Turnip yellow mosaic virus and cell-free protein synthesis

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December, 1972.

TURNIP YELLOW MOSAIC VIRUS and CELL-FREE PROTEIN SYNTHESIS

A THESIS.
Submitted in accordance with the
requirements of the University of Durham
for the degree of
Doctor of Philosophy

by J.W. Liddell, B.Sc. (Dunelm)

Abstract.

A polypeptide - synthesising cell-free system directed by Poly-U or Turnip Yellow Mosaic Virus-RNA was derived from imbibed seeds of Phaseolus aureus; this in vitro system was dependent upon exogenous tRNA. The Poly-U-directed system functioned in the presence of tRNAs from P.aureus, Vicia faba and yeast, whereas TYMV-RNA was translated only in the presence of tRNAs from P.aureus or V.faba. This translation barrier was related to the inability of the P.aureus high-speed supernatant enzyme fraction to charge various "protein" amino acids to yeast tRNA under uniform conditions. Such incompatibility did not exist in the Poly-U system where incubation conditions were constructed to favour only one amino acid, i.e. phenylalanine. The P.aureus enzyme fraction promoted esterification of valine to TYMV-RNA. More rigorous conditions were required for the translation of Poly-U in a Transfer System with yeast tRNA than with V.faba tRNA. Poly-U and TYMV-RNA competed for ribosomal binding sites. The characteristics of synthetic and natural templates in amino acid incorporation were correlated.

CONTENTS

Abbreviations.

The majority of abbreviations, symbols and conventions used in this thesis are in accordance with the recommendations of the 'Biochemical Journal', (1972), 126, 4. For typographical reasons the following were used in place of those recommended:-

CreP	Creatine phosphate
g	unit of gravitational field
hr	hour(s)
PCK	Phosphocreatine kinase
Poly-U	Polyuridylic acid
s	Svedberg unit
s'	sedimentation coefficient
sec	second(s) (time)
TYMV	Turnip Yellow Mosaic Virus
TYMV-RNA	RNA derived from TYMV

Occasional abbreviations are notated where they occur in the text.

In addition the following convention has been adopted in the Results Section; Transfer System:-

$14\left[U^{14}C\right]$ AA-V.faba tRNA	$14\left[U^{14}C\right]$ amino acid mixture + $6\left[^{12}C\right]$ amino acid mixture charged to V.faba tRNA to form an aminoacyl-tRNA complex.
$\left[^{14}C\right]$ Phe-V.faba tRNA	$\left[^{14}C\right]$ Phenylalanine + $19\left[^{12}C\right]$ amino acid mixture charged to V.faba tRNA to form an aminoacyl-tRNA complex.

and similarly with the various $\left[^{14}C\right]$ labelled aminoacyl-yeast tRNA complexes.

Introduction.

It is generally held that the process of protein synthesis in living cells consists of two stages, Transcription and Translation. First, DNA is transcribed into a RNA intermediate. The RNA has ribonucleotide sequence complementary with a deoxyribonucleotide sequence of one of the strands of the DNA. In this transcription stage the DNA is acting as a template, and the RNA as a messenger. In the translation stage, the mRNA attaches to ribonucleoprotein particles, the ribosomes, which are the sites of protein synthesis. There, the mRNA determines the order of linkage of amino acids into protein. (Attardi, 1967; Matthaei, Sander, Swan, Kreuzer, Caffier and Parmeggiani, 1968; Ochoa, 1968).

mRNA is translated in the 5' to 3' direction. (Ochoa, 1968). Thus, synthesis of a protein is initiated at the amino-terminal amino acid and proceeds towards the carboxy-terminal amino acid (Attardi, 1967; Bishop, Leahy and Schweet, 1960; Dintzis, 1961; Matthaei, Sander, Swan, Kreuzer, Caffier and Parmeggiani, 1968; Ochoa, 1968). In the process of translation a group of three adjacent nucleotides (the codon) in the mRNA, determines which amino acid is to be attached to the forming peptide chain. It has been established which codons specify each of the 20 "protein" amino acids (Cold Spring Harbor Symposium, 1966). This is the Genetic Code. It would appear that the sequence of amino acids in a polypeptide chain possesses all the information required for the production of the three-dimensional structure of the native

protein molecule (Anfinsen, 1967).

The discrete mechanisms of protein biosynthesis have been elucidated using cell-free in vitro protein-synthesising systems. Nirenberg (1964) has summarised that these systems may be prepared by disintegrating cells in aqueous media, removing unbroken cells and debris by low-speed centrifugation and small molecules by dialysis. Demonstration of protein synthesis with these extracts requires the presence of ATP, GTP, an ATP regenerating system, a monovalent cation either K^+ or NH_4^+ and the divalent ion Mg^{2+}, sulphydryl compounds which stabilise the system, and amino acids some of which are usually radioactively labelled. In such a system mRNA can translate into protein, when the translation is assayed by following the incorporation of labelled amino acids into protein.

The presence of a mRNA is essential. The mRNA may be present in the extract when it is the endogenous messenger or it may be added as an exogenous messenger. The exogenous messenger can be either a natural messenger or a synthetic polyribonucleotide. (Lengyel, Speyer and Ochoa, 1961; Nirenberg and Matthaei, 1961; Nishimura, Jones, Ohtsuka, Hayatsu, Jacob and Khorana, 1961).

The cell extract may be fractionated further by high-speed centrifugation to yield a microsomal pellet and a high-speed supernatant fraction. The microsomal pellet contains ribosomes and proteins required for peptide chain initiation. The supernatant fraction contains transfer RNA, aminoacyl-transfer RNA synthetases and proteins involved in the elongation and

termination of the peptide chain. (Lengyel and Soll, 1969). 120 different macromolecules are known to be involved in translation. (Lengyel and Soll, 1969). Each in vitro system may require supplementation of some of the components to witness the synthesising activity more clearly, so that Boulter (1970) describes a typical plant-derived "complete" cell-free ribosomal system as containing K^+, Mg^{2+}, ATP, ATP regenerating system, GTP, tRNAs, supernatant enzyme fraction and ribosomes (microsomes), one labelled and the other 19 unlabelled "protein" amino acids, buffered for autoxidation reaction and pH, together with an appropriate mRNA.

Consideration of the various steps in protein synthesis can conveniently start with the ribosome. The ribosome is the site of both in vivo and in vitro protein synthesis (Littlefield, Keller, Gross and Zamecnik, 1955). In the bacterial cell the ribosome sediments at a coefficient of approximately 70s whilst an s value of 80 is held for eukaryotic cytoplasmic ribosomes. However, in the higher organisms, the organelles such as mitochondria and chloroplasts have ribosomes at 70s. Separation of ribosomes into these 2 broad groups is convenient though perhaps arbitrary (Boulter, Ellis and Yarwood, 1972). The ribosome consists of 2 unequal subunits 30s and 50s in 70s ribosomes (Tissieres and Watson, 1958) and 40s and 60s in 80s ribosomes (Chao, 1957). A great proportion of information about in vitro protein synthesis pertains to the 70s prokaryotic-derived ribosome.

In a consideration of the translation of mRNA to protein

it is convenient to divide the process into:

(i) amino acid activation and formation of aminoacyl-tRNA,

(ii) peptide chain initiation,

(iii) peptide chain elongation,

(iv) peptide chain termination and release.

(i) Amino acid activation and formation of aminoacyl-tRNA:

The formation of the aminoacyl-tRNA catalysed by the aminoacyl-tRNA synthetase is a two-step reaction involving activation (Stulberg and Novelli, 1962; Allende, Allende, Gatica, Celis, Mora and Matamala, 1966; Rouget and Chapeville, 1968; Cassio, 1968) and transfer (Allende and Allende, 1964; Norris and Berg, 1964). The product of the activation step is the enzyme-bound aminopyrophosphate. In the transfer step, the activated amino acid is transferred onto the tRNA (a transacylation) to form aminoacyl-tRNA.

The generally accepted reaction can be expressed:-

Activation:

$$\text{amino acid} + \text{ATP} + \text{enzyme} \rightleftharpoons \overbrace{\text{aminoacyl-AMP}}^{\text{enzyme}} + \text{pyrophosphate}$$

Transfer:

$$\overbrace{\text{aminoacyl-AMP}}^{\text{enzyme}} + \text{tRNA} \rightleftharpoons \text{aminoacyl-tRNA} + \text{enzyme} + \text{AMP}$$

Rouget and Chapeville (1971) have observed that this reaction pattern seems oversimplified. With the leucyl-tRNA synthetase of Escherichia coli the binding of ATP to the enzyme is the first step of leucine activation and the binding of the amino acid occurs after the formation of an enzyme-ATP complex.

Allende, Chaimovich, Gatica and Allende (1970) using

threonyl-tRNA synthetase, Berry and Grunberg-Manago (1970) with lysyl-tRNA synthetase, Parin, Savelyev and Kisselev (1970) with tryptophanyl-tRNA synthetase, and Papas and Mehler (1971), using prolyl-tRNA synthetase, obtained similar results.

A prerequisite for accurate translation is the high specificity of aminoacyl-tRNA synthetases. (Loftfield and Eigner, 1966; Novelli, 1967; Peterson, 1967). Specificity is exhibited at both steps of the aminoacyl reaction. Using material from E.coli, it was shown that besides activating their cognate amino acids, isoleucyl-tRNA synthetase activates valine, and valyl-tRNA sythetase activates threonine. These irrelevant amino acids are not then transferred to the tRNA. (Baldwin and Berg, 1966; Bergman, Berg and Dieckmann, 1961; Hirsh and Lipmann, 1968).

Misrecognition between the enzyme and tRNA has been observed in heterologous reactions, for example the esterification of $tRNA^{Ala}$ and $tRNA^{Val}$ from E.coli by the phenylalanyl-tRNA synthetase from Neurospora crassa (Holten and Jacobson, 1969) and yeast (Taglang, Waller, Befort and Fasiolo, 1970) or with valine by the valyl-tRNA synthetase from E.coli on various tRNAs from yeast (Giege, Kern, Ebel and Taglang, 1971). Lack of specificity of the isoleucyl-tRNA synthetase from Bacillus stearothermophilus at higher temperatures has been reported by Arca et al. (1965, 1967). These investigators have shown that at temperatures above 75^{o} the charging of isoleucine onto tRNA decreases rapidly, and that valine, serine and threonine are transferred, most probably, to $tRNA^{Ile}$.

Calendar and Berg (1966) showed that certain amino acid analogues acylate $tRNA^{Tyr}$ with tyrosyl-tRNA synthetase from E.coli and Bacillus subtilis.

Azetidine-2-carboxylic acid, a lower homologue of proline, is incorporated into mung bean proteins when seedlings are grown in its presence. Where the proline homologue occurs naturally in a plant, e.g. Convalaria and Polygonatum, the prolyl-tRNA synthetase does not acylate the homologue with the proline-accepting tRNA. (Peterson and Fowden, 1963).

Phenylalanyl-tRNA synthetases from a number of plants have proved less specific than other synthetases. Phaseolus aureus and Leucaena leucocephala yield phenylalanyl-tRNA synthetase which activates mimosine. (Smith and Fowden, 1968).

Using E.coli, rat liver and the fungus Coprinus lagophus, Fowden, Lewis and Tristram (1968) showed that methionyl-tRNA synthetases acylate $tRNA^{Met}$ with ethionine.

In E.coli a single leucyl-tRNA synthetase is capable of activating different $tRNA^{Leu}$ species (Kan and Sueoka, 1971). The latter result is in sharp contrast to the studies of Anderson and Cherry (1969) who have demonstrated six different leucine-accepting tRNA species, chargeable with homologous synthetase in soy bean. Four of the $tRNA^{Leu}$ species were acylated by the soy bean hypocotyl synthetases and the other two by cotyledon synthetases. Further, soy bean leucyl-tRNA synthetases have been elucidated as three distinct groups - one group found in the cotyledons and two groups in the hypocotyl. (Kanabus and Cherry, 1971).

Of particular interest to those working with cell-free *in vitro* systems is the degree of specificity between aminoacyl-tRNA synthetases and tRNA derived from different species of organism. Ermokhina, Stambolova, Zaitseva and Belozerskii (1965) examined phenylalanyl-tRNA synthetase in pea, yeast and algae where the $tRNA^{Phe}$ and synthetase were interchangeable. Though the $tRNA^{Met}$ and methionyl-tRNA synthetases of pea and algae were interchangeable, the relevant components of yeast gave only 40%-50% charging in mixed systems.

Yot, Pinck, Haenni, Duranton and Chapeville (1970) when acylating *E.coli* tRNA with 15 separate $[^{14}C]$ amino acids, found that the *E.coli* enzymes would acylate about half these labels to Chinese cabbage tRNA. Hayashi (1966) found there was no charging in heterologous systems using yeast and *E.coli* tyrosyl-tRNA synthetases and purified $tRNA^{Tyr}$.

Allende (1969) reports work using wheat embryo enzymes and tRNA. Enzymes from a heterologous source charged the plant tRNA fully as in the case of yeast seryl-tRNA synthetase. The seryl-tRNA synthetase of wheat embryo charged *E.coli* tRNA partially whilst the *E.coli* synthetase had no charging ability on the wheat tRNA.

In the case of methionyl-tRNA synthetases the *E.coli* enzyme charged only 50% of the wheat tRNA, the explanation being that *E.coli* synthetase recognises only one of the two species of $tRNA^{Met}$ present in wheat embryo. Similar findings were presented by Yarwood, Boulter and Yarwood (1971) using *V.faba* $tRNA^{Met}$ and *E.coli* enzyme. The same authors subsequently

showed that V.faba synthetases aminoacylated both E.coli $tRNA^{Met}$ fractions. (1971).

The rates of acylation may vary between the homologous and the heterologous system. Enzymes from pea seed and wheat germ catalysed esterification of amino acids at a faster rate with homologous tRNA than with the heterologous tRNA (Moustafa, 1965).

It is clear that for in vivo protein biosynthesis the appropriate tRNA-tRNA synthetase apparatus must be available to the cell, if the message is to be translated successfully. If the cell were subject to messages where this apparatus is not conducive, it follows that such messages would be unacceptable and protein synthesis would not occur.

It has been shown that when E.coli B. is infected with bacteriophage T_2 or T_4 changes in host $tRNA^{Leu}$ occur (Sueoka and Kano-Sueoka, 1964; Kano-Sueoka and Sueoka, 1966; Kan, Kano-Sueoka and Sueoka, 1968; Kano-Sueoka, Nirenberg, Sueoka, 1968). These changes may be described as: (i) inactivation of the host tRNA; Waters and Novelli (1967, 1968) suggested that this inactivation is promoted by a phage-induced nuclease specific for host $tRNA^{Leu}$, (ii) production of a bacteriophage coded tRNA; Weiss, Hsu, Foft and Scherberg (1968) (using a technique of hybridising charged tRNA at low temperature) showed that at least one species of $tRNA^{Leu}$ and $tRNA^{Pro}$ was coded for by T_2 phage.

Herpes simplex virus has been shown to specify at least

one species of $tRNA^{Arg}$ (Subak-Sharpe and Hay, 1965; Subak-Sharpe, Shepherd and Hay, 1966).

Differences in tRNA species and in tRNA methylase activities between neoplastic and normal tissues of human and animal origin have been reported by many investigators. (Tsutsui, Srinivasan and Borek, 1966; Taylor, Buck, Grange and Holland, 1968; Baliga, Borek, Weinstein and Srinivasan, 1969; Yang, Hellman, Martin, Hellman and Novelli, 1969; Gallo and Pestka , 1970).

Alterations in tRNA species were studied by Sekiya and Oda (1972) in Simian virus [SV40] infected and transformed cells, focussing particularly on the role of the virus in the process. These workers concluded that none of the altered species of tRNA are encoded in the SV40 genome, rather the alterations were produced by derepressed synthesis of the host genome (after viral infection) caused by some cellular function, not directly by a viral gene product.

Rous sarcoma virus-RNA has four classes of intervirion RNA: 7s, small quantities of 18s and 28s, 70s and a substantial amount of 4s (Bishop et al., 1970). The 4s of the virus and its host cell have identical electrophoretic mobilities in 10% polyacrylamide gels and are equally methylated. However, significant differences in nucleotide compositions are detectable. Purification of RNA from Avian myeloblastosis virus (a tumour virus) gave Erikson (1969) a 4s component similar to host cell tRNA. The results indicated that the viral 4s RNA was not simply a contaminant derived from cellular débris. Apparently

unbeknown to him (Erikson), Bonar, Sverak, Bolognesi, Langlois, Beard and Beard (1967) arrived at the same conclusions concerning Avian myeloblastosis virus, and had aminoacylated, with a [^{14}C] amino acid mixture, the 4s component. Bonar et al. speculated that ribosomal-RNAs and tRNAs present and acting simultaneously with the virus-specific genome are required for initiating the preliminary stages of infection. Erikson and Erikson (1972) showed the Avian myeloblastosis virus to have arginine, tryptophan, cystine and lysine synthetase activities for chick embryo tRNA. The synthetases are membrane-bound or contained within membranes but these workers concluded that there is no supporting biological evidence to suggest an essential role for aminoacylation of specific tRNA in the replication cycle of this virus.

A valine-specific tRNA-like structure was demonstrated in Turnip Yellow Mosaic Virus-RNA by Yot, Pinck, Haenni, Duranton and Chapeville (1970). They concluded that this tRNA was an integral part of the viral-RNA, located in the vicinity of 3' terminus of the viral-RNA. The viral acylation could be catalysed by Chinese cabbage, E.coli, yeast and rat liver valyl-tRNA synthetases. Yot et al. also make the observation that all bacteriophage and viral RNAs examined to date contain identical sequence(-CpCpA or -CpC) at their 3' terminus, suggesting that these RNAs could also accept an amino acid under appropriate conditions, or at least have retained the vestige of a tRNA-like structure. In this case only a knowledge of the sequence of about the last 50 nucleotides at the 3' terminus of

these RNAs would reveal this vestige.

Aviv, Boime and Leder (1971) have shown that small amounts of Encephalomyocarditis viral-RNA direct a 50-fold increase in amino acid incorporation, in appropriately supplemented ascites tumour cell extracts under conditions that give rise to authentic viral polypeptides. Incorporation in these crude extracts is almost entirely dependent upon the addition of exogenous tRNA. Further, this incorporation is restricted to that tRNA derived from ascites tumour cells or from rat liver, whereas tRNA from yeast and E.coli does not permit translation of the viral-RNA. The authors claim that the translational barriers are due partly to an incompatability between the tRNA of yeast and E.coli and tRNA synthetases of the ascites tumour cells. However, Aviv et al. showed that E.coli tRNA synthetases do not restore viral-directed protein synthesis in the presence of E.coli tRNA. Using the synthetic messenger polyuridylic acid in the ascites system, E.coli tRNA did not support polyphenylalanine synthesis whereas yeast tRNA did. The synthesis of polyphenylalanine could be restored by using E.coli tRNA. Finally, tRNA from ascites tumour cells and E.coli could be aminoacylated by the ascites tumour cell enzyme but tRNA from yeast could not. These workers suggest that, although compatability of tRNA and synthetases is important, the restriction imposed by tRNA in this system possibly involves the favoured use of specific degenerate codon classes by the virus and the ascites tumour cell.

The specific recognition site on the tRNA for its relevant aminoacyl-tRNA synthetase is still problematical. The large degree of homology in the structures of the various tRNAs makes

it difficult to distinguish a specific continuous nucleotide in an identical position in all tRNAs as a recognition site for the aminoacyl-tRNA synthetase. Cramer, Doepner, v.d. Haar, Schlimme and Seidel (1968) have suggested that the process may involve the recognition of features of tertiary structure.

Cassio (1968), Iaccarino and Berg (1969), Yaniv and Gros (1969), Papas and Mehler (1968), Parin and Kisselev (1969) with several synthetases have clearly demonstrated that the activation and the recognition sites for tRNA are distinct. Yarus and Berg (1969); Helene, Brun and Yaniv (1971), have suggested an interaction between the two catalytic sites of these enzymes.

(ii) Peptide chain initiation:

Polypeptide chain initiation in bacteria seems so clearly established that only a rough outline will appear here. About 40% of the E.coli proteins have methionine as the N-terminal amino acid, although methionine constitutes only 2.5% of the total amino acid in proteins. Methionine, alanine, serine and threonine together account for 95% of the N-terminal amino acids in these proteins. (Waller, 1963). Over 60% of the methionine residues attached to E.coli tRNA have their α-amino groups formylated. No other N-formylated aminoacyl-tRNA has been detected. The formylation of the α-amino group of methionine takes place after esterification by $tRNA^{Met}$. (Marcker and Sanger, 1964).

The formyl donor is N^{10}-formyltetrahydrofolate (Adams and Capecchi, 1966; Dickerman, Steers, Redfield, Weissbach, 1967; Marcker, 1965). The formylating enzyme, methionyl-tRNA

transformylase, has been purified from E.coli (Dickerman et al. 1967). There are two classes of methionine-accepting E.coli tRNA species, namely $tRNA_F^{Met}$ and $tRNA_M^{Met}$. Methionyl-$tRNA_F$ can be formylated by an enzyme; methionyl-$tRNA_M$ cannot. (Clark and Marcker, 1965, 1966). The $tRNA_F^{Met}$ has unusual base pairing near the 3' and 5' ends (Dube, Marcker, Clark and Cory, 1968) when compared with all other tRNAs of known sequence.

The RNA of bacteriophage (coliphage) f2 and R17, acting as a messenger, directs the synthesis of at least three virus-specific proteins in an in vitro E.coli cell-free amino acid-incorporating system. (Capecchi and Gussin, 1965; Nathans, Notani, Schwartz and Zinder, 1962).

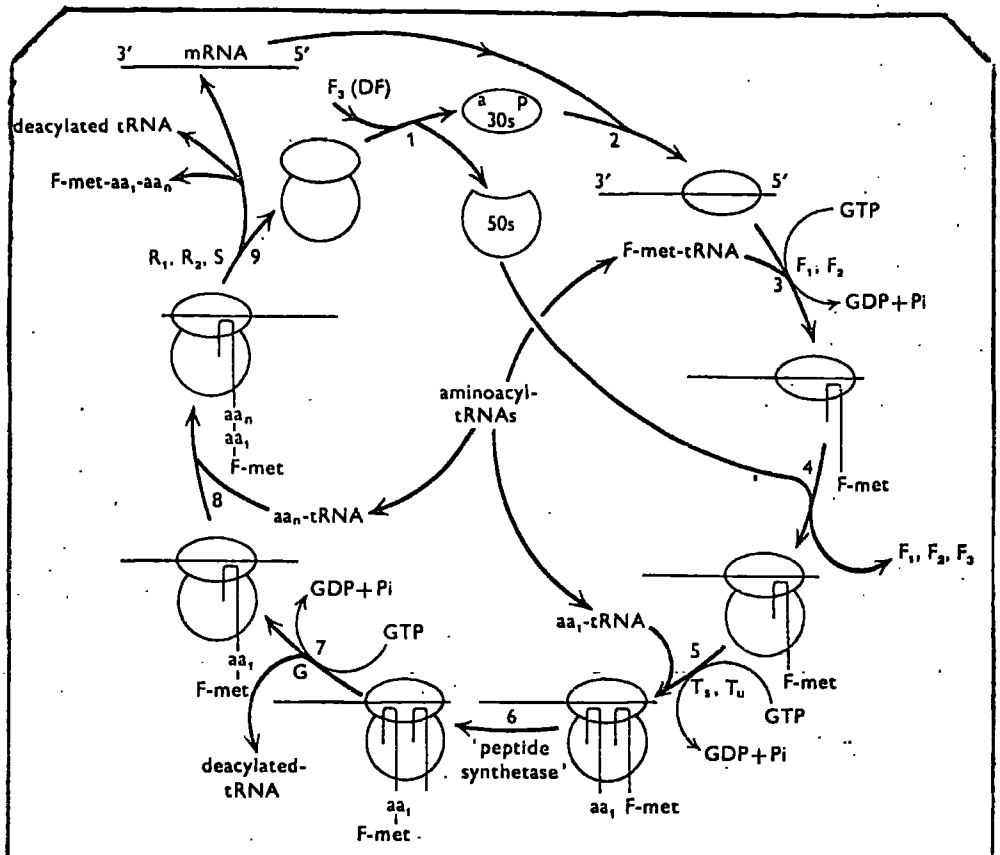
Each of these proteins has N-formyl-methionine as the N-terminal amino acid. (Adams and Capecchi, 1966; Lodish, 1968; Vinuela, Salas and Ochoa, 1967; Webster, Engelhardt and Zinder, 1966). This indicates that fMet-$tRNA_F$ can serve as a peptide chain initiator for each of the proteins programmed by polygenic mRNA. The dependence of in vitro protein synthesis directed by f2-RNA on fMet-$tRNA_F$ was shown by Burchall and Hitchings (1965), and Eisenstadt and Lengyel (1966) showed that amino acid incorporation directed by f2-RNA depends upon either added fMet-$tRNA_F$ or formyltetrahydrofolate. This dependence is found only at low Mg^{2+} concentration (4-8 mM). At high Mg^{2+} concentration, the incorporation is only slightly or not at all dependent on fMet-$tRNA_F$ (Eisenstadt and Lengyel, 1966; Kolakofsky and Nakamoto, 1966). Capecchi (1966) demonstrated, in a cell-free extract of E.coli in which protein synthesis was directed by endogenous mRNA, that one formyl-methionine residue was incorporated for approximately 150 amino acids and formyl-methionine was the only formyl-amino acid.

The work of Clark and Marcker (1966); Ghosh, Soll and Khorana (1967); Salas, Hille, Last, Wahba, Ochoa (1967), showed that fMet-$tRNA_F$ serves as a source of N-terminal methionine residues and methionyl-$tRNA_M$ supplies methionine residues for internal and C-terminal positions of the polypeptide chains. The codons specifying $tRNA_F^{Met}$ are AUG and GUG, whilst for $tRNA_M^{Met}$ the codon is AUG. (Clark and Marcker, 1965; Ghosh et al., 1967; Salas et al., 1967; Sundararajan and Thach, 1966).

A convenient hypothesis is that the binding sites on the ribosome are an A-site (aminoacyl site) and a P-site (peptidyl site). Given that this premise is controversial as to number and arrangement of sites, it has been shown that fMet-$tRNA_F$ may bind to either site. Tetracycline, an antibiotic inhibiting the binding of aminoacyl-tRNA to the A-site (Gottesman, 1967), was found to block the binding of fMet-$tRNA_F$ to ribosomes in the presence of GTP, mRNA and initiation factors. Puromycin inhibits fMet-$tRNA_F$ at the P-site (Bretscher and Marcker, 1966; Leder and Bursztyn, 1966; Ohta, Sarkar and Thach, 1967; Salas et al., 1967). Thus fMet-$tRNA_F$ may enter at the A-site and move to the P-site or it may enter at the P-site.

Boulter, Ellis and Yarwood (1972) have proposed the following diagram and legend, summarising the knowledge to-date of protein synthesis on the 70s ribosome. Fig: Page 15.

Plant viral-RNAs are capable of promoting biosynthesis in the E.coli in vitro cell-free system. (Voorma et al., 1965; Verhoef et al., 1967, 1968; Van Ravenswaay Claasen et al., 1967; Van Duin et al., 1968; Albrecht et al., 1969 i; Hoogendam et al., 1968; Reinecke et al., 1968). However, the biosynthetic products lack



Text-fig. . Mechanism of protein synthesis on the 70s microbial ribosome.

Initiation

1, 2: Dissociation of monomer into native subunits requires initiation factor F_3 (dissociation factor DF), which also promotes binding of mRNA to 30s subunit. 'a' and 'p' indicate relative positions of aminoacyl- and peptidyl-sites respectively.

3: Formation of initiation complex requires factors F_1 and F_2, GTP and f-met-tRNA. The latter is shown entering directly into the 'p' site, alternatively it may enter the 'a' site and then move to the 'p' site.

4: Functional ribosome formed by addition of 50s subunit; initiation factors released.

Chain elongation

5: Transfer factors T_s and T_u and GTP required for binding of aminoacyl-tRNA in 'a' site.

6: Peptide bond formation requires peptide synthetase (peptidyl-transferase) thought to be a function of the ribosome.

7: GTP and transfer factor G are required for displacement of discharged tRNA and translocation of peptidyl-tRNA to the 'p' site. Although often referred to as the 'translocase', factor G appears to promote the release (uncoupled from translocation) of the deacylated tRNA molecule from the 'a' site, while translocation *per se* seems to be a function of the 50s subunit (Roufa, Skogerson & Leder, 1970; Tanaka, Lin & Okuyama, 1971; however, cf. Lucas-Lenard & Haenni, 1969).

8: Chain elongation proceeds by repetition of steps 5, 6 and 7.

Chain termination

9: Requires release factors R_1, R_2 and S. A specific peptidyl-tRNA hydrolase may also be required to give the free polypeptide (Cuzin *et al.* 1967; Vogel, Zamir & Elson, 1968; de Groot, Panet & Lapidot, 1968). Ribosome may be released in form of subunits, but in absence of F_3 these will spontaneously recombine.

any resemblance to (Aach et al., 1964) or show a limited similarity to the authentic coat protein, (Van Ravenswaay Claasen et al., 1967, Clark et al., 1965) so that it seems possible that different mechanisms underlie protein synthesis initiation in the homologous 70s compared with the heterologous 70s systems. Albrecht et al., (1969i) have shown that plant viral-RNA can promote the binding of fMet-tRNA$_F$ to E.coli ribosomes as does coliphage. The binding and incorporation were strictly dependent on "crude" or fractionated ribosomal initiation factors.

Outstanding differences between the homologous and heterologous systems were as follows:-

(i) Plant viral-RNA can bind to E.coli ribosomes in the absence of initiation factors, whereas the binding of MS$_2$-RNA is fully dependent on these factors (Albrecht et al., 1969,i and ii).

(ii) Binding of aminoacyl-tRNA to the phage-RNA-ribosome complex requires the previous binding of fMet-tRNA$_F$ (Voorma et al., 1969) whereas plant viral-RNA can promote the binding of aminoacyl-tRNA to ribosomes in the complete absence of fMet-tRNA$_F$.

Verhoef et al., 1968, using alfalfa mosaic viral [AMV]-RNA, have demonstrated that the viral-RNA-ribosome complex permits the binding of only three aminoacyl-tRNAs (i.e. phenylalanyl-, isoleucyl- and valyl-tRNA). Phenylalanyl-tRNA and isoleucyl-tRNA are directed by adjacent codons on the AMV-RNA chain (Verhoef, Lupker, Cornelissen and Bosch, 1971). Verhoef and Bosch (1971) also showed that both these species of aminoacyl-tRNA, when N-acetylated, can function as chain initiators in protein synthesis in cell-free systems of E.coli, programmed with AMV-RNA.

Many proteins carrying a N-terminal acetyl group have been known for some time, but in general the origin of the acetyl group and the mechanism of acetylation remains obscure.

Pearlman and Bloch (1963) showed that N-acetyl-L-tyrosine could be transferred to tRNA; subsequently, deacylation of the tRNA yielded N-acetyl-L-tyrosine. Indications were also obtained by these authors that the N-acetylated forms of tryptophan, alanine, glutamic acid and histidine might also be chargeable to tRNA. Haenni and Chapeville (1966) attempted to repeat this work with E.coli enzymes and E.coli tRNA species charging for N-acetyl-phenylalanine and N-acetyl-leucine, but were unsuccessful. N-acetyl-phenylalanyl-tRNA (produced by chemically acetylating phenylalanyl-tRNA) when stripped of the acetylated amino acid, was then capable of esterifying phenylalanine.

An enzyme has been isolated from chicken reticulocytes catalysing the transfer of the acetyl residue from acetyl coenzyme-A to certain proteins (foetal and chicken haemoglobins). This finding indicates that masking of the α-group of the N-terminal amino acid may occur after protein synthesis (Marchis-Mouren and Lipmann, 1965).

There is no consensus on the universality of peptide chain initiation on the 80s ribosomes, i.e. cytoplasmic ribosomes of eukaryotes. The 70s ribosome in eukaryotes is found only in the mitochondria and chloroplasts of these organisms, and it is now generally held that the protein-synthesising mechanism of these organelles is that of the bacterial 70s ribosome (Ref.

Boulter, Ellis and Yarwood, 1972).

Caskey, Redfield and Weissbach (1967), demonstrated two species of methionine-accepting tRNA in guinea-pig liver, one of which was formylatable by E.coli transformylase. However, no homologous transformylase was found. A similar situation obtained with yeast (a unicellular eukaryote). Takeishi, Ukita and Nishimura (1968) showed that the formylated methionyl-tRNA (tRNA designated $tRNA_{F*}^{Met}$) could replace the E.coli fMet-$tRNA_F$ in an E.coli in vitro system.

Kewar, Spears and Weissbach (1970) separated two species of methionine-accepting tRNA from rabbit liver. Drews, Hogenauer, Unger and Weil (1971) separated two species from mouse liver. In both cases, one species of $tRNA^{Met}$ was formylated by E.coli transformylase. Three methionine-accepting tRNAs were isolated from crude rabbit liver tRNA by Gupta, Chatterjee, Bose, Bhaduri and Chung (1970). Only one of these species could be charged by the E.coli synthetase and could be formylated by the E.coli transformylase.

Results of incorporation experiments using tRNA carrying modified valine residues indicated that no special initiating tRNA exists other than the valyl-tRNA translating the N-terminal codon in rabbit haemoglobin synthesis. (Rich, Eikenberry and Malkin, 1966; Arnstein and Rahamimoff, 1968; Rahamimoff and Arnstein, 1969). Confirming this were reports that newly synthesised haemoglobin and also the nascent chains on reticulocyte ribosomes have valine as the only detectable N-terminal residue. (Rahamimoff, Arnstein, 1969; Gonano and

and Baglioni, 1969; Mosteller, Culp and Hardesty, 1968).

However, subsequent work has demonstrated that methionyl-$tRNA_{F*}$ is incorporated in the N-terminal position of both α and β chains of rabbit haemoglobin during the initiation process of protein synthesis and is removed during the early stages of peptide chain growth. (Jackson and Hunter, 1970; Housman, Jacobs-Lorena, Rajbhandary and Lodish, 1970; Wilson and Dintzis, 1970; Bhaduri, Chatterjee, Bose and Gupta, 1970; Gupta et al., 1970; Smith and Marcker, 1970). Jackson and Hunter (1970) have suggested that any real initiation by the valine residue must be abnormal, and it is therefore of interest that only a small proportion of chains were initiated in this way.

A model proposed by Smith and Marcker (1970) demands that the methionine residues which initiate rabbit globin chains are unmodified and are furnished by a particular species of tRNA. Caffier, Raskas, Parsons and Green (1971) using cultured human cells (KB) infected with human adenovirus type 2 showed that:

(i) extracts from such infected cells synthesise structural viral polypeptides,

(ii) these extracts initiate protein synthesis with methionine in vitro,

(iii) the relative rate of initiation by methionine and chain elongation is the same in vitro and in vivo,

(iv) a special species of yeast $tRNA^{Met}$ preferentially responds to specific N-terminal codons in these extracts.

The methionine initiator has also been recorded in the

synthesis of protamine in trout testis cells (Wigle and Dixon, 1970).

Galper and Darnell (1969) reported that inhibition of formylation by aminopterin did not affect the growth of HeLa cells. Brown and Smith (1970), using a mouse ascites system, have found that initiation by methionyl-$tRNA_{F*}$ is not dependent upon formylation at low Mg^{2+}. Indeed, in this system formylation of methionyl-$tRNA_{F*}$ destroys its initiating ability.

From wheat germ, Leis and Keller (1970) demonstrated 3 chromatographically distinct methionine-accepting tRNAs of which two species, one major and one minor, appear to function in protein chain initiation, as demonstrated by the AUG-dependent reaction with puromycin on wheat germ ribosomes. The minor methionyl-tRNA (which probably initiates in the organelles), was formylatable by a transformylase in wheat germ extracts, but the initiating major methionyl-tRNA was not formylatable.

These results have been confirmed in wheat germ by Marcus, Weeks, Leis and Keller (1970), Tarrago, Monasterio and Allende (1970), and Ghosh, Grishko and Ghosh (1971). Yarwood, Boulter and Yarwood (1971) have derived similar conclusions from V.faba studies.

Initiation in E.coli requires at least three protein factors (FI, FII and FIII) for natural mRNA translation. These initiation factors are normally associated with the ribosome (Brawerman and Eisenstadt, 1966; Eisenstadt and Brawerman, 1966; Revel and Gros , 1966; Stanley, Salas, Wahba, Ochoa, 1966; Salas, Hille, Last, Wahba, Ochoa, 1967; Salas, Miller,

Wahba, Ochoa, 1967).

Marcus (1970) using tobacco mosaic virus-RNA in a wheat embryo system has demonstrated an initiation complex requiring ATP and 2 protein factors, (isolated from the high-speed supernatant). ATP was thought to be an absolute requirement but further work indicates that it may be replaceable by GTP (J.D. Bewley, private communication, 1972).

(iii) Peptide chain elongation

Elongation of the peptide chain requires K^+, Mg^{2+}, GTP, charged-tRNA, and a soluble enzyme fraction containing the "Transfer factors".

Transfer factors have been resolved clearly in E.coli and Pseudomonas fluorescens and were designated Tu, Ts and G. (Kaziro and Inoue, 1968; Parmeggiani, 1968; Lucas-Lenard and Lipmann, 1966). The factors from Bacillus stearothermophilus S1, S2, S3, (Skoultchi, Ono, Moon and Lengyel, 1968) correspond to Ts, G and Tu respectively. Yeast transfer enzymes were resolved into 2 fractions comparable with Tu and G (Richter and Klink, 1967; Ciferri, Parisi, Perani, Grandi, 1968).

The need for GTP and factors in attaching aminoacyl-tRNA to the mRNA-ribosome complex was first established in a cell-free system from reticulocytes (when 2 transfer factors were recorded) by Arlinghaus, Schaeffer and Schweet (1964). These requirements for bacterial factors were not established until much later.

Chain elongation was studied mainly with Poly-U which promoted poly phenylalanine synthesis at "high" Mg^{2+} con-

centration. At high Mg^{2+} there was a strong aminoacyl-tRNA binding which is independent of factors and GTP. (Kurland, 1966; Ravel, 1967). Subsequently, work in the E.coli system (Ravel, 1967) and the yeast system (Ayuso and Heredia, 1968; Richter and Klink, 1968) using the synthetic messenger at lower Mg^{2+} levels, demonstrated the need for GTP and transfer factors in binding phenylalanyl-tRNA to the ribosome-Poly-U complex. Pestka (1968) and Ravel (1967) using E.coli ribosomes and Poly-U, have shown that a ribosome dipeptidyl-phenylalanyl-tRNA may be formed which may or may not be catalysed enzymatically. Diphenylalanyl-tRNA is an analogue of fMet-$tRNA_F$ and may serve as a chain initiator. (Lipmann,1967; Nakamoto and Kolakofsky, 1966). Although Siler and Moldave, (1969) using rat liver, and Busiello, di Girolamo and Felicetti (1971) using reticulocytes, showed that little or no diphenylalanyl-tRNA was formed when these ribosomes were incubated with phenylalanyl-tRNA and Poly-U, Rahamimoff, Baksht, Lapidot and de Groot (1972) report that incubation of rabbit reticulocyte ribosomes with purified phenylalanyl-tRNA in the absence of transfer factors and GTP gave considerable amounts of di-phenylalanyl-tRNA.

Transfer factors from plants have been recorded. Allende (1969) reported partial purification of wheat embryo factors. Legocki and Marcus (1970) described partial purification of two soluble transfer factors from wheat germ. App (1969) has isolated two factors from the high-speed supernatant from rice embryos.

Ciferri, Parisi, Perani and Grandi (1968), working with an E.coli transfer system, have indicated that yeast factors are able to substitute for Tu and G factors of E.coli. Ts from E.coli could not be replaced by the yeast subfraction. Allende (1969), employing components from wheat embryo, guinea-pig liver, E.coli and tobacco leaf chloroplasts (70s ribosomes) notes that the transfer enzymes from plant systems can function with ribosomes from animal sources. The reverse obtains, but these components cannot be exchanged with their bacterial counterparts.

Drews, Hogenauer, Unger and Weil (1971) and Chatterjee, Bose, Woodley and Gupta (1971) have indicated that the methionyl-$tRNA_{F*}$ from mammalian cells may have a dual function in protein synthesis. Methionyl-$tRNA_{F*}$ was found to donate methionine into internal positions of nascent peptide chains.

Using systems from mouse liver and ascites tumour cells, Drews, Grasmuk and Weil (1972) report results that T-factor does not only promote the binding of methionyl-$tRNA_M$ to ribosomes but can also catalyse the ribosomal attachment of methionyl-$tRNA_{F*}$ very efficiently. However, the preferential incorporation of methionine from methionyl-$tRNA_M$ in peptide chain elongation, which becomes evident when both methionine-accepting tRNAs are introduced into a cell-free system in saturating conditions, is collateral with the finding that T-factor, when provided with both iso-accepting tRNAs, displays a strong preference for the ribosomal binding of methionyl-$tRNA_M$. It might be that structural features inherent in the

nucleotide sequences of methionyl-$tRNA_M$ and methionyl-$tRNA_{F*}$ are responsible for quantitative differences in the capacities of these tRNAs to associate with GTP, T-factors and the ribosomal A-site. These authors also showed that the formylation of the α-amino group of methionyl-$tRNA_{F*}$ is completely prohibitive to the recognition of this tRNA by T-factor.

Richter, Lipmann, Tarrago and Allende (1971) have demonstrated the formation of an unstable ternary complex of GTP, methionyl-$tRNA_{F*}$ from yeast or wheat embryo and transfer factor preparations from these organisms. The ternary complex from yeast was non-functional when introduced into a cell-free protein-synthesising system containing yeast ribosomes and Poly(AUG) whereas the analogous complex with methionyl-$tRNA_M$ was very active in polymethionine synthesis.

(iv) Polypeptide chain termination and release:

Chain termination in E.coli extracts occurs following translation of one of the three terminator codons UAA, UAG or UGA. (Capecchi, 1967; Caskey, Tompkins, Scolnick, Caryk and Nirenberg, 1968; Last, Stanley, Salas, Hille, Wahba and Ochoa, 1967). Milman, Goldstein, Scolnick and Caskey (1969) have differentiated 3 release factors of E.coli. The factors are designated R_1, R_2 and S, where R_1 recognises UAA and UAG, R_2 recognises UAA and UGA and S accelerates the release rates induced by R_1 or R_2.

Beaudet and Caskey (1971) using rabbit reticulocyte ribosomes and release factors from reticulocytes, Chinese hamster liver and guinea-pig liver, have demonstrated that these

terminator codons probably act in the same way in mammals (eukaryotes). Results are not available for polypeptide chain termination in plants. For more comprehensive treatments of chain termination see: Basilio, Bravo and Allende (1966), Soll, Cherayil and Bock (1967), Caskey, Beaudet and Nirenberg (1968), Lengyel and Soll (1969), Allende (1969), von Ehrenstein (1970) and Nirenberg (1970).

Turnip Yellow Mosaic Virus:

TYMV is a RNA virus first described by Markham and Smith (1946). The ability of TYMV to infect plants appears to be confined almost entirely to the Cruciferae, and the common experimental host is Chinese cabbage, Brassica pekinensis. (For a comprehensive review of TYMV see: Matthews and Ralph, 1966).

It seems probable that infectious TYMV-RNA, like the RNA of tobacco mosaic virus, consists of one polynucleotide chain representing the full RNA complement of the intact virus. Klug, Longley and Leberman (1966) suggest that TYMV-RNA exists in situ in 32 packets. The RNA is enclosed within a single icosahedral protein shell comprising 180 structure units. (Harris and Hindley, 1961; Symons, Rees, Short and Markham, 1963). The 180 units are clustered in groups of 5 and 6 to produce 32 capsomeres. (Nixon and Gibbs, 1960; Huxley and Zubay, 1960).

Bound to the RNA is virus-specific polyamine. (Johnson and Markham, 1962). Subsequently, Beer and Kosuge (1970), examining the polyamine, found that spermidine accounted for approximately 1% virus weight while spermine accounted for less

than 0.04%.

A survey of the peptide fragments, derived from the protein components following its digestion with trypsin, led to the isolation and characterisation of 11 unique peptide sequences, which collectively account for the entire composition of the virus protein. The protein of the virus has thus been shown to be composed of identical polypeptide chains each containing 189 amino acid residues. (Harris and Hindley, 1965). Valine, isoleucine and leucine account for 25% of the total residues.

It has been deduced that there is sufficient RNA in a single TYMV complement to code for 10-12 proteins about the size of the viral coat protein. However, if any other proteins coded for by the viral-RNA were of average size, fewer than 10 (probably 2-4) could be coded for in addition to the viral coat. One or more of these additional proteins will presumably be the viral polymerase or polymerases. Others may be concerned with the synthesis of the polyamine found in TYMV. It seems most probable that TYMV coat protein derives from the 80s (cytoplasmic) ribosomes (Matthews and Ralph, 1966).

A double-stranded TYMV-RNA is associated with the chloroplast fraction from infected leaves (Ralph and Clark, 1966). Ralph, Bullivant and Wojcik (1971) confirmed this report and have postulated that viral-RNA replicates itself in the chloroplast vesicles, which were first demonstrated by Ushiyama and Matthews (1970). The nascent TYMV-RNA from these vesicles moves to "pockets" between chloroplasts where the virus is

rapidly assembled.

Astier-Manifacier and Cornuet (1971) have demonstrated a RNA-dependent RNA-polymerase from the chloroplastic fraction of Chinese cabbage leaves. Although the enzyme is found in apparently healthy plants, its specific activity increases during the first week of infection by TYMV.

In summary the mechanism for translation on the 70s prokaryotic ribosome is known in some detail. Much less is known of the mechanism on the 80s cytoplasmic ribosome of animals and even less for plants.

The present investigation is an attempt to determine some facets of the translation mechanism of a plant-derived cell-free cytoplasmic ribosomal polypeptide-synthesising system, directed by synthetic and viral messenger molecules. To further this, an attempt is made to characterise a crude, homologous cell-free 80s ribosomal system from Phaseolus aureus and to substitute tRNAs from a closely related legume, Vicia faba, and a unicellular eukaryote, yeast, when the system is directed by Poly-U or RNA from Turnip Yellow Mosaic Virus. The viral-RNA may be regarded as non-homologous to the Phaseolus aureus in vitro system since the virus is not known to infect that plant.

It should be noted that yeast tRNA is a commercial preparation.

Biological Materials.

A) Brassica pekinensis var Wong Bok seeds were obtained from Thompson and Morgan, London Road, Ipswich, Suffolk, and grown at the University of Durham Science Site greenhouse in an artifical soil mixture in 4" plastic pots at 21° - 25° Natural light was supplemented, if necessary, with fluorescent lighting to give a day length of 15-17 hr. Plants were watered twice a day if warranted.

B) Turnip Yellow Mosaic Virus was obtained from Mr. I.M. Thompson and was the Cambridge strain propagated by Dr. R.E.F. Matthews in New Zealand.

(1) Inoculum: A TYMV-infected leaf of Brassica pekinensis var Wong Bok was ground in a mortar with a small amount of carborundum and sterile water.

(2) Inoculation: Plants were finely sprinkled with carborundum. An index finger was dipped into the inoculum and rubbed lightly over a leaf. Plants at the 4-6 leaf stage were used, but more mature plants could be used.

(3) Infection: About 2 weeks after inoculation the mosaic syndrome was apparent. Harvesting of leaves took place at 4-8 weeks after inoculation. Approximately 4% of plants refused infection.

C) Phaseolus aureus seeds were obtained from the Tyneside Seed Company, Gateshead, Co. Durham, and were used either imbibed or in a germinating state.

(1) Imbibition: Dry seeds were soaked in intermittent running tap water for 24-36 hr, rinsed in several changes of sterile distilled water at room temperature, and dried on absorbent paper.

(2) Germination: Seeds to be germinated were treated first as in C(1). The imbibed seeds were placed in 70% (V/V) ethanol-sterilised plastic seed trays lined with dampened absorbent paper, and kept in the dark at room temperature under an intermittent overhead water spray. After 6 days, when harvested, the emergent plumules were approximately 10 cm long.

Microbial contamination was not apparent.

D) Vicia faba var Triple White seeds were obtained from the Tyneside Seed Company, Gateshead, Co. Durham. The seeds were used either in a germinating or developing state.

(1) Germination: Dry seeds were soaked for 24 hr in intermittent running tap water. The imbibed seeds were immersed in 10% calcium hypochlorite solution (3.5% available chlorine). After 3 min contact the seeds were repeatedly rinsed in sterile distilled water at room temperature until the odour of chlorine was not detectable. The seeds were planted in moistened Vermiculite in 70% (V/V) ethanol-sterilised plastic seed trays, which were kept in the dark under an intermittent overhead water spray. After 6-8 days, when harvested, the plumules were approximately 5 cm long.

Microbial contamination was not apparent.

(2) Developing: Seeds were grown in the open at the University of Durham Science Site garden, and were harvested 50 days after first flowering. Some beans were processed at once, the rest frozen under liquid nitrogen, to be stored.

Chemicals and Reagents:

Adenosine 5' triphosphate, disodium salt, from equine muscle; Guanosine 5' triphosphate, sodium salt, from equine muscle; Creatine phosphokinase, from rabbit muscle; Phosphocreatine, disodium salt; Polyuridylic acid, potassium salt; L-amino acids; Reduced glutathione; Bovine serum albumin.	Sigma Chem. Co., London.
Yeast tRNA.	Boehringer Corporation, London.
2, 5, Diphenyloxazole (PPO)	Koch-Light,
1, 4, bis 2-(5-phenyloxazolyl) benzene (POPOP)	Colnbrook, Bucks.
$[^{14}C]$ amino acids:	
DL-leucine 55 mCi/mmol.	Radiochemical Centre, Amersham, Bucks.
DL-phenylalanine 59 mCi/mmol	
DL-valine 34 mCi/mmol	
DL-alanine 23.4mCi/mmol	
DL-glycine 41.4mCi/mmol	
DL-glutamic acid 23.6mCi/mmol	
$[U^{14}C]$ amino acid mixture (CFB 104)	

Composition by activity of [^{14}C] amino acid mixture:

L-alanine	10%	L-lycine	5.5%
L-arginine	6.5%	L-phenylalanine	7.0%
L-aspartic acid	9.0%	L-proline	6.0%
L-glutamic acid	12.5%	L-serine	5.0%
glycine	5.0%	L-threonine	6.0%
L-leucine	12.0%	L-tyrosine	3.5%
L-isoleucine	5.0%	L-valine	7.0%

Specific activity 52 mC/m Atom carbon

All other chemicals were obtained from B.D.H. Ltd., Poole, Dorset, England, and were analytical grade, where available.

Artificial Soil:

Loam	7 bushels
Sphagnum peat	4 bushels
Sharp sand	2 bushels
John Innes Base Fertiliser	52 oz
Chalk	13 oz

Scintillation fluid:

PPO	4.5 g
POPOP	0.1 g
AnalaR Toluene to 1 litre	

Microsomal extractant:

Sucrose	0.4M
Magnesium chloride	0.005M
Potassium chloride	0.016M
Tris-HCl buffer, pH 7.6 at 0^{o}	0.05M

Microsomal Resuspension medium:

Magnesium chloride	0.001M
Potassium chloride	0.001M
Tris-HCl buffer, pH 7.6 at 0^{o}	0.01M

Dialysis Buffer for High-Speed Supernatant:

Magnesium chloride	0.005M
Potassium chloride	0.016M
Tris-HCl buffer, pH 7.6 at 0^{o}	0.05M

Transfer RNA extractant:

Magnesium chloride	0.003M
Potassium chloride	0.024M
Tris-HCl buffer, pH 7.6 at 20^{o}	0.1M

TYMV extractants:

Magnesium bentonite was prepared by the method of Dunn and Hitchborn (1965). Final concentration: 50 mg bentonite/ml. Stored at 5^{o}.

Disodium hydrogen phosphate Potassium dihydrogen phosphate	0.01M
Magnesium sulphate	0.005M

TYMV-RNA extractant: (Dunn and Hitchborn)

Sodium chloride	0.5M
Disodium EDTA	0.01M
Tris-HCl buffer, pH 7.6 at 20^{o}	0.5M

Sodium bentonite suspension was prepared by the method of Dunn and Hitchborn (1966). Final concentration approx. 26 mg/ml. Stored at 5^{o}.

Solutions required for Polyacrylamide gel electrophoresis:

Acrylamide and bisacrylamide were recrystallised by the method of Loening (1968).

2-5% stock acrylamide solution:

Recrystallised acrylamide	15 g
Recrystallised bisacrylamide	0.75g
Distilled water to	100 ml

Stock 5 x concentrated buffer, pH 7.6 - 7.7

Tris	21.8 g	= final running concn.	36 mM
$NaH_2PO_4 2H_2O$	23.4 g	= " " "	30 mM
Disodium EDTA $2H_2O$	1.85 g	= " " "	1 mM
Distilled water to	1 litre		

$[^{12}C]$ amino acid mixture:

Alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine were the 20 L- amino acids used in the preparation of the $[^{12}C]$ amino acid mixtures.

The solutions were made to 1 mM with respect to each amino acid. Whichever $[^{14}C]$ label was used, the equivalent $[^{12}C]$ amino acid was omitted from the mixture, thus for one $[^{14}C]$ label a 19 $[^{12}C]$ mix was prepared; for multi-$[^{14}C]$ labels a 6 $[^{12}C]$ mix was prepared, consisting of L-methionine, L-cysteine, L-glutamine, L-asparagine, L-histidine and L-tryptophan.

Methods

1. Determination of protein in TYMV-RNA preparations:

Protein was determined by the method of Bramhall, Noack, Wu and Loewenberg (1969). This method avoids RNA complications. Bovine serum albumin was used as reference standard. Ranges of 5-25 µg protein were stained with Xylene Brilliant Cyanin G and the absorbance was determined at 610nm on the Uvispek spectrophotometer (Hilger and Watts). Ranges of 50-200 µg protein were stained with Naphthalene blue black (Microme No. 1113) at 10 mg/ml in acetic acid:methanol:water (1:4:5) and the absorbance determined at 620nm.

2. Estimation of TYMV and RNA from absorbance measurements at 260nm:

All samples were scanned spectrophotometrically from 190nm - 400nm enabling various ratios to be calculated.

(A) TYMV. Determinations of virus concentrations assumed an extinction coefficient of 70 at $E^{1\%}_{1cm}$ (Goffeau and Bové, 1965) although numerous varying extinction coefficients appear to be used (Kaper and Litjens, 1966).

(B) TYMV-RNA. Determination of viral-RNA concentration assumed an extinction coefficient of 233±2 at $E^{1\%}_{1cm}$ (Haselkorn, 1962).

(C) tRNA. Determination of tRNA concentration assumed an extinction coefficient of 240 at $E^{1\%}_{1cm}$ (Yarwood, 1968).

(D) Microsomes. Determination of microsomal concentration assumed an extinction coefficient of 113 at $E^{1\%}_{1cm}$ (T'so and Vinograd, 1961).

