (Klug et al, 1974). The molecule is L-shaped, with Loop II at one end of the L, and the CCA_{OH} end at the other end. Loops I and IV interact with each other at the corner. Each arm of the L contains a column of stacked bases. Part of the stacking is due to the fact that the helical stem regions are aligned along the arms of the L, but some contribution is also made by the loop regions.

The helix of stem b is augmented by base pairs involving some of the bases in Loop I. A14, A21 and U8 are involved in a base triple interaction and the helix is further augmented by a non-Watson-Crick base pair involving G15 and C48. Stacked on top of this helix is a base from Loop IV, either U59 or m'A58. Loop II is stacked on stem c, in the manner suggested by Fuller and Hodgson (1967), on the 3' side of the loop. The two bases of A44 and G45 appear to be stacked at the other end of stem b. Robertus et al, (1974a) propose that m_2^2 G26 intercollates between these two bases, while Kim et al, (1974b) propose a base pair involving m_2^2G26 and A44 .

FIG. 3.



Schematic representation of the tertiary structure of Yeast $tRNA^{Phe}$. (Kim et al, 1974a).

— — Proposed region of interaction with the cognate ligase (Rich, 1974).

The interactions stablising the conformation of the central region of the molecule include three base triple interactions. These involve A14, A21 and U8, A9, A12 and A23, and C13, G22 and m^7G46. The non-standard base pair involving G15 and C48 also helps to maintain the integrity of this region. Kim et al, (1974b) also propose a base pair involving m_2^2G26 and G45.

There is some disagreement about the exact nature of the interactions holding Loops I and IV together. The arrangement of bases is probably as shown in the schematic diagram, FIG.3. The helix of stem d is extended by a base pair involving T54 and m'A 58. Kim et al, (1974b), have suggested that 55 and G18 form a base pair. C56, at the extreme end of Loop IV is close enough to G18 and G19 to form a base pair with one of them. Robertus et al, (1974) postulate the involvement of G18 in such a base pair, while Kim et al, (1974b) favour the involvement of G19. Thus Loop IV is narrow and tightly knit, all the bases protruding inwards, and the distance between the polynucleotide chains being little greater than that expected in an RNA double helix.

1.3.3.6. Correlation of the Evidence Available about the Tertiary Structure of tRNA.

The tertiary structure of all tRNAs can be assumed to be similar to that determined for yeast $tRNA^{Phe}$. The results of chemical modification, enzyme dissection and oligonucleotide binding studies discussed earlier in this chapter indicate that in the teritary structure of tRNA:-

(i) The CCA_{OH} end and the anticodon are exposed. This is consistent with the models proposed by Robertus et al (1974) and Kim et al (1974b). The fact that three of the other

four bases in Loop II point inwards (see schematic diagram, FIG.3) explains their partial reactivity to chemical reagents.

(ii) The bases of Loop IV are buried. This is consistent with the models proposed for yeast $tRNA^{Phe}$.

(iii) The variable (α and β) regions of Loop I are exposed, while the rest of the bases of this Loop are buried. In the models proposed from the results of X-ray analysis the variable bases are the only ones that point outwards into the medium.

(iv) The extra loop Loop III is partially exposed. The models proposed by Robertus et al (1974a) and Kim et al (1974b) have tertiary interactions involving some bases of this loop. These bases have been found not to be chemically reactive. However, in Yeast $tRNA^{Phe}$ at least, the rest of the bases in this loop are available for reaction.

(v) In the models proposed, U8, A9 and m_2^2G26 are involved in tertiary structure interactions. This is consistent with the lack of reactivity of the nucleotide between stems a and b (m_2^2G26 in Yeast $tRNA^{Phe}$)and A9. However, in E. coli tRNAs, S^4U8 does appear to be available for modification. This can be explained by the fact that the base triple U8, A14, A21 does not involve the carbonyl group attached to carbon 4 of the uracil ring (Kim et al, 1974a), and so, in E. coli tRNAs, where this is replaced by a thiono group, this group is available for chemical modification.

N.M.R. studies on $tRNA^{Phe}$ have indicated that of the various modified bases, m_2^2G and T, are less mobile than would be expected from the cloverleaf model. (Kan et al, 1974). In the models

proposed for Yeast $tRNA^{Phe}$ by Robertus et al (1974a), and Kim et al (1974b) both of these bases are involved in tertiary structure hydrogen bonding interactions.

The models suggest that U8 and C13 stack on each other and therefore S^4U8 and C13 in E. coli $tRNA^{Val}$ and $tRNA^{Met}_f$ would be close enough to allow the photodimerisation reation reported by Favre et al (1969) and Berthelot et al (1972). The CCA_{OH} end and anticodon are about 77 Å apart (Rich, 1972) which is greater than the 40 Å required by the singlet - singlet energy transfer between the Y base of Yeast $tRNA^{Phe}$ and flourescent compounds attached to the 3' terminus, and thus is consistent with results of Beardsley & Cantor (1970).

A number of tertiary structure interactions in the model involve constant features of tRNA structure (FIG. 1). These are the base pairs involving G15 and C48 and T54 and m^1A58, the base triple involving U8, A14 and A21, and the interactions between G18, G19 and Ψ55 and C56 of Loop IV.

A number of Mg^{2+} ions are known to be very strongly bound to tRNA. This number has been quoted as 4 (Danchin & Gueron, 1970) and 3 (Willick & Kay, 1971; Wolfson & Kearns, 1974). Mg^{2+} ions have been postulated to be associated with Loop III in Yeast $tRNA^{Phe}$ (Beardsley et al, 1970). There is evidence that a Mg^{2+} is associated with the s^4U region of several E. coli tRNAs (Pal et al, 1972; Jones & Kearns, 1974).

The implications for structure - function relationships suggested by the newly elucidated 3-dimensional structure are discussed in Section 1.4.

1.4. THE RELATIONSHIP OF STRUCTURE TO FUNCTION IN TRANSFER RNA.

Many attempts have been made to relate the structure of tRNA to its functions, particularly to the functions of tRNA during protein synthesis. Each tRNA must possess unique features which enable it to be recognised and charged only by its cognate aminoacyl-tRNA ligase. Transfer RNAs involved in peptide chain elongation must also possess common structural features which enable them to recognise and bind to the elongation factor - GTP complex. These features must be absent from initiator tRNAs which are unable to bind to this complex. Similarly, initiator tRNAs must possess features recognisable by initiation factors that are absent from tRNAs involved in chain initiation. All tRNAs capable of binding to ribosomes must possess common features that enable them to do so. Transfer RNAs that do not take part in protein synthesis need not have the features responsible for elongation factor and ribosome binding.

During the process of tRNA biosynthesis, enzymes involved in cleavage of pre-tRNA and in the synthesis of modified nucleosides must also be able to recognise specific sites on the tRNA molecule. The only regions of tRNA structure which have been definitely assigned a role, as yet, are the $-CCA_{OH}$ terminus, which accepts the cognate amino acid, and the anticodon, which interacts by hydrogen bonding with the complementary codon on the mRNA.

1.4.1. Specific Recognition of the Cognate Aminoacyl - tRNA Ligase.

The aspect of tRNA structure - function relationships most relevant to this thesis is that of specific recognition of the cognate aminoacyl - tRNA ligase. This subject has been reviewed

by Chambers (1971), Yarus (1969) and Zachau (1969). Some of the techniques used to investigate the structural features responsible for this specific recognition are discussed in this Section. Some of these techniques have also proved useful in investigation of the structural features of tRNA important in interactions with initiation and elongation factors, ribosomes and maturation enzymes.

1.4.1.1. Chemical Modification.

An attempt is made to correlate structural changes that occur on modification with loss of amino acid accepting ability. Thus areas essential for this particular function can be distinguished from non-essential areas. However, various criteria must be satisfied before chemical modification can be used as an effective probe of structure - function relationships (Chambers, 1971).

a) The modification must be well defined, and it must be possible to locate the position of modifications in the tRNA molecule.
b) It must be possible to separate active and inactive molecules, so that modifications responsible for loss of function can be pinpointed.
c) To get an accurate picture of the changes that occur, and which of these can be associated with loss of activity, it is necessary to start with pure, fully active tRNA.
d) It is important to know whether the modifications that occur alter the tertiary structure of the tRNA e.g. by the incorporation of bulky groups into the tRNA. If such a conformational change does occur, due account must be taken of this when attempts are made to correlate loss of function with modification.

However, even when these criteria have been satisfied, much of the data obtained from chemical modification of tRNA has proved difficult to interpret. In some cases, all types of modification that occur are found in both active and inactive fractions, indicating that the modifications have caused an alteration of the kinetic parameters Km and V_{MAX} rather than a total loss of activity. In such a situation, two problems arise.

a) It is not possible to say that the specific recognition site has been inactivated. It may be that part of the tRNA, not at the specific recognition site, which normally comes into contact with the ligase has been altered (chemically or conformationally), so that close contact of the tRNA with the ligase is inhibited.

b) Without further investigation, it is difficult to say which of the many modifications that may have occurred is responsible for the altered kinetic parameters.

Most of the reagents that have been used for modification of tRNA are specific for single stranded regions and are only likely to destroy the ligase recognition site if it involves a single stranded region. It is possible that the reason why chemical modification has been able to provide relatively few answers about the position of the ligase recognition sites is that these are present in double stranded regions. In general, the data from chemical modification experiments has made it possible to discount certain regions of tRNA as positions of recognition sites.

1.4.1.2. Enzymatic Dissection.

Transfer RNA can be dissected into fragments of various sizes by nuclease digestion under a variety of conditions. Fragments and combinations of fragments may then be tested for amino acid

accepting activity e.g. Seno et al (1969) and Schmidt et al (1970). Fragments may also be tested as competitive inhibitors of the charging reaction (Stulberg & Isham, 1967). The major disadvantage of this approach is that it is impossible to guarantee that the fragments assume the same conformation as in the native tRNA molecules. A fragment may contain the ligase recognition site, but because of a non-native conformation, this may not be available for interaction with the enzyme.

1.4.1.3. Comparison of the Primary and Secondary Structures of Isoacceptors.

Many organisms possess enzymes that will in vivo charge two different tRNAs (isoacceptors) with the same amino acid. The structual features common to these isoacceptors, after excluding features common to all tRNAs, are possible components of the specific ligase recognition sites (see Chambers, 1971). The technique can be further extended by comparing the structures of tRNAs from several different organisms that can be charged by the same enzyme in vitro (heterologous charging) (see Roe & Dudock (1972)). Using highly purified components it is possible in vitro to obtain intra-specific non-cognate charging (Yarus, 1972).

1.4.1.4. Isolation of Mutant tRNAs.

Supressor tRNA genes allow the possibility of genetic analysis so that the effect of sequence changes on tRNA function can be studied (Smith, 1972). The only supressor tRNA that has been used extensively in this way as yet is E. coli $tRNA^{Tyr}_{su^+_3}$. It is possible to select for tyrosine supressor mutants that can be

charged with another amino acid. Many amber mutations are known that are not supressed by $tRNA^{Tyr}_{su_3^+}$. Mutants of su_3^+ that can supress such amber mutations must contain altered $tRNA^{Tyr}_{su_3^+}$ molecules that are chargeable with another amino acid, this amino acid being acceptable, when inserted in response to UAG, in the functional gene product. The mutant tRNA may be isolated and the sequence changes responsible for altered recognition determined. The region of the tRNA molecule in which sequence changes have occurred can thus be implicated in the ligase recognition site.

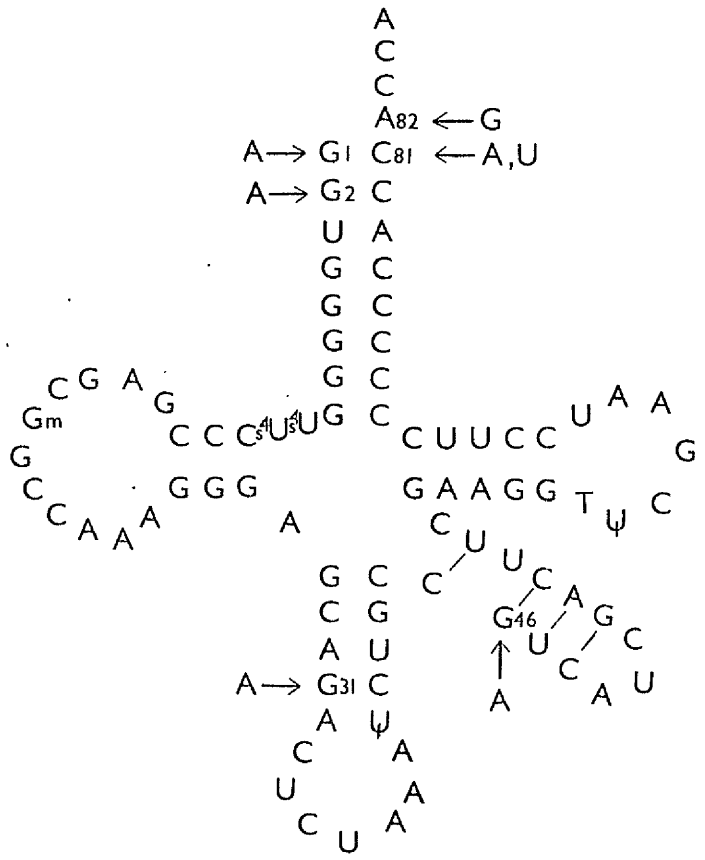
1.4.1.5. Complexes of aminoacyl - tRNA Ligases with tRNAs.

When tRNA forms a specific complex with its cognate aminoacyl-tRNA ligase, the regions of tRNA in contact with the ligase are protected from nuclease attack, and are also unavailable for the binding of complementary oligonucleotides. Although the protected regions of tRNA probably contain the ligase recognition site, the technique is primarily useful in determining the overall topography of the tRNA - ligase complex (Hörz & Zachau 1973; Yaniv & Gros, 1969; Dube, 1973b; Schoemaker & Schimmel, 1974).

1.4.1.6. The Ligase Recognition Site.

A vast amount of data has accumulated from experiments designed to locate the ligase recognition site in various tRNAs. In interpretation of such data, it is important to remember that the form and location of the site on a tRNA molecule, recognised by its cognate ligase, may vary from tRNA to tRNA. Therefore, it is important to glean as much information as possible about the interactions between each tRNA and its cognate aminoacyl-tRNA ligase. Two systems that have been studied in detail are discussed below.

FIG. 4.



Mutants of E.coli $tRNA^{Tyr}_{su_3^+}$ deficient in tyrosine acceptor activity.

1.4.1.6.1. E. Coli $tRNA^{Tyr}_{su_3^+}$, E. coli tyrosyl-tRNA Ligase.

Mutants of $tRNA^{Tyr}_{su_3^+}$ deficient in tyrosine acceptor activity have been isolated and are indicated in FIG 4. Mutant tRNAs with the base substutions G31 ⟶ A31 (Abelson et al, 1970) and G46 ⟶ A46 (Smith, 1972) have altered kinetics of aminoacylation. This has been attributed to an altered conformation of the tRNA. Some mutants of su_3^+ are able to supress amber mutations by the insertion of an amino acid other than tyrosine i.e. mutants containing the single base substitutions G1 ⟶ A1, G2 ⟶ A2, C81 ⟶ A81, C81 ⟶ U81, A82 ⟶ G82 (Celis et al, 1973). Four of these mutant tRNAs are able to insert either tyrosine or glutamine in response to the codon UAG. The mutation A82 ⟶ G82 alters the specificity of the tRNA from tyrosine to glutamine.

Cashmore (1970) has shown that modification of a large number of cytosine residues in $tRNA^{Tyr}_{su_3^+}$ does not abolish tyrosine accepting activity. Thus the only region implicated in ligase recognition is the amino acid acceptor stem (stem a).

1.4.1.6.2. Yeast $tRNA^{Phe}$, Yeast phenylalanyl-tRNA Ligase.

Roe & Dudock (1972) have compared the structures of eight tRNAs chargeable by yeast phenylalanyl-tRNA ligase and it has emerged that ten nucleotides are common to each of these tRNAs. These are

```
C U C G A
| | | |
G A G C
```

in stem b, and the adenosine residue at the fourth position from the 3' end. Kem et al (1972) suggest that the extra loop may also be important in interaction with Yeast phenylalanyl-tRNA ligase as seven tRNAs chargeable by this enzyme have the common sequence R Ψ G^*U^* C in the extra loop.

Chemical modification of yeast $tRNA^{Phe}$ with Kethoxal (Litt,

1971) and sodium borohydride (Igo-Kemenes & Zachau, 1969, 1971) indicate that G20 and G34 (see FIG 2) may be involved in ligase recognition, but the W and two D residues are not. That W is not involved in ligase recognition has been confirmed by the work of Thiebe & Zachau (1968).

Cleavage of yeast $tRNA^{Phe}$ in Loops I and IV (Schmidt et al, 1970; Thiebe et al, 1972) or in Loop II (Phillipsen et al, 1968) does not completely abolish phenylalanyl accepting activity. Fragments of yeast $tRNA^{Phe}$ have been tested for phenylalanine accepting activity (Thiebe et al, 1972). Removal of parts of stems b and e causes complete loss of activity as does the removal of G19, G20 and W37 in the same molecule.

When complexed with yeast phenylalanyl tRNA ligase, Loops I and II are protected from nuclease attack (Hörz & Zachau, 1973). The regions implicated in ligase recognition are thus stem b, the base fourth from the 3' terminus and possibly Loop I, Loop II and Loop III, and stem e.

1.4.1.6.3. A General Discussion of Specific Ligase Recognition.

In neither of the two examples discussed above can the ligase recognition site be unambiguously pinpointed. For most other tRNAs, evidence is even more scanty, and often conflicting.

The regions of tRNA that would seem initially to be the most suitable condidates for the ligase recognition site are those regions which have different structures in different tRNAs, namely the anticodon, Loop III and the α and β regions of Loop I. However, there are several reasons for discounting each of these regions as recognition sites in all tRNAs, although there is some evidence that they may be important in ligase recognition in some tRNAs. In many cases, tRNAs specific for the same amino acid with

different anticodons may be charged by the same enzyme. The anticodon may be cleaved enzymatically, or its bases chemically modified without total loss of amino acid accepting activity in many cases (Thiebe & Zachau, 1969; Hashimoto et al, 1969), while in other cases excision of anticodon bases (Mirzabekov et al, 1971) or modification of anticodon bases (Squires & Carbon, 1971a, 1971b; Schulman & Goddard, 1973; Chambers et al, 1973 has been found to destroy amino acid accepting activity.

While the extra loop (Loop III) has been implicated in the recognition of yeast phenylalanyl-tRNA ligase, (Kern et al, 1972), this loop must be discounted as a universal recognition site, because E. coli glutaminyl-tRNA synthetase can recognise and charge with glutamine both E. coli $tRNA^{Gln}$, which has a small Loop III, and mutants of E. coli $tRNA^{Tyr}_{su_3^+}$ with single base substitutions, all of which have a large Loop III (Celis et al, 1973). Chemical modification of bases in the α and β regions of Loop I does not usually result in loss of amino acid accepting activity (Igo-Kemenes & Zachau, 1969; Cashmore, 1970), but in at least one case (Shugart & Stulberg, 1969) such a loss of activity does result.

Crothers et al (1972) have suggested that the nucleotide fourth from the 3' terminus of tRNA may constitute a discriminator site in tRNA. Examination of the sequences of several tRNAs from different species led to the conclusion that this nucleotide is likely to be the same in tRNAs coding for chemically similar amino acids. This nucleotide has been implicated in the synthetase recognition site of yeast phenylalanyl-tRNA ligase (Roe & Dudock, 1972; Kern et al, 1972), and it has been found that a base change in this position from A to G in E. coli

$tRNA^{Tyr}_{su3^+}$ leads to altered recognition, from tyrosyl-tRNA ligase to glutaminyl-tRNA ligase. This change in specificity is consistent with Crothers' suggestion, because tRNAs specific for glutamine usually have a G residue in this position.

A variety of intraspecific misacylations can occur with highly purified components in vitro. Yarus & Mertes (1973) have found that there is a tendency for tRNAs to be misacylated with amino acids that are chemically similar to the cognate amino acid, and have suggested that an appreciable part of the free energy of binding of the synthetase to the tRNA comes from interaction with structural features common to at least several tRNAs, coding for chemically similar amino acids. In a similar study, Pachmann et al (1973) have studied the binding of yeast $tRNA^{Ser}$ and yeast $tRNA^{Phe}$ to cognate and non-cognate ligases. They found that yeast phenylalanyl-tRNA ligase has one site for the binding of both $tRNA^{Phe}$ and $tRNA^{Ser}$, and seryl-tRNA ligase has two sites for the binding of both tRNAs. They concluded that unspecific interaction may be an important initial step preceeding the specific binding and recognition of tRNA by the synthetase. Myers et al (1971) have compared the K_ms of several aminoacyl-tRNA ligases for their cognate tRNAs and found them to be numerically similar. They have inferred from this that the interactions involved in the binding of each of these tRNAs to its cognate ligase must be very similar and that electrostatic interaction between the enzyme and the phosphate groups of the tRNA accounts for a major portion of the binding energy of these molecules.

These observations, particularly the fact that intraspecific misacylation can occur in vitro, raises the question of whether recognition of a tRNA by its cognate ligase is as specific as was

at first thought. However, if this is not the case, for faithful translation to occur, there must be some mechanism *in vivo* for recognising and deacylating incorrectly charged tRNAs. The ability of aminoacyl-tRNA ligase to deacylate incorrectly charged cognate tRNAs, and tRNAs charged incorrectly with the cognate amino acid, observed by several workers (Yarus, 1972; Ritter & Jacobson, 1972; Bonnet *et al*, 1972) may possibly fulfill this role *in vivo*.

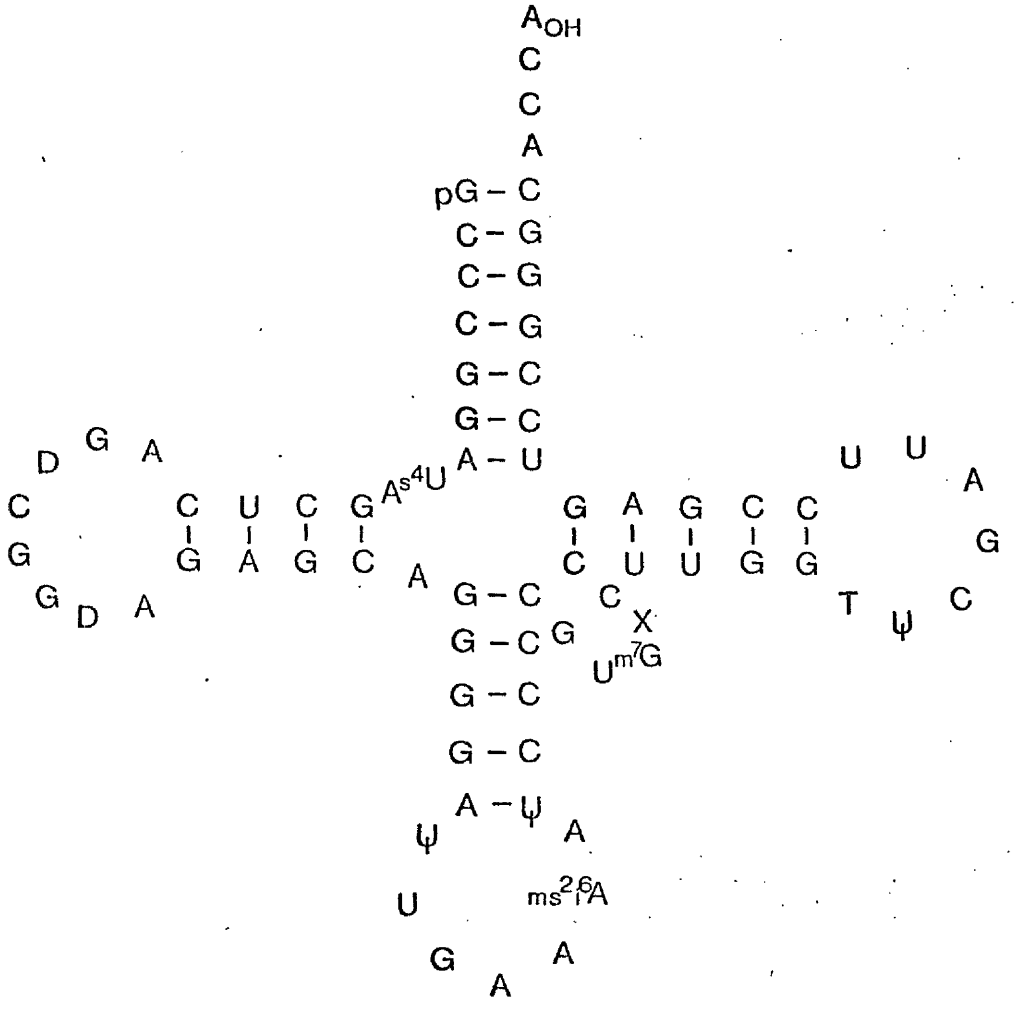
1.5. THE AIMS OF THE PROJECT.

The original aims of the project described in this thesis were as follows:-

(i) To use bisulphite modification in order to discover which cytidine residues are present in exposed single stranded regions of native *E. coli* $tRNA^{Phe}_2$.

(ii) To compare the cytidine residues available for modification in charged and uncharged *E. coli* $tRNA^{Phe}_2$, in an attempt to detect any conformational changes that may occur on aminoacylation.

(iii) To investigate the effects of bisulphite modification on the ability of $tRNA^{Phe}_2$ to be recognised and charged by its cognate aminoacyl-tRNA ligase, and if possible, to determine which of the modifications that occur are responsible for the loss of amino acid accepting activity, in an attempt to pinpoint the features responsible for recognition of the tRNA by its cognate aminoacyl-tRNA ligase.



E. coli $tRNA^{Phe}$

Barrell & Sanger (1969).

FIG. 5.

1.5.1. E. coli $tRNA^{Phe}_{2}$.

Pure E. coli $tRNA^{Phe}_{2}$ has been isolated by several methods:-

a) Reverse phase chromatography on RPC-1 and gel filtration on Biogel P2 (Kelmers, 1966).

b) Ion exchange chromatography on DEAE - Sephadex A-50 and reverse phase chromatography on RPC-1 (Nishimura et al, 1967).

c) Reverse phase chromatography on RPC-1 and RPC-2 (Shugart et al, 1968).

d) By making use of its specific binding to ribosomes in the presence of polyuridylic acid (Nirenberg & Leder, 1964).

e) Chromatography on benzoylated DEAE-cellulose and DEAE-Sephadex A-50 (Brown et al, 1972).

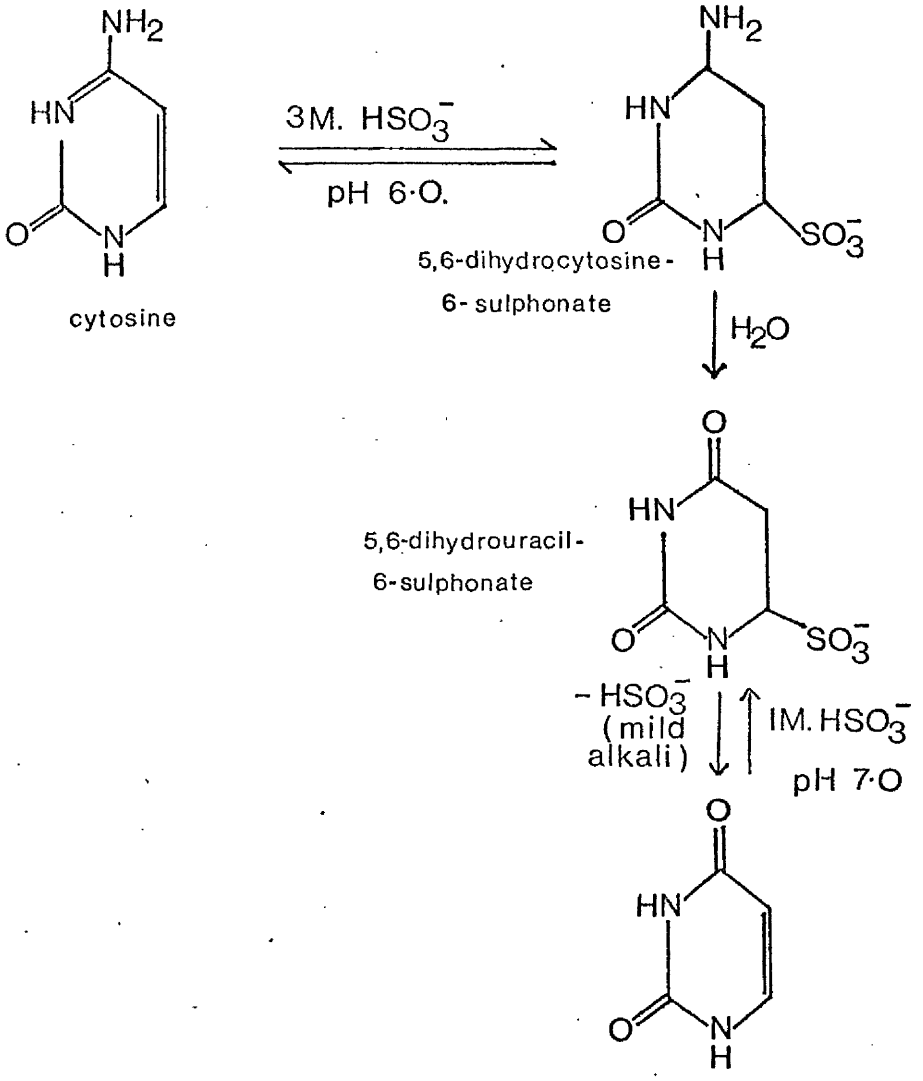
f) Chromatography on benzoylated DEAE-cellulose and reverse phase chromatography on RPC-5 (Huang & Mann, 1974).

The existence of two or three isoacceptor phenylalanine tRNAs in E. coli cells grown under normal conditions has been described (Pearson et al, 1971; Muench & Berg, 1966; Roy & Söll, 1968). Some purification procedures fractionate the isoacceptors better than others. The isoacceptor used in this project was E. coli $tRNA^{Phe}_{2}$ i.e. the phenylalanine isoaccepting tRNA eluted second from a benzoylated DEAE-cellulose column. Uziel & Gassen (1969) published the sequence of this tRNA, but this sequence has since been proved to be incorrect. Barrel & Sanger (1969) have determined the correct sequence. This is illustrated in FIG 5.

1.5.2. Chemical Modification Using Bisulphite.

In 1970, two different groups described the use of bisulphite as a modifying agent for cytosine, uracil and their derivatives (Hayatsu et al, 1970a, b; Shapiro et al, 1970a). The series of

FIG. 6.



Bisulphite modification of cytosine and uracil.

reactions involved for cytosine and uracil are indicated in FIG 6. The reaction between uracil and bisulphite to give 5, 6 dihydrouracil-6-sulphonate is optimal at pH 7, while formation of 5, 6 dihydrouracil sulphonate from cytosine is optimal at pH 6. The formation of the bisulphite adduct facilitates nucleophilic substitution at the exocyclic amino group, allowing substitution of -OH for - NH_2. The bisulphite adduct may be removed by treatment with weak alkali. In this way, bisulphite at pH 6 may be used to deaminate cytidine residues in RNA (Shapiro et al, 1970b). This method has been used in structural and functional studies of tRNA (Singhal, 1971; Kućan et al, 1971; Schulman & Goddard, 1973; Goddard & Schulman, 1972; Chambers et al, 1973; Singhal, 1974; Seno, 1975).

Goddard & Schulman (1972) reported that bisulphite mediated deamination of cytidine residues in RNA is strongly inhibited by ordered structure, and in almost all cases where bisulphite has been used to modify tRNA at temperatures lower than 37°C, only bases in single stranded regions have reacted. (The exception is C72 in yeast $tRNA^{Val}$ (Chambers et al, 1973)).

Bisulphite (1M bisulphite, pH7, 37°C) has also been used to modify uridine residues in tRNA (Furuichi et al, 1970). Some minor nucleosides that are found in tRNA have been reported to react with bisulphite. These include N^6-isopentenyladenosine (Furuichi et al, 1970; Hayatsu et al, 1972), pseudouridine (Singhal 1971, 1974), 4-thiouridine (Hayatsu & Inoue, 1971; Rao & Cherayil, 1974), 5-methylcytidine (Hayatsu et al, 1970) and 5-methylaminomethyl-2-thiouridine and 2-thiocytidine (Rao & Cherayil, 1974). The formation of a N^6-isopentenyladenosine-bisulphite adduct appears to occur in both 1M bisulphite, pH7,

at 37°C (Furuichi et al, 1970) and in 3M bisulphite, pH6, at 25°C (Kucan et al, 1971). Pseudouridine only reacts with bisulphite at high concentrations and at tempratures above 60°C. 5-methylcytidine, 5-methylaminomethyl-2-thiouridine, and 2-thiocytidine appear to be modified by bisulphite under conditions suitable for cytidine modification.

The sulphydryl group of 4-thiouridine may be removed by incubation in low concentrations of bisulphite (about 10^{-2} M) followed by treatment with mild acid or alkali (Hayatsu & Inoue, 1971). The reaction is dependent on oxygen, and is inhibited by high concentrations of bisulphite (> 1M) and pHs lower than 7. This reaction and several others including the oxygen-dependent cleavage of the glycosidic linkage of pyrimidine nucleosides (Kitamura & Hayatsu, 1974), the cleavage of phosphodiester bonds in DNA (Hayatsu & Miller, 1972), and the formation of a N^6 - isopentenyladenosine-HSO_3^- adduct. (Hayatsu et al, 1972) are thought to involve the $\cdot SO_3^-$ radical, formed from bisulphite in the presence of oxygen.

$$HSO_3^- + O_2 \longrightarrow \cdot SO_3^- + \cdot HO_2.$$

In high concentrations of bisulphite (> 1M), this free radical is probably quickly destroyed by the bisulphite ions surrounding it.

2.1. MATERIALS.

2.1.1. Chemicals.

Adenosine-5'-triphosphate (disodium salt), glutathione (reduced form), sodium bisulphite, streptomycin sulphate, L-phenylalanine and Trizma base were purchased from the Sigma Chemical Co. Ltd., Norbiton Station Yard, Kingston-upon-Thames, Surrey. 2-mercaptoethanol was purchased from Koch-Light Laboratories Ltd., Kolnbrook, Bucks. Bovine Serum Albumin was obtained from the Armour Chemical Co. Ltd., All other chemicals were of Analar grade and were obtained from B.D.H. Chemicals Ltd., Poole, Dorset.

2.1.2. Radiochemicals.

L-Phenyl (2,3-^{3}H) alanine (20 Ci/mmole) L-(U-^{14}C) Phenylalanine (450 mCi/mmole) and sodium hydrogen (^{35}S) sulphite (10·8-12·7 Ci/mmole) were obtained from The Radiochemical Centre, Amersham, Bucks.

