Turnip yellow mosaic virus: biochemical aspects of the infection process

Thompson, I. M.

TURNIP YELLOW MOSAIC VIRUS :

BIOCHEMICAL ASPECTS OF THE INFECTION PROCESS

A Thesis -
submitted in accordance with the
requirements of the University of
Durham for the degree of Doctor
of Philosophy

By

I.M. THOMPSON, B. Pharm., M.P.S.

Department of Botany

September 1973

ABSTRACT

Investigators have demonstrated that the RNA from a variety of plant viruses can function as mRNA in *in vitro* protein synthesising systems. An active homologous plant cell-free amino acid incorporating system has been isolated from *Brassica chinensis* L., leaves, and partially characterised employing polyuridylic acid(poly(U)) and turnip yellow mosaic virus(TYMV) RNA as messengers. Activity of this system has been compared with an active system isolated from developing seeds of *Vicia faba* L., and with mixed *B. chinensis* - *V. faba* systems. Bacterial contamination was found to be low in such incubations.

$[^{14}C]$ phenylalanine aminoacylation of *B. chinensis* tRNA was maximal at 17.5 mM Mg^{2+} under which conditions TYMV RNA was found to accept $[^{14}C]$ valine.

Maximum $[^{14}C]$ phenylalanine poly(U) directed incorporation occurred at 8 mM Mg^{2+} in the *V. faba* and 6 mM Mg^{2+} in the *B. chinensis* system. TYMV RNA was relatively inactive when employing a single $[^{14}C]$ amino acid, but was active in the *B. chinensis* system when employing a $[^{14}C]$ amino acid mixture (maximum incorporation at 6mM Mg^{2+} and 70 mM K^{+}).

Partial characterisation of TYMV RNA (and other system components) was carried out using polyacrylamide gel and analytical ultracentrifugation techniques, indicating the infectious viral RNA to have a high sedimentation coefficient (c. 33 S).

Various extraction procedures of plant and viral components are

discussed with reference to ease of isolation and resultant biological activity. Microsomes isolated rapidly from young B. chinensis leaves were found to be most active in amino acid incorporation.

The application of these experiments to studies on biochemical aspects of the TYMV infection process are considered in the light of other work in this field.

It would appear that resistance of V. faba to infection by TYMV does not reside at the ribosomal level, and that TYMV protein synthesis occurs, at least in vitro, on the '80 S' rather than the '70 S' ribosome.

CONTENTS

INTRODUCTION

The biochemistry of virus infection in a sense is synonymous with the biochemistry of virus synthesis. The typical plant virus has been shown to consist of two chemical components, the infectious ribonucleic acid (RNA)* and virus protein. This RNA and protein do not occur in uninfected tissue. The protein has not exhibited any known enzymic activity, cannot by itself initiate infection, and appears to serve only as the protecting coat for the more delicate RNA. The RNA on the other hand possesses the capability of causing infection that results in the formation of typical virus particles. It therefore appears to carry the genetic information to reproduce not only itself but also the virus protein.

Most early workers believed that the multiplication of viruses did not differ essentially from that of microorganisms, i.e. the mechanism of reproduction resided within the virus particles and, given the proper medium, new progeny would arise from the parent. The final step of reproduction could be longitudinal or transverse fission followed by growth to maturity. The current view is that virus protein and nucleic acid are formed by two separate systems, the protein and nucleic acid then combine to form the stable, complex nucleoprotein particle.

There appears to be no alternative at present but to consider the biochemistry of virus multiplication to be intimately associated with two separate but interrelated biosynthetic mechanisms of the host;

---

* The abbreviations and conventions used throughout this thesis are those recommended in "The Biochemical Journal - Instructions to Authors" (Biochem. J. 121, 1-19, 1971).

the RNA involved with the activity of the nucleus or its immediate environment and the protein with the ribosomal system. It may be asked whether the virus influences or alters the genetic coding of the nuclear DNA to make an obviously foreign RNA or whether, given the proper substrates, energy, and enzymes, viral RNA serves as its own template. It may also be asked how the viral RNA participates in the synthesis of yet another foreign product, virus protein.

To virologists these are questions of paramount importance for in their solution may well be the answer to the direct control of virus diseases. The following is a short review of some of the literature pertaining to the subject, keeping these questions in mind.

It has been shown that the RNA moiety of tobacco mosaic virus (TMV) (Gierer and Schramm, 1956) and of several other plant viruses (Diener and Weaver, 1959; Harrison and Nixon, 1959; Kaper and Steere, 1959a, 1959b; Kassanis, 1960; Reichmann and Stace-Smith, 1959; Rushizky and Knight, 1959) is capable of initiating infection on its own and is also able to trigger the synthesis of complete virus particles. It is therefore implied that the genetic information for the complete virus particle is contained within its RNA.

If this is so, the nucleic acid of an RNA virus must be able to perform a number of essential functions, namely:

(1) Before an infecting RNA can act as a template for its own replication, or as a mRNA for protein synthesis, it

must be released from its viral coat. This separation is therefore of prime importance.

(2) Once in the host cell in a free state, plant virus RNA must induce the synthesis of complete virus particles. It must therefore be capable of self-reproduction, so as to direct the synthesis of copies of itself.

(3) In turn it must be able to direct the synthesis of coat and other virus specific proteins, and

(4) Finally there must be transport of the information for these processes to a new host cell, in the form of mature virions.

Uncoating of the parental viral RNA:

Unequivocal evidence of uncoating is at present lacking for plant viruses, although both direct and indirect experiments suggest it does occur. Shaw (1967) reported that almost all TMV particles retained by leaves after inoculation are at least partially uncoated.

The indirect experiments depend on comparing the susceptibility of infections made with whole virus and naked RNA to various inactivating agents. Exposure to ultraviolet light inactivates TMV and its RNA, or prevents successful infection if inoculated leaves are irradiated soon after inoculation (Siegel *et al.*, 1957). It was found that infectious RNA was more sensitive *in vitro*, or very soon after inoculation, than the intact virus. There was a lag period *in vivo* of resistance to the effect of irradiation on the virus, the lag period being reduced when the RNA was employed. This delay *in vivo* was

interpreted as being the time taken for the protein to be removed from the virus.

Bawden and Kleczkowski (1955) distinguished three changes of state with respect to photoreactivation of ultraviolet inactivated potato virus X particles infecting Nicotiana glutinosa. In the first state, exposure to visible light had no effect, and lesion number was not increased. In the second, the virus could be photoreactivated. If at the end of this period the leaf was not exposed to visible light, the particles became refractory to photoreactivation. The first stage might represent entry and uncoating of the virus, the second the establishment of free nucleic acid in the epidermal cells and the third, the destruction of inactive RNA by host nucleases (Bawden, 1957).

Various workers have found that the maximum number of local lesions appear in the infected tissues earlier upon inoculation with the viral RNA than with the intact virus; Fraenkel-Conrat et al., (1958), Kassanis, (1960) with both TMV and tobacco necrosis virus (TNV), Schramm and Engler, (1958), with infective TMV-RNA in a free state, Kassanis (1960) infective intact TNV.

Bawden and Kleczkowski (1960) and Bawden (1964) have questioned the validity of assuming that the time differences correspond to the time periods needed by the viral RNA to separate from its protein coat. Bawden (1964) found that, for both TMV and TNV intact virus as inoculum survived on the leaf and began to initiate infection over a considerably longer period than the naked RNA. Infection by RNA is better

synchronized because RNA complements that do not establish themselves successfully soon after infection become inactivated.

Infiltration of tobacco leaves with RNase prior to, or within two hours after inoculation with TMV leads to abortion of the infection (Hamers-Casterman and Jeener, 1957), suggesting that approximately two hours are required to free the RNA from its protein mantle and during this time it is vunerable to the action of the infiltration enzyme. However, if the infiltration takes place after two hours liberated RNA will have become protected either by its association with the host or polymerisation with virus protein.

Reddi(1966) inoculated tobacco leaves with $\left[^{32}P\right]$-labelled intact TMV and followed the time course of RNase-sensitive RNA appearance. About 1% was sensitive at zero time, a significant rise (to 2.5%) being detected at three hours after inoculation. After six hours, 5% was sensitive the rate of increase levelling off. The size of the RNase-sensitive RNA was not established, and there was therefore no indication to what extent the rise was due to the abortive partial uncoating of virus rods.

In another direct approach, Shaw (1967,1969) inoculated tobacco leaves with reconstituted TMV (prepared from non-radioactive RNA and $\left[^{14}C\right]$-labelled protein subunits). Employing this technique he avoided complications due to labelled degredation products of the RNA. In leaf extracts made at ten minutes after inoculation, about 25% of the virus protein was found in the top zone on a sucrose density

gradient, clearly separated from the main virus zone, and this amount increased over five to nine hours. The initial rapid release of coat protein was not affected by low temperatures or cycloheximide and so the process did not seem to depend on pre-existing or induced enzymes.

The major difficulty with both Reddi's and Shaw's experiments is that at best, only about 0.01% of the inoculum successfully infects cells. Thus, the RNA or protein studied is mainly the excess inoculum that cannot be removed by washing. Attempts have been made, so far unsuccessfully, to observe the uncoating process by electron microscopy (Cocking and Pojnar, 1968).

The mechanism of the *in vivo* separation of the viral nucleic acid from the protein coat of the virus particles is not known. The results of Gordon and Smith (1960,1961), however, suggest that the enzymatic machinery of the host cells may be involved.

Viral RNA reproduction:

Siegel *et al.*,(1957) suggested that after TMV RNA has separated from its protein coat, it is absorbed by some "receptor" in the host cell, probably the host cell nucleus. Examination of nuclei from $\left[^{32}P\right]$ -labelled TMV infected tobacco leaves, revealed the presence of a radioactive RNA fraction of similar nucleotide composition to that of TMV RNA, thus suggesting the presence of parental TMV RNA (Reddi, 1966).

Various lines of cytological evidence have suggested that there is an RNA accumulation in the nucleus of cells shortly after infection

with TMV (Bald and Solberg, 1961; von Wettstein and Zech, 1962; Zech, 1952,1954; Zech and Vogt-Kŏhne, 1955).

Cytochemically, Hirai and Wildman (1963) showed, in tomato stem hair cells, that RNA rather than DNA accumulated in the nuclei of TMV infected cells. Examining several strains of TMV, Bald (1964) using phase contrast microscopy and RNase digestion, came to the same conclusion.

Yasuda and Hirai (1964) suggested that, on examination of $\left[{}^{3}H\right]$ -labelled uracil incorporation into TMV infected tobacco leaf epidermis, although there was no differential labelling in the cytoplasm, the rate of incorporation into the nuclei of TMV-infected cells was higher than in healthy ones.

Hirai and Nakagaki (1966) found a transitory increase, of about 20%, in the RNA content of the nuclei of N.tabacum epidermal cells, six to eight hours after inoculation with TMV.

The steps involved in RNA-synthesis in the nucleus have been examined. Sänger and Knight (1963) demonstrated that actinomycin D, a potent inhibitor of DNA-dependent RNA synthesis, inhibited normal nucleic acid metabolism in plant cells, but not TMV RNA synthesis. Hiruki and Kaesberg (1965) obtained similar results when examining brome mosaic virus RNA, in barley tissue. Semal (1967) reinvestigating the effects of actinomycin D on brome mosaic virus and TMV synthesis, found the effect on TMV production varied according to the time of application of the actinomycin D. It is suggested that there may be some DNA-dependent

host cell function required for a very early step in virus establishment, as also suggested by Lockhart and Semancik (1968) working with cowpea yellow mosaic virus in cowpea (and as noted for certain animal and bacterial RNA viruses).

Smith and Schlegel (1965) and Schlegel and Smith (1966) showed that tritiated uridine was selectively incorporated into the nucleus and nucleolus of actinomycin D TMV infected tobacco leaf disks, and Vicia faba root tips infected with clover yellow mosaic virus. RNase and DNase treatment removed the label from uninfected cells, but only reduced the label in infected cells. It is suggested that virus directed RNA synthesis takes place in the nucleolus and that at least some of this is viral RNA. Laflèche and Bové (1968) obtained similar results with turnip yellow mosaic virus (TYMV) in Chinese cabbage, finding that tritiated uridine accumulated in the nucleolus (as well as in the spaces between clumped chloroplasts) in actinomycin D treated tissues.

Not all viruses, however, show localisation of actinomycin D resistant incorporation in the nucleolus. De Zoeten and Schlegel (1967) found that broad bean mottle virus in V.faba roots gave rise to actinomycin D resistant incorporation in the cytoplasm, particularly in the endoplasmic reticulum and the Golgi apparatus. Their preliminary results suggest a similar situation with tomato ringspot virus and tomato spotted wilt virus.

Babos and Shearer (1969) showed tobacco leaf RNA synthesis was inhibited by actinomycin D and that application of the optimal

conditions of treatment for the suppression of cellular RNA synthesis to infected tissue enhanced the synthesis of TMV RNA. Moreover, infected tissue exhibited an excess AMD-resistant RNA synthesis not accounted for by the RNA incorporated into virus particles. In view of the existence of this non-viral RNA synthesis in AMD-treated infected cells, it is considered impossible to know which kind of RNA is synthesised in the nucleolus in such tissue.

Shipp and Haselkorn (1964) first reported a double-stranded or replicative form of TMV RNA, which was resistant to RNase and DNase and capable of heat denaturation and reannealment. Burdon et al.,(1964) confirmed and further characterised this double-stranded form. Similarly double-stranded TYMV RNA was isolated from Chinese cabbage leaves infected with TYMV (Gigot et al., 1965; Mandel et al.,1964; Ralph et al., 1965). Weissmannet al., (1965,1966) was able to isolate the TMV minus strand and showed it to have a base composition complementary to that of TMV RNA. The double-stranded form of the viral RNA occurs in low concentrations in the plant (about 2 μg/g fresh leaf weight for TYMV in Chinese cabbage - Bockstahler (1967)).

Ralph and Clark (1966) were unable to demonstrate the presence of double-stranded TMV RNA in the cell nuclei, and suggest that the viral duplex RNA may be associated with the mitochondria. Although particles of one isolate of tobacco rattle virus have been found in regular contact with mitochondria, Harrison and Roberts (1968) consider there is no convincing evidence for chloroplasts or mitochondria as viral assembly sites. Ralph and Wojcik (1969) found that double-stranded RNA synthesised

in cell-free extracts from TMV-infected tobacco leaves, is associated with some cytoplasmic structure and not with the nuclei. It is suggested therefore that infection with TMV induces an increased synthesis in the nucleus of a kind of host RNA that is relatively resistant to actinomycin D inhibition (e.g. tRNA).

TYMV duplex RNA has been shown to replicate viral RNA by an asymmetric semi-conservative mechanism (Ralph et al., 1965) and to appear in TYMV-infected Chinese cabbage leaves three days after inoculation, i.e. during the latent period preceding the appearance of complete virus particles (Ralph and Clark, 1966).

It has been observed that during extraction of viral RNA, the double -stranded structure may be formed by base-pairing between the minus strand and the partially completed plus strands attached to it, (Weissmannet al., 1968).

From this consideration of viral RNA biosynthesis, it becomes apparent that, as with every process in a living organism, the biosynthesis has to be catalyzed by specific enzymes. The genetic code for these enzymes has to be contained in the single stranded plant virus RNA, since it alone is able to initiate the entire infection process.

The introduction of the virus RNA on infection, represents the introduction of one single-stranded polycistronic message into an environment already containing between 1,000 and 10,000 endogenous cellular mRNA's (Spiegelman and Haruna, 1966). Thus, the infecting RNA must contain or develop some selective advantages over the endogenous

messengers, if the infection is to succeed. In the case of single-stranded bacterial RNA viruses (and probably those of other species), this advantage is derived through the synthesis of a unique new enzyme. The translational machinery of the infected cell translates a portion of the infecting RNA to yield the new specific enzyme(s). This new enzyme(s), a specific RNA replicase, utilises nucleoside-5'-triphosphates to carry out the synthesis of many new single-stranded virus RNA molecules identical to the infecting RNA (Spiegelman et al., 1965). The enzyme produced is specific for the RNA of the infecting virus, not reproducing other or endogenous RNA's in the infected cell (Fiers et al.,1967; Haruna et al., 1963; Haruna and Spiegelman, 1965; Spiegelman and Haruna 1966). This unique feature establishes an autocatalytic situation, rapidly resulting in a selective advantage for the infecting single-stranded RNA virus.

The size and structure of TMV RNA are such as to allow for the coding of a number of 'proteins'. Since the protein subunits of TMV consist of 158 amino acid residues (Anderer et al., 1960; Tsugita et al., 1960), assuming a triplet code, 474 nucleotides out of the 6500 nucleotides present in TMV RNA are enough to code for the virus protein. The rest would suffice to code for a number of enzymes. No virus specific enzymes have been found so far in virus infected plants, except for a synthetase in TYMV infected Chinese cabbages (Astier-Manifacier and Cornuet, 1965 a,b;1966 a,b).

Several workers using cell free extracts of fractions from infected leaves have claimed the synthesis in vitro of material having some of the

properties of viral RNA. No one, as yet, has achieved the unambiguous synthesis of new infective viral RNA as has been done for bacterial RNA virus Qβ (Spiegelman et al., 1965). Ralph and Wojcik (1966a) found that chloroplast and nuclei preparations from both healthy and TYMV infected Chinese cabbage leaves, with the addition of the four ribonucleoside triphosphates and an ATP generating system, synthesised single-stranded RNA, and that this synthesis could be inhibited by actinomycin D. They provided no evidence for the synthesis of complete intact TYMV RNA in their system. Similar results have been obtained for extracts of brome mosaic virus infected barley leaves (Semel and Hamilton, 1968). Suggestions that cell-free systems of infective viral RNA has been achieved (e.g. Kim and Wildman, 1962; Balandin et al., 1964; Cochran, 1963; Cornuet and Astier-Manifacier, 1962; Karasek and Schramm, 1962; Tongur et al., 1963) are of doubtful significance (Matthews, 1970; McLaren, 1967; Ralph and Matthews, 1963; Takahashi, 1964). Karasek and Schramm (1962) consider that the in vitro system may be capable of 'finishing off' some of the unfinished TMV RNA molecules present in the system, and thus produce an increase in infectivity.

Astier-Manifacier and Cornuet (1964, 1965 a,b, 1966 a,b) claim that their isolated enzyme is able to synthesise in vitro single-stranded polynucleotides which are identical with TYMV RNA. An RNA-dependent RNA polymerase which incorporates nucleotides from ribonucleoside triphosphates into an acid insoluble polymer has been purified from the chloroplastic fraction of Chinese cabbage leaves (Astier-Manifacier and Cornuet, 1971).

One of the reasons for the failure to find virus specific enzymes in infected cells may be that they occur in such small quantities. Macquaire (1965) used DEAE cellulose chromatography to fractionate proteins from extracts of TYMV infected leaves, but in these attempts to isolate RNA-synthetases specific for TYMV, the levels have been very low. An increase in RNA-polymerase activity has been observed in cucumbers infected with cucumber mosaic virus (Gilliland and Symons, 1968).

No definite answer can be given as yet to the origin of the 'building blocks' of the newly synthesised virus specific nucleic acids. Fry and Matthews (1963) followed the change in total RNA content of tobacco leaf epidermis in the early stages after inoculation with TMV. The mechanical inoculation process induces a rise and fall in RNA content over the first few hours. At about six to eight hours after inoculation, there was a rapid rise of about 10% in the RNA content of the TMV inoculated epidermis. Taken together with the evidence of Hirai and Nakagaki (1966), it is suggested that there is a burst of RNA synthesis in the nucleus at about five to six hours and that this RNA later migrates to the cytoplasm. This general picture would fit with cytological observations. Ralph and Wojcik (1970) found good correlation between chlorophyll content and double-stranded RNA content in various fractions, but no correlation with DNA content.

Kubo et al., (1965) using MAK column chromatography, showed that the overall chromatographic pattern of nucleic acids from healthy and infected tissues was the same, except for the occurrence of TMV RNA in the

latter. Reddi (1963 a) has shown, however, that there is a rapid degradation of tobacco leaf ribosomal RNA upon infection with TMV, concomitant increase in TMV RNA. Reddi (1963 b) suggests from $\left[^{32}P\right]$ -labelling experiments that ribonucleosides are used in TMV RNA synthesis. Kubo (1966) and Röttger (1965) noted a dramatic increase in RNA with a base composition similar to normal leaf RNA, in TMV-infected tobacco leaves at early infection stages. Babos (1966) showed that the decrease of ribosomal content was independent of the amount of virus synthesised in the infected leaves, and states that the variance of his results with those of Reddi may be due to the fact that "the source of materials for the synthesis of virus RNA is variable and depends on the physiological state of the cells in which the virus multiplies".

Shigematsu et al., (1966) did not find any reduction in the amount of ribosomal RNA, two days after infecting young tobacco leaves with TMV, yields of s-RNA and 17S RNA even showing an increase.

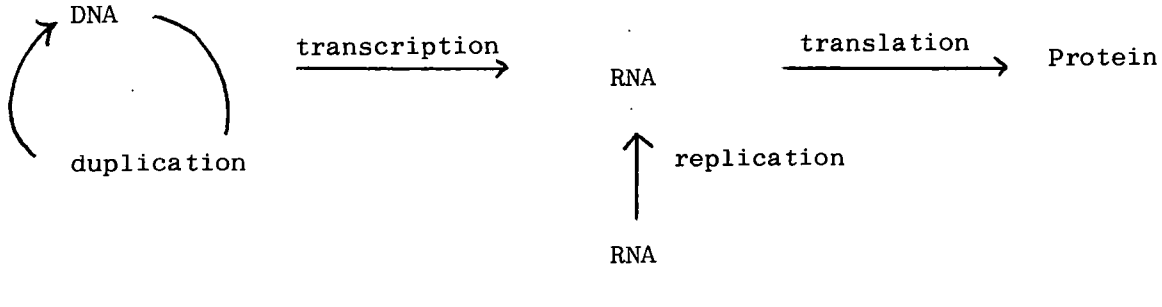
Certain isolates of tobacco rattle virus are defective in that they initiate an infection in which viral RNA is produced, but no intact virus rods are formed. Cadman (1962) suggested that nuclei were the most likely site for the accumulation of this viral RNA.

It has been reported that both wound tumor virus (Black and Markham, 1963) and rice dwarf virus (Miura et al., 1966) are double-stranded, and so multiplication of these viruses will involve stages differing from those for TMV biosynthesis.

Very little is known about the details of plant virus protein synthesis *in vivo*, most of our knowledge of protein biosynthesis being based on information obtained from other systems. It would appear, however, that protein synthesis follows somewhat universal lines, a review of which would be advantageous before considering viral protein synthesis in particular.

Protein Synthesis

For some time it has been realised that nucleic acids play an important role in protein biosynthesis and work in this field has been inseparably linked with studies of the nucleic acids and their interactions with proteins. The amino acid sequence of a particular protein is specified by the sequence of nucleotides (comprising adenine, thymine, guanine and cytosine) in a particular segment of the deoxyribonucleic acid (DNA) molecule. The process of protein biosynthesis consists essentially of two stages. Firstly, the DNA is transcribed into a ribonucleic acid intermediate, messenger RNA (mRNA) which has a ribonucleotide sequence complementary to that of the deoxyribonucleotide sequence of one of the strands of the DNA serving as template (transcription) (Geiduschek and Haselkorn, 1969). The mRNA becomes attached to cytoplasmic ribonucleoprotein particles (ribosomes) which are the sitesof protein synthesis, and there it determines the order of linkage of amino acids into a specific protein (translation), (Attardi, 1967; Matthaei *et al.*, 1968; Ochoa, 1968).



In vivo and in vitro studies of amino acid incorporation in a wide range of species have led to the acceptance of a general scheme of protein biosynthesis for all organisms.

Hultin (1950) working with chicks and Keller (1951) with rats studied in vivo incorporation of labelled amino acids, and found that liver microsomes were more highly labelled than any other fraction. Disintegration with deoxycholate (an anionic detergent) indicated the radioactivity to be in the " deoxycholate-insoluble fraction", which Littlefield et al., (1955) showed, by ultracentrifugal analysis and electron microscopy to consist chiefly of ribosomes.

In vitro studies followed, such systems developed becoming major tools for the examination of all aspects of the mechanism of protein biosynthesis.

Siekevitz (1952) using the first in vitro system from rabbit liver containing a microsome fraction, mitochondria with energy substrates, soluble cytoplasm and a labelled amino acid, reported evidence for peptide bond formation. Zamecnik and Keller (1954) showed that the mitochondria were unnecessary, and could be replaced by ATP and a source of high energy phosphate, e.g. phosphoenol pyruvate plus pyruvic kinase, to regenerate ATP.

Stephenson et al., (1956) using tobacco leaf extracts, reported the first cell-free system to incorporate amino acids from plant material. A wide range of plant material has been employed since, to provide active amino acid incorporating systems (reviewed in Mans, 1967; Boulter, 1970).

Protein biosynthesis involves the formation and stepwise elongation of peptide chains from the amino-terminal amino acid, and proceeds towards the carboxy-terminal amino acid (Attardi, 1967; Bishop et al., 1960; Dintzis, 1961; Matthaei et al., 1968, Ochoa, 1968). Amino acid addition is under genetic control, and a group of three adjacent nucleotides in the mRNA specifies which amino acid is to be linked to the growing peptide chain.

Synthetic polynucleotides of known composition, but of random sequence, were employed initially as messengers in in vitro incorporation in Escherichia coli systems. Problems in determining the actual sequence of the bases in the triplets resulted, however, in some ambiguities. Unambiguous code elucidation resulted from the triplet binding experiments of Nirenberg et al., (1966) and the employment of synthetic polynucleotides of known repeating sequence in in vitro incorporations(Khorana et al., 1966).

All 64 possible sequences of the three nucleotides have been assigned to amino acids, with the exception of UAA, UAG and UGA (known as ochre, amber and opal respectively). It appears that the sequence of amino acids in a polypeptide chain contains all of the information required for generating the three-dimensional structure of the native protein molecule(Anfinsen, 1967).

Crick (1957, 1958) postulated the existence of small "adaptor" molecules capable of recognising and binding the amino acids and interacting with specific nucleotide sequences of the template mRNA.

Hoagland et al., (1957) showed the presence of an aminoacyl-RNA complex in an in vitro rat liver system. This complex dissociated in the presence of a microsomal suspension, with the incorporation of the amino acid into the microsomal fraction. The RNA fraction was termed transfer RNA (tRNA). Cell extracts contain about 40 species of tRNA ,(Lengyel and Söll, (1969) and there is at least one specific tRNA for each of the common amino acids employed in protein biosynthesis.

The tRNA's that accept the activated amino acids are amino acid-specific (Doctor et al., 1961) and are characterised by the presence of at least four functional sites:-

(1) the 75 to 85 nucleotides comprising the overall structures always end in a 3' sequence of CCA - the amino acid acceptor site;

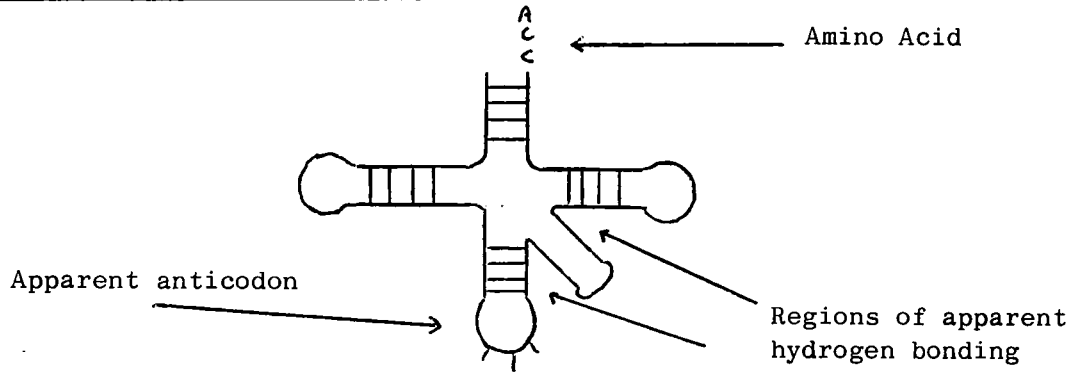
(2) the tRNA structure contains a sequence of nucleotides that is complementary (in the hydrogen bonding sense) to a trinucleotide sequence of mRNA's that is known to code for the amino acid accepted by the specific tRNA (the sequence being termed the anticodon);

(3) a complex site which is recognised by the aminoacyl tRNA synthetase enzyme specific to that species of tRNA, and

(4) the ribosome interaction site.

There must be, in addition, regions in the molecule which are recognised by tRNA pyrophosphorylase and the various enzymes which generate the unusual bonds, such as the tRNA methylases. Some tRNA's may be involved in regulatory processes which may require special structural features(von Ehrenstein, 1970).

Generalised two-dimensional structure of tRNA's



The first primary structure of a tRNA species to be elucidated was alanine tRNA from yeast, (Holley et al., 1965). Holley et al., (1965) proposed the "clover leaf model" for the structure of tRNA molecules, and although the tertiary structure of tRNA has not been fully elucidated it is thought that the loops of the "clover leaf" are folded over (in a two-dimensional sense), into structures featuring complementary hydrogen bonding in similar regions of the molecule. A compact structure is thus formed, held together by base pairing and hydrogen bonding, and from which the anticodon and the amino acid carrying the 3' terminal adenosine moiety protrude. The complete sequence of several of these amino acid specific tRNA's has been determined (Holley et al., 1964; Madison et al., 1966; Zachau et al., 1966).

At a molecular level, mRNA translation or protein synthesis begins with the activation of the free amino acids (AA) to form enzyme-bound amino-acyladenylates (Enz-AMP-AA) (Equation i),(Demoss et al., 1956), followed by the formation of aminoacyl tRNA complexes (tRNA-AA) (Equation ii),(Hoagland et al., 1957). Both reactions are catalysed by aminoacyl tRNA synthetase enzymes (Davie et al., 1956) that are specific

for each tRNA species, (Doctor et al., 1961) and presumably link the correct amino acid to the 3'-hydroxyl of the terminal adenosine of a specific tRNA molecule (McLaughlin and Ingram, 1964; Sonnenbichler et al., 1963). There are at least 20 different aminoacyl-t-RNA synthetases, one for each of the 20 natural amino acids (Berg, 1961; Hoagland, 1960).

$$\text{Enzyme} + \text{AA} + \text{ATP} \rightleftharpoons \text{Enzyme} - \text{AMP} - \text{AA} + \text{PPi} \quad \underline{\text{Activation}} \text{ (i)}$$

$$\text{Enzyme} - \text{AMP} - \text{AA} + \text{tRNA} \rightleftharpoons \text{tRNA} - \text{AA} + \text{Enzyme} + \text{AMP} \quad \underline{\text{Transfer}} \text{ (ii)}$$

There is some evidence that ATP is bound to the enzyme before the amino acid (Allende et al., 1966; Rouget and Chapeville, 1968) and that the site for amino acid activation is different from the site for aminoacyl transfer.

Although most of the work on aminoacyl tRNA synthetase enzymes has been done with material from bacterial or animal sources, there has been recent progress in work on purifed plant synthetases (e.g. Peterson and Fowden, 1965; Smith and Fowden, 1968). The specificity of aminoacyl-tRNA synthetases, a prerequisite for faithful translation of the genetic message, must be very high (Loftfield and Eigner, 1966; Novelli, 1967; Peterson, 1967), and is exercised at the level of amino acid activation as well as in the transfer step, (Lengyel and Söll, 1969). There is no subsequent check on whether the tRNA is carrying the correct amino acid during the formation of peptide bonds on the ribosome.

The mechanism by which the aminoacyl tRNA synthetase enzymes recognise

their relevant species of tRNA is unknown and it may be that the process varies between tRNA and its cognate aminoacyl-tRNA synthetase, (Lengyel and Söll, 1969).

The subsequent steps of protein synthesis involve mRNA and ribosomes. The ribosome, a large ribonucleoprotein particle, is the site of translation of both in vivo and in vitro protein synthesis. Ribosomes are differentiated into two size classes, 70S occurring in procaryotic organisms, mitochondria and chloroplasts and 80S occurring in eucaryotic cytoplasm. All ribosomes consist of two unequal subunits, 30S and 50S in the 70S (Tissières and Watson, 1958; Tissières et al., 1959) and 40S and 60S in 80S ribosomes,(Chao, 1957). The smaller subunit contains a single 16S or 18S RNA molecule and about 20 different proteins, while the larger subunit contains one molecule of 23S, 25S or 28S, one 5S RNA molecule and 30 to 40 different proteins (Craven and Kurland, 1969; Petermann,1964; Spirin and Gavrilova, 1969). The two subunits differ in stability, the smaller unit being more labile due to the latent RNase associated with it (Elson, 1959; Tal and Elson, 1963).

Cotter et al., (1967) working with yeast, have suggested a model for the ribosome in which the helical regions of the r RNA project from the surface of the ribosome, and the amorphous, non-helical regions with their associated proteins project into the ribosome centre. Protection of about 30 - 35 amino acids of the growing polypeptide chain from proteolytic enzymes,(Malkin and Rich, 1967) and the inaccessibility of a segment of the mRNA of about 30 nucleotides length (Takanami and Zubay, 1964; Takanami et al., 1965) suggest that there is a protected

region inside the ribosome where peptide bond formation and polypeptide elongation occur, (Redman and Sabatini, 1966). Similar conclusions have been formed from electron microscopic studies of ribosomes from a wide range of sources (Bruskov and Kiselev, 1968a,b; Nanninga, 1963) and supported by hydrodynamic measurements, (Petermannand Pavlovec, 1969).

Mangiarotti and Schlessinger (1966) and Cundcliffe (1968) have suggested that free 70S ribosomes do not occur in E.coli in vivo, and all the ribosomes exist as free subunits or as parts of polysomes. They suggest that 70S ribosomes are artifacts resulting from polysomal breakdown during ribosome preparation. Ribosomal subunit exchange has been demonstrated in vivo (Kaempfer et al., 1968) and in vitro (Kaempfer, 1968) and it was found to be coupled with protein synthesis.

The sequence of events occurring during protein synthesis may be summarised as follows:-

1. First there is the initiation step in which a specific aminoacyl-tRNA associates in a hydrogen-bond-mediated process with a trinucleotide sequence (codon) of mRNA upon a ribosomal subunit. The sequence in E. coli may be summarised as a 30S subunit (Eisenstadt and Brawerman, 1967; Nomura et al., 1967; Nomura and Lowry, 1967) forms a complex with mRNA codon unit (AUG) or (GUG) (Thach et al., 1966) and formylmethionyl tRNA, (Capecchi, 1966). The formation of this complex requires GTP, (Arlinghaus et al., 1964) and one or more enzymatic "initiation factors" (Lengyel and Söll, 1969; Stanley et al., 1966).

2. The 'initial complex' is completed with the uptake of a 50S ribosomal subunit and a second aminoacyl-tRNA, to yield a 70S ribosome containing two bound aminoacyl-tRNA's, established by the mRNA established codon-anticodon interaction (Arlinghaus et al., 1964; Igarashi and Kaji, 1967; Nomura et al., 1967).

3. Once the protein is completed, the 70S ribosome dissociates into 30S and 50S subunits.

Similar cycling of the 80S ribosomal subunits has been reported by Hogan and Korner (1968), Kabat and Rich (1969), Kaempfer (1969) and Heywood (1970). Heywood suggests that a similar sequence of events to that suggested by Guthrie and Nomura (1968) occurs in eucaryotic cells.

As noted, new aminoacyl residues enter the polypeptide chain attached to tRNA, so that during the process of protein synthesis, the C' end of the polypeptide chain is not free, but attached to tRNA (Bretscher, 1963,1965; Gilbert, 1963; Nathans and Lipmann, 1961; Takanami, 1962). Peptide bond formation results therefore from a reaction between peptidyl tRNA and aminoacyl tRNA, the peptidyl tRNA being increased by one aminoacyl residue, and the free tRNA released. This peptide bond formation is followed by rearrangement of the mRNA upon the ribosome so as to facilitate the binding of another aminoacyl tRNA,(Arlinghaus, et al., 1964). Repetition of the transfer of the formylmethionine containing component (now a peptide) to the amino acid of the adjacent aminoacyl-tRNA continues the cycle. The simultaneous presence of peptidyl tRNA and aminoacyl tRNA in the functioning ribosome suggests that there are two separate tRNA binding sites on the ribosome and their presence has

been confirmed experimentally (see Spirin, 1968). The mRNA binding centre and the aminoacyl tRNA binding site are located on the smaller subunit, (Okamoto and Takanami, 1963) and isolated 30S subunits show the same capacity for selective binding of aminoacyl tRNA in the presence of a template polynucleotide as the complete ribosome(Kaji et al., 1966; Pestka and Nirenberg, 1966; Suzuka et al., 1965). The peptidyl binding site which has an increased affinity for N-blocked aminoacyl tRNA's, and which only binds aminoacyl or deacylated tRNA weakly, is located on the larger subunit(Bretscher and Marcker, 1966; Cannon, 1967; Gilbert, 1963). If the functioning ribosome is dissociated into subunits, the peptidyl tRNA remains firmly bound to the larger subunit, and Schlessinger et al., (1967) have shown that the peptidyl tRNA is the major contributor to the stabilisation of the subunit association into a complete ribosome. The peptidyl-transferase centre is also located exclusively on the 50S subunit, and is an inherent part of it (Monro, 1967; Traut and Monro, 1964). The associated state of subparticles in a complete ribosome is stable only while the ribosome is engaged in the process of polypeptide synthesis, i.e. retains the growing polypeptide, (Mangiarotti and Schlessinger, 1966).

Translation of the mRNA therefore falls into three phases, initiation, elongation and termination. High magnesium levels, where the need for "proper" chain initiation is eliminated, were employed in much of the original work on in vitro amino acid incorporation , (Eisenstadt and Lengyel, 1967). Presumably under such conditions, aminoacyl tRNA was attached to both aminoacyl and peptidyl ribosomal sites, peptide bond formation occurring between them. Mosteller et al.,

(1968) working with the rabbit reticulocyte system found that the magnesium optimum for $[^{14}C]$ valine incorporation into globin was 3mM, but for polyuridylic acid (poly-U) directed polyphenylalanine synthesis, it was 10mM. This could, however, be reduced to 4mM by preincubating the ribosomes with poly-U, deacylated tRNA, KCl and reduced glutathione, i.e., to about the same magnesium optimum as globin synthesis by natural messenger. $tRNA^{phe}$ was found to be responsible for this shift in the magnesium optimum, (Culp et al., 1968).

Most of the work on chain initiation has been carried out using bacterial systems (especially E.coli), and a mechanism involving formylmethionyl tRNA as the only natural chain initiating tRNA in protein synthesis, for procaryotic organisms, is now generally accepted.

Waller (1964) observed that although methionine constituted only 2.5% of the total amino acids in E.coli protein, about 40% of the protein had methionine as the N-terminal amino acid, suggesting the role of methionine in polypeptide chain initiation. Clark and Marcker, (1966a) reported two methionine accepting tRNA's in E.coli which could both be charged by the same methionyl tRNA synthetase. However, whereas the methionine residue of one of them can be formylated the methionine of the other cannot. The former is referred to as $tRNA_F^{met}$ and the latter as $tRNA_M^{met}$. The formylation of $tRNA_F^{met}$ is catalysed by a transformylase with formyl tetrahydrofolic acid acting as the formyl donor (Marcker, 1965). The transformylase is specific for met-$tRNA_M$, and will not formylate any other aminoacyl tRNA's, indicative of recognition of a structural region unique to $tRNA_F^{met}$.

Both $tRNA^{met}$ respond to the codon AUG, but $tRNA_F^{met}$ also responds to GUG (which is the code for valine). It has been suggested that GUG codes for valine when it occurs intramolecularly in mRNA and for fMet-tRNA when it is terminal (see Ochoa, 1968). fMet-tRNA is always incorporated into the N' terminal position in proteins, never internally, (Clark and Marcker, 1966 a,b) and it has been suggested that this is due to its inability to form a complex with the ribosome transfer factor FII and GTP, in contrast to all other aminoacyl tRNA's (Ono et al., 1968).

$tRNA_F^{met}$ has been reported present in many other species of bacteria (see Marcker and Smith, 1969 and Lengyel and Söll, 1969 for references), in the mitochondria of yeast and rat liver (Smith and Marcker, 1968) and in the mitochondria of HeLa cells (Galper and Darnell, 1969). N-formylmethionine was reported as the N-terminal residue of the viral coat protein synthesised in a system containing Euglena gracillis chloroplast ribosomes, E. gracillis high-speed supernatant fraction and f2 bacteriophage RNA (Schwartz et al., 1967).

FMet-tRNA has not been detected in the cytoplasmic system (Lengyel and Söll, 1969). Little work has been carried out on chain initiation in the 80 S ribosome, although Rich et al., (1966) and Arnstein and Rahamimoff (1968) have indicated that valyl tRNA is the chain initiator for haemoglobin synthesis. This is now proven to be incorrect and initiation of haemoglobin is by unformylated $tRNA^{met}$ (Bhaduri et al., 1970; Wilson and Dintzis, 1970).

Although the actual initiator molecule may be different, it seems

likely that the mechanism of chain initiation on both 70 and 80S ribosomes is similar, in that the role of the subunits is common to both and initiating factors and GTP are required, (Heywood, (1970). Information is available on two plant systems, wheat germ (Ghosh et al., 1971; Leis and Keller, 1970; Marcus et al., 1970; Tarrago et al., 1970) and V. faba, (Yarwood et al., 1971).

It appears that there are three main transfer factors involved in chain elongation in procaryotic organisms, and they have been isolated from a range of micro-organisms, e.g. from E.coli, (Lucas-Lenard and Lipmann, 1966), from Pseudomonas fluorescence and Bacillus stearothermophilus, (Skoultchi et al., 1968). Transfer factors have also been reported from yeast extract (Ayuso and Heredia, 1968; Richter and Klink, 1968) a rat liver system, (Ibuki and Moldave, 1968) and the reticulocyte system, (Arlinghaus et al., 1964). Allende (1969) has reported the partial purification of the transfer factors from wheat embryo and App, (1969) has isolated two factors, required for in vitro polyphenylalanine synthesis, from rice embryos. GTP and all three protein factors are necessary for the occurrence of chain elongation, although the necessity for GTP was obscured in early work in which high magnesium concentrations were employed (Kurland, 1966; Ravel, 1967).

De Groot et al., (1967) demonstrated GTP dependent enzymatic binding of phenylalanyl tRNA to wheat ribosomes and Jerez et al., (1969) showed the formation of a GTP-protein complex from extracts of wheat embryos that will bind unblocked aminoacyl tRNA's, although little work has been performed on the binding of tRNA to plant 80S ribosomes.

Denatured tRNA will not react, suggesting the necessity for the correct tRNA conformation, as well as the unblocked aminoacyl group.

After the attachment of the aminoacyl tRNA to the ribosome, peptide bond formation occurs between the carboxylic group of the terminal aminoacyl residue of the peptidyl tRNA and the α -amino group of the aminoacyl tRNA, the reaction being catalysed by peptidyl transferase (a constituent of the 50S subunit) (Monro, 1967; Monro and Marcker, 1967; Monro et al., 1968). A similar sequence is proposed for mammalian ribosomes, (Skogerson and Moldave, 1968). GTP and the supernatant proteins do not appear to be involved in the reaction, but monovalent and divalent cations (K^+ or NH_4^+ and Mg^{2+}) are required, (Monro and Marcker, 1967; Traut and Monro, 1964). The 3' terminal nucleotide sequence (CCA) of both the peptidyl and aminoacyl tRNA molecules seem to be involved (Monro et al., 1968; Rychlik et al., 1963).

After peptide bond formation, the newly formed peptidyl-tRNA is located in the aminoacyl site on the ribosome (Erbe et al., 1969) and the discharged tRNA on the peptidyl site, (Lucas-Lenard and Haenni 1969). Translocation, involving the movement of the peptidyl-tRNA from the aminoacyl to the peptidyl site and the movement of the ribosome along the mRNA by the length of one codon, requires GTP (which is apparently cleaved to GDP and Pi), (Lengyel and Söll, 1969) and enzyme factor FII (Pestka, 1968, 1969; Ono et al., 1969a). No further peptide bond formation can occur in the absence of translocation, (Lucas-Lenard and Haenni, 1969).

During chain elongation, the growing polypeptide chain remains linked to tRNA and bound to the mRNA-ribosome complex. After completion

the peptide chain is released from both of these bonds in the course of a composite process known as chain termination (Ganoza, 1966; Kössel, 1968; Last et al., 1967; Takanami and Yan, 1965; Zinder et al., 1966). Termination apparently occurs when the ribosome and its attached peptidyl tRNA reach a termination codon in its progress along the messenger. It is believed that the codons UAA, UAG and UGA (which are designated as nonsense codons) may serve as chain termination signals (Brenner et al., 1965; Sambrook et al., 1967; Weigert and Garen, 1965; Weigert et al., 1967). These terminator codons occur in E.coli, UAA being the one most frequently used in vivo,(Garen 1968).

Formylmethionine release is promoted by the presence of two release factors (R_1 and R_2). R_1 is specific for termination coded by UAA or UAG, and R_2 for that coded by UAA or UGA (Capecchi, 1967; Caskey et al., 1968; Scolnick et al., 1968). A release factor-terminator codon-ribosome intermediate, before formylmethionine release, has been reported by Scolnick and Caskey (1969). They suggest that the role of release factors may be to recognise termination codons.

Milman et al., (1969) have identified a protein factor S, from an E. coli B supernatant fraction, which stimulates the release of the polypeptide chain from the ribosome, possible by stimulating terminator codon recognition.

Phillips (1971) has noted that a temperature sensitive mutant of E. coli K12 is defective in a previously uncharacterised component essential for protein synthesis. A non-ribosomal component (Z factor) which rescues the protein synthesising activity of the mutant crude extract has been partially purified from a parental strain. Z factor is

shown to be distinct from G and T translocation factors, and evidence suggests it is not one of the initiation factors, aminoacyl-tRNA synthetases, the release factor R_2 or the S factor.

After release of the polypeptidyl-tRNA, the mRNA ribosome complex falls apart, giving rise to subunits (Schlessinger and Aspirion, 1969; Webster and Zinder, 1969) although there are some indications that ribosomes may be released from the complex in free 70S ribosomes which are subsequently dissociated into subunits, (Lengyel and Söll 1969).

Ribosomes exist in two states in the cell, free in the cytoplasm, or attached to the endoplasmic reticulum. The proportion of the two classes varies from cell to cell, or even in the same cell at different stages of its development. Cells which are actively dividing tend to contain mainly free ribosomes, whereas undividing, differentiated cells contain mainly membrane bound ribosomes. Secretory cells, e.g. liver, pancreas etc., contain a much higher proportion of membrane bound ribosomes than non-secreting cells (Blobel and Potter, 1967; Henshaw et al., 1963; Loeb et al., 1967; Palade and Siekevitz,1956) and this had led to the suggestion that the free ribosomes synthesise protein for intracellular purposes, while the membrane bound ribosomes synthesise proteins for export (Birbeck and Mercer, 1961; Campbell and Sargent, 1967; Siekevitz and Palade, 1960).

The protein synthetic capacity of both free and membrane ribosomes has been compared in vivo, (Sellinger and Ohlsson, 1969) and in vitro (Redman, 1968; Priestly et al., 1969; Takagi and Ogata, 1968) and both are found to be equally active.

Although the ribosomes are the chief sites of protein synthesis in the cell, evidence has been accumulating that organelles such as the chloroplast and mitochondrion are also capable of limited protein synthesis (see Boulter, 1970 for review and references). It seems, however, that these organelles are only semi-autonomous in that they are responsible only for the synthesis of certain proteins, the rest being under genetic control and synthesis on the cytoplasmic ribosomes, (Kirk and Tilney-Bassett, 1967).

Viral protein synthesis

Before the bulk of infective, complete virus particles can be detected, protein-free RNA accumulatesin the infected cells in large amounts. Evidence also points to the fact that the protein of plant viruses also accumulates at a certain stage of the infection process, and it is not associated with RNA in this stage. Takahashi and Ishii (1952) demonstrated the accumulation of a protein, absent from healthy tobacco leaves, in TMV-infected leaves. This protein has been termed X-protein,(Takahashi and Ishii, 1952), abnormal protein and soluble antigen (Commoner et al., 1953; Jeener and Lemoine, 1953). Takahashi and Ishii (1953) found the X-protein to consist of RNA-free spherical particles, having an antigenic structure similar to that of intact TMV. TMV protein and X-protein have the same amino acid composition, (Newmark and Fraser, 1956) and the electrophoretic mobility of the X-protein is characteristic of the strain producing it,(Takahashi 1955), indicating X-protein to be viral specific. The host does not appear to influence the serological propreties of X-protein,(Takahashi and

Ishii 1953). van Rysselberge and Jeener (1957) observed that the abnormal protein undergoes a rapid turnover in the early stages of infection and concluded that it is actually the immediate precursor of the protein part of the virus (the specific radioactivity of the virus and that of the X-protein being in agreement with this hypothesis).

From these results it appeared that TMV biosynthesis occurred in two separate stages, viral RNA and protein accumulation and finally assembly. Takahashi (1959) demonstrated reconstitution of anomalous protein, isolated from infected plants, and nucleic acid isolated from TMV, into highly infectious TMV particles *in vitro*. It was suggested that TMV could assemble *in vivo* by a similar mechanism.

Markham and Smith (1949) demonstrated that purified preparation of TYMV contained two types of particles, one consisting of a protein shell enclosing RNA, the other, a protein shell containing no RNA. The protein shell was not found to be infective and this was perhaps the first indication in plant virus work that the nucleic acid moiety of the virus was responsible for its infective property. It was suggested that synthesis of viral particles does not necessarily imply the simultaneous presence of proportionate amounts of both RNA and protein. It may involve a mechanism in which viral protein is produced in excess. Neither with TMV nor with TYMV were pools of detectable quantities of X-protein (Commoner *et al.*, 1953; Jeener, 1954) and top component, respectively, found before the appearance of the virus. Subunits of the protein of TYMV are able to form virus-like particles *in vivo*, giving rise to the top component, whereas subunits of TMV protein are unable to

do so, and occur as non-aggregated X-protein.

Matthews (1960) demonstrated the presence of five virus-like nucleoprotein fractions (in addition to the top component) with approximate RNA contents of 6%, 10%, 20%, 35% and 36% respectively. Only the fraction containing 36% RNA was infectious. When the virus infected plants were labelled with $\left[^{32}P\right]$ for short periods, the specific radioactivity of the RNA in the individual fractions decreased with increasing RNA content,(Matthews et al., 1963). It is possible that the fractions may represent steps in the assembly of the complete virus nucleoprotein.

In general, the results suggest that viral nucleic acid and protein synthesis are not synchronized , and are probably separately controlled. It may be assumed that the TYMV fractions are formed owing to some disturbance in RNA biosynthesis, partly formed RNA strands being released and coated by the excess protein. This interpretation, however, would not give any explanation as to the results of the labelling experiments mentioned above. Bollard and Matthews (1966) stated that there was no conclusive evidence as to the role of any of the anomalous virus proteins in the infected cell.

Macromolecules varying in their RNA content have also been found in plants infected with squash mosaic virus,(Mazzone et al., 1962), cherry yellow and necrotic ring spot viruses,(Willison et al., 1961) and wild cucumber mosaic virus,(Yamazaki and Kaesberg, 1961).

There is also evidence to indicate that in some cases plant viral RNA multiplies in the infected cell without assembly or inducing the formation of its protein coat. Cadman (1962) showed that the NM form of

tobacco rattle virus, although causing severe symptoms, exists in the form of free nucleic acid strands (proably attached to cell nuclei). Babos and Kassanis (1962) described unstable variants of tobacco necrosis virus which may also exist as free RNA. Siegel et al., (1962) reported the isolation of two defective nitrite mutants of TMV, one of which (PM_1) failed to induce protein synthesis, the other (PM_2) inducing synthesis of a virus-like protein which, however, failed to aggregate in vivo with viral nucleic acid to form complete virus particles.

The site of virus coat protein synthesis within the cell has not been established for any plant virus. Schramm and Röttger (1959) using fluorescent antibodies for in situ investigations showed that TMV protein appeared first in a zone around the nucleus, and then spread all over the cytoplasm, in infected tobacco plants. In this later stage of infection, most virus protein was associated with the microsomal fraction, no virus protein being demonstrated from either the nuclei or the chloroplasts. They concluded that virus protein was synthesised in the microsomes. Hirai and Hirai (1964) also using fluorescent antibody staining, followed the appearance of TMV antigen in leaf hair cells of tomato, and also observed the spread into the cytoplasm from the nucleus.

Reddi (1964) claimed to have been able to isolate virus particles from the nuclei of infected cells, but found the chloroplast fraction of TMV-infected tobacco leaves to be free from TMV. In contrast, Zaitlin and Boardman (1958) demonstrated the release of small amounts of TMV from the chloroplast fraction of TMV-infected tobacco leaves, by extraction with a suitable buffer. Labelling experiments with $\left[^{14}C\right]$

aspartic acid suggested that TMV could be synthesised within the chloroplasts, moving then into the cytoplasm. Matsushita (1965) substantiated this suggestion by demonstrating that most of the infection was observed in the chloroplast fraction forty hours after inoculation, after which infectivity in the chloroplast fraction decreased and that of the cytoplasm increased with time.

Nagaraj (1965) studied TMV antigen in N.glutinosa leaves. Antigen was first detected as tiny specks of fluorescent material in the cytoplasm, later forming larger masses around the chloroplasts. No antigen was detected within the nuclei or chloroplasts.

Shalla and Amici (1967) using electron microscopy to examine cells treated with ferritin-labelled antibody, found that while most of the TMV protein and all the virus rods assembled in the ground cytoplasm, there was some viral protein antigen (but no rods) inside the infected cell nuclei. They concluded that TMV RNA and protein are formed in the nuclei, but that assembly into nucleoprotein particles occurs in the cytoplasm.

Langenberg and Schlegel (1969) detected virus protein in both the nucleus and cytoplasm, about six hours after TMV inoculation of tobacco leaves.

Nuclei, microsomes and chloroplasts have therefore been equally suggested to be the organelles in which TMV particles or their protein coats are synthesised. The discrepancies may simply be due to difficulties encountered in distinguishing between the site of viral protein synthesis

and the site of virus assembly.

Spencer and Wildman (1964) demonstrated that 80% of the amino acid incorporating activity of cell-free extracts of tobacco leaves was associated with a fraction consisting of chloroplasts and nuclei. Most of the activity was associated with the chloroplasts, Boardman et al., (1965) demonstrating that this ability was due to ribosomes in the chloroplasts.

Amino acid activating enzymes were found to be mainly in the supernatant fraction of tobacco leaf homogenates, as measured by hydroxamate formation, (Hayashi, 1962), but a significant amount was present in the nuclei and in the chloroplast fraction. In leaves that had been infected with TMV for 3 days, there was a 50% increase in amino acid activation in the supernatant fraction. Takahashi and Hirai (1966) found that isolated mitochondria from TMV infected tobacco leaves incorporated more amino acids into protein than did those from healthy leaves. At present their results are difficult to interpret in relation to virus synthesis.

There are two classes of leaf ribosomes, the cytoplasmic with $S_{20,w}$ 80 and the chloroplastic with $S_{20,w}$ 68 (Lyttleton, 1962; Clark et al., 1964) but the possible role of these two classes, in plant virus protein synthesis, has not been clearly established.

Boardman et al., (1966) demonstrated that 70 S ribosomes were ten to twenty-fold more active in in vitro than 80 S.

Van Kammen (1963) in experiments with TMV in tobacco leaves, suggested that uncoated TMV RNA may be associated with the 80 S ribosomes.

He employed antibodies purified from TMV antiserum to remove TMV from the ribosome fraction (which in turn was isolated by a lengthy sedimentation procedure). The infectivity of the material in the ribosome fraction was destroyed by RNase at high concentration. There is some doubt as to whether van Kammen was studying naked RNA as associated with the ribosomes in vivo, or contamination of the ribosome fraction with TMV which had become uncoated during the isolation procedure, (Matthews, 1970).

Similarly with Chinese cabbage, the two species of ribosomes have been demonstrated, (Clark et al., 1964). Reid and Matthews (1966) and Ushiyama and Matthews (1970) have shown that TYMV infection of Chinese cabbage has little effect on the concentrations of 83 S ribosomes and fraction II protein (both cytoplasmic) but the concentration of fraction I protein (from the chloroplasts) is reduced, and 68 S ribosomes are eliminated. Cytologically, TYMV causes marked abnormalities in the chloroplast ultrastructure of infected cells, without much affecting other cell structures (Chalcroft and Matthews, 1966; Chalcroft and Matthews, 1967 a,b; Gerola et al., 1966; Miličič and Štefanac, 1967; Reid and Matthews, 1966). Ushiyama and Matthews (1970) observed small vesicles in cells infected with TYMV, and suggest that the replicative form of the viral RNA is located in the peripheral vesicles of the chloroplasts and that RNA complements move from the vesicles to the cytoplasm where coat protein synthesis and particle assembly take place.

One of the best ways to more clearly understand biological processes is to study them under the simplest and most clearly defined conditions

(Fraenkel-Conrat and Weissmann, 1968). Single cells are therefore preferred to organisms or organs, and cell extracts are often of even more value. When Matthaei and Nirenberg (1961) established reproducible conditions for the in vitro study of the incorporation of $[^{14}C]$ amino acids into polypeptides or proteins, an avenue for progress was opened. The concept that certain types of RNA were required as templates or messengers to stimulate and direct such a system was quickly confirmed by their cell-free system and the synthetic polynucleotide, polyuridylic acid. These authors then attempted to employ the most readily available pure natural messenger RNA, namely, TMV RNA,(Nirenberg and Matthaei 1961). The finding that this RNA effectively stimulated amino acid incorporation in the cell-free Escherichia coli extracts, led to repeated attempts to detect among the proteinaceious products the only then known protein coded by TMV RNA, namely, the TMV coat protein (Aach et al., 1964; Tsugita et al., 1962).

In vitro plant viral protein synthesis:-

Nirenberg and co-workers developed an efficient, cell-free, protein synthesising system from E. coli and were the first to employ plant viral RNA as a messenger in such systems. After Nirenberg and Matthaei's (1961a) initial observation that TMV RNA supported cell-free protein synthesis, Tsugita et al., (1962) studied this system in some detail. They concluded, on the basis of the coincidence of chromatographic profiles of tryptic peptides of the $[^{14}C]$ -phenylalanine labelled, TMV RNA dependent, in vitro product and the known location of phenylalanine within TMV coat protein tryptic peptides (and similarly

for tyrosine), that the in vitro product(s) included TMV coat protein. However, it was noted that wild-type TMV had no histidine in its coat protein, (Knight, 1947), yet similar levels of $[^{14}C]$ histidine incorporation were obtained with wild-type TMV RNA and TMV RNA from a mutant containing histidine in its coat protein. No label was found in the N-terminal peptide, N-acetyl-seryl-tyrosine, when incorporation of $[^{14}C]$ tyrosine into the TMV RNA-dependent product, was followed by the addition of TMV coat protein, chymotryptic digestion, and isolation of this peptide. Only 10% of the in vitro synthesised protein was taken up during reconstitution of TMV from TMV coat protein and RNA. The authors interpreted this finding as support for TMV coat protein synthesis, by reasoning that partially synthesised coat protein molecules specifically inhibited virus reconstitution.

TMV RNA was also employed by other workers as a messenger for cell-free protein (e.g. Nathans et al., 1962). Aach et al., (1964) found no clear evidence that the TMV RNA-dependent in vitro product protein, produced by E. coli systems, was TMV coat protein. This observation was subsequently confirmed (Schwartz, 1967). Although f2 virus RNA directed the synthesis of a protein with some of the properties of f2 coat protein in a cell-free system derived from Euglena gracilis, TMV RNA directed the synthesis of a product which was other than TMV coat protein, (Schwartz et al., 1965).

Ofengand and Haselkorn (1962) first reported that TYMV RNA serves as a messenger in an E. coli cell-free system. Kolakofsky and Nakamoto (1966) and Voorma and Bosch (1965) have subsequently confirmed this finding. To date, no workers have, however, been able to characterise

the TYMV RNA-dependent *in vitro* product protein produced by an *E. coli* system as TYMV coat protein, or any other known protein, (Haselkorn, 1966).

Similarly, TYMV enhances amino acid incorporation in a cell-free system derived from rabbit reticulocytes, (Betheil and Arnstein 1965). The TYMV RNA-dependent product produced by this system had the 1:1, valine to isoleucine ratio characteristic of TYMV coat protein.

Rolleston *et al.*, (1970) employed TYMV RNA in a cell-free system derived from rat skeletal muscle, and found that ribosomes from diabetic rats were less active than normal rat ribosomes, under TYMV direction in their system.

Satellite tobacoo necrosis virus (STNV) provided the first example where a plant viral RNA could be shown to code for the synthesis of a specific protein. Studies of the RNA and coat protein of STNV suggested that it was composed of homogeneous coat protein molecules and a single-stranded monocistronic mRNA (containing only enough information to code for no more than two proteins), (Reichmann 1964). Clark *et al.*, (1965) confirmed this theory *in vitro*, employing STNV RNA in an *E. coli* system the polypeptide material obtained, on tryptic digestion, giving a series of peptides similar to, but not identical with, those found in the virus coat protein.

Bosch *et al.*, (1966) demonstrated that alfalfa mosaic virus (AMV) RNA derived from the top component, stimulated amino acid incorporation into polypeptides, in a cell-free *E.coli* system. Characterisation of the *in vitro* products revealed a striking similarity to the viral coat protein.

The RNA fraction, however, appeared also to govern the synthesis of other polypeptide material, possibly indicative of further messenger activity.

Stubbs and Kaesberg (1967) demonstrated that unfractionated bromegrass mosaic virus (BMV) RNA and each of its three naturally occurring components stimulated incorporation of amino acids into peptides in an E. coli cell-free system, although the majority of the products were not coat-protein like.

Spencer and Wildman (1964) and van Kammen (1967) were unable to stimulate amino acid incorporation in cell-free plant systems (tobacco leaves) using TMV RNA although polyuridylic acid was active.

Sela and Kaesberg (1969) presented evidence that TMV coat protein was synthesised in a ribosomal cell-free system isolated from tobacco chloroplasts, the product formed combining with TMV RNA to form a nucleoprotein body, having physical, chemical and biological properties of authentic TMV.

Marcus (1969, 1970a,b,c) working with a wheat embryo system, found that TMV RNA stimulated amino acid incorporation. He suggests that a TMV RNA-ribosomal complex is a direct precursor of the polyribosome and that the conversion of this complex into polyribosomes requires aminoacyl transfer.

From the general pattern developing, it has been attempted to explain the results obtained with in vitro protein synthesis directed by plant virus messengers.

The RNA's of all bacterial virus so far examined contained a

triphosphate in the 5' position (Glitz, 1968; Roblin, 1968; Takanami 1967). Initial studies on the nucleotide sequences of the 5' terminal ends of plant viral RNA indicate some variation in the original 5' terminal nucleotide sequence. TMV RNA contains an unphosphorylated 5' terminal adenosine (Fraenkel-Conrat and Singer, 1962) whilst tobacco necrosis virus (Lesnaw and Reichmann,1970) and STNV RNA contains a 5' terminal sequence of ppApGpUp... (Reichmann et al., 1966; Wimmer and Reichmann, 1968; Wimmer et al., 1968). There is some indication that initiation of protein synthesis need not begin with the first trinucleotide encountered at the 5' end of the mRNA. Specifically, MS2, an RNA phage of E. coli known to initiate protein synthesis on all of its cistrons with formylmethionyl tRNA (Viñuela et al., 1967) has a 5' terminal sequence of pppGpGpUp... rather than the expected ApUpGp... or GpUpGp..., (Glitz, 1968). The identity of the 5' terminal sequence is considered to be important from the standpoint of their role as a recognition site for the virus specific RNA replicase, (Lenshaw and Reichmann, 1970).

Most of the in vitro studies on protein biosynthesis, employing plant viral RNA as messenger, have utilised E. coli systems. E. coli systems are known to initiate, in vivo, with formylmethionyl tRNA, (Capecchi, 1967) and it is probable therefore that formylmethionyl tRNA also initiates in E. coli in vitro systems.

The N-terminal group of the specific protein(s) synthesised in the presence of TMV RNA, by an E. coli system have been analysed. Schwartz (1967) found that a high percentage of these specific molecules

terminated at their N-terminal ends with formylmethionine or formylmethionine containing peptides. A high proportion of the remaining molecules synthesised *in vitro* lacked the formyl group, but were otherwise indistinguishable from the formylated product(s).

Hoogendam *et al.*, (1968) and Reinecke *et al.*, (1968) have demonstrated that a high percentage of the coat protein molecules of AMV, synthesised by an *in vitro* *E. coli* system, originate at their N-terminal end with formylmethionine. They demonstated that *in vitro* translation of the top component, *a*, of AMV by a reconstituted *E. coli* system is dependent upon N^5-formyltetrahydrofolic acid.

Kolakofsky and Nakamoto (1966) first demonstrated that TYMV RNA *in vitro* translation, by an *E. coli* system was enhanced by the addition of formylmethionyl tRNA. Albrecht *et al.*, (1969) found that binding of formylmethionine to ribosomes by TYMV was dependent on the presence of ribosomal factors.

Schwartz (1967) demonstrated similarly that *in vitro* translation of TMV RNA was enhanced by the addition of N^5-formyltetrahydrofolic acid.

Albrecht *et al.*, (1969a) demonstrated that ribosomal factors F_1, F_2 and F_3 are required for the binding of formylmethionyl tRNA to *E. coli* ribosomes in the presence of AMV RNA. AMV RNA can bind to *E. coli* ribosomes in the complete absence of initiation factors, (Albrecht, 1969, 1969a) such binding not requiring binding of formylmethionyl tRNA either (addition of factors even lowering the binding of the plant viral messenger).

E. coli cells depleted of formyltetrahydrofolate as a result of

exposure to trimethoprim, a specific inhibitor of dihydrofolate reductase, yield cell-free extracts demonstrating a requirement for N^5- formyltetrahdyrofolate during STNV RNA translation (Clark, 1968).

Both the ability of E. coli extracts to synthesise, in vitro, a material initiating with formylmethionine and the ability of E. coli extracts to demonstrate a dependency upon the formylmethionyl tRNA or N^5- formyltetrahydrofolic acid are a function of the magnesium concentration employed. These specific syntheses and dependencies upon formyl or formylmethionyl donors occur, in general, at the 'low magnesium' levels of 6 to 8 mM Mg^{2+} (Clark et al., 1965; Hoogendam et al., 1968; Kolakofsky and Nakamoto, 1966; Reinecke et al., 1968; Schwartz, 1967). Higher Mg^{2+} levels result in less dependency upon specific donors and in vitro synthesis of proteins having less clearly defined N-terminal groups.

Developing seeds of V. faba contain two major and one minor $tRNA^{Met}$ species, only one of the major species ($tRNA_1^{Met}$) being changed by E. coli enzyme, and neither being formylatable. The minor species ($tRNA_3^{Met}$) is changed or formylated by bean or E. coli enzyme. Results implicate $tRNA_1^{Met}$ in protein chain initiation ($tRNA_3^{Met}$ possibly being the initiator tRNA of cell organelles), (Yarwood et al., 1971).

It may be that in vitro translation of plant viral RNA's in E. coli cell-free systems forces the in vitro translation of plant viral messengers by E. coli rules. It is suggested therefore, that at low Mg^{2+} concentration, the E. coli system scans down from the 5' end of the plant viral RNA until the first E. coli

initiator codon is found (AUG or GUG). If this codon is in phase, correct reading frame is established and a recognisable protein is produced. If incorrect (missence) reading frame is established, an unrecognisable protein is produced. Higher Mg^{2+} enhance mistakes in this system. Two kinds of mistake are suggested, namely that the system will either initiate at the first AUG or GUG with methionyl tRNA rather than formylmethionyl tRNA or that the system will initiate randomly with various aminoacyl or N-acetyl-aminoacyl tRNA's,(Reichmann and Clark 1968).

Experimental evidence somewhat supports these hypotheses in that TMV and TYMV RNA's in E. coli systems yield materials that cannot be characterised as the coat protein of the respective virus. The products could be missence products derived from forced initiation at the first AUG or GUG, but they could be, as suggested by Schwartz (1967), products representing preferential translation of cistrons not coding for coat protein.

It has been assumed that it is the plus virus strand that acts as the messenger in vivo, rather than the complementary minus strand, in protein biosynthesis. Schwartz (1967) suggested that in vitro synthesis of coat proteins may be impossible in a system lacking a replicase, due to the fact that the coat protein cistrons are a component of the minus strand of the RNA replicative form. Partial characterisation of a protein synthesised in vitro as viral coat (van Ravenswaay Claasen et al., 1967) does suggest that it is the plus strand that is read as messenger, however. Double-stranded viral RNA's would not be expected to act as messenger RNA, and this lack of activity has been shown for rice dwarf virus RNA, (Miura

and Muto, 1965).

In contrast to the TMV and TYMV systems, STNV and AMV top component yield, *in vitro*, in *E. coli* systems, materials characterised as the coat protein of the respective virus (Clark et al., 1965; van Ravenswaay Claasen *et al.*, 1967). These could reflect the fortunate occurrence where scanning of the RNA's from their 5' ends in search of the first AUG or GUG leads to the establishment of correct reading frame and synthesis of a recognisable coat protein.

The above theory is also generally supported by the various effects noted as a function of the *in vitro* Mg^{2+} ion concentration. Dependence of the *in vitro* translation upon formyl or formylmethionyl donors decreases with higher Mg^{2+} concentrations (Clark, 1968; Hoogendam *et al.*, 1968; Kolakofsky and Nakamoto, 1966; Reinecke *et al.*, 1968; Schwartz,1967) suggesting that initiation 'mistakes' involving initiations other than those employing formylmethionyl tRNA are encouraged. Studies with AMV RNA of the top component indicate that specific mistakes such as binding of N-acetylphenylalanyl tRNA (i.e. formation of initial N-aminoacyl tRNA, mRNA, ribosome complexes) and incorporation of N-acetylphenylalanine are increased at higher Mg^{2+} concentrations,(Verhoef *et al.*, 1967).

Reinecke (1967) found that the label was incorpoated into polypeptide during incubation of N-acetyl- $\left[^{14}C\right]$ phenylalanyl-tRNA with a cell free system of *E. coli* programmed with top *a* AMV RNA. STNV RNA failed to promote incorporation of label from N-acetyl- $\left[^{14}C\right]$ phenylalanyl-tRNA but did stimulate that from $\left[^{14}C\right]$ phenylalanyl-tRNA, indicating that deacylation does not occur under the conditions of the incubations

(12 mM Mg^{2+}). TYMV RNA binds to a number of aminoacyl-tRNA's whereas AMV RNA only binds phenylalanyl and valyl-tRNA's, and STNV does not bind any,(Bosch, 1967).

With recent advances, our present knowledge of the interaction of viruses and cells has developed to the point where attention is now being focused on particular events at the level of virus and host cell macromolecules. To this end, in vitro experimental systems have been developed to examine such problems as viral inhibition of host macromolecular synthesis, replication of viral nucleic acids, translation of the viral genome into functional proteins and the assembly of viral polypeptides into infectious particles.

Although much of the early work on morphology and chemical composition of viruses was carried out with plant viruses, because of inherent difficulties only bacterial and animal viruses have been employed to advantage in in vitro protein synthesising systems. The widespread presence of phenolic material, nucleases, strong cellulose walls and difficulties in uniformly infecting and labelling large numbers of plant cells, have limited plant systems in such investigations. Most workers therefore, in attempting to demonstrate plant viral coat protein synthesis in vitro have used ribosomes, tRNAs and enzyme from a bacterial source (usually E. coli). At least some (but not all) of the proteins made in vitro have been shown to be similar to coat proteins of the respective virus used.

Recently a further step has been taken in regards to the study of

assembly *in vitro*, (Sela and Kaesberg, 1969), by utilising protein synthesised *in vitro* and studying its further assembly *in vitro*. A further advance on these lines would be the coupling of an *in vitro* RNA-synthesising system to such a protein synthesising system, to determine if both viral RNA and protein synthesised *in vitro* can be assembled into intact infective virus.

As a preliminary to such experiments and the possible elucidation of some of the factors affecting the infection process, an *in vitro* protein synthesising system has been developed employing TYMV RNA and *Brassica chinensis* ribosomes, enzyme and tRNA.

TYMV was chosen as the source of viral RNA after a review of the literature to find a virus that could be both easily cultured and isolated.

The following pages outline some of the problems encountered in the attainment and development of this *in vitro* system.

MATERIALS AND METHODS

1. Biological Materials:

(a) Chinese cabbage seed (Brassica chinensis L., var. Chihli, Pte Sai and Wong Bok) was obtained from Thompson and Morgan (Ipswich) Limited. Plants were grown from seed, in an insect-proofed area under normal greenhouse conditions, at the University of Durham Botanic Gardens. Storage of the seed at room-temperature for three years had little effect on viability.

(i) The seeds were sown either singly into sterile John Innes Number 1 potting compost, or 'broadcast' in John Innes seedling compost. Seedlings from 'broadcast' sowings were 'pricked-out', at a suitable stage in development, into individual pots containing John Innes Number 1 or Number 2 potting compost (growth in Number 1 or 2 composts showed little variation and therefore Number 1 was used routinely).

Plants sown singly were found to take longer to grow and produce sturdy plants than those broadcast sown and subsequently planted-out individually. The former method was used whenever possible, however, to reduce the time and effort involved in plant cultivation.

Growth in plastic pots was found to be better than growth in traditional clay pots (probably as the round or square plastic pots retained soil moisture longer than did the clay). Adequate watering was found to be essential to maintain sturdy growth and overwatering was not found to be a problem.

The plants were grown at a temperature of 20-25^{o}C, in a heated greenhouse, throughout the year, the light being supplemented

artificially from November to May. Under artificial light conditions, growth was retarded (especially in infected plants) until natural daylight increased.

Plants, or leaf material, were harvested in the morning, and the age as given in the text refers to the age of the plants from the time of sowing. Plant material of the same age and of similar leaf size was taken to aid in standardisation.

Growth of uninfected plants out of doors was attempted, during the summer months, but was abandoned after the first few crops were eaten by rabbits.

(ii) Certain plants were allowed to go to seed, and the seed pods harvested immediately before use. The seeds were removed from the pods and used for experimental purposes (although not for the growth of stock plants).

(iii) Dry seeds of B. chinensis var. Wong Bok were washed in tap water and then rinsed three times in sterile water, before being sown directly onto absorbent paper towelling (which lined the bases of sterilised plastic trays). The seeds were covered with a further layer of paper which was moistened with sterile water, by means of a fine spray.

The seeds were allowed to germinate in the dark at 25°C, for varying periods, until the cotyledons had developed (but before they had changed colour from yellow to green). A fairly constant humidity was maintained throughout germination by covering the trays with a black

polythene-sheeting enclosure.

Any material showing the slightest sign of infection was discarded (although infection was very rarely noted during the incubation period, or for some time afterwards).

After five to six days, the seedlings were 2-3 cm in length, and the first pair of true leaves were just becoming evident.

(iv) Dry seeds of B. chinensis var. Wong Bok, were washed and rinsed in sterile water prior to being sown directly into vermiculite (contained in sterilised plastic trays). The vermiculite was subsequently moistened with sterile water, and covered with absorbent paper towelling.

The trays were covered with black polythene-sheeting and the temperature of incubation was maintained at 25°C.

(v) In some experiments, procedure (iv) was modified in that absorbent paper towelling was not placed directly over the seeds (i.e., on the vermiculite surface), but was suspended some 5 cm above the seeds. This facilitated removal of the seedlings from the vermiculite, whereas in method (iv) they tended to become attached to, or interwoven in, the paper towelling. Better germination resulted, however, when method (iv) was employed.

(b) Seeds of Vicia faba L. var. Triple White were obtained from the Tyneside Seed Company, Gateshead, and plants were again grown at the University of Durham Science Laboratories.

(i) Seeds were sown in pots during February and were maintained in heated greenhouses until the young plants were transplanted into the open ground, at the end of March. Any sowings made after this date were made directly into the open ground. Plants grown out of doors were used as a source for large scale extraction procedures.

The plants were observed daily, and to indicate the age of the pods, the flowers were date-labelled on the first day they were fully open. Pollination was assumed to occur on the same day as the flower opening. Previous work (Wheeler, 1965) has shown that this method gives material which is on average of the same age.

Immediately before use the pods were harvested, care being taken to sample pods of similar size. Standardisation was carried further by selecting seeds of the same size from the collected pods. The age of the pods given in the text refers to the number of days of pod development after the full opening of the flower.

(ii) Using standard growth conditions, pot grown plants were maintained under greenhouse conditions. Under these conditions, whilst growing in plastic plant pots, the bean plants flowered and set seed. These plants were used for inoculation experiments.

When grown under a mixture of natural and artificial light, bean plants were not affected to the same extent as B. chinensis, and their growth was readily maintained throughout the winter months.

(iii) Dry seeds of V. faba var. Triple White were washed to remove surface dirt. They were imbibed in running tap-water for 24 h prior to

surface 'sterilisation' by shaking, for two min in chlorinated lime solution. The lime wash was decanted and the seeds washed with sterile water until the smell of chlorine was just perceptible. The seeds were then sown in rows on absorbent paper (which lined the bases of sterile plastic trays). The seeds were covered with paper and thoroughly soaked with sterile water. The tray was covered with black polythene sheeting and the germination temperature was maintained at 25°C.

Germination was allowed to proceed for five days, care being taken to remove any seeds showing signs of infection. After five days the plumules emerged from beneath the testas and were about 5 mm in length.

(c) Samples of turnip yellow mosaic virus (TYMV) infected material were kindly supplied as follows:-

(i) Sample from Dr. J. Bové,
Chef du Service de Biochimie,
I.F.A.C.,
Station Centrale de Physiologie Végétale,
Route de St. Cyr,
Versailles, FRANCE.

This sample of infected Chinese cabbage plant readily infected test plants, the systemic symptoms appearing first in the hearts of the rosette. Infection produced by this 'isolate' appeared sooner and killed the plants quicker than the other isolates (the other isolates very rarely killing their hosts).

(ii) Sample from Dr. G. Belli,

Istituto di Patologia Vegetale,

Milan, ITALY.

This sample, cultured in Italy, originally came from Dr. K.M. Smith when working in Cambridge.

(iii) Sample from Dr. E.M.J. Jaspers,

Biochemisch Laboratorium Rijksunivesiteit,

Leiden, THE NETHERLANDS.

This strain is identical with the 'type-strain' of Symons et al., (1963).

(iv) Sample from Professor R.E.F. Matthews,

Department of Cell Biology,

The University of Auckland,

NEW ZEALAND.

This strain came from the stock culture in Cambridge (in 1956).

Most samples appeared to have come originally from the type strain as described by Markham and Smith (1949) (but see Matthews and Ralph, 1966).

(v) Sample from Professor R. Markham,

John Innes Institute,

Colney Lane,

Norwich, ENGLAND.

This is the original 'type strain', or one closely resembling it (Matthews and Ralph, 1966).

Turnip yellow mosaic virus (synonyms, Newcastle (turnip) virus - Croxall et al., 1953; Brassicavirus octahedron - Roland, 1959) is serologically related to wild cucumber mosaic and cacao yellow viruses (Martyn, 1968).

Most experiments were performed using cultures originating from the New Zealand isolate. This isolate gave symptoms indistinguishable from those originally described for the virus (Markham and Smith, 1949; Reid and Matthews, 1966).

TYMV stock cultures were maintained by inoculating B. chinensis plants grown under standard conditions. About two to three weeks after sowing, when the first true leaves of the seedlings were rapidly expanding, the seedlings were inoculated mechanically with a suspension of TYMV.

The inoculum used was either a purified viral suspension or crude infected leaf- sap. Prior to any inoculation, the leaves of the plants were lightly sprinkled with fine carborundum powder (to act as a mild abrasive and so to allow easier penetration of the leaves by the virus), after preliminary cleaning of the leaves with sterile water. It was found more efficient to sprinkle the leaves than to grind the inoculum with carborundum as suggested by Jaspars (1969).

The inoculum was applied directly to the leaf surface with the forefinger. It was found that consistent results could be obtained using the same forefinger (probably as experience was gained as to the degree of pressure to apply so as not to damage the leaves too seriously).

After applying the inoculum, there was a two min time lapse before lightly spraying the leaves, for 10 sec, with sterile water (to remove excess inoculum).

When employing purified TYMV suspensions, it was found necessary to well rinse the leaves to prevent scorching by NaCl or Tris buffers. Aqueous suspensions (without buffer) did not cause scorching.

TYMV RNA was similarly employed as an inoculum. A soaked absorbent gauze pad was used to apply the nucleic acid to avoid RNase contamination (likely to occur if the RNA was applied with the forefinger).

The virus and nucleic acid were similarly applied to the leaves of 45 - 50 day-old (from planting) V. faba plants grown in the greenhouse. Inoculation was performed by gently rubbing the leaf surface, by dipping the damaged leaf into the inoculum or by injection of the material into a leaf vein or into the main plant stem. Viral RNA was applied to broken leaf edges or to the leaf laminae by means of an absorbent gauze pad.

Various combinations of bean and Chinese cabbage leaf extracts, infected and uninfected, were used as inoculum sources, these combinations being discussed further under later headings.

All apparatus was thoroughly cleaned and whenever practicable heat sterilised, to ensure no accidental carry-over of TYMV between test plants.

Throughout all inoculations, for control purposes, comparable plants were rubbed with buffer or uninfected leaf sap only, to simulate inoculation.

It was found that at least 50% of the virus suspensions used as sources of inocula were viable after 2 yr storage at $4^{o}C$. The degree of infectivity was, however, somewhat reduced. A six-month old freeze-dried

infected leaf sample provided by Dr. Jaspars in 1969, was found to be infective.

Freezing of infected leaf samples, although suggested as a means of viral storage (Belli, 1969) was only used as a safeguard over the winter months, to maintain the stock cultures in the event of total plant loss. Fresh viral extracts were normally used for all other experimental purposes.

(d) Leaves from radish plants, Raphanus sativus var. Scarlet Marvel (R. & G. Cuthbert, Goff's Oak, Herts.) were employed in one experiment.

2. Chemicals and Reagents

Except where listed, chemicals were obtained from British Drug Houses (BDH) Chemicals Limited, Poole, Dorset, and were of analytical grade when available.

Adenosine-5'-triphosphate (disodium salt) (ATP)

Bovine serum albumin (fraction V)

Cadaverine dihydrochloride

Creatine phosphokinase

Guanosine-5'-triphosphate (sodium salt) (GTP)

Phosphocreatine (disodium salt)

Polyuridylic acid (potassium salt)

Putrescine dihydrochloride

Ribonuclease-A (75 units/mg from bovine pancreas, 5 x crystallised)

Spermidine trihydrochloride

Spermine tetrahydrochloride

were obtained from Sigma Chemical Company Limited, London.

2,5-diphenyloxazole (PPO)

L-glutathione (reduced)

1,4-di- [2-(5-phenyloxazolyl)] benzene (POPOP)

were obtained from Koch-light Laboratories, Colnbrook, Bucks.

L-amino acids ('A' grade) were obtained from California Corporation for Biochemical Research, Los Angeles, U.S.A.

Orcinol was obtained from Hopkins and Williams Limited, Chadwell Heath, Essex.

Bakers yeast soluble RNA was obtained from Boehringer and Soehne GmbH, Mannheim, Germany.

Napthalene blue black (Michrome no. 1113)

Xylene brilliant cyanin G (Michrome no. 1224)

were obtained from Edward Gurr Limited, London.

Ferric chloride ($FeCl_3 \cdot 6H_2O$) was obtained from May and Baker Limited, Dagenham, Essex.

Labelled amino acids were obtained from the Radiochemical Centre, Amersham, Bucks. and were

Amino acid-C14(U) mixture (Table 1), specific activity 52mCi/mAtom Carbon

DL-Arginine-1-C14 monohydrochloride,sp. activity 10.7 mCi/mM

Table 1

Composition of Amino Acid-C14(U) Mixture

L-amino acids isolated from Chlorella protein hydrolysate and purified by ion exchange and paper chromatography. The amino acid-C14(U) mixture compounded from the individual amino acids was composed of (composition by activity):

L-alanine	10%	L-lysine	5.5%
L-arginine hydrochloride	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%

Code CFB.104 Radioactive dilution 16 ml/mCi.

