## *Nucleic acids and proteins during development of leaves of festuca pratensis*

Diana Jupp

# Nucleic Acids and Proteins during development of Leaves of *Festuca pratensis*.

By

Diana Jupp B. Sc. (Dunelm)



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

## DECLARATION.

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

Signed: . . . . . .

Date: . . December 6th 1979 . . .

# CONTENTS.

ABSTRACT.

Two developmental systems for leaves of Festuca pratensis were compared, namely development along the leaf (base to apex) and development of the whole leaf with time. The methods for examination of nucleic acids from leaves of F. pratensis were optimised and the changes occurring in rRNA fractions, tRNAs and polyribosomes during both developmental systems were recorded. These changes were related to changes in levels and syntheses of soluble and particulate proteins. Activities of enzymes intimately involved in these relationships, namely RNAses, ATPases, phosphatases, pyrophosphatases and phosphodiesterases were monitored during development. The metabolic status was further examined by the study of the polypeptide - synthesizing capacity of the ribosomes at different stages as revealed in wheatgerm-derived cell-free systems. Three phases of increased metabolism were apparent in both developmental systems but only the initial and final phases involved increased RNA synthesis which was correlated with increased protein synthetic capacity.

Work involving "greening-up" and excision showed some similarities to the normal courses of development but also important differences particularly with respect to the magnitude of the response.

ACKNOWLEDGEMENTS.

I wish to express my grateful thanks to my supervisor, Dr. J. A. Pearson, for his friendly encouragement, patience and helpful comments during the course of this work and, particularly, for the support shown through the difficulties of the last months; to my supervisor at the Welsh Plant Breeding Station, Dr. H. Thomas, for enthusiastic interest in the work and good-humoured guidance; to Dr. J. L. Stoddart, Head of the Department of Plant Biochemistry, for becoming more than a surrogate supervisor during Dr. Thomas's absence and for all the support he has shown me over the last four years; to the other members of the Plant Biochemistry laboratory, Dr. T. W. A. Jones, Dr. C. J. Pollock, Dr. G. Riley, and E. J. Lloyd, for their help and endurance, and to Mrs. M. Lloyd-Evans and Miss D. Mills for their patient washing-up and particularly to Miss A. Thomas for her cheerful technical help.

I would also like to thank Professor D. Boulter for provision of research facilities in the Department of Botany at the University of Durham, Professor J. P. Cooper for the provision of superb facilities and additional financial support during my time working at the Welsh Plant Breeding Station, and the Science Research Council for financial support.

My thanks go most especially to my father, Mr. E. F. Parker, for his hard work and patience in the typing of this work, and finally to my husband, Barry, for his consideration, warm encouragement and long-suffering support during the months of separation during 1978 and, more recently, months of unusual mealtimes. My thanks also to my friends and relations who have all provided such a tremendous source of encouragement.

## ABBREVIATIONS.

The following abbreviations are used in the text of this thesis:-

| | |
|---|---|
| A | Absorbance. |
| A | Ampere. |
| AMP | Adenosine monophosphate. |
| ADP | Adenosine diphosphate. |
| ATP | Adenosine triphosphate. |
| ATPase | Adenosine triphosphatase. |
| BSA | Bovine serum albumin. |
| CBB | Coomassie Brilliant Blue. |
| cpm | counts per minute. |
| Cyt C | cytochrome C. |
| DNA | deoxyribonucleic acid. |
| DEP | diethylpyrocarbonate. |
| DTT | dithiothreitol. |
| E. coli | Escherichia coli |
| EDTA | ethylene diamine tetraacetate. |
| fresh wt. | fresh weight. |
| GTP | guanosine triphosphate. |
| GF/C | glass fibre disc. |
| xg. | acceleration due to gravity. |
| h. | hour. |
| HVPE | high voltage paper electrophoresis. |
| HEPES | N-2-hydroxyethylpiperazine-$N^1$-2 ethanesulphonic acid. |
| $K_m$ | Michaeli's constant. |
| min. | minute(s). |
| MW | molecular weight. |

Abbreviations (contd.)

| | |
|---|---|
| NAD(H) | nicotinamide adenine dinucleotide (reduced form). |
| OA | ovalbumin. |
| Pa | Pascal(s). ($m^{-1}.kg.s^{-2}$). |
| PVP | polyvinylpyrrolidone |
| PPO | 2,5 diphenyloxazole. |
| PAGE | polyacrylamide gel electrophoresis. |
| RNA | ribonucleic acid. |
| rRNA | ribosomal RNA. |
| mRNA | messenger RNA. |
| tRNA | transfer RNA. |
| RNAse | ribonuclease. |
| s | sedimentation coefficient. |
| SDS | sodium dodecyl sulphate. |
| temp. | temperature. |
| TEMED | $N:N:N^1:N^1$-tetramethyl 1,2 diaminoethane. |
| TCA | trichloracetic acid. |
| t.l.c. | thin layer chromatography. |
| TM virus | tobacco mosaic virus. |
| Tris | tris(hydroxymethyl)aminomethane. |
| U | units of enzyme activity. |
| uv | ultraviolet. |
| vol | volume. |
| V/V | volume/volume. |
| W/V | weight/volume. |

## CHAPTER 1.

I. General Introduction. Developmental control on many levels.

II. General Materials and Methods.

- i. Biological material.
- ii. Chemicals.
- iii. Sterile technique.
- iv. Chlorophyll estimation.
- v. Experimental observation of the two developmental systems.

III. General development data.

- i. Growth and fresh weight changes.
- ii. Chlorophyll levels during development.
- iii. Morphology and fibre content during development.

# CHAPTER 1.

## I. General Introduction.

### Development control on many levels.

The immobility of higher plants causes them to be particularly vulnerable to environmental stress, thus, unlike animals which can often dissociate themselves from the point of stress, they must rely more heavily on their inherent genetic plasticity. This may be a contributory factor in the maintenance of adaptability throughout the development of higher plants and a justification for even the ageing process generally being under positive control, unlike the equivalent process in animals where the accumulation of random errors in the cellular physiology contributes to the ageing condition (Orgel, 1970). Unlike characteristic animal ageing, the ageing of higher plants is frequently restricted to particular organs; for example, cotyledonary leaves senesce following production of secondary leaves (Cherry, 1967) or seasonal senescence of the aerial parts of many herbaceous plants. In these instances, a control in senescence is required for the maximal redeployment of metabolites to growing (Thrower, 1967) or storage tissues or seeds (Sinclair and de Wit, 1975). Leopold (1961) has further hinted at the ecological advantages inherent in a high turnover of generations to encourage genetic selection of adaptive qualities so important to the immobile plant. The adaptability of earlier phases of development is in the reception of environmental stimuli on which the subsequent development of the plant or particular organ will depend. Thus, the daylength prevailing during early stages of leaf development may influence the onset and course of senescence (Krizek *et al.*, 1966; Schwabe, 1970; Spencer and Titus, 1972).

Investigations into the development of higher plants are complicated by their lack of uniformity and because ageing of individual organs is not simultaneous. To impose uniformity, for example by the use of excised organs in experiments, immediately removes the correlative influences of the remaining tissues and the

course of development will be disturbed (Lewington et al., 1967; Simon, 1967). This points to another experimental problem in that the course of development under different conditions may appear to be the same but may, in fact, result from several different patterns of metabolism (Simon, 1967).

The inherent adaptability in directing subsequent development poses experimental problems of replication. It is vital to development studies that constant conditions are maintained for the growth of the plant which must be as genetically uniform as possible. Most environmental influences (for example, light, temperature, water stress, infection) and even the position on the plant (for example, Hedley and Stoddart, (1972b) found differences in the development of third and fifth leaves of Lolium temulentum) affect the course of development of the leaf. In addition, total leaf measurements may give a false impression of the metabolic status of the leaf as the direction of further development might result from catastrophic biochemical changes confined to a small area (Hardwick et al, 1968)

The leaf is, therefore, subject to an enormous variety of stimuli on emergence. Light is the most obvious influence both in its quality (intensity and spectral composition) and duration. Phytochrome has been frequently implicated in the mediation of the reaction to light. Its function has been related to increased enzyme syntheses and activities (for example, Graham et al., 1968; Schopfer and Mohr, 1972), increased accumulation of RNAs (for example, Thien and Schopfer, 1975), chlorophyll synthesis (for example, Nadler and Granick, 1970), increased hormonal levels (for example, Black and Vlitos, 1971) and numerous other developmental components which lead to profound effects on leaf morphology (Sanchez and Cogliati, 1975), inter-organ correlations (De Greef and Caubergs, 1972) and developmental life span (Singh, 1975). However, these are regarded as secondary effects. Despite reports of its physical association with nucleic acids (Galston, 1968; Quail, 1975) the level at which phytochrome control acts seems to be concentrated at the membranes (Hendricks and Borthwick, 1967; Tanada, 1968). This concept

eliminates the assumption that organismal development primarily involves the selective control of gene expression. Post-transcriptional control, for example through the activation of existing inactive enzymes, may appear energetically expensive but in view of the value of rapid adaptability, already expounded, this level of control may represent a very important asset. Synthesis of enzymes from transcriptional level takes perhaps several hours so that the presence of an inactive pool of potentially active enzymes which could be rapidly mobilized would be a great advantage. However, those responses which do not require immediate interpretation may, indeed, be mediated through nuclear channels of control. At least one other photoreceptive substance, a blue-light absorbing pigment, is important in the mediation of photomorphogenesis, thus providing a greater variety of the capacity to adapt.

During ageing, leaves retain the same elements of metabolic flexibility allowing continued modification to adapt to prevailing conditions. This is perhaps best exemplified in the regreening property of many senescent leaves under favourable conditions (Wollgiehn, 1967; Simon, 1967) and the rooting of excised leaves (Woolhouse, 1967).

The effects of growth regulators at all phases of development have been widely reported but the primary expression of their effect remains speculative, ranging from direct effects on nucleic acids (Woolhouse, 1967; Dyer and Osborne, 1971) to inhibition of protein hydrolysis (Kuraishi, 1968; Tavares and Kende, 1970). The realisation that effects of exogenously applied hormones whilst imitating normal processes superficially frequently acted via completely different channels (Woolhouse, 1967) has devalued the use of hormones in development work. However, out of such work has come the confirmation that development can follow a number of different courses with the same ultimate physiological expression.

The possibilities of pools of inactive enzymes and long-lived mRNAs tend to lessen the importance of nuclear control since the pools could be established during early development. Even work with inhibitors need not necessarily imply requirement for mRNA transcription although rRNA and tRNA transcription control may

provide important restraints on metabolism. The observation of at least one gene which is ~~expressed only~~ most obvious at senescence, namely that for chlorophyll degradation (Thomas and Stoddart, 1975) should provide a useful probe into the nuclear control of the later stages of development.

Even with minimal contribution from the nuclear-based nucleic acid in the actual control of development there is still enormous scope for control at the post-transcriptional level, many aspects of which have already been mentioned. One, which is perhaps less obvious, is the possibility of changes in translational rate. Thus, even exposing polyribosomes in the process of polypeptide synthesis to a cell-free system need not necessarily imply that the rate of protein production echoes the in vivo situation. In this way, the retention of active polyribosomes at, for example, senescence need not imply that a high rate of protein synthesis exists but could be further insurance against environmental change.

The phenomena of delayed or reversed senescence would also tend to favour a non-reliance on genetically pre-programmed development. Amongst others, the adherents to the theories of photoreceptive substances mediating photomorphogenic change at the gene level would support the idea of continued nucleic activity even at late stages of development. However, if gene level control is not the major process through which mediators of environmental influence act then the alternative may well be the provision of a sufficiently large programme at the onset of development to accommodate these developmental possibilities. The work of Hedley and Stoddart (1972b) showed three phases of increased protein synthesis during the development of leaves of Lolium temulentum. The first and last phases were influenced by environmental conditions operating during early development which would suggest the presence of a long-term influence on ~~the~~ senescence ~~signal~~.

The control of development need not, of course, be exclusive to one system or another but involve a subtle co-operative interplay of a number of systems which need not be temporally, organismally or interspecifically uniform. (A form of endogenous senescence may

also be important for example in determining the life span of particularly long-lived organs, for example, conifer leaves). For these reasons it is necessary to observe development of a particular organ on a number of different ~~cellular~~ levels. Only by such observation will a clear, overall picture emerge of the subtleties of developmental control. The paradox of experimental work in development is the need to remove correlative influences for the observation at particular levels but having achieved this, extrapolation to the natural state is not necessarily valid. In the words of Wareing and Phillips (1970), "the plant body at any given stage is the resultant of interaction between the inherent (genetic) potentialities of the species and the external factors of the environment."

Leaves of grass species are particularly useful for such study as they not only age as a whole with time but, by virtue of their basal meristems, sequentially along the leaf from base to apex. In addition, in theory, the problems of variable patterns of early growth are to some extent overcome by reference to the emergence of the ligule as an indicator of leaf maturity. (However, as has already been mentioned, environmental conditions affecting early stages may also affect the subsequent course of senescence.) This work was originally conceived to investigate the senescence phenomena in leaves of Festuca pratensis with a view to probing the triggering and sustaining mechanisms. As the work progressed it became clear that it was necessary to review this particular developmental phase in the context of the complete development of the leaf in order to derive critical qualitative and quantitative comparisons. Despite the complexities of developmental control suggested above, it is reasonable to compare the status of the well-established tenets of the central dogma at different phases. Examination of the key components would be more likely to reveal specific aspects of the course of development than the observation of more secondary factors such as hormonal balance, enzyme activities

and ultrastructural characteristics. Nevertheless, these latter may be important pointers to the underlying control but may not necessarily be indicative of the means of achievement. To this end, some of the qualitative and quantitative characteristics of nucleic acids were investigated throughout leaf development in relation to the content and synthesis of proteins. Enzymes of phosphorus metabolism were examined since they were considered to be intimately involved with the presumptive major basis of control. Bearing in mind the caution with which results from cell-free polypeptide-synthesizing systems must be interpreted (see above), the polypeptide-synthesizing capacity of polyribosomes at different developmental stages was examined in conjunction with the other cellular characteristics. Preliminary work on the comparison of two artificial developmental systems, namely 'greening-up' and excision, with the natural course of leaf development was also carried out.

## II. General Materials and Methods.

### i. Biological materials.

Seeds of meadow fescue (Festuca pratensis L) were provided by J. Lewis (Seed Multiplication Department, Welsh Plant Breeding Station) and stored in sealed containers at 4°C. Originally a variety within the type S-215 was used, namely Festuca pratensis L. cv Aberystwyth Perdita. From 1976, work was restricted to the use of more robust, larger and faster growing Dutch variety, Kweekbedrijf CID Langoed Zelder Ottersum, known as cv Rossa.

Seeds were sown in John Innes No. 1 compost in seed trays and grown in a glasshouse at 21-25°C. Supplementary lighting was provided, when necessary, by fluorescent lights maintaining a daylength of 15 - 17 h. At the fourth leaf stage the plants were transplanted into individual 10 cm. diameter pots.

At the Welsh Plant Breeding Station, seeds were planted in shallow boxes of John Innes No. 1 compost either in the greenhouse under normal daylight, supplemented when necessary with high pressure mercury vapour lighting to give a daylength of 16 h. or in a growth room at 20°C. providing 16 h. photoperiod at an intensity of 22,000 - 25,000 lux. One week after sowing, 15 g. Fisons 52 fertilizer (nitrogen:phosphate:potassium, 20:10:10) per $15.2 \times 10^3$ $cm^3$ compost were sprinkled evenly over the box and was gradually washed into the compost by daily watering.

In one instance (plants used for polyribosome extraction along the leaf and for material for excising) plants were transferred to the laboratory and maintained under a 16 h. photoperiod by a fluorescent light at 5,600 lux and 23°C.

Seeds of Pisum sativum cv Meteor or Pisum sativum cv Feltham First were soaked in tap water for 24 or 48 h. prior to germination in seed trays lined with damp paper towel or absorbent cotton wool saturated with tap water. Trays were covered in aluminium foil and seed germination proceeded for a further 48 h. at 25°C.

Wheatgerm extracts were prepared from untoasted wheatgerm obtained from Niblack's Inc., Rochester, New York.

ii. Chemicals.

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

Radiochemicals were obtained from the Radiochemical Centre, Amersham, Bucks.:-

(a) 8-$^{3}$H adenine in aqueous solution (22 Ci/mmol.)(1mCi./µl.)

(b) $^{14}$C-labelled amino acids in aqueous solution containing 2% (V/V) ethanol (50 µCi./ml.). Individually purified L-amino acids uniformly labelled and mixed in the proportions of a typical protein hydrolysate as follows,

| | % total activity |
|---|---|
| L-Ala | 10.0 |
| L-Arg | 6.5 |
| L-Asp | 9.0 |
| L-Glu | 12.5 |
| Gly | 5.0 |
| L-Isoleu | 5.0 |
| L-Leu | 12.0 |
| L-Lys | 5.5 |
| L-Phe | 7.0 |
| L-Pro | 6.0 |
| L-Ser | 5.0 |
| L-Thr | 6.0 |
| L-Tyr | 3.5 |
| L-Val | 7.0 |

(c) L- U-$^{14}$C leucine in aqueous solution containing 2% (V/V) ethanol (354 mCi./mol.)(50 µCi./ml.).

Aurin tricarboxylic acid was a gift from Aldrich Chemical Co. Ltd., Gillingham, Dorset.

Cytochrome C from Boehringer Mannheim, Mannheim, Germany.

Fluram from F. Hoffmann - La Roche and Co., Ltd., Diagnostica, Basle, Switzerland.

Oligo(dT)-cellulose from Collaborative Research Inc., Waltham, Mass., U.S.A.

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

Sephadex G-25, G-100 from Pharmacia Fine Chemicals, Uppsala, Sweden.

## iii. Sterile technique.

Experiments frequently required maintenance of sterile techniques when glassware and solutions (where appropriate) were autoclaved. Particular care was observed in maintaining sterile conditions for the wheatgerm-derived cell-free system; after autoclaving, solutions were stored at -20°C. and glassware (notably Durham Tubes) was kept in sealed containers. Hamilton syringes were rinsed thoroughly in ethanol followed by sterilized water, or disposable tipped automatic syringes were used. Solutions were continually checked for contamination by routine inclusion of controls in cell-free system experiments. Sterility was further ensured by regular filtration through millipore filters (0.2 μm.). Whenever leaf sections were to be incubated, either in radioisotope labelling experiments or excision experiments, they were first washed for 3 min. in a sterilizing solution consisting of

10 ml. sodium hypochlorite
0.1 ml. Nonidet P42
} in 230 ml. distilled water.

or

3 ml. sodium hypochlorite
0.05 ml. Nonidet P40
} in 100 ml. distilled water.

followed by at least three rinses with sterilized water. All such manipulations were carried out under a laminar flow clean air cabinet maintaining sterility by UV light.

All Sephadex or Sepharose columns were stored at 4°C., saturated with sodium cyanide (0.02% W/V) solutions to maintain sterility.

### iv. Chlorophyll estimation.

Pellets from centrifugations were extracted at least seven times with equal volumes of acetone, "whirlimixed" and centrifuged between extractions at 2,000 g. for 2 - 3 min. Alternatively, pooled chloroform:methanol washes from the extraction procedure for particulate protein (Chapter 3, II i.) were thoroughly mixed and 0.25 ml. were taken up in 2.5 ml. acetone. Chlorophyll was estimated by the method of Mackinney (1940). $A_{645}$ and $A_{663}$ were recorded using a Pye-Unicam SP800 spectrophotometer against an acetone blank.

### v. Experimental observation of the two developmental systems.

The ageing of the whole leaf with time was investigated by following the development of the fourth leaf (which emerged at approximately day 23 from sowing) from emergence through to day 70 when it showed advanced senescence.

The ontogenic sequence along the leaf was investigated by dividing the mature leaf into eight proportionally-sized sections which assumed that the rate of development is uniform throughout the leaf. Maturity was judged by the emergence of the ligule.

## III. General development data.

### i. Growth and fresh weight changes.

The change in leaf length (Figure 1.1) follows the classical sigmoid growth curve in which the exponential growth is followed by a fall in growth rate. Leaf fresh weight follows a similar pattern to the increase in length but falls off dramatically with senescence presumably due to the translocation of products of catabolism to other parts of the plant. Figure 1.2 shows the fresh weights of equivalent length sections of leaf tissue from the base to the apex. Fresh weight per unit section drops presumably as a result of extension growth rather than cell division. Nearer the apex the fall in fresh weight may also represent loss of catabolic products by translocation. If the fresh weights of different aged whole leaves (Figure 1.1 A) are expressed on a per unit area basis then it can be seen that a massive increase in fresh weight occurs between day 24 and day 29 as a result of high mitotic activity, which falls off due to a predominance of extension growth by vacuolation over the following 20 days. A further increase, but less dramatic than the earlier increase, is apparent at day 49 when the leaf has reached maximum length, perhaps due to increased fibre content, and this is followed by a decrease which confirms the speculative decrease indicated at senescence in Figure 1.1 A. Day 49 can be regarded as the time at which full maturity has been reached and senescence commences.

### ii. Chlorophyll levels during development.

The chlorophyll content per unit area increases (Figure 1.1 B) to a maximum at day 42 (a). The fall between a and b is not due to senescent degradation, as confirmed by expressing the chlorophyll content on a per leaf basis, but rather reflects continued leaf growth before reaching maximal growth. Actual chlorophyll loss is apparent between b and c as confirmed by expressing chlorophyll content on a per leaf basis and per unit DNA basis. Day 49 is the turning point for the start of senescence.

Chlorophyll content increases along the leaf from the base. Degradation occurs beyond point 6. Comparison of chlorophyll status in the two ageing systems would therefore suggest that point 6 along the leaf could be considered equivalent to ageing between day 49 and day 57.

### iii. Morphology and fibre content during development.

Fibre and silica contents increase with age in fescue varieties (for example, Archer and Decker, 1977) and with high temperatures and nitrogen stress. Photoperiod also has profound effects on cell wall components (Bowman and Law, 1964; Allinson, 1971). As a result problems of cellular component extraction of plant material arise as the severity of required procedure will differ between young and mature tissue and from tissue exposed to different temperatures or daylengths. Fibre content of F. pratensis was subjectively assessed as increasing to a maximum at day 57 and portion 7 along the leaf.

Figure 1.1

A. Changes in leaf length and fresh weight during development of the fourth leaf of *F. pratensis.*

B. Changes in chlorophyll content per unit area during development of the fourth leaf of *F. pratensis.*

FIGURE 1.1

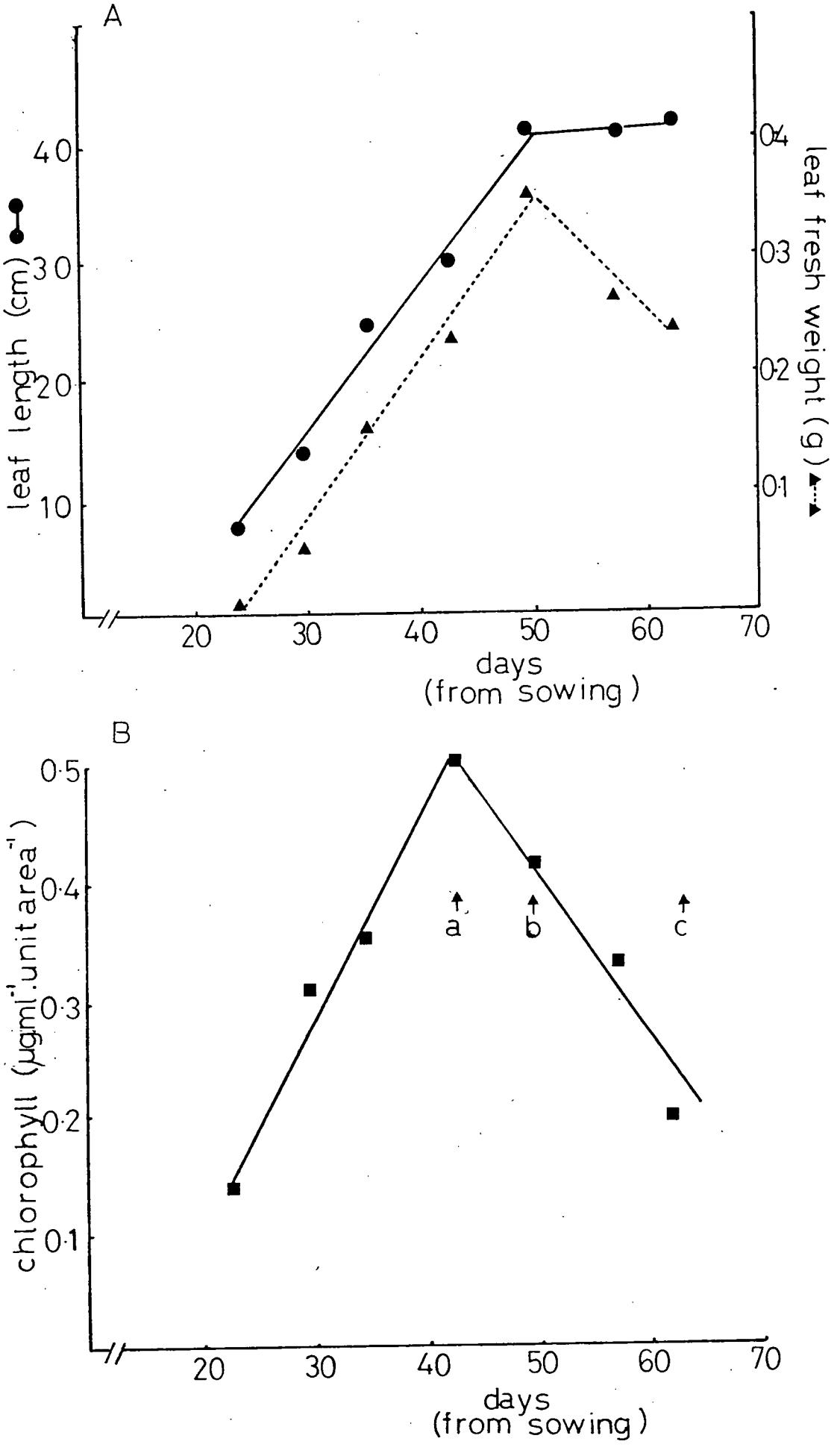
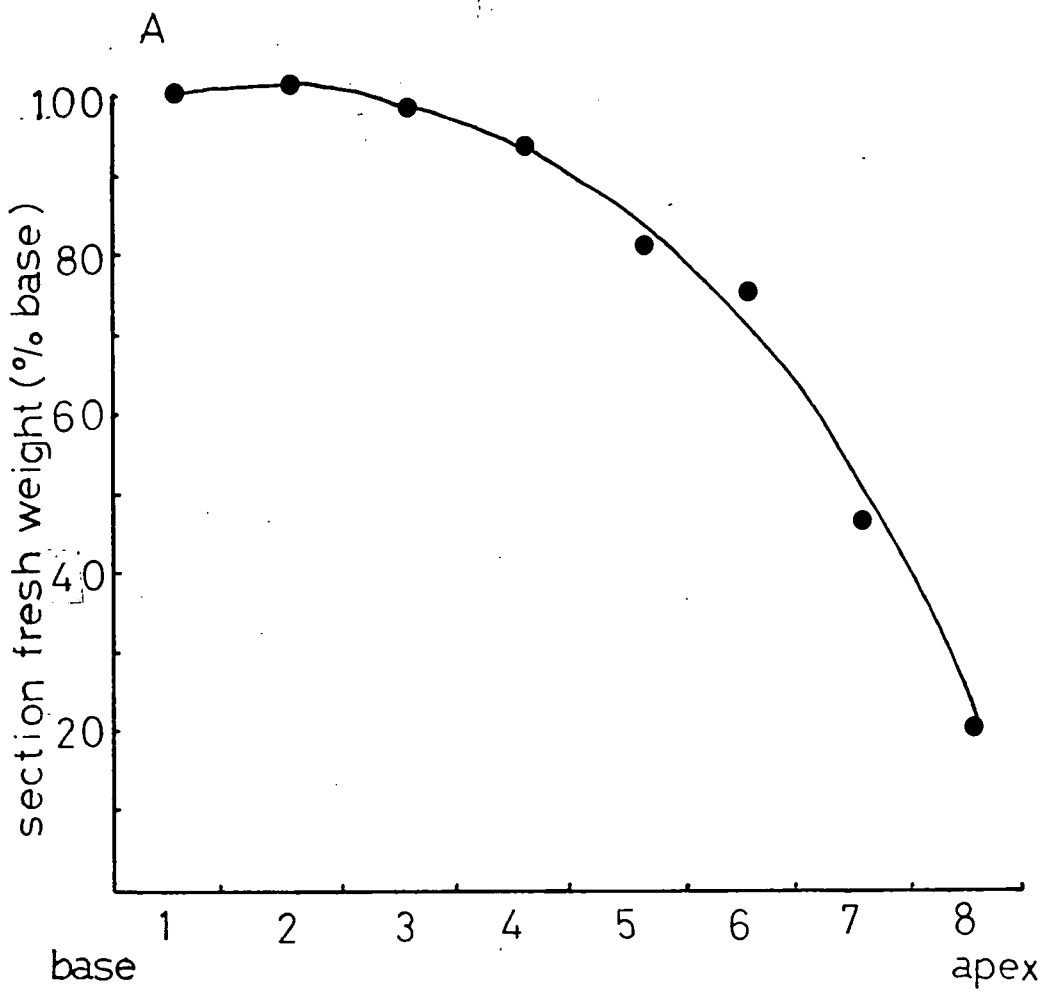

A
leaf length (cm)
40
30
20
10
leaf fresh weight (g)
0·4
0·3
0·2
0·1
20
30
40
50
60
70
days
(from sowing)
B
chlorophyll (µgml⁻¹.unit area⁻¹)
0·5
0·4
0·3
0·2
0·1
a
b
c
20
30
40
50
60
70
days
(from sowing)

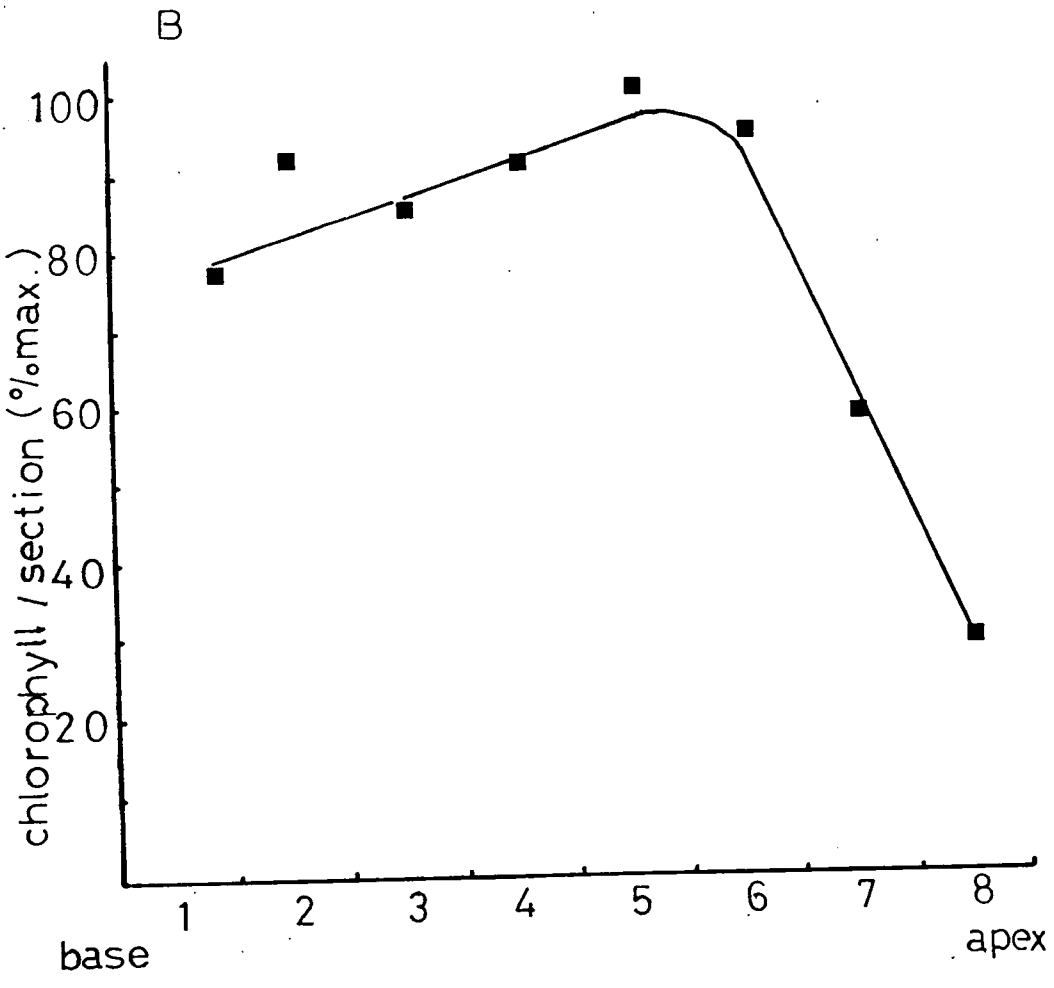
Figure 1.2

A. Changes in fresh weight per section along the axis of a mature leaf of F. pratensis.

B. Changes in chlorophyll content expressed as % of the maximum, per section along the axis of a mature leaf of F. pratensis.

FIGURE 1.2


A
100
80
60
40
20
section fresh weight (% base)
1
2
3
4
5
6
7
8
base
apex


B
100
80
60
40
20
chlorophyll / section (%max.)
1
2
3
4
5
6
7
8
base
apex

# CHAPTER 2. NUCLEIC ACIDS AND DEVELOPMENT OF LEAVES OF F. PRATENSIS.

1. General Introduction.
   - i. Early events.
   - ii. Effect of Light.
   - iii. Phase of Maturity.
   - iv. Senescence.

2. Methods.
   - i. Aims in Analysis of RNA.
   - ii. Extraction of RNA
     - a. Phenol method.
     - b. Limitations in the phenol method.
     - c. DEP method.
     - d. Limitations in the DEP method.
     - e. Spectrophotometric estimation of RNA.
     - f. Comparison of phenol and DEP methods.
   - iii. PAGE
     - a. PAGE system used.
     - b. Molecular weight determination.
     - c. Some effects of $Mg^{2+}$ and EDTA.
     - d. Optimum electrophoresis conditions.
       1. EDTA.
       2. $Mg^{2+}$.
       3. Electrophoresis time.
       4. Temperature.
       5. Use of $Ca^{2+}$.
     - e. 'Split' gels.
   - iv. Radioisotope labelling of RNA
     - a. Time course of uptake.
     - b. Calculation of specific activities.
   - v. Nucleotide extraction and estimation.
   - vi. Ribosome extraction
     - a. Methods used.
     - b. Spectrophotometric estimation of ribosomes.

vii. Sucrose gradient centrifugation.

viii. PAGE

ix. Some quantitative and qualitative variations in ribosome yield,

- a. Homogenization.
- b. Effect of ionic strength.
- c. Effect of PVP.
- d. Effect of incorporating a 'Lyphogel' purification step.
- e. Effect of centrifugation time.
- f. Effect of RNAse and EDTA.
- g. Addition of 'Nonidet' in the extraction.

x. Radio-isotope labelling of ribosomes.

xi. Extraction of mRNA.

xii. PAGE.

xiii. Radio-isotope labelling of mRNA.

3. Experimental.

i. Total nucleic acids

- a. Quantitative estimation of total nucleic acids.
- b. Radio-isotope labelling of total nucleic acids.
- c. Relative changes in specific fractions.
- d. Radio-isotope labelling of specific fractions.

ii. Nucleotides

- a. Radio-isotope labelling of nucleotides.

iii. Ribosomes

- a. Quantitative estimation of ribosomes.
- b. Comparison of 'free' and 'membrane bound.'
- c. Radio-isotope labelling of ribosomes.

iv. Poly(A)-containing mRNA.

## 1. General Introduction.

The vehicle for transforming genetic information into cellular realisation is the ribosome (Wettstein et al., 1963). It is commonly regarded as a RNA-protein complex through which mRNA passes with consequent translation of genetic codons into polypeptides, by sequential amino acid assembly (Burka and Marks, 1964). The rRNA may serve only to provide a template for ribosomal protein assembly, since partial destruction of the rRNA need not lead to loss of ribosomal function (Furano and Harris, 1971). In addition, the regulation of ribosome formation seems to be limited by the availability of ribosomal proteins (Shulman et al., 1973; Warner, 1974), with consequent wastage of excess precursoral rRNA (Cooper and Gibson, 1971; Grierson et al., 1976). Although the functional importance of rRNA in the actual translation of mRNA may have diminished in the light of these observations it is nevertheless a central pivot in ontogeny. It follows that its synthesis, presence and activity will be intimately involved. These processes in themselves may well represent further modes of developmental control, apart from those occurring at the transcription level, i.e., in the nucleus. Since no functioning ribosomes have been found in the nucleus (Hamkalo and Miller, 1973), the cytoplasmic location of the protein-synthesizing machinery, both from physical and temporal considerations, supports the concept of ribosome assembly and function being responsible for the more rapid and subtle re-establishment of cellular homeostasis following environmentally-caused imbalance. For example, it is difficult to imagine the phytochrome-mediated stimulation of phenylalanine ammonia-lyase activity in pea (which is apparent within 1 h. (Smith and Attridge, 1970) ) going through nuclear channels of control whereas cytoplasmic-controlled de novo synthesis could be feasible.

Improvements in the extraction and monitoring techniques over the last 15 years have provided greater opportunities to examine the role of all the nucleic acids and their involvement in development. It has been realised that quite subtle differences in synthesis of particular RNA species may have profound effects

on the ordering of developmental sequences; for example, the light-stimulated assembly of Fraction I protein is realised through an increase in chloroplast rRNA synthesis (Cohen and Schiff, 1976; Ingle, 1968 a).

It was not until 1962 that two types of ribosome in plants were distinguished (Lyttleton, 1962), on the basis of their behaviour in sucrose density gradients. Clark et al. (1964) isolated two ribosomal species from Chinese cabbage with sedimentation coefficients of 83S and 68S. The origin of the latter was attributed to the chloroplast and amounted to 20-35% (W/W) of the total ribosome population. A variety of other photosynthetic plants were examined and nearly all yielded two distinct ribosome types with sedimentation coefficients of about 80S and 70S (Boardman et al., 1965; Sissakian et al., 1965; Sager and Hamilton, 1967). Most of the cases where such distinction was not observed have since been shown to be due to a disregard of species-specific ribosome stabilities on extraction; for example, Brawerman and Eisenstadt (1967) failed to find two clases of ribosome in Euglena, but in 1969 Rawson and Stutz, by appreciation of the requirements for stabilizing cytoplasmic ribosomes, established sedimentation coefficients for two classes which were consistent with those found in other plants.

It followed that since there were distinct sizes of ribosome, the constituent RNAs would probably be of different and identifiable sizes. Stutz and Noll (1967) were the first to distinguish cytoplasmic and chloroplastic rRNAs on the basis of their sedimentation rates in sucrose density gradients. With the subsequent development of PAGE, Loening and Ingle (1967) provided further and more elegant evidence of these different rRNAs. In further studies, rRNAs of many green and non-green plant tissues were examined (Ingle et al., 1970; 1971). Their heterogeneity in RNA content was also found to be reflected in the proteins associated with the ribosomal complex which were not only dependent on ribosome size but varied from species to species (Gualerzi and Cammarano, 1970).

Methods are therefore presently available to analyse the ribosome, both intact and reduced to its constituent parts. Their relationship to the developmental process can now be assessed quite profitably.

Whereas the ribosome was found to be extractable by relatively simple methods, since it is a discrete particle with a sedimentation coefficient quite different from other cell organelles and particles, polyribosome extraction required more care. These aggregates of ribosomes are visualized as linked by mRNA (Wettstein et al., 1963). The exposed linking mRNA is very susceptible to shearing forces and RNAse attack (Thomas, 1976b).

Plants present particularly difficult material for polyribosome extraction because of their rigid cell walls, presence of fibres and high levels of endogenous RNAse (Lyndon, 1966; Weeks and Marcus, 1969). Early polyribosome preparations from plant tissue failed to yield polymers greater that tetramers (Pearson and Wareing, 1970) but more recently reports of decamers (Anderson and Key, 1971) and even dodecamers (Davies et al., 1972; Evans et al., 1979; Saadi, 1978 personal communication) have been made. With establishment of methods thought to maintain the polyribosomes in their in vivo configuration (for example, by comparison with electron micrograph studies of polyribosomes in situ (Eilam et al., 1971; Schiff, 1974) ) comparisons based on polymer size ratios can be made of the protein synthetic capabilities at any one time in development (Beachy et al., 1978).

Although mRNA was thought to have been isolated by Ishihama et al. in 1962, its heterologous molecular weight presents particular problems in its purification (Grierson and Loening, 1974). The realisation that at least some of the mRNA possessed 'Poly-(A) tails' (Matthews, 1973; Brawerman, 1974; Sagher et al., 1974) provided an elegant basis for one step isolation by affinity chromatography. More recently, messenger ribonucleoprotein has been removed from polyribosome preparations essentially by heat shock, thus allowing isolation of all mRNAs in the process of being translated whether poly-(A)-containing or not (Liautard

and Liautard, 1977). Once these methods were developed, closer investigation of the qualitative and quantitative properties of certain mRNA could proceed, with particular reference to development.

Until development of all the above techniques, nucleic acid measurements had been on a gross scale (for example: Smillie and Krotov, 1961; Key, 1964; Weidner and Mohr, 1967). These provided limited information concerning the real roles of nucleic acids in developmental control, particularly in the light of evidence of long-lived mRNA (Jachymczyk et al., 1974; Payne, 1976; Taneja and Sachar, 1976), inactive ribosomes (Kabat and Rich, 1969; Wareing and Phillips, 1970; Beevers and Poulson, 1972) and preferential synthesis or loss of different tRNA species (Kaneko and Doi, 1966; Strehler, 1967; Bick et al., 1970; Burkard et al., 1970; Sacher, 1973; Wright et al., 1973).

i. Early events.

Several reports suggest that RNA synthesis is not required for the progression of the early stages of seed germination (Ihle and Dure, 1969; Walbot, 1972; Stoddart et al., 1973). Germination in rice has been shown to be independent of RNA synthesis 18-24 h. after the start of imbibition although synthesis of RNA could be detected 9 h. after the start of imbibition (Bhat and Padayatty, 1974). Wheat embryos show formation of mature ribosomes within 6 h. of imbibition (Dobrzanska et al., 1973). Ribosomal proteins are synthesized within 12 h. of the start of imbibition in rice (Bhat and Padayatty, 1975) and other protein syntheses are detectable within a few hours of imbibition (Wareing and Phillips, 1970). All these observations require that ribosomes and mRNA were stored in the seed and made available immediately on imbibition. There have been a number of reports of ribosome presence in seeds (Weeks and Marcus, 1971; Schultz et al., 1972; Spiegel and Marcus, 1975; Osborne et al., 1977) but Cherry (1967) and Marcus and Feeley (1965) found them to be non-functional.

Osborne et al.(1977) and Bray and Chow (1976), however, related the viability of rye and pea seeds, respectively, to the degree of fragmentation of the rRNAs in the dry seed. There have also been a wealth of observations of mRNA in the dormant seed (Barker et al., 1971; Bhat and Padayatty, 1974; Jachymczyk et al., 1974; Grierson and Covey, 1975; Payne, 1976; Osborne et al., 1977; Peumans et al., 1978). Such stored mRNA may be spatially separated from the translational components (Giles et al., 1977) or it may be in an inactive form such as ribonucleoprotein (Spirin, 1969; Weeks and Marcus, 1971; Jachymczyk et al., 1971, 1974) or stabilized by the possession of poly-(A) tails (Huez et al., 1974; Osborne et al., 1977). Some of these stored mRNAs have been shown to be translated in vitro (Gordon and Payne, 1976; Peumans and Carlier, 1977; Osborne et al., 1977).

Significant increases in nucleic acid synthesis are not really apparent in the seeds of most plants until 24 h. after imbibition. All classes of RNA are synthesized in this phase which is associated with cell division (Grierson et al., 1976).

The order of RNA synthesis, and its significance, following the use of stored protein-synthesizing apparatus (i.e. in the first 8 - 12 h. (Stoddart et al., 1973)) has been much in debate. Bhat and Padayatty (1975) reported tRNA synthesis within 6 h. of imbibition of rice seeds (but they admitted that the incorporation of radioactive label could have been due to turnover of their exposed CCA ends.). rRNA synthesis followed between 6 and 12 h. and mRNA synthesis between 12 and 24 h. Dobrzanska et al., (1973) showed mRNA to be the first synthesized class of RNA in wheat germination if the seeds were pre-soaked. Where seeds were not pre-soaked rRNA synthesis was found to be the earliest transcriptional event (Chen et al., 1971). Work on higher plants is fragmentary but other systems such as early development of the fungal spore have been closely examined. Tanaka et al. (1966) observed a rRNA - tRNA - mRNA synthetic sequence within 1 h. of Aspergillus oryzae conidia germination, whereas Roheim et al. (1974) found that all classes of RNA were synthesized within 15 min. of germination of spores of Rhizopus stolonifer. On the basis of Greenberg and Penman's (1966) calculation for the time

required for transcription and processing of rRNA leading to functional ribosome production in HeLa cells, 15 min. is inadequate to allow for de novo synthesis and assembly of ribosomes. Roheim et al. must rather have been observing post-transcriptional modification. There are reports which suggest that poly-(A) containing RNA is synthesized immediately upon imbibition (Ajtkhozhin et al., 1973; Spiegel et al., 1975; Doshchanov et al., 1975). Caers et al. (1979) suggest from their work with germinating wheat embryos that there is no preferential employment of pre-formed or newly synthesized mRNA as templates for polypeptide synthesis and there are no qualitative differences in the polypeptide products. However, these observations hinge on the belief that the inhibitor of mRNA synthesis used, cordycepin, has no other in vivo effect and that radio-labelling is not merely a result of post-transcriptional modification. Spiegel and Marcus (1975) found that cordycepin had no measurable effect on polyribosome formation in wheat embryos during the first 40 min. of germination, indicating the minor importance of newly synthesized mRNA within this period.

Probably the precise ordering of RNA synthesis is species-specific and is, to some extent, affected by environmental conditions. Presumably the information available for translation by stored RNAs of the seed is sufficient to allow germination under favourable conditions but further growth requires feedback of environmental conditions on which the translational and transcriptional controls can react. Doubtless the sequences of RNA syntheses are significant but since all classes are synthesized in large quantities within 24 h. and all classes are present until this time, it is more likely that their relative proportions are of greater importance in controlling development than their sequential synthesis.

Analysis of the stages following germination is complicated by the development of different organs, their inherent developmental patterns and the inter-organ relationships necessarily invoked. By the use of autoradiography, Masuda et al. (1966) found that the bulk of RNA was formed prior

to cell expansion in oat coleoptiles. Tester (1977), on the other hand, demonstrated an increase in synthesis of RNA in the oat coleoptile during expansion which declined with maturity. At this point there is a shift of developmental emphasis on to the emerging primary leaves which show a 400% increase in DNA and tRNA and a 320% increase in rRNA. 82 h. after germination the developmental patterns of these two tissues, as evidenced by their nucleic acid complements, are quite different. RNA/DNA ratios have frequently been considered valuable in comparison of different regions of differentiation since the level of DNA per cell should remain constant (Brown and Naylor, 1965; Gyldenholm, 1968). Woodstock and Skoog (1960) found a decrease in this ratio over two days in corn root tips. Whereas, Brown and Naylor (1965) found in Mimosa root tips an increase in the ratio followed by a decrease after six days but, during the corresponding period, there was a steady increase in the epicotyls. Likewise, cotyledons, whether hypogeal or epigeal, showed the classic symptoms of senescence in their loss of protein and RNA following full expansion (Payne and Boulter, 1974) and this can be considered consistent with redeployment of metabolic precursors in actively growing regions.

ii. Effect of light.

The illumination of dark grown aerial tissue has frequently been shown to elicit an increase in total RNA (Bogorad, 1967; Filner and Klein, 1968; Gyldenholm, 1968; Poulson and Beevers, 1970; Harel and Bogorad, 1973; Tester, 1977). There are, however, isolated reports of light-induced inhibition of RNA synthesis (for example, Mathew et al., 1976). Grierson and Covey (1975) demonstrated a tenfold increase in RNA accumulation in leaves of Phaseolus aureus occurring between 48 and 120 h. after the onset of illumination. 70% of this was rRNA. However, the dark grown seedling also accumulates RNA to the same extent but this accumulation appears to lag behind light-treated seedlings by approximately 24 h. (Grierson et al., 1970). Alternatively, the period of processing may be longer in the dark (Ingle, 1968b). Care must be exercised in the interpretation of results of radioisotope-labelling of RNA

since an increase in labelling may rather reflect an increased uptake of label (causing a higher specific activity of nucleotide pool) rather than increased synthesis (Woolhouse, 1967; Grierson, 1972). Pulse-labelling of RNA has revealed the occurrence of high molecular weight precursors of rRNA which are processed via several intermediates over several hours (Greenberg and Penman, 1966; Rogers et al., 1970; Leaver and Key, 1970; Grierson et al., 1970, 1976; Grierson and Loening, 1972, 1974; Hartley and Ellis, 1973). The ratio of radioactively-labelled precursor RNA to the radioactively-labelled product, i.e., mature RNA species, provides an estimate of RNA processing independent of uptake (Grierson et al., 1976). Experiments have indicated that larger amounts of precursor RNA are converted into rRNA within 2 or 3 h. of illumination compared with dark controls.

The increase in RNA following exposure to light has been attributed to a massive net increase in chloroplast rRNA (Ingle, 1968 b; Bogorad, 1968; Treharne et al., 1970; Paranjothy and Wareing, 1971; Harel and Bogorad, 1973; Mikulovich, 1978) with turnover but no increase in cytoplasmic rRNA (Dyer et al., 1971; Cohen and Schiff, 1976). Some reports suggest that the dark grown plant material contains no plastid rRNA (Pollack and Davies, 1970; Heizmann,1970; Scott et al., 1971; Brown and Haselkorn, 1971). However, this seems unlikely in view of the biochemical (for example, Granick, 1959) and ultrastructural (Jacobson et al., 1963) changes apparent during early development of the proplastid which must require protein synthesis, not all of which could be processed by cytoplasmic ribosomes (Smith, 1970; Siddell and Ellis, 1975; Leech, 1976; Chelm et al., 1977). Plastid rRNA has been identified in dark grown tissue by sucrose density gradient analysis (Boardman, 1967), PAGE (Ingle, 1968 a; Smith, 1970), electron microscopy (Schiff, 1974; Jacobson et al., 1963) and hybridization with plastid DNA (Chelm et al., 1977); furthermore its synthesis has been shown to occur at this time (Wollgiehn and Parthier, 1977). All these observations evaluated the etioplast rRNA contribution to total RNA as about 2% (W/W). This rises to between 20% and 30% (W/W) between 48 - 120 h. after light treatment (Rhodes and Yemm, 1963; Parthier and

Wollgiehn, 1966; Gyldenholm, 1968; Grierson and Covey, 1975; Cohen and Schiff, 1976; Chelm et al., 1977) lagging behind maximal cytoplasmic rRNA synthesis by between 12 and 48 h. (Ingle, 1968 a; Poulson and Beevers, 1970; Grierson et al., 1970; Cohen and Schiff, 1976). Very little polycistronic precursor RNA ($2.9x10^6$, Grierson and Loening, 1974; Hartley and Ellis, 1973) is synthesized in dark grown seedlings but those grown in the light show enhanced synthesis which is indicative of the increase in chloroplast rRNA which follows (Grierson et al., 1976). Synthesis of chloroplast RNA seems to be restricted to a comparatively short period in development and the subsequent turnover is very low (Ingle, 1968 a). Although Actinomycin D and a number of other nucleic acid synthesis inhibitors have been shown to prevent normal greening (Bogorad and Jacobson, 1964; Beridze et al., 1966), increased chloroplast RNA synthesis does not appear to be a pre-requisite for all aspects of further chloroplast development; for example, chlorophyll production continues when chloroplast RNA synthesis is blocked by rifampin (Bogorad and Woodcock, 1971). Furthermore, Poulson and Beevers (1970) have shown that chlorophyll production occurs during the first 16 h. of illumination of barley leaves when there is no increased chloroplast RNA synthesis.

An increase in the percentage of ribosomes occurring as polyribosomes, following illumination, has been reported by Clark et al. (1964); Williams and Novelli (1968); Pearson and Wareing (1970); Travis et al. (1970) and Poulson and Beevers (1970). This may partly be due to assembly of existing free monosomes. Smith (1976) has shown that RNA synthesis is not required within the first four hours of light-induced polyribosome level increase. The rapid polyribosome formation (40 min.) in chloroplasts of Chinese cabbage (Clark et al., 1964) is also consistent with an "assembly" rather than a "synthesis" interpretation. However, Harel and Bogorad (1973) have shown a preferential labelling of chloroplast rRNA and subsequently polyribosomal RNA within 2 h. of light treatment. Work with inhibitors of RNA and protein

syntheses has confirmed that these syntheses are required for prolonged polyribosome increase (Smith, 1976). In fact, newly labelled rRNA seems to be preferentially associated with polyribosomes as opposed to monosomes (Cohen and Schiff, 1976) suggesting that newly synthesised rRNA is immediately involved in protein production. However, light is again regarded as hastening rather than inducing the processing of rRNA. Additionally, Williams and Novelli (1964) found ribosomes of maize leaves to be more active than dark grown leaves in their capacity to incorporate amino acids into polypeptides 2 h. after the start of light treatment, suggesting that light affects some intrinsic property of the ribosomes. Travis et al. (1972) showed that light lowered the magnesium requirement for polyribosome-directed cell-free polypeptide synthesis and experiments using puromycin suggested that light enhances the occupation of ribosomal p-sites by peptidyl tRNAs.

The protein synthetic capacity, as judged by in vitro analysis of chloroplastic and cytoplasmic ribosomes from young leaves of Perilla, showed the former to be twice as active as the latter (Callow et al., 1972). Boardman et al. (1966) also noted a comparatively low rate for cytoplasmic ribosomes from tobacco leaves.

Poly(A)-containing mRNA, like other classes of RNA, seems to be accumulated more rapidly in light-treated leaves than in dark controls but with no net synthetic increase (Giles et al., 1977). Furthermore, poly(A)-containing mRNAs for all light-induced polypeptides are present in dark grown tissue, though not associated with polyribosomes. In fact, Grierson et al., (1976) suggest that dark grown seedlings of Phaseolus aureus actually contain more cytoplasmic rRNA and poly(A)-containing mRNA than light grown leaves if the amounts are expressed on a per cell basis. It must be remembered, however, that there are substantial quantities of poly(A)-minus RNA (up to 50% (W/W): Grierson et al., 1976) which have not been accounted for in these reports. Unlike the conclusions of Harel and Bogorad (1973) it seems that light mobilizes mRNA from an inactive form to a translatable form. Grierson et al. (1976) concluded that light

either modifies the processing or synthesis of RNA or affects its metabolic stability.

There is a gradual decline in both cytoplasmic and chloroplastic rRNA following their attainment of maximum accumulation. This decline is associated with completion of cell division (Grierson et al., 1976). Poly(A)-containing RNA declines rapidly at this stage (Grierson and Covey, 1975) which may be due to degradation or merely the loss of poly(A)-tails (Grierson et al., 1976).

Despite the subtleties of sequential occurrence of various classes of RNA and their precursors at the early stage of development which have now been reported, it is still of value to investigate the relative accumulation of these classes particularly by radioisotope labelling, providing due consideration of nucleotide pool sizes is taken.

iii. Phase of maturity.

The intermediate phase following germination and greening but preceding senescence has been largely ignored by the research worker. Not only is it a phase which is difficult to define but it does not have an experimental equivalent which is easily manipulated as the early and late phases have in the "greening up" and "excision" experimental equivalents (see Chapter 5.). The little information that is available generally involves protein rather than nucleic acid estimations (for example, Hedley and Stoddart, 1971b) which need not necessarily be closely related in this developmental period. The phase has been regarded as one of moderate turnover of metabolites with overall synthesis equalling degradation (Thomas, 1976b). Beyond the stage of full leaf expansion, chloroplast rRNA appears to be made at a very low rate or not at all (Ingle, 1968a; Treharne et al., 1970; Paranjothy and Wareing, 1971; Callow et al., 1972). The protein synthetic capacity in chloroplasis of Perilla decreased at full leaf expansion whereas the activity of cytoplasmic ribosomes expressed per unit area of leaf was greatest at this time (Callow et al., 1972). At variance with this, Boardman et al. (1966) showed that chloroplast

ribosomes isolated from mature tissue were more efficient at protein synthesis than cytoplasmic ribosomes. However, the observation, by Mans and Novelli (1961), that cytoplasmic ribosome activity could be improved by washing the ribosomal preparations in sodium deoxycholate may point to the need to remove ribosomal-bound nucleases which otherwise interfere with template activity. Boardman et al. (1966) also inferred that some inhibitory factor was involved which could be removed by buffer washes.

iv. Senescence.

As already outlined in Chapter 1, foliar senescence can result from a number of causes which may superficially appear to elicit similar cellular processes but which, in fact, may have quite different modes of implementation (Lewington and Simon, 1968; Wareing and Phillips, 1970). In order to isolate the plant's own control applied to senescence from external interference (by environmental (e.g., by hormones) or secondary factors (e.g., "wounding" response on excision)) the biochemistry of the intact leaf must be investigated where possible. The role of nucleic acids under these conditions is of particular interest. Are they central to the implementation of this developmental phase as in earlier phases or is the burden of control now on cytoplasmic events such as protein synthesis and enzyme activation?

There is much evidence to suggest that the overall cellular content of RNA and DNA declines gradually following full expansion of the leaf (Lindner et al., 1956; Bottger and Wollgiehn, 1958; Smillie and Krotkov, 1961; Kessler and Engelberg, 1962; Hardwick et al., 1968; Woolhouse, 1967; Callow, 1971). However, a slight rise prior to a final rapid decline has been reported (Woolhouse, 1967). Generally, the cytoplasmic and chloroplastic rRNA losses occur at a similar rate (Dyer and Osborne, 1971) but in those species which do not show re-greening (e.g., Xanthium) the chloroplast rRNA declines much faster than the cytoplasmic rRNA and may provide the reason for their inability to re-green.

Although Srivastava (1972) failed to find any difference in RNA synthesized by old and young barley leaves by the technique of hybridization of DNA and RNA, some information on the changes in particular RNA fractions during senescence is available; for example, the $1.1x10^6$ chloroplast rRNA tends to be more labile with age (Ingle, 1968a;Callow et al., 1972) as does the $0.7x10^6$ component of cytoplasmic ribosomes (Dyer and Osborne, 1971). (However, the lability of the $1.1x10^6$ rRNA of Chinese cabbage is reduced by extraction in DEP and is apparent even in the senescence (Strangeway, 1977)). Synthesis of $0.56x10^6$ rRNA apparently continues beyond that of $1.1x10^6$ rRNA in Perilla (Callow et al., 1972). This is at variance with the speculation that both subunits are derived from the same polycistronic precursor (Grierson et al., 1976).

The declines in cytoplasmic ribosome populations (Shaw and Manocha,1965; Barton, 1966; Butler, 1967; Srivastava and Atkin, 1968) and chloroplastic ribosome populations (Mittleheuser and Van Steveninck, 1970; Eilam et al., 1971) as observed by electron microscopy are both regarded as early symptoms of senescence. In Perilla the quantity of polyribosomes extractable from chloroplasts and the proportion of polyribosomes to monoribosomes decreases with age more rapidly than the quantity and proportion of polyribosomes from cytoplasmic origins (Callow et al., 1972). "Free" ribosomes are lost before those which are "membrane bound" (Butler and Simon, 1970; Eilam et al., 1971). If these two populations of polyribosome differ in the polypeptide product made (see Chapter 2, part IX g.) then their differential loss may be quite significant in the control of senescence.

The capacity of polyribosomes for directing protein synthesis as demonstrated in in vitro studies is reduced more rapidly in those derived from chloroplasts than from the cytoplasm of Perilla by 80% and 50% respectively, over the same period of time (Callow et al., 1972).

A rapidly labelled RNA fraction associated with polyribosomes showed considerable reduction in its synthesis (Wollgiehn, 1967). If this represents mRNA this observation is indicative of the reduction in its synthesis with ageing.

De novo synthesis of proteins, particularly of hydrolytic enzymes, is required for the normal process of senescence (McHale and Dove, 1968; Udvardy et al., 1969; Martin and Thimann, 1972) and this is supported by the fact that inhibitors of protein synthesis tend to inhibit the senescence phenomenon (Thomas, 1975). This implies the need for functional ribosomes but does not necessitate RNA synthesis. However, Wollgiehn and Parthier (1964) reported that inhibitors of RNA synthesis accelerated senescence. Wollgiehn (1967) reported significant synthesis of RNA in tobacco leaf discs six days after excision as evidenced by incorporation of radioactively labelled adenine. Callow et al. (1972) demonstrated synthesis of cytoplasmic, but not chlorplastic, rRNA following full expansion of the leaf and well into senescence. This RNA labelling, as suggested to account for the early RNA synthesis apparent at germination, may also represent post-transcriptional modification of pre-functional RNA. One of the reactions to treatment with ABA, which promotes senescence, is inhibition of RNA synthesis (Villiers, 1968; Beevers et al., 1970; Paranjothy and Wareing, 1971) though the relationship of these is not established. Whether RNA synthesis occurs or not, and the work with Actinomycin D carried out by Thomas (1975) would suggest that if it does occur it does not affect the course of senescence, retention of functional ribosomes is essential. The ability of most leaves, in quite late stages of senescence, to re-green on decapitation of the plant (Wollgiehn, 1967) or in response to light (Takegami, 1975) or kinetin (Richmond and Lang, 1957) treatments, all of which seem to involve increases in RNA and protein and are prevented by inhibitors of RNA and protein syntheses, must require that both the transcriptional and translational capabilities of the cell have remained unimpaired. In fact, Eilam et al., (1971) noted an increase in the proportion of polyribosomes at incipient senescence which indicates that protein synthesis can occur at a very late stage in senescence. Polyadenylation of mRNA appears to be required for the normal progress of senescence as experiments using cordycepin suggest (Takegami and Yoshida, 1975) but this need not require transcriptional events.

## 2. Methods.

### i. Aims in investigating the RNA complement and its relationship to development in Festuca pratensis.

It was decided to follow RNA synthesis by radioisotope labelling in both developmental systems in Festuca pratensis, i.e., sequential ageing along the leaf and temporal whole leaf ageing. Most fractions, except precursoral ones (labelling time was too long for observation of these), were examined in an attempt to map their synthetie relationship and possible roles in control. It was also important to establish a link between these two developmental systems. Hedley and Stoddart (1972b) established the relationship between protein synthesis and enzyme activities in Lolium temulentum, but could this be shown in nucleic acid content and synthesis in F. pratensis?

### ii. Extraction of RNA.

Since over 80% (W/W) of cellular RNA is intimately associated with protein, mainly in the form of ribosomes, the strategy for its purification must involve effective deproteinization, particularly with regard to inhibition of the ubiquitous endogenous and exogenous RNAses. The remaining uncomplexed RNA is mostly low molecular weight soluble tRNA which, by virtue of these characteristics, is less difficult to extract (Hoagland et al., 1958) but, by the same token, is easily lost due to its high solubility. Less than 2% (W/W) of the cellular RNA is mRNA of heterogenous molecular weight and therefore difficult to isolate and identify.

#### a. Phenol methods.

These methods, based on those developed by Kirby (1965;1968), include 8-hydroxyquinoline which prevents oxidation of phenol, has some inhibitory effect on RNAse (Kirby, 1962) and may also be

valuable in removing metal ions involved in binding RNA to protein (Barlow and Mathias, 1966). Tri-isopropylnaphthalene sulphonate also acts as an RNAse inhibitor and sodium chloride is added to reduce the solubility of phenol in water and to precipitate solubilized protein.

Phenol method used.

This method was adapted from that described by Leaver and Ingle (1971) from the original technique of Kirby (1965).

Chopped leaf tissue was homogenized in a pestle and mortar following freezing with liquid nitrogen. Equal volumes of "detergent mix" and "phenol mix", amounting to approximately five times the volume of leaf tissue, were added sequentially and were thoroughly mixed.

"detergent mix."

| | |
|---|---|
| 10g./l. | tri-isopropylnaphthalene sulphonate |
| 60g./l. | p-aminosalicylate (sodium salt) |
| 50mM. | sodium chloride |
| 10mM. | magnesium chloride |
| 10mM. | Tris-HCl pH 7.4 |
| 3% (V/V) | "phenol mix" (to dissolve p-aminosalicylate) |

"phenol mix."

| | |
|---|---|
| Re-distilled phenol | |
| 10% (V/V) | m-cresol |
| 0.1% (V/V) | 8-hydroxyquinoline |
| saturated with 10mM tris-HCl | pH 7.4 |

The homogenate was centrifuged at 2,500g in a MSE Minor bench centrifuge for 10 min. at room temperature. The lower phenol layer was discarded. Sodium chloride was added to the aqueous layer and interphase material to a final concentration of 0.5M, and thoroughly mixed. A second equal volume of phenol was added, mixed thoroughly and then separated by centrifugation as before. The aqueous phase was retained and the nucleic acids precipitated from it by the addition of three volumes of ice cold absolute alcohol, followed by overnight storage at -20°C. The precipitated nucleic acids were collected by centrifugation at 2,500g at room

temperature followed by at least three washes with 70% (V/V) ethanol; the first containing 2g./l. SDS. The final pellet was either resuspended in a small volume of appropriate buffer and used immediately or stored under 70% (V/V) ethanol at -20°C.

Although operations were not carried out in a cold room the RNA extracts were kept on ice between centrifugations and the homogenization in liquid nitrogen contributed to maintenance of low temperatures throughout.

b. Limitations.

The high U.V. absorption of phenol interferes with spectrophotometric estimation of the nucleic acids and effective washing reduces the yield. RNA may be lost in precipitates of denatured protein (Poulson, 1973). Although phenol denatures many proteins it does not completely inactivate nucleases (Littauer and Sela, 1962; Kidson et al., 1963; Rushizky et al., 1963). Most rRNA and tRNA will be extracted by this method but some mRNA may be retained at the organic/aqueous interface (Kidson and Kirby, 1964; Perry et al., 1972; see Section XII.). Phenol extraction can cause aggregation as well as loss of RNA (Sedat and Sins-heimer, 1970; Schechter, 1973). Furthermore, phenol extraction may result in contamination with pectic substances which co-migrate with RNA in PAGE and hence interfere with quantitation (Leaver and Ingle, 1971).

c. DEP methods.

The observation that DEP acts by causing irreversible structural modification in proteins (Rosén and Fedorcsàk, 1966; Wolf et al., 1970) prompted its use as a powerful enzyme inhibitor and use in nucleic acid extraction (Fedorcsàk and Ehrenberg, 1966). The method compared favourably with the phenol methods in nearly all cases in RNA extractions from plant material (Solymosy et al., 1968; 1970; Lazar et al., 1969).

DEP method used.

The method of Solymosy et al. (1970) as modified by Strangeway (1977) was used with minor alterations.

The tissue was ground in a pestle and mortar following freezing in liquid nitrogen with a total of 13.6 ml. "extraction medium" per g. fresh weight of tissue.

"extraction medium"

| | |
|---|---|
| 2.9% (V/V) | DEP (stored at 4°C.) |
| 30 mM | SDS (stored as 100g./l. stock at 4°C.) |
| 100 mM | Tris-acetate pH 7.2 |
| 53 mM | sodium acetate |
| either 2.6 mM | EDTA (Disodium salt) |
| or 8.8 mM | magnesium acetate |

DEP is unstable in aqueous solution and so was added separately, as was the SDS.

In early experiments incubation at 37°C. for 5 min. was performed followed by centrifugation at 2,500g in a MSE Minor bench centrifuge. 10% (W/V) sodium chloride was added to the decanted supernatant and the mixture was incubated at 37°C. for a further 5 min. The incubation steps were later dropped as they provided no significant increase in yield, providing the mixing with sodium chloride was thorough. The extract was centrifuged at 10,000g for 20 min. at 4°C. in a MSE 18 centrifuge.

Three volumes of ice cold absolute ethanol were added to the supernatant followed by storage at -20°C. overnight. The precipitated nucleic acids were collected by centrifugation and resuspended in a small volume of appropriate buffer without further purification.

d. Limitations.

Although DEP extraction has been shown to give RNA products which retain template activity (Fedorcsak et al., 1969), transfer activity (Abadom and Elson, 1970; Fedorcsak et al., 1969) and infectivity (Oxelfelt and Arstrand, 1970) and DNA products which retain their infectivity (phage DNA, Kondorosi et al., 1972) and transforming ability (bacterial DNA, Fedorcsak and Turtoczky, 1966) nevertheless there has been criticism levelled at the use of DEP based on various reported detrimental effects. Solymosy et al. (1970) and Oxelfelt and Arstrand (1970) observed inactivation of

purified TMV-RNA. Denic et al. (1970) and Ortwerth (1971) noted loss of tRNA acceptor activity. Oberg (1970) reported inactivation of single-stranded poliovirus but not of the double stranded replicative form (Oberg, 1971). This latter observation pointed to some form of complexing of the nucleic acid with DEP and further reports suggest that DEP interacts with adenine molecules (Leonard et al., 1970). However, for rRNA extraction procedures none of these criticisms seem valid particularly in view of the consistent and high yields even with small quantities of plant material.

e. Spectrophotometric estimation of RNA.

The concentration of RNA resuspended in buffer was estimated from the U.V. absorption spectrum of samples diluted appropriately in distilled water, assuming the following relationship:

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

(Tester and Dure, 1966)

Measurements were made using a Perkin-Elmer 402 or a Pye-Unicam SP 800 spectrophotometer employing a distilled water blank.

f. Comparison of phenol and DEP methods.

Comparisons of overall nucleic acid yield and behaviour under PAGE (section iii) were made following phenol or DEP extraction from F. pratensis leaves (Table 2.1; Figure 2.1). These show that DEP extraction increases the yield without affecting the electrophoretic behaviour of the rRNAs. Table 2.2 suggests that there is loss in purity as a result of DEP extraction.

Phenol extraction results in an $E_{max}$ of 260 nm and an $E_{min}$ of 232 nm whereas for DEP extractions, whilst the $E_{max}$ remains the same, the $E_{min}$ is shifted to 237 nm. Such a shift has been noted by Strangeway (1977) and Solymosy et al. (1972) and has been attributed to carboxymethylation of the nucleic acid bases by the latter, although this might also arise from protein contamination.

TABLE 2.1

Comparison of the yield of nucleic acids from leaves of F. pratensis following extraction with phenol or DEP. The results are expressed as µg. nucleic acid yielded per g. fresh weight of tissue.

FIGURE 2.1

Comparison of the spectrophotometric scans at 265 nM. of PAGE gels of phenol (——) and DEP (-------) extracted nucleic acid. Approximately 15 µg. nucleic acid were electrophoresed in each case for 3 h. at room temperature at 50 V on 2.6% (W/V) acrylamide gels in Loening's 3'E' buffer minus $Mg^{2+}$.

TABLE 2.1

| | $\mu g.g^{-1}$ | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Phenol | 390 | 475 | 242 |
| DEP | 810 | 930 | 725 |
| %increase yield | 52 | 49 | 67 |

FIGURE 2.1

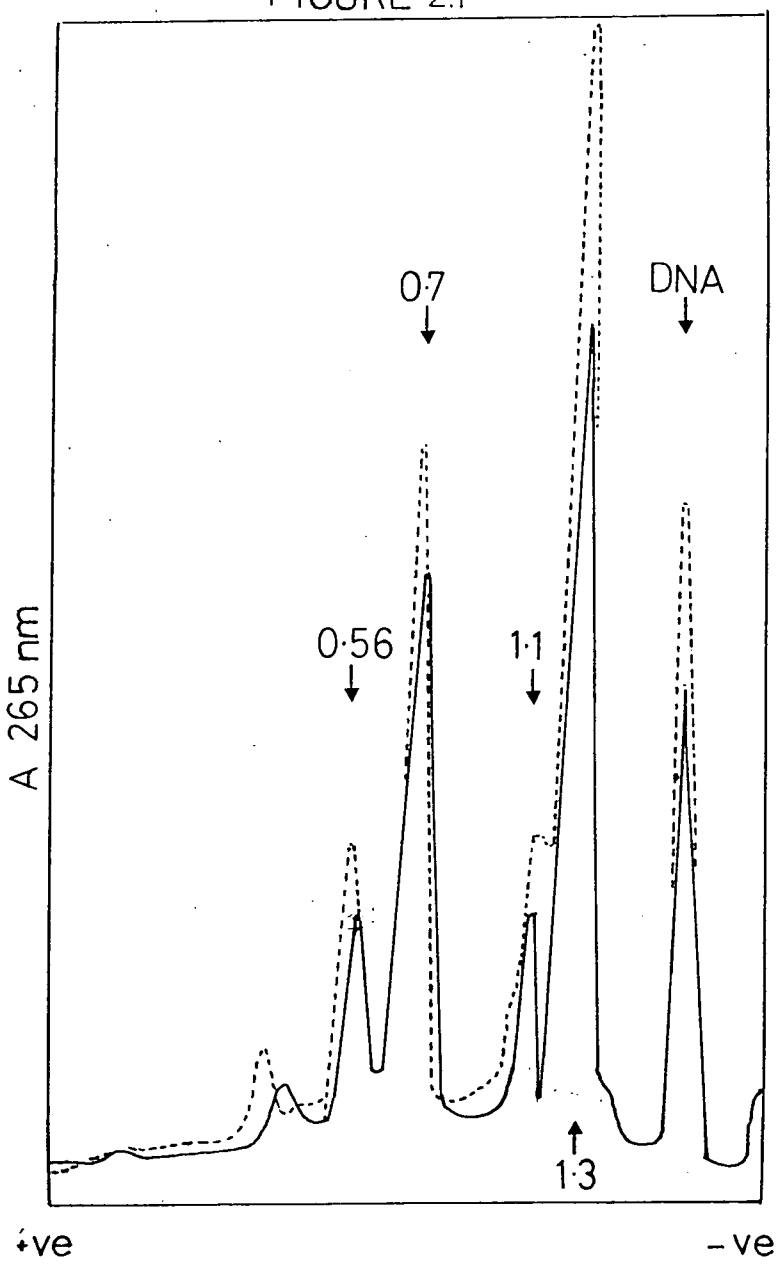


TABLE 2.2

Comparison of purity of nucleic acids prepared by phenol or by DEP methods. The purity is judged by comparison of spectral ratios as indicated.
DEP 2nd. wash values are those achieved following a further ethanolic wash which reduces the yield but improves purity.

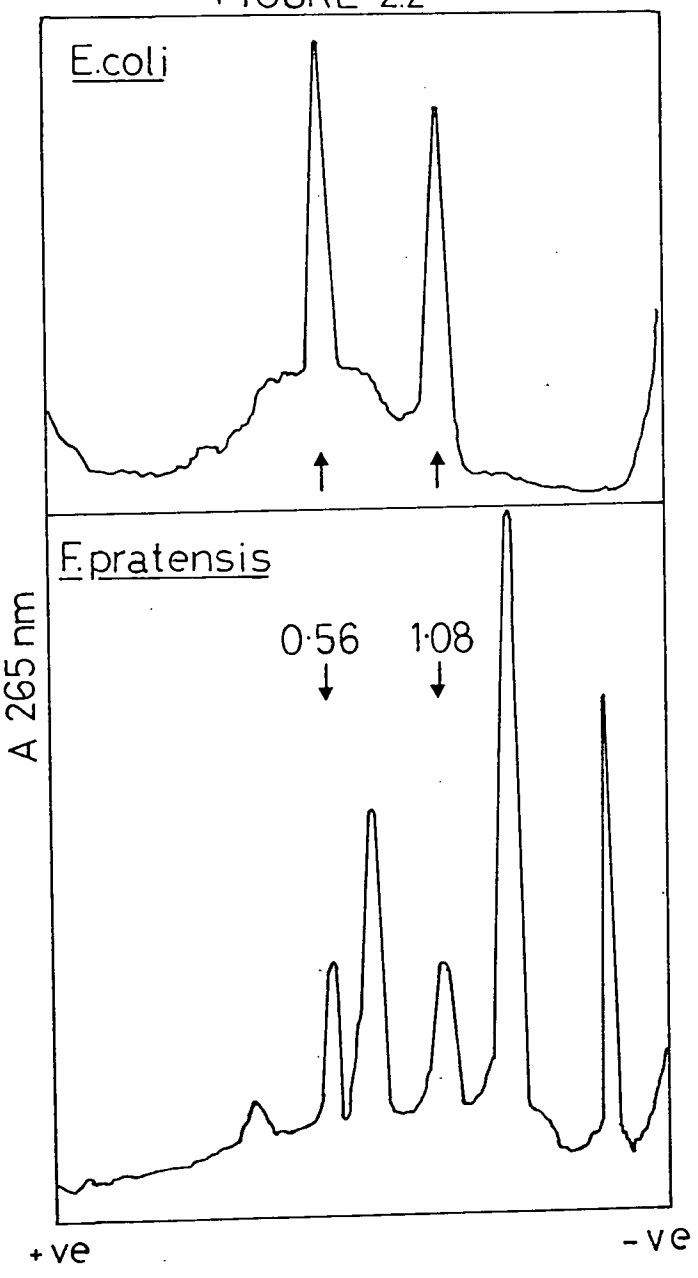
Figure 2.2

Comparison of PAGE of *E. coli* and *F. pratensis* extracted by DEP. Electrophoresis and gel buffers include EDTA.

TABLE 2.2

| | $\frac{E\,max}{E\,min}$ | $\frac{E\,max}{E\,280}$ |
|---|---|---|
| Phenol | 2·04 | 1·87 |
| DEP | 1·63 | 1·72 |
| DEP 2nd wash | 1·93 | 1·89 |

FIGURE 2.2



Where $E_{max}/E_{min}$ and $E_{max}/E_{280}$ ratios approach the value 2.0 ± 0.1 a good degree of RNA purity can be inferred (Peterman, 1964). Table 22shows that $E_{max}/E_{min}$ and $E_{max}/E_{280}$ for DEP-extracted RNA were lower than the corresponding values for phenol-extracted RNA but following further purification by further precipitation with ethanol these values did improve although this caused a reduction in yield. Although this implies that the DEP method suffers from contamination of the RNA extract, the contamination is not such that it interferes with PAGE or spectrophotometric estimation.

Sensu stricto RNA extraction is not improved by DEP use but its use does allow for a reduction in purification steps and therefore an increase in yield.

Using half leaves of Chinese cabbage, Strangeway (1977) showed that the DEP method resulted in an increase in yield of nearly 60% compared with the phenol method, with no observable loss in purity as judged by similar spectral ratios. The losses in the phenol method were attributed to the need for extensive purification steps. Solymosy et al. (1968) reported higher yields of RNA using DEP in extractions from tobacco and bean. Again the lower yields with phenol were attributed to the necessity for cleaning-up steps for U.V. spectrophotometric estimation. Melera et al. (1970) compared these two extraction procedures using Physarum and showed there was no difference in electrophoretic behaviour of the differently extracted rRNAs. Strangeway (1977) showed that all aspects of electrophoretic behaviour of RNA extracted from Chinese cabbage were similar following extraction by either method.

The elevated temperatures used by Strangeway (1977), and originally used here, in the DEP method did not result in high molecular weight aggregates as the criticisms of Lovett and Leaver (1969) suggest.

Not only were DEP extracted RNA yields appreciably (at least 50%) greater than those achieved with phenol extraction, but subsequent resolution in PAGE was markedly improved providing $Mg^{2+}$ ion requirements were observed (see section II, iii c.).

Addition of $Mg^{2+}$ to the phenol extraction medium led to contamination of the RNA with non-nucleic acid material. This interfered with subsequent electrophoresis but resolution was partially restored by inclusion of EDTA at a later stage in extraction (Leaver and Ingle, 1971). However, as fully explained in section II, iii c., this results in loss of integrity of the $1.1 \times 10^6$ RNA. Since inclusion of $Mg^{2+}$ in the DEP extraction procedure does not cause co-precipitation of non-nucleic acid material and requires no further clarification by EDTA addition, the integrity of the $1.1 \times 10^6$ RNA can be maintained with no loss in resolution. The DEP method has the further advantage of being rapid and relatively straightforward.

iii. Polyacrylamide Gel Electrophoresis (PAGE).

Sucrose density gradient analysis of rRNA was the most widely used method until 1967. The results from its use were variable (Baltus and Quertier, 1966; Pollard et al., 1966; Spencer and Whitfield, 1966) partly due to hydrostatic pressure-induced degradation (Infante and Baierlein, 1971). Methylated serum albumin supported on Kieselguhr columns were also used with limited success (Ishihama et al., 1962; Loening and Ingle, 1967).

Loening (1967) modified the PAGE technique previously only used for low molecular weight RNA (Richards et al., 1965) or partial digests (Gould, 1966) to allow separation of high molecular weight RNA from a number of different sources. The superiority of PAGE over previous methods was further established by Bishop et al. (1967), Peacock and Dingman (1967) and Grossbach and Weinstein (1968). Richards et al. (1965) had already established the relationship of migration of low molecular weight RNAs in PAGE as being inversely related to their sedimentation coefficients. Loening and Ingle (1967) showed this to be true of high molecular weight RNAs also. In reports in 1968 and 1969, Loening attributed apparent molecular weights to the resolved rRNAs. Thus cytoplasmic rRNAs have apparent

molecular weights of $1.3 \times 10^6$ (25S) and $0.7 \times 10^6$ (18S) and chloroplastic rRNAs of $1.11 \times 10^6$ (23S) and $0.56 \times 10^6$ (16S) (Mache et al., 1978).

a. PAGE system used.

Gels were prepared essentially by the method of Loening (1967, 1968).

For 2.6% (W/V) acrylamide gels a stock monomer solution containing 150 g./l. acrylamide and 7.5 g./l. bis-acrylamide was made. For 7.5% (W/V) gels the bis-acrylamide concentration was lowered to 3.25 g./l. These monomer stocks were stirred for over 1 h., filtered through Whatman No. 1 filter paper and stored in the dark at room temperature for not more than two months.

Ammonium persulphate (100 g./l.) stock was generally made up fresh immediately prior to use, although occasionally it was stored at $0^{\circ}$C. for a few days.

Electrophoresis buffers were:-

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

| | | |
|---|---|---|
| 120 mM. | tris-acetate | pH 7.2 or 7.8 |
| 60 mM. | sodium acetate | |
| either 3 mM. | EDTA (disodium salt) | |
| or 10 mM. | magnesium acetate | |

(B) '3E' buffer (Loening, 1969)

| | | |
|---|---|---|
| 108 mM. | tris-phosphate | pH 7.6 |
| 90 mM. | sodium dihydrogen phosphate | |
| 3 mM. | EDTA (disodium salt) | |

All these buffers were diluted 2:1 with distilled water and 2 g./l. SDS added prior to electrophoresis.

The desired acrylamide concentrations in the gel were obtained by mixing appropriate proportions of reagents as follows:

| Gel strength (% acrylamide) | 2.6 | 7.5 |
|---|---|---|
| stock monomer (ml.) | 4.33 | 12.50 |
| water (ml.) | 12.11 | 3.94 |
| '3E' buffer | 8.32 | 8.32 |

20 µl. TEMED and 200 µl. ammonium persulphate (stock) were added simultaneously, mixed quickly but gently (so as to prevent undue $O_2$ incorporation which inhibits polymerization).