2.1.3. Transfer RNA.

Soluble (^{32}P) ribonucleic acid from E. coli K12 CA265 was obtained from the Radiochemical Centre, and crude transfer RNA from E. coli K12 CA265 was supplied by the Microbiological Research Establishment, Porton Down, Salisbury, Wiltshire. Purified phenylalanine specific transfer RNA ($tRNA^{Phe}$) from E. coli MRE 600 (RNase negative) 15276 was purchased from the Boeringer Corporation, Lewes, East Sussex. This purified $tRNA^{Phe}$ incorporated 900-1100 pmoles phenylalanine per A_{260} unit.

2.1.4. Materials for Electrophoresis.

Whatman chromatography paper DE 81 (DEAE - cellulose paper) was obtained from McCulloch Bros., Glasgow. Whatman 52 and 3MM papers were purchased from Reeve-Angel Scientific Ltd., London. Cellulose acetate electrophoresis strips were purchased from Oxoid Ltd., Southwark Bridge Road, London. The constituents of the marker dye were supplied by Searle Scientific Services, High Wycombe, Bucks.

2.1.5. Materials for Autoradiography.

Kodirex X-ray film, 35x43 cm., was obtained from Kodak Ltd., Wythenshawe, Manchester.

2.1.6. Enzymes.

Ribonuclease T_1 (crystalline), manufactured by the Sankyo Company Ltd., Tokyo, Japan, was purchased through Calbiochem Ltd., 10 Wyndham Place, London. Pancreatic ribonuclease was obtained from the same source.

E. coli phenylalanyl-tRNA ligase was prepared as described by Stulberg (1967), the Hydroxylapatite Chromatography stages being omitted.

2.1.7. Column Chromatography Media.

Preswollen DEAE-cellulose (DE 52) was purchased from Whatman Biochemicals Ltd., Maidstone, Kent. Benzoylated DEAE-cellulose was supplied by the Boeringer Corporation (London)Ltd., Bell Lane, Lewes, East Sussex. RPC-5 was purchased from Miles Laboratories Ltd., Stoke Court, Stoke Poges, Slough. Sephadex G-100 was obtained from Pharmacia Fine Chemicals, 75 Uxbridge Road, London.

2.1.8. Scintillation Fluids and Other Materials for Scintillation Spectrometry.

Analar grade toluene was supplied by Koch-Light Ltd., Colnbrook, Bucks, as were 2,5 - diphenyloxozole (PPO) and 1,4 - di 2-[(5-diphenyloxazolyl)] benzene (POPOP). Hyamine hydroxide (a 10% solution in methanol) was obtained from Nuclear Enterprises, Sighthill, Edinburgh. Whatman 3MM paper discs (2·5 cm.) were obtained through Reeve Angel Scientific Ltd., London.

Toluene/PPO/POPOP was 5g. of PPO and 0·3g POPOP dissolved in 1 litre of Analar toluene.

Toluene/PPO was 5g of PPO dissolved in 1 litre of Analar toluene.

2.1.9. Miscellaneous.

E. coli MRE 600 Cells, used for the preparation of phenylalanyl-tRNA ligase, were purchased from the Microbiological Research Establishment. The cells were stored at -70°C prior to use.

Visking tubing was obtained from The Scientific Instrument Centre, 1 Leeke Street, London. Before use it was treated in the following way. The tubing was cut into suitable lengths and boiled twice in 50g/l sodium carbonate, then once in 0·05M EDTA pH 7·0 and once in distilled water. Each piece was autoclaved separately in distilled water in a small glass bottle.

2.2. METHODS.

2.2.1. Precautions against Nuclease Contamination.

The following precautions were observed:-

(i) Disposable plastic gloves were worn.

(ii) Buffers and other solutions were sterilised, either by autoclaving at 15 p.s.i for 20 mins., or by filtration.

(iii) Glassware and other apparatus in contact with RNA solutions was sterilised either by autoclaving (as above) or by immersion for 20 mins., in 15% w/v hydrogen peroxide, followed by ten washes with sterile distilled water.

(iv) Column chromedia were assumed to be sterile.

(v) Dialysis tubing was treated as described in Section 2.1.9.

2.2.2. Assay for Phenylalanine Accepting Activity.

Assays for phenylalanine accepting activity in fractions from columns, or chemically modified tRNAs, were routinely done in a total volume of 150 µl. The assay mixture contained 100 mM tris-HCl pH 7·5, 10mM $MgCl_2$, 10mM KCl, 10mM NH_4Cl, 4m M reduced glutathione, 2mM ATP, 6·7 mM radioactively labelled phenylalanine, 20 µg purified phenylalanyl-tRNA ligase and tRNA containing up to 300 pMoles of phenylalanine tRNA. Blanks containing water instead of tRNA were included with each batch of tRNA solutions being assayed.

The reaction was started by addition of the enzyme (diluted as required with Bovine Serum Albumin solution 6mg/ml.). After incubation at 37°C for 20 min., a 100 µl aliquot was removed from each tube onto Whatman 3MM filters. The RNA was precipitated onto the filters by immersing them in ice-cold 10% (w/v) trichloracetic acid for 10 min. Excess phenylalanine was removed by washing the filters twice for 20 mins in ice-cold 5%

trichloracetic acid. The filters were finally washed in methylated spirits and then dried.

When ^{14}C labelled phenylalanine was used, the specific activity was 50-200 Ci/Mole, and the filters were counted in a scintillation counter after addition of 5Ml. of toluene/PPO. When ^{3}H labelled phenylalanine was used, the specific activity was 1000 Ci/Mole, and the filters were counted after solubilisation (incubation with 0·5 ml. 10% hyamine hydroxide at 60°C for 20 min.) and addition of 10ml. of toluene/PPO/POPOP.

2.2.3. Preparation of Phe-tRNA^{Phe}.

Purified E. coli tRNA^{Phe} was charged with ^{3}H or ^{14}C labelled phenylalanine by using a scaled up version of the Assay technique described above. Phe-tRNA^{Phe} and uncharged tRNA were separated from the other components of the assay mixture using a DEAE-cellulose column.

The reaction was stopped after 20 min. by addition of $^{1}/10$ of the assay volume of 1M sodium acetate, pH 5·0, and immersion of the tube in ice. The assay mixture was applied to a DEAE-cellulose column which had been equilibrated with 50mM sodium acetate, pH 5·0, 0·1M NaCl. Up to 50 A_{260} units of tRNA were applied per cm^3 of packed DEAE-cellulose. The phenylalanyl-tRNA ligase and excess phenylalanine were eluted from the column by application of 50mM sodium acetate, pH 5·0, 0·35 M NaCl. When no further radioactivity or U.V. absorbing material could be detected in the eluate, a solution of 0·1·M sodium acetate, pH 5·0, 2·0M NaCl, 30% ethanol was applied to the column to elute the tRNA.

2.2.4. RNA Fingerprinting.

This has been reviewed by Brownlee (1972).

2.2.4.1. Enzymatic Digestion of RNA.

Aliquots of tRNA solution, containing 10-20 µg. of tRNA, were desalted by exhaustive dialysis against distilled water and lyophilised in siliconised tubes. The RNA was digested with either T_1 ribonuclease, to obtain oligonucleotides terminating in guanosine-3'-phosphate, or Pancreatic ribonuclease, to obtain oligonucleotides terminating in a pyrimidine nucleoside-3'-phosphate. The conditions for enzymatic digestion were the same for both T_1 and Pancreatic ribonucleases. An enzyme:substrate ratio of between 1:10 and 1:20 was used in 10mM tris-HCl buffer, pH 7·4, containing 1mM EDTA. The digestion was carried out in the drawn-out tip of a capillary tube in a volume of 5 µl for 30 min. in a humidified oven, at 37°C.

2.2.4.2. Two-dimensional Ionophoresis Fractionation Procedure.

The procedure described by Sanger *et al* (1965) was followed. A cellulose acetate strip (3 x 98cm.) was moistened with buffer (pH 3·5) containing 7M Urea, 5% (w/v) acetic acid, adjusted to pH 3·5 with pyridine. The point of application, about 10cm from the cathode end of the strip was blotted free of excess liquid and the digest applied as a spot. Spots of marker dye (a mixture of 2% Xylene cyanol F.F. (blue), 2% acid fuchsin (red), and 9% methyl orange (yellow)) were applied on each side of the digest. The remainder of the strip was then blotted, placed on the perspex supporting rack in the electrophoresis tank, and subjected to

electrophoresis at 4·5 Kilovolts until the blue dye had moved 40-45cm. for a pancreatic RNase digest or 55-60cm for a T_1 RNase digest. The tank contained white spirit as an insulator between the anode and cathode buffers. After electrophoresis, the cellulose acetate strip was removed from the tank, and excess white spirit was allowed to drip off. Oligonucleotides produced by T_1 and Pancreatic RNase digestion of E. coli $tRNA^{Phe}$ could be detected between the origin and the slowest pink marker dye.

The cellulose acetate strip was placed on the DEAE paper sheet (62 x 92cm.) along an origin line 10cm. from one end. A pad of 5 strips of Whatman 3MM paper (4 x 64cm), that had been soaked in distilled water, was then placed on top of the cellulose acetate strip and a glass plate placed on top of these in order to press the strips together evenly. Water from the paper pad passed through the cellulose acetate strip, carrying with it the negatively charged oligonucleotides. These bind strongly to the positively charged DEAE-cellulose paper. Cellulose acetate strips containing the products of pancreatic RNase degestion were positioned on the DEAE-cellulose paper with the origin of the cellulose acetate strip about 4cm. from the end of the DEAE-cellulose paper. The products of T_1 RNase digestion stretched for a distance of 60cm. along the cellulose acetate strip. It was therefore necessary to divide the strip into a short section (2·5cm. behind the origin to 12·5cm. in front) and a longer section. These two pieces were applied to two separate pieces of DEAE-cellulose paper.

After the transfer had been allowed to proceed for 20-30 min., the cellulose acetate and 3MM strips were removed, and the

DEAE-cellulose paper was dried. Urea was removed from the origin area by washing in ethanol for 2 min. The paper was dried in air and marker dye spots were applied to the origin on the DEAE-cellulose paper. The paper was completely wetted with 7% (v/v) formic acid. Due to the extreme fragility of wet DEAE-cellulose paper, this process was performed after the paper had been placed on a rack. The rack was placed slowly into the electrophoresis tank, the origin area being near the cathode compartment. A voltage of 1·1 Kilovolts was applied until the blue marker had travelled about 30-35cm (for a T_1 RNase digest) or 20-25cm (for a Pancreatic RNase digest) As considerable heat was generated during this process, the white spirit was cooled by the passage of tap water through cooling coils for the whole time. After electrophoresis, the papers (still on the racks) were removed from the tanks and dried thoroughly to ensure complete removal of formic acid, which tends to 'fog' photographic film.

2.2.4.3. Autoradiography.

The papers were marked with ^{35}S labelled ink, and cut to a suitable size for autoradiography with Kodirex X-ray film, i.e. no larger than 42x35cm. The marks made by the radioactive ink served both to identify the autoradiograph and to enable it to be aligned accurately with the DEAE-cellulose paper, so that the oligonucleotide spots could be located and excised.

The cut DEAE-cellulose papers were taped in contact with Kodirex X-ray film and stored in lead backed folders (0·5mm of lead) in a light-proof cabinet. Where more than 0·2 μCi of ^{32}P tRNA had been used, the films were developed after 24 hrs., and where less ^{32}P tRNA had been used, a longer time was allowed

before the films were developed.

The oligonucleotide spots were excised and counted using toluene/PPO (or toluene/PPO/POPOP for samples containing ^{35}S) scintillant, in a scintillation counter.

2.2.4.4. Estimation of the Percentage Molar Veild of each Oligonucleotide on a T_1 and Pancreatic RNase Fingerprint.

The number of phosphorus atoms in each oligonucleotide spot on a fingerprint containing a total of "n" spots can be calculated.

X = number of counts per minute of ^{32}P in a given oligonucleotide spot.

There are 76 P atoms per Molecule of E. coli $tRNA^{Phe}_2$.

Y = number of P atoms in an oligonucleotide

$$= \frac{X \times 76}{\sum X_{1 \rightarrow n}}$$

Some oligonucleotides may be present in greater than 100% yield (if there are more than one such oligonucleotide per molecule of tRNA), or less than 100% yeild, (the tRNA is less than 100% pure, or depurination has occurred during the fingerprinting procedure). When the composition of an oligonucleotide has been determined, the percentage molar yield of this oligonucleotide can be calculated. If Z is the expected number of P atoms in the oligonucleotide.

$$\text{Percentage Molar yield} = \frac{Y \times 100}{Z}$$

All of the oligonucleotides on each fingerprint, including the possible contaminants, were considered.

2.2.5. Determination of Oligonucleotide Composition.

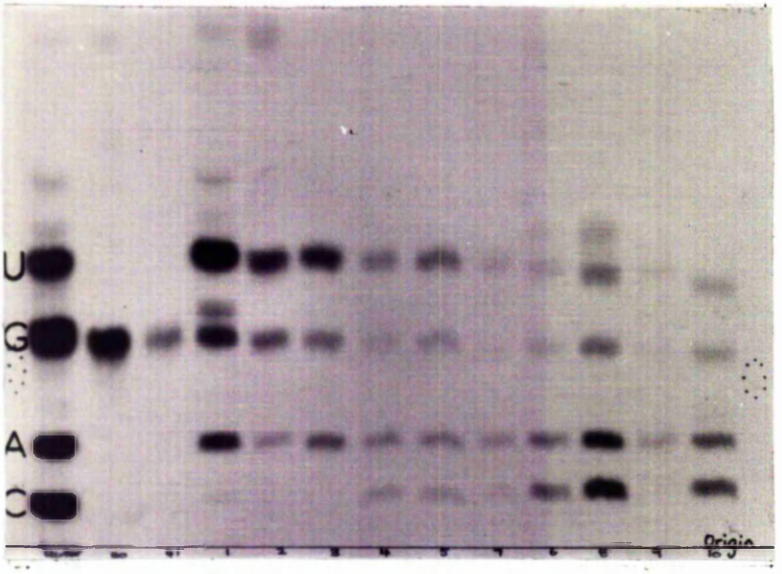
2.2.5.1. Elution of Oligonucleotide Spots from DEAE-cellulose Paper.

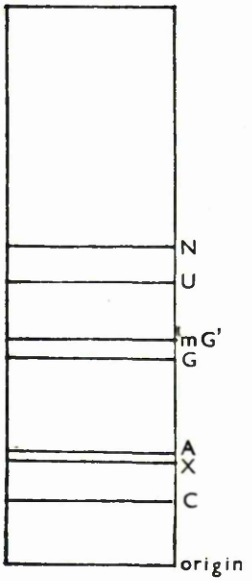
In order to identify the oligonucleotides, they were eluted from the DEAE-cellulose paper and further digested with alkali, pancreatic ribonuclease (in the case of T_1-oligonucleotides) or T_1 ribonuclease (in the case of pancreatic-oligonucleotides). The oligonucleotides were ionically bound to the DEAE-cellulose paper and so could not be eluted with water, but were eluted by 30% (v/v) triethylamine carbonate. This was prepared by passing carbon dioxide through a mixture of 30% triethylamine, 70% water until two phases could no longer be distinguished, and then adjusting the pH to $10{\cdot}0 \pm 0{\cdot}2$ by addition of more triethylamine or carbon dioxide. Elution was carried out as described by Sanger & Tuppy (1951), the eluate volume being between 50 and 100 µl. The eluate was placed on a PVC sheet annealed to a labelled glass plate and allowed to evaporate at 60°C. In order to ensure complete removal of triethylamine carbonate, water was added to the residue and then evaporated, several times.

2.2.5.2. Alkaline Hydrolysis.

Each eluted, dried down, oligonucleotide was dissolved in 10-20 µl of 0·2M sodium hydroxide. The solution was drawn into a capillary tube, both ends of which were then sealed. The hydrolysis was carried out at 37°C for 16 hrs.

FIG. 7.





KEY.

N.B. Nomenclature of nucleotides is defined on p.iii.

Separation of nucleotides by electrophoresis at pH 3·5.

2.2.5.3. Digestion with Enzymes.

The oligonucleotide was dissolved in 10-20 μl of enzyme solution and digestion was carried out in a capillary tube as described for alkaline hydrolysis. If the incubation time exceeded 30 min., the tubes were sealed. The condition for digestion by the various nucleases were:-

(i) Pancreatic Ribonuclease.

10 μl of 1mM EDTA, 10mM tris-HCl (pH 7·4) containing 0·1mg pancreatic RNase per ml. at 37°C for 30 min.

(ii) T_1 Ribonuclease.

As for Pancreatic RNase, substituting T_1 RNase.

2.2.5.4. Electrophoresis.

After hydrolysis with either alkali, or an appropriate enzyme, the material was applied as a streak about 2cm. long, to Whatman 52 paper for ionophoresis at pH 3·5 (5% acetic acid, 0·5% pyridine (v/v). Electrophoresis was carried out at 4.5 kilovolts for about 45 min., until the leading pink marker dye approached the anode buffer compartment.

The four major mononucleotides can be well separated using this system, (see FIG 7). The relative amounts of each mononucleotide could be estimated by excision and liquid scintillation counting of the oligonucleotide spots after autoradiography. This enabled the composition of each oligonucleotide to be determined.

2.2.6. Bisulphite Modification of tRNA.

E. coli $tRNA^{Phe}$ was incubated in 1M bisulphite, pH 7·0, or 3M bisulphite, pH 6·0, for various lengths of time as described in the Results Section. After this time, removal of bisulphite, and destruction of bisulphite adducts was carried out in the following manner:-

(i) An equal amount of distilled water was added to the tRNA/bisulphite solution to dilute the solution and thus stop the reaction.

(ii) Removal of bisulphite involved dialysis against 0·15M NaCl, 20mM tris-HCl, pH 7·5, and then against 0·15M NaCl, 5mM tris-HCl, pH 7·5, each for 2hrs., at room temperature.

(iii) Removal of the bisulphite adducts involved dialysis against 0·1M tris-HCl, pH 9·0 at 37°C for 9 hrs.

(iv) Finally the tRNA was neutralised by dialysis against 10mM tris-HCl, pH 7·0, 10mM $MgCl_2$, and then 2mM tris-HCl, pH 7·0, 10mM $MgCl_2$, each for 2 hrs., at 4°C.

3.1. PURIFICATION OF ACTIVE E. COLI $tRNA^{Phe}_2$ AND ITS FINGERPRINT ANALYSIS.

A number of methods have been used to separate the very similar components of a crude tRNA mixture. Methods used have involved those based on the distribution between two phases e.g. countercurrent distribution (Apgar et al, 1962; Karau & Zachau, 1964) and reverse phase chromatography (Kelmers, 1965), or chromatography on columns of hydroxylapatite (Meunch & Berg, 1966), methylated albumin kieselguhr (Sueoka & Yamane, 1962), DEAE-cellulose (Cherayil & Bock, 1965), DEAE-Sephadex (Nishimura et al, 1967) or benzoylated DEAE-cellulose (Gillam et al, 1967). The covalent coupling of aminoacyl-tRNA to a modified cellulose has also been described as a method for purification of tRNA (Bartkowiak et al, 1974).

Methods used for the purification of E. coli $tRNA^{Phe}_2$ have been described, (see Section 1.5.1.).

For the purpose of the work described in this thesis, an attempt was made initially to isolate E. coli $tRNA^{Phe}_2$ in two stages. These were first, chromatography of crude E. coli tRNA on a benzoylated DEAE-cellulose column, followed by further chromatography of the fraction containing $tRNA^{Phe}$ on a benzoylated DEAE-cellulose column, after it had been charged to the maximum extent with phenylalanine. A similar purification method has since been described by Brown et al (1972).

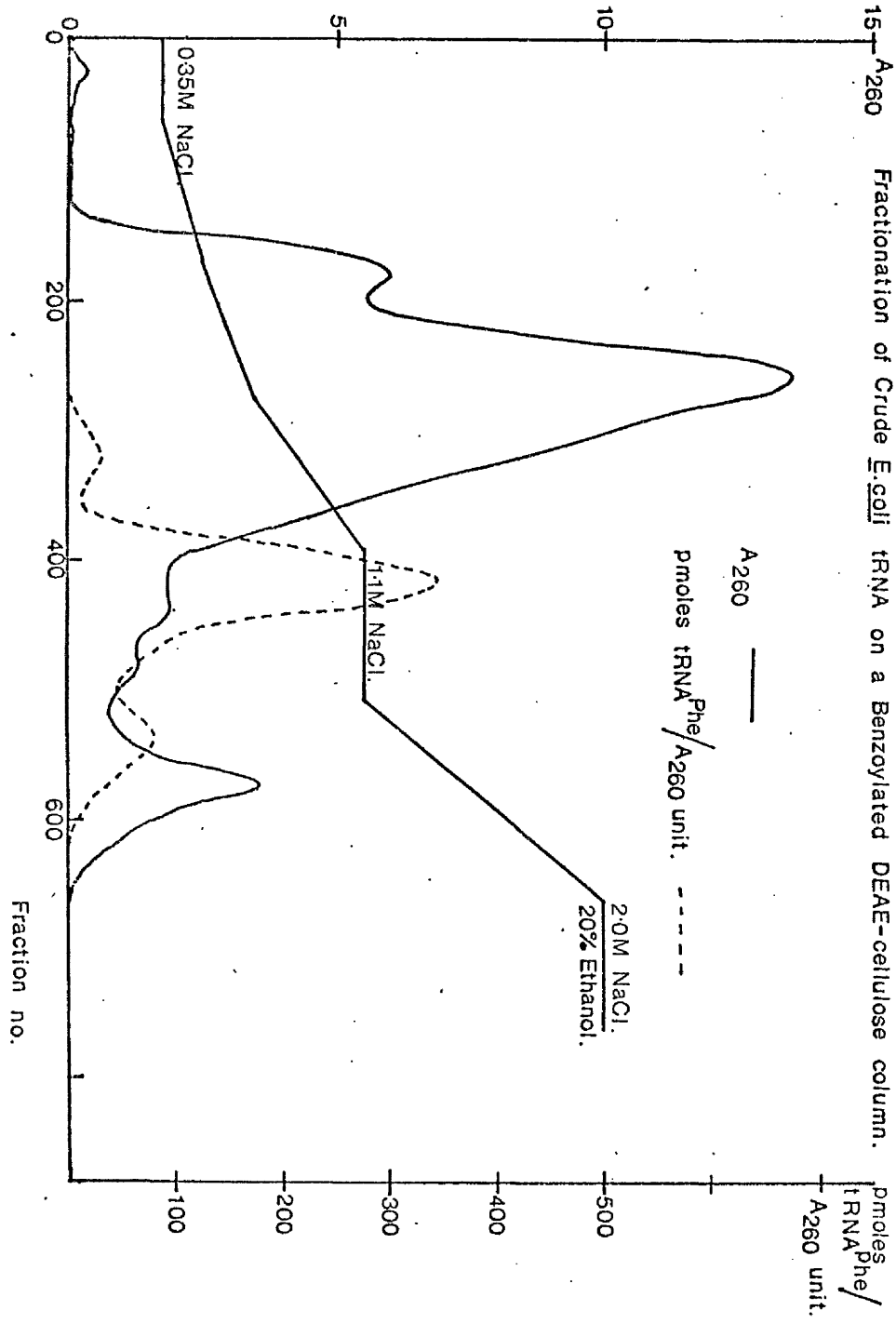


FIG. 8.

Fractionation of Crude E.coli tRNA on a Benzoylated DEAE-cellulose column.

3.1.1. Fractionation of Crude E. coli tRNA on a Benzoylated DEAE-cellulose column.

The column was equilibrated with 0·35M NaCl, 10mM $MgCl_2$, 2mM $Na_2S_2O_3$. Crude E. coli tRNA, accepting approximately 25 pmoles of phenylalanine per A_{260} unit, was dissolved in equilibration buffer and applied to the column. The crude tRNA was either unlabelled, or uniformly labelled with ^{32}P (activity approximately 40 μCi per mg. tRNA). Routinely, about 20 A_{260} units of crude tRNA were applied per $cm.^3$ of packed benzoylated DEAE-cellulose. A series of NaCl gradients were applied (see FIG. 8) until phenylalanine tRNA was eluted. Each solution applied to the column contained 10mM $MgCl_2$, 2mM $Na_2S_2O_3$. The total volume of the NaCl gradients was approximately 10ml. per cm^3 of packed benzoylated DEAE-cellulose.

The fractions were assayed for phenylalanine accepting activity as described in Section 2.2.2. When the second peak of phenylalanine accepting activity began to be eluted, the NaCl concentration of the eluting solution was kept constant, in order to ensure the maximum purification. An NaCl/ethanol gradient was necessary to elute the remaining tRNA from the column. A third fraction of $tRNA^{Phe}$ was eluted by this gradient. A typical fractionation is shown in FIG. 8. Recovery of $tRNA^{Phe}$ from the column was of the order of 75-80%. $tRNA^{Phe}_2$ comprised approximately 85% of the total $tRNA^{Phe}$. Fractions of $tRNA^{Phe}_2$ of phenylalanine accepting activity greater than 150 pmoles per A_{260} unit were pooled. The pooled fractions accepted 200-250 pmoles of phenylalanine per A_{260} unit, a 10 - fold purification having been achieved.

Purification of Phe-tRNA^{Phe} on a Benzoylated-DEAE-cellulose column.

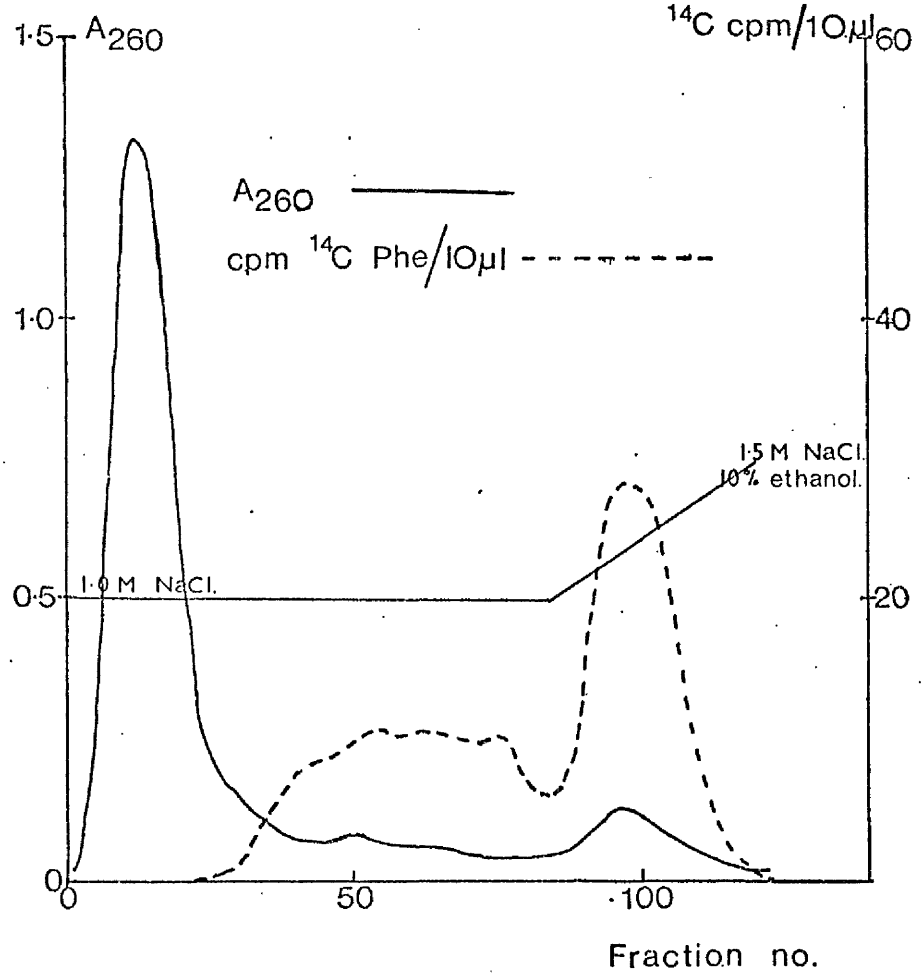


FIG. 9.

3.1.2. Further Purification of $tRNA^{Phe}_2$ using a Benzoylated DEAE-cellulose column.

Litt (1968) found that yeast phenylalanyl-$tRNA^{Phe}$ had a greater affinity for benzoylated DEAE-cellulose than uncharged $tRNA^{Phe}$, requiring a higher concentration of ethanol for its elution. This property of phenylalanyl-$tRNA^{Phe}$ was used to enable the isolation of highly purified yeast $tRNA^{Phe}$. Brown *et al* (1972) have used a similar method for the purification of *E. coli* $tRNA^{Phe}$.

Fractions from the first benzoylated DEAE-cellulose column, containing $tRNA^{Phe}_2$, were pooled and concentrated by addition of two volumes of absolute ethanol, standing overnight at -20°C, centrifuging at -10°C, and resuspension of the tRNA precipitate in 10mM $MgCl_2$. The tRNA was aminoacylated as described in Section 2.2.3., using ^{14}C-labelled phenylalanine of specific activity 15 cpm per pmole. The phenylalanyl-$tRNA^{Phe}_2$ was applied to a second benzoylated DEAE-cellulose column in equilibration buffer (0·3 M NaCl, 10mM $MgCl_2$, 2mM $Na_2S_2O_3$, 10mM sodium acetate, pH 5·0). This column was run at 4°C to retard deacylation. Buffer containing 1·0M NaCl was applied to the column to elute any uncharged $tRNA^{Phe}_2$ and other contaminating tRNAs. An NaCl/ethanol gradient was then applied to elute phenylalanyl-$tRNA^{Phe}_2$. The total gradient volume was 15ml. per cm^3 packed volume of benzoylated DEAE-cellulose. The eluted tRNA was charged with between 1000 and 1500 pmoles of phenylalanine per A_{260} unit of tRNA. FIG. 9 shows a typical fractionation. The recovery of purified phenylalanyl-$tRNA^{Phe}_2$ from such a column was 65-75%. This low recovery was due partly to deacylation of

phenylalanyl-$tRNA^{Phe}_2$ during the fractionation. Methods that have been used to deacylate the purified phenylalanyl-$tRNA^{Phe}_2$ are indicated in the table below (TABLE 3).

TABLE 3.

Deacylating medium	pmoles of Phe per A_{260} unit of tRNA before deacylation	Phe accepting activity of tRNA after deacylation (pmoles/A_{260} unit)
(i) 10mM $MgCl_2$, 0·15M tris-HCl, pH9·0, 25°C.	1250	810
(ii) 10mM $MgCl_2$, 50mM tris-HCl, pH7·5, 37°C.	1250	725
(iii) 10mM $MgCl_2$, 50mM $(NH_4)_2CO_3$, pH7·9, 37°C.	1250	805

Complete deacylation was found to occur after incubation of phenylalanyl-$tRNA^{Phe}_2$ in any of these media for 2 hours. After deacylation, the free phenylalanine was removed by dialysis against 10mM $MgCl_2$, 10mM tris-HCl, pH 7·5, twice, each time for two hours.

As can be seen from the above table, after deacylation the tRNA could not be charged to the original extent with phenylalanine. This indicated that the tRNA had been inactivated during the deacylation step. In order to overcome this problem, another method was tried for the further purification of the $tRNA^{Phe}_2$-containing pool obtained from the first benzoylated DEAE-cellulose column.

Fractionation of Partially Purified $tRNA^{Phe}$ on an RPC-5 column.

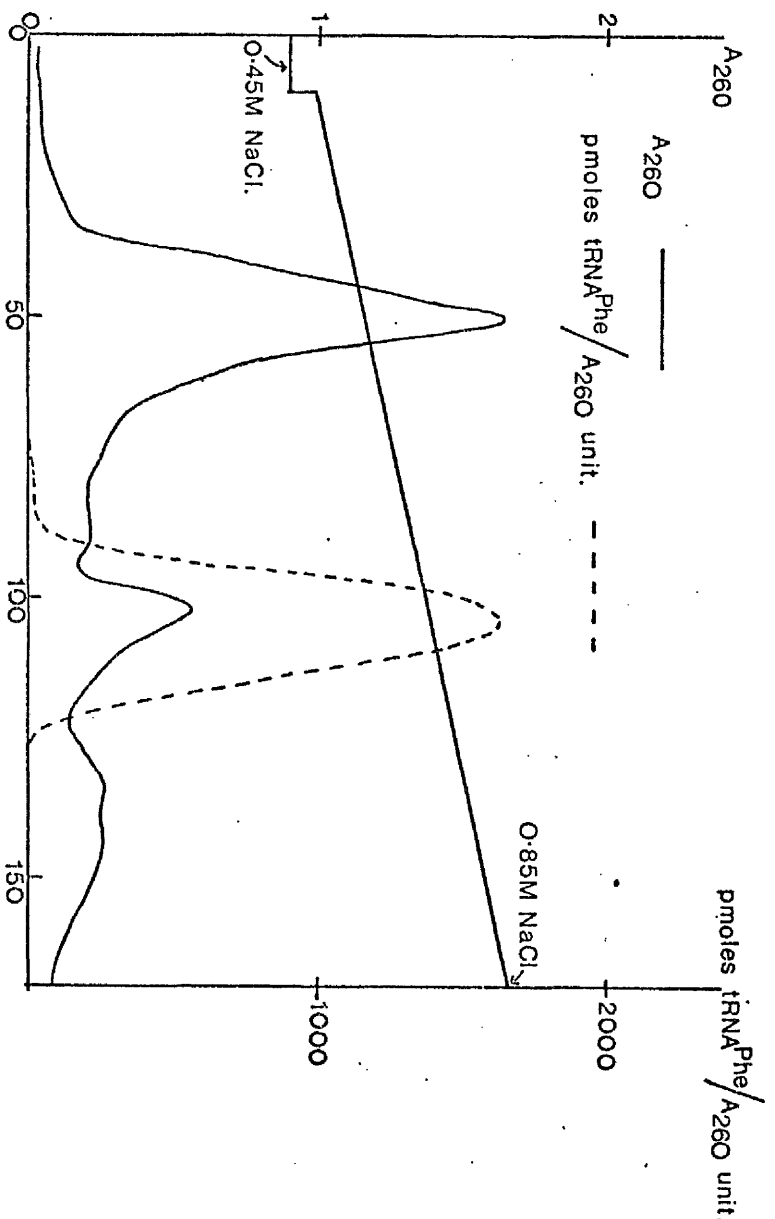


FIG. 10.

3.1.3. Further Purification of $tRNA^{Phe}_2$ using Reverse Phase Chromatography on an RPC-5 Column.

The use of RPC-5 for the purification of E. coli $tRNA^{Phe}$ has been described by Pearson et al (1971).

