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Nucleic Acids in Ageing Leaves of

Chinese Cabbage (*Brassica pekinensis* Rupr.)

BY

Geoffrey Strangeway B.Sc. (Dunelm)

A Thesis submitted
to the University of Durham
for the degree of Doctor of
Philosophy

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DECLARATION

The work described in this thesis has been carried out by the undersigned at the Department of Botany of Durham University and has not been previously submitted for any other diploma or degree.

Signed:

G. Stronge

Date:

16th December 1977

ERRATUM

The values quoted for the sedimentation coefficients throughout this thesis should read S not s.

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ABSTRACT

Methods for the extraction of uncomplexed host RNA from turnip yellow mosaic virus infected chinese cabbage plants and for the electrophoretic fractionation of ribosomes, ribosomal sub-units, polyribosomes and virus particles are described and characterised. The changes that occurred in ribosomal RNA fractions and in polyribosome status of sequentially ageing leaves of uninfected and TYMV-infected chinese cabbage plants were examined using these techniques. The peak accumulation of chloroplast and cytoplasmic ribosomal RNA occurred at different times in the young leaves but following leaf maturity both fractions declined at a similar rate. The high levels of polyribosomes observed in the youngest leaves fell during leaf expansion to a level that was largely maintained after full leaf expansion was achieved. The effects of the virus on these patterns of change were attributable to its effect on chloroplast metabolism and periods of multiplication.

The senescence of excised leaf discs was also studied and differences in the patterns of change of ribosomal



RNA were found between different incubation treatments. Retardation of senescence of the leaf discs by light or kinetin principally affected levels of cytoplasmic ribosomal RNA. A different pattern of senescence in excised leaf discs and attached leaves was observed with an accelerated rate of loss of chloroplast ribosomal RNA in the former. Kinetin was seen to ^{el}~~il~~licit a different pattern of response in TYMV-infected discs compared to uninfected discs.

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ABBREVIATIONS

The following abbreviations are used in the text of this thesis.

RNA	=	Ribonucleic Acid
DNA	=	Deoxyribonucleic Acid
RNAase	=	Ribonuclease
mRNA	=	Messenger RNA
rRNA	=	Ribosomal RNA
sRNA	=	Soluble RNA
tRNA	=	Transfer RNA
DEP	=	Diethylpyrocarbonate
SDS	=	Sodium dodecyl sulphate
EDTA	=	Ethylene diamine tetra acetic acid (disodium salt)
DOC	=	Sodium deoxycholate
TEMED	=	$N:N:N^1:N^1$ -tetra-methyl-1:2- diamino ethane
TCA	=	Trichloroacetic acid
PPO	=	2,5-diphenyloxazole
Cetrimide	=	Cetyltrimethylammonium bromide

CK	=	6-furfuryl amino purine
u.v.	=	Ultra-violet
s	=	sedimentation coefficient
A	=	Absorbance at a designated wavelength
E	=	Extinction at a designated wavelength
O.D.	=	Optical Density
PAGE	=	Polyacrylamide gel electrophoresis
ATP	=	Adenine triphosphate ^{os} /
E.coli	=	<u>Escherichia coli</u>
mol.wt.	=	molecular weight
F.wt.	=	fresh weight
CPM	=	counts per minute
p.p.m.	=	parts per million
MAK	=	Methylated albumin-kieselguhr

1. GENERAL INTRODUCTION

The final stage in the natural life cycle of plants is death with the deteriorative processes which lead to this termination of the functional life of the organism (or organ, cell etc.,) being termed senescence. It is difficult to determine exactly which of the processes that occur in old tissue specifically result in the termination of life and which are simply reflections of changes associated with the passing of time, i.e. ageing. It is possibly because of this and also because senescence is not a well-defined developmental stage compared with, for example, germination or flower initiation that has caused plant senescence to be a relatively neglected field of study. However, since all stages of development are of equal intrinsic interest and also because of the practical implications in terms of post-harvest physiology of fruits and vegetables senescence warrants further study. In addition further interest arises because senescence in plants, in contrast to most animals, appears to have a number of positive functions (Leopold, 1961). An example of this is the onset of senescence in old leaves which is correlated with mobilisation of large amounts of organic materials out of the leaf to younger parts of the plant, particularly

flowers and fruits (Thrower, 1967). Fruit ripening is another form of senescence which is advantageous in terms of seed distribution either by animals or more directly by dehiscence. The advantages of senescence need not be confined to individual plant organs since Leopold (1961) has suggested that plant senescence may have functions relating to ecological adaptation and also facilitating turnover of generations which may be of evolutionary advantage to the species.

Senescence in plants can be divided into a number of general types. In monocarpic species such as cereals the entire plant may die following flowering and fruiting (overall senescence), in other species only the above-ground parts of the shoot may senesce whilst the roots and underground systems remain viable (top senescence). Less drastic forms of senescence are seen in the annual senescence of just the leaves of deciduous trees (deciduous senescence) or the progressive senescence of a particular leaf in response to the formation of young leaves (sequential senescence). That these types of senescence may involve different patterns of metabolism has been proposed by Simon (1967) from a review of the literature

concerning protein synthesis in each of these different cases. Simon also indicated that there was still insufficient information on the subject particularly with regard to the control mechanisms involved, a situation which still holds today.

In this study the sequential senescence pattern was examined and all leaves along the gradient of ageing, from young to senescent, were sampled on single plants. This enabled age related changes in all the chosen parameters to be studied with the possibility that ageing changes as opposed to specific senescence effects might be distinguished. Additionally some studies were also performed using excised leaf discs. The use of leaf discs or excised leaves for the study of senescence is common in the literature as it provides a convenient system in which the incubation conditions can be carefully controlled and the complicating effects of correlative changes in other parts of the plant are avoided. Lewington and Simon (1967) and Simon (1967) however consider that the senescence of excised tissues should be considered a unique form of ageing not necessarily reproducing the same changes as in the intact plant. A comparison of the ageing of the intact leaf and of

excised discs was made to see if this was true for the experimental plant used in this study - chinese cabbage (Brassica pekinensis var. chihili). Chinese cabbage is a vegetable species and as a member of the genus Brassica is closely related to a number of other important vegetable species (e.g. cabbage, cauliflower, brusselsprout, turnip and swede) it is also important as the experimental host for turnip yellow mosaic virus (TYMV) (see below).

In considering leaf senescence certain experimental observations have suggested that it is not due to irreversible changes, at least in the early stages. For example the leaves of many monocarpic species can be prevented from senescing and often have their life spans considerably extended if the young flower buds are removed (Murneek, 1926; Leopold et al., 1959); the old leaves of many plants which have begun to exhibit symptoms associated with senescence, e.g. yellowing, decline in photosynthetic rate, can recover and show renewed synthesis or activity if the plant is decapitated (Das, 1968; Woolhouse 1967). In these instances senescence

appears to have arisen through a physiological imbalance in the plants, rather than through irreversible damage due either to mutations in the genome (Szilard, 1959) or through an accumulation of errors at transcription or translation (Orgel, 1963, 1970; Holliday, 1969). These irreversible changes may however be contributory to the 'endogenous senescence' of individual cells and could be of importance in determining the life span of particularly long-lived organs such as the leaves of conifers though this has yet to be established.

If leaf senescence is the result of a switch in the physiology of the plant or organ it is thus a stage in differentiation similar to other developmental stages and can be characterised by the fact that this switch leads to a series of deteriorative steps which ultimately end in death. The trigger which results in this change can either be environmental or internal, for example, temperature (Mothes and Baudisch, 1958), nutrient deficiency (Williams, 1936) and drought (Gates, 1955) can all lead to leaf senescence. Light, too, can play an important role with light intensity (Brougham, 1958), darkness (Vickery et al., 1937) and particular photoperiods (Lockhart and Gottschall, 1961; Krizek et al., 1966; Spencer and Titus, 1972) all possibly having an effect.

Internal events happening in other parts of the plant can cause senescence of leaves, particularly when flowering and fruiting occur, though even the production of new leaves can lead to senescence of the older leaves in the case of sequential senescence.

During the course of natural leaf ageing and senescence a number of physiological changes have been observed. The rate of photosynthesis frequently declines with age from full leaf expansion (Smillie, 1962; Hardwick et al., 1968; Peat, 1970; Ludlow and Wilson, 1971) though it is not always clear whether this is through actual changes in the dark or light reactions or whether there is some other constraint upon the system e.g. restricted gas flow caused by a change in the stomatal opening. Respiration may also gradually decline with leaf age in some species (Yemm, 1956; Smillie, 1962) but in others a fall in the rate of respiration may not occur until late in the life cycle of the leaf some time after the beginning of the photosynthetic decline (Woolhouse, 1967). A rise in the rate of respiration prior to abscission similar to the respiratory climateric of fruit ripening is observed in some leaves (Eberhardt,

1955; Yemm, 1956; Woolhouse, 1967). Total chlorophyll content of the leaf may also decline though there may be no correlation with the changing photosynthetic rate since different patterns of leaf yellowing are observed. Callow (1969) has demonstrated that in sequentially ageing leaves of Perilla frutescens there is a decline with age in the capacity to carry out cyclic photophosphorylation but in tomato leaves Peat (1970) found that the photochemical efficiency did not change with time except when the leaves showed visible senescent symptoms although the rate of photosynthesis declined after full leaf expansion.

Also with increasing leaf age the total soluble protein content of the leaves declines. In Pisum arvense electrophoresis of the soluble proteins suggests that all fractions decrease in amount with increasing leaf age once growth of the leaf has ceased (Carr and Pate, 1967) whilst in P. frutescens and barley (Woolhouse, 1967; Atkin and Srivastava, 1970) there appears to be a preferential decrease in Fraction 1 of the soluble protein, a chloroplast component. Overall protein synthesis can also decline with leaf age and in P. frutescens there is

a greater loss of capacity for protein synthesis in in vitro systems using chloroplast components (Callow et al., 1972). Simon (1967) and Atkin and Srivastava (1970) have found that the incorporation of radioactively labelled amino acids into proteins is maintained in ageing cucumber cotyledons and barley leaves respectively. In this sort of work treatment of the results is important as is illustrated by the work of Parthier (1964) on leaves of tobacco. He observed that the total incorporation of ^{14}C - leucine into protein was lowest in the old leaves and that there was a decline with age in incorporation when related to uptake, the specific activity however was greatest in the old leaves. Difficulties like this may arise because the overall level of both protein and RNA is much lower in old leaves than in young leaves. Additional problems arise with regard to insufficient information on the turnover and size of precursor pools and the degree to which components of these pools are available for synthesis within the leaf in view of the possible effects of mobilisation and translocation to other parts of the plant which is a marked physiological process in the senescence of attached leaves. Atkin and Srivastava (1970)

have also suggested that the pattern of labelling observed in the protein fractions can vary according to the length and conditions of incubation employed. There are indications that leaves can often retain the ability to synthesise protein even if synthesis may be markedly reduced (Simon, 1967). Protein synthesis could also be affected if the structural components of protein synthesis deteriorated thus ultra-structural studies have shown that a decline in the ribosome population is one of the first indications of the degeneration of the cell (Butler, 1967). The free ribosomes generally appear to decline before the membrane-bound ribosome population and if these two ribosome populations are responsible for the synthesis of different proteins (Setterfield, 1961; Wright, 1963; Payne and Boulter, 1969 a,b) then a significant effect on the metabolism of the cell could result as a consequence of a differential loss between these two ribosome populations.

As suggested by the ultrastructural observation on the ribosome populations RNA content of the leaf also declines with age and appears to be a general characteristic of leaf ageing (Bottger and Wollgiehn, 1958). The bulk of the RNA decline is a result of a loss of ribosomal RNA, the total soluble RNA fraction showing no marked change (Wollgiehn, 1967; Knight and Quick, 1969). In Xanthium pensylvanicum (Dyer and Osborne, 1971), P. frutescens (Callow et al., 1972)

and cucumber the rRNA from the chloroplast declines faster than that of the cytoplasm but in Vicia faba (Dyer and Osborne, 1971) these two fractions decrease at similar rates. Evidence from P.frutescens suggests that there is a differential loss in the capacity for synthesis between chloroplast and cytoplasmic rRNA. Individual rRNA fractions may show age-related changes particularly in the heavy rRNA molecule of chloroplasts (1.1×10^6 mol.wt) which frequently shows an increased lability with age (Ingle, 1968) and Dyer and Osborne (1971) have also found evidence for a preferential breakdown of the 0.7×10^6 mol.wt. component of the cytoplasmic ribosomes in senescing leaves of X. pensylvanicum and tobacco. Bick et al., (1970) and Wright et al., (1973) have found that particular species of transfer-RNA may show marked changes during the course of senescence which may have an influence on the control of protein synthesis (Strehler, 1967; Sacher, 1973). In contrast to the RNA changes in many species there is no significant alteration in the DNA content.

Ultrastructural changes with regard to the cell ribosome population have already been mentioned but a number of other ultrastructural changes are associated with leaf senescence and have been reviewed by Butler and Simon (1971). The typical changes observed in a

range of species are similar and can be summarised as follows. Early senescent changes include the decline of the ribosome population, reduction of the membranes of the endoplasmic reticulum and golgi apparatus and the commencement of chloroplast deterioration. The mitochondria are generally more resistant and often persist until a late stage in the deterioration of the cell as does the nucleus. The tonoplast ruptures before the organelles have completely degenerated but the plasmalemma is often one of the last recognisable components. An increase in permeability of the leaf cells with age was first observed by Sacher (1957) and further work by Eilam (1965) attributed this to an increase in the apparent free space of the cells implying an increase in leakiness of the membranes. This is consistent with the observed deterioration of the cell membrane systems but recent evidence (Wittenbach, 1977) suggests that membrane permeability is not the cause of senescence. Whilst these structural changes are suggestive they do not directly indicate the physiological capabilities of the cell nor the relative importance to the life of the cell of the observed deteriorative changes. Butler and Simon (1971) suggest that the

degenerative changes may become irreversible once the membrane system and in particular the tonoplast has ruptured releasing lysosomal enzymes. An investigation of the ultrastructural changes occurring in sequentially ageing leaves of chinese cabbage and ageing leaves of chinese cabbage infected with TYMV is in progress and preliminary results can be found in appendix 1.

Plant growth hormones have frequently been implicated as important regulators of the senescence associated changes indicated above. Chibnall (1954) observed that Phaseolus leaves that were allowed to root had their life span considerably extended which lead to the suggestion that a factor from the roots may be involved in the regulation of leaf metabolism. The subsequent demonstration by Richmond and Lang (1957) that the application of kinetin (a synthetic cytokinin) to detached leaves of X.pensylvanicum could delay the onset of senescence prompted suggestions that similar compounds may be produced in the roots. In 1962 Kulaeva demonstrated that xylem sap exudates of tobacco had senescence retarding activity and the presence of naturally occurring cytokinin in the xylem sap was subsequently confirmed by Kende (1965). In addition to cytokinins it is now known that the other main plant growth regulators can affect leaf senescence. Thus

gibberellic acid is effective in the retardation of senescence in Taraxacum officinale and Rumex (Fletcher and Osborne, 1965; Whyte and Luckwill, 1966; Goldthwaite and Laetsch, 1967), and auxins are effective in Prunus serrulata (Osborne, 1959; Osborne and Hallaway, 1964), whilst in some species senescence can be enhanced by application of abscisic acid or ethylene (Aspinall et al., 1967; El-Antably et al., 1967; Wareing et al., 1968; Burg 1968). Senescence can also be affected by interactions between these hormones, thus excised leaves of tobacco require both auxin and cytokinin for senescence to be retarded (Engelbrecht and Conrad, 1961), and an interaction between naphthalene acetic acid and kinetin has been observed in detached leaves of broccoli (von Abrams and Pratt, 1966). Back and Richmond (1969) also found interrelationships between gibberellic acid and cytokinins when applied to leaf discs of Taraxacum and Tropaeolum. Antagonistic effects between senescence inhibiting and senescence promoting hormones may also occur (e.g. Back and Richmond, 1971). In situations in which more than one hormone has been shown to affect senescence there are indications that the points of

action are different, for example the interactions between gibberellin, kinetin and abscisic acid in Rumex (Back and Richmond, 1971; Back et al., 1972), which suggests that the different hormones may not affect senescence by the same mechanism and that therefore a number of different potential sites for regulatory action may exist.

The retardation of leaf senescence by plant growth regulators is frequently associated with an improvement in the RNA and protein content of the leaves, and this is also observed when leaf senescence is deferred by decapitation of the plant (Mothes and Baudisch, 1958) or by allowing the leaf to root (Wollgiehn, 1961). The results of early work using radioactive precursors suggested that cytokinin (Sugiura et al., 1962), gibberellin (Fletcher and Osborne, 1965) and auxin (Osborne and Hallaway, 1964; Osborne, 1967) maintained RNA and protein levels by a stimulation of synthesis. Kuraishi (1968) however presented evidence that in Brassica leaf discs cytokinin treatment lead to a decrease in the rate of protein breakdown and therefore suggested that cytokinin action is by the inhibition of protein hydrolysis. Similar conclusions were reached by Tavares and Kende (1970) who found that cytokinin treatment of corn leaf

discs slowed the loss of radioactive label from protein although the specific activity did not change.

Whilst the overall level of protein synthesis in senescing leaves may decline de novo synthesis of specific hydrolytic enzymes has been reported including ribonuclease (McHale and Dove, 1968; Udvardy et al., 1969; Lontai et al., 1971), protease (Martin and Thimann, 1972) and β ,1-3 gluc^aonase (Moore and Stone, 1972). Martin and Thimann (1972) have concluded that protein synthesis, probably of protease enzymes, is important to the development of senescence since the use of inhibitors of proteinsynthesis inhibits senescence in detached oat leaves. Wollgiehn and Parthier (1964) however have found that inhibitors of RNA and protein synthesis accelerate leaf senescence. The mode of action of the growth hormones may be related to the regulation of these hydrolytic enzymes, a hypothesis that is supported by the fact that levels of protease (Anderson and Rowan, 1965; Balz, 1966; Atkin and Srivastava, 1969; Martin and Thimann, 1972) and RNAase (Srivastava and Ware, 1965; Balz, 1966; Srivastava, 1968a; Sodek and Wright, 1969; Wyen et al., 1972; Arad et al., 1973) are lower in detached leaves treated with cytokinins as compared to untreated controls.

Similar retarding effects by auxin on ribonuclease levels have been reported by Sacher (1969), de Leo and Sacher (1970) and Pilet (1970). Stimulation of ribonuclease activity in ageing oat leaves by abscisic acid and in petals of Ipomoea tricolor by ethylene have been observed by Udvardy and Farkas (1972) and Kende and Baumgartner (1974) respectively. If the different hormones can affect these specific enzymes then an alteration in the hormone balance could bring about senescent changes and there are indications that such interactions could occur. Thus a reduction in the rate of root growth at the time of flowering has often been noted (Leonard, 1962) which may be related to the production of IAA at the plant apex (Beever and Woolhouse, 1975), this reduction may lead to decreased cytokinin production in the roots which in turn could have important consequences with regard to shoot senescence (Sitton et al., 1967). In abscission also there appears to be an interaction between auxin and abscisic acid possibly involving ethylene production (Abeles, 1973), and histochemical (Webster, 1968) and biochemical studies (Abeles and Holm, 1967) of the abscission zone have suggested that RNA and protein may be involved. In this work, however, it should

be noted that the measured hydrolytic activities do not necessarily reflect the in vivo degradative activities of the enzymes since the enzymes may be confined to lytic compartments.

It has been indicated above that changes in physiology, biochemistry and ultrastructure are associated with the development of leaf senescence. At present there is no general theory of senescence which adequately correlates these diverse changes, indeed it is not yet established which reactions or loss of reactions are the key factors that lead to senescence. Various means of retarding senescence have been mentioned and the possible regulatory role of plant growth hormones has been outlined both of which are associated with changes in nucleic acids and protein metabolism although the specificity of some of the observed changes is in doubt. Detailed studies on the qualitative changes in rRNA and ribosomes during senescence of the intact leaf have been infrequent (Callow et al., 1972; Callow, 1974; Dyer and Osborne, 1971). Because of this and also because of the possibly central role played by nucleic acids various aspects of RNA and ribosome changes occurring during leaf ageing in chinese cabbage were examined in this study.

One reason for the choice of chinese cabbage as the experimental plant was that it is the experimental host for turnip yellow mosaic virus. This virus was first described by Markham and Smith (1946, 1949) and its host range is restricted to the Cruciferae in which a number of important vegetable crops are susceptible (Broadbent and Heathcote, 1958) though it is particularly virulent in turnip (Brassica rapa) and chinese cabbage (Brassica pekinensis) and in the latter it accumulates in amounts rarely achieved by other plant viruses (Markham, 1959). TYMV is a well studied member of the group of 'spherical plant viruses' and Matthews and Ralph (1966) consider that the contribution made by the study of this virus to our understanding of the structure and function of plant viruses is second only to that of tobacco mosaic virus (TMV). The typical effects of TYMV on the structure and physiology of the host (chinese cabbage) have been reviewed by Ralph and Matthews (1966) and Matthews (1973) so only the main features of TYMV infection will be outlined here.

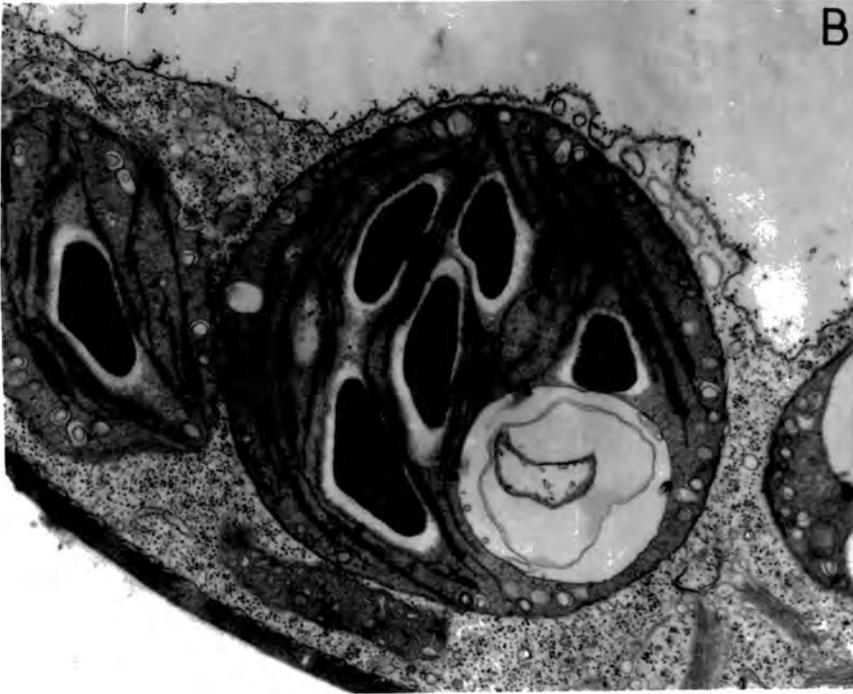
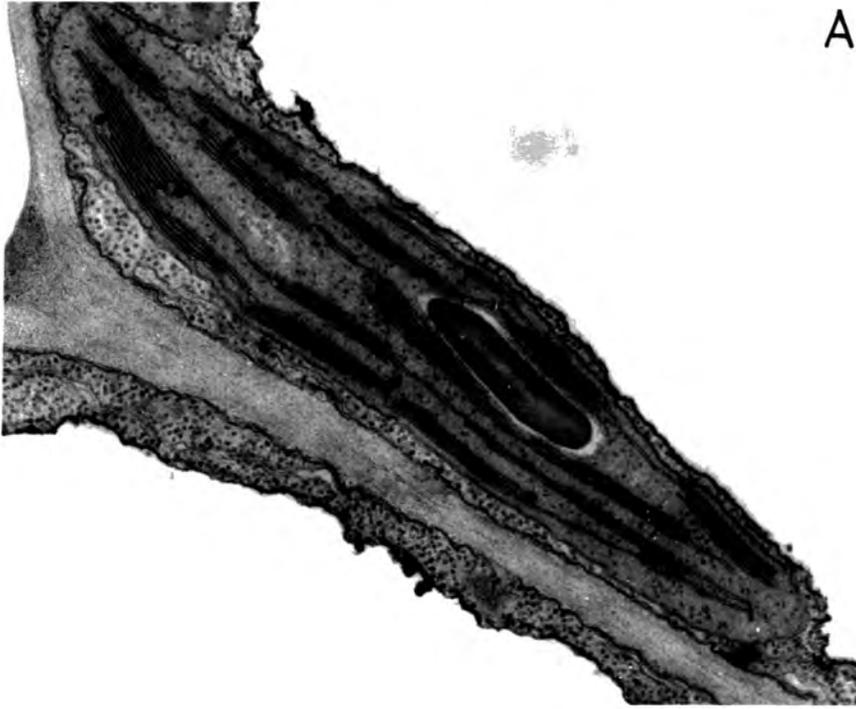
In mechanically inoculated leaves TYMV causes local chlorotic or whitish lesions though their formation is variable and thus the assay of the virus is difficult. Infection results in small stunted plants and the systemically infected leaves show a pronounced mosaic

of dark green, yellow-green and yellow areas the development of which has been described by Reid and Matthews (1966). Cytological studies have shown that the dark green areas are normal but that the other areas of the mosaic show a number of abnormalities of the chloroplast (Chalcroft and Matthews, 1966). The chloroplasts become swollen and rounded and frequently aggregate together but remain separated by a narrow gap. Within the chloroplasts there is a characteristic formation of membrane-bound vesicles, these are of two sorts, small flask-shaped vesicles arranged near the exterior of the chloroplasts and larger vesicles which cause distortion of the stroma lamellae. The number and size of the grana in the chloroplast are also generally reduced (figure 1.1). The nucleus and mitochondria of infected cells appear to be of normal character. Further examination of ultrastructural aspects of TYMV infection of chinese cabbage can be found in appendix 1. Physiologically TYMV infection causes an increase in the Hill reaction and a faster rate of ATP formation by both cyclic and non-cyclic phosphorylation (Goffeau and Bove, 1965). Also during the period of rapid virus synthesis TYMV infection appears to divert the flow of carbon

Figure 1.1

Electron micrographs of chloroplasts

- A. From young uninfected leaf of chinese cabbage
(x 35,500)
- B. From a TYMV-infected leaf of chinese cabbage
(x 14,500)



fixed by the Calvin pathway from sugars to acids by increasing the rate of carboxylation of phosphoenol pyruvate (Bedbrook and Matthews 1972, 1973). Whilst it is known that the chloroplast ribosome population is greatly reduced in systemically infected leaves (Reid and Matthews, 1966) further detailed studies of nucleic acid metabolism in the host plant have been hampered because of the readiness with which TYMV-RNA complexes with the rRNA of the host (Matthews and Ralph, 1966). Difficulties in studying the ribosome population also arise because the presence of TYMV which cosediments with the polyribosomes interferes with measurement of the polyribosomes by analytical centrifugation (Matthews and Ralph, 1966). Clark et al (1964) also experienced problems in obtaining satisfactory sucrose density gradient fractionations of polyribosomes from chinese cabbage because of u.v. absorbing material diffusing or sedimenting into the gradients. In order that a closer examination of the effects of TYMV on nucleic acid metabolism during leaf ageing might be investigated a method of RNA extraction which prevents the formation of the TYMV-RNA-chinese cabbage rRNA complex was devised and the details and characterisation of this are described

in section 3.

2. GENERAL MATERIALS AND METHODS

2.1 Biological materials

2.11 Seeds of chinese cabbage (Brassica pekinensis Rupr. var. chihili) were obtained from Thompson and Morgan, London Road, Ipswich, Suffolk. Seeds were germinated in John Innes compost in seed trays. After two weeks the seedlings were pricked out and potted into 4" plastic pots in a green house at 21°-25°C. Natural light was supplemented in winter with fluorescent lighting to give a day length of 15-17 hours.

2.12 Turnip yellow mosaic virus was the cambridge strain propagated by Dr. R.E.F. Matthews in New Zealand. An inoculum was prepared by grinding leaves of TYMV-infected chinese cabbage showing a uniform yellow mosaic in a mortar with a small amount of carborundum and water. Inoculation was achieved by dipping an index finger into the inoculum and then lightly rubbing the upper surface of the leaves. Plants at the 2-3 leaf stage were normally used. After 10-14 days symptoms of systemic TYMV-infection were apparent on at least 95% of the inoculated plants.

2.13 Seeds of Pisum sativum var. feltham first were obtained from Thompson and Morgan. To obtain imbibed embryos the dry seeds were soaked under running tap water overnight, the testas were then removed from the swollen seeds and the imbibed embryos dissected out. After freezing in liquid air the embryos were stored at -20°C until required.

2.2 Chemicals and Reagents

With the exception of those chemicals listed below all reagents were purchased from British Drug Houses Ltd., or Sigma Chemicals Ltd., and were of analytical grade when necessary.

³H-Uridine from The Radiochemical Centre, Amersham, Bucks.
Triton X-100 from Rohm and Haas U.K. Ltd., Lenig House, Croydon, Surrey.

Agarose from Research Division, Miles Laboratories Ltd.,

TPNS from Kodak Ltd., Kirkby, Liverpool.

PPO and Piperidine from Koch-Light Laboratories Ltd., Colnbrook, Bucks.

2.3 Quantitative Extraction of Cellular Components

The quantitative amounts of the following components in plant samples were determined by modifications of the methods of Osborne (1962).

2.31 Chlorophyll

The tissue was extracted for five minutes in boiling 80% ethanol and then twice in boiling absolute ethanol. The three alcohol extracts were combined and made up to a standard volume. Chlorophyll content was estimated by measuring the peak absorbance at 665 nm in a Perkin-Elmer 402 spectrophotometer (figure 2.1).

2.32 RNA

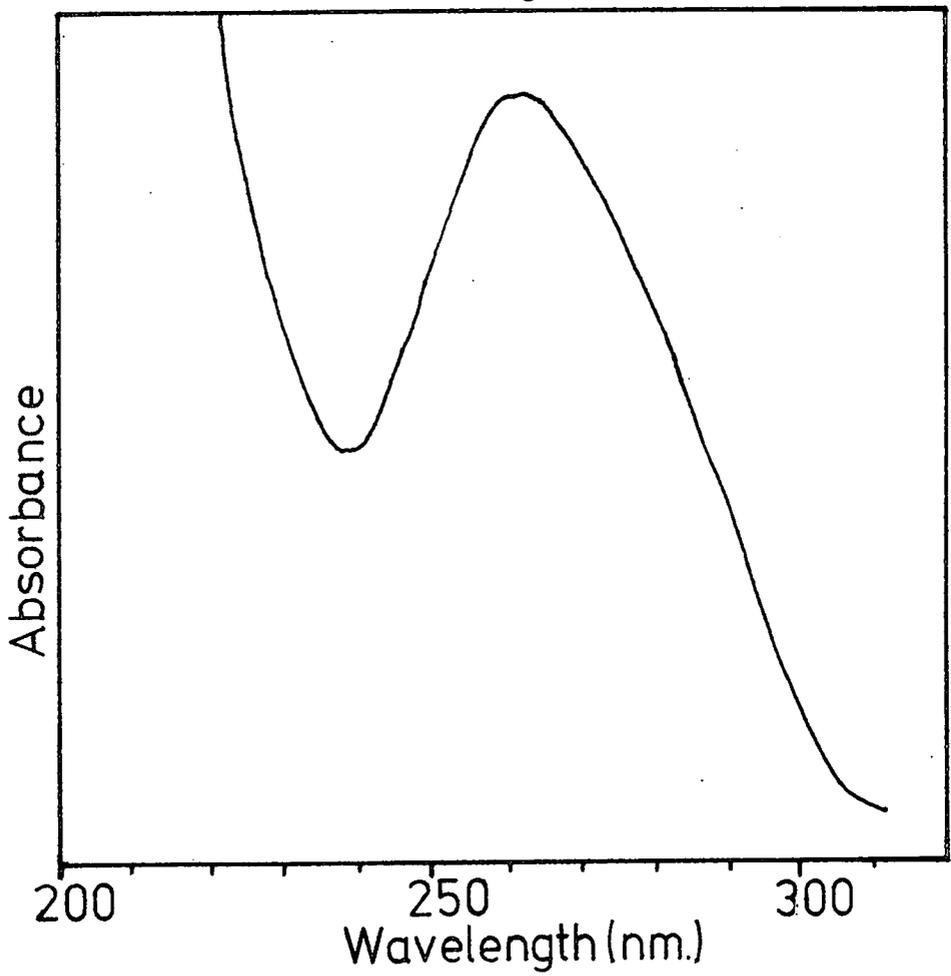
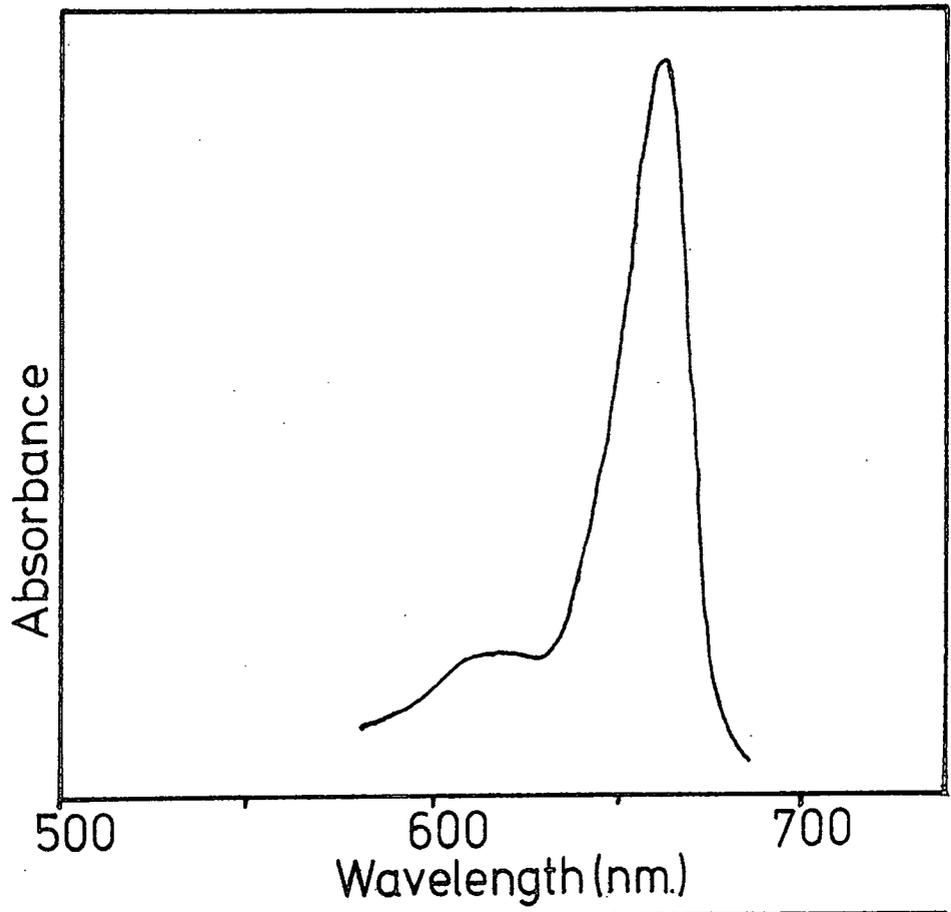
To obtain a quantitative estimate of RNA content the decolourised tissue remaining after the ethanolic chlorophyll extraction was ground in a mortar with a small volume of absolute ethanol. The homogenate was then centrifuged for five minutes at top speed (2,500g.av.) in a MSE minor bench centrifuge, (all subsequent centrifugations were as described here). The pellet was washed twice in absolute alcohol, to remove soluble pools, by resuspension and centrifugation. The final washed pellet was resuspended in 5% TCA and incubated for five minutes at 0°C, after centrifuging the pellet was resuspended in 5% TCA and the incubation repeated. The TCA residue was then pelleted and extracted once in cold absolute alcohol and twice in ethanol : ether : chloroform

Figure 2.1

Absorption spectrum of an ethanolic extract
of chlorophyll.

Figure 2.2

u.v. absorption spectrum of TCA-extracted RNA.



(1:1:1 v/v). The pellet was then resuspended in 0.3M KOH and incubated at 37°C for 16 hrs. After centrifuging and retaining the supernatant the residue was twice resuspended in water and centrifuged. The three supernatants were combined and the pH lowered to approximately 2.0 by addition of HClO₄. The precipitated DNA and insoluble KClO₄ were removed by centrifugation and the supernatant was made up to a standard volume. The ribonucleotide content of the supernatant was then estimated from the u.v. absorption spectrum as recorded with either a Perkin-Elmer 402 or a Pye Unicam SP800 spectrophotometer (figure 2.2). For the purposes of determining the ribonucleotide concentration the following relationship was assumed:

for a 1 cm light path if O.D.260-O.D.290 = 22 O.D. units
then the solution contains 1mg/mlRNA (Tester and Dure,
1966).

2.33 DNA

The residue containing DNA remaining from the quantitative RNA extraction was washed twice by resuspension and centrifugation in cold 5% TCA, once in absolute ethanol and once in ethanol/ether (3:1 v/v). The final washed pellet was resuspended in 0.5M HClO₄ and incubated at 90°C for seven minutes. After centrifuging the supernatant was retained and the pellet washed twice with distilled water. The combined supernatants were made up to a standard volume and the u.v.

absorption spectrum recorded with a Perkin-Elmer 402 or Pye Unicam SP800 spectrophotometer. The concentration of deoxyribonucleotides was determined using the same formula as for ribonucleotides since thymidine has essentially the same extinction coefficient as uridine (Markham, 1955).

3. RNA AND RIBOSOME STUDIES - CHARACTERISATION

3.1 Introduction

It has been established that two different ribosome populations exist in plant leaf tissue (Lyttleton, 1962; Clark et al., 1964; Sissakian et al., 1965; Boardman et al., 1966; Stutz and Noll, 1967), an 80s species similar to animal ribosomes and a 70s species similar to bacterial ribosomes and found in cell organelles. The occurrence of different sized ribosomes suggested that they would contain different sized ribosomal RNA molecules. Fractionation of RNA by MAK chromatography demonstrated that in green radish cotyledons there were one or two more ribosomal RNA species than found in root tips or other non-green tissues (Loening and Ingle, 1967) but these were only partially resolved. Sucrose density gradient fractionation of rRNA gave varying results in different tissues, for example two chloroplastic and two cytoplasmic rRNA species of similar size were found in Acetabularia (Baltus and Quertier, 1966) but lettuce chloroplast rRNA was found to be indistinguishable from cytoplasmic rRNA by Pollard et al., (1966). Spencer and Whitfield (1966) separating chloroplast rRNA from isolated chloroplasts of a number of plant species on sucrose density gradients demonstrated only one RNA species from the chloroplasts and this was of a similar size to the smaller of the two cytoplasmic rRNA species found.

The technique of polyacrylamide gel electrophoresis had at this time only been successfully applied to low molecular weight RNA species (Richards et al., 1965) or to partial digests of ribosomal RNA (Gould, 1966). In 1967 Loening, by the use of purified reagents, managed to apply the technique to high molecular weight RNA from pea seedling root tips, E.coli and rabbit reticulocytes (Loening, 1967). The same year Loening and Ingle (1967) demonstrated that a range of high molecular weight rRNA species existed in green plant tissues. They showed that compared to non-green plant tissues (root-tips and tubers) in green tissues (leaves and cotyledons) two main peaks and a few minor peaks were always present in addition to the two cytoplasmic rRNA peaks. These additional fractions were shown to be associated with the chloroplast fraction. This early work of Loening and others (Mills et al., 1967; Bishop et al., 1967; Peacock and Dingman, 1967; Grossbach and Weinstein, 1968) established the superiority of polyacrylamide gel electrophoresis for the fractionation of high molecular weight rRNA over previously used methods. Dyer and Leech (1968) have also established, by polyacrylamide gel electrophoresis, that the low molecular weight RNA from chloroplasts is distinct from similar material originating in the cytoplasm. These differences were also

evident by MAK column chromatography but not by sucrose density gradient centrifugation.

Richards et al., (1965) had shown that the mobility of low molecular weight RNA in polyacrylamide gels was inversely related to the sedimentation coefficient of the molecule. Loening and Ingle (1967) demonstrated that this relationship held for high molecular weight RNA, and it therefore followed that the mobility should be inversely related to the log (molecular weight). This was shown to be the case for the RNA from a number of virus species (Bishop et al., 1967) and for rRNA from a wide range of procaryotic and eucaryotic species (Loening, 1968c, 1969). Peacock and Dingman (1968) showed that the same relationship was true in composite agarose-acrylamide gels and also that the migration of the RNA species was linear with time, an essential prerequisite for RNA molecular weight determination by polyacrylamide gel electrophoresis. Using the electrophoretic method of molecular weight determination Loening (1968c) established that the high molecular weight RNA from higher plant chloroplasts had approximate molecular weight values of 1.11×10^6 and 0.56×10^6 . These values were similar to those of RNA from bacterial ribosomes and distinct from cytoplasmic rRNA species (1.3×10^6 and 0.7×10^6 molecular weight).

A consistent feature of the RNA fractionation of green tissue from higher plants was that unlike the cytoplasmic rRNA components the expected ratio, based on a mass, of approximately 2:1 between the heavy and the light chloroplast rRNA species was very rarely achieved. The 1.1×10^6 molecular weight RNA was usually considerably depleted and in some species, e.g. swiss chard, was virtually absent (Leaver and Ingle, 1971). These results had in fact lead to a suggestion that chloroplast ribosomes contained only one high molecular weight species of RNA (Spencer and Whitfield, 1966). The low and variable ratio obtained for the chloroplast rRNA species was, however, shown to be the result of the instability of the 1.1×10^6 molecular weight chloroplast rRNA fraction (Loening and Ingle, 1967). The synthesis and stability of this chloroplast rRNA fraction in radish cotyledons was examined by Ingle (1968) who found that the 1.1×10^6 molecular weight RNA was specifically cleaved into two fragments of molecular weights 0.7×10^6 and 0.4×10^6 . If the amount of the 1.1×10^6 molecular weight fraction observed was corrected, allowing for this breakdown, the ratio of the two chloroplast rRNA species was close to the theoretical value based on their molecular weights. Ingle also observed by incorporation studies that newly synthesised 1.1×10^6 molecular weight RNA was stable and

that it was only the older accumulated 1.1×10^6 molecular weight RNA which was susceptible to breakdown. More extensive studies showed that under normal extraction conditions only the 1.1×10^6 molecular weight rRNA was unstable and that the stability varied in different species as did the size and number of breakdown fragments produced (Ingle et al., 1970; Leaver and Ingle, 1971).

Consideration of the conditions of extraction and fractionation of the RNA revealed that the presence of EDTA was concerned with the observed lability of the 1.1×10^6 molecular weight fraction. Replacement of the EDTA by magnesium in the extraction and fractionation buffers resulted in a higher yield of intact 1.1×10^6 molecular weight rRNA (Ingle et al., 1970; Leaver and Ingle, 1971). The presence of magnesium during extraction and fractionation did however have the disadvantage that the RNA obtained contained more non-nucleic acid material than that prepared in EDTA and also the final resolution in the electrophoresis gels was poorer. The breaks that occur in the chloroplast 1.1×10^6 molecular weight RNA could be caused by ribonuclease activity during extraction, however for each plant species the positions of the breaks are very specific and also only one of the high molecular weight rRNA fractions is affected which argues against a random

ribonuclease action. The evidence from the synthesis of this chloroplast rRNA fraction (Ingle, 1968) suggests that the cleavage of the molecule occurs in vivo in the ribosome, presumably at points rendered vulnerable to ribonuclease due to the conformation of the RNA within the ribosomal structure. The ability of the magnesium ions to maintain the integrity of this labile rRNA fraction is also dependant upon the maintained conformation of the RNA within the ribosome since addition of magnesium after extraction fails to reconstitute the dissociated molecule. In the ribosome the broken ends of the polynucleotide chain would be held together thus enabling the magnesium ions to bridge the cleavage point.

While under normal electrophoretic separation of plant rRNA only the 1.1×10^6 molecular weight chloroplast species is unstable it is now apparent that other rRNA species may have 'hidden breaks' in their structure. These fragments can be released from the parent molecule under conditions which break hydrogen bonds, e.g. heating at 60°C for 10 minutes, and then rapidly cooling (Leaver, 1973). This treatment releases a low molecular weight RNA fraction which has been shown to be released from the 25s cytoplasmic RNA of a wide range of plants (Payne and Dyer, 1972; Grierson, 1974) but is not detected in preparations of RNA from

chloroplasts or procaryotic organisms. This RNA species was previously found in animal RNA preparations (Pene et al., 1968; Sy and McCarty, 1970; King and Gould, 1970) and is designated 5.8s RNA (though the terms 6s RNA, 7s RNA, 28s_A RNA and '1'-RNA have also been used). Heat denaturation has also been reported by Higo et al., (1971) and Grierson (1974) in pea and by Yokoyama et al., (1973) in soybean to result in the cleavage of plant 25s rRNA into an 18s product. Reijnders et al., (1973) have also demonstrated marked differences in the apparent size of native and denatured RNA by polyacrylamide gel electrophoresis, though in this instance the changes were probably due to changes in secondary structure and hence electrophoretic mobility. Hepburn and Ingle (1976) have recently disputed some results of conversion of the 25s RNA to a 16-18s RNA product on the grounds that the denaturing conditions were too severe and hence unnatural cleavage products were produced.

The methods used for extracting RNA for qualitative analysis in most common use are based on those of Kirby (1965, 1968) utilising phenol in combination with a detergent. Phenol is an efficient protein denaturant (Kirby 1956, 1957; Pusztai, 1966) which partially inhibits ribonuclease activity. The addition of 8-hydroxyquinoline helps to prevent oxidation of the phenol and has the added

advantage that it has some inhibitory effect on ribonuclease activity (Kirby, 1962). Detergents commonly used in conjunction with phenol are sodium trimisopropyl naphthalene sulphonate (TPNS) and sodium-4-aminosalicylate (PAS) (both used in this present study) which are capable of dissociating nucleic acid bound protein. The nucleic acid is finally obtained from the aqueous solution by precipitation with ethanol. However the use of phenol as an extraction medium for obtaining RNA has certain disadvantages. Phenol has a high absorption in the ultraviolet wavelengths which interferes with spectrophotometric assay of the RNA and can produce high background absorptions on polyacrylamide gel electrophoresis. This contamination can be removed by washing of the nucleic acid though the final RNA yield is reduced, in addition RNA may also be lost during phenolic extraction in the precipitates of denatured proteins (Poulson, 1973). The incomplete inhibition of ribonuclease activity (Rushizky et al., 1963) is a further drawback.

In 1938 Boehm and Metha first synthesised the bacteriocidal agent diethylpyrocarbonate (DEP), a substance which reacts with proteins resulting in irreversible structural modifications (Rosen and Fedorcsak, 1966; Wolf et al., 1970) and because of this it is a powerful inhibitor of enzyme activity. The demonstration that this inhibitory

activity extended to nucleases led to the suggestion that it might be used successfully for the extraction of nucleic acid (Fedorcsak and Ehrenberg, 1966). Solymosy and co-workers (Solymosy et al., 1968, 1970) subsequently used DEP in combination with sodium dodecyl sulphate (SDS) for the extraction of RNA from tobacco, barley, pea and bean leaves. Solymosy et al., (1968) and Lazar et al., (1969) compared the DEP-SDS RNA extraction method with RNA extraction by the phenol-SDS method of Itai and Hirai (1966). In young leaves the DEP-SDS method was observed to give a higher yield and better protection against breakdown by ribonuclease but in ageing tobacco leaves this method gave a lower yield of heavy ribosomal RNA as compared to the phenol method (Lazar et al., 1969). Modifications of the original method (Solymosy et al., 1970) eradicated this preferential loss and higher yields of both chloroplast and cytoplasmic rRNA were obtained by the DEP-SDS method when compared with the more quantitative detergent-phenol extraction procedure of Ingle and Burns (1968) though this latter method was carried out at room temperature which may have led to some degradation of the RNA. The electrophoretic behaviour of DEP-SDS extracted rRNA appeared to be similar to that of phenol extracted rRNA though this was not expressly examined. Melera et al., (1970) have compared

the electrophoretic characteristics of DEP and phenol extracted rRNA from Physarum polycephalum and obtained similar results with both extraction methods. The hybridisation of RNA to DNA from E.coli has also been reported to be unaffected when the RNA was extracted in the presence of DEP (Summers, 1970).

The use of DEP for the extraction of RNA has now been reported to be successful in animal tissues (Abadom and Elson, 1970), Algae (Cattolico and Jones, 1972), Fungi (Chet and Rusch, 1970; Forrester et al., 1970), bacteria (Summers, 1970) and viruses (Gulyas and Solymosy, 1970; Oberg, 1970; Oxelfelt and Arstrand, 1970) in addition to plant tissues. However caution in the use of DEP has been expressed following reports that chemical modification of nucleic acids can occur, probably by carboxymethylation of the bases (Denic et al., 1970; Oberg, 1971(b); Leonard et al., 1970; Solymosy et al., 1971; Henderson et al., 1973). DEP extraction of ribonucleic acids has been shown to give products retaining their template activity (Fedorcsak et al., 1969), transfer activity (Abadom and Elson, 1970; Fedorcsak et al., 1969) and infectivity (Oxelfelt and Arstrand, 1970). In contrast, however, under certain conditions DEP does have a detrimental effect; Gulyas and Solymosy (1970) and Oxelfelt and Arstrand (1970)

observed that DEP treatment inactivated purified TMV-RNA, Denic et al., (1970) and Ortwerth (1971) both found a loss of tRNA acceptor activity, and single-stranded poliovirus RNA is also inactivated (Oberg, 1970). The transforming ability of bacterial DNA (Fedorcsak and Turtoczky, 1966), the infectivity of double-stranded phage DNA (Kondorosi et al., 1972) and the double-stranded replicative form of polio virus RNA (Oberg, 1971a) however are unaffected.

Whilst one is aware of the possible adverse effects of the use of DEP in RNA extraction on the biological activity of RNA the method does give good recoveries and is very effective in yielding relatively undegraded RNA. Also the simpler procedure of extraction suggested that this method might be advantageous in situations in which only small quantities of starting material were available. For these reasons the DEP-SDS method was tested and characterised with chinese cabbage leaf material.

Studies of the effects of turnip yellow mosaic virus on the nucleic acid metabolism of its host have been greatly hampered by the formation of an aggregation complex between the host RNA and TYMV-RNA on extraction of the total RNA from infected leaves (Matthews and Ralph, 1966). Matus et al., (1964) reported that the typical green leaf RNA profiles obtained from uninfected chinese cabbage leaves by both

analytical centrifugation and sucrose density gradient centrifugation were not observed with TYMV-infected leaf extracts in which a rapidly sedimenting aggregate was apparent. The high purity ratios obtained from the ultra-violet absorption profiles of infected-leaf extracted RNA suggested that virus induced impurities were not the cause of this aggregation. They have suggested that the complex formation is the result of non-specific aggregation probably related to the unusually high cytosine content of TYMV-RNA (38%), possibly by hydrogen bonding between the cytosine residues and available bases of the host RNA that are not internally base paired. That an open secondary structure is required for this aggregation to occur is suggested by the fact that low molecular weight 4s RNA (mainly tRNA) is not complexed. Attempts to break the aggregate by treatment with ribonuclease, EDTA or by including urea in the isolation or purification stages were unsuccessful. MAK column chromatography also failed to separate the host and viral nucleic acids because the aggregate could not be eluted from the column (Matus et al., 1964). Non-aqueous extraction of the nucleic acid in formamide-chloroform or the use of heat-shock to break the complex have also proved unsuccessful (Pearson, unpublished data) likewise fractionation of the complex by polyacrylamide gel electrophoresis does not

dissociate the complex (this thesis). The use of the DEP-SDS RNA extraction method to obtain uncomplexed host RNA from TYMV-infected leaves is described and characterised below.

Ribosomes are relatively stable particles and have sedimentation properties that are quite different from other cell organelles and particles, therefore provided steps are taken to maintain their stability (e.g. including magnesium ions in the buffers) they can be obtained by fairly simple procedures. Extraction of polyribosomes is more difficult as the exposed strands of messenger RNA between the ribosomes are particularly susceptible to shearing forces and ribonuclease attack. Obtaining good yields of intact polyribosomes from plant tissues has particular difficulties in that the presence of tough cell walls require harsher homogenisation (and therefore greater shearing forces) and also because of the high levels of ribonuclease activity usually present. A wide range of possible nuclease inhibitors have been used in conjunction with ribosome isolation from plant tissues, such as Zn^{2+} (Barker and Rieber, 1967), Cu^{2+} (Hall and Cocking, 1966), polyvinyl sulphate (Clark et al., 1964), Macloid (Marcus et al., 1967), Bentonite (Watts and Mathias, 1967) and DEP (Weeks and Marcus, 1969; Anderson and Key, 1971). Payne and Loening (1970) have systematically studied a wide range of substances used as ribonuclease inhibitors

(but not DEP) in pea root microsome isolation. Bentonite was found to be the most effective inhibitor but as already found by Tester and Dure (1966) extensive ribosome binding by the bentonite was in evidence; a property utilised by Dunn and Hitchborn (1965) to purify some plant viruses. The preparation of bentonite is also tedious as exact processing and grading of the particles is critical for maximum activity (Watts and Mathias, 1967). In chinese cabbage leaf tissue Clark et al., (1964) found that the use of polyvinyl sulphate as a nuclease inhibitor gave some protection to polyribosome isolates, however, satisfactory sucrose density gradient fractionations of the polyribosomes could not be obtained because the polyvinyl sulphate interfered with the ultra-violet monitoring of the gradients. Weeks and Marcus (1969) using wheat embryos and roots, and Anderson and Key (1971) using soybean hypocotyls and mung-bean embryonic axes have successfully used DEP as a nuclease inhibitor during polyribosome extraction. This inhibitor was also found to be successful in protecting polyribosomes extracted from cotyledons of broad bean (Vicia faba) in which there is a high proportion of membrane-bound polyribosomes (Lonsdale and Boulter, 1973), but under certain conditions the use of DEP has been reported to result in the dissociation of the ribosomes into sub-units (Anderson and Key, 1971;

Huvos and Solymosy, 1971). The use of DEP for the isolation of polyribosomes from green leaf tissue was therefore examined in chinese cabbage.

The separation of ribosome fractions is most commonly performed by sucrose density gradient centrifugation (see reviews of Peterman, 1964; Birnie et al., 1969) and with extreme care in preparation and monitoring excellent resolution can be obtained (Noll, 1969). Stutz and Noll (1967) have also achieved resolution between 80s cytoplasmic and 70s chloroplastic ribosomes from pinto beans on a preparative scale by the use of isokinetic sucrose density gradients but there are indications that under the usual conditions for sucrose density gradient centrifugation degradation of the profiles can occur, possibly as a result of hydrostatic pressures (Infante and Baierlein, 1971). As an alternative to centrifugation methods the use of polyacrylamide gel electrophoresis for the separation of ribosome fractions has been described by Hjertens et al., (1965) but although a number of reports have since appeared in the literature the technique is not widely used. Because the parameters on which electrophoretic separation is based are different to those operative during centrifugation techniques it might be expected that additional information on ribosome properties could be obtained by the use of this method. That this was

so has been demonstrated by Dahlberg et al., (1969) who showed that there was a heterogeneity of electrophoretic migration of the 30s sub-unit of E.coli ribosomes, a heterogeneity not previously evident by centrifugation methods of analysis. This electrophoretic heterogeneity has subsequently been shown to extend to both the 50s sub-unit and intact 70s ribosomes of E.coli (Talens et al., 1970, 1973). The excellent resolution of the polyacrylamide gel electrophoresis method has now been utilised to study the effects of the protein factors necessary for protein synthesis on the behaviour of E.coli ribosomes (Talens et al., 1970; Vermeer et al., 1971; Bauer and Keuchler, 1974). The nature of sub-unit association examined by Van Diggelen and Bosch (1973) in E.coli and von der Decken et al., (1970) in rat tissues also involved the use of polyacrylamide gel electrophoresis of the ribosome fractions, likewise the technique has been utilised to distinguish between the 70s ribosomes of the blue-green alga Anabaena flosaquae and those from E.coli (Carlton and Herson, 1972). Using conventional centrifugation techniques the sedimentation coefficients for ribosomes extracted from mitochondria are in the range 40s to 80s or more. In their review Ashwell and Work (1970) have indicated that the size distribution of mitochondrial ribosomes may be related to phylogenetic factors where

higher organisms such as mammals, amphibians, birds and insects appeared to contain mitochondrial ribosomes with sedimentation values of 50s-60s and lower eucaryotes (yeast and fungi) had mitochondrial ribosomes with sedimentation values in the range 70s-80s. The use of polyacrylamide gel electrophoresis for the fractionation of mitochondrial ribosomes has revealed that the 55s ribosomes of rat liver mitochondria had a larger volume than 70s E.coli ribosomes but were smaller than rat liver cytoplasmic ribosomes (De Vries and van Der Koogh-Schuuring, 1973), also for the ciliate Tetrahymena pyriformis it had been possible to separate 80s mitochondrial ribosomes from the cytoplasmic ribosomes with which they co-migrate during sucrose density gradient centrifugation (Curgy et al., 1974). In a further extension of the method it has been demonstrated that electrophoresis in polyacrylamide gels is also capable of separating poly-ribosomes. Dahlberg et al., (1969, 1973) were able to discern ribosome polymers from E.coli up to the octamer and Ledoigt et al., (1975) published profiles indicating polymers up to the tetramer from Tetrahymena pyriformis.

Although the potential usefulness of the electrophoretic method of ribosome separation has been demonstrated its application has been almost entirely confined to lower

organisms and reports of polyacrylamide gel electrophoresis of ribosomes from higher plants are rare except for those reported by Thomas (1973) and Ledoigt et al., (1975). In view of this and because of the general advantages inherent in polyacrylamide gel electrophoresis as an analytical technique a method of polyacrylamide gel electrophoresis suitable for the fractionation of higher plant polyribosomes was developed from that described by Thomas (1973) for the fractionation of pea seed ribosomes. The description and characterisation of this method follows below.

In TYMV-infected chinese cabbage plants the presence of TYMV, with a sedimentation value of 114s, in the infected leaves has made study of the host ribosomes difficult because it interferes with the measurement of ribosomes using Schlieren optics in the analytical ultracentrifuge (Matthews and Ralph, 1966). Use of sucrose density gradients has also proved unsatisfactory since Clark et al., (1966) were unable to obtain polyribosome profiles from chinese cabbage that were free of non-ribosomal ultra-violet absorbing material diffusing or sedimenting into the gradients. Whilst satisfactory sucrose density gradients were obtained from chinese cabbage leaves in this study interference by the presence of TYMV in extracts from infected leaves was

confirmed. The application of the polyacrylamide gel electrophoresis method of ribosome fractionation as a possible means of examining host polyribosomes extracted from TYMV-infected tissue is also described.

3.2 Methods

3.21 Qualitative Extraction of Ribonucleic Acid

3.21.1 Detergent-phenol extraction

The method used was adapted from the detergent-phenol method (Kirby, 1965; Loening, 1967) as described by Leaver and Ingle (1971).

The tissue was homogenised either with a pestle and mortar or for 1 minute in a Virtis '45' homogeniser, set at full speed, with five volumes of detergent medium consisting of:

1% tri-isopropylphthalene sulphonate

6% p-aminosalicylate

50mM NaCl

10mM MgCl₂

10mM Tris-HCl pH 7.4

The homogenate was then mixed with an equal amount of phenol mixture consisting of:

Redistilled phenol containing

10% m-Cresol

0.1% 8-hydroxyquinoline

saturated with 10 mM tris-HCl pH 7.4

followed by blending using a 'whirlimixer' (Fison Scientific Apparatus Ltd., Loughborough) and was centrifuged for 10 minutes at top speed in an MSE minor bench centrifuge at room temperature. The lower phenol layer was removed using

a Pasteur pipette and the upper aqueous layer and interphase material was made 0.5M with respect to NaCl. This aqueous fraction was then extracted a second time by addition of an equal volume of phenol mixture. The two phases were separated by centrifugation, as above, and the upper aqueous layer was removed with a pasteur pipette, care being taken not to disturb the interphase material. The aqueous supernatant was made 10 mM with respect to EDTA (by adding an appropriate volume of a 0.25M stock solution). Following a final phenol extraction the aqueous phase was removed and the nucleic acid precipitated from it by addition of two volumes of absolute ethanol and storage overnight at -20°C . The precipitated nucleic acid was pelleted by centrifugation for 10 minutes at full speed in a bench centrifuge. The pellet was washed once, by resuspension and centrifugation, in 80% ethanol containing 0.5% SDS and then dissolved in 0.2M sodium acetate + 0.5% SDS and the nucleic acid was again pelleted by centrifugation and then resuspended in 0.2M sodium acetate. 1% cetrimide was added (0.5ml per 1.0ml sodium acetate) (Ralph and Bellamy, 1964). After 2 hours at 0°C the insoluble cetab.salt of RNA was pelleted by centrifugation in a bench centrifuge (10 mins. full speed). The pellet was washed three times in 70% ethanol containing 0.1M sodium acetate to convert CTA-RNA to the sodium salt and to remove CTA-acetate. The final RNA

pellet was normally redissolved in a small volume of the appropriate electrophoresis buffer (see 3.22.2) and either used immediately or stored at -20°C .

All the above operations were carried out at $0-4^{\circ}\text{C}$ and the various solutions stored at 4°C .

3.21.2 DEP-SDS extraction

The method used was modified from that of Solymosy et al., (1968, 1970).

The tissue was either ground by hand with a pestle and mortar or at full speed in a Virtis '45' homogeniser for 90 seconds with 12 ml of extractant buffer per gram fresh weight of tissue:

0.12M tris-acetate pH 7.2

10 mM Magnesium acetate

60 mM SDS

0.4 ml DEP

the SDS was added separately from a stock solution. DEP which is unstable in aqueous solution, was also added separately to the buffer. The homogenate was incubated at 37°C for 5 minutes and then centrifuged at 2.500g. in a MSE minor bench centrifuge for 10 mins. The pale yellow supernatant was decanted off and retained. This supernatant was made 10% with respect to sodium chloride and incubated at 37°C for 5 minutes. The homogenate was then centrifuged at 10,000 g. for 20 mins. at 4°C in a MSE 18 centrifuge.

Nucleic acid was precipitated from the resulting colourless supernatant by the addition of two volumes of cold absolute ethanol and overnight storage at -20°C . The precipitated nucleic acid was pelleted by centrifugation and dissolved in a small volume of an appropriate electrophoresis buffer. Further purification of this first ethanol precipitate was found to be unnecessary for subsequent electrophoresis.

3.22 Fractionation and Characterisation of RNA

3.22.1 Spectrophotometric estimation of RNA

The concentration of RNA resuspended in buffer was estimated from the u.v. absorption spectrum assuming the following relationship:

$$\text{OD}_{260} - \text{OD}_{290} = 22 \text{ OD units} = 1\text{mg/ml RNA}$$

(Tester and Dure, 1966).

The u.v. absorption spectrum was recorded with a Perkin-Elmer 402 or a Pye Unicam SP 800 spectrophotometer using a distilled water blank.

3.22.2 Polyacrylamide gel electrophoresis of nucleic acids

Polyacrylamide gels were prepared essentially by the methods of Loening (1967, 1968a). Acrylamide and methylene bisacrylamide were recrystallised from chloroform and acetone respectively (Loening, 1967). For gels of an acrylamide proportion of less than 5% a stock monomer solution of 15% acrylamide and 0.75% bisacrylamide was prepared, for gels of a greater acrylamide percentage the

the monomer stock solution contained 15% acrylamide and 0.325% bisacrylamide. These stock solutions were stored in the dark at room temperature. Ammonium persulphate (10%) was stored at 0 °C and was made up fresh every two weeks.

The buffers used for electrophoresis were:

'3E' buffer of Bishop et al., (1967)

0.12M tris-acetate pH 7.2

0.06M sodium acetate

0.003M disodium EDTA

'3E' - magnesium buffer (derived from '3E' buffer by replacing the EDTA with magnesium acetate)

0.12M tris-acetate pH 7.2

0.06M sodium acetate

0.01M magnesium acetate

'E' buffer of Loening (1969)

0.036M tris-HCl pH 7.7

0.03M sodium dihydrogen phosphate

0.001M disodium EDTA

This buffer was prepared as a three-fold concentration stock solution. The desired acrylamide concentrations in the gel were prepared by using the below proportions of reagents.

	Gel strength (% acrylamide)				
	2.2	2.4	2.6	3.0	7.5
Stock monomer (ml)	3.66	4.0	4.33	5.0	12.5
Water (ml)	12.78	12.44	12.11	11.44	3.94

8.32ml of buffer (or three-fold buffer stock solution) and 0.02ml of TEMED were then added and the solution mixed and degassed under vacuum. 0.2ml of 10% ammonium persulphate was then added to the solution and after mixing the gels were cast from the mixture in vertically held 'plexiglass' tubes (9cm x 0.6cm intl. diam.) to a depth of 8.0cm. To support the gels the lower end of the 'plexiglass' tubes were sealed with dialysis membrane held in place with a rubber ring. To prevent the gel solution passing through the membrane before it had set a closed rubber tube was fitted over the membrane thus forming an air seal. Polymerisation was normally complete in about 20 minutes after which the air seals were removed before transferring the gels, in their tubes, to the electrophoresis tank. With 7.5% polyacrylamide gels the surface of the gel solution was carefully layered with a small amount of water applied to the meniscus of the solution by means of a syringe to ensure a flat surface to the gel. This was not a problem with gels of lower acrylamide concentration. The gel running buffer was prepared by diluting the buffer or stock solution 3:1 with distilled water and adding SDS to a

final concentration of 0.2%.

The electrophoresis was carried out at room temperature using a Vokam power pack set on constant current mode. The gels were pre-run at 6mA/gel (50-55v total voltage) for 30 minutes prior to loading the samples. 15-25 μ g of RNA in 10-15 μ l of buffer plus approximately 10% sucrose were loaded onto each gel and electrophoresed at 6 mA/gel for 3 hours. On completion of electrophoresis the dialysis membrane was removed from the bottom of the tube and the gels were gently blown into a petri dish. The gels were then washed for two hours in 7% acetic acid to remove the strong u.v. absorbing material present at the top of the gel. Swelling of these low acrylamide percentage gels was not appreciable during this short washing period. RNA bands were located by scanning the gels at 265 nm in a Joyce-Loebl Polyfrac (fitted with a 265 interference filter) linked to a Servoscribe chart recorder. The area under peaks on gel traces was measured by weighing tracings of the peaks.

RNA bands were also located by staining for 2 hours in 0.02% toluidine blue. Destaining was achieved with several changes of distilled water.

3.22.3 Molecular weight determinations

The molecular weights of the various RNA fractions were determined from a plot of log (molecular weight) against mobility using a standard marker (Peacock and Dingman, 1968;

Loening, 1968, 1969; Bishop et al., 1967). The standard used was E.coli ribosomal RNA (obtained from British Drug Houses) using values of 1.08×10^6 and 0.56×10^6 (Loening, 1969) for the two major peaks.

3.23 Preparation of chloroplasts and chloroplast RNA

3.23.1 Isolation of chloroplasts

The method of chloroplast isolation adopted was modified from that of Kirk and Pyliotis (1972).

8 - 10g quantities of leaf lamina were surface sterilised for 30 seconds in 70% ethanol, 5 minutes in 3% hypochlorite and then rinsed in sterile distilled water and blotted dry. The leaves were chopped into small pieces and mixed with 50 ml of chilled extraction medium:

0.02M tris-HCl pH 7.2

0.5M Sucrose

5mM $MgCl_2$

The tissue was blended in a Virtis '45' homogeniser for 5 seconds at full speed then 3 seconds also at full speed. The homogenate was strained through two layers of 'miracloth' (Calbiochem) and the filtrate centrifuged for 7 minutes at 1,250g. av. in a bench centrifuge. The chloroplast pellet was then gently resuspended in 20 ml of fresh extraction medium and centrifuged again (7 mins. 1250 g. av.) to give a final washed chloroplast preparation.

3.23.2 Extraction of RNA from isolated chloroplasts

The washed chloroplast pellet was resuspended in 5.0ml of the DEP-SDS RNA extraction buffer (see 3.21.2) without the SDS and DEP but including 0.5M sucrose. (Sucrose was included to permit examination of the intact chloroplasts under the light microscope prior to extraction). The chloroplast membranes were then solubilised by the addition of 20% Triton X-100 to a final concentration of 2% (Leaver and Ingle, 1971) and mixing on a 'whirlimixer'. After centrifugation at 10000g. for 10 minutes at 4°C (MSE 18 centrifuge) the green supernatant was extracted for RNA by addition of 10% SDS, to a final concentration of 1%, and 0.2ml DEP and continuing the DEP-SDS RNA extraction as described in 3.21.2.

The final supernatant was a green colour and a green precipitate resulted on ethanolic precipitation of the nucleic acids, this problem is discussed in 3.45. Chloroplast RNA was fractionated by electrophoresis in polyacrylamide gels as detailed in 3.22.2.

3.24 Preparation and Fractionation of Ribosomes

3.24.1 Isolation of ribosomes

Polyribosomes were extracted essentially by the method of Jachymczyk and Cherry (1968) as described by Pearson (1969b).

Leaf laminae were washed in distilled water, blotted dry and immediately frozen in liquid air to prevent polyribosome loss by wilting and also to aid homogenisation. The frozen

leaves were ground to a powder in a pre-cooled mortar and then further ground after the addition of 3ml per gram fresh weight of extractant buffer:

- 0.02M tris-HCl pH 8.0
- 0.25M Sucrose
- 0.01M Magnesium chloride
- 0.015M Potassium chloride
- 0.005M β -mercaptoethanol
- 0.5% Sodium deoxycholate

The resulting brei was strained through one layer of 'miracloth' and the filtrate centrifuged at 20,000g. for 15 mins. at 4^oC in an MSE 'High Speed' 18 centrifuge. 3.0 ml aliquots of the post-mitochondrial supernatant were layered over 1.0ml 0.5M and 3.0ml 1.6M sucrose made up in 0.02M tris-HCl pH 8.0, 0.01M magnesium chloride, 0.075M potassium chloride, 0.005M β -mercaptoethanol and centrifuged at 105,000 g.av. for 3 hours at 0^oC in the 10 x 10ml titanium angle rotor in an MSE 'Superspeed 65' ultracentrifuge (Wettstein et al., 1963). Following centrifugation the supernatant was decanted off and the tubes drained by inversion. The walls of the centrifuge tubes were then wiped dry with absorbant tissue paper. The resulting clear or pale yellow polyribosome pellet was either used immediately or stored at -20^oC. It was essential for good preparations that the extraction was performed rapidly and that the

operations were carried out at 0-4°C.

3.24.2 Spectrophotometric estimation of ribosomes

Estimation of ribosomal RNA in polyribosomes was determined from the u.v. spectrum by the method of Tester and Dure (1966) where

$$OD_{260} - OD_{290} = 11 = 1 \text{ mg/ml ribosomes}$$

3.24.3 Fractionation

(a) Sucrose Density Gradient Centrifugation

Polyribosomes were fractionated on 16ml 15-34% (w/v) linear sucrose density gradients prepared by modification of the method of Britten and Roberts (1960).

Stock solutions of 15% and 35% sucrose were made up in buffer:

0.02M tris-HCl pH 8.0

0.01M Magnesium chloride

0.02M Potassium chloride

A 2.0ml pad of 50% sucrose was pipetted into a centrifuge tube to act as a cushion layer. With the connection between the chambers of the gradient former closed 8.0ml of the 15% sucrose was pipetted into the outer chamber and 8.0ml of 34% sucrose was pipetted into the mixing chamber. The centre valve was then opened and the solution allowed to flow through the exit tubing and down the wall of the centrifuge tube thus building up the gradient. To ensure mixing of the two sucrose solutions a magnetic stirrer was introduced into the mixing

chamber of the gradient former.

150-300 μ g ribosomes resuspended in approximately 0.5ml of buffer were carefully layered onto the surface of the gradient which was then centrifuged at 95,000 g.av. for 90 minutes at 2^oC in a 3 x 23ml swing-out rotor in an MSE 'superspeed-65' ultracentrifuge. The centrifuge was allowed to slow down without the use of the brake. The gradients were fractionated using an Isco density gradient fractionator, model 180, in conjunction with a tube piercing device (Lonsdale, 1972). The gradient was displaced with 50% (w/v) sucrose pumped into the bottom of the centrifuge tube at a rate of 0.5ml per minute and was monitored at 254 nm with an Isco optical unit, model UA, fitted directly to the top of the tube containing the gradient. The absorption of the gradient was recorded on a Servoscribe chart recorder.

(b) Polyacrylamide Gel Electrophoresis

(i) Polyribosomes

The conditions for the electrophoresis of polyribosomes were modifications of the method used by Thomas (1973) for the electrophoresis of pea seed ribosomes.

2.2% polyacrylamide gels were prepared as described in 3.22.2 but five-fold concentration stock solutions of buffer were employed (Loening, 1968a). The buffer used in the gels and as electrophoresis running buffer (diluted five times) was:

0.1M tris-acetate pH 8.0

75mM potassium acetate

37.5mM Magnesium acetate

The gels were pre-run for 30 minutes at 13v per gel (approximately 10 mA per gel) using a Vokam power pack set on constant voltage mode. Polyribosome samples containing 5-15 μ g ribosomes in 5-40 μ l of electrophoresis buffer plus 10% sucrose were loaded onto the gels. Electrophoresis was carried out at 13 v per gel for 2 hours at 2^oC. The buffer in the electrophoresis tank reservoirs (approximately 1.5 litres total volume) was circulated from the anode chamber to the cathode chamber using a peristaltic pump (circulated at a rate of 5.0ml per min.) in order to prevent the depletion of magnesium in the gels by accretion of magnesium ions at the cathode.

On completion of electrophoresis the gels were washed for 2 hours in 7% acetic acid prior to location of the polyribosome bands by scanning at 265 nM in a Joyce-Loebl Polyfrac. Polyribosome fractions were also visualised by staining for RNA with 0.02% toluidine blue (destaining with several changes of distilled water) and by staining for protein with 0.1% amido black in 7% acetic acid (destaining with several changes of 7% acetic acid).

Extreme care was required in the handling of these very low acrylamide content gels which had little mechanical

rigidity. This was particularly so with stained gels which swelled considerably during the staining procedure.

(ii) Ribosomal Sub-units

The ribosomes present in the 105,000g. pellet were dissociated into their constituent sub-units by resuspension in a buffer containing pyrophosphate:

20mM tris-acetate pH 8.0

50mM potassium acetate

5mM tetrasodium pyrophosphate (Thomas, 1973)

Ribosomal sub-units were fractionated in 3.0% polyacrylamide gels prepared as previously described (3.22.2), but using a five-fold concentration buffer solution (Loening, 1968a).

The buffer was:

0.125M tris-HCl pH 8.0

5mM Magnesium chloride

and was diluted five-fold for the gel running buffer. Gels were pre-run for 30 minutes at 13v per gel (approx. 5mA per gel). 10-40µg of ribosomal sub-units in resuspension buffer plus 10% (w/v) sucrose were carefully loaded onto the tops of the gels. Electrophoresis was carried out at 13v per gel for 3 hours at 2^oC. Buffer recirculation and the large capacity electrophoresis tank were employed.

Ribosomal sub-unit fractions were located by u.v. scanning and by staining as described for polyribosome gels.

Fractionation of ribosomal sub-units could also be

achieved by direct electrophoresis of samples previously resuspended in ribosome resuspension buffer. In this situation the resuspended ribosomes were electrophoresed in 3.0% gels prepared using an EDTA-containing buffer:

0.125M tris-HCl pH 8.0

12.5mM EDTA

The gel running buffer was a five-fold dilution of this buffer. The other conditions of electrophoresis were as described above except that buffer recirculation was unnecessary.

(iii) Fractionation in composite Agarose-Acrylamide Gels
Ribosomes were fractionated in gels of lower acrylamide concentration by introducing an agarose support. The gels were prepared in gel tubes by the method of McDevitt and Muir (1971).

Agarose was dissolved in a sufficient volume of water at 100°C to give a final concentration in the gel of 0.5%. The solution was stirred continuously until the agarose had fully dissolved and was then cooled to 40°C. At the same time the proportions of buffer, monomer mixture and TEMED required for a 2.0% acrylamide gel were mixed and heated to 40°C. When both solutions were at the same temperature they were mixed together and shaken. The ammonium persulphate was then added and after mixing the solution was pipetted into vertically held 'plexiglass' tubes as previously described.

A lowered persulphate concentration (1.6%) was used to ensure that the agar support would set before the acrylamide polymerised. The tubes were placed in a cold room (4°C) for about 10 minutes to hasten the gelation of the agar. The surface of the agar was then carefully layered with a small drop of water and the gels left at room temperature until the polyacrylamide had set.

The buffers used and the conditions of electrophoresis were as described previously for ribosome electrophoresis.

3.24.4 Extraction of RNA from isolated ribosomes

RNA was prepared from the 105,000g. ribosome pellets by resuspending them in 3.0ml of the DEP-SDS extraction buffer (including the DEP and SDS) (see 3.21.2). The resuspension was incubated at 37°C for 5 mins. and the RNA was precipitated by addition of two volumes of absolute ethanol and storage at -20°C. The nucleic acids were pelleted by centrifugation and dissolved in a small volume (approximately 0.2ml) of buffer. The nucleic acid solution was then fractionated by polyacrylamide gel electrophoresis as described in 3.22.2. This method was also suitable for extraction of RNA from ribosome preparations which had already been resuspended and used for fractionation into polyribosomes or ribosomal sub-units.

The following methods of RNA isolation were also used but were found to be less satisfactory for reasons indicated in 3.9.

a) The detergent-phenol method was used as described in 3.21.1 with the ribosome pellet being resuspended in 3.0ml detergent medium. The procedure was then continued as previously described for RNA extraction from plant tissue.

(b) Resuspended ribosomes were deproteinised by making the resuspension 1% with respect to SDS and incubation at 37°C for 5 minutes. The resuspension was then loaded directly on to the polyacrylamide gel (Kurland, 1960; Staehelin et al., 1964).

(c) Treatment with guanidinium chloride to dissociate ionically ribosomal particles into their constituent components (Cox and Arnstein, 1963; Thomas, 1973) was also used to obtain RNA from ribosomes. The resuspended ribosomes were mixed with two volumes of 6M guanidinium chloride and incubated on ice for 15 minutes. The RNA was then precipitated by addition of an equal volume of absolute ethanol and overnight storage of the solution at -20°C. This ethanol precipitate was then dissolved in electrophoresis buffer plus 10% sucrose and used directly for electrophoresis.

3.25 Determination of Radioactivity in Samples Fractionated by PAGE

To determine the radioactivity in samples in polyacrylamide gels the gels were sliced transversely but because of their softness slicing was facilitated by initially freezing the gels as described below. An aluminium trough was formed in

which the gel was retained at its running length by rubber bungs at either end. A freezing mixture, formed by mixing dry-ice and hexane, was sluiced over the gel until it was completely frozen. The opaque frozen gel was then transferred to the cutting block of a Mickle Tissue Slicer on which a damp filter paper strip had been placed and was frozen in position by covering it with crushed dry-ice. The tissue slicer was then used to cut standard sized slices (normally 1.0 mm), each slice being immediately transferred to a clean, dry scintillation vial. 0.5ml of a 10% (v/v) aqueous piperidine solution containing 1 mM EDTA was then added to each vial and the gel slices dried down at 60°C for about 5 hours during which time the ribosomal RNA was hydrolysed. 0.5ml of water was added to each of the dried slices which were then allowed to swell. 5.0 ml of scintillation cocktail were added to each vial. The cocktail used had the following composition (Patterson and Green, 1965):

1000 ml Toluene

500 ml Triton X-100

4 g PPO

The vials were stoppered, shaken and stored for 24 hours in the dark before counting to allow subsidence of chemiluminescence. The scintillant became cloudy on addition to the vials but subsequently cleared as water was removed from the gels by the Triton X-100. Counting of the samples was

performed in a Beckman LS-200 B scintillation counter with counting efficiencies of 30% for ^3H and 85% for ^{14}C .

3.26 Preparation of Magnesium Bentonite

Magnesium bentonite was prepared from bentonite powder by the method of Dunn and Hitchborn (1965). Bentonite powder was resuspended in 0.01M phosphate buffer pH 7.4 containing 0.01M magnesium sulphate in the proportion of 1 gram powder to 20 ml buffer. After shaking for two hours the suspension was centrifuged for one minute at 400g. in a bench centrifuge. The resulting supernatant was then centrifuged for 15 minutes at 17,000g. in an MSE 'high speed' 18 centrifuge at room temperature. The pellet was then resuspended in half the original volume of 1mM phosphate buffer pH 7.4 containing 1mM magnesium sulphate and shaken again for 2 hours. The low and high speed centrifugations were repeated and the final pellet was resuspended in 1mM phosphate buffer pH 7.4 plus 1 mM magnesium sulphate to a final concentration of approximately 50 mg/ml bentonite (estimated from dry weight determination). The bentonite suspension was stored at 4°C until required.

3.3 Detergent-Phenol method of RNA extraction

3.31 U.V. spectral characteristics

The typical u.v. absorption spectrum of detergent-phenol extracted chinese cabbage leaf RNA showed an absorption maximum at 258 nm. and a minimum at 230 nm. (Figure 3.1). The ratios E258/E230 and E258/E280 routinely had the value of 2.0 ± 0.1 indicating essentially pure RNA preparations (Peterman, 1964).

The yield of RNA varied with the age and physiological state of the leaf tissue used for extraction (See 4.3) however with mature fully expanded leaves of chinese cabbage a yield of 0.5 - 1.0 mg RNA per gram fresh weight of leaf lamina was usual with this method.

3.32 PAGE and molecular weight determination

Detergent-phenol extracted RNA was resuspended in Loening 'E' buffer (Loening, 1969) plus 10% sucrose and fractionated in 2.6% polyacrylamide gels with Loening 'E' buffer used as the running buffer (figure 3.2). The electrophoretograms reveal principle peaks which are derived from the large and small sub-units of the cytoplasmic 80s and the chloroplastic 70s ribosomes. In order of increasing electrophoretic mobility these have been characterised as the 25s cytoplasmic, 23s chloroplastic, 18s cytoplasmic and 16s chloroplastic ribosomal RNA species respectively (Loening and Ingle, 1967). Fractionation on a gel of a

Figure 3.1

u.v. absorption spectrum of RNA extracted by the
detergent-phenol method.

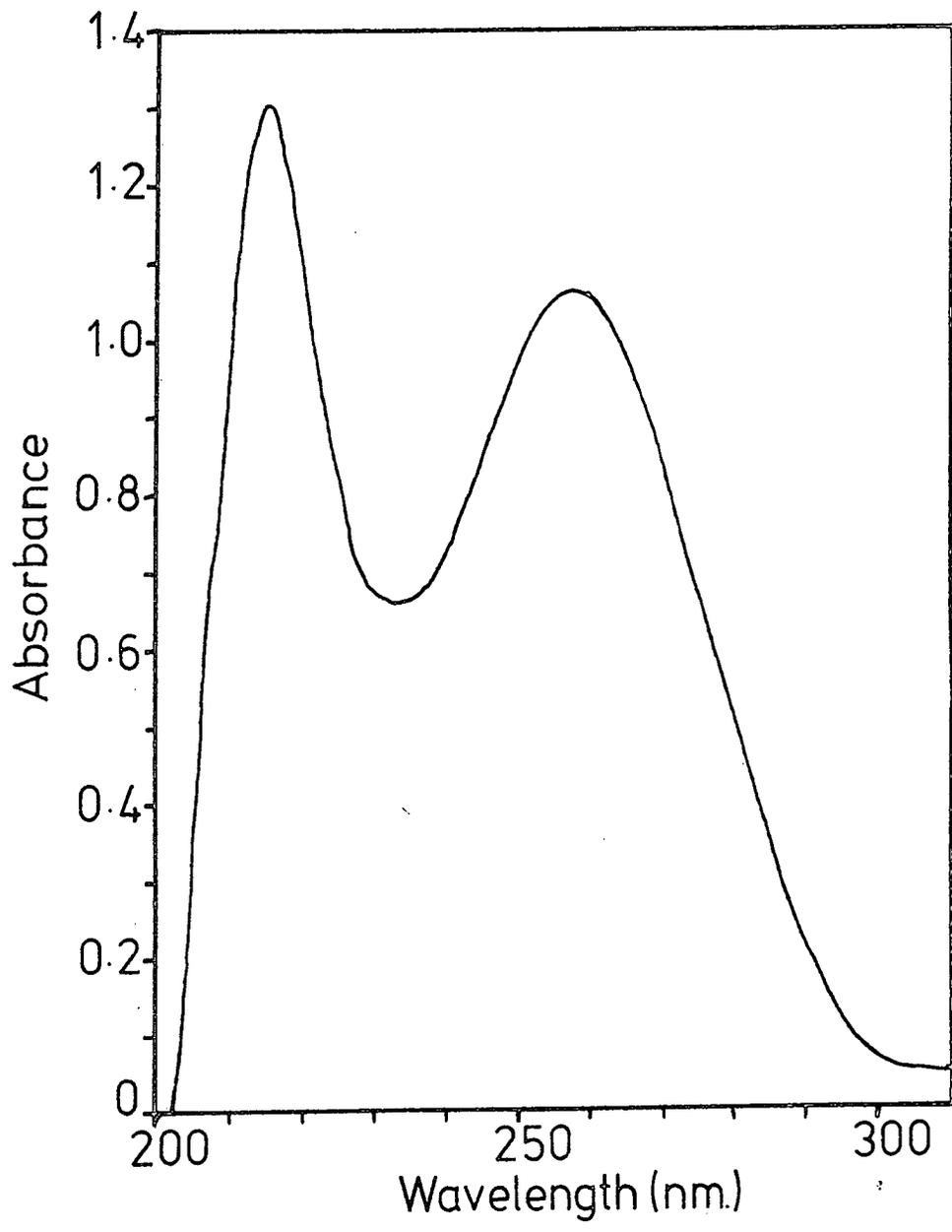


Figure 3.2

PAGE of high molecular weight RNA extracted from
leaves by the detergent-phenol method

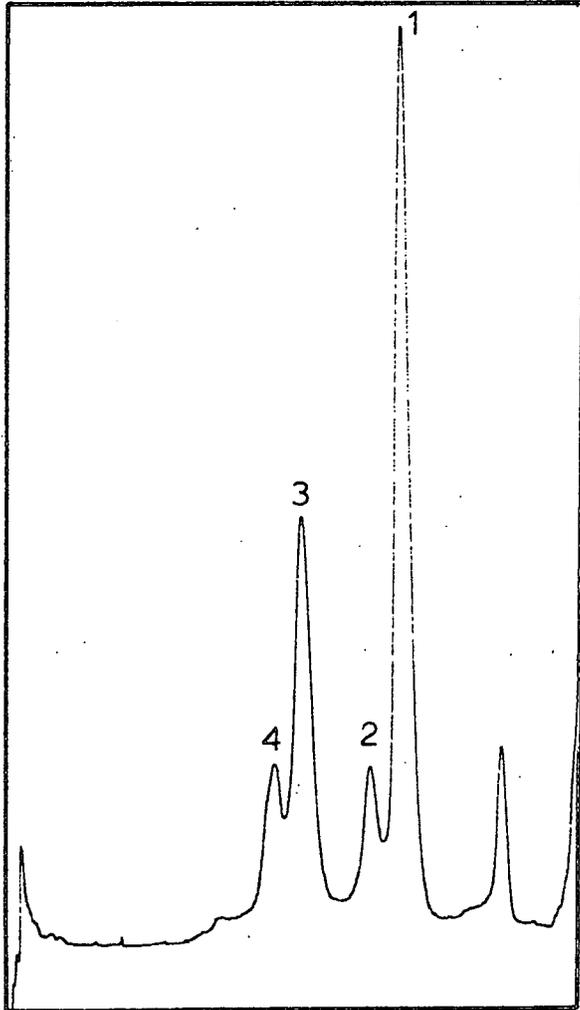
Peak 1 : 25s cytoplasmic rRNA
Peak 2 : 23s chloroplastic rRNA
Peak 3 : 18s cytoplasmic rRNA
Peak 4 : 16s chloroplastic rRNA

Figure 3.3

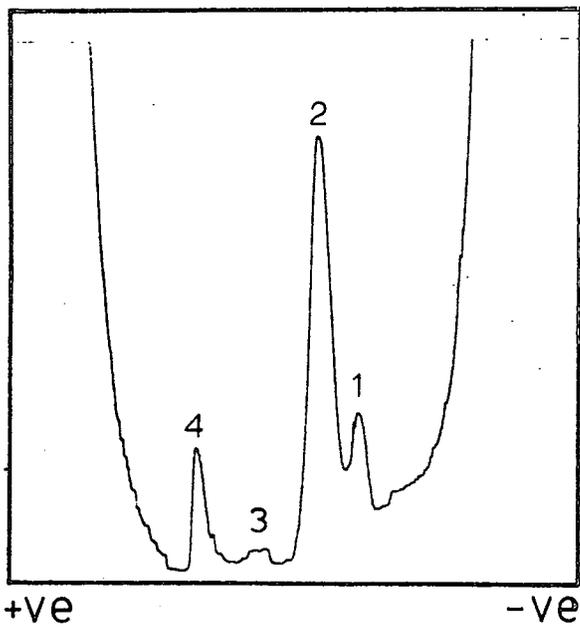
PAGE of low molecular weight RNA extracted from
leaves by the detergent-phenol method

Peak 1 : 5s
Peak 2 : 4s
Peak 3 : nucleotides
Peak 4 : ion front

A 265nm.



A 265nm.



+ve

-ve

smaller pore size (7.5% acrylamide) results in the exclusion of these high molecular weight ribosomal RNA fractions and resolves low molecular weight components (Loening, 1968a). The two RNA fractions observed (figure 3.3) are the 4s (comprising mainly the tRNA) and the 5s rRNA. The latter consists of two components (not resolved) derived from the large ribosomal subunits of both 80s and 70s ribosomes (Payne and Dyer, 1971). Also apparent was an additional faster migrating peak which could be washed out from the gels very easily and which probably represented free nucleotides (Pearson, 1969a).

It has been demonstrated that there exists a linear relationship between mobility and log (molecular weight) for electrophoretically separated RNA species (Bishop et al., 1967; Loening, 1968c ; Peacock and Dingman, 1968). Utilising this relationship the molecular weights of the high molecular weight chinese cabbage leaf ribosomal RNA components were determined. Highly polymerised E.coli RNA (obtained from British Drug Houses) was used as a standard molecular weight marker assuming values of 1.08×10^6 and 0.56×10^6 for the 23s and 16s fractions respectively (Loening, 1969; Payne and Loening, 1970). Leaf RNA extracts and the E.coli RNA standards were resuspended and electrophoresed in Loening 'E' buffer. Samples and standard were

fractionated, singly or mixed together, in the same electrophoresis apparatus under the same conditions (figure 3.4). Estimates obtained for the molecular weight of the chinese cabbage rRNA components are detailed below:

Cytoplasmic 25s = 1.3×10^6

18s = 0.7×10^6

Chloroplastic 23s = 1.05×10^6

16s = 0.56×10^6

These values are in good agreement with other published molecular weights for higher plant rRNA species (Loening, 1968c).

3.33 Some effects of the presence of magnesium ions and EDTA during extraction

Leaver and Ingle (1971) found that the quality of electrophoretic fractionation of RNA from radish cotyledons was influenced both by the presence of EDTA or magnesium ions in the detergent medium during the extraction and also in the electrophoresis buffer. Figure 3.5 shows electrophoretograms of chinese cabbage leaf RNA extracted with either EDTA (figure 3.5a) or magnesium chloride (figure 3.5b) added to the detergent medium. Fractionation was performed in Loening 'E' buffer (Loening, 1969).

The low yield of intact chloroplast 23s rRNA and the presence of breakdown fractions indicated the lability of this RNA species in EDTA, as previously described for other

Figure 3.4

PAGE of chinese cabbage rRNA to determine molecular weight.

A. Chinese cabbage rRNA extracted by the detergent-phenol method.

Peak 1 : 1.3×10^6 mol. wt.
Peak 2 : 1.05×10^6 mol. wt.
Peak 3 : 0.7×10^6 mol. wt.
Peak 4 : 0.56×10^6 mol. wt.

B. E.coli rRNA standard

Peak 1 : 1.08×10^6 mol. wt.
Peak 2 : 0.56×10^6 mol. wt.

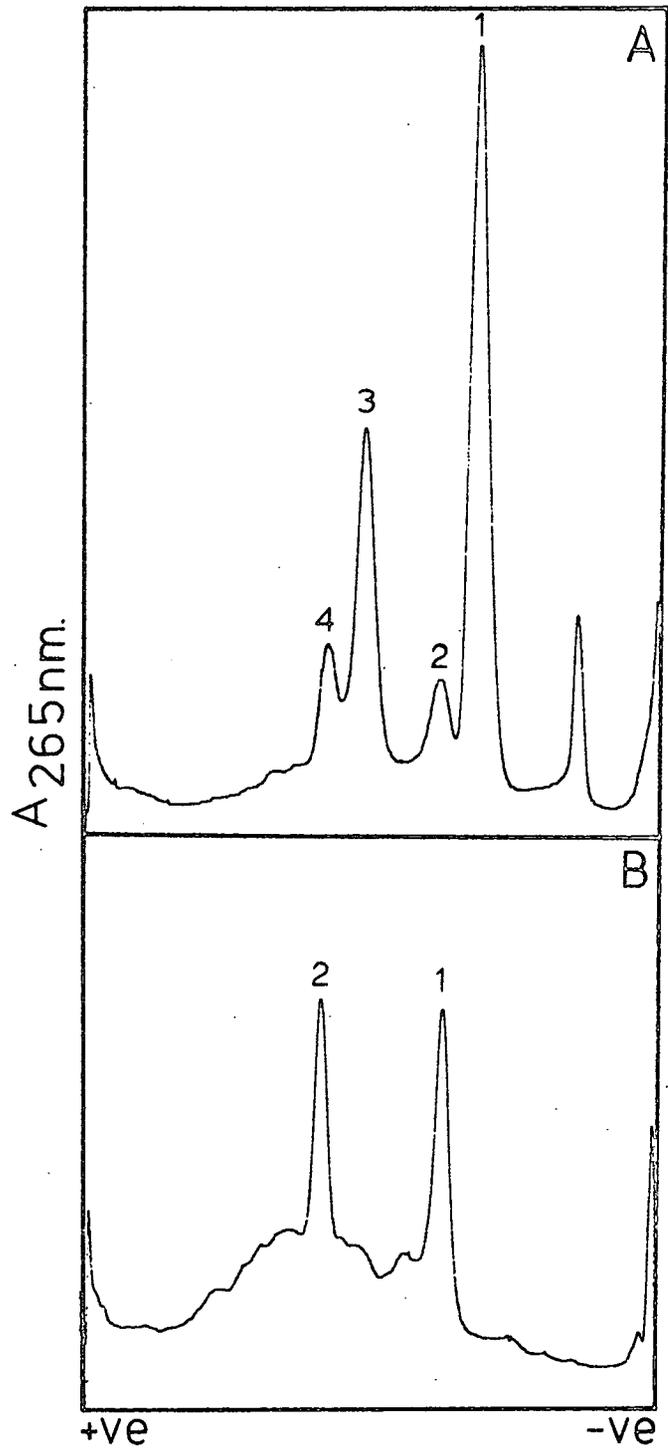
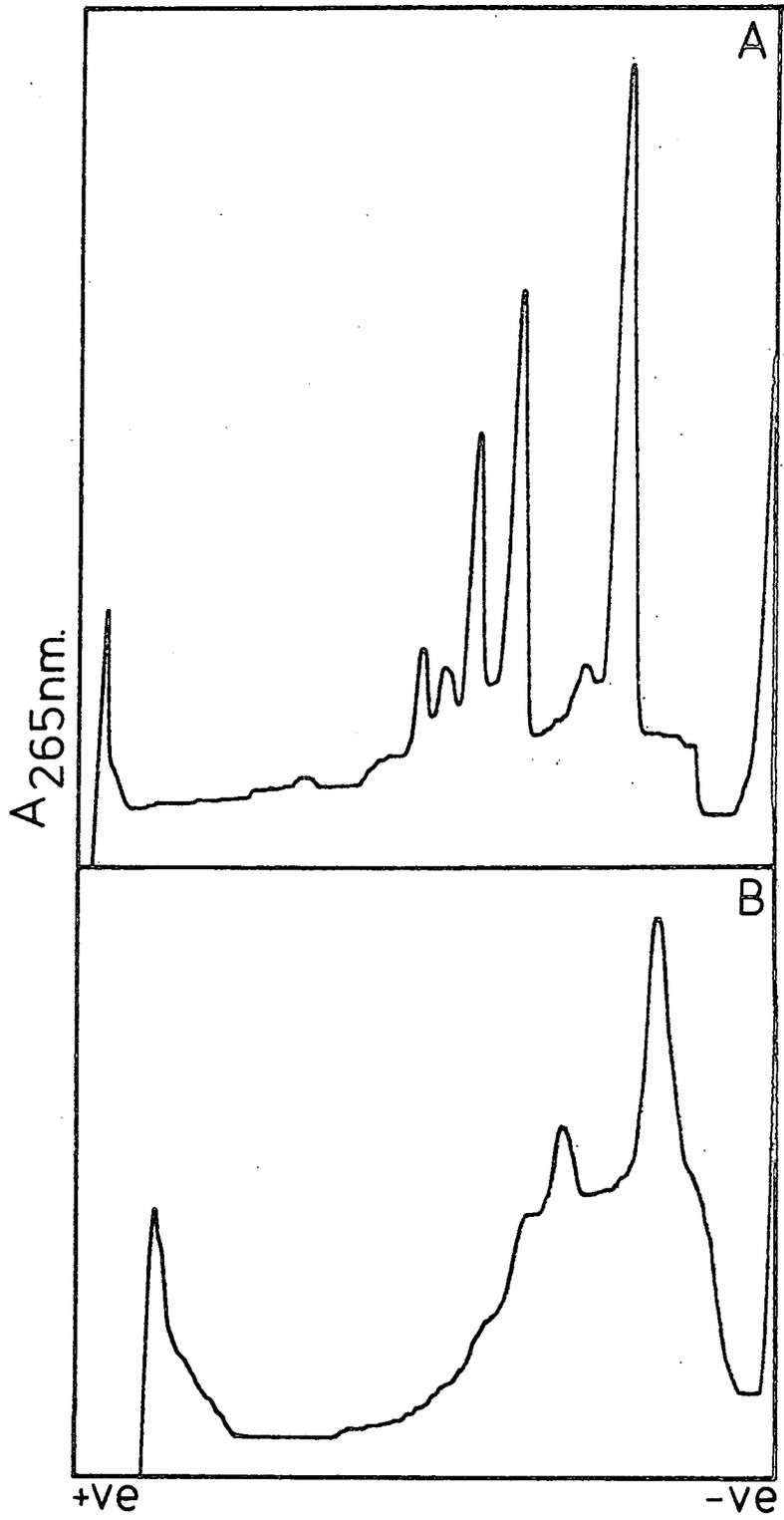


Figure 3.5

- A. PAGE of chinese cabbage leaf RNA extracted by the detergent-phenol method with EDTA included in the detergent medium.

- B. PAGE of chinese cabbage leaf RNA extracted by the detergent-phenol method with magnesium chloride included in the detergent medium.



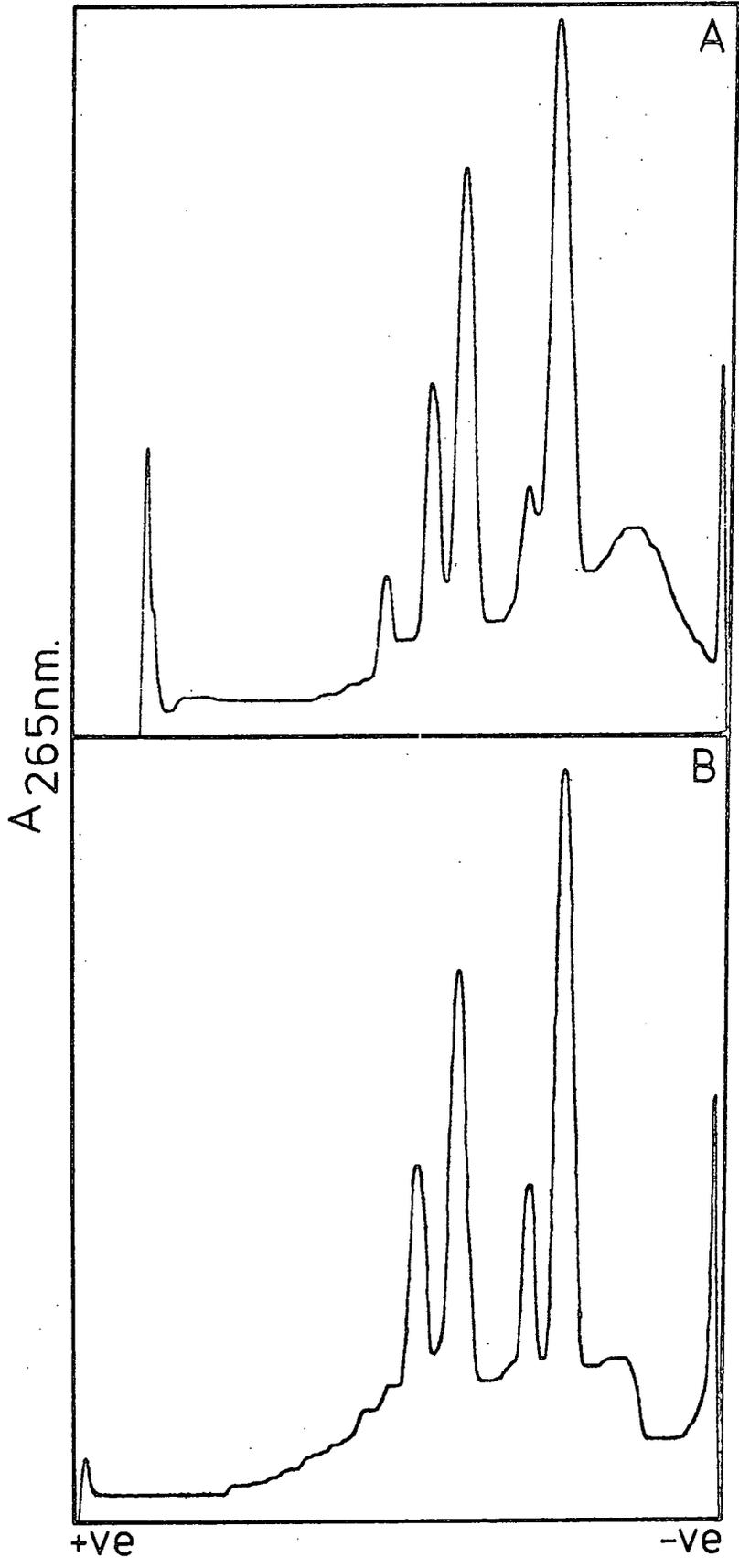
species by Ingle (1968) and Ingle et al., (1970). This instability is due to 'hidden breaks' in the polynucleotide chain being revealed when the RNA molecule unfolds in the presence of EDTA (Ingle et al., 1970). Extraction in the presence of magnesium gives a sample contaminated with non-nucleic acid material, as evidenced by low values obtained for the spectral ratios E_{260}/E_{230} and E_{260}/E_{280} , this results in poor resolution of the RNA peaks similar to that described by Leaver and Ingle (1971).

An attempt was made to improve the extraction method by either reducing the breakdown apparent in the EDTA extracted material or improving the resolution of the magnesium prepared RNA. This was done by adding magnesium chloride (to a final concentration of 10 mM) to the EDTA extraction, and by adding EDTA (to a final concentration of 10mM) to the magnesium extraction. In both cases the addition was made to the aqueous phase immediately prior to the final phenol deproteinisation step (see 3.21.1). Extraction with EDTA plus added magnesium ions showed some improvement over extraction with EDTA alone, addition of magnesium at a late stage in the extraction procedure thus having a small effect in preventing the dissociation of the chloroplast 23s rRNA (figure 3.6(a)). RNA prepared in the presence of magnesium with added EDTA showed a marked increase in definition of

Figure 3.6

- A. PAGE of chinese cabbage leaf RNA extracted by the detergent-phenol method including EDTA with magnesium chloride added at a late stage in the extraction.

- B. PAGE of chinese cabbage leaf RNA extracted by the detergent-phenol method including magnesium with EDTA added at a late stage in the extraction.



the ribosomal RNA fractions on electrophoresis (Figure 3.6(b)) and there was also improved preservation of the 23s rRNA compared to EDTA-prepared samples. This qualitative improvement in the integrity of the chloroplast high molecular weight rRNA is reflected in the increased ratio of heavy to light rRNA components (Table 1).

Table 1

Extraction	*Cytoplasmic rRNA 25s/18s	Chloroplastic rRNA 23s/16s
EDTA	1.45	0.54
Magnesium	1.55	- I
EDTA + magnesium	1.67	0.64
Magnesium + EDTA	1.82	1.17

* Peak area ratios

I Definition of electrophoretogram too poor to measure accurately

The two high molecular weight rRNA components are present in equimolar amounts in each of the two ribosome types (70s and 80s) (Leaver and Ingle, 1971). Using the molecular weights determined for chinese cabbage rRNA (see above) then the expected ratios for the heavy to light rRNA components are 1.86 and 1.88 for the cytoplasmic and chloroplastic rRNA species respectively. Whilst there is good agreement with this theoretical value for the cytoplasmic

RNA peaks the value for the chloroplast component is low. Dissociation of the 23s chloroplast RNA induced by resuspension and electrophoresis in EDTA-containing buffer (Loening 'E') could account for this though a species specific low degree of stability of this RNA fraction could also be contributory (Leaver and Ingle, 1971).

3.34 RNA extracted from leaves of TYMV-infected plants

The u.v. absorption spectrum of total nucleic acid extracted from TYMV-infected plants shows the same maxima and minima as healthy plant preparations (figure 3.7). The spectral ratios E_{258}/E_{230} and $E_{258}/280$ both gave values of 2.0 ± 0.1 in good agreement with the degree of purity obtained with healthy leaf extracts.

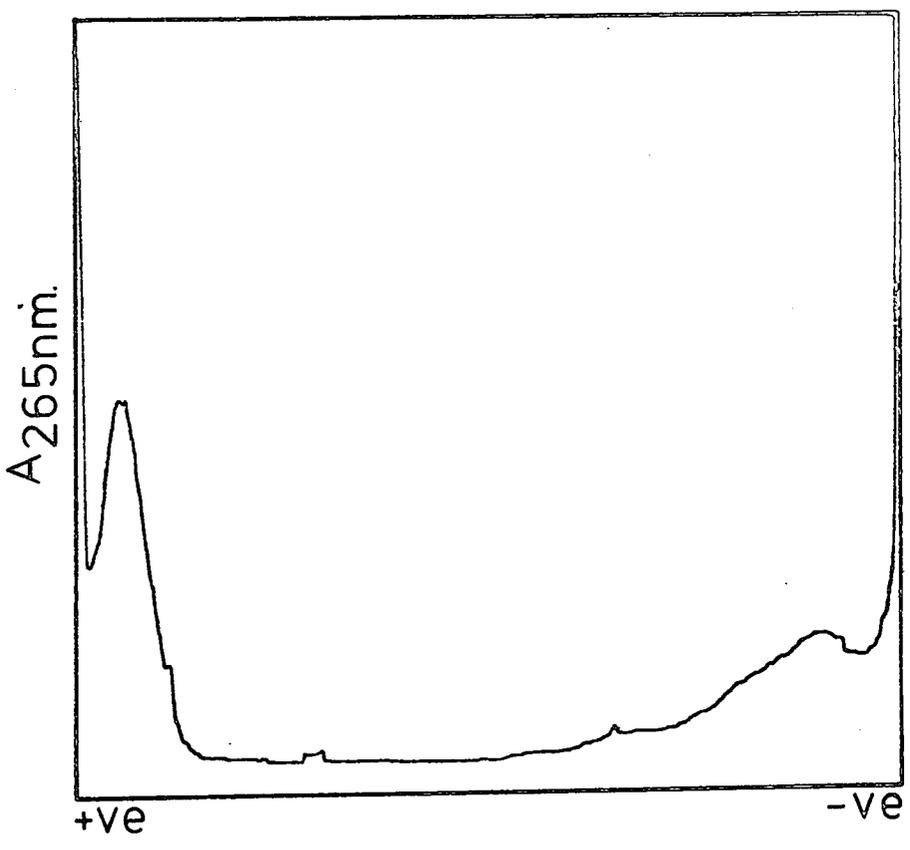
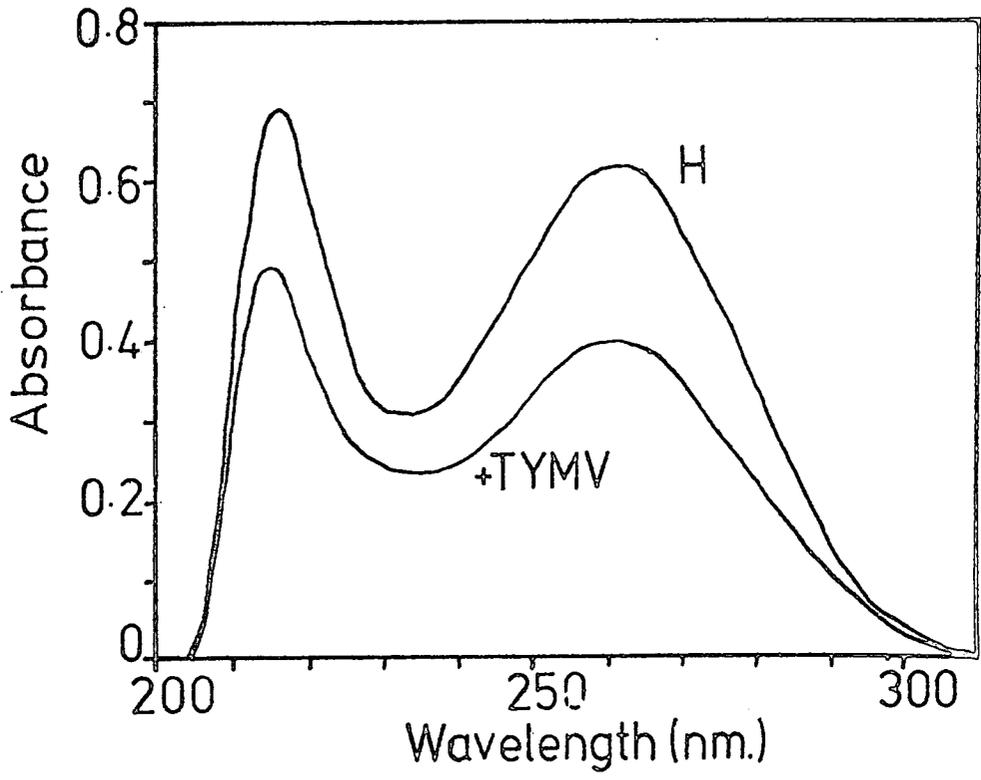
On electrophoresis of detergent-phenol RNA extracts from TYMV-infected plants no peaks corresponding to high molecular weight rRNA were observed (figure 3.8). At the top of the gel was a strongly u.v. absorbing band which stained positively on treatment with toluidine blue thus indicating that this fraction had a nucleic acid nature. In the fractionation of nucleic acids by sucrose density gradient centrifugation or by chromatography in methylated albumin kieselguhr (MAK) columns, Matus et al., (1964) have observed that with nucleic acid extracts from TYMV-infected leaves a large RNA aggregate interfered with the separations. This

Figure 3.7

u.v. absorption spectrum of RNA extracted from healthy (H) and TYMV-infected (TYMV) chinese cabbage leaves by the detergent-phenol method.

Figure 3.8

PAGE of rRNA extracted from TYMV-infected chinese cabbage leaves by the detergent-phenol method.



aggregate was the result of a complex formation between TYMV-RNA and the host rRNA. The result obtained with polyacrylamide gel electrophoresis was consistent with the work of Matus et al., (1964) and suggested that the host-virus RNA complex had been formed and was being retained at the surface of the gel during electrophoresis. As far as the author is aware this is the first report of the application of polyacrylamide gel electrophoresis, with its good resolution of high molecular weight RNA components, to the problem of TYMV-host rRNA interactions.

This phenomenon of complex formation is limited to the high molecular weight fractions. Electrophoresis of the low molecular weight RNA resulted in a profile essentially similar to that obtained with healthy leaf RNA extracts (figure 3.9). This suggested that an open secondary structure is required for the aggregation to occur though possibly these low molecular weight RNA fractions have an unsuitable base structure for complexing to occur.