"Plexiglass" tubes (9 cm. x 0.6 cm. internal diameter) were supported vertically in the electrophoresis tank. The lower ends of the tubes were sealed with pre-soaked dialysis tubing secured by small rubber rings. Closed rubber tubes were fitted over the membranes to form an air seal to prevent the unpolymerized gel passing through the membrane before setting. The gel mix was pipetted gently down the sides of the tubes, so as not to trap air bubbles, to a depth of 8.00 cm. A small amount of distilled water (approx. 100 µl.) was immediately layered over 7.5% gels via a syringe in order to flatten the gel surface.

"Split" gels were made by pipetting 7.5% gel mix to a depth of 5 cm. and flattening the surface with distilled water. After 5 - 10 min. this water layer was removed and the 7.5% portion was overlaid with 3 cm. of 2.6% gel mix, which was allowed to polymerize in the normal way.

The air seals were removed and the tank reservoirs were filled with appropriate buffer so that the tops and bottoms of the tubes were completely covered. Electrophoresis was carried out using a Vokam power pack set on constant current mode, either at room temperature or at 4°C. Gels were pre-run at 6mA/gel for at least 30 min. to remove free acrylamide, ammonium persulphate and other impurities (Poulson and Beevers, 1970). Samples containing between 10 - 30 µg. RNA in varying amounts of buffer (up to 100 µl.) and containing 100 g./l. sucrose were loaded. Electrophoresis was performed at 6 mA/gel for between 2.5 and 4 h.

On completion of electrophoresis the dialysis membrane was removed and the gels were either gently dislodged by pressure from a pipette bulb in the case of low percentage acrylamide gels or by means of a syringe adaptation filled with distilled water for high percentage gels. Particular care was required in handling "split" gels. To ensure removal of these gels intact a compromise between the two methods of gel release was employed.

Gels were generally washed in 7% (V/V) acetic acid for at least 1 h. prior to scanning, in order to remove U.V. absorbing debris, particularly from the top of the gel.

RNA bands were visualised by scanning the gels in a Joyce-Loebl Polyfrac at 265 nm., linked to a Servoscribe chart recorder, or with a Vitatron UPS recording at 254 nm.

Confirmation of band location was achieved by staining with toluidine blue (0.2 g./l.) for at least 1 h. followed by successive washes of distilled water.

b. Molecular weight determination.

Apparent molecular weights were calculated by reference to the electrophoretic behaviour of highly polymerized E. coli RNA of assumed molecular weights of $1.08 \times 10^6$ and $0.56 \times 10^6$ (Loening, 1969; Payne and Loening, 1970). The estimates obtained for molecular weights of RNA from F. pratensis are in good agreement with those obtained for other plant RNA species (Loening, 1968) (figure 2. 3).

c. Some effects of $Mg^{2+}$ and EDTA on PAGE.

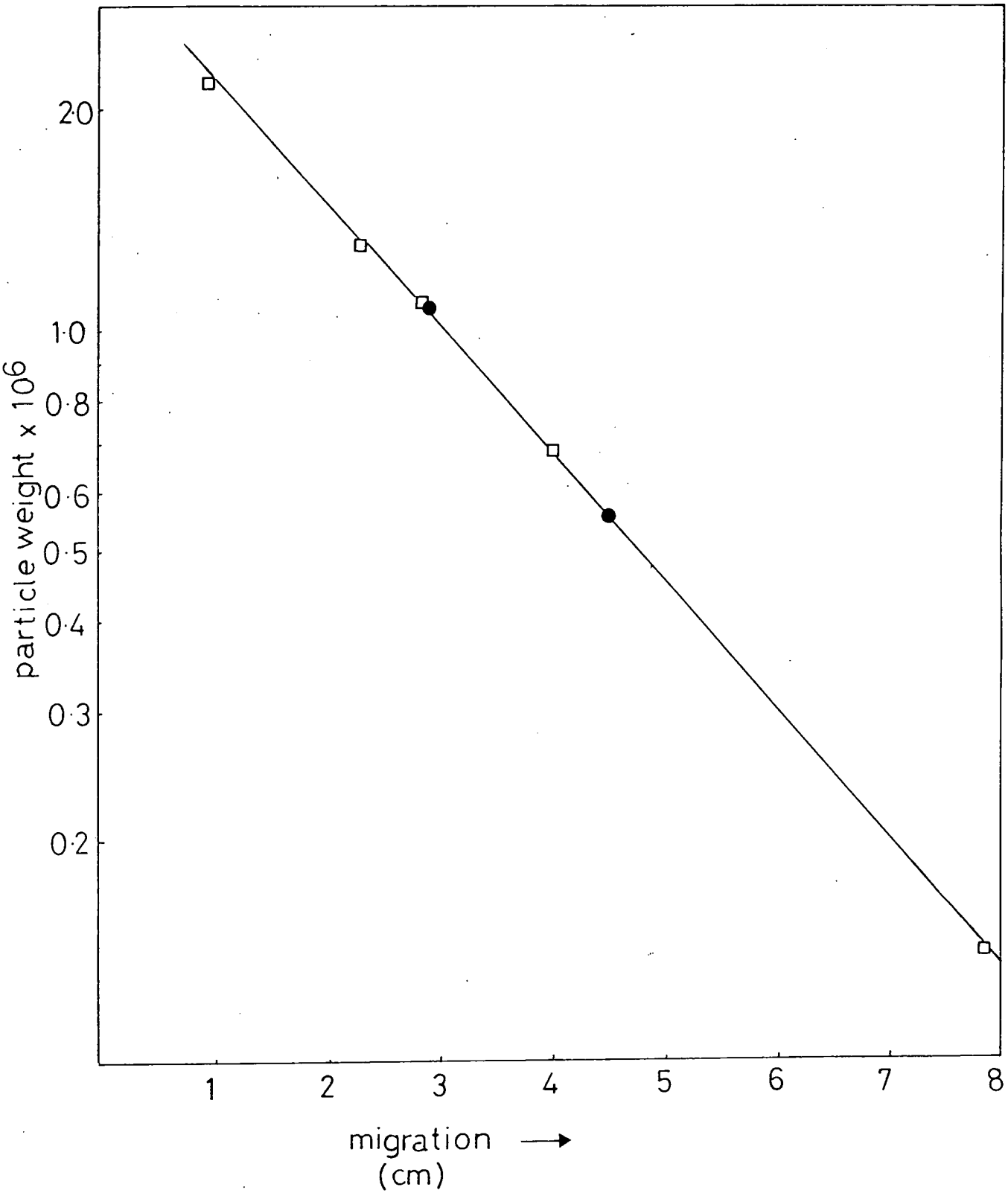
Unlike the ratio of 2:1 of the large and small cytoplasmic rRNA subunits, based on their mass, a similar ratio for chloroplastic rRNAs has been rarely achieved. The ratio, more often than not, approached 1:1 due to depletion of the $1.1 \times 10^6$ rRNA (Ingle and Burns, 1968; Ingle, 1968a; Fraser, 1969; Payne and Loening, 1970; Leaver and Ingle, 1971; Dyer et al., 1971). In fact, Leaver and Ingle (1971) reported that this fraction was absent from Swiss chard, and Spencer and Whitfield (1966) suggested that only one high molecular weight rRNA was present in the chloroplasts of a number of species. Ingle (1968a) found that newly synthesized $1.1 \times 10^6$ rRNA was stable but soon broke down into two fragments. This stability was found to vary with different species and different ages (Ingle et al., 1970; Leaver and Ingle, 1971; Grierson, 1974). This led to the suggestion that RNAse action might cause these fragmentations. Since only one rRNA was affected it was unlikely to be due to random RNAse activity. Ingle (1968a) claimed that the cleavage occurred in vivo and was due to the

FIGURE 2.3

Calibration for determination of molecular weights for electrophoretically separated RNA species. The relationship between mobility and log.(molecular weight) has been demonstrated by Bishop *et al.*, 1967; Loening, 1968; Peacock and Dingman, 1968 .

- ● *E. coli* markers.
- □ Migration of *F. pratensis* RNAs related to their theoretical molecular weights.

FIGURE 2.3


2·0
1·0
0·8
0·6
0·5
0·4
0·3
0·2
particle weight x 10^6
1
2
3
4
5
6
7
8
migration →
(cm)

ribosomal conformation which provided specific areas of exposure for RNAse attack. The inclusion of $Mg^{2+}$ ions from the onset of extraction stabilized these cleavage points but addition following extraction in their absence failed to maintain rRNA integrity. Leaver and Ingle (1971) proposed the presence of "hidden breaks" in the rRNA which were revealed by extraction in EDTA but could be protected by $Mg^{2+}$. Atchison et al. (1973) suggested that rather than the presence of "hidden breaks" there might be "hidden nucleases." They co-extracted RNA from tobacco leaves and E. coli, reasoning that any treatment which only affected the $1.1x10^6$ rRNA from tobacco leaf would be evidence of its inherent instability. Degradation of E. coli RNA did occur when mixed with leaf rRNA but it remained intact if extracted alone. Inclusion of guanidium chloride, a strong dissociating agent, was the only means of "hidden nuclease" removal but resulted in aggregation of RNA which could be reversed by heating at $95^{o}C$. in the presence of EDTA. Dyer and Payne (1974) proposed that there was a RNAse which was latent under extraction conditions in which the integrity of the ribosomes from bean root was maintained. However, disruption caused preferential absorption of the RNAse on to ribosomes and low ionic conditions caused its activation. Perhaps a similar situation exists for chloroplast ribosomes. Bourque et al. (1973) described a much improved extraction of RNA from Jack bean, tobacco and pea leaves using 25 mM $Mg^{2+}$ in the extraction medium. Their results suggested that $Mg^{2+}$ was not required for stabilizing the $1.1x10^6$ rRNA but rather as an inhibitor of RNAse activity since no breakdown was observed in subsequent PAGE in the presence of EDTA and without $Mg^{2+}$ in the cold. This result was quite at variance with the observation of Leaver and Ingle (1971) that EDTA added to $Mg^{2+}$-extractions at a later stage, whilst preventing some of the non-nucleic acid contamination associated with $Mg^{2+}$ inclusion, caused breakdown of the $1.1x10^6$ rRNA. However, electrophoresis in this instance was carried out at room temperature and may have stimulated RNAse activity. Grierson (1974) showed that the absence of $Mg^{2+}$ alone in PAGE was sufficient to reduce the integrity of the $1.1x10^6$ rRNA. It does seem likely that the use of such high $Mg^{2+}$ levels by

Bourque et al. (1973) allowed sufficient co-precipitation with the nucleic acids for subsequent maintainence of the $1.1x10^6$ rRNA in PAGE. $Mg^{2+}$ addition to RNA causes an increase in absorbance at 300 nm. but no increase at 258 nm. The inhibition of RNAse activity could be attributed to a change in secondary structure by an increase in H-bonding which might confer some sort of resistance to RNAse ~~stock~~ (Morrill and Reiss, 1969). However, Atchison et al. (1973) regard such binding of divalent cations as minimal according to their measurements of hypochromicity.

The requirement for EDTA in PAGE stemmed from the need to "clarify" the $Mg^{2+}$-extracted nucleic acid (see above). Nucleic acids run on EDTA-containing PAGE not only displayed greater resolution but migrated approximately 10% further along the gel with no evidence of aggregation. Gagnon and de Lamirande (1972) observed 60% autodegradation caused by EDTA in RNA preparations from tobacco leaves. The DEP method of extraction does not suffer from the $Mg^{2+}$-related PAGE interference so EDTA is not required. Consequently, chloroplast rRNA can be maintained without loss or loss of resolution on PAGE (Strangeway, 1977).

Extractions of nucleic acids from F. pratensis were carried out in the presence and absence of $Mg^{2+}$ and EDTA as described in the legend to Figure 2.4. The investigation was designed to establish the stability of the chloroplast rRNA and to examine the claim of Bourque et al. (1973). It was considered important to optimize the extraction and separation of nucleic acids particularly in view of further work involving the monitoring of nucleic acids from leaves of different ages.

The results from several experiments are summarized in Figure 2.4. The importance of $Mg^{2+}$ in maintaining $1.1x10^6$ rRNA integrity is clear. It must be present during cell disruption since its subsequent addition fails to confer stability on the $1.1x10^6$ rRNA and merely confers the electrophoretic behaviour characteristic of $Mg^{2+}$-containing PAGE systems. This is consistent with the work of Ingle (1968a) which suggested fragmentation was an in vivo event. However, providing the $Mg^{2+}$ concentration is sufficiently high ( $\geq$ 10 mM.) during extraction, and by implication in excess of the

FIGURE 2.4

PAGE of rRNA extracted from *F. pratensis* by the DEP method. The figure at the top of each profile represents the buffer used in extraction (either containing 10 mM. $Mg^{2+}$ or 3 mM. EDTA) and the figure beneath this indicates the electrophoresis and gel buffer used. Electrophoresis was carried out for 3 h. either at room temperature (rt) or at $4^{o}$C. as indicated.

FIGURE 24

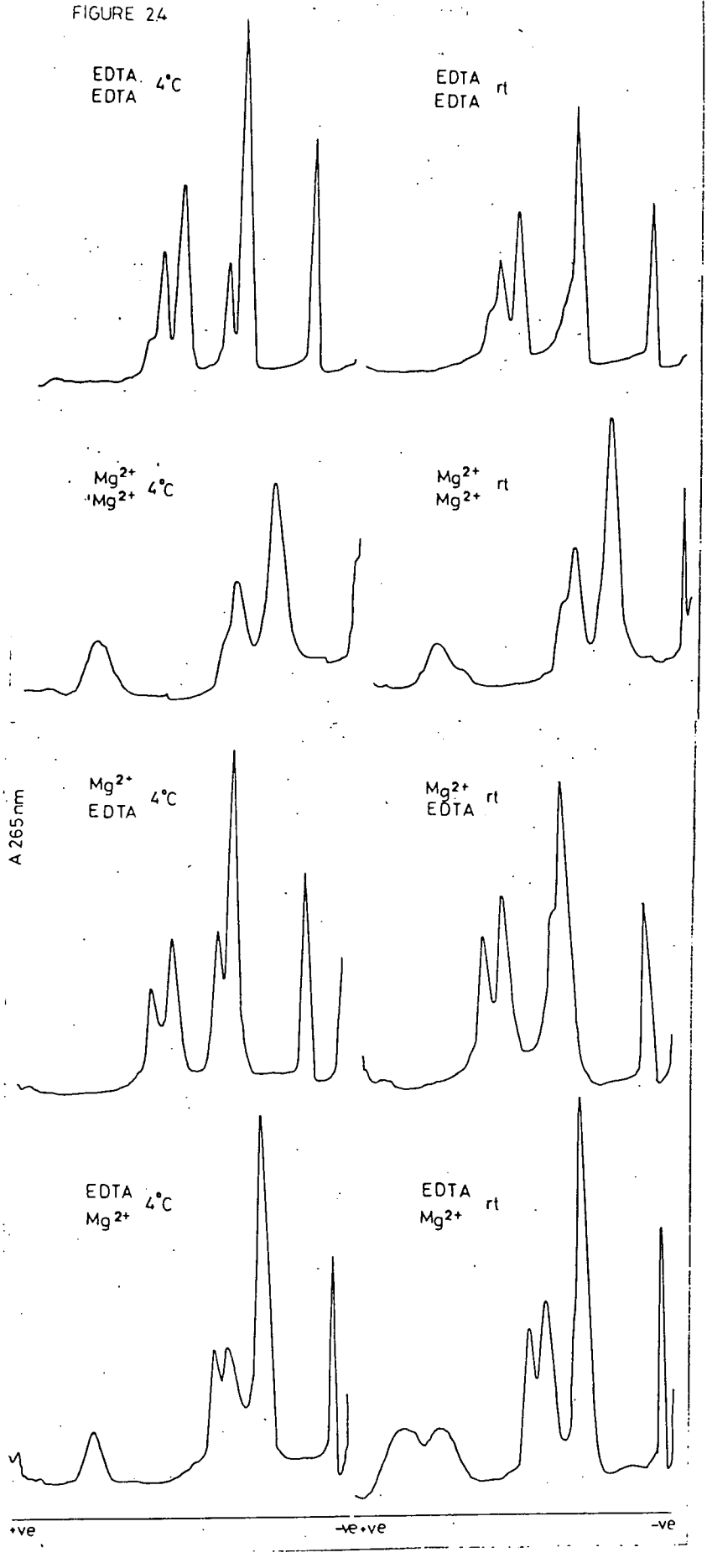

EDTA
EDTA 4°C
EDTA
EDTA rt
Mg2+
Mg2+ 4°C
Mg2+
Mg2+ rt
A 265 nm
Mg2+
EDTA 4°C
Mg2+
EDTA rt
EDTA
Mg2+ 4°C
EDTA
Mg2+ rt
+ve
-ve+ve
-ve

TABLE 2.3

Effect of $Mg^{2+}$ concentration during extraction and subsequent PAGE of rRNAs from F. pratensis. Concentrations of extraction buffers and PAGE buffers used were 2.5 mM., 5.0 mM. and 10 mM. as indicated in the Table

a. peak area ratios 25S/18S (cytoplasmic)

b. peak area ratios 23S/16S (chloroplastic)

The high values for 'a' on electrophoresis and correspondingly low values for 'b' may result from some form of aggregation.

TABLE 2.3

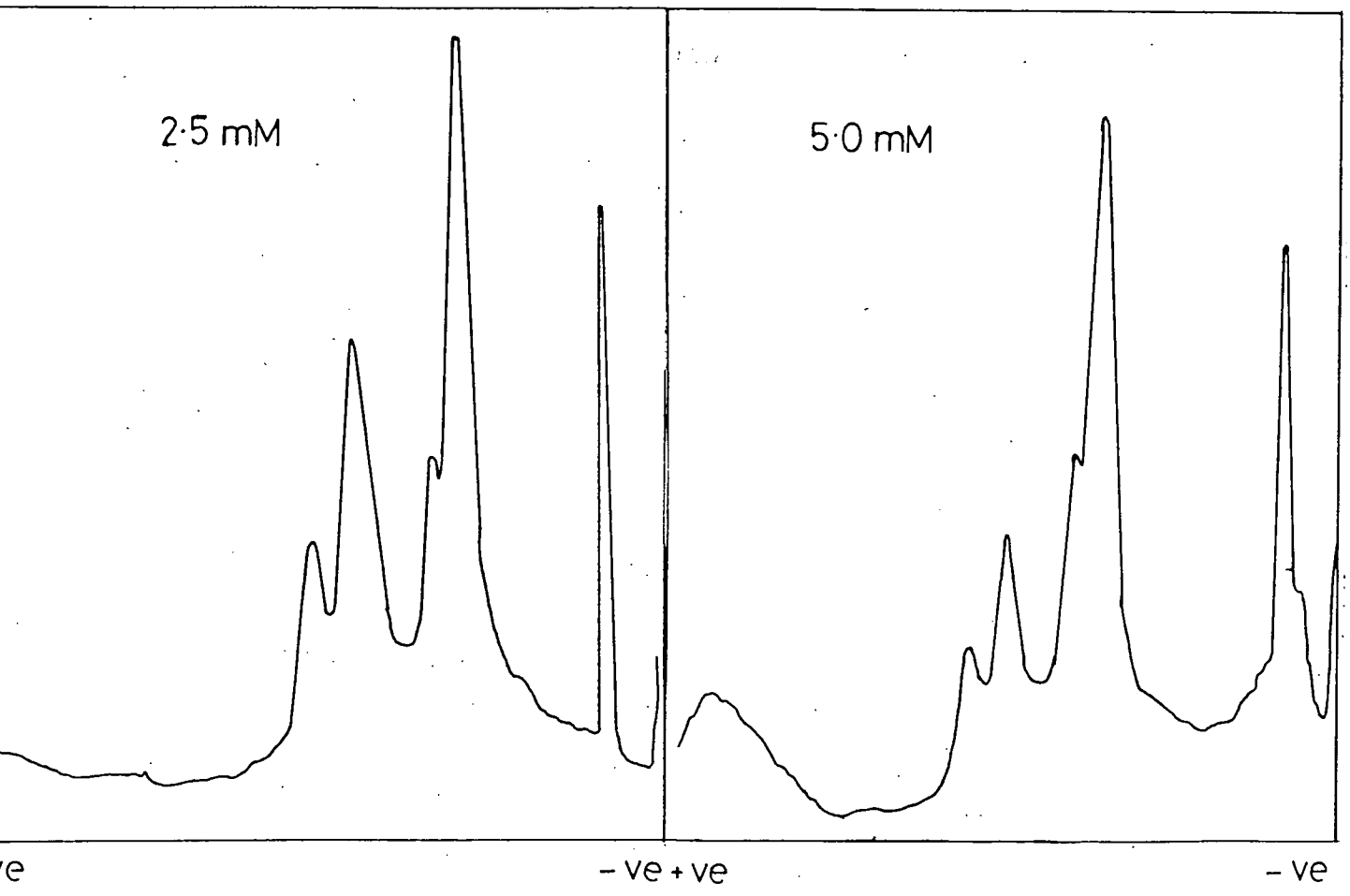
| PAGE → | 2·5 | 5·0 | 10·0 | ratio ↓ | |
|---|---|---|---|---|---|
| extraction ↓ | 1·58 | 2·01 | 2·60 | 25S/18S | a |
| 2·5 | 1·66 | 1·57 | 1·45 | 23S/16S | b |
| 5·0 | 2·27 | 2·11 | 2 68 | 25S/18S | a |
| | 2·07 | 2·03 | 1·78 | 23S/16S | b |
| 10·0 | 2·11 | 2·18 | 2·33 | 25S/18S | a |
| | 1·97 | 1·88 | 1·67 | 23S/16S | b |

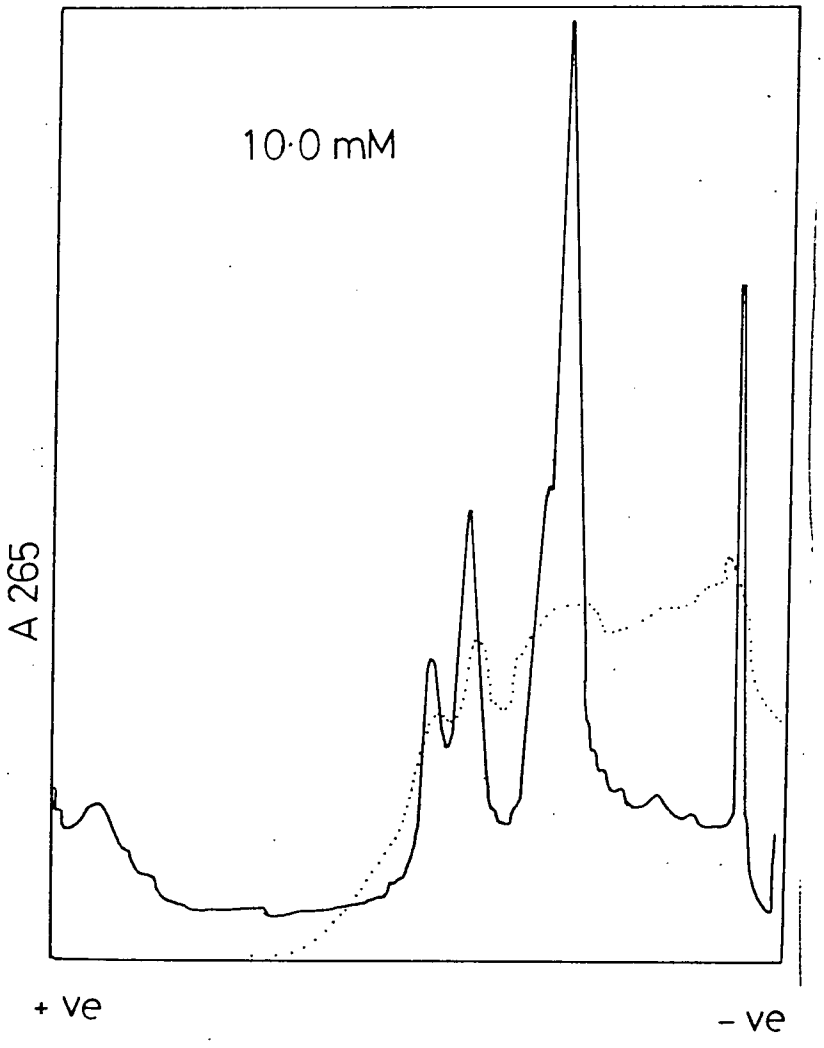
FIGURE 2.5

PAGE of rRNA from *F. pratensis* extracted by the DEP method at different concentrations of $Mg^{2+}$ and electrophoresed at that concentration also.

..... 10 mM. extraction run on 20 mM. PAGE gels.

FIGURE 2.5



chelating capacity of the EDTA concentration, its presence does not seem to be required during subsequent PAGE, particularly if this is carried out at 4°C. However, these observations do not preclude the possibility of "hidden breaks" if $Mg^{2+}$ could be conceived of as binding with the rRNA and if subsequent chelation by EDTA was insufficient to cause reversal of the $Mg^{2+}$-imposed stability. The most likely explanation involves a return to the interpretation of Ingle (1968a) which is essentially a compromise between the two alternative proposals of Atchison et al. (1973) and Leaver and Ingle (1971).

d. Optimal electrophoresis conditions.

The optimal conditions can be summarized as follows:-

1. EDTA.

   3mM. gave the best resolution. 1mM. seemed to cause breakdown of $0.7x10^6$ rRNA and 6mM. caused the appearance of a peak between $1.3x10^6$ and $1.1x10^6$ rRNAs.

2. $Mg^{2+}$

   Aggregation seems to occur at high concentrations ( > 20mM.) but below this $Mg^{2+}$ has little apparent effect on resolution (Figure2.5). Table 5 indicates that some degree of aggregation occurs on PAGE gels containing 10mM. $Mg^{2+}$ as evidenced by the high $1.3x10^6$ : $0.7x10^6$ rRNA ratios. The highest degree of integrity was obtained when extraction and PAGE were carried out at 5mM. At 2.5mM. some breakdown of $1.1x10^6$ rRNA occurred which elevated the value for $0.7x10^6$ rRNA resulting in a lower cytoplasmic rRNA ratio. The deleterious effects arising from extraction in low or high $Mg^{2+}$ buffers could be compensated for by subsequent PAGE $Mg^{2+}$ concentrations.

3. Electrophoresis time.

   At 1 h. the rRNA has barely entered the gel; by 2 h. the $0.56x10^6$ and $0.7x10^6$ peaks are resolved but the $1.1x10^6$ remains a shoulder on the $1.3x10^6$ rRNA peak. By 2 h. 30 min.

the $1.1x10^6$ rRNA is resolved from the $1.3x10^6$ rRNA, by 3 h. the peaks have broadened and any low molecular weight RNA has migrated off the gel.

4. Temperature.
   Leaver (1973) demonstrated the beneficial effect on maintenance of the chloroplast $1.1x10^6$ rRNA of lowered temperatures. This was demonstrated for F. pratensis (Figure 2.4)and subsequently routinely used.
5. $Ca^{2+}$
   Substituting $Ca^{2+}$ for $Mg^{2+}$ did not result in maintenance of the $1.1x10^6$ rRNA to the same extent. Furthermore, electrophoresis had to be carried out in the absence of SDS since precipitation of a calcium salt resulted from its inclusion.

e. "Split" gels.

Since the speed of migration was affected by the "split" gel arrangement a tracker dye of bromophenol blue was always included in sample loading. Although good separations could be achieved with economy of time and materials (Figure 2, 6) the gels required much more care in handling. As a result, the practicality of handling large numbers of gels was in doubt. However, as a reliable and rapid "look-see" procedure it was highly successful.

iv. Radio-isotope labelling of RNA.

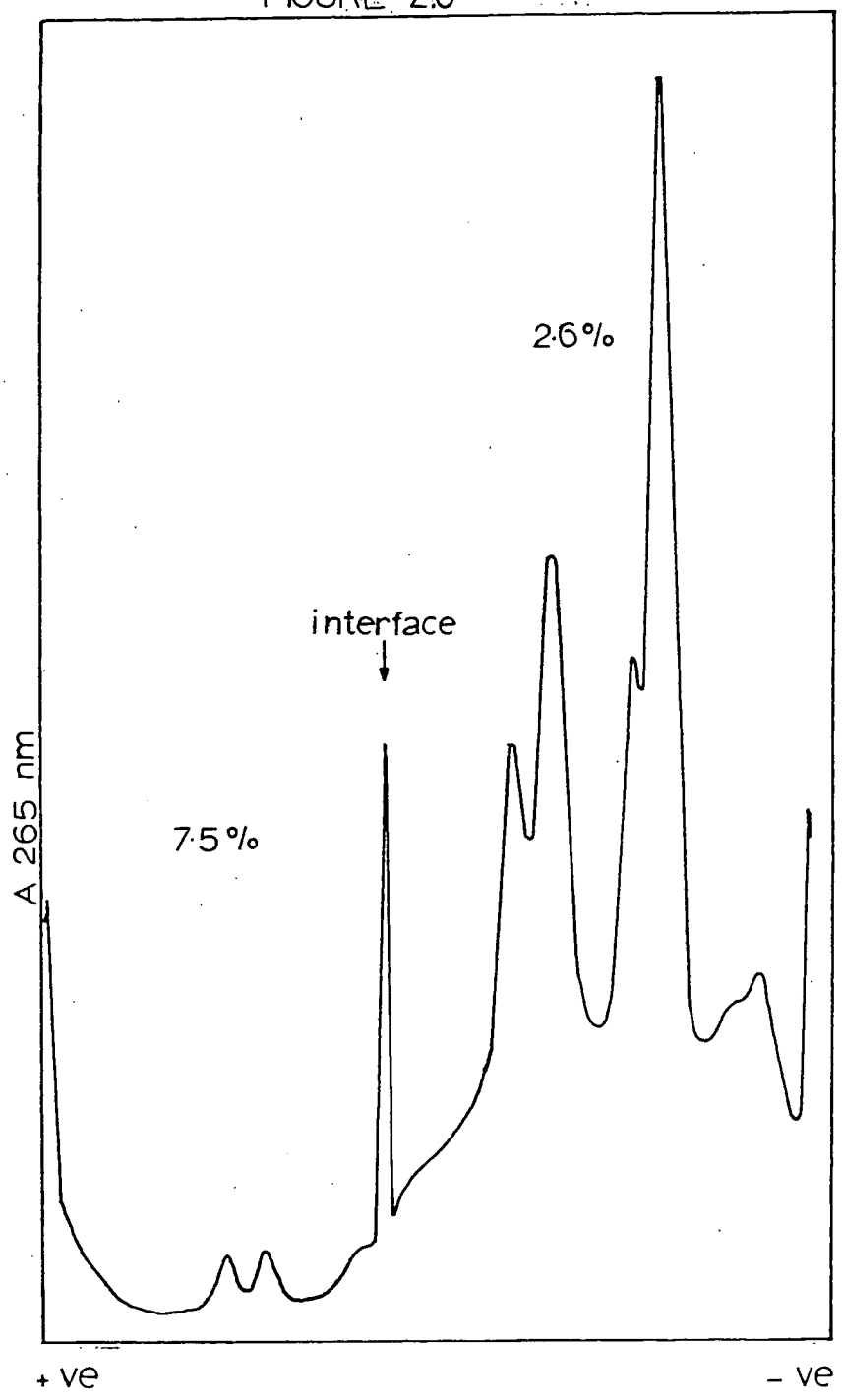
Sterilized sections (general methods) from four complete leaves or portions of leaves were floated lower side down on 10 ml. sterilized water containing 50 µl. 3H-adenine to give a final concentration of 5 µCi/ml. in sterile Petri dishes. These were directly illuminated at 2,000 lux for 22 h. at 23°C.

Incorporation was measured by taking small aliquots (e.g., 50 µl.) of resuspended RNA in clean glass scintillation vials or polypropylene inserts and adding 5 ml. or 2 ml. of scintillation fluid respectively. The scintillation fluid used was that of Patterson and Green (1965) and consisted of:-

FIGURE 2.6

Spectrophotometric profile of RNA from leaves of *F. pratensis* electrophoresed on a 'split' gel.

FIGURE 2.6



4 g. PPO
1,000 ml. toluene
500 ml. Triton X-100.

The vials were shaken, then stored in the dark for 24 h. prior to counting to allow for subsidence of chemiluminescence.

Where radioactivity was to be measured in PAGE gels the following procedure was adopted. Gels were placed in troughs formed of aluminium foil of the exact length of the gel as determined by placing rubber bungs at either end. The trough was laid on a bed of crushed solid $CO_2$ until the gel was completely frozen. The opaque frozen gel was then transferred to the cutting block of a Mickle Tissue Slicer (Mickle Laboratory Engineering Co., Gomshall, Surrey) which was lined by a strip of damp filter paper which immediately provided firm adhesion. Standard 1.0mm. slices were cut and immediately transferred to scintillation vials or inserts. 0.5 ml. piperidine (100 g./l.) were added to each slice. Each slice was dried down at 60°C. for at least 5 h. to allow hydrolysis of the RNA. 0.5 ml. water were then added and the slices allowed to swell for 1 h. prior to the addition of 5 ml. or 2 ml. of scintillation fluid.

Counting was performed in a Beckman LS-200B scintillation counter with maximum counting efficiency of 30% for $^{3}H$ and 85% for $^{14}C$ or in a Tracerlab Corumatic 200 scintillation counter with counting efficiency of 43% for $^{3}H$ and 90% for $^{14}C$ or in a LKB Rackbeta 1215 scintillation counter with counting efficieney of at least 45% for $^{3}H$ and 98% for $^{14}C$.

a. Time course of radioisotopic uptake.

A time course of label uptake was conducted (Figure 27) and although uptake appeared to plateau after 10 h. incubations were performed for 22 h. to ensure labelling of all synthesized fractions.

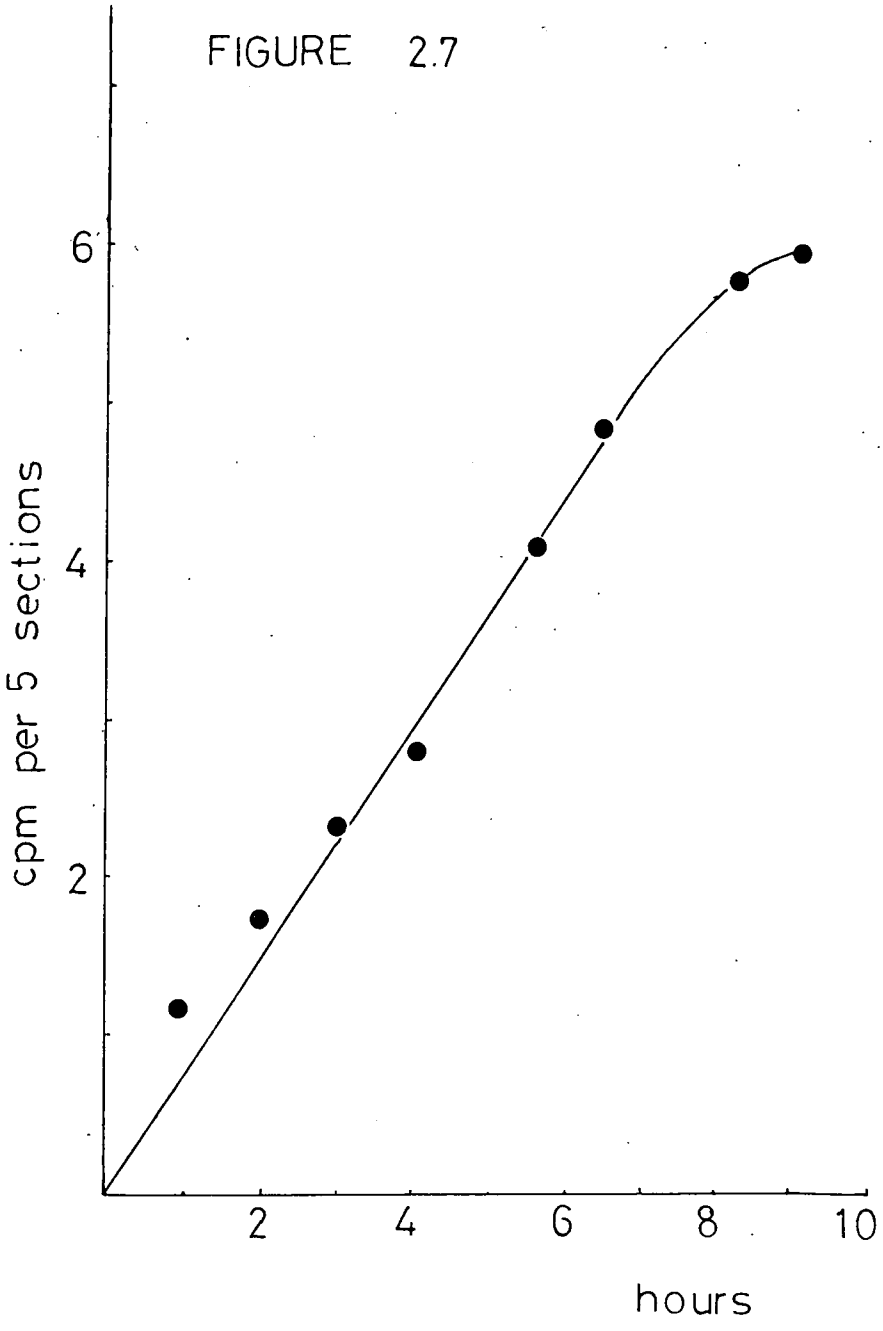
b. Calculation of specific activities.

Results were expressed as specific activities in order to compensate for differences in RNA extraction or extraction requirements. These were calculated as cpm. per µg. RNA counted.

FIGURE 2.7

Time course of uptake of $^{3}$H-adenine (5 uCi./ml.). Following thorough washing, 5 sections (1 cm.) of F. pratensis leaves which had been incubated in radioisotope were ground in 1 ml. 5% (W/V) trichloroacetic acid, centrifuged at 2,500 g. for 5 min. and 100 µl. aliquots were taken for scintillation counting. The points are an average of three such values.

FIGURE 2.7



v. Nucleotide extraction and estimation.

Ethanol supernatants left after precipitation of RNA were concentrated at least ten times by blowing down with compressed air and gentle heating at 40°C. in a Grant BT 3 block thermostat (Grant Instruments (Cambridge) Ltd., Cambridge). The concentrated samples were applied as spots to the origin on a 72 x 24 cm. Whatman No. 3 MM chromatography paper which was subsequently saturated with 8% (V/V) formic acid. Electrophoresis was conducted using a Shandon Southern Model L 24 high voltage electrophoresis apparatus in 8% (V/V) formic acid with a p.d. of 2 - 3 KV (≏ 90 mA) for 1 h. The platen was cooled to minimise resistive heating. On completion of electrophoresis, the electrophoretograph was hung up to air dry and the positions of the nucleotides recorded under U.V. light. Usually, only the standards were of sufficient concentration to be visualized in this way. These areas were marked and equivalent areas for the samples were cut out for elution in 1.0 ml. distilled water and spectroscopic estimation at $A_{260}$ and $A_{290}$ using a Pye Unicam SP 800 spectrophotometer linked to a Servoscribe chart recorder to allow for scale expansion.

Estimation of radioisotope-labelling of nucleotides was carried out by transferring aliquots (50 µl. - 100 µl.) of elutant to scintillation vial insets, adding 2 ml. scintillation fluid and following the counting procedure outlined in section iv.

vi. Polyribosome extraction.

Wettstein et al. (1963) described ribosomal aggregates from rat liver which directed $^{14}$C-leucine incorporation into protein proportionally to the size of the aggregate. Two important methodological considerations arose from this work, namely that it was necessary to keep extraction temperature at about 0°C. in order to favour heavy aggregates and that isolation required $Mg^{2+}$. High temperatures led to degradation to inactive monomers possibly as a result of enhancement of RNAse activity (Arnstein, 1961).

As already mentioned, plants are particularly difficult source material because of the harsh methods required for their

homogenization. In addition, the ubiquity of RNAse requires that steps be taken to inhibit its action in polyribosome extraction (Weeks and Marcus, 1969; Anderson and Key, 1971; Roberts et al., 1973). Payne and Loening (1970) described a number of inhibitors used in ribosome isolation from pea roots. Of these, bentonite provided the best protection but caution in its use had already been expressed by Tester and Dure (1966) who had shown it to cause extensive ribosome binding. Loening (1968) had also indicated that a combination of high $Mg^{2+}$ and bentonite in the extraction caused losses, in particular of chloroplast RNA. This was also indicated in the work of Bourque et al. (1973). Weeks and Marcus (1969), Travis et al. (1970), Anderson and Key (1971) and Alscher et al. (1978) employed DEP as an effective RNAse inhibitor and routinely obtained increased proportions of polyribosomes. However, in view of some of the limitations outlined in section II, ii d. and reports suggesting that under some circumstances DEP causes ~~reduction~~ separation of ribosomes to their subunits (Huvos and Solymosy, 1971; Anderson and Key, 1971), reduction in polyribosome yield and the percentage of large polyribosomes (Davies et al., 1972) and also aggregation in PAGE (Strangeway, 1977) its usefulness in polyribosome extraction is in doubt. Certainly it cannot be used in isolation of polyribosomes destined for in vitro protein synthetic activity measurements (Weeks and Marcus, 1969; Anderson and Key, 1971).

The use of the RNAse inhibitor, polyvinyl sulphate, is limited by its interference in U.V. monitoring of separated ribosome fractions (Clark et al., 1964) and at high concentrations causes dissociation into polydisperse (Hsiao, 1968) or discrete (Vanyushin and Dunn, 1967) RNA products.

The use of $Cu^{2+}$ (Hall and Cocking, 1966) and $Zn^{2+}$ (Barker and Rieber, 1967) as RNAse inhibitors was shown by McGown et al. (1971) to cause anomalies (notably aggregation) in subsequent gradient analysis.

Davies et al. (1972) described a method for polyribosome extraction from pea stems avoiding the use of RNAse inhibitors with their concomitant dubious side-effects. This involved the use of

buffers of high concentration (>200 mM., but less than 400 mM.) and high pH (pH 8.5 - 9). $K^+$ concentrations over the range 0 - 100 mM. and $Mg^{2+}$ concentrations over the range 10 - 50 mM. were found not to affect the yield. However, absence or high molarity ( > 80 mM.) of $Mg^{2+}$ obliterated polyribosome appearance on sucrose density gradients. Breen et al. (1972) found that polyribosome isolation in high salt and high pH was effective in barley also. The improvement conferred by raising the pH may be due to the removal of RNAse associated with ribosomes which remains absorbed and active at pH below 8.5 (Hsiao, 1968). Beachy et al. (1978) established that for isolation of polyribosomes from soybean, pH 9 buffers yielded poor polyribosome patterns and buffers containing high $K^+$ to $Mg^{2+}$ ratios (greater than 6 : 1) were better than lower ratios. Gray and Cashmore (1976) had already reported this in their polyribosome isolation using buffers of pH 8.5 and containing $K^+$ and $Mg^{2+}$ in the ratio 20 : 1. The criticism that high salt removes constituent proteins can be ignored from the functional aspect as polyribosomes isolated from pea in high salt conditions have been shown to be ~~fully~~ active in in vitro protein synthesis (Cammarano et al., 1972; Beachy et al., 1978).

a. Methods used.

The method originally adopted was essentially that of Jachymczyk and Cherry (1968) as described by Pearson (1969). However, modifications were made which provided both higher yields and qualitative reproducibility for F. pratensis. The original method and that used finally will be described separately (A., B. respectively). Factors in the course of development of the method are described in

A. Plant tissue was ground to a fine powder following freezing in liquid nitrogen. This not only aided homogenization but also prevented polyribosome loss usually associated with excision and subsequent wilting

Three times the tissue volume of extraction buffer were added with or without Nonidet P40 or P42 (0.4% V/V) (see ix. g.)

The extraction buffer contained:-

| | | |
|---|---|---|
| 20 mM. | Tris-HCl | pH 8.0 |
| 250 mM. | sucrose | (RNAse free) |
| 10 mM. | magnesium chloride | |
| 15 mM. | potassium chloride | |
| 5 mM. | β-mercaptoethanol | |

The resulting brei was centrifuged at 10,000 g. for 20 min. at 4°C. in a MSE "High Speed" centrifuge. 3 - 4 ml. of post-mitochondrial supernatant were carefully layered over 4 ml. of 1.5M sucrose dissolved in extraction buffer. Centrifugation followed at 105,000 g. for at least 3 h. (see ix. e.) in a 10x10 ml. titanium angle rotor in a MSE "Superspeed" 65 ultracentrifuge at 4°C. Following centrifugation the supernatant was decanted and the tubes inverted over absorbent paper for several minutes. The tube walls were wiped with absorbent paper and the opalescent pellet was then either resuspended in extraction buffer (50 - 200 µl.) or immediately frozen in liquid nitrogen and stored at -20°C.

B. The final method adopted for polyribosome extraction was essentially that of Jackson and Larkins (1976) and Larkins et al. (1976) as modified by Beachy et al. (1978).

Plant tissue was ground following freezing in liquid nitrogen. After thawing at 4°C., 0.1 g. PVP (see ix. c.) per g. tissue were added followed by extraction buffer amounting to five times the volume of tissue, with or without Nonidet P42 (0.4% V/V).

The extraction buffer contained:-

| | | |
|---|---|---|
| 200 mM. | Tris-HCl | pH 8.5 |
| 200 mM. | sucrose (RNAse free) | |
| 400 mM. | potassium chloride | |
| 50 mM. | magnesium chloride | |
| 5 mM. | DTT | |

Insoluble material was removed by centrifugation at 10,000 g. for 20 min. in a 10x10 ml. angle rotor in a MSE "Superspeed" 50 TC at 4°C. The resulting supernatant was layered over 4 ml. 1.5M sucrose dissolved in extraction buffer (A.) and the procedure outlined in (A.) was pursued.

Extracts destined for introduction into the wheatgerm cell-free protein-synthesizing system or for sucrose density gradient analysis were transferred to capped conical microcentrifuge tubes and clarified by centrifugation at 13,000 g. for 1.5 min. at 4°C. in a Quickfit microcentrifuge (12x1.5 ml.) (Quickfit Instrumentation, England).

b. Spectrophotometric estimation of ribosomes.

The U.V. spectrum was recorded using a Perkin Elmer 402 or a Pye Unicam SP 800 spectrophotometer. Quantitative estimation was made using the principle of Tester and Dure (1966),

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

vii. Sucrose density gradient centrifugation.

This has been the most favoured ribosome fractionation technique (Peterman, 1964) and excellent resolution can be obtained (Noll, 1969; Beachy et al., 1978; Evans et al., 1979).

The method of Britten and Roberts (1960) was used initially in a modified form, involving the use of 20 ml. gradients from 15 - 34% (W/V) sucrose. 1 ml. aliquots of gradient buffer increasing by 1.2% sucrose content were layered over each other in 23 ml. centrifuge tubes. Equilibration of the gradients was allowed to take place (to smooth the step gradient into a linear one by diffusion) overnight at 2°C.

The gradient buffer contained:-

20 mM. Tris-HCl pH 8.0
10 mM. magnesium chloride
20 mM. potassium chloride

100 µg. ribosomes in approximately 200 µl. extraction buffer were layered on to the gradients which were centrifuged for 1 h. 30 min. at 85,000 g. in a 3x23 ml. swing out rotor on a MSE "Superspeed" 65 ultracentrifuge at 4°C. The gradients were fractionated by pumping sucrose (500 g./l.) into the bottom of the centrifugation tube and monitoring the absorption at 265 nm. of the displaced gradient through a flow-through attachment in the Joyce Loebl polyfrac (Figure 2.8 a).

Polyribosome separations obtained in this way were improved upon by modification of the method adopted by Beachy et al. (1978). Clarified polyribosome suspensions were applied to 12.5 - 50% (W/V) sucrose linear gradients made using a Buchler auto-densiflow apparatus (Buchler Instruments, Nuclear Chicago, Fort Lee, N.J., U.S.A.) and a two-chambered Gradient maker with constant stirring. Gradients were centrifuged at 75,000 g. for 1 h. 45 min. at 4°C. in a 3x25 ml. swing out rotor on a MSE "Superspeed" 50 TC centrifuge. Fractionation was achieved by reversal of the auto-densiflow apparatus and passage of the gradient through a LKB 4701 A Uvicord ultraviolet absorptiometer by means of a LKB Perpex peristaltic pump. The transmittance at 254 nm. was recorded on a servoscribe chart recorder moving at 200 mm./h. To check gradient linearity, fractions (0.5 ml.) were collected after flowing through the Uvicord and the sucrose densities were measured with refractometers (Figure 2.8 b). Narrowing the percentage range of gradients did not provide an appreciably improved separation (Figure 2.9), neither did prolonging centrifugation (Figure 2.10).

Since the Uvicord recorder records transmittance the extinction curve (which is related to sample concentration) was constructed by the relationship $E = \log \frac{1}{T}$, so that quantitative comparisons of different ribosome size classes could be assessed. This was achieved by weighing delineated areas under particular peaks.

FIGURE 2.8

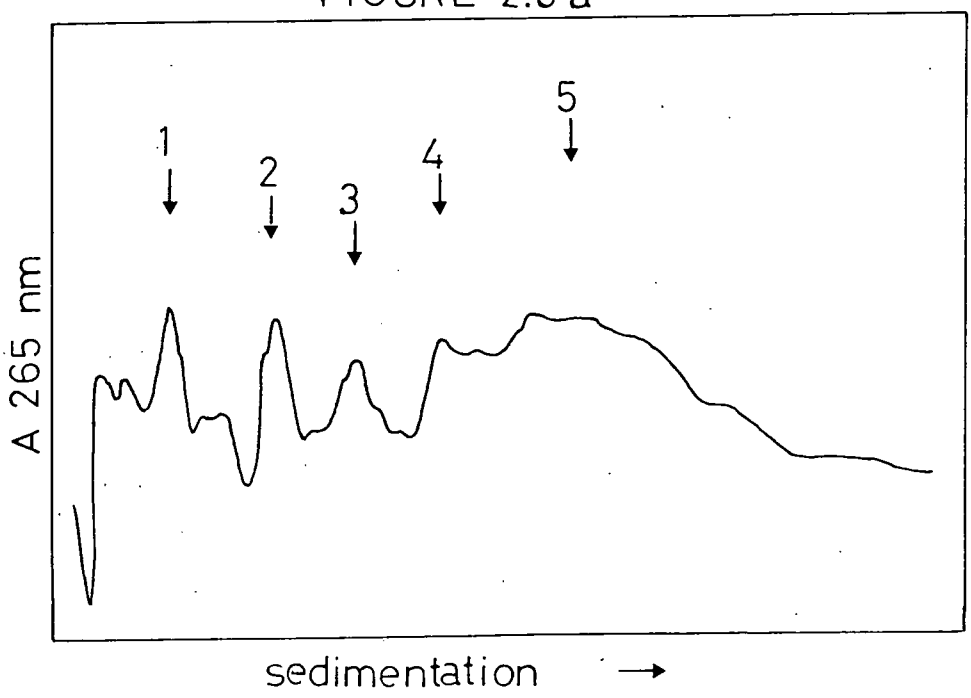
a. Spectrophotometric profile obtained from ribosomes extracted from leaves of F. pratensis separated on a step sucrose density gradient.

b. Spectrophotometric profile obtained from ribosomes extracted from leaves of F. pratensis separated on a linear sucrose density gradient, together with the % sucrose concentrations as recorded by refractometer.

1 subunit
2 monoribosome
3 dimer
4 trimer
5 tetramer
6 pentamer.

.-.-.- Background density of blank gel.

FIGURE 2.8 a


1
2
3
4
5
A 265 nm
sedimentation

b.

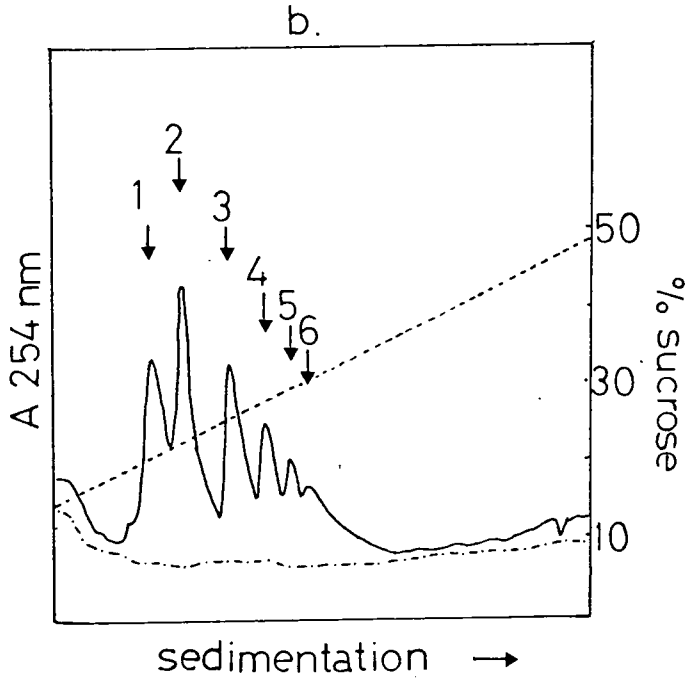

1
2
3
4
5
6
A 254 nm
50
30
10
% sucrose
sedimentation

FIGURE 2.9

Spectrophotometric profile of polyribosomes extracted from leaves of F. pratensis on a 20 - 45% sucrose density gradient.

FIGURE 2.10

Spectrophotometric profile of polyribosomes extracted from leaves of F. pratensis separated on 12.5 - 50% sucrose density gradient for 2 h. 15 min.

FIGURE 2.9

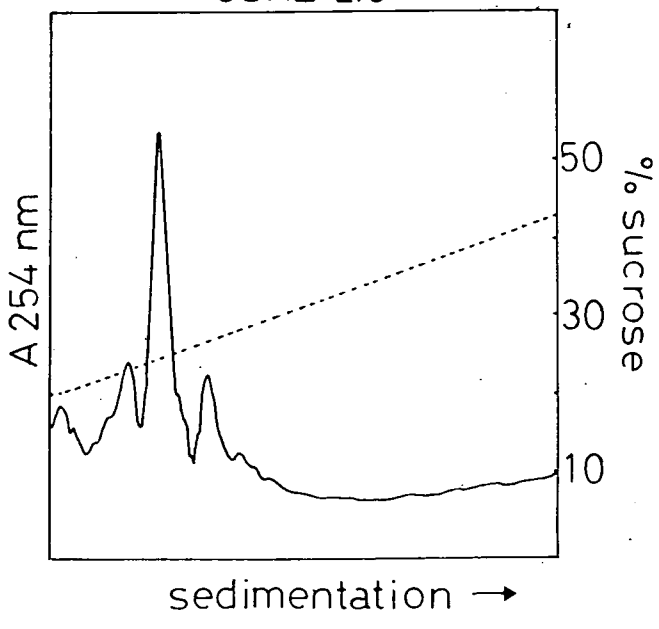
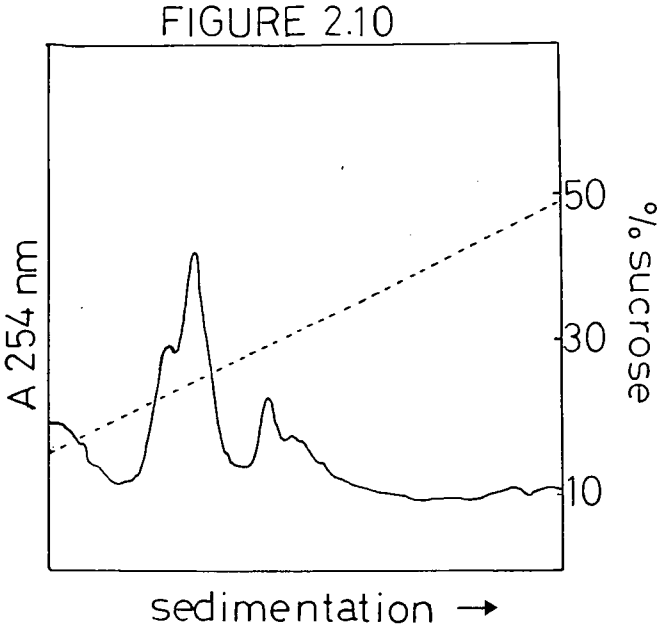



FIGURE 2.10



viii. PAGE.

Criticism has been levelled at the use of sucrose density gradient method of fractionation because of profile distortion by hydrostatic pressures (Infante and Baierlein, 1971), reproducibility of gradients, the need for sample clarity and the large volume of sample required for loading. All these problems are overcome by the use of PAGE. The use of PAGE in separation of ribosome fractions was first described by Hjertens et al. (1965) and in the separation of polyribosomes by Dahlberg et al. (1969; 1973). Despite the elegance of the technique there have been few reports of its successful utilization for higher plant ribosomes (Thomas, 1973; Ledoigt et al., 1975; Strangeway, 1977).

The method used was a modification of that described by Thomas (1973) for the electrophoresis of pea seed ribosomes.

2.2% polyacrylamide gels were made using the following proportions of reagents:-

| | |
|---|---|
| stock monomer (150 g./l. acrylamide : 7.5 g./l. bis-acrylamide | 3.66 ml. |
| buffer | 8.32 ml. |
| water | 12.78 ml. |
| TEMED | 0.02 ml. |
| ammonium persulphate (100 g./l.) | 0.20 ml. |

The buffer used in this instance was:-

60 mM. Tris-acetate pH 8.0
45 mM. potassium acetate
22.5 mM. magnesium acetate

This buffer was diluted 2 : 1 with distilled water for use as electrophoresis running buffer. Electrophoresis was carried out at 2°C. with continual circulation of the running buffer between the upper and lower tanks using a peristaltic pump with a flow rate of 5.0 ml. per min. This was necessary to counteract the concentration of $Mg^{2+}$ ions at the cathode and the concomitant depletion of $Mg^{2+}$ in the gels. Gels were pre-run for at least 30 min. at 13 V. per gel (5.75 mA) using a Vokam power pack set on constant voltage mode. 5 - 15 µg. polyribosomes in 10 - 100 µl. extraction buffer were loaded on to gels (no sucrose was added as the amount adhering to the 105,000 g. pellet after centrifugation through sucrose was sufficient to "weight" the sample on application to the gel surface).

Electrophoresis was carried out for between 1 h. 45 min. and 2 h. 15 min. Gels were removed and washed in 7% (V/V) ethanol for at least 1 h. to remove U.V. absorbing debris that prevailed in the first 4 cm. of the gel. Polyribosome bands were visualized by scanning at 265 nm. in a Joyce Loebl polyfrac.

ix. Some quantitative and qualitative variations in polyribosome yield.

The conditions for polyribosome extraction show an apparent dependence on the species used as starting material. This may partly be due to differences in endogenous RNAse content, differences in cell wall rigidity, presence of fibres and compartmentation. Thus, for each species, optimal polyribosome isolation conditions must be ascertained.

a. Homogenization.

Liquid $N_2$ vs. "Polytron" vs. electric chopper.

Green tissue is highly fibrous (Chapter 1.) and so poses particular problems in choice of suitable homogenization procedure. Liquid $N_2$ was the obvious choice since it is rapid, subsequent grinding is independent of fibre content of the tissue and it is applicable to small quantities of starting material.

However, in recent reports the "Polytron" (Willems) has been used with success (Giles et al., 1977; Evans et al., 1979). Its great advantage lies in the speed with which extractions can be carried out (3 - 30 s.). Although exceptional polyribosome isolations were performed on pea embryonic axes and cotyledon tissue, the fibrous nature of F. pratensis was not suited to the "Polytron." At best the yield was in the region 35 - 40 µg. per g. fresh weight. In addition large quantities of tissue (5 g.) were required which limited its usefulness in this instance. Finally, homogenization was attempted using an electric carving knife adapted to chop the tissue by razor blades at high speed and in a confined area. This cutting method was not hampered by the fibre content of F. pratensis but the length of time required for complete homogenization was detrimental to the final yield (Figure 2.11).

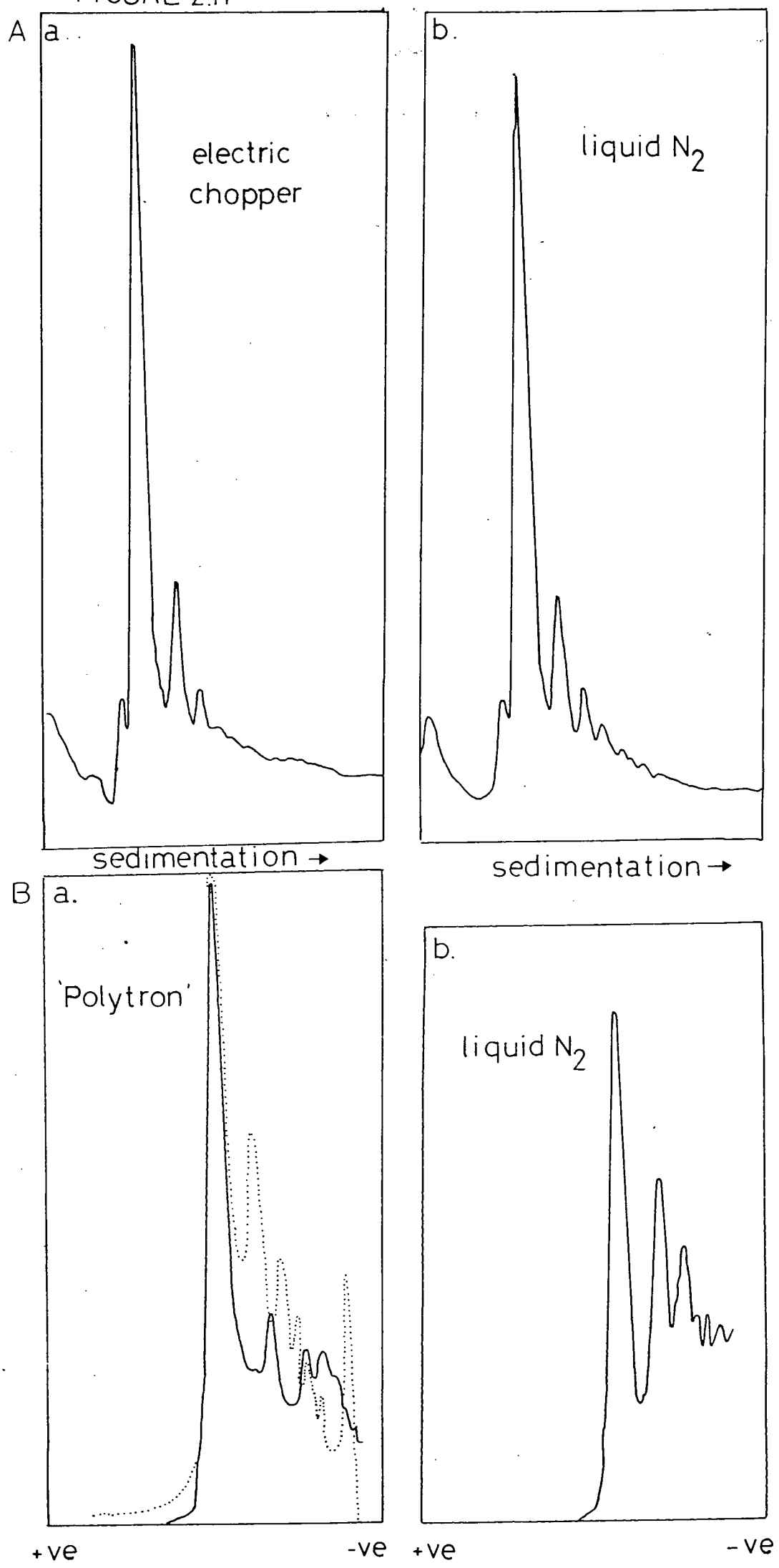
FIGURE 2.11 A.

Comparison of sucrose density gradient analysis of polyribosomes extracted by (a) electric chopper and (b) liquid $N_2$ from F. pratensis leaves. Although there is virtually no qualitative difference in the behaviour of the extracts on sucrose density gradients, quantitatively the liquid $N_2$ method resulted in a 30% increase in yield over the electric chopper method. (a) 80 µg. polyribosomes loaded; (b) 60 µg. polyribosomes.

FIGURE 2.11 B.

Comparison of PAGE of polyribosomes extracted by (a) 'Polytron' 36 µg./g. fwt. 18.2 µg. loaded; (b) liquid $N_2$ 108 µg./g. fwt. 10 µg. from F. pratensis leaves and P. sativum (10 µg.). (Yield 520 µg./g. fwt.) - increase in yield of 300%.

FIGURE 2.11


A
a.
electric
chopper
sedimentation →
b.
liquid $N_2$
sedimentation →
B
a.
'Polytron'
+ve
-ve
b.
liquid $N_2$
+ve
-ve

Liquid $N_2$ was the most effective homogenization aid for *F. pratensis* and was finally adopted for use throughout this work unless otherwise indicated.

b. Effect of ionic strength.

Bolton (1966) noted that polyribosome extraction from *E. coli* required observation of $Mg^{2+}$ optima. Beyond the range of $Mg^{2+}$ optimal concentration, polyribosome aggregation occurred and below this range, dissociation of ribosomes into sub-units and the release of latent RNAses. Gray and Cashmore (1976) and Beachy *et al.* (1978) established the importance of ionic composition and Jackson and Larkins (1976), Breen *et al.* (1972) and Davies *et al.* (1972) the importance of high ionic strength and pH of the buffers used in polyribosome isolation. High levels of $K^+$ (> 600 mM.) were shown to lead to dissociation of polyribosomes into sub-units as a result of replacement of $Mg^{2+}$ by the monovalent ion (Breen *et al.*, 1972). Wilson (1968) showed that at $K^+$ concentrations above 500 mM. RNAses were solubilized from microsomal suspensions of corn tissue which may account for polyribosome dissociation.

Further to the advantages of using high pH buffers (see introduction) Wilson (1968) also found that RNAse activity declined at pH above 7.2.

Figure 2.12shows the results of extractions of polyribosomes from *F. pratensis* leaves at different pH and different $K^+$ : $Mg^{2+}$ ratios.

These results indicate that high pH and a high $K^+$ : $Mg^{2+}$ ratio were required for good yields and larger range of polymers. The buffer system of Beachy *et al.* (1978) was finally adopted.

c. Effect of PVP.

Since *Festuca pratensis* was known to contain high quantities of phenolics, particularly in association with ageing, steps were taken to reduce their interference in polyribosome isolation. Tannins frequently complex with proteins and so may indirectly complex with polyribosomes. PVP has been shown to dissociate such complexes even to the extent of reversing imbibition of enzyme

FIGURE 2.12

PAGE profiles of polyribosomes from leaves of F. pratensis extracted under variable conditions.

a. pH 8.0 with original extraction medium ($Mg^{2+}$:$K^{+}$, 1:1.5).
   15 µg. polyribosomes electrophoresed.

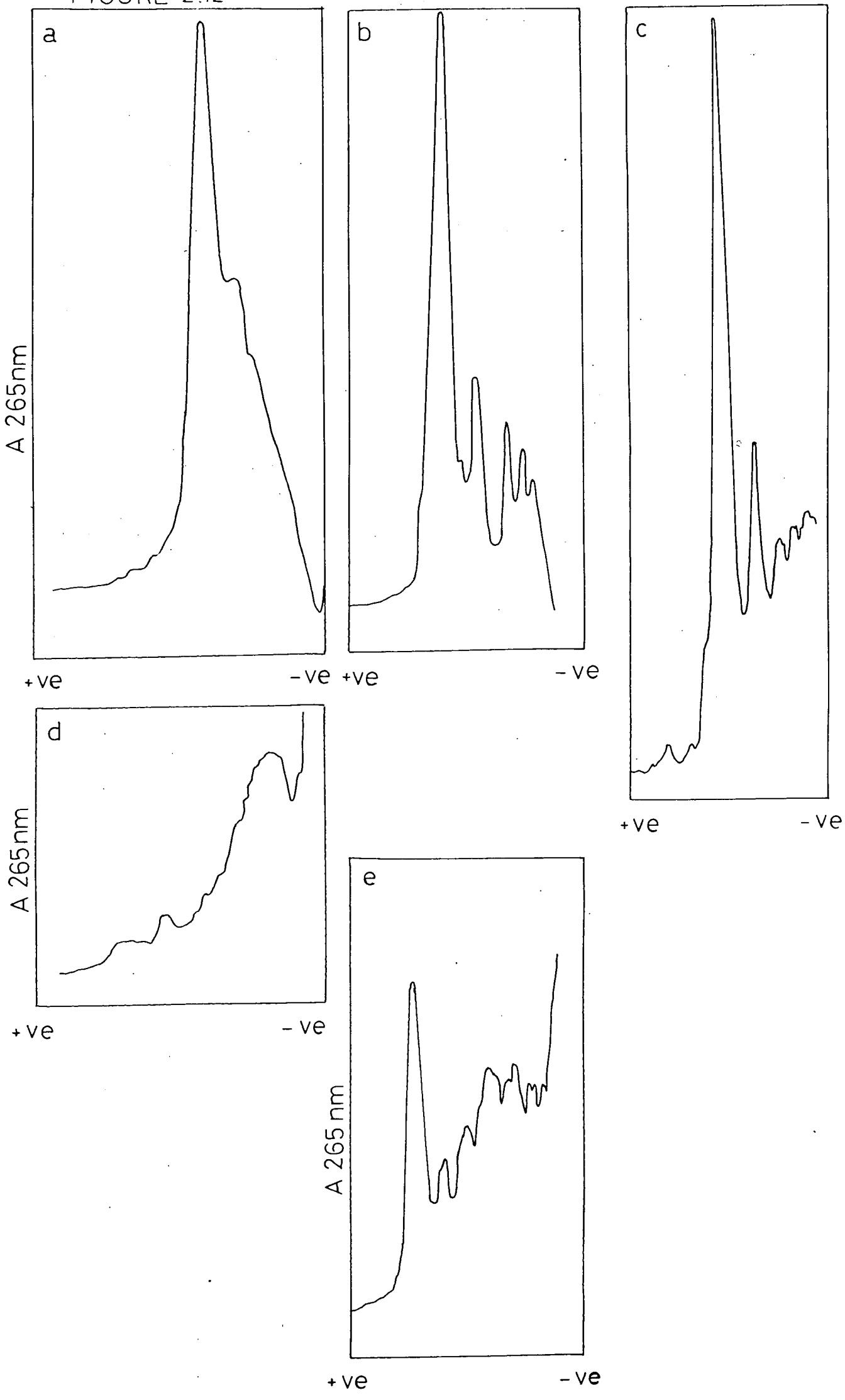
b. pH 8.5 with original extraction medium ($Mg^{2+}$:$K^{+}$, 1:1.5).
   18 µg. polyribosomes electrophoresed.

c. pH 8.5; 200 mM. Tris ($Mg^{2+}$:$K^{+}$, 1:1.5).

d. pH 8.5; 200 mM. Tris, 30 mM. $Mg^{2+}$ ($Mg^{2+}$:$K^{+}$, 1:0.5)

e. pH 8.5; 200 mM. Tris, 50 mM. $Mg^{2+}$ ($Mg^{2+}$:$K^{+}$, 1:8)

FIGURE 2.12



activity (Goldstein and Swain, 1965). Insoluble PVP was added to the homogenate prior to the first centrifugation (insoluble PVP is 70% as effective as soluble PVP but has the advantage of being separable by centrifugation.). Figure 2.13 shows the improvement on polyribosome yield and pattern when PVP was included in the extraction.

d. Effect of incorporating a "Lyphogel" step in the extraction.

The greened tops and unemerged base of leaves of F. pratensis were extractedseparately in the normal way. However, following the 10,000 g. centrifugation the supernatants were mixed with 15% (W/V) "Lyphogel" beads and kept at -4°C. for 20 min., followed by centrifugation at 1,000 g. for 3 min. The supernatant was layered on 4 ml. sucrose cushion and the normal procedure was carried out. At least a 30% decrease in yield occurred in both extractions probably as a result of losses related to the further manipulation steps. (The polyribosomes extracted were fully functional in directing protein synthesis in a wheatgerm derived cell-free system. The acrylamide of the "Lyphogel" did not apparently poison the system. This adds weight to the feasibility of taking particular polymeric fractions from PAGE separated polyribosomes for analysis of protein synthetic capacity.)

e. Effect of centrifugation time.

Leaver and Dyer (1974) criticised the interpretation of polyribosome size class ratios obtained by previous workers without consideration of the preferential sedimentation of these different classes by different centrifugation times. In order to establish the centrifugation conditions that would provide an accurate indication of size class ratios a number of centrifugation times were analysed. Figure 2.14 indicates that by 3 h. 30 min. there is little enhancement of the monoribosome peak by further centrifugation. For in vitro studies (Chapter 6) where monoribosomes are not functional, 3 h. centrifugations were considered adequate. However, arising from the question of

FIGURE 2.13

Sucrose density gradient profiles of polyribosomes extracted in buffer 2 in the presence and absence of PVP. 150 µl. polyribosomes centrifuged in each case (containing a. 110 µg., b. 120 µg.). The polyribosomes were extracted from young, emerging leaves of *F. pratensis*.

FIGURE 2.13

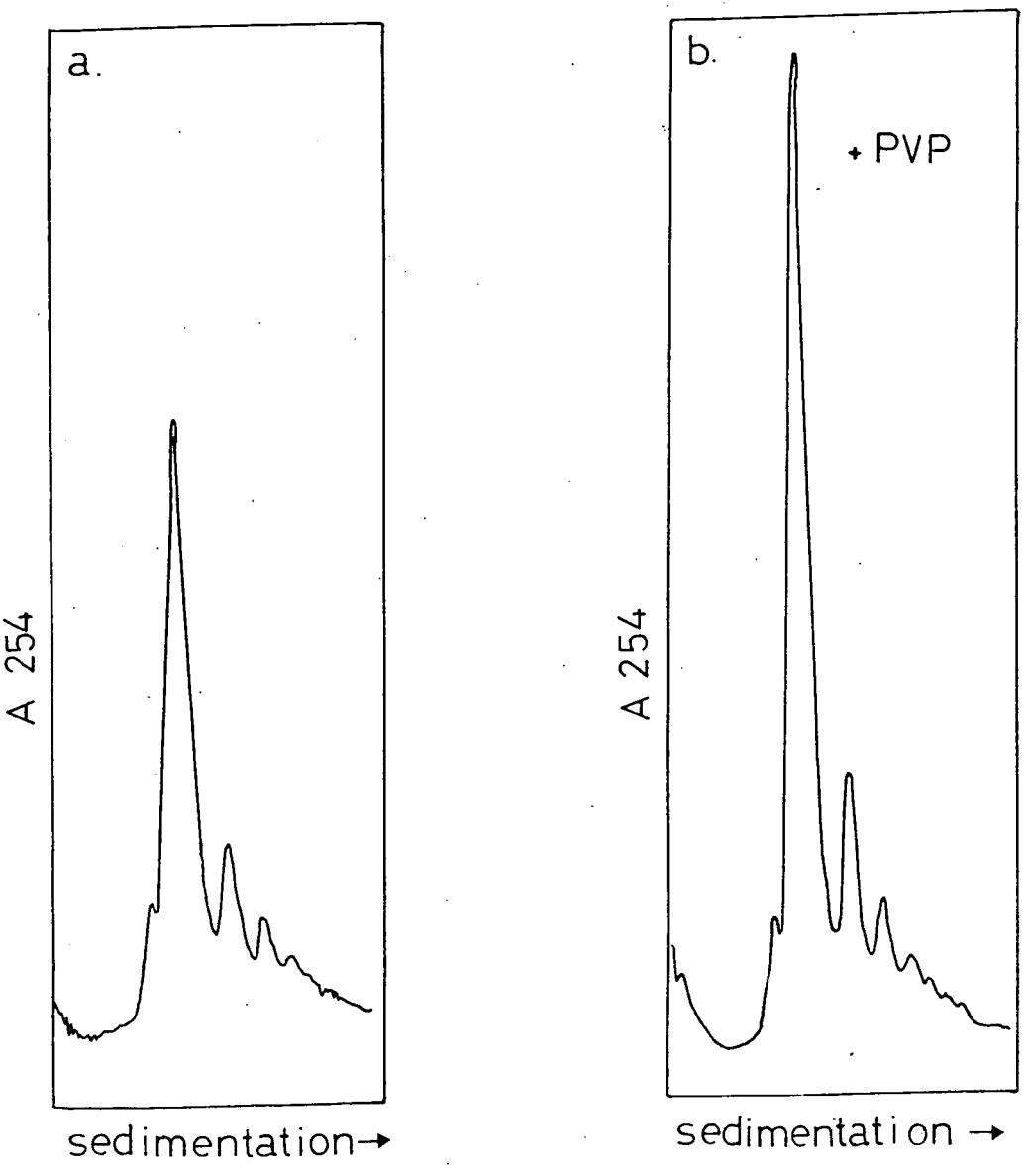
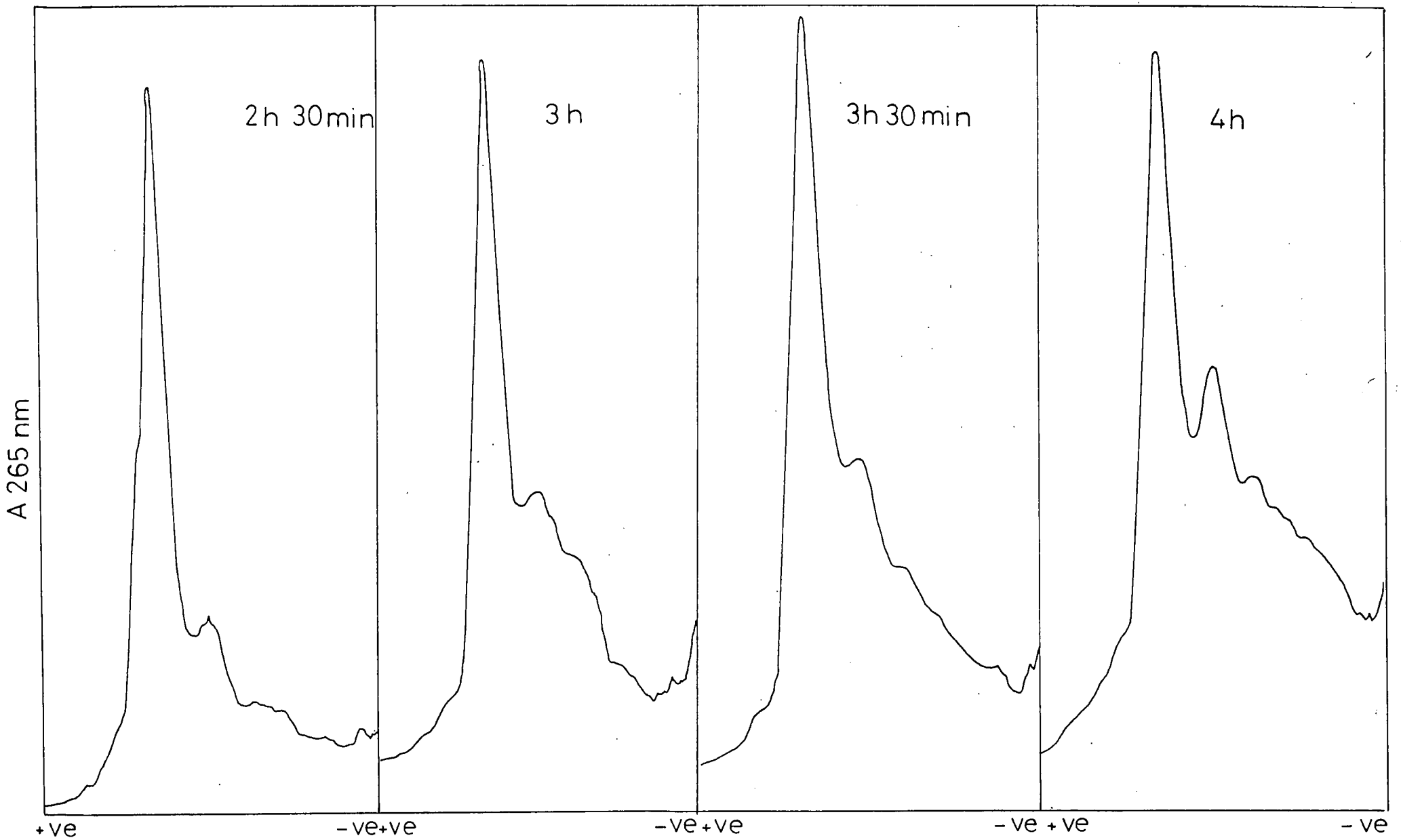


FIGURE 2.14

PAGE profiles of polyribosomes extracted from F. pratensis following 105,000 g. centrifugations for different lengths of time. 50 µl. loadings in each case.

FIGURE 2.14



centrifugation time is the speculation of how accurately extracted ribosomes characterize the status of ribosomes *in situ*. Phillips *et al*. (1969) regarded a proportion of monoribosomes of 20-25% as being rather high to reflect the actual cell complement, but this assumed that monoribosomes arising from protein synthesis completion are spontaneously broken down. However, Kabat and Rich (1969) observed monoribosomes in cells not actively engaged in protein synthesis and were able to distinguish active monoribosome from inactive or procedurally altered ones (also, Blobel, 1971). Comparison with electronmicrographs has confirmed that extracted polyribosomes do appear to reflect the *in situ* situation, assuming that the manipulations required for electronmicroscopy do not disturb the cellular composition.

### f. The effect of RNAse and EDTA.

In order to establish the validity of polyribosome preparations and to rule out the possibility of ribosomal aggregation, EDTA or RNAse were added to the resuspended polyribosome pellets (Nemer *et al*., 1974; Beachy *et al*., 1978). Figure2.15shows the effects of incubation with both these denaturants as established by both sucrose density gradient analysis and PAGE. 5 µg. bovine pancraetic RNAse were added to 100 µl. resuspended polyribosomes and incubated at 37°C. for 30 min. This resulted in the loss of polyribosomes and concomitant enhancement of monoribosome and sub-unit peaks. EDTA was added to a final concentration of 20 mM. and incubated at 0°C. for 30 min. which resulted in degradation of larger polyribosomes limiting maximum size to that of tetramers with a concomitant enhancement of monoribosome and sub-unit peaks.