3. Estimation of radioactivity in 5%(W/V) trichloroacetic acid insoluble (peptidyl) material (90°):

Incorporation of [^{14}C] amino acids into peptidyl material was determined by the method of Mans and Novelli (1960, 1961), using 3 MM Whatman 2.2 cm diam. filter-paper discs. The discs were dried with a Morphy-Richards hair dryer and washed by the method of Mans and Novelli (1961). The discs were placed in vials containing 10 ml scintillation fluid and the radioactivity estimated with a Beckman Liquid Scintillation counter. Quench curves, calculated from pipetting known amounts of [^{14}C] amino acids onto discs, indicated an approximate efficiency of 90%. All samples were counted to a confidence limit of ± 3%, a function of c.p.m. and time. External standards were recorded with every sample count.

Blank discs i.e. discs which had accompanied the sample discs through the washing procedure, were always included in sample counting at the rate of 10% sample total or 10, whichever was the less. Vials of scintillant were always counted before use and only those having values of between 50-60 c.p.m. were used.

4. Estimation of [^{14}C] aminoacylation of tRNA:

tRNA acylation by [^{14}C] amino acids was estimated as in 3, with the exception of the 90°, 5%(W/V) trichloroacetic acid wash.

5. Presentation of Results:

(A) Peptidyl incorporation: When a single [^{14}C] label

was used the results are expressed as picomol $[^{14}C]$ amino acid incorporated/mg microsomes, or as c.p.m./mg microsome. Relevant component quantities of the incubation are included.

(B) Acylations: Results are presented as c.p.m./mg tRNA or c.p.m./mg Viral-RNA. Component quantities are indicated.

6. Sterility precautions:

Washed glassware, except pipettes and volumetric flasks, was kept permanently in an oven at 160°. Pipettes (0.1 ml and 0.2 ml) were washed with detergent, rinsed in distilled water and dried in an oven at 60° for 2 hr. Distilled water, used in incubations and solutions, was autoclaved and stored at 4°. Volumetric glassware was autoclaved, dried and stored, sealed. Spatulas, forceps, etc. were burnt off with ethanol before use.

Essential components of the cell-free system were omitted as a monitoring control of microbial contamination effects.

7. Isolation of microsomes from imbibed seeds of Phaseolus aureus:

Phaseolus aureus seeds were imbibed as described in 'Biological Materials'. The beans were shaken in sterile distilled water to remove the testas, as far as possible, and dried on absorbent paper. The following procedure was carried out at 2-4°: 100 g beans were ground in a mortar with 75 ml microsomal extractant plus mercaptoethanol (0.035 ml mercaptoethanol : 100 ml microsomal extractant).

In preparative work, the centrifuges used were:

(i) MSE MISTRAL 4L (4L)

(ii) MSE HIGH SPEED 18 (H.S.18)

(iii) MSE SUPER SPEED 65 (S.S.65)

The brei was centrifuged at 2,300g* (4L) for 30 min. The supernatant was decanted through 8 layers of sterile gauze. The pelleted cell débris was reground with 25 ml microsomal extractant plus mercaptoethanol and centrifuged as before. The supernatant was filtered and pooled with the first supernatant obtained. The supernatant was centrifuged at 38,000g (H.S.18) for 15 min and the post-mitochondrial supernatant was decanted through sterile gauze and centrifuged at 205,000g (S.S.65) for 2 hr, to pellet the ribonucleoprotein particles. The high-speed supernatant was decanted and stored at -20° for dialysis. The centrifuge tubes were inverted to drain off remaining supernatant, the sides wiped carefully with absorbent tissue and the surface of each pellet washed with 1 ml microsomal resuspension medium. The ribonucleoprotein particles were resuspended in the microsomal resuspension medium at a concentration of 20 mg/ml microsomes but later in this work concentrations of 50 mg/ml microsomes were used. The yield of microsomes was of the order of 1 mg microsome/1 g imbibed beans.

* The calculation of the centrifugal field is based on the average radius of rotation of the column of liquid in the rotor tubes.

8. Dialysis of high-speed supernatant:

The following procedure was carried out at 2-4°
The high-speed supernatant was dialysed in Visking tubing against dialysis buffer. Five changes of buffer at hourly intervals were used at the rate of 100 ml buffer to 1 ml high-speed supernatant. The high-speed supernatant was centrifuged at 4,000g (4L) for 15 min at 4° to pellet globulins. The high-speed supernatant was tubed in 0.5 ml amounts in sterile tubes and stored at -70°

9. Extraction of transfer Ribonucleic Acids:

The following procedure was applied to imbibed Phaseolus aureus seeds, germinating Phaseolus aureus, germinating Vicia faba and developing Vicia faba. Imbibed Phaseolus aureus seeds (and imbibed Vicia faba seeds) gave tRNA which had very low acylation rates, consequently only germinating and developing material was used. The excised plumules or developing seeds (500-1000 g) were blended by a Townsend and Mercer macerator in 333 ml tRNA extractant : 666 ml 90% phenol (V/V), until a suitable homogenate was produced. The homogenate was stirred at room temperature for 60 min and then cooled to 5° The aqueous phase, phenol phase and cell débris were separated by centrifugation at 2,300g (4L) for 30 min at 4° The aqueous phase was pipetted off and centrifuged at 23,000g (H.S.18) for 30 min at 4° to complete separation of the phases. The aqueous phase was taken and adjusted to 0.1M-potassium acetate using 1M-potassium acetate, pH 6. Two vol. absolute ethanol (at -20°)

were added and the whole stored at -20° for at least 1 hr. The resulting precipitate was recovered by centrifugation at 2,300g (4L) for 15 min at 4° and the pelleted precipitate suspended in 100 ml 1M-NaCl, shaken for 30 min at 0°, when the low mol.wt. RNA was extracted. The suspension was centrifuged at 23,000g (H.S.18) for 45 min at 4° when the high mol.wt. RNA was pelleted. Supernatant was taken and precipitated with 2 vol. absolute ethanol (at -20°) and stored at - 20° for at least 1 hr. The precipitate was recovered by centrifugation at 2,300g (4L) for 15 min at 4° then dissolved in 1.8M-Tris-HCl buffer, pH 9 at 37° and incubated at 37° for 45 min, when the tRNA was deacylated (Mosteller, Culp and Hardesty, 1967). The solution was precipitated with 2 vol. absolute ethanol (at -20°) and stored at -20° for at least 1 hr. The precipitate was recovered by centrifugation at 2,300g (4L) for 15 min at 4° and the pelleted precipitate was evacuated and dissolved in 0.05M-Tris-HCl buffer, pH 7.6 at 20° Insoluble material was removed by centrifugation at 4,000g (4L) for 20 min and the supernatant chromatographed on a DEAE 52 column.

10. Preparation of DEAE 52 column:

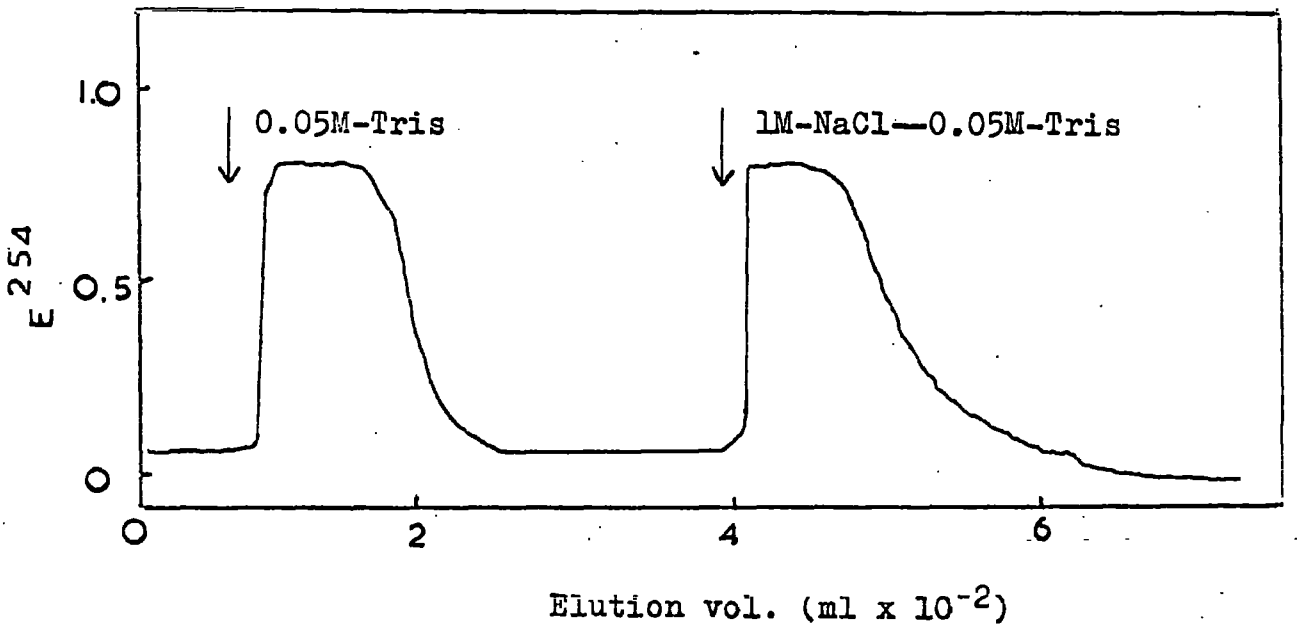
30 g Whatman preswollen DEAE 52 cellulose and 180 ml 0.05M-Tris-HCl buffer, pH 7.6 at 20° were stirred and the pH adjusted to 7.6 with 5N-HCl. The DEAE 52 settled and the supernatant was discarded. Tris-HCl buffer was added to original volume and stirred, and pH checked. This was repeated until pH 7.6 obtained. The suspension was poured into a 500 ml

measuring cylinder and the fines were decanted after 20 min. 0.05M-Tris-HCl buffer, pH 7.6 was added, equalling half the volume of slurry. The suspension was agitated and poured into a 1.5 cm diam. column. The excess buffer was run off the column, compacting the DEAE 52.

11. Chromatography of transfer RNA: (See FIG.1)

This procedure was carried out at 2-4^{o} using the method of Smith and Fowden (1968). tRNA in 0.05M-Tris-HCl buffer, pH 7.6 was absorbed onto the column, the column being connected to an ISCO model 222 ultra-violet 254nm absorbency analyser and a chart recorder. Step-wise elution was carried out using a first step of 0.05M-Tris-HCl buffer, pH 7.6 to remove extraneous material, and a second step of 1M-NaCl in 0.05M-Tris-HCl buffer, pH 7.6 to displace tRNA. The NaCl eluate was adjusted to 0.1M with respect to potassium acetate and precipitated with 2 vol. absolute ethanol (at -20^{o}) and stored at -20^{o} for at least 1 hr. The tRNA was recovered by centrifugation at 4,000g (4L) for 30 min at 4^{o} The pellet was evacuated and dissolved in a minimum volume of sterile distilled water. The material was stored in sterile tubes at -70^{o}. Spectrophotometry determined the tRNA concentration. The yields were not predictable. Germinated material yielded between 5-100 mg/kg of starting material. tRNA from germinating material had a characteristic pink colour from Phaseolus aureus and a variable intense blue colour from Vicia faba.

Fig.1. DEAE52 chromatography of tRNA from 50-day developing seed of V. faba.



This trace is qualitatively characteristic of all tRNA material processed in this investigation independent of source.

12. Extraction of Turnip Yellow Mosaic Virus:

TYMV was cultivated in Brassica pekinensis as described in 'Biological Material'. The method of extraction was that of Dunn and Hitchborn (1965). 100 g of leaf tissue were ground with 50 ml magnesium bentonite and 5 ml 0.1M-$MgSO_4$. The sap expressed from the macerate was centrifuged at 30,000g (S.S.65) for 20 min at 4^{o}. The supernatant was taken and centrifuged at 150,000g (S.S.65) for 1 hr at 4^{o}. The pellets from this centrifugation were suspended in 0.005M-$MgSO_4$—0.01M-phosphate, using a volume equal to approximately 10% that of the sap, and 0.1 ml bentonite preparation was added per 1 ml of suspension. The 30,000g centrifugation step was repeated to remove bentonite and the supernatant was centrifuged at 150,000g (S.S.65) for 1 hr at 4^{o} to pellet the virus. The viral pellet was stored at 4^{o} and RNA extraction was accomplished within 4 days' storage, in view of evidence that RNA degrades within stored virus (Haselkorn, 1962). The yield was estimated spectrophotometrically and was of the order of 1 mg virus/1 g infected leaves.

13. Extraction of RNA from Turnip Yellow Mosaic Virus:

Two methods were employed in the extraction of RNA from TYMV. The first method exactly followed Dunn and Hitchborn (1966) i.e. when TYMV is treated with 33% (V/V) ethanol in the presence of sodium chloride and at neutral pH, the protein is precipitated and the RNA remains in solution. Only small quantities of virus could be processed at one time and scaling-up of the technique led either to use of vast amounts of glassware and the

accompanying centrifugation problems, or to denaturing of the RNA product when larger volumes were processed in one vessel.

The second method was based on the phenol extraction procedures of Haselkorn (1962) and Fraenkel-Conrat (1969). The virus was dissolved in 0.01M-NaCl to give a 1-3 mg/ml concentration. This was gently shaken with an equal volume of 90% (V/V) redistilled phenol for 15 min at room temperature. After centrifugation at 4,000g (4L) for 20 min at 4° the aqueous layer was taken and added to an equal volume of 90% (V/V) phenol. Shaking and centrifugation were repeated. The aqueous layer was washed with several changes of ether until no opacity was apparent. For every 1 ml of solution 1 drop of 3M-sodium acetate was added, together with 2½ vol. absolute ethanol (at -20°). The whole was stored at -20° for 1 hr, when RNA precipitation was accomplished.

The precipitate was recovered by centrifugation at 4,000g (4L) for 20 min at 4°. The ethanol precipitation and centrifugation were repeated. The pelleted precipitate was evacuated and dissolved in 0.01M-NaCl (see 14). The yield of viral-RNA was estimated spectrophotometrically and was of the order of 20-25 mg/100 mg virus.

14. Viral-RNA Storage:

All viral-RNA preparations were stored at -20° in 0.3 ml quantities. In the first stages of the work, viral-RNA was stored in 0.01M-potassium acetate, pH 6, and later in 0.01M-Tris-HCl, pH 7.6 or 0.01M-NaCl. Viral-RNA was used once from

0.01M-potassium acetate or twice from salt or Tris buffer from the same storage tube. Control incubations without viral-RNA contained the same amount of "viral buffer" as viral-RNA experiments.

15. Polyacrylamide gel preparations:

Gels were prepared after the method of Loening (1968,i) Briefly, 5.0 ml stock acrylamide solution was mixed with stock buffer solution and water to give the desired gel concentration as follows:

Gel conc. %	2.0	2.2	2.4	2.6
Buffer (ml)	7.5	6.8	6.25	6.0
Water (ml)	24.7	22.0	19.7	17.8

The solution was degassed under vacuum for 15 sec and 25 μl of Temed (N.N.N'.N'. tetramethylethylenediamine) and 0.25 ml freshly dissolved 10% ammonium persulphate were added.

The conditions of electrophoresis followed Loening's method (1968,i), where operations were carried out at room temperature, using in the buffer compartments a five times dilution of the stock buffer solution, in which sodium dodecylsulphate was dissolved at 2 g/l. Gels were pre-electrophoresed at 5mA/tube 0.25 in diam. and 8V/cm length of gel, for 1 hr. RNA samples (up to 2 mg/ml) were dissolved in the stock buffer containing 5% sucrose and 0.2% (W/V) sodium dodecylsulphate material was layered on to gels (generally 2.2% and 2.4%) up to 50 μl. Gels were run for 2.5 - 3 hr at the current and voltage used in pre-electrophoresis. After running, gels were

soaked in stock buffer for 1 hr before scanning on the Joyce-Loebl chromoscan at 265nm using a 265nm interference filter with a medium pressure Hg lamp and a light path of 50-150μm x 1-2 mm. Molecular weights were calculated according to Loening (1968,ii,1969).

16. Sedimentation coefficients determined by analytical ultracentrifugation:

Two analytical ultracentrifuges were used. (i) Griffin-Christ Omega II with Schlieren optics, (ii) Beckman Spinco E with Schlieren optics. The details for operation of these machines are given in the Manuals supplied by the manufacturers.

Centrifugation Data:

	Omega II	Spinco E
Rev./min a)TYMV	35,000	30,000
b)RNA	—	60,000
Temperature ^{o}	20	25(RNA); 20(TYMV)
Cell.	16 mm, single sector	12 mm, single sector
Rotor	Omega 1 cell/1 ref.cell.	An/D
Photographed	Known intervals	Known intervals
Exposure	4 seconds	4 seconds
Bar angle ^{o}	70	

The calucation of Sedimentation coefficient(s') is based upon the proposition that $s' = \frac{1}{w^2 x} \cdot \frac{dx}{dt}$

where w = angular velocity of rotor in radians/second.

$= \frac{2\ \Pi\ \text{rev./min}}{60}$

and x = distance of boundary (particle) from rotor centre

$\frac{dx}{dt}$ = rate of change of x with respect to t (seconds)

The equation may be alternatively expressed (Schachman, 1959) as $s' = \frac{2(x_2 - x_1)}{(x_2 + x_1)\ w^2\ (t_2 - t_1)}$

where x_1 = position of boundary at time t_1 from rotor centre

x_2 = position of boundary at time t_2 from rotor centre

1 Svedberg unit = 10^{-13} seconds.

x_2 and x_1 are corrected according to photographic magnification values of the equipment.

TYMV, TYMV-RNA and E.coli RNA were centrifuged at various concentrations, details of which appear in the "Results" section. The buffer used was 0.1M-NaCl-0.02M-Tris-HCl, pH 7.5 at 20^{o}.

17. Incubations:

Thin-walled sterile 4 in long narrow-bore test tubes were used for all incubations. Incubation ingredients stored at 0^{o} and sub-0^{o} were thawed and maintained in an ice-bath. Incubation mixtures were prepared in tubes in the ice bath, and transferred to a constant temperature water bath, usually at 30^{o}, although this temperature was varied when necessary. Incubation tubes were gently agitated at short intervals and prior to aliquots being removed.

18. tRNA amino acyl acceptor capacity assay:

The capacity for amino acylation of tRNA, and therefore by definition activity of the high-speed supernatant dialysed enzyme fraction, were estimated in a system containing Tris-HCl buffer, $MgCl_2$, GSH, ATP, a $[^{14}C]$ amino acid, and a dialysed high-speed supernatant fraction with 1 mg tRNA/1 ml incubation.

Since the quantities of the ingredients vary considerably in the incubations, exact details are presented in legends and tables in "Results" section.

At various time intervals aliquots of the mixture were removed, placed on filter paper discs, and processed as described in (4).

19. Cell-free amino acid incorporation systems:

(A) The Complete system:

Incubations were of total volume 1 ml, 0.5 ml or 0.25 ml depending on requirements. Ingredients, quantities and reaction times vary in the incubations. Exact details are presented in

legends and tables in "Results" section.

The incubations contained: Tris-HCl buffer, $MgCl_2$, KCl, GSH, GTP, an energy system comprising ATP, PCK, CreP, a $[^{14}C]$ amino acid, a 19 $[^{12}C]$ amino acid mixture, a synthetic or viral message, if required, tRNA, microsomes at 1 mg/ml incubations and a high-speed supernatant dialysed fraction.

Aliquots removed were normally 0.05 ml/disc. The discs were dried, washed and counted as described in (3).

(B) The Transfer system:

The incubations contained Tris-HCl buffer, $MgCl_2$, KCl, GSH, GTP, tRNA previously charged with 1 $[^{14}C]$ amino acid + 19 $[^{12}C]$ amino acids, or tRNA previously charged with a 14 $[U^{14}C]$ amino acid mixture (CFB 104) + 6 $[^{12}C]$ amino acid mixture, a synthetic or viral message if required, and microsomes. Quantitative details of the incubation mixtures accompany the legends and tables in the "Results" section.

Aliquots were removed and processed as in (3).

20. Preparation of charged tRNA:

tRNA was aminoacylated, after the method of Ravel, Mosteller and Hardesty (1966). Single $[^{14}C]$ or multi $[^{14}C]$ amino acid labels were used to charge Vicia faba tRNA and yeast tRNA.

(A) Charging of Vicia faba tRNA with $[^{14}C]$ phenylalanine:

tRNA from 50 day old developing V. faba seed was used.

The optimal conditions for charging in a 1 ml incubation 1 mg V. faba tRNA with $[^{14}C]$ phenylalanine were:

100 µmol Tris-HCl buffer pH 7.8 at 30°, 20 µmol GSH, 2 µmol ATP, 16 µmol $MgCl_2$, a high-speed dialysed supernatant at optimal concentration (in this case 0.16 ml), 0.02 µmol [^{14}C] phenylalanine, 0.02 µmol of the [^{12}C] amino acid mixture, the whole to 1 ml sterile distilled water.

The 1 ml incubation was scaled up to 20 ml (viz. 20 mg tRNA) and incubated in a sterile conical flask for 20 min at 30°. At this time, an equal vol. of 90% (V/V) phenol was added. The flask was shaken for 15 min at room temperature and then cooled in ice for 1 hr, when a temperature of 5° obtained. The mixture was centrifuged at 4,000g (4L) for 30 min at 4°. The upper aqueous phase was taken, made 0.1M with respect to potassium acetate, 2 vol. absolute ethanol (at -20°) added, and the whole stored at -20° for at least 1 hr. The phenol phase was re-extracted with an equal vol. of 0.1M-potassium acetate, centrifuged and precipitated with absolute ethanol as before. The precipitates were recovered by centrifugation at 4,000g (4L) for 30 min at 4°.

Ethanol precipitations and centrifugations were repeated 3 more times and the final precipitate dissolved in water. The tRNA was dialysed in Visking tubing against 5 hourly changes of sterile distilled water at the rate of 200 ml water : 1 ml tRNA, the operations being carried out at 2-4°.

The tRNA was made 0.1M with respect to potassium acetate, 2 vol. absolute ethanol (at -20°) were added, and tRNA was precipitated by storing at -20° for at least 1 hr. The tRNA was recovered by centrifugation at 4,000g (4L) for 30 min at 4°.

Ethanol precipitations and centrifugations were repeated 3 more times and the final precipitate was dissolved in water. The tRNA was dialysed in Visking tubing against 5 hourly changes of sterile distilled water at the rate of 200 ml water : 1 ml tRNA, the operations being carried out at 2-4°.

The tRNA was made 0.1M with respect to potassium acetate and 2 vol. absolute ethanol (at -20°) were added, and tRNA was precipitated by storing at -20° for at least 1 hr. The tRNA was recovered by centrifugation at 4,000g (4L) for 30 min at 4°. The pellet was evacuated and dissolved in a minimum volume of sterile distilled water. The concentration of tRNA was determined spectrophotometrically and the level of charging determined by the scintillation counter. V. faba tRNA material charged to 35,00 c.p.m./mg tRNA.

(B) Charging of V. faba tRNA with $[U^{14}C]$ amino acid mixture:

tRNA from 50 day developing V.faba seed was used. The conditions and ingredients of the charging technique were almost identical to the single $[^{14}C]$ phenylalanine charging of (20A), except that the 19 $[^{12}C]$ amino acid mixture was replaced by the 6 $[^{12}C]$ amino acid mixture, and 1 ml of the CFB 104 $[U^{14}C]$ amino acid mixture/20 ml incubation replaced the $[^{14}C]$ phenylalanine.

V. faba tRNA material charged to a level of 450,000 c.p.m./ mg tRNA.

(C) Charging of Yeast tRNA with $[^{14}C]$ phenylalanine:

The procedure for charging Yeast tRNA was that described in (20A), except that the incubation time was 40 min at 37°.

Yeast tRNA charged to 24,000 c.p.m./mg tRNA.

(D) Charging of Yeast tRNA with [^{14}C] leucine:

The procedure was that described in (20C), except that [^{14}C] leucine was substituted for [^{14}C] phenylalanine, the 19 [^{12}C] amino acid mixture contained [^{12}C] phenylalanine as substitute for [^{12}C] leucine, and $MgCl_2$ was 12 mM.

Yeast tRNA charged to 27,000 c.p.m./mg tRNA.

(E) Charging of Yeast tRNA with [U^{14}C] amino acid mixture:

The procedure was that described in (20B), except that the incubation time was 45 min, and $MgCl_2$ was 12 mM.

Yeast tRNA charged to 250,000 c.p.m./mg tRNA.

21. The zero-time sample:

The zero-time sample represented the time required: (i) to remove an aliquot from the tube in the ice-bath by pipette; (ii) transfer the pipette to the disc; (iii) pipette the sample onto the disc; (iv) dry the disc; (v) place the disc in 10% (W/V) tricholoroacetic acid.

These operations take up to 45 sec to complete and in that time the temperature rises. Thus, zero-time sample is not necessarily a true representation of zero-time which implies zero temperature. In some instances, particularly plant tRNA acylations, the subtraction of zero-time figures would give erroneously low results. In such cases, the blank disc plus background counts were usually substituted for zero-time.

Results

1. Characterisation of TYMV and various RNA preparations

(A) Analytical ultracentrifugation:

The sedimentation coefficients of TYMV and TYMV top component (T) were calculated.

TYMV: $114.7s_{20\ BUFFER}$; fiducial limits of error, P = 95%: 95.2 - 104.8%

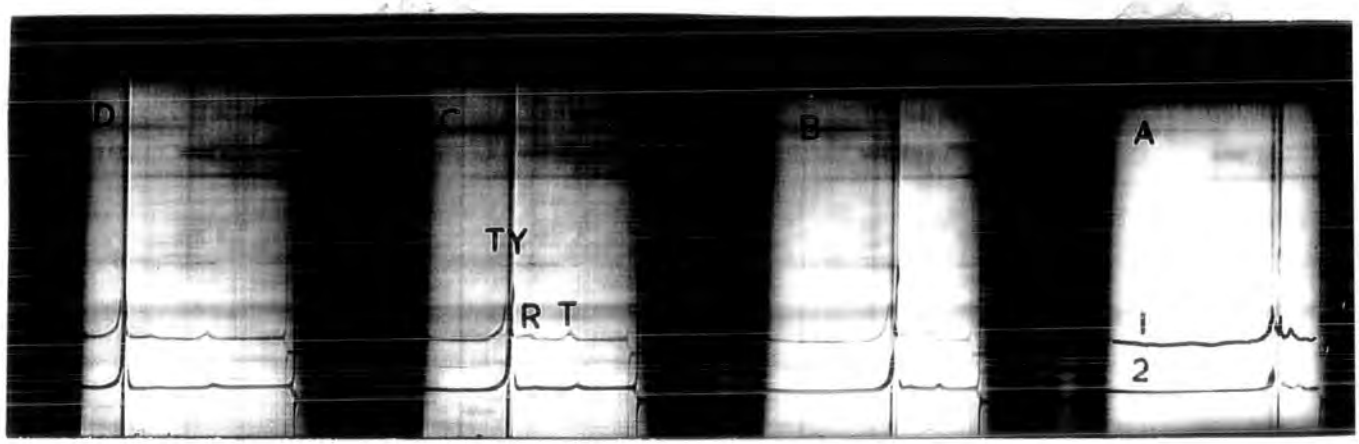
T : $52.1s_{20\ BUFFER}$; fiducial limits of error, P = 95%: 95.5 - 105.5%

Most of this investigation was carried out on the Omega II ultracentrifuge, situated in the Botany Dept., Univ. Durham, but the results were confirmed using the Spinco E of the Biochemistry Dept., Univ. Newcastle. (Plate 1) Analysis of the Schlieren optical film profiles showed that the extraction procedure of TYMV from Chinese cabbage allowed minimal contamination with plant ribosomal material.

Spinco E Schlieren profiles showed that TYMV-RNA prepared by the exact alcohol method of Dunn and Hitchborn (1965) was similar to that obtained by the phenol method of Haselkorn (1962). When the alcohol method was scaled up 20x severe degradation of that TYMV-RNA occurred. Phenol-extracted TYMV-RNA sedimented more quickly than E.coli total RNA. (Plates 2,3) It was concluded that the exact method of Dunn and Hitchborn must be followed to provide TYMV-RNA at least comparable with that RNA provided by the phenol method.

Measurements and observations were made from film negatives and not from the type of prints presented here, which are of poor quality.

Plate 1. Schlieren profiles of TYMV.

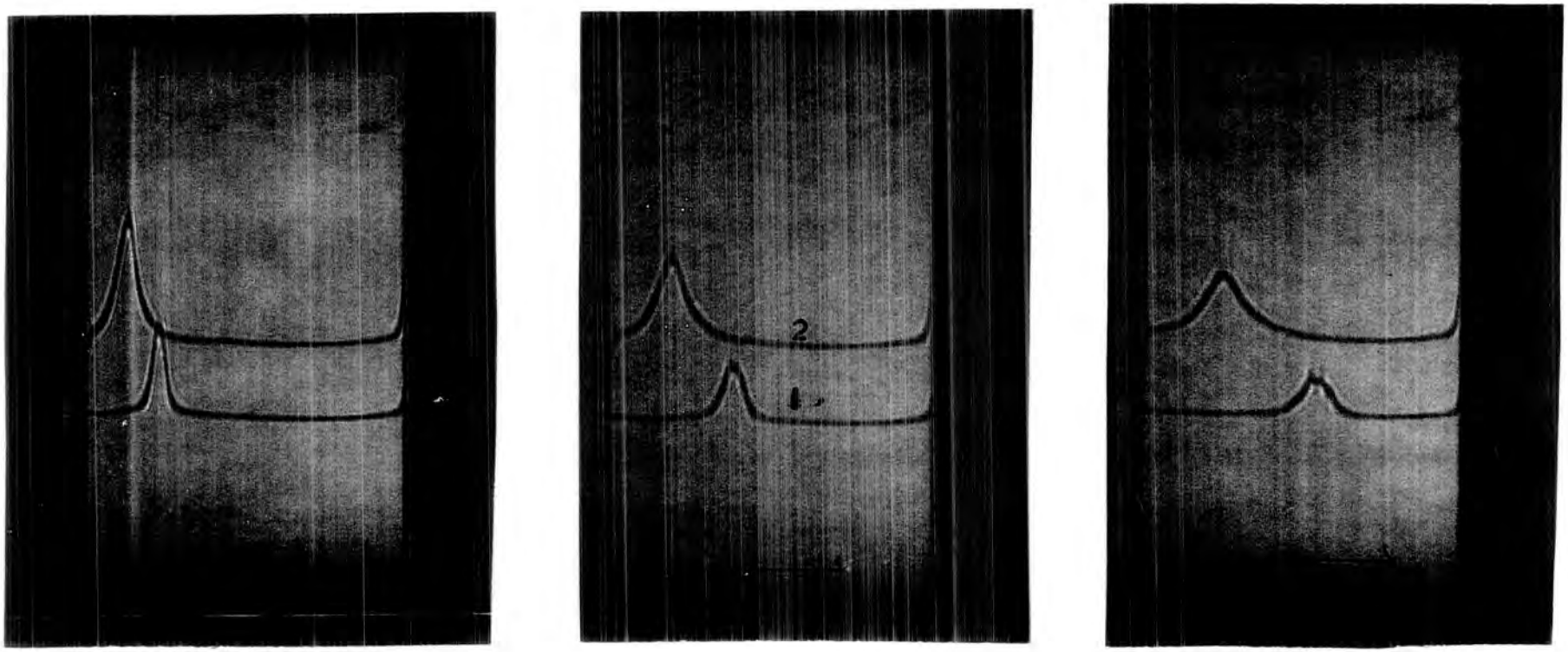


TY-TYMV; R-Ribosomal material; T-Top component.

Photographs were taken at (A) when Spinco E centrifuge had reached 30,000 rev./min, (B) after 4 min, (C) after 8 min, (D) after 16 min.

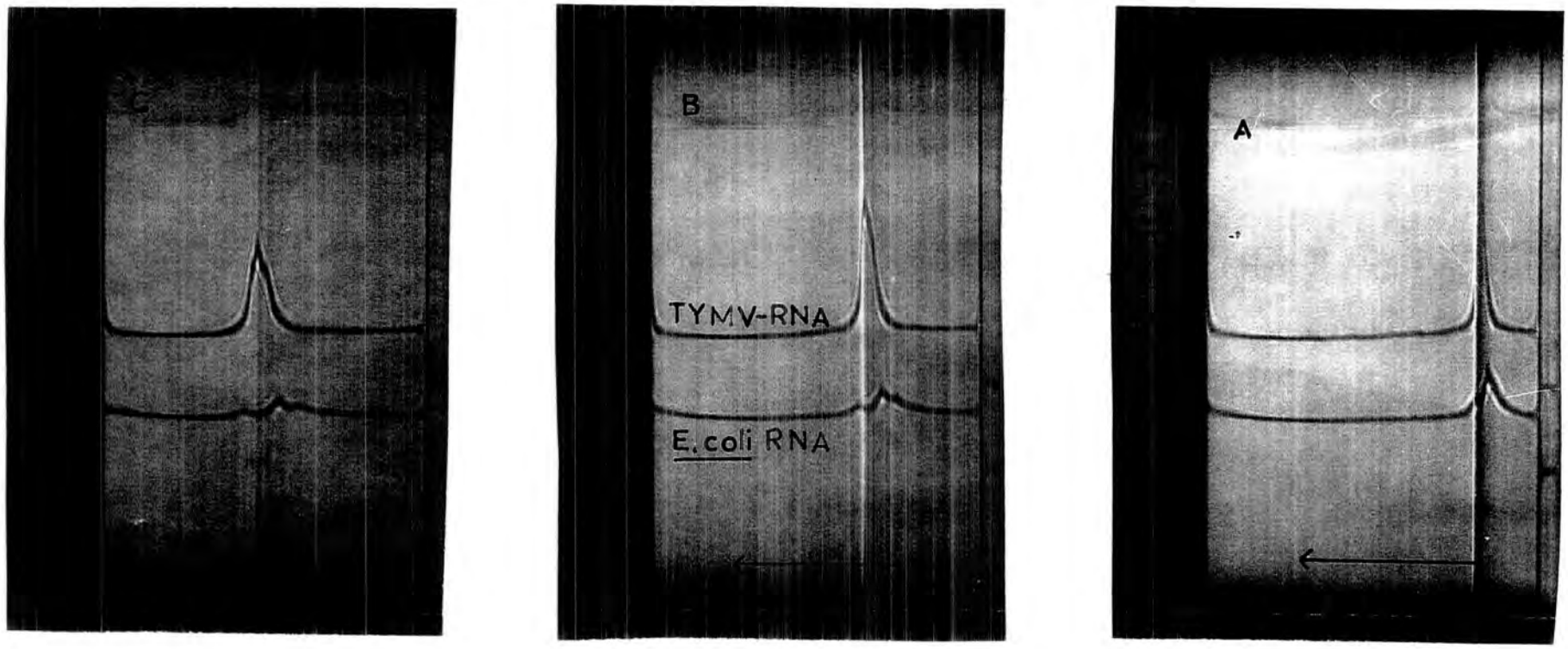
2 concentrations of TYMV were used. 1) 20 mg/ml, 2) 10 mg/ml.

Plate 2. Schlieren profiles TYMV-RNA (alcohol-extracted)



(1) TYMV-RNA extracted by exact method of Dunn and Hitchborn, (2) that method scaled up 20x. Photographs were taken at (A) when Spinco E centrifuge had reached 60,000 rev./min, (B) after 8 min, (C) after 16 min. Concentrations of TYMV-RNAs were 10 mg/ml.

Plate 3. Schlieren profiles TYMV-RNA (phenol-extracted)



Photographs were taken at (A) when Spinco E centrifuge had reached 60,000 rev./min, (B) after 8 min, (C) after 16 min. Concentrations of TYMV-RNA and E.coli RNA were 5 mg/ml.

(B) Polyacrylamide gel electrophoresis:

This technique allowed a calculation of the molecular weight of TYMV-RNA to be made, based upon the premise that the 23s and 16s components of E.coli RNA have a molecular weight of 1.07×10^{-6} daltons and 0.56×10^{-6} daltons respectively. The value for TYMV-RNA was 2.5×10^{-6} daltons (FIG.2).

The electrophoretic traces presented (FIGS.3,4) are of alcohol-extracted viral-RNA; phenol-extracted material was identical. Minimal viral-RNA degradation is indicated. The traces of Chinese cabbage RNA and TYMV-RNA + Chinese cabbage RNA indicate the resolution of this electrophoretic technique. Plant-and-viral RNAs were applied in rapid sequence to the gel and not mixed prior to admission. (FIGS.5,6)

(C) Determination of protein content of TYMV-RNA preparations:

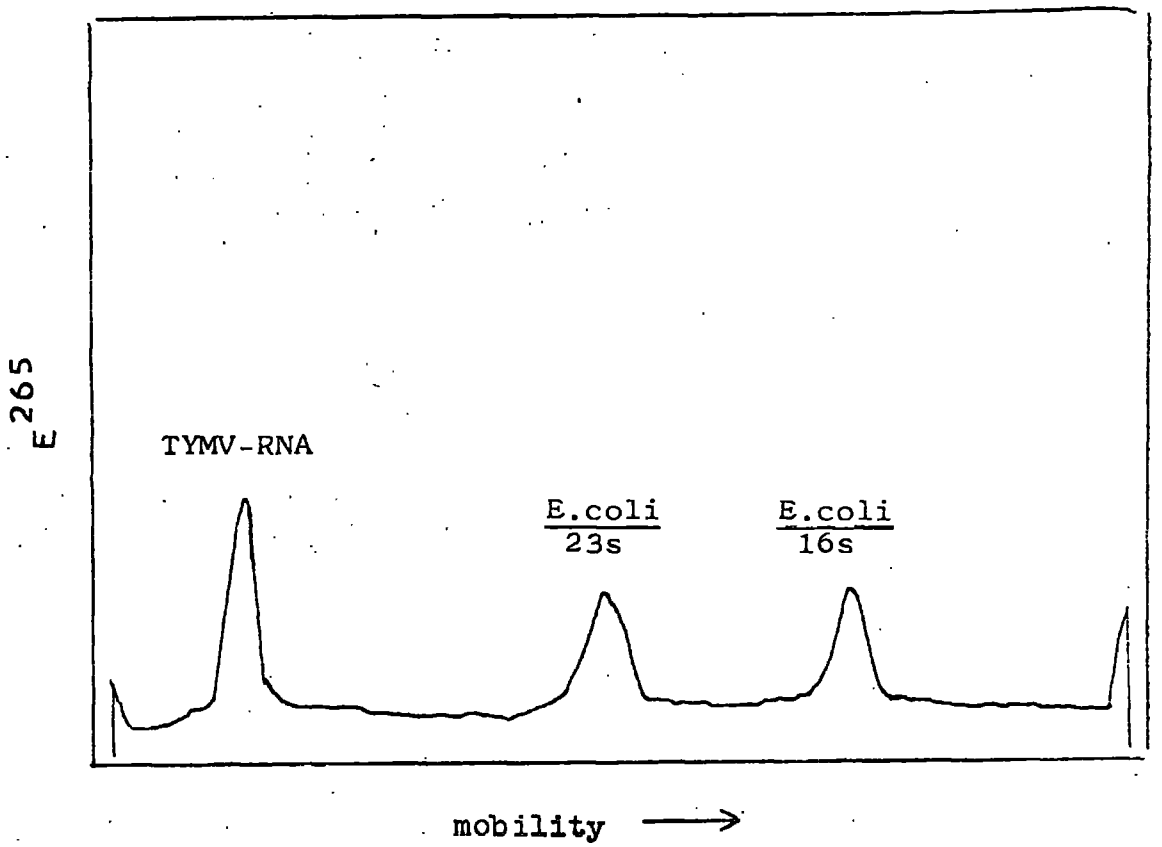
The protein content of TYMV-RNA preparations was estimated against the Xylene Brilliant Cyanin-Bovine Serum Albumin calibration curve (FIG.7). The Naphthalene Blue Black calibrated range (FIG.8) was not suitable for the low content of protein found in the viral-RNA preparations.

Values of 0.5 - 2% protein were determined in a series of viral-RNA preparations. Total RNA of E.coli and yeast had protein contents of 0.7% and 1.3% respectively.

(D) Spectrophotometry:

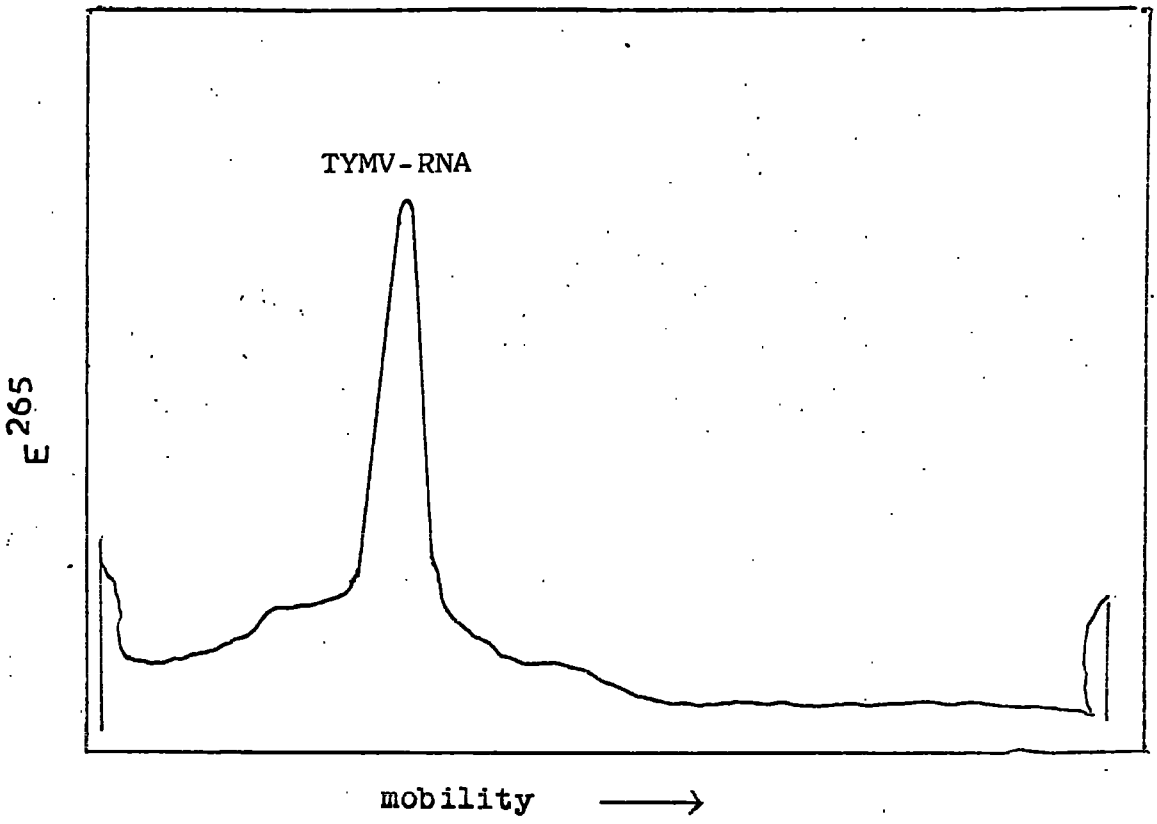
No differences were observed in the spectral characteristics

Fig.2. Polyacrylamide gel electrophoresis: TYMV-RNA and *E. coli* RNA.



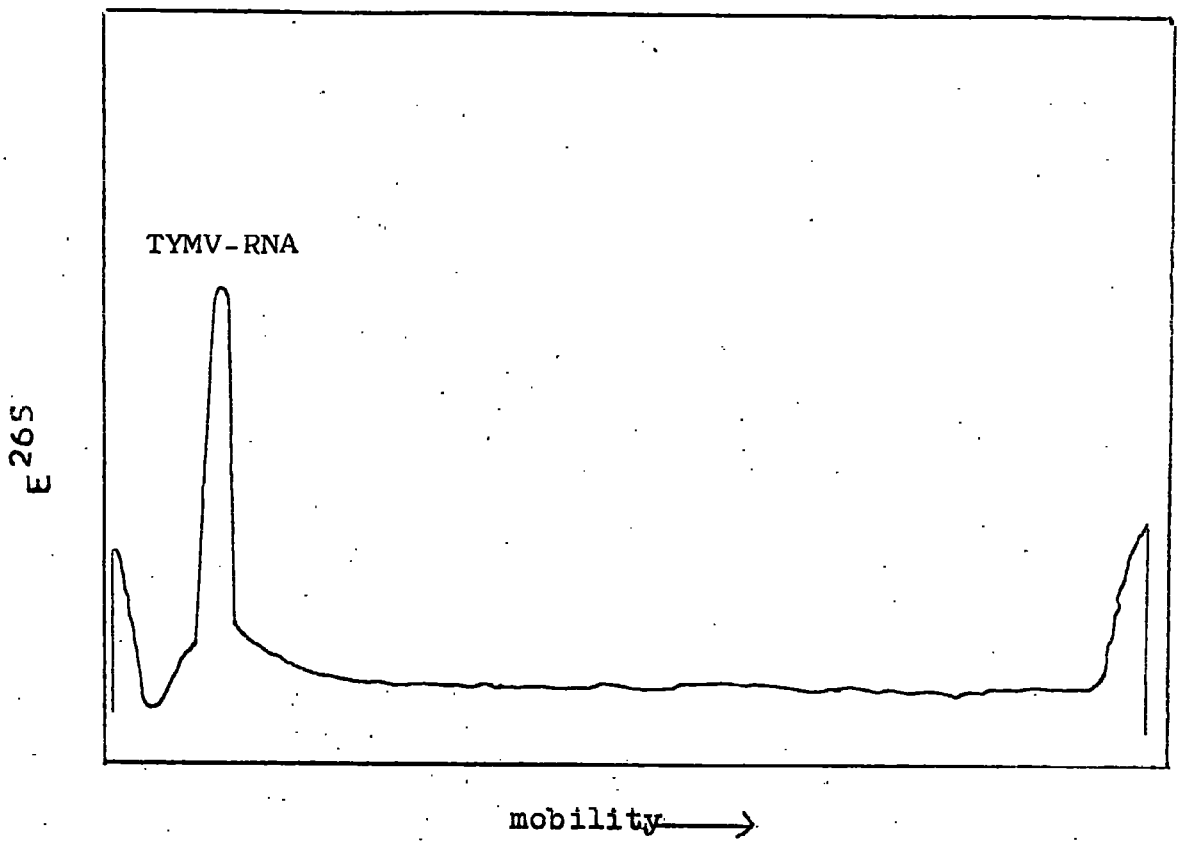
10 µl TYMV-RNA (1.0 mg/ml), 10 µl *E. coli* RNA (2 mg/ml) electrophoresed for 2.5 hr on 2.2% (w/v) gel.

Fig.3. Polyacrylamide gel electrophoresis: TYMV-RNA.



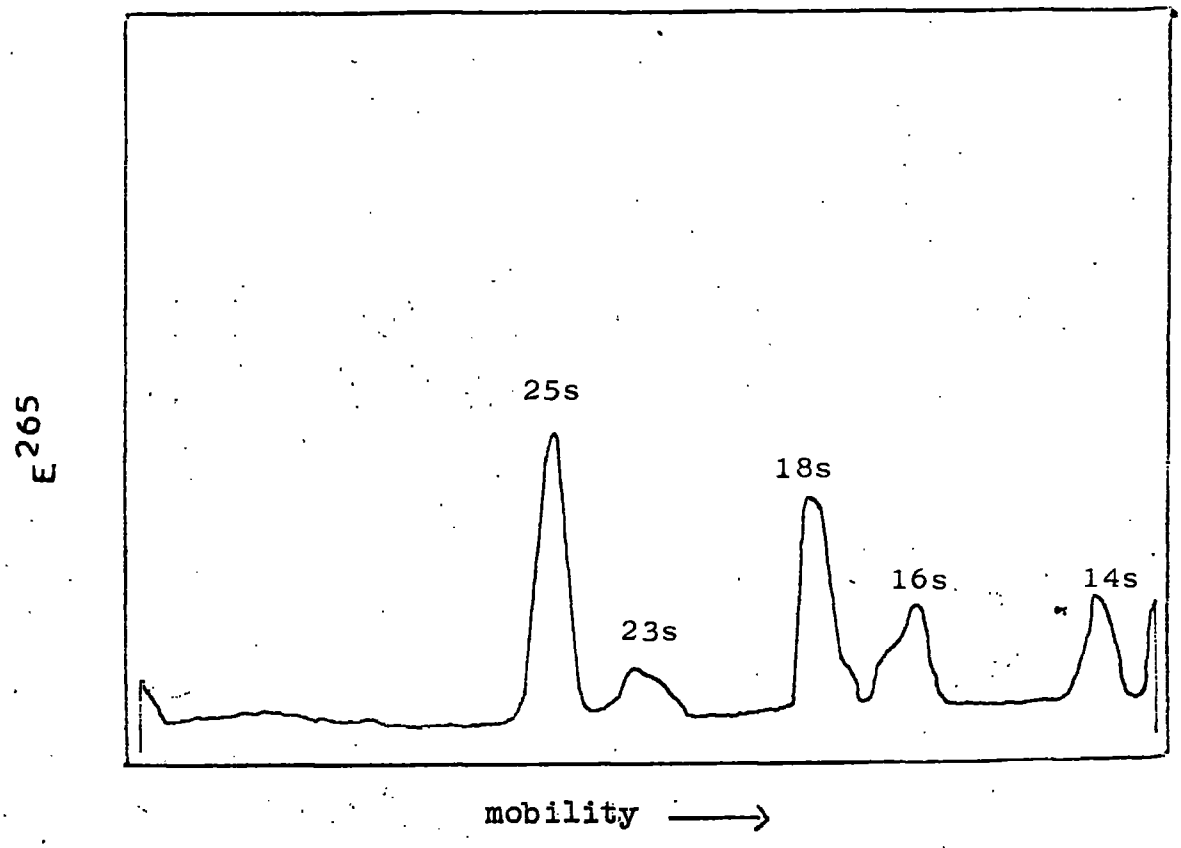
20 µl TYMV-RNA (1 mg/ml) electrophoresed for 3 hr on 2.2% (w/v) gel.

Fig.4. Polyacrylamide gel electrophoresis: TYMV-RNA



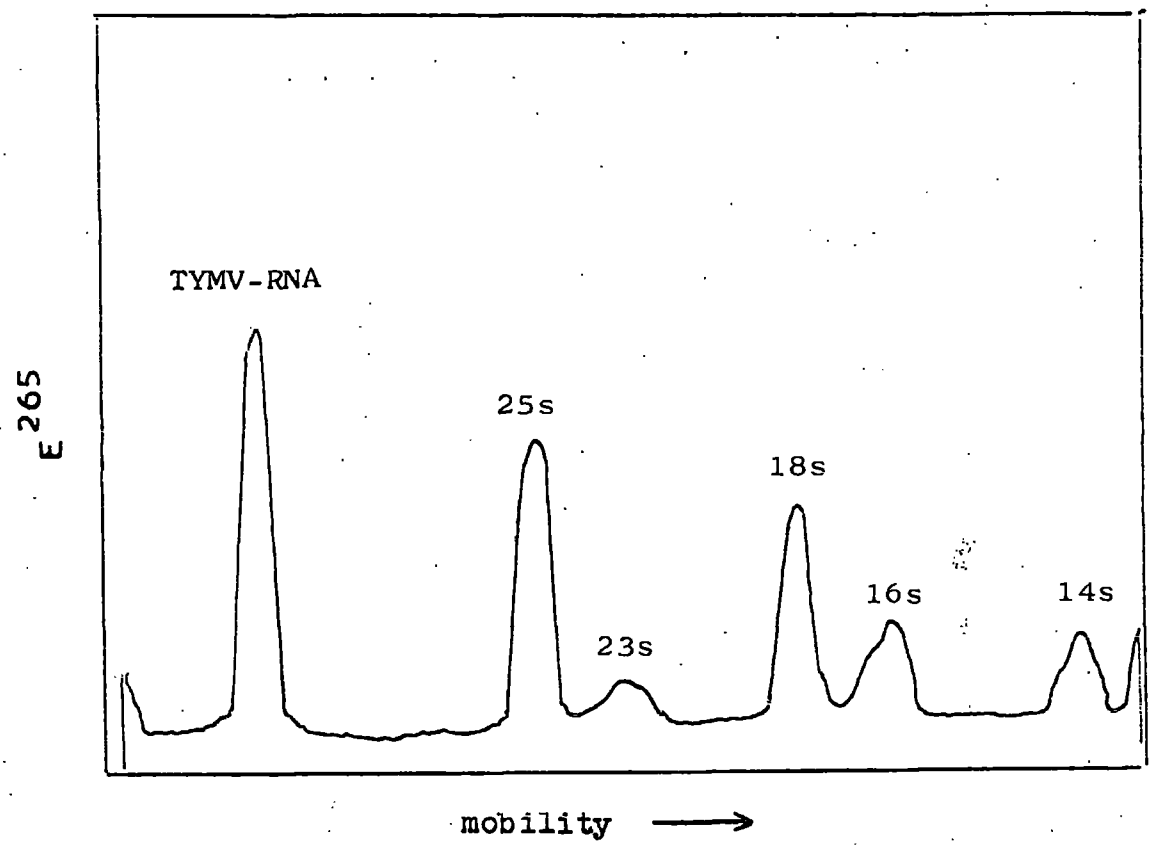
20 µl TYMV-RNA (1 mg/ml) electrophoresed for 3 hr on 2.4% (w/v) gel.

Fig.5. Polyacrylamide gel electrophoresis: Chinese Cabbage RNA.



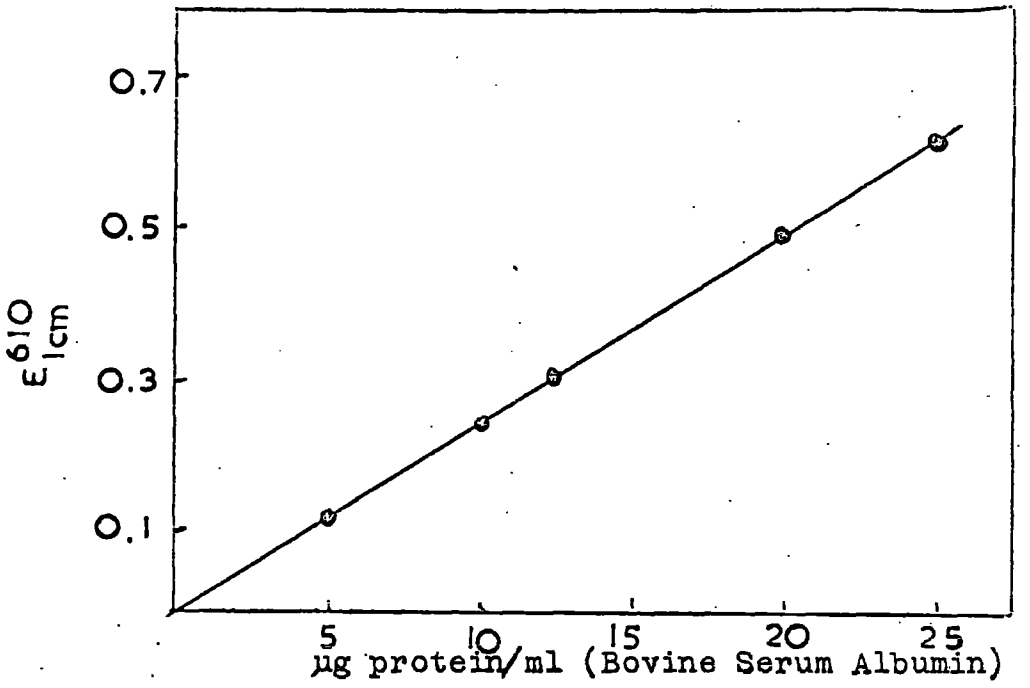
10 µl Chinese Cabbage RNA (1.5 ug/ml) electrophoresed for 2.5 hr in 2.2% (w/v) gel.