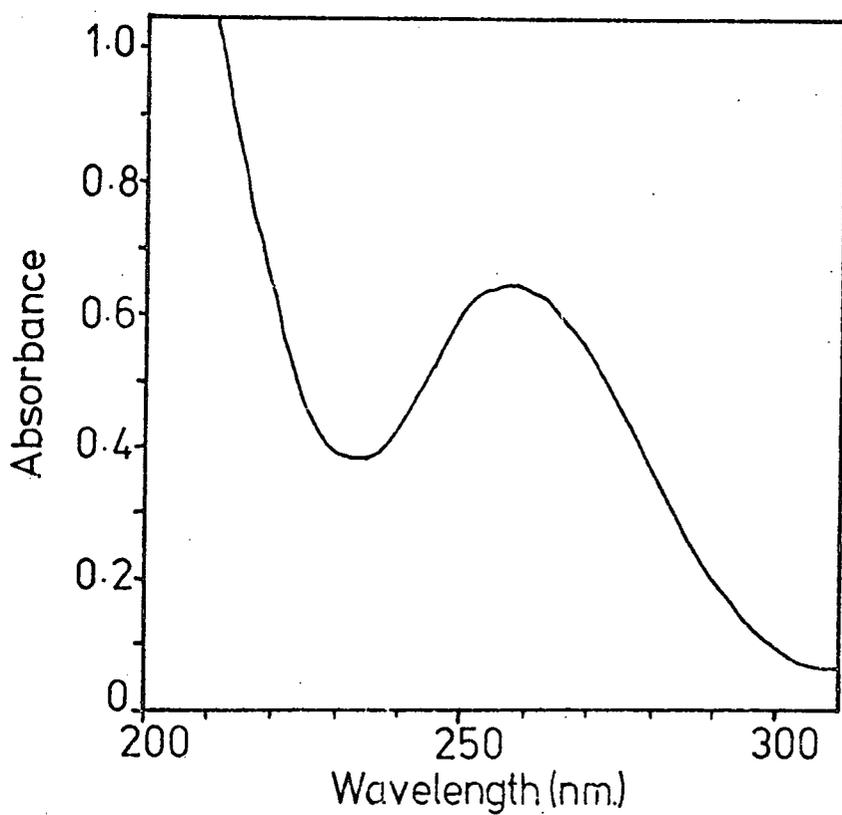
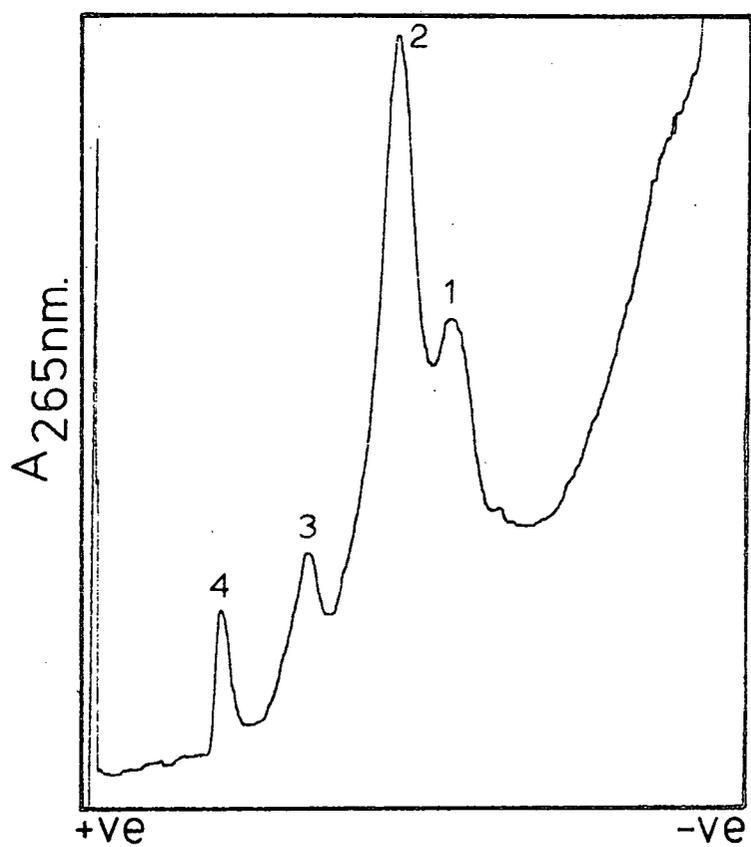
Figure 3.9

PAGE of low molecular weight RNA extracted from TYMV-infected leaves by the detergent-phenol method.

Peak 1 : 5s
Peak 2 : 4s
Peak 3 : nucleotides
Peak 4 : ion front

Figure 3.10

u.v. absorption spectrum of RNA extracted from chinese cabbage leaves by the DEP-SDS method.



3.4 DEP-SDS method of RNA extraction

3.41 U.V. spectral characteristics

The u.v. absorption spectrum of RNA extracted from chinese cabbage leaves by the DEP-SDS method has a maximum absorption of 260 nm and a minimum at 235 nm (figure 3.10). The shift in the absorption minimum compared to detergent-phenol extracted RNA is probably attributable to carboxy-methylation of the nucleic acid bases (Solymosy et al., 1972). The average ratios for $E_{\text{max}}/E_{\text{min}}$, and E_{260}/E_{280} had values of 1.78 and 1.86 respectively, values lower than the corresponding values for detergent-phenol extracted RNA. However these values were obtained from RNA following only one ethanol precipitation, further purification steps did improve these values but there was a reduction in yield. Similar estimates with detergent-phenol extracted RNA are not possible because phenol contamination always obscures the u.v. spectrum of the RNA obtained directly from a single ethanol precipitation (see 3.6).

3.42 PAGE and molecular weight determination

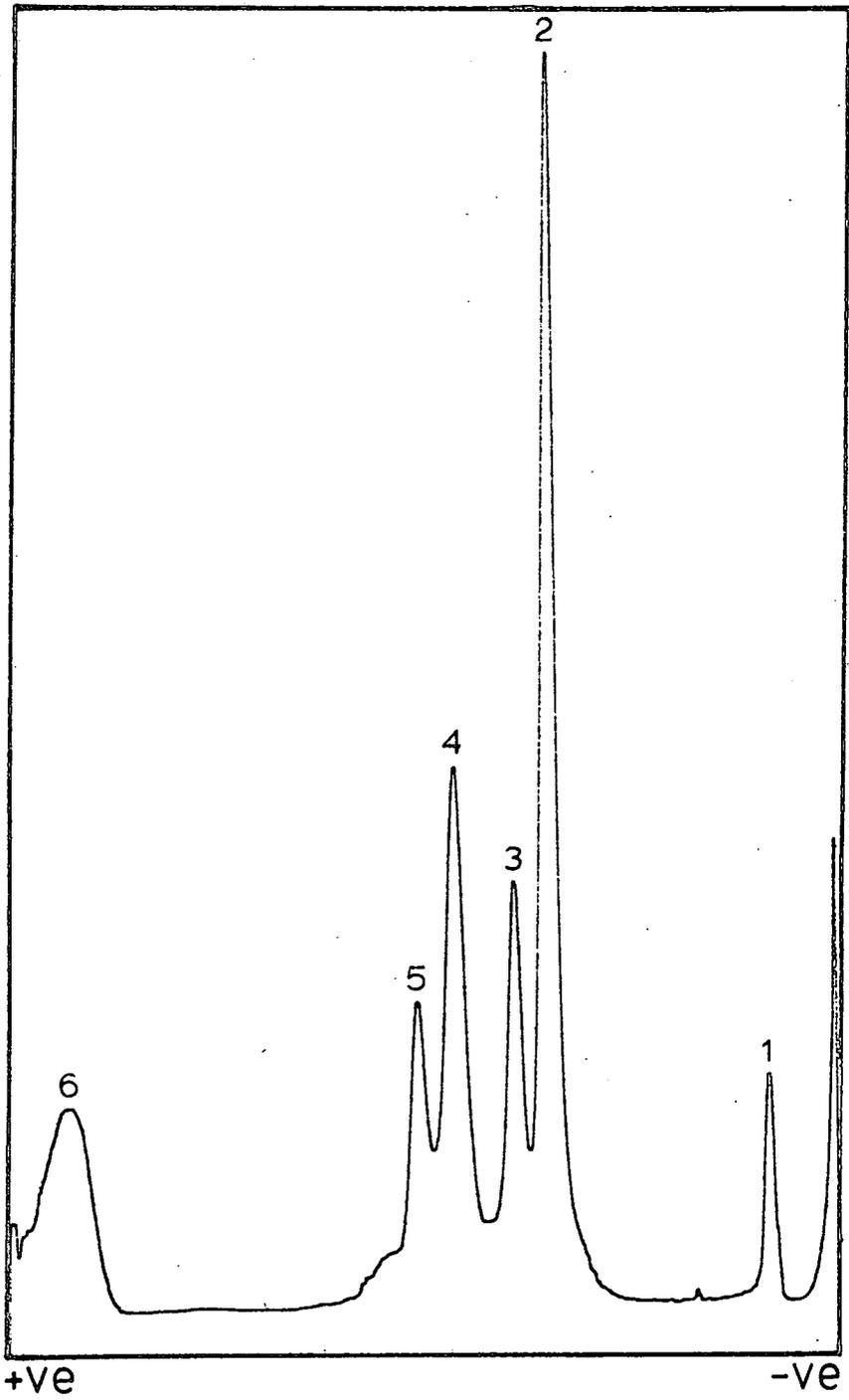
RNA extracted by the DEP-SDS method was resuspended in the magnesium-containing buffer derived from the electrophoretic buffer of Bishop et al., (1967) (see 3.22.2) and this buffer was also used as the electrophoresis buffer. Figure 3.11 illustrates DEP-SDS extracted RNA electrophoresed under these conditions. The four major high-molecular weight

Figure 3.11

PAGE of rRNA extracted from chinese cabbage leaves by the DEP-SDS method.

- Peak 1 : DNA
- Peak 2 : 25s cytoplasmic rRNA
- Peak 3 : 23s chloroplastic rRNA
- Peak 4 : 18s cytoplasmic rRNA
- Peak 5 : 16s chloroplastic rRNA
- Peak 6 : low molecular weight RNA

A 265nm.



ribosomal RNA fractions derived from cytoplasmic 80s and chloroplastic 70s ribosomes are clearly defined and there is good preservation of the labile 23s chloroplast RNA. Compared to detergent-phenol extracted RNA, extraction in the presence of magnesium alone did not produce 'dirty' profiles on subsequent electrophoresis of the RNA. Consequently the labile chloroplast 23s RNA can be fractionated intact which is not as readily achieved with the detergent-phenol method since exposure to EDTA is necessary to clarify the extract (see 3.33).

The molecular weights of the high molecular weight RNA fractions obtained with the DEP-SDS method were determined by a plot of mobility against log (molecular weight) as previously described (3.32). DEP-SDS extracted RNA was resuspended and electrophoresed in Loening 'E' buffer in which maximum unwinding of the tertiary structure of the molecules might be expected. E.coli 23s and 16s rRNA was used as a standard molecular weight marker. Exposure to EDTA-containing buffer caused some dissociation of the chloroplast 23s rRNA (figure 3.12). The estimated value for the molecular weights of the four major rRNA species were:

$$\text{Cytoplasmic 25s} = 1.3 \times 10^6$$

$$18\text{s} = 0.7 \times 10^6$$

$$\text{Chloroplastic 23s} = 1.05 \times 10^6$$

$$16\text{s} = 0.55 \times 10^6$$

Figure 3.12

PAGE of chinese cabbage rRNA to determine molecular weight.

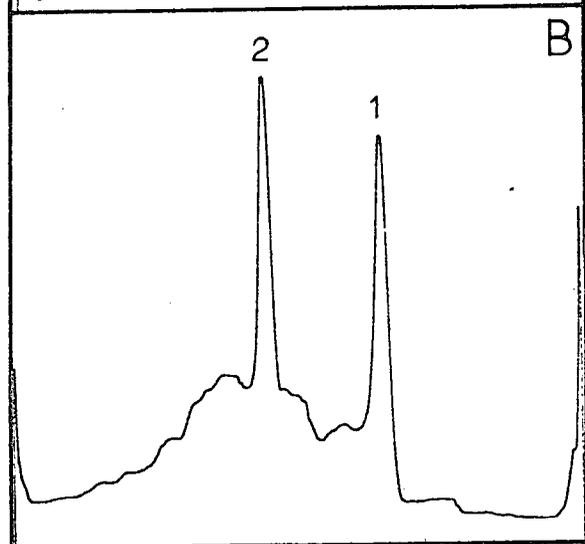
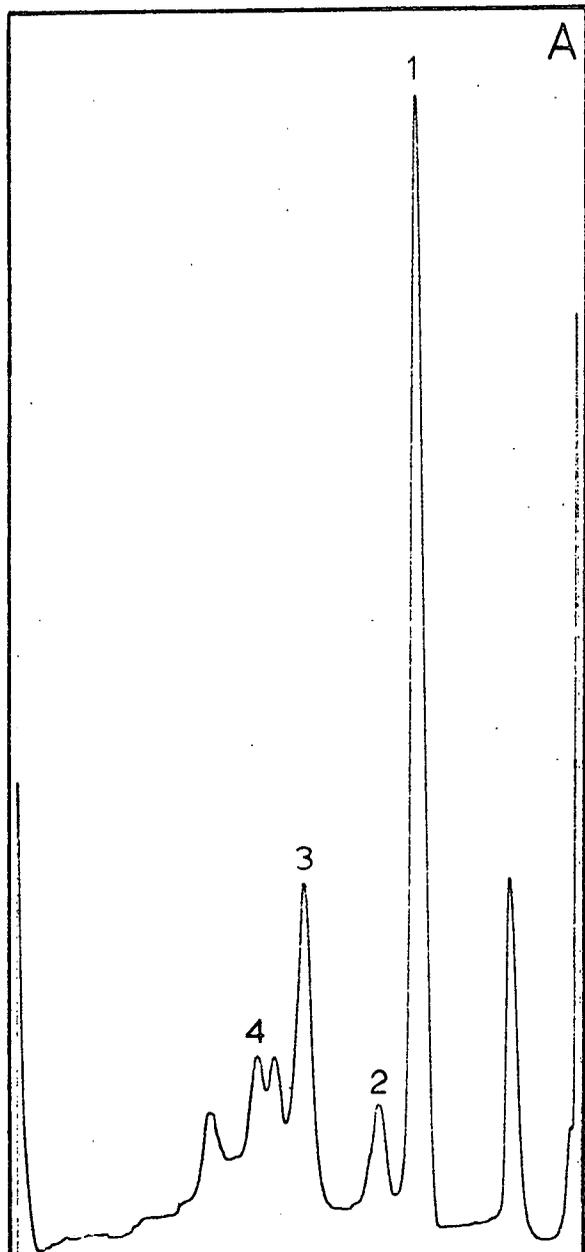
A. Chinese cabbage rRNA extracted by the DEP-SDS method.

Peak 1 : 1.3×10^6 mol. wt.
Peak 2 : 1.05×10^6 mol. wt.
Peak 3 : 0.7×10^6 mol. wt.
Peak 4 : 0.55×10^6 mol. wt.

B. E.coli rRNA standard

Peak 1 : 1.08×10^6 mol. wt.
Peak 2 : 0.56×10^6 mol. wt.

A 265nm.



+ve

-ve

These results agree with values obtained for detergent-phenol extracted RNA, except for the chloroplast 16s RNA species which gave a marginally lower estimate. Extraction of the RNA in the relatively 'high-salt' conditions of the DEP-SDS method thus does not appear to affect the conformation, and therefore electrophoretic mobility, of the molecules when resuspended and electrophoresed in EDTA buffer, at least as judged by molecular weight determination.

Electrophoresis of the RNA in 7.5% polyacrylamide gels give a similar profile to that obtained with detergent-phenol extracted RNA with peaks of low molecular weight species designated 4s and 5s (figure 3.13).

3.43 Choice of extraction buffer for the DEP-SDS method

An initial extraction using the DEP-SDS method (Solyosy et al., 1968, 1970) was carried out using a three-fold dilution of '3E'-magnesium buffer (see 3.22.2) as the extractant buffer. Electrophoresis of the DEP-SDS extracted RNA was performed using this magnesium buffer, thus homogeneity of environment was maintained throughout the extraction and fractionation. The RNA profile on gels obtained using this system showed very good resolution between the high-molecular weight RNA species (figure 3.14) though the ratios of heavy to light ribosomal RNA components from the cytoplasm and chloroplast were 1.47 and 0.99 respectively (based on peak

Figure 3.13

PAGE of low molecular weight RNA extracted from
chinese cabbage leaves by the DEP-SDS method.

Peak 1 : 5s
Peak 2 : 4s
Peak 3 : nucleotides
Peak 4 : ion front

A 265nm

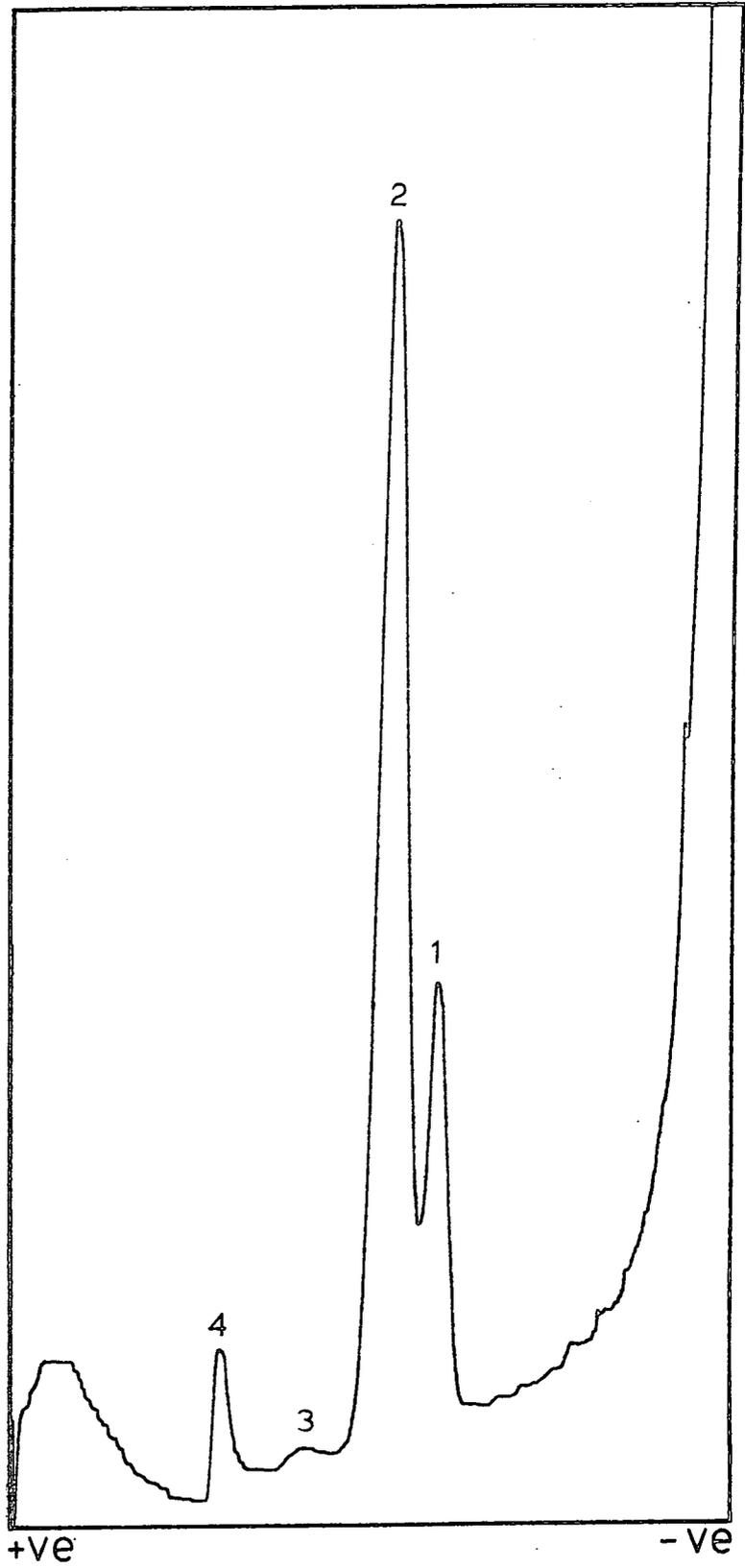


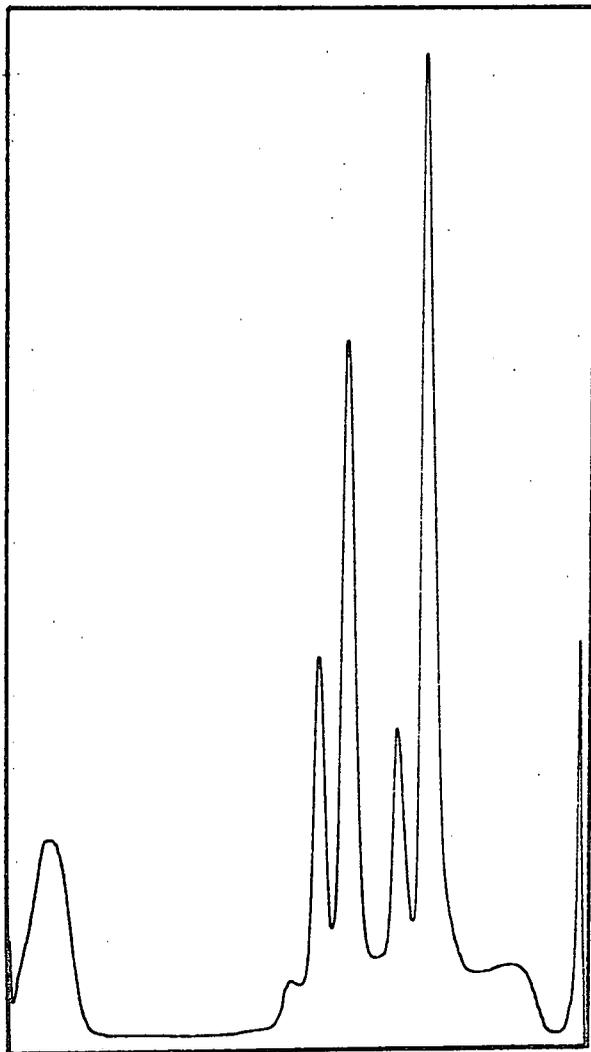
Figure 3.14

PAGE of rRNA extracted from healthy chinese cabbage leaves by the DEP-SDS method using a three-fold dilution of '3E'-magnesium buffer as extractant buffer.

Figure 3.15

PAGE of rRNA extracted from TYMV-infected chinese cabbage leaves by the DEP-SDS method using a three-fold dilution of '3E'-magnesium buffer as extractant buffer.

A 265nm.



A 265nm.



+ve

-ve

area weights). Lazar et al., (1969) reported that under certain conditions there was a preferential loss of heavy rRNA when the RNA was extracted in the presence of DEP and a similar response here could explain the low ratios observed. Only small amounts of 23s chloroplast RNA degradation products were present suggesting that breakdown of this fraction was not contributing substantially to the poor ratios obtained.

Use of this buffer to extract RNA from a TYMV-infected plant showed complex formation (Matus et al., 1964) as evidenced by strong u.v. absorption at the top of the gel (figure 3.15). Some uncomplexed host RNA had entered the gel though the yield was very low. The fact that complex formation had occurred suggested that TYMV-RNA had been released from the intact viral particle, this contrasted with the results of Bagi et al., (1970) who found that TMV-RNA was not released during DEP-SDS extraction of TMV-infected tobacco plants.

The result obtained by Bagi et al., (1970) and the fact that a small proportion of the host RNA was not complexed during DEP-SDS RNA extraction suggested that under altered conditions of RNA extraction isolation of the total host rRNA from infected tissue might be possible.

Further extractions were performed in which undiluted '3E'-magnesium buffer stock solution ('high-salt buffer')

was used as the extractant buffer. The increased ionic strength and magnesium concentration would help to preserve the RNA and would also aid in reducing nuclease activity (Davies et al., 1972). Healthy leaf RNA profiles obtained following extraction in this buffer showed excellent definition of the RNA peaks and the ratios of cytoplasmic 25s : 18s and chloroplast 23s : 16s RNA were 1.75 and 1.49 respectively (figure 3.16). The cytoplasmic ratio was in close agreement with that obtained with the detergent-phenol method whilst the chloroplastic value was somewhat better. Solyomosi et al., (1970) similarly found that in tobacco leaves DEP-SDS extraction gave less degradation of the chloroplast rRNA when compared to the detergent-phenol method of Ingle and Burns (1968).

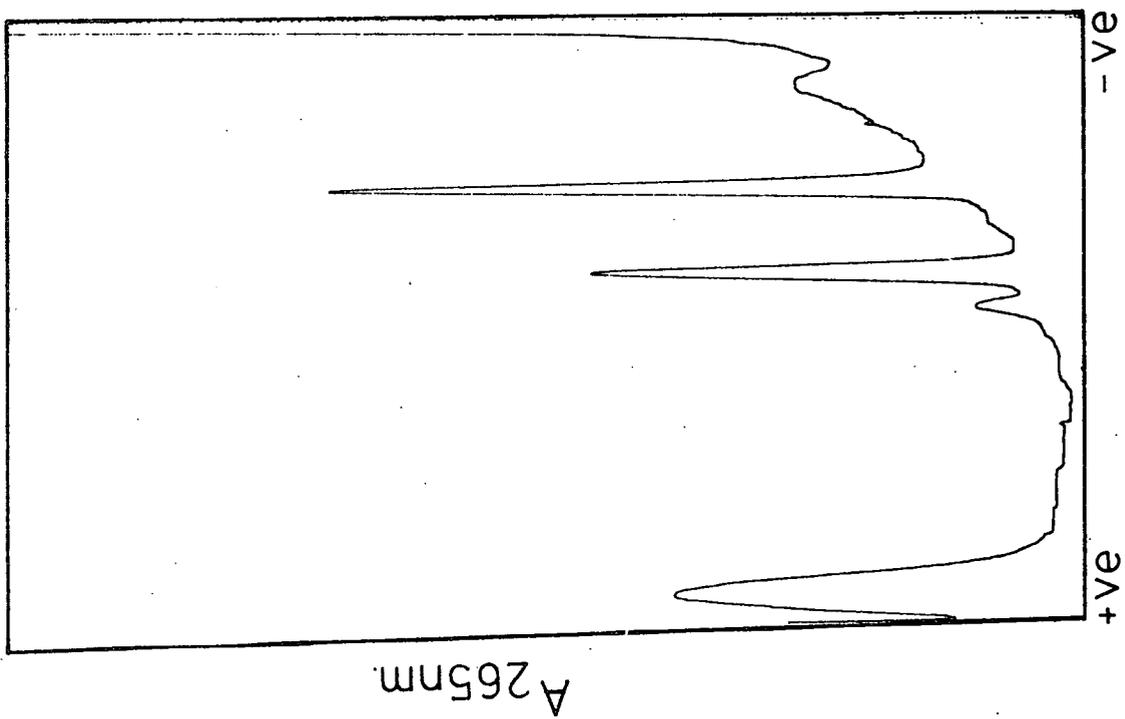
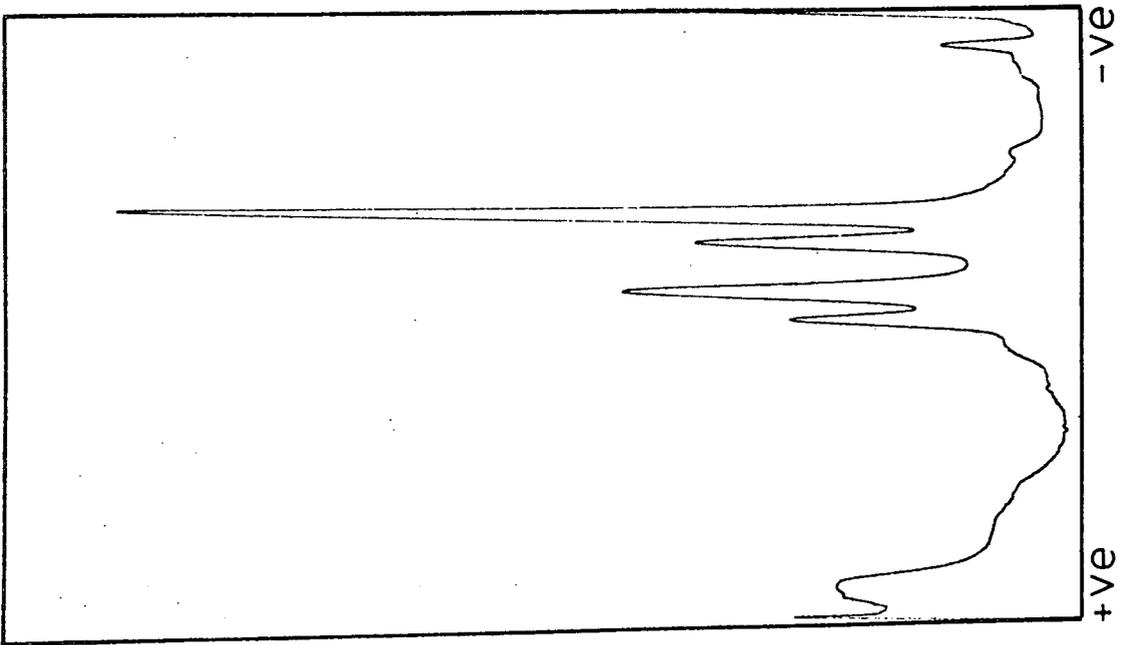
TYMV-infected tissue extracted with this 'high-salt buffer' resulted in the profile illustrated in figure 3.17, distinct cytoplasmic rRNA peaks were observed but with only small amounts of chloroplast material. Cells infected with TYMV have very abnormal chloroplasts (Chalcroft and Matthews, 1966). Reid and Matthews (1966) found that it was not possible to detect any 68s chloroplast ribosomes in extracts from yellow-green (i.e. infected) areas of chinese cabbage leaves, dark green areas of the mosaic pattern appeared normal and probably contained no virus. It thus seemed likely that a substantial proportion of the chloroplast rRNA

Figure 3.16

PAGE of rRNA extracted from healthy chinese cabbage leaves by the DEP-SDS method using the normal 'high-salt' buffer.

Figure 3.17

PAGE of rRNA extracted from TYMV-infected chinese cabbage leaves by the DEP-SDS method using the normal 'high-salt' buffer.



observed in the DEP-SDS extract was derived from these uninfected areas within the mosaic of infection since whole leaves were used in the extraction.

Compared to the 'E'-magnesium buffer extract of infected tissue the total absorbance on the gel trace observed using '3E' buffer represented a 3.5 - fold increase. Variation in yield, either inherent in the extraction method or between the tissue samples used, would not have accounted for this degree of difference. Under these conditions of relatively high ionic strength with increased magnesium ion concentration complex formation was prevented but it was not possible to establish if this inhibition of complex formation was total since the amount of virus present in the sample was not known.

DEP-SDS extracted RNA was mixed with an equal volume of 10µg/ml RNAase and was incubated at room temperature for 10 minutes prior to electrophoresis. Electrophoresis of RNA from TYMV-infected or uninfected plants both showed only a peak of DNA (figure 3.18), the RNA being susceptible to the added RNAase. Protection of the RNA against nuclease attack by possible complexing between DEP and the adenine residues (Henderson et al., 1973) did not therefore occur.

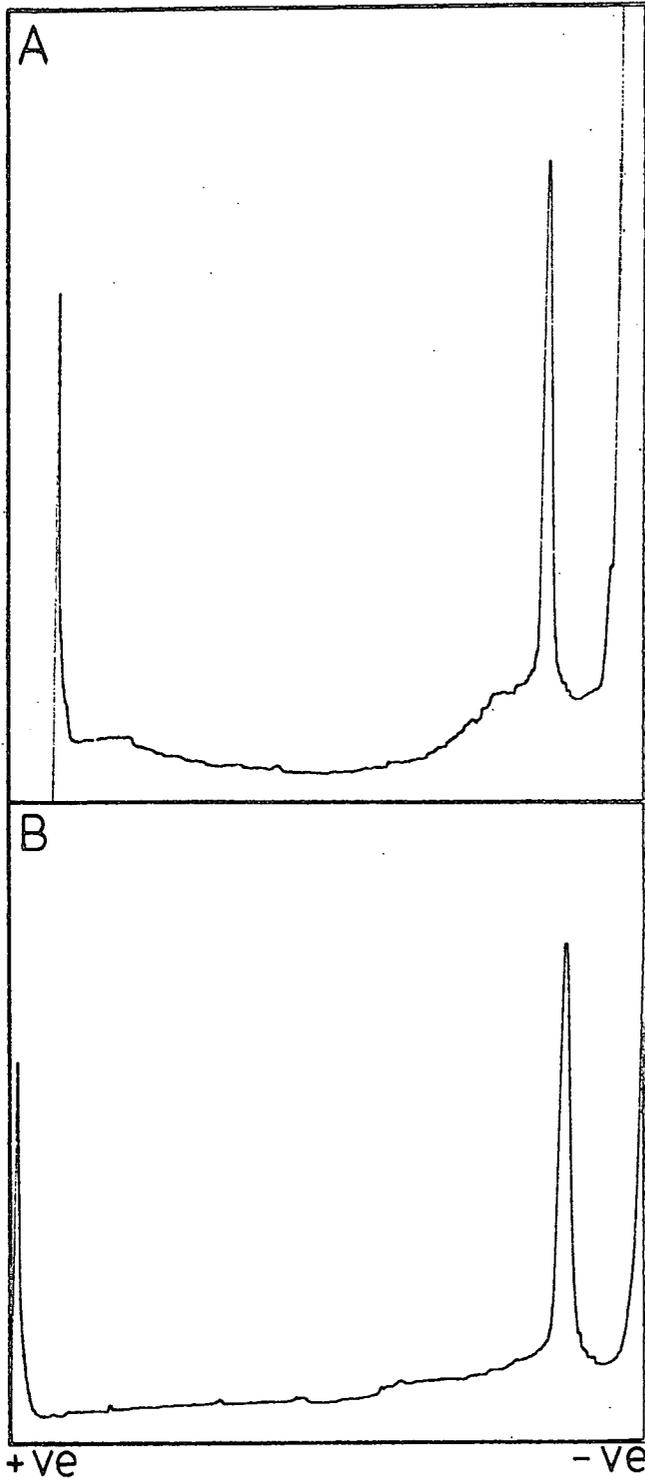
A third extraction was performed using the '3E' buffer of Bishop et al., (1967) as extractant buffer. This buffer was the same as the '3E' magnesium buffer used above except

Figure 3.18

PAGE of rRNA extracted by the DEP-SDS method after incubation for 10 minutes with 10 μ g/ml RNAase.

- A. RNA extracted from healthy chinese cabbage leaves.
- B. RNA extracted from TYMV-infected chinese cabbage leaves.

A 265nm.



that the magnesium was replaced by disodium-EDTA. The RNA extracted with this buffer from TYMV-infected plants had a similar electrophoretic profile to extracts obtained using 'E' magnesium buffer during the extraction (figure 3.20). Thus increased ionic strength not associated with an increase in magnesium ions was insufficient to prevent complex formation between TYMV-RNA and host RNA. Uninfected plants extracted with this EDTA-containing buffer showed a greater degree of degradation of the 23s chloroplast rRNA than was evident with either of the magnesium buffers (figure 3.19).

In an examination of the characteristics of the complex formed between TYMV-RNA and chinese cabbage RNA Matus et al., (1964) observed that the complex was evident on fractionation of the RNA by sucrose density gradient centrifugation, analytical centrifugation and also on MAK column chromatography. They found that attempts at separating the aggregate by adding EDTA to the RNA solution prior to fractionation were unsuccessful as were attempts to prevent the complex forming by including urea in the extraction medium. Similarly the use of conditions of extraction in which hydrogen-bonding would be reduced, e.g. non-aqueous extraction in chloroform-formamide (Pearson, unpublished data), have not resulted in the prevention of complex formation. Modification of the DEP-SDS method of RNA extraction (Solyosy et al., 1968, 1970),

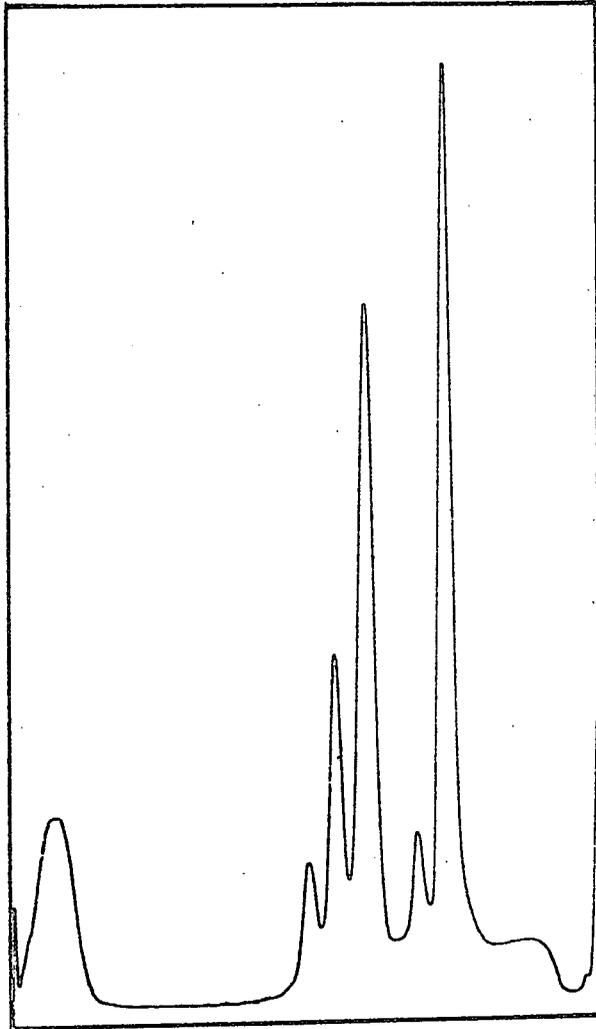
Figure 3.19

PAGE of rRNA extracted from healthy chinese cabbage leaves by the DEP-SDS method using the '3E' buffer of Bishop et al., (1967) as extractant buffer.

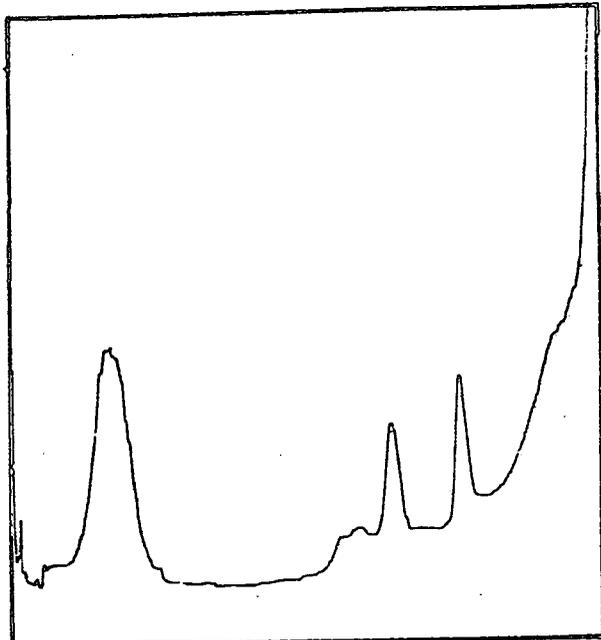
Figure 3.20

PAGE of rRNA extracted from TYMV-infected chinese cabbage leaves by the DEP-SDS method using the '3E' buffer of Bishop et al., (1967) as extractant buffer.

A 265nm.



A 265nm.



+ve

-ve

as described above, resulted in the successful isolation of host RNA from TYMV-infected plants without the characteristic host RNA-TYMV-RNA complex being formed. This had not previously been possible with phenol-based RNA extraction procedures. In view of the release of uncomplexed host RNA from TYMV-infected tissue and also the good quality polyacrylamide gel electrophoresis fractionations possible '3E'-magnesium buffer was chosen as the extractant buffer when using the DEP-SDS method of RNA extraction.

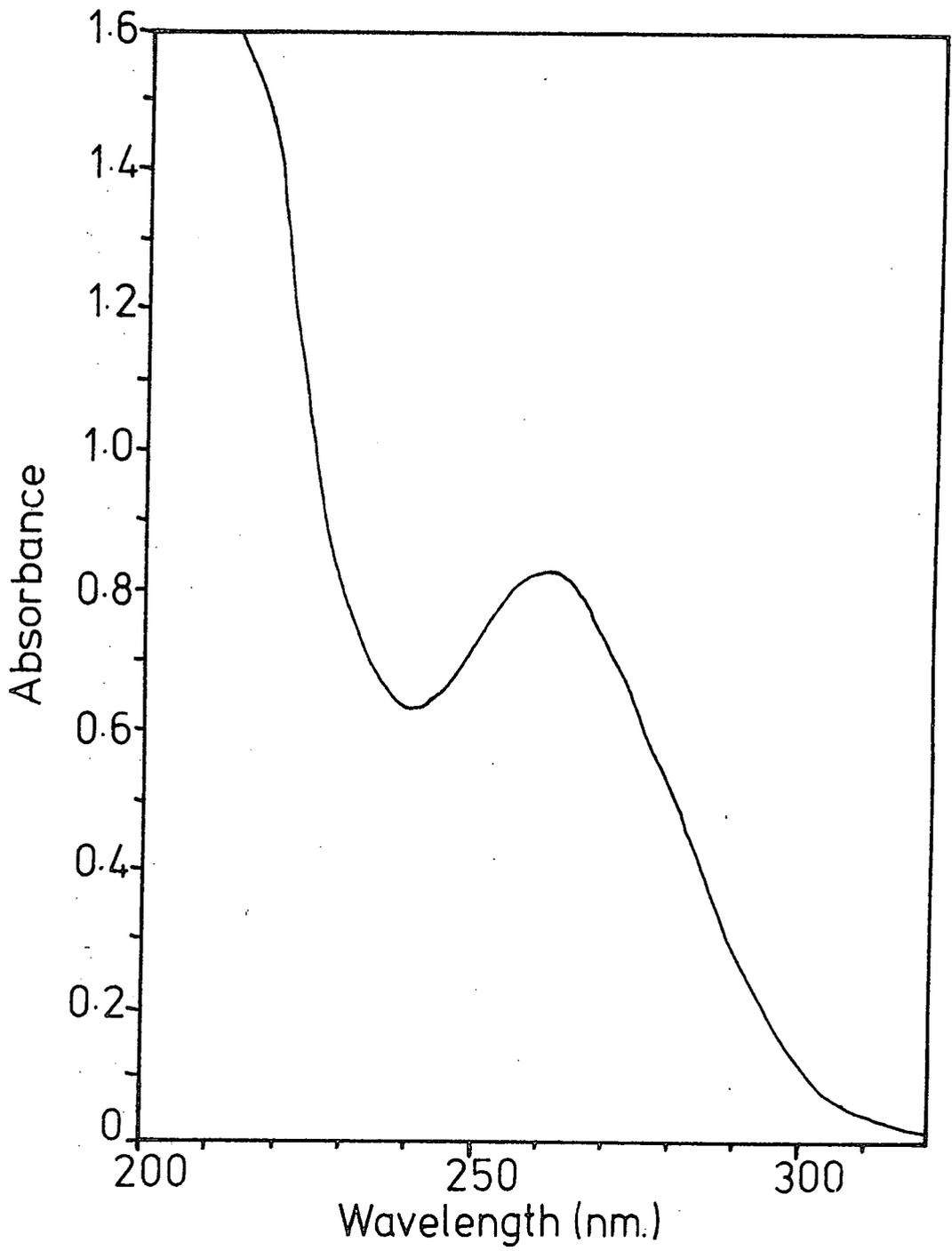
3.44 Characteristics of RNA extracted from TYMV-infected plants

The u.v. absorption spectrum of total leaf RNA extracted by the DEP-SDS method from TYMV-infected chinese cabbage leaves is illustrated in figure 3.21. The absorption maximum was at 260 nm, in contrast to uninfected leaf extracts the absorption minimum was in the range 239nm-242nm. The ratios E_{max}/E_{min} and E_{max}/E_{260} had the average values 1.27 and 1.64 respectively.

TYMV-RNA has an u.v. absorption maximum at 260nm and a minimum at 230nm, whereas intact TYMV has its minimum absorption shifted to 240nm, (Liddell, 1972). If under the conditions of RNA extraction used the complex was not formed because the viral RNA was not being stripped of the protein coat then the shift in u.v. absorption minimum of the RNA

Figure 3.21

u.v. absorption spectrum of RNA extracted from TYMV-
infected chinese cabbage leaves by the DEP-SDS method.



extracts might be expected if intact virus was present. The variation in the wavelength of minimum absorption could thus be explained in terms of differing amounts of virus being present in any given extract. Supporting evidence that the RNA extracts from TYMV-infected plants contained intact TYMV was provided by examining the effects of prior freezing of the tissue before RNA extraction. Kaper and Altling Siberg (1969b) have found that if TYMV is frozen in vitro and thawed then the RNA is released from the protein coat. Samples were taken from healthy and TYMV-infected chinese cabbage leaves, one sample of each of the two tissues was then extracted for RNA by the DEP-SDS method. Duplicate samples were also taken and were frozen in liquid air prior to RNA extraction with DEP-SDS. The uninfected leaf RNA profiles were not affected by the process of freezing (figure 3.22). With TYMV-infected material freezing of the tissue markedly reduced the amount of ribosomal RNA observed on the gels as compared to tissue that had been extracted without prior freezing (figure 3.23). It appeared that freezing the infected tissue had released the TYMV-RNA from the intact virus and under these conditions the DEP-SDS method did not prevent complex formation between the host and TYMV-RNA. The method thus prevents complex formation in the presence of the virus either by preventing or being

Figure 3.22

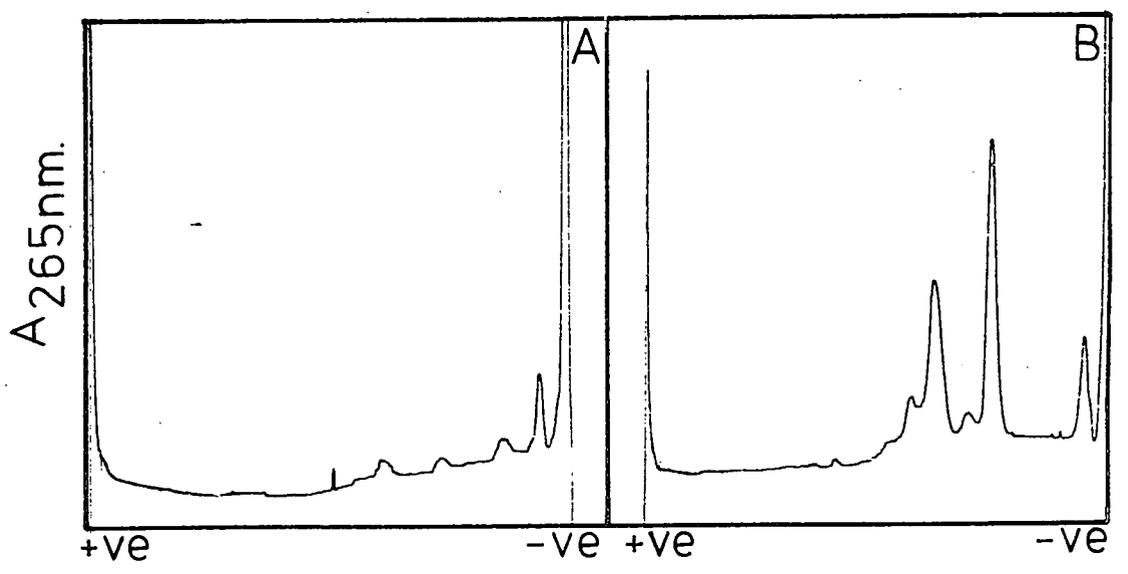
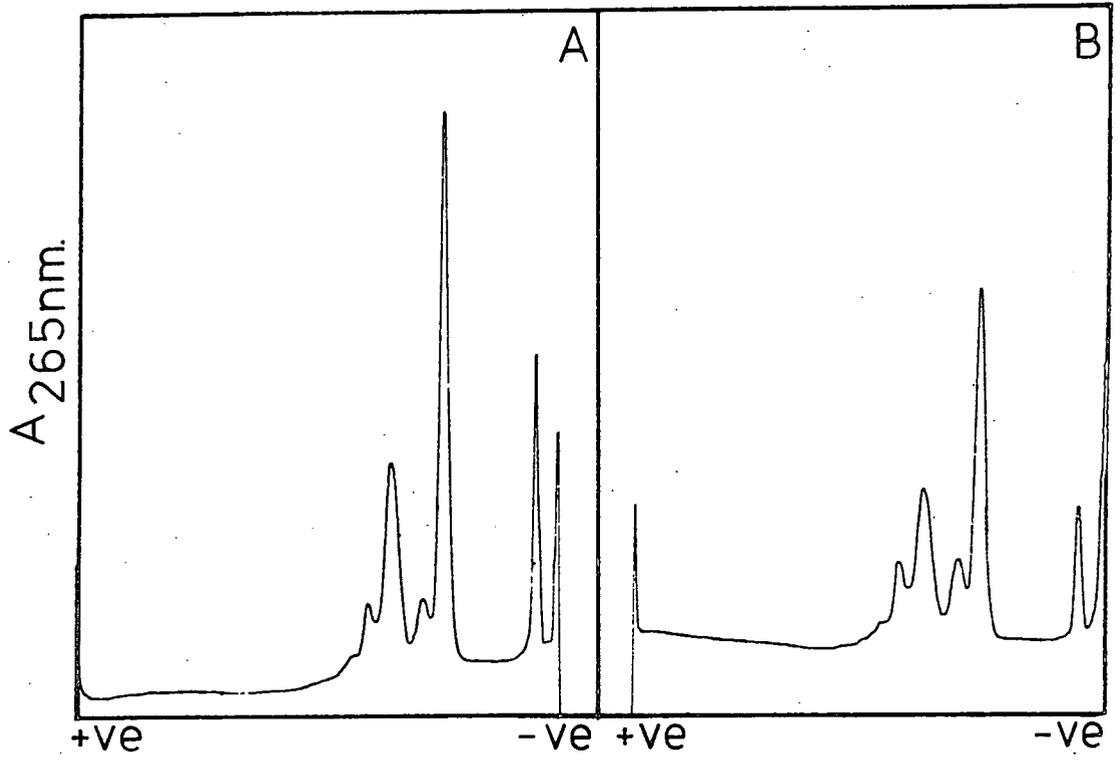
The effect of freezing of healthy leaf tissue on the electrophoretic profile of DEP-SDS extracted RNA.

- A. Leaf tissue frozen in liquid air prior to extraction.
- B. Leaf tissue extracted without prior freezing.

Figure 3.23

The effect of freezing of TYMV-infected leaf tissue on the electrophoretic profile of DEP-SDS extracted RNA.

- A. Leaf tissue frozen in liquid air prior to extraction.
- B. Leaf tissue extracted without prior freezing.



incapable of dissociating the protein coat from the intact virus.

It was also observed that a slight positive protein staining (amido black) could be detected on the surface of gels used to fractionate RNA from TYMV-infected tissue. This would be consistent with undissociated virus being present, though slight protein contamination of the samples would produce the same result.

The molecular weights of the RNA peaks observed on electrophoresis of RNA extracted from TYMV-infected plants were determined from a plot of log (molecular weight) against mobility as previously described (see 3.32). E.coli ribosomal RNA was used as a standard molecular weight marker. The values estimated for the cytoplasmic and chloroplastic high molecular weight ribosomal RNA species were the same as those previously determined for the same RNA species obtained from uninfected plants (see 3.42).

Fractionation of RNA from TYMV-infected plants on 7.5% polyacrylamide gels gave the same pattern of low molecular weight RNA peaks as found with healthy tissue (figure 3.24).

3.45 Extraction of RNA from cotyledons and isolated chloroplasts

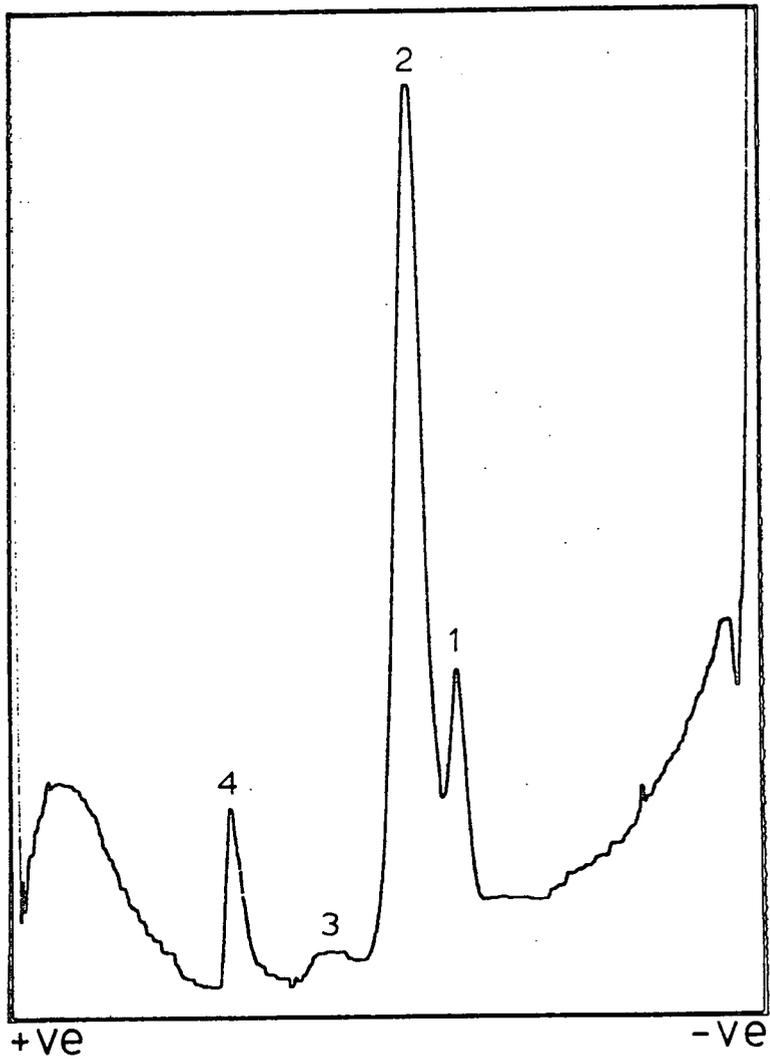
The DEP-SDS method was used to extract RNA from dark-grown and light-grown cotyledons and also from etiolated hypocotyls. The u.v. spectra obtained using resuspensions of

Figure 3.24

PAGE of low molecular weight RNA extracted from TYMV-infected leaves by the DEP-SDS method.

Peak 1 : 5s
Peak 2 : 4s
Peak 3 : nucleotides
Peak 4 : ion front

A 265nm.



the first ethanol precipitate were slightly poorer than those obtained for leaves. The E^{260}/E^{280} ratios for dark-grown cotyledons, light-grown cotyledons and hypocotyls being 1.41, 1.53 and 1.45 respectively, though this possible contamination did not interfere with subsequent electrophoresis. The yields obtained from these materials were 1.3 mg RNA/gram fresh weight for dark-grown cotyledons, 0.4 mg RNA/gram fresh weight for light-grown cotyledons and 0.15 mg RNA/gram fresh weight for the hypocotyls.

The high molecular weight RNA profiles for these different tissues (figure 3.25) showed differing proportions of the main RNA components (Table 2).

Table 2

	% Cytoplasmic RNA	% Organelle RNA
Light-grown cotyledons	59.7	40.4
Dark-grown cotyledons	71.1	28.9
Hypocotyls	92.1	7.9

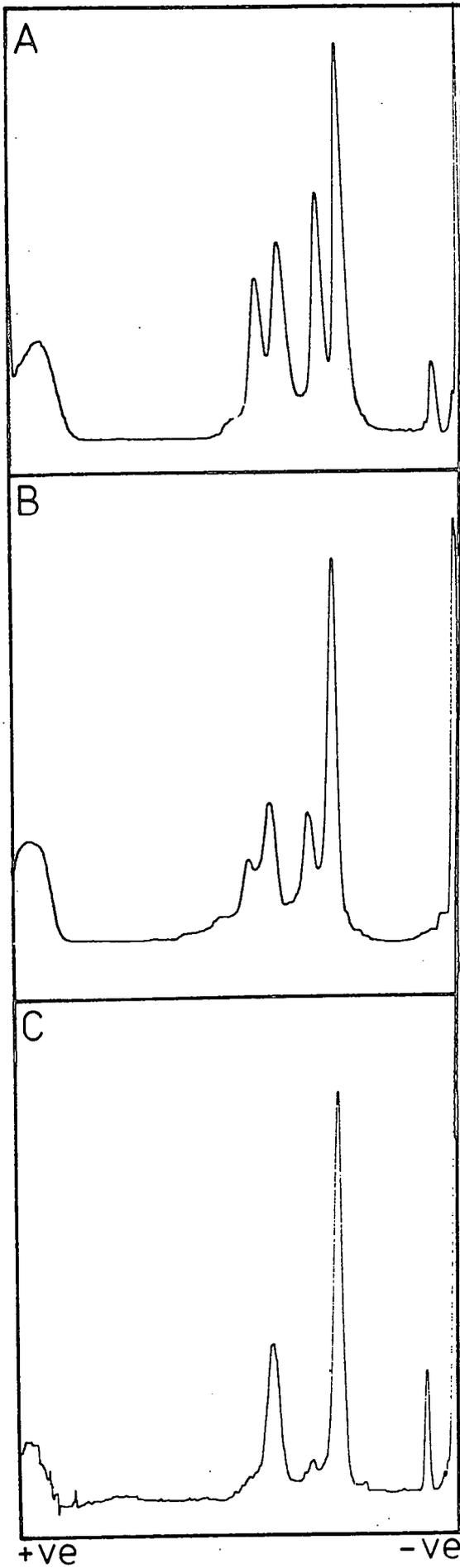
The proportion of organelle RNA (mainly chloroplasts) found in the light-grown cotyledons is slightly higher than that found by Heber (1963) for leaves of rye, broad bean, tobacco and spinach though it is similar to the value found for chinese cabbage leaves (see 4.3). There was a substantial amount of RNA of the same size as chloroplast RNA

Figure 3.25

PAGE of rRNA extracted from non-leaf tissues by the
DEP-SDS method.

- A. Light-grown cotyledons of chinese cabbage
- B. Dark-grown cotyledons of chinese cabbage
- C. Etiolated hypocotyls of chinese cabbage

A 265nm.



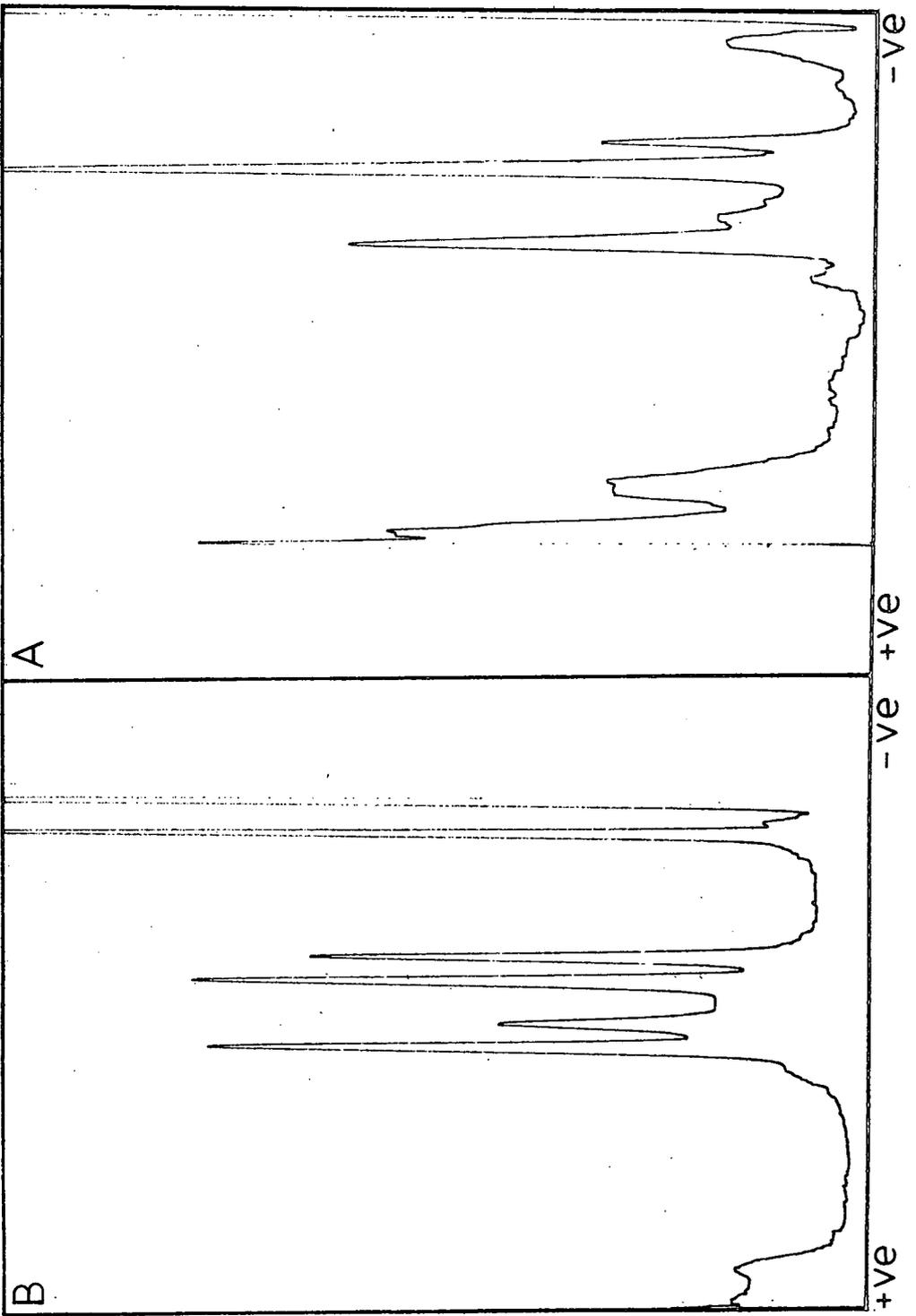
in the dark-grown cotyledons even though chloroplast development is retarded under these growth conditions. The hypocotyl ribosomal RNA consists almost entirely of cytoplasmic material though a small amount of material of organelle rRNA size was observed.

Chloroplasts were prepared by a modification of the method of Kirk and Pyliotis (1972) as described in 3.23, and the chloroplasts thus prepared appeared to be intact as judged by light microscopy. Slight contamination of the preparation with cytoplasmic components was observed, Clark et al., (1964) similarly found that by differential centrifugation alone it was not possible to obtain wholly clean chloroplast preparations from chinese cabbage leaves. Attempts to improve the method of chloroplast isolation by using discontinuous sucrose density gradients (Harvey and Brown, 1969) were not satisfactory. The use of Triton-X100 to lyse the chloroplast membranes (Stutz and Noll, 1967; Leaver and Ingle, 1971) and removal of the residue by centrifugation prior to RNA extraction served to significantly reduce the degree of contamination in the final chloroplast RNA electrophoretograms (figure 3.26). Extraction of nucleic acids from the pellet resulting from Triton-X100 treatment showed that some chloroplast material was lost at this stage though cytoplasmic RNA and DNA were present in excess (figure 3.27).

Figure 3.26

PAGE of RNA extracted from isolated chinese
cabbage leaf chloroplasts by the DEP-SDS method.

- A. Triton X-100 used to lyse chloroplast membranes.
- B. Triton X-100 omitted from the extraction.



A_{265nm}.

A

B

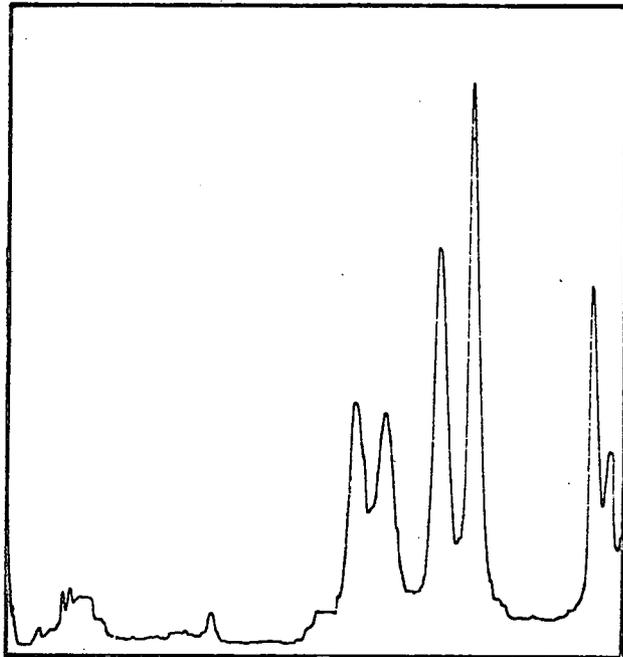
Figure 3.27

PAGE of RNA in the residual pellet remaining after Triton X-100 treatment of isolated chloroplasts. RNA extracted by the DEP-SDS method.

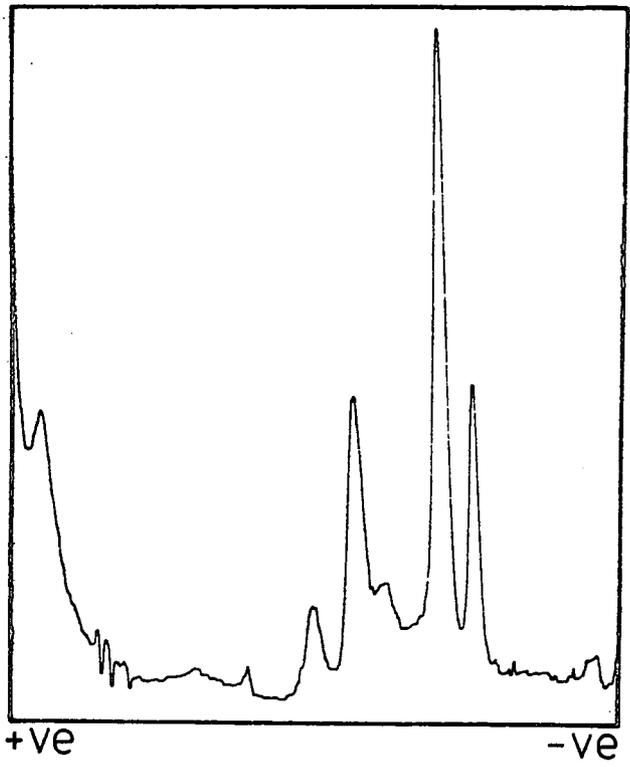
Figure 3.28

PAGE of DEP-SDS extracted RNA from chloroplasts isolated from TYMV-infected chinese cabbage leaves.

A 265nm.



A 265nm.



A disadvantage of the use of Triton-X100 to disrupt chloroplasts was that the final supernatant of the extraction procedure (containing the nucleic acids) was a dark green colour, precipitation of the nucleic acids with two volumes of ethanol then resulted in a green precipitate which obscured the u.v. spectrum. Further purification steps (ethanol-SDS washes and precipitation with cetrimide) did not remove the pigmentation, estimation of RNA content of the samples by their u.v. spectrum was therefore not possible. On electrophoresis this pigmentation was apparent as bands at the bottom of the gel which obscure the low molecular weight RNA.

Extraction of RNA from isolated chloroplasts by the method of Leaver and Ingle (1971), using double-strength detergent medium and phenol, was not found to be successful. Also large quantities of starting material were required to compensate for the loss of RNA during the purification steps that are necessary to eliminate phenol contamination.

The electrophoretic profile of RNA extracted by the DEP-SDS method from isolated chloroplasts (figure 3.26a) shows good preservation of the labile 1.05×10^6 mol.wt. chloroplast RNA when resuspended and electrophoresed in magnesium-containing buffer. The amount of cytoplasmic rRNA evident was comparable to that found in chloroplast preparations from

certain other plant species (Ingle et al., 1970) and presumably reflects a degree of association of cytoplasmic ribosomes with the outer membrane of the chloroplast. Extraction of RNA from isolated chloroplasts of TYMV-infected plants showed a similar amount of cytoplasmic RNA as that found in healthy preparations but there was proportionally far less chloroplast rRNA (figure 3.28). Again in a magnesium environment the integrity of the 1.05×10^6 mol. wt. fraction was preserved. It is not possible in these profiles to assess what proportion of the observed chloroplast rRNA is derived from infected cells as opposed to uninfected cells within the mosaic pattern of TYMV infection. There was no evidence of the virus being present in these isolated chloroplast preparations though this might have been expected if replication of the virus RNA was associated with the chloroplasts (Ralph and Clark, 1966; Ushiyama and Matthews, 1970; Lafleche and Bove, 1971; Ralph et al., 1971). However if the replication was occurring in the peripheral vesicles found in the chloroplast of TYMV-infected plants (Ushiyama and Matthews, 1970) then it was possible that any possible viral RNA was being sedimented along with the membranes in the clarificatory centrifugation following Triton-X100 disruption of the chloroplasts.

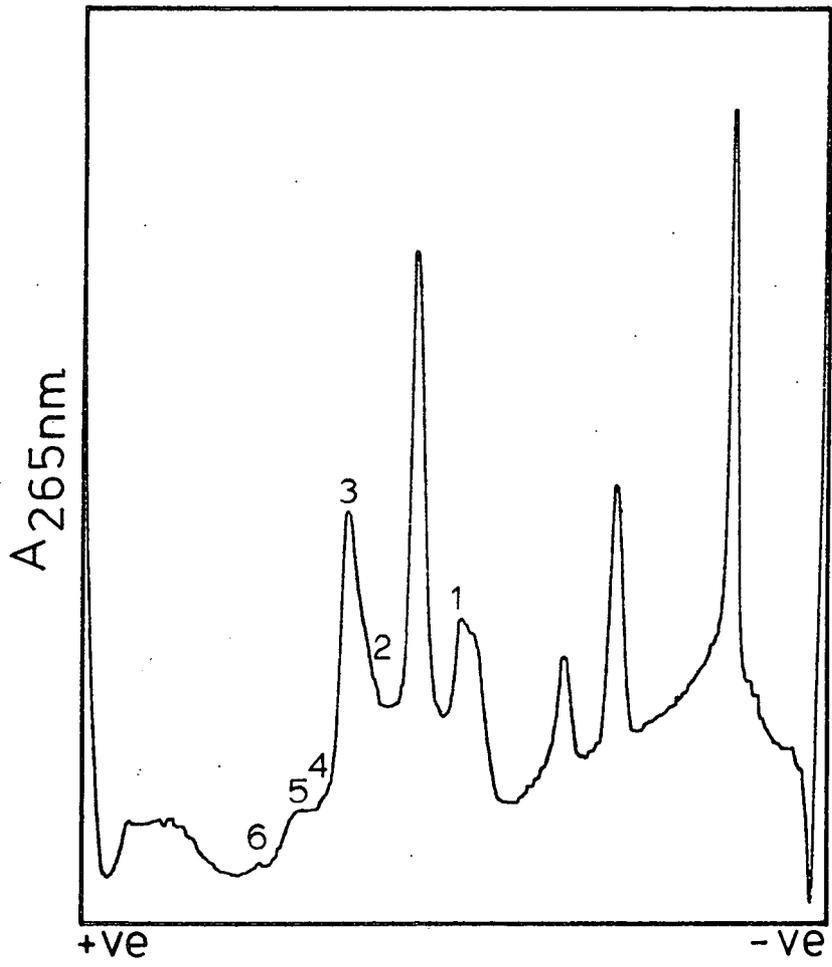
Resuspension and electrophoresis of chloroplast rRNA in EDTA buffer results in the dissociation of the labile

1.05×10^6 mol. wt. fraction (Ingle, 1968; Leaver and Ingle, 1971) with the associated appearance of a number of breakdown fractions (figure 3.29). The smaller ribosomal RNA species from the chloroplast (0.56×10^6 mol. wt.) and the cytoplasmic ribosomal RNA components (1.3×10^6 , 0.7×10^6 mol. wt.) are unaffected under these conditions (Leaver and Ingle, 1971). The molecular weights of the breakdown fractions were estimated from a plot of log (molecular weight) against migration using the 1.3×10^6 , 1.05×10^6 , 0.7×10^6 and 0.56×10^6 fractions as internal standards. The principle breakdown fractions had apparent molecular weights of 0.59×10^6 , 0.42×10^6 and 0.35×10^6 , other observed fractions gave values of 0.48×10^6 , 0.37×10^6 and 0.29×10^6 . These breakdown fractions were also present on EDTA electrophoresis of total leaf rRNA extracts (see 3.52).

Figure 3.29

PAGE of RNA extracted from isolated chloroplasts by the DEP-SDS method and resuspended and electrophoresed in EDTA-containing buffer (Loening 'E' buffer).

Peak 1	:	0.59 x 10 ⁶	mol. wt.
Peak 2	:	0.48 x 10 ⁶	mol. wt.
Peak 3	:	0.42 x 10 ⁶	mol. wt.
Peak 4	:	0.37 x 10 ⁶	mol. wt.
Peak 5	:	0.35 x 10 ⁶	mol. wt.
Peak 6	:	0.29 x 10 ⁶	mol. wt.



3.5 Some Effects of Magnesium and EDTA on Electrophoretic Fractionation of RNA

3.51 Molecular weight determination

In the course of these investigations three different buffers have been used for the electrophoresis of RNA - namely Loening 'E' buffer (Loening, 1969), the EDTA buffer of Bishop et al., (1967) and a magnesium containing buffer derived from the buffer of Bishop et al., (see 3.22.2). All molecular weights quoted in this thesis were determined using Loening 'E' buffer which has been demonstrated as giving molecular weight determinations that are probably close to the true molecular weight (Loening, 1969). Loening (1969) has noted that differences in ionic environment produced changes in electrophoretic mobility and hence apparent molecular weight and the possibility of such effects were looked at with regard to the buffer systems cited above.

As indicated previously (3.32) detergent-phenol extracted RNA resuspended and electrophoresed in Loening 'E' buffer gave the following molecular weight values for the 'high' molecular weight rRNA components of chinese cabbage when compared to E.coli rRNA used as a standard molecular weight marker:

Cytoplasmic rRNA	1.3×10^6	0.7×10^6
Chloroplastic rRNA	1.05×10^6	0.56×10^5

Similar values were obtained for DEP-SDS extracted rRNA

(see 3.42). If the cytoplasmic rRNA peaks were used as internal standards for molecular weight determinations then both RNA extraction methods gave the same result for the molecular weights of the chloroplast rRNA peaks i.e. 1.05×10^6 and 0.56×10^6 . These values were taken as the actual molecular weight values with which to compare the various electrophoretic conditions.

The same RNA extracts as used for molecular weight determination by electrophoresis in Loening 'E' buffer (Loening, 1969) were subsequently re-electrophoresed in the EDTA buffer of Bishop et al., (1967) and in the derived magnesium buffer. The molecular weights of the rRNA species were determined by comparison with E.coli rRNA. In the EDTA buffer of Bishop et al., (1967) both the phenol and the DEP-SDS RNA extracts gave lower molecular weight estimates for the rRNA species than those found using Loening 'E' buffer (Table 3). The difference in the molecular weight estimations of the chloroplast rRNA found with the EDTA buffer of Bishop et al., (1967) as compared with the Loening buffer was largely removed if the cytoplasmic rRNA species were used as internal molecular weight markers. The magnesium buffer also resulted in a changed estimate of the molecular weight of the rRNA species.

Thus differences induced in the conformation and/or the effective charge on the RNA molecules were observed with the

Table 3

Electrophoresis buffer	Sample	Mol. wt. $\times 10^6$ of the major rRNA species							
		<u>E. coli</u> rRNA as standard			Plant Cytoplasmic rRNA as standard				
'E' (Loening, 1969)	Phenol-Extracted RNA	1.30	1.05	0.70	0.56	1.3	1.05	0.70	0.56
'E' (Loening, 1969)	DEP-Extracted RNA	1.30	1.05	0.70	0.55	1.3	1.05	0.70	0.56
EDTA (Bishop et al., 1967)	Phenol-Extracted RNA	1.25	1.03	0.69	0.56	1.3	1.04	0.70	0.57
EDTA (Bishop et al., 1967)	DEP-Extracted RNA	1.28	1.02	0.69	0.54	1.3	1.05	0.70	0.56
Magnesium buffer	Phenol-Extracted RNA	1.27	1.01	0.73	0.60	1.3	1.01	0.70	0.57
Magnesium buffer	DEP-Extracted RNA	1.26	1.01	0.70	0.56	1.3	1.04	0.70	0.55

three different electrophoretic buffers used, this resulted in a changed electrophoretic mobility and a difference in the apparent molecular weight of a particular RNA species. The change in apparent molecular weight obtained with the EDTA buffer of Bishop et al., (1967) was not expected since this buffer was similar to Loenings 'E' buffer except that it is acetate based rather than phosphate (see 3.22.2). The slightly higher values obtained with Loening 'E' buffer probably represent a more accurate estimate of the molecular weight in that the lower migration is suggestive of a greater unfolding of the RNA molecule (although a change in effective charge may also be involved). The increase in electrophoretic mobility observed with the magnesium buffer was as anticipated if the RNA was in a more compact configuration. Loening (1969) found that the increase in electrophoretic mobility in magnesium was not as great as was expected from the changes observed in sedimentation coefficients in the analytical ultracentrifuge. A masking of the negative charge on the RNA molecule by the magnesium ions may in part account for this.

With the magnesium buffer it was observed that the 1.3×10^6 and 1.05×10^6 rRNA fractions had slightly greater relative mobilities when compared with E.coli rRNA than when electrophoresed in Loening 'E' buffer, the 0.7×10^6 and 0.56×10^6 fractions, however, had a slightly lower relative

mobility. This discontinuity in magnesium buffer between the large and small ribosomal RNA components has also been noted by Loening (1969), who suggested that some structural difference between the components may be the explanation.

The above fractionations were repeated with duplicate RNA samples that had been resuspended in the magnesium buffer. Contrary to the results of Loening (1969) it was found that the buffer in which the RNA was resuspended did influence electrophoretic migration and consequently the apparent molecular weight. The values obtained for magnesium resuspended RNA from either detergent-phenol or DEP-SDS extracted material were similar when fractionated in Loening 'E' buffer (Table 4).

Table 4

	Apparent mol. wt. relative to <u>E.coli</u> rRNA				Apparent mol. wt. relative to Plant cytoplasmic rRNA			
Phenol Extracted RNA	1.29	1.08	0.71	0.58	1.3	1.07	0.7	0.57
DEP-SDS Extracted RNA	1.29	1.07	0.71	0.56	1.3	1.05	0.7	0.56

Relative to the E.coli rRNA markers the larger cytoplasmic rRNA species (1.3×10^6) shows a greater electrophoretic mobility than when resuspended in EDTA buffer. The remaining rRNA species exhibited a relative decrease in mobility. A differential effect of magnesium on the different RNA components was thus observed and it took a slightly different form from magnesium electrophoresis of RNA

resuspended in EDTA-containing buffer in that the larger chloroplast rRNA species now showed a lower mobility and hence a greater apparent weight. The conformation of the RNA molecules in magnesium buffer might be expected to be more compact, an increase in relative mobility would therefore be expected. As this was not observed another factor opposing the compactness appears to be operating, possibly a masking of the net negative charge on the RNA molecules by binding of the positively charged magnesium ions. Differences in relative mobility were also extended to standard E.coli rRNA resuspended in either EDTA or magnesium containing buffer, slightly different migrations were evident on both EDTA or magnesium electrophoresis. For molecular weight determinations EDTA-resuspended E.coli rRNA was always used.

Magnesium electrophoresis of RNA resuspended in magnesium buffer resulted in molecular weight estimations distinctly different to those obtained by electrophoresis of EDTA resuspensions in Loening 'E' buffer. Generally the estimates, relative to E.coli rRNA as a standard marker, were lower in the magnesium system. The molecular weight estimations in magnesium also showed a degree of variability. If the binding of magnesium ions to the RNA was not specific then variations in the conformation and overall charge of the RNA

molecules could occur which could account for variability in electrophoretic migration in turn giving rise of a changed molecular weight estimation.

In view of the above results it would seem that the presence of magnesium ions has a greater influence on electrophoretic molecular weight estimation than has hitherto been suggested. Also similar EDTA-containing buffers can give rise to slightly differing molecular weight estimations. Caution in the use of electrophoresis of RNA as a means of molecular weight estimation has also been expressed in the denaturation studies of Reijnders et al., (1973) and of Pring and Thornbury (1975).

Throughout the above results detergent-phenol and DEP-SDS extracted RNA showed the same pattern of change in response to the different conditions of electrophoresis. The extraction conditions of high salt and magnesium concentration used in the DEP-SDS method do not appear to have had any great influence on the electrophoretic behaviour of the rRNA species compared to the more usual detergent-phenol method of RNA extraction.

3.52 Breakdown fractions of rRNA

Under conditions of EDTA electrophoresis RNA fractions in addition to the four major high-molecular weight ribosomal RNA species were present. Under these electrophoretic conditions the ratio of the two chloroplast rRNA species, as

observed in electrophoretograms, is not equimolar (Loening and Ingle, 1967; Ingle et al., 1970; Ingle, 1968; Leaver and Ingle, 1971). The low amount of the chloroplast large ribosomal sub-unit RNA and the presence of additional RNA fractions of lower molecular weight suggested a selective breakdown of this chloroplast fraction. This has now been demonstrated in a number of plant species (Ingle, 1968; Leaver and Ingle, 1971; Grierson, 1974). The pattern of breakdown of this labile chloroplast rRNA fraction varies from species to species (Leaver and Ingle, 1971) and can also vary within a species under different conditions of RNA extraction and fractionation (Grierson, 1974). The pattern of breakdown products obtained from the dissociation of chinese cabbage chloroplast 1.05×10^6 RNA was examined under varying conditions of electrophoresis.

The greatest number of additional breakdown fractions was observed on EDTA electrophoresis of RNA samples resuspended in EDTA buffer. The degradation product present in the largest amount had an apparent molecular weight of 0.42×10^6 when compared with E.coli rRNA standards, other breakdown fractions that were observed had molecular weights of 0.59×10^6 , 0.48×10^6 and 0.37×10^6 . In addition to these fractions further minor breakdown peaks were sometimes observed with molecular weights of 1.0×10^6 , 0.9×10^6 , 0.35×10^6 and 0.29×10^6 . The appearance of these latter

components was not consistent and may have depended on the physiological state of the tissue or on slight variations in extraction. The amount of chloroplast 1.05×10^6 rRNA present was always greatly reduced under these conditions. EDTA electrophoresis of RNA from isolated chloroplasts gave a similar pattern of breakdown products (see figure 3.29) suggesting that the observed fractions were primarily derived from the labile chloroplast rRNA fraction.

In neither EDTA electrophoresis of total leaf RNA nor the EDTA electrophoresis of isolated chloroplast rRNA was a 0.7×10^6 molecular weight RNA fraction observed (as evidenced by a reduction in the ratio of $1.3 \times 10^6 : 0.7 \times 10^6$ cytoplasmic RNA species) though this is a common breakdown fraction in many other plant species (Ingle, 1968; Leaver and Ingle, 1971).

The pattern of breakdown products indicated above could be significantly changed by the presence of magnesium ions either in the resuspension buffer or in the electrophoresis buffer. Total leaf RNA extracts resuspended in magnesium buffer and fractionated in the presence of EDTA showed a marked reduction in the number of breakdown fractions observed. Only one major breakdown peak consistently appeared, the 0.42×10^6 molecular weight fraction, other fractions that appeared on some gels had molecular weights of 0.9×10^6 ,

0.59×10^6 , 0.48×10^6 , 0.37×10^6 and 0.29×10^6 . These fractions occurred primarily when using the EDTA buffer of Bishop et al., (1967) and were less evident in Loening 'E' buffer (Loening, 1969).

The above molecular weights are quoted as the expected molecular weight value taken from the homogenous EDTA buffer system. The influence of magnesium resuspension on molecular weight determination has been indicated previously (see 3.51).

Electrophoresis in magnesium buffer gave a similar reduction in the number of breakdown products detected. Again the 0.42×10^6 component was the main breakdown fraction observed, some of the other breakdown fractions noted above were present in very small amounts on some gels. The lack of breakdown fractions detectable on the electrophoretograms was reflected in an increased amount of intact 1.05×10^6 chloroplast rRNA present. The ratio of 1.05×10^6 : 0.56×10^6 (taken from peak area weights) was close to 2.0 in these gels. Unexpectedly the resuspension buffer did not appear to influence the pattern of breakdown products observed on magnesium electrophoresis. No consistent qualitative or quantitative differences were observed with regard to the pattern of minor breakdown products when magnesium resuspended RNA was compared to RNA resuspended in EDTA buffer. The above results are summarised in Table 5.

Table 5

Pattern of rRNA breakdown fractions under varied electrophoresis conditions

RNA resuspension buffer	Electrophoresis buffer	Major breakdown fractions (mol. wt $\times 10^6$)		Minor breakdown fractions (mol. wt. $\times 10^6$)					
EDTA	EDTA	0.59	0.48	0.42	0.37	1.0	0.9	0.35	0.29
Magnesium	EDTA		0.42			0.9	0.59	0.48	0.37
EDTA	Magnesium		0.42			0.9	0.59	0.37	0.29
Magnesium	Magnesium		0.42			0.9	0.59	0.37	0.29

Differences were looked for in the pattern of breakdown products obtained between the detergent-phenol and DEP-SDS RNA extraction methods, but RNA prepared by either method behaved as described above. There were some slight variations in the particular complement of minor breakdown components present in any particular gel, however since these fractions tended to vary just as much within an extraction method it was thought that the variation observed was due rather to differences in the tissue extracted. The high-salt, high magnesium concentration buffer used in the DEP-SDS method did not therefore seem to confer any greater stability on the labile 1.05×10^6 rRNA species than did the modified detergent-phenol method used in this work.

3.53 Heat-treated rRNA

The 23s rRNA of chloroplast ribosomes is extremely labile and normal conditions of RNA extraction and fractionation aim to preserve the integrity of this fraction. Under conditions of mild heat shock the 'hidden breaks' in the 23s rRNA species are released (Grierson, 1974) and the dissociation fragments are observed on subsequent electrophoresis. Electrophoresis in the presence of magnesium ions does not result in the reconstitution of these breakdown fractions. Figure 3.30 shows total leaf RNA that has been heat-treated at 60°C for 5 minutes prior to electrophoresis, the RNA was resuspended and



electrophoresed in Loening 'E' buffer (Loening, 1969). The molecular weights of the fractions observed were estimated by a plot of log (molecular weight) against electrophoretic mobility using E.coli ribosomal RNA as a standard molecular weight marker. Conformational changes induced in the RNA fractions by the heat treatment gave rise, in some instances, to slightly differing molecular weight values to those obtained on undenatured RNA. The probable correspondence with breakdown fractions already described is seen in Table 6.

Heat treatment of EDTA - resuspended RNA samples resulted in the complete dissociation of the labile 23s rRNA fraction (figure 3.30). The breakdown products observed on EDTA electrophoresis of undenatured RNA were all observed and in addition there were further fractions present in the heat-treated sample (Table 6). These extra-fragments had apparent molecular weights of 1.11×10^6 , 0.33×10^6 and 0.23×10^6 . A major change was observed in the ratio of the 1.3×10^6 : 0.7×10^6 cytoplasmic rRNA peaks, in native RNA the ratio between these two components was approximately 2 : 1 (based on peak area weights), but in electrophoretograms of heat-treated RNA the ratio was 1 : 1 or less. Comparison of equivalent sample loadings of heated and control material showed that this change in ratio was due to a decrease in absorbance of the 1.3×10^6 peak, the absorbance present in

Table 6 Breakdown fractions produced by heat treatment

Resuspension Buffer	Treatment	Breakdown fractions mol. wt. x 10 ⁶ relative to <u>E.coli</u> rRNA							
EDTA	Native	1.0	0.9	0.59	0.48	0.42	0.37	0.35	0.29
Magnesium	Native		0.9	0.6		0.42	0.37		0.29
EDTA	60°C, 5 mins.	1.11	0.98	0.61	0.5	0.44	0.39	0.35	0.3
Magnesium	60°C, 5 mins.		0.9	0.62	0.48	0.44	0.39		0.23

Figure 3.30

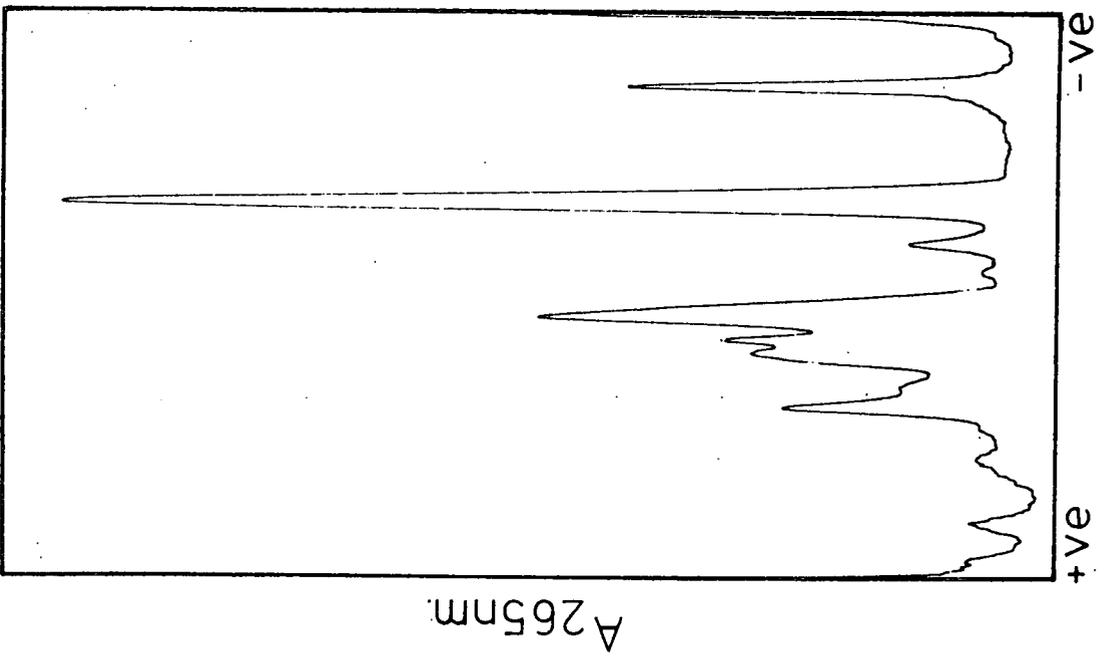
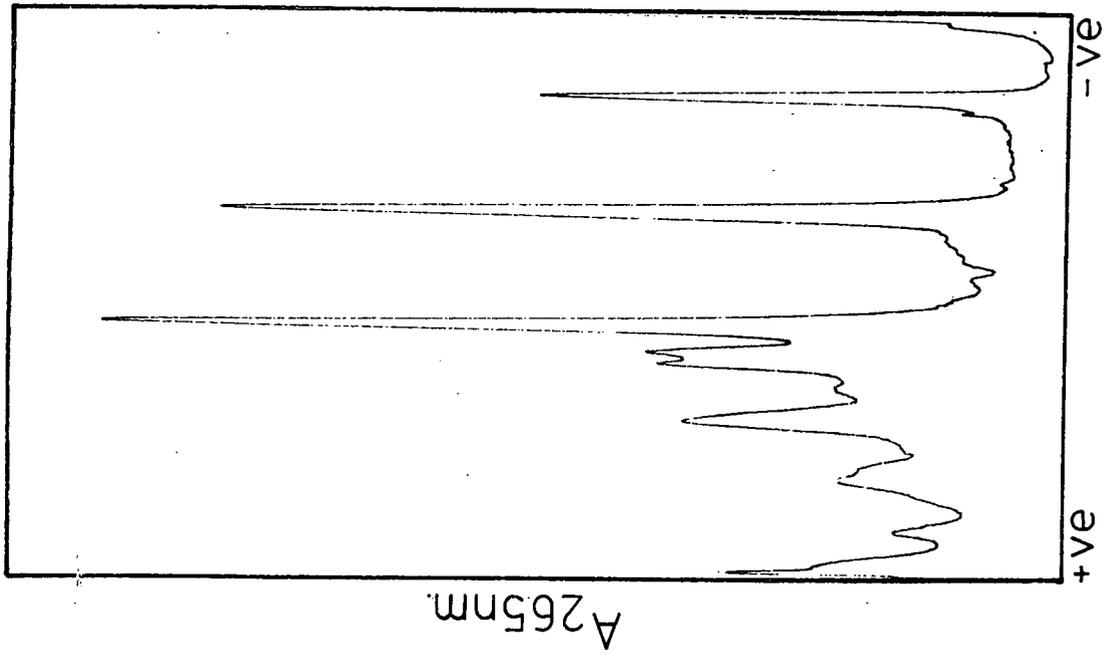
PAGE of rRNA heat-treated (60°C for 5 minutes) prior to electrophoresis.

RNA resuspended and electrophoresed in Loening 'E' buffer.

Figure 3.31

PAGE of rRNA heat-treated (60°C for 5 minutes) prior to electrophoresis.

RNA resuspended in magnesium buffer and electrophoresed in Loening 'E' buffer.



the 0.7×10^6 peak was unchanged. This suggests a lability of the cytoplasmic large ribosomal sub-unit RNA under the conditions of heat treatment used. The specific dissociation of the 1.3×10^6 rRNA on heat-treatment has previously been described in mung-beans (Grierson, 1974) and peas (Higo et al., 1971). The extra breakdown products indicated above may possibly be derived from the cytoplasmic 1.3×10^6 rRNA rather than from the chloroplast 1.05×10^6 rRNA. However heat-treatment of RNA resuspended in magnesium buffer still resulted in the production of the 0.33×10^6 and 0.23×10^6 components although the 2 : 1 ratio of 1.3×10^6 : 0.7×10^6 rRNA species was maintained, (figure 3.31). The higher molecular weight breakdown products (1.11×10^6 and 0.98×10^6) are not seen in the profile of magnesium resuspended RNA, the small amount of chloroplast 1.05×10^6 rRNA present, stabilised against dissociation by the presence of magnesium ions, may however have been masking the detection of these breakdown species. Heat-treatment of rRNA from isolated chloroplasts also gave rise to fragments with apparent molecular weights of 0.33×10^6 and 0.23×10^6 further suggesting that these fragments are not necessarily from cytoplasmic rRNA.

Further comparisons between magnesium and EDTA - resuspended, heat treated RNA did not reveal any other possible fragments as possible cytoplasmic rRNA breakdown products.

The 0.35×10^6 component found in EDTA resuspended material but absent from the magnesium resuspended RNA was also found in undenatured EDTA - resuspended RNA. Under these latter conditions the 1.3×10^6 cytoplasmic rRNA is stable and this fraction is therefore probably not of cytoplasmic origin. It thus appeared that the induced breakdown of this cytoplasmic rRNA species gave rise to fragments of similar size to RNA components already described (either breakdown products or native RNA) and therefore could not be resolved by the methods used. For the unequivocal determination of the specific dissociation products of this RNA species experiments in which this RNA component was isolated prior to heat treatment would need to be carried out.

The presence of magnesium in the RNA resuspension buffer has less of a stabilising effect on dissociation during heat-treatment than it does on native RNA (Table 6). Fragments of apparent molecular weight 1.11×10^6 , 0.98×10^6 and 0.35×10^6 were not detected in the electrophoretograms of magnesium-resuspended heat-treated leaf RNA (figure 3.31), the former two RNA species may however have been masked by the presence of some intact 1.05×10^6 chloroplast rRNA.

For both magnesium and EDTA - resuspended leaf RNA the effects of heat treatment were the same whether the RNA was extracted by the detergent-phenol or by the DEP-SDS method.

A further RNA species was released upon heat-treatment of leaf ribosomal RNA. This low molecular weight component was readily distinguished from 4s and 5s species when electrophoresed in 7.5% polyacrylamide gels (figure 3.32). This RNA species, designated 5.8s RNA (Payne and Dyer, 1972), has been found in a wide range of species and is characteristic of cytoplasmic ribosomes of eucaryotes (Payne and Dyer, 1972). The TYMV-host RNA complex formed during detergent-phenol RNA extraction does not inhibit the release of 5.8s RNA upon heat treatment. Heat treatment does not however permit the release of any other fragments from the complex.

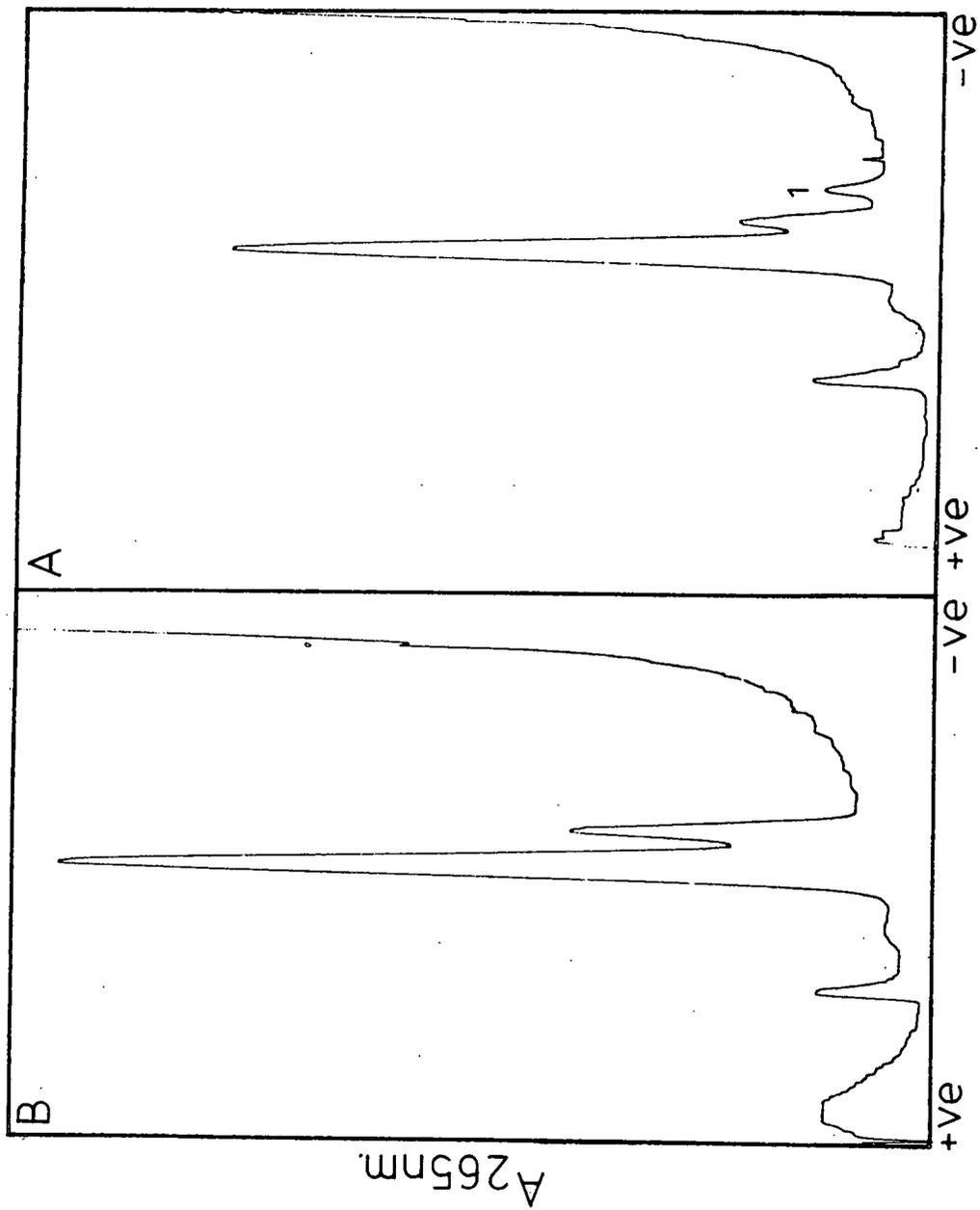
Figure 3.32

PAGE of low molecular weight RNA.

- A. RNA heat-treated (60°C for 5 minutes) prior to electrophoresis.

Peak 1 : 5.8s

- B. Control.



3.6 Comparison of Detergent-Phenol and DEP-SDS methods of RNA Extraction

Previous sections have described various aspects of RNA extraction and fractionation involving RNA extracted by two different methods, here a comparison of the detergent-phenol and DEP-SDS methods of RNA extraction is made to illustrate the distinctive features of the two methods.

Characteristics of the u.v. absorption spectra of RNA isolated by the two methods have been detailed previously (see 3.31 and 3.41) and are summarised in Table 7.

Table 7

	λ Max	λ Min	E_{max}/E_{min}	E_{max}/E_{280}
Detergent-phenol	258nM	230nM	2.0	2.0
DEP-SDS	260nM	235nM	1.78	1.86

There was a shift in the wavelengths of the absorption maximum and minimum of DEP-SDS extracted RNA as compared to detergent-phenol prepared material. A similar change in u.v. absorption characteristics has been noted by Solymosy et al., (1972) for DEP treated yeast RNA and has been attributed to carboxymethylation of the nucleic acid bases.

The values for the spectral ratios of DEP-SDS extracted RNA are lower than those obtained with detergent-phenol extracted material. However the results for DEP-SDS RNA were obtained on resuspension of the RNA after only one alcohol precipitation, similar figures could not be obtained with the

phenol method since traces of phenol contaminated the RNA at this stage. This contamination obscured the u.v. absorption spectrum of the RNA by shifting the absorption maximum to 270nm and giving a further 'shoulder' of absorption at approximately 280nm (figure 3.33). The effectiveness of the steps taken to remove phenol contamination were examined by recording the u.v. absorption spectra of a sample removed at different stages throughout the purification procedure. RNA that had been extracted by the DEP-SDS method was similarly treated. The stages of purification and the spectral ratios obtained at each particular step are detailed in Table 8.

Table 8

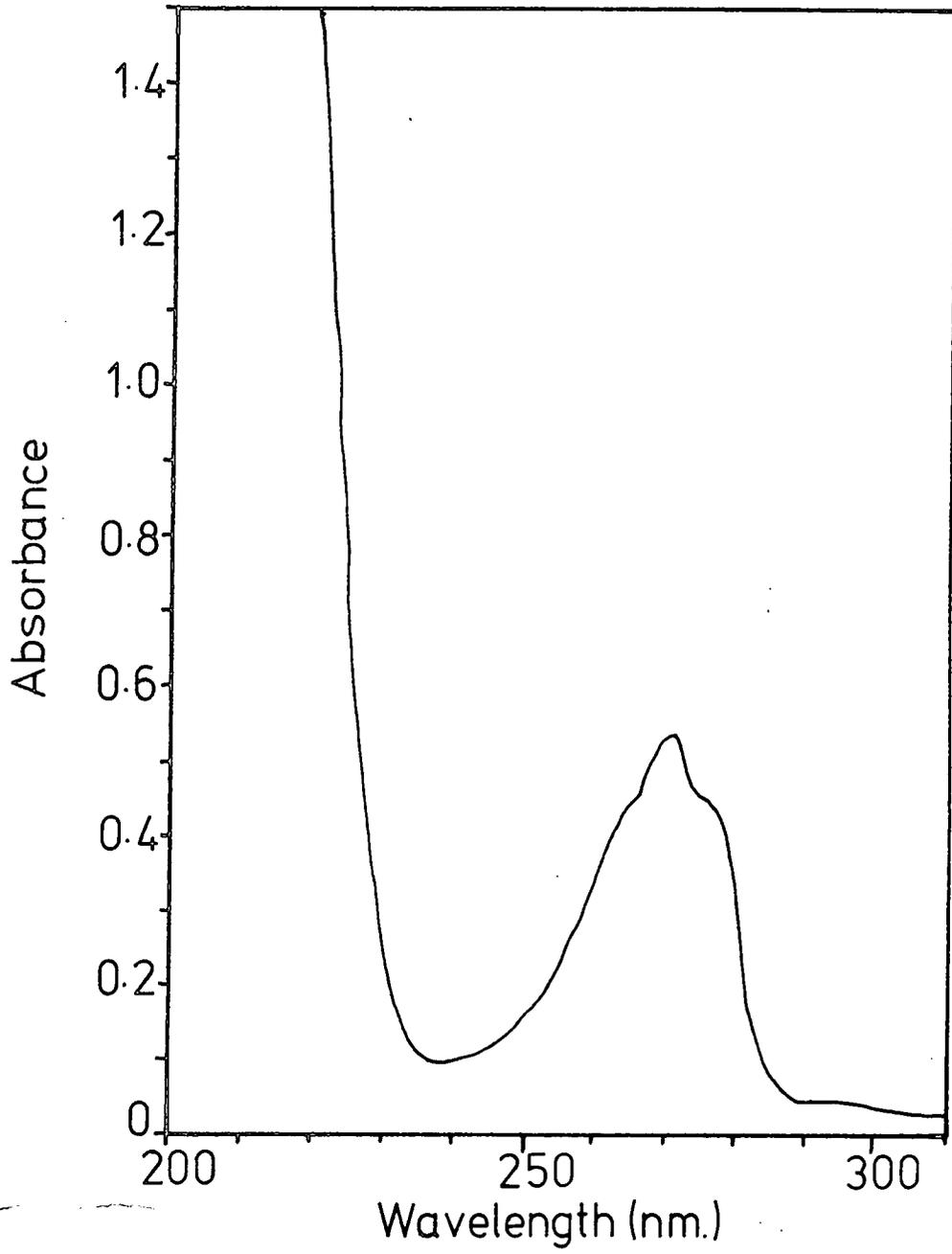
Stage of purification	<u>Phenol</u>		<u>DEP-SDS</u>	
	E258/ E230	E258/ E280	E260/ E235	E260/ E280
After first ethanol precipitation	--	-- *	1.65	1.79
After washing in 80% EtOH + 0.5% SDS	1.80	1.80	1.74	1.80
After second ethanol precipitation	2.03	1.84	1.82	1.74
After cetab precipitation	1.56	1.50	1.80	1.50
Final preparation	2.00	1.96	1.91	1.89

* phenol contamination obscured u.v. spectrum

There was an improvement in the spectral ratios for detergent-phenol prepared RNA with successive purification steps. The low ratios obtained after the precipitation with

Figure 3.33

u.v. absorption spectrum of detergent-phenol extracted
chinese cabbage leaf RNA showing phenol contamination.



cetrimide are because the RNA exists at this stage as the cetab-salt (Ralph and Bellamy, 1964), subsequent conversion of the RNA back to the sodium salt gives the ratios obtained with the final preparation. Although there is some improvement in the ratios for the purposes of electrophoresis two ethanolic precipitation steps are sufficient, no great improvement in purity being apparent by utilising precipitation with cetrimide. The purity of DEP-SDS extracted RNA also improves slightly with successive purification steps. As has been shown previously good electrophoretic profiles can be achieved after only one ethanol precipitation of DEP-SDS prepared material even though the spectral ratios can be improved by further cleaning. In part these lower ratios may be a result of carboxymethylation reactions between the nucleic acid bases and DEP (Solymosy et al., 1972).

An important point that was affected by the purification steps used was the effect on the final yield of RNA. Solymosy et al., (1968) have reported that the DEP-SDS extraction method gave a higher yield of RNA from tobacco and bean than phenol methods, particularly with ageing material. It was found in this work that a lower yield with the detergent-phenol method was associated with the necessity of using purification steps to clean up the RNA.

Table 9

Extraction method	RNA yield ($\mu\text{g/g}$ f.wt.)	$E_{\text{max}}/$ E_{min}	$E_{\text{max}}/$ E_{280}
1. Detergent-phenol, full purification	505	1.98	1.94
2. DEP-SDS, full purification	545	1.93	1.93
3. Detergent-phenol, full purification	580	2.02	1.98
4. DEP-SDS, 1st ethanol precipitate	914	1.85	1.98

2g fresh weight of chinese cabbage leaves were taken and split into half-leaves, one set of half leaves was extracted by the detergent-phenol method and the remainder by the DEP-SDS method. In both cases the RNA extracted was purified using the full purification procedure normally used with detergent-phenol RNA extractions (see 3.21.1). The yield obtained with the two methods was comparable (Samples 1 and 2, Table 9). A second 2g fresh weight batch of leaves was similarly divided into half-leaves and the RNA extracted by each of the two methods. The full purification procedure was again performed on the detergent-phenol extracted RNA and the yield obtained (Sample 3, Table 9) was close to that achieved in samples 1 and 2 with leaves of the same age. The yield for the second DEP-SDS RNA extract was determined from the u.v. absorption spectrum of the RNA after just one ethanolic

precipitation of the RNA. The spectral ratios indicated that the RNA was of a similar degree of purity to the detergent-phenol extracted RNA, but the yield obtained (914 $\mu\text{g/g}$ f.wt.) represented an increase of nearly 60% compared to the detergent-phenol extracted RNA. These results demonstrated that the purification steps required to eliminate phenol contamination resulted in a loss of RNA. Because extensive purification steps were not necessary with the DEP-SDS method of RNA extraction there was a greater conservation of material which was of advantage when only limited amounts of starting tissue were available. The ability to use smaller initial amounts of material has led to the successful use of the DEP-SDS method for the extraction of nucleic acids from isolated chloroplasts (see 3.45), extracted ribosomes (see 3.9) and also from ageing or senescent leaf material in which decreased amounts of RNA are present (see 4.3).

In order to preserve the integrity of the labile chloroplast 23s RNA magnesium ions have to be included in the RNA extraction medium. With the detergent-phenol method this leads to contamination of the extracted RNA with non-nucleic acid material which results in poor resolution of the RNA fractions on subsequent electrophoresis as described by Leaver and Ingle (1971). The addition of EDTA at a late stage in the extraction procedure overcame this to some extent

(see 3.33) but adversely affected the integrity of the chloroplast 1.05×10^6 rRNA. The problem of 'dirty' profiles was not encountered with the DEP-SDS method even though the extraction buffer had the same magnesium concentration as that used in the detergent-phenol method and because EDTA was not needed to clarify the extract with the DEP-SDS method the integrity of the rRNA fractions was maintained with only slight evidence of breakdown or dissociation.

That RNA extracted by either the detergent-phenol or DEP-SDS methods behaved similarly on electrophoresis has been indicated previously (see 3.5). With regard to the pattern of breakdown products apparent under different conditions of electrophoresis, the effects of heat shock and the electrophoretically estimated molecular weights, RNA obtained by either method gave the same results. Thus, from the point of view of electrophoresis, the two methods of RNA extraction used on chinese cabbage were equivalent, but the DEP-SDS method held certain advantages regarding speed and ease of extraction, and also in the greater conservation of RNA.

The DEP-SDS method however had a major advantage with regard to the extraction of RNA from TYMV-infected chinese cabbage leaves. In this material the detergent-phenol method of RNA extraction was not satisfactory because on extraction a complex was formed between TYMV-RNA and the host rRNA

(Matus et al., 1964), this complex was not formed under the conditions employed for the DEP-SDS extraction method (see 3.43). It has already been demonstrated that DEP-SDS extracted RNA and detergent-phenol extracted RNA from healthy tissue behave similarly on electrophoresis (see 3.5). The electrophoretic behaviour of RNA extracted from infected tissue was compared to that of RNA obtained from uninfected tissue also extracted by the DEP-SDS method. The RNA from infected and control leaf material was resuspended and subsequently electrophoresed in Loening 'E' buffer (Loening, 1969). Molecular weights of the RNA fractions were estimated graphically from a plot of log (molecular weight) against migration using the E.coli rRNA as a molecular weight standard. The estimated molecular weights obtained were 1.3×10^6 and 0.7×10^6 for the cytoplasmic rRNA species, and 1.05×10^6 and 0.56×10^6 for the chloroplastic rRNA species, breakdown products of 0.42×10^6 and 0.59×10^6 were also observed. In some gels further breakdown fractions could be detected if the sensitivity of recording of the gel trace was increased, fractions of estimated molecular weight 0.9×10^6 , 0.37×10^6 and 0.35×10^6 were then observed. The lower amount of dissociation products found on EDTA electrophoresis of RNA extracted from infected leaf tissue was correlated with the reduced amount of chloroplast RNA present in the tissue.