The RPC-5 was suspended in equilibration buffer in a jacketted column (50cm x 1cm diameter) maintained at 37°C. Equilibration buffer (0·45 M NaCl) and all subsequent buffers contained 10mM $MgCl_2$, 1mM β-mercaptoethanol, 10mM tris-HCl, pH 7·0. 80 A_{260} units of $tRNA^{Phe}_2$ rich tRNA, obtained by benzoylated DEAE-cellulose chromatography, were applied to the column in equilibration buffer. When a 0·5 - 0·9M NaCl gradient was applied (total volume, 200mls), $tRNA^{Phe}_2$ was eluted at 0·7M NaCl at a maximum specific activity of 1600 pMoles $tRNA^{Phe}_2$ per A_{260} unit of tRNA. Fractions with a specific activity greater than 1000 pmoles per A_{260} unit were pooled. A typical fractionation is shown in FIG. 10.

On storage of the purified $tRNA^{Phe}_2$ in the elution buffer at -10°C, it gradually lost phenylalanine accepting activity. After four weeks, only 25% of the accepting activity was retained. At this stage, attempts were made to reactivate the deactivated tRNA and to find conditions under which $tRNA^{Phe}_2$ was stable. Initially however, the tRNA was tested for the presence of a nuclease.

3.1.4. Test for the Presence of Nucleases in tRNA.

The method used was essentially that of Stern & Littauer (1971). Equal volumes of tRNA solution and 0·2M tris-HCl, pH8·8

were incubated at 37°C for 25 hours. Stern & Littauer (1971) regarded samples which lost more than 15% of their amino acid accepting activity over this period, as contaminated with nucleases. However, it was found that $tRNA^{Phe}_2$ which was nuclease-free by this criterion was not stable when stored in the elution buffer at -10°C over a period of several weeks.

3.1.5. Attempts to "Renature" Inactivated $tRNA^{Phe}_2$.

The existence of a tRNA species in two forms, one capable of being charged by its cognate amino acid ("native form") and the other not chargeable ("denatured form"), has been described (Lindahl et al, 1966; Fresco et al, 1966; Gartland & Sueoka, 1966; Lindahl et al, 1967; Reeves et al, 1970; Ishida et al, 1971). The inactive form is presumed to differ in tertiary and/or secondary structure from the active form. Methods of interconversion of the two forms have been published (Lindahl et al, 1966; Fresco et al, 1966; Reeves et al, 1970; Ishida et al, 1971).

An attempt was made to "renature" inactive $tRNA^{Phe}_2$ by heating it at 50°C for 10 min. in 50mM tris-HCl pH7·5, 20mM $MgCl_2$ (see Ishida et al, 1971) but this was not successful, no significant change in the phenylalanine accepting activity occuring on heat treatment.

3.1.6. The effect of Enzyme Concentration of Phenylalanine Accepting Activity.

In many cases, it has been shown that aminoacylation reactions of tRNA lead to plateau values which reflect incomplete

reactions, and are a function of the enzyme concentration (Bonnet & Ebel, 1972). Renaud et al, (1974) have postulated the existence of two interconvertible forms of Yeast $tRNA^{Phe}$ to explain the observed phenomenon of an initial slow aminoacylation rate with some batches of Yeast $tRNA^{Phe}$. This initial slow reaction was no longer observed if the tRNA had been preincubated with its cognate ligase. It was postulated that preincubation with the ligase allowed conversion of an inactive form of Yeast $tRNA^{Phe}$ to an active, rapidly chargeable one.

As such an explanation may be possible for E. coli $tRNA^{Phe}_2$, attempts were made to fully charge the inactive $tRNA^{Phe}_2$ by:-

(i) Doubling the enzyme concentration in the assay.

(ii) Preincubating the $tRNA^{Phe}_2$ with its cognate ligase.

However, in neither case was a significant increase in the amount of phenylalanine accepted per A_{260} unit of tRNA observed.

3.1.7. Optimal Storage Conditions for E. coli $tRNA^{Phe}_2$.

The effect of storage of E. coli $tRNA^{Phe}_2$ in various solutions at, -20°C, 4°C and 25°C, was investigated. The solutions were:-

(A) RPC-5 elution buffer (0·7M NaCl, 10mM $MgCl_2$, 1mM β-mercaptoethanol, 10mM tris-HCl, pH 7·0).

(B) Distilled water.

(C) 10mM $MgCl_2$.

(D) 10mM $MgCl_2$, 10mM tris-HCl, pH 7·0.

(E) 10mM $MgCl_2$, 2mM $Na_2S_2O_3$, 50mM tris-HCl, pH 7·5.

(F) 10mM $MgCl_2$, 1mM β-mercaptoethanol, 0·1M NaCl, 10mM tris-HCl, pH 7·3.

FIG. 11.

STORAGE OF *E. COLI* $tRNA_2^{Phe}$ UNDER VARIOUS CONDITIONS.

A. RPC-5 elution buffer (0·7M NaCl, 10mM $MgCl_2$, 1mM β-mercaptoethanol, 10 mM tris-HCl, pH 7·0.

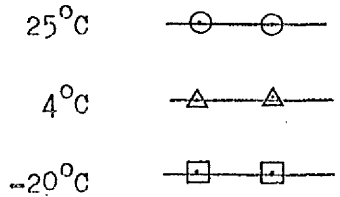
B. Distilled water.

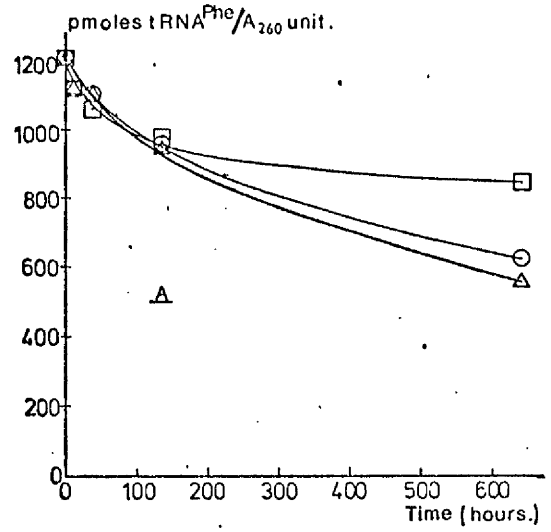
C. 10mM $MgCl_2$.

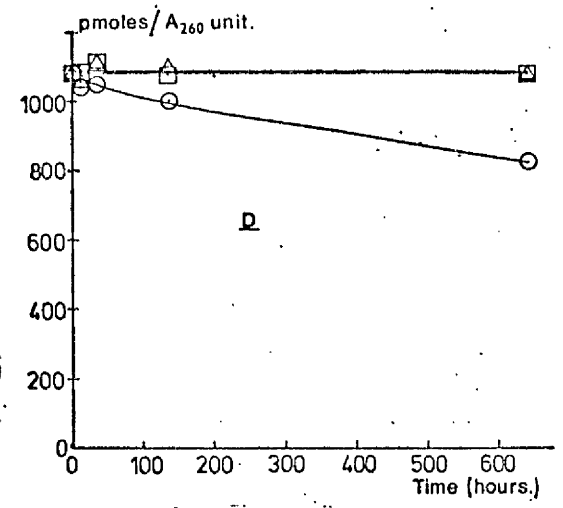
D. 10mM $MgCl_2$, 10mM tris-HCl, pH 7·0.

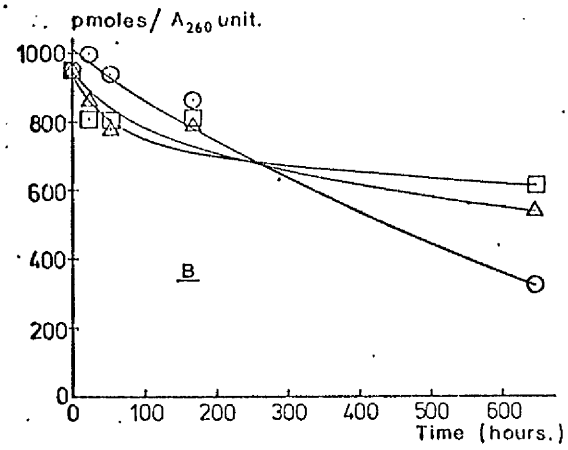
E. 10mM $MgCl_2$, 2mM $Na_2S_2O_3$, 50mM tris-HCl, pH 7·5.

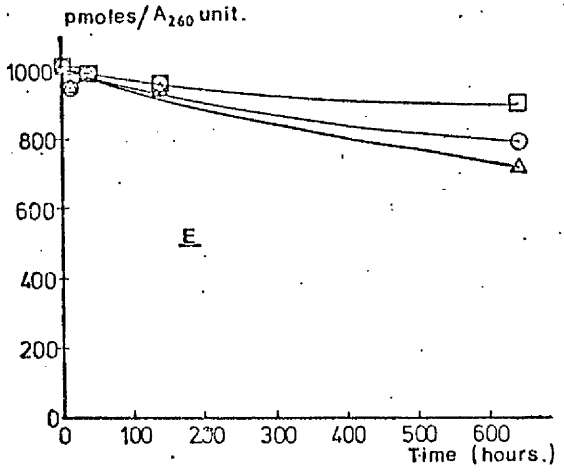
F. 10 mM $MgCl_2$, 1mM β-mercaptoethanol, 0·1M NaCl, 10mM tris-HCl, pH 7·3.

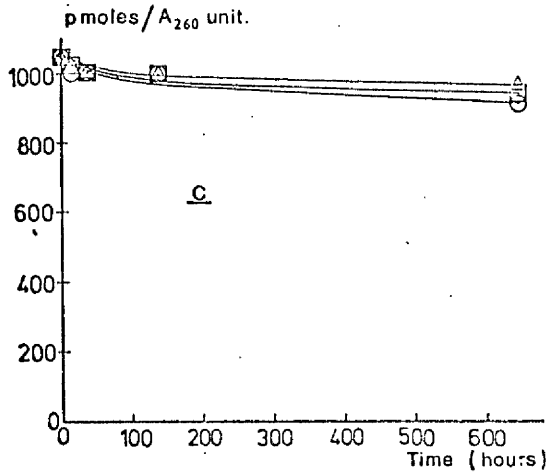


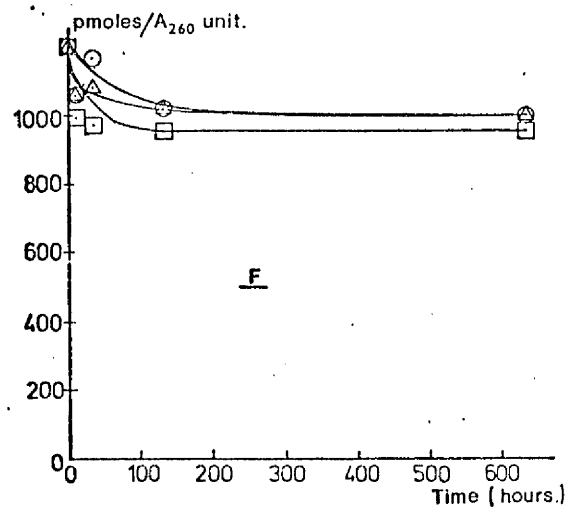

pmoles tRNA^{Phe}/A_{260} unit.
1200
1000
800
600
400
200
0
0 100 200 300 400 500 600
Time (hours.)
A


pmoles/ A_{260} unit.
1000
800
600
400
200
0
0 100 200 300 400 500 600
Time (hours.)
D


pmoles/ A_{260} unit.
1000
800
600
400
200
0
0 100 200 300 400 500 600
Time (hours.)
B


pmoles/ A_{260} unit.
1000
800
600
400
200
0
0 100 200 300 400 500 600
Time (hours.)
E


pmoles/ A_{260} unit.
1000
800
600
400
200
0
0 100 200 300 400 500 600
Time (hours)
C


pmoles/A_{260} unit.
1000
800
600
400
200
0
0 100 200 300 400 500 600
Time (hours.)
F

Solutions E and F were recommended by the Microbiological Research Establishment, Porton Down, Wiltshire, and Miles Laboratories Ltd., for the storage of E. coli $tRNA^{Phe}$ (personal communications). Aliquots of $tRNA^{Phe}_2$ solutions, of a size suitable for assay, were prepared and stored at -20°C, so that repeated thawing and refreezing of the $tRNA^{Phe}_2$ solutions was not necessary.

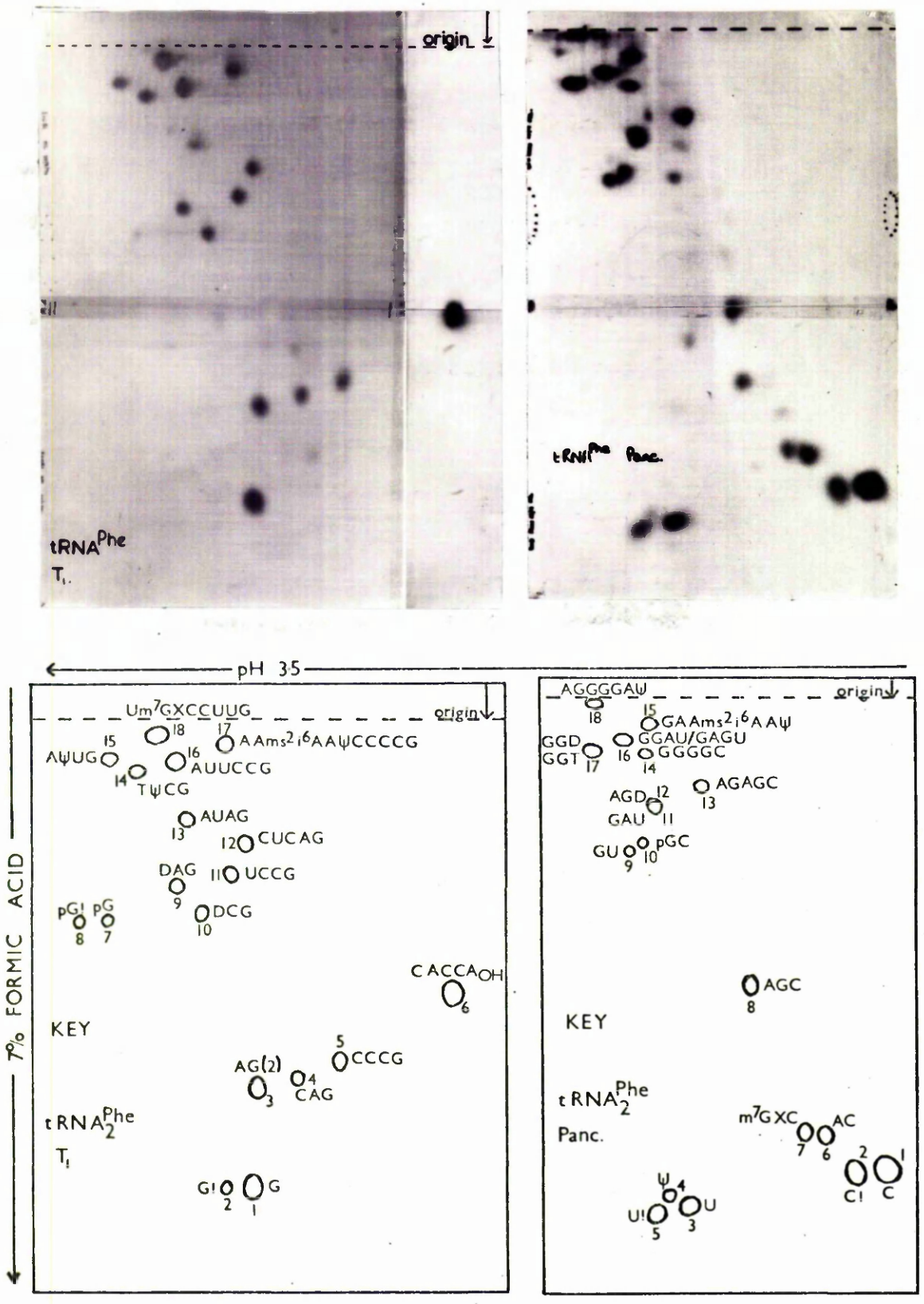
The results are indicated in FIG. 11. E. coli $tRNA^{Phe}_2$ appears to be most stable in 10mM $MgCl_2$, 10mM tris-HCl, pH7·5, at 4°C or -20°C.

3.1.7.2. The Effect of Addition of Crude E. coli tRNA to Purified $tRNA^{Phe}_2$.

E. coli $tRNA^{Phe}_2$, in pooled fractions obtained from the first benzoylated DEAE-cellulose column, was stable (i.e. did not lose phenylalanine accepting activity), over long periods at 4°C or -20°C. It is possible, therefore, that the loss of activity might be a function of the purity of the $tRNA^{Phe}_2$ preparation. The instability of some tRNAs when highly purified, but not when partially purified, has been observed by other workers (A. Atkinson, Microbiological Research Establishment, Porton Down, Wiltshire; personal communication).

Where purified ^{32}P-labelled $tRNA^{Phe}_2$ had been prepared, it was possible to add an equal amount of crude E. coli tRNA (unlabelled), without reducing the purity of the ^{32}P-labelled $tRNA^{Phe}_2$ and so without affecting the fingerprints. This procedure was found to stabilise purified $tRNA^{Phe}_2$, enabling it to be stored for long periods without loss of phenylalanine accepting activity.

FIG. 12.



T_1 and Pancreatic RNase Fingerprints of E. coli $tRNA^{Phe}_2$.

3.1.7.3. Routine Storage Conditions.

After addition of unlabelled, crude tRNA to purified $tRNA^{Phe}_2$ containing fractions from an RPC-5 column, the tRNA was dialysed twice for two hours against 10mM $MgCl_2$, 10mM tris-HCl, pH7·0, and stored at -20°C. Care was taken to avoid exposure of the purified tRNA to light, in order to prevent photochemical crosslinking of s^4U8 and C13 which might lead to loss of amino acid accepting activity (Favre et al 1971; Carré et al, 1974).

3.1.8. Fingerprinting of E. coli $tRNA^{Phe}_2$.

Purified ^{32}P-labelled E. coli $tRNA^{Phe}_2$ was fingerprinted as described in Section 2.2.4. The T_1 and Pancreatic RNase fingerprints are shown in FIG. 12, and are similar to those published for E. coli $tRNA^{Phe}$ by Barrel & Sanger (1969). Transference of the origin parts of the cellulose acetate strips has allowed the inclusion of $CACCA_{OH}$, on the T_1 RNase fingerprint, and C on the Pancreatic RNase fingerprint. The slightly different positions of the oligonucleotides on the Pancreatic RNase fingerprint are due to the use of 7% formic acid, rather than pyridine/acetate buffer, pH 1·9 for electrophoresis in the second dimension. The nucleotide composition of each oligonucleotide was determined after alkali digestion, as described in Section 2.2.5. TABLES 4 and 5 indicate the composition of each oligonucleotide, which is consistent with their identification by Barrel & Sanger (1969). As^4UAG and $GGAs^4U$ were not detected, only AUAG and GGAU, indicating that s^4U had been converted to U, either during preparation of the tRNA for fingerprinting or during the fingerprinting procedure.

TABLE 4.

OLIGONUCLEOTIDES ON A T_1 RNASE FINGERPRINT OF E. COLI $tRNA_2^{Phe}$.

OLIGONUCLEOTIDE	SEQUENCE DETERMINED BY BARRELL & SANGER (1969)	RELATIVE PROPORTIONS OF EACH NUCLEOTIDE						
		A+	C	G	U*	'mG'	X	pG
1	G	-	-	1·00	-	-	-	-
2	G!	-	-	1·00	-	-	-	-
3	AG(2)	0·63	-	1·00	-	-	-	-
4	CAG	0·88	0·97	1·00	-	-	-	-
5	CCCG	-	2·91	1·00	-	-	-	-
6	$CACCA_{OH}$	1·00	2·63	-	-	-	-	-
7	pG	-	-	-	-	-	-	1·00
8	pG!	-	-	-	-	-	-	1·00
9	DAG	1·12	-	1·00	1·08	-	-	-
10	DCG	-	1·09	1·00	0·82	-	-	-
11	UCCG	-	1·86	1·00	0·93	-	-	-
12	CUCAG	0·65	1·89	1·00	0·86	-	-	-
13	AUAG	2·22	-	1·00	1·19	-	-	-
14	TΨCG	-	0·94	1·00	2·21	-	-	-
15	AΨUG	1·06	-	1·00	2·89	-	-	-
16	AUUCCG	0·93	2·22	1·00	1·61	-	-	-
17	$AAms^2i^6AA\Psi CCCG$	3·55	3·78	1·00	1·18	-	-	-
18	$Um^7GXCCUUG$	-	1·68	1·00	2·67	1·10	0·80	

Oligonucleotides obtained by T_1 RNase digestion were assumed to contain one G. Dashes indicate that the proportion of a particular nucleotide in the oligonucleotide was less than 0·25. Each oligonucleotide was analysed at least three times, and the results shown are mean values.

'mG' is the product of alkali treatment of m^7G.

\+ including ms^2i^6A.

* including Ψ, rT, D.

TABLE 5.

OLIGONUCLEOTIDES ON A PANCREATIC RNASE FINGERPRINT OF E. COLI $tRNA^{Phe}_{2}$.

OLIGONUCLEOTIDE	SEQUENCE DETERMINED BY BARREL & SANGER (1969)	RELATIVE PROPORTIONS OF EACH NUCLEOTIDE						
		A+	C	G	U*	'mG'	X	pG
1	C	-	1·00	-	-	-	-	-
2	C!	-	1·00	-	-	-	-	-
3	U	-	-	-	1·00	-	-	-
4	Ψ	-	-	-	1·00	-	-	-
5	U!	-	-	-	1·00	-	-	-
6	AC	1·24	1·00	-	-	-	-	-
7	m^7GXC	-	1·00	-	-	1·14	0·66	-
8	AGC	0·66	1·00	1·14	-	-	-	-
9	GU	-	-	0·97	1·00	-	-	-
10	pGC	-	1·00	-	-	-	-	1·18
11	GAU	0·93	-	1·05	1·00	-	-	-
12	AGD	-	-	-	-	-	-	-
13	AGAGC	2·16	1·00	2·80	-	-	-	-
14	GGGC	-	1·00	2·79	-	-	-	-
15	GAAms^2i^6AAΨ	3·64	-	1·24	1·00	-	-	-
16	GAGU/GGAU	1·06	-	2·16	1·00	-	-	-
17	GGD/GGT	-	2·06	-	1·00	-	-	-
18	AGGGGAΨ	1·90	-	3·05	1·00	-	-	-

Oligonucleotides obtained by Pancreatic RNase digestion were assumed to contain one pyrimidine nucleotide. Dashes indicate that the proportion of particular nucleotide in the oligonucleotide was less than 0·25. Each oligonucleotide was analysed at least twice, and the results shown are mean values.

'mG' is the product of alkali treatment of m^7G.

\+ including ms^2i^6A.

* including Ψ , rT, D.

TABLE 6.

ELECTROPHORETIC MOBILITIES OF CONSTITUENT NUCLEOTIDES OF E. COLI $tRNA^{Phe}_{2}$.

NUCLEOTIDE	Ru DETERMINED	BROWNLEE (1969)
A	0·40	0·41
C	0·21	0·21
G	0·73	0·74
U	1·00	1·00
Ψ	with U	0·98
D	with U	1·00
rT	with U	0·98
'mG'	0·81	0·82
ms^2i^6A	with A	just ahead of A
X	0·36	-
pG	1.29	-

Ru = electrophoretic mobility of a nucleotide relative to the mobility of U on Whatman No. 52 paper at pH 3·5.

'mG' is the product of alkali treatment of m^7G.

The results shown are each mean values from at least three determinations.

A typical electrophoretic separation of nucleotides on Whatman No. 52 paper at pH 3·5 is shown in FIG. 7.

TABLE 7.

PERCENTAGE MOLAR YIELDS OF OLIGONUCLEOTIDES ON A T_1 RNASE FINGERPRINT OF E. COLI $tRNA^{Phe}_2$.

OLIGONUCLEOTIDE	PERCENTAGE MOLAR YIELD
G] G!]	84
AG (2)	125
CAG	103
CCCG	95
$CACCA_{OH}$	94
pG] pG]	78
DAG	111
DCG	104
UCCG	111
CUCAG	95
AUAG	61
TΨCG	136
AΨAG	110
AUUCCG	113
$AAms^2i^6AA\Psi CCCCG$	72
$Um^7GXCCUUG$	97

The results shown are mean values from three fingerprints of E. coli $tRNA^{Phe}_2$, of average phenylalanine acceptor activity, 1250 pmoles / A_{260} unit.

TABLE 8.

PERCENTAGE MOLAR YIELDS OF OLIGONUCLEOTIDES ON A PANCREATIC RNASE FINGERPRINT OF E. COLI $tRNA^{Phe}$.

OLIGONUCLEOTIDE	PERCENTAGE MOLAR YIELD
C C !	126
Ψ	70
U U !	130
AC	89
m^7GXC	99
AGC	86
GU	106
pGC	77
GAU / AGD	97
AGAGC	75
GGGC	86
$GAAms^2i^6AA\Psi$	80
GGT / GGD	112
AGGGGAΨ	75

These results were obtained from a $tRNA^{Phe}_2$ sample capable of accepting 1100 pmoles of phenylalanine per A_{260} unit.

Of the various modified nucleotides in E. coli $tRNA^{Phe}_2$, the mobilities of Ψ, D, T, ms^2i^6A, and 'mG'(the product of alkali treatment of m^7G) have already been described (Brownlee, 1972). The R_us of X and pG were determined. TABLE 6 indicated the R_us of all of the component nucleotides of E. coli $tRNA^{Phe}_2$ as determined, together with the values cited by Brownlee (1972), where applicable.

TABLE 7 and 8 indicates the relative molar yield of each oligonucleotide on the T_1 RNase and Pancreatic RNase fingerprints.

3.1.9. Discussion.

3.1.9.1. Inactive Forms of E. coli $tRNA^{Phe}_2$.

The inactivation of E. coli $tRNA^{Phe}_2$ described in this Section must be due to some change in $tRNA^{Phe}_2$ that reduces its affinity for the cognate ligase. There have been many reports of the existence of tRNA in forms which are inactive in aminoacylation assays. Lindahl et al (1966) described the existence of active (native) and inactive (denatured)forms of Yeast $tRNA^{Leu}$. Gartland and Sueoka (1966) have described a similar situation in the case of E. coli $tRNA^{Trp}$. The native and denatured forms of these two tRNAs can be interconverted. When denatured forms of these two tRNAs are heated in the presence of 20mM Mg^{2+} at 50-60°C at pH 7·5 - 8·0 for 5-10 mins., they are converted to the active forms. Conversion of the native to the denatured form may be accomplished by heating in the presence of EDTA at 50-60°C at pH 7·5 - 8·0 at 5-10 mins. (Lindahl et al, 1966; Ishida et al, 1971).

However, as described in Section 3.1.5., such treatment is

not capable of reactivating E. coli $tRNA^{Phe}_2$. This is consistent with the results of Lindahl et al (1966) who failed to detect such a renaturable species of $tRNA^{Phe}$ in crude E. coli tRNA. Furthermore, Mg^{2+} was present during the purification and storage procedures employed, and at no stage was the tRNA subjected to the presence of chelating agents which might remove the Mg^{2+} and thus allow the formation of such a denatured tRNA.

The loss of amino accepting activity of Yeast $tRNA^{Ala}$ on the formation of dimers has been described Loehr & Keller, (1968). The formation of dimers is thought to be facilitated by low temperatures (Lindahl et al, 1966), by the action of heat on concentrated tRNA solutions (15 -40 mg/ml.) and certain types of column chromatography (Loehr & Keller, 1968). However, purified E. coli $tRNA^{Phe}_2$ was never present in solution in such concentrations as these, and inactivation occurred after, not during column chromatography. In addition, the heat treatment described in Section 3.1.5. should be capable of destroying such dimers, (Lindahl et al, 1965).

Singhal & Best (1973) have shown that during purification of E. coli tRNAs containing s^4U, up to 35% of the tRNA molecules may become photochemically cross-linked, and have pointed out that such tRNAs are unsuitable for structural studies. There is some evidence to suggest that such a cross-link in E. coli $tRNA^{Phe}_2$ does alter its phenylalanine accepting activity (Carré et al, 1974) and for this reason, during the purification and storage of E. coli $tRNA^{Phe}_2$, it was protected from exposure to light as much as possible. Such a covalent cross-link would give rise to A s^4UAG in the T_1 RNase fingerprint with loss of A s^4UAG and CUCAG

CUCAG. Although AUAG was present in low yield in the T_1 RNase fingerprint, CUCAG was not present in such low yield, and an oligonucleotide with the composition As^4UAG was not detected.

CUCAG

Renaud et al (1974) have described an inactive form of Yeast $tRNA^{Phe}$, convertible to an active form by incubation with the cognate ligase. However, as indicated in Section 3.1.6., I was not able to convert inactive E. coli $tRNA^{Phe}_2$ to an active form by such a procedure.

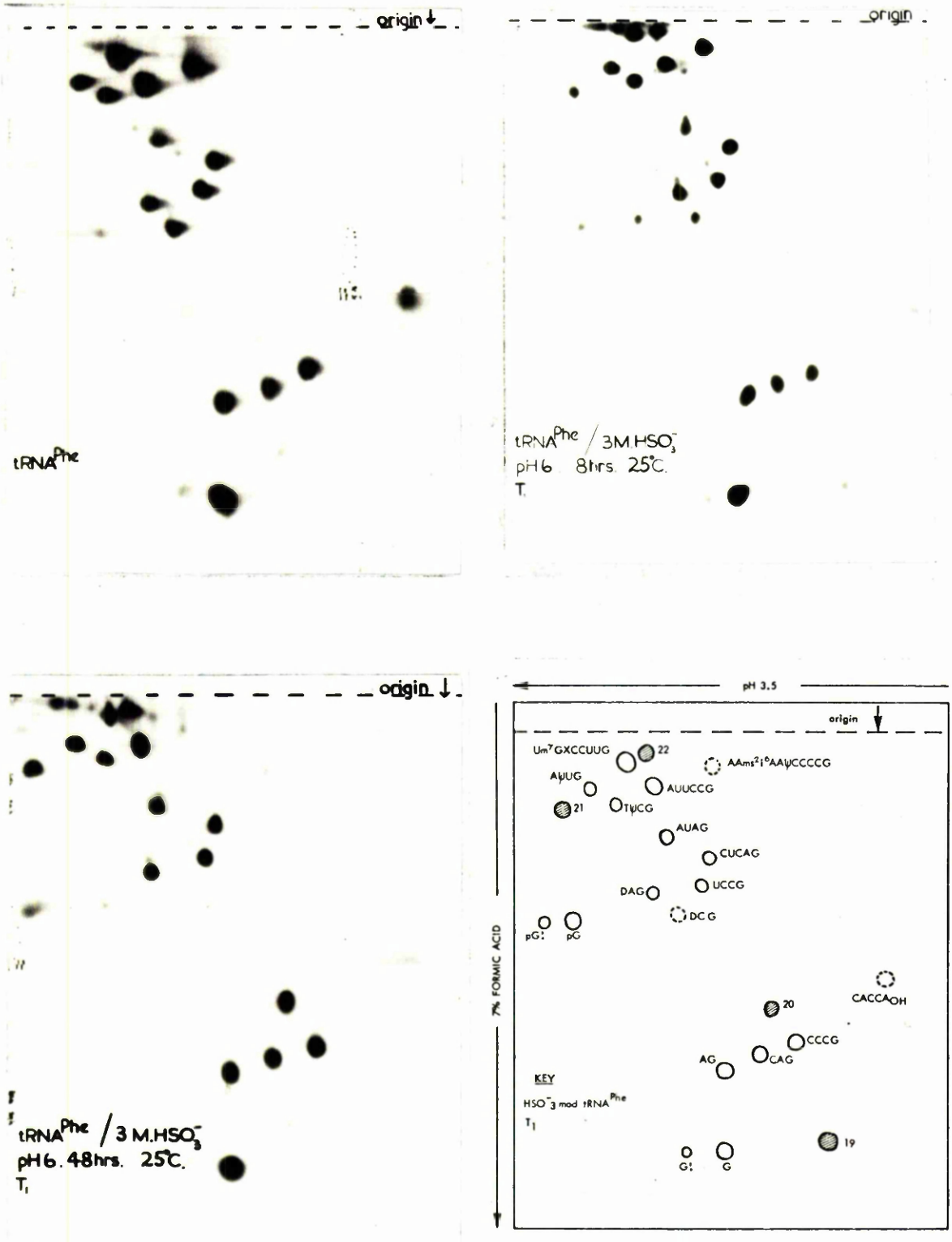
3.1.9.2. Fingerprints of E. coli $tRNA^{Phe}_2$.

The results obtained on fingerprinting E. coli $tRNA^{Phe}$ were consistent with the primary structure described by Barrel & Sanger (1969), but cannot be reconciled with that proposed by Uziel & Gassen (1969). The results, though not sufficient to provide an independent determination of the sequence of E. coli $tRNA^{Phe}_2$ did allow identification of this tRNA as the $tRNA^{Phe}$ whose sequence was described by Barrel & Sanger (1969). This sequence has been assumed to be correct throughout the rest of the thesis.

As can be seen from TABLES 7 and 8, not all of the nucleotides were present in 100% yield. This is due to the fact that the $tRNA^{Phe}_2$ was not 100% pure. Sequences common to many tRNAs, e.g. TΨCG, AG, were thus present in greater than 100% yield, while those common to only a small number of tRNAs e.g. $AAms^2i^6A$ AΨCCCCG, were present in less than 100% yield. Another possible reason for low yield of oligonucleotides containing modified bases, is that the $tRNA^{Phe}_2$ sample, as isolated, was not fully modified. The extent of modification may depend on the

growth conditions of the cells (Shugart et al, 1968; Huang & Mann, 1974). The absence of As^4UAG and $GGAs^4U$ from T_1 and Pancreatic RNase fingerprints is probably due to conversion of s^4U to U in the acid conditions of electrophoresis employed during fingerprinting (Barrel & Sanger, 1969). However, it is possible that the E. coli $tRNA^{Phe}_2$, as isolated, contained a low proportion of s^4U in position 8 due to the growth conditions.

FIG. 13.



T_1 RNase Fingerprint of E. coli $tRNA^{Phe}$, unmodified and modified for 8 and 48 hours in 3M $NaHSO_3$, pH 6·0 at 25°C.