Specific activity 52 mCi/mAtom carbon.

L-Aspartic acid-C14(U), specific activity 8.7 mCi/mM

DL-Glutamic acid-1-C14, specific activity 23.6 mCi/mM

DL-Leucine-1-C14, specific activity 55 mCi/mM

L-Lysine-C14(U) monohydrochloride, sp activity 301 mCi/mM

DL-3-phenyl(alanine-1-C14), specific activity 48 mCi/mM

DL-Valine-1-C14, specific activity 48 mCi/mM

Chloramphenicol was obtained from Parke, Davis and Company, Pontypool, Mon.

Cetyltrimethlyammonium bromide (Cetrimide) was obtained from I.C.I. Limited, Macclesfield, Cheshire.

Oxoid Nutrient Broth No. 2 was obtained from Oxoid Limited, London, and Davis Standard Agar, from Davis Gelatine Limited, Warwick.

Vermiculite (Veri-Gro) was obtained from Pan Britannica Industries Limited, Waltham Abbey, Essex.

Visking dialysis tubing was obtained from Scientific Instrument Centre Limited, London.

Whatman 3MM paper and DEAE cellulose were obtained from H. Reeve Angel and Company, London.

Reagents and Solutions:

Phosphate buffer refers to an 8:2 mixture of Na_2HPO_4:KH_2PO_4 unless otherwise stated (pH 7.4 at 20°C).

Chlorinated lime solution was prepared fresh for each operation, and contained approximately 2,500 ppm of available chlorine.

Chlorinated lime		1.25 g
Boric acid		1.25 g
Water	to	100 ml

Solutions for Lowry's Folin Method for Protein Determination

(a) Freshly prepared for each set of determinations, a working solution containing

1ml 1% (w/v)	copper sulphate
1ml 2% (w/v)	sodium potassium tartrate
50ml 2% (w/v)	sodium carbonate in 0.1N NaOH

(b) Folin-Ciocalteu's phenol reagent (used diluted 1:1 with water)

Solutions for the Orcinol Determination of RNA

5% (w/v)	trichloroacetic acid
10% (w/v)	recrystallised orcinol in ethanol
0.1% (w/v)	$FeCl_3 \cdot 6H_2O$ in concentrated HCl

Scintillation Fluid

PPO		4.5 g
POPOP		0.1 g
Toluene	to	1 l

Solutions for Bramhall's Method of Protein Determination

(a) 10mg/ml xylene brilliant cyanin G in 7% (v/v) acetic acid

Destain solution: 66 ml methanol: 34ml water:1ml concentrated ammonia.

(b) 10mg/ml napthalene blue black in 1:4:5 (v:v:v) acetic acid: methanol:water.

Die release solution: 0.05 N NaOH.

Nutrient Agar

Oxoid nutrient broth No. 2		25 g
Davis standard agar		20 g
Water	to	1 l

Malt Agar

Malt extract		20 G
Davis Standard agar		18 G
Water	to	1 l

Solutions for Polyacrylamide Gel Electrophoresis

Acrylamide and bisacrylamide were recrystallised by the method of Loening (1967, 1968) and stored in sealed containers in the absence of light.

(a) 2-5% Stock Acrylamide Solution

Recrystallised acrylamide		15 g
Recrystallised bisacrylamide		0.75 g
Water	to	100 ml

(b) Stock 'E' Buffer (5x concentrated)

$NaH_2PO_4 \cdot 2H_2O$	150 mM
disodium EDTA.$_2H_2O$	5 mM
Tris-acetic acid buffer	180 mM (pH 7.6 at room temp)

(c) Preparation of Gels

	2.2%	2.4%
Stock acrylamide soln.	5.0 ml	5. 0 ml
Stock 'E' buffer	6.8 ml	6.25 ml
Water	22.0 ml	19. 7 ml

3. General Procedures Followed During Preparation of Materials, and Extraction Procedures:

Unless otherwise stated, the following points should be borne in mind when reading the text.

Water used was distilled, sterilised by autoclaving at 115°C (10 lb. per sq. in), for a time sufficient to ensure that the whole of the water in the sealed container was maintained at that temperature for 30 min.

Solutions were prepared, aseptically, using this water, and were stored at 0-5°C whenever practicable.

Glassware and apparatus used to prepare or to store solutions, was sterilised by an appropriate method, e.g., glassware and wedgewood mortars were dried and then heated to and maintained at 160°C for 2-3 hr.

Operations and procedures, where practicable, were carried out in a cold-room maintained at 4°C, or in ice-baths at c. 0°C (pH being determined at 4°C).

Generally, centrifugations were carried out at 2-4°C and all 'g'

values refer to values determined at the base of the container.

Where appropriate, disposable polythene gloves were worn to minimise contamination of extracts etc., with exogenous RNase.

Determination of Protein:

Protein was determined either by the method of Lowry et al., (1951) (calibration graph fig. 1) or by the method of Bramhall et al., (1969)(calibration graphs figs. 2A, 2B) using bovine serum albumin as a calibration standard.

Determination of Ribonucleic Acid:

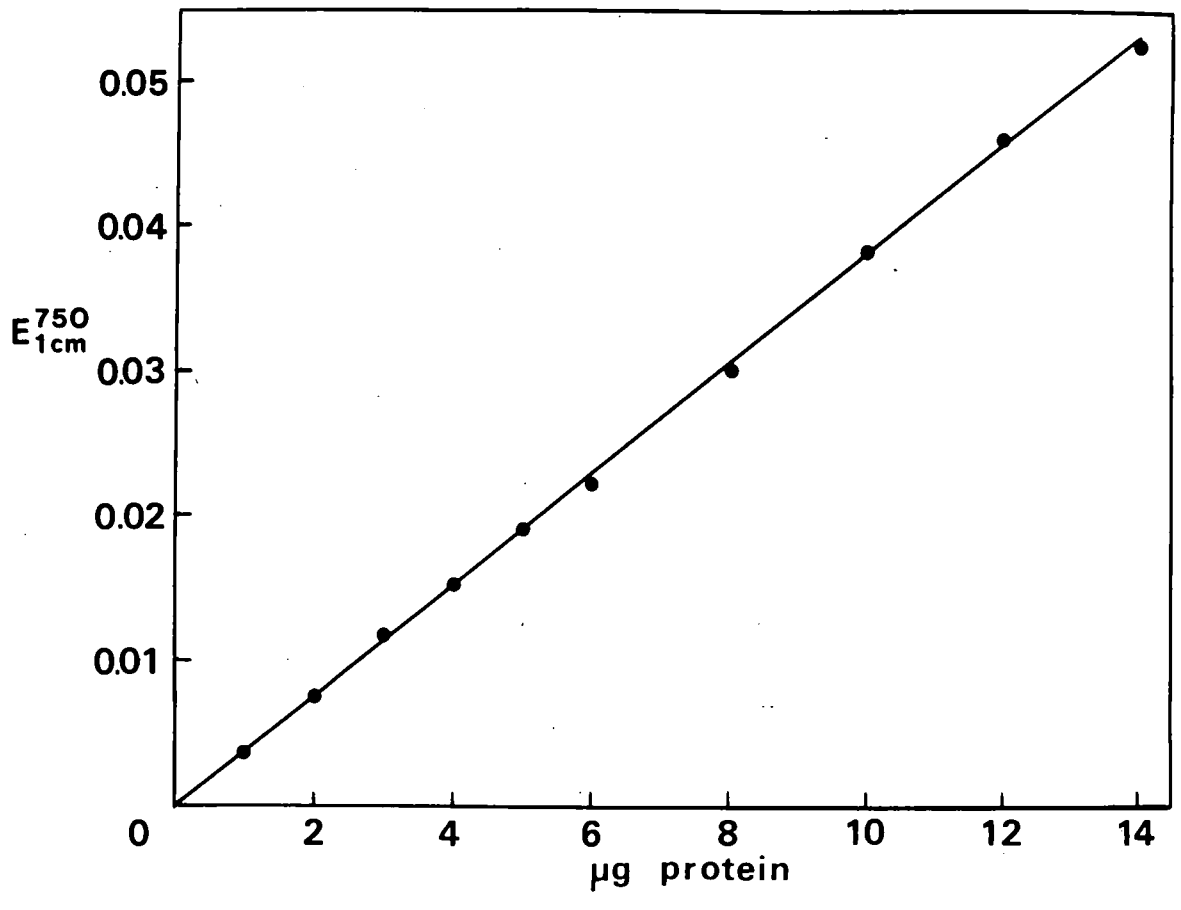
RNA was determined by the orcinol method of Mejbaum (1939) as described by Campbell and Sargent (1967) using E. coli RNA as calibration standard, (fig. 3).

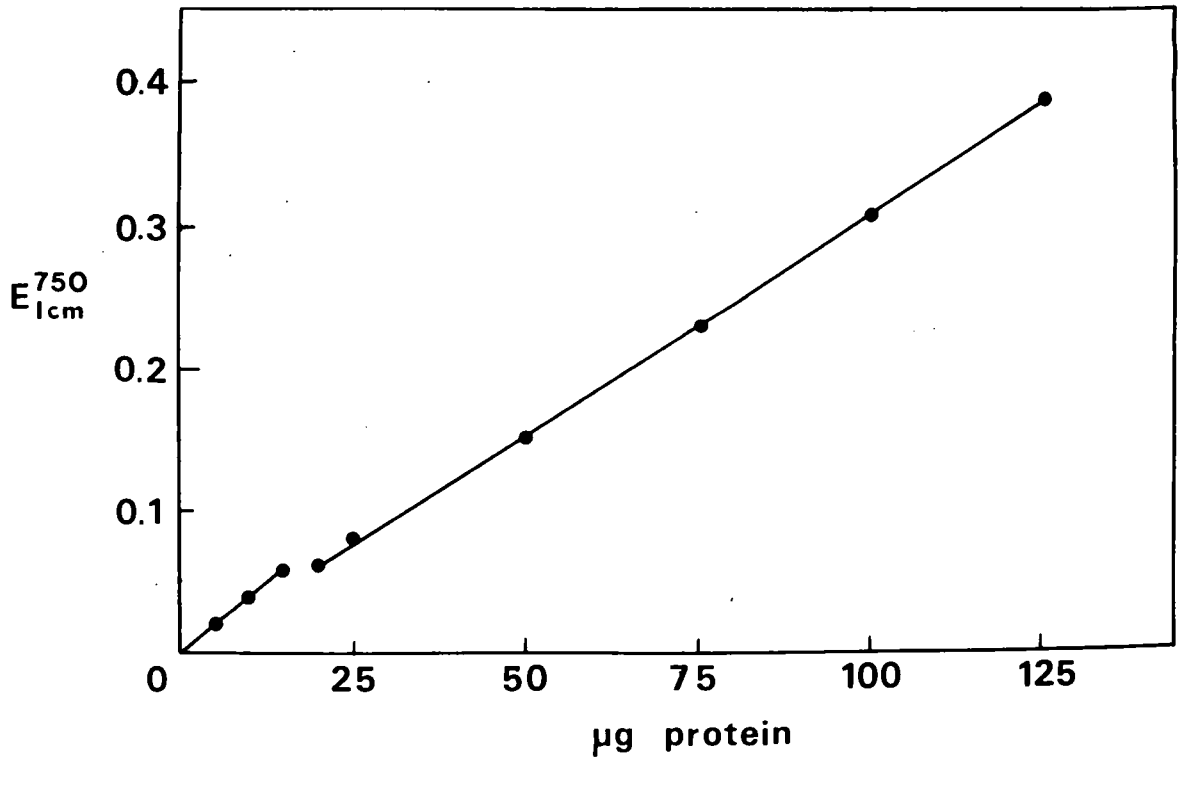
The concentration of microsomal suspensions, solutions of RNA etc., were also determined from E^{260}_{1cm} measurements of suitable dilutions (assuming an optical density of 11.3 for a 1 mg/ml suspension of microsomes (T'so and Vinograd, 1961), an optical density of 24 for a 1 mg/ml solution of tRNA (Yarwood, 1968) and an optical density of 23 for a 1 mg/ml solution of TYMV RNA (Haselkorn, 1962)).

Counting of Bacteria:

Samples of solutions, reaction mixtures etc., were taken and diluted, where necessary, with $\frac{1}{4}$ strength Ringers solution. Inoculations were made by standard techniques onto nutrient agar and malt agar plates, incubations being at 30°C and 22°C respectively. Plate examination was made after 48 h incubation and then at 24 hour intervals for 5 days.

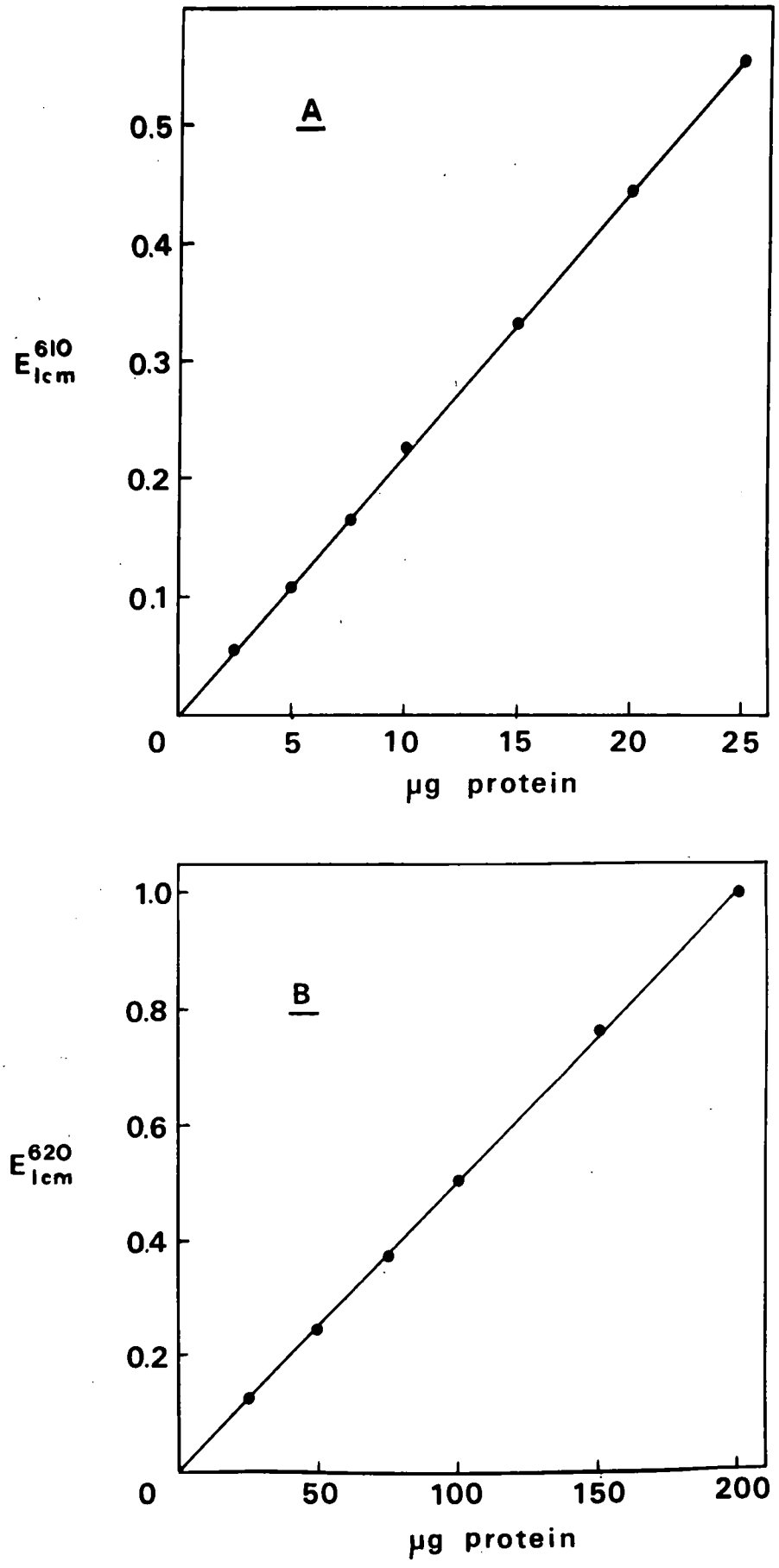
Fig. 1 . Calibration curves for the estimation of protein by the method of Lowry et al. (1951)





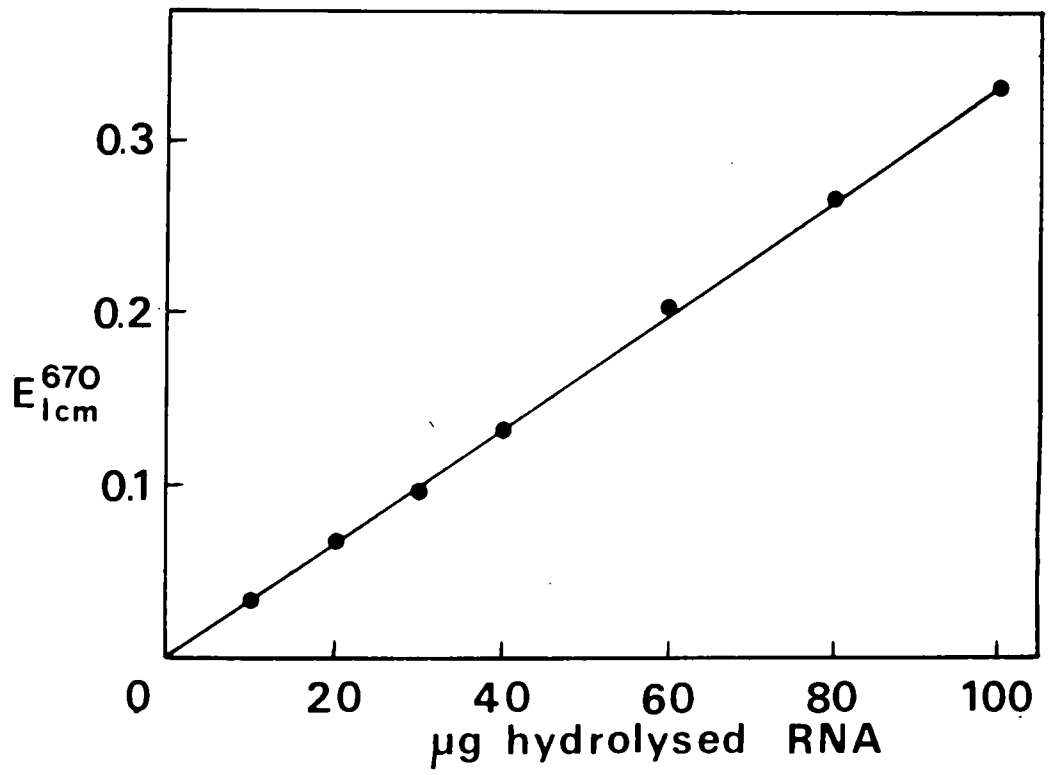
Typical calibration curves for the determination of protein, standard solutions of bovine serum albumin being routinely tested to confirm calibrations under test conditions.

Fig. 2 . Calibration curves for the determination of protein by the method of Bramhall et al. (1969)



A. Xylene brilliant cyanin G series.
B. Naphthalene blue black series.

Fig. 3 . Calibration curve for the Mejbaum method of RNA determination as described by Campbell and Sargent(1967)



Typical calibration curve for the Orcinol determination of RNA samples. Standard RNA solutions were routinely tested to confirm calibration curves under test conditions.

Preparation of Magnesium Bentonite:

(a) Magnesium bentonite was prepared from technical grade bentonite powder by a modification of the method of Petermann and Pavlovec (1963). The powder was dried at 100°C and sterilised by dry heat before use. Bentonite (100 g) was sprinkled onto the surface of 2l of 10^{-2} M $MgSO_4$ - 10^{-2} M phosphate buffer ph 7.4 (20°C), and allowed to sink into the liquid. The suspension was shaken on a vibratory-mixer for at least 2 h, and then centrifuged at 400g for 2 min. The pellet was discarded and the supernatant centrifuged at 17,000g for 15 min. The supernatant was discarded and the resulting pellet suspended in 1 l of 10^{-3} M $MgSO_4$ - 10^{-3} M phosphate buffer pH 7.4 (20°C) and vibratory-shaken for 2 h. The low and high-speed centrifugations were then repeated. The final high-speed pellets were suspended in 300-400 ml of the 10^{-3} M buffer.

Dry-weight determinations were performed on samples of the suspension to ascertain the actual bentonite content.

(b) A second method was employed to produce a more homogenous product. Bentonite (100 g) was suspended in 2 l of 10^{-2} M phosphate buffer pH 7.4 (20°C) by means of an Ato-Mix blender. The suspension was centrifuged at 450g for 5 min and the black pellets discarded. A M solution of $MgSO_4$ was added to the supernatant to give a final concentration of 10^{-2} M $MgSO_4$. This suspension was shaken for 2 h before centrifuging at 18,000g for 15 min. The supernatant was re-centrifuged at 18,000g, and the pellets resulting from both centrifugations suspended in 1 l of 10^{-3} M $MgSO_4$ - 10^{-3} phosphate buffer pH 7.4 (20°C). Mixing was accomplished by means of an Ato-Mix blender

and the suspension was then centrifuged at 18,000g for 15 min (repetition of the low-speed centrifugation being found unnecessary). The resultant pellet was suspended in 500 ml of the 10^{-3} buffer mixture prior to assay.

Assay of Magnesium Bentonite:

A 5 ml sample of the suspension was taken and the bentonite precipitated by the addition of an equal volume of 90% ethanol. The bentonite was pelleted by centrifuging in a Piccolo Bench Centrifuge at top speed, 2,500g, for 15 min. The pellets were washed twice with 90%ethanol and dried to constant weight at 105°C. Yields were usually in the region of 30% of the starting material.

The bentonite suspension was adjusted to a working concentration of 50 mg magnesium bentonite per ml, and stored at 5°C until required. Unless otherwise stated, all references to magnesium bentonite are to magnesium bentonite prepared by method (b) and all volumes are given as volumes of this preparation containing 50 mg/ml (designated MAGBENT for simplicity).

Preparation of Sodium Bentonite:

Sodium bentonite was prepared from sterilised technical grade bentonite, by a method based on that used by Barr and Guth (1951). Bentonite powder (50 g) was sprinkled onto the surface of 1 l of M NaCl - 0.01 M Na_3EDTA - 0.01 M phosphate buffer, pH 7.4 (20°C) and was readily dispersed by means of an Ato-Mix blender (the higher ionic

concentration aiding hydration and flocculation of the powder). The suspension was centrifuged at 1,300g for 20 min and the opalescent supernatant discarded. The pellets were resuspended in 1 l of the buffer solution and shaken for 3h before re-centrifugation.

The resulting pellets were suspended in nine times their volume of water and shaken on a vibratory-mixer for 15 min, or until evenly dispersed. The resulting suspension was centrifuged at 720g for 10 min and the sediment rejected. The bentonite in the supernatant was precipitated by the addition of 1.5 vol. of 90% (v/v) ethanol and pelleted by centrifugation at 1,500g for 15 min at 5-10^{0}C (the ethanol/bentonite/water mixture being too viscous to allow ready pelleting of the bentonite at a lower temperature).

The pellets resulting were approximately equal in volume to the supernatant (the total volume equalling c. 2l). An equal volume of 90% (v/v) ethanol was added to the pellets, and after mixing, centrifugation at 1,500g was repeated.

The pellets from this centrifugation were similarly resuspended in alcohol and recentrifuged. The final pellets were suspended in 1 l absolute ethanol, prior to assaying.

After assaying, the suspension was centrifuged at 1,500g for 15 min and the pellets resuspended in an equal volume of water. The final concentration of the sodium bentonite was adjusted to give a concentration of 18 mg/ml of sodium bentonite, by the addition of 50% (v/v) ethanol.

Assay of Sodium Bentonite:

A 5 ml sample of the suspension was taken and centrifuged at 3/4 top-speed of a Piccolo Bench Centrifuge for 15 min, and then for 2 min at top-speed (to compact the pellets). The ethanol was decanted and the pellets gently dried at first, to remove excess alcohol, before being dried to constant weight at 105°C. Initial slow drying was found necessary with both magnesium and sodium bentonite pellets to prevent 'spitting' and subsequent loss of material.

The bentonite suspension, adjusted to a working concentration of 18 mg/ml sodium bentonite, (designated SODBENT) was stored at 5°C until required. Samples were brought to room temperature immediately before use in extraction procedures.

The yield of sodium bentonite represented, on average, 44% of the starting material.

4. Isolation Procedures:

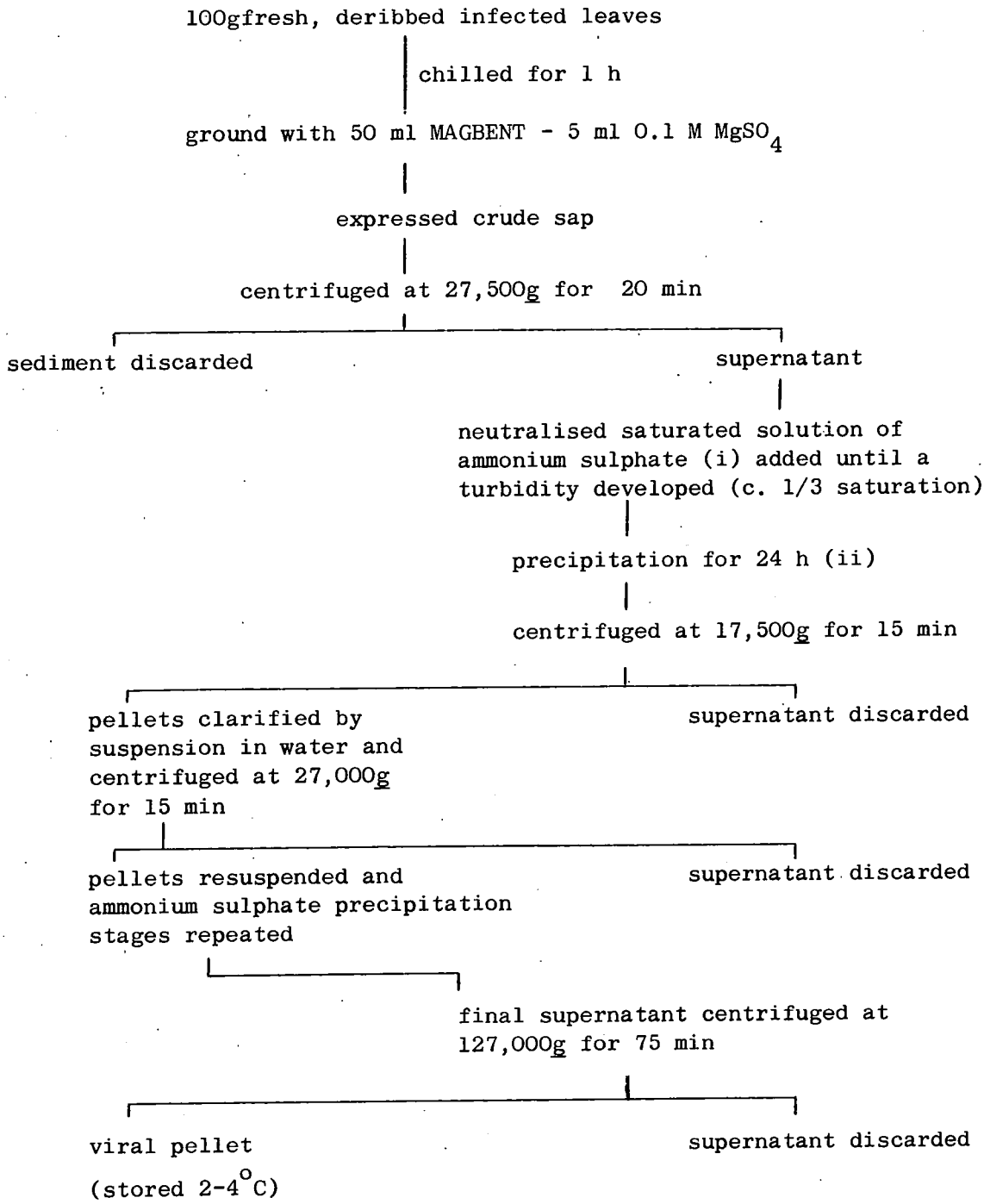
(a) Turnip Yellow Mosaic Virus:

Turnip yellow mosaic virus was isolated from Brassica chinensis leaves by ammonium sulphate precipitation (Table 2) or by differential centrifugation (Table 3).

A sample of virus in 5 x 10^{-3} $MgSO_4$ - 10^{-2} phosphate buffer, pH 7.4 (20°C) was shaken with MAGBENT (resulting in a final concentration of 5 mg bentonite/ml). Bentonite was removed by centrifugation at 27,500g for 15 min to leave the 'purified' viral suspension. This test was carried out to test the adsorptive power of the magnesium bentonite suspension.

Table 2

Isolation of Turnip Yellow Mosaic Virus from Brassica chinensis by Ammonium Sulphate Precipitation (i) (ii)



(i) Markham and Smith, 1949

(ii) Dunn and Hitchborn, 1966

Table 3

Isolation of Turnip Yellow Mosaic Virus from Brassica chinensis by Differential Centrifugation (i)

100 g fresh, deribbed infected leaves

↓ chilled for 1 h

ground with 50 ml MAGBENT - 5 ml 0.1 M $MgSO_4$

↓

expressed crude sap

↓

centrifuged at 27,200g for 15 min

sediment discarded	supernatant centrifuged at 160,700g for 60 min
pellets suspended in 5×10^{-3} M $MgSO_4$ - 10^2 phosphate buffer pH 7.4 (20^oC) volume equal to 1/10 original sap volume). 0.1 ml MAGBENT added per ml of suspension	supernatant discarded

↓

centrifuged at 27,200g for 20 min

pellet discarded	supernatant centrifuged at 160,700g for 60 min
viral pellet (stored at 2-4^oC or used immediately)	supernatant discarded

(i) Dunn and Hitchborn, 1965

The purification procedures were followed by determining the absorbence profiles of the virus containing preparations by means of a scanning spectrophotometer. Those final preparations prepared by differential centrifugation, which were analysed in the analytical ultracentrifuge, yielded only the Schlieren-optical peaks corresponding to reported S values for TYMV components (Matthews and Ralph, 1966). Yields of 250-1,250 mg extracted virus per kg leaf material were obtained (cf. Beer and Kosuge, 1970).

Dry Weight Determination of Turnip Yellow Mosaic Virus:

Samples of TYMV, extracted by differential centrifugation were exhaustively dialysed against water before being dried to constant weight at 60°C.

From the optical density of the suspensions prior to drying, the extinction coefficient at 260 nm was found to be 7.0 ± 0.1 cm^2 mg^{-1} (which is in agreement with the value as determined by Goffeau and Bové, 1965).

(b) Turnip Yellow Mosaic Virus Ribonucleic Acid:

Viral nucleic acid was prepared from less than one week-old TYMV, prepared by differential centrifugation. (If filtered through a bacteria-proof filter, however, the virus preparation may be maintained for longer periods, at low temperatures).

(i) Phenolic Extraction based on the method of Haselkorn (1962):

One ml of a 0.1 to 1.0% viral suspension in 0.01 M NaCl - 0.002 M Tris-HCl buffer, pH 7.5 was vibrated for 60 s with an equal volume of

90% (w/w) analytical grade phenol and centrifuged at 1,750g for 20 min (in an MSE Minor centrifuge). The supernate was removed and an equal volume of 90% (w/w) phenol added. This mixutre was vibrated for 30 s before centrifuging at 1,750g for 20 min.

The aqueous phase was washed three times with ether, any ether remaining after the final wash being removed by blowing nitrogen into the solution.

The RNA was precipitated by the addition of an equal volume of 90% (v/v) ethanol, the precipitation being allowed to proceed at $-20^{o}C$ overnight. The RNA was centrifuged down at 1,750g for 20 min, and taken up in 0.01 M sodium acetate, pH 5.5. The RNA was reprecipitated with alcohol before being finally stored under ethanol at $-20^{o}C$.

Samples of viral RNA were centrifuged and reprecipitated before use, to remove any attendant haze.

It has been observed that freezing and thawing do not appear to alter the sedimentation pattern of the RNA, or lower its infectivity (Haselkorn, 1962).

(ii) Ethanol Precipitation based on the method of Dunn and Hitchborn(1966)

The virus was suspended in water at a concentration of 0.5 - 1.0 mg/ml To 0.8 ml of this suspension was added 0.1 ml of 0.5 M NaCl - 0.5 M Tris-HCl buffer, pH 7.5 - 0.01 M Na_3EDTA, 0.15 ml SODBENT and absolute ethanol to provide a final alcohol concentration of 40% (v/v).

The mixture was shaken and allowed to stand at room temperature for

15 min before cooling in ice for 2 min. The mixture was then centrifuged at 1,750g for 20 min (at 5-10°C, as use of a lower temperature is precluded, the viscosity of the alcohol/bentonite suspension being too great to allow adequate separation at lower temperatures).

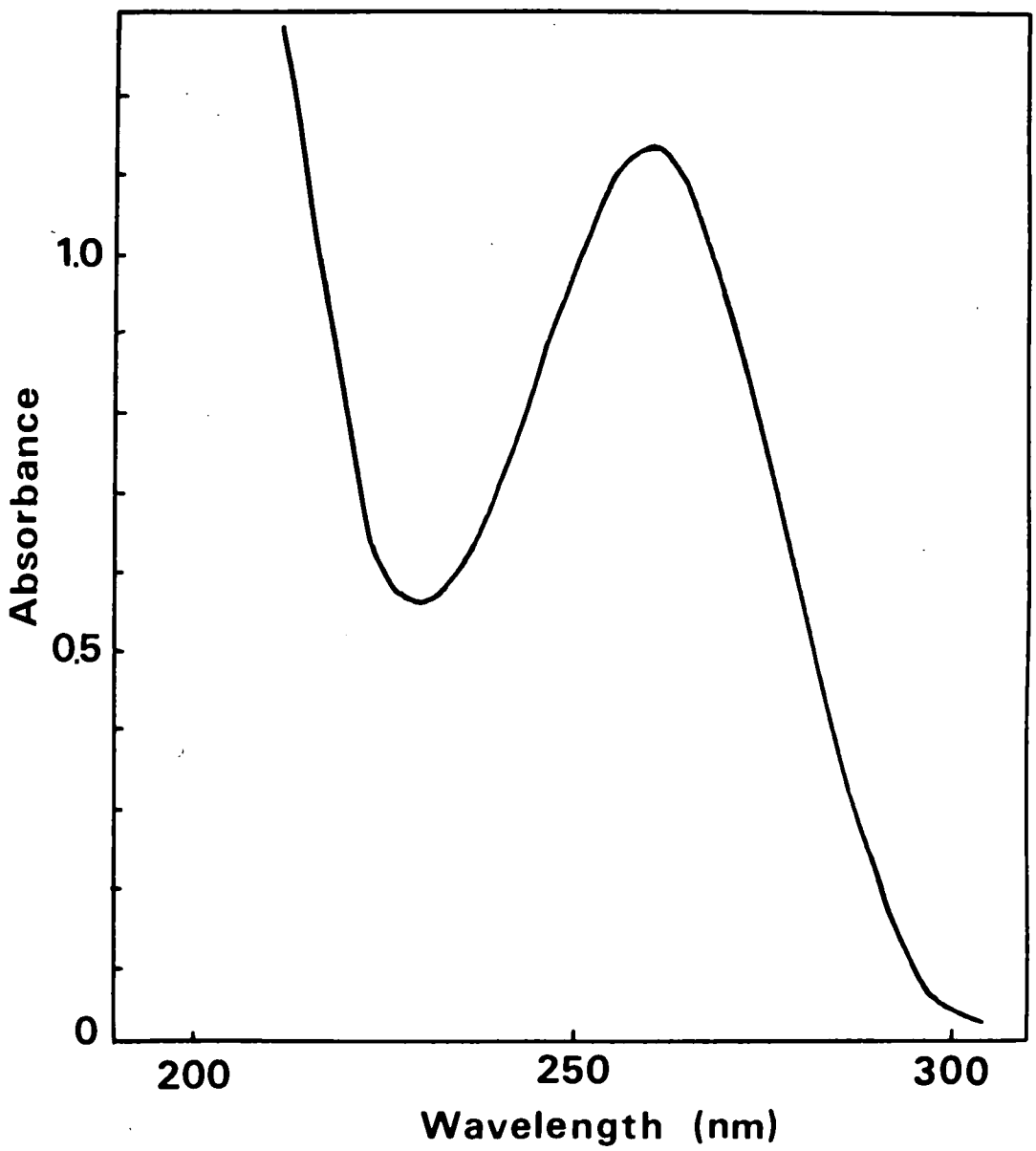
To complete the removal of the bentonite and viral protein, the supernatant was carefully taken and 0.1 ml extraction buffer and 0.1 ml 90% (v/v) ethanol added. After shaking the mixture, it was centrifuged at 1,750g for 20 min (at 5°C).

To the supernatant was added an equal volume of 90% (v/v) ethanol to precipitate the RNA. Precipitation was allowed to proceed overnight at -20°C before centrifugation and decantation of excess alcohol from the nucleic acid pellet. The pellets were stored under a small volume of alcohol at -20°C. At this temperature, as reported by Dunn and Hitchobrn (1966) the viral nucleic acid was found to be stable for at least two years. Prior to use samples were dissolved in a suitable buffer and examined spectrophotometrically (fig. 4).

Effect of Ribonuclease on the Infectivity of TYMV and TYMV RNA:

A sample of TYMV RNA prepared by method (ii) and a sample of the virus, prepared by differential centrifugation, were incubated, at the concentration at which they were to be inoculated, in 0.01 M Tris-HCl buffer, pH 7.5 with 5 µg RNase per litre, for 2h, at 20°C. The infectivities of these preparations and similar unincubated samples were compared by inoculating Chinese cabbage plants. Control and RNase-treated

Fig. 4 . Absorption spectrum of TYMV RNA



TYMV RNA prepared by the method of Dunn and Hitchborn(1966) and dissolved in 0.1 M NaCl-10^{-2} phosphate buffer, pH 7.4(20°C). The absorption spectrum of a suitable dilution was determined in water.

samples were applied to opposite halves of ten leaves.

Lesion assays were not routinely employed as they have been found not to be very successful (Matthews and Ralph, 1966). Under the conditions of growth employed, even with the nitrogen content of the growing medium limited (Diener and Jenifer, 1964), systemic infection followed the local lesions rapidly (and even when using very dilute inocula, the results were often ambiguous).

(c) Total Leaf RNA Extraction:

Total Leaf RNA was extracted from B. chinensis leaves by the method of Ralph and Bellamy (1964) (Table 4), and examined spectrophotometrically before use (fig. 18).

(d) Isolation of tRNA from Brassicachinensis var. Wong Bok:

Initially, in an attempt to aid ease of tRNA extraction, the Chinese cabbage plants were grown in the dark, under black polythene sheeting, for varying periods immediately prior to leaf harvesting. Periods of 12, 24, 36, 48 and 72 h were employed in an attempt to reduce leaf starch/polysaccharide levels.

Harvested leaves were then treated as described in Table 5 to isolate tRNA.

The nature of the tRNA was determined by showing that its ability to accept amino acids was dependent on the addition of amino acid activating enzymes, ATP and Mg^{2+} ions. Furthermore, the bond between the

Table 4

Isolation of Total Ribonucleic Acid from Brassica chinensis var. Wong Bok Leaves (Ralph and Bellamy, 1964)

1 g, fresh deribbed, uninfected six week old leaves

↓ immersed in ice-water, blotted dry

Homogenised with 1.2 ml extractant (equal parts 0.5% aqueous sodium napthalenedisulphonate solution and 90% (w/v) aqueous phenol, containing 0.1% 8-hydroxquinoline) in a cooled MSE Top-Drive Homogeniser at ¾ full speed for 10 min

↓

centrifuged at 5,000g for 5 min

- Pellet discarded
- supernatant - two vol. 95% (v/v) ethanol added

↓

White, flocculent ppt. washed once with 70% (v/v) ethanol, twice with 95% (v/v) ethanol, twice with acetone and twice with ether

↓

Powder dried over $CaCl_2$

↓

Dissolved in 0.02 M Tris-HCl buffer pH 8.1, containing 0.025 M NaCl (at 4°C). Equal volumes 2.5 M potassium phosphate buffer, pH 8.0 and 2-methoxyethanol added

↓

mixture shaken vigorously for 2 min

↓

centrifuged at 5,000g for 2 min

- Pellet discarded
- Interphase discarded
- Supernatant - equal volume of 0.2 M sodium acetate added

↓

RNA precipitated with 1% (w/v) Cetrimide solution (0.5 ml soln. per ml supernatant)

↓

chilled at 4°C for 1 h, centrifuged at 5,000g for 5 min

- Ppt. washed three times with 70% (v/v) ethanol (containing 0.1 M sodium acetate)
- Supernatant discarded

↓

centrifuged off and washed with 70 and 95% (v/v) ethanol, acetone, and ether as above

↓

Pellet stored dry at - 20°C

Table 5

Isolation of tRNA from Leaves of Six to Eight Week Old Plants of Brassica chinensis var. Wong Bok

leaves harvested into ice-cold water, washed three times, blotted dry (if stored, weighed, packed into polythene bags, stored at - 20°C)

↓

ground (dependent on wt./volume) by means of pestle and mortar or household meat grinder. Frozen leaves first allowed to thaw to room temp.

↓

1 g ground material mixed with equal volume of 90% (w/w) aqueous phenol, 0.25 ml MAGBENT and 0.25 ml 0.1 Tris-HCl buffer, pH 7.5 (5°C) (containing 3 mM $MgCl_2$ and 24 mM KCl)

↓

shaken on orbital shaker or stirred (200 rev./min for 1 h, 250 rev./min for 2 h, at room temp.)

↓

centrifuged at 2,400g for 1 h at 5°C

Pellet discarded

aqueous supernatant made 0.1 M with respect to sodium acetate, pH 5.5 (0°C) 2.5 vol. absolute ethanol added, with stirring. Stored 24 h at - 20°C.

alcoholic suspension retained, centrifuged 2,400g for 1 h

Supernatant decanted/discarded

Pellet taken up in 50% original fresh leaf wt. of 1.8 M Tris-HCl buffer, pH 8.9. Incubated, 37°C for 1 h (hydrolysis of amino acids linked to tRNA). Sodium acetate added to final concn. of 0.1 M 2.5 vol. absolute ethanol added, with stirring. Stored at - 20°C for 12 h

Supernatant discarded

↓

centrifuged at 2,400g for 1 h

Pellets taken up in 1.0 M NaCl (50% original fresh leaf wt.) Shaken 30 min at 0°C.

Supernatant discarded

↓

centrifuged off undissolved material re-extracted pellets with 1.0 M NaCl (25% original fresh leaf wt.) - re-centrifuged

Pellets discarded

Combined supernatants precipitated with acetate/ethanol

↓

centrifuged at 7,000g

Pellets dissolved in min. 0.05 M Tris-HCl buffer, pH 7.5

Supernatant discarded

↓

DE 52 chromatography

amino acid and the RNA was not resistant to hot trichloroacetic acid treatment.

(e) Isolation of tRNA from Developing Seeds of Vicia faba:

The pods were harvested as described above (page 52), and the intact seeds collected.

The seeds were blended in an Ato-Mix blender, for 2 min, in a mixture of 1 part 0.003 M $MgCl_2$ - 0.024 M KCl - 0.1 M Tris-HCl, pH 7.5 at 20°C: 2 parts of 90 (w/w) aqueous phenol (employing 1.5 ml extractant per g seeds).

The homogenate was shaken for 2 h at room temp. on an orbital shaker (250 rev/min), and the mass then centrifuged at 2,400g for 1 h. The aqueous supernatant was strained through four layers of sterile gauze and adjusted to 0.1 M sodium acetate, pH 5.5. Absolute ethanol (2.5 vol.) was then added, to precipitate the nucleic acid, precipitation being allowed to proceed for 24 h at - 20°C. The procedure outlined in Table 5 for the extraction of tRNA from Brassica chinensis leaves was followed from this stage, until the final acetate/ethanol precipitation. The pellet obtained by centrifuging at 7,000g was drained of alcohol, any alcohol remaining being removed under vacuum. The dry pellet was dissolved in a suitable solvent and the solution stored at - 20°C or - 70°C. The yield of tRNA was 2-3 mg per g of seed.

Unchromatographed V. faba tRNA from 40 day-old beans was routinely used in incorporation experiments, samples being assayed spectrophotometrically

before use.

(f) Ion-Exchange Column Chromatrography of Transfer Nucleic Acid:

(i) The chromatography column for tRNA purification was prepared as described in Table 6.

(ii) Chromatography of Brassica chinensis Leaf tRNA:

Chromatography was carried out in a cold-room maintained at $4^{o}C$. The column was connected to an Isco model 220 ultraviolet liquid flow analyser, which was attached to a Servoscribe potentiometric recorder (type RE 511.20), set to run at 30 mm/h (at a calibration of 10 mV).

The sample was applied carefully to the column, and allowed to run into the column at its own rate. If necessary, the tRNA sample was centrifuged prior to column application. Extraneous material was eluted with 0.05 Tris-HCl buffer, pH 7.5 ($0^{o}C$) causing an increase in the 254 nm absorbancy of the eluate. On the return of the base-line to its original position the Tris-HCl buffer was replaced by 0.2 M NaCl - 0.05 M Tris-HCl.

After collecting the eluate containing 254 nm absorbing material, and on return of the base-line, the tRNA was eluted from the column by 1.0 M Nacl - 0.05 M Tris-HCl buffer, pH 7.5 ($0^{o}C$) (fig. 5).

The eluates from each stage were adjusted to 0.1 M with respect to sodium acetate, pH 5.5 and 2.5 volumes of absolute ethanol were added to each fraction, with constant stirring. Precipitation was allowed to proceed for 12 h at - $20^{o}C$, after which time the tRNA and other

Table 6

Preparation of the Chromatography Column

30 g Whatman's microgranular-form diethylaminoethyl cellulose 52 (DE 52) stirred rapidly into 180 ml 0.05 M Tris-HCl buffer, pH 7.5. pH adjusted to 7.5 by the addition of 5 M HCl. All operations at room temp.

left for 10 min

Sediment — supernatant/fines discarded

Tris-HCl buffer added to volume, slurry rapidly stirred and pH re-adjusted to 7.5

left for 5 min

procedure repeated

resultant slurry stirred vigorously, then allowed to stand for 20 min

slurry — supernatant discarded

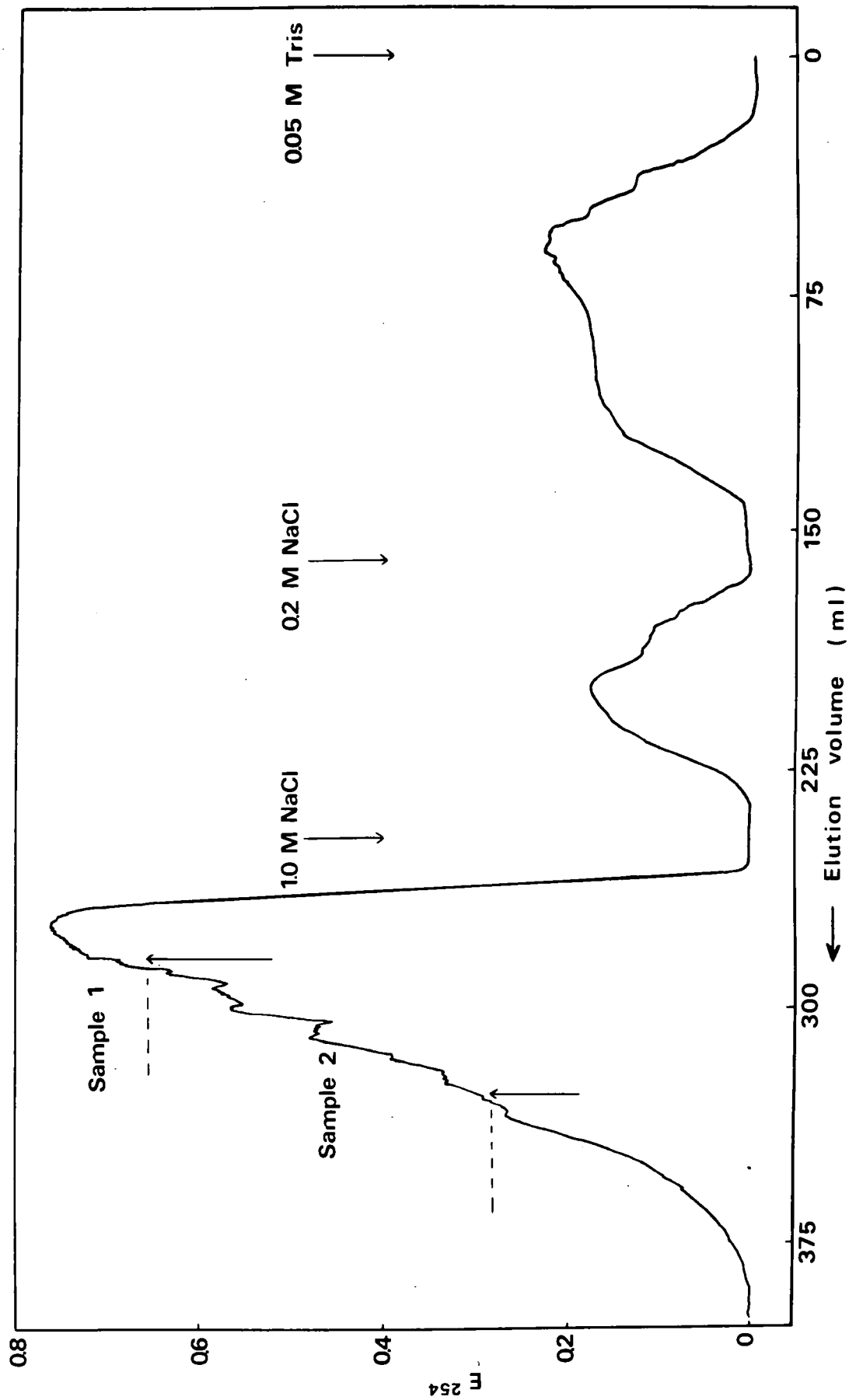
a half-volume of 0.5 M Tris-HCl buffer added, the mixture well shaken and poured immediately into a chromatography column (internal diameter 1.5 cm, length 30 cm)

DE 52 allowed to settle for 1 h, excess Tris buffer flowing out and compacting the column

fresh buffer added to top of column, stored at 2^{o}C. (Compacted column 12.5 cm in length)

Fig. 5 Servoscribe trace of chromatography of Brassica chinensis tRNA on DE 52 cellulose

Elution as described on page 82.



fractions were collected by centrifugation at 4,000g for 30 min.

The ethanol was decanted, traces of ethanol being removed from the centrifuge walls be means of paper towelling. Any alcohol remaining was removed under vacuum.

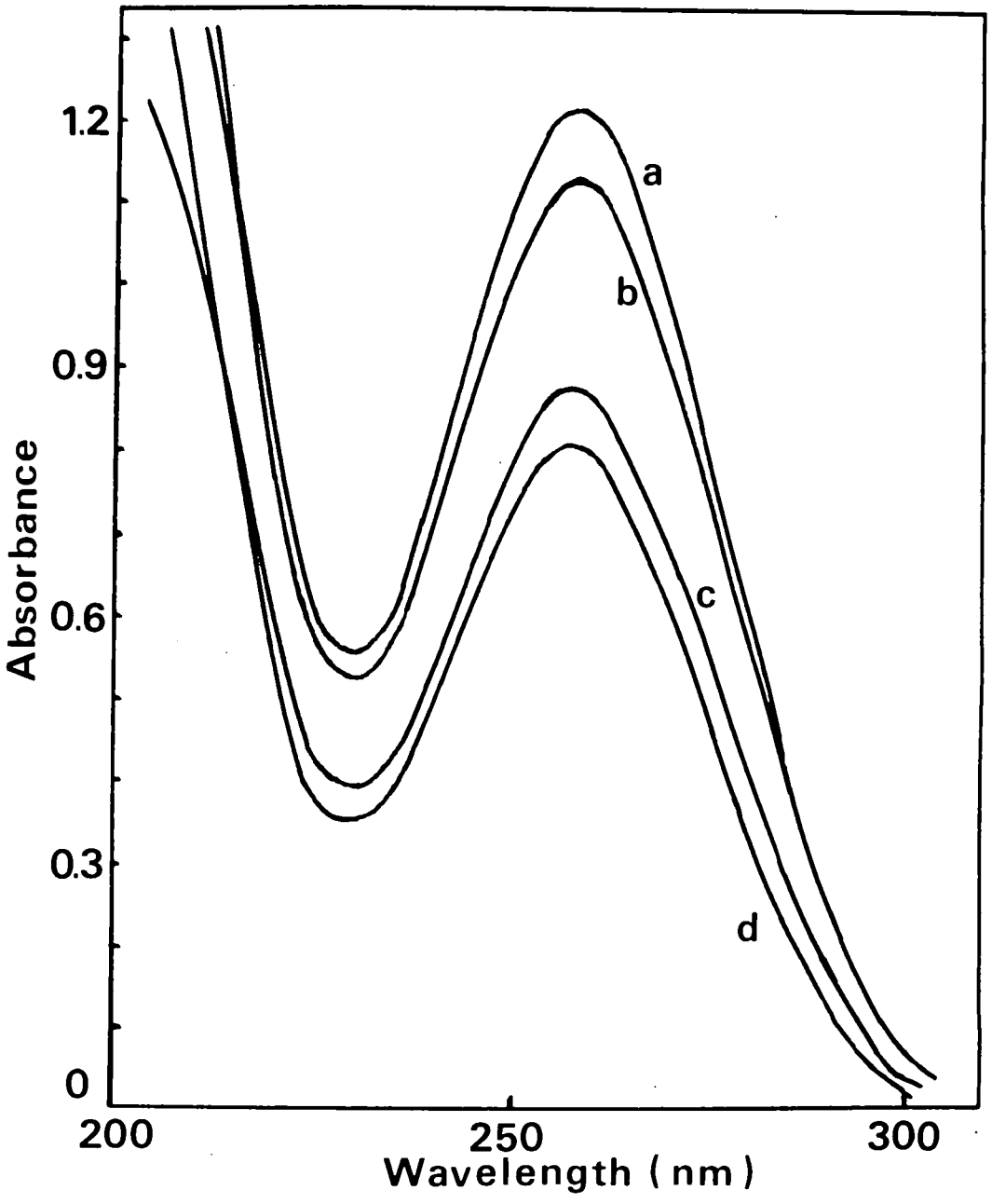
Chromatography produced a more homogenous product which dissolved readily in the minimum of 0.01 M sodium acetate, pH 5.5 ($0^{o}C$). After spectrophotometric assay (fig. 6) 0.25 ml volumes were stored at - $70^{o}C$. Storage over two years at this temperature did not affect acylation etc., nor did freezing and thawing.

Initial attempts to employ EDTA buffers (as suggested by Dunn, 1969p) were found difficult in that such buffers possess an inherent high uv absorbance at 254 nm. This necessitated extra column washings to 'normalise' the base-line between each elution stage. Complete removal of unwanted material at each elution stage was therefore difficult to ascertain.

(g) Isolation of Microsomal Particles:

(i) Microsomal particles were obtained by a number of experimental procedures, but only the principle methods are outlined here. Brief details of minor variations are given in Table 7 , and the basic method in Table 8 . All preparations were examined spectrophotometrically and their ability to incorporate amino acids into hot trichloroacetic acid stable material determined.

Fig. 6 . tRNA absorption spectra



Absorption spectra of suitable dilutions of tRNA samples(dissolved in 0.1 M NaCl-phosphate buffer) in water.

a. B.chinensis tRNA sample 1(see fig. 5).
b. B.chinensis tRNA sample 2(see fig. 5).
c. Yeast tRNA.
d. V.faba tRNA(see page 81).

Table 7

Brief Outline of Methods Employed for Microsome Isolation

Number	Grinding Method	Centrifugation
1	Pestle/mortar (± sand)	1000g x 10 min; 4000g x 7 min; 23000g x 20 min; 105000g x 2 h
2	"	1000g x 10 min; 20000g x 20 min; 35000g x 30 min; 105000g x 2 h
3	Razor chopped	"
4	Pestle/mortar (± liquid N_2)	4000g x 5 min; 22500g x 15 min; 35000g x 20 min; 105000g x 2 h
5	Pestle/mortar	1000g x 10 min; sample layered over 1 ml 0.5 M and 3 ml 1.6 M sucrose and 20000g x 15 min; 105000g x 2 h
6	Pestle/mortar	25000g x 5 min; 38000g x 10 min; 105000g x 2.5 h

Clarification: 23000 - 30000 g x 10-15 min

Usual Procedures: Methods 2 and 'quick' method 6

Table 8

Isolation of Microsomes from One to Two-week Old *Brassica chinensis* var. Wong Bok Leaves

leaves harvested into ice-cold water, blotted dry, deribbed.
1 g leaf tissue ground with 2-3 ml extractant (0.45 M sucrose - 0.01 M magnesium acetate - 0.01 M KCl - 0.05 M Tris-HCl buffer, pH 7.5 at 0°C - 5 mM mercaptoethanol) gently for 2-3 min, in a pre-cooled mortar

↓

cell mass pressed through four layers of sterile gauze, and centrifuged at 1,000g for 10 min

- pellet discarded
- supernatant re-centrifuged at 20,000g for 20 min
  - pellet discarded
  - post-mitochondrial supernatant re-centrifuged at 35,000g x 30 min
    - pellet discarded
    - final supernatant centrifuged at 105,000g x 2.5 h
      - excess extractant drained from pellets
        - surface of pellets washed with 0.5 ml volumes of 0.005 M magnesium acetate - 0.01 M KCl - 0.01 M Tris-HCl buffer, pH 7.5 (at 0°C) - 5 mM mercaptoethanol x five
          - pellets resuspended in above medium
            - suspension clarified by centrifugation at 20,000g x 10-15 min
              - pellets discarded
              - suspension assayed spectrophotometrically by reading absorbancy of a water diluted sample against an appropriate blank, or by Orcinol RNA determination
                - isolates used immediately or stored at - 70°C in small aliquots
          - washings stored at - 20°C
      - high-speed supernatant decanted, stored at - 20°C
        - dialysed for use as 'soluble' or enzyme fraction in *in vitro* amino acid incorporation experiments

Tata and Williams-Ashman (1967) observed that the optimal concentration of homogenate for studies on amino acid incorporation in vitro by microsomes was 2.5 ml/g of rat liver. This volume was found to produce a suitable homogenate, which was easily 'worked-up' and corresponds to the volume of extractant found suitable and used in the present work.

The yield of microsomes depended upon the age of the leaves but was fairly constant for any particular leaf 'age'. Usually a concentration of 0.5 mg microsomes/g fresh weight of leaf material was obtained.

(ii) Isolation of Microsomes from Brassica chinensis var. Wong Bok Plumules:

The seedlings were germinated as described above (page 50), and any testas remaining removed.

The small seedlings were collected into liquid nitrogen cooled, pre-weighed tubes. A 1:1 ratio of extractant to seedlings was used (with a small quantity of acid-washed sand). Method 4, as outlined in Table 7 , was then followed.

(iii) Isolation of Microsomes from Brassica chinensis var. Wong Bok Seeds:

The seeds were collected as described above (page 50), and microsomes were extracted by the method described for plumule extraction.

(iv) Isolation of Microsomes from Brassica chinensis var. Wong Bok Leaves by a Rapid Extraction Procedure:

Leaves were harvested rapidly and packed between layers of crushed

ice. The leaves were quickly blotted dry and weighed into 10 g quantities.

The procedure outlined above for normal leaf extraction was then employed, using the centrifugation conditions listed under Method 6 in Table 7 . Ribosomes so prepared were used immediately for experimental purposes.

(v) Isolation of Microsomes from the Cotyledons of Developing Seeds of Vicia faba:

Pods were harvested as outlined on page 52 and the seeds extracted and the testas removed with the aid of sterile scalpel and forceps.

One vol. microsomal extractant to 1 g of seeds was used, together with a little acid-washed sand. Grinding was accomplished either using a pestle and mortar or an Ato-Mix blender, depending on material volume.

Extraction procedure was basically as outlined for normal B. chinensis leaf extraction (Table 8) except that at the first grinding, after the low-speed centrifugation, the pellet resulting was re-extracted with a fresh volume of extractant (equal to half the original volume of extractant). The final microsomal suspension was stored, in small volumes at - 70°C.

A single batch of (60-65 day) bean microsomes was used, together with the dialysed enzyme, for the majority of in vitro experiments involving bean material. These isolates maintained their activity for over 7 months. Even after freezing and thawing, their activity was not greatly reduced (cf. cabbage microsomes where the activity varied greatly

after prolonged storage at - 70°C).

(vi) Isolation of Microsomes from the Plumules of Germinating Seeds of Vicia faba:

The plumules were carefully excised and ground under liquid nitrogen, to a fine powder. The powder was then homogenised in a glass hand-homogeniser, with one vol. extractant. The ground material was treated as described in Table 8 , and the final microsomal suspension stored at - 70°C in small volumes.

(h) Preparation of Enzyme for Incorporation Studies:

The soluble or enzyme fractions used in incorporation experiments consisted of the final high-speed supernatants derived from the last stage in microsome isolation.

The enzyme was used as such, or was first dialysed against extractant buffer (minus sucrose) for 5 h, during which time the dialysis buffer solution was constantly stirred and replaced hourly. Dialysis was performed to remove sucrose and contaminating endogenous amino acids (Allende, 1969).

A fine flocculent precipitate occasionally formed during dialysis and this was removed before storage of the enzyme at - 70°C.

Samples of enzyme stored at - 20°C were comparable with - 70°C stored samples with regard to their enzymatic activity. No attempt was made to concentrate the amino acid activating activity of the enzyme fractions.

Liquid Scintillation Counting:

(a) The 2 σ counting error, which represents the 95% confidence limit, was determined automatically for every sample. This value is related to the activity of the sample and to the length of time of counting, decreasing rapidly with increase in counting time. A standard counting time of 50 min was usually employed, thus allowing even low activity samples to be counted with an error not greater than 5%. Background radiation varied between 50 to 100 c.p.m. and agreement between filter paper discs bearing similar samples was found to be reasonably constant.

The liquid scintillation counter employed (Beckman, Model LS- 200B) had automatic external ratio standardisation, and this facility was employed to indicate the efficiency of the counting procedure throughout the experimental work. On repeat counts the efficiency was found to vary little over a 24 h period of counting.

The counting efficiency obtained under standard experimental conditions was determined using a standard filter paper disc impregnated with a standard sample of $[^{14}C]$ phenylalanine, and put through the standard washing procedure for discs. The efficiency of counting was determined by employment of the external ratio standardisation, and variation of the volume of scintillation fluid in the counting vial. It is reported that for solid supports not releasing material into the scintillation fluid, variation in volume gives an indication of counting efficiency, (Mans and Novelli, 1961). The efficiency of the test disc counting increased with increasing scintillation fluid volume up to a volume of 8 ml, above which apparent efficiency was not linearly

dependent on volume. Variable results were obtained although a high degree of efficiency was indicated. A standard 8 ml volume of scintillation fluid for each counting vial was therefore employed routinely.

Under normal experimental conditions counting efficiency was 70% and variation in sample volume from 0.05 to 0.1 ml did not significantly alter counting results.

(b) Results of $[^{14}C]$ Amino Acid Incorporation:

Results of experiments using single amino acids are expressed as pmole $[^{14}C]$ amino acid incorporated/mg microsome or per mg microsomal RNA.

Results using $[^{14}C]$ mixture are expressed as c.p.m./mg microsomes or per mg microsomal RNA.

Results for tRNA charging are expressed as c.p.m./mg tRNA.

Determination of the Aminoacyl Acceptor Activity of tRNA and the Aminoacyl Synthetase Activity of the Enzyme Preparations:

All incubations were performed using small, rimless, glass test-tubes (Pyrex 7.5 cm. x 0.7 cm. internal dimensions) which were capped throughout the preparative and incubation stages (to reduce dust and air-borne microbial contamination to a minimum). The incubation mixes were prepared and maintained at $0^{o}C$ until the addition of the enzyme. They were then transferred, after mixing of the tube contents with the

aid of a vibratory mixer ('buzzing'), to a constant-temperature water-bath maintained at the temperature of the experiment. During the course of the experiment the tubes were agitated manually, at intervals, to ensure adequate mixing. On removal of the tubes prior to sampling, they were again 'buzzed'.

The formation of aminoacyl-tRNA and the aminoacyl synthetase activity of the enzyme fractions were determined using an incubation system containing a radioactively labelled amino acid.

The typical incubation medium contained (per ml) 100 μmoles of Tris-HCl buffer, pH 7.8, 17.5 μmoles magnesium acetate, 20 μmoles reduced glutathione, 2 μmoles ATP, 0.02 μmoles of the amino acid to be tested (and $[^{14}C]$ labelled), 1.0 mg tRNA and a variable volume of enzyme (dependent on the volume required to provide maximum acceptor activity, or 'charging', of the tRNA under the conditions of the experiment).

Incubations were normally performed at 25^{o}C, and at the selected sampling times, 0.05 or 0.1 ml samples were removed from the 0.5 ml incubation mixture and pipetted onto Whatman 3MM filter-paper discs of diameter 24 mm. The discs were then dried for 5 s (Noll, 1969) in a stream of warm air (to improve reproductibility) before being washed and counted as described below (Table 9).

In Vitro Amino Acid Incorporating Systems:

The incubation mixes were prepared and maintained at 0^{o}C until the microsome particles were added. They were then transferred to the constant-temperature water-bath as outlined above.

The complete incorporation mixture contained in the typical 0.5 ml incubation, 25 μmoles Tris-HCl buffer, pH 7.8 at 25^{o}C, 3 μmoles magnesium acetate, 35 μmoles KCl, 5 μmoles reduced glutathione, 0.1 μmoles GTP, 2 μmoles ATP, 5 μmoles creatine phosphate, 10 μg phosphocreatine kinase, 0.2 mg tRNA, 0.5 mg microsomes (as determined by absorption spectrophotometry) enzyme, the volume being that giving maximum activity as determined by assay (in this incorporating system), and 100 μg messenger (poly(U) or TYMV RNA). The radioactive tracer used was either a single $\left[^{14}C\right]$ amino acid (0.02 μmoles/ml) or a $\left[^{14}C\right]$ amino acid mixture (0.01 ml) and dependent upon which a suitable supplement of the remaining $\left[^{12}C\right]$ amino acids, was added.

The method of determination of the incorporation of $\left[^{14}C\right]$ amino acids into peptidyl material is based on the method of Mans and Novelli (1960, 1961) samples of the incubation mixture being pipetted onto Whatman 3MM filter-paper discs (diameter 24 mm) and dried prior to immersion in ice-cold 10% w/v trichloroacetic acid to terminate the reaction.

The discs were prepared for scintillation counting as described by Mans and Novelli (1961) washing being performed as described in Table 9.

Assays were conducted in triplicate, as were samplings, and each experiment included control tubes which were treated in an identical manner except that the $\left[^{14}C\right]$ amino acid was absent, or was not added until the end of the incubation period (to measure 'noise' and other background interference).

The prepared discs, from the incorporation or charging experiments, were placed carefully in scintillation vials containing 8 ml of scintillation fluid. The radioactivity present was determined using the Beckman Liquid Scintillation Spectrometer with automatic internal standard setting. Sample counting time was varied to suit the activity present in the discs, so as to obtain statistically acceptable results.

Preparation of Filter-Paper Discs for Radioassay:

The washing procedure adopted may be conveniently described in tabular form (Table 9)

Polyacrylamide Gel Electrophoresis:

Polyacrylamide gel electrophoresis (PAGE) was carried out using Perspex tubes of internal diameter 7 mm and length 115 mm, as gel supports, in a two-compartment electrophoresis chamber. Platinum electrodes were used in each buffer compartment.

Acrylamide stock solution, undiluted electrophoretic buffer ('E' buffer) and water were mixed to give the desired gel concentration, dissolved air being removed rapidly by vacuum.

In order, 25 μl of N, N, N', N', tetramethyl-ethylenediamine (Temed) and 0.25 ml 10% (w/v) ammonium persulphate (freshly prepared) were added. Mixing was accomplished by swirling to avoid aeration. The gels were allowed to set for at least 30 min and then used immediately (as storage was not found to be practicable).

Table 9

Preparation of Samples for Radioassay

	tRNA/Enzyme Assay Discs		Amino Acid Incorporation Discs	
1.	60 min	10% TCA (10 ml/disc)	$0^{\circ}C$	A.
2.		Decant and wash with 5% TCA		B.
3.	15 min	5% TCA (10 ml/disc)	$20^{\circ}C$	C.
4.		Decant and wash with 5% TCA		D.
		30 min 5% TCA (10 ml/disc)	$90^{\circ}C$	E.
		Decant, rinse twice with 5% TCA		F.
5.		Rinse with Ether - Ethanol (1 : 1)		G.
6.	30 min	Ether - Ethanol (1 : 1) (5 ml/disc)	$37^{\circ}C$	H.
7.	Decant, wash with Ether - Ethanol (1 : 1) and then with Ether			I.
8.	15 min	Ether (5 ml/disc)	$20^{\circ}C$	J.
9.		Rinse with Ether		K.
10.	5 min	Ether (5 ml/disc)	$20^{\circ}C$	L.

Air dry and put in 8 ml scintillation fluid, in vials showing little residual radiation (i.e. less than 50 c.p.m.)

TCA - trichloracetic acid

Electrophoresis was carried out at room temperature, using a five times dilution of the stock 'E' buffer in the buffer compartments (the buffer containing 2g/l sodium lauryl sulphate). Pre-electrophoresis was carried out for 60 min (to remove any ultra-violet absorbing materials from the gels and to introduce the nuclease inhibitor, sodium lauryl sulphate into the gels) at 5 mA per tube and at 8 volts per cm of gel length.

RNA samples, dissolved in diluted 'E' buffer containing sodium lauryl sulphate and 6% (w/v) sucrose, were layered onto the gel surface at 'zero time', with no current passing, (25 µg of RNA sample/gel).

Electrophoresis was continued for from two to three hours, according to gel length and the resolu tion desired. After electrophoresis, the gels were carefully removed from their supports and soaked in 'E' buffer for 60 min to aid in lowering background absorption (Loening, 1968, 1969).

The gels were scanned in a parallel sided quartz container, in a Joyce-Loebl Chromoscan Mark II (a double beam recording and integrating densitometer) at 265 nm, using a light slit 50-150 wide by 1 to 2 mm.

It has been shown that the mobility of the low molecular weight RNA in PAGE is inversely related to the sedimentation coefficient (Richards et al., 1965) and also for ribosomal RNA (Loening and Ingle, 1967). From this it follows that the relative mobility should be inversely related to the log molecular weight. This was found to be the case for a range of viral RNA species (Bishop et al., 1967).

The distances moved by the RNA species studied (or their mobilities) were therefore expressed graphically to determine molecular weights and sedimentation coefficients.

Analytical Ultracentrifugal Analysis:

Analytical ultracentrifugal analysis was carried out, employing an Omega II Preparative Ultracentrifuge with Analytical Attachment, standard Analytical rotor and standard cell (See Appendix), using Schlieren optics. A small number of determinations, for comparison purposes were carried out employing the Beckman Spinco E Analytical Ultracentrifuge (Pain, 1970), using Schlieren and ultra-violet optical systems. Where possible samples were run in suitable buffers containing 100 mM KCl to aid in suppressing possible charge effects (Möller et al., 1969).

With the moving boundary method, sedimentation coefficients are generally evaluated from the equation (iii).

$$\underline{s} = \frac{dx/dt}{\omega^2 x} \qquad \text{Equation (iii)}$$

where, $\omega^2 x$ is the centrifugal field and dx/dt is the rate of change of particle distance from the centre of the rotor with respect to time.

This approach to the sedimentation coefficient is based on the equation defined by Svedberg (1940). The sedimentation coefficient, $\underline{s}$, is thus a measurement of the transport of the material across the cell under the influence of a centrifugal field (and has units of cm/sec/dyne/g

or sec). Sedimentation coefficients are often reported in Svedbergs(S) where one Svedberg is equal to 10^{-13} sec.

Equation (iii) may be written

$$\underline{s} = \frac{d(\ln x)}{dt} \cdot \frac{1}{\omega^2} \qquad \text{Equation (iv)}$$

i.e., $$\underline{s} = \frac{2.303}{\omega^2 . 60} \cdot \frac{d(\log x)}{dt} \qquad \text{Equation (v)}$$

Equation (v) holds true only if there is no increase in temperature during the run. From this equation the sedimentation coefficient of the macromolecule under investigation was determined. Determinations were made by plotting 'log x' against time 't' (in minutes) for all values for one experiment. The slope of the graph was determined and substituted in equation (v) , to determine $\underline{s}$.

The $\underline{s}$ as determined, should be corrected to standard conditions, i.e., $\underline{s}_{20,w}$, to a medium of specific gravity 0.9982 and viscosity 1,0050 centipoise (i.e., water at $20^{o}C$).

This correction can be made in two stages as a convenient and adequate approximation (Markham, 1967). The $\underline{s}$ value is multiplied by the relative viscosity of water at the temperature of the run. In dilute salt solutions this correction is often adequate by itself. Secondly, the $\underline{s}$ is multiplied by the relative viscosity of the buffer solution used and by the factor

$$\frac{(1 - 0.998\,\bar{V})}{(1 - V\rho\, t^{o})}$$

where, $\bar{V}$ is the specific volume of the material under investigation and ρt the density of the solvent at temperature, t^{o}. The latter may usually be taken as the density of the buffer at $20^{o}C$ multiplied by the density of water at $t^{o}C$. The last factor usually involves a very small or negligible correction if aqueous solvents are used. If one uses 0.1 M NaCl buffered if necessary with 0.01 M buffer, at a temperature of $20^{o}C$, the overall correction for solvent viscosity and density with a nucleoprotein virus is of the order of x 1.015 or less, and so may usually be neglected.

In the text therefore, sedimentation coefficient values are given as determined graphically and corrected according to the following equation (as suggested by Pain, 1970).

$$s_{20^{o}} = s_{t^{o}.\ buffer} \times (\eta \text{ rel.buffer } 20^{o}C) \times \left(\frac{\eta\ t^{o}}{\eta\ 20}\right) H_2O$$

where, η is the viscosity of the buffer or water at the stated temperature ($20^{o}C$ or t^{o}). This equation does not take into account interparticulate interaction or the effect of concentration on the sedimentation coefficient.

Measurements were made from photographic prints or from the actual negatives held flat in an enlarger carrier. From the idea of Markham, (1967) a template was devised and drawn on graph paper, at a times ten magnification. The negative was projected onto this template and the negative reference points lined up with those on the template. Relatively easy reading off of peak movement distances was thus facilitated.

RESULTS

1. Biological Materials:

The plants grown as Brassica chinensis var. Wong Bok bore a close resemblance to the description of B. chinensis given by Bailey (1961). The seed used was found to be almost 100% pure and to have an 85-90% germination rate.

Similar good germination was obtained with Vicia faba seeds grown both under greenhouse and field conditions.

Typical symptoms resulted from inoculating Chinese cabbage plants with TYMV. Occasionally uninfected plants were seen which exhibited some of the symptoms of infected plants. Back-inoculations from such specimens did not result, however, in infected test plants, suggesting a genetic or nutritional factor. Seed from infected plants did not produce infected plants on germination.

Local chlorotic lesions sometimes developed from points of infection following mechanical inoculation of younger plants, followed by outstanding mottling (because of which Markham and Smith (1949) gave the name turnip yellow mosaic to the virus). About 8 to 10 days after inoculation, marked yellowing and discolouration of the younger leaves resulted, with gradual even vein yellowing in the older leaves. In the final stages of infection the mosaic was very pronounced, resembling a variegation of intense yellow-green or cream patches, intermingled with dark green areas (figs. 7, 8, 9).

Fig. 7 Uninfected and TYMV infected Brassica chinensis plants to show marked mosaic symptoms

1. B. chinensis var. Wong Bok infected with 'New Zealand' sample of TYMV. Plant on right uninfected.

2. B. chinensis var. Chihli infected with 'New Zealand' sample of TYMV. Plant on right uninfected.

3. B. chinensis var. Pte Sai infected with 'New Zealand' sample (front left) and 'French' sample (front right) of TYMV. Plant in middle uninfected.

1.

2.

3.

Samples taken from each of these coloured areas produced similar coloured infections in new plants. Sub-culturing, however eventually resulted in general mixed colourations returning.

The French isolate produced more rapid leaf discolouration and was more necrotic than the other isolates, usually killing the young infected plants. Plants infected by the other samples were very rarely killed and often flowered and set seed if allowed to do so. The white 'break' in the yellow flowers (as noted by Markham and Smith, 1949) was occasionally observed.

Chinese cabbage plants inoculated with TYMV RNA exhibited exactly the same symptoms as produced by the intact virus, although the infection time varied from 8 to 15 days.

The infection exhibited similar symptoms in all three varieties of Chinese cabbage, although the pale green leaves of the Pte Sai variety were always yellower. Uninfected plants of the Chihli and Wong Bok varieties were a darker green in colour, and so the yellowing was less marked in infected test plants.

Control plants (similarly treated to test plants but without infective agent) never exhibited any symptoms resembling infection by TYMV.

Initial experiments using the Markham strain of TYMV in an attempt to inoculate V. faba plants were apparently unsuccessful in that no symptoms of infection were noted in the bean. Chinese cabbage

plants inoculated with the same inocula at the same time showed typical symptoms after 9 days.

Initial experiments using the New Zealand strain were more successful. Various methods of inoculation were attempted including carborundum aided leaf abrasion, dipping of damaged leaf edges into viral suspensions and injection of suspensions into leaf veins or the main plant stem. Infection was not readily apparent in the bean test plants although readily apparent in the Chinese cabbage controls.

The white flowers of the inoculated beans aborted before development, however, to a greater extent than in the control bean plants. Fourteen days after 'carborundum abrasion' inoculation, a slight red mottling on the surfaces of the inoculated bean leaves became apparent, especially at the sites of inoculation. Re-infection of healthy Chinese cabbage plants with leaf macerates of the red mottled areas was attempted. In three cases out of three, typical TYMV infection symptoms were produced (but only 15 to 21 days after inoculation of the cabbages). Re-infection attempts using macerates of non-mottled bean leaves were only partially successful in that only one test cabbage plant in three showed symptoms of infection. Attempted isolation of virus from the mottled bean leaves proved unsuccessful.

No unusual symptoms were noted in the bean plants that had been leaf-dipped or injected with TYMV suspensions.

The results of a final series of experiments to implicate TYMV as an agent of infection in the broad bean are presented in Table 10. (the New Zealand sample being used in this and subsequent inoculations).

Fig. 8 Samples of Brassica chinensis and Vicia faba leaves to show variation in mosaic symptoms of TYMV infection

1. Various ages of Brassica chinensis var Wong Bok leaves infected with TYMV ('New Zealand' sample). Leaf at bottom right uninfected.

   Two V. faba leaves (top right), leaf on right uninfected and leaf on left 'infected' with TYMV.

2. Old, tall flower stem leaves of Brassica chinensis var Wong Bok showing marked 'colour-breaking'.

1.

2.

Fig. 9 Brassica chinensis leaves and Vicia faba leaves and plant infected with TYMV

1. B. chinensis older flower stalk leaves exhibiting blooming (also seen on younger leaves).

2. V. faba plant on right inoculated by leaf abrasion with TYMV. Plant on left uninfected.

1.

2.

Table 10

Results of inoculating Vicia faba with TYMV and Brassica chinensis with V. faba isolates

	Inoculum Source	Back-inoculation Result
A.		
1.	Inoculated *V. faba* leaf	NVS No TYMV found on examining under E.M.
2.	Leaves immediately above inoculated *V. faba* leaf	NVS
3.	Leaves immediately below inoculated *V. faba* leaf	NVS
4.	Leaves at apex of inoculated *V. faba* plant	NVS
5.	Infected *B. chinensis* and uninfected *V. faba* leaf	Infection after 11 days
6.	Uninfected *V. faba* leaf	NVS
7.	Infected *B. chinensis*	Infection after 10 days
8.	Leaves immediately above inoculated *V. faba* leaf + uninfected *B. chinensis*	Symptoms of infection noted after 15 days
9.	Uninfected *B. chinensis*	NVS

NVS - No visible symptoms of infection.

Examination and sampling (back-inoculation) after 21 days.

Three test plants employed for each inoculation and back-inoculation.

Table 11

Results of inoculating Vicia faba with TYMV and Brassica chinensis with V. faba isolates

B.

	Inoculum Source	Back-inoculation Result
1.	Inoculated V. faba leaf	NVS
2.	Inoculated V. faba leaf + uninfected B. chinensis	Grey 'blooming' of central rosette. Plant unhealthy.
3.	Leaves from above inoculated V. faba leaf	NVS
4.	Leaves from above inoculated V. faba leaf + B. chinensis	'Blooming' greater and unhealthier than B.2
5.	Uninfected B. chinensis	NVS
6.	Infected B. chinensis	10 days after inoculation 2 plants showing typical infection symptoms., 1 plant exhibiting 'blooming' and slight lesioning

NVS - No visible symptoms of infection.

Examination and sampling (back-inoculation) after 21 days.

Three test plants employed for each inoculation and back-inoculation.

The bean plants were inoculated by leaf abrasion with a suspension of TYMV, 21 days prior to sampling. Inoculated bean plants did not exhibit any noticeable symptoms of infection at the time of sampling.

To confirm the results from the above series (Table 10) and to ensure that infection of the inoculated Chinese cabbage plants was not due to contamination the experiment was repeated (Table 11).

To compare the effect, a number of bean plants were inoculated with TYMV RNA (Table 12). Fourteen days after inoculation, (and subsequently after 21 days) the bean plants were examined (Table 13). In general RNA inoculated bean plants grew taller and spindlier than virus inoculated specimens. Samples inoculated from the beans, 28 days after the initial inoculations exhibited only mild discolouration of Chinese cabbage test plants. "Abortion" of the bean flowers ceased after this time and subsequent flowering was normal, the seed pods showing no abnormal symptoms.

Using the bean plants examined in Tables 12, 13, and inoculating fresh Chinese cabbage plants, the day after the first series of inoculations were performed, similar results to Tables 12, 13 were obtained (Table 14).

Radish plants are reported to be a host for TYMV (Markham and Smith,

Table 12

Results of Inoculating Vicia faba with TYMV and TYMV RNA

	Inoculum	Host	Result
1.	TYMV	B. chinensis	Symptoms after 12 days
2.	TYMV	V. faba	No obvious symptoms, slightly etiolated.
3.	TYMV RNA	B. chinensis	Symptoms after 13 days
4.	TYMV RNA	V. faba	As for 2
5.	TYMV & bean leaf (uninfected)	B. chinensis	Typical symptoms
6.	TYMV RNA & bean leaf (uninfected)	B. chinensis	Typical symptoms, not so pronounced as 3

Three test plants used for each inoculum source.

Table 13

Results of Back-inoculating Brassica chinensis from Vicia faba Isolates

	Inoculum Source	Back-Inoculation Result
1.	Virus inoculated V.faba leaf	NVS
2.	Virus inoculated V.faba leaf + uninfected B. chinensis	Blooming of leaves; non-inoculated plant in same pot NVS.
3.	Virus/leaf above inoculated V. faba leaf	Slight mottling of leaves
4.	Uninfected B. chinensis + 3	Uncertain
5.	Uninfected V. faba and B. chinensis leaves	NVS
6.	Uninfected V. faba leaf	NVS
7.	Uninfected B. chinensis leaf	NVS
8.	Virus infected B. chinensis	Typical symptoms after 8 days
9.	RNA infected B. chinensis	Typical symptoms after 10 days
10.	RNA inoculated V. faba above inoculated point	Very slight blooming/mottling
11.	Uninfected B. chinensis + 10	Blooming on central rosette
12.	Virus inoculated V. faba + radish leaf	Blooming after 10 days

NVS - no visible symptoms

Sampling after 14 days and 21 days onto B. chinensis. Three test plants used for each inoculation and back-inoculation.