Fig.6. Polyacrylamide gel electrophoresis: TYMV-RNA and Chinese Cabbage RNA.



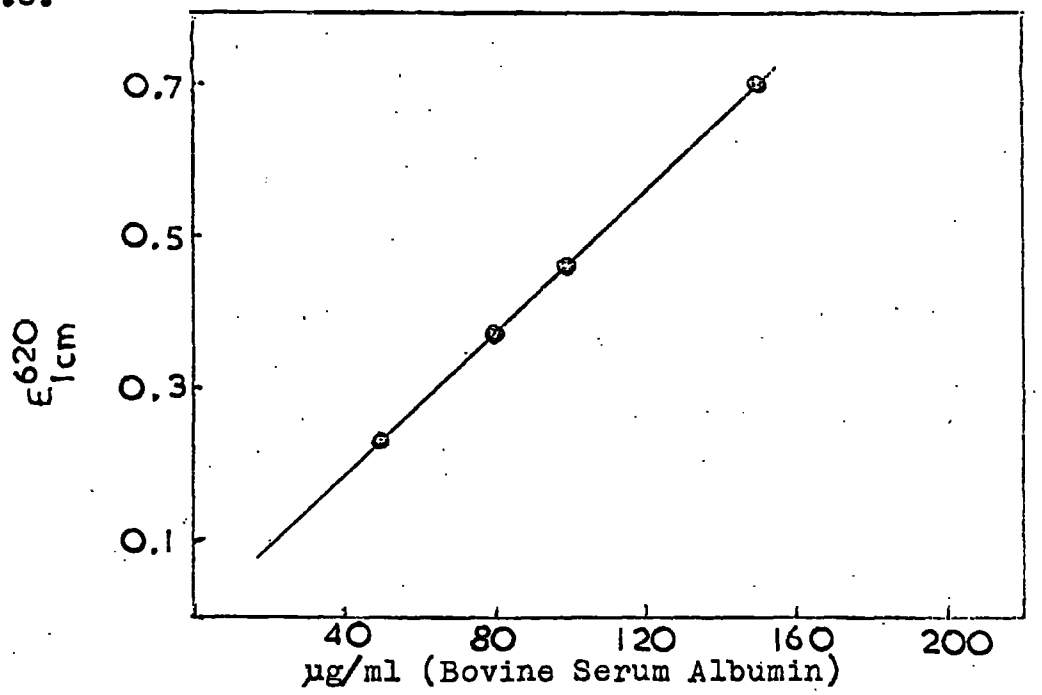
10 µl TYMV-RNA (1.5 ug/ml), 10 µl Chinese Cabbage RNA (1.5 mg/ml) electrophoresed for 2.5 hr on 2.2% (w/v) gel.

Fig.7. Calibration Graphs for Protein Content Determination of TYMV-RNA preparation (Bramhall et al., 1969)



Dye: Xylene Brilliant Cyanin.

Fig.8.



Dye: Naphthalene Blue Black.

of TYMV-RNA prepared by the two procedures (FIG.9). The characteristic 225-240nm absorption shift between TYMV and TYMV-RNA is shown in FIG.10.

The effect of DEAE 52 chromatography of tRNA from V.faba and P.aureus on the spectral characteristics, is shown in FIGS.11 and 12. Such traces were found to give no indication of the potential biological activity of the tRNA.

The necessity for dialysis of precharged tRNA for use in the Transfer System is shown in FIG.13. Non-dialysed material gave 260:280nm = 3-4. Dialysis resulted in a ratio much closer to that of the non-charged tRNA, i.e. 2.

FIGS.14 and 15 represent the typical absorption profiles of yeast tRNA and P.aureus microsomal suspension respectively.

2. Acylation of tRNAs

The maximum acylation rates in optimum conditions are given in FIGS.16, 17 and 18. Whilst P.aureus and V.faba* tRNAs were acylated by Val, Phe and Leu in 3-18 min, maximum acylation of yeast tRNA occurred after 40 min.

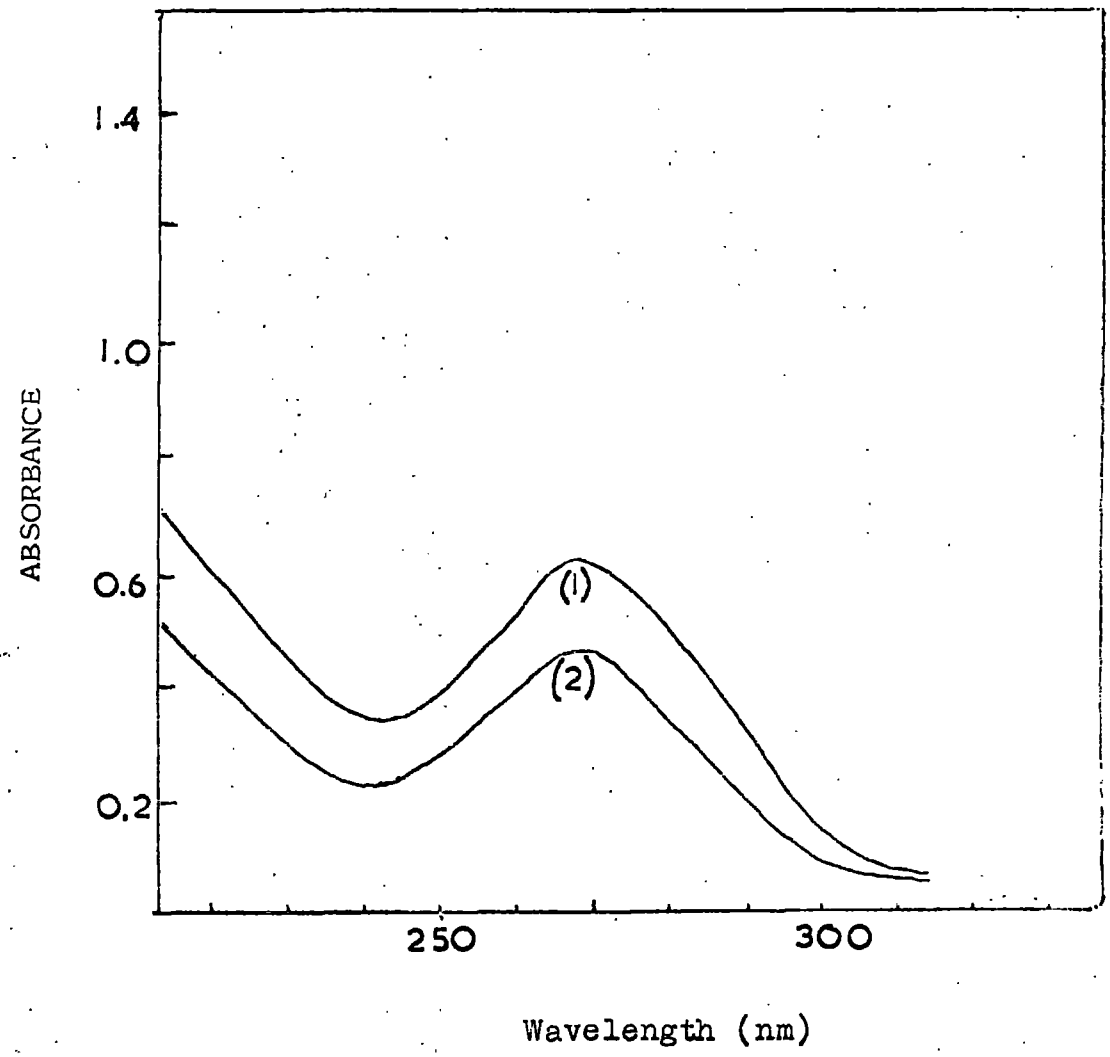
The effect of pH on this process was related rather to the source of tRNA than to individual amino acids (FIGS.19, 20,21) and although definite pH optima may be determined, there is a broad range of pH values giving satisfactory aminoacylation.

* V.faba tRNA is that material derived from 50-day developing seeds except if otherwise stated.

Fig.9. ABSORPTION SPECTRUM: TYMV-RNA:

(1) Ethanolic extraction

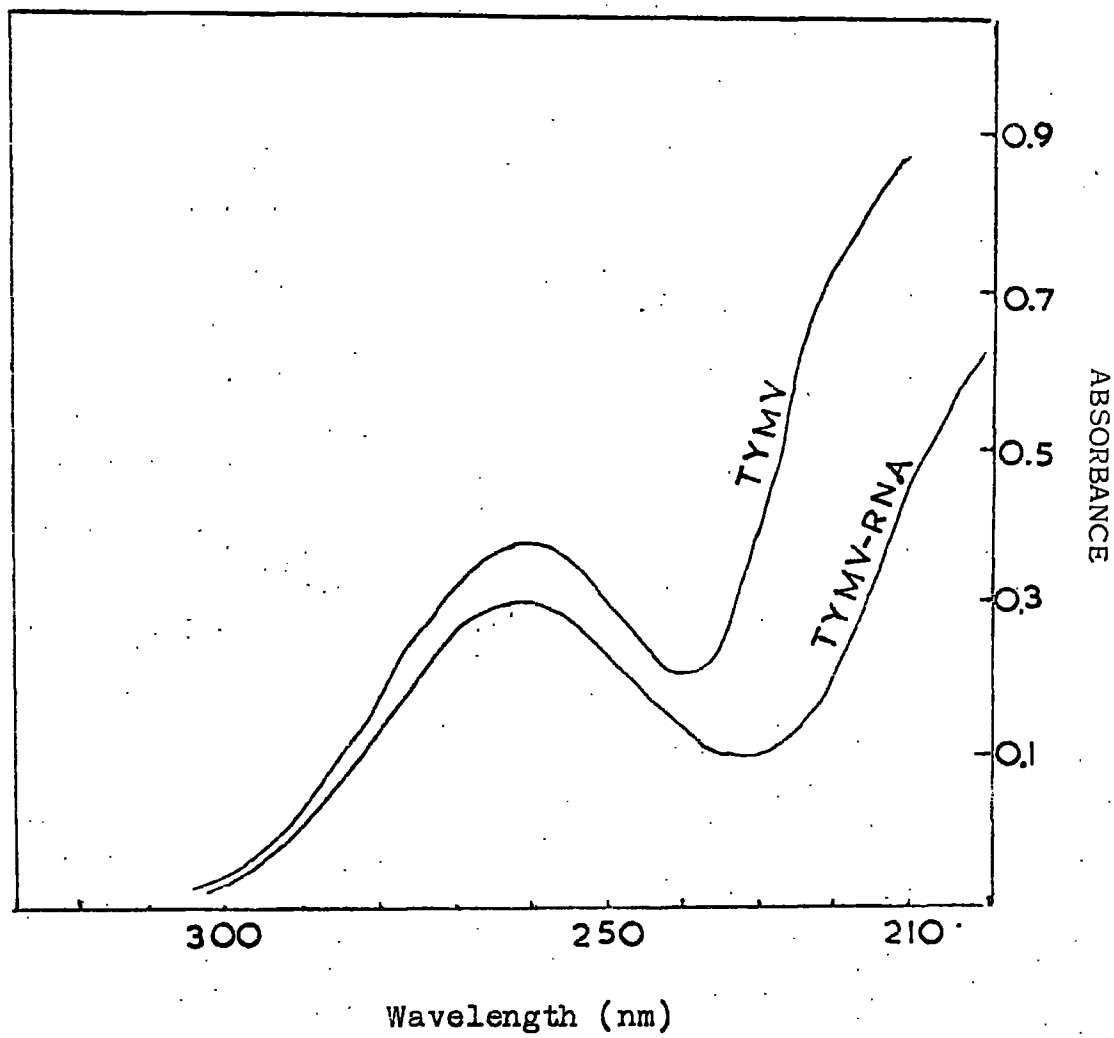
(2) Phenolic extraction



Determined in 0.01 Tris-HCl pH 7.8 at 30°, at room temperature.

Spectrophotometer: Hilger and Watts Ultrascan II.

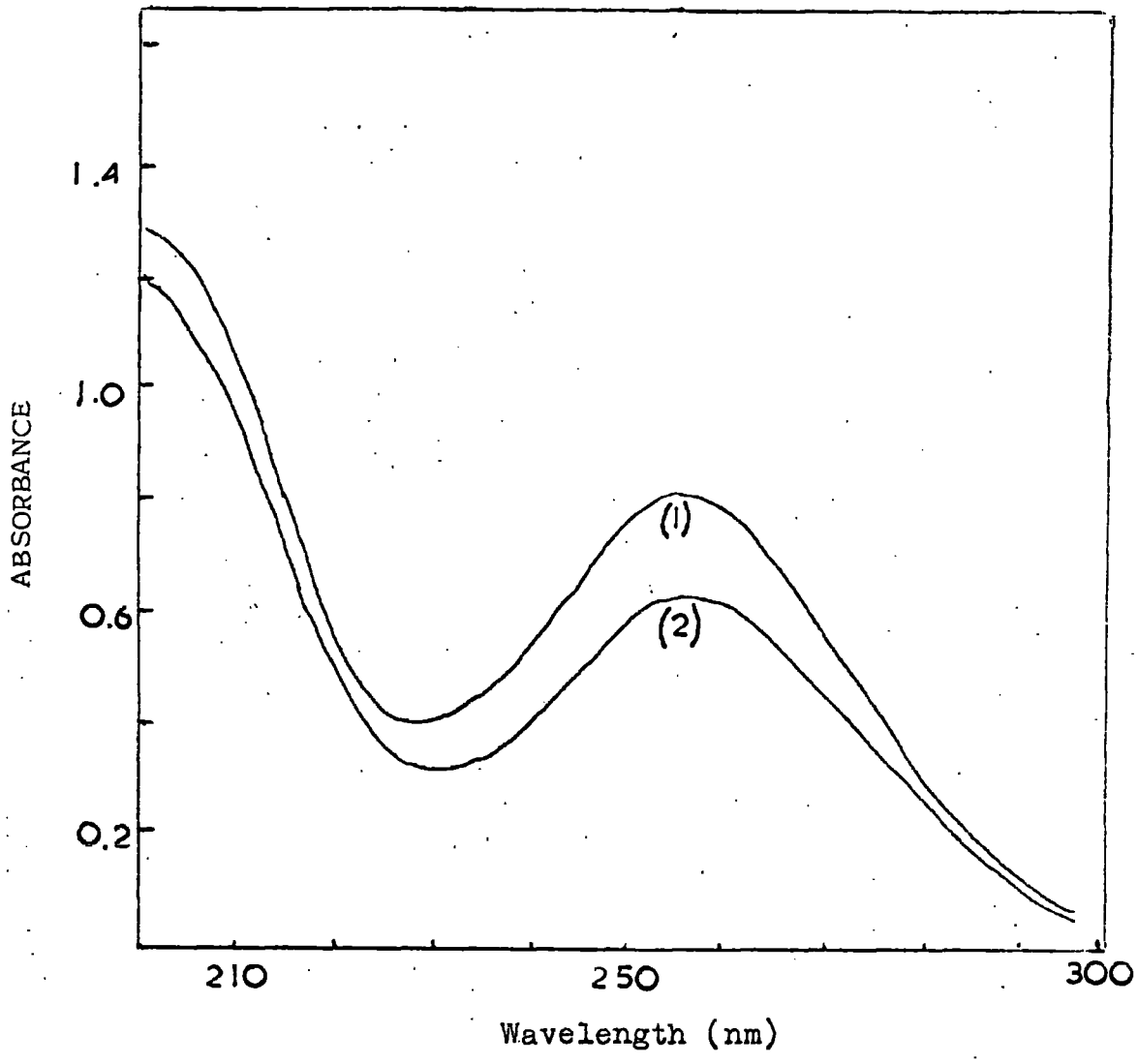
Fig.10. ABSORPTION SPECTRUM: TYMV and TYMV-RNA (phenolic extraction).



Determined in 0.01M-NaCl at room temperature.

Spectrophotometer: Perkin Elmer 402.

Fig. 11. ABSORPTION SPECTRUM: V. faba tRNA (germinating).



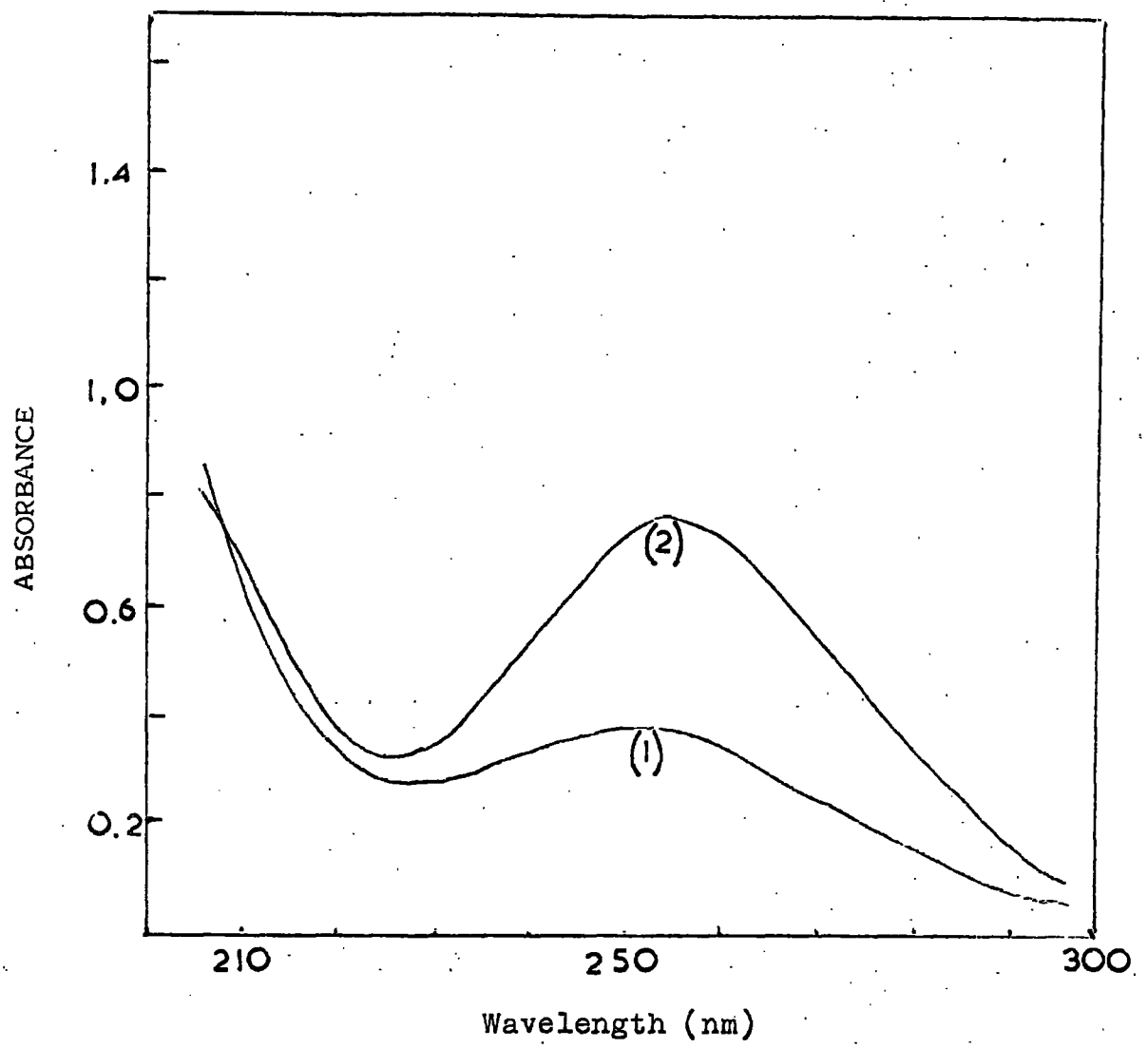
Determined in water at room temperature

(1) Before DEAE 52 chromatography

(2) After DEAE 52 chromatography

Spectrophotometer: Pye Unicam SP800.

Fig.12. ABSORPTION SPECTRUM: P. aureus tRNA.



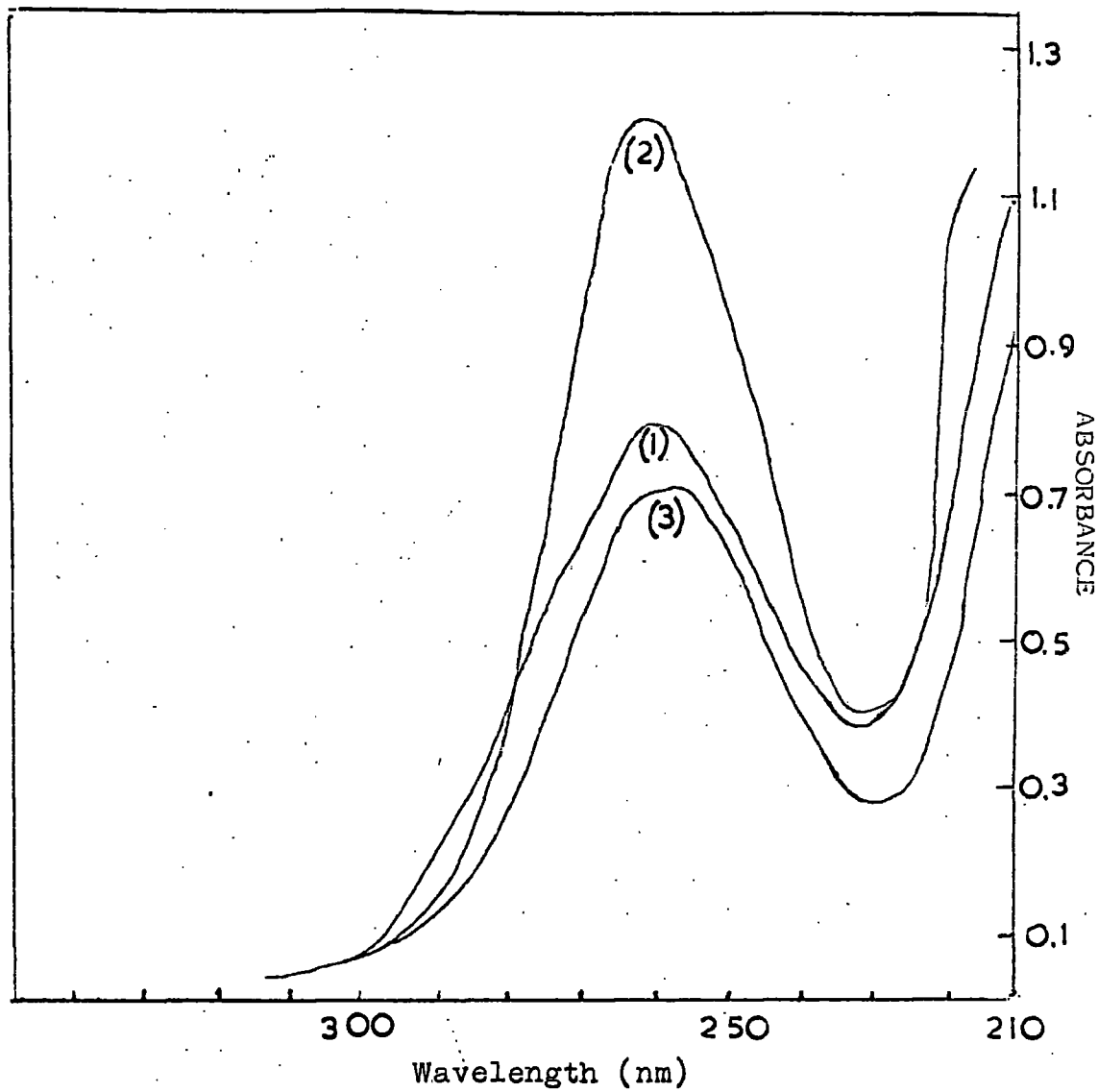
Dilutions determined in water at room temperature

(1) Before DEAE 52 chromatography.

(2) After DEAE 52 chromatography.

Spectrophotometer: Pye Unicam SP800.

Fig.13. ABSORPTION SPECTRUM: V. faba tRNA.



Determined in water at room temperature.

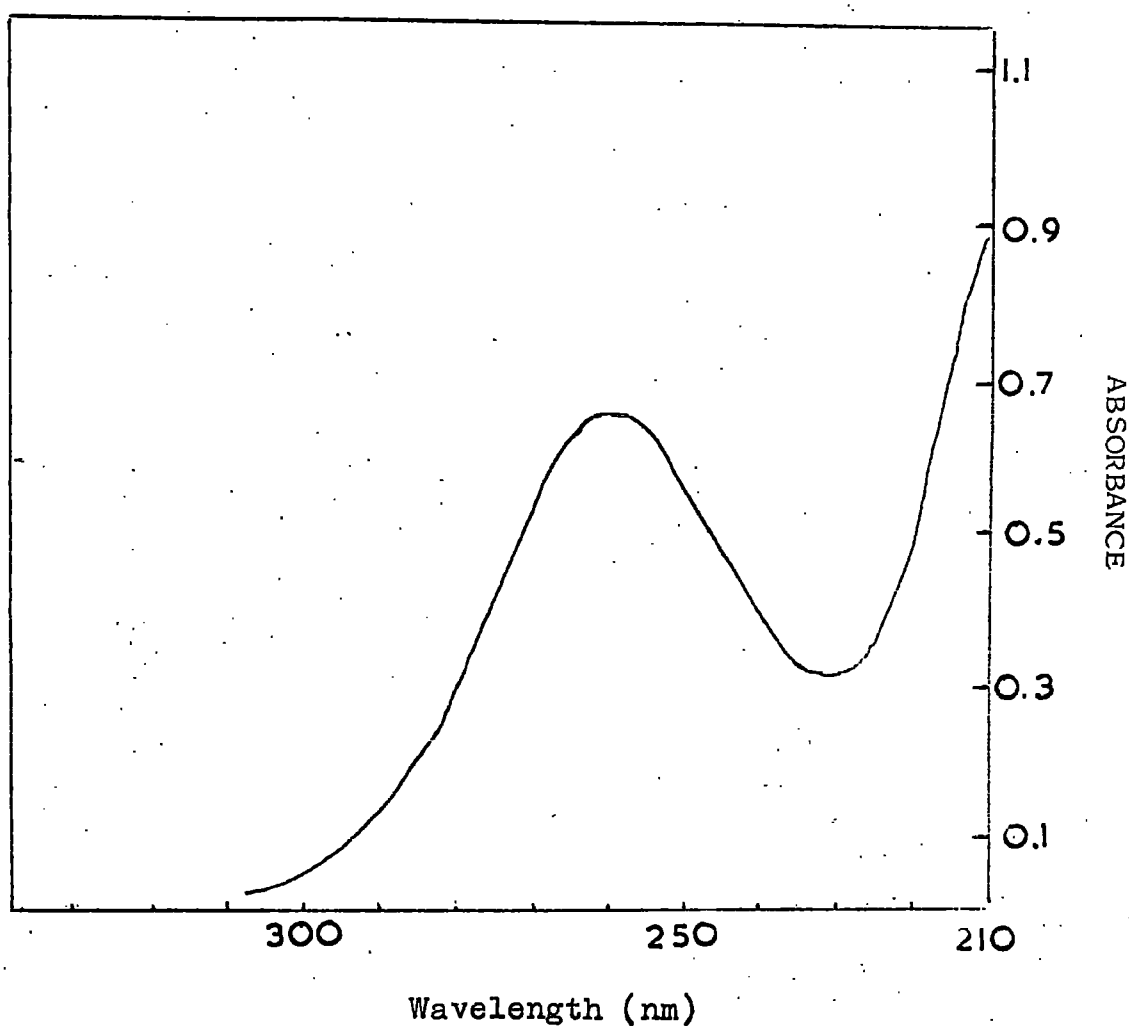
(1) V. faba tRNA (developing)

(2) V. faba tRNA acylated with 20 amino acids but not dialysed.

(3) V. faba tRNA acylated with 20 amino acids and then dialysed.

Spectrophotometer: Perkin Elmer 402.

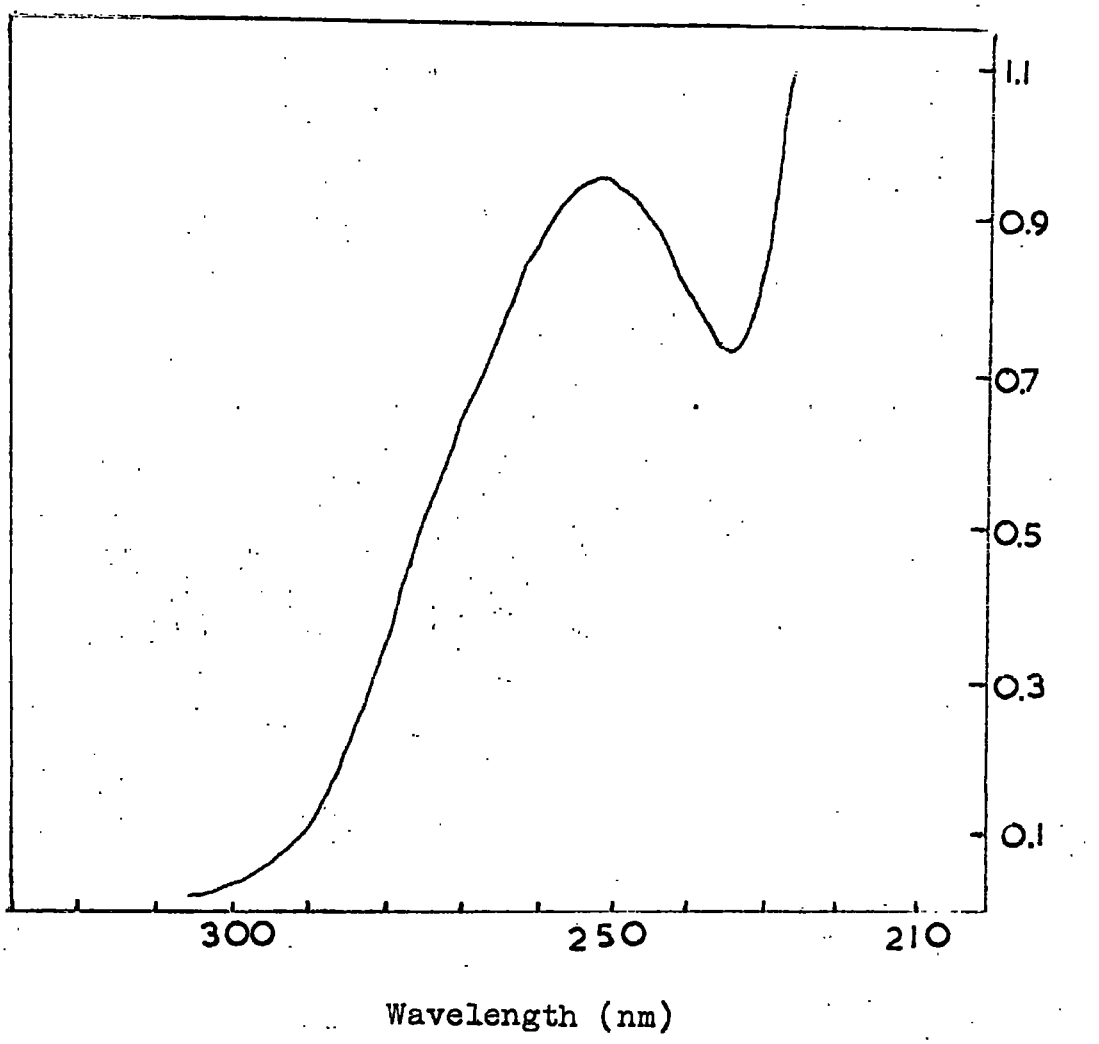
Fig.14. ABSORPTION SPECTRUM: Yeast tRNA



Determined in water at room temp.

Spectrophotometer: Perkin Elmer 402.

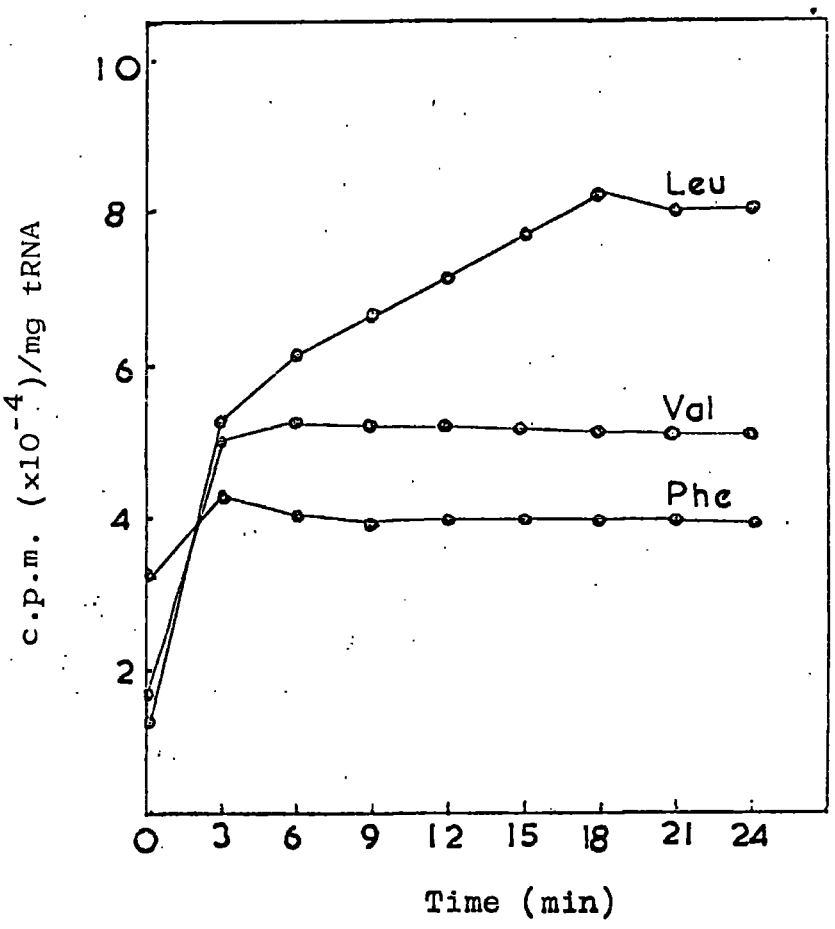
Fig.15. ABSORPTION SPECTRUM: P. aureus Microsomal preparation.



Determined in water at room temperature.

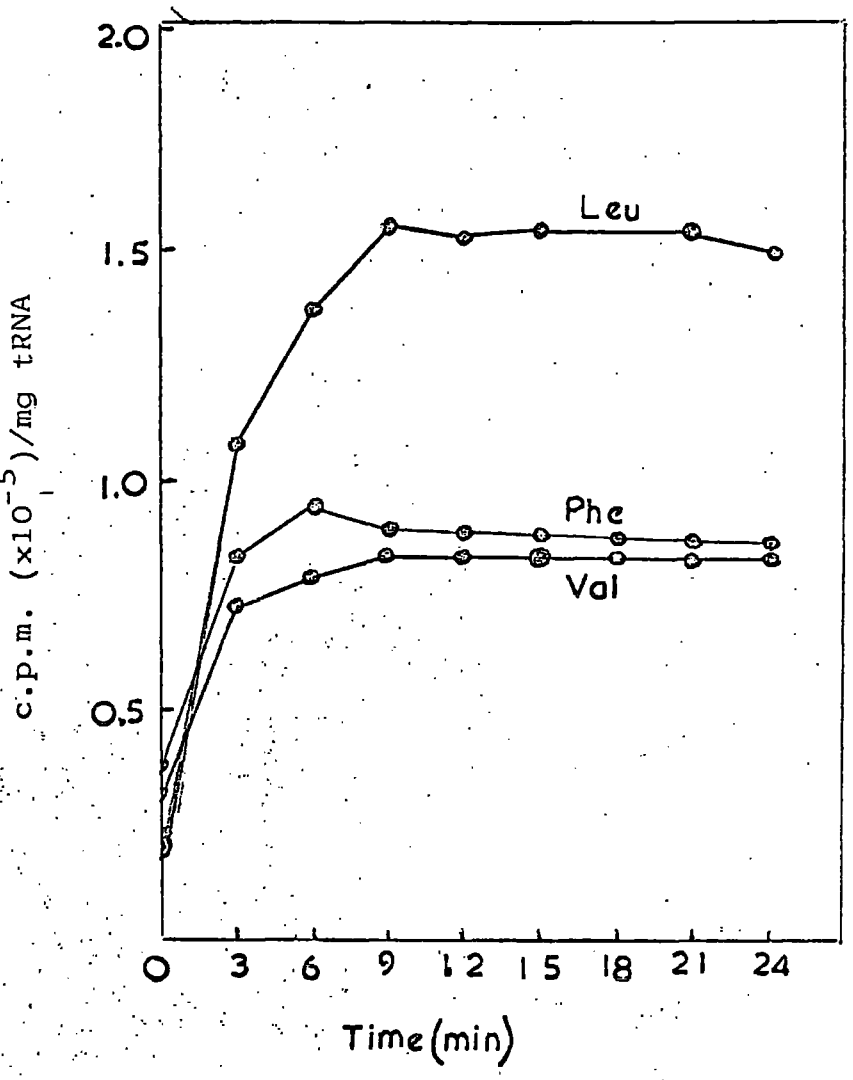
Spectrophotometer: Perkin Elmer 402.

Fig.16. Time courses of P. aureus tRNA acylation.



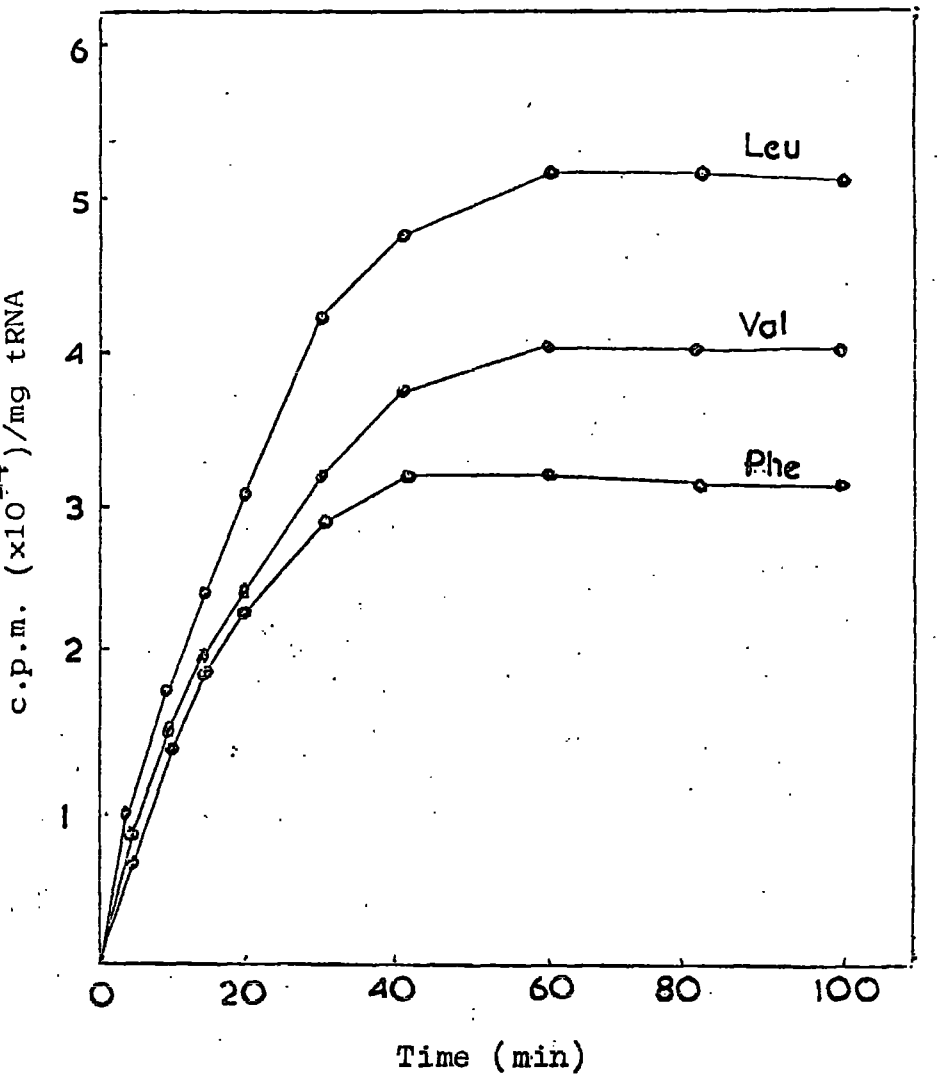
0.5 ml incubations contained: 50 µmol Tris-HCl pH 7.8 at 30°, 1 µmol ATP, 10 µmol GSH, 0.12 ml high-speed supernatant, 0.5 mg tRNA, and either 0.01 µmol [^{14}C] Phe with 3 µmol $MgCl_2$, or 0.01 µmol [^{14}C] Leu with 3 µmol $MgCl_2$, or 0.01 µmol [^{14}C] Val with 2 µmol $MgCl_2$. Incubation was at 30° and 0.05 ml samples assayed at times indicated. Radioactivity/disc determined as in Methods 4.

Fig.17. Time courses of V. faba tRNA acylation.



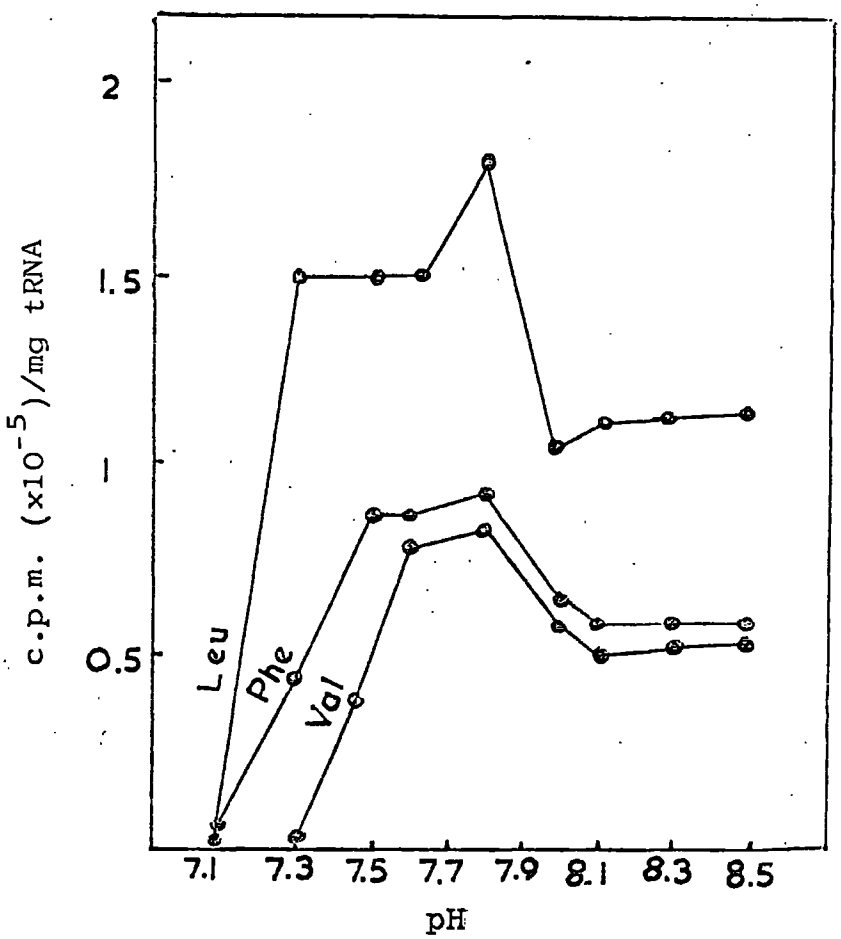
0.5 ml incubations contained: 50 µmol Tris-HCl pH 7.8 at 30°, 1 µmol ATP, 10 µmol GSH, 0.08 ml high-speed supernatant, 0.5 mg tRNA and either: 0.01 µmol [^{14}C] Leu with 3 µmol $MgCl_2$, or 0.01 µmol [^{14}C] Val with 3 µmol $MgCl_2$ or 0.01 µmol [^{14}C] Phe with 8 µmol $MgCl_2$. Incubation was at 30° and 0.05 ml samples assayed at times indicated. Radioactivity/disc determined as in Methods 4.

Fig.18. Time course of Yeast tRNA acylation.



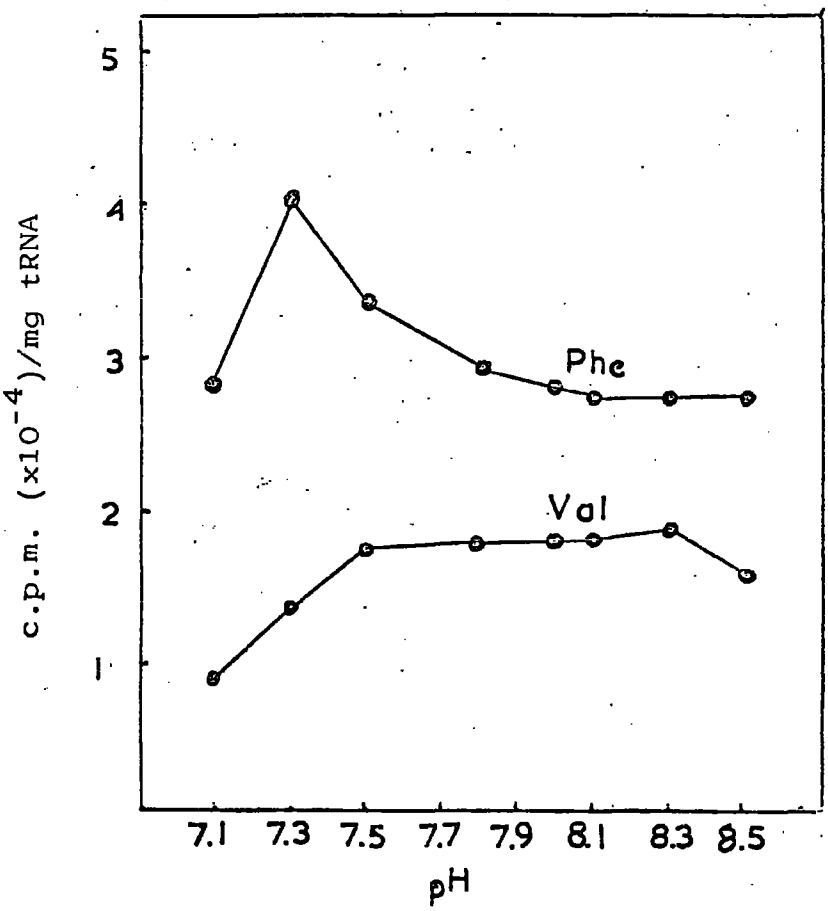
1 ml incubations contained: 100 µmol Tris-HCl pH 7.8 at 37°, 2 µmol ATP, 20 µmol GSH, 0.16 ml high-speed supernatant, 1 mg tRNA and either 0.02 µmol [^{14}C] Val and 4 µmol $MgCl_2$, or 0.02 µmol [^{14}C] Leu and 12 µmol $MgCl_2$ or 0.02 µmol [^{14}C] Phe and 15 µmol $MgCl_2$. Incubation was at 37° and 0.1 ml samples assayed at time intervals indicated. Radioactivity/disc determined as in Methods 4.

Fig.19. Effect of pH on V. faba tRNA acylation.



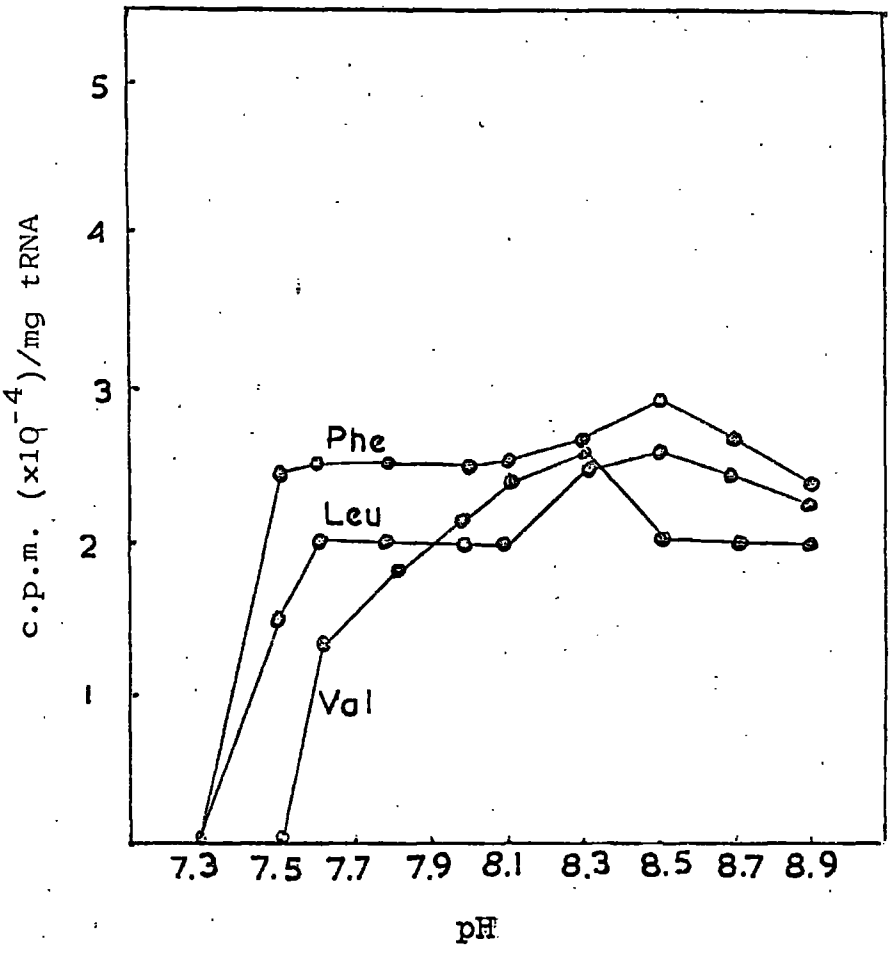
0.25 ml incubations contained: 25 µmol Tris-HCl at varying pH at 30°, 0.5 µmol ATP, 5 µmol GSH, 0.05 ml high-speed supernatant, 0.25 mg tRNA, either 0.005 µmol [^{14}C] Val with 0.15 µmoles $MgCl_2$, or 0.005 µmol [^{14}C] Phe with 4 µmol $MgCl_2$, or 0.005 µmol [^{14}C] Leu with 0.15 µmol $MgCl_2$. Incubations were at 30° and 0.05 ml samples assayed at 0, 15, 30, 45 min. Radioactivity/dise determined as in Methods 4. 15 min assays are reported.

Fig.20. Effect of pH on P. aureus tRNA acylation.



0.25 ml incubations contained: 25 µmol Tris-HCl at varying pH at 30°, 0.5 µmol ATP, 5 µmol GSH, 0.06 ml high-speed supernatant, 0.25 mg tRNA either [^{14}C] Val and 1 µmol $MgCl_2$, or [^{14}C] Phe and 2.5 µmol $MgCl_2$. Incubation was at 30° and 0.05 ml samples were assayed at 0, 15, 30, 45 min. Radioactivity/disc determined as in Methods 4. 15 min assays are reported.

Fig.21. Effect of pH on Yeast tRNA acylation.



0.25 ml incubations contained: 25 µmol Tris-HCl at varying pH at 30°, 0.5 µmol ATP, 5 µmol GSH, 0.04 ml high-speed supernatant, 0.25 mg tRNA; either 0.005 µmol [^{14}C] Val and 1 µmol $MgCl_2$; or 0.005 µmol [^{14}C] Leu and 3 µmol $MgCl_2$; or 0.005 µmol [^{14}C] Phe and 4 µmol $MgCl_2$. Incubation was at 30° and 0.05 ml samples assayed at 0, 15, 30, 45 min. Radioactivity/disc determined as in Methods 4. 30 min assays are reported.

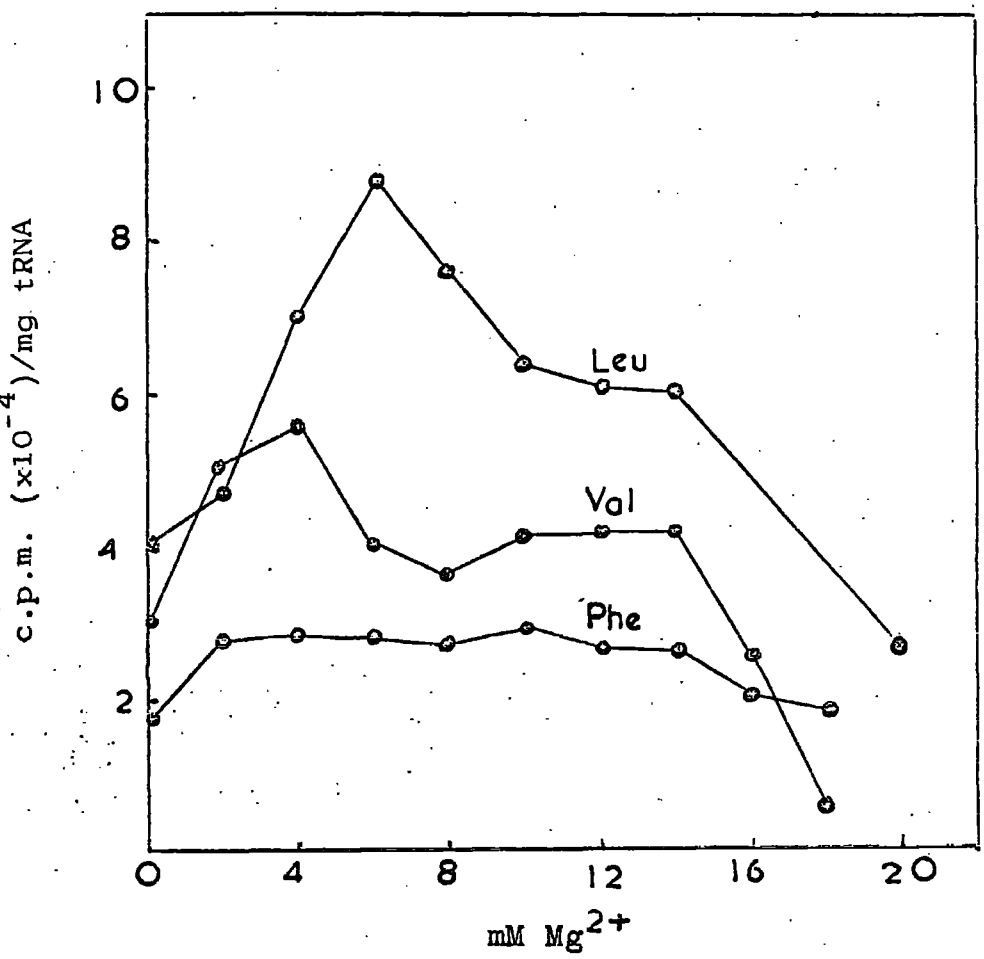
Mg^{2+} concentration optima for acylation by Val of the 3 tRNAs was almost the same, i.e. 4mM. V.faba tRNA and P.aureus tRNA were similar in showing no sharp Mg^{2+} optimum for Phe esterification, whereas yeast tRNA had a marked requirement of 15mM for this amino acid. The Mg^{2+} requirements for Leu esterification were different for each tRNA (FIGS.22, 23,24).