### g. Addition of "Nonidet."

Many reports indicate that inclusion of detergent in the extraction medium for polyribosome isolation leads to an increased yield (Lonsdale and Boulter, 1973; Larkins and Davies, 1973). This is attributed to the release of "membrane-bound" polyribosomes (Eilam *et al*., 1971; Beachy *et al*., 1978). Some workers have attributed

FIGURE 2.15

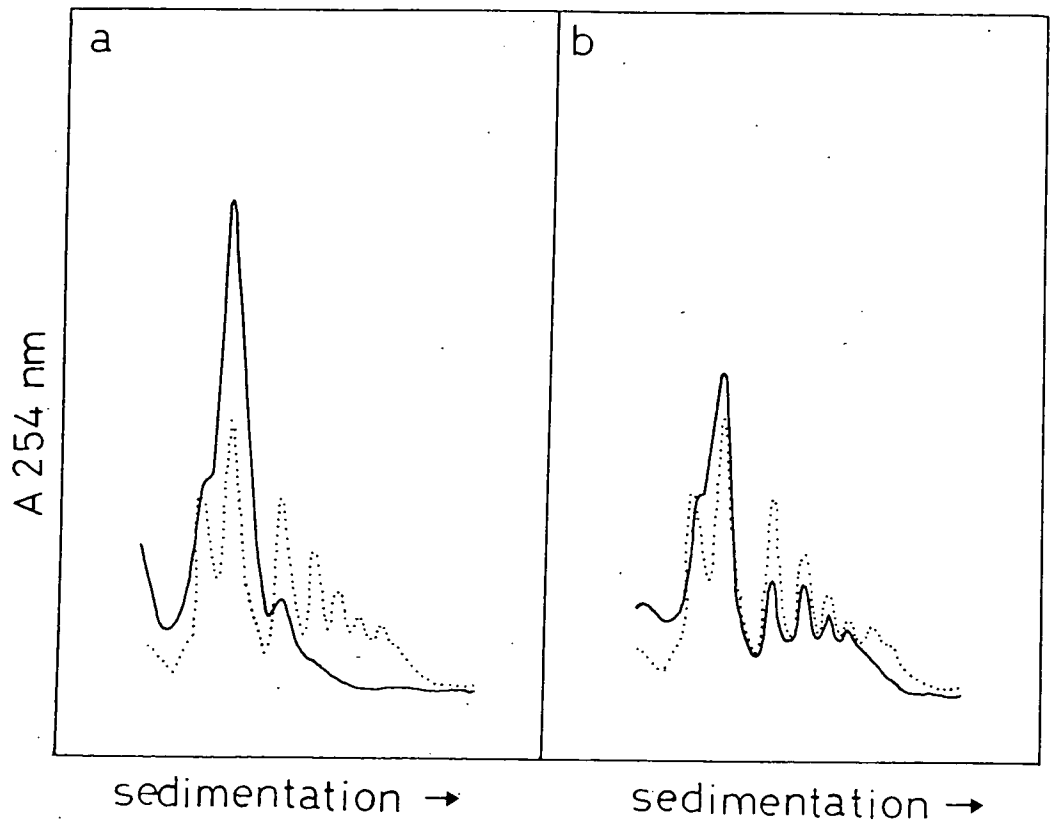
Sucrose density gradient and PAGE profiles of polyribosomes from leaves of F. pratensis

a. incubated in RNAse prior to separation ( —— )

b. incubated in EDTA prior to separation ( —— )

..... normal separation.

FIGURE 2.15


a
b
A 254 nm
sedimentation →
sedimentation →

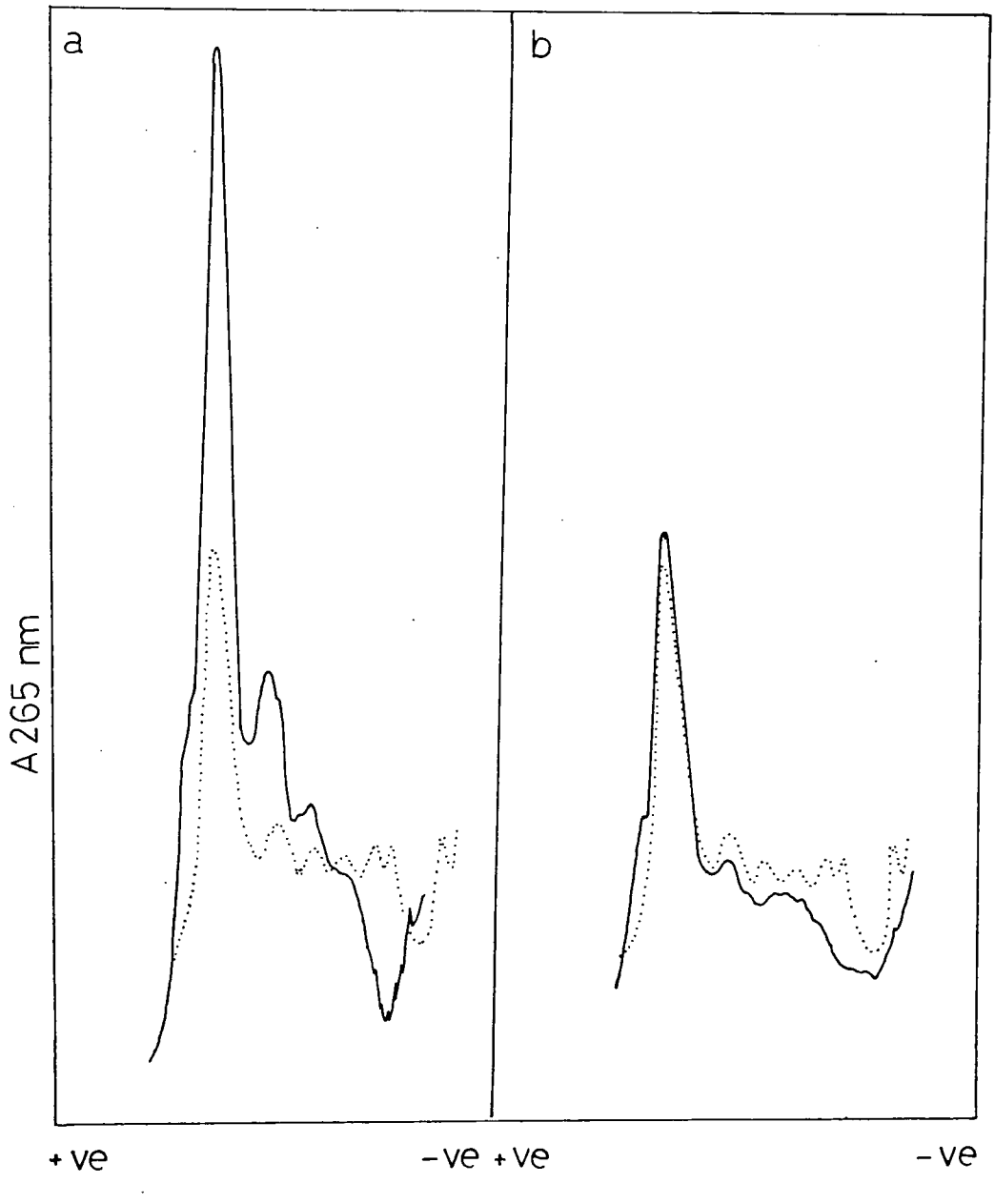

a
b
A 265 nm
+ ve
- ve + ve
- ve

FIGURE 2.16

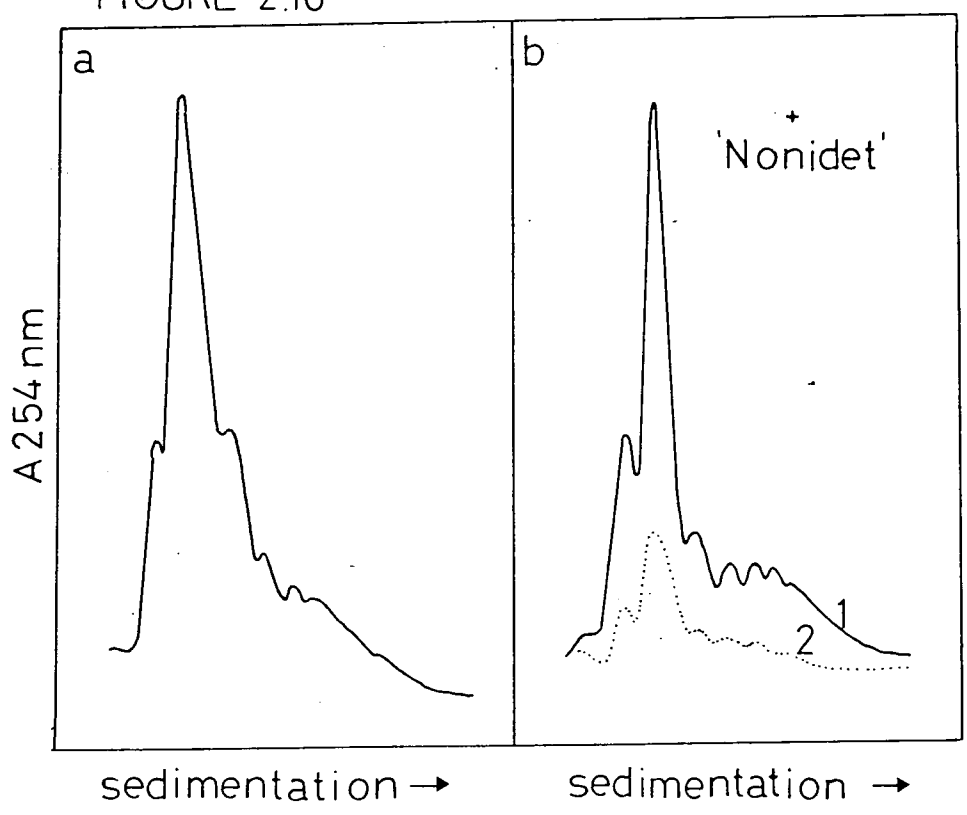
Sucrose density gradient and PAGE profiles of polyribosomes from F. pratensis in the absence (a) or presence (b) of 0.4% (V/V) 'Nonidet' P40.

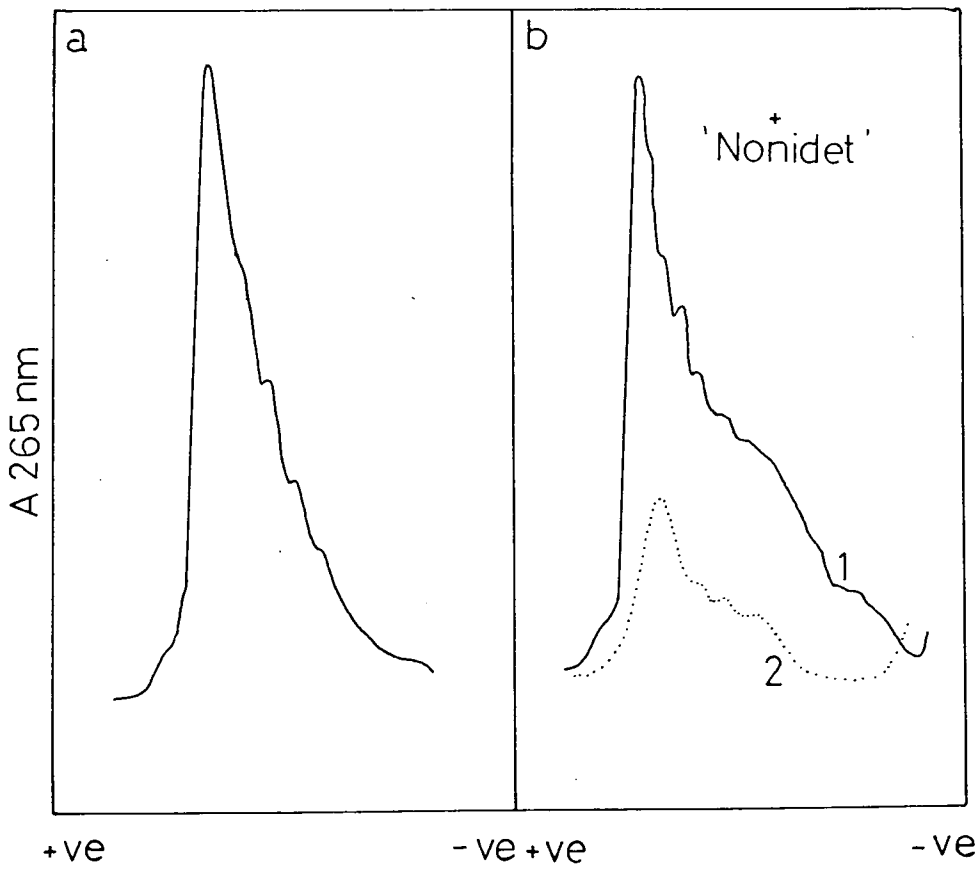
In b, Line 1 (——— ) represents the total polyribosomes extracted in the presence of 0.4% (V/V) 'Nonidet' P40; Line 2 (.....) represents re-extraction of the 10,000 g. pellet from a with 0.4% (V/V) 'Nonidet' P40.

140 µl. were loaded onto sucrose density gradients.

25 µl. were loaded onto PAGE gels.

FIGURE 2.16


a
b
'Nonidet'
A 254 nm
1
2
sedimentation →
sedimentation →


a
b
'Nonidet'
A 265 nm
1
2
+ve
-ve
+ve
-ve

functional significance to these two classes (Setterfield, 1961; Wright, 1963; Payne and Boulter , 1969). Electronmicroscopic studies of plant and animal tissue have suggested that "free" and "membrane-bound" ribosomes might synthesize different proteins (for example, Opik (1968) suggested preferential synthesis of storage proteins by "membrane-bound" ribosomes in seeds of Phaseolus vulgaris.). There are a number of reports of specific polypeptides formed on particular polyribosomes. Synthesis of zein is confined to polyribosomes associated with protein bodies in corn (Burr and Burr, 1976; Larkins et al., 1976); synthesis of the large sub-unit of Fraction I (and not structural proteins) of the chloroplast is generally attributed to free and not thylakoid-bound polyribosomes (Ellis , 1975 ; 1976.).

This polyribosomal preference appears to be, to some extent, species-specific. For example, there is no difference in the polypeptides synthesized in vitro by either "free" or "membrane-bound" polyribosomes from soybeans (Beachy et al., 1978) and although over 50% of tobacco chloroplast ribosomes were found to be associated with the thylakoid they were not responsible for the large sub-unit of Fraction I nor structural protein synthesis (Chen and Wildman, 1970). "Free" ribosomes have frequently been shown to be more active in in vitro protein-synthesizing systems than "membrane-bound" polyribosomes (for example, Rolleston , 1974).

This has led to the suggestion that "membrane-bound" polyribosomes are a store of relatively inactive message. The significance of these two populations is not fully known but their existence and their relationship during development cannot be ignored as their differential functions may provide a means of control during development.

Gordon et al. (1975) found DOC and Triton X-100 equally effective in providing substantially increased yields of polyribosomes. The use of Nonidet has most recently been preferred since it is a relatively mild detergent and of much greater purity than Triton X-100.

A 30 - 40% increase in yield following Nonidet inclusion in the extraction procedure was usually achieved from leaves of Festuca pratensis. The polyribosomal profiles indicated a slight preferential increase in polyribosomes relative to monoribosomes (Figure 2; 16).

x. Radio-isotope labelling of polyribosomes.

This was carried out as described in Section iv. Particular care was taken in handling 2.2% PAGE gels for slicing to prevent their fragmentation and distortion on freezing.

xii. Extraction of mRNA.

It has already been stated (General Introduction) that mRNA extraction has proved the most difficult to achieve. However, many mRNAs contain sequences of poly(A) which can be exploited in order to purify the mRNA (Aviv and Leder, 1972; Matthews, 1973; Higgins et al., 1973; Sagher et al., 1974; Brawerman, 1974; Verma et al., 1974). Histone mRNA (Adesnik et al., 1972), spinach chloroplast mRNA (Hartley, 1976) and yeast mitochondria mRNA (Groot et al., 1974) lack these sequences. Other chloroplasts have been shown to contain a small proportion of poly(A)-containing mRNA; for example maize chloroplasts contain 6% (Haff and Bogorad, 1976). Evidence also suggests that there are a number of cytoplasmic mRNAs which lack poly(A) (Nemer et al., 1974; Milcarek et al., 1974; Fraser, 1975; Grierson and Covey, 1975; Covey and Grierson, 1976; Gray and Cashmore, 1976). Poly(A)-containing mRNA can be extracted by a number of methods based on its affinity binding to a purine base bound to a supporting matrix, for example oligo-dT cellulose (Aviv and Leder, 1972; Fraser, 1975), poly(U)-sepharose (Haff and Bogorad, 1976), millipore filters (Sagher et al., 1974).

The significance of the poly(A) sequence is still much in debate. 70% of HeLa mRNAs and 40% of sycamore mRNAs carry poly(A) sequences (Milcarek et al., 1974; Covey and Grierson, 1976, respectively). Matthews (1973) concluded from studies of the rate of labelling of the poly(A) region compared with the remaining portion of the molecule that poly(A) was added post-transcriptionally. Grierson (1975) and Haff and Bogorad (1976) provided evidence for this in Phaseolus aureus and maize respectively by the inability to detect poly d(T) sequences in the DNA. Milcarek et al (1974) suggest that a large proportion of the poly(A) material in HeLa cells is associated with the membrane even after removal of the associated polyribosomes. Could the poly(A) sequences provide some sort of directive, indicating the site of particular messages and their translational apparatus? The observation of a considerable degree of variation in poly(A) length (for example, Sagher et al., 1974) and reports suggesting that poly(A)-plus and poly(A)-minus mRNAs are

not necessarily functionally different (Lodish et al., 1974; Gray and Cashmore, 1976) have lent support to the suggestion that ordered shortening of these sequences may provide some sort of timing control mechanism (Sheiness and Darnell, 1973). On this basis, estimates of poly(A)-containing mRNA, whilst providing evidence of newly polyadenylated (not necessarily newly synthesized) message need not reflect the translational capacity of the cell since mRNAs containing only short poly(A) sequences (and therefore excluded from affinity chromatography) or without poly(A) may nevertheless be functional. In fact, polyadenylation has been shown not to be a prerequisite for polyribosome formation in early germination (Spiegel and Marcus, 1975).

Whatever the function of the poly(A) sequence and the significance of poly(A)-containing mRNA, it was considered of interest to compare the poly(A) content of F. pratensis at different times during development.

a. Method used.

DEP complexes with adenine residues (Leonard et al., 1970) thus prohibiting its use in the isolation of poly(A)-containing mRNA. The total RNA extraction was performed using the phenol method outlined in Section 2 ii. a. This, too, has been criticised on the basis of differential affinity of the two ends of the mRNA between the phenolic and aqueous phases thus removing the poly(A)-containing mRNA. Special attention was, therefore, paid to retaining the interface region with the aqueous phase. The ethanol precipitated 2,500 g. pellet did not require washing since contaminating phenol was removed in the following procedure modified from that of Fraser and Carter (1976). The pellet was resuspended in 1 ml. "binding buffer" which consisted of:-

| | | |
|---|---|---|
| 10 mM. | Tris-HCl | pH 7.8 |
| 400 mM. | sodium chloride | |
| 1 mM. | EDTA (disodium salt) | |
| | SDS (2 g./1,000 ml.) | |

This was shaken with 20 mg. oligo-dT cellulose for 30 - 60 min. at room temperature, followed by centrifugation at 2,500 g. for 1 min. The pellet was washed four times with 0.5 ml. "binding buffer" and the washes were pooled. Initially, this was then followed by four

successive washes with 0.5 ml. "elution buffer" which consisted of:-

10 mM. Tris-HCl pH 7.8
1 mM. EDTA
SDS (2 g./1,000 ml.)

These washes were pooled and combined with 50 µg. yeast "carrier" s-RNA. Precipitation of the "carrier" RNA and poly(A)-containing RNA was achieved by addition of three times the volume of ethanol and storing at -20°C. overnight.

Additionally, an intermediate elution step was introduced between the "binding buffer" and "elution buffer" washes. This "intermediate buffer" contained 200 mM. sodium chloride.

xiii PAGE.

Pellets obtained after centrifugation of the ethanol precipitate were resuspended in "elution buffer" containing 10% (W/V) sucrose and were applied to 2.6% (W/V) polyacrylamide gels which were electrophoresed according to the method described in section II iii. a. PAGE was only used to demonstrate the distribution of radioactive label entering poly(A)-containing RNA since the carrier RNA would interfere with any spectrophotometric measurements and the levels of mRNA would probably be below the limits of conventional spectrophotometric scanning capabilities. Gels were sliced for radioactive counting as described in Section IV. Figure 2.17 shows a typical distribution of label associated with mRNA and comparison with the yeast "carrier" RNA spectrophotometric scan shows that the criticism of association even under PAGE is not valid.

xiv. Radio-labelling of mRNA.

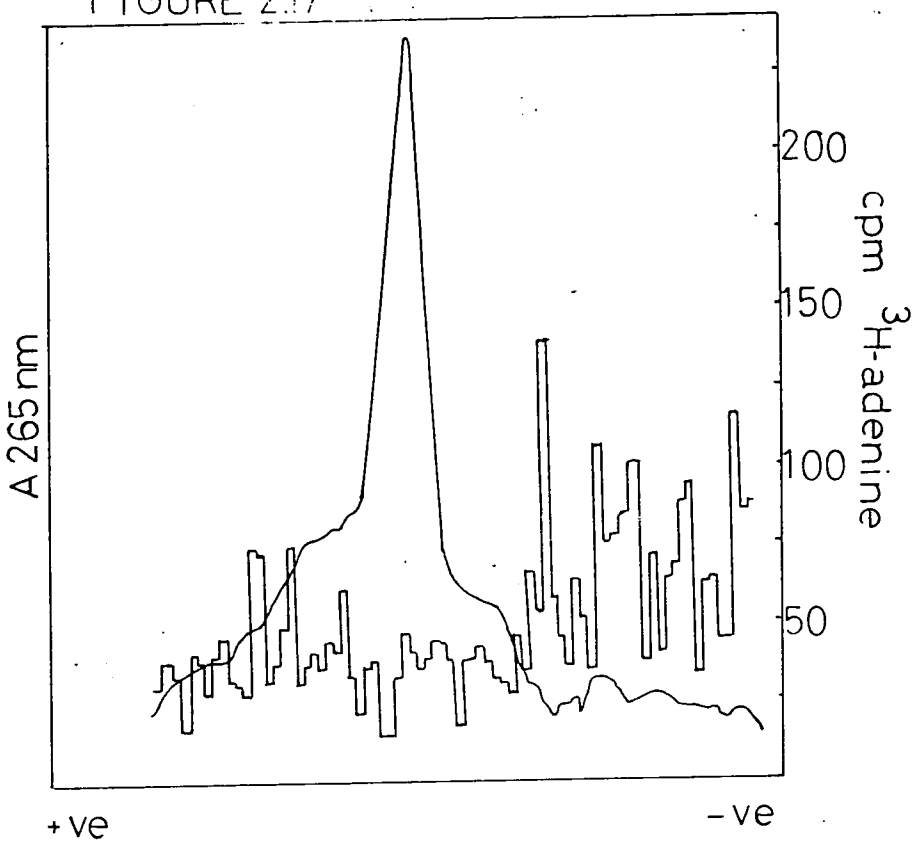
Except when poly(A) was to be used for *in vitro* studies, labelling was carried out as a matter of course. This was because the low levels of poly(A)-containing RNA were below the limits of normal spectrophotometric estimation. Labelling was carried out by floating 1 cm. sterilized leaf sections lower side down in dishes containing 5 µCi/ml. H-adenine in 10 ml. sterilized water for 17 h. at 25°C. in the light.

Small aliquots (50 µl.) of "binder buffer" washes, "intermediate buffer" washes and "elution buffer" washes were counted as described in Section IV.

FIGURE 2.17

PAGE profile of yeast 'carrier' RNA superimposed on the $^{3}$H-adenine distribution of label in poly(A)-RNA (histogram).

FIGURE 2.17



## 3. Experimental.

### i. Total Nucleic Acids.

#### a. Quantitative estimation of total nucleic acids.

Figure 2.18 shows the yield of nucleic acid obtained per leaf at different stages following emergence of the fourth leaf ( ≙ Day 20). Nucleic acid levels rose rapidly during early growth, levelling out and finally decreasing during senescence. If the data are expressed on a unit area basis (Figure 2.18 B) the decline in nucleic acid content appears to occur earlier. However, comparison with Figure 1.1, Chapter 1, would suggest that elongation rather than cell division occurred at (a) and would therefore account for the apparent decline in nucleic acid levels. Comparison of Figures 2.18 A and B would imply that at (b) genuine degradation of nucleic acid has occurred.

Figure 2.19 shows the yield of nucleic acid extracted from proportional sections from mature leaves of F. pratensis. Three other experiments all showed similar peaks of increased yield of the same order but these were either shifted acropetally or the width of the peak varied slightly. Since these experiments were done with plants grown under different conditions, these slight deviations can be regarded as arising from this (see Chapter 1). The decline at point 3 in Figure 2. 19 may also be an artifact of elongation rather than cell division.

#### b. Incorporation of radio-isotope label into nucleic acid.

The incorporation of $^{3}$H-adenine into nucleic acid, as a measure of nucleic acid synthesis, was followed in development of the fourth leaf and development along the fully mature leaf of F. pratensis. With reference to Figure 2.20, it can be seen that both developmental systems displayed at least two (and probably three) peaks of increased specific activity. With reference to chlorophyll levels (Figures 1.1 B and 1.2 B) and cellular morphology (as visualized by light microscopy and electronmicroscopy) these

FIGURE 2.18

Quantitative estimation of nucleic acid at different stages in development of the fourth leaf of F. pratensis expressed as

A. µg. per leaf, and

B. µg./unit area.

FIGURE 2.18

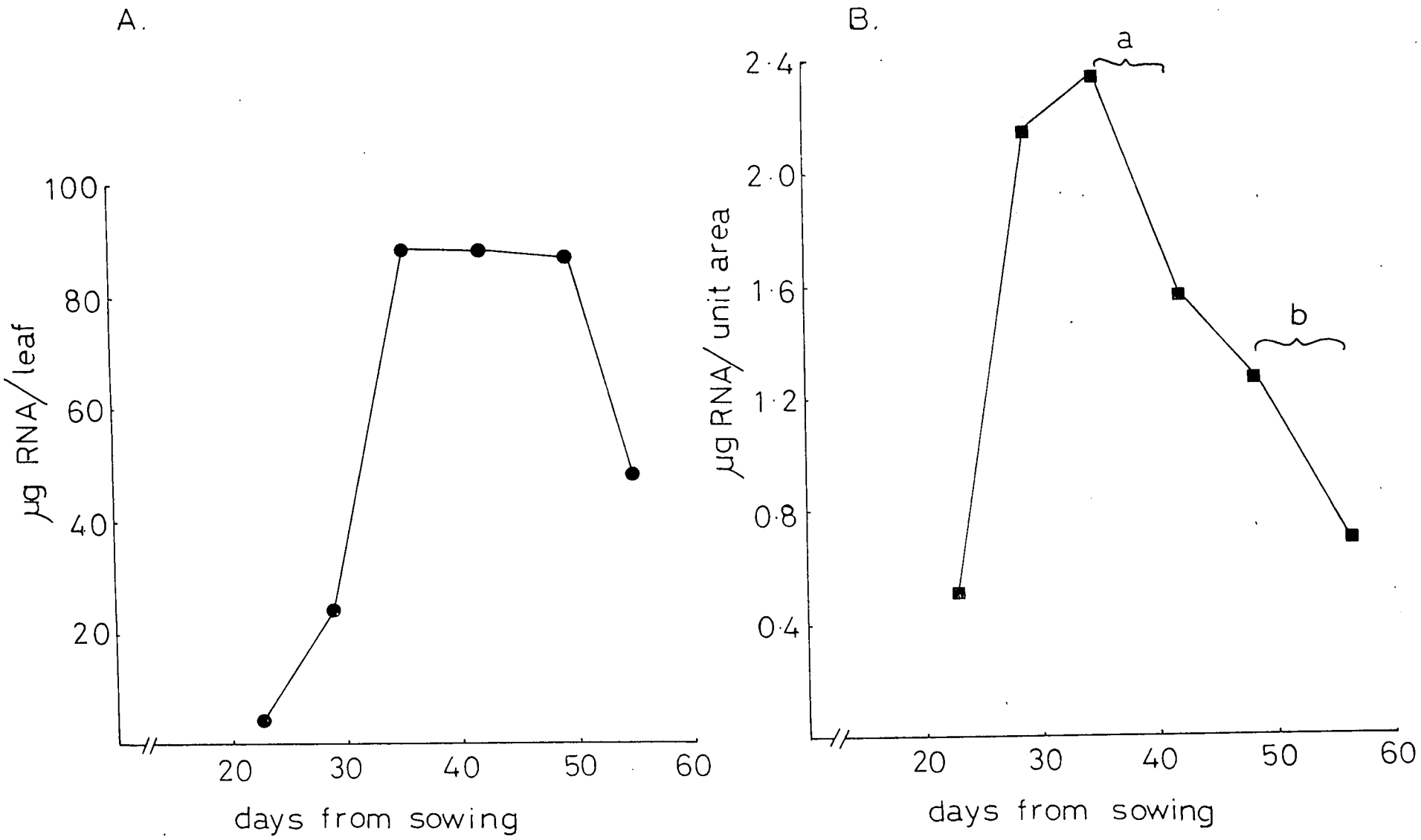

A.
100
80
60
40
20
µg RNA/leaf
20
30
40
50
60
days from sowing
B.
a
b
2·4
2·0
1·6
1·2
0·8
0·4
µg RNA/unit area
20
30
40
50
60
days from sowing

FIGURE 2.19

Quantitative estimation of nucleic acid along a fully developed leaf of *F. pratensis*.

FIGURE 2.19

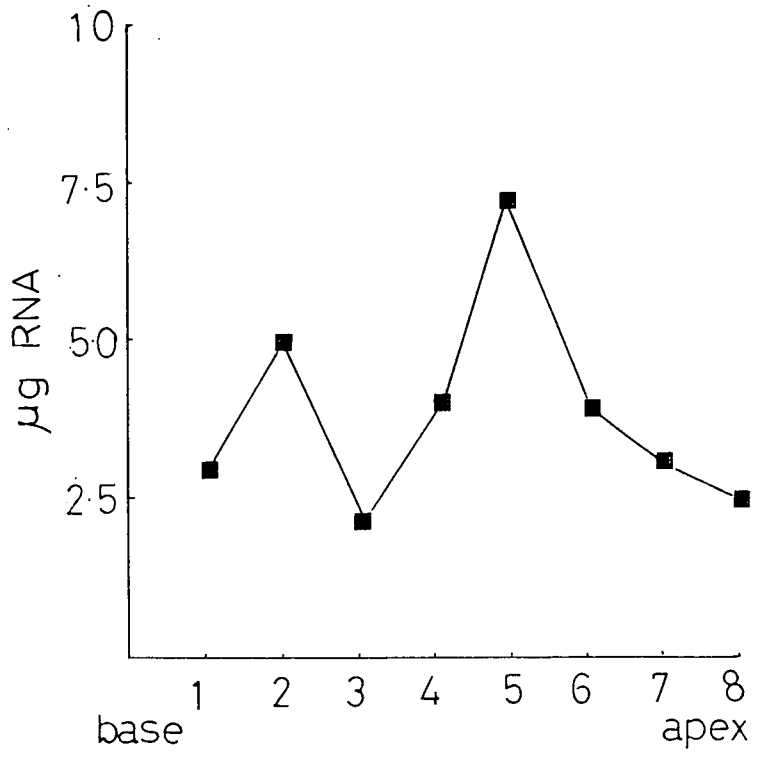


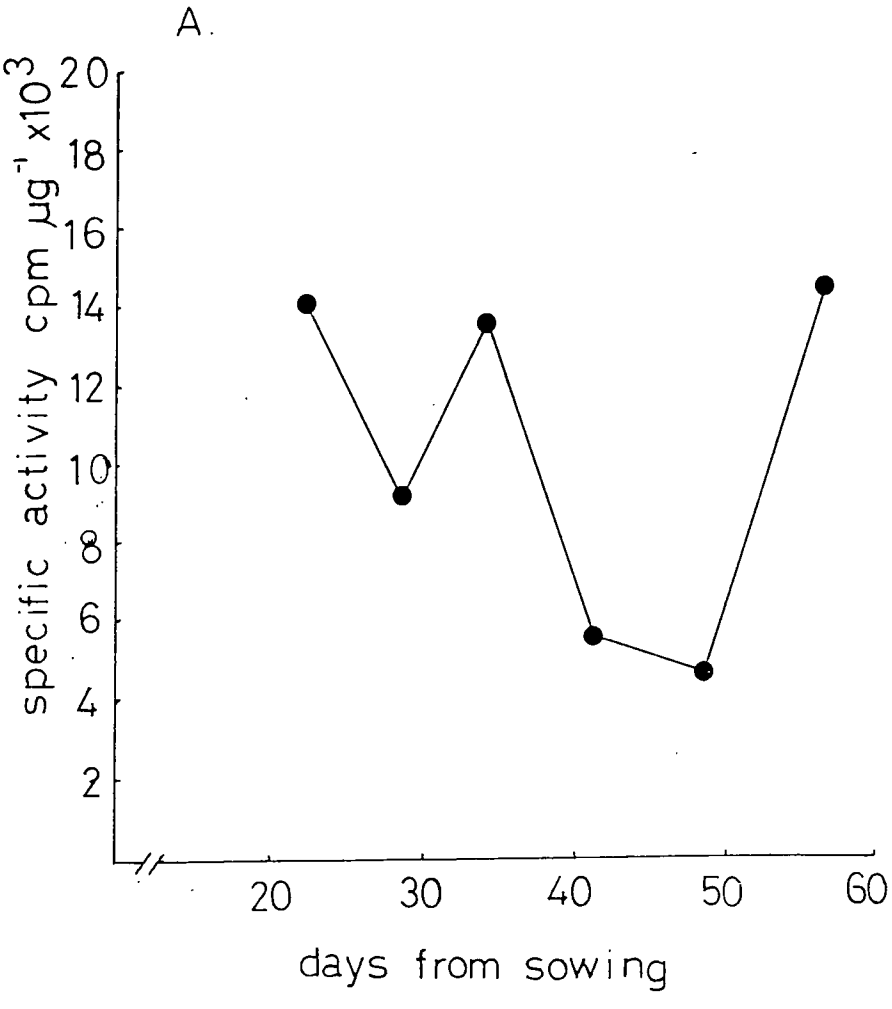
FIGURE 2.20

Specific activities of radioisotope labelling of RNA in development leaves of F. pratensis.

A. fourth leaf at different stages in development.

B. along a fully mature leaf.

FIGURE 2.20


A.
specific activity cpm µg⁻¹ x10³
20
18
16
14
12
10
8
6
4
2
20
30
40
50
60
days from sowing

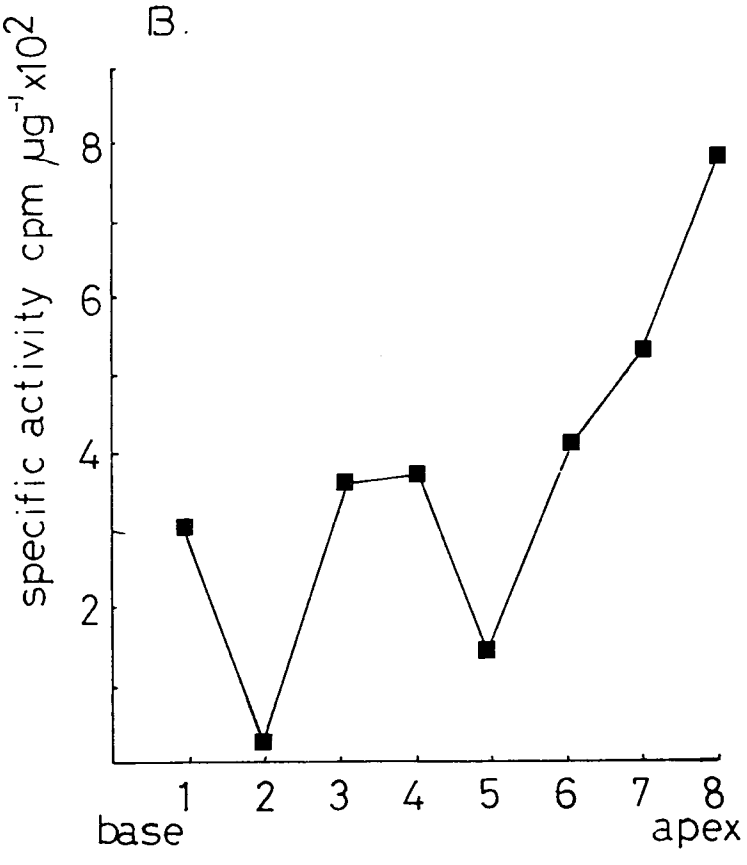

B.
specific activity cpm µg⁻¹x10²
8
6
4
2
1
2
3
4
5
6
7
8
base
apex

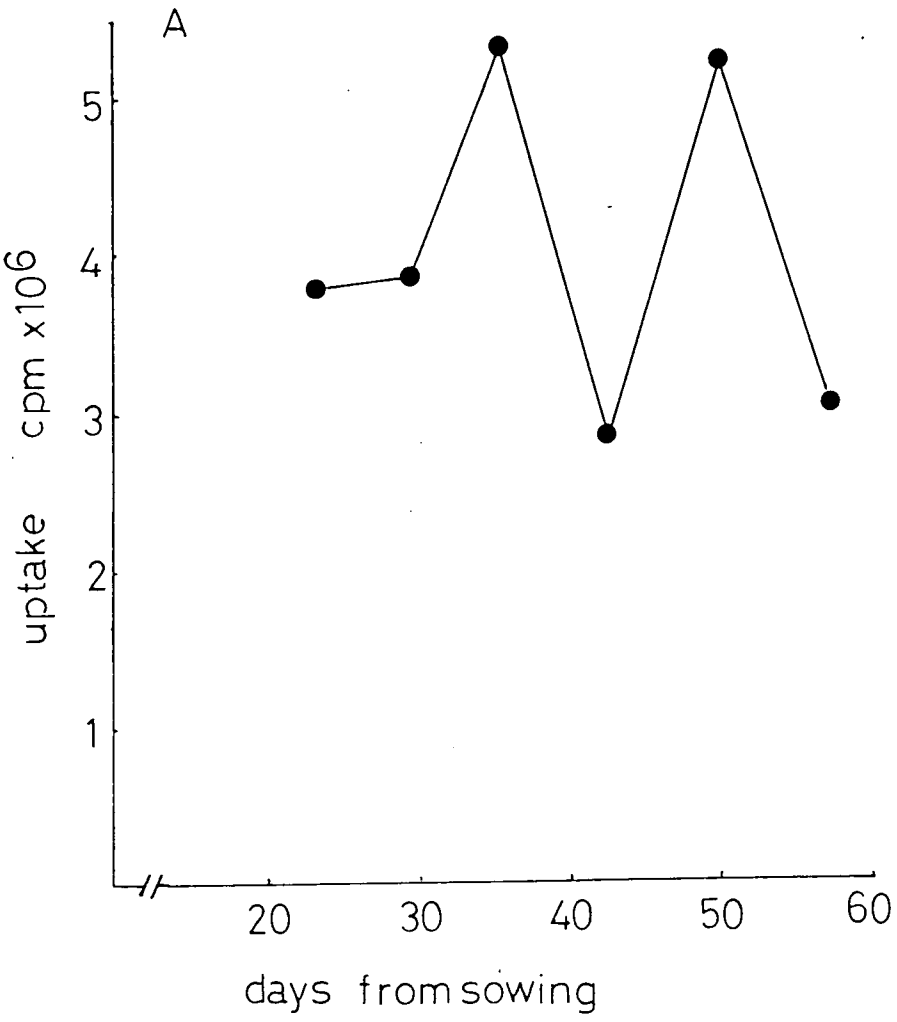
FIGURE 2.21

Absolute levels of uptake of radioisotope.

A. the fourth leaf of *F. pratensis* during development

B. along the fully mature leaf.

FIGURE 2.21


A
uptake cpm x10^6
5
4
3
2
1
20
30
40
50
60
days from sowing

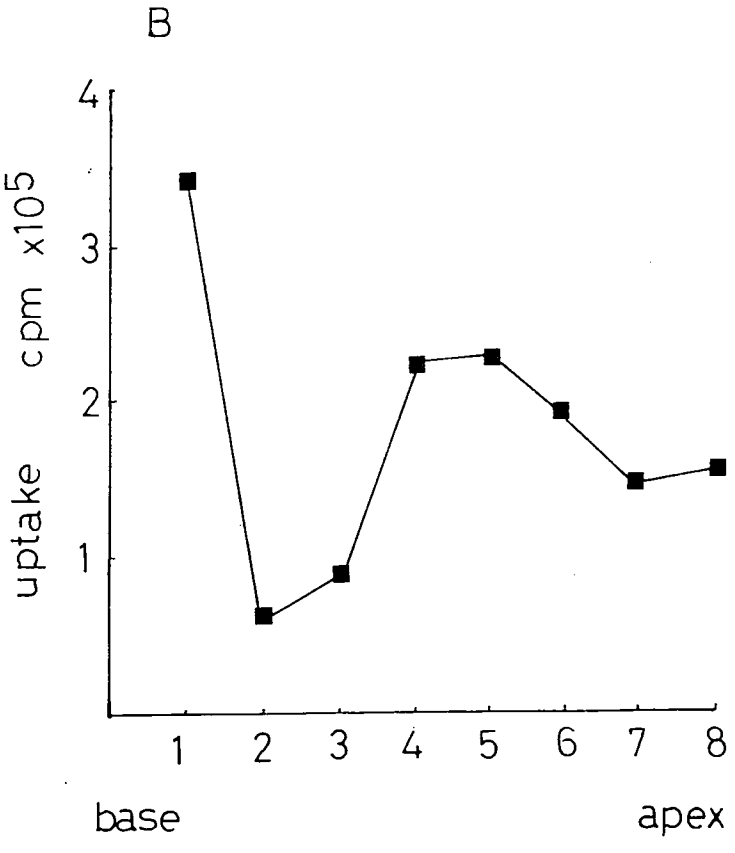

B
uptake cpm x10^5
4
3
2
1
1
2
3
4
5
6
7
8
base
apex

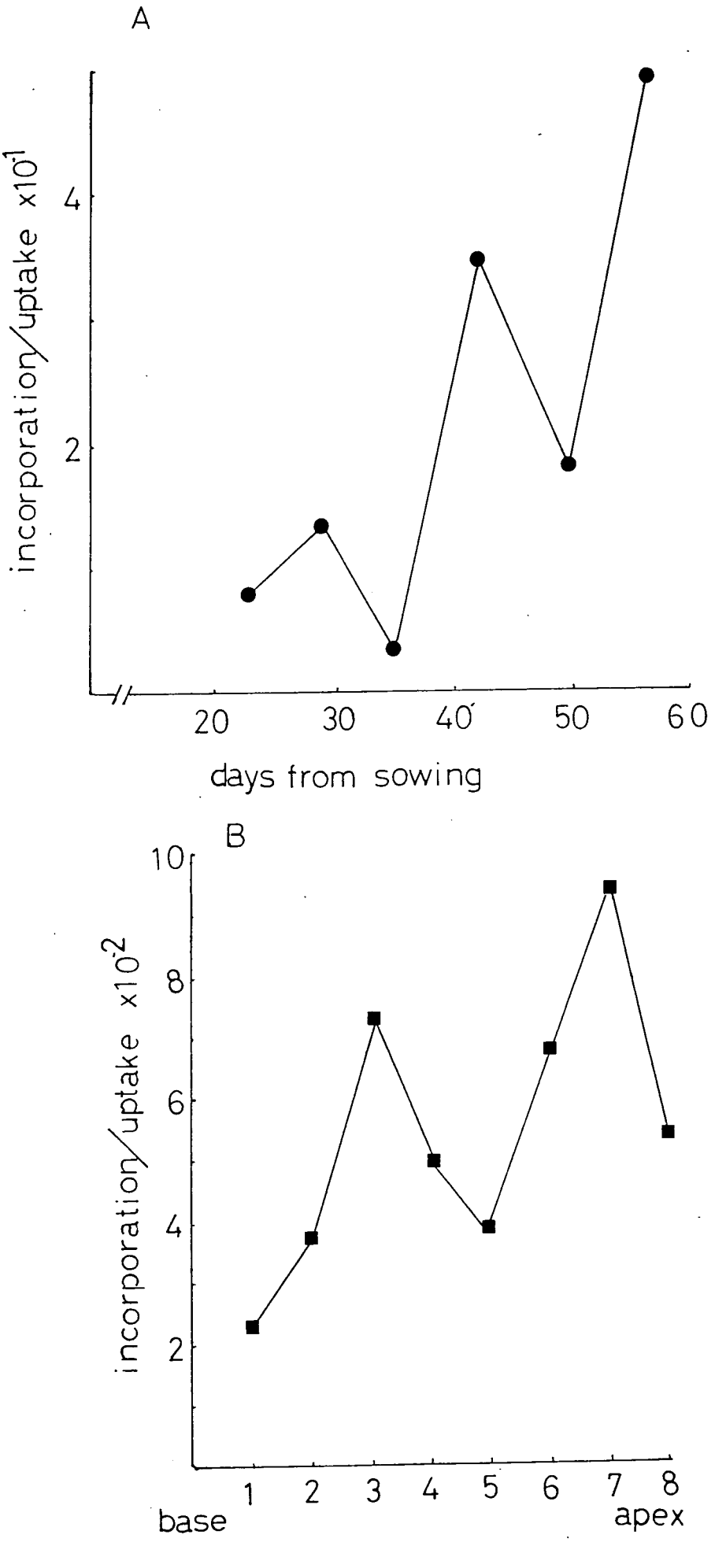
FIGURE 2.22

Ratio of incorporation to uptake of radioisotope label

A. in the fourth leaf in *F. pratensis* during development

B. along the fully mature leaf.

FIGURE 2.22


A
incorporation/uptake x10⁻¹
4
2
20
30
40
50
60
days from sowing
B
incorporation/uptake x10⁻²
10
8
6
4
2
1
2
3
4
5
6
7
8
base
apex

peaks can be attributed to more or less similar periods in development.

The first peaks, coincident with whole leaf emergence or greening of the leaf base, were consistent with many reports of light enhanced RNA synthesis (Chapter 2 I, ii.).

The probable second peak was less familiar but may reflect the synthesis of RNA required for the coding and translation of proteins associated with maturity (for example, some of those involved in secondary product metabolism.).

The third peak was consistent with a number of reports, see (Chapter 2 I, iv.). In this instance the final peaks are large in comparison to the previous two. This may be partly due to a decrease in RNA content which may reflect in vivo loss or increased RNAse activity as a result of wounding (Pitt, 1974). Nevertheless, RNA levels at senescence were of the order apparent at early stages of development (Figures 2.18 and 2.19) and these values may be underestimates in view of phenolic contamination in extracts from older material, so synthetic activity is justifiably implicated. This challenges the suggestion that senescence occurs independently of RNA synthesis. It could be argued that membranes of older tissue become "leaky" (Simon, 1974) thus providing increased pools of radio-isotopically labelled precursor for RNA synthesis. However, from the values for total uptake of radio-isotope (Figure 2.21) and ratio of incorporation to uptake (Figure 2 2 2) this does not provide a valid interpretation of the enhanced incorporation at incipient senescence.

Criticism can be levelled at the interpretation of radio-isotope labelling through specific activities, not least of which is the order of magnitude involved in each piece of data. Since different conditions necessarily prevailed for different experiments specific activities provide the least controversial basis for comparisons.

c. Relative changes in specific RNA fractions.

Using 2.6% (W/V) PAGE gels more detailed information concerning the metabolism of particular ribosomal fractions was obtained.

Figures 223 and 224 show the percentage of each fraction of rRNA as a function of the total rRNA (and breakdown products). Point 8 along the leaf was not recorded since the levels of rRNA were below the sensitivity of the spectrophotometer. In addition, the increased levels of endogonous phenolics in older tissue (apical and Day 56) prevented conclusive estimation of peak areas from being made.

In different aged tissue the cytoplasmic rRNAs declined with age with a consequent increase in breakdown products. Ageing along the leaf showed an overall decline in cytoplasmic rRNAs apart from two points where an increase was apparent (Points 5 and 7). It is interesting to note that at these points the $0.38 \times 10^5$ rRNA also seemed to increase. Since the inclusion of the $0.38 \times 10^5$ rRNA in the ribosome has been regarded as conferring functional status on the ribosome (Pearson, personal communication) its presence would suggest that at Points 5 and 7 the ribosomes were active or at least have had the capacity to be so. There is no such discontinuity in the differently aged tissue either in the $1.3 \times 10^6$, $0.7 \times 10^6$, or the $0.38 \times 10^5$ rRNAs.

The $1.1 \times 10^6$ rRNA declined earlier than the $1.3 \times 10^6$ rRNA in the ontogenic sequence along the leaf. The proportion of $0.56 \times 10^6$ rRNA appeared to increase slightly but this was probably due to inflation by breakdown products. In the development with time there was also a decrease in $1.1 \times 10^6$ rRNA which preceded the $1.3 \times 10^6$ rRNA loss. Again, the $0.56 \times 10^6$ rRNA increase might have reflected breakdown of other rRNAs, particularly when compared with the high proportion of other breakdown products.

The early decline in $1.1 \times 10^6$ rRNA could have reflected a shift in the optimal conditions required for the maintainance of its integrity, for example $Mg^{2+}$ requirements. Other results where variable ~~light~~ $Mg^{2+}$ concentrations were employed showed no enhancement of the $1.1 \times 10^6$ rRNA from senescent tissue. However, the possibility of some other non-optimal factor in extraction cannot be excluded; for example RNAse levels may be raised above those successfully inhibited by DEP in the extraction. Although specific RNAses have

FIGURE 2.23

Yield of $1.3 \times 10^6$ rRNA (25S) and $0.7 \times 10^6$ rRNA as % of the total RNA extracted, calculated from 2.6% PAGE gel profiles

A. in different aged leaves of *F. pratensis*

B. in sections along mature leaves of *F. pratensis*.

▲·····▲ } refer to the percentage
▼·····▼ } of breakdown products at each phase.

FIGURE 2.23

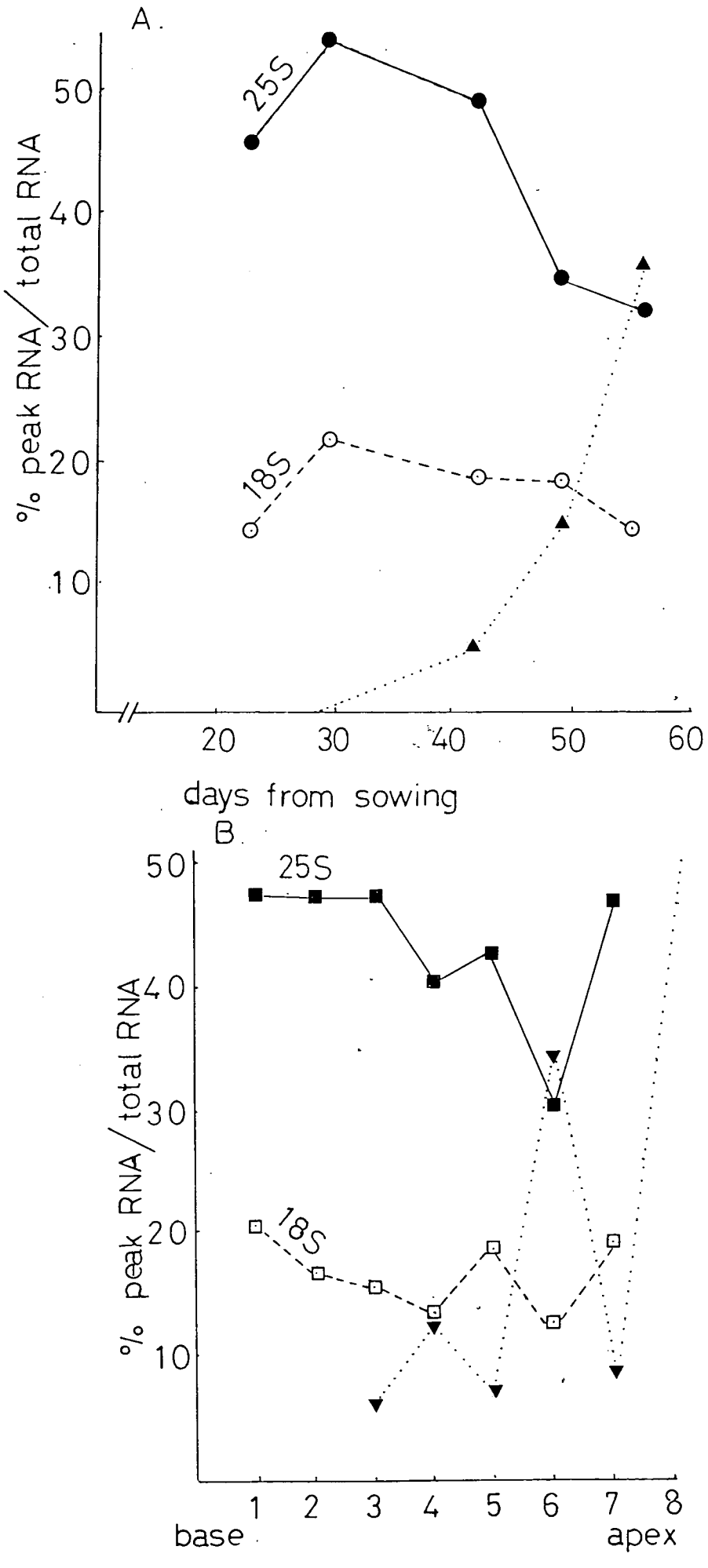

A.
25S
18S
% peak RNA / total RNA
50
40
30
20
10
20
30
40
50
60
days from sowing
B.
25S
18S
% peak RNA / total RNA
50
40
30
20
10
1
2
3
4
5
6
7
8
base
apex

FIGURE 2.24

Yield of $1.1 \times 10^6$ (23S) and $0.56 \times 10^6$ (16S) rRNAs. rRNA as % of the total RNA extracted, calculated from 2.6% PAGE gel profiles

A. in different aged leaves of F. pratensis

B. in sections along mature leaves of F. pratensis.

▲.....▲ ▼.....▼ refer to the percentage of breakdown products at each phase.

FIGURE 2.24

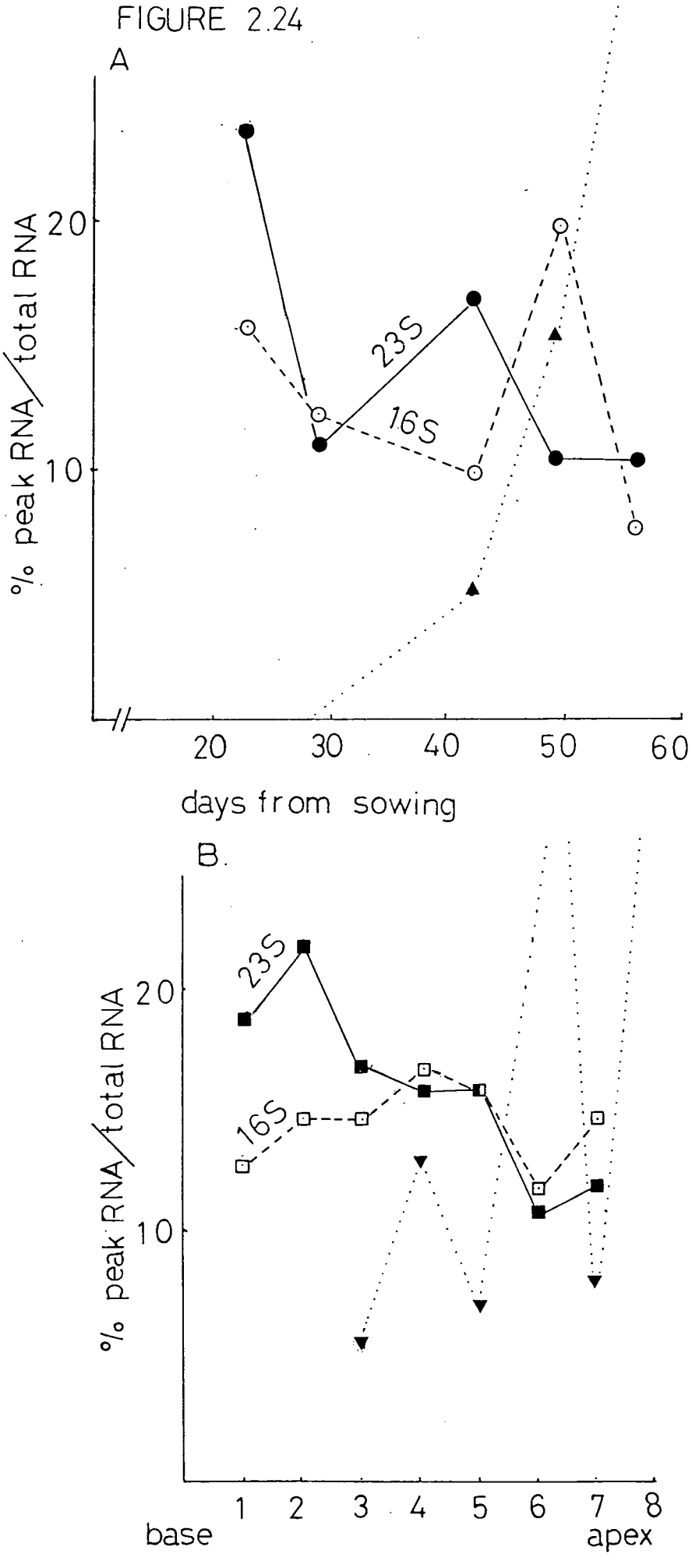

A
% peak RNA / total RNA
20
10
23S
16S
20
30
40
50
60
days from sowing
B.
% peak RNA / total RNA
20
10
23S
16S
1
2
3
4
5
6
7
8
base
apex

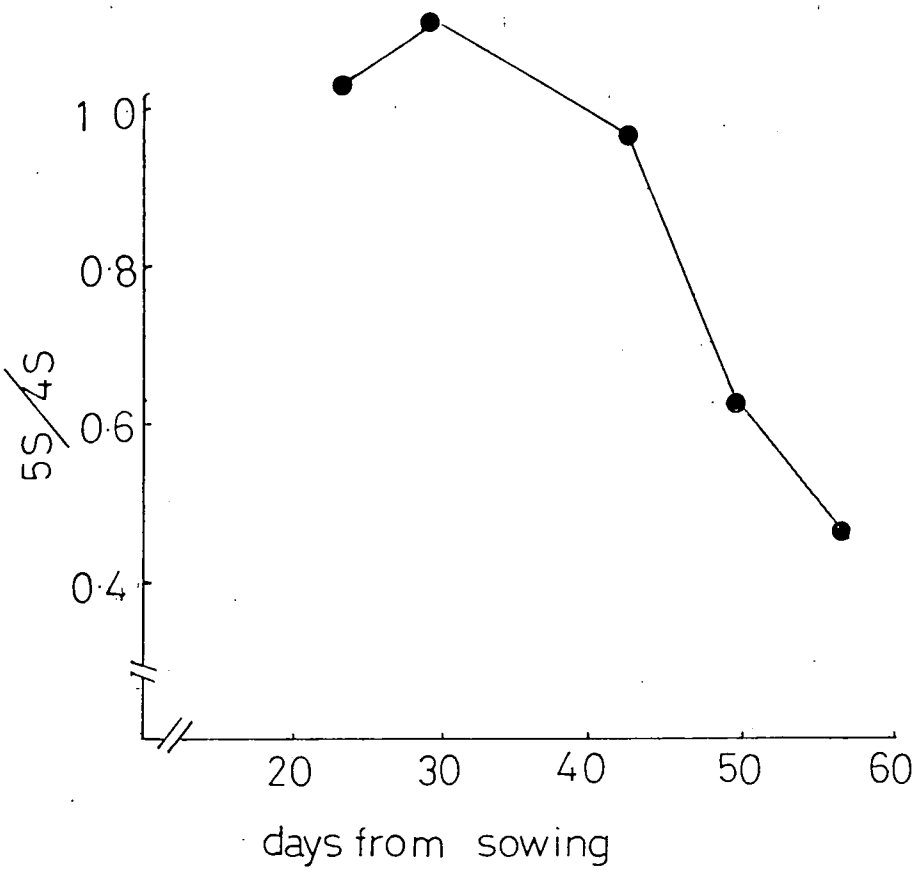
FIGURE 2.25

Ratio of (5S):(4S), $0.38 \times 10^5 : 0.25 \times 10^5$ rRNAs as calculated from 7.5% PAGE gel profiles

A. in different aged leaves of *F. pratensis*

B. in sections along mature leaves of *F. pratensis*.

FIGURE 2.25


1·0
0·8
0·6
0·4
5S/4S
20
30
40
50
60
days from sowing

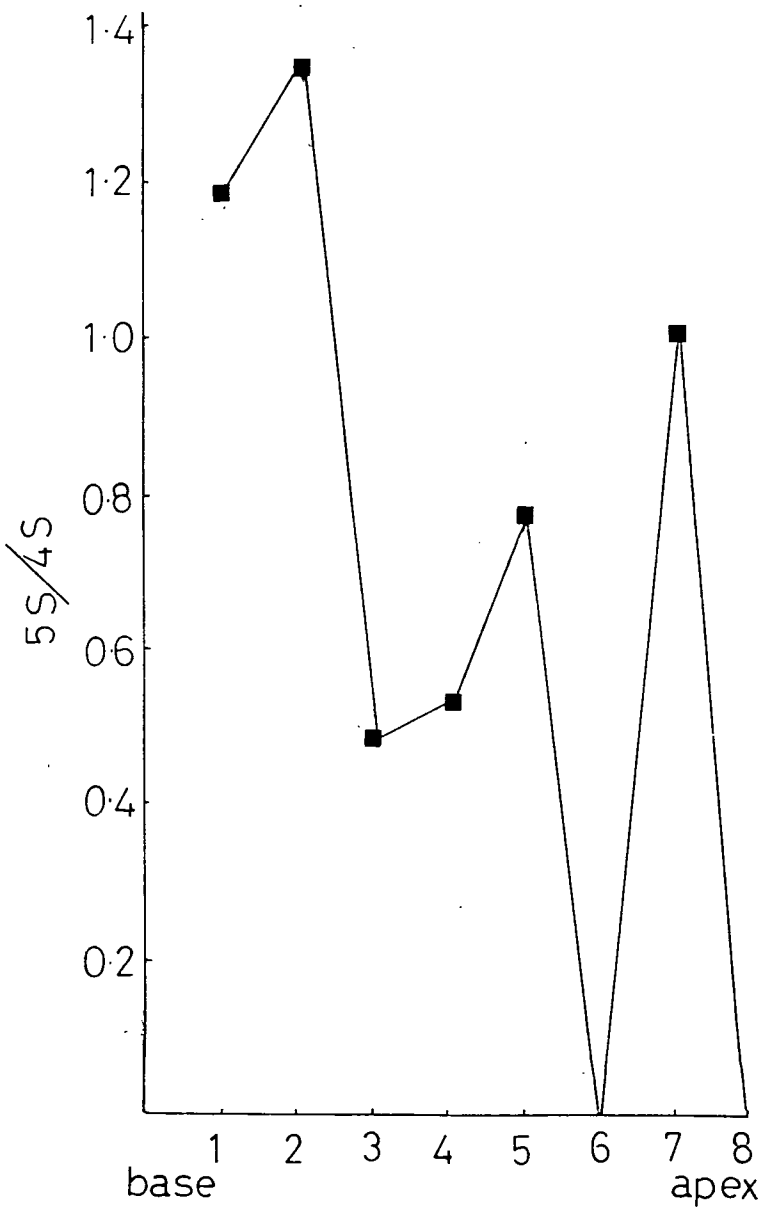

1·4
1·2
1·0
0·8
0·6
0·4
0·2
5S/4S
1
2
3
4
5
6
7
8
base
apex

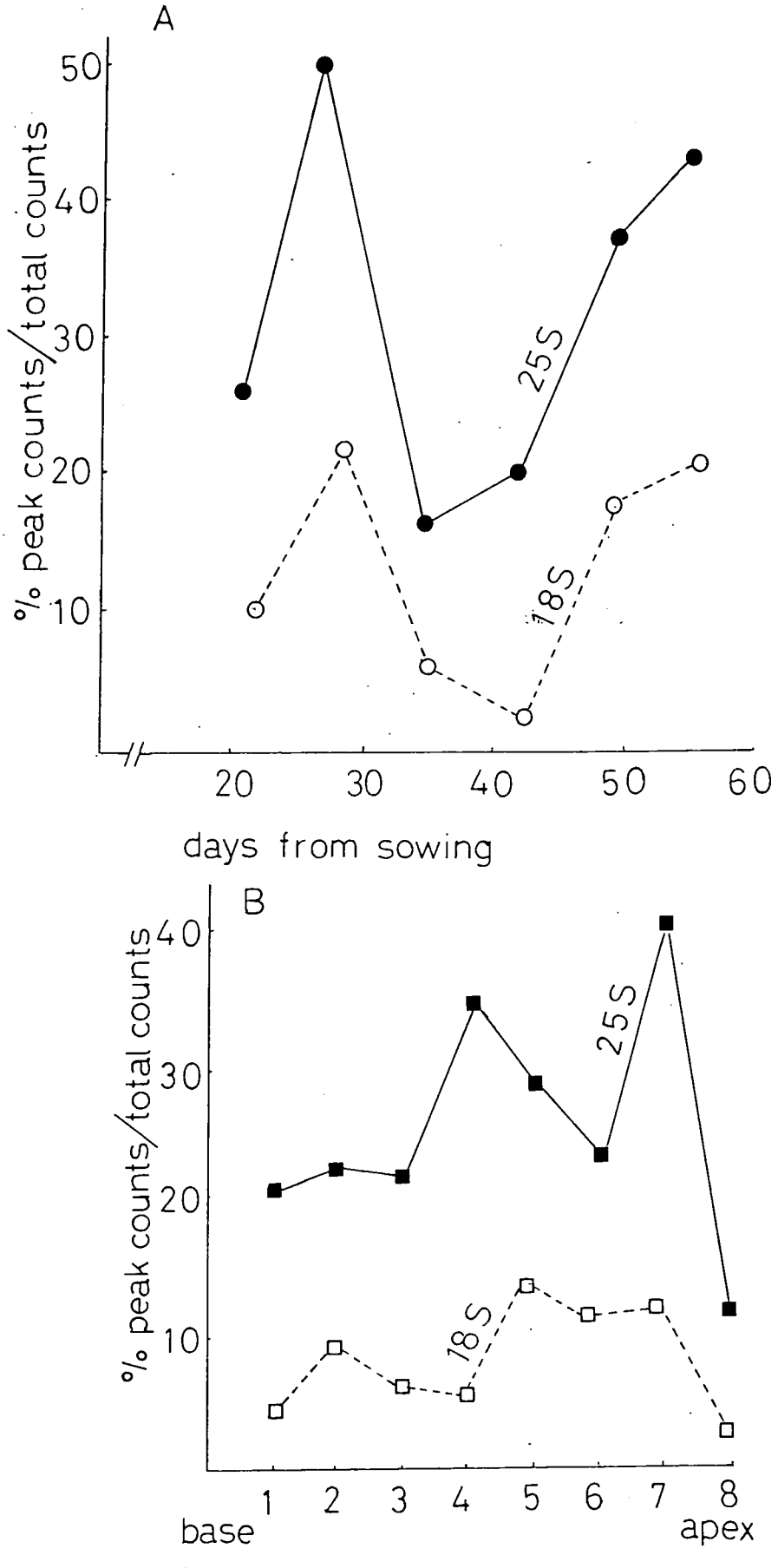
FIGURE 2.26

Cpm. incorporated into $1.3 \times 10^6$ (25S) and $0.7 \times 10^6$ (18S) cytoplasmic rRNAs as percentages of the total cpm. entering the 2.6% PAGE gel

A. in different aged leaves of *F. pratensis*

B. in sections along mature leaves of *F. pratensis*.

FIGURE 2.26


A
50
40
30
20
10
% peak counts/total counts
25S
18S
20
30
40
50
60
days from sowing
B
40
30
20
10
% peak counts/total counts
25S
18S
1
2
3
4
5
6
7
8
base
apex

been observed those resulting from wounding and senescence are generally regarded as being ubiquitous in their effect. It therefore seems unlikely that only chloroplast rRNA would be thus affected by their release in extraction, unless the RNAse is attached to the $1.1x10^6$ RNA (Hsaio, 1968). Even if the conditions required for extraction have changed this would still be evidence of an age-related change in chloroplast rRNA. It would seem however that the breakdown of $1.1x10^6$ rRNA was an in vivo event probably resulting from the disruption of chloroplast structure.

d. Radio-isotope labelling of specific RNA fractions.

As a result of discrepancies arising from phenolic contamination in spectrophotometrically estimating RNAs from older tissue and in order to create uniformity in data it was decided to compare the radio-isotope labelling of different fractions by reference to the total radio-isotope label entering the gel. Both ageing systems showed considerable RNA synthesis of both cytoplasmic and chloroplastic rRNAs quite late in development. In both, the early maximal synthesis of chloroplastic rRNA preceded maximal synthesis of cytoplasmic rRNA. The synthesis of $1.3x10^6$ and $0.7x10^6$ rRNA appeared to be closely linked in both developmental systems but the link between $1.1x10^6$ rRNA and $0.56x10^6$ rRNA synthesis seemed more tenuous. This independence is in accord with other work which has suggested that the $1.1x10^6$ and $0.56x10^6$ rRNAs are not derived from the same precursor (eg. Callow et al, 1972).

The increased levels of rRNAs apparent in Figure 2.23 B were mirrored by increased synthesis (Figure 2.26 B) thus further confirming the real nature of these increases. The increased synthesis of rRNAs at incipient senescence in development with time was not mirrored by any increase in percentage of these fractions. This, however, probably indicates rapid turnover of rRNA at this stage as also evidenced by an increase in breakdown products over that expected for the observed decline in rRNA. Furthermore, since the whole leaf is taken for extraction any increase in RNA, which

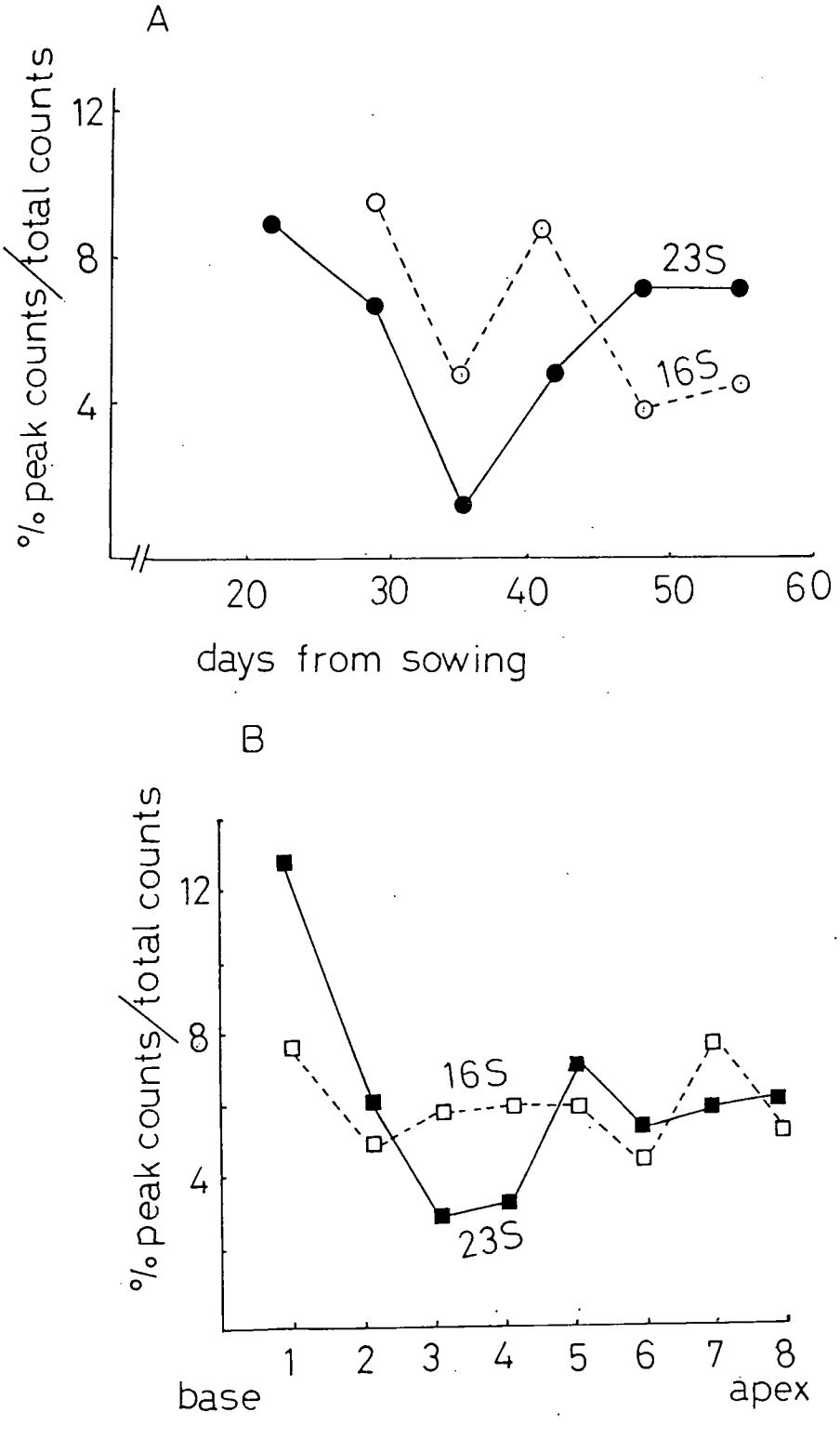
FIGURE 2.27

Cpm. incorporated into $1.1 \times 10^6$ (23S) and $0.56 \times 10^6$ (16S) chloroplastic rRNAs as percentages of the total cpm. entering the 2.6% PAGE gel

A. in different aged leaves of *F. pratensis*

B. in sections along mature leaves of *F. pratensis*.

FIGURE 2.27


A
% peak counts/total counts
12
8
4
23S
16S
20
30
40
50
60
days from sowing
B
% peak counts/total counts
12
8
4
16S
23S
1
2
3
4
5
6
7
8
base
apex

may in fact be localised (for example as apparent in points 5 and 7 along the leaf), may be diluted by the high levels elsewhere.

The fact that the apparently increased levels of $0.56 \times 10^6$ rRNA at Day 49 were not reflected by increased synthesis may provide further evidence that this fraction has been artificially inflated by breakdown products of non-radioisotope-labelled older rRNAs.

ii. Radiolabelling of nucleotides: estimation of changes in the pool.

The relative amounts of radio-isotope associated with various adenine derivatives were obtained. Spectrophotometric estimations were frequently at the limits of instrument reliability and so, whilst informative to some extent (for example, high levels of labelled adenine and adenosine were present in young and old tissue, with a concomitant decline in labelled nucleotides), were not used for specific activity calculations. Comparison of elutable counts from the HVPE paper were considered a more reliable indicator of metabolic pools. However, whilst these data may provide some indication of available radioisotope-containing pool they do not take into account any compartmentalization that may limit the accessibility of some or all of these precursors. These precursors may be so located as to predetermine their metabolic pathway, possibly in such a way as to preclude entry into nucleic acids. Pertinent to the question of compartmentalization is the observation of Nierlich (1967; 1968) that there is a preferential incorporation of endogenous products of RNA breakdown rather than use of exogenous precursors in bacteria. If this were so in the case of higher organisms then at a time when turnover was high the resultant incorporation of radioisotopically-labelled precursors might be low. Radio-isotopic labelling, under these circumstances, would not provide a good indication of synthetic activity.

The levels of labelled AMP and ATP appeared to be inversely related until the oldest tissue when label appeared to be preferentially incorporated into ADP. It is possible that where AMP was

FIGURE 2.28

Cpm. entering nucleotide fractions as percentage of the total counts entering the pool.

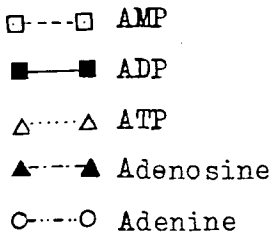
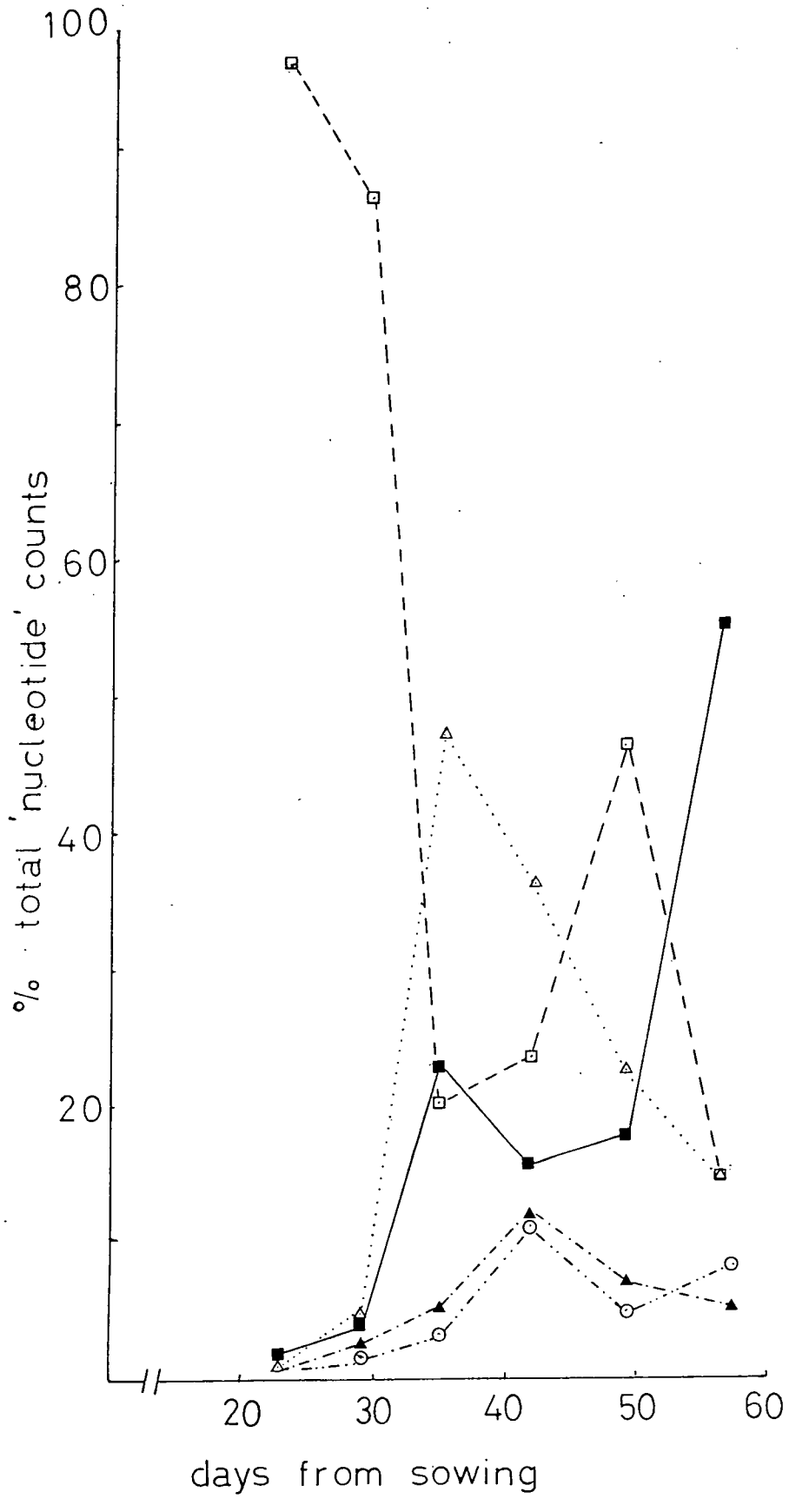


FIGURE 2.28


100
80
60
40
20
% total 'nucleotide' counts
20
30
40
50
60
days from sowing

labelled preferentially this indicated high metabolic activity, radioisotope label passing through ATP and ADP, and that stages with high radioisotopically-labelled ATP levels (Day 35 - 49) indicated a build-up of energetic capacity, only possible when the leaf stopped growing and prior to senescence involving high metabolic activity once again.

iii. Ribosomes.

a. Quantitative estimation of ribosomes.

Ribosomes were extracted by method I using 10 mM. $Mg^{2+}$ and by method 2 (see Chapter 2, 2 vi. a) from proportional sections along the leaf. It was found to be necessary to carry out the extractions and estimations simultaneously; freezing of the leaf tissue for 24 h. storage at -20°C and freezing the ribosome 100000 g pellet resulted in lowered yields. The yield per section is shown in Figure 2.29 (each section was between 3 - 4.5 cm. long.). There were two peaks of increased yield as was apparent for total nucleic acid yield (Figure 2.19). The second peak was slightly shifted acropetally in the total ribosome estimations, which may result from differences in the ease with which membrane-bound RNA was released by the different methods of extraction since free ribosomes are unlikely to be lost. For example, at point 5 (Figure 2.19) there was an increase in yield probably deriving from bound ribosomes (Figure 2.30) which were, perhaps, incompletely released by the milder detergent, Nonidet, used in ribosome extractions. Alternatively this shift could result, as in other experiments, from even slight differences in growth conditions to which the plants have been subjected which resulted in differential development.

Figure 2.30 shows the ratio of free to membrane-bound ribosomes (extracted by method 2) from proportional sections along the leaf of F. pratensis. The number of free ribosomes increased dramatically with incipient senescence. There was also an increase midway along the leaf which corresponded to a period of increased synthesis of nucleic acid (Figures 2.20 and 2. 26) and also one where a number of ribosome breakdown products were in evidence (Figures 2.23 and 2.24). This may be symptomatic of a period which follows completion of cell division, of high turnover or replacement of ribosomes which have been previously associated with early growth. It is at this time that the ratio of polyribosomes to monoribosomes dropped (Figure 2.31) as would be expected if the

FIGURE 2.29

Yield of polyribosomes per section along the mature leaf of F. pratensis

1. Ribosomes extracted by method 1.
2. Ribosomes extracted by method 2.

FIGURE 2.29

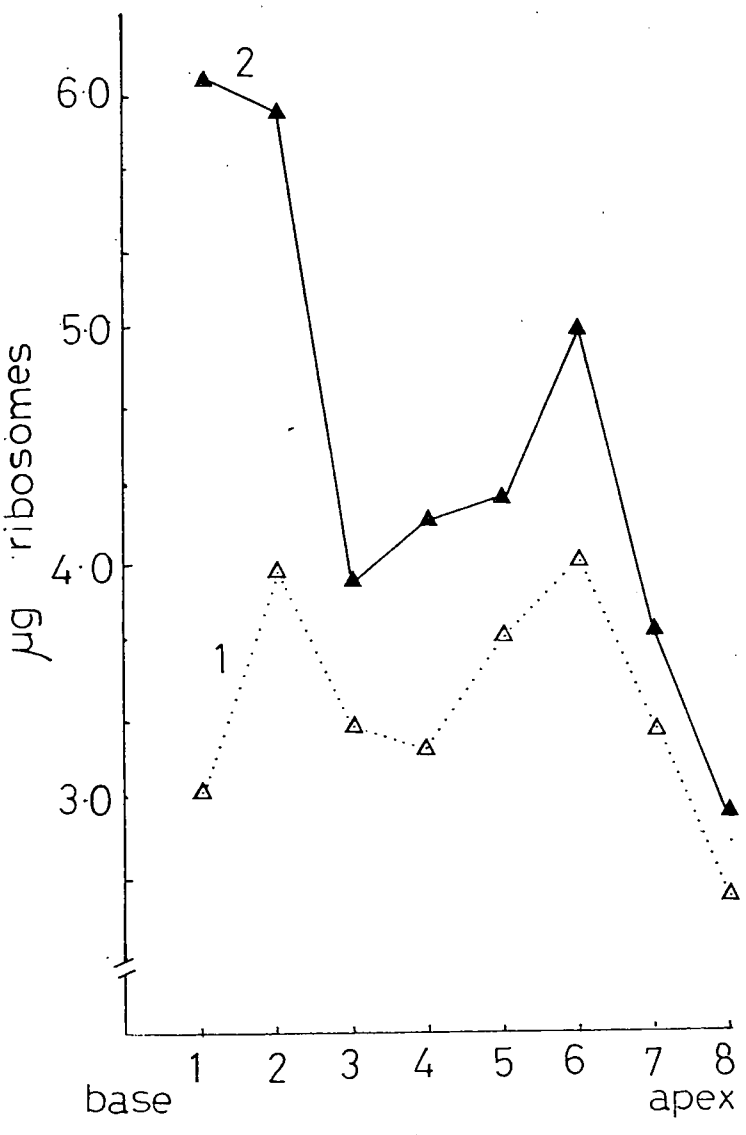


FIGURE 2.30

The ratio of free to membrane bound ribosomes from sections across the mature leaf of F. pratensis.