Table 14

Results of Inoculating Brassica chinensis with Vicia faba Leaf Isolates

	Inoculum Source	Back-Inoculation Result
1.	Virus/Leaf above inoc. *V. faba* leaf	Slight blooming
2.	Uninfected *B. chinensis* + 1.	Greater blooming and mottling
3.	Uninfected Radish leaf + 1.	Greater blooming and mottling
4.	Uninfected Radish leaf	NVS
5.	Uninfected *B. chinensis*	NVS

NVS - no visible symptoms

Sampling (back-inoculation) fifteen days after original inoculation

1949). An inoculum was prepared by grinding uninfected radish leaves with Table 12 TYMV inoculated bean leaves. On application to Chinese cabbage test plants, after ten days, "blooming" of the leaves of the central rosettes resulted (to a greater extent than on applying a "pure" bean inoculum). Similar enhanced "infection" resulted on mixing the Table 12 TYMV inoculated bean leaves with uninfected cabbage leaves before applying to test plants (Table 14).

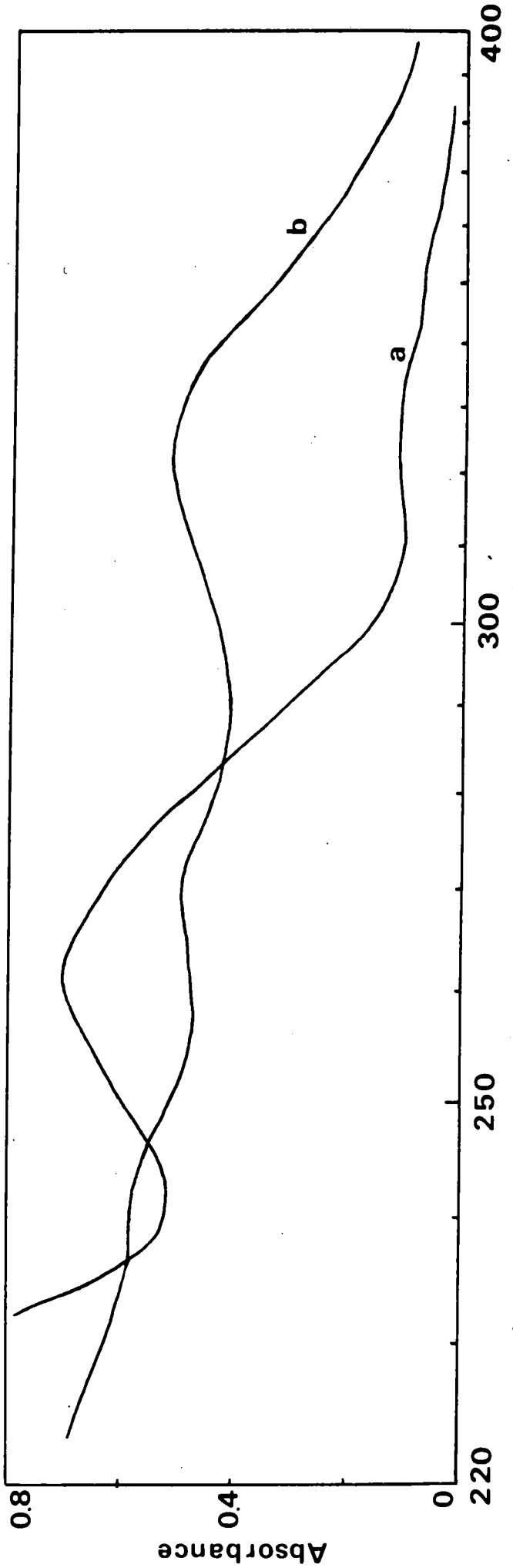
During the course of these experiments samples of bean leaves examined under the electron microscope failed to show the presence of typical TYMV crystalline aggregates, as seen in infected Chinese cabbage leaves (page 317).

Back-inoculation from Chinese cabbage test plants exhibiting "blooming" (inoculated with bean isolates) onto fresh uninfected Chinese cabbage resulted in the production of similar "blooming". Cabbage plants showing the more typical symptoms of TYMV infection when back-inoculated, occasionally produced "blooming" but more often the typical symptoms of TYMV infection.

Viral Extraction:

(a) The ammonium sulphate precipitation procedure for the isolation of TYMV (page 72), was found to be an easy but time consuming method. Samples prepared by this method exhibited absorption spectra indicative of contamination by plant material (fig. 10). The sedimentation pattern of such samples was similar to that shown by infected plant

Fig. 10 . Absorption spectra of TYMV and infected Brassica chinensis leaf sap



Samples from the ammonium sulphate extraction procedure for TYMV, suitably diluted in water.

a. TYMV from the second ammonium sulphate precipitation stage.
b. Infected cell sap supernatant prior to first ammonium sulphate precipitation stage.

sap (fig. 22), but the contaminants were in lower concentration. The sedimentation pattern of infected plant sap bore a close resemblance to analytical ultracentrifuge pictures by Dunn and Hitchborn (1965) and other workers of infected sap.

Each precipitation step helped to reduce contamination, but electron microscopic examination of a typical isolate indicated the presence of 18 S protein. Ammonium sulphate precipitation removed most of the extraneous material having an absorption maximum at c. 315-320 nm.

Viral isolates extracted by this method were infective, and isolates examined after one week of storage at $4^{o}C$ had no apparent change in absorption spectrum (and were still infective).

Extraneous plant material was removed from precipitated samples by treatment with MAGBENT, resulting in an almost pure preparation of top and bottom components of TYMV (with little loss in concentration of the virus).

On treating the purified sample of virus with further magnesium bentonite, it was found that under viral extraction conditions only 1-2% of the virus was adsorbed. This compared favourably with the magnesium bentonite prepared and used by Dunn and Hitchborn (1965).

After bentonite suspensions had been centrifuged, in some salt solutions, the optical density of the supernatant was slightly higher than that of the corresponding salt solution. Normally the centrifugation times and speeds were sufficient to eliminate this as a major source of contamination of either the virus or its nucleic acid.

(b) Magnesium bentonite treatment and differential centrifugation proved a more rapid and convenient TYMV isolation procedure than ammonium sulphate precipitation, yielding 'clean' viral samples. Such samples yielded only the Schlieren-optical peaks corresponding to reported S values for TYMV components (Matthews and Ralph, 1966), and had typical absorption spectra. Samples prepared by this method were found to readily re-infect B. chinensis.

Treatment of the purified viral suspension with magnesium bentonite, other than a slight increase in optical density, did not significantly alter the absorption spectrum or relative infectivity of the sample (fig. 11), after bentonite removal.

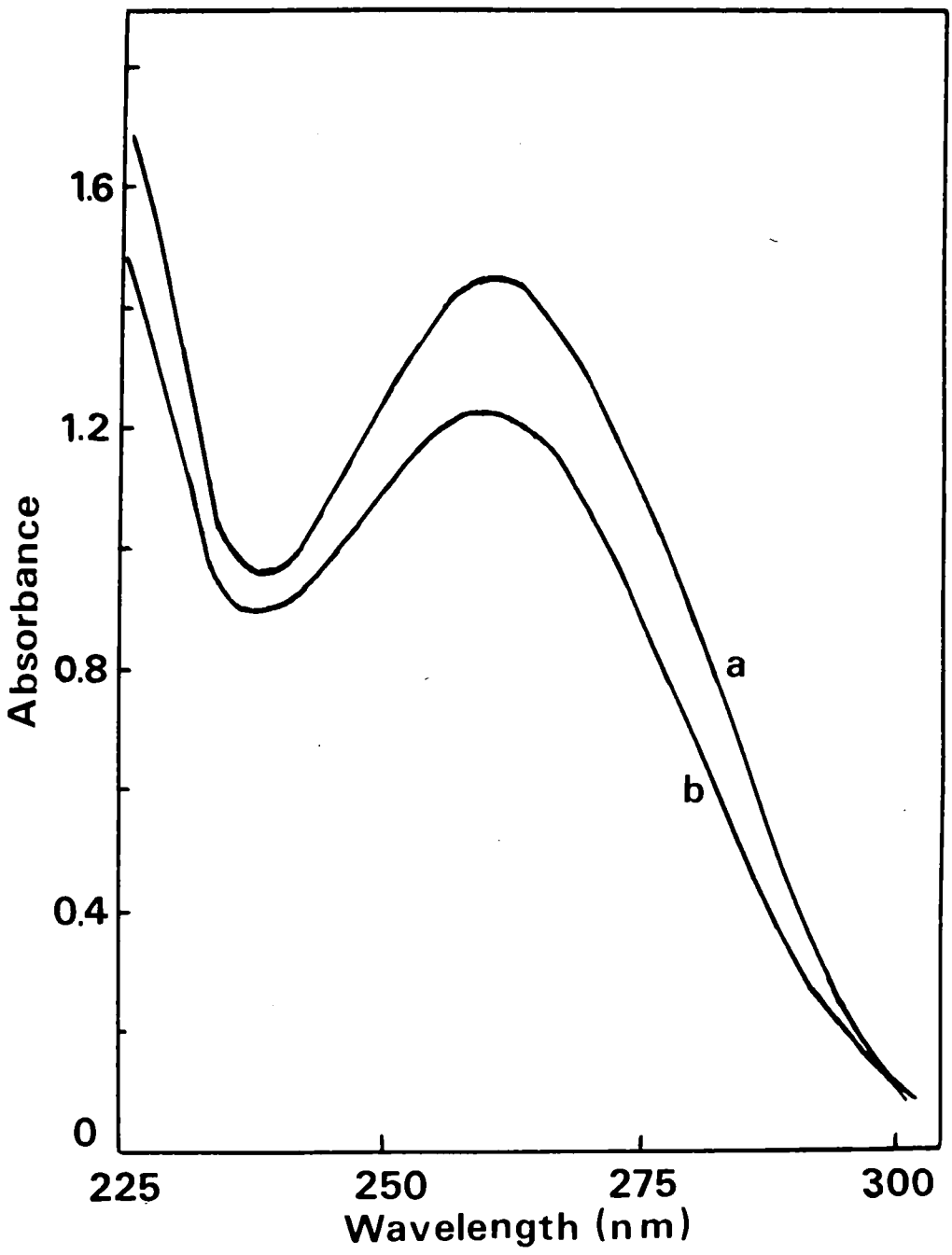
Comparative Viral Extractions:

Initial experiments indicated that the 'strain' of TYMV provided by Professor Markham and Professor Matthews, produced the most vigorous infection under the growth conditions employed.

These two 'strains' were inoculated onto three varieties of B. chinensis and were subsequently isolated, by differential centrifugation (method (b) above), to indicate the best host/strain combination (Table 15).

In infected plant sap there appeared to be a slight increase in a component(s) exhibiting maximum absorption at c. 315 nm (possibly indicative of an increase in phenolic products). No final pellet or any u.v. absorbing material was obtained when uninfected plant leaves were subjected to virus extraction procedures (centrifuge tube washings

Fig. 11 . Absorption spectrum of TYMV



TYMV prepared by the method of Dunn and Hitchborn(1965), suspended in 5 x 10^{-3} M $MgSO_4$-10^{-2} M phosphate buffer, pH 7.4(20°C).

a. A suitable dilution in water.
b. A suitable dilution of a sample treated with 0.1 vol. magnesium bentonite suspension(50 mg/ml) and clarified by centrifugation.

Table 15

Comparative Viral Extractions from Brassica chinensis Varieties

Strain	Brassica chinensis var.		
	Pte Sai	Wong Bok	Chihli
	(mg virus/Kg fresh leaf wt*)		
New Zealand	500	640	400
Markham	450	600	625
Uninfected	0	0	0

* determined by optical density measurement

Results are the average of three experiments

exhibiting no absorption over the range 200- 450 nm).

Viral RNA Isolation:

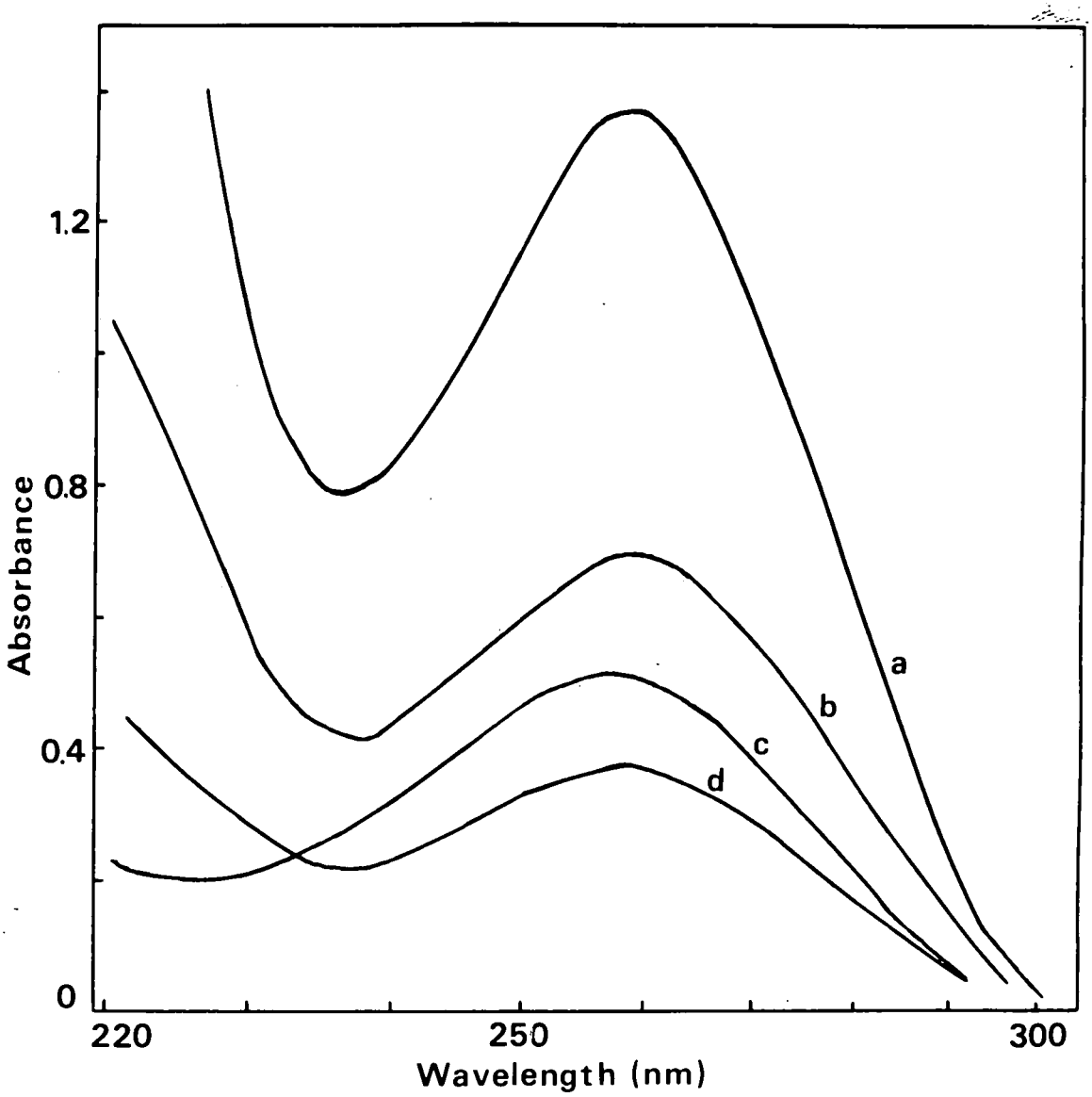
Samples of virus prepared by ammonium sulphate precipitation were not found to be suitable for RNA extraction unless extensively treated with bentonite. Degradation of the isolated RNA readily occurred, samples being unstable on storage for even short periods. TYMV RNA was therefore routinely isolated from TYMV extracted by bentonite treatment with differential centrifugation (Table 3).

Infectious RNA was prepared from TYMV by phenol extraction and ethanol precipitation techniques. The phenol extraction procedure resulted, however, in rather low yields, probably as a result of losses occurring at the interphases. No attempt was made to reduce these losses, and this method was employed to prepare a small number of samples for comparative purposes only.

Absorption spectra of ethanol precipitated RNA samples (from Table 15 , TYMV samples) indicated that virus from the New Zealand 'strain' cultured on B. chinensis var. Wong Bok had a typical TYMV RNA spectrum, and that such samples were 'cleaner' preparations (fig. 12).

TYMV RNA at 50 μg per ml produced from 20 to 30 lesions per half leaf, and TYMV applied to the opposite half leaves gave an average of 56 per 2.5 μg, 25 per 0.5 μg and 7 per 0.1 μg TYMV per ml, respectively. Assays were hampered by the rapid onset of systemic infection.

Fig. 12. Absorption spectra of TYMV and TYMV RNA



TYMV prepared by the method of Dunn and Hitchborn(1965), from infected Brassica chinensis var. Wong Bok leaves

a. Sample from R.E.F. Matthews, New Zealand.
b. Sample from R. Markham, England.

TYMV RNA prepared by the method of Dunn and Hitchborn(1966) from the above TYMV isolates.

c. RNA from TYMV sample originally supplied from New Zealand(a).
d. RNA from TYMV sample originally supplied from England(b).

All samples were suspended in water and the absorption spectra of suitable dilutions determined in water.

A sample of TYMV RNA incubated with 5 µg RNase per litre was found to be inactive, whereas a sample of TYMV similarly treated produced typical symptoms of infection (and average number of lesions).

Analytical Ultracentrifugation Analysis of Turnip Yellow Mosaic Virus and its Nucleic Acid:

Virus and viral RNA preparations subjected to analytical ultracentrifugation were found to exhibit varying sedimentation characteristics, dependent somewhat on the buffer employed and partly on the concentration of the material under investigation.

TYMV bottom component was found to have a sedimentation coefficient varying between 107 - 118 S and the top component between 50 - 57 S (in 0.1 M NaCl - 0.01 M PO_4 buffer)(fig. 13). Insufficient determinations were performed to extrapolate the values obtained to zero concentration. Comparable values were obtained using both the Omega and Spinco Analytical Ultracentrifuges (employing Schlieren optics) (Table 16).

Variations in values obtained for virus extracted from different ages of plants were not greater than the normal variations between different viral samples.

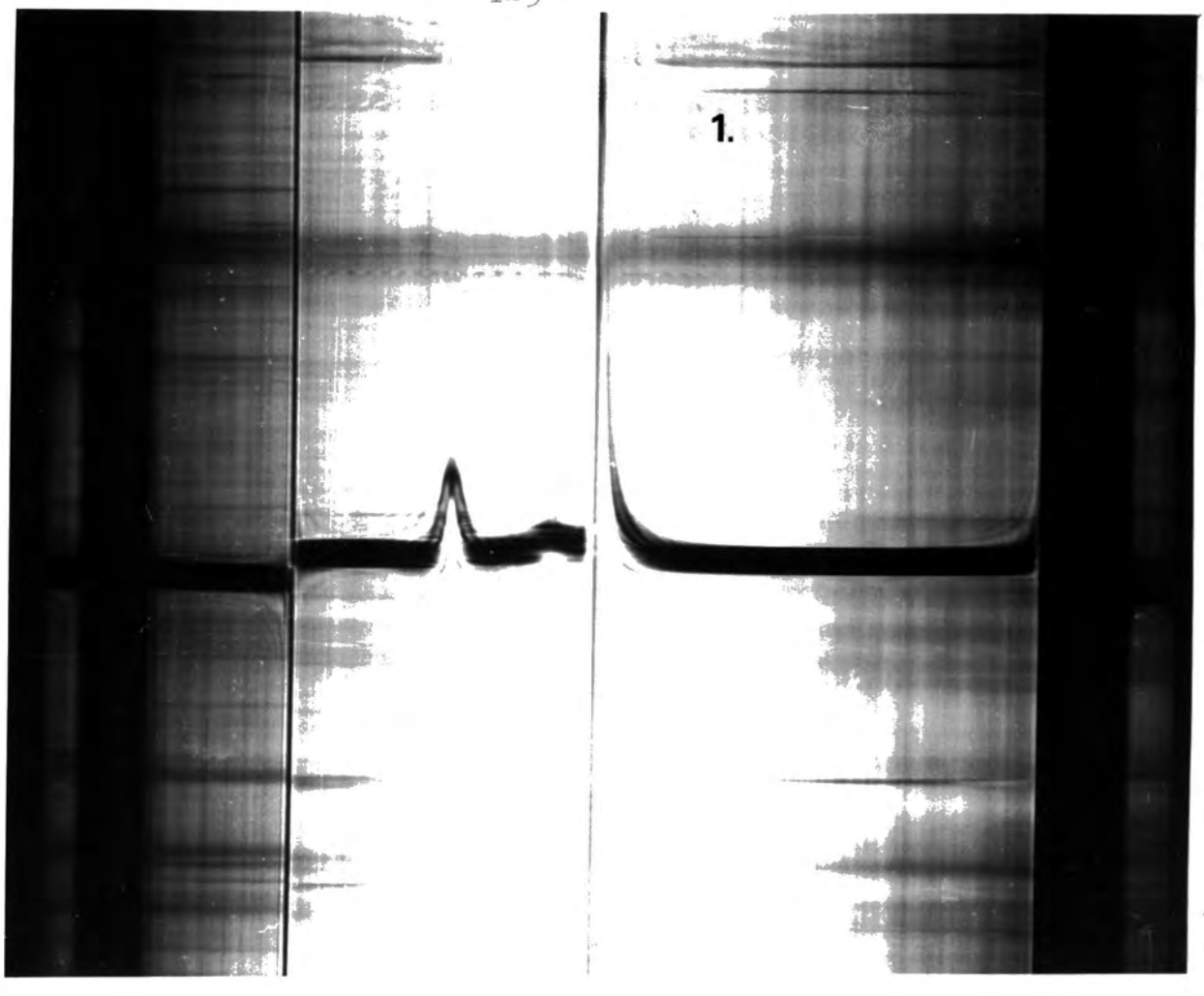
Occasionally a small 'peak' was observed between the top and bottom viral components, with a sedimentation coefficient of c. 80 S, possibly indicative of incomplete removal of plant ribosomal material. From 'area under peak' measurements, this represented c. 1% of 260 nm absorbing material. Treatment of such preparations with magnesium bentonite removed this 'peak' with little loss of bottom component and

Fig. 13 Schlieren patterns of two TYMV preparations to show leaf microsomal contamination

1. TYMV suspension exhibiting marked concentration of 'middle' component ('80 S' Brassica chinensis leaf leaf ribosomes), between bottom component (107 - 118 S) and top component (50 - 57 S).

2. TYMV suspension with low '80 S' component contamination

Omega rotor speed	40,000 rev./min
Temperature	20°C
Bar angle	60°
Time (after attaining speed)	6 min
Buffer	0.1 M NaCl - 0.01 M phosphate buffer pH 7.4
Concentration	3 mg/ml 260 nm absorbing material
Direction of sedimentation	left to right


1.

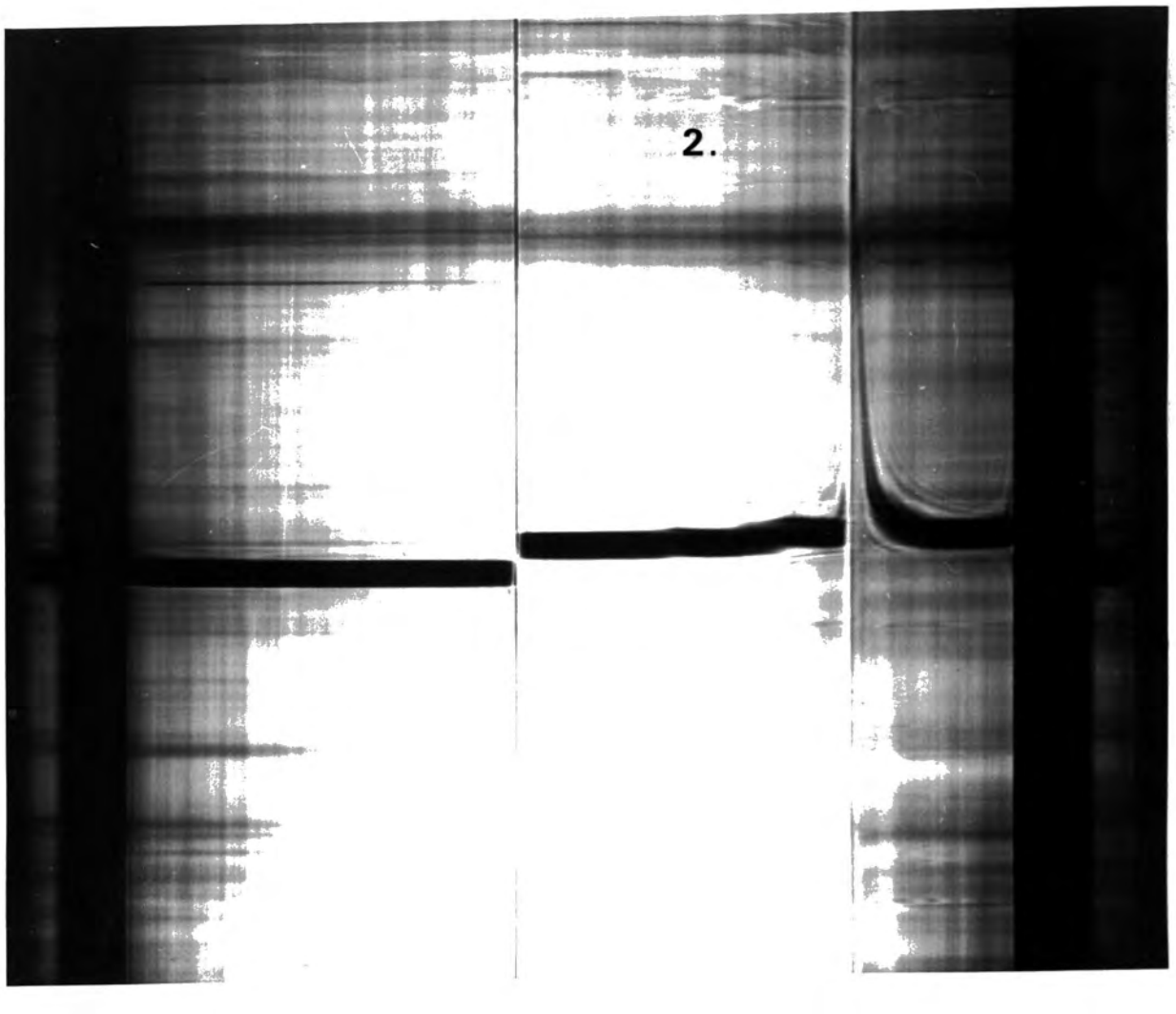

2.

Table 16

Comparison of Sedimentation Coefficients Determined Using Omega and Spinco E Analytical Ultracentrifuges(Schlieren Optics)

	S_{20} Coefficients of TYMV* Components	
Instrument	Top Component	Bottom Component
Omega	53	116
Spinco E	54	118

*TYMV concentration: 5 mg per ml

Buffer: 0.1 M NaCl - 0.02 M Tris Hydrochloride, pH 7.5

(some contamination in both determinations by an 82 S component)

only slight loss of top component. No peak due to 18 S plant protein was evident in purified viral extracts.

TYMV nucleic acid prepared by ethanol precipitation, was found to have a sedimentation coefficient of 32.5 S in 0.2 M NaCl - 0.02 M EDTA - 0.05 M Tris Hydrochloride, pH 7.5 (Spinco E, u.v. optics, at 40 μg per ml). Heating at 65^{o}C for 3 min reduced the concentration of RNA sedimenting at 32.5 S by c. 40%(similar to the findings of Dunn and Hitchborn, 1966).

When greater concentrations of RNA were examined by Schlieren optics, ambiguous results were obtained. E. coli highly polymerised ribosomal RNA (in 0.1 M KCl - 0.01 M phosphate buffer, pH 7.4) exhibited two components sedimenting at 12.9 S and 10.4 S at 10 mg per ml, and 8.3 S and 5.5 S at 15 mg per ml. Reported values for these components are 23.5 and 16.3 (Kurland, 1960).

Under similar conditions TYMV RNA (10 mg per ml) exhibited a (fig. 14) single component, sedimenting at 12.7 S. In an attempt to relate the two determinations, a mixture of TYMV RNA (2.5 mg per ml) and E. coli RNA (5.0 mg per ml) was examined. After a short period of centrifugation however, the mixture set solid in the analytical cell, forming an opaque gel. From readings obtained before gel formation, the TYMV RNA component was sedimenting at 17.5 S and the only E. coli component observed, at 16 S. Assuming a simple concentration dependence and that the E. coli and TYMV RNA's were behaving similarly under the experimental conditions employed (and using only coefficients determined for three concentrations), a simple extrapolation to zero concentration

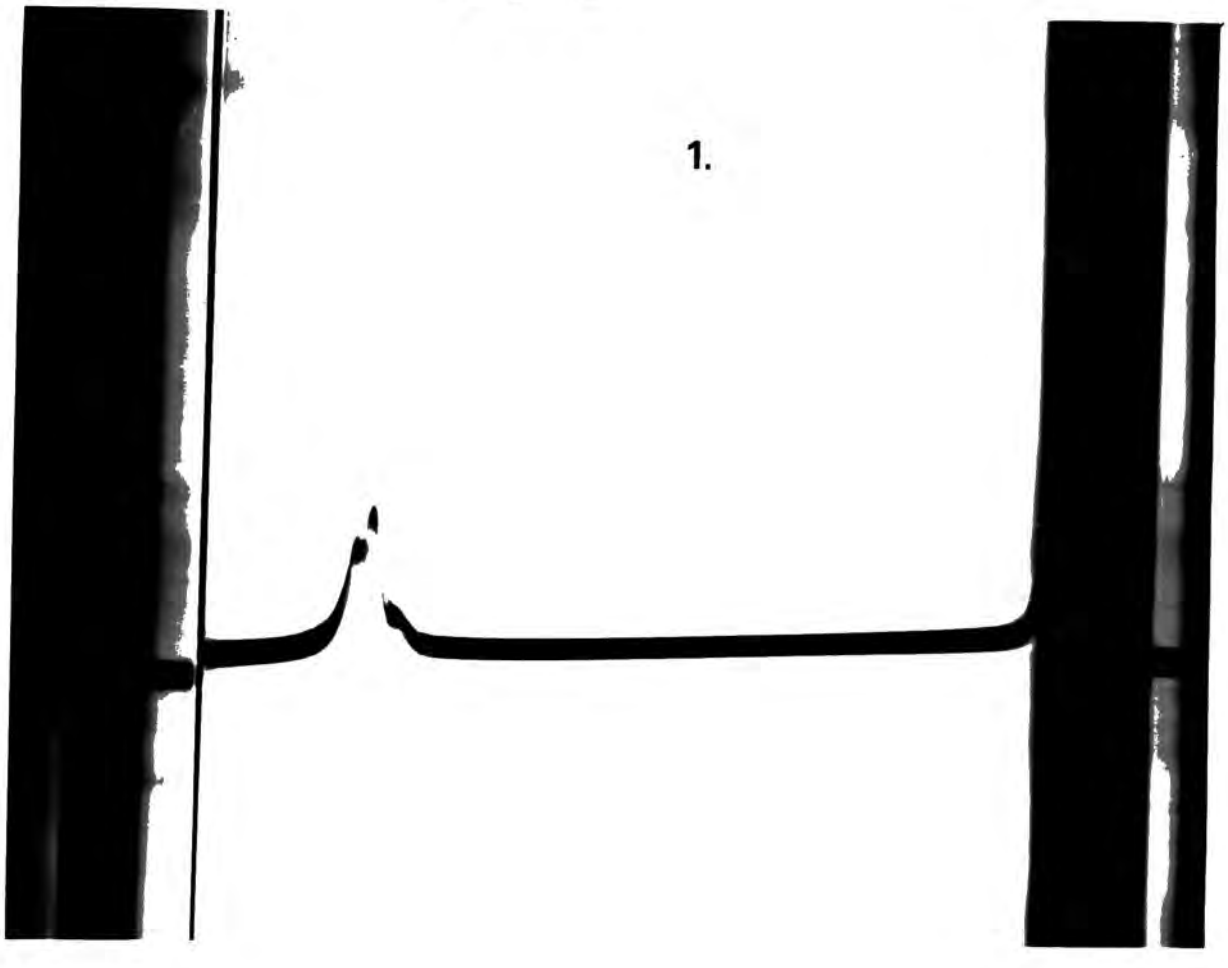
Fig. 14 Schlieren patterns of Escherichia coli and TYMV RNA's

1. *E. coli* highly polymerised ribosomal RNA.

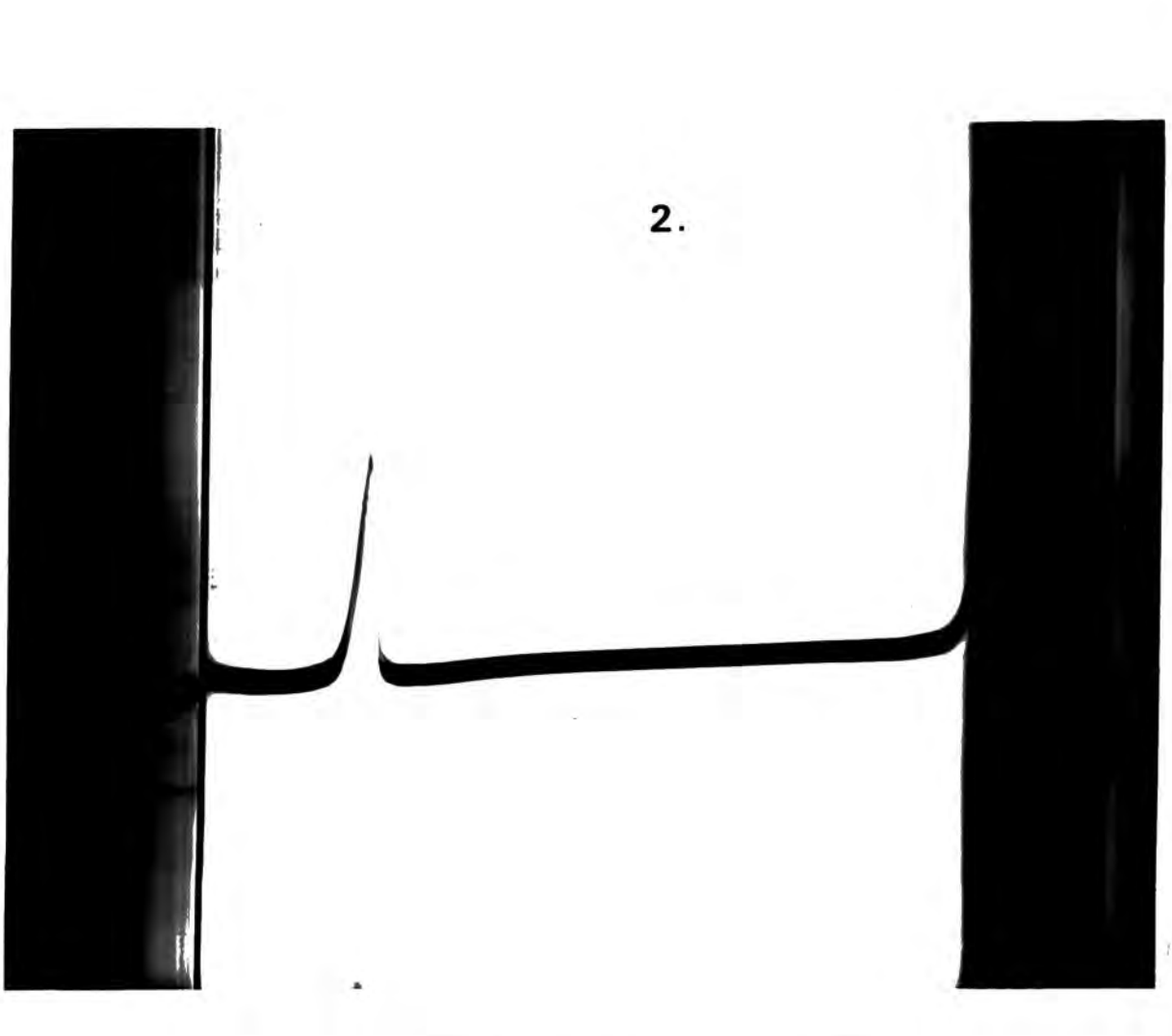
2. TYMV RNA extracted by the method of Dunn and Hitchborn (1966).

Omega rotor speed	40,000 rev/min
Temperature	$5^{o}C$
Bar angle	70^{o}
Time (after attaining speed)	45 min
Buffer	0.1 M KCl - 0.01 M phosphate buffer, ph 7.4
Concentration	10 mg/ml 260 nm absorbing material
Direction of sedimentation	left to right

1.



2.



gave an approximate value of 23 S for the TYMV RNA component. Reported values for TYMV RNA range from 22 - 28 S but are very dependent on concentration and buffer (Dunn and Hitchborn, 1966; Haselkorn, 1962; Hitchborn, 1968).

Spinco E determinations gave similar results for both ethanol precipitated and phenol extracted samples. At a concentration of 5 mg per ml, E. coli components sedimented at 16.9 and 9.8 S and TYMV RNA at 16 S. RNase degraded samples of TYMV RNA resulted in broad spreading peak, with an average sedimentation coefficient of c. 5 S.(fig.15)

Frozen and thawed samples of TYMV RNA had similar sedimentation characteristics to fresh material.

At the higher concentrations, some TYMV RNA samples exhibited a number of other, rather indeterminite, peaks with sedimentation coefficients ranging from 14 - 18 S. At no time, however, were any peaks noted which would have indicated the presence of complete virus or empty coat protein shells.

All TYMV RNA samples examined in the ultracentrifuge, with the exception of the RNase degraded preparation, were found to produce typical symptoms of infection in B. chinensis, before and after centrifugation.

Polyacrylamide Gel Electrophoresis of TYMV RNA:

TYMV RNA, commercial samples of highly polymerised E. coli and yeast ribosomal RNA's, and prepared total B. chinensis var. Wong Bok

Fig. 15 Spinco E schlieren patterns of TYMV, and Escherichia coli and TYMV RNA

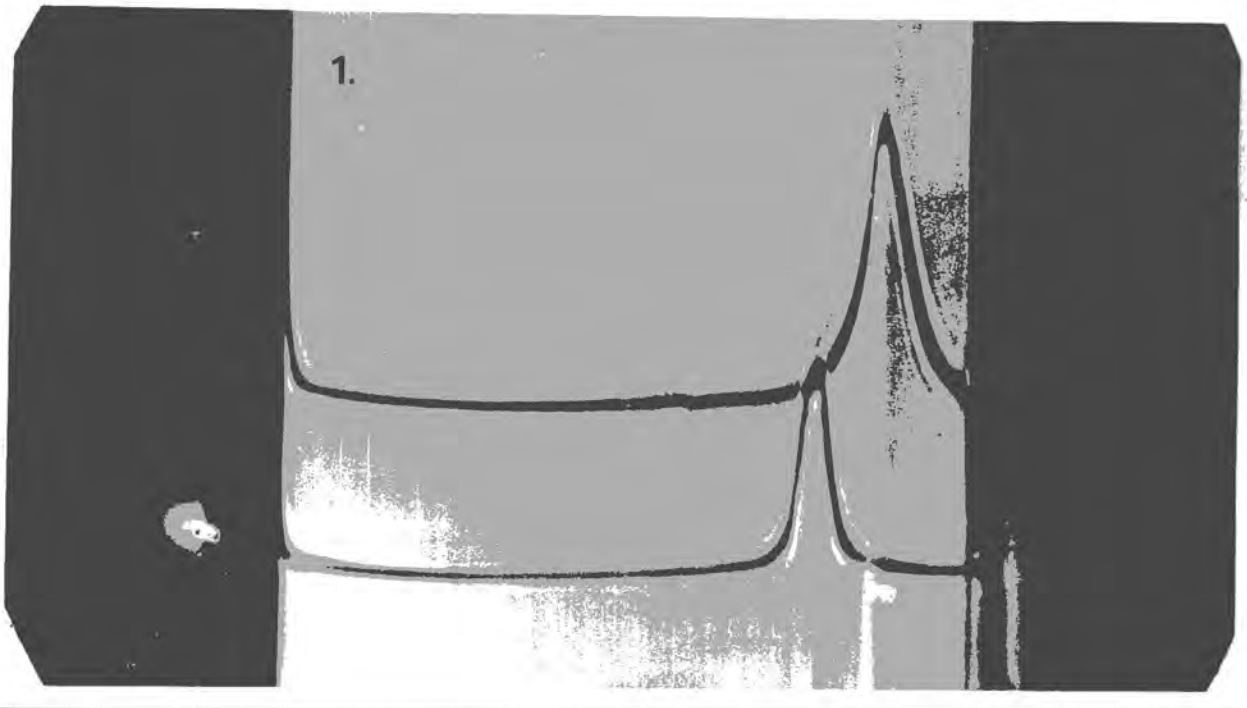
1. Upper - Degraded TYMV RNA (10 mg/ml)
   Lower - TYMV RNA (10 mg/ml)
   Prepared by the Dunn and Hitchborn (1966) method.

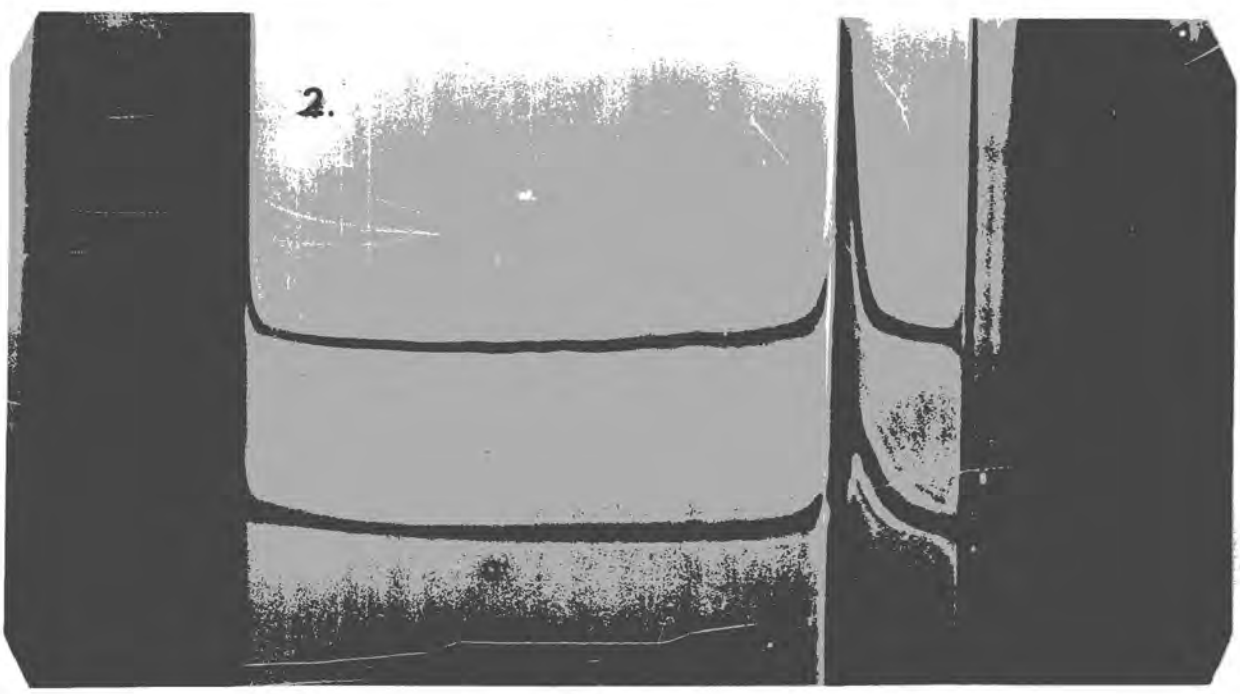
2. Upper - TYMV RNA (5 mg/ml) prepared by the method of Haselkorn (1962)
   Lower - E. coli highly polymerised ribosomal RNA (5 mg/ml)

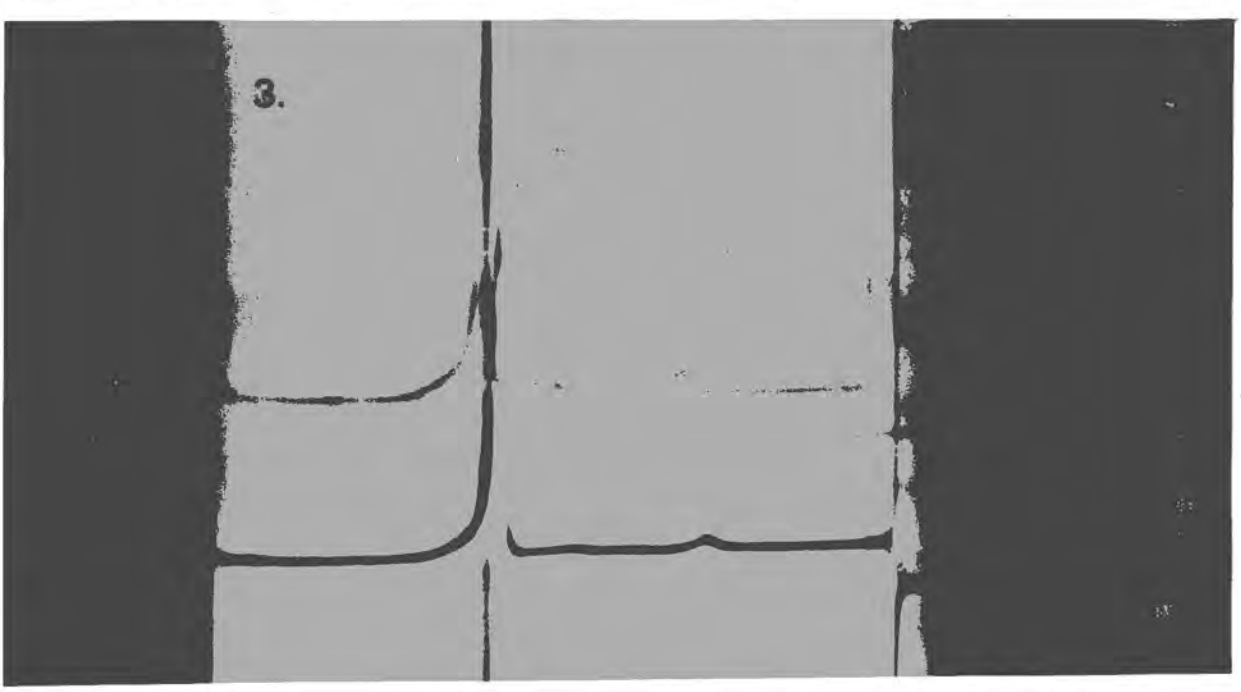
3. Upper - TYMV (20 mg/ml)
   Lower - TYMV (10 mg/ml)
   Prepared by the method of Dunn and Hitchborn (1965)
   (To illustrate slight concentration dependence)

Spinco E rotor speed	1. + 2. 60,000 rev./min; 3. 36,000 rev./min
Temperature	1. + 2. 25°C; 3. 22°C
Bar angle	70°
Buffer	1. + 2. 0.1 M NaCl - 0.02 M Tris HCl, buffer pH 7.5 3. 5×10^{-3} $MgSO_4$ - 10^{-2} M phosphate, pH 7.4

Direction of sedimentation right to left


1.


2.


3.

uninfected leaf RNA, were examined by polyacrylamide gel electrophoresis (PAGE) (figs. 16,17) and by absorption spectroscopy (fig. 18).

From the literature, values of 23.5 and 16.3 S were assigned to the two components of E. coli RNA (Kurland, 1960) and molecular weights of 1.08 and 0.56 respectively (Stanley and Bock, 1965). Total leaf RNA from B. chinensis was found to have components sedimenting at c. 25, 23, 18 and 16 S when examined on gels (fig. 16). Matus et al., (1964) reported values of 24, 16, 12 and 4 S, the 12 S being DNA and the 4 S tRNA.

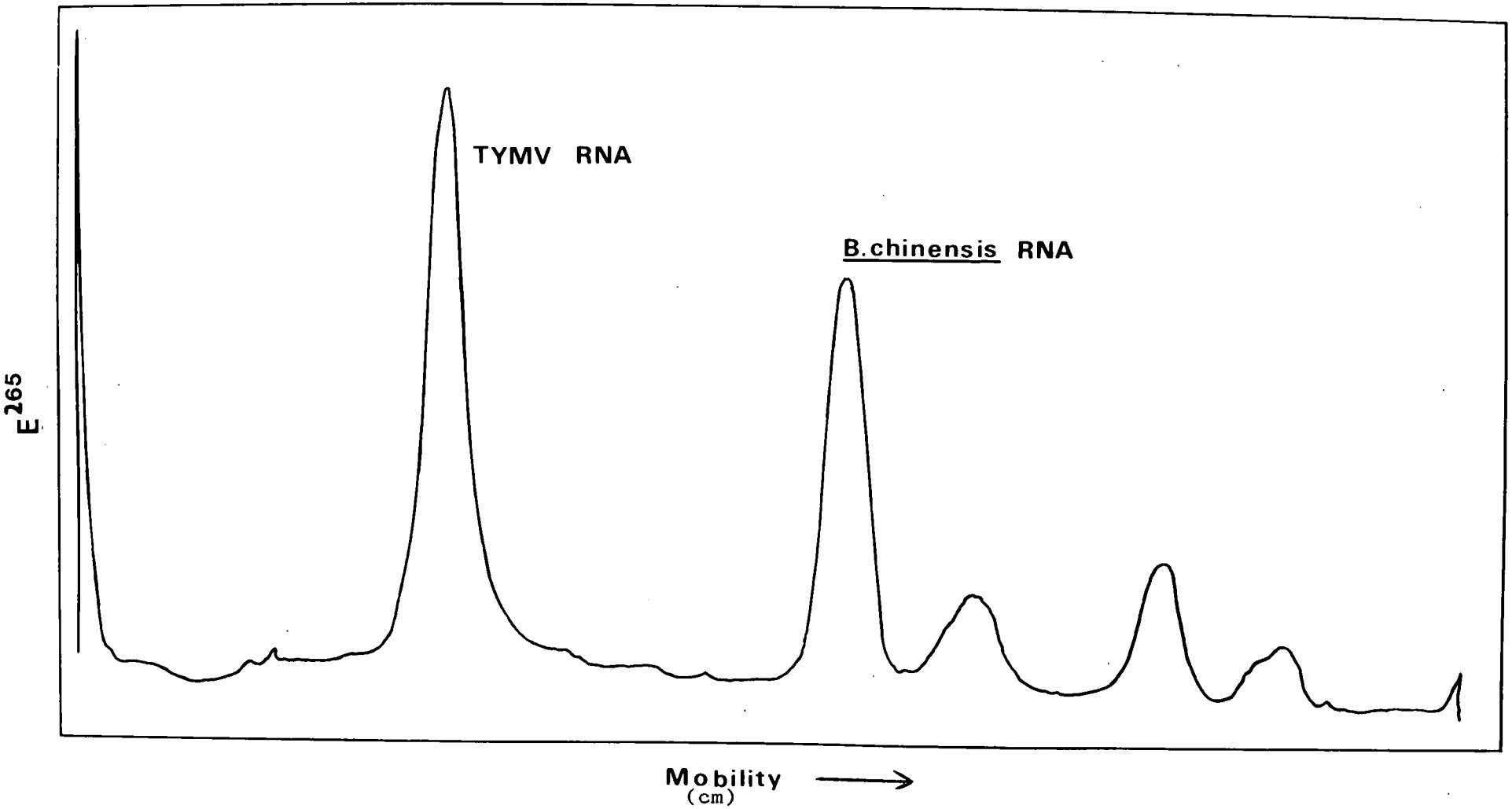
Yeast RNA was not employed routinely, either alone or in mixtures as it gave very variable results, the baselines of scanned gels appearing uneven and raised.

Commercial samples of E. coli RNA were often found to be grossly degraded, a number of batches being necessarily rejected before obtaining the sample employed for the present work.

Storage of gels prior to use was found to be unsatisfactory, often resulting in ambiguous determinations. Samples were run on 2.2% and 2.4% gels, as 2.8% gels were found to be too concentrated, preventing adequate penetration and separation of the viral and standard components.

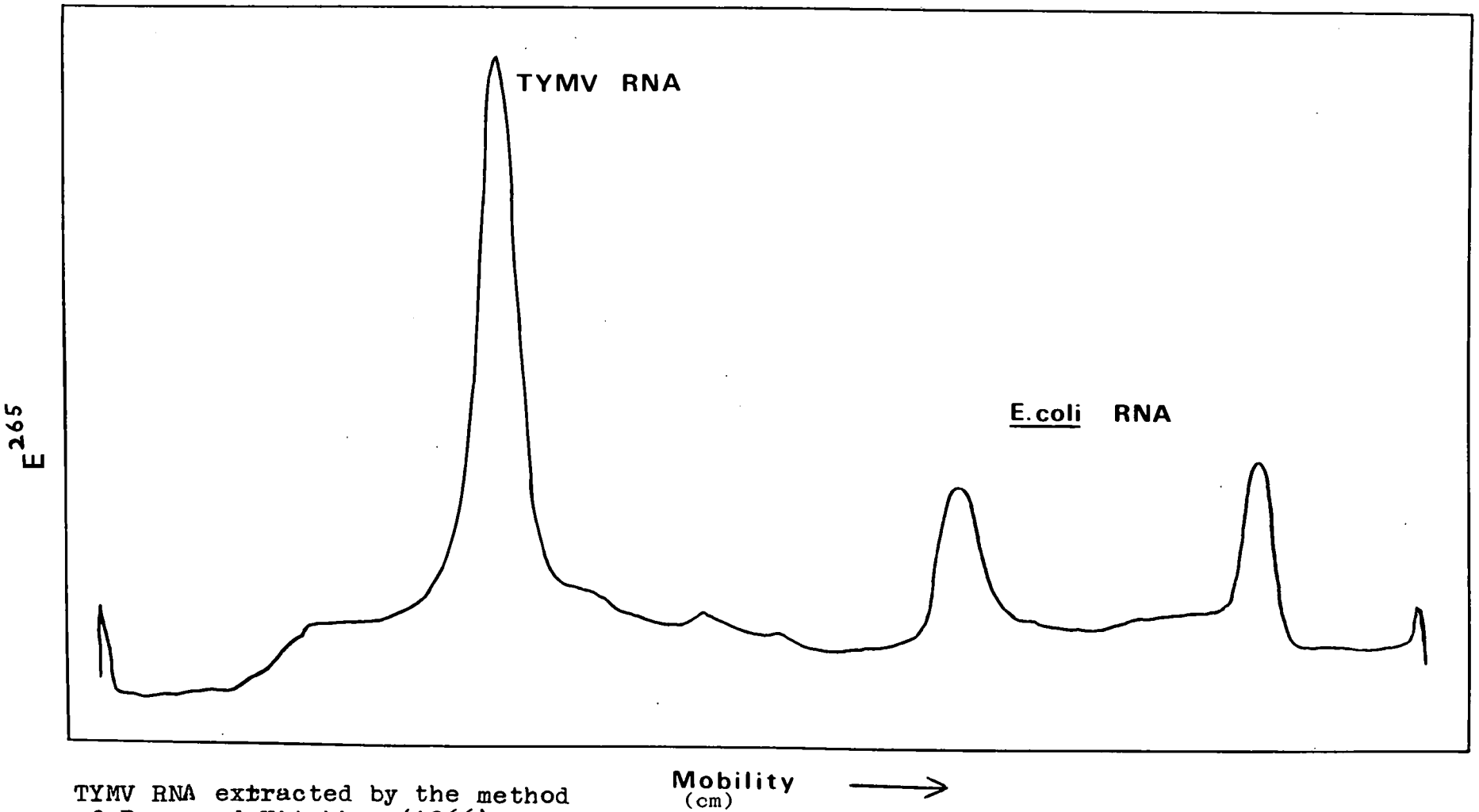
When run on 2.4% gels, the mixture of viral and E. coli RNA's exhibited some form of complexing, which was not apparent on the 2.2% gels (fig. 17). When viral and B. chinensis total leaf RNA were applied to the same gel, no complexing resulted (fig. 16).

Fig. 16. Polyacrylamide gel electrophoresis of TYMV and Brassica chinensis leaf RNAs



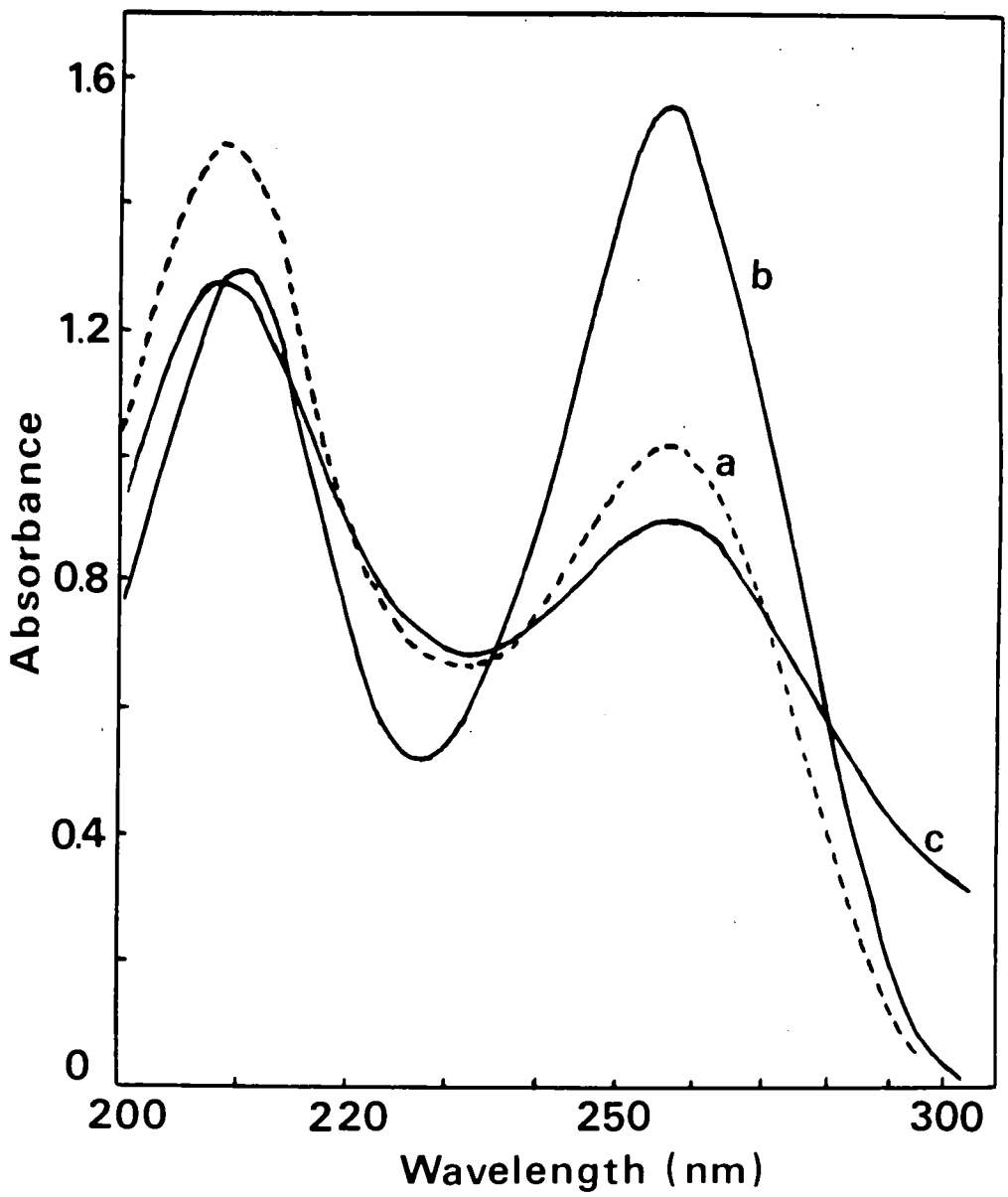
Brassica chinensis var. Wong Bok total leaf RNA extracted by the method of Ralph and Bellamy(1964). TYMV RNA extracted by the method of Dunn and Hitchborn(1966). Electrophoresis on 2.2% gels as described in Methods. Expanded Chromoscan trace(x 3).

Fig. 17. Polyacrylamide gel electrophoresis of TYMV and Escherichia coli ribosomal RNAs



TYMV RNA extracted by the method of Dunn and Hitchborn(1966).
Electrophoresis on 2.2% gel, as described in Methods. Expanded Chromoscan trace(x 3).

Fig. 18. RNA absorption spectra



RNA samples dissolved in diluted polyacrylamide gel electrophoresis buffer(page 62)the absorption spectra of suitable dilutions being determined in water.

a. TYMV RNA prepared by Dunn and Hitchborn method(1966).
b. E.coli highly polymerised ribosomal RNA.
c. B.chinensis leaf RNA prepared by the method of Ralph and Bellamy(1964).

All TYMV RNA samples examined were shown to contain no extraneous RNA material. Slight 'shouldering' of the viral peak was noted with most samples.

Yeast and viral RNA's run together altered the migration pattern of the viral RNA (as compared to lone runs). This was not observed with E. coli/TYMV RNA mixtures, traces of the individual components being readily superimposable on mixed run traces.

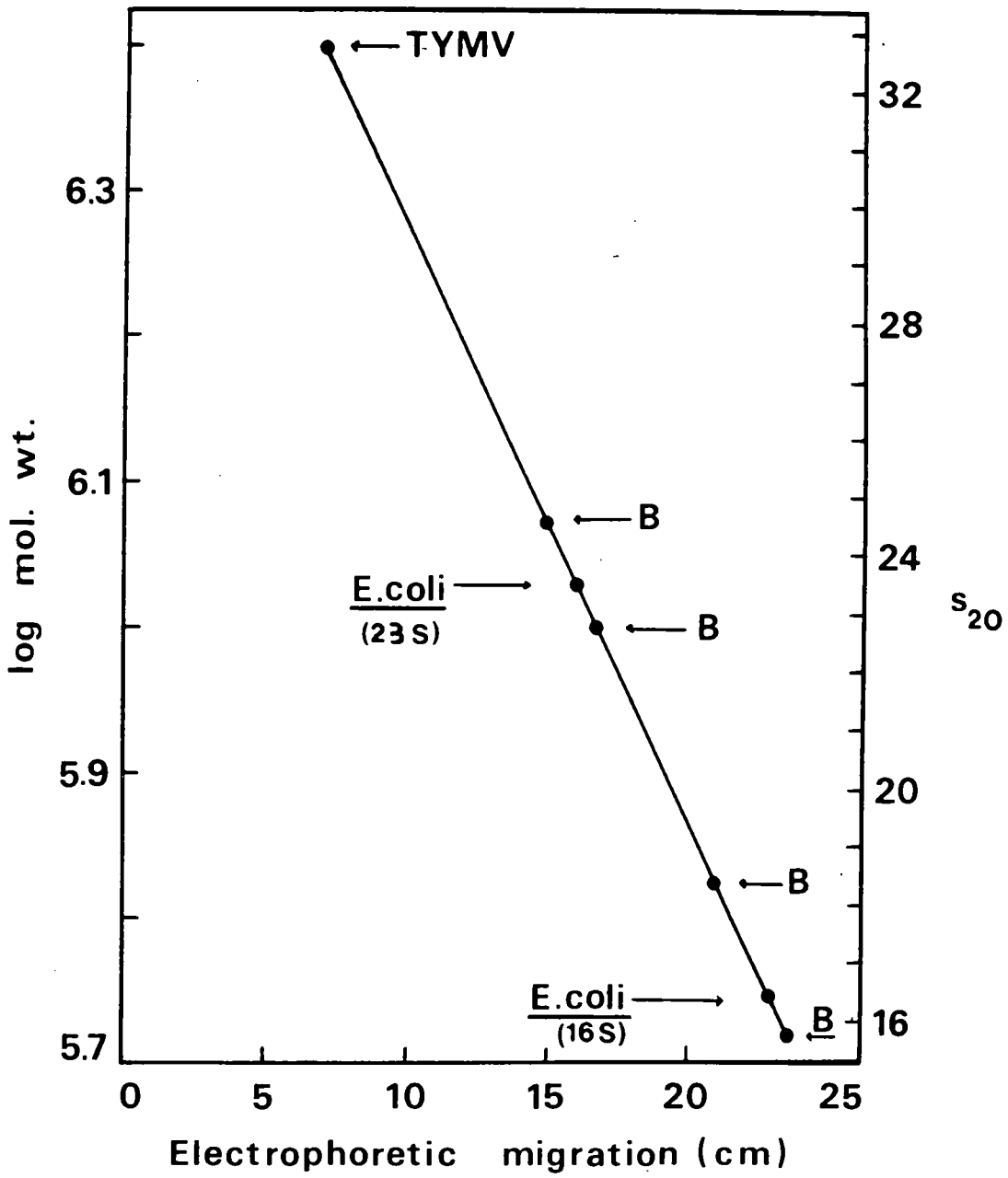
From a graph of log mol wt x mobility in the gel (cm), the average value for the molecular weight of TYMV RNA was found to be 2.5×10^6 (fig. 19) with a corresponding sedimentation coefficient of 32.75 S. Frozen and thawed samples of TYMV RNA gave similar results.

Protein Determination:

Protein determinations performed on simple viral RNA preparations by the Lowry method gave spurious results, indicating a 'background' protein content of 40 µg protein per 100-250 µg viral RNA. Determinations performed on commercial samples of E. coli and yeast ribosomal RNA's indicated a 'background' protein content of 5 µg per 10-75 µg ribosomal RNA. A sample of guanine similarly treated was shown to contain 10 µg 'protein' per µg guanine.

On examination of the samples by the method of Bramhall et al., (1969), guanine was found to be free of detectable protein (in a guanine concentration range of from 5 to 50 µg), commercial samples of RNA were shown to contain less than 1% protein and TYMV RNA from 0-2.5% protein. On reprecipitation or centrifugation of viral RNA samples to remove any attendant 'haze', the protein contamination level

Fig. 19 . Semi-log plot of molecular weight and plot of sedimentation coefficient against electrophoretic migration of RNA samples in 2.2% polyacrylamide gel



Electrophoresis carried out as described in methods, RNA samples being isolated by normal procedures. The results are of a typical determination, E.coli and TYMV RNAs and B.chinensis and TYMV RNAs being run on separate gels. The combined results are presented above. Gel length was routinely 7-8 cm, the above migration figures being measured directly from the expanded densitometer tracing.

B ... B.chinensis leaf RNA

was reduced to less than 0.5% (without any apparent reduction in RNA concentration or activity).

Microsomal Preparations:

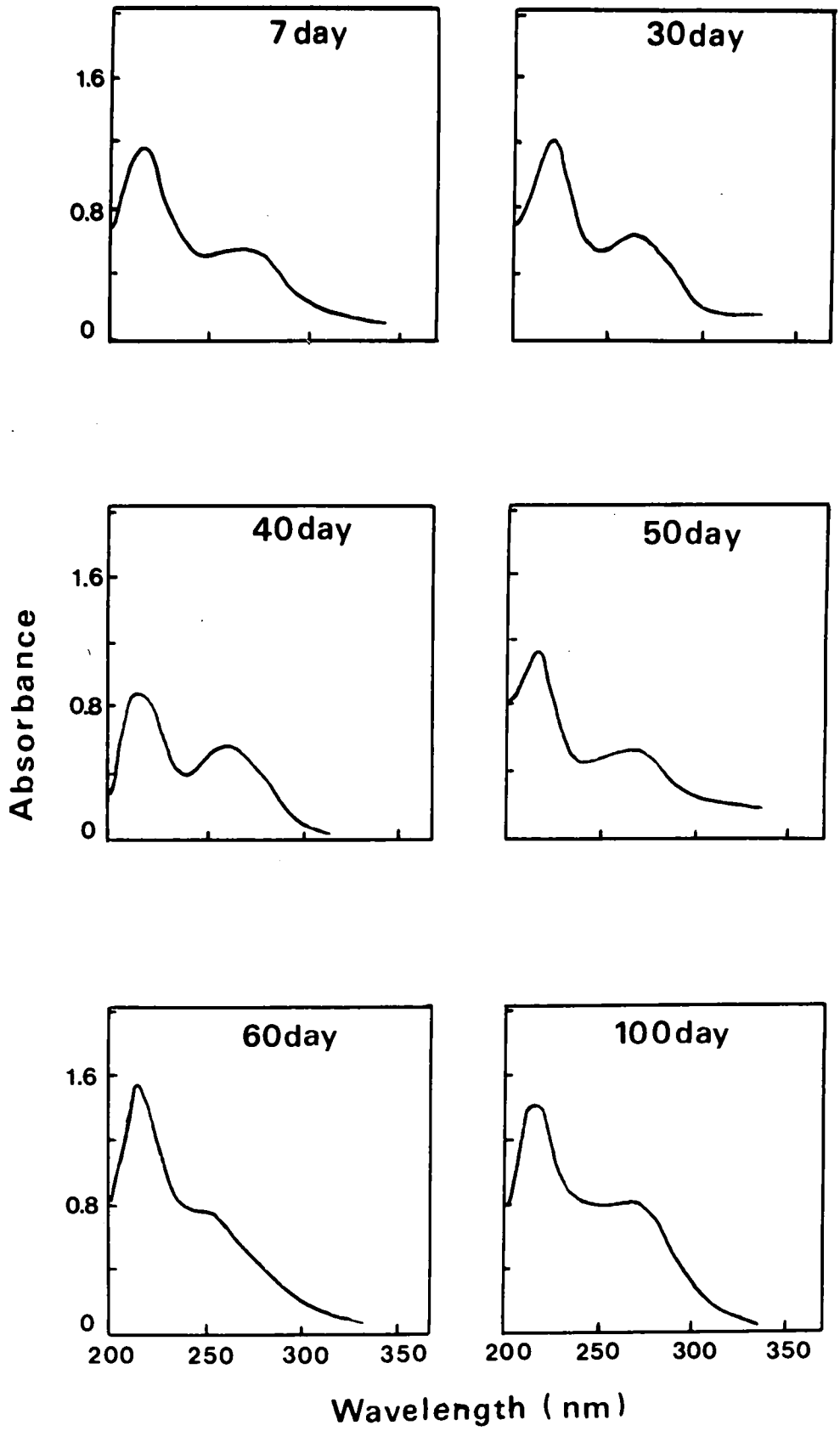
Variation in u.v. absorption spectra of B. chinensis microsomal preparations from different ages of leaf (fig. 20) resulted in difficulties in assessing amino acid incorporation simply on an optical density estimated 'ribosome' content basis. The RNA content of each isolate was therefore determined and calculations based on a standard mg ribosomal RNA.

Although somewhat dependent upon method of extraction, young B. chinensis leaf material (from plants 8 to 30 days old from seed) provided more active microsomal isolates for amino acid incorporation (fig. 21). Similarly enzyme preparations were more active from leaf material within this age group. Deoxycholate (Doc) treatment was not found to improve incorporation ability to any great extent. Microsomal isolation procedures attempted but not routinely employed, and the activity of the resultant isolates, are mentioned briefly in the Discussion Section.

Microsomal protein:RNA ratios varied little over the main range of ages, being in the order of 2:1. Very young leaves, up to c. 11 days from planting, had a 1:2 ratio, and older leaves, showing an increased protein content, from 15:1 to 40:1 (the latter in 100+ day leaves) (fig. 21).

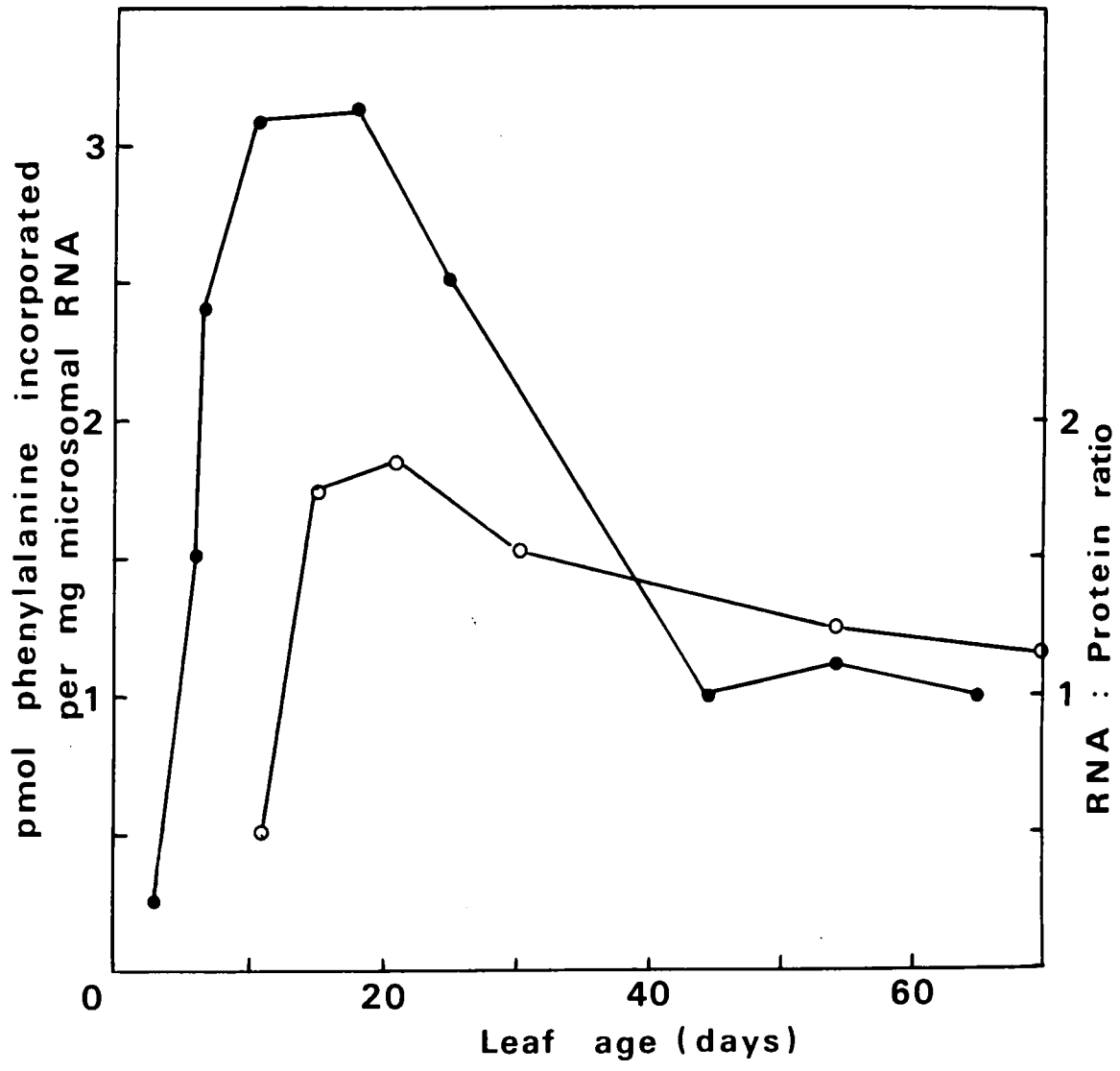
Analytical ultracentrifugation of the various B. chinensis isolates

Fig.20 . Absorption spectra of microsome preparations from Brassica chinensis var. Wong Bok leaves



Microsome preparations isolated from B.chinensis leaves by the rapid extraction procedure as described in Methods. Absorption spectra are of suitable dilutions in water.

Fig.21 . Variation in RNA:protein ratio and in vitro activity of Brassica chinensis leaf microsomes prepared from different ages of leaves



Incubation conditions as described in Methods, samples being taken after 40 min(poly(U) being used as messenger). Microsomes isolated by 'quick' method, RNA being determined by the Orcinol method and protein by the Lowry method.
RNA:protein results are the average of three determinations, $[^{14}C]$ phenylalanine incorporation results, the average of two.

●——● amino acid incorporation

○——○ RNA:protein ratio

indicated typical microsomal preparations, with mainly 80-83 S and to a lesser extent 67-70 S, polyribosomal components and 18 S plant protein (fig. 22). A microsome isolate from a TYMV infected B. chinensis plant (absorption spectrum fig. 23) had a reduced 70 S component, but was found to incorporate amino acids to the same level as an isolate from the same age of uninfected plant, under TYMV RNA direction (uninfected 1450 c.p.m. per sample, and infected 1400 c.p.m. per sample).

Electron microscopic examination of a typical B. chinensis microsome preparation indicated the presence of free and, to a lesser extent, membrane-bound ribosomes with little chloroplastic contamination (Cobb, 1970).

Ribosomes from 65 day old developing V. faba seeds were found to be fairly active in amino acid incorporation, as previously reported (Payne, 1970), although microsomes from germinating seeds were not.

Large-scale isolates from bean material were found to have little activity, probably the length of time of extraction contributing to ribosomal denaturation.

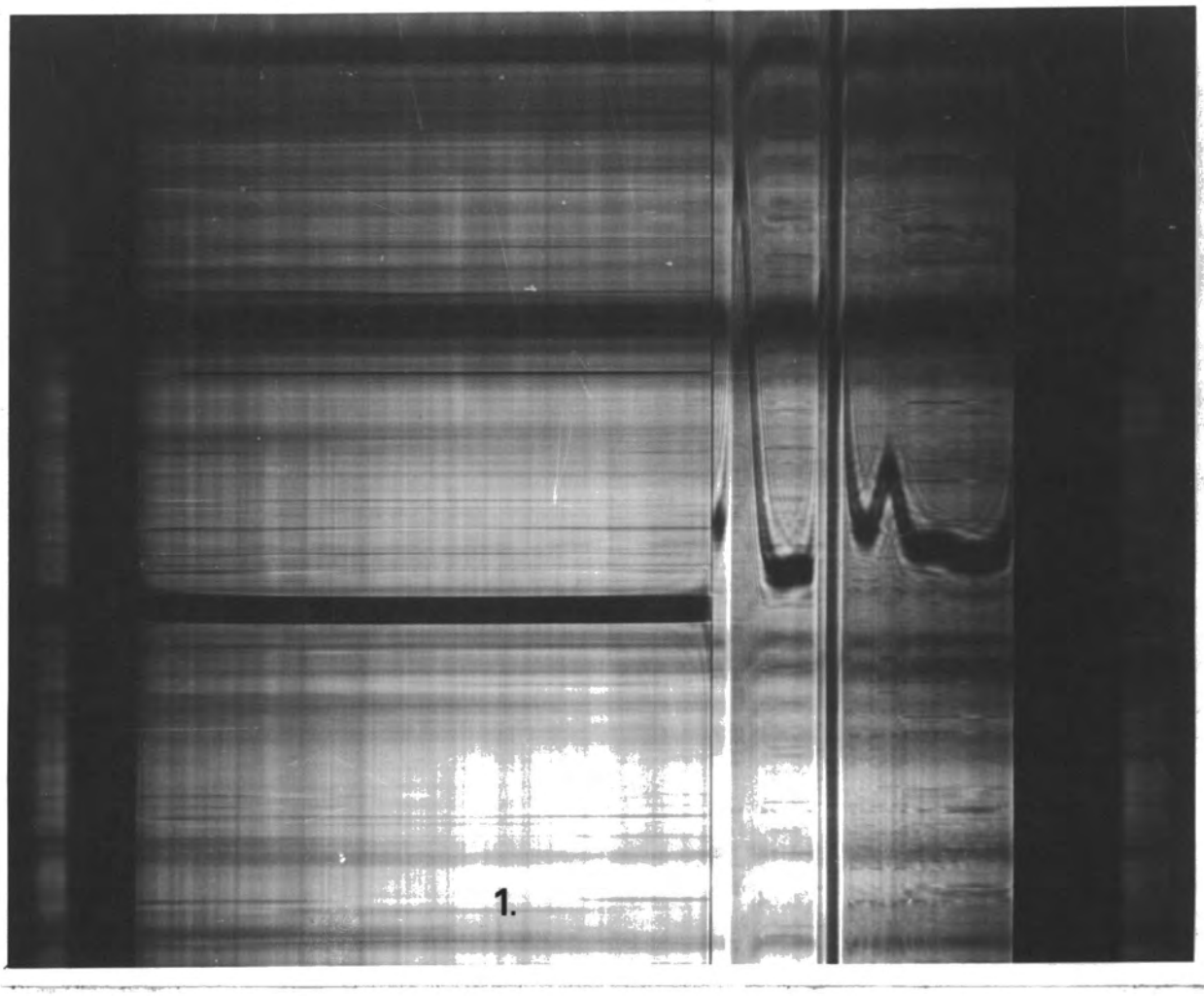
Analytical ultracentrifugation of bean plumule microsomes indicated the presence of 8.5, 26, 31, 45, 54, 58, 62, 70-75, 96 and 120-130 S components. Large-scale isolates from developing bean yielded only an inactive 79 S component (bean plumule and developing bean buffer being 0.01M KCl - 0.005M Mg^{2+} - 0.01 M Tris Hydrochloride, ph 7.4). (fig. 26)

Fig. 22 Schlieren patterns of a Brassica chinensis leaf microsome preparation and TYMV-infected leaf sap to show component variation as a result of infection

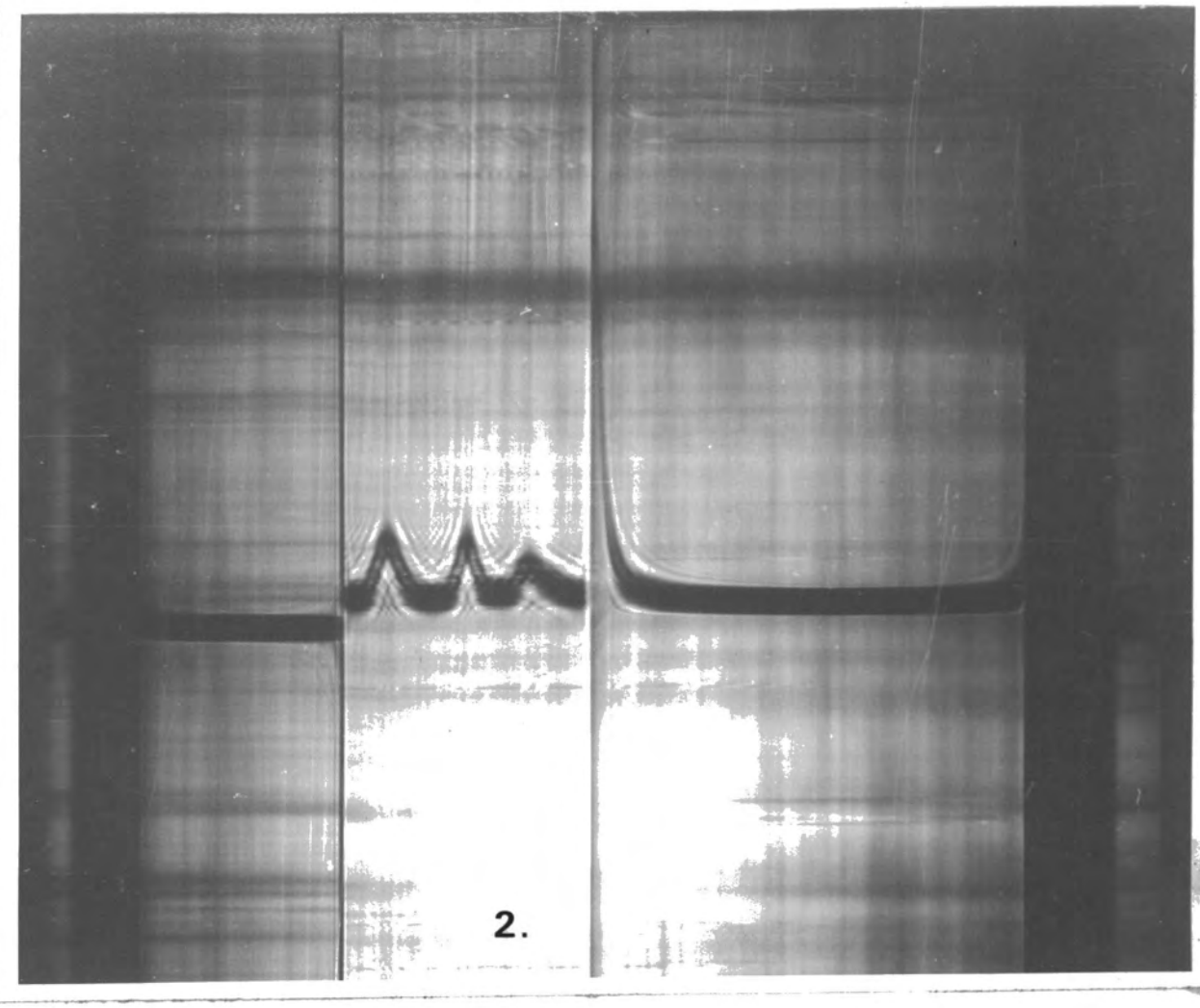
1. Microsome preparation from Brassica chinensis leaves (plants under 1 month old) containing c. 25 mg/ml 260 nm absorbing material (suspended in 0.005 M magnesium acetate - 0.01 M KCl - 0.01 M Tris HCl, pH 7.4)

2. Infected leaves subjected to TYMV extraction procedure of Dunn and Hitchborn (1965). Supernatant from first high speed spin, (page 73)

Omega rotor speed	40,000 rev/min
Temperature	$20^{o}C$
Bar angle	65^{o}
Time (after attaining speed)	1. 3 min; 2. 5 min
Direction of sedimentation	left to right

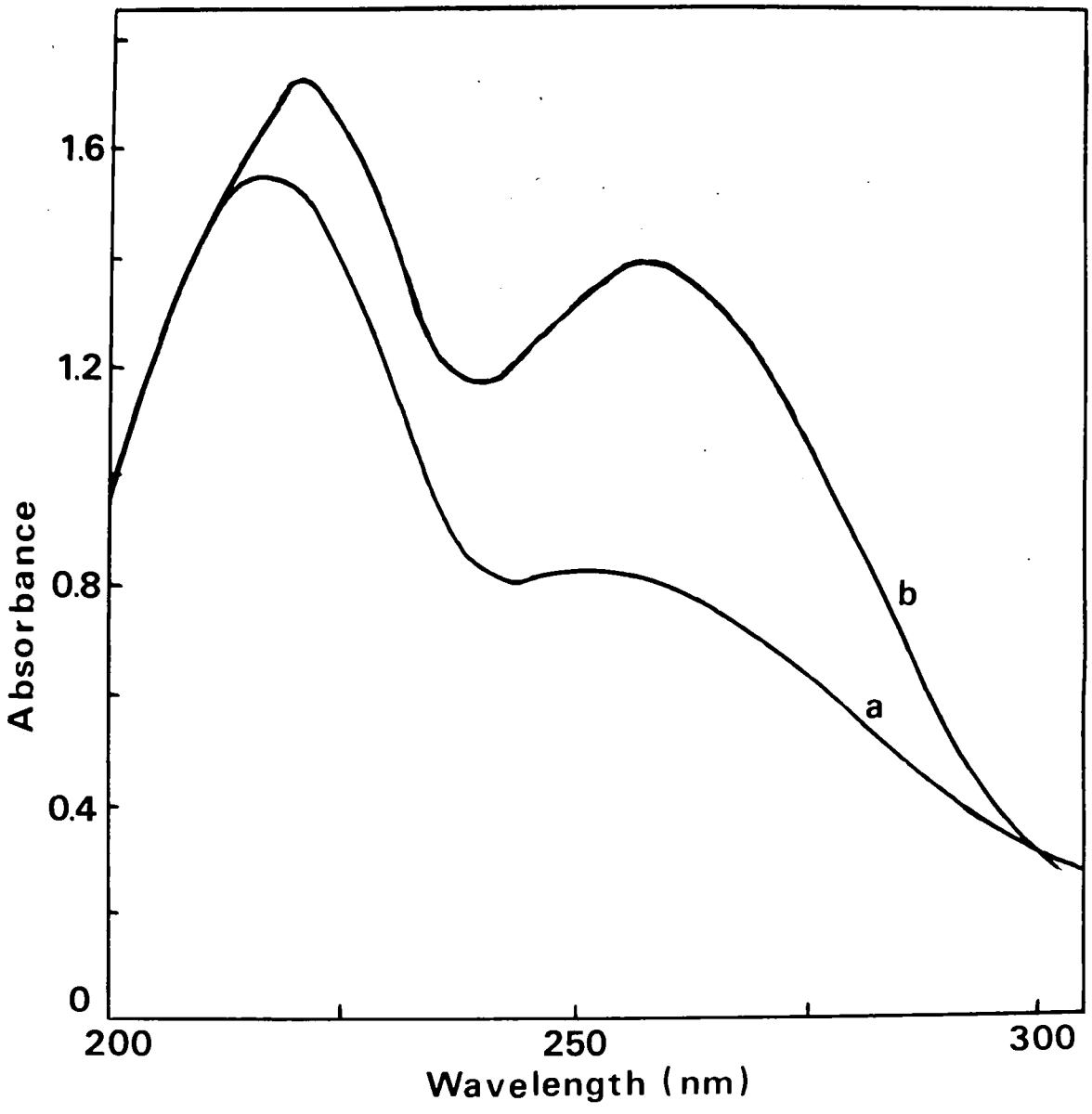


1.