FIG.25 shows the concentration effects of the high-speed supernatant enzyme fraction in tRNA acylation by Val. No sharp optimum is apparent and the results given in this graph could also represent the concentration effects when Leu and Phe were used. All preparations of the high-speed supernatant during this investigation showed this characteristic absence of sharp concentration optimum.

FIG.26 shows the rates and amounts of acylation achieved by yeast tRNA in relation to temperature and time. The rate of acylation increased at higher temperature although at 45^{o} and 37^{o} there appeared to be some breakdown of the reaction after 40 min.

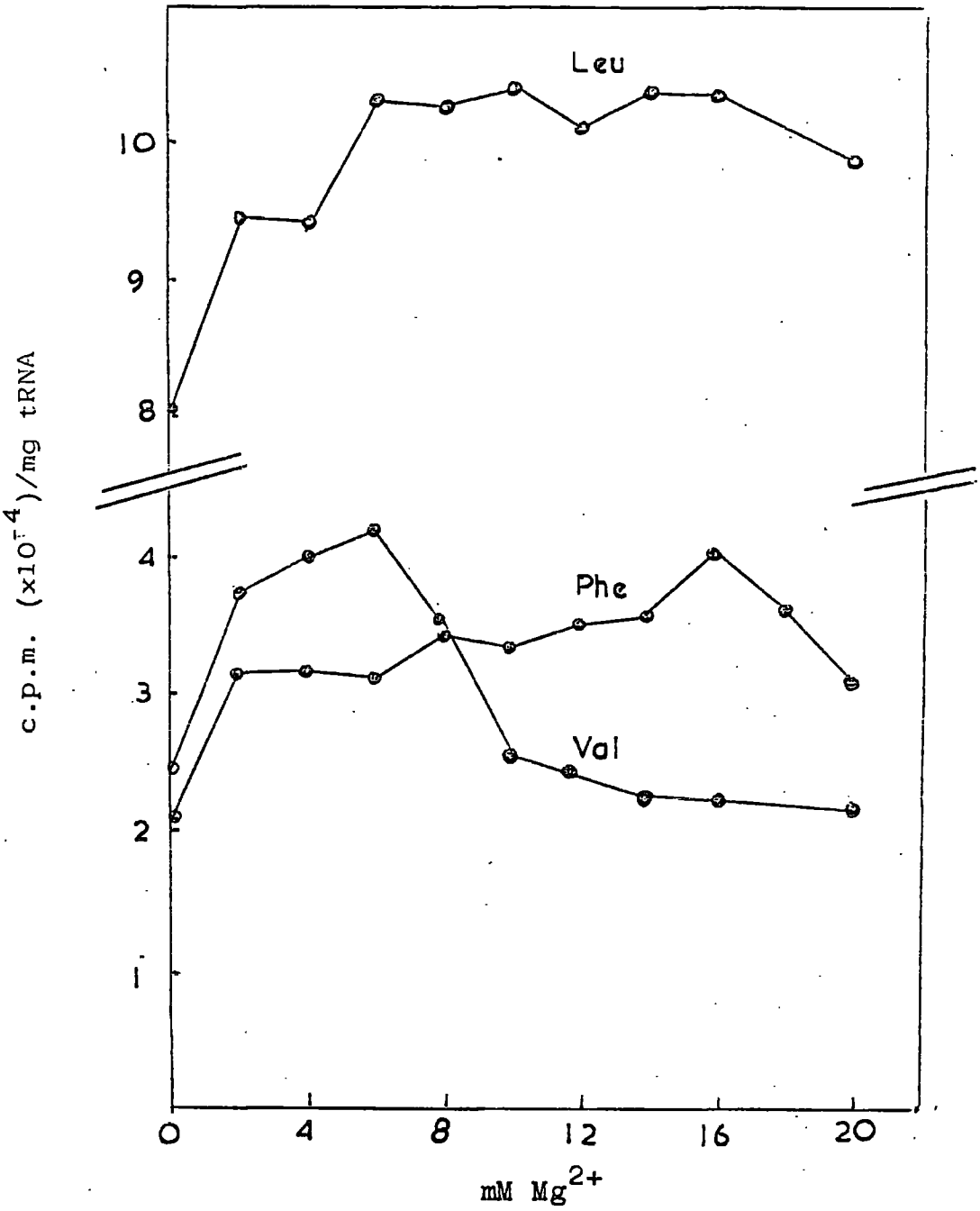
Aminoacylation by the 14 $[U^{14}C]$ amino acid mixture (FIG.27) showed the same optimal concentration of the mixture for both V.faba and yeast tRNAs. However, there was a difference of 300,000 c.p.m/mg tRNA between the total acylations of the 2 tRNAs. The time course of acylation indicated maximum acceptance at 15 min for V.faba tRNA and between 30-60 min for yeast tRNA.

Fig.22. Effect of Mg^{2+} concentration on P. aureus tRNA acylation.



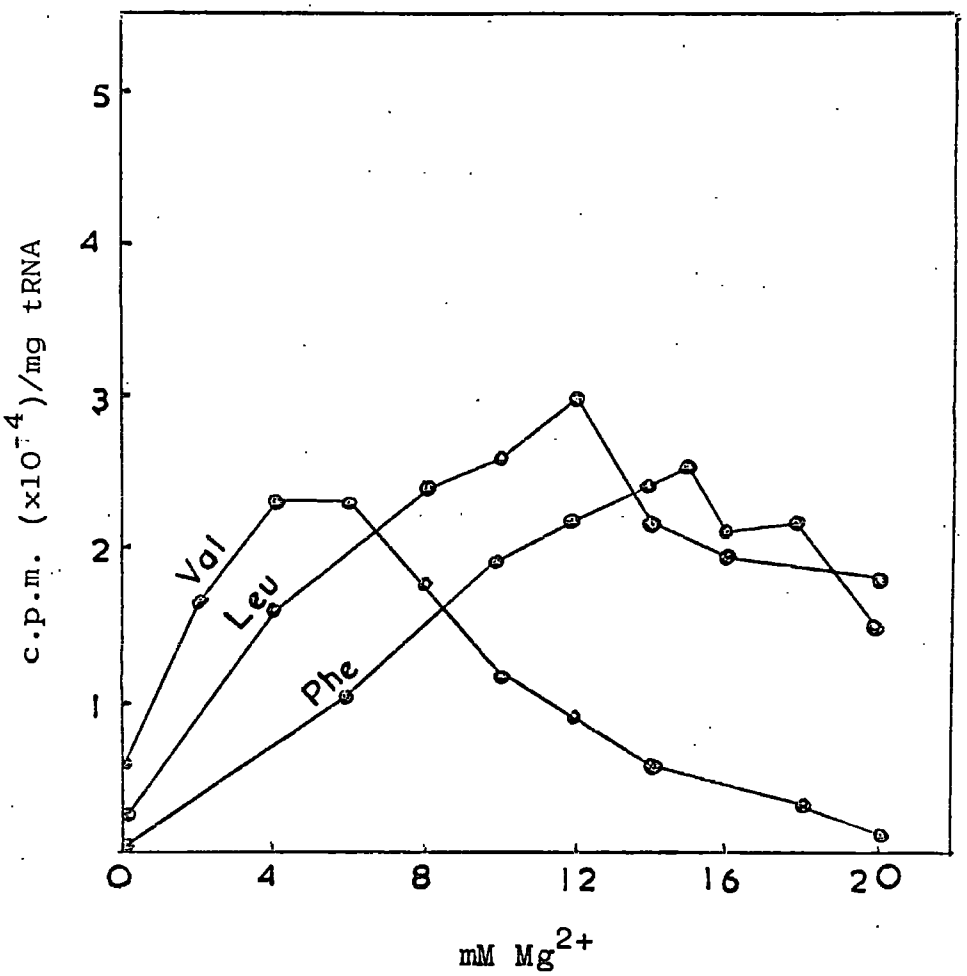
0.25 ml incubations contained: 25 µmol Tris-HCl pH 7.8 at 30°, 0.5 µmol ATP, 5 µmol GSH, 0.04 ml high-speed supernatant, 0.25 mg tRNA, varying µmol $MgCl_2$, either 0.005 µmol [^{14}C] Leu, or 0.005 µmol [^{14}C] Val, or 0.005 µmol [^{14}C] Phe. Incubation was at 30° and 0.05 ml samples assayed at 0, 15, 30, 45 min. Radioactivity/disc determined as in Methods 4. 15 min assays are reported.

Fig.23. Effect of Mg^{2+} concentrations on V. faba tRNA acylation.



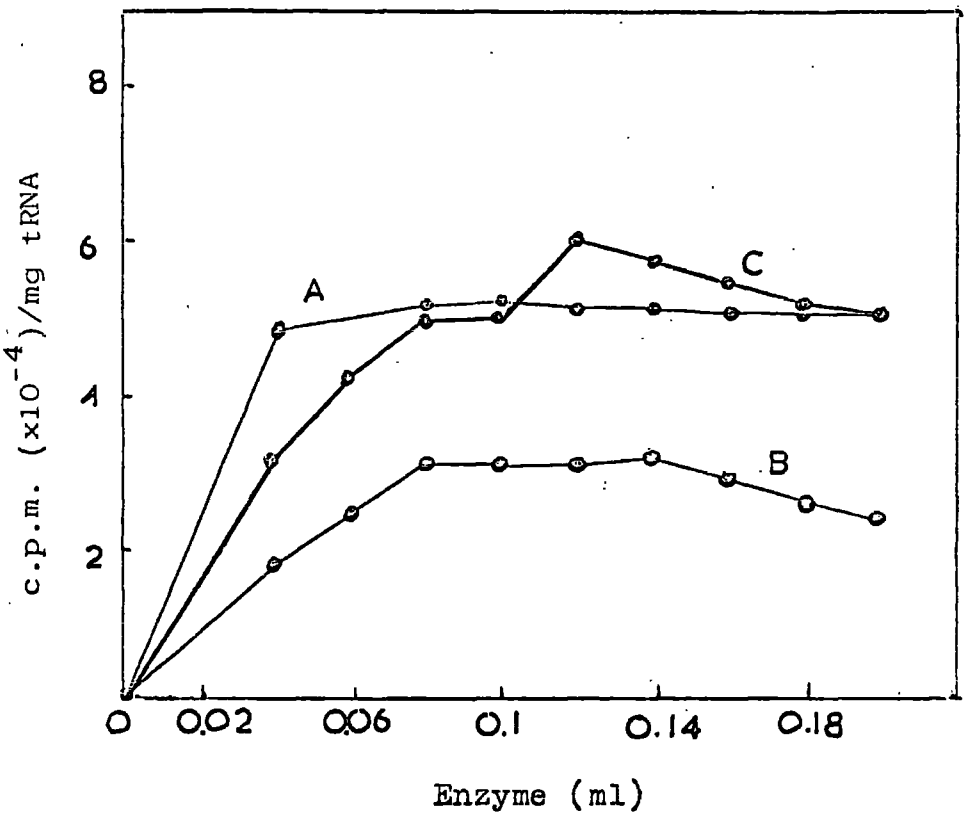
0.25 ml incubations contained: 25 µmol Tris-HCl pH 7.8 at 30°, 0.5 µmol ATP, 5 µmol GSH, 0.04ml high-speed supernatant, 0.25 mg tRNA, varying µmol $MgCl_2$, either 0.005 µmol $[^{14}C]$ Leu, or 0.005 µmol $[^{14}C]$ Val, or 0.005 µmol $[^{14}C]$ Phe. Incubation was at 30° and 0.05 ml samples assayed at 0, 15, 30, 45 min. Radioactivity/disc determined as in Methods 4. 15 min assays are reported.

Fig.24. Effect of Mg^{2+} concentration on Yeast tRNA acylation.



0.5 ml incubations contained: 50 µmol Tris-HCl pH 7.8 at 37°, 1 µmol ATP, 10 µmol GSH, 0.08 ml high-speed supernatant, 0.5 mg tRNA, either 0.01 µmol [^{14}C] Val, or 0.01 µmol [^{14}C] Leu, or 0.01 µmol [^{14}C] Phe. Incubations were at 37° and 0.1 ml samples assayed at 0, 20, 40, 60 mins. Radioactivity/disc determined as in Methods 4. 20 min assays are reported.

Fig.25. Effect of P. aureus high-speed supernatant enzyme fraction on esterification of [^{14}C] Leu by tRNAs.



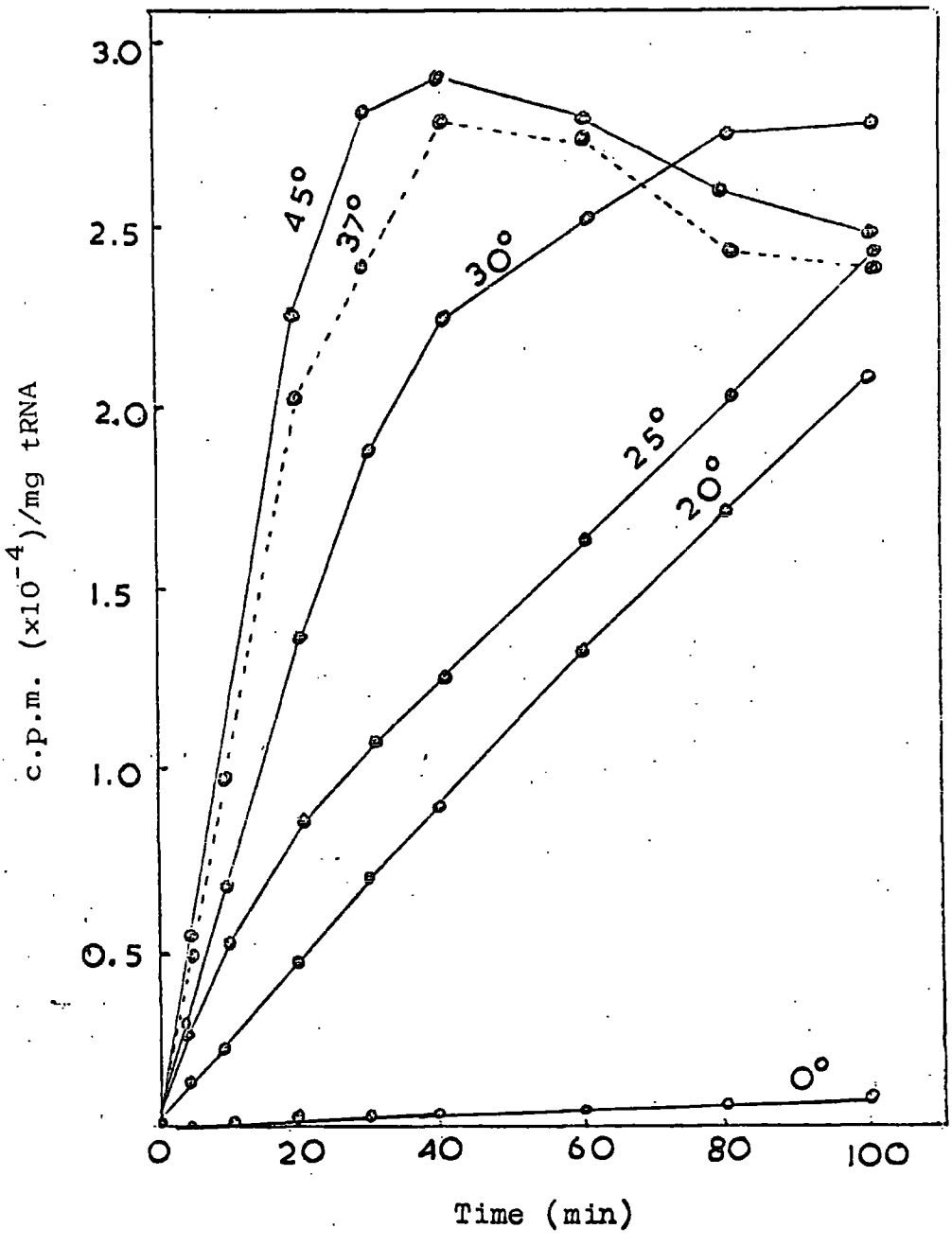
0.5 ml incubations contained: 50 µmol Tris-HCl pH 7.8 at 30° or 37°, 1 µmol ATP, 10 µmol GSH, 0.01 µmol [^{14}C] Leu, 0.5 mg tRNA and varying vol. high-speed supernatant. Yeast tRNA 37° incubation included 6 µmol $MgCl_2$; V. faba tRNA 30° incubation included 4 µmol $MgCl_2$; P. aureus tRNA 30° incubation included 6 µmol $MgCl_2$. 0.1 ml samples assayed at 0, 20, 40, 60 min. Radioactivity/disc determined as in Methods 4. V. faba tRNA and P. aureus tRNA 20 min assays and Yeast tRNA 40 min assays are reported.

A: V. faba tRNA.

B: Yeast tRNA.

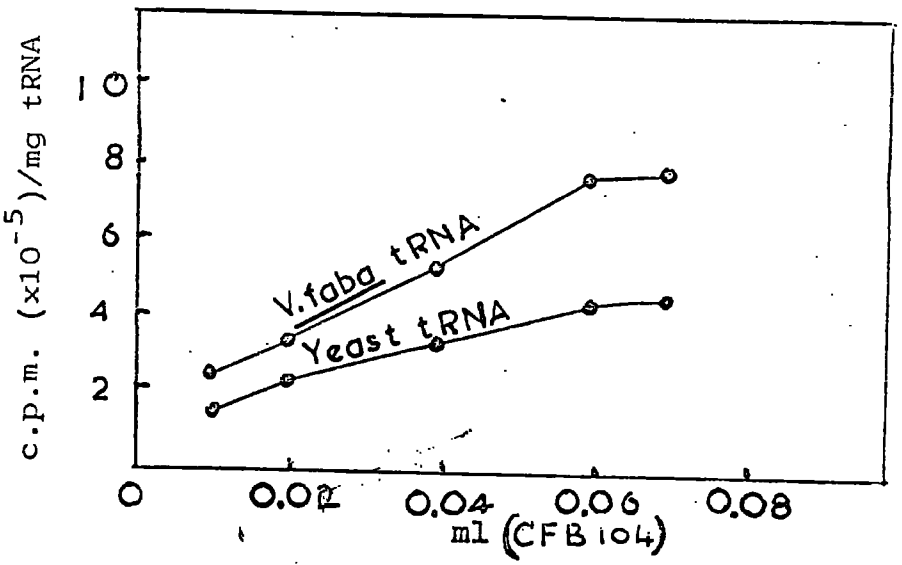
C: P. aureus tRNA.

Fig. 26. Effect of Temperature on Yeast tRNA acylation by Val.



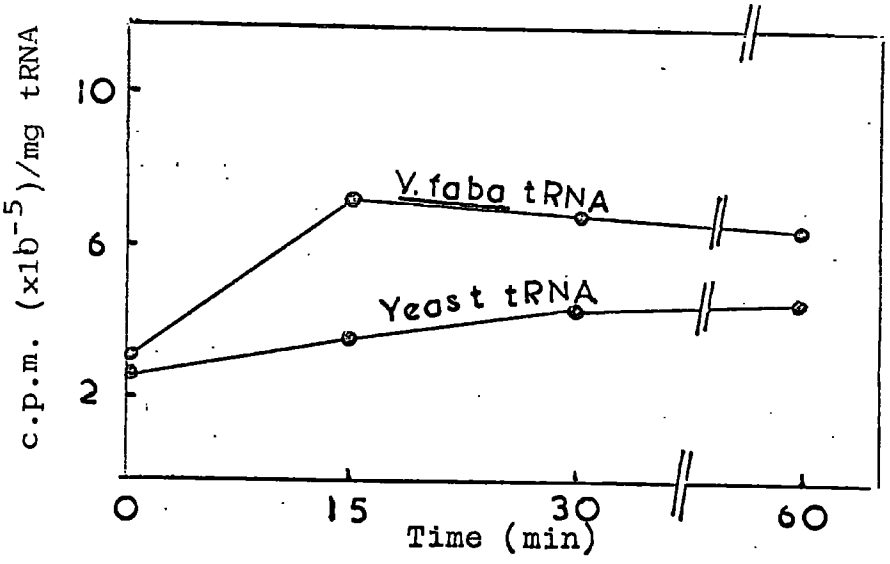
0.5 ml incubations contained: 50 µmol Tris-HCl pH 7.8, 1 µmol ATP, 10 µmol GSH, 0.01 µmol [^{14}C] Val, 2 µmol $MgCl_2$, 0.5 mg yeast tRNA, 0.08 ml high-speed supernatant. 0.05 ml samples assayed at temperatures and times indicated. Radioactivity/disc determined as in Methods 4.

Fig.27. Effect of concentration of 14 [U^{14}C] amino acid mixture (CFB104) on tRNA acylation.



Yeast tRNA - c.p.m. after 30 min incubation.

V. faba tRNA - c.p.m. after 15 min incubation.



Time courses based on 0.06 ml CFB104/0.25 ml incubation.

TABLE 1 illustrates the acylation of the various sources of tRNA using identical conditions, other than the temperature of incubation. Overall, yeast tRNA was acylated at a lower level than the other tRNAs.

The ATP requirement for Val esterification by 3 tRNAs is demonstrated in TABLE 2. Given a constant level of endogenous contaminating ATP, Val, Leu and Ala had quantitatively differing ATP requirements. (TABLE 3).

tRNA preparations were stored at -70^{o} for at least 2 yr without apparent loss of activity.

3. Esterification of Val to TYMV-RNA*

The time course of Val esterification under optimal conditions appeared complete at 18 min at 30^{o} (FIG.28). There was a sharp pH optimum at 7.8 (FIG.29), a sharp Mg^{2+} optimum at 12mM (FIG.30) and a temperature optimum at 30^{o} (FIG.31).

There was no sharp concentration optimum for the high-speed supernatant, similar activity being obtained from 0.05 - 0.12ml supernatant/0.5ml incubation (FIG.32). ATP was an absolute requirement for the reaction (FIG.33) and GTP was not an acceptable alternative.

* TYMV-RNA was extracted by the phenol method except if otherwise stated.

TABLE 1. Esterification of aminoacids using P.aureus high-speed supernatant enzyme fraction with various tRNAs and TYMV-RNAs.

[^{14}C] amino acid	Yeast tRNA c.p.m./mg	V.faba tRNA (germinating) c.p.m./mg	V.faba tRNA (developing) c.p.m./mg	P.aureus tRNA (germinating) c.p.m./mg	TYMV-RNA (phenolic extraction) c.p.m./mg	TYMV-RNA (alcoholic extraction) c.p.m./mg
Leu	58,632	188,080	104,060	62,060	200	300
Val	38,222	75,720	64,500	44,780	13,028*	11,820*
Phe	36,700	180,420	109,500	22,840	0	100
Glu	3,120	Not done	16,164	17,160	0	0
Gly	12,998	Not done	58,468	21,166	0	0
Ala	11,480	42,200	25,726	15,220	0	0

0.5ml incubations contained; 50 µmol Tris-HCl pH 7.8 at 30° or 37°, 1 µmol ATP, 10 µmol GSH, 6 µmol $MgCl_2$, 0.08ml high-speed supernatant and respectively 0.01 µmol [^{14}C] amino acid and 0.5mg tRNA. Incubation as directed.† Yeast tRNA assayed at 40 min (0.1ml sample) and other tRNAs at 20 min (0.1ml sample). Radioactivity/disc determined as in Methods 4.

* EXPERIMENTS with high-speed supernatant and [^{14}C] Val showed no charging.

† Yeast tRNA at 37°, other RNAs at 30°

TABLE 2. ATP dependence of acylation of tRNA with Val.

tRNA	ATP c.p.m./mg tRNA	%	-ATP c.p.m./mg tRNA	%
P.aureus	53,674	100	40,300	73
V.faba	72,610	100	23,064	32
Yeast	24,046	100	10,442	43

0.5ml incubations contained: 50 µmol Tris-HCl pH 7.8 at 30°, 1 µmol ATP, 10 µmol GSH, 0.01 µmol [^{14}C] Val, 0.08ml high-speed supernatant and either 0.5mg P.aureus tRNA with 2 µmol $MgCl_2$ or 0.5mg V.faba tRNA, 3 µmol $MgCl_2$ or 0.5mg yeast tRNA with 2 µmol $MgCl_2$. Incubation was at 30° and 0.1ml samples assayed at 0, 15, 30 min. Radioactivity/disc determined as in Methods 4. 30 min assays are reported.

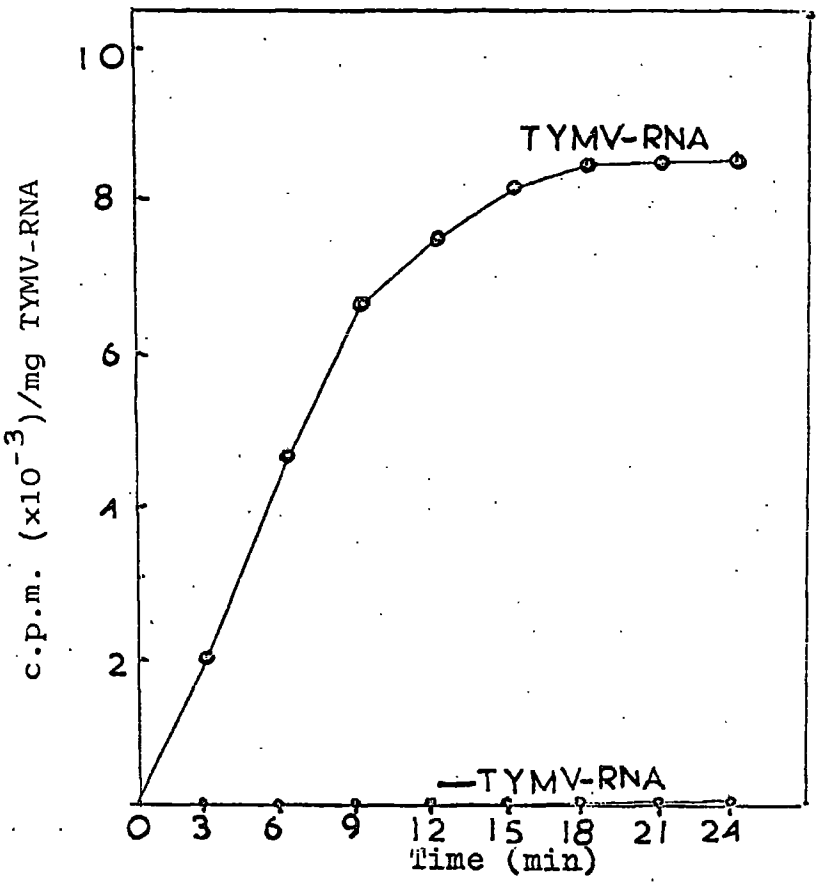
TABLE 3. ATP dependence of acylation of tRNA

(V.faba - germinating)

[^{14}C] amino acid	ATP c.p.m./mg tRNA	%	-ATP c.p.m./mg tRNA	%
Val	80,768	100	25,628	32
Leu	156,632	100	10,220	6
Ala	52,200	100	5,400	10

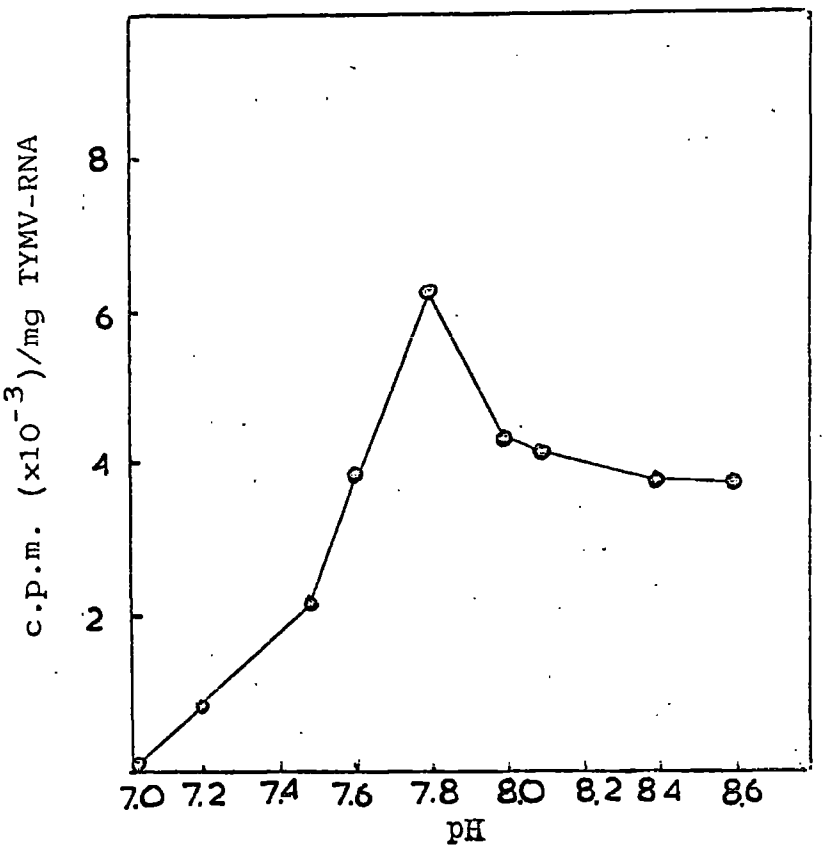
0.25ml incubations contained: 25 µmol Tris-HCl pH 7.8 at 30°, 3 µmol $MgCl_2$, 0.5 µmol ATP, 5 µmol GSH, 0.05ml high-speed supernatant, 0.25mg tRNA and either 0.005 µmol [^{14}C] Val, Leu or Ala. Incubation was at 30° and samples assayed at 0, 15, 30, 45 min. Radioactivity/disc determined as in Methods 4. 45 min assays are reported.

Fig.28. Time course of TYMV-RNA esterification of Val.



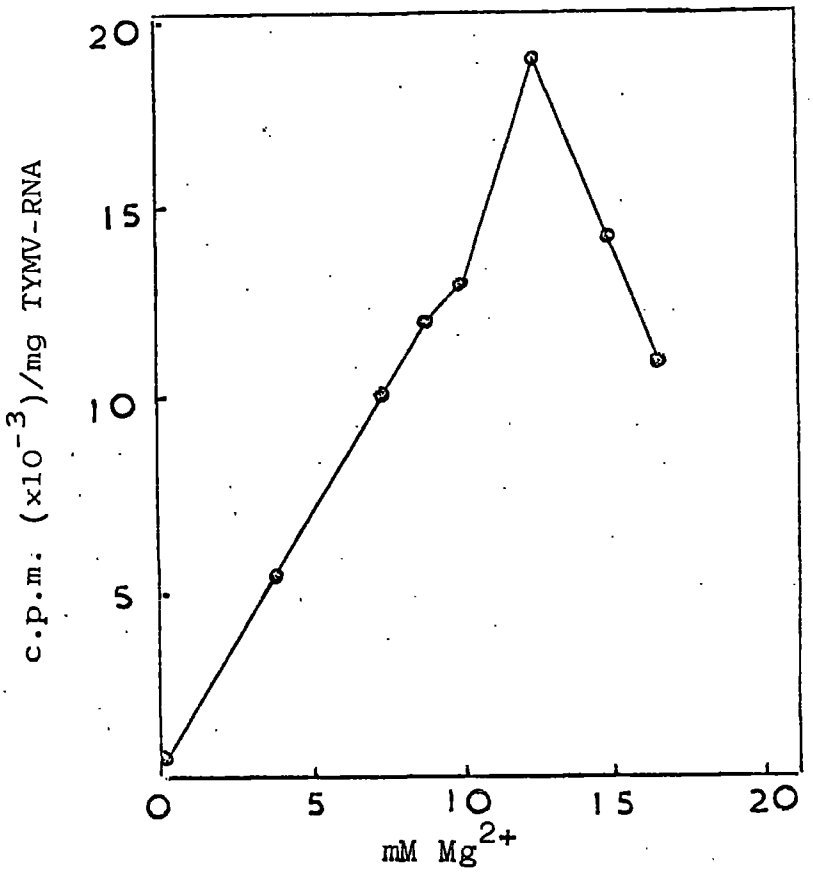
1 ml incubations contained: 100 µmol Tris-HCl pH 7.8 at 30°, 20 µmol GSH, 2 µmol ATP, 12 µmol $MgCl_2$, 0.02 µmol [^{14}C] Val, 0.12 ml high-speed supernatant and 1 mg TYMV-RNA. Incubation was at 30° and 0.1 ml samples assayed at times indicated. Radioactivity/disc determined as in Methods 4.

Fig.29. Effect of pH on TYMV-RNA esterification of Val.



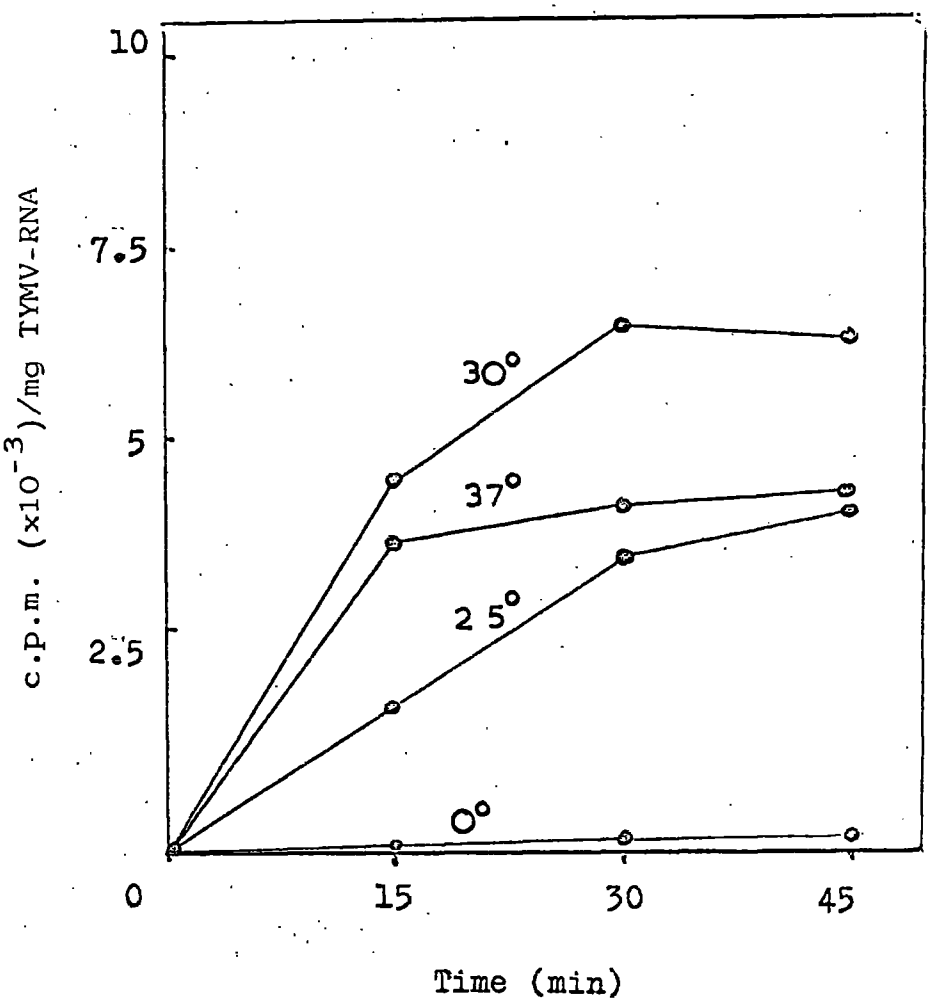
0.25 ml incubations contained: 25 µmol Tris-HCl at 30°, 0.5 µmol ATP, 5 µmol GSH, 3 µmol $MgCl_2$, 0.005 µmol [^{14}C] Val, 0.03 ml high-speed supernatant, and 0.25 mg TYMV-RNA. Incubations were at 30° and 0.05 ml samples assayed at 0, 10, 20, 40 min. Radioactivity/disc determined as in Methods 4. 10 min assays are reported.

Fig.30. Effect of Mg^{2+} concentration on TYMV-RNA esterification of Val.



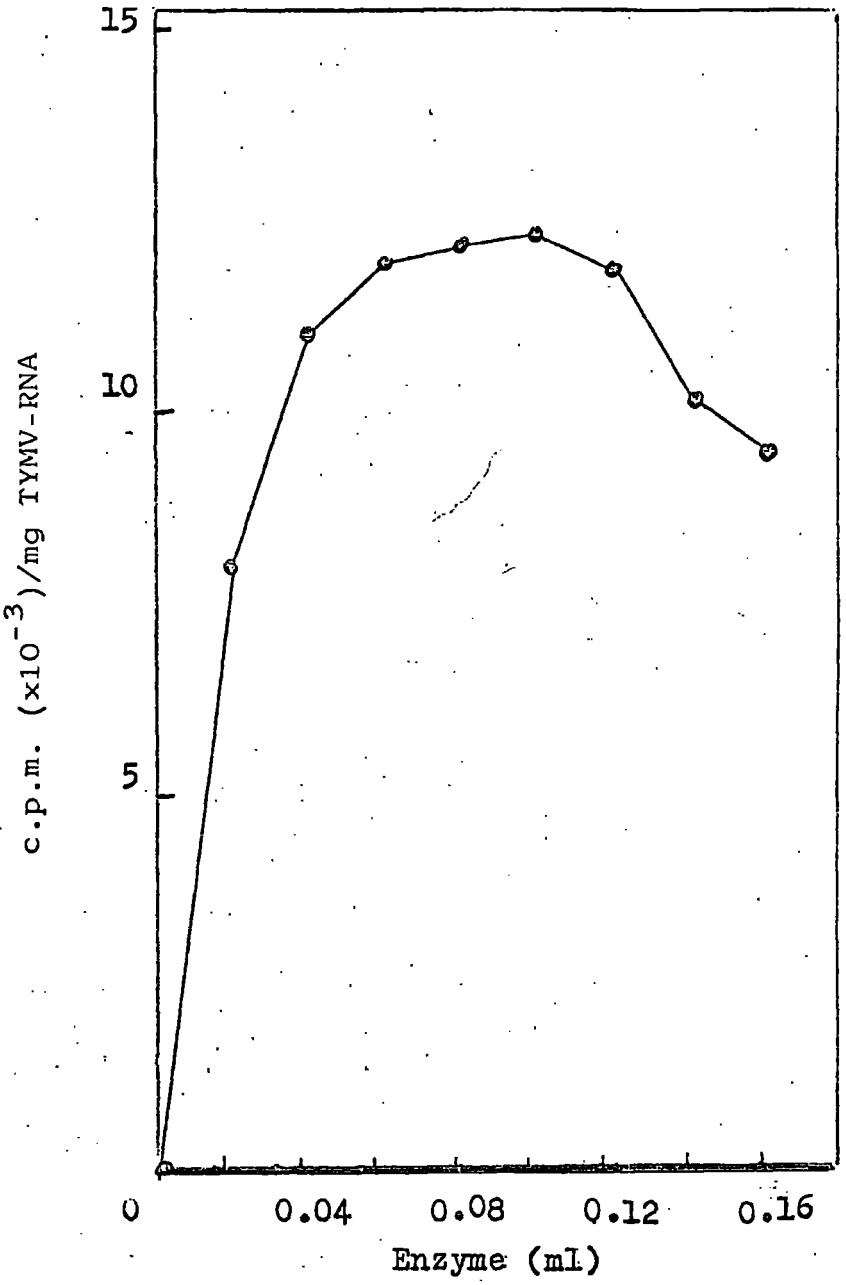
0.5 ml incubations contained; 50 µmol Tris-HCl pH 7.8 at 30°, 1 µmol ATP, 10 µmol GSH, 0.01 µmol [^{14}C] Val, 0.5 mg TYMV-RNA, 0.04 ml high-speed supernatant and varying µmol $MgCl_2$. Incubation was at 30° and 0.1 ml samples assayed 0, 10, 20, 40 min. Radioactivity/disc determined as in Methods 4. 20 min assays are reported.

Fig.31. Effect of temperature on TYMV-RNA esterification of Val.



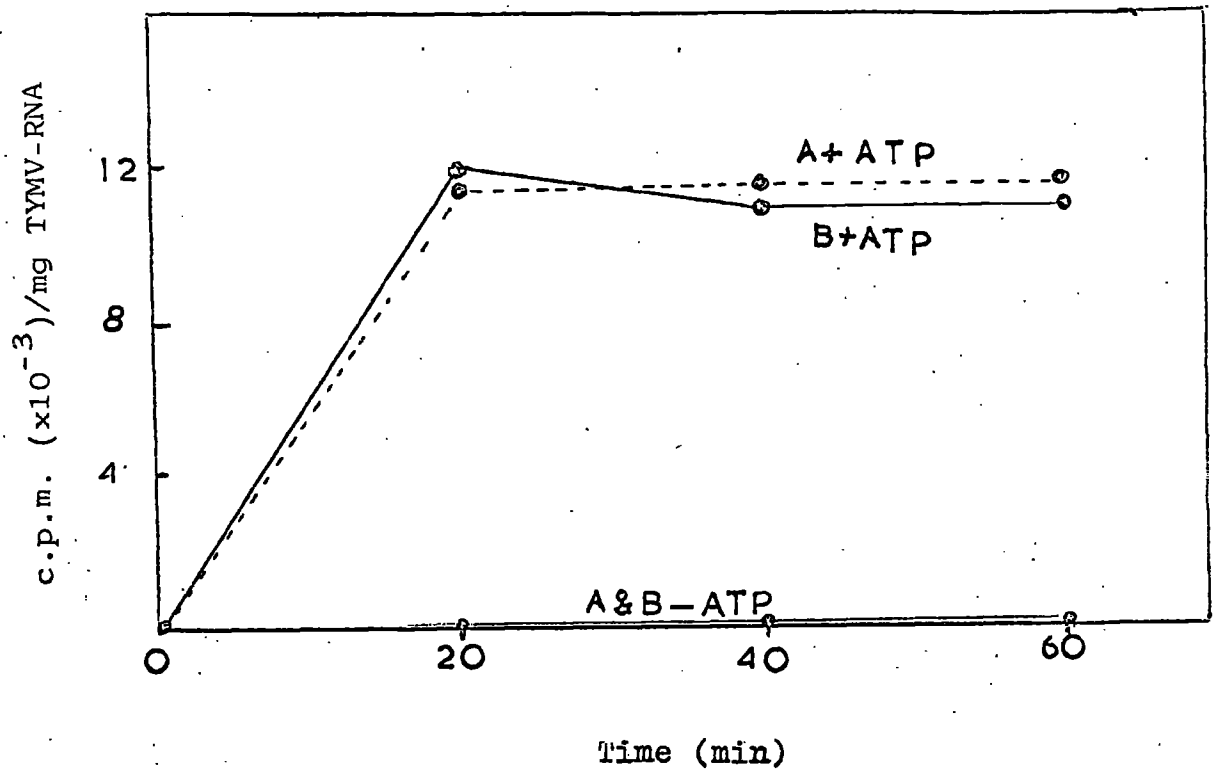
0.5 ml incubations contained: 50 µmol Tris-HCl pH 7.8, 1 µmol ATP, 10 µmol GSH, 0.01 µmol [^{14}C] Val, 6 µmol $MgCl_2$, 0.5 mg TYMV-RNA, and 0.04 ml high-speed supernatant. 0.1 ml samples assayed at temperatures and times indicated. Radioactivity/disc determined as in Methods 4.

Fig.32. Effect of P. aureus high-speed supernatant enzyme fraction on esterification of Val by TYMV-RNA.



0.5 ml incubations contained: 50 µmol Tris-HCl pH 7.8 at 30°, 1 µmol ATP, 10 µmol GSH, 6 µmol $MgCl_2$, 0.01 µmol [^{14}C] Val, 0.5 mg TYMV-RNA, varying vol. high-speed supernatant; incubation was at 30°. 0.1 ml samples assayed at 0, 10, 20, 40 min. Radioactivity/disc determined as in Methods 4. 10 min assays are reported.

Fig.33. Esterification of Val by TYMV-RNA: ATP dependence.



0.25 ml incubations contained: 25 µmol Tris-HCl pH 7.8 at 30°, 0.5 µmol ATP, 5 µmol GSH, 3 µmol $MgCl_2$, 0.005 µmol [^{14}C] Val, 0.03 ml high-speed supernatant and 0.25 mg TYMV-RNA. Incubation was at 30° and 0.5 ml samples assayed at times indicated. Radioactivity/disc determined as in Methods 4.

A: TYMV-RNA, extracted by Alcoholic procedure.

B: TYMV-RNA, extracted by Phenolic procedure.

Each method of extraction produced TYMV-RNA with similar charging capacities (FIG.33, conjoint TABLE 1). It was possible to deacylate valyl - TYMV-RNA by the hot 5% (W/V) trichloroacetic acid procedure (TABLE 4).

4. Complete System

(A) Poly-U-directed incorporation of [^{14}C] Phe:

In the presence of tRNA from yeast, P.aureus or V.faba*, Poly-U would direct poly-Phe synthesis in the Complete System (FIG.34). With V.faba tRNA the incorporation was linear for 2 hr and terminated at 2.5 hr; with yeast and P.aureus tRNAs linearity obtained for 1.5 hr and terminated at 2.5 hr.

The requirement for the energy system - ATP, PCK and CreP - is demonstrated in FIGS.35, 36 and TABLE 5. Since microsomes and high-speed supernatant enzyme fractions were common components of these systems, the results were complex. Whereas systems containing V.faba tRNA or P.aureus tRNA were only partially dependent on the energy system, the yeast tRNA system was totally dependent. Supplementation of ATP alone allowed 60% activity in the system containing yeast tRNA, and the same condition for V.faba tRNA allowed 87% activity.

Mg^{2+} concentration greatly influenced poly-Phe synthesis. Optima for systems containing tRNA from V.faba, P.aureus and yeast were 10mM, 12mM and 12mM respectively. These optima were sharp and there was little incorporation without exogenously-supplied Mg^{2+} (FIG.37).

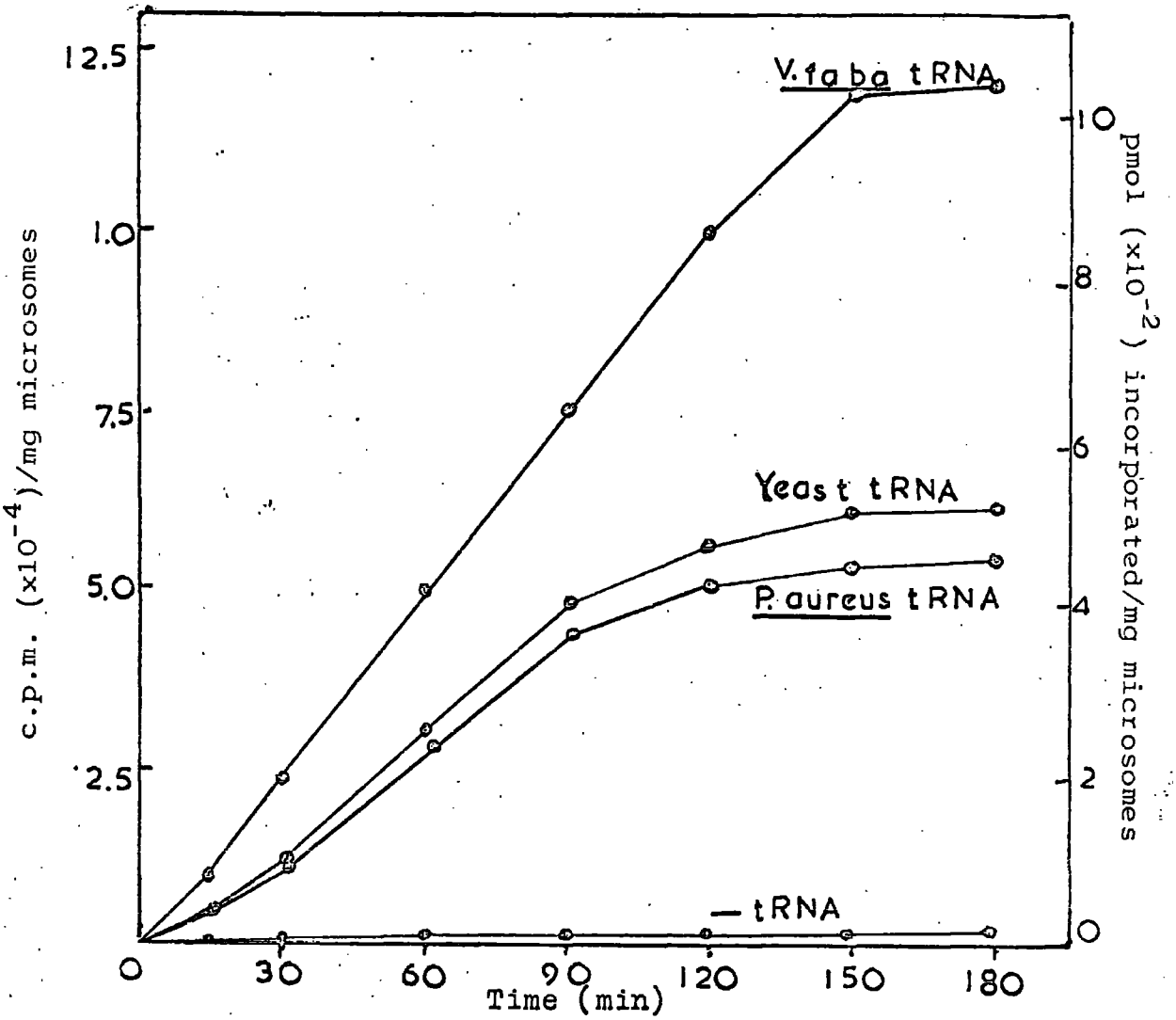
* In all in vitro incorporation systems V.faba tRNA is from 50-day developing seeds.

TABLE 4. Deacylation of TYMV-RNA

Acylation (Set 1) c.p.m./mg TYMV-RNA	Deacylation (Set 2) c.p.m./mg TYMV-RNA
9,866	350

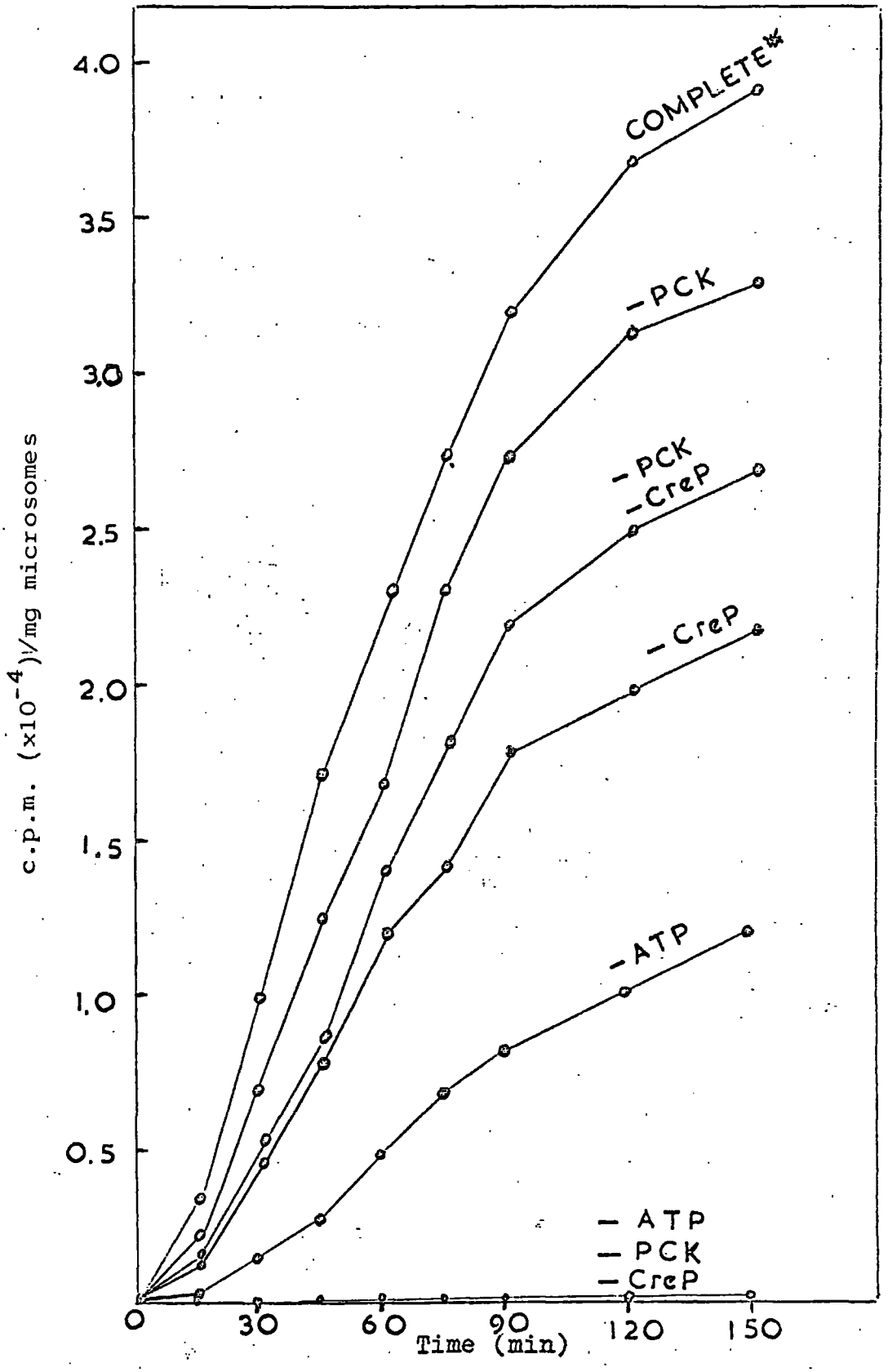
1ml incubations contained: 100 µmol Tris-HCl pH 7.8 at 30°, 12 µmol $MgCl_2$, 2 µmol ATP, 20 µmol GSH, 1mg TYMV-RNA, 0.02 µmol [^{14}C] Val, and 0.08ml high-speed supernatant. Incubation was at 30° and 2 x 0.1ml samples assayed at 0, 10, 20, 30 min. Set 1 discs were treated as in Methods 3, i.e. 5% (W/V) hot TCA wash, and Set 2 as in Methods 4.

Fig.34. COMPLETE SYSTEM: Poly-U-directed [^{14}C] Phe incorporation with tRNAs from V. faba, P. aureus and yeast.