FIGURE 2.30

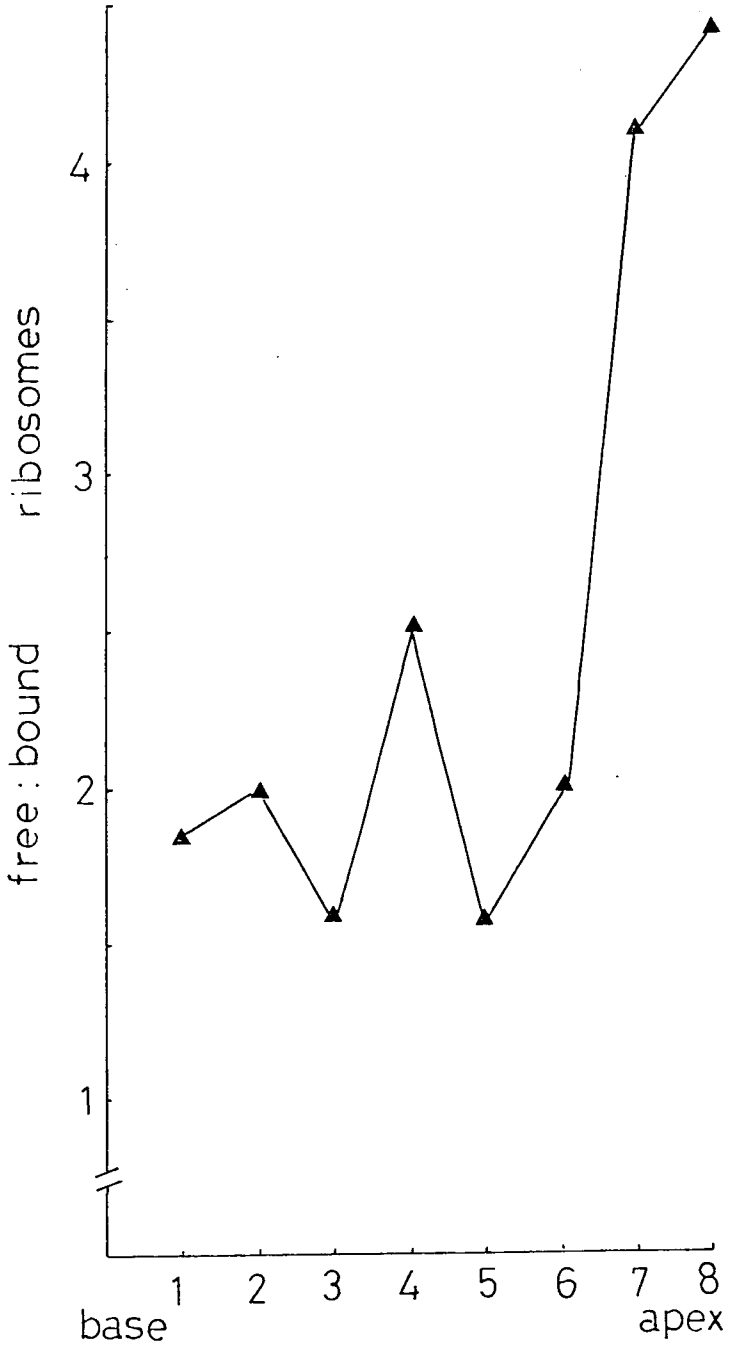
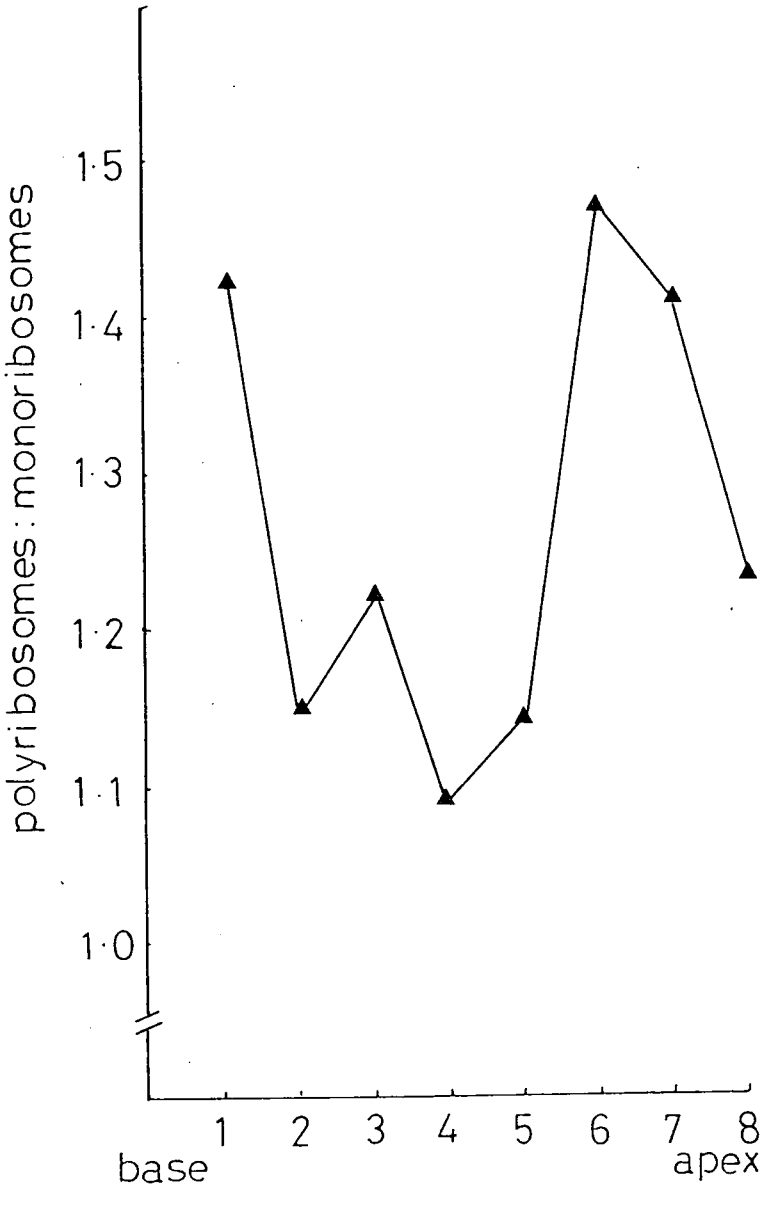


FIGURE 2.31

The ratio of polyribosomes to monoribosomes from sections along the mature leaf of F. pratensis.

FIGURE 2.31


polyribosomes : monoribosomes
1·5
1·4
1·3
1·2
1·1
1·0
1
2
3
4
5
6
7
8
base
apex

emphasis had been shifted from protein synthetic activity to degradation and replacement of existing protein synthetic machinery. The ratio of polyribosomes to monoribosomes mirrored the data for free and membrane-bound ribosomes, thus at senescence monoribosomes increased corresponding to the increase in free ribosomes. The two Figures taken together would tend to suggest that membrane-bound ribosomes were polyribosomic whilst free ribosomes may have been polyribosomic but the yields were elevated by loss of bound ribosomes in the form of monoribosomes.

b. Radio-isotope labelling of ribosomes.

Proportional sections of mature leaves of F. pratensis were labelled with $^{3}$H-adenine. The specific activities obtained from radioisotope-labelling of the ribosomes are shown in Figure 2.32. It would appear that at points 4 and 5 a considerable amount of synthesis has occurred, since both the yield and counts incorporated were high. However, at point 4, 2.2% PAGE gels provided evidence of subunits with as much as 10% of the total ribosomal counts appearing in these. It is more likely that these were evidence of synthesis, but they could result from breakdown either due to rapid turnover or to some metabolic change which caused the degradation of ribosomes in extraction. The appearance of subunits may have been a function of the increased levels of free monoribosomes which may have been more susceptible to dissociation. There seems to have been considerable ribosome synthesis at senescence, in accord with the results in Figures 2.20 and 2.23.

Figure 2.33 shows the ratio of counts incorporated per µg. of polyribosomes to those incorporated per µg. of monoribosome. Comparison with Figure 2.32 indicates that at both points where specific activity was high, i.e. at point 4 and at the apex, the counts seem to have been preferentially entering the monoribosome fraction. Once again, bearing in mind the long incubation in radioisotope (22 h.), this could have resulted from rapid turnover or have been indicative of synthesis per se. However, it does not diminish the fact that considerable synthesis must have occurred at both these times.

FIGURE 2.32

Specific activity of incorporation of cpm. into ribosomes from sections along the mature leaf of F.pratensis.

FIGURE 2.32

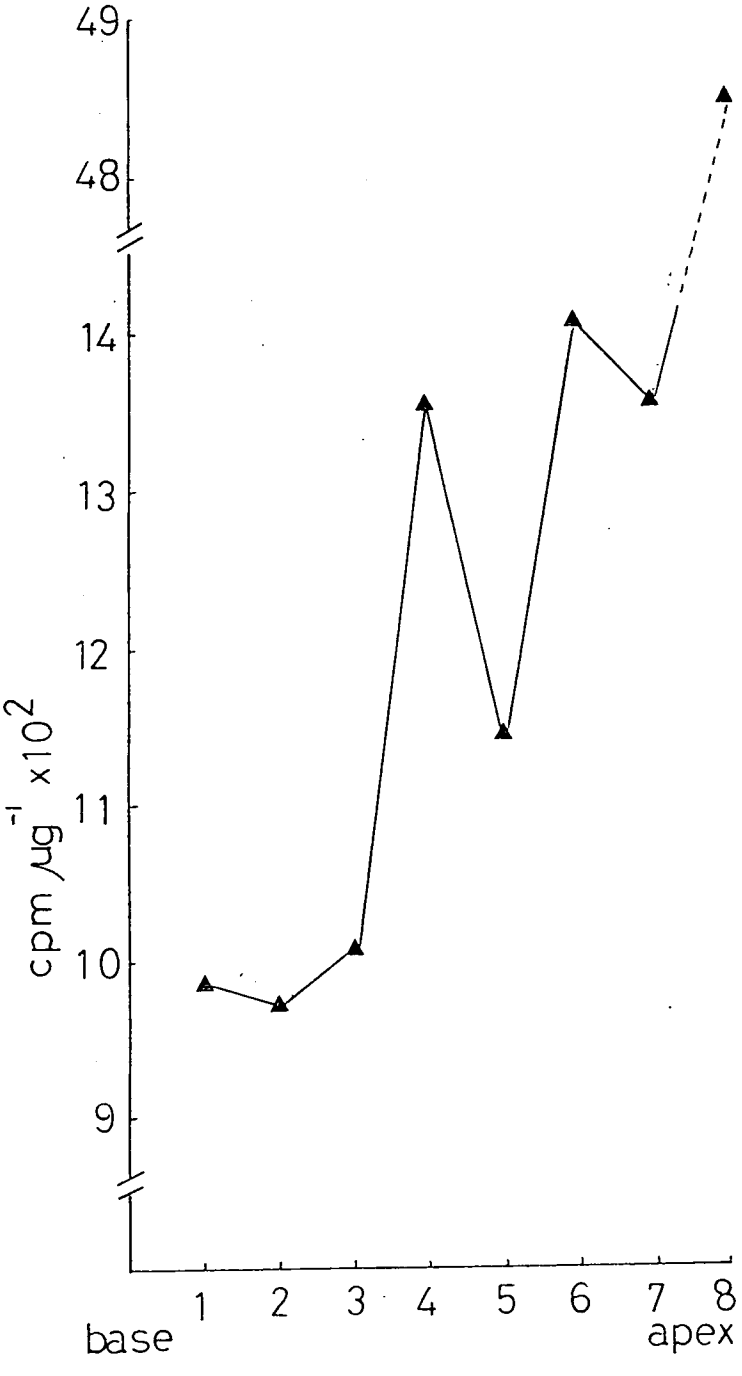
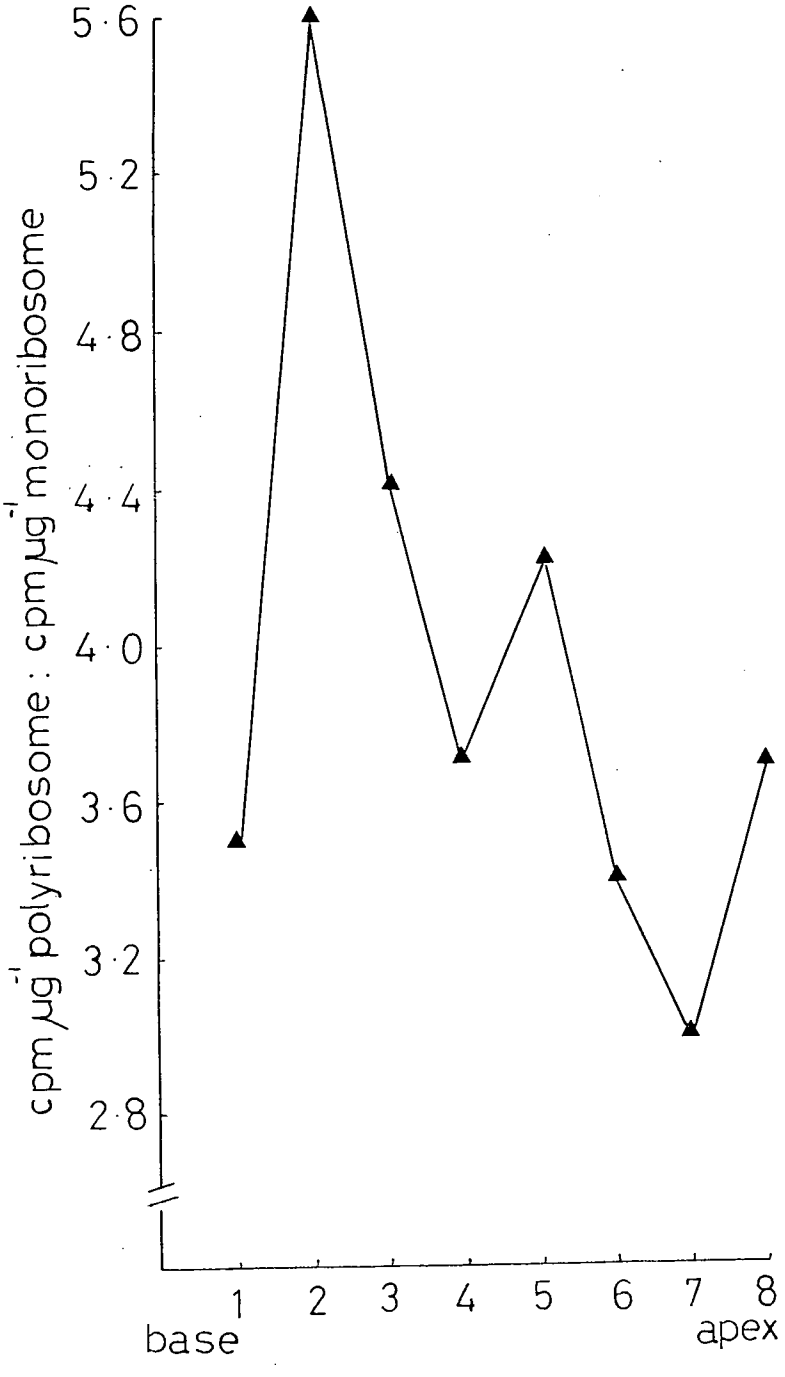


FIGURE 2.33

Ratio of the specific activity of counts incorporated per µg. of polyribosomes to the counts incorporated per µg. of monoribosomes from sections along the mature leaf of *F. pratensis*.

FIGURE 2.33


5·6
5·2
4·8
4·4
4·0
3·6
3·2
2·8
cpm µg⁻¹ polyribosome : cpm µg⁻¹ monoribosome
1
2
3
4
5
6
7
8
base
apex

iv. Poly(A)-containing mRNA.

Poly(A)-containing mRNA from proportional sections along mature tissues of F. pratensis was labelled with $^{3}$H-adenine for 17 h. in the light. Extraction involved three washes of the oligo(dT) cellulose-poly(A)-mRNA complex with "binding buffer" followed by one of "intermediate buffer" and finally dissociation by washing with elution buffer. The counts obtained from resuspended mRNA, following alcoholic precipitation with a carrier RNA, were recorded on a per section basis (Figure 2.34). Three areas of high poly-adenylation were apparent, which seemed to precede the high polyribosome to monoribosome syntheses (Figure 2.33) and coincided with high levels of polyribosomes (Figure 2.31).

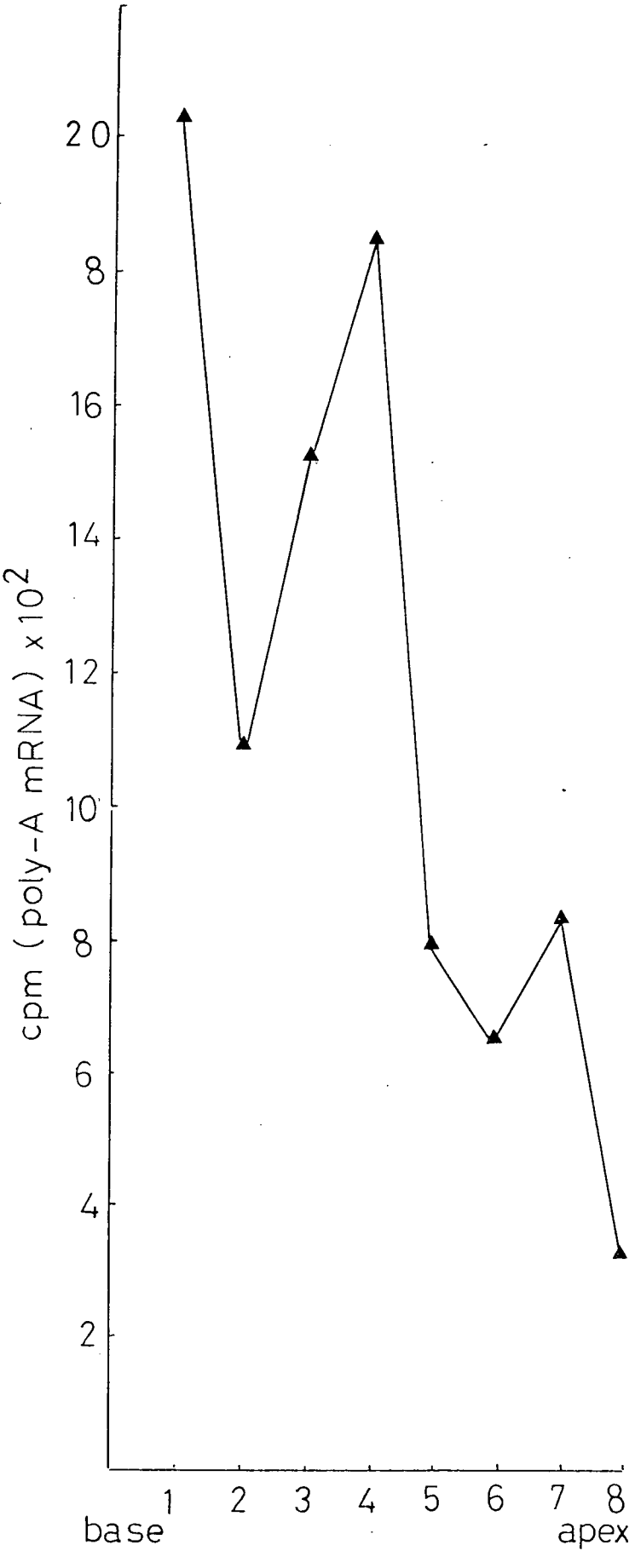
Since no estimate of yield of mRNA could be obtained from such small quantities of material, these results were of limited value. Furthermore, it is not possible to ascertain whether poly(A)-containing mRNA in the process of being translated is as easily extracted as "free" mRNA either due to the inaccessability of the poly(A) tail or reduction of its size.

It does seem that low levels of polyadenylation do occur even quite late in development.

FIGURE 2.34

Cpm. incorporated into poly(A)-containing mRNA from sections along mature leaf of *F. pratensis*.

FIGURE 2.34



## Chapter 3. Proteins and development of leaves of Festuca pratensis.

I. Introduction

- i. Early development
- ii. Light effects.
- iii. Phase of maturation.
- iv. Senescence.
- v. Aims in investigating proteins in development of F. pratensis.

II. Methods

- i. Extraction of proteins and amino acids.
- ii. PAGE.
- iii. Estimation of protein.
- iv. Estimation of free amino acids.
- v. Radioisotope-labelling of proteins and amino acid pools.

III. Experimental

- i. Quantitative estimation of protein during development.
- ii. Radioisotope-labelling of protein during development.
- iii. Quantitative estimation of the free amino acid pool.
- iv. Radioisotope-labelling of the free amino acid pool.
- v. Radioisotope-labelling of protein fractions.
- vi. Summary.

## I. Introduction.

Estimates of total protein content and synthesis during development have largely been ignored in favour of examination of particular enzymes (see Chapter 4) or structural proteins. Where such work has been reported the cellular proteins have usually been split into two general classes, "soluble" and "particulate." The former are free in the cytosol and are easily extracted whereas the latter are either integral parts of membranes or associated with cell organelles and require more vigorous extraction, for example with detergent. Since the extraction procedures are relatively straightforward it is surprising how little information on the synthesis and turnover of these classes of protein is available. This information, particularly in conjunction with concomitant nucleic acid estimations, could provide important clues to the factors involved in the displacements of synthesis and degradation which lead to growth and finally to the death of the plant.

Early workers (for example, Gregory and Sen, 1937) assumed that there was a continual turnover of proteins. With the introduction of radioisotope precursors, this was confirmed; net protein content was found to be remarkably stable but concurrent with this was a continual synthesis and degradation. The balance between anabolic and catabolic processes seems to be largely responsible for the progress of development (Mothes, 1926; Walkley, 1940; Vickery *et al.*, 1940; Chibnall and Wiltshire, 1954; Dedeken-Grenson, 1954).

There is little recent work concerning total protein metabolism during the development of attached leaves. Most workers have adopted the approach of probing into the control mechanisms via artificial systems which eliminate correlative influences, for example by following the early metabolism through greening of etiolated tissue and late metabolism through excision and applying external conditions of suppression or stimulation (see Chapter 5); whilst these are useful model systems they need not necessarily relate to the processes of the intact plant (for examples, see Chapter 5). This Chapter is concerned with the protein complement of intact leaves of *Festuca pratensis*.

## i. Early development.

Appreciable protein synthesis is apparent within a few hours of imbibition of seeds (Marcus and Feeley, 1964; Hallam et al., 1972; Bhat and Padayatty, 1975; Stoddart et al., 1973) with little or no net protein content change. Protein synthesis is obligatory for the progress of germination as work with inhibitors has suggested (Stoddart et al., 1973; Bhat and Padayatty, 1975). This is unlike the independence of germination from the early synthesis of RNA.

Stoddart et el. (1973) observed two phases of increased protein synthesis: the first maximum occurring between 4 and 5 hours following the onset of imbibition and independent of RNA synthesis, and the second at 8 h. and at least partially dependent on RNA synthesis. Jachymczyk et al. (1974) showed that protein synthesis could proceed without RNA synthesis for up to 40 h. in pea embryonic axes. This and many other observations (for example, Marcus and Feeley, 1964; Bhat and Padayatty, 1975; Osborne et al., 1977) necessarily required viable pre-existing mRNA, rRNA and other components of translation (see Chapter 2, Introduction).

Observation of high levels of the enzymes required for RNA synthesis (Barker and Reiber, 1967) and of a number of other enzymes (Hedley and Stoddart, 1972a) at the start of germination suggests that the early synthesized proteins are not concerned with resynthesis of the enzyme complement. The existing or activated enzyme complement frequently consists of high levels of soluble hydrolases which provide precursors for the early synthesis of protein (and RNA) from the breakdown of the storage materials of the seed endosperm (Larson and Beevers, 1965). It would seem likely that the early synthesized proteins are those of a structural kind and/or those required for the more subtle aspects of metabolic control produced in response to environmental conditions.

## ii. Light effects.

Total leaf protein increases rapidly (2 -3 h.) when etiolated leaves are placed in the light (De-Deken Grenson, 1954; Mego and Jagendorf, 1961; Rhodes and Yemm, 1963). As already observed for RNA synthesis (see Chapter 2), light is not a prerequisite for protein accumulation either; for example, Fraction I synthesis is merely accelerated by light treatment (Ellis, 1969 ). Fraction I content has been the most studied soluble protein with relation to development. Its synthesis is restricted to the early phase of leaf development corresponding with leaf expansion (Woolhouse, 1967; Smillie, 1969). Following this it turns over at a very low rate and declines gradually.

The increased capacity of ribosomes from light treated leaves to direct poly-(U) incorporation into phenylalanine as a result of accelerated acceptance of initiation factors (Travis et al., 1972) may provide at least part of the reason for light-accelerated, but not necessarily enhanced, protein synthesis in vivo. Other results confirm that light is associated with the development and maintenance of the protein-synthesizing apparatus (Williams and Novelli, 1964, 1968; Travis et al., 1970) rather than being involved specifically with the induction of particular proteins or stabilisation of mRNA (Pine and Klein, 1972).

## iii. Phase of maturation.

Hedley and Stoddart (1972b) reported a peak of protein synthesis which correlated with attainment of maximum chlorophyll content and occurred approximately midway through the total life span of attached leaves of Lolium temulentum. The enzyme activities associated with this phase varied depending on photoperiod and leaf position. Nevertheless, this peak bore a constant temporal relationship to leaf emergence and no visible effect of photoperiod on this phase was detectable.

Environmental factors which tended to delay the onset of senescence apparently did so by extension of the interval between the second and third incorporation maxima and partly by delaying initiation of the first. Environmental factors prevailing in the early stages of development can affect the onset and course of senescence (Schwabe, 1970). It is possible that this predestined second protein synthetic phase merely has a maintenance function rather than one in which important control factors might be operating and, as such, need not be flexible.

### iv. Senescence.

There are many reports which suggest that senescence requires synthesis of protein (Wollgiehn, 1967; Woolhouse, 1967; McHale and Dove, 1968; Udvardy et al., 1969; Hedley and Stoddart, 1971 b; Martin and Thimann, 1972; Baumgartner et al., 1975; Thomas, 1976a) although the net levels of protein decline (Carr and Pate, 1967; Woolhouse, 1967). This decline may result from degradation or decreased synthesis. Synthesis may be limited by amino acid availability due to export to younger parts of the plant or by reduced protein synthetic capacity. The former suggestion loses credibility in the light of work with detached leaves where protein declines even in the presence of high levels of amino acid (Vickery et al., 1937) and with its reliance on the assumption of continual turnover of protein. Fraction I, for example, does not turn over appreciably following maturation yet it declines quite rapidly at senescence. Proteases (Martin and Thimann, 1972) and RNAses (Dove, 1973; Baumgartner et al., 1975; Thomas, 1975) are synthesized at senescence and obviously require a low threshold of protein synthetic activity. Once degradative enzymes have been maximally induced or activated senescence will proceed unabated and it is at this point that senescence becomes irreversible.

## v. Aims in investigating proteins in development of Festuca.

Syntheses of soluble and particulate protein fractions were quantified from leaves of F. pratensis throughout development, both sequentially along the leaf and temporally. Estimates of free amino acids were also carried out in order to provide some indication of amino acid flux and pool size but without consideration of compartmentation. Would the three phases of maximal protein synthesis in development of leaves of Lolium temulentum (Hedley and Stoddart, 1972b) be reflected not only in another species, Festuca pratensis, but also in the development sequentially along the leaf axis ?

## II. Methods.

### i. Extraction of protein and amino acids.

Protein extraction is relatively straightforward providing precautions are taken to buffer the extract at approximately neutrality, to protect the easily oxidized sulphydryl groups with suitable reducing agents

Leaf material (0.16 - 2.0 g.) was homogenized in 2 ml. extraction buffer with acid-washed sand at room temperature. The extraction buffer consisted of

40 mM. Tris-HCl pH 7.5
10 mM. magnesium sulphate
250 mM. EDTA (disodium salt)
2 mM. mercaptoethanol.

The homogenate was centrifuged at 13,000 g. for 6 min. in a Quickfit microcentrifuge. 0.5 ml. supernatant were mixed with 0.5 ml. 'denaturation buffer' and heated in a water bath at 100°C. for 2 min. to give a crude denatured soluble protein extract. The 'denaturation buffer' consisted of

62.5 mM. Tris-HCl pH 6.8
5% (W/V)glycerol
250 mM. mercaptoethanol
20 g./l.SDS.

0.25 ml. supernatant were added to 0.25 ml. trichloroacetic acid (100 g./l.) and centrifuged at 13,000 g. for 6 min. to remove precipitated soluble protein. The supernatant was taken for free amino acid estimation.

The 13,000 g. pellet from the first centrifugation was re-extracted with 1 ml. extraction buffer followed by another 13,000 g. spin. This supernatant was discarded and the pellet was washed twice with 1 ml. chloroform:methanol mix (2:1 V/V) followed by one wash with anhydrous methanol to remove remaining lipids. The 13,000 g. supernatants were pooled and kept in the dark at 4°C.

for later chlorophyll estimations.

2 ml. 'denaturation buffer' were added to the final 13,000 g. white pellet which was then incubated at 100°C. for 2 min. Clarification was carried out by centrifugation at 13,000 g. for 6 min. The supernatant represented the crude particulate protein fraction.

ii. PAGE

The polyacrylamide rod gel system used was essentially that of Henriques and Park (1975) and employed a 1 cm. stacking gel of 5% (W/V) polyacrylamide and a 9 cm. running gel of 15% (W/V) polyacrylamide. The acrylamide was made up in

190 mM. Tris-HCl pH 8.8
1 g./l. SDS
20 mM. EDTA (disodium salt)
with 4 g./l. bis-acrylamide.

The final pH of the stacking gel was 7.0 This resulted from it being made up with gel buffer diluted with two volumes of diluting solution consisting of 1 g./l. SDS and 20 mM. EDTA (disodium salt) at pH 6.5. The gels were prepared in perspex tubes of internal diameter 0.6 cm. The surfaces of both the running and stacking gels were levelled by careful overlaying with about 100 µl. distilled water which were removed following gel polymerization. The running buffer consisted of

50 mM. Tris-HCl pH 8.2
2 mM. EDTA (disodium salt)
1 g./l. SDS
380 mM. glycine.

The electrophoresis tanks were set up as described in Chapter 2, II iii. 50 µl. 'desalted' protein extract (see following section) were loaded onto gels and run at 3 mA. per gel. Electrophoresis was carried out, at room temperature, until the bromophenol blue tracker dye, which marked the solvent front, was approximately 0.5 cm. from the bottom of the tube.

Gels were removed with a syringe device which released them from the tubes by pressure and circulation of water. Gels were immediately stained with CBB stain made up as a 37.5 g./l. solution in 12.5% (V/V) acetic acid and 31.25% (V/V) propanol, for at least 3 h. (Fairbanks et al., 1971). Destaining was achieved by immersion in

3.75 g./l. CBB
12.5% (V/V) acetic acid
12.5% (V/V) propanol

for 1 - 2 h., followed by

1.875 g./l. CBB
12.5% (V/V) acetic acid.

for 3 h. and, finally, several changes of 10% (V/V) acetic acid.

Gels were scanned at 640 nm. using the densitometer attachment of a Vitatron MPS spectrophotometer.

Molecular weights were estimated by comparison of migration of cytochrome C (12,270 MW), ovalbumin (43,000 MW) and BSA (66,000 MW) (Henriques and Park, 1975; Weber and Osborn, 1969).

### iii. Estimation of protein.

Following comparison with the Biuret method for protein estimation, the method of Lowry et al. (1951) was finally adopted. Prior to quantitation, the crude extracts (both soluble and particulate) were subjected to a 'desalting' step involving passage through Sephadex G-25 columns supported vertically in Pasteur pipettes, by elution with distilled water without restricting flow. This was essential since SDS and mercaptoethanol both interfere with the colour production in the Lowry method (Vallejo and Lagunas, 1970; Geiger and Bessman, 1972; Ross and Schatz, 1973). Other methods of sulphydryl contamination removal were also tried, namely by carboxymethylation with excess iodoacetate (Ross and Schatz, 1973), oxidation by addition of hydrogen peroxide (Geiger and Bessman, 1972) and dialysis, but these were not as successful as G-25 columns. The 'desalted' extracts were suitably diluted (usually 60 µl. in a total of 600 µl. water or buffer) and brought to 37°C. 3 ml. copper solution (freshly made by the

FIGURE 3.1

Calibration of Lowry estimation of protein with different concentrations of BSA.

FIGURE 3.2

Calibration of fluorescence method of amino acid estimation using different concentrations of glycine.

FIGURE 3.1

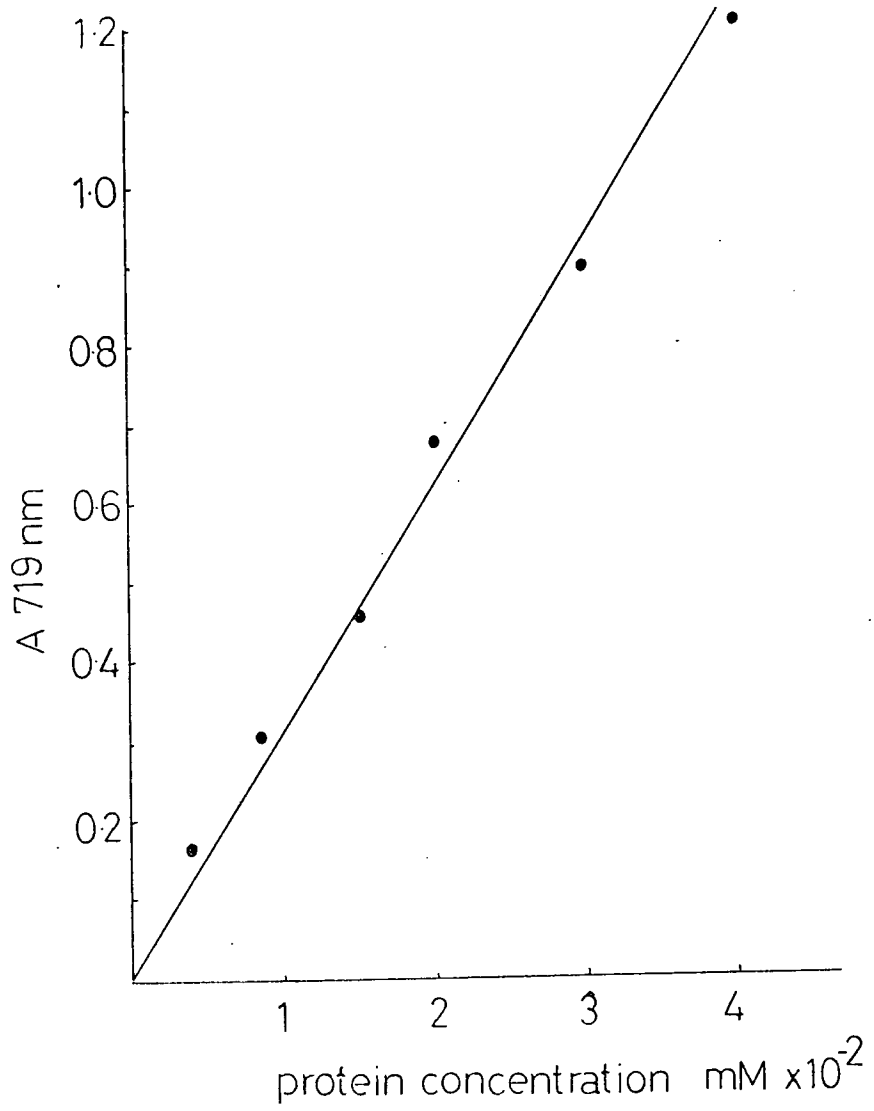
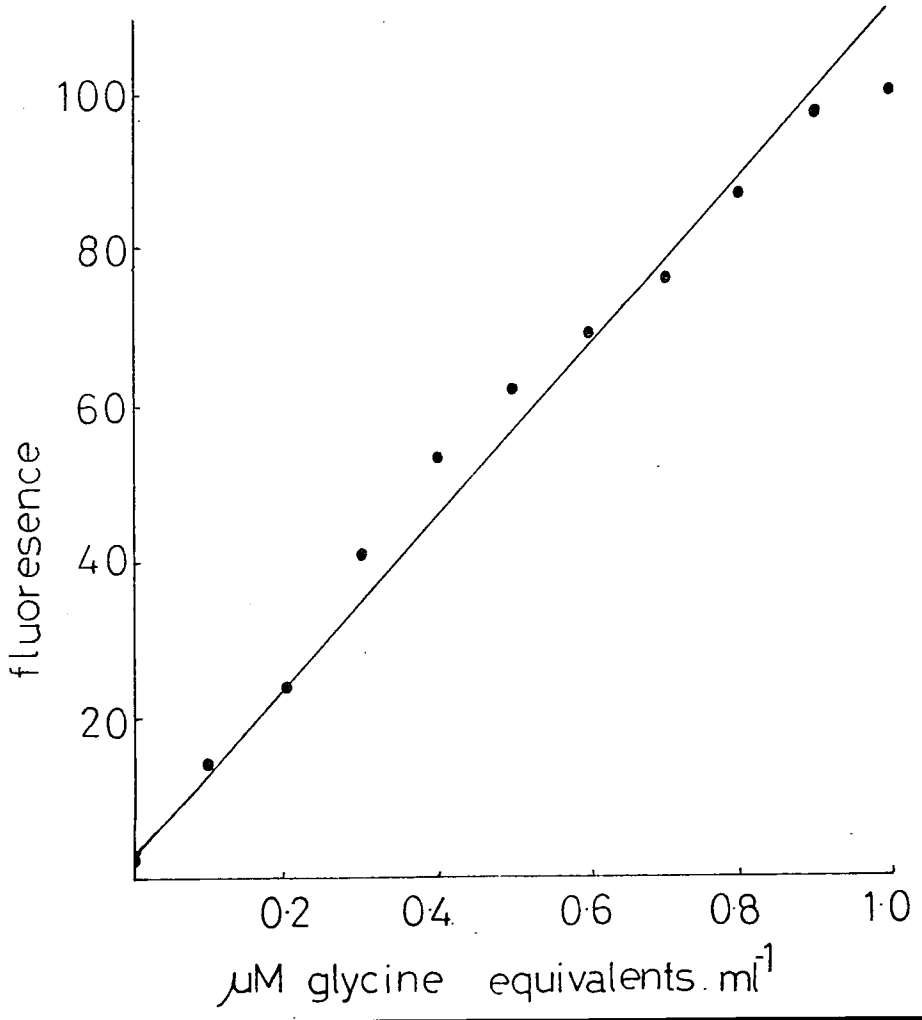



FIGURE 3.2



addition of 1 ml. of $CuSO_4.5H_2O$ stock (20 mM.) to 100 ml. of sodium carbonate (anhydrous) stock (70 mM.) in 100 mM. sodium hydroxide, and equilibrated at 37°C.) were added and incubated for exactly 10 min. at 37°C. 300 µl. Folin-Ciocalteau reagent were then added with thorough and immediate mixing. Exactly 15 min. later the absorbance at 719 nm. was measured using a Vitatron colorimeter (Vitatron U.K. Ltd.) in conjunction with a Fisons autodispenser. Following correction for the blank (prepared by substituting water or buffer for the protein extract) the protein content was estimated by reference to a standard calibration curve (Figure 3.1). Errors due to sulphydryl group reactions were further minimized by assaying in chromic acid washed tubes. Where samples were not 'desalted', protein was precipitated with two and a half times sample volume with trichloroacetic acid (100 g./l.) at 4°C. for 30 min., centrifuged at 13,000 g. for 6 min. at 4°C. in a Quickfit microcentrifuge and the pellets digested with 250 µl. 100 mM. sodium hydroxide overnight at 40°C. in a Grant BT 3 block thermostat. Suitable aliquots of this digest were then used in Lowry estimations.

iv. Estimation of free amino acids.

Amino acid estimation was achieved by fluorimetric measurement of the reaction of Fluram with primary amines. Providing fluophor concentration does not become self quenching this method is highly reproducible, sensitive to picomole amounts, requires no heating or harsh conditions, thus having many advantages over more traditional methods.

Estimations were carried out using a Vitatron MPS with the fluorimeter mode. 0.1 ml. sample (suitably diluted, if necessary) was mixed with 1.5 ml. 200 mM. borate buffer (pH 9.0). 0.5 ml. Fluram (15 mg./100 ml. acetone) was added with instantaneous mixing. The fluorescence was recorded with reference to a blank of 0.1 ml. 80% (V/V) ethanol and a standard glycine solution (1 mM. in 80% (V/V) ethanol). The concentrations of amino acids were estimated by reference to a calibration curve obtained for glycine (Figure 3.2).

## v. Radioisotope labelling of proteins and amino acid pools.

Following sterilisation, 1 cm. sections of 4 leaves were incubated with shaking for 16 h. at room temperature in 4.1 ml. buffer (pH 7.5) containing 1.25 µCi per ml. L-($^{14}$U-C) amino acid and 5.5 mM. ATP, in light from an Angle-poise lamp at 5,000 lux. The buffer was that used as extraction buffer (Section II. i). Estimation of amino acid incorporation involved harvesting the leaf sections and, following profuse washing to remove surface radioactivity, homogenization as described in Section II. i. The radioactivity incorporated into protein was calculated from the total counts which had entered the PAGE gel following electrophoresis, by counting 1.8 mm. slices obtained using a wire slicer and metal guide. The first 3.6 mm. of the gel were not included in this estimation assuming this to contain residual lipids and non-protein material. Gel slices were treated as for RNA gel slices (see Chapter 2, II iv) and counted in a Trace-Lab Corumatic 200 scintillation counter with 98% efficiency for $^{14}$C, using toluene: Triton X 100 : PPO scintillation fluid (Chapter 2, II iv).

## III. Experimental.

### i. Quantitative estimation of protein during development.

Soluble and particulate protein fractions were estimated in both developmental systems. Figures 3.3 and 3.4 show the protein yields obtained. Figure 3.3 A shows that soluble protein increases to a maximum by Day 49 (full expansion) and is then degraded. Particulate protein is also maximal by Day 49 but seems to decline prior to this, perhaps as a result of membrane maturation associated with extension growth. The early decline in soluble protein content if expressed per unit area (Figure 3.3 B) is probably due to vacuolation. Figure 3.4 shows a similar trend in protein content to Figure 3.3 B, with an early increase which declines with age to the initial level.

### ii. Radioisotope labelling of proteins during development.

Figures 3.5 and 3.6 show the results of several experiments designed to investigate the levels of protein synthesis with development. In this instance the labelling patterns are very different (cf. similarities in radioisotope-labelling RNA, Chapter2, Figure 20). However, in both developmental systems there are high rates of soluble protein synthesis at early and senescent stages of development. The high rate of protein synthesis at extreme senescence (Day 62) is consistent with a number of reports of de novo synthesis of degradative enzymes (Chapter 3, I iv).

In Figure 3.6 there is a third peak of increased specific activity of soluble protein approximately mid-way in development. Not only is this consistent with the results of Hedley and Stoddart (1972b) for temporal development of Lolium temulentum but also it coincides with a period of increased RNA synthesis (Chapter 2, Figure 2.20). If this phase is short-lived (2 -4 days in L. temulentum : Hedley and Stoddart, 1972b) then it is possible that

FIGURE 3.3

Soluble (S) and particulate (P) protein contents of fourth leaves of different ages expressed

A. on a per leaf basis

B. on a per unit area basis.

FIGURE 3.3

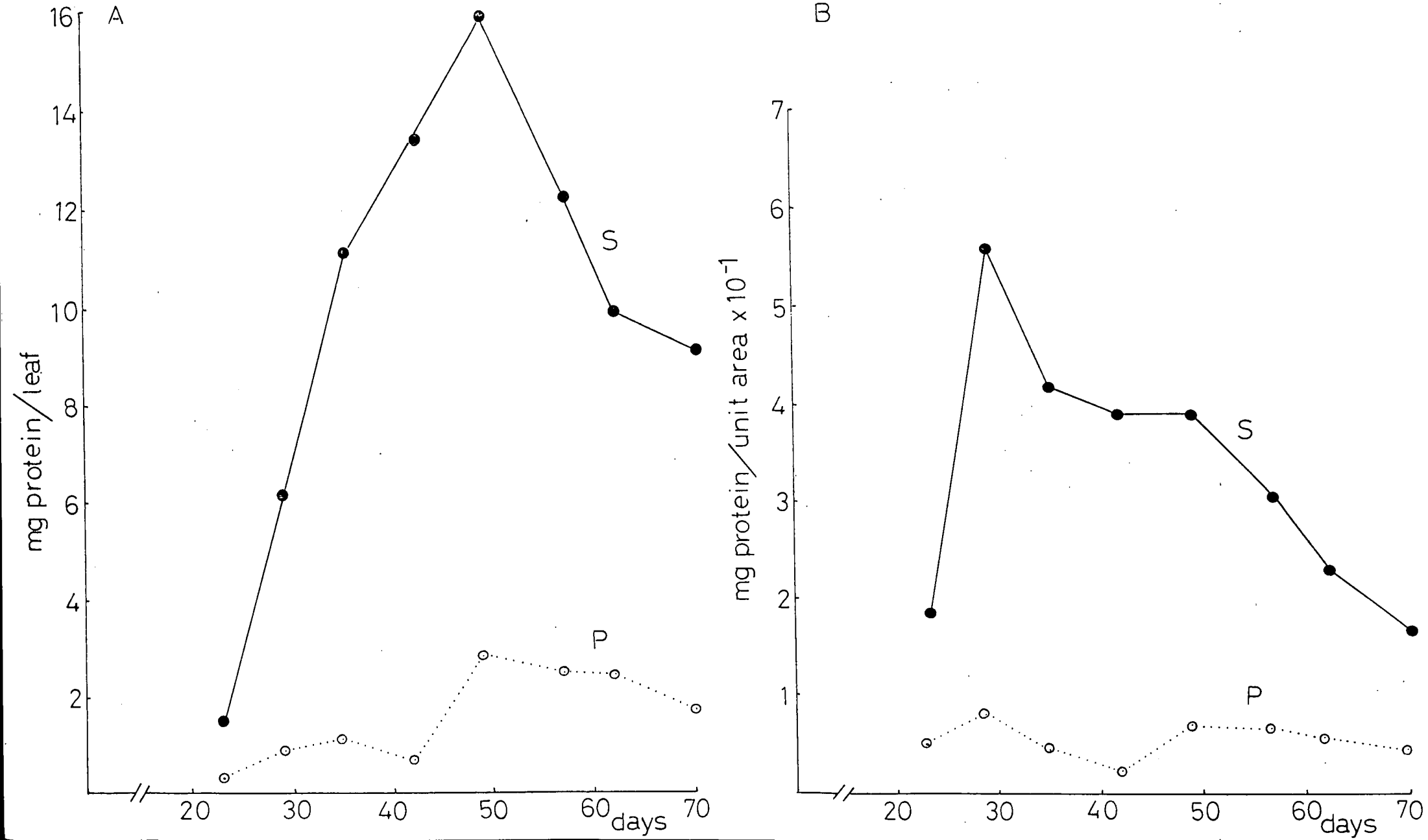

A
B
mg protein/leaf
mg protein/unit area x 10⁻¹
S
P
days
20
30
40
50
60
70
2
4
6
8
10
12
14
16
1
3
5
7

FIGURE 3.4

Soluble (S) and particulate (P) protein contents of sections along the mature leaf.

FIGURE 3.4

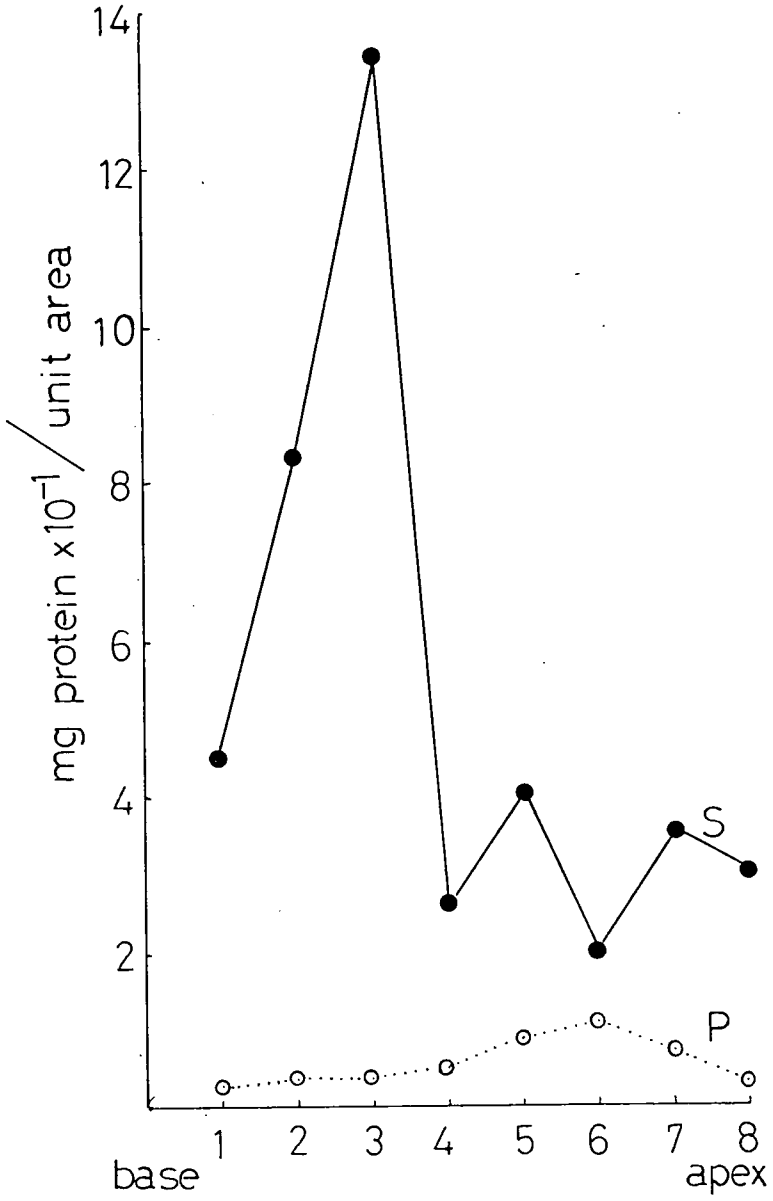


FIGURE 3.5

Specific activity of radioisotope-labelling of soluble (S) and particulate (P) proteins during development of the fourth leaf with time.

FIGURE 3.5

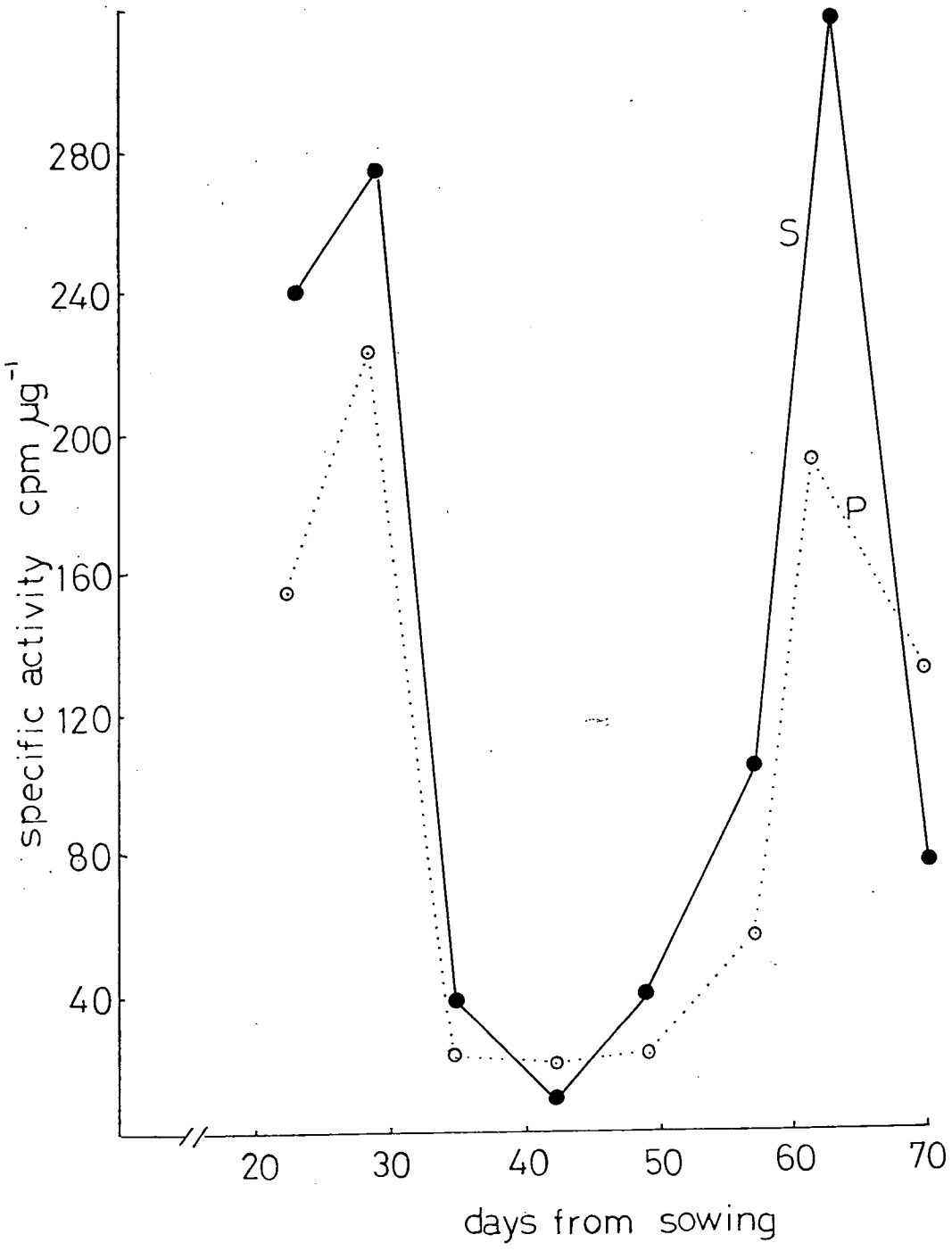

280
240
200
160
120
80
40
specific activity cpm µg⁻¹
S
P
20
30
40
50
60
70
days from sowing

FIGURE 3.6

Specific activity of radioisotope-labelling of soluble (S) and particulate (P) proteins in sections along the mature leaf.

FIGURE 3.6

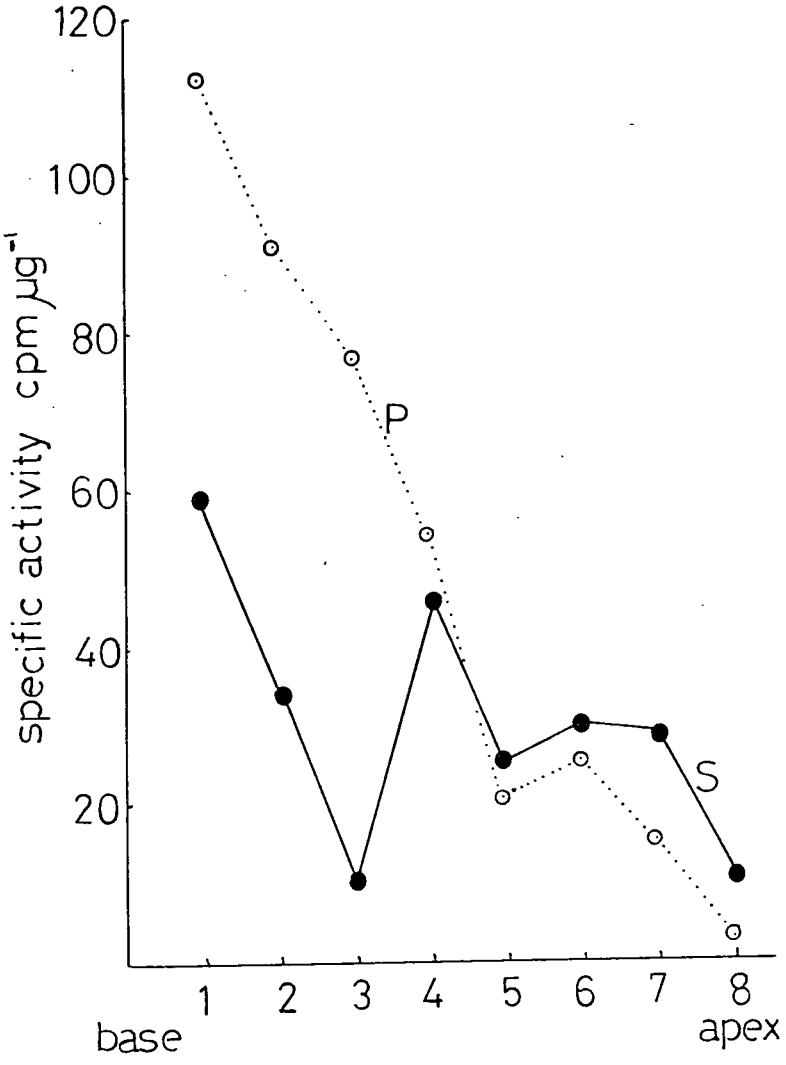

120
100
80
60
40
20
specific activity cpm µg⁻¹
P
S
1
2
3
4
5
6
7
8
base
apex

FIGURE 3.7

Amino acid content of fourth leaf of *F. pratensis* expressed

A. on a per leaf basis

B. on a per unit area basis.

FIGURE 3.7

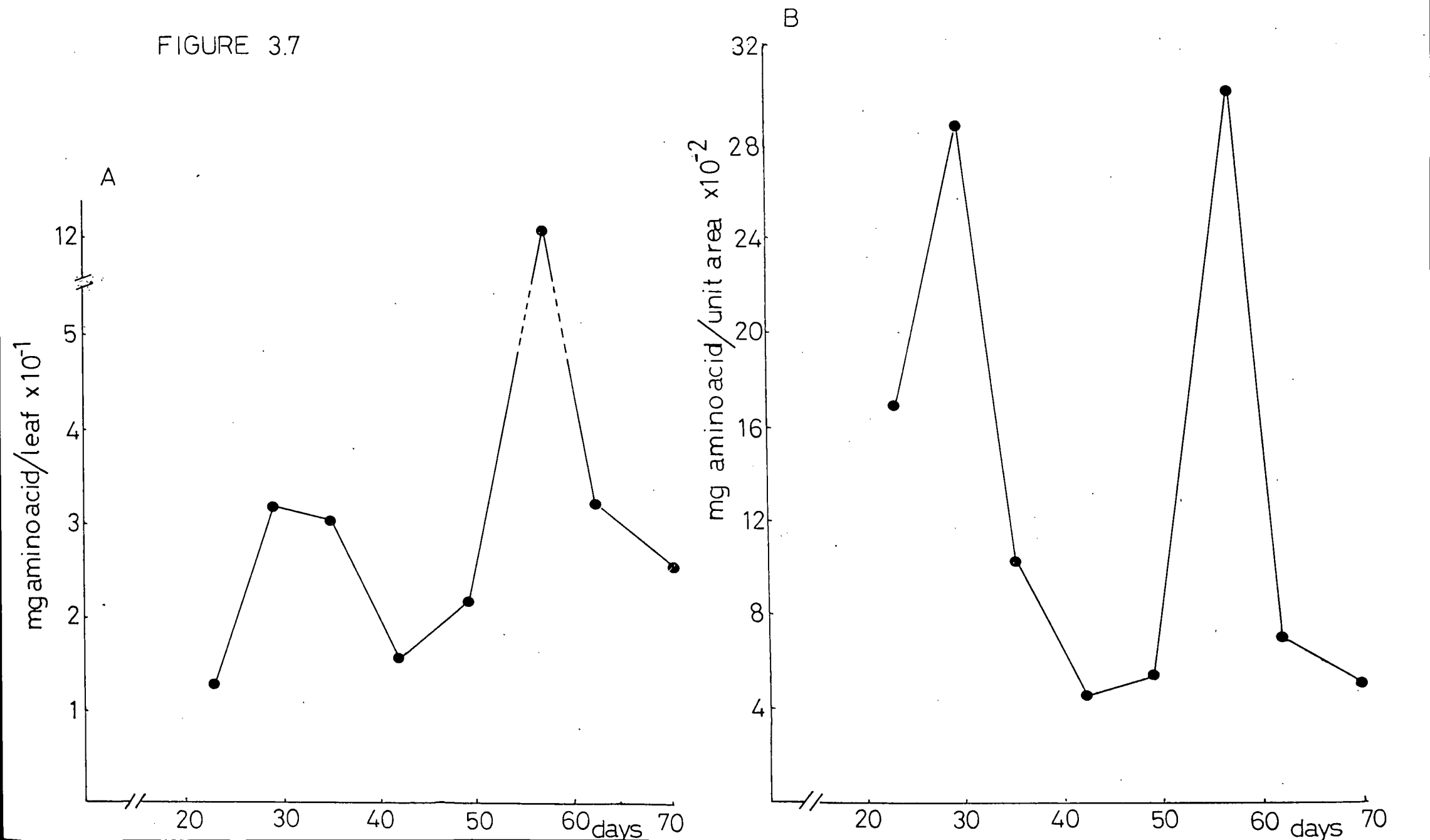

A
mg amino acid/leaf x10⁻¹
12
5
4
3
2
1
20
30
40
50
60
days
70
B
mg amino acid/unit area x10⁻²
32
28
24
20
16
12
8
4
20
30
40
50
60
days
70

FIGURE 3.8

Amino acid content of sections along the mature leaf.

FIGURE 3.9

Specific activities of radioisotope-labelling of amino acid pools in sections along the mature leaf.

FIGURE 3.8

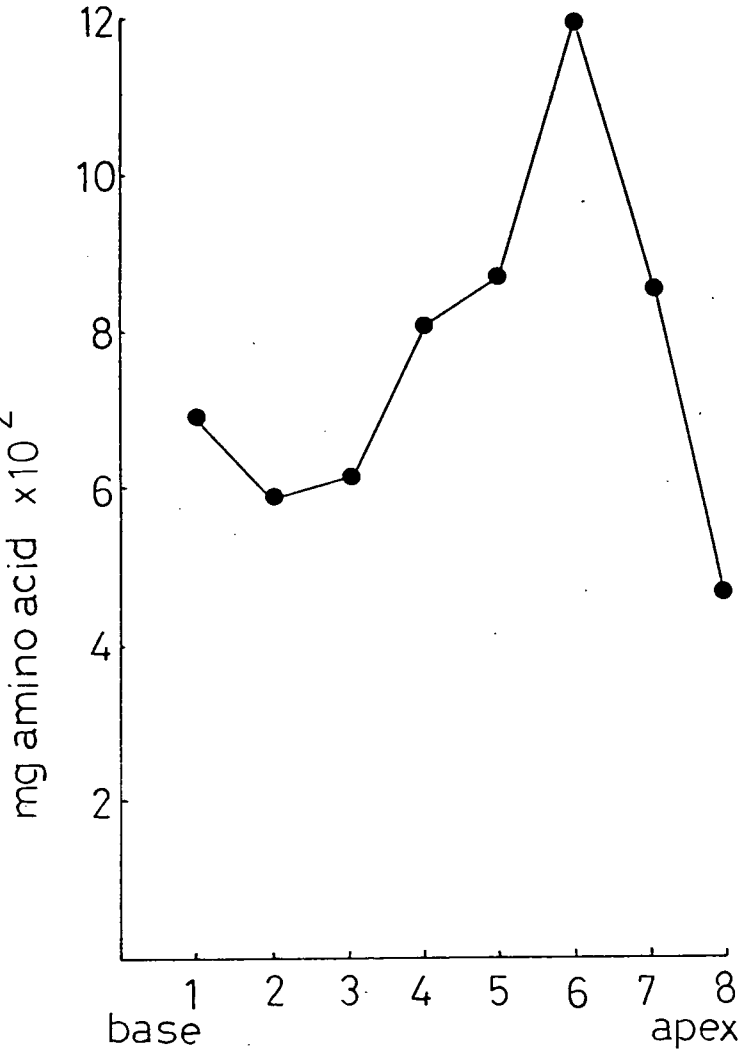



FIGURE 3.9

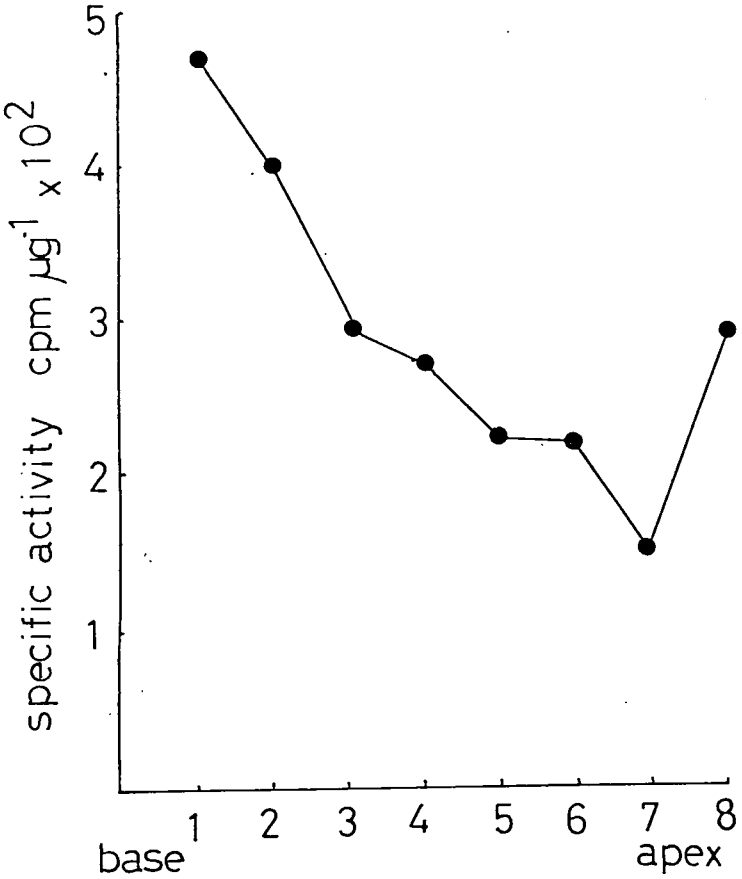
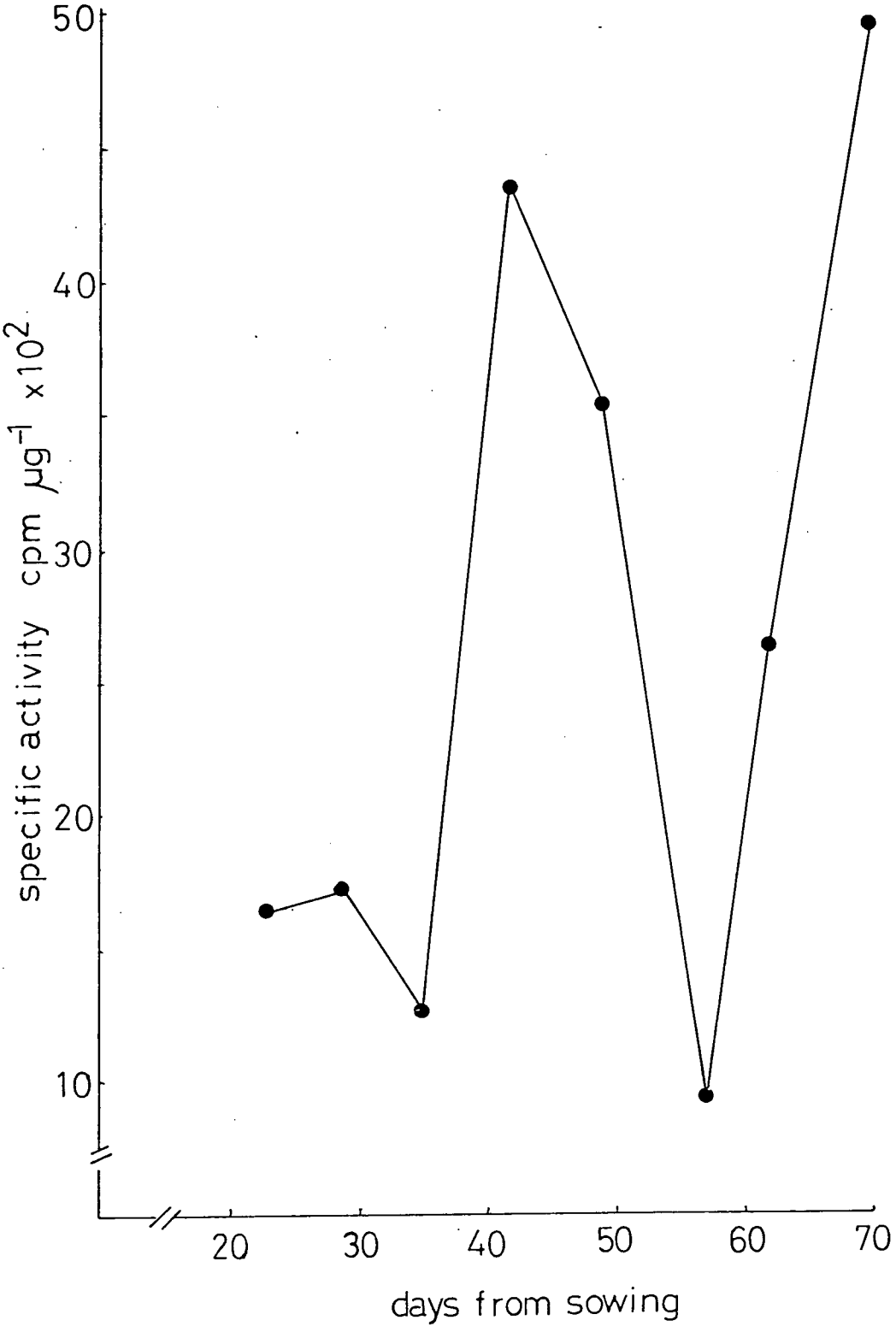


FIGURE 3.10

Specific activities of radioisotope-labelled pools in the development of the fourth leaf.

FIGURE 3.10


50
40
30
20
10
specific activity cpm μg⁻¹ x10²
20
30
40
50
60
70
days from sowing

in the sampling of different aged leaves from F. pratensis it was missed, since there were up to 7 days between samples. Alternatively, it may be such a localized resurgence of synthetic activity that the relative inactivity of other areas may dilute out its effect when expressed as total leaf specific activities. The synthetic pattern for particulate protein is very different along the leaf (Figure 3.6) when compared with temporal development (Figure 3.5). This could also be explained if the laying down of particulate protein was localized in very young tissue, i.e., the effect of elongation and maturation with lower levels of particulate protein synthesis effectively dilutes out the high localized activity.

### iii. Quantitative estimation of the free amino acid pool.

In both developmental systems there is an increase in free amino acids at incipient senescence. This may result from an increased degradation of proteins in excess of synthesis (even though this is enhanced at this time). The amino acids may accumulate prior to translocation and redeployment to younger parts of the plant. Following the increase is a final decline which coincides with the final stages of senescence when protein synthesis is very low. The early increase in free amino acid pool (particularly noticeable at Day 29, Figure 3.7) may result from increased metabolism of protein.

### iv. Radioisotope labelling of the free amino acid pool.

The increase in specific activity of the amino acid pool at very late senescence is shown by both developmental systems (Figures 3.9 and 3.10). It has been suggested for bacteria that anabolism preferentially utilizes the breakdown products of catabolism (Nierlich, 1967; see Chapter 2, III ii). If this could be extended to the preferential translocation (for redeployment) of the products of

catabolism then the elevated specific activities of radioisotope-labelled amino acid pools could be thus explained. The increased specific activity at Day 42 (Figure 3.10) may be indicative of this precursoral preference and may, in addition, provide the answer to the missing peak of protein synthetic activity occurring, as it does, approximately mid-way in development, corresponding to the increased RNA synthetic levels shown in Figure 2.20 and indicated equivocally in Figure 3.11 (all particulate and one soluble peak showing increased relative synthesis).

### v. Radioisotope labelling of protein fractions.

Four major soluble proteins and three major particulate proteins were clearly distinguished at all ages. Their relative syntheses were estimated by measurement of the radioisotope label present in them compared with the total radioisotope label entering the gel. The profiles show striking similarities. There are two predominant types of phasic synthesis apparent for these proteins, both consisting of two periods of increased synthesis. One shows early and late phases, as exemplified by soluble proteins 1, 2 and 4, and the second shows an early and a mid-developmental phase, as exemplified by all the particulate proteins and by soluble protein 3. These results may be indicative of three synthetic phases, the first corresponding to overall cellular synthesis and provision for normal metabolism, the second maybe representing a maintenance and replacement of existing protein constituents (mostly particulate) and the third phase representing a *de novo* synthesis of degradative soluble enzymes.

### vi. Summary.

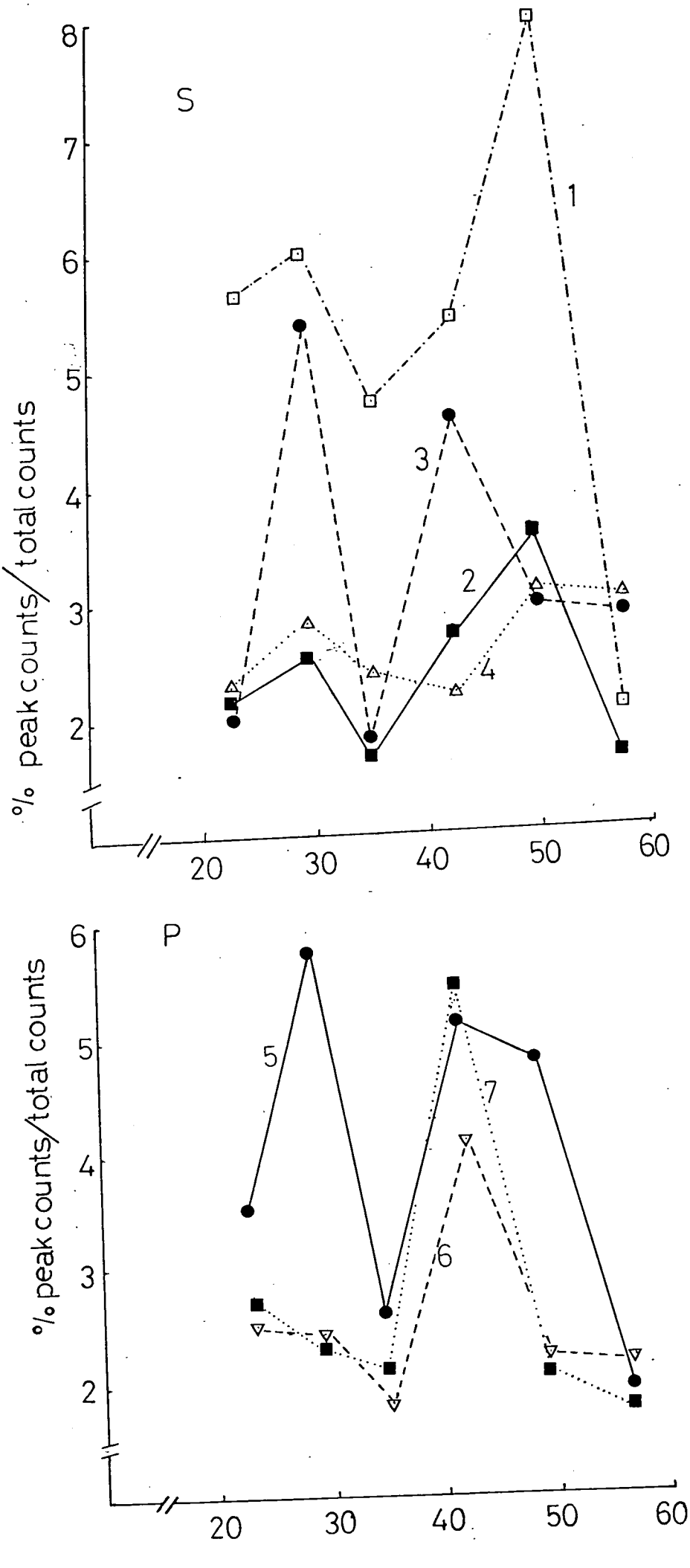
Although three peaks of increased protein synthesis were not apparent in whole leaf ageing (Figure 3.5) the relative syntheses of particular proteins would suggest three phases of increased synthetic activity (Figure 3.11). In the development along the leaf evidence

FIGURE 3.11

Radioisotope-labelling of soluble (S) and particulate (P) protein fractions expressed as % counts entering the PAGE gel with development of the fourth leaf.

| | | |
|---|---|---|
| Soluble fractions | 1 | 57,000 MW |
| | 2 | 20,000 MW |
| | 3 | 18,000 MW |
| | 4 | 15,000 MW |
| Particulate fractions | 5 | 37,000 MW |
| | 6 | 15,000 MW |
| | 7 | 12,000 MW |

FIGURE 3.11


S
P
% peak counts/total counts
% peak counts/total counts
1
2
3
4
5
6
7
8
7
6
5
4
3
2
20
30
40
50
60

is presented of three phases of increased protein synthesis in soluble protein at least (Figure 3.6). Amino acids increase prior to translocation at early senescence (Figures 3.7, 3.8). The specific activities of the amino acid pools increased at late senescence (Figures 3.9, 3.10) perhaps as a result of preferential translocation of products of catabolism. At levels of decreased incorporation of radioisotopically-labelled amino acids into protein the sizes of amino acid pools were not the limiting factors.

## Chapter 4. Enzymes and development of leaves of Festuca pratensis.

I. Introduction

- i. Early events
- ii. Light effects
- iii. Phase of maturity
- iv. Senescence
- v. Enzyme location.

II. Methods

- i. Extraction for assay of soluble enzymes
- ii. PAGE of isoenzymes
- iii. ATPase
  - A. a. test-tube assay
    - b. calculation of enzyme activity
    - c. divalent ion requirement
    - d. pH optimization
    - e. specificity towards ATP
    - f. calculation of $K_m$ and $V_{max}$
    - g. optimal $Mg^{2+}$
    - h. response to enzyme concentration
  - B. PAGE assay.
- iv. RNAse
  - A. a. test-tube assay
    - b. calculation of enzyme activity
    - c. pH optimization
    - d. substrate optimization
    - e. response to enzyme concentration
  - B. PAGE assay.

v. Pyrophosphatase

A. a. test-tube assay

b. calculation of enzyme activity

c. pH optimization and $Mg^{2+}$ requirement

d. optimal $Mg^{2+}$

e. calculation of $K_m$ and $V_{max}$

f. response to enzyme concentration

B. PAGE assay.

vi. Phosphatase

A. a. test-tube assay

b. calculation of enzyme activity

c. pH optimization and $Mg^{2+}$ requirement

d. calculation of $K_m$ and $V_{max}$

e. response to enzyme concentration

B. PAGE assay

a. acid phosphatase

b. alkaline phosphatase.

vii. Phosphodiesterase

A. a. test-tube assay

b. calculation of enzyme activity

c. pH optimization

d. calculation of $K_m$ and $V_{max}$

viii. Location of enzymes

a. sucrose density gradient centrifugation

b. differential centrifugation.

ix. Assays of other enzymes used as cytological markers

a. catalase

b. NAD-cytochrome C oxidoreductase

c. succinate dehydrogenase

d. uricase.

III. Experimental work.

i. Enzymes of phosphorus metabolism during development

ii. Isoenzyme patterns during development

iii. Enzyme location.

## I. Introduction.

Enzymes are necessarily involved in developmental control and have taken on an even more central role with the realisation that the rates of catabolism are as important as the rates of anabolism in the regulation of gene action (Trewavas, 1976). Nucleases will therefore be implicated in control of gene expression. Nucleic acids turn over at different rates in different species (Lin et al., 1976; Trewavas, 1970; Grierson, 1976), and Dove (1973) has suggested that different nucleases are responsible for the regulation of different nucleic acids. Ribonucleases may act in a degradative (Kessler and Engelberg, 1962; Bagi and Farkas, 1967; Dove, 1967; Lewington et al., 1967) or synthetic fashion (Barker and Douglas, 1960; Reddi, 1959) and may act at a number of different levels or may be compartmented such that their action is restricted. In addition to ribonucleases a number of other enzymes may regulate nucleic acid levels by hydrolysis (for example, phosphatases and phosphodiesterases). In most studies the latter has not been distinguished from RNA-specific enzymes (Wyen et al., 1969). Pyrophosphatases have been implicated as important in development in that their activities result in shifts in the production of ATP. Kornberg (1962) noted that biosynthetic pathways for nucleic acids, proteins, lipids and carbohydrates had at least one synthetic reaction releasing pyrophosphate. Acid pyrophosphatase has been associated with catabolic processes and alkaline pyrophosphatase with anabolic processes (Naganna and Sripathi, 1954; Parups, 1976). ATPase, with its critical role in membrane-mediated transport and ATP provision (Raghavendra and Das, 1978) is also likely to have important consequences in development. Although a number of other enzymes have been shown to play important roles in the determination of development - for example, glutanine synthetase activity is important in its role of converting nitrogen released from protein hydrolysis into translocatable glutanine (Skorey and Beevers, 1978) and the concomitant change in leaf status from sink to source (Ryle and Powell, 1972) - the relationship of phosphorus metabolism to ontogenetic control must be crucial.

## i. Early events.

Germination involves rapid increases in a number of enzyme activities resulting from activation and induction (see Chapter 3). Thomas (1976a) found that the increase in phosphatase during germination was partially insensitive to cyclohexamide and that this form of enzyme was particle-bound. Yatsu and Jacks (1968) noted the presence of acid phosphatase in protein bodies of dry cotton seeds. Hydration may result in its activation. De novo synthesis does occur concurrently with this activation (Shain and Mayer, 1968; Thomas, 1976a). All but one of the nine isoenzymes of acid phosphatase in germinating lettuce seeds were present in the dry seeds (Meyer et al., 1971). Stoddart et al. (1973) observed an acid phosphatase isoenzymic shift during the first 12 h. of barley germination from a slow moving form to two faster-moving forms (in electrophoretic separation) suggestive of mobilization by dissolution of high molecular weight aggregates or associations. Naganna and Sripathi (1954) found increases in acid phosphatase and both acid and alkaline pyrophosphatases in germinating wheat presumed to have a catabolic function in the mobilization of food reserves for germination. A new soluble ATPase appears 24 - 48 h. after lettuce seed germination (Meyer et al., 1971) but independent of protein synthesis. Meyer et al. (1971) provide equivocal suggestions that this arises from release of a partially inactive particulate phosphatase. RNAse activity has been shown to increase in germinating peas (Barker and Douglas, 1960) and barley coleoptiles (Ledoux et al., 1962); however, Grellet et al., (1968) noted simultaneous increase in RNA and RNAse in Vicia sativa embryos with an increase in RNAse but decline in RNA of the cotyledons.

## ii. Light effects.

There are a number of novel enzymes which arise from light treatment and the activities of many others are modified drastically by light (Marcus, 1960; Keister *et al.*, 1962; Kagawa *et al.*, 1973). One of the most striking increases in activity is that of polyribosome associated RNAse in lupin seedlings which may be up to a twofold increase (Acton *et al.*, 1970). This observation is consistent with the degradative function of RNAse since growth has been inhibited by light. Udvardy *et al.* (1967) found light inhibited particle-bound RNAses but stimulated total RNAse activity in *Avena* leaves. The particulate RNAse was mostly associated with chloroplasts. ATPase activity increased by 72% in greening bean leaves and developed a requirement for $Mg^{2+}$ (Gregory and Bradbeer, 1975) resembling the activity reported for greening (McCarty and Racker, 1968) but the short time required for this increase suggests that it resulted from activation rather than *de novo* synthesis. Alkaline pyrophosphatase activity correlates with increased protein synthesis in bean leaf discs and is associated with chlorophyll increase in cucumber cotyledons (Rauser, 1971). Acid phosphatase activity doubled in the first 10 h. of illumination in leaves of *L. temulentum* (Hedley and Stoddart, 1972).