2.

Fig. 23 . Absorption spectra of microsome isolates from TYMV infected and uninfected Brassica chinensis leaves



Microsomes prepared by the rapid extraction procedure as described in Methods, from 27 day B.chinensis var. Wong Bok leaves. The absorption spectra of suitable dilutions were determined in water.

a. Microsomes isolated from uninfected plants.

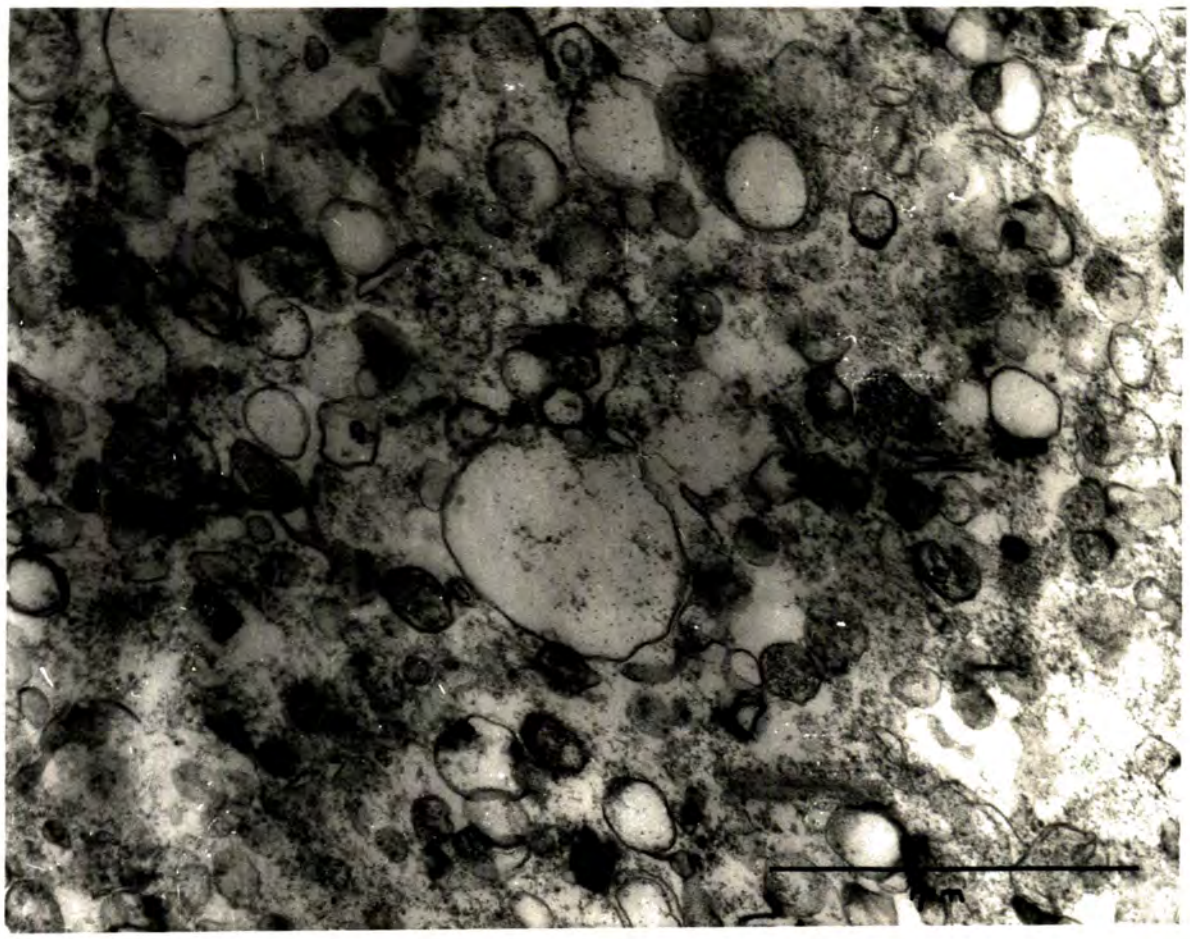
b. Microsomes isolated from infected plants.

Fig. 24 a Electron micrograph of Brassica chinensis leaf microsomal material (to show the absence of chloroplastic contamination)

b. Electron micrograph of a negatively stained sample of Turnip Yellow Mosaic Virus

Stained with phosphotungstic acid in an attempt to show complete virus particles and empty protein shells.

a.



b.

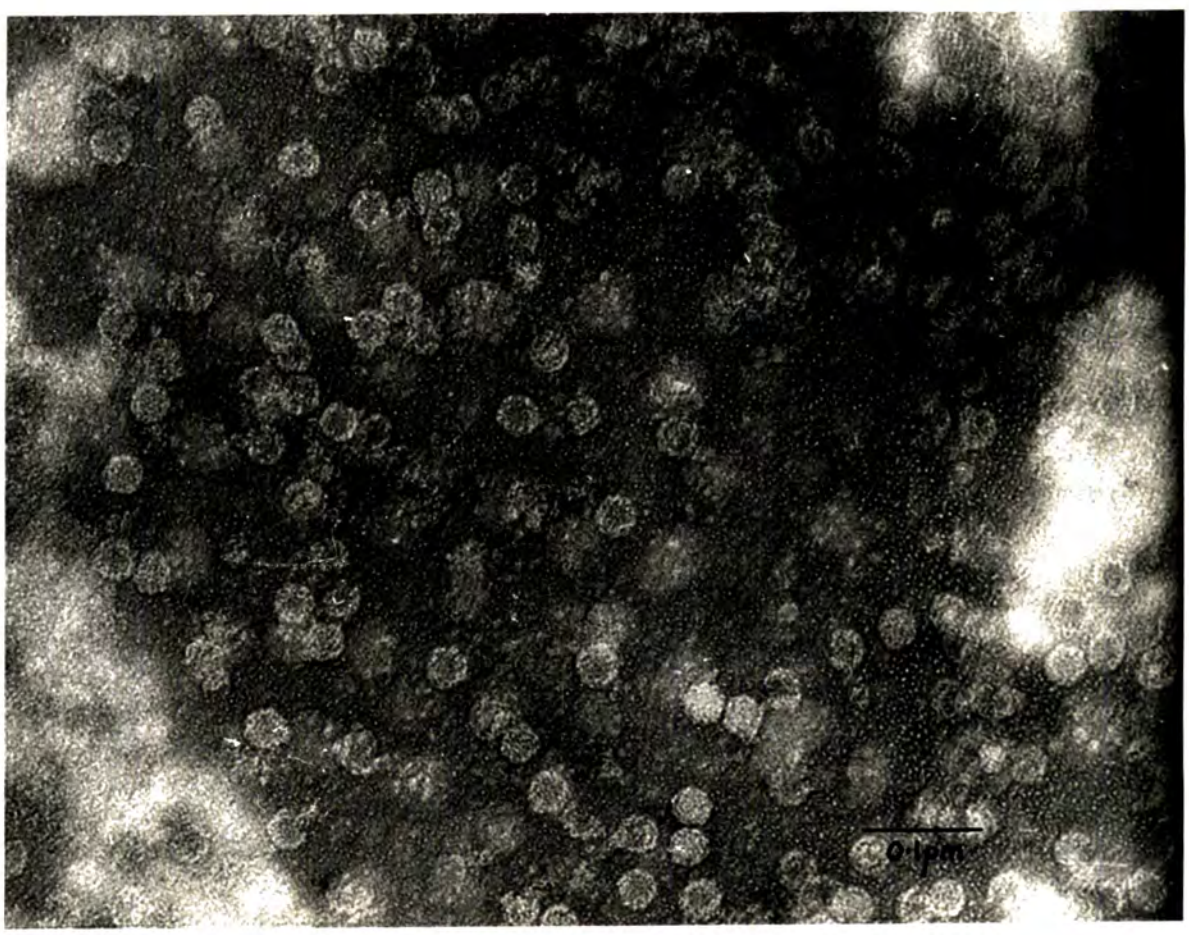
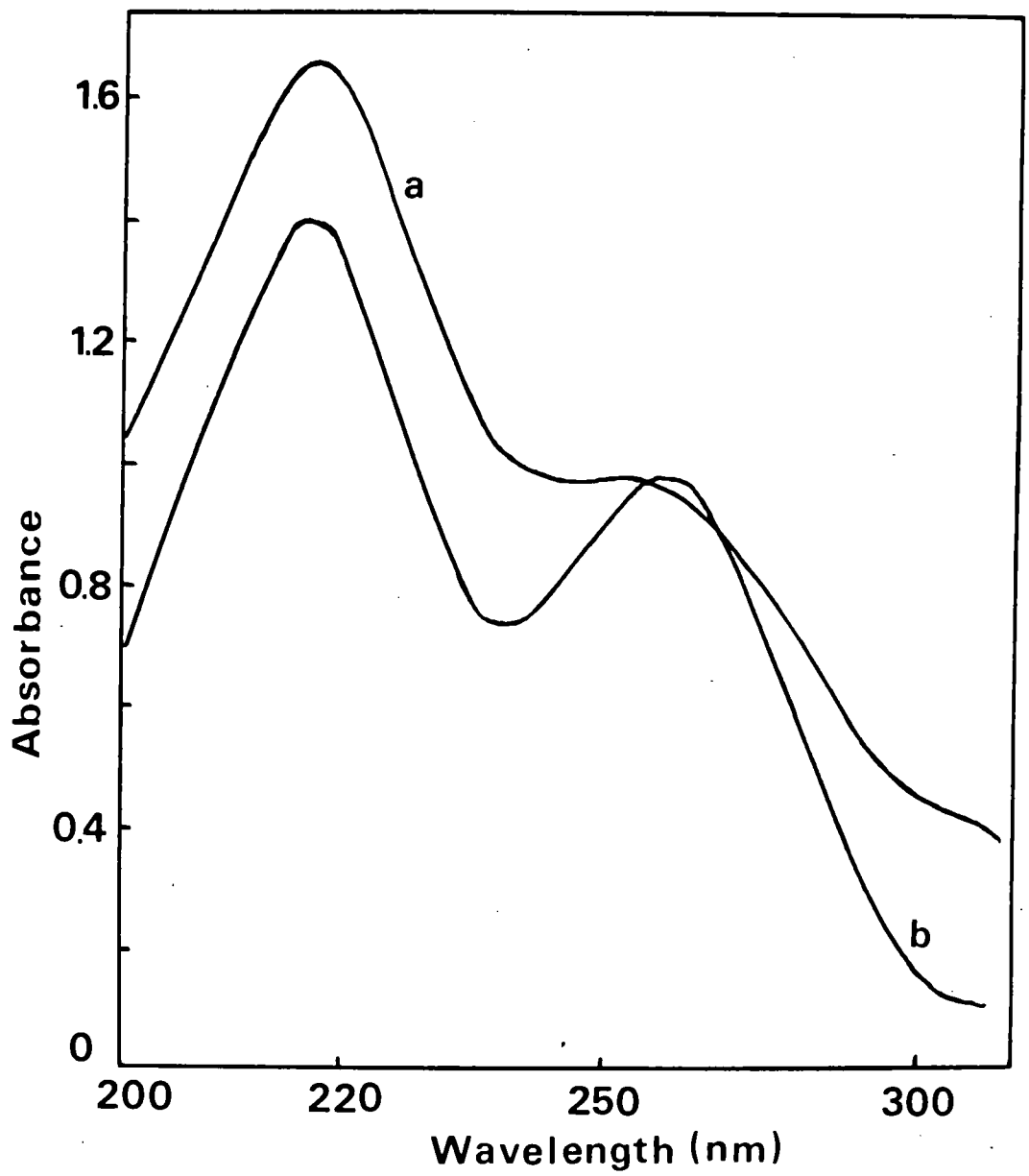


Fig. 25. Absorption spectra of microsome preparations from germinating and developing Vicia faba seeds



Microsome preparations isolated as described in Methods, the absorption spectra of suitable dilutions being determined in water.

a. Microsomes isolated from 7 day old plumules.

b. Microsomes isolated from 65 day old developing bean seeds.

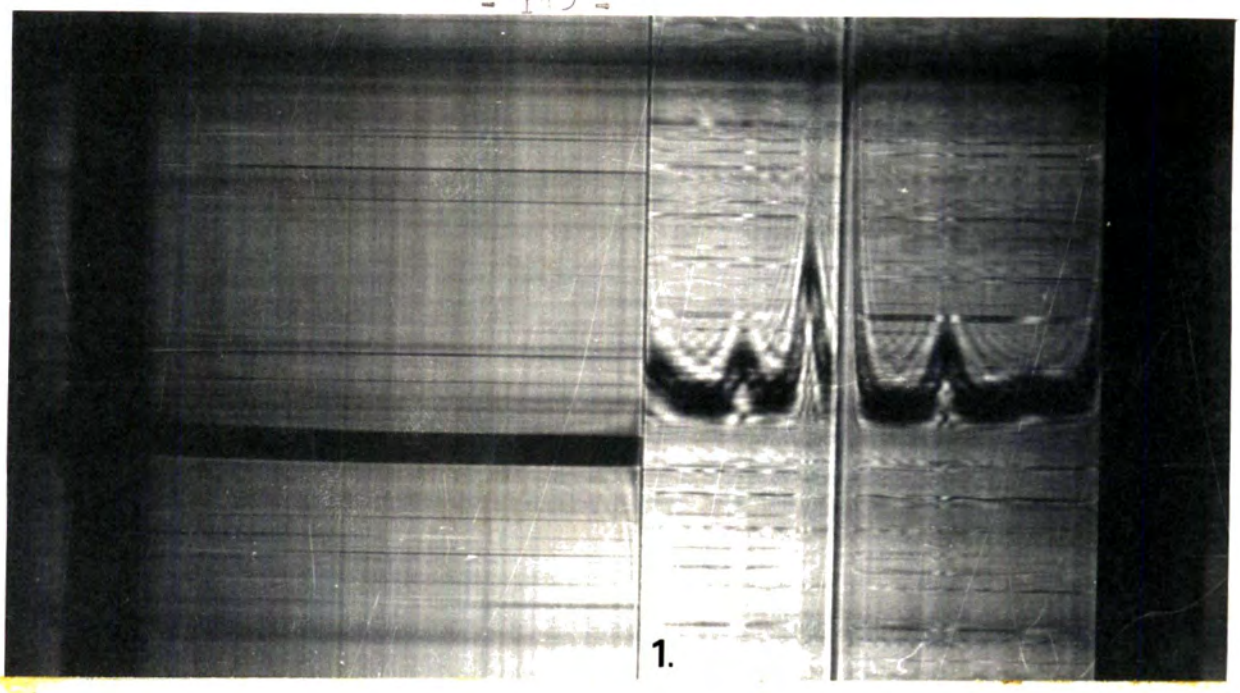
Fig. 26 Schlieren patterns of microsome preparations from Vicia faba and Brassica chinensis

1. Microsome preparation from V. faba 48 h plumules (c. 20 mg/ml 260 nm absorbing material), (fig. 25).

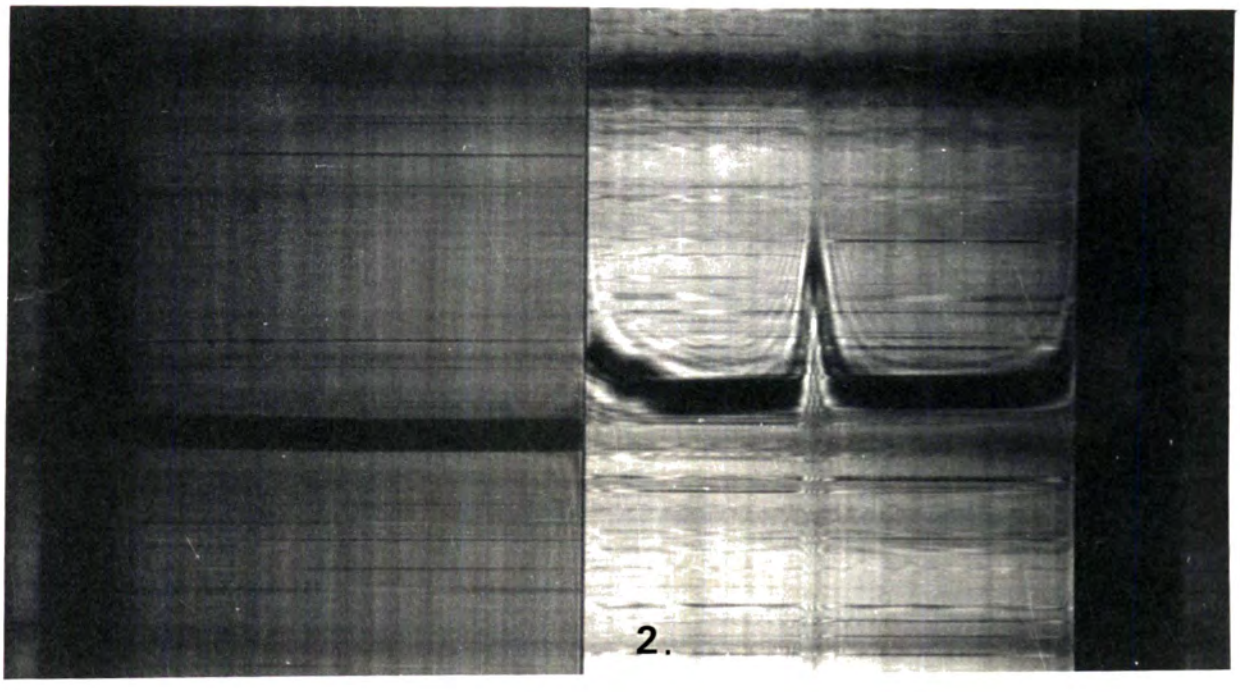
2. Microsome preparation from V. faba 65 day old developing beans (c. 25 mg/ml 260 nm absorbing material), (fig. 25).

3. Microsome preparation from B. chinensis leaves (plants less than one month old) (c. 25 mg/ml 260 absorbing material), (fig. 20).

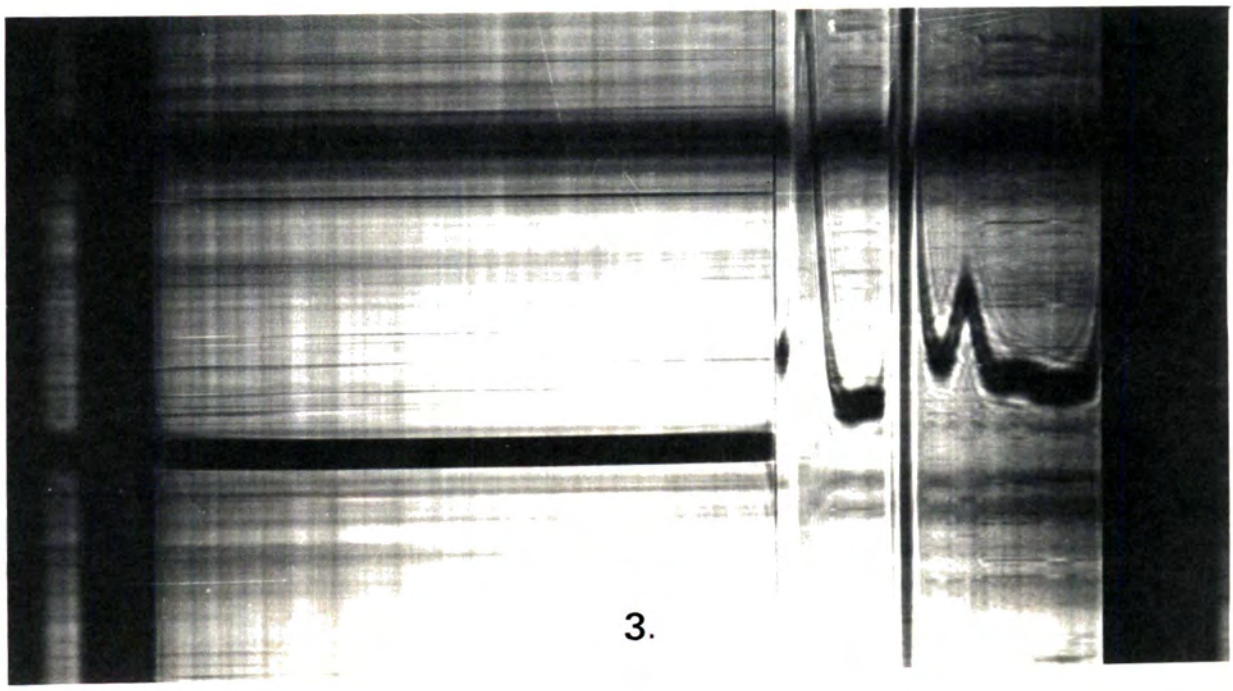
Omega rotor speed	40,000 rev./min
Temperature	$20^{\circ}C$
Bar angle	1. and 2. 60° 3. 65°
Time (after attaining speed)	1. and 2. 5 min; 3. 3 min
Buffer	0.005 M magnesium acetate - 0.01 M KCl - 0.01 M Tris HCl, pH 7.4
Direction of sedimentation	left to right



1.



2.



3.

Small scale preparations from developing bean, examined in the above buffer yielded only an active 79 S component.

The fine white cloud covering the final high-speed pellets of bean microsome preparations was found to have no incorporating activity under test conditions. Similar, but green flocculent coverings of B. chinensis high-speed microsome pellets were found to have activity, in some poly(U) incorporation experiments, almost to the same level as the microsome pellets themselves.

Microsomes prepared by grinding B. chinensis leaves in 0.005 M magnesium acetate - 0.1 M phosphate buffer (Vanyushin and Dunn, 1967), sedimented at 83 S and 117 S in this buffer, but at 105, 84 and 66 S in 0.01 M magnesium acetate - 0.05 M phosphate. These ribosomes were not active in amino acid incorporation, (fig. 27)

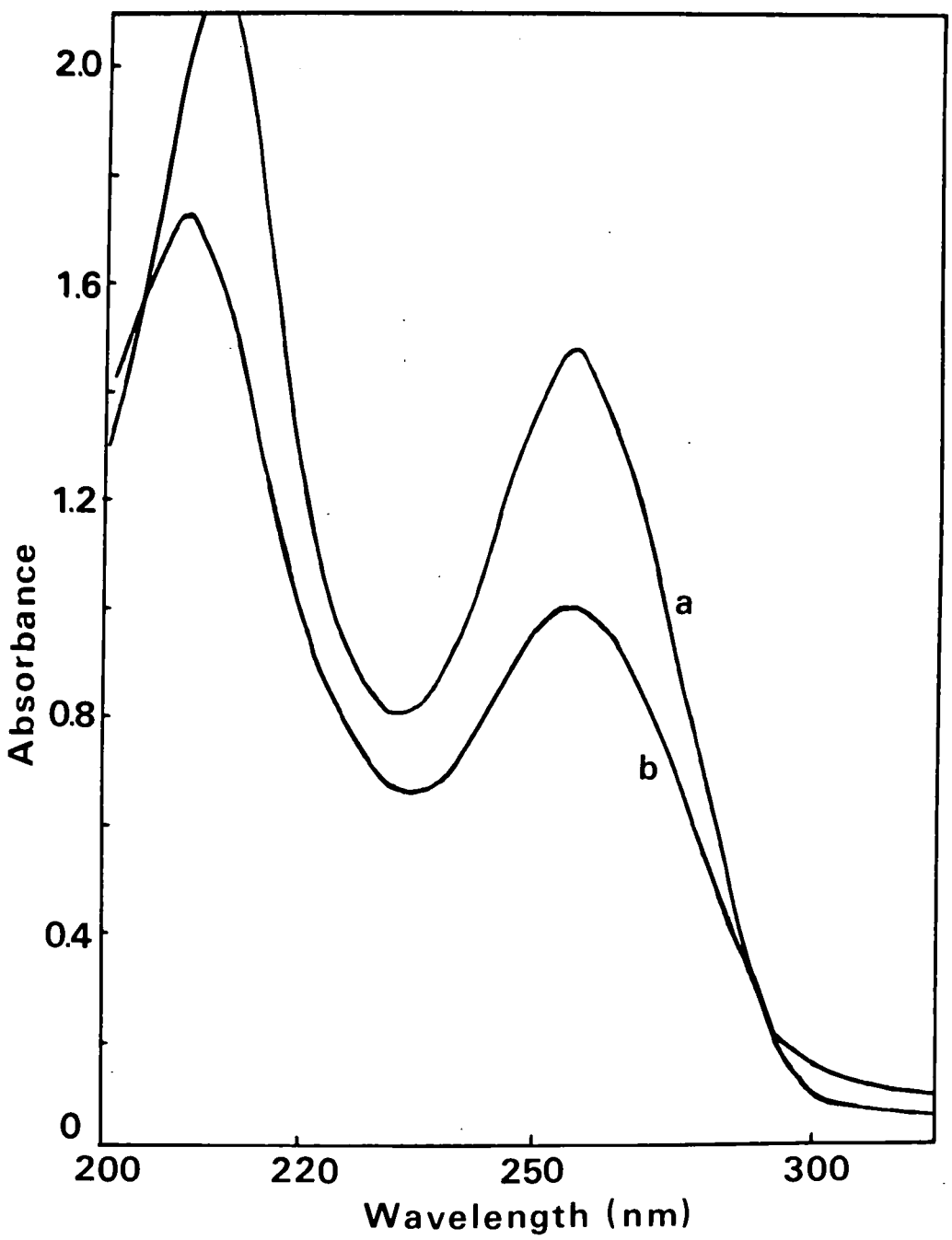
Analytical Ultracentrifugation of Chinese Cabbage Plant Sap:

Samples of B. chinensis leaves extracted as for viral extraction, without the aid of bentonite, were examined in the Omega Ultracentrifuge, using Schlieren optics. Typical profiles resulted as seen in fig. virus particles being completely absent from this uninfected material.

Infected leaves extracted similarly were seen to contain viral components, and occasionally a ribosome dimer of 120 S.

Comparison of fig.28,1 (uninfected sap), fig. 28,3 (infected sap) and fig. 28, 2 (prepared virus) indicate clearly the absence of plant components in purifed viral extracts.

Fig. 27 . Absorption spectra of Brassica chinensis var. Wong Bok leaf microsome preparations



Microsome preparations isolated by the method of Vanyushin and Dunn(1967).

a. Suspended in 0.01 M $MgSO_4$-0.05 M phosphate buffer, pH 7.4(20°C).
b. Suspended in 0.005 M $MgSO_4$-0.01 M phosphate buffer, pH 7.4(20°C).

Absorption spectra of suitable dilutions in water.

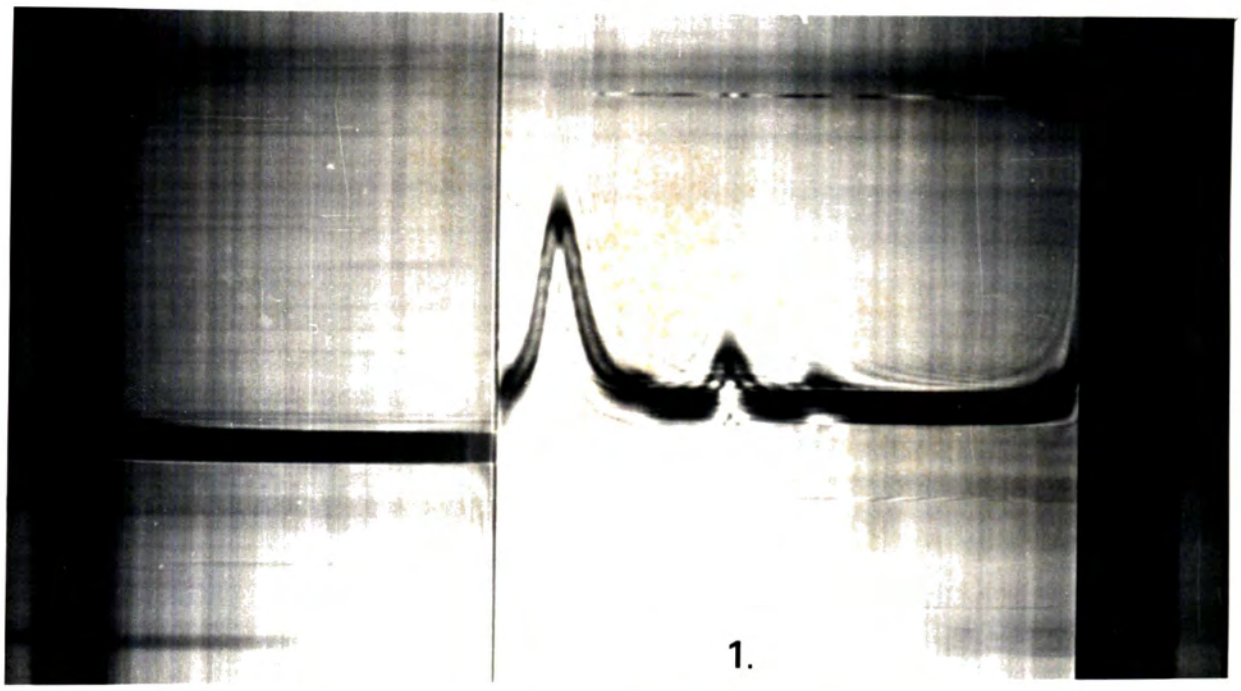
Fig. 28 Schlieren patterns of TYMV, uninfected and TYMV-infected Brassica chinensis leaf sap

1. Uninfected leaves subjected to TYMV extraction procedure of Dunn and Hitchborn (1965). Supernatant from first high-speed spin, (page 73).

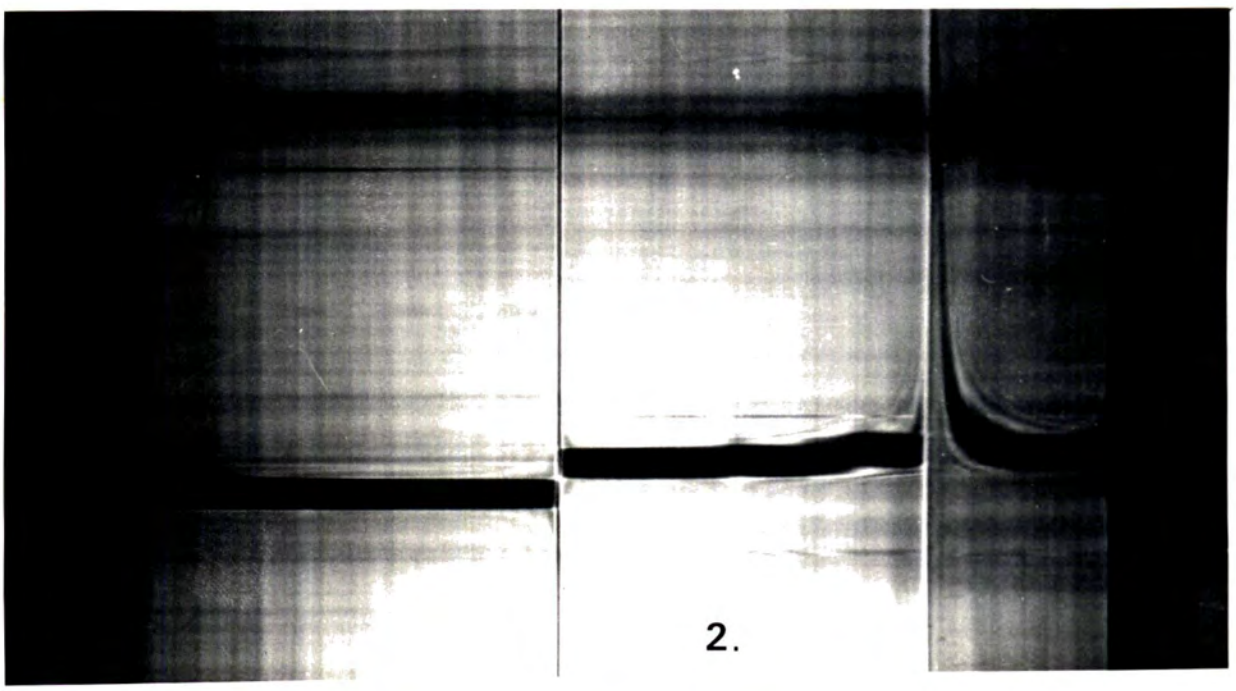
2. TYMV extracted by the procedure of Dunn and Hitchborn (1965). Concentration 3.0 mg/ml in 0.1 M NaCl - 0.01 M phosphate buffer 7.4

3. Infected leaves subjected to TYMV extraction procedure of Dunn and Hitchborn (1965). Supernatant from first high-speed spin, (page 73).

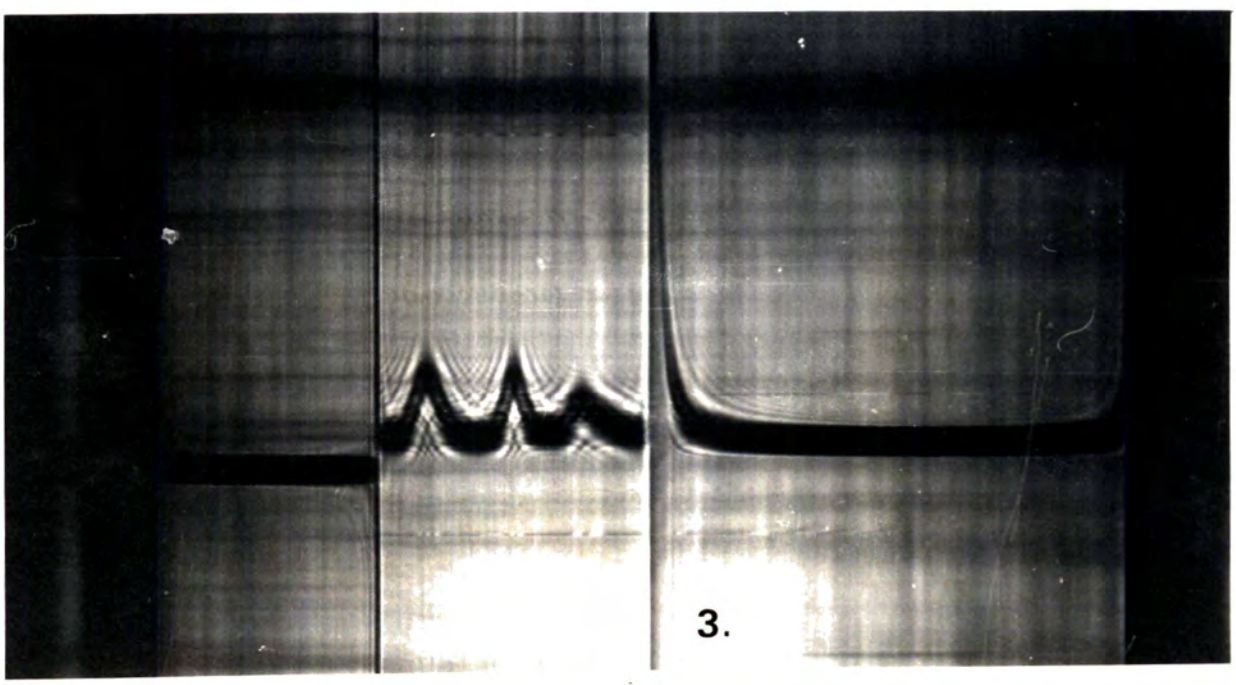
Omega rotor speed	40,000 rev/min
Temperature	$20^{o}C$
Bar angle	1. 55^{o}; 2. 60^{o}; 3. 65^{o}
Time (after attaining speed)	1. 5 min; 2. 6 min; 3. 5 min
Direction of sedimentation	left to right



1.



2.



3.

Brassica chinensis Transfer Nucleic Acid:

(a) From a typical 1 kg sample of Brassica chinensis var. Wong Bok leaves (of various ages), 170 mg of 260 nm absorbing material was isolated, which on chromatography yielded 85-90% of the original material. The chromatographed nucleic acid was taken to be tRNA from its absorption spectrum (fig. 6) and its ability to accept amino acids (Table 17). Deacylation was found to improve the amino acylation capacity of tRNA isolates (Table 17).

Material prepared without the use of magnesium bentonite in the initial stages of isolation exhibited a much greater absorption at 260 nm, than bentonite prepared material, at the 1.0 M NaCl stage in chromatography.

The preparations isolated by ethanol precipitation varied in colour from white to a pale fawn. Storage at -70°C for over one year did not affect the activity of the tRNA, nor did freezing and thawing.

Redistilled phenol was found to result in a degraded tRNA product when employed in the isolation procedure, whereas analytical grade phenol, plus distilled water, stored under nitrogen at -20°C did not. Analytical grade phenol solutions were found to be stable at room temperature, whereas redistilled phenol/water mixtures readily turned red.

(b) Aminoacylation was found to be dependent on the presence of tRNA (and tRNA concentration, fig. 29), ATP and enzyme (Tables 18 and 20) and also on method of extraction and treatment (Table 17). The absence in the incubation mix of the remaining complement of $\left[^{12}C\right]$ amino acids reduced the charging capacity of the enzyme and the c.p.m. per tRNA sample by c. 5%.

Table 17

Comparison of Aminoacylation Capacity of Various tRNA Samples

Method of Isolation	tRNA Type	c.p.m./mg tRNA
Simple grinding/separate phenol extraction, no magnesium bentonite	B. chinensis	971
	V. faba(i)	12,547
Grinding with phenol only, no magnesium bentonite	B. chinensis	1,185
Normal extraction with separate phenol and bentonite treatments	B. chinensis	2,150
Combined phenol/bentonite extraction	B. chinensis(ii)	3,434
Subsequent Treatment		
Before chromatography	B. chinensis(ii)	3,200
After chromatography (and prior to deacylation)	B. chinensis(ii)	18,200
Prior to deacylation	V. faba (i)	12,547
	Yeast(iii)	3,775
After deacylation	B. chinensis (ii)	22,445
	V. faba (i)	24,300
	Yeast(iii)	5,270

Aminoacylation with $[^{14}C]$ phenylalanine was carried out under standard experimental conditions as described in Methods(and in Table 18).

A tRNA concentration of 0.5 mg of 260 nm absorbing material,and 0.1 ml Vicia faba 'enzyme' per 0.5 ml incubation were employed.

Numbers refer to the same samples at different stages of treatment

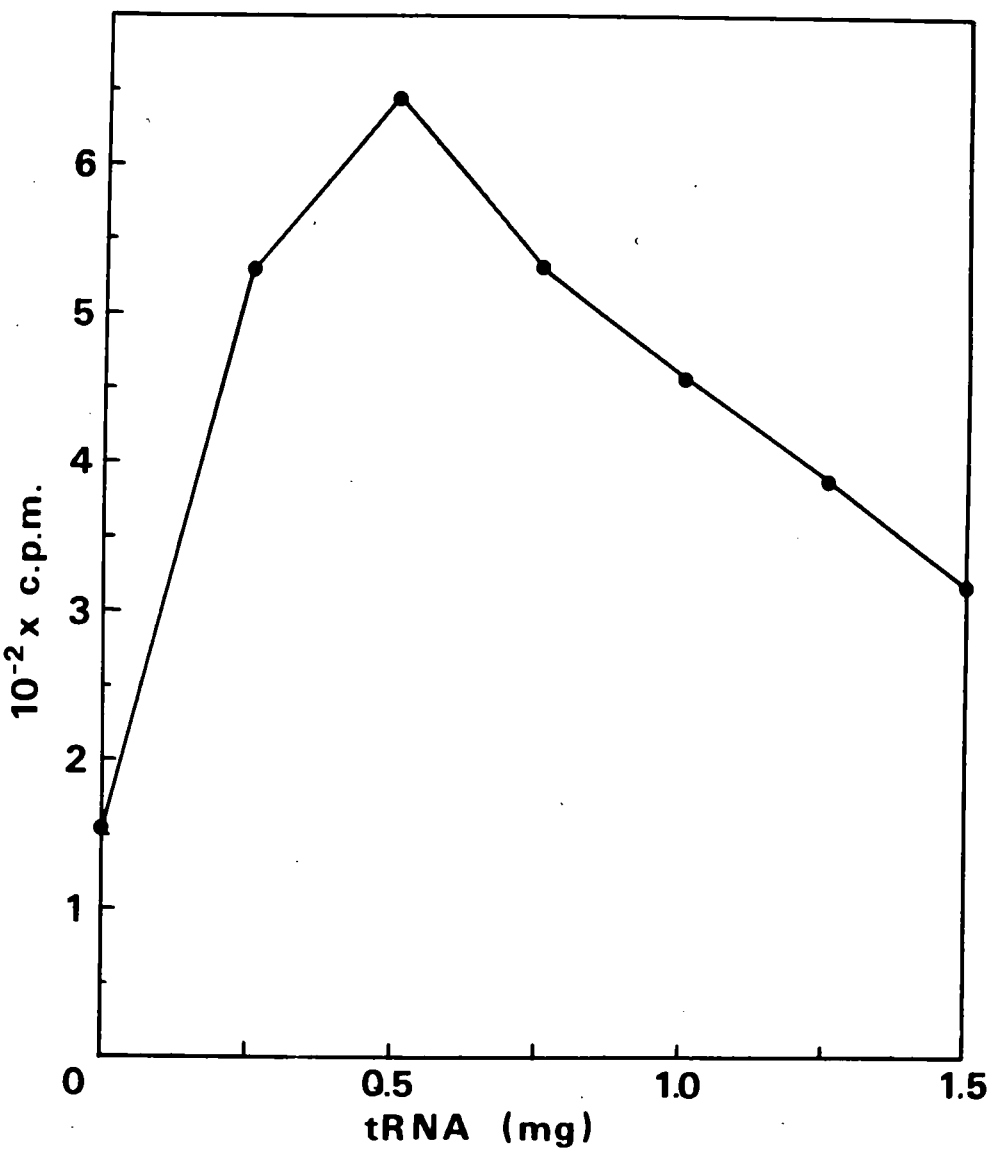
Table 18

Requirements for phenylalanine acylation of Brassica chinensis tRNA

Additions or omissions	Normal washing c.p.m./mg tRNA	Hot trichloroacetic acid wash for 30 min c.p.m./mg tRNA
Complete		
(- [^{12}C] amino acids)	21,445	200
+ [^{12}C] amino acids	22,580	231
- Enzyme	1,392	184
- tRNA	Background only	Background only
- ATP	1,200	178

The acylation of Brassica chinensis tRNA was carried out in 0.5 ml of a mixture containing 50 μmol Tris-HCl, pH 7.8, 8.75 μmol magnesium acetate, 10 μmol GSH, 2 μmol ATP, 0.5 mg tRNA, 0.15 ml dialysed B. chinensis high-speed supernatant ('enzyme preparation') and 0.01 μmol [^{14}C] Phe (specific activity 48 mCi/mmol). The incubation was for 20 min at 25^{o}C, after which time four, 0.1 ml samples were taken and added by sorption to filter paper discs. Two discs were washed normally (page 97), two were subjected to 5% (w/v) trichloroacetic acid at 90^{o}C for 30 min. Background 80 c.p.m. Results average of three determinations.

Fig. 29 . Effect of tRNA concentration on the aminoacylation of Brassica chinensis tRNA with [^{14}C] phenylalanine



Incubation conditions as described in Methods, samples being taken after 25 min incubation. Magnesium concentration 10 mM.
The results are the average of three determinations, being the c.p.m. per 0.1 ml sample of incubation mix. tRNA concentrations are per 0.5 ml incubation.

The aminoacyl-tRNA complexes were found to be hydrolysed by 5% (w/v) trichloroacetic acid at 90°C (Table 18).

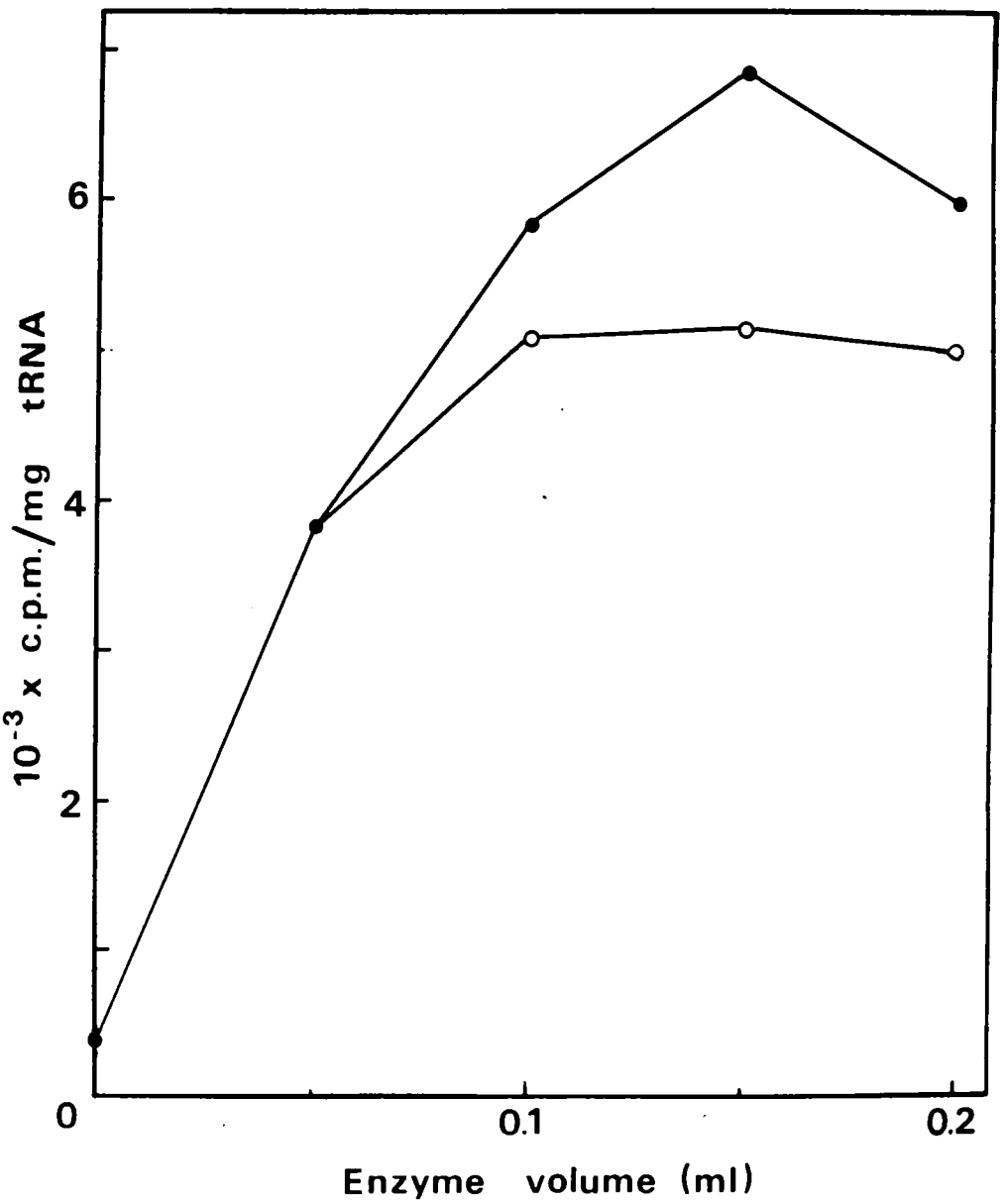
The optimum concentration of enzyme was determined experimentally for each 'batch', the results of a typical enzyme assay being expressed graphically in fig. 30 . In general the volume of enzyme promoting maximum charging with phenylalanine of B. chinensis tRNA was found to be 0.15 ml of B. chinensis enzyme (i.e. final high-speed supernatant), and 0.1 ml of V. faba 'enzyme' (per 0.5 ml incubation volume). The amount of enzyme varied slightly with the age of the plant source and to a lesser extent with the method of extraction.

Magnesium ion concentration was found to have an effect on charging capacity. On charging B. chinensis tRNA with phenylalanine, maximum charging occurred at a Mg^{2+} concentration of 17.5 mM, although there was little variation over the range 10-25 mM (fig. 31).

The time course of tRNA aminoacylation varied somewhat with the source of the tRNA, the source of the enzyme (figs. 32, 33) and to a lesser extent with the $\left[^{14}C\right]$ amino acid employed (Table 19) Prolonged incubations were not routinely performed, samples normally being taken after 20 to 40 min. to indicate the degree of aminoacylation as a reference point for incorporation experiments.

Total amino acid acceptor activity and total aminoacyl synthetase activity of the enzyme preparations were determined using a $\left[^{14}C\right]$ amino acid mixture, and comparison made with single amino acid aminoacylations (Table 19). Charging was again found to be dependent upon the addition of tRNA and enzyme (Table 20).

Fig. 30 . Effect of enzyme concentration on the aminoacylation of tRNA with [^{14}C] phenylalanine

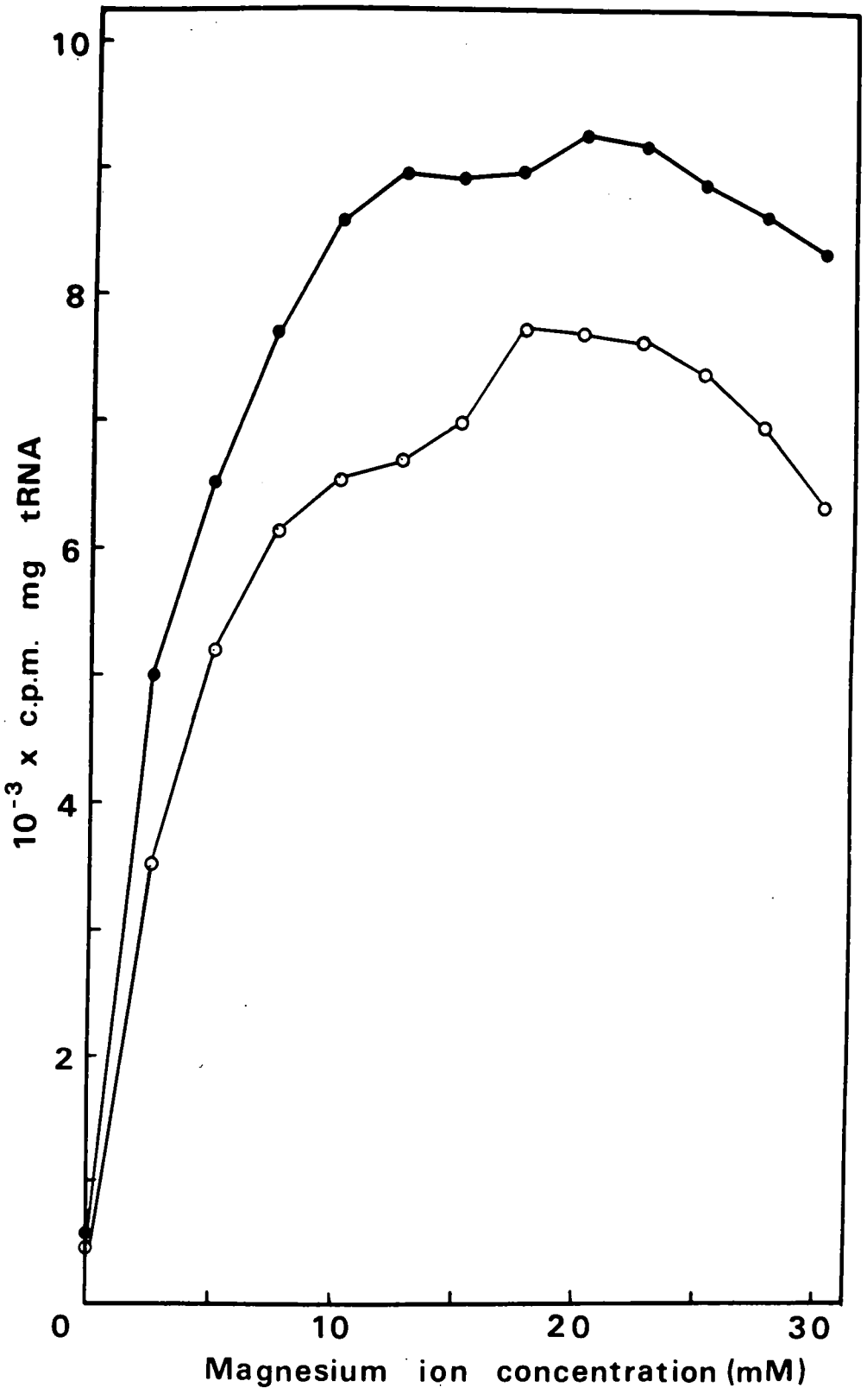


Incubation conditions are as described in Methods except that a Mg^{2+} concentration of 10 mM was employed. Samples were taken at 20 min, and discs washed normally. The results are the average of three determinations.

●——● Brassica chinensis tRNA

○——○ Yeast tRNA

Fig. 31. Effect of magnesium acetate concentration on the aminoacylation of Brassica chinensis tRNA with [^{14}C] phenylalanine

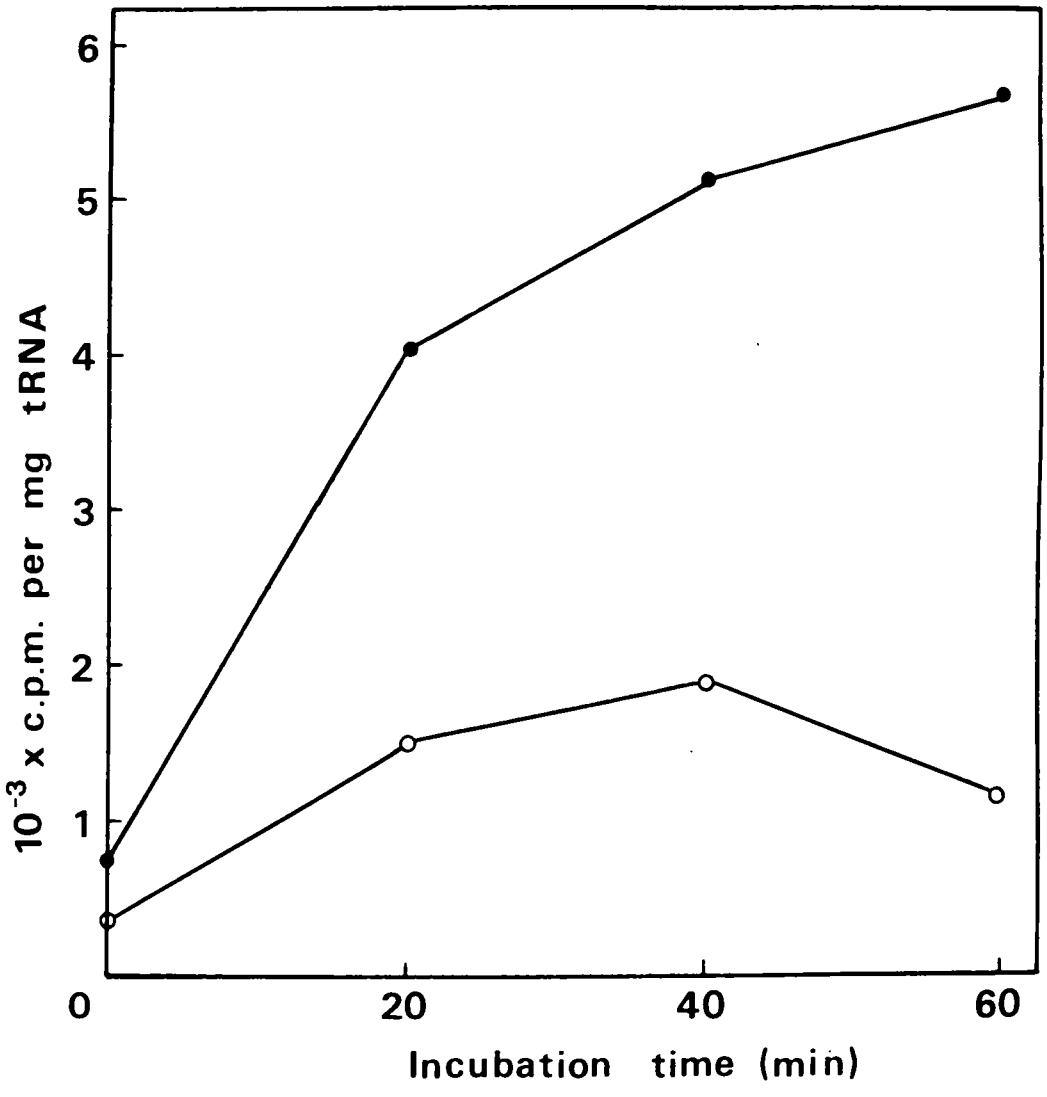


Incubation conditions are as described in Methods.
The results are the average of four determinations.

●——● sample taken after 20 min

○——○ sample taken after 40 min

Fig. 32 . Time course of aminoacylation of Brassica chinensis tRNA with [^{14}C] phenylalanine: Substitution of tRNA with yeast tRNA

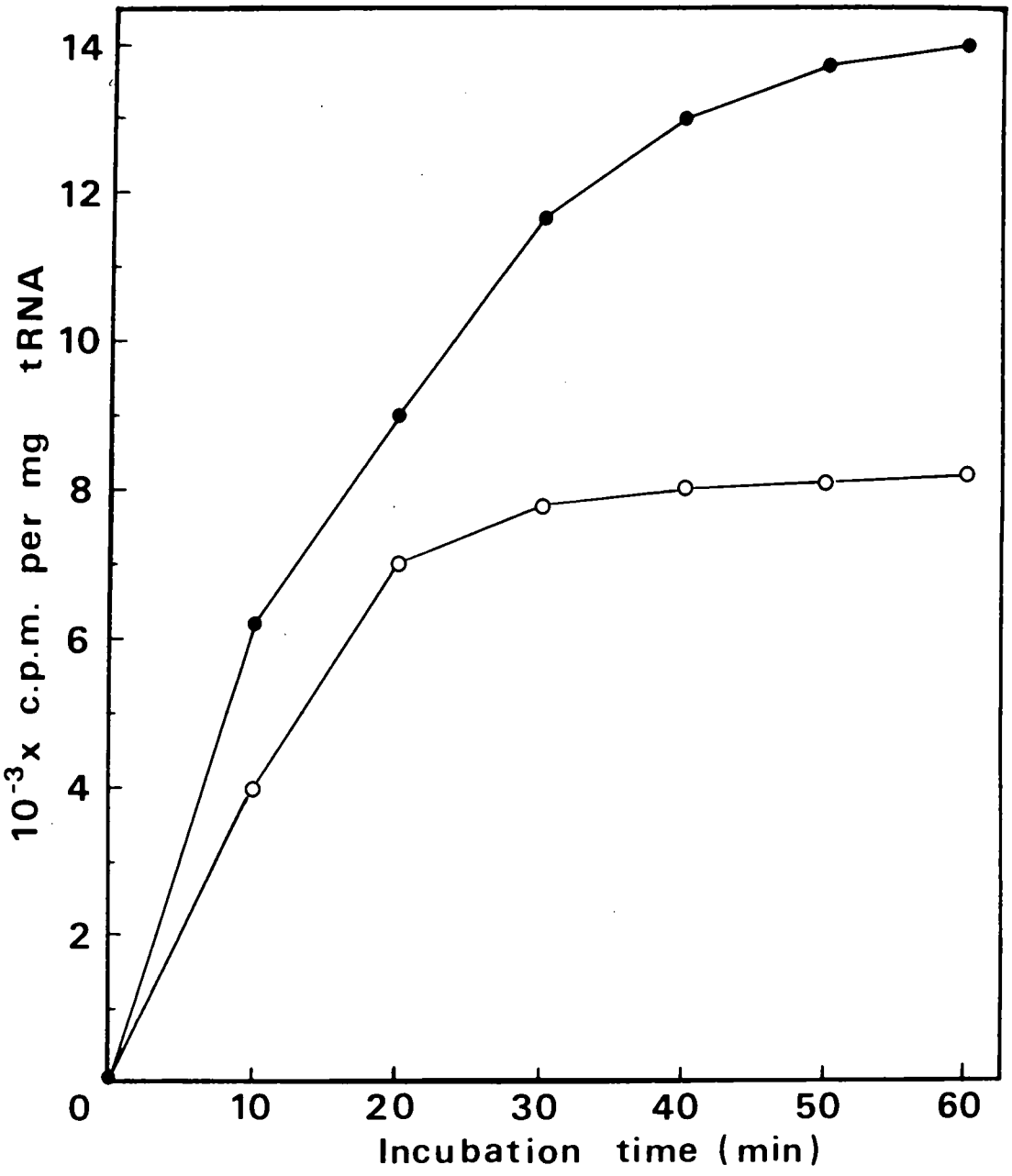


Incubation conditions as described in Methods, except that a magnesium acetate concentration of 10 mM was employed, and an enzyme volume of 0.1 ml per ml incubation. The results are the average of three determinations.

●——● Yeast tRNA

○——○ B.chinensis tRNA

Fig. 33 . Time course of aminoacylation of Brassica chinensis tRNA with [^{14}C] phenylalanine: Use of Brassica chinensis and Vicia faba enzymes



Incubation conditions as described in Methods, except that a magnesium acetate concentration of 10 mM was employed and an enzyme volume of 0.1 ml per ml incubation. The results are the average of three determinations.

●——● V.faba enzyme

○——○ B.chinensis enzyme

Table 19

Comparison of charging capacity of Brassica chinensis tRNA by various $[^{14}C]$ amino acids

$[^{14}C]$ Amino Acid	c.p.m. per mg tRNA
Mixture	43,017
Phe	16,900
Leu	7,218
Val	9,800

Incubation conditions as described in Methods (and Table 18)

Individual amino acids, 0.01 μmol per 0.5 ml incubation. Specific activities Phe - 48 mCi/mmol; Leu - 55mCi/mmol; Val - 33.9 mCi/mmol.

Amino acid mixture, 0.01 ml per 0.5 ml incubation, and specific activity 52 mCi/mAtom.

Table 20

Requirements for aminoacylation of Brassica chinensis tRNA with $[^{14}C]$ amino acid mixture

Incubation	Normal Washing c.p.m. per mg tRNA	Hot trichloracetic acid wash c.p.m. per mg tRNA
Complete	43,017	1,300
- tRNA	Background count only	
- Enzyme	1,980	760

Incubation conditions as described in Methods(and Tables 18 and 19)

Transfer RNA stored at -70°C remained active over a twelve month period, and two samples of B. chinensis tRNA, one stored at -20°C and the other at -70°C, were found to have similar activities after ten months storage. Repeated freezing and thawing of these samples or storage under alcohol did not adversely affect tRNA activity.

Although at least two separate tRNA peaks were obtained at the 1.0 M NaCl stage of tRNA chromatography, the activity of the peaks varied by only 5-10% and so a mixture of the two peaks was routinely used for experimental purposes.

Aminoacylation of Turnip Yellow Mosaic Virus Nucleic Acid:

Two samples of TYMV RNA examined were found to have $\left[^{14}C\right]$ valine acceptor activity under optimum phenylalanine aminoacylation conditions for B. chinensis tRNA.

TYMV RNA charging continued for up to 50 min, then levelled off, whereas B. chinensis tRNA levelled off after c. 75 min (fig. 34).

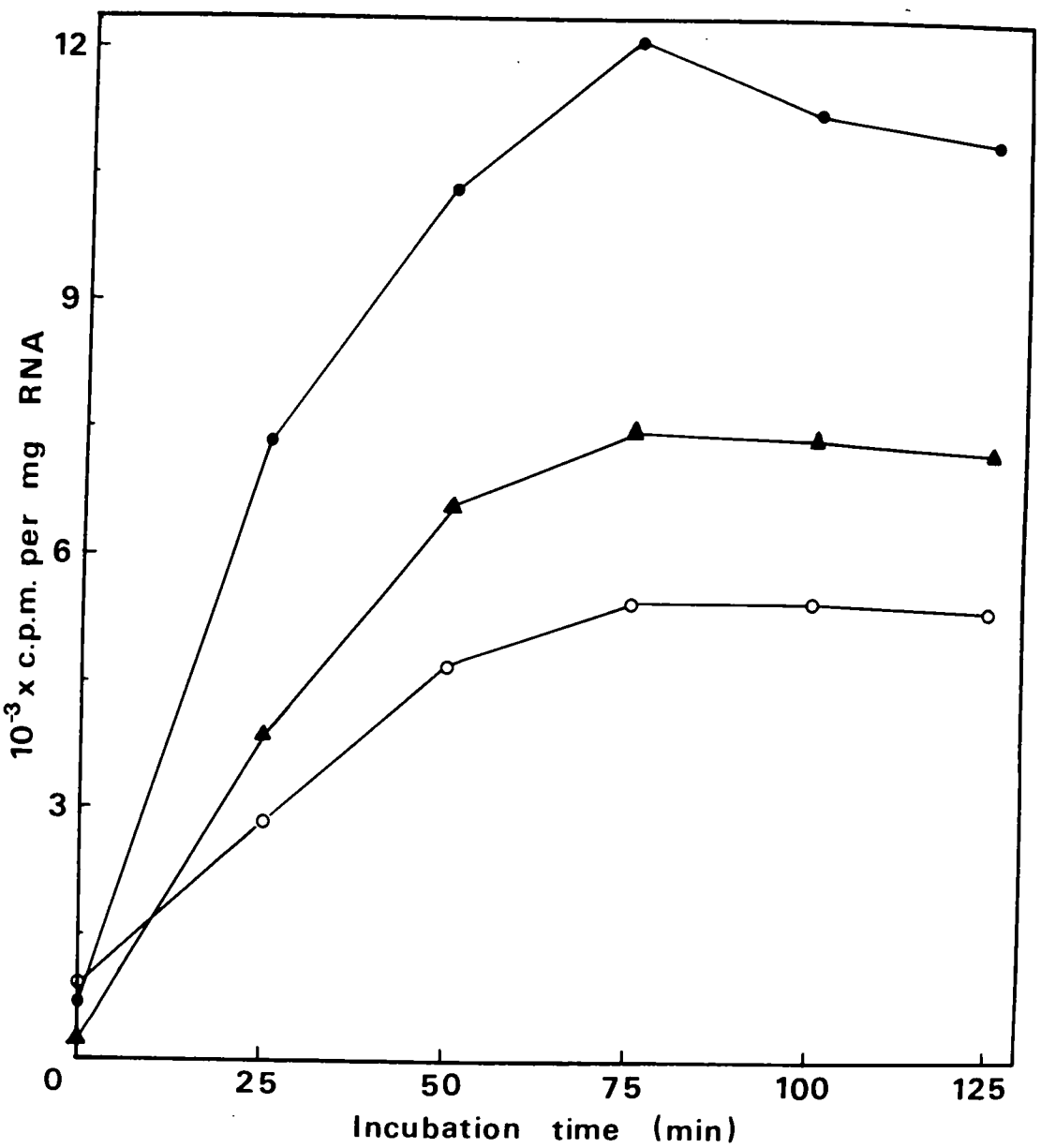
TYMV RNA was found not to accept phenylalanine under test conditions and the aminoacyl-TYMV RNA complex formed with valine was found to be hydrolysed by 5% (w/v) trichloracetic acid at 90°C.

Cell-free Amino Acid Incorporation:

Free amino acids were incorporated into hot (90°C) trichloroacetic acid insoluble material in the presence of tRNA, microsomes and enzyme.

Various combinations of tRNA, microsomes and enzyme from Brassica

Fig. 34. Aminoacylation of Brassica chinensis tRNA and TYMV RNA with [^{14}C] valine



Incubation conditions as described in Methods, with RNA concentration of 1 mg/ml in each case. Results are from a single experiment.

●——● B.chinensis tRNA (sample 1 from chromatography)

▲——▲ TYMV RNA prepared by Dunn and Hitchborn (1966) method. Two

○——○ samples prepared from two batches of TYMV prepared by usual methods.

chinensis and V. faba (and yeast tRNA) were employed, incorporation being stimulated by either the artificial messenger poly(U), promoting polyphenylalanine synthesis, or by TYMV RNA.

(a) Vicia faba System:

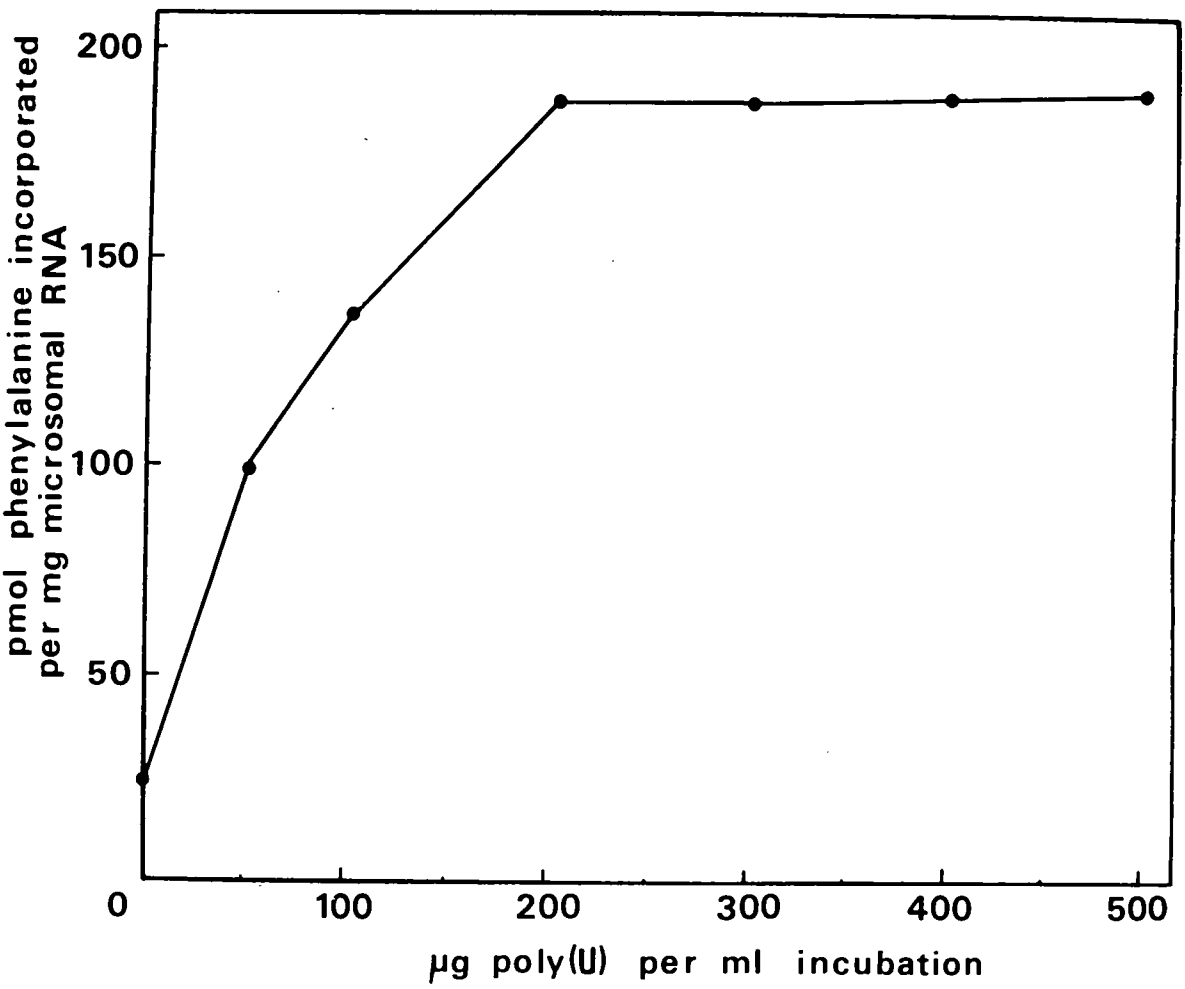
Preliminary experiments using a 'standard' Vicia faba system (comprising enzyme and microsomes from 65-day old and tRNA from 40-day old developing bean seeds) were performed to determine the activity of an equivalent Brassica chinensis leaf system. Germinating bean experiments (as with germinating B. chinensis experiments) resulted in little activity.

In the bean system, phenylalanine incorporation was found to be dependent upon the addition of poly(U), maximum incorporation occurring at a poly(U) concentration of 200 μg per ml incubation (fig. 35). This concentration was employed as standard in all other incorporation experiments involving phenylalanine and poly(U). Under standard conditions, after incubation at 25^{o}C for 40 min, 200-250 pmol of phenylalanine were incorporated. In the absence of poly(U), incorporation was reduced to 10-25 pmol.

Incorporation was found to be dependent upon the addition of microsomes and enzyme (Table 21).

In most experiments a single batch of V. faba high speed supernatant was employed (from 65-day old developing beans), 0.1 ml of this preparation stimulating maximum incorporation.

Fig. 35. Effect of poly(U) concentration on [^{14}C] phenylalanine incoorporation in the Vicia faba in vitro system



Incubation conditions were as described in Methods, samples being taken after 40 min. The results are derived from a single experiment.

Table 21

Effect of co-factors on [^{14}C] phenylalanine incorporation in the Vicia faba system

Incubation	pmol [^{14}C] phe incorporated per mg microsomal RNA
Complete + poly(U)	178
- poly(U)	22
- microsomes	Background count (c. 50 c.p.m./disc)
- enzyme	28

Incubation conditions as described in Methods, samples being taken after 40 min incubation. tRNA from 40-day old and enzyme and microsomes from 65-day old developing V. faba seeds.

Maximum incorporation occurred when employing a microsome concentration of 250-500 μg microsomal RNA per ml incubation (fig. 36) although the lower concentration was routinely used.

Poly(U) directed incorporation was found to be influenced by temperature, maximum incorporation occurring at 25°C (fig. 37). A slight decrease in the optimum temperature of incubation was noted with increase in length of time of incubation. In the absence of poly(U), temperature change had little influence on the level of incorporation.

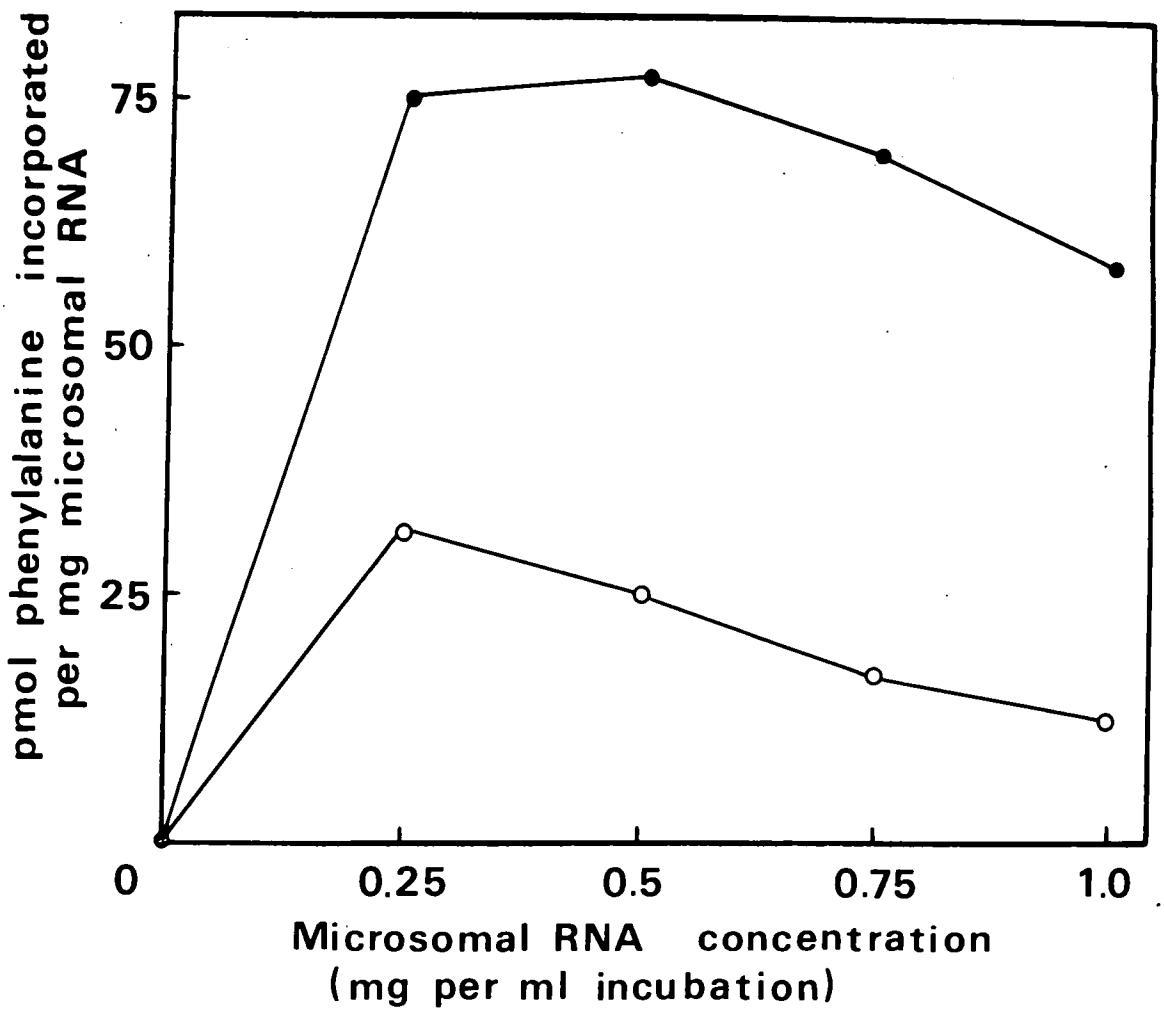
Incubations performed at 25°C (pH 7.8) were found to be significantly affected by the magnesium concentration. Maximum incorporation occurred at 8 mmol per l Mg^{2+}, although a second maximum was observed at 20 mM (fig. 38). The lower Mg^{2+} concentration was used routinely.

(b) Brassica chinensis System:

Phenylalanine incorporation in the B. chinensis system was found to be dependent upon the addition of poly(U), enzyme (and enzyme concentration, fig. 39) and microsomes (Table 22).

Magnesium ion concentration exhibited a pronounced effect on the level of incorporation, maximum incorporation occurring at 6 mmol/l Mg^{2+} at pH 7.8. As with the bean system, a second optimum was noted (16 mM) (fig. 40) although again the lower concentration was used routinely. Even when incorporating maximally, the cabbage system had only 10% of the activity of the similar bean system.