3.2. BISULPHITE MODIFICATION OF E. COLI $tRNA_2^{Phe}$.

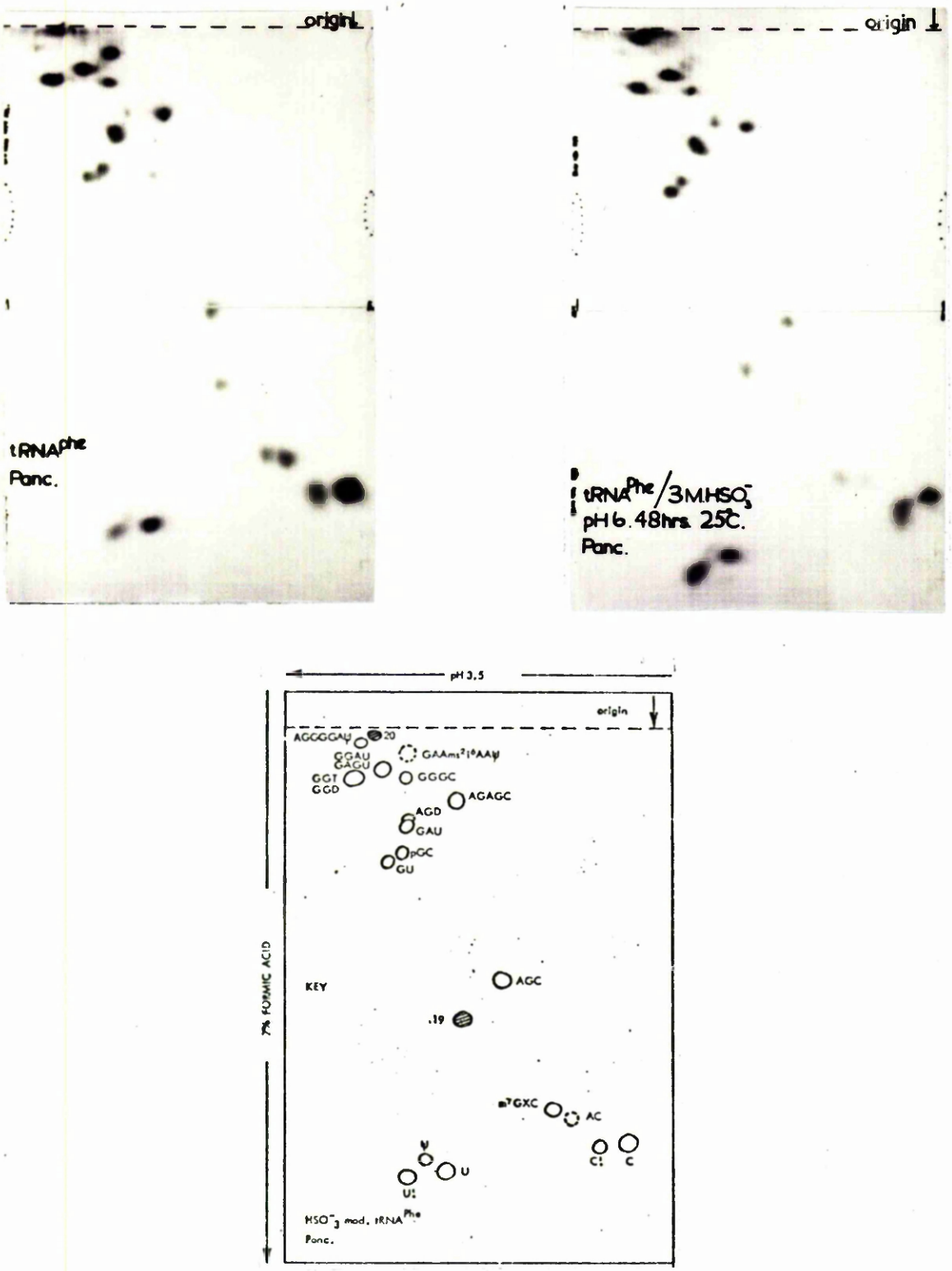
3.2.1. Modification in 3M Bisulphite pH 6·0.

Purified, ^{32}P-labelled E. coli $tRNA_2^{Phe}$, capable of accepting 1100 pmoles of phenylalanine per A_{260} unit, was incubated in 10mM $MgCl_2$, 3M sodium bisulphite, pH 6·0 at 25°C. Aliquots were withdrawn, immediately after addition of the bisulphite, and at intervals up to 48 hours. Each aliquot was treated as described in Section 2.2.6., to destroy the bisulphite adducts, and then fingerprinted. FIG. 13 shows representative T_1 RNase fingerprints of aliquots withdrawn (A) before addition of bisulphite, (B) after incubation in bisulphite for 8 hours, (C) after incubation for 48 hours. FIG. 14 shows Pancreatic RNase fingerprints of aliquots taken before and after 48 hours modification.

Over a period of 48 hours, three oligonucleotides disappeared from the T_1 fingerprint, i.e. DCG, $CACCA_{OH}$ and $AAms^2i^6AA\psi CCCCG$. These are represented by broken circles in FIG. 13. The shaded spots in the KEY in FIG. 13 represent the four new nucleotides that appeared on bisulphite modification (numbered 19, 20, 21 and 22). One of these oligonucleotides (numbered 19) appeared soon after bisulphite was added, but had disapreared after 24 hours modification.

Two oligonucleotides disappeared from the Pancreatic RNase fingerprint, i.e. AC and $GAAms^2i^6AA\psi$, after bisulphite modification for 48 hours. These are represented by broken circles in FIG 14. Over this period, two new oligonucleotide spots, numbered 19 and 20 appeared on the Pancreatic RNase fingerprint.

FIG. 14.



Pancreatic RNase Fingerprints of unmodified $tRNA_2^{Phe}$, and $tRNA_2^{Phe}$ modified for 48 hours in 3M $NaHSO_3$, pH 6·0 at 25°C.

TABLE 9.

COMPOSITION OF NEW T_1 AND PANCREATIC RNASE OLIGONUCLEOTIDES PRODUCED ON BISULPHITE MODIFICATION OF E. COLI $tRNA^{Phe}_2$.

NEW OLIGONUCLEOTIDE	NUCLEOTIDE COMPOSITION					PROBABLE SEQUENCE
	A	C	G	U	N	
T_1 RNASE						
19	1·00	2·31	-	1·25	-	$CA(C,U)A_{OH}$
20	1·10	1·00	-	2·20	-	$CAUUA_{OH}$
21	-	-	1.01	1·81	-	DUG
22	2·74	3·93	1·00	1·17	0·75	AANA Ψ CCCCG
PANCREATIC RNASE						
19	3·22		1.20	1·00	0·70	GAANAΨ
20	1·33			1·00		AU

N is the nucleotide produced by bisulphite modification of ms^2i^6A.

Oligonucleotides produced by T_1 RNase digestion of $tRNA^{Phe}_2$ were assumed to contain one G, and those produced by Pancreatic RNase digestion, one pyrimidine nucleotide. Dashes indicate nucleotides that were present in amounts relative to G or pyrimidine nucleotides of less than 0·25. The results are mean values of at least three determinations.

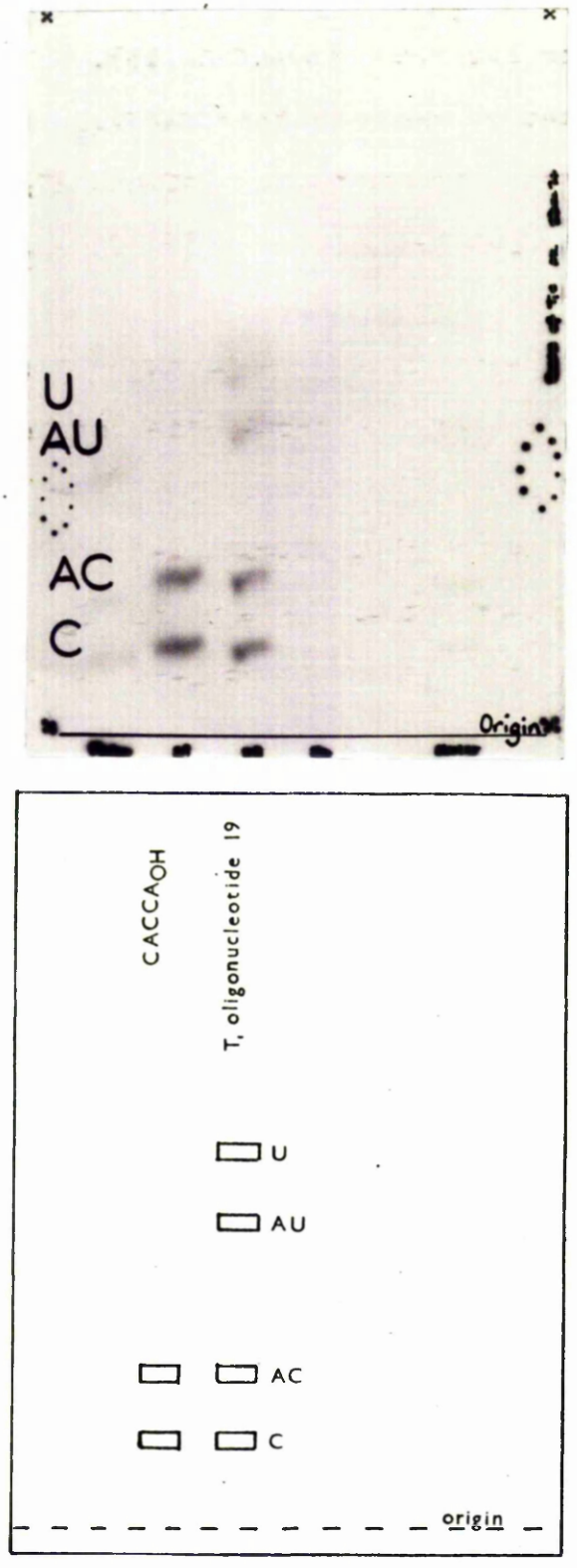
The nucleotide compositions of the new oligonucleotides were determined after alkaline hydroylsis as described in Section 2.2.5. (see TABLE 9).

Oligonucleotides 19 and 20 from T_1 RNase fingerprints did not contain G, indicating that these had been produced by modification of the 3' terminal sequence $CACCA_{OH}$. (See FIG. 18 for a cloverleaf representation of *E. coli* $tRNA^{Phe}_2$). Oligonucleotide 19, composition A, 2C, U, must have been produced by the modification of one cytidine residue and oligonucleotide 20, composition A, C, 2U, by the modification of two cytidine residues.

Determination of which two cytidine residues of the sequence $CACCA_{OH}$ had been modified was accomplished by examination of the Pancreatic RNase fingerprint of *E. coli* $tRNA^{Phe}_2$ which had been modified with bisulphite for 48 hours, and also by Pancreatic RNase digestion of oligonucleotides 19 and 20 from T_1 RNase fingerprints. Oligonucleotide GGGC (containing C72) was still present on a Pancreatic RNase fingerprint after 48 hours modification. This indicated thatthe two cytidine residues modified in the sequence $CACCA_{OH}$ must be C74 and C75 and not C72. Oligonucleotide 19 on the Pancreatic RNase fingerprint of *E. coli* $tRNA^{Phe}_2$ modified for 48 hours was found to have the composition 1A, 1U, and must therefore have been AU produced by modification of AC (C74). AC was totally absent from this fingerprint, indicating that C74 had been completely deaminated on treatment of *E. coli* $tRNA^{Phe}_2$ with bisulphite for 48 hours.

Pancreatic ribonuclease digestion of oligonucleotide 19 from the T_1 ribonuclease fingerprint of *E. coli* $tRNA^{Phe}_2$, modified with bisulphite for 8 hours (see Section 2.2.5.3.) produced a mixture

FIG. 15.



Pancreatic RNase digestion products of T_1 oligonucleotides $CACCA_{OH}$ and 19.

of C, AC, and AU. These were separated by electrophoresis as described in Section 2.2.5.4. (See FIG. 15). These results are indicative of the presence of both $CACUA_{OH}$ and $CAUCA_{OH}$. Estimation of the relative amounts of AC and AU, indicated that the oligonucleotide spot 19 contained 65% $CACUA_{OH}$ and 35% $CAUCA_{OH}$.

Oligonucleotide spot 21, on the T_1 RNase fingerprints, was DUG which must have arisen from bisulphite modification of C17 in the sequence DCG.

After alkaline hydrolysis, oligonucleotide 22 on the T_1 RNase fingerprints was found to contain A, C, G, U and N (an unknown nucleotide with an Ru of 1·14 - see FIG. 7) in the ratios 4:3:1:1:1. This oligonucleotide was most likely to have been derived from bisulphite modification of the sequence $AAms^2i^6AA\psi CCCCG$, although no cytidine residues appeared to have been modified.

Hayatsu et al (1972) have reported bisulphite modification of N^6-isopentenyladenosine, resulting in the formation of a bisulphite adduct, stable in mild alkali (pH 9·0 buffer). A similar adduct may have been produced with ms^2i^6A in this case. Support for this is lent by the fact that after a period of 48 hours of bisulphite modification, the oligonucleotide $GAAms^2i^6AA\psi$ was lost from a Pancreatic RNase fingerprint of E. coli $tRNA^{Phe}_2$, and a new oligonucleotide (20), with the nucleotide composition 3A, G, U, N (See TABLE 9) appeared.

The percentage yields of each of the oligonucleotide spots on each T_1 RNase fingerprint, of $tRNA^{Phe}_2$ samples at different stages of modification, were determined (TABLE 10). The disappearances of $CACCA_{OH}$, DCG and $AAms^2i^6AA\psi CCCCG$ together

TABLE 10

PERCENTAGE YIELDS OF OLIGONUCLEOTIDES PRODUCED BY T_1 RNASE DIGESTION OF E. COLI $tRNA_2^{Phe}$ AT VARIOUS STAGES OF BISULPHITE MODIFICATION.

OLIGONUCLEOTIDE	PERCENTAGE YIELD OF OLIGONUCLEOTIDE								
	BEFORE ADDITION OF BISULPHITE	TIME AFTER ADDITION OF BISULPHITE (HOURS) 0	$\frac{1}{2}$	$1\frac{1}{2}$	$3\frac{1}{2}$	$5\frac{1}{2}$	8	24	48
G + G!	102	110	115	96	112	107	93	90	117
AG (2)	128	133	133	121	120	117	115	127	123
CAG	97	109	87	99	88	96	102	86	105
CCCG	86	94	85	99	86	96	102	87	101
pG + pG!	90	86	76	92	96	85	82	77	88
DAG	101	119	115	113	117	120	116	107	104
UCCG	105	109	110	106	104	104	110	99	112
CUCAG	83	87	79	81	93	82	92	80	90
AUAG	52	60	53	50	59	54	60	50	62
TΨCG	131	136	128	128	128	123	125	112	130
AΨUG	123	132	122	120	130	115	125	127	130
AUUCCG	117	110	105	100	117	111	107	113	114
$Um^7GXCCUUG$	87	90	76	80	83	80	80	85	87
$CACCA_{OH}$	96	93	93	48	39	45	24	0	0
19	0	0	20	-	22	-	18	13	0
20	0	0	0	9	15	27	-	78	89
DCG	124	120	94	78	54	25	20	14	2
21	0	0	17	39	65	97	86	126	126
$AAms^2i^6AAΨCCCCG$	86	88	72	74	66	50	42	18	11
22	0	0	16	16	26	45	50	67	90

- indicates not measured.

The results are the mean of two separate Bisulphite modification experiments.

FIG. 16.

LOSS OF OLIGONUCLEOTIDES $CACCA_{OH}$, DCG, AND $AAms^2i^6AA\Psi CCCCG$ FROM A T_1 RNASE FINGERPRINT OF E. COLI $tRNA_2^{Phe}$ ON BISULPHITE MODIFICATION.

(a). CONVERSION OF $CACCA_{OH}$ TO $CA(C,U)A_{OH}$.

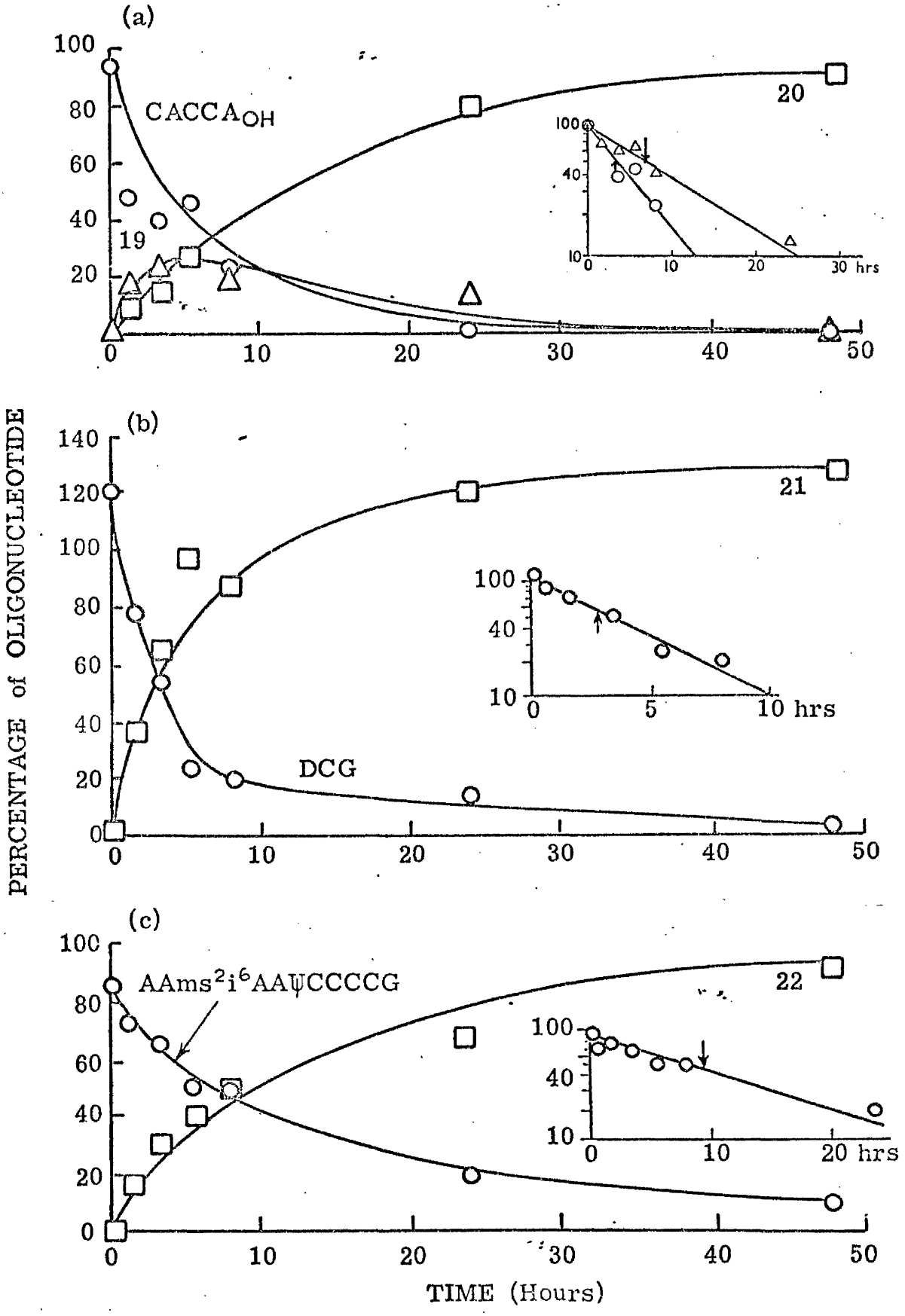
The inset shows plots of Log Percentage Yield of $CACCA_{OH}$ (○—○), and $CACCA_{OH}$ + $CA(C,U)A_{OH}$ (△—△) against Time. The $t_{\frac{1}{2}}$ for disappearance of $CACCA_{OH}$ was 3·25 hours, and for the disappearance of $CACCA_{OH}$ + $CA(C,U)A_{OH}$, 7·0 hours. Therefore, the $t_{\frac{1}{2}}$ for the modification of each of C74 and C75 must have been between 3·25 and 7·0 hours.

(b) CONVERSION OF DCG TO DUG.

The inset shows a plot of Log Percentage Yield of DCG against Time. The $t_{\frac{1}{2}}$ for disappearance DCG, and therefore for modification of C17 was 2·25 hours.

(c). CONVERSION OF $AAms^2i^6AA\Psi CCCCG$ TO $AANA\Psi CCCCG$.

The inset shows a plot of Log Percentage Yield of $AAms^2i^6AA\Psi CCCCG$ against Time. The $t_{\frac{1}{2}}$ for disappearance of $AAms^2i^6AA\Psi CCCCG$ and therefore for modification of ms^2i^6A37 was 9·5 hours.


(a)
CACCA$_{OH}$
19
20
100
80
60
40
20
0
10
20
30
40
50
100
40
10
0
10
20
30 hrs
(b)
21
DCG
140
120
100
80
60
40
20
0
10
20
30
40
50
100
40
10
0
5
10 hrs
(c)
AAms^{2}i^{6}AAψCCCCG
22
100
80
60
40
20
0
10
20
30
40
50
100
40
10
0
10
20 hrs
PERCENTAGE of OLIGONUCLEOTIDE
TIME (Hours)

with the appearances of oligonucleotides 19 and 20, 21 and 22 respectively are indicated in FIG. 16.

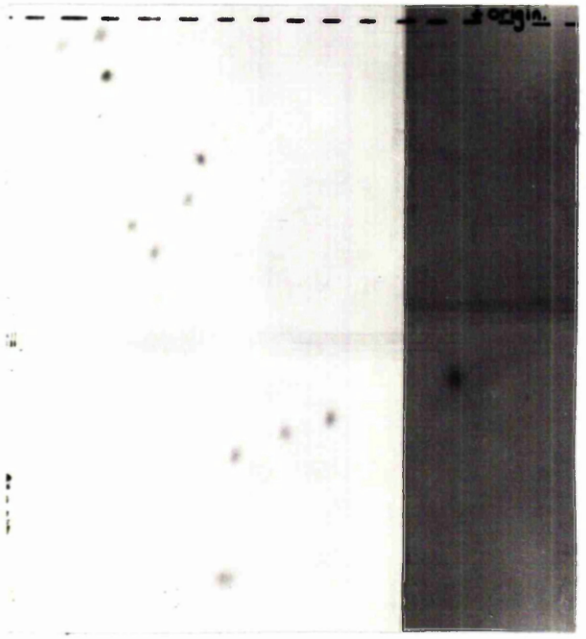
3.2.2. Modification of E. coli $tRNA^{Phe}_2$ with 1M Bisulphite, pH 7·0.

Furuichi et al,(1970) have reported that modification of N^6-isopentenyladenosine in yeast $tRNA^{Tyr}$ is possible in 1M sodium bisulphite, pH 7·0 at 37°C. Modification of cytidine residues is much less favourable under these conditions (Hayatsu et al, 1970a). An attempt was made to modify E. coli $tRNA^{Phe}_2$ under such conditions, using ^{35}S-labelled bisulphite, to determine whether any $(^{35}S)HSO_3^-$ incorporated into the tRNA remained after pH 9·0 treatment.

^{32}P-labelled $tRNA^{Phe}_2$ was incubated in 10mM $MgCl_2$, 1M sodium bisulphite, pH 7·0 at 37°C for 24 hours. ^{35}S-labelled sodium bisulphite of the maximum specific activity available (12·7 Ci/mMole) from the Radiochemical Centre, Amersham, Bucks, was used to prepare 1M sodium bisulphite, pH 7·0. Nevertheless, to get a reasonable ratio of counts of ^{35}S to ^{32}P in any oligonucleotide it was found necessary to add purified unlabelled $tRNA^{Phe}_2$ to ^{32}P-labelled $tRNA^{Phe}_2$ as isolated. The ^{32}P-labelled $tRNA^{Phe}_2$, thus prepared, contained approximately 4×10^4 cpm ^{32}P per A_{260} unit, having a phenylalanine accepting activity of 1100 pmoles per A_{260} unit. The 1M bisulphite, pH 7·0 contained approximately $1{\cdot}6 \times 10^{12}$ cpm ^{35}S per mole.

After incubation in 1M sodium bisulphite, pH 7·0 at 37°C for 24 hours, the $tRNA^{Phe}_2$ was treated to remove any bisulphite adducts of uridine residues (Section 2.2.6.) and then fingerprinted (Section 2.2.4.) The T_1 RNase fingerprint is

FIG. 17.



T_1 RNase Fingerprint of E. coli $tRNA_2^{Phe}$, modified for 24 hours in 1M $NaHSO_3$, pH 7·0 at 37°C.

shown in FIG. 17.

The oligonucleotide spots were excised from the fingerprint and the amount of ^{35}S and ^{32}P in each determined by liquid scintillation counting. The counter was set so that ^{35}S could be counted with as little flowover of ^{32}P counts as possible and vice versa. It was possible to count ^{32}P without any flowover of ^{35}S counts but there was flowover of 11% of ^{32}P counts into the ^{35}S channel. The ^{35}S counts were corrected for this flowover.

TABLE 11 shows the percentage molar yields of each oligonucleotide on a T_1 RNase fingerprint of modified $tRNA^{Phe}_2$ (calculated from ^{32}P counts as described in Section 2.2.4.4.), compared with these of unmodified *E. coli* $tRNA^{Phe}_2$. The only oligonucleotide whose yield decreased significantly on modification of $tRNA^{Phe}_2$ with 1M sodium bisulphite, pH 7·0, was $AAms^2i^6AA\psi CCCCG$. A new oligonucleotide, oligonucleotide 22 (compare FIG. 13, Section 3.2.1.), appeared. This has been assigned the structure $AANA\psi CCCCG$ (Section 3.2.1.) and was the only oligonucleotide to contain ^{35}S (0·76 Moles of $(^{35}S)HSO_3^-$ per Mole of oligonucleotide). The ratio of ^{35}S ; ^{32}P counts in this oligonucleotide was of the order of 1 : 3, so the amount of $(^{35}S)HSO_3^-$ present could be estimated with reasonable accuracy.

Only one oligonucleotide, i.e. $GAAms^2i^6AA\psi$, disappeared from Pancreatic RNase fingerprints of modified $tRNA^{Phe}_2$. A new spot corresponding to oligonucleotide 20 (FIG. 14, Section 3.2.1.) appeared, containing 0·85 Moles of $(^{35}S)HSO_3^-$ per Mole of oligonucleotide.

TABLE 11

COMPARISON OF PERCENTAGE MOLAR YIELDS OF T_1 RNASE NUCLECTIDES OF E. COLI $tRNA^{Phe}_2$, UNMODIFIED, AND MODIFIED IN 1M $NaHSO_3$ FOR 24 HOURS AT 37°C.

OLIGONUCLECTIDE	PERCENTAGE MOLAR YIELD	
	UNMODIFIED $tRNA^{Phe}_2$	MODIFIED $tRNA^{Phe}_2$
G + G!	102	115
AG (2)	112	122
CAG	98	103
CCCG	98	109
$CACCA_{OH}$	96	102
pG + pG!	90	82
DAG	104	105
DCG	120	120
UCCG	102	112
CUCAG	82	84
AUAG	64	60
TΨCG	126	120
AΨUG	100	92
$Um^7GXCCUUG$	89	96
$AAms^2i^6AAΨCCCCG$	88	13
22	0	85

3.2.3. Discussion.

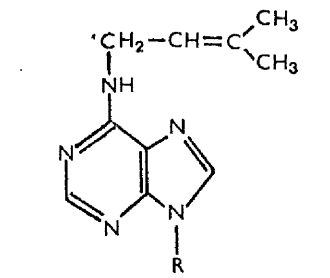
As indicated in Section,1.5.2., bisulphite has been used to selectively convert cytidine residues in exposed single stranded regions of tRNA into uridine residues. During such modifications, T_1 RNase oligonucleotides containing modifiable cytidine residues will gradually disappear from the T_1 RNase fingerprint, a new oligonucleotide appearing, in a one-higher graticule on modification of one cytidine residue, or a two higher graticule on modification of two cytidine residues.

In a situation involving conversion of cytidine to uridine residues, T_1 RNase fingerprints are capable of providing much more information about the position of modifiable cytidine residues than Pancreatic RNase fingerprints. If a modifiable cytidine residue is situated between pyrimidine residues the modification will only be identifiable as an increase in the U spot and a decrease in the C spot, whereas such a residue is probably part of a unique T_1 RNase oligonucleotide, whose disappearance from a T_1 RNase fingerprint can easily be noticed. (For an example consider the bisulphite modification of C17 in the sequence DCG.) For this reason, T_1 RNase fingerprints have been the primary source of modification data in this case of bisulphite modification of E. coli $tRNA^{Phe}_2$. Data from Pancreatic RNase fingerprints, while confirming modifications suggested by T_1 RNase fingerprint data, may also be useful in deciding which residue has been modified, when there are more than one modifiable residue in a particular T_1 RNase oligonucleotide (consider the cases of modification of $CACCA_{OH}$ and $AAms^2i^6AA\Psi CCCCG$ described in Section 3.2.2.).

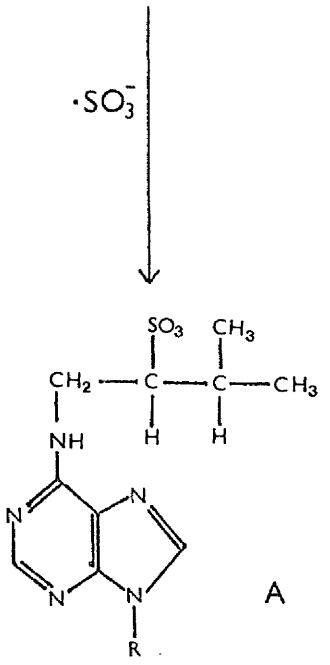
It can be seen from FIG. 16 that the appearance of oligonucleotide 21 corresponded well with the disappearance of DCG as did the appearance of oligonucleotide 22 with the dissappearance of $AAms^2i^6AA\Psi CCCCG$ and the appearance of oligonucleotide 20 with the disappearance of oligonucleotides 19 and $CACCA_{OH}$. In each case, this provides confirmatory evidence that one oligonucleotide is the bisulphite modification product of the other. Modification of C17 and ms^2i^6A 37 appeared to follow first order kinetics. The disappearance of $CACCA_{OH}$ and $CACCA_{OH}$ + $CA(C,U)A_{OH}$ also appeared to follow first order kinetics. Modification of C17 appeared to be the fastest reaction, DCG having a $t_{\frac{1}{2}}$ of 2·25 hours. Modification of C74 and C75 proceeded more slowly, $CACCA_{OH}$ having a $t_{\frac{1}{2}}$ of 3·25 hours and $CACCA_{OH}$ + $CA(C,U)A_{OH}$ a $t_{\frac{1}{2}}$ of 7·0 hours. Modification of ms^2i^6A 37 appeared to proceed most slowly $AAms^2i^6AA\Psi CCCCG$ having a $t_{\frac{1}{2}}$ of approximately 9·5 hours. The fact that loss of each of the oligonucleotides $CACCA_{OH}$ + $CA(C,U)A_{OH}$; DCG and $AAms^2i^6AA\Psi CCCCG$ form T_1 RNase fingerprints appeared to follow first order kinetics is indicative of the fact that the $tRNA_2^{Phe}$ did not undergo any conformational changes (caused by partial bisulphite modification, or prolonged immersion in 3M sodium bisulphite, pH 6·0) which would make certain residues more, or less, reactive. If something of this nature had happened, the plots of Log Percentage Yield of Oligonucleotide versus Time would be likely to be biphasic, and not to follow simple first order kenetics.

Singhal & Best (1973) have suggested that up to 40% of any isolated tRNA, containing s^4U, is present in a cross-linked form, due to the photoactivated cross linking of C13 and s^4U8 (Favre et al, 1969). The low yield of AUAG (derived from As^4UAG) on

FIG. 18.



R= β-D- ribofuranosyl.



A

A was found to be the major product of the reaction of isopentenyladenosine with 1M $NaHSO_3$ at pH 7·0, 0° C, (Hayatsu et al, 1972).

REACTION OF BISULPHITE WITH ISOPENTENYLADENOSINE.

T_1 RNase fingerprints throughout the period of bisulphite modification might imply that some molecules were present in this cross-linked form, which might have a non-native conformation. If this were the case, the oligonucleotide As^4UAG should have been present (in the top left hand corner
CUC AG
of the T_1 RNase fingerprint) and AUAG and CUCAG would be present in correspondingly low yields. However, the percentage yield of CUCAG was never as low as that of AUAG, and no spot corresponding to the oligonucleotide As^4UAG was
CUCAG
detected on any of the fingerprints in significant amounts (i.e. > 10% yield). The $tRNA^{Phe}_2$ used for bisulphite modification did not, therefore, contain significant amounts of the cross-linked form. The low percentage yield of AUAG was probably due to incomplete conversion of As^4UAG to AUAG, and 'streaking' on the fingerprint.

Addition of SO_3^{--} to N^6- isopentenyladenosine has been suggested to take place with the participation of the free radical $\cdot SO_3^{--}$ (Hayatsu et al, 1972) as shown in FIG. 18. Formation of this free radical requires oxygen, and the free radical is quickly destroyed in solutions containing higher concentrations of bisulphite than about 10^{-2} M (Hayatsu & Inoue, 1971). However, there must be enough free radical available to allow slow reaction with ms^2i^6A 37 in 3M bisulphite, pH 6·0 or 1M bisulphite, pH 7·0. The low values obtained for Moles of (^{35}S) HSO_3^{--} per Mole of ms^2i^6A 37 - containing oligonucleotide (Section 3.2.2.) might be due to the presence of a small proportion of $tRNA^{Phe}_2$ molecules containing A37 rather

than ms^2i^6A 37. The extent of modification of some tRNAs has been shown to depend on the growth conditions. Modification of *E. coli* $tRNA^{Phe}_2$ with ^{35}S - labelled 3M bisulphite, pH 6·0 proved impractical because ^{35}S - labelled sodium bisulphite of adequate specific activity to allow a reasonable ratio of ^{35}S to ^{32}P counts could not be obtained.

Hayatsu & Inoue (1971) have described the conversion of 4 - thiouracil to uracil derivatives by the free radical $\cdot SO_3^-$. Since *E. coli* $tRNA^{Phe}_2$ contains s^4U, and the conditions used for bisulphite modification must have allowed the production of $\cdot SO_3^-$ (because of the reaction with ms^2i^6A 37), then conversion by bisulphite, of s^4U8 to U8, may have occurred. However, using the fingerprinting procedure described (Section 2.2.4.) it would not be possible to detect such a change, as s^4U8 appeared to be converted to U8 on fingerprinting of unmodified *E. coli* $tRNA^{Phe}_2$. This was presumably due to the acid conditions of electrophoresis in the second dimension (Barrel & Sanger, 1969).