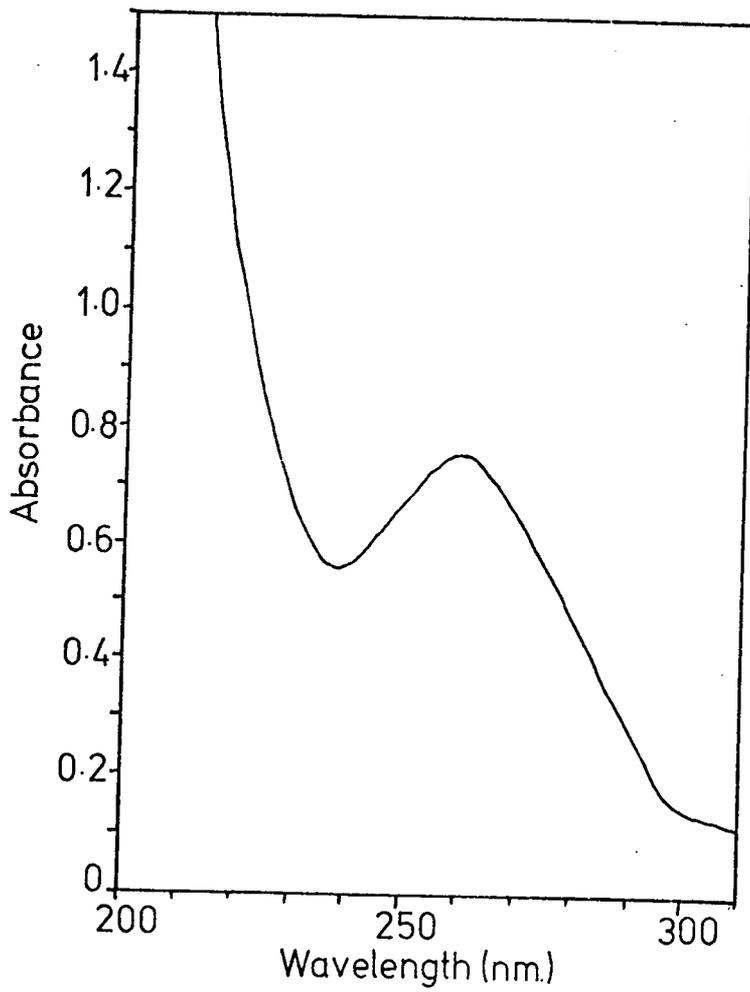
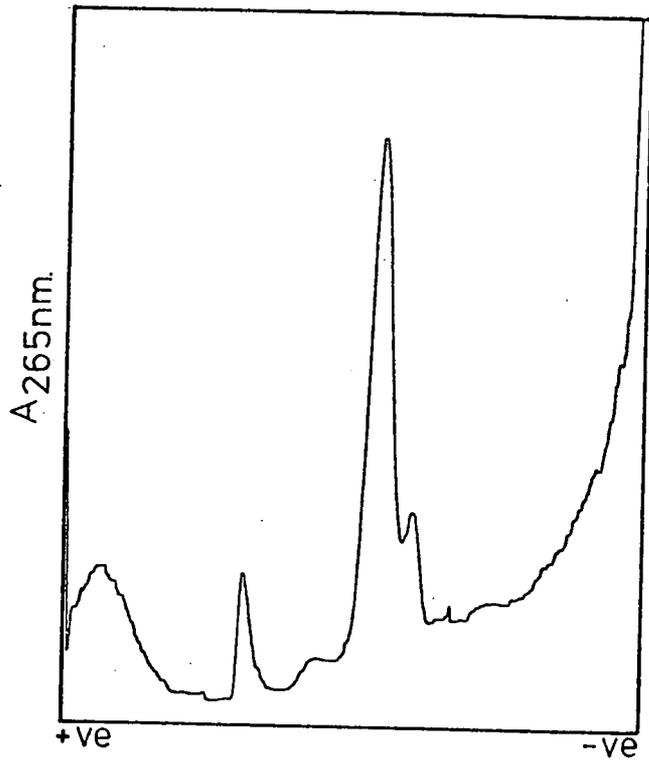
The behaviour of the high molecular weight rRNA fractions from TYMV-infected material behaved similarly to RNA extracted from healthy leaf tissue when subjected to the different conditions of electrophoresis previously described (see 3.5) and to heat shock. Fractionation of low molecular weight RNA extracted from TYMV-infected leaves showed the same differentiation into 4s and 5s RNA as found with uninfected leaf RNA (figure 3.34). Thus from the above results with regard to electrophoretic criteria the RNA extracted from TYMV-infected chinese cabbage plants behaved in exactly the same manner as RNA obtained from uninfected plants. The only marked difference being the large reduction in the proportions of chloroplast rRNA which is consistent with previously reported chemical and cytological abnormalities in the chloroplasts of TYMV-infected plants (Reid and Matthews, 1966; Chalcroft and Matthews 1966, 1967; Gerola et al., 1966; Milicic and Stefanac, 1967).

Figure 3.34

PAGE of low molecular weight RNA extracted from TYMV-infected leaves by the DEP-SDS method.

Figure 3.35

u.v. absorption spectrum of a total ribosome extract from chinese cabbage leaves.



3.7 Ribosomes and Polysomes

3.71 U.V. spectral characteristics

The u.v. absorption spectrum of total ribosome extracts from uninfected chinese cabbage leaves showed a maximum absorption at 258nm and a minimum at 238nm (figure 3.35) in agreement with the values found by Kliffen (1970) for pea ribosomes. With extracts from TYMV-infected leaves there was a shift in the wavelengths of maximum and minimum absorption to 259-261 nm and 239-240 nm respectively. The degree of change of these maxima and minima appeared to be related to proportion of virus present in the extract.

The average value for the ratio $E_{\text{max}}/E_{\text{min}}$ was 1.32 for healthy plant preparations and 1.40 for preparations from TYMV-infected plants suggesting slight protein contamination in some samples (Odintsova et al., 1964; Peterman, 1964). The average value for the ratio E_{260}/E_{280} was 1.71 for both healthy and TYMV-infected leaf extracts. This figure is intermediate between the values of 1.8-1.85 for cytoplasmic ribosomes and 1.56-1.67 for chloroplast ribosomes found by Odintsova and Yurina (1969) but the chinese cabbage leaf extracts were of total leaf ribosomes. The possible protein contamination indicated in the $E_{\text{max}}/E_{\text{min}}$ values may also have been contributory to this lower value.

The yield of ribosomes from leaf tissue was found to vary with the age of the leaf extracted (see 4.4).

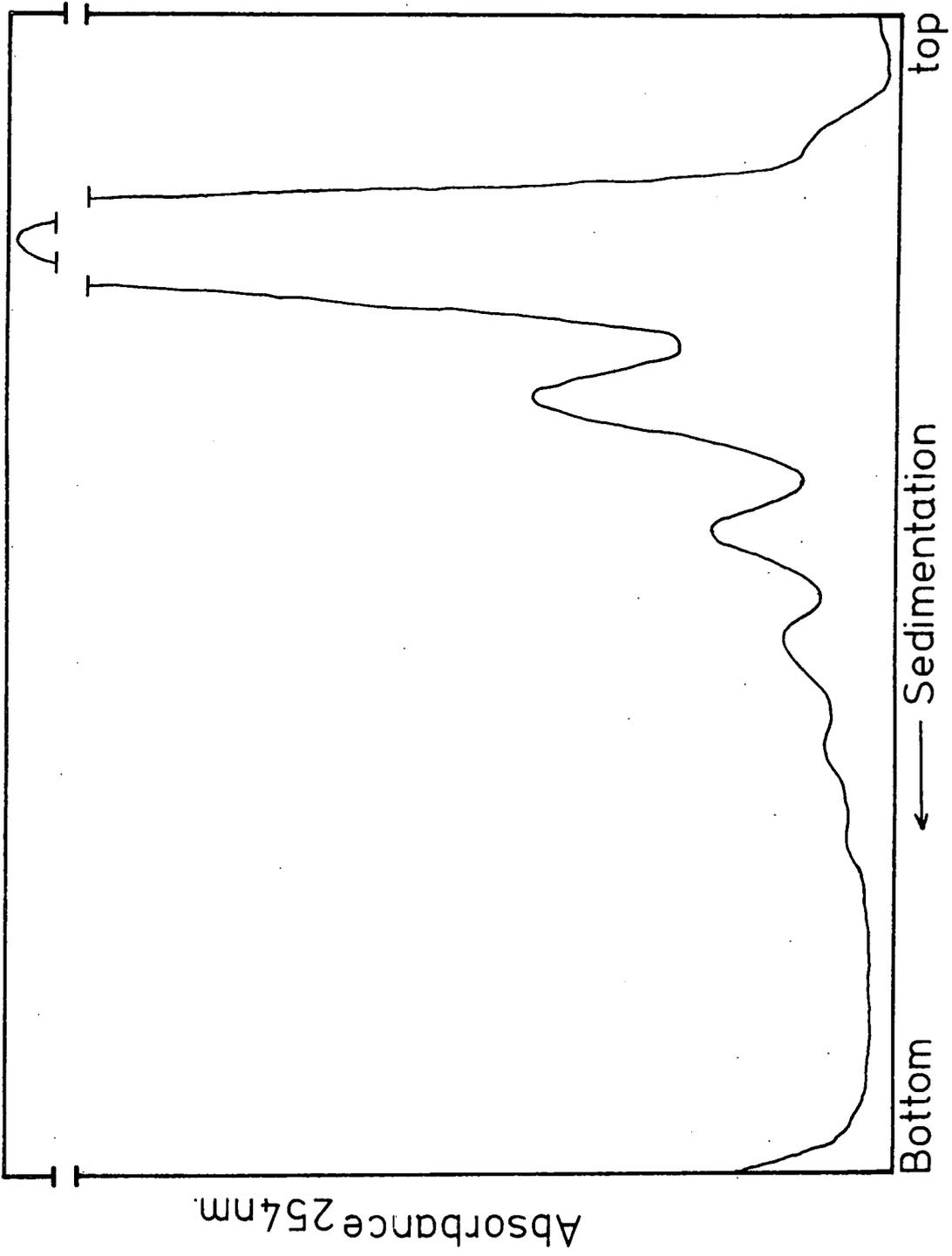
3.72 Sucrose Density Gradient Fractionation

The 105,000g. ribosome pellets from young leaves were re-suspended in ribosome buffer, layered onto linear 15-34% sucrose density gradients and centrifuged as described in 3.24.3. The fractionated ribosomes, monitored at 254nm, typically showed six peaks (figure 3.36). The fraction showing the strongest u.v. absorption was taken to be the monoribosomes with the subsequent peaks representing increasing numbers of ribosomes attached to mRNA^o (dimers → hexamers) (Dass and Bayley, 1965). Clark et al., (1964) obtained polyribosomes to the level of pentamer from young chinese cabbage leaves, their result being obtained in the analytical ultracentrifuge as they were unable to achieve satisfactory sucrose density gradient separations. By using higher salt concentrations and a raised pH Davies et al., (1972) have managed to extract polyribosomes from etiolated pea stems consisting of at least nine ribosomes attached to mRNA species. However the use of their method of extraction in this present work did not result in any further increase in the size of polymers observed with chinese cabbage leaf material.

Total ribosome extracts from TYMV-infected leaves were similarly fractionated by sucrose density gradient centrifugation but the presence of TYMV in these preparations interfered with the resolution of the dimer and trimer

Figure 3.36

Polyribosomes extracted from young leaves of chinese
cabbage fractionated on a 15-34% linear sucrose density
gradient.



polyribosome fractions (figure 3.37), resulting in difficulties with regard to examining the status of the host ribosome population. The measurement of polyribosome concentration in infected tissue by analytical centrifugation is also affected (Matthews and Ralph, 1966).

Figure 3.38 shows a sucrose density gradient fractionation of polyribosomes extracted from etiolated pea stems. Separation of fractions to the nonamer demonstrates that the method can resolve larger polymers than those routinely obtained from chinese cabbage leaves, therefore the profiles achieved are probably limited by the difficulties of using leaves as a source material rather than by the analysis.

3.73 Polyacrylamide gel electrophoresis

3.73.1 Standard electrophoretic system

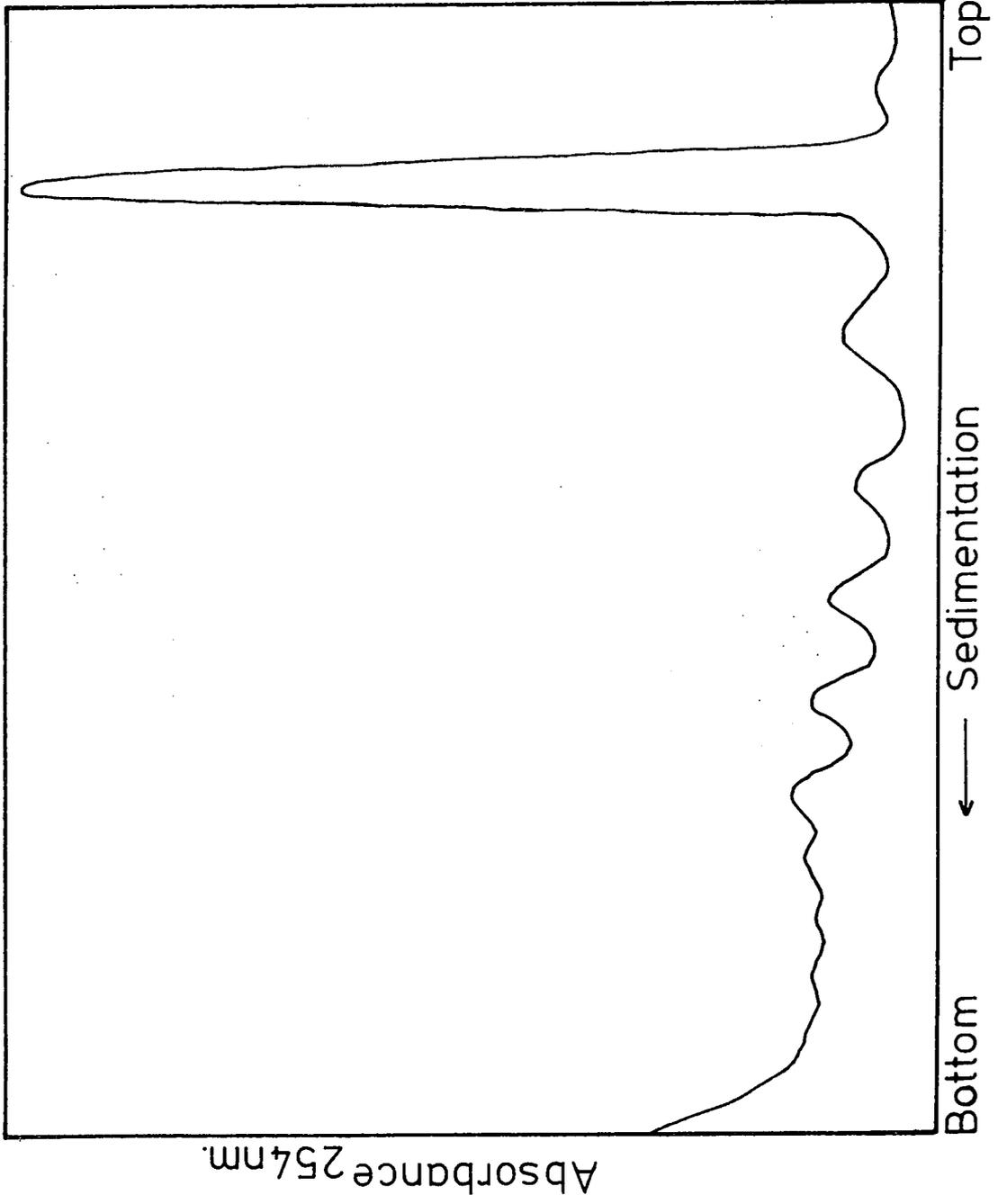
Aliquots of ribosome preparations resuspended in ribosome buffer plus approximately 10% sucrose were carefully loaded on to the surface of 2.2% polyacrylamide gels. The conditions for electrophoresis were as described in 3.24.3. A constant voltage mode was selected on the power pack as this was found to provide the most stable conditions throughout the electrophoresis. A small drop in the amperage occurred during the 30 minute pre-run from approximately 70 mA to 66 mA per electrophoresis tank containing 1.5 litres of buffer with seven 8 cm gels. (The absolute values varied slightly

Figure 3.37

Polyribosomes extracted from TYMV-infected leaves of
chinese cabbage fractionated on a 15-34% linear sucrose
density gradient.

Figure 3.38

Polyribosomes extracted from etiolated pea stems
separated on a 15-34% linear sucrose density gradient.



with different batches of buffer but a drop of 4 mA per tank was usual. During the electrophoresis itself the current was more stable fluctuating by no more than 2 mA per tank).

In the course of the electrophoretic run accretion of a magnesium oxide at the cathode (upper buffer reservoir) occurred thus affecting the stability of the ribosomes. This accretion was markedly reduced by circulating the electrophoresis running buffer, at a rate of 5.0 ml per min., between the two reservoirs of the gel tank by means of a peristaltic pump. With the normal two hour duration of electrophoresis it was not necessary to replace the running buffer in the manner described by Dahlberg et al., (1973).

The results of an electrophoretic separation of ribosomes extracted from young chinese cabbage leaves is illustrated in figure 3.39. The peak exhibiting the greatest u.v. absorption has been designated the monoribosome peak with the remaining peaks being successive polyribosome fractions. That the designated monoribosome peak was not a ribosome sub-unit was confirmed by co-electrophoresis with polyribosomes that had been resuspended in either EDTA or tetrasodium pyrophosphate - containing buffer. Under these conditions magnesium ions are removed from the ribosomes and they dissociate into their constituent sub-units (Spirin and Gavrilova, 1969).

Figure 3.39

PAGE of polyribosomes extracted from young leaves of chinese cabbage.

↓ Large ribosomal sub-unit peak

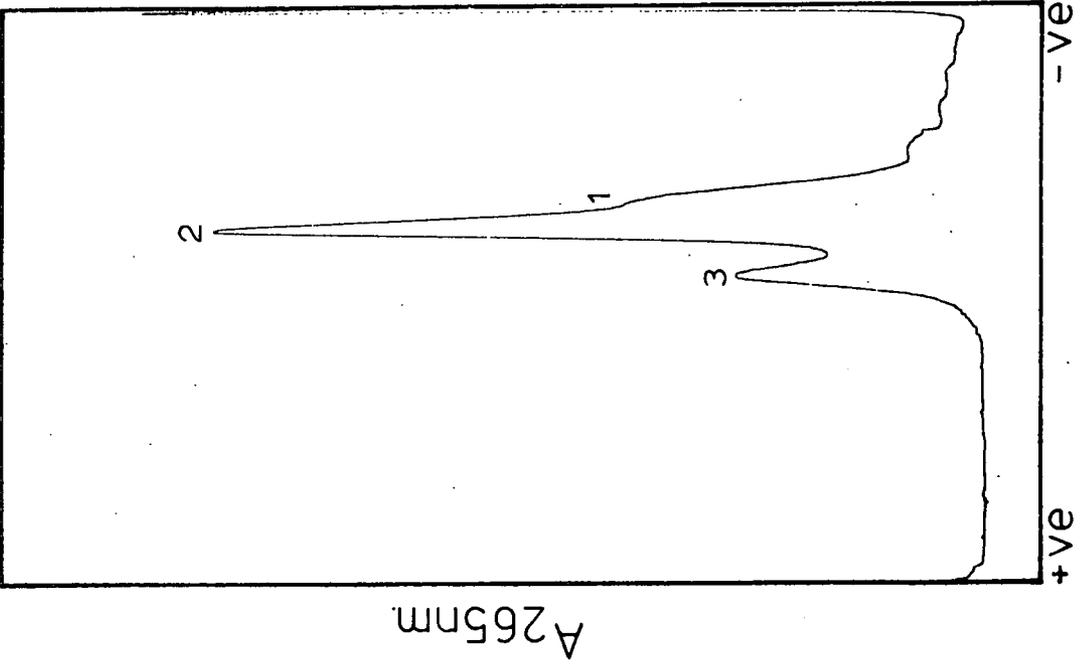
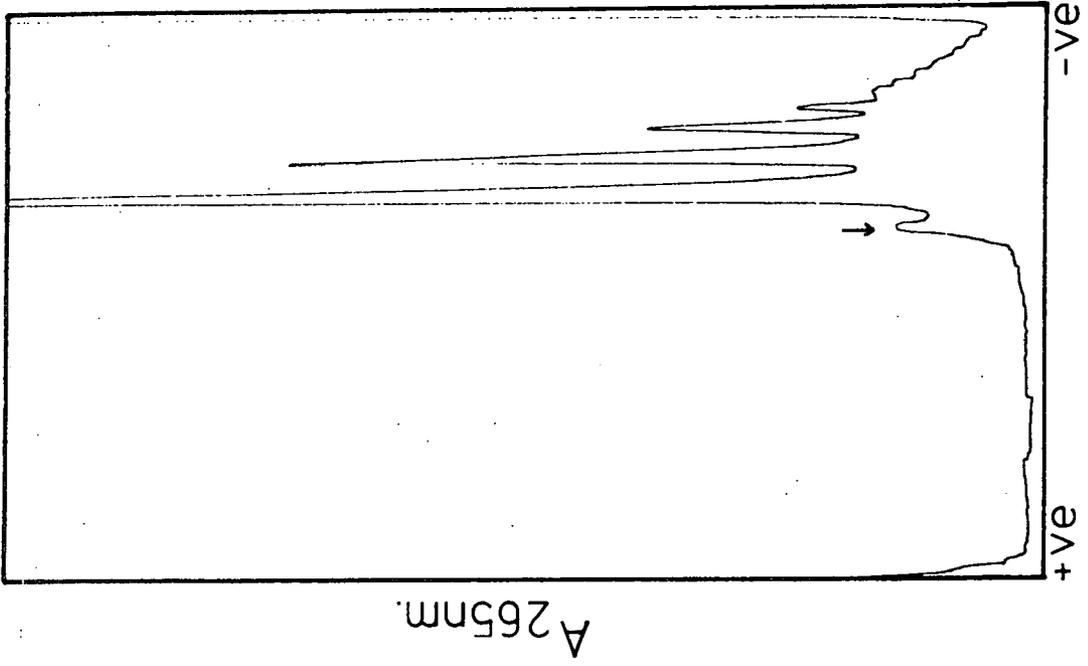
Figure 3.40

PAGE of a mixture of polyribosomes extracted from young leaves of chinese cabbage and of polyribosomes dissociated into ribosomal sub-units.

Peak 1 : Monosomes

Peak 2 : Large ribosomal sub-unit

Peak 3 : Small ribosomal sub-unit



Ribosomes treated thus gave two peaks of greater electrophoretic mobility than that indicated to be the monoribosome (figure 3.40). The electrophoretic mobility of the large ribosomal sub-unit corresponded to that of the small peak arrowed in figure 3.39. Thus free ribosomal sub-units were present in the polyribosome extracts, either as a natural component of the ribosome population or as a result of dissociation of some ribosomes during extraction and/or fractionation. In the electrophoretic gel ribosomal polymer peaks to at least the septamer were clearly resolved and there were 'shoulders' suggesting that higher polymers were also present. This result compares very favourably with the profiles obtained by sucrose density gradient fractionation of polyribosomes extracted from leaves of a similar age (figure 3.36). No indication of chloroplast ribosome components were evident in the gel profiles, nor were they apparent when ribosomes were electrophoresed in smaller pore polyacrylamide gels. The sharpness of the polyribosome peaks obtained by electrophoretic fractionation suggests that chloroplast components might be readily resolved if they were present in larger concentrations.

Under these conditions of electrophoresis the quantity of ribosomes loaded onto the gel was important. 5-15 μ g ribosomes proved to be optimum with heavier loadings resulting

in aggregation of the ribosomes, possibly caused by the concentrating of the sample on the surface of the gel (figure 3.41). This loading quantity of ribosomes is less than that which can be practicably separated by sucrose density gradient centrifugation.

3.73.2 Characterisation of electrophoresed polyribosomes

Gels in which ribosomes had been fractionated were stained for RNA (toluidine blue) or for protein (amido black). In both instances bands corresponding to those located by scanning in the u.v. stained positively indicating that they were of nucleoprotein character. A clear zone at the top of the gel did not take up either stain suggesting that all the material had migrated into the gel. The presence of further bands in this region not detectable by staining was unlikely because the very sensitive (down to 0.01 O.D. units) u.v. scanning procedures similarly did not locate any absorption in this zone.

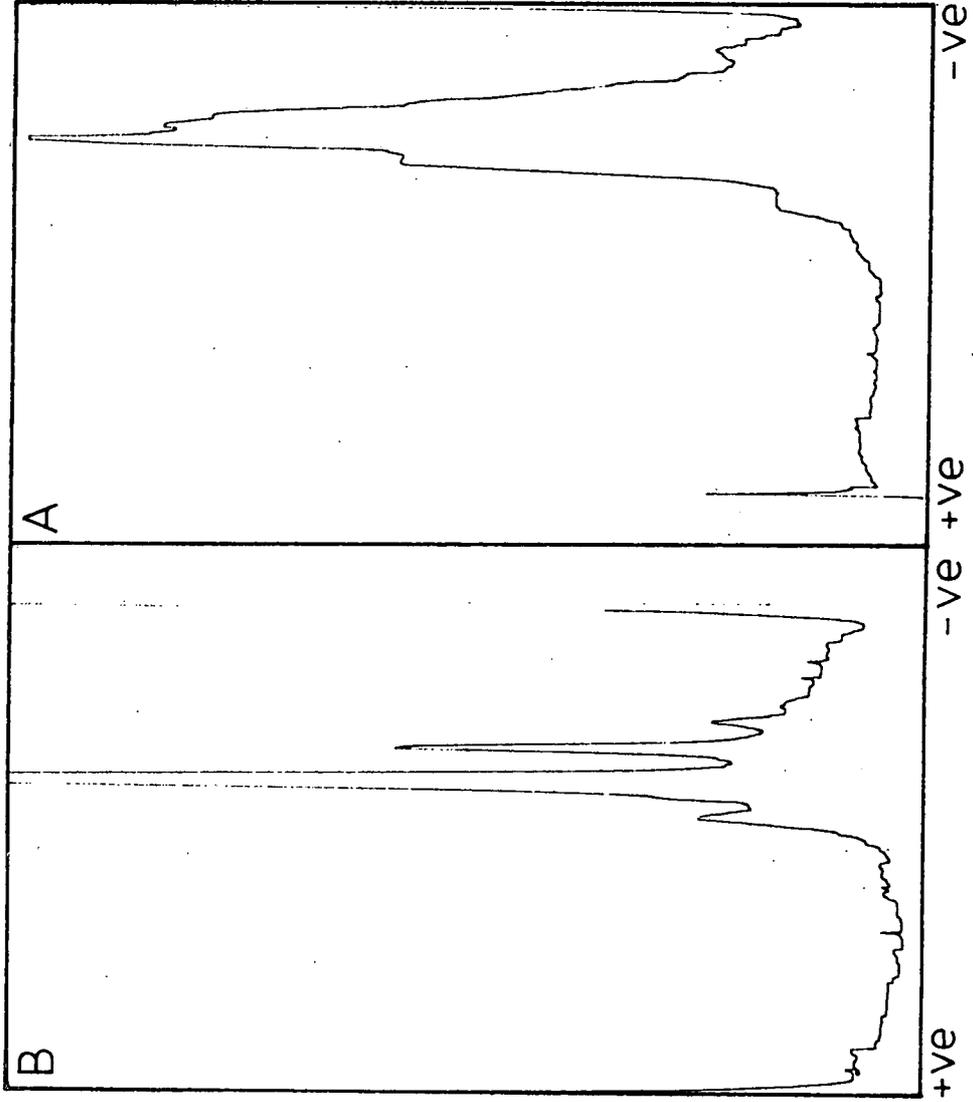
A sample of a polyribosome resuspension was mixed with an equal volume of a solution of 10 μ g/ml ribonuclease and the mixture was incubated for 10 minutes at room temperature prior to electrophoresis. Comparison of the ribonuclease treated sample with an untreated control revealed a loss of the higher polymers with a corresponding increase in the absorption observed in the monoribosome peak (figure 3.42) (Gilbert, 1963;

Figure 3.41

PAGE of polyribosomes illustrating aggregation of
fractions

A. 20 μ g loading

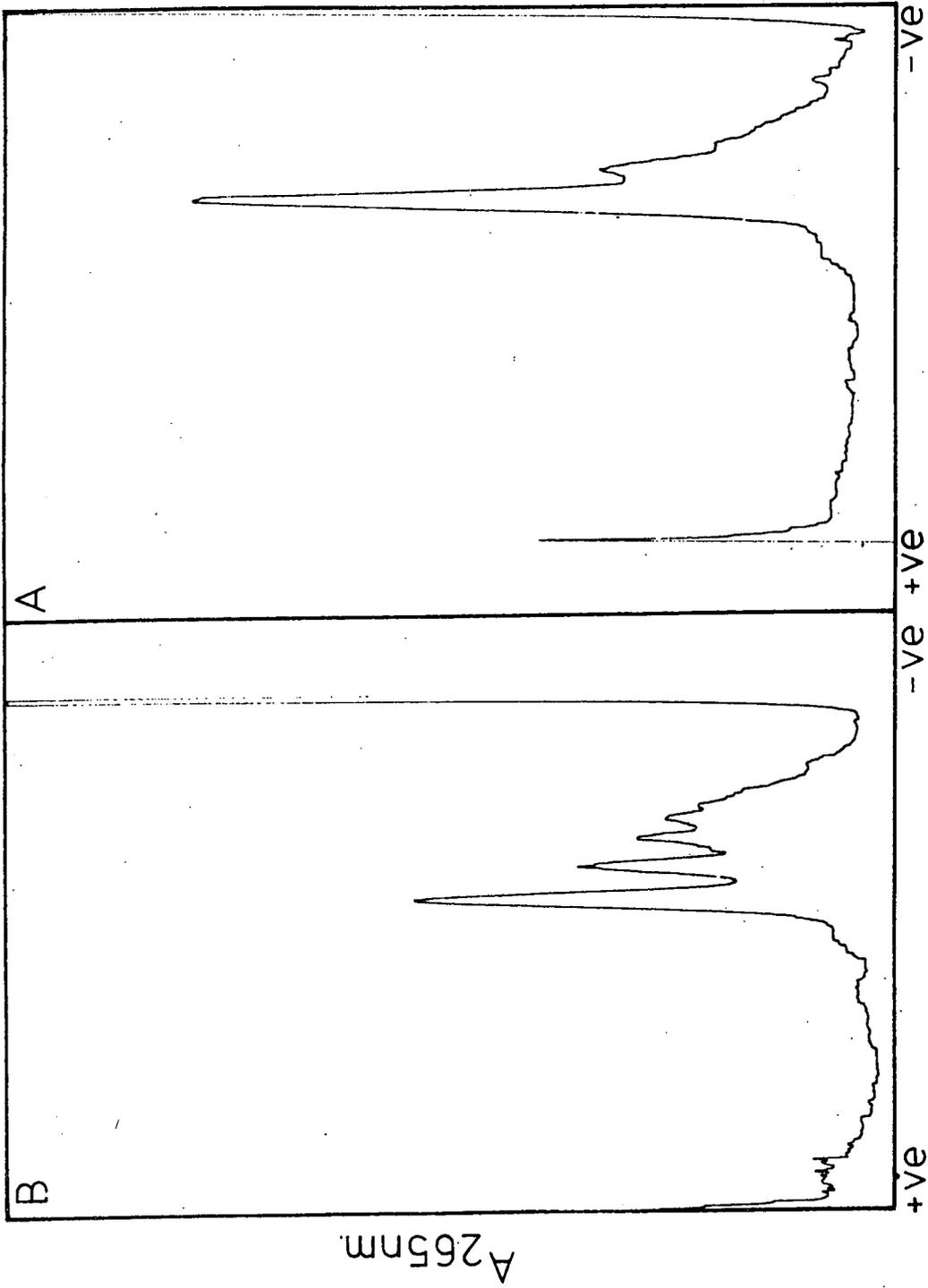
B. 5 μ g loading of same sample as in A.



A_{265nm}.

Figure 3.42

- A. PAGE of polyribosomes treated with 10 μ g/ml ribonuclease for 10 minutes prior to electrophoresis.
- B. Control - PAGE of untreated polyribosomes.



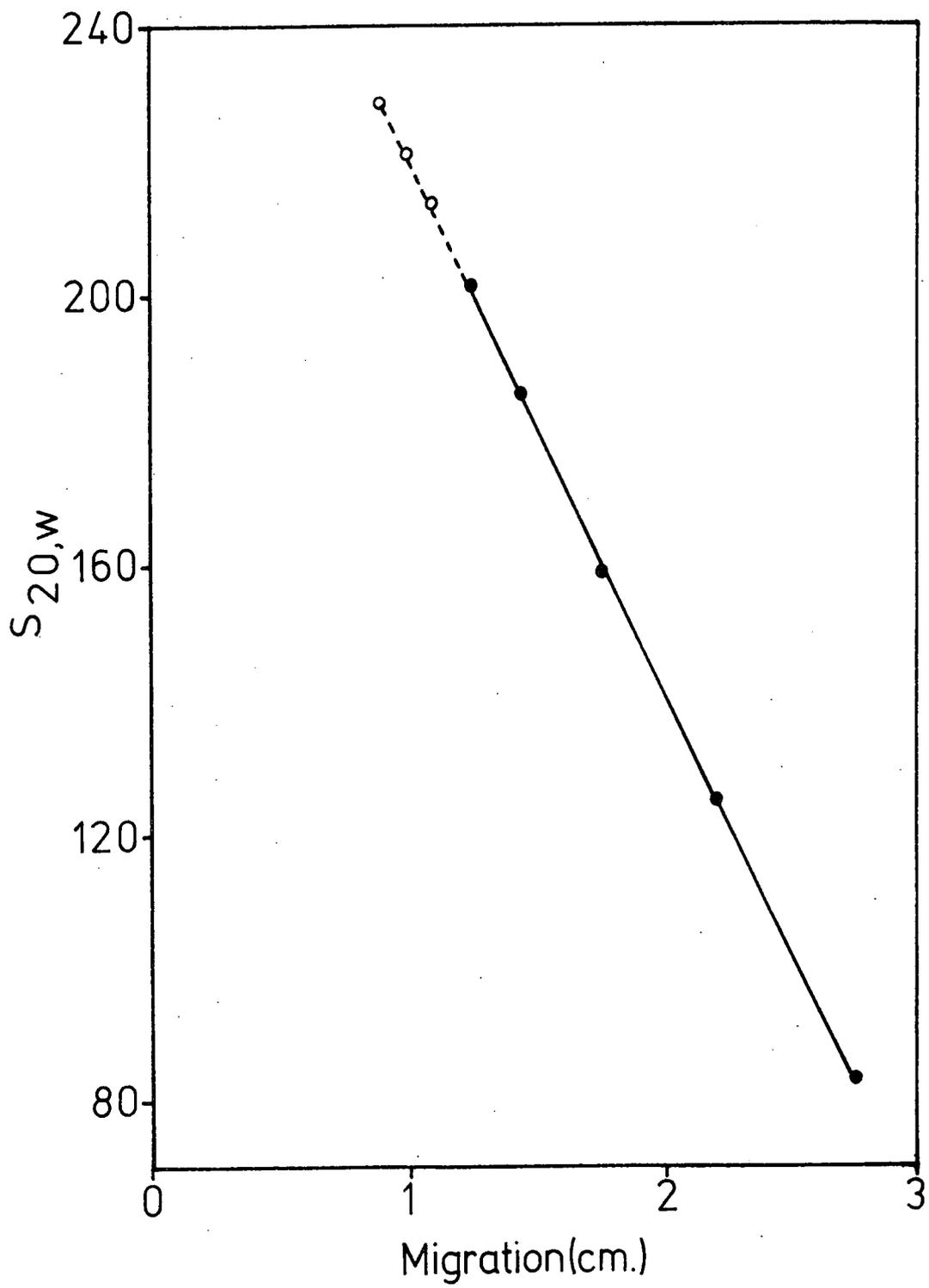
Warner et al., 1963; Levine et al., 1965). This was consistent with the degradation of the polymers by the digestion of the exposed mRNA linking the ribosome units (Warner et al., 1963). In the ribonuclease-treated material the dimer fraction persisted. The occurrence of ribosome dimers resistant to mild nuclease attack has previously been reported by Ling and Dixon (1970).

It has been shown that for electrophoretically separated RNA species there is a linear relationship between migration in the gel and the sedimentation values of the various RNA species (Richards et al., 1965; Loening and Ingle, 1967). Like RNA, ribosomes have a constant charge to mass ratio (Peterman, 1964) and consequently a similar relationship was looked for in this electrophoretic system. Based on the results of Clark et al., (1964) sedimentation values of 83s, 125s, 159s, 186s and 202s were assigned to the monomer to pentamer respectively. When these values were plotted against electrophoretic migration an extremely good straight line fit resulted which enabled values of 215s, 221s and 229s to be estimated for the larger polymers observed on electrophoresis (figure 3.43). The electrophoretic technique thus appears to be a rapid method by which sedimentation co-efficients of ribosomes and ribosomal polymers may be estimated. However further comparisons between electrophoretic mobility and

Figure 3.43

Plot of sedimentation coefficient against electrophoretic migration of polyribosome fractions

- Values of sedimentation coefficients based on those obtained for chinese cabbage by Clark et al., (1964).
- Predicted values for hexamer, septamer and octamer fractions.



sedimentation coefficient for polyribosomes of other species need to be carried out before the validity of this system can be fully assessed.

Dahlberg et al., (1969) demonstrated an inverse relationship between the logarithm of the particle weight of E.coli polyribosomes and their migration in composite agarose-acrylamide gels. From results of the various methods used the molecular weight of the plant 80s ribosome appears to be $4.1 - 4.6 \times 10^6$ (Spirin and Gavrilova, 1969). The molecular weights of the polyribosomes were assigned as the appropriate multiple of the monoribosome weight. No allowance was made for the presence of mRNA as it was thought that the weight contribution would not be significant. The plot of log (molecular weight) against electrophoretic migration approximated closely to a straight line suggesting the presence of a polymeric series (figure 3.44).

3.73.3 Effects of storage on polyribosome pellets

The 105,000g. polyribosome pellet could be stored for several weeks at -20°C with little degradation of the polymeric units being apparent, as assessed from the electrophoretic profile. However, once a preparation had been resuspended in a buffer it had to be used almost immediately since prolonged maintenance of the sample at 0°C , or cycles of freezing and thawing induced dissociation of the ribosomes (Table 10).

2

Figure 3.44

Plot of particle weight against electrophoretic migration of polyribosome fractions.

The limits on the points indicate the possible range of particle weights assuming a weight for 80s plant ribosomes to be $4.1 - 4.6 \times 10^6$ (Spirin and Gavrilova, 1969).

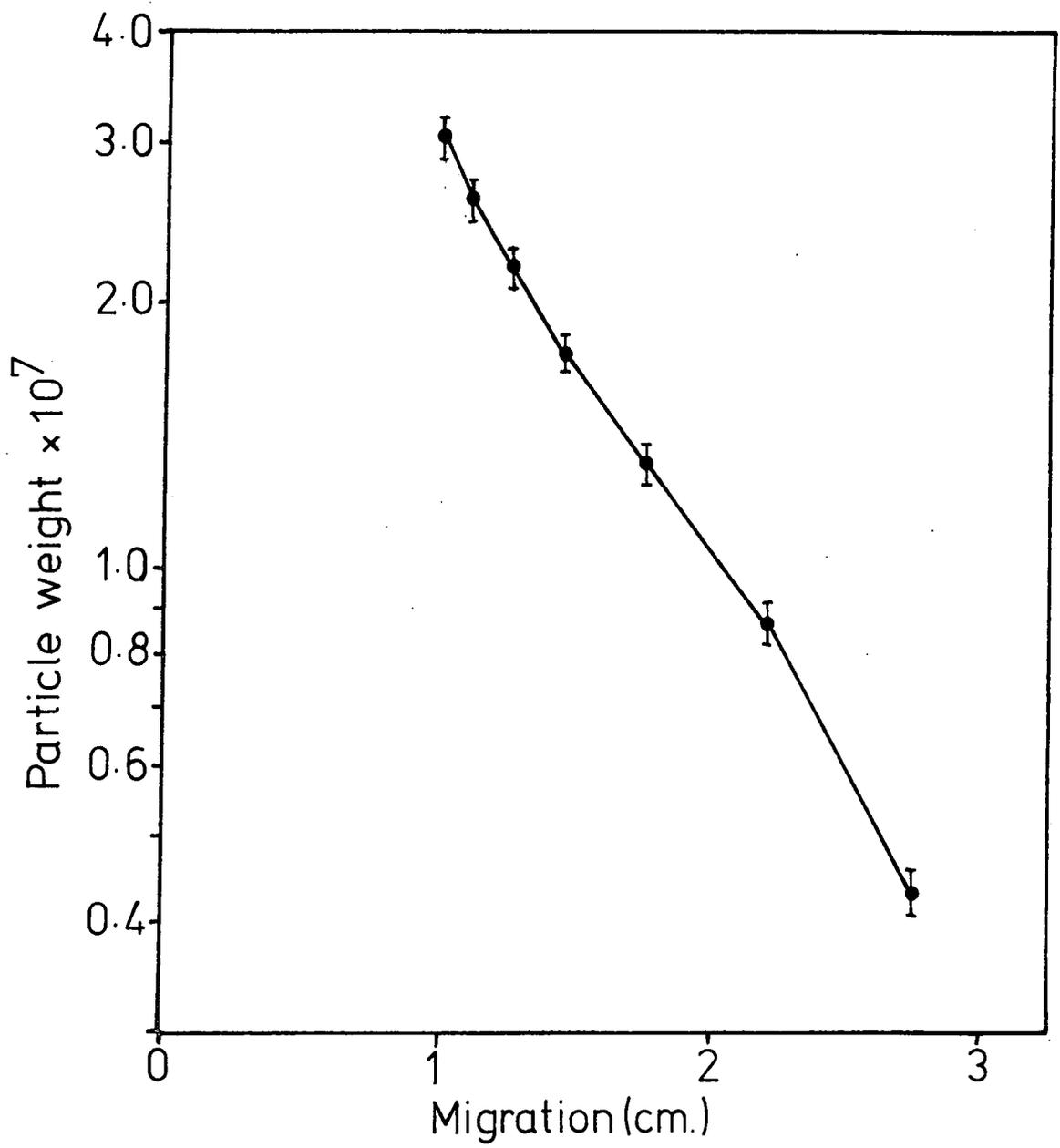


Table 10

	% Ribosomes (Monosomes + polysomes)	% Ribosomal sub-units
Control	94.9	5.1
Control after 2 cycles of freezing and thawing	79.0	21.0
Duplicate pellet stored at -20°C, 3 weeks	93.7	6.2

This dissociation was evident as an increase in the proportion of ribosomal sub-units observed in the profiles. These sub-units were primarily derived from the polyribosomes, distinct increases in the monoribosome fraction, indicative of ribonuclease activity (see figure 3.42) were not observed. The changes apparent on freezing and thawing thus appeared to be a result rather of mechanical factors such as rupture of the polymeric formation by ice crystal formation.

3.73.4 Effects of duration of electrophoresis

An investigation was made into the effects of increasing the duration of electrophoresis in an attempt to separate more distinctly the larger polymers. Electrophoresis was performed in the normal manner with replicate samples of the same healthy leaf polyribosome preparation loaded onto each gel. At hourly intervals, from two to five hours inclusive, a gel tube was raised within the electrophoresis tank such that its top was above the surface of the buffer in the upper reservoir. The electrophoretic separation for that sample

was thus terminated but the gel was maintained under similar conditions to the remaining samples still being fractionated. At each hourly sampling the power source was switched off whilst the gel tube was being manipulated and then re-set so as to maintain a constant potential difference of 13 volts per gel for the remaining gels. The whole manipulation took only about 30 secs. and therefore the interruption should not have affected migration rates significantly.

Examination of the electrophoretograms (figure 3.45) showed that the polyribosome peaks became slightly less sharp with increasing length of time of electrophoresis. Slight variations in ribosome structure or contamination with non-ribosomal particles which would mean a range of molecular weight may have been contributory to this broadening of the peaks. The relative sharpening of the peaks at two hours enabled the higher polymers to be resolved more clearly than after longer electrophoretic periods. Loading of greater amounts of material on to the gels to counteract effects of band spreading were ineffective due to aggregation effects mentioned previously (see figure 3.41).

It was observed that with prolonged electrophoresis, under the conditions used, the migration of the polymer bands was not strictly linear with time, there being a decrease in migration at longer time periods (figure 3.46). The relative

Figure 3.45

PAGE of replicate polyribosome samples electro-
phoresed for increasing periods of time

- A. 2 hour electrophoresis
- B. 3 hour electrophoresis
- C. 4 hour electrophoresis
- D. 5 hour electrophoresis

A_{265nm}.

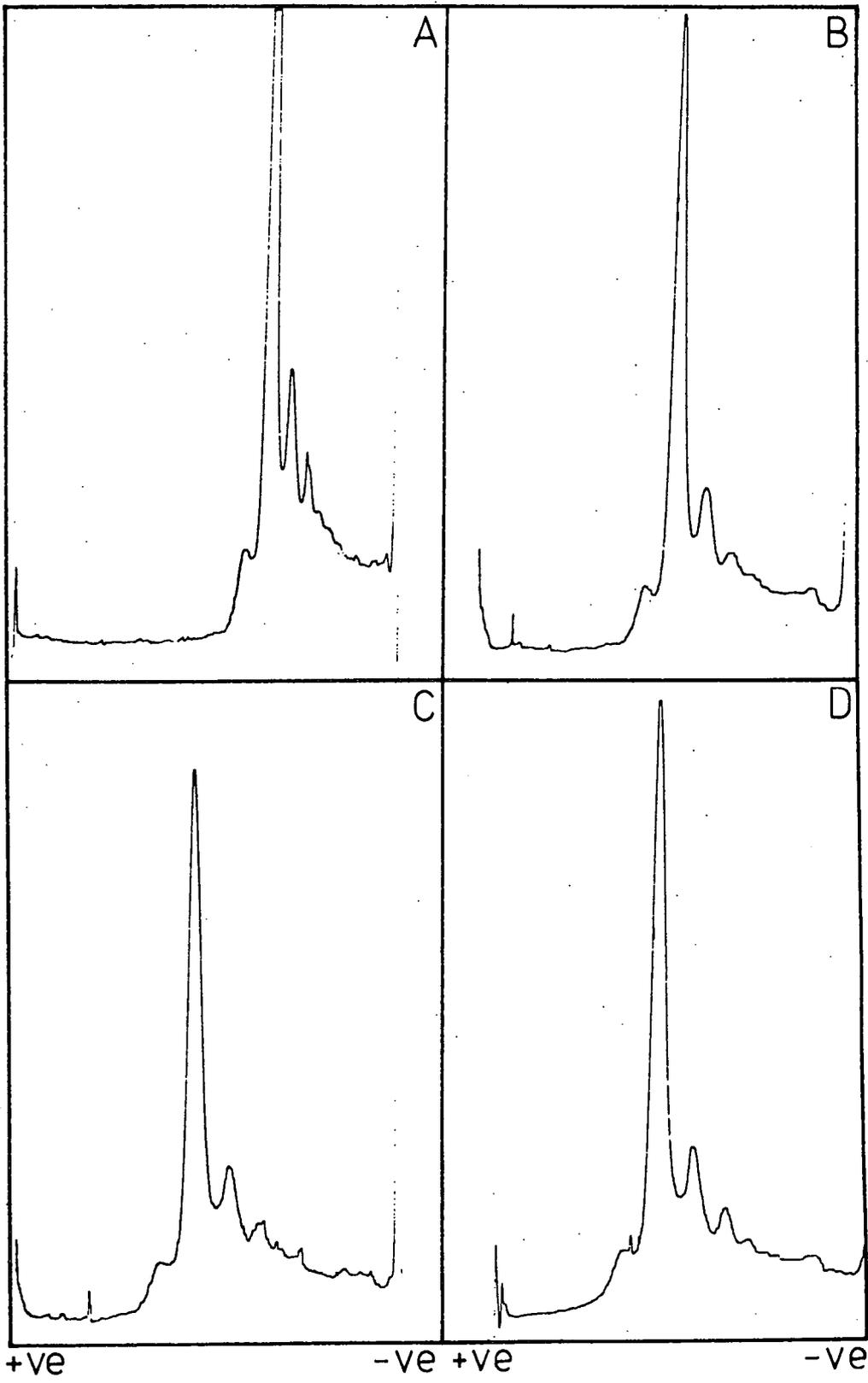
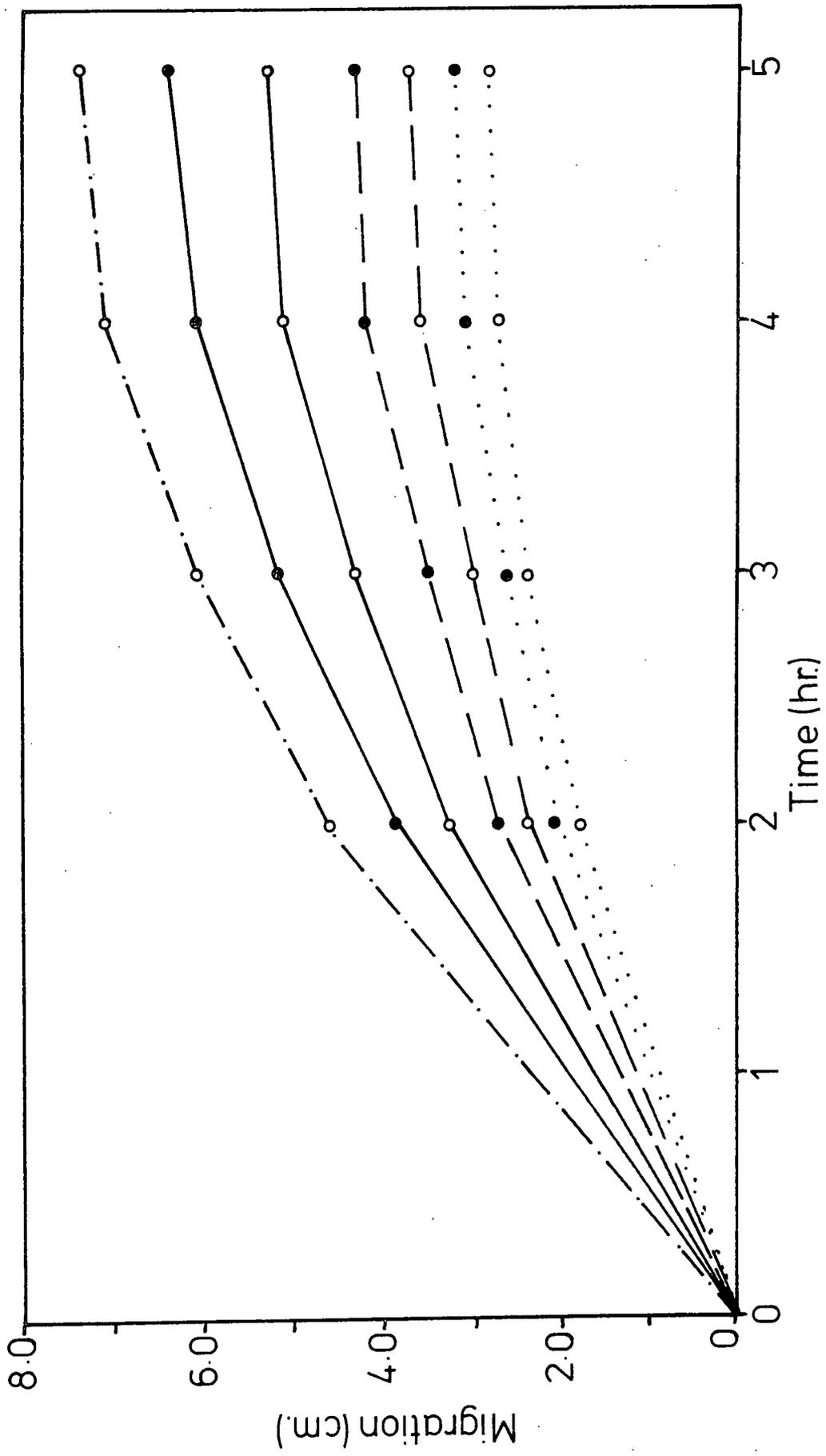


Figure 3.46

Plot of electrophoretic migration against time

- Large sub-unit
- Monomer
- Dimer
- Trimer
- Tetramer
-● Pentamer
-○ Hexamer



migrations of the different fractions, however, remained constant (Table 11) indicating that all the components were similarly affected.

Table 11

	Migration relative to migration of large sub-unit peak			
	2 hours	3 hours	4 hours	5 hours
Monomer	0.85	0.85	0.86	0.85
Dimer	0.71	0.70	0.72	0.72
Trimer	0.59	0.57	0.59	0.58
Tetramer	0.52	0.49	0.51	0.50
Pentamer	0.46	0.43	0.44	0.43
Hexamer	0.39	0.39	0.38	0.38

3.73.5 Effects of alterations of the electric current

Further attempts to improve the resolution of the electrophoretic system involved alterations in the electric current being applied to the gels. An increase in the voltage to 15 volts per gel resulted in no significant increase in peak migration and the resolution was similar to that obtained with the standard electrophoretic conditions. Even though faster migration rates, induced by additional increases in the voltage, would give less time for nucleases to act on the samples because of the possibility of physical shearing effects on the polyribosomes further increases in voltage were not performed.

Fractionations were also carried out by lowering the electric current and increasing the duration of the electrophoresis. Gels were run at 5mA per gel (approximately 8 volts per gel) for periods up to eight hours (Dahlberg et al., 1973). Polyribosome samples electrophoresed under these conditions showed decreased resolution compared to the normal electrophoretic separations (figure 3.47).

In both instances in which prolonged electrophoresis was employed, either with the normal electric current conditions or as above, the higher polymers were poorly resolved. This suggests some degradative process acting upon the ribosomes. This may be a purely physical phenomenon resulting from the movement of the polyribosomes through the 'molecular sieve' formed by the gel or alternatively under prolonged electrophoresis the recirculation of buffer by a peristaltic pump may not be sufficient to prevent the depletion of magnesium thus giving rise to instability of the ribosomes. Accretion of magnesium at the cathode in these longer electrophoretic runs was greater than that observed after the usual two hours. Dahlberg et al., (1973), in addition to recirculating the buffer, found it necessary to change the running buffer every two hours when performing an eight hour long electrophoresis of bacterial polyribosomes.

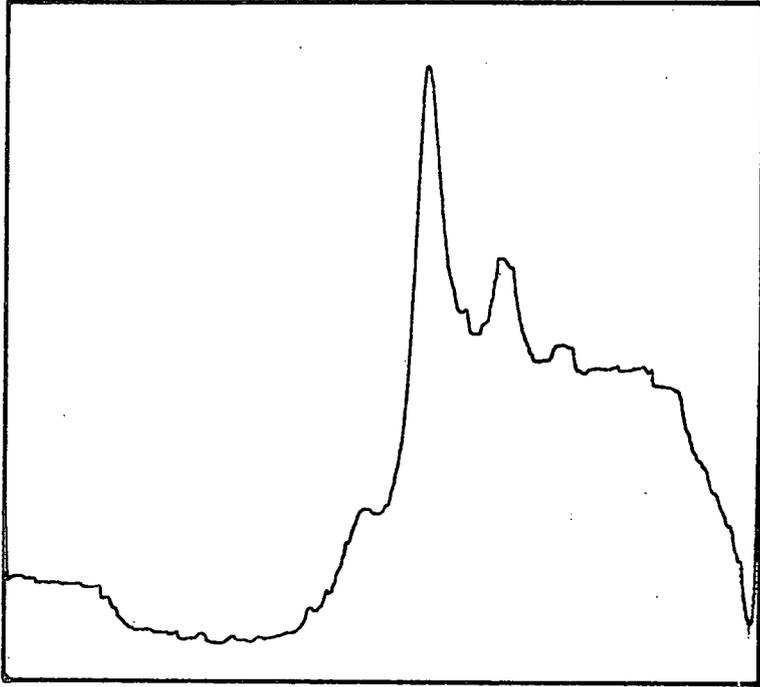
Figure 3.47

PAGE of polyribosomes electrophoresed at a reduced electric current (8 volts per gel) for a longer period of time (6 hours) than the standard electrophoretic system.

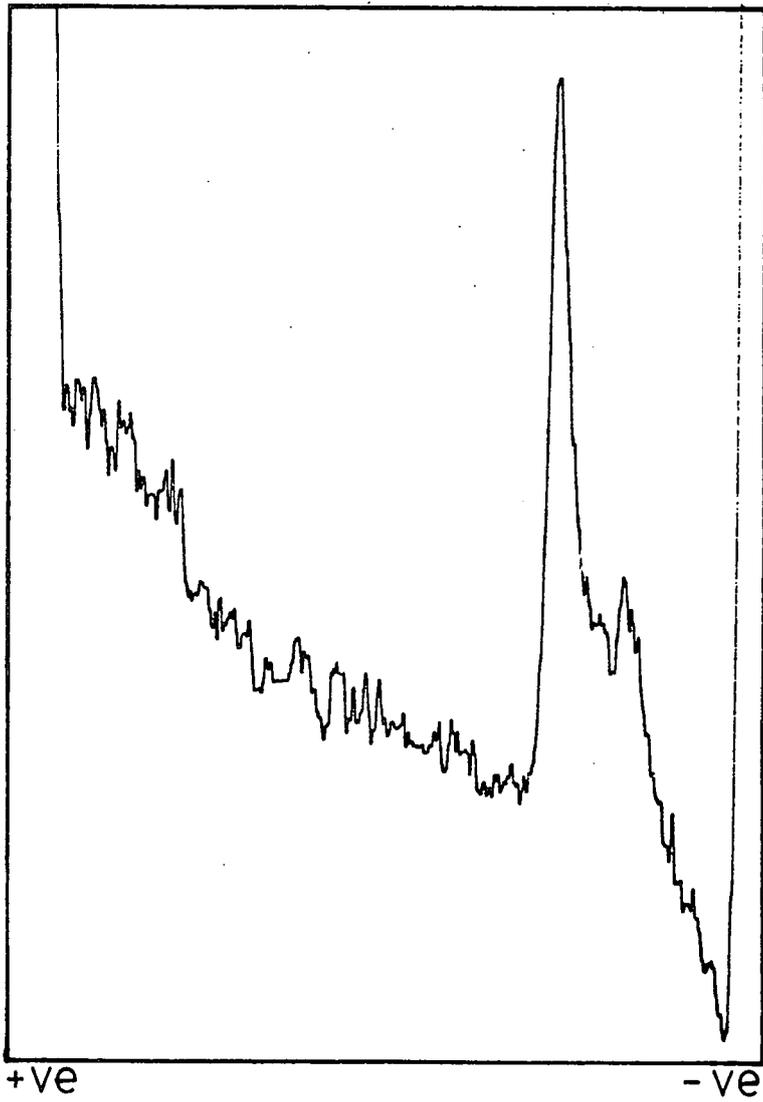
Figure 3.48

Polyribosomes electrophoresed in a 2.0% acrylamide - 0.5% agarose composite gel.

A 265nm.



A 265nm.



3.73.6 The use of agarose-acrylamide composite gels

In achieving an electrophoretic separation of bacterial polyribosomes Dahlberg et al., (1969) used 2.25% acrylamide gel slabs supported by 0.5% agarose, an attempt was made to reproduce this system in gel tubes. 2.0% acrylamide gels with 0.5% agarose supports were prepared essentially by the method of McDevitt and Muir (1971) using the ribosome electrophoresis buffers (see 3.24.3). A reduced acrylamide concentration was used to try and gain increased migration into the gel. Without agarose supports 2.0% acrylamide gels are too fluid to be handled easily, they also swell considerably on washing which may result in distortion of the bands when the gel is scanned in the u.v. The other conditions of electrophoresis were as normally employed.

On the resulting electrophoretograms monomer and dimer ribosome peaks were observed but other polymer bands were obscured by the irregular u.v. background introduced by the agarose used (Miles Laboratories Ltd.) (figure 3.48). Staining of the gel located further bands but the resolution was not as good as that obtained in unsupported 2.2% acrylamide gels. These results were less satisfactory than those achieved by Dahlberg et al., (1969) who located polyribosome bands to the octamer by staining, and may be attributable to the unsuitability of the particular agarose used (Peacock and

Dingman, 1968) and the high sensitivity of the scanning procedure.

3.73.7 Electrophoresis in buffers of varying magnesium concentration

Magnesium appears to be essential for the stability of ribosomes (Peterman, 1964) so an attempt to examine the effect of different magnesium concentrations during electrophoresis on plant ribosomes was made. The buffers used for this investigation were based on that Dahlberg et al., (1969) found to be suitable for the fractionation of E.coli polyribosomes:-

5mM tris-HCl pH 8.0

1mM magnesium chloride

This buffer was used as the electrophoresis running buffer and, as a five-fold concentration stock solution, for formation of 2.2% polyacrylamide gels. Individual gel runs varied in the concentration of magnesium chloride present in the buffer. By the use of this simpler buffer system the effects of magnesium ions could be looked at in the absence of other possible stabilising influences e.g. potassium ions (Naslund and Hultin, 1970). Ribosome preparations were resuspended in buffer at the same magnesium ion concentration as that in which they were subsequently electrophoresed.

Electrophoresis in buffer containing no magnesium caused the ribosomes to precipitate out of solution near the top of the gel (figure 3.49), the precipitated ribosomes being

evident as white opaque bands in the gel. A similar precipitation effect was observed if phosphate buffers were used for electrophoresis. To prevent this precipitation and allow migration of the ribosomes in the gel only a low concentration of magnesium (0.07 mM) needed to be present in the electrophoresis buffer (figure 3.50), however resolution was poor with only a broad peak being discernable. On increasing the level of magnesium in the buffer to 0.2mM it was possible to observe some intact ribosomes (both as monomers and small polysomes) though ribosomal sub-units were present in excess (figure 3.51). Further increases in magnesium concentration tended to result in a decrease in the proportion of ribosomes present as sub-units. At a magnesium ion concentration of 1.0 mM the degree of dissociation of ribosomes into sub-units was found to be variable. All electrophoretic profiles showed some dissociation which could vary between complete dissociation into two discrete sub-unit peaks to a situation in which sub-units could only be detected as a 'shoulder' on the monoribosome peak (figure 3.52). Increasing the magnesium concentration to 2.0 mM did not prevent this variation although the extremes of dissociation were not observed. This variability was not apparent under the standard conditions of ribosome electrophoresis in which a more stable environment for the extracted

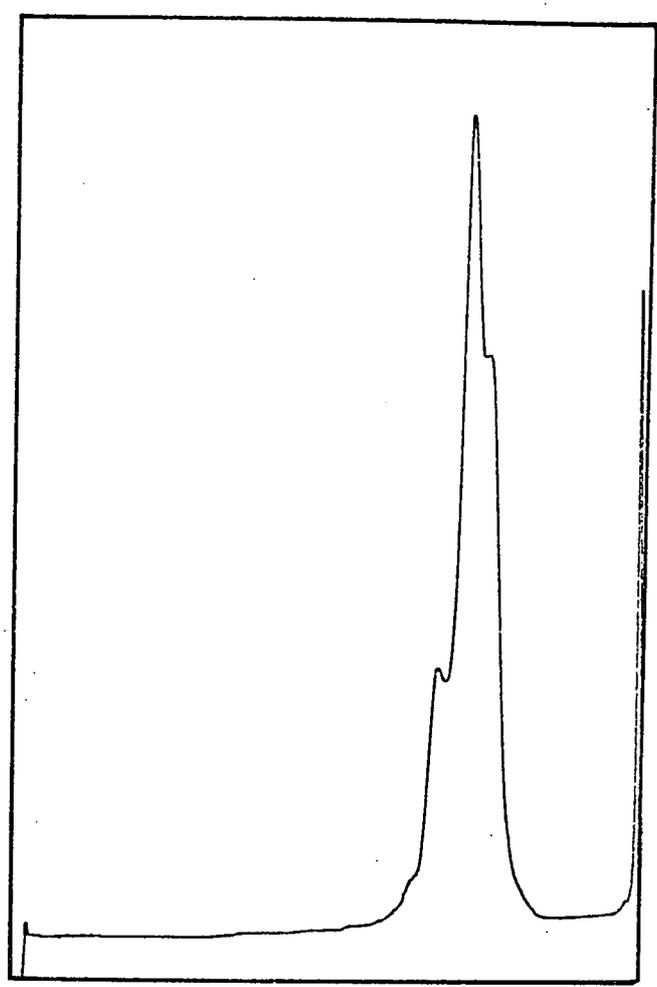
Figure 3.49

PAGE of polyribosomes electrophoresed in the absence of magnesium ions.

Figure 3.50

PAGE of polyribosomes electrophoresed in a buffer containing 0.07 mM MgCl_2 .

A 265nm.



A 265nm.

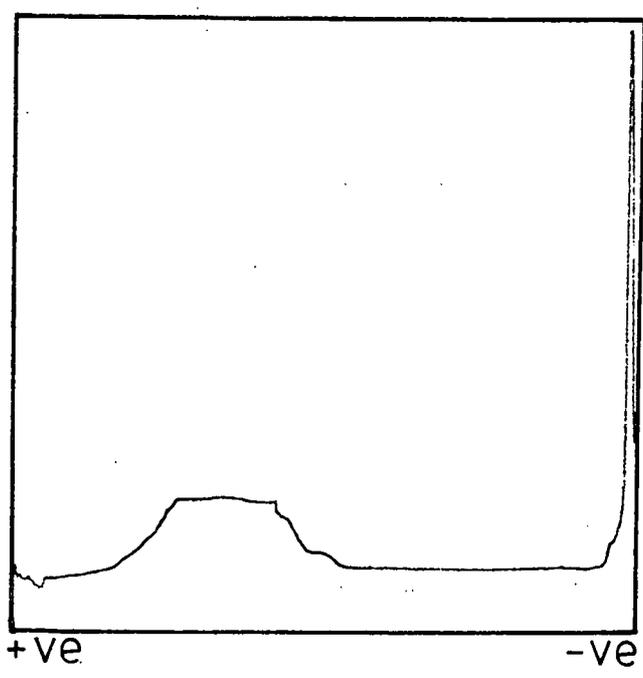


Figure 3.51

PAGE of polyribosomes electrophoresed in a buffer containing 0.2 mM $MgCl_2$.

Peak 1 : Monoribosomes
Peak 2 : Large sub-unit
Peak 3 : Small sub-unit

A_{265nm}.

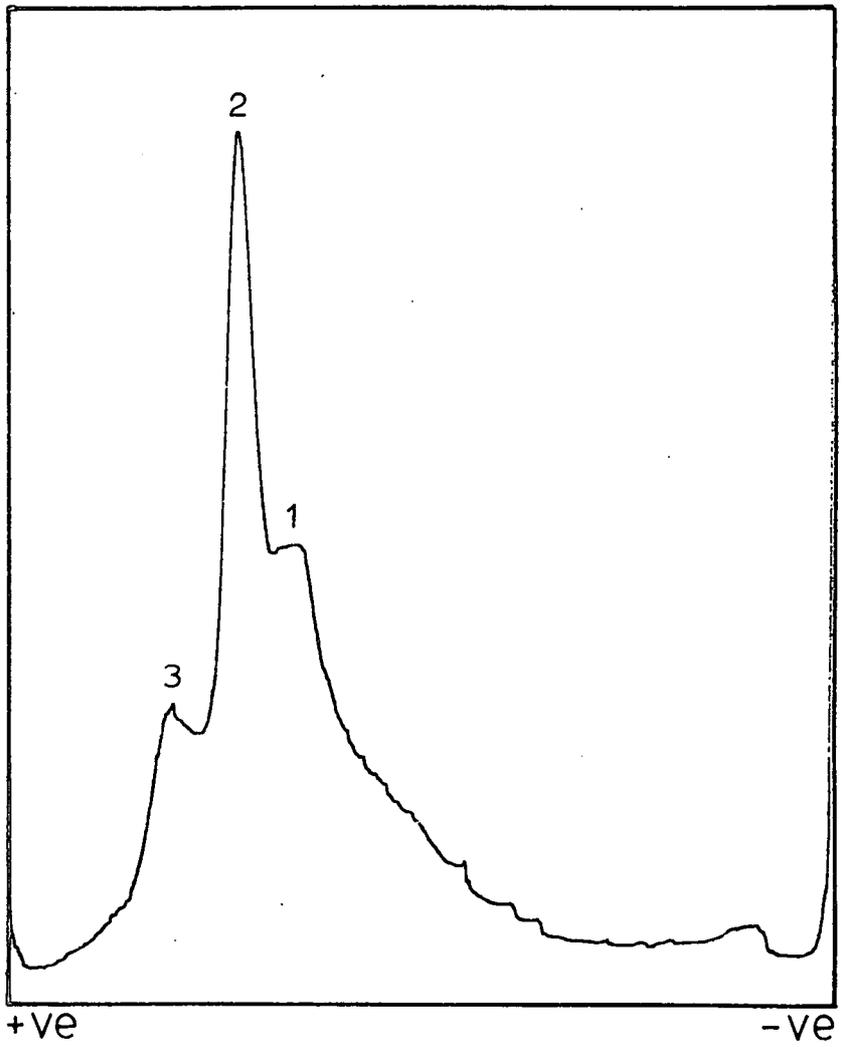
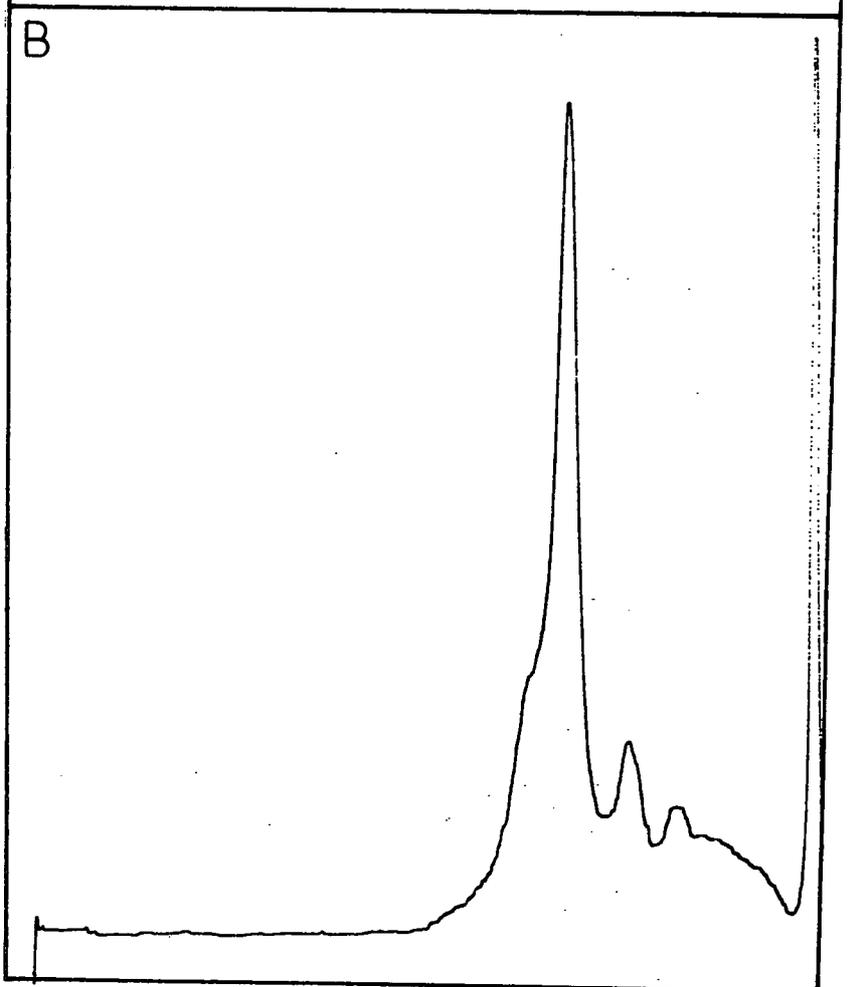
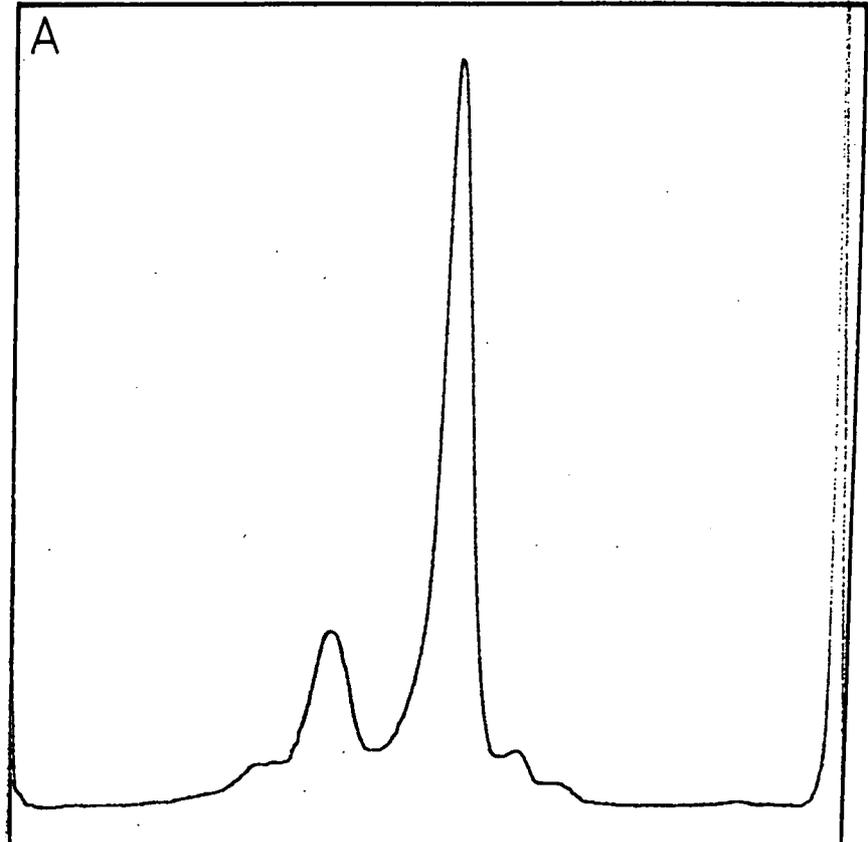


Figure 3.52

PAGE of polyribosomes in buffer containing 1.0 mM
MgCl₂.

- A. Showing almost complete dissociation into the
ribosomal sub-units.
- B. Showing only slight dissociation into ribosomal
sub-units.

A 265nm.



+ve

-ve

ribosomes was used (higher magnesium ion concentration, potassium ions present). This was shown by electrophoresis of duplicate polyribosome preparations prepared from the same starting material. One sample was electrophoresed under the standard electrophoretic conditions whereas the other was electrophoresed in 2.0 mM magnesium-tris-HCl buffer. There was a marked difference in the proportion of ribosomal subunits present on electrophoresis, more dissociation being apparent on electrophoresis in 2.0 mM magnesium (figure 3.53). That the 2.0 mM magnesium buffer could give satisfactory results with some preparations is shown in Figure 3.54. The fact that the dissociation of ribosomes could be prevented under certain conditions of electrophoresis suggested that the observed changes were not a consequence of an altered status of the ribosome population (i.e. proportion of subunits and polysomes) in the material used or of a changed susceptibility to the conditions of extraction, a possible variation in response to magnesium concentration is indicated.

Chinese cabbage plants grown under the conditions described for this study (see 2.11) showed a seasonal variation in growth, plants growing in the winter being smaller and producing fewer leaves than plants grown in the summer. It appeared that the changing susceptibility to magnesium also showed a seasonal variation, which may possibly

Figure 3.53

PAGE of duplicate polyribosome preparations.

- A. Electrophoresis using buffer containing 2.0 mM MgCl_2 .
- B. Electrophoresis using the standard buffer system.

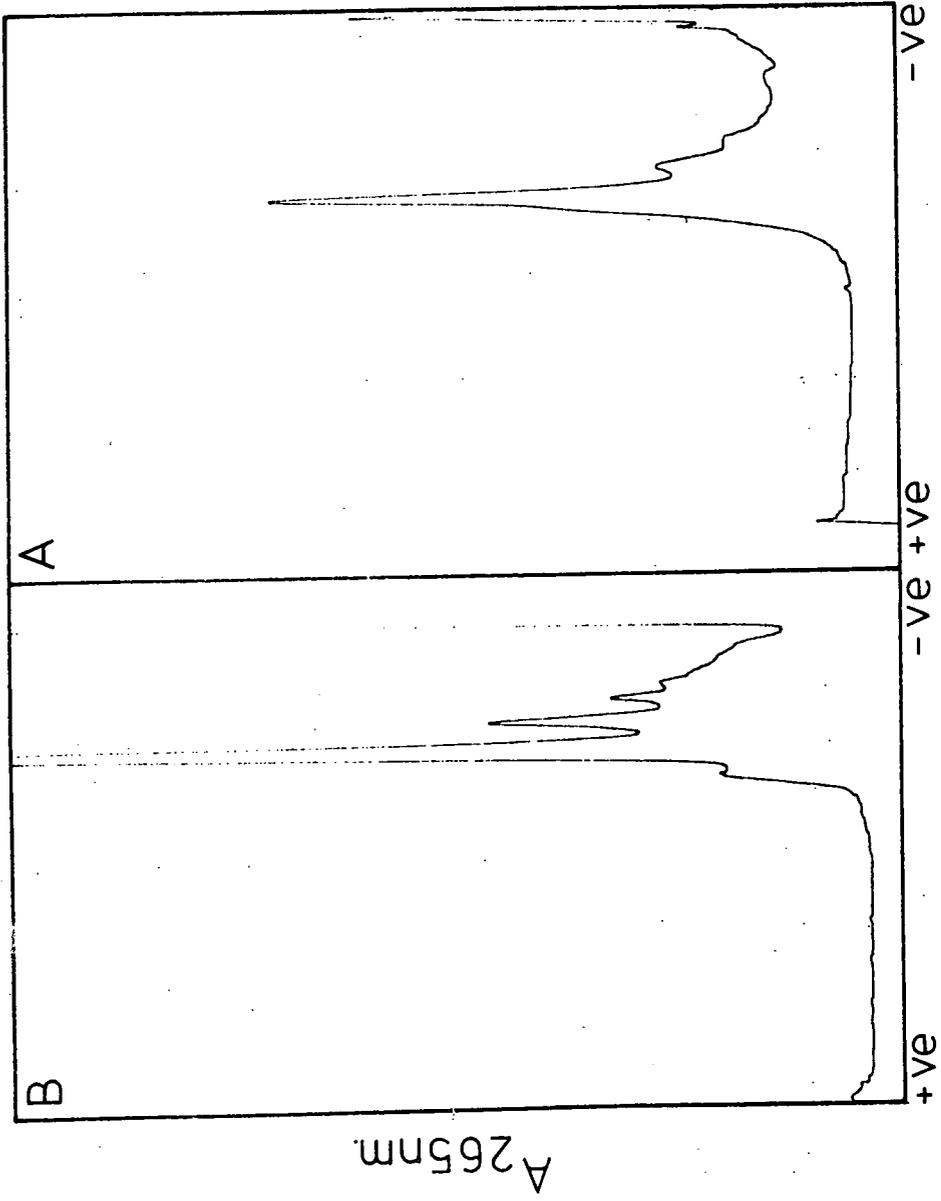
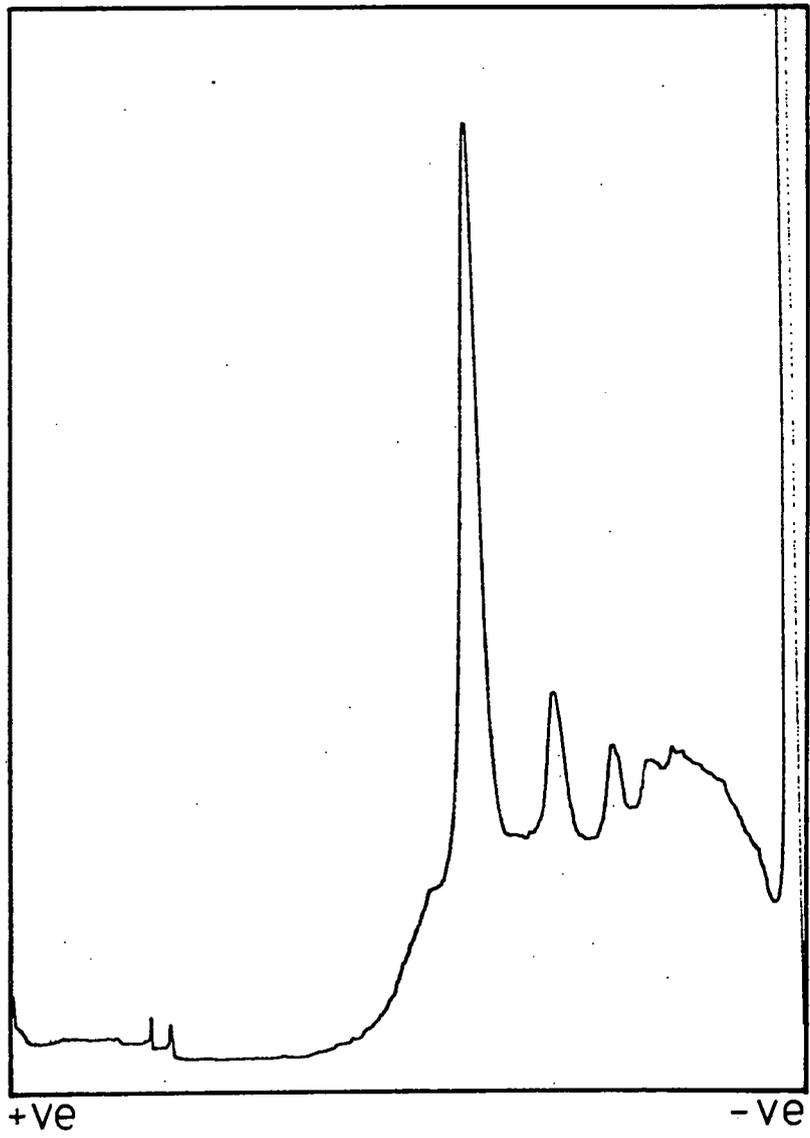


Figure 3.54

PAGE of polyribosomes using buffer containing 2.0 mM MgCl_2 illustrating good preservation of polyribosome fractions.

A 265nm.



be related to this annual cycle of growth. The greatest degree of dissociation was observed in the autumn (September-October) and the least about early spring (March), periods that corresponded to the times at which the changed growth rate in chinese cabbage plants occurred.

Variations in physiological activities in terms of circadian rhythms have been reviewed by Sweeney (1969). Seasonal variation in physiological properties have also been noted by Manus and Goldthwaite (1975) with regard to the rate of senescence of detached leaves of Rumex, similarly there is a change in the ease of enzymic release of protoplasts from leaves of tobacco (Power and Cocking, 1970) and chinese cabbage (Strangeway and Pearson unpublished data).

3.73.8 Incorporation of ³H-uridine into leaf polyribosomes

Tritiated uridine was incorporated into leaf polyribosomes in the following manner. A chinese cabbage plant had all its leaves, except the youngest, removed. A small volume of the uridine was taken up into a sterile syringe and the labelled precursor was introduced into the plant by inserting the syringe into the petiole of the leaf. The leaf was allowed to take up the uridine over a period of 24 hours. This mode of incorporation was adopted because when detached leaves were used slight wilting occurred which could lead to a loss of polyribosomes (Clark et al., 1964). After the incubation

polyribosomes were extracted from the leaf and fractionated by the methods previously described. Following the u.v. scanning of the polyacrylamide gel it was frozen, cut into slices and the radioactivity in each slice assessed as detailed in 3.25. The greatest activity was found in the ribosome region of the gel and differentiation of the labelling pattern into discrete peaks was possible to at least the tetramer with further activity evident in the zone of the higher polymers (figure 3.56). The electrophoretic separation method thus seems suitable for incorporation studies using specific radioactive precursors. The electrophoretic separation method would also have the added advantage that any loosely bound labelled precursors associated with the ribosomes would be removed very quickly during the course of the electrophoresis. With sucrose density gradients any loosely bound precursors would not readily be removed.

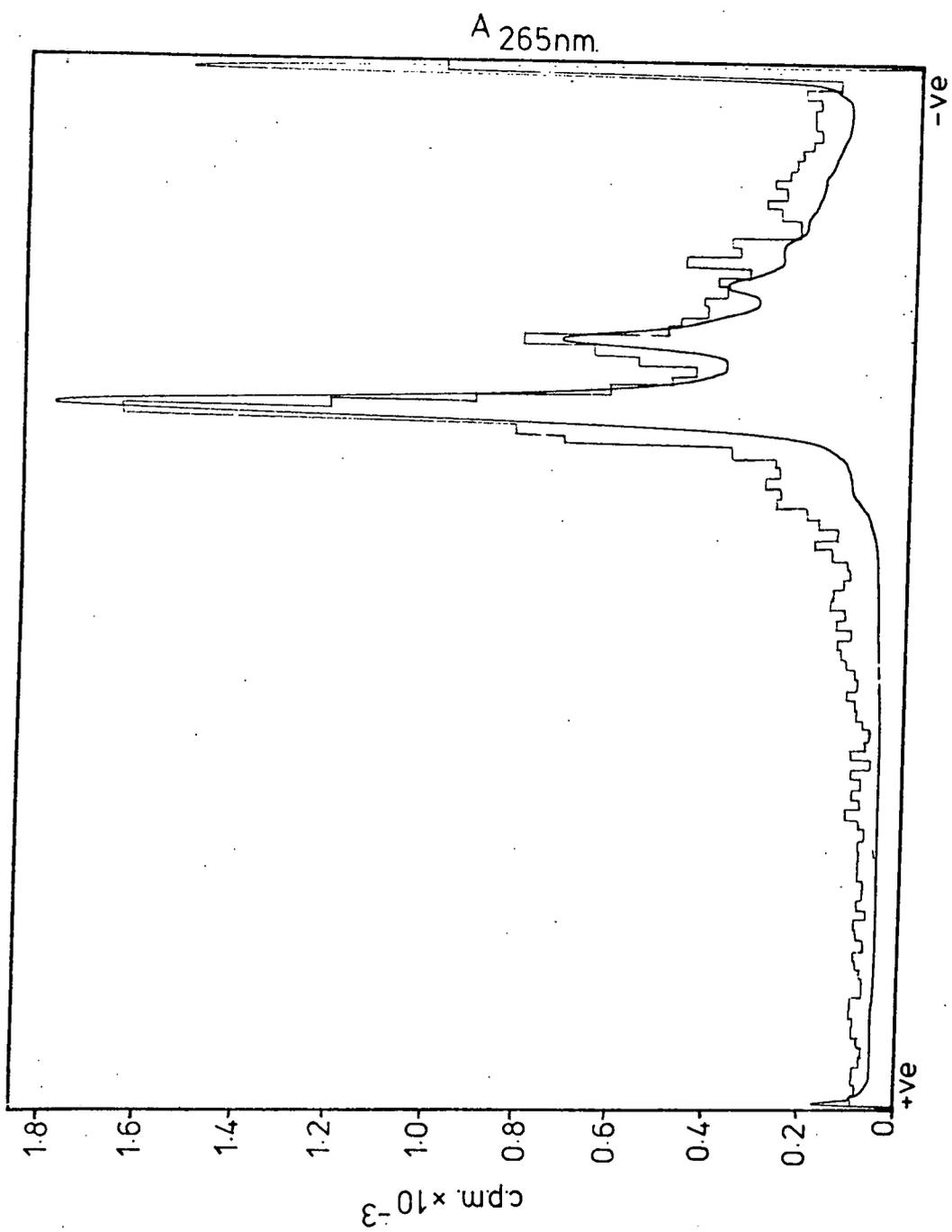
3.73.9 Electrophoresis of ribonucleoprotein preparations from TYMV-infected chinese cabbage plants

Electrophoresis of ribosome extracts from TYMV-infected chinese cabbage leaves revealed additional peaks to those observed in healthy leaf preparations. A major fraction was seen to migrate between the monomer and the dimer ribosome peaks and this was shown to be the virus by comparison with electrophoresis of the virus in crude sap extracts (Pearson

Figure 3.56

Incorporation of ^3H -uridine into chinese cabbage leaf
polyribosomes separated by PAGE