Fig. 36 . Effect of microsome concentration on [^{14}C] phenylalanine incorporation in the Vicia faba in vitro system

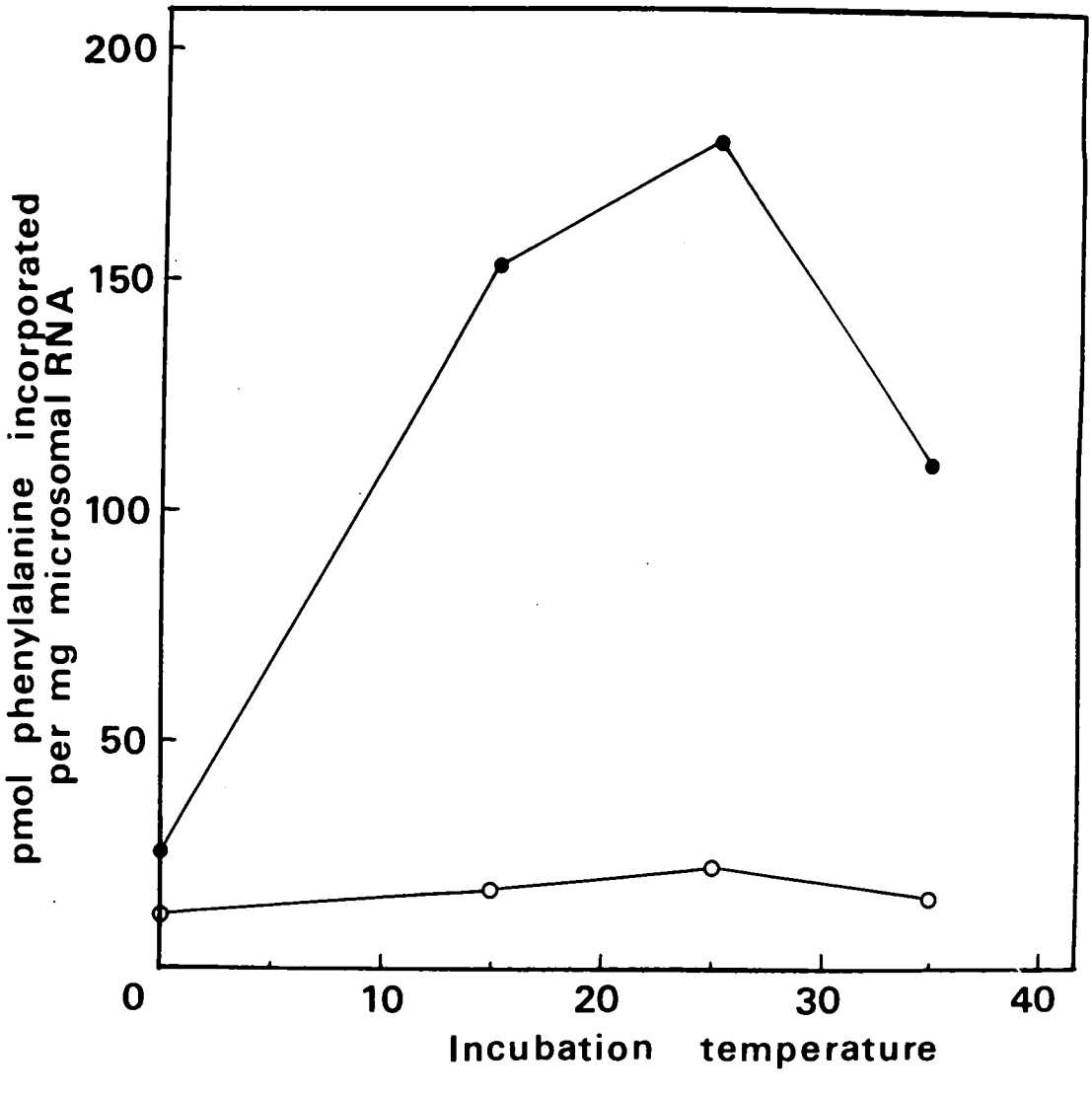


Incubation conditions as described in Methods, employing a poly(U) concentration of 200 µg/ml, and a magnesium concentration of 8 mM.
The values are the average results from three experiments, samples being taken after 40 min.

●——● plus poly(U)

○——○ minus poly(U)

Fig. 37 . Effect of temperature on [^{14}C] phenylalanine incorporation in the Vicia faba in vitro system

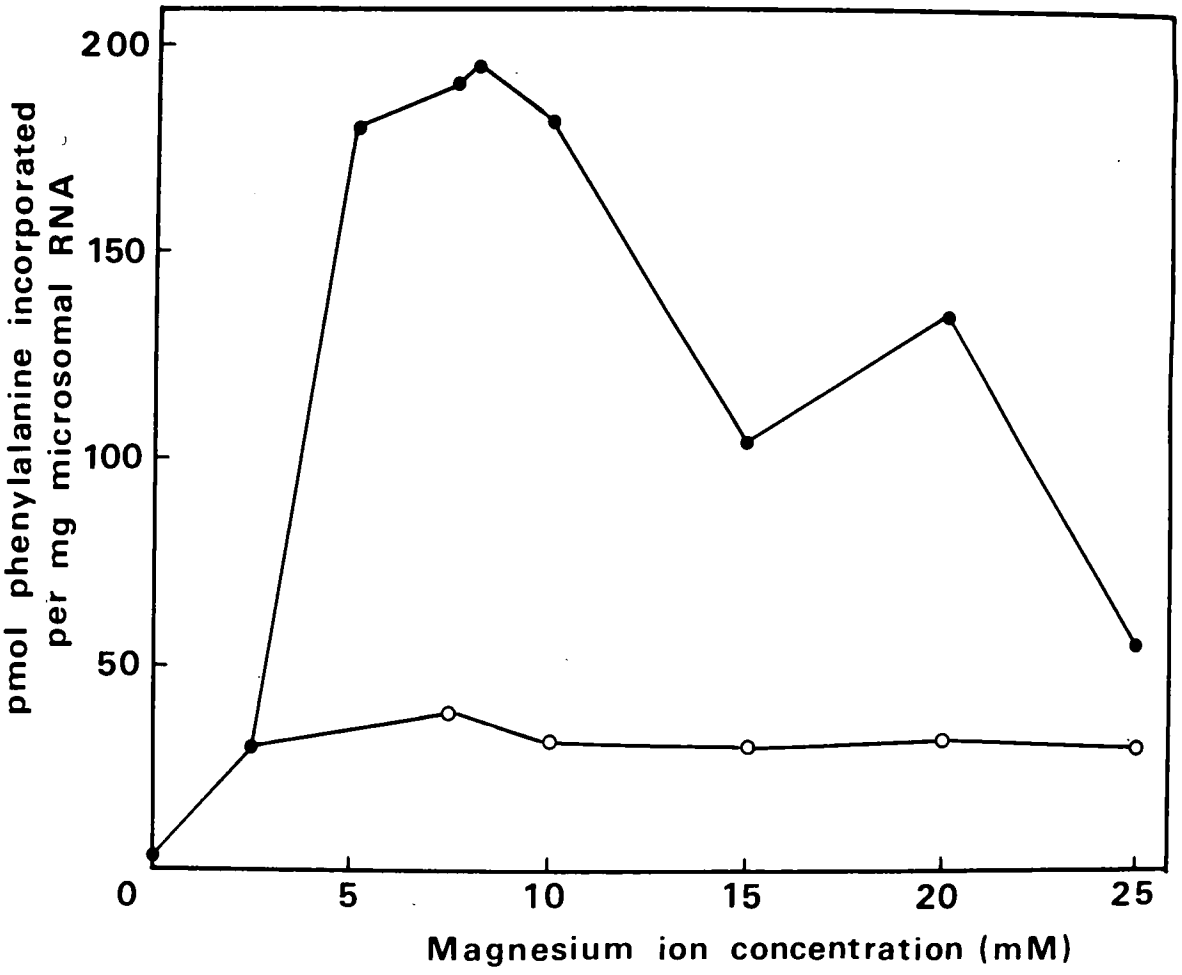


Incubation conditions as described in Methods, samples being taken after 40 min. The results are the average of two determinations.

●——● Incubations with poly(U)

○——○ Incubations minus poly(U)

Fig. 38 . Effect of magnesium acetate concentration on [^{14}C] phenylalanine incorporation in the Vicia faba in vitro system



Incubation conditions as described in Methods, employing 200 µg/ml incubation poly(U). Samples taken at 40 min, the values being the average of three determinations.

●——● plus poly(U)

○——○ minus poly(U)

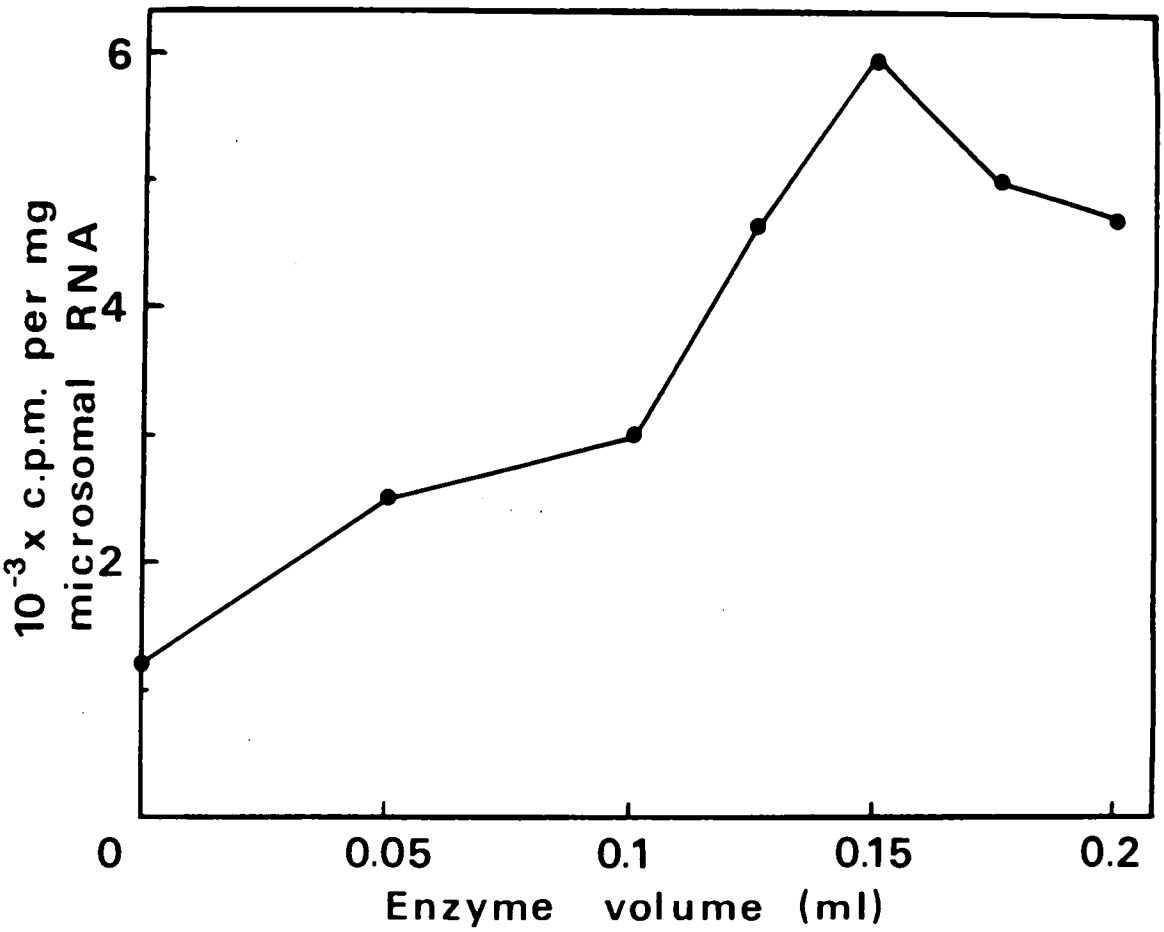
Table 22

Effect of co-factors on [^{14}C] phenylalanine incorporation in the Brassica chinensis system

Incubation	pmol [^{14}C] phe incorporated per mg microsomal RNA
Complete + poly(U)	16
- poly(U)	2
- enzyme	4
- microsomes	Background count only (c. 50 c.p.m./disc)
Complete + poly(U) + 0.2 mM Spermine	35

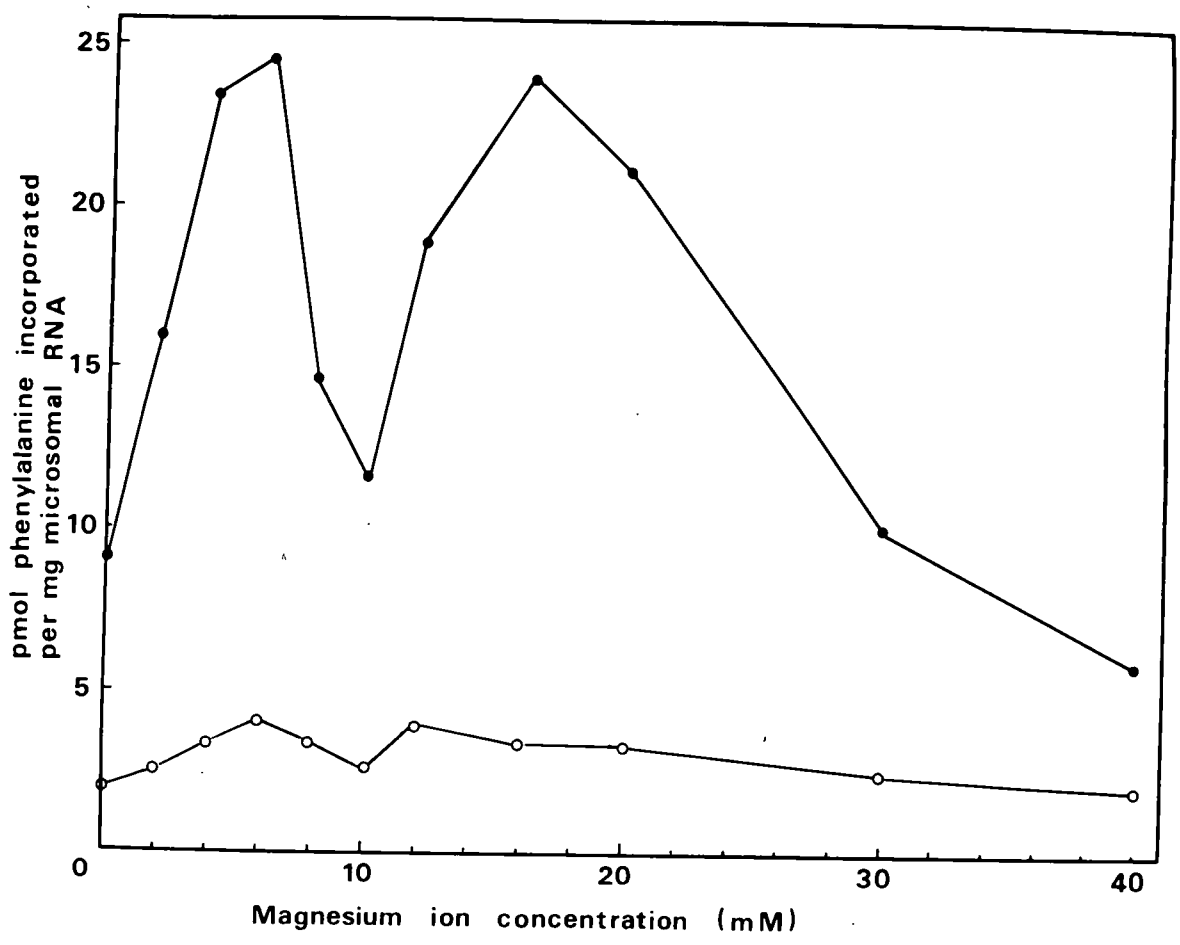
Incubation conditions as described in Methods, samples being taken after 20 min at 25^{o}C.

Fig. 39 . Effect of enzyme concentration on [^{14}C] phenylalanine incorporation in the Brassica chinensis in vitro system



Incubation conditions as described in Methods. Samples taken after 40 min, poly(U) concentration 200 µg/ml incubation. The results are the average of three determinations.

Fig. 40. Effect of magnesium acetate concentration on [^{14}C] phenylalanine incorporation in the Brassica chinensis in vitro system



Incubation conditions as described in Methods, employing a poly(U) concentration of 200 µg/ml incubation. The results are the average of three determinations, samples being taken after 40 min.

●——● plus poly(U)

○——○ minus poly(U)

Under standard conditions, with 'good' preparations, after 20 min incubation at 25°C, 10-20 pmol of phenylalanine were incorporated under poly(U) direction. After 40 min, 20-25 pmol per mg microsomal RNA were incorporated. Further addition of 200 µg poly(U) per ml incubation, after 40 min of incubation, resulted in 20% increase in incorporation. In the absence of poly(U), incorporation was 5-10 pmol.

In the majority of experiments, results were much more variable often only 5-10 pmol phenylalanine being incorporated per mg microsomal RNA (above background).

In one experiment, in which 16 pmol phenylalanine were incorporated (minus poly(U) equalling 2 pmol) under poly(U) direction, incorporation was doubled to 35 pmol in the presence of 0.2 mM spermine.

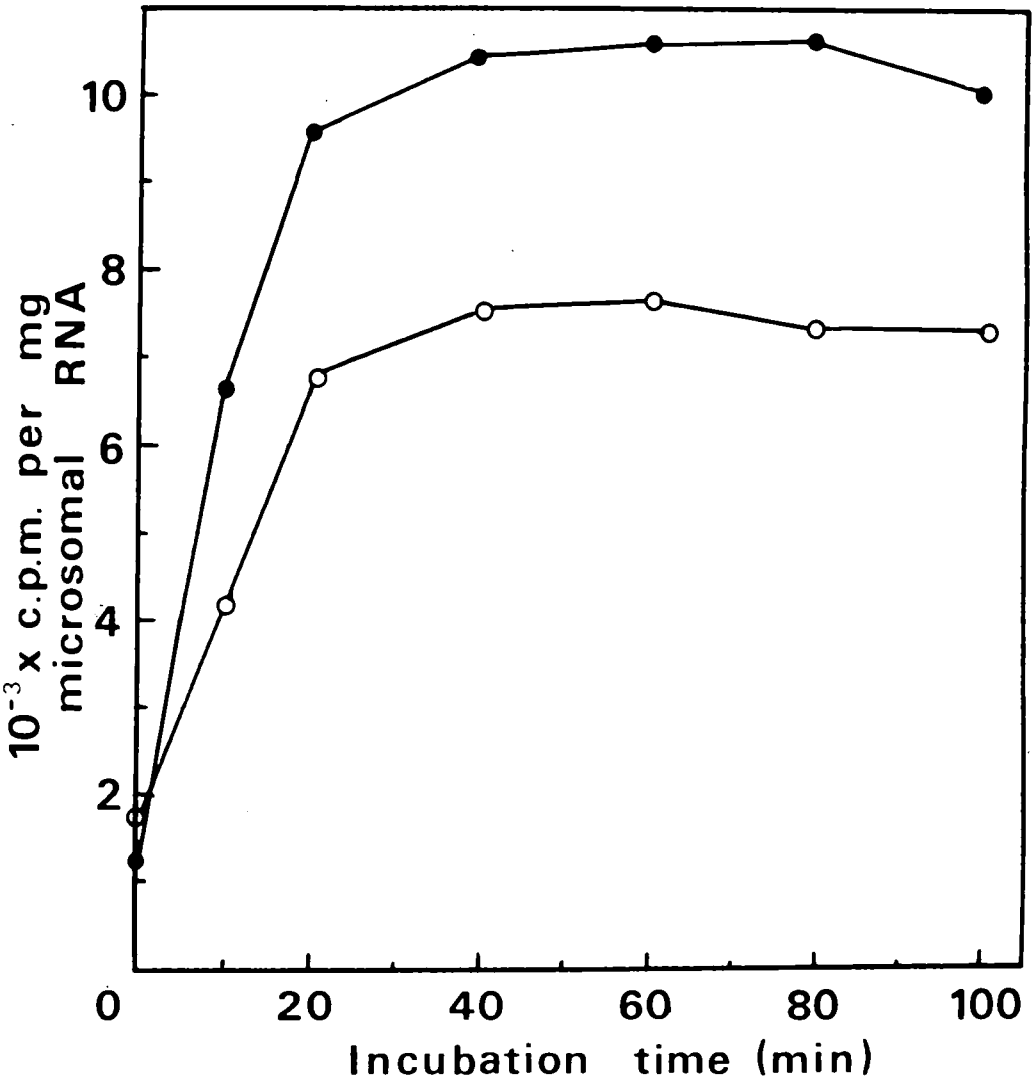
(c) Mixed Systems:

Although B. chinensis and V. faba systems incorporated phenylalanine in vitro, to varying extents under the direction of poly(U), the time courses of incorporation at 25°C were somewhat similar (fig. 41). Linearity was maintained for up to 30 to 40 min in both systems.

Various combinations of enzyme and ribosomes from the two systems were employed to test components and in an attempt to determine if poly(U) was active in a heterologous plant system (Table 23).

On substituting B. chinensis tRNA in the bean system, there

Fig. 41 . Time courses of $[^{14}C]$ incorporation in the Brassica chinensis and Vicia faba systems under TYMV RNA direction



Incubation conditions as described in Methods, employing microsomes from 10 to 20 day B.chinensis leaves and from 60-65 day developing V.faba seeds.
The results are the average of two determinations.

●——● B.chinensis system

○——○ V.faba system

Table 23

Effect of mixing Brassica chinensis and Vicia faba amino acid incorporating systems on $[^{14}C]$ phenylalanine incorporation under poly(U) direction

	Incubation			pmol $[^{14}C]$ phe incorporated per mg microsomal RNA
	Enzyme	Microsomes	tRNA	
a)	V	V	V	108
	V	V	B	109
	B	B	B	17
b)	V	V	V	139
	V	V	B	28
c)	V	B	V	20
	B	B	Y	84
	V	V	Y	2
	B	V	Y	10
	V	B	Y	11

where B = Brassica chinensis
V = Vicia faba
Y = Yeast

Incubation conditions (25°C) as described in Methods, samples being taken after 40 min.

was little change in activity. Incorporation depended mainly on the sample of B. chinensis tRNA used. On substituting either B. chinensis enzyme or microsomes, incorporation usually fell by up to 60%.

Yeast tRNA was found to have a much lower activity than native tRNA in the bean system (Table 23).

In a mixed bean and cabbage system (with either bean or cabbage microsomes) with bean or cabbage enzyme, activity was reduced to 12.5% the activity of the complete cabbage system. The activity, was however, five times the activity of a yeast tRNA substituted bean system.

It was noted that the degree of incorporation in minus poly(U) incubations was reduced in those incubations apparently approximating more to 'optimum' conditions for in vitro incorporation.

(d) Systems Employing Natural Messenger:

As a development from the above experiment, TYMV RNA was introduced into the bean and cabbage systems, in place of poly(U).

Viral RNA showed no apparent activity in a bean system, which under poly(U) direction incorporated 183 pmol phenylalanine per mg microsomal RNA (5 mmol/l Mg^{2+}) . Background incorporation was, however, reduced in the presence of the viral RNA (Table 24).

Similarly, in mixed bean/cabbage systems, except when bean microsomes were employed with cabbage tRNA and enzyme, no activity was evident. In the active system, viral RNA directed the incorporation of 9 - 10 pmol of phenylalanine per mg microsomal RNA. In a yeast tRNA

Table 24

Incorporation in in vitro systems under TYMV RNA and Poly(U) direction

System	Messenger	mM Mg^{2+}	pmol $[^{14}C]$ phe incorporation *
V. faba	+ poly(U)	5	183
V. faba	+ TYMV RNA	5	13.2
V. faba	- message	5	13.6
V. faba, yeast tRNA	+ poly(U)	5	94
V. faba, yeast tRNA	+ TYMV RNA	5	9.2
V. faba, yeast tRNA	- message	5	9.4
B. chinensis	+ poly(U)	5	33.1
B. chinensis	+ TYMV RNA	5	6.3
B. chinensis	- message	5	2.8
B. chinensis	+ poly(U)	12	27
B. chinensis	+ TYMV RNA	12	29.6
B. chinensis	- message	12	4.2
B. chinensis	+ poly(U)	16	16.2
B. chinensis	+ TYMV RNA	16	22
B. chinensis	- message	16	3.4

* pmol phenylalanine incorporated per mg microsomal RNA

Incubation conditions (at 25°C) as described in Methods, with samples taken after 40 min.

substituted bean system, viral RNA had low activity (Table 24).

Viral RNA exhibited no activity when incubated in a bean system at 5, 10 and 15 mmol/l Mg^{2+}, with $[^{14}C]$ lysine or at 5 mmol/l Mg^{2+} with $[^{14}C]$ arginine or glutamic acid. On substituting yeast tRNA in the bean system, at 5 mM Mg^{2+}, and employing $[^{14}C]$lysine, 600 c.p.m. above background were recorded. Employing $[^{14}C]$ aspartic acid in either a bean tRNA or yeast tRNA bean system, resulted in counts of 300 and 400 c.p.m. above background respectively. On incubation with a mixture of the $[^{14}C]$amino acids (arginine, aspartic acid, glutamic acid and lysine), viral RNA produced an 850 c.p.m. increase above a background count of 3450 c.p.m. in a yeast tRNA bean system (5 mM Mg^{2+}).

In a Chinese cabbage system at higher Mg^{2+} levels, TYMV RNA exhibited a greater activity (Table 24) which was not, however, consistent. Poly(U) added to such systems was active. On reaching an incorporation plateau with TYMV RNA, addition of poly(U) resulted in a measurable degree of incorporation (c. 1-2 pmol).

Only slight activity (in the order of 1 pmol) activity was obtained when employing TYMV RNA with $[^{14}C]$ valine or $[^{14}C]$ leucine, with added B. chinensis tRNA, and even less activity (above background count) with tRNA.

The variable and low activity of single amino acid incorporation experiments led to the use of a $[^{14}C]$amino acid mixture for the majority of experiments with the B. chinensis system.

(e) Systems Employing $[^{14}C]$ Amino Acid Mixture

$[^{14}C]$ amino acid mixture incorporation was found to be dependent upon the addition of viral RNA (Table 25). Incorporation was also found to be influenced by temperature (figs. 42,43). maximum incorporation occurring at 25°C. It was observed that an initial lag period of 4-5 min often occurred prior to incorporation in the Chinese cabbage system, when employing TYMV RNA.

Maximum incorporation occurred when employing a microsome concentration of 150 µg microsomal RNA per 0.5 ml incubation (fig. 44) although this was to some extent dependent upon the preparation. A chloroplast extract was found to be inactive in the system.

Incorporation was also found to be dependent upon the presence (and concentration) of microsomes, enzyme, tRNA, GTP, ATP and an ATP generating system (Table 25) and to be influenced somewhat by the addition of polyamines (Table 26). Addition of ATP and ATP generating system after 20 min incubation, resulted in a slight increase in $[^{14}C]$ amino acid incorporation, although occasionally a decrease resulted.

Incorporations were carried out at pH 7.8, and at this pH, magnesium and potassium levels exhibited noticeable effects on incorporation levels, (fig. 45). Maximum incorporation resulted with a magnesium concentration of 6 mmol/l and a potassium concentration of 70 mmol/l.

Analytical ultracentrifugation of an incorporation mixture (after incorporation) and comparison with the microsomal suspension employed (before incorporation), indicated that the 80 S component originally present was reduced on incorporation, but that the 70 S

Table 25

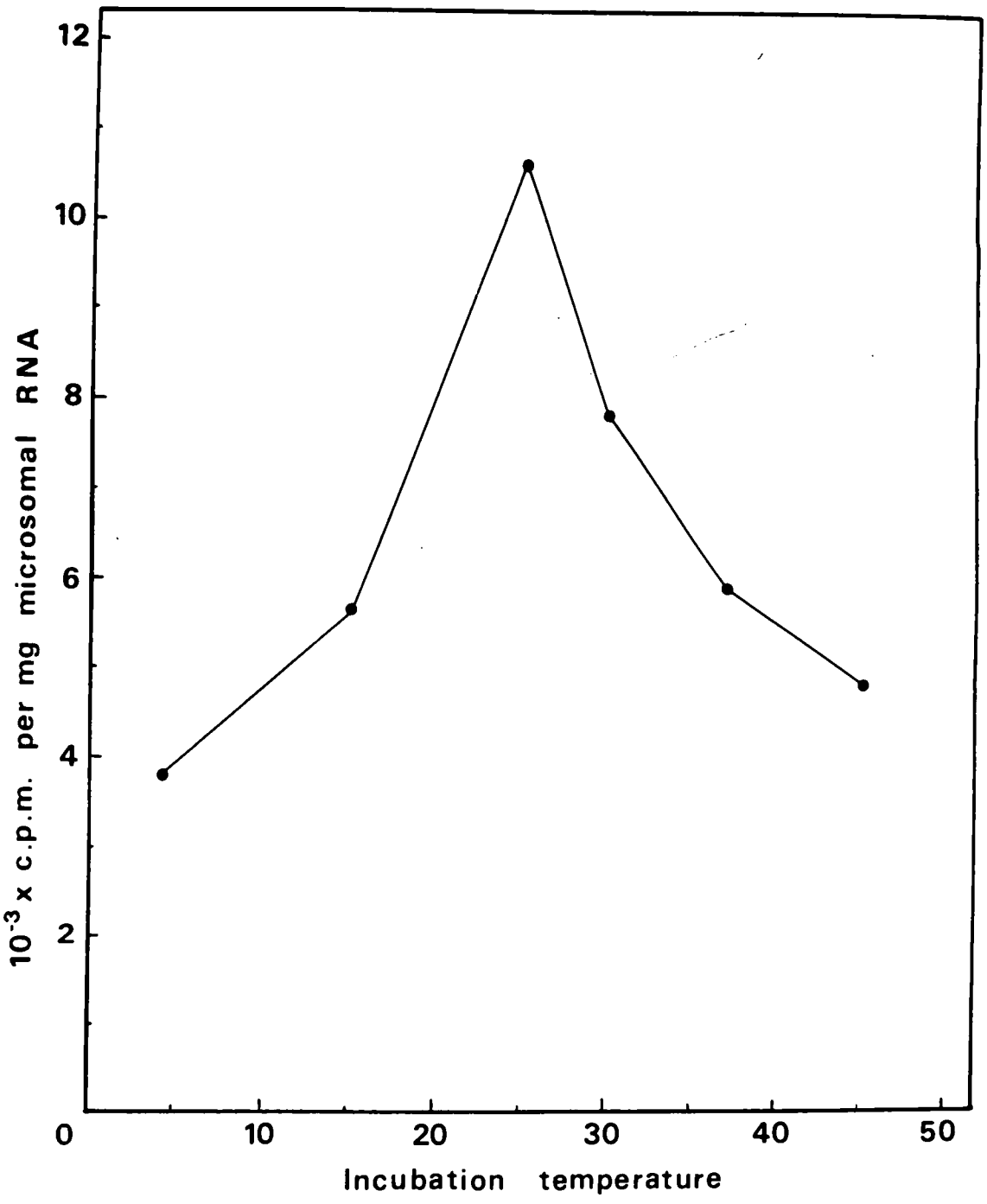
Characteristics of $[^{14}C]$ amino acid mixture incorporation in the complete Brassica chinensis in vitro system

Incubation	c.p.m.*	% Complete
Complete (+ TYMV RNA)	12,800	
- TYMV RNA	1,200	
- enzyme	3,390	26.5
- microsomes	3,000	23.4
- tRNA	4,400	34.4
- GSH	6,570	51.3
- GTP	5,250	41
+ ATP (- ATP system)	3,900	30.5
- ATP (- ATP system)	1,060	8.3
- ATP (+ ATP system)	4,500	35.2
+ RNase (0.5 µg/ml)	140	1.1
+ enzyme from infected plant	5,280	41.3
(i) -ATP(-ATP system) at 20 min	650	
(ii) -ATP(-ATP system) at 40 min	1,060	
(iii) Addition of ATP system + ATP after 20 min to (i)	2,430	

* c.p.m. per mg microsomal RNA

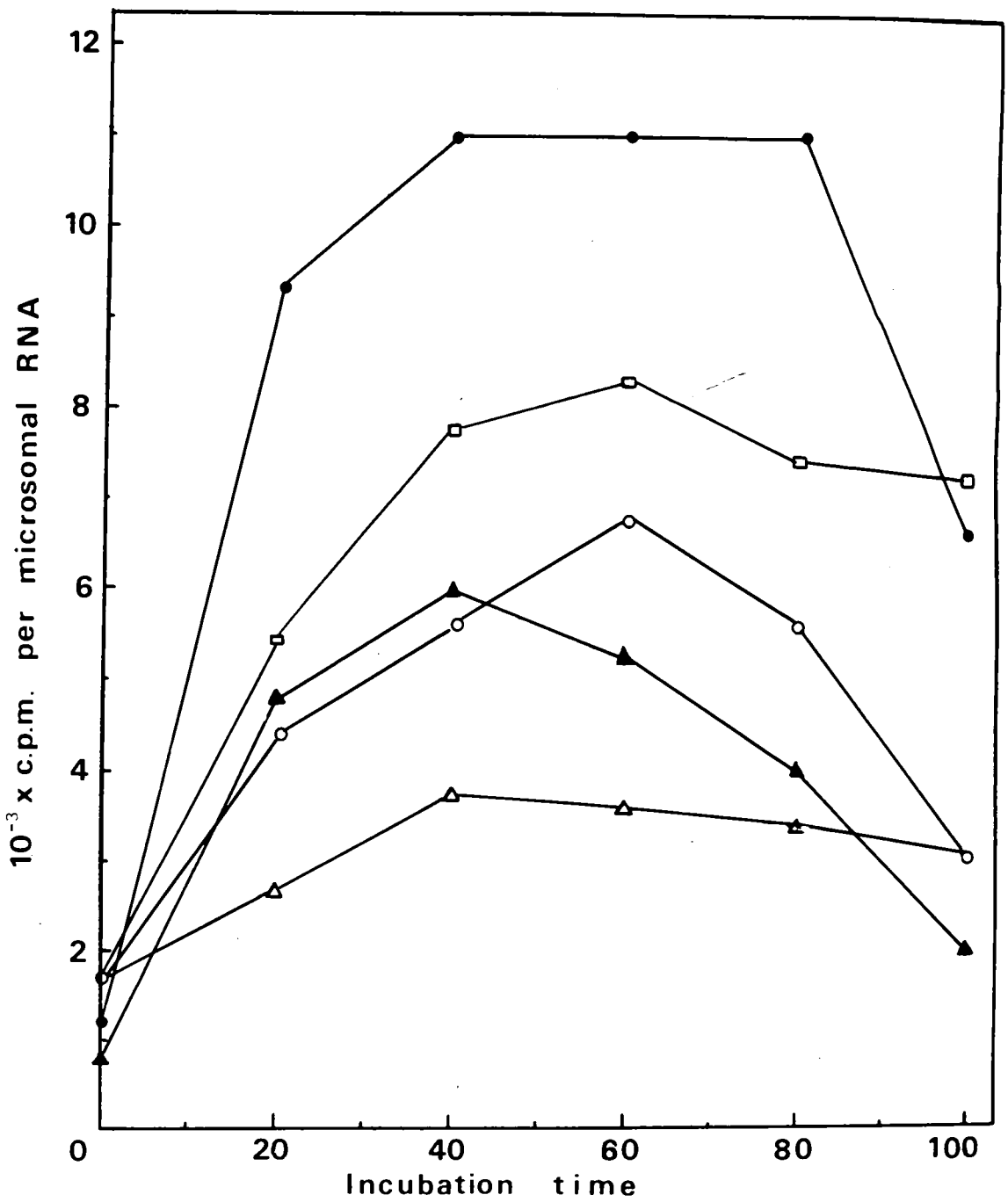
Incubation conditions (at 25°C) as described in Methods, with 6 mM Mg^{2+} and 100 µg TYMV RNA per 0.5 ml incubation (Samples taken after 40 min)

Fig.42 . Effect of temperature on amino acid incorporation in the Brassica chinensis in vitro system employing TYMV RNA as natural messenger



Incubation conditions as described in Methods, employing [^{14}C] amino acid mixture. Magnesium concentration 6 mM, potassium concentration 70 mM, and samples taken after 40 min. Results are the average of two experiments.

Fig. 43 . Time curves to show the effect of temperature on amino acid incorporation in the Brassica chinensis in vitro system employing TYMV RNA as natural messenger



Incubation conditions as described in Methods, employing [^{14}C] amino acid mixture. Magnesium acetate concentration 6 mM, potassium chloride concentration 70 mM, the results being the average of two determinations.

△——△ 0° □——□ 30°

○——○ 15° ▲——▲ 37°

●——● 25°

Table 26

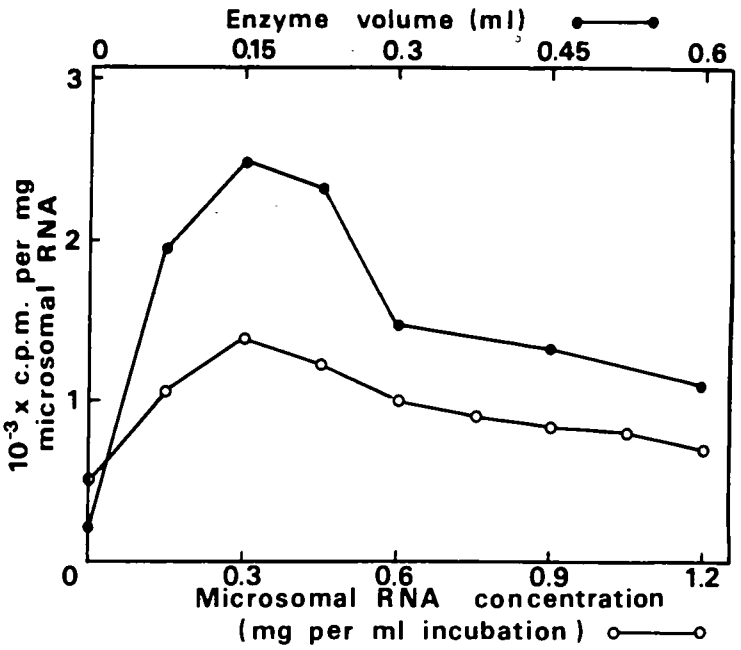
Effect of various additives on $[^{14}C]$ amino acid incorporation in the Brassica chinensis system

Incubation	Additive	c.p.m.*
Complete (+ TYMV RNA)		9,250
Complete (- TYMV RNA)		500
Complete (+ TYMV RNA)	0.2 mM Spermine	8,500
	0.4 mM Spermine	7,000
	1.0 mM Spermidine	10,000
	2.0 mM Spermidine	12,500
	1.0 mM Putrescine	11,500
	10.0 mM Putrescine	10,500
	1.0 mM Cadaverine	9,400
	2.0 mM Cadaverine	9,400
Complete (+ TYMV RNA)	- tRNA	6,300
	-tRNA (+ 0.2 mM Spermine)	8,000
	-tRNA (+ 1.0 mM Spermine)	6,500
Complete (+TYMV RNA)	2.0 mM Spermidine	12,500
	(2mM Mg^{2+}) 2.0 mM Spermidine	5,000
Complete (+ TYMV RNA)		7,900
	10 µg Chloramphenicol	7,750
	50 µg Chloramphenicol	8,000
Complete (- TYMV RNA)	TYMV (250 µg)	675

* c.p.m. per mg microsomal RNA

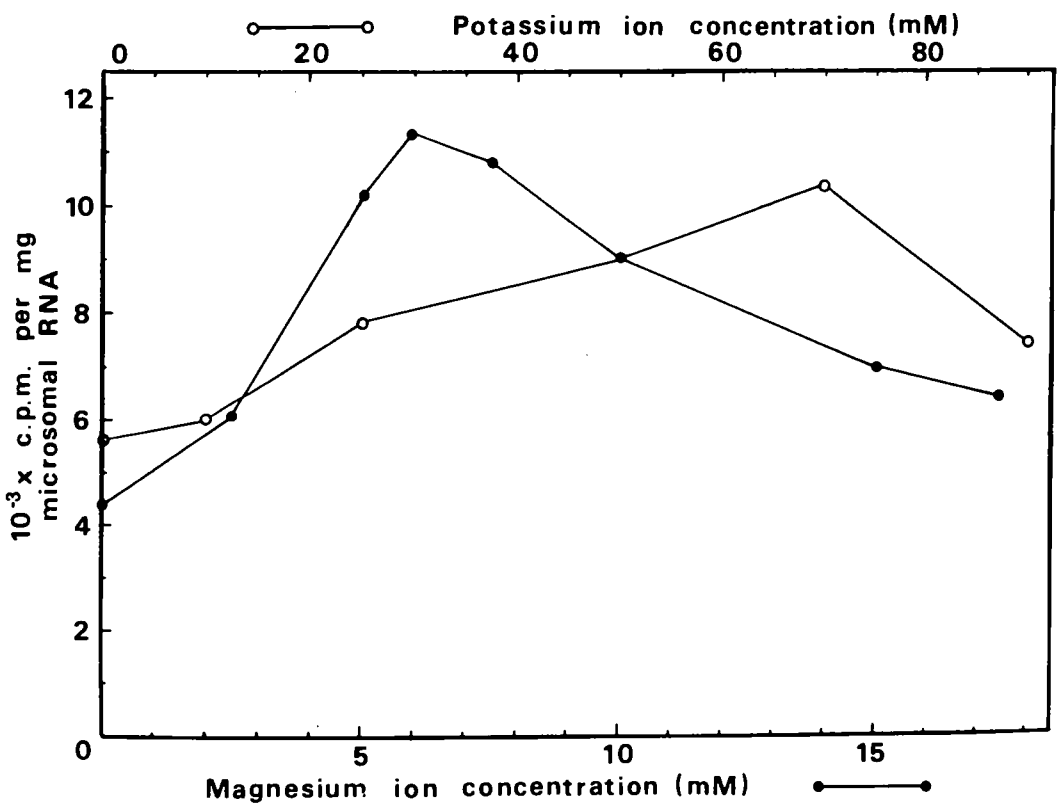
Incubation conditions (at 25°C) as described in Methods, with TYMV RNA concentration of 100 µg/0.5 ml incubation. Samples taken after 40 min.

Fig.44 . Effect of microsome and enzyme concentrations on [^{14}C] amino acid incorporation in the Brassica chinensis in vitro system



Incubation conditions as in Methods, employing [^{14}C] amino acid mixture, and TYMV RNA as natural messenger. The values are the average of three determinations.

Fig. 45 . Effect of magnesium and potassium ion concentrations on amino acid incorporation in the Brassica chinensis in vitro system



Incubation conditions as described in Methods, employing [^{14}C] amino acid mixture and TYMV RNA as natural messenger. Magnesium series employing 70 mM potassium and potassium series, 6 mM magnesium. The values are the average of three determinations.

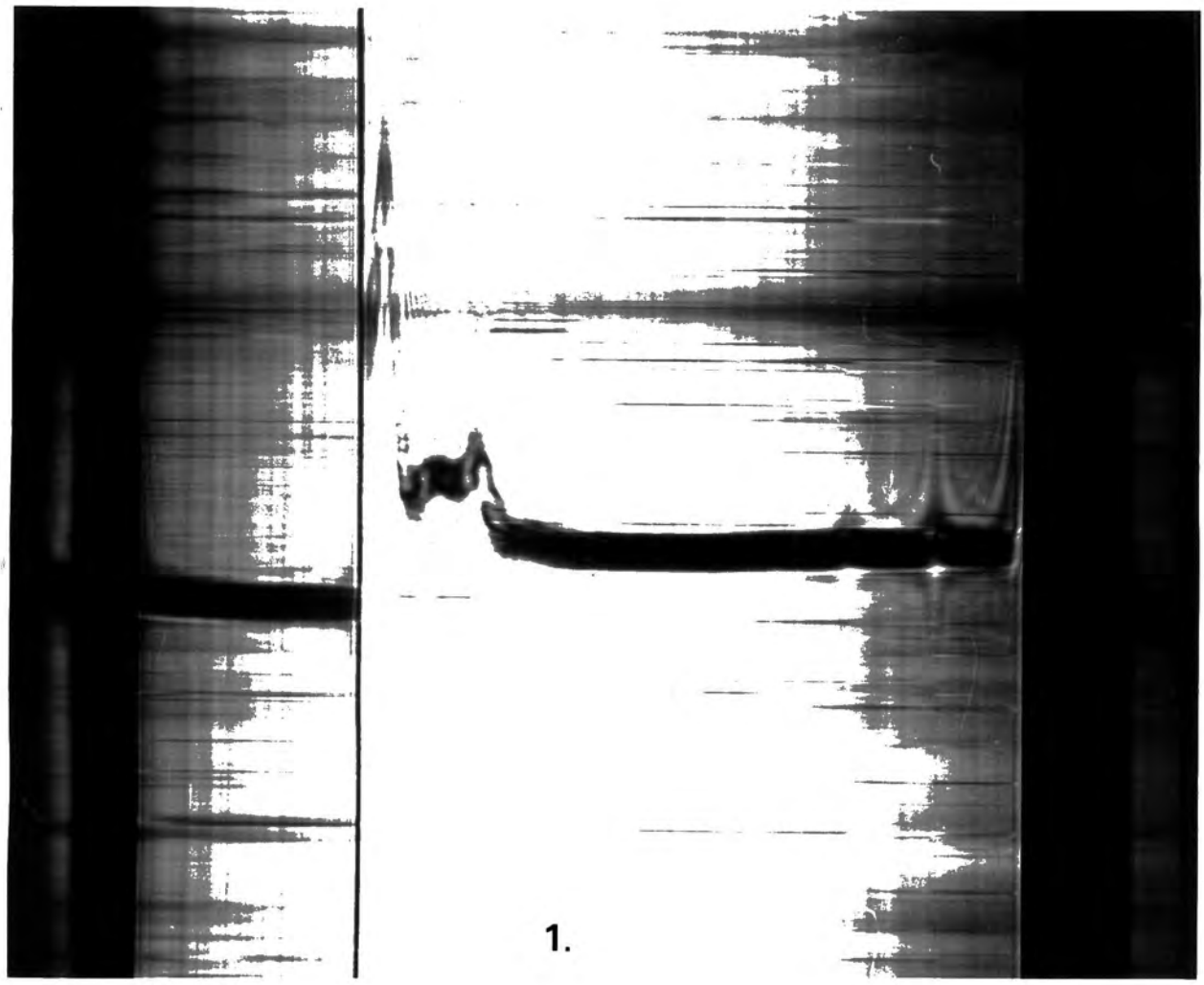
component was still present after incorporation (and in approximately the concentration expected from the dilution of the original microsomal suspension). A number of components with higher sedimentation coefficients were observed in the incubation mixture (fig. 46).

$[^{14}C]$ amino acid mixture incorporation in the bean system, under TYMV RNA direction, was not as great as in the cabbage system (Table 27). On substituting cabbage enzyme or ribosomes into the bean system, the activity was reduced even further, although a Chinese cabbage tRNA substituted bean system was more active than the complete bean system.

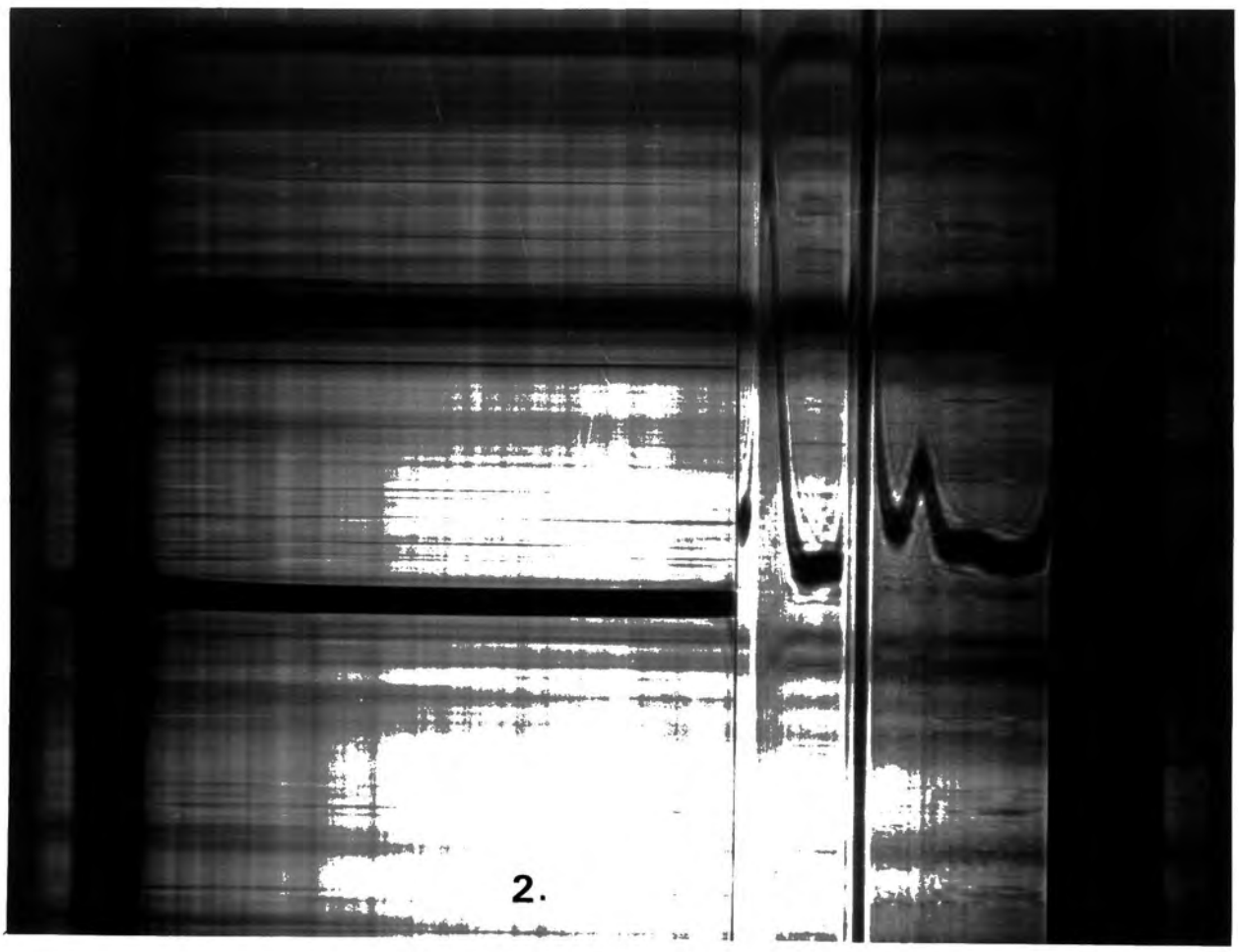
Bacterial Counting

Samples taken from incubation mixtures indicated a bacterial content of from 1000 to 2000 bacteria per ml, the larger counts resulting from samples taken from uncapped incubation tubes. No fungal colonies were obtained on plating out incubation mix samples.

Electron microscopic examination of ribosomal pellets did not demonstrate the presence of bacteria which were also found to be absent on phase contrast examination of viral suspensions.



1.



2.

Fig. 46 Schlieren patterns of Brassica chinensis leaf microsome preparations, before and after in vitro amino acid incorporation (in an attempt to show removal of '80 S' component)

1. Brassica chinensis microsome preparation, diluted by amino acid incorporation mixture (as described in Methods), after amino acid incorporation.

2. Brassica chinensis microsome preparation (25 mg/ml of 260 nm absorbing material) suspended in 0.005 M magnesium acetate - 0.01 M KCl - 0.01 M Tris HCl, pH 7.4

Omega rotor speed	40,000 rev./min
Temperature	20°C
Bar angle	1. 40°; 2. 65°
Time(after attaining speed)	3 min
Direction of sedimentation	left to right

Table 27

Characteristics of $[^{14}C]$ amino acid mixture incorporation in mixed in vitro amino acid incorporating systems

Incubation	c.p.m.*	Message (TYMV/RNA)
B. chinensis complete	11720	+
	1400	-
V. faba complete	7200	+
	850	-
V. faba system, B. chinensis tRNA	8100	+
	950	-
V. faba system, B. chinensis enzyme	5200	+
V. faba system, B. chinensis microsomes	3000	+

*c.p.m. per mg. microsomal RNA

Incubation conditions (25°C) as described in Methods, with 6 mM Mg^{2+} and samples taken after 40 incubation.

DISCUSSION

The importance of determining the mechanism of protein synthesis to biologists in general, is reflected in the vast amount of literature that has accumulated in recent years. The use of cell-free systems of protein synthesis derived from various sub-cellular organelles, together with genetic studies, has resulted in remarkable advances of our knowledge of the mechanism of protein biosynthesis and the genetic code.

Such cell-free systems are dependent on the presence of messenger RNA (mRNA) for activity, either endogenous or exogenous. They may be used for the translation of messenger with some fidelity, as witnessed by the *in vitro* synthesis of coat and other viral protein, under phage RNA direction. Several attempts have been made to isolate from higher organisms natural mRNA. The range of materials which have been studied includes peanut cotyledons (Jachymczyk and Cherry, 1968), wheat embryos (Chen *et al.*, 1968; Marcus and Feeley, 1964), rat liver (Kempf and Mandel, 1969), calf lens cells (Konings and Bloemendal, 1969), epidermis of the giant silk moth (Howells and Wyatt, 1969) and phytohemagglutinin stimulated human leucocytes (Silber *et al.*, 1968). The results obtained are, however, confusing and evade critical comparison mainly because of differences in the cell-free systems and experimental conditions, methods of mRNA extraction and the absence of proper standards, such as poly(U) or viral RNA (Fedorcsák *et al.*, 1969).

Cell-free systems may be used in translating synthetic polyribonucleotides, such systems being well developed as a result of the difficulties encountered in isolating natural mRNA's.

Much of our present knowledge of the biochemical mechanics involved in protein synthesis has been gained by the intensive study of systems isolated from bacteria and mammals, although the study of amino incorporation in systems derived from the cytoplasm of plant cells has been greatly simplified by this previous work. Many of the essential features have been found to be similar in the species tested.

The interest in extending our knowledge of protein synthesis to other organisms is readily apparent, since these studies could give insight into the mechanics of macromolecular synthesis that organisms have necessarily adapted because of differing living conditions.

In vitro systems involving plant viruses have recently attracted great interest, because of the difficulties encountered in obtaining endogenous mRNA. Such systems provide an access for examination of mRNA structure and function and viral reproduction and proliferation. Although plant viruses more than other systems, except certain RNA phages of *Escherichia coli*, offer the best readily available supply of well-characterised viruses for study of the basic principles of RNA replication and translation, *in vitro* systems have, as yet, provided little useful information about the synthesis of plant virus coat proteins.

Various conditions have been employed in attempts to develop *in vitro* plant virus systems:

1. systems that include surviving membrane-bound organelles;
2. unpurified systems, not including membrane-bound organelles;
3. systems containing partially purified enzymes and nucleic acids from infected plants, and
4. *in vitro*, synthetic systems, from organisms other than plants (often bacteria) employing plant virus RNA.

Several factors have probably contributed to these systems having provided little definite information, namely:

(a) the phenolic materials that are widespread in plant tissues and which frequently cause difficulties in virus isolation also inactivate enzymes in tissue extracts.

(b) nucleases are widespread in plant tissues and rapidly inactivate RNA unless removed or inhibited;

(c) the presence of a cellulose wall means that fairly severe methods of cell breakage are employed. Chloroplasts are fragile organelles, and chloroplast fragments of various sizes are difficult to avoid in many preparations;

(d) cells in expanded leaves contain a large vacuole, the contents of which may damage cytoplasmic constituents during cell breakage (before any protective materials in the extracting medium can be effective).

Bearing in mind these difficulties an attempt has been made to develop cell-free systems from Vicia faba and Brassica chinensis, using Turnip Yellow Mosaic Virus (TYMV) RNA as 'natural' messenger, and poly(U) as synthetic messenger.

In part due to the reasons outlined above, although 'working' cell-free systems have been established with plant material, net protein synthesis has not been conclusively shown,(Boulter, 1970). Lack of protein synthesis may also be due to the use of an 'incomplete' messenger when employing extracted endogenous or exogenous natural messengers.

There may be three specific objects when carrying out in vitro

amino acid incorporation experiments:

1. the determination of the relative activity of the protein synthesising machinery of tissues under specific conditions. (The obvious disadvantages of using cell-free systems for these studies are counterbalanced by the elimination of factors such as the permeability barriers, cellular pools etc,)
2. the study of the properties of the components that intervene in the processes of protein synthesis, and
3. the achievement of the synthesis of a specific protein.

The pursuit of the first object requires that the investigator attempts to develop a system that interferes as little as possible with the components involved. It is important to retain the integrity of the system, if a specific protein is to be produced by a particular source tissue. The synthesis of specific proteins has been achieved *in vitro* in several animal systems, (Bishop *et al.*, 1960; Campbell and Kernot, 1962), but specific plant protein synthesis has not been achieved. Schwartz *et al.*, (1965) however, have produced bacteriophage coat protein in an *Euglena gracilis* system, purified to reduce the endogenous incorporation and to enhance the effect of added viral mRNA.

If it is required to study the properties of the components involved in the processes of protein synthesis, a simplified system with isolatedcomponents and the best attainable incorporation is to be preferred. Such systems have usually made use of poly(U), directing the synthesis of polyphenylalanine,(Nirenberg and Mathaei, 1961).

A number of characteristics of a system must be demonstrated in order

to establish that any observed amino acid incorporation by isolated ribosomal preparations is due to the ribosomes themselves. Hoagland (1960) has suggested the following criteria for equating amino acid incorporation with protein synthesis:

1. incorporation of the L-amino acid(s) should be irreversible once the amino acid has entered the protein. Subsequent continued incubation in the presence of excess of the same $\left[^{12}C\right]$ amino acid should not reduce the total amount of the $\left[^{14}C\right]$ amino acid in the protein;
2. more or less complete dependence of incorporation on the presence of a metabolic energy source - ATP and an ATP-generating system;
3. incorporation of the $\left[^{14}C\right]$ amino acid should be shown to be in a true peptide linkage in the protein, as seen by its appearance in identifiable peptides upon partial protein hydrolysis;
4. the amino acid should be located within the peptide chain and not solely in the terminal position;
5. the amino acid should appear in a single specific isolatable protein of the cell of origin, e.g. haemoglobin in the rabbit reticulocyte system,(Bishop et al., 1961).

In addition the system should show sensitivity to added ribonuclease (Allende and Bravo, 1966; Ellis and MacDonald, 1967; Parisi and Ciferri 1966).

All the above criteria have seldom been satisfied in any one investigation, requirement (5) having been demonstrated in relatively few.

The present investigation is therefore, mainly methodological

in character, tests not being performed to determine the polypeptide nature of the trichloroacetic acid precipitable products formed by the cell-free systems, under either poly(U) or TYMV RNA influence. The formation of trichloroacetic acid precipitable material is however, for reasons of brevity, referred to as 'protein synthesis' and the activity of poly(U) or viral RNA referred to as 'template' or 'messenger' activity.

The reaction mixture of cell-free systems for <u>in vitro</u> protein synthesis is based on the requirements for the three main reactions outlined below (see also page 19 for further clarification).

Enzyme + Amino Acid + ATP $\longrightarrow$ Enzyme-AMP-Amino Acid + PPi

Enzyme-AMP-Amino Acid + tRNA $\longrightarrow$ tRNA-Amino Acid + Enzyme + AMP

n(tRNA-Amino Acid) ——— messenger RNA $\longrightarrow$ POLYPEPTIDE + ntRNA

Such cell-free systems usually contain the following components:

1. A particulate component (i.e. microsome, polysome or ribosome), usually prepared from fresh material by differential centrifugation (similar in fact to methods employed in viral purification);
2. Amino acids - twenty of the L-series are used as the substrate for polypeptide synthesis, one or more of which is $\left[^{14}C\right]$ labelled. Acid hydrolysates of radioactive algal protein may also be employed.
3. Activating and transfer enzymes prepared from the high speed supernatant after pelleting the ribosomes. The supernatant may be used as such or partially purified by dialysis, ammonium

sulphate precipitation or iso-electric precipitation at pH 5;

4. tRNA - prepared from fresh material, often by phenolic extraction;
5. GTP, ATP and an enzymatic regenerating system, e.g. pyruvate and pyruvate kinase or creatine phosphate and phosphocreatine kinase;
6. Magnesium ions, to preserve ribosome integrity, and monovalent cations, e.g. NH_4^+ or K^+;
7. A sulphydryl compound, e.g., dithiothreitol, GSH or mercaptoethanol, to buffer auto-oxidation reactions;
8. Messenger RNA, natural or in the form of synthetic polynucleotides. Early workers used no messengers and it may be assumed that these were present in the natural mixture or that partially synthesised polypeptides were completed.

The complete reaction mixture is usually buffered, often with tris hydroxymethyl amino methane hydrochloride (Tris-HCl), at pH values between 7 and 7.8. The length and temperature of incubation are altered to suit the system under study.

The first cell-free system derived from plants that was reported to incorporate amino acids into protein was described by Stephenson et al., (1956). Following this publication a large number of systems from differing sources were reported (see references in Allende, 1969; Boulter 1970; Mans 1967; Rowan, 1966).

The kinetics of the incorporation vary from system to system, but usually incorporation is completed before 150 pmol of labelled amino acid

have been incorporated per mg ribosomal RNA (rRNA), (Boulter 1970). Two types of system have been employed to study incorporation, (a) the 'complete' system , measuring total amino acid incorporation from charging tRNA with amino acids, binding of aminoacyl-tRNA to the ribosomes and transfer of the aminoacyl moiety to the growing polypeptide chain, and (b) the 'transfer' system, in which $[^{14}C]$ aminoacyl-tRNA is added to the other components.

The advantage of the latter system is that fewer radioactive counts need be added than in the complete system. In general, however, such a system is less active thanthe 'complete', Allende and Bravo (1966) finding, for example, that 135 pmol of label was incorporated per mg rRNA by a complete wheat system, whereas the transfer system only incorporated 15.3 pmol. Comparable values for the 4-day-old broad bean plumule system are 280 and 40 pmol respectively, (Yarwood and Boulter, 1970).

Because of this inherent lower activity in 'transfer' systems a 'complete' system was employed throughout the present work.

An important concern of investigators studying amino acid incorporation is whether the results can be attributed to bacterial contamination, (Allende, 1969). Since, even under careful aseptic conditions, bacteria have been found in components that intervene in incorporating systems (Hall and Cocking, 1966), it is important to determine whether the number of bacteria present can explain or significantly contribute to the results.

Preparations from plant sources are likely to be contaminated with micro-organisms from the leaf surface, whereas preparations from animal sources are usually derived from the 'bacteria-free, inner animal'.

Contamination in animal systems is therefore more likely to be operator introduced.

Yarwood (1968) showed that most of the bacteria were sedimented with the mitochondria during V.faba ribonucleoprotein particle preparation. With suitable aseptic precautions, therefore, it should be possible to maintain bacterial participation to a minimum, even in plant in vitro systems.

Bacteria differ markedly in the amounts of radioactivity they incorporate on a per cell basis,(Davies and Cocking 1967). Sandell et al., (1967) found that the incorporation of amino acids into protein in mitochondrial preparations was dependent on the bacterial growth during incubation rather than on the number of cells present, again suggesting the need for sterile conditions, especially during prolonged incubation experiments.

The time curves of bacterial incorporation are, except at very high contamination (greater than 10^7 bacteria/ml), quite different from those of ribosomal incorporation. The bacterial curve is biphasic, there being little incorporation in the first 60 min incubation period, followed by a period of rapidly accelerating incorporation. True ribosomal incorporation, dependent somewhat on the source material and incubation conditions, proceeds linearly over the first minutes of incubation, (often from 20 to 60 min), after which time the rate of incorporation may fall (due in part to exhaustion of substrates and product accumulation). The non-biphasic time courses for the in vitro bean (fig. 41 , page 171) and Chinese cabbage (fig. 43 , page 179) systems are clearly seen to be ribosomal in nature.

Davies and Cocking (1967) suggested that in incubations with less than 10^3 bacteria/ml, incorporation due to bacteria is negligible. App and Jagendorf (1964), however, reported that there was no linear relationship between levels of bacterial contamination and amino acid incorporation by chloroplast preparations, although different micro-organisms differ greatly in their ability to incorporate amino acids in in vitro incubations.

Bacterial counts of incubation samples are by themselves unreliable unless taken in conjunction with time course data. They are suspect in that they are greatly affected by operator technique, plating medium constitution, temperature and time of incubation of the inoculated plates etc., (Thompson 1967). The nature of the amino acid incorporation mixture may also alter the results by inhibiting or potentiating bacterial growth. Lack of correspondence between amino acid incorporation and bacterial counts is not however, considered to be evidence of their non-participation, (Boulter 1970).

Bacterial counts from samples of Chinese cabbage incubation mixtures indicated that contamination was 1,000 - 2,000 organisms/ml. Such low counts are in agreement with amino acid incorporation time courses, as noted above. No fungal contamination was found in the samples tested. The higher bacterial counts were obtained from those incubation tubes which had been left uncapped during the period of incorporation, suggesting operator/aerial contamination.

No bacterial contamination was observed in electron micrographs of bean and cabbage microsome preparations, material similar to which was used in incorporation experiments.

Swain and Dekker (1969) consider that compliance with the following points rules out significant microbial contamination:

1. the plants used in any study should be germinated under essentially sterile conditions;
2. the requirement for GTP and for poly(U) is strict, and
3. the incorporation of a labelled amino acid into protein, as a function of length of time of incubation, does not increase indefinitely, but reaches a finite level after a period of time.

Bacterial incorporation of amino acids is independent of added GTP and/or ATP and the energy generating system, and the partial dependence of the Chinese cabbage system on added energy source and messenger tends to suggest low bacterial participation. Demonstration of a particular requirement for any component is not always possible in a microsomal system, in that the microsomes and enzyme preparations may well be contaminated with significant amounts of the component(s) under investigation.

Although bacterial incorporation can be eliminated by the addition of antibiotics, their use, as pointed out by Boulter (1965) and Lado and Schwendimann (1966) cannot be wholly relied upon. Identification of the final product protein is undoubtedly the ultimate criterion. If the product protein is easily identifiable, the problem of bacterial participation is eliminated,(Allende 1969).

With lightly infected starting material it was found possible therefore, by the usual ultracentrifugation techniques, to prepare particulate and other preparations relatively free of bacterial

contamination. Such preparations, supplemented by carefully stored, aseptically prepared, sterile solutions were employed throughout this work, producing mixtures in which most (if not all) the observed activity was due to the ribosomes themselves. To assist sterility, all incubation tubes were lightly capped throughout the incorporation experiments.

Experimental Procedures

i) Brassica chinensis:

Chinese cabbage is the common experimental host for TYMV, but according to Bailey (1949), there are two species of Brassica, B.chinensis L and B.pekinensis Lour. Rupr. with this common name. Brassica petsai Parl. is said to be a synonym of B.pekinensis. Menage (1966) gives Chinese cabbage as Brassica cernua, listing varieties as, Pte-Sai, Wong Bok and Chihli.

The plants grown as B.chinensis var Wong Bok bear the closest resemblence to the description of B.chinensis as outlined by Bailey (1949), and are from the same source as the variety used by Haselkorn (1969), namely B.chinensis var. Pte Sai ("pe tsai" being the Chinese phrase for cabbage). Matthews and Ralph (1966) noted, however, that the B.pekinensis var. Wong Bok used in their laboratory, and obtained from plants grown from local commercial seed, showed noticeable variation in leaf shape and colour. This they suggested indicated some genetically based variation within normal varieties. In all three varieties grown at Durham these variations were also seen, and so only plants showing the same general characters were used for experimental purposes. It may

well be that the Chinese cabbage used in the present work as the experimental host for TYMV, and named B.chinensis is synonymous with B.pekinensis. In fact the samples of infected leaf supplied by Professor Matthews bore a close resemblence to infected leaves of B.chinensis var Wong Bok.

As noted by Markham and Smith (1949) a white "break" in the yellow flowers of TYMV infected B.chinensis occasionally occurred, and in general the test plants were small and stunted (especially if infected young).

ii) Microsomal Extraction:

Unlike nuclei and mitochondria, the term 'microsome' does not refer to a discrete cytoplasmic organelle, being an operational term denoting the particulate fraction sedimenting upon centrifugation at speeds exceeding 10000 g (de Duve, 1964; Reid, 1967). Different types of microsomal fractions can therefore vary both in structure and biochemical properties, not only according to intrinsic features such as species, age and tissue type, but also to extraneous conditions such as nutritional and hormonal status,(Tata, 1969). The term 'microsomes' however, often refers to a mixture of membraneous elements and ribosomes (or polysomes) present either as separate entities or as complexes (derived from the smooth, or agranular, and the rough, or granular, endoplasmic reticulum), and it is to such a mixture that the term refers in this text.

Although the isolation of microsomes was described by Claude in 1941, the most successful isolation procedure is that of differential centrifugation devised by Palade and Siekevitz (1956). Most procedures

employ a modification of their original method, involving separation of nuclei and mitochondria from the tissue homogenate by low-speed centrifugation, followed by ultra-centrifugation of the mitochondria-free supernatant. Ribosomes or polysomes may be prepared from the microsomal pellet by treating with detergent, e.g. sodium deoxycholate, to free the membranes. Bloemendal et al., (1964, 1967) have described a detergent free method in which centrifugation through a discontinuous sucrose density gradient allows the separation of free polysomes (active in incorporating amino acids into protein).

Ribosomes from different sources have varying requirements for certain factors in order to sustain their integrity and hence protein synthesising capability. Extraction media and procedures have therefore to be chosen so as to meet, as far as possible, these individual requirements.

Perzynski et al., (1969) observed that the method of preparation of Bacillus stearothermophilus ribosomes played an important role in the fidelity of poly(U) translation, the highest ambiguity resulting when the ribosomes were dissociated into subunits and purified in sucrose. Similar dependence on isolation procedure was noted by Lamfrom and Grunberg-Manago (1967) in reticulocyte ribosomes. Loss of important associated factors may have accounted for activity reduction.

Means and Baker (1969) showed that the yield of polysomes from baboon liver (Papio papio) and their activity in cell-free systems was dependent on the method of homogenisation and concentration of deoxycholate. Zamecnik (1953), Zamecnik and Keller (1954) and Ganoza et al., (1965) demonstrated the importance of homogenising conditions in the preparation

of active ribosomes from rat liver.

That extraction media composition and procedures need to be varied to suit the use to which the preparations are to be put, and to suit the nature of the tissue has been found to be especially true for plant ribosomes (Arglebe and Hall, 1969).

In the present work typical microsomal preparations were prepared by simple grinding of B.chinensis leaves in 0.005 M magnesium acetate - 0.01 M phosphate buffer (Vanyushin and Dunn, 1967). Such preparations however, were inactive in amino acid incorporation although components in such samples sedimented at 83 S and occasionally 117 S (when examined in the above buffer). On increasing the magnesium concentration, and examining in 0.01 M magnesium acetate - 0.05 M phosphate, the components sedimented at 105, 84 and 66 S (the increased magnesium concentration apparently causing a splitting of the ribosomes into subunits). Inactivity in these preparations was probably due to ribosome damage as no protective agents (e.g., sucrose, MCE) were employed. Degradation or removal of associated factors could also have resulted in activity loss.

Various methods of homogenisation and extraction media were therefore employed in an attempt to obtain active bean and cabbage preparations. Heavy grinding of leaf material was avoided as it reduced the cabbage leaf chlorophyll pigmented tissues to such a fine state as to prevent sedimentation on centrifuging. Microsomes prepared with the aid of heavy comminution were invariably inactive, probably as a result of physical damage. No obvious components were observed when such preparations were examined in the analytical ultracentrifuge.

Chinese cabbage ribosomes prepared by the method of van Kammen (1967) gave poor incorporation of amino acids, although this was improved by adopting a razor blade as the means of comminution. Although the ribosomes were more active, the method was lengthy and yields low.

A 'quick method' was therefore devised, giving the best preparations, this method being used whenever possible for obtaining B.chinensis ribosomes. Although still active after freezing quickly and storage at $-70^{\circ}C$, Chinese cabbage microsomes gave better and more consistent results when used immediately after preparation. Van Kammen (1967) observed that although tobacco leaf ribosomes retained their activity during dialysis, they did not if frozen.

For good incorporation, the age of the leaves was found to be of importance. Extraction of microsomes from B.chinensis plumules, seeds and seedlings resulted in poor preparations, possibly due to physical damage during isolation. Developing bean preparations from V.faba were however, active and used routinely. The extraction of bean microsomes by the method of Payne (1970) was found to be satisfactory for producing active preparations from this source, slight changes in the extraction media having apparently little or no effect on the efficiency of the isolates.

Preparations from young leaves were found to be much more active than from older leaves, possibly as a result of increased RNase in the older leaves. Rapid isolation of microsomes is also important from the fact that leaf wounding can cause a rapid, prolonged and non-specific increase in the RNase activity of the leaves (Diener, 1961; Bagi and Farkas, 1967), which would be deleterious in an in vitro system.

Babos (1966) reported that there was no difference in the ribosomal content of TMV infected and healthy tobacco leaves. In the present work microsomes from infected and uninfected samples of similar leaves of the same age of B.chinensis plants gave similar levels of incorporation, suggesting little alteration of the ribosomal status quo in infected tissue or at least in the ribosome status required for in vitro activity.

One of the most important factors in microsome isolation has been found to be the concentration of magnesium ions. The lower the concentration in the preparative stages, and the lower the concentration in the resuspending medium, the clearer the determination of the magnesium level required to promote maximum incorporation. Offsetting this is the fact that the stability of ribonucleoprotein particles is decreased at low Mg^{2+} concentration and in the absence of bivalent ions, the particles disintegrate into smaller subunits (Petermannet al., 1958). The particles however, recombine on the addition of Mg^{2+} thereby regaining their incorporating activity (Takanami, 1960).

Varying Mg^{2+} optima have been reported, e.g. for ribosomes from the first seedling leaves of barley, 1-2 mM Mg^{2+} (Srivastava and Arglebe, 1967), from wheat plumules, 1 mM Mg^{2+} (Arglebe and Hall, 1969). However, Clark et al., (1964) found 10 mM Mg^{2+} to be optimal for B.pekinensis ribosomes, and this concentration was employed in the present work.

The Mg^{2+} concentration in the resuspending medium was reduced to assist in determining optimal Mg^{2+} concentration for amino acid incorporation, from the relatively high level of 10 mM in the extraction medium.

The inclusion of various other 'protecting' agents in the extraction medium has also been found important. In microsomal systems, a sucrose concentration of 0.45 M (as employed in this work) has been found to preserve structural integrity (Hogeboom et al., 1948), although very active preparations of bacterial ribosomes have been prepared in the absence of sucrose (Matthaei and Nirenberg, 1961).

Leslie and Mansbridge (1970) noted that sedimentation of rat liver microsomes through dense layers of sucrose, greatly reduced their activity. Similarly Chinese cabbage microsomes prepared by the procedure of Pearson (1969), in the absence of deoxycholate, and employing such a procedure were found to have low activity. Twice-washed, DOC prepared microsomes were, however, active, although less so than microsomes prepared by the 'quick extraction' method. Undoubtedly further work on ribosomes and associated co-factors would help to elucidate the factors affecting stability and subsequent activity in the in vitro system.

Several investigators have included a thiol source in their extraction medium (Cleland, 1964; Devenuto and Lange, 1967; Kaji, 1968; Liao and Williams-A, 1962) in order to protect ribosomes and cell sap enzymes. Mercaptoethanol was used by Parish (1969) in an attempt to stabilise E.coli polysomes as it has been shown that thiols protect E. coli monosomes against dissociation.

Mercaptoethanol was used routinely in extracting both bean and cabbage microsomes, although its absence in one assay did not significantly alter the results. On dialysis of high-speed supernatant, mercaptoethanol was included in the dialysing solution, as a protective agent.

When microsomes are prepared for use in incorporation experiments it is often preferred to use the final incubation medium throughout. Where this is not practicable, the extraction medium is supplemented with Tris-HCl buffer , KCl (or NaCl or NH_4Cl) in varying amounts, as employed here.

The widespread occurrence of RNase in plant tissues has hampered the isolation of polysomes, messenger and other RNAs. A rise in RNase activity on injury or detachment of leaves increases the problem, the rise being accountable for by either induced enzyme activity, or by new enzyme synthesis (Hadziyev et al., 1969).

Furthermore young tissues are often extracted and Phillips and Fletcher (1969) observed in Phaseolus vulgaris that RNase levels increased in expanding and maturing tissue, declining in senescing tissue. McHale and Dove (1968) observed that RNase activity increased almost linearly in older tomato leaves, the activity in younger leaves increasing to an 'RNase plateau'. Santilli et al., (1962) found a decrease in RNase activity in leaves of Pinto bean but Reddi (1959) described an increase in the activity of the enzyme in ageing tobacco leaves. It has been suggested that such conflicting results could possibly be resolved on the basis that RNase levels depend on the physiological age of the tissue (Phillips and Fletcher, 1969).

RNase inhibitors such as Zn^{2+} (Barker and Rieber, 1966,1967), Cu (Hall and Cocking, 1966), polyvinyl sulphate (Clark et al., 1964), Macaloid (Marcus et al., 1967), and bentonite (Brownhill et al., 1959; Dunn and Hitchborn, 1965,1966; Fraenkel-Conrat et al., 1961; Petermann and Pavlovec, 1963; Watts and Mathias, 1967) have been employed in ribosome

extraction procedures, the presence of these inhibitors being thought to minimise damage to ribosomes and polysomes. Their use in homogensising media has not always been effective (Clark et al., 1964; Hsiao, 1968; Shinozawa et al., 1968) and in addition may result in considerable loss of polyribosomes (Hsiao, 1968; Tester and Dure, 1966). Bentonite was found to considerably reduce the size of the microsomal pellet in Chinese cabbage procedures.

More recently, diethylpyrocarbonate has been employed (Fedorcsák and Ehrenberg, 1966; Solymosy et al., 1968) but has been found to have a deleterious effect on in vitro ribosomal amino acid incorporation (Weeks and Marcus, 1969), probably by inactivating enzymes (e.g., ribosome associated protein, essential for peptide synthesis).

RNase inhibitors were not therefore employed routinely in the extraction of B.chinensis or V.faba microsomes. It is interesting to note that addition of RNase to amino acid incorporating mixtures containing B.chinensis greatly reduced the level of incorporation. It may be inferred that such a large reduction in activity by a low concentration of added RNase indicates a negligible quantity of RNase being present in the microsome preparation itself.

The pH of the microsome extraction medium was adjusted to 7.5 at $0^{o}C$ as this is considered to be close to the optimum pH for ribosome extraction (Arglebe and Hall, 1969; Good et al., 1966).

No attempt was made to study the affect of variation of pH in the extraction medium or incubation mixtures, although it has been reported that this factor may have a marked effect on incorporation levels (Payne, 1970).

Enzyme dialysis was performed to remove low molecular weight contaminants, counterions and solvent components. Endogenous amino acid removal was considered necessary so as to prevent swamping of added $[^{14}C]$ amino acid(s) with resultant apparent low *in vitro* activity. The dialysis tubing employed was soaked to remove plasticizers (e.g. glycerin), traces of sulphurous compounds and heavy metal ions that may have affected incorporation.

iii) Total Leaf RNA Extraction:

The procedures employed in the isolation and purification of plant nucleic acids from whole tissues vary in detail according to the nature of the material used. The method of Ralph and Bellamy (1964) was employed in the present work to obtain total leaf RNA from *B.chinensis*. This method involves preferential precipitation of the RNA with cetyltrimethylammonium bromide. Ralph and Bellamy (1964) reported that TMV RNA prepared by this method was biologically active.

Matus *et al.*, (1964) isolated total Chinese cabbage leaf RNA by this method, and on examination by analytical ultracentrifugation, four components were identified with sedimentation co-efficients of 4 S, 12 S, 16 S and 24 S as previously reported (Ralph and Bellamy, 1964). The 12 S component was later identified as DNA, the 4 S tRNA and the 16 and 24 S components as ribosomal RNAs. The best preparation obtained by Matus *et al.*, (1964) gave an O.D. 260/280 = 1.96, this preparation containing little impurities. Total *B.chinensis* leaf RNA isolated for the present work had an O.D. ratio of 1.56 and components of 25, 23, 18 and 16 S (any 4 S component not being detectable under test conditions).

iv) tRNA Extraction:

Hoagland et al., (1957,1958) first observed that when amino acids are incubated with ATP and the nonparticulate or soluble fraction of rat liver cytoplasmic extracts, they are bound to an RNA component with a low molecular weight. They demonstrated that the RNA-bound amino acids are incorporated into protein on further incubation with GTP and the microsomal fraction of the cytoplasm, and concluded that these complexes are intermediate in protein synthesis.

The RNA component of low molecular weight was named soluble or transfer RNA, being a mixture of species of molecular weight about 27,000 (70-80 nucleotides) and sedimentation constant about 4. A number of procedures for the isolation of tRNA have been devised and described in the literature (see reviews by Brown, 1963; Staehelin, 1963; Weiss and Kelmers, 1967), the method employed being largely dependent on source material. Since tRNA does not appear to be complexed with protein, its separation mainly requires the removal of soluble protein (including RNase) and polysaccharides. Phenol extraction is commonly used, together with high-speed centrifugation for long periods to obtain an aqueous phase to extract. Prolonged centrifugation is, however, inconvenient, tending to degredation (Holden and Pirie, 1955). To avoid this methods have been devised to extract total cell RNA with subsequent tRNA isolation, by means of concentrated salt solutions.

Further developments have led to methods in which very little ribosomal RNA is extracted originally, as such rRNA may "register" as tRNA or may be active in an in vitro system or bind to and block ribosomes.

Holley et al., (1961a) used DEAE cellulose to free tRNA preparations of polysaccharides, proteins and small oligonucleotides by adsorbing the tRNA on the ion exchange column material at low salt concentrations, and eluting with solutions containing 1.0 M NaCl. Such step elution yields a mixture containing all the tRNAs.

In the present work, V.faba tRNA was extracted by the method of Payne (1970), but this method proved unsatisfactory for the isolation of tRNA from B.chinensis, low yields of low activity resulting. From studies on the use of magnesium bentonite in the preparation of plant viruses (Dunn and Hitchborn, 1965) a method was developed which avoided the necessity for high-speed centrifugation (and incorporated a means of inhibiting RNase from the time of tissue maceration).

Transfer nucleic acid was found to be easier to extract from plants grown normally, rather than those subjected to varying periods of darkness before extraction (the fractions being less viscous and easier to handle). With the plants grown in the dark, very viscous extracts were obtained which resulted in large losses of tRNA at each purification stage.

Simple grinding of B.chinensis leaves with magnesium bentonite and $MgSO_4$ (as for viral extraction, page 73) with subsequent clarification and tRNA precipitation (Dunn, 1965) resulted in very low yields (in the order of 1mg/100g leaves). The tRNA so isolated did have a characteristic uv absorption profile however, although charging ability was poor.

To increase the yield, a second phenol extraction of the interphase material was attempted, but this resulted in little improvement, and a

degraded final product. Pellets were 'sticky', difficult to re-dissolve and adhered readily to glass vessels during transfers. Such samples charged with phenylalanine at only 10% the efficiency of V.faba tRNA (the V.faba tRNA charging at 12500 c.p.m./mg V.faba tRNA).

Losses of tRNA by adsorption onto the bentonite was precluded by the fact that similar yields were obtained in the absence of bentonite.

To speed the extraction process, the bentonite grinding and phenol extraction stages were combined. This resulted in a better yield and a more active product. Apparently such mixed extraction procedures have been successfully employed by other workers (Rogg et.al., 1969).

It is important to inhibit/remove RNase, as although in extracting RNA, RNase is inactive and largely extracted by phenol, some does remain in the aqueous phase. On removal of the phenol, the enzyme again becomes active and the RNA is rapidly degraded (Huppert and Pelmont, 1962; Littauer and Sela, 1962). Hall and Doty (1959) found dodecylsulphate was insufficient with phenol to inhibit RNase. In vitro use of polyvinyl-sulphate (PVS) by Shinozawa et al., (1968) found that aminoacylation of tRNA was reduced whereas Ilan and Ilan (1969) found an increase. Bentonite, as it is physically removed and does not alter biological activity has proved to be a better inhibitor (Brownhill et al., 1959; Fraenkel-Conrat et al., 1961).

The tRNA obtained by the phenol extraction procedures is partially and variably charged with amino acids, and additional treatment is usually required to remove them (Monier et al., 1960; Lipmannet al., 1959).

Amino acid stripping in the extraction stages resulted in great improvement, as did chromatography, in charging ability. Step-wise elution in chromatography yielded a mixed product. Two separate peaks were obtained at the 1.0 NaCl stage and collected on chromatography of B. chinensis tRNA. The activity of the two peaks were found to be slightly different, probably due to a variance of the tRNA species contained in each fraction. No attempt was made to investigate this aspect further,

At low temperatures, elution was slow, although diffusion in the column, bacterial growth and degradation of labiles are diminished. The reverse is true at higher temperatures. For this reason all chromatograpy was carried out at $4^{o}C$. Dunn (1970) however, conducted tRNA separations at room temperatures on regenerated chromatographic supports.

Aminoacylation was found to be typical being dependent upon the presence of tRNA, ATP and enzyme. Maximum charging of B.chinensis tRNA with phe was found to occur at 17.5mM Mg^{2+}, this figure being close to the higher of the two Mg^{2+} optima for amino acid incorporation in the presence of ribosomes (the efficient charging of the tRNA possibly aiding in incorporation or in misreading).

v) TYMV Extraction:

Isolation and purification of viruses are rarely performed as goals in themselves. To study the basic properties of a virus, it is essential to isolate preparations, more or less free of host material but retaining infectivity. No one general procedure is applicable to all cases, and even different strains of the same virus may require different

procedures for effective isolation. Lister and Bancroft (1970) noted that tobacco streak virus component ratios were influenced by host and extraction procedure. For plant viruses, purity and homogeneity are operational terms defined by the virus and the methods of isolation used. A viral preparation may be considered 'pure' if the impurities or variations present do not interfere with the particular property being studied. Effective isolation procedures have now been developed for a number of plant viruses as can be seen in the reviews of Steere (1959, 1964).