Summarising the results described in this Section, modification of *E. coli* $tRNA^{Phe}_2$ with 3M sodium bisulphite, pH 6·0, in the presence of 10mM $MgCl_2$, has been shown to produce the following nucleotide modifications:-

a) C17 $\longrightarrow$ U17

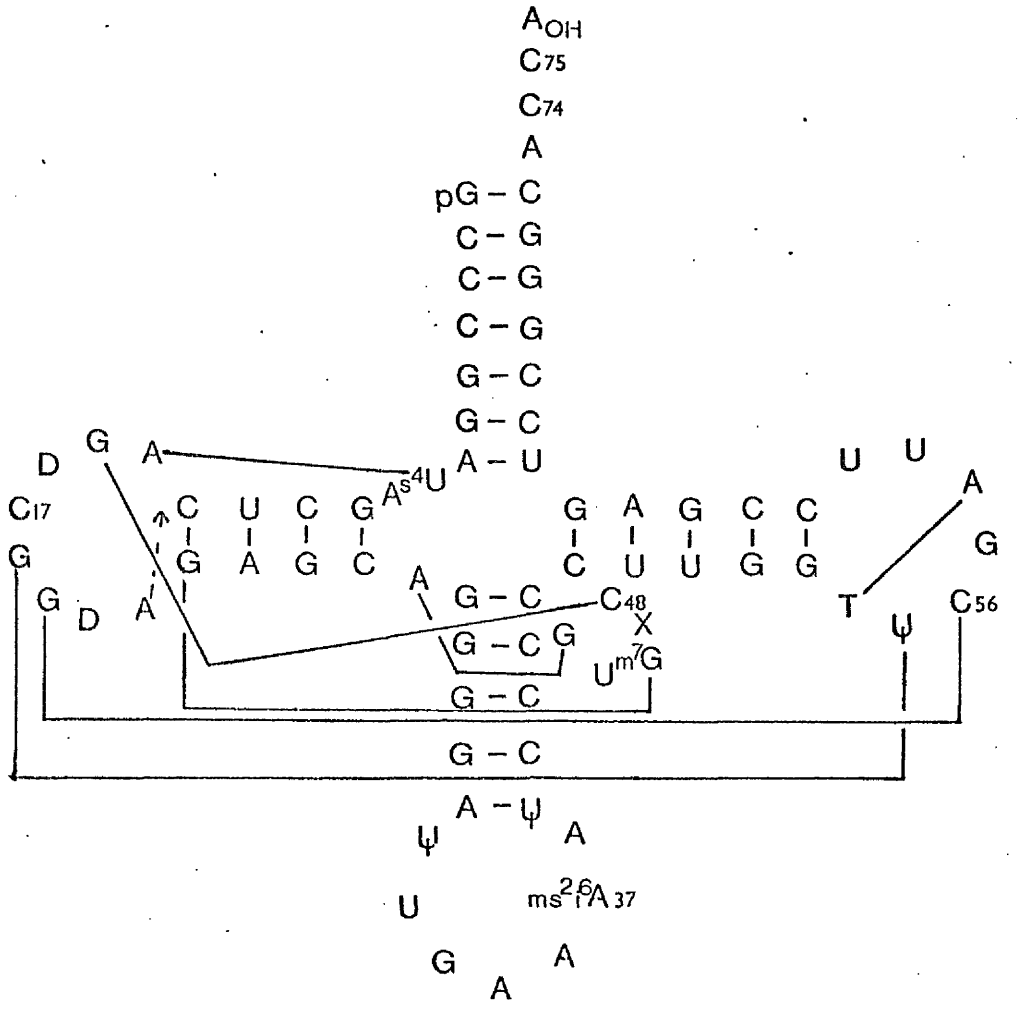
b) C74 $\longrightarrow$ U74

c) C75 $\longrightarrow$ U75

d) ms^2i^6A 37 $\longrightarrow$ $ms^2i^6A - HSO_3^-$ 37.

These modifications were complete after treatment at 25°C for 48 hours. No other modifications were observed under these conditions.

FIG. 19.



E.coli $tRNA_2^{Phe}$ with the tertiary interactions described for Yeast $tRNA^{Phe}$ by Ladner et al (1975b).

N.B. There is some direct evidence for the existence of a hydrogen bond between s^4U8 and A14 in E. coli $tRNA^{Phe}$, (Reid et al , 1975).

FIG. 19 is a cloverleaf representation of E. coli $tRNA^{Phe}_2$, with the tertiary interactions suggested by the results of Kim et al (1974b), Robertus et al (1974a) and Ladner et al (1975b), indicated. It can be seen that there are five cytidine residues in single stranded regions of the cloverleaf structure, i.e. C17, C48, C56, C74 and C75. Of these, only three appeared to be available for bisulphite modification, i.e. C17, C74 and C75. If the structure proposed by Kim et al (1974b) and Robertus et al (1974a) for yeast $tRNA^{Phe}$ is common to all tRNAs, then C48 is probably involved with G15 in a tertiary structure hydrogen bonding interaction, thus making it unavailable for modification by bisulphite. Similarly, C56 is probably hydrogen bonded to G19 and therefore unavailable for reaction with bisulphite (Ladner et al, 1975b). The only other nucleotide modified by bisulphite, i.e. ms^2i^6A 37 is also present in a single stranded region, adjacent to the 3' end of the anticodon.

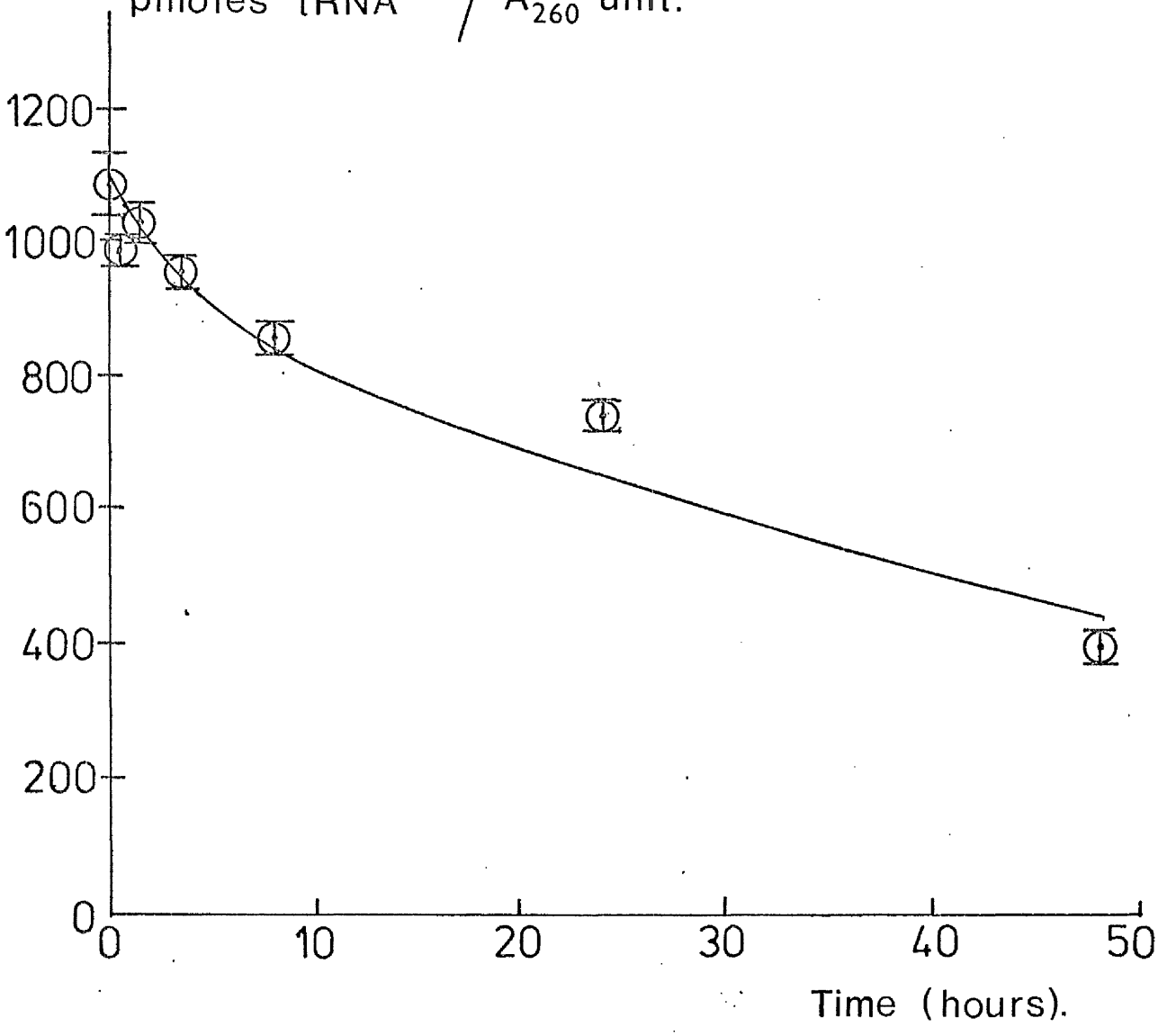
The fact that the fastest bisulphite modification reaction is the conversion of C17 to U17 is consistent with the suggestion of Ladner et al (1975b) that the bases of the region of Loop I are fully exposed. This reaction takes place even faster than that of C74 or C75. Through the amino acid acceptor end of the tRNA Molecule is not buried in the structure and can extend into the solvent, its position is not fixed like that of the α region of Loop I, and it is free to stack on stem a or to fold over and interact with the helix of stem a. This probably accounts for the lower reactivity of C74 or C75 compared with C17. The fact that oligonucleotide 19, on T_1 RNase fingerprints of bisulphite modified $tRNA^{Phe}_2$, contained

65% $CACUA_{OH}$ and 35% $CAUCA_{OH}$ suggests that C75 was more reactive than C74. However, to prove this, it would be necessary to examine Pancreatic RNase fingerprints of $tRNA^{Phe}_2$ samples taken at intervals during modification. The percentage yields of AC at various times of bisulphite modification would indicate the rate of reaction of C74. Since bisulphite modification of ms^2i^6A 37 involves an entirely different reaction mechanism, its rate cannot be directly compared with the rates of reaction of C17, C74 and C75.

The results obtained by bisulphite modification of E. coli $tRNA^{Phe}_2$ are consistent with the 3-dimensional structure of tRNA suggested by Robertus et al (1974a) and Kim et al (1974b). Bisulphite modification appears to be an effective probe for cytidine residues, not involved in base pairing interactions, either of secondary or tertiary structure. The results obtained are also strongly suggestive of the fact that E. coli $tRNA^{Phe}_2$ maintains its native structure under the conditions of modification used.

FIG. 20.

Effect of bisulphite modification on the phenylalanine accepting activity of E.coli $tRNA^{Phe}$.



N.B. The zero time sample was taken immediately after addition of bisulphite. Before bisulphite modification the $tRNA_2^{Phe}$ accepted 1100 pmol. Phe per A_{260} unit.

3.3. THE EFFECT OF BISULPHITE MODIFICATION ON THE PHENYLALANINE ACCEPTING ACTIVITY OF E. COLI $tRNA^{Phe}_2$.

3.3.1. Inactivation of E. coli $tRNA^{Phe}_2$.

Aliquots of *E. coli* $tRNA^{Phe}_2$, removed during bisulphite modification as described in Section 3.2.1., were assayed for phenylalanine accepting activity as described in Section 2.2.2., after destruction of bisulphite adducts (Section 2.2.6). The values obtained for pmoles phenylalanine accepted per A_{260} unit of tRNA were corrected, to allow for the presence of crude tRNA which had been added in order to stabilise $tRNA^{Phe}_2$ (Section 3.1.7.3). As can be seen from FIG. 20, bisulphite modification caused a progressive loss of phenylalanine accepting activity.

As the changes in the $tRNA^{Phe}_2$ molecule, caused by bisulphite modification, might have caused it either to be unchargeable, or more slowly chargeable with phenylalanine, an attempt was made to discover whether the charging reaction was complete under the assay conditions after 20 mins.

Samples of unlabelled *E. coli* $tRNA^{Phe}_2$ were incubated with 3M sodium bisulphite, 10mM $MgCl_2$, pH 6·0 at 25°C for 24 and 48 hours. The bisulphite adducts were destroyed, and the modified tRNA dialysed finally against 10mM tris-HCl, 10mM $MgCl_2$, pH 7·0. The extent of charging with phenylalanine of unmodified, 24 hour and 48 hour modified $tRNA^{Phe}_2$ samples, under standard assay conditions (Section 2.2.2), was examined over a period of one hour. Exactly the same amount (400 pmoles) of $tRNA^{Phe}_2$ (either unmodified, or modified with bisulphite for 24 or 48 hours) was used in each case, in a total charging mixture

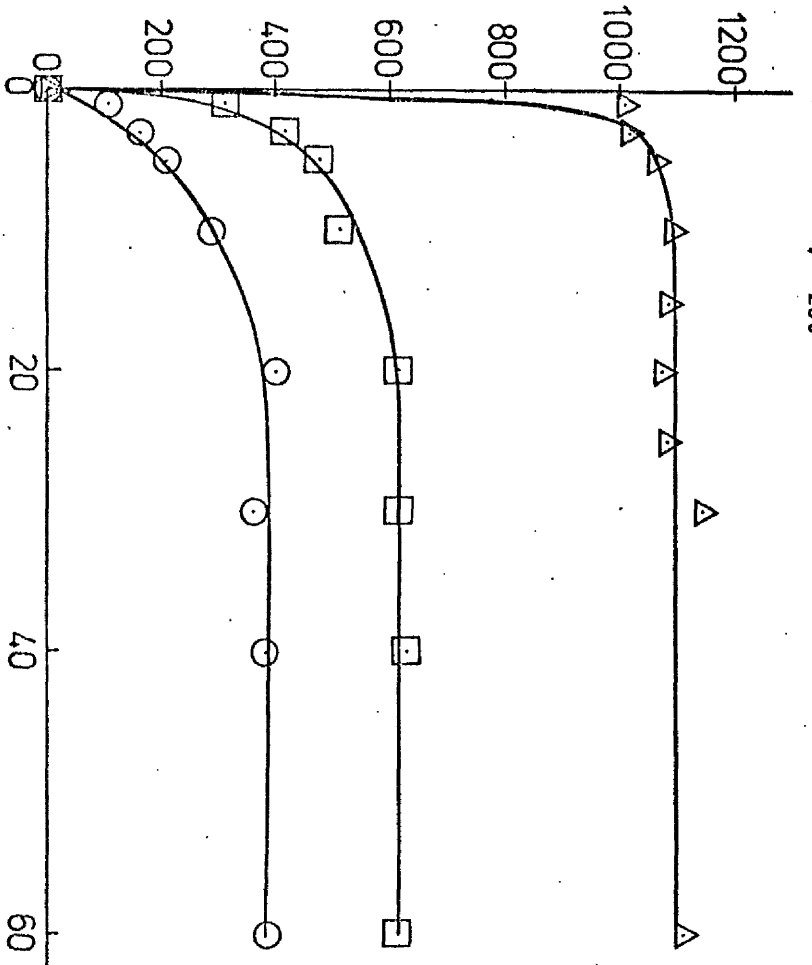


FIG. 21.

Extent of phenylacylation of unmodified and bisulphite modified $tRNA_2^{Phe}$.

volume of 2mls. The reaction was started by addition of purified phenylalanyl-tRNA ligase. Duplicate samples of 100 µl were taken at various times after the reaction was started and treated as described in Section 2.2.2. The zero time sample was taken, before addition of the ligase, in quadruplicate and this was regarded as the blank. The extent of charging of the different $tRNA^{Phe}_{2}$ samples with time is shown in FIG. 21. It can be seen from this graph that although the modified tRNA samples appeared to be charged more slowly with phenylalanine, they were charged to the maximum extent after 20 mins.

3.3.2. Effect of Phenylalanyl-tRNA Ligase Concentration on the Extent of Charging of Modified E. coli $tRNA^{Phe}_{2}$.

Renaud et al (1974) found that the extent of aminoacylation of yeast $tRNA^{Phe}$ depended on the concentration of phenylalanyl-tRNA ligase. With this in mind, an attempt was made to discover the effect of increasing the ligase concentration on the extent of charging of E. coli $tRNA^{Phe}_{2}$ which had been modified with bisulphite for a period of 48 hours.

A total reaction mixture of 2mls. was used, containing 400 pMoles of modified $tRNA^{Phe}_{2}$. The reaction mixtures were set up and 100 µl samples were taken as described in the previous Section. The ligase concentrations in the charging mixtures were

A) 135 µg / ml (Standard assay conditions).

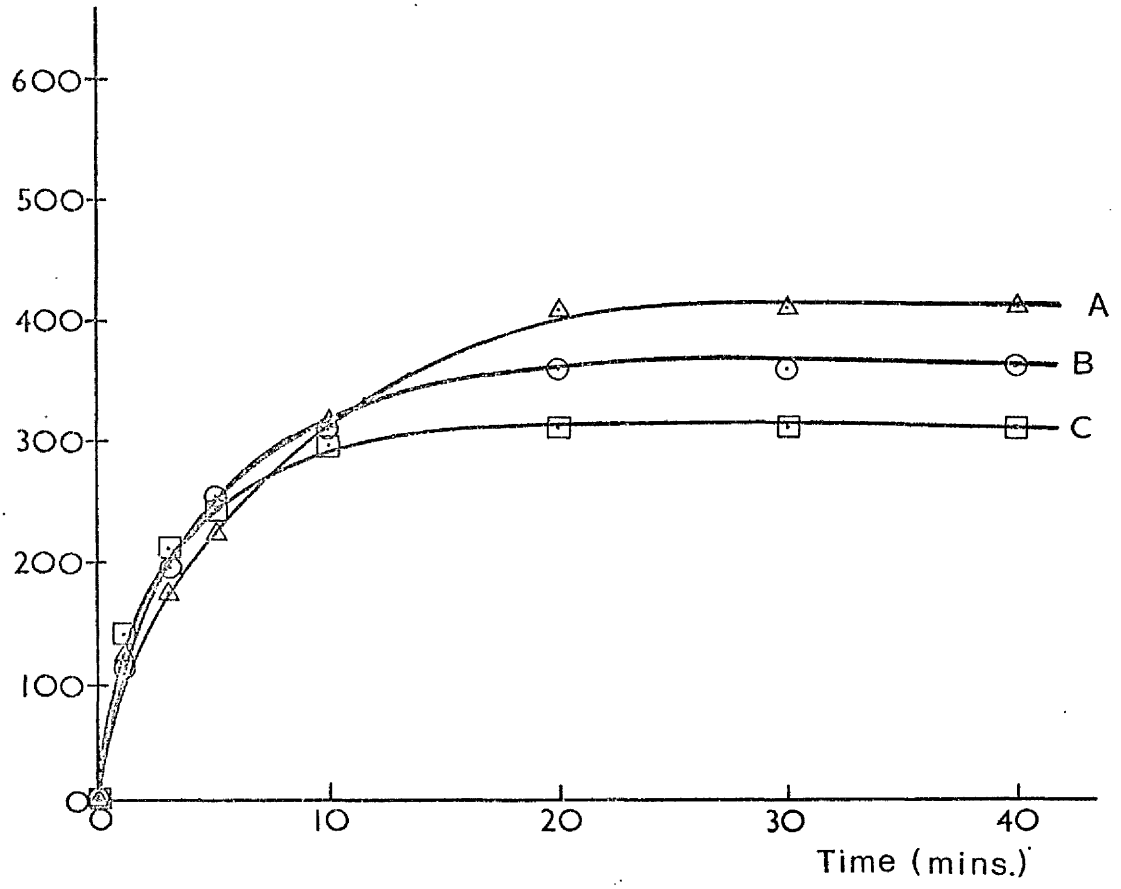
b) 270 µg / ml.

c) 405 µg / ml.

The results shown in FIG. 22 indicate that increasing the enzyme concentration caused a decrease in the extent to which

FIG. 22.

Effect of enzyme concentration on extent of charging of $tRNA^{Phe}$.



A. normal concentration of ligase.

B. 2x normal enzyme concentration.

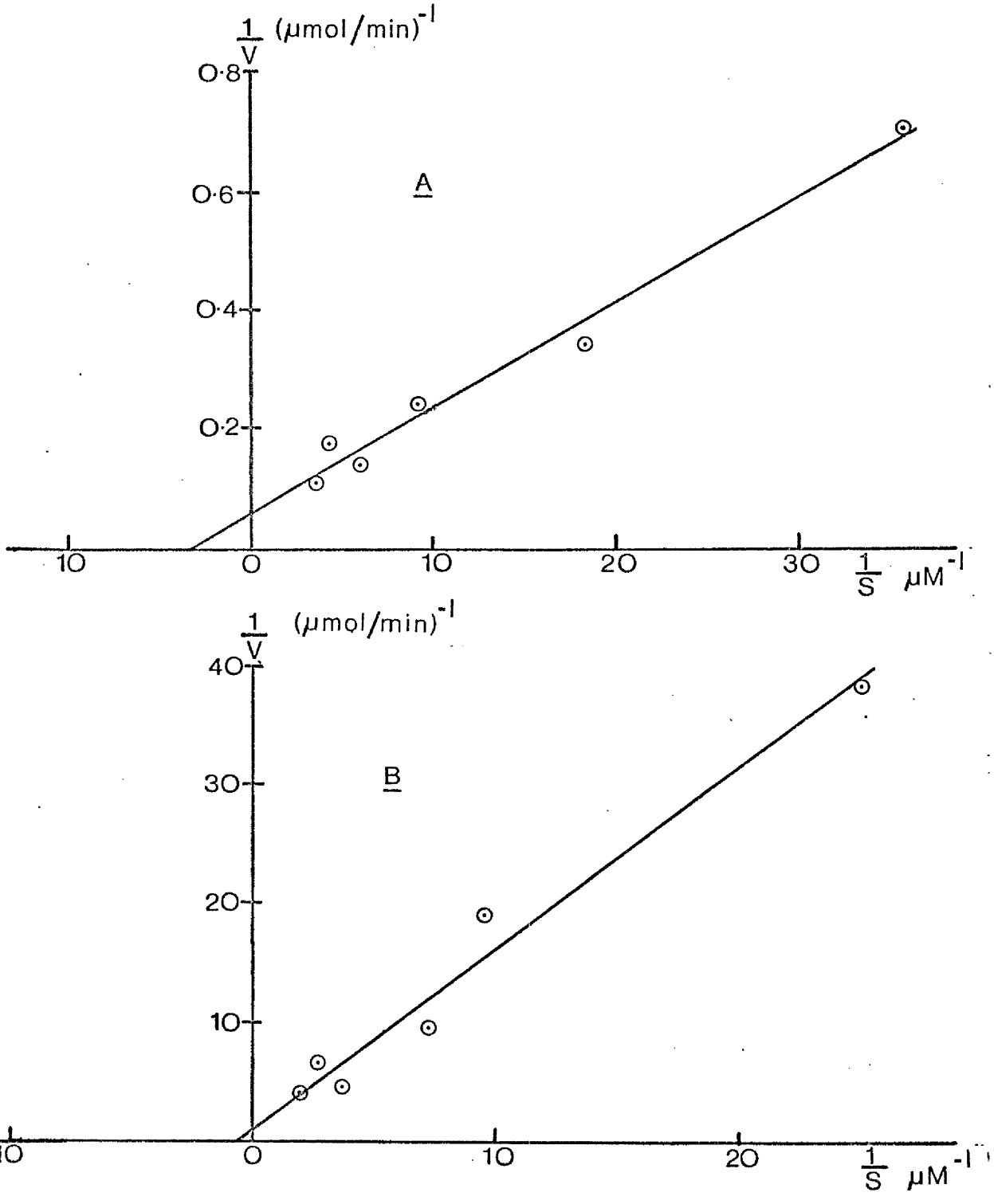
C. 3x normal enzyme concentration.

FIG. 23.

Lineweaver – Burk plots for :-

A. Unmodified $tRNA_2^{Phe}$.

B. 48 hour bisulphite modified $tRNA_2^{Phe}$.



fully bisulphite modified $tRNA^{Phe}_2$ could be charged with phenylalanine.

3.3.3. The Effect of Bisulphite Modification on Km and V_{MAX} for $tRNA^{Phe}_2$ in the Charging Reaction.

The initial rates of aminoacylation of *E. coli* $tRNA^{Phe}_2$ (unmodified and bisulphite modified for 48 hours) were measured with various different concentrations of $tRNA^{Phe}_2$ in the reaction mixture. The concentrations of the other components of the charging mixture were as described in Section 2.2.2., except that one tenth of the concentration of phenylalanyl-tRNA ligase was used. As the concentrations of the other substrates (ATP and phenylalanine) were kept constant, while the concentration of $tRNA^{Phe}_2$ was varied, it was possible from these results to derive apparent K_ms and V_{MAX}s for the two species of $tRNA^{Phe}_2$. The reaction was carried out in a total of 2mls., and started by the addition of ligase as described in Section 3.3.1. Duplicate 100 μl aliquots were taken every 30 seconds after addition of the ligase. In all cases the initial rates were found to be constant for at least the first 3·5 minutes.

FIG. 23 shows Lineweaver-Burk plots for unmodified *E. coli* $tRNA^{Phe}_2$ and tRNA that had been modified with bisulphite for 48 hours. From these results, the K_m and V_{MAX} for each of the two species of $tRNA^{Phe}_2$ were determined, and are shown below

	K_m (37°C.)	V_{MAX} (37°C.)
Unmodified $tRNA^{Phe}_2$	3×10^{-7}M	18·0 μMoles / min.
Fully bisulphite modified $tRNA^{Phe}_2$	1.6×10^{-6}M	1·3 μMoles / min.

These results indicate that complete conversion of C17 $\longrightarrow$ U17, $ms^2i^6A37 \longrightarrow ms^2i^6A - HSO_3^- 37$, C74 $\longrightarrow$ U74 and C75 $\longrightarrow$ U75 in E. coli $tRNA^{Phe}$ caused a decrease in the V_{MAX} and an increase in K_m for the $tRNA^{Phe}_2$.

3.3.4. Separation of Active and Inactive Forms of Bisulphite Modified $tRNA^{Phe}_2$.

In order to discover which of the changes occurring on bisulphite modification were responsible for loss of phenylalanine accepting activity, an attempt was made to separate active and inactive forms of bisulphite modified $tRNA^{Phe}_2$. Active bisulphite modified $tRNA^{Phe}_2$ molecules are chargeable with phenylalanine, while inactive ones are not. Charged and uncharged molecules may be separated on a Benzoylated DEAE-cellulose column in a manner similar to that described in Section 3.1.2. This separation depends on the fact that phenylacylated $tRNA^{Phe}_2$ molecules have a greater affinity for the hydrophobic benzoyl groups of Benzoylated DEAE-cellulose than uncharged molecules. Changes in structure, caused by bisulphite modification, which are present in inactive molecules but not in active molecules, must be responsible for the loss of phenylalanine accepting activity.

^{32}P-labelled, purified E. coli $tRNA^{Phe}_2$ (see Section 3.1.7.3.) was incubated in 3M sodium bisulphite, 10mM $MgCl_2$ pH 6·0 at 25°C for 12 hours. Bisulphite modification for this length of time reduced the phenylalanine accepting activity to about 60% of its original value, when assayed by the method described in Section 2.2.2. The bisulphite adducts were destroyed (Section 2.2.6.) and the $tRNA^{Phe}_2$ phenylacylated to the

Separation of active and inactive bisulphite-modified $tRNA_2^{phe}$

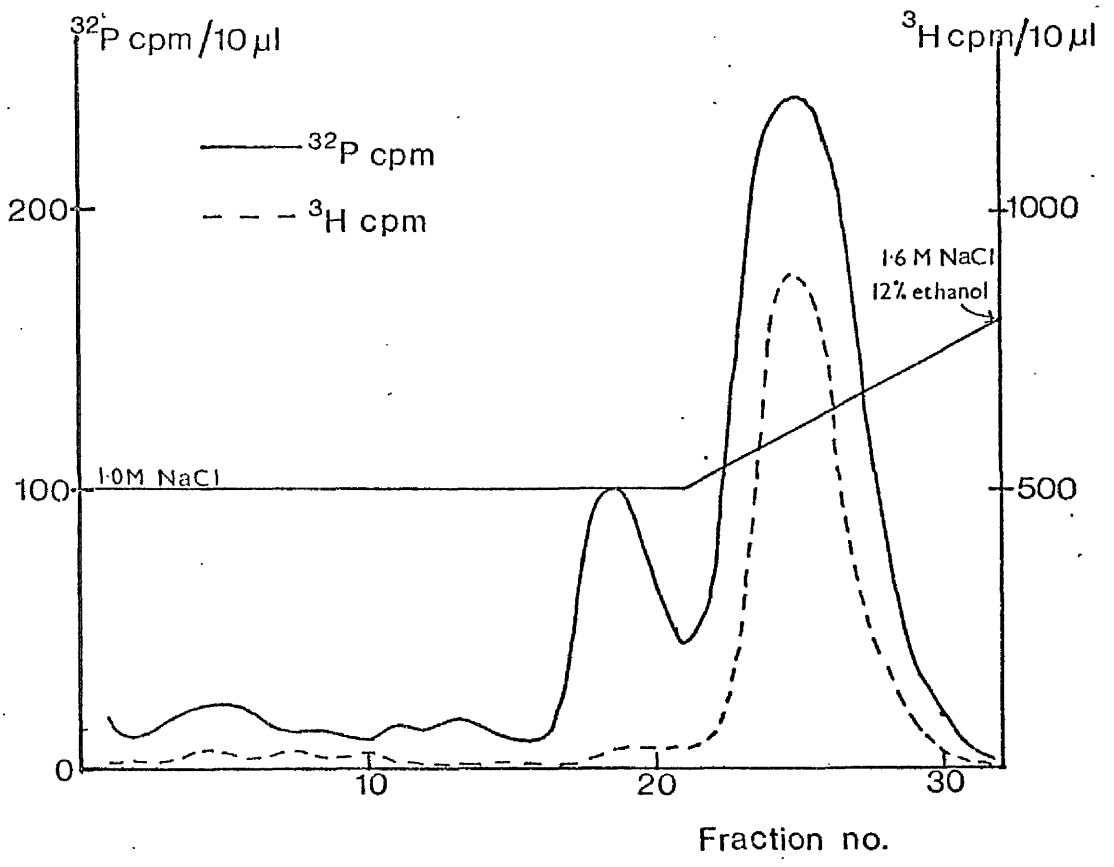
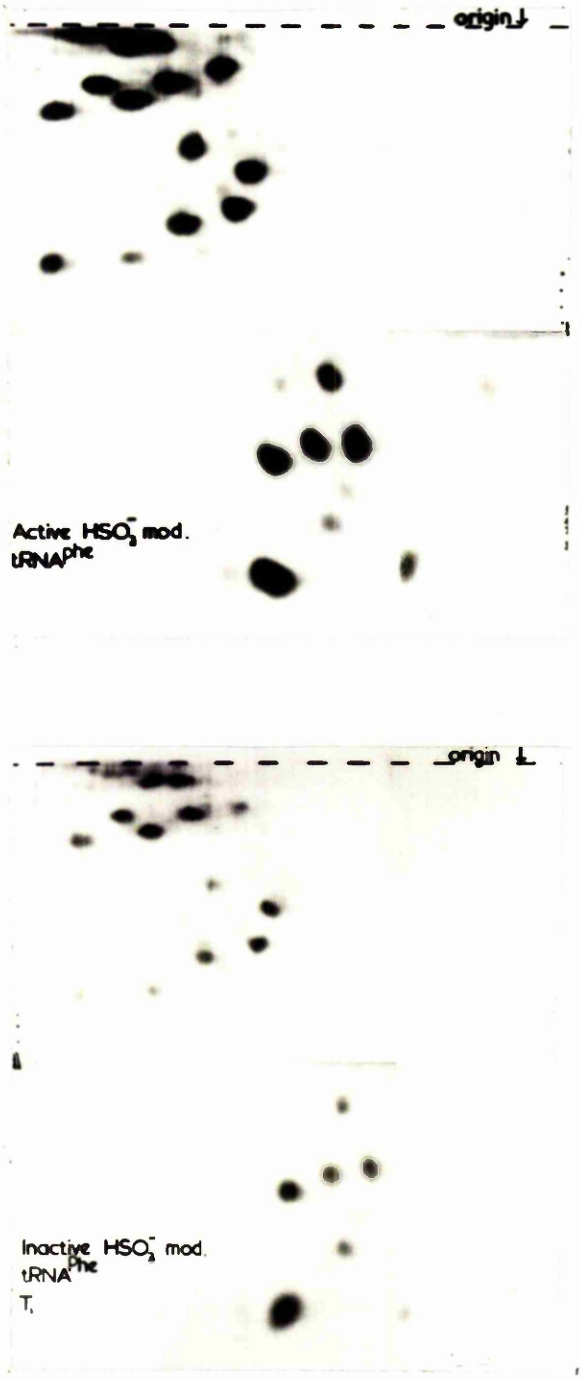


FIG. 24.

maximum extent as described in Section 2.2.3. Separation of charged and uncharged $tRNA^{Phe}_2$ molecules was performed exactly as described in Section 3.1.2. Such a separation is shown in FIG. 24. The first peak of ^{32}P - containing material was uncharged modified $tRNA^{Phe}_2$, while the second ^{32}P - containing peak contained charged modified $tRNA^{Phe}_2$. From the ratio of ^{3}H to ^{32}P counts, it was calculated that the second peak contained 950 - 1250 pmoles phenylalanine per A_{260} unit of tRNA.

The two fractions, phenylalanyl-$tRNA^{Phe}_2$, and uncharged $tRNA^{Phe}_2$, were pooled separately. Phe - $tRNA^{Phe}_2$ was deacylated by dialysis against 0·1M tris - HCl, pH 9·0 buffer at 37°C for 2 hours, followed by dialysis against 20mM tris - HCl, 10mM $MgCl_2$, pH 7·0, to restore the pH to neutrality. Both fractions were desalted by exhaustive dialysis against distilled water, lyophilised, and fingerprinted. FIG. 25 shows T_1 RNase fingerprints of active and inactive bisulphite modified forms of $tRNA^{Phe}_2$. TABLE 12 compares the percentage molar yields of the oligonucleotides on fingerprints of active and inactive fractions of $tRNA^{Phe}_2$. An examination of the fingerprints in FIG. 25 reveals that both active and inactive fractions contain $tRNA^{Phe}_2$ molecules with all of the possible bisulphite modifications described in Section 3.2. A comparison of the percentage molar yields of each oligonucleotide in the two fingerprints indicates that the amounts of "modified oligonucleotides", i.e. DUG, AANAΨCCCCG, $CA(C,U)A_{OH}$ and $CAUUA_{OH}$, are slightly higher in the inactive fraction than in the active fraction, with correspondingly lower values for DCG,

FIG. 25.



T_1 RNase Fingerprints of Active and Inactive Fractions of Bisulphite Modified $tRNA_2^{Phe}$.