## iii. Phase of maturity.

Enzymes associated with rapid growth and high metabolic activity decline at maturation. Acid RNAse declines with cessation of protein synthesis in first seedling leaves of barley (Lazar and Farkas, 1970). Alkaline pyrophosphatase ~~loses its dominance over~~ decreases relative to acid pyrophosphatase in fully grown bean leaves as subsequent leaves develop (Naganna and Sripathi, 1954). Leaves developing translocation function show increased acid pyrophosphatase activities (Naganna and Sripathi, 1954; Kar and Mishra, 1975).

## iv. Senescence.

Although caution has already been applied to interpretation of the results obtained in ageing studies particularly concerning the relationship of detached and attached tissue, it is even more critical to the interpretation of enzyme activities (see Chapter 5). *In vivo* results would suggest that RNAses and acid phosphatases increase markedly at senescence (Lazar and Farkas, 1970; McHale and Dove, 1968; Parish, 1968 ; Hedley and Stoddart, 1972b; Yatsu and Jacks, 1972).

Chromatin associated RNAse increased with the progress of senescence in barley first leaves as the second leaves started to grow (Srivastava, 1968 a) whilst soluble RNAse decreased and only increased at very late stages of senescence. Baumgartner and Mahle (1977) observed dramatic changes in the proportions of isoenzymes of RNAse during the course of morning glory petal senescence with two isoenzymes increasing and two disappearing. The RNAse increase is at least partially due to *de novo* synthesis as shown by density labelling studies (Baumgartner *et al.*, 1975; Sacher and Davies, 1974) and work with inhibitors (DeLeo and Sacher, 1970; Thomas, 1975).

Both alkaline and acid pyrophosphatases decline with age in sunflower and tobacco leaves and cucumber cotyledons, but the alkaline pyrophosphatase decline showed a closer correlation with chlorophyll decline (Rauser, 1971) and with increases of catabolic enzymes (Lewington *et al.*, 1967). Kar and Mishra (1975) reported a concomitant increase in acid pyrophosphatase with a decrease in alkaline pyrophosphatase in senescence of both attached and excised rice leaves. Kisban *et al.* (1964) also found a reduced alkaline to acid pyrophosphatase ratio in wheat and barley leaf senescence.

## v. Enzyme location.

There is evidence to suggest that the primary cytological location of many enzymes changes during development or more specifically that different isoenzymes, which are differently located, are dominant at

different stages. As has already been mentioned, chromatin-associated RNAse is more active than soluble RNAse in senescence (Srivastava, 1968 b) and dominant ATPase activity changes from a particulate to a soluble cellular location during the first 72 h. of germination of lettuce seeds (Meyer et al., 1971).

Similarly, the increase in acid phosphatase with age has been found to be restricted to the particulate fraction (Udvardy et al., 1969). The cellular location of the enzymes under investigation was established in order to distinguish age-related shifts in location. Neutral ATPases are generally considered to be associated with the plasma membrane (for example, Winter-Sluiter et al., 1977) and alkaline ATPases may be of mitochondrial origin (Leonard and Hotchkiss, 1978).

Approximately 80% RNAse is soluble (Lyndon, 1966; Gibson and Paleg, 1972). Vacuoles or lysosome-like vesicles have been implicated as the site for soluble acid RNAses. Acid RNAses have also been observed in mitochondrial location (Wilson and Shannon, 1963; Mahle, 1966; Mahle and Moor, 1968; Leigh, 1978 personal communication). The alkaline RNAses have frequently been observed as being particulate (Matsushita and Ibuki, 1960; Reddi and Mauser, 1965; Semadeni, 1967).

The latency of enzymes at different stages may alter. Parish (1968) and Pirie (1959) noted that peroxidase activity could be raised in young plant tissue to almost mature levels by freezing and thawing or application of anionic detergents.

## II. Methods.

### i. Extraction for assay of soluble enzymes.

Leaf tissue was homogenized in a pestle and mortar with three times its volume of extraction buffer with approximately 16% (W/V) sand and 1.6% (W/V) PVP. The extraction buffer consisted of

50 mM. Tris-HCl pH 7.6
5 mM. magnesium sulphate
15 mM. potassium chloride
2 mM. mercaptoethanol.

The homogenate was centrifuged at 1,000 g. for 10 min. at 4°C. in a MSE bench centrifuge. Aliquots of the supernatant were used for ATPase estimation without further purification. The 1,000 g. supernatant was centrifuged at 20,000 g. for 20 min. in an IEC International refrigerated centrifuge Model PR-2 (International Equipment Co., Needham Hts. Mass., USA) using a 6x7 ml. angle rotor (295 head). Aliquots of this supernatant were used directly on PAGE after making to 10% (W/V) with sucrose. Further aliquots were used in Lowry protein estimation following precipitation with trichloroacetic acid and digestion with sodium hydroxide (as described in Chapter 3, II iii).

The supernatants were desalted by passage through Sephadex G-25 (medium) columns (13x0.8 cm.; void volume = 4 ml.), without restricting flow then eluted with buffer containing

10 mM. Tris-HCl pH 7.6
1 mM. magnesium sulphate
3 mM. potassium chloride

and collected in four times the original volume. Desalting provided up to 400% improvement in enzyme activity. These eluants were used undiluted for all other enzyme assays. The 20,000 g. pellet was combined with the 1,000 g. pellet for chlorophyll estimation.

ii. PAGE of isoenzymes.

A modified slab gel system, similar to that described by Price (1968) and illustrated and described in detail by Hedley (1971) was adopted. The 7% (W/V) acrylamide gel solution was made up by mixing

| | | |
|---|---|---|
| 7% (W/V) | "Cyanogum 41" | adjusted to pH 9 with 1.0 M. citric acid |
| 76.3 mM. | Tris | |
| 0.5% (V/V) | TEMED | |
| 0.1% (W/V) | ammonium persulphate. | |

Slabs (7.5x7.5x0.3 cm.) were poured (whilst horizontal) and contained by perspex 'formers' made water-tight with silicone grease. At the eventual upper end, the perspex former was slotted so as to provide six lanes (0.8 cm. wide) for sample application. A glass front plate was placed carefully over the setting gel so as to exclude air bubbles. The front and back plates were clamped together. Once the gel had polymerized the upper and lower 'formers' were removed and excess acrylamide scraped away or washed off with 'running buffer' containing

30 mM. boric acid adjusted to pH 9.0 with 2.5 M. NaOH.

Strips of acrylamide gel the width of the slab and 0.5 cm. wide were layered across the top of the slotted end of the slab gel so as to form enclosed compartments for sample application. The PAGE gels were held vertically between two perspex troughs, the lower end immersed in 'running buffer' containing an anodic platinum electrode. 13 cm. wicks of three layers of Whatman No. 3 chromatography paper (7.5 cm. wide) were soaked in 'running buffer' and then eased between the front and back plates until flush with the strip of gel overlaying the slab. The other ends of the wicks were immersed in 'running buffer' contained in the cathodic chamber.

50 - 100 µl. samples were applied to the compartmented surface of the gel. Electrophoresis was carried out using a power pack set at constant current mode providing 15 mA. per gel (resulting in a

potential difference of about 320 V.) at 4°C., until the ionic front (visualized by co-electrophoresis with bromophenol blue) was 1 cm. from the lower end of the gel (usually about 3 h.).

Prior to electrophoresis, gels could be stored for up to 36 h. with the 'formers' still in position, in closed containers at 4°C.

### iii. ATPase (EC 3. 6. 1. 3)

$$ATP \xrightleftharpoons{ATPase} ADP + P_i$$

A. a. Test-tube assay

For each sample two tubes were prepared containing

| | | final concentration |
|---|---|---|
| 150 µl. | 100 mM. citrate buffer pH 5 (Sørensen) | 60 mM. |
| 50 µl. | 50 mM. ATP | 10 mM. |
| 25 µl. | 100 mM. calcium nitrate | 10 mM. |
| or 25 µl. | 100 mM. magnesium sulphate | 10 mM. |

or neither of these last two.

The ATP was made as a 5 ml. stock solution of 50 mM. in water saturated with sodium bicarbonate to pH 6.

To one of the duplicate tubes 25 µl. enzyme extract (crude 1,000 g. supernatant) were added, to give a final assay volume of 250 µl. Both tubes were incubated at 37°C. for 10 min. The reaction was terminated by addition of 500 µl. ammonium molybdate (2 mM. in 5 M. sulphuric acid). 25 µl. enzyme extract was added to the second assay tube (control) following the addition of the ammonium molybdate. 200 µl. Fiske SubbaRow reducer (Bartlett, 1959 - see below) were added and the mix was made up to 5.0 ml. with distilled water. The $A_{719}$ was recorded. Estimation of ATPase activity was expressed as $P_i$ released with reference to a calibration curve constructed using $Na_3PO_4.12H_2O$ (Figure 4.1).

FIGURE 4.1

Calibration of absorbance at 719 nm. for released phosphorus ($P_i$).

TABLE 4.1

Divalent ion requirement. Left hand column indicates the assay conditions. $A_{719}$ is the absorbance of the assay to which enzyme extract has been added. $A_{719}^{Blk}$ is the control, enzyme added after completion of incubation, and $A_{719}$ is the difference between these two values.

FIGURE 4.1

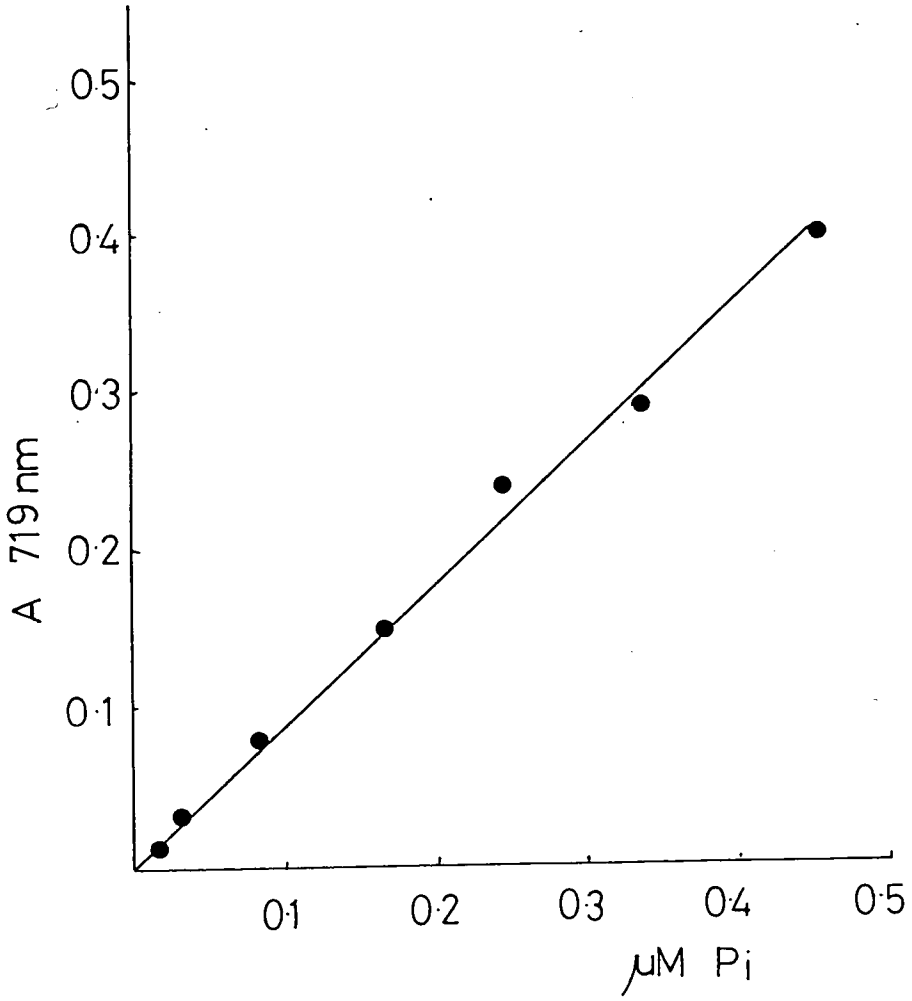



TABLE 4.1

| | A 719 | A 719 Blk. | Δ A 719 |
|---|---|---|---|
| buffer blank | 0·10 | 0·10 | 0 |
| - $Mg^{2+}$ - $Ca^{2+}$ | 0·31 | 0·25 | 0·06 |
| + $Mg^{2+}$ - $Ca^{2+}$ | 0·45 | 0·25 | 0·20 |
| - $Mg^{2+}$ + $Ca^{2+}$ | 0·45 | 0·26 | 0·19 |
| + $Mg^{2+}$ + $Ca^{2+}$ | 0·40 | 0·24 | 0·16 |

The Fiske-SubbaRow reducer consisted of

10 mM. aminonaphthol sulphonic acid

40 mM. sodium sulphite

made up in 790 mM. sodium metabisulphite.

The reducer was filtered through Whatman No. 1 chromatography paper prior to use and was made up fresh every fortnight.

b. Calculation of enzyme activity.

1 µmole $P_i$ in 5.0 ml. gives $A_{719} = 0.875$

Since $ATP + H^+ + OH^- \rightleftharpoons ADP + P_i$

ATPase activity can be obtained as follows:

$$U.ml^{-1} = \frac{40 \times x}{10 \times 0.875}$$

$$= 4.57x$$

where $x = A_{719}$ assay.

c. Divalent ion requirement.

Assays were carried out in the presence or absence of 10 mM. magnesium suphate or 10 mM. calcium nitrate as shown in Table 4.1. All subsequent assays included 10 mM. magnesium sulphate. A certain amount of competitive inhibition occurred when both catalytic ions were added at 5 mM.

d. pH optimization.

Using buffers ranging from pH 3 to pH 10 assays were carried out at final pH 4 to pH 8 (Table 4.2, Figure 4.2).

All subsequent assays were conducted at pH 5.0 using 100 mM. citrate buffer (Sørenson).

e. Specificity towards ATP.

50 mM. solutions of ADP and AMP were prepared in a similar way to the preparation of stock ATP solutions and were used in place of ATP in incubations as indicated in Table 4.3.

f. Calculation of $K_m$ and $V_{max}$.

Several ATP concentrations were introduced into the assay (Figure 4. 3 A). A Lineweaver-Burk plot (Lineweaver and Burk, 1934) was constructed (Figure 4.3 B) and the $K_m$ found to equal 3.6 mM. and the $V_{max}$ = 4.7 $Uml.^{-1}$. All subsequent assays included 10 mM. ATP.

TABLE 4.2

ATPase activity as estimated by $P_i$ release under different pH assay conditions (final pH). The different compositions of buffers had different effects on the final pH as indicated.

FIGURE 4.2

ATPase activity under different pH assay conditions.

●——● the results of the use of Sørensen citrate and Gomori Tris-HCl buffers

Δ---Δ the results from the use of Sørensen glycine.NaOH buffers.

TABLE 4.2

| buffer pH | final pH | Δ $A_{719}$ | enzyme activity $U.ml^{-1}$ |
|---|---|---|---|
| 3·0 | 4·0 | 0·60 | 2·74 |
| 4·0 | 4·5 | 0·74 | 3·38 |
| 5·0 | 5·0 | 1·27 | 5·80 |
| 6·0 | 6·0 | 0·85 | 3·38 |
| 7·0 | 6·5 | 0·58 | 2·65 |
| 8·0 | 8·0 | 0·29 | 1·33 |
| 8·5 | 6·0 | 0·54 | 2·47 |
| 9·5 | 8·0 | 0·13 | 0·59 |
| 10 | 8·5 | 0·11 | 0·50 |

citrate (Sørensen)
Tris.HCl (Gomori)
glycine NaOH (Sørensen)

FIGURE 4.2

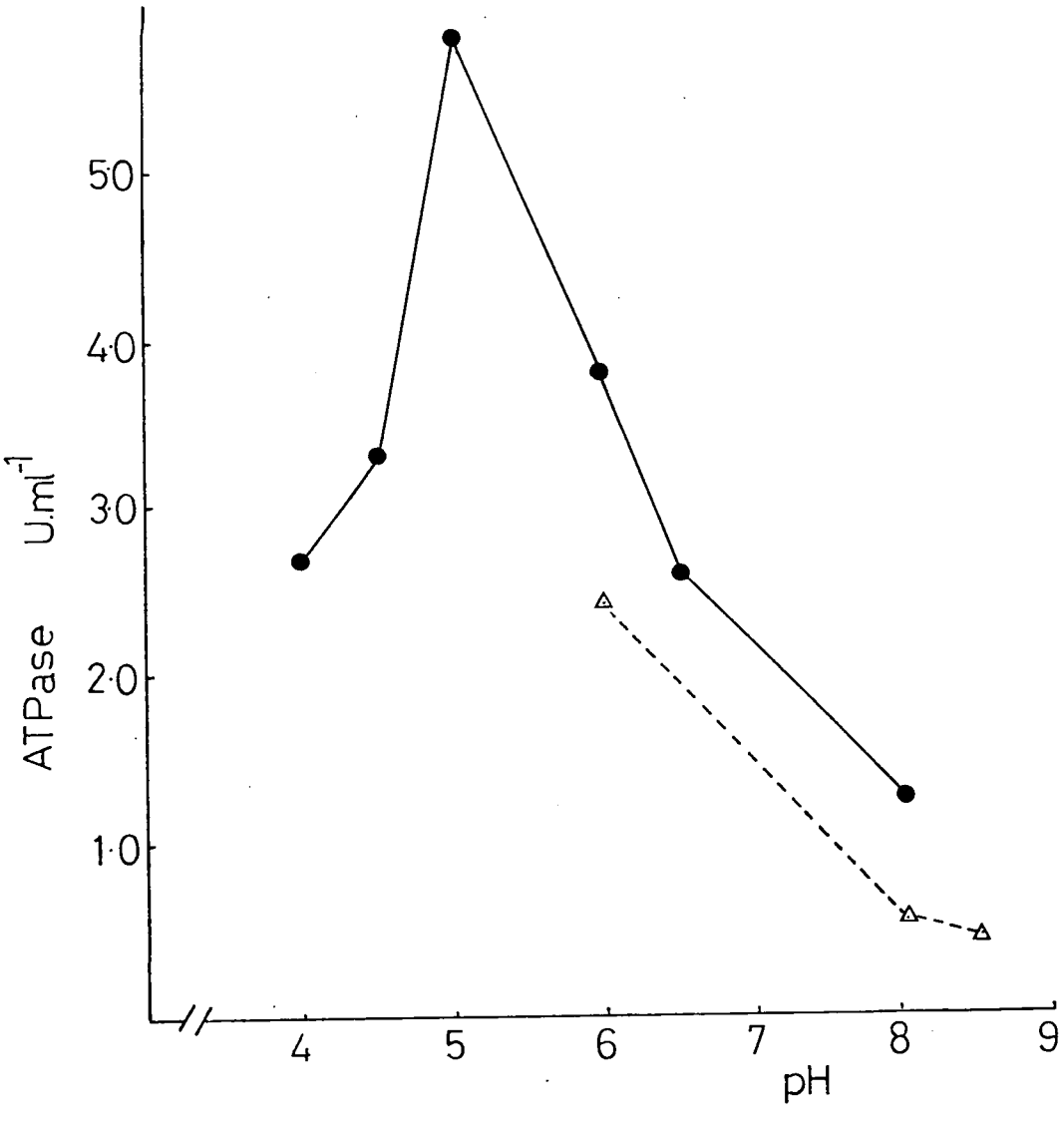


TABLE 4.3

Specificity of ATPase effect of enzyme extract on ATP, ADP and AMP.

FIGURE 4.3 A.

Effect of substrate (ATP) concentration on ATPase activity.

TABLE 4.3

| substrate | ΔA719 | enzyme activity $Uml^{-1}$ |
|---|---|---|
| ATP | 0·65 | 2·97 |
| ADP | 0·33 | 1·51 |
| AMP | 0·15 | 0·69 |

FIGURE 4.3

A.

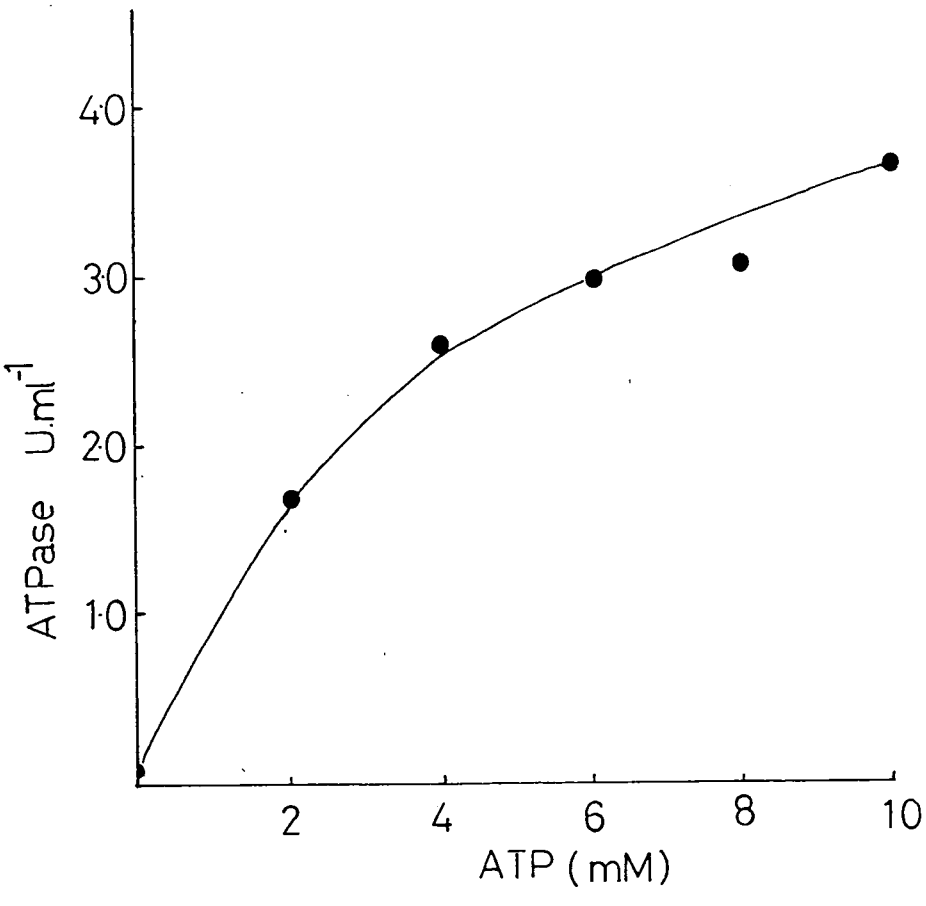


FIGURE 4.3 B.

Lineweaver-Burk plot of ATPase activity.

V = $U.ml^{-1}$

S = substrate concentration in moles.

FIGURE 4.4

Effect of magnesium concentration on ATPase activity.

FIGURE 4.3
B.

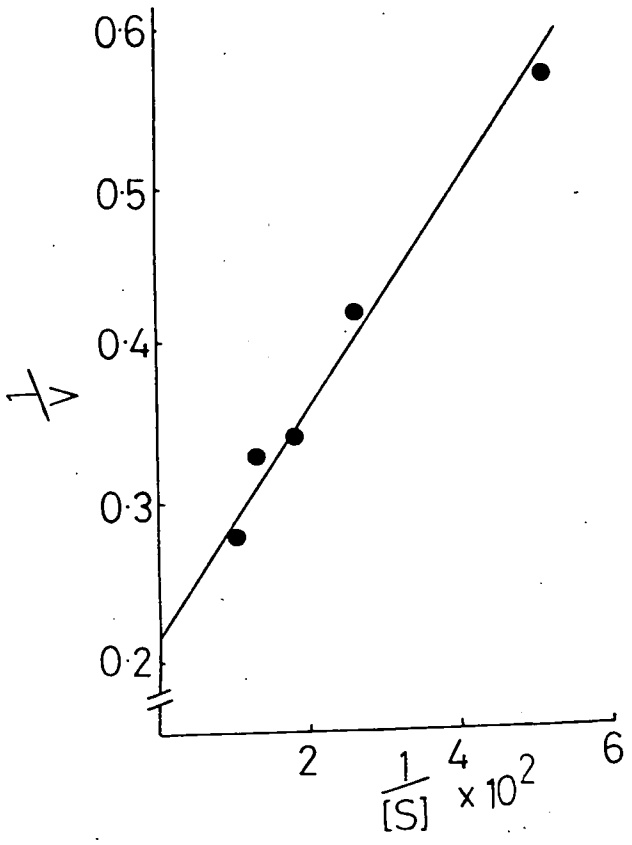



FIGURE 4.4

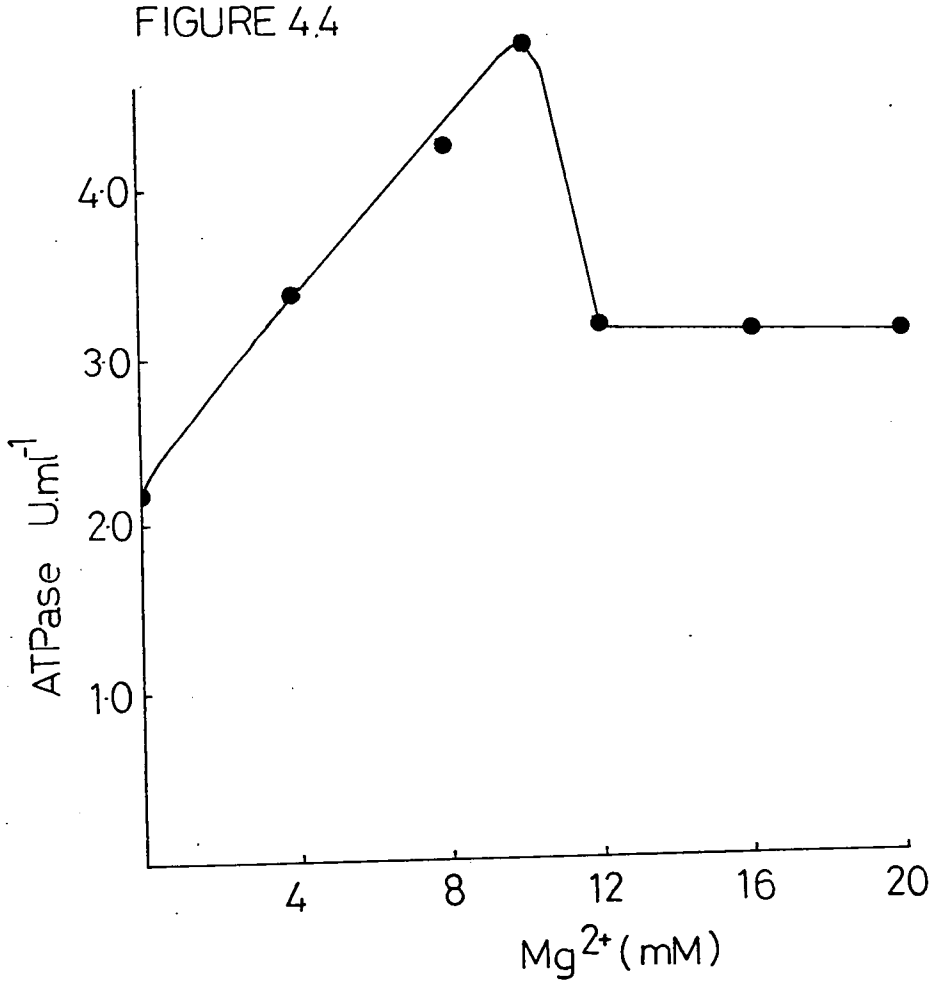


FIGURE 4.5

Effect of enzyme extract concentration on the assay for ATPase.

FIGURE 4.6

Photograph of PAGE gel stained for ATPase activity (d). Tracks 1 & 2 are stained for AMPase and ADPase respectively.

FIGURE 4.5

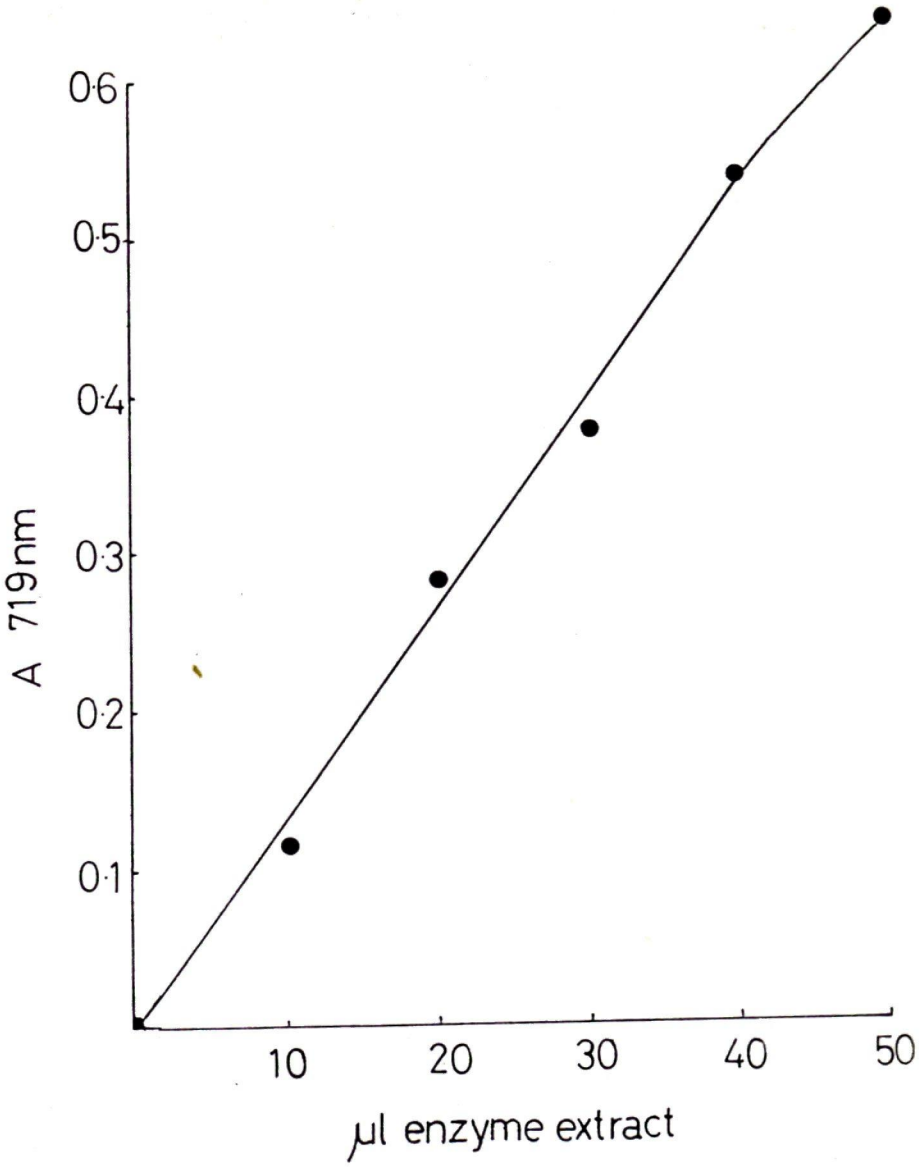
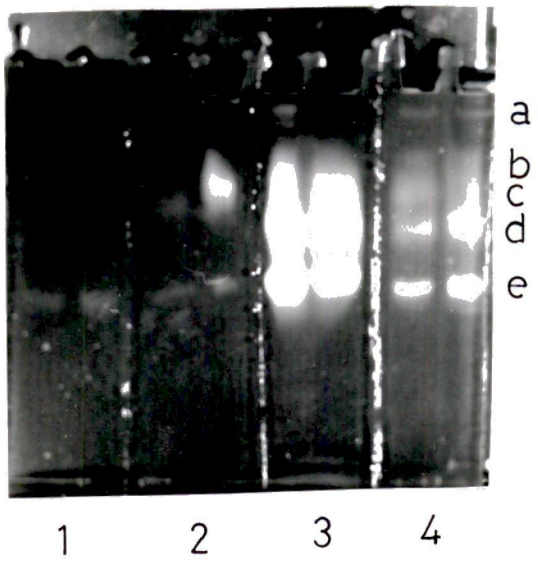



FIGURE 4.6



g. Optimal $Mg^{2+}$ concentration.

Having established the divalent ion requirement (see c.) the optimal concentration was ascertained using a range of concentrations (Figure 4.4). Subsequent assays were carried out in the presence of 10 mM. magnesium sulphate.

h. Response to enzyme concentration.

A number of enzyme concentrations were added to the assay in order to confirm classic enzymatic characteristics (Figure 4.5).

B. PAGE assay.

Following electrophoresis the slab gels were incubated overnight in a mixture of

| | | |
|---|---|---|
| 9.4 | mM. | ATP |
| 1 | mM. | magnesium sulphite |
| 50 | mM. | calcium nitrate |
| 20 | mM. | Tris-HCl pH 7.5 |
| 5 | mM. | magnesium sulphate |
| 125 | mM. | EDTA (disodium salt) |
| 1 | mM. | mercaptoethanol. |

A stock of ATP and magnesium sulphite was made to pH 7.5 with NaOH and was added in equal volume to a stock containing 100 mM. calcium nitrate in protein extraction buffer (see Chapter 3. II i). The ATPase isoenzymes appeared as white bands of calcium phosphate. Neither the inclusion of lead nitrate in the incubation (Plant Biochemistry, Jodrell Laboratory, Kew, 1978; personal communication) nor the inclusion of ammonium sulphate (10 g./l.) provided any improvement in the technique or visualization of the bands. The specifity of this location porcedure was tested by substituting ADP and AMP for the ATP in the incubation (Figure 4.6). In the enzyme extract there is one general phosphatase (e) another non-specific phosphatase (c) and ATPase (d). The general phosphatases are probably responsible for the activity recorded in Table 4.3.

## iv. RNAse. (EC 2. 7. 7. 16/17)

RNAse disrupts all internucleotide linkages in RNA via intramolecular transphosphorylation.

The assay was based on the release of soluble nucleotides by the action of the RNAses on RNA. The amount of activity can be assessed by the increase in absorbance at $A_{260}$ due to soluble nucleotides after precipitation of the oligonucleotides.

A. a. Test-tube assay.

The incubation contained

125 µl. buffer (either Tris-HCl pH 7.5 (Gomori) or citrate pH 5.0 (Sørenson))

125 µl. yeast ribonucleic acid (sodium salt) (made up as 0.8% (W/V) solution at pH 5.2 with 0.1 M. NaOH)

50 µl. enzyme extract.

Incubation at 37°C. was terminated after 45 min. by the addition of an equal volume (i.e., 300 µl.) of 400 mM. cadmium sulphate followed by a further incubation at 37°C. for 15 min. The precipitated oligonucleotides were removed by centrifugation at 1,000 g. for 5 min. in a MSE bench centrifuge. 100 µl. of the supernatant were diluted 25 times with distilled water and the $A_{260}$ recorded on a Pye-Unicam SP 800 spectrophotometer with reference to both "water blanks" and "no substrate blanks."

b. Calculation of enzyme activity.

It was found that 1 µmole NTP in an equivalent assay volume of 0.6 ml., diluted and measured in the manner described, gave an $A_{260} = 0.26$.

Thus, if $A_{260}$ of sample = x,

$$\text{enzyme activity } (U.ml^{-1}) = \frac{20 \times x}{0.26 \times 45}$$

$$= 1.709x.$$

c. pH optimization.

Using buffers ranging from pH 3.2 to pH 10, assays were carried out as shown in Figure 4.7.

pH 5 was found to be optimal for the assay of RNAse but as there are numerous reports of an alkaline RNAse (for example, Reddi, 1966;

FIGURE 4.7

Effect of pH on RNAse activity.

FIGURE 4.8

Effect of substrate concentration (expressed as % W/V of total assay since yeast RNA is of heterologous molecular weight) on RNAse activity.

FIGURE 4.7

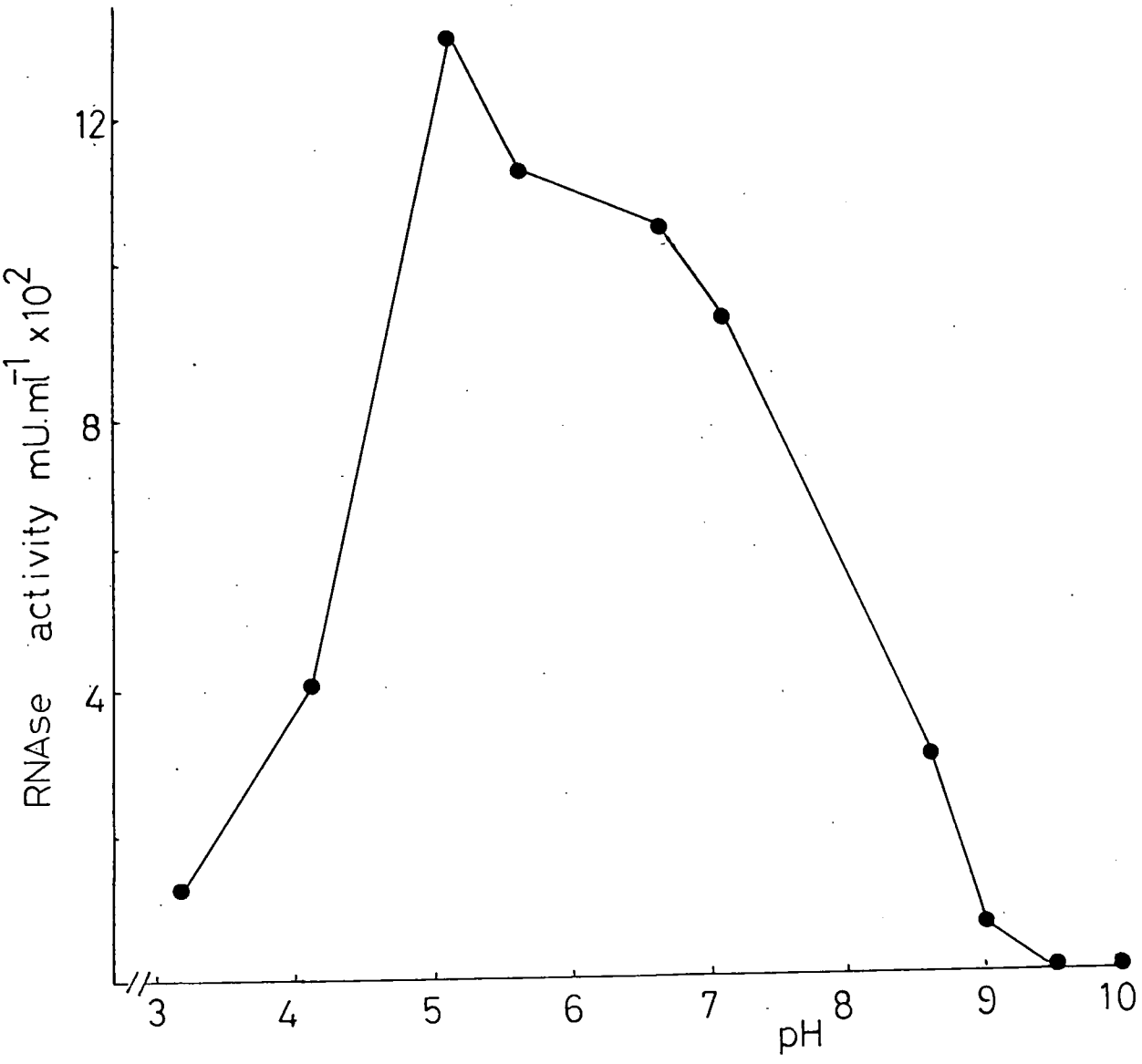
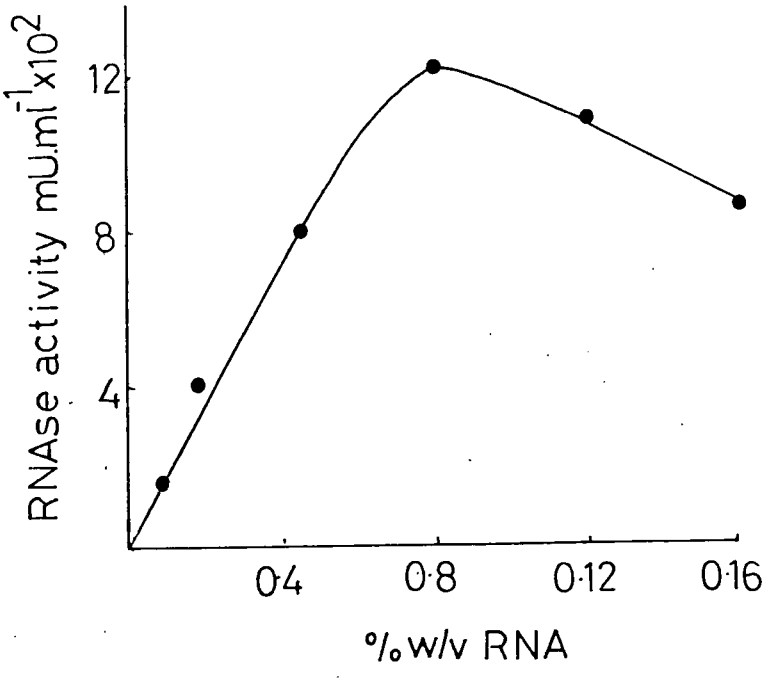



FIGURE 4.8



Wilson, 1968; Birmingham and Maclachlan, 1972; Dove, 1973) estimations were also made at pH 7.0 on the principle that it might manifest itself at different stages of development (Sahulka, 1971), a possibility which may have been obscured by using extracts of complete, fully matured leaves.

d. Substrate optimization.

Several assays were carried out at different substrate concentrations to find the concentration at which maximal activity ($V_{max}$) occurred, i.e., saturation. All subsequent assays used 0.8% (W/V) ribonucleic acid solutions (Figure 4.8).

B PAGE assay.

The RNAse isoenzyme detection method was essentially that of Wilson (1969).

Following electrophoresis, slab gels were incubated at 4°C. in 5 mM. citrate buffer (pH 5.0) for 15 min. to bring the pH of the gel to that for optimal RNAse activity. The gels were then incubated for a further 30 min. in 0.4 % (W/V) yeast RNA in citrate buffer (pH 5.0) at room temperature. The gels were then immersed for 30 s. with constant agitation in toluidine blue solution (0.2 g. in 0.5% (V/V) acetic acid). The gels were then plunged into a large volume of de-ionized water to dilute the stain rapidly. Destaining was achieved by several washes of 7% (V/V) acetic acid. Subsequent soaking in 5% (V/V) perchloric acid (Wilson, 1969) did not enhance the banding pattern. Isoenzymes appeared as clear bands against a background of blue-black RNA stained gel (Figure 4.10).

The RNAse locating method of Rosenthal and Lacks (1977) was tested. Although little success was achieved with slab gels, the method was successful when applied to rod gels. This may be due to the increased intensity of background staining in the rod gels by virtue of their thickness which allowed clearer delineation of the unstained areas. The method works on the principle that RNAse activity can be restored after electrophoresis in SDS, i.e., denaturing conditions. RNA substrate is immobilised on the gel supports by

FIGURE 4.9

Effect of enzyme extract concentration on the assay for RNAse.

FIGURE 4.9

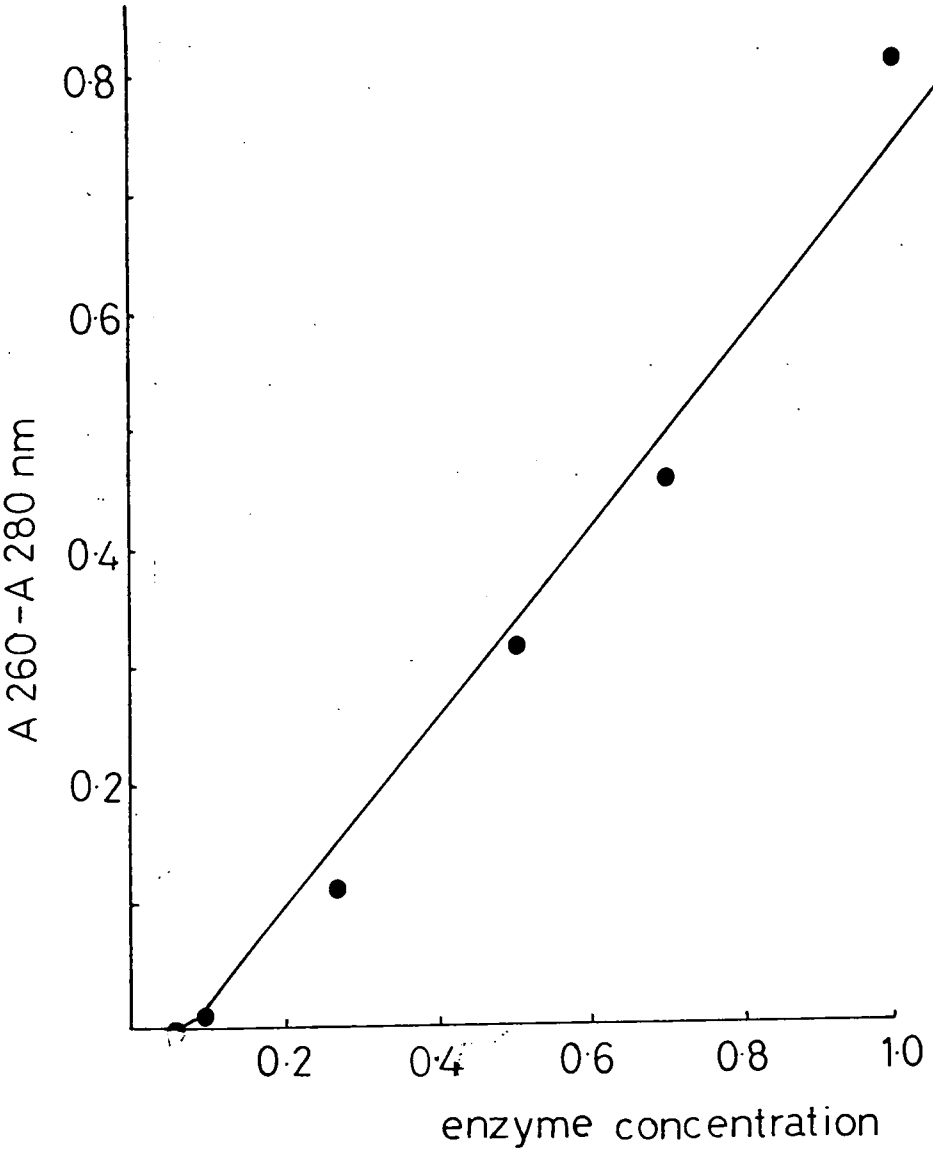


FIGURE 4.10

Photograph of RNAse isoenzymes as visualized by the method modified from Wilson (1969) as applied to slab PAGE gels.

FIGURE 4.11

Diagram of RNAse location on rod gels as visualized by the method of Rosenthal and Lacks (1977).

EB = stained by ethidium bromide

CBB = protein stained by coomassie Brilliant Blue

← presumptive RNAse

areas of CBB stain

fluorescence

clear areas of RNAse action

opaque band

dark opaque band.

FIGURE 4.10

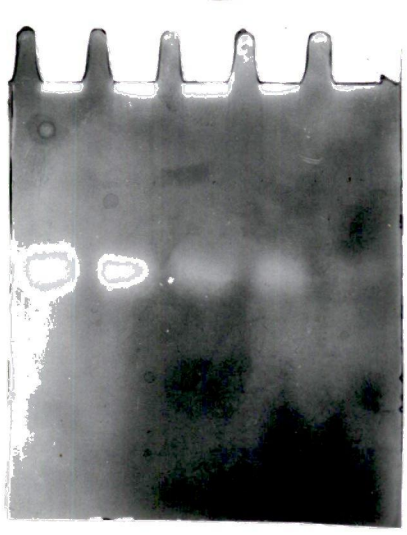
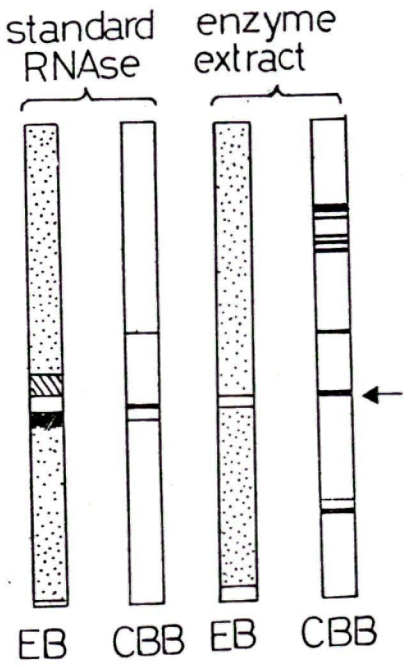


FIGURE 4.11



inclusion prior to polymerization of the gel (unlike the previously described method where RNA substrate is only allowed to penetrate the gel after electrophoresis). Rod or slab gels were made including 0.002% (W/V) yeast RNA. Following electrophoresis in appropriate running buffers containing 0.1% (W/V) SDS and 2 mM. EDTA (disodium salt) the gels were washed in distilled water followed by incubation for 1 h. in a mixture of

40 mM. Tris-HCl pH 7.6
2 mM. magnesium chloride
0.02% (W/V) sodium azide

then a second distilled water wash, and incubation overnight at 30°C. to allow for reconstitution of the RNAse. The gels were then transferred to another mix containing in addition 2 mM. calcium chloride and were incubated for a further 3 h. at 30°C.
1 µg./ml. ethidium bromide was then added and the development of fluorescence under a U.V. source was monitored over 1 h. Figure 4.11 shows a typical band distribution as recorded from rod gels. (These gels could not be photographed easily because of the necessity for U.V. light.).

### v. Pyrophosphatase (EC 3. 6. 1. 1)

Pyrophosphatase hydrolyses pyrophosphate to orthophosphate.

A. a. Test-tube assay.

The determination of acid and alkaline inorganic pyrophosphatase activities was carried out essentially by the method of Kar and Mishra (1975) in conjunction with the method of Stanton (1968) for the determination of liberated phosphorus as modified from Bartlett (1959). The methods of Jaffe and Galston (1966) and Lowry and Lopez (1946) for phosphorus measurement were compared but the Stanton procedure proved the most reproducible and straightforward.

The assay mix included

150 µl. citrate buffer pH 5
50 µl. 75 mM. sodium pyrophosphate
50 µl. enzyme extract

or 100 µl. Tris-HCl buffer pH 8
50 µl. 75 mM. sodium pyrophosphate
50 µl. 750 mM. magnesium chloride
50 µl. enzyme extract.

Incubation was carried out at 37°C. for 10 min. and was terminated by addition of 500 µl. 20 mM. ammonium molybdate in 5 M. sulphuric acid. Initially, 1 ml. 20% (V/V) perchloric acid was used to stop the reaction and after centrifugation, aliquots of the clarified supernatant were taken for phosphorus estimation. The method was simplified by the addition of ammonium molybdate directly to the incubation. 200 µl. Fiske-SubbaRow reducer (see II iii, A a) were added and the incubation was made up to 5 ml. before centrifugation at 3,500 g. in a MSE Multex centrifuge. The developed colour was measured at $A_{719}$ in a Vitatron colorimeter and the phosphorus concentration calculated by reference to a standard curve constructed using $Na_3PO_4.12H_2O$ (Figure 4.1) after adjustment for "no enzyme" and "water blanks."

b. Calculation of enzyme activity.

1 µmole $P_i$ in 5.0 ml. gives $A_{719}$ = 0.875

$$U.ml^{-1} \text{ pyrophosphatase} = \frac{20 \times x}{10 \times 0.875}$$

$$= 2.29\ x \text{ where } x = A_{719} \text{ assay.}$$

c. pH optimization and $Mg^{2+}$ requirement.

The assays were carried out at a range of pH in the presence or absence of 200 mM. magnesium chloride. From Figure 4.12 it can be seen that magnesium favours alkaline pyrophosphatase activity and inhibits the activity at lower pH (Rauser, 1971).

d. Optimal $Mg^{2+}$

Assays were carried out at pH 8.0 in the presence of a range of magnesium chloride concentrations (Figure 4.13). Subsequent assays included 750 mM. $Mg^{2+}$.

e. Calculation of $K_m$ and $V_{max}$.

Several sodium pyrophosphatase concentrations were introduced into the assay (Figure 4.14 A). A Lineweaver-Burk plot was constructed (Figure 4.14 B) and the $K_m$ for acid pyrophosphate was found to equal 9.0 mM. and the $V_{max}$ = 1.69; $K_m$ for alkaline pyrophosphatase

FIGURE 4.12

Effect of pH on the assay for pyrophosphatase in the presence ( ●——● ) or absence ( △----△ ) of 200 mM. $MgCl_2$.

FIGURE 4.12

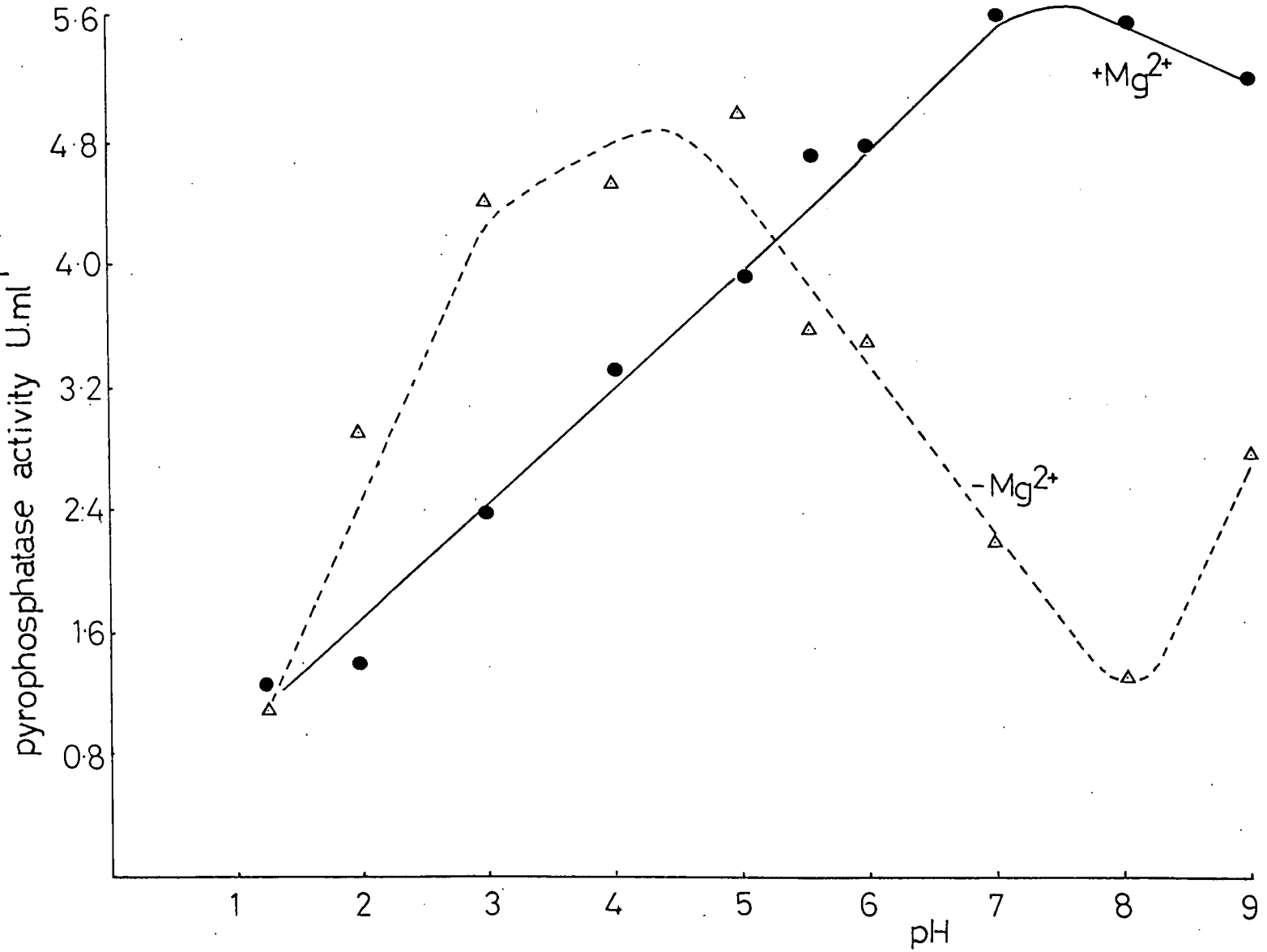

5·6
4·8
4·0
3·2
2·4
1·6
0·8
pyrophosphatase activity U.ml$^{-1}$
+Mg$^{2+}$
-Mg$^{2+}$
1
2
3
4
5
6
7
8
9
pH

FIGURE 4.13

Effect of $Mg^{2+}$ concentration on alkaline pyrophosphatase activity (pH 8.0).

FIGURE 4.14 A.

Effect of sodium pyrophosphate concentration on pyrophosphatase activity.

●—● plus $Mg^{2+}$ at pH 8.0

△---△ minus $Mg^{2+}$ at pH 5.0.

FIGURE 4.13

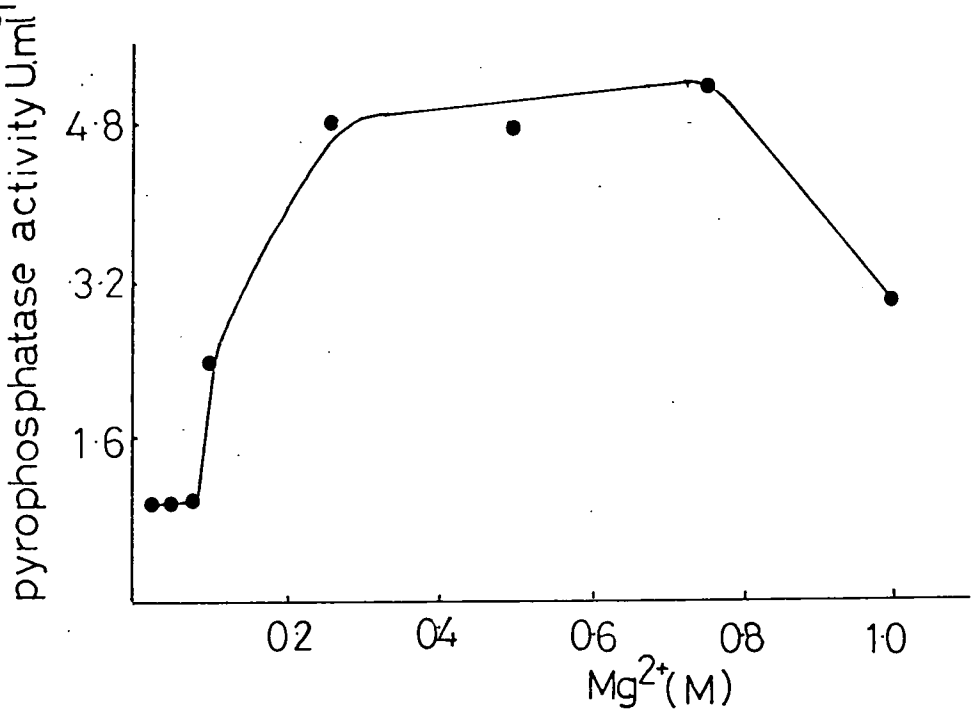



FIGURE 4.14

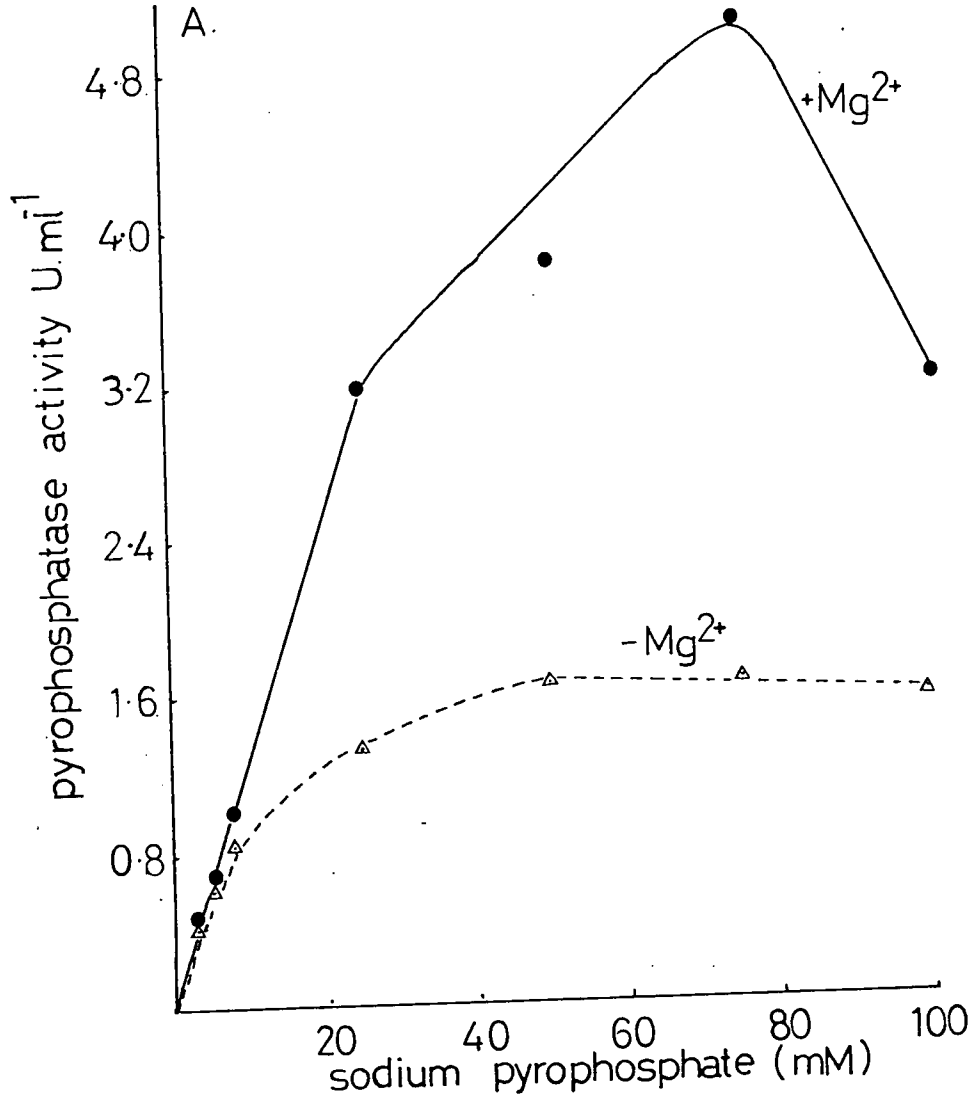
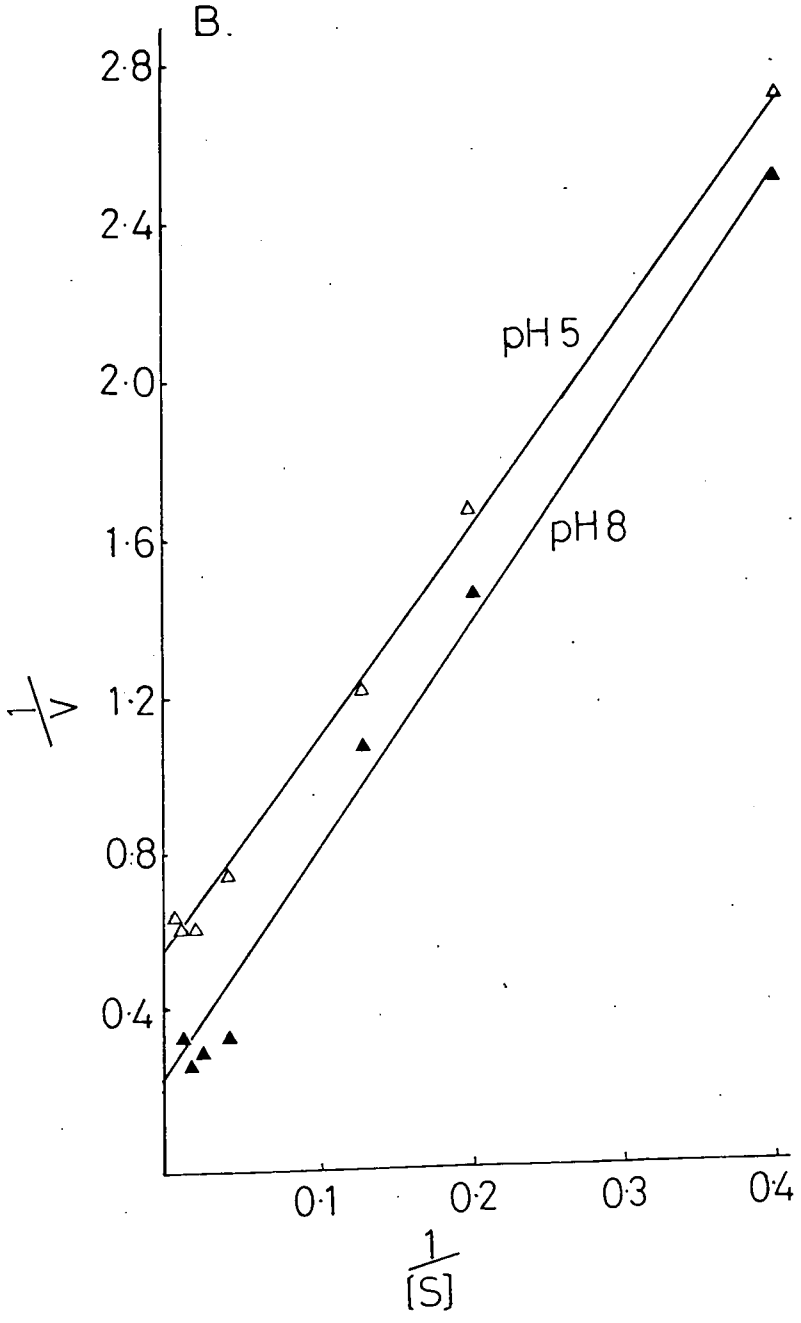


FIGURE 4.14 B.

Lineweaver-Burk plot for acid ( △——△ ) and alkaline (▲——▲ ) pyrophosphatases. The alkaline pyrophosphatase assay included 750 mM. $Mg^{2+}$.

FIGURE 4.14

B.



was found to equal 27.3 mM., $V_{max}$ = 4.55.

All subsequent assays included 75 mM. sodium pyrophosphate.

f. Response to enzyme concentration.

Figure 4.15 shows the results of a number of assays carried out using various enzyme concentrations to confirm classic enzymatic characteristics.

B. PAGE assay.

The identification of pyrophosphatase isoenzymes on PAGE slab gels was based on the test-tube method as suggested by Shaw and Prasad (1970). Following electrophoresis the gels were incubated in

15 ml. citrate buffer pH 5.0
5 ml. 75 mM. sodium pyrophosphate

or 10 ml. Tris-HCl pH 8.0
5 ml. 750 mM. magnesium chloride
5 ml. 75 mM. sodium pyrophosphate

for 1 h. 30 min. at room temperature. 10 ml. 200 mM. ammonium molybdate in 5 M. sulphuric acid were added followed by 4 ml. Fiske-SubbaRow reducer. Blue bands appeared almost immediately. Recording of these had to be carried out within 1 h. due to their transient nature. The bands were never particularly well defined as in other enzyme location procedures, tending to appear diffused (Figure 4.16).

vi. Phosphatase.

(Acid phosphatase EC 3. 1. 3. 2;
Alkaline phosphatase EC 3. 1. 3. 1)

The assay is based on the amount of 4-nitrophenol liberated per unit time from 4-nitrophenylphosphate, as determined in alkaline solution at 410 nM. (Walter and Schütt, 1974).

$$\text{4-nitrophenylphosphate} + H_2O \xrightleftharpoons{\text{phosphatase}} \text{4-nitrophenol} + H_3PO_4$$

A. a. Test-tube assay.

The assay mix contained

250 µl. acetate buffer pH 5
or 250 µl. glycine buffer pH 10
250 µl. 15.2 mM. p-dinitrophenol phosphate
100 µl. enzyme extract (diluted tenfold)

FIGURE 4.15

Effect of enzyme extract concentration on the assay for pyrophosphatase.

△——▲ alkaline pyrophosphatase

△——△ acid pyrophosphatase.

FIGURE 4.15

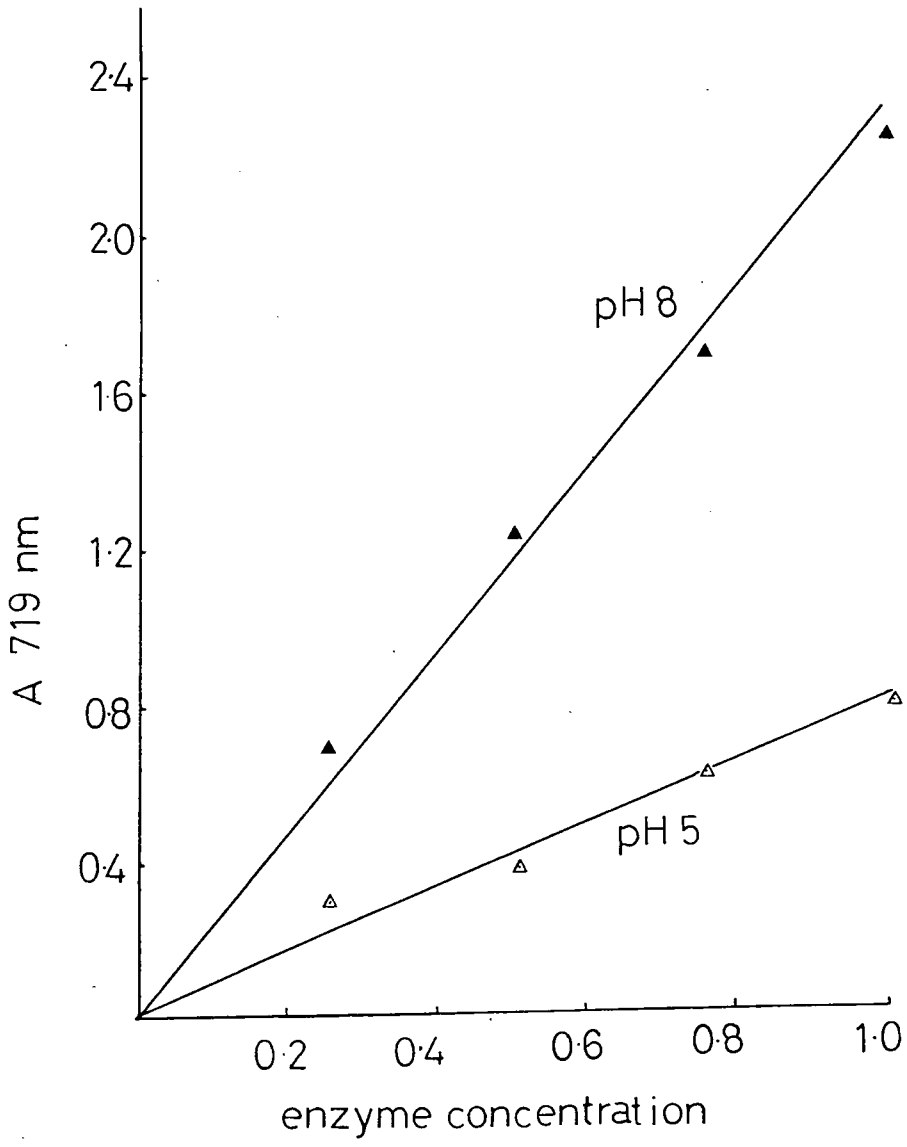
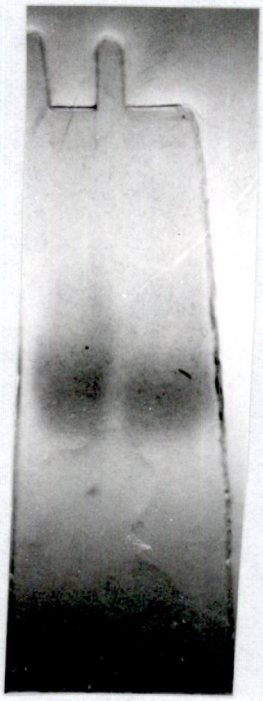

2·4
2·0
1·6
1·2
0·8
0·4
A 719 nm
pH 8
pH 5
0·2
0·4
0·6
0·8
1·0
enzyme concentration

FIGURE 4.16

Photograph of alkaline pyrophosphatase isoenzymes visualized by the method of Shaw and Prasad (1970) as applied to slab PAGE gels.

FIGURE 4.16



Incubation was for 10 min. at 37°C. and was terminated by addition of 2.5 ml. 100 mM. sodium hydroxide. The resultant colour development was measured at $A_{410}$ with a Vitatron colorimeter. Enzyme activity was calculated by reference to a standard curve constructed for p-nitrophenol (Figure 4.17) after adjustment for "no enzyme" and "water" blanks.

b. Calculation of enzyme activity.

The unit of enzyme is that amount of enzyme releasing 1.0 umole of p-nitrophenol per min. at 37°C.

Figure 4.17 shows the calibration curve constructed for p-nitrophenol diluted as for the enzyme assay. From this it can be extrapolated that 1 µmole p-nitrophenol has an $A_{401} = 6.75$.

$$\text{therefore, } U.ml^{-1} = \frac{100 \times x}{10 \times 6.75} = 1.5x$$

where $x = A_{401}$ assay.

c. Optimization of pH and $Mg^{2+}$ requirement.

The effect of 200 mM. and 20 mM. magnesium chloride concentrations on the assay were examined at a range of pH. There was no preferential enzyme activation with either concentration of magnesium (Figure 4.18) at pH 5.0, but there was slight activation at higher pH.

d. Calculation of $K_m$ and $V_{max}$

Several concentrations of p-nitrophenol phosphate were included in assays for acid phosphatase (Figure 4.19 A). A Lineweaver-Burk plot was constructed (Figure 4.19 B). $V_{max}$ was found to equal 1.1 $U,ml^{-1}$ and $K_m = 1.9$ µmole.

e. Response to enzyme concentration.

Figure 4.20 shows the results of a number of assays carried out using various enzyme concentrations to confirm classic enzymatic characteristics.

FIGURE 4.17

Calibration of absorption at 410 nm.
of p-nitrophenol in alkaline solution.

FIGURE 4.17

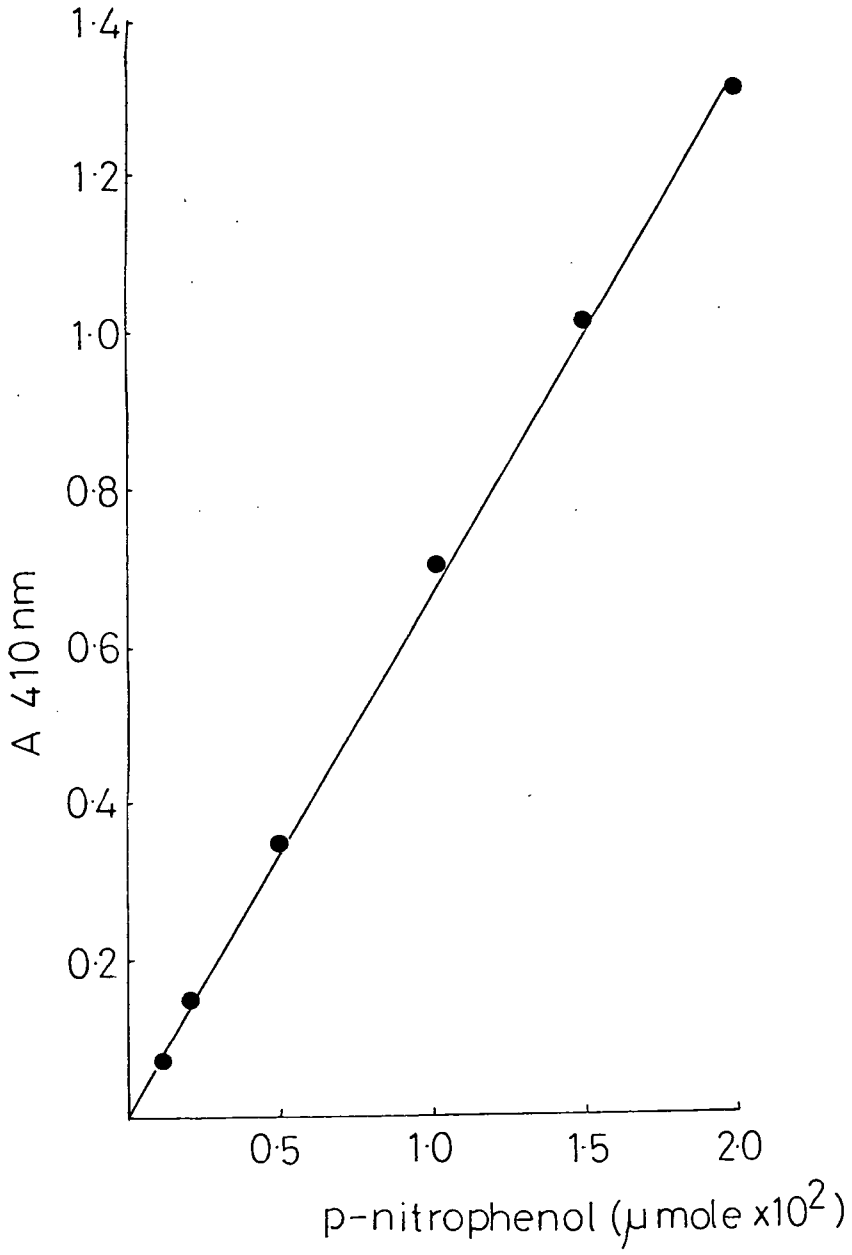


FIGURE 4.18

Effect of pH and $Mg^{2+}$ on phosphatase assay.

●—● No $Mg^{2+}$

△--△ 20 mM. $Mg^{2+}$

□····□ 200 mM. $Mg^{2+}$.

FIGURE 4.18

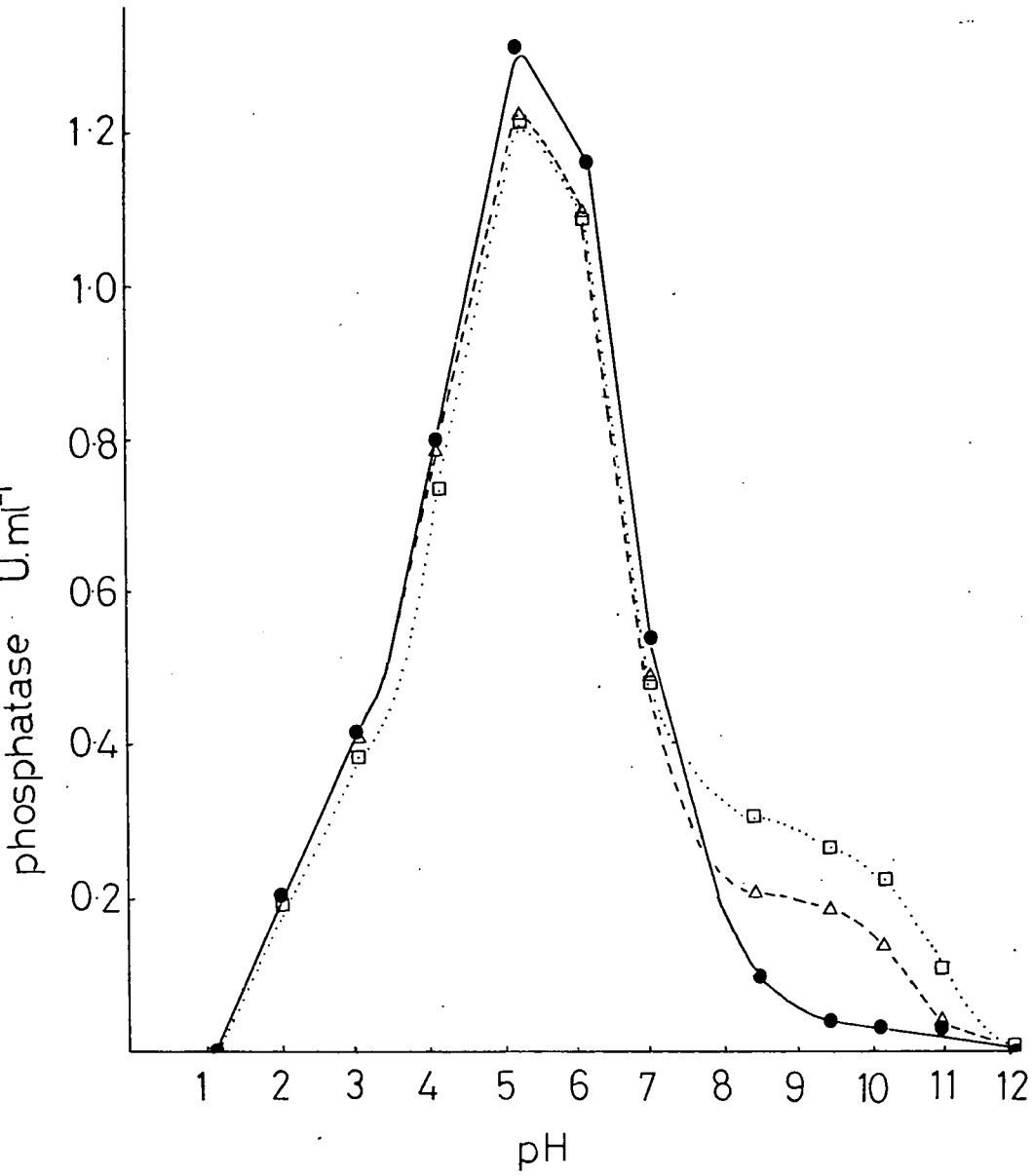


FIGURE 4.19 A.

Effect of p-nitrophenol phosphate concentration on acid phosphatase activity.

FIGURE 4.19 B.

Lineweaver-Burk plot for acid phosphatase.