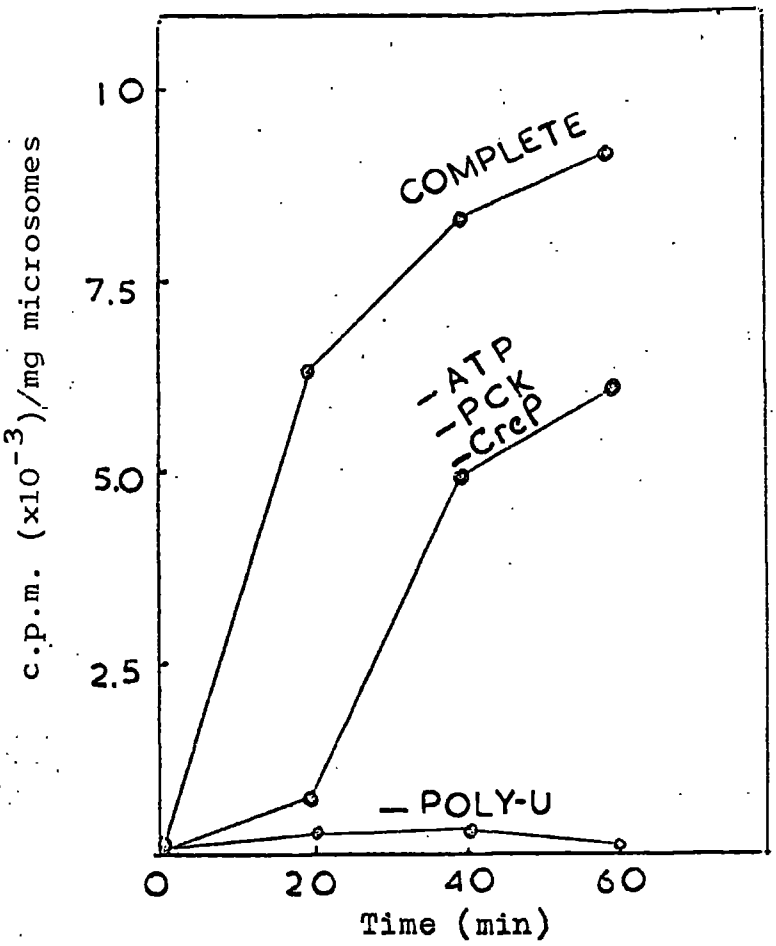
0.5 ml incubations contained: 30 µmol Tris-HCl pH 7.8 or 8.1 at 30°, 35 µmol KCl, 2 µmol ATP, 5 µmol CreP, 10 µg PCK, 0.1 µmol GTP, 5 µmol GSH, 0.01 µmol [^{14}C] Phe, 0.01 µmol 19 [^{12}C] amino acid mixture, 0.5 mg microsomes, 0.1 mg Poly-U, either 0.2 mg yeast tRNA with 6 µmol $MgCl_2$ and 0.04 ml high-speed supernatant, or 0.2 mg V. faba tRNA with 5 µmol $MgCl_2$ and 0.02 ml high-speed supernatant, or 0.2 mg P. aureus tRNA with 6 µmol $MgCl_2$ and 0.04 ml high-speed supernatant. Bean-tRNA systems at pH 7.8; yeast tRNA system at pH 8.1. Incubation was at 30° and 0.05 ml samples assayed at times indicated. Radioactivity/disc determined as in Methods 3.

Fig. 35. COMPLETE SYSTEM: ATP, PCK and CreP in Poly-U-directed [^{14}C] Phe incorporation (Yeast tRNA)



* Complete System without Poly-U: 600 c.p.m. per mg microsomes at 15 min. No activity at other times.

Fig.36. COMPLETE SYSTEM: ATP and ATP regenerating system in Poly-U-directed [^{14}C] Phe incorporation (P. aureus tRNA).



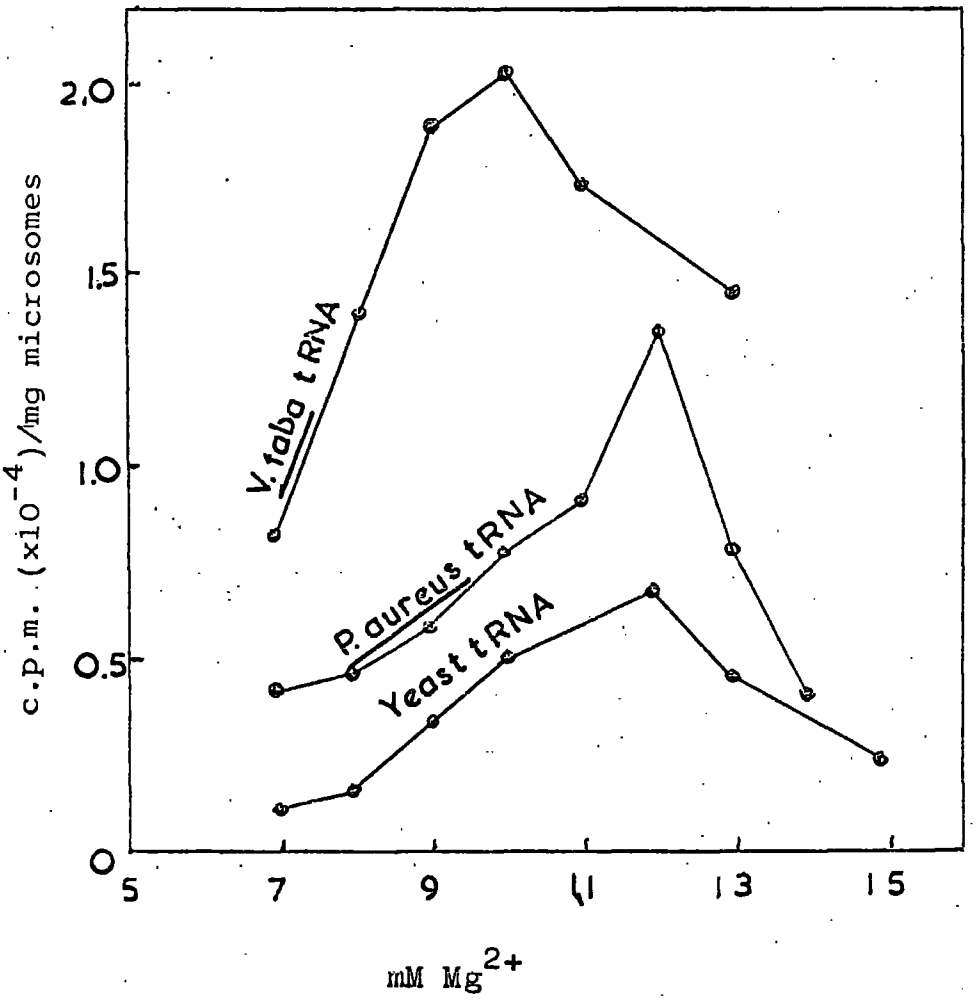
0.5 ml incubations contained: 30 µmol Tris-HCl pH 7.6 at 30,° 35 µmol KCl, 2 µmol ATP, 5 µmol CreP, 10 µg PCK, 0.1 µmol GTP, 5 µmol GSH, 0.01 µmol [^{14}C] Phe, 0.01 µmol 19 [^{12}C] amino acid mixture, 0.5 mg microsomes, 6 µmol $MgCl_2$, 0.2 mg tRNA, 0.1 mg Poly-U and 0.04 ml high-speed supernatant. (ATP, CreP, PCK, omitted as required). Incubation was at 30° and 0.1 ml samples assayed at times indicated. Radioactivity/disc determined as in Methods 3.

TABLE 5. COMPLETE SYSTEM: ATP, PCK and CreP in Poly-U-directed [^{14}C] Phe incorporation (V.faba tRNA).

	c.p.m./mg microsomes	%
Complete	10,700	100
— ATP	7,466	60
— ATP-PCK-CreP	7,440	60
— PCK-CreP	9,384	87
— Poly-U	200	1.9
— Microsomes	-	-

0.5ml incubations contained: 30 µmoles Tris-HCl pH 7.8 at 30°, 35 µmol KCl, 2 µmol ATP, 5 µmol CreP, 10 µg PCK, 0.1 µmol GTP, 5 µmol GSH, 5 µmol $MgCl_2$, 0.01 µmol [^{14}C] Phe, 0.01 µmol 19 [^{12}C] amino acid mixture, 0.5mg microsomes, 0.2mg tRNA, 0.1mg Poly-U and 0.04ml high-speed supernatant. (Omissions made as indicated). Incubation was at 30° and 0.05ml samples assayed at 0, 15, 30, 60 mins. Radioactivity/disc determined as in Methods 3. 15 min assays are reported.

Fig.37. COMPLETE SYSTEM: Effect of Mg^{2+} concentration on Poly-U-directed $[^{14}C]$ Phe incorporation; (*V. faba* tRNA, *P. aureus* tRNA, Yeast tRNA)



0.5 ml incubations contained: 30 µmol Tris-HCl pH 7.8 at 30° or 37°, 35 µmol KCl, 2 µmol ATP, 5 µmol CreP, 10 µg PCK, 0.1 µmol GTP, 5 µmol GSH, 0.1 mg Poly-U 0.01 µmol $[^{14}C]$ Phe, 0.01 µmol 19 $[^{12}C]$ amino acid mixture, 0.5 mg microsomes and either 0.2 mg yeast tRNA, *V. faba* tRNA or *P. aureus* tRNA, 0.06 ml high-speed supernatant and varying µmol $MgCl_2$. Incubation was 30° for *V. faba* and *P. aureus* tRNAs and 37° for yeast tRNA. 0.1 ml samples were assayed at 0, 15, 30, 60 min. Radioactivity/disc determined as in Methods 3. 30 min assays are reported.

The fact that the microsomal preparations were not free of enzymatic activity is shown by the results given in FIGS. 38 and 39. However, the optimal concentration for exogenously-added high-speed supernatant was 0.04ml for 0.5ml incubations involving yeast tRNA and P.aureus tRNA, and 0.02ml/0.5ml incubation for V.faba tRNA. The high-speed supernatants could be stored for at least 6 months at -70° without loss of activity.

FIG.34 (see also FIG.73) shows that Poly-U-directed systems were virtually totally dependent on exogenously-supplied tRNA.

The effect of pH on the Complete System showed an optimum at pH 7.8 with V.faba tRNA and a less sharp optimum at pH 8.1 with yeast tRNA (FIG.40). When incubations containing yeast tRNA were carried out at 30° there was an obvious lag phase, which was greatly reduced when the incubation temperature was raised to 37°. The overall incorporation of this system was greater at 30° than at 37° (FIG.41).

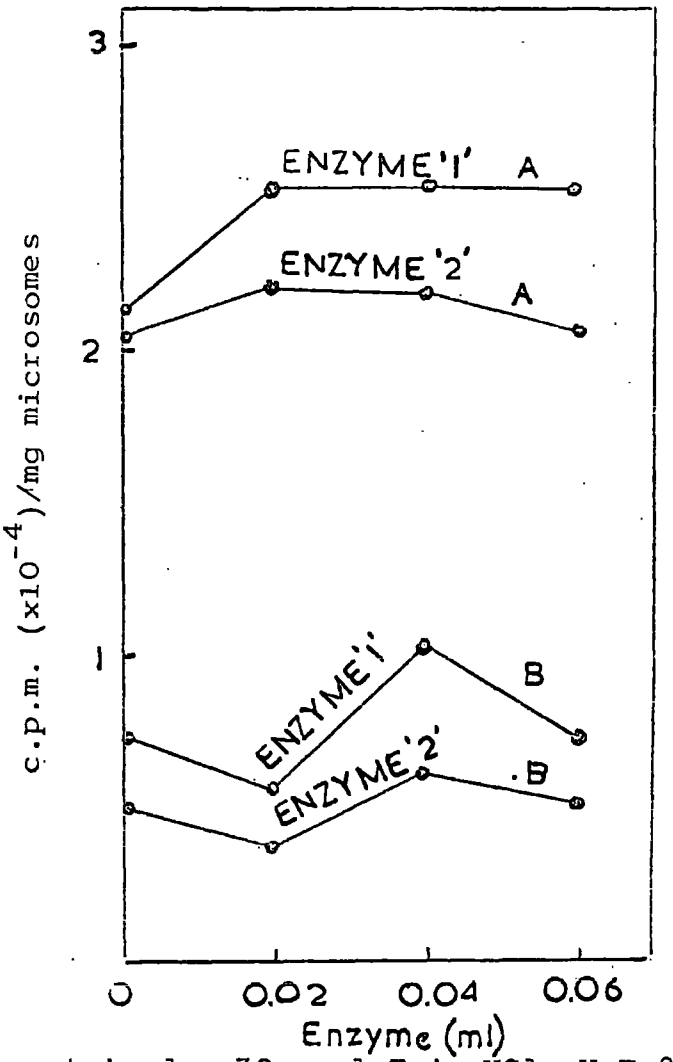
The results given in TABLE 6 and FIG.42 demonstrate the almost total failure to implicate GTP in these Poly-U-directed Complete Systems.

(B) TYMV-RNA-directed incorporation of $[^{14}C]$ Leu:

In the Complete System, which included P.aureus tRNA, TYMV-RNA* directed the incorporation of $[^{14}C]$ Leu (FIG.43). The Mg^{2+} concentration optimum for viral-RNA direction was 5mM, whilst endogenous activity was maximal at 10mM. This

* In all in vitro incorporation systems TYMV-RNA was that material obtained by phenol extraction.

Fig.38. COMPLETE SYSTEM: Effect of 2 P. aureus high-speed supernatant enzyme fractions on Poly-U-directed [^{14}C] Phe incorporation (V. faba tRNA, P. aureus tRNA).

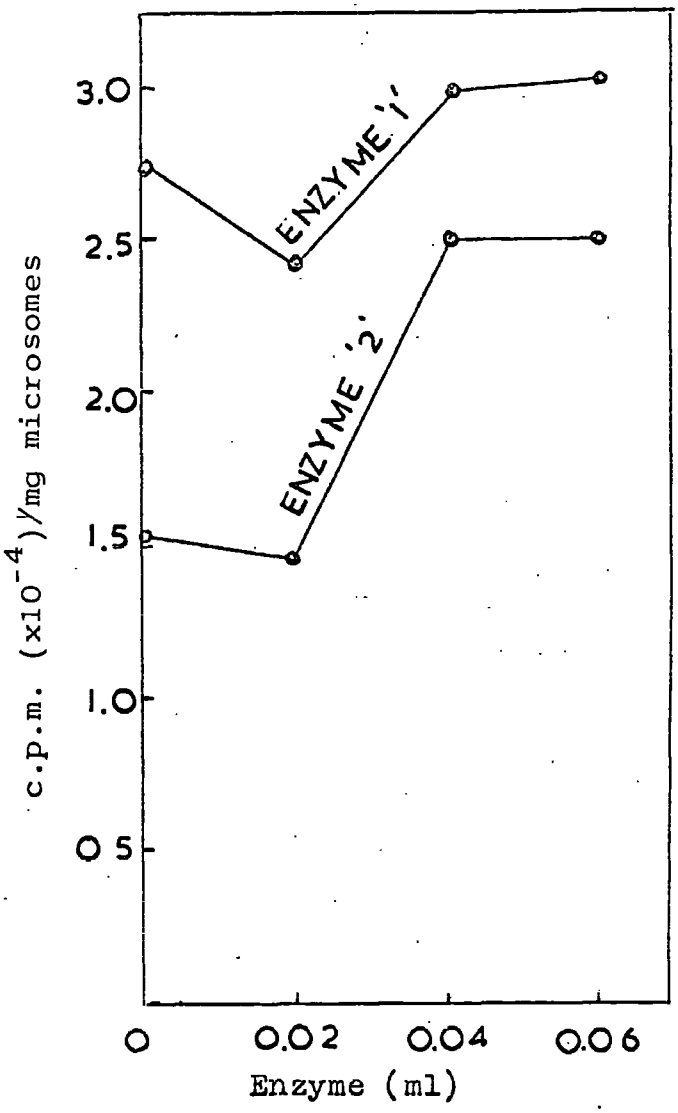


0.5 ml incubations contained: 30 µmol Tris-HCl pH 7.8 at 30°, 35 µmol KCl, 2 µmol ATP, 5 µmol CreP, 10 µg PCK, 0.1 µmol GTP, 5 µmol GSH, 0.01 µmol [^{14}C] Phe, 0.01 µmol 19[^{12}C] amino acid mixture, 0.5 mg microsomes, 0.1 mg Poly-U, either 0.2 mg V. faba tRNA with 5 µmol $MgCl_2$, or 0.2 mg P. aureus tRNA with 6 µmol $MgCl_2$, and varying vol. high-speed supernatant. Incubation was at 30° and 0.05 ml samples assayed at various time intervals. Radioactivity/disc determined as in Methods 3. 30 min assays are reported.

A: V. faba tRNA.

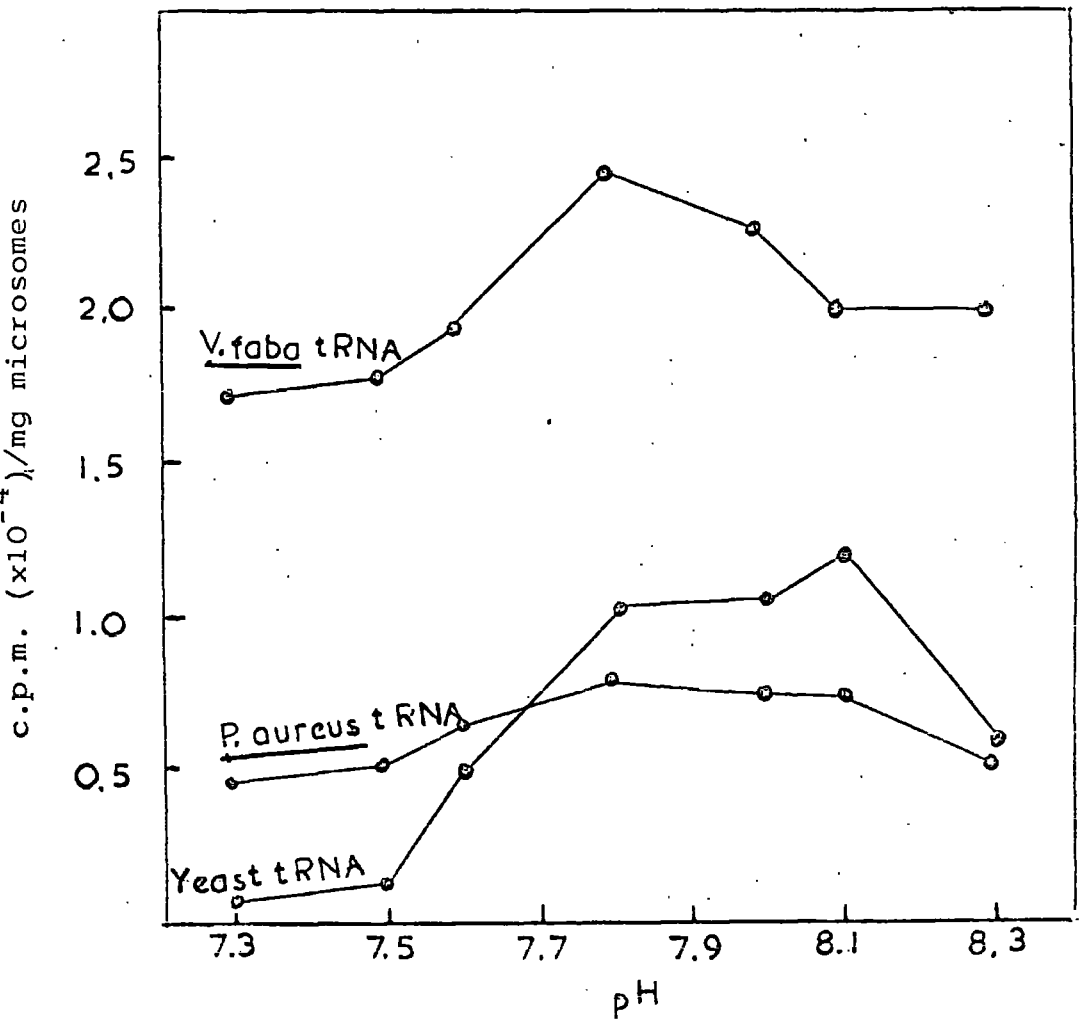
B: P. aureus tRNA.

Fig.39. COMPLETE SYSTEM: Effect of 2 P. aureus high-speed supernatant enzyme fractions on Poly-U-directed [^{14}C] Phe incorporation: (Yeast tRNA).



0.5 ml incubations contained: 30 µmol Tris-HCl pH 7.8 at 30°, 35 µmol KCl, 2 µmol ATP, 5 µmol CreP, 10 µg PCK, 0.1 µmol GTP, 5 µmol GSH, 0.01 µmol [^{14}C] Phe, 0.01 µmol 19 [^{12}C] amino acid mixture, 0.5 mg microsomes, 0.2 mg tRNA, 6 µmol $MgCl_2$, 0.1 mg Poly-U. and varying vol. high-speed supernatant. Incubation was at 30 min and 0.05 ml samples assayed at various time intervals. Radioactivity/disc determined as in Methods 3. 60 min assays are reported.

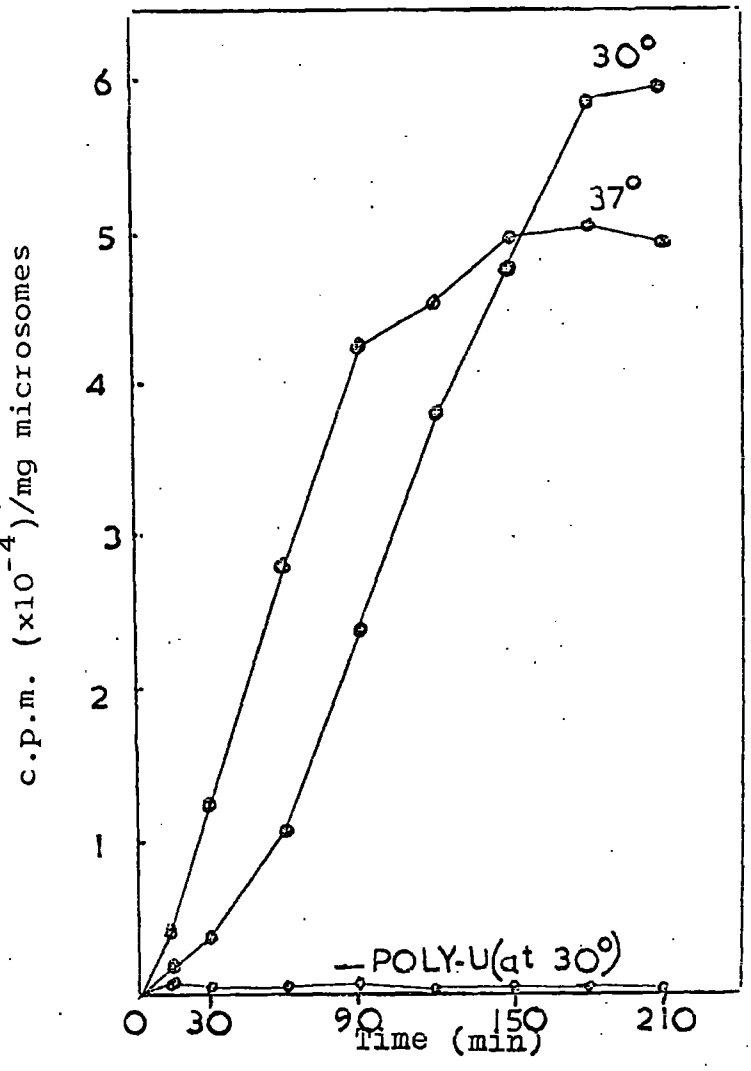
Fig.40. COMPLETE SYSTEM: Effect of pH on poly-U-directed [^{14}C] Phe incorporation. (*V. faba* tRNA, yeast tRNA, *P. aureus* tRNA).



0.25 ml incubations contained: 15 µmol Tris-HCl at varying pH at 30°, 17.5 µmol KCl, 1µmol ATP, 2.5 µmol CreP, 5 µg PCK, 0.05 µmol GTP, 0.005 µmol [^{14}C] Phe, 0.005 µmol 19 [^{12}C] amino acid mixture, 0.25 mg microsomes 0.05 mg Poly-U, 0.03 ml high-speed supernatant, and either 0.1 mg yeast tRNA* with 3 µmol $MgCl_2$ or 0.1 mg *V. faba* tRNA with 2.5 µmol $MgCl_2$. Incubation was at 30° and 0.05 ml samples assayed at 0, 30, 60, 120 min. Radioactivity/disc determined as in Methods 3. 30 min assays are reported.

*(or *P. aureus* tRNA).

Fig.41. COMPLETE SYSTEM: Effect of temperature on Poly-U-directed [^{14}C] Phe incorporation (Yeast tRNA).



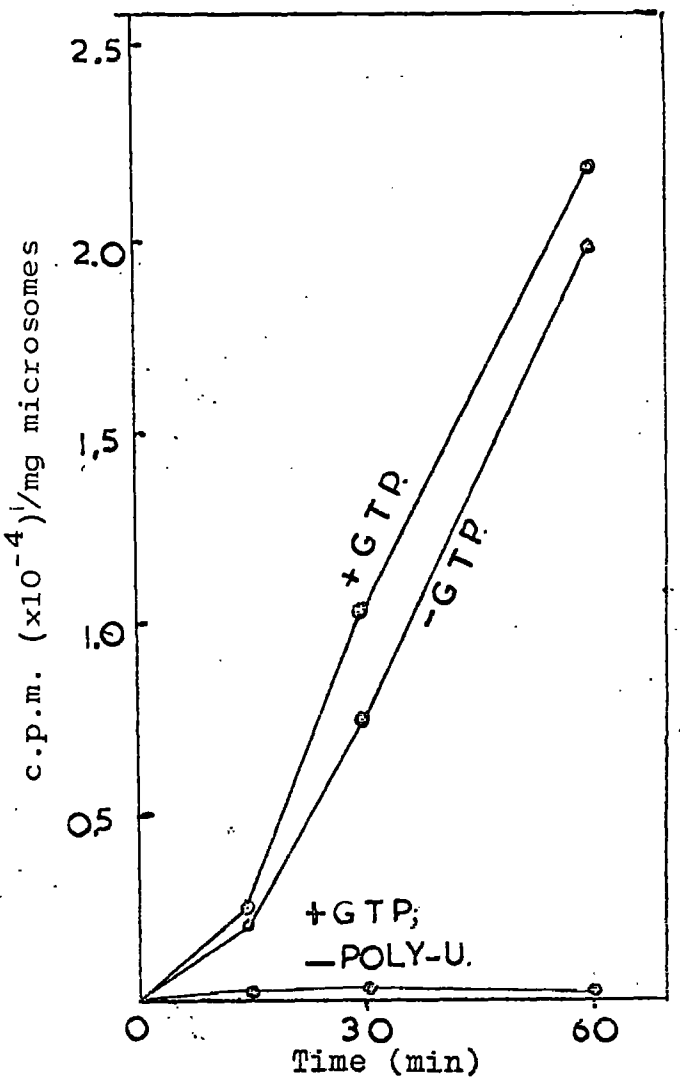
0.5 ml incubations contained: 30 µmol Tris-HCl pH 7.8 at 30° or 37°, 35 µmol KCl, 6 µmol $MgCl_2$, 2 µmol ATP, 5 µmol CreP, 10 µg PCK, 0.1 µmol GTP, 5 µmol GSH, 0.01 µmol [^{14}C] Phe, 0.01 µmol 19 [^{12}C] amino acid mixture, 0.1 mg Poly-U, 0.2 mg tRNA, 0.5 mg microsomes and 0.06 ml high-speed supernatant. Incubation was at 30° or 37° and 0.05 ml samples assayed at times indicated. Radioactivity/disc determined as in Methods 3.

TABLE 6. COMPLETE SYSTEM: GTP dependence in Poly-U-directed [^{14}C] Phe incorporation and TYMV-RNA-directed [^{14}C] Leu incorporation (V.faba tRNA)

tRNA	COMPLETE c.p.m./mg microsomes	%	COMPLETE — GTP c.p.m./mg microsomes	%
V.faba	10,700	100	10,800	100.9
P.aureus	3,556	100	3,502	98
TYMV-RNA	1,820	100	1,800	99

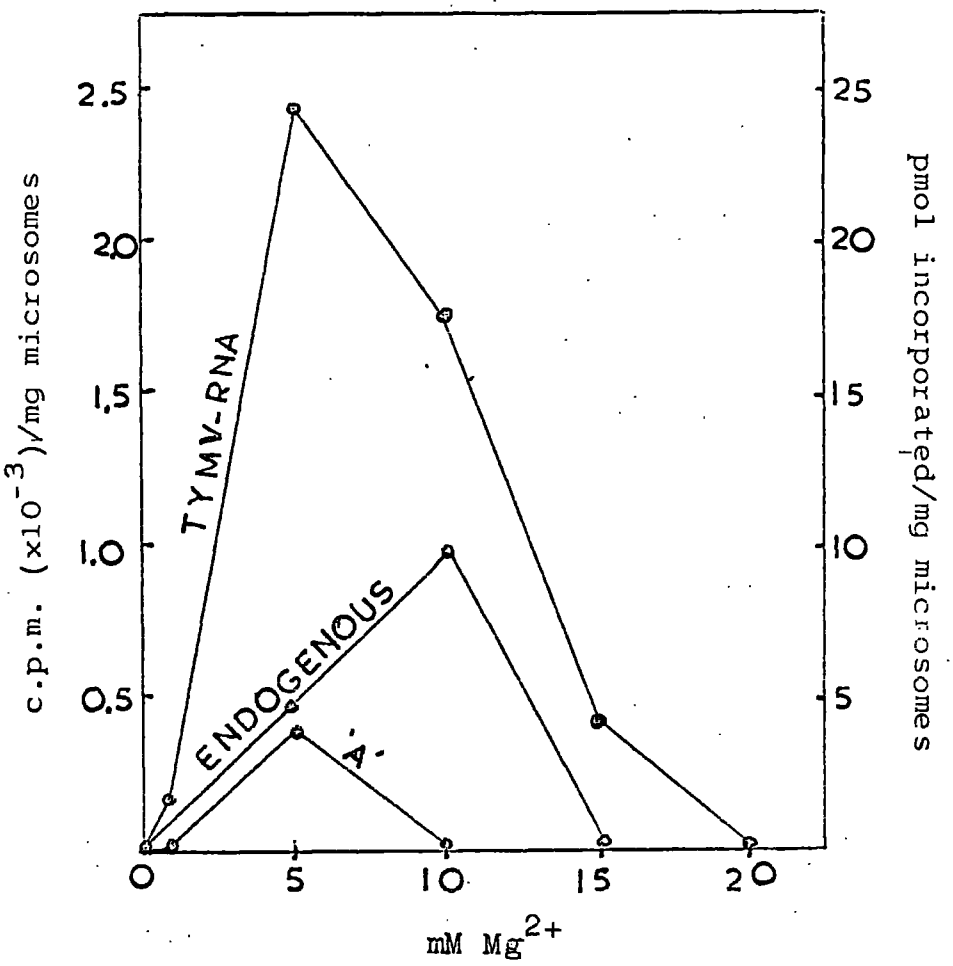
Incubation details: V.faba and P.aureus as in TABLE 5 with appropriate omissions and substitutions. TYMV-RNA system: 0.5ml incubations contained: 30 umol Tris-HCl pH 7.8 at 30°, 35 µmol KCl, 2 µmol ATP, 5 µmol CreP, 10 µg PCK, 0.1 µmol GTP, 2.5 µmol $MgCl_2$, 5 µmol GSH, 0.01 µmol [^{14}C] Leu, 0.01 µmol 19 [^{12}C] amino acid mixture, 0.2mg tRNA, 0.5mg microsomes, 0.25mg TYMV-RNA and 0.04ml high-speed supernatant. Incubation was at 30° and 0.05ml samples assayed at 0, 10, 15, 30, 45, 60 min. Radioactivity/disc determined as in Methods 3. 30 min assays are reported.

Fig.42. COMPLETE SYSTEM: GTP dependence in Poly-U-directed [^{14}C] Phe incorporation (Yeast tRNA).



0.5 ml incubations contained: 30 µmol Tris-HCl pH 7.8 at 30°, 35 µmol KCl, 2 µmol ATP, 5 µmol CreP, 10 µg PCK, 0.1 µmol GTP, 5 µmol GSH, 0.01 µmol [^{14}C] Phe, 0.01 µmol 19[^{12}C] amino acid mixture, 0.5 mg microsomes, 6 µmol $MgCl_2$, 0.2 mg tRNA, 0.1 mg Poly-U and 0.06 ml high-speed supernatant. Incubation was at 30° and 0.05 ml samples assayed at times indicated. Radioactivity/disc determined as in Methods 3.

Fig.43. COMPLETE SYSTEM: TYMV-RNA-directed [^{14}C] Leu incorporation (P. aureus tRNA).



'A' : TYMV-RNA without tRNA supplementation.

0.5 ml incubations contained: 30 µmol Tris-HCl pH 7.8 at 30°, 35 µmol KCl, 2 µmol ATP, 5 µmol CreP, 10 µg PCK, 0.1 µmol, GTP, varying µmol $MgCl_2$, 5 µmol GSH, 0.01 µmol [^{14}C] Leu, 0.01 µmol 19[^{12}C] amino acid mixture and 0.5 mg microsomes, 0.2 mg P. aureus tRNA, 0.1 mg TYMV-RNA, 0.08 ml high-speed supernatant. Incubation was at 28° and 0.1 ml samples assayed at 0, 30, 60 min. Radioactivity/disc determined as in Methods 3. 30 min assays are reported.

graph also records the marginal activity promoted by TYMV-RNA in the absence of exogenously-supplied tRNA, but this was an exceptional result.

In the Complete System which included V.faba tRNA, optimal TYMV-RNA direction was at 2mM Mg^{2+} whilst the endogenous activity was again more pronounced at 10mM (FIG.44). There was no activity in the absence of tRNA supplementation. The time course of incorporation was complete at 30 min (FIG.45).

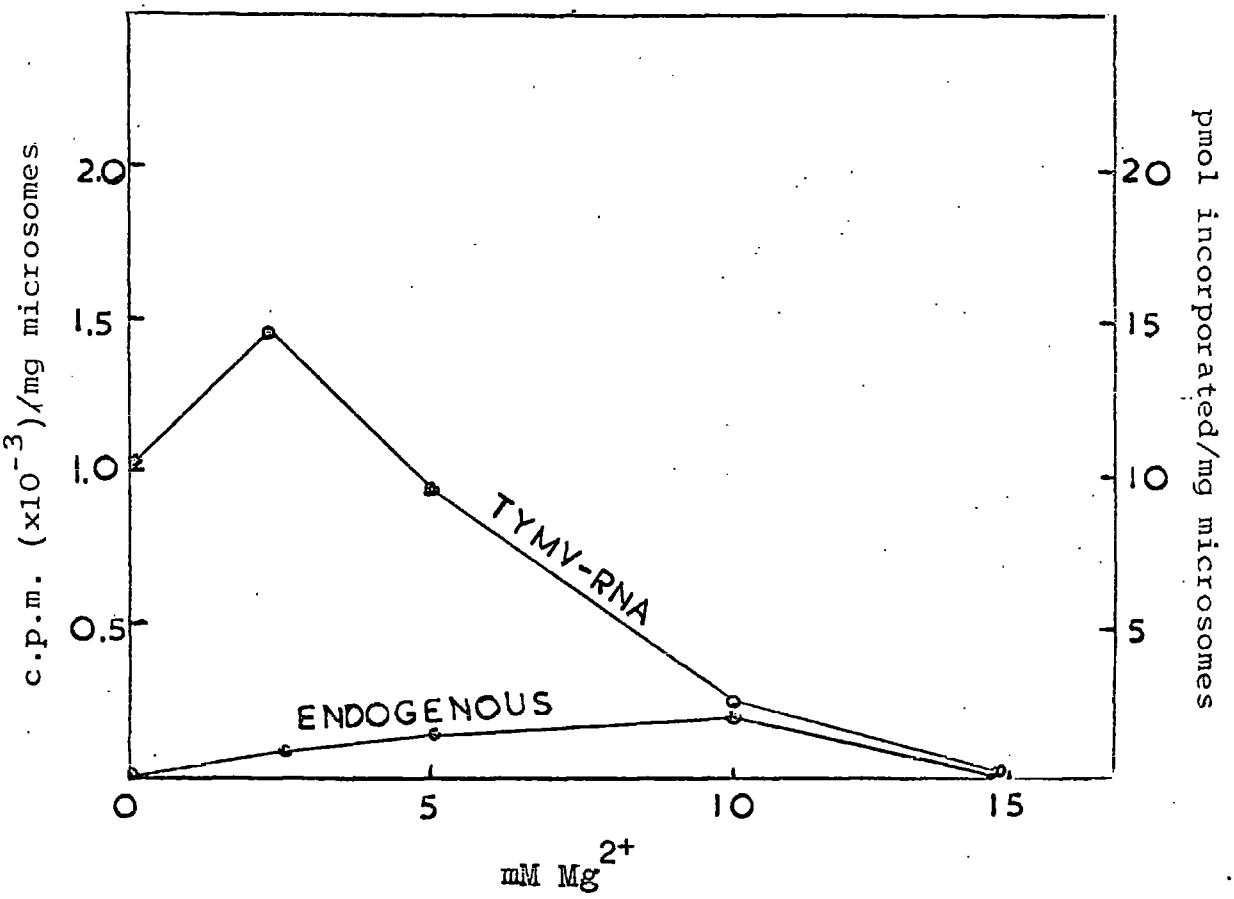
Dependence of the incorporation upon ATP, PCK and CreP is shown in TABLE 7; GTP dependence is shown in conjoint TABLE 6.

In general, the Complete System with yeast tRNA showed no activity in the presence of TYMV-RNA. Variations of parameters having failed to promote viral-RNA direction, a higher specific activity L-[^{14}C]Leu was used. The results are shown in FIGS. 46 and 47. The recorded response contrasts sharply with the presented profiles of viral-RNA direction in the presence of bean tRNAs.

In the Complete System, TYMV-RNA was presented in 0.01M-potassium acetate buffer. Viral-RNA failed to direct incorporation if thawed and frozen more than once, but repeated freezing and thawing did not affect the charging properties of the RNA. Viral-RNA preparations were stored at -20°.

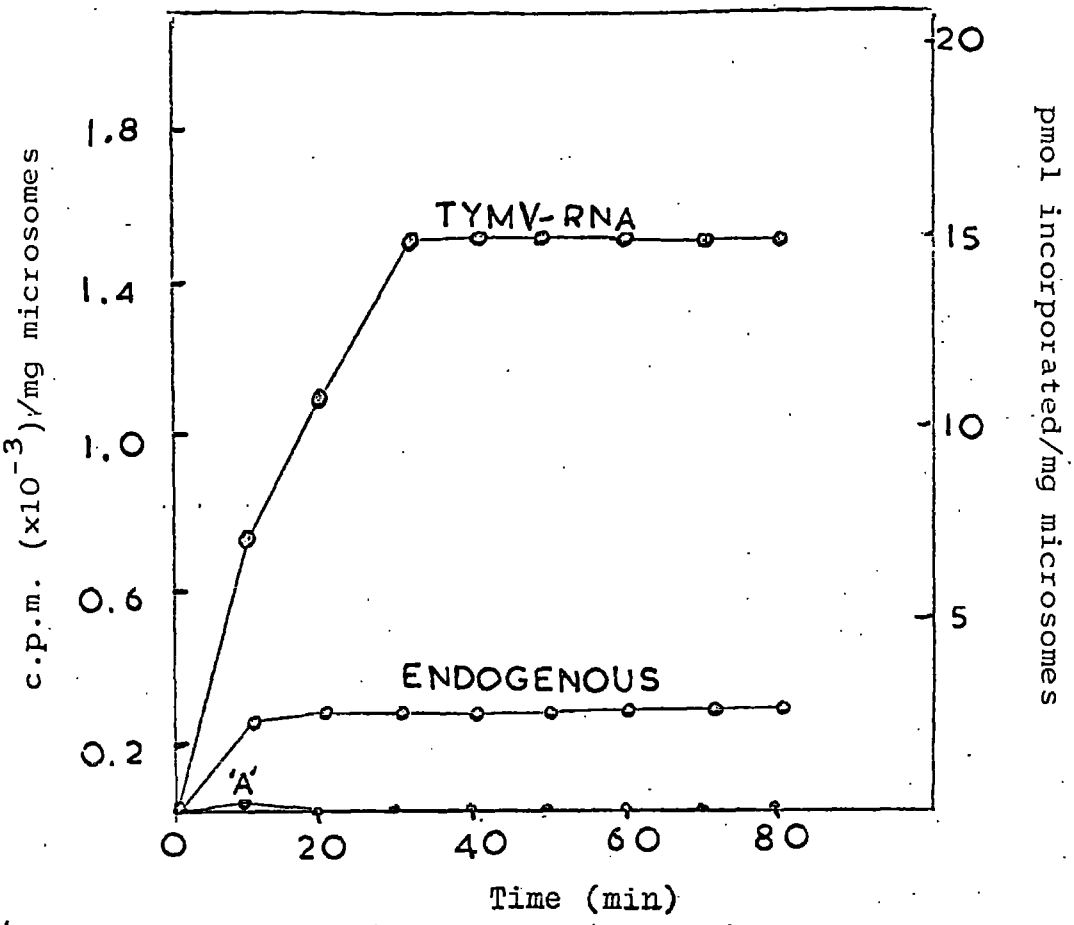
In Poly-U- and TYMV-RNA-directed systems, microsomes were stored at -70° at a concentration of 20mg/ml, maintaining their activity for at least 6 months with repeated freezing and thawing. The endogenous levels of activity, however, were not reproducible and once-thawed microsomal preparations had a

Fig.44. COMPLETE SYSTEM: TYMV-RNA-directed [^{14}C] Leu incorporation (V. faba tRNA).



0.5 ml incubations contained: 30 µmol Tris-HCl pH 7.8 at 30°, 35 µmol KCl, 2 µmol ATP, 5 µmol CreP, 10 µg PCK, 0.1 µmol GTP, varying µmol $MgCl_2$, 5 µmol GSH, 0.01 µmol [^{14}C] Leu, 0.01 µmol 19 [^{12}C] amino acid mixture, 0.5 mg microsomes, 0.13 mg TYMV-RNA, 0.2 mg V. faba tRNA and 0.04 ml high-speed supernatant. Incubation was at 30° and 0.05 samples assayed at 0, 10, 20, 30, 40, 60 min. Radioactivity/disc determined as in Methods 3. 20 min assays are reported.

Fig.45. COMPLETE SYSTEM: Time course of TYMV-RNA-directed [^{14}C] Leu incorporation (*V. faba* tRNA).



'A': TYMV-RNA without tRNA supplementation.

0.5 ml incubations contained: 30 µmol Tris-HCl pH 7.8 at 30°, 35 µmol KCl, 2 µmol ATP, 5 µmol CreP, 10 µg PCK, 0.1 µmol GTP, 2.5 µmol $MgCl_2$, 5 µmol GSH, 0.01 µmol [^{14}C] Leu, 0.01 µmol 19 [^{12}C] amino acid mixture, 0.5 mg microsomes, 0.13 mg TYMV-RNA, 0.2 mg *V. faba* tRNA and 0.04 ml high-speed supernatant. Incubation was at 30° and 0.05 ml samples assayed at times indicated. Radioactivity/disc determined as in Methods 3.

TABLE 7. COMPLETE SYSTEM: ATP, PCK, Cre P in TYMV-RNA-directed [^{14}C] Leu incorporation (V.faba tRNA)

	Complete		Complete - ATP, PCK, Cre P	
	c.p.m./mg microsomes	%	c.p.m./mg microsomes	%
TYMV-RNA	820	100	766	93

0.5ml incubations contained: 30 µmol Tris-HCl pH 7.8 at 30°, 35 µmol KCl, 2 µmol ATP, 5 µmol CreP, 10 µg PCK, 0.1 µmol GTP, 2.5 µmol $MgCl_2$, 5 µmol GSH, 0.01 µmol [^{14}C] Leu, 0.2mg tRNA, 0.01 µmol 19 [^{12}C] amino acid mixture, 0.5mg microsomes, 0.25mg TYMV-RNA and 0.04ml high-speed supernatant (ATP, PCK, CreP omission as indicated). Incubation was at 30° and 0.05ml samples assayed at 0, 10, 15, 30, 45, 60 min. Radioactivity/disc determined as in Methods 3. 30 min assays are reported.

Fig.46. COMPLETE SYSTEM: Time course of TYMV-RNA-directed $[^{14}C]$ Leu* incorporation (Yeast tRNA).

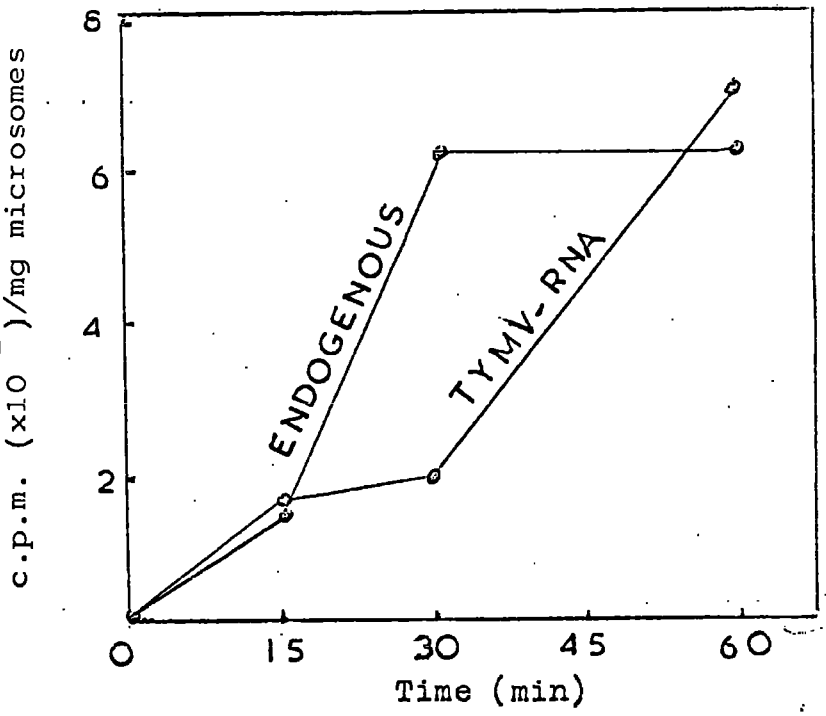
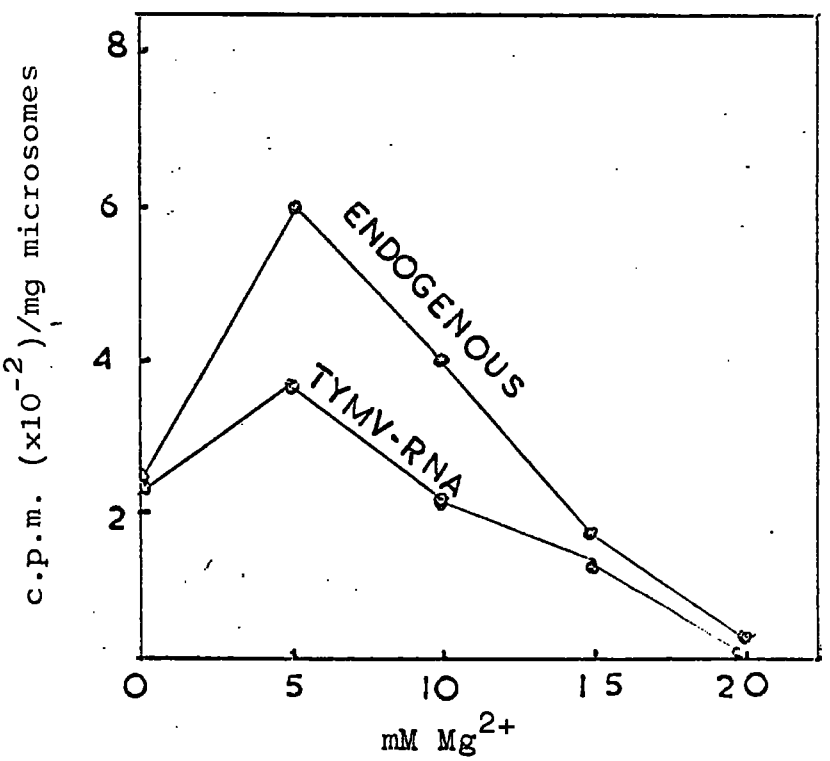


Fig.47. COMPLETE SYSTEM: Effect of Mg^{2+} concentration on TYMV-RNA-directed $[^{14}C]$ Leu incorporation (Yeast tRNA).



greater apparent endogenous activity than repeatedly frozen and thawed preparations.

5. Transfer System

Yeast tRNA was precharged with [^{14}C] Leu, [^{14}C] Phe or the 14 [U^{14}C] amino acid mixture (Methods 20). V.faba tRNA was precharged with [^{14}C] Phe or the 14 [U^{14}C] amino acid mixture (Methods 20).

(A) Poly-U-directed incorporation of [^{14}C] Phe:

A sharp optimum at 80mM K^+ was shown for the [^{14}C] Phe-yeast tRNA Transfer System, whereas the optimum for the 14 [U^{14}C] AA-V.faba tRNA system at 80mM was much less critical (FIGS.48, 49).

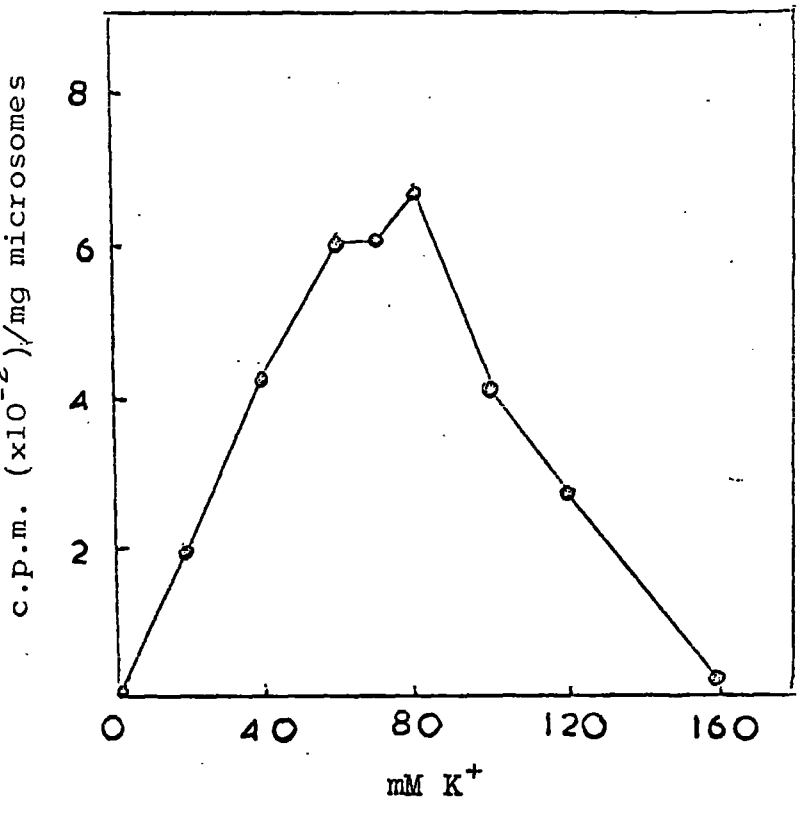
The Mg^{2+} optimum in each Transfer System was 8mM (FIGS.50, 51), though the yeast system showed a more critical response than the V.faba. The pH effect contrasted sharply in the two systems (FIGS.52, 53). In the V.faba system there was no optimum between pH 7.3 - 8.5; in the yeast system there was activity in the range pH 7.3 - 8.1 with a discernible optimum at pH 7.6.

The enzymatic properties of the microsomal preparation are illustrated by FIGS.54 and 55. Addition of the high-speed supernatant enzyme fraction did not enhance incorporation.

Dependence of the Transfer System on GTP was partial (FIGS.56, 57) and some depression of activity was observed when ATP was added to the [^{14}C] Phe-yeast tRNA system (FIG.57).

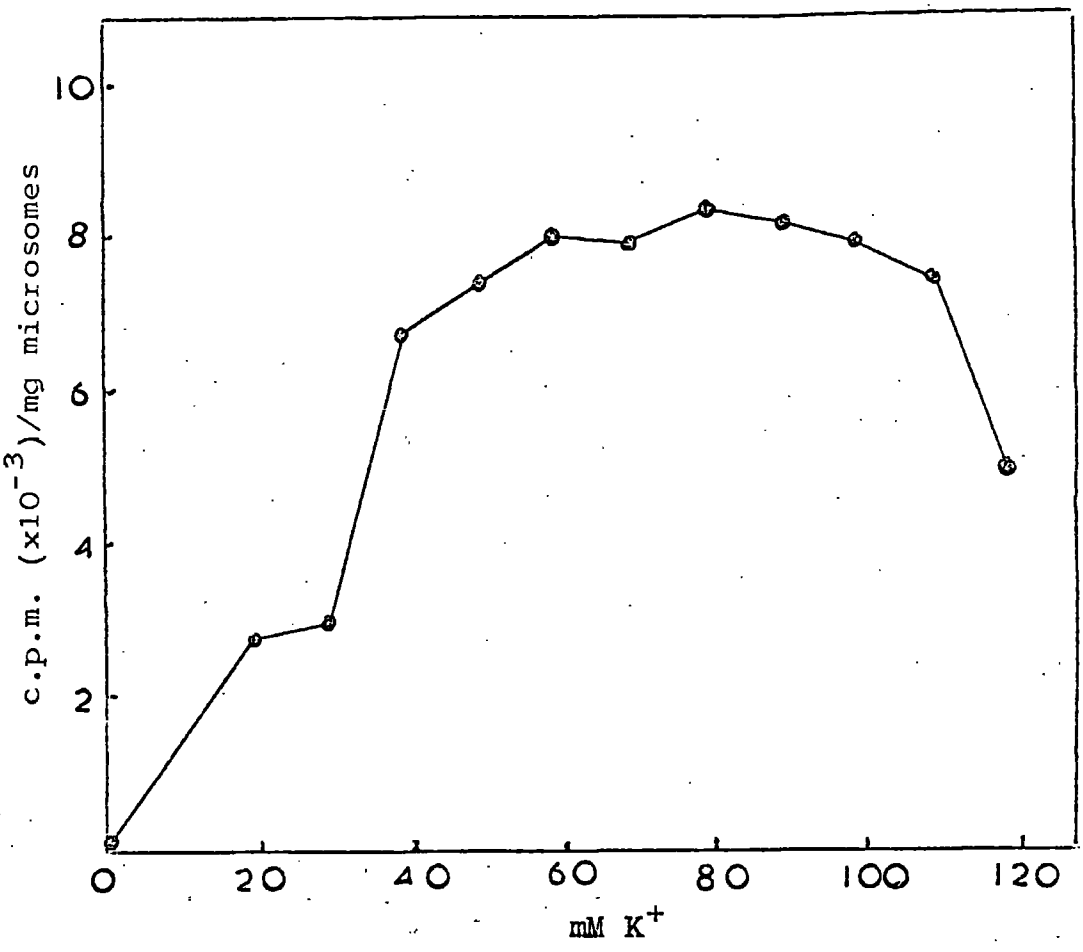
Most of the graphs show the Poly-U dependence of the incorporations and endogenous activity was not apparent in systems using [^{14}C] Phe-yeast tRNA and 14 [U^{14}C] AA-yeast tRNA (FIGS.55, 57, 58). Transfer from [^{14}C] Phe-

Fig.48. TRANSFER SYSTEM: Effect of K^+ concentration on Poly-U-directed Phe incorporation from [^{14}C] Phe-Yeast tRNA.