Factors affecting isolation include methods of infectivity assay, choice of suitable host plant, conditions of host growth and time of harvesting. The choice of extraction medium is also important as is the method of comminution of the infected tissue, in order to retain the virus in an infective unaggregated state.

To this end, TYMV was grown on its commonest experimental host, B. chinensis. Suitable growth conditions for the plants were determined by trial and error, as were methods of inoculation and choice of the most 'effective' host variety. From experimental determinations, it was found that B. chinensis var. Wong Bok gave the sturdiest plants and yielded the highest viral titre by the method of isolation employed. The comparative viral extractions were however rather subjective, 'biological' factors not being taken into account. Repeated extractions of the different isolates of TYMV from the three varieties of Chinese cabbage resulted in similar results, but values frequently overlapped.

TYMV has been purified by other workers from clarified plant sap by ethanol precipitation and subsequent ammonium sulphate crsytallisation

(Markham and Smith, 1949), by alternate cycles of centrifugation (Cosentino et al., 1956) and where only the active nucleoprotein was required, by emulsification with n-butanol and chloroform (Steere, 1956). Magnesium pyrophosphate gel, previously used for the purification of animal viruses and bacteriophages, has also been employed (Rialdi and Zunino, 1969).

Dunn and Hitchborn (1965) however devised a method of TYMV isolation using bentonite, the main advantages being a saving in time and a reduction or elimination of RNase contamination. Bentonite is an effective inhibitor of RNase activity (Brownhill et al., 1959; Fraenkel-Conrat et al.,1961) and has been frequently employed in the preparation of both RNA and ribosomes. It evidently functions by binding the nuclease (Brownhil et al., 1959; Jacoli, 1968) and is capable of adsorbing other proteins (Brownhill et al., 1959).

Employing the method of Markham and Smith (1949) contamination by plant materials (e.g. plant protein and ribosomal material) was evident, such preparations requiring further purification procedures. From analytical ultracentrifugation analysis of viral preparations (page 122)it was found that the method of Dunn and Hitchborn (1965) produced a 'cleaner' preparation. As a result, this latter method was employed routinely (with only a slight modification in the method of preparation of the magnesium bentonite).

vi) TYMV RNA Extraction:

Although the RNA of TYMV is readily freed from the protein by treatment with 33% ethanol at neutral pH (Markham and Smith, 1949), RNA

made this way has a much lower molecular weight (Cohen and Schachman, 1957) than that prepared by the use of phenol (Cheng, 1959; Haselkorn, 1962). One possible explanation of these results is that viral preparations, like the TMV preparations of Pirie (1956), are contaminated with RNase, which is inactivated by phenol but not by ethanol.

To eliminate RNase activity in TYMV preparations when extracting viral RNA, the method of Dunn and Hitchborn (1966) employing ethanol precipitation in the presence of sodium bentonite was used. Using this method Dunn and Hitchborn (1966) obtained 22 S heat stable TYMV RNA, contrasting to the low molecular weight material prepared by other workers, and employing this method in the present work, TYMV RNA with a high (c. 33S) sedimentation coefficient was routinely obtained.

On examination by gel electrophoresis it was found that the molecular weight was 2.5×10^6, slightly higher than the reported value 2.3×10^6 (Haselkorn, 1962). It has, however, been reported that TMV RNA exhibits a slightly excessive molecular weight when examined by gel electrophoresis (Loening, 1969). It is possible that the molecule is relatively unfolded compared with rRNA and has a relatively low sedimentation coefficient (Boedtker, 1960). Although no clear indication of the various components of TYMV RNA was seen under analytical ultracentrifugation, slight 'shouldering' was observed on gel examination of the RNA. These shoulders may have been indicative of the various components of TYMV RNA, or an indication of inherent 'gel drag'.

Variation in analytical ultracentrifuge figures is possibly the result of the fact that sedimentation rate and sedimentation heterogeneity of TYMV are sometimes unpredictable and are quite dependent on factors such

as, for example, the metal content of the virus (Johnson, 1964).

Sedimentation heterogeneity has been emphasised in a study by Matthews (1960) and Hitchborn (1968) observed that the change in the sedimentation coefficient of RNA (from heat treated TYMV) from 28 S to 22 S, as the ionic strength of the medium was decreased, paralleled the behaviour of RNA from TMV and TYMV under similar circumstances. (Boedtker, 1960; Dunn and Hitchborn, 1966; Haselkorn, 1962).

Haselkorn (1962) showed that preparations of TYMV containing heat stable 22 S material were infectious when prepared by a phenol extraction procedure. Isolation in the present work of TYMV RNA by this method yielded preparations comparable with bentonite prepared samples. Samples prepared by the two methods gave similar levels of amino acid incorporation, appeared to be equally infective on back inoculation and to be relatively free of contaminants. No intact virus was detected in such RNA preparations when examined in the analytical ultracentrifuge and absorption spectra of phenol and ethanol prepared RNA samples exhibited typical 'clean' TYMV RNA profiles, with maximum absorption at 260 nm and minimum at 232 nm (the max:min ratio being invariably over 2).

The infectivity of the RNA as compared to the intact virus was found to be similar to that found by Dunn and Hitchborn (1966), and Kaper and Steere (1959 a,b) (the latter preparing the RNA by a heating method from TYMV purified by the use of the butanol-chloroform procedure of Steere (1956)). It was indicated that TYMV RNA has approximately 1% the infectivity of the same weight of virus, or 0.3% (w/v) of the infectivity of the RNA of the intact virus.

Kaper and Siberg (1969) have isolated infectious TYMV RNA by freezing and thawing in vitro and Hitchborn (1968) obtained the release of 28 S RNA from TYMV heated in vitro. The RNA obtained by Kaper and Siberg (1969) sedimented at 33.5-34.3 S comparable with values obtained in the present work.

Experimentally degraded TYMV RNA was found to be non-infectious, to sediment at c. 4-5 S and to be inactive in in vitro amino acid incorporation.

TYMV RNA heated at about 40°C was found by Lyttleton and Matthews (1958) to sediment at 2-5 S. Such RNA would almost certainly be extensively broken down and non-infectious (Hitchborn, 1968), for there is much evidence that whole infectious TYMV RNA sediments at c. 28 S (Dunn and Hitchborn, 1966; Haselkorn, 1962). Kaper and Steere (1959b) found that the RNA from heated TYMV was infectious, suggesting that degredation of the RNA was not intrinsic to the process of disruption of the virus by heat. There is evidence that degredation of RNA may take place within stored virus particles (Haselkorn, 1962), and for this reason all the present experiments were carried out on freshly prepared virus. The speed of extraction necessary suggests that RNase is one of the principal factors involved in degradation, possibly adsorbed onto coat protein. On storage this RNase may be released in an active state, attacking the RNA when it is liberated.

It was shown by the absence of local lesions or systemic infection that the infectivity of TYMV RNA preparations was completely destroyed by RNase, but that the infectivity of the virus was unaffected. This indicates that the intact virus was not responsible for infectivity of the isolated

nucleic acid, or activity in amino acid incorporation. Evidence from protein analysis of RNA samples also precludes the presence of protein, either of viral or plant origin.

Matus et al., (1964) found that TYMV RNA aggregated into stable complexes with ribosomal RNA from Chinese cabbage leaves. When examined on PAGE mixtures of Chinese cabbage total leaf RNA and TYMV RNA gave good separation and were not apparently complexed. Neither were E. coli ribosomal RNA and TYMV RNA (although a gel formation was noted on analytical ultracentrifugation of such a mixture). Matus et al., (1964) suggested that this complexing is concerned with metal ions present, and it is possible that the EDTA present in the electrophoretic buffer solution minimised this effect, allowing ready separation.

Characterisation of the In Vitro System:

The demonstration for any particular component for amino acid incorporation, in a microsomal system, is not always possible. Appreciable concentrations of the components under investigation often contaminate the microsomes and enzyme fractions. Even minor alterations in, for example, the ionic composition can greatly alter the activity of such a system. It may be remembered also, that any change in the concentration of one component affects the environment of the whole system and may be counterbalanced or precipitate other unforeseen changes.

Absolute dependence on any one component is, therefore rarely shown unless steps are taken to remove 'contaminants'. Such 'purification' can lead to ready loss in activity, by the untoward removal of 'essential factors'. If known, these 'factors' may be isolated and added to the 'purified' system, thus restoring activity.

In developing an amino acid incorporating system from B. chinensis it was decided not to 'over-purify' the various fractions, and so only elementary de-contamination techniques were employed. Further purification in an active, characterised system would undoubtedly be advantageous, but it was not considered to be a worthwhile proposition at this stage.

The activity of in vitro incorporating systems from various sources has been expressed in a number of ways. Results vary depending upon whether they are expressed on a microsome content based on E^{256}_{1cm} spectrophotometric determination or on rRNA content determined independently. Protein content interferes with spectrophotometric

determinations of RNA and as this can vary between preparations, the Orcinol method for RNA determination was performed routinely. The results are therefore usually expressed as pmol (or c.p.m.) of label incorporated per mg. ribosomal RNA as determined by the Orcinol method.

Comparison of results between systems is often difficult in that conditions employed in the systems vary greatly. Amino acid incorporation in the presence of endogenous messenger also varies between systems, probably being dependent upon the amount of endogenous messenger and functional polysomes present. Such incorporation may also be due, in part, to completion of existing, rather than initiation of new chains.

The rate of amino acid incorporation in in vitro systems is influenced to some extent by the presence of RNase in particulate and enzyme fractions. Improved techniques of ribosomal extraction could lead to better polysome and mRNA preservation, thus leading to better amino acid incorporation. In such systems, endogenous messenger activity could be studied with greater ease. RNase activity was not found, however, to be a significant problem in the Chinese cabbage or V. faba systems.

The levels of amino acid incorporation in the viral systems were found to be relatively low, apparent in vivo incorporation being always greater than the 'equivalent' in vitro system. This is to be expected in that the in vitro system is entirely artificial and a poor imitation of the natural state. During extraction from their natural environment, some degradation undoubtedly occurs in ribosome and other preparations. Messenger RNA, for example, is known to be extremely labile and its

isolation from plant sources has not been tremendously successful.

Coleman (1969) observed that the in vitro system is less efficient than the living cell in which there is a constant environment, difficult if not impossible to achieve in vitro. In an attempt to overcome the difficulties of maintaining constant optimal levels of precursors, and the removal of unwanted accumulation products, Coleman (1969) developed a novel method for achieving cell-free synthesis of protein, based on a "Sephadex" column technique.

Aminoacylation of Chinese cabbage tRNA was found to be dependent upon the presence of tRNA, ATP and enzyme, the aminoacyl-tRNA complexes formed being hydrolysed by 5% (w/v) trichloroacetic acid at 90°C. The effect of variation of tRNA concentration on aminoacylation incorporation may be dependent upon the fact that ATP is a necessary cofactor for the process. The optimum concentration for aminoacylation varies for each tRNA species, and this in turn is dependent upon the Mg^{2+} level. Variation in tRNA concentration may therefore take the system through a range of Mg^{2+}/ATP optima for some species, which may in turn inhibit the activities of others (Rubin et al., 1967).

Rubin et al., (1967) found that for crude and purified tRNA samples, the quantitative esterification of tRNA depends not only on pH but also on the kind of buffer employed.

As will be noted in the sections on amino acid incorporation, monovalent and divalent cations affect incorporation levels. The effects

of various divalent cations on the aminoacylation of tRNA from several sources have been reported (Allende et al., 1965; Berg and Ofengand, 1958; Devi and Sarkar, 1963; Kalousek and Rychlik, 1965; Schweet and Allen,1958). The presence of K^+ was found important in the Chinese cabbage incorporating system, but its usefulness in the aminoacylation of tRNA depends on the specific system under investigation (Kalousek and Rychlik, 1965; Schweet and Allen, 1958).

The degree of incorporation of amino acid into $tRNA^{aa}$ is dependent upon the molar ratio of amino acid to tRNA, but in crude tRNA preparations, total aminoacylation is less sensitive to minor changes in solution variables. In the case of cabbage tRNA, charging was found to be proportional to the volume of amino acid added in the case of the $\left[^{14}C\right]$ amino acid mixture, and the time course of aminoacylation varied somewhat with enzyme source and to a lesser extent with the $\left[^{14}C\right]$ amino acid in the case of single charging.

Aminoacyl-tRNA synthetases (amino acid activating enzymes) catalyse the esterifications of certain amino acids with the corresponding species of tRNA. The complexity of activating enzymes is not described entirely by a geometric pattern of binding at catalytic sites; there is compelling evidence for the conclusion that conformational changes essential for catalysis result from the interaction of enzyme with substrate (Mehler,1970).

The aminoacyl-tRNA synthetases all require the presence of a divalent cation for activity, but show considerable variation in many other properties (including stability, especially on storage, activity with pH and the effects of univalent cations). In solution many of the

enzymes are unstable and lose their activity within a few hours. Chinese cabbage and bean enzyme preparations were stored frozen and found to retain their activity over prolonged storage.

Bichowsky-Slomnicki (1969) observed that an inhibitor for polyphenylalanine synthetases in an E. coli cell-free system was present in crude yeast tRNA preparations. This inhibitory activity was decreased with purification of the tRNA. The low activity of yeast tRNA in the bean and cabbage systems was, however, probably not a direct result of such inhibitors, but a function of aminoacylation enzymes in the supernatant fraction.

Enzyme concentration was found to affect amino acid incorporation in the cabbage system, an optimum volume being employed for maximum incorporation. Protein concentrations were not routinely determined as they have been found to vary with the source of material and to be unreliable guides to the activity of enzyme supernatants (Payne, 1970). Rubin et al., (1967) noted that there is a relationship between the quantity of crude aminoacyl-tRNA synthetase mixture, added as total protein, and the amount of aminoacylation. Above a certain quantity, inhibition may result.

The pH of incorporation mixes was kept constant, although in general, amino acid activating enzymes display a great dependency of activity on pH (with a relatively sharp optimum). The precise shape and position of the curve appears to depend on the reaction that is measured and many other environmental factors e.g., Mg^{2+} concentration.

Normal buffer ions may cause aminoacyl-sRNA synthetase enzyme inhibition (Fowden and Frankton, 1968), and although not generally recognised as a substrate, Mg^{2+} is essential for at least some of the partial reactions of all aminoacyl-tRNA synthetases. There are differences of opinion over the role of metal ions in reactions of tRNA, but there is a general agreement that an Mg.ATP complex is the form in which ATP reacts with these enzymes. It is surprising that so many different ratios of Mg^{2+} to ATP have been described as optimal for various enzymes of this group (the answer not lying in buffer, pH or other factors).

Cross reactions of an enzyme with tRNA designed for another amino acid do not occur except in cases of heterologous reactions involving similar structures of tRNA accepting different amino acids in different organisms. The non-accepting tRNAs of homologous systems apparently are not bound to any enzyme to an appreciable extent since they do not act as competitive inhibitors. The only known specific inhibitors of tRNA binding are fragments derived from that tRNA.

Correct translation of genetic information depends on the correct aminoacylation of tRNA by the aminoacyl-tRNA synthetase. Faulty cross reactions could be the reason for non-infection by 'foreign' viruses in a non-host plant. Similarly, interuption of the normal mechanism could occur by the viral RNA having a similar structure to the tRNA, thus infiltrating into the normal cycle. The fact that TYMV RNA may act at is own tRNA is discussed in a later section.

Use of Synthetic Message (poly(U)):

Initial experiments were performed with poly(U) to establish working systems having the characteristics of cell-free amino acid incorporating systems. The Chinese cabbage and V. faba incorporating systems were found, as might be expected, to be active for differing periods of time and at slightly different rates when employing poly(U) as messenger. This variation is to be expected in that the components of the two systems come from differing biological sources and were not isolated by identical procedures. Variation in activity may also result from the availability of essential enzymes, saturation of the system by product formation resulting in blockage and the presence of degraded ribosomes (as a result of poor preparation or unsuitable storage conditions).

Variation in the activity of the systems could also have been due partially to the presence of membrane-bound ribosomes which may or may not be involved in protein synthesis in vivo. The presence of membrane-bound ribosomes in one system and not in the other, and a variation in the proportion of cytoplasmic to chloroplastic ribosomes would undoubtedly influence reaction kinetics in vitro.

The microsomes from Chinese cabbage were probably not membrane-bound to any extent, being free as expected in actively growing leaves. Older leaves provide a larger percentage of membrane-bound ribosomes and as a result are probably less active. Whaley et al., (1960) found that merestematic cells which are rapidly dividing have mostly free ribosomes, which become bound to the cell on maturation.

The microsomes from the 65-day old beans probably contained an

appreciable proportion of membrane-bound ribosomes as previously reported (Payne, 1970). At this age in the developing bean, the storage proteins, vicilin and legumin are being synthesised rapidly, and it is at this stage that the membrane-bound ribosomes are most abundant. Payne (1970) found that maximum *in vitro* poly(U) incorporation occurred using microsomes from this age of bean and paralleled the *in vivo* activity.

The fact that microsomes were isolated from developing bean seeds for the *V. faba* system and from leaves for the *B. chinensis* system may also be reflected in the effective *in vitro* activity. This aspect of microsome variation warrents further investigation, but was not considered extensively in the present work.

Under poly(U) stimulation, bean microsomes incorporated amino acids for up to 50 to 60 min (at 25^{o}C), and the cabbage microsomes for up to 40 to 50 min. Incorporation was essentially linear for up to 30 to 40 min in both systems, the resultant fall-off in activity after this period possibly being due to lack of substrate, product accumulation or microsomal breakdown. There was no pronounced fall-off in pre-formed 'protein' after the linear period suggesting low proteolytic activity and the activity of added RNase suggests low endogenous RNase activity in the *B. chinensis* system.

Microsomes are not usually considered to be active for more than 15 to 20 min at 37^{o}C (Zamecnic and Keller, 1954), amino acid incorporation being completed, under standard conditions, in 20 to 30 min (Boulter,1970). An exception has been noted with red beet microsomes (Ellis and MacDonald

1967) where incorporation was linear for 1h.

Korner (1961) considered that rat liver ribosomal systems could last longer, for even up to three hours, the time being somewhat dependent upon the preparational procedures employed.

In other systems it has been found that incorporation with poly(U) may be linear up to 30 min (Matthaei *et al.*, 1962; Nirenberg *et al*,1963) then ceasing. Matthaei *et al.*, (1962)suggested that the poly(U) was broken down by nucleases in their *E. coli* extracts, or that the polypeptides were not released from the ribosomes when the poly(U) was read to the end. In the latter case the system becomes saturated.

Variation of microsome concentration or temperature of incubation altered rates and degree of amino acid incorporation, the effects of temperature being discussed more fully under a later heading. Saturation of the *in vitro* system with messenger undoubtedly affects *in vitro* activity, which in turn will be reflected by altering the microsome concentration. Alteration will also affect other factors, including microsome-bound enzyme/co-factor concentration. Any non-specific binding of *in vitro* system components by microsomes will be affected by alteration in microsome concentration, which in turn will affect the *in vitro* activity. Variation in bound endogenous messenger or RNase will also play a part.

Levels of amino acid incorporation recorded in vitro are never as high as the rates of *in vivo* protein synthesis. This fact probably stems from the isolation of the various components for protein synthesis and their subsequent assay in a purely artificial system. A certain amount

of degradation and loss of activity is bound to occur during microsome extraction and enzyme and RNA isolations.

The V. faba system developed in the present work was found to be comparable with the system developed by Payne (1970). Optimal activity was at 25°C with a poly(U) concentration of 200 µg/ml incubation. There appeared to be two Mg^{2+} optima, one at 8 mmol and the other at 20 mmol of added Mg^{2+}. The former level was used routinely, the latter probably being a reflection of accentuated misreading as a result of excess Mg^{2+}.

Payne (1970) in the bean system found a sharp Mg^{2+} optimum at 10 mmol, although the optimum for incorporation by endogenous messenger was lower at 6 mM. Similar results to this were reported by Mosteller et al., (1968) for amino acid incorporation by a system from rabbit reticulocytes.

Unpublished work by Boulter and Yarwood has shown that the magnesium optimum for phenylalanine incorporation by V. faba microsomes can be reduced by pre-incubating the microsomes with poly(U) in a system similar to that reported by Mosteller et al., (1968).

Allende and Bravo (1966) noted a 10 mM optimum for Mg^{2+} in their wheat germ embryo system when employing poly(U), and these variations may reflect inherent variations in the different biological systems, or the variation in Mg^{2+} already present from extraction procedures etc.

Maximum incorporation employing 250 µg microsomal RNA per ml incubation resulted in 200 to 250 pmol of phenylalanine incorporated. Payne (1970) obtained similar levels of incorporation in a comparable system, as did Allende and Bravo (1966) (160 pmol/mg wheat ribosomal RNA)

and Leaver and Key (1967) (100 pmol/mg carrot root ribosomal RNA). At some point ribosomes will become saturated with messenger and from this it may be seen that microsome and messenger concentrations are interrelated and will affect resultant *in vitro* activity (together with other variables).

Activity was found in general to be dependent upon the addition of ATP and in the absence of added enzyme source, the activity was considerably less than that of the complete system. Bacterial incorporation is independent of added ATP and the generating system, and the ATP requirement for the *V. faba* system suggests low bacterial participation. The activity of the system in the absence of added enzyme (or ATP) was probably due to the presence of contaminating enzyme (or ATP) in the microsome preparations.

Endogenous RNA activity (i.e. in the absence of added poly(U)) was usually less than 10 pmol phenylalanine per mg microsomal RNA. This is to be expected as endogenous mRNA is extremely labile and no special precautions were taken to preserve it during isolation procedures. The activity in the absence of poly(U) may have been due in part to completion of chains, rather than to pronounced endogenous activity.

After establishing a working *V. faba* system using poly(U) as artificial messenger, the system conditions were adopted for *B. chinensis* components.

Although incorporation levels were lower than the equivalent bean system, maximum incorporation at 6 mmol/l Mg^{2+} was usually in the order of 20 pmol phenylalanine incorporated per mg microsomal RNA. Further

addition of poly(U) during the course of the incubation resulted in a 20% increase in activity, suggesting that the low initial activity was not due to lack of substrate. Many factors could have contributed to the relatively low activity of the cabbage system, the main source of inhibition probably resulting from the enzyme fraction.

No attempt was made to purify the enzyme fraction, other than dialysis. Fractionation and subsequent recombination may have improved *in vitro* activity.

Chinese cabbage microsomes may be more labile than bean ribosomes, their degradation resulting in low activity of the *in vitro* system.

As with the bean system, a second magnesium optimum was found in the cabbage system, at 16 mmol/l added Mg^{2+}. Again this may have resulted from increased misreading of the artifical messenger.

The presence of 0.2 mmol spermine increased incorporation, possibly stabilising the system or aiding in initiation/ translation processes (the effects of added polyamines are discussed further under a later heading).

Mixed Systems:

Combined bean and cabbage systems (or the use of yeast tRNA) were never as good as complete bean systems, suggesting some incompatability of components or the presence/absence of inhibitors/essential components.

Chinese cabbage tRNA in the bean system resulted in similar order incorporation, but substitution of cabbage enzyme caused a marked decrease in activity. It would appear that the enzyme fraction was not providing

the requisite factors for incorporation or that components present in the cabbage leaf enzyme were inhibitory. The latter is most likely, although substitution of cabbage microsomes in the bean system also drastically reduced activity, suggesting some complex factor(s) affecting interchangeability (their examination being beyond the scope of the present work).

Yeast tRNA was not as active as native tRNA in the bean and cabbage systems, secondary structure, enzyme or inhibitor availability possibly causing this effect. The use of yeast tRNA was therefore reserved to test bean or cabbage systems if native tRNA was of suspect activity.

The reduction in incorporation activity in incubations in the absence of poly(U) (under optimum conditions for plus poly(U) incubations) possibly resulted from a reduction in non-specific binding.

Further aspects on mixed systems, with emphasis on the viral message are discussed later.

Use of Natural Message (TYMV RNA):

(i) Single $[^{14}C]$ Amino Acid Systems:

Synthetic polynucleotides, including poly(U), have been widely employed as 'messengers' in in vitro amino acid incorporating systems. The use of such messengers is always open to suspect unless end product analysis can confirm the expected result. The first synthetic polyribonucleotide shown to act as a messenger in an in vitro cell-free

system was poly(U) (Nirenberg and Matthaei, 1961). It was shown that this polymer was highly specific for the conversion of l- phenylalanine into an acid-precipitable form in an E. coli pre-incubated system. The product of the incorporation had properties similar to those of polyphenylalanine. Similarly in the B. chinensis and V. faba systems developed in the present work, poly(U) was employed and assumed to be programming polyphenylalanine synthesis.

Ambiguity of poly(U) translation was however demonstrated, first in an E. coli cell-free system by Matthaei et al., (1962) and Bretscher and Grunberg-Manago (1962). These workers showed that poly(U) can stimulate incorporation for leucine and valine as well as phenylalanine. Ambiguity in translation has also been demonstrated for other synthetic polynucleotides (Friedman and Weinstein, 1964,1965; Davies, 1966; Nishimura et al., 1969). It has been shown to be dependent upon environmental conditions such as temperature, magnesium concentration (Szer and Ochoa, 1964), addition of ethanol (So and Davie, 1965), polyamines (Friedman and Weinstein, 1964), aminoglycoside antibiotics (Davies et al., 1964), concentration of sRNA, pH of the medium (Grunberg-Manago and Dondon, 1965) and the origin of the isolated system (Friedman et al., 1968). Preparations obtained from different bacteria translate synthetic polynucleotides with different fidelity, and it is suggested that the accuracy of translation is dependent upon the presence of components involved in protein biosynthesis, characteristic of that given organism.

It may also be noted that various workers have reported the inhibition of amino-acylation of tRNA by synthetic polynucleotides and Letendre et al., (1969) have shown that poly(U) inhibits the charging

of yeast tRNA by purified yeast lysyl tRNA synthetase, the inhibition being competitive.

Where possible therefore, the use of natural messengers is to be preferred, possibly tending more to in vivo conditions. Endogenous plant mRNAs are very labile and so many workers have employed 'semi-endogenous' mRNAs in the form of plant viral RNA.

To this end, TYMV RNA was employed in the present work, and after developing the working V. faba/B. chinensis poly(U) systems, attempts were made to introduce it in place of the synthetic polynucleotide. These attempts were only partially successful.

As with the 'synthetic' systems mentioned above, amino acid incorporation under TYMV RNA stimulation varied with microsome content and temperature of incubation. The latter also altered the kinetics of incorporation as observed by Katz and Moss (1969) and Wettstein et al.,(1964)

In the main, TYMV RNA showed no apparent activity in the bean system (which was fairly active under poly(U) stimulation) when employing the single $[^{14}C]$ amino acid phenylalanine. Background incorporation was however reduced in the presence of viral RNA (as noted with poly(U) systems above), possibly suggesting binding and competitive or non-competitive inhibition of endogenous mRNA if present. Sela (1969) noted that amino acid incorporation can occur with $[^{12}C]$ amino acids which would not be detected by the assay system used in the present work and this is another possible cause of apparent inactivity in the TYMV RNA/bean polyphenylalanine system.

Many other factors could have been preventing amino acid incorporation in the bean system, including the absence of specific tRNAs or enzyme factors. Little activity was noted in mixed bean and cabbage component systems when employing TYMV RNA and $[^{14}C]$ phenylalanine. When B. chinensis tRNA and enzyme were used with bean ribosomes, activity was noted suggesting that the viral RNA was unable to use bean tRNA, or that the Chinese cabbage enzyme contained essential factors missing from the bean isolate.

It would appear that the bean ribosomes were not the main source of inactivity, being active in the mixed system. This may suggest that in vivo, bean ribosomes are not the level at which TYMV is prevented from infecting bean plants.

In a yeast tRNA substituted bean system, viral RNA was inactive, again suggesting the presence of some essential factor in Chinese cabbage tRNA for TYMV RNA amino acid incorporation. Conditions may however have been simply unsuitable for yeast tRNA activity (although yeast tRNA was shown to be active in bean and cabbage systems with poly(U)). The importance of tRNA and factors concerning it and viral protein synthesis are discussed later.

Other single $[^{14}C]$ amino acids, at various magnesium levels, were also employed in the bean/viral system, but were found inactive unless combined.

Viral RNA was found to be inactive in the complete single $[^{14}C]$ amino acid cabbage system, except at high magnesium levels (suggesting forced misreading of the viral message) although added poly(U) resulted in

incorporation. This activity did however suggest that the cabbage system was functional, and that possibly more $[^{14}C]$ amino acids were required to record any viral activity.

The variable and low activity of single amino acid incubations led therefore to the employment of a $[^{14}C]$ amino acid mixture, with consequent improvement of results.

(ii) $[^{14}C]$ Amino Acid Mixture System:

In the B. chinensis system, a requirement for $[^{12}C]$ amino acids was not absolutely demonstratable, probably due to the presence of endogenous amino acids in the various fractions. Sela (1969) reported the non-requirement of exogenous amino acids for protein synthesis in plant cell-free systems. Allende and Bravo (1966) suggested that it may be necessary to pass the enzyme fraction through a Sephadex G25 column to remove contaminating amino acids. In the present work, however, enzyme preparations were dialysed to eliminate endogenous amino acids, as suggested by Allende (1969). It is probable that this was insufficient to remove all contaminants.

Van Kammen (1967) and Allende and Bravo (1966) observed that the omission of the mixture of unlabelled amino acids from their systems had no significant effect on amino acid incorporation. Korner (1961), Staehelin et al., (1967) and Wannemacher et al., (1968) however, reported the requirement for added amino acids to obtain maximal incorporation. Huston et al., (1970) found excess amino acids were not inhibitory in their

rat liver polyribsomal system, whereas Wannemacher et al., (1968) found the opposite when employing total ribonucleoprotein particles.

In the B. chinensis system there was frequently a lag of about five minutes before incorporation followed a linear course for 25 to 30 min. This lag has also been noted for TYMV RNA promoted polypeptide synthesis in an E. coli system (Voorma, 1965; Voorma and Bosch, 1965; Voorma et al., 1965).

In their system, after a lag of about 5 min, incorporation of $[^{14}C]$ amino acid followed a linear course for up to about 24 min of incubation. The reaction was found to stop due to messenger degredation, incorporation being optimal at 18 μmol Mg^{2+} per ml (cf. Chinese cabbage system, optimum 6 mmol/l Mg^{2+}). In the Chinese cabbage system, (employing $[^{14}C]$ phenylalanine) incorporation may have been limited by messenger breakdown as addition of poly(U) resulted in further activity. This activity suggested the system to be still active, and that either TYMV and poly(U) act at different ribosomal sites or that TYMV can be readily replaced or removed by poly(U).

Activity in the Chinese cabbage system was found in general to be dependent upon the addition of ATP and was further enhanced by the presence of an ATP-generating system. Omission of ATP reduced incorporation by 65%, and its addition during the course of the incubation, doubled the incorporation level at the time of addition. It is clear that although incorporation was energy dependent, added ATP was not the only energy source. In particular, the energy generating system appears to meet only part of the energy requirement. Presumably the ribosomal fraction contained

sufficient AMP or ADP, or both, which together with the generating system could have supplied the necessary energy. The dependence, at least in part, on added ATP does suggest ribosomal rather than bacterial incorporation, the latter being independent of ATP.

The requirement for ATP was not found to be consistent. On occasion, addition of ATP resulted in a reduction in incorporation. This could have been due in part to the binding of magnesium ions by the added ATP, ATP being a very strong chelating agent for Mg^{2+} (Martell and Schwarzenbach, 1956). Zamecnik and Keller (1954) observed that ATP added in high amounts (0.01M) to microsomal systems did act as a chelating agent and inhibited amino acid incorporation. Korner (1961) noted however that purified ribosomes are not as sensitive to high concentrations of ATP when added together with equimolar amounts of $MgCl_2$.

GTP is necessary for maximal activity in virtually all cell-free incorporating systems, the Chinese cabbage system being no exception. Incorporation was however only reduced by 59% in the cabbage system in the absence of added GTP. Some peptide synthesis did occur, possibly arising from the presence of small amounts of ribosome bound GTP and to traces of guanosine nucleotides and nucleoside diphosphate-nucleotide triphosphate transphosphorylase activity in the system (not removed during microsome isolation) (Means and Baker, 1969). Such endogenous material could be hydrolysed to GDP, and the GDP reconverted to the trinucleotide by the ATP generating system.

The lack of absolute requirement for GTP is not unusual in the unfractionated system (Marcus and Feeley, 1965; Means and Baker, 1969;

Robinson and Novelli, 1962; So and Davie, 1963). The extent of inhibition due to the absence of this nucleotide varies considerably (Campagnoni and Mahler, 1967; Spector and Travis, 1966; Teng and Hamilton, 1967), Arlinghaus et al.,(1964) being the first to report the requirement for GTP and an enzymatic factor in the binding of aminoacyl-tRNA in the presence of mRNA in the rabbit reticulocyte system. The binding of aminoacyl-tRNA to E. coli ribosomes also involves GTP (Ertel et al., 1968; Lucas-Lenard and Haenni, 1968; Ravel et al., 1967,1968). Hydrolysis of this nucleotide allows translocation of peptidyl-tRNA from the donor to the acceptor site in the ribosome (Ohta and Thach, 1968).

The use of high magnesium concentrations in incorporation mixtures can also mask GTP requirements (Kurland,1966; Ravel, 1967), and cessation of incorporation may be aided by ATP and GTP breakdown by triphosphatases.

Although incorporation was dependent upon the addition of microsomes and tRNA in the cabbage system, a certain amount of incorporation was recorded in their absence. This was probably again due to the presence of these as enzyme contaminants. Variation in microsome concentration, altered the level of incorporation, excess microsomes being inhibitory (probably partly due to magnesium and other co-factor binding).

The concentration of magnesium is critical for optimum activity in the in vitro amino acid incorporating system. An optimum of 8 mmol/l Mg^{2+} was found for the poly(U) directed and 6 mmol/l Mg^{2+} for the viral RNA directed Chinese cabbage systems (magnesium concentrations referring to added Mg^{2+}). In the viral RNA system, excess magnesium was found to be inhibitory, probably combining with other essential factors in the incubation mix.

The stability of ribosomes is decreased at low magnesium concentrations, Mg^{2+} ions being essential to maintain ribosomal integrity (Tissières and Watson, 1958). In the complete absence of bivalent ions, the particles disintegrate into smaller subunits (Petermann et al., 1958). Activity is restored with recombination, occurring on the addition of Mg^{2+} (Takanami, 1960). Excess Mg^{2+} may have resulted in ribosome precipitation as noted by Takanami (1960).

Mg^{2+} ions are an integral part of protein biosynthesis, being required for the formation of Enzyme-Amino-Acid-AMP (fig.1p20), for binding the messenger to the ribosome (Ravel and Shorey, 1969), and for peptide bond formation etc. Amino acid incorporating systems vary in their Mg^{2+} requirements, material from differing plant sources having different Mg^{2+} requirements. This varies from 5 mmol in tobacco leaf cytoplasmic ribosome systems (Boardman et al., 1966) and pea epicotyl systems (Davies and Maclachlan, 1969) to 20 mmol in a castor bean embryo system (Parisi and Ciferri, 1966). Levels between 5 and 10 mmol Mg^{2+} appear to be the most commonly employed.

The different magnesium optima may reflect the different levels of magnesium employed in the preparation and storage of the ribosomes. The concentration of Mg^{2+} ions in the extraction medium can influence incorporation levels of the extracted ribosomes considerably (Huston et al., 1970). Ribosomes are known to adsorb magnesium ions and high levels of magnesium in the extractant during isolation allow such preparations to be resuspended and stored at lower Mg^{2+} (without apparent loss of activity).

The 1:1 complex formation between organic phosphates and Mg^{2+} is well recognised (Manchester, 1970). The concentration of free Mg^{2+} is

therefore dependent upon the other components of the incubation mix. Media for incubation of ribosomes are often made up on the principle of a requirement for ATP and GTP, Mg^{2+} present in an amount sufficient to chelate the nucleoside triphosphates present plus the quantity normally required, an ATP generating system, monovalent salt and Tris buffer. All these components affect each other and in turn the overall picture.

For several ribosomal systems, optimal Mg^{2+} concentrations have been stated and such figures may be empirically correct. If we assume however that it is the value of Mg^{2+} that is critical in determining the behaviour and activity of the system, it is impossible to make adequate assessment or comparisons of the significance of these figures without determination of the concentration of the free ion. Insofar as it is reasonable to analyse published work, optimal Mg^{2+} concentrations for incorporation by endogenous messenger in vitro appears to be 2-3 mM, but this figure is likely to be an upper limit in that it takesno account of Mg^{2+} binding by RNA and protein, which may be appreciable (Petermann, 1960; Warren, 1964). It comes near to what might be considered a reasonable figure for free Mg^{2+} concentration in vivo (England et al., 1967; Kerson et al., 1967; Rose, 1968). Conversely attachment of ribosomes to poly(U) requires higher concentrations of Mg^{2+} (Williamson , 1969).

No attempt was made in the present work to analyse free Mg^{2+}. It was however assumed that as standard conditions of extraction and standard volumes of enzymes etc., were employed (as far as practicable), Mg^{2+} added to the amino acid incorporation incubation reflected the effect

of Mg^{2+} ions on the activity of such systems, and allowed some comparison between them.

Although cell-free systems in general exhibit a requirement for Mg^{2+}, some preparations exhibit maximal activity over a wide range of Mg^{2+} concentration (Campagnoni and Mahler, 1967; Zomzely et al., 1966), others requiring a more definite amount for maximal activity (Ganoza et al., 1965; Means and Baker, 1969; Teng and Hamilton, 1967). The Chinese cabbage and bean systems fall more or less into the latter category, the optimum depending upon messenger type.

A certain dependence upon added K^+ for amino acid incorporation was noted in the cabbage system, K^+ appearing to be necessary for maximal activity in some cell-free systems (Hoagland et al., 1956; Teng and Hamilton, 1967), others requiring no added K^+ (Means and Baker, 1969; Spector and Travis, 1966; Wettstein et al., 1963). There appears to be some link between ribosome stability and Mg^{2+}/K^+ concentrations (Sátake et al., 1965), and its part in supporting ribosomal fractions has been considered (Hultin, 1964). Holley et al., (1961b) reported that the activity of isolated tyrosine tRNA synthetase was stimulated by K^+.

The level of K^+ in the extraction medium can play an important part in determining activity of the resultant ribosomes (Huston et al., 1970). In the cabbage and bean systems, the K^+ level was maintained constant in extraction stages, being only varied in the incubation mixture. The requirement for K^+ was not as definite as that for Mg^{2+} in the cabbage system, ranging over 50 to 90 mmol/l, although the optimum appeared to be 70 mmol/l. This range and optimum level corresponds with the 75 mMof the

rat liver polyribosomal system of Huston et al., (1970) and the 60 mM of the castor bean embryo system of Parisi and Ciferri (1966). Although Cooper et al., (1968) obtained similar results these high ranges are considerably in excess of the 20 to 25 mM employed by several other investigators (Campbell et al., 1967; Stahl et al., 1968; Wunner et al., 1966; Zamecnik and Keller, 1954).

It has been demonstrated that in the rat liver system, Na^+ is inhibitory to protein synthesis (Tillotson et al., 1969). In the cabbage incubation medium, Na^+ was added in the form of disodium creatine phosphate (effectively increasing the total monovalent cation concentration). Rather than an inhibitory effect, Na^+ may have had an additive effect with K^+ in the 20 to 25 mM K^+ range systems.

It may be noted that several workers have used NH_4^+ either in part (von der Decken, 1967) or as the sole source (Cooper et al., 1968; Staehlin, et al., 1967) of monovalent cation for in vitro systems.

In the Chinese cabbage system, amino acid incorporation was reduced to 50% by the omission of GSH from the incubation mixture. Allende and Bravo (1966) in their wheat germ embryo system found that they did not require GSH. Microsomal systems from mice are stimulated by GSH whereas rat liver systems are inhibited by concentrations above 0.0025 M (Sachs, 1957). In the mouse system, no inhibition was observed, even at concentrations of 0.04 M (Robinson and Novelli, 1962).

Von der Decken (1968) showed a requirement for GSH added directly to the assay medium, using total ribonucleoprotein ribosomes rather than

polysomes. Other workers (Baliga et al., 1968; Garren et al., 1967; Sidransky et al., 1968) have added thiol groups to the assay medium, but not to the extraction medium. Huston et al., (1970) noted no further stimulation of incorporation on addition of extra GSH to their assay medium, and concluded that a source of sulphydryl was better utilised in the isolation steps in order to protect sulphydryl sites on cell sap enzymes.

Henshaw et al., (1963) obtained high activity in preparing rat liver microsomes in the presence of 0.006 M MCE.

Van Kammen (1967) observed that amino acid incorporation was very sensitive to the addition of a small amount of pancreatic RNase, 0.1 μg giving 60% inhibition and 1.0 μg reducing incorporation to a negligible level in a tobacco leaf system. In the Chinese cabbage system, 0.5 μg per ml RNase reduced incorporation to 1% of the control. The sensitivity to RNase, as noted previously, could indicate that the amount of contaminating active nucleases in the ribosomal and enzyme preparations is small, amounting to only a fraction of the concentration added. Similar inhibition by RNase in various systems has been reported by other workers (see Mehta et al., 1969).

In the Chinese cabbage system, incorporation was found to be dependent upon the addition of viral RNA, the concentration of which was maintained constant. Poly(U) tends to saturate in vitro systems at rather low concentrations (c. 100-200 mμ mol/ml) (Jones et al., 1964; Lengyel et al., 1961; Matthaei et al., 1962). At greater concentrations it may become inhibitory, but it is uncertain why (Jones et al., 1964 ;

Lengyel et al., 1961). Viral RNAs appear to saturate the in vitro system in a similar range of concentrations. TMV RNA saturates at c. 200 mμ mol, f2 RNA at 300 mμ mol (Nathans et al., 1962; Tsugita et al., 1962), and TYMV RNA at 70 mμ mol (Ofengand and Haselkorn, 1962). In the Cabbage system, stimulated by TYMV RNA, poly(U) addition resulted in increased incorporation suggesting that either the system was not saturated by TYMV RNA, or that the poly(U) acted at different sites.

Maximum incorporation occurred at 25°C in the Chinese cabbage TYMV RNA directed system, the effect of temperature on incorporation being discussed further under a later heading, as is the effect of polyamines on incorporation.

During microsome extraction, a similar volume of extractant was employed to extract similar weights of similar leaf samples. The volume of enzyme promoting maximum amino acid incorporation was therefore similar in all preparations. This may be indicative of a homogenous enzyme distribution or the saturation of the supernatant by the requisite factors. Bearing the latter point in mind, extraction of similar ages of leaves may also be a relevant factor. No survey was made of the effect of leaf enzyme age on incorporation activity, but it is probable that enzyme environment alters with leaf age.

Increase in enzyme volume beyond the optimum resulted in inhibition of incorporation. This may have been due to the presence of endogenous amino acid(s) isotopically diluting out the $[^{14}C]$ amino acids. Alternatively inhibition may have been due to increasing concentrations of proteases and RNases, or even to the presence of multiple (degenerate) species of tRNA which would block incorporation.

Employment of enzyme from infected B. chinensis plants resulted in a reduction in incorporation by over 50%, possibly as essential factors were absent as a result of viral infection (or as a result of increased RNase concentration).

It was observed that TYMV RNA in the bean system was not as active as in the cabbage system. The activity, however, does suggest that TYMV could infect, or at least produce coat protein in vivo in the bean if the RNA was able to reach the ribosomes. On substituting cabbage tRNA into the bean system, incorporation was increased, but addition of Chinese cabbage enzyme or ribosomes decreased activity, suggesting some incompatibility, presence of inhibitors or a combination of such factors.

The phenomenon of species specificity between aminoacyl-tRNA synthetases and tRNA preparations from different organisms is well documented (Doctor and Mudd, 1963). Little work has been done however to compare the compatibility of plant synthetases and tRNAs with components from other sources.

Since the initial formulation of the concept of mRNA binding to ribosomes, it has generally been assumed that ribosomes cannot selectively recognise the specific mRNAs and therefore represent a non-specific machinery for translation of genetic messages (Lipmann, 1963). Evidence has been presented, however, suggesting the differential affinities of animal ribosomes for certain classes of informational macromolecules (Carter and Levy, 1967; Naora and Naora, 1967). In previous work, failure to demonstrate the ability of ribosomes to

distinguish certain messages may have been partially due to the use of the heterologous system, i.e., the binding of TYMV RNA or synthetic polymers by E. coli ribosomes (Dahlberg and Haselkorn, 1965, 1967; Moore, 1966; Weksler and Gelboin, 1967). Recently studies on interferon have clearly revealed that ribosomes do distinguish the specific RNA in the homologous system for binding (Carter and Levy, 1967, 1968; Marcus and Salb, 1966). Since the binding of RNA by ribosomes takes place at a certain region on the RNA chain (Dahlberg and Haselkorn, 1966; Takanami et al., 1965; van Duin et al., 1966), it appears likely that a certain nucleotide sequence situated at/or near the binding site on the RNA chain is responsible for recognition by specific ribosomal particles (Naora and Kodaira, 1969). It is possible that the viral RNA must first 'tune-in' to the cells system, or alter the environment of the cell to its own benefit for activity. Apparently TYMV is active in the bean system in vitro, but it may not be able to adapt for in vivo activity.

The mechanism of polypeptide chain elongation is functionally analagous in mammalian and bacterial systems, but there is evidence against the complete interchangeability of supernatant factors. Although bacterial factors are interchangeable (Parisi et al., 1967; Salas et al., 1965) and yeast ribosomes and factors can complement mammalian ribosomes and factors (Nathans and Lipmann, 1961), Rendi and Ochoa (1962) reported that no protein synthesis occurred when mammalian ribosomes were used with bacterial supernatant factors and vice versa. Canning and Griffen (1965) however found that E. coli system fraction could substitute for tumor supernatant fraction on tumor ribosomes. Similarly, So and Davie (1963) found that yeast ribosomes were supplemented by E. coli supernatant fraction.

Krisko et al., (1969) suggested that E. coli elongation factor T can replace the corresponding mammalian T_1 factor but that factor G cannot substitute for mammalian factor T_2. Obviously in the bean system some supplementation by cabbage enzyme was possible, as was supplementation of the bean system by cabbage ribosomes. Although there is species specificity in the action of amino acid synthetases, the amino acid from aminoacyl-tRNA is incorporated into protein irrespective of source of the tRNA (Moldave, 1965; Schweet and Heintz, 1966). In the use of Chinese cabbage and yeast tRNAs, therefore, it is the enzyme fraction that dictates primary activity, the ability to charge any particular tRNA with the requisite amino acid depending on the species of synthetase present.

Novelli (1967) stressed the need to ascertain the optimal Mg^{2+}/ATP concentration ratio for aminoacyl-sRNA synthetases and even for the same enzyme obtained from different species. Thus when employing mixed systems, this factor along with others could alter the efficiency.

A release factor, probably a protein of molecular weight 40,000-50,000 has been recognised as necessary for the release of newly formed polypeptide chain from the messenger RNA-peptidyl-tRNA complex (Capecchi, 1967). The possible specificity of such factors in different groups of organisms remains to be assessed. If such specificity exists it might play some part in the inefficient translation of plant viral RNAs in in vitro systems derived from bacteria, or for that matter, from any heterologous source. This does not appear to be a problem in the bean/Chinese cabbage systems, where the message is apparently active, although the products were not isolated and analysed.

In the cabbage *in vitro* system D-threo chloramphenicol (10 and 50 μg/ml incubation) had little effect on incorporation levels, suggesting incorporation to be largely cytoplasmic in nature.

Several antibiotics have been tested for inhibition of protein synthesis, one possible approach to the study of protein synthesis having been the use of inhibitors which specifically inhibit only one step among the several that are necessary for the biosynthesis of proteins.

Chloramphenicol is a potent inhibitor of bacterial protein synthesis *in vivo* (Gale and Folkes, 1953; Wisseman *et al.*, 1954) although *in vitro* results have varied depending on the assay used (Bresler *et al.*, 1968; Julian, 1965; Kucan and Lipmann, 1964). While protein synthesis in most non-bacterial systems is only marginally affected by chloramphenicol (Gale, 1963), chloroplasts constitute a notable exception. Thus the incorporation of amino acids into protein by ribosomes isolated from chloroplasts is sensitive to chloramphenicol (Spencer, 1965) and the development of chloroplasts in *Euglena gracilis* is prevented by chloramphenicol at concentrations which have little effect on essential cytoplasmic protein synthesis (Smillie *et al.*, 1963). These effects have been attributed to the greater capacity of chloroplast ribosomes to bind chloramphenicol (Anderson and Smillie, 1966).

Chloroplasts from higher plants contain ribosomes resembling bacterial ribosomes in their sedimentation behaviour (Boardman *et al.*, 1966; Lyttleton, 1962), in the sizes of their RNA components (Loening and Ingle, 1967; Stutz and Noll, 1967) and in the inhibition of their

amino acid incorporating ability by chloramphenicol (App and Jagendorf, 1963; Spencer and Wildman, 1964). In contrast, cytoplasmic ribosomes from both green and non-green tissues of higher plants are insensitive to chloramphenicol both *in vivo* and *in vitro* (Ellis, 1964; Ellis and MacDonald, 1967; Marcus and Feeley, 1965; Parisi and Ciferri, 1966).

It has been shown for Euglena (Eisenstadt and Brawerman, 1964) and *Nicotiana tabacum* (Ellis, 1969) that the cytoplasmic and chloroplast ribosomes from the same plant cells differ in their response to chloramphenicol. There are four stereoisomers of chloramphenicol (D- and L-threo and D- and L-erythro); the naturally occurring antibiotic being the D-threo isomer. The inhibition by chloramphenicol of amino acid incorporation is highly specific for the D-threo isomer (Rendi and Ochoa, 1962) in bacterial ribosomes and a similar stereospecificity has been reported for chloroplast ribosomes in *Nicotiana tabacum* (Ellis, 1969).

It may be noted however, that other processes, including ion uptake (Ellis, 1963) and oxidative phosphorylation (Hanson and Krueger, 1966) are inhibited by several isomers including the D-threo form; in bacteria these processes are not inhibited by chloramphenicol (Brock and Moo-pen, 1962; Kaback and Stadtman, 1966).

Application of In Vitro Amino Acid Incorporation Experiments to Biochemical Aspects of the Infection Process:

Although *in vitro* systems, to date, have given us little information concerning the synthesis of plant virus coat proteins, attempts to determine the sites of coat protein synthesis using labelled amino acids *in vivo* have been no more successful. The unsuccessful nature of the *in vivo* experiments has been due in main to the high level of incorporation throughout the entire cell, and to lack of inhibitors specific to host protein synthesis.

No viral proteins other than the coat protein and tentative identification of virus-specific synthetase have been isolated from virus-infected plants, or recognised among the products of *in vitro* protein synthesising systems using plant viral RNAs as message. These proteins may well occur in very low concentrations, and since they are of unknown function, are difficult to assay (Matthews, 1970). Other workers have made unsuccessful attempts to identify new non-coat proteins in TYMV-infected leaves using chromatography and microimmunological methods to separate isotopically labelled proteins (Matthews, 1970).

In vitro experiments with isolated material from *Brassica chinensis* and *Vicia faba* have therefore been performed in an attempt to elucidate some of the simple and basic stages of the TYMV infection process. The following discussion therefore is a review of present knowledge of the infection process (and coat protein synthesis), related where possible to the present work on the bean and cabbage amino acid incorporating systems.

Recent work on protein synthesis is consistent with the proposal of Jacob and Monod (1961) that the information for specifying protein structure is carried from DNA to the site of protein synthesis, the ribosome, by mRNA. Their proposal further required that the ribosome be an unspecialised structure capable of making any protein with a suitable mRNA. This proposal would negate this level as a source of host specificity (although possible enzyme differences must be borne in mind). TYMV RNA was active in bean and cabbage single and mixed systems, incorporating amino acids, thus furthering the view of the unspecialised nature of the ribosome. The artificial nature of the in vitro system, however, may preclude such unspecificity in vivo.

When an infectious RNA molecule penetrates a cell it behaves as a mRNA, coding for the synthesis of the coat protein as well as for the synthesis of one, or two enzymes, necessary for the replication of the virus RNA itself (Weissmann, 1965). According to this view, synthesis of the virus should be independent of the genetic material of the cell, namely the DNA in the nucleus. Indeed Sänger and Knight (1963) have shown in vivo, with tobacco leaves, that TMV RNA is still synthesised when the synthesis of cellular RNA is almost completely inhibited by actinomycin-D. In the uninfected cell, the synthesis of normal RNA occurs on a DNA template. Leaf RNA synthesis also continues during TYMV infection of leaves (Ralph et al., 1965) so that TYMV probably competes with host mRNA for host ribosomes. New RNA-synthesising machinery must be induced in the host after infection with an RNA virus.

This indicates that the step of transcription as a controlling element of protein synthesis is by-passed, and is probably not involved

in host specificity. The regulation of viral protein synthesis must be provided by other means, and the survival of viral RNA in the DNA-governed environment of the host cell raises the problem of protecting this molecule against the normally rapid inactivation of mRNA molecules.

Francki and Matthews (1962) found that in TYMV infected Chinese cabbage leaves, treated with 2-thiouracil, virus nucleoprotein production was suppressed while the production of empty protein shells was greatly stimulated. It is suggested that virus coat protein synthesis can proceed in the absence of viral RNA synthesis and must be produced on a viral RNA message that is relatively stable in the cell.

TYMV RNA appeared to be reasonably stable in the in vitro cabbage and bean systems, being active for the 'expected' period of time for such microsomal systems.

Hiruki (1969) showed that host rRNA and BMV RNA were synthesised concurrently and, under their experimental conditions, at about the same rate. Rapid degredation of mRNA after bromegrass mosaic virus infection was not detected, although it has been reported to occur in tobacco leaves after infection with TMV (Reddi, 1963 a,b).

The mechanisms of viral coat protein synthesis resembles in all aspects studied so far, the synthesis of normal cellular proteins. The replicated progeny viral RNA acts as a messenger for this synthetic process. In picorna virus infected cells, the time course of coat protein synthesis follows that of the production of progeny RNA, although in Newcastle disease virus and Sendai virus-infected cells, virus specific RNA complementary to parental RNA is synthesised and attached to polysomes

(Blair and Robinson, 1968; Bratt and Robinson, 1967; Kingsbury, 1966). It is possible that parental paramyxovirus RNA might however take part in the formation of virus-specific polysomes synthesising early viral proteins (Bukrinskaya et al., 1969).

In the poliovirus HeLa cell system (Summers et al., 1965) other polypeptides, besides virus proteins, were synthesised under viral control, indicating that the function of progeny RNA was not restricted to coat protein synthesis. Their experiments did not however consider whether the parental RNA also participated in coat protein synthesis. An elucidation of this would answer the question of whether there is a functional difference between parental and progeny RNA on the translational level. Bukrinskaya et al., (1969) suggest that parental paramyxovirus RNP can direct the protein synthesis at the early stages of infection.

In the synthesis of viral coat protein, in vivo, it would be theoretically possible for the message to be read either from the viral strand or the minus strand (which has a sequence complementary to the strand found in the virus). It has been assumed in the present in vitro work, that it was the plus strand, rather than the complementary minus strand that is active in protein synthesis in vivo. This assumption received strong support from the studies on nitrous acid induced mutants of TMV, as Wittmann (1962) demonstrated that the frequency of occurrence of certain amino acid changes made it virtually certain that it was the viral strand that was used as message in protein synthesis. Until recently (van Ravenswaay Claasen et al., 1967) partial characterisation of alfalfa mosaic virus protein synthesised in vitro as viral coat protein, however, there has been no direct evidence that the viral plus strand is the

one that is read as messenger. Double-stranded RNAs would not be expected to act as messengers and this lack of activity has been shown by Miura and Muto (1965) for rice dwarf virus RNA.

The key event in protein biosynthesis is the association of the viral RNA with the ribosomes of the host cell and the production of early proteins. Shaw (1967) reported that almost all TMV particles retained by leaves after inoculation are at least partially uncoated. Kiho (1969) studied the fate of this parental TMV-RNA and found that infecting TMV RNA was associated with host cell components, forming structures believed to be de novo polysomes.

The next step is the actual trigger for the replication of viral RNA: the displacement of the parental RNA strand from the ribosome.

It is suggested that an early event in infection is virus induced inhibition of cell protein synthesis, which apparently functions by producing a change in the host cell mRNA molecule so that new ribosomes cannot be attached to it. This inhibitory role may also be involved in the liberation of the parental RNA strand from the ribosome. Höfschneider and Häusen (1968) have speculated that this is the role of early protein in viral replication. Alternatively the viral RNA replicase may start to produce a double strand from the parental RNA which still has ribosomes attached to it.

In either case, the hypothesis that the process of displacement is a function of a viral gene product, offers the advantage that the time period during which viral RNA acts as a messenger can be controlled. This may ensure that the viral RNA replicase is present in sufficient

concentration to allow for replication.

Two problems affecting *in vitro* amino acid incorporating systems have so far been discussed, the possibility of the plus or minus strand being responsible for protein synthesis and the possible presence of an 'inhibitory' role *in vivo*, which may not be able to function *in vitro*.

The inhibitory role may be necessary for release and movement of viral RNA along the ribosome. This may however only be an important factor *in vivo*, the *in vitro* system precluding the need for such a mechanism. This factor did not appear to prevent incorporation in the cabbage and bean *in vitro* systems, but may have been involved in termination of incorporation (release of synthesised material being prevented).

It may be remembered that attachment of viral RNA to the ribosomes in the *in vitro* systems may be occurring non-specifically or by simple adsorption without concurrent polypeptide synthesis. The fact that incorporation did occur suggests a 'normal' ribosome-mRNA attachment with resultant amino acid incorporation. Whether the plus-strand viral RNA provided could have produced coat protein *in vivo* is immaterial at this stage in that any incorporation ('protein synthesis') *in vitro* is a distinct improvement in work on viral protein synthesis. The cabbage and bean system were not sufficiently advanced to allow product analysis, which might have shed light on the 'correctness' of employing native TYMV RNA as coat protein messenger.

Incorporation levels in the bean and cabbage systems were found to be low in comparison to poly(U) directed systems. It may have been however

that actual polypeptide synthesis was triggered by the natural messenger (i.e. TYMV RNA), but that the in vitro environment was not entirely satisfactory for high levels of activity. It is possible that the viral RNA extracted from the isolated TYMV was incomplete in some vital part, even though analytical ultracentrifugation indicated it to have a relatively high sedimentation coefficient. A high sedimentation coefficient is usually indicative of an infectious particle in vivo, although the in vivo state may more readily accept a damaged RNA and allow normal infection to proceed. A damaged message in vitro may block the ribosomes thus leading to low incorporation levels. On the other hand, the presence of the complementary strand may be a pre-requisite for activity, a point discussed further under the question of the 'split message'.

The use of a simple in vitro system cannot hope to answer all of the problems associated with plant virus protein synthesis, but may elucidate some and be pointer to the answer of others. For example, as noted above, if the ribosome has the ability to make any kind of protein, the fact that TYMV RNA was active in both bean and cabbage systems may give an insight into the level(s) at which host specificity functions.

Since no alternate mechanism for protein synthesis has been established, the universal occurrence in animals, micro-organisms and higher plants of ribosomes must be assumed (Petermann, 1964). Ribosomes of plants have not been studied extensively as those of animals or micro-organisms. In general they resemble animal rather than bacterial ribosomes in composition and size (Ts'o, 1962). They contain about 40% RNA, and have sedimentation

coefficients for intact particles and subunits close to 80 S, 60 S and 40 S. An additional component, from chloroplasts (Lyttleton, 1962) was reported in spinach, white clover and several other plants (Lyttleton, 1960).

Association of parental viral RNA with ribosomes has not been demonstrated (Matthews and Ralph, 1966). This situation however was adopted in the present work, in that TYMV RNA was presented to plant ribosomes in the in vitro systems. True association cannot be assumed however, although the activity of the bean and cabbage systems does suggest a functional association between parental viral RNA and ribosomes. Whether or not this occurs in vivo cannot be answered from these simple experiments.

Leaves of B. chinensis contain '83S' ribosomes free in the cytoplasm, and '68S' ribosomes occurring largely, if not entirely, in the chloroplasts (Clark et al., 1964). Examination of B. chinensis isolates by analytical centrifugation confirmed these findings, similar isolates being used for the in vitro experiments (these isolates containing only a small percentage of chloroplast ribosomes).

Cytoplasmic ribosomes may occur singly or as aggregates, polysomes. In cells containing endoplasmic reticulum, most ribosomes are attached to the membrane, but some are present free in the cytoplasm (Benedetti et al., 1966). In in vitro experiments with rabbit reticulocyte ribosomes, Schreml and Burka (1968) found that the specific activity of bound and free ribosomes was roughly equal, although the average activity of the membrane bound was 21% of the free.

In animals, the nutritional state (Sidransky et al., 1969) and age (Campbell and Lawford, 1968) have been reported as likely to determine the relative proportion of bound vs. free ribosomes. Payne and Boulter (1969) observed changes in the cotyledons of developing bean seeds. During the phase of storage protein synthesis, free and membrane bound ribosomes do not interchange, and as both classes synthesise protein in vivo it is possible that they may synthesise different groups of proteins. This may have some relevance on viral protein synthesis.

In the germinating bean in vitro system, a large proportion of the ribosomes were probably membrane bound (Payne, 1970). As the system was less active than the cabbage system under TYMV RNA stimulation, bearing in mind the many other variables, it may be postulated that TYMV protein is synthesised on free rather than bound ribosomes. Young actively growing leaves contain a high percentage of free ribosomes which could account for the high activity in the cabbage system.

The presence of bound and free ribosomes which may synthesise different classes of proteins presents a further question. It is possible that the viral RNA may be read from a different starting point by the different types of ribosome (free and bound), the product of one possibly being coat protein, the product of the other being 'viral specific'. Any 'wrongly' directed protein could block/inhibit the synthesis of 'true' coat material. The increase in bound ribosomes and the decrease in viral concentration in older plants may be connected, or may be simply coincidental. On the other hand the two types of ribosome may be necessary for biosynthesis of different viral 'factors'.

The currently accepted model of protein synthesis holds that the majority of proteins are formed on the multi-ribosomal aggregates, polysomes (Arnstein, 1969). For a monocistronic message, the ribosome attaches to one end of the mRNA and moves along the strand as the template code directs sequential binding of aminoacyl-tRNA molecules, with attendant incorporation of the amino acids into a polypeptide chain. When the other end of the mRNA has been attained, the ribosome detaches from the template strand and the completed polypeptide strand is released. This process may be carried out simultaneously by more than one ribosome on the same strand (Gierer, 1963; Gilbert, 1963; Rich et al., 1963).

Polysomes have been characterised in a number of animal (Gierer, 1963; Warner et al., 1962; Wettstein et al., 1963) and bacterial systems (Gilbert, 1963), but the principal difficulty in their study appears to be to high susceptibility of the mRNA strand to RNase. Nucleases appear to be particularly active in leaves, and the various extraction media used for the isolation of animal polysomes have been reported inadequate. Rapid production of double-stranded viral RNA possibly aids the virus in overcoming RNase attack.

The major environmental influence on polysome levels is a diurnal cycle, in which light is reported to be the dominant factor. Polysomes decrease in amount during the night, reaching their lowest level before dawn. Two to three hours after sunrise, Clark et al., (1964) report that 80-90% of 83 S Chinese cabbage ribosomes may be in the form of polysomes. This diurnal variation is consistent with a number of earlier observations made by other methods, indicating that protein synthesis in leaves is greater in the light (Chibnall, 1924; Parthier, 1961).

The work of Clark et al., (1964) suggests that cytoplasmic protein-synthesising is just as sensitive to diurnal control by light as that in Chinese cabbage chloroplasts. Williams and Novelli (1964) demonstrated that illuminating dark grown seedlings of Zea mays enhanced the amino acid incorporating activity of ribosomes isolated from them. Mans (1966), inferred that the increase in activity resulted from an increase in the level of mRNA associated with the ribosomes isolated from the illuminated seedlings.

Chinese cabbage leaves collected at different times of day and grown under differing light intensities, did not demonstrate any noticeable variation in incorporating activity in vitro (although this aspect was not studied extensively). This could suggest low polysome concentrations, or the fact that polysomal activity was insignificant in the in vitro cabbage system.

The presence of considerable quantities of polysomes is in doubt, in that intact active polysomes are difficult to extract from leaf material. Although rapid extraction procedures were employed, no other method for polysome 'protection' was employed. Polysomes if present, may not have been able to accept the viral RNA, and if isolated ribosomes were present, these may have been unable to form new polysomes under the conditions employed. In either case low incorporation would result.

Dunn et al., (1963) found that young Chinese cabbage leaves had a ribosomal content five to ten times greater than older leaves. Clark et al., (1964) determined that 8 to 9 day old Chinese cabbage leaves contained the greatest concentration of 83 S ribosomes. Their measurements of

polysomes in leaf extracts possibly indicates the proportion of *in vivo* protein synthesising machinery which is active at a given time. Young leaves were therefore extracted for the *in vitro* Chinese cabbage systems, assuming that this active *in vivo* state would be reflected *in vitro*.

Srivastava (1969) found that incorporation of phenylalanine into the protein of mouse skeletal muscle decreased with the age of the mouse, this change being accompanied by a progressive decline in the concentration of polysomes. Similarly Murthy (1966) found phenylalanine incorporation into protein by rat brain ribosomes decreased with the age of the rat, although incorporation into rat liver ribosomes increased with age. Mehta *et al.*, (1969) found that polysome preparations from wheat leaves were markedly affected by the age of the leaves, ribosomes from younger leaves being more active than those from older leaves.

In vivo TYMV rapidly invades the younger leaves, and ribosomal concentration and activity probably play major roles. Possibly to reproduce this active state *in vitro*, active polysomes are initially required.

In animal cells, cellular polysomes disappear under the influence of an inhibitory protein, a new class of 'viral' polysome appearing later. This may mean that the inhibitory protein is no longer functioning or that the inhibitor is able to distinguish between cellular and viral mRNA (apparently analagous to the action of interferon in which viral and host cell mRNA are also distinguishable).

Haselkorn and Fried (1964) noted that TYMV RNA and TMV RNA associate with *E. coli* ribosomes *in vitro* to form complexes (monosomes) containing

single ribosomes. Poly(U) associates with ribosomes to form polysomes, but the ribosomes in such polysomes participate in the rate-limiting step of polyphenylalanine synthesis as individuals. The ability of polynucleotides to form monosomes or polysomes in vitro appears to be correlated with the secondary structure of the polynucleotide. In vitro TYMV RNA may not have had the 'correct' secondary structure resulting in low amino acid incorporation ('coat protein' synthesis possibly being the limiting step in this case).

Biswas (1969) noted that in seeds , all the metabolic activities are highly suppressed, metabolic activity beginning as they imbibe water. Low amino acid incorporation by ribosomes from ungerminated wheat embryos was reported by Allende and Bravo (1966). Marcus and Feeley (1964) reported that the entire apparatus necessary for protein synthesis is functional in the cotyledon of the ungerminated peanut and that mRNA is limiting. Marcus and Feeley (1965) and Marcus et al., (1966) found that protein synthesising capacity of imbibing wheat embryos was accompanied by an increase in polysomes (which accounted for most of the amino acid incorporation). Sturani et al., (1968) found very little activity in the ribosomes of dry castor bean endosperms, even in the presence of poly(U), and suggested that their structure had been affected. Dormant spores of Bacillus cereus and B. megaterium yield ribosomes low in amino acid incorporating activity and bind aminoacyl-tRNA poorly (Idriss and Halvorson, 1969). In contrast, polysomes and active ribosomes from germinated spores were obtainable. After germination, the dormant spore ribosomes regained their ability to bind tRNA and to function in poly(U) phenylalanine incorporation. The impaired activity in dormant

spores was not attributed to RNase activity. Linskens (1969) reported that changes in the ribosomal pattern in Fucus eggs upon fertilisation reflected the time course of protein synthesis.

Extracted ribosomes from Chinese cabbage seed pods and germinating seeds were found to be inactive in amino acid incorporation, even in the presence of poly(U). Damage to the ribosomes during extraction, or their immature nature could have been the cause of this. Similarly ribosomes isolated from germinating beans were found to be relatively inactive (although found by Payne (1970) to be active).

TYMV is not reported to be seed borne (although throughout the present work a number of control plants exhibited typical symptoms of infection - attempts at back inoculation were unsuccessful). This may be a result of an inability of TYMV RNA to enter the requisite tissues, or if able, to be adversely affected by storage. Degradation of TYMV on storage is well documented, and storage combined with RNase activity and drying out of seed tissues could enhance this. It is possible that seed ribosomes are in some way different to normal leaf ribosomes, and are thus unable to bind TYMV RNA. This would appear unlikely as poly(U) was inactive also. It is possible that heterologous mRNAs (natural or artificial) are unable to react with such ribosomes before a certain stage in their development. Close association of nascent endogenous messenger with seed ribosomes could also prevent acceptance of a 'foreign' message.

Chloroplastic or Cytoplasmic Ribosomes?:

Rezende-Pinto and Borges (1952) found no change in the granular structure of the chloroplasts from the green areas of Chinese cabbage leaves systemically infected with TYMV. In the 'chlorotic' areas, many chloroplasts had lost their granular structure. Chalcroft and Matthews (1966) found that dark green areas of leaves showing mosaic pattern appeared normal, whereas all other types of tissue in the mosaic area had markedly abnormal chloroplasts (swollen and frequently aggregated into large clumps). They observed no particles of the size of 83 S ribosomes of TYMV within the chloroplasts, the nucleus and mitochondria also appearing normal.

Clark (1964) observed that the 68 S ribosomes of Chinese cabbage leaves appear to be confined to the chloroplasts. In TYMV inoculated leaves, the concentration of 68 S ribosomes in developing lesions fell rapidly and in yellow-green or white areas were completely absent. They were still found to be present at normal concentrations in the dark green areas, probably allowing normal plant functions to continue thus preventing the rapid death of the plant. Complete removal of the 68 S ribosomes would undoubtedly precipitate the rapid death of the plant, which would not be advantageous to the virus. It may be conjectured that mosaic formation is in fact intended by the viral genome, so as to prolong its hosts existence. Conversely, the plant may have areas more resistant to viral attack.

If there is a variation in ribosomes with respect to sensitivity to viral attack, total cytoplasmic ribosome isolation (as in the present work)

may result in a 'dilution' of the sensitive types with consequent poor *in vitro* activity.

Clark *et al.*, (1964) observed that in healthy leaves, the concentration of 83 S ribosomes fell steadily during leaf expansion. In developing lesions in leaves inoculated with TYMV, the normal fall in concentration was halted about the same time the lesions first became visible. From then they observed that local lesion tissue showed a higher concentration of 83 S ribosomes than uninfected, In leaves showing full mosaic symptons however, there was no marked difference between the dark green and yellow tissues in the concentration of these ribosomes. In white areas they noted an increase in concentration.

Matthews (1958) and Francki and Matthews (1962) found that as TYMV increased in concentration, the total RNA of the leaf also increased by an amount equivalent to that found in the virus. Analysis of the overall base composition of the RNA showed no significant change in the host RNA of the infected leaf, indicating that TYMV infection does not have any major effect on the overall amount or composition of host RNA in infected tissues. However, as noted by Matthews and Ralph (1966) in these analyses the normal leaf RNA measured would have been largely 83 S ribosomal and any significant changes in RNA fractions present in smaller amounts (e.g., 68 S ribosomal or tRNA) would not have been detected.

Because the proportion of bases in TYMV RNA deviate widely from unity, it is possible to distinguish clearly between viral and duplex RNA by nucleotide analysis. After labelling infected plants, Ralph *et al.*, (1965) found that the isolated duplex RNA had an apparent base composition

like that of viral RNA, but as time of labelling increased from 30 min to 8 days, the base composition became like that expected for the TYMV duplex. Similar results were obtained with TMV double-stranded RNA. These results suggest that the viral RNA strand of the duplex is replicating more rapidly than the minus strand, and that the newly formed viral RNA strands are displaced from the duplex. Such an asymmetric semi-conservative replication mechanism has been proposed for several RNA viruses (Fenwick et al., 1964; Ochoa et al., 1964; Weissmannet al., 1964).

Single-stranded TYMV has not been shown to occur free in infected cells, or associated with ribosomes either in monosome or polyribosome form in such cells (Matthews, 1970).

Van Kammen (1963) carried out experiments with TMV in tobacco leaves which suggested that uncoated TMV RNA may be associated with the 80 S ribosomes. There is some doubt however (Matthews, 1970), as to whether van Kammen was studying naked RNA as associated with the ribosomes in vivo, or contamination of the ribosome fraction with TMV which had become uncoated during the isolation procedure.

By analogy with other protein synthesising systems it is probable that TYMV RNA does become associated with one or more ribosomes in vivo to produce viral coat protein. The effect of TYMV infection on ribosomes and polysomes has been shown by workers to vary according to the tissue examined. The implication of these various changes in relation to the synthesis of viral protein are therefore obscure.

Reid and Matthews (1966) found that material assumed to be ribosomes

and sedimenting at c. 83 S, in extracts from inoculated leaves, reacted with TYMV rabbit antiserum. This may be interpreted to suggest that these ribosomes have associated with them serologically reactive subunits of the virus capsid. However, 83 S ribosomes in systemically infected leaves did not react detectably. It may be that 'protein' formed in systemically infected leaves is more viral 'specific' protein than true 'coat' protein, thus a detectable reaction might not be expected.

The possible role of the two classes of plant ribosomes has not therefore been clearly established for any plant virus. Hirai and Wildman (1967) in unpublished experiments observed that 70 S chloroplast ribosomes decreased in amount and in in vitro amino acid incorporating activity, compared to healthy leaves, at about the same time when the TMV growth curve departed markedly from the exponential condition. In contrast the 80 S cytoplasmic ribosomes did not seem to change. They suspected that the disappearance of chloroplast ribosomes is a result of competition for substrates by the TMV system.

Hirai and Wildman (1969) found that during early systemic infection by TMV in tobacco, chloroplast ribosome and protein synthesis was inhibited, while cytoplasmic ribosome synthesis was not. However, in the zone of active mesophyll cells surrounding the necrotic centre of local lesions induced by TMV in Nicotiana glutinosa, Israel and Ross (1967) observed a marked increase in the amount of endoplasmic reticulum and associated ribosomes. Likewise the chloroplasts in the cells in this zone appeared to contain more ribosomes than usual.

Chloroplast ribosomes in Chinese cabbage have also been reported

to decrease in amount in extracts of virus infected tissue (Reid and Matthews, 1966), and are no longer detectable in tissue in which the virus is still being produced. If this represents a total loss of 68 S ribosomes rather than a change to some inextractable form, then chloroplast ribosomes are unlikely to be involved in virus coat protein synthesis.

The extent to which the chloroplast ribosomes are reduced depends largely on the strain of TYMV and increases with time of infection. Ushiyama and Matthews (1970) have observed that loss of ribosomes more or less parallels the loss of chlorophyll, 'white strains' causing the most severe losses.

TYMV infection has apparently comparatively little effect on the concentration of 83 S ribosomes. Randles and Matthews (1970) noted that as TYMV infects young Chinese cabbage leaves systemically, there is a temporary rise in the concentration of 83 S ribosomes seen in extracts made just before the virus becomes detectable in density gradients. They attributed this rise to a virus induced increase in the extractability of the ribosomes, rather than to an increase in synthesis.

Further to the fact that membrane-bound and free ribosomes may represent different protein synthesising systems, Mehta et al., (1968) showed that wheat leaves contain two distinct classes of polysomes, and the co-existence of these polysomal classes in the same cell suggested to them the presence of two protein synthesising systems. Van Kammen (1967) also suggested the presence of two different amino acid incorporating systems in tobacco leaves. Mehta et al., (1969) observed that in comparison

with similar preparations from wheat leaf chloroplasts, the cytoplasmic polysomes showed much less amino acid incorporation, even in the presence of poly(U).

Boardman, et al., (1966) demonstrated that the 70 S ribosomes in a tobacco leaf extract were 10 to 20 fold more active in protein synthesis than the 80 S ribosomes in a cytoplasmic extract. Isolation of the 70 S ribosomes from the chloroplasts reduced their activity, whereas purification of the 80 S increased their activity.

Eisenstadt and Brawerman (1964) found that cytoplasmic ribosomes from Euglena gracilis cultures harvested at stages beyond the log phase of growth were the most active in amino acid incorporation (activity being enhanced by glutathione and by 5% ethanol).

Nicholson and Flamm (1964) found that in tobacco cell cultures, the cytoplasmic membrane bound ribosomes were the major site of protein synthesis, nuclear ribosomes being inactive (Flamm and Birnstiel, 1964).

Although these various observations allow no unequivocal conclusion, it is probable that TYMV protein is made on 83 S ribosomes. Attempts to demonstrate the presence of TYMV RNA in poly- or monosomes in leaf extracts have given no useful results to confirm this. This has been due in part to the instability of polysomes in leaf extracts, the continued synthesis of plant proteins during virus infection and the presence of many contaminating labelled materials in the polysome fraction (Clark et al., 1964).

According to Jacob and Monod (1961) the ribosome being an

unspecialised structure would suggest that viral protein could be synthesised on either 68 S or 83 S. Whether this is a correct supposition has yet to be confirmed. Undoubtedly with the correct *in vitro* environment, amino acid incorporating activity could be obtained with both species. Product analysis would be necessary to ascertain the nature of the synthesised product to compare it with native coat protein.

Little activity was obtained here in an *in vitro* Chinese cabbage 'chloroplast' amino acid incorporating system, and this section of the work was not pursued further. The attempt at developing an 80 S system was therefore partly arbitary and partly based on the evidence outlined above.

It may be noted, however, that Sela and Kaesberg (1969) employed a tobacco leaf chloroplast system and claimed the synthesis of tobacco mosaic virus coat protein.

Certain viral proteins may therefore be made *in vivo* on one class of ribosomes, the other class being involved at certain stages. It may be that the 80 S ribosome is in some way better adapted for the task of 'coat protein' synthesis, *in vitro* and possibly also *in vivo*.

It is interesting to note that analytical ultracentrifugation of a microsome suspension before and after incorporation of amino acids indicated that the 80 S component disappeared on incorporation. Bearing in mind dilution factors, the concentration of the 68 S component did not appear to vary, although there were a number of higher sedimenting components after incorporation. It may be tentatively suggested that this shows that,

at least *in vitro*, TYMV RNA associates with 80 S ribosomes and not with 68 S ribosomes (under the experimental conditions employed). This suggestion is furthered by the fact that a microsomal suspension from TYMV infected *B. chinensis* leaves (with a reduced 70 S content) and a microsomal suspension from uninfected leaves (with a normal 70 S and 80 S content) produced similar levels of amino acid incorporation.

Amino acid incorporation into protein by a higher plant cell-free system occurs at much lower rates than animal or bacterial systems. The demonstration of TMV coat protein synthesis by Sela and Kaesberg (1969) however, using a tobacco leaf ribosomal system suggests the apparent rates of amino acid incorporation calculated from the specific radioactivity of supplied amino acids may represent only a fraction of the true activity of the system. An examination of the rates of aminoacyl-tRNA synthesis indicates that cell-free systems from plants may be quite active but that endogenous concentrations of amino acids dilute supplied tracer amino acids (Hall and Tao, 1970).

Ofengand and Haselkorn (1962), Haselkorn *et al*, (1963) and Haselkorn and Fried (1964) have suggested from studies with a TYMV RNA primed *in vitro* *E. coli* protein synthesising system that protein synthesis occurred on complexes of TYMV RNA with single *E. coli* ribosomes. Voorma *et al*., (1964, 1965) however, with essentially the same system, obtained polyribosomes in the presence of 1.8×10^{-2} M Mg^{2+}. The extent of amino acid incorporation per ribosomal aggregate increased when the number of ribosomes associated with the message RNA molecule increased.

Nirenberg and Matthaei (1961) described stimulation of protein synthesis by RNA from E. coli ribosomes and from TMV in an E. coli cell-free system. Preliminary experiments showed that only a fraction of the RNA was active and in the majority of experiments the product did not resemble the coat protein (Aach et al., 1964). Nathans et al., (1962) found, however, that f2 RNA in an E. coli system stimulated the production of the corresponding viral coat protein (as did Schwartz et al.,(1965) in the heterologous system containing f2 RNA and Euglena chloroplast ribosomes). Similarly Clark et al., (1965) found this with satellite tobacco necrosis virus (which probably codes for no more than two proteins). The polypeptide material formed in their in vitro system yielded on tryptic digestion a series of peptides similar but not identical with those found in the virus coat protein. It has not been absolutely established that the f2 product was functionally competent to the extent of being assembled into phage or phage-like particles. Knowle (1969) demonstrated that E. coli cell-free extracts were capable of incorporating amino acids into phage-like particles, albeit in low yield.

These experiments at least suggest that plant viral RNA in contrast to phage RNA confronts us with some particular problems so far as its translation in vitro is concerned and possibly these difficulties are unrelated to the type of ribosome employed.

The lack of demonstration of the synthesis of TMV coat protein in E. coli cell-free systems raised the question of whether none of the plant messages could be read in a bacterial system, or whether the information transfer barrier was related particularly to features of TMV RNA.

Van Kammen (1967) observed that in his tobacco leaf system, the failure of TMV RNA to stimulate amino acid incorporation may be ascribed to faulty interaction between RNA and ribosomes.

More generally however it can be surmised that some of the problems with the relatively large messengers like TMV and TYMV RNAs are related to their polycistronic nature. Genetic analysis has identified at least three cistrons in the viral genome of R 17, the small RNA virus infecting E. coli. The 'A' cistron specifies a protein necessary for the synthesis of infective phage particles, the 'B' cistron specifies the virus coat protein and the 'C' cistron codes for the virus-specific RNA synthetase involved in the synthesis of the new viral RNA (Gussin,1966).

It is clear that TYMV RNA is able to code for more than the virus shell subunit alone and consequently it may be considered as a polycistronic messenger. Assuming triplet coding ratio of three nucleotides per amino acid, TYMV RNA should be able to code for about ten polypeptides of the size of the shell subunit.

The small RNA molecule isolated from the non-infectious top component of alfalfa mosaic virus (AMV) served as a message for AMV coat protein in the E.coli system (van Ravenswaay-Claasen et al., 1967) whereas the large RNA molecule from the infectious bottom component was relatively inactive. Separation of cistrons is therefore suggested.

It would appear that such a separation of cistrons may be a fairly common phenomenon in plant viruses. Cowpea mosaic virus (CMV) gives rise to three components, only two containing nucleic acid (Bruening and

Agrawal, 1967). Wood and Bancroft (1965) demonstrated that the bottom component, containing the full complement of nucleic acid, was infectious, whereas the middle component was not. Addition of the middle component to the bottom component, greatly enhanced the infectivity of the latter.

Similarly van Kammen (1968) demonstrated that middle and bottom cowpea components were not infectious when inoculated alone, but that infectivity was restored on mixing the two components. This suggests that the genetic information for functions necessary for the establishment of infection is distributed between the two RNAs and that there is mutual dependence and complementation. This complementation is strain specific, or at least dependent on a certain close relationship of the isolates used (De Jager and van Kammen, 1970).

Tobacco rattle virus (TRV) has three distinct nucleoprotein particles two of which contain nucleic acid. The larger one presumably regulates the production of the viral nucleic acid, while the shorter rod codes for the production of infectious RNA. Infection with only long rods results in the production of infectious RNA, or the unstable form of TRV, whereas typical nucleoprotein particles, or the stable form of TRV are recovered only after infection by both particles (Semancik and Reynolds, 1969).

Such complementation due to the distribution of genetic information between the RNAs of two or more nucleoprotein components has also been demonstrated for TRV by Frost et al., (1967) and Lister, (1968), for alfalfa mosaic virus by van Vloten-Doting et al., (1968), for the bean pod mottle virus by Wood and Bancroft (1965) and for tobacco streak virus by

Fulton (1967).

Wood and Bancroft (1965) demonstrated that particles with partially damaged RNA in the bottom component of bean pod mottle, although non-infectious by themselves, became infectious in the presence of some middle component. This activation is presumably due to the middle component supplementing the bottom component particle with the damaged cistron in the nucleic acid (Reichmann and Clark, 1968).