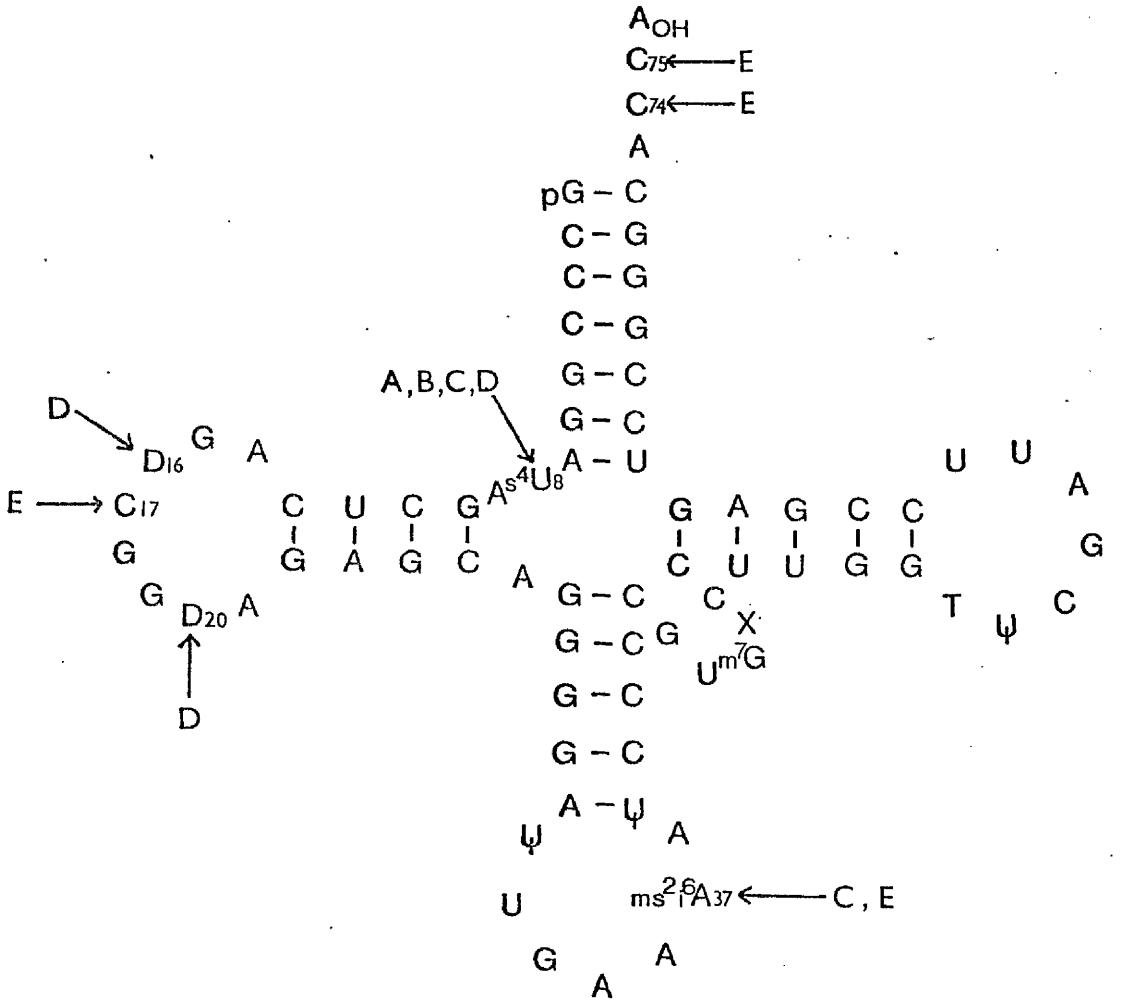
TABLE 12.

PERCENTAGE MOLAR YIELDS OF OLIGONUCLEOTIDES ON T_1 RNASE FINGERPRINTS OF ACTIVE AND INACTIVE FRACTIONS OF BISULPHITE MODIFIED E. COLI $tRNA^{Phe}_2$.

OLIGONUCLEOTIDE	PERCENTAGE MOLAR YIELD	
	ACTIVE FRACTION	INACTIVE FRACTION
G + G!	99	120
AG (2)	103	128
CAG	103	109
CCCG	103	109
$CACCA_{OH}$	8	4
pG + pG!	95	102
DAG	90	99
DCG	46	33
UCCG	101	100
CUCAG	77	79
AUAG	61	60
TψCG	130	150
AψUG	117	113
AUUCCG	100	123
$AAms^2i^6AA\psi CCCCG$	43	14
$Um^7GXCCUUG$	86	75
19 ($CA(C,U)A_{OH}$)	36	37
20 ($CAUUA_{OH}$)	58	63
21 (DUG)	48	65
22 (AANAψCCCG)	38	56

The $tRNA^{Phe}_2$ had been modified in 3M $NaHSO_3$, 10mM $MgCl_2$, pH 6·0 for 12 hours before separation of active and inactive fractions.

FIG.26.



A Parachloromercuribenzoate

B Cyanogen Bromide

C Iodine

D Sodium Borohydride

E Sodium Bisulphite

Chemical Modification of E. coli $tRNA^{Phe}$.

$AAms^2i^6AA\psi CCCCG$ and $CACCA_{OH}$.

3.3.5. Discussion.

There have been several attempts to pinpoint the ligase recognition site of E. coli $tRNA^{Phe}_2$. Chemical modification with (A) Parachloromercuribenzoate (Pal et al, 1972), (B) Cyanogen bromide (Carré et al, 1974), (C) Iodine (Faulkner & Uziel, 1971) and (D) Sodium borohydride (Shugart & Stulberg, 1969) has been shown to cause modification of various nucleotides as indicated in FIG. 26. Modification of s^4U8 with Parachloromercuribenzoate did not prove to be a useful probe of the ligase recognition site, as the product, 4 - [(p - carboxyphenyl) mercurithio] uridine, was found to be labile in the presence of Mg^{2+} ions, which were a component of the phenylalanine accepting assay. However, cyanogen bromide modification of s^4U in E. coli $tRNA^{Phe}$ did not significantly affect its phenylalanine accepting activity.

Modification of E. coli $tRNA^{Phe}$ with sodium borohydride for $3\frac{1}{2}$ hours caused a complete loss of phenylalanine accepting activity, together with hydrogenation of s^4U8, D16 and D20, suggesting that one or all of these nucleotides, or this region of the molecule, is involved in the synthetase recognition site. Iodine modification of E. coli $tRNA^{Phe}$ resulted in modification s^4U8 (probably conversion to the sulphonate) and ms^2i^6A37 (probably formation of iodine addition products). However, molecules with both of these modifications were still active in phenylalanine accepting assays. Taking into account both the results of Shugart & Stulberg (1969) and Faulkner & Uziel (1971), D16 and / or D20 seem to be the most likely contenders for the

ligase recognition site.

Stulberg & Isham (1967) have tested various products of limited snake venom phosphodiesterase digestion of E. coli $tRNA^{Phe}$, as inhibitors of $tRNA^{Phe}$ aminoacylation. One region of $tRNA^{Phe}$ was implicated in synthetase recognition, i.e., the region from G19 to U33. Considering the results so far described, D20 is the most likely nucleotide to be involved in the ligase recognition site. Undermethylation of $tRNA^{Phe}$ (Shugart et al, 1968) has been found to decrease the phenylalanine accepting activity of $tRNA^{Phe}$. Formation of a photochemically induced cross-link between s^4U8 and C13 has been found to completely destroy phenylalanine accepting activity (Carré et al, 1974).

The results described in Sections 3.3.1. and 3.3.2., indicate that bisulphite modification of E. coli $tRNA^{Phe}_2$ causes a loss of phenylalanine accepting activity. While bisulphite modification of $tRNA^{Phe}_2$ for 48 hours caused complete conversion of C17 to U17, ms^2i^6A37 to $ms^2i^6A - HSO_3^-37$, C74 to U74 and C75 to U75, some phenylalanine accepting activity remained. This suggests that these modifications were not directly responsible for the loss of phenylalanine accepting activity. Comparison of active and inactive forms, separated as described in Section 3.3.4., indicates that both forms contain all the possible bisulphite modifications. These results confirm that no single bisulphite modification was responsible for the loss of phenylalanine accepting activity. Complete bisulphite modification did alter the Km and V_{MAX} of E. coli phenylalanyl - tRNA ligase for $tRNA^{Phe}_2$ (Section 3.3.3.). However, it has been proved in Sections 3.3.1. and 3.3.2., that even when charging

of 48 hour bisulphite modified $tRNA^{Phe}_2$ is complete, a proportion of the molecules remain uncharged.

A possible explanation for the results described could be that while the modifications were not directly responsible for loss of phenylalanine accepting activity, they made the $tRNA^{Phe}_2$ molecules more susceptible to denaturation, producing an inactive form. Denaturation could have occurred on removal of bisulphite adducts by pH 9·0 treatment. Denaturation has been shown to occur on deacylation of Phe-$tRNA^{Phe}$ under similar conditions (TABLE 3, Section 3.1.2.). However, the $tRNA^{Phe}_2$ that was used in bisulphite modification had been stabilised by the addition of crude tRNA (Section 3.1.7.3.), and the phenylalanine accepting activity of $tRNA^{Phe}_2$ removed immediately after addition of bisulphite, and treated with pH 9·0 buffer to remove bisulphite adducts, was not reduced compared with that of unmodified, untreated $tRNA^{Phe}_2$.

Another possible explanation for the results is that some chemical modification was caused by bisulphite that was not detected by the methods described in Section 3.2.1, and that this modification was responsible for loss of phenylalanyl accepting activity. As discussed in Section 3.2.3., bisulphite is capable of the conversion of s^4U to U, and this modification would not be detected on the fingerprints if it occurred on bisulphite modification of *E. coli* $tRNA^{Phe}_2$. This nucleotide is probably not a component of the ligase recognition site, as it can be modified with cyanogen bromide (Carré *et al*, 1974) or iodine (Faulkner & Uziel, 1971) without loss of activity.

The introduction of a cross-link between C13 and s^4U8 in *E. coli* $tRNA^{Phe}$ has been shown to destroy its phenylalanine

accepting activity (Carre et al, 1974). However, there was no oligonucleotide on the T_1 RNase fingerprint of inactive form of $tRNA_2^{Phe}$, and not in that of the active form, that would correspond to As^4UAG, nor is there any difference in the

CUCAG

yield of AUAG and CUCAG between the active and inactive forms (see TABLE 12).

Rich (1974) has proposed a general model for the tRNA - aminoacyl - tRNA ligase interaction. In this model the ligase interacts with a relatively large area of the tRNA molecule i.e. stem a, part of stem b, stem c and in some cases, the anticodon (see FIG. 3). Several lines of evidence have led to this proposal. The ligase must interact with the 3' -OH terminus of the molecule, as it is here that the cognate amino acid is attached. The anticodon has proved to be important in recognition of some tRNAs by their cognate aminoacyl - tRNA ligases but not all (see Section 1.4.1.6.3.). Rich has proposed that in some cases there is interaction between the ligase and the anticodon of the cognate tRNA, but not in all cases. There is some evidence that stem b is important in recognition of tRNA by its cognate ligase (Roe & Dudock, 1972; Kern et al, 1972).

Rich has suggested that the variable regions of tRNA structure (α and β regions of Loop I and Loop III) are not involved in interaction with the ligase. Evidence for this comes from the fact that tRNAs with the same nucleotide sequences in the α and β regions of Loop I are recognised by different ligases (Kim et al, 1974a). Also, two tRNAs with

different Loop III structures (*E. coli* $tRNA^{Gln}$ and a mutant *E. coli* $tRNA^{Tyr}_{su_3^+}$) have been shown to be charged by the same ligase (Celis *et al*, 1973). The facts that the K_ms of many aminoacyl - tRNA ligase for their cognate tRNAs are numerically similar (Myers *et al*, 1971) and that a number of tRNAs may be aminoacylated *in vitro* by the same ligase (Roe & Dudock 1972; Yarus & Mertes, 1973) have suggested that an appreciable part of the free energy of binding of tRNA to its cognate ligase comes from interactions common to many tRNA - ligase pairs. These interactions are probably those between the ligase and the ribose - phosphate backbone, the specificity of interaction depending on particular specific sequences.

If the aminoacyl - tRNA ligase interacts closely with such a large area of the tRNA, as described by Rich (1974), the interaction is bound to be affected by minor changes in the conformation of the tRNA, the correct conformation being essential for close interaction. The loss of phenylalanine accepting activity observed on bisulphite modification of *E. coli* $tRNA^{Phe}_2$ was most probably due to the fact that modifications introduced into the $tRNA^{Phe}_2$ molecules facilitated their denaturation to an inactive form.

The alteration in the values of Km and V_{MAX} of phenylalanyl-tRNA ligase for $tRNA^{Phe}_2$ might not be due to lower affinity of modified $tRNA^{Phe}_2$ for the ligase, but simply due to the fact that there is a significant proportion of unchargeable $tRNA^{Phe}_2$ molecules in the 48 hour modified $tRNA^{Phe}_2$ sample, which could possibly act as competitive or uncompetitive inhibitors. In order to discover the true effect of conversion of C17 to

U17, $ms^2i^6A_{37}$ to $ms^2i^6A - HSO_3^-$ 37, C74 to U 74 and C75 to U75 on Km and V_{MAX} for $tRNA^{Phe}_2$, it would be necessary to carry out the kinetic studies on active $tRNA^{Phe}_2$ isolated from bisulphite modified $tRNA^{Phe}_2$ by the method described in Section 3.2.4.

3.4. ISOLATION AND BISULPHITE MODIFICATION OF Phe-tRNA$^{Phe}_{2}$.

Aminoacyl-tRNA has been shown to interact in a different way to tRNA, with the E. coli elongation factor, Tu (Ono et al, 1968), aminoacyl-tRNA ligases (Lagerkvist et al, 1966), the histidine operon repressor (Lewis & Ames, 1972) and ribosomal binding sites (Grajevskaja et al, 1972). To account for these differences, it has been suggested that tRNA molecules undergo a conformational change on aminoacylation (Sarin & Zamecnik, 1965; Schofield, 1970; Woese, 1970). C.D. and U.V. studies of E. coli tRNA^{Val} and tRNA$^{Met}_{f}$ (Adler & Fasman, 1970), partial nuclease digestion studies of E. coli tRNA^{Phe}, Yeast tRNA^{Phe} and Yeast tRNA^{Ser} (Hänngi & Zachau, 1971), small angle X-ray scattering studies of E. coli tRNA^{Val} (Ninio et al, 1972), tritium exchange studies of E. coli tRNA$^{Met}_{f}$ (Englander et al, 1972), P.M.R. studies of yeast tRNA^{Phe} (Wong et al, 1973), and Raman spectroscopy studies of yeast tRNA^{Phe} (Thomas et al, 1973b) did not show any detectable change in conformation on aminoacylation.

However, small conformational changes were detectable on aminoacylation by other C.D. and U.V. studies of E. coli tRNA$^{Met}_{f}$ (Wanatabe & Imahori, 1971), kinetic binding to unfractionated tRNA (Tritton & Mohr, 1973), and increased binding of Mn^{2+} and oligo-C to aminoacylated tRNA (Cohn et al, 1969; Danchin & Grunberg - Manago, 1970). Most of the available evidence implies that if any changes do occur on aminoacylation, they are small, being restricted to slight changes in tertiary structure or minor rearrangements of the secondary structure.

Forget & Weissmann (1967) suggested that binding of aminoacyl-tRNA to the ribosome might involve interactions between the TψCG region of Loop IV (a constant feature of tRNA structure) and a CGAAC sequence contained in 5S RNA of the large ribosomal subunit. It is interesting to note that the TψCG sequence is absent from some tRNAs not involved in ribosome-mediated protein synthesis, i.e. glycine tRNAs of some species of Staphylococcus involved in cell wall biosynthesis (Roberts, 1972). Other evidence supporting this hypothesis has been presented by Richter et al (1973), who have shown that the tetranucleotide TψCG will bind to E. coli 50S ribosomal subunits, thus inhibiting elongation factor Tu - dependent aminoacyl-tRNA binding, and Erdmann et al (1973) who have shown that this tetranucleotide binds to a specific 5S RNA - protein complex derived from E. coli 50S ribosomal subunits. This binding is abolished on chemical modification of the two adenines in the sequence CGAAC of 5S RNA. Also in support of this hypothesis, Dube (1973a) has shown that E. coli 70S ribosomes, but not 30S ribosomal subunits, will protect Loop IV of E. coli $tRNA^{Met}_{f}$.

While the results described above suggest the importance of an interaction between Loop IV of aminoacyl-tRNA and 5S RNA in the binding of aminoacyl-tRNAs to the ribosome, recent results of the X-ray analysis of yeast $tRNA^{Phe}$ have indicated that none of the bases of the sequence TψCG would be available for base pairing to the sequence CGAAC in 5S RNA, except perhaps ψ55 (Ladner et al, 1975b). There is evidence for the "buried" nature of these bases from chemical

modification and enzyme dissection data described in Section 1.3., and from the results of bisulphite modification of *E. coli* $tRNA^{Phe}_2$ described in Section 3.2. It is possible, however, that on aminoacylation, a conformational change exposing the TψCG region, occurs. If such a conformational change were to occur on aminoacylation of *E. coli* $tRNA^{Phe}_2$, it ought to be detectable by bisulphite modification, because C56 would become exposed and therefore available for modification. Bisulphite modification of Phe - $tRNA^{Phe}_2$ would also reveal whether any other cytosine residues become exposed on aminoacylation.

Bisulphite modification of *E. coli* Phe $tRNA^{Phe}_2$ was complicated, practically, by the fact that deacylation was found to occur under the modification conditions normally used (3M sodium bisulphite, 10mM $MgCl_2$, pH 6.0 at 25°C). Under these conditions, Phe - $tRNA^{Phe}_2$ was found to have a half life of 3.5 hours. Therefore, in order to unambiguously identify which cytosine residues were available for modification in Phe-$tRNA^{Phe}$, it was necessary to separate modified Phe - $tRNA^{Phe}_2$ from modified deacylated $tRNA^{Phe}_2$ after a period of modification. This separation was effected on a Benzoylated DEAE-cellulose column.

3.4.1. Preparation and Bisulphite Modification of *E. coli* Phe - $tRNA^{Phe}_2$.

Purified ^{32}P - labelled *E. coli* $tRNA^{Phe}_2$, capable of accepting 800 pmoles of phenylalanine per A_{260} unit, was charged to the maximum extent with ^{3}H - labelled phenylalanine

Separation of Bisulphite Modified $tRNA_2^{Phe}$ and Phe-$tRNA_2^{Phe}$.

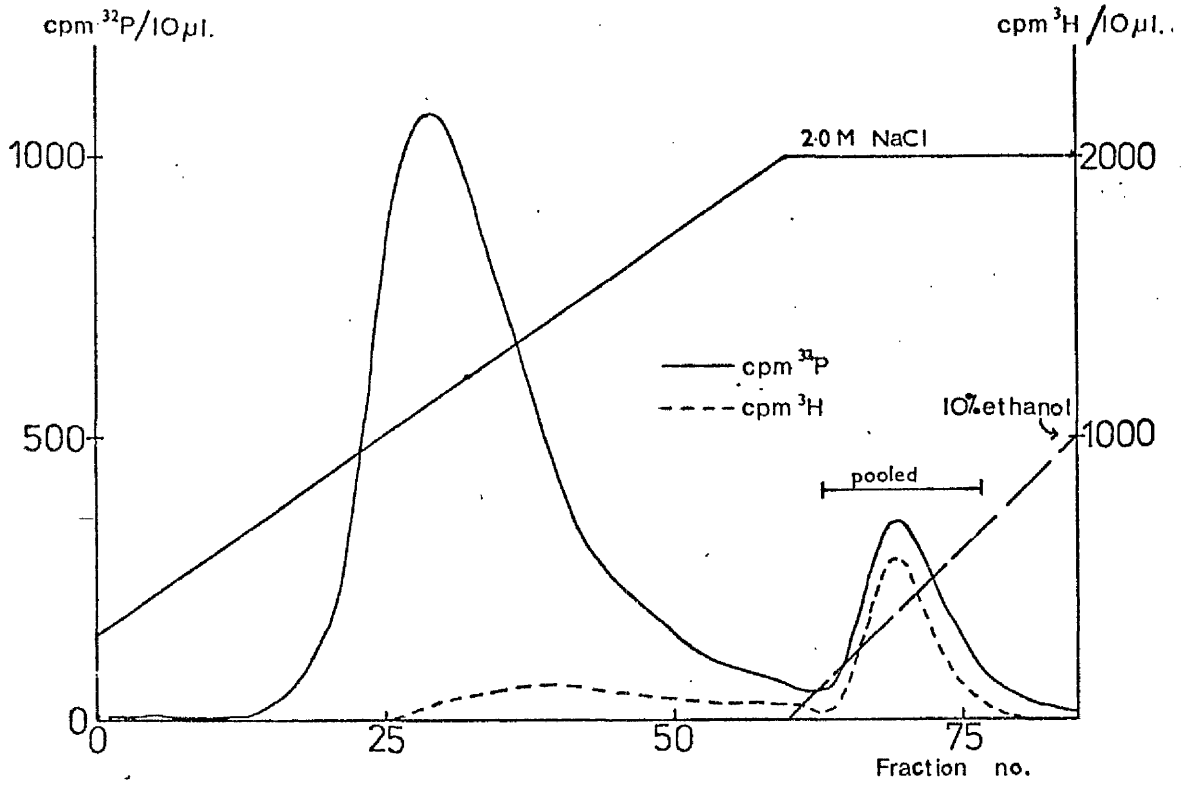


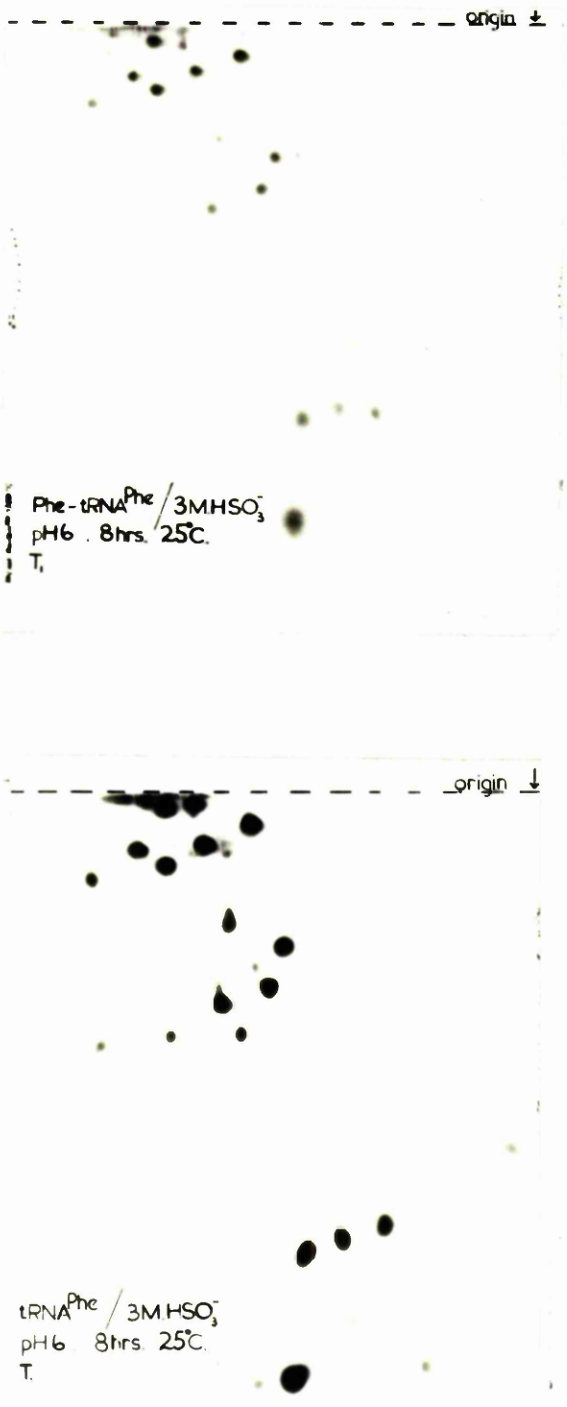
FIG. 27.

as described in Section 2.2.3. The charged tRNA was suspended in 3M sodium bisulphite, 10mM $MgCl_2$, pH 6·0 at 25°C for 8 hours. At the end of this time, the modified tRNA was dialysed to remove excess bisulphite, first against 0·1 M sodium acetate, 10mM $MgCl_2$, pH 5·0, and then twice against 10mM sodium acetate, 10mM $MgCl_2$, pH 5·0, each time for 1 hour at 4°C.

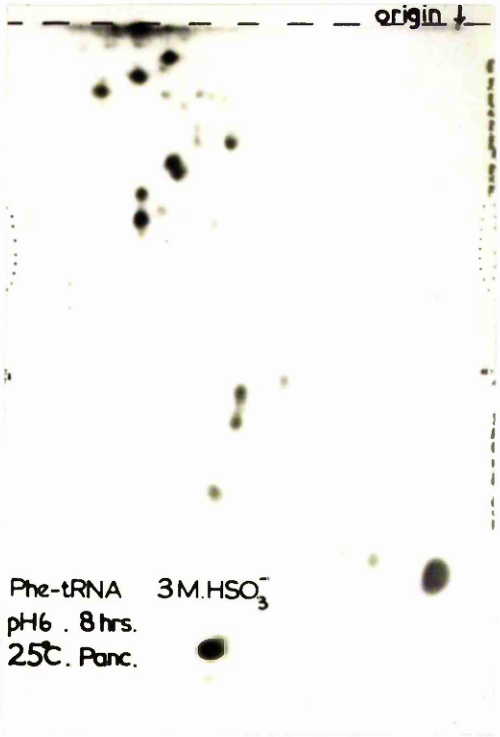
After dialysis, the modified tRNA was applied to a Benzoylated DEAE-cellulose column which had been pre-equilibrated with 0·3 M NaCl, 10mM $MgCl_2$, 10mM sodium acetate, pH 5·0, at 4°C. The ratio of tRNA applied to packed bed volume of the column was the same as described in Section 3.1.1. The column was run at 4°C in pH 5·0 buffer to retard deacylation of the modified Phe-$tRNA^{Phe}_2$. A 0·3 M to 2·0 M NaCl gradient was applied to the column in order to elute deacylated $tRNA^{Phe}_2$ molecules containing bisulphite adducts and any other tRNA species, which were contaminants of the original $tRNA^{Phe}_2$ preparation. Because of the more hydrophobic nature of Phe - $tRNA^{Phe}_2$ (containing bisulphite adducts), the application of an ethanol gradient was required for its elution from the column. A 2·0 M NaCl, 0% Ethanol to 2·0 M NaCl, 20% Ethanol gradient was employed. The fractionation is shown in FIG. 27. The second peak contained $tRNA^{Phe}_2$ charged to an extent of 1150 pmoles ^3H - labelled phenylalanine per A_{260} unit of tRNA.

The whole of the second peak, between the limits shown on FIG. 27, was pooled and dialysed for 9 hours against 0·1 M tris - HCl, pH 9·0 at 37°C. This procedure served both to remove bisulphite adducts and deacylate the Phe-$tRNA^{Phe}_2$. The

FIG. 28.



Comparison of T_1 RNase Fingerprints of E. coli $tRNA_2^{Phe}$, modified with 3M $NaHSO_3$, pH 6·0 at 25°C, in the charged and uncharged form.



Comparison of Pancreatic RNase Fingerprints of E. coli $tRNA_2^{Phe}$, modified with 3M $NaHSO_3$, pH 6.0 at 25°C, in the charged and uncharged form.

TABLE 13.

PERCENTAGE YIELDS OF OLIGONUCLEOTIDES ON T_1 RNASE FINGERPRINTS OF E. COLI $tRNA_2^{Phe}$, BISULPHITE MODIFIED FOR 8 HOURS, IN THE CHARGED AND UNCHARGED FORMS.

OLIGONUCLEOTIDE	PERCENTAGE YIELD	
	UNCHARGED FORM	CHARGED FORM
G + G!	90	90
AG (2)	110	119
CAG	98	102
CCCG	98	95
$CACCA_{OH}$	24	15
pG + pG!	102	108
DAG	104	104
DCG	20	17
UCCG	102	106
CUCAG	82	85
AUAG	64	55
TψCG	126	134
AψUG	103	110
AUUCCG	101	100
$AAms^2i^6AA\psi$ CCCCG	49	73
Um^7GXCCUUG	89	86
19 ($CA(C,U)A_{OH}$)	18	11
20 ($CAUUA_{OH}$)	35	48
21 (DUG)	86	82
22 (AANAψCCCCG)	50	28

pH was restored to neutrality by dialysis against 10mM tris-HCl, 10mM $MgCl_2$, pH 7·0 and then against 2mM tris - HCl, 10mM $MgCl_2$, pH 7·0, each for two hours at room temperature. The modified $tRNA^{Phe}_2$ was prepared for fingerprinting by exhaustive dialysis against water, followed by lyophilisation.

The T_1 and Pancreatic RNase fingerprints of E. coli $tRNA^{Phe}_2$, bisulphite modified in the charged form for 8 hours is shown in FIG. 28, together with fingerprints of uncharged $tRNA^{Phe}_2$ of comparable phenylalanine accepting activity (i.e. 1100 pmoles per A_{260} unit, see Section 3.2.1.) which had been modified for 8 hours with bisulphite. TABLE 13 compares the percentage molar yields of the oligonucleotides in both T_1 RNase fingerprints.

3.4.2. Discussion.

Because separation of bisulphite modified charged and uncharged forms of $tRNA^{Phe}_2$ on Benzoylated DEAE - cellulose was performed before removal of bisulphite adducts, and the presence of these might increase the affinity of the tRNA for Benzoylated DEAE - cellulose, a 0·3 to 2·0 M NaCl gradient was used to ensure complete elution of the modified uncharged $tRNA^{Phe}_2$, before application of the ethanol gradient to elute modified Phe - $tRNA^{Phe}_2$. Separation of the two forms appeared to be successful. Considering FIG. 27, there was no significant elution of ^3H counts before application of the ethanol gradient. The small amount of ^3H phenylalanine eluted was probably due to deacylation of modified Phe - $tRNA^{Phe}_2$ on the column. Elution of modified Phe - $tRNA^{Phe}_2$ charged with

1150 pmoles phenylalanine per A_{260} unit showed that the charged $tRNA^{Phe}_2$ had been purified by chromatography on Benzoylated DEAE - cellulose (in the same way as described in Section 3.1.2.).

Comparison of the fingerprints in FIG. 28 indicates that no new modified oligonucleotides appeared after bisulphite modification of Phe-$tRNA^{Phe}_2$ for 8 hours, that did not appear on similar bisulphite modification of uncharged $tRNA^{Phe}_2$. However, all of the new oligonucleotides that appeared on bisulphite modification of uncharged $tRNA^{Phe}_2$ also appeared on modification of Phe-$tRNA^{Phe}_2$. Comparison of the percentage yields of oligonucleotides in each of the two T_1 RNase fingerprints (TABLE 13) does not indicate any major differences in the composition of the two modified $tRNA^{Phe}_2$ species. In particular, modification of C56, which would be indicated by a decreased yield of $T\Psi CG$, did not aprear to have occurred. Small differences in the percentage molar yields of two oligonucleotides in the two T_1 RNase fingerprints are noticeable, however. The yield of $CAUUA_{OH}$ on the T_1 RNase fingerprint of $tRNA^{Phe}_2$ modified in the charged form was increased, compared with yield of this oligonucleotide on a fingerprint of bisulphite modified uncharged $tRNA^{Phe}_2$, and the yield of $CACCA_{OH}$ correspondingly decreased, implying faster modification of C74 and C75 in Phe - $tRNA^{Phe}_2$ than in $tRNA^{Phe}_2$. In addition, there appeared to be less modification of ms^2i^6A 37, over the period of 8 hours, in Phe - $tRNA^{Phe}_2$ than in $tRNA^{Phe}_2$.

Faster modification of C74 and C75 in Phe - $tRNA^{Phe}_2$

than in $tRNA^{Phe}_2$ must be due to the fact that these cytidine residues became more available for modification, on aminoacylation of the tRNA. As already discussed in Section 3.2.3., these residues are not fully exposed in $tRNA^{Phe}_2$ as they are modified more slowly than C17. The addition of phenylalanine to the $-CCA_{OH}$ end of $tRNA^{Phe}_2$ probably encourages stacking of the $-CCA_{OH}$ end on stem a, and this may allow C74 and C75 to be more exposed than in other possible conformations i.e., interacting with stem a or regions of other tRNA molecules.

The reason for slower modification of ms^2i^6A37 in Phe - $tRNA^{Phe}_2$ than in $tRNA^{Phe}_2$ is more difficult to explain. It cannot be due to localised alteration of structure on aminoacylation (as in the case of C74 and C75) because the anticodon is probably about 80Å distant from the $-CCA_{OH}$ end. It may be that the process of aminoacylation caused an alteration in the conformation of the anticodon loop, making the isopentenyl adenosine side chain of ms^2i^6A37 less accessible to bisulphite modification. There is no evidence for any other conformational change in E. coli $tRNA^{Phe}_2$ on aminoacylation, from the results described in this Section.

The fact that TΨCG did not appear to become exposed on aminoacylation (at least of E. coli $tRNA^{Phe}_2$) does not disprove the theory of Forget & Weissman (1967). Schwartz et al (1974) have proposed that such a conformational change occurs on interaction of the anticodon of the aminoacyl-tRNA with its complementary anticodon on mRNA on the ribosome. They have presented some evidence for the occurence of such a

conformational change, in an *E. coli* system, which appears to require the mediation of elongation factor Tu, GTP, a template and 30S ribosomal subunits. There is some evidence that interaction of aminoacyl-tRNA with elongation factor Tu (*E. coli* system) does not involve the destruction of any base pairing interactions (Schulman *et al*, 1974a), and it is therefore unlikely that this interaction causes exposure of the TΨCG sequence in aminoacyl-tRNA. Rich (1974) has proposed that exposure of the TΨCG sequence may occur during translocation of the tRNA on the ribosome, and that interaction of this sequence with 5S RNA may be involved in translocation.

3.5. THERMAL DENATURATION OF E. COLI $tRNA_2^{Phe}$ STUDIED BY BISULPHITE MODIFICATION.

Conformational changes in tRNA have been postulated, on aminoacylation (Sarin & Zamecnik, 1965; Schofield, 1970; Woese, 1970), and on enzymic binding of aminoacyl-tRNA to the ribosome - mRNA complex (Schwartz et al, 1974). Certain tRNAs may exist in two different conformations, one active, and one inactive in aminoacylation. Therefore, it is of interest to examine some of the conformations in which tRNA molecules may exist, and one way of doing this is to examine the intermediate conformations which occur on thermal denaturation of the tRNA (e.g. Cole et al, 1972; Reisner et al, 1973; Crothers et al, 1974; Wong et al, 1975).