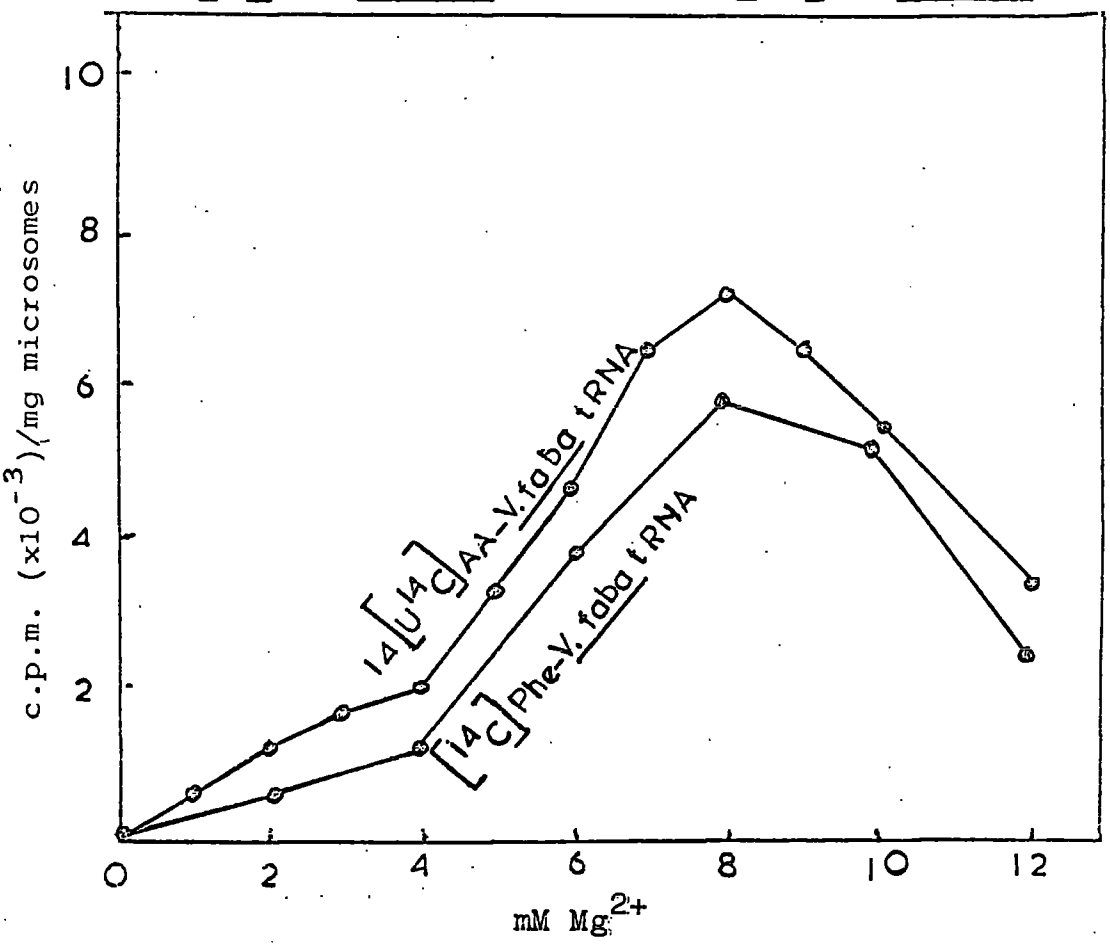
0.25 ml incubations contained: 15 µmol Tris-HCl pH 7.8 at 30°, varying µmol KCl, 2 µmol $MgCl_2$, 0.05 µmol GTP, 2.5 µmol GSH, 0.05 mg Poly-U, 0.25 mg microsomes, 0.1 mg yeast tRNA precharged with [^{14}C] Phe. Incubation was at 30° and 0.05 ml samples assayed at 0, 15, 30, 60 min. Radioactivity/disc determined as in Methods 3. 30 min assays are reported.

Fig.49. TRANSFER SYSTEM: Effect of K^+ concentration on Poly-U-directed Phe incorporation from 14 [U-^{14}C] AA-*V. faba* tRNA.



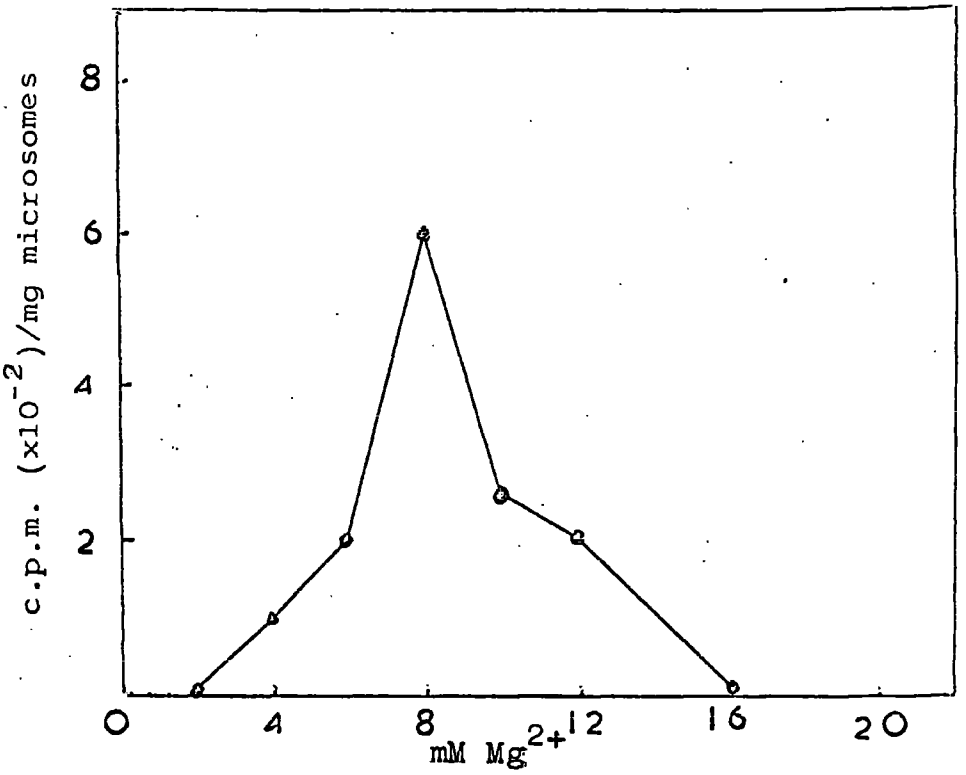
0.25 ml incubations contained: 15 µmol Tris-HCl pH 7.8 at 30°, varying µmol KCl, 2 µmol $MgCl_2$ 0.05 µmol GTP, 2.5 µmol GSH, 0.05 mg Poly-U, 0.25 mg microsomes, 0.1 mg *V. faba* tRNA precharged with 14 [U-^{14}C] AA. Incubation was at 30° and 0.05 ml samples assayed at 0, 15, 30, 60 min. Radioactivity/disc determined as in Methods 3. 30 min assays are reported.

Fig.50. TRANSFER SYSTEM: Effect of Mg^{2+} concentration on Poly-U direction of Phe incorporation from $[^{14}C]$ Phe-*V. faba* tRNA and $^{14}[U^{14}C]$ AA-*V. faba* tRNA.



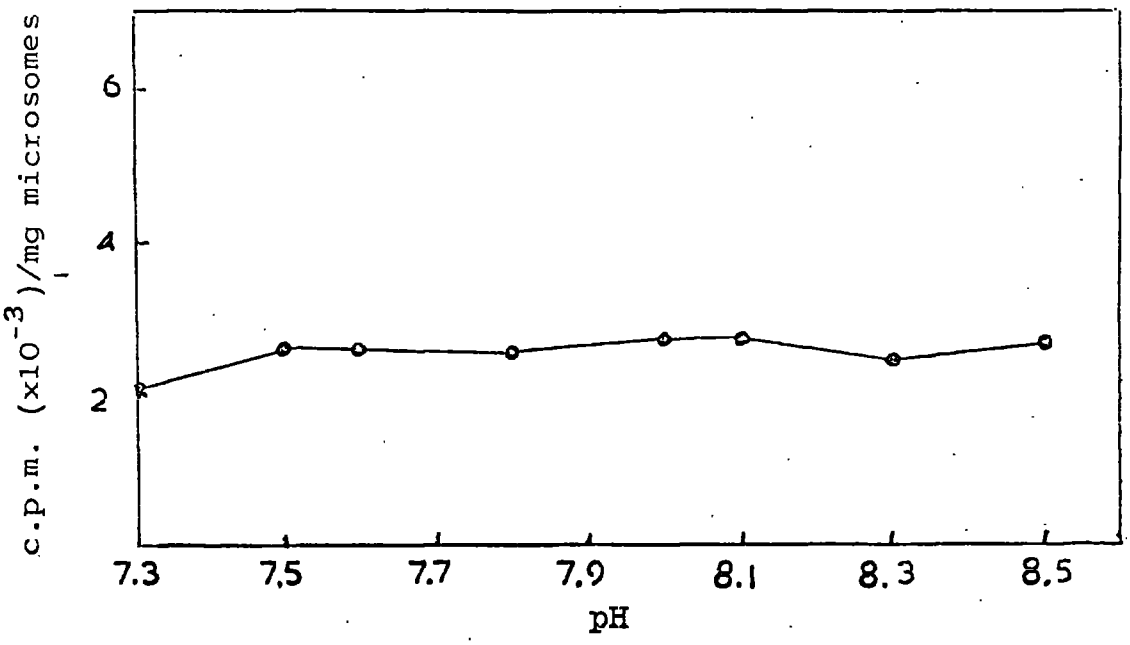
0.25 ml incubations contained: 15 µmol Tris-HCl pH 7.8 at 30°, 20 µmol KCl, varying µmol $MgCl_2$, 0.05 µmol GTP, 2.5 µmol GSH, 0.05 mg Poly-U, 0.25 mg microsomes, and 0.1 mg *V. faba* tRNA precharged with either $[^{14}C]$ Phe or $^{14}[U^{14}C]$ AA. Incubation was at 30° and 0.05 ml samples assayed at 0, 15, 30, 60 min. Radioactivity/disc determined as in Methods 3. 30 min assays are reported.

Fig.51. TRANSFER SYSTEM: Effect of Mg^{2+} concentration of Poly-U direction of Phe incorporation from $^{14}[U^{14}C]$ AA-Yeast tRNA.



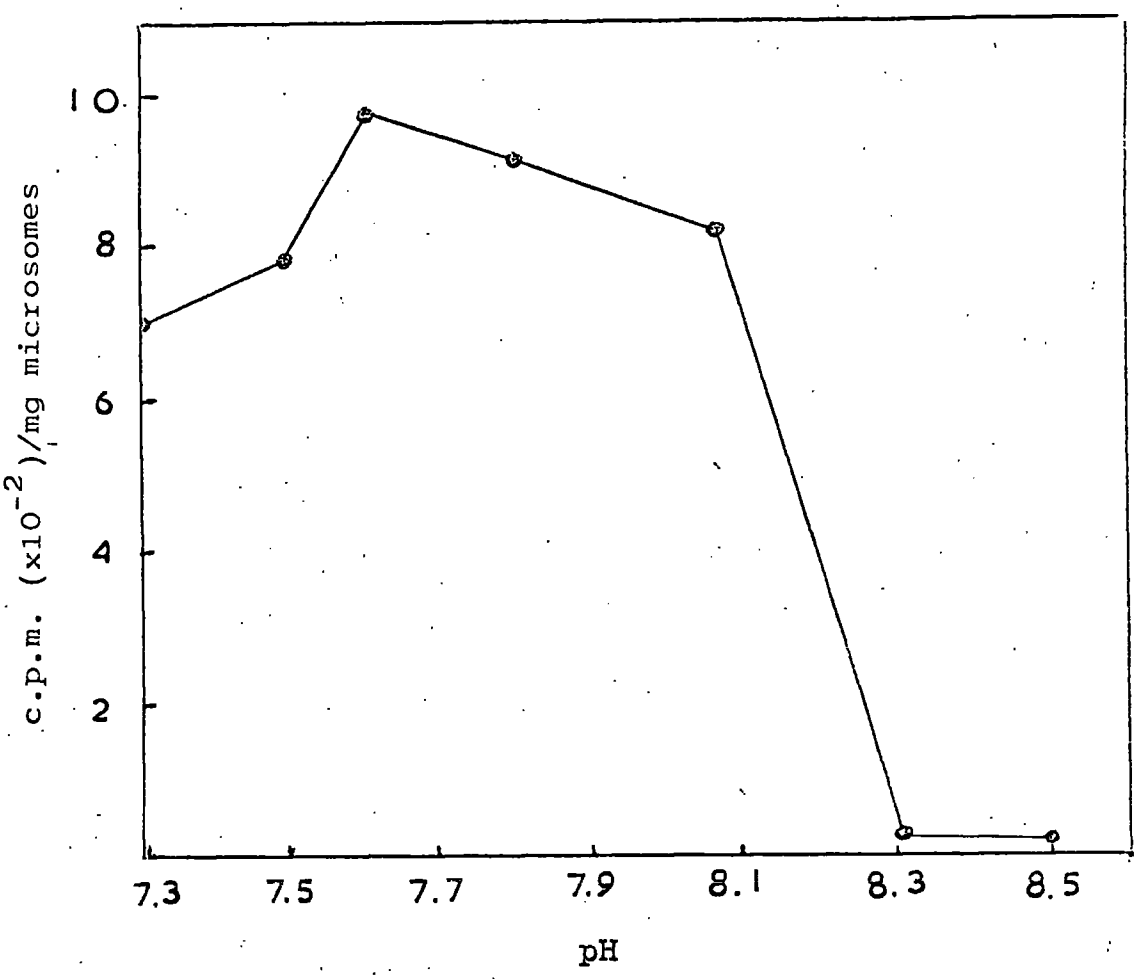
0.25 ml incubations contained: 15 µmol Tris-HCl pH 7.8 at 30°, 20 µmol KCl, varying µmol $MgCl_2$, 0.05 µmol GTP, 2.5 µmol GSH, 0.05 mg Poly-U, 0.25 mg microsomes and 0.1 mg yeast tRNA precharged with $^{14}[U^{14}C]$ AA. Incubation was at 30° and 0.05 ml samples assayed at 0, 20, 40, 80 min. Radioactivity/disc determined as in Methods 3. 40 min assays are reported.

Fig.52. TRANSFER SYSTEM: Effect of pH on Poly-U-directed Phe incorporation from [^{14}C] Phe — V. faba tRNA.



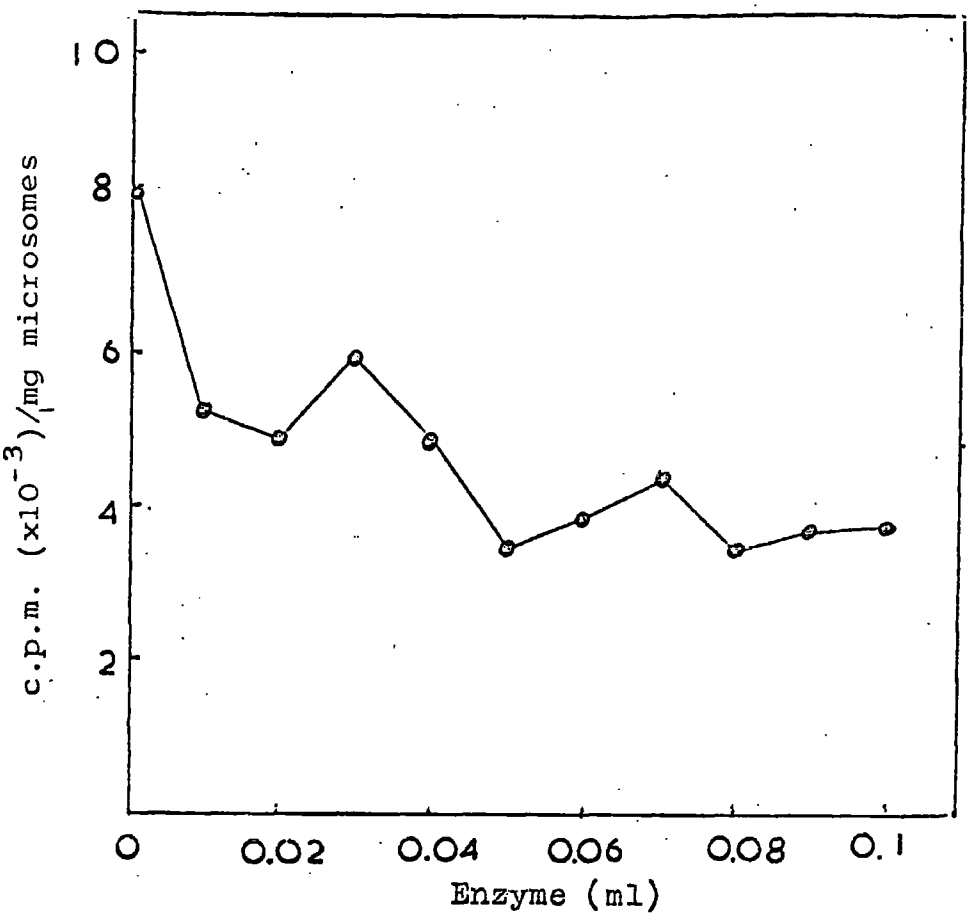
0.25 ml incubations contained: 15 µmol Tris-HCl varying pH at 30°, 15 µmol KCl, 2 µmol $MgCl_2$, 0.05 µmol GTP, 2.5 µmol GSH, 0.05 mg Poly-U, 0.25 mg microsomes and 0.1 mg V. faba tRNA precharged with [^{14}C] Phe. Incubation was at 30° and 0.05 ml samples assayed at 0, 15, 30, 60 min. Radioactivity/disc determined as in Methods 3. 15 min assays are reported.

Fig.53. TRANSFER SYSTEM: Effect of pH on Poly-U-directed Phe incorporation from [^{14}C] Phe-Yeast tRNA.



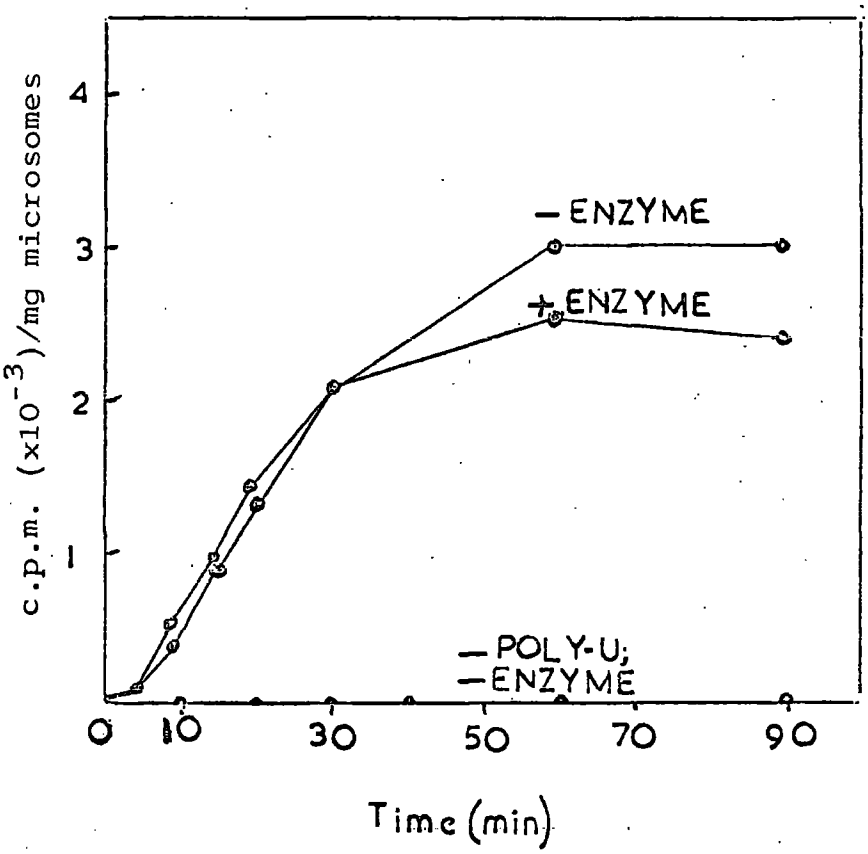
0.25 ml incubations contained: 15 µmol Tris-HCl varying pH at 30°, 20 µmol KCl, 2 µmol $MgCl_2$, 0.05 µmol GTP, 2.5 µmol GSH, 0.05 mg Poly-U, 0.25 mg microsomes and 0.1 mg yeast tRNA precharged with [^{14}C] Phe. Incubation was at 30° and 0.05 ml samples assayed at 0, 15, 30, 60 min. Radioactivity/disc determined as in Methods 3. 30 min assays are reported.

Fig.54. TRANSFER SYSTEM: Effect of supplementation of P. aureus high-speed supernatant enzyme fraction on Poly-U-directed Phe incorporation from [^{14}C] Phe-V. faba tRNA.



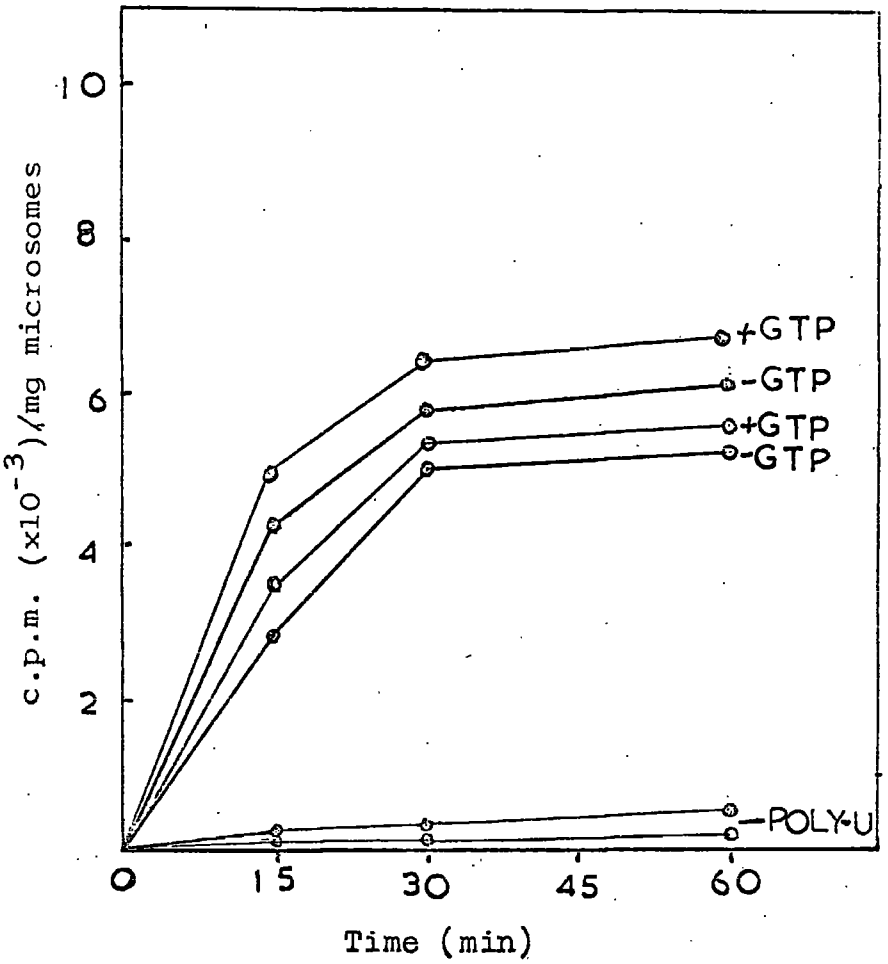
0.25 ml incubations contained: 15 µmol Tris-HCl pH at 30°, 15 µmol KCl, 2 µmol $MgCl_2$, 0.05 µmol GTP, 2.5 µmol GSH, 0.05 mg Poly-U, 0.25 mg microsomes, 0.1 mg V. faba tRNA precharged with [^{14}C] Phe, and varying vol. high-speed supernatant. Incubation was at 30° and 0.05 ml samples assayed at 0, 15, 30, 60 min. Radioactivity/disc determined as in Methods 3. 30 min assays are reported.

Fig.55. TRANSFER SYSTEM: Effect of supplementation of P. aureus high-speed supernatant enzyme fraction on Poly-U-directed Phe incorporation from [^{14}C] Phe-Yeast tRNA.



0.5 ml incubations contained: 30 µmol Tris-HCl pH 7.8 at 30°, 40 µmol KCl, 4 µmol $MgCl_2$, 0.1 µmol GTP, 5 µmol GSH, 0.5 mg microsomes, 0.1 mg Poly-U, 0.1 mg yeast tRNA precharged with [^{14}C] Phe, with and without 0.08 ml high-speed supernatant. Incubation was at 30° and 0.05 ml samples assayed as indicated. Radioactivity/disc determined as in Methods 3.

Fig.56. TRANSFER SYSTEM: GTP dependence on Poly-U-directed Phe incorporation from [^{14}C] Phe-*V. faba* tRNA and 14 [U^{14}C] AA-*V. faba* tRNA.

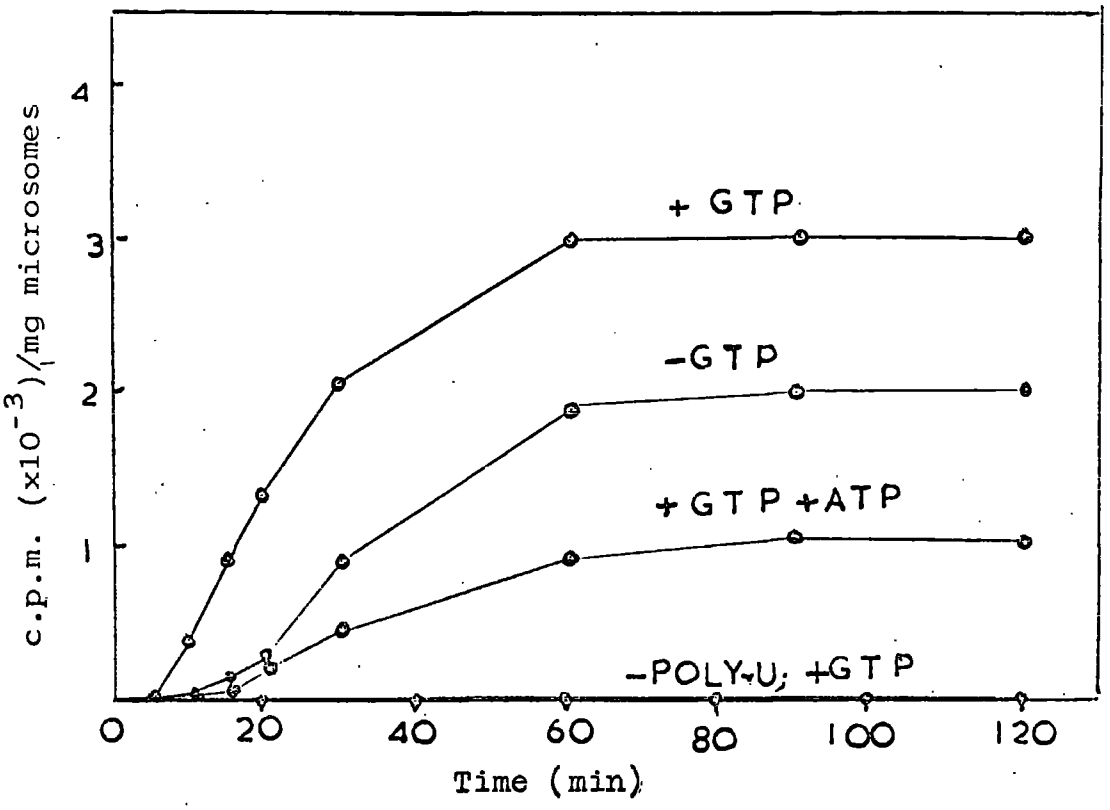


0.25 ml incubations contained: 15 µmol Tris-HCl pH 7.8 at 30°, 0.05 µmol GTP, 2.5 µmol GSH, 2 µmol $MgCl_2$, 20 µmol KCl, 0.05 mg Poly-U, 0.25 mg microsomes and 0.2 mg *V. faba* tRNA precharged with [^{14}C] Phe or 14 [U^{14}C] AA. Incubation at 30° and 0.05 ml samples assayed at times indicated. Radioactivity/disc determined as in Methods 3.

—●—●— : 14 [U^{14}C] AA-*V. faba* tRNA.

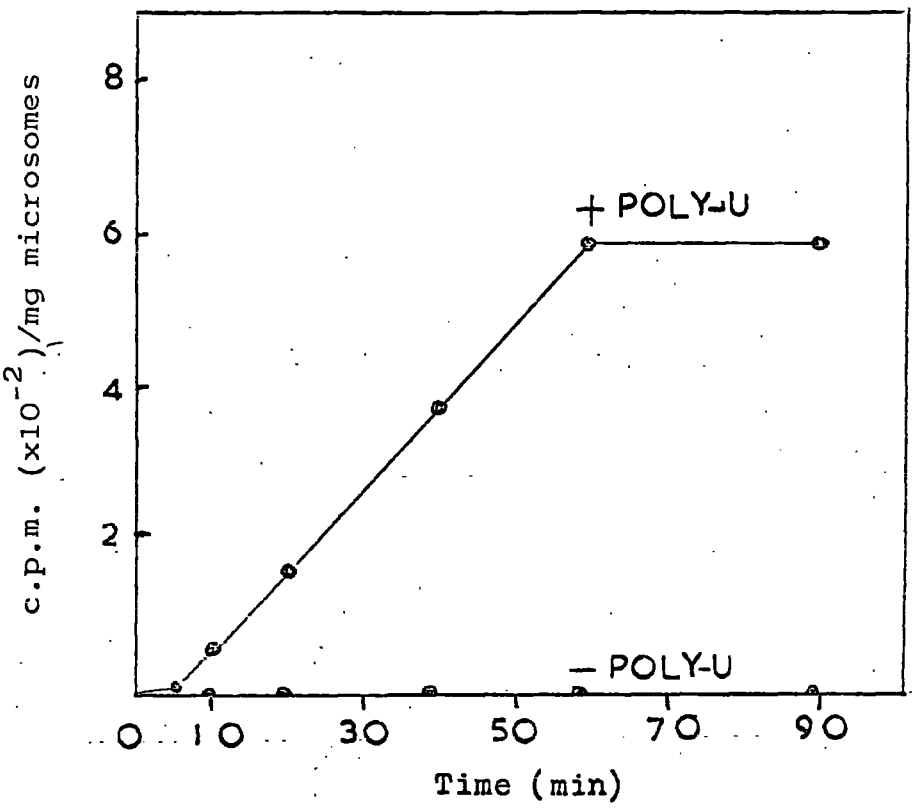
—○—○— : [^{14}C] Phe-*V. faba* tRNA.

Fig.57. TRANSFER SYSTEM: GTP dependence of Poly-U-directed Phe incorporation from [^{14}C] Phe-Yeast tRNA.



0.5 ml incubations contained: 30 µmol Tris-HCl pH 7.8 at 30°, 0.1 µmol GTP, 5 µmol GSH, 4 µmol $MgCl_2$, 40 µmol KCl, 0.1 mg Poly-U, 0.5 mg microsomes and 0.2 mg tRNA precharged with [^{14}C] Phe. Addition 2 µmol ATP as indicated. Incubation was at 30° and 0.05 ml samples assayed at times indicated. Radioactivity/disc determined as in Methods 3.

Fig.58. TRANSFER SYSTEM: Poly-U-directed Phe incorporation from 14[U^{14}C]AA-Yeast tRNA.



0.5 ml incubations contained: 30 µmol Tris-HCl pH 7.8 at 30°, 0.1 µmol GTP, 5 µmol GSH, 4 µmol $MgCl_2$, 40 µmol KCl, 0.1 mg Poly-U, 0.5 mg microsomes, and 0.2 mg yeast tRNA precharged with 14[U^{14}C]AA. Incubation was at 30° and 0.05 ml samples assayed at times indicated. Radioactivity/disc determined as in Methods 3.

V.faba tRNA and 14 [U^{14}C] AA-V.faba tRNA showed incorporation in the absence of Poly-U (FIGS.56,59).

There was an obvious lag phase in incorporation from precharged yeast tRNA Transfer Systems (FIGS.57,58). This 5 min lag was not apparent in precharged V.faba tRNA systems.

Levels of incorporation activity differed considerably between 14 [U^{14}C] AA-V.faba tRNA and its yeast counterpart - the latter only achieving some 10% activity. Incorporation from [^{14}C] Phe-precharged tRNAs from yeast and V.faba were of the same order.

The rates and completion of incorporation varied between the yeast and V.faba precharged system. Generally, the yeast system had terminated at 1 hr whereas V.faba gave a linear response to about 30 min, terminating at 30 - 60 min.

The optimal concentration of precharged tRNA in the Transfer System was 0.4mg tRNA/1ml incubation. (FIG.60).

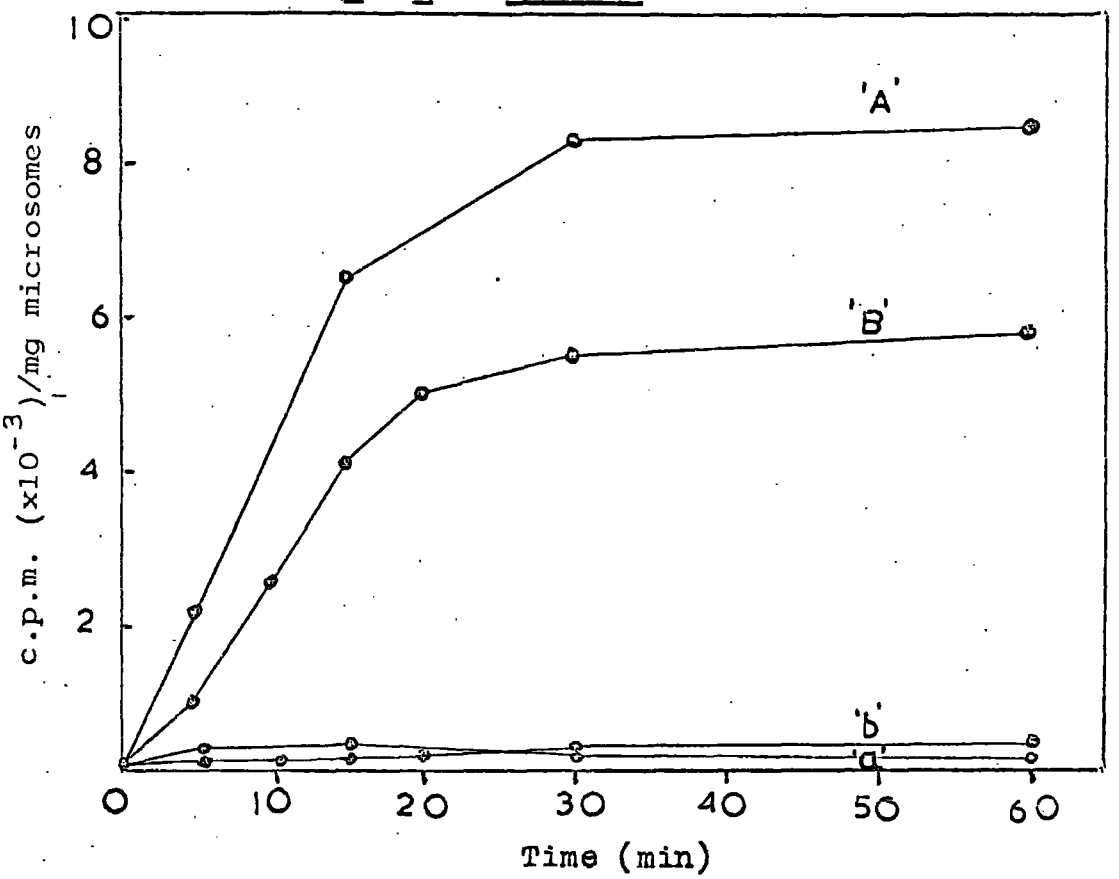
(B) TYMV-RNA-directed incorporation of [^{14}C] amino acids:

No viral-RNA activity was demonstrated in Transfer Systems containing [^{14}C] Phe-V.faba tRNA, [^{14}C] Phe-yeast tRNA or [^{14}C] Leu-yeast tRNA.

In the Transfer System from 14 [U^{14}C] AA-V.faba tRNA, TYMV-RNA directed incorporation of [^{14}C] label at a Mg^{2+} optimum of 2mM (FIG.68). The reaction was partially GTP dependent (FIG.61) and the temperature response of incorporation was observed as in FIG.62.

The time-course of TYMV-RNA-directed incorporation was

Fig.59. TRANSFER SYSTEM: Poly-U direction of Phe incorporation from [^{14}C] Phe-*V. faba* tRNA and 14[U ^{14}C] AA-*V. faba* tRNA.



0.5 ml incubations contained: 30 µmol Tris-HCl pH 7.8 at 30°, 40 µmol KCl, 0.1 µmol GTP, 5 µmol GSH, 4 µmol $MgCl_2$, 0.1 mg Poly-U, 0.5 mg microsomes and 0.2 mg *V. faba* tRNA precharged with either [^{14}C] Phe or 14[U ^{14}C] AA. Incubation was at 30° and 0.05 ml samples assayed at times indicated. Radioactivity/disc determined as in Methods 3.

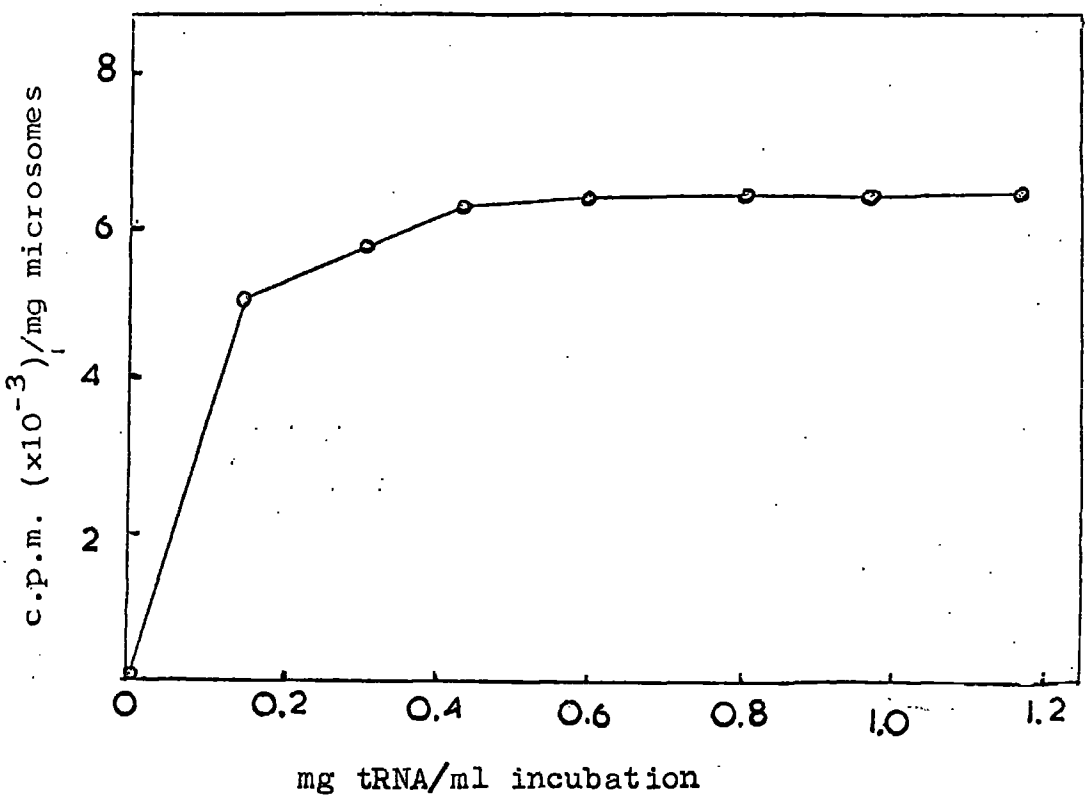
'A': 14[U ^{14}C] AA-*V. faba* tRNA + Poly-U

'a': 14[U ^{14}C] AA-*V. faba* tRNA - Poly-U

'B': [^{14}C] Phe-*V. faba* tRNA + Poly-U

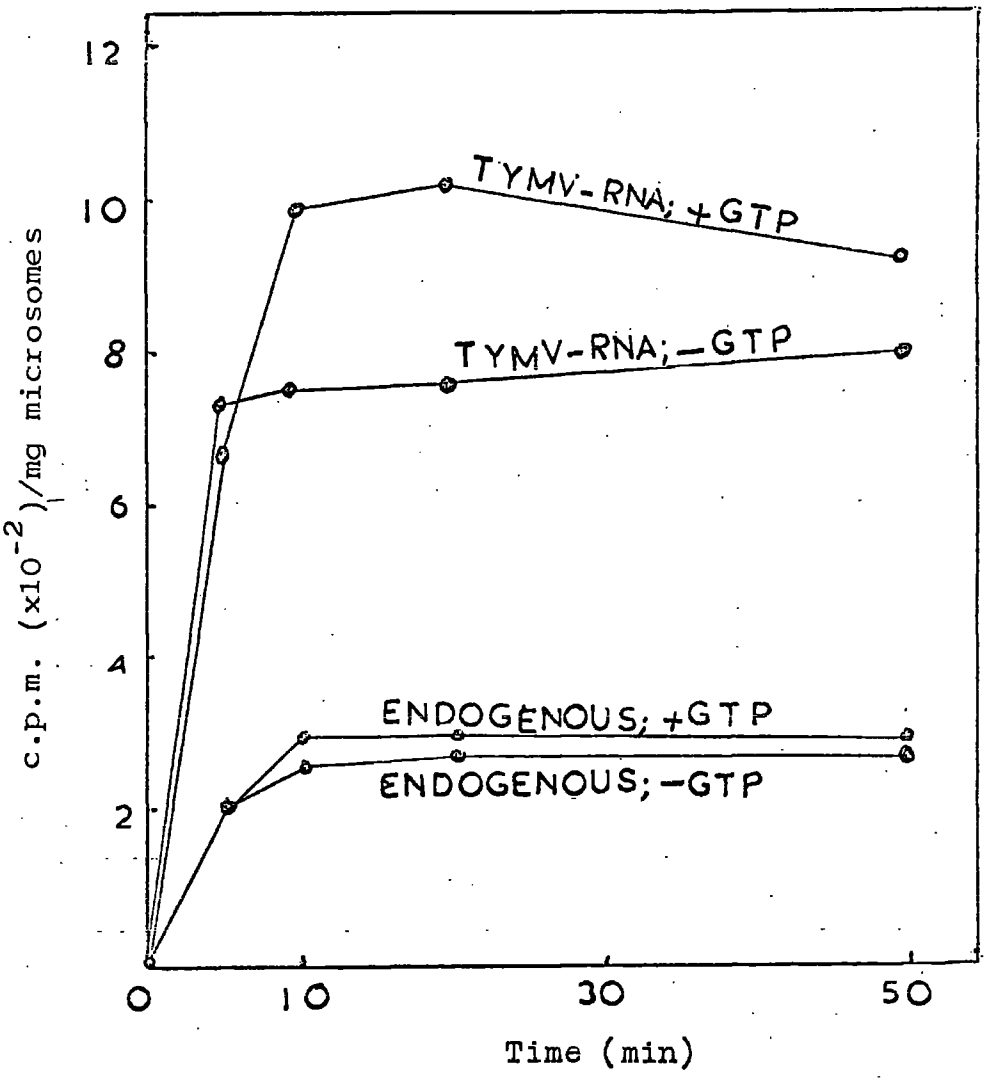
'b': [^{14}C] Phe-*V. faba* tRNA - Poly-U

Fig.60. TRANSFER SYSTEM: Saturation curve for [^{14}C] Phe—*V. faba* tRNA in Poly-U-directed Phe incorporation.



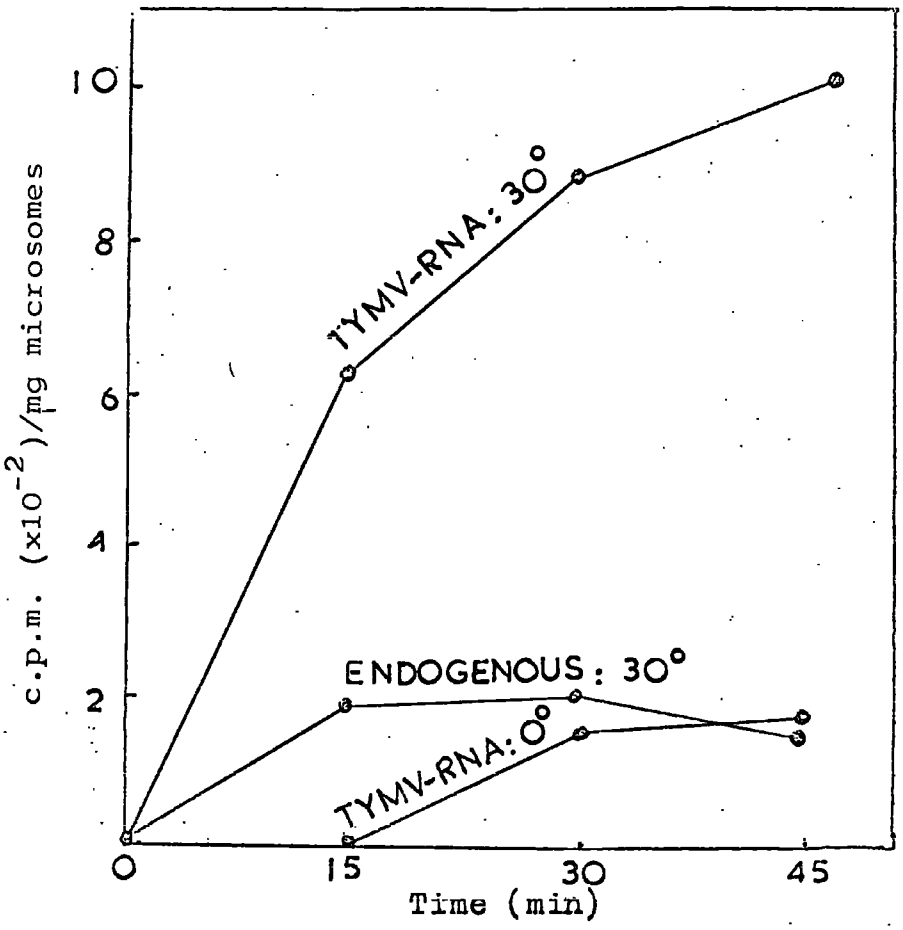
0.25 ml incubations contained: 15 µmol Tris-HCl pH 7.8 at 30°, 15 µmol KCl, 2 µmol $MgCl_2$, 0.05 µmol GTP, 2.5 µmol GSH, 0.05 mg Poly-U, varying mg *V. faba* tRNA precharged with [^{14}C] Phe and 0.25 m'somes. Incubation was at 30° and 0.05 ml samples assayed at 0, 15, 30, 60 min. Radioactivity/disc determined as in Methods 3. 30 min assays are reported.

Fig.61. TRANSFER SYSTEM: GTP dependence of TYMV-RNA-directed incorporation from 14 [U^{14}C] AA - V. faba tRNA.



0.25 ml incubations contained: 15 µmol Tris-HCl pH 7.8 at 30°, 20 µmol KCl, 0.5 µmol $MgCl_2$, 0.05 µmol GTP, 2.5 µmol GSH, 0.25 mg microsomes, 0.2 mg TYMV-RNA and 0.1 mg V.faba tRNA precharged with 14 [U^{14}C] AA. Incubation was at 30° and 0.05 ml samples assayed at times indicated. Radioactivity/disc determined as in Methods 3.

Fig.62. TRANSFER SYSTEM: Effect of temperature on TYMV-RNA-directed incorporation from 14 [U^{14}C] AA-*V. faba* tRNA.



0.25 ml incubations contained: 15 µmol Tris-HCl pH 7.8 at 0° and 30°, 20 µmol KCl, 0.5 µmol $MgCl_2$, 0.05 µmol GTP, 2.5 µmol GSH, 0.25 mg microsomes, 0.2 mg TYMV-RNA and 0.1 mg *V.faba* tRNA precharged with 14 [U^{14}C] AA. Incubation was at 0° and 30° and 0.05 ml samples assayed at times indicated. Radioactivity/disc determined as in Methods 3.

variable (FIGS.61,62). When full time-courses are not reported in the figures and tables, results are recorded at a linear or sub-linear level.

A competitive effect between Poly-U- and TYMV-RNA-direction in the Transfer System was observed. Thus at 8mM Mg^{2+} (Poly-U, Mg^{2+} optimum), incorporation was reduced in the presence of viral-RNA. The time curve of this reaction shows a lag phase not present with Poly-U alone. There was little incorporation directed by TYMV-RNA (FIG.63).

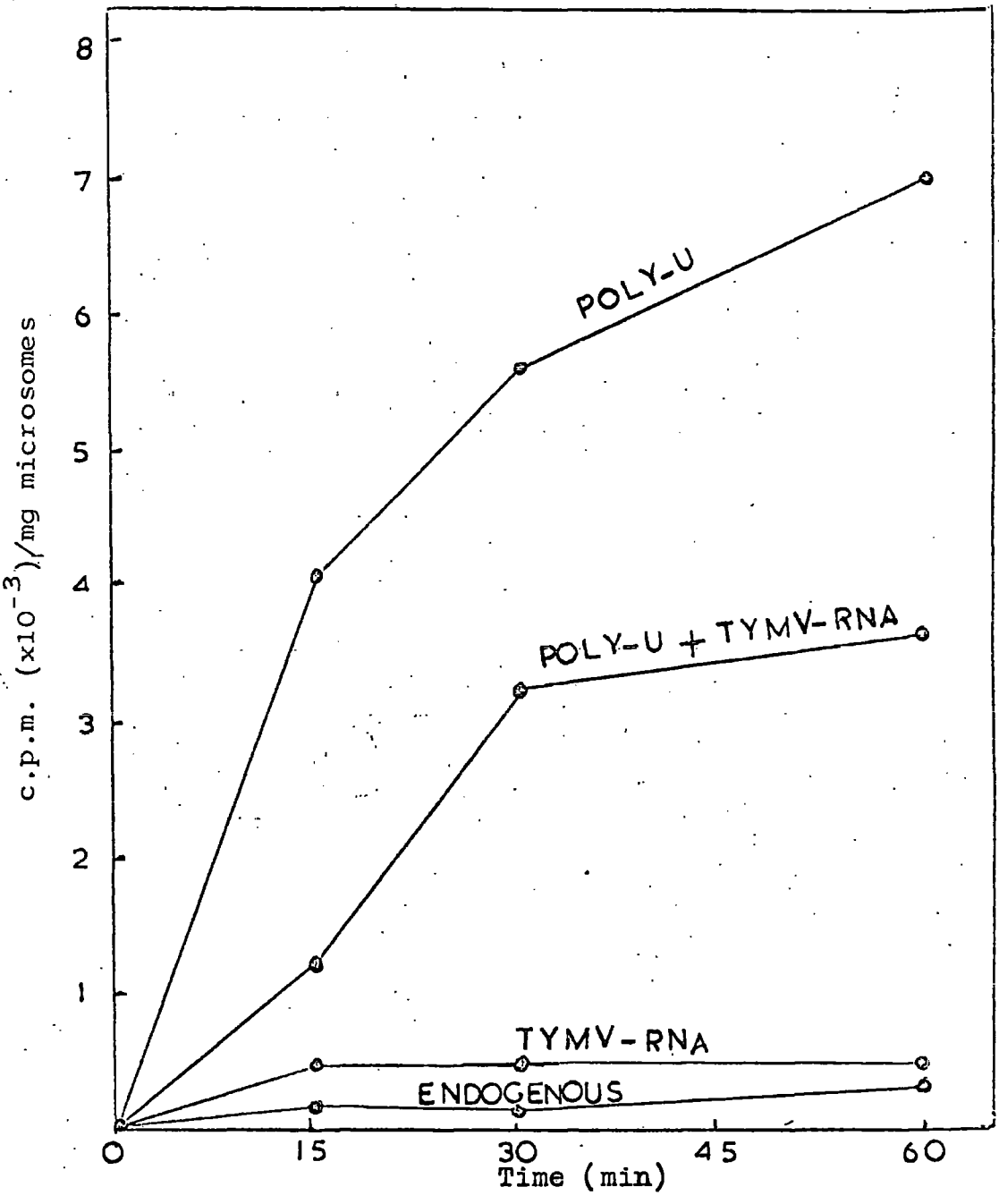
At a Mg^{2+} optimum suitable for TYMV-RNA incorporation direction, an additive effect was shown when both messenger molecules were present; the incorporation response was biphasic, unlike the results obtained with Poly-U and viral-RNA alone (FIG.64).

The 14 $\left[U^{14}C\right]$ AA-yeast tRNA Transfer System showed no significant activity with regard to viral-RNA direction. The results recorded in FIGS.65,66 represent the greatest response achieved in repeated attempts to demonstrate significant activity.

TYMV-RNA directed incorporation from 14 $\left[U^{14}C\right]$ AA-V.faba tRNA only in the presence of K^{+}. There was no critical optimum of concentration, since 20 - 80mM maintained the integrity of the system (FIG.67). Endogenous activity showed optimal K^{+} effects at 60 - 100mM.