———— u.v. absorption
~~~~~ C.P.M.  $^3\text{H}$ -uridine



unpublished) by the method of Wolf and Casper (1971). A minor fraction is also observed between the dimer and trimer (figure 3.57). Compared to sucrose density gradient fractionation (figure 3.37) the virus was now fully separated from the dimer polyribosome fraction though it partially obscured the monoribosome peak. In samples containing proportionally more virus than the example above this problem was more acute. By separation on polyacrylamide gels with a smaller pore size a more satisfactory resolution between the viral components and the polyribosomes can be obtained, although the larger polymers are excluded from the gel (figure 3.58).

Magnesium-treated bentonite has been shown to absorb to itself ribosomes and some virus particles, though TYMV is not affected. These properties have led to the utilisation of magnesium bentonite in the preparation of pure TYMV and its 'top-component' (Dunn and Hitchborn, 1965). The remaining polyribosomes from the fractionation illustrated above (figure 3.58) were mixed with an equal volume of magnesium bentonite resuspended in magnesium-phosphate buffer. After incubation for 5 minutes at 0°C the samples were clarified in a bench centrifuge (5 mins, 2500g. av.) prior to electrophoresis. The magnesium bentonite treatment resulted in the complete removal of the ribosomal material from the electrophoretic profiles leaving the virus and presumed 'top component'

Figure 3.57

- A. PAGE of polyribosomes extracted from healthy chinese cabbage leaves.
- B. PAGE of polyribosomes extracted from TYMV-infected chinese cabbage leaves.

Peak 1 : TYMV

Peak 2 : 'top-component'

A<sub>265nm</sub>.

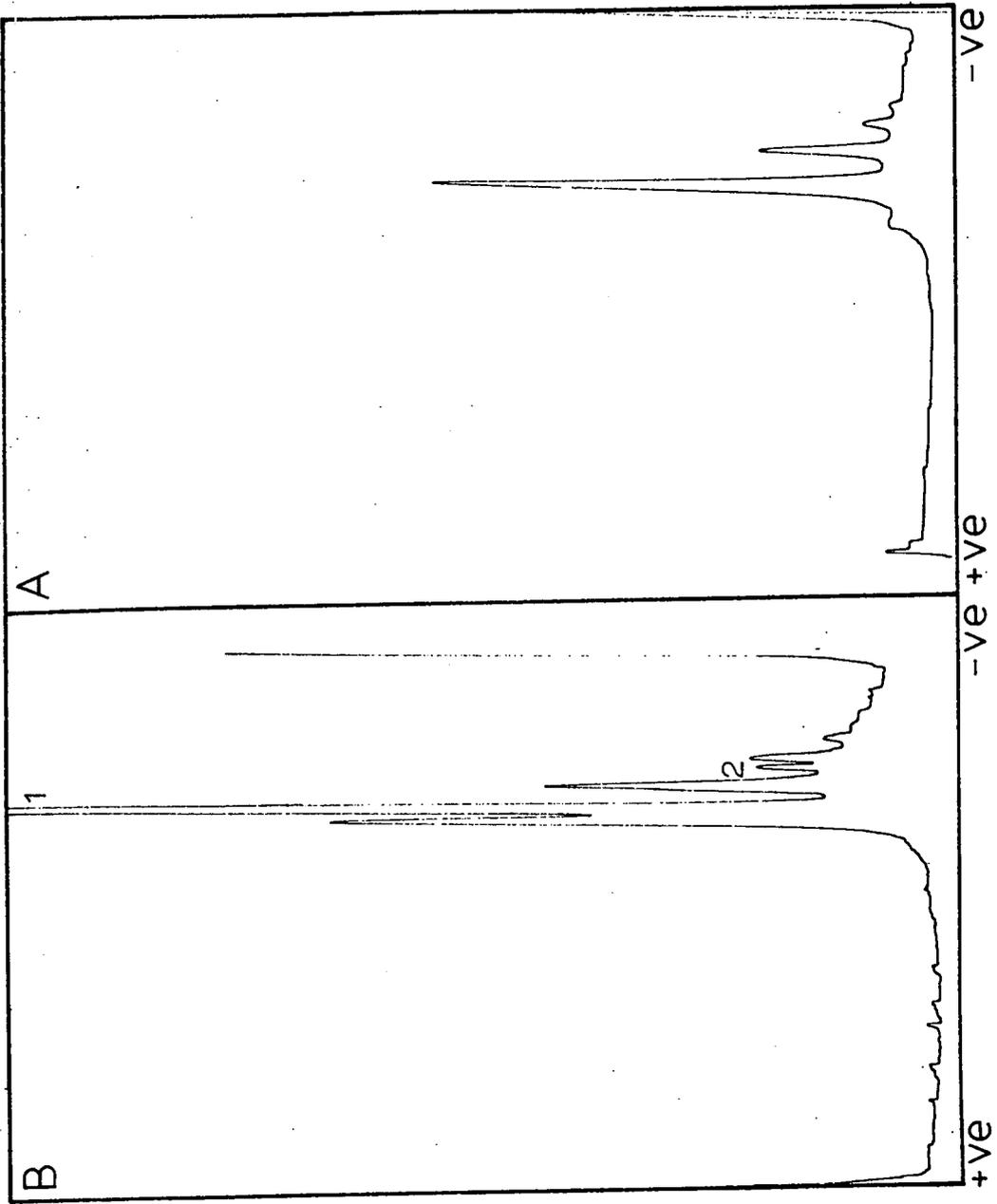
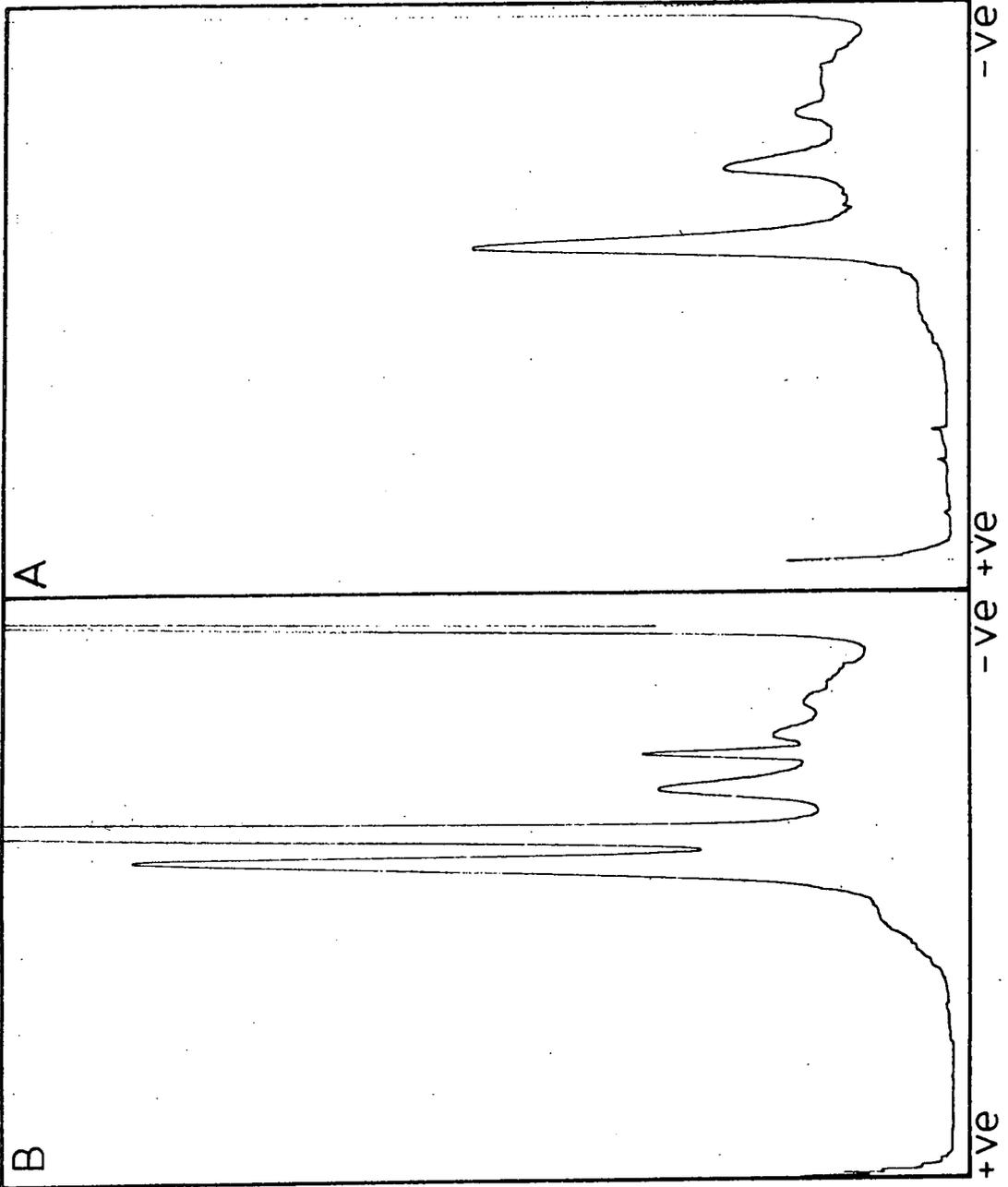


Figure 3.58

PAGE of polyribosomes in gels of smaller pore size  
(2.6% acrylamide).

- A. Polyribosomes extracted from healthy chinese  
cabbage leaves.
- B. Polyribosomes extracted from TYMV-infected chinese  
cabbage leaves



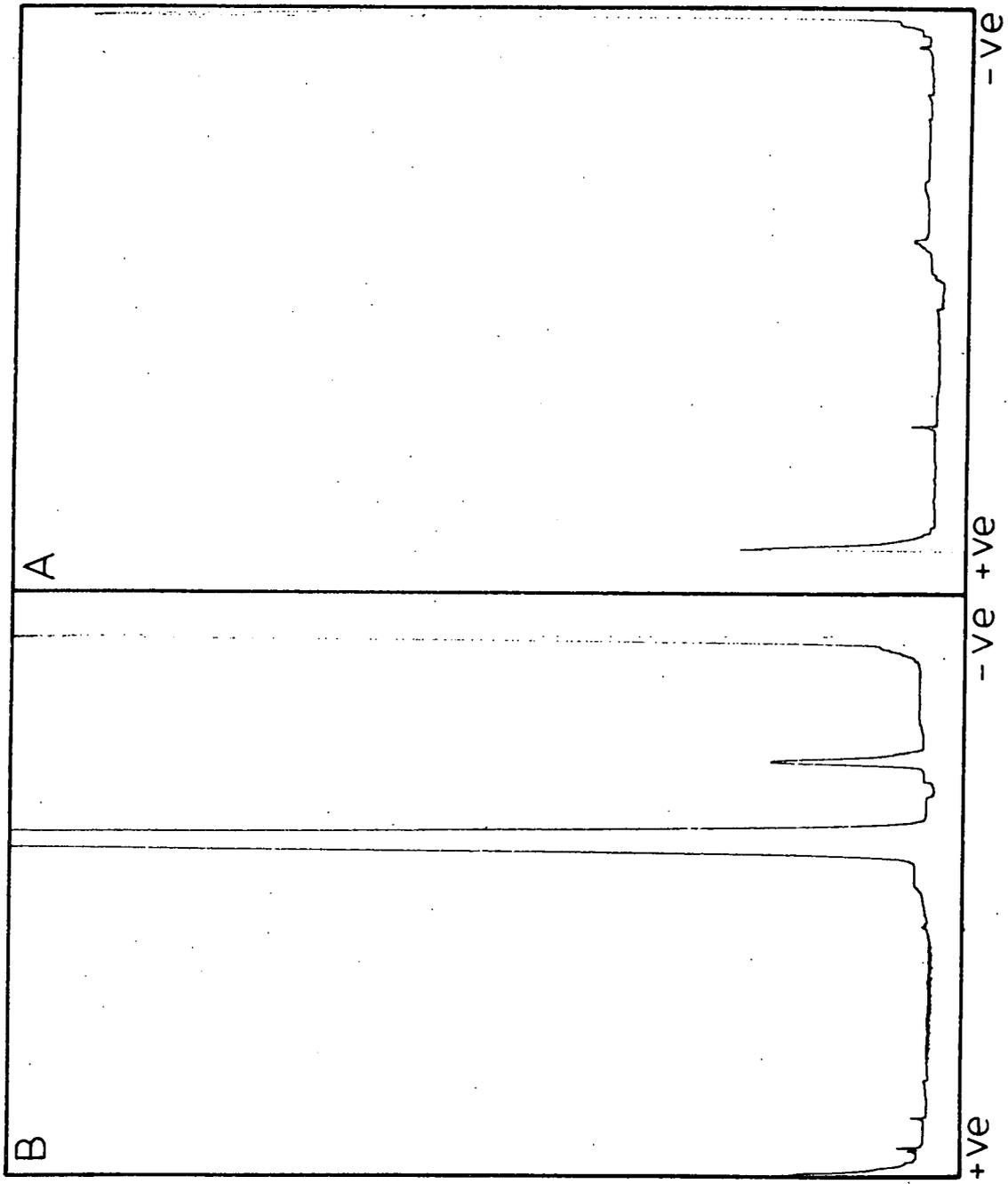
$A_{265nm}$

Figure 3.59

PAGE of samples shown in figure 3.58 treated with magnesium bentonite (5 minutes at 0°C) prior to electrophoresis.

- A. Polyribosomes extracted from healthy chinese cabbage leaves
- B. Polyribosomes extracted from TYMV-infected leaves

A 265nm.



unaffected in the infected leaf extract (figure 3.59). The TYMV peak stained positively for both protein and nucleic acid. The minor virus associated fraction was also proteinaceous but stained only weakly for nucleic acid. This slight staining however suggested that this fraction did not consist solely of the empty viral protein coats ('top component') but that other minor TYMV fractions may also have been present (Matthews and Ralph, 1966). Wolf and Casper (1971) also resolved TYMV into two fractions by electrophoresis of clarified sap extracts from chinese cabbage leaves.

Taking the polyribosome peaks as standards the sedimentation co-efficients ( $S_{20w}$ ) of the two viral components were estimated from a plot of sedimentation value against migration in the electrophoretic gel and values of 95s and 146s were obtained for the virus and 'top-component' respectively. These estimates differ markedly from the published values of 117s for TYMV and 53-54s for the 'top component' obtained by analytical centrifugation (Matthews and Ralph, 1966). The dissimilarity in the results may have been due to a different mass : charge ratio in the viral components as compared to the ribosomes and polyribosomal aggregates, if this was the case then a different migration rate would be expected.

The electrophoretic method of ribosome fractionation offers a means by which TYMV components can be unequivocally separated from the host ribosome population without

eradicating the latter. This is not readily achieved by the use of sucrose density gradient centrifugation.

### 3.73.10 Electrophoresis of ribosomes extracted from other species

Ribosomes in dry pea seeds exist in the monomeric condition (Barker and Reiber, 1967; Thomas, 1973) and this is probably general for all seeds (Thomas 1972a). This observation was confirmed for dry pea seeds with the electrophoretic method used here. Ribosomes extracted from dry pea seeds migrated as a single band in the polyacrylamide gel to a position equivalent to that of the monoribosome peak of leaf preparations (figure 3.60).

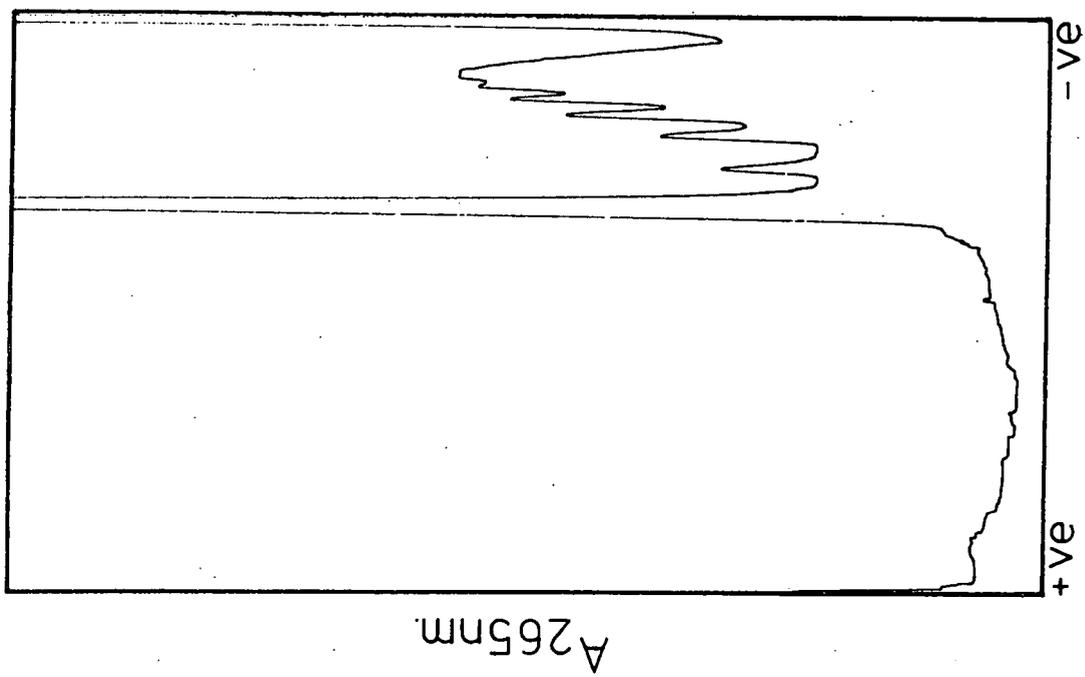
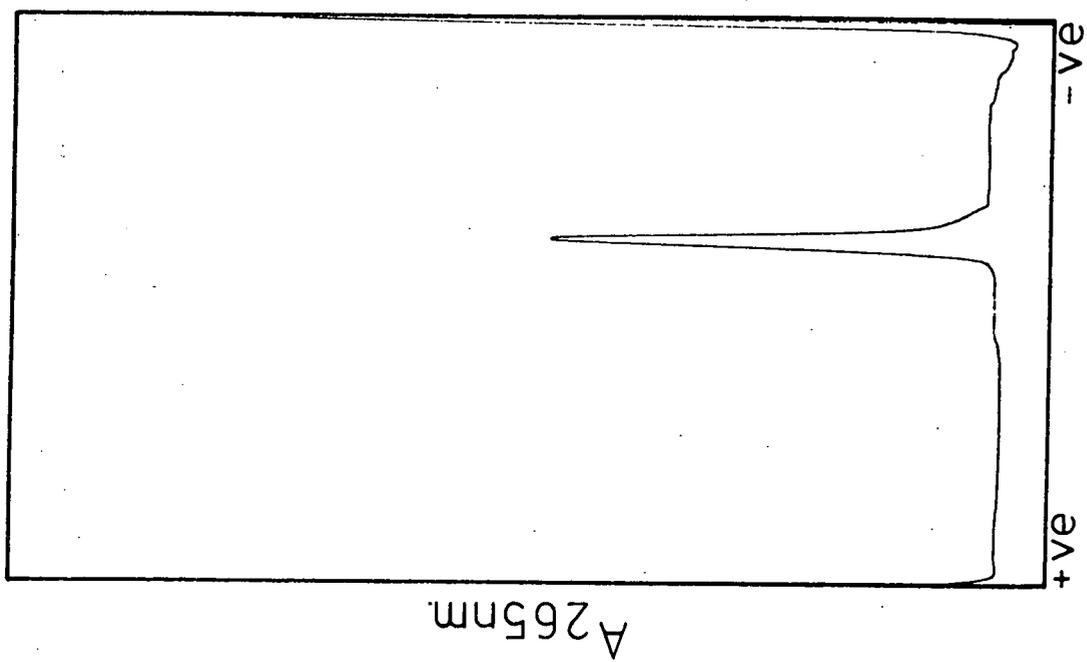
Large quantities of polyribosomes of a greater average length than those found in chinese cabbage leaves can easily be obtained from actively growing pea shoots and stems. It was thought that ribosomes extracted from such tissue would serve as a useful test to the electrophoretic system. Dry pea seeds were imbibed overnight under running tap water, the testas were removed and ribosomes were extracted from the excised embryonic axes. Electrophoresis of the isolated pea polyribosomes showed distinct ribosome peaks to the octamer with an undifferentiated zone of larger polymers (figure 3.61). The greatest u.v. absorbance was found in the septamer and octamer polyribosome peaks in contrast to leaves in which the smaller polymers were present in greatest amounts. Thus

Figure 3.60

PAGE of ribosomes extracted from dry pea seeds.

Figure 3.61

PAGE of ribosomes extracted from imbibed pea embryos (2 $\mu$ g ribosomes loaded onto gel).



polymers larger than those routinely found in chinese cabbage leaves can freely enter the gels under the conditions of electrophoresis used. This suggests that shearing effects on the larger polymers were probably not significant in the leaf material. The electrophoresis of long chain polymers from pea does however demonstrate a restriction in the use of the electrophoretic method. Because of the logarithmic relationship between migration and particle weight of the polyribosomes the larger polymers migrate very close together, this results in a lack of distinction between the higher polymers as seen in the pea profile. While this is no drawback if overall polyribosome levels are required it is a disadvantage if individual large polyribosome peaks are being investigated.

Polyribosomes have also been extracted and fractionated with success from the fungus Phycomyces blakesleanus. A two day old liquid culture of the fungus was harvested by vacuum filtration through a millipore filter. The yellow pad of mycelium was frozen in liquid air and the polyribosomes were extracted by the methods described for leaf material. The quality of the extracted ribosomes, as judged by their u.v. spectrum, was comparable to leaf ribosome extracts with values for the ratios  $E^{260}/E^{230}$  and  $E^{260}/E^{280}$  being 1.25 and 1.58 respectively. The yield obtained was 2.25mg ribosomes per gram fresh weight of mycelium. Electrophoretic fractionation showed polymers clearly defined to the septamer with some

larger polysomes also being present (figure 3.62).

3.74 Polyribosomes extracted in the presence of Triton X-100

Berridge et al., (1970) obtained an increased yield of 68s chloroplast ribosomes in chinese cabbage leaf ribosome preparations when the extraction was performed in the presence of Triton X-100. A normal extraction of ribosomes from chinese cabbage leaves was therefore carried out but with the DOC replaced by 5% Triton X-100. The post-mitochondrial supernatant obtained was a dark green colour, in contrast to the pale yellow supernatant usual with the normal procedure. This coloured supernatant gave rise to a dark green 105,000g. ribosome pellet. The resuspended pellet was clarified by centrifugation in a bench centrifuge prior to estimation of the sample in the u.v. (see Table 12) and electrophoresis.

Table 12

| Sample                            | u.v. spectral ratio |                    | Yield - $\mu$ g<br>ribosomes per<br>gram f.wt. |
|-----------------------------------|---------------------|--------------------|------------------------------------------------|
|                                   | $E_{\max}/E_{\min}$ | $E_{\max}/E_{280}$ |                                                |
| DOC extract<br>(control)          | 1.33                | 1.70               | 377                                            |
| Triton X-100<br>extract           | 1.19                | 1.40               | --                                             |
| Clarified Triton<br>X-100 extract | 1.48                | 1.77               | 330                                            |

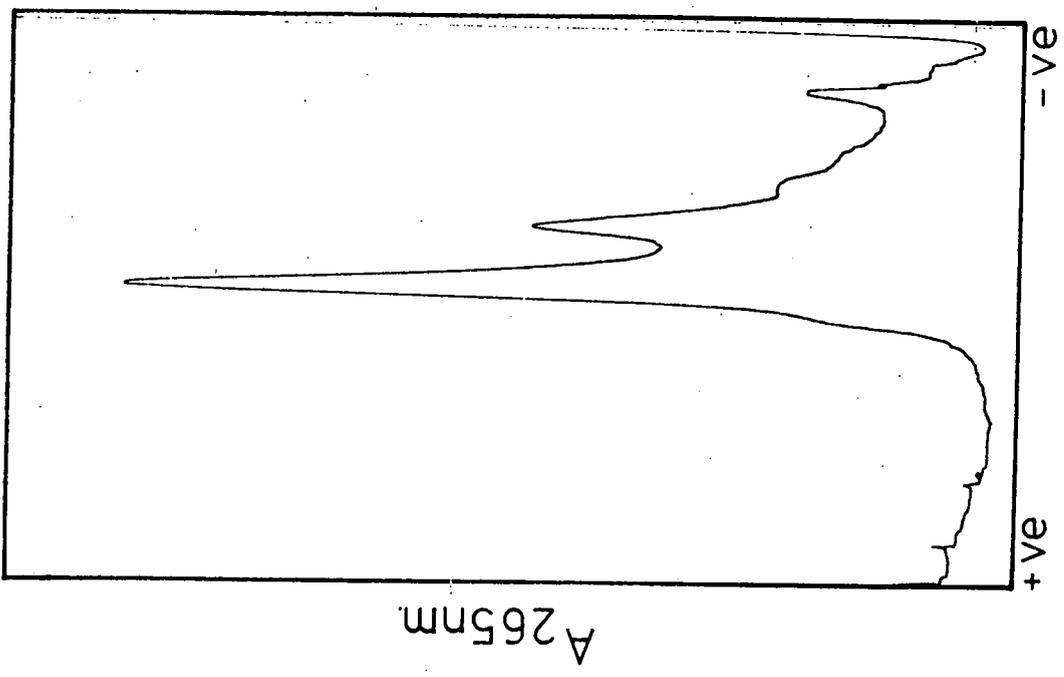
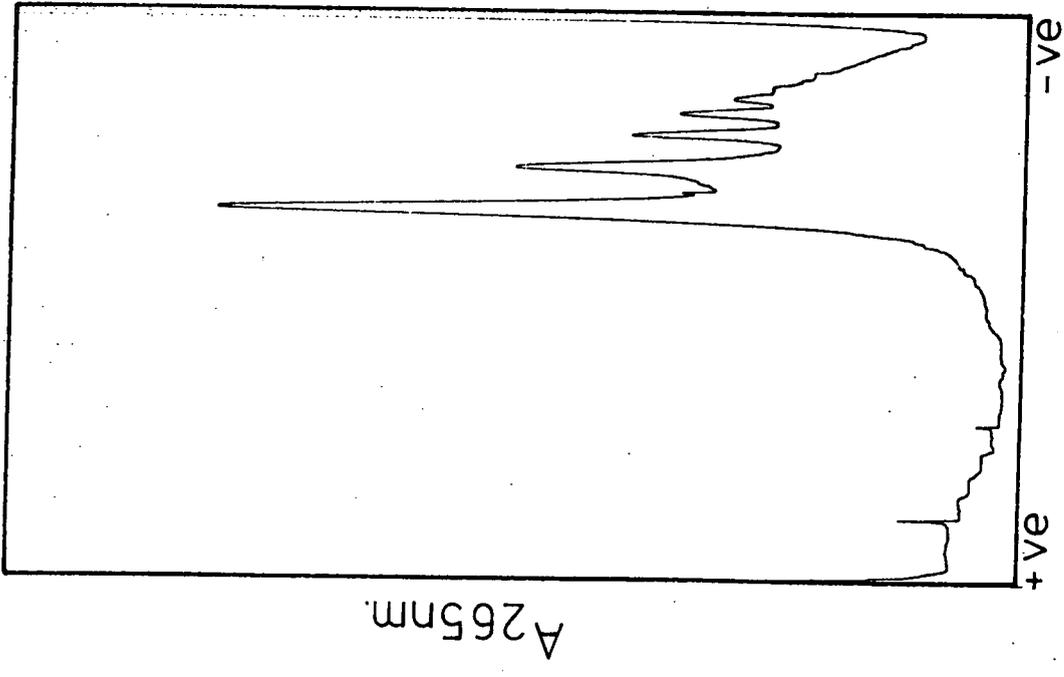
The total yield of ribosomes from leaf material was similar regardless of whether DOC or Triton X-100 was present in the

Figure 3.62

PAGE of ribosomes extracted from the mycelium of  
Phycomyces blakesleanus.

Figure 3.63

PAGE of ribosomes extracted from chinese cabbage  
leaves in the presence of Triton-X100.



extraction. Gordon et al., (1975) similarly found that DOC or Triton gave similar yields when extracting ribosomes from radish cotyledons and that either gave substantially greater yields than extractions in their absence.

The electrophoretic profiles obtained for the Triton extracted material were similar to those for normal extracts (figure 3.63). There was no evidence of additional chloroplast ribosome components, similarly fractionation of the RNA from Triton X-100 extracted ribosomes showed no increase in the chloroplast fractions when compared to the profiles obtained from normally extracted ribosomes. Because no advantages were evident by using Triton X-100 instead of DOC in the extraction medium no further use was made of this detergent for ribosome extraction.

### 3.75 Polyribosomes extracted in the presence of DEP

The nuclease inhibitor DEP has been used successfully in the isolation of polyribosomes from wheat embryos and corn root tips (Weeks and Marcus, 1969) and from cotyledons of developing seeds of Vicia faba (Lonsdale and Boulter, 1973) although under certain conditions DEP-induced dissociation of ribosomes can occur (Anderson and Key, 1971; Huvos and Solymosy, 1971).

A normal leaf ribosome extraction was carried out in which DEP was added (final concentration 1.5%) to the initial grinding buffer. The extraction was then carried out in the

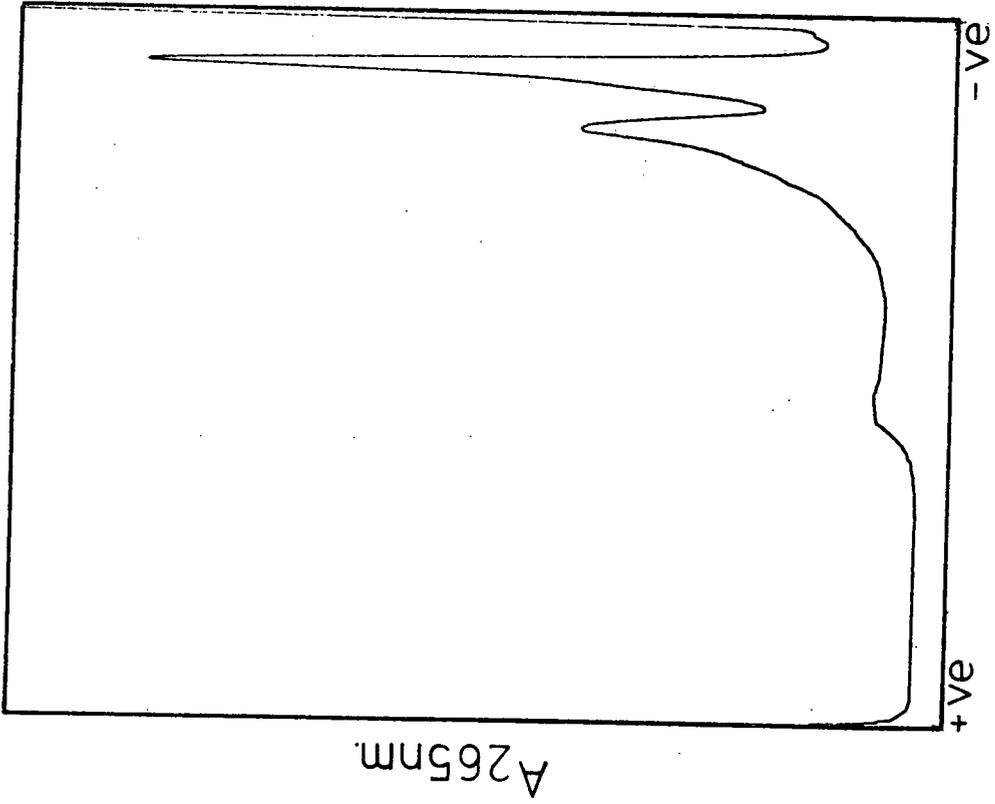
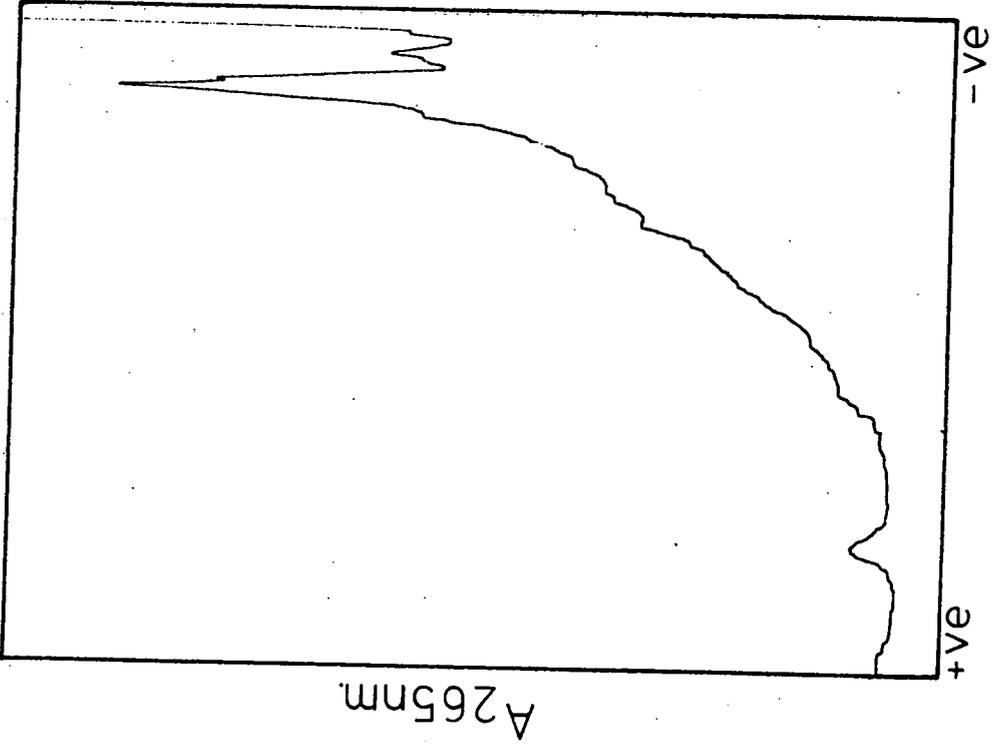
usual way producing a pale yellow ribosome pellet. The u.v. spectral characteristics of the DEP-treated ribosome extract were similar to those of untreated controls except that there was a slight shift in the wavelength of minimum absorption to 242 nm. The yield of ribosomes from leaf material was reduced by approximately 50% compared to controls when DEP was present in the extraction. Fractionation of these ribosomes showed a large peak near the top of the gel (figure 3.64) suggesting aggregation of the ribosomes, preferential dissociation into ribosomal sub-units was not observed. In view of these unsatisfactory results DEP was not included as a nuclease inhibitor during ribosome extraction.

Figure 3.64

PAGE of ribosomes extracted from chinese cabbage leaves in the presence of DEP.

Figure 3.65

PAGE of ribosomal sub-units in 3.0% acrylamide gels using the electrophoresis buffer of Thomas (1973).



### 3.8 Ribosomal sub-units

#### 3.81 U.V. absorption spectrum characteristics

Ribosomal sub-units were obtained by resuspension of the 105,000g. ribosome pellet in tris-acetate buffer containing the chelating agent tetrasodium pyrophosphate (Thomas, 1973). The u.v. absorption spectrum of resuspended sub-units was similar to that of intact ribosomes with a maximum absorption at 258 nm and a minimum at 238 nm. Ribosomal sub-unit resuspensions from infected tissue showed the same shifts in the wavelengths of maximum and minimum absorption as observed in polyribosome preparations. The average values for the ratios  $E_{max}/E_{min}$  and  $E_{max}/E_{280}$  were 1.33 and 1.68 respectively for sub-units from healthy tissue, and 1.37 and 1.74 respectively for infected extracts. These values are in good agreement with those obtained for polyribosomes.

#### 3.82 PAGE of ribosomal sub-units

Ribosomal sub-units were initially fractionated by the method of Thomas (1973) but in 3.0% acrylamide gels (Dahlberg et al., 1969). Ribosomal sub-units from a number of species have been successfully separated in gels of a similar acrylamide concentration (Talens et al., 1970; Carlton and Herson, 1972; de Vries and van der Koogh-Schuuring, 1973; Curgy et al., 1974; Ledoigt et al., 1975). Resolution was obtained between the large and small sub-unit fractions and changes of slope on the leading edges of these two main peaks

indicate the presence of minor fractions (figure 3.65). Migration into the gel was low and attempts were made to further resolve the components observed by either increasing the time of electrophoresis to five hours or by increasing the current to 8mA per gel. In both instances the sub-units proved to be unstable and a single broad peak was all that was obtained (figure 3.66). Electrophoresis in gels of lower acrylamide concentration (2.6%) did allow greater migration into the gel but the extra components noted above were not seen.

Greater electrophoretic mobility was obtained when the running buffer was replaced by an EDTA-containing buffer (Dahlberg et al., 1969):

25mM tris-HCl pH 8.0

2.5mM disodium EDTA

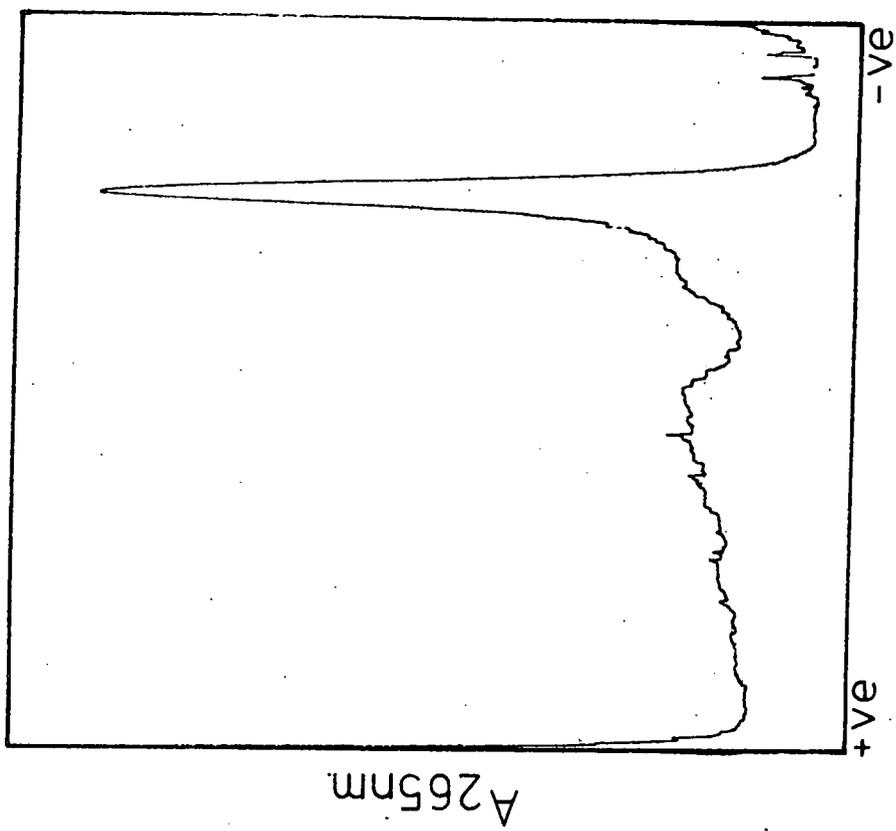
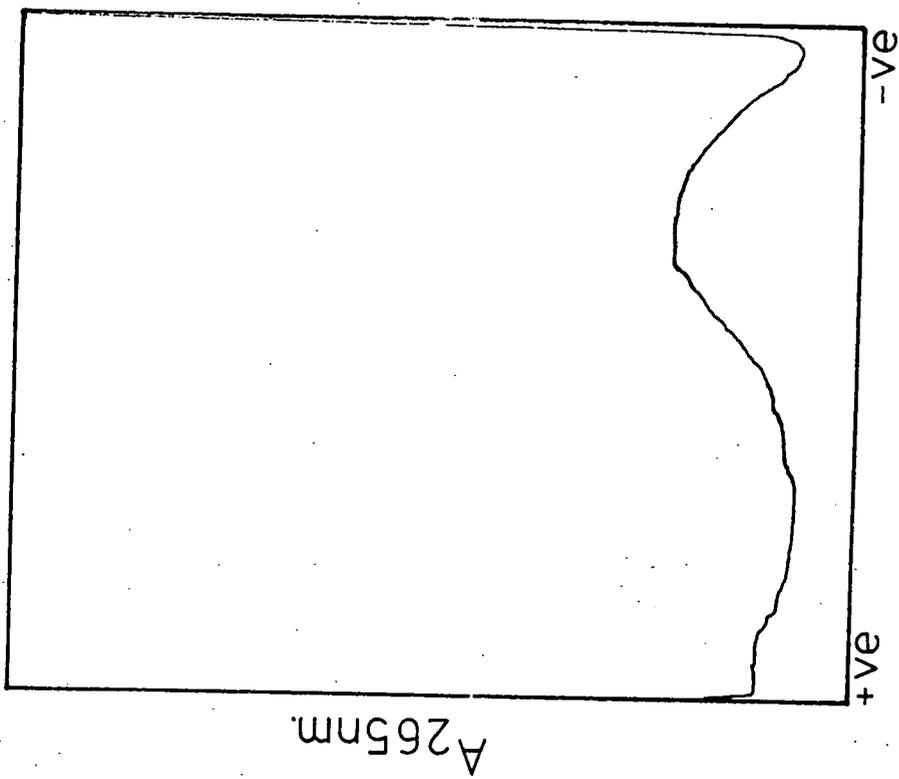
However, the small ribosomal sub-unit peak was poorly resolved (figure 3.67). When ribosomes are induced to dissociate by the use of chelating agents (e.g. pyrophosphate, EDTA) to remove magnesium ions the released sub-units often suffer structural changes (e.g. Tashiro and Siekevitz, 1965; Lamfrom and Glowacki, 1962). The possibility of these effects occurring in addition to simple dissociation may account for the results with the EDTA buffer. Because of this ribosomes resuspended in sub-unit buffer were electrophoresed in the

Figure 3.66

PAGE of ribosomal sub-units electrophoresed under the same conditions as figure 3.65 but at an increased current (8mA per gel).

Figure 3.67

PAGE of ribosomal sub-units using the EDTA-containing buffer of Dahlberg et al., (1969).



presence of magnesium ions. The gel running buffer used was:

25mM tris-HCl pH 8.0

1.0mM magnesium chloride

Recirculation of the buffer between the reservoirs of the electrophoresis tank was employed to reduce the effects of magnesium accretion. Under these conditions of electrophoresis mobility of the sub-unit into the gel was obtained and at the same time their integrity was preserved (figure 3.68).

Secondary components are evident as 'shoulders' on the main sub-unit peaks.

Heterogeneity of the sub-units of 70s ribosomes from procaryotic species (Dahlberg et al., 1969; Talens et al., 1970; Ledoigt et al., 1975) has not been observed during electrophoresis of eucaryotic 80s ribosomes (von der Decken et al., 1970; Thomas, 1973; Ledoigt et al., 1975). Because of this and the fact that the sub-units were obtained from total leaf extracts it is probable that these extra fractions are the sub-units derived from 70s chloroplast ribosomes.

It was apparent then that low concentrations of magnesium ions were required to maintain the structural integrity of ribosomal sub-units obtained by dissociation of ribosomes with chelating agents. Electrophoresis of dissociated ribosomes in the presence of higher magnesium concentrations resulted in aggregation and/or reconstitution of the sub-

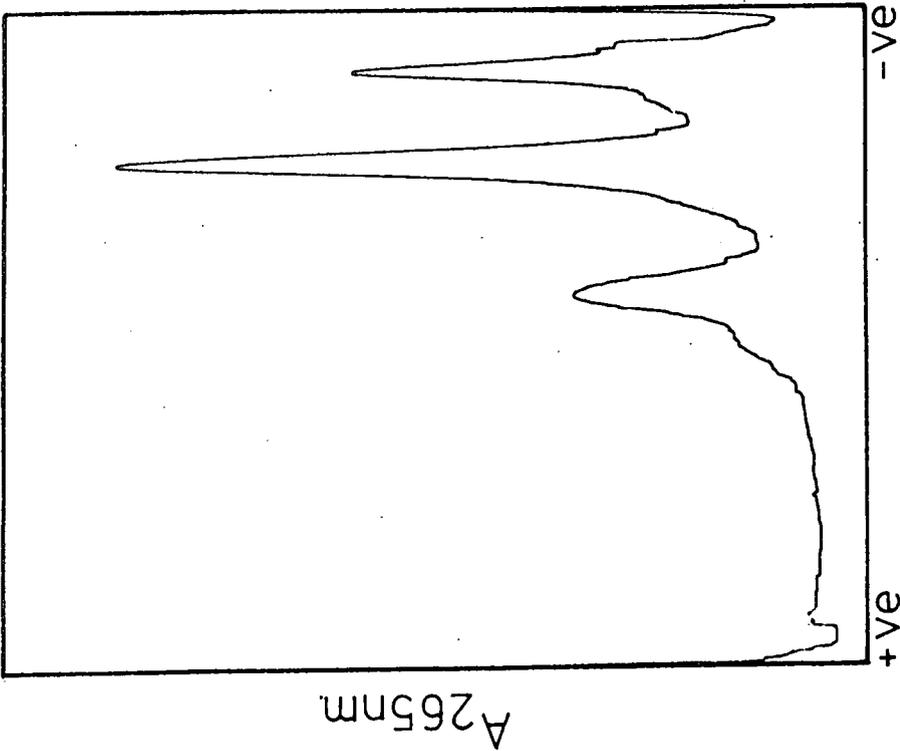
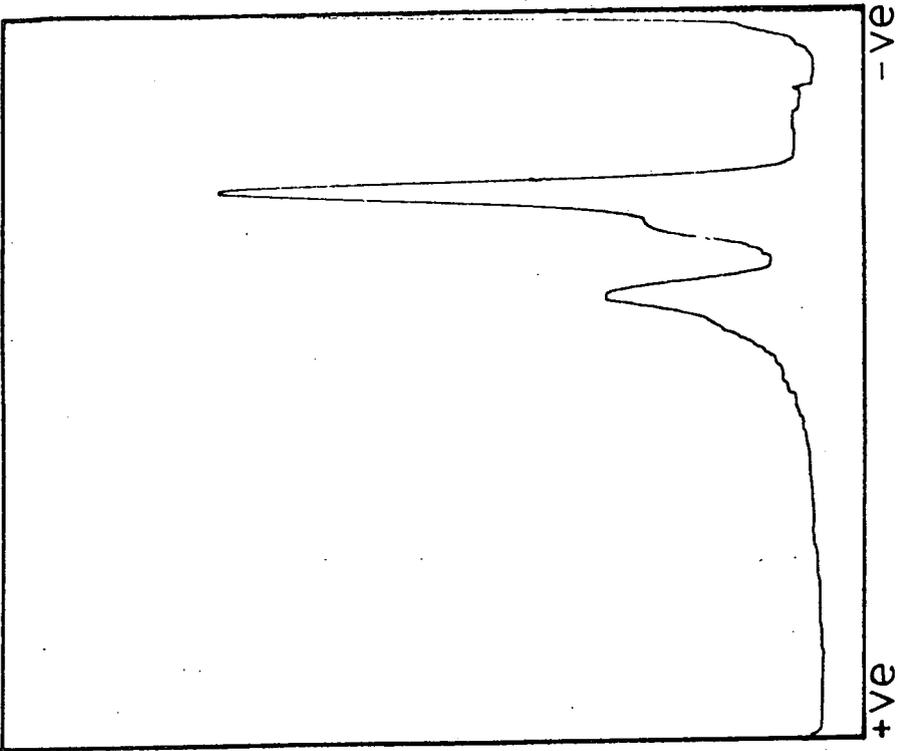


Figure 3.68

PAGE of ribosomal sub-units using tris-HCl buffer  
(pH 8.0) containing 1.0 mM  $\text{MgCl}_2$  as the electrophoresis  
buffer.

Figure 3.69

PAGE of ribosomal sub-units using tris-HCl buffer  
(pH 8.0) containing 2.0 mM  $\text{MgCl}_2$  as the electrophoresis  
buffer.



units into particles of lower electrophoretic mobility (figure 3.69).

It was assumed that the ribosomal sub-units would show a similar linear relationship between sedimentation coefficient and migration in the polyacrylamide gel as that demonstrated for ribosomes and polyribosomes (see 3.73.1). Ribosomal sub-units obtained from imbibed pea embryos were used as standards and values of 60s and 40s were assigned to the large and small sub-units respectively (Bayley, 1964). Using these standards the sedimentation coefficient for the chinese cabbage ribosomal sub-unit peaks were determined graphically and values of 56s and 36s were obtained. Analytical ultracentrifugation of the sub-units of chinese cabbage 80s ribosomes gave figures of 55s and 40s for the large and small sub-units respectively (Clark et al., 1964). Using these estimates for the size of the main sub-unit peaks an estimate was made of the sedimentation value of the secondary components observed in the ribosomal sub-unit electrophoretograms. Figures of 48-50s and 30-35s were obtained for the larger and smaller of the two components respectively. These values are close to those published for the sub-units of chloroplast ribosomes obtained by analytical ultracentrifugation - 47s and 33s (Lyttleton, 1962). This indicates that these fractions may indeed be of chloroplastic origin as suggested above.

3.83 PAGE of polyribosome resuspensions to yield ribosomal sub-units

Samples previously used for polyribosome analysis could subsequently be fractionated as ribosomal sub-units by electrophoresis under conditions inducing ribosome dissociation. Electrophoresis in EDTA-containing buffer:

25mM Tris-HCl pH 8.0

2.5 mM EDTA

resulted in the dissociation of the ribosomes but lack of resolution of the small sub-unit, as observed previously, again occurred (figure 3.70). The presence of magnesium in the resuspension buffer was insufficient to counteract the effects of the chelating agent. Electrophoresis of re-suspended ribosomes in buffers of low magnesium concentration did not produce a consistent degree of dissociation. That this may be connected with the seasonal growth cycle of chinese cabbage has already been mentioned (see 3.73.7).

3.84 PAGE of ribosomal sub-units from TYMV-infected plants

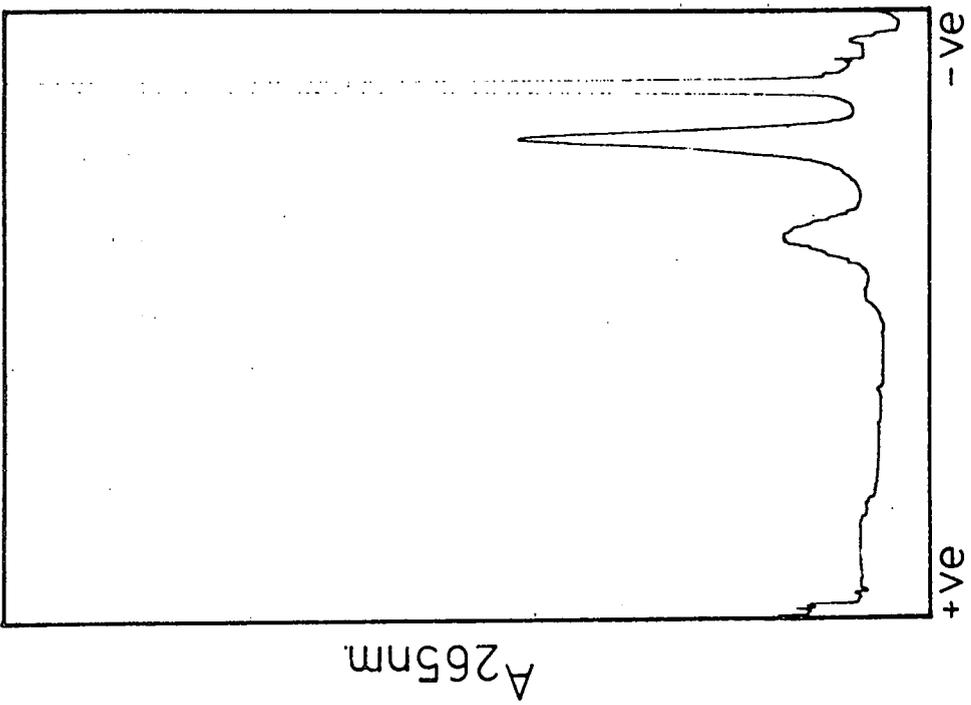
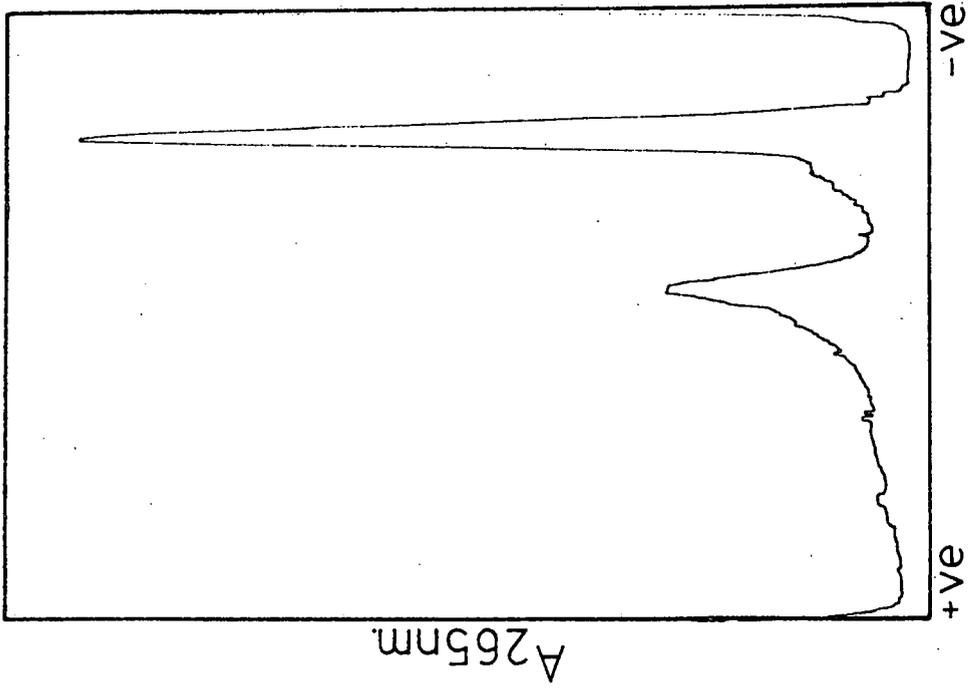
The electrophoretic separation of ribosomal sub-units obtained from TYMV-infected plants can be achieved by the same means as for healthy tissue. Samples resuspended in sub-unit buffer and electrophoresed in low magnesium concentration buffer showed distinct sub-unit peaks with, in addition, a virus band of lower electrophoretic mobility

Figure 3.70

PAGE of ribosomes (resuspended in ribosome buffer) in the EDTA-containing buffer of Dahlberg et al., (1969) to yield ribosomal sub-units.

Figure 3.71

PAGE of ribosomal sub-units obtained from TYMV-infected leaves of chinese cabbage (conditions as figure 3.68).



(figure 3.71). The viral 'top component' can also be observed near the top of the gel.

EDTA electrophoresis of infected samples resuspended in ribosome buffer produced a changed relative migration between the virus and the large sub-unit. The virus under these conditions of electrophoresis had an electrophoretic mobility greater than that of the large ribosomal sub-unit peak (figure 3.72), again the viral 'top component' was present. A differential effect of EDTA on the conformation of the large sub-unit as compared to the virus could explain this change in relative mobility. Also a change in the effective charge on the particles brought about by the removal of  $Mg^{2+}$  ions by the chelating agent could be contributory.

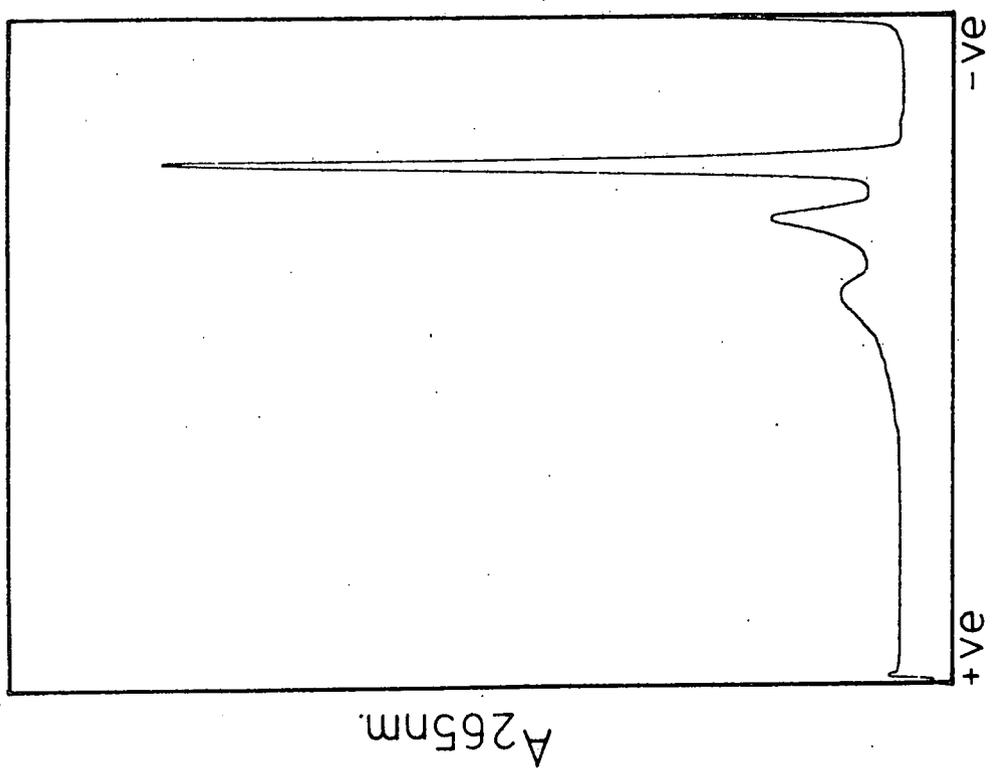
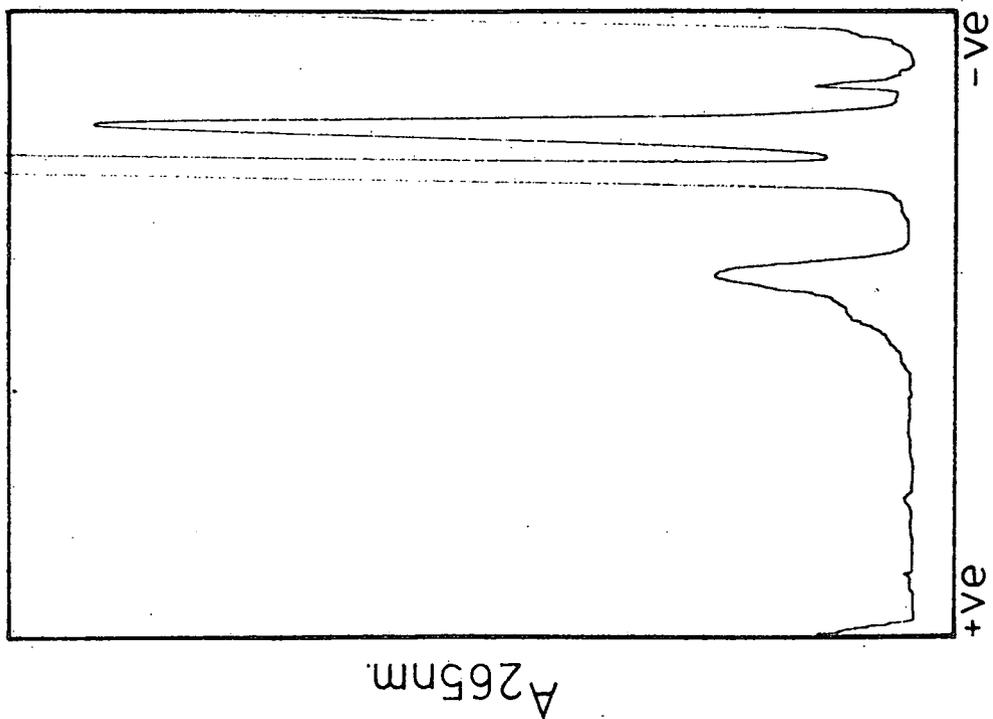
The use of the pyrophosphate buffer as the electrophoresis buffer did not produce this change in relative migration. Using this buffer gels of a lower acrylamide content (2.4%) had to be used to achieve sufficient migration into the gel without the breakdown of the sub-units indicated in 3.82 (figure 3.73). The use of these larger pore gels may mean that the conformation effects of the chelating agent are less evident.

Figure 3.72

PAGE of ribosomal sub-units obtained from TYMV-infected leaves of chinese cabbage in the EDTA-containing buffer of Dahlberg et al., (1969).

Figure 3.73

PAGE of ribosomal sub-units obtained from TYMV-infected leaves of chinese cabbage in 2.6% acrylamide gels using the buffer of Thomas (1973).



### 3.9 RNA from isolated ribosomes

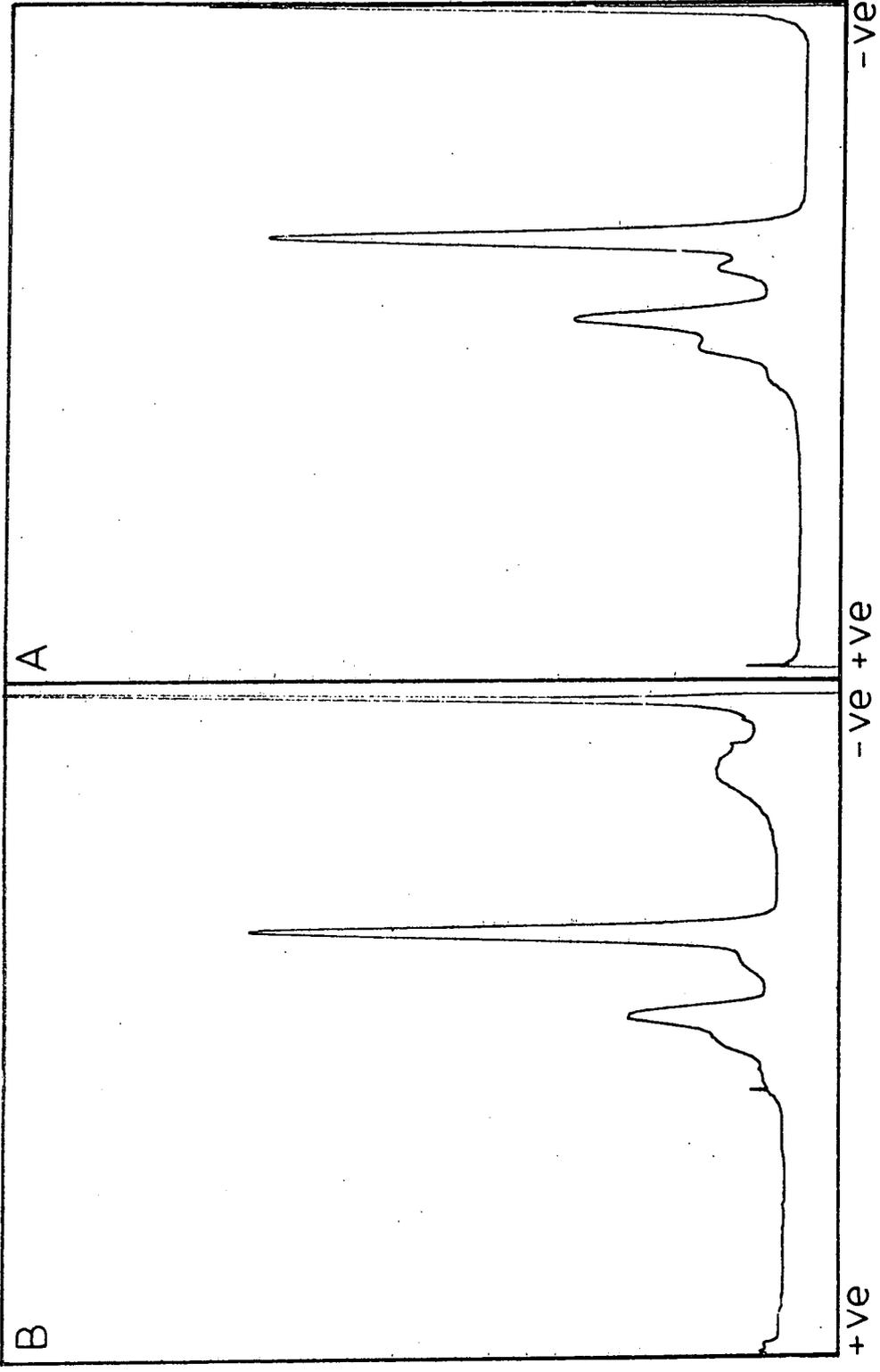
#### 3.91 DEP-SDS method of RNA extraction

Figure (3.74a) shows a magnesium electrophoresis of RNA extracted from a total ribosome preparation from uninfected leaves of chinese cabbage by the method described in 3.24.4. The four major rRNA species are present. Resuspension of the sample in EDTA-containing buffer has resulted in some dissociation of the labile 23s chloroplast fraction. Compared to total RNA extracts from green leaves the proportions of chloroplast components relative to the cytoplasmic ones is low. It thus appears that the method of ribosome extraction employed does not release total leaf ribosomes. Lyttleton (1967) similarly found a low release of chloroplast ribosomes in Chenopodium album. With chinese cabbage Berridge et al., (1970) found that only about 11% of the ribosomes extracted were of the 70s type, inclusion of Triton-X100 in the extraction increased this proportion to 33%. Extraction of RNA from ribosomes extracted in the presence of Triton-X100 in this work failed to show an increase in the proportion of chloroplast components. Using an essentially similar extraction method to that used here Pearson (1969b) did not observe a low chloroplast ribosome recovery from radish leaves as judged by rRNA content but here distinction between peaks was low. This suggests that there may be some species specificity with regard to the ease of chloroplast

Figure 3.74

PAGE of RNA extracted from isolated ribosomes by the DEP-SDS method.

- A. RNA from ribosomes isolated from healthy chinese cabbage leaves
- B. RNA from ribosomes isolated from TYMV-infected chinese cabbage leaves



A265nm.

A

B

-ve

-ve +ve

+ve

ribosome extraction.

This method of RNA extraction was also found to be suitable for ribosome extracts from infected tissue, complex formation was not induced and the host rRNA was clearly defined (figure 3.74b). The extra fraction of low electrophoretic mobility on this gel is the virus.

### 3.92 SDS method of RNA extraction

This method was tried because without any alcohol precipitation steps the conservation of material should be higher and thus may be of advantage when only small samples were available. The results obtained were inconsistent and breakdown of the major RNA fractions frequently occurred but complex formation was not induced in extracts from infected plant samples (figure 3.75). Ribosome-associated ribonuclease activity (Hsaio, 1968) may be partly responsible for this breakdown which was not evident when DEP was present. SDS however has been reported as being a mild nuclease inhibitor (Poulson, 1973) suggesting that the DEP in conjunction with the high salt buffer used may confer some structural stability on the RNA molecules.

### 3.93 Detergent-phenol method of RNA extraction

Using this method to extract RNA from ribosomes profiles essentially similar to those achieved with DEP-SDS were obtained for healthy leaf extracts (figure 3.76) and again the low proportion of chloroplast rRNA species was observed. With

Figure 3.75

PAGE of RNA extracted from isolated ribosomes by treatment with SDS.

- A. RNA from ribosomes isolated from healthy chinese cabbage leaves
- B. RNA from ribosomes isolated from TYMV-infected chinese cabbage leaves

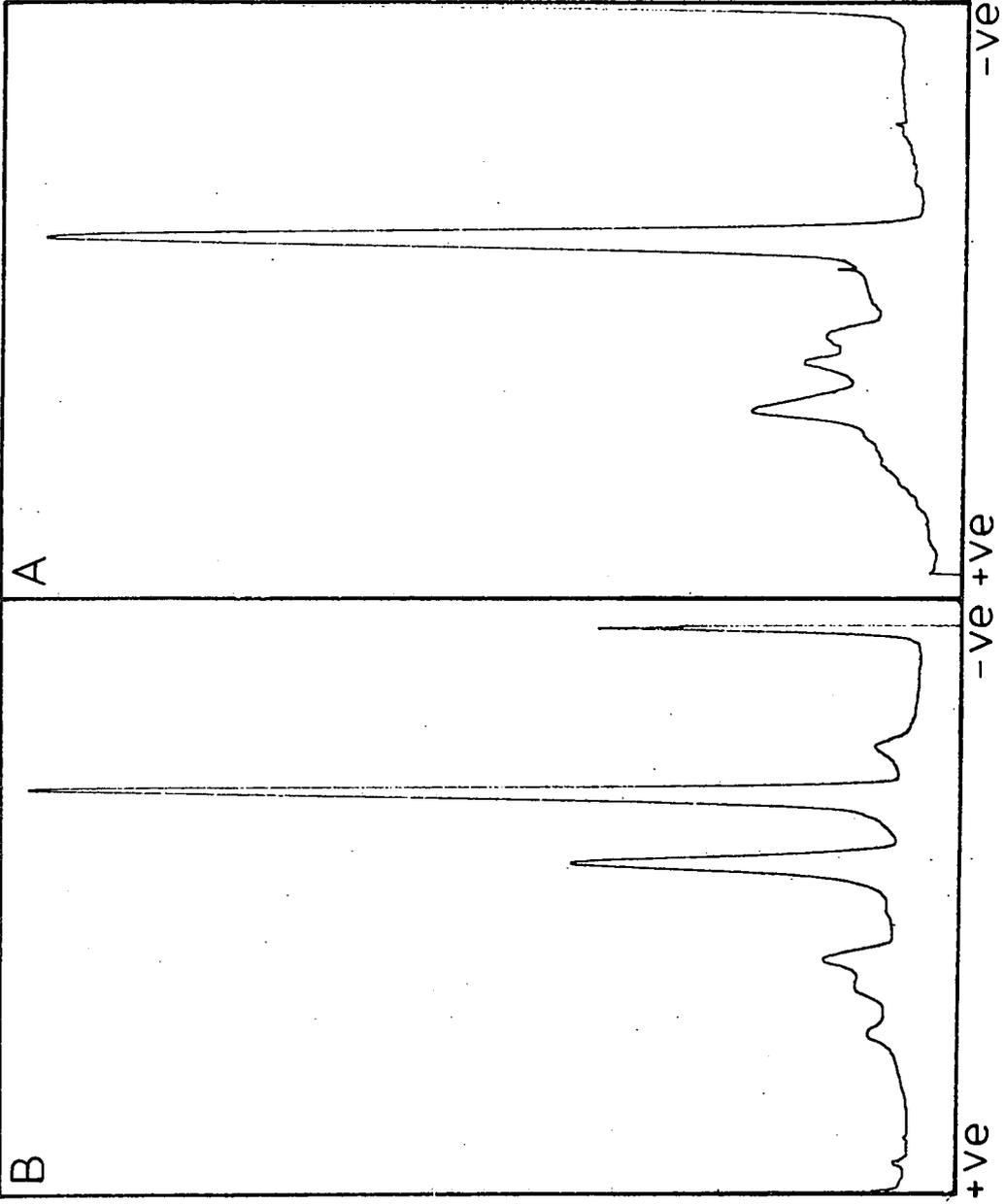
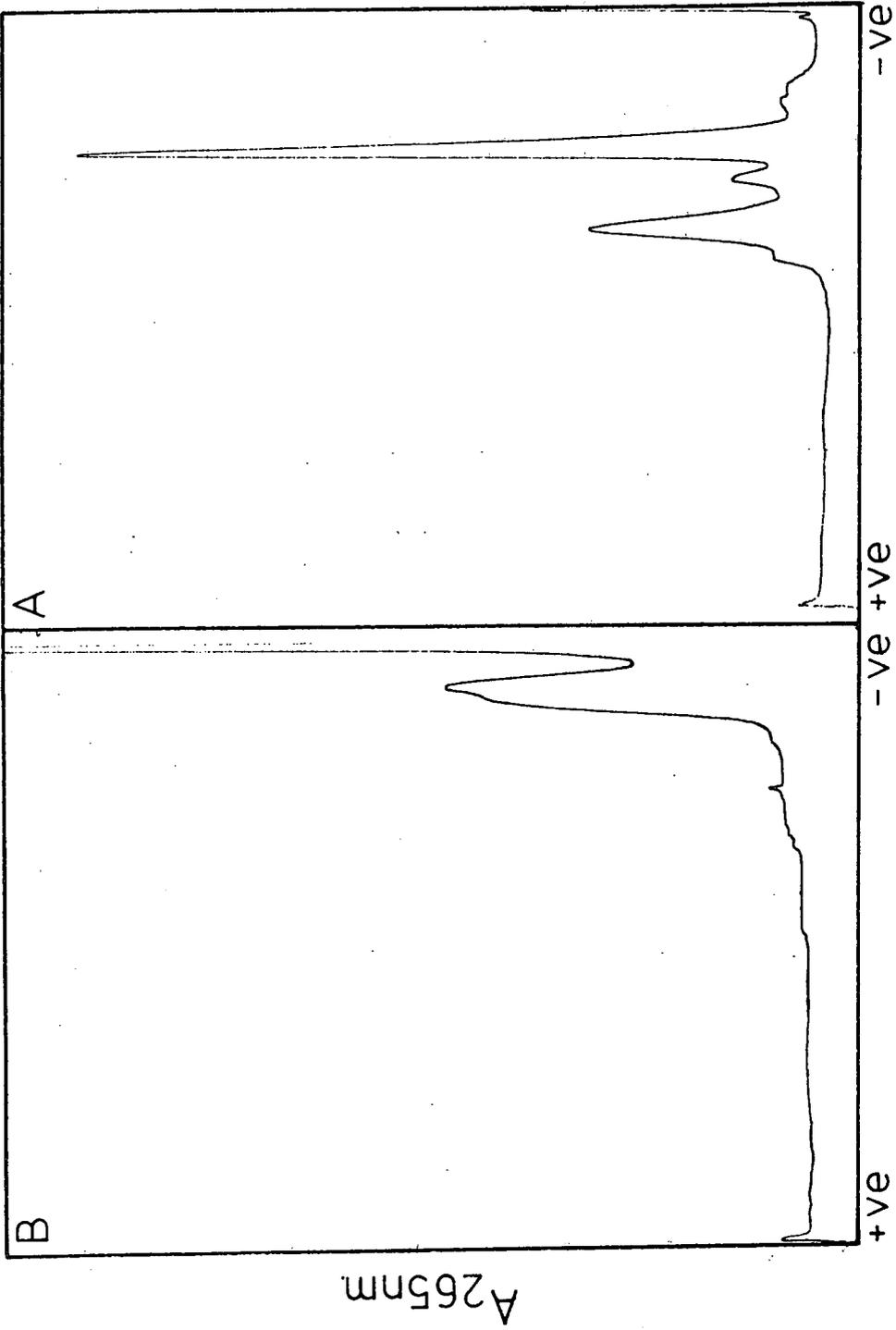


Figure 3.76

PAGE of RNA extracted from isolated ribosomes by the detergent-phenol method.

- A. RNA from ribosomes isolated from healthy chinese cabbage leaves
- B. RNA from ribosomes isolated from TYMV-infected chinese cabbage leaves



ribosomes extracted from TYMV-infected leaves the viral RNA-host RNA complex was formed and for this reason this method was again found to be unsatisfactory for chinese cabbage. The usual drawbacks of the detergent-phenol method still prevailed but were now more disadvantageous because of the small amounts of starting material used.

### 3.94 Guanidinium chloride method of RNA extraction

The use of guanidinium chloride has been reported as being suitable for the isolation of RNA from ribosomes (Cox and Arnstein, 1963) and has been successfully applied to plant ribosomes (Thomas, 1973). Extraction of RNA from ribosome preparations from TYMV-infected tissue resulted in the formation of the virus-host RNA complex, in view of this observation this method was discontinued.

### 3.95 Characterisation of RNA extracted from ribosomes by the DEP-SDS method

#### 3.95.1 U.V. absorption spectra

The u.v. absorption profiles of RNA extracted from ribosomes showed the same features as found for leaf total RNA extracts. There was a shift in the absorption minima attributable to interaction of the RNA with DEP (Solymosy et al., 1972), also there was a shift in the absorption maximum and minimum of the infected ribosomal extracts which could be accounted for by the presence of intact TYMV. The average

ratios for  $E_{\max}/E_{\min}$  and  $E_{\max}/E_{280}$  were:

Table 13

|                                  | $E_{\max}/E_{\min}$ | $E_{\max}/E_{280}$ |
|----------------------------------|---------------------|--------------------|
| RNA from uninfected ribosomes    | 1.16                | 1.74               |
| RNA from TYMV-infected ribosomes | 1.20                | 1.85               |

The  $E_{\max}/E_{280}$  ratios are in close agreement with the values obtained for total leaf RNA extracts but the  $E_{\max}/E_{\min}$  values are lower.

The average recovery of RNA from ribosomes was 403 $\mu$ g RNA per mg ribosomes. This is consistent with an RNA : protein ratio of 40:60 (wt/wt) in plant ribosomes (Tso et al., 1956, 1958). For TYMV-infected ribosome extracts the average RNA yield was 280 $\mu$ g RNA per mg ribosomes plus virus. This estimate is lower than that for either ribosomes (40% RNA) or TYMV (34% RNA) (Kaper and Litjens, 1966). If, as has been suggested above, the virus is not being deproteinised in these extracts then this yield may represent only that RNA present in the ribosomes in the sample and the recovery here may be the same as for healthy ribosomes. This could be confirmed if the proportion of virus present in the sample were known.

### 3.95.2 Molecular weight determination

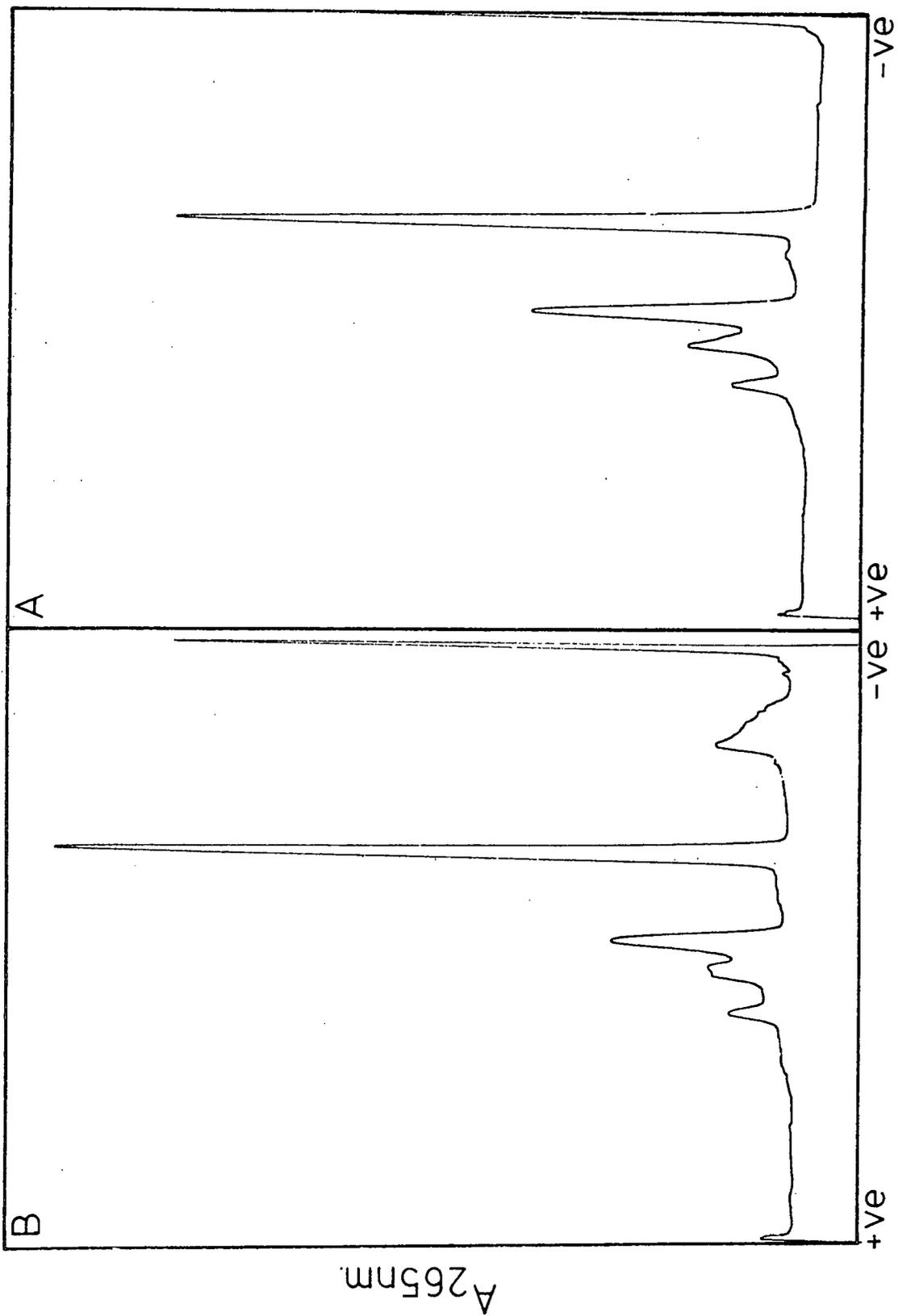
As already indicated magnesium electrophoresis showed four main rRNA peaks corresponding to the ribosomal RNA

species from the large and small sub-units of cytoplasmic 80s and chloroplastic 70s ribosomes. The molecular weights of the RNA fractions were determined from a plot of log (molecular weight) against migration in the gel. The cytoplasmic rRNA species were used as molecular weight standards with values of  $1.3 \times 10^6$  and  $0.7 \times 10^6$  as previously determined (3.32 and 3.42). The RNA samples were resuspended and fractionated in EDTA buffer which resulted in the dissociation of the chloroplast 23s fraction with a <sup>concomittant</sup> ~~commensurate~~ appearance of breakdown products (figure 3.77). The chloroplast rRNA species were estimated to have a molecular weight of  $1.05 \times 10^6$  and  $0.56 \times 10^6$  with breakdown peaks which had values of  $0.58 \times 10^6$ ,  $0.43 \times 10^6$ ,  $0.37 \times 10^6$  and  $0.32 \times 10^6$ . These values are in close agreement with those for total leaf RNA extracts and the slight difference was the result of using the EDTA buffer of Bishop et al., (1967) rather than that of Loening (1969). Degradation products specific to RNA extraction of isolated ribosomes (Labrie, 1969; Dingman et al., 1970; Payne and Loening, 1970; Gaskill and Kabat, 1971; Takagi et al., 1971; Lonsdale and Boulter, 1974) were not observed though their presence may have been masked by the instability of the  $1.05 \times 10^6$  fraction giving rise to a number of dissociation products.

Figure 3.77

PAGE of RNA extracted from isolated ribosomes and electrophoresed using '3E' buffer of Bishop et al., (1967).

- A. RNA from ribosomes isolated from healthy chinese cabbage leaves
- B. RNA from ribosomes isolated from TYMV-infected chinese cabbage leaves



### 3.10 Discussion

The difficulty in obtaining intact and resolving the labile 23s chloroplast rRNA from plant tissues when using phenol methods of RNA extraction have been indicated by Leaver and Ingle (1971). Under conditions in which the dissociation of the chloroplast rRNA fraction was reduced to a minimum by the presence of magnesium during both extraction and fractionation the total RNA preparation was contaminated with non-nucleic acid material and 'dirty', poorly resolved gel electrophoretograms were obtained. This result for phenol extraction was confirmed for chinese cabbage leaf RNA and attempts were made to improve the method. By adding EDTA to clarify the extract at a late stage during magnesium extraction the purity of the RNA obtained, the integrity of the 23s rRNA and the quality of the resolution achieved were all improved. Some dissociation of the 23s rRNA was still evident, induced by the presence of the EDTA, but this was unavoidable if clarification of the sample was to be obtained. The addition of magnesium to an EDTA extraction was less effective since it appeared that magnesium was unable to bridge breaks in the polynucleotide chain once the fragments have been released from the conformation conferred upon the RNA in the intact ribosome. Electrophoretic characterisation of the phenol extracted RNA from chinese cabbage leaves resulted in the 'typical' profiles expected for green leaf tissue (Loening

and Ingle, 1967; Loening, 1967), and electrophoretic estimation of the molecular weights of the main fractions observed were also consistent with previously published results for higher plant rRNA species (Loening, 1968c).

On the electrophoretic criteria of qualitative profiles and estimated molecular weight of the fractions, the RNA from chinese cabbage leaves extracted under the 'high-salt' conditions for the DEP-SDS method behaved similarly to phenol extracted RNA. Nucleic acids extracted by DEP-methods from a number of sources have similarly been shown to be indistinguishable analytically from phenol extracted nucleic acids with regard to sedimentation properties (Lazar et al., 1969; Solymosy et al., 1970), behaviour on MAK columns (Lazar et al., 1969), hybridisation with DNA (Summers, 1970) and also electrophoretically (Lazar et al., 1969; Melera et al., 1970; Solymosy et al., 1970). The biological activity of DEP-extracted RNA from chinese cabbage was not examined but evidence from other sources suggests that DEP used under normal extraction conditions does not influence the activity of RNA species (Fedorcsak et al., 1969; Abadom and Elson, 1970) though extensive exposure to DEP of isolated RNA can lead to a loss of biological activity (Gulyas and Solymosy, 1970; Oxelfelt and Arstrand, 1970; Oberg, 1970). The loss of activity is probably a result of carboxymethylation of the nucleic acid bases (Leonard et al., 1970; Oberg, 1971(b); Henderson et al., 1973) which gives rise to a characteristic

change in the u.v. absorption spectrum of the nucleic acids and their bases (Solymosy et al., 1972) and such a change in the wavelength of minimum u.v. absorption was observed for the DEP-SDS extracted RNA from chinese cabbage leaves.

Using the original DEP-SDS extraction method Lazar et al., (1969) observed that there was a preferential loss of the heavy ribosomal RNA components in mature tobacco leaf tissue. A similar reduced yield of heavy ribosomal RNA components from chinese cabbage leaves was observed when the RNA was extracted using the 'low-salt' buffer (see 3.43), this effect being abolished when the 'high-salt' environment was employed. Why this should have occurred was not clear though it may reflect differences in the RNA-protein interactions between the large and small ribosomal sub-units. In the work of Solymosy et al., (1968, 1970) and Lazar et al., (1969) using the DEP-SDS extraction for obtaining RNA from green leaves of pea, tobacco, bean and barley a greater yield of RNA was obtained than with phenol-based extraction methods. In this study an increased yield with the DEP-SDS method was only apparent because the number of washing steps following alcohol precipitation of the nucleic acids could be reduced (see 3.6), the yields being similar if a full purification procedure was used with both methods. The difference <sup>from</sup> ~~to~~ the previously published results may be because the detergent-phenol

extraction method employed in this study gave a more quantitative yield than the phenol methods (Itoh and Hirai, 1966; Ingle and Burns, 1968) used by Solymosy and co-workers.

In addition to the increased recovery possible the DEP-SDS method was also superior because it was possible to obtain and resolve intact 23s chloroplast rRNA by magnesium extraction and fractionation. With the phenol method electrophoretic profiles with high background absorbances were obtained if only magnesium was present during the extraction and fractionation of the RNA, addition of EDTA did improve the resolution possible but some dissociation of the 23s rRNA still occurred. Thus with the DEP-SDS method it was possible to obtain all the high molecular weight rRNA fractions intact and fully resolved without the complicating effect of the presence of a number of dissociated fragments. The reason for this may be related to the high capacity of DEP to denature proteins and precipitate them from solution (Rosen and Fedorcsak, 1966) thus effectively clarifying the RNA containing solution and counteracting any binding capacity that the inclusion of the magnesium produces. In addition the high inhibitory effect of DEP on ribonuclease activity may also have been contributory.

The number and size of the dissociation fragments of the labile 23s rRNA varies in different plant species. Leaver and Ingle (1971) have compared the cleavage products from a number

of plant species and have suggested that there are three particular regions or 'hot-spots' at which the 23s rRNA may break. The approximate molecular weights of the components that cleavage at these sites can yield are  $0.7 \times 10^6$  and  $0.4 \times 10^6$ , or  $0.9 \times 10^6$  and  $0.2 \times 10^6$ , or, if there is a median cleavage, two molecules of  $0.5 - 0.6 \times 10^6$  molecular weight. Cleavage at more than one site (Grierson, 1974) would produce additional fragments. In chinese cabbage the small size of the dissociation fractions observed indicated that possibly cleavage was occurring at more than one labile spot. For example the main breakdown fraction observed had a molecular weight of  $0.42 \times 10^6$ , thus a component of approximately  $0.7 \times 10^6$  would also have been expected. Such a fragment was not detected suggesting that a second labile site in the remainder of the molecule existed giving smaller dissociation fractions such as those described. It has been assumed that the dissociation fragments observed were derived from the labile chloroplast  $1.05 \times 10^6$  molecular weight fraction. This was because the appearance of these fractions was correlated with conditions in which the  $1.05 \times 10^6$  molecular weight peak on electrophoretograms was markedly reduced and also because these fractions were observed in preparations of rRNA from isolated chloroplasts (though slight cytoplasmic rRNA contamination occurred). Because of

of this evidence and by analogy to published reports for other plant species (Ingle, 1968; Ingle et al., 1970; Leaver and Ingle, 1971) it is believed that the fractions observed were of chloroplastic origin. The pattern of breakdown of the chloroplast  $1.05 \times 10^6$  molecular weight RNA was the same for both detergent-phenol and DEP-SDS extracted RNA, the higher ionic strength conditions of the DEP-SDS extraction buffer did not therefore confer a greater stability on this labile rRNA species.

It has already been noted that electrophoresis in magnesium-containing buffer largely maintains the integrity of the  $1.05 \times 10^6$  molecular weight fraction. It was also observed that merely resuspending the extracted RNA in a magnesium-containing buffer prior to electrophoresis was sufficient to reduce the degree of dissociation of the  $1.05 \times 10^6$  molecular weight RNA. Magnesium ions present during fractionation also influenced the electrophoretic mobility of the high molecular weight rRNA fractions. Relative to standard molecular weight markers (E.coli rRNA) anomalous molecular weight values were obtained from a plot of log (molecular weight) against migration following magnesium electrophoresis, a change in electrophoretic mobility was also apparent if the RNA was ~~just~~ resuspended in magnesium-containing buffer and subsequently electrophoresed in EDTA buffer. A differential

effect on relative migration was observed between the larger and smaller ribosomal RNA components, a phenomenon that has previously been reported by Loening (1969) who has suggested structural differences between the components as the explanation of the effect. The fact that just resuspension of the RNA sample in magnesium buffer could lead to an effect on electrophoretic mobility illustrates how important the conformation (or charge) of the RNA molecular is to electrophoretic migration and hence molecular weight determination, a point also made by Fisher and Dingman (1971). The effects of various ionic environments on electrophoretic mobility have shown that certain RNA species show anomalous migrations compared to their known molecular weight (Loening, 1969) similarly Bishop et al., (1967) have also noted that single stranded  $\phi$ x 174 and Q $\beta$  RNA did not migrate in accordance with other viral RNA species. The overall content of the bases guanine and cytosine in the RNA molecule appear to influence the degree of unfolding and hence the observed electrophoretic mobility (Loening, 1969) and because of results like these the suitability of electrophoretic estimation of nucleic acid molecular weights has been questioned (Boedtker, 1971; Reijnders et al., 1973; Spohr et al., 1976). The doubts about this method have arisen particularly because of the discrepancy between sedimentation values and

the electrophoretic mobilities of mitochondrial ribosomal RNA species which are highly susceptible to changes in ionic environment and temperature (Grivell et al., 1971; Groot et al., 1970; Forrester et al., 1970; Pring and Thornbury, 1975). The use of polyacrylamide gel electrophoresis for molecular weight estimation under conditions of RNA denaturation <sup>has</sup> been advocated, and various denaturing conditions have been proposed e.g. 8M urea at 60°C (Reijnders et al., 1973), use of formaldehyde (Boedtke, 1971), formamide at 45-55°C (Spohr et al., 1976). All these systems can give rise to a greater unfolding of RNA molecules but have the added disadvantage that 'hidden breaks' in the polynucleotide chains are revealed and fragmentation of the molecules can occur. Similar effects of a heat-shock treatment were described in this study (see 3.53). In all the studies the anomalous migration behaviour appears to occur in RNA species of unusual base composition or configuration, the behaviour of the major plant rRNA fractions were mutually consistent under varying conditions suggesting that the migration-molecular weight relationship can be used with these molecules (Hepburn and Ingle, 1976). Fisher and Dingman (1971) have suggested that the mobilities observed reflect molecular radii rather than molecular weight, and since it has been shown that RNA molecules of the same molecular weight can have different

molecular radii (Gesteland and Boedtger, 1964) it may be better to state that electrophoretically determined molecular weights are only apparent values, which can act as a means of identification, until confirmation by other techniques is obtained.

It has been noted that the heat-shock treatment used in this study (heating the RNA suspension at 60°C for 5 min.) gave rise to conformational changes in the molecules (resulting in a changed electrophoretic mobility) and also revealed 'hidden breaks'. It has been reported by Bostock et al., (1971) for Tetrahymena pyriformis, Ishikawa and Newburgh (1972) for the silk moth, Shine and Delgarno (1973) for a number of insect species and also in peas and soybean by Higo et al., (1971) and Yokoyama et al., (1973) respectively that the normally stable rRNA from the large sub-unit of 80s ribosomes in fact contains more than one polynucleotide fragment. One fragment of low molecular weight size (5.8s) has been characterised from a number of plant species and is found only in 80s ribosomes (Payne and Dyer, 1972), a similar fraction was also found in heat-treated chinese cabbage RNA by electrophoresis in 7.5% polyacrylamide gels. Higo et al., (1971), Yokoyama et al., (1973) and Grierson (1974) demonstrated a lability of plant 25s rRNA under denaturing conditions with products of 18s-16s being those formed in the greatest amount and molecular weights of 0.7 and 0.6 x 10<sup>6</sup> were assigned to these fragments in

Phaseolus aureus (Grierson, 1974). A decrease in the amount of the  $1.3 \times 10^6$  molecular weight rRNA was observed in heat treated chinese cabbage leaf RNA but breakdown fragments resulting from this dissociation could not be determined by the methods used, however if fractions of a similar size to those found by Grierson were produced their presence on the electrophoretograms would have been masked by other rRNA fractions. Interestingly the resuspension of the RNA in magnesium-containing buffer prior to heat treatment maintained the integrity of the  $1.3 \times 10^6$  rRNA presumably by bridging any breaks. Hepburn and Ingle (1976) examining rates of hydrolysis of 25s rRNA from pea epicotyls and artichoke tubers under conditions of thermal denaturation concluded that the 16s - 18s fractions observed were a consequence of hydrolysis of phosphodiester bonds. They therefore suggest that in at least some reports the presence of these fractions are the result of too severe denaturation conditions and that 25s rRNA does exist as a unique molecular species.

It has been demonstrated by Matus et al., (1964) and in this study that phenol extraction of nucleic acids from leaves of TYMV-infected chinese cabbage results in the aggregation of the host rRNA and TYMV-RNA. Various conditions under which hydrogen bonding is disrupted have proved unsuccessful in breaking this complex (see 3.1). Prevention of complex

formation would therefore seem a more fruitful means of enabling examination of host RNA in infected plants and this appeared to occur when using the DEP-SDS method of RNA extraction. Evidence that the viral RNA was not being released from the intact virion has been described (see 3.44) and included u.v. absorption studies and the response of the tissue to freezing prior to extraction. The structure of TYMV is stabilised primarily by protein-protein interactions with some contribution from pH-dependent protein-RNA linkages (Kaper, 1971). The relatively less important role of protein-RNA linkages confer upon the particle great stability to dissociation upon treatment with the ionic detergent SDS which cleaves these bonds (Boatman and Kaper, 1976). It is therefore suggested that the successful use of the DEP-SDS method of RNA extraction to obtain uncomplexed host RNA from TYMV infected plants was related to this resistance to dissociation by SDS of the virus. The extreme sensitivity of TYMV to phenol (Diener and Scheider, 1968) causing the release of TYMV-RNA and thus aggregation with the host RNA explains the failure of conventional phenol methods of RNA extraction to obtain uncomplexed host RNA from infected plants. It was observed that the DEP-SDS method of RNA extraction failed to prevent complexing when the 'low-salt' or EDTA-containing buffers were used (see 3.43). It is thought that the protein-protein

interactions in TYMV are primarily hydrophobic and high salt concentrations therefore stabilise capsid structure (Kaper, 1971), thus the lower ionic strength conditions in these two buffers may permit dissociation of the otherwise stable protein-protein bonds with the consequent release of TYMV-RNA and hence complex formation with the host RNA.

Evidence now suggests that the site of TYMV-RNA synthesis is in the peripheral vesicles observed in the chloroplasts of infected plants (Ralph and Clark, 1966; Ralph and Wojcik, 1966; Ralph et al., 1971; Lafleche and Bove, 1971). Now that it was possible to obtain uncomplexed host RNA from infected plants RNA was extracted from chloroplasts isolated from TYMV-infected leaves. Electrophoretic fractionation of the RNA from these chloroplasts did not reveal any components in addition to chloroplast rRNA and some contaminating cytoplasmic rRNA species. It was possible however that the vesicles containing the double stranded replicative form of TYMV-RNA may have sedimented with the membrane fraction following disruption of the chloroplast with Triton X-100. Using the method of Wolf and Casper (1971) to electrophorese viral particles from crude plant homogenates also failed to produce a TYMV fraction when applied to isolated chinese cabbage chloroplasts (Pearson, unpublished data).

Characterisation of chinese cabbage RNA extracted from TYMV-infected plants revealed that the RNA obtained had the same electrophoretic characteristics as RNA obtained from uninfected leaves. Qualitatively the infected leaf extracts showed a marked reduction in the proportion of chloroplast rRNA, an effect that might have been anticipated from the cytological abnormalities of the chloroplast induced by TYMV (Chalcroft and Matthews, 1966, 1967; Gerola et al., 1966; Milicic and Stefanac, 1967). It was probable that a substantial proportion of the observed chloroplast rRNA had been extracted from dark-green areas of the mosaic pattern of infection which appear cytologically normal. Further studies on host RNA metabolism in TYMV-infected plants need to be undertaken now that a method of differential extraction of host nucleic acid is available.

In previous work on chinese cabbage leaf polyribosomes (Clark et al., 1964) difficulties were experienced both in the preservation of polyribosomes (due to ribonuclease activity) and their fractionation on sucrose density gradients. Clark et al., (1964) managed a degree of stability by the use of polyvinyl sulphate as a nuclease inhibitor but this interfered with subsequent sucrose density gradient fractionation of the isolated polyribosomes. In this study polyribosomes were

obtained without the use of added nuclease inhibitors and they were satisfactorily fractionated by both sucrose density gradient centrifugation and polyacrylamide gel electrophoresis. By sucrose density gradient centrifugation polyribosomes up to at least the hexamer could be clearly resolved in extracts from leaves that were not fully expanded. Whilst the resolution that can be achieved by sucrose density gradient centrifugation can be very high (Noll, 1969) there are certain disadvantages in its use for the fractionation of polyribosomes. For example breakdown of the polyribosomes can occur under the conditions used for sucrose density gradient analysis (Thomas, 1972b) also the methods employed for monitoring of the gradients result in their destruction. As an alternative to sucrose density gradient centrifugation a method of polyacrylamide gel electrophoresis of polyribosomes has been described and characterised. It was found that for chinese cabbage leaf material fractionation of the polyribosomes by polyacrylamide gel electrophoresis gave a resolution of individual fractions at least comparable to the use of sucrose density gradients. With TYMV-infected leaves sucrose density gradient separation of polyribosome extracts were unsatisfactory because the virus sedimented into the polyribosome region and obscured some of the ribosome fractions. Polyacrylamide gel electrophoresis of similar

extracts also resulted in the virus particle migrating with the ribosomes and there was a partial peak overlap with the monoribosome fraction. The virus could be fully resolved from the monoribosomes by use of smaller pore gels. Evident from polyacrylamide gel electrophoresis but not from sucrose density gradients was an additional virus-associated fraction tentatively identified as viral 'top component'. This fraction also migrated with the ribosome peaks and could interfere with the resolution of the polyribosomes when the virus was present in a proportionally high concentration in the extract.

A number of factors were found to be of importance in obtaining the greatest resolution by the electrophoretic method of polyribosome fractionation. A relatively high current for a short period of time for electrophoresis gave optimum resolution whilst increasing the duration of electrophoresis either at the same or reduced voltage gradients resulted in a degree of band spreading, a result contrary to the findings of Ledoigt et al., (1975) who found that the resolving power increased as a function of time. The cause of the peak spreading was not obvious but may have been related to the different conformations of the polyribosomes either inherent or as a result of the influence of magnesium ions. In this respect it was necessary to circulate the buffer between the tank reservoirs during electrophoresis to

prevent depletion of magnesium by accretion at the cathode. This possible magnesium depletion did not appear to be significant during short electrophoretic runs but the amount of accretion that was seen to occur during long electrophoresis runs suggested that it was significant under these latter conditions. Dahlberg et al., (1969) also found it necessary to recirculate their electrophoresis buffer through both reservoirs. Treatment of the polyribosome pellet prior to fractionation was also important for the final resolution on fractionation. The 105,000g. pellets, either freshly prepared or that had been stored at  $-20^{\circ}\text{C}$ , were resuspended in buffer and fractionated immediately to obtain maximum preservation of the polyribosomes. Subsequent freezing and thawing of resuspended ribosomes lead to a degradation of the polyribosomes to ribosomal sub-units indicative of mechanical rather than enzymic degradation. The resuspended ribosomes were layered directly onto the surface of the polyacrylamide gel, it was not found necessary to gel the polyribosome sample in place to prevent possible streaking effects (Dahlberg et al., 1969).

In addition to analytical fractionation other information could also be obtained from electrophoretically separated polyribosomes. It has been demonstrated that, at least for native plant rRNA species, there is a linear relationship between the electrophoretic mobility of the molecule and its

sedimentation value (Loening and Ingle, 1967) or log (molecular weight) (Loening, 1968c). Since ribosomes like RNA have a substantial negative charge and a constant charge to mass ratio (Peterman, 1964) the possibility of similar relationships were examined. Under the conditions of electrophoresis used a linear relationship between migration and the sedimentation values for chinese cabbage polyribosomes published by Clark et al., (1964) was demonstrated which enabled sedimentation values of 215s, 221s and 229s to be predicted for the hexamer, septamer and octamer polyribosomes of chinese cabbage. Ledoigt et al., (1975) have also found that there is a linear relationship between sedimentation value and electrophoretic migration for the ribosomes of Tetrahymena pyriformis and the monoribosomes and sub-units of Euglena gracilis, rat and E.coli. In this study an inverse relationship between migration and log (molecular weight) was also demonstrated thus confirming for a primarily 80s ribosome population what Dahlberg et al., (1969) have shown for 70s E.coli polyribosomes. In these analyses no evidence for a specific retardation of the larger polymers was observed on migration through the gels, also the relative migrations of the different polymers appeared to remain constant with time throughout the electrophoretic run (see 3.73.4). Ledoigt et al., (1975) however did observe that in low magnesium

concentrations (1 of 2 mM) migration of the larger polymers was slower than was expected from the measured rates of migration of the smaller polymers. That is the migration of the polymers was not proportional to the number of ribosomes they contained. This led them to the conclusion that in the conditions they used the polyribosomes were present as compact clusters rather than as chains, a conclusion confirmed by electronmicroscopy. Induced conformational effects giving a change in migration of the polyribosomes in the gels were observed by Dahlberg et al., (1973) following treatment of E.coli polyribosomes with antibiotics. In view of these reports a more extensive study of the effects of various conditions on polyribosome conformation and hence electrophoretic mobility need to be carried out before the use of the electrophoretic method to measure size can be advocated. In both of these migration-size relationships the migration of the viral components did not correspond with their known sedimentation values or molecular weights. A different mass to charge ratio for these components as compared to ribosomes could be the reason for this.

To obtain ribosomal sub-units the 105,000g. ribosome pellet was resuspended in a buffer containing the chelating agent tetrasodium pyrophosphate which removes magnesium ions and causes the ribosomes to dissociate into their constituent

sub-units. For successful fractionation of the sub-units by electrophoresis in 3.0% acrylamide gels it was necessary to have low concentrations of magnesium (1.0 mM) in the gel and electrophoresis buffers. Use of the resuspension buffer as the electrophoresis buffer resulted in a very low mobility of the sub-units in the 3.0% gels or under slightly different conditions loss of integrity of the sub-units. This loss of integrity was also apparent if an EDTA-containing buffer was used instead of the pyrophosphate buffer. At these low magnesium ion concentrations recombination of sub-units was not observable though this did occur at higher magnesium levels. The reason for this susceptibility of the sub-units to chelating agents was not determined, but the possibility that it was due to structural changes in the released sub-units was suggested by the dissociation studies of Lamfrom and Glowacki, (1962) and Tashiro and Siekevitz (1965) using chelating agents, also there have been a number of reports of ribonuclease activity being associated with ribosome preparations from plants (Hsaio, 1968; Wyen and Farkas, 1971; Gagnon and de Lamirande, 1972; Dyer and Payne, 1974). In at least some species the activity of this ribosome associated nuclease activity is latent and is activated under conditions in which ribosome structure is disrupted e.g. by removal of divalent cations by chelating agents (Wyen and Farkas, 1971;

Gagnon and de Lamirande, 1972; Dyer and Payne, 1974).