FIGURE 4.19

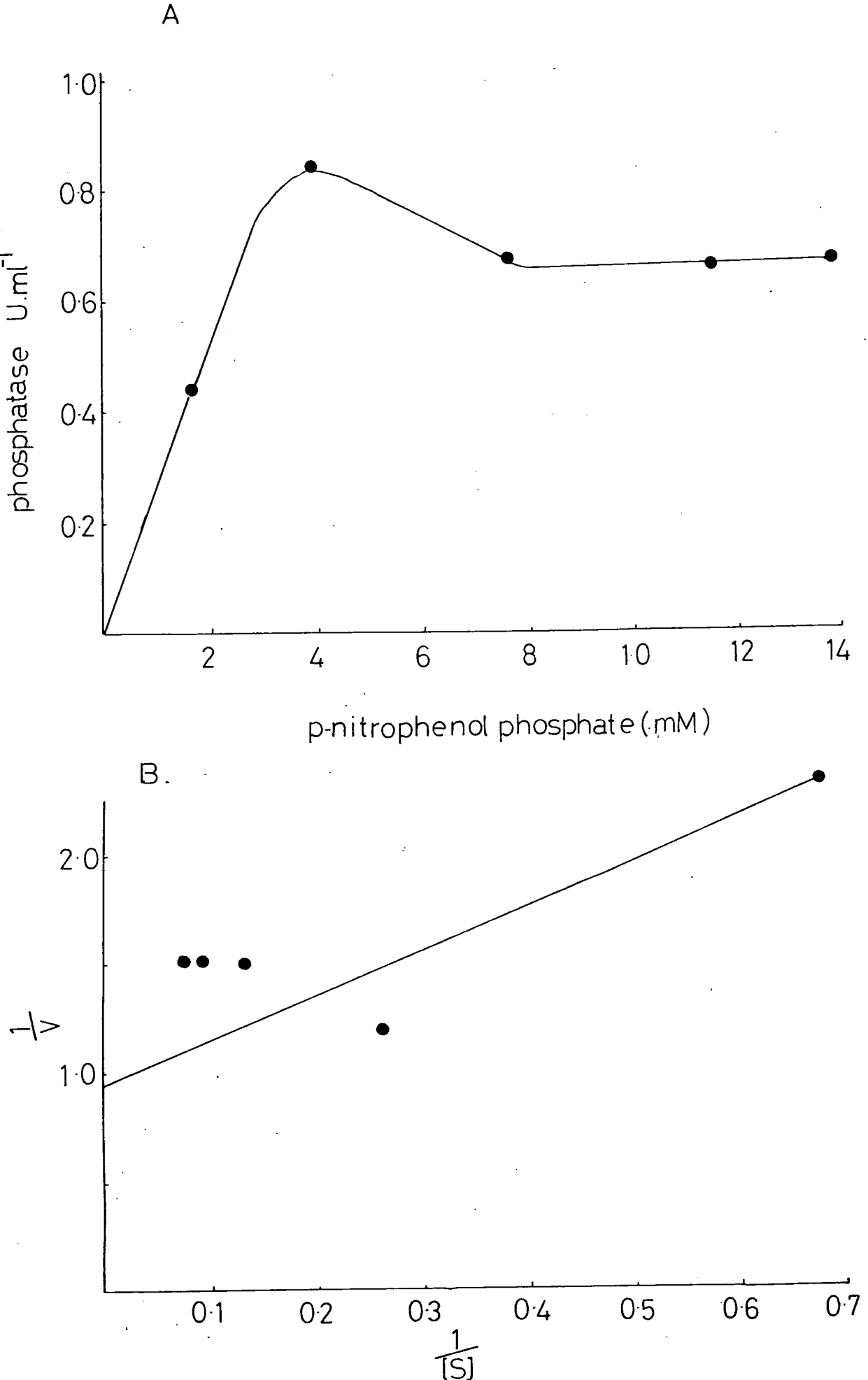

A
1·0
0·8
0·6
0·4
0·2
phosphatase U.ml⁻¹
2
4
6
8
10
12
14
p-nitrophenol phosphate (mM)
B.
2·0
1·0
1/v
0·1
0·2
0·3
0·4
0·5
0·6
0·7
1/[S]

FIGURE 4.20

Effect of enzyme extract concentration on the assay for acid phosphatase.

FIGURE 4.21

Photograph of acid phosphatase isoenzymes separated on slab PAGE gels.

FIGURE 4.20

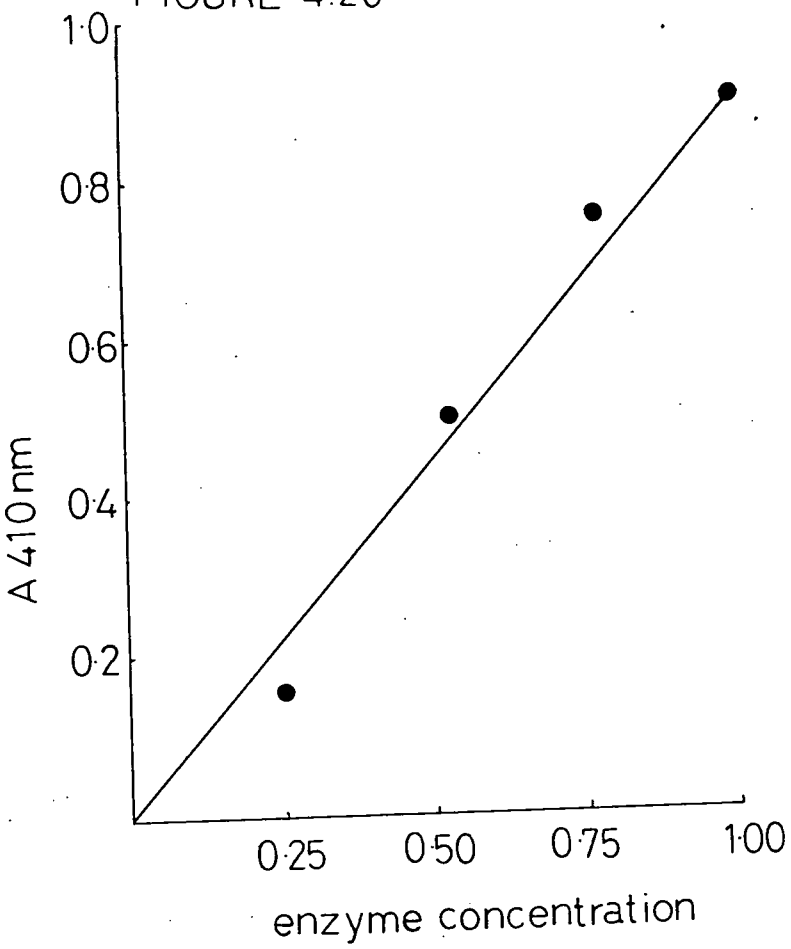
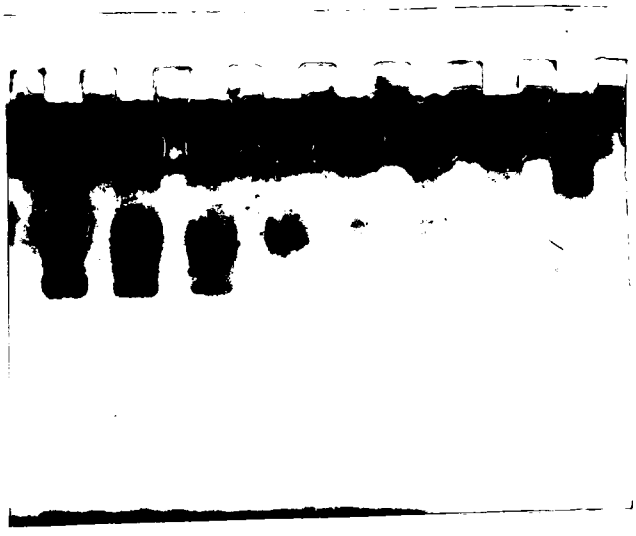



FIGURE 4.21



B. PAGE assay.

a. Acid phosphatase.

Following electrophoresis, the slab gels were soaked in 400 mM. sodium acetate pH 5.0 for 30 min. at 4°C. The gels were then transferred to a 100 ml. mix consisting of

6 mM. sodium-alpha-naphthyl phosphate (made up as a stock of 300 mM. in acetone:water 1:1).
2.2 mM. Fast Garnet GBC salt
2.5 mM. magnesium chloride
200 mM. sodium acetate pH 5.0.

Bands were allowed to develop over several hours in the dark at room temperature.

An alternative method developed by Allen et al. (1963) was tested. In essence it was very similar to the above method except in the use of Black K salt instead of Fast Garnet and omitting the magnesium chloride. Bands were obtained following incubation at room temperature for several hours, but their clarity and the homogeneity of the stain were inferior to the above method.

b. Alkaline phosphatase.

The method used above was adapted for alkaline phosphatase substituting Tris-HCl buffer (pH 9.0) for the acetate buffer. Bands were never as intense as those obtained for acid phosphatase ( and therefore did not photograph well).

vii. Phosphodiesterase. (EC 3. 1. 4. 1)

The assay for 'non-specific' phosphodiesterase involves the liberation of p-nitrophenol from sodium bis-(p-nitrophenyl)phosphate.

A. a. Test-tube assay. (Koerner and Sinsheimer, 1957)

The assay contained

100 µl. citrate buffer pH 5.0
or 100 µl. Tris-HCl pH 8.0
100 µl. 10 mM. bis-p-nitrophenylphosphate
100 µl. enzyme extract (diluted tenfold).

The incubation at 37°C. was terminated after 10 min. by the addition of 2.5 ml. 100 mM. sodium hydroxide. The resulting colour was measured at $A_{410}$. The enzyme activity was calculated after due adjustment for "no enzyme" and "water" blanks, with reference to a standard curve constructed for p-nitrophenol phosphate (Figure 4.17).

b. Calculation of enzyme activity.

As for phosphatase (vi. A a).

i.e., $U.ml^{-1} = 1.5x$ where $x = A_{410}$ assay.

c. pH optimization.

The effect of pH on the assay for phosphodiesterase was examined over a range of pH (Figure 4.22). Subsequent assays were carried out at pH 5 and pH 8.

d. Calculation of $V_{max}$ and $K_m$.

Several concentrations of bis-p-nitrophenyl phosphate were introduced into the assay and enzyme activity recorded (Figure 4.23 A). A Lineweaver-Burk plot gave a $V_{max} = 0.14\ U.ml^{-1}$ and $K_m = 4.2$ mM.

## viii. Location of enzymes.

Preliminary work was carried out on the cellular location of the enzymes under investigation, with a view to comparison at different ages. This was carried out in two ways, as follows:-

(a). Sucrose density gradient.

Separation of cellular organelles was achieved by centrifugation on a 25 - 57% (W/V) sucrose density gradient (see Chapter 2). 3 - 5 g. F. pratensis leaf tissue were chopped by an electric chopper in 7 ml. buffer consisting of

100 mM. $NaH_2PO_4$
1.2 mM. EDTA (disodium salt)
15% (W/V) sucrose.

Following centrifugation at 5,000 g. to remove excess plastid and debris material, 4.5 ml. supernatant were loaded onto the gradient, which was centrifuged at 90,000 g. for 15 h. in a 3x25 ml. swing-out rotor on a MSE Superspeed 50 TC at 4°C. The separation into discrete organellar bands was visible by virtue of their associated pigments (Figure 4.24).

FIGURE 4.22

Effect of pH on the assay for phosphodiesterase.

FIGURE 4.22

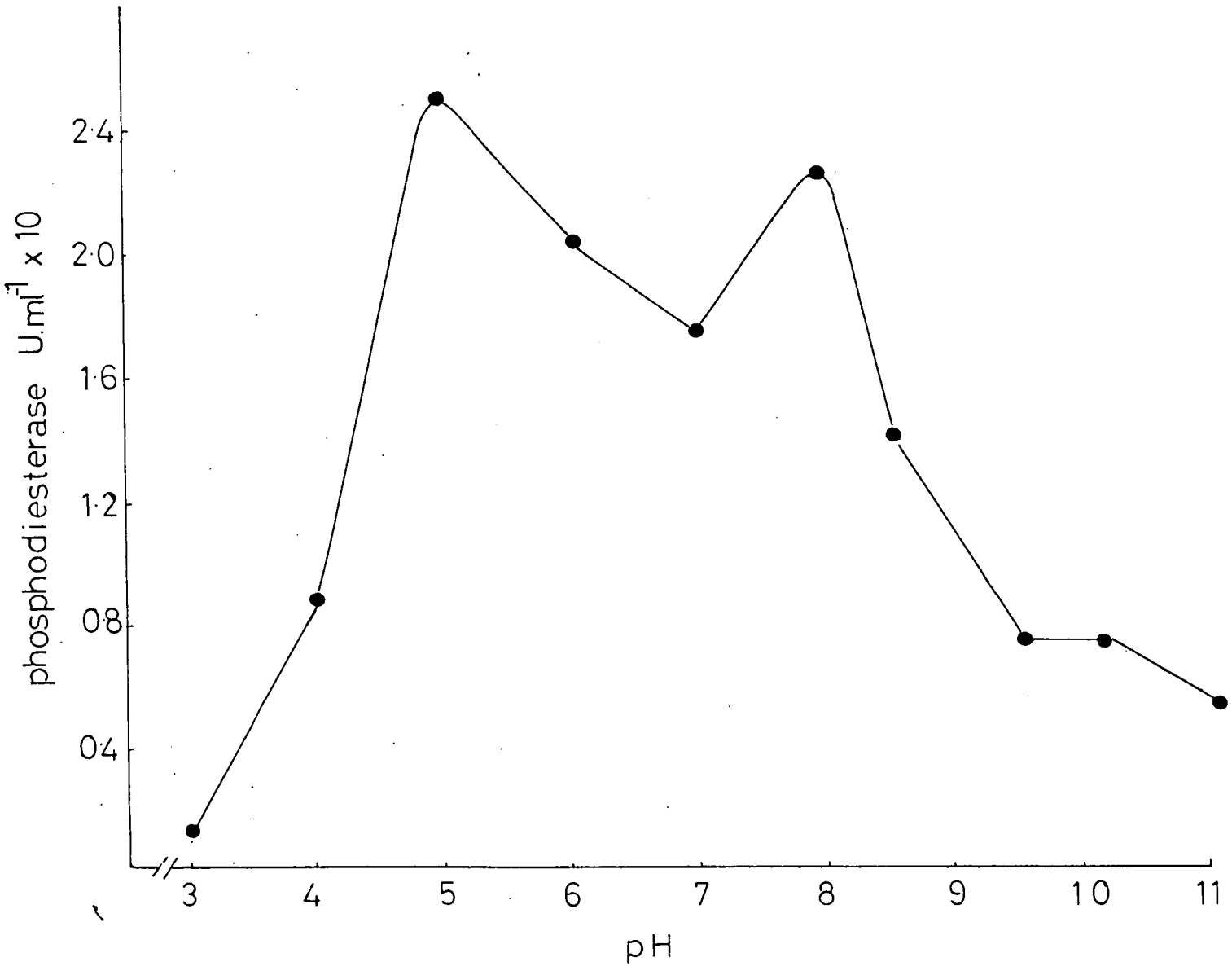


FIGURE 4.23 A.

Effect of bis-nitrophenyl phosphate concentration on phosphodiesterase activity at pH 8.0

FIGURE 4.23 B.

Lineweaver-Burk plot of alkaline phosphodiesterase activity.

FIGURE 4.23

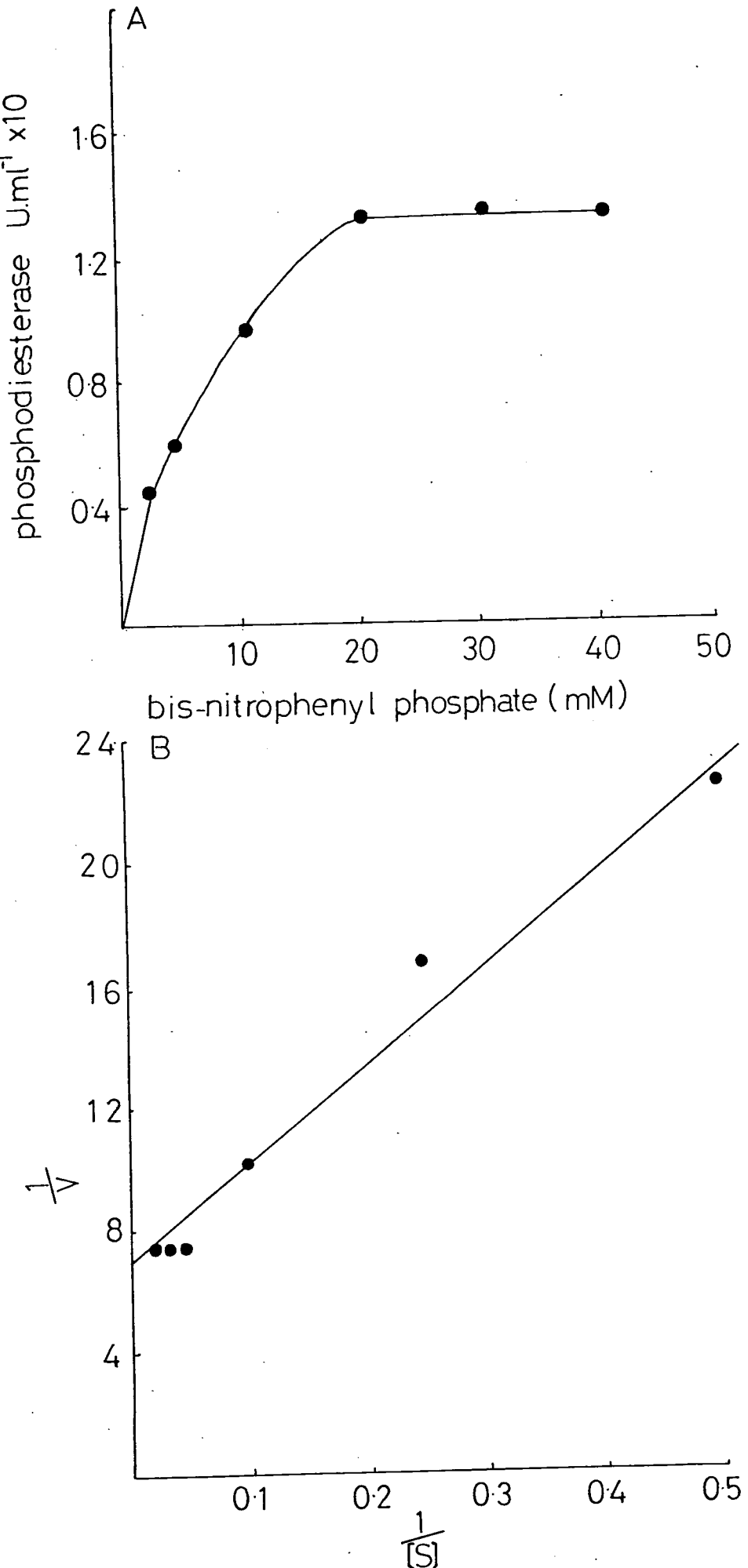
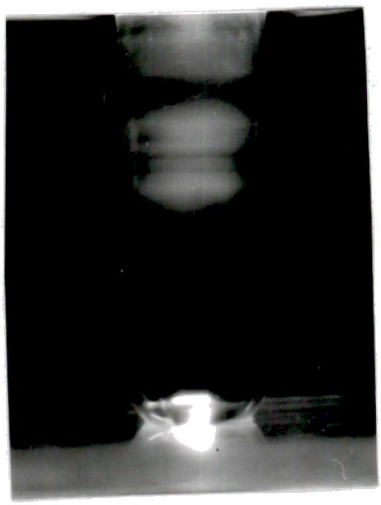

A
phosphodiesterase U.ml$^{-1}$ x10
1·6
1·2
0·8
0·4
10
20
30
40
50
bis-nitrophenyl phosphate (mM)
B
24
20
16
12
8
4
$\frac{1}{v}$
0·1
0·2
0·3
0·4
0·5
$\frac{1}{[S]}$

FIGURE 4.24

Photograph of sucrose density gradient (25 - 57% (W/V)) separation of cell fractions of leaves of *F. pratensis*.

FIGURE 4.24



750 µl. fractions were collected using an auto-densiflow apparatus (Buchler Instruments, USA) linked to a LKB Perpex peristaltic pump. Enzyme assays, including those for marker enzymes (see following section) were carried out on these fractions.

(b). Differential centrifugation.

3.0 g. F. pratensis leaf tissue were chopped in 13 ml. buffer containing 50 mM. Tris-HCl (pH 7.6) and 250 mM. sucrose, using an electric chopper at 4°C. The brei was passed through two layers of Miracloth and centrifuged at 400 g. for 10 min. in a MSE bench centrifuge. The resultant pellet was washed with 2 ml. extraction buffer and re-centrifuged at 400 g. for 10 min. (fraction 1). The supernatants were pooled and centrifuged at 4,000 g. in a 10x10 ml. angle rotor in a MSE superspeed 50 TC for 10 min. The pellet was washed with 2 ml. extraction buffer and re-centrifuged (fraction 2). The pooled supernatants were centrifuged at 17,000 g. and, following retention and washing of the pellet (fraction 3), at 160,000 g. to give a final pellet (fraction 4) and supernatant (fraction 5). Enzyme and enzyme marker assays were performed on the five fractions. To ascertain whether activity was reduced by compartmentation of some kind (whether real or induced by extraction conditions), 0.1% (V/V) Triton X 100 was added to duplicate assays. Alternatively, enzyme fractions were sonicated at an amplitude of 3.5 µ for 30 s. in a MSE Ultrasonicator prior to introduction into the assay systems.

## ix. Assays of other enzymes used as cytological markers.

There has been considerable debate as to the value of marker enzymes in the identification of cell constituents (for example, 6th International Sub-cellular Methodology Forum, 1978). Problems evolve from contamination of preparations by other cellular or extra-cellular components, the lack of absolute markers (i.e., those whose distribution is restricted to a single cell component), the transitory appearance of markers elsewhere (for example, in early cellular development) and membrane leakiness.

Succinate dehydrogenase is the enzyme marker of choice for intact mitochondria. Catalase and uricase are also recommended as general markers of mitochondria and their fragments (6th International Sub-cellular Methodology Forum, 1978).

NAD-cytochrome C oxidoreductase, which is Antimycin A insensitive, occurs in endoplasmic reticulum but is not an absolute marker since it is present in small amounts in the nuclear envelope, Golgi apparatus and plasma membrane. Despite these complications of secondary location it is nevertheless considered as a useful marker (6th International Sub-cellular Methodology Forum, 1978).

Since all these marker enzymes have been frequently measured at the Welsh Plant Breeding Station, optimal conditions were assumed without re-assessment.

a. Catalase. (EC 1. 11. 1. 6)

The assay was performed in a quartz cuvette in a Pye-Unicam SP 800 spectrophotometer. The change in $A_{230}$ was monitored over at least 5 min.

The assay mix consisted of

| | | | |
|---|---|---|---|
| 3 mM. | phosphate buffer | pH 7.5 | in |
| 0.05% (V/V) | hydrogen peroxide | | 2.9 ml. |
| 50 µl. | enzyme extract | | |

On addition of the enzyme and following thorough mixing, the fall in $A_{230}$ was immediately recorded so as to provide as close an estimation of initial reaction rate as possible. The production of bubbles in the cuvette brought the reaction rapidly to non-linearity.

Calculation of enzyme activity:

$$U.ml^{-1} = A_{230} \times 1.2$$

A = change in absorbance per min.

b. NAD-cytochrome C oxidoreductase. (EC 1. 6. 2. 1)

The assay mix in a 3 ml. glass cuvette set in a Pye-Unicam SP 800 spectrophotometer consisted of

| | | | |
|---|---|---|---|
| 50 mM. | phosphate buffer | pH 8.0 | in 2 ml. |
| 0.75 mM. | NADH | | |
| 25 mM. | potassium cyanide | | |
| 20 mM. | Antimycin A | | |

Immediately prior to the addition of enzyme extract, 200 µl. 0.4 mM. cytochrome C solution were added, followed by 100 µl. enzyme extract. The change in $A_{550}$ was followed over several minutes. As with the catalase assay, speed was important in order to obtain as near to initial reaction rates as possible.

Calculation of enzyme activity:

$$U.ml^{-1} = A_{550} \times 3.39$$

$A_{550}$ = change in absorbance per min.

c. Succinate dehydrogenase. (EC 1. 3. 99. 1)

The assay consisted of

| | |
|---|---|
| 250 µl. | 200 mM. phosphate buffer pH 7.4 containing 12 mM. MTT tetrazolium |
| 500 µl. | enzyme extract |
| 250 µl. | 300 mM. sodium succinate pH 7.4 or 300 mM. sodium malonate. |

Following incubation at 37°C. for 10 min., 6 ml. "stopping reagent" were added, shaken and centrifuged at 1,000 g. for 5 min. to remove denatured protein. "Stopping reagent" consisted of

| | |
|---|---|
| 36% (V/V) | ethanol |
| 57% (V/V) | ethyl acetate |
| 0.6% (W/V) | trichloroacetic acid |
| 6.4% (V/V) | water. |

The resultant coloured supernatant was recorded at $A_{560}$.

d. Uricase. (EC 1. 7. 3. 3)

$$\text{Urate} + O_2 + H_2O \overset{\text{uricase}}{\rightleftharpoons} \text{Allantoin} + H_2O_2 + CO_2$$

The assay is based on the principle that urate has a high U.V. absorption (at 290 nm.) whereas the product of the reaction, allantoin, does not absorb at this wavelength.

The assay was carried out in 3 ml. quartz cuvettes at room temperature. The assay contained

| | | |
|---|---|---|
| 1.0 ml. | 8 uM. uric acid | |
| 1.0 ml. | 200 mM. borate buffer | pH 8.5 |
| 500 µl. | enzyme extract | |
| 500 µl. | distilled water. | |

The decrease in $A_{290}$ was recorded relative to a control including 200 ul. 0.1% (W/V) potassium cyanide (in place of some of the distilled water addition) over several minutes.

## III. Experimental Work.

### i. Enzymes of phosphorus metabolism during development.

Three separate experiments were conducted; one using the whole leaves of different ages, and two using sections along the leaf. The enzyme activities from each experiment are linked in Figures 4.25 to 4.33 so that discontinuities due to seasonal variations or growth conditions would not obscure the salient trends.

ATPase and alkaline and acid pyrophosphatases show similar patterns of activity in whole leaf ageing, displaying an early peak of increased activity between 35 and 42 days and a later phase at 55 to 62 days (Figures 4.25, 4.28, 4.29). Alkaline pyrophosphatase is about four times as active as acid pyrophosphatase in the first phase of increased activity. The second phase reveals a reduction in the alkaline to acid pyrophosphatase ratio by half, which is consistent with the reports in the literature which attribute an anabolic function to alkaline pyrophosphatase and a catabolic function to acid pyrophosphatase. Kar and Mishra (1975) emphasised the importance of the ratio of these two enzymes rather than their absolute activities. Furthermore, the peak of acid pyrophosphatase activity at senescence is broader than for alkaline pyrophosphatase which resembles the ATPase pattern slightly better. Ageing along the leaf shows differences in the pattern of all these three enzyme activities compared with the development of the whole leaf. However, all show high activities at the base and reduction in activity in senescent apical sections with an increased phase of activity at Section 5-6. The ratio of alkaline pyrophosphatase to acid phosphatase (Figures 4.28 B and 4.29 B) does not decline at senescence as observed for whole leaf ageing. This may be indicative of very localized areas of increased acid pyrophosphatase activity which would function in catabolism perhaps by redeployment of precursors to growing regions. This may be the cause of the peak at Section 5 which corresponds to high metabolic activity as indicated by ATPase activity.

FIGURE 4.25 A.

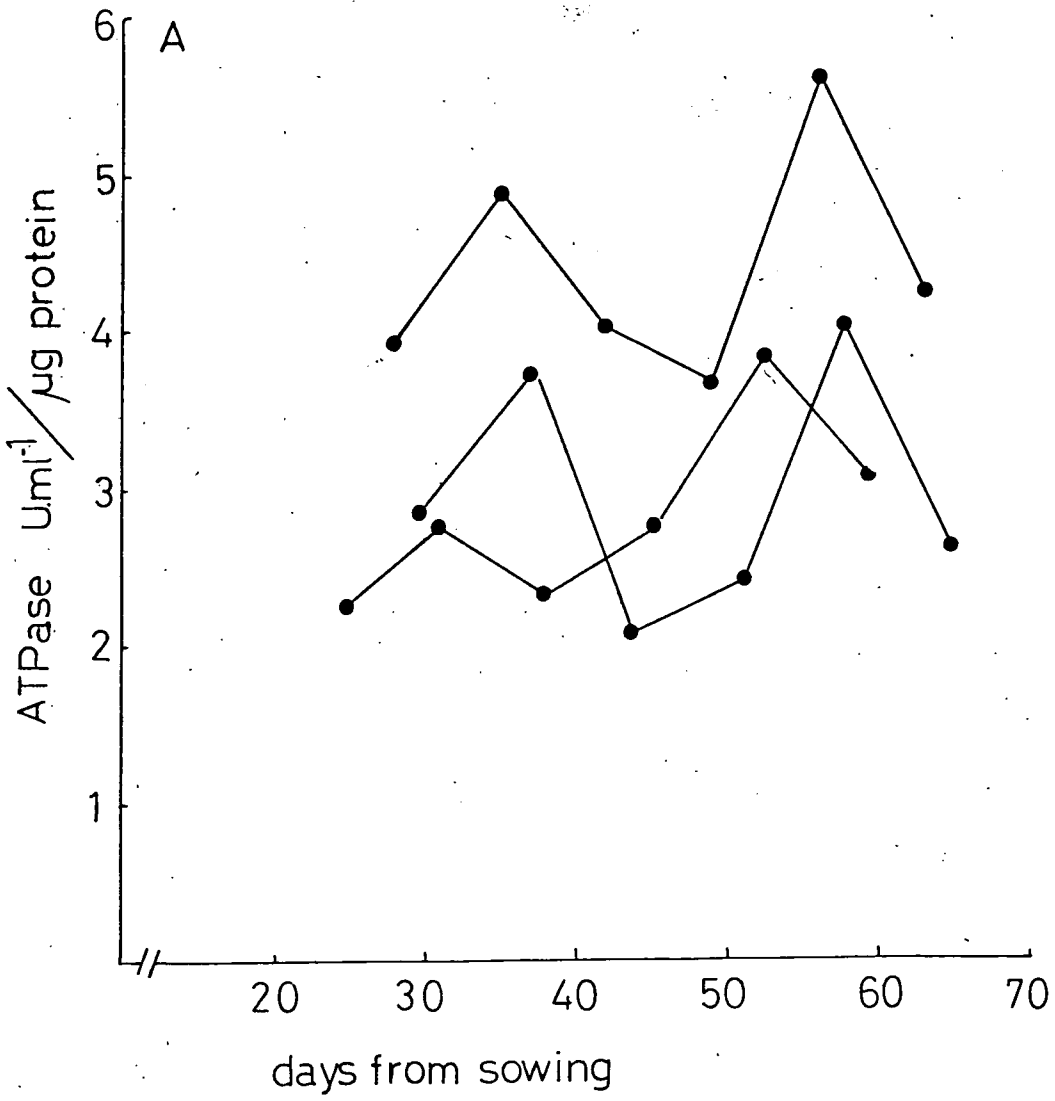
ATPase activity expressed per µg. protein in enzyme extract of fourth leaves of *F. pratensis* at different ages.

FIGURE 4.25 B.

ATPase activity expressed per µg. protein in the enzyme extract, from sections along the leaf.

Each •—• represents a different experiment.

FIGURE 4.25


A
6
5
4
3
2
1
ATPase Uml-1/µg protein
20
30
40
50
60
70
days from sowing

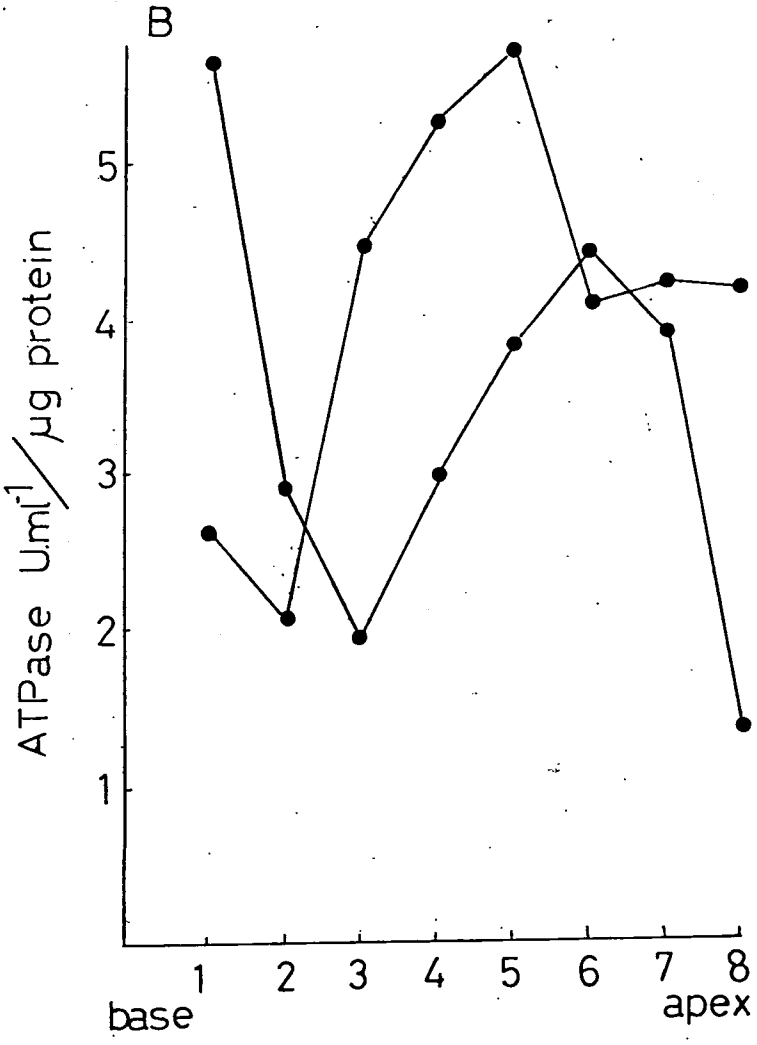

B
5
4
3
2
1
ATPase Uml-1/µg protein
1
2
3
4
5
6
7
8
base
apex

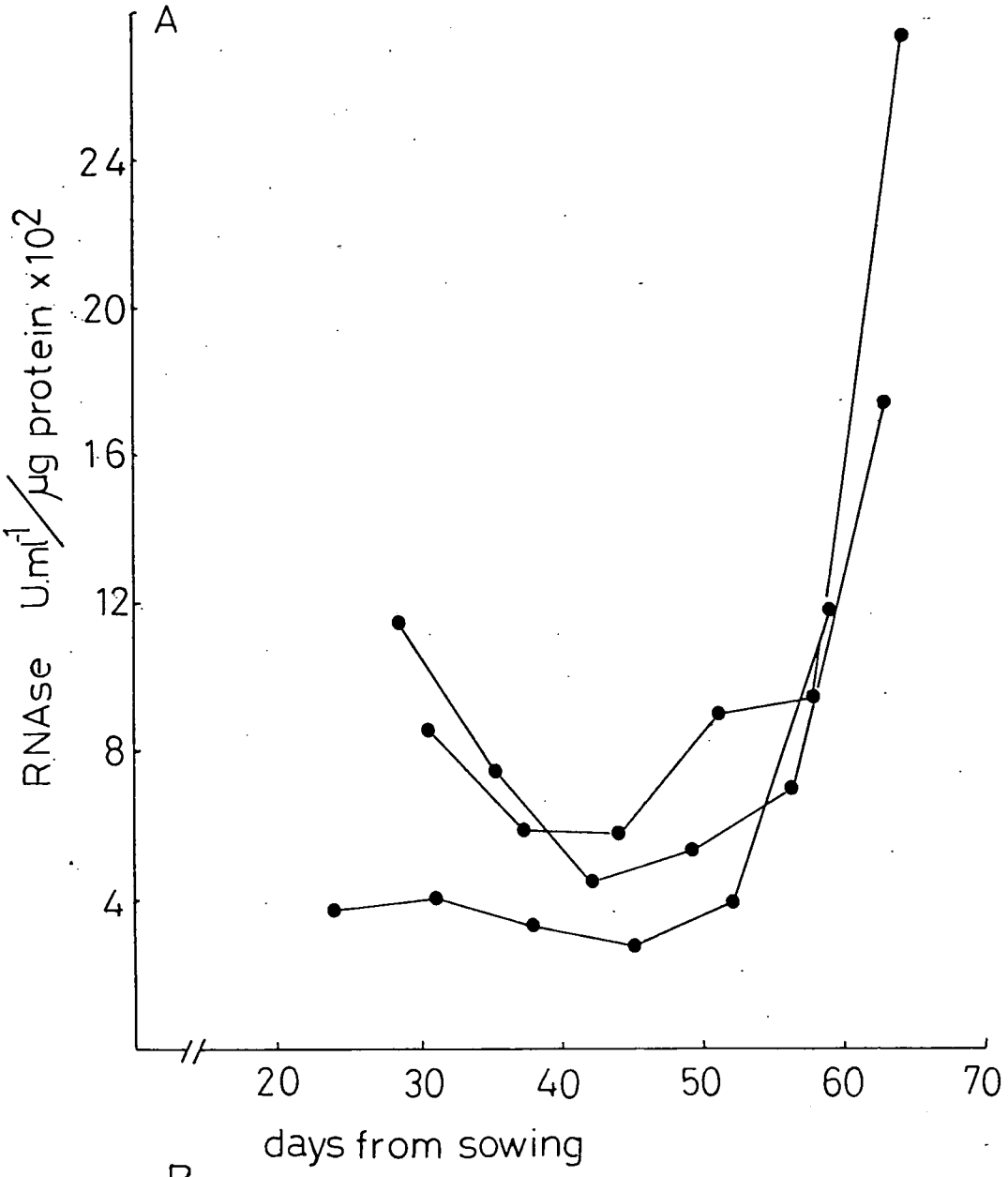
FIGURE 4.26

Acid RNAse activities expressed per µg. protein in enzyme extract,

A. of fourth leaves of F. pratensis at different ages

B. from sections along the leaf.

FIGURE 4.26


A
RNAse U.ml-1/µg protein x10²
24
20
16
12
8
4
20
30
40
50
60
70
days from sowing

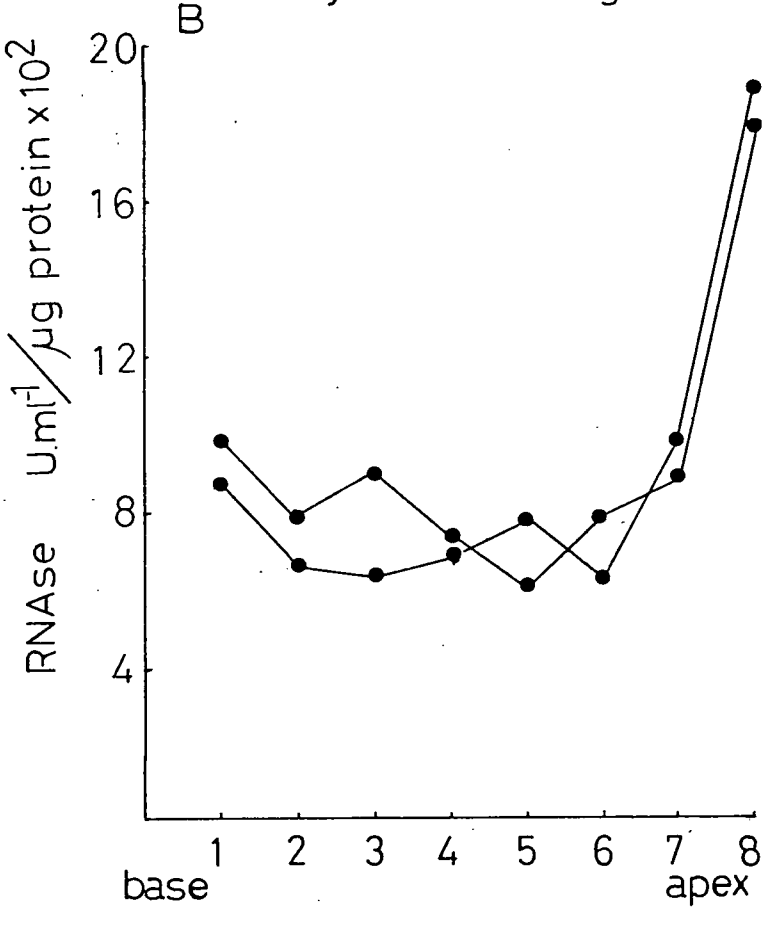

B
RNAse U.ml-1/µg protein x10²
20
16
12
8
4
1
2
3
4
5
6
7
8
base
apex

FIGURE 4.27

Alkaline RNAse activities expressed per µg. protein in enzyme extract of

A. fourth leaves of *F. pratensis* at different ages

B. sections along the leaf.

FIGURE 4.27

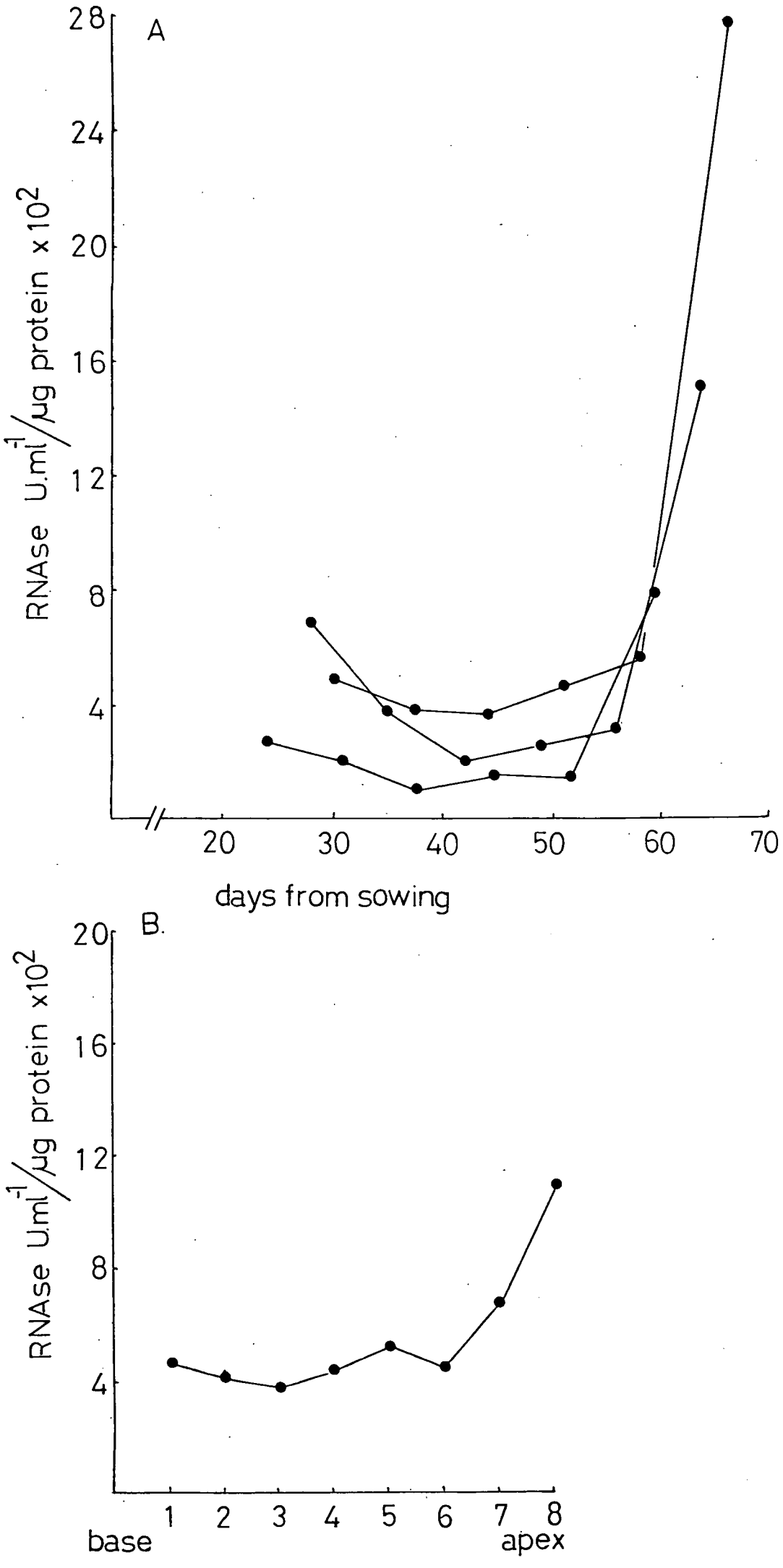

A
28
24
20
16
12
8
4
RNAse U.ml-1/µg protein x10²
20
30
40
50
60
70
days from sowing
B.
20
16
12
8
4
RNAse U.ml-1/µg protein x10²
1
2
3
4
5
6
7
8
base
apex

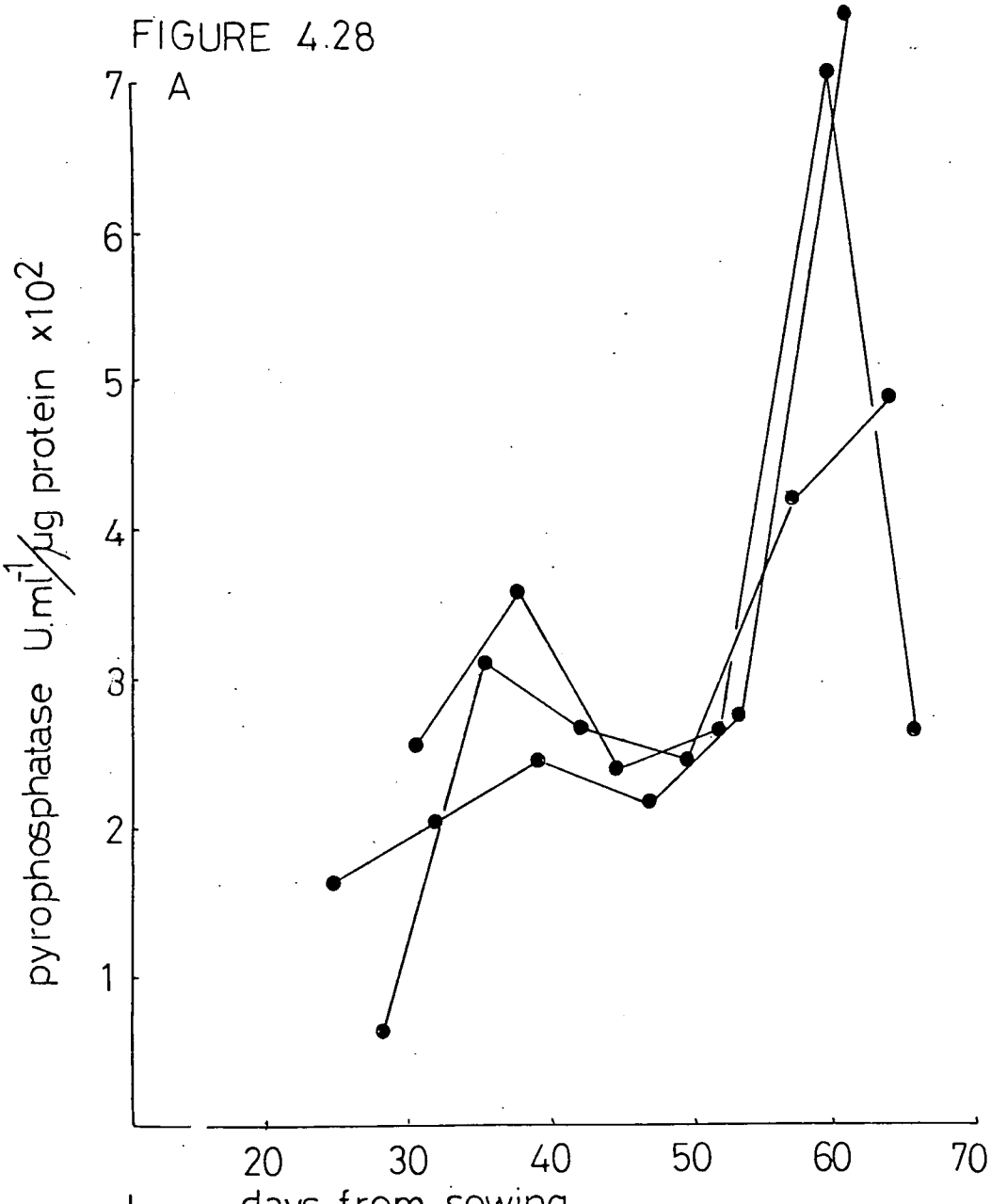
FIGURE 4.28

Acid pyrophosphatase activities expressed per µg. of protein in enzyme extract of

A. fourth leaves of *F. pratensis* at different ages

B. sections along the leaf.

FIGURE 4.28


A
pyrophosphatase U.ml-1/µg protein x10²
7
6
5
4
3
2
1
20
30
40
50
60
70
days from sowing

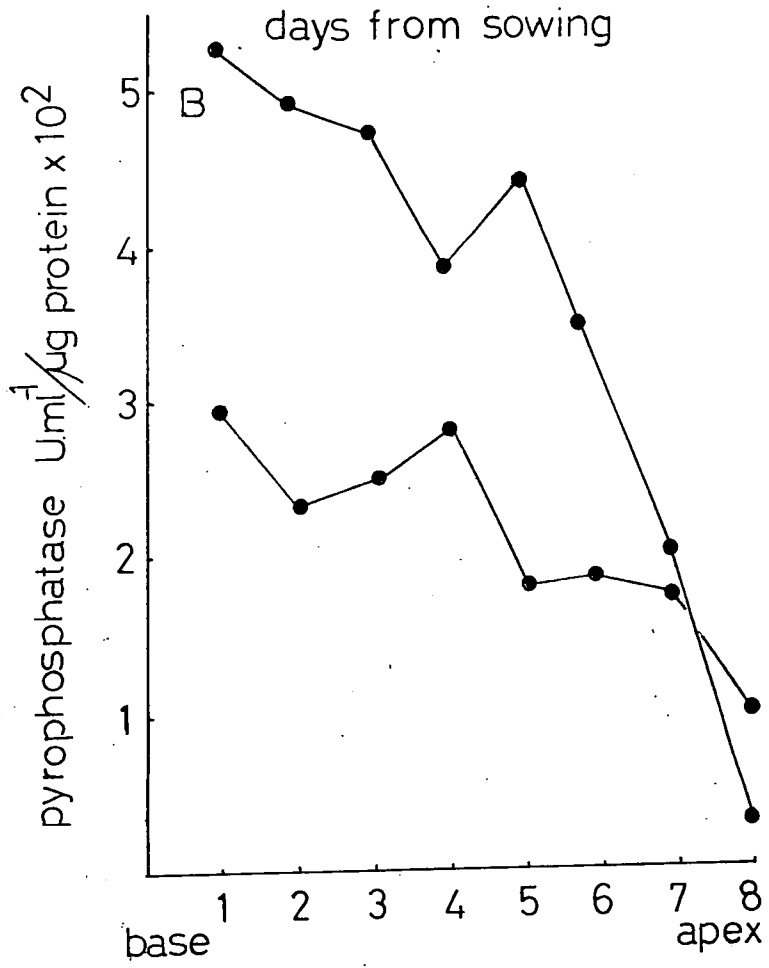

B
pyrophosphatase Uml-1/µg protein x 10²
5
4
3
2
1
1
2
3
4
5
6
7
8
base
apex

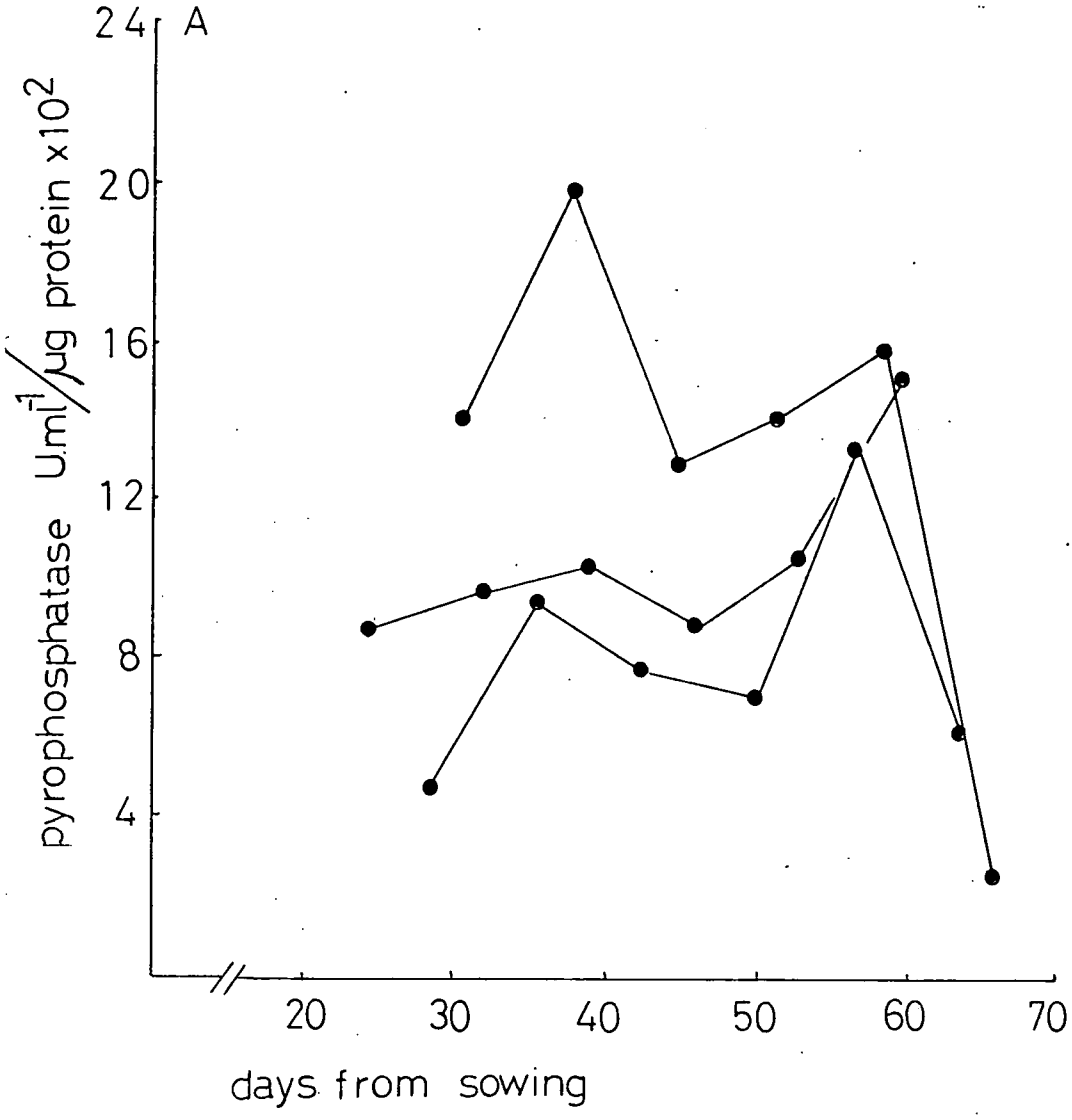
FIGURE 4.29

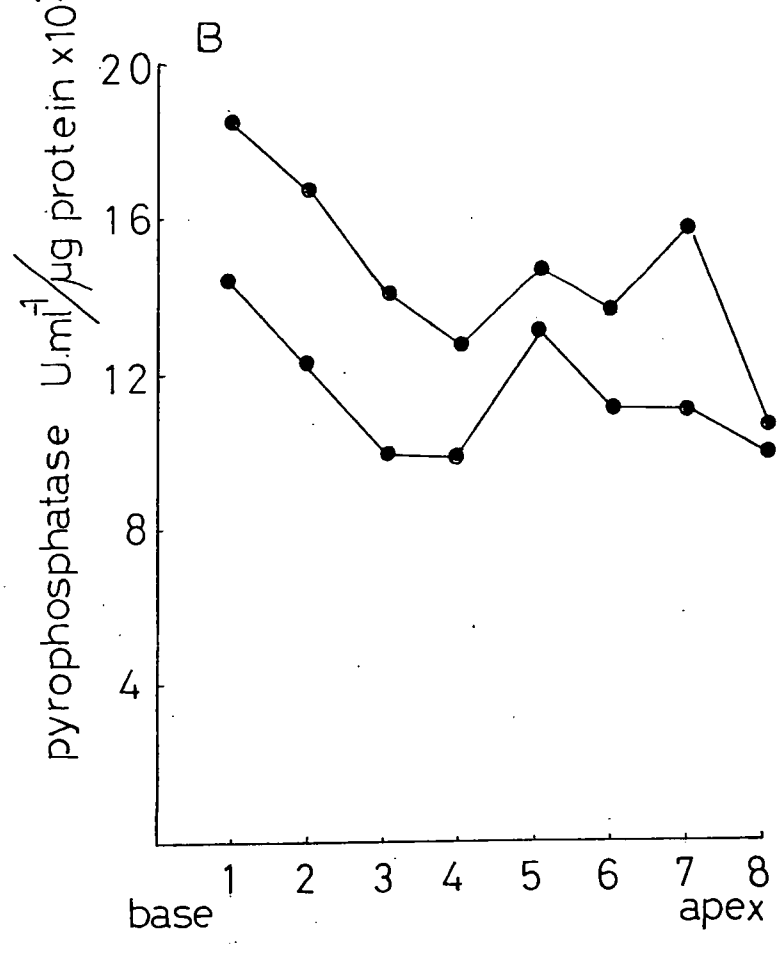
Alkaline pyrophosphatase activities expressed per µg. protein of enzyme extract of

A. fourth leaves of F. pratensis at different ages

B. sections along the leaf.

FIGURE 4.29


A
24
20
16
12
8
4
pyrophosphatase U.ml-1/µg protein x10²
20
30
40
50
60
70
days from sowing


B
20
16
12
8
4
pyrophosphatase U.ml-1/µg protein x10²
1
2
3
4
5
6
7
8
base
apex

RNAses and phosphodiesterases follow similar patterns in both developmental systems (Figures 4.26, 4.27, 4.32, 4.33) displaying a classic large increase in activity at senescence. RNAse activities are slightly enhanced at early development amounting to approximately a quarter of the senescent activity in the whole leaf ageing and half the senescent activity in development along the leaf. Phosphodiesterase activities at the base of the leaf are of a similar order to apical activities. Acid phosphodiesterase is approximately twice as active as alkaline phosphodiesterase but the developmental pattern displayed by both is similar. Acid RNAse is approximately 30% more active at all development stages until senescence when the activities are similar.

Phosphatase activity displays further different patterns (Figures 4.30, 4.31). Alkaline phosphatase activities would tend to suggest a three peak pattern with a peak of activity at 40-45 days in whole leaf ageing (Figure 4.31 A) and at Section 5 - 6 in development along the leaf (Figure 4.31 B). Acid phosphatase activity indicates a slightly earlier mid-peak at day 35-40 (Figure 4.30 A) and Section 3 - 4 (Figure 4.30 B).

The early and late phases of increased enzyme activity correspond to the increased levels of soluble protein synthesis along the leaf (Figure 3.6) and in the development of the whole leaf (Figure 3.5). Ageing along the leaf shows an increased phase of synthesis at Section 4 which would correspond to the increase in acid phosphatase activity (Figure 4.30) and precedes the increase in alkaline phosphatase activity (Figure 4.31).

Only the phosphatases displayed any sort of three peak pattern of activity. Comparison with Figure 3.11 would suggest that increased synthesis during mid-development tends to be associated with particulate proteins rather than soluble ones; only one of the soluble proteins monitored showed any increased relative synthesis at this phase (Day 42).

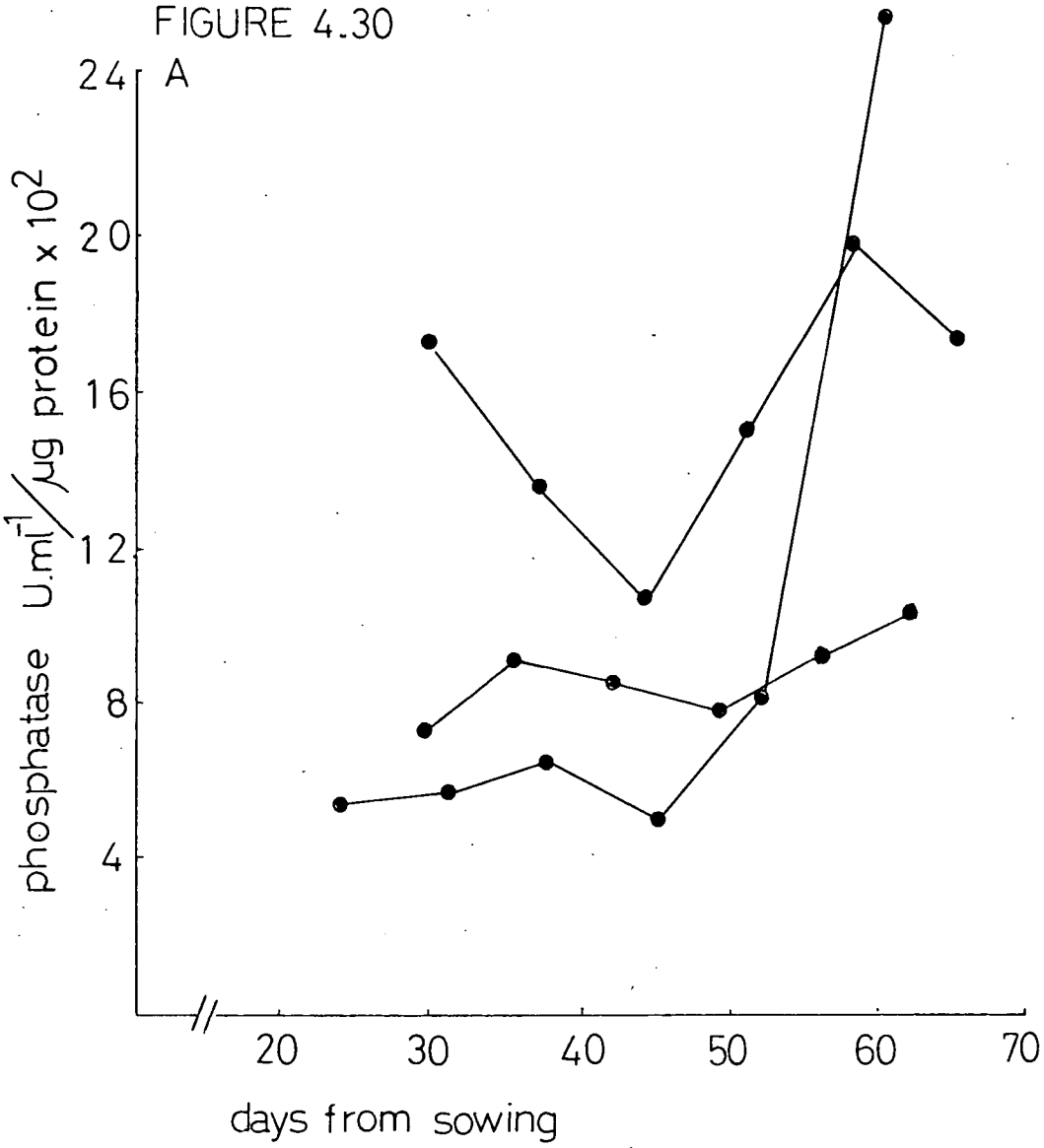
FIGURE 4.30

Acid phosphatase activities expressed per µg. protein in enzyme extract of

A. fourth leaves of F. pratensis at different ages

B. sections along the leaf.

FIGURE 4.30


A
24
20
16
12
8
4
phosphatase U.ml$^{-1}$/µg protein x 10$^2$
20
30
40
50
60
70
days from sowing

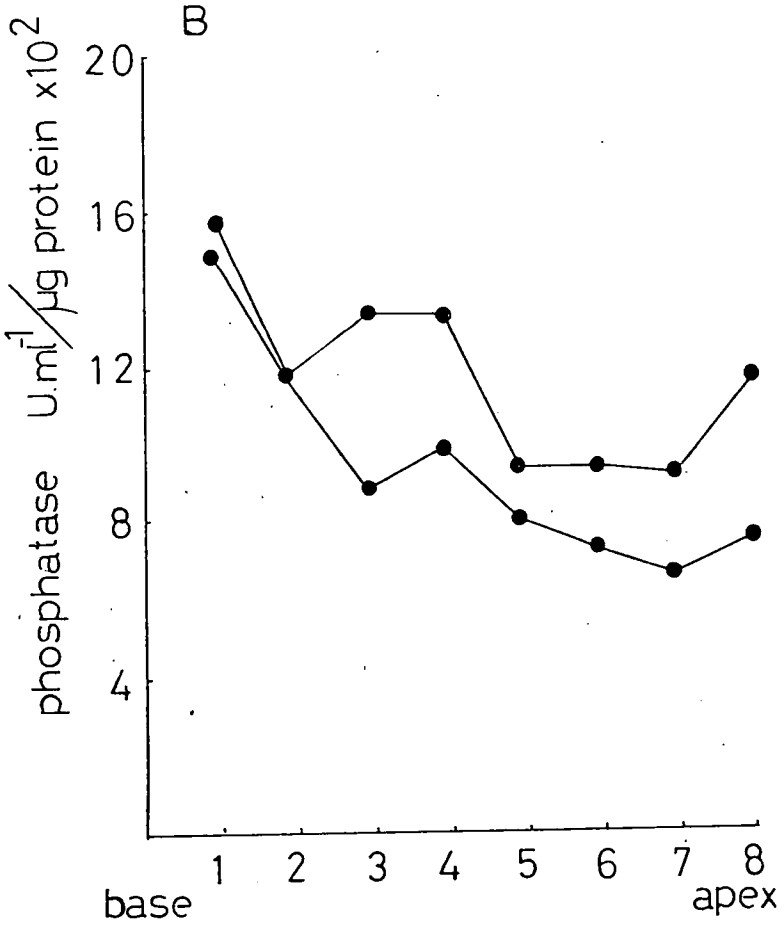

B
20
16
12
8
4
phosphatase U.ml$^{-1}$/µg protein x10$^2$
1
2
3
4
5
6
7
8
base
apex

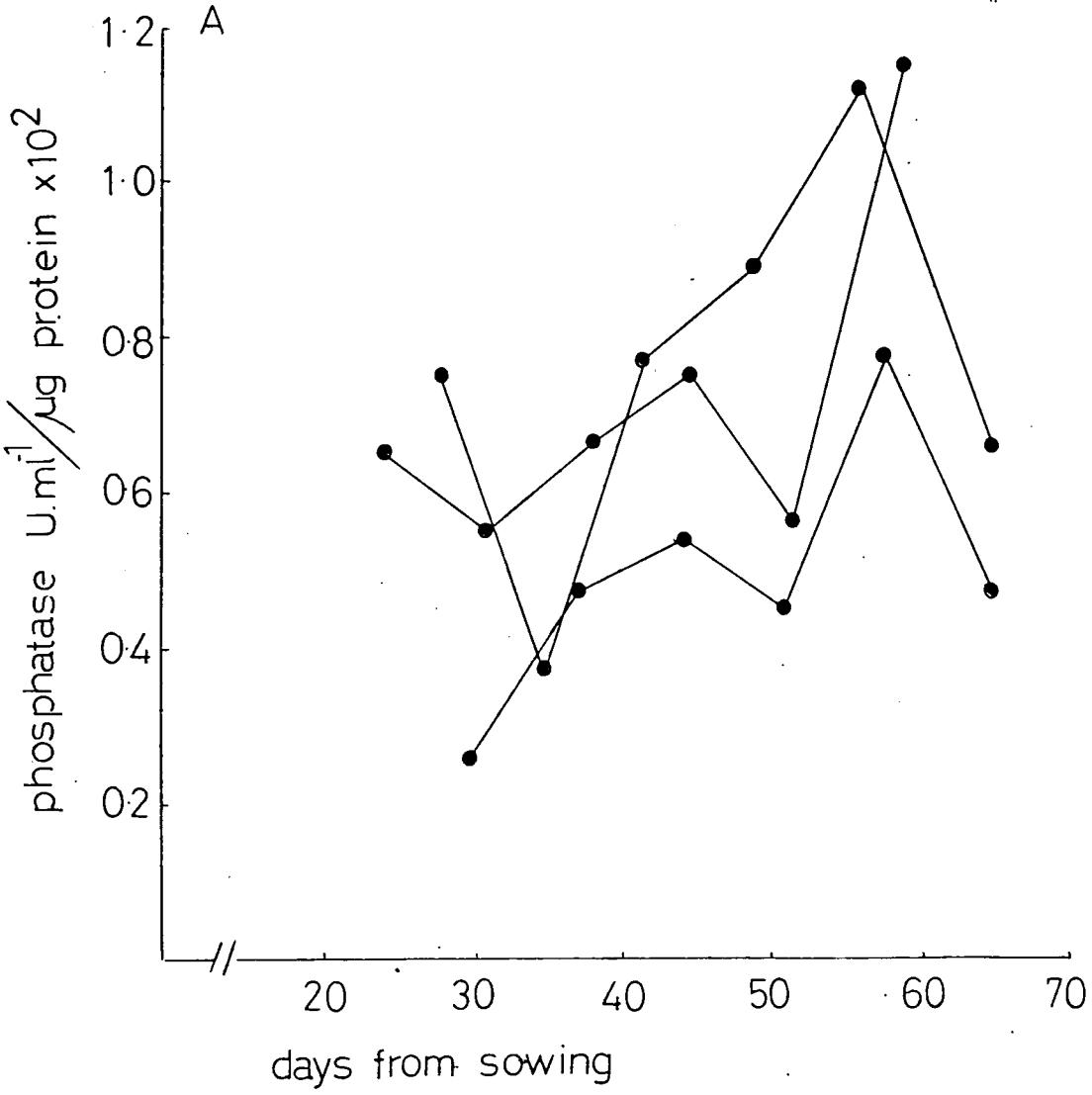
FIGURE 4.31

Alkaline phosphatase activities expressed per µg. protein in enzyme extract of

A. fourth leaves of F. pratensis at different ages

B. sections along the leaf.

FIGURE 4.31


A
1·2
1·0
0·8
0·6
0·4
0·2
phosphatase U.ml-1/µg protein x10²
20
30
40
50
60
70
days from sowing

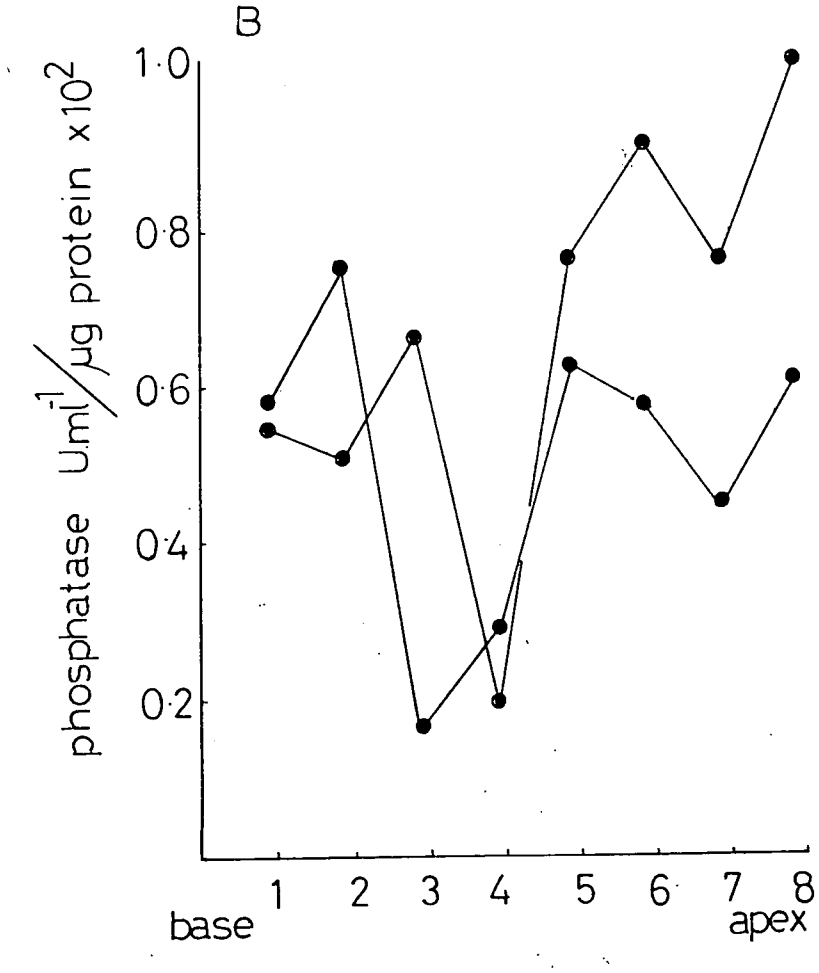

B
1·0
0·8
0·6
0·4
0·2
phosphatase U.ml-1/µg protein x10²
1
2
3
4
5
6
7
8
base
apex

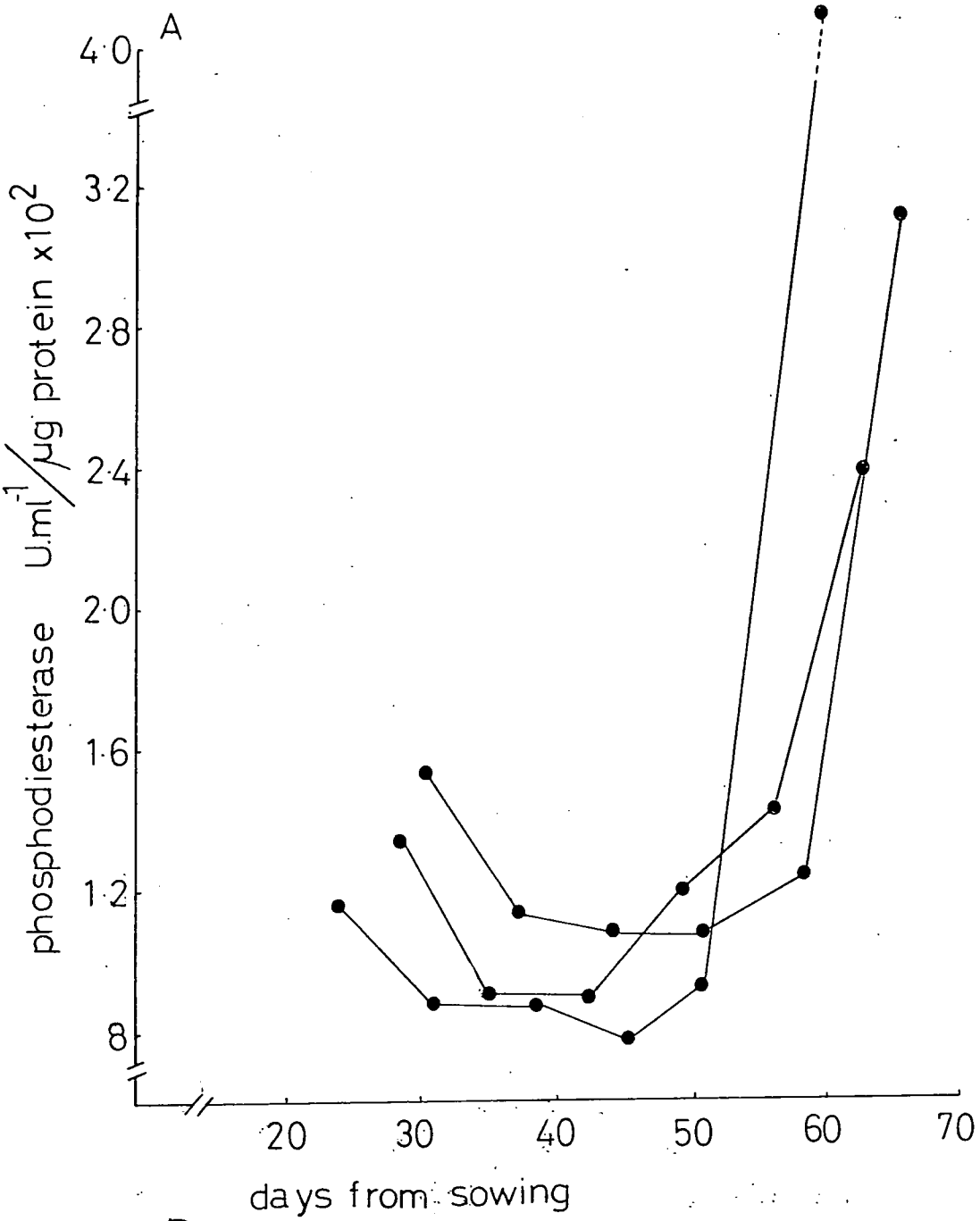
FIGURE 4.32

Acid phosphodiesterase activities expressed per µg. protein in enzyme extract of

A. fourth leaves of *F. pratensis* at different ages

B. sections along the leaf.

FIGURE 4.32


A
4·0
3·2
2·8
2·4
2·0
1·6
1·2
8
phosphodiesterase U.ml-1/µg protein x10²
20
30
40
50
60
70
days from sowing

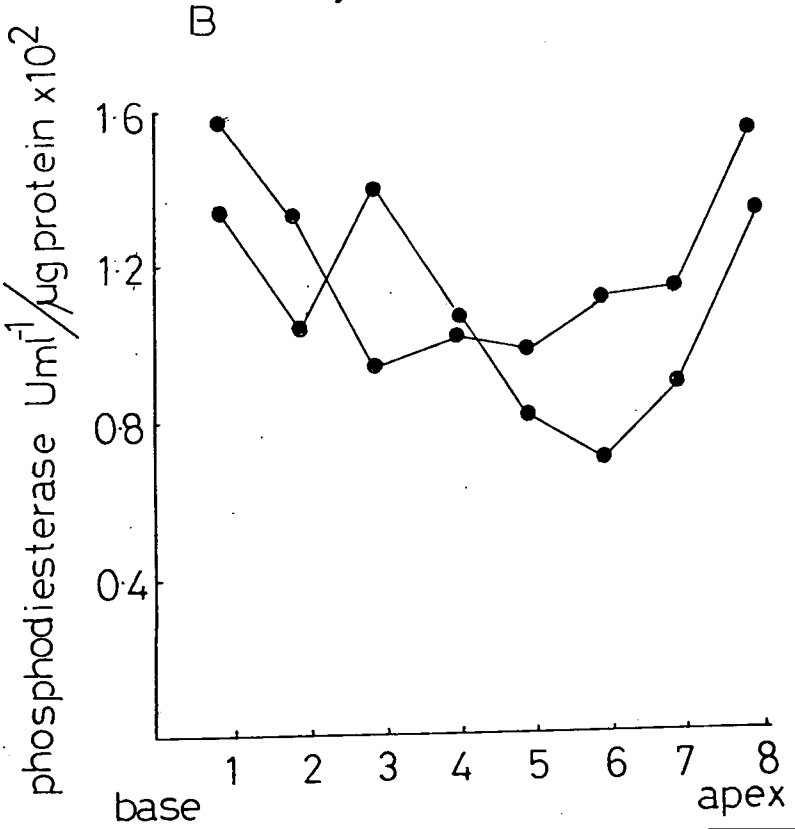

B
1·6
1·2
0·8
0·4
phosphodiesterase Uml-1/µg protein x10²
1
2
3
4
5
6
7
8
base
apex

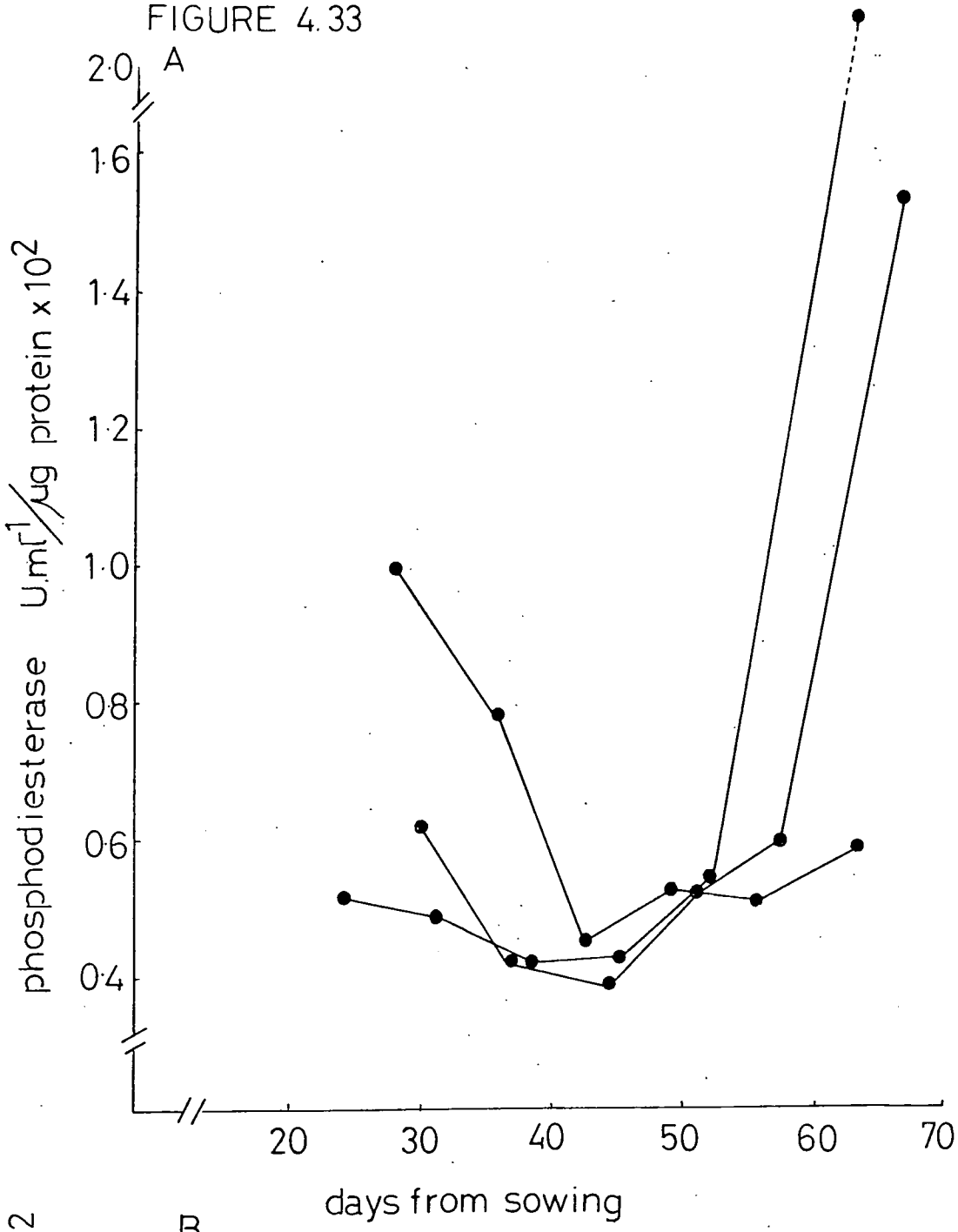
FIGURE 4.33

Alkaline phosphodiesterase activities expressed per µg. protein of enzyme extract of

A. fourth leaves of *F. pratensis* at different ages

B. sections along the leaf.

FIGURE 4.33


A
2·0
1·6
1·4
1·2
1·0
0·8
0·6
0·4
phosphodiesterase U.ml⁻¹/µg protein x10²
20
30
40
50
60
70
days from sowing

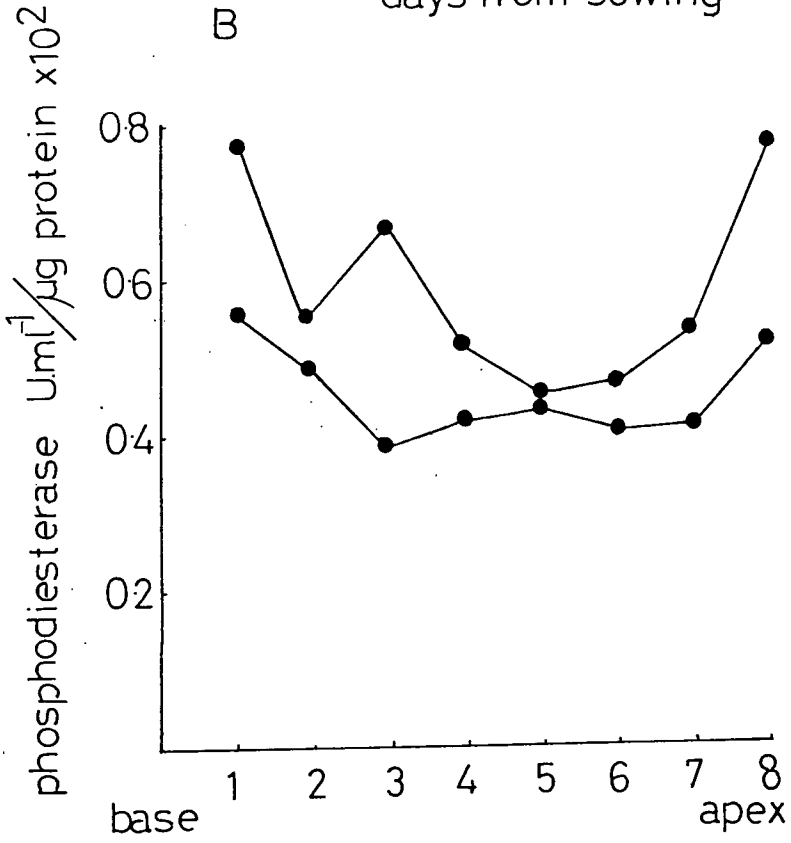

B
0·8
0·6
0·4
0·2
phosphodiesterase U.ml⁻¹/µg protein x10²
1
2
3
4
5
6
7
8
base
apex

FIGURE 4.34

Sucrose density gradient centrifugation location of enzymes and marker enzymes.