If it is assumed that the initial requirement for infectivity is the replication of the genome (RNA), it must be concluded that in CMV experiments, the replicase must be encoded in at least two cistrons (Reichmann and Clark, 1968). This requires that the replicase be made up of at least two different enzymes or parts of the same enzyme. If separation of cistrons is confirmed it provides the possibility of a separate investigation of various functions required for virus multiplication and gives rise to questions concerning the significance of this division for the evolution of the virus, the interaction of the components in the process of multiplication, the connection between the possibility of complementation and the relationship of strains, and the similarity of RNAs, if any (De Jager and van Kammen, 1970).

It is possible that some viruses with apparently only one kind of particle may in fact consist of two dependent particles that cannot be separated at present by physical means. In all the multi-particle viruses described to date, the particles involved differ in size (or molecular weight) because they contain different amounts of RNA. The RNA of the particles is of similar but not necessarily identical, base composition.

For tobacco rattle and alfalfa mosaic virus there is good evidence that the RNA of the smallest particle contains the coat protein cistron used by the other particles. This is sufficient reason, but may not be the only reason, why the larger particle needs the smaller one.

It is possible that there is no duplication of function in the RNAs of the two particles, and that each depends on the other for all the functions they provide. The available evidence suggests that the RNA of the various classes of particle in these multi-particle viruses may be synthesised independently in the cell. Infectious RNA of TRV long rods can be synthesised in the absence of short rods. Van Kammen (1967) noted that the middle and bottom components of CMV increase at independent rates, and he has demonstrated the presence of two pieces of double-stranded RNA of appropriate size in nucleic acid preparations made from infected plants (van Griensven and van Kammen, 1969). It remains to be determined whether all the RNA pieces in any virus use the same RNA synthetase provided by one of them. If they do they all presumably have the same 'recognition site' in the RNA.

The multi-particle arrangement of genetic material must have some survival value. The most probable advantage is that of increased genetic flexibility over a virus with all its genetic material in one piece. In several RNA containing animal viruses (e.g. reovirus and influenza virus (Davies and Barry, 1966)) the RNA exists within the virus particle in several pieces. The multi-particle plant viruses may represent a morphological variation of essentially the same combination - a virus consisting of several pieces of genetic material. In viruses with several pieces of RNA in one particle, genetic reassortment can

presumably take place during virus replication. In addition to reassortment at this stage the existence of the RNA in several particles might allow selection and reassortment at other stages, e.g., during transmission, entry into the cell and movement through the plant.

Experiments by van Vloten-Doting et al., (1968) showed that new 'strains' of AMV can arise from the mixing of top 'a' from one strain and bottom from another. Van Vloten-Doting (1968) showed that all four particles may influence disease symptoms, which may allow rapid adaption to new hosts and to changing conditions in a given host.

The separation of the genome may have some advantage with respect to the initiation of early events in virus replication with the cell, if some functions need to be carried out at one site and some at another.

The tobacco rattle group are transmitted by nematodes although the relationship with their vectors is not established. They consist of single thick rods, and as such might be susceptible to breaking by shear forces either in the nematode or the plant. The two-particle system might be an adaption that avoids excessive random breaks in the viral rods.

A disadvantage of the multi-particle system, at least in theory, is that the probability of establishing a successful infection is reduced, since two or more particles must co-operate.

Matthews (1960) noted the presence of at least four minor components in addition to the RNA-devoid top component, in TYMV preparations. Three of these minor components had lower sedimentation rates, and were found

to contain lesser amounts of RNA. Infectious virus was designated B_1 and empty protein shells, T.

The fastest minor component, B_2, contains the same amount of RNA, of the same characteristic base composition as B_1, although the RNA is polydisperse of S_{20w} = 26-29. It is the minor component occurring in the largest amount and is of low infectivity. The lack of infectivity is considered to be probably due to some defect in the RNA (Matthews and Ralph, 1966).

B_0 is a minor component, containing 24% by weight RNA, and with a characteristic TYMV base composition. The RNA has S_{20w} = 21, but is not infectious.

B_{00} is a non-infectious component containing 10% RNA, and component B_{000} consists of material sedimenting between T and B_{00}.

The non-infective nucleoprotein fractions B_{00}, B_0 or B_2 added to purified B_1 in approximately equal concentrations do not appear to bring about any increase in the number of local lesions produced by the B_1 fraction in Chinese cabbage plants. Nor is the infectivity of isolated B_1 RNA increased by the addition of minor component RNA (Faed and Matthews, 1970), and it does not therefore appear to be a two particle virus.

The various components however could have regulatory roles, binding to ribosomes to alter or aid the speed or time of occurrence of any reaction.

Total extracted viral RNA was employed in the Chinese cabbage <u>in vitro</u>

system, with the RNA sedimenting at c. 30 S. No attempt was made to separate the various components. The low activity of the system may therefore have been due to binding of the smaller RNA components, possibly preferentially, to the exclusion of 'infective' moieties. The slight delay in some experiments before amino acid incorporation was observed could be a result of initial binding of the smaller components, prior to removal/displacement by the 'correct' messenger. The smaller components could in fact be nonsense or lethal in the in vitro and/or in vivo states.

The minor TYMV components could come from assembly steps for the complete particle, from in vivo degradation products or from errors at the particle assembly stage of virus synthesis.

There appears to be no reason why some satellite virus should not use the coat protein of the virus upon which it is dependent. Multi-particle satellite viruses might exist, and a multiparticle satellite using the same coat protein as the virus upon which it depended might be worth searching for among the viruses with defective particles of unknown function (Matthews, 1970).

Spindle tuber disease of potato is incited by free RNA and neither conventional virions nor proteins that could be construed as viral coat proteins are synthesised in infected plants. It is too small to contain the genetic information necessary for self-replication and must rely for its replication mainly on biosynthesis systems already operative in the uninoculated plant (Diener, 1971). It may be one of the RNAs which are the 'missing link' between viruses and genes (Temin, 1970). Use of the

biosynthetic system of a plant might in such cases be a source of plant host specificity. Certain characteristics in the genetic 'make-up' of the plant could determine which viruses could invade and multiply in the host tissues.

It is possible therefore that the principles of separated cistrons apply in the case of TYMV to a greater or lesser extent. This could account for low activity in the in vitro systems. The requisite component(s) for initiation etc. may be absent or damaged or the concentration of the required component(s) could be insufficient to allow measurable incorporation in a crude system. Analytical ultracentrifugation and polyacrylamide gel electrophoresis only indicated the general nature of the viral RNA employed, and it would appear that separation, purification and subsequent recombination of the various components, with subsequent product analysis, could go far to indicate requisite mRNA conditions. Whether parental RNA or progeny RNA or negative or positive RNA strands are employed has already been discussed, although a further possibility is the necessity for a combination of positive and negative strands, for activity.

The question is still open to what degree secondary structure, or intramolecular sequence complementarity, is a prerequisite for the function of viral and mRNA molecules (Mundry, 1969). Haselkorn and Fried (1964) noted that RNAs such as those from TMV and TYMV have a significant amount of secondary structure in solution, which may limit the readout of the message in vitro to non-representative regions. STV and similar smaller RNAsmay possess less secondary structure in vitro. Voorma et al.,(1971) noted that the secondary and tertiary structure of bacteriophage RNA is

possibly concerned in the regulation of the synthesis of the three proteins encoded by MS2-RNA. Spirin (1963,1964) has comprehensively reviewed the literature pertaining to the structure of RNA in solution and has concluded that it is dependent on the ionic environment and temperature. The in vitro conditions will therefore reflect these factors and can only be an approximation to those found in vivo.

Boedtker and Stumpp (1964) using thermally degraded RNA, found that the amount of amino acid incorporated in the in vitro E. coli system was independent of the RNA molecular weight about 400,000. Incorporation was gradually reduced as the molecular weight fell below 300,000.

Yamazaki and Kaesberg (1966) showed that although a product similar to R17 coat protein was made in their system, the major products were very basic polypeptides resistant to trypsin and soluble in 5% trichloroacetic acid. Moreover in a crude cell-free system there occurs endogenous incorporation of amino acids which has often been considered to be polypeptide synthesis. The evidence however has shown that this amino acid incorporation consists of single amino acid additions to either the N-termini (Momose and Kaji, 1966) or to the C-termini (Atherly and Imsande, 1967) of some pre-existing proteins in the cell-free system. Barondes and Nirenberg (1962) observed the degredation of synthetic mRNA in their system before significant polyphenylalanine synthesis had occurred. Igarashi and Paranchych (1967) found their system had appreciable RNase activity, removal increasing poly(U) activity but reducing viral activity (as it removed important factors from the ribosomes). Addition of RNase restored activity, suggesting that viral RNA fragments had messenger activity.

Phage f2 contains three cistrons, one for coat protein, one for maturation or 'A' protein (a minor component of the virus particle) and one for an RNA polymerase (Horiuchi, Lodish and Zinder, 1966; Gussin, 1966). When purified f2 RNA is used as messenger in a cell-free system, the predominant product is coat protein (Nathans et al., 1962). Lesser amounts of non-coat material are also synthesised and several workers have concluded that phage RNA polymerase protein is synthesised in vitro (Capecchi, 1966; Viñuela et al., 1968).

Lodish and Robertson (1969) have demonstrated the formation of a maturation protein and suggest that synthesis of maturation protein is independent of translation both of coat and polymerase genes, a result which implies that there must be at least two sites on f2 RNA to which ribosomes can attach independently. The notion that ribosomes can attach to sites at the interior of a mRNA is also supported by the experiments of Bretscher (1968) and Webster and Zinder (1969), and could lead to coding and other errors.

The initiation factor required for binding natural mRNA to ribosomes could have a role in this (choice of product) process by binding specifically to certain regions of the phage RNA. Lodish and Robertson (1969) investigated the role of secondary structure and of possible differences in nucleotide sequences at the beginning of the genes as an explanation of this phenomenon.

The synthesis of maturation protein in the phage infected cell (i.e. in vivo) may be different from in vitro. The multi-stranded replicative intermediate RNA found in f2 infected cells is localised on

polysomes and may represent the predominant species of f2 messenger RNA during much of the infectious cycle. Engelhardt et al., (1968) have shown that in a cell-free system the single-stranded tails of these structures (representing nascent RNA strands) direct synthesis of coat protein but not of RNA polymerase. It is suggested likely that the relative amounts of maturation protein produced, as well as the mechanism of regulation of protein synthesis, is different for replicative intermediate and for whole single-stranded phage RNA molecules.

Matus et al., (1964) observed in complexing experiments with TYMV and plant ribosomal RNA that preferential binding of host ribosomes by viral RNA might confer an advantage in the production of viral over host proteins. This complexing of TYMV RNA with Chinese cabbage RNA (Matus et al., 1964) may be of significance although it appears to be a relatively non-specific phenomenon (complexing by hydrogen bonding with unpaired bases in the ribosomal RNA). Matus et al., (1964) suggest the phenomenon appears to be related to the base composition of the aggregating species rather than to metal ions or polyamines although Mitra and Kaesberg (1963) reported aggregation of TYMV RNA with polyamines under certain conditions.

Staehlin et al., (1964) also reported the interaction of mRNA and ribosomal RNA from rat liver and suggested that this may have functional significance in vivo although Matus et al., (1964) found an absence of interaction of TMV RNA and tobacco ribosomal RNA. This suggested that this type of interaction may have no functional significance in the cell. In vitro there may be extensive non-functional

binding of TYMV RNA with ribosomes, the in vitro environment being unsuitable for polypeptide synthesis, and reflected in the low activity of the B. chinensis system.

Bosch et al., (1966) found that isolated 70 S E. coli ribosomes associated with TYMV RNA at $0^{o}C$ in the complete absence of protein synthesis. The relative abundance of aggregates was strongly dependent on the ribosomes/messenger ratio and was affected by Mg^{2+} concentration of the medium. The conditions favouring ribosome/TYMV-RNA association were also optimal for polypeptide synthesis. The aggregates formed by mixing ribosomes and viral RNA at $4^{o}C$ were able to initiate polypeptide synthesis when supplemented with $\left[^{14}C\right]$ amino acids etc., It was noted that the plateau of RNA/ribosomes occurred at 10-15 ribosomes/TYMV RNA, the same level as for maximal incorporation. It was surmised that monosomes may be formed primarily, subsequently aggregating to form 'polymonosomes'.

Ribosomes centrifuged through 0.5 M NH_4Cl showed a degradative activity towards viral RNA. This may be an important factor in vitro, although in the present work a fairly specific KCl concentration was required for optimal activity in the cabbage system.

Bosch et al., (1966) concluded from their work that either each TYMV RNA molecule is able to bind a limited number of ribosomes in vitro before the onset of polypeptide synthesis. The polysomal aggregates thus formed are able to bind various aminoacyl-tRNAs and will initiate polypeptide synthesis

Or each TYMV RNA molecule associates with only one ribosome, the

monosomes being able to aggregate and yield rapidly sedimenting complexes. It remains to be explained why various processes reach a maximum at 10-15 ribosomes/messenger molecule.

Ribosomes programmed with TYMV RNA are able to bind different species of aminoacyl-tRNA and intact TYMV RNA has about four ribosome-binding sites (Dahlberg and Haselkorn, 1966). If it is assumed that a ribosome-binding site in TYMV RNA represents a potential peptide chain initiation site, then the four regions in TYMV RNA defined by the sites could be translated independently. Four viral functions in the infection process could then be preferentially expressed in the correct sequence. As such, the polycistronic TYMV RNA would also be a polyfunctional mRNA (Dahlberg and Haselkorn, 1966). Summers et al., (1965) observed in poliovirus infected HeLa cells that all of the cistrons were not translated at the same rate or at the same time.

If monosomes were the only aggregates active in protein synthesis, the attachment of only two species of tRNA to the corresponding two adjacent triplets for the messenger chain would be expected. This might constitute the 'block' in the in vitro system, polysomes not being able to form in the conditions employed.

TYMV RNA often contains some fragmented material, and as noted above, these fragments may offer different and more binding sites for ribosomes than the complete messenger. Binding of these could result in delay in protein synthesis proper.

Van Knippenberg et al., (1964, 1965 a,b) performed experiments with streptomycin which indicated that the messenger is never completely

read. Streptomycin was able to relieve a rate-limiting step in the translation process. Although streptomycin stimulated in vitro protein synthesis programmed by TYMV or AMV RNA, Gorini and Kataja (1964) noted that the conformation of a sensitive ribosome may be altered in such a way that the messenger is read in a different manner (which could lead to ambiguity).

Van Knippenberg assumed that stimulation of TYMV RNA promoted protein synthesis was a consequence of misreading. Streptomycin probably reduces the specificity of the codon-anticodon interaction. When poly(U) is used as messenger, the incorporation of phenylalanine is accomplished through phenylalanine specific tRNA. Streptomycin accelerates this process by rendering the specific requirement less stringent. In the case of a co-polymeric messenger like viral RNA, various tRNAs are involved. Due to code degeneracy, more than one specific tRNA is able to carry a single amino acid. Some of the tRNAs may be scarcely available in the in vitro system. If required by the messenger, delay would thus result. Streptomycin apparently circumvents these blocks by allowing other tRNAs to fill the sites.

In the Chinese cabbage in vitro system the lack of any tRNA species should be of only small importance as total cell tRNA was used. When tRNAs from either the first or second stages of Chinese cabbage tRNA collection were examined independently, or together, there was no significant difference in activities in the in vitro system. Any difference may have been masked by the use of the $\left[^{14}C\right]$ amino acid mixture, rather than the use of a single $\left[^{14}C\right]$ amino acid. It is still possible however that a minimal concentration of some vital tRNA species was absent.

The amount of tRNA present in tobacco leaves 48 hours after inoculation with TMV has been reported to increase about four-fold (Shigematsu et al., 1966) but using similar methods, Kubo et al., (1965) found no such increase.

Johnson and Young (1969) used aminoacyl-tRNA synthetases from E. coli to assay for tRNAs fractionated from healthy and TMV-infected tobacco leaves. When young plants were used, no quantitative differences were detected for seven amino acids.

The tRNA environment of the cell may be altered on infection possibly as a result of direct influence. The use of tRNA from uninfected leaves in the in vitro system, may not therefore compare exactly to the in vivo state. The fact that TYMV RNA can be charged and possibly act as its own 'tRNA' may also be of vital significance in initial and other stages of the biochemical process.

Transfer Nucleic Acids:

The existence of multiple species of tRNA for a single amino acid was first shown by Doctor et al., (1961) for the tRNAs isolated from yeast. Since then heterogeneity in tRNA species for a single amino acid has been demonstrated in other micro-organisms and mammalian cells (Novelli, 1967; Yang and Novelli, 1968). The separation and study of these various tRNA species appears to be of importance therefore because of recent suggestions that tRNAs may play a substantial role in cell differentiation and cellular regulatory mechanisms (Ames and Hartman, 1963;

Legocki and Wojciechowska, 1970; Stent, 1964). These hypotheses require that the population of some tRNA molecules in an organism changes as the physiological state of the organism alters. That alterations of specific tRNAs occur in bacterial and higher organisms during virus infection and differentiation has been well documented.

The role that such altered species of tRNA may play in macromolecular regulation and differentiation has been further discussed by Kano-Sueoka and Sueoka (1966), Holland et al.,(1967) and van Etten et al., (1969) who suggest that a modification of specific species of tRNA may alter the translation of certain mRNA molecules, thus allowing differential gene expression. The report of Anderson (1969) clearly demonstrates that the rate of mRNA translation may be regulated by the quantity of specific aminoacyl-tRNAs and if the control of protein synthesis occurs at the level of mRNA readout, both specific tRNAs and aminoacyl-tRNA synthetases may have a regulatory function. Certain models have been proposed to account for cellular ageing as a result of differentiation, and one such model (Strehler et al., 1967) implicates tRNAs and aminoacyl synthetases as limiting components in translation.

Changes in the distribution of certain tRNA species have been reported in phage-infected bacteria (Kano-Sueoka and Sueoka, 1966; Kan et al., 1968; Gefter and Russell, 1969; Waters and Novelli, 1967; Weiss et al., 1968), virus infected animal cells (Hay et al., 1967), bacterial cells under different growth conditions (Doi et al., 1968; Kaneko and Doi, 1966; Sueoka and Kano-Sueoka, 1964; Kano-Sueoka and Sueoka, 1966; Lazzarini, 1966; Yegian and Stent, 1969 a,b), bacterial cells undergoing sporulation (Kaneko and Doi, 1966; Lazzarini, 1966),

differentiation of animal cells (Axel et al., 1967; Holland et al., 1967; Marshall and Nirenberg, 1969; Mushinski and Potter, 1969; Strehler et al., 1967; Yang and Novelli, 1968), in erythrocytes of developing chicks (Lee and Ingram, 1967), during differentiation of wheat seedlings (Vold and Sypherd, 1968) and in other higher plants (Anderson and Cherry, 1969).

Data on the differentiation of tRNA in plants has tended to be scanty and concerning rather short times of development (e.g., seed germination) (Legocki and Wojciechowska, 1970).

Although it has been shown that tRNAs may differentiate as plants develop (as observed by Brown (1969) in mimosa epicotyl tissues) the nature of this differentiation is not known at present. Some degenerated tRNA species may be formed or disappear in the course of development, and the species affinity of aminoacyl-tRNA synthetases to certain tRNA particles may change. Brown and Novelli (1968) and Epler (1969) showed that mitochondrial and cytoplasmic tRNAs of Neurospora crassa differ among each other.

The possibility of regulation of protein synthesis at the level of translation has thus been suggested by several workers (Ames and Hartman, 1963; Armstrong, 1966; Axel et al., 1967; Kano-Sueoka and Sueoka, 1966; Sueoka and Kano-Sueoka, 1964; Stent, 1964; Sueoka et al., 1966; Strehler et al., 1967). It is obvious that by limiting the complement of tRNAs and/or synthetases available for translation, protein synthesis can be controlled at the level of mRNA readout (Anderson, 1969).

Although changes in specific tRNAs have been noted, little except for the demonstration by Strehler et al., (1967) is known of changes in synthetases. Several leucyl-tRNA synthetases in the rabbit differ quantitatively between different specialised cells. Anderson and Cherry (1969) showed that there were six leucyl-acylated tRNAs from soyabean cotyledons, but only four in the hypocotyl system, tentatively suggesting variation in the synthetases present. They also demonstrated that cytokinin had an influence on acylation of tRNA, which may have some bearing on the regulation of protein biosynthesis.

The base composition and amount of RNA present in the leaves of healthy and TMV infected tobacco plants have been investigated by Röttger (1965), and the RNAs present in tobacco leaves following infection with TMV were fractionated by Kubo et al., (1965). Johnson and Young (1969) isolated and fractionated on benzoylated DEAE-cellulose, the tRNAs of healthy and TMV-infected leaves of Nicotiana tabacum.

Changes in the population of tRNA molecules following infection of E. coli with T2 and T4, and herpes virus infection of animal cells, have been observed. In the case of E. coli, phage infection has shown to cause methylation (Boezi et al., 1967) and thiolation (Hsu et al., 1967) of tRNA. The changes may be a result of modifications of pre-existing tRNA species or from de novo phage-induced tRNA synthesis. Recent work suggests that at least some of the modified tRNA is coded for by the phage (Daniel et al., 1968; Tillack and Smith, 1968; Weiss et al., 1968) or by the viral genome (Hay et al., 1967; Suback-Sharpe et al., 1965).

Infection of E. coli by RNA phage R17 does not result in any change in tRNA synthesis (Hudson and Paranchych, 1968). Changes in the tRNA

populations following infection by this RNA phage would be unexpected since the phage genome contains information sufficient for the synthesis of only three proteins (Gussin, 1966).

TMV RNA however, consisting of 6400 nucleotides, may well carry information specifying new tRNA species. The alteration in the elution profile of a tRNA species accepting phenylalanine after infection of tobacco leaves with TMV (Johnson and Young, 1969) thus appears to parallel the results obtained with the DNA phages and the DNA viruses.

It should be noted, however, that Schlegel and Smith, (1966) observed differences in behaviour between older and younger leaves in their studies on actinomycin D inhibition of cellular RNA synthesis in plant cells. This apparent dependence of the alteration in tRNA elution profile on the physiological state of tobacco leaves may mean that such a change is not the direct biochemical result of TMV infection.

It is possible that damage caused by the virus particle or RNA results in an alteration of tRNA population pattern as a result of 'leakage' between cell organelles. On extraction therefore a false impression could be obtained. Alternatively during viral development a second specific species of tRNA may develop exhibiting itself as a large shift in the normal population when compared with young leaves (Johnson and Young, 1969). In older samples, this new species may decrease and thus decrease the shift when compared with older leaves.

This latter possibility may suggest that viral infection, damages and alters many of the important cell organelles and functions to such an extent as to precipitate premature ageing (and in some cases death) of the host plant).

Chinese cabbage tRNA profiles in the uninfected and infected states were not investigated. It may therefore be only tentatively proposed that the nature of the tRNA population in this particular case plays an important and direct role in determining the efficiency of viral infection *in vivo* and protein synthesis *in vitro*. The low activity of the experimental systems may be related to the isolation of the tRNA from the wrong 'age' of leaf, or to the fact that for higher activity, tRNA from infected plants is required. Such tRNA may have been specially prepared/modified by the virus.

It would be interesting to investigate the effect of tRNA isolated from different ages of infected Chinese cabbage on *in vitro* activity, and to try to relate this to the constitution of the tRNA population. Investigations of mixtures of isolated tRNA species may also aid investigations and a combination of results may suggest if actual chemical/structural alteration is the cause/effect of viral activity.

It is interesting to note that Pinck *et al.*,(1970) observed that, in the presence of cell-free extracts and ATP, TYMV RNA can interact with one amino acid, valine, which becomes attached to the 3' terminal nucleotide of the RNA molecule, in a manner analagous to that encountered in aminoacyl-tRNAs. They observed, however, that during incubation some degredation of TYMV RNA occurred. They were unable to bind any other amino acids, and unpublished results demonstrated certain similarities between $tRNA^{Val}$ and the TYMV polynucleotide moiety which accepts valine (Yot *et al.*, 1970). Their results and the fact that all bacteriophages and viral RNAs examined to date contain identical sequences

(-CpCpA or CpC) at their 3' termini, suggest that under appropriate conditions these RNAs could also accept an amino acid.

The TYMV RNA routinely employed in the cabbage and bean *in vitro* systems was also found to bind valine (the extent being somewhat dependent on the viral sample), although no incorporation of [^{14}C] valine resulted in a complete system (in the presence or absence of added tRNA).

RNA from avian myeloblastosis virus can be resolved into two major fractions (Carnegie *et al.*, 1969). The low molecular weight RNA fraction amounts to about 20% of the viral RNA and a little less than half of the RNA in this fraction appears to be tRNA. RNA from the virus attached amino acids and transferred the amino acids to peptide chains under the experimental conditions Carnegie *et al.*, (1969) employed. It is suggested that the viral tRNA is not of cellular origin, but a selective mechanism could be operative during the maturation of the virus, incorporating one species of cellular tRNA into the particles and excluding others(Trávníček, 1968). The functional tRNA appears to be part of the virus particle and the work of Carnegie *et al.*,(1969) supports the contention of Trávníček (1968).

The experiments of Vescia *et al.*, (1968) suggest that tRNA from tumor tissue was structurally different from tRNA from normal tissue. The circumstances of tRNA being present in a tumor virus is of interest since the oncogenic property of the virus implies that a mechanism of continuous translational control is established in the host cell after infection.

It is possible that 'tRNA-like' molecules may be identifiable in other plant viruses, these 'molecules' playing an important role in establishing the virus and in subsequent translational control of its reproduction. That fact that $[^{14}C]$ valine was not incorporated in the complete cabbage system possibly suggests dilution by endogenous amino acids or the absence of some requisite factor(s).

Polyamines - In Vitro and In Vivo:

In the Chinese cabbage in vitro system, spermine had a slight inhibitory action, cadaverine had little effect but spermidine increased incorporation by 3000 c.p.m. per mg ribosomal RNA. In the absence of tRNA, the activity of the system was reduced by over 30%. In the absence of tRNA but with the addition of spermidine there was little change in activity, but on replacing the spermidine with spermine there was a marked increase in incorporation of 2000 c.p.m. per mg ribosomal RNA. In the latter case it is possible that the configuration of the native viral RNA was so altered to enable it to act as its own 'initiator' tRNA.

The polyamines, putrescine $[NH_2(CH_2)_4NH_2]$, spermidine $[NH_2(CH_2)_3NH(CH_2)_4NH_2]$ and spermine $[NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2]$ are widely distributed in nature (Tabor and Tabor, 1964). They have been found in most animal tissues, bacteria, fungi, bacteriophages, wheat and tomato and as a major component of several plant alkaloids.

Johnson and Markham (1962) reported isolating a compound from TYMV

they identified as bis(3-aminopropyl)amine, also detected in turnip crinckle, broad bean mottle and tobacco mosaic viruses. They were unable to demonstrate it, or other triamines in uninfected Chinese cabbage. Beer and Kosuge (1970) however, isolated spermidine and spermine from purified preparations of TYMV, and were unable to detect bis(3-aminopropyl)amine.

The physiological role of the polyamine is not known with certainity, but by virtue of their highly cationic nature (all the amino groups being protonated at physiological pH values), they bind strongly to nucleic acids (Hirschman et al., 1967; Tabor and Tabor, 1964).

The role of polyamines in protein synthesis has been studied by several workers and it has been shown that polyamines aggregate ribosomal subunits into 70 S and 100 S ribosomal particles (Cohen and Lichtenstein, 1960; Colbourn et al., 1961; Martin and Ames, 1962), inhibit enzymatic degredation of ribosomes (Erdmann et al., 1968) and stimulate the incorporation of amino acids into polypeptides in vitro (Bretthauer et al., 1963; Martin and Ames, 1962; Nathans and Lipmann, 1961).

It has been shown that a critical level of magnesium is required for the maintenance of ribosome function, and that some of this magnesium can be replaced by polyamines (Weiss and Morris, 1970). Polyamines can replace Mg^{2+} in cell-free systems of E. coli directed both by poly(U) (Takeda, 1969a) and MS2 phage RNA (Takeda, 1969b). Since E. coli cells contain polyamines it is suggested that polyamines probably exhibit similar effects on protein synthesis in vivo (Takeda, 1969a). Hurwitz and Rosano (1967) even proposed that polyamines might be more important for protein synthesis in vivo than Mg^{2+} ions.

That polyamines can stimulate aminoacyl-tRNA formation (Takeda and Igarashi, 1969) and replace Mg^{2+} ions in the binding of mRNA to ribosomes (Takeda, 1969c; Tanner, 1967) may suggest possible roles for the polyamine(s) found in TYMV. They may be present to aid structural intregity, to form a ribosome-virus complex, to act on TYMV RNA when acting as a 'tRNA' or to act on other 'modified' tRNAs.

In addition to known effects of spermidine on RNA synthesis *in vitro* (Fuchs *et al.*, 1967), spermidine has been indicated as a controlling element in RNA synthesis *in vivo*. That spermidine can serve to organise helical structure in both RNA (Szer, 1966) and DNA (Liquori *et al.*, 1967) is known. Indeed, it has been shown that polyamines can help to effect a conversion *in vitro* of the inactive configuration of tRNA to the active form (Fresco *et al.*, 1966; Ishida and Sueoka, 1966; Muench and Safille, 1968). Spermidine appears to be the most active organic cation in this conversion (Cohen *et al.*, 1969), and may have been functioning in this way in the cabbage system.

Takeda and Igarashi (1969) found that putrescine stimulated aminoacyl-tRNA, but in the TYMV system, there was only a slight improvement in incorporation in the presence of this polyamine. This may suggest that polyamines (if involved) are involved at earlier or later stages in the biosynthetic process, or that excess Mg^{2+} present in the incorporation mix was masking any 'polyamine' effect.

In a system derived from *Rhizoctonia solani* (Obrig *et al.*, 1969) found a lack of stimulation by polyamines. This was unexpected as polyamine is required for optimum activity in amino acid incorporation

by both yeast and Botryodiplodia cell-free systems (Bretthauer et al., 1963; Downey et al., 1965; van Etten, 1968).

Recently, polyamines have been shown to displace Mg^{2+} ions from ribosomal RNA and to increase binding of certain soluble RNA species to the ribosomes (Choi and Carr, 1967; Tanner, 1967). The net effect in both cases is that it lowers the Mg^{2+} concentration that is required. Thus in Rhizoctonia solani it may be that it can displace Mg^{2+} but not replace it.

In the B. chinensis system, at optimum Mg^{2+} level of 6 mM, spermidine increased incorporation by 3000 c.p.m. At low Mg^{2+} (2mM), incorporation was however approximately halved. This may suggest that the addition at 'optimum Mg^{2+}' reduced the Mg^{2+} concentration required, but still allowed incorporation to proceed. At low Mg^{2+} it again lowered the Mg^{2+} concentration required, but at this new level was unable to replace it, and so incorporation was inhibited.

The inconclusive effect of polyamines on the TYMV in vitro system may be due in part to the in vitro 'environment'. Takeda and Igarashi (1970) found that aminoacylation of tRNA with leucine, isoleucine and valine stimulated by either magnesium acetate or spermine was sensitive to NaCl and that KCl, NH_4Cl and NaCl inhibited isoleucyl-tRNA formation. Phenylalanyl-tRNA formation was not however inhibited by NaCl, KCl and NH_4Cl in the presence of magnesium acetate, but was inhibited by these salt solutions in the presence of spermine. It was suggested that the inhibitory effect of salt solutions on aminoacyl-tRNA formation might be due to the inhibition of the binding of spermine to tRNA. In the in vitro

cabbage system, the position would appear to be further complicated by the use of a $[^{14}C]$ amino acid mixture, instead of a single $[^{14}C]$ amino acid (a number of aminoacylations being studied rather than a single one).

Temperature Effects:

Various incubation temperatures have been employed in the wide range of *in vitro* protein synthetic studies. The temperature range of from 25° to 37°C appears to be the most frequently employed in cell-free systems derived from plant material, 35-37°C being adopted for most bacterial and mammalian systems.

On incubating the *V. faba* *in vitro* system with poly(U), the optimum temperature of incorporation was determined to be c. 25°C. Similarly, using TYMV RNA as messenger in the *B. chinensis* system (employing $[^{14}C]$ amino acid mixture) maximum incorporation occurred at 25°C.

Temperature plays an important role in determining the activity of the artificial *in vitro* system, and although at present it is not possible to provide in such systems the conditions prevailing *in vivo*, some correlation between the two states may be made. Interpretations of the effect of temperature are, however, tentative unless confirmed by *in vivo* observations.

The effect of temperature may be divided into two, i.e., the effect

on the in vitro system and protein biosynthesis in general and the effect on the virus.

(a) Temperature Effect on In Vitro Systems and Protein Biosynthesis:

Increase in temperature can cause increased incorporation by a simple enzymatic effect. This effect, however, is offset by decreased template and ribosome stability, enzyme denaturation and tRNA structure variation at higher than 'normal' temperatures.

It is known that several physical properties of tRNA are altered between 35° and 45°C, these changes being attributed to unfolding of the tertiary structure. Schlimme et al., (1969) noted a decrease in the rate of charging of yeast tRNA with increase in temperature, the effects observed not being due to a change or irreversible inactivation of purified aminoacyl synthetases (since their inactivation followed a normal pattern above 43°C). The amino acid activation reaction, measured by pyrophosphate formation, had a normal temperature profile up to 50°C, and only at this temperature was the enzyme denatured. They concluded that the observed changes in aminoacylation caused by temperature changes were due to changes in the physical properties of the tRNA, such changes yielding a molecule having a non-aminoacylatable conformation.

In Bacillus stearothermophilus it has been suggested that maintenance of the correct secondary structure of sRNA in the intact thermophile (a necessary requisite for biological activity) could be achieved by polycation stabilisation, rather than by alterations in the primary structure (Friedman and Weinstein, 1966; Mangiantini et al., 1965).

Using crude extracts of B. stearothermophilus strain 2184, Friedman and Weinstein (1966) studied the effect of temperature on leucyl and phenylalanyl tRNA synthetases, and suggested that the high temperature optima (55-65^{o}C) were primarily a function of the thermophile enzymes. Bubela and Holdsworth (1966) studying an unclassified strain of the same organism obtained similar results.

A high temperature optimum for enzymatic activity does not necessarily imply the presence of a heat-stable enzyme per se, but may reflect an elevated heat of activation for the substrate or co-factor -stabilised enzyme complex.

Results on work in this field have been interpreted to indicate that native tRNA possesses a conformation which is essential for synthetase recognition and that this can be reversibly lost on raising the temperature.

Similarly Brown and Zubay (1960) and Takanami et al., (1961) observed that the amino acid incorporating ability of tRNA is not destroyed by heat treatment followed by quick cooling. They suggested that a specific secondary structure was unnecessary for the enzymatic formation of aminoacyl-tRNA. It is possible however that the portion of the RNA molecule reacting with the enzyme is renatured by cooling (Arcà et al., 1963).

Such conformational changes may therefore play a part in the effect of temperature in mesophile in vitro amino acid incorporating systems.

Coding errors brought about through anomalous charging of tRNA

at elevated temperatures may also be instrumental in defining the maximum growth temperatures of thermophiles (Friedman, 1968). Anomalous charging behaviour begins at temperatures corresponding to the onset of hyperchromic changes in the tRNA melting curve.

Fan et al., (1964) found that there was a relatively constant relationship between the rate of mRNA degradation and protein synthesis in B. subtilis, for a wide temperature range.

It has also been observed that ribosomes of the ciliated protozoan Tetrahymena pyriformis, show significant denaturation close to each strain's maximum growth temperature (Byfield et al., 1969). Protein synthesis was reduced at 34°C to 30% of that at 29°C, the temperature effect being strain specific (Byfield and Scherbaum, 1967 a). The same temperature levels initiating in vitro ribosomal melting caused significant in vivo reduction in messenger translation efficiency, possibly resulting from template hydrolysis without coincident translation (Byfield and Scherbaum, 1966, 1967 a,b). Lipid biosynthesis was increased under raised temperature conditions, suggesting that reduction in protein synthesis was not due to an underlying reduction in energy sources (Byfield and Scherbaum, 1967 c).

Schiebel et al., (1969) noted in the slime mould Physarum polycephalum a decreased protein synthesis and polysome breakdown at temperatures above the growth optimum.

Mouse liver ribosomal RNA undergoes thermal denaturation at temperatures slightly above a mouse's normal body temperature (Byfield et al., 1969).

Employing cultures of a Saccharomyces cerevisiae mutant, Hutchison et al., (1969) reported that at temperatures above the normal growth temperature (23°C), the rate of protein synthesis decreased slowly, paralelling the decay of polysomes to monosomes. They suggest that heat-shock decreases polyribosome intregity, possibly by mRNA destruction (as suggested by Byfield and Scherbaum, 1966 etc.) by simple separation of ribosomes from mRNA or as the result of inhibition of one or more steps in the initiation of protein synthesis.

Hutchison et al., (1969) found that polysomes remaining after 30 min at 36°C were active in in vitro polypeptide synthesis, monoribosomes formed by polysome decay being inactive. Ribosomes isolated from cultures maintained at 36°C for 1 h were inactive in vitro, unless poly(U) was added as messenger. They suggest that the mRNA of the yeast is metabolically unstable, having a half-life of 23 min at 36°C.

Rosen et al., (1967) observed that the heat lability of a cell-free amino acid synthesising system derived from trout liver was localised in the aminoacyl transferase activity of the cell sap and that the heat lability of the microsomes may be related to transferase activity. Although initial rates of incorporation at 30°C or 37°C were similar to those at 23°C, more phenylalanine incorporation occurred at 23°C after 35 min than at 30° or 37°C. Aminoacyl synthetases and transferases were not found to be temperature sensitive and they suggest that the loss of aminoacyl transfer activity was not due to GTP hydrolysis. This latter occurrence would appear as a loss in transfer activity, GTP being necessary for the formation of the peptide bond. Neither deacylation of phe-tRNA or mRNA

hydrolysis were found to be significantly involved in loss of activity.

Moldave and co-workers(Fessenden and Moldave, 1962, 1963; Gasior and Moldave, 1965) separated the aminoacyl transferase activity of rat liver into two soluble protein fractions, both necessary for the transfer of amino acid from tRNA to ribosomes. One, however, was less stable than the other and these findings may be applicable to other systems. Support for their interpretations of their findings has come from studies in preparations from rabbit reticulocytes, E. coli and Pseudomonas fluorescens.

The heat stability of ribosomes from B. stearothermophilus has been demonstrated in comparative studies with E. coli ribosomes utilising thermal denaturation profiles (Friedman et al., 1967; Mangiantini et al., 1965; Sanders and Campbell, 1966). The greater resistance of thermophilic ribosomes than E. coli ribosomes to heat denaturation suggested a possible correlation with the maximal growth temperature of these organisms (Mangiantini et al., 1965).

Friedman and Weinstein (1966) in a sub-cellular protein synthesising system derived from B. stearothermophilus, observed that over the range 30-70°C native mRNA optimally directed $[^{14}C]$ phenylalanine, lysine and proline into polypeptides at 55-60°C. Incorporation was greater at 65°C than at 37°C, although in an E. coli system, $[^{14}C]$ phenylalanine incorporation at 65°C was 10% of that at 37°C. A poly(U) directed B. stearothermophilus system showed greater activity at 37°C than at 65°C suggesting that the in vitro system provided an essential component, not available at this temperature in vivo, or the inability of the organism to

grow at 37°C resided in an aspect of cell metabolism not directly related to protein synthesis. Leucine-phenylalanine ambiguity tests demonstrated that low temperature and high magnesium ion concentration greatly enhanced the incorporation of leucine in the presence of poly(U). Such temperature variation could therefore similarly lead to active though ambiguous incorporation in the mesophile system with similar variations in ribosomal activity as a result of temperature variation (Friedman and Weinstein, 1964, 1966).

While temperatures which induce in vitro melting appear to correspond to those which inhibit protein synthesis, there is no direct demonstration that this is the real mechanism of action. Pace and Campbell (1967) and Mangiantini et al., (1965) suggest, however, that the thermostability of ribosomes could be the limiting factor in determining the upper growth temperature for organisms. It is possible that homeothermy has evolved as a protection against thermal impairment of mRNA translation in higher forms.

(b) Temperature Effect on Viruses:

It may also be noted that the quantity and quality of some viruses is affected by the temperature at which the infected plants are maintained (Kassanis, 1952, 1957 a; Lebeurier and Hirth, 1966). Most strains of TMV produce less infective virus in plants kept at high temperatures (32-36°C) than at normal glasshouse temperatures (20-25°C). Lebeurier and Hirth (1966) suggested that RNA replication was inhibited at temperatures far removed from optimal (28°C) in TMV-LB strains.

Kassanis and McCarthy (1967) suggested, however, that low infectivity need not necessarily have resulted from inhibition of RNA replication. It is interesting to note that Kassanis and Bastow (1971) compared the multiplication of four strains of TMV at temperatures of 20-25°C and at 35°C. The concentration of total infective virus RNA, intact virus, virus antigen and insoluble virus protein in plants at different times after inoculation were observed. The concentration of total infective RNA of all strains reached its maximum about a week after inoculation and was ten times greater at 20°C than at 35°C. The intact virus and virus antigen concentration were similarly reduced. Virus multiplication was affected by increased temperature in two ways,
(i) the replication of the RNA of all strains was inhibited, and
(ii) the RNA of those strains which had defective protein was degraded. when infected plants were transferred from 35° to 20°C, infectivity of the type strain was increased.

Excluding the behaviour of the defective strains, different plant viruses react to elevated temperatures in three ways; (1) the amount of virus and its specific activity is the same at 20° and 35°C, e.g. bromegrass mosaic virus (BGMV) (Kassanis and Lebeurier, 1969); (2) much less virus is produced at 35°C than at 20°C as found in type strain of TMV (Kassanis and Bastow, 1971) and (3) multiplication is completely inhibited at 35°C e.g. tomato bushy stunt virus (TBSV) (Kassanis, 1954) - plants infected with such viruses can be freed from infection by maintaining them at 35°C.

Kassanis and Lebeurier (1969) observed that infectivity of TBSV

was lost *in vivo* and *in vitro* without any apparent change in the physical properties of the particles or of the nucleic acid. Inactivation of BGMV, but not TBSV was suggested to reflect breaking of the nucleic acid.

Heat therapy has been found applicable more generally with isometric viruses rather than to those with elongated particles (Kassanis, 1954, 1957 b). Lyttleton and Matthews (1958) found that TYMV RNA can be released without gross denaturation of the protein shell, which remains antigenically active, by heating the preparation at 45°C for 10 to 30 min. They suggested that this was a possible mechanism for virus inactivation in plants at high temperatures, but disproved the idea by showing that virus does not multiply at 33°C and virus already in the plants loses its infectivity without releasing its RNA (Matthews and Lyttleton, 1959). Matthews and Lyttleton, (1959) observed that proteins and TYMV disappear in Chinese cabbage at 33°C at about the same rates, and that TYMV in plants held at 35°C lost infectivity without significant changes in other virus properties. Uncoating of the virus may prevent infectivity, but once uncoated increase in temperature may cause breakdown of the RNA. Several viruses having isometric particles lose infectivity between 30°C and 36°C and Dunn and Hitchborn (1966) suggest that degradation of the RNA of the virus may be involved.

Probably all viruses can be inactivated *in vivo* with the right combination of temperature, time and attention to all factors favouring plant survival (Bawden, 1966), this principle of heat therapy being readily employed in Man at the onset of viral attack.

The most plausible hypothesis for the mechanism of heat therapy is that high temperatures cause the destruction of essential chemical activities in both virus and host, but that the host is not as sensitive or is better able to recover from any damage, i.e. the temperature coefficient of thermal inactivation for the host exceeds that of the virus at certain temperatures (Geard, 1958). The inactivation process *in vivo* may be purely physical, as it appears to be *in vitro*, or it may be aided by biological changes induced in the host metabolism by high temperature (Gay and Kuhn, 1968).

No systematic study has been made of the time course of TYMV development in Chinese cabbage plants grown under various strictly controlled environmental conditions. Dunn and Hitchborn (1966) did observe that virus purified from infected plants grown in the open during the summer yielded preparations containing more heat stable 22 S RNA than did virus purified from infected plants grown in the greenhouse during the same period. They suggested there was a correlation between the conditions under which the plants were grown and the proportion of virus particles which contained completely undegraded RNA. High temperatures slow virus multiplication and in plants held at $33^{o}C$ no virus increase occurs (Matthews and Lyttleton, 1959). The Chinese cabbage plants used in the present work were grown at $25^{o}C$ which appeared to be the optimum for sturdy and rapid growth. The fact that the optimum temperature for *in vitro* activity of the viral RNA was the same as this optimum growth temperature may, however, have been purely coincidental. *In vitro* protein synthesis may, however, reflect the optimum temperature of plant growth, the optimum temperature of *in vivo* virus reproduction, any combination of these factors, or be simply a

summation of the physical and chemical properties resulting from the use of an artificial system.

Temperature rise may alter TYMV RNA physical structure with resultant loss in biological activity, as has been shown for other RNA species, but further experimental work is needed before the precise 'cause and effect' of temperature on viral *in vitro* protein synthesis can be ascertained.

Host Specificity:

The ability of TYMV to infect plants appears to be confined entirely to the Cruciferae, attempts by Markham and Smith (1949) to infect species in other families by means of dodder (*Cuscuta* spp.) being unsuccessful. Markham and Smith found the virus occurring naturally on turnips, swedes and broccoli. Borges (1947) and Broadbent and Heathcote (1958) transmitted the virus to a series of cruciferous hosts. Of 21 species from other families tested, TYMV was found only to infect *Reseda odorata*, where it produced vein yellowing or banding and chlorotic or necrotic spots. Sander and Schramm (1963) reported that their strain C of TYMV would infect *Nicotiana tabacum* var. Xanthi, visible chlorotic lesions being produced but only during the winter.

Croxall *et al.*, (1953) attempted to inoculate *V. faba* var. Early Longpod with TYMV isolates with no apparent success. As it was intended to use an *in vitro* protein synthesising system derived from *V. faba* with TYMV RNA as 'messenger' an attempt was made in the present work to infect

V. faba var. Triple White in order to compare the results with these earlier observations.

Initial experiments to infect V. faba were apparently unsuccessful when employing the "Markham strain". The "New Zealand strain" however caused early "abortion" of the white bean flowers (although this could have been simply a sign of a metabolic disorder).

Red-mottling of the bean leaves after TYMV inoculation was considered to be a sign of infection in the test bean plants, although typical infected Chinese cabbage symptoms were absent. It is possible that some "infection" was occurring either as a definite (although rather symptomless) infection or as a "local" infection, the bean leaves acting as local storage organs for the virus. Control bean plants were not affected and it is interesting to note that Drijfhout (1968) observed similar red-mottling with pea leaf-roll virus, which produced small brown dots in peas and caused premature leaf shedding in the Vicia-bean.

Repeat experiments in the present work, with Chinese cabbage plants, gave similar results, and it was evident that back-inoculated plants were not infected as a result of contamination, but by a causative organism from the bean plants. Uninfected radish leaves ground with inoculated/'infected' bean leaves increased the blooming in cabbage plants inoculated with this mixture, possibly suggesting that the radish leaves provided some factor which aided the re-infection process.

It would appear also that some symptoms became evident after inoculating Chinese cabbage plants with inocula prepared by grinding together leaves from an inoculated bean plant, and leaves from an uninfected

Chinese cabbage plant. The 'blooming' exhibited in such cases was a greying of the central rosette of the younger leaves, advancing outwards with age (and often accompanied by slight lesioning of the older leaves). This 'blooming' was occasionally seen as a symptom on normally inoculated and infected Chinese cabbage plants (maintained as source plants for TYMV).

It is interesting to note that back-inoculation from TYMV RNA inoculated bean plants produced typical symptoms in Chinese cabbage up to about 28 days after initial bean inoculation. After this time, only mild discolouration of back-inoculated Chinese cabbage leaves resulted and bean flower abortion ceased. Possibly at this time, after the initial inoculation of the bean leaves, the 'infective agent' was no longer viable. It is also possible that either the bean had 'killed' the virus, or if the 'infective agent' had never in fact taken hold in the bean plant, it was no longer able to lead an individual existence away from its true host (i.e. Chinese cabbage).

Failure to observe crystalline TYMV aggregates under electron microscopic examination may simply have been due to a very low/local concentration of the virus in the bean leaves.

Some viruses, under appropriate conditions, may infect a plant without producing obvious signs of disease, or lead to the rapid death of the plant. TYMV normally produces some of the most obvious symptoms of infection in the development of a pattern of light and dark green areas, giving a mosaic effect in infected leaves (often approaching genetic variegation). The macroscopic symptoms induced reflect histological

changes within the plant.

When no distinguishable symptoms appear but there is an infection, the plant is referred to as having masked symptoms.

In some diseases the virus infects a given host and increases therein but no visible signs appear over the entire range of environmental conditions to which the host is usually exposed. This condition prevails in many varieties of potato infected with the latent-mosaic virus.

In other cases a host may shown signs of the disease for a relatively short time after it becomes infected, but the symptoms gradually disappear, and clones derived from such a plant continue to contain the virus but show no symptoms. The plant which is a symptomless carrier is distinguished from one regarded as having masked symptoms by the fact that in the latter instance, the signs are not visible in a particular sector of the usual range of environment, while in the former case the plant is symptomless throughout the entire range of environment.

Many strains of a virus may have very similar host ranges, while others may differ considerably. Only a relatively small proportion of host-virus combinations have been tested and our knowledge of the occurrence and distribution of viruses in plants is fragmentary. In many investigations on host ranges, only positive results have been recorded and back inoculations have not been performed to test for masked infections. It is also important to decide what constitutes multiplication over and above the inoculum remaining on the 'infected' leaf.

The manner of inoculation can alter results, many plants containing

inhibitors that prevent mechanical inoculation to the species, from the species, or both. Often only one set of conditions of inoculation are employed, which may hide a possible host. Often closely related strains differ in the range of plants that they will infect.

Evidence available for small viruses suggests that host range is a property of the RNA rather than the protein coat, although attempts to extend host range by using infectious RNA rather than virus have generally been unsuccessful (e.g. Haselkorn, 1962 b). The host range of viral RNA cannot be extended by coating the RNA in the protein of a virus which can attack the host, and coating with a protein from a virus unable to attack a host does not prevent the RNA from becoming established. This does not, however, preclude the possibility that there is one small protein or peptide molecule firmly bound to the RNA and involved in host specificity and virus establishment.

Sander and Schramm (1963) observed that in experiments using the natural host and the host of the other virus reciprocally, and comparing the infectivities of the complete virus and of the free RNA of TMV and TYMV, that the coat protein of the complete virus enhances the infectivity in comparison to the free RNA, only in the host to which the virus is adapted.

Mechanical inoculation involves the introduction of infective virus or viral RNA into a wound made through the plant surface. Infection occurs when the virus has established itself successfully in the cell. Severe abrasion has been found more effective in some cases (Louie and Lorbeer, 1966) as has rubbing of an infected leaf onto a leaf surface

(Murakishi, 1963). These methods and injection were attempted to instigate TYMV infection in the bean with only a small degree of success.

The form in which a new virus spreads from cell to cell within leaves is not known, the first crop of virus passing unnoticed in infectivity assays. It is only at the time when some hundreds of cells at each infection site have become infected that multiplication becomes detectable (Harrison, 1958). The concept of virus diseases as disturbances of the host's nucleoprotein metabolism almost necessitates the conclusion that the ability of virus to infect, multiply and cause symptoms will vary greatly with changes in the physiological conditions of the host. Almost every change in growing conditions that affects plant growth or cellular metabolism also effects the behaviour of the virus. This aspect was not considered further in the present work, but it is possible that further symptoms of infection could have been produced in the bean plants by varying growth conditions.

Information concerning the functions performed by a plant virus or a plant host in the course of infection and the molecular events that accompany the replication of plant viruses is scanty. A plant infected with one strain of a virus is usually protected from infection by other related strains of the same virus (Fulton, 1951). The subject of interference has been reviewed by Ross (1959) and Kassanis (1963). It is widely assumed that the interference phenomenon might be explained by competition between plant viruses for the theoretical adsorption sites (receptors) of the plant cell (Rappaport and Wu, 1962; Siegel, 1959; Wu and Hudson, 1963; Wu and Rappaport, 1961). It is supposed that when

the saturation of cell receptors by the interfering virus (or protein) occurs, particles of the second virus cannot participate in the infection. It is possible that the bean plants employed were infected by a symptomless virus, preventing TYMV invasion and infection. If infected, the infection was not transmitable from 'uninfected' test bean plants to Chinese cabbage.

Cell receptors play an important role in determining cell susceptibility to animal viruses and bacteriophages (for review see Fenner, 1968; Stent, 1963). Non-host animal and bacterial cells are often insusceptible to a certain virus because they fail to absorb it and do not allow penetration. Unfortunately, the data we have at present concerning virus-specific receptors of plant cells are difficult to interpret (Novikov and Atabekov, 1970). We know nothing definite about the chemical nature of cell receptors and their localisation in the plant cell. As V. faba ribosomes and TYMV RNA were active together in vitro, it would appear that resistance to infection (assuming the bean inoculation experiments to be either negative or at the least inconclusive) lies at another point in vivo. Whether this 'resistance' occurs at the viral protein synthesis stage or some other is open to speculation. Possibly if the TYMV viral RNA were able to attach to bean ribosomes in vivo typical symptoms and typical infection would ensue.

It is known that the resistance of animal cells to the virus can in some cases be due to the inability of the cell to uncoat the adsorbed virus (Darnell and Sawyer, 1960; Holland, 1962). Enders et al., (1967) showed, however that the coat protein can be removed from

poliovirus particles which entered cells resistant to poliovirus. Thus, the virus uncoating mechanism operates non-specifically in this system. Information concerning the uncoating mechanisms operating in plant cells infected with virus is scanty (Reddi, 1966; Shaw, 1967, 1969). Hiebert et al., (1968) showed that protein from a virus normally unable to infect a certain host does not restrict the infectivity of a foreign viral RNA enclosed in the hybrid spherical virus obtained in vitro. Atabekov et al., (1970) present evidence to suggest that the virus uncoating mechanism of the plant cell in some cases does not control the host range of plant viruses.

It is suggested that the mechanism controlling the host range does not operate at the first stages of plant virus replication. In other cases, the first stage barrier(s) is operative, but it can be overcome by using free RNA for inoculation. Examples of this kind are not numerous for plant viruses (Humm and Humm, 1968; Süss et al., 1965). Inoculation of bean leaves with TYMV RNA did not, however, produce a ready infection.

It has been known for some time that attachment and uncoating are necessary, but are not always sufficient conditions for virus replication (see review by Fenner, 1968). Free viral RNA is not necessarily capable of infecting cells that are not susceptible to the native virus (Holland et al., 1959; Holland and Hoyer, 1962; Agol, 1964; St. Geme et al., 1967). In particular cases the cell is abortively infected even after successful adsorption, penetration and uncoating (e.g., Buck et al., 1967).

Atabekov et al., (1970) showed that RNAs from four plant viruses were completely inert in plants that are not susceptible to the virus

itself i.e. the host range of the viruses coincided with that of the free RNA. Their observations provide evidence suggesting the existence of one (or more) intra-cellular barrier(s) operating at the later stages of viral replication. Such barriers may be mechanisms controlling either translation or replication of the viral RNA responsible for the specific defence of the plant cell against the foreign genetic material. It is not excluded that multiple cellular barriers controlling the host range of plant viruses at different stages of infection may exist in the plant cell, and investigations into this aspect of host specificity are necessarily difficult.

A number of 'mixed' systems have apparently been successful however, including the replication of an infectious form of TMV in chicken embryos (Cochran et al., 1969), and the binding and translation of TYMV RNA by skeletal muscle ribosomes from normal and diabetic rats (Rolleston et al., 1970).

Cochran et al., (1969) found that a small RNA (4-6 S) isolated from a 75 hour TMV infection in Turkish tobacco, appeared to be the most potent embryo inoculum, and Cochran has theorised that TMV RNA is a polymer composed of linked infectious monomeric units (and that the 4-6 S RNA may be this unit). This work may lead to further investigations of other viral RNAs in an attempt to isolate such small active units.

From these observations it is clear that further work is necessary before any clear proposals as to the level(s) of host specificity can be made.

Electron Microscopy:

There has been little study of small isometric plant viruses by electron microscopy (Hills and Plaskitt, 1968). Under staining conditions as applied to plant tissues by Kolehmainen et al., (1965) only the nucleic acid is deeply stained, and the nucleic acid core of a small spherical virus and a ribosome are nearly indistinguishable (Chalcroft and Matthews, 1966; Edwardson et al., 1966; Gerola et al., 1965).

Virus particles in sections lose their characteristic angular profile and appear smaller than they do in vitro in negative stain (Milne, 1967). Shikata and Maramorosch (1966) showed that pea enation mosaic virus particles occur in the nuclei, cytoplasm and central vacuoles of infected cells. They were able to use the large size of this virus as a criterion to distinguish between virus particles and ribosomes. Milne (1967) attempted to distinguish between ribosomes and virus particles by the irregular ribosome outline and the more regular appearance of the virus particles. Edwardson et al.,(1966) reported that in sections of centrifuge pellets, southern bean mosaic virus and tobacco necrosis virus were dissimilar in appearance to that of ribosomes from tissue sections.

Milne (1967) noted that it was difficult to distinguish the small isometric virus particles of TYMV from ribosomes, although it was sometimes possible if the virus was crystallised. Pre-treatment of tissues with permanganate or EDTA (for sowbane mosaic virus) appeared to destroy the ribosomes but also resulted in excessive disorganisation of the tissues.

Prior to noting the observations of this paper (Milne, 1967) a similar pre-treatment of B. chinensis leaves was employed. The leaves were floated/soaked in 0.01 M EDTA (pH 7.4) solution for 24 h prior to examination. It was anticipated that the virus would be 'stable' to such treatment, but that the ribosomes would not. On examination of infected and uninfected tissues similarly treated, no conclusive information was however obtained. Bean leaves treated and examined in like manner were no more informative.

Milne (1967) did find TYMV free in the cytoplasm but absent from the nuclei, chloroplasts and mitochondria.

Infected B. chinensis leaves in the present work did however show markedly abnormal chloroplasts as reported by Chalcroft and Matthews (1966), but star shaped bodies found by them in infected cytoplasm, and other abnormalities, were not encountered.

Ushiyama (1971) found on electron microscopy of Chinese cabbage leaves systemically infected with TYMV, numerous particles assumed to be virus. These were present in the cytoplasmic spaces between clumped chloroplasts, but in the general ground cytoplasm TYMV particles were indistinguishable from the ribosomes. When detached whole leaves from intact plants were held at 60°C for 5 min and kept at 20°C for 18 h, particles located in the spaces between chloroplasts and in the general cytoplasm of the infected leaves were apparently crystallised. No such crystalline arrangements were observed by him in healthy leaves.

These experiments were repeated in the present work using

B. chinensis test plants and typical crystallisation was observed (fig.47) in infected leaves, but not in uninfected. On examination of bean leaves, however, no crystallisation was observed. A number of bean leaves were examined, and even those exhibiting 'TYMV symptoms' of infection did not exhibit this phenomenon. It is possible that the TYMV, if present, was in so small a concentration as to prevent observable crystallisation.

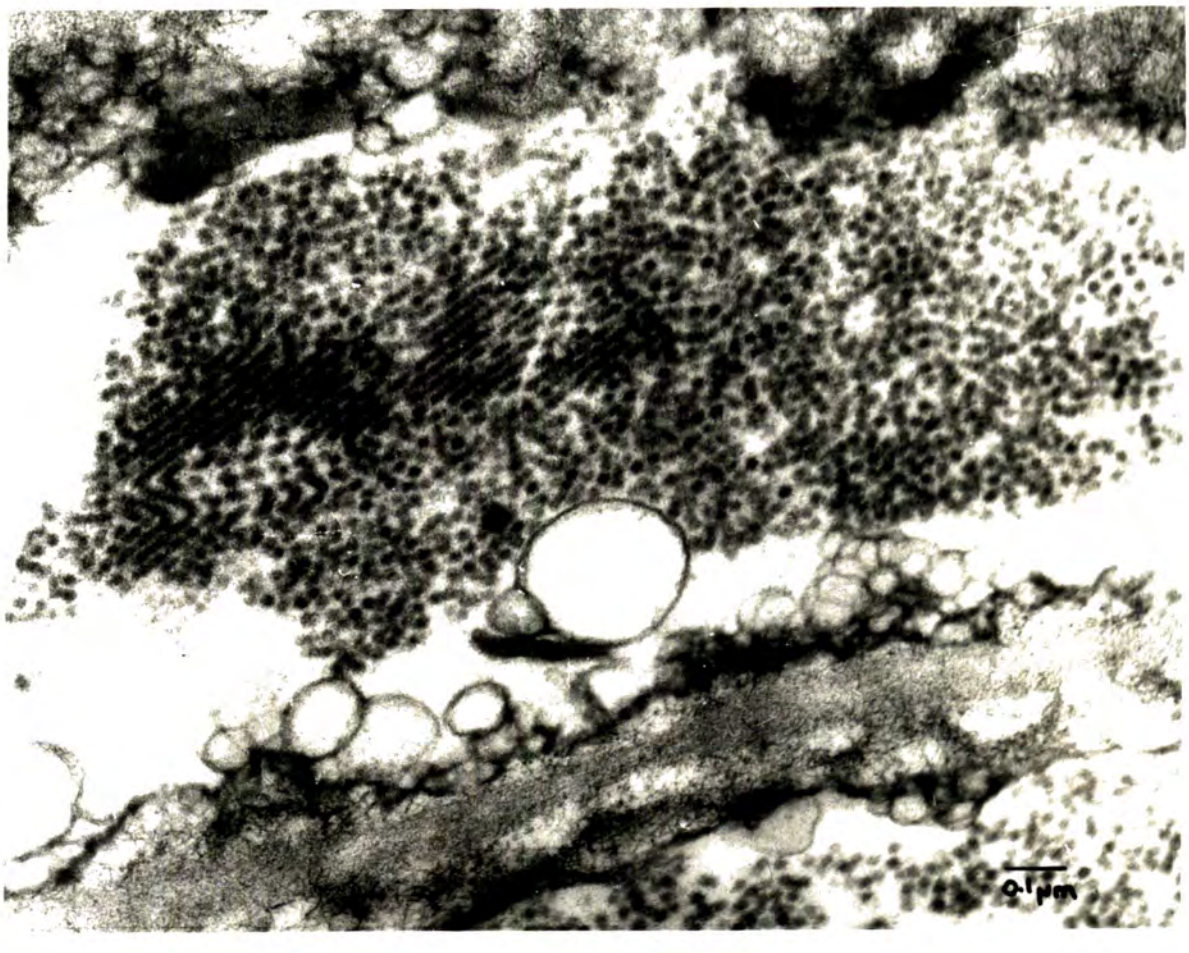
The virus crystals appeared in sections of the wilted leaves, either as cubic or hexagonal close-packed structures. Although crystalline arrays of intra-cellular virus have been observed in cells of host plants infected with isometric viruses (Edwardson et al., 1966 Gerola et al., 1965; Russo et al., 1968; Shikata and Maramorosch, 1966), no crystals of TYMV particles are normally seen in situ (Chalcroft and Matthews, 1966; Gerola et al., 1966; Milne, 1967).

Wherever the components of TYMV may be synthesised, Milne (1967) observed their completed virions only in the cytoplasm, and only exceptionally confined within vesicles or membranes. Martelli and Castellano (1969) noted that intra-nuclear occurrence of pelargonium leaf curl virus was not simply due to a redistribution of particles to equilibrate virus concentration throughout the cell. De Zoeten and Gaard (1969) seemed inclined to attribute the intra-nuclear occurrence of southern bean mosaic virus to particle translocation from the cytoplasm, rather than to in situ virus replication or assembly. Shikata and Maramorosch (1966) suggested that pea enation mosaic virus multiplied in nuclei of pea plants before invading the ctyplasm of infected cells.

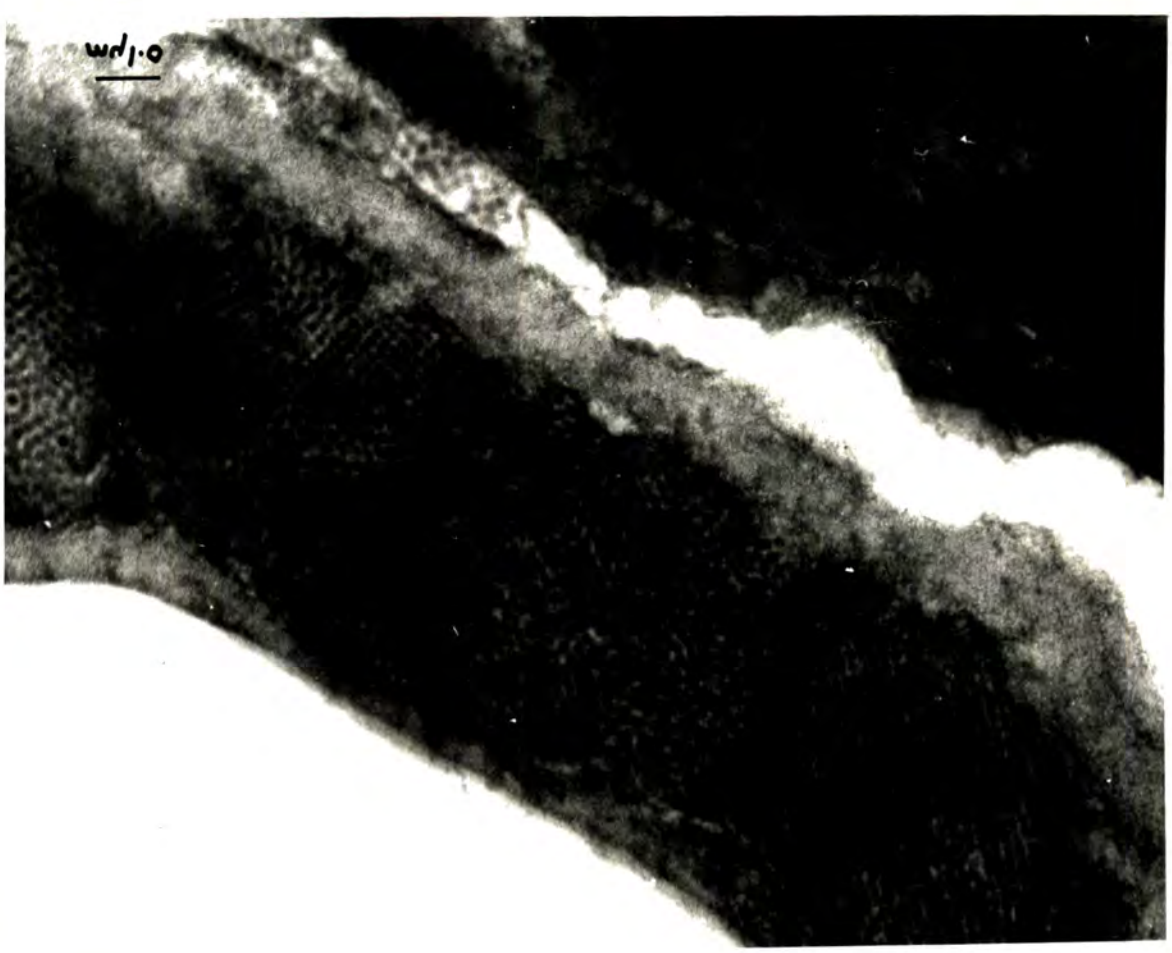
Kitajima and Costa (1969) noted an association of pepper ringspot

Fig. 47 Electron micrographs of Turnip Yellow Mosaic Virus systemically infected Brassica chinensis leaf material (after wilting, according to the procedure of Ushiyama, 1971) to show virus crystallisation(located in the spaces between the chloroplasts and in the general cytoplasm).

a.



b.



virus with mitochondria. The significance of this is uncertain, but there are some animal viruses in which mitochondria seem to be one of the centres of virus synthesis e.g., avian erythroblastic leukemia (Benedetti and Bernhard, 1958), avian myeloblastosis (Bonar et al., 1959) and rinderpest (Breese and De Boer, 1962). Whereas animal viruses appear within the mitochondria, pepper ringspot virus particles are arranged on the mitochondrial surface. This close topological relation between pepper ringspot and mitochondria might result from a high energy demand during the synthesis or assembly of this virus and dependence of some phase of viral synthesis on mitochondrial DNA is also possible.

In zinnia leaf tissue infected with dahlia mosaic virus numerous Golgi bodies (dictyosomes) and thick-walled microvesicles were observed by Kitajima et al., (1969) in close proximity to virus inclusions. The topological association of dahlia mosaic virus-infected inclusions with the Golgi complex suggests that the latter is involved in their formation. Kitajima and Lauritis (1969) observed the presence of intact virions within the plasmodesmata. Mature particles free in the cytoplasm were suggested to probably originate from inclusions, which are apparently the site of synthesis and assembly of this virus.

Electron microscopic work has therefore provided varied information on the position of virus particles within some plants, but the use of this information to specify points of virus synthesis, assembly etc., is open to question. If the results of such work, however, can be confirmed by biochemical observations, much may be learnt concerning these important topics.

In vitro experimental systems involving plant viruses have attracted great interest in recent years and have become so refined that much information has been gained about biological processes formerly considered inaccessible to study except *in vivo*.

It has been noted at various points throughout this thesis, however, that such *in vitro* experiments are not without their shortcomings. Reconstitution of a virus particle from its component parts does not establish *a priori* the mechanism of virus assembly *in vivo*, nor does protein synthesis *in vitro* necessarily reflect the *in vivo* mechanism. The isolated synthesis of viral protein *in vitro* tends to obscure the fact that protein synthesis, nucleic acid replication and other metabolic reactions are closely coupled processes *in vivo*. The separation of these biological processes often obfuscates their inter-relationship, and may lead to an apparent inactivity in any one of the systems. This may have been the case in the present work, in that vital factors for 'good' *in vitro* amino acid incorporation were missing. 'Good' activity is of course difficult to define, especially when dealing with systems derived from plant material, which tend to have more inherent problems than systems derived from other sources.

Although one must recognise the artificiality of the *in vitro* experiment, and that it often may not reflect accurately on living processes, it is equally apparent that many valuable facts concerning a variety of biological reactions can be discovered.

The advantage of using defined components in studying a biological reaction is self-evident, and the present work was based on

this concept. The attempt to produce an active incorporating system from Chinese cabbage, programmed by TYMV RNA was successful in that the system was partially characterised. The use of this system, with related observations, to determine biochemical aspects of the infection process has only been partially successful, but has suggested many avenues for investigation along the way. It has been tentatively proposed that the system isolated indicates the participation of the '80 S' ribosome in viral protein synthesis and that TYMV host specificity does not appear to be at the protein biosynthetic level.

Further purification of the system components and isolation and characterisation of the system product(s) would undoubtedly shed light on the feasibility of comparing the observations made on the in vitro system to the actual in vivo state.

It is to be hoped that this and similar work will be continued as knowledge evolving from this type of investigation and from similar experiments is of fundamental importance to all branches of virology and in fact to biology in general. The information obtained deals with such problems as the replication, transcription and translation of genetic material, the regulation of these processes and the generation of biologically active structures. Results from such work are applicable to a multitude of problems being investigated today.

APPENDIX

Analytical Ultracentrifugal Analysis:

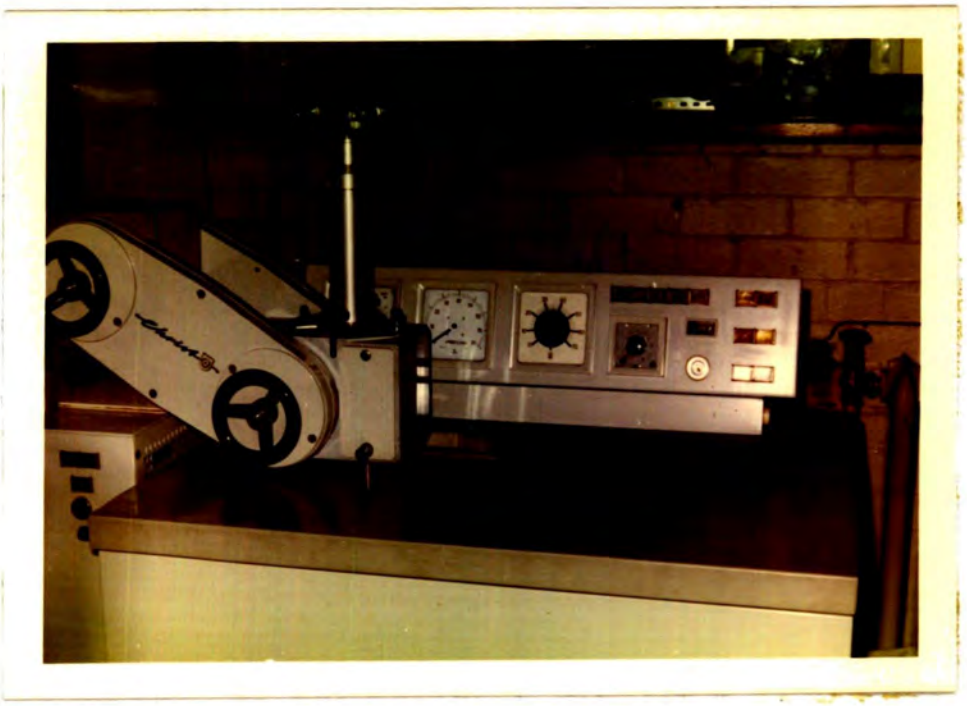
Analytical ultracentrifugal analysis was performed using an Omega II Preparative Ultracentrifuge with the Analytical Attachment, standard Analytical Rotor and standard Analytical Cell.

The Schlieren optical system, normally an integral part of the analytical ultracentrifuge, is used in this system as an accessory with an analytical rotor, for both direct observation and photography of boundary movements of macromolecules, in the preparative Omega II ultracentrifuge. With this analytical accessory attached, sedimentation velocity studies for the determination of sedimentation coefficients may be performed.

The Schlieren optical accessory is attached to the side of the ultracentrifuge (fig. 48) and is swung into position on two twin-arm parallelogram suspensions, supported on hinge points. This ensures the perfectly vertical position of the assembly. The 'optical box' contains all the necessary components for photographic recording of the sedimentation process (i.e., water cooled mercury high-pressure light source, lenses, Schlieren diaphragm and camera). Deflection of the light beam on to the camera is accomplished by pre-adjusted deflection mirrors, bolted to the base of the centrifuge chamber, prior to rotor installation. Round plane-parallel quartz covers protect the deflection mirrors from condensation.

Assembly of the cell and installation in the rotor are performed as with similar analytical apparatus (Omega accessories being almost

Fig. 48 Omega II Preparative Ultracentrifuge with Analytical Attachment.



identical with comparable Beckman Spinco E apparatus). The standard Omega analytical rotor is similar to the Spinco Analytical-D-rotor, and has two cell holes. In the Omega rotor, one hole houses the analytical cell, the other the counterbalanced reference cell, which produces the reference image. The standard analytical cell was used in conjunction with a 12 mm. thick, aluminium, single-sector centre-piece (of 4^{o} sector angle) and plain quartz windows.

Using this apparatus, for an average sample, suitable light intensity settings and exposure times were determined. By experiment (fig. 49) rotor acceleration was found to be uniform over most of the range from zero to the required rotor speed. For all determinations the recommended Schott OG 530 filter was used in the optical system.

Once the pre-selected speed is reached, a bright rectangular central field can be observed in the viewer of the reflex camera attachment, which is formed by the light beams passing the analytical cell. Right and left of the central field there are wide black stripes produced by the covers of the reference holes and in between bright bands of light from the right and left-hand reference holes of the counterbalance cell (fig. 50). The edge of the right-hand light band forms the reference line of the meniscus (RKM). The distance between rotor centre (RZ) and this line is 5.700 cm. The boundary zone between air and fluid inside the cell is seen as a vertical line, the meniscus (M). Once a certain centrifugation period has elapsed the gradient starts separating from the meniscus in the direction of centrifugation, to the bottom of the cell (B) in the shape of a Gaussian distribution curve. The solvent is to the right of the gradient, and to the left the plateau zone. The

Fig.49 . Acceleration curve of the analytical rotor in the Omega II analytical ultracentrifuge

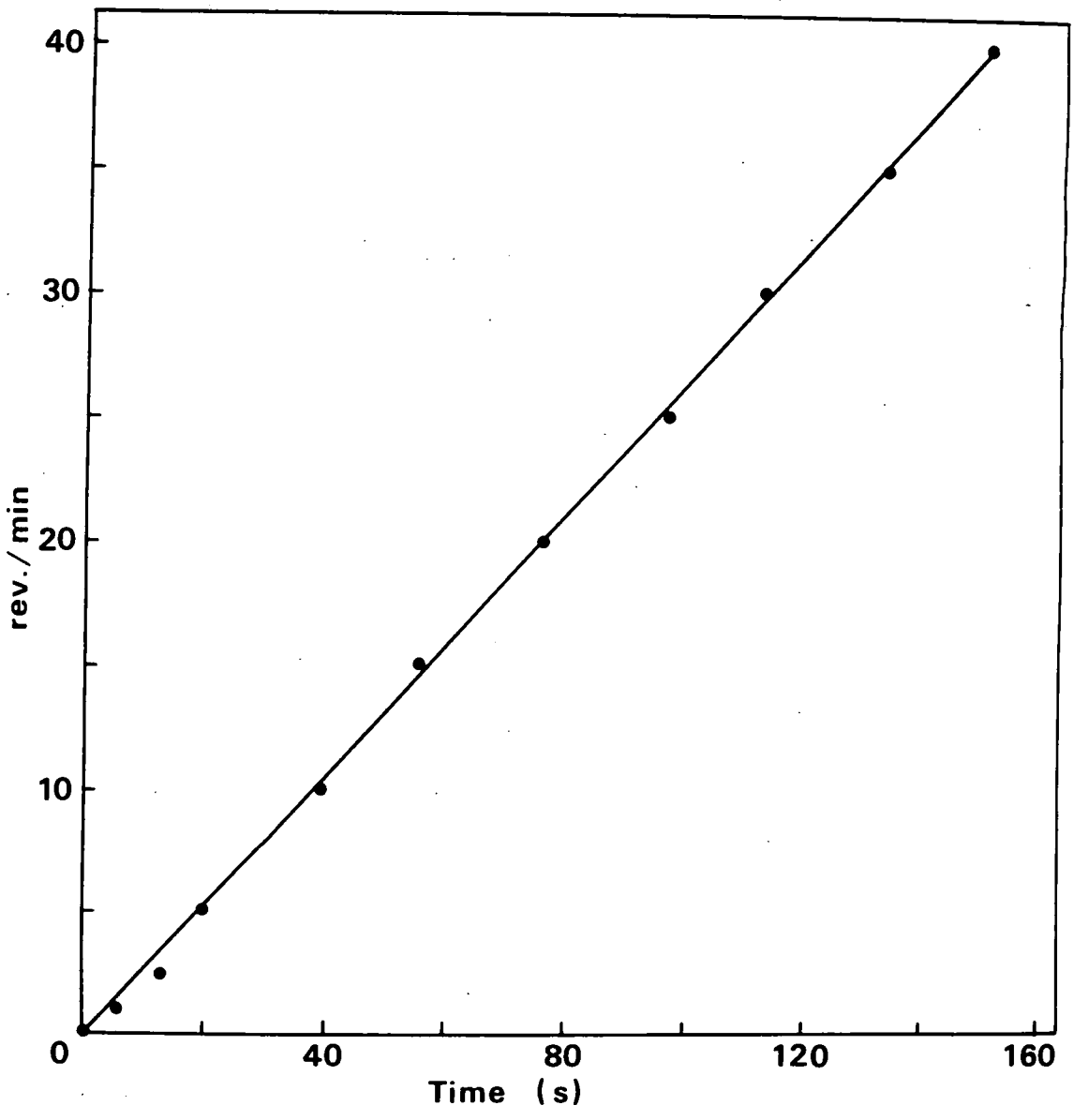
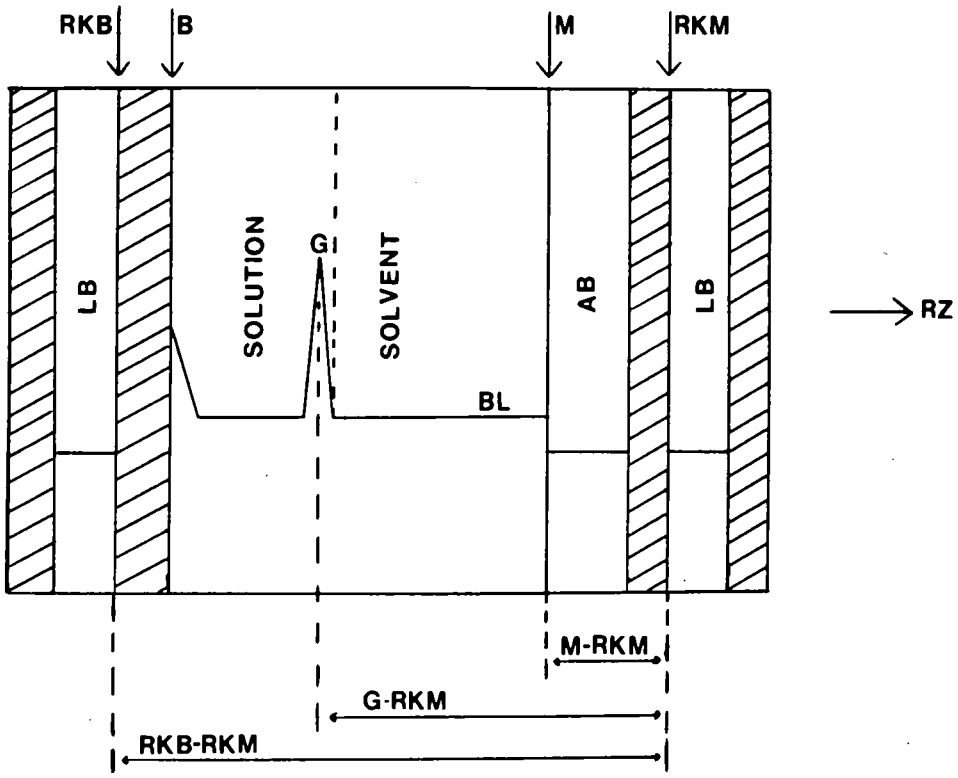


Fig. 50. Diagrammatic representation of image viewed through the reflex attachment of the camera affixed to the Omega analytical ultracentrifuge



Direction of sedimentation is from right to left.

AB	air bubble	M	solution meniscus
B	cell bottom	RKB	reference edge bottom
BL	base line	RKM	reference edge
G	gradient	RZ	rotor center
LB	light band(reference cell)		

increase in thickness of the base line at the cell bottom (B) is due to deposition of sedimenting material. Left of the plateau zone is another light band bordered by two black bars. The left-hand edge of this light band (RKB) forms the reference line. The distance between RKB and the rotor centre is 7,300 cm.

NOTES ADDED IN PROOF:

Tobacco mosaic virus (Öberg and Philipson, 1972) and bromegrass mosaic virus (Hall et al., 1972) can enzymatically bind histidine and tyrosine respectively to the 3' end of the molecule. No aminoacylation was observed with poliovirus RNA (Öberg and Philipson, 1972). Sela (1972) demonstrated that fragments of TMV RNA can accept serine and methionine, but it appears that acylation cannot take place on the intact molecule. It is suggested that aminoacylation may be a regulatory function or may be involved in the recognition of viral RNA replicase (Littauer and Inouye, 1973).

A review on protein synthesis by Haselkorn and Rothman-Denes (1973) has been published covering material published, for the most part, subsequent to the review on protein synthesis by Lucas-Lenard and Lipmann, (1971).

Hall, T.C., Shih, D.S., Kaesberg, P. (1972) Biochem. J. 129 969

Haselkorn, R., Rothman-Denes, L.B. (1973) A. Rev. Biochem. 42 397

Littauer, U.Z., Inouye, H., (1973) A. Rev. Biochem. 42 439

Lucas-Lenard, J., Lipmann, F., (1971) A. Rev. Biochem. 40 409

Öberg, B., Philipson, L., (1972) Biochem. biophys. Res. Commun 48 927

Sela, I., (1972) Virology 49 90

BIBLIOGRAPHY

Abbreviations of journal titles are based on those listed in "Abbreviated Titles of Biological Journals" 3rd edn. (1968) issued by The Biological Council (Inst. Biol., London).