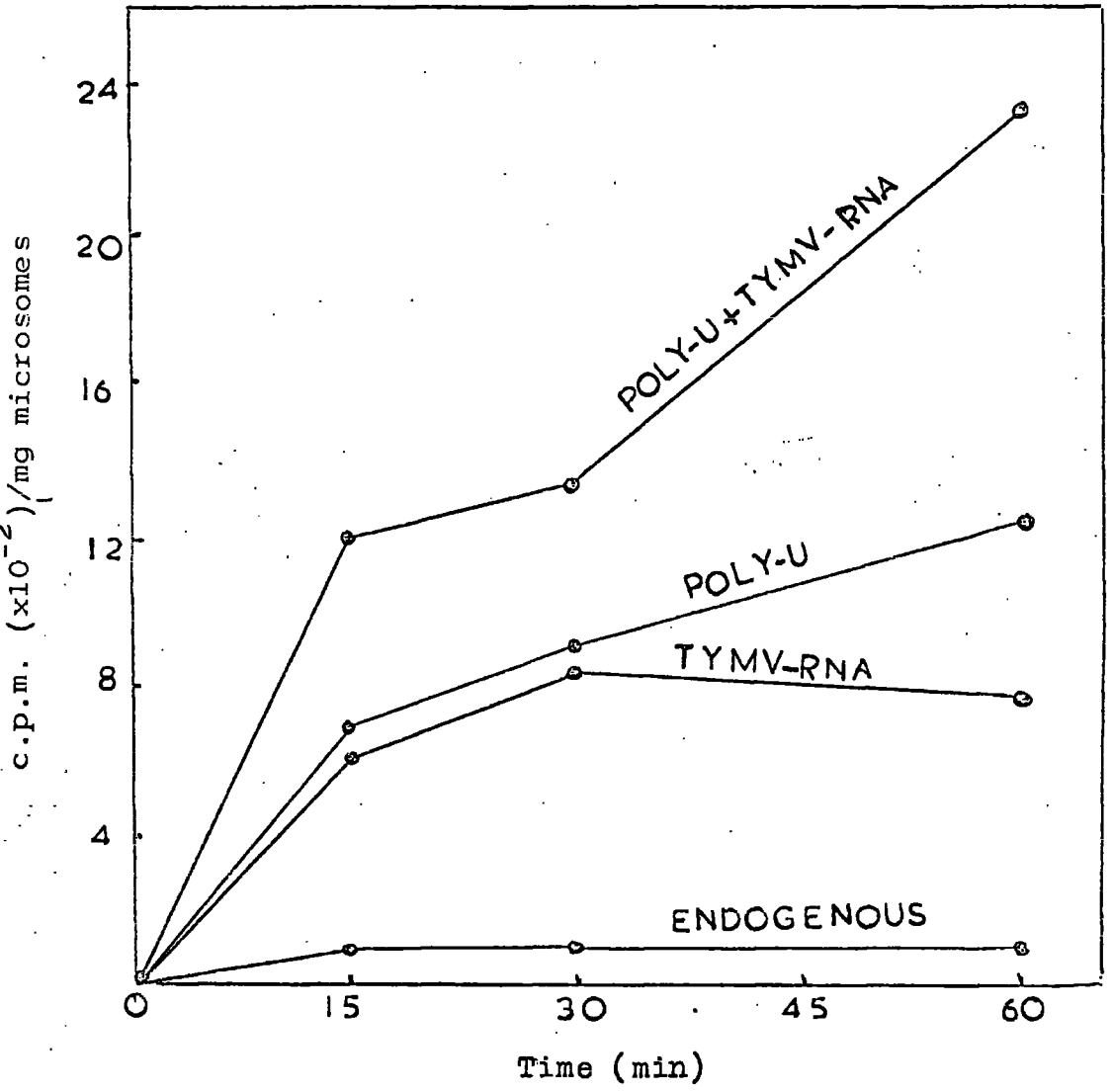
The endogenous activity in the precharged V.faba tRNA Transfer System, unlike viral-RNA and Poly-U direction, varied

Fig.63. TRANSFER SYSTEM: Competitive effect of poly-U and TYMV-RNA at 8mM Mg^{2+} (^{14}[U^{14}C] AA-V. faba tRNA).



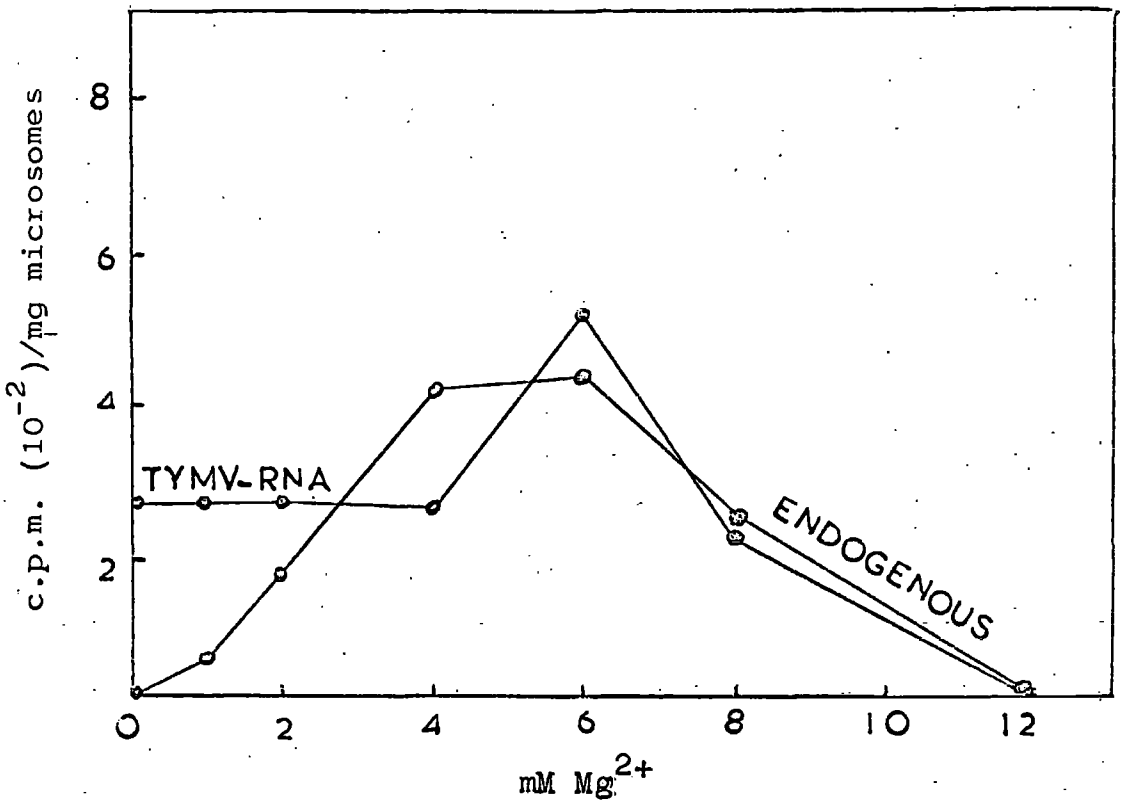
0.25 ml incubations contained: 15 µmol Tris-HCl pH 7.8 at 30°, 20 µmol KCl, 2 µmol $MgCl_2$, 0.05 µmol GTP, 2.5 µmol GSH, 0.25 mg microsomes, either 0.2 mg TYMV-RNA, and/or 0.05 mg Poly-U and 0.1 mg V. faba tRNA precharged with ^{14}[U^{14}C] AA. Incubation was at 30° and 0.05 ml samples assayed at times indicated. Radioactivity/disc determined as in Methods 3.

Fig.64. TRANSFER SYSTEM: Competitive effect of Poly-U and TYMV-RNA at 2mM Mg^{2+} (^{14}[U^{14}C] AA – V. faba tRNA).



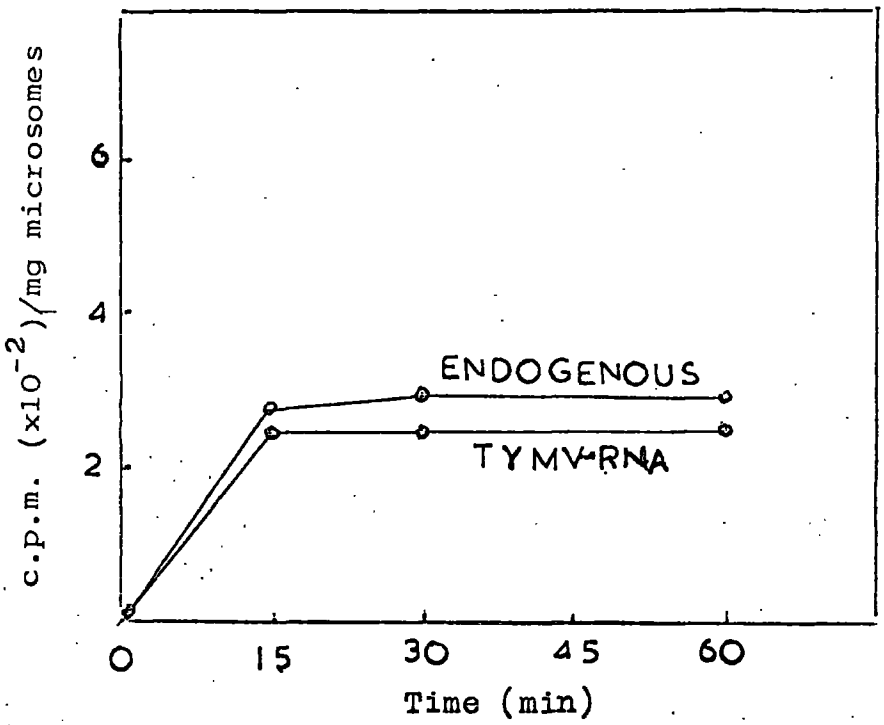
0.25 ml incubations contained: 15 µmol Tris-HCl pH 7.8 at 30°, 20 µmol KCl, 0.5 µmol $MgCl_2$, 0.05 µmol GTP, 2.5 µmol GSH, 0.25 mg microsomes, either 0.2 mg TYMV-RNA and/or 0.05 mg Poly-U and 0.1 mg V. faba tRNA precharged with ^{14}[U^{14}C] AA. Incubation was at 30° and 0.05 ml samples assayed at times indicated. Radioactivity/disc determined as in Methods 3.

Fig.65 TRANSFER SYSTEM: TYMV-RNA-directed incorporation from 14 [U^{14}C] AA-Yeast tRNA, at various Mg^{2+} concentrations.



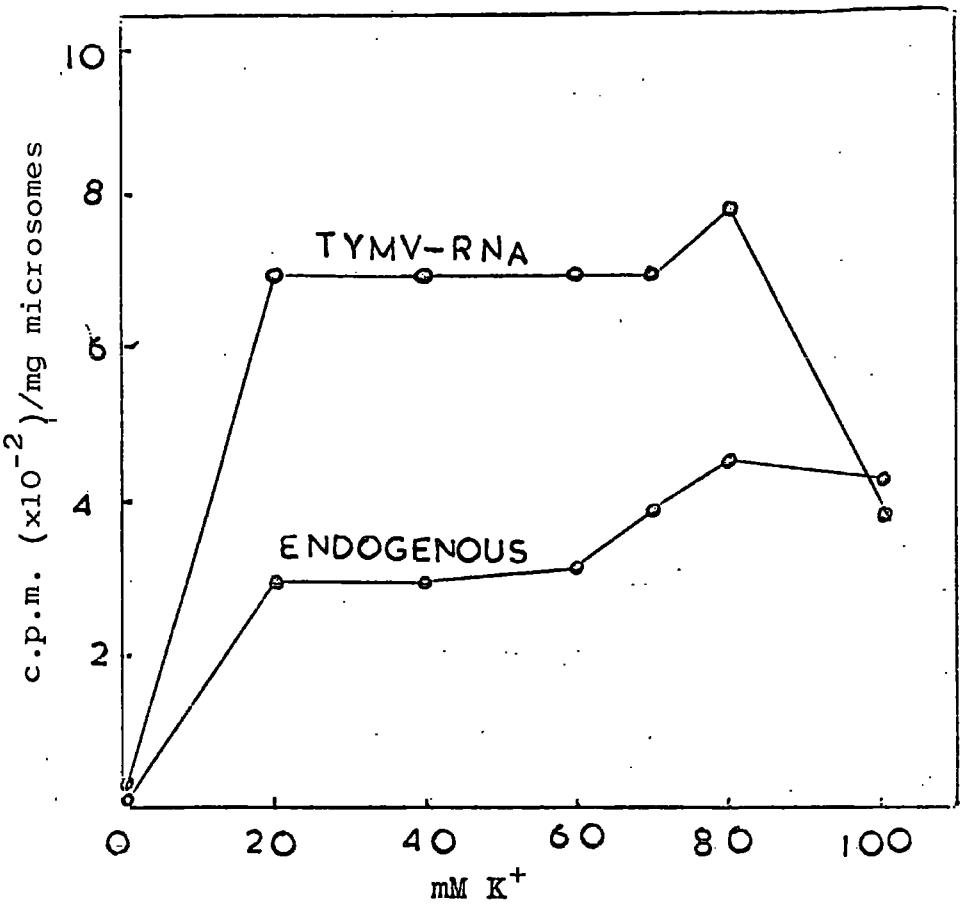
0.25 ml incubations contained: 15 µmol Tris-HCl pH 7.8 at 30°, 20 µmol KCl, 0.25 µmGTP, 2.5 µmol GSH, 0.25 mg microsomes, varying µmol $MgCl_2$, 0.2 mg TYMV-RNA and 0.1 mg yeast tRNA precharged with 14 [U^{14}C] AA. Incubation was at 30° and samples assayed at 0, 15, 30, 60 min. Radioactivity/disc determined as in Methods 3. 30 min assays are reported.

Fig.66. TRANSFER SYSTEM; TYMV-RNA-directed incorporation from 14 [U ^{14}C] AA-Yeast tRNA.



0.25 ml incubations contained: 15 µmol Tris-HCl pH 7.8 at 30°, 20 µmol KCl, 1.0 µmol $MgCl_2$, 0.5 µmol GTP, 2.5 µmol GSH, 0.25 mg microsomes, 0.2 mg TYMV-RNA and 0.1 mg yeast tRNA precharged with 14 [U ^{14}C] AA. Incubation was at 30° and samples assayed at times indicated. Radioactivity/disc determined as in Methods 3.

Fig.67. TRANSFER SYSTEM: Effect of K^+ on TYMV-RNA-directed incorporation from 14 $[U^{14}C]$AA-*V. faba* tRNA.



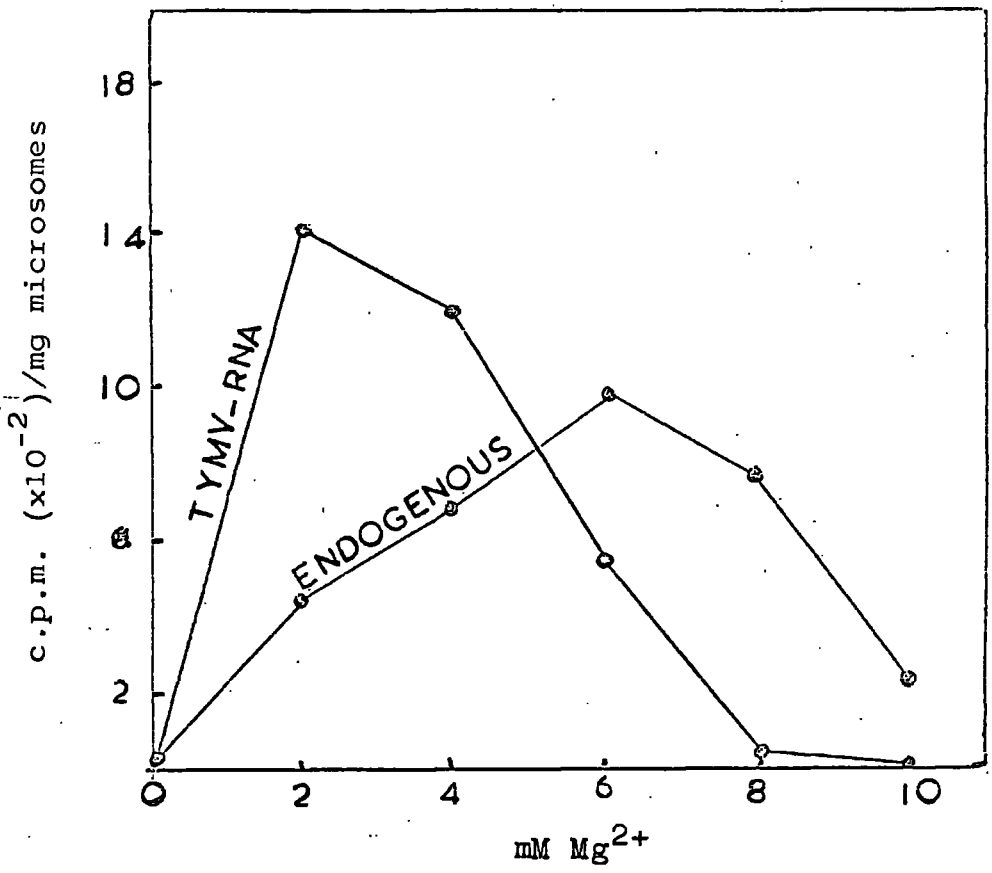
0.25 ml incubations contained: 15 µmol Tris-HCl pH 7.8 at 30°, varying µmol KCl, 0.5 µmol $MgCl_2$, 0.05 µmol GTP, 2.5 µmol GSH, 0.25 mg microsomes, 0.2 mg TYMV-RNA, and 0.1 mg *V. faba* tRNA precharged with 14 $[U^{14}C]$ AA. Incubation was at 30° and 0.05 ml samples assayed at 0, 15, 30, 60 min. Radioactivity/disc determined as in Methods 3. 30 min assays are reported.

from preparation to preparation. Most significant endogenous activity was shown by microsomal preparations which had been thawed once prior to use. The Mg^{2+} optimum for endogenous was 6mM (FIG.68). The graph shows suppression of endogenous activity in the presence of TYMV-RNA at Mg^{2+} concentrations not conducive for viral-RNA promotion of amino acid incorporation. The profile also shows the general relationship of Mg^{2+} to viral-RNA direction and endogenous incorporation. These results are more clearly illustrated in FIGS.69,70,71 and 72.

The results of adding ATP and Val to the TYMV-RNA-directed 14 $[U^{14}C]$ AA-V.faba tRNA Transfer System are demonstrated in TABLE 8. Complete suppression of the system occurred with ATP and ATP + Val. Little suppression occurred with Val alone. The microsomal preparation described in TABLE 8 was used in a Poly-U-directed Complete System without supplementation with the high-speed supernatant. This system was devoid of endogenous tRNA (i.e. Phe-accepting-tRNAs), and was enhanced by addition of ATP; conversely the activity was reduced (though not eliminated) in the absence of ATP (FIG.73).

In the Transfer System, TYMV-RNA was presented in 0.01M-NaCl or 0.01M-Tris-HCl pH 7.8 at 30^{o}, and viral-RNA would promote incorporation if thawed and frozen more than once. Microsomal preparations were stored at -70^{o} at a concentration of 50mg/ml in contrast to the storage at 20mg/ml used in the Complete System.

Fig.68. TRANSFER SYSTEM: Effect of Magnesium concentration on TYMV-RNA-directed incorporation and Endogenous incorporation from 14 [U^{14}C] AA-V. faba tRNA.



0.25 ml incubations contained: 15 µmol Tris-HCl pH 7.8 at 30°, 20 µmol KCl, varying µmol $MgCl_2$, 0.05 µmol GTP, 2.5 µmol GSH, 0.25 mg microsomes, 0.2 mg TYMV-RNA and 0.1 mg V. faba tRNA precharged with 14 [U^{14}C] AA. Incubation was at 30° and 0.05 ml samples assayed at 0, 15, 30, 60 min. Radioactivity/disc determined as in Methods 3. 30 min assays are reported. (See Figs. 69, 70, 71, 72)

Fig.69. TRANSFER SYSTEM: TYMV-RNA-directed incorporation and Endogenous incorporation at 6 mM Mg^{2+} from 14 [U ^{14}C] AA-V. faba tRNA.

Incubation: as Fig.68.

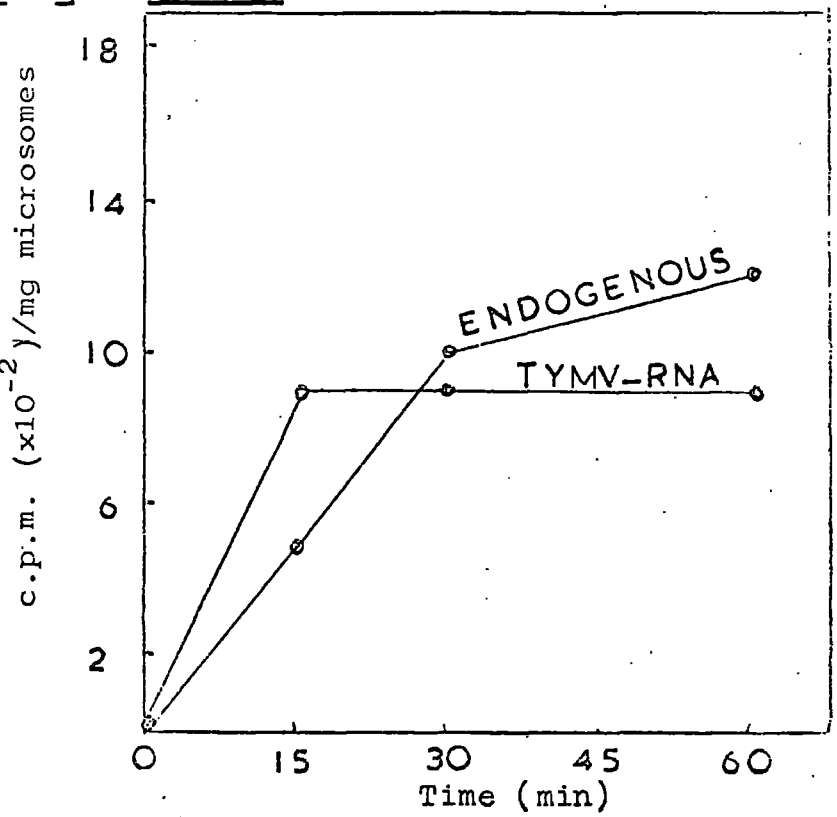


Fig. 70. TRANSFER SYSTEM: TYMV-RNA-directed incorporation and Endogenous incorporation at 8 mM Mg^{2+} from 14 [U ^{14}C] AA-V. faba tRNA.

Incubation: as Fig. 68.

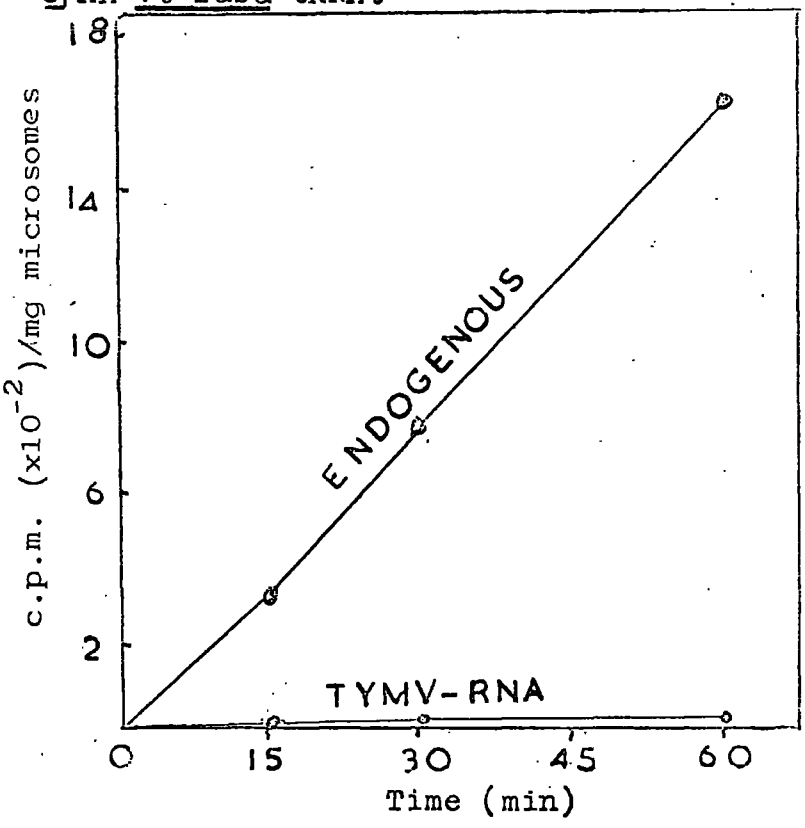


Fig.71. TRANSFER SYSTEM: TYMV-RNA-directed incorporation and Endogenous incorporation at 2 mM Mg^{2+} from 14 [U ^{14}C] AA-V. faba tRNA.

Incubation: as Fig. 68.

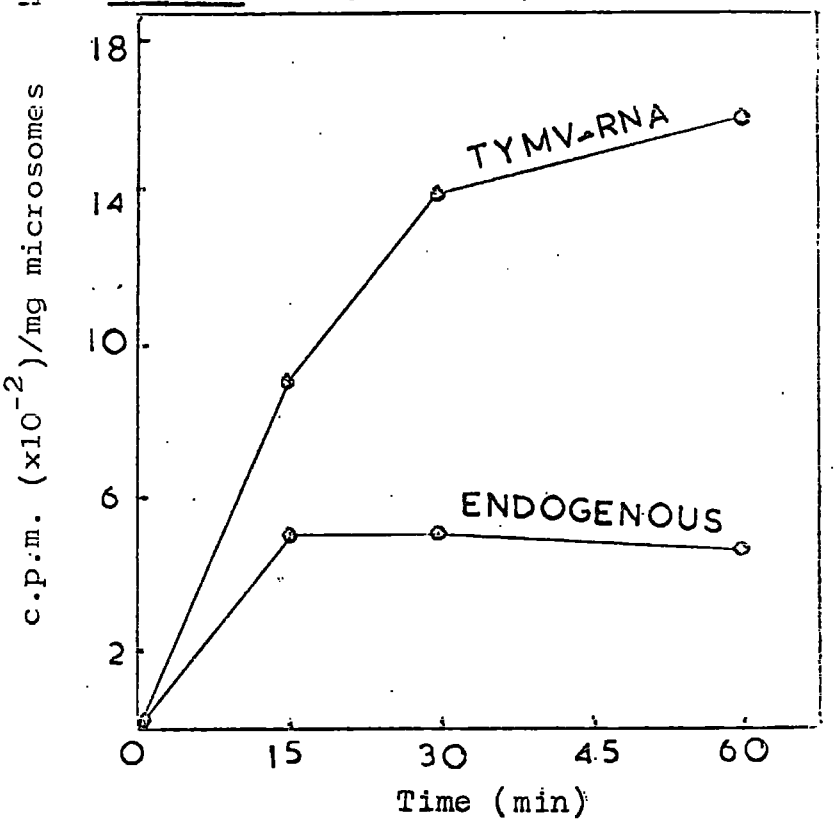


Fig.72. TRANSFER SYSTEM: TYMV-RNA-directed incorporation and Endogenous incorporation at 4 mM Mg^{2+} from 14 [U ^{14}C] AA-V. faba tRNA.

Incubation: as Fig. 68.

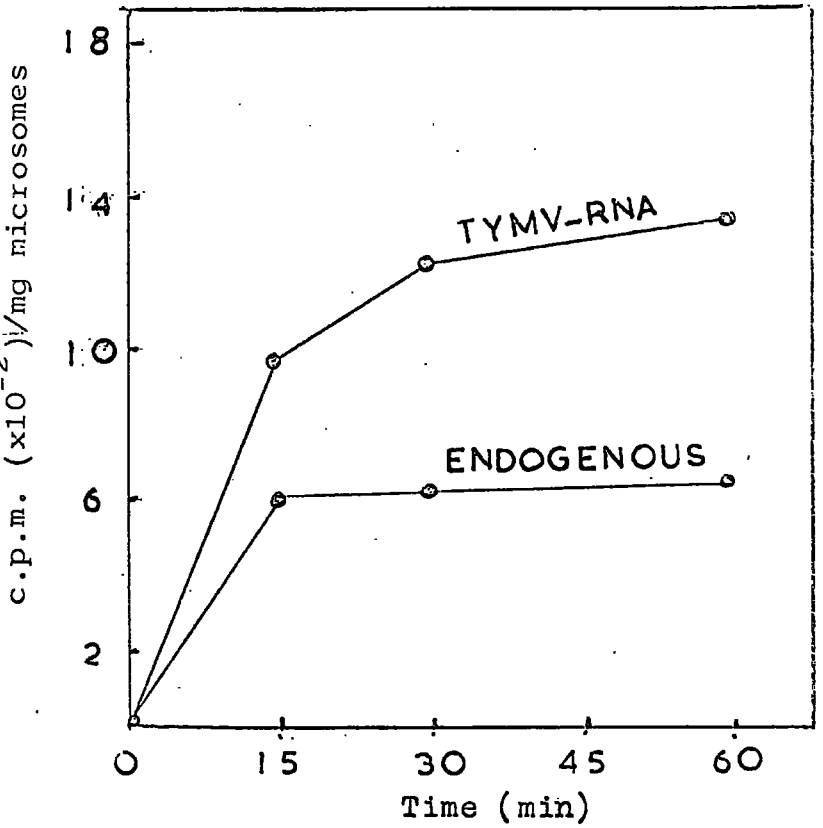
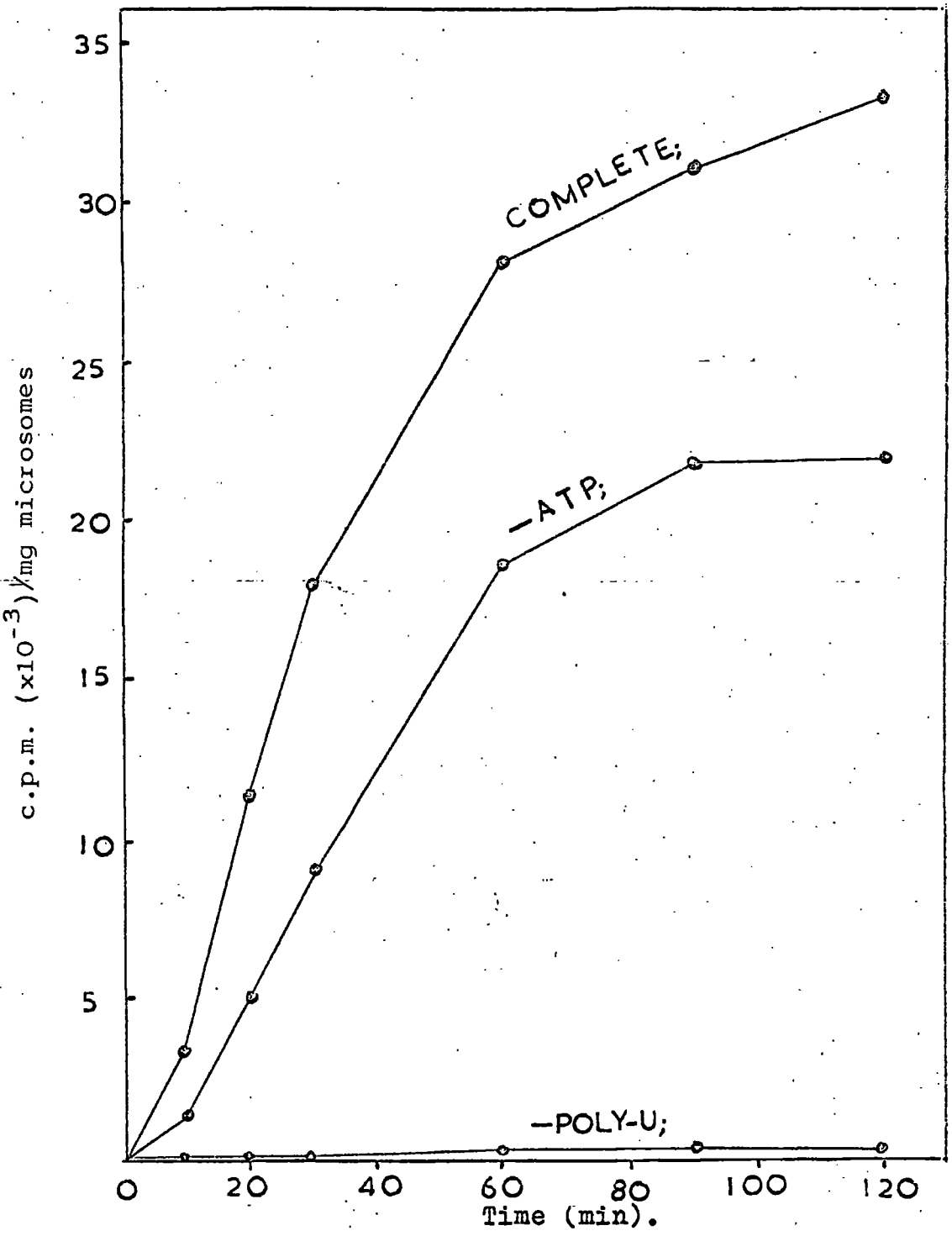


TABLE 8. TRANSFER SYSTEM: Effect of ATP and $[^{12}C]$ Val addition to TYMV-RNA-directed incorporation from 14 $[U^{14}C]$ AA-V.faba tRNA.

	TRANSFER c.p.m./mg microsomes	+ Valine c.p.m./mg microsomes	+ ATP c.p.m./mg microsomes	+ Val + ATP c.p.m./mg microsomes
TYMV-RNA	1,892	1,360	-	60
Endogenous	240	560	-	20
TYMV-RNA — microsomes	-	-	-	-
Endogenous — microsomes	-	-	-	-

0.25ml incubations contained: 15 µmol Tris-HCl pH 7.8 at 30^{0}, 20 µmol KCl, 0.5 µmol $MgCl_2$, 0.05 µmol GTP, 2.5 µmol GSH, 0.25mg microsomes, 0.2mg TYMV-RNA, 0.1mg V.faba tRNA precharged with 14 $[U^{14}C]$ AA and (as indicated) 1 µmol ATP and 0.01 µmol $[^{12}C]$ Val. Incubation was at 30^{o} and 0.05ml samples assayed at 0, 15, 30, 60 min. Radioactivity/disc determined as in Methods 3. 30 min assays are reported.

Fig.73. COMPLETE SYSTEM: Poly-U-directed incorporation of [^{14}C] Phe. System not supplemented with ATP regenerating system or high-speed supernatant enzyme fraction.



Discussion.

Three objectives of *in vitro* amino acid-incorporating experiments have been outlined by Allende (1969):

(1) the determination of the relative activity of the protein-synthesising machinery of tissues under specific conditions;

(2) study of the component properties of protein synthesis;

(3) the achievement of the synthesis of a specific protein.

Certain experimental conditions are necessary for attainment of these objectives. The first may be accomplished in the development of a system that interferes as little as possible with the components involved. Such a system functions, by definition, under the direction of an endogenous mRNA and may not need supplementation with all the known required components some of which may be present as contaminants.

The second objective requires a simplified system with isolated components and the maximum incorporation attainable. Nirenberg and Matthaei (1961) described such a system using Poly-U to direct a polyphenylalanine synthesis although for a complete understanding of the second objective several different systems are necessary.

The third objective is more difficult to achieve; a system developed to synthesise a specific protein must be carefully maintained to preserve its integrity.

We are much closer to achieving the first and second objectives in cell-free amino acid incorporating-systems from plant sources, witness the extensive reviews of Holley (1965),

Mans (1967), Allende (1969) and Boulter (1970). The third objective has not been unequivocably accomplished in plant systems to-date. Schwartz, Eisenstadt, Brawerman and Zinder (1965) synthesised a 'phage coat protein in a Euglena gracilis chloroplastic system directed by f2 bacteriophage; Sela and Kaesberg (1969) using tobacco chloroplast ribosomes directed by tobacco mosaic viral-RNA, claim to have synthesised the viral coat protein; Klein, Nolan, Lazar and Clark (1972) have synthesised viral coat protein in a wheat embryo system directed by satellite tobacco necrosis viral-RNA. It is significant that the products are not plant protein. It should be noted however, that the second and third objectives cannot be so readily separated and analysed in different systems as may become evident during the Discussion.

A main concern of investigators working with cell-free systems is that the amino acid incorporation, particularly when determined as a hot trichloroacetic acid precipitable material, results from the intended director component and not from microbial interference in the in vitro system.

Much has been written on the effects of microbial contamination in in vitro systems (Mans and Novelli, 1964; App and Jagendorf, 1964; Hall and Cocking, 1966; Allende, 1969; Boulter, 1970; Beevers and Poulson, 1972) and criteria are prescribed for recognition of unwanted microbial participation. In general the criteria are: a dependence upon exogenously-supplied components, particularly the energy system; a short

time-course of incorporation; a sharp Mg^{2+} optimum.

Contradictory opinions are held on the definition "short time course". Boulter (1970) states that most cell-free systems are unstable and amino acid incorporation is completed under standard conditions in 20 - 30 min or less. Beevers and Poulson (1972) agree with this observation and add that the causes of the limitation of reaction (for Poly-U direction) are not known. Allende (1969) maintains that if incorporation continues at a good rate after 1.5 - 2 hr bacterial contamination should be suspected, since all cell-free systems are usually exhausted by that time and need complementation with energy sources, mRNA or even with fresh enzymes. Because wild-type bacteria have minimal requirements, the more ingredients that are found to be necessary for incorporation the less likely it is that the activity is due to microbial effects.

Care must be taken to reveal any essential components present in the cell-free system in order to confirm, for example, that endogenous ATP is not supplying the requirements of an apparently non-ATP-dependent system. A Poly-U-directed system can be shown not to be microbially affected by the control experiment which omits Poly-U. Since Poly-U might be the source of microbial contamination systematic omission of Mg^{2+} or tRNA or energy system and omission of selected and permutated components will elucidate information required.

A consideration of the kinetics of the <u>in vitro</u> system under various conditions will assist in diagnosing microbial

effects. Mans and Novelli (1964) describing the typical biphasic curve of bacterial incorporation, illustrate the method by which total incorporation promoted by micro-organisms may be demonstrated. There is little incorporation for the first 60 min compared with the subsequent rapid increase in incorporated label.

The various criteria offer a guide which must be specifically interpreted in the context of the in vitro system under investigation. The ultimate criterion of in vitro amino acid incorporation may be the identification of the final product, arguably the most elusive factor of plant-derived cell-free systems.

In this present investigation, microbial effects in the in vitro systems were monitored in every set of incubations. Monitoring was accomplished by a process of essential component omission and the construction of a kinetic curve of incorporation, made possible by sampling the incubation at various times. The construction of time courses, though tedious, did afford considerations of the kinetic linearity for each incorporation experiment, so that assumptions necessitated by "one-time assays" were not required. It is concluded from the results obtained that the incorporation experiments described in this investigation did not result from microbial activity.

Before discussing the main results in detail it is appropriate here to comment on the characteristics of the natural template, TYMV-RNA, which was used.

TYMV was characterised by analytical ultracentrifugation. The sedimentation coefficients obtained for virus and T component (TYMV protein without RNA) agree with the published figures of:

		TYMV	T
Lyttleton and Matthews	(1958)	108s	50s
Haselkorn	(1962)	116s	-
Kaper, Kupke, Ulrich and Weber	(1966)	116-117s	53-54s

Schlieren film profiles showed patterns similar to those published by Dunn and Hitchborn (1965). Virus preparations showed T component and a small amount of plant ribosomal material but since viral-RNA preparations had a very low protein content it was concluded that the viral-RNA extraction procedures would eliminate unwanted protein occurring in the virus preparations.

Haselkorn (1962) estimated the molecular weight of TYMV-RNA as $2.3 \pm 0.15 \times 10^{-6}$ daltons using analytical ultracentrifugation techniques. Kaper and Litjens (1966) have suggested that 1.9×10^{-6} daltons is a more appropriate value; Mitra (1964) from sedimentation viscosity studies and Hirth, Horn and Strazielle (1965) from light-scattering measurements of the isolated RNA concur with the latter figure.

The molecular weight of 2.5×10^{-6} daltons, recorded by polyacrylamide gel electrophoresis in this investigation is in agreement with Haselkorn's (1962) figure. However, Loening (1969) showed that the molecular weight of tobacco mosaic viral-RNA was overestimated 10 -20% by polyacrylamide gel electrophoresis.

Bishop, Claybrook and Spiegelman (1967) also found that the molecular weight of tobacco mosaic viral-RNA was overestimated about 12% when related to electrophoretic mobilities of bromegrass mosaic viral-RNA and E.coli RNA. Tobacco mosaic viral-RNA has, to some extent, the properties of DNA, in which the mobility is almost independent of the molecular weight and does not vary much with gel concentration (Loening,1967). However, the results presented in FIGS.3 and 4 show an obvious difference in TYMV-RNA mobility on gels of different concentration.

Matthews and Ralph (1966) record that TYMV-RNA has an unusual capacity to combine in solution with other nucleic acids, such as plant ribosomal RNA. This tendency appears to be a function of the high cytosine content of TYMV-RNA since the phenomenon was not observed with tobacco mosaic viral-RNA (Matus, Ralph and Mandel, 1964). The resolving of TYMV-RNA and Chinese cabbage RNA (FIG.6) into distinct electrophoretic peaks may seem to contradict this report but since the viral- and Chinese cabbage RNA were mixed only at the top of the gel it is possible that combination was prevented by immediate application of electrical current. Pearson (1972 - private communication) has obtained similar resolving of TYMV-RNA and Chinese cabbage RNA in the one gel.

Pinck, Yot, Chapeville and Duranton (1970) have shown that TYMV-RNA, when incubated with ATP and nucleic acid-free extracts of E.coli, binds valine. The amino acid is bound by an ester linkage to the 3' terminal nucleotide of the RNA molecule in a

manner analogous to that encountered in aminoacyl- tRNA. Yot, Pinck, Haenni, Duranton and Chapeville (1970) demonstrated that the nucleotide composition near the valine - binding site is different for TYMV-RNA and $tRNA^{Val}$ from Chinese cabbage, consequently host $tRNA^{Val}$ is not involved in the observed charging of TYMV-RNA with valine. Further, the TYMV-RNA appears to have a tRNA - like structure at or near its 3' end that is recognised by three different enzymes which specifically catalyse reactions involving tRNA. In other experiments Yot et al.(1970) found that partially purified valyl-tRNA synthetases from yeast and rat liver were also active in binding valine to TYMV-RNA. When E.coli valyl synthetase was highly purified TYMV-RNA was not charged. However, if tRNA nucleotidyltransferase was included in the reaction mixture AMP was incorporated at the 3' end of the RNA and valine was then attached to TYMV-RNA. The purified valyl-tRNA synthetase sufficed to charge valine to both E.coli and Chinese cabbage tRNAs without a nucleotidyltransferase involvement.

In the present investigation, the high-speed supernatant enzyme fraction of P.aureus was capable of promoting the binding of valine to RNA extracted from TYMV by the phenol and alcohol procedures. The rates and levels of viral-RNA charging were similar, irrespective of the extraction procedure. Endogenously-supplied ATP was necessary for the reaction. The enzyme did not promote viral-RNA charging with 5 other $[^{14}C]$ amino acids, although it was capable of charging these amino acids to P.aureus tRNA, V.faba tRNA and, to a lesser extent, yeast tRNA (TABLE 1).

Treatment of valyl — TYMV-RNA with hot trichloroacetic acid resulted in loss of bound radioactivity analogous to the hydrolysis of the ester bond which takes place when charged tRNA is treated in this manner.

Although no appreciable differences were detected between TYMV-RNA extracted by either procedure, the analytical ultracentrifuge Schlieren patterns for a scaled-up alcohol procedure showed RNA that was severely degraded (when Chinese cabbage plants were challenged with this preparation no infection resulted). Since it appears that the component quantities of the Dunn and Hitchborn (1966) method are critical with respect to volume as well as to proportion, the phenol method of extraction was preferred; comparatively large quantities of TYMV up to 100mg could be processed in one flask to yield suitable RNA.

It was clear from the molecular characteristics and valine-charging properties that the TYMV-RNA preparations used here were comparable with those of other workers.

The present work is concerned with the incorporation of Poly-U- and TYMV-RNA-directed amino acids into peptidyl material on P.aureus ribosomes; it is convenient to consider first Poly-U direction.

A lag phase was a characteristic feature of the kinetics of Poly-U direction of polyphenylalanine synthesis on the P.aureus ribosome in the Complete System. The duration of the lag phase depended on the source of tRNA, incubation temperature and presence of a suitable concentration of components essential to

the system. Nevertheless when optimal conditions pertained a lag phase was still generally discernible. However, FIG.36 does show a linear response from O-time. That the absence of a lag phase here was an artefact may be explained on the basis that insufficient samples were assayed at different times in the linear phase. Thus, by graphically connecting the O-time point to the point where linearity was terminating, a response curve was produced without lag.

A more or less prolonged lag phase, lag period or induction period is a feature of *in vitro* systems where the template polynucleotide does not bear an initiating codon. Spirin and Gavrilova (1969) have pointed out that the main cause of the lag lies in the fact that all the usual aminoacyl - tRNAs, being well retained in the A-site of the ribosome, are not retained easily in the P-site and in such a case the formation of the first peptide bond occurs only occasionally and is of relatively low probability. Even though this probability is of low order, the initiation of translation into polypeptide is achieved and therefore it is possible to induce translation without an initiating codon.

The lag phase is characteristic of the Poly-U systems of Nakamoto, Conway, Allende, Spyrides and Lipmann (1963), Allende, Monro and Lipmann (1964), Nishizuka and Lipmann (1966), and Sander and Matthaei (1969). Only after 30 sec to several min have elapsed does polyphenylalanine synthesis take place, it then increases rapidly and reaches a linear rate. In an *in vitro* system with Poly-U it is precisely initiation that is hindered since the first peptide bond is a prerequisite of translation.

When the first peptide bond is formed i.e. diphenyl-phenylalanyl-tRNA, then the course of translation proceeds vigorously.

One of the main factors affecting the lag phase is the Mg^{2+} concentration, though Mg^{2+} must not be considered to act solely in this manner. The ion maintains the integrity of the ribosome (Tissieres and Watson, 1958), binds mRNA to the ribosome (Moore and Asano, 1966) and binds aminoacyl-tRNA to the ribosome (Ravel and Shorey, 1969). Sharp Mg^{2+} optima (FIG.37) were obtained in the Poly-U-directed in vitro Complete Systems in the present investigation; where tRNA from yeast or P.aureus were included in the Mg^{2+} optimum was 12mM and V.faba tRNA systems were 10mM. Strictly, the Mg^{2+} optima refer to that quantity of Mg^{2+} ion added specifically to the incubation so that the Mg^{2+} present in other ingredients of the incubation, such as the microsomal preparation and the high-speed supernatant enzyme fraction, is ignored. Boulter (1970)has pointed out that the various Mg^{2+} optimal levels reported for plant-derived cell-free systems are in part a reflection of the different Mg^{2+} levels used in preparations of ribosomes and enzyme fractions and subsequent storage since ribosomes are known to absorb Mg^{2+} in the isolation procedure.

It seems that approximately 10mM Mg^{2+} are commonly used in Poly-U-directed plant-derived cell-free systems (Payne 1970). Prokaryotic systems utilise very approximately 15 - 20 mM Mg^{2+} (Spirin and Gavrilova, 1969). These levels of Mg^{2+} are generally referred to as "high" when compared with natural

messenger systems and the initiation as not "proper" when compared with systems possessing initiating codons (Lengyel and Soll, 1969). It has been found that "high" levels of Mg^{2+} are only required at the beginning of polypeptide synthesis for the rapid passage through the lag phase; when translation is proceeding fully the Mg^{2+} concentration can be lowered to e.g. 7 - 10mM in prokaryotic cell-free systems, (Revel and Hiatt, 1965; Nakamoto and Kolakofsky, 1966). An explanation for the initiating role of Mg^{2+} is that at increased concentration of that ion the ternary complex of ribosome — template — aminoacyl-tRNA becomes sufficiently stable even without initiator tRNA, and at the same time the affinity of the aminoacyl-tRNA for the P-site is increased. Thus, the probability of an appropriate neighbouring arrangement of the two aminoacyl-tRNAs on the ribosome increases, leading to the corresponding comparatively rapid formation of the first peptide bond. Dipeptidyl-tRNA, analogue of fMet-$tRNA_F$, may serve as a chain initiator (Lengyel and Soll, 1969) and when such modified aminoacyl-tRNAs are added to the system the Mg^{2+} concentration requirement is reduced (Igarashi, 1970). This author also demonstrated that at Mg^{2+} concentrations conductive for Poly-U direction, without initiator, the addition of modified aminoacyl-tRNAs decreased overall incorporation. Nakamoto and Kolakofsky (1966) obviated the lag phase in a Poly-U system by the exogenous supplementation of diphenyl-phenylalanyl-tRNA. It is evident that exogenous peptidyl-tRNA can readily occupy

the peptidyl-tRNA binding site as a result of its great affinity for it (Rychlik, 1966 i and ii). Further, Springer and Grunberg-Manago (1972), using the fMet-tRNA$_F$ analogue N-acetyl-Phenylalanyl-tRNA in a Poly-U E.coli cell-free system, have shown that at 10mM Mg^{2+} the analogue binds poorly to the ribosome, but at 5mM the binding is strongly enhanced.

In considering the following it is implicit that Poly-U codes for [^{14}C] phenylalanine and only those parameters which govern phenylalanyl-tRNA need be considered.

The optimal conditions for the Poly-U-directed Complete Systems varied according to the source tRNA used. It must be premised that the parameters which govern the tRNA ^{Phe} aminoacylation reaction, when matching the parameters which govern those other reactions in the Complete System, must result in maximal incorporation. Conversely, if the parameters governing the aminoacylation reaction do not conform with those other reactions of the Complete System, then a condition obtains whereby incorporation is dependent upon a compromise between these 2 sets of reactions; then the incorporation is below the arbitrary maximum. Indeed, the sharper the parameter optima the greater the degree of compromise.

The condition of "matching" was illustrated by the identical optimal responses of the reactions of aminoacylation and incorporation with V.faba tRNA ^{Phe} to variations in pH. (FIGS. 19,40). A mutual concession was exemplified by the dissimilar optimal responses of the reactions of aminoacylation and

incorporation with yeast tRNA ^{Phe} (FIGS.21, 40) and P.aureus tRNA ^{Phe} (FIGS.20, 40) to variations in pH. Notwithstanding these discernible pH optima, it should be noted that in the Poly-U-directed Complete Systems a broad pH range (related specifically to the source tRNA) allowed significant polyphenylalanine synthesis, characteristic of other plant-derived in vitro systems (Payne, 1970; Beevers and Poulson, 1972).

No critical Mg^{2+} optima within the range of 2 - 16mM were recorded for the esterification of phenylalanine to tRNAs from V.faba and P.aureus, therefore the Mg^{2+} requirement of the latter reaction would not appear to compromise the Mg^{2+} requirement of the Complete System (FIG.37). The Mg^{2+} optimum for the esterification of phenylalanine to yeast tRNA was sharper at 15mM, whereas the Mg^{2+} optimum for the Complete System with that source tRNA was 12mM; possibly an example of concession.

The comparable rates of polyphenylalanine synthesis in the Complete System with yeast tRNA or P.aureus tRNA did not parallel the rates of acylation by phenylalanine of these 2 source tRNAs; yeast tRNA required about 40 min for total acylation whereas P.aureus tRNA was totally acylated in 5 min. The rate of aminoacylation in these Complete Systems was not, therefore, limiting.

It is an attractive proposition that the charging capacity of V.faba tRNA ^{Phe}, being much greater than that capacity for yeast and P.aureus tRNAs (TABLE 1) was reflected in the greater incorporation of the Poly-U-directed Complete System containing

V.faba tRNA (FIG.34). Although this may be true, Sueoka and Kano-Sueoka (1970) have pointed out that chromatography of tRNA preparations may lead to modifications of tRNA, which though chargeable, are not capable of transferring the amino acid to the polypeptide chain.

It would appear, therefore, that for maximal synthesis of polyphenylalanine by Poly-U direction in the P.aureus microsomal system, a heterologous tRNA is better able to meet the requirements necessary for the synthesis than the homologous tRNA. The significance of this finding may simply be that, artefactually, the synthetic messenger imposes on the system a set of non-physiological parameters to which V.faba tRNA conforms more easily than do the other source tRNAs. On the other hand, it is possible that a preparation of tRNA from developing P.aureus seed might allow increased polyphenylalanine synthesis in the system. Payne (1970), using tRNA derived from ranges of 30—100-day developing seed of V.faba, has shown that 60-day material showed a greater capacity for accepting $[^{14}C]$ phenylalanine and the 14 $[^{14}C]$ amino acid mixture. In the present investigation, tRNA material from 50-day V.faba developing seed was not found to have superior phenylalanine - charging properties, compared with tRNA material derived from germinating V.faba (TABLE 1).

Other workers quote such a variety of incubation times that it is difficult to compare them. Allende and Bravo (1966), using a wheat embryo system, reported 160 pmol phenylalanine incorporated/mg RNA in 45 min. Leaver and Key (1967), using carrot root ribosomes, achieved 100 pmol phenylalanine

incorporated/mg RNA in 30 min. Payne, et al.(1971), with a V.faba Complete System, recorded 900 pmol phenylalanine incorporated/mg RNA in 40 min and 1,500 pmol in 120 min. The time-courses of phenylalanine incorporation presented here (FIG.34) make possible a corellation between each of the times mentioned above. Since these results are expressed as pmol/mg microsomes each value may be doubled, on the assumption that RNA forms 50% of the microsomes. Thus, the Complete System with V.faba tRNA at 30, 45 and 120 min recorded 400, 600 and 1,800 pmol phenylalanine incorporated/mg RNA, respectively. For yeast tRNA and P.aureus tRNA systems corresponding values were 200, 300, 800-900 approximately.

All poly-U-directed Complete Systems were dependent upon an exogenously-supplied energy system. In considering the Complete System with yeast tRNA the following situation obtained. No activity was promoted in the absence of ATP, PCK and CreP, indicating complete dependence of the system on an exogenously-supplied energy source. About 30% activity compared with that of the Complete System was allowed in the absence of ATP, implying that the ATP regenerating system, i.e. PCK and CreP, was able to meet part of the system's energy requirements. Similar findings have been reported for a wheat embryo ribosomal system (Allende, 1969) and a tobacco leaf ribosomal system. (Van Kammen, 1967).

In the present work ATP supplementation alone allowed 60% activity, and the addition of CreP enhanced that activity a further 20%, illustrating its function in the regenerating system.

The combination of ATP and PCK did not allow greater activity than did ATP alone. Indeed, after 40 min incubation the rate with ATP and PCK fell away in comparison to the rate with ATP alone, so it may be that prolonged incubation times lead to PCK becoming inhibiting in the absence of CreP.

Creatine phosphate (phosphocreatine) is a high energy phosphate compound, first shown to be necessary, together with ATP, in the microsomal system by Zamecnik and Keller (1954). It is assumed that the purpose of the high energy phosphate compound is to regenerate ATP either from ATP or from AMP resulting from amino acid activation. Thus it is usual to add phosphocreatine kinase to the system. (Phosphoenolpyruvate [PEP] and pyruvate kinase [PK] are also commonly used as a regenerating system).

Using an <u>in vitro</u> rat liver system, Todd and Campbell (1969) showed the complexity of the relationship between concentrations of ATP, CreP with PCK, PEP with PK, and Mg^{2+}. Unlike PEP, CreP has poor chelating properties so that increased Mg^{2+} concentrations inhibitory to an <u>in vitro</u> system using ATP + CreP may not be inhibitory to a system using ATP + PEP. (Staehelin, (1969) found that 10 µmol PEP binds 5 µmol Mg^{2+}). Further increasing of Mg^{2+} concentration in the absence of PEP led to an increase of incorporation in presence of ATP alone, though the level of incorporation allowed by ATP + PEP was never attained. Todd and Campbell (1969) also demonstrated that a myokinase is necessary for the regeneration of ATP and it is suggested that this enzyme

be sought in other *in vitro* systems. They concluded that an energy regenerating system is necessary in microsomal systems and that PEP is the most active, but for polysomal systems an energy-regenerating system is not required, providing extra ATP and Mg^{2+} ions are added. It is worth noting, however, that Zamecnik and Keller (1954) showed CreP and PEP to have equivalent activity in their *in vitro* system.