It is also possible therefore that latent nuclease activity associated with the ribosome preparations may have been activated by the exposure to chelating agents which in turn lead to a degradation of the sub-units, however the ribosomal proteins might be expected to hold the sub-unit conformation together even if the RNA was degraded (Dahlberg et al., 1969). The sub-unit instability thus requires further study before the cause can be elucidated.

Electrophoresis of sub-units from TYMV-infected plants in either magnesium-containing or EDTA-containing buffer also had a differential effect on the relative migrations of the sub-units and the virus. With magnesium electrophoresis the relative migrations of the sub-units and virus were similar to those seen on polyribosome fractionation with the virus having a slower electrophoretic mobility than the sub-unit fractions. With EDTA electrophoresis, however, the virus had an intermediate mobility between that of the large and small sub-units. Sub-unit instability in EDTA was also observed with the infected-leaf extracts and because of the reduced levels of chloroplastic ribosomes in TYMV-infected plants this would suggest that this instability is associated with the 40s cytoplasmic sub-unit (though similar effects in the chloroplast ribosomal sub-units are not eliminated).

It is possible that EDTA-induced structural changes may result in the change in relative mobilities between the virus and the sub-units though a differential change in the effective charge on the particles by removal of magnesium ions might also produce the observed response.

The use of polyacrylamide gel electrophoresis to fractionate procaryotic ribosomal sub-units lead to the discovery that there was a heterogeneity of the sub-units at least with regard to electrophoretic behaviour (Dahlberg et al., 1969; Talens et al., 1970, 1973). This heterogeneity was not observed with the sub-units of chinese cabbage leaf ribosomes, which consisted primarily of 80s cytoplasmic ribosomes, a result consistent with the work of Ledoigt et al., (1975) who found that sub-unit heterogeneity was confined to procaryotic 70s type ribosomes.

Another effect of magnesium that was noted was the susceptibility of polyribosomes to dissociate into sub-units at low magnesium ion concentrations. Despite being prepared by the same method different batches of polyribosome extracts from chinese cabbage leaves showed a variable response when electrophoresed in buffers containing 1 or 2 mM magnesium. This was particularly evident with the 1 mM magnesium buffer in which quite good resolution of polyribosome peaks was observed in some instances whilst in other preparations

complete dissociation into ribosomal sub-units occurred. In 2 mM magnesium buffer variations were still observed but not to the extreme degree that was apparent with 1 mM magnesium buffer. Electrophoresis of the same samples by the standard method in which a more stable environment for the ribosomes was provided did not result in this dissociation phenomenon. This observation together with the fact that both the 1 mM and 2 mM magnesium buffer could support intact polyribosome profiles suggested that there was a variation in the response of the ribosomes rather than the effect being an artefact of the fractionation. Though the evidence was only circumstantial it appeared that the response of the ribosomes was possibly related to the observed change in the seasonal growth habit of chinese cabbage, thus some change in the physiology of the plants were also reflected in the stability of the ribosomes. What form this change takes is not readily apparent since the 'susceptible' ribosomes could be fractionated quite satisfactorily by the standard electrophoretic method. The dissociation to sub-units instead of just monoribosomes would indicate that ribonuclease activity was probably not the main cause but the effect might reflect a change in the binding of divalent cations such as magnesium or calcium, a reduced level of these elements bound to the ribosomes if it was not supplemented by the electrophoresis buffers could result in

dissociation. However since the buffer in which the ribosomes were extracted contained 10 mM magnesium possibly it was due to a failure to bind magnesium. These comments can only be regarded as speculative until a more rigorous investigation of the phenomenon is undertaken.

Payne and Loening (1970) have characterised specific rRNA breakdown fractions that occur following RNA extraction of isolated pea root microsomes. They suggest that tissue homogenisation damages some of the ribosomes causing certain regions to become exposed to nuclease attack, this results in a characteristic pattern of breakdown fragments not observed after normal RNA extraction from the tissue. In this work the RNA was extracted from total leaf ribosome extracts and on electrophoresis in EDTA the chloroplast 23s rRNA dissociated into a number of smaller fragments. No breakdown fragments specific to RNA extraction from isolated ribosomes were observed, however these chloroplast dissociation fragments may have masked their appearance, though even after magnesium electrophoresis additional products were not observed. In these circumstances the magnesium ions may bridge 'hidden breaks' caused by ribosome extraction in the same way that the  $1.05 \times 10^6$  molecular weight chloroplast fraction is maintained intact.

Dahlberg et al., (1969) first demonstrated that poly-ribosomes of E.coli consisting of up to eight ribosomes linked together by mRNA could penetrate and be resolved on composite agarose-acrylamide gels since then the use of poly-acrylamide gel electrophoresis for ribosome fractionation has been almost solely confined to the study of monoribosomes and ribosomal sub-units, primarily from procaryotes. The use of an electrophoretic method of polyribosome fractionation has certain advantages over other methods, because the components are separated on the basis of charge possible dissociation due to hydrostatic pressures generated by sucrose-density gradient centrifugation (Infante and Baierlein, 1971; Subramanian and Davis, 1971) is avoided. The number of different samples that can be fractionated at one time can be much greater than that normally possible by centrifugation methods and because of the sharpness of the bands that can be achieved and the sensitive monitoring facilities available very small quantities of material can be satisfactorily fractionated (e.g. figure 3.61). During the ultra-violet monitoring the gels are maintained intact and can be subsequently specifically stained or sliced for radioactive incorporation studies. Also with regard to radioactive work the electrophoretic process may serve to remove non-incorporated label associated with the extracted ribosomes.

There are indications that under defined conditions an electrophoretic method may provide a rapid and simple technique for the measurement of sedimentation values or molecular weights of ribosomes and ribosomal aggregates. Finally the fixation and storage of the gel is also possible. A limitation of electrophoresis is that it is primarily an analytical method, preparative scale separations being better accommodated by conventional centrifugation procedures. The properties of ribosomes which lead to an electrophoretic separation are different <sup>from</sup> ~~to~~ those involved in sedimentation separations, therefore different information about ribosomes might be expected to be obtained following an electrophoretic separation, instances where this has been demonstrated have already been detailed (see 3.1).

It has been shown that electrophoresis in polyacrylamide gels can be applied to chinese cabbage leaf polyribosomes with a degree of resolution at least comparable with that obtained using sucrose density gradient centrifugation. The use of this method in the study of ribosome changes occurring during leaf ageing of both uninfected and TYMV-infected chinese cabbage leaves is described below where it enabled the polyribosome patterns of individual aged leaves to be analysed in which the ribosome content was extremely low.

#### 4. AGEING STUDIES

##### 4.1 Introduction

In plants a number of different forms of leaf ageing or senescence can occur and these have been mentioned in the general introduction (1.) The leaves of chinese cabbage exhibit a sequential pattern of ageing though a precise relationship with regard to the number of leaves that a plant could support as described by Woolhouse (1967) for Perilla frutescens does not occur. Grown under the described conditions the chinese cabbage plants used in this study showed a marked seasonal variation in growth with plants grown in the winter producing fewer leaves and growing slower than similar plants cultivated in the summer. Chinese cabbage plants infected with turnip yellow mosaic virus also exhibited this seasonal variation and a further retardation of growth occurred as a consequence of the virus infection. To examine the changes in various cellular components that occurred during leaf ageing the approach used was to take individual plants and analyse each leaf along the phyllotaxy, in this way a spectrum of leaf ages, from young through mature to old, was obtained. Difficulties in precisely ageing individual leaves were therefore avoided and plants grown at different seasons of the year could then be compared in that the same continuum of leaf physiological states was examined though the actual number of samples used along that

continuum would be dictated by the leaf number.

The use of leaf discs or detached leaves in studies of senescence is common since it is often assumed that the changes observed are similar to those occurring in attached leaves except greatly accelerated (e.g. Biswal and Mohanty, 1976). The losses of components such as chlorophyll, protein and nucleic acids, and structural symptoms of senescence are usually extensive after only a few days detachment of leaf discs taken from most plant species (e.g. Back and Richmond, 1971; Fletcher and Osborne, 1966; Beevers, 1966; Goldthwaite and Laetsch, 1967, 1968; Dennis et al., 1967; Phillips et al., 1969; Atkin and Srivastava, 1969) and these changes are generally more extensive if the discs are incubated in darkness rather than illuminated (Dale, 1967; Goldthwaite and Laetsch, 1967; Lewington and Simon, 1969; Simon, 1967). A number of reports have appeared in which differences between the patterns of senescence of attached leaves and leaf discs have been observed (Lewington and Simon, 1969; Lewington et al., 1967; Muller and Leopold, 1966; Simon, 1967; Wollgiehn, 1967; Krul, 1974; Spencer and Titus, 1973) and because of these differences <sup>from</sup> ~~to~~ normal attached leaf senescence it has been suggested that the senescence of detached leaves or discs can be considered as a separate form of senescence (Lewington and Simon, 1969; Simon, 1967). Differences might be expected since detached leaves or discs are not connected to the

vascular system of the whole plant and consequently are unable to export or import metabolites (Muller and Leopold, 1966). With discs an additional factor that may be induced is a 'wound response' affecting metabolism arising as a result of excision, this being particularly significant at the edges of the discs. The importance of this effect, especially with regard to the uptake of applied radioactive precursors, has been indicated by Hardwick and Woolhouse (1968) and Pratt and Matthews (1971). Whilst this kind of evidence illustrates that the metabolism of leaf discs may be different to that of the intact plant their use does have certain advantages over using whole plants, for example different conditions can be chosen for their incubation and these conditions can be closely controlled. Also for investigating the possible effects of applied substances, particularly growth hormones, the use of discs or detached leaves in which the target tissue can be defined often makes them the material of choice compared to the intact plant.

Very few studies have been published in which the qualitative RNA changes occurring in the intact plant during ageing have been compared with similar changes in excised leaf discs. For this reason an examination of the RNA changes occurring in leaf discs of chinese cabbage incubated under different conditions was undertaken and the patterns of change that emerged were compared to those observed in the

intact plant.

Each of the main classes of plant growth regulator substances are able to affect senescence in at least some species. Since Richmond and Lang (1957) showed that treatment of detached leaves of Xanthium pensylvanicum with kinetin delayed senescence similar results have been obtained by treating detached leaves of Prunus serrulata with auxin (Osborne and Hallaway, 1960, 1964) and in Taraxecum officinale gibberellin has the same effect (Fletcher and Osborne, 1965), whilst the hormone abscisic acid and also ethylene are both able to stimulate leaf senescence (El-Antably et al., 1967; Burg, 1968). Of the hormones that are capable of delaying senescence it is the cytokinins which appear to have the greatest retarding effect in the widest range of plant species, whereas the other growth substances may have a variable effect or no effect at all, indeed in certain circumstances auxin has been shown to actually induce or accelerate senescence (Osborne, 1967). In conjunction with the use of leaf discs to look at ageing changes the effect of applying cytokinin to the incubated discs was also examined. The application of cytokinin had possibly an additional interest in view of the report of Berridge et al., (1970) who found that cytokinin bound to purified chinese cabbage ribosomes and that there was a positive correlation between the extent of the binding and the biological activity

of the cytokinins or cytokinin analogues.

The effect of TYMV-infection on chinese cabbage leaves is to produce a mosaic pattern of infection in the leaves containing both infected and uninfected cells (Chalcroft and Matthews, 1966). This mosaic pattern can vary markedly between different leaves with the result that in the sampling of all leaves of a single infected plant a uniform tissue type is not being examined. Because of this sampling problem the use of discs offered an experimental situation in which a more uniformly affected type of tissue could be examined. Therefore characterisation of the changes occurring in incubated leaf discs taken from uninfected plants was necessary so as to provide suitable control material with which to compare the metabolism of discs excised from TYMV-infected leaves. In this way the use of discs may provide a useful means of demonstrating differences in host metabolism between TYMV-infected and uninfected leaf material which are not so readily recognised in the intact plant because of the effects of the mosaic pattern of infection.

There have been few reports of the effects of growth substances applied to virus-infected plant tissues; Cheo (1971) has examined the virus-replicating capacity of tobacco mosaic virus in cotton when kinetin, IAA or 2,4-D was applied to the plants, Russell and Kimmins (1971) assayed the levels of endogenous gibberellins and looked at the effects of

applied gibberellin on barley plants infected with barley yellow dwarf virus, the effect on tobacco mosaic virus multiplication of abscisic acid application to ageing tobacco leaves has been examined by Balazs et al., (1973) and Pearson and Thomas (unpublished data) have investigated the ultrastructural features of abscisic acid treated TYMV-infected chinese cabbage leaves. TYMV infection leads to the disruption of the structural integrity of the chloroplasts (Chalcroft and Matthews, 1966), plant leaf senescence also results in the structural degradation of the cells (Butler and Simon, 1971) with the chloroplast being particularly susceptible (Shaw and Manocha, 1965; Barton, 1966). In the retardation of leaf senescence the application of cytokinins appears to be correlated with a maintenance of chloroplast integrity (Dennis et al., 1967) and because of this some effect of cytokinin application might be expected in ageing TYMV-infected leaf material. This possibility of a further insight into virus-host interactions was another reason why cytokinin application to leaf discs was of potential interest.

#### 4.2 Sequential leaf ageing - Quantitative changes

The changes that occurred in certain cellular components during the ageing of chinese cabbage leaves were examined in single plants. The leaves on each plant used were numbered successively along the spiral phyllotaxy from the youngest leaf that could be practically analysed to the eldest. Each leaf in turn was then removed, weighed and samples of known weight taken and extracted for total DNA, RNA and chlorophyll content by the methods of Osborne (1962) as described in 2.3. Duplicate plants of similar size which were average representatives of the plants present in the population were used when the first leaf produced by the plant was yellow and senescent. Thus leaves showing a spectrum of physiological states from young rapidly growing leaves, through mature leaves to old leaves were examined.

The pattern of fresh weight changes observed in the laminae of the leaves is illustrated in figure 4.1. From the youngest leaf and continuing for seven or eight leaves there was an increase in fresh weight which was followed by a decline in size in subsequent leaves. It should be noted, however that this decline in fresh weight was not solely correlated with the leaves being older, as observed by Clark et al., (1964), because the first few leaves produced by these chinese cabbage plants do not achieve the large size of subsequently formed leaves. This point is illustrated in figure 4.2 where leaf

Figure 4.1

The pattern of change in fresh weight of the leaf laminae of successive leaves along the phyllotaxy.

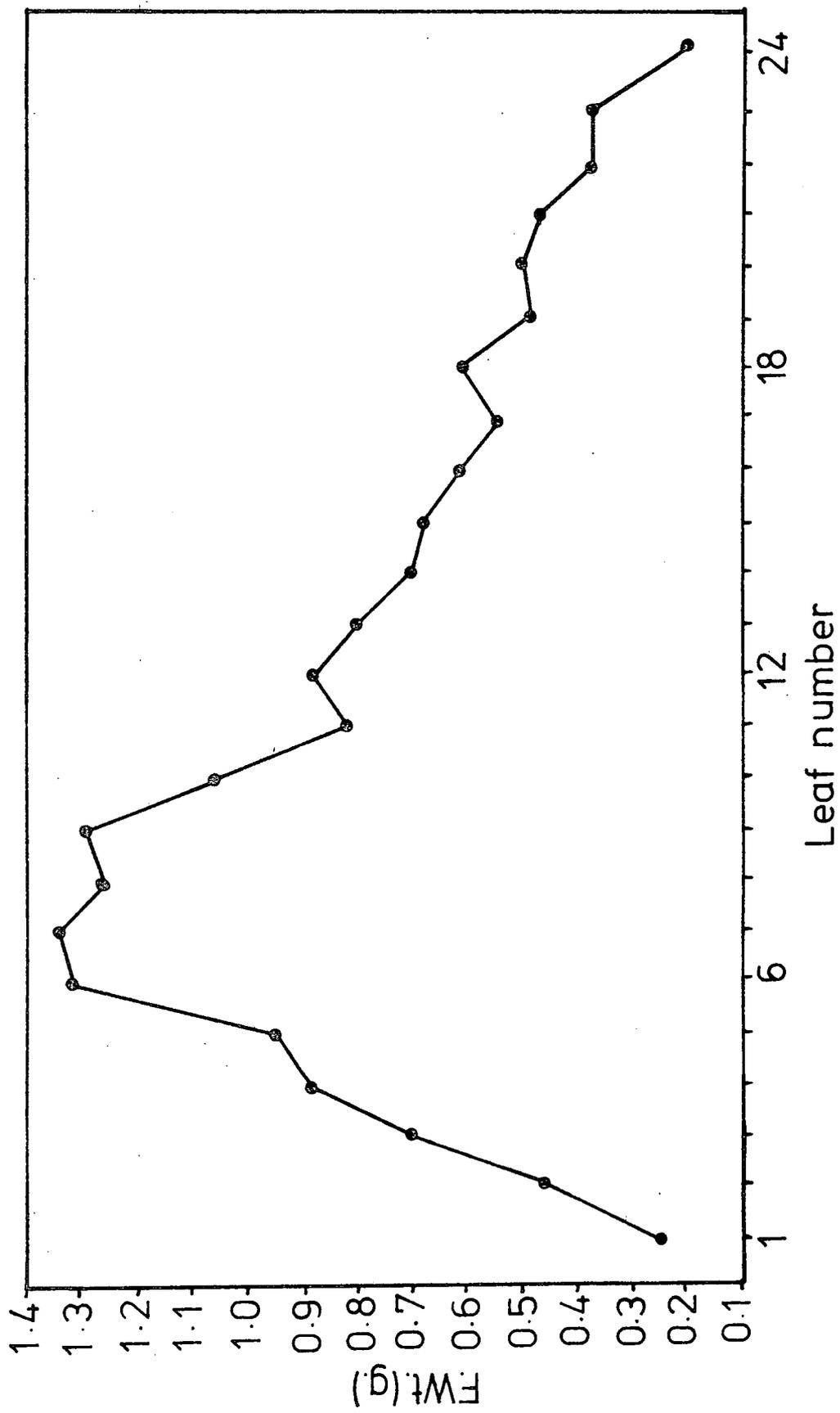
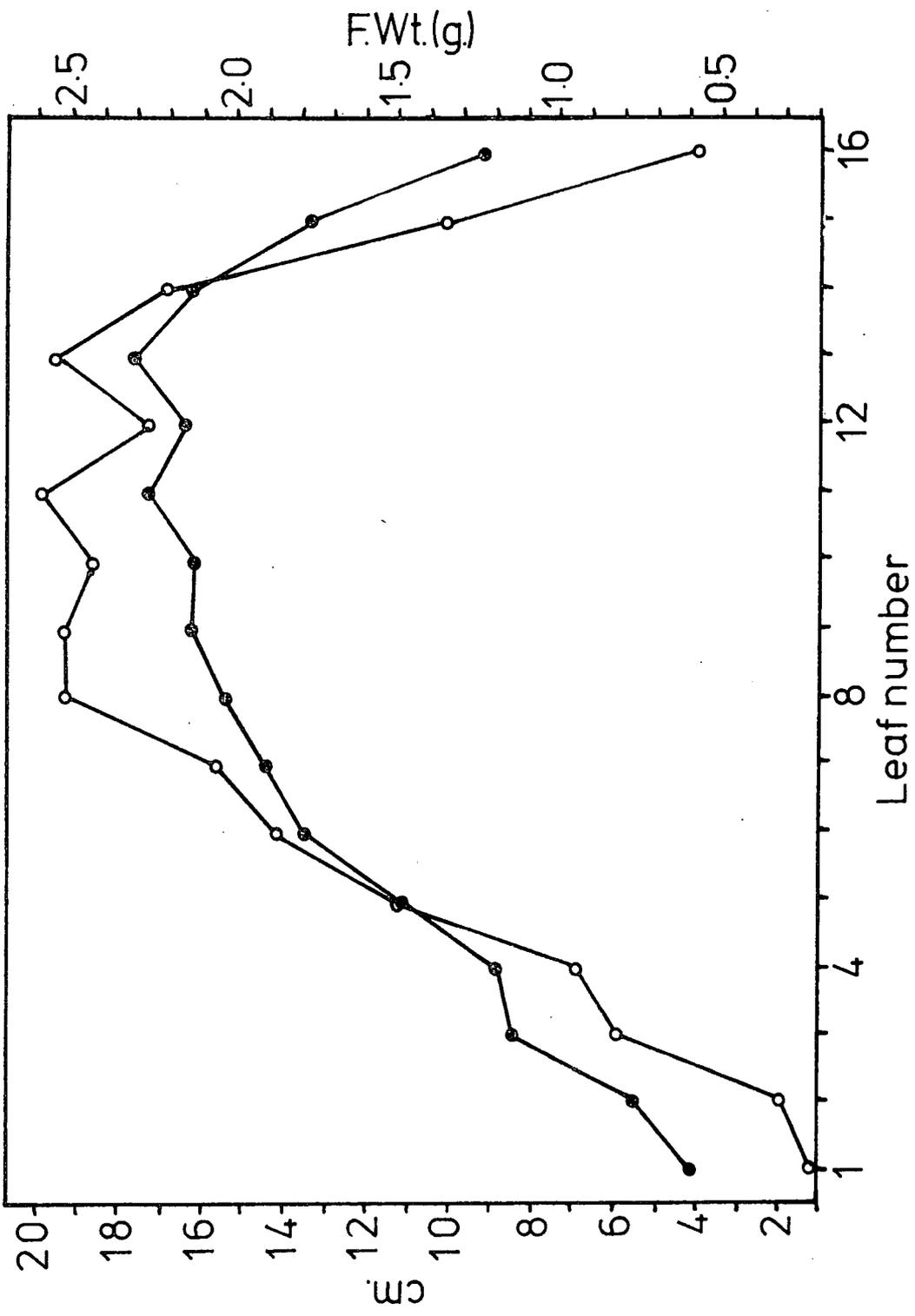


Figure 4.2

The pattern of change in leaf length and fresh weight along the phyllotaxy.

- leaf length (cm)
- fresh weight (g)



length has been used as an index of growth. The average values for the daily growth (in terms of leaf length) of individual leaves from a population of young plants showed that individual leaves exhibited a sigmoid type of growth curve (figure 4.3).

#### 4.21 DNA content

Analysis of the total DNA content in each leaf of a plant (figure 4.4) showed a similar trend to the fresh weight changes, there was an initial rise in the amount of DNA extracted per leaf reaching a maximum in the seventh or eighth leaf (as numbered, see above), there then followed a steady decline in successively older leaves. If these DNA yields were expressed in terms of the amount of DNA extracted per gram fresh weight of leaf lamina then the pattern of change illustrated in figure 4.5 was observed. There was a sharp decline in DNA concentration in the first five or six leaves which then markedly levelled off. Over the first six leaves there was a drop in yield of DNA per gram fresh weight of lamina of approximately 60% as compared to the yield obtained in the youngest leaf sampled, whilst in the remaining eighteen leaves there was only an additional drop of 5.0% in the DNA yield per gram fresh weight. An increase in leaf size by rapid cell extension growth in the younger leaves of the plant (Maksymowych, 1973) could explain this decrease in DNA concentration even though the absolute level of DNA in

Figure 4.3

The average change in length of successive leaves produced on a plant following germination.

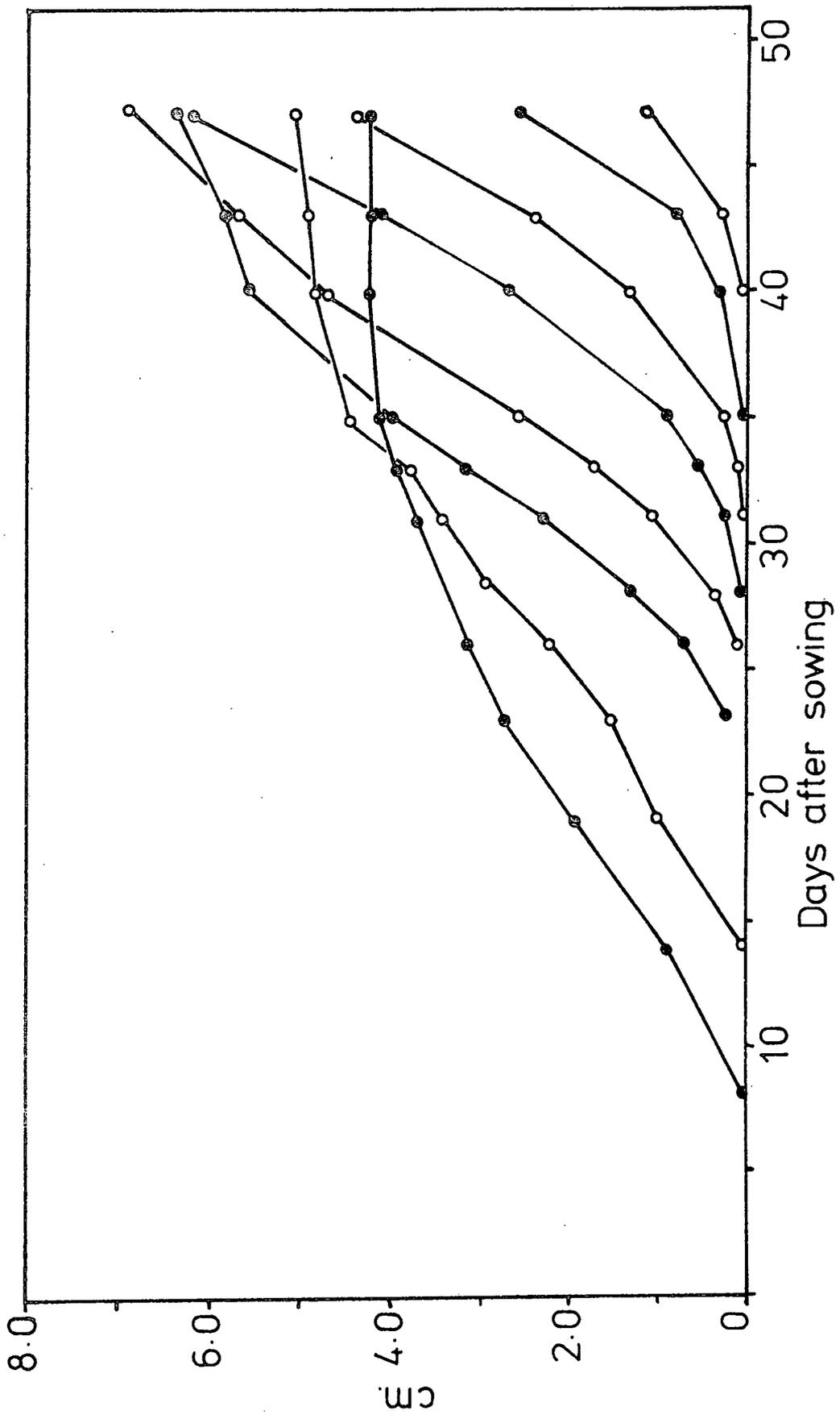


Figure 4.4

The change in total DNA content per leaf along the phyllotaxy.

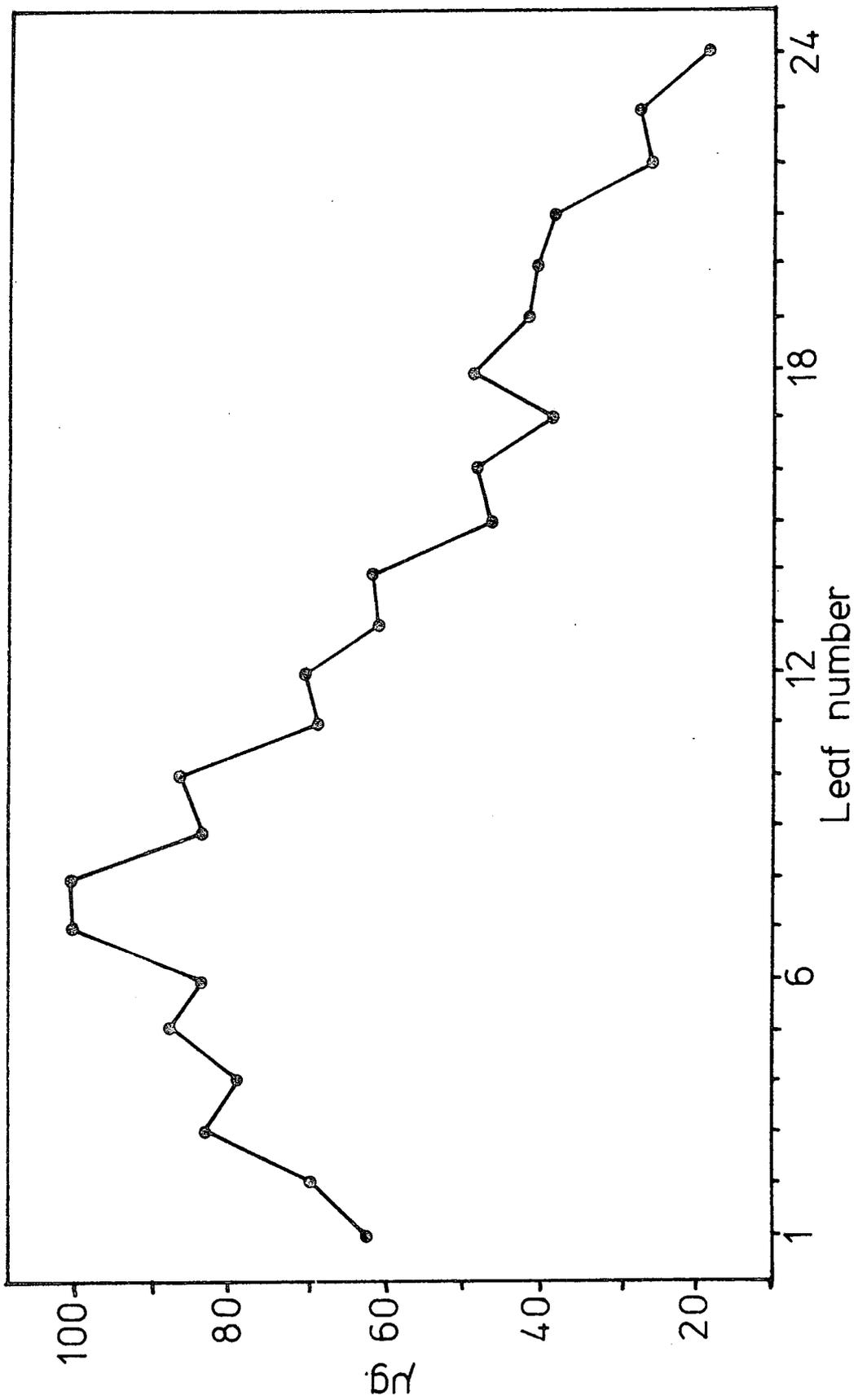
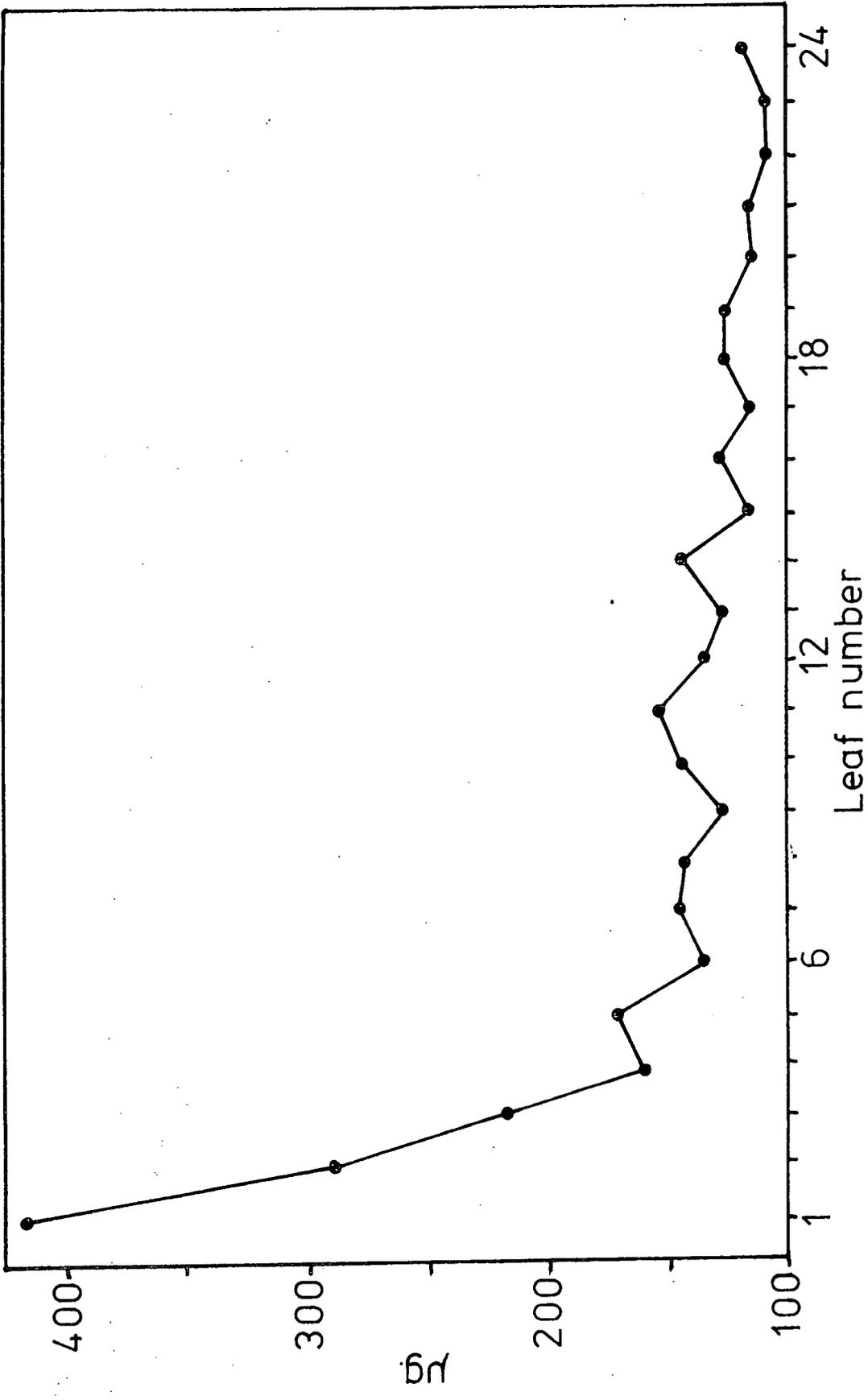


Figure 4.5

The change in DNA content per gram fresh weight of leaf lamina along the phyllotaxy.



the successive leaves was increasing (figure 4.4). Also of interest was the fact that in the oldest leaves there was no marked decline in the concentration of DNA in terms of the yield per gram fresh weight. A concomitant decline in the fresh weight of individual leaves, as suggested by Clark et al., (1964), may have served to offset a fall in the amount of DNA thus resulting in the apparent maintenance of DNA levels in the leaves.

#### 4.22 RNA content

The total yield of RNA per leaf in contrast to the corresponding data for DNA showed a steady decline in amount from the youngest to the oldest leaf (figure 4.6) although the irregularity of the graph suggested that there may have been a slight increase in the youngest two or three leaves. Thus the total RNA content of the leaves began to decline before the maximum size of the leaves was reached. The rate of decline observed in the older leaves was probably exaggerated because the smaller physical size attained by these leaves would result in a lower RNA yield than the larger, younger leaves in addition to any age-induced changes.

Expression of the RNA content on a yield of RNA per gram fresh weight of leaf lamina basis showed a similar trend to that observed for DNA (figure 4.7), there was an initial phase of rapid decline in RNA concentration in the youngest five or six leaves in which there was a drop of approximately 75% from

Figure 4.6

The change in total RNA content per leaf along the phyllotaxy.

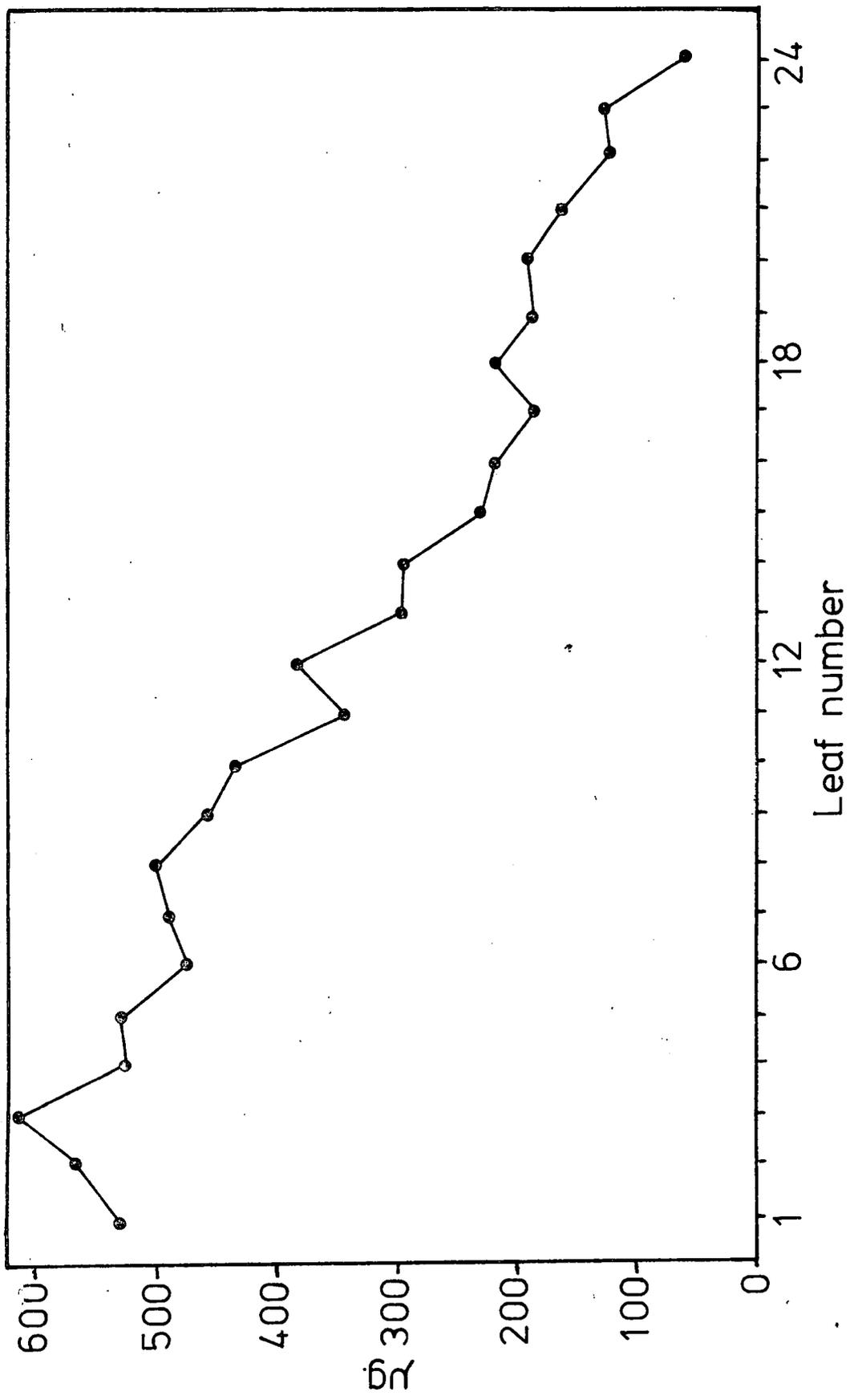
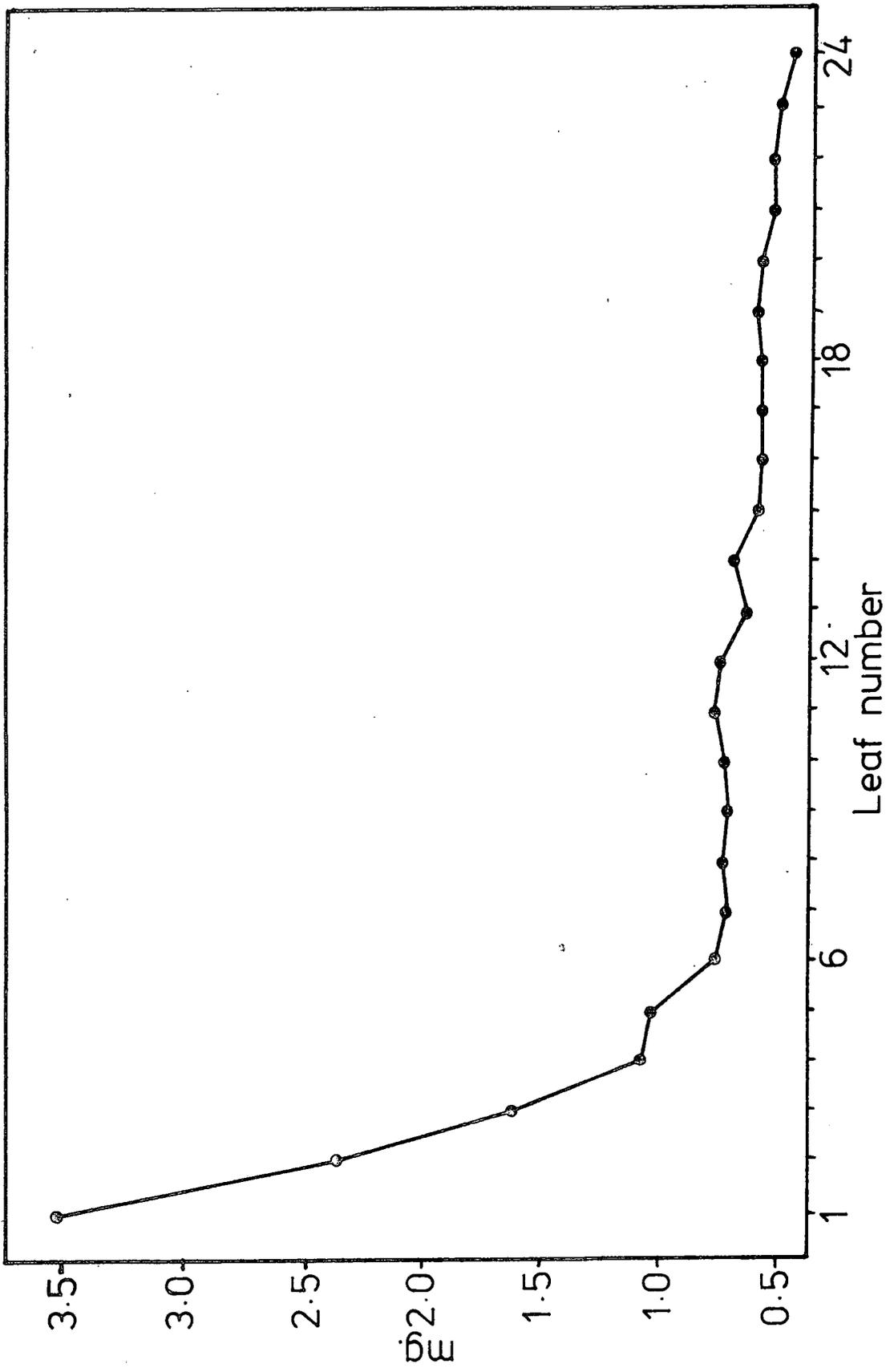


Figure 4.7

The change in RNA content per gram fresh weight of leaf lamina along the phyllotaxy.



the concentration observed in the youngest leaf. In the remaining leaves the total drop was only an additional 12% with a suggestion in the graph of a slight acceleration of RNA loss relative to fresh weight in the oldest few leaves. Decreases in the fresh weight of individual leaves may have led to the apparent maintenance of RNA levels in the older leaves.

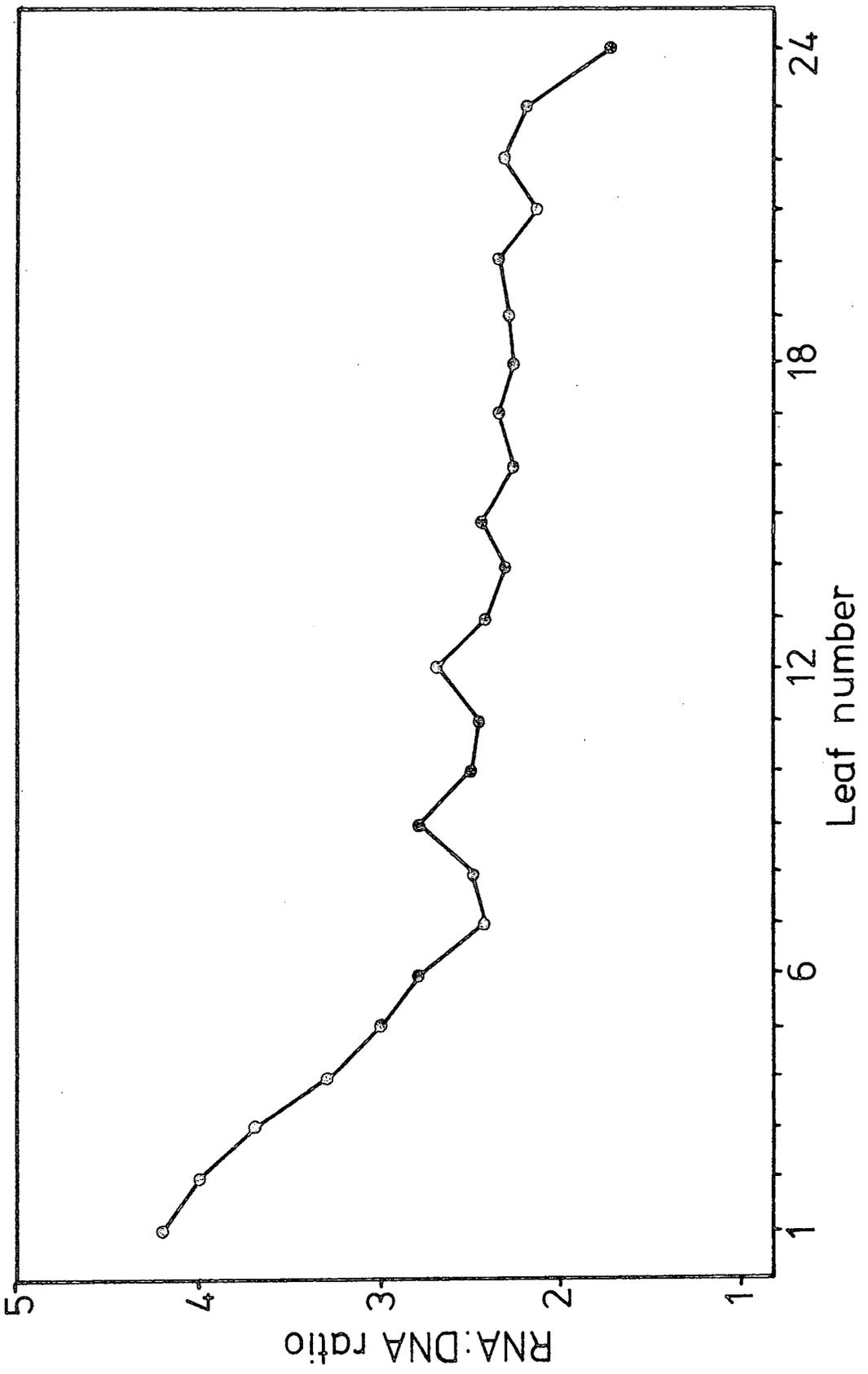
The possible differences in the relative declines of RNA and DNA during ageing suggested by the above data was examined by consideration of the ratio of RNA to DNA in each leaf. In the youngest leaves the ratio RNA content : DNA content had a value of 4.0 - 4.5 which fell to 2.0 - 2.5 in the seventh/eighth leaves indicating a relatively faster decrease in RNA as compared to DNA content. This proportion of the two components was then maintained until in the oldest leaves there was a further decrease in the value of the RNA : DNA ratio (figure 4.8). Therefore there was a relative decline in RNA during the period of leaf expansion which flattened off at a point corresponding to maximum leaf growth in the plants used, then in the oldest leaves there was another relatively faster decline in RNA content.

#### 4.23 Chlorophyll content

With regard to the chlorophyll content of the leaves, on a yield per leaf basis a pattern of change similar to that of leaf lamina fresh weight was observed. There was an increase

Figure 4.8.

The change in the RNA:DNA ratio of the leaves along the phyllotaxy.



in total chlorophyll yield with age reaching a maximum at a point just after the maximum leaf fresh weight was achieved following by a steady decline in chlorophyll yield (figure 4.9). The chlorophyll content expressed as a yield per gram fresh weight of lamina exhibited a different trend to that seen with both DNA and RNA and there was a steady rise in chlorophyll concentration with age which continued beyond the point at which maximum leaf size was achieved (figure 4.10), the chlorophyll concentration was then maintained until in the oldest leaves there was observed a drop in chlorophyll content of up to 40% of the average level found in mature leaves. The amount of chlorophyll extracted was also considered relative to the amount of DNA extracted from the same leaf thus giving an indication of the chlorophyll content per cell. On this basis the quantity of chlorophyll per unit amount of DNA was found to rise steeply in the youngest few leaves, a rise at a lower rate was then evident until in the oldest four or five leaves a slight decline occurred (figure 4.11). Thus chlorophyll levels seemed to be maintained above that of any DNA changes until late in the leaf life cycle at which stage there was a comparatively accelerated loss.

As has been previously stated the first leaves produced by the plant and hence the oldest leaves investigated in the

Figure 4.9

The change in total chlorophyll content per leaf along the phyllotaxy.

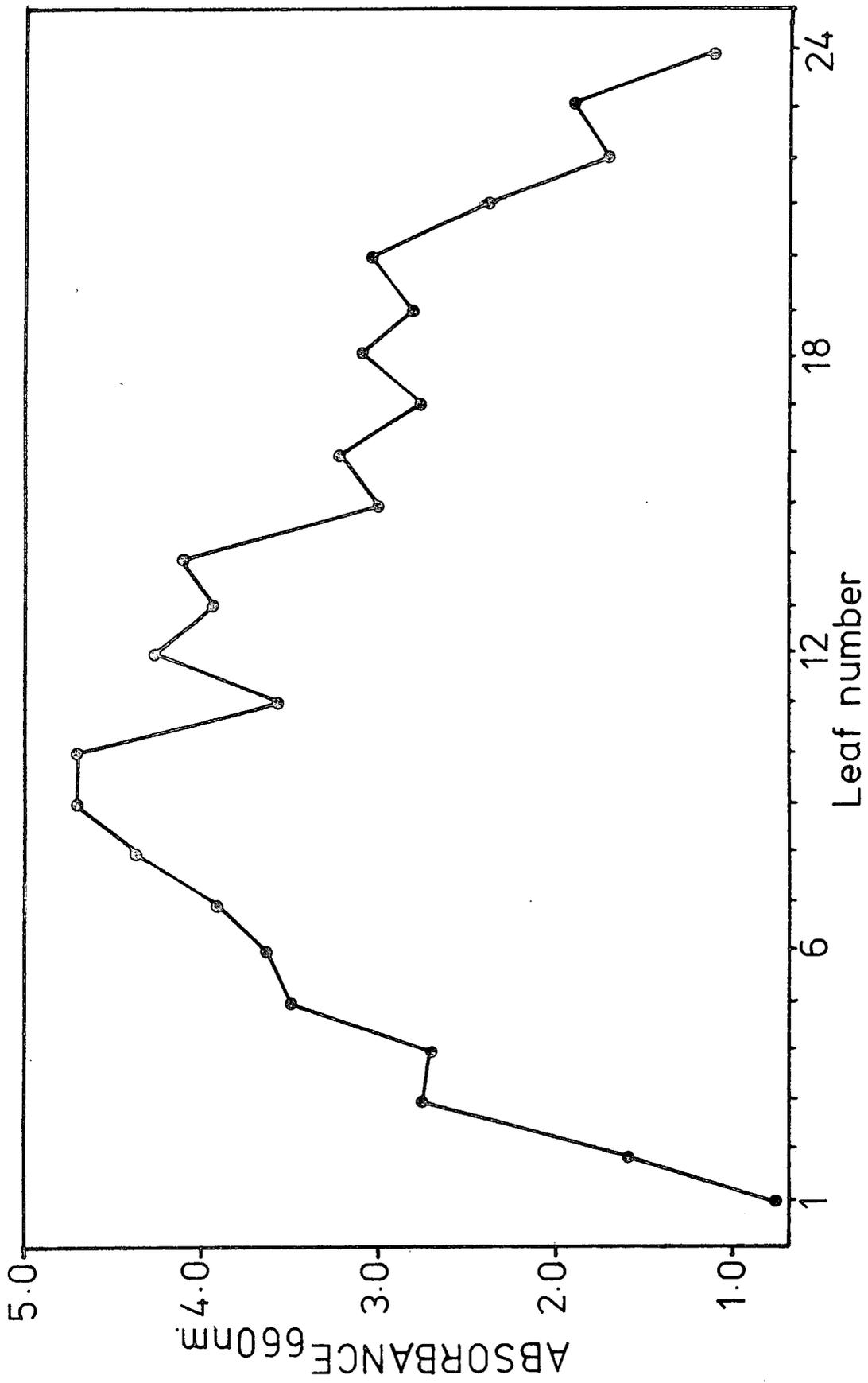


Figure 4.10

The change in the chlorophyll content per gram fresh weight of leaf lamina along the phyllotaxy.

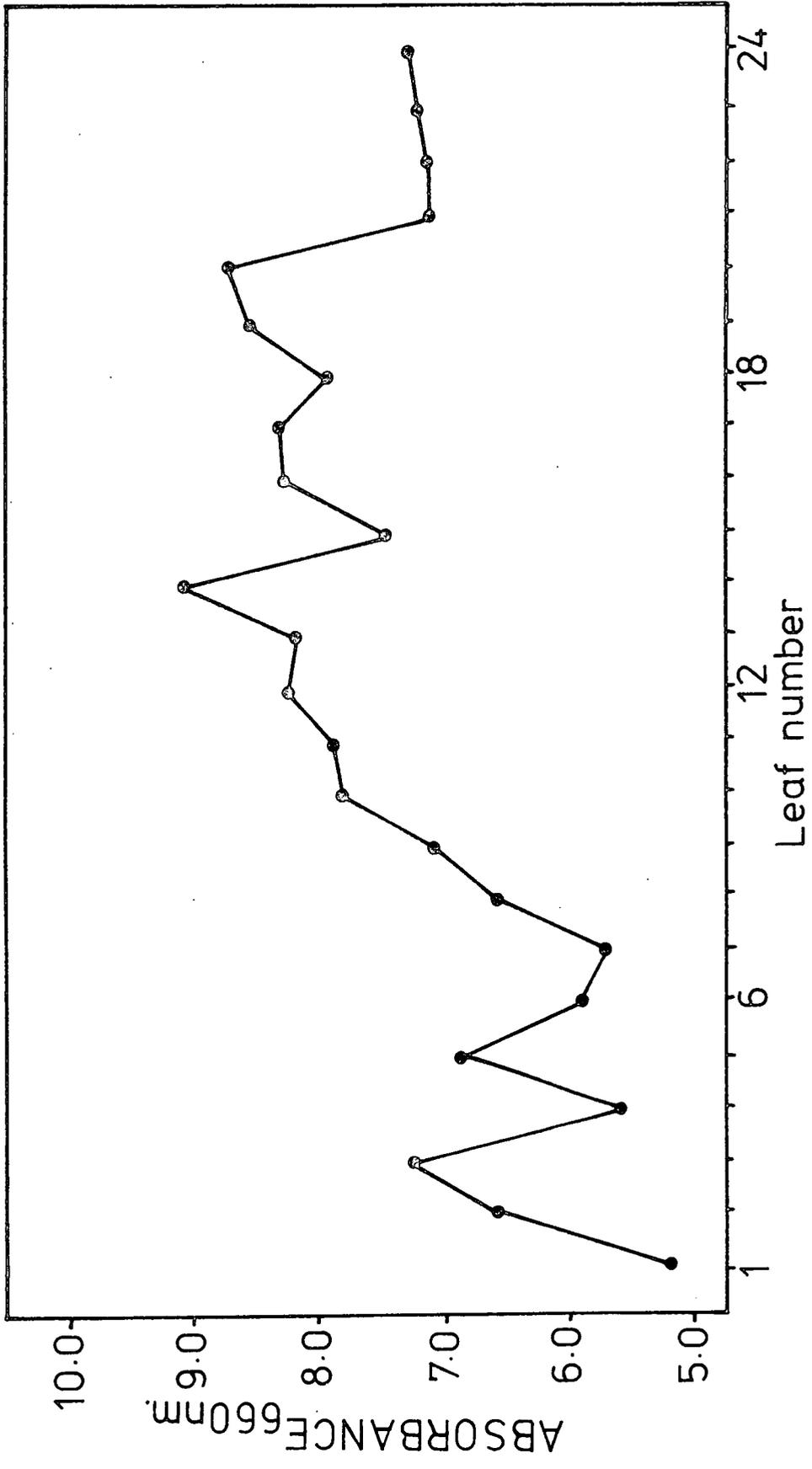
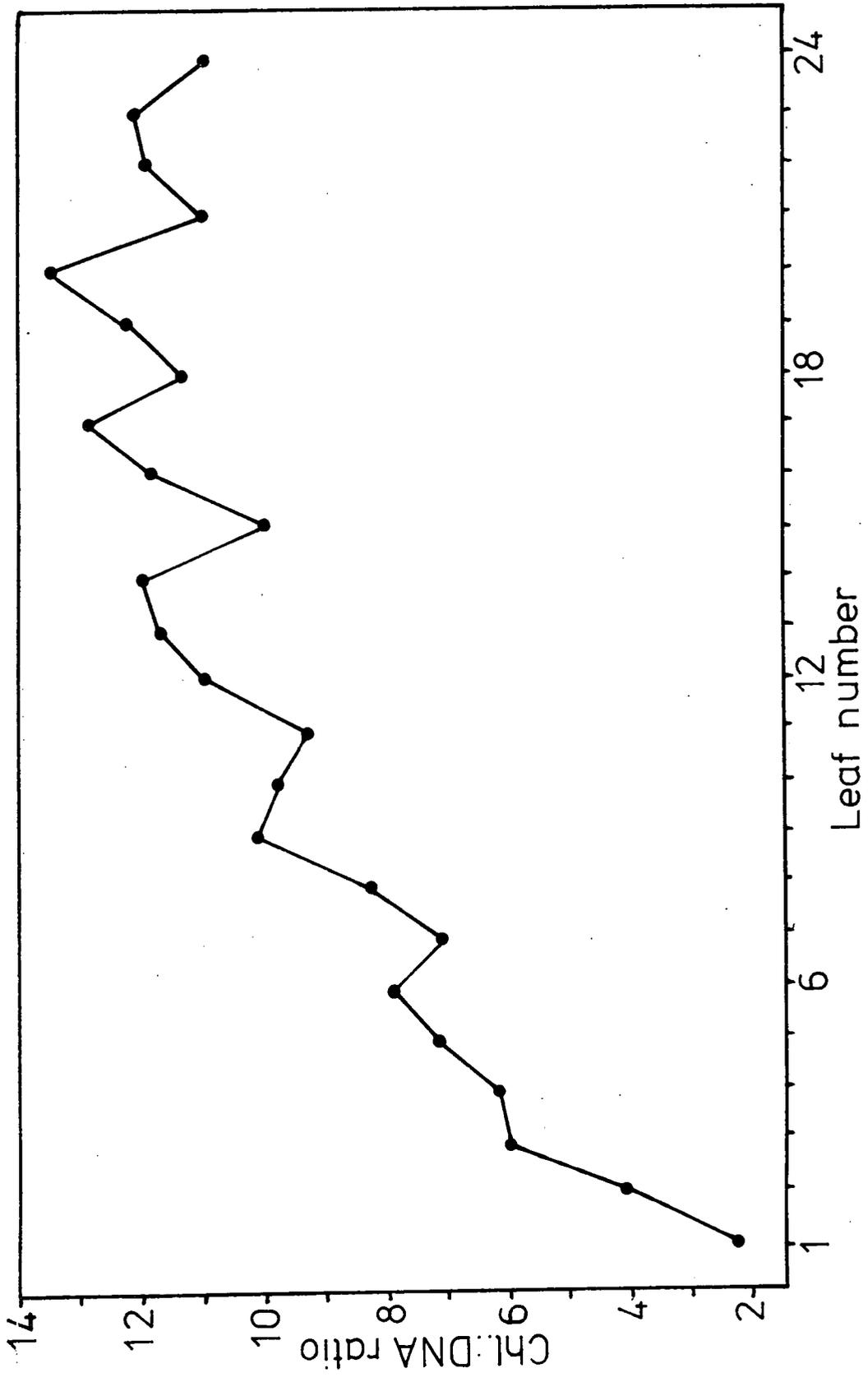


Figure 4.11

The change in the chlorophyll : DNA ratio of the leaves  
along the phyllotaxy.



above study were atypical in that they did not achieve the physical size attained by subsequent leaves. Thus comparison back to the levels of the various cell components found in mature leaves was difficult except on a yield per gram fresh weight basis, because of this the changes taking place in one particular leaf through the latter stages of its life cycle, i.e. from just prior to achieving maximum size (fresh weight and leaf length) until senescence, were studied. It was decided to follow the changes occurring with age in the fifth leaf produced by the plant as this was the first leaf in which the grosser differences in leaf size were absent. A population of chinese cabbage plants were cultivated under the described glasshouse conditions and each plant was individually numbered. Sampling commenced on the same date for all plants and began at a stage just prior to maximum size attainment by the fifth leaf. Each sample consisted of fifteen discs taken from the leaves using a 0.7 cm diameter cork borer. Fifteen different plants were used for each sample and for each of two replicates, and the forty-five different plants to be used on each sampling were chosen by utilising random number tables. This procedure was employed to reduce any possible effects of different rates of growth of individual plants within the population. The leaf disc samples, once taken, were weighed and extracted for cellular components as previously described (see 2.3) and the lengths

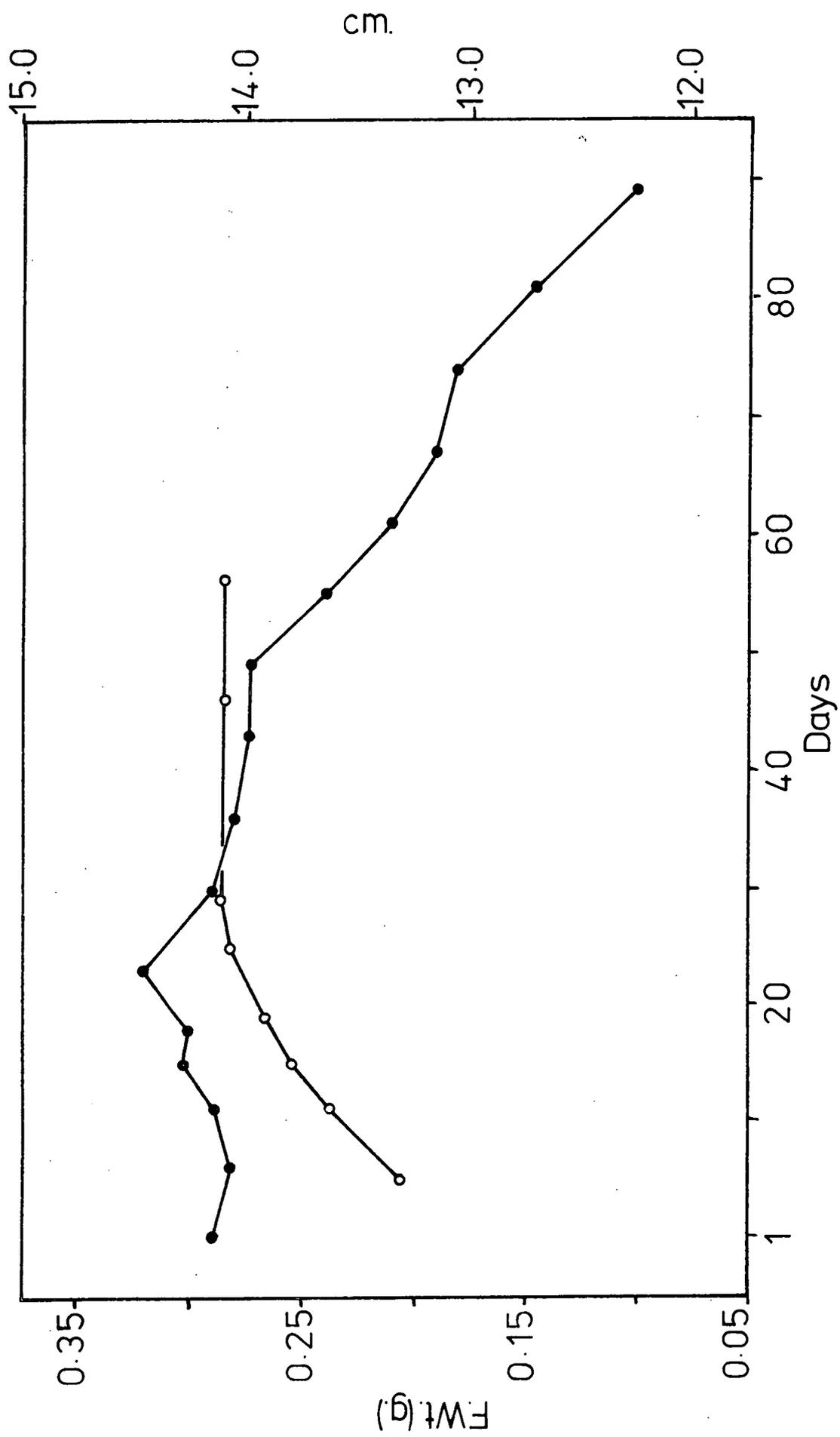
of the fifth leaves were also measured over the sampling period. The experiments were continued until all 5th leaves had abscised<sup>edd</sup>.

Figure 4.12 illustrates the changes seen in the growth parameters leaf length and fresh weight for a typical experiment. The pattern of change for leaf length showed the final phase of a sigmoid curve type of growth already demonstrated for chinese cabbage leaves (see figure 4.3 and Clark et al., 1964). The average leaf length was still increasing at the beginning of the sampling period and a maximum length was eventually attained which was subsequently maintained. Fresh weight was also increasing at the beginning of the sampling period reaching a maximum value at the same time as maximum leaf length was achieved, there then followed a steady decline in fresh weight which continued until sampling ceased. These results confirmed the loss in fresh weight of older leaves of chinese cabbage as previously reported (Clark et al., 1964). As described above the data obtained from sampling successive leaves on a single plant showed that the DNA changes per leaf closely following the leaf fresh weight changes (figure 4.4) whereas with RNA content a markedly different relationship to fresh weight was observed (figure 4.6). Because of the reduced size attained by the first leaves produced by the plant clarification of what was solely an ageing change in RNA content was necessary.

Figure 4.12

The change with time in the average length of the 5th leaf and fresh weight of discs excised from the 5th leaf of a population of chinese cabbage plants.

- Fresh weight (g)
- Leaf length (cm)



Plotting the changes in RNA content during ageing of the fifth leaf showed that the RNA yield was declining before maximum leaf length, and hence maximum fresh weight, was achieved (figure 4.13). This result was thus consistent with the RNA content per leaf data from the sequential senescence analysis in which it was also observed that RNA yields were declining before maximum leaf size was reached (figure 4.6).

The chlorophyll levels observed during fifth leaf ageing showed a pattern of change closely following that of fresh weight (figure 4.14), with a decline in chlorophyll content beginning at the same point at which fresh weight began to decrease. Thus in the fifth leaf senescence a much stronger correlation between fresh weight and chlorophyll content was apparent than that observed in the sequential senescence analysis.

Figure 4.13

The change with time in the average length of the 5th leaf and total RNA content of discs excised from the 5th leaf of a population of chinese cabbage plants.

- RNA content ( $\mu\text{g}$ )
- Leaf length (cm)

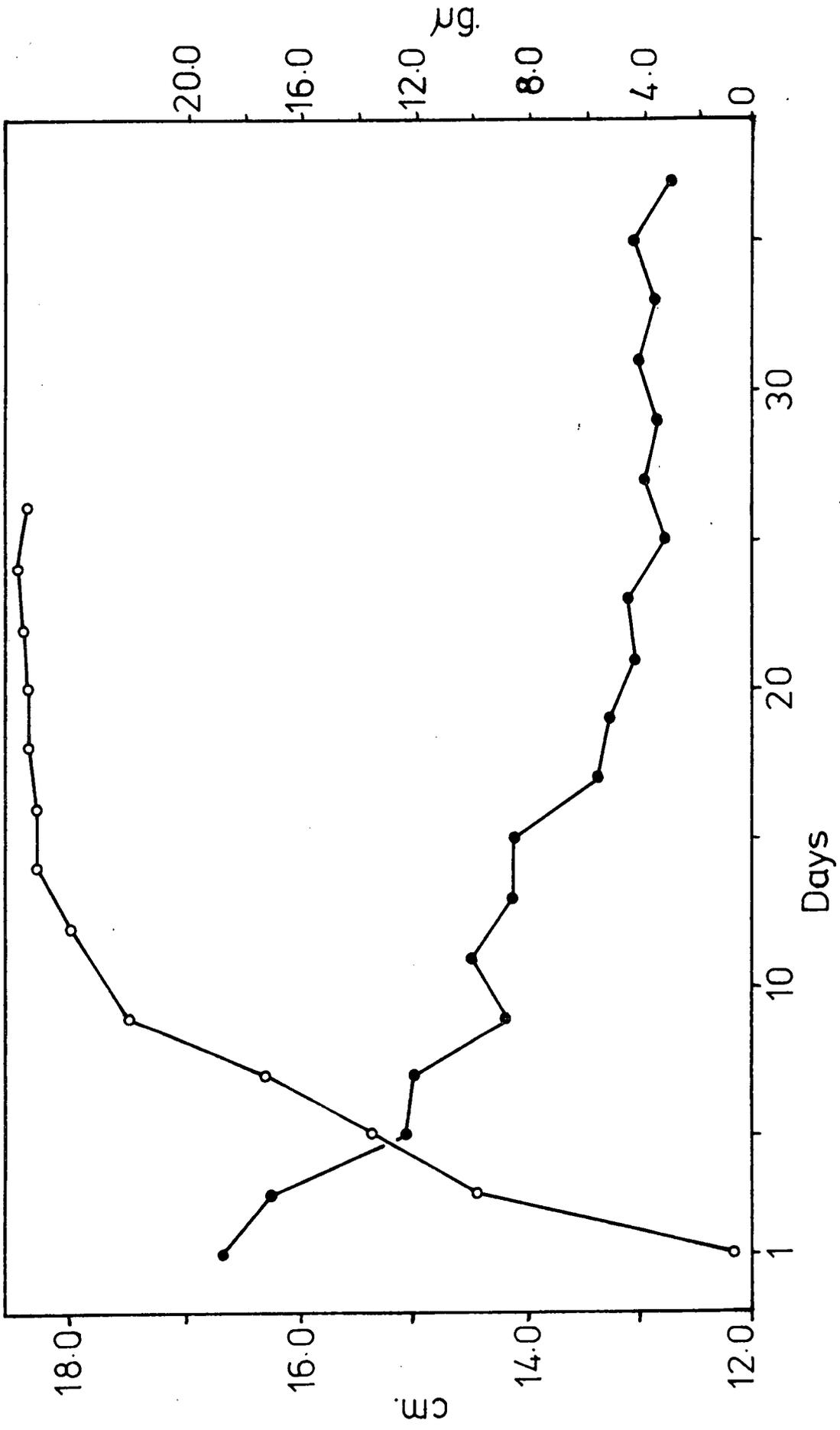
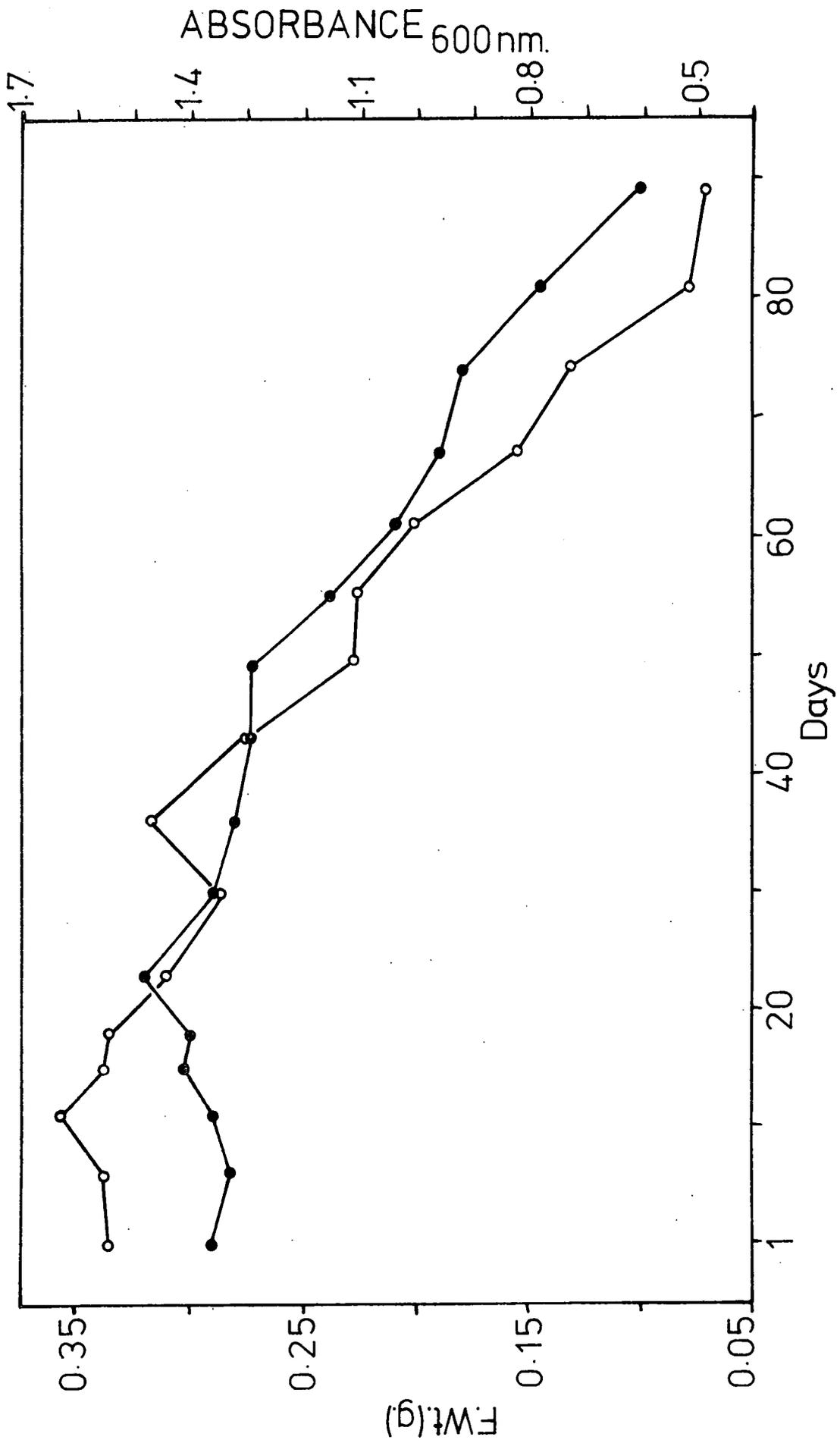


Figure 4.14

The average change with time in fresh weight and chlorophyll content of the 5th leaf of a population of chinese cabbage plants.

- Fresh weight (g)
- Chlorophyll



### 4.3 Sequential leaf ageing - Qualitative RNA changes

#### 4.3.1 RNA prepared by the DEP-SDS method

To complement the quantitative changes already described the qualitative RNA changes occurring during leaf ageing were also examined by performing qualitative extractions on successive leaves along the phyllotaxy of an individual plant. All leaves on a plant from the youngest leaf that was practicable to use to the oldest senescent leaf were numbered along the spiral phyllotaxy. Each leaf was weighed and a sample extracted for total DNA, RNA and chlorophyll content (see 4.2), the remainder of the leaf lamina was extracted by the DEP-SDS extraction method. The RNA precipitate obtained following one ethanol precipitation was used for subsequent analysis thus enabling the low amounts of total RNA present in the oldest leaves (see 4.22) to be conserved and fractionated.

With increasing leaf age there was a slight decline in the spectral ratio  $E_{260}/E_{280}$  of the RNA samples (figure 4.15), a similar trend was evident with the ratio  $E_{max}/E_{min}$  but the values were less consistent. The decline in the apparent purity of the RNA preparations may have been attributable to the accumulation of secondary metabolites, such as phenolics, in the older leaves. There was also a slight decline with age in the recovery of RNA from the leaf material, as judged by the yield obtained by qualitative extraction expressed as

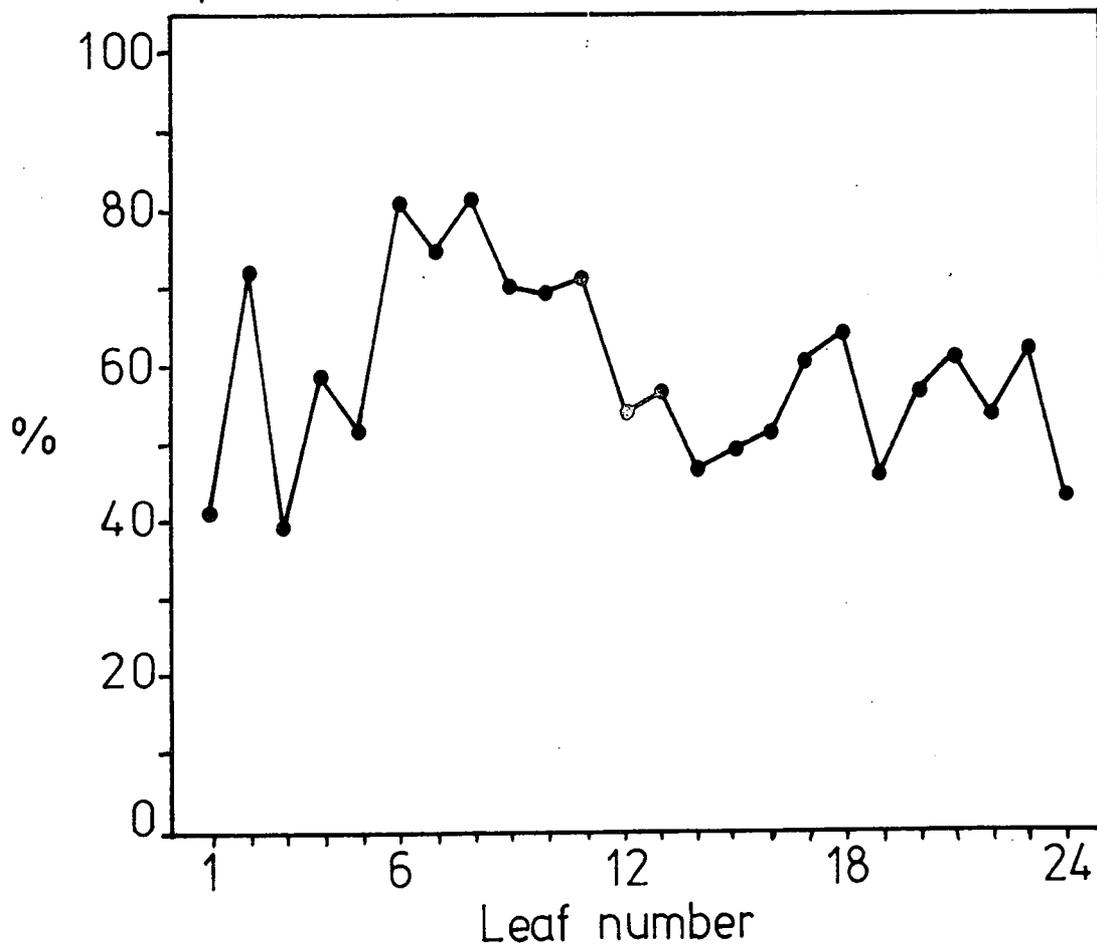
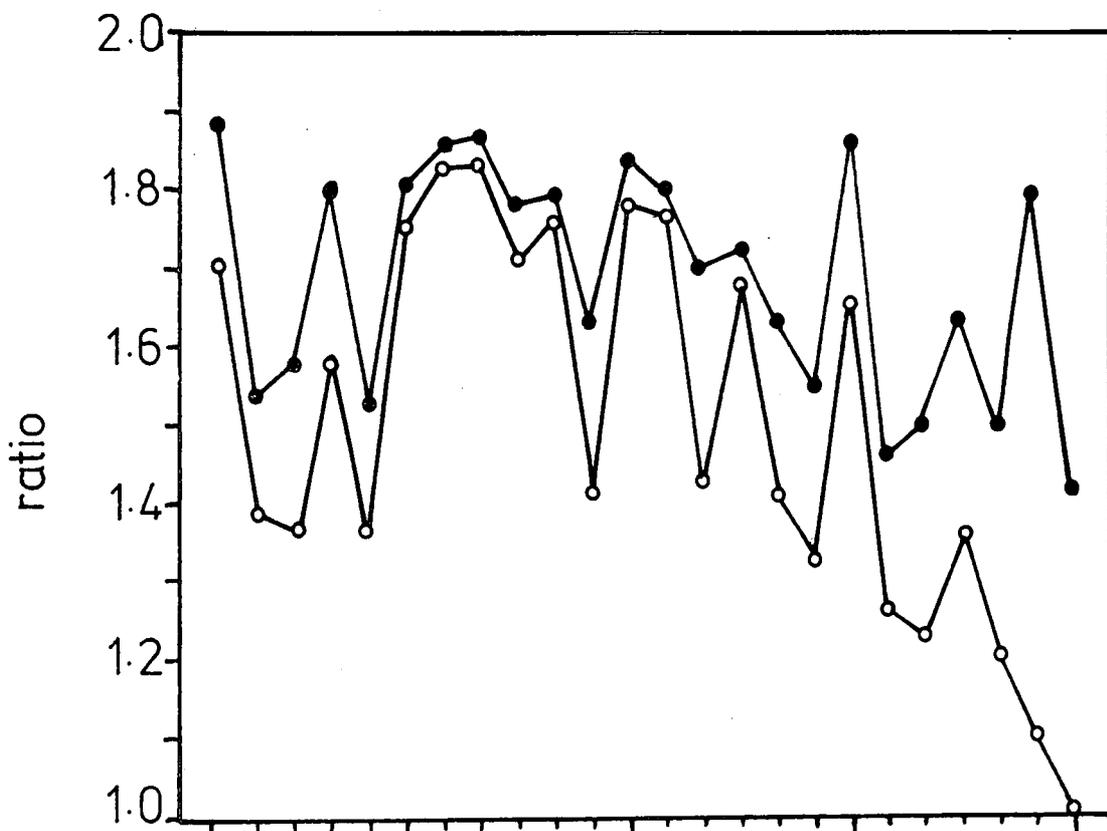
Figure 4.15

The change in the value of the u.v. absorption spectrum ratios of RNA extracted by the DEP-SDS method from successive leaves along the phyllotaxy.

- Ratio  $E_{260}/E_{280}$
- Ratio  $E_{max}/E_{min}$

Figure 4.16

The change in recovery of RNA extracted by the DEP-SDS method, expressed as a percentage of the total yield obtained by TCA extraction, from successive leaves along the phyllotaxy.



as a percentage of the yield obtained by quantitative TCA RNA extraction (figure 4.16). Lazar et al., (1969) using the DEP-SDS method of RNA extraction found that with ageing barley leaves the percentage recovery of nucleic acid remained constant but with ageing tobacco leaves a loss with age was observed. This loss was apparent as a preferential decrease in the recovery of heavy ribosomal RNA and subsequent improvement of their extraction procedure (Solymosy et al., 1970) prevented this loss. As judged by the gel electrophoretograms in this study the decreased RNA recovery from chinese cabbage leaves observed using the DEP-SDS method was not a result of a preferential loss of particular high molecular weight rRNA components but seemed to be the result of a change in the state of the extracted tissue (i.e. a quantitative rather than a qualitative change). Bawden and Pirie (1972) have also reported a change in the recovery of nucleic acid in old leaves of tobacco and have suggested that 'fixation' of the nucleic acid to the leaf fibre is an important factor. In chinese cabbage there was also a proportionally lower recovery in the youngest leaves. This was seen in the young not fully expanded leaves which had a fairly high density of vascular tissue which may therefore, have presented a tissue more resistant to homogenisation with the pestle and mortar used. These were also the leaves in which there was less fresh weight of material from which to extract RNA, thus

inconsistencies in the degree of homogenisation of the tissue may be the main contributory factor in the observed decrease in recovery of RNA in the youngest leaves. Attempts to improve the standardisation of homogenisation by using available commercial tissue homogenisers were unsuccessful because the small amounts of material available with the younger leaves could not be handled efficiently.

On a yield per gram fresh weight basis the RNA showed a rapid decline to approximately 30% of its initial concentration over the youngest few leaves followed by a more gradual decline (figure 4.17). This was similar to the quantitative result using TCA although the initial decline was more rapid and the subsequent loss, once the initial rate of decline had fallen, was greater. The lowered recovery of RNA in the oldest and youngest leaves noted above will have influenced this result. Similarly there was a deviation from the result found with the TCA extracted RNA yield when the DEP-SDS yields were expressed on the basis of the yield per leaf, the steady decline in RNA content observed with the quantitative extracts was seen to flatten off in the youngest leaves with the qualitative results (figure 4.18). Again this difference was probably influenced by the decreased RNA recovery in the youngest leaves with the DEP-SDS extraction.

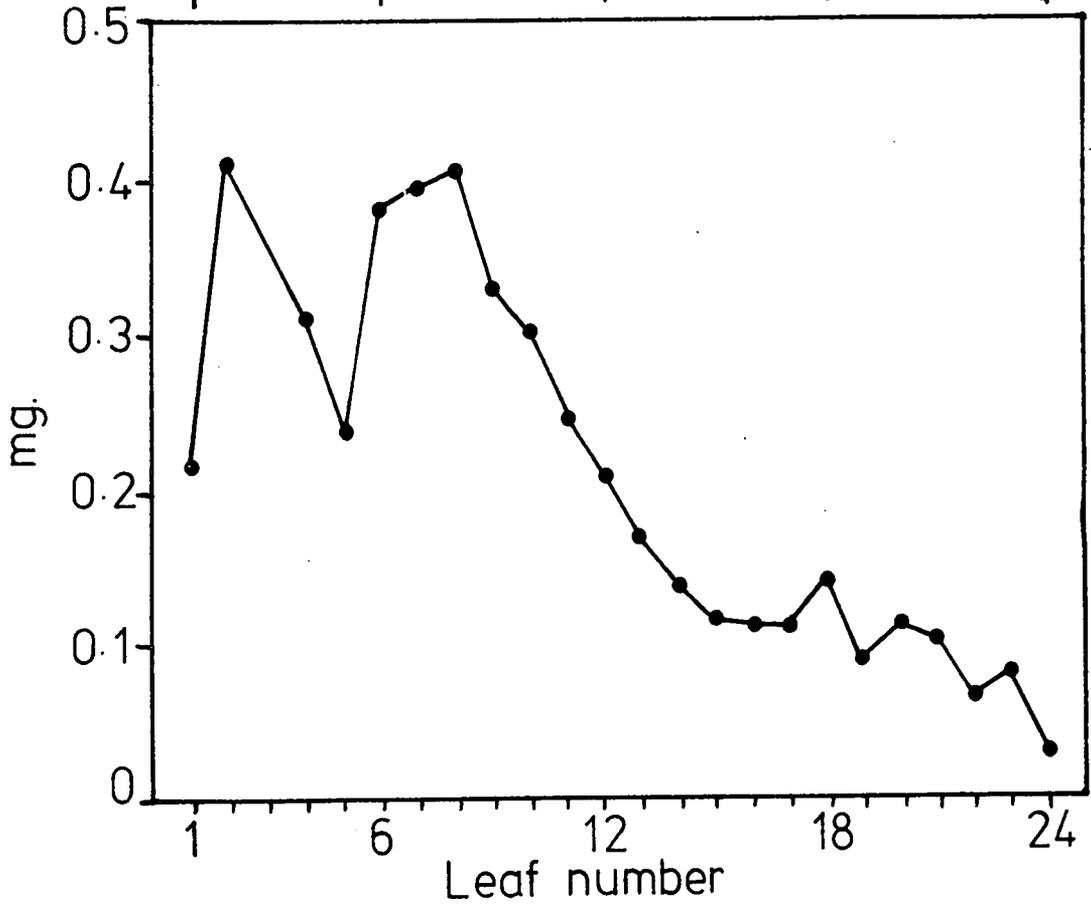
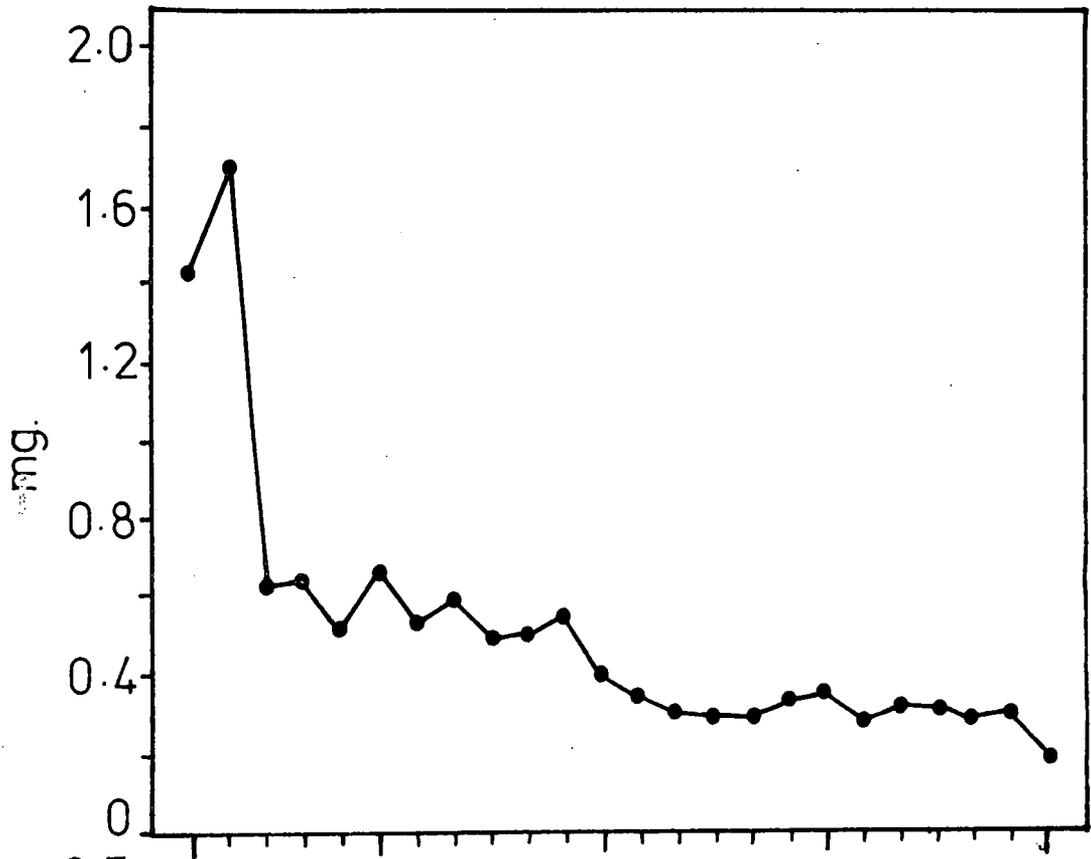
As has been previously shown the electrophoresis of chinese cabbage high molecular weight RNA in EDTA-containing

Figure 4.17

The yield of RNA per gram fresh weight of leaf lamina from successive leaves along the phyllotaxy. RNA extracted by the DEP-SDS method.

Figure 4.18

The total yield of RNA per leaf from successive leaves along the phyllotaxy. RNA extracted by the DEP-SDS method.



buffer systems results in the dissociation of the labile  $1.05 \times 10^6$  molecular weight chloroplast rRNA into a number of breakdown fractions (see 3.52). With a magnesium electrophoretic system the dissociation of the  $1.05 \times 10^6$  molecular weight fraction was reduced to a minimum with just the  $0.42 \times 10^6$  molecular weight breakdown component being evident in a reduced amount. In contrast to phenolic RNA extraction however, the DEP-SDS RNA extraction method did not give electrophoretograms with a high background absorption following magnesium electrophoresis. Thus in order to examine possible changes in the different high molecular weight rRNA fractions extracted by the DEP-SDS method from sequentially ageing leaves the magnesium electrophoresis buffer modified from the electrophoretic buffer of Bishop et al., (1967) was used (see 3.22.2). The only correction that was then necessary to estimate the content of chloroplast  $1.05 \times 10^6$  molecular weight RNA was to add the area measured for the  $0.42 \times 10^6$  molecular weight fraction to that measured for the  $1.05 \times 10^6$  molecular weight species.

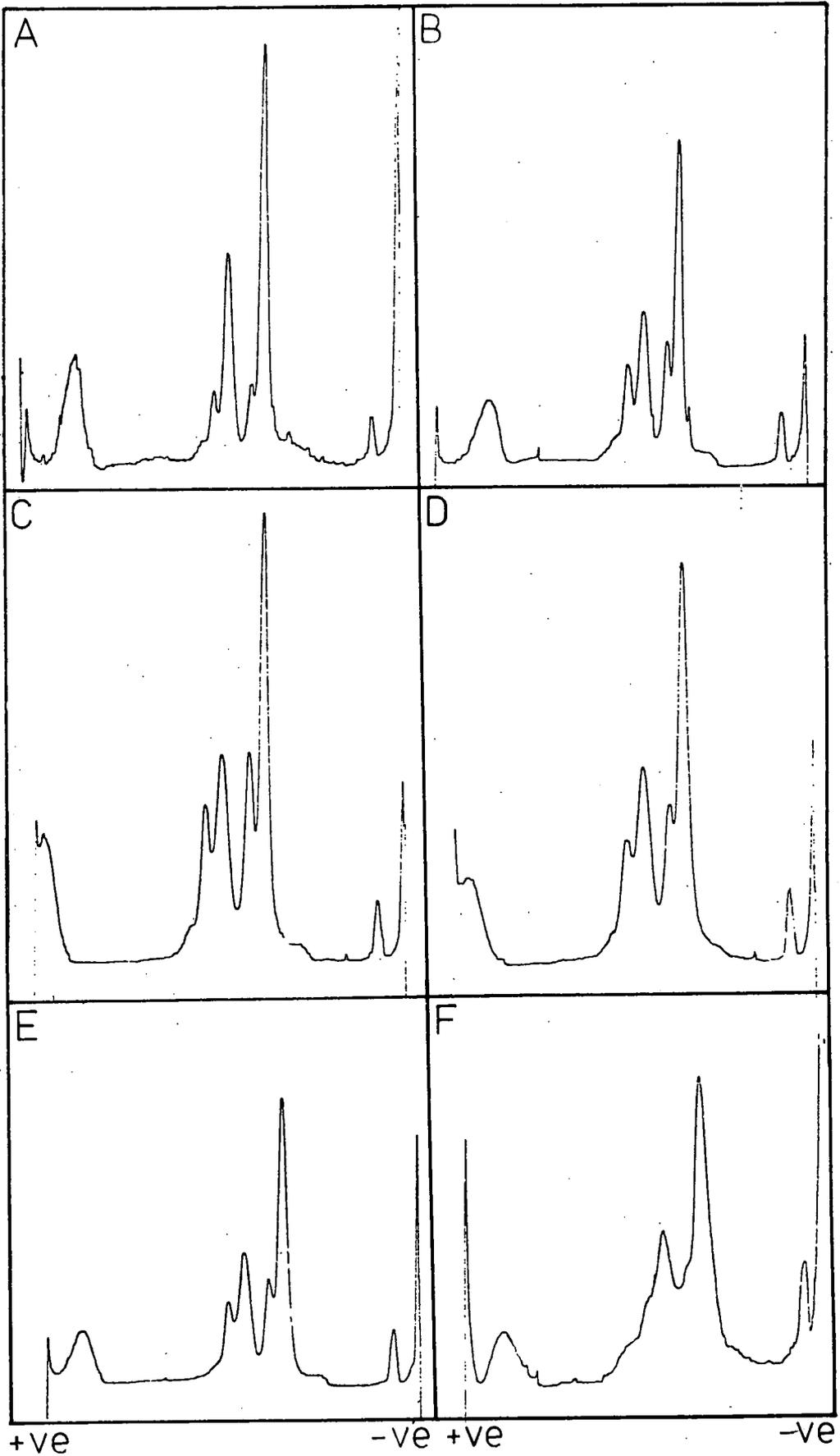
Electrophoretograms of RNA extracted from different levels in the ageing sequence are illustrated in figure 4.19. From observation of the gel profiles there appeared to be an increase in the relative proportion of chloroplast RNA in the youngest leaves which then remained at an approximately constant level. Even in the oldest leaves significant amounts of

Figure 4.19

PAGE of RNA extracted by the DEP-SDS method from different leaves along the phyllotaxy

- A. Leaf 1 (young)
- B. Leaf 4
- C. Leaf 9
- D. Leaf 14
- E. Leaf 21
- F. Leaf 24 (old)

A<sub>265nm</sub>.



chloroplast material were still present, in contrast to the greatly reduced levels found by Callow et al., (1972) for attached leaves of Perilla frutescens. 10 - 20  $\mu\text{g}$  rRNA were loaded on the gels and a broadening of the RNA peaks in the samples from older leaves was evident which could be attributed to the larger loading volumes necessary because of the reduced quantity of RNA present in the tissue extracts. Alternatively this peak broadening may indicate that in the older leaves there are changes in the conformation of the RNA molecules resulting in a population of fractions of slightly different electrophoretic mobilities.

In order to examine the proportions of the different RNA components more closely the peak area weight of each fraction present on the electrophoretogram were determined as indicated in section 3.22.2 and the amount of each RNA fraction was then expressed as a percentage of the total high molecular weight rRNA (figure 4.20). The observed <sup>apparent</sup> subjective change in the total chloroplast rRNA (i.e. the sum of the percentage areas of the  $1.05 \times 10^6$ ,  $0.42 \times 10^6$  and  $0.56 \times 10^6$  molecular weight fractions) in the youngest leaves was clearly demonstrated. The proportion of chloroplast rRNA rose from about 22% in the youngest leaf sampled to approximately 39% in the eighth and ninth leaves, after this there was a slight, but steady, decline in the relative proportion of chloroplast rRNA to a final level of 29% in the oldest leaf. No accelerated reduction

of chloroplast rRNA relative to cytoplasmic rRNA occurred in the latest leaves. The pattern of change in the relative proportion of total cytoplasmic rRNA was naturally complementary to that for the chloroplastic fraction. Figure 4.21 shows the change in the relative proportions of each of the major ribosomal-RNA fractions. The percentage values plotted for the  $1.05 \times 10^6$  mol.wt. chloroplast rRNA include the value for the  $0.42 \times 10^6$  mol.wt. fraction which contributed 2-3% to the value plotted. The two chloroplast components ( $1.05 \times 10^6 + 0.42 \times 10^6$  and  $0.56 \times 10^6$ ) had closely similar changes in proportion which paralleled the changes for total chloroplast rRNA. A similar situation was evident for the two cytoplasmic fractions ( $1.3 \times 10^6$  and  $0.7 \times 10^6$ ) though the parallel fit between the  $1.3 \times 10^6$  and  $0.7 \times 10^6$  changes was less good. These graphs suggest that the relative changes indicated above were effected through a change in either total cytoplasmic or total chloroplastic rRNA rather than by a marked change in a single component of either fraction.

From the percentage proportions of the different high molecular weight rRNA components the yield in each fraction was computed. Because of the changes in percentage recovery with leaf age (figure 4.16) the basis for these calculations was the yield of total RNA obtained by TCA extraction, in this way the absolute levels in each fraction could be more accurately ascertained. Expressed as a milligram yield of RNA in each fraction per leaf the total cytoplasmic rRNA

Figure 4.20

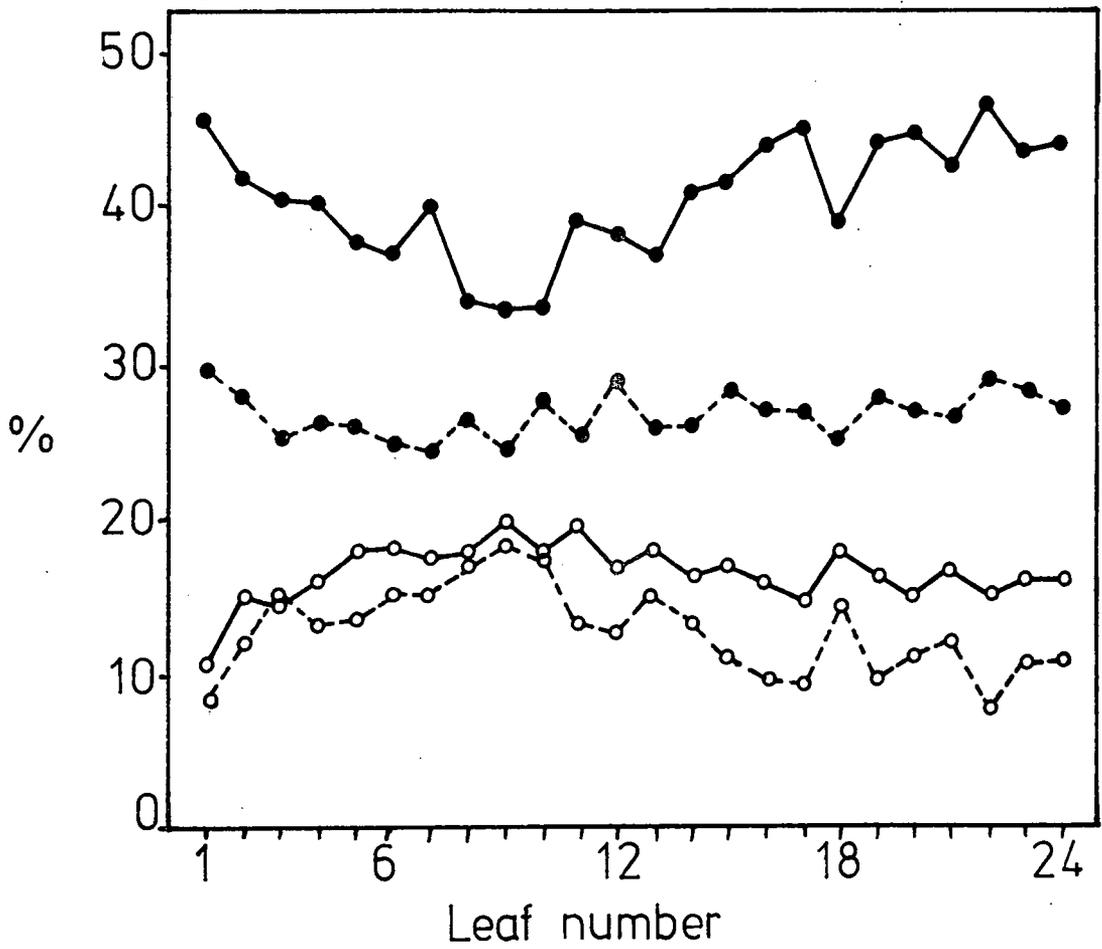
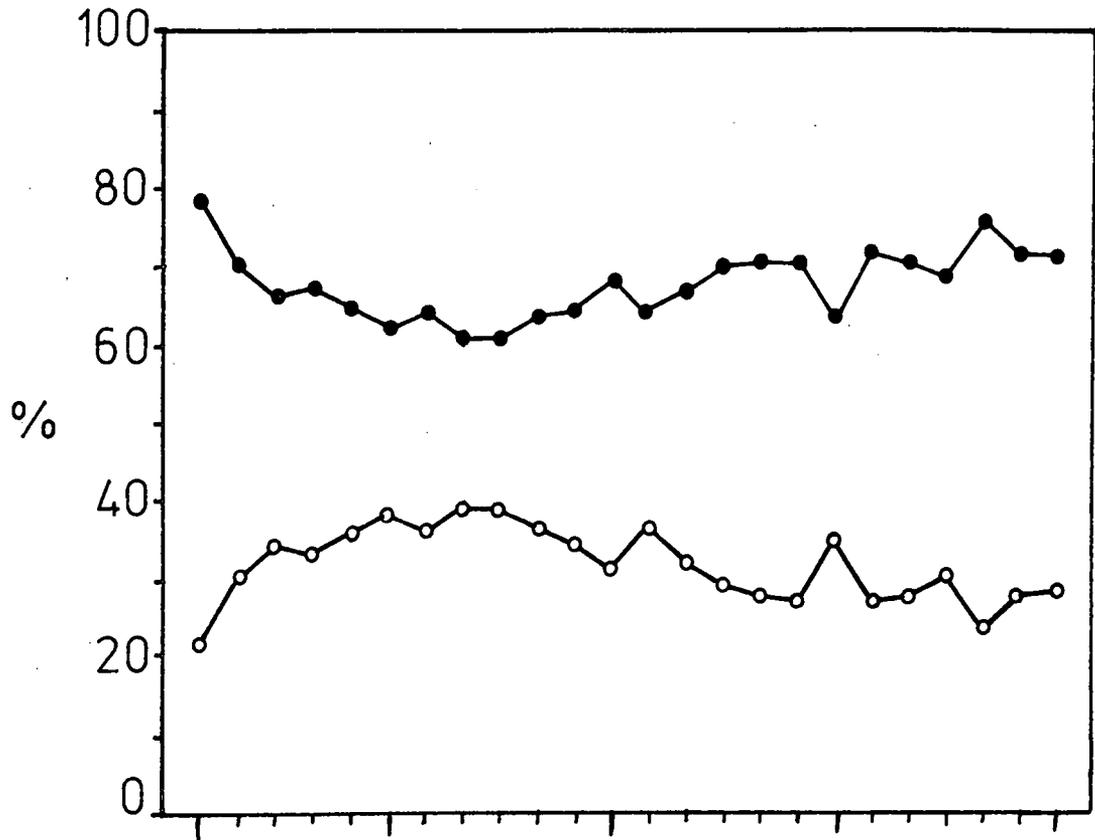
The change in the proportion of cytoplasmic and chloro-  
plastic rRNA extracted by the DEP-SDS method from  
successive leaves along the phyllotaxy.

- Cytoplasmic rRNA
- Chloroplastic rRNA

Figure 4.21

The change in the proportion of the component cytoplasmic  
and chloroplastic rRNA fractions extracted by the DEP-SDS  
method from successive leaves along the phyllotaxy.

- ⊖—⊖  $1.3 \times 10^6$  mol. wt. (cytoplasmic)
- ⊖—⊖  $1.05 \times 10^6$  mol. wt. (chloroplastic)
- ⊖---⊖  $0.7 \times 10^6$  mol. wt. (cytoplasmic)
- ⊖---⊖  $0.56 \times 10^6$  mol. wt. (chloroplastic)



showed a similar trend to that for total RNA as determined by TCA extraction (figure 4.6) in that there was a steady decline in yield from the youngest leaf to the oldest (figure 4.22). The total chloroplastic rRNA however showed an initial rise in yield per leaf which flattened off and subsequently declined steadily. The changes taking place in the individual components of either the cytoplasmic ( $1.3 \times 10^6$  and  $0.7 \times 10^6$ ) or chloroplastic ( $1.05 \times 10^6 + 0.42 \times 10^6$  and  $0.5 \times 10^6$ ) fractions paralleled the changes that occurred in total cytoplasmic or total chloroplastic rRNA respectively. There was thus a net synthesis of chloroplast rRNA per leaf occurring in the youngest leaves though this was not the case with cytoplasmic rRNA. Expressed as a milligram yield of RNA per gram fresh weight the total cytoplasmic rRNA again showed a trend similar to that observed for total RNA from the TCA quantitative results. There was an initial steep decline in cytoplasmic rRNA concentration which then flattened off markedly and declined only slightly (figure 4.23), total chloroplastic rRNA showed a similar trend but because the starting yield was much smaller the initial concentration decline occurring in the first few leaves was less pronounced. As was the case for the yields per leaf the changes taking place in the constituent components of the cytoplasmic and chloroplastic fractions showed a parallel trend to that of their respective total cytoplasmic or chloroplastic rRNA.

Figure 4.22

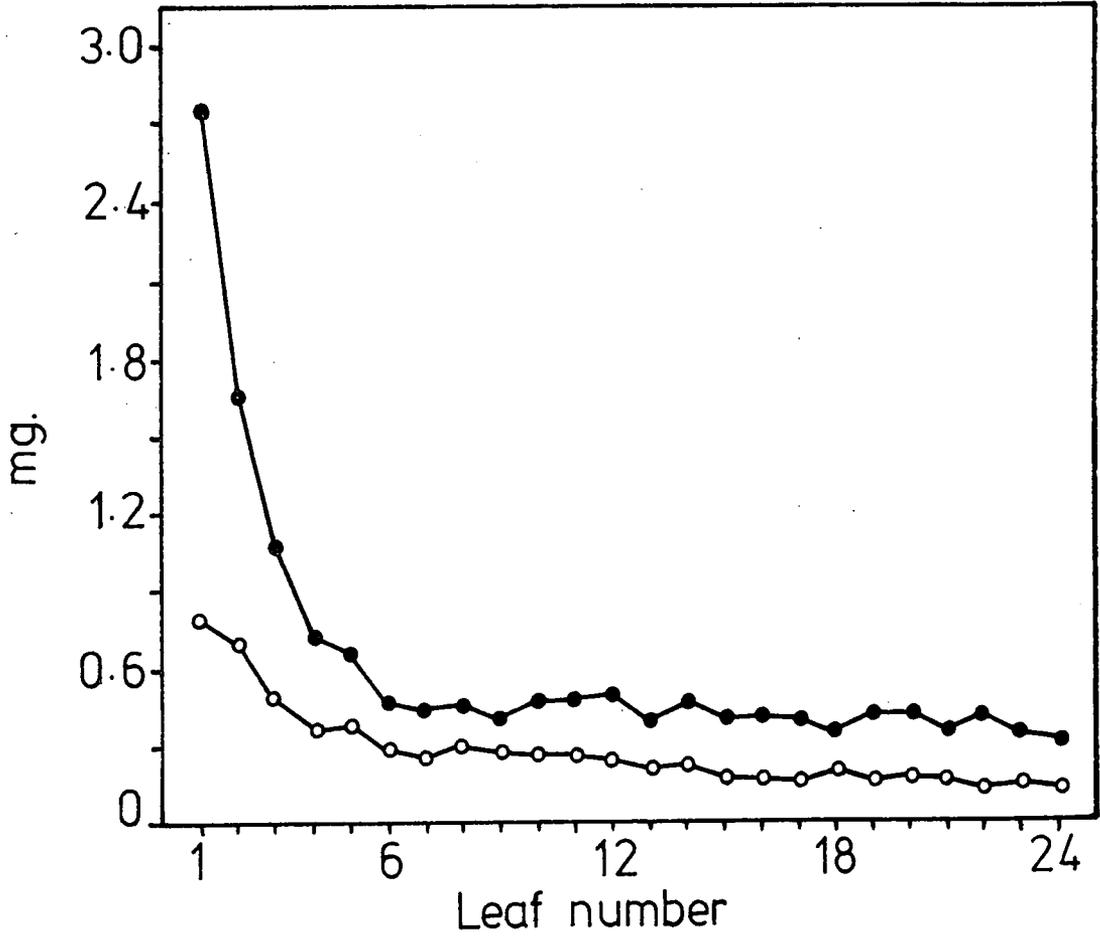
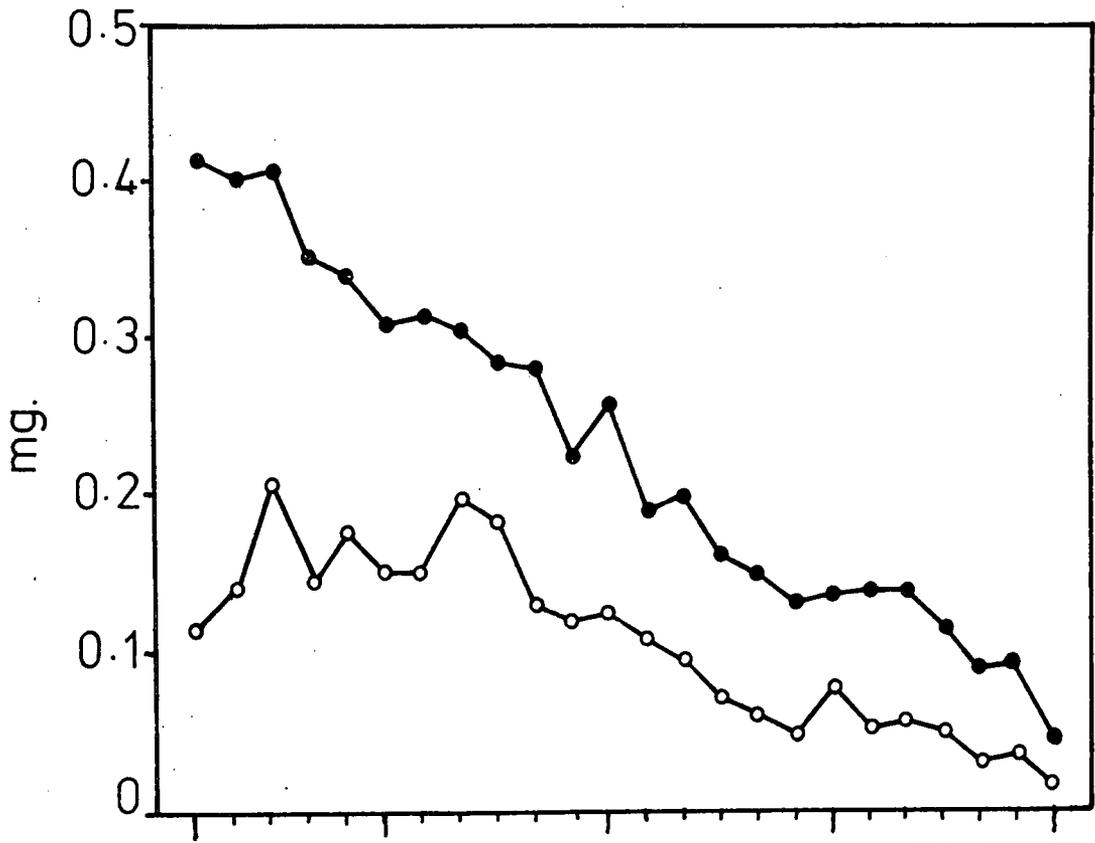
The change in total yield per leaf of cytoplasmic and chloroplastic rRNA from successive leaves along the phyllotaxy. RNA extracted by the DEP-SDS method.

- Cytoplasmic rRNA
- Chloroplastic rRNA

Figure 4.23

The change in yield per gram fresh weight of leaf lamina of cytoplasmic and chloroplastic rRNA from successive leaves along the phyllotaxy. RNA extracted by the DEP-SDS method.

- Cytoplasmic rRNA
- Chloroplastic rRNA



The low molecular weight RNA present in the DEP-SDS extracted RNA was observed by fractionation of the samples in 7.5% polyacrylamide gels (Loening, 1968). For all leaves both 4s and 5s components could be discerned (figure 4.24) and an examination of the electrophoretograms did not reveal any obvious trends with increasing leaf age. The peak areas of the 'soluble' (4s) RNA and of the low molecular weight rRNA (5s) were measured by the method previously described (see 3.22.2) and were expressed as a percentage of the total area measured for each low molecular weight RNA electrophoretogram. The relative proportions of these two low molecular weight RNA fractions remained approximately constant throughout the profiles of leaves along the sequential senescence (figure 4.25). By correction of the peak areas measured on the low molecular weight RNA gel profiles to an equivalent sample loading and gel trace amplification to the high molecular weight RNA gels of the same leaf it was possible to estimate the percentage of 4s, 5s and high molecular weight rRNA for individual leaves. It was observed that the proportions of high molecular weight rRNA remained at about the same level throughout the ageing sequence (figure 4.26). The pattern of change in the 5s rRNA appeared to correlate with the changes seen in the 4s RNA <sup>not</sup> rather than with the high molecular weight rRNA as might have been expected. The total levels of 5s rRNA were very low though (approximately 3% average) and

Figure 4.24

PAGE of low molecular weight RNA extracted by the  
DEP-SDS method from different leaves along the  
phyllotaxy.

- A. Leaf 2
- B. Leaf 8
- C. Leaf 14
- D. Leaf 21

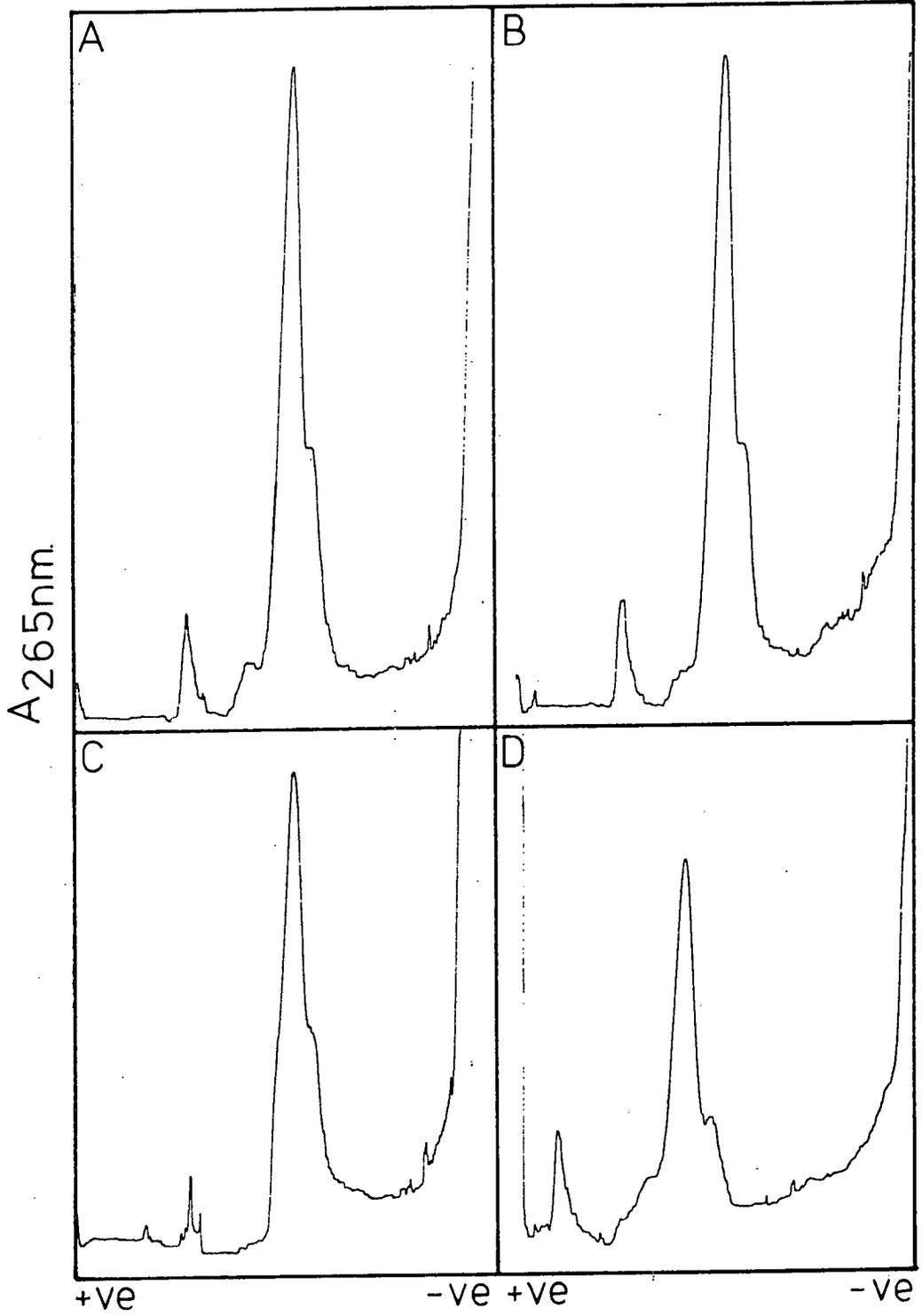


Figure 4.25

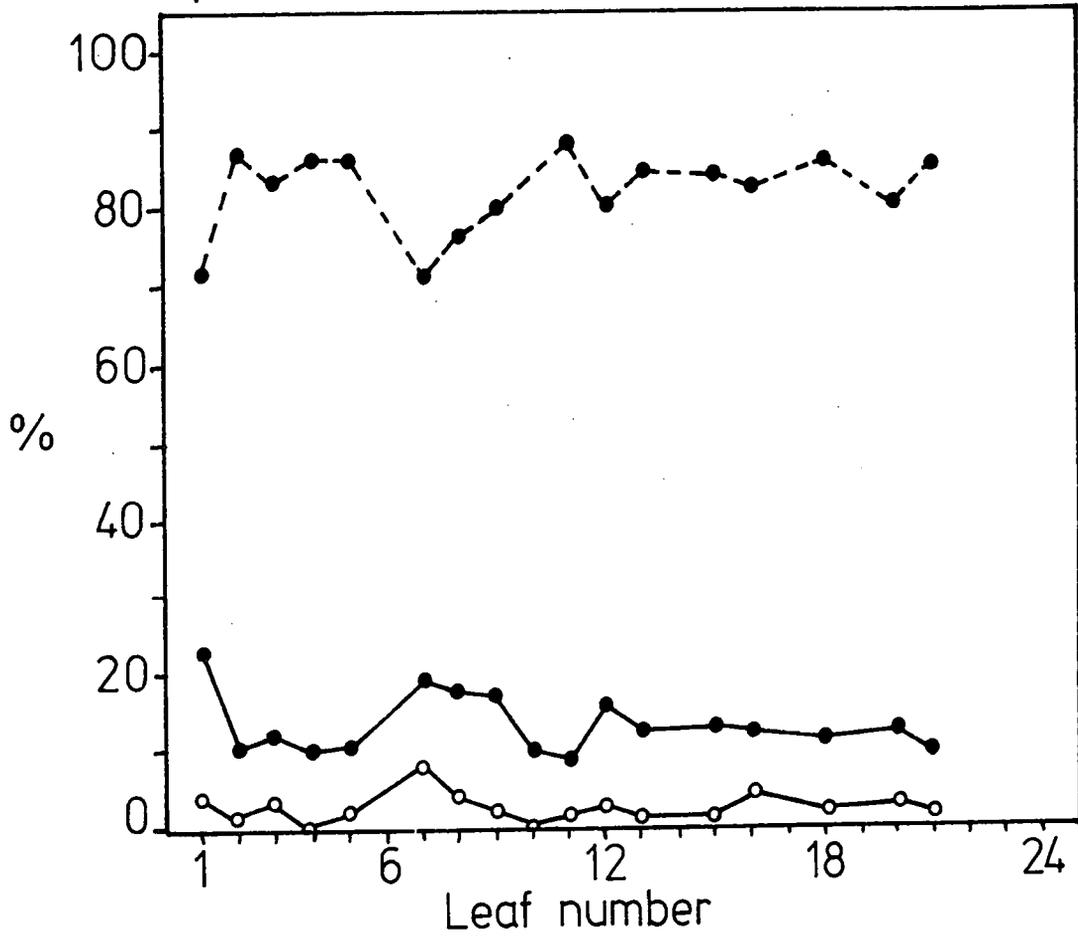
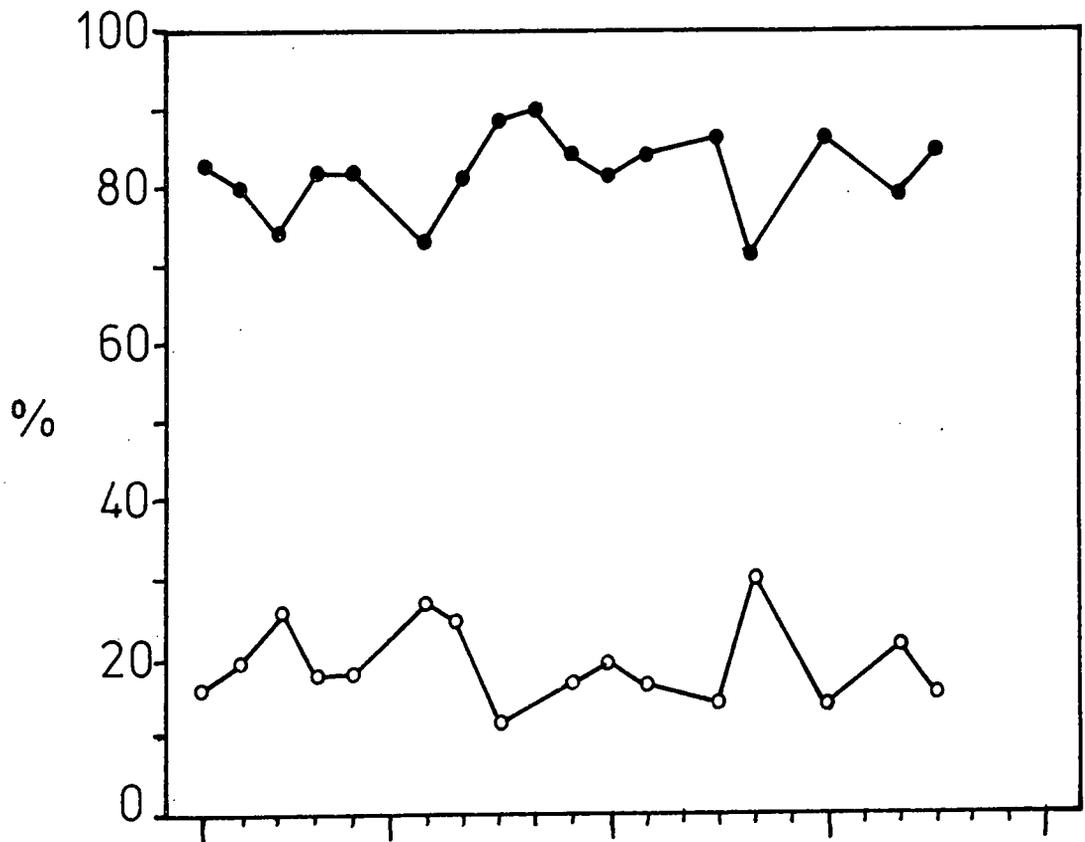
The change in proportion of low molecular weight RNA components extracted by the DEP-SDS method from successive leaves along the phyllotaxy.

- 4s RNA
- 5s RNA

Figure 4.26

The change in proportion of total RNA extracted by the DEP-SDS method from successive leaves along the phyllotaxy

- High molecular weight rRNA
- 4s RNA
- 5s RNA



the fluctuations observed may not be significant being rather an artefact of the measurements, however a recent report has indicated that 5s rRNA metabolism may show a different pattern of behaviour to that of high molecular weight rRNA (Miller, 1974).

The RNA, resuspended in magnesium buffer, was also electrophoresed on 2.6% polyacrylamide gels in the EDTA-containing buffer of Bishop et al., (1967), this buffer being chosen because of its similar constitution to the magnesium buffer used for resuspension of the RNA. Electrophoresis under these conditions resulted in the dissociation of the labile  $1.05 \times 10^6$  molecular weight chloroplast rRNA fraction (see 3.52). The pattern of dissociation products thus released was examined in the leaves of different age along the phyllotaxy. The condition of the RNA being resuspended in magnesium buffer resulted in a constraint on the system in that the presence of magnesium ions reduced the number of possible dissociation fragments that could be observed (see 3.52). Also with the older leaves the larger sample loading volumes necessary because of reduced yields of RNA in the extracts resulted in a broadening of the RNA peaks which tended to obscure some of the possible breakdown fractions. The molecular weights of the observed fractions were estimated from a plot of log (molecular weight) against migration using the cytoplasmic rRNA fractions as internal standard molecular weight markers.

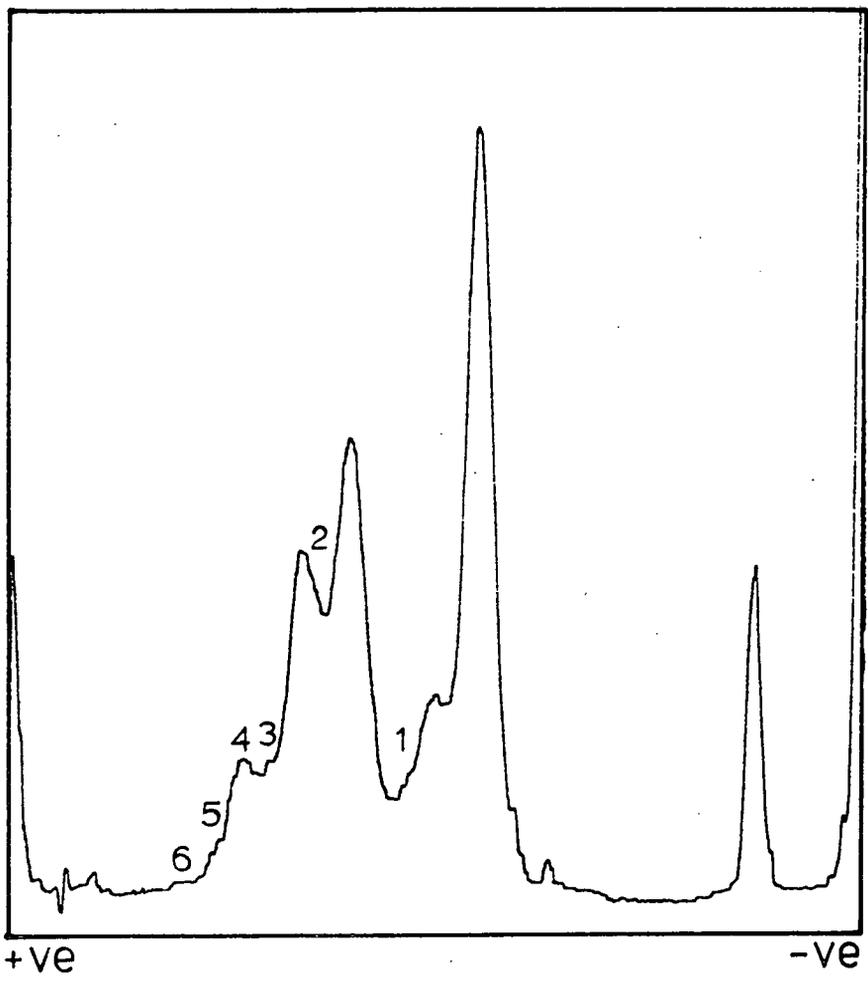
By this method breakdown fractions of the following average apparent molecular weights were observed in most samples:  $0.96 \times 10^6$ ,  $0.6 \times 10^6$ ,  $0.49 \times 10^6$ ,  $0.43 \times 10^6$ ,  $0.38 \times 10^6$  and  $0.32 \times 10^6$  (figure 4.27). As previously described (see 3.51) the presence of magnesium in the resuspension buffer would have influenced the migration of these RNA species, thus giving rise to anomalous molecular weights by the method of determination used. Therefore these values are only relative and the species observed above probably correspond to the fractions of  $1.0 \times 10^6$  or  $0.9 \times 10^6$ ,  $0.59 \times 10^6$ ,  $0.48 \times 10^6$ ,  $0.42 \times 10^6$ ,  $0.37 \times 10^6$  and  $0.35 \times 10^6$  or  $0.29 \times 10^6$  molecular weight respectively as determined with an homogenous EDTA-containing electrophoresis system and using E.coli rRNA as a standard molecular weight marker (see 3.52). The absence of any of the above breakdown fractions did not follow a consistent pattern with regard to leaf age. Quantitatively, from the peak areas measured following magnesium gel electrophoresis, the amount of RNA present in the  $0.42 \times 10^6$  molecular weight breakdown fraction followed the same pattern of change as total chloroplastic rRNA both on a per gram fresh weight or on a per leaf basis. Thus within the stated limitations of this examination (i.e. the presence of magnesium ions) there was no evidence of qualitative or quantitative differences in the stability of the  $1.05 \times 10^6$  molecular weight chloroplast rRNA fraction with leaves of

Figure 4.27

PAGE of high molecular weight rRNA in Loening 'E'  
buffer (Leaf 14 in ageing sequence).

|        |   |                    |          |
|--------|---|--------------------|----------|
| Peak 1 | : | $0.96 \times 10^6$ | mol. wt. |
| Peak 2 | : | $0.6 \times 10^6$  | mol. wt. |
| Peak 3 | : | $0.49 \times 10^6$ | mol. wt. |
| Peak 4 | : | $0.43 \times 10^6$ | mol. wt. |
| Peak 5 | : | $0.38 \times 10^6$ | mol. wt. |
| Peak 6 | : | $0.32 \times 10^6$ | mol. wt. |

A<sub>265nm</sub>.



different ages.

#### 4.32 RNA prepared by the detergent-phenol method

The results detailed above on qualitative RNA changes occurring during sequential ageing of chinese cabbage leaves were all obtained using a relatively novel method of RNA extraction - the DEP-SDS method. This method has already been characterised and compared to the more common detergent-phenol method of RNA extraction (see 3.6) and been shown to give the same qualitative results as the latter method. As a check on this characterisation in an experimental situation a plant was chosen from the same population as used for the DEP-SDS qualitative RNA determinations. Quantitative analysis of the chlorophyll, RNA and DNA contents of each leaf had shown that the plant exhibited very similar quantitative characteristics to the plant used for DEP-SDS qualitative RNA extraction. This having been established each leaf was subsequently extracted by the detergent-phenol method in order to examine the qualitative RNA characteristics.

It was apparent from both the DEP-SDS extraction and from the quantitative determinations that the potential total yield of RNA from the oldest leaves on the plant would be very low. As has been shown the yield of RNA possible with the detergent-phenol method of extraction is reduced because of the number of cleaning-up steps required to completely remove contaminating phenol (see 3.6), consequently to ensure

electrophoretic profiles of all leaves sampled the purification steps employed were reduced to one ethanolic precipitation followed by one wash in 80% ethanol plus 0.5% SDS. This resulted in poorer spectral ratios from the u.v. absorption profiles than those quoted in the detergent-phenol extracted RNA characterisation (see 3.31).

Electrophoretograms of detergent-phenol extracted RNA from different leaves along the sequential senescence are illustrated in figure 4.28. As for the DEP-SDS extracted material breakdown of the labile  $1.05 \times 10^6$  molecular weight chloroplast fraction was minimal due to electrophoresis in magnesium buffer. By measurement of the peak area weights of the gel traces (as described in 3.22.2) the changing proportions of the different high molecular weight RNA fractions along the age sequence of leaves was examined, the areas of individual high molecular weight fractions being expressed as a percentage of the total area measured for all fractions. Values for the large chloroplast rRNA fraction included the areas of both the  $1.05 \times 10^6$  molecular weight and  $0.42 \times 10^6$  molecular weight components. The youngest leaves were again seen to have proportionally less chloroplast rRNA with the youngest leaf having approximately 20% of its high molecular weight RNA as chloroplast rRNA, this proportion rose to a maximum of 43% and there followed a gradual decline in proportion to a final level of about 30% (figure 4.29).

Figure 4.28

PAGE of RNA extracted by the detergent-phenol method  
from successive leaves along the phyllotaxy.

- A. Leaf 1 (young)
- B. Leaf 10
- C. Leaf 21
- D. Leaf 24 (old)

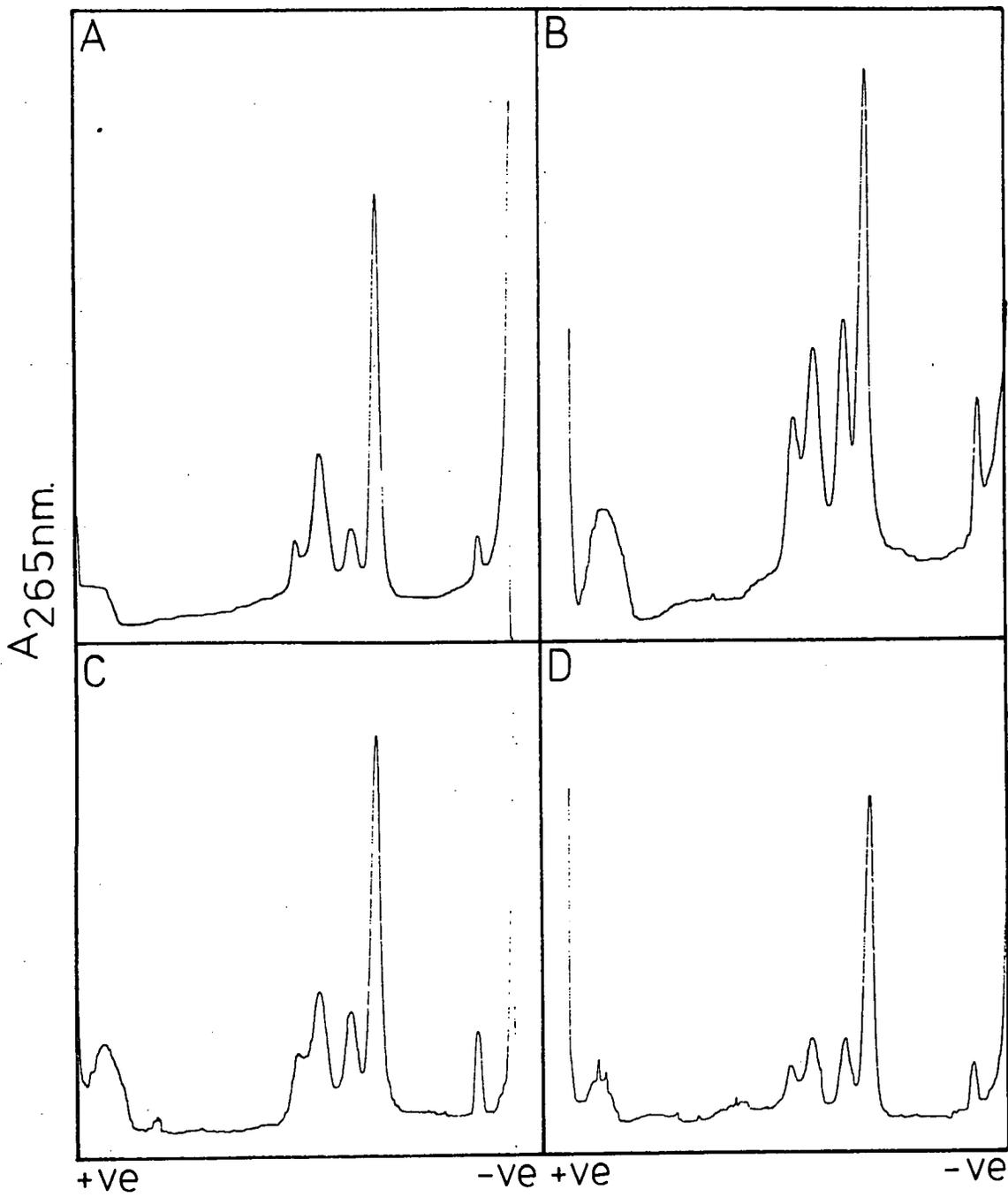


Figure 4.29

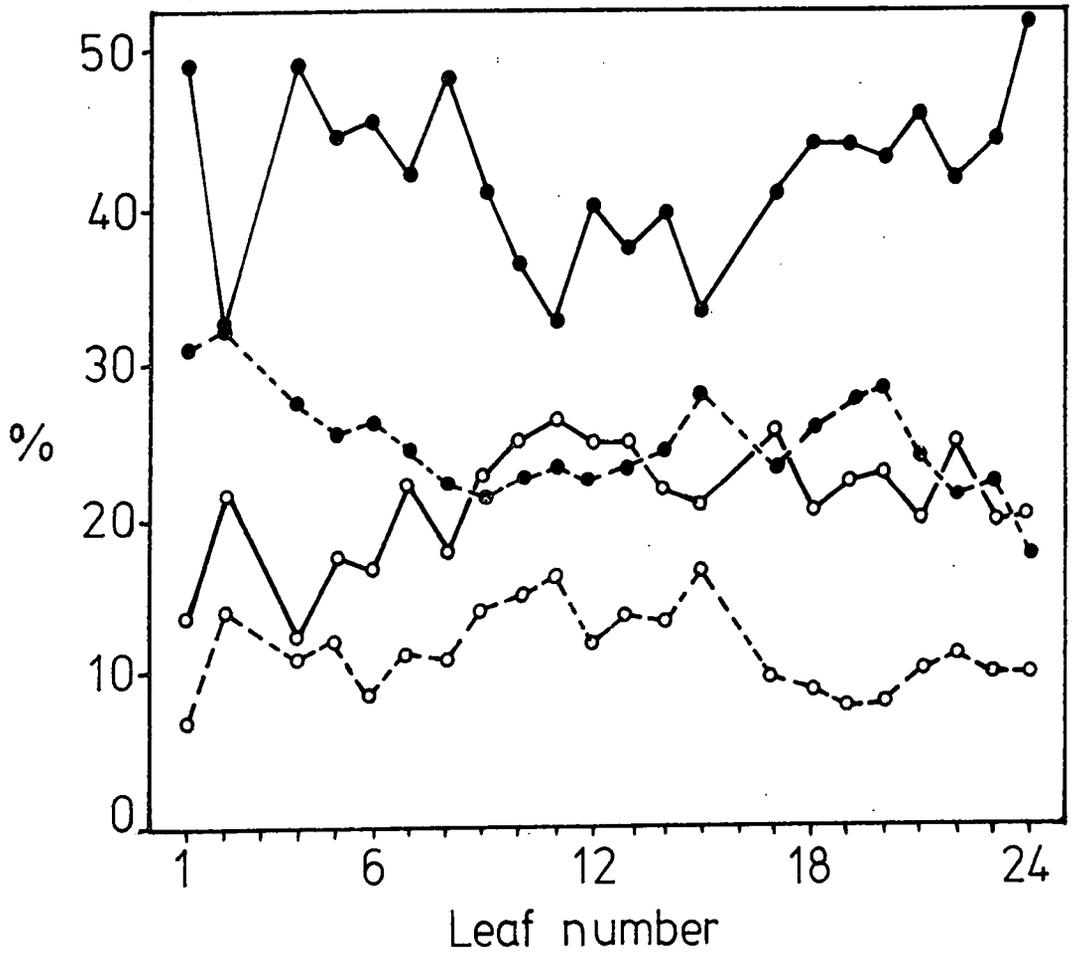
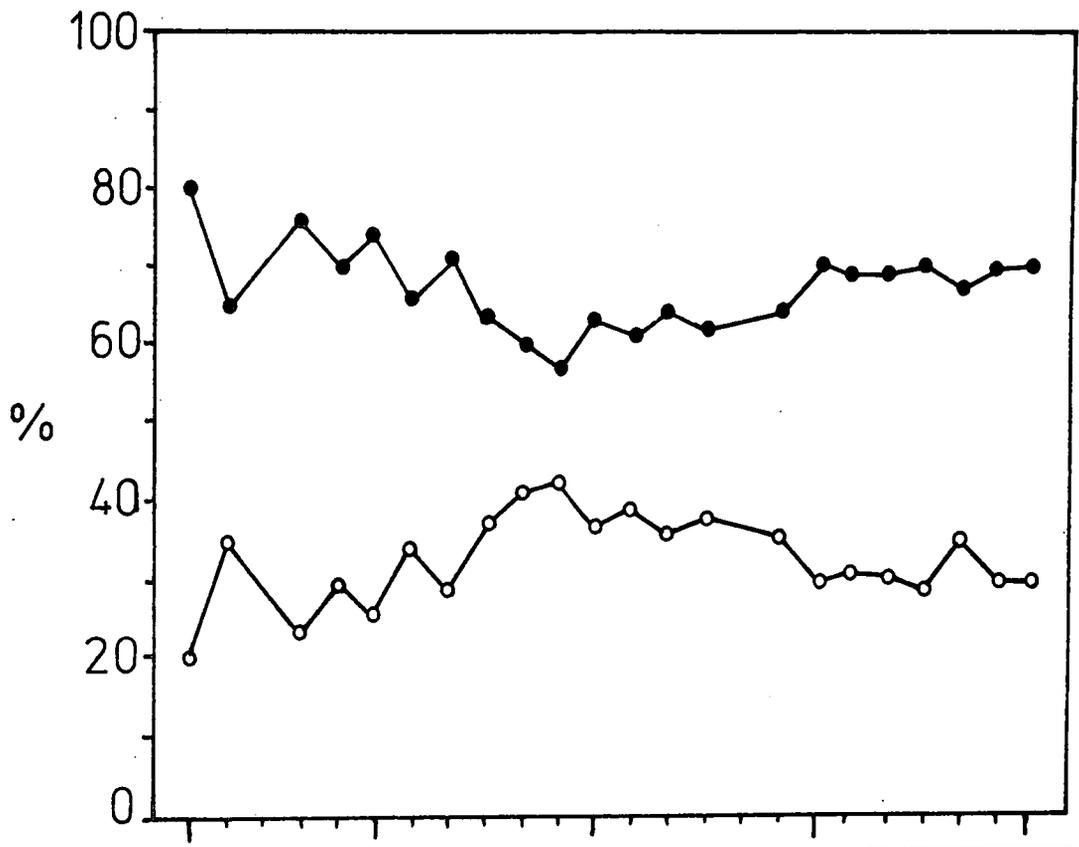
The change in the proportion of cytoplasmic and chloro-  
plastic rRNA extracted by the detergent-phenol method  
from successive leaves along the phyllotaxy.

- Cytoplasmic rRNA
- Chloroplatic rRNA

Figure 4.30

The change in the proportion of the component cytoplasmic  
and chloroplatic rRNA fractions extracted by the detergent-  
phenol method from successive leaves along the phyllotaxy.

- $1.3 \times 10^6$  mol. wt. (cytoplasmic)
- $1.05 \times 10^6$  mol. wt. (chloroplatic)
- $0.7 \times 10^6$  mol. wt. (cytoplasmic)
- $0.56 \times 10^6$  mol. wt. (chloroplatic)



This pattern of change, as well as the order of magnitude involved, was very similar to that evident under DEP-SDS RNA extraction and again no accelerated loss of chloroplast rRNA was observed in the oldest leaves. With regard to individual high molecular weight rRNA fractions the constituent cytoplasmic and chloroplastic components each followed trends similar to those indicated above for total cytoplasmic and total chloroplastic rRNA respectively (figure 4.30) as was also the case with the DEP-SDS extracted material. The percentage proportions of the different high molecular weight fractions were converted to milligram yields in the same manner as that described for DEP-SDS extracted RNA, using the TCA- extracted RNA yield as the basis for the calculations. When these results were expressed as either a yield per leaf or yield per gram fresh weight the overall changes in total cytoplasmic or total chloroplastic rRNA showed similar trends to those previously observed for these fractions in the DEP-SDS extracted RNA (figures 4.31, 4.32). The changes in total cytoplasmic rRNA were similar to the changes in total RNA as observed in the quantitative TCA extracts. With the chloroplast rRNA presumed net synthesis in the youngest leaves caused less pronounced changes in amount in these leaves though this effect was less evident than with the DEP-SDS extracted material.

The phenol extracted RNA resuspended in magnesium containing buffer was also fractionated in EDTA-containing

Figure 4.31

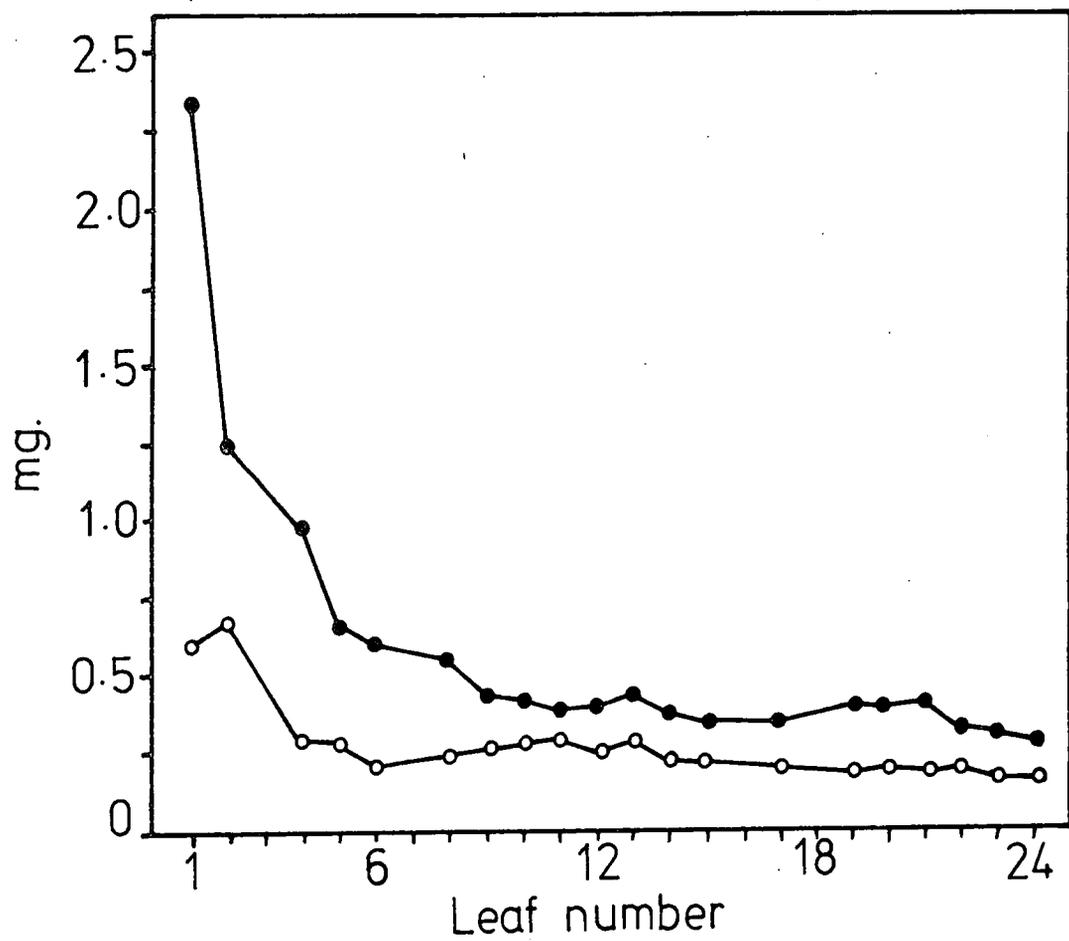
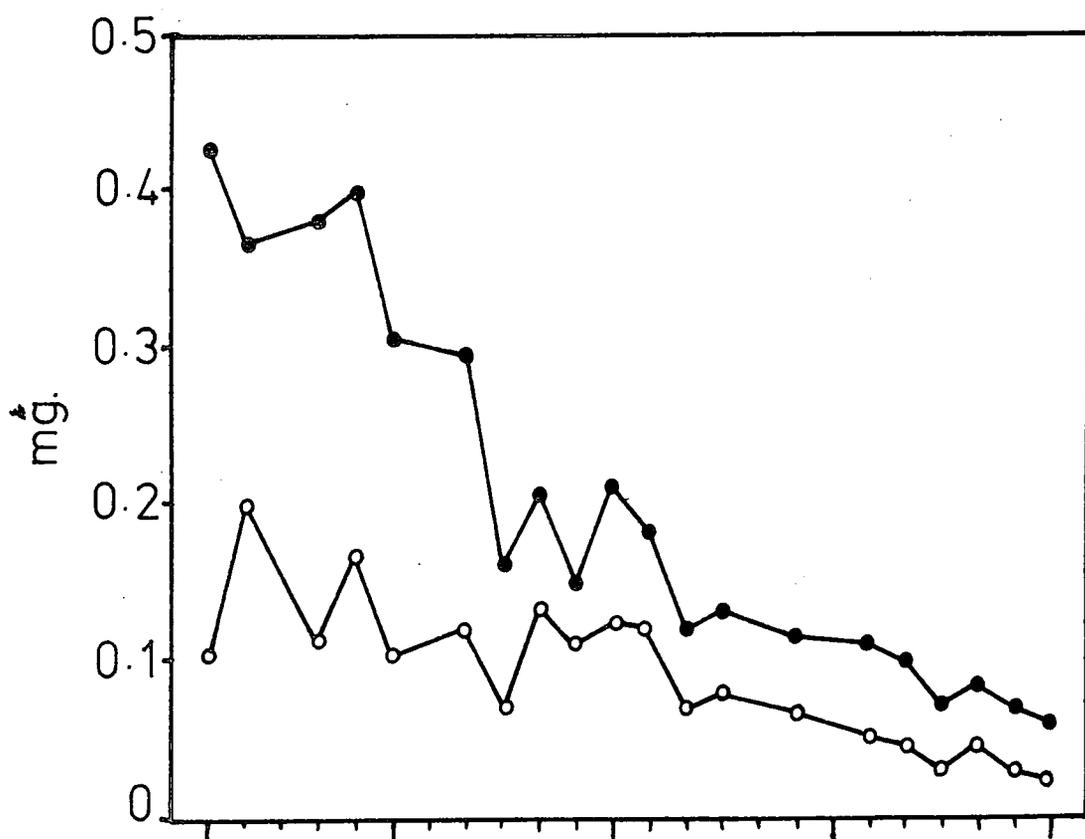
The change in total yield per leaf of cytoplasmic and chloroplastic rRNA from successive leaves along the phyllotaxy. RNA extracted by the detergent-phenol method.

- Cytoplasmic rRNA
- Chloroplastic rRNA

Figure 4.32

The change in yield per gram fresh weight of leaf lamina of cytoplasmic and chloroplastic rRNA from successive leaves along the phyllotaxy. RNA extracted by the detergent-phenol method.

- Cytoplasmic rRNA
- Chloroplastic rRNA



buffer. The labile  $1.05 \times 10^6$  molecular weight chloroplast fraction was unstable under these conditions and further dissociation products, in addition to the  $0.42 \times 10^6$  molecular weight fraction seen on magnesium electrophoresis, were observed on the gels. Using the  $1.3 \times 10^6$  and  $0.7 \times 10^6$  molecular weight cytoplasmic rRNA peaks as internal molecular weight standards the apparent molecular weights of all the dissociation fractions were determined graphically from a plot of log (molecular weight) against migration. The average apparent molecular weight values obtained for these fractions were  $0.95 \times 10^6$ ,  $0.59 \times 10^6$ ,  $0.49 \times 10^6$ ,  $0.42 \times 10^6$ ,  $0.38 \times 10^6$ ,  $0.32 \times 10^6$  and  $0.27 \times 10^6$  (figure 4.33). These fractions could be discerned in most samples from leaves along the ageing sequence. The consistent presence of a fraction of apparent molecular weight of  $0.27 \times 10^6$  was additional to those fractions previously observed with EDTA electrophoresis of the DEP-SDS RNA extracts from leaves during sequential ageing. This may have arisen by ribonuclease action possibly as a result of activation of the enzyme in the presence of EDTA (Gagnon and de Lamirande, 1972; Dyer and Payne, 1974), the presence of DEP in the original extractions would have inhibited this activity. The absence of any of these components from particular samples did not show a consistent pattern with leaf age and was therefore probably more dependant on the extraction and/or fractionation conditions. Thus as

Figure 4.33

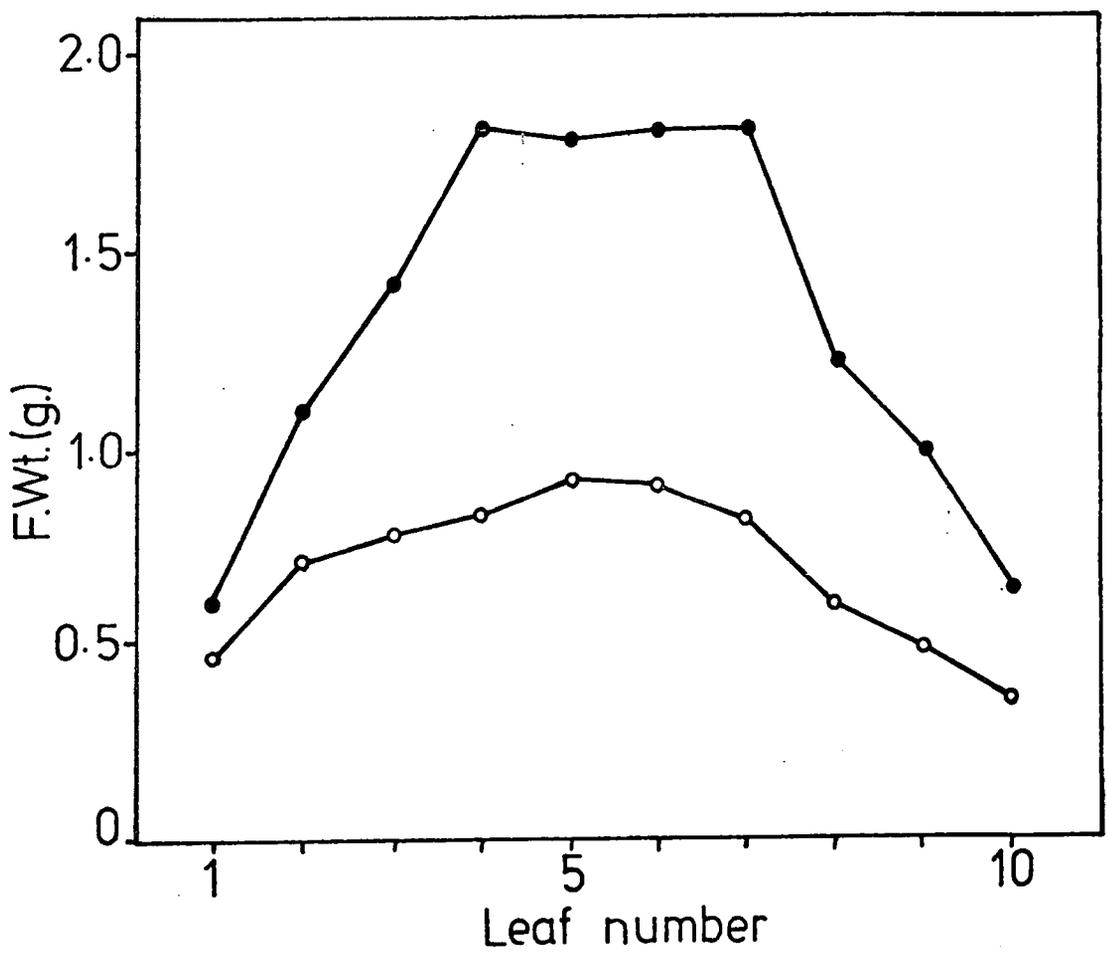
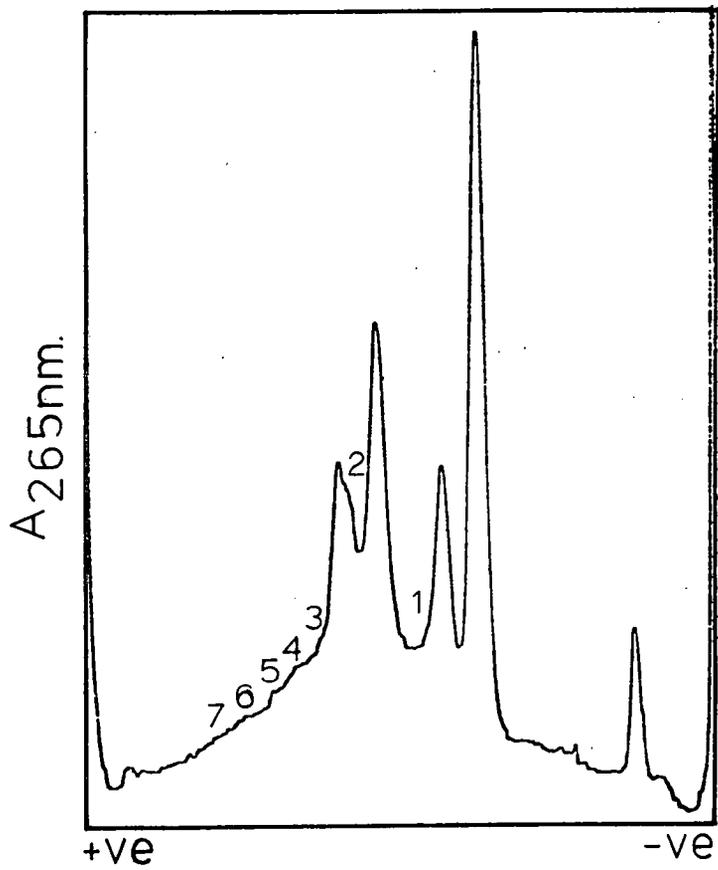
PAGE of high molecular weight rRNA in Loening 'E' buffer  
(Leaf 14 in ageing sequence).

|        |   |                    |          |
|--------|---|--------------------|----------|
| Peak 1 | : | $0.95 \times 10^6$ | mol. wt. |
| Peak 2 | : | $0.59 \times 10^6$ | mol. wt. |
| Peak 3 | : | $0.49 \times 10^6$ | mol. wt. |
| Peak 4 | : | $0.42 \times 10^6$ | mol. wt. |
| Peak 5 | : | $0.38 \times 10^6$ | mol. wt. |
| Peak 6 | : | $0.32 \times 10^6$ | mol. wt. |
| Peak 7 | : | $0.27 \times 10^6$ | mol. wt. |

Figure 4.34