FIGURE 4.34

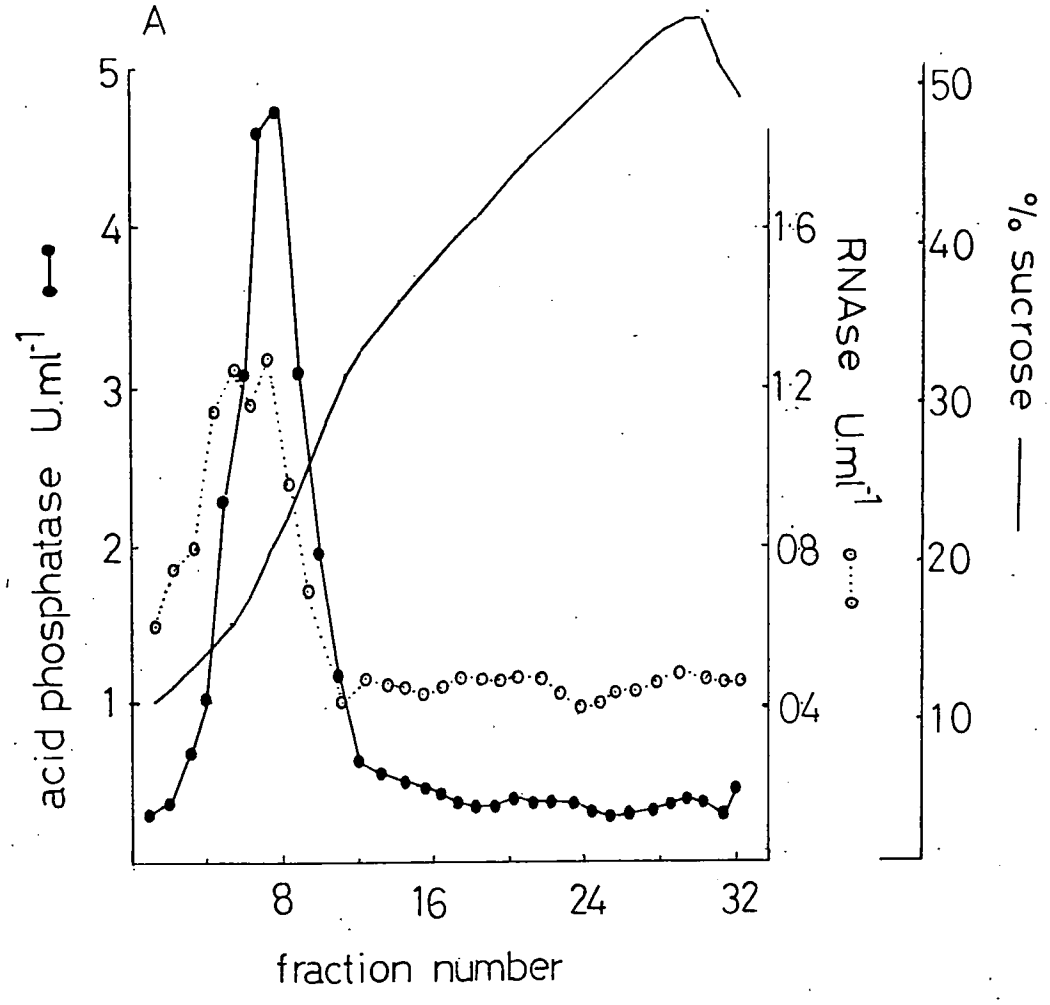
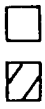

A
acid phosphatase U.ml-1
RNAse U.ml-1
% sucrose
fraction number
B
catalase U.ml-1 x 10-2
NAD cytochrome C oxidoreductase U.ml-1 x 10-2
a
b
c
fraction number

FIGURE 4.35

Relative enzyme activities and chlorophyll content from the five fractions resulting from differential centrifugation.



enzyme activity

enzyme activity following sonication at 3.5 μ for 30 s.

FIGURE 4.35

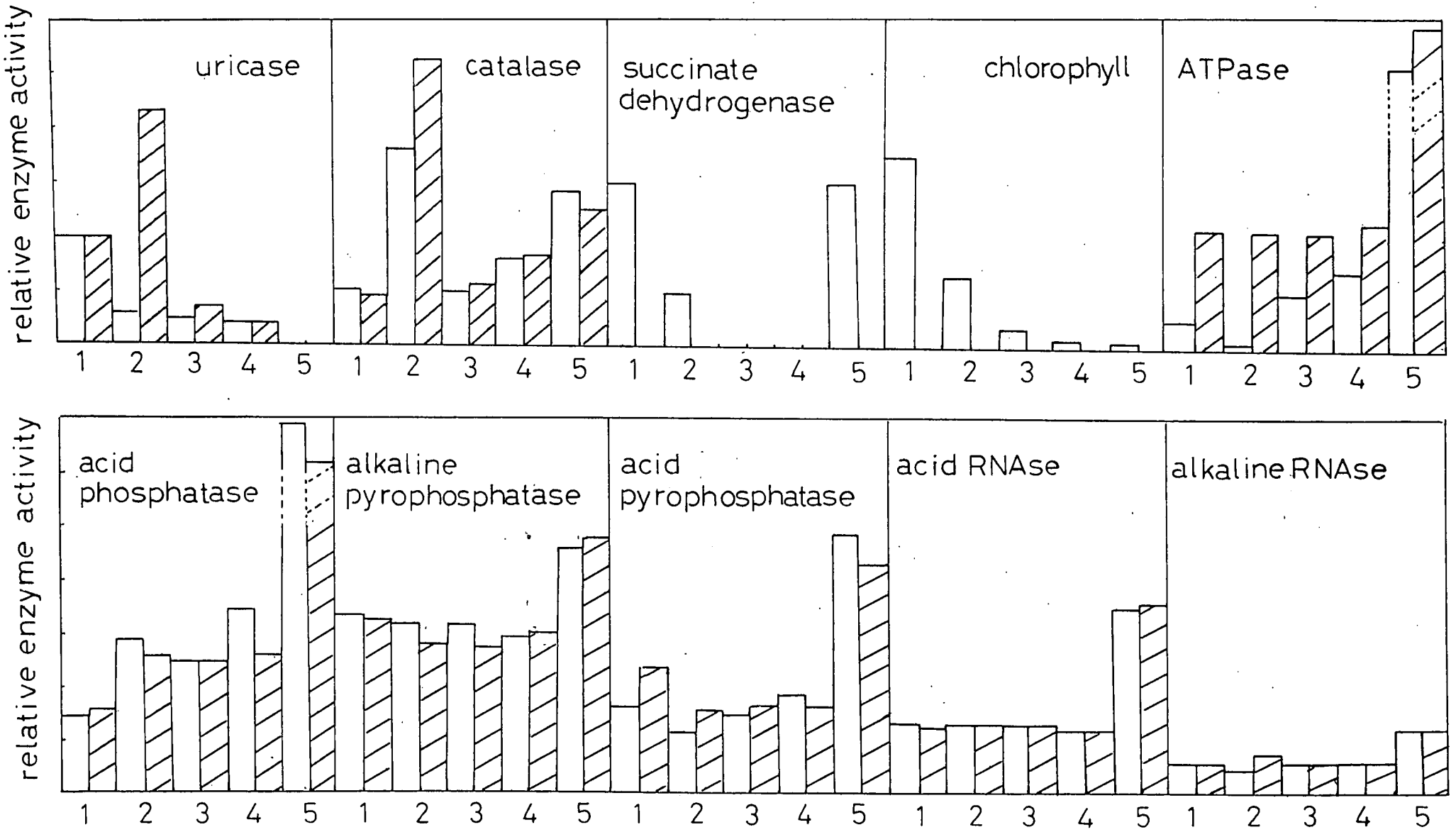
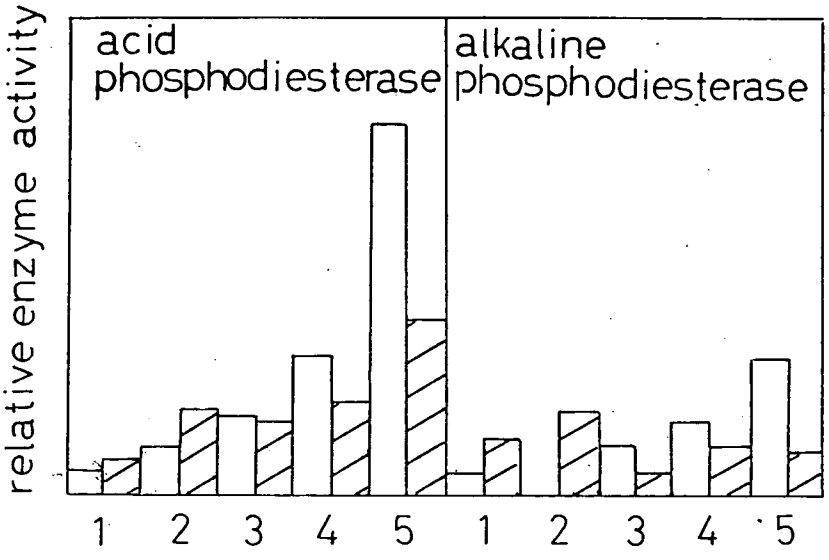

relative enzyme activity
uricase
catalase
succinate dehydrogenase
chlorophyll
ATPase
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
relative enzyme activity
acid phosphatase
alkaline pyrophosphatase
acid pyrophosphatase
acid RNAse
alkaline RNAse
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5

FIGURE 4.35 cont.



ii. Isoenzyme patterns during development.

PAGE of acid phosphatase isoenzymes showed a shift in isoenzyme proportions with development along the leaf. Low molecular weight isoenzymes were prevalent in early development and a novel high molecular weight isoenzyme appeared at incipient senescence intensifying with the course of senescence (see Figure 4.36).

ATPase, RNAse and pyrophosphatase isoenzyme patterns showed no such observable shift in emphasis with development.

iii. Enzyme location.

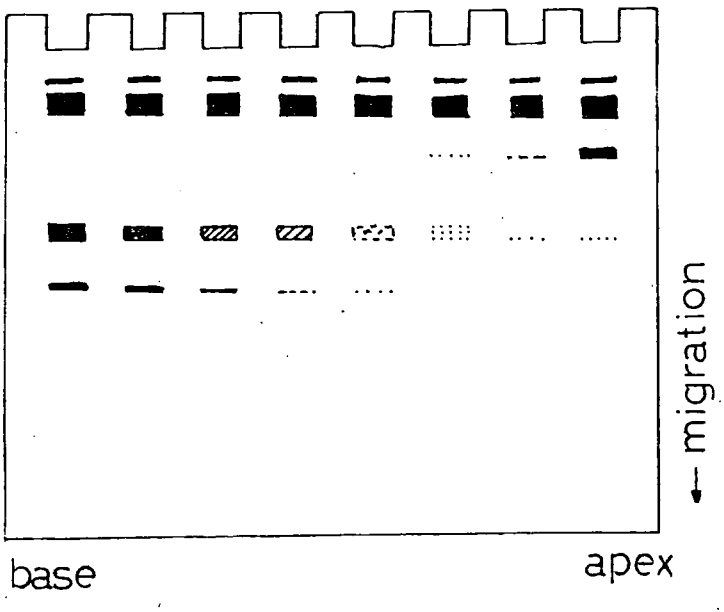
Figure 4.34 shows the results from monitoring of fractions from sucrose density centrifugation for enzyme activity associated with particular cell fractions. Acid RNAse activity was found to be firmly associated with both peaks (a and b) of catalase activity and acid phosphatase was associated with peak b only. Neither enzyme corresponded with NAD cytochrome C oxidoreductase activity (peak c). These results could be tentatively regarded as providing evidence for the mitochondrial location of both acid phosphatase and acid RNAse.

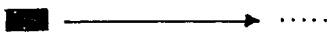
Figure 4.35 shows the enzyme activities associated with the five fractions obtained by differential centrifugation. The fractions were sonicated to release latent enzymes. Uricase was primarily associated with fraction 2 but was only released after sonication. There was no soluble activity and fraction 1 displayed considerable activity without sonication. Catalase was primarily associated with fraction 2 but, unlike uricase, there was activity associated with all the other fractions including soluble fraction 5. The distribution of succinate dehydrogenase, uricase and catalase would suggest that mitochondria and mitochondrial fragments are present in fractions 1 and 2. All phosphorus metabolism enzymes investigated showed major activity associated with soluble fraction. ATPase, alkaline phosphodiesterase and acid pyrophosphatase showed increased activities following sonication of fractions 1 and 2. ATPase showed increased activity following sonication in all fractions suggesting that it is

FIGURE 4. 36.

Acid phosphatase isoenzymes from sections along the mature leaf of F. pratensis.

FIGURE 4.36



decreasing
intensity of
band staining

membrane bound. Clearly, the acid and alkaline isoenzymes of pyrophosphatase and the acid and alkaline isoenzymes of phosphodiesterase are not similarly located in membranes. The results from differential centrifugation do not indicate a close association of catalase succinate dehydrogenase or uricase activities with acid RNAse or acid phosphatase activities although acid phosphatase does show some association with membrane fractions.

Having established the distribution of enzyme activities at maturity it would be interesting to compare these with later and earlier stages in development. For example, does ATPase activity become more labile as the membranes become disorganized with senescence ?

## Chapter 5. Two experimental probes into development: 'Greening-up' and Excision.

I. Introduction: 'Greening-up' and excision.

II. 'Greening-up.'

1. Methods
2. Nucleic acids and 'greening-up'
   i. Qualitative and quantitative comparison.
   ii. Radio-isotope labelling.
3. Proteins and 'greening-up.'
4. Enzymes and 'greening-up.'

III. Excision.

1. Methods
2. Nucleic acids and excision.
3. Proteins and excision.
4. Enzymes and excision.

## I. Introduction: 'Greening-up' and Excision.

Inherent in the model systems of 'greening-up' and excision are problems of interpretation in relation to the normal course of ontogeny. 'Greening-up' involves an enforced period of etiolation when potential for subsequent light-triggered biochemical changes may be enhanced or reduced and the time scale of succeeding events need not reflect the normal course of events. Nevertheless, the actual manifestation of light-induced changes need not be affected and, at least, artificial 'greening-up' can be used to establish trends and can be manipulated to provide important clues to the basis of the response, for example regarding possible photoreceptors. Excision, whilst useful in providing a defined zero point from which subsequent events can be followed (Thomas, 1975) has the major disadvantage of incurring concomitant induction of wound responses (Bagi and Farkas, 1967; Lewington et al., 1967; Pitt and Galpin, 1971; Spencer and Titus, 1973; Pitt, 1974; Macnicol, 1976; Sacher and DeLeo, 1977). In addition, excretion of enzymes or other metabolites into the suspension medium may lead to observation of apparent decreases in content (Atkin and Srivastava, 1969; Lazar and Farkas, 1970). Furthermore, the problems associated with edge-effects should not be overlooked (Hardwick et al., 1968). Pratt and Matthews (1971) observed a geographic gradient of RNA precursor penetration from the cut edge of leaf discs of tobacco and Chinese cabbage towards the centre, thus outer areas appeared to synthesize RNA more efficiently. Macnicol (1976) noted metabolic changes localized in leaf disc outer regions resulting from membrane damage and consequent ionic imbalance. He also suggested that the fact that photosynthetic metabolism of leaf discs appears to conform with attached leaves may only be a reflection of the relative autonomy of the photosynthetic apparatus. Choe and Thimann (1974) provided support for this in their observation that chloroplasts isolated from oat leaves at incipient senescence retained their photosynthetic capacity for a number of days.

In both 'greening-up' and excision, the contribution made by the plant's response to stress alone must be recognised.

'Greening-up.'

It has already been mentioned (Chapter 2,I.ii) that light tends to accelerate biochemical changes rather than enhance them: for example, the nucleic acid content of light-treated bean leaves increases approximately 24 h. in advance of dark-grown plants (Grierson et al., 1970). However, there are a number of novel biochemical changes which are triggered by light. These may result from de novo syntheses or activation, and inhibitors of RNA and protein syntheses have been widely used in attempts to distinguish these possibilities. Eytan and Ohad (1970) demonstrated co-operation between cytoplasmic and chloroplastic ribosomes in the provision of lamellar proteins for photosynthesis. Over 50% total soluble protein increase during greening can be attributed to Fraction I protein of which the small sub-unit has been shown by genetic analysis and use of inhibitors to be synthesized by chloroplast ribosomes (Chen and Wildman, 1970). The light effects on specific RNA fractions, proteins and enzymes of phosphorus metabolism have been outlined in the introductions to the relevant Chapters.

Excision.

Many workers have compared the ageing of intact leaves with the stress-induced ageing created by excision (e.g., Lewington et al., 1967; Spencer and Titus, 1973). Chlorophyll loss in discs of both cucumber cotyledons (Lewington et al., 1967) and apple leaves (Spencer and Titus, 1973) preceded protein loss whereas it followed protein decline in attached leaves. Protein and RNA losses were less pronounced in excised cucumber cotyledons (Lewington et al., 1967). Such differences may, in part, be associated with the disconnection by excision from the transport systems and whole plant correlative influences (Müller and Leopold, 1966; Wollgiehn, 1967). However, such isolation has resolved some questions of senescent events; for example, protein synthesis declines with excision even though amino acid pools would not be limiting due to mobilization to actively growing regions (Vickery et al., 1940; Mothes and Engelbrecht, 1956). Nevertheless, retention of the petiole in

excised leaves, through which translocates would pass, enhances senescence (Misra and Biswal, 1973). In fact, the capacity for RNA and protein syntheses in excised tissue has been found to be less than in ageing attached leaves (Wollgiehn, 1967). Thomas and Stoddart (1975) observed a 75% loss in protein content in leaf sections of F. pratensis over 6 days. Most of this can be attributed to a rapid degradation of Fraction I which may be inhibited by cyclohexamide (Peterson and Huffaker, 1975). Click and Hackett (1963) have observed an initial increase in RNA and protein synthesis resulting from mechanical damage

RNAse activity increases on excision as it does in response to other stresses (for example, viral infection (Randles, 1968)). This has been demonstrated in many species including tomato (McHale and Dove, 1969; Dove, 1971), wheat (Sodek and Wright, 1969), tobacco (Lazar and Farkas, 1970), barley (Atkin and Srivastava, 1970) and Avena (Udvardy et al., 1967). Two phases may be distinguished, firstly, an immediate and rapid increase (wound response?) which subsequently declines (DeLeo and Sacher, 1970; Pitt and Galpin, 1971), and a long term increase which probably contributes to the death of the isolated plant material (Dove, 1973). Both the rapid and the long term responses are inhibited by cyclohexamide (DeLeo and Sacher, 1970), chloramphenicol (Bagi and Farkas, 1967; McHale and Dove, 1968) and actinomycin D (Bagi and Farkas, 1967). However, Thomas (1976a) found that the increase in RNAse activity could only be inhibited by D-MDMP and not by inhibitors of transcription, suggesting that translation events were responsible either directly or indirectly. Sacher and Davies (1974) reported de novo synthesis of RNAse as implied by density-labelling experiments in excised Rheo leaves. Pitt (1971, 1974) reported that only 25% RNAse activity increase in excised potato leaves resulted from de novo synthesis and that the rest derived from activation of pre-formed RNAse. The characteristic RNAse changes in senescing plant tissues are sufficiently similar to changes following leaf excision that it has often been assumed that detachment merely accelerates the normal senescence patterns of intact leaves (Dove, 1973). However, Wyen et al. (1971)

found different RNAse patterns in intact and excised Avena leaves.

Acid phosphodiesterase activity increases within 8 h. of detachment (Udvardy et al., 1969) but alkaline phosphodiesterase remains unaffected (Wyen et al., 1971). Pitt and Galpin (1971) noted a minor increase in acid phosphodiesterase in potato leaves as a result of mechanical damage.

Macnicol (1976) noted a rapid rise in ATP levels and ATP:ADP ratios following excision, implying the involvement of ATPase.

Parish (1968 ) noted an increase in acid phosphatase activity in senescing tobacco leaf discs which could be reversed by hydroxyproline, suggesting synthesis was involved in its increase.

Figure 5.1

Relative chlorophyll levels of shoots of *F. pratensis* resulting from different length exposures to white light.

FIGURE 5.1

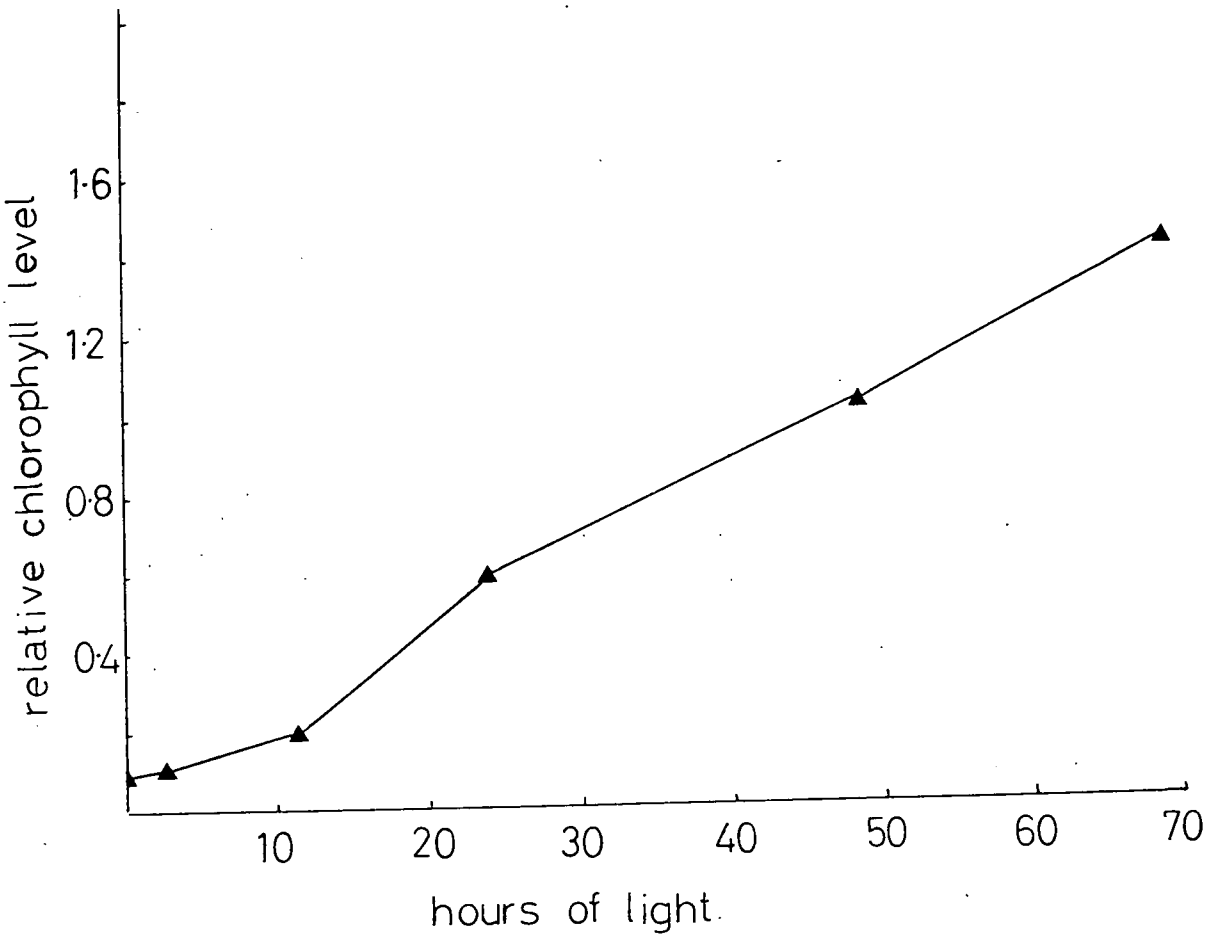
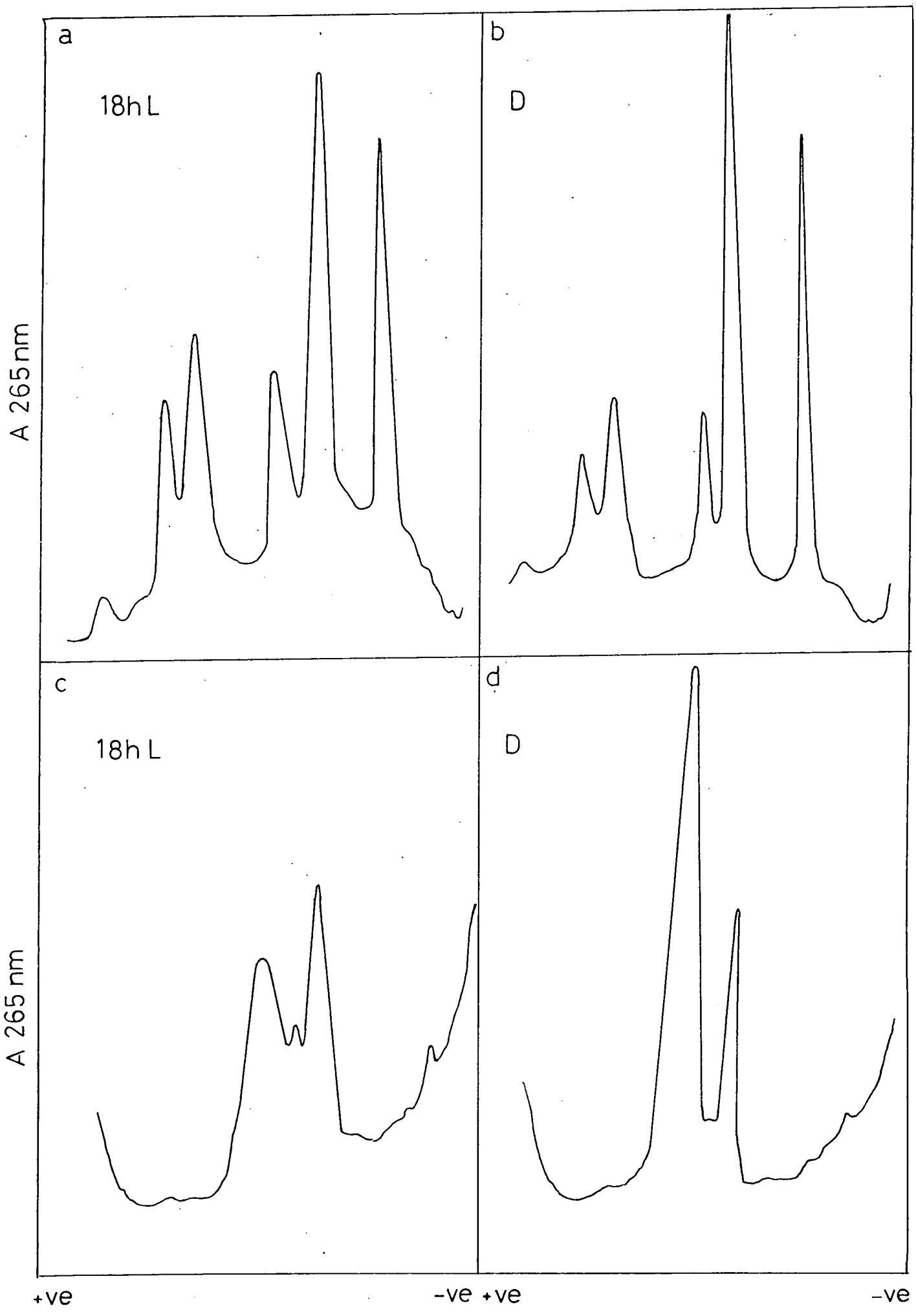

relative chlorophyll level
1·6
1·2
0·8
0·4
10
20
30
40
50
60
70
hours of light

Figure 5.2

Spectrophotometric scans at 265 nm. of separation of RNA from leaves of F. pratensis by PAGE. Electrophoresis at 4°C. in EDTA buffer (Loenings).

a. 2.6% (W/V) PAGE gel loaded with 15 µg. RNA from 18 h. light treated leaves.
b. 2.6% (W/V) PAGE gel loaded with 15 µg. RNA from dark-grown leaves.
c. 7.5% (W/V) PAGE gel loaded with 30 µg. RNA from 18 h. light treated leaves.
d. 7.5% (W/V) PAGE gel loaded with 30 µg. RNA from dark grown leaves.

FIGURE 5.2



## II. 'Greening-up.'

### 1. Methods.

Seeds of F. pratensis were sown in approximately 1.5 cm. depth acid-washed sand in either glass Petri-dishes or perspex lidded boxes and kept in a cupboard in a dark room at 28°C., watered daily with 1 or 5 ml. water respectively. All manipulations were conducted using a green safelight (40 lux). 'Greening-up' of etiolated tissue was achieved by transferring the seedlings to a Fisons Growth Cabinet with incident white light of 6200 lux or by placing beneath a fluorescent light of 180 lux or at 6000 lux.

Radioisotope labelling of greening tissue was achieved by excising fourteen day old dark grown shoots, sterilizing them, and floating them on 10 ml. sterile water containing 5 μCi/ml. $^3$H-adenine. Petri dishes containing shoots which were to be kept in the dark were wrapped in aluminium foil and maintained at the same conditions as the light-treated shoots.

Chlorophyll content was measured and recorded in a relative manner (Figure 5.1). Over 70 h. exposure to light chlorophyll content showed a geometric increase.

### 2. Nucleic acids and 'greening-up.'

#### i. Qualitative and quantitative comparison.

Fourteen day old dark grown seedlings of F. Pratensis were given 18 h. white light at 6000 lux. RNAs, extracted by Method 2 (Chapter 2,2.ii.c) were spectrophotometrically estimated and fractionated on 2.6% (W/V) and 7.5% (W/V) PAGE gels (Figure 5.2). There were no qualitative differences in the rRNA complement of light-treated and dark-grown shoots; chloroplast rRNAs were clearly present in dark-grown shoots as suggested by the work of Smith, 1972.

There was an overall drop in RNA content of between 10 - 12% (W/W) as a result of light treatment, indicative of light induced use and subsequent destruction. Light enhanced the level of chloroplast rRNAs and the ratio of large to small ribosomal subunits came closer to that for functional ribosomes. More striking differences were apparent in the low molecular weight RNAs. The dark grown shoots contained larger amounts of compared with the light-treated shoots perhaps as a result of the mobilisation of tRNAs in response to light. A third peak of RNA is apparent in light-treated shoots. The mean size of chloroplast tRNAs is greater than for cytoplasmic tRNAs (Whitfield, 1973) and this intermediate peak may represent increased chloroplast tRNA in response to light. This may even provide the key to light enhanced protein syntheses, particularly as it only appeared in early phases of normal growth, i.e. the basal section of a leaf and at early emergence of the leaf. The '5S' rRNA peak broadened on light treatment, suggestive of increased chloroplast '5S' rRNA which is larger than the cytoplasmic. This may indicate the enhanced functional capacity of the chloroplast ribosome (Whitfield, 1973)

Polyribosomes increased on exposure to light but after 48 h. reverted to pre-light treatment amounts when expressed on a per g. fresh weight basis (Figure 5.3). This pattern was apparent for the early stages of development along the leaf (Chapter 2, Figure 2.29). Their polypeptide synthetic capacity was observed by introduction into a wheatgerm cell-free polypeptide-synthesizing system. The capacity declined with the length of light treatment (Figure 5.3) as apparent in early stages of development along the leaf (Chapter 3, Figure 3.7). This may result from increased levels of monoribosomes due to higher levels of polyribosome turnover as a consequence of both synthesis and breakdown. Without fractionation of the polyribosomes, the reason for the apparent decrease in capacity must remain speculative.

Figure 5.3

A. Ribosome content of shoots of *F. pratensis* given different lengths of white light treatment.

B. Polypeptide-synthetic capacity of ribosomes expressed as cpm. of radioisotopically-labelled amino acid incorporated into polypeptide per µg. ribosome introduced into the wheatgerm cell-free system.

FIGURE 5.3

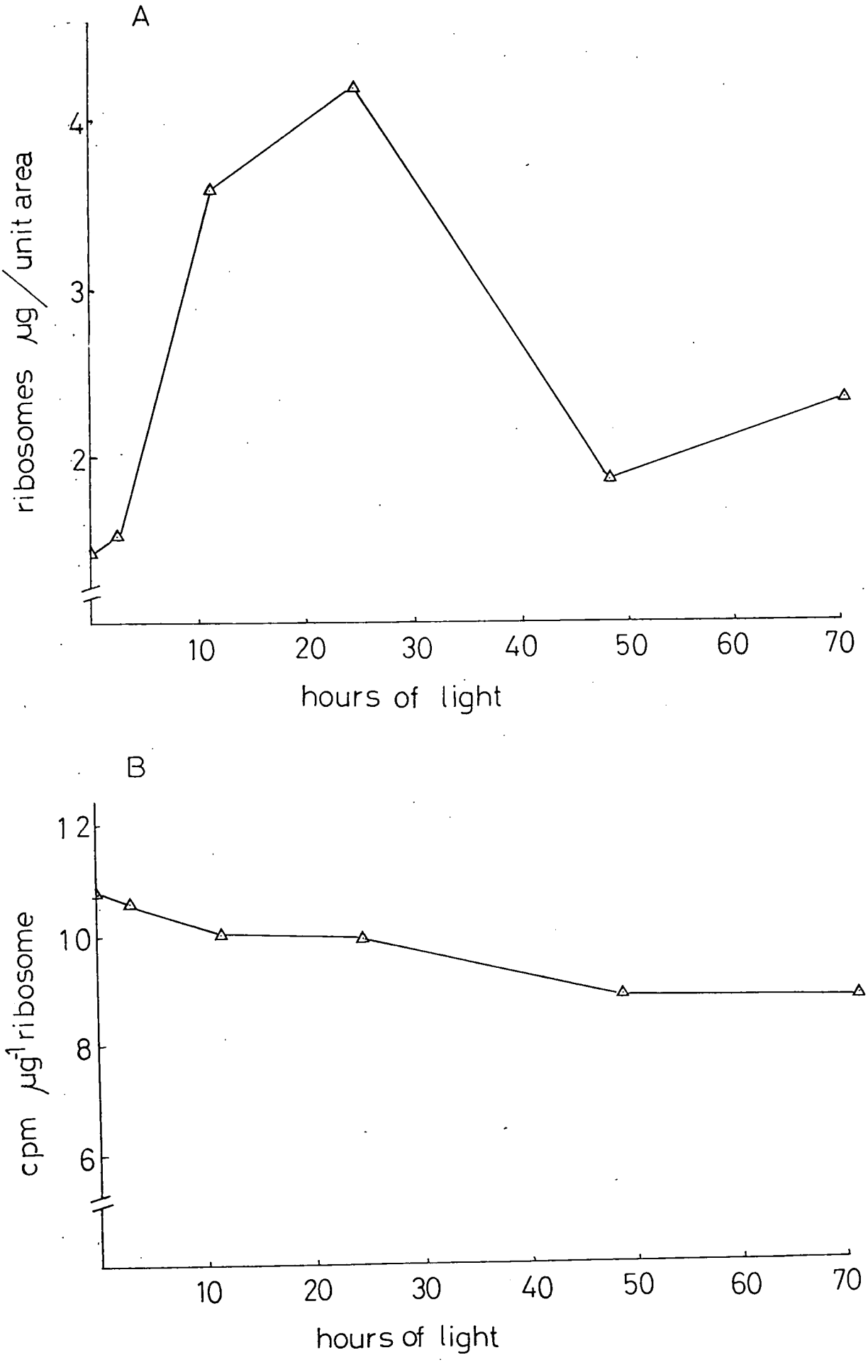

A
ribosomes µg / unit area
4
3
2
10
20
30
40
50
60
70
hours of light
B
cpm µg$^{-1}$ ribosome
12
10
8
6
10
20
30
40
50
60
70
hours of light

Figure 5.4

Spectrophotometric scan of 2.6% (W/V) PAGE gel fractionation of RNAs superimposed on the radioisotope label distribution

a. from shoots exposed to 18 h. white light (184L).

b. from darkened shoots (D).

FIGURE 5.4


a
18h L
A 265 nm -----
cpm $^3$H-adenine x10$^2$
8
7
6
5
4
3
2
1
b
D
A 265nm -----
cpm $^3$H-adenine x10$^2$
8
7
6
5
4
3
2
1

Figure 5.5

Soluble protein content expressed on a per unit area basis of shoots of *F. pratensis* given different lengths of white light treatment.

FIGURE 5.5

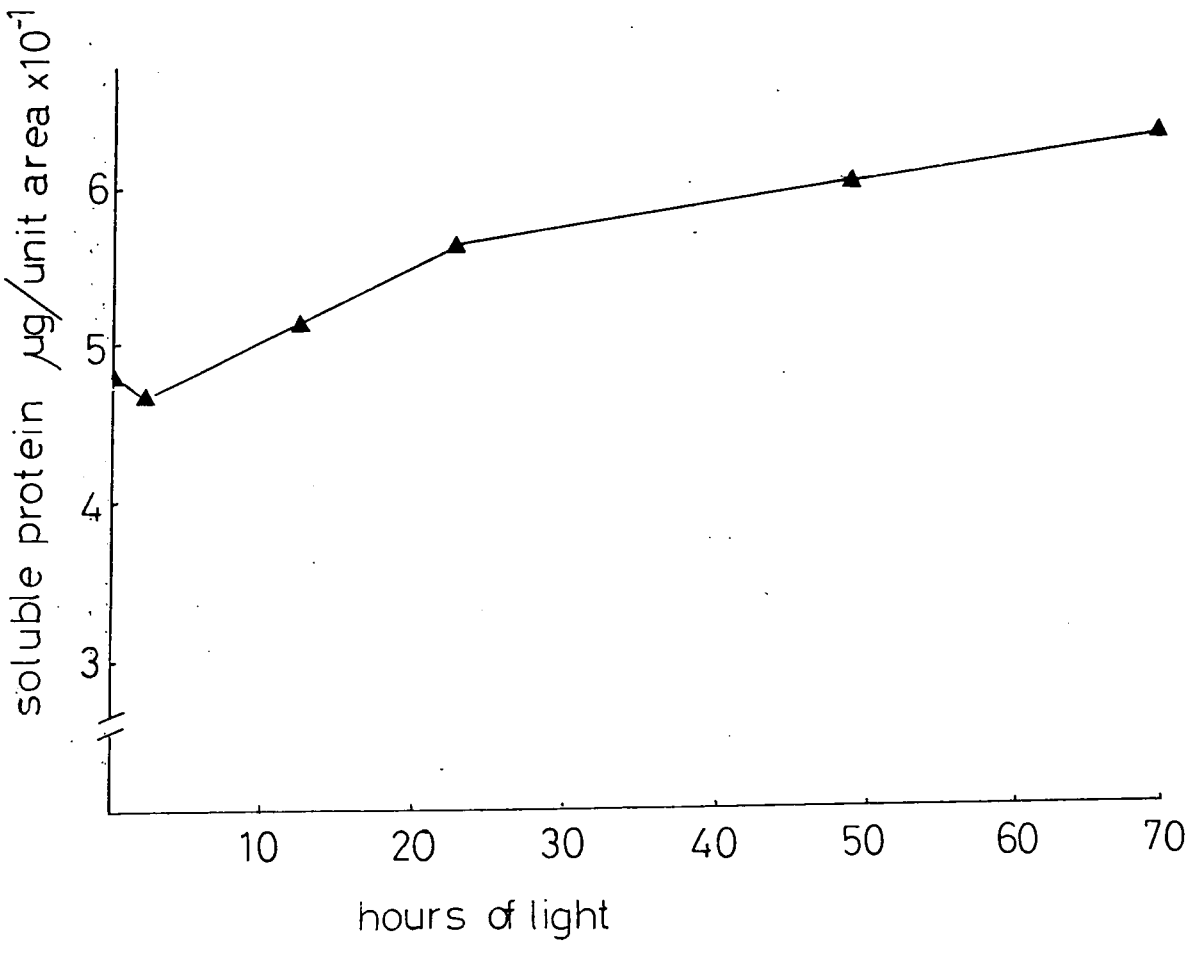

soluble protein µg/unit area x10⁻¹
6
5
4
3
10
20
30
40
50
60
70
hours of light

Figure 5.6

Activities of different enzymes expressed per µg. protein of shoots of F. pratensis exposed to different lengths of white light.

FIGURE 5.6

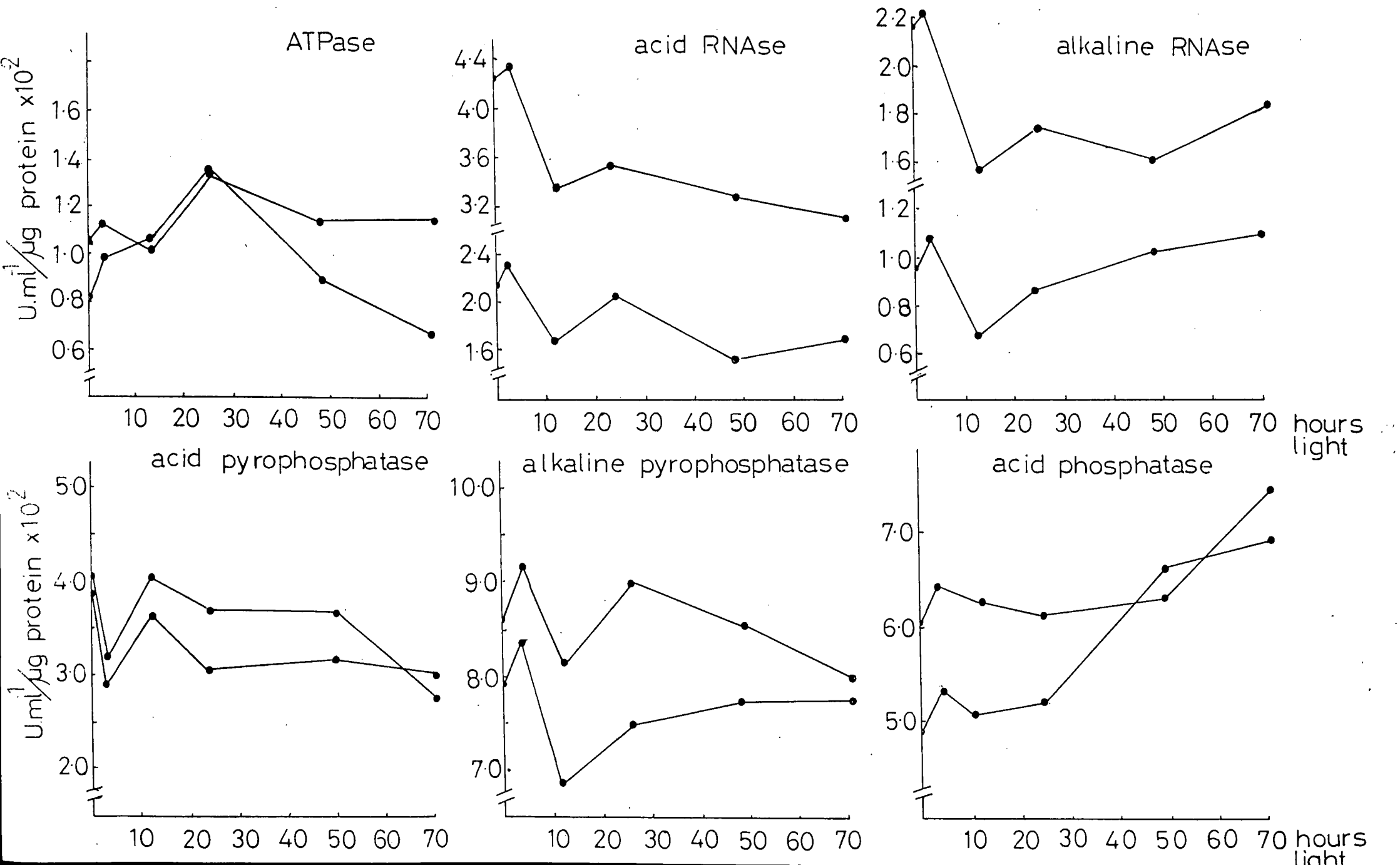
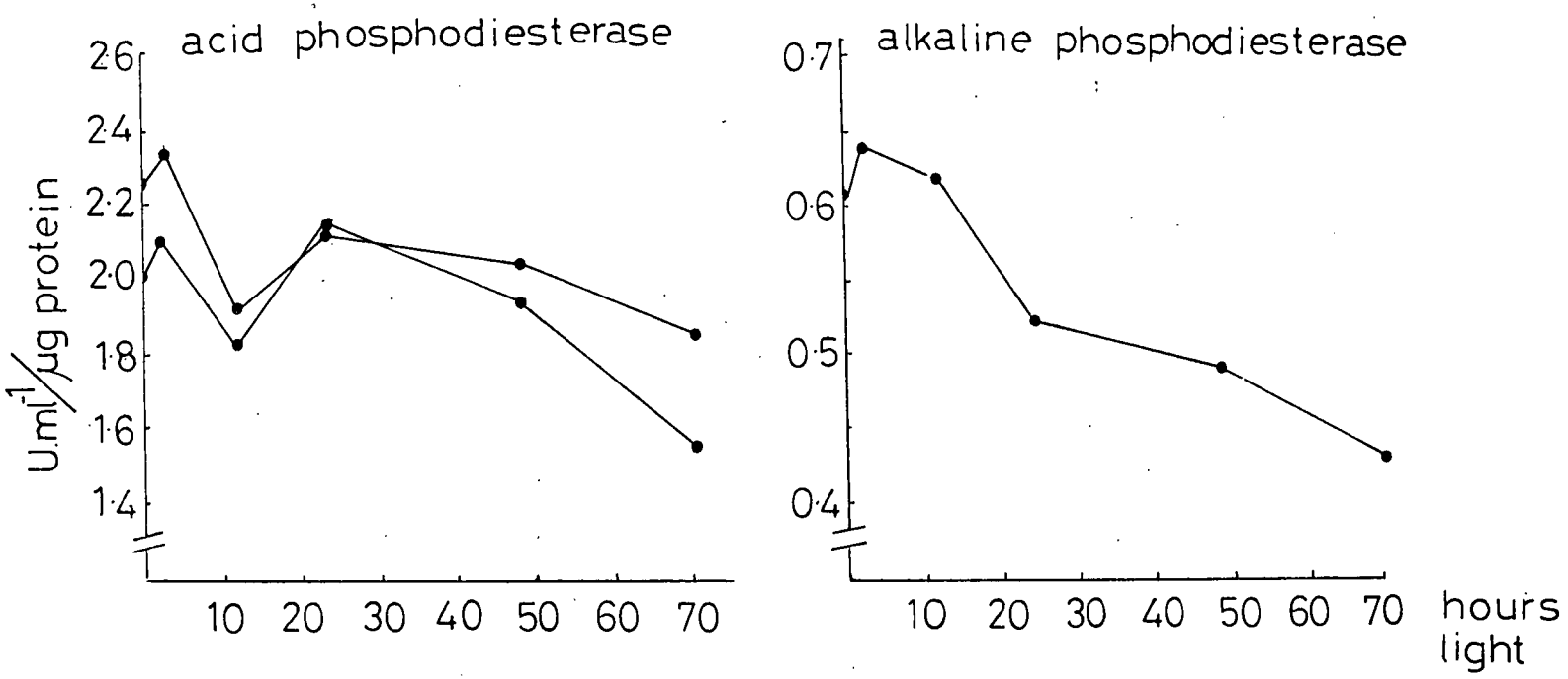

ATPase
acid RNAse
alkaline RNAse
acid pyrophosphatase
alkaline pyrophosphatase
acid phosphatase
U.ml⁻¹/µg protein x10⁻²
hours light
10 20 30 40 50 60 70

FIGURE 5.6 cont.



ii. Radio-isotope labelling.

The pattern of radio-isotope labelling of specific fractions of rRNA is shown in Figure 5.4. The synthesis of chloroplast rRNAs is enhanced by light, particularly $1.1x10^6$ RNA, but synthesis of this fraction did occur in dark grass shoots, confirming that light is not a prerequisite for chloroplast rRNA formation. The slight shoulder apparent in Figure 5.2 b, slightly heavier than $0.7x10^6$ rRNA, is echoed by an increased level of radio-isotope incorporation (Figure 5.4 b). It is possible that this is some precursoral RNA, perhaps of $0.56x10^6$ rRNA, which requires light for maturation.

Preliminary work involving radio-isotope labelling of the poly(A)-containing RNAs suggested that light enhanced the synthesis or post-transcriptional modification of these RNAs by at least fivefold, notably of the presumptive mRNAs of high molecular weight, as visualized on 2.6% (W/V) PAGE gels.

3. Proteins and 'greening-up.'

As reported for many species (for example, De-Deken Grenson, 1954; Mego and Jagendorf, 1961; Rhodes and Yemm, 1963; Smillie, 1969) soluble protein content increased as a result of light treatment (Figure 5.5). This is consistent with the early phases of normal development as shown in Chapter 3, Figures 3.3 and 3.4 where the early increase is followed by a decline.

4. Enzymes of phosphorus metabolism and 'greening-up.'

All the enzymes examined in Chapter 4 were observed in relation to 'greening-up' of fourteen day old dark grown tissue (Figure 5.6). There were essentailly two patterns of enzyme response to light. Type 1 showed an immediate increase (within 2 h.) followed by a decrease and then another increase to a maximum at 24 h., as exemplified by acid RNAse, alkaline pyrophosphatase and acid phosphodiesterase. The early increase, which was also apparent in ATPase and acid

phosphatase, was unlikely to involve nucleic acid de novo synthesis suggesting cytoplasmic control operated at this time at translational or activation levels. The subsequent decline probably resulted from a delayed lag in the synthesis of the particular enzyme, during which time other enzymes (type 2) may have been preferentially synthesized, for example, ATPase, acid pyrophosphatase, alkaline phosphodiesterase and acid phosphatase. Acid phosphatase, and to a lesser extent, RNAse and alkaline pyrophosphatase, are unusual in that they continue to show an increase after 70 h. light. In the case of acid phosphatase this is consistent with the early increase within six days and at section 3 (Figure 4.30) whilst other enzymes mostly decrease in this period.

The responses of these enzymes to light do mirror the levels at early development (Figures 4.25 - 4.33) although the time scale is effectively reduced in the artificial system perhaps as a result of a build up of the capacity to respond to light during enforced etiolation.

Figure 5.7

Relative chlorophyll levels of excised sections of leaves of *F. pratensis* incubated for different lengths of time.

FIGURE 5.7

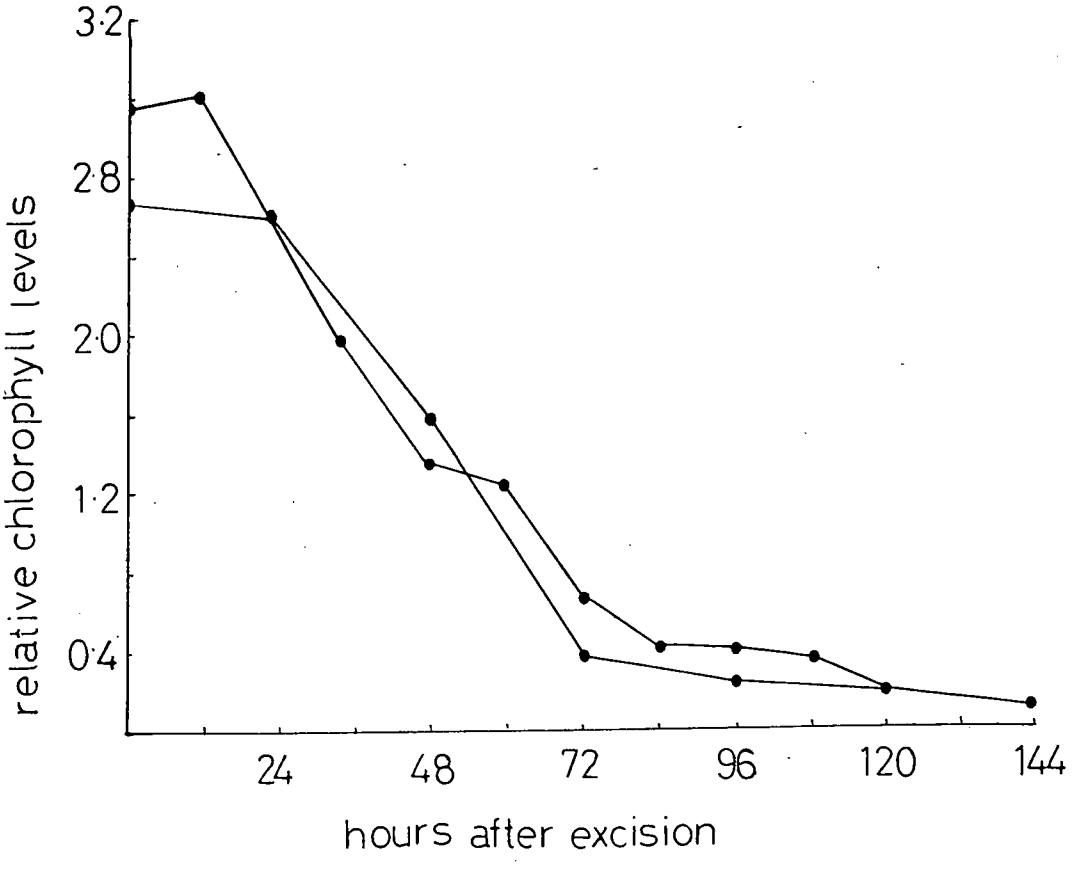

3·2
2·8
2·0
1·2
0·4
relative chlorophyll levels
24
48
72
96
120
144
hours after excision

Figure 5.8

A. Ribosome content of excised sections of leaves of F. pratensis at different lengths of incubation.

B. Polypeptide-synthesizing capacity of ribosomes expressed as cpm. of radioisotopically-labelled amino acid incorporated into polypeptide per µg. ribosomes introduced into the wheatgerm cell-free systems.

FIGURE 5.8

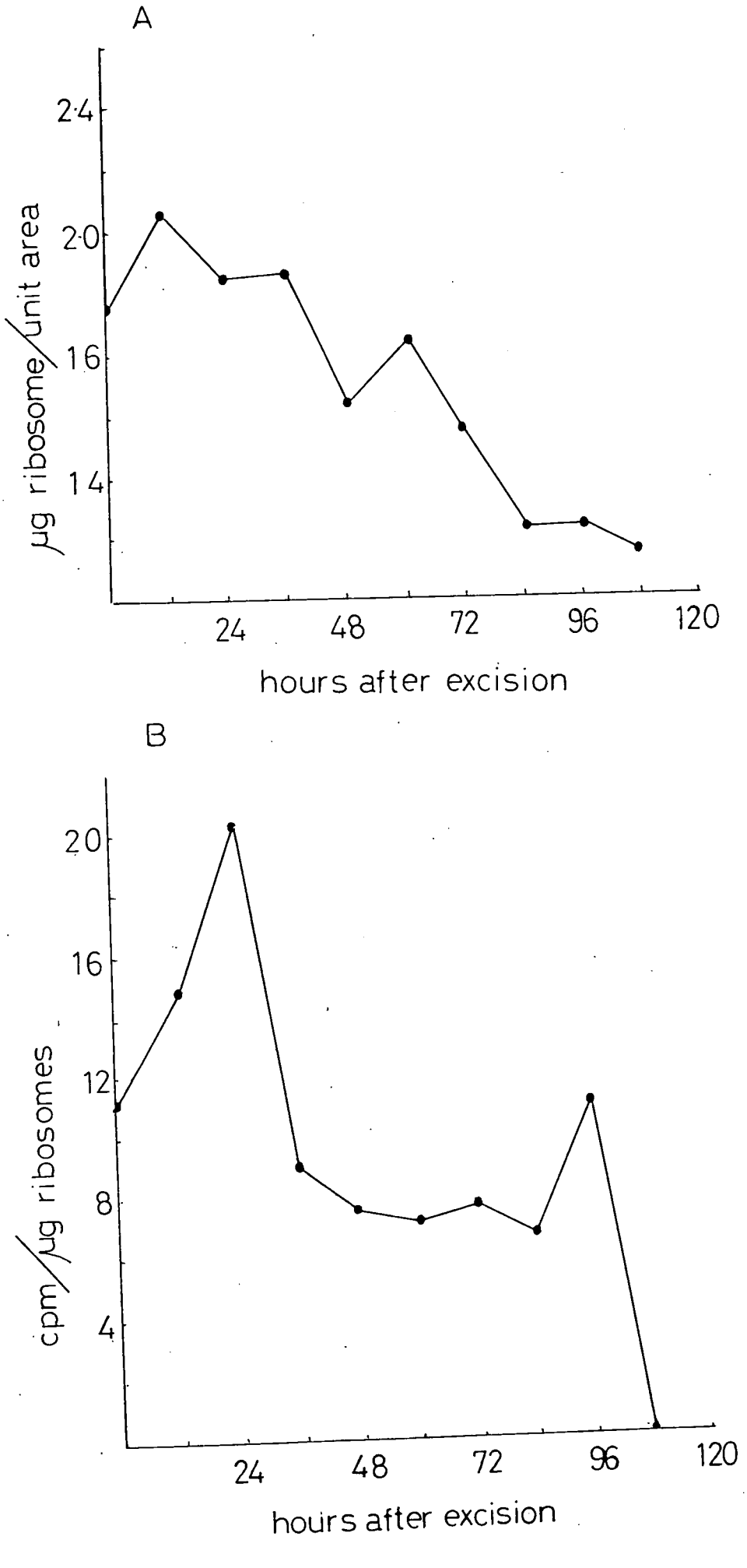
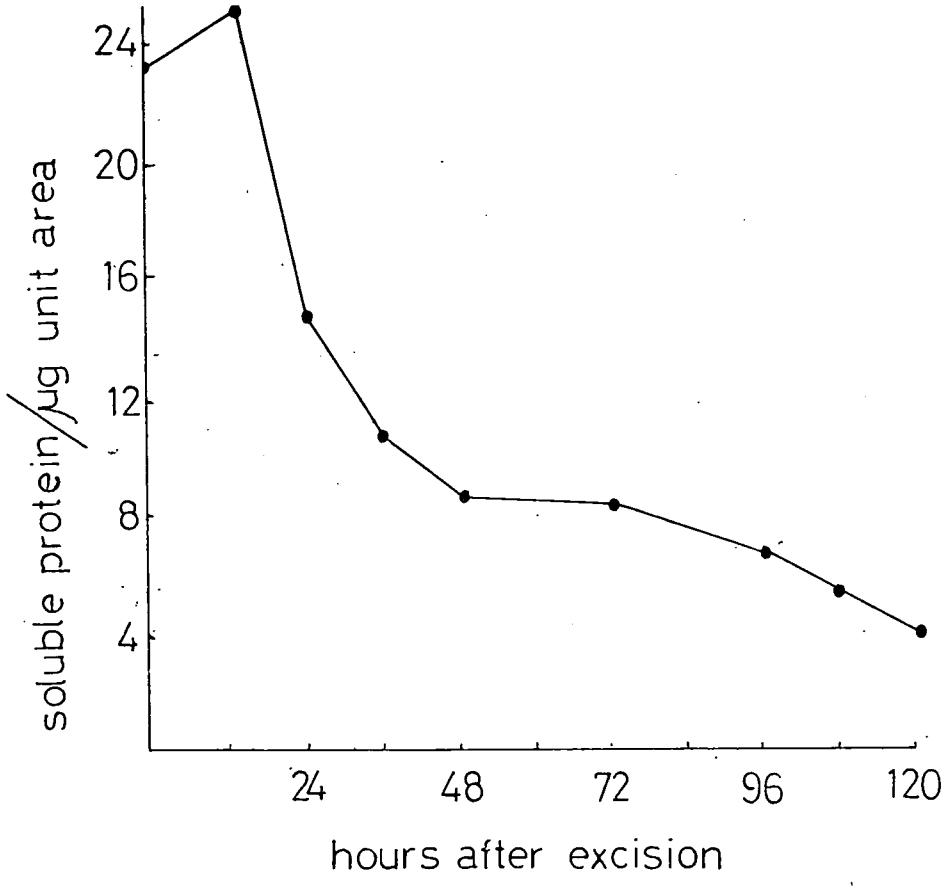

A
2·4
2·0
1·6
1·4
µg ribosome/unit area
24
48
72
96
120
hours after excision
B
20
16
12
8
4
cpm/µg ribosomes
24
48
72
96
120
hours after excision

Figure 5.9

Soluble protein content expressed per unit area of excised sections of leaves of *F. pratensis* incubated for different lengths of time.

FIGURE 5.9


soluble protein/ug unit area
24
20
16
12
8
4
24
48
72
96
120
hours after excision

## III. Excision.

### 1. Methods.

Mature leaves of comparable age and length were cut into 1 cm. sections after removal of the top 3 cm. and basal 1 cm. Following surface sterilization they were placed lower side down on heat-sterilized filter papers moistened with 5 ml. sterile water in Petri dishes. Following incubation for an appropriate time in the dark at $28^{0}$C., sections were blotted dry and extractions were carried out in the normal way.

The chlorophyll content was found to decline rapidly with incubation (Figure 5.7) following excision. 96 h. represented an advanced stage of senescence on grounds of chlorophyll content. Loss of chlorophyll was faster than recorded by Thomas and Stoddart (1975) who noted a 50% decrease by 72 h. Sections were completely yellow by 120 h.

### 2. Nucleic acids and excision.

Only ribosome levels were observed in the course of senescence following excision (Figure 5.8 a). After an initial increase in ribosome content the level decreased with further incubation. The polypeptide-synthesizing capacity (Figure 5.8 b) as judged by introduction of ribosomes into a wheatgerm-derived cell-free system increased over the first 24 h. following excision but subsequently declined with a small increase prior to death. The initial increase is consistent with increased protein synthetic capacity at incipient senescence but it might equally result from a wound response.

### 3. Proteins and excision.

Figure 5.9 shows an overall decline in soluble protein content following excision. An initial increase (12 h.) may result from contribution by the wound response. The decline is consistent with those observed in Figures 3.3 and 3.4 at late stages of development.

4. Enzymes of phosphorus metabolism and excision. (figure 5.10)

Most of the enzymes examined showed an overall increase in activity over the incubation period with a decline apparent only beyond 96 - 108 h. Acid RNAse and ATPase both showed a decrease in activity at 96 h. followed by a return to earlier levels of activity. This corresponds to the turning point for activity of other enzymes and may be the result of proteolytic action prior to re-establishment of enzyme levels. This phase also corresponds to the increased protein synthesizing capacity (Figure 5.8 b). Perhaps increased acid RNAse and ATPase levels result from synthesis at this time. In one result for acid phosphatase an increase in activity was observed at 96 h. The slightly more rapid fall in the level of pyrophosphatase activity is consistent with the pattern of decline in the normal ageing systems (Figure 4.28 and 4.29).

Summary.

Although overall patterns of metabolism in the artificial development systems and the in vivo systems of development are similar, the magnitude and timing of these changes vary considerably. It would seem that these model systems are only reliable as indicators of the general trends in development.

Figure 5.10

Enzyme activities expressed per µg. protein of sections of leaves of *F. pratensis* incubated for different lengths of time. The results of two separate experiments are given.

FIGURE 5.10

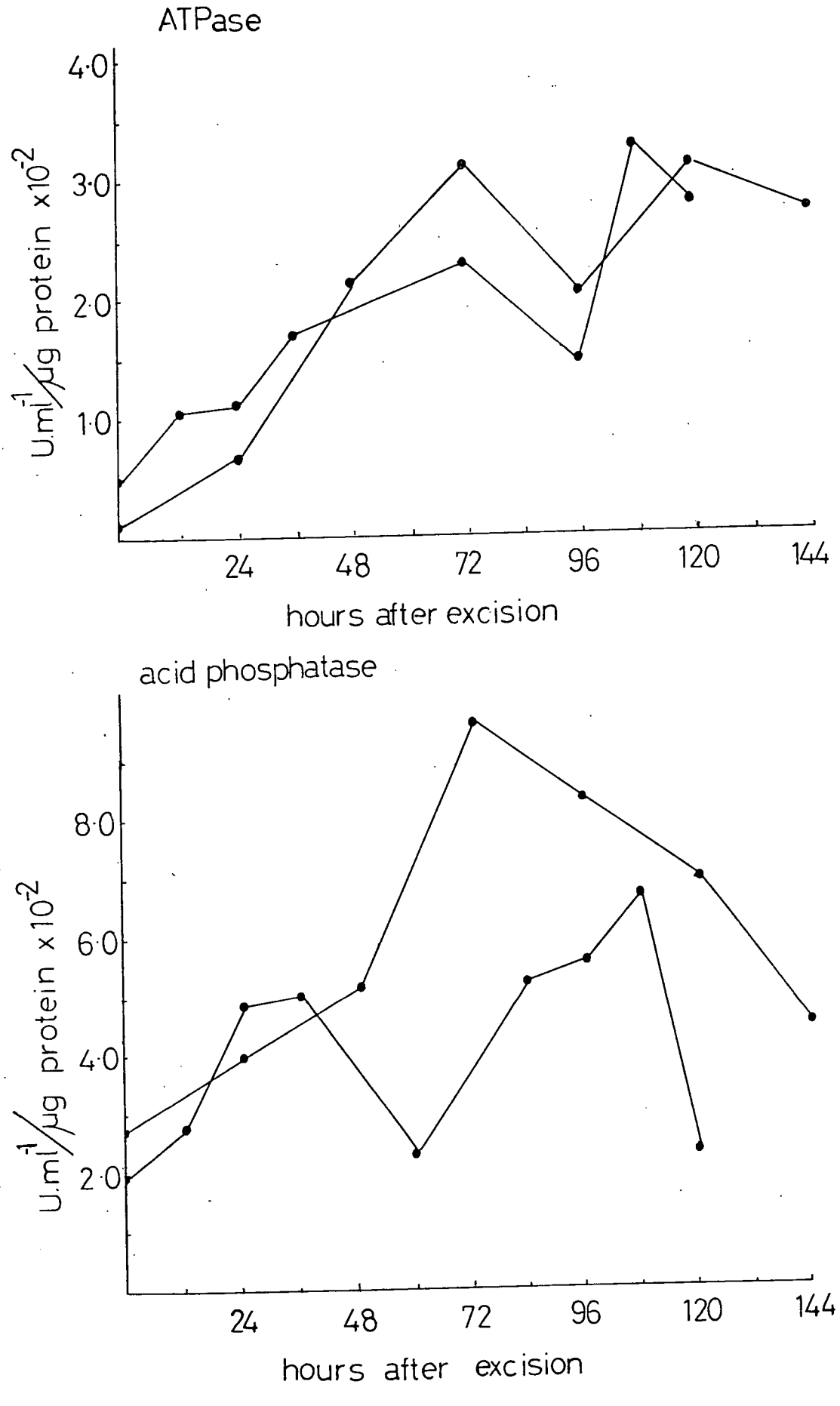
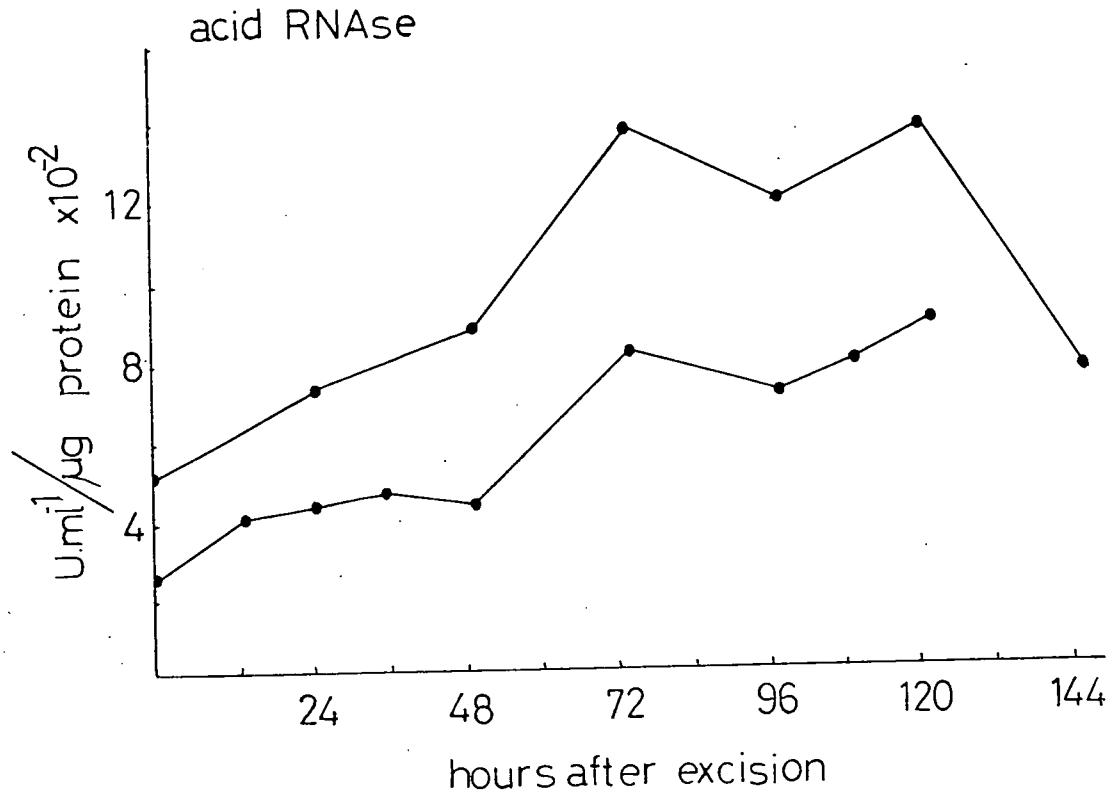

ATPase
4·0
3·0
2·0
1·0
U.ml$^{-1}$/µg protein x10$^{-2}$
24
48
72
96
120
144
hours after excision
acid phosphatase
8·0
6·0
4·0
2·0
U.ml$^{-1}$/µg protein x10$^{-2}$
24
48
72
96
120
144
hours after excision

FIGURE 5.10 cont.



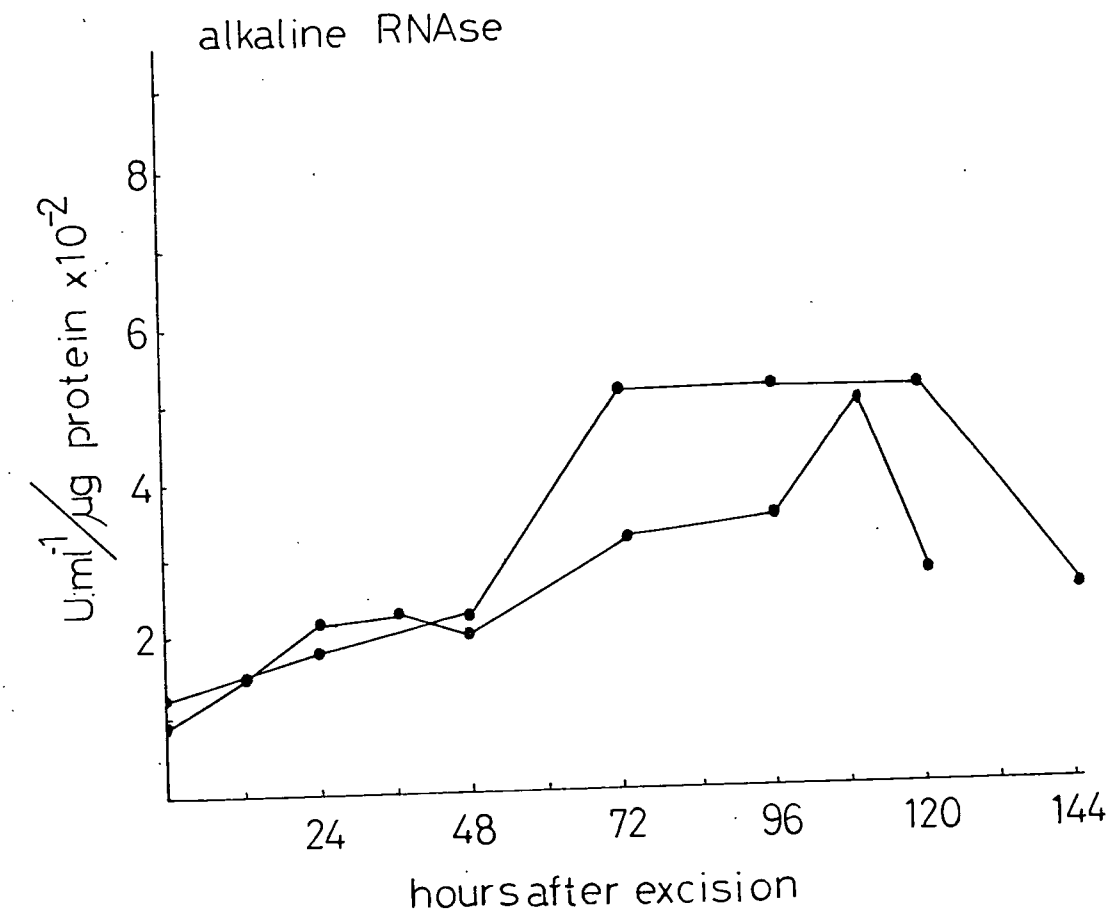
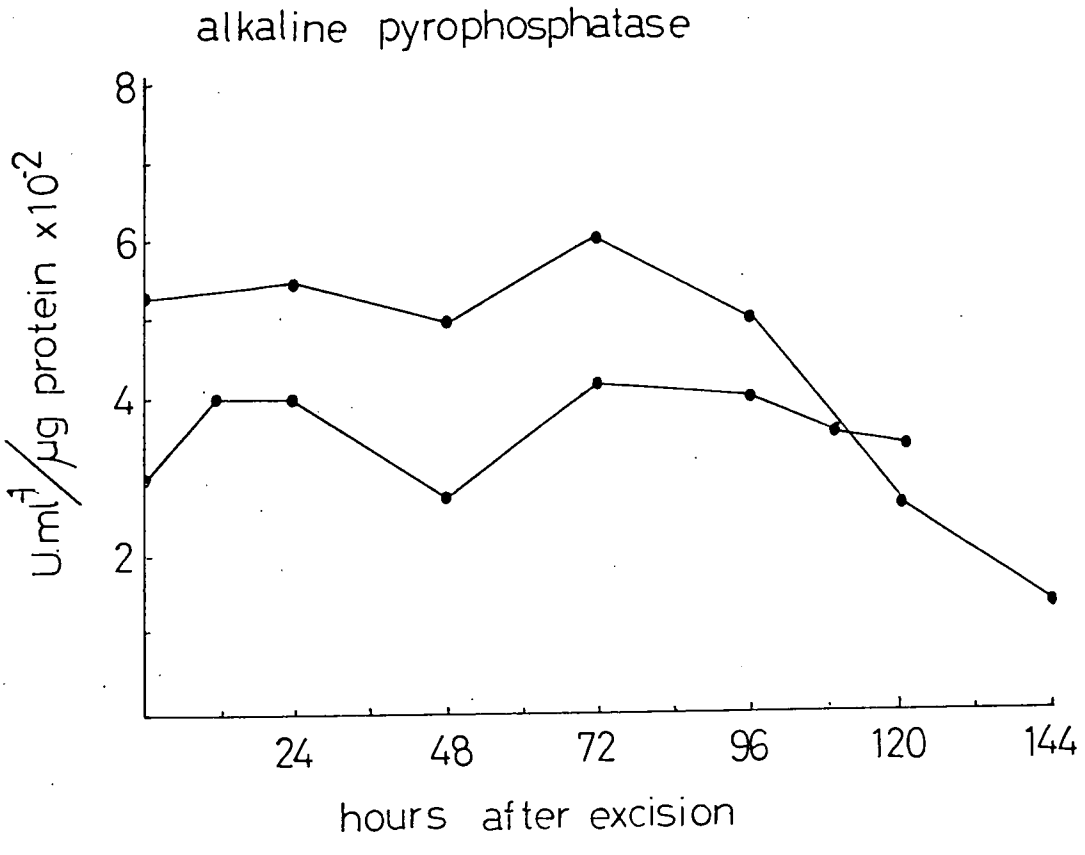



FIGURE 5.10 cont.



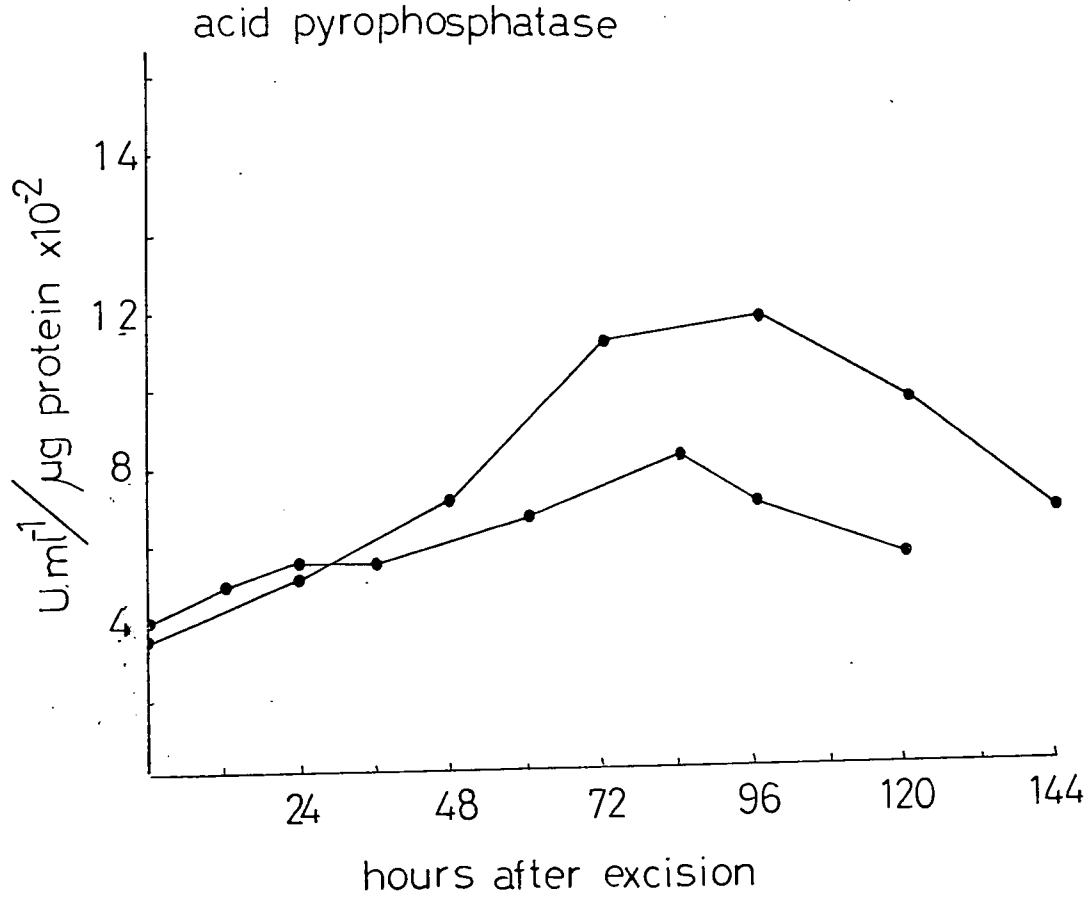
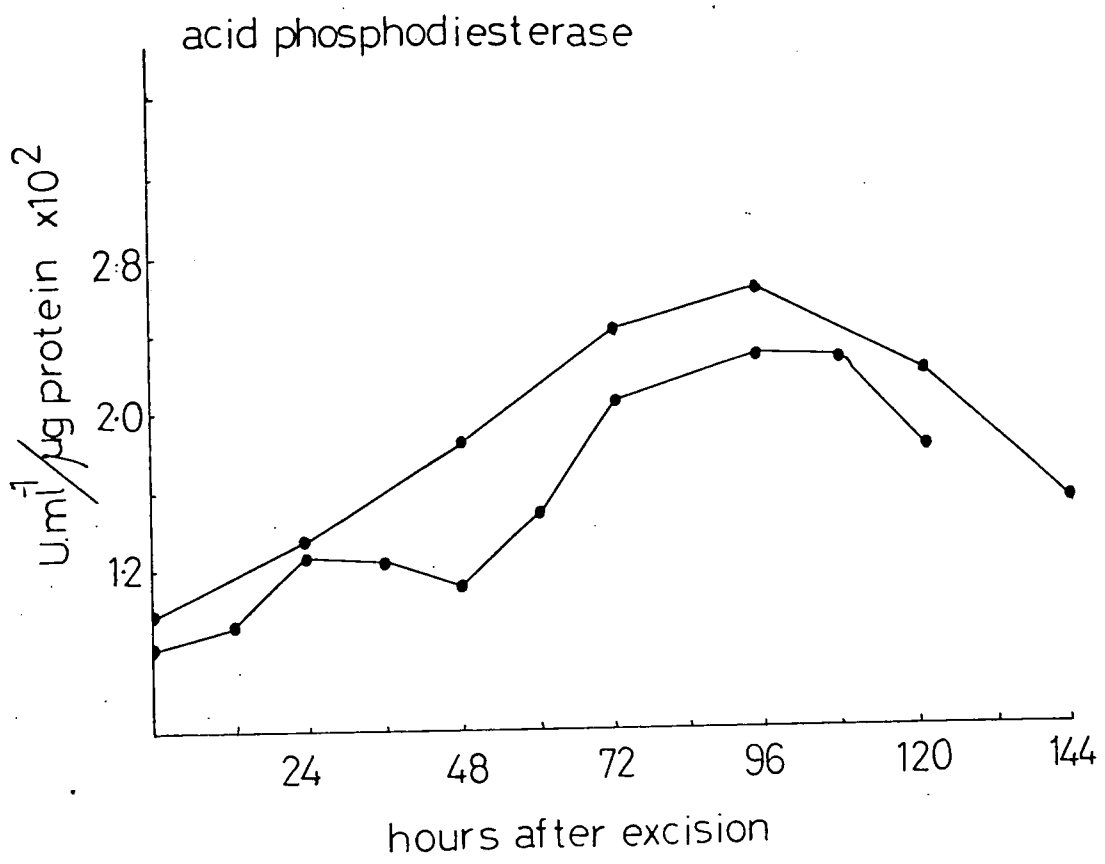
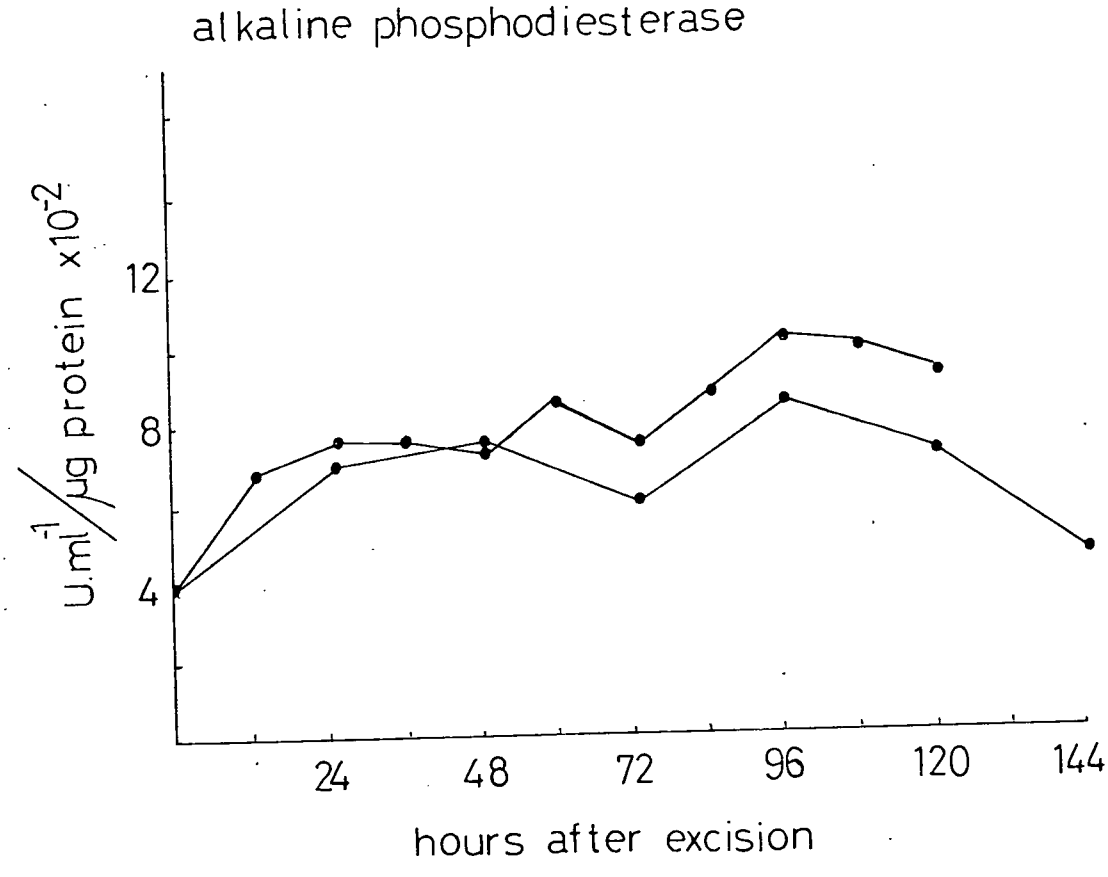



FIGURE 5.10 cont.