Aach, H.G., Funatsu, G., Nirenberg, M.W., Fraenkel-Conrat, H. (1964) Biochemistry, N.Y. 3 1362

Agol, V.I. (1964) Vop. Virusol. 9 718

Albrecht, J., Hoogendam, B.W., Rozenboom, W., Verhoef, N.J., Voorma, H.O., Bosch, L. (1969) Biochim. biophys. Acta 190 504

Albrecht, J., Rozenboom, W., Vermeer, C., Bosch, L. (1969a) FEBS Lett. 5 313

Allende, C.C., Allende, J.E., Gatica, M., Celis, J., Mora, G., Matamala, M. (1966) J. biol. Chem. 241 2245

Allende, J.E., (1969) In "Techniques in Protein Biosynthesis". Vol. 2. (Ed. Campbell, P.N. and Sargent, J.R.) pp 55-100 Academic Press Inc., New York.

Allende, J.E., Bravo, M. (1966) J. biol. Chem. 241 5813

Allende, J.E., Mora, G., Gatica, M., Allende, C.C. (1965) J. biol. Chem 240 PC 3229

Ames, B.N., Hartman, P.E. (1963) Cold Spring Harb.Symp. quant. Biol. 28 349

Anderer, F.A., Uhling, H., Weber, E., Schramm, G. (1960) Nature, Lond. 186 922

Anderson, L.A., Smillie, R.M. (1966) Biochem. biophys. Res. Commun. 23 535

Anderson, M.B., Cherry, J.H. (1969) Proc. natn. Acad. Sci. U.S. 62 202

Anderson, W.F., (1969) Proc. natn, Acad. Sci. U.S. 62 566

Anfinsen, C.B., (1967) In "Harvey Lectures Ser. 61" pp. 95-116. Academic Press Inc., New York.

App, A.A. (1969) Pl. Physiol. 44 1132

App, A.A. Jagendorf, A.T., (1963) Biochim, biophys. Acta 76 286

App, A.A. Jagendorf, A.T. (1964) Pl. Physiol. 39 772

Arcà, M., Calvori, C., Frontali, L., Tecce, G. (1963) Biochem. biophys. Res. Commun. 10 117

Arglebe, C., Hall, T.C., (1969) Pl. Cell Physiol. 10 171

Arlinghaus, R.G., Schaeffer, J., Schweet, R.S. (1964) Proc. natn. Acad. Sci. U.S. 51 1291

Armstrong, D.J. (1966) Proc. natn. Acad. Sci. U.S. 56 64

Arnstein, H.R.V. (1969) In "Subcellular Components. Preparation and Fractionation". (Ed. Birnie, G.D. and Fox, S.M.) pp 109 - 115 Butterworths, London

Arnstein, H.R.V., Rahamimoff, H. (1968) Nature, Lond. 219 942

Astier-Manifacier, S., Cornuet, P. (1964) Compt. Rend. 259 4401

Astier-Manifacier, S., Cornuet, P. (1965a) Biochem. biophys. Res. Commun. 18 283

Astier-Manifacier, S., Cornuet, P. (1965b) Ann. Epiphyties 16 13

Astier-Manifacier, S., Cornuet, P. (1966a) Bull. Soc. Franc. Physiol. Vegetale 12 20

Astier-Manifacier, S., Cornuet, P. (1966b) Ann. Epiphyties 17 191

Astier-Manifacier, S., Cornuet, P. (1971) Biochim. biophys. Acta 232 484

Atabekov, J.G., Novikov, V.K., Vishnichenko, V.K., Javakhia, V.G. (1970) Virology 41 108

Atherly, A.G., Imsande, J. (1967) Biochim. biophys. Acta 145 491

Attardi, G. (1967) A. Rev. Microbiol. 21 383

Axel, R., Weinstein, I.B., Farber, E. (1967) Proc. natn. Acad. Sci. U.S. 58 1255

Ayuso, M.S., Heredia, C.F. (1968) Eur. J. Biochem. 7 111

Babos, P. (1966) Virology 28 282

Babos, P., Kassanis, B. (1962) Virology 18 206

Babos, P., Shearer, G.B., (1969) Virology 39 286

Bagi, G., Farkas, G.L. (1967) Phytochemistry 6 161

Bailey, L.H. (1949) (1961) In "Manual of Cultivated Plants" 2nd Ed. 1961 p 438 Macmillan Co., New York

Balandin, I.G., Khoroshutina, E.B., Tongur, V.S., (1964) Dokl. - Biochem. Sect. (English Transl.) 155 201

Bald, J.G., (1964) Virology 22 377

Bald, J.G., Solberg, R.A. (1961) Nature, Lond. 190 651

Baliga, B.S., Pronczuk, A.W., Munro, H.N. (1968) J. molec. Biol. 34 199

Barker, G.R., Rieber, M. (1966) Biochem, J. 98 4p

Barker, G.R., Rieber, M. (1967) Biochem. J. 105 1195

Barondes, S.H., Nirenberg, M.W. (1962) Science, N.Y. 138 810

Bawden, F.C. (1957) In "Ciba Found. Symp. Nature Viruses" pp 170 - 190

Bawden, F.C. (1964) "Plant Viruses and Virus Diseases" 4th Ed. Ronald Press, New York

Bawden, F.C. (1966) Ann. appl. Biol. 58 1

Bawden, F.C., Kleczkowski, A. (1955) J. gen. Microbiol. 13 370

Bawden, F.C., Kleczkowski, A. (1960) Virology 10 163

Beer, S.V., Kosuge, R. (1970) Virology 40 930

Belli, G. (1969) Private communication

Benedetti, E.L., Bernhard, W. (1958) J. Ultrastruct. Res. 1 309

Benedetti, E.L.,Bont, W.S., Bloemendal, H. (1966) Lab. Invest. 15 196

Berg, P. (1961) A. Rev. Biochem. 30 293

Berg, P., Ofengand, E.J. (1958) Proc. natn, Acad. Sci. U.S. 44 78

Betheil, J.J., Arnstein, H.R.V. (1965) Biochem. biophys. Res. Commun. 21 323

Bhaduri, S., Chatterjee, N.K., Bose, K.K., Gupta, N.K., Biochem. biophys. Res. Commun. 40 402 (1970)

Birbeck, M.S.C., Mercer, E.H. (1961) Nature, Lond. 189 558

Bishop, D.H.L., Claybrook, J.R., Spiegelman, S. (1967) J. molec. Biol. 26 373

Bishop, J., Favelukes, G., Schweet, R., Russell, E. (1961) Nature, Lond. 191 1365

Bishop, J., Leahy, J., Schweet, R. (1960) Proc. natn. Acad. Sci. U.S. 46 1030

Bichowsky-Slomnicki, L. (1969) Biochem. biophys. Res. Commun. 37 750

Biswas, B.B. (1969) Arch. Biochem. Biophys. 132 198

Black, L.M. Markham, R. (1963) Neth. J. Plant Pathol. 69 215

Blair, C.D., Robinson, W.S. (1968) Virology 35 537

Blobel, G., Potter, V.R., (1967) J. molec. Biol. 26 279

Bloemendal, H., Bont, W.S., Benedetti, E.L. (1964) Biochim. biophys. Acta 87 171

Bont, W.S.,
Bloemendal, H., de Vries, M., Benedetti, E.L. (1967) Biochem. J. 103 177

Boardman, N.K., Francki, R.I.B., Wildman, S.G., (1965) Biochemistry, N.Y. 4 872

Boardman, N.K., Francki, R.I.B., Wildman, S.G. (1966) J. molec. Biol. 17 470

Bockstahler, L.E., (1967) Molec. Gen. Genetics 100 337

Boedtker, H. (1960) J. molec. Biol. 2 171

Boedtker, H., Stumpp, S. (1964) Biochem. biophys. Acta 87 247

Boezi, J.A., Armstrong, R.L., De Backer, M. (1967) Biochem. biophys. Res. Commun. 29 281

Bollard, E.G., Matthews, R.E.F., (1966) In "Plant Physiology. A Treatise" Vol. IV B. (Ed. Steward, F.C.) pp 417-550 Academic Press Inc. New York

Bonar, R.A., Parsons D.F., Beaudreau, G.S., Becker, C. Beard, J.W., (1959) J. natn. Cancer Inst. 23 199

Borges, M de L.V. (1947) Agron. Lusitana 9 253

Bosch, L. (1967) In "Regulation of Nucelic Acid and Protein Biosynthesis" (Ed. Koningsberger, V.V. and Bosch, L.)pp 259-271. Elsevier, Amsterdam

Bosch, L., van Knippenberg, P.H., Voorma, H.O., van Ravenswaay Claasen, J.C., (1966) In "Viruses of Plants" (Ed. Beemster, A.B.R. and Dijkstra, J.) pp 275 - 294 North Holland Publishing Co. Amsterdam

Boulter, D. (1965) In "Biosynthetic Pathways of Higher Plants" (Ed. Pridham, J.B. and Swain, T.) pp 101 - 115 Academic Press Inc., New York.

Boulter, D. (1970) A. Rev. Pl. Physiol. 21 91

Bramhall, S., Noack, N., Wu, M., Loewenberg, J.R. (1969) Analyt. Biochem 31 146

Bratt, M.A., Robinson, W.S. (1967) J. molec. Biol 23 1

Breese, S., De Boer, C. (1962) Proc. 5th. Int. Conf. Electron Microscopy 2 V-7

Brenner, S., Stretton, A.O.W., Kaplan, S. (1965) Nature, Lond. 206 994

Bresler, S., Grajevskaja, R., Kirilov, S., Saminski, E. (1968) Biochim. biophys. Acta 155 465

Bretscher, M.S. (1963) J. molec. Biol. 7 446

Bretscher, M.S. (1965) J. molec. Biol. 12 913

Bretscher, M.S. (1968) Nature, Lond. 220 1088

Bretscher, M.S., Grunberg-Manago, M. (1962) Nature, Lond. 195 283

Bretscher, M.S., Marcker, K.A. (1966) Nature, Lond. 211 380

Bretthauer, R.K., Marcus, L. Chaloupka, J., Halvorson, H.O., Brock, R.M. (1963) Biochemistry, N.Y. 2 1079

Broadbent, L., Heathcote, G.D. (1958) Ann. appl. Biol. 46 585

Brock, T.D. Moo-Penn G. (1962) Arch. Biochem. Biophys. 98 183

Brown, D.H., Novelli, G.D., (1968) Biochem. biophys. Res. Commun. 31 262

Brown, G.L. (1963) In "Progress in Nucelic Acid Research". Vol. 2 (Ed. Davidson, J.N. and Cohn, W.E.) pp 259-310. Academic Press Inc., New York

Brown, G.N. (1969) Pl. Physiol. 44 272

Brown, L., Zubay, G., (1960) J. molec. Biol. 2 287

Brownhill, T.J., Jones, A.S., Stacey, M. (1959) Biochem. J. 73 434

Bruening, G., Agrawal, H.O. (1967) Virology 32 306

Bruskov, V.I., Kiselev, N.A. (1968a) J. molec. Biol. 37 367

Bruskov, V.I., Kiselev, N.A. (1968b) J. molec. Biol. 38 443

Bubela, B., Holdsworth, E.S. (1966) Biochim. biophys. Acta 123 376

Buck, C.A., Granger, G.A., Taylor, M.W., Holland, J.J. (1967) Virology 33 36

Bukrinskaya, A.G., Bykovsky, A.P.H., Zhdanov, V.M. (1969) Virology 39 705

Burdon, R.H., Billeter, M.A., Weissmann, C., Warner, R.C., Ochoa, S., Knight, C.A. (1964) Proc. natn. Acad. Sci. U.S. 52 768

Byfield, J.E., Lee, Y.C., Bennett, L.R. (1969) Biochem. biophys. Res. Commun. 37 806

Byfield, J.E., Scherbaum, O.H. (1966) Life Sci. 5 2263

Byfield, J.E., Scherbaum, O.H. (1967a) Proc. natn. Acad. Sci. U.S. 57 602

Byfield, J.E., Scherbaum, O.H. (1967b) J. Cell Physiol. 70 265

Byfield, J.E., Scherbaum, O.H. (1967c) Science, N.Y. 156 1504

Cadman, C.H. (1962) Nature, Lond. 193 49

Campagnoni, A.T., Mahler, H.R. (1967) Biochemistry, N.Y. 6 956

Campbell, P.N., Kernot, B.A., (1962) Biochem. J. 82 262

Campbell, P.N., Lawford, G.R. (1968) In "Structure and Function of the Endoplasmic Reticulum in Animal Cells". (Ed. Gran, F.C.) p. 57. Academic Press Inc., New York

Campbell, P.N., Lowe, E., Serck-Hanssen, G. (1967) Biochem. J. 103 280

Campbell, P.N., Sargent, J.R. (1967) In "Techniques in Protein Biosynthesis". Vol. 1 (Ed. Campbell, P.N. And Sargent, J.R.) pp 1-63, 301 Academic Press Inc., New York

Canning, L., Griffen, A.C. (1965) Biochim, biophys. Acta 103 522

Cannon, M. (1967) Biochem. J. 104 934

Capecchi, M.R., (1966) Proc. natn. Acad. Sci. U.S. 55 1517

Capecchi, M.R., (1966) J. molec. Biol. 21 173

Capecchi, M.R., (1967) Proc. natn. Acad. Sci. U.S. 58 1144

Carnegie, J.W., Deeney, A.O'C., Olson, K.C., Beaudreau, G.S., (1969) Biochim. biophys. Acta 190 274

Carter, W.A., Levy, H.B. (1967) Archs. Biochem. Biophys. 120 563

Carter, W.A., Levy, H.B., (1968) Biochim, biophys. Acta 155 437

Caskey, C.T., Tompkins, R., Scolnick, E., Caryk, T., Nirenberg, M. (1968) Science, N.Y. 162 135

Chalcroft, J.P., Matthews, R.E.F., (1966) Virology 28 555

Chalcroft, J.P., Matthews, R.E.F., (1967a) Virology 33 167

Chalcroft, J.P., Matthews, R.E.F., (1967b) Virology 33 659

Chao, F.C. (1957) Arch. Biochem. Biophys. 70 426

Chen, D., Sarid, S., Katchalski, E. (1968) Proc. natn. Acad. Sci. U.S. 60 902

Cheng, P.Y. (1959) Proc. natn. Acad. Sci. U.S. 45 1557

Chibnall, A.C. (1924) Biochem.J. 18 387

Choi, Y.S., Carr, C.W., (1967) J. molec. Biol. 25 331

Clark, B.F.C., Marcker, K.A., (1966a) J. molec. Biol. 17 394

Clark, B.F.C., Marcker, K.A., (1966b) Nature, Lond. 211 378

Clark, J.M. jun. unpublished. Reported on p.308, Reichmann, M.E. and Clark, J.M. jun. (1968) A. Rev. Phytopathology 6 295

Clark, J.M. jr., Chang, A.Y., Spiegelman, S., Reichmann, M.E. (1965) Proc. natn. Acad. Sci. U.S. 54 1193

Clark, M.F. (1964) Biochim, biophys. Acta 91 671

Clark, M.F., Matthews, R.E.F., Ralph, R.K. (1964) Biochim, biophys. Acta 91 289

Claude, A. (1941) Cold Spring Harb. Symp. quant. Biol. 9 263

Cleland, W.W. (1964) Biochemistry, N.Y. 3 480

Cobb, A. (1970) Private communication

Cochran, G.W., (1963) Symp. Fundamental Cancer, Res., 17th Houston, 252

Cochran, G.W., Fister, G.M., Shigematsu, A., Smart, R.A. Le Grande Shupe, J., Chidester, J.L., (1969) Nature, Lond. 224 1312

Cocking, E.C., Pojnar, E. (1968) J. gen. Virol. 2 317

Cohen, S.S., Lichtenstein, J. (1960) J. biol. Chem. 235 2112

Cohen, S.S., Morgan, S., Streibel, E. (1969) Proc. natn. Acad. Sci. U.S. 64 669

Cohen, S.S., Schachman, H.K. (1957) Virology 3 575

Colbourn, J.L., Witherspoon, B.H., Herbst, E.J., (1961) Biochim, biophys. Acta. 49 422

Coleman, G. (1969) Nature, Lond. 222 666

Commoner, B., Yamada, M., Rodenberg, S.D., Wang, T-Y., Basler, E. (1953) Science, N.Y. 118 529

Cooper, W.K., Muranatsu, K., Wannemacher, R.W. (Jr.)(1968) Biochim. biophys. Acta 169 269

Cornuet, P., Astier-Manifacier, S. (1962) Compt. Rend. 255 3076

Cosentino, V., Paigen, K., Steere, R.L. (1956) Virology, 2 139

Cotter, R.I., McPhie, P., Gratzer, W.B., (1967) Nature, Lond. 216 864

Craven, G.R., Kurland, C. (1969) Unpublished. Reported on p. 273
Lengyel, P. and Söll, D. (1969) Bact. Rev. 33 264

Crick, F.H.C. (1957) Biochim, Soc. Symp. 14 25

Crick, F.H.C., (1958) Symp. Soc. exp. Biol. 12 138

Croxhall, H.E., Gwynne, D.C., Broadbent, C. (1953) Pl. Path. 2 122

Culp, W.J., Mosteller, R.D., Hardesty, B. (1968) Arch. Biochem, Biophys. 125 658

Cundecliffe, E. (1968) Biochem. biophys. Res. Commn. 33 247

Dahlberg, J.E., Haselkorn, R. (1965) Science, N.Y. 149 78

Dahlberg, J.E., Haselkorn, R. (1966) Proc. natn. Acad. Sci. U.S. 55 1269

Dahlberg, J.E., Haselkorn, R. (1967) J. molec. Biol. 24 83

Daniel, V., Sarid, S., Littauer, U.Z. (1968) Proc. natn. Acad. Sci. U.S. 60 1403

Darnell, J.E., Sawyer, T.K., (1960) Virology 11 665

Davie, E.W., Koningsberger, V.V., Lipmann, F. (1956) Arch. Biochem. Biophys. 65 21

Davies, E., Maclachlan, G.A. (1969) Arch. Biochem. Biophys. 129 581

Davies, J. (1966) Cold Spring Harb. Symp. quant. Biol. 31 665

Davies, J., Gilbert, W., Gorini, L. (1964) Proc. natn. Acad. Sci. U.S. 51 883

Davies, J.W., Cocking, E.C. (1967) Biochem, J. 104 23

Davies, P., Barry, R.D. (1966) Nature, Lond. 211 384

de Duve, C. (1964) J. theor. Biol. 6 33

De Groot, N., Kaufmann, Y., Shafrir, I. (1967) Biochem. biophys. Res. Commun. 26 691

De Jager, C.P. van Kammen, A. (1970) Virology 41 281

Demoss, J.A., Genuth, S.M., Novelli, G.D. (1956) Proc. natn. Acad. Sci. U.S. 42 325

Devenuto, F., Lange, R.J.G. (1967) Biochim. biophys. Acta 134 443

Devi, A., Sarkar, N. (1963) Biochim. biophys. Acta 68 254

De Zoeten, G.A, Gaard, G. (1969) J. Cell Biol. 40 814

De Zoeten, G.A., Schlegel, D.E., (1967) Virology 32 416

Diener, T.O. (1961) Virology 14 177

Diener, T.O. (1971) Virology 45 411

Diener, T.O., Jenifer, F.G., (1964) Phytopathology 54 1258

Diener, T.O., Weaver, M.L. (1959) Virology 8 531

Dintzis, H.M., (1961) Proc. natn. Acad. Sci. U.S. 47 247

Doctor, B.P., Apgar, J., Holley, R.W. (1961) J. biol. Chem. 236 1117

Doctor, B.P., Mudd, J.A. (1963) J. biol. Chem. 238 3677

Doi, R.H., Kaneko, I., Igarashi, R.T., (1968) J. biol. Chem. 243 945

Downey, K.M., So, A.G., Davie, E.W. (1965) Biochemistry, N.Y. 4 1702

Drijfhout, E. (1968) Euphytica 17 224

Dunn, D.B., (1965) Communications of the 2nd FEBS Symp. Vienna p. 275

Dunn, D.B., (1969p) Private communication

Dunn, D.B., (1970) Private communication

Dunn, D.B., Hitchborn, J.H. (1965) Virology 25 171

Dunn, D.B., Hitchborn, J.H. (1966) Virology 30 598

Dunn, D.B., Hitchborn, J.H., Trim, A.R. (1963) Biochem, J. 88 34P

Edwardson, J.R., Purcifull, D.E., Christie, R.G., (1966) Can. J. Bot. 44 821

Eisenstadt, J.M., Brawerman, G. (1964) J. molec. Biol. 10 392

Eisenstadt, J.M., Brawerman, G. (1964) Biochim. biophys. Acta 80 463

Eisenstadt, J.M., Brawerman, G. (1967) Proc. natn. Acad. Sci. U.S. 58 1560

Eisenstadt, J.M., Lengyel, P. (1967) Science, N.Y. 154 524

Ellis, R.J., (1963) Nature, Lon. 200 596

Ellis, R.J., (1964) Phytochemistry 3 221

Ellis, R.J., (1969) Science, N.Y. 163 477

Ellis, R.J., MacDonald, I.R. (1967) Pl. Physiol. 42 1297

Elson, D. (1959) Biochim. biophys. Acta 36 372

Enders, J.F., Holloway, A., Grogan, E.A., (1967) Proc. natn. Acad. Sci. U.S. 57 637

Engelhardt, D.L., Robertson, H.D., Zinder, N.D. (1968) Proc. natn. Acad. Sci. U.S. 59 972

England, P.J., Denton, R.M., Randle, P.J., (1967) Biochem, J. 105 32C

Epler, J.L. (1969) Biochemistry, N.Y. 8 2285

Erbe, R., Nau, M., Leder, P. (1969) J. molec. Biol. 39 441

Erdmann, V.A., Thomas, G.A., Norton, J.W., Herbst, E.J. (1968) Biochim. biophys. Acta 157 43

Ertel, R., Brot, N., Redfield, B., Allende, J.E., Weissbach, H., (1968) Proc. natn. Acad. Sci. U.S. 59 861

Faed, Matthews, (1970) Unpublished Reported on p. 234 "Plant Virology" Matthews, R.E.F., Academic Press Inc., New York

Fan, D.P., Higa, A., Levinthal, C. (1964) J. molec. Biol. 8 210

Fedorcsák, I., Ehrenberg, L. (1966) Acta chem. scand. 20 107

Fedorcsák, I., Natarajan, A.T., Ehrenberg, L., Europ. J. Biochem. 10 450 (1969)

Fenner, F. (1968) "Biology of Animal Viruses Vol. 1. Molecular and Cellular Biology". Academic Press, New York.

Fenwick, M.L., Erikson, R.L., Franklin, R.M., (1964) Science, N.Y. 146 527

Fessenden, J.M., Moldave, K. (1962) Biochemistry, N.Y. 1 485

Fessenden, J.M., Moldave, K. (1963) J. biol. Chem. 238 1479

Fiers, W., Verplancke, H., van Styvendaele, B. (1967) In "Regulation of Nucleic Acid and Protein Biosynthesis" (Ed. Koningsberger, V.V. and Bosch, L.) Elsevier, Amsterdam pp. 154-166

Flamm, W.G., Birnstiel, M.L., (1964) Biochim. biophys. Acta 87 101

Fowden, L., Frankton, J.B. (1968) Phytochemistry, 7 1077

Fraenkel-Conrat, H., Singer, B. (1962) Biochemistry, N.Y. 1 120

Fraenkel-Conrat, H., Singer, B., Tsugita, A. (1961) Virology 14 54

Fraenkel-Conrat, H., Singer, B., Veldee, S. (1958) Biochim. biophys. Acta 29 639

Fraenkel-Conrat, H., Weissmann ,C., (1968) In "Molecular Basis of Virology" (Ed. Fraenkel-Conrat, H.) pp 209-221 Reinhold Book Corp.,New York

Francki, R.I.B., Matthews, R.E.F., (1962) Virology, 17 367

Fresco, J.R., Adams, A., Ascione, R., Henley, D., Lindahl, T. (1966) Cold Spring Harb. Symp. quant. Biol. 31 527

Friedman, S.M. (1968) Bact. Rev. 32 27

Friedman, S.M., Axel, R., Weinstein, I.B., (1967) J. Bact. 93 1521

Friedman, S.M., Berezney, R., Weinstein, I.B., (1968) J. biol. Chem. 243 5044

Friedman, S.M., Weinstein, I.B., (1964) Proc. natn. Acad. Sci. U.S. 52 988

Friedman, S.M., Weinstein, I.B., (1965) Biochem. biophys. Res. Commun. 21 339

Friedman, S.M., Weinstein, I.B., (1966) Biochim. biophys. Acta 114 593

Frost, R.R., Harrison, B.D., Woods, R.D., (1967) J. gen. Virol. 1 57

Fry, P.R., Matthews, R.E.F., (1963) Virology 19 461

Fuchs, E., Millette, R.L., Zillig, W., Walter, G., (1967) Europ. J. Biochem. 3 183

Fulton, R.W., (1951) Phytopathology 41 579

Fulton, R.W., (1967) Virology 32 153

Gale, E.F. (1963) Pharmac. Rev. 15 481

Gale, E.F., Folkes, J.P., (1953) Biochem. J. 53 493

Galper, J.B., Darnell, J.E. (1969) Biochem. biophys. Res. Commun. 34 205

Ganoza, M.C. (1966) Cold Spring Harb. Symp. quant. Biol. 31 273

Ganoza, M.C., Williams, C.A., Lipmann, F. (1965) Proc. natn. Acad. Sci. U.S. 53 619

Garen, A. (1968) Science N.Y. 160 149

Garren, L.D., Richardson, A.P., Crocco, R.M. (1967) J. biol Chem. 242 650

Gasior, E., Moldave, K. (1965) J. biol. chem. 240 3346

Gay, J.D., Kuhn, C.W., (1968) Phytopathology 58 1609

Geard, I.D. (1958) J. Aust. Inst. agric. Sci. 24 312

Gefter, M.L., Russell, R.L. (1969) J. molec. Biol. 39 145

Geiduschek, E.P., Haselkorn, R. (1969) A. Rev. Biochem. 38 647

Gerola, F.M., Bassi, M., Belli, G. (1965) Caryologia 18 353

Gerola, F.M., Bassi, M., Giussani, G. (1966) Caryologia 19 457

Ghosh, K., Grishko, A., Ghosh, H.P., (1971) Biochem. biophys. Res. Commun. 42 462

Gierer, A. (1963) J. molec. Biol. 6 148

Gierer, A., Schramm, G. (1956) Z. Naturf. 11b 138

Gigot, C., Philipps, G., Hirth, L. (1965) Bull. Soc. Chim. biol. 47 1667

Gilbert, W. (1963) J. molec. Biol. 6 389

Gilliland, J.M., Symons, R.H., (1968) Virology 36 232

Glitz., D.G. (1968) Biochemistry, N.Y. 7 927

Goffeau, A., Bové, J.M. (1965) Virology 27 243

Good, N.E., Winget, G.D., Winter, W., Connolly, T.N., Izawa, S., Singh, R.M.M. (1966) Biochemistry, N.Y. 5 467

Gordon, M.P., Smith, C. (1960) J. biol. Chem. 235 28pc

Gordon, M.P., Smith, C. (1961) J. biol. Chem. 236 2762

Gorini, L., Kataja, E. (1964) Proc. natn. Acad. Sci. U.S. 51 487

Grunberg-Manago, M., Dondon, J. (1965) Biochem. biophys. Res. Commun. 18 517

Gussin, G.N., (1966) J. molec. Biol. 21 435

Guthrie, C., Nomura, M., (1968) Nature, Lond. 219 232

Hadziyev, D., Mehta, S.L., Zalik, S. (1969) Can. J. Biochem. 47 273

Hall, B.D., Doty, P. (1959) J. molec. Biol. 1 111

Hall, T.C., Cocking, E.C. (1966) Biochim. biophys. Acta 123 163

Hall, T.C., Tao, K.L. (1970) Biochem. J. 117 853

Hamers-Casterman, C., Jeener, R. (1957) Virology 3 197

Hanson, J.B., Krueger, W.A., (1966) Nature, Lond. 211 1322

Harrison, B.D., (1958) J. gen. Microbiol. 15 210

Harrison, B.D., Nixon, H.L., (1959) J. gen. Microbiol. 21 591

Harrison, B.D., Roberts, I.M., (1968) J. gen. Virol. 3 121

Haruna, I., Nozu, K., Ohtaka, Y., Spiegelman, S., (1963) Proc. natn. Acad. Sci. U.S. 50 905

Haruna, I., Spiegelman, S., (1965) Proc. natn. Acad. Sci. U.S. 54 579

Haselkorn, R. (1962) J. molec. Biol. 4 357

Haselkorn, R. (1962b) In "Acids Ribonucleiques et Polyphosphates. Structure, synthese et Fonctions" Colloq. Intern. Centre Nat. Rech. Sci. (Paris) 106 489

Haselkorn, R. (1966) Private communication in Boedtker, H., Stumpp,S. Biochim. biophys. Acta 114 355

Haselkorn, R. (1969) Private communication

Haselkorn, R., Fried, V.A. (1964) Proc. natn. Acad. Sci. U.S. 51 308

Haselkorn, R., Fried, V.A., Dahlberg, J.E. (1963) Proc. natn. Acad. Sci. U.S. 49 511

Hay, J., Subak-Sharpe, H., Shepherd, W.M. (1967) Biochem. J. 103 69P

Hayashi, Y. (1962) Virology 18 140

Henshaw, E.C., Bojarski, T.B., Hiatt, H.H. (1963) J. molec. Biol. 7 122

Heywood, S.M., (1970) Nature, Lond. 225 696

Hiebert, E., Bancroft, J.B., Brakee, C.E. (1968) Virology, 34 492

Hills, G.J., Plaskitt, A. (1968) J. Ultrastruct. Res. 25 323

Hirai, A., Wildman, S.G., (1967) Virology 33 467

Hirai, A., Wildman, S.G., (1969) Virology 38 73

Hirai, T., Hirai, A., (1964) Science, N.Y., 145 589

Hirai, T., Nakagaki, Y., (1966) In "Viruses of Plants". (Ed. Beemster A.B.R., and Dijkstra, J.) pp. 90-93. North-Holland Publishing Co., Amsterdam.

Hirai, T., Wildman, S.G., (1963) Pl. Cell Physiol. 4 265

Hirschman, S.Z., Leng, M. Felsenfeld, G., (1967) Biopolymers 5 227

Hiruki, C. (1969) J. Virol. 3 498

Hiruki, C., Kaesberg, P., (1965) Phytopathology 55 1061

Hitchborn, J.H. (1968) J. gen. Virol. 3 137

Hoagland, M.B., (1960) In "The Nucleic Acids" Vol. 3. (Ed. Chargaff E. and Davidson, J.N.) p 349-408 Academic Press Inc. New York

Hoagland, M.B., Keller, E.B., Zamecnik, P.C. (1956) J. Biol. Chem. 218 345

Hoagland, M.B., Stephenson, M.L., Scott, J.F., Hecht, L.I., Zamecnik, P.C. (1958) J. biol. Chem. 231 241

Hoagland, M.B., Zamecnik, P.C., Stephenson, M.L. (1957) Biochim. biophys. Acta 24 215

Höfschneider, P.H., Hâusen, P. (1968) In "Molecular Basis of Virology" (Ed. Fraenkel-Conrat, H.) pp 169-208. Reinhold Book Corp., New York

Hogan, B.L.M., Korner, A., (1968) Biochim. biophys. Acta 169 129

Hogeboom, G.H., Schneider, W.C., Palade, G.E. (1948) J. biol.Chem. 172 619

Holden, M., Pirie, N.W., (1955) Biochem. J. 60 39

Holland, J.J. (1962) Virology 16 163

Holland, J.J., Hoyer, B.H., (1962) Cold Spring Harb. Symp. quant. Biol. 27 101

Holland, J.J., McLaren, L.C., Syverton, J.T., (1959) J. exp. Med. 110 65

Holland, J.J., Taylor, M.W., Buck, C.A. (1967) Proc. natn. Acad. Sci. U.S. 58 2437

Holley, R.W., Apgar, J., Doctor, B.P., Farrow, J., Marini, M.A., Merrill, S.H. (1961 a) J. biol. Chem. 236 200

Holley, R.W., Apgar, J., Everett, G.A., Madison, J.T., Marquisee, M., Merrill, S.H., Penswick, J.R., Zamir, A. (1965) Science N.Y. 147 1462

Holley, R.W., Brunngraber, E.F., Saad, F., Williams, H.H, (1961b) J. biol. chem. 236 197

Holley, R.W., Everett, G.A., Madison, J.T., Zamir, A. (1964) J. biol. Chem. 240 2122

Hoogendam, B.W., van Ravenswaay Claasen, J.C., Bosselaar, A., Voorma, H.O., Bosch, L., (1968) Biochim. biophys. Acta 157 579

Horiuchi, K., Lodish, H.F., Zinder, N.D., (1966) Virology 28 438

Howells, A.J., Wyatt, G.R., (1969) Biochim. biophys. Acta 174 86

Hsiao T.C.(1968) Pl. Physiol. 43 1355

Hsu, W.T., Foft, J.W., Weiss, S.B., (1967) Proc. natn. Acad. Sci. U.S., 58 2028

Hudson, J.B., Paranchych, W. (1968) J. Virol. 2 124

Hultin, T., (1950) Expl. Cell. Res. 1 376

Hultin, T. (1964) Int. Rev. Cytol. 16 1

Humm, D.G., Humm, J.H., (1968) Fed. Proc. 27 772

Huppert, J., Pelmont, J., (1962) Arch. Biochem. Biophys. 98 214

Hurwitz, C., Rosano, C.L., (1967) J. biol. Chem. 242 3719

Huston, R.L., Schrader, L.E., Honold, G.R., Beecher, G.R., Cooper, W.K., Sauberlich, H.E., (1970) Biochim. biophys. Acta 209 220

Hutchison, H.T., Hartwell, L.H., McLaughlin, C.S., (1969) J. Bact. 99 807

Ibuki, F., Moldave, K., (1968) J. biol. Chem. 243 791

Idriss, J.M., Halvorson, H.O., (1969) Arch. Biochem. Biophys. 133 442

Igarashi, K. , Kaji, A., (1967) Proc. natn. Acad. Sci. U.S. 58 1971

Igarashi, S., Paranchych, W., (1967) J. Virol. 1 529

Ilan, J., Ilan, J. (1969) Science, N.Y. 164 560

Ishida, T., Sueoka, N., (1966) J. biol. Chem. 243 5329

Israel, H.W., Ross, A.F. (1967) Virology 33 272

Jachymczyk, W.J. Cherry, J.H. (1968) Biochim. biophys. Acta 157 368

Jacob, F., Monod, J., (1961) J. molec. Biol. 3 318

Jacoli, G.G. , (1968) Can. J. Biochem. 46 1237

Jaspars, E.M.J., (1969) Private Communication

Jeener, R. (1954) Biochim. biophys. Acta 13 307

Jeener, R., Lemoine, P. (1953) Nature, Lond. 171 935

Jerez, C., Sandoval, A., Allende, J., Henes, C., Offengand, J., (1969) Biochemistry, N.Y. 8 3006

Johnson, M.S., Young, R.J., (1969) Virology 38 607

Johnson, M.W. (1964) Virology 24 26

Johnson, M.W., Markham, R. (1962) Virology 17 276

Jones, O.W., Townsend, E.E., Sober, H.A., Heppel, L.A., (1964) Biochemistry, N.Y. 3 238

Julian, G.R., (1965) J. molec. Biol. 12 9

Kaback, H.R., Stadtman, E.R., (1966) Proc. natn, Acad. Sci. U.S. 55 920

Kabat, D., Rich, A., (1969) Biochemistry, N.Y. 8 3742

Kaempfer, R., (1968) Proc. natn. Acad. Sci. U.S. 61 106

Kaempfer, R., (1969) Nature, Lond. 222 950

Kaempfer, R., Meselson, M., Raskas, H.J., (1968) J. molec. Biol. 31 277

Kaji, H., (1968) Biochemistry, N.Y. 7 3844

Kaji, H., Suzuka, I., Kaji, A. (1966) J. biol. Chem. 241 1251

Kalousek, F., Rychlik, I. (1965) Collń. Czech. chem. Commun. 30 3909

Kan, J., Kano-Sueoka, T., Sueoka, N., (1968) J. biol. Chem. 243 5584

Kaneko, I., Doi, R.H., (1966) Proc. natn. Acad. Sci. U.S. 55 564

Kano-Sueoka, T., Sueoka, N., (1966) J. molec. Biol. 20 183

Kaper, J.M., Siberg, R.A., (1969) Virology 38 407

Kaper, J.M., Steere, R.L., (1959a) Virology 7 127

Kaper, J.M., Steere, R.L., (1959b) Virology 8 527

Karasek, M., Schramm, G. (1962) Biochem. biophys. Res. Commun. 9 63

Kassanis, B. (1952) Ann. appl. Biol. 39 358

Kassanis, B., (1954) Ann. appl. Biol. 41 470

Kassanis, B., (1957a) Virology 4 187

Kassanis, B., (1957b) Advan. Virus. Res. 4 221

Kassanis, B. (1960) Virology 10 353

Kassanis, B., (1963) Advan. Virus, Res. 10 219

Kassanis, B., Bastow, C. (1971) J. gen. Virol. 11 157

Kassanis, B., Lebeurier, G. (1969) J. gen. virol. 4 385

Kassanis, B., McCarthy, D., (1967) J. gen. Virol. 1 425

Katz, E., Moss, B. (1969) J. Virol. 4 416

Keller, E.B., (1951) Fed. Proc. 10 206

Kempf, J., Mandel, P., (1968) Bull. Soc. Chim. biol. 50 773

Kerson, L.A., Garfinkel, D., Mildvan, A.S. (1967) J. biol. Chem. 242 2124

Khorana, H.G., Büchi, H., Ghosh, H., Gupta, N., Jacob, T.M., Kössel, H., Morgan, R., Narang, S.A., Ohtsuka, E., Wells, R.D. (1966) Cold Spring Harb. Symp. quant. Biol. 31 39

Kiho, Y. (1969) Japan. J. Microbiol. 13 312

Kim, Y.T., Wildman, S.G., (1962) Biochem. biophys. Res. Commun. 8 394

Kingsbury, D.W., (1966) J. molec. Biol. 18 204

Kirk, J.T.O., Tilney-Bassett, R.A.E. (1967) "The Plastids" Freeman and Co., London

Kitajima, E.W., Costa, A.S., (1969) J. gen. Virol. 4 177

Kitajima, E.W., Lauritis, J.A., (1969) Virology 37 681

Kitajima, E.W., Lauritis, J.A., Swift, H., (1969) Virology 39 240

Knight, C.A., (1947) J. biol. Chem. 171 297

Knowle, P., (1969) Biochim. biophys. Acta 190 496

Kolakofsky, D., Nakamoto, T., (1966) Proc. natn. Acad. Sci. U.S. 56 1786

Kolehmainen, L. , Zech, H.m Wettstein, D., (1965) J. Cell Biol. 25 77

Konings, R.N.H., Bloemendal, H., (1969) Europ. J. Biochem. 7 165

Korner, A. (1961) Biochem. J. 81 168

Kössel, H., (1968) Biochim, biophys. Acta 157 91

Krisko, I., Gordon, J., Lipmann, F., (1969) J. biol. Chem. 244 6117

Kubo, S. (1966) Virology 28 229

Kubo, S., Tomaru, K., Nitta, T., Shiroya, T., Hidaka, Z., (1965) Virology 26 406

Kućan, Z., Lipmann, F. (1964) J. biol. Chem. 239 516

Kurland, C.G., (1960) J. molec. Biol. 2 83

Kurland, C.G., (1966) J. molec. Biol. 18 90

Lado, P., Schwendimann, M. (1966) Ital. J. Biochem. 15 279

Laflèche, D., Bové, J.M., (1968) Compt. Rend. Acad. Sci. Paris 266 1839

Lamfrom, H., Grunberg-Manago, M. (1967) Biochem, biophys. Res. Commun. 27 1.

Langenberg, W.G., Schlegel, D.E., (1969) Virology 37 86

Last, J.A., Stanley, W.M., Salas, M.S., Hille, M.B., Wahba, A.J., Ochoa, S., (1967) Proc. natn. Acad. Sci. U.S. 57 1062

Lazzarini, R.A., (1966) Proc. natn. Acad. Sci. U.S., 56 185

Leaver, C.J., Key, J.L. (1967) Proc. natn. Acad. Sci. U.S. 57 1338

Lebeurier, G., Hirth, L., (1966) Virology 29 385

Lee, J.C. Ingram, V.M. (1967) Science, N.Y. 158 1330

Legocki, A., Wojciechowska, K., (1970) Bull. Acad. Polon. Sci. Sér. Sci. Biol. 18 7

Leis, J.P., Keller, E.B., (1970) Biochem. biophys. Res. Commun. 40 416

Lengyel, P., Söll, D. (1969) Bact. Rev. 33 264

Lengyel, P. , Speyer, J.F., Ochoa, S., (1961) Proc. natn. Acad. Sci. U.S., 47 1936

Leslie, R.A., Mansbridge, J.N., (1970) Biochem. J. 117 893

Lesnaw, J.A., Reichmann, M.E., (1970) Proc. natn. Acad. Sci. U.S. 66 140

Letendre, C.H., Humphreys, J.M., Grunberg-Manago, M. (1969) Biochim, biophys. Acta 186 46

Liao, S., Williams, Ashman, H.G., (1962) Proc. natn. Acad. Sci. U.S. 48 1956

Linskens, H.F., (1969) Planta 85 175

Lipmann, F., (1963) Prog. Nucleic Acid Res. 1 135

Lipmann, F., Hülsmann, W.C., Hartmann, G., Boman, H.G., Acs, G., (1959) J. cell. comp. Physiol. 54 75

Liquori, A.M., Costantino, L., Crescenzi, V., Elia, V., Giglio, E., Puliti, R., De Santis Savino, M., Vitagliano, V., (1967) J. molec. Biol. 24 113

Lister, R.M., (1968) J. gen. Virol. 2 43

Lister, R.M., Bancroft, J.B., (1970) Phytopathology 60 689

Littauer, U.Z., Sela, M., (1962) Biochim. biophys. Acta 61 609

Littlefield, J.W., Keller, E.B., Gross, J., Zamecnik, P.C., (1955) J. biol. Chem. 217 111

Lockhart, B.E.L., Semancik, J.S. (1968) Virology 36 504

Lodish, H.F., Robertson, H.D., (1969) J. molec. Biol. 45 9

Loeb, J.N., Howell, R.R., Tomkins, G.M., (1967) J. biol. Chem. 242 2069

Loening, U.E. (1967) Biochem. J. 102 251

Loening, U.E., (1968) J. molec. Biol. 38 355

Loening, U.E., (1969) Biochem. J. 113 131

Loening, U.E., Ingle, J., (1967) Nature, Lond. 215 363

Loftfield, R.B., Eigner, E.A. (1966) Biochim. biophys. Acta 130 426

Louie, R., Lorbeer, J.W., (1966) Phytopathology 56 1020

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, E.J. (1951) J. biol. Chem. 193 265

Lucas-Lenard, J., Haenni, A. (1968) Proc. natn. Acad. Sci. U.S. 59 554

Lucas-Lenard, J., Haenni, A., (1969) Proc. natn. Acad. Sci. U.S. 63 93

Lucas-Lenard, J., Lipmann, F., (1966) Proc. natn. Acad. Sci. U.S. 55 1562

Lyttleton, J.W., (1960) Biochem. J. 74 82

Lyttleton, J.W., (1962) Expl. Cell. Res. 26 312

Lyttleton, J.W., Matthews, R.E.F. (1958) Virology 6 460

McHale, J.S., Dove, L.D., (1968) New. Phytol. 67 505

McLaren, G.D., (1967) J. gen. Virol. 1 243

McLaughlin, C.S., Ingram, V.M., (1964) Science, N.Y., 145 942

Macquaire, G., (1965) Ann. Epiphyties 16 27

Madison, J.T., Everett, G.A., Kung, H.K., (1966) Cold Spring Harb. Symp. Quant. biol. 31 409

Malkin, L.I., Rich, A(1967) J. molec. Biol. 26 329

Manchester, K.L., (1970) Biochim, biophys. Acta 213 532

Mandel, H.G., Matthews, R.E.F., Matus, A.I., Ralph, R.K., (1964) Biochem. biophys. Res. Commun. 16 604

Mangiantini, M.T., Tecce, G., Toschi, G., Trentalance, A. (1965) Biochim. biophys. Acta 103 252

Mangiarotti, G., Schlessinger, D., (1966) J. molec. Biol. 20 123

Mans, R.J., (1966) In "Biochemistry of Chloroplasts" Vol II. (Ed. Goodwin T.W.) pp 351-369. Academic Press Inc. New York

Mans, R.J., (1967) A. Rev. Pl. Physiol. 18 127

Mans, R.J., Novelli, G.D., (1960) Biochem. biophys. Res. Commun. 3 540

Mans, R.J., Novelli, G.D., (1961) Arch. Biochem. Biophys. 94 48

Marcker, K., (1965) J. molec. Biol. 14 63

Marcker, K.A., Smith, A.E., (1969) Bull. Soc. Chim. biol. 51 1453

Marcus, A., (1969) Symp. Soc. Exptl. Biol. Symp. XXIII, 143

Marcus, A., (1970b) J. biol. Chem. 245 955

Marcus, A., (1970c) J. biol. Chem. 245 962

Marcus, A., Feeley, J., (1964) Proc. natn. Acad. Sci. U.S., 51 1075

Marcus, A., Feeley, J., (1965) J. biol. chem. 240 1675

Marcus, A., Feeley, J., Volcani, T., (1966) Pl. Physiol. 41 1167

Marcus, A., Weeks, D.P., Leis, J.P., Keller, E.B., (1970) Proc. natn. Acad. Sci. U.S., 67 1681

Marcus, L., Ris, H., Halvorson, H.O., Bretthauer, R.K., Bock, R.M., J. Cell Biol. 34 505 (1967)

Marcus, P.I., Salb, J.M. (1966) Virology 30 502

Markham, R. (1967) In "Methods in Virology" Vol. II (Ed. Maramorosch, K. and Koprowski, H.) p. 1-39. Academic Press Inc., New York.

Markham, R., Smith, K.M. (1949) Parasitology 39 330

Marshall, R.E., Nirenberg, M. (1969) Devl. Biol. 19 1

Martell, A.E., Schwarzenbach, G. (1956) Helv. chim. Acta 39 653

Martelli, G.P., Castellano, M.A., (1969) Virology 39 610

Martin, R.G., Ames, B.N. (1962) Proc. natn. Acad. Sci. U.S. 48 2171

Martyn, E.B., (1968) In "Plant Virus Names" (Ed. Martyn, E.B.) p.122 Commonwealth Mycological Inst. Kew, Surrey, England

Matsushita, K., (1965) Plant and Cell Physiol. 6 1

Matthaei, J.H., Jones, O.W., Martin, R.G., Nirenberg, M.W. (1962) Proc. natn. Acad. Sci. U.S. 48 666

Matthaei, J.H., Nirenberg, M.W.(1961) Proc. natn. Acad. Sci. U.S. 47 1580

Matthaei, H., Sander, G., Swan, D., Kreuzer, T., Caffier, H., Parmeggiani, A. (1968) Nátúrwissenschaften 55 281

Matthews, R.E.F, (1958) Virology 5 192

Matthews, R.E.F., (1960) Virology 12 521

Matthews, R.E.F., (1970) "Plant Virology" Academic Press Inc. New York

Matthews, R.E.F., Bolton, E.T., Thompson, H.R., (1963) Virology 19 179

Matthews, R.E.F., Lyttleton, J.W. (1959) Virology 9 332

Matthews, R.E.F., Ralph, R.K., (1966) Advan. Virus Res. 12 273

Matus, A.I., Ralph, R.K., Mandel, H.G., (1964) J. molec. Biol 10 295

Mazzone, H.M., Incardona, N.L. Kaesberg, P. (1962) Biochim. biophys. Acta 55 164

Means, A.R., Baker, C.A., (1969) Biochim. biophys. Acta 182 461

Mehler, A.H., (1970) Prog. Nucleic Acid Res. and Molec. Biol. 10 1

Mehta, S.L., Hadziyev, D., Zalik, S., (1968) Biochim. biophys. Acta 169 381

Mehta, S.L., Hadziyev, D., Zalik, S. (1969) Biochim. biophysc. Acta 195 515

Mejbaum, W (1939) Hoppe-Seyler's Z. physiol. Chem 258 117

Menage, R.H. (1966) In "Gardening for Adventure" Phoenix House Press, London.

Miličič, D., Stefanac, Z., (1967) Phytopath. Z 59 285

Milman, G., Goldstein, J., Scolnick, E., Caskey, T., (1969) Proc. natn. Acad. Sci, U.S. 63 183

Milne, R.G., (1967) Virology 32 589

Mitra, S., Kaesberg, P. (1963) Biochem. biophys. Res. Commun. 11 146

Miura, K.I., Kimura, I., Suzuki, N., (1966) Virology 28 571

Miura, K.I., Muto, A. (1965) Biochim, biophys. Acta 108 707

Moldave, K., (1965) A. Rev. Biochem. 34 419

Möller, W., Amons, R., Groene, J.L., Garrett, R.A., Terhorst, C.P., (1969) Biochim. biophys. Acta 190 381

Momose, K., Kaji, A. (1966) J. biol. Chem. 241 3294

Monier, R., Stephenson, M.L., Zamecnik, P.C., (1960) Biochim. biophys. Acta 43 1.

Monro, R.E., (1967) J. molec. Biol. 26 147

Monro, R.E., Cerná, J., Marcker, K.A., (1968) Proc. natn. Acad. Sci. U.S. 61 1042

Monro, R.E., Marcker, K.A., (1967) J. molec. Biol. 25 347

Moore, P.B. (1966) J. molec. Biol. 18 8

Mosteller, R.D., Culp, W.J., Hardesty, B., (1968) Biochem. biophys. Res. Commun. 30 631

Muench, K.H., Safille, P.A. (1968) Biochemistry, N.Y. 7 2799

Mundry, K.W., (1969) Molec. Gen. Genetics 105 361

Murakishi, H.H., (1963) Nature, Lond. 198 312

Murthy, M.R.V. (1966) Biochim. biophys. Acta 119 599

Mushinski, J., Potter, M., (1969) Biochemistry, N.Y. 8 1684

Nagaraj, A.N., (1965) Virology 25 189

Nanninga, N., (1963) J. Cell Biol. 33 C1

Naora, H., Kodaira, K., (1969) Biochim. biophys. Acta 182 469

Naora, H., Naora, H., (1967) Biochim. biophys. Acta 134 277

Nathans, D., Lipmann, F. (1961) Proc. natn. Acad. Sci. U.S. 47 497

Nathans, D., Notani, G., Schwartz, J.H., Zinder, N.D., (1962) Proc. natn. Acad. Sci. U.S. 48 1424

Newmark, P. Fraser, D., (1956) J. Am. chem. Soc. 78 1588

Nicholson, M.O., Flamm, W.G., (1964) Fed. Proc. 23 316

Nirenberg, M.W., Caskey, C.T., Marshall, R.E., Brimacombe, R., Kellogg, D., Doctor, B.P., Hatfield, D., Levin, J., Rottman, F., Pestka, S., Wilcox, M., Anderson, F. (1966) Cold Spring Harb. symp. quant. Biol. 31 11.

Nirenberg, M.W., Jones, O.W., Leder, P., Clark, B.F.C., Sly, W.S., Pestka, S., (1963) Cold Spring Harb. Symp. quant. Biol. 28 549

Nirenberg, M.W., Matthaei, J.H., (1961) Proc. natn. Acad. Sci. U.S. 47 (1961) 1588

Nishimura, S., Harada, F., Hirabayashi, M., (1969) J. molec. Biol. 40 173

Noll, H., (1969) In "Techniques in Protein Biosynthesis" Vol. 2. (Ed. Campbell, P.N. and Sargent, J.R.) pp 101 - 179 Academic Press Inc., New York

Nomura, M., Lowry, C.V., (1967) Proc. natn. Acad. Sci. U.S. 58 946

Nomura, M., Lowry, C.V., Guthrie, C., (1967) Proc. natn. Acad. Sci. U.S 58 1487

Novelli, G.D., (1967) A. Rev. Biochem. 36 449

Novikov, V.K., Atabekov, J.G., (1970) Virology 41 101

Obrig, T.G., Cerna, J., Gottlieb, D., (1969) Phytopathology 59 187

Ochoa, S., (1968) Nátürwissenschaften 55 505

Ochoa, S., Weissmann, C., Borst, P.H., Burdon, R.H., Billeter, M.A., (1964) Fed. Proc. 23 1285

Offengand, J., Haselkorn, R., (1962) Biochem. biophys. Res. Commun. 6 469

Ohta, T., Thach, R.E., (1968) Nature, Lond. 219 238

Okamoto, T., Takanami, M., (1963) Biochim. biophys. Acta 76 266

Ono, Y., Skoultchi, A., Klein, A., Lengyel, P. (1968) Nature, Lond.220 1304

Ono, Y., Skoultchi, A., Waterson, J., Lengyel, P., (1969a) Nature, Lond. 222 645.

Pace, B., Campbell, L.L. (1967) Proc. natn. Acad. Sci. U.S. 57 1110

Pain, R.H., (1970) Private communication

Palade, G.E., Siekevitz, P., (1956) J. biophys. biochem. Cytol. 2 171 , 671

Parish, J.H., (1969) Biochim, biophys. Acta 182 454

Parisi, B., Cifferri, O., (1966) Biochemistry, N.Y. 5 1638

Parisi, B., Milanesi, G., Van Etten, J.L., Perani, A., Cifferri, O., (1967) J. molec. biol. 28 295

Parthier, B., (1961) Flora, Jena, 151 368

Payne, E.S. (1970) Ph.D. Thesis, University of Durham

Payne, P.I. , Boulter, D., (1969) Planta 84 263

Pearson, J.A., (1969) Expl. Cell. Res. 57 235

Perzyński, S., Chomczyński, P., Szafrański, P., (1969) Biochem. J. 114 437

Pestka, S., (1968) Proc. natn. Acad. Sci. U.S. 61 726

Pestka, S., (1969) J. biol. Chem. 244 1533

Pestka, S., Nirenberg, M.W., (1966) J. molec. Biol. 21 145

Petermann, M.L., (1960) J. biol. Chem. 235 1998

Petermann, M.L., (1964) "The Physical and Chemical Properties of Ribosomes" Elsevier Publishing Company, Amsterdam.

Petermann, M.L., Hamilton, M.G., Balis, M.E., Samarth, K., Pecora, P., (1958) In "Microsomal Particles and Protein Synthesis" (Ed. Roberts, R.B.) p. 70 Pergamon Press N.Y. and Lond.

Petermann, M.L., Pavlovec, A. (1963) J. biol. Chem. 238 318

Petermann, M.L., Pavlovec, A., (1969) Biopolymers, 7 73

Peterson, P.J., (1967) Biol. Rev. 42 552

Peterson, P.J., Fowden, L., (1965) Biochem. J. 97 112

Phillips, D.R., Fletcher, R.A. (1969) Physiologia Pl. 22 764

Phillips, S.L., (1971) J. molec. Biol. 59 461

Pinck, M., Yot, P., Chapeville, F., Duranton, H.M. (1970) Nature, Lond. 226 954

Pirie, N.W. (1956) Biochem. J. 63 316

Priestly, G.C., Pruyn, M.L., Malt, R.A., (1969) Biochim. biophys. Acta 190 154

Ralph, R.K., Bellamy, A.R., (1964) Biochim. biophys. Acta 87 9

Ralph, R.K., Clark, M.F., (1966) Biochim. biophys. Acta 119 29

Ralph, R.K., Matthews, R.E.F. (1963) Biochem. biophys. Res. Commun. 12 287

Ralph, R.K., Matthews, R.E.F., Matus, A.I., Mandel, H.G., (1965) J. molec. Biol. 11 202

Ralph, R.K., Wojcik, S.J., (1966a) Biochim. biophys. Acta 119 347

Ralph, R.K., Wojcik, S.J., (1969) Virology 37 276

Ralph, R.K., Wojcik, S.J., (1970) Personal Communication reported on p.191 "Plant Virology", Matthews, R.E.F., Academic Press Inc. New York

Randles, and Matthews, (1970) Unpublished. Reported on p.326 "Plant Virology" Matthews R.E.F., (1970) Academic Press Inc., New York

Rappaport, I., Wu, J.H., (1962) Virology 17 411

Ravel, J.M. (1967) Proc. natn. Acad. Sci. U.S. 57 1811

Ravel, J.M., Shorey, R.L. (1969) J. cell. Physiol. 74 103S

Ravel, J.M., Shorey, R.L., Froehner, S., Shive, W. (1968) Arch. Biochem. Biophys. 125 514

Ravel, J.M., Shorey, R.L., Shive, W., (1967) Biochem. biophys. Res. Commun. 29 68

Reddi, K.K. (1959) Biochim. biophys. Acta 33 164

Reddi, K.K., (1963a) Proc. natn. Acad. Sci. U.S. 50 75

Reddi, K.K., (1963b) Proc. natn. Acad. Sci. U.S. 50 419

Reddi, K.K., (1964) Proc. natn. Acad. Sci. U.S. 52 397

Reddi, K.K., (1966) Proc. natn. Acad. Sci. U.S. 55 593

Redman, C.M. (1968) Biochem. biophys. Res. Commun. 31 845

Redman, C.M., Sabatini, D.D., (1966) Proc. natn. Acad. Sci. U.S. 56 608

Reichmann, M.E., (1964) Proc. natn. Acad. Sci. U.S. 52 1009

Reichmann, M.E., Chang, A.Y., Faiman, L., Clark, J.M. Jr. (1966) Cold Spring Harb. Symp. quant. Biol. 31 139

Reichmann, M.E., Clark, J.M. Jr. (1968) A. Rev. Phytopathology 6 295

Reichmann, M.E., Stace-Smith, R., (1959) Virology 9 710

Reid, E., (1967) In "Enzyme Cytology" p. 321 (Ed. Roodyn, D.) Academic Press. Inc., New York

Reid, M.S., Matthews, R.E.F. (1966) Virology 28 563

Reinecke, C.J., (1967) Reported on page 266 "Regulation of Nucleic Acid and Protein Biosynthesis" (Ed. Koningsberger, and Bosch, L) Elsevier Publishing Co. Amsterdam.

Reinecke, C.J., van Reisen, R., Voorma, H.O., Bosch, L., (1968) Biochim. biophys. Acta 157 566

Rendi, R., Ochoa, S., (1962) J. biol. Chem. 237 3707, 3711

Rezende-Pinto, M.C., Borges, M de L.V. (1952) Agron. Lusitana 14 259

Rialdi, G., Zunino, F., (1969) J. gen. Virol. 5 105

Rich, A., Eikenberry, E.F., Malkin, L.I. (1966) Cold Spring Harb. Symp. quant. Biol. 31 303

Rich, A. Warner, J.R., Goodman, H.M. (1963) Cold Spring Harb. Symp. quant. Biol. 28 269

Richards, E.G., Coll, J.A., Gratzer, W.B. (1965) Analyt. Biochem. 12 452

Richter, D., Klink, F. (1968) FEBS Letters 2 49

Robinson, C.L., Novelli, G.D. (1962) Arch. Biochem. Biophys. 96 459

Roblin, R. (1968) J. molec. Biol. 31 51

Rogg, H., Wehrli, W., Staehelin, M. (1969) Biochim. biophys. Acta 195 13

Roland, G. (1959) Taxon 8 126

Rolleston, F.S., Wool, I.G., Martin, T.E. (1970) Biochem. J. 117 899

Rose, I.A. (1968) Proc. natn. Acad. Sci. U.S. 61 1079

Rosen, L., Murray, E.L., Novelli, G.D. (1967) Canad. J. Biochem. 45 2005

Ross, A.F. (1959) In "Plant Pathology - Problems and Progress 1908-1958" (Ed. Holton, C.S.) pp. 511-520 Univ. Wisconsin Press, Madison, Wisconsin

Röttger, B. (1965) Biochim. biophys. Acta 95 525

Rouget, P., Chapeville, F. (1968) Eur. J. Biochem 4 310

Rowan, K.S. (1966) Int. Rev. Cytol. 19 301

Rubin, I.B., Kelmers, A.D., Goldstein, G. (1967) Analyt. Biochem. 20 533

Rushizky, G.W., Knight, C.A. (1959) Virology 8 448

Russo, M., Martelli, G.P., Quacquarelli, A. (1968) Virology 34 679

Rychlík, I., Chládek, S., Žemlička, J. (1963) Biochim. biophys. Acta 138 640

St. Geme, G.W., Yoyama, P.S., Brumbaugh, J.L., Dietz, M.E. (1967) Proc. Soc. exp. Biol. Med. 125 58

Sachs, H. (1957) J. biol. Chem. 228 23

Salas, M., Smith, M.A., Stanley, W.M. jun., Wahba, A.J., Ochoa, S. (1965) J. biol. Chem. 240 3988

Sambrook, J.F., Fan, D.P., Brenner, S. (1967) Nature, Lond. 214 452

Sandell, S., Löw, H., von der Decken, A. (1967) Biochem. J. 104 575

Sander, E., Schramm, G. (1963) Z. Naturf. 18b 199

Sänger, H.L., Knight, C.A. (1963) Biochem. biophys. Res. Commun. 13 455

Santilli, V., Nepokroeff, C.M., Gagliardi, N.C. (1962) Nature, Lond. 193 656

Satake, M., Takahashi, Y., Mase, K., Ogata, K. (1965) J. Biochem., Japan 57 184

Saunders, G.F., Campbell, L.L. (1966) J. Bact. 91 332

Schiebel, W., Chayka, T.G., de Vries, A., Rusch, H.P. (1969) Biochem. biophys. Res. Commun. 35 338

Schlegel, D.E., Smith, S.H. (1966) In "Viruses of Plants" (Ed. Beemster, A.B.R. and Dijkstra, J.) pp. 54-65. North-Holland Publ., Amsterdam

Schlessinger, D., Aspirion, D. (1969) A. Rev. Microbiol. 23 387

Schlessinger, D., Mangiarotti, G., Aspirion, D. (1967) Proc. natn. Acad. Sci. U.S. 58 1782

Schlimme, E., v.d. Haar, F., Cramer, F. (1969) Z. Naturf. 24b 631

Schramm, G., Engler, R. (1958) Nature, Lond. 181 916

Schramm, G., Röttger, B. (1959) Z. Naturf. 14b 510

Schreml, W., Burka, E.R. (1968) J. biol. Chem. 243 3573

Schwartz, J.H. (1967) J. molec. Biol. 30 309

Schwartz, J.H., Eisenstadt, J.M., Brawerman, G., Zinder, N.D. (1965) Proc. natn. Acad. Sci. U.S. 53 195

Schwartz, J.H., Meyer, R., Eisenstadt, J.M., Brawerman, G. (1967) J. molec. Biol. 25 571

Schweet, R., Heintz, R. (1966) A. Rev. Biochem. 35 723

Schweet, R.S., Allen, E.H. (1958) J. biol. Chem. 233 1104

Scolnick, E., Caskey, T. (1969) Proc. natn. Acad. Sci. U.S. 64 1235

Scolnick, E., Tompkins, R., Caskey, T., Nirenberg, M.W. (1968) Proc. natn. Acad. Sci. U.S. 61 768

Sela, I. (1969) Arch. Biochem. Biophys. 133 454

Sela, I., Kaesberg, P. (1969) J. Virol. 3 89

Sellinger, O.Z., Ohlsson, W.G. (1969) Life Sci. 8 1083

Semal, J. (1967) Phytopath. Z. 59 55

Semal, J., Hamilton, R.I. (1968) Virology 36 293

Semancik, J.S., Reynolds, D.A. (1969) Science, N.Y. 164 559

Shalla, T.A., Amici, A. (1967) Virology 31 78

Shaw, J.G. (1967) Virology 31 665

Shaw, J.G. (1969) Virology 37 109

Shigematsu, A., Mizusawa, Y., Hirai, T. (1966) Virology 28 331

Shikata, E., Maramorosch, K. (1966) Virology 30 439

Shinozawa, T., Yahara, I., Imahori, K. (1968) J. molec. Biol. 36 305

Shipp, W., Haselkorn, R. (1964) Proc. natn. Acad. Sci. U.S. 52 401

Sidransky, H., Sarma, D.S. R., Bongiorno, M., Verney, E. (1968) J. biol. Chem. 243 1123

Sidransky, H., Verney, E., Shinozuka, H. (1969) Expl. Cell Res. 54 37

Siegel, A. (1959) Virology 8 470

Siegel, A., Ginoza, W., Wildman, S.G. (1957) Virology 3 554

Siegel, A., Zaitlin, M., Sehgal, O.P. (1962) Proc. natn. Acad. Sci. U.S. 48 1845

Siekevitz, P. (1952) J. biol. Chem. 195 549

Siekevitz, P., Palade, G.E. (1960) J. biophys. biochem. Cytol. 7 619

Silber, R., Unger, K.W., Ellman, L. (1968) Br. J. Haemat. 14 261

Skogerson, L., Moldave, K. (1968) Arch. Biochem. Biophys. 125 497

Skoultchi, A., Ono, Y., Moon, H.M., Lengyel, P. (1968) Proc. natn. Acad. Sci. U.S. 60 675

Smillie, R.M., Evans, W.R., Lyman, H. (1963) Brookhaven Symp. Biol. 16 89

Smith, A.E., Marcker, K.A. (1968) J. molec. Biol. 38 241

Smith, I.K., Fowden, L. (1968) Phytochem. 7 1065

Smith, S.H., Schlegel, D.E. (1965) Virology 26 180

So, A.G., Davie, E.W. (1963) Biochemistry, N.Y. 2 132

So, A.G., Davie, E.W. (1965) Biochemistry, N.Y. 4 1973

Solymosy, F., Fedorcsak, L, Gulyás, A., Farkas, G.L., Ehrenberg, L. (1968) Europ. J. Biochem. 5 520

Sonnenbichler, J., Feldman, H., Zachau, H.G. (1963) Z. Physiol. Chem. 334 283

Spector, A., Travis, D. (1966) J. biol. Chem. 241 1290

Spencer, D. (1965) Arch. Biochem. Biophys. 111 381

Spencer, D., Wildman, S.G. (1964) Biochemistry, N.Y. 3 954

Spiegelman, S., Haruna, I. (1966) Proc. natn. Acad. Sci. U.S. 55 1539

Spiegelman, S., Holland, I.B., Beaudreau, G., Mills, D., Haruna, I. (1965) Proc. natn. Acad. Sci. U.S. 54 919

Spirin, A.S. (1963) In "Progress in Nucleic Acid Research." (Ed. Davidson, J.N. and Cohn, W.E.) Vol. 1 p. 301. Academic Press Inc., New York

Spirin, A.S. (1964) "Macromolecular Structure of Ribonucleic Acids" (Translation Ed. Stekol, J.A.) Reinhold Pub. Corp. New York

Spirin, A.S. (1968) In "Currents in Modern Biology" Vol. 2 pp. 115-127 North-Holland Publishing Co., Amsterdam

Spirin, A.S., Gavrilova, L.P. (1969) Molecular Biology, Biochemistry and Biophysics. Vol. 4. The Ribosome. Springer-Verlag, Berlin

Srivastava, B.I.S., Arglebe, C. (1967) Pl. Physiol. 42 1497

Srivastava, U. (1969) Arch. Biochem. Biophys. 130 129

Staehelin, M. (1963) In "Progress in Nucleic Acid Research." Vol. 2. (Ed. Davidson, J.N. and Cohn, W.E.) pp. 169-195 Academic Press Inc., New York.

Staehelin, T., Verney, E., Sidransky, H. (1967) Biochim. biophys. Acta 145 105

Staehelin, T., Wettstein, F.O., Oura, H., Noll, H. (1964) Nature, Lond. 201 264

Stahl, J., Lawford, G.R., Williams, B., Campbell, P.N. (1968) Biochem. J. 109 155

Stanley, W.M., Bock, R.M. (1965) Biochemistry, N.Y. 4 1302

Stanley, W.M. jun., Salas, M., Wahba, A.J., Ochoa, S. (1966) Proc. natn. Acad. Sci. U.S. 56 290

Steere, R.L. (1956) Phytopathology 46 60

Steere, R.L. (1959) In "Advances in Virus Research" (Ed. Smith, K.M. and Kauffer, M.A.) 6 1 Academic Press Inc., New York

Steere, R.L. (1964) In "Plant Virology" (Ed. Corbett, M.K. and Sisler, H.D.) pp. 211-234 Univ. of Florida Press, Gainesville, Florida

Stent, G.S. (1963) "Molecular Biology of Bacterial Viruses" Freeman and Co., San Fransisco, California

Stent, G.S. (1964) Science, N.Y. 144 816

Stephenson, M.L., Thimann, K.V., Zamecnik, P.C. (1956) Arch. Biochem. Biophys. 65 194

Strehler, B.L., Hendley, D.D., Hirsch, G.P. (1967) Proc. natn. Acad. Sci. U.S. 57 1751

Stubbs, J.D., Kaesberg, P. (1967) Virology 33 385

Sturani, E., Cocucci, S., Marrè, E. (1968) Plant and Cell Physiol. 9 783

Stutz, E., Noll, H. (1967) Proc. natn. Acad. Sci. U.S. 57 774

Subak-Sharpe, H., Hay, J. (1965) J. molec. Biol. 12 924

Sueoka, N., Kano-Sueoka, T. (1964) Proc. natn. Acad. Sci. U.S. 52 1535

Sueoka, N., Kano-Sueoka, T., Gartland, W.J. (1966) Cold Spring Harb. Symp. quant. Biol. 31 571

Summers, D.F., Maizel, J.V., Darnell, J.E. (1965) Proc. natn. Acad. Sci. U.S. 54 505

Süss, R., Sander, E., Röttger, B., Senger, H. (1965) Biochim. biophys. Acta 95 388

Suzuka, I., Kaji, H., Kaji, A. (1965) Biochem. biophys. Res. Commun. 21 187

Svedberg, T., Pedersen, K.O. (1940) The Ultracentrifuge. Clarendon Press, Oxford.

Swain, R.R., Dekker, E.E. (1969) Pl. Physiol. 44 319

Symons, R.H., Rees, M.W., Short, M.N., Markham, R. (1963) J. molec. Biol. 6 1

Szer, W. (1966) Biochem. biophys. Res. Commun. 22 559

Szer, W., Ochoa, S. (1964) J. molec. Biol. 8 823

Tabor, H., Tabor, C.W. (1964) Pharmac. Rev. 16 245

Takagi, M., Ogata, K. (1968) Biochem. biophys. Res. Commun. 33 55

Takahashi, T., Hirai, T. (1966) Physiologia Pl. 19 888

Takahashi, W.N. (1955) Virology 1 393

Takahashi, W.N. (1959) Virology 9 437

Takahashi, W.N. (1964) In "Plant Virology" (Ed. Corbett, M.K. and Sisler, H.D.) p. 315 Univ. of Florida Press, Gainesville, Florida

Takahashi, W.N., Ishii, M. (1952) Nature, Lond. 169 419

Takahashi, W.N., Ishii, M. (1953) Am. J. Bot. 40 85

Takanami, M. (1960) Biochim. biophys. Acta 39 318

Takanami, M. (1962) Biochim. biophys. Acta 61 432

Takanami, M. (1967) J. molec. Biol. 23 135

Takanami, M., Okamoto, T., Watanabe, I. (1961) J. molec. Biol. 3 476

Takanami, M., Yan, Y. (1965) Proc. natn. Acad. Sci. U.S. 54 1450

Takanami, M., Yan, Y., Jukes, T.H. (1965) J. molec. Biol. 12 761

Takanami, M., Zubay, G. (1964) Proc. natn. Acad. Sci. U.S. 51 834

Takeda, Y. (1969a) J. Biochem., Tokyo 66 345

Takeda, Y. (1969b) Biochim. biophys. Acta 179 232

Takeda, Y. (1969c) Biochim. biophys. Acta 182 258

Takeda, Y., Igarashi, K. (1969) Biochem. biophys. Res. Commun. 37 917

Takeda, Y., Igarashi, K. (1970) Biochim, biophys. Acta 204 406

Tal, M., Elson, D. (1963) Biochim. biophys. Acta 76 40

Tanner, M.J.A. (1967) Biochemistry, N.Y. 6 2686

Tarragó, A., Monasterio, O., Allende, J.E. (1970) Biochem. biophys. Res. Commun. 41 765

Tata, J.R. (1969) In "Subcellular Components: Preparation and Fractionation." (Ed. Birnie, G.D. and Fox, S.M.) pp. 83-107 Butterworths, London

Tata, J.R., Williams-Ashman, H.G. (1967) Europ. J. Biochem. 2 366

Temin, H.M. (1970) Perspect. Biol. Med. 14 11

Teng, C-S., Hamilton, T.H., (1967) Biochem. J. 105 1091

Tester, C.F., Dure, L. (III). (1966) Biochem. biophys. Res. Commun. 23 287

Thach, R.E., Dewey, K.F., Brown, J.C., Doty, P. (1966) Science, N.Y. 153 416

Thompson, I.M. (1967) Unpublished observations

Tillack, T.W., Smith, D.W.E. (1968) Virology 36 212

Tillotson, J.A., Schrader, L.E., Huston, R.L. (1969) Fed. Proc. 28 755

Tissières, A., Watson, J.D. (1958) Nature, Lond. 182 778

Tissières, A., Watson, J.D., Schlessinger, D., Hullingworth, B.R. (1959) J. molec. Biol. 1 221

Tongur, V.S., Balandin, I.G., Vyshepan, E.D., Khoroshutina, E.B. (1963) Vop. Virusol. 8 (2) 142

Traut, R.R., Monro, R.E. (1964) J. molec. Biol. 10 63

Trávníček, M. (1968) Biochim. biophys. Acta 166 757

Ts'O, P.O.P. (1962) A. Rev. Pl. Physiol. 13 45

Ts'O, P.O.P., Vinograd, J. (1961) Biochim. biophys. Acta 49 113

Tsugita, A., Fraenkel-Conrat, H., Nirenberg, M.W., Matthaei, J.H. (1962) Proc. natn. Acad. Sci. U.S. 48 846

Tsugita, A., Gish, D.T., Young, J., Fraenkel-Conrat, H., Knight, C.A., Stanley, W.M. (1960) Proc. natn. Acad. Sci. U.S. 46 1463

Ushiyama, R. (1971) Jap. J. Microbiol. 15 257

Ushiyama, R., Matthews, R.E.F. (1970) Virology 42 293

Van Duin, J., Pley, C.W., Bonnet-Smits, E.M., Bosch, L. (1968) Biochim. biophys. Acta 155 444

Van Etten, J.L. (1968) Arch. Biochem. Biophys. 125 13

Van Etten, J.L., Koski, R.K., El-Olemy, M.M. (1969) J. Bact. 100 1182

van Griensven, L.J.L.D., van Kammen, A. (1969) J. gen. Virol. 4 423

van Kammen, A. (1963) Meded. LandbHoogesch. Wageningen 63 1

van Kammen, A. (1967) Virology 31 633

van Kammen, A. (1967') Arch. Biochem. Biophys. 118 517

van Kammen, A. (1968) Virology 34 312

van Knippenberg, P.H., Grijm-Vos, M., Veldstra, H., Bosch, L. (1965b) Biochem. biophys. Res. Commun. 20 4

van Knippenberg, P.H., van Ravenswaay-Claasen, J.C., Grijm-Vos, M., Veldstra, H., Bosch, L. (1965a) Biochim. biophys. Acta 95 461

van Knippenberg, P.H., Veldstra, H., Bosch,L. (1964) Biochim. biophys. Acta 80 526

van Ravenswaay-Claasen, J.C., van Leeuwen, A.B.J., Duijts, G.A.H., Bosch, L. (1967) J. molec. Biol. 23 535

van Rysselberge, C., Jeener, R. (1957) Biochim. biophys. Acta 23 18

van Vloten-Doting, L. (1968) Ph.D Thesis. University of Leiden

van Vloten-Doting, L., Kruseman, J., Jaspars, E.M.J. (1968) Virology 34 728

Vanyushin, B.F., Dunn, D.B. (1967) Biochim. biophys. Acta 134 91

Verhoef, N.J., Reinecke, C.J., Bosch, L. (1967) Biochim. biophys. Acta 149 305

Vescia, A., Giordano, G.G., Hermann, G. (1968) J. molec. Biol. 33 625

Viñuela, E., Algranati, I., Felix, G., Garwes, D., Weissmann, C., Ochoa, S. (1968) Biochim. biophys. Acta 155 558

Viñuela, E., Sălas, M., Ochoa, S. (1967) Proc. natn. Acad. Sci. U.S. 57 729

Vold, B.S., Sypherd, P.S. (1968) Proc. natn. Acad. Sci. U.S. 59 453

von der Decken, A. (1967) J. Cell Biol. 33 657

von der Decken, A. (1968) Europ. J. Biochem. 4 87

von Ehrenstein, G. (1970) In "Aspects of Protein Biosynthesis" Part A (Ed. Anfinsen, C.B., jun.,) pp. 139-214 Academic Press Inc., New York

von Wettstein, D., Zech, H. (1962) Z. Naturf. 17b 376

Voorma, H.O. (1965) Ph.D Thesis. University of Leiden

Voorma, H.O., Benne, R., Den Hertog, T.J.A. (1971) Europ. J. Biochem. 18 451

Voorma, H.O., Bosch, L. (1965) Biochem. biophys. Res. Commun. 18 420

Voorma, H.O., Gout, P.W., van Duin, J., Hoogendam, B.W., Bosch, L. (1964) Biochim. biophys. Acta 87 691

Voorma, H.O., Gout, P.W., van Duin, J., Hoogendam, B.W., Bosch, L. (1965) Biochim. biophys. Acta 95 446

Waller, J.P. (1964) J. molec. Biol. 10 319

Wannemacher, R.W. jun., Cooper, W.K., Muramatsu, K. (1968) Biochim. biophys. Acta 169 269

Warner, J.R., Rich, A., Hall, C.E. (1962) Science, N.Y. 138 1399

Warren, R.L. (1964) Biochem. J. 92 234

Waters, L.C., Novelli, G.D. (1967) Proc. natn. Acad. Sci. U.S. 57 979

Watts, R.L., Mathias, A.P. (1967) Biochim. biophys. Acta 145 828

Webster, R.E., Zinder, N.D. (1969) J. molec. Biol. 42 425

Weeks, D.P., Marcus, A. (1969) Pl. Physiol. 44 1291

Weigert, M.G., Garen, A. (1965) Nature, Lond. 206 992

Weigert, M.G., Lanŭka, E., Garen, A. (1967) J. molec. Biol. 23 391

Weiss, J.F., Kelmers, A.D. (1967) Biochemistry, N.Y. 6 2507

Weiss, R.L., Morris, D.R. (1970) Biochim. biophys. Acta 204 502

Weiss, S.B., Hsu, W.T., Foft, J.W., Scherberg, N.H. (1968) Proc. natn. Acad. Sci. U.S. 61 114

Weissmann, C. (1965) Conf. on Plant Viruses. Symp. V., Wageningen, July 1965

Weissmann, C., Billeter, M.A., Schneider, M.C., Knight, C.A., Ochoa, S. (1965) Proc. natn. Acad. Sci. U.S. 53 653

Weissmann, C., Billeter, M.A., Viñuela, E., Libonati, M. (1966) In "Viruses of Plants" (Ed. Beemster, A.B.R. and Dijkstra, J.) pp. 249-274 North-Holland Publishing Co., Amsterdam

Weissmann, C., Borst, P., Burdon, R.H., Billeter, M.A., Ochoa, S. (1964) Proc. natn. Acad. Sci. U.S. 51 682

Weissmann, C., Felix, G., Slor, H. (1968) Cold Spring Harb. Symp. quant. Biol. 33 83

Weksler, M.E., Gelboin, H.V. (1967) J. biol. Chem. 242 727

Wettstein, F.O., Noll, H., Penman, S. (1964) Biochim. biophys. Acta 87 525

Wettstein, F.O., Staehelin, T., Noll, H. (1963) Nature, Lond. 197 430

Whaley, W.G., Mollenhauer, H.H., Leech, J.H. (1960) Am. J. Bot. 47 401

Wheeler, C.T. (1965) Ph. D. Thesis. University of Liverpool

Williams, G.R., Novelli, G.D. (1964) Biochem. biophys. Res. Commun. 17 23

Williamson, A.R. (1969) Biochem. J. 111 515

Willison, R.S., Tremaine, J.H., Weintraub, M. (1961) Can. J. Bot. 39 1447

Wilson, D.B., Dintzis, H.M. (1970) Proc. natn. Acad. Sci. U.S. 66 1282

Wimmer, E., Chang, A.Y., Clark, J.M. jun., Reichmann, M.E. (1968) J. molec. Biol. 38 59

Wimmer, E., Reichmann, M.E. (1968) Science, N.Y. 160 1452

Wisseman, C.L., Smadel, J.E., Hahn, F.E., Hopps, H.E. (1954) J. Bact. 67 662

Wittmann, H.G. (1962) Z. VererbLehre 93 491

Wood, H.A., Bancroft, J.B. (1965) Virology 27 94

Wu, J.H., Hudson, W. (1963) Virology 20 158

Wu, J.H., Rappaport, I. (1961) Virology 14 259

Wunner, W.H., Bell, J., Munro, H.N. (1966) Biochem. J. 101 417

Yamazaki, H., Kaesberg, P. (1961) Biochim. biophys. Acta 51 9

Yamazaki, H., Kaesberg, P. (1966) Proc. natn. Acad. Sci. U.S. 56 624

Yang, W-K., Novelli, G.D. (1968) Proc. natn. Acad. Sci. U.S. 59 208

Yarwood, A. (1968) Ph.D Thesis. University of Liverpool

Yarwood, A., Boulter, D. (1970) Unpublished. Reported in Boulter, D. A. Rev. Plant Physiol. 21 p.94

Yarwood, A., Boulter, D., Yarwood, J.W. (1971) Biochem. biophys. Res. Commun. 44 353

Yasuda, Y., Hirai, T. (1964) Expl. Cell Res. 34 210

Yegian, C.D., Stent, G.S. (1969a) J.molec. Biol. 39 45

Yegian, C.D., Stent, G.S. (1969b) J. molec. Biol. 39 59

Yot, P., Pinck, M., Haenni, A.L., Duranton, H.M., Chapeville, F. (1970) Proc. natn. Acad. Sci. U.S. 67 1345

Zachau, H.G., Dütting, D., Feldmann, H. (1966) Angew. Chem. 78 392

Zaitlin, M., Boardman, N.K. (1958) Virology 6 743

Zamecnik, P.C. (1953) Fed. Proc. 12 29S

Zamecnik, P.C., Keller, E.B. (1954) J. biol. Chem. 209 337

Zech, H. (1952) Planta 40 461

Zech, H. (1954) Expl. Cell Res. 6 560

Zech, H., Vogt-Köhne, L. (1955) Naturwissenschaften 42 337

Zinder, N.D., Engelhardt, D.L., Webster, R.E. (1966) Cold Spring Harb. Symp. quant. Biol. 31 251

Zomzely, C.E., Roberts, S., Brown, D.M., Provost, C. (1966) J. molec. Biol. 19 455

ACKNOWLEDGEMENTS

I am indebted to my supervisor Professor D. Boulter, for his continued help and guidance at all stages of this work, and for use of the facilities of the Botany Department, University of Durham. I should like to thank members of the department for helpful criticism and discussion of this work, including A.R. Cobb and R. Swincoe for the electron microscopy (and G.H. Banbury for interpretation of the electron micrographs).

I should like to thank Doctors G. Belli, J. Bove, E.M.J. Jaspars and Professors R. Markham and R.E.F. Matthews for supplies of Turnip Yellow Mosaic Virus and for advice on inoculation procedures, R. Haselkorn for information on the source of supply of Chinese cabbage seed, Dr. D.B. Dunn and staff of the John Innes Institute for advice and encouragement on various aspects of this work and Dr. R.H. Pain, Department of Biochemistry, University of Newcastle upon Tyne, for Spinco E analytical ultracentrifugal analysis of viral samples.

Finally, I acknowledge receipt of a Science Research Council Research Studentship.