The pattern of change in fresh weight of the leaf laminae  
of successive leaves along the phyllotaxy.

- Healthy plant
- TYMV-infected plant



with DEP-SDS extracted RNA no discernable correlation between the breakdown fractions observed on EDTA electrophoresis and leaf age was observed.

Overall the pattern of changes that emerged from detergent-phenol RNA extraction from leaves along the sequential senescence of chinese cabbage plants was the same as that previously obtained by DEP-SDS RNA extraction. The result therefore confirmed the characterisation studies already described (see 3.6).

#### 4.33 Comparison of uninfected and TYMV-infected plants

The system of sampling each leaf along the spiral phyllotaxy of an individual plant was applied to a comparison of the qualitative changes occurring in a TYMV-infected chinese cabbage plant and a control uninfected plant. A TYMV-infected plant was used from a population that was 60-days old, at this stage the elder of the two leaves that had been inoculated with virus had become senescent. The control plant was taken from a similar but uninfected population cultivated at the same time under the same greenhouse conditions and of the same age. The leaves of the plant were numbered along the spiral phyllotaxy from the youngest leaf sampled to the oldest, and the individual leaf laminae were weighed prior to extraction of the RNA by the DEP-SDS method. The difference in lamina fresh weight between TYMV-infected and uninfected plants plotted in figure 4.34 clearly shows the stunting effect on

growth caused by TYMV-infection. It should be noted that the plants used in this comparison had fewer leaves than those used in the proceeding ageing study (4.31 and 4.32), this was because there was a distinct seasonal change in growth rate apparent in the chinese cabbage plants grown under the conditions used in this study and plants planted in the winter months had a much slower rate of growth than plants grown at other times of the year.

The ratio  $\frac{E_{max}}{E_{min}}$  and  $\frac{E_{max}}{E_{280}}$  taken from the u.v. absorption spectra of the RNA extracts from the leaves of the virus-infected and uninfected plants are compared in Table 14.

Table 14

| Leaf No.  | $\frac{E_{max}}{E_{min}}$ |               | $\frac{E_{max}}{E_{min \ 280 \ nm}}$ |               |
|-----------|---------------------------|---------------|--------------------------------------|---------------|
|           | Uninfected                | TYMV-infected | Uninfected                           | TYMV-infected |
| 1 (young) | 1.37                      | 1.24          | 1.85                                 | 1.51          |
| 2         | 1.52                      | 1.27          | 1.78                                 | 1.63          |
| 3         | 1.43                      | 1.27          | 1.74                                 | 1.63          |
| 4         | 1.35                      | 1.24          | 1.72                                 | 1.65          |
| 5         | 1.44                      | 1.25          | 1.69                                 | 1.63          |
| 6         | 1.31                      | 1.21          | 1.68                                 | 1.61          |
| 7         | 1.35                      | 1.85          | 1.67                                 | 1.58          |
| 8         | 1.27                      | 1.18          | 1.65                                 | 1.60          |
| 9         | 1.19                      | 1.11          | 1.72                                 | 1.56          |
| 10 (old)  | 1.12                      | 1.07          | 1.81                                 | 1.63          |

For each ratio the values for extracts from uninfected leaves were slightly higher than the corresponding value for infected leaf extracts, this was the same relationship as described in the characterisation of the DEP-SDS RNA extraction method (see 3.41). The decline in the value of the spectral ratios with leaf age described above (see 4.31, figure 4.15) was here seen only in the ratio  $E_{\max}/E_{\min}$  and was evident in both infected and uninfected extracts. The value of the wavelength of minimum absorption in the TYMV-infected extracts was slightly variable within the range 239-242nm with a shift towards 242nm occurring in the older leaves.

From the u.v. absorption spectrum RNA yields in the extracts were calculated and the values for the yields per leaf and per gram fresh weight of leaf lamina are plotted in figures 4.35 and 4.36. On a per leaf basis the extracts from uninfected tissue showed a similar trend to that previously observed for total yields of RNA from qualitative RNA extraction (see figure 4.18) with a steady decline in the RNA content of each leaf following on from a relatively constant level. The low yield obtained in the youngest leaf was a consequence of inefficient homogenisation of this small tissue sample by the 'Virtis -45' tissue homogeniser used. The infected leaf extracts showed a generally similar trend though higher RNA levels were recorded despite the leaves being physically

Figure 4.35

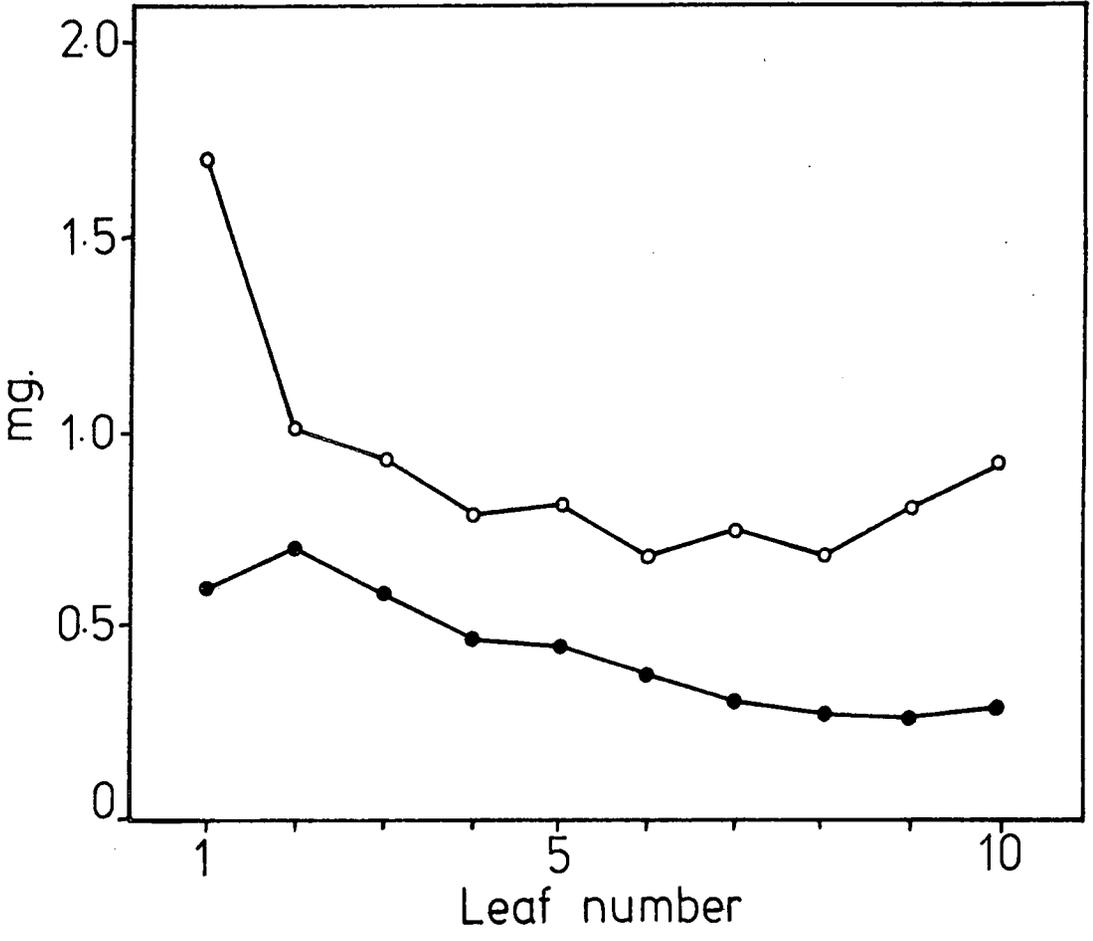
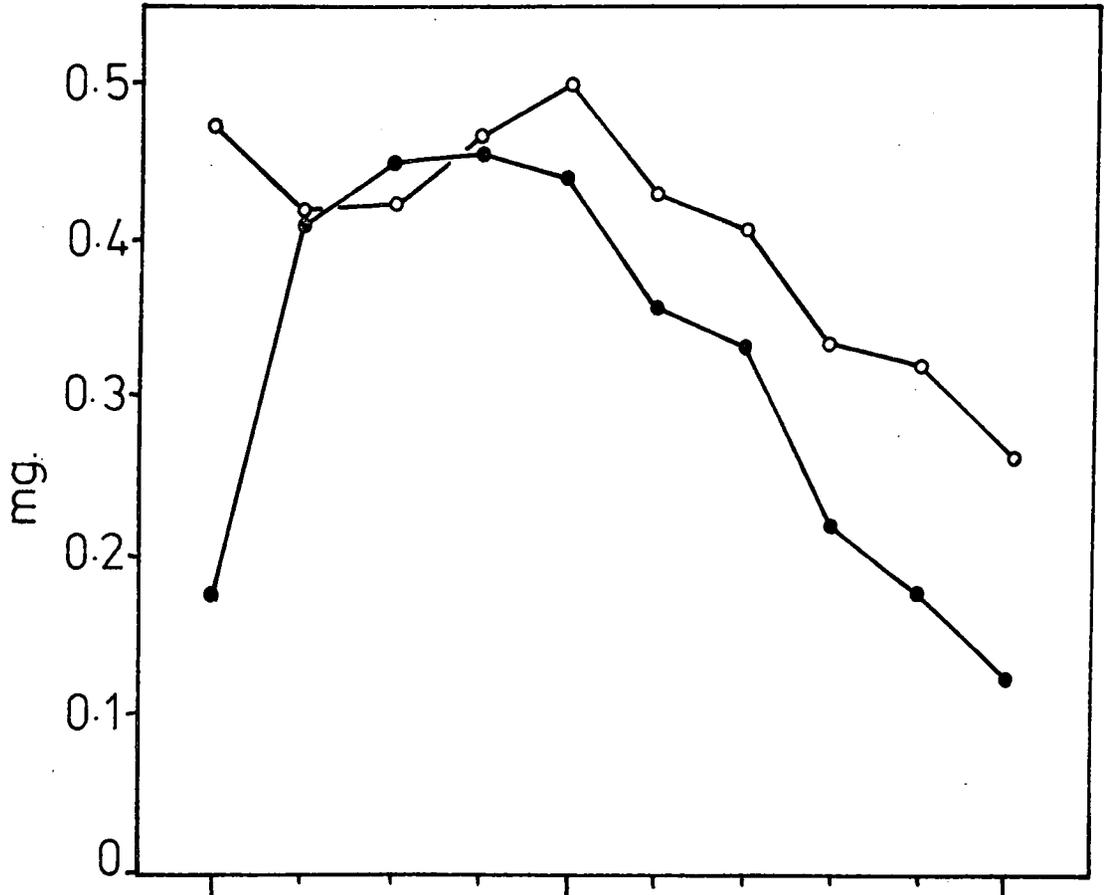
The total yield of RNA per leaf from successive leaves along the phyllotaxy. RNA extracted by the DEP-SDS method.

- Healthy plant
- TYMV-infected plant

Figure 4.36

The yield of RNA per gram fresh weight of leaf lamina from successive leaves along the phyllotaxy. RNA extracted by the DEP-SDS method.

- Healthy plant
- TYMV-infected plant

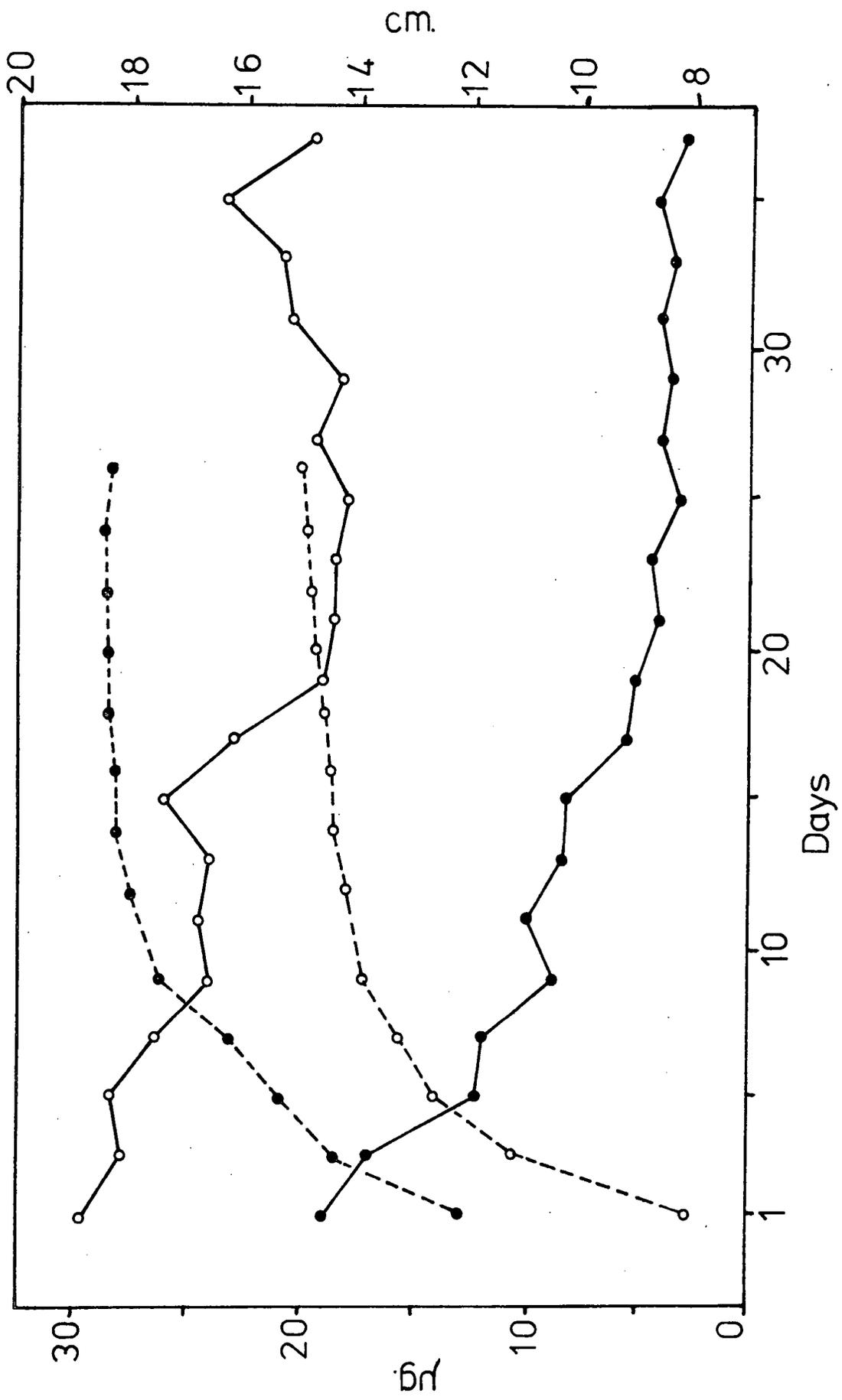


smaller, this increase in RNA level can be attributed to the contribution to the u.v. absorption of the extract made by the presence of the virus. The concentration of RNA in the leaves (figure 4.36) again showed similar curves of decline to those previously described, and the increased level of RNA plus virus in the infected leaf extracts was again apparent. Also noticeable in the oldest leaves was an increase in RNA/virus concentration, a similar increase in yield in ageing TYMV-infected leaves was observed when TCA extracts of total RNA were made from infected leaves when following the ageing changes in an individual leaf. The total RNA changes occurring in the fifth leaf of two populations of plants (TYMV-infected and uninfected) were examined in a similar manner to that already described in 4.2. Figure 4.37 shows the RNA yields that were obtained and clearly indicates the rise in RNA yield in the last stage of the life cycle of the TYMV-infected leaves. Measurements of leaf length are included in the figure to show the RNA changes in the context of the growth of the leaf. A possible reason for the rise in yield which is apparent in the infected leaf extracts is that membrane degradation in these older leaves increases the recovery of material, particularly perhaps of the virus, from these leaves. A reduction in fresh weight could also have resulted in this effect but this was less likely in view of the fact that a similar change was not observed in uninfected leaf extracts.

Figure 4.37

The change with time in the average length of the 5th leaf and RNA content of discs excised from the 5th leaf of populations of chinese cabbage plants.

- RNA content, healthy plants
- RNA content, TYMV-infected plants
- leaf length (cm), healthy plants
- leaf length (cm), TYMV-infected plants



Also from the ageing of the fifth leaf in which the yields were not expressed relative to fresh weight an absolute increase in yield was evident. A difficulty that existed in considering yields from extracts of leaves systemically infected with TYMV was the absence of suitable control material to enable determination of the proportion of the total yield observed that was attributable to the virus. With inoculated half-leaves Matthews (1958) and Francki and Matthews (1962) found that as TYMV increased in amount so the level of total RNA increased by an amount equivalent to that found in the virus.

The qualitative RNA extracts were fractionated into high molecular weight rRNA components by polyacrylamide gel electrophoresis using a magnesium buffer system (see 3.22.2), this buffer being used to maintain the integrity of the chloroplast  $1.05 \times 10^6$  fraction thus facilitating estimation of the various high molecular weight rRNA fractions. Typical gel electrophoretograms from different leaves along the ageing sequence of healthy and TYMV-infected plants are illustrated in figures 4.38 and 4.39 respectively. The uninfected leaf sequence appeared to show little qualitative difference with age, any increase in the proportion of chloroplast rRNA due to synthesis of this fraction in the youngest leaves was not as obvious as that previously described for sequential leaf ageing. In the oldest leaf a substantial proportion of chloroplast rRNA remained. In the infected plant leaf sequence (figure 4.39)

Figure 4.38

PAGE of RNA extracted by the DEP-SDS method from different leaves along the phyllotaxy of a healthy chinese cabbage plant.

- A. Leaf 1 (young)
- B. Leaf 4
- C. Leaf 7
- D. Leaf 10 (old)

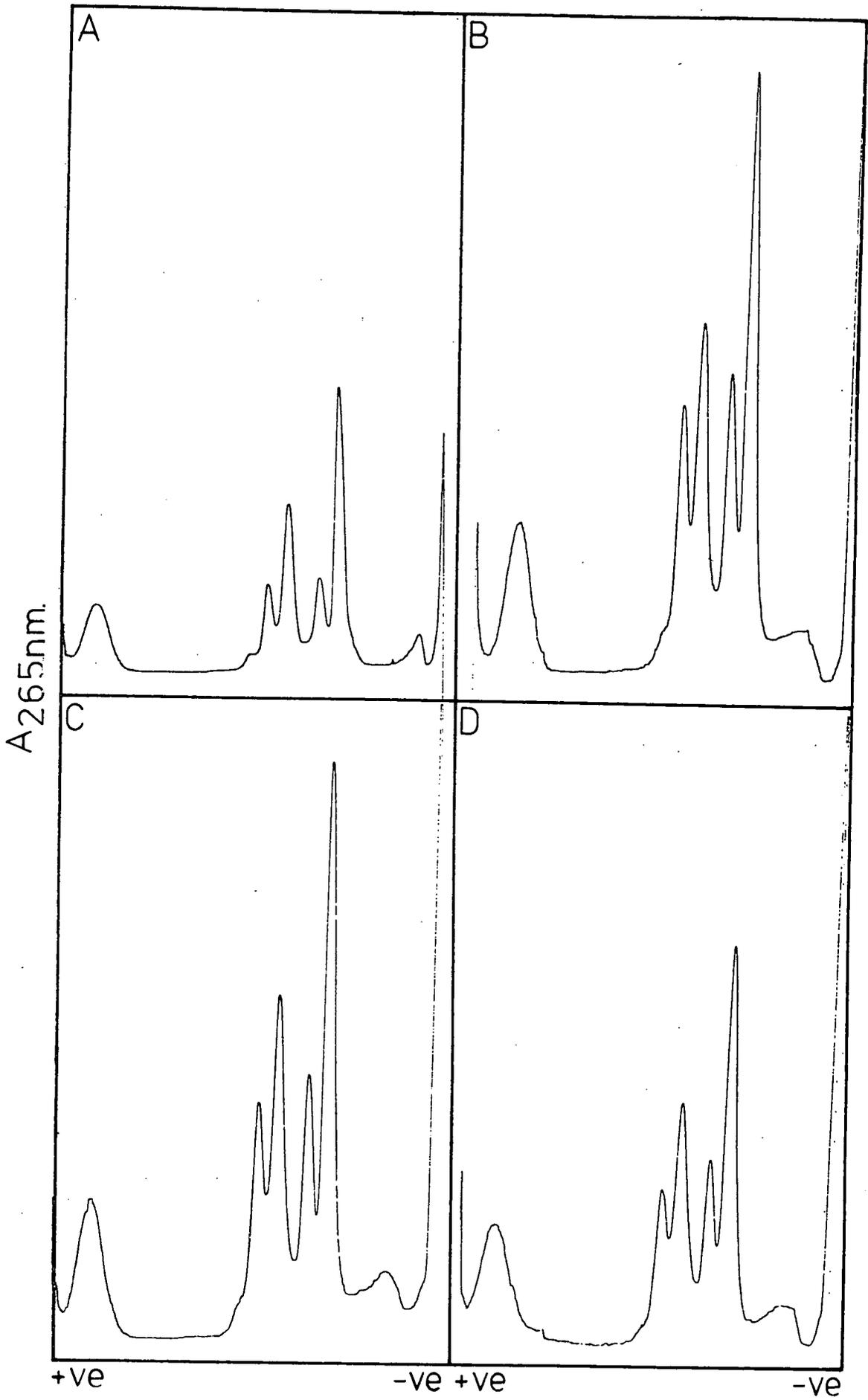
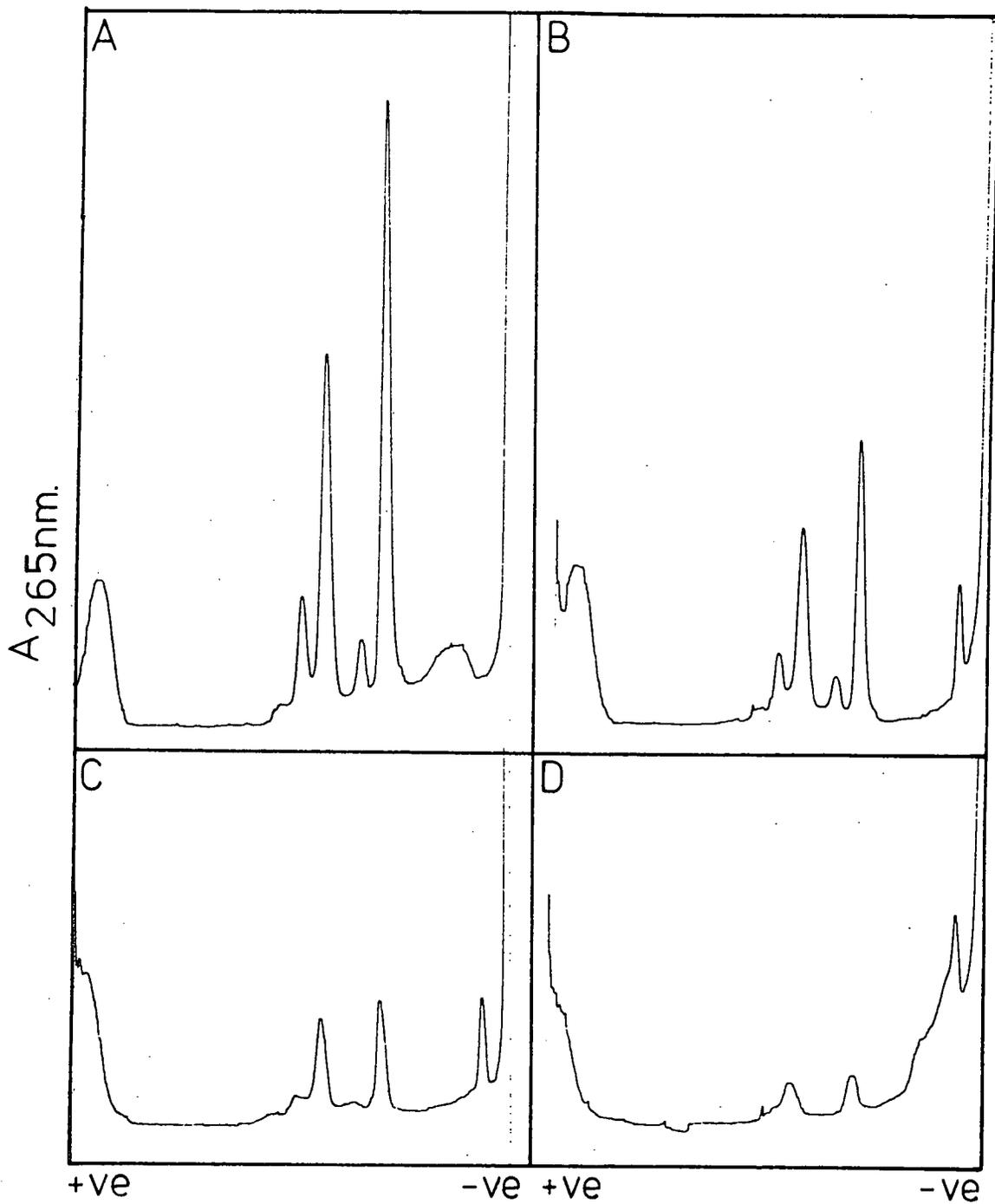


Figure 4.39

PAGE of RNA extracted by the DEP-SDS method from different leaves along the phyllotaxy of a TYMV-infected chinese cabbage plant.

- A. Leaf 1 (young)
- B. Leaf 4
- C. Leaf 7
- D. Leaf 10 (old)



distinct high molecular weight rRNA peaks were evident on all gels. In all cases the chloroplastic components represented a smaller proportion of the total high molecular weight rRNA than in the uninfected leaf extracts and the  $1.05 \times 10^6$  chloroplast rRNA also seemed to be dissociated to a greater degree in the infected leaf samples. These subjective observations were quantified by the measurement of the peak area weights of the gel traces. In the uninfected extracts there was an initial small rise (6%) in the proportion of total chloroplast ribosomal RNA in the youngest leaves, this level was then maintained in subsequent leaves with possibly an indication of a slight decline in the oldest leaves (figure 4.40a). The changes that occurred in the constituent cytoplasmic ( $1.3 \times 10^6$  and  $0.7 \times 10^6$ ) and chloroplast ( $1.05 \times 10^6$  plus  $0.42 \times 10^6$ , and  $0.56 \times 10^6$ ) fractions paralleled the changes in total cytoplasmic and total chloroplastic rRNA respectively. The overall pattern of changes in the uninfected leaf extracts were therefore similar to those previously described. The proportional changes observed in the TYMV-infected leaf samples (figure 4.40b) showed certain differences to the uninfected results with the average proportion of chloroplast rRNA being approximately 15% below that in the uninfected samples and there was also a decline in the proportion of chloroplast rRNA in the older leaves. The magnitude of this decline in the oldest two leaves may have been exaggerated due to difficulties in defining the

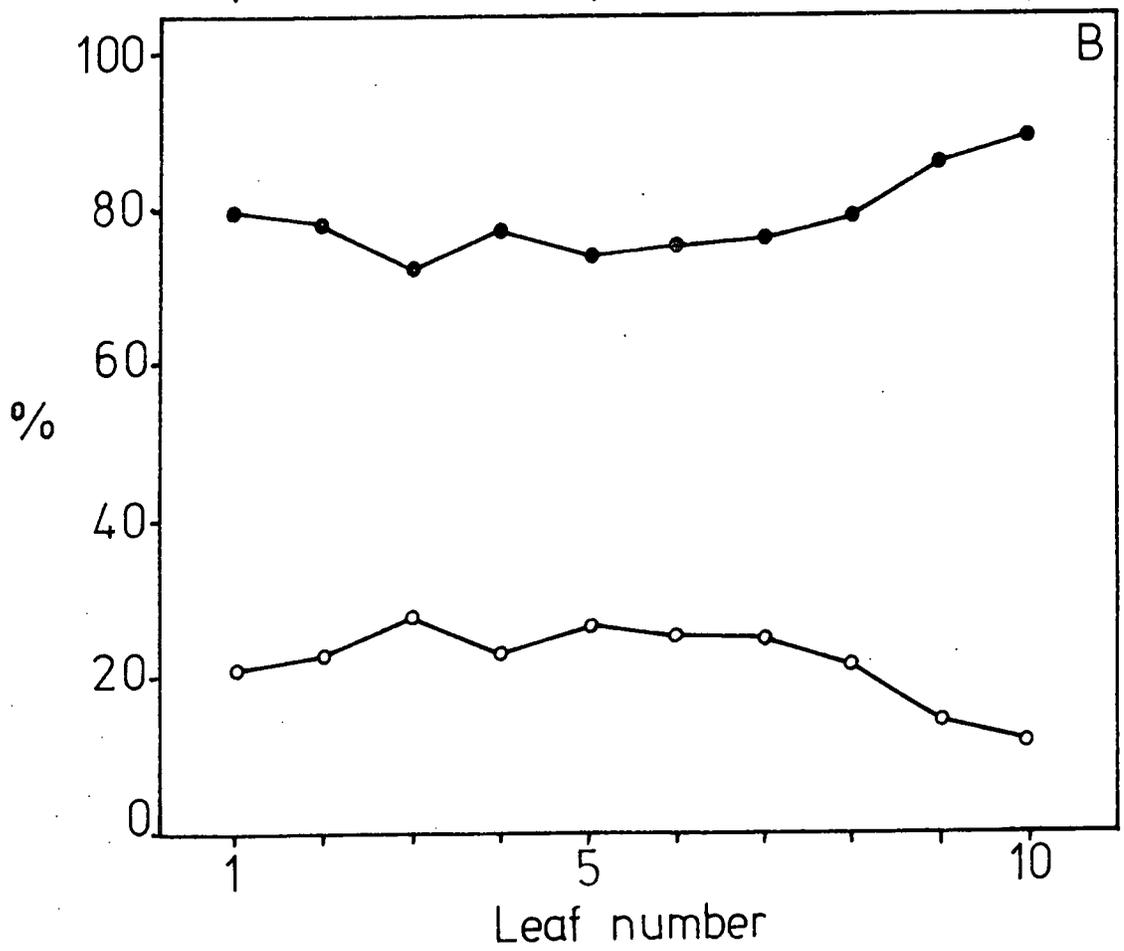
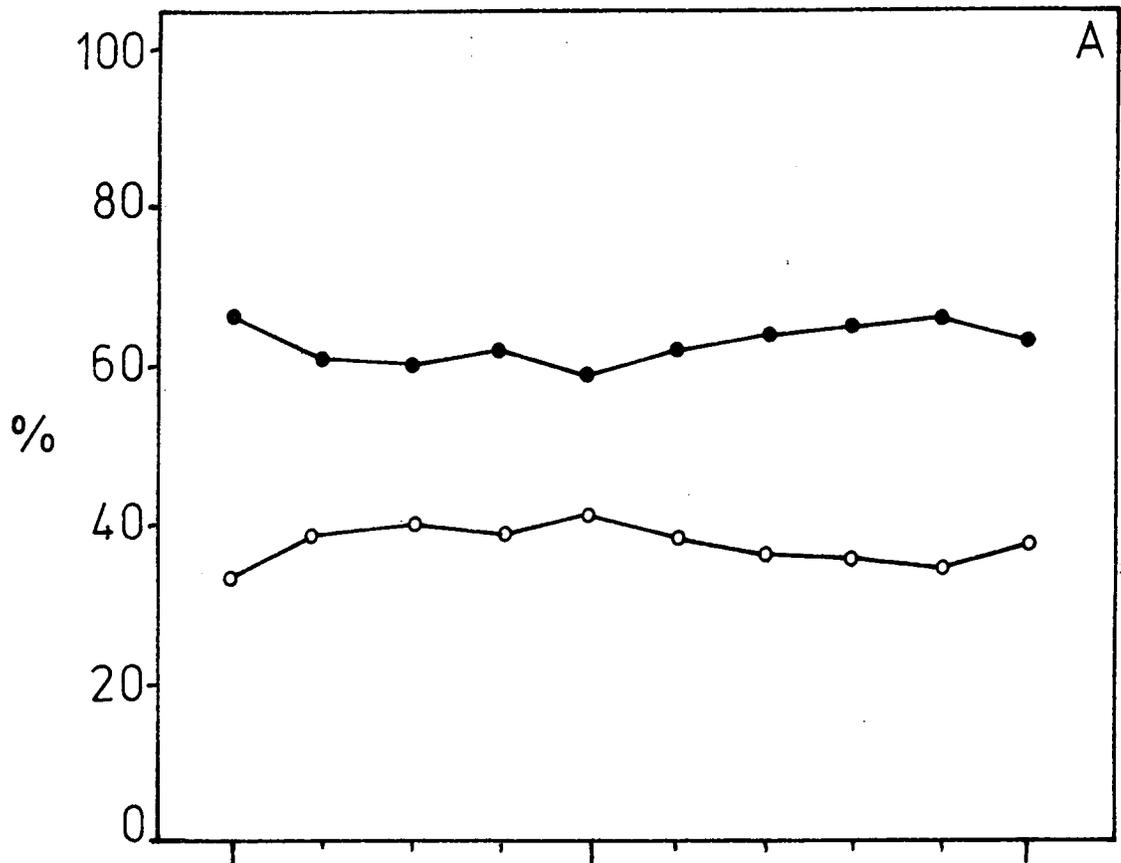
Figure 4.40

The change in proportion of cytoplasmic and chloro-  
plastic rRNA extracted by the DEP-SDS method from  
successive leaves along the phyllotaxy

A. Healthy plant

B. TYMV-infected plant

- cytoplasmic rRNA
- chloroplastic rRNA



peak areas on the gel traces in which the amount of chloroplast rRNA was very low (figure 4.39). A further difference to the uninfected samples was in the proportions of the constituent cytoplasmic and chloroplastic fractions. The changes in the  $1.3 \times 10^6$  and  $0.7 \times 10^6$  or the  $1.05 \times 10^6$  plus  $0.42 \times 10^6$ , and  $0.56 \times 10^6$  fractions paralleled the trends observed in the total cytoplasmic or chloroplastic rRNA respectively, however, the heavy and light rRNA species in both the cytoplasmic and chloroplastic fractions were present in an approximately 1 : 1 ratio (average ratios of 1.06 and 0.96 respectively). The stability of the  $1.3 \times 10^6$  cytoplasmic high molecular weight rRNA and the correction applied to the measured area of the  $1.05 \times 10^6$  chloroplasts rRNA (by addition of the area of the dissociation product) suggested that breakdown of these two fractions was not the contributory cause for the observed low ratios. The possibility existed, therefore, that there was a preferential loss of the high molecular weight rRNA species in the extracts of RNA from TYMV-infected leaves which may have been the result of some residual complexing.

The peak area weight proportions for the uninfected leaf samples were converted to milligram yields in each high molecular weight rRNA fraction using the total RNA yield per leaf or per gram fresh weight obtained for the DEP-SDS qualitative RNA extraction. A similar treatment was not possible for the infected leaf samples because in addition to the rRNA proportions

the proportion of the total yield attributable to the virus was required. This information was not available from the gel traces as a constant proportion of the virus could not be assumed because of variations in the mosaic in the different leaves along the ageing sequence.

In the uninfected leaves the changes that occurred in both total cytoplasmic and total chloroplastic rRNA content per leaf were similar in that both showed an increase in total yield in the youngest leaves followed by a steady decline in yield in the remaining leaves (figure 4.41), this decline commencing in leaves that had not achieved their maximum size. A preferential net increase in chloroplast rRNA as was noticed previously (see figure 4.22) was not evident, the low recovery of RNA from the youngest leaf (noted above) may have influenced this. The changes in the constituent cytoplasmic and chloroplastic ribosomal RNA species were parallel to those of total cytoplasmic and chloroplastic ribosomal RNA respectively. On a yield per gram fresh weight basis the total cytoplasmic and total chloroplastic rRNA again showed similar overall changes (figure 4.42) and parallel trends were also evident in the component cytoplasmic and chloroplastic rRNA species. The pattern of RNA yield seen here was similar to that previously described (see figure 4.23). The low yield in the youngest leaf was again due to the problems incurred on homogenisation as indicated above.

The magnesium resuspended RNA samples from both TYMV-

Figure 4.41

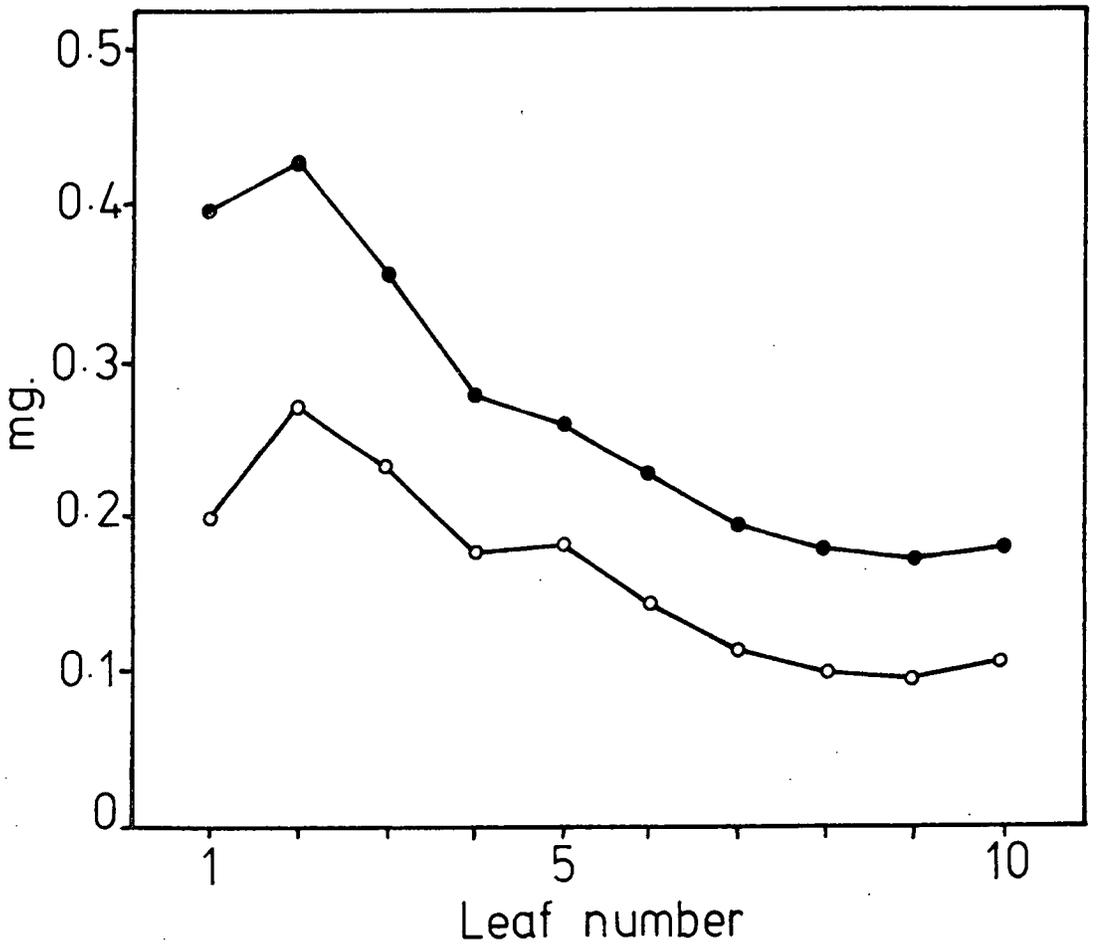
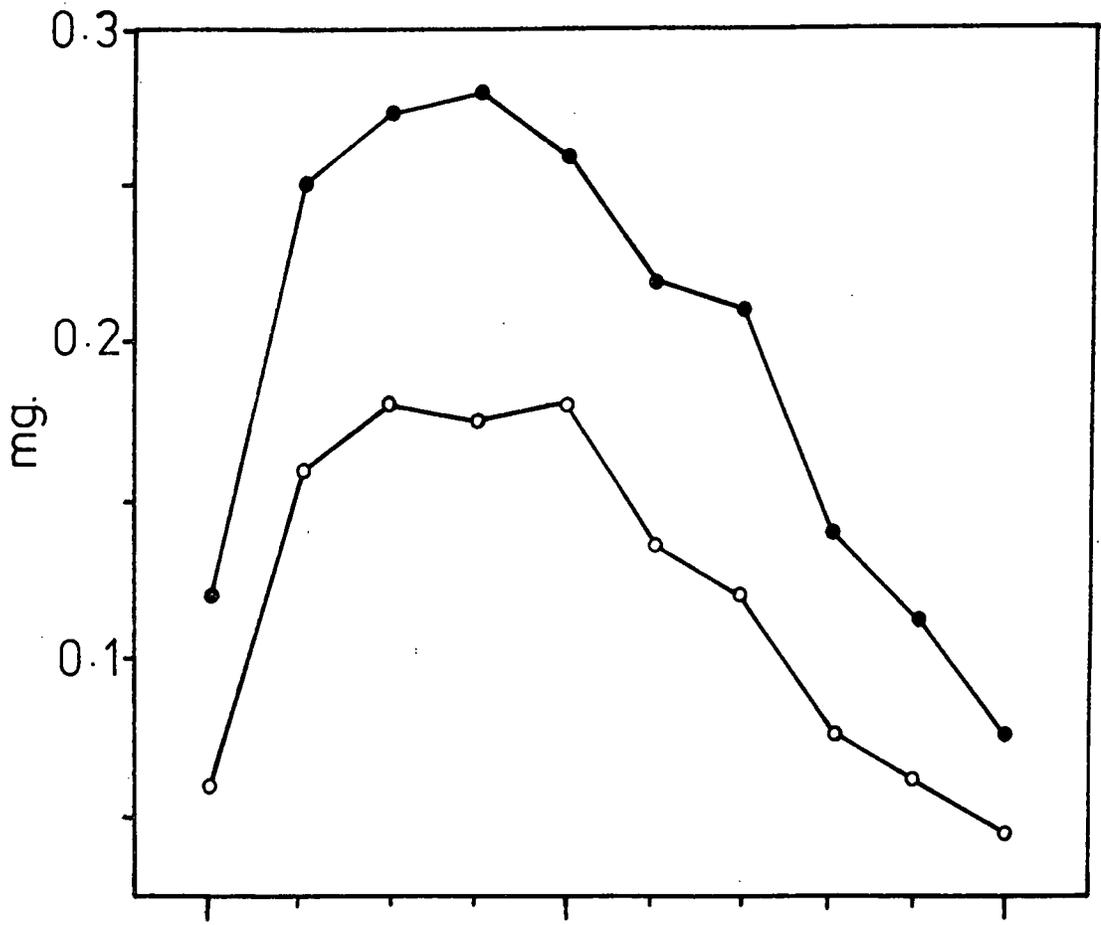
The change in total yield per leaf of cytoplasmic and chloroplastic rRNA from successive leaves along the phyllotaxy. RNA extracted by the DEP-SDS method.

- Cytoplasmic rRNA
- Chloroplastic rRNA

Figure 4.42

The change in yield per gram fresh weight of leaf lamina of cytoplasmic and chloroplastic rRNA from successive leaves along the phyllotaxy. RNA extracted by the DEP-SDS method.

- Cytoplasmic rRNA
- Chloroplastic rRNA



infected and uninfected leaves were fractionated by electrophoresis in EDTA-containing buffer in order to compare the pattern of dissociation products produced by this treatment and gel profiles typical of those obtained are illustrated in figure 4.43. Because of the presence of magnesium the appearance and apparent molecular weight of individual dissociation products were a little variable. The range of fractions observed in the uninfected leaf extracts were the same as that described in 4.31 and again no difference in the pattern of dissociation products with leaf age was discernable. The extracts from the younger TYMV-infected leaves showed a similar pattern of dissociation fractions as that observed in the uninfected samples. Fewer breakdown fractions were observed in the older leaves but this was probably a result of a failure to detect minor fractions owing to the decreased absolute yield of chloroplast rRNA in these leaves.

Fractionation of the leaf samples on 7.5% polyacrylamide gels in order to examine the low molecular weight RNA revealed no qualitative difference between TYMV-infected and uninfected leaf extracts (figure 4.44). In neither the uninfected nor the TYMV-infected samples was there any indication of qualitative changes in the low molecular weight RNA fractions with ageing, a result consistent with that previously described (see 4.31).

The major qualitative difference between uninfected and TYMV-infected leaf RNA extracts in the above results was a marked

Figure 4.43

PAGE of high molecular weight rRNA in Loening 'E'  
buffer (Leaf 4 in ageing sequence).

- A. Healthy plant
- B. TYMV-infected plant

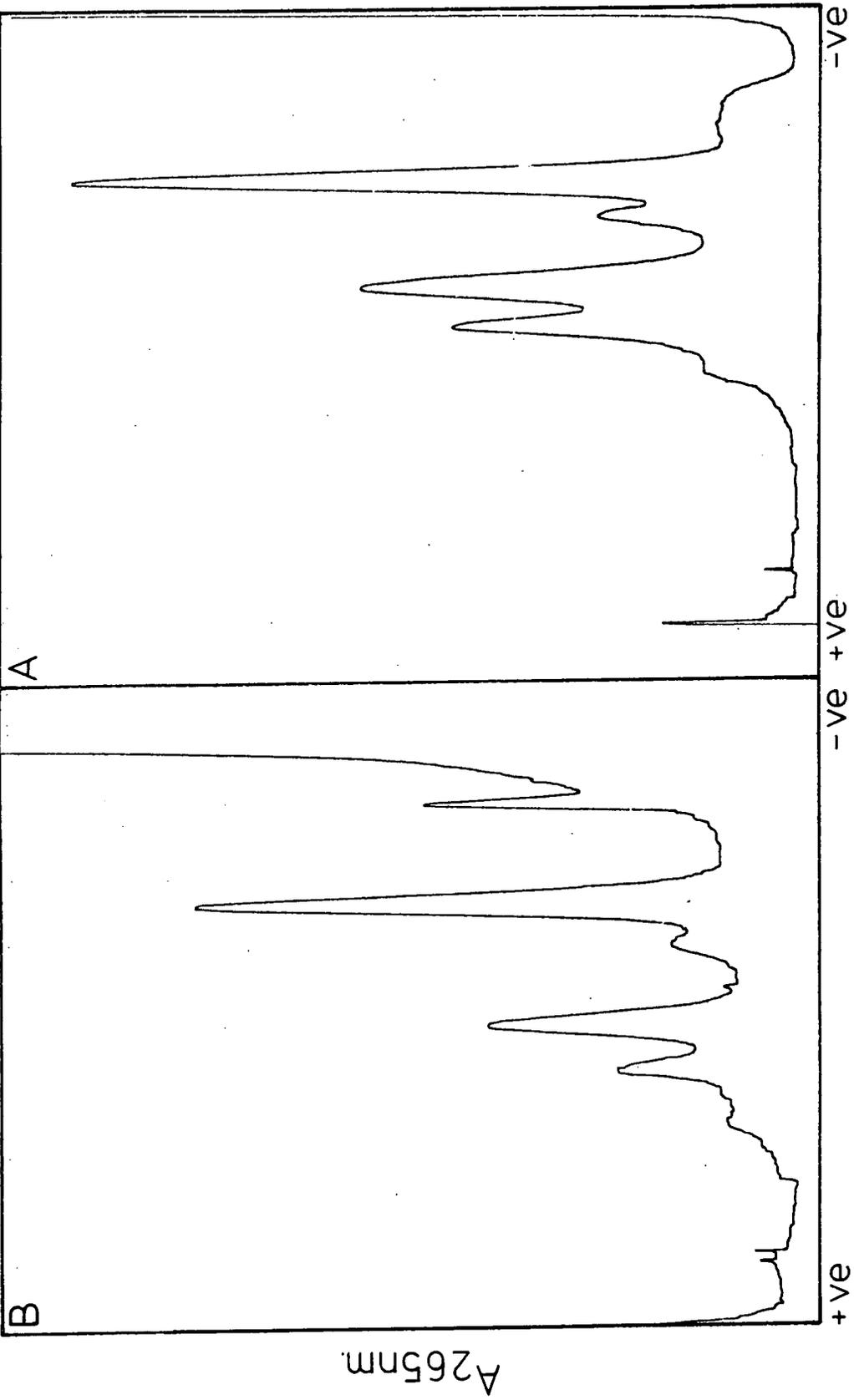
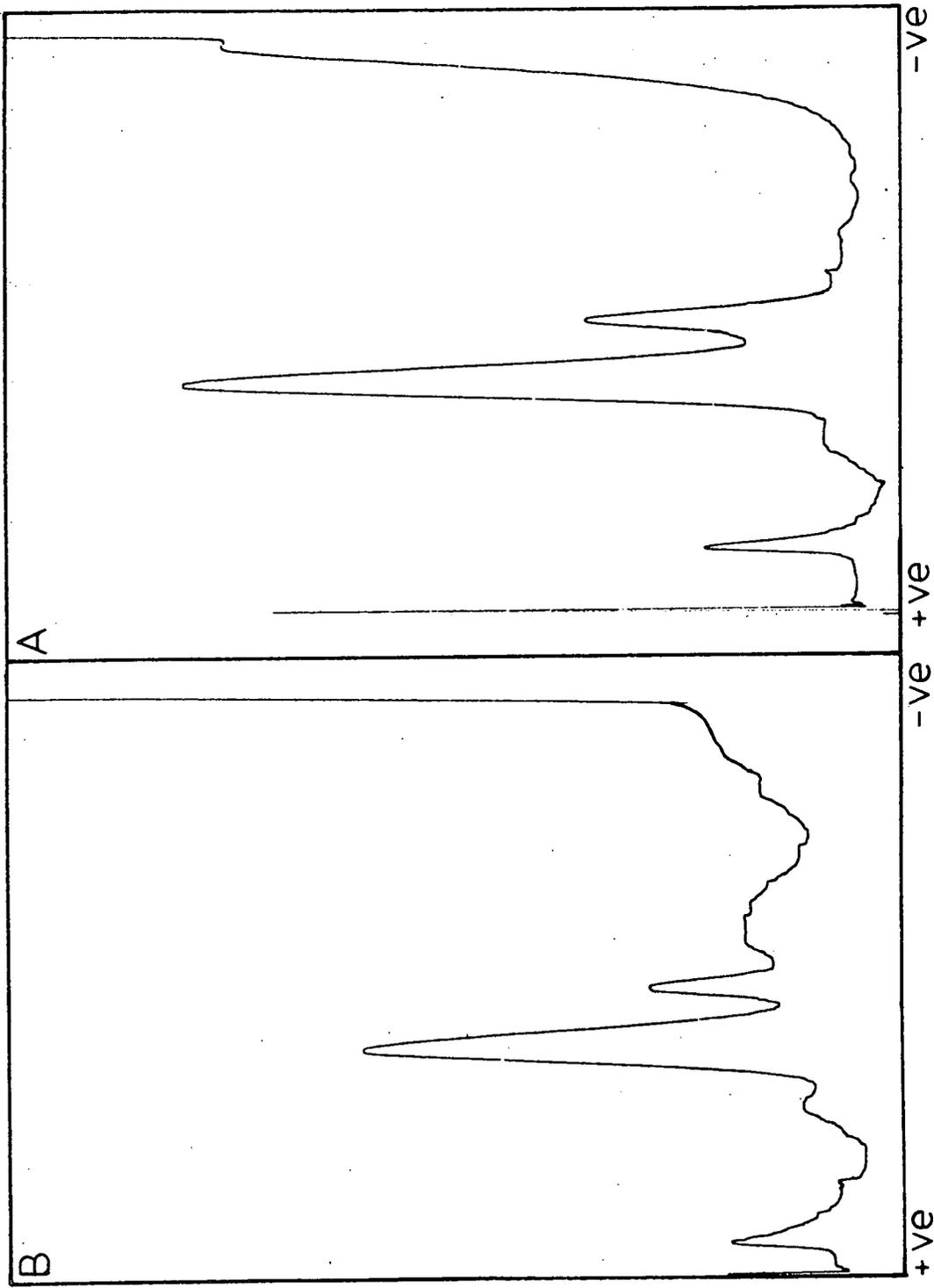


Figure 4.44

PAGE of low molecular weight RNA.

- A. Healthy plant
- B. TYMV-infected plant

A<sub>265nm</sub>.



reduction in the proportion of chloroplast rRNA and this difference did not appear to change with leaf age. The results also highlighted the problems of quantitative estimation of host RNA in the TYMV-infected tissue. Despite the disadvantages indicated this type of treatment has not previously been possible with TYMV-infected plant material because of the occurrence of TYMV-RNA-host RNA complex formation during the usual RNA extraction procedures.

#### 4.4 Sequential leaf ageing - Ribosome changes

The changes that occurred in the ribosome population during leaf ageing were examined in a similar way to that in which nucleic acid changes had been studied. Plants were chosen that were average members of either a three month old TYMV-infected or uninfected population of chinese cabbage plants grown under glasshouse conditions. The leaves on the plants were numbered along the spiral phyllotaxy from the youngest leaf on the plant that could be practically handled to the oldest. The plants were used when the leaves used for inoculation of the virus in the infected population were senescing; uninfected plants of the same age were used as controls.

Each leaf was measured and weighed prior to extraction and the characteristic smaller size of the first leaves produced by the plant were clearly evident in the uninfected leaf lamina fresh weight changes (figure 4.45). The inoculated leaves (i.e. the oldest two leaves) of the infected plant showed a similar size increase as corresponding leaves on the uninfected plant but in subsequent leaves the typical stunting effect of TYMV infection was evident. Changes in leaf length paralleled the fresh weight changes described.

The extraction of polyribosomes from the leaves was carried out as previously detailed (see 3.24) from 1.0g samples of leaf lamina or the whole leaf lamina, whichever was the least.

Figure 4.45

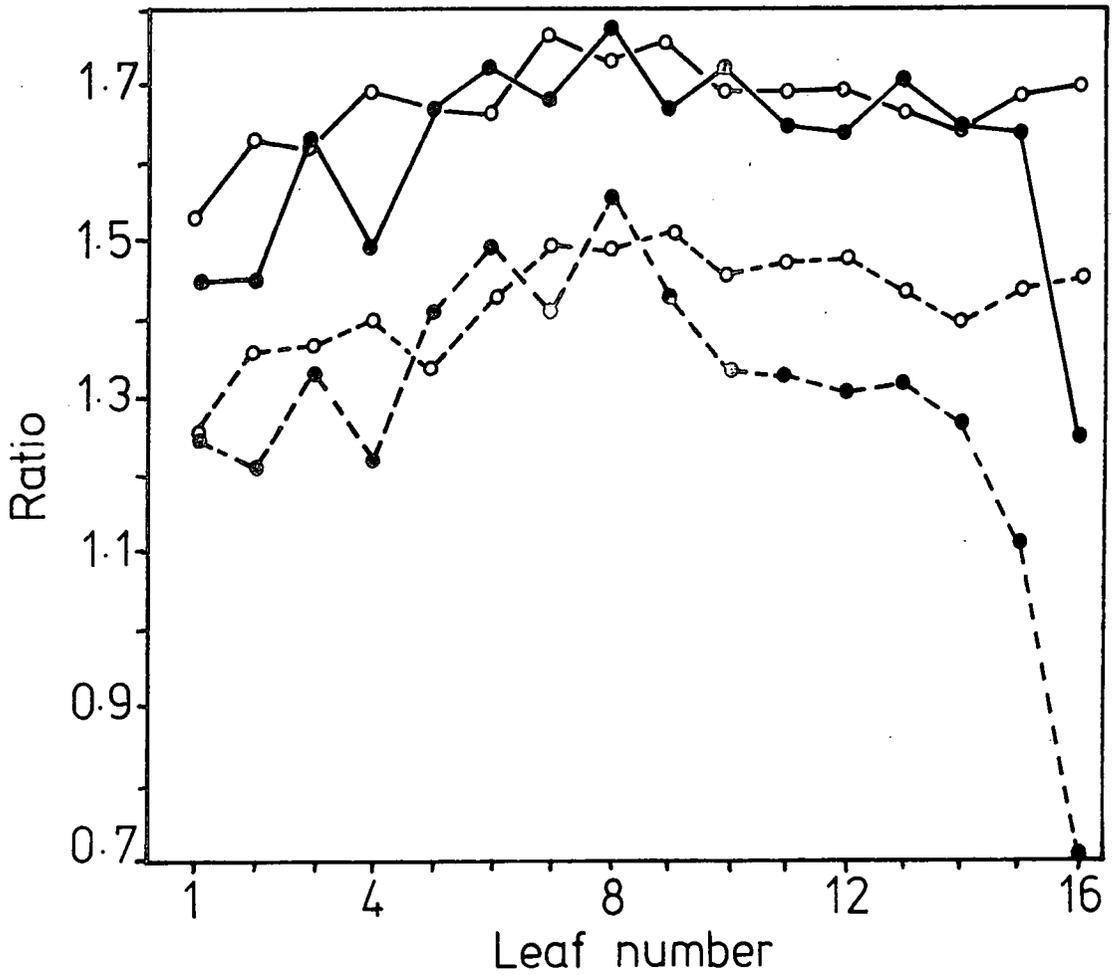
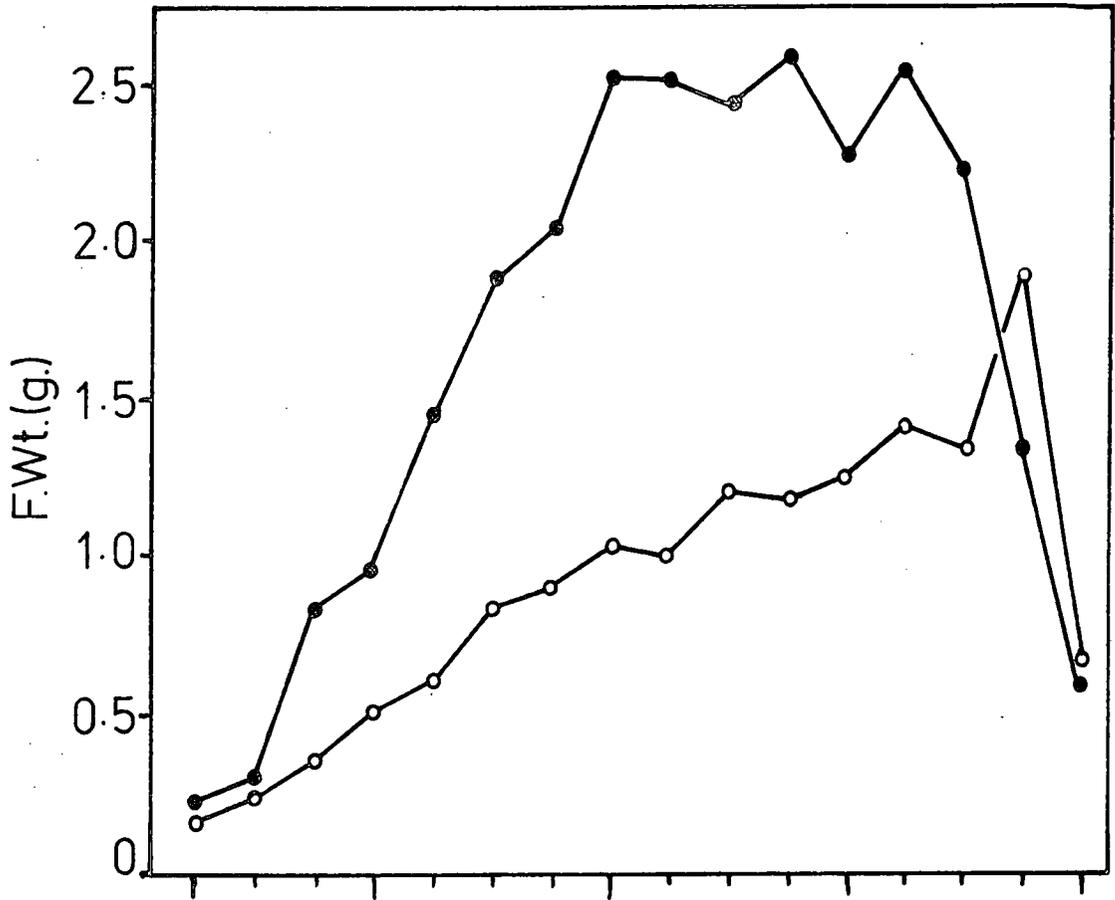
The pattern of change in fresh weight of the leaf laminae of successive leaves along the phyllotaxy.

- Healthy plant
- TYMV-infected plant

Figure 4.46

The change in the value of the u.v. absorption spectrum ratios of ribosome extracts from successive leaves along the phyllotaxy.

- E260/E280 healthy plant
- E260/E280 TYMV-infected plant
- Emax/Emin healthy plant
- Emax/Emin TYMV-infected plant



After extraction the 105,000 g. ribosome pellets were stored at  $-20^{\circ}\text{C}$  until they were fractionated. Prior to fractionation the pellets were resuspended in a small known volume of ribosome buffer (see 3.24) and the u.v. absorption spectrum of an aliquot recorded. The values for the spectral ratios  $E_{\text{max}}/E_{280}$  and  $E_{\text{max}}/E_{\text{min}}$  were seen to change during the course of leaf ageing (figure 4.46). The values of the ratios for the uninfected extracts increased from the youngest leaf to a maximum at leaf 8, there was subsequently a decline which was markedly accelerated in the oldest leaf. This decline was seen to be of a greater proportion in the  $E_{\text{max}}/E_{\text{min}}$  ratio values suggesting contamination with u.v. absorbing secondary metabolites in extracts from the older leaves. The rise in the value of the spectral ratios in the younger leaves was also evident in extracts from TYMV-infected tissue, but in contrast to the uninfected ratios, these were seen not to decline greatly in the older leaves. The presence of the virus particles in the extracts of these older leaves may have helped to maintain the spectral ratio values.

Estimates of the total yield of ribosomes in each sample were determined from the u.v. absorption spectra, the value for TYMV-infected tissue including in the total yield the contribution made by the presence of the virus in the extracts. In terms of the yield of ribosomes per leaf the uninfected leaves showed a similar trend to that of total ribosomal RNA

already described (see figure 4.18) with the greatest absolute level of ribosomes present in the youngest leaf and this level steadily declining until in the oldest leaves there was an accelerated loss (figure 4.47). This accelerated rate of loss was not previously observed with RNA extractions and may reflect a change in the recovery of ribosomes during extraction from these older leaves. The pattern of change observed in TYMV-infected leaves was different to that from uninfected material where here an initial increase in, then a maintenance of the level of ribosomes plus virus was evident with a drop in yield in the oldest leaf. The changes in the infected leaf material were more irregular than those in the healthy tissue, a result probably due to an uneven distribution of the virus in the leaves of the infected plant which was apparent from the differences in mosaic pattern induced in the leaves by the presence of the virus. This again illustrates a problem incurred when dealing with TYMV-infected chinese cabbage leaves. The yield of ribosomes per gram fresh weight from uninfected leaves showed a pattern of change similar to that previously observed with RNA, there was an initial fast rate of decline in ribosome concentration in the younger leaves which then levelled off to a much slower rate that persisted into the oldest leaves in which there was a slight increase in rate of decline (figure 4.48). The pattern of change from TYMV infected leaves showed an essentially similar

Figure 4.47

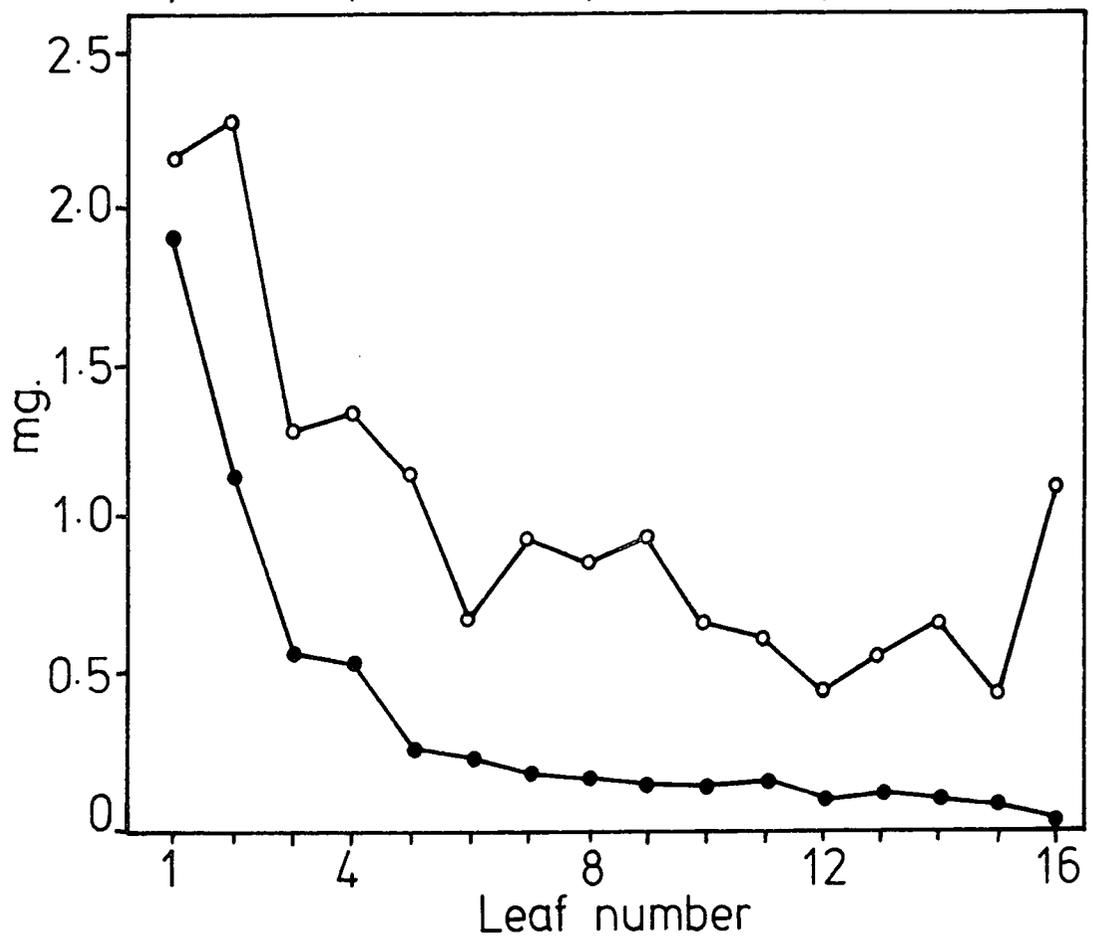
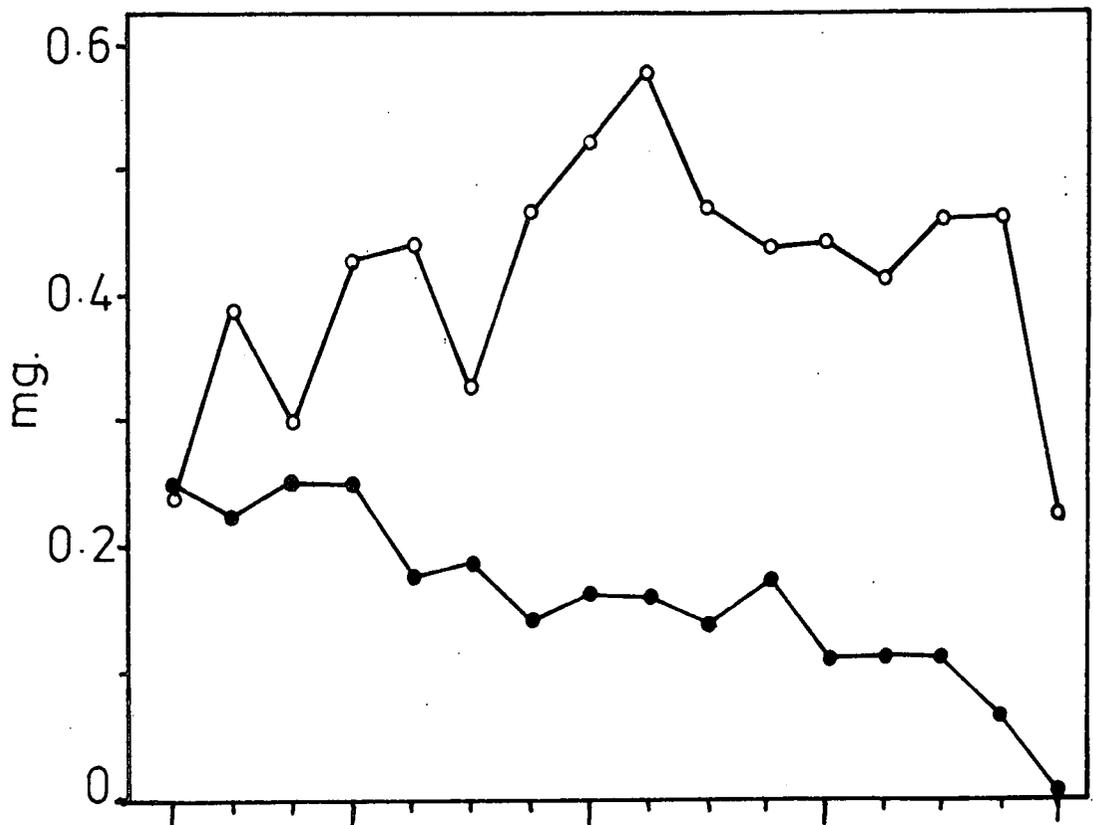
The yield of ribosomes (or ribosomes plus virus) per leaf from successive leaves along the phyllotaxy.

- Healthy plant
- TYMV-infected plant

Figure 4.48

The yield of ribosomes (or ribosomes plus virus) per gram fresh weight of leaf lamina from successive leaves along the phyllotaxy.

- Healthy plant
- TYMV-infected plant



trend though greater irregularity in the curve was observed, again attributable to a variable virus distribution between leaves. There was also a rise in the concentration of ribosomes plus virus particles in the oldest leaf, this was similar to the RNA change observed in infected leaves described above (see 4.33) and can probably be explained by the same reasons as those previously proposed.

Electrophoretograms of separated polyribosomes from different uninfected leaves along the ageing sequence are illustrated in figure 4.49. It was apparent from these gel traces that there were high levels of polyribosomes compared to monoribosomes in the youngest, most rapidly metabolising leaves which declined in successive leaves to a level that was maintained throughout the older leaves. No ribosomes were detected on the electrophoretogram of the extract from the oldest leaf, this was because the yield obtained was too low to be detected by the method of fractionation used. The trend empirically outlined above was quantified by measuring the peak area weights of the electrophoretograms and expressing the peak area weights of individual fractions as a percentage of the total area measured. The proportion of ribosomes as polyribosomes were initially high (approximately 70%) but declined to a level of 43-44%, this level being maintained until in the oldest leaves a rise in polysome proportion was observed (figure 4.50). Because of the difficulty of determining

Figure 4.49

PAGE of polyribosomes extracted from different leaves  
along the phyllotaxy of a healthy chinese cabbage plant.

- A. Leaf 1 (young)
- B. Leaf 6
- C. Leaf 11
- D. Leaf 15 (old)

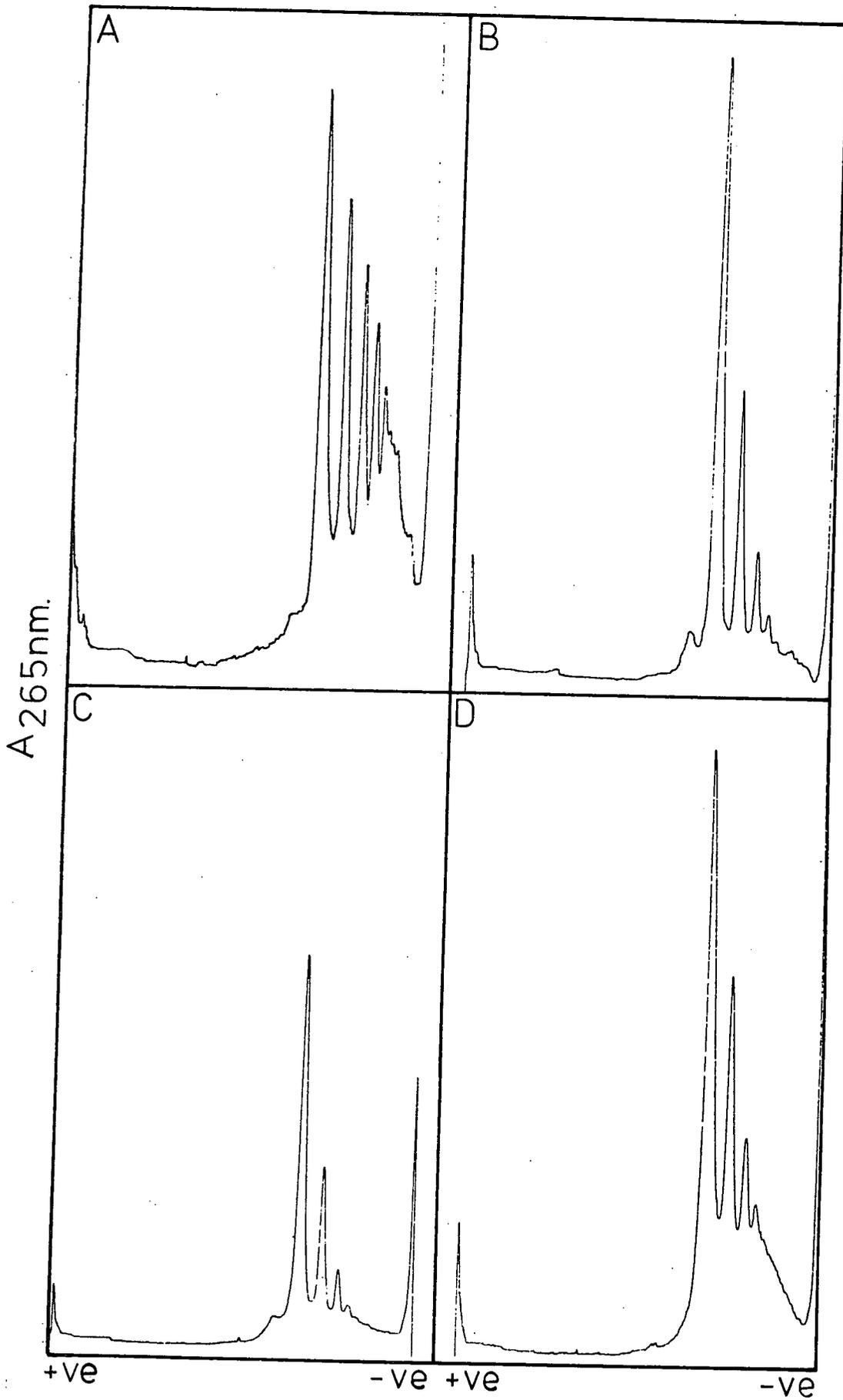


Figure 4.50

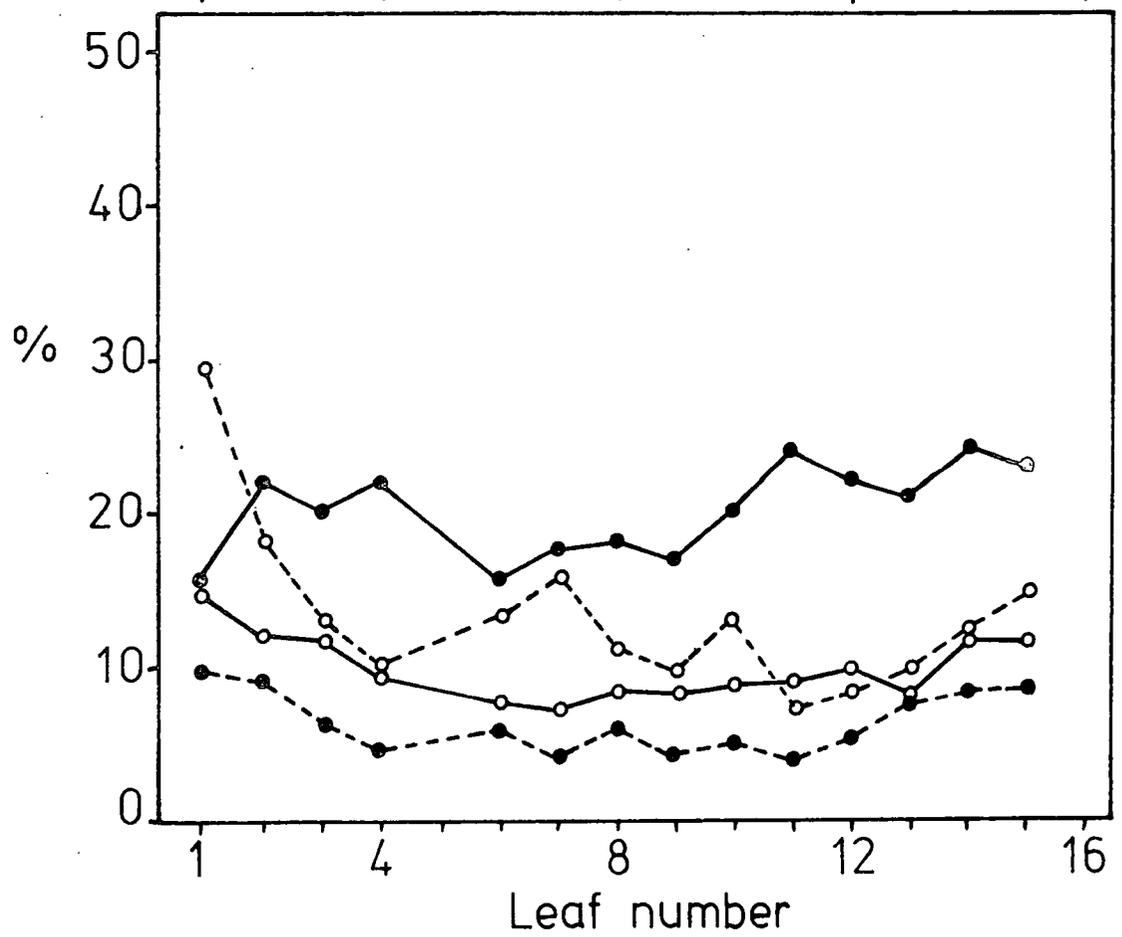
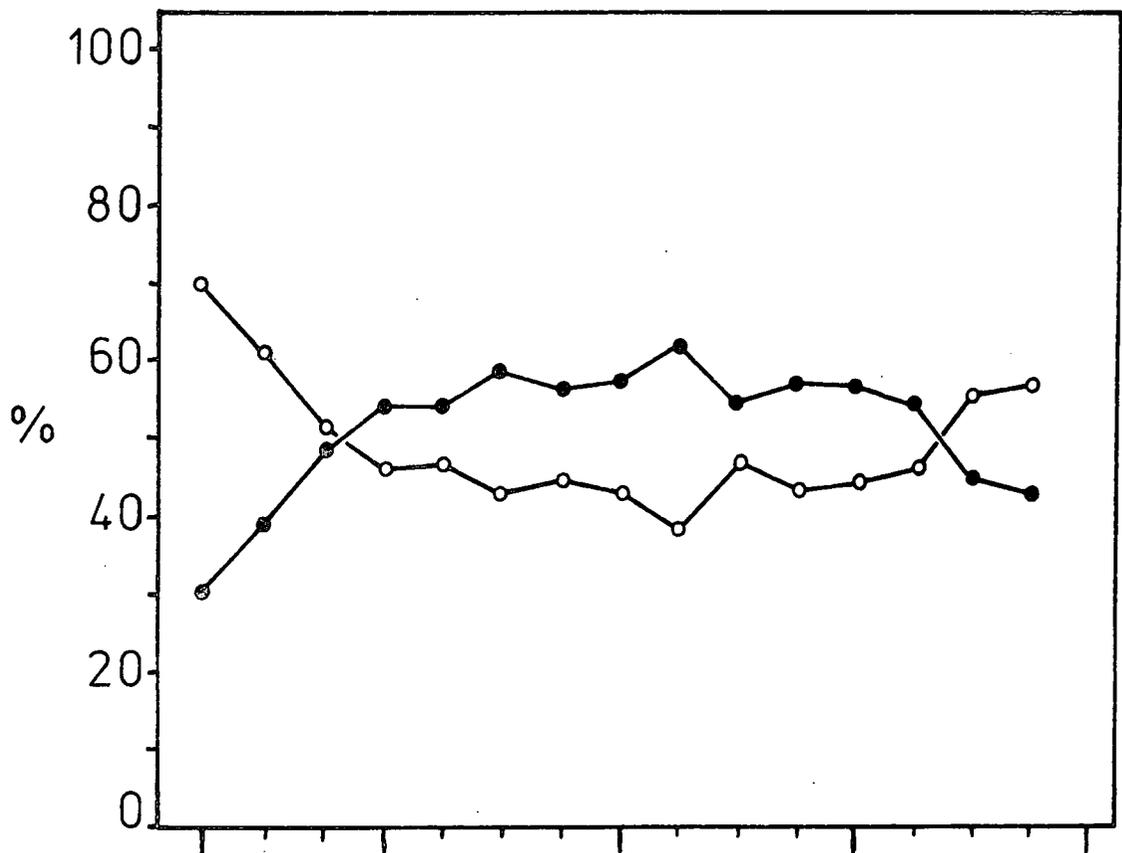
The change in the proportion of polyribosomes and mono-ribosomes (plus ribosomal sub-units) extracted from successive leaves along the phyllotaxy of a healthy plant.

- o Polyribosomes
- ⊙ Monoribosomes (plus ribosomal sub-units)

Figure 4.51

The change in the proportion of the component polyribosome fractions extracted from successive leaves along the phyllotaxy of a healthy plant.

- Dimer
- Trimer
- Tetramer
- Pentamer plus larger polyribosomes



separately the peak areas of the monoribosomes and ribosomal sub-units from the electrophoretograms these two fractions were combined and the area measured for these fractions constituted the remaining absorption peaks on the gel traces. The change in the proportion of monoribosomes plus ribosomal sub-units consequently showed an opposite trend to that described for polyribosomes. From figure 4.49 it was evident that free sub-units represented only a small proportion of the absorption on the traces and that therefore the changes ascribed to the combined fraction were due primarily to changes in the monoribosomes. It was notable that the observed fall in polyribosome level occurred before the completion of leaf extension had occurred.

The peak areas of individual polyribosome fractions, expressed as a percentage of the total area on the electrophoretograms, were also measured but only the dimer, trimer and tetramer peaks could be unequivocally defined on all the gel traces and therefore polyribosomes of a larger size (i.e. pentamer and greater) were measured together and classed as 'large polyribosomes'. The changes observed in the trimer, tetramer and 'large polyribosome' fractions were similar to those for total polyribosomes (figure 4.51). The dimer fraction, however, increased in the youngest leaves as opposed to the decline evident with the other polyribosome fractions but following this initial increase a pattern of change

similar to that of total polyribosomes was apparent.

Using the percentage proportions of the different ribosome fractions the yields of each fraction per leaf and per gram fresh weight were calculated from the respective total ribosome yield values for each leaf. The yield of total polyribosomes per leaf was then seen to fall rapidly in the younger leaves (up to the seventh leaf) with a slower rate of decline in the subsequent leaves. The yield of monoribosomes (plus ribosomal sub-units), however, increased in the youngest leaves then, after reaching a maximum value, a decline occurred which paralleled closely that of the polyribosome fraction (figure 4.52). For the individual polyribosome fractions the changes that occurred in the trimer, tetramer and 'large polyribosome' fractions again reflected those described for total polyribosomes (figure 4.53). An atypical rise in level of the dimer was seen in the youngest four leaves but the subsequent pattern of change was the same as that for the larger polyribosome fractions. The initial rise in yield of the dimer fraction occurred in the same leaves as those in which the monoribosome plus sub-unit yield increased.

On a yield per gram fresh weight basis the total polyribosome concentration fell rapidly in the youngest few leaves eventually reaching a level that fell only slightly in the remaining leaves (figure 4.54). The change in the concentration of monoribosomes plus sub-units was similar though

Figure 4.52

The change in total yield per leaf of polyribosomes and monoribosomes (plus ribosomal sub-units) extracted from successive leaves along the phyllotaxy of a healthy plant.

- o Polyribosomes
- ⊙ Monoribosomes (plus ribosomal sub-units)

Figure 4.53

The change in total yield per leaf of the component polyribosome fractions extracted from successive leaves along the phyllotaxy of a healthy plant.

- ⊙—⊙ Dimer
- ⊙—⊙—⊙ Trimer
- ⊙--⊙ Tetramer
- ⊙--⊙ Pentamer plus larger polyribosomes

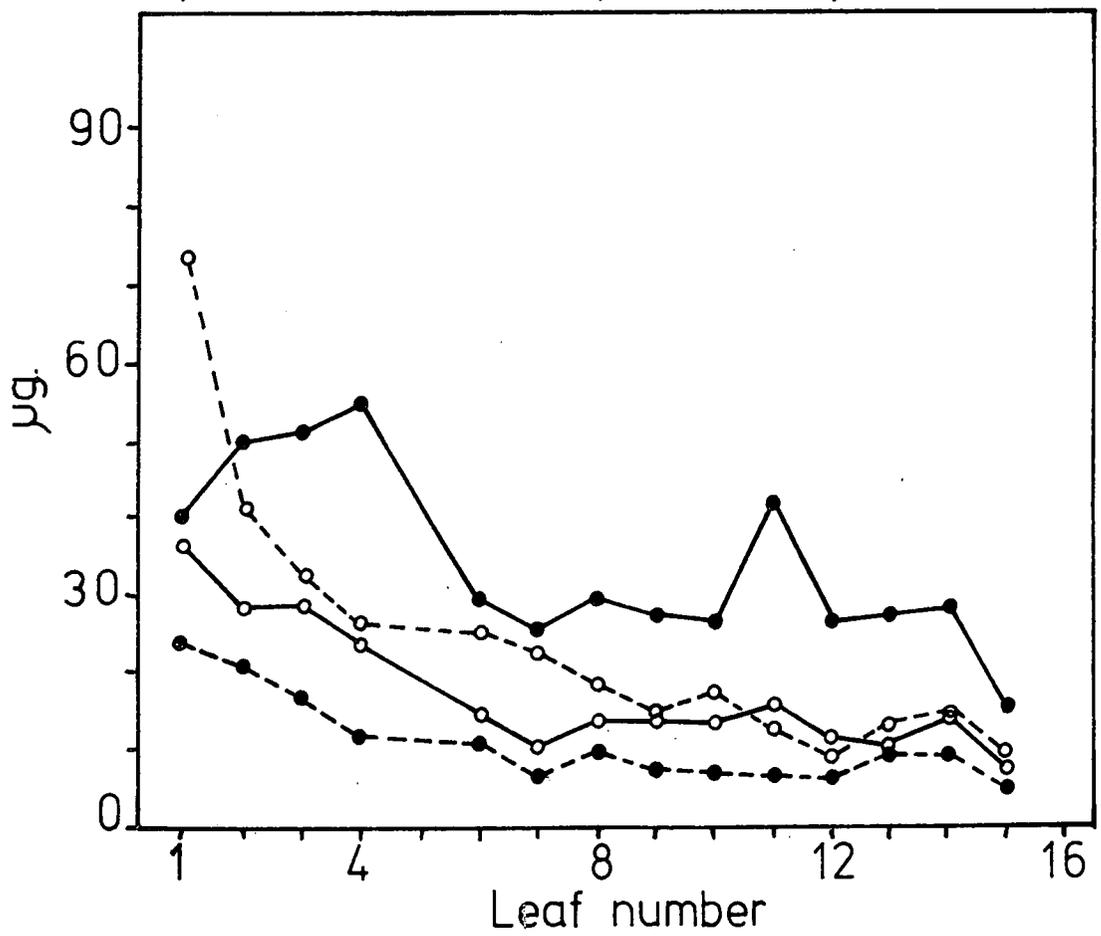
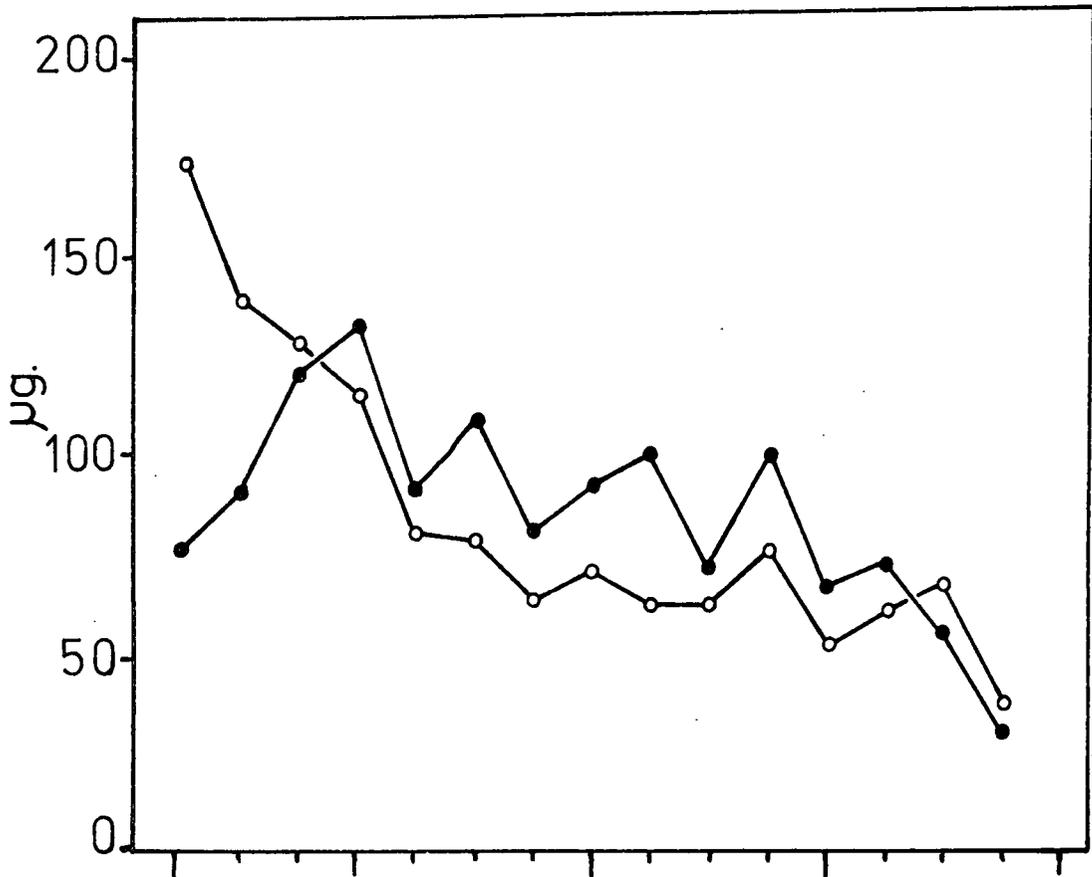


Figure 4.54

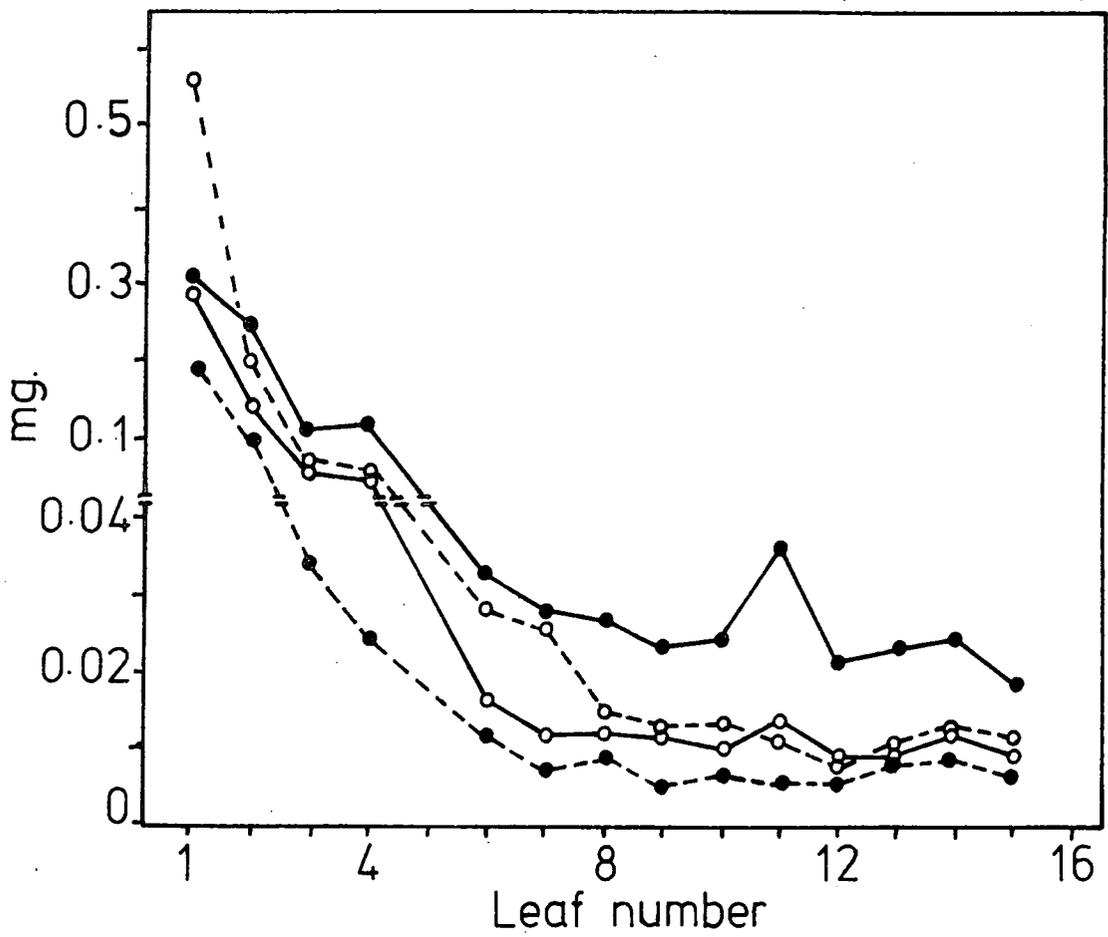
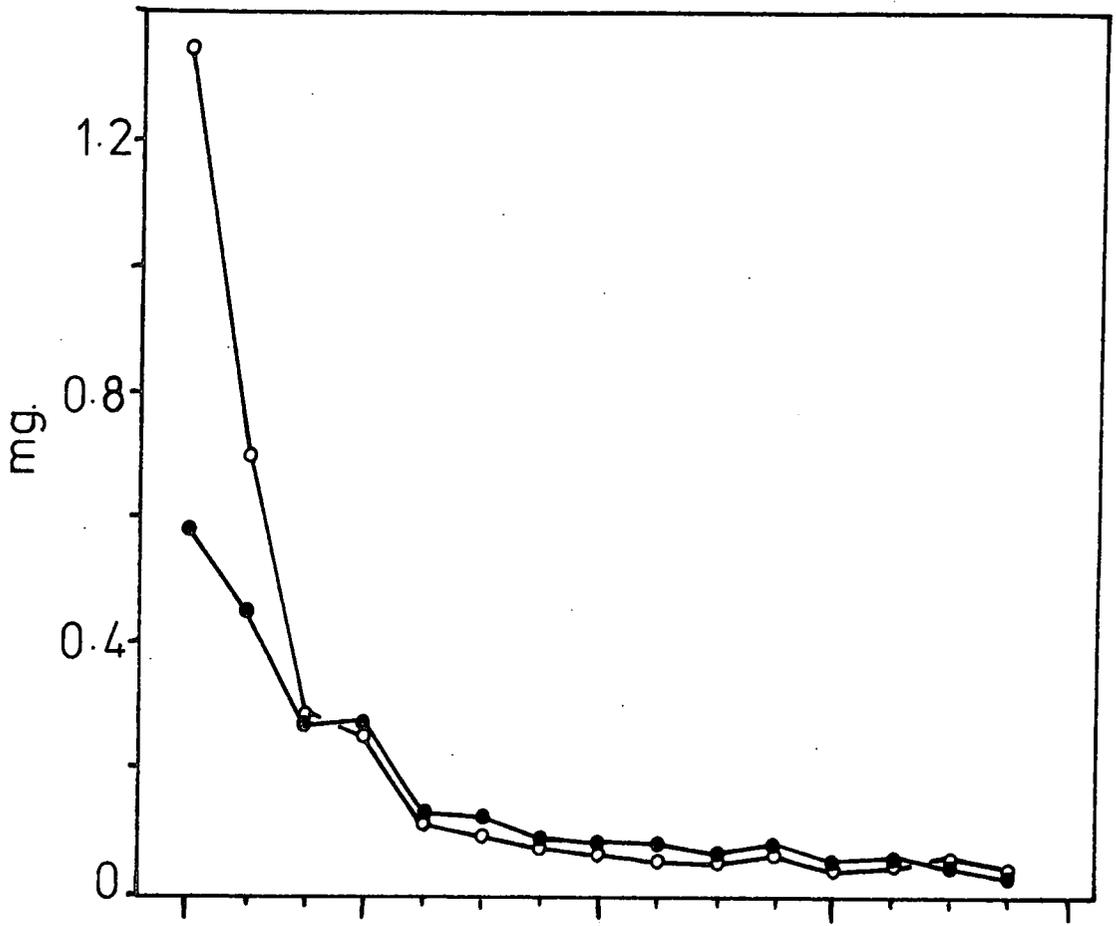
The change in yield per gram fresh weight of leaf lamina of polyribosomes and monoribosomes (plus ribosomal sub-units) extracted from successive leaves along the phyllotaxy of a healthy plant.

- Polyribosomes
- Monoribosomes plus ribosomal sub-units

Figure 4.55

The change in yield per gram fresh weight of leaf lamina of the component polyribosome fractions extracted from successive leaves along the phyllotaxy of a healthy plant.

- Dimer
- Trimer
- Tetramer
- Pentamer plus larger poly-ribosomes



the initial decline was at a much slower rate. All the constituent polyribosome fractions showed a similar pattern of change (figure 4.55) though again the change in the younger leaves of the dimer fraction had a closer affinity to the changes evident in the monoribosome fraction rather than that of total polyribosomes. It is possible that, in addition to being the result of the formation of new polyribosomes, some of the increase observed in the monoribosome and dimer fractions was a consequence of a breakdown of the longer chain polyribosomes abundant in the younger leaves. If this was the case then the increase in the dimer fraction as well as the monoribosomes would suggest the presence of a ribonuclease resistant dimer, evidence for the existence of such a dimer has been presented by Ling and Dixon (1970) in trout testis during maturation.

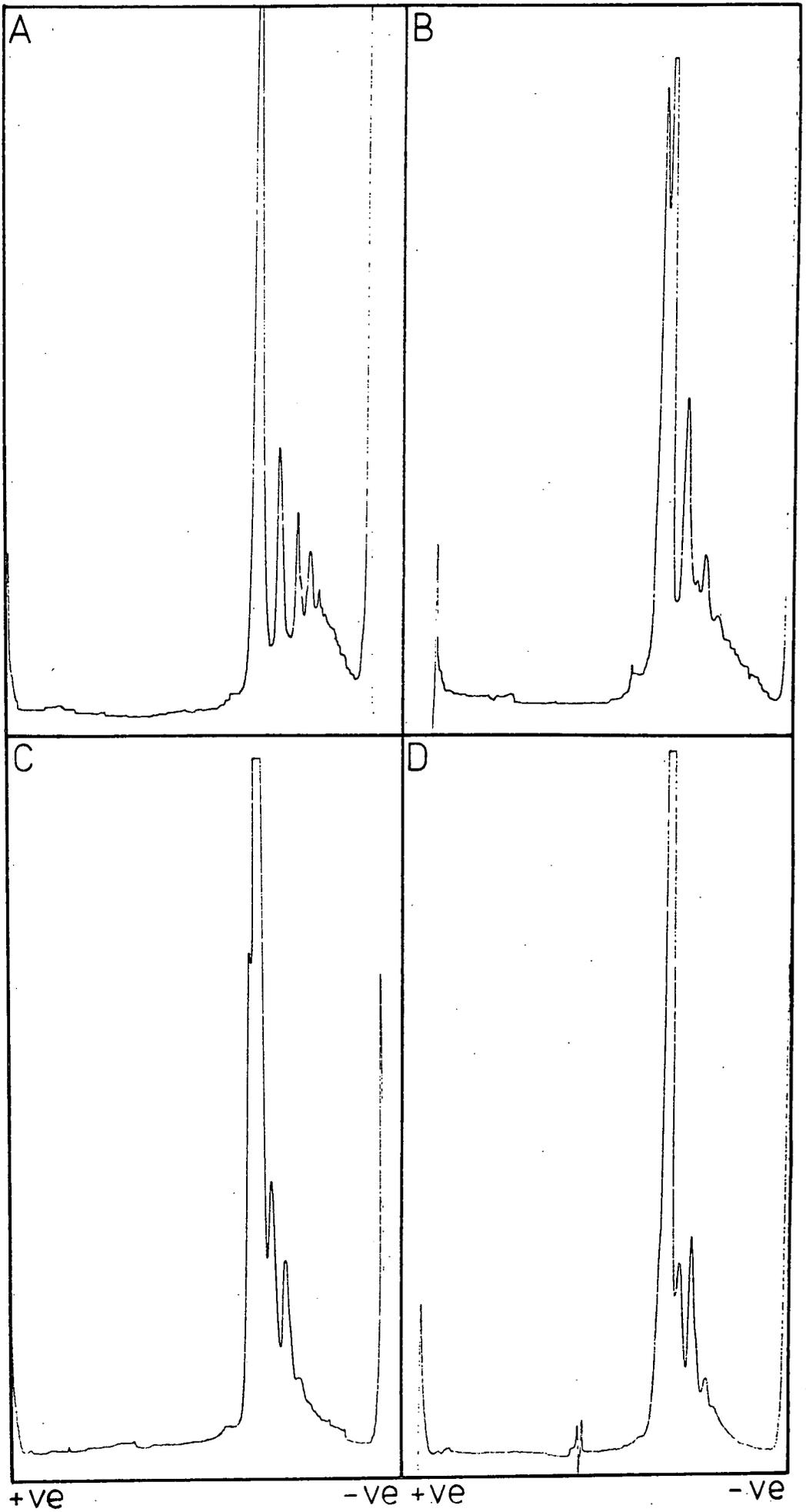
The changing pattern of polyribosome electrophoretograms with age for extracts from TYMV-infected leaves is illustrated in figure 4.56 where the influence of the presence of the virus in the extracts on the gel profiles is clearly seen. In the youngest leaves although the virus is present (as evidenced by the mosaic pattern of the leaves extracted, and see also the ribosome sub-unit electrophoretograms below) its appearance on the gel traces was masked by the high levels of host ribosomes (leaves 1 to 5), the virus was only evident on some of these gel traces by the 'shoulder' in the slope of the

Figure 4.56

PAGE of polyribosomes plus virus extracted from different leaves along the phyllotaxy of a TYMV-infected chinese cabbage plant.

- A. Leaf 1 (young)
- B. Leaf 6
- C. Leaf 11
- D. Leaf 15 (old)

A<sub>265nm</sub>.



monoribosome peak (leaves 4 and 5). From leaf 6 the virus and the 'top-component' fraction were identifiable as discrete peaks on the electrophoretograms, this condition existed until the virus was present in excess of the host ribosomes where the absorption of the viral components was sufficiently large to obscure some of the ribosome fractions (leaves 10 to 16). Because standard amounts of u.v. absorbing material (i.e. ribosomes plus viral particles) were loaded on to each gel the change in relative proportions of the viral components and the host ribosomes described reflects the state of these elements in the sequence of infected leaves on the plant. It was not possible to determine from the electrophoresis data how much of this change was attributable to decreased levels of host ribosomes and how much to an increase in the amount of the virus. A fall in ribosome concentration in leaves of increasing age from uninfected material suggested that a decline in the amount of host ribosomes could make a significant contribution to the observed proportional change between the virus components and the host ribosomes. The ribosome changes in uninfected leaf samples reflected total alterations in content of both cytoplasmic and chloroplastic ribosomes whereas the infected leaf ribosome changes would primarily be the result of changes in the cytoplasmic ribosomes due to the severe reduction in chloroplast material (see 4.34). However the qualitative ribosomal RNA changes indicated that there were similar quantitative changes in both cytoplasmic and chloroplastic fractions therefore the inference on the behaviour

of the host ribosomes in infected tissue based on the changes observed in uninfected tissue was probably correct.

Because of the marked overlapping of u.v. absorption by the virus and 'top-component' with the ribosome peaks accurate peak area measurements of the gel traces were not possible. However, from the electrophoretograms it was evident that in the youngest leaves high levels of polyribosomes were present and that the proportion of these to the monoribosome fraction was reduced in the subsequent leaves (figure 4.56). In the older leaves estimation of the proportion of ribosomes as polyribosomes was difficult because the increased proportion of virus in a given sample resulted in fewer ribosomes being loaded on to the gel. Therefore the absence of long chain polymers from the electrophoretograms may be the result of a failure to detect these fractions in the reduced amounts loaded on to the gels rather than because they were absent from the host. As far as could be ascertained from comparison of the electrophoretograms the gross changes in the polyribosome population from TYMV-infected leaves did not show marked differences with leaf age from those extracted from equivalent uninfected tissue, however the described interference effects of the virus precluded a closer comparison.

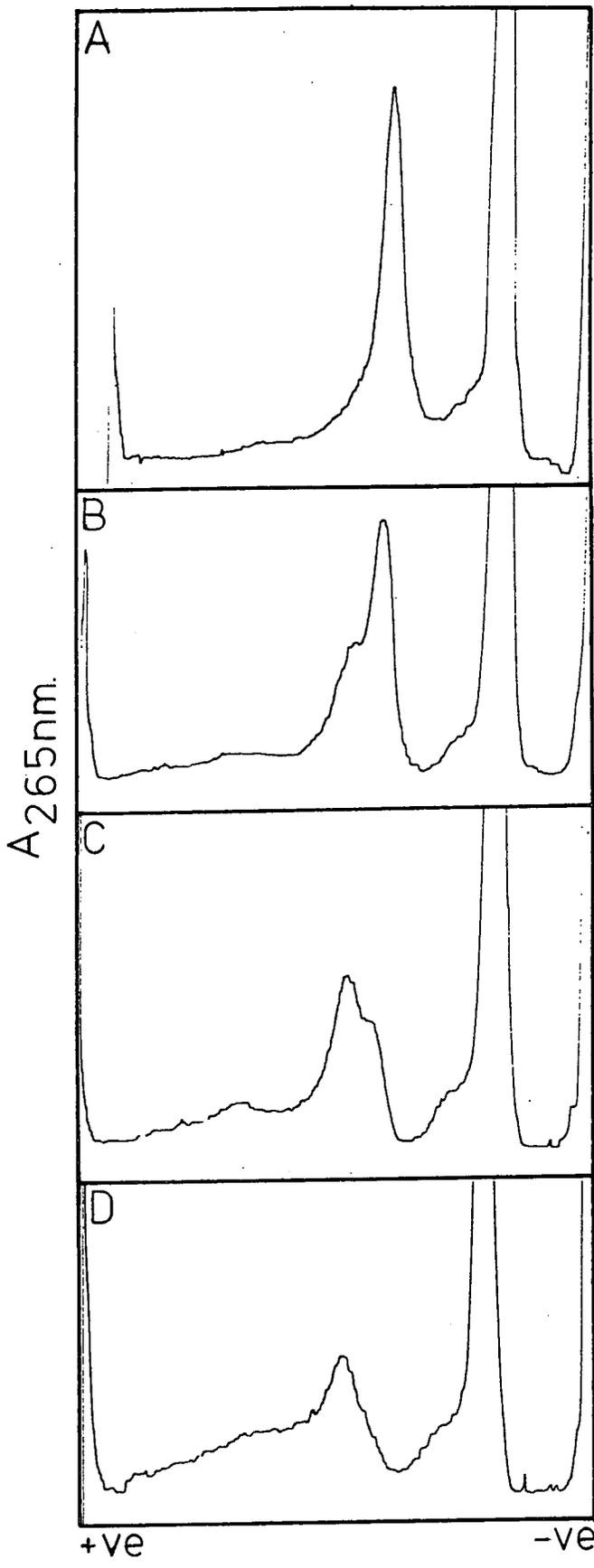
The ribosome extracts from both TYMV-infected and uninfected leaves were also electrophoresed in an EDTA-containing buffer (see 3.24.3) in order to examine the ribosomal

sub-units released by the dissociation of the ribosomes. With the electrophoretograms of the ribosomal sub-units from the uninfected leaf extracts a distinct variation in the stability of the small sub-unit fraction with age was apparent (figure 4.57). The gel profiles obtained from the younger leaves (numbers 1 to 5) had two sharply defined sub-unit peaks with changes of slope on the leading edges of the peaks in the expected position for chloroplast ribosomal sub-units (see 3.82). In successive leaves the fraction represented by the 'shoulder' on the small sub-unit peak becomes proportionally larger and could be identified as a distinct peak on the gel trace, e.g. leaf 7, which eventually became the major small sub-unit fraction (leaf 9). In the older leaves only one small sub-unit peak was evident and by measurement of the migration relative to the large sub-unit it was established that the single small sub-unit peak on the electrophoretograms from older leaves had the same relative migration as this second sub-unit fraction. The qualitative rRNA data indicated that changes in the proportion of the small sub-unit of the 70s ribosome was unlikely to explain the observed change, it therefore appeared that there was a leaf-age associated change in the susceptibility to EDTA of the small sub-units or that there was an inherent age change in the structure or stability of these small ribosomal sub-units that was observable by EDTA electrophoresis. If this was the case then it is likely that

Figure 4.57

PAGE of ribosomal sub-units obtained from polyribosome extracts from successive leaves along the phyllotaxy of a healthy plant illustrating the change in migration of components of the small ribosomal sub-unit peak.

- A. Leaf 2
- B. Leaf 7
- C. Leaf 9
- D. Leaf 11



such a change would not have been observable by sucrose density gradient fractionation of the sub-units.

Electrophoretograms of the dissociated ribosomes from TYMV-infected leaf extracts are illustrated in figure 4.58. The change in the proportional amounts of virus particles and host ribosomes, noted above with the electrophoresis of polyribosomes, was seen more clearly in the sub-unit fractionations in which the virus was separated more distinctly from the ribosome components. In the young leaves there was a clear excess of ribosomal sub-units over the virus, with successively older leaves the proportion of virus increased and the viral 'top-component' peak was also apparent. In the oldest leaves the proportion of virus had risen so much that very little, or no, host ribosomal sub-units were detected on the gels. The change in the form of the small ribosomal sub-unit described above for uninfected extracts was also apparent in the infected leaf extracts though the change could not be followed through to the oldest leaves because of the greatly reduced amounts of total ribosomal sub-units on the gels. However, for those gels in which it could be determined the values for the migration relative to that of the large ribosomal sub-unit peak of the two small sub-unit fractions were the same as those found for the healthy leaf extracts. Because of the greatly reduced amounts of chloroplast ribosomal material in the TYMV-infected leaf tissues the appearance of

Figure 4.58

PAGE of ribosomal sub-units plus virus obtained from polyribosome extracts from successive leaves along the phyllotaxy of a TYMV-infected plant.

- A. Leaf 1 (young)
- B. Leaf 6
- C. Leaf 11
- D. Leaf 15 (old)



similar changes in the small ribosomal sub-unit fraction to those in uninfected tissue again suggested that these changes were primarily associated with the small cytoplasmic ribosomal sub-units.

In studies on ribosomal changes during sequential ageing of leaves on the intact plant the technique of ribosome fractionation by polyacrylamide gel electrophoresis proved to be very consistent and reproducible. Because of the small amounts of material necessary and the high sensitivity of the recording equipment it was possible to examine changes in old, senescent leaves in which the recoveries of polyribosomes were very low. Examination of ribosome changes in TYMV-infected tissue was also possible but the migration of the viral components interfered with a full elucidation of the different polyribosome peaks, this being a particular problem when the relative proportion of the virus was high. Similar problems with TYMV-infected tissue were also encountered when the ribosomes were fractionated by sucrose density gradient centrifugation.

#### 4.5 Leaf Disc Ageing - Quantitative Changes

##### 4.51 Uninfected plants

The leaf discs used in this study were taken only from mature, fully expanded leaves of healthy chinese cabbage plants thus eliminating the possibility of different rates of ageing in young and old tissue having a complicating effect on the final results. Discs of 1.0 cm diameter were cut from the leaf laminae with a cork borer taking care to avoid the heavily veined areas. The discs were surface sterilised by soaking for 30 seconds in 70% ethanol, then transferred to 3% hypochlorite solution for 5 minutes followed by a final thorough rinsing in sterile distilled water. All discs cut were collected and mixed in sterile distilled water prior to setting up the incubation treatments. Four different incubation treatment were used on batches of the leaf discs, these were:

- i) floated on water in the light
- ii) floated on water in the dark
- iii) floated on a solution of 5 p.p.m. kinetin (6-furfurylamino purine) in the light
- iv) floated on a solution of 5 p.p.m. kinetin in the dark

To set up these treatments a random ten discs were transferred, using sterile forceps, to a petri-dish containing 30 ml of the appropriate sterile incubation solution and floated on the surface adaxial side uppermost. The incubation solutions also had the antibiotic neomycin added (5 p.p.m.) to ensure that

no bacterial growth occurred. All the above operations were carried out under a sterile inoculating hood. The dishes of leaf discs to be illuminated were placed under cool fluorescent strip lights (280 lumens per sq. ft.) in a growth room maintained at 22°C. The remaining dishes were incubated in the dark in a growth room also held at 22°C and were covered with a black polythene sheet to exclude any stray light. At twenty-four hour intervals one dish from each treatment was removed and the discs frozen in liquid air and stored at -20°C until they were extracted for DNA, RNA and chlorophyll content by the methods previously described (see 2.3). At the beginning of the experiment batches of ten discs were frozen with liquid air and stored, these were the zero time controls.

#### 4.51.1 DNA content

In all four treatments the changes in DNA content were a little irregular but there did appear to be a differential response to the different treatments (figure 4.59). The greatest decline in DNA level occurred in the discs floated on water and kept in the dark, both illumination or kinetin treatment retarded this loss though in the case of kinetin the initial retardation was followed by a rapid decline after about five days to final levels at the end of the incubation period (ten days) similar to the water-treated discs. When both light and kinetin were provided the levels

Figure 4.59

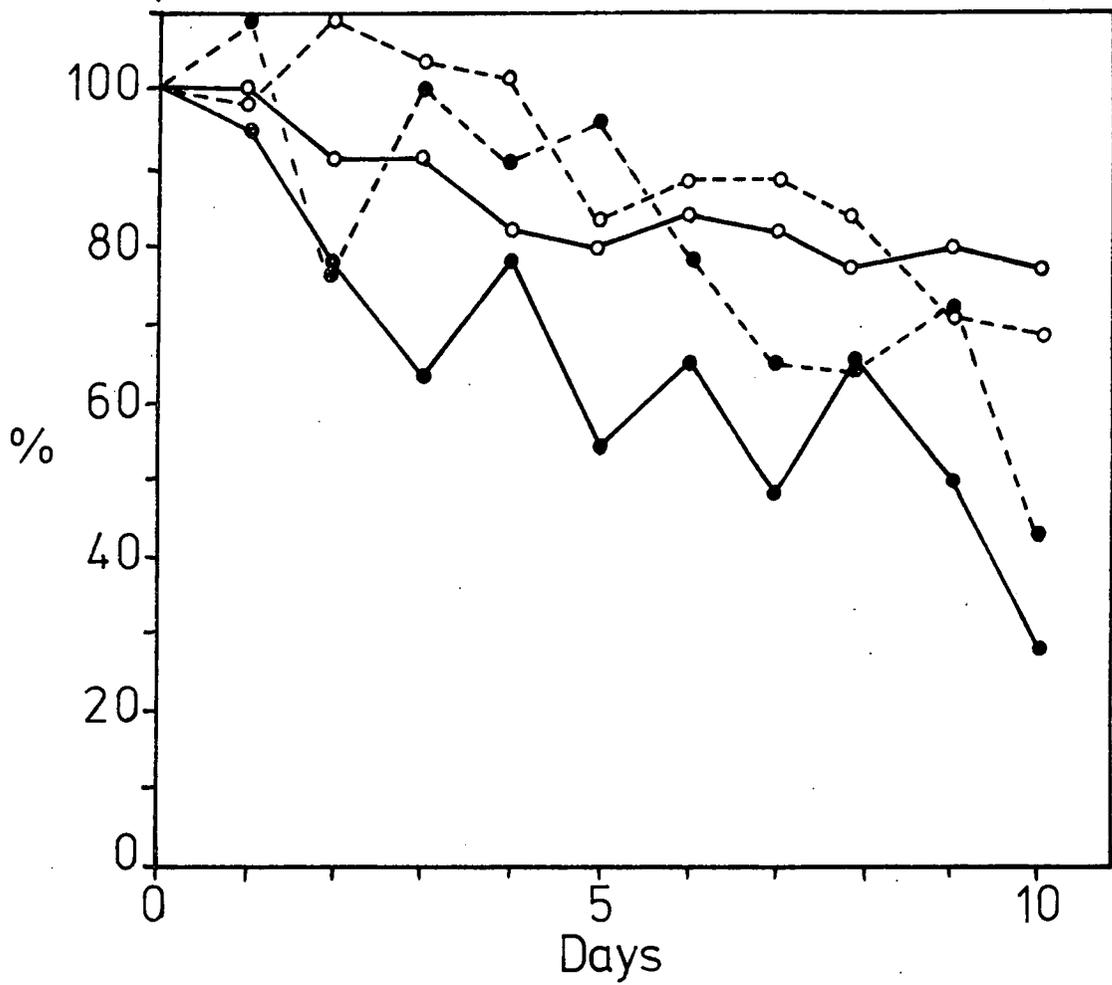
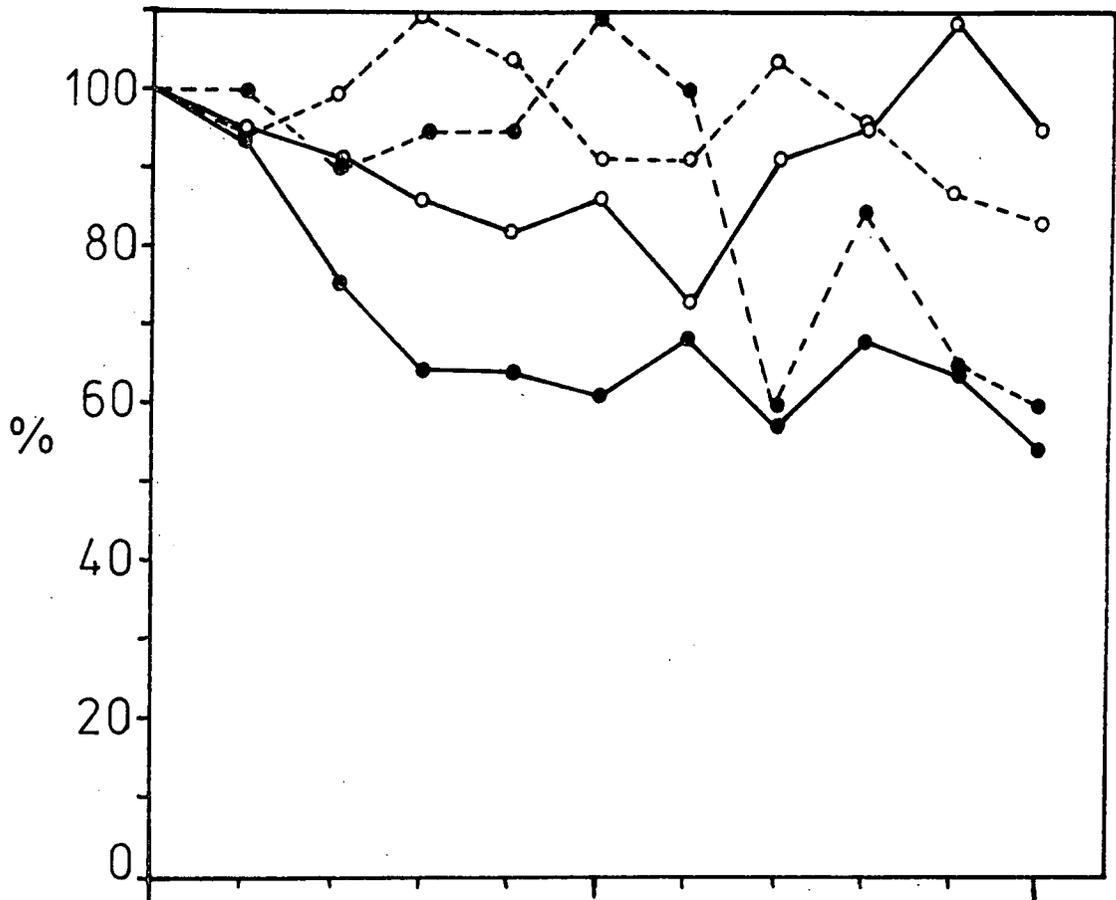
The change with time of the DNA content of leaf discs excised from healthy leaves of chinese cabbage. (Values expressed as a percentage of the yield obtained from the zero time controls)

- Discs incubated on water in the dark
- Discs incubated on water in the light
- Discs incubated on kinetin solution in the dark
- Discs incubated on kinetin solution in the light

Figure 4.60

The change with time of the RNA content of leaf discs excised from healthy leaves of chinese cabbage. (Values expressed as a percentage of the yield obtained from the zero time controls)

- Discs incubated on water in the dark
- Discs incubated on water in the light
- Discs incubated on kinetin solution in the dark
- Discs incubated on kinetin solution in the light



of DNA were maintained throughout most of the incubation period in contrast to the water plus light treatment in which there was indications of a recovery of DNA level after an initial fall in content.

#### 4.51.2 RNA content

In all the four treatments used the RNA level was seen to fall with increasing time of incubation (figure 4.60) though at different rates. Discs floated on water in the dark showed the most rapid rate of decline which was again retarded by light or kinetin. Application of 5 p.p.m. kinetin to the discs incubated in the dark maintained the RNA level initially until about day five when the content fell and final RNA levels were similar to those of the water treatment after ten days. The rate of decline in RNA content observed in the illuminated discs was lower over the latter part of the incubation period whilst cytokinin treatment of the illuminated discs maintained higher levels of RNA in all but the longest incubation periods (figure 4.61). In this latter treatment there was also a slight increase in the RNA content over the first few days of incubation, the subsequent rate of fall being greater than that observed in the illuminated discs floated on water. The observed patterns of change were seen to be more irregular in the dark-treated discs than in those that were incubated in the light and this was evident in both the water and kinetin treatments.

Figure 4.61

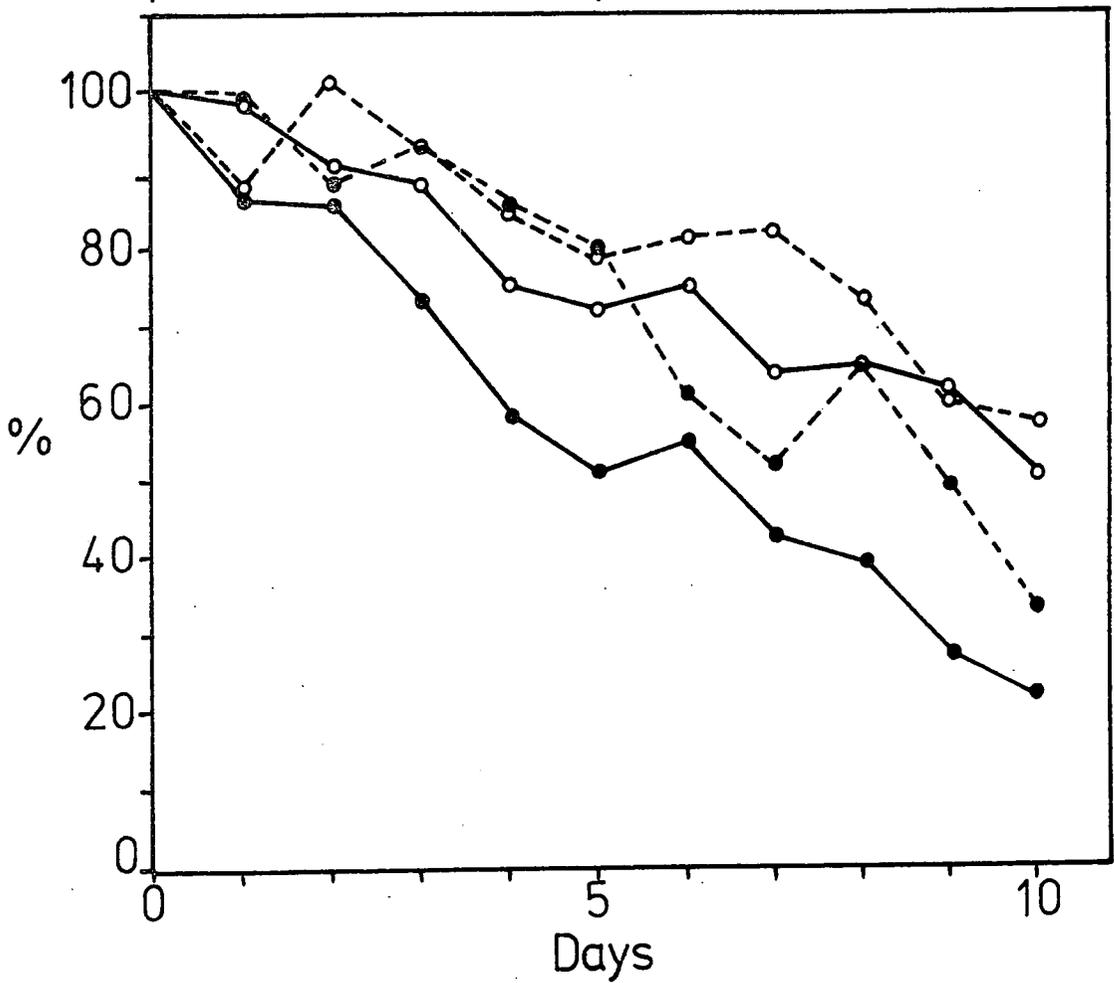
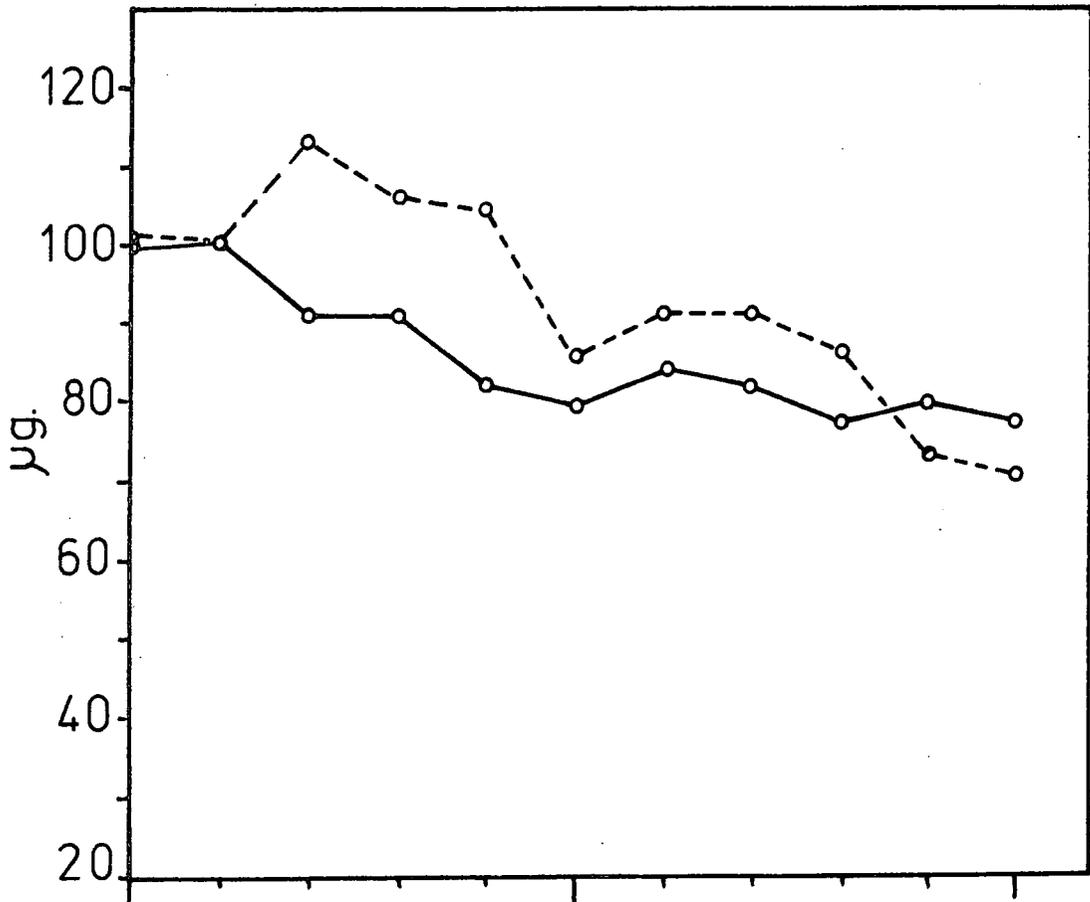
The changes with time of the RNA content of leaf discs excised from healthy leaves of chinese cabbage and incubated in the light.

- Discs floated on water
- Discs floated on kinetin solution

Figure 4.62

The change with time of the chlorophyll content of leaf discs excised from healthy leaves of chinese cabbage. (Values expressed as a percentage of the yield obtained from the zero time controls)

- Discs incubated on water in the dark
- Discs incubated on water in the light
- Discs incubated on kinetin solution in the dark
- Discs incubated on kinetin solution in the light



#### 4.51.3 Chlorophyll content

With the changes in total chlorophyll content of the leaf discs a similar pattern of change in response to the different treatments were observed to that described for RNA (figure 4.62). Again the greatest rate of decline was seen to occur in the discs floated on water in the dark and retardation of this decline occurred when the water was replaced by a 5 p.p.m. kinetin solution or when the discs were illuminated. Treatment of the discs with both kinetin and light resulted in a maintenance of chlorophyll content at a higher level than that found with the other treatments for most of the period of incubation used.

#### 4.52 TYMV-infected plants

To examine the changes with age of excised leaf discs of TYMV-infected chinese cabbage leaves the same experimental procedure as used for discs from uninfected leaves was employed. When cutting the discs from the infected leaves, in addition to avoiding heavily veined areas of the leaf, dark green areas of the mosaic pattern of infection were also avoided, thus all the discs were taken from as uniform as possible yellow-green areas of the mosaic. As with the uninfected discs the TYMV-infected leaf discs were thoroughly mixed and a random ten discs selected and placed adaxial side uppermost in each petri dish. The incubation treatments and the procedure of sampling and extraction was the same as that used with the uninfected

leaf discs.

#### 4.52.1 DNA content

Compared to the uninfected leaf disc material the initial starting yields of DNA in the time zero controls were approximately 30% higher in the TYMV-infected leaf disc samples (28 $\mu$ g per 10 discs in the infected compared to 21 $\mu$ g per 10 discs in the uninfected). This was probably a reflection of the stunting effect of TYMV on the growth of chinese cabbage resulting in a greater density of cells in the given area of the leaf discs. As was the case with uninfected material the DNA content was found to show irregular changes with increasing incubation period, a problem possibly complicated by an uneven distribution of viral symptoms amongst the discs despite taking measures to reduce this. The greatest rate of DNA decline occurred in those discs floated on water in the dark and illumination of the discs resulted in a retardation of this rate of fall (figure 4.63). Treatment of the discs with kinetin had a retarding effect on DNA loss both in the dark and in the light. This kinetin effect was not evident until after three days incubation when the DNA content was observed to recover and be maintained in the illuminated discs whilst in the dark incubated discs the fall in DNA content was retarded relative to the water control. In these TYMV-infected leaf disc samples, therefore, illumination of the discs retarded DNA loss as it did in the uninfected material, in addition

Figure 4.63

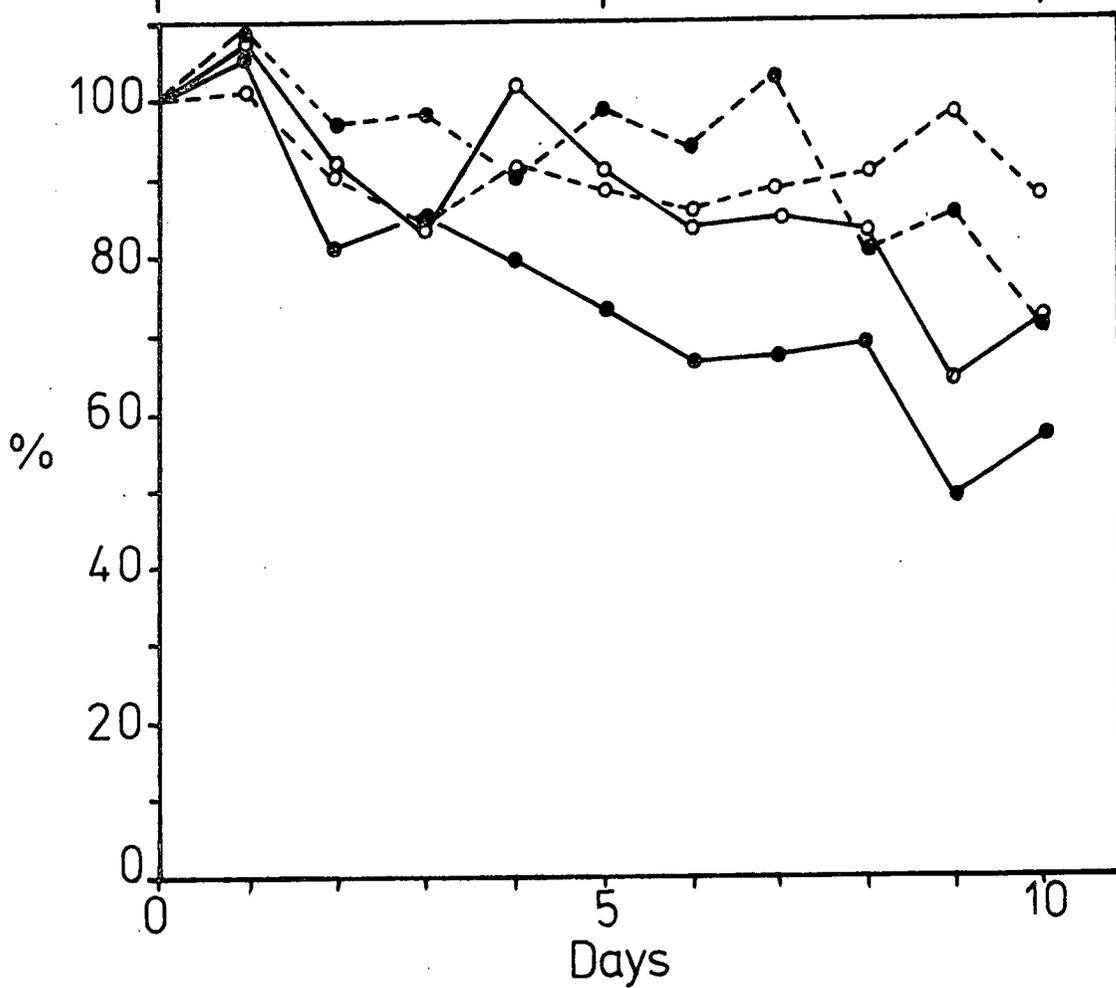
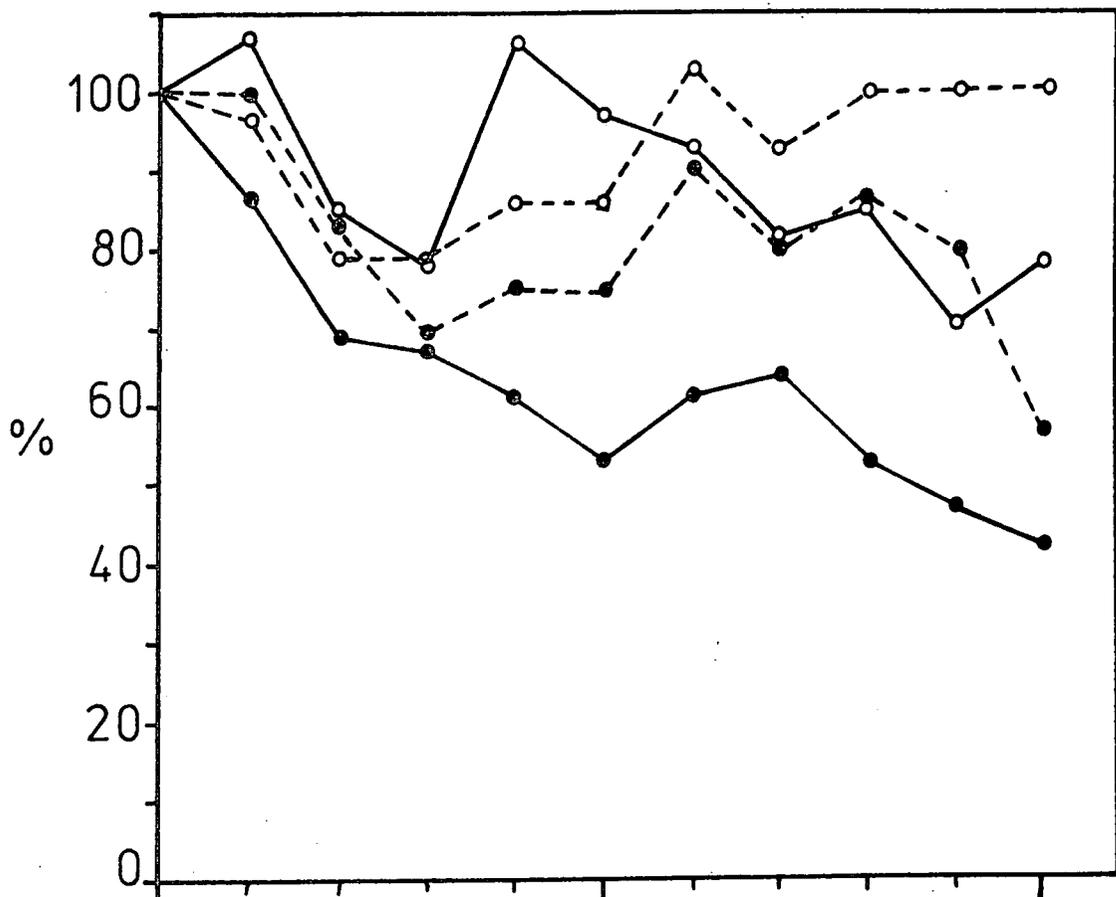
The change with time of the DNA content of leaf discs excised from TYMV-infected leaves of chinese cabbage. (Values expressed as a percentage of the yield obtained from the zero time controls)

- Discs incubated on water in the dark
- Discs incubated on water in the light
- Discs incubated on kinetin solution in the dark
- Discs incubated on kinetin solution in the light

Figure 4.64

The change with time of the RNA content of leaf discs excised from TYMV-infected leaves of chinese cabbage. (Values expressed as a percentage of the yield obtained from the zero time controls)

- Discs incubated on water in the dark
- Discs incubated on water in the light
- Discs incubated on kinetin solution in the dark
- Discs incubated on kinetin solution in the light



there appeared to be a more pronounced retardation by kinetin treatment. These relative effects of the incubation treatments were also compared by looking at the initial and final DNA levels in the uninfected and TYMV-infected leaf disc samples. At time zero the DNA content was approximately 30% higher in the infected disc material, after ten days incubation on water, either in the light or in the dark, the DNA yields from the infected discs were the same as found in the correspondingly treated uninfected disc incubations, this would indicate a greater relative loss of DNA in the infected material. Those infected leaf discs treated with kinetin, however, had a final DNA content approximately 40-45% higher than the corresponding uninfected discs, thus kinetin had a proportionally greater retarding effect on DNA loss in the TYMV-infected material.

#### 4.52.2 RNA content

Considering RNA content the initial yields in the zero time control samples for TYMV-infected discs were approximately three times greater than those in the corresponding uninfected discs (an average yield of 32.2 $\mu$ g per disc in infected material and 10.3 $\mu$ g per disc in uninfected material). Also, compared to the uninfected results, the RNA content of the discs did not show an even rate of change, an effect which was possibly the result of varying amounts of virus in the different samples. The pattern of change of RNA content in the infected disc incubations showed similar trends to those described for

DNA content above (figure 4.64). The greatest overall fall in RNA level occurred in the discs floating on water in the dark, a similar pattern of decline but at a lower rate being evident for the discs floated on water and illuminated. An effect of applying kinetin to the illuminated discs was only apparent at the longer incubation periods where the overall RNA level was higher than in the water treated discs.

Retardation of the deleterious dark effects was also seen upon incubation of the discs with kinetin applied and again this was most evident in the latter half of the incubation period used. Because of the presence of the virus the total RNA yield from the time zero controls was approximately three times greater in the infected samples compared to corresponding uninfected tissue. After ten days incubation the relative level of RNA content in the infected discs floated on water and illuminated remained the same indicating a similar overall rate of RNA decline in both tissue types for this period of incubation. The remaining treatments all resulted in a relatively slower rate of RNA decline in the infected tissues as evidenced by a higher relative level of RNA after ten days. This was most marked in the dark treated discs in which the final RNA content in the infected discs was six times greater than that of correspondingly treated uninfected discs whilst with the kinetin treatments a final RNA content four times greater than that found in uninfected

discs incubated under similar conditions was obtained.

#### 4.52.3 Chlorophyll content

As was the case with DNA and RNA the pattern of change observed in the chlorophyll content of the discs was more variable than that obtained with uninfected leaf discs, an effect probably attributable to the virus-induced alteration of the photosynthetic pigments (Crosbie and Matthews, 1974a). As for DNA and RNA the greatest decline in chlorophyll content occurred in those discs floated on water and incubated in the dark (figure 4.65). The infected discs floated on water and illuminated showed a chlorophyll decline of a slower rate than that of the dark incubated discs and application of kinetin resulted in a maintenance of chlorophyll levels above those of the corresponding water treatments. In the dark kinetin maintained chlorophyll at a similar level to the water plus light treatment and showed a similar rate of chlorophyll decline. With the discs incubated with kinetin in the light after an initial fall the chlorophyll content recovered and the amount extracted at the end of the incubation period was greater than that in any other treatment. Thus in contrast to leaf discs taken from healthy chinese cabbage leaves kinetin treatment of illuminated discs only produced an effect in the longer incubation periods. The total chlorophyll content in the zero time controls of the TYMV-infected leaf discs was reduced to about one third (35%) of

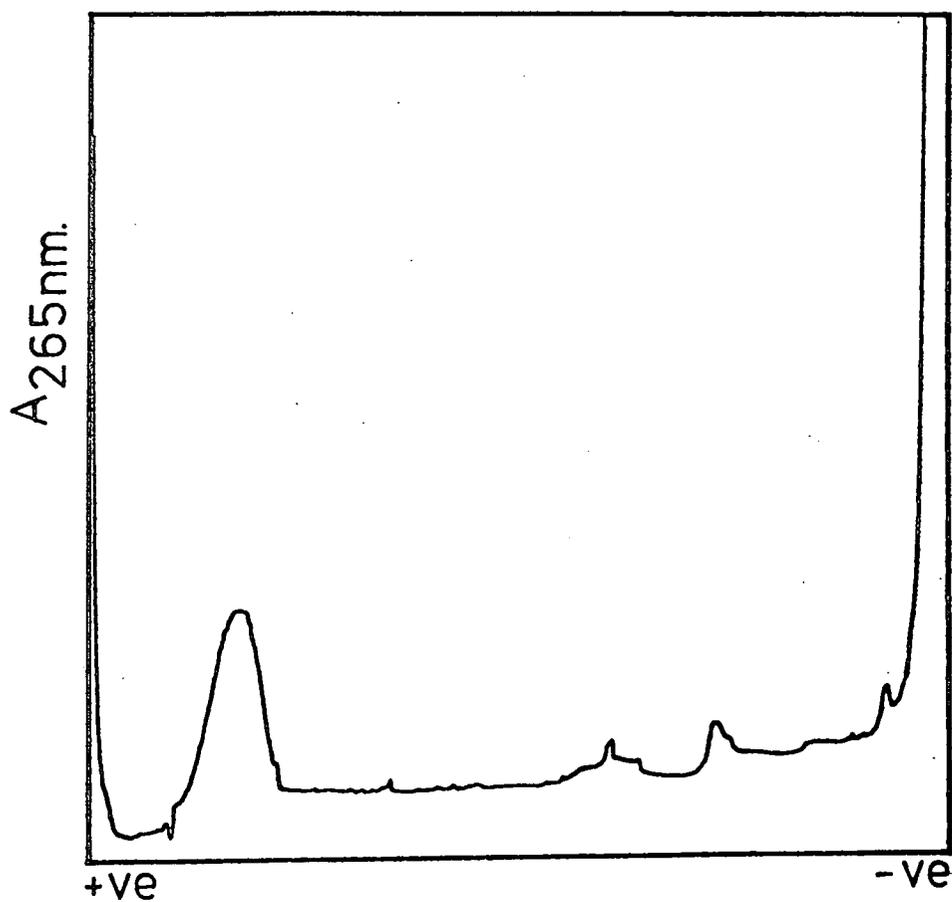
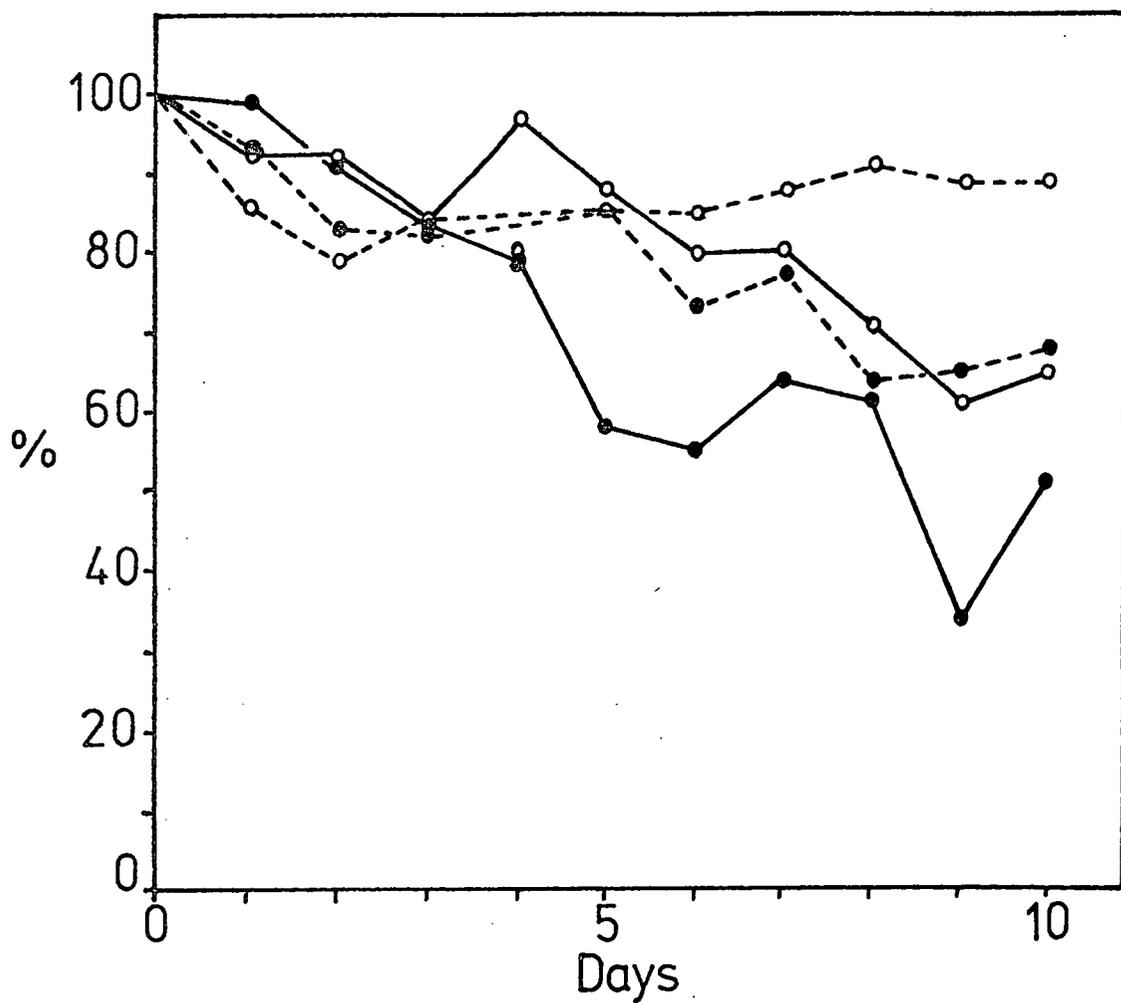
Figure 4.65

The change with time of the chlorophyll content of leaf discs excised from TYMV-infected leaves of chinese cabbage. (Values expressed as a percentage of the yield obtained from the zero time controls)

- Discs incubated on water in the dark
- Discs incubated on water in the light
- Discs incubated on kinetin solution  
in the dark
- Discs incubated on kinetin solution  
in the light

Figure 4.66

PAGE of RNA extracted by the DEP-SDS method from incubated leaf discs excised from TYMV-infected chinese cabbage leaves. Complex formation between TYMV-RNA and chinese cabbage rRNA occurred as a result of freezing of the leaf discs prior to RNA extraction.



that obtained from the corresponding uninfected leaf discs.

As was the case for RNA the relative level remained unchanged after ten days in those discs incubated in the light and floated on water. With the remaining treatments a slower rate of chlorophyll decline was obtained with the infected discs in which the final levels of chlorophyll were approximately 65% of that obtained from similarly incubated uninfected leaf discs.

#### 4.53 Summary

From the above results regarding the senescing of leaf discs excised from uninfected and TYMV-infected leaves of chinese cabbage both similarities and differences in the response to the incubation treatments were apparent. A difference was immediately apparent in the initial yields of DNA, RNA and chlorophyll obtained from the zero time control discs which could be explained in terms of the physiological effects induced by the virus upon the host. As judged by the yields obtained after ten days incubation there were also differences in the overall loss of the cellular components in response to the different treatments employed though the graphs of the changes suggested that the rates of change were not necessarily constant over the entire ten days. For both types of tissue the greatest declines in the content of DNA, RNA and chlorophyll occurred in those discs floated on water and kept in the dark. This rate of loss was retarded by

light or by floating the discs on a solution of kinetin. Although kinetin treatment retarded the deleterious changes the pattern of response seemed to be different in uninfected and TYMV-infected tissue. In the healthy discs the retarding effect of kinetin appeared to fall off after about five days in the dark and after a little longer in the light, whilst in the infected discs the cytokinin effect persisted until the end of the incubation period and there also appeared to be a greater proportional retardation by kinetin than in the uninfected material. The interpretation of this was difficult, however, because there was some variability in the pattern of change observed particularly in the results for the infected material and in the dark treatments of both tissue types. For this reason a statistical analysis of the results was undertaken in order to examine more closely the possible differences in response to the incubation treatments of the two types of experimental tissue.

#### 4.54 Statistical analysis

An initial approximation of the trends shown by the different cellular components in response to the incubation treatments employed was made by linear regression analysis, however, not all the lines gave a significant result either because there was no significant linear trends with time or because, as suggested above, the trends were non-linear. Therefore the 'curvefit' computer program implemented by