Partial dependence on ATP and the regenerating system was shown in the Poly-U-directed Complete Systems with *V.faba* tRNA or *P.aureus* tRNA. It would seem that there must be high contaminating levels of ATP in these systems. Since the tRNAs are the only materials of differing source in the *P.aureus* microsomal Complete System, it is arguable that the contaminating ATP was not present in the microsomal and/or the *P.aureus* high-speed supernatant enzyme preparations. It will be convenient to enlarge upon this phenomenon in a later part of the Discussion.

GTP was not a dependent factor in the Poly-U-directed Complete System with *V.faba* or *P.aureus* tRNAs (TABLE 6). The Complete System with yeast tRNA showed some dependence though marginal (FIG.42). Similar findings were reported by Parisi and Ciferri (1966) and Payne (1970). It was assumed by these authors that GTP was present as a contaminant of the microsomal and high-speed supernatant enzyme fractions.

The notable incorporation achieved without exogenously-supplied high-speed supernatant enzyme fraction (FIGS.38, 39) is not unusual. Allende (1969), Payne (1970), Marei, Gadallah and Kilgore (1972) have reported similar findings in their

respective wheat germ, V.faba and Fig fruit in vitro Poly-U-directed systems. It is interesting that the present work showed a depression of incorporation between endogenously - and exogenously - supplied enzyme when the latter was not optimal. This depression, apparent in the Complete Systems having yeast or P.aureus tRNAs present was absent in the V.faba tRNA system. This may have resulted from not adding the exact amount of high-speed supernant enzyme fraction to promote this incorporation depression. No simple explanation of the depression response seems available.

The temperature of all incorporation experiments was arbitrarily fixed at 30°, consequent upon the fact the TYMV is particularly sensitive to temperatures above 30°. Matthews and Lyttleton (1959) showed that when Chinese cabbage plants in which TYMV was multiplying were held at 33°, virus multiplication ceased. These authors concluded that the lack of infectivity of virus at 33° was due to breaks occurring in the viral-RNA. Matthews and Ralph (1966) stated the probability that heat inactivation in vivo is due to breaks in the RNA, with loss of RNA secondary structure. This premise was first proposed by Haselkorn (1962) from heat inactivation studies on TYMV-RNA in vitro. The charging of TYMV-RNA by valine optimal at 30° (FIG.31) may therefore be significant.

Payne (1970) has observed that the optimum temperature of a Poly-U-directed system is dependent upon a compromise between 2 sets of reactions - the first, a simple thermo-dynamic effect

of increased incorporation with increasing temperature and a second, counterbalancing effect, the decreasing stability of ribosomes and mRNA at higher temperature. Krahn and Paranchynch (1970) have shown that in the binding of Poly-U to E.coli ribosomes, the polynucleotide attaches more efficiently at a critical temperature consistent with the ordered-state (a helix) to random coil transition. The affinity of Poly-U for E.coli ribosomes is greatest at 10^{o}, more than 5 times greater than at 37^{o}. Nuclease experiments indicated that the reduction at 37^{o} was not due to degradation effects. Above 10^{o} the reduction in binding was analogous to "melting" curves for double-stranded (complementary) RNA. These authors conclude that the binding of Poly-U to ribosomes, completed in 30 sec, is not a rate-limiting parameter for temperature optima of polypeptide synthesis (where optima for that condition are above 10^{o}). Spirin and Gavrilova (1969) record that increased temperature promotes the formation of the complex 30s (E.coli) -- template -- aminoacyl-tRNA whether the template be polynucleotide or oligonucleotide. At 37^{o} the rate of binding of aminoacyl-tRNA is double that at 24^{o}; at 15^{o} it is many times lower than at 24^{o}, while at temperatures from $0\text{-}5^{o}$ binding is very difficult to observe.

In the Poly-U-directed Complete System with yeast tRNA 2 distinct time-courses of phenylalanine were seen (FIG.41). At 30^{o} the incorporation had a pronounced lag phase, then linearity obtained until a plateau was reached, which indicated either that a constant equilibrium of the systems obtained or that the system

was exhausted of essential component(s). At 37° the lag phase was much reduced, suggesting a simple thermo-dynamics effect in accelerating the speed of the initiating reaction i.e. increasing the random chance probability of the formation of the first peptide bond. The subsequent linearity was slightly steeper than at 30° and the plateau was reached earlier i.e. the overall incorporation was lower. Unlike the system demonstrated by Krahn and Paranchynch (1970) the P.aureus high-speed supernatant enzyme fraction and microsomal preparation used here are not guaranteed nuclease-free, so that increased nuclease activity concomitant with the higher temperature may play a part in terminating polyphenylanine synthesis earlier at 37° rather than 30°.

Ribosome dissociation may be influenced by temperature. Peterman (1964) demonstrated dissociation (and association) of Jensen Sarcoma ribosomes to be temperature-influenced. Bodley (1969) reported denaturation of E.coli ribosomes with increasing temperature. Schiebel, Chayka, de Vries and Rusch (1969) and Byfield and Scherbaum (1966, 1967 i and ii) have implicated mRNA breakdown at increased temperature in their in vitro systems.

Using a trout liver cell-free system, Rosen, Murray and Novelli (1967) detected a transferase fraction of the enzyme to be heat-labile. This lability was related to time and temperature, since at 23° and 37° the initial rates of polyphenylalanine synthesis were comparable but after 35 min more polypeptide was incorporated at 23° than at 37°. Mangiantini, Tecce, Toschi and Trentalance (1965) and Friedman (1968) have suggested that the

optimum temperature of amino acid incorporation reflects the temperature at which the organism lives. Further, the thermostability of the ribosomes may be a limiting factor in determining the upper growth temperature of the organism.

The effect of temperature on acylation of $tRNA^{Phe}$ was not considered in the present work. Payne (1970) showed that V.faba $tRNA^{Phe}$ charging between 15 - 37° was more or less independent of temperature, and similar findings were reported by Thiebe and Zachau (1968) for charging of $tRNA^{Phe}$ from wheat and yeast. However, the effects of temperature on charging yeast tRNA with valine were considered (FIG.26). It is clear that the rates of acylation were a function of increased temperature from 0 - 45°. At 40 min the acylations at 20° and at 25° were still linear, indicating that total acylation has still to be achieved. At 30°, total acylation was obtained at 80 min (linearity to 40 min). At 37° and 45° acylation rates increased but after 40 min bound radioactivity was diminished. This may indicate that at these times and temperatures tRNA was no longer capable of acylation and charged tRNA was de-acylating.

Sarin, Zamecnik, Bergquist and Scott (1966) have shown that several physical properties in tRNA are changed between 35 - 45°. These changes are attributed to unfolding of tertiary structure. Schlimme, v.d. Haar and Cramer (1969) correllated these changes with the rate of aminoacylation of purified yeast $tRNA^{Ser}$ and $tRNA^{Phe}$. They concluded that the sharp drop in charging exhibited at 39° was due to conformational changes in the tRNA molecule, but that an equilibrium between the ordered and partially-unfolded

structure existed.

Fresco, Adams, Ascione, Henley and Lindahl (1966) demonstrated that E.coli valyl-tRNA formation was quantitative up to 55°, was about 80% at 60° and dropped to 0 at 65°. The aminoacylating activity lost at 65° was fully recovered upon lowering the temperature.

Novelli (1967) summed up the position with regard to heat inactivation of tRNAs when he stated that different tRNAs can be denatured under a variety of conditions. In some cases the conditions leading to denaturation can be stabilised in the denatured configuration and as such are incapable of becoming aminoacylated. In other cases the conditions leading to denaturation are so mild that they preclude any interruption of secondary structure.

The time required for heat inactivation at 60° varied with two different aminoacyl-tRNA synthetases studied by Reznikova (1965) Addition of the appropriate amino acid partially stabilised the enzyme, while addition of amino acid together with ATP did not further increase stability. A purified isoleucyl-tRNA synthetase was stabilised at 55° by addition of saturating amounts of amino acid, ATP, amino acid + ATP, listed in order of stability which suggests that the conformation of the enzyme is most stable when the enzyme occurs as its aminoacyladenylate complex (Baldwin and Berg, 1966).

In order to interact with the ribosome and perform its template role in polypeptide synthesising systems, the template polynucleotide evidently should satisfy a single necessary condition - it should NOT possess a perfect secondary structure or a large fraction of especially stable co-operative helical regions. Thus neither the usual native DNAs nor double-stranded viral-RNAs, nor helical complexes of synthetic polynucleotides of the type of the Poly-A — Poly-U complex as well as synthetic polynucleotides with an especially large G content, possess template activity and they are generally incapable of combining with the ribosomes (Takanami and Okamoto, 1963, i and ii; Nirenberg and Matthaei, 1961; Singer, Jones and Nirenberg, 1963; Nirenberg, Jones, Leder, Clark, Sly and Pestka, 1963). Nevertheless natural mRNA and single-stranded viral-RNAs e.g. TYMV-RNA, are ideal templates although they possess a rather developed secondary structure (Spirin, 1963; Bautz, 1963; Haselkorn, Fried and Dahlberg, 1963). In order to associate with the ribosome, the polynucleotide primarily must possess single-stranded non-helical regions. It is especially important that the 5' terminal nucleotides should not be included within the helix, since the ribosome is preferentially attached to the 5' end of the polynucleotide.

In the present investigations, TYMV-RNA was found to promote the incorporation of a $[^{14}C]$ amino acid, determined as a hot trichloroacetic acid precipitable material, in the Complete System with V.faba or P.aureus tRNAs. (FIGS.44, 43).

The time-course of TYMV-RNA-direction (FIG.45), showing no

apparent lag phase, is typical of a natural messenger-RNA, with initiating codon and/or initiating conditions present in the incubation. (Spirin and Gavrilova, 1969).

$[^{14}C]$ leucine was used in the experiments since leucine together with isoleucine and valine account for 25% of the residues found in the protein shell of the virus (Matthews and Ralph, 1966). It was thought advisable not to use $[^{14}C]$ valine, since this amino acid binds to the 3' terminus of TYMV-RNA, even though valyl — TYMV-RNA was deacylated by the hot trichloroacetic acid procedure (TABLE 4).

In the Complete System with P.aureus or V.faba tRNAs the Mg^{2+} optima for viral-RNA direction were 5mM and 2mM respectively. These concentrations parallel the Mg^{2+} optima for Poly-U direction which were correspondingly 12mM and 10mM. In the Complete System with V.faba tRNA considerable incorporation was promoted by viral-RNA in the absence of exogenously-supplied Mg^{2+} but as previously discussed an amount of Mg^{2+} was present in the microsomal and high-speed supernatant enzyme preparations.

No absolute statement may be made with regard to a universal concentration requirement for Mg^{2+} for natural messenger-directed in vitro systems. Spirin and Gavrilova (1969) have stated that the optimum concentration of Mg^{2+} is from 5mM to 15 - 20mM; in the presence of increased concentrations of monovalent cations (K^+, NH_4^+, $Tris^+$) somewhat larger concentrations of Mg^{2+} are required. It has been shown that at concentrations greater than 9mM the association of the ribosome with the template and the

usual tRNAs becomes stronger and the ribosomes begin to form the initial complex less specifically, independent of whether there is an initiating codon present analogous to the situation with Poly-U (Nakamoto and Kolakofsky, 1966; Kolakofsky and Nakamoto, 1966; Sundararajan and Thach,1966).

The work of Verhoef and Bosch (1971) shows how a viral-RNA messenger can initiate at 2 Mg^{2+} optima. Using E.coli ribosomes, alfalfa mosaic viral-RNA required initiating factors, GTP and fMet-$tRNA_F$ to promote incorporation at low Mg^{2+} concentrations. When N-acetylaminoacyl-tRNAs were present in the system alfalfa mosaic viral-RNA promoted incorporation in the absence of factors, GTP and fMet-$tRNA_F$ at relatively high Mg^{2+} concentrations. Since these workers did not identify the polypeptides produced under the two conditions it remains to be determined whether both initiating systems have relevance in vivo. The failure to-date to demonstrate unequivocably a mechanism for acetylation of aminoacyl-tRNAs, other than the established fMet-$tRNA_F$ mechanism, must caution statements which regard in vitro incorporation promoted by viral-messengers at high Mg^{2+} concentrations as a reflection of an in vivo situation.

The Mg^{2+} optima required for TYMV-RNA direction reported in this present investigation (FIGS.43, 44) compared with those shown by Marcus (1970) in a tobacco mosaic viral-RNA wheat embryo ribosomal system i.e. 3.4mM, and by Klein, Nolan, Lazar and Clark (1972) in a satellite tobacco necrosis viral-RNA wheat embryo ribosomal system i.e. 3mM.

It is worth noting that the comparatively low Mg^{2+} requirements for these 80s ribosomal systems, compared with 70s systems, may be a consequence of the fact that bacterial ribosomes can be dissociated readily at 1mM Mg^{2+} into sub-units and mRNA (Gros, Hiatt, Gilbert, Kurland, Risebrough and Watson, 1961; Schlessinger and Gros , 1963). Eukaryotic ribosomes appear more stable at Mg^{2+} concentrations lower than 1mM Mg^{2+} (Arnstein, 1963).

The TYMV-RNA-directed Complete System with V.faba tRNA showed little GTP dependence (TABLE 6) paralleling the same lack of dependence exhibited by the Poly-U-directed Complete System with the same tRNA. The ATP, PCK, CreP dependence of viral-RNA direction in the Complete System was also minimal (TABLE 7) but, as previously discussed, the Complete System with V.faba tRNA was contaminated with ATP capable of supplying the requirements of the viral-RNA-directed system.

In Complete Systems involving natural messengers it is essential that the full aminoacyl-tRNA apparatus be present, since natural mRNAs code for all the usual 20"protein"amino acids. Consequently, the Complete Systems used in this investigation were exogenously-supplied with these 20 amino acids, despite the fact that many workers have reported a sufficiency of contaminating amino acids present in microsomal systems (Matsushita, Mori and Hata, 1968; Parisi and Ciferri, 1966, and Pearson, 1969). It may be added that in Poly-U systems only phenylalanine need be supplemented (Allende, 1969) although Spirin and Gavrilova (1969) do point out that the presence of other aminoacyl-tRNAs in

Poly-U systems may influence the rate of synthesis.

A general survey of the Results Section would show that maximum incorporation promoted by approximately 0.1mg TYMV-RNA in the Complete System with tRNAs from P.aureus or V.faba was respectively 25 and 15 pmol $[^{14}C]$ leucine incorporated/mg microsomes. No significant incorporation was promoted by the viral-RNA in the Complete System with yeast tRNA. These results contrast with Poly-U direction of the Complete System where much higher rates of incorporation were obtained (of the order 10- to 40- fold) and where V.faba was the best source of tRNA used, rather than P.aureus as in the viral-RNA system.

At equivalent concentrations of the 2 messenger molecules, it might be expected that TYMV-RNA direction would result in a lower incorporation value than Poly-U direction because TYMV-RNA directs 20 amino acids and the $[^{14}C]$ leucine label is distributed amongst these in the polypeptide chain. In a synthesised polypeptide chain of arbitrary unit length, the amount of leucine must perforce be less than the amount present in a polyphenylalanine chain. There are other reasons why viral-RNA direction would lead to lower incorporation, for example breaks in the mRNA molecule. (Haselkorn, 1962).

TYMV-RNA failed to direct polypeptide synthesis in the Complete System with yeast tRNA. This suggests that the amino-acylation of that source tRNA by P.aureus high-speed supernatant enzyme fraction was not conducive to the translation of the natural messenger molecule, contrasting with the satisfactory performance

of yeast tRNA in the Complete System directed by Poly-U, using the same high-speed supernatant enzyme fraction. Subsequent experimentation was made to compare the activity of the high-speed supernatant enzyme fraction in acylation of the different source tRNAs and various amino acids. It may be judged empirically that the same volume of P.aureus high-speed supernatant enzyme fraction (at 12mM Mg^{2+}, at a constant ATP concentration and at a constant $Tris^{+}$ concentration) charged yeast tRNA less well than V.faba or P.aureus tRNAs (TABLE 1), with one exception, namely that there was 30% greater formation of yeast phenylalanyl-tRNA compared with P.aureus phenylalanyl-tRNA.

When V.faba tRNA and yeast tRNA were charged with the 14[U ^{14}C] amino acid mixture under the same conditions, the label bound to yeast tRNA was about half that bound to V.faba tRNA. Allowing that the commercially-obtained yeast tRNA preparation was not just simply damaged in extraction, several explanations are feasible:

1. V.faba tRNA material possesses more tRNA species cognate with the particular 14[U ^{14}C] amino acids found in the mixture than does yeast tRNA material;

2. Fewer species of tRNA are charged by the P.aureus high-speed supernatant enzyme fraction in the yeast preparation than in the V.faba preparation;

3. Individual species of tRNA are not charged by the P.aureus high-speed supernatant enzyme fraction as successfully in the yeast as in the V.faba material.

At present, little data is available on the exact tRNA complement of any source tRNA. Some information has been

published which indicates the proportion and activity of some tRNAs change during organism development, but these are comparative rather than absolute values. (Yang and Comb, 1968; Zeikus, Taylor and Buck, 1969; Kaneko and Doi, 1966; Doi, Kaneko and Igarashi, 1968; Anderson and Cherry, 1969).

Although parameters pertaining to phenylalanine acylation have been referred to in a consideration of the Poly-U-directed Complete System, it is useful to revise them here when discussing the parameters affecting acylation of valine, and leucine with the source tRNAs. In the acylation of these amino acids to tRNAs, whereas the P.aureus high-speed supernatant fraction promoted rapid rates with V.faba and P.aureus tRNAs, the rates with yeast tRNA were decreased considerably (FIGS.16, 17, 18). Lower acylation rates have been recorded in heterologous reaction by a number of workers, Loftfield and Eigner (1963), Lagerkvist and Waldenstrom (1964), Kalousek and Rychlik (1965), Kalousek, Cerna, Rychlik and Sorm (1966), and Moustafa (1966). E.coli valyl-tRNA synthetase is known to react very slowly with yeast tRNA ^{Val}, but Loftfield and Eigner (1963) have shown that the Michaelis constants are remarkably similar for the heterologous and homologous systems. These authors concluded that the difference between the rates was probably due to structural distinctions affecting valyl-tRNA formation.

A general comment on the effect of pH on acylation of valine, leucine and phenylalanine to the different source tRNAs could be that pH was optimal above 8 for yeast tRNA, and below 8 for tRNAs

from V.faba and P.aureus (FIGS. 19, 20, 21). In nearly all respects the ranges of pH and optima were different for the esterification of each amino acid to each tRNA.

Information about pH effects on aminoacyl-tRNA synthetases is limited when compared with the data available on other governing parameters. The aminoacyl-tRNA ester bond does, however, become increasingly unstable at high pH values, for example at pH 8 a half-reaction time of 9 min occurs for spontaneous splitting of lysine from the ester bond. (Kalousek and Rychlik, 1965). An examination of the effect of pH on synthetase activity in extracts of wheat germ has shown that the optimum varies with the enzyme, the values veering towards the alkaline side of neutrality. (Moustafa and Lyttleton, 1963). Optimal pH values for purified enzymes can vary from 6.8 - 7.1 for aspartyl-tRNA synthetase from yeast (Moustafa and Peterson, 1962), to 8.5 - 9 for the valyl-tRNA synthetase from E.coli (Bergman, Berg and Dieckmann, 1961). In an early report, Davie et al (1956) showed that tryptophanyl-tRNA synthetase from pancreas did not exhibit an optimum pH value. Makman and Cantoni (1966) demonstrated that the conditions necessary for yeast seryl-tRNA synthetase to esterify serine were more stringent with E.coli tRNA than with yeast tRNA as substrate. In the report of these authors the pH optimum was higher with E.coli tRNA as substrate.

Mg^{2+} is necessary for the aminoacylation reaction. Peterson (1967) summarised that the optimum concentration of Mg^{2+} and the relative activity on either side of the optimum varies with each

enzyme. Mans (1967) emphasised that the optima for aminoacyl-tRNA synthetases with regard to pH, ionic strength and especially the Mg^{2+}/ATP ratio, would be different for each activating enzyme, both within species and between species.

The Mg^{2+} optima for valine, leucine and phenylalanine acylation of yeast tRNA had a relatively smaller activity either side the optimum than the tRNAs from V.faba or P.aureus (FIGS.22, 23, 24). This is particularly emphasised, since in the absence of exogenously supplied Mg^{2+} the yeast tRNA is scarcely acylated whilst the activation of V.faba and P.aureus tRNAs is still marked - these acylations being dependent on contaminating Mg^{2+} present in the incubations. In the V.faba tRNA and P.aureus tRNA acylations the relative activity either side of the optimum was so great that the high-speed supernatant enzyme fraction was capable of promoting marked esterification of valine and phenylalanine at the Mg^{2+} optimum for leucine, and vice versa. A similar situation obtained with P.aureus tRNA, but not with yeast tRNA. At the Mg^{2+} optimum for phenylalanyl-yeast-tRNA formation, the enzyme fraction allowed only poor esterification of valine, and the converse held. Leucine, however, could be charged reasonably well at the Mg^{2+} optimum for phenylalanine and valine. This implies a situation whereby an optimum condition for one amino acid-tRNA esterification is inhibiting to another, which may have been the reason why yeast tRNA charged glutamic acid so poorly at 12mM Mg^{2+} (TABLE 1). The inference may also be drawn that in the TYMV-RNA-directed Complete System containing yeast tRNA, lack

of activity was due to a constraint placed upon the system; for example, Mg^{2+} requirements for reactions other than aminoacylation precluded satisfactory acylation of the yeast tRNA to the usual 20 "protein" amino acids. This limiting condition would not apply to tRNAs from V.faba and P.aureus, since they could function under the restraints of the Complete System.

It has been previously deduced that ATP was a contaminant in the Poly-U-directed Complete System with V.faba or P.aureus tRNAs, by virtue of the activity allowed in the absence of exogenously-supplied ATP. In the acylation activity of the 3 source tRNAs by valine using P.aureus high-speed supernatant enzyme fraction in the absence of exogenously-supplied ATP, various reductions were recorded (TABLE 2). At the simplest evaluation, the results indicated that there was contaminating ATP present in the reaction, if the reaction were a true theoretical enzymatically-promoted aminoacylation. The contaminating ATP might well be associated with tRNA having survived the extraction process, or it might be present in the high-speed supernatant enzyme fraction, even though these fractions had been dialysed to remove ATP. The results presented in Table 3 show the relative reduction in acylation activity with leucine, valine and phenylalanine with one source tRNA - in this case tRNA from V.faba germinating seed. Again at the simplest evaluation, this result showed that different amino acids required different amounts of ATP for esterification to tRNAs, since the level of contaminating ATP in the reaction was the same although of unknown quantity.

Nevertheless, as Mans (1967) has pointed out, it is the ratio between Mg^{2+} and ATP, and not Mg^{2+} alone which conditions the parameters of Mg^{2+} optima for specific aminoacylation reactions. In this present investigation, exogenously-supplied ATP was kept constant but since the concentration of contaminating ATP was obviously unknown the Mg^{2+} optimum obtained could be said to be arbitrary. In the different reductions of activity among the three amino acids with the same source of tRNA, the absence of exogenously-supplied ATP may merely have led to a reduction in activity consequent upon the alteration of the Mg^{2+}/ATP ratio. The values presented in Table 3 show that the three amino acids have differing Mg^{2+}/ATP optimal ratios.

Mans (1967) reported that the optimum ratio of Mg^{2+}/ATP for E.coli leucyl-tRNA synthetase is 10, while that for prolyl-tRNA synthetase is 30. Further, comparing the leucyl-tRNA synthetase of E.coli and rat liver, the optimum of the former is 10, and of the latter is 1. Indeed, at 10, rat liver leucyl-tRNA synthetase shows hardly any discernible reaction.

In summary, when optimal conditions have been determined for reactions containing ATP and Mg^{2+}, the omission of ATP, resulting in a depression or absence of activity indicates ATP dependence. It should be emphasised that the presence of the remaining endogenous ATP constitutes a Mg^{2+}/ATP ratio, non-optimal for the reaction. Therefore, it is advisable to determine the Mg^{2+} optimum in terms of the contaminating ATP, when the percentage differences in activity between exogenously-added ATP in the

presence of contaminating ATP, and contaminating ATP alone, are then more meaningful.

There are innumerable instances quoted where the heterologous synthetase does not recognise a tRNA species. This is claimed to be an absolute phenomenon, not simply a question of providing the correct pH, ionic and Mg^{2+}/ATP ratio environment. (For ref. see Novelli, 1967, and Boulter, 1970). To demonstrate such phenomena, it is necessary to fractionate the high-speed supernatant enzyme into individual aminoacyl-tRNA synthetases, in order that cross-reactivity with purified tRNA species may be investigated. It was not feasible to enter upon such a project in the context of the present work, but it is a possibility that one or more aminoacylating tRNA synthetases of P.aureus high-speed supernatant enzyme fraction did not recognise yeast tRNA species. It should be borne in mind that V.faba tRNA — P.aureus high-speed supernatant enzyme fraction is a heterologous system, which does not inhibit the TYMV-RNA-directed Complete System, as does its yeast tRNA counterpart.

It was apparent that the conditions were different for the formation of aminoacyl-tRNA with each source tRNA. If these conditions were constraining on the Poly-U-directed P.aureus Complete Systems, and in the case of yeast tRNA, inhibiting in the TYMV-RNA-directed P.aureus Complete System, rather than allowing the aminoacyl-tRNA complex to be formed in situ the provision of aminoacyl-tRNA was likely to overcome these barriers. The provision of such preformed aminoacyl-tRNA to the polypeptide-synthesising in vitro system constitutes the Transfer System.

P.aureus tRNA was becoming increasingly difficult to produce practicably so it was decided not to employ that material in the Transfer System investigations.

Although viral-RNA direction of the Complete System gave clearly discernible and reproducible activity it was hoped to magnify the amount of radioactivity from the Transfer System by using a multi-$[^{14}C]$ label, which would also facilitate the identification of a viral-RNA-synthesised product by various radiochemical techniques available. There are also indications that single labels on a Transfer System derived from plant sources and directed by viral-RNA caused complications because the shortness of viral-synthesised peptide chains leads to difficulties in discerning the single label activity (van Kammen, 1967; J. Bewley, 1972, private communication). Indeed Matthews and Korner (1970), using a viral-RNA-directed mammalian cell-free system demonstrated an initial lag phase of 4 min which was entirely artefactual since at that time the particularly short peptide chain length was acid soluble.

The Transfer System was investigated using both single- and multi- labelled aminoacyl-tRNA.* It should be noted that the $14[U^{14}C]$ amino acid mixture (CFB 104) is composed of 14 individually purified L- amino acids - $[U^{14}C]$ mixed in approximately the same proportions as a typical algal protein

* All aminoacyl-tRNAs were constructed from the 20 usual amino acids. Single-label means one $[^{14}C]$ amino acid + 19 $[^{12}C]$ amino acids; multi-label means $14[U^{14}C]$ amino acids + 6 $[^{12}C]$ amino acids (See Methods).

hydrolysate.. It is not that material obtained by acid hydrolysis of algal protein (CFB 25) which the Radiochemical Centre, Amersham, Bucks, recommend as not being suitable for protein synthesising systems.

Since it is generally held that the function of ATP and the regenerating system is in aminoacylation of tRNAs these components were omitted from the constitution of the Transfer System, a procedure commonly adopted (Igarashi and Paranchynch, 1967; Payne, 1970) although some workers include PEP and PK or CreP and PCK in Transfer Systems to regenerate GTP (Allende, 1969; Marcus, 1970).

The Transfer System was first characterised in terms of Poly-U direction using both single- and multi- $[^{14}C]$ labelled aminoacyl-tRNAs derived from yeast or V.faba tRNAs. The single-$[^{14}C]$ label was phenylalanyl-tRNA. Although not every Poly-U experiment discussed here was performed with single- and multi-$[^{14}C]$ labels, sufficient were performed to allow an assumption that the parameters conditioning polyphenylalanine synthesis were the same with each type of label.

Aspects of the synthesis of polyphenylalanine in the Transfer System were markedly different from the synthesis in the Complete System. The overall activity promoted by Poly-U was much lower and the time-course of incorporation much shorter in the Transfer System (FIGS.34, 55 - 59). Allende and Bravo (1966) and Payne (1970) have reported similar contrasts between Transfer and Complete Systems. This reduction of activity may be consequence

of the phenylalanine transferred from the aminoacyl-tRNA complex being rate-limiting, whereas in the Complete Systems $[^{14}C]$labelled amino acids were in excess. Alternatively, since the aminoacyl-tRNA preparation might contain non-acylated tRNA (Allende, 1969) this might become charged in the incubation with contaminating $[^{12}C]$ phenylalanine and any ATP present, resulting in $[^{14}C]$ polyphenylalanine synthesis becoming diluted by its $[^{12}C]$ counterpart with a consequent reduction of radioactivity/unit peptide chain. When ATP was added to the yeast aminoacyl-tRNA Transfer System a marked depression of activity occurred, illustrating that such a phenomenon was possible (FIG.57) or that the supplementation of ATP induced Mg^{2+} absorption rendering sub-optimal the available Mg^{2+}. It was unlikely that tRNA was a contaminant of the microsomal preparation (FIG.73).

The significance of the different rates of incorporation of polyphenylalanine from yeast and V.faba aminoacyl-tRNAs in the Transfer System may also be a reflection of varying amounts of non-acylated tRNA present in the respective preparations, since Kaji and Kaji (1964), Kurland (1966), Levin (1966), Seeds and Conway (1966) have all shown that free tRNA is well-bound with the ribosome in the presence of template. This characteristic may also account for the overall lower incorporation capacity of the Transfer as compared with the Complete System.

The lack of enhancement when exogenously-supplying high-speed supernatant enzyme fraction to the Transfer System may have been due to an unwanted supplementation of $[^{12}C]$ amino acids but this

seems unlikely since these enzyme fractions were extensively dialysed, ostensibly to remove such contaminants (FIGS.54, 55). Alternatively, exogenous supplementation of enzyme may merely have increased the total concentration of enzyme above its optimum. A similar situation, though a little more complex, was obtained in the Complete System already discussed. It is obvious that the microsomal preparation requires much more thorough washing to rid preparations of this enzymatic influence but this was deliberately avoided in case essential co-factors necessary for viral-RNA-direction were lost. Since the Mg^{2+} optimum for the yeast aminoacyl-tRNA Transfer System was critical (FIG.51) it was also possible that the Mg^{2+} present in the high-speed supernatant enzyme fraction rendered the total Mg^{2+} concentration non-optimal. In all Transfer System incubations the enzyme, therefore, was not supplemented exogenously.

The Mg^{2+} requirement i.e. exogenously-supplemented, in the Poly-U-directed Transfer System with each source aminoacyl-tRNA complex was optimal at 8mM, contrasting with the Complete System at 12mM (yeast tRNA) and 10mM (V.faba tRNA). Allende (1969) reported a similar reduction in Mg^{2+} requirement between Complete and Transfer System derived from wheat embryo. An explanation for this reduction is that in the Complete System the extra Mg^{2+} was necessary to meet aminoacylation reaction requirements. Igarashi and Paranchynch (1967) record identical Mg^{2+} optimal for Poly-U-directed E.coli - derived Complete and Transfer Systems but the range allowing polyphenylalanine synthesis was much less in their

Transfer than in their Complete System.

K^+ was an absolute requirement in yeast or V.faba aminoacyl-tRNA Transfer System though the optimum for the former was much more critical (FIGS.48, 49). The high concentrations of K^+ needed for maximal activity are again reminiscent of the Allende (1969) wheat embryo Transfer System, and as that author points out these were very similar for the E.coli - derived Transfer System of Nakamoto, Conway, Allende, Spyrides and Lipmann (1963).

GTP could be clearly implicated in the Poly-U-directed Transfer System using yeast or V.faba aminoacyl-tRNA complexes (FIGS.56, 57). This increased dependence compared with the Complete Systems may be a consequence of the absence of high-speed supernatant enzyme fraction contributing GTP.

Striking differences between the yeast and V.faba aminoacyl-tRNA complexes in the Transfer System were found in their response to pH variation (FIGS.52, 53). There was no optimum for incorporation in the range pH 7.3 - 8.5 for the V.faba complex, contrasting with the range of pH 7.3 - 8.1 with a discernible optimum at pH 7.6, for the yeast complex. This indicated that the P.aureus transfer enzymes common to each Transfer System were much more stringent in their pH requirements for the transfer of the yeast aminoacyl-tRNA. Secondly, the transfer enzymes were much less stringent in their pH requirements than the aminoacylation enzymes in the in vitro systems containing V.faba tRNA, since in the Transfer System with V.faba aminoacyl-tRNA there was no

optimal response in the range of pH 7.3 - 8.5, contrasting with the discernible optimum of pH 7.8 in the Complete System with that source tRNA. Thirdly, in *in vitro* systems containing yeast tRNA there appeared 2 divergent optimal pH responses for transfer enzymes and aminoacylation enzymes, - the former at pH 7.6 and the latter at pH 8.1.

Evidently, in the Complete System containing yeast tRNA there is a mutual concession, towards optimal incorporation, between the aminoacylation reaction stage and the transfer stage, a condition not apparent in the corresponding *V.faba* tRNA system.

The various time-courses of incorporation in the Transfer System (FIGS. 54 - 59), show lag phases concomitant with Poly-U direction. The higher level of activity recorded with the multi-labelled *V.faba* aminoacyl-tRNA compared with the single label (FIG.59) may simply signify that more [^{14}C] phenylalanine charged to *V.faba* tRNA using the 14[^{14}C] amino acid mixture than using [^{14}C] phenylalanine, which would be reflected in incorporation if the aminoacyl-tRNA transference of phenylalanine were rate-limiting for peptidyl incorporation. On the other hand, data has been published showing that Poly-U can direct the incorporation of substantial amounts of leucine in an *E.coli* - derived *in vitro* system (Bretscher and Grunberg-Manago, 1962; Matthaei, Jones, Martin, and Nirenberg, 1962). This anomalous Poly-U-directed incorporation of leucine has also been demonstrated in 80s ribosomal *in vitro* systems as well as in other 70s systems. (Sager, Weinstein and Ashkenazi, 1963; Bretscher and Jones, 1967;

Stavy, 1968; Friedman, Berezney and Weinstein, 1968). This effect is explicable in terms of translational errors induced by the non-physiological nature of *in vitro* systems (Boulter, Ellis and Yarwood, 1972). Serine, isoleucine and leucine were shown to be directed into peptidyl material by Poly-U but at concentrations below 10mM Mg^{2+} i.e. more closely resembling physiological conditions, the anomalous incorporation was eliminated (Szer and Ochoa, 1964). All the anomalous amino acids referred to above were present in the $^{14}[U^{14}C]$ amino acid mixture.

It must be remembered that aminoacyl-tRNAs labelled with $^{14}[U^{14}C]$ amino acids were essentially for use in TYMV-RNA-directed Transfer Systems, so that the yeast tRNA was charged at 12mM Mg^{2+} with the multi-label, on the understanding that the yeast-aminoacyl-tRNA preparation would contain representatives of all 20 "protein" amino acids. As mentioned previously, if the material had been charged at the Mg^{2+} optimum for phenylalanine it was probable that valine, at least, would have esterified to tRNA at a low level and this would not be conducive to a natural messenger-coding propensity. Since the Mg^{2+} optimum was not so critical for production of *V.faba* aminoacyl-tRNA, this condition was not relevant. The single label yeast i.e. $[^{14}C]$ phenylalanine, aminoacyl-tRNA preparation was charged at the phenylalanine Mg^{2+} optimum to facilitate Poly-U direction in the Transfer System. This would explain the higher levels of polyphenylalanine synthesis in the single-labelled yeast aminoacyl-tRNA Transfer System, compared with its multi-labelled counterpart.

Despite the fact that each source aminoacyl-tRNA functioned in the Transfer System, the ionic and pH parameters governing the reaction with yeast aminoacyl-tRNA were more stringent than those with the V.faba complex. Since the provision of ready-formed aminoacyl-tRNA still permitted differences between yeast and V.faba complexes, it would appear that certain quantitative restrictive influences lay beyond the aminoacylation stage. Parisi et al.(1967), Klink and Richter (1966), Ilan and Lipmann (1966), Allende (1969) and Ciferri and Parisi (1970) have all reported results which show incompatibilities within heterologous ribosome-transfer enzyme systems, although Boulter, Ellis and Yarwood (1972) have concluded that there is a far greater degree of interchangeability of components from different eukaryotic systems than there is between those of eukaryotes and prokaryotes.

In the Transfer System, containing 14 $[U^{14}C]$ V.faba aminoacyl-tRNA, TYMV-RNA directed the synthesis of polypeptide by a temperature-dependent reaction (FIG.62). The Mg^{2+} optimum for this reaction was 2mM, ostensibly the same as in the Complete System, but completely dependent upon exogenously-added Mg^{2+} i.e. $MgCl_2$, since no viral-RNA activity was observed without that salt supplementation. Since the Complete System was exogenously-supplemented with the high-speed supernatant enzyme fraction (containing Mg^{2+}), the Transfer System optimum constituted a reduction in the Mg^{2+} requirements, corresponding to similar

reductions in the Poly-U Transfer Systems*. This was taken to indicate that a portion of the Mg^{2+} supplied to the Complete System was required for aminoacylation reactions.

Dependence on GTP was shown, the partiality again demonstrating that the microsomal preparation used here was contaminated with GTP (FIG.61). K^{+} ion was an absolute requirement for viral-RNA-directed (and Poly-U) incorporation (FIG.67). The formation of the ternary complex, ribosome — template — aminoacyl-tRNA, is specifically stimulated by K^{+} or NH^{4} cations (Spirin and Gavrilova, 1969). Stimulation may range from strong (Spyrides, 1964) to slight (Pestka and Nirenberg, 1966). The Mg^{2+} ion is essential for the formation of the ternary complex; from the Mg^{2+} optimum determined in the present work, it may be deduced that binding of the aminoacyl-tRNAs with ribosomes would use GTP and Transfer factors. Only <u>in vitro</u> systems which function above 10mM Mg^{2+}, a somewhat arbitrary figure, are GTP and Transfer factors not required. (Spyrides and Lipmann, 1962; Conway, 1964; Kaji and Kaji, 1964; Spyrides, 1964; Nirenberg and Leder, 1964; Nishizuka and Lipmann, 1966 ii, Kurland, 1966).

Spirin and Gavrilova (1969) have pointed out that the K^{+} ion serves another useful purpose in the suppression of "non-specific binding" of tRNA or aminoacyl-tRNA species.

* Microsomes for the Transfer System were stored at 50 mg/ml, compared with those for the Complete System stored at 20 mg/ml. Their presence in the incubation at 1 mg/ml represents a further reduction in Mg^{2+}.

to the ribosome in the absence of template, or where template is not ribosome-bound. This non-specific complex, readily formed at 0 - 4° needs neither energy source nor protein factors for formation. In the absence of K^+, in the work reported were, it was noted in experiments with 14 $[U^{14}C]$ - V.faba aminoacyl-tRNAs that 0-time samples showed high radioactivity, possibly owing, in part, to non-specific aminoacyl-binding. Using the Nirenberg and Leder (1964) modified binding technique (i.e. no aminoacyl-tRNA present), Dahlberg and Haselkorn (1967) investigated the binding properties of TYMV-RNA to E.coli ribosomes and showed that the viral-RNA is attached to the 30s ribosomal subunit; this could take place at 1mM Mg^{2+}, the reaction being temperature-dependent.

Cations are required to neutralise the phosphate groups of TYMV-RNA and the ribosomal RNA (Goldberg, 1966), thereby allowing the two structures to interact. This necessity for charge neutralisation, before interaction can occur, indicates that the viral-RNA approaches a region of high negative charge during the binding process. The complex, once formed, is stabilised by divalent cations, Mg^{2+}. However, Dahlberg and Haselkorn (1967) showed that the stabilisation of the complex could not be due to charge neutralisation alone, since K^+ tended to antagonise Mg^{2+} and de-stabilise the complex when Mg^{2+} was above 5mM. Below 5mM, Mg^{2+} and K^+ did not compete but assisted in neutralising the charge. It was concluded that the association of viral template to the ribosome was stabilised both on account of non-specific neutralisation of negative charges and of direct participation of divalent cations.

The competition between Poly-U and TYMV-RNA direction was recorded in the V.faba aminoacyl-tRNA-containing Transfer System (FIGS.63, 64). This reaction has been reported previously. (Haselkorn and Fried, 1964 i and ii; Dahlberg and Haselkorn, 1965, 1967). In summary E.coli ribosomes were not divided into 2 classes, one binding Poly-U and the other TYMV-RNA, therefore the two polynucleotides competed for the same ribosomal site. The equilibrium constants for ribosomal association were of the same order of magnitude so that when TYMV-RNA was in excess in the incubation, Poly-U depressed TYMV-RNA binding. When Poly-U and TYMV-RNA were used together to direct incorporation of amino acids, $[^{14}C]$ proline incorporation was depressed, compared with TYMV-RNA-directed incorporation of that amino acid. $[^{14}C]$ phenylalanine was depressed slightly when a mixture of TYMV-RNA and Poly-U was used.

In incorporation and binding experiments, both messengers were capable of both activities, at the same Mg^{2+} optimum. A considerable lag phase (3 min) was observed in TYMV-RNA-promoted incorporation which the authors attributed to (a) association of RNA and ribosomes proceeding in the absence of protein synthesis, and (b) to peptide chain initiation. This lag was not obvious with Poly-U-directed phenylalanine incorporation which was 10x greater than the proline incorporations.

In observing the competitive effects of Poly-U and TYMV-RNA in the Transfer System with V.faba aminoacyl-tRNA, there was one major parameter difference from the system of Haselkorn et al. The Mg^{2+} requirements were not equivalent for Poly-U-and TYMV-RNA-

directed incorporation. At 2mM Mg^{2+} viral-RNA promoted significant incorporation of amino acids and at that concentration Poly-U-direction was markedly sub-optimal. At 8mM Mg^{2+}, Poly-U optimally directed polyphenylalanine synthesis whereas there was little viral-RNA-directed incorporation. In the presence of TYMV-RNA at the Poly-U Mg^{2+} optimum, polyphenylalanine synthesis was reduced by half and a considerable lag phase was observed. The reason for the lag phase, with both mRNAs present, may have been that the lag was more apparent at the lower level of polyphenylalanine synthesis, or that the binding rate of viral-RNA was more rapid than that of Poly-U in the initial stages of binding.

The hot trichloroacetic acid process (Methods, 3) allows recovery of minimal polypeptide chains; it is not diagnostic of binding. With this in mind, the inhibition of polyphenylalanine synthesis was seen to indicate that TYMV-RNA was binding to ribosomal sites occupiable by Poly-U. If the general premise is accepted that mRNA-binding to ribosomes is the first step in translation, it is perplexing to note the ability of TYMV-RNA to bind but not to promote polypeptide synthesis. Yet, E.coli ribosomes have been shown to associate readily with TYMV-RNA at 0^{o} in the complete absence of protein synthesis. (Bosch, van Knippenberg, Voorma and van Ravenswaay Claasen, 1966; Voorma, Gout, van Duin, Hoogendam and Bosch, 1965). Simple mixing of the washed ribosomes at the appropriate Mg^{2+} concentration resulted in heavy complexes, characterised by sucrose density centrifugation, which sedimented much more quickly than single 70s particles. The relative

abundance of these complexes was strongly dependent on the ribosome : viral-RNA ratio and Mg^{2+} concentration. These authors also pointed out that in a range of plant viral messengers studied this was a unique property of TYMV-RNA.

At 2mM Mg^{2+}, TYMV-RNA promoted polypeptide synthesis and Poly-U sub-optimally promoted polyphenylalanine synthesis. In the presence of both mRNAs, an increased recoverable radioactivity was noted, representing viral-RNA-directed incorporation of 14 $\left[U^{14}C\right]$ amino acids, including phenylalanine, and Poly-U-directed $\left[^{14}C\right]$ polyphenylalanine synthesis. There was apparently no depression of Poly-U synthesis of polyphenylalanine in the presence of TYMV-RNA, and the converse held.

It is possible that, at a Mg^{2+} concentration suitable for TYMV-RNA direction of polypeptide synthesis, only the appropriate initiation site on the TYMV-RNA molecule is bound to the ribosome. Dahlberg and Haselkorn (1966) have shown that each intact molecule of TYMV-RNA contains 4 ribosome-binding sites, one with a stronger affinity for the ribosome than the other three.

In the present work, the incorporation promoted by TYMV-RNA terminated at 30 min, whereas an increase of activity with Poly-U alone was still being recorded between 30-60 min. In the presence of both messengers, the incorporation response seemed purely additive up to 30 min, followed by an increase in incorporation the rate of which between 30-60 min was

greater than that directed by Poly-U at that time. The rate of increase after 30 min with both messengers was parallel to that obtained with Poly-U alone, from 0-15 min. It must be concluded that the increase of polypeptide synthesis after 30 min in the presence of both messengers was in effect the result of Poly-U direction only. It is implicit that the TYMV-RNA messenger molecule, having been translated, was released from the ribosome, thereby allowing fresh ribosomes to bind Poly-U to induce further polyphenylalanine synthesis. This demonstrated a recycling of ribosomes in peptide chain synthesis.

Revel, Greenshpan and Herzberg (1970) have demonstrated that phage T4 -RNA is translated by E.coli ribosomes in preference to most synthetic templates. This preference is attributed to initiation factors and not simply to the presence of the initiating codon AUG, since blocked copolynucleotides containing the codon AUG had no competitive activity. Lodish (1969) reported species specificity in the recognition of the different 'phage f2 RNA cistrons.

When endogenous activity was recorded in the Transfer System, TYMV-RNA inhibited that activity at 8 - 10 mM Mg^{2+} (FIGS.68, 70), and suppressed it at 6mM Mg^{2+} since in the equivalent TYMV-RNA incubation, activity was less than in the endogenous control (FIG.69). At the Mg^{2+} optimal for TYMV-RNA-direction of incorporation, the endogenous controls indicated less activity than in the incubations containing TYMV-RNA.

The suppression of endogenous activity by TYMV-RNA may be analogous with the situation obtaining with Poly-U added to TYMV-RNA. Any control incubations, therefore, performed at the Mg^{2+} optimum for viral-RNA direction but excluding viral-RNA, must be treated circumspectly. That is to say, such endogenous activity may not necessarily occur in the presence of the viral-RNA.

For this reason, the practice of subtracting endogenous activity from viral-RNA-promoted activity has been avoided in this thesis. To evaluate fully the competition effects shown by TYMV-RNA, it will be necessary to consider *in vitro* systems where the promoters of activity are in saturating conditions. The isolation of endogenous mRNAs is essential to demonstrate any preferential selection by the ribosome of viral- or homologous-mRNAs.

An attempt to investigate the role of valyl — TYMV-RNA in the Transfer System led to complete inhibition of the system (TABLE 8). This could be interpreted that charged TYMV-RNA was incapable of promoting polypeptide synthesis, but it was significant that the endogenous activity recorded in the absence of ATP was also inhibited with ATP present. This indicated that inhibition was a general property of the *in vitro* system, rather than a specific viral-RNA function. The inhibition was probably induced by ATP either absorbing Mg^{2+} from the system rendering it non-optimal or promoting charging of non-acylated tRNA which ultimately led to isotopic dilution of the $[^{14}C]$ label. Non-acylated tRNA, if present, would be found in the aminoacyl-tRNA preparation for it was unlikely there was tRNA in the microsomal preparation (FIG.73). The activity ··

recorded in this Fig. in the absence of ATP indicated that ATP was contaminating either in the microsomal fraction and/or the V.faba tRNA. High-speed supernatant enzyme fraction was not added, so that fraction was eliminated as a contaminating source of ATP.

The problem of contaminating -ATP and -GTP has been a constant problem in this investigation; future work must ensure that such endogenous components are removed from the microsomal preparation by means of thorough washing, column chromatographic procedures or sucrose gradient techniques. Thorough washing of the microsomal preparations also validates the claim that elongation factors are found only in the high-speed supernatant enzyme fraction. (Nathans and Lipmann, 1961; Lengyel and Soll, 1969).

It is doubtful whether the viral-RNA was uncharged in the Complete System. The parameters governing esterification of valine to TYMV-RNA (FIGS.28, 29, 30, 32) match those required by the Complete System directed by Poly-U, although in the TYMV-RNA-directed Complete System the Mg^{2+} requirements for optimal formation of valyl-TYMV-RNA were relatively higher. A more successful approach must be through the provision of pre-charged TYMV-RNA to the polypeptide-synthesising system, where the effect of charged or non-charged viral-RNA might be evaluated.

The true biological significance of tRNA-like structures in viral-RNAs is not known at present, but it is known that specific amino acids either have an inhibitory effect or are essential to viral multiplication (Eaton, Scala and Low, 1964; Medvedkina, Maslova and Shirman, 1969, 1972, and Obert, Tripier, Bingen and

Guir, 1971).

When yeast 14 [U^{14}C] aminoacyl-tRNA was used in the Transfer System neither was there significant incorporation promoted by TYMV-RNA nor endogenously-promoted activity. The conclusion must be that the provision of yeast aminoacyl-tRNA did not overcome the barrier observed in the TYMV-RNA-directed Complete System with yeast tRNA. As already discussed, yeast tRNA in the Complete and Transfer Systems directed by Poly-U showed marked parameter differences from the corresponding V.faba tRNA systems. It would be interesting to see whether a yeast high-speed supernatant enzyme fraction would permit TYMV-RNA direction of a P.aureus microsomal system with yeast tRNA. If it should not be effective then other factors may be necessary for successful viral-RNA translation. Aviv, Boime and Leder (1971) have postulated the use of specific degenerate codon classes by viral-RNA; they suggest that the barriers of heterology may extend to less closely related higher organisms, to specialised organs of a single species, to organs at various stages of development or even to certain viruses and their hosts.

It was to be expected that no activity was recorded with single [^{14}C] labelled phenylalanyl-yeast tRNA and the corresponding leucyl-tRNA. It was rather problematical however, that V.faba [^{14}C] phenylalanyl-tRNA present in the Transfer System directed by TYMV-RNA allowed no activity. Possibly the amount of [^{14}C] phenylalanine incorporated into acid - insoluble material was not sufficient to be recorded. Other workers generally use much higher specific activity labels than were used here. Klein, Nolan, Lazar

and Clark (1972) showed that the use of $[^{14}C]$ algal hydrolysate mixture limited translation in an *in vitro* Complete System directed by satellite tobacco necrosis viral-RNA. These workers claimed that such mixtures created size non-uniformities in the product proteins which hindered characterisation of the translation product. The problem was overcome by using a mixture of 15 $[^{14}C]$ amino acids at equivalent concentrations supplemented with the appropriate $[^{12}C]$ amino acids.

Boulter (1970) has observed that the establishment of a natural messenger system is necessary before the understanding of the mechanism of protein synthesis on plant ribosomes is complete. Certainly the use of synthetic messengers has rendered comprehensible many of the broad principles of protein biosynthesis, but the conditions they require for promotion of polypeptide synthesis may not be a true reflection of the physiological state. An examination of the conditions required, *in vitro*, by a natural messenger molecule derived from a plant cell, should more accurately reflect the *in vivo* situation. Since the isolation of plant mRNAs is yet to be achieved, then RNAs derived from viruses which infect plants are regarded as a viable substitute. Comparisons have been made in this discussion between the characteristics of Poly-U direction and TYMV-RNA direction of polypeptide synthesis; it is, perhaps necessary at this point to consider the relation between the plant mRNA and viral mRNA.

In this investigation a viral-RNA has been used in an *in vitro* system derived from a plant which the virus does not infect. The main practical reason for the selection of this plant-derived

system was that Durham University's Department of Botany had already considerable experience with *in vitro* systems utilising components from legumes. However, it might be argued that the conditions necessary for the promotion by TYMV-RNA of polypeptide synthesis in a legume-derived cell-free system, are artefactual by definition since a corresponding *in vivo* state does not, in this case, exist. It is necessary to recall some facets of host-specificity of viruses to gain a clearer understanding of this apparent artefact. Fraenkel-Conrat (1969) pointed out that host-specificity is, in part, a property of the viral coat protein; infectious RNA has been shown in several instances to be capable of infecting tissue not susceptible to the intact virion.

Indeed, when the replication of viruses is under consideration, host-specificity may be a function of one or more of the following stages. Replication is preceded by the entry of the virus into a host cell, the removal of the viral envelope and/or capsid protein. Then viral nucleic acid is transported to the sites where it is replicated, transcribed to RNA if need be, and translated. Finally, the progeny components mature to virus particles and usually leave the host cell through budding, extrusion or lysis of the cell. Relatively little is known about the earlier and later stages of the process. Double-stranded DNA viruses have mechanisms of replication most closely resembling those which occur in the normal cell. In the replication of the simplest viruses (such as the Picorna

group)*, containing linear RNA regarded as analogous to the mRNA of the normal cell, two-thirds of the replication process are considered equivalent to those occurring in the healthy cell: nucleic acid replication and translation. (Fraenkel-Conrat, 1969).

In considering initiation of protein biosynthesis in vivo, the question must be raised whether the ribosome preferentially binds viral-RNA rather than the host RNA. There were indications during the present investigation that TYMV-RNA prevented endogenous incorporation and Poly-U direction, though all reactions were conditioned by various Mg^{2+} concentrations. However, as stated previously, amongst viral-RNAs TYMV-RNA has unique binding properties which may indeed make it an unsuitable choice for studies designed to clarify a general statement on viral-RNA preferential ribosome binding, should such a phenomenon exist. It is generally held that one of the early effects of viral-infection in animal cells is the arrest of host RNA- and protein-synthesis. A similar situation obtains after 'phage infection of bacterial cells.

To account for this inhibition induced by the 'phage particle, two mechanisms have been proposed. The first involves a protein synthesised after infection under 'phage control. Ennis (1970) has

* The Picorna group contains the viruses of poliomyelitis, encephalomyocarditis, foot-and-mouth disease; the f2 class of bacteriophage. TYMV is also a member of this group.

demonstrated such a protein in a 'phage T4 - E.coli B cell-free system. This worker concluded that either this protein, necessary for the maximum termination of host RNA synthesis, may be labile or its formation and accumulation may be regulated. The second mechanism (Rouviere, Wyngaarden, Cantoni, Gros and Kepes, 1968) demands no 'phase-induced protein to shut off host synthesis. Indeed, protein synthesis plays no part at all; it is related entirely to the multiplicity of infection, i.e. the greater the multiplicity, the greater is the depression of synthesis. Fraenkel-Conrat (1969) in agreement with this premise, has stated the opinion that the shut-off of host synthesis in viral-infected animal cells is a coincidental result of early viral development and not an intrinsic activity of viral infection.

In conclusion, many questions remain to be answered with regard to the functioning of viral-RNAs in cell-free systems, and caution must be exercised in the general assumption that mRNA derived from plant viruses behaves in exactly the same manner as mRNA found in healthy plant cells. A full understanding of viral-RNA promotion of protein biosynthesis may not be reached until the isolation and characterisation of a plant messenger molecule has been achieved; then the properties of these messenger molecules may be correlated.

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Acknowledgements.

I am indebted to Professor D. Boulter for his counsel and the use of the facilities of the Department of Botany, University of Durham. I thank Dr. R. Pain, Department of Biochemistry, University of Newcastle, for the use of the Spinco E ultracentrifuge, and Mr. I.M. Thompson for the provision of Chinese cabbage RNA.

Finally, I am grateful to the Science Research Council for financial support.