It has been postulated that, on thermal denaturation of tRNA, tertiary structure interactions are destroyed first, followed by the interactions stabilising the helical stem regions (Fresco et al, 1966) and there is evidence for this in the cases of some tRNAs e.g. Yeast $tRNA^{Phe}$ (Reisner et al, 1973) and E. coli $tRNA_1^{Tyr}$, $tRNA_f^{Met}$, $tRNA_2^{Phe}$ and $tRNA_1^{Val}$ (Cole et al, 1972). There is some evidence for simultaneous melting of stem b and the tertiary structure in the case of E. coli $tRNA_f^{Met}$. Magnesium ions have been found to stabilise the tertiary structure (Cole et al, 1972; Wong et al, 1975) and secondary structure (Kearns et al, 1971; Cole et al, 1972; Levy et al, 1972) against thermal denaturation.

This Section is an account of the changes in bisulphite pattern of E. coli $tRNA_2^{Phe}$ which occur on modification at elevated temperatures.

3.5.1. Melting Curves of E. coli $tRNA^{Phe}_2$ in the Presence and Absence of Mg^{2+} Ions.

Initially, an attempt was made to discover at what temperature E. coli $tRNA^{Phe}_2$ melts in the presence of 3M sodium bisulphite, pH 6·0, i.e. the conditions necessary for bisulphite modification. However, the U.V. absorbance melting curve could not be determined directly in 3M sodium bisulphite, pH 6·0, as bisulphite under these conditions, forms adducts with cytidine and uridine residues, resulting in the loss of U.V. absorbance of these residues (Shapiro et al, 1970a; Hayatsu, 1970). Therefore, when any cytidine or uridine residues became exposed on thermal denaturation, although an increase in absorbance at 260nm would occur because of the melting, a decrease would also occur because of bisulphite adduct formation, and a typical absorbance melting profile would not be obtained. For this reason, $tRNA^{Phe}_2$ melting was followed, (A) in the absence of Mg^{2+} (in 3M NaCl, 10mM sodium phosphate buffer, pH 6·0), and (B) in the presence of Mg^{2+} (in 3M NaCl, 10mM $MgCl_2$, 10mM sodium phosphate buffer, pH 6·0). 3M sodium bisulphate, pH 6·0, could not be used, as it was insoluble under these conditions.

No attempt was made to remove strongly bound magnesium ions from $tRNA^{Phe}_2$ by dialysis against a Mg^{2+} chelating agent, such as EDTA. In order to obtain $tRNA^{Phe}_2$ in a solution containing no magnesium ions, purified unlabelled $tRNA^{Phe}_2$ (which had been taken straight from an RPC - 5 column, without addition of crude tRNA) was ethanol precipitated (see Section 3.1.2.) from a solution which had been dialysed, five times over a period of

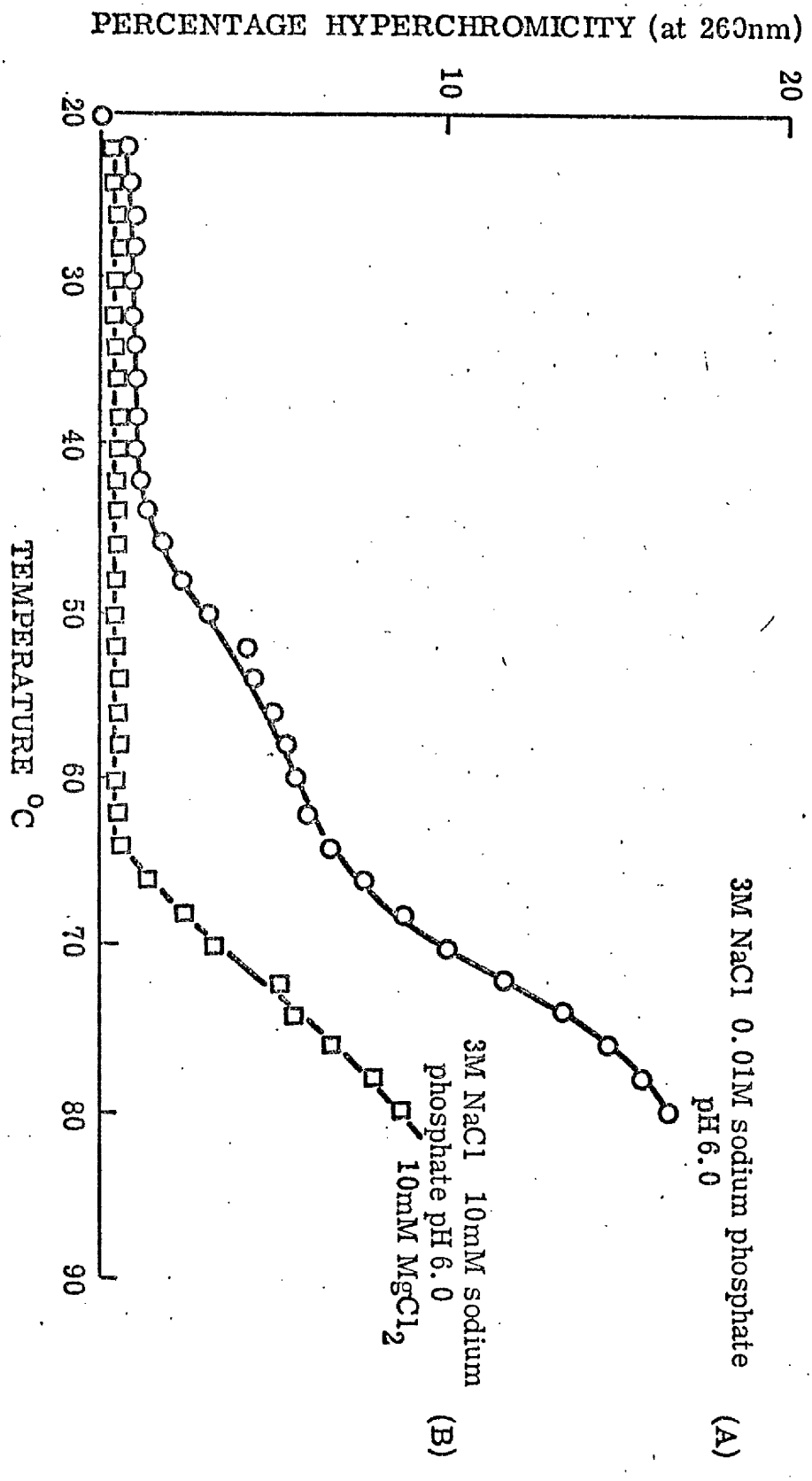

PERCENTAGE HYPERCHROMICITY (at 260nm)
0
10
20
20
30
40
50
60
70
80
90
TEMPERATURE °C
MELTING CURVES OF E. coli tRNA$_2^{Phe}$
(A) 3M NaCl 0.01M sodium phosphate pH 6.0
(B) 3M NaCl 10mM sodium phosphate pH 6.0 10mM $MgCl_2$

FIG. 29.

12 hours against 0·5 M NaCl, tris - HCl pH 7·0 (containing no added Mg^{2+}), and then resuspended in the appropriate solution. Melting of $tRNA^{Phe}_2$ was followed, at 260 nm., using a Pye Unicam SP 8000 spectrophotometer equipped with a heating block.

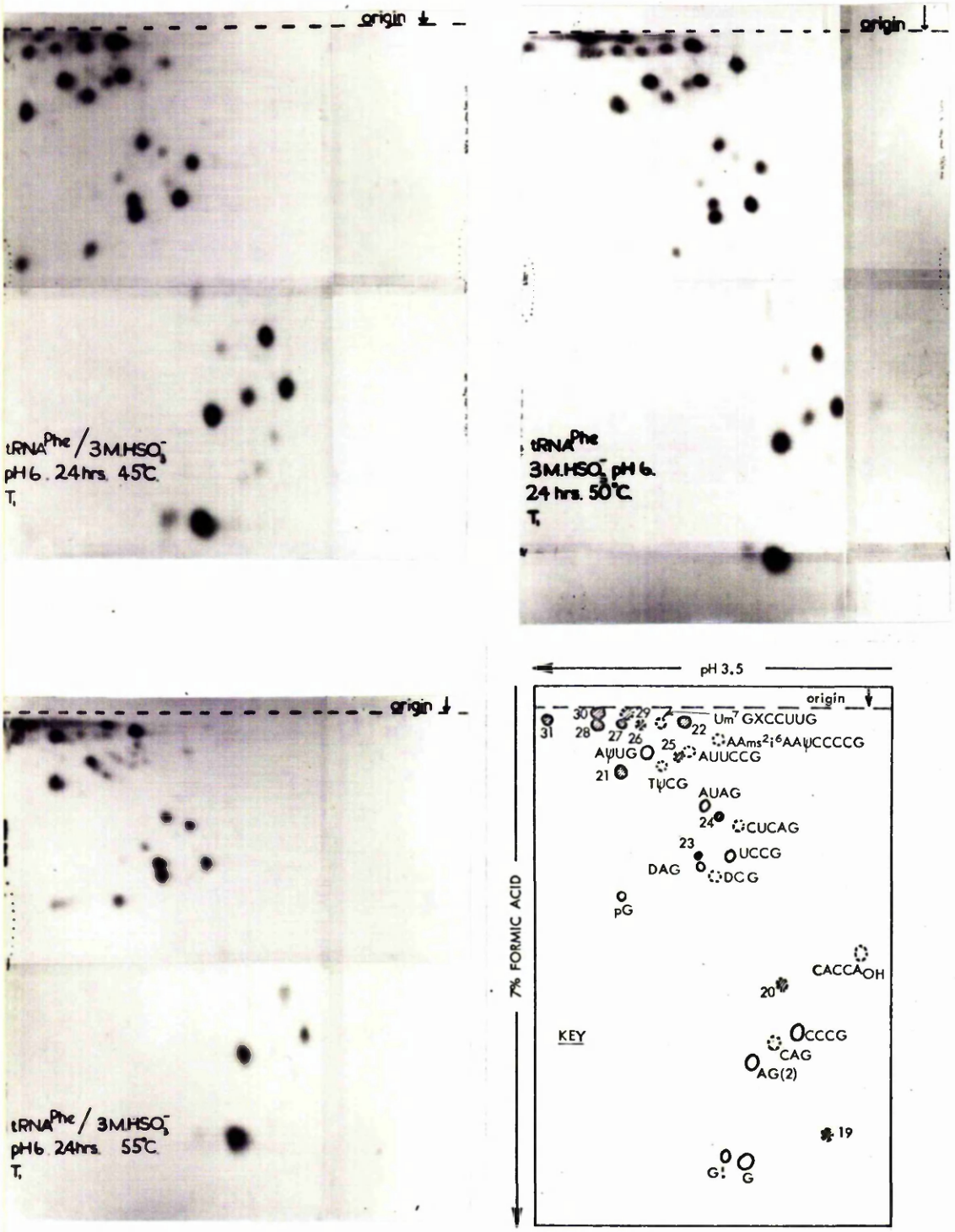
The A_{260} melting curves of E. coli $tRNA^{Phe}_2$, (A) in the absence of Mg^{2+} ions, and (B) in the presence of Mg^{2+} ions are shown in FIG. 29. Melting began at about 50°C in the absence of Mg^{2+}, and at about 60°C in the presence of Mg^{2+}.

3.5.2. Bisulphite Modification of E. Coli $tRNA^{Phe}_2$ at Elevated Temperatures.

In order to avoid the complications of bisulphite modification of pseudouridine at temperatures higher than 60°C (Singhal, 1974), it was decided to modify $tRNA^{Phe}_2$ in the absence of Mg^{2+} at elevated temperatures. Another advantage in performing the experiments in the absence of Mg^{2+} is that, for some tRNAs, that melting seems to take place over a broader temperature range in the absence of Mg^{2+} (Cole et al, 1972; Levy et al, 1972; Wong et al, 1975), because a series of discrete conformational transitions occur, rather than the more co-operative melting encouraged by the presence of Mg^{2+}.

Purified, ^{32}P - labelled E. coli $tRNA^{Phe}_2$ (capable of accepting 1150 pmoles of phenylalanine per A_{260} unit), to which cold crude tRNA had been added (Section 3.1.7.2.), was ethanol precipitated from a Mg^{2+} free solution, as described earlier, and then suspended in 3M sodium bisulphite, pH 6·0, for 24 hours at (A) 45°C, (B) 50°C, or (C) 55°C. T_1 RNase

FIG. 30a.



T_1 RNase Fingerprints of E. coli $tRNA_2^{Phe}$, after modification with 3M $NaHSO_3$, pH 6.0 at elevated temperatures, in the absence of Mg^{2+}, for 24 hours.

FIG. 30b.

T_1 RNase Fingerprint of E. coli $tRNA_2^{Phe}$, after modification with 3M $NaHSO_3$, pH 6·0 at 25°C, in the presence of 10mM Mg^{2+}, for 24 hours.

TABLE 14.

NEW OLIGONUCLEOTIDES APPEARING ON T_1 RNASE FINGERPRINTS OF E. COLI $tRNA_2^{Phe}$ AFTER BISULPHITE MODIFICATION AT ELEVATED TEMPERATURES.

OLIGONUCLEOTIDE	RELATIVE PROPORTION OF EACH NUCLEOTIDE							PROBABLE SEQUENCE
	A^+	C	G	U	'mG'	X	pG	
23	0·96	-	1·00	1·08	-	-	-	UAG
24	1·00	-	-	3·17	-	-	-	$UAUUA_{OH}$
25	1·00	0·93	1·00	1·77	-	-	-	(U,U,C)AG
26	0·71	0·83	1·00	3·21	-	-	-	A UU(C,U)G
27	1·00	-	1·00	2·61	-	-	-	UUUAG
28	0·56	-	1·00	3·50	-	-	-	AUUUUG
29	-	1·00	1·00	3·67	0·78	0·67	-	$Um^7GX(C,U)UUG$
30	-	-	1·00	4·38	0·86	0·75	-	$Um^7GXUUUUG$
31	-	-	1·00	2·40	-	-	-	TψUG

A^+ includes ms^2i^6A

U includes ψ, D, T.

'mG' is the product of alkali treatment of m^7G.

The nucleotide compositions of all oligonucleotides was determined at least twice, except in the cases of oligonucleotides 28 and 31 and the values shown are mean values.

- indicates that the proportion of a particular nucleotide in an olignucleotide, with respect to G, was less than 0·25.

fingerprints of $tRNA^{Phe}_2$ samples modified at 45°C, 50°C, and 55°C are shown in FIG. 30a. New oligonucleotide spots (numbered, and represented as shaded spots in the KEY in FIG. 30a) had appeared, and some oligonucleotide spots (represented by broken circles in the KEY) had disappeared from the T_1 RNase fingerprints, when they were compared with the T_1 RNase fingerprint of unmodified $tRNA^{Phe}_2$ (FIG. 12) and that of $tRNA^{Phe}_2$ (of comparable phenylalanine accepting activity 1100 pmoles (A_{260}unit) which had been modified in 3M sodium bisulphite, 10mM $MgCl_2$, pH 6·0, at 25°C for 24 hours (Section 3.2., FIG. 30b).

The nucleotide compositions of the new oligonucleotides were determined after alkaline hydrolysis, as described in Section 2.2.5. (TABLE 14). Oligonucleotide spots normally present in T_1 RNase fingerprints of unmodified $tRNA^{Phe}_2$, which had disappeared from, or diminished in intensity on T_1 RNase fingerprints of $tRNA^{Phe}_2$ which had been bisulphite modified at elevated temperatures, are shown in TABLE 15 together with identification of the cytidine residues that they contain. The last column in TABLE 15 suggests probable products (new oligonucleotide spots) of bisulphite modification of these oligonucleotides. TABLE 16 shows the percentage yields of oligonucleotides on each of the T_1 RNase fingerprints of *E. coli* $tRNA^{Phe}_2$ modified at elevated temperatures for 24 hours, compared with those of $tRNA^{Phe}_2$ modified in 3M sodium bisulphite, 10mM $MgCl_2$, pH 6.0 for 24 hours (Section 3.2.).

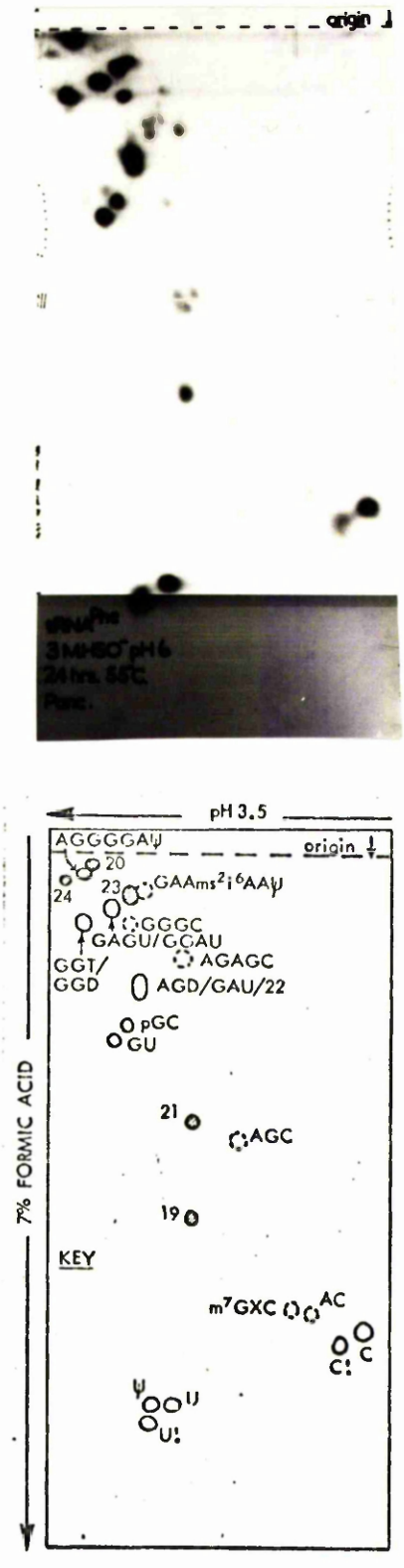
The Pancreatic RNase fingerprint of $tRNA^{Phe}_2$, bisulphite modified at 55°C for 24 hours is shown in FIG. 31. Spots that

TABLE 15.

OLIGONUCLEOTIDES THAT DISAPPEARED FROM T_1 RNASE FINGERPRINTS AFTER MODIFICATION OF $tRNA^{Phe}_2$ AT ELEVATED TEMPERATURES.

OLIGONUCLEOTIDE	C RESIDUES CONTAINED	PROBABLE PRODUCTS OF BISULPHITE MODIFICATION
CAG	C25	UAG
$CACCA_{OH}$	C72, C74, C75	$CAUUA_{OH}$ $UAUUA_{OH}$
DCG	C17	DUG
CUCAG	C11, C13	(U,U,C)AG UUUAG
TΨCG	C56	TΨUG
AUUCCG	C61, C62	AUU(U,C)G AUUUUG
$AAms^2i^6AA\,\Psi CCCCG$	ms^2i^6A37, C40, C41, C42, C43	AANAΨCCCCG
$Um^7GXCCCCG$	C48, C49	$Um^7GX(C,U)UUG$ $Um^7GXUUUUG$

FIG. 31.



Pancreatic RNase Fingerprint of E. coli $tRNA^{Phe}$, after modification in 3M $NaHSO_3$, pH 6.0 at 55°C, in the absence of Mg^{2+}, for 24 hours.

TABLE 16.

PERCENTAGE YIELDS OF OLIGONUCLEOTIDES ON T_1 RNASE FINGERPRINTS OF E. COLI $tRNA_2^{Phe}$ AT ELEVATED TEMPERATURES.

OLIGONUCLEOTIDE	PERCENTAGE YIELD			
	25°C	45°C	50°C	55°C
G + G!	90	98	105	89
AG (2)	127	134	124	140
CAG	86	58	42	45
CCCG	87	93	96	85
$CACCA_{OH}$	0	0	0	0
pG + pG!	77	90	106	86
DAG	107	85	96	101
DCG	14	0	0	0
UCCG	99	80	89	75
CUCAG	80	44	34	4
AUAG	50	52	49	46
TΨCG	112	78	40	31
AΨUG	127	94	93	87
AUUCCG	113	60	27	12
$AAms^2i^6AAΨCCCCG$	18	13	32	26
$Um^7GXCCUUG$	85	40	22	9
19	13	0	0	0
20	78	92	70	82
21	126	109	104	110
22	67	70	50	52
23	0	40	50	80
24	0	10	15	23
25	0	26	20	19
26	0	28	24	31
27	0	20	37	77
28	0	13	36	57
29	0	17	34	19
30	0	23	34	62
31	0	40	65	90

TABLE 17.

NEW OLIGONUCLEOTIDES APPEARING ON PANCREATIC RNASE FINGERPRINTS OF E. COLI $tRNA_2^{Phe}$ AFTER BISULPHITE MODIFICATION AT ELEVATED TEMPERATURES.

OLIGONUCLEOTIDE	RELATIVE PROPORTION OF EACH NUCLEOTIDE							PROBABLE SEQUENCE
	A^+	C	G	U	'mG'	X	pG	
21	-	-	-	1·00	0·67	0·48		m^7GXU
22	Ran with AGD / GAU							AGU
23	2·31	-	1·67	1·00	-	-	-	AGAGU
24	-	-	2·50	1·00	-	-	-	GGGU

A^+ includes ms^2i^6A

U includes Ψ, rT, D.

\- indicates that the proportion of a particular nucleotide in an oligonucleotide of less than 0·25, with respect to the pyrimidine nucleotide.

have disappeared from the fingerprint compared with that of unmodified $tRNA^{Phe}_2$ (FIG. 12) are shown as broken circles, and the new oligonucleotides are shown as shaded spots. The nucleotide compositions of the new oligonucleotides were determined after alkaline hydrolysis (TABLE 17). TABLE 18 indicates the oligonucleotide spots which disappeared from Pancreatic RNase fingerprints, together with identification of the cytidine residues contained, and their probable products.

T_1 RNase fingerprints were used as the principal source of data about high temperature modification of $tRNA^{Phe}_2$. Elevation of the temperature to $55^{o}C$ resulted in bisulphite modification of C11, C13, C25, C48, C49, C56, C61, C62 and C72, as well as those nucleotides modified at $25^{o}C$ (C17, C74, C75 and ms^2i^6A37). Of these, the extents of modification of C25, C56 and C72 could be determined directly by the extents of modification of CAG, TΨCG and $CAUUA_{OH}$, respectively. After bisulphite modification at $25^{o}C$ for 24 hours, C74 and C75 had been completely modified, while C72 was not modified at all (Section 3.2.). It was therefore assumed that C74 and C75 were modified faster at higher temperatures than C72, and that the T_1 oligonucleotide with the composition 1A, 1C, 2U, was $CAUUA_{OH}$. The extents of modification of C11 and C13, C48 and C49, and C61 and C62 were not obvious from examination of the extents of modification of CUCAG, $Um^7GXCCUUG$ and AUUCCG as each of these oligonucleotides contained two cytidine residues.

Pancreatic RNase fingerprints were a relatively poor source of data about the residues modified at high temperatures,

TABLE 18.

OLIGONUCLEOTIDES THAT DISAPPEARED FROM PANCREATIC RNASE FINGERPRINTS OF E. COLI $tRNA_2^{Phe}$, BISULPHITE MODIFIED AT ELEVATED TEMPERATURES.

OLIGONUCLEOTIDE	C RESIDUES CONTAINED	PROBABLE PRODUCTS OF BISULPHITE MODIFICATION
AC	C74	AU
m^7GXC	C48	m^7GXU
AGC	C11	AGU
AGAGC	C25	AGAGU
GGGC	C72	GGGU
GAAms^2i^6AA Ψ CCCCG	ms^2i^6A37	GAANAΨ CCCCG

partly due to the fact that some oligonucleotides which were products of bisulphite modification, ran with oligonucleotides already present, i.e. AGU with CAU/AGD and AGAGU with GAAms^{2}i^{6}AAψ, and partly because some of the residues modified, i.e. C49, C13, C61 and C62 occurred in pyrimidylcytidine sequences and could only be detected as a decrease in the C pool and increase in the U pool. However, it was possible to determine the extent of modification of C48, after 24 hours at each of the four temperatures, by determining the extent of modification of m^7GXC. Confirmation of the extent of modification of C72, by determining the extent of modification of GGGC was also possible. Because of contamination of AGU (product of bisulphite modification of ACC containing C11) with GAU and ACD, the extent of modification of C11 could not be determined.

The case of C48 and C49 is the only one in which the relative extents of modification of two cytidine residues, in the same T_1 RNase oligonucleotide, have been elucidated after reference to the Pancreatic RNase fingerprint. The yield of m^7GXU on the Pancreatic RNase fingerprints of E. coli $tRNA^{Phe}_2$ modified at elevated temperatures was calculated as a percentage of the total yield of m^7GXC + m^7GXU. These were compared with the relative percentage yields of Um^{7}GXCCUUG, Um^{7}GX(C,U)UUG and Um^{7}GXUUUUG on T_1 RNase fingerprints of the same modified $tRNA^{Phe}_2$ samples, as shown in TABLE 19 below.

TABLE. 19.

Nuclease	Oligonucleotide	Percentage yield at various temperatures (to the nearest 5%)		
		45°C	50°C	55°C
Pancreatic RNase	Um^7GXC	60	25	10
Pancreatic RNase	Um^7GXU	40	75	90
T_1 RNase	$Um^7GXCCUUG$	50	20	10
T_1 RNase	$Um^7GX(C,U)UUG$	20	40	20
T_1 RNase	$Um^7GXUUUUG$	30	40	70

The yield of m^7GXU indicates the extent of modification of C48, and the yield of $Um^7GXUUUUG$ indicates the extent of modification of both C48 and C49 in the same $tRNA^{Phe}_2$ molecule. At each temperature, the percentage yield of m^7GXU was almost equivalent to the percentage yield of $Um^7GX(C,U)UUG$ + $Um^7GXUUUUG$. This indicates that C48 was modified before C49 in the same $tRNA^{Phe}_2$ molecule.

FIG. 32 is a diagrammatic representation of E. coli $tRNA^{Phe}_2$ with the tertiary interactions suggested by Kim et al (1974b) and Ladner et al (1975b), showing the percentages of modification of each of the various cytidine residues at 25°C, 45°C, 50°C and 55°C.

3.5.3. Melting of E. coli $tRNA^{Phe}_2$ Followed by Bisulphite Adduct Formation.

It is obvious from the results described in Section 3.5.2.

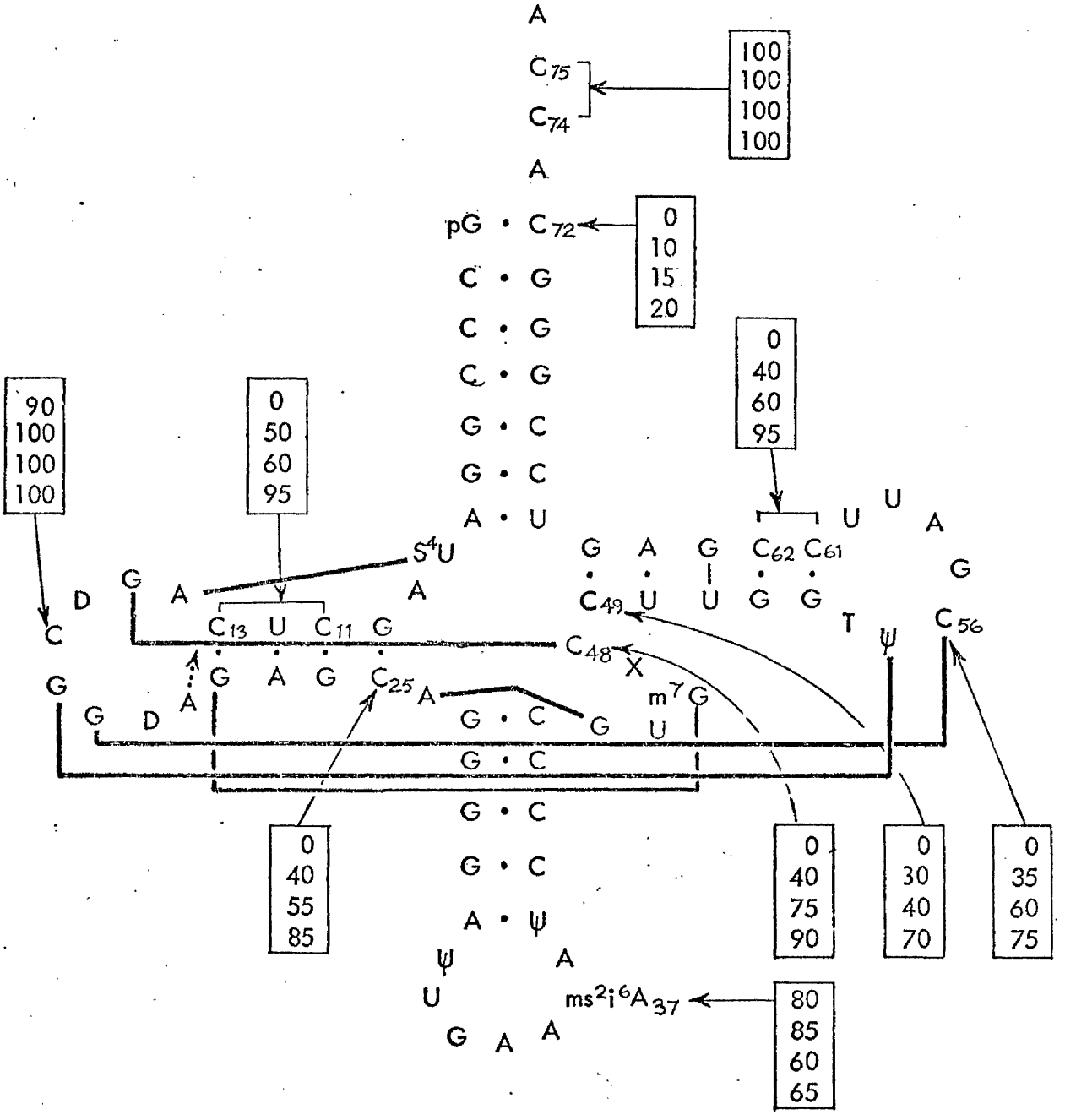
FIG. 32.

CLOVERLEAF REPRESENTATION OF E. COLI $tRNA_2^{Phe}$ INDICATING THE EXTENTS OF BISULPHITE MODIFICATION OF VARIOUS CYTIDINE RESIDUES AFTER MODIFICATION FOR 24 HOURS AT ELEVATED TEMPERATURES.

The figures in the boxes indicate the percentage of $tRNA_2^{Phe}$ molecules in which bisulphite modification of particular nucleoside residues had occurred, after modification in 3M sodium bisulphite, 10mM $MgCl_2$, pH 6·0 at 25°C, and after modification in 3M sodium bisulphite, pH 6·0 at 45°C, 50°C, and 55°C respectively.

In some cases, it proved impossible to determine the relative extents of modification of two cytidine residues in the same T_1 RNase oligonucleotide, i.e. in the cases of C11 and C13, C62 and C63, and C74 and C75. In these cases, the figures in the boxes indicate the percentage of $tRNA_2^{Phe}$ molecules in which modification of one of the two cytidine residues had occurred.

The tertiary interactions described by Ladner et al (1975b) for Yeast $tRNA^{Phe}$, as they would apply to E. coli $tRNA_2^{Phe}$, are also indicated.


A
C75
C74
100 100 100 100
A
pG • C72
0 10 15 20
C • G
C • G
C • G
G • C
G • C
A • U
0 40 60 95
U U A
G A G C62 C61
C49 U U G G
T ψ C56
G
90 100 100 100
0 50 60 95
S4U
D G A A
C13 U C11 G
C48
X
G A G C25
A
m7G
G • C
G U
G D
A
G • C
G • C
G • C
A • ψ
ψ A
U
ms2i6A37
G A A
0 40 55 85
0 40 75 90
0 30 40 70
0 35 60 75
80 85 60 65

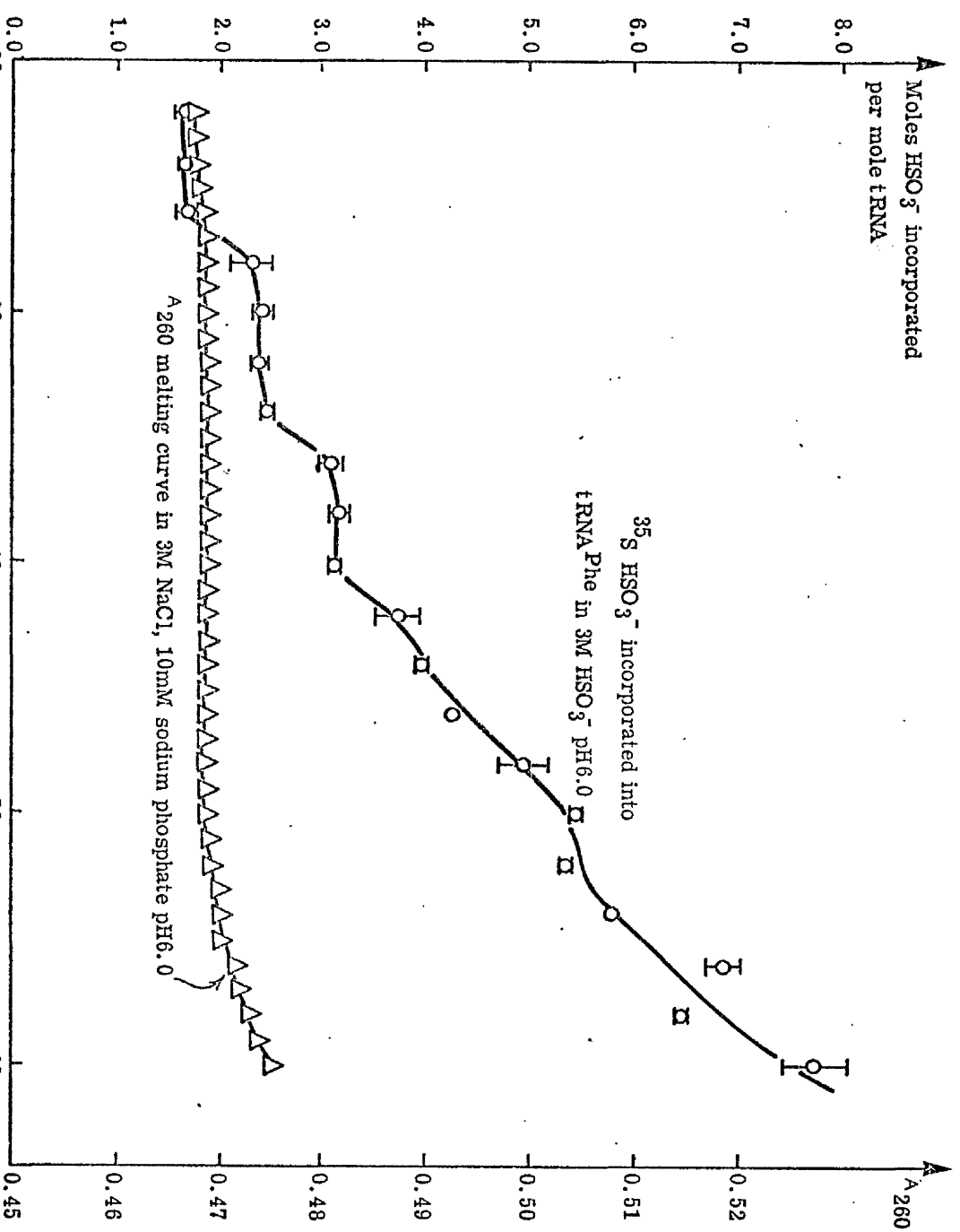
that although the absorbance at 260nm of $tRNA^{Phe}_2$ only began to increase when the temperature reached $50^{o}C$, some destruction of secondary and tertiary structure must have occurred at temperatures lower than this. An attempt was made to estimate the number of cytidine and uridine residues exposed in the tertiary structure of $tRNA^{Phe}_2$ at various temperatures, by the extent of bisulphite adduct formation.