Durham University Computer Unit was used to fit both linear and quadratic regressions to the raw data points by the criterion of 'least squares fit'. To test the significance of the linear and quadratic regressions the F-test and the technique of analysis of variance was used. The analysis of variance tables are included in Appendix 2 and Table 15 summarises the values of F and their level of significance for the different components examined (DNA, RNA and chlorophyll) for each incubation treatment for both healthy and TYMV-infected leaf discs.

From Table 15 it is apparent that in most instances significant linear changes with time occur and the negative slopes of the linear regressions (see Appendix 2) confirms the observed declines in yield of the cellular components with time of incubation. With TYMV-infected tissue significant linear trends were not observed for any of the components measured in those discs floated on kinetin and illuminated. The quadratic regression applied to these treatments was significant at the 5% level for RNA and chlorophyll which suggests that the observed recovery of these two components was probably significant. With the DNA content of the discs neither linear or quadratic regressions adequately accounted for the variation observed.

For uninfected material significant linear trends were observed for all incubation treatments when RNA and chlorophyll

Table 15

| Cellular Component | Incubation treatment | Uninfected |              |           |              |        |              | TYMV-infected |              |           |              |  |  |
|--------------------|----------------------|------------|--------------|-----------|--------------|--------|--------------|---------------|--------------|-----------|--------------|--|--|
|                    |                      | Linear     |              | Quadratic |              | Linear |              | Linear        |              | Quadratic |              |  |  |
|                    |                      | F          | Significance | F         | Significance | F      | Significance | F             | Significance | F         | Significance |  |  |
| DNA                | H <sub>2</sub> O+ Dk | 27.24      | 0.1%         | 8.16      | 5%           | 52.41  | 0.1%         | 0.35          | NS           |           |              |  |  |
|                    | H <sub>2</sub> O+ Lt | 0.37       | NS           | 9.75      | 5%           | 5.97   | 5%           | 0.26          | NS           |           |              |  |  |
|                    | CK + Dk              | 112.93     | 0.1%         | 26.69     | 0.1%         | 4.52   | 10%          | 0.13          | NS           |           |              |  |  |
|                    | CK + Lt              | 5.26       | 10%          | 2.84      | NS           | 2.26   | NS           | 3.13          | NS           |           |              |  |  |
| RNA                | H <sub>2</sub> O+ Dk | 32.04      | 0.1%         | 1.23      | NS           | 64.43  | 0.1%         | 0.86          | NS           |           |              |  |  |
|                    | H <sub>2</sub> O+ Lt | 69.86      | 0.1%         | 8.32      | 5%           | 18.68  | 1%           | 2.16          | NS           |           |              |  |  |
|                    | CK + Dk              | 15.42      | 1%           | 3.32      | NS           | 12.64  | 1%           | 2.16          | NS           |           |              |  |  |
|                    | CK + Lt              | 36.77      | 0.1%         | 4.39      | 10%          | 2.16   | NS           | 6.4           | 5%           |           |              |  |  |
| Chloro-<br>phyll   | H <sub>2</sub> O+ Dk | 340.48     | 0.1%         | 1.34      | NS           | 60.64  | 0.1%         | 0.86          | NS           |           |              |  |  |
|                    | H <sub>2</sub> O+ Lt | 187.25     | 0.1%         | 0.67      | NS           | 49.47  | 0.1%         | 2.02          | NS           |           |              |  |  |
|                    | CK + Dk              | 97.75      | 0.1%         | 1.64      | NS           | 45.33  | 0.1%         | 1.12          | NS           |           |              |  |  |
|                    | CK + Lt              | 58.07      | 0.1%         | 2.69      | NS           | 0.75   | NS           | 5.99          | 5%           |           |              |  |  |

df = degrees of freedom

NS = not significant

contents of the discs were considered. The 5% significance of the quadratic regression applied to the RNA content of the leaf discs floated on water and illuminated was in accord with the observed change in the rate of RNA decline under this treatment. With the DNA content of the uninfected leaf discs both linear and quadratic trends were found to be significant for the dark treatments whilst for those discs floated on water in the light only the quadratic regression was significant. Similarly to the infected tissue neither linear or quadratic regressions were significant for the kinetin treated discs kept in the light. These different trends evident for the DNA content may in part be a reflection of the variability of the yields within individual treatments that was observed.

In order to evaluate if there was a different response to kinetin between the two types of tissue, as suggested above, it was necessary to combine the data points, i.e. the yields obtained in the two kinetin treatments for a given cellular component (DNA, RNA or chlorophyll) were treated as one set of data points and linear and quadratic curves again fitted using the computer program. The effect of light and any possible interaction between light and kinetin was also assessed in a similar way. The details of the analysis of variance of these regressions can be found in Appendix 2 and the significance level of the F values obtained are detailed

in Table 16.

The effect of light on the rate of change was significant to at least the 1% level for the linear regression for both types of tissue and for all the cellular components examined. This would indicate an immediate response to light by the tissue and from the previous descriptions (see 4.52 and 4.53) the response to supplying light was to lower the rate of decline that occurred in the contents of DNA, RNA or chlorophyll in dark incubated discs. Whilst the effect of light was similar for both uninfected and TYMV-infected tissue the effect on the rate of change induced by kinetin treatment was different. In the TYMV-infected discs there was again a significant linear effect, that is, there was an immediate response to supplied kinetin which again was a retardation of the decline of the cellular components examined compared to the water treatments. In the discs excised from uninfected tissue there was no significant linear effect with DNA and RNA but there was a significant quadratic effect which, from examination of the quadratic regression coefficients (see Appendix 2), took the form of an initial retardation by kinetin which subsequently declines relative to the water controls. The chlorophyll content of the uninfected leaf discs showed both a linear and a quadratic effect in response to kinetin significant at the 5% level. From the shape of

Table 16

| Cellular Component | df   | Effect              | Uninfected |              |        | TYMV-infected |              |           |      |     |
|--------------------|------|---------------------|------------|--------------|--------|---------------|--------------|-----------|------|-----|
|                    |      |                     | F          | Significance | Linear | F             | Significance | Quadratic |      |     |
| DNA                |      | H <sub>2</sub> O/CK | 0.43       | NS           | 20.07  | 0.1%          | 14.22        | 0.1%      | 0.84 | NS  |
|                    | 1,32 | Lt/Dk               | 16.56      | 0.1%         | 0.02   | NS            | 15.23        | 0.1%      | *    | NS  |
|                    |      | Interaction         | 3.09       | 10%          | 0.24   | NS            | 0.01         | NS        | 1.06 | NS  |
| RNA                |      | H <sub>2</sub> O/CK | 0.47       | NS           | 9.43   | 1%            | 16.83        | 0.1%      | *    | NS  |
|                    | 1,32 | Lt/Dk               | 8.81       | 1%           | 0.01   | NS            | 10.25        | 1%        | 0.70 | NS  |
|                    |      | Interaction         | 3.72       | 10%          | 0.59   | NS            | 0.08         | NS        | 6.46 | 5%  |
| Chloro-<br>phyll   |      | H <sub>2</sub> O/CK | 6.97       | 5%           | 6.05   | 5%            | 31.74        | 0.1%      | 1.81 | NS  |
|                    | 1,32 | Lt/Dk               | 44.40      | 0.1%         | 0.08   | NS            | 25.61        | 0.1%      | 0.53 | NS  |
|                    |      | Interaction         | 1.81       | NS           | 0.07   | NS            | 0.36         | NS        | 3.22 | 10% |

\* Because of rounding errors no 'F' value obtained, see Appendix 2.

df = degrees of freedom

NS = not significant

the curves it would appear that this meant that kinetin was more effective in retarding the deteriorative changes in the presence of light at the longer incubation times.

#### 4.6 Leaf Disc Ageing - Qualitative RNA changes

In order to examine the qualitative rRNA changes taking place during ageing of excised leaf discs an experimental procedure the same as that described for the quantitative changes in cell components was employed. Preparation of the leaf discs from healthy or TYMV-infected chinese cabbage plants and the conditions of incubation and sampling were as previously described (see 4.51) except that 20 x 1 cm diameter leaf discs were placed in each petri dish. Sterile conditions were again maintained throughout the preparation and incubation of the leaf discs. At time zero and at 24 hour intervals thereafter one petri dish from each treatment was taken and the leaf discs transferred to a specimen tube, frozen with liquid air and stored at  $-20^{\circ}\text{C}$  until extracted. Extraction of the RNA for qualitative examination was by the DEP-SDS method. Electrophoresis of the extracted RNA was in 2.6% polyacrylamide gels using the magnesium-containing electrophoresis buffer (see 3.22.2) to maintain the integrity of the chloroplast  $1.05 \times 10^6$  molecular weight rRNA fraction.

Fractionation of rRNA extracted from discs obtained from TYMV-infected leaves resulted in gel profiles in which virtually no high molecular weight rRNA of the host was present (figure 4.66). This result was unexpected in view of the apparent prevention of TYMV-RNA-host RNA complex formation when using the DEP-SDS method therefore a repeat extraction

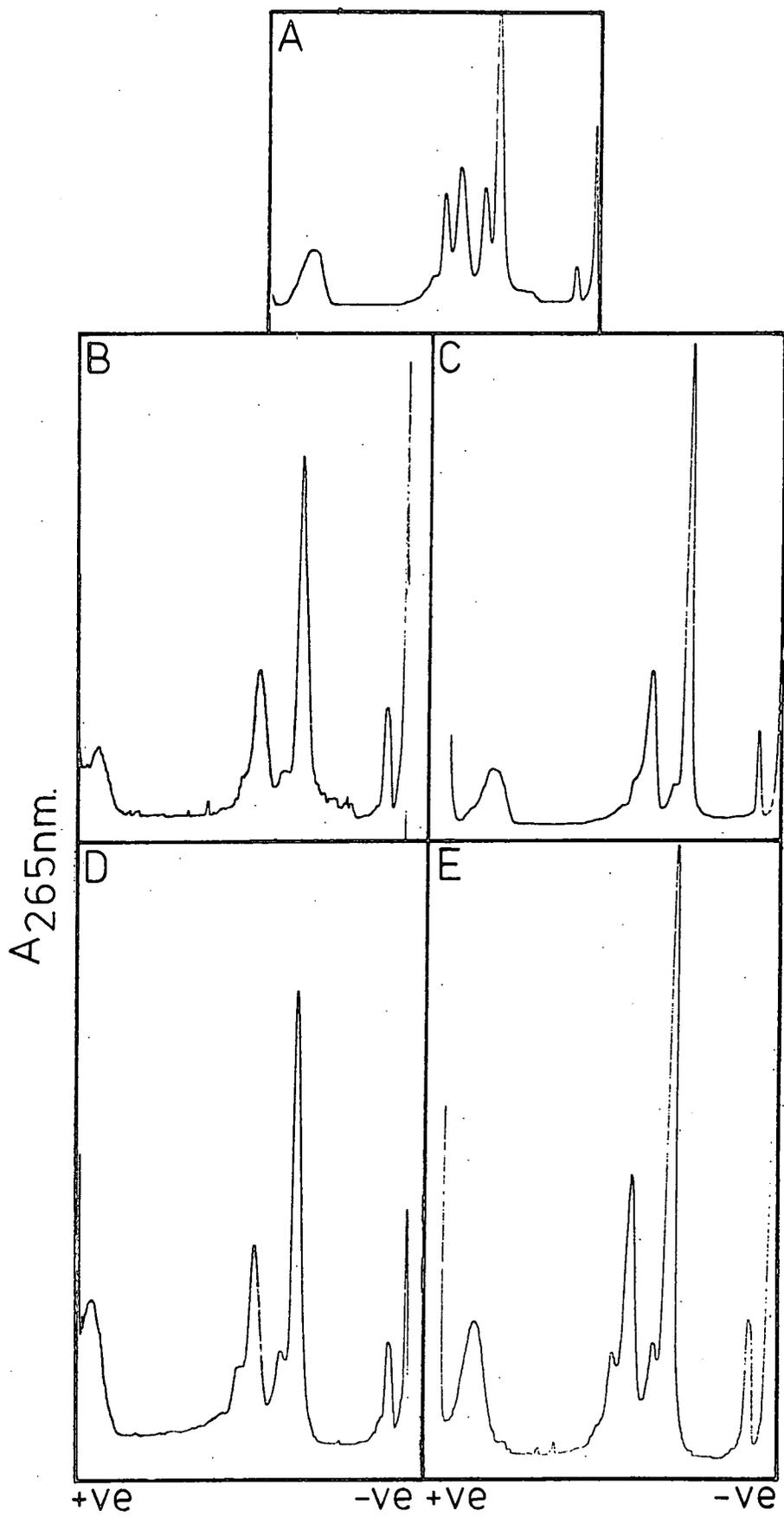
and electrophoretic separation was performed on TYMV-infected leaf tissue. Half the material used was frozen in liquid air prior to homogenisation (as were the leaf discs) while the remainder was homogenised without prior freezing. Control extractions of fresh and frozen leaves of uninfected chinese cabbage were also performed. The results of this experiment have been detailed above (see Section 3.44 and figures 3.22 and 3.23) and it was found that freezing of TYMV-infected leaf tissue before homogenisation resulted in the formation of TYMV-RNA-host RNA complex formation, presumably by the release of TYMV-RNA from the intact viral particles (Kaper and Alting-Siberg, 1969a,b). For this reason no qualitative rRNA data on the ageing of leaf discs excised from TYMV-infected chinese cabbage leaves was obtained.

With the uninfected leaf disc extracts all the incubation treatments used appeared to give a similar pattern of qualitative change (figure 4.67). Compared to the time zero control electrophoretogram (figure 4.67a) the electrophoretograms of extracts from aged leaf discs all showed a proportional decrease in chloroplast rRNA regardless of the conditions under which the leaf discs were incubated. The decrease in amount of rRNA resulted in a less distinct separation of the chloroplast rRNA peaks from the cytoplasmic rRNA peaks, this was particularly a problem in those discs floated on water and incubated in the dark in which the greatest change

Figure 4.67

PAGE of RNA extracted by the DEP-SDS method from incubated leaf discs excised from healthy leaves of chinese cabbage

- A. Zero time control
- B. Discs incubated on water in the dark for 9 days
- C. Discs incubated on water in the light for 10 days
- D. Discs incubated on kinetin solution in the dark for  
10 days
- E. Discs incubated on kinetin solution in the light for  
10 days



in chloroplast rRNA appeared to take place. A deterioration in the quality of the gel traces was evident in all treatments after 5 to 6 days incubation but was less so with extracts from leaf discs floated on the 5 p.p.m. kinetin solution and illuminated. From measurement of the peak area weights the proportional amounts of cytoplasmic and chloroplastic rRNA were determined. For all treatments a relative decrease in the proportion of chloroplast rRNA (or increase in cytoplasmic rRNA) was observed with this change being of a lesser extent in the cytokinin treated incubations than in the corresponding water controls (figure 4.68). The constituent cytoplasmic ( $1.3 \times 10^6$  and  $0.7 \times 10^6$  mol.wt.) and chloroplastic ( $1.05 \times 10^6$  plus  $0.42 \times 10^6$  and  $0.56 \times 10^6$  mol.wt.) rRNA components showed parallel changes to total cytoplasmic and chloroplastic rRNA respectively. (Certain points on the graph for the dark water incubations are omitted because of the difficulty in accurately determining the peak areas of the chloroplast fractions in those incubations in which this fraction was greatly reduced).

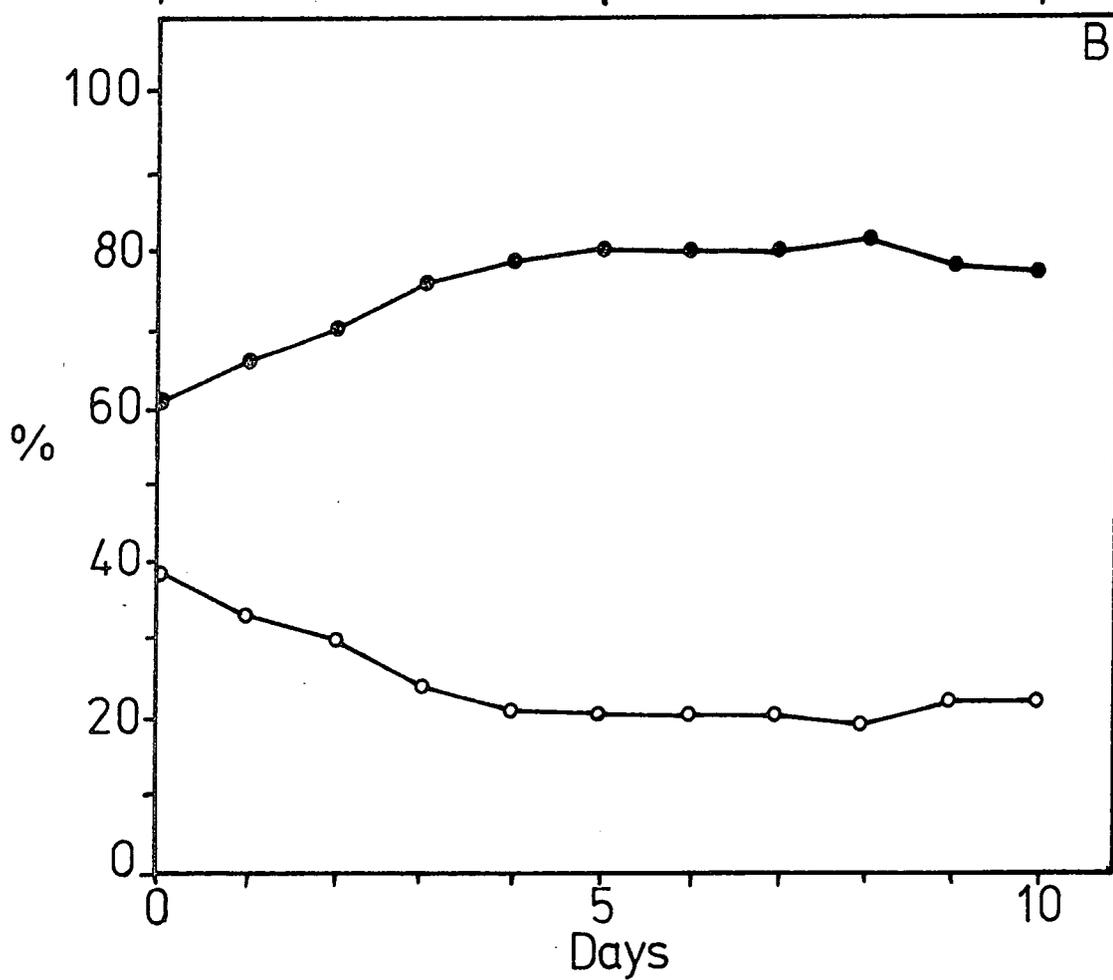
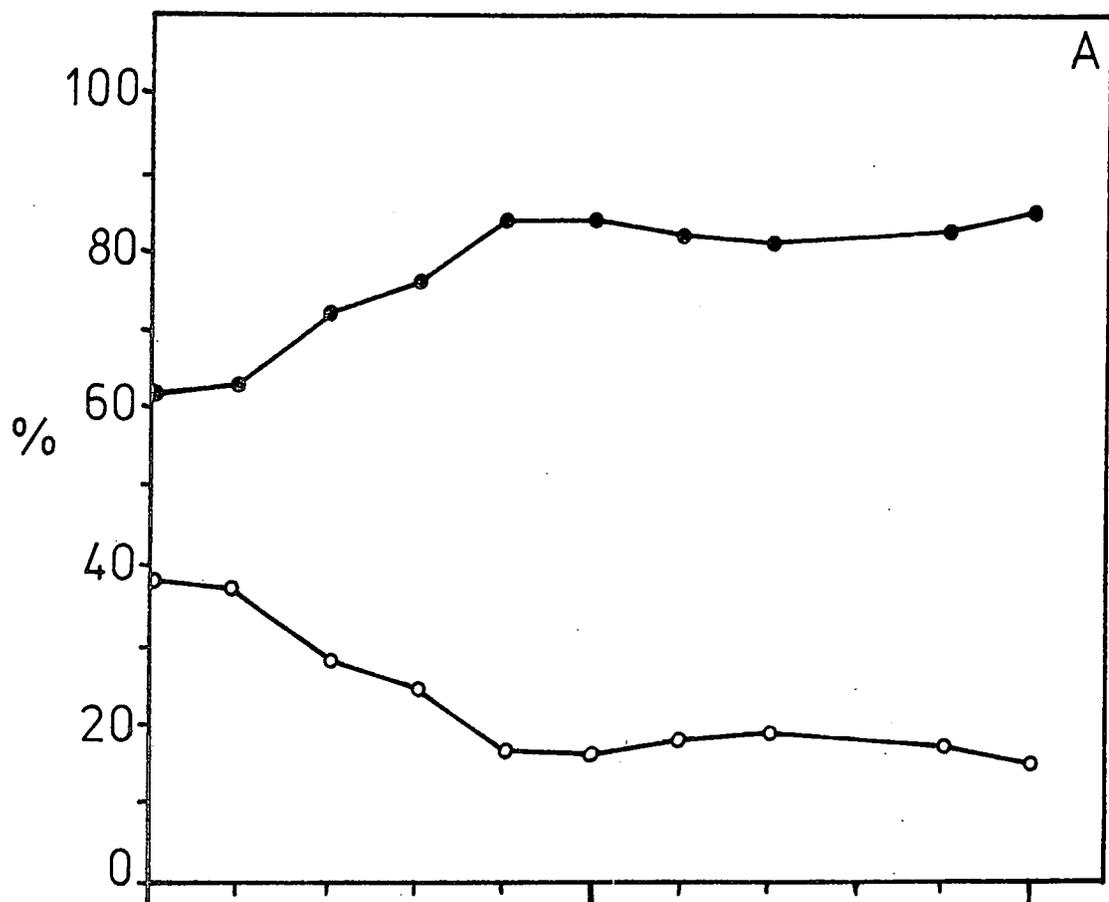
From the peak area proportions the yield of RNA in each high molecular weight rRNA fractions was calculated. The total RNA yield obtained by TCA extraction of the discs was used for these computations in order to avoid any possibility of a variable recovery of RNA by the DEP-SDS extraction method from discs aged for differing periods. In those discs floated on

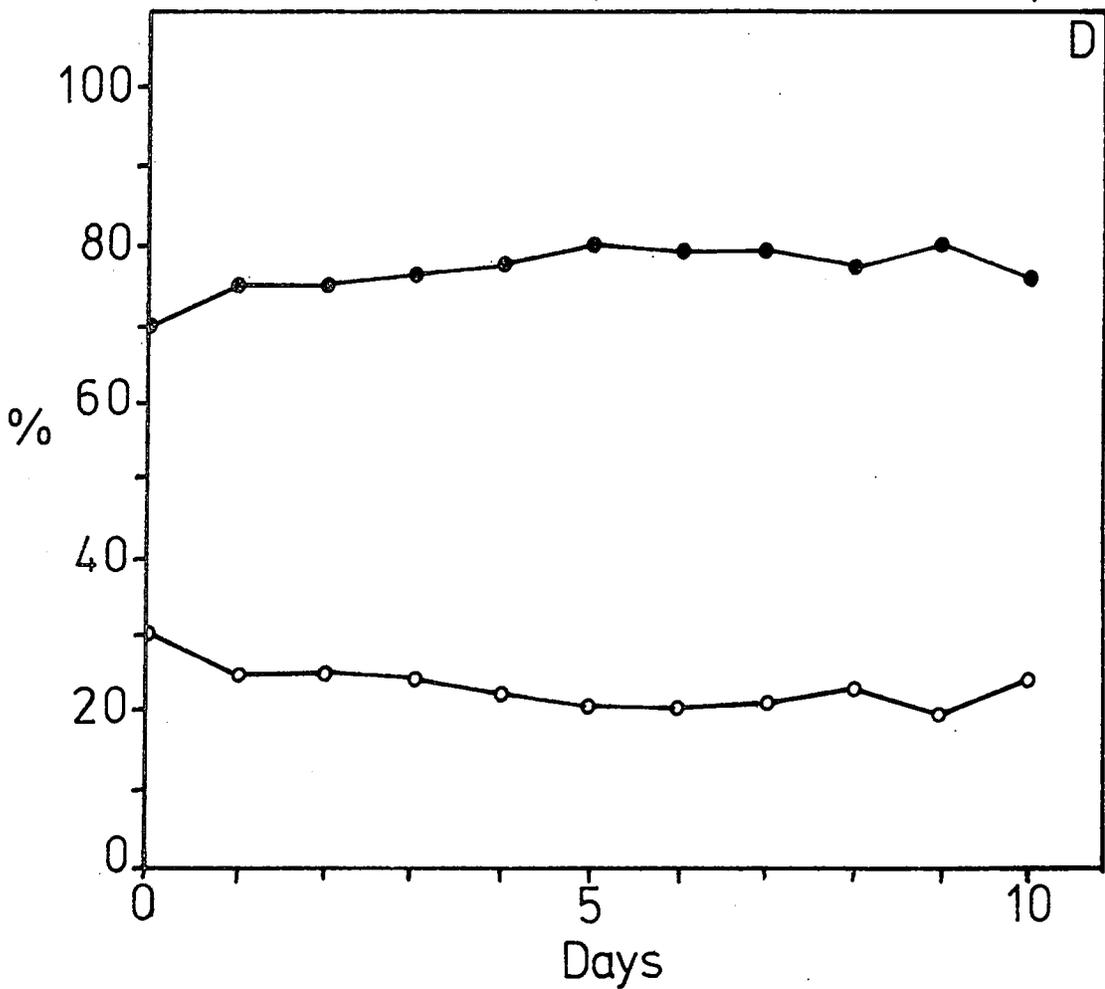
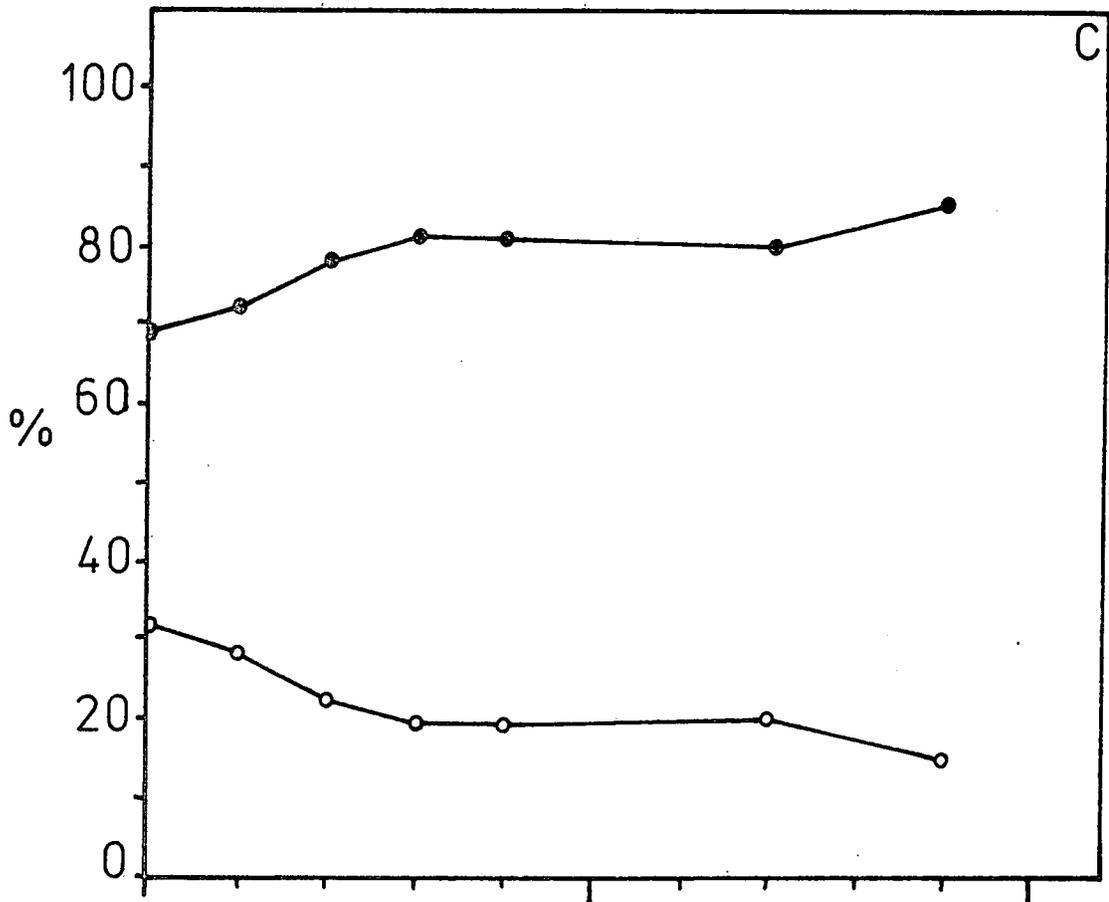
Figure 4.68

The change with time in the proportion of cytoplasmic and chloroplastic rRNA extracted by the DEP-SDS method from leaf discs excised from healthy leaves of chinese cabbage.

- Cytoplasmic rRNA
- Chloroplastic rRNA

- A. Discs incubated on water in the light
- B. Discs incubated on cytokinin solution in the light
- C. Discs incubated on water in the dark
- D. Discs incubated on cytokinin solution in the light





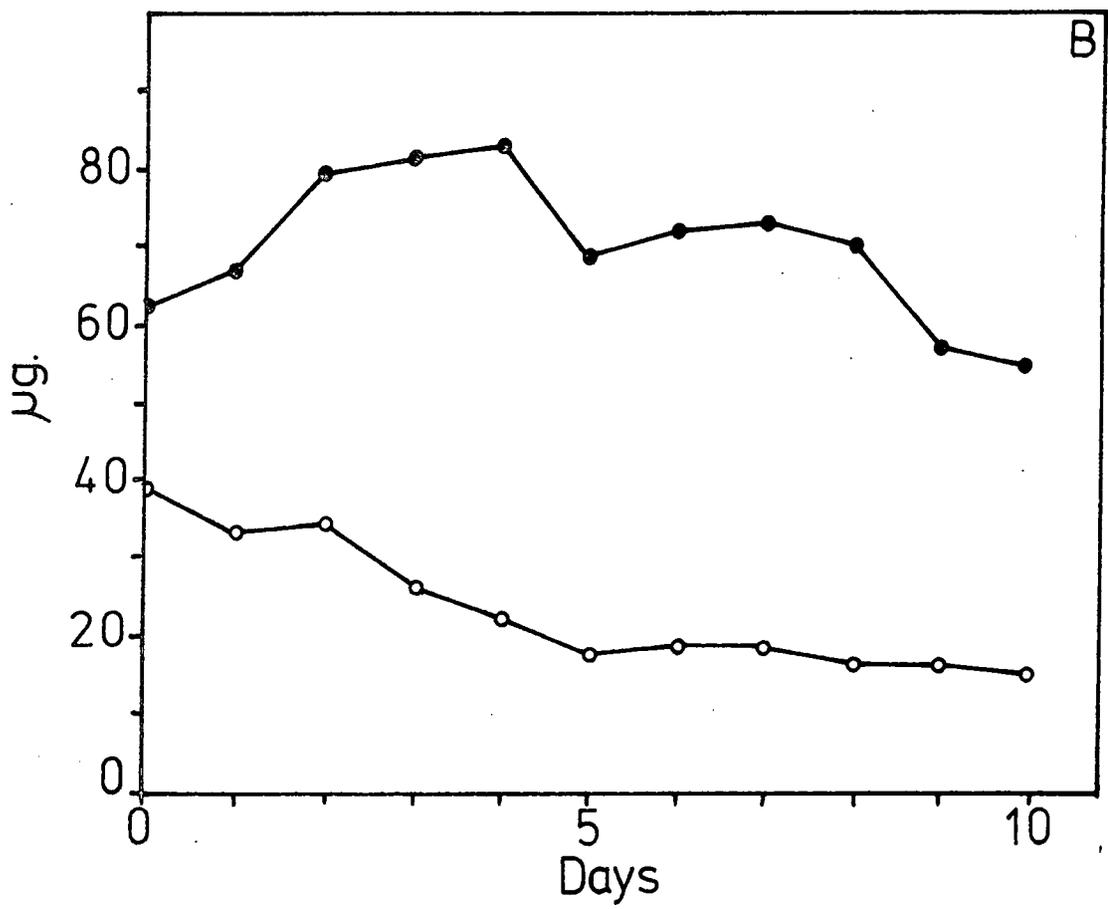
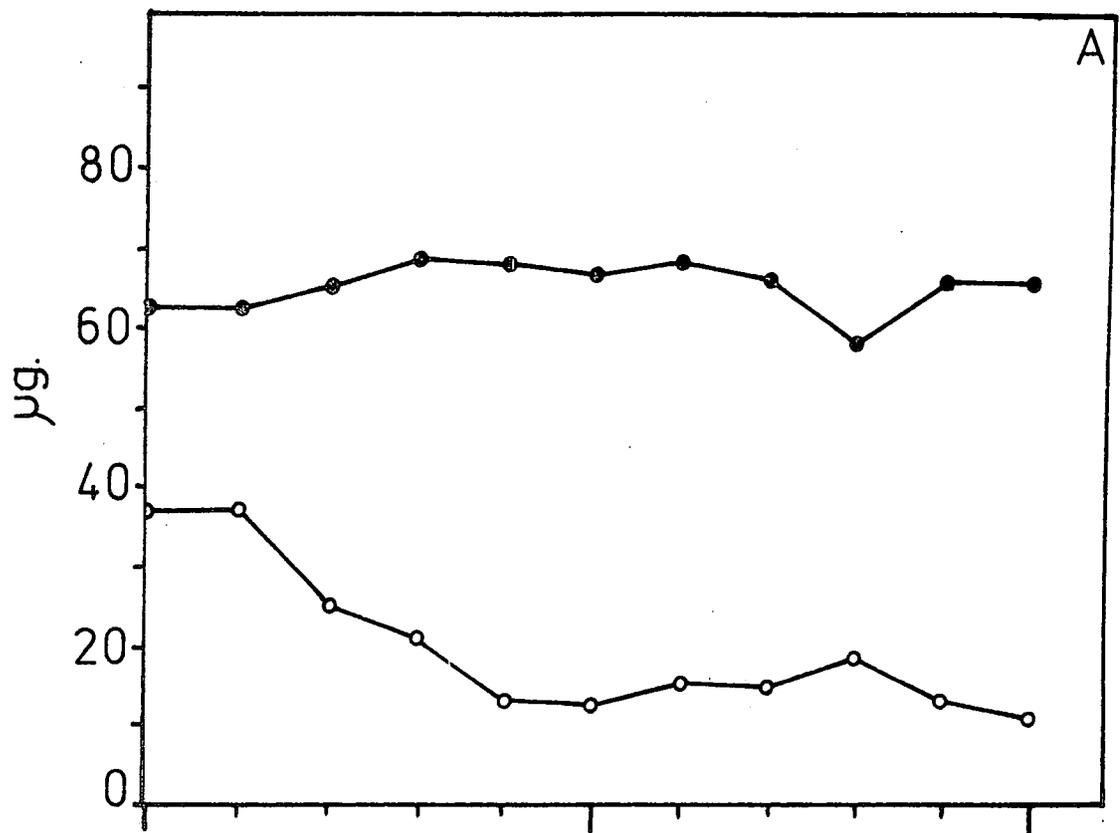
water and illuminated the total yield of cytoplasmic rRNA remained the same throughout the ten day period of incubation (figure 4.69a), the total chloroplast rRNA, however, showed a marked decline in yield during the early period of incubation, this decline then being largely arrested after the fifth day. The component fractions of total cytoplasmic and total chloroplastic rRNA showed the same respective changes. Application of kinetin to light incubated discs resulted in little change in the pattern of loss of total chloroplast rRNA (figure 4.69b) but total cytoplasmic rRNA showed an initial increase in yield, this new level of cytoplasmic rRNA declining after four days until the end of the incubation period. The rise in total cytoplasmic rRNA was evident in both the  $1.3 \times 10^6$  and  $0.7 \times 10^6$  mol.wt. cytoplasmic rRNA fractions. With dark incubated leaf discs a similar pattern of decline of the total chloroplast rRNA was observed in those discs floated on water (figure 4.69c) whilst in contrast to the light-treated discs the total cytoplasmic rRNA was not maintained but exhibited a decline in yield. The changes observed in the  $1.3 \times 10^6$ ,  $1.05 \times 10^6$  plus  $0.42 \times 10^6$ ,  $0.7 \times 10^6$  and  $0.56 \times 10^6$  mol.wt. fractions reflected the changes described for total cytoplasmic and total chloroplastic rRNA. Kinetin treatment of the dark incubated discs retarded the initial rate of loss of the chloroplast rRNA although the overall loss in chloroplast rRNA after ten days incubation was similar to that in the

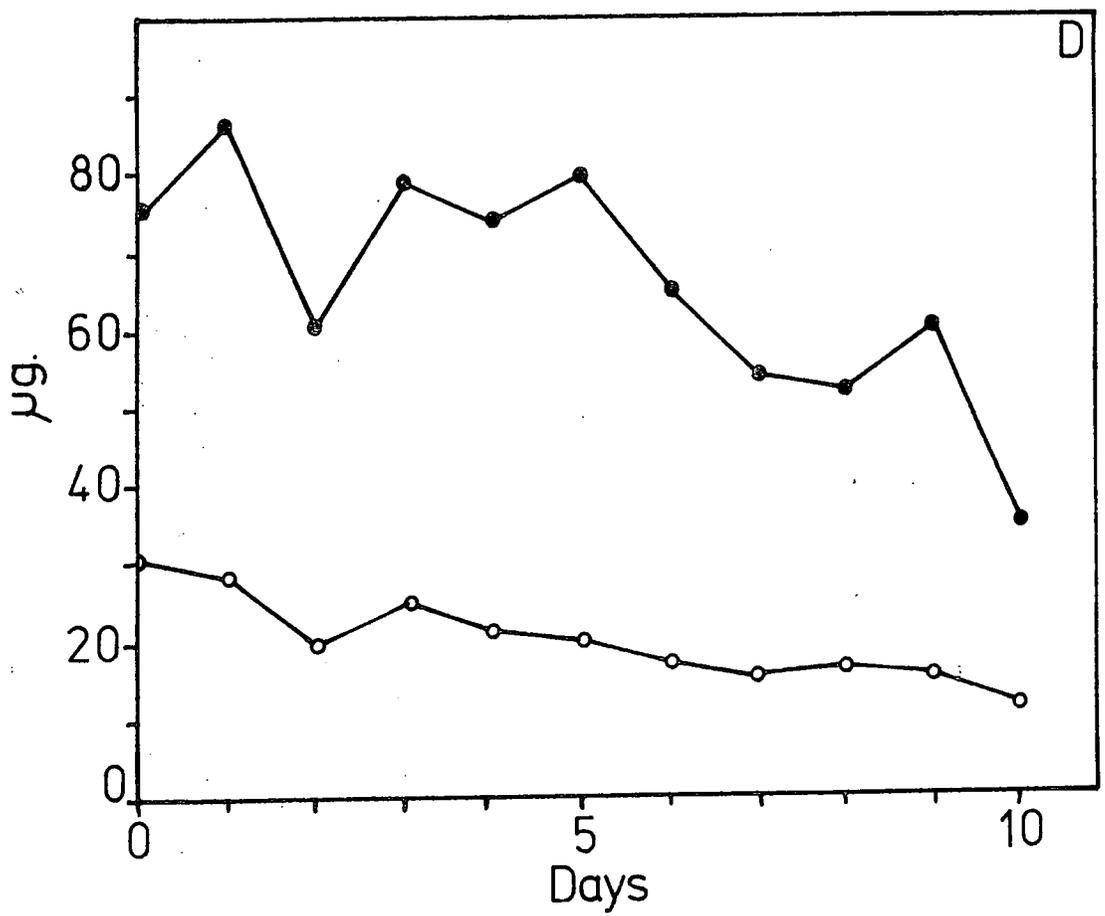
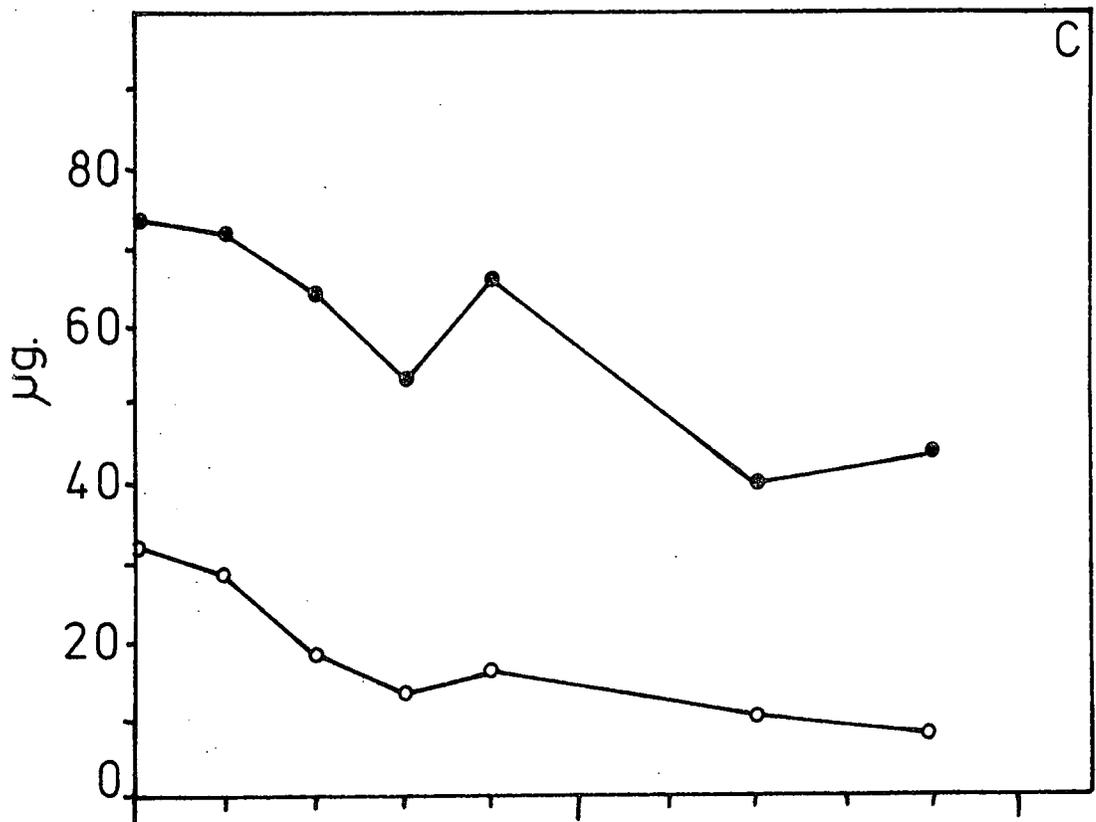
Figure 4.69

The change with time of the total yield of cytoplasmic and chloroplastic rRNA extracted by the DEP-SDS method from leaf discs excised from healthy leaves of chinese cabbage.

- Cytoplasmic rRNA
- Chloroplastic rRNA

- A. Discs incubated on water in the light
- B. Discs incubated on kinetin solution in the light
- C. Discs incubated on water in the dark
- D. Discs incubated on kinetin solution in the dark





light-treated discs (figure 4.69d). An increase in the yield of cytoplasmic rRNA was not apparent in these kinetin-treated discs as it was in the light-incubated kinetin treated discs but compared to the dark, water control, however, the rapid decline in total cytoplasmic rRNA was delayed for five days. The component fractions of the cytoplasmic and chloroplastic rRNA behaved in a similar way to that described for total cytoplasmic and chloroplastic rRNA respectively.

In all the incubation treatments, therefore, a similar decline in total chloroplast rRNA occurred with only the application of kinetin to leaf discs incubated in the dark appearing to have an effect on retarding the rate of decline of the chloroplast fraction. More marked changes were observed in the behaviour of cytoplasmic rRNA. The rapid decline in yield of this fraction in those discs incubated in the dark was eliminated upon illumination; kinetin treatment also retarded the fall in cytoplasmic rRNA but only for a short period of incubation, a decline then occurring. In the light kinetin was seen to have a slight promotory effect on cytoplasmic rRNA content at short periods of incubation, a decline again occurring after a few days. Thus each of the four incubation conditions used had its own characteristic pattern of qualitative rRNA change.

The DEP-SDS extracted ribosomal RNA was also fractionated in an EDTA-containing buffer system (see 3.33.2). The range

of rRNA dissociation fragments produced by this treatment were the same as those previously found with EDTA electrophoresis of rRNA resuspended in a magnesium-containing buffer (see 3.52) and no qualitative differences were observed with varying periods of incubation of the discs, nor between different incubation treatments. However the data for all incubation periods was incomplete because of the large reduction of chloroplast rRNA (the primary source of the breakdown fractions) in those leaf discs incubated for the longest periods of time. Failure to detect dissociation fragments in these extracts did not therefore necessarily imply a qualitative change in the rRNA of the sample but rather a failure to detect fractions present in only minute amounts.

## 5. GENERAL DISCUSSION

Studies on the ageing of attached leaves have largely been confined to an examination of the changes taking place after full leaf maturity has been attained i.e. the changes leading to senescence, in this study the pattern of change of various molecular components in attached leaves were monitored throughout leaf development. Considering DNA, it was found that the total content per leaf closely paralleled the changes in fresh weight whilst DNA concentration showed a rapid decline in the youngest leaves with only a small further decline in concentration in the older leaves (see figures 4.4, 4.5). Except during interphase of cell division the DNA content per nucleus and hence, essentially, per cell remains constant (Howard and Pelc, 1953), the DNA changes observed therefore suggested that both cell number and cell size were increasing in the youngest leaves, this conclusion being in accord with the measurements on cell size and cell number in expanding leaves of spinach made by Detchon and Possingham (1972). There was no accelerated rate of loss of DNA observed in the oldest leaves of chinese cabbage and this relative stability of DNA with age was also apparent from the DNA : RNA ratio of the different leaves (see figure 4.8).

Though the pattern of change of total RNA or ribosome concentration was similar to that described for DNA (see

figures 4.7, 4.48) the yield per leaf showed a different pattern with the content steadily declining from the youngest to the oldest leaf sampled (see figures 4.6, 4.47). Thus in chinese cabbage the maximum net accumulation of RNA and ribosomes occurred at an early stage in the development of the leaf before full leaf expansion was achieved. In cucumber cotyledons there is also a peak net accumulation of total RNA before maximum size is achieved (Lewington et al., 1967). This high level of RNA might be anticipated because at this stage of leaf development the greatest amount of net synthesis would be occurring. Similarly the polyribosome content of many cells has been shown to be directly correlated with the rate of protein synthesis occurring in those cells (Wettstein et al., 1963; Payne et al., 1971; Travis and Key, 1971; Beevers and Poulson, 1972) and it was also in the youngest leaves of chinese cabbage that the highest proportions of ribosomes present as polyribosomes was recorded (70%), during leaf expansion this proportion fell to approximately 45% and remained at this level in the mature leaves. This change supports the view that the highest net synthesis was occurring in the youngest leaves and that there was a subsequent decline in overall protein synthesis during leaf extension.

Although total RNA content was declining in the youngest leaves sampled the net accumulation of cytoplasmic and chloroplastic rRNA did not occur at the same time, cytoplasmic

rRNA followed the same pattern as total RNA but chloroplastic rRNA showed a net increase during leaf expansion and declined only after maximum leaf size was achieved (see figure 4.22). This timing of the accumulation of chloroplast rRNA was also reflected in the increase in the content per leaf of chlorophyll during leaf expansion (see figure 4.9) and, though not specifically examined, it would be anticipated from the work of Clark et al. (1964) on chinese cabbage and of Eilām et al. (1971) on cucumber leaves that the chloroplast ribosome population would also show a similar increase in the young leaves. A similar differential timing of the accumulation of cytoplasmic and chloroplastic rRNA occurs in radish cotyledons (Ingle, 1968) but in cucumber leaves both cytoplasmic and chloroplastic rRNA accumulate throughout leaf expansion (Callow, 1974) whilst in Perilla frutescens (Callow et al., 1972) and tobacco (Takegami, 1975) the peak accumulation of chloroplast rRNA is seen to occur before that of cytoplasmic rRNA. The reason for these differences could be environmental resulting from the conditions under which the plants were grown with light regime possibly being of particular importance (Grierson et al., 1976), the anatomy of the shoot apex could influence the pattern of accumulation e.g. the tight head in which young leaves of chinese cabbage develop may shield the young developing leaf from light, or it could be a developmental step the timing of which may be genetically determined and species specific.

This is a problem which requires further investigation and in which the biochemical changes could be linked to an examination of the development of the proplastid into the mature chloroplast. The net accumulation of the chloroplast rRNA in the young leaves of chinese cabbage was consistent with incorporation studies on radish cotyledons (Ingle, 1968; Paranjothy and Wareing, 1971), Pea (Treharne et al., 1970) and cucumber leaves (Callow, 1974) in which marked synthesis of plastid rRNA was confined to the period of expansion with very little or no incorporation of labelled precursors into chloroplast rRNA after full leaf expansion. Incorporation into cytoplasmic rRNA can continue until late in the life cycle of the leaf which suggest a greater rate of turnover of the cytoplasmic ribosomes. Incorporation studies on chinese cabbage would therefore be of interest in view of the differing accumulation patterns of cytoplasmic and chloroplastic rRNA and also in conjunction with systemic turnip yellow mosaic virus infection since the virus primarily affects chloroplast structure and metabolism. The lack of a technique for obtaining uncomplexed host rRNA from TYMV-infected plants has previously precluded such studies.

Following full leaf expansion there was a decline in the total yield per leaf of both cytoplasmic and chloroplastic rRNA (see figure 4.22) until in the oldest leaf sampled there

remained 14.8% and 9.3% respectively of the yield obtained at full leaf expansion. There was thus only a slight difference between the rates of loss of cytoplasmic and chloroplastic rRNA and peaks of chloroplast rRNA were quite distinct on electrophoretograms of RNA extracted from the oldest leaves (see figure 4.19). The presence of chloroplast rRNA in these oldest leaves was in accord with the electron microscopy of old leaves in which the chloroplast was often the residual organelle of the cell (see Appendix 1), it was also consistent with the typical pattern of leaf ageing in chinese cabbage in which the leaves stay green until just prior to abscission when they rapidly dry, this pattern of ageing also being reflected in the estimates of chlorophyll content per cell (see figure 4.11). Vicia faba which has a similar conservation of chloroplast rRNA to chinese cabbage also shows little yellowing of the leaf with age (Dyer and Osborne, 1971), in cucumber leaves (Callow, 1974) and leaves of Xanthium pennsylvanicum (Dyer and Osborne, 1971), however, the proportion of chloroplast rRNA falls rapidly with increasing leaf age. These latter two species have long-lived yellow leaves which may explain the difference observed in the retention of chloroplast rRNA though tobacco also has long-lived yellow leaves but has no accelerated loss of chloroplast rRNA (Dyer and Osborne, 1971). In tobacco, V. faba and chinese cabbage the lamellae of the chloroplasts remain largely intact

in cells of old leaves indicating a prolonged integrity of the chloroplasts which may account for the maintained level of chloroplast rRNA. Thus chlorophyll content need not be indicative of the physiological state of the leaf and changes in chlorophyll content may not be an inevitable part of the ageing process, as is also indicated by the senescence of non-yellowing mutants of Festuca pratensis (Thomas and Stoddart, 1975).

The yield of ribosomes obtained fell with increasing leaf age, however, polyribosomes were found in all the leaves sampled except the oldest in which no ribosomes were detected even by the sensitive methods used. Eilam et al. (1971) similarly found that polyribosomes were present at all stages of ageing of cucumber leaves except possibly when the leaves were about to dry out as then the majority of ribosomes were lost. These authors experienced difficulties, however, in obtaining sufficient yields of ribosomes for analytical centrifugation which illustrates the usefulness of the PAGE technique of polyribosome fractionation which enabled the handling of very small quantities of material.

It has been noted above that the proportion of ribosomes as polyribosomes fell during leaf expansion to approximately 45%, this level was then maintained until in the oldest leaves an increase in the proportion of polyribosomes occurred (see

figure 4.50). During the course of senescence the free ribosome population usually declines before the membrane-bound ribosomes (Butler and Simon, 1971; Eilam et al., 1971) thus a loss of membrane integrity at this stage of senescence may result in membrane-bound polyribosomes becoming more accessible to extraction. The proportional increase in polyribosomes might also arise if there was a preferential loss of monoribosomes by degradative enzymes possibly released by rupture of the lysosomal or tonoplast membranes (Butler and Simon, 1971; Appendix 1). The observed increase in the proportion of polyribosomes could also reflect a change in metabolism, the polyribosomes possibly being involved in the synthesis of senescence specific proteins. Attempts to test directly if the increase in polyribosomes was a result of increased protein synthesis, rather than say an artefact of extraction, by the use of labelled precursors have inherent difficulties in that old leaves undergoing sequential senescence lose substrates and nutrients by sink effects to younger parts of the plant. The old leaves therefore present a difficult target tissue into which to introduce labelled substrates and a negative result need not necessarily reflect the level of protein synthesis occurring. The use of cell free extracts might also give an incorrect assessment of the in vivo situation because senescing leaves may not have lost synthetic

capabilities even though no such synthesis might be occurring in vivo (Simon, 1967).

Extrapolating from the ribosomal RNA results it would be expected that the populations of cytoplasmic and chloroplastic ribosomes would decline with leaf age at similar rates, differences could however arise in the polyribosome status of these two ribosome populations. Such a difference has been observed in P. frutescens by Callow et al. (1972) who found that with increasing leaf age an earlier and greater decline in the proportion of polyribosomes occurred in the chloroplast ribosome population compared with the changes observed in the cytoplasmic ribosome population. However, in P. frutescens the yield of chloroplast ribosomes declined faster than that of cytoplasmic ribosomes which would not be anticipated in chinese cabbage, the trend in polyribosome status of the two ribosome populations may therefore show a different pattern to P. frutescens.

The decline in ribosome, and hence rRNA, content discussed above is a characteristic feature of ageing leaves and could arise through an accelerated breakdown of existing ribosomes, slower formation of new ribosomes or both. The latter alternative could arise by a number of mechanisms; a change in the nature of the DNA by, for example, an age-dependent

alteration of histone binding (von Hahn, 1964/5) could lead to a decrease in the proportion of DNA template transcribed; a decrease in the synthesis of nucleotide triphosphates or other building blocks of the ribosome or a lowered activity of RNA polymerase could also lead to fewer ribosomes. Regarding breakdown Srivastava (1968b) has suggested that chromatin-associated nucleases may play a significant role in the observed declines. The changing patterns of ribonuclease activity have been reviewed by Dove (1973) who has indicated that a number of different ribonuclease fractions are present in plants and that conflicting evidence exists between the activity of ribonucleases and the amount of RNA in ageing leaves (e.g. Kessler and Engelberg, 1962; Phillips and Fletcher, 1969; Phillips et al., 1969) possibly, therefore, only specific ones may be involved in the age related RNA decline (e.g. Hadziyev et al., 1969). Eilam et al. (1971) have expressed the view that only the later stages of ribosome decline are related to increases in ribonuclease activity and that this is not the primary cause of the initial decline.

Considering more closely the individual RNA components it was seen that the ratio of heavy to light rRNA did not change with increasing leaf age for either chloroplastic or cytoplasmic rRNA. This was in contrast to the situation found in tobacco and X. pennsylvanicum in which indications of a selective

breakdown of the  $0.7 \times 10^6$  mol. wt. fraction has been observed (Dyer and Osborne, 1971). That such an asynchronous breakdown by endogenous ribonucleases may occur in vivo was indicated by Payne and Loening (1970) who demonstrated that the  $0.7 \times 10^6$  mol. wt. cytoplasmic rRNA species was degraded more readily than the  $1.3 \times 10^6$  mol. wt. species during the isolation of pea root microsomes, similar observations also being made by Roberts et al. (1973) with ribosomes isolated from rye embryos. Attempts to detect accumulated RNA breakdown products by such a breakdown in vivo have not been successful (Dyer and Osborne, 1971; Callow and Woolhouse, 1973; Payne and Boulter, 1974) nor were they observed in this study. This suggests that RNA degradation may proceed rapidly to fractions not distinguished by electrophoresis and this may possibly involve transfer of the ribosomes into lysosomes and/or vacuoles (Payne and Boulter, 1974).

An age-associated change characteristic of chloroplast rRNA is the accumulation of 'nicks' in the  $1.1 \times 10^6$  mol. wt. chloroplast rRNA first described by Ingle (1968) in radish cotyledons. Knight and Quick (1969) using MAK column chromatography to separate the RNA fractions from radish leaves only detected the  $0.4 \times 10^6$  mol. wt. breakdown fraction resulting from the in vivo 'nicking' of the  $1.1 \times 10^6$  mol. wt. rRNA after the total RNA content of the leaves had begun to

decline. Similarly Rosner et al. (1974) only detected this breakdown fraction in the older fronds of Spirodela. In chinese cabbage this lability of the  $1.05 \times 10^6$  mol. wt. chloroplast rRNA fraction was evident in leaves of all ages but no age-related increase in the amount of  $0.42 \times 10^6$  mol. wt. RNA was observed. The lability of the  $1.05 \times 10^6$  mol. wt. chloroplast rRNA was observed despite the use in the extraction procedure of the powerful ribonuclease inhibitor DEP which thus confirms these cleavages as the result of hidden breaks rather than as a consequence of ribonuclease damage during extraction. This early detection of 'hidden breaks' in chinese cabbage suggests that turnover of this fraction may have commenced in the very young leaves before the peak accumulation of chloroplast rRNA has occurred.

A further age-associated change has been found in the proportion of low molecular weight 'soluble-RNA' which was seen to increase in the yellow senescent leaves of X. pennsylvanicum (Dyer and Osborne, 1971) and in ageing radish leaves (Knight and Quick, 1969) but was not observed with chinese cabbage (see figure 4.26). The reason for the difference may be related to the fact that in X. pennsylvanicum and radish there is a preferential decline in the chloroplast fractions of high molecular weight rRNA which may result in an apparent increase in sRNA whilst in chinese cabbage, in

which no preferential loss of chloroplast rRNA was observed, the overall level of the high molecular weight RNA would be maintained and thus would not give rise to an apparent change in sRNA. However whilst overall levels of sRNA may not show marked changes during senescence alterations in amount of specific t-RNA species, which largely constitute the sRNA fraction, have been implicated as being a possibly significant controlling factor in senescence (Bick et al., 1970; Wright et al., 1973; Nathan and Richmond, 1974), though the precise mechanism of possible control is not known.

A striking age-related change occurred in the electrophoretic mobility of the small ribosomal sub-units (see figure 4.58), it thus seemed that a change in the physiology of the leaf was reflected in a change in the physical characteristics of the ribosomal sub-units. This possibility has previously been suggested by Barker and Hollinshead (1965) working on the changing magnesium stability of pea ribosomes, Biswas (1969) also noted that in mung beans the ease of dissociation of ribosomes into sub-units increased with increasing age of the cotyledons. These observations could be related to endogenous magnesium levels changing which may result in a different net charge on the extracted sub-units thus giving rise to the observed changes in electrophoretic mobility. An alteration of the net charge on the sub-units might also

result if the protein complement of the ribosomes was changed, such an age-related change has been described by Srivastava and Arglebe (1967) for barley leaf ribosomes. The mobility change might also result if there was a conformation change in the sub-units, Dahlberg et al. (1973) using PAGE detected such conformation changes induced by certain drugs in polyribosomes extracted from E. coli.

The change in the quantitative yields of DNA, RNA and chlorophyll that occurred in the course of the incubation of leaf discs excised from mature healthy leaves of chinese cabbage showed patterns of change similar to those expected from the literature (see 4.1). The period of incubation employed (ten days) was longer than that generally used and the rate of change for a particular component was not necessarily constant for a given incubation treatment. Although statistically significant losses occurred with all the incubation treatments used (except for the DNA content of illuminated discs) different rates of change and patterns of loss were apparent, thus the greatest deterioration occurred in the dark and retardation of the loss of the cellular components occurred when kinetin or light were supplied. Suggestions of a quadratic element (significant at the 5% level) in the effect of light on discs floated on

water were seen with DNA and RNA content indicating, from the regression coefficients (Appendix 2), that there was a greater retardation of the deteriorative changes by light at the longer incubation periods. This was probably because the light permitted continued photosynthetic activities whilst the dark-treated discs had only stored food reserves which would have been rapidly utilised (see Appendix 1). Supporting this idea is the recent report from Thimann et al. (1977) who have shown that for detached oat leaves light delays senescence but can be substituted to some extent by supplying sucrose or glucose.

Considering the combined data the overall retardation of the deteriorative changes by light was a linear effect, whilst retardation by kinetin was not constant over the whole of the incubation period as there was a decline in the kinetin effect at the longer incubation periods. A similar response in the levels of chlorophyll and RNA to cytokinin treatment has been observed in detached barley leaves (Biswal and Mohanty, 1976), but as this appears to be an effect which is only evident after at least four to six days of incubation its occurrence in other tissues may not have been observed because of the generally shorter incubation periods used. In these instances, as in the early part of the incubation period with chinese cabbage, an essentially linear effect would be apparent (e.g. Osborne, 1962; Takegami, 1975). The

observed reduction in the retarding effect of kinetin could be a real effect on the physiology of the leaf discs arising through a raising of the metabolic functions of the cells to a supra-optimal level which cannot be maintained and consequently declines as observed. There is also a possibility that it could be an artefact of the experimental system if the supplied kinetin had been metabolised or for some other reason became unavailable to the experimental tissue e.g. by physical obstruction to uptake by death of cells at the cut edges of the excised discs. These are potentially more serious problems when long incubation periods are used and simple experiments can be designed to assess their possible significance. The statistical analysis gave no indication of any interaction between the effects of light and kinetin on the levels of the cellular components examined.

Although the quantitative results are of interest it is difficult to fully equate them with similar analyses on the intact plant because the time scale over which the changes occur and the absolute levels of the cellular components were different, and also because the leaf discs are removed from the influence of other parts of the plant thus precluding export of substrates such as occurs in attached leaves. Therefore though broadly similar changes with regard to declines in total chlorophyll and RNA content occurred, and the DNA

content was seen to be more stable, comparison of the qualitative RNA changes occurring in the discs was easier and possibly more meaningful.

In the attached leaves of chinese cabbage following leaf maturity the content of both cytoplasmic and chloroplastic rRNA declined at similar rates with increasing leaf age. Similarly both cytoplasmic and chloroplastic rRNA were seen to decline from the beginning of the incubation period in those excised leaf discs floated on water in the dark (see figure 4.70) however the rates of decline were different and after nine days 39% of the cytoplasmic rRNA content of the discs had been lost whilst for chloroplastic rRNA the figure was 76%. This preferential decline was more marked in the remaining leaf disc incubation treatments in which similar declines in chloroplast rRNA were observed but either no marked decline in cytoplasmic rRNA occurred or the decline was delayed until a few days after excision. Thus there was a qualitative difference between the patterns of rRNA decline in attached leaves and excised leaf discs, differences were also apparent at the ultrastructural level with regard to vesiculation of the grana in the chloroplasts and the form of the accumulated plastoglobuli (see Appendix 1). These results thus indicate that attached leaf senescence and excised leaf disc senescence in chinese cabbage are different

for the parameters examined. It is more difficult, however, to assess if these differences represent fundamentally different courses of physiological ageing or whether they are different secondary features of an otherwise common ageing mechanism.

Light treatment of the discs floated on water prevented the decline in cytoplasmic rRNA, similarly retardation by kinetin treatment acted principally on cytoplasmic rRNA causing a delay in its decline though a slight retardation of the initial rate of loss of chloroplastic rRNA also occurred. If both light and kinetin were supplied to the discs the decline in cytoplasmic rRNA was not only delayed but there was an initial increase in the content of cytoplasmic rRNA. A stimulation of RNA level by cytokinin treatment has previously been observed in leaf discs of tobacco (Sugiura et al., 1962) and X. pennsylvanicum (Osborne, 1962) and in excised barley leaves (Srivastava and Ware, 1965). The work of Berridge et al. (1970), who found that kinetin appeared to bind preferentially to 80s cytoplasmic ribosomes rather than to the 70s chloroplastic ribosomes of chinese cabbage, suggests a possible direct relationship with this observed effect on cytoplasmic rRNA levels and would also suggest that retardation of senescence is not necessarily dependent upon maintenance of chloroplast rRNA. There is,

however, conflicting evidence, for example Dyer and Osborne (1971) have noted that leaves of Nicotiana tabacum and Vicia faba which can be regreened retain chloroplast rRNA whereas X. pennsylvanicum leaves lose chloroplast rRNA on yellowing and cannot be regreened. In experiments on excised leaf discs of N. tabacum floated on water for four days Takegami (1975) observed a preferential decline of chloroplast rRNA whilst cytoplasmic rRNA remained unchanged in the dark and increased in the light. This result was similar to chinese cabbage but in contrast application of cytokinin (benzylaminopurine) to the discs was seen to have virtually no effect on cytoplasmic rRNA but chloroplast rRNA loss was inhibited, particularly in the dark. This indication of an inter-generic difference between the effect of cytokinin on the pattern of qualitative rRNA changes has also been shown to be interspecific (Sugiura et al., 1962). The above results suggest that senescence in different species can be associated with different patterns of change of the rRNA fractions and that these changes may therefore be a secondary rather than a primary effect of cytokinin application. This opinion has also been expressed by Paranjothy and Wareing (1971) who found that kinetin treatment did not stimulate chloroplast rRNA synthesis in detached radish leaves and also

that senescence retardation by kinetin still occurred if cytoplasmic rRNA synthesis was suppressed by 5-fluorouracil. They suggested that the principal effect may be on particular mRNA and sRNA fractions though other explanations are possible, particularly in terms of suppression of breakdown by inhibition of proteolysis (Srivastava and Ware, 1965; Balz, 1966, Kuraishi, 1968; Tavares and Kende, 1970) or ribonuclease (Srivastava and Ware, 1965; Balz, 1966; Udvardy et al., 1967; Shibaoka and Thimann, 1970; Wyen et al., 1972), Thimann and co-workers have also suggested a specific action on a serine containing protease (Shibaoka and Thimann, 1970; Martin and Thimann, 1972; Choe and Thimann, 1974).

Work on the effects of plant virus infection on host metabolism frequently involve examination of the changes occurring following inoculation of the leaves (e.g. Frazer, 1969, 1972; Nakagaki and Hirai, 1971; Balazs et al., 1973; Aldwinkle, 1975; Kasamo and Shimomura, 1977), sham inoculation of similar leaves or half-leaves then provides suitable control material. However inoculation of leaves can result in wound effects (Diener, 1961; Bagi and Farkas, 1967; Randles, 1968) in addition to virus induced responses and in this study therefore systemically infected leaves were investigated and uninfected plants of a similar age were used as controls. Examination of chinese cabbage leaves systemically infected with TYMV had inherent

difficulties not encountered when dealing with uninfected tissue; because of the mosaic pattern of infection in which areas of cells that do not show symptoms of virus are present (Chalcroft and Matthews, 1966) different leaves sampled along the phyllotaxy of a plant in sequential ageing studies could have quite different proportions of the leaf apparently affected by the virus, also areas of the leaf which macroscopically appear to be uniformly infected can consist of horizontal layers of cells of quite different degrees of infection (Chalcroft and Matthews, 1967). As a consequence of this lack of uniformity in the tissue sampled the results obtained from TYMV-infected material were more irregular than from comparable uninfected tissue, this still being the case when using leaf discs in which smaller areas of more uniform appearing tissue were used. A further problem was encountered when assaying the levels of RNA or ribosomes since estimation of these components using absorbance in ultraviolet light also included contributions to the observed absorbance made by the presence of the virus in the extracts. Estimation of the amount of virus present is difficult because lesion assays require carefully defined conditions and can still be variable (Diener and Jenifer, 1964; Pleij et al., 1977), quantitative comparisons between TYMV-infected and uninfected material was

therefore difficult though qualitative comparisons could be made.

The yield of RNA plus virus showed essentially similar changes per leaf and per gram fresh weight with increasing leaf age as did the RNA content of leaves of uninfected plants (see figures 4.35, 4.36), however there was an increased recovery in the oldest leaves of TYMV-infected plants that did not occur in healthy plants and this was also apparent in the ribosome extracts. This feature may be the result of easier extraction of the RNA and ribosomes plus virus because of deterioration of the cells, alternatively this increase might represent increased assembly of the virus possibly through the declining integrity of the cells, particularly of the chloroplasts, resulting in a lowered resistance of the host to the virus. In this connection Kiraly et al. (1968) have reported that susceptibility of tobacco and bean plants to TMV infection is increased by host senescence, possibly through degradation providing substrates for virus synthesis (Nakagaki and Hirai, 1971). Correlated with this change in yield was an accelerated rate of loss of chloroplast rRNA in the oldest leaves (see figure 4.40) and as it is the chloroplast that is primarily affected by TYMV infection then it might be anticipated that it would be this organelle that would be chiefly affected if increased virus production

was occurring. The change in the ribosome plus virus content of the leaves of infected plants showed a lowered rate of decline in concentration compared with healthy plants (see figure 4.48) and there was a delayed decline in the content per leaf (see figure 4.47), this could be attributable to virus multiplication and a distinct change in the proportion of virus to total ribosomes was seen in these younger leaves (see figure 4.57). Because of co-migration of virus components with the polyribosome fractions estimation of these fractions was not possible though a similar pattern of change in the proportion of ribosomes as polyribosomes appeared to occur with age in the TYMV-infected leaves as in uninfected leaves except that the level of polyribosomes in the youngest infected leaves was lower. The qualitative rRNA data showed that in uninfected plants there was a marked increase in the chloroplast fractions in the youngest leaves, therefore a significant proportion of the polyribosomes present were probably concerned with the synthesis of chloroplast specific proteins. Because TYMV mainly affects the chloroplasts the lowered level of polyribosomes observed in the infected leaves may have been a consequence of inhibition of chloroplast associated syntheses.

With leaf disc ageing the general effects of the incubation treatments were seen to be similar with both TYMV-infected and uninfected material, that is the greatest deterioration

occurred in the discs floated on water in the dark and these changes retarded by both kinetin and light, in detail, particularly with kinetin treatment, difference were observed. There was, for example, a recovery of the decline in content of chlorophyll and RNA in those discs floated on kinetin and illuminated. Regarding the overall data the effects of both light and kinetin were linear whereas for uninfected discs kinetin treatment had a quadratic effect. This meant that the effect of kinetin persisted throughout the incubation period in the infected whereas in the healthy discs a loss of the retarding effect of kinetin occurred. The reason why this should have occurred can only be speculated at present without further investigation but the continued retardation of the senescent changes by kinetin could be because the virus infection prevents the supra-optimal levels of metabolism postulated for the healthy discs being reached and thus the degenerative changes are retarded but at a level of metabolism that can be accommodated by the cells. The statistical analysis also suggested that there was an additive interaction effect between light and kinetin in the infected discs that was not evident in the uninfected material, this may have arisen because the light maintained the integrity of the chloroplasts thus resisting the effects of the virus and permitting continued chloroplast based synthesis not possible in the dark. These

differences between the senescence of TYMV-infected and uninfected chinese <sup>c</sup>abbage leaf discs were observed on examination of total RNA changes, further study of particular RNA fractions in ageing leaf discs may therefore provide a valuable system in which to look at TYMV-host interactions.

From this study it was apparent that, with regard to changes in the cellular components monitored, attached leaves of chinese cabbage had a characteristic pattern of change that was not necessarily the same as that found for other species. These inter-specific differences also extended to the excised leaf disc ageing system in which marked intra-specific differences were also observed. Previous work has implicated the possibly important role in senescence of the loss of chloroplast components (e.g. Mittelheuser and von Steveninck, 1971; Callow et al., 1972; Takegami, 1975) however in ageing chinese cabbage leaves chloroplast rRNA was retained and the retardation of senescence in chinese cabbage leaf discs was associated primarily with changes in cytoplasmic rRNA. In terms of RNA and ribosome content, therefore, there is not a single type of leaf ageing, does this then imply that a number of different possible ageing mechanisms can be operative, or is it that the parameters examined are not

adequate indicators of the causal processes involved in ageing? To elucidate problems of this sort attention should perhaps now be focused on aspects of the possible control mechanisms that could be involved. Such investigations could include aspects of both transcription and translation level control, e.g. RNA polymerase activity, changes in ~~enzyme~~ <sup>en</sup> isozymes, changes in t-RNA species and specific amino acid t-RNA synthetase activities, and also the possible role of plant growth regulators in affecting these functions.

From the preliminary examination of the effect of TYMV-infection on chinese cabbage it was evident that it was mainly chloroplastic components that were affected, as would be anticipated from the ultrastructural effects induced by the virus (Appendix 1). Other alterations in the pattern of change of the RNA and ribosome content of the host were attributable to probable periods of virus synthesis. Radioactive incorporation studies might help resolve this and the DEP-SDS method of RNA extraction, which permits extraction of uncomplexed host RNA from infected plants, now makes such experiments possible. Additionally investigation of possible control mechanisms in ageing leaves applied to TYMV-infected plants may provide useful information on the mode of virus infection. It is interesting in this connection to speculate on the possible significance of a t-RNA like structure at the 3' end of TYMV-RNA (Yot et al., 1972).

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APPENDIX 1.

ULTRASTRUCTURAL STUDIES ON CHINESE CABBAGE

K. Thomas and G. Strangeway

In conjunction with the biochemical studies on ageing of the intact plant and of excised leaf discs of chinese cabbage on examination of the ultrastructural changes occurring has also been initiated with particular attention being paid to the chloroplast. These investigations are still in progress and preliminary findings are detailed below.

Materials and Methods

Chinese cabbage plants were grown as described in 2.11 and small pieces of leaf were taken from selected leaves of different ages along the spiral phyllotaxy from a young not fully expanded leaf to an old senescent leaf. Also samples were taken from the centre portion of leaf discs which had been prepared and incubated under the same conditions as those described in 4.5.

The leaf pieces were fixed overnight at 4°C in 2.5% glutaraldehyde plus 1.5% formaldehyde in 0.1 M phosphate buffer, pH 7.0. After washing in phosphate buffer the tissue was post-fixed in 2.0% osmium tetroxide. Dehydration was through a graded ethanol series beginning at 25% ethanol, the specimens then being embedded in Spurr's resin. Sections were cut with an LKB ultramicrotome and subsequently stained in

uranyl acetate and lead citrate. Specimens were examined with an AEI EM6B electron microscope.

## Results

### 1. The Intact Plant

#### (a) Uninfected chinese cabbage leaves

The ultrastructural changes observed with increasing leaf age and senescence in chinese cabbage were generally similar to those reported for attached leaves of Phaseolus (Barton, 1965), cotyledons of cucumber (Butler, 1967) and wheat leaves (Mittelheuser and van Steveninck, 1971). In a typical young vacuolated cell the chloroplasts were arranged peripherally in a dense ground cytoplasm (Plate 1). The thylakoid membranes of the chloroplast were largely parallel and well-defined grana were present, also small starch grains were observed in the chloroplasts as were a few small plastoglobuli (Plate 2). In some chloroplasts the presence of electron clear areas indicated the possible location of chloroplast DNA. As the leaf matured the chloroplasts remained peripherally arranged in the cell but became larger and rounded as a result of an increase in the size of the starch grains (Plate 3). The larger starch grains caused a distortion of the parallel pattern of the thylakoid membranes (Plate 4) and an increase in the number of plastoglobuli found in the chloroplast was also apparent. At this stage the mitochondria and nuclei appeared normal and ribosomes were still evident but with increasing age a progressive degradation of the cell occurred with the density of both

PLATE 1

Peripherally arranged, elongate chloroplasts of a cell from a young leaf of chinese cabbage. (x 9,500)

PLATE 2

Typical appearance of chloroplasts from young leaves of chinese cabbage. (x 35,500)

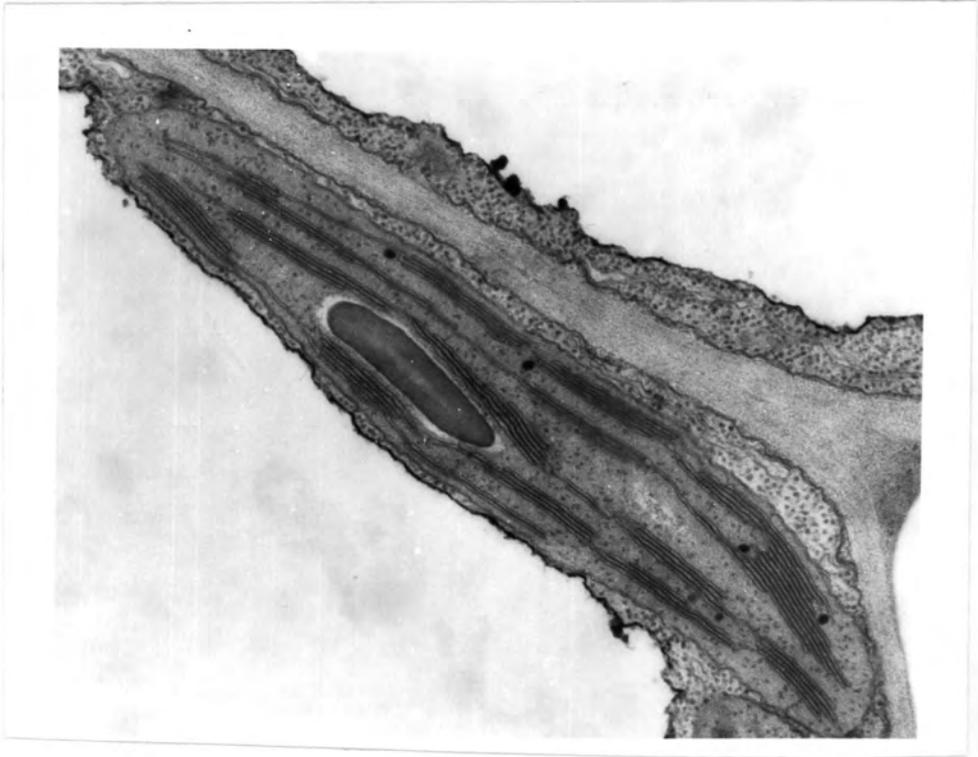
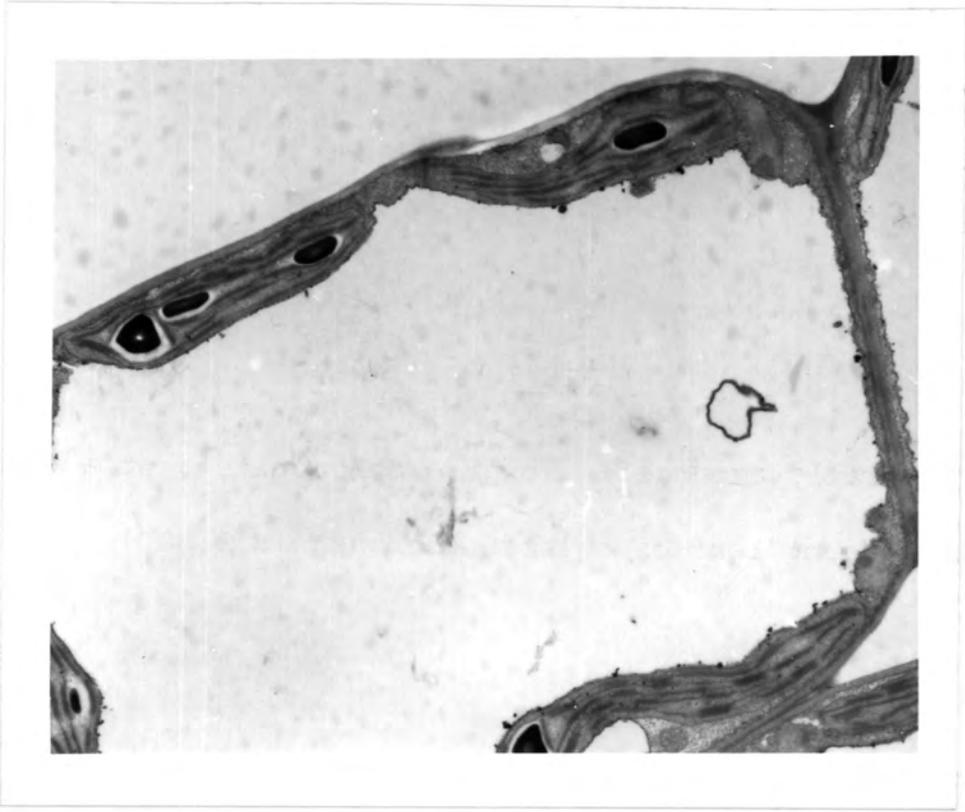
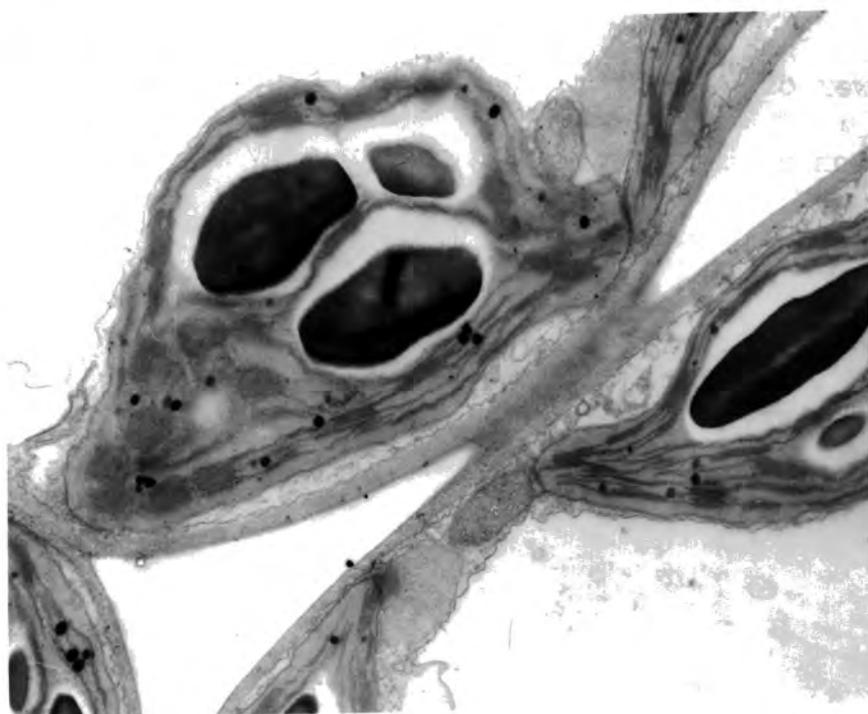
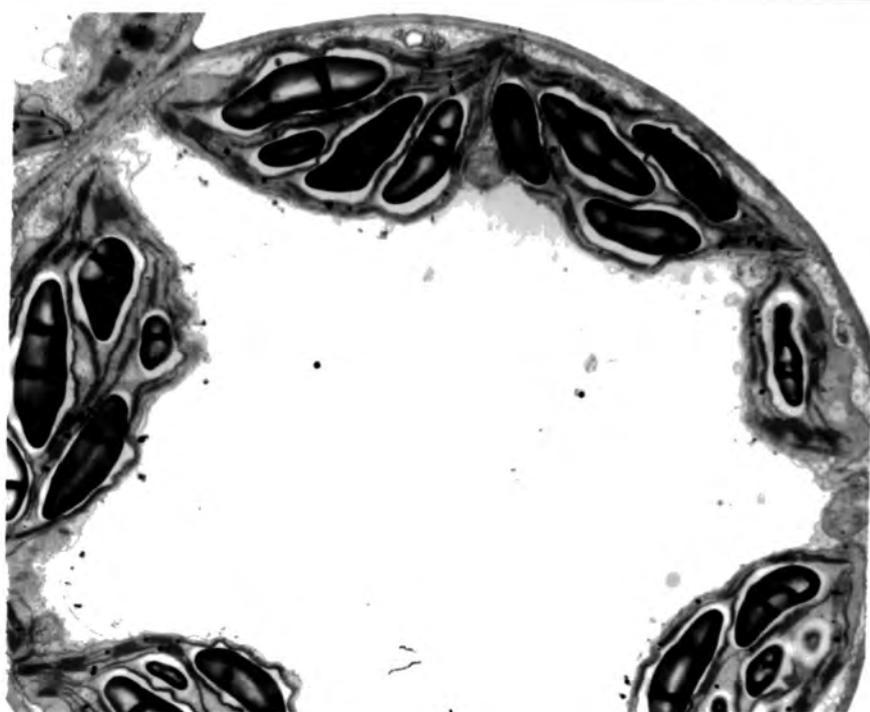


PLATE 3

Chloroplasts of a mature leaf which have swollen as a result of enlargement of the starch grains. (x 9,000)

PLATE 4

High power of a chloroplast from a mature leaf illustrating distortion of the thylakoid membranes by enlargement of the starch grains. (x 14,500)



the cytoplasm and the stroma of the chloroplasts decreasing and it became difficult to locate ribosomes. Within the chloroplasts the starch grains were lost by gradual shrinkage but the plastoglobuli increased in size (Plate 5), this swelling of the plastoglobuli being accompanied by a decrease in size of the grana and a reduction of the thylakoid membrane system. In the oldest leaves sampled in which the internal membrane system of the chloroplasts was considerably reduced there was a massive increase in the size of the plastoglobuli (Plate 6) suggesting that the plastoglobuli may be found as a result of accumulation of lipid derived from the breakdown of the membrane system (Ikeda and Ieda, 1964; Barton, 1966; Butler, 1967; Mittelheuser and van Steveninck, 1971). Whilst degeneration of the internal structure of the chloroplast was evident, the outer membrane remained intact until after the tonoplast membrane had ruptured, the mitochondria and nuclei having burst before. This evidence of a long lasting chloroplast is in accord with the RNA results in which chloroplast RNA was found in even the oldest leaves sampled.

(b) Chinese cabbage leaves systemically infected with TYMV

The cytological abnormalities induced in the leaf cells of the host by turnip yellow mosaic virus are confined to the chloroplasts (Chalcroft and Matthews, 1966; Gerola et al., 1966; Milicic and Stefanac, 1967). In contrast to

PLATE 5

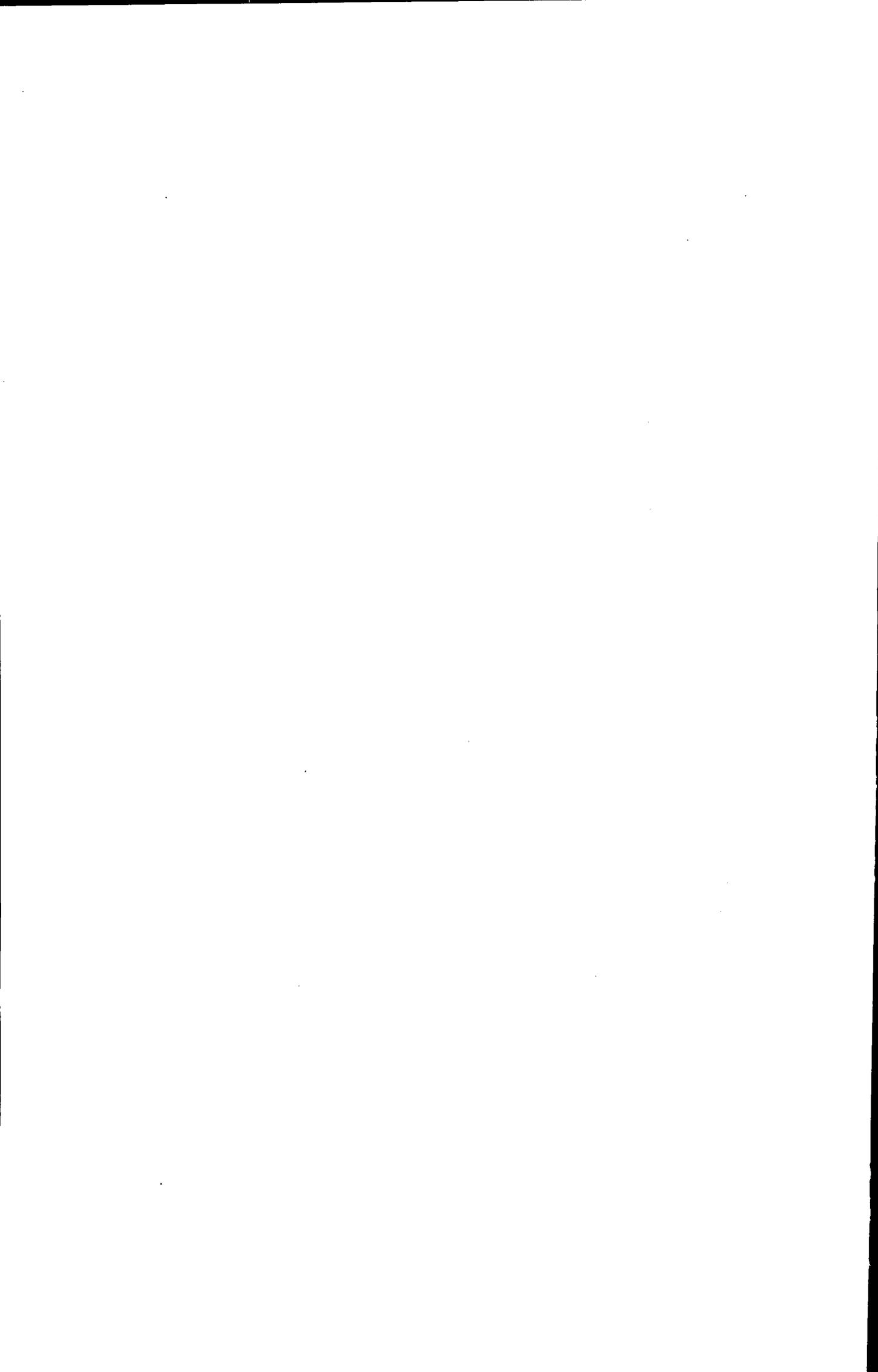
Chloroplast from an old leaf showing enlarged plastoglobuli.

(x 24,000)

PLATE 6

Chloroplast from a senescent leaf in which the membrane system is considerably reduced and many large plastoglobuli are present. (x 36,000)





uninfected tissue the chloroplasts become rounded, clump together and may fragment. Although clumped together the chloroplasts remain separated by a narrow channel continuous with the ground cytoplasm and where the chloroplast envelope membranes are adjacent they often stain darker (Plate 7). The gap between the chloroplasts can become enlarged and such areas seem to contain accumulated material staining in a similar fashion to ribosomes or virus particles though it was not possible to distinguish which they were (Plate 8). Within the chloroplasts and associated with TYMV-infection were two types of membrane-bound vesicle, a large form which may contribute to the fragmentation of the chloroplasts and a small, often flask-shaped, vesicle found at the periphery of the chloroplasts. These latter vesicles, which are characteristic of the TYMV group viruses in any host species, appear to be invaginations of the chloroplast envelope (Hatta et al., 1973). Evidence suggests that they might be the site of synthesis of the viral RNA (see review, Matthews, 1973) and it has been proposed that the protein coat of the virus is assembled around the emerging double-stranded RNA at the neck of these peripheral vesicles (Hatta and Matthews, 1976).

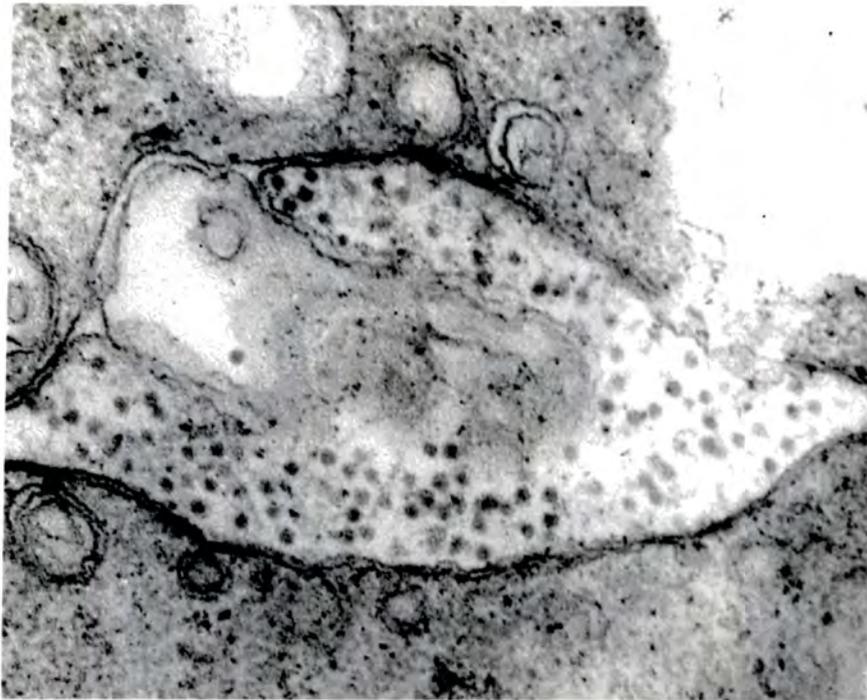
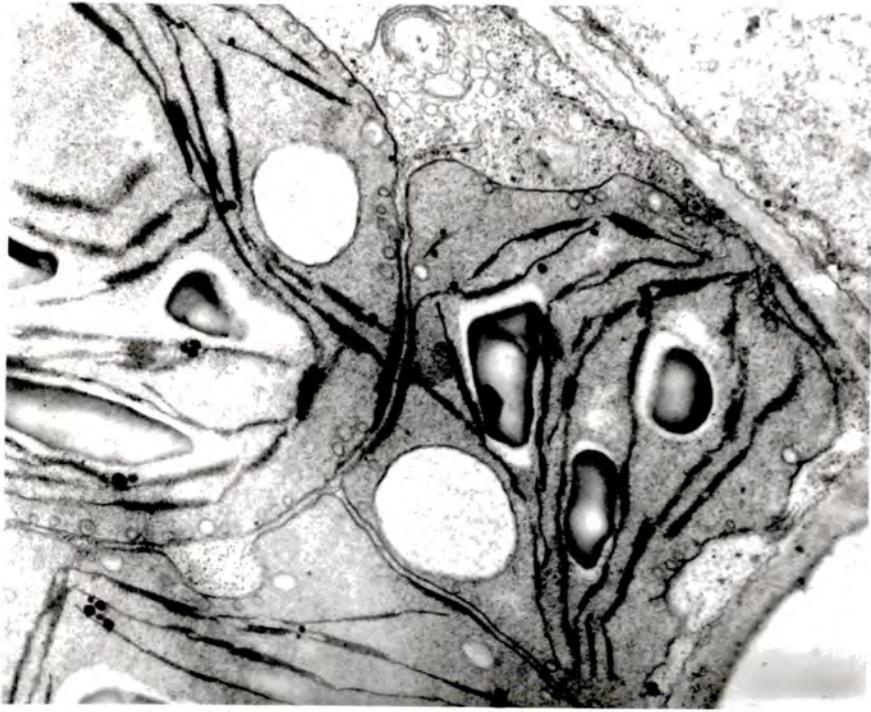
These characteristics of TYMV-infection were observed in cells of all ages but were often less severe in the cells of young leaves in which the chloroplasts tended to show less

PLATE 7

Chloroplasts clumped together as a result of TYMV-infection. Note the narrow channel separating the aggregated chloroplasts and the darkening of regions of adjacent chloroplast envelopes. (x 20,500)

PLATE 8

A pocket between adjacent chloroplasts in which dark staining ribosome or virus-like particles are evident. (x 100,000)



clumping, fragmentation and large vesicle production than in the older leaves of the plant (Plate 9). This however could be variable and was possibly due to the presence of different strains of the virus which are able to induce a varying severity of infection (Chalcroft and Matthews, 1966). In cells of older leaves the typical aggregation of the chloroplasts into clumps (Plate 10) or lined in rows (Plate 11) was apparent. Small starch grains were present in cells of young leaves but in mature leaves these grains were comparatively smaller than those found in the chloroplasts of leaves of a similar age taken from uninfected plants (Plate 12). With maturity there was also an increase in the number of plastoglobuli and ribosome-like particles were still clearly resolved in both the cytoplasm and the chloroplasts. The ultrastructural appearance of uninfected and TYMV-infected mature leaf tissue was similar except for the typical cytological changes induced by the virus on the host, in older tissue further differences were apparent. In TYMV-infected cells though the plastoglobuli became slightly larger they did not swell to the large proportions typical of uninfected cells, instead myeline figures (coils of membranes) were evident which may have formed as a result of the degeneration of the thylakoid system within the chloroplast (Plate 13). The starch grains become reduced in size but in TYMV-infected plants this reduction appeared to

PLATE 9

Cell from a young leaf of a TYMV-infected plant. (x 5,500)

PLATE 10

Cell from a mature leaf of a TYMV-infected plant  
illustrating aggregation of the chloroplasts into a clump.  
(x 7,000)

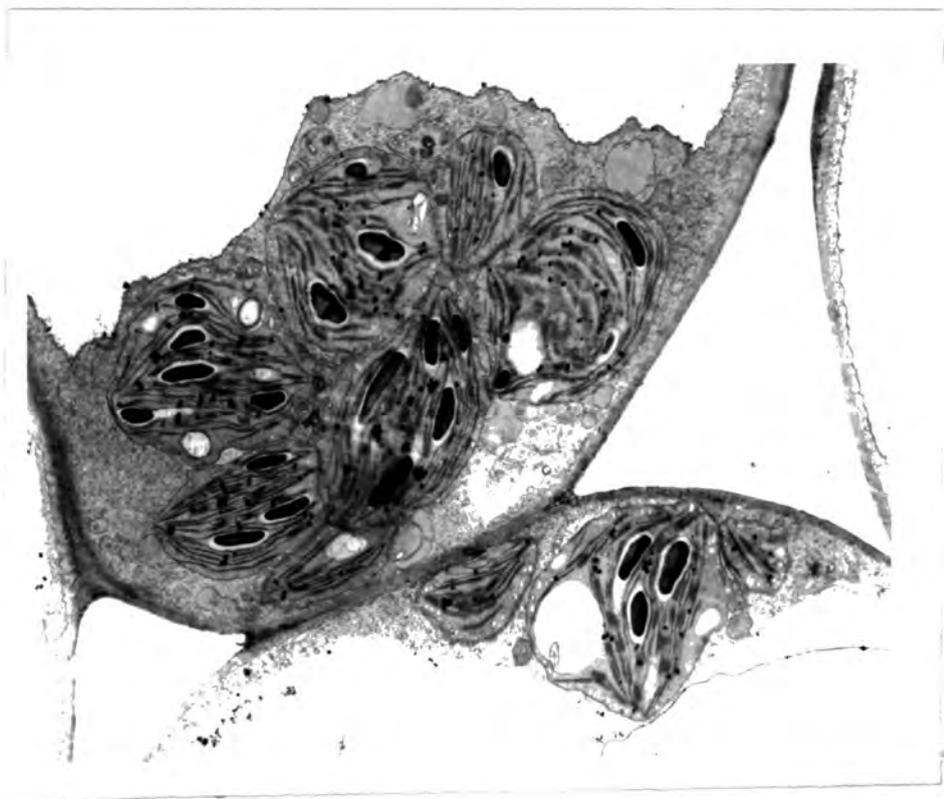
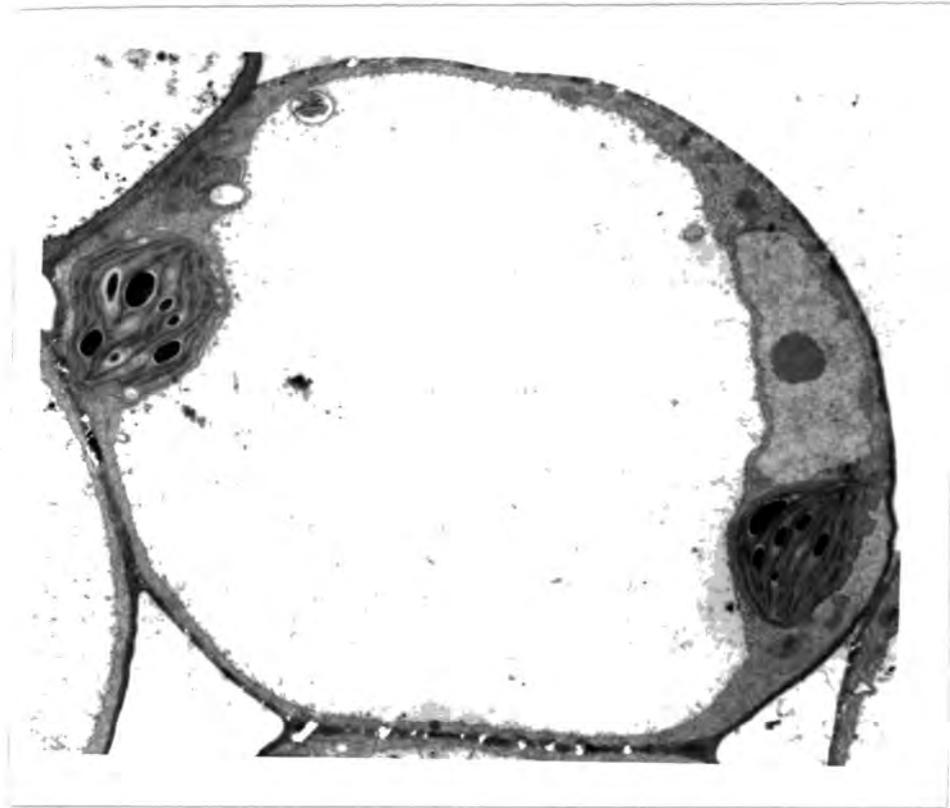


PLATE 11

Cell from a mature leaf of a TYMV-infected plant with  
the chloroplasts aggregated in a row. (x 9,000)

PLATE 12

Chloroplasts from a mature leaf infected by TYMV.  
(x 27,500)

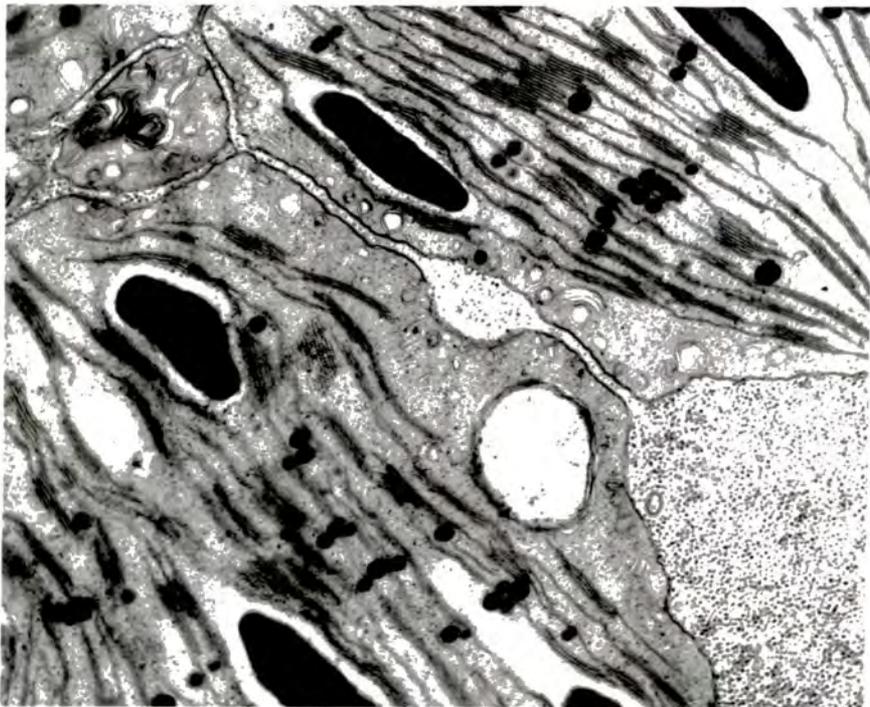


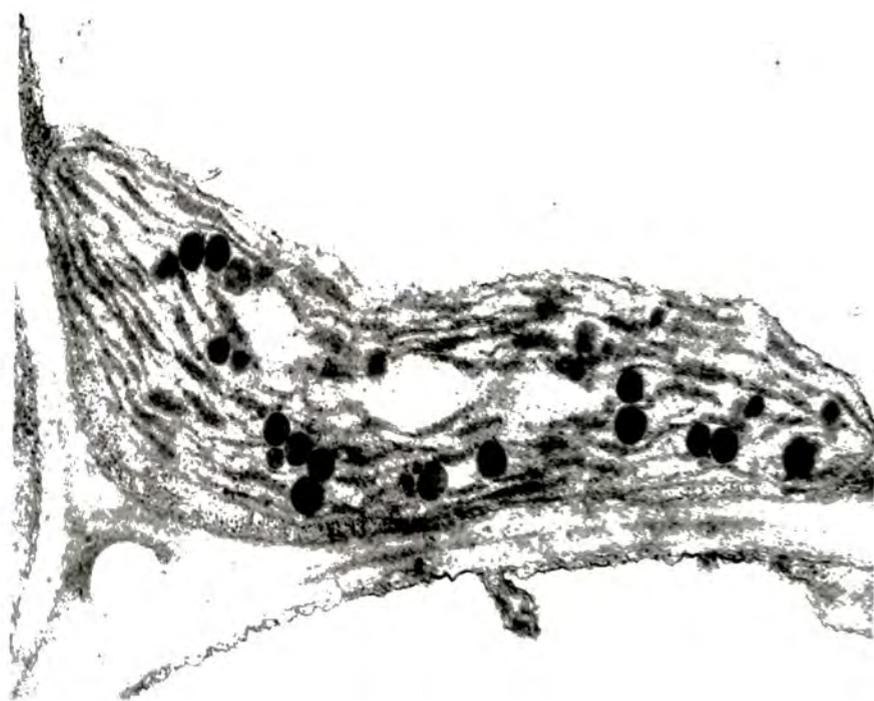
PLATE 13

Cell from an old leaf of a TYMV-infected plant.

(x 12,500)

PLATE 14

Chloroplast from leaf discs floated on water for  
four days in the dark. (x 24,000)



be by a process of autolysis rather than by the gradual shrinkage observed in uninfected cells. The number and size of the large vesicles typical of TYMV-infection increased in the older cells which may also have been a senescent effect though similar vesicle production did not occur in uninfected cells.

## 2. Leaf Discs

### (a) Excised from uninfected chinese cabbage leaves

Samples for ultrastructural examination were taken from leaf discs floated on water and kept in the dark and after four days incubation the principle senescent features that were evident in the attached leaf were observed in the disc material. There was an increase in the number of plastoglobuli in the chloroplast and there was a loss of the starch grains by a process of gradual shrinkage (Plate 14), at this stage the cytoplasm was usually still intact although the tonoplast had ruptured in some cells. A unique feature of disc senescence became apparent in the chloroplasts after six days, a vesiculation of the grana (Plate 15), this phenomenon has also been noticed in the chloroplasts of detached leaves of wheat (Shaw and Manocha, 1965) and Festuca pratensis (Thomas, unpublished data) but was not observed in attached leaf material. The appearance of the cells after six days was similar to that at four days but the cytoplasm was thinner and the grana of the chloroplasts were further reduced, rupture of the tonoplast was common but the organelle envelope membranes were still intact. The stroma of the chloroplast was dense, the nuclei were still intact and cristae were still evident in the mitochondria (Plate 16). This contrasted with detached wheat leaves in which the cells were ultrastructurally degenerate

PLATE 15

Chloroplast from leaf discs floated on water for six days in the dark illustrating vesiculation of the grana.

(x 19,000)

PLATE 16

Chloroplasts and mitochondria from leaf discs floated on water for six days in the dark. (x 18,000)



after five days (Mittelheuser and van Steveninck, 1971).

If the discs were kept in the dark but floated on a solution of kinetin essentially similar changes to the water treatment were observed but they were delayed. For example after six days the tonoplast was often still intact and many chloroplasts were still in a good condition (Plate 17) though this could be variable and pronounced vesiculation of the grana was evident in some cells (Plate 18). Further increases in the number of plastoglobuli were noticeable after eight days and the tonoplast membrane was generally ruptured. Continued deterioration of the chloroplast thylakoid membranes occurred but the mitochondria still had distinct cristae.

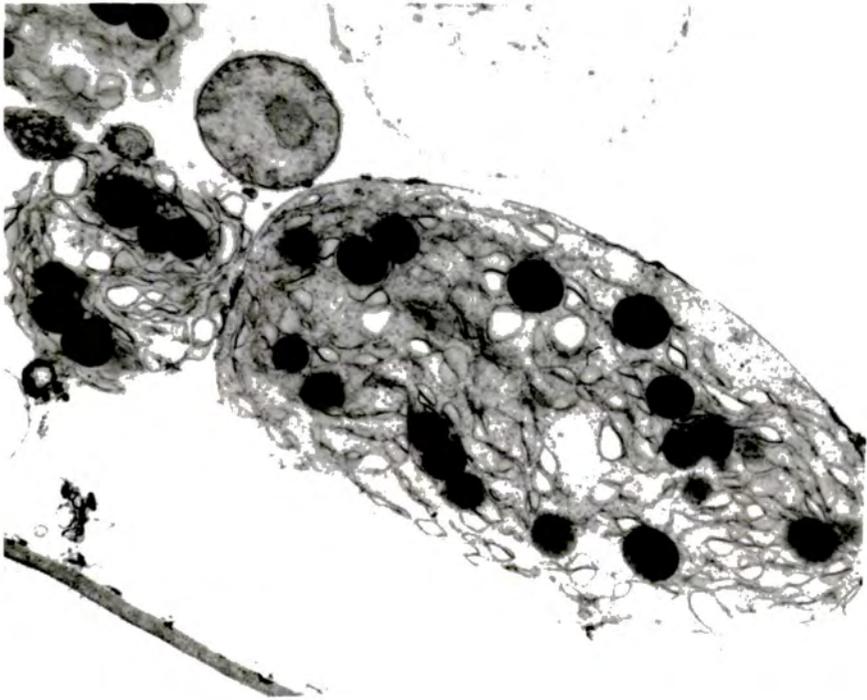
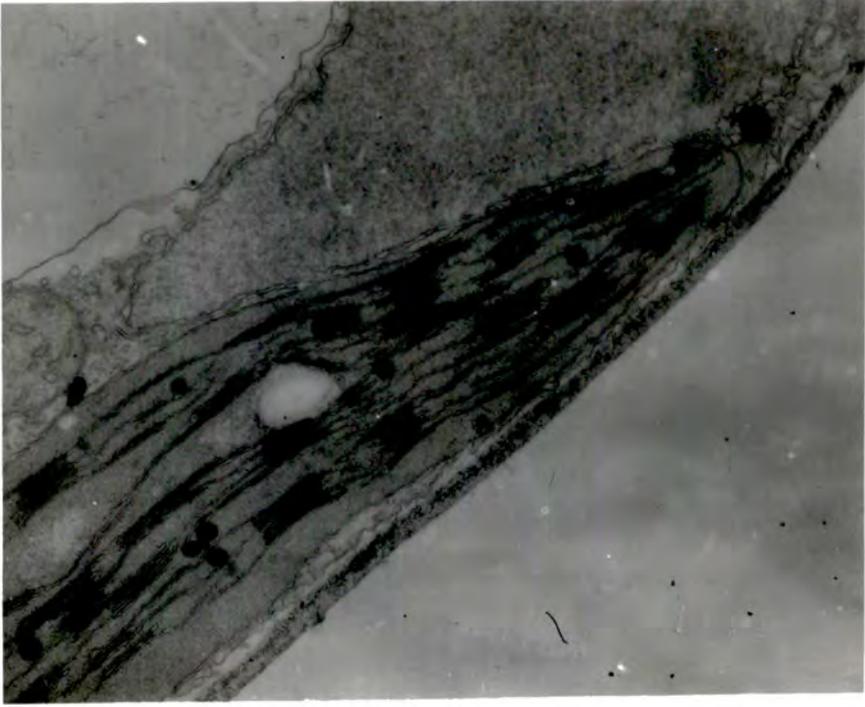
Compared with the pattern of change observed in intact leaves leaf discs showed similar degenerative changes but differences were also observed, most noticeably the pronounced vesiculation of the chloroplast. Also the number of plastoglobuli within the chloroplast increased but by eight days incubation they had not achieved the large size that was attained in the intact leaf. The mitochondria and nuclei also seemed to show a greater stability in the excised material relative to the chloroplasts compared to the intact plant. No qualitative changes were observed on cytokinin treatment which appeared only to delay the changes occurring on discs floated on water, a result consistent with the work of Harding et al., (1968) working on Brassica juncea and Mittelheuser and

PLATE 17

Chloroplast from leaf discs floated on kinetin solution  
for six days in the dark. (x 27,500)

PLATE 18

Chloroplasts in which greater deterioration has occurred  
after six days incubation of discs floated on kinetin  
in the dark. (x 18,500)



van Steveninck (1971) working on wheat leaves.

(b) Excised from TYMV-infected chinese cabbage leaves

Control samples of the leaf discs taken from TYMV-infected leaves showed well-developed symptoms of infection such as rounding and clumping of the chloroplasts and the presence of both large and small vesicles within the chloroplasts (Plate 19).

After three days incubation of discs floated on water and kept in the dark the appearance of the cells had changed considerably, there was a marked increase in the number and size of the large vesicles within the chloroplasts some of which becoming free in the cytoplasm. This release of the vesicles resulted in fragmentation of the chloroplasts which also became dispersed rather than clumped together (Plate 20). There was a reduction in the chloroplast thylakoid membrane system but no significant increase in the size or number of plastoglobuli, nor were any myeline figures evident as they had been in intact tissue, also in the chloroplasts the starch grains had been lost. The cytoplasm was swollen and less dense than in the controls and by six days the tonoplast of many cells had ruptured. The degeneration of the chloroplasts continued and the thylakoids were markedly reduced after six days and an increase in the numbers of plastoglobuli occurred (Plate 21). Expulsion of the large vesicles from the chloro-

PLATE 19

Cells from zero time control leaf discs excised from  
TYMV-infected leaves. (x 5,500)

PLATE 20

Cell from leaf discs excised from TYMV-infected leaves  
and floated on water for three days in the dark. (x 8,500)

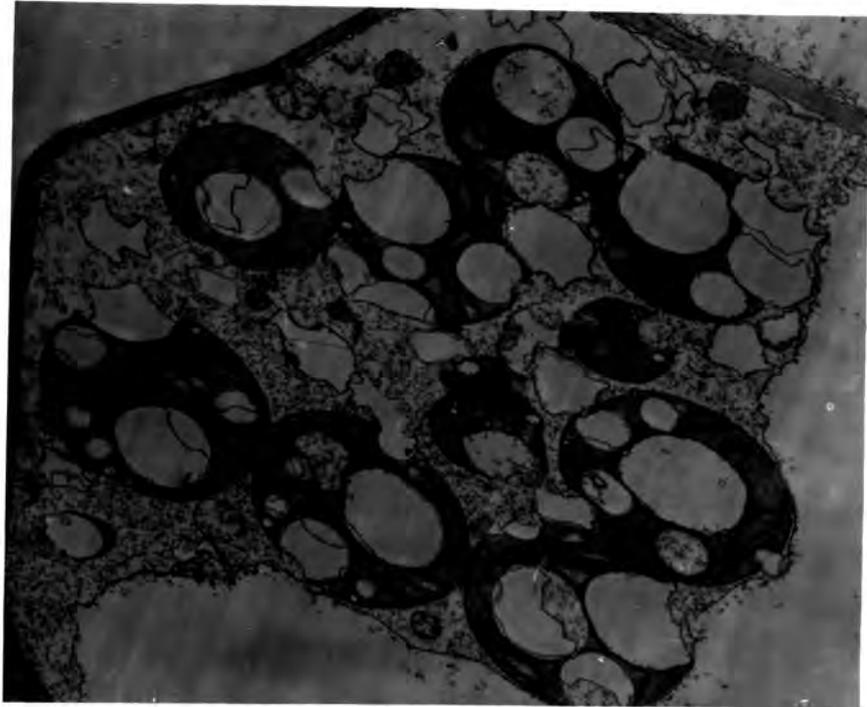
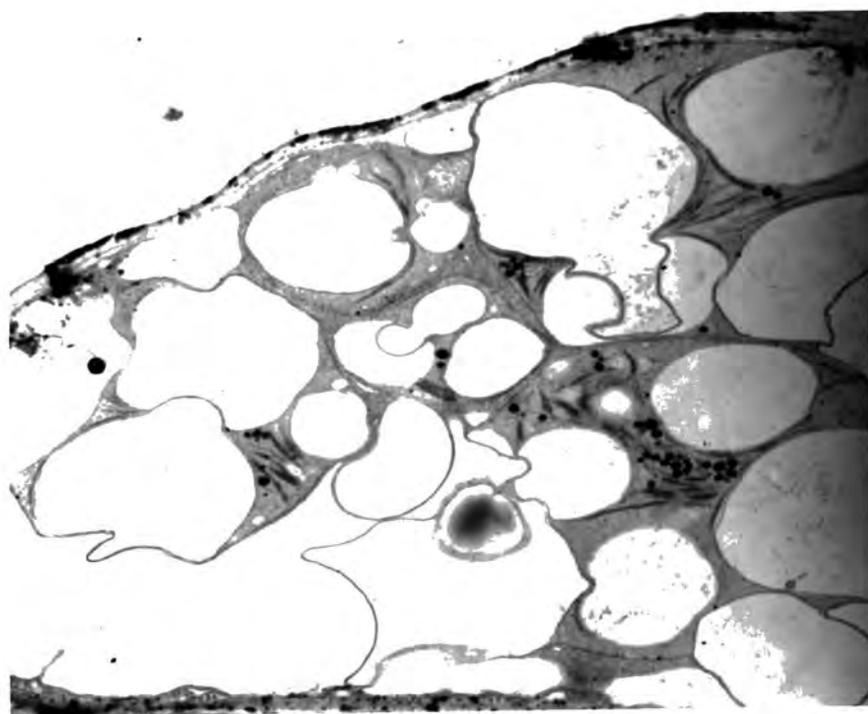
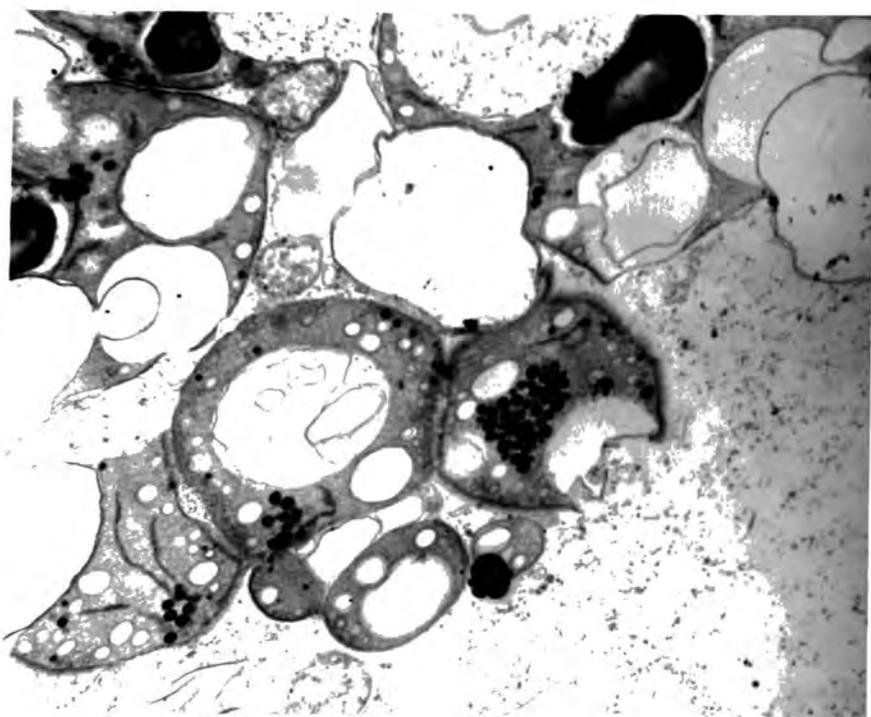


PLATE 21

Cell from leaf discs excised from TYMV-infected leaves and floated on water for six days in the dark. (x 13,500)

PLATE 22

Cell from leaf discs excised from TYMV-infected leaves and floated on water for eight days in the dark. (x 11,500)



plast continued giving further fragmentation of these organelles which by eight days had become highly distorted (Plate 22). Although there was considerable distortion of the chloroplasts cristae were still evident in the mitochondria after eight days incubation.

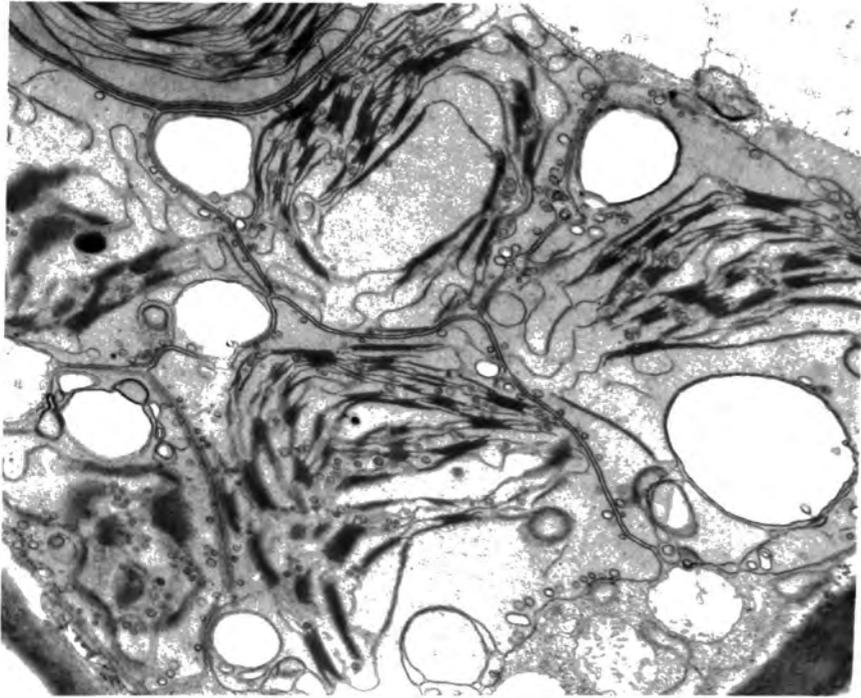
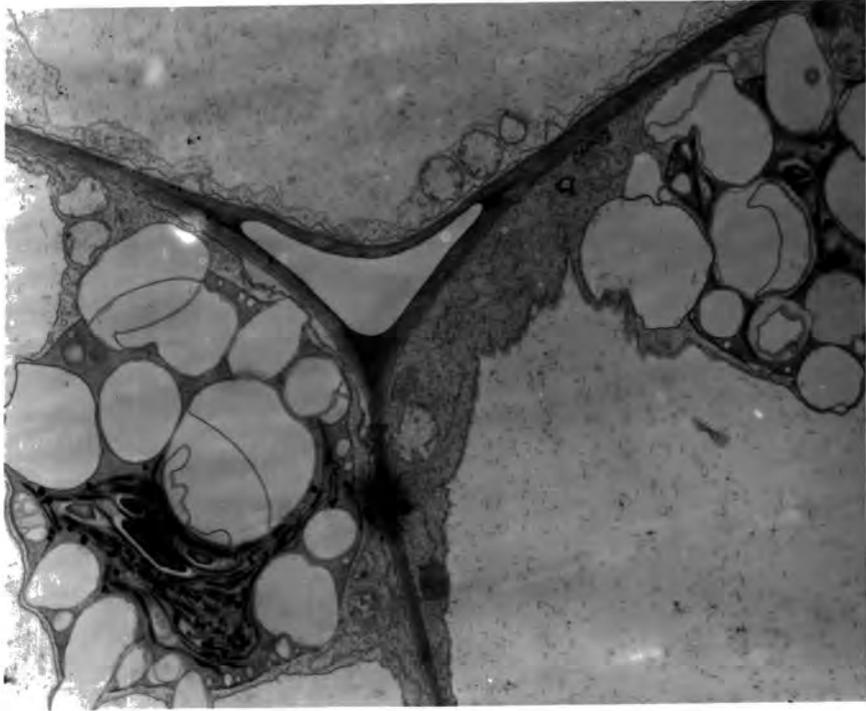
The changes that occurred in discs that were illuminated were similar to those that are described above but their appearance was delayed, thus after three days incubation the cytoplasm was more dense and exhibited less swelling than was evident in the dark treated discs. There was an increase in the number of large vesicles but this did not result in as much dispersion as occurred in the dark, also starch grains were still present in the chloroplasts (Plate 23) and could also be found up to six days after excision of the discs. This retardation of the deteriorative changes by light was particularly manifest after longer periods of incubation, even after eight days, in contrast to the dark-incubated discs, some cells showed a marked retention of the chloroplast thylakoid membranes and resistance to deterioration (Plate 24). This response could be variable though and dispersion and fragmentation of the chloroplasts accompanied by a reduction in the thylakoids and increases in plastoglobuli was observed in some cells. The reason for the wide range of physical appearance in response to the incubation conditions may be

PLATE 23

Cells from leaf discs excised from TYMV-infected leaves  
and floated on water for three days in the light. (x 9,000)

PLATE 24

Cell from leaf discs excised from TYMV-infected leaves  
and floated on water for eight days in the light. (x 14,000)



related to varying degrees of severity of infection due to different strains of the virus being present. Again, as in the dark, cristae were evident within the mitochondria after eight days.

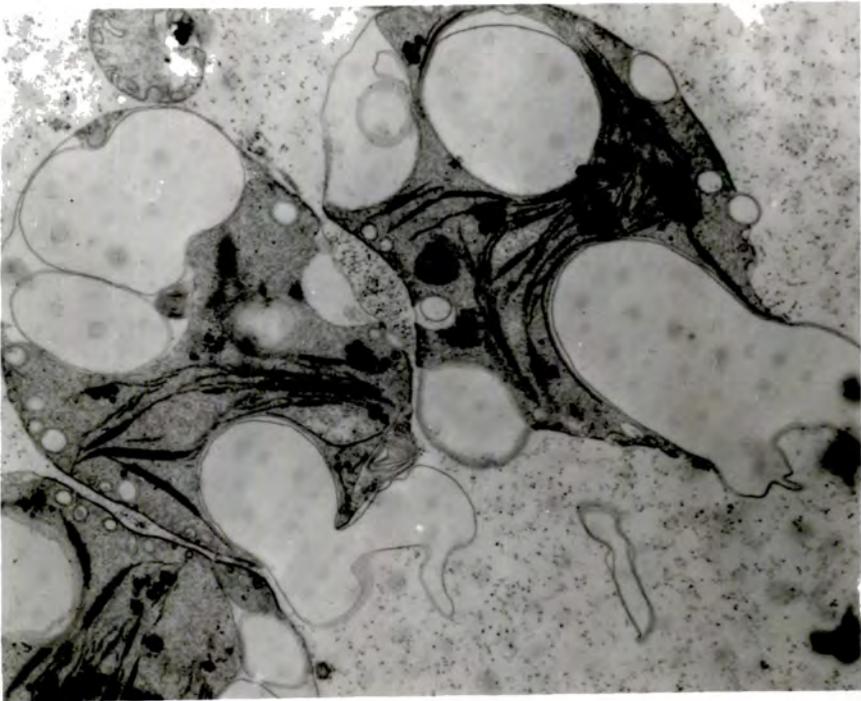
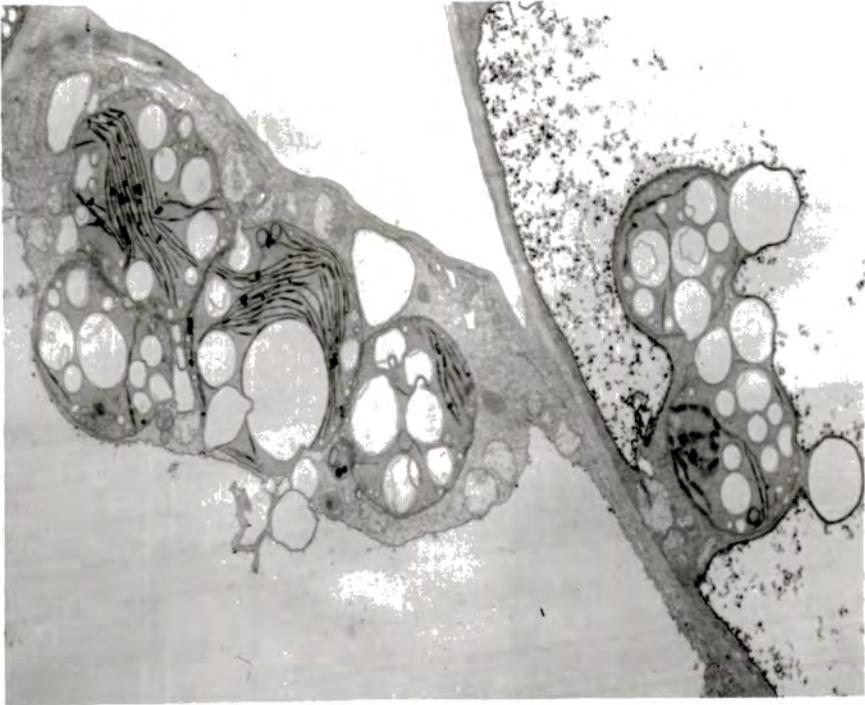
If the discs were floated on cytokinin solution rather than water and then incubated in the dark there was a retardation of the deteriorative changes. After three days the chloroplasts were not as widely dispersed as those in the cells of discs floated on water even though large vesicles were being formed giving rise to some distortion of the chloroplasts (Plate 25). Some residual starch was also present even though the discs were not illuminated, also there was less swelling of the cytoplasm which was therefore more dense than the corresponding water treatments. The typical degenerative changes described above continued with longer incubations, after eight days further fragmentation and dispersal of the chloroplasts had occurred (Plate 26) but less deterioration had occurred at this time than in the water treatments in which there was a great distortion of the chloroplasts after eight days. Thylakoid membranes were still present though reduced and the numbers of plastoglobuli had increased. The cytoplasm had become quite diffuse and swollen but the mitochondria in the cytoplasm had not greatly degenerated structurally.

PLATE 25

Cells from leaf discs excised from TYMV-infected leaves and floated on kinetin solution for three days in the dark ( x 5,000)

PLATE 26

Cell from leaf discs excised from TYMV-infected leaves and floated on kinetin solution for eight days in the dark. (x 14,500)



The retardation of senescence in cytokinin treated discs was greater if the discs were incubated in the light. After three days there was a smaller increase in the numbers of large vesicles in the chloroplasts than occurred under the other incubation conditions and consequently the chloroplasts were clumped together with the thylakoid membranes still largely intact. Quite large amounts of starch remained at this stage and again the cytoplasm was dense and not swollen (Plate 27). Further increases in size and number of the large chloroplast vesicles lead to more dispersed and swollen chloroplasts but this deterioration was not as great as in other incubations at corresponding periods of time. The thylakoids were less reduced than in other treatments (except possibly the light-treated infected discs floated on water) and increases in the number of plastoglobuli were not great (Plate 28). Starch grains were still evident in some chloroplasts at six days though they were not observed after eight days. As with the other incubation treatments mitochondria were still well preserved after eight days as were the nuclei.

The qualitative changes that occurred during the incubation of discs excised from TYMV-infected leaves were similar in all conditions. There was a swelling and thinning of the cytoplasm and rupture of the tonoplast and within the

PLATE 27

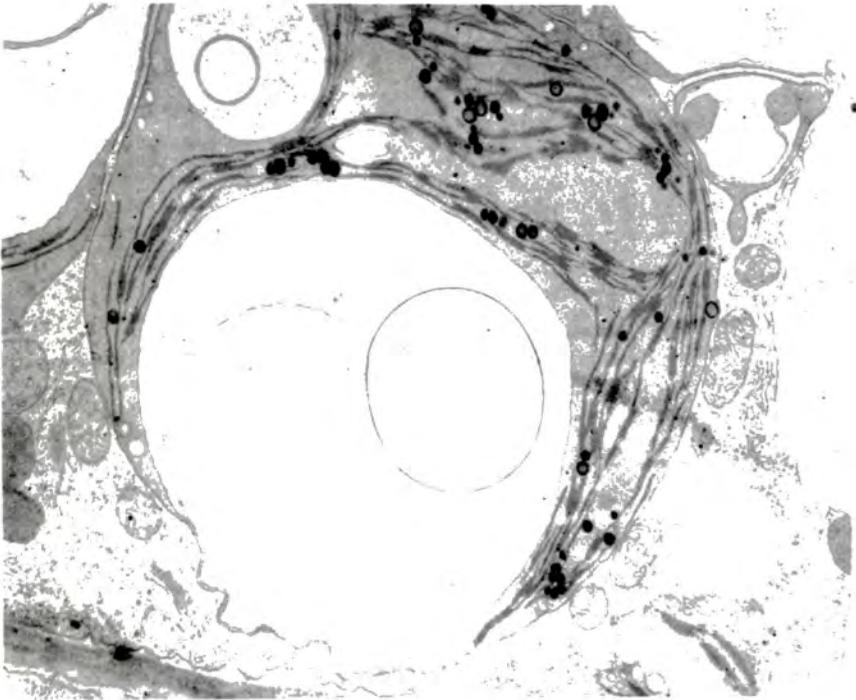
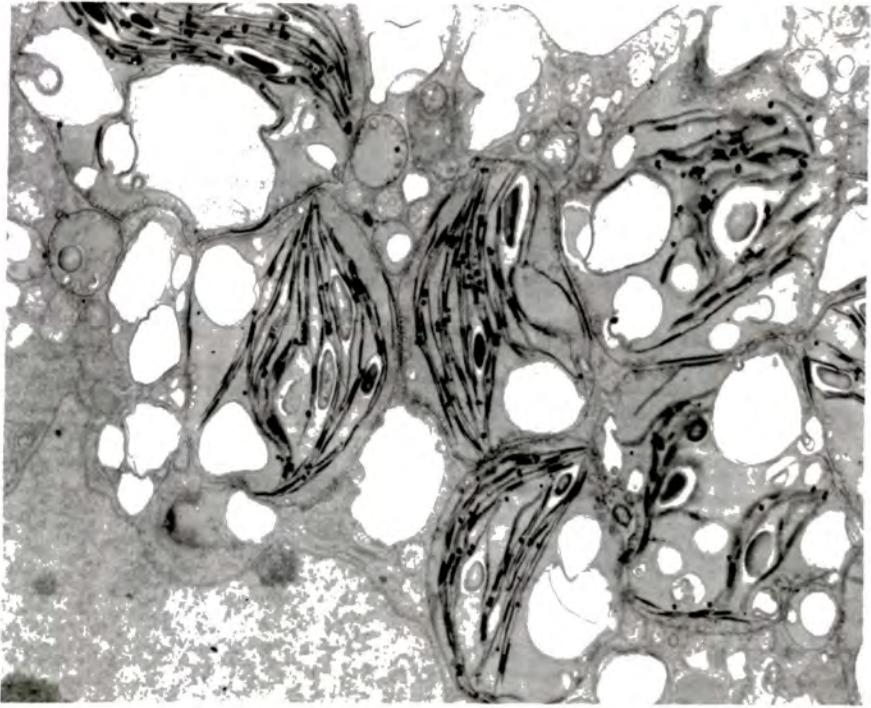
Cell from leaf discs excised from TYMV-infected leaves and floated on kinetin solution for three days in the light.

(x 9,000)

PLATE 28

Cell from leaf discs excised from TYMV-infected leaves and floated on kinetin solution for six days in the light.

(x 12,500)



chloroplast the number and size of the large vesicles increased which lead to a disruption of the chloroplasts. Also within the chloroplasts there was a loss of starch and an increase in the number of plastoglobuli but no myeline figures were observed though these were distinct in the old leaves of the intact plant. No vesiculation of the grana were observed though this did occur in discs excised from uninfected leaves. The pattern of changes occurred most rapidly in those discs floated on water and incubated in the dark and was retarded if the discs were illuminated or treated with cytokinin, with slightly greater retardation of the deteriorative changes occurring when both light and cytokinin were supplied. Though senescence was retarded under these conditions neither light nor cytokinin appeared to reduce the cytological symptoms characteristic of TYMV-infection.

APPENDIX 2

STATISTICS

The Durham University Computer Unit 'Curvefit' program was used to fit curves of the general form  $y = A_0 + A_1x + A_2x^2 \dots \dots \dots A_nx^n$  to given data points. This program was used to fit linear and quadratic curves to the raw data obtained by the quantitative analysis of the incubated leaf discs (see Table A1) where the values of x were the time intervals of sampling (0 to 10 days inclusive) and the y values the yields of DNA, RNA or chlorophyll (measured in O.D. units) obtained for a particular incubation treatment. Table A2 summarises the intercept and regression coefficients of y upon x obtained with the computer program and also the sums of squares of the errors (residual sum-of-squares). The technique of analysis of variance was applied to the regression lines to test the significance of the regressions.

The total variation of y (as the sum-of-squares) is:

$$\sum (y - \bar{y})^2$$

which is equivalent to  $\sum y^2 - \frac{1}{n} (\sum y)^2$

The amount of this variation which is accounted for by the linear regression of y upon x is:

$$\frac{[\sum (x - \bar{x})(y - \bar{y})]^2}{\sum (x - \bar{x})^2} \quad \text{(Regression sum-of-squares)}$$

which is equivalent to  $\frac{(\sum xy - \frac{\sum x \sum y}{n})^2}{\sum x^2 - \frac{(\sum x)^2}{n}}$

The residual variation not accounted for by the linear regression of y upon x is therefore the total sum-of-squares minus the regression sum-of-squares,

$$\text{i.e. } \sum y^2 - \frac{(\sum y)^2}{n} - \frac{(\sum xy - \frac{\sum x \sum y}{n})^2}{\sum x - \frac{(\sum x)^2}{n}}$$

The value of these various sums-of-squares for the linear regression were calculated from the raw data, (the residual sum-of-squares agreeing in each case with that obtained from the computer program). The computer program also provided the value of the residual sum of squares after fitting a quadratic regression. The difference between the residual sums-of-squares for the linear and quadratic regressions provided a measure of the amount of variation accounted for by the quadratic regression (i.e. the quadratic regression sum-of-squares) that was not accounted for by the linear regression. The values of the linear and quadratic regression sums-of-squares and the residual sum-of-squares were converted to mean-square terms by dividing each by its number of degrees of freedom. The F-statistic was then obtained by dividing the mean-square for each regression by the mean-square of the residual variation. The analysis of variance tables for the regressions of the individual incubation treatments are given in Table A3.

The above regression curves were fitted to the data points for the individual incubation treatments, to evaluate the particular effects of light and kinetin the data from different treatments were considered in combinations as outlined below.

A linear regression of the total data for a given cellular component (i.e. 44 points) was calculated and the value of the sum-of-squares of the regression of the overall data obtained (Reg.  $SS_{\text{overall}}$ ). This gave a measure of the variation of the total data that could be explained by fitting one line to the data and has one degree of freedom. The amount of variation in the total data that could be explained by fitting four linear regressions (i.e. one line for each of the four incubation treatments) is given by summing the individual regression sum-of-squares terms for each of the four lines (4 Reg. SS) and this has four degrees of freedom. The difference between these two values (Reg. difference SS) represents the amount of variation that is not explained by fitting one line which is explained when the four lines are fitted and has three degrees of freedom. These three degrees of freedom can be resolved into their constituent components which are the effect of kinetin-v-water, the effect of light-v-dark and the interaction between these. To do this for each of the cellular components examined a

a linear regression was fitted to the water treatments (i.e. discs floated on water in the dark and discs floated on water in the light) and the regression sum-of-squares on the 22 points calculated, and similarly for the kinetin treatments (i.e. discs floated on kinetin in the dark and discs floated on kinetin in the light). The sum of these two regression sum-of-squares is thus the amount of variation of the total data explained by fitting two lines relating to water and kinetin ( $H_2O/CK$  2 Reg. SS) and has two degrees of freedom. The difference between fitting one line and these two lines (i.e.  $H_2O/CK$  2 Reg. SS<sub>overall</sub>) gives the regression difference sum-of-squares of water/kinetin ( $H_2O/CK$  Reg. diff. SS) with one degree of freedom. These calculations were repeated on the light ( $H_2O$  + light and kinetin + light) and dark ( $H_2O$  + dark and kinetin + dark) treatments to obtain the regression difference sum-of-squares of light/dark (Lt./Dk. Reg. diff. SS) also with one degree of freedom. The interaction regression sum-of-squares was then obtained by subtraction of the  $H_2O/CK$  Reg. diff. SS and Lt./Dk. Reg. diff. SS from the Reg. difference SS. To obtain quadratic evaluations of the effects of water-v-kinetin, light-v-dark and the interaction the Durham Computer Unit 'Curvefit' program was run to fit linear and quadratic curves to the data combined in the same way as above thus

obtaining the value of the residual sums-of-squares for these lines (Table A4). The amount of variation explained by fitting one quadratic curve to all forty-four points for a given cellular component compared to fitting one linear curve is then given by:

$$\text{Linear Residual SS}_{\text{All}} - \text{Quadratic Residual SS}_{\text{All}} (Q_1)$$

Similarly values for the amount of variation explained by fitting quadratic curves rather than straight lines for two lines relating to Water/Kinetin or light/dark and for four lines (one for each incubation treatment) can also be found by summing the appropriate linear residual sums-of-squares and subtracting the appropriate summed quadratic residual sums-of-squares (giving  $Q_{2.1}$  for  $H_2O/CK$ ,  $Q_{2.2}$  for  $lt/dk$  and  $Q_4$  for the four incubation treatments). The regression sums-of-squares with one degree of freedom for fitting quadratic curves were then found from the following formulae:

$$\text{Quadratic } H_2O/CK \text{ Reg. SS} = Q_{2.1} - Q_1$$

$$\text{Quadratic } lt/dk \text{ Reg. SS} = Q_{2.2} - Q_1$$

$$\text{Quadratic interaction Reg. SS} =$$

$$Q_4 - (Q_{2.1} - Q_1) - (Q_{2.2} - Q_1) - Q_1$$

The 'explained' residual deviation was then obtained from the sum of the quadratic residual sums-of-squares for the

four curves of the individual treatments. The linear and quadratic regression sums-of-squares for  $H_2O/CK$ ,  $lt/dk$  and interaction (one degree of freedom each) and the residual sum-of-squares (thirty-two degrees of freedom) were converted to mean-square values and the F-statistic calculated (Table A5).

Table A1

## 1. Uninfected Plant

| Cellular Component | Incubation Treatment     | Days  |       |       |       |       |       |       |       |       |       |       |
|--------------------|--------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|                    |                          | 0     | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     | 10    |
| DNA                | H <sub>2</sub> O + Dark  | 0.140 | 0.130 | 0.105 | 0.090 | 0.090 | 0.085 | 0.095 | 0.080 | 0.095 | 0.090 | 0.075 |
|                    | H <sub>2</sub> O + Light | 0.110 | 0.105 | 0.100 | 0.095 | 0.090 | 0.095 | 0.080 | 0.100 | 0.105 | 0.120 | 0.105 |
|                    | CK + Dark                | 0.100 | 0.100 | 0.090 | 0.095 | 0.095 | 0.100 | 0.100 | 0.060 | 0.085 | 0.065 | 0.060 |
|                    | CK + Light               | 0.115 | 0.110 | 0.115 | 0.130 | 0.120 | 0.105 | 0.105 | 0.120 | 0.110 | 0.100 | 0.095 |
| RNA                | H <sub>2</sub> O + Dark  | 0.265 | 0.220 | 0.180 | 0.145 | 0.180 | 0.125 | 0.150 | 0.110 | 0.150 | 0.115 | 0.065 |
|                    | H <sub>2</sub> O + Light | 0.220 | 0.220 | 0.200 | 0.200 | 0.180 | 0.175 | 0.185 | 0.180 | 0.170 | 0.175 | 0.170 |
|                    | CK + Dark                | 0.195 | 0.250 | 0.175 | 0.230 | 0.210 | 0.220 | 0.180 | 0.150 | 0.150 | 0.165 | 0.100 |
|                    | CK + Light               | 0.225 | 0.220 | 0.250 | 0.235 | 0.230 | 0.190 | 0.200 | 0.200 | 0.190 | 0.160 | 0.155 |
| Chlorophyll        | H <sub>2</sub> O + Dark  | 1.060 | 0.910 | 0.900 | 0.775 | 0.620 | 0.545 | 0.585 | 0.460 | 0.410 | 0.290 | 0.230 |
|                    | H <sub>2</sub> O + Light | 0.900 | 0.880 | 0.815 | 0.790 | 0.680 | 0.645 | 0.675 | 0.575 | 0.585 | 0.555 | 0.460 |
|                    | CK + Dark                | 0.945 | 0.935 | 0.830 | 0.875 | 0.805 | 0.760 | 0.580 | 0.490 | 0.610 | 0.460 | 0.315 |
|                    | CK + Light               | 0.910 | 0.805 | 0.925 | 0.850 | 0.790 | 0.710 | 0.740 | 0.750 | 0.665 | 0.555 | 0.525 |

Table A1 (cont.)

2. TYMV-infected plants

|             |                          |       |       |       |       |       |       |       |       |       |       |       |
|-------------|--------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|             | H <sub>2</sub> O + Dark  | 0.180 | 0.155 | 0.125 | 0.120 | 0.138 | 0.119 | 0.110 | 0.115 | 0.095 | 0.085 | 0.075 |
| DNA         | H <sub>2</sub> O + Light | 0.135 | 0.145 | 0.115 | 0.105 | 0.144 | 0.131 | 0.125 | 0.110 | 0.115 | 0.095 | 0.105 |
| OD260-      | CK + Dark                | 0.150 | 0.150 | 0.125 | 0.105 | 0.113 | 0.113 | 0.135 | 0.120 | 0.130 | 0.120 | 0.085 |
| OD290       | CK + Light               | 0.145 | 0.140 | 0.115 | 0.115 | 0.125 | 0.125 | 0.150 | 0.135 | 0.145 | 0.145 | 0.145 |
|             | H <sub>2</sub> O + Dark  | 0.750 | 0.790 | 0.610 | 0.640 | 0.595 | 0.550 | 0.495 | 0.505 | 0.515 | 0.370 | 0.425 |
| RNA         | H <sub>2</sub> O + Light | 0.655 | 0.700 | 0.600 | 0.545 | 0.670 | 0.595 | 0.545 | 0.555 | 0.545 | 0.420 | 0.475 |
| OD260-      | CK + Dark                | 0.690 | 0.750 | 0.665 | 0.670 | 0.620 | 0.680 | 0.650 | 0.715 | 0.560 | 0.590 | 0.480 |
| OD290       | CK + Light               | 0.735 | 0.745 | 0.665 | 0.630 | 0.670 | 0.645 | 0.635 | 0.655 | 0.655 | 0.725 | 0.650 |
|             | H <sub>2</sub> O + Dark  | 0.335 | 0.330 | 0.305 | 0.280 | 0.265 | 0.195 | 0.185 | 0.215 | 0.205 | 0.115 | 0.170 |
| Chlorophyll | H <sub>2</sub> O + Light | 0.325 | 0.300 | 0.300 | 0.270 | 0.315 | 0.285 | 0.260 | 0.260 | 0.230 | 0.200 | 0.210 |
| OD660       | CK + Dark                | 0.330 | 0.310 | 0.270 | 0.270 | 0.265 | 0.280 | 0.240 | 0.255 | 0.210 | 0.215 | 0.225 |
|             | CK + Light               | 0.340 | 0.290 | 0.270 | 0.285 | 0.280 | 0.290 | 0.290 | 0.300 | 0.310 | 0.305 | 0.305 |

Table A2

Intercept, regression co-efficients and sums of squares of the errors.

1. Uninfected Plant

| Cellular Component | Incubation treatment     | Linear                        |                | SS        | Quadratic                     |                |                | SS        |
|--------------------|--------------------------|-------------------------------|----------------|-----------|-------------------------------|----------------|----------------|-----------|
|                    |                          | A <sub>0</sub><br>(intercept) | A <sub>1</sub> |           | A <sub>0</sub><br>(intercept) | A <sub>1</sub> | A <sub>2</sub> |           |
| DNA                | H <sub>2</sub> O + Dark  | 0.12                          | -0.48          | 0.001515  | 0.13                          | -0.014         | 0.00094        | 0.0007499 |
|                    | H <sub>2</sub> O + Light | 0.098                         | -0.00045       | 0.001100  | 0.11                          | -0.0079        | 0.00084        | 0.0004958 |
|                    | CK + Dark                | 0.11                          | -0.0038        | 0.001515  | 0.096                         | 0.0028         | -0.00066       | 0.001136  |
|                    | CK + Light               | 0.12                          | -0.0017        | 0.0006764 | 0.11                          | 0.0028         | -0.00045       | 0.0004991 |
| RNA                | H <sub>2</sub> O + Dark  | 0.22                          | -0.014         | 0.006785  | 0.24                          | -0.025         | 0.0010         | 0.005883  |
|                    | H <sub>2</sub> O + Light | 0.21                          | -0.005         | 0.0006543 | 0.22                          | -0.011         | 0.00062        | 0.0003207 |
|                    | CK + Dark                | 0.23                          | -0.0098        | 0.007787  | 0.21                          | 0.0065         | -0.0016        | 0.005503  |
|                    | CK + Light               | 0.24                          | -0.0079        | 0.002319  | 0.23                          | 0.0019         | -0.00098       | 0.001497  |
| Chlorophyll        | H <sub>2</sub> O + Dark  | 1.01                          | -0.080         | 0.01915   | 1.04                          | -0.097         | 0.0018         | 0.01641   |
|                    | H <sub>2</sub> O + Light | 0.90                          | -0.042         | 0.009008  | 0.91                          | -0.051         | 0.0009         | 0.008308  |
|                    | CK + Dark                | 1.0                           | -0.061         | 0.04030   | 0.95                          | -0.032         | 0.0028         | 0.003345  |
|                    | CK + Light               | 0.93                          | -0.036         | 0.02617   | 0.88                          | -0.0083        | -0.0028        | 0.01959   |

2. TYMV-Infected Plant

| Cellular Component | Incubation treatment     | Linear                        |                | Quadratic      |                |                | SS       |           |
|--------------------|--------------------------|-------------------------------|----------------|----------------|----------------|----------------|----------|-----------|
|                    |                          | A <sub>0</sub><br>(intercept) | A <sub>1</sub> | A <sub>0</sub> | A <sub>1</sub> | A <sub>2</sub> |          |           |
| DNA                | H <sub>2</sub> O + Dark  | 0.16                          | -0.0085        | 0.001261       | 0.17           | -0.011         | 0.00025  | 0.001208  |
|                    | H <sub>2</sub> O + Light | 0.14                          | -0.0033        | 0.001623       | 0.13           | -0.00080       | -0.00025 | 0.001571  |
|                    | CK + Dark                | 0.14                          | -0.0034        | 0.002338       | 0.14           | -0.0055        | 0.00021  | 0.002307  |
|                    | CK + Light               | 0.13                          | -0.0016        | 0.001372       | 0.14           | -0.0051        | 0.00067  | 0.0009862 |
| RNA                | H <sub>2</sub> O + Dark  | 0.75                          | -0.036         | 0.01961        | 0.77           | -0.051         | 0.0015   | 0.01770   |
|                    | H <sub>2</sub> O + Light | 0.68                          | -0.021         | 0.02149        | 0.66           | -0.0096        | -0.0011  | 0.02042   |
|                    | CK + Dark                | 0.73                          | -0.017         | 0.02597        | 0.69           | 0.0082         | -0.0025  | 0.02045   |
|                    | CK + Light               | 0.70                          | -0.0045        | 0.01459        | 0.74           | -0.032         | 0.032    | 0.008099  |
| Chloro-<br>phyll   | H <sub>2</sub> O + Dark  | 0.34                          | -0.020         | 0.007054       | 0.35           | -0.029         | 0.00089  | 0.006372  |
|                    | H <sub>2</sub> O + Light | 0.33                          | -0.011         | 0.002922       | 0.31           | -0.0032        | -0.00083 | 0.002334  |
|                    | CK + Dark                | 0.31                          | -0.010         | 0.002376       | 0.32           | -0.016         | 0.00058  | 0.002085  |
|                    | CK + Light               | 0.29                          | -0.00041       | 0.003445       | 0.31           | -0.013         | 0.0013   | 0.001970  |

Table A3

Analysis of variance tables

1. Uninfected Plant

| Cell Component | Incubation Treatment     | Source    | df | Reg SS    | MS         | F      | Significance |
|----------------|--------------------------|-----------|----|-----------|------------|--------|--------------|
|                | H <sub>2</sub> O + Dark  | Linear    | 1  | 0.0025536 | 0.0025536  | 27.24  | 0.1%         |
|                |                          | Quadratic | 1  | 0.0007651 | 0.0007651  | 8.16   | 5%           |
|                |                          | Residual  | 8  | 0.0007499 | 0.00009373 |        |              |
|                |                          | Total     | 10 | 0.0040686 |            |        |              |
|                | H <sub>2</sub> O + Light | Linear    | 1  | 0.0000227 | 0.0000227  | 0.37   | NS           |
|                |                          | Quadratic | 1  | 0.0006042 | 0.0006042  | 9.75   | 5%           |
|                |                          | Residual  | 8  | 0.0004958 | 0.00006197 |        |              |
|                |                          | Total     | 10 | 0.0011227 |            |        |              |
| DNA            | CK + Dark                | Linear    | 1  | 0.0016036 | 0.0016036  | 112.93 | 0.1%         |
|                |                          | Quadratic | 1  | 0.0003790 | 0.0003790  | 26.69  | 0.1%         |
|                |                          | Residual  | 8  | 0.0001136 | 0.0000142  |        |              |
|                |                          | Total     | 10 | 0.0020962 |            |        |              |

Table A3 (cont.)

|             |                          |           |    |                  |            |       |      |
|-------------|--------------------------|-----------|----|------------------|------------|-------|------|
| DNA (Cont.) | CK + Light               | Linear    | 1  | 0.0003282        | 0.0003282  | 5.26  | 10%  |
|             |                          | Quadratic | 1  | 0.0001773        | 0.0001773  | 2.84  | NS   |
|             |                          | Residual  | 8  | <u>0.0004991</u> | 0.00006238 |       |      |
|             |                          | Total     | 10 | 0.0010046        |            |       |      |
| RNA         | H <sub>2</sub> O + Dark  | Linear    | 1  | 0.0235645        | 0.0235645  | 32.04 | 0.1% |
|             |                          | Quadratic | 1  | 0.000902         | 0.000902   | 1.23  | NS   |
|             |                          | Residual  | 8  | <u>0.005883</u>  |            |       |      |
|             |                          | Total     | 10 | 0.0303495        |            |       |      |
|             | H <sub>2</sub> O + Light | Linear    | 1  | 0.0028002        | 0.0028002  | 69.86 | 0.1% |
|             |                          | Quadratic | 1  | 0.0003336        | 0.0003336  | 8.32  | 5%   |
|             |                          | Residual  | 8  | <u>0.0003207</u> | 0.00004008 |       |      |
|             |                          | Total     | 10 | 0.0034545        |            |       |      |

Table A3 (Cont.)

|             |                        |           |    |           |            |        |      |
|-------------|------------------------|-----------|----|-----------|------------|--------|------|
| RNA (cont.) | CK + Dark              | Linear    | 1  | 0.0106036 | 0.0106036  | 15.42  | 1%   |
|             |                        | Quadratic | 1  | 0.002284  | 0.002284   | 3.32   | NS   |
|             |                        | Residual  | 8  | 0.005503  | 0.00068787 |        |      |
|             |                        | Total     | 10 | 0.0183906 |            |        |      |
| Chlorophyll | CK + Light             | Linear    | 1  | 0.0068809 | 0.0068809  | 36.77  | 0.1% |
|             |                        | Quadratic | 1  | 0.000822  | 0.000822   | 4.39   | 10%  |
|             |                        | Residual  | 8  | 0.01641   | 0.00205125 |        |      |
|             |                        | Total     | 10 | 0.0091999 |            |        |      |
| Chlorophyll | H <sub>2</sub> O+ Dark | Linear    | 1  | 0.6984111 | 0.6984111  | 340.48 | 0.1% |
|             |                        | Quadratic | 1  | 0.00274   | 0.00274    | 1.34   | NS   |
|             |                        | Residual  | 8  | 0.01641   | 0.00205125 |        |      |
|             |                        | Total     | 10 | 0.7175611 |            |        |      |

Table A3 (Cont.)

|                          |           |    |            |            |        |      |
|--------------------------|-----------|----|------------|------------|--------|------|
| H <sub>2</sub> O + Light | Linear    | 1  | 0.1944602  | 0.1944602  | 187.25 | 0.1% |
|                          | Quadratic | 1  | 0.0007     | 0.0007     | 0.67   | NS   |
|                          | Residual  | 8  | 0.008308   | 0.0010385  |        |      |
|                          | Total     | 10 | 0.2034682  |            |        |      |
| CK + Dark                | Linear    | 1  | 0.4087002  | 0.4087002  | 97.75  | 0.1% |
|                          | Quadratic | 1  | 0.00685    | 0.00685    | 1.64   | NS   |
|                          | Residual  | 8  | 0.03345    | 0.00418125 |        |      |
|                          | Total     | 10 | 0.4490002  |            |        |      |
| CK + Light               | Linear    | 1  | 0.14220023 | 0.14220023 | 58.07  | 0.1% |
|                          | Quadratic | 1  | 0.00658    | 0.00658    | 2.69   | NS   |
|                          | Residual  | 8  | 0.01959    | 0.00244875 |        |      |
|                          | Total     | 10 | 0.16837023 |            |        |      |

Chlorophyll  
(Cont.)

Table A3 (cont.)

2. TMV-infected plant

|                          |           |    |           |             |       |      |
|--------------------------|-----------|----|-----------|-------------|-------|------|
| H <sub>2</sub> O + Dark  | Linear    | 1  | 0.0079135 | 0.0079135   | 52.41 | 0.1% |
|                          | Quadratic | 1  | 0.000053  | 0.000053    | 0.35  | NS   |
|                          | Residual  | 8  | 0.001208  | 0.000151    |       |      |
|                          | Total     | 10 | 0.0091745 |             |       |      |
| H <sub>2</sub> O + Light | Linear    | 1  | 0.0011716 | 0.0011716   | 5.97  | 5%   |
|                          | Quadratic | 1  | 0.000052  | 0.000052    | 0.26  | NS   |
|                          | Residual  | 8  | 0.001571  | 0.000196375 |       |      |
|                          | Total     | 10 | 0.0027946 |             |       |      |
| CK + Dark                | Linear    | 1  | 0.0012989 | 0.0012989   | 4.52  | 10%  |
|                          | Quadratic | 1  | 0.000037  | 0.000037    | 0.13  | NS   |
|                          | Residual  | 8  | 0.002301  | 0.000287625 |       |      |
|                          | Total     | 10 | 0.0036369 |             |       |      |

DNA

Table A3 (Cont.)

|                          |            |           |           |           |             |      |    |
|--------------------------|------------|-----------|-----------|-----------|-------------|------|----|
| DNA (Cont.)              | CK + Light | Linear    | 1         | 0.0002784 | 0.0002784   | 2.26 | NS |
|                          |            | Quadratic | 1         | 0.0003858 | 0.0003858   | 3.13 | NS |
|                          |            | Residual  | 8         | 0.0009862 | 0.000123275 |      |    |
|                          |            | Total     | 10        | 0.0016504 |             |      |    |
| H <sub>2</sub> O + Dark  | Linear     | 1         | 0.14256   | 0.14256   | 64.43       | 0.1% |    |
|                          | Quadratic  | 1         | 0.00191   | 0.00191   | 0.86        | NS   |    |
|                          | Residual   | 8         | 0.01770   | 0.0022125 |             |      |    |
|                          | Total      | 10        | 0.16217   |           |             |      |    |
| H <sub>2</sub> O + Light | Linear     | 1         | 0.0476736 | 0.0476736 | 18.68       | 1%   |    |
|                          | Quadratic  | 1         | 0.00107   | 0.00107   | 0.42        | NS   |    |
|                          | Residual   | 8         | 0.02042   | 0.0025525 |             |      |    |
|                          | Total      | 10        | 0.0691636 |           |             |      |    |

RNA

Table A3 (Cont.)

|             |                         |           |    |           |             |       |      |
|-------------|-------------------------|-----------|----|-----------|-------------|-------|------|
| RNA Cont.   | CK + Dark               | Linear    | 1  | 0.032302  | 0.0323020   | 12.64 | 1%   |
|             |                         | Quadratic | 1  | 0.00552   | 0.00552     | 2.16  | NS   |
|             |                         | Residual  | 8  | 0.02045   | 0.00255625  |       |      |
|             |                         | Total     | 10 | 0.058272  |             |       |      |
| Chlorophyll | CK + Light              | Linear    | 1  | 0.0021827 | 0.0021827   | 2.16  | NS   |
|             |                         | Quadratic | 1  | 0.006491  | 0.006491    | 6.4   | 5%   |
|             |                         | Residual  | 8  | 0.008099  | 0.001012375 |       |      |
|             |                         | Total     | 10 | 0.0167727 |             |       |      |
| Chlorophyll | H <sub>2</sub> O + Dark | Linear    | 1  | 0.0483002 | 0.0483002   | 60.64 | 0.1% |
|             |                         | Quadratic | 1  | 0.000682  | 0.000682    | 0.86  | NS   |
|             |                         | Residual  | 8  | 0.006372  | 0.0007965   |       |      |
|             |                         | Total     | 10 | 0.0553542 |             |       |      |

Table A3 (Cont.)

|                          |           |    |           |             |       |      |
|--------------------------|-----------|----|-----------|-------------|-------|------|
| H <sub>2</sub> O + Light | Linear    | 1  | 0.0144327 | 0.0144327   | 49.47 | 0.1% |
|                          | Quadratic | 1  | 0.000588  | 0.000588    | 2.07  | NS   |
|                          | Residual  | 8  | 0.002334  | 0.00029175  |       |      |
|                          | Total     | 10 | 0.0173547 |             |       |      |
| CK + Dark                | Linear    | 1  | 0.0118145 | 0.0118145   | 45.33 | 0.1% |
|                          | Quadratic | 1  | 0.000291  | 0.000291    | 1.12  | NS   |
|                          | Residual  | 8  | 0.002085  | 0.000260625 |       |      |
|                          | Total     | 10 | 0.0141905 |             |       |      |
| CK + Light               | Linear    | 1  | 0.0000184 | 0.0000184   | 0.75  | NS   |
|                          | Quadratic | 1  | 0.001475  | 0.001475    | 5.99  | 5%   |
|                          | Residual  | 8  | 0.001970  | 0.00024625  |       |      |
|                          | Total     | 10 | 0.0034634 |             |       |      |

NS = Not significant

Table A4

1. Uninfected Plant

| Cellular  |                  | Residual sum-of-squares                     |           |          |
|-----------|------------------|---------------------------------------------|-----------|----------|
| Component | Treatment        | Linear                                      | Quadratic |          |
| DNA       | H <sub>2</sub> O | (H <sub>2</sub> O/DK + H <sub>2</sub> O/LT) | 0.004185  | 0.002820 |
|           | Kinetin          | (CK/DK + CK/LT)                             | 0.005623  | 0.005086 |
|           | Dark             | (DK/H <sub>2</sub> O + DK/CK)               | 0.003685  | 0.003652 |
|           | Light            | (LT/H <sub>2</sub> O + LT/CK)               | 0.002693  | 0.002629 |
|           | ALL              |                                             | 0.009847  | 0.009752 |
| RNA       | H <sub>2</sub> O | (H <sub>2</sub> O/DK + H <sub>2</sub> O/LT) | 0.01872   | 0.01755  |
|           | Kinetin          | (CK/DK + CK/LT)                             | 0.01271   | 0.009787 |
|           | Dark             | (DK/H <sub>2</sub> O + DK/CK)               | 0.02050   | 0.02035  |
|           | Light            | (LT/H <sub>2</sub> O + LT/CK)               | 0.004897  | 0.004843 |
|           | ALL              |                                             | 0.03722   | 0.03702  |

Table A4 (Cont.)

|                               |                  |                                             |          |          |
|-------------------------------|------------------|---------------------------------------------|----------|----------|
| Chlorophyll                   | H <sub>2</sub> O | (H <sub>2</sub> O/DK + H <sub>2</sub> O/LT) | 0.1334   | 0.1303   |
|                               | Kinetin          | (CK/DK + CK/DK)                             | 0.1183   | 0.1049   |
|                               | Dark             | (DK/H <sub>2</sub> O + DK/CK)               | 0.1093   | 0.1088   |
|                               | Light            | (LT/H <sub>2</sub> O + LT/CK)               | 0.05732  | 0.05583  |
|                               | ALL              |                                             | 0.3187   | 0.3169   |
| <u>2. TYMV-infected Plant</u> |                  |                                             |          |          |
| DNA                           | H <sub>2</sub> O | (H <sub>2</sub> O/DK + H <sub>2</sub> O/LT) | 0.004384 | 0.004384 |
|                               | Kinetin          | (CK/DK + CK/LT)                             | 0.005977 | 0.005647 |
|                               | Dark             | (DK/H <sub>2</sub> O + DK/CK)               | 0.005037 | 0.004947 |
|                               | Light            | (LT/H <sub>2</sub> O + LT/CK)               | 0.005454 | 0.005377 |
|                               | ALL              |                                             | 0.01387  | 0.01370  |

Table A4 (Cont.)

|             |                  |                                             |         |         |
|-------------|------------------|---------------------------------------------|---------|---------|
| RNA         | H <sub>2</sub> O | (H <sub>2</sub> O/DK + H <sub>2</sub> O/LT) | 0.05394 | 0.05388 |
|             | Kinetin          | (CK/DK + CK/LT)                             | 0.05497 | 0.05495 |
|             | Dark             | (DK/H <sub>2</sub> O + DK/CK)               | 0.09608 | 0.09562 |
|             | Light            | (LT/H <sub>2</sub> O + LT/CK)               | 0.1073  | 0.1062  |
|             | ALL              |                                             | 0.2286  | 0.2285  |
| Chlorophyll | H <sub>2</sub> O | (H <sub>2</sub> O/DK + H <sub>2</sub> O/LT) | 0.01968 | 0.01968 |
|             | Kinetin          | (CK/DK + CK/LT)                             | 0.01930 | 0.01776 |
|             | Dark             | (DK/H <sub>2</sub> O + DK/CK)               | 0.01780 | 0.01687 |
|             | Light            | (LT/H <sub>2</sub> O + LT/CK)               | 0.01848 | 0.01838 |
|             | ALL              |                                             | 0.05928 | 0.05846 |

Table A5

## Analysis of Variance Tables

1. Uninfected Plant

Cellular

| Component | Source                        | df | SS         | MS          | F     | Significance |
|-----------|-------------------------------|----|------------|-------------|-------|--------------|
| DNA       | H <sub>2</sub> O/CK Linear    | 1  | 0.00003843 | 0.00003843  | 0.43  | NS           |
|           | H <sub>2</sub> O/CK Quadratic | 1  | 0.001807   | 0.001807    | 20.07 | 0.1%         |
|           | LT/DK Linear                  | 1  | 0.00149116 | 0.00149116  | 16.56 | 0.1%         |
|           | LT/DK Quadratic               | 1  | 0.000002   | 0.000002    | 0.02  | NS           |
|           | Interaction Linear            | 1  | 0.0002784  | 0.0002784   | 3.09  | 10%          |
|           | Interaction Quadratic         | 1  | 0.0000216  | 0.0000216   | 0.24  | NS           |
|           | Residual                      | 32 | 0.0028808  | 0.000090025 |       |              |

Table A5 (Cont.)

|             |                               |    |            |               |       |      |
|-------------|-------------------------------|----|------------|---------------|-------|------|
| RNA         | H <sub>2</sub> O/CK Linear    | 1  | 0.00019381 | 0.00019381    | 0.47  | NS   |
|             | H <sub>2</sub> O/CK Quadratic | 1  | 0.003893   | 0.003893      | 9.43  | 1%   |
|             | LT/DK Linear                  | 1  | 0.00363688 | 0.00363688    | 8.81  | 1%   |
|             | LT/DK Quadratic               | 1  | 0.000004   | 0.000004      | 0.01  | NS   |
|             | Interaction Linear            | 1  | 0.00153401 | 0.00153401    | 3.72  | 10%  |
|             | Interaction Quadratic         | 1  | 0.0002446  | 0.0002446     | 0.59  | NS   |
|             | Residual                      | 32 | 0.0132037  | 0.00041261562 |       |      |
| Chlorophyll | H <sub>2</sub> O/CK Linear    | 1  | 0.01693845 | 0.01693845    | 6.97  | 5%   |
|             | H <sub>2</sub> O/CK Quadratic | 1  | 0.0147     | 0.0147        | 6.05  | 5%   |
|             | LT/DK Linear                  | 1  | 0.10789117 | 0.10789117    | 44.40 | 0.1% |
|             | LT/DK Quadratic               | 1  | 0.00019    | 0.00019       | 0.08  | NS   |
|             | Interaction Linear            | 1  | 0.00439108 | 0.00439108    | 1.81  | NS   |
|             | Interaction Quadratic         | 1  | 0.00018    | 0.00018       | 0.07  | NS   |
|             | Residual                      | 32 | 0.077758   | 0.0024299375  |       |      |

Table A5 (Cont.)

2. TYMV-infected Plant

## Cellular

| Component | Source                        | df | SS          | MS            | F     | Significance |
|-----------|-------------------------------|----|-------------|---------------|-------|--------------|
| DNA       | H <sub>2</sub> O/CK Linear    | 1  | 0.0026952   | 0.0026952     | 14.22 | 0.1%         |
|           | H <sub>2</sub> O/CK Quadratic | 1  | 0.00016     | 0.00016       | 0.84  | NS           |
|           | LT/DK Linear                  | 1  | 0.0028867   | 0.0028867     | 15.23 | 0.1%         |
|           | LT/DK Quadratic               | 1  | - 0.000003* |               |       | NS           |
|           | Interaction Linear            | 1  | 0.00000096  | 0.00000096    | 0.01  | NS           |
|           | Interaction Quadratic         | 1  | 0.0002008   | 0.0002008     | 1.06  | NS           |
|           | Residual                      | 32 | 0.0060662   | 0.00018956875 |       |              |
| RNA       | H <sub>2</sub> O/CK Linear    | 1  | 0.0341264   | 0.0341264     | 16.38 | 0.1%         |
|           | H <sub>2</sub> O/CK Quadratic | 1  | - 0.00002*  |               |       | NS           |
|           | LT/DK Linear                  | 1  | 0.0213505   | 0.0213505     | 10.25 | 1%           |
|           | LT/DK Quadratic               | 1  | 0.00146     | 0.00146       | 0.70  | NS           |
|           | Interaction Linear            | 1  | 0.0001719   | 0.0001719     | 0.08  | NS           |
|           | Interaction Quadratic         | 1  | 0.013451    | 0.013451      | 6.46  | 5%           |
|           | Residual                      | 32 | 0.066669    | 0.00208340625 |       |              |

Table A5 (Cont.)

|             |                               |    |           |               |       |      |
|-------------|-------------------------------|----|-----------|---------------|-------|------|
| Chlorophyll | H <sub>2</sub> O/CK Linear    | 1  | 0.0126582 | 0.0126582     | 31.74 | 0.1% |
|             | H <sub>2</sub> O/CK Quadratic | 1  | 0.00072   | 0.00072       | 1.81  | NS   |
|             | LT/DK Linear                  | 1  | 0.0102146 | 0.0102146     | 25.61 | 0.1% |
|             | LT/DK Quadratic               | 1  | 0.00021   | 0.00021       | 0.53  | NS   |
|             | Interaction Linear            | 1  | 0.0001420 | 0.001420      | 0.36  | NS   |
|             | Interaction Quadratic         | 1  | 0.001286  | 0.001286      | 3.22  | 10%  |
|             | Residual                      | 32 | 0.012761  | 0.00039878125 |       |      |

\* Negative values obtained because of rounding errors produced by the computer program

NS = Not significant