Purified ^{32}P - labelled E. coli $tRNA^{Phe}_2$ was prepared, and instead of addition of crude tRNA, a quantity of purified cold $tRNA^{Phe}_2$ was added, so that the tRNA contained approximately 5×10^{3} cpm ^{32}P per A_{260} unit. $10A_{260}$ units of this $tRNA^{Phe}_2$, which had a phenylalanine accepting activity of approximately 1000 pmoles per A_{260} unit, was suspended in 1·5 ml of 3M ^{35}S - labelled sodium bisulphite, pH 6·0. The ^{35}S - labelled bisulphite contained approximately 1000 cpm ^{35}S per nMole.

The temperature of incubation of the $tRNA^{Phe}_2$ solution was raised, from $20^{o}C$ to $60^{o}C$ by $0·25^{o}C$ per minute, using a Haake Bath Circulator, Model F.S., containing water, coupled to a Haake Electronic Digital Temperature Control Programmer, Model P.G., set at $1^{o}C$ per 4 mins. 50 µl samples of $tRNA^{Phe}_2$ were removed at 8 min. intervals, and dialysed overnight against five changes of buffer (0·1M sodium acetate, pH 6·0), to remove excess bisulphite. After this time, two aliquots were taken from each dialysed $tRNA^{Phe}_2$ sample, and spotted onto 3MM filter discs (2cm diameter). The filter papers were washed once for 30 mins. in ice-cold 10% (W/V) trichloracetic acid, to precipate the tRNA, and twice for 30 mins. in ice-cold 5% trichloracetic acid to remove any remaining free $^{35}S\text{-}HSO_3^-$. Finally, they were washed in methylated spirit, dried and

FIG. 33.

"MELTING CURVES" OF E.coli $tRNA^{Phe}$



counted in toluene PPO/POPOP for ^{35}S and ^{32}P, and the amount of bisulphite incorporated per Mole of tRNA in each sample was calculated.

FIG. 33 shows the incorporation of bisulphite into the tRNA as the temperature was increased, compared with the A_{260} melting curve in 3M NaCl, 10mM sodium phosphate buffer, pH 6.0.

3.5.4. Discussion.

The results described in this Section clearly demonstrate that quite extensive destruction of secondary and tertiary structure interactions occurred on raising the temperature of $tRNA^{Phe}_2$ solution (in the absence of magnesium ions) from 25°C to 55°C. Of the nucleotides available for bisulphite modification at 25°C, in the presence of Mg^{2+}, C17, C74 and C75 also appeared to be fully available for modification at 45°C and higher temperatures in the absence of Mg^{2+}. However, ms^2i^6A 37 appeared to be less available for bisulphite modification at 50°C and 55°C. Other cytidine residues that became available for bisulphite modification at elevated temperatures were those of C11, C13, C25, C48, C49, C56, C61, C62 and C72. Modification of C72, even at 55°C, appeared to be slow indicating that the base pair involved, G1 - C72 was not significantly disrupted at these temperatures. Modification of the other cytidine residues appeared to be quite extensive, even at 45°C.

The results indicate some destruction of tertiary structure interactions, between G19 and C56, and G15 and C48 at 45°C. C56 and C48 became more available for

modification on raising the temperature to $50^{\circ}C$ and $55^{\circ}C$.

C11, C13 and C25, which are involved in base pairs of stem b were also available for bisulphite modification at $45^{\circ}C$ and even more so at $50^{\circ}C$ and $55^{\circ}C$. Some melting of stem b must therefore, have occurred at temperatures below $45^{\circ}C$. Similarly, C49, C61 and C62, involved in Watson - Crick base pairing in stem e were available for bisulphite modification at $45^{\circ}C$ and more so at $50^{\circ}C$ and $55^{\circ}C$. The results indicate that on heating solutions of *E. coli* $tRNA^{Phe}_2$ in the absence of magnesium ions some destruction of tertiary structure interactions, and secondary structure interactions of stems b and e occurred, even at temperatures below $45^{\circ}C$. At $55^{\circ}C$, quite extensive destruction of these interactions had taken place. However, even at $55^{\circ}C$, no reaction of bases involved in base pairing interactions in stems a and c was observed, except for slight modification of C72 which is involved in the terminal base pair of stem a. This indicated that no appreciable melting of stems a and c occurred at temperatures of $55^{\circ}C$ and below.

Proton nuclear magnetic resonance has been used in quite a few cases to study the thermal denaturation of various tRNAs. Crothers *et al* (1974) and Wong *et al* (1975) have used this technique to study the thermal denaturation of *E. coli* $tRNA^{Met}_f$. Both groups agree that, in the absence of Mg^{2+} ions, destruction of tertiary structure and the secondary structure base pairs of stem b took place initially, although they suggest different temperatures for these transitions. Stem e was the next to melt, with stems a and c melting out at higher temperatures.

The addition of Mg^{2+} ions (Wong et al, 1975) raised the temperature at which melting commenced and the temperature range over which melting occurred was narrower than in the absence of Mg^{2+} ions.

N. M. R. studies on the thermal denaturation of E. coli $tRNA^{Glu}$ (in the presence of Mg^{2+} ions) have suggested a similar pattern of melting, i.e. initial melting out of tertiary structure interactions and the base pairs of stem b, followed at higher temperatures by melting of stems a and e, and finally stem c (Hilbers & Schulman, 1974). Kastrup & Schmidt (1975) have observed initial melting of stem b, in the thermal denaturation of E. coli $tRNA^{Val}_1$ in the absence of Mg^{2+} ions, followed at higher temperatures by melting of stems c and e. Hilbers et al (1973), using N. M. R. to study the thermal denaturation of Yeast $tRNA^{Phe}$, have also reported melting of stem b together with parts of stems a and c before melting of the rest of the molecule. However, Reisner et al (1973) using a differential melting technique and fragments of Yeast $tRNA^{Phe}$ suggest initial melting of tertiary structure, then melting of stems a and c, and finally of the more stable stems b and e, on thermal denaturation.

Caron & Dugas (1976) have used a spin labelling technique to investigate the transitions involved in the melting of three E. coli tRNAs, $tRNA^{Glu}$, $tRNA^{Met}_f$ and $tRNA^{Phe}$. The thermal denaturation behaviour of these tRNAs appeared to be similar. Initial destruction of tertiary structure and the base pairs of stem b occurred in each case. Melting of stems c and d appeared to occur next, with the last stage being

melting of stems a and e.

A general feature of the results from the thermal denaturation studies described above is the initial melting of tertiary structure. This was proposed as the initial stage of thermal denaturation of tRNA as long ago as 1966 (Fresco et al 1966). Another feature that emerges from a study of the secondary structures of the various tRNAs and their patterns of melting is that, as would be expected, stems containing large numbers of G - C base pairs (thermodynamically more stable than A - U base pairs) melt later than those containing fewer G - C base pairs, or a mixture of G - C and A - U base pairs. This observation is consistent with the results obtained for thermal denaturation of E. coli $tRNA^{Phe}_{2}$, as studied by Bisulphite modification, described in this thesis. The most stable helices, stems a and c contain seven and four G - C base pairs respectively. Each of these stems also has an A - U or A - ψ base pair at one end of the helix. Melting of these weaker base pairs would not be detected using bisulphite modification as only cytidine residues are modified by bisulphite, but it is probable that melting of these A - U and A - ψ base pairs does occur at elevated temperatures.

The helices which appeared to disrupt readily, stems b and e, each contain only three G - C base pairs and one A - U base pair. Therefore, they would be expected to be less stable than stems a and c. Stem e also contains a G - U base pair. Such a G - U base pair in stem a of Yeast $tRNA^{Phe}$ has been shown to require a slight distortion of the

phosphodiester backbone to accomodate it (Ladner et al 1975b). This distortion may weaken the helix, in this case stem e of E. coli $tRNA^{Phe}_2$, making it more susceptible to thermal denaturation.

The results described in this Section are obviously preliminary. Although, Bisulphite modification at elevated temperatures was allowed to proceed for 24 hours, maximal modification of some cytidine residues might not have occurred in this time. Determination of the maximum extent of modification of each particular residue at each temperature would make the results more definitive. The kinetics of modification of each residue would indicate whether a residue was available for modification from the beginning or only after modification of other residues, thus revealing whether any conformational changes in the tRNA molecule, due to Bisulphite modification, occurred. However, the results do give some indication of the availability of the cytidine residues of E. coli $tRNA^{Phe}_2$ for modification at various stages of its thermal denaturation. At $45^{o}C$ in the absence of Mg^{2+} ions, quite extensive denaturation of E. coli $tRNA^{Phe}_2$ had occurred. This is borne out by the fact that at temperatures as low as $27^{o}C$, more cytidine and/or uridine residues were available for bisulphite adduct formation than at $25^{o}C$ (see FIG. 33). It would be interesting to modify $tRNA^{Phe}_2$ at lower temperatures to see which tertiary or secondary interactions are destroyed first.

Bisulphite adduct formation (described in Section 3.5.3.) appears to be an effective probe of tRNA melting. The results shown in FIG. 33, however, are anomalous. At $25^{o}C$, when three

cytidine resudes (C17, C74 and C75), four uridine residues (U33, U45, U59 and U60) and ms^2i^6A 37 are theoretically available for bisulphite adduct formation, only 1·65 moles of (^{35}S) HSO_3^- appear to be bound per mole of tRNA. This is probably due to a) the fact that bisulphite adduct formation is fairly slow and the temperature is increasing by 0·25°C per minute so that maximal adduct formation at a particular temperature is not possible, and b) the fact that HSO_3^- adducts may be lost during the dialysis step.

The fact that a large increase in the number of bisulphite adducts formed is accompanied by only a small increase in absorbance can be explained by the fact that the optical melting phenomenon depends both on breaking base pairs and unstacking of bases in single stranded regions. Some of the bases available for adduct formation with bisulphite may still be stacked, although no longer base paired.

4. CONCLUSION.

Two methods have been described for the purification of E. coli $tRNA^{Phe}_2$. Loss of phenylalanine accepting activity was observed both on deacylation of Phe-$tRNA^{Phe}_2$ and on storage of purified $tRNA^{Phe}_2$ under some conditions. The loss of activity could not be attributed to the presence of nucleases and must therefore have been due to some chemical and/or conformational change in the $tRNA^{Phe}_2$ molecule. The existence of certain species of tRNA molecules in both active and inactive conformations has been described before, (Lindahl et al, 1966; Gartland & Sueoka, 1966; Renaud et al, 1974), but methods that have been described as capable of converting the inactive tRNA molecules to active molecules were not found to be effective in the renaturation of $tRNA^{Phe}_2$ (Lindahl et al, 1966; Ishida et al, 1971).

Investigation of the stability of E. coli $tRNA^{Phe}_2$ under various conditions led to the discovery of the optimal storage conditions, under which inactivation was negligible. Addition of unlabelled, unpurified tRNA to ^{32}P-labelled purified $tRNA^{Phe}_2$ was found to be very effective in stabilisation of the active form. Particular care was exercised to ensure that the $tRNA^{Phe}_2$ was protected from light during purification and storage to prevent the formation of a photochemically induced cross-link between s^4U8 and C13, which probably causes loss of phenylalanine accepting activity in E. coli $tRNA^{Phe}$ (Carré et al, 1974). The ease of denaturation of $tRNA^{Phe}_2$ was borne in mind in later chemical modification studies. The activity of $tRNA^{Phe}_2$ was determined directly before chemical modification,

and such studies were always carried out in the presence of an equal amount of unlabelled, unpurified tRNA, to discourage denaturation.

The T_1 and Pancreatic RNase fingerprints of E. coli $tRNA^{Phe}_2$ shown in FIG. 12, were directly comparable to those obtained by Barrel & Sanger (1969), indicating that their E. coli $tRNA^{Phe}$ is identical to E. coli $tRNA^{Phe}_2$. There was no evidence to support the structure described by Uziel & Cassen (1969) for E. coli $tRNA^{Phe}$.

Bisulphite modification was used as a probe of the tertiary structure of E. coli $tRNA^{Phe}_2$. At this point, it is of value to discuss results published recently on the tertiary structure of Yeast $tRNA^{Phe}$. The tertiary structure of Yeast $tRNA^{Phe}$ (presumed to be common in most respects to all tRNAs), determined by X-ray diffraction studies, to a resolution of $3\overset{\circ}{A}$ has been described in Section 1.3.3.5. However, recent papers have described further details of tertiary structure after examination of diffraction data at a resolution of $2{\cdot}5\overset{\circ}{A}$ (Ladner et al, 1975b, Quigley et al, 1975). Although previous papers had described only tertiary interactions involving hydrogen bonds between nucleotide bases, these two papers reveal the importance of hydrogen bonding interactions of -OH groups of ribose molecules and the oxygen atoms of phosphate groups to each other and to nucleotide bases, in the maintenance of tertiary structure.

Ladner et al (1975b) and Quigley et al (1975) have confirmed the existence of hydrogen bonding interactions between G18 and Ψ55 (G18 is bonded to the base and ribose of Ψ55 and also to

ribose 58) and C56 and G19 (the only Watson-Crick base pair found in the tertiary structure interactions), which were suggested by Kim et al (1974b). Certain invariant or semi-invariant bases have been ascribed the role of fixing the ribose - phosphate backbone in strained regions (Ladner et al, 1975b). N4 of the invariant base of C61 hydrogen bonds to an oxygen of phosphate 60. This phosphate group is also anchored to the 2' -OH group of m^1A58. The semi-invariant base of C11 in Yeast $tRNA^{Phe}$ forms a hydrogen bond between N4 of C11 and 2'OH of ribose 9. Such an interaction would also be possible if U were present in this position instead of C, as it is in some tRNAs. The invariant base of A21 has been found to hydrogen bond, not to the bases of the base pair U8 - A14, but rather to riboses 8 and 48 Ladner et al, 1975b; Quigley et al, 1975). Further interactions include hydrogen bonding between G57 and the 2' -OH group of ribose 8 and 1' -OH group of ribose 19, which helps to hold together Loops I and IV, and a hydrogen bond between the 2' -OH groups of riboses 58 and 59, which helps to stabilise a strained region of the ribose phosphate backbone.

There is some evidence for the existence of Mg^{2+} binding sites between phosphates 8 and 9, and between residue 17 of one molecule and residues 20 and 21 of the adjacent molecule in the cystal lattice (Quigley et al, 1975), where they probably stabilise strained regions of the ribose - phosphate backbone. There is also some evidence for the presence of a magnesium ion adjacent to U59 and C60, again in a strained region (Ladner et al, 1975b).

In this thesis Bisulphite modification of E. coli $tRNA^{Phe}_2$ has been described. The $tRNA^{Phe}_2$ used in these studies was between 65% and 75% pure. The $tRNA^{Phe}_2$ did not undergo any detectable conformational change during modification, making certain residues more or less reactive. The $tRNA^{Phe}_2$ concentration was kept below 5 A_{260} units per ml. to prevent possible dimerisation of $tRNA^{Phe}_2$ molecules. The results obtained on Bisulphite modification of E. coli $tRNA^{Phe}_2$ described in this thesis are consistent with a tertiary structure for $tRNA^{Phe}_2$ similar to that determined, by X-ray diffraction studies, for Yeast $tRNA^{Phe}_2$. It is of interest that bisulphite modification of C17 (in a region fully exposed in Yeast $tRNA^{Phe}$) occurred with a $t_{\frac{1}{2}}$ for disappearance of C17 of 2·25 hours, faster than modification of poly C (4·3 hours (Goddard & Schulman, 1972)) under similar conditions. This indicates the extremely exposed nature of C17. Modification of C74 and C75 occurred with a $t_{\frac{1}{2}}$ for each cytidine residue of between 3·25 and 7·0 hours. Thus C74 and C75 were modified more slowly than C17, probably at a similar rate to cytidine residues in poly C.

Examination of the results of chemical modification of Yeast $tRNA^{Phe}_2$ (Ladner et al, 1974b) has shown that they are consistent with the tertiary structure determined by X-ray diffraction, and has thus demonstrated the efficacity of chemical modification in tRNA tertiary structure studies. This had previously been doubted because of the possibility of tRNA denaturation during chemical modification. The results of this thesis indicate that Bisulphite is a useful reagent for

chemical modification of tRNA, as it does not cause a conformational change, at least in the case of E. coli $tRNA^{Phe}_2$, during modification.

Taking into account the nature of the Bisulphite modification reaction (see FIG. 6), it is likely that any cytosine base in tRNA involved in hydrogen bonding interactions would be less reactive than an unbonded cytosine base. The two cytidine residues of E. coli $tRNA^{Phe}_2$, in single stranded regions of the cloverleaf structure, but unreactive because they are involved in tertiary structure interactions, are C48 and C56. If direct comparison with Yeast $tRNA^{Phe}$ is valid, O2 and N3 of C48 are probably hydrogen bonded to G15 and O2, N3 and N4 of C56 hydrogen bonded to G19. In addition, bisulphite modification appears to be inhibited by stacking (modification of C17 was faster than modification of C74 and C75 and cytidine residues in poly C).

By the standard fingerprinting procedure, it was not possible to investigate the effect of Bisulphite of s^4U8 in E. coli $tRNA^{Phe}_2$. However, such a modification could probably be followed by other methods, i.e. by the comparison of the ratio of A_{335} to A_{260} before and after modification, or by comparison of the nucleoside composition of modified and unmodified $tRNA^{Phe}_2$. A chromatographic method of separating uridine and 4-thiouridine has been described by Singhal & Best (1973).

Bisulphite modification did not prove to be a very effective probe of the specific ligase recognition site of E. coli $tRNA^{Phe}_2$. There was found to be no difference in the bases modified in active and inactive fractions of Bisulphite

modified $tRNA^{Phe}_2$. Using the methods described in this thesis, it was not possible to determine whether s^4U8 was modified by Bisulphite. However, as already discussed in Section 3.2.3., such a modification would be unlikely to cause a loss of phenylalanine accepting activity. Investigation of the nucleoside composition of active and inactive bisulphite modified $tRNA^{Phe}_2$ for relative amounts of uridine and 4-thiouridine would prove this point.

On examination of the 3-dimensional structure of Yeast $tRNA^{Phe}$, determined by X-ray diffraction, Robertus et al (1974a) and Ladner et al (1975b) have suggested that bases in the α and β regions of Loop I, because of their variability in different tRNAs, and extremely exposed nature, may act as specific ligase recognition sites. There is some doubt about these sites being specific ligase recognition sites, however, as some tRNAs have common sequences in these regions (Kim et al, 1974a), and usually, chemical modification of bases in these regions does not lead to loss of amino acid accepting activity (see Section 1.4.1.C.3.)

Considering the particular case of E. coli $tRNA^{Phe}$, Shugart & Stulberg (1969) have described inactivation on reduction of s^4U8, D16 and D20. The results obtained on chemical modification of s^4U8 in E. coli $tRNA^{Phe}$ by several methods (Section 3.3.5.) indicate that this is an unlikely candidate for a component of the ligase recognition site, so attention must focus on the α and β regions. The results described in this thesis, however, indicate that the conversion of C17 to U17 (C17 is a component of the α region of E. coli $tRNA^{Phe}_2$) with

bisulphite did not destroy specific ligase recognition, suggesting that C17, at least, is not an essential part of the specific ligase recognition site of E. coli $tRNA^{Phe}_2$.

The anticodon loop is another region of tRNA structure that has been considered as a component of the specific ligase recognition site (See Section 1.4.1.6.3.). One nucleotide of the anticodon loop of E. coli $tRNA^{Phe}_2$ was found to be reactive with Bisulphite, i.e. ms^2i^6A37. While the inactive fraction of Bisulphite modified $tRNA^{Phe}_2$ contained a slightly higher proportion of modified ms^2i^6A37 than the active fraction, there is certainly no conclusive evidence that modification of this nucleotide caused loss of phenylalanine accepting activity. Modification of E. coli $tRNA^{Phe}_2$ with 1M sodium bisulphite, pH 7·0 at 37°C for 24 hours caused complete modification of only one nucleotide, i.e. ms^2i^6A37 (Section 3.2.2.). Examination of the phenylalanine accepting activity of this modified $tRNA^{Phe}_2$ would be helpful in deciding whether the results obtained, of the greater extent of modification of ms^2i^6A37 in the inactive fraction, are significant.

It might be possible to replace the three nucleotides at the 3'-OH terminus of fully bisulphite modified E. coli $tRNA^{Phe}_2$ (after modification in 3M sodium bisulphite, pH 6·0 at 25°C for 48 hours) with the $-CCA_{OH}$ repair enzyme described by Deutscher (1973), so that $tRNA^{Phe}_2$ containing only the modifications C17 ⟶ U17 and $ms^2i^6A37 \longrightarrow ms^2i^6A - HSO_3 - 37$ would be obtained. The phenylalanine accepting activity of this modified $tRNA^{Phe}_2$ could then be determined, and this would help to elucidate the effects of modification of C74 and C75 on phenylalanine accepting activity.

As the results described in Section 3.3. stand, it seems most likely that inactivation of E. coli $tRNA^{Phe}_2$ was due to a conformational change, facilitated in Bisulphite modified $tRNA^{Phe}_2$. The occurrence of such a conformational change could possibly be confirmed by structural studies (using one of the physical techniques described in Section 1.3.3.5.) on unmodified and fully Bisulphite modified $tRNA^{Phe}_2$. As already suggested in Section 3.3.5., in order to exhaustively define the effect of Bisulphite modification on the phenylalanine accepting activity of E. coli $tRNA^{Phe}_2$, it would be necessary to investigate the apparent Km and V_{MAX} of the ligase for fully Bisulphite modified $tRNA^{Phe}_2$. To obtain active $tRNA^{Phe}_2$ molecules with 100% modification of all the four reactive bases, it would be necessary to separate active from inactive molecules after modification in 3M sodium bisulphite, pH 6·0 at 25^{0}C, for 48 hours.

As discussed in Section 1.4.1.1., the use of chemical modification to investigate specific ligase recognition sites of tRNA molecules is fraught with difficulties. Before a particular modification can be correlated with loss of amino acid acceptor activity, active and inactive forms must be separated. This has not always proved possible (Chang et al, 1972), and incomplete separation of active and inactive forms has been described (Cashmore, 1970).

Litt (1971) has reported results on the Kethoxal modification of Yeast $tRNA^{Phe}$ analagous to those described in this thesis for Bisulphite modification of E. coli $tRNA^{Phe}_2$. Both possible Kethoxal modifications (of G20 and G24) were

present in both active and inactive modified $tRNA^{Phe}$ molecules. While Litt has suggested that these results may be explained by the stereoisomerism of Kethoxal adduct formation, they could equally well be explained by a conformational change in Yeast $tRNA^{Phe}$ molecules which was facilitated in Kethoxal modified Yeast $tRNA^{Phe}$.

Cytosine specific reagents usually cause modification of the two cytidine residues, in the 3' terminal $-CCA_{OH}$ sequence (Cashmore, 1970; Kućan et al, 1971; Chambers et al, 1973; Chang, 1973; Schulman & Goddard, 1973; Chang & Ish - Horowicz, 1974; Rhodes, 1975). Alteration of the chemical structure of this region, because of its position close to the point where the amino acid is attached, is bound to affect tRNA - ligase interaction, and may thus alter the rate or extent of aminoacylation. However, because the $-CCA_{OH}$ sequence is common to all tRNAs, the altered rate or extent of aminoacylation cannot be due to destruction of a specific ligase recognition site. An additional problem has been encountered with Bisulphite modification of cytidine residues in the $-CCA_{OH}$ sequence of Yeast $tRNA^{Tyr}$. Kućan et al, (1971) have reported the presence of a nuclease in the crude ligase preparation employed for aminoacylation of modified $tRNA^{Tyr}$, which removed the sequence HUA_{OH} from modified $tRNA^{Tyr}$, leading to the production of $tRNA^{Tyr}$ molecules incapable of accepting tyrosine. I found no evidence for the presence of a similar nuclease in the purified phenylalanyl - tRNA ligase preparation used to phenylacylate Bisulphite modified E. coli $tRNA^{Phe}_2$. The inactive modified $tRNA^{Phe}_2$ contained a total yield of the 3' terminal T_1 oligonucleotides, $CACCA_{OH}$, $CA(C,U)A_{OH}$

and $CAUUA_{OH}$ comparable to that found in active modified $tRNA^{Phe}_2$ (see TABLE 12).

It is obvious that the utmost care must be exercised in the use of chemical modification to examine the ligase recognition sites of tRNA molecules. While all of the criteria described in Section 1.4.1.1. should be observed, an essential requirement, that can not always be easily satisfied, and is difficult to prove, is that chemical modification must not alter the conformation of the tRNA molecule, or facilitate a conformational change. In my opinion, this is one of the biggest stumbling blocks in the use of chemical modification for such structure function studies. Another big drawback is that most modifying agents used are only capable of reacting with bases in single stranded regions of tRNA, whereas it is quite likely that the ligase recognition site may include helical stem regions (Roe & Dudock, 1972; Kern et al, 1972; Rich, 1974).

Bisulphite modification of E. coli Phe-$tRNA^{Phe}_2$ has been described in Section 3.4. This was an attempt to discover whether there are any conformational differences between charged and uncharged $tRNA^{Phe}_2$. Obviously, only the conformation of nucleoside residues which react with Bisulphite could be investigated, i.e. cytidine and 2-methyl thio - N^6 - isopentenyl adenosine residues. In particular, it was thought that C56 might become available for Bisulphite modification, due to exposure of the sequence GTΨCG on aminoacylation. However, no reaction of C56 was detected, either before or after aminoacylation of E. coli $tRNA^{Phe}_2$. As already discussed in Section 3.4.3., this does not necessarily mean that the

TΨCG region of $tRNA^{Phe}_2$ is not involved in binding to 5S RNA of the ribosome. Exposure of this sequence might occur on interaction of aminoacyl-tRNA with the elongation factor, GTP, template and 30S ribosomal sub-units, as suggested by Schwarz et al (1974).

The only detectable difference in the Bisulphite modification pattern of Phe - $tRNA^{Phe}_2$ as compared to $tRNA^{Phe}_2$ was that ms^2i^6A 37 was less modified after 8 hours in the charged form of $tRNA^{Phe}_2$, than in the uncharged form. There are several possible explanations for this result. It could be due to a local conformational change of Loop II, the anticodon loop, on aminoacylation of E. coli $tRNA^{Phe}_2$, that made the isopentenyl adenoside side chain of ms^2i^6A 37 less exposed, and therefore less available for Bisulphite modification. However the anticodon must remain exposed, as it is the aminoacyl form of tRNA that interacts with the codon on mRNA, or become exposed (e.g. on interaction with the elongation factor) before interaction with the codon.

Although Robertus et al (1974a)have determined that the bases of the anticodon of Yeast $tRNA^{Phe}$ are stacked on the 3' side of Loop II (the F H conformation described by Fuller & Hodgson (1967)), there is some evidence from chemical modification (Chang & Ish-Horowicz, 1974) and oligonucleotide binding studies (Uhlenbeck, 1972; Schimmel et al, 1972) that different tRNAs may have different anticodon loop conformations, the bases of some tRNAs being stacked on the 5' side of the anticodon loop, i.e. the hf conformation (see Woese (1970)). A conformational change in the anticodon loop of E. coli

$tRNA^{Phe}_2$ from FH to hf on aminoacylation would explain the results obtained for modification of ms^2i^6A 37, the base of this nucleotide becoming exposed on such a conformational change.

The result could also be explained if Phe-$tRNA^{Phe}_2$ containing modified ms^2i^6A 37 was more rapidly deacylated than Phe-tRNA containing unmodified ms^2i^6A 37. If pure $tRNA^{Phe}_2$ had been used in this experiment, the deacylated bisulphite modified $tRNA^{Phe}_2$ could have been fingerprinted, and if modification of ms^2i^6A 37 facilitated deacylation, this $tRNA^{Phe}_2$ fraction would have contained a greater proportion of tRNA molecules with modification of ms^2i^6A 37.

A simpler explanation for the slower modification of ms^2i^6A 37 in charged *E. coli* $tRNA^{Phe}_2$ would be possible if modification was able to continue during dialysis of tRNA to remove Bisulphite. As Bisulphite modification of ms^2i^6A 37 is a free radical reaction (Hayatsu *et al*, 1972), it is able to take place in low concentrations of Bisulphite ($\sim 1 \times 10^{-2}$M (Hayatsu & Inoue, 1971)). After Bisulphite modification of $tRNA^{Phe}_2$, it was dialysed against pH 7·0 buffer, while after modification of Phe-$tRNA^{Phe}_2$ dialysis against pH 5·0 buffer was employed, prior to fractionation on a Benzoylated DEAE - cellulose column. There is some evidence that the rate of Bisulphite modification of isopentenyladenosine is optimal at pH 7·0, and is reduced on lowering the pH (Hayatsu *et al*, 1972). If some modification of ms^2i^6A 37 was able to continue during the dialysis stage, it would proceed faster in pH 7·0 buffer than in pH 5·0 buffer i.e. faster in the uncharged form of $tRNA^{Phe}_2$ than in the charged form. However, there is some evidence described in this thesis

that modification of ms^2i^6A37 did not continue during dialysis. A sample of $tRNA^{Phe}_2$ removed immediately after suspension in 10mM $MgCl_2$, 3M sodium bisulphite, pH 6·0 was found to contain a similar percentage yield of the T_1 RNase oligonucleotide $AAms^2i^6AA\Psi CCCCG$ to that of the same $tRNA^{Phe}_2$ sample before Bisulphite modification, implying that no modification of ms^2i^6A37 occurred during dialysis.

Chemical modification has not previously been used as a probe of the conformation of aminoacyl-tRNA, probably mainly because of the fact that deacylation often occurs during the conditions of chemical modification used. In this thesis, a method has been described for the saparation of charged and uncharged modified $tRNA^{Phe}_2$, so that the modifications which occur in charged $tRNA^{Phe}_2$ could be pinpointed. This method could be extended to other tRNA species if phenoxyacetylation of the aminoacyl-tRNA was performed after chemical modification and before Benzoylated DEAE-cellulose chromatography. The separation of phenozyacetylated aminoacyl-tRNA from tRNA, in this way, has been described by Gillam & Tener (1971). Alternatively, modified aminoacyl-tRNA and modified uncharged tRNA could be separated by chromatography on Dihydroxyboryl substituted cellulose (McCutcheon et al, 1975).

Bisulphite modification of E. coli $tRNA^{Phe}_2$ at elevated temperatures has shown that even at $45^{\circ}C$, in the absence of added Mg^{2+}, some destruction of the tertiary interactions involving C56 and G19, and C48 and G15 had occurred. In a proportion of $tRNA^{Phe}_2$ molecules, destruction of the secondary structure of stem b and/or stem e had also occurred. Cytidine residues in the helical regions of stems a and c

were not modified, even at 55°C (except for C72 to a small extent), indicating that these stems are more heat stable than stems b and e. This can be explained by the fact that there are a greater number of thermodynamically more stable G - C base pairs in stems a and c than in stems b and e.

It is interesting to note that destruction of the base pair C49 - G65 appeared to occur only in molecules in which destruction of the tertiary structure base pair C48 - G15 had occurred. This is probably due, at least partly, to the fact that the C48 - G15 base pair is weaker than the C49 - G65 base pair, involving only two hydrogen bonds (Robertus et al, 1974b) instead of the three hydrogen bonds in the standard Watson - Crick base pair C49 - G65.

A similar explanation may be valid for breakage of the C56 - G19 Watson - Crick tertiary structure base pair less readily than the C48 - G15 tertiary structure base pair. However, account must be taken of the other base pairing interactions in the regions of G - C base pairs which appear to be destroyed on elevation of the temperature. If a G - C base pair is present in a region whose conformation is fixed by the participation of other base pairing interactions, it will be less easily broken than an isolated G - C base pair holding two parts of the tRNA molecule together. By analogy with Yeast $tRNA^{Phe}$ (Ladner et al, 1975b), in E. coli $tRNA^{Phe}_2$ only one base pair is responsible for holding together Loops I and III, i.e. C48 - G15. However there are several interactions holding together Loops I and IV, the standard Watson - Crick G19 - C56 base pair, hydrogen bonds between the base and ribose of Ψ55 and the ribose of A53 with the

base of G18, and a hydrogen bond between the base of G57 and the 1' -OH group of the ribose of G19. Therefore, it is unstandable that the G19 - C56 base pair is less readily disrupted than the G15 - C48 base pair.

The early melting of stem b of E. coli $tRNA_2^{Phe}$ which has also been described in thermal denaturation studies of other tRNA molecules (Hilbers et al, 1973; Crother et al, 1974; Hilbers & Schulman, 1974; Kastrup & Schmidt, 1975; Wong et al, 1975; Caron & Dugas, 1976) is of interest in that this region has been postulated to contain the synthetase recognition site of Yeast $tRNA^{Phe}$ (Roe & Dudock, 1972). However, there is evidence from N.M.R. studies on the interaction of E. coli $tRNA^{Glu}$ with glutamyl - tRNA synthetase, that the interaction does not involve breakage of any base pairs in helical regions of the tRNA molecules (Schulman et al, 1974).

It is obvious that a number of factors contribute to the stability of any base pairing interaction in tRNA. The results obtained on thermal denaturation of E. coli $tRNA_2^{Phe}$, as studied by Bisulphite modification, indicate that tertiary structure interactions are not necessarily "weak" interactions destroyed first on thermal denaturation as postulated by Tresco et al (1966). They may be as difficult to destroy as some secondary interactions, as illustrated by the breakage of the base pairs of stem b at similar temperatures to the breakage of the tertiary structure base pairs C48 - G15 and C56 - G19.

In conclusion, the stability of different regions of a tRNA molecule on thermal denaturation must depend on its unique secondary structure and may therefore differ from tRNA to tRNA.

Bisulphite modification at elevated temperatures has proved an effective probe of the thermal denaturation of *E. coli* $tRNA_2^{Phe}$ in a preliminary study.

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