# CHAPTER 6. WHEAT GERM CELL-FREE POLYPEPTIDE-SYNTHESIZING SYSTEM.

1. Introduction.
2. Wheatgerm cell-free system used.
3. Time courses for polypeptide synthesis directed by
   - poly U.
   - TMV-RNA
   - pea embryonic axis polyribosomes
   - F. pratensis polyribosomes
4. Wheatgerm-minus system.
5. Effect of polyribosome concentration.
6. Optimization of cell-free system
   - i. pH.
   - ii. $Mg^{2+}$ concentration
   - iii. $K^{+}$ concentration
   - iv. creatine phosphokinase concentration
   - v. spermine requirements.
7. Charging of tRNAs.
8. Effect of ATA.
9. Effects of the use of "Nonidet" in polyribosome extraction.
10. Comparison of polyribosome polypeptide-synthesis capacities.
11. Comparison of "free" and "membrane-bound" polyribosome polypeptide-synthesis capacities.
12. Summary.

## 1. Introduction.

It has long been known that *in vitro* systems can incorporate amino acids into polypeptides and that these depend on the presence of $Mg^{2+}$, GDP or GTP and an energy generating system such as that created by the combination of phosphocreatine and creatine kinase (Keller and Zamecnik, 1956; Rabson and Novelli, 1960). In addition, certain soluble factors, some of which may be loosely bound in polyribosomes *in vivo* but which may be easily removed during extraction, are also required (Ochoa, 1968). It is the loss of these soluble factors which precludes the direction of polypeptide synthesis by purified polyribosomes *in vitro* even when they are supplied with all other cofactors. A supply of these soluble factors, which include tRNAs and enzymes (for example, those required for acylation and initiation of polypeptide synthesis) can be achieved by the inclusion of a "wheatgerm S23 extract" in the cell-free polypeptide-synthesizing system. Wheatgerm-derived cell-free systems were first used successfully in the translation of viral mRNA (Roberts and Patterson, 1973; Davies and Kaesberg, 1973). More recently refinement of the system has led to successful translation of mRNAs from a number of different plant sources - developing bean leaves (Giles *et al.*, 1977), *Lemna gibba* (Tobin and Klein, 1975), soybean (Gordon and Payne, 1976; Verma *et al.*, 1974) and pea epicotyls (Verma *et al.*, 1975) - even to the extent of producing identifiable products (Verma *et al.*, 1974, 1975; Larkins *et al.*, 1976; Sun *et al.*, 1975; Muntz, 1978; Evans *et al.*, 1979). The wheatgerm polypeptide-synthesizing system has distinct advantages over other cell-free systems (for example, rabbit reticulocyte, frog oocyte) in its low cost, ease in preparation and its high translational efficiency.

The introduction of polyribosomes into such a system does not require the occurrence of initiation events. However, Sun *et al.* (1975) showed (by inclusion of T-2 toxin, an inhibitor of initiation, in a bean polyribosomal RNA-directed wheatgerm cell-free system) that the polypeptide synthesis was reduced by 30%. This implies that associated with polyribosomes are mRNAs not yet in the process of

being translated or, alternatively, assuming the possibility of repeated translation, that some mRNAs, having been translated, require initiation events for further translation. However, since monoribosomes are necessarily included in total ribosomal RNA preparations, neither of these possibilities requires the functioning of the wheatgerm derived ribosomes.

Introduction of poly(A)-containing RNA from F. pratensis into the wheatgerm cell-free system provided inconclusive results and in no instance did efficient translation occur. However, introduction of ribosomal RNA into a similar system was successful. This was probably a reflection of the greater quantity of mRNA introduced, the functional capacity of which was protected by extraction as a component of polyribosomes. In addition, it is likely that translation would be more successful in a system requiring only the soluble factors derived from another source, i.e., the wheatgerm, than one requiring the full function of all the components of translation to be provided by the wheatgerm. It was decided to compare the capacities for directing cell-free polypeptide synthesis of ribosomes extracted from leaf tissue from different stages in ontogeny and to relate these to in vivo events (Beevers and Poulson, 1972).

## 2. Wheatgerm cell-free system used.

### i. Wheatgerm extract "S23".

Following the recommendations of Marcu and Dudock (1974) extracts were made from Niblack wheatgerm (see Biological materials). The desirability of using a wheatgerm with low endosperm content and low endogenous RNA template activity was detailed by Senger and Gross (1976), and Niblack wheatgerm fulfils these requirements.

The method of obtaining the S23 extract was largely that of Marcu and Dudock (1974). All solutions (minus DTT), glassware and pestles and mortars were autoclaved prior to use.

The complete extraction was carried out at 4°C. 2 g. raw wheatgerm were ground in a mortar, with an equal weight of crushed Pasteur pipettes, for approximately 60 s. 4 ml. of extraction

buffer consisting of:-

| | | |
|---|---|---|
| 20 mM. | HEPES | pH 7.6 (KOH) |
| 100 mM. | potassium chloride | |
| 1 mM. | magnesium acetate | |
| 2 mM. | calcium chloride | |
| 7 mM. | DTT | |

were added and gently swirled for a further 30 s. The resultant thick paste was scraped into tubes and centrifuged at 22,000 g. for 12 min. in a MSE "Superspeed" 50 TC centrifuge using a 8X50 ml. angle rotor at 4°C. The $A_{260}/A_{280}$ ratios of the supernatant were in accordance with those of Marcu and Dudock (1974), i.e., approximately 1.4. The supernatant was applied to a 21x1 cm. Sephadex G-25 (medium) column and eluted with "column buffer" consisting of:-

| | | |
|---|---|---|
| 20 mM. | HEPES | pH 7.6 (KOH) |
| 100 mM. | potassium chloride | |
| 3 mM. | magnesium acetate | |
| 1 mM. | DTT | |

without restricting the flow. The fast moving beige turbid region (which separated from a slow moving yellow region) was collected in 10 drop fractions from which 30 µl. were diluted in 3 ml. water for spectrophotometric estimation. Fractions giving a diluted $A_{260}$ of greater than 0.9 units were pooled and centrifuged as before for 20 min. The $A_{260}$ of the resultant supernatant was in the region 75 - 90 units. 200 µl. aliquots were stored under liquid nitrogen until required. Since prolonged storage at -70°C. impairs the activity (Marcu and Dudock, 1974) new batches were prepared at least every three weeks.

ii. Cofactor "mix".

Stock solutions were made up under a laminar flow clean air cabinet, as follows, and stored at -20°C. for up to 6 weeks. Where appropriate solutions were filtered using millipore (0.2 µ) filters at weekly intervals to ensure sterility.

(a) 20 mM. ATP
2 mM. GTP
160 mM. creatine phosphate
40 mM. DTT
} kept as stock in 10 ml.

(b) spermine 1.01 mg./ml.

(c) creatine phosphokinase 4 mg./ml. 50% (V/V) glycerol

(d) 1 M. HEPES pH 7.9 (with KOH)

(e) 100 mM. magnesium acetate

(f) 1 M. potassium chloride

(g) amino acid stock containing 1 mM. of the L-isomers of

alanine
arginine
asparagine
aspartic acid
cysteine
cystine
glutamic acid
glutamine
glycine
histidine
isoleucine
lysine
methionine
proline
serine
threonine
tryptophan
tyrosine
valine

In order to solubilize tryptophan, tyrosine and cystine, the solution was brought to pH 8 with KOH. Stocks were stored in 80 µl. aliquots so that amounts used did not have to go through more than one cycle of freezing and thawing.

The following volumes of stock solution were mixed so that their contribution to the final concentration (i.e., in the 50 µl. assays) would be those figures noted in brackets:-

| | | | Volume mixed. | Concentration in final assay. |
|---|---|---|---|---|
| (a) | stock | ATP | 2.5 µl. | (1 mM.) |
| | | GTP | | (0.1 mM.) |
| | | creatine phosphate | | (8 mM.) |
| | | DTT | | (2 mM.) |
| (b) | spermine | | 2.5 µl. | (0.25 mM.) |
| (c) | creatine phosphokinase | | 1.5 µl. | (120 µg./ml.) |
| (d) | HEPES | | 0.3 µl. | (6 mM.) |
| (f) | potassium chloride | | 1.07 µl. | (21.7 mM.) |
| (g) | amino acids | | 1 µl. | (0.02 mM. each) |
| (h) | water | | 3.13 µl. | |
| (i) | $^{14}$C-leucine | | 3 µl. | (1.5 µCi.) |
| or (j) | $^{14}$C-amino acids | | 3 µl. | (3 µCi.) |
| | | | Total 15 µl. | |

Ribosomal pellets were resuspended in "column buffer" (100 - 200 µl.). 20 µl. were added simultaneously with 15 µl. wheatgerm extract S23 to 15 µl. aliquots of the "mix" in sterilized Durham tubes. These were immediately sealed with plungers from disposable 1 ml. syringes, shaken and then transferred to pre-incubated test tubes (dry inside) and allowed to incubate for 40 min. at 30°C.

The final assay concentrations of those constituents also contributed by the wheatgerm extract and the polyribosome suspension were:-

| | | Contributions from | | |
|---|---|---|---|---|
| | Total. | Wheatgerm | "mix" | ribosome |
| HEPES | 20 mM. | 6 mM. | 6 mM. | 8 mM. |
| magnesium acetate | 2.1 mM. | 0.9 mM. | 0 | 1.2 mM. |
| potassium chloride | 112 mM. | 30.3 mM. | 21.7 mM. | 40 mM. |
| | | | +20 mM. in adjusting HEPES to pH 7.9. | |

Termination of the assay was achieved by pipetting 20 or 40 µl. aliquots on to GF/C discs simultaneously with 200 µg. BSA (in 50 µl.) and immediately plunging the discs into 10% (W/V)

trichloroacetic acid. The discs were then "washed" in several volumes of 5% (W/V) trichloroacetic acid. Removal of charged tRNAs was achieved by heating at 80 - 85°C. in 5% (W/V) trichloroacetic acid for at least 15 min. Finally the discs were washed in ethanol : acetone (1:1, V/V) three times and twice in acetone prior to drying at 60°C. for a few minutes. The discs were transferred to scintillation vial inserts and following addition of 2 ml. scintillation fluid were counted as described previously (Chapter 2, 2 iv.). The scintillation fluids were either

0.5% W/V PPO
10% W/V naphthalene
} in 1,4-dioxan

or Triton X : toluene 1:2 V/V
containing 0.2% PPO.

4. Time course and efficiency of translation.

i. Ribosomes were extracted by method 1 (Chapter 2, 2 vi. a.) at pH 8.5 from 0.5 g. of 24 h. germinated embryonic axes. 90 µg. ribosomal RNA were introduced into a wheatgerm cell-free polypeptide-synthesizing system. (Since estimates for the percentage of mRNA present in polyribosomes are between 1 - 3% (Marcu and Dudock, 1974; Grierson et al., 1976) this represents between 1.1 - 3.3 µg. mRNA which is well within the range of translatable capability of the 50 µl. assay system: for example, Marcu and Dudock reported saturation by addition of 4.5 µg. TMV-RNA; Giles et al. (1977) used 5 µg. poly(A)-containing RNA.) The incorporation above background achieved after 30 min. amounted to a tenfold stimulation.

ii. Polyribosomes from complete leaves of F. pratensis were extracted by method 1 at pH 8.5. 5 µg. were introduced into the wheatgerm cell-free polypeptide-synthesizing system. As in the pea polyribosome-directed system incorporation plateaued at 30 min. These incorporation rates are characteristic of cell-free amino-acid-incorporating systems free from bacterial contamination (Boulter, 1970) (Figure 6, 1)

FIGURE 6.1

Incorporation of $^{3}$H-leucine into hot trichloroacetic acid insoluble material in wheatgerm cell-free polypeptide-synthesizing system as directed by

A. 90 µg. ribososmes from pea embryonic axis

B. 5 µg. ribosomes from F. pratensis.

FIGURE 6.1

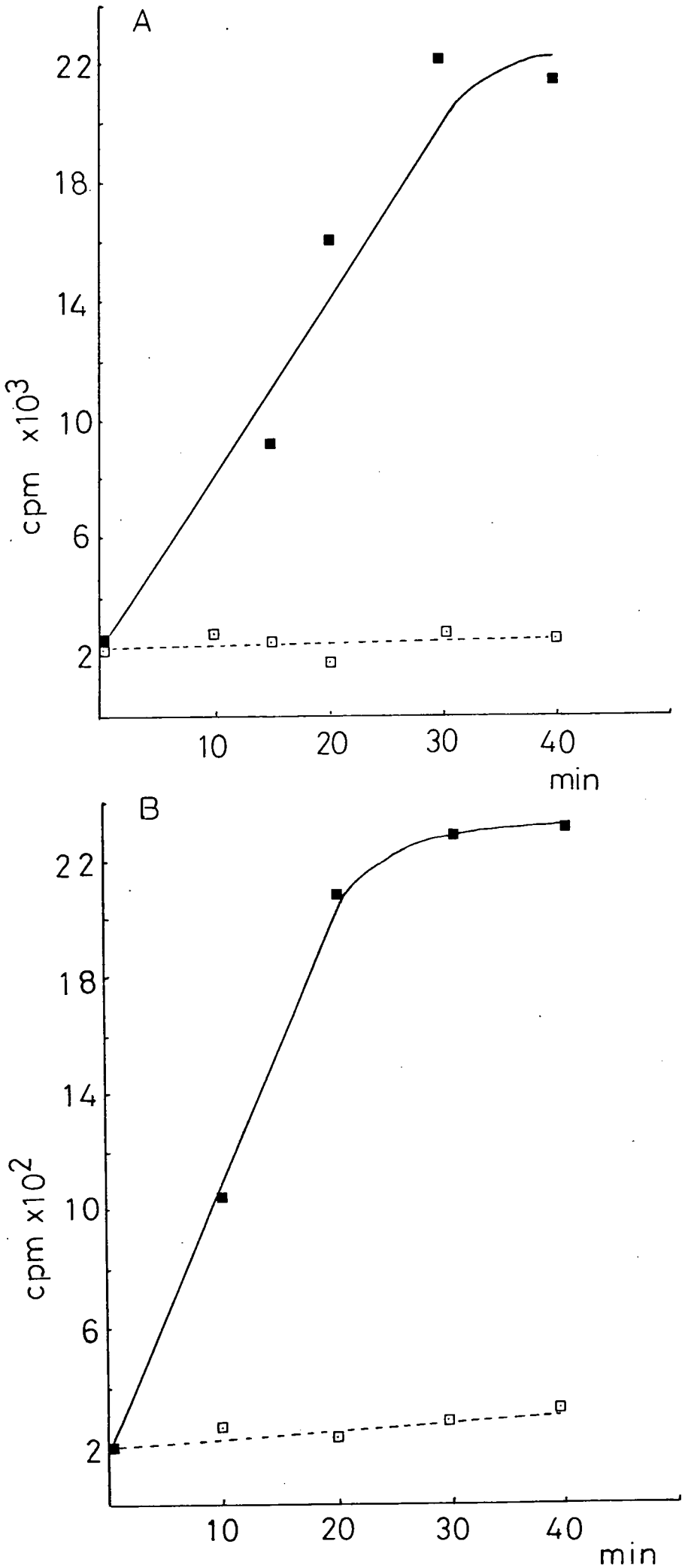

A
22
18
14
10
6
2
cpm x10^3
10
20
30
40
min
B
22
18
14
10
6
2
cpm x10^2
10
20
30
40
min

TABLE 6.1

The requirement for soluble factors derived from the wheatgerm S23 extract. The polypeptide synthesizing system was set up minus the wheatgerm and minus the added ribosomes.

FIGURE 6.2

The relationship between quantity of ribosomes introduced into the wheatgerm cell-free system and the radioisotope incorporated into polypeptides.

TABLE 6.1

| | cpm | stimulation over background |
|---|---|---|
| complete system (18µg) | 393±10 | 6 |
| minus wheatgerm | 75±3 | 1·2 |
| minus ribosomes | 70±3 | 1·0 |
| estimated polypeptide made | 90µg | |

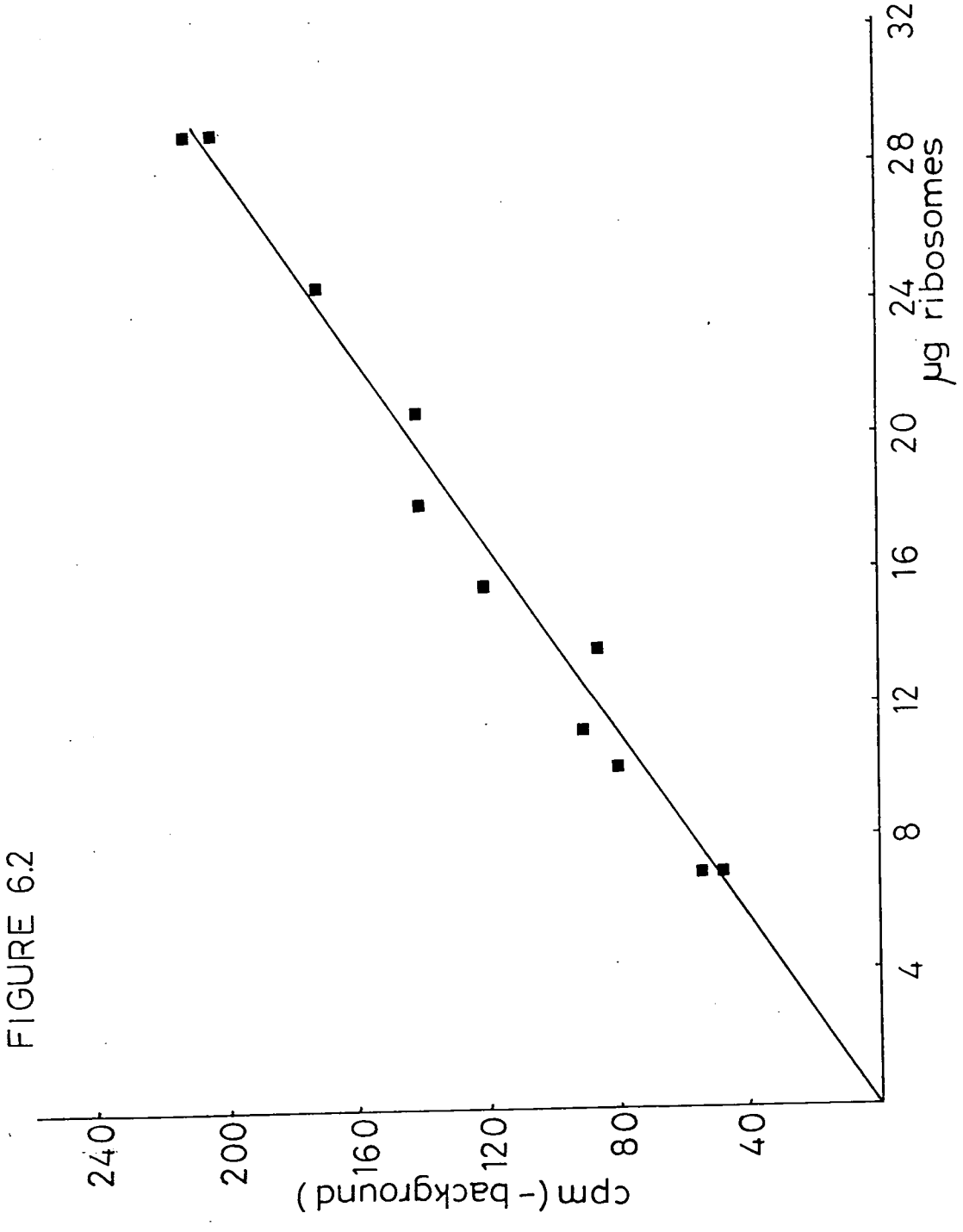



FIGURE 6.2

TABLE 6.2

The effect of pH on the wheatgerm cell-free polypeptide synthesizing system when supplied with polyribosomal RNA from F. pratensis. The complete systems included 2.1 mM. $Mg^{2+}$, 92 mM. $K^{+}$, 0.25 mM. spermine. Incubation was for 30 min. and 30 µg. polyribosomes were introduced into the system.

FIGURE 6.3

The effect of $Mg^{2+}$ concentration on the efficiency of the wheatgerm cell-free polypeptide synthesizing system containing 92 mM. $K^{+}$ at pH 7.4 in the presence ( □----□ ) or absence ( ■——■ ) of 0.25 mM. spermine.

TABLE 6.2

| | cpm | stimulation over background |
|---|---|---|
| complete pH 7·4 | 295 ± 5 | 9·8 |
| minus mRNA | 30 ± 2 | |
| complete pH 6·5 | 70 ± 2 | 3·5 |
| minus mRNA | 20 ± 1 | |
| complete pH 7·9 | 3343 ± 90 | 3·1 |
| minus mRNA | 1090 ± 30 | |

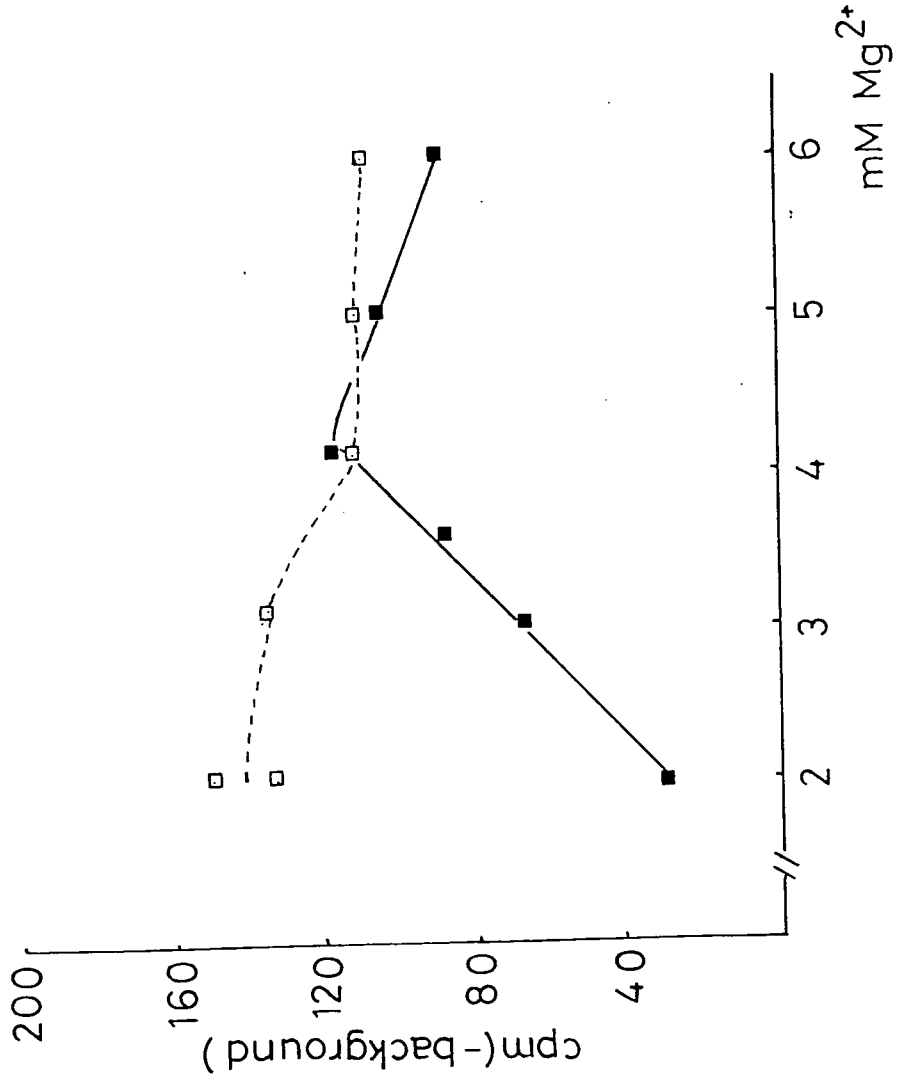



FIGURE 6.3

4. Wheatgerm-minus system.

To confirm the need for the "soluble factors" contributed by the S23 extract in polyribosome-directed polypeptide synthesis, assays were carried out substituting 15 µl. "column buffer" for the wheatgerm extract. Table 6. 1 shows that no incorporation of amino acids occurred in the absence of the wheatgerm extract.

5. Effect of polyribosome concentration.

The incorporation was linearly related to the ribosomes added, as was previously found for polyribosomes of French bean and soybean by Sun et al. (1975) and Beachy et al. (1978) respectively. None of the concentrations appeared to saturate the polypeptide-synthesizing system (Figure 6, 2). Tobin and Klein (1975) demonstrated saturation of the wheatgerm cell-free system on introduction of 5 µg. poly(A)-containing RNA. Regardless of whether this saturation was as a result of exhaustion of ribosomes or soluble components, it would follow that ribosome introduction would not be saturating below 500 µg. This is consistent with the findings of Sun et al. (1975) where saturation was apparent at $A_{260}$ of 7 units (approximately 600 µg.).

6. Optimization of the system.

i. pH.

Marcu and Dudock (1974) noted that pH was critical in the operation of the wheatgerm dell-free system, recording a loss of efficiency of 30% if the pH was raised or lowered 0.3 pH units above or below the optimum of pH 7.4. The observations recorded in Table 6. 2 bear out these findings. The high values for the counts obtained using pH 7.9 result from work carried out at Durham using $^{3}$H-leucine of high specific activity.

ii. $Mg^{2+}$ concentration.

The translation of mRNA is very sensitive to $Mg^{2+}$ concentration but the optimal concentration appears to be species-specific. Dependence on wheatgerm ribosomes for polypeptide synthesis, i.e. by supplying poly(A)-containing RNA or viral RNA, requires

a $Mg^{2+}$ concentration of 2 - 2.5 mM. (Marcu and Dudock, 1974; Tobin and Klein, 1975; Giles et al., 1977; Beachy et al., 1978). However, in the absence of spermidine the optima for French bean polyribosome and soybean ribosome -directed polypeptide syntheses are 6 mM. and 4 mM. respectively (Sun et al., 1975; Beachy et al., 1978, respectively). Beachy et al. (1978) also noted a shift in $Mg^{2+}$ optimum on addition of 0.4 mM. spermidine, from 4.0 mM. to 2.5 mM. Evans et al. (1979) noted an optimum of 3.0 mM. for polyribosomes from pea embryonic axes in the presence of spermidine. In the light of the report by Yarwood et al. (1971) suggesting that p-site attachment requires high $Mg^{2+}$ levels, it is surprising that the $Mg^{2+}$ requirement of a system requiring p-site occupation (i.e., on introduction of mRNA) should be lower than required by a system mostly completing initiated polypeptides (i.e., on introduction of polyribosomes).

The $Mg^{2+}$ optimum for F. pratensis ribosomes was found to be 4 - 5 mM. shifting to 2 - 3 mM. in the presence of 0.25 mM. spermine (Figure 6, 3).

iii. $K^+$ concentration.

$K^+$ concentration is also critical for cell-free polypeptide synthesis but seems to have a broader optimal range. Again, the requirement is species-specific, Poly(A)-containing RNA from Lemna gibba having an optimum of 80 mM. (Tobin and Klein, 1975), TMV-RNA of 90 - 110 mM. (Marcu and Dudock, 1974), soybean poly(A)-containing RNA 105 - 135 mM., soybean polyribosomal RNA 55 - 85 mM. (Beachy et al., 1978) and pea embryonic axis polyribosomal RNA 80 mM. (Evans et al., 1979) which suggests no particular difference in the mRNA and polyribosomal requirements.

The $K^+$ optimum for F. pratensis ribosomes lies in the range 85 - 110 mM. (Figure 6, 4).

FIGURE 6.4

The effect of $K^+$ concentration on the efficiency of the wheatgerm cell-free polypeptide synthesizing system containing 2.1 mM. $Mg^{2+}$, 0.25 mM. spermine at pH 7.4.

FIGURE 6.4

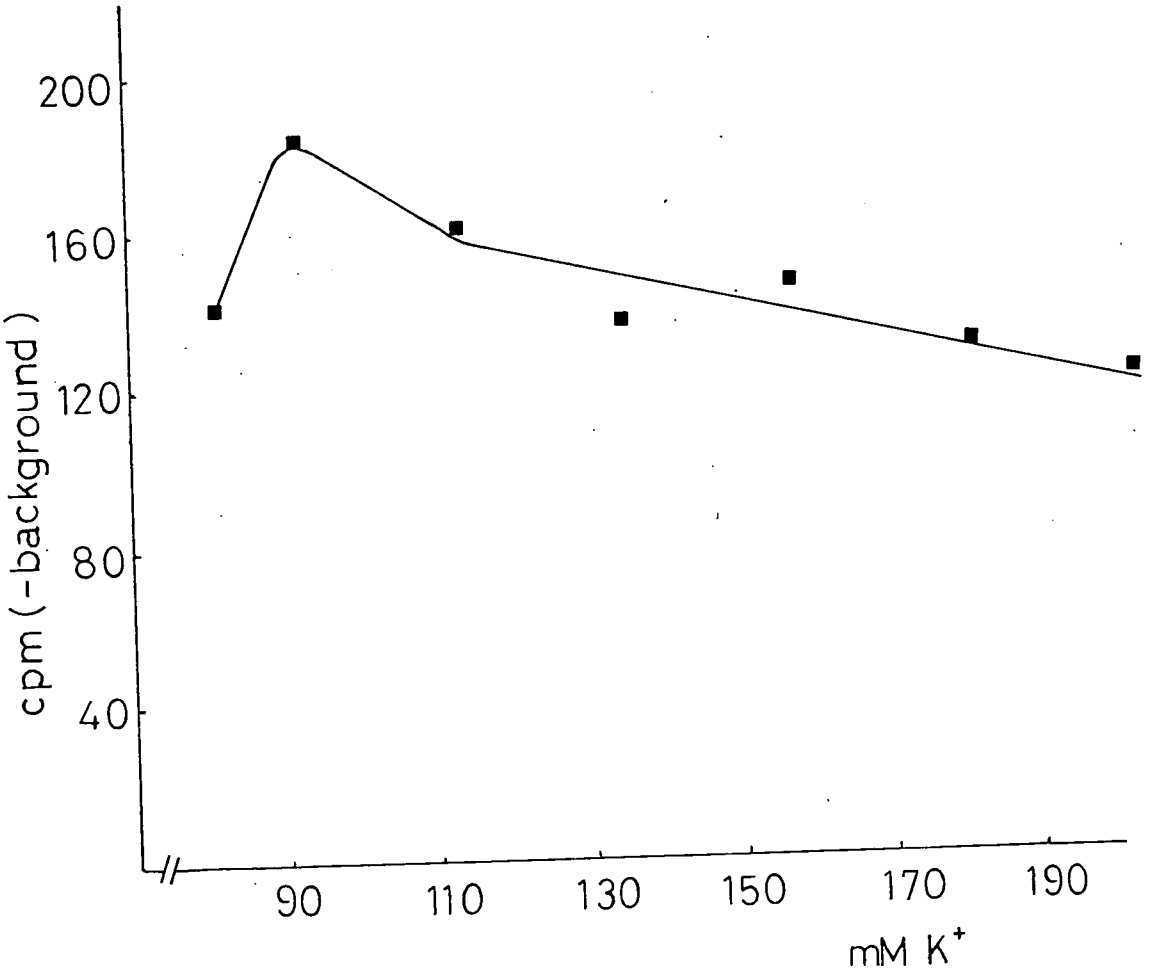


TABLE 6.3

The tRNA charging ability of the wheatgerm cell-free system

a. without ribosomes added,

b. with ribosomes from *F. pratensis* added, and

c. with ribosomes from pea added

after 30 min. incubation under standard conditions.

+cyclohex. refers to the inclusion of 10 µg. of cycloheximide in the incubation.

TCA = trichloroacetic acid.

TABLE 6.3

| | cpm | incorpn over equivt. zero. |
|---|---|---|
| a. minus ribosomes | 2120±20 | 26 |
| " " + cyclohex. | 2225±20 | 25 |
| " " hot TCA insol. | 83±3 | 1·3 |
| " " zero time | 83±3 | — |
| " " + cyclohex. zero time | 110±5 | — |
| " " hot TCA insol. zero time | 65±2 | — |
| b. plus F. pratensis ribosomes | 1550±15 | 25 |
| " " hot TCA insol. | 425±8 | 6·8 |
| % counts unused | 72·6% | — |
| c. plus pea ribosomes | 1424±12 | 28 |
| " " hot TCA insol. | 780±10 | ·16 |
| % counts unused | 45·2% | — |

iv. Creatine phosphokinase concentration.

Creatine phosphokinase is essential to the energy generating capacity of the cell-free system. Originally added at a concentration of 40 µg./ml., several reports indicated the requirement for greater concentrations. Addition of 120 µg./ml. showed very slight enhancement of polypeptide synthesis ( $< 5\%$). However, this was maintained to ensure that energy generation was not limiting.

v. Spermine requirements.

Spermine and spermidine have regularly been used in cell-free systems as they often stimulate amino acid incorporation. Their mode of action is still obscure. A polyamine similar to spermine has been found associated with TMV-virions (Johnson and Markham, 1962) and spermidine has been found to be a normal constituent of some ribosomes, apparently functioning in maintenance of rRNA integrity (Ulbr cht and Szer, 1967). Spermidine has been shown to provide as much as 46% inhibition of RNAse (Payne and Loening, 1970). Marcu and Dudock (1974) reported that spermine-induced stimulation was only apparent in the TMV-RNA directed system and in fact some inhibition was noted on its inclusion in a hen oviduct polyribosomal RNA-directed system.

Addition of spermine to F. pratensis ribosome-directed wheatgerm cell-free system did not enhance amino acid incorporation at its optimal $Mg^{2+}$ concentration (4 mM.) (Figure 6, 3), but it stimulated amino acid incorporation more than fourfold at 2 mM. $Mg^{2+}$.

7. Charging of tRNAs.

In order to eliminate the possibility of polypeptide synthesis being limited by availability of tRNA, controls were simultaneously carried out with routine experiments. These involved recording the incorporated amino acids which had not been solubilized by heating in 5% (W/V) trichloroacetic acid for 15 min. A measure of the charging of undirected tRNAs could be assessed by subtracting the value for hot trichloroacetic acid insoluble incorporation from these control values (Table 6, 3). 10 µg. cyclohexamide were

FIGURE 6.5

The effect of including 0.1 mM. ATA in the wheatgerm cell-free polypeptide-synthesizing system directed by ribosomes from F. pratensis.

▲——▲ plus ribosomes

■---■ plus ribosomes + ATA

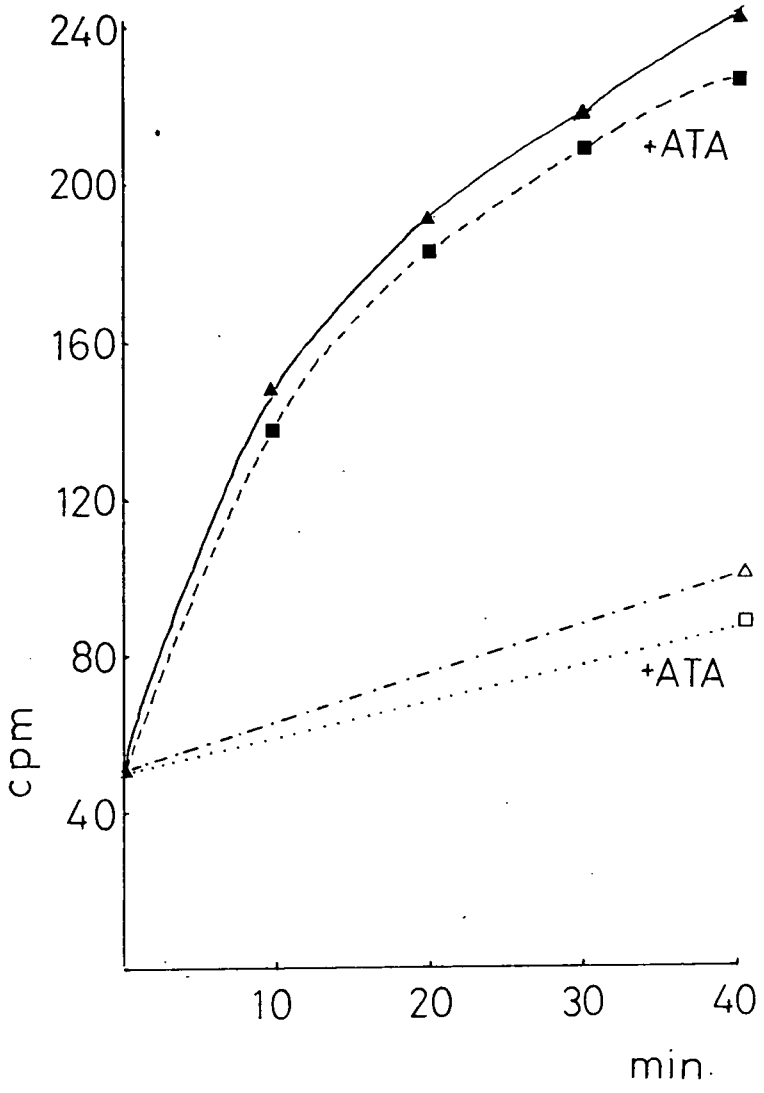
△···△ minus ribosomes, i.e., endogenous

□·····□ Minus ribosomes + ATA.

TABLE 6.4

Results of three separate experiments giving the incorporations of radioactivity into trichloroacetic acid insoluble material in cell-free polypeptide synthesizing systems directed by ribosomes from F. pratensis leaves extracted in the presence or absence of 'Nonidet' or re-extracted in its presence.

FIGURE 6.5




| extraction conditions | 1 | | 2 | | 3 | |
|---|---|---|---|---|---|---|
| | cpm | µg | cpm | µg | cpm | µg |
| plus 'Nonidet' | 515 | 22 | 685 | 27 | 393 | 18 |
| minus 'Nonidet' | 385 | 14·5 | 520 | 18 | 280 | 12 |
| re-extracted plus 'Nonidet' | 130 | 7·3 | 190 | 9 | 110 | 6·5 |

TABLE 6.4

included in a control lacking introduced mRNA. Its presence did not reduce the incorporation into hot trichloroacetic acid soluble material, further implying that these were the result of tRNA charging. Table 6, 3 shows that regardless of the mRNA introduced there was an excess of charged tRNAs available at all times.

8. Effect of ATA in the incubation.

ATA is an inhibitor of initiation events (Marcus et al., 1970) and has been shown to prevent TMV-RNA directed polypeptide synthesis in a wheatgerm cell-free system (Marcus et al., 1970; Beachy et al., 1978). Its effect on polyribosome directed amino acid incorporation has been found to be negligible (Beachy et al., 1978) or mild (Marcus et al., 1970). This presumably confirmed that only nascent polypeptides were being completed in vitro. This is in contrast to the report of an up to 30% stimulation of incorporation by the addition of bean polyribosomes which was attributable to reinitiation (Sun et al., 1975; see Chapter 6.5) but this could reflect the different efficiencies of the initiation inhibitors used. Since translational control mechanisms operating in vivo might be lost in the programming of a cell-free system, the lack of effect of inclusion of ATA also confirms that the synthetic state of the polyribosomes in vivo is maintained in vitro and only those polypeptides already initiated are completed.

2.5 µl. ATA were added to the "mix" to give a final concentration of 0.1 mM., replacing some of the water addition. The time courses for incorporation of amino acids into polypeptides were unaffected by ATA addition, suggesting that the incorporation was a result of completion of nascent polypeptides (Figure 6, 5). From endogenous control data it can be seen that the 6% difference, presumably due to initiation events, could all be attributed to endogenous activity.

9. Effect of "Nonidet" P40 extractior of ribosomes on subsequent in vitro activity.

Ribosomes were prepared from leaves of F. pratensis in the presence and absence of "Nonidet" P40, and subsequently introduced into the cell-free system. The incorporation achieved by ribosomes extracted with "Nonidet" P40 present at the onset equalled that achieved by summation of the incorporations of "Nonidet"-minus extracted and "Nonidet"-plus re-extracted ribosomes (i.e., "free" plus "bound") (Table 6, 4). This would suggest that "Nonidet" does not interfere with in vitro activity. Sun et al. (1975) observed no interference in in vitro activity when bean polyribosomes were isolated in the presence of 0.4% (W/V) "Nonidet" P40 and Beachy et al. (1978) even found extraction in 1% (V/V) Triton X-100 did not have a detrimental effect on subsequent in vitro behaviour.

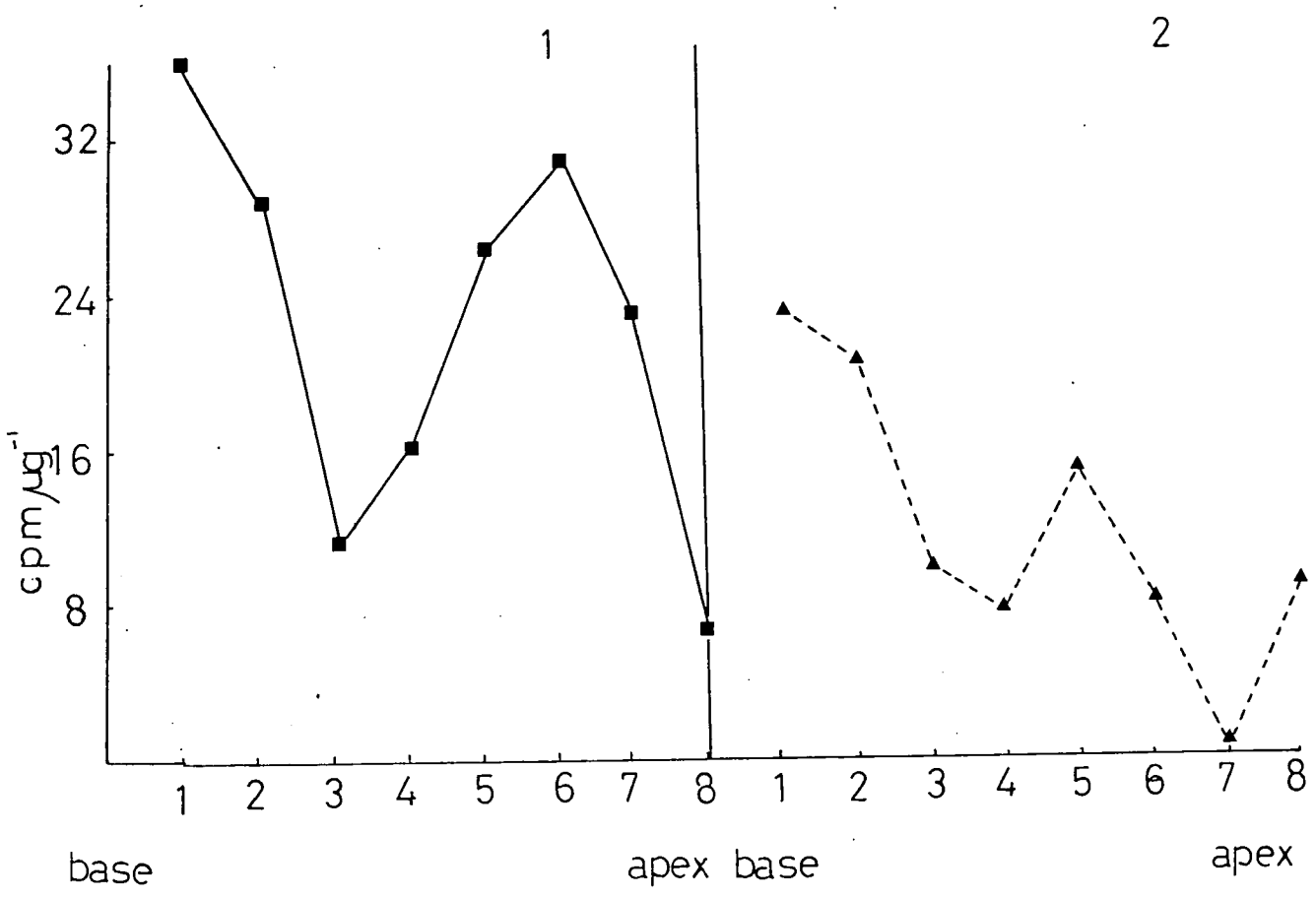
10. Comparison of ribosome capacity along the leaf.

Ribosomes were extracted by methods 1 and 2 (Chapter 2, 2 vi a). The 100,000 g. pellets were re-suspended in wheatgerm "column buffer" and clarified by centrifugation at 13,000 g. for 1.5 min. 20 µl. aliquots were introduced into wheatgerm cell-free systems and were incubated for 40 min. at 30°C. Trichloroacetic acid-precipitated materials were counted and related to the quantity of polyribosomes introduced. Figure 6. 6 shows the results of four such experiments expressed as specific activities, i.e., cpm. incorporated into polypeptide per µg. ribosomes added. The variable results are, once again, probably due to the lack of reproducibility amongst F. pratensis populations and the differences in growth pattern incurred by slight differences in growing conditions. Furthermore, although fully developed leaves were used in all instances, the time following attainment of full maturity was not taken into account. From morphological records made it is feasible to shift the ontogenic sequence of ① back (these leaves were harvested in the winter of 1978: plants were slow growing during this period and therefore slow to senesce; the apical sections of these mature leaves were still 90%

FIGURE 6.6

The synthetic capacity of ribosomes extracted from sections along the mature leaf of *F. pratensis* on introduction into the wheatgerm cell-free system.

FIGURE 6.6


1
2
32
24
16
8
cpm µg⁻¹
1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8
base
apex base
apex

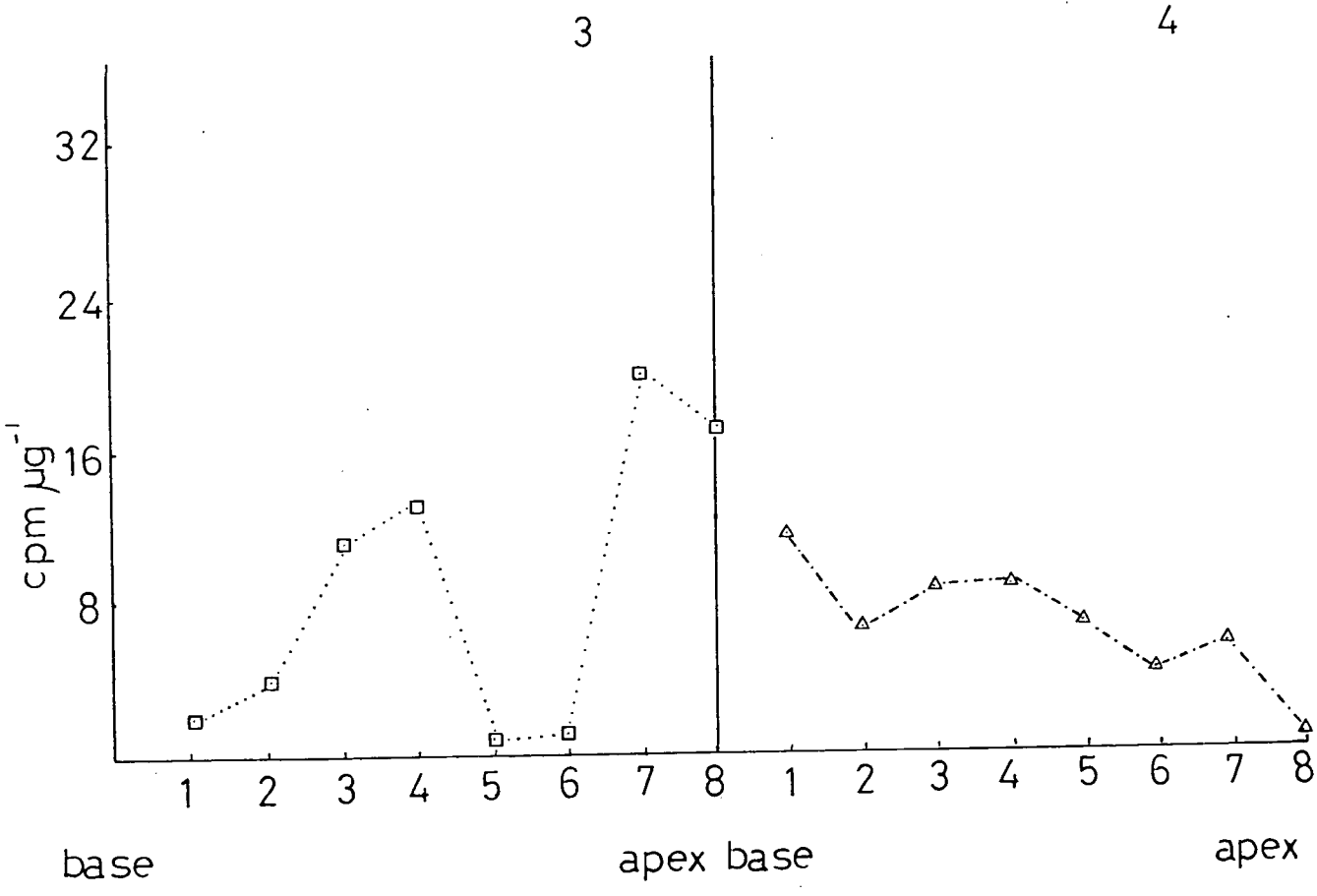

3
4
32
24
16
8
cpm µg⁻¹
1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8
base
apex base
apex

FIGURE 6.7

The synthetic capacities of ribosomes extracted from sections along the leaf of F. pratensis shown in Figure 6.6, re-aligned on the basis of morphological development.

FIGURE 6.8

Shows the synthetic capacities of polyribosomes extracted from sections along the leaf of F. pratensis. µg. polyribosomes were estimated from the ratio of polyribosomes to monoribosomes obtained from 2.2% PAGE gel profiles.

FIGURE 6.7

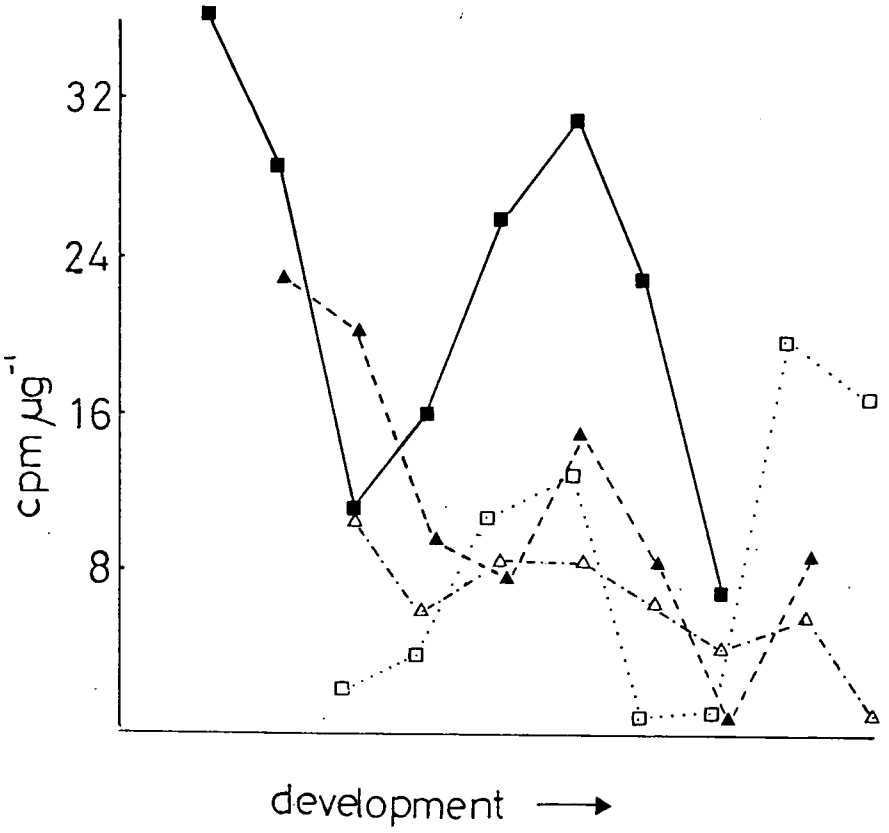
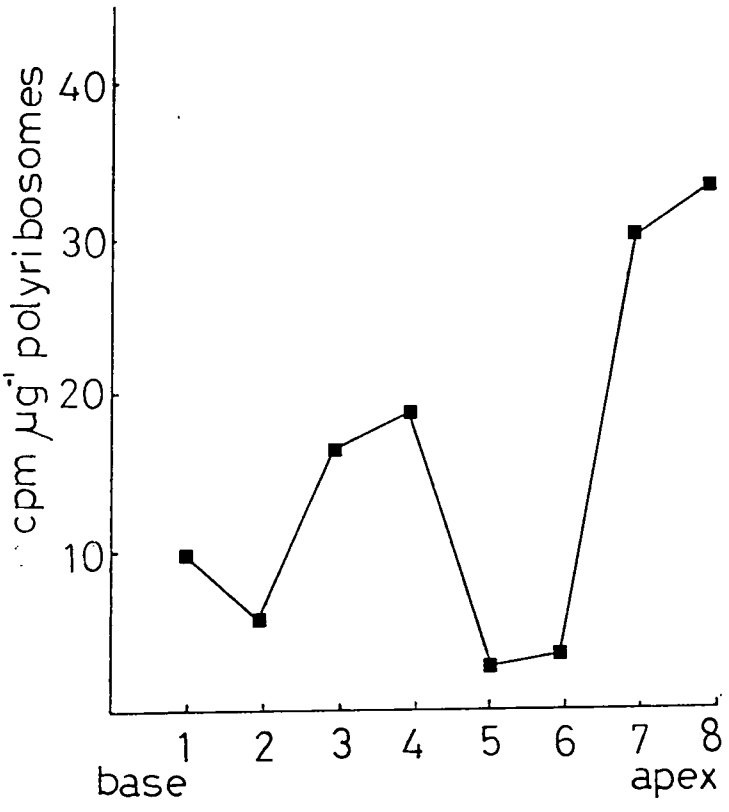



FIGURE 6.8



green), and ③ and ④ can be regarded as having developed one stage further (the apical sections were 90% yellow). These were from summer grown plants, the leaves of which tended to grow to a greater length than winter grown leaves and, by maturity, the apical sections always showed advanced senescence. Regardless of this re-alignment for ontogenic comparison purposes (Figure 6, 7) it can be seen that in each case there are at least two peaks of increased capacity for protein synthesis and depending on the exact stage of development of the leaf these are shifted accordingly along the leaf. Taking the results together, it would seem likely that there are three periods of raised synthetic capacity and these appear to correspond with the phases of increased protein synthesis already noted (Chapter 3). However, because of the variability both seasonal and experimental, these results are necessarily only tentative. The most notable criticism must lie in the low levels of polyribosomes extracted and the low levels of amino acid incorporation which they direct. Furthermore, the percentage of non-functional monoribosomes was not recorded in these instances. Figure 6. 8 shows one experiment where the specific activities were corrected for polyribosomes alone. This must necessarily provide a better indicator of polypeptide synthetic capacity but requires very much increased yields of ribosomes for simultaneous PAGE or sucrose density gradient analysis. The problems associated with trying to increase the yield of ribosomes from F. pratensis leaf tissues have already been outlined (Chapter 2, 2 vi.), but until this is achieved and the precise timing of leaf development recorded the results must remain speculative albeit consistent with the proposed course of leaf development. Lin et al. (1973) observed a shift in $Mg^{2+}$ requirement for amino acid incorporation into polypeptides as directed by polyribosomes from young and old corn leaves. The higher $Mg^{2+}$ requirement for ribosomes from old tissue was interpreted as resulting from increased amounts of unoccupied p-sites. This implies that a consideration of the $Mg^{2+}$ optima not only in the cell-free protein-synthesizing system but also in the ribosome

extraction may be critical in comparison of the synthetic capacity of polyribosomes from different aged tissue. For unequivocal comparison, full assessment of these possibilities would have to be made.

11. Comparison of polypeptide-synthesizing capacity of "membrane-bound" and "free" ribosomes.

Ribosomes were extracted according to method 2 (Chapter 2, 2 vi a) without detergent. The 10,000 g. pellets were re-extracted with equal volumes of buffer containing 0.4% "Nonidet" P40. The first and second supernatants were regarded as containing "free" and "membrane-bound" ribosomes respectively and subsequent extractions were as usual. 20 µl. aliquots of resuspended ribosome pellets were introduced into the wheatgerm cell-free system. Figure 6. 9 indicates quite different capacities for these two classes of ribosome. The "membrane-bound" ribosomes appear to be more active in polypeptide synthesis at senescence although it must be remembered that the "free" ribosome population is much inflated by monoribosomes derived from degradation of polyribosomes and, since these will be inactive, the specific activity will be artificially low this time (Chapter 2, Figure 30). The membrane-bound nature of the polypeptide-synthesizing polyribosomes at senescence may be designed to ensure protection, both of the polyribosomes and products, from the degradative enzymes in abundance at this stage. Alternatively they may be so located as to release the products, which may be degradative enzymes, in a compartmentalized fashion.

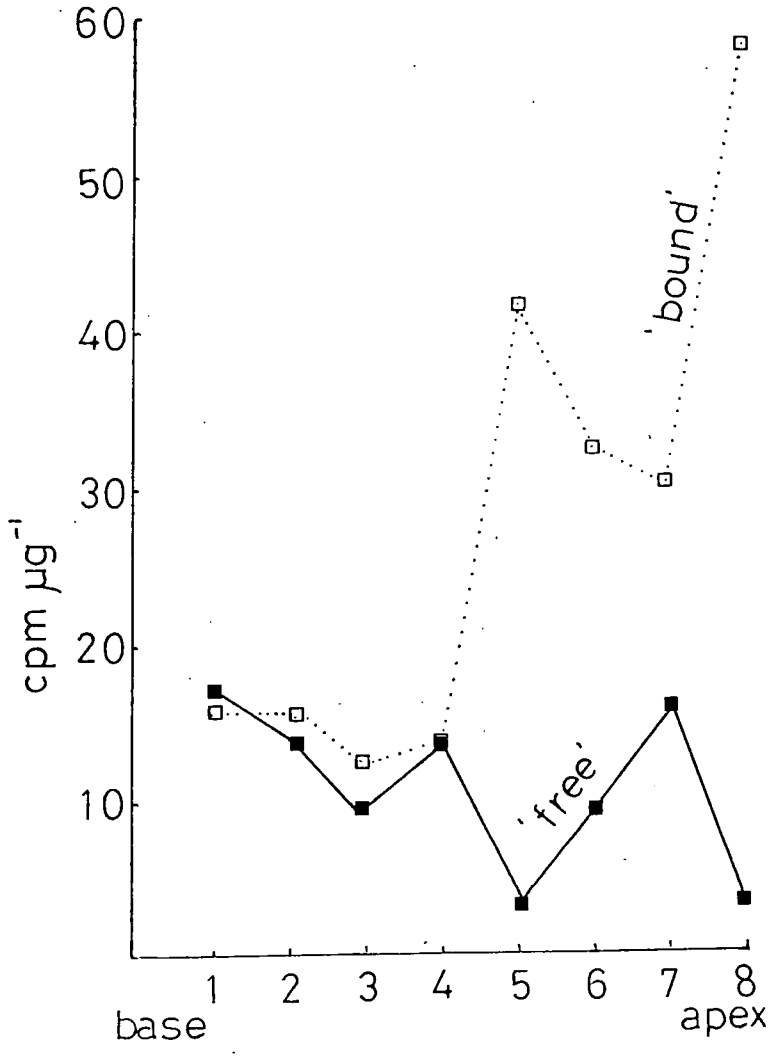
12. Summary.

In a general way, the periods when there is high ribosomal synthetic activity (Chapter 2, Figures 20, 26) are consistent with periods of increased polypeptide synthetic ability (Figure 6. 8), i.e., at points 4 and 7 along the leaf. These are also points where there is a high "free" to "bound" ratio amongst the ribosome populations (Chapter 2, Figure 30). Since there is a high percentage of monoribosomes at point 4 (Chapter 2, Figure 31) the actual

FIGURE 6.9

The synthetic capacities (expressed as cpm. $\mu g^{-1}$) of the 'free' and 'membrane-bound' populations of ribosomes extracted from sections along the leaf of *F. pratensis*.

FIGURE 6.9



specific activity of synthetic capacity of free ribosomes may be much greater than implied in Figure 6. 9. It therefore further suggests that point 4 represents a period of high turnover and high synthetic activity of both RNA and polypeptides which is further confirmed by the high incorporation of radio-isotope label into soluble proteins in vivo (Chapter 3, Figure 6 ). The high amounts of $0.38 \times 10^5$ rRNA, which are supposed to be associated with active ribosomes, correspond, to some degree, with the periods of increased synthetic capacity (Chapter 2, Figure 25). Furthermore, high poly(A)-containing mRNA syntheses also coincide with the high activity of polyribosomes at points 4 and 7 (Chapter 2, Figure 35). Although it may be argued that the high ribosome content at points 2 and 6 (Chapter 2, Figure 29) may be the cause of the apparently lowered synthetic capacities, these do not correspond to periods of high monoribosome percentages (Chapter 2, Figure 31) and the actual values obtained do not support this suggestion since the levels of incorporation were by no means constant.

The empirical evidence would suggest that there are at least two areas of high metabolic activity, points 4 and 7, and probably a third at the base of the leaf.

## General Discussion.

It is clear that the course of development of leaves of F. pratensis results from highly involved metabolic correlations and no one factor can be exclusively regarded as having primary control. There is a constant overall pattern in their development, presumably of genetic origin, which is modified and adapted by prevalent cellular and extracellular conditions. The repetition of the three phases of increased metabolic activity with ageing throughout this work would suggest that these are symptomatic of an underlying genetic programme. However, the timing and magnitude of these phases is variable (for example, the variations due to seasonal growth differences amongst the plant material).

The early phase of increased activity has been well documented and results from the high metabolic activity associated with cell division and exposure to light. The phase of increased activity associated with senescence has also received much attention, particularly in conjunction with the debate as to its positive contribution to plant development. The results obtained here serve to confirm both these phases and, in addition, have indicated that ageing along the axis of a mature leaf can be used, with some reservations, as a mirror for whole leaf ageing with time. Full maturity was reached by day 49 and this may be correlated with point 6 along the leaf using a large number of different parameters. At these points metabolic activity is switched to catabolism which has been shown to necessarily involve the synthesis of new protein synthetic machinery, increased activities of catabolic enzymes and even the appearance of novel isoenzymes. Having achieved the new cellular emphasis, the protein synthetic machinery itself comes under the degradative hammer. Thus, nucleotides and amino acids accumulate briefly prior to translocation and redeployment and all but purely degradative enzymes decline in activity. The death of the leaf is programmed

to result in the mimimum loss of resources.

The phase of increased activity midway in development has not received the same attention as these other two phases and may well turn out to be critical in the timing of subsequent events. Although evidence would suggest that such a phase is present in whole leaf development it was more easily pinpointed and examined in development along the leaf. Point 4 sees an increase in nucleic acid synthesis (Figures 2.20, 2.26), maximal ribosome synthesis (Figure 2.32), an increase in soluble protein synthesis (Figure 3.6), a high 'free':'membrane bound' ratio of ribosomes (Figure 2.30), high ribosome polypeptide-synthesizing capacity (Figure 6.8) and high polyadenylation of mRNA (Figure 2.35). The polyribosome proportion of ribosomes increases compared to monoribosomes subsequently (Figure 2.31) as does the $0.36X10^{6}$ rRNA content (Figure 2.25). There is an increase in ribosomal breakdown products (Figures 2.23, 2.24) and a high preferential incorporation of radioisotopically-labelled precursor into monoribosomes rather than polyribosomes (Figure 2.33). All the evidence would suggest that this is a period of rapid turnover and replacement of cellular constituents associated with early growth.

A number of possibilities for further investigation arise from this preliminary work. It would be profitable to work to increase the yield of ribosomes obtainable from portions along the leaf of F. pratensis such that sufficient radioisotopically-labelled amino acids may be incorporated into products of cell-free polypeptide-synthesizing systems directed by them. In this way particular products may be identified electrophoretically or, more conclusively, by immunochemistry. It would also be interesting to compare the products of cell-free polypeptide-synthesizing systems directed by different size classes of polyribosome. This would be feasible even with relatively low yields of ribosome following electrophoresis and direct use of eluted slices of acrylamide gel

since it has been shown that acrylamide does not poison the cell free system (Chapter 2. 2 ix. d.).

An interesting comparison may be obtained from introducing ribosomes from different aged whole leaves into a cell-free polypeptide-synthesizing system.

An alternative approach to the investigation of the timing of particular enzyme syntheses would involve the positive identification of protein fractions labelled *in vivo*. This would require refinement of the electrophoretic identification procedure and the application of this to complete or partially purified radioisotopically-labelled protein extracts.

Figures GD 1 and 2 summarize some of the major changes in metabolic activity associated with development.

FIGURE GD 1.

Periods of high metabolic activity (shaded) during the development of the complete 4th. leaf of F. pratensis.

A. Total nucleic acid synthesis.
B. Cytoplasmic RNA synthesis.
C. Chloroplast RNA synthesis.
D. Soluble protein synthesis.
E. Particulate protein synthesis.
F. Relative synthesis soluble 57,000 MW protein.
G. Relative synthesis of soluble 18,000 MW protein.
H. Relative synthesis of soluble 15,000 MW protein.
I. Relative synthesis of soluble 20,000 MW protein.
J. Relative synthesis of particulate 37,000 MW protein.

Enzyme activities:-

K. RNAse.
L. Acid pyrophosphatase.
M. Alkaline pyrophosphatase.
N. ATPase.
O. Alkaline phosphatase.
P. Phosphodiesterase.
Q. Chlorophyll level.

FIGURE GD.1

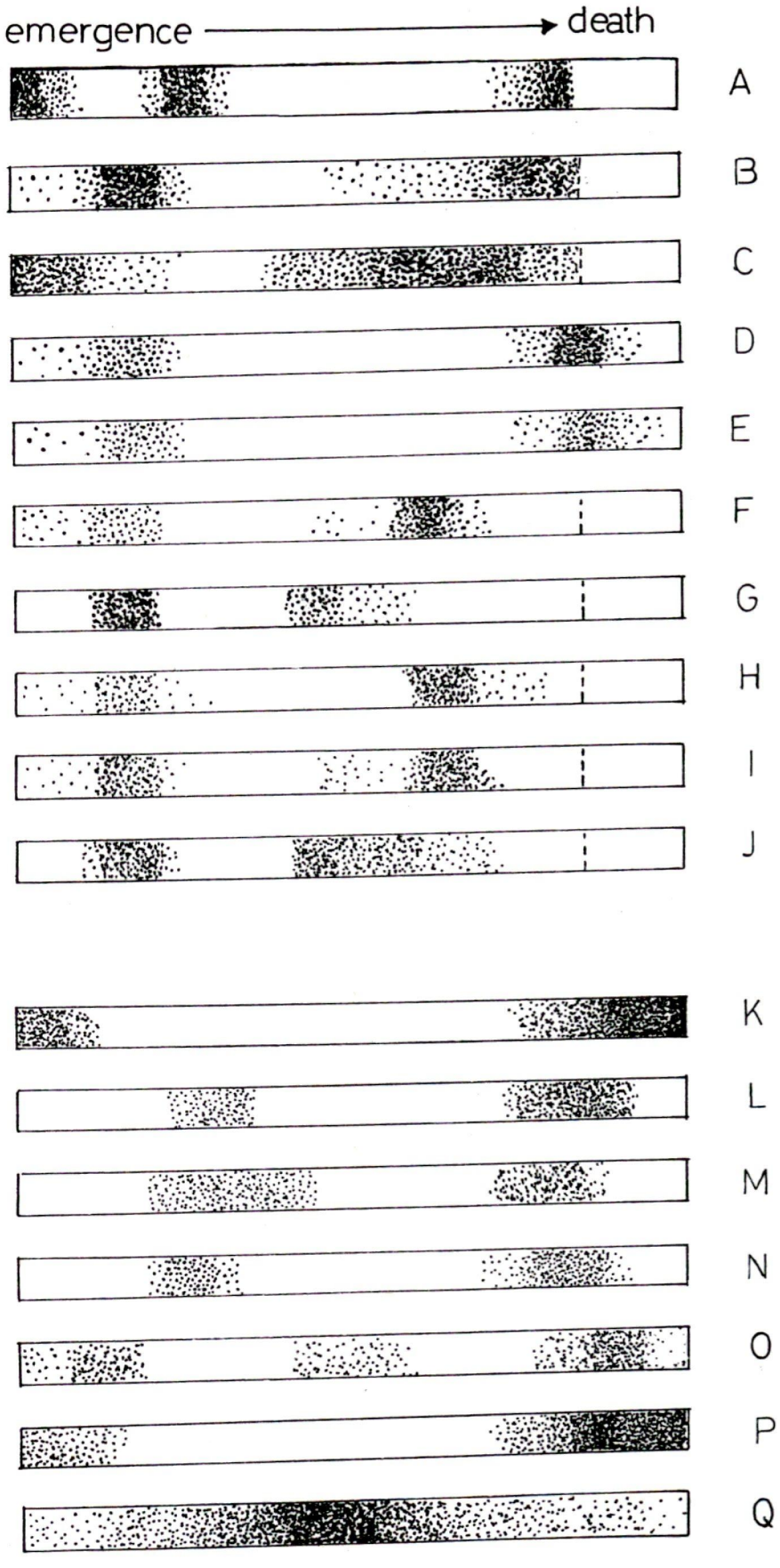


FIGURE GD 2.

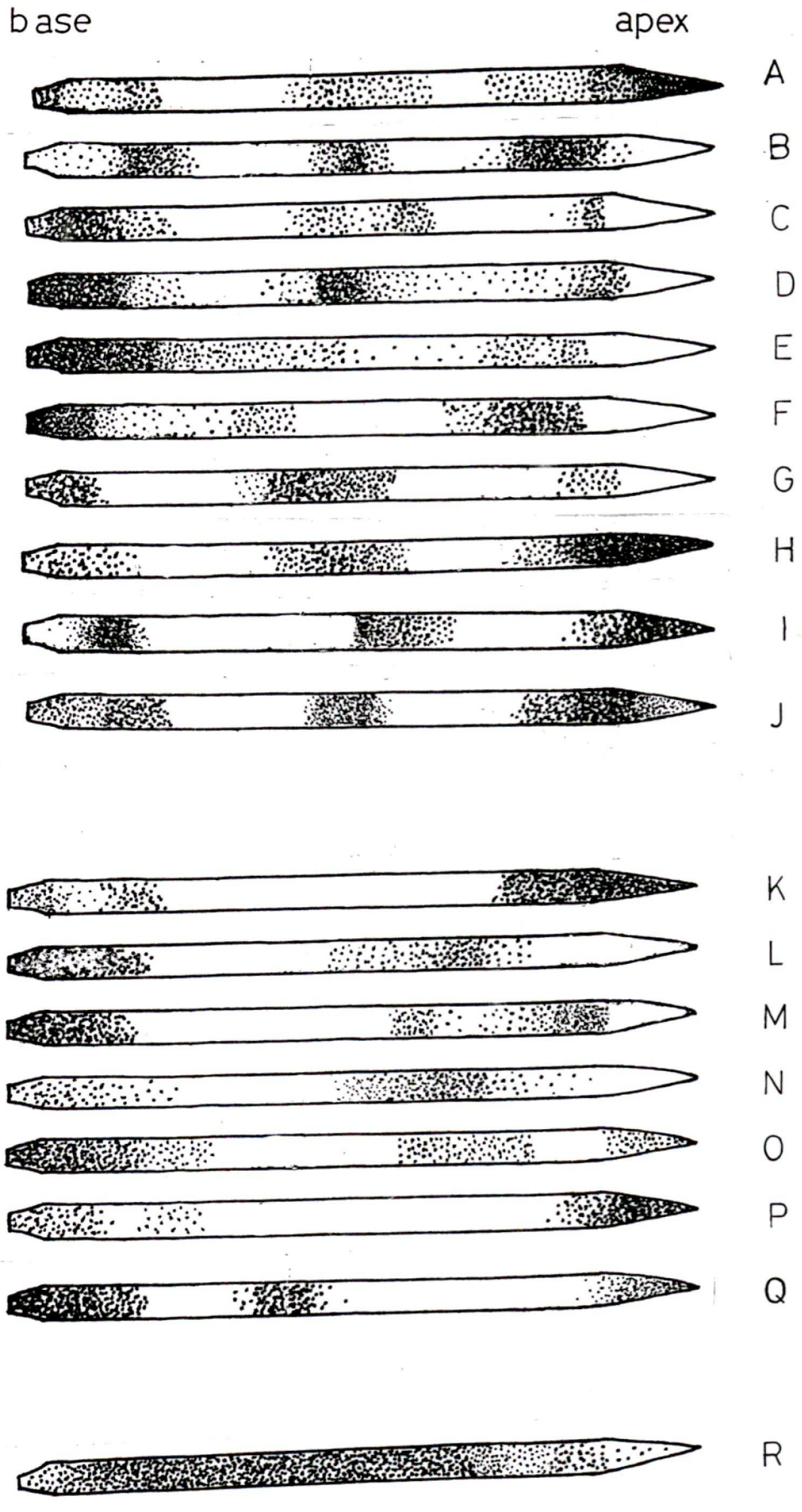
Areas of high metabolic activity (shaded) during the development along the mature leaf of F. pratensis.

A. Total nucleic acid synthesis.
B. Cytoplasmic RNA synthesis.
C. Chloroplast RNA synthesis.
D. Soluble protein synthesis.
E. Particulate protein synthesis.
F. Polyribosome : monoribosome ratio.
G. Poly(A)-containing RNA synthesis.
H. Free : membrane bound ribosome ratio.
I. Polyribosome : monoribosome synthesis ratio.
J. Protein synthetic capacity of ribosomes.

Enzyme activities:-

K. RNAse.
L. Acid pyrophosphatase
M. Alkaline pyrophosphatase
N. ATPase.
O. Alkaline phosphatase.
P. Phosphodiesterase.
Q. Acid phosphatase.
R. Chlorophyll level.

FIGURE GD.2



## SUMMARY


1. Chapter 1, III i.

Changes in leaf length and fresh weight have classic growth curve characteristics. A fall in fresh weight is apparent between day 29 and day 49 presumably due to vacuolation. A decrease due to senescence occurs after day 49 when the leaf has reached full length.

2. Chapter 1, III ii.

Maximum chlorophyll content occurs at day 42. Loss of chlorophyll due to senescence occurs beyond day 49.

Maximum chlorophyll content occurs at point 5 along the leaf and degradation occurs beyond point 6.

3. Chapter 1, III iii.

Fibre content was subjectively assessed at a maximum at day 57 and point 7 along the leaf.

4. Chapter 2, 2 ii. f.

Extraction of nucleic acids using DEP increases the yield (by at least 50%) over that obtained using traditional phenol methods. It also results in improved subsequent electrophoretic separation of rRNAs.

5. Chapter 2. 2 iii. c.

$Mg^{2+}$ must be present in the nucleic acid axtraction buffer in order to maintain the integrity of the $1.1x10^6$ rRNA. If the $Mg^{2+}$ concentration is sufficiently high ( $\geqslant$ 10 mM.) during extraction its presence during subsequent electrophoresis is not vital particularly if this is carried out at $4^{o}C$.

6. Chapter 2. 2 iii d.

3 mM. EDTA or 5 mM. $Mg^{2+}$ electrophoresis buffers give the best resolution in electrophoretic separation of RNAs. 2h. 30min. electrophoresis time is optimal and lowered temperatures ($4^{o}C$.) provide the best conditions for maintenance of the $1.1x10^6$ rRNA.

7. Chapter 2. 2 vi. a.

Liquid $N_2$ is the most effective homogenization procedure for leaves of F. pratensis.

8. Chapter 2. 2 vi. b.

The most suitable extraction buffers for polyribosome extraction contain high $K^+$:$Mg^{2+}$ ratio at pH 8.5.

9. Chapter 2. 3 i. a.

RNA content of the whole leaf of F. pratensis increases with age to a maximum at day 35. Decline due to senescence is apparent after day 49.

RNA content of portions along the leaf is maximal at point 5. Senescent breakdown occurs beyond point 6.

10. Chapter 2. 3 i. b.

Three peaks of increased total nucleic acid synthesis are observed in both developmental systems. These occur at day 24, day 35 and day 56, points 1, 3-4 and 7-8.

11. Chapter 2. 3 i. c.

Cytoplasmic rRNAs decline with age beyond day 42 with a concomitant increase in breakdown products.

Cytoplasmic rRNAs decline beyond point 3. At points 5 and 7 synthesis increases with concomitant reduction in appearance of breakdown products.

Chloroplastic rRNAs decline more rapidly than cytoplasmic rRNAs in both developmental systems. The increases in $0.56 \times 10^6$ rRNA may reflect enhancement by breakdown products.

12. Chapter 2. 3 i. d.

The early maximal syntheses of chloroplastic rRNAs (point 1 and day 24) precede the early maximal syntheses of cytoplasmic rRNAs (point 2 and day 24) in both developmental systems.

Syntheses of $1.3 \times 10^6$ and $0.7 \times 10^6$ rRNAs are closely linked but the syntheses of the $1.1 \times 10^6$ and $0.56 \times 10^6$ rRNAs do not correlate suggesting that, unlike the cytoplasmic rRNA subunits, chloroplastic rRNA subunits are not derived from the same high molecular weight precursor.

There is considerable synthesis of all rRNA fractions at senescence (day 57 and point 7).

13. Chapter 2. 3 iii. a.

Ribosome content was maximal at two points along the leaf corresponding to the two points of maximal total nucleic acid content (point 2 and points 5-6).

The ratio of free to membrane-bound ribosomes increases at senescence and also at point 4. The ratio of polyribosomes to monoribosomes is high at three periods in development along the leaf, at points 1, 3 and 6-7. At point 4 there is a drop in this ratio.

14. Chapter 2. 3 iii. b.

Ribosome synthesis is high at point 4 along the leaf and a dramatic increase in synthesis occurs at senescence, points 7-8. At both points radioisotopically-labelled precursors enter the monoribosome fraction in preference to the polyribosome fraction. Periods of apparent increased polyribosome assembly occur at points 2, 5 and 8.

15. Chapter 2. 3 iv.

Elevated periods of polyadenylation of mRNA occur at points 1, 4 and 7 along the leaf.

16. Chapter 3. III. i.

Soluble and particulate protein increases to a maximum at day 49 beyond which senescent decline ensues.

Soluble and particulate protein content also increases along the leaf, declining with senescence beyond point 6.

17. Chapter 3. III. ii.

Both developmental systems show high rates of soluble protein synthesis at early and senescent stages of development (points 1 and 7, day 24-29 and day 62). Ageing along the leaf shows a decline in particulate protein synthesis from early development and only a slight increase at point 6, whereas particulate protein synthesis in temporal ageing shows a direct correlation with soluble protein synthesis.

In addition, ageing along the leaf shows a third phase of increased soluble protein synthesis at point 4.

18. Chapter 3. III. v.

The relative syntheses of four major soluble proteins and three major particulate proteins display essentially two patterns during development of the whole leaf. The soluble proteins (57,000 MW, 20,000 MW and 15,000MW) show an early and late phase of increased relative synthesis (day 29 and day 49).. The particulate proteins (37,000 MW, 15,000 MW and 12,000 MW) and one soluble protein (18,000 MW) show early and mid-development synthetic phases (day 29 and day 42).

19. Chapter 4. II.

Optimal conditions for assay of ATPase, acid and alkaline RNAse, acid and alkaline pyrophosphatase, acid and alkaline phosphatase and acid and alkaline phosphodiesterase are recorded.

20. Chapter 4. III. i.

ATPase and alkaline and acid pyrophosphatases show similar patterns of activity in whole leaf ageing, displaying an early

peak (day 35-42) and a late peak (day 55-62) of activity.

Alkaline pyrophosphatase (to which an anabolic function is attributed) activity is four times that for acid pyrophosphatase (to which a catabolic function is attributed) during the early peak, but this drops by half in the later peak.

All three enzymes show high activity at the base of the leaf and increased activity at points 5-6. The ratio of alkaline pyrophosphatase to acid pyrophosphatase does not decline at senescence as observed for whole leaf ageing.

RNAses and phosphodiesterases follow similar patterns in both developmental systems. All show a large increase in activity at senescence and an increase at early development. Acid RNAse is approximately 30% more active than alkaline RNAse at all phases until senescence when the activities are similar.

Alkaline phosphatase shows three peaks of increased activity at early development (point 2-3, day 24-29), mid-development (Point 5-6, day 40-49) and at senescence (point 8, day 56-59). Acid phosphatase displays a slightly earlier mid-peak (point 3-4, day 35-40).

21. Chapter 4. III. ii.

Acid phosphatase shows a shift in emphasis of isoenzymes with development. A novel isoenzyme of high molecular weight appears at incipient senescence (point 6).

22. Chapter 4. III. iii.

All the enzymes associated with phosphorus metabolism investigated show major activity associated with the soluble fraction. ATPase has some association with membranes. The acid

and alkaline isoenzymes of phosphodiesterase and the acid and alkaline isoenzymes of pyrophosphatase are not similarly located in the cell.

23. Chapter 5. II. 2 i.

There are no qualitative differences in the rRNA complement of light-treated and dark grown shoots of F. pratensis. RNA content drops (by 10-12%) as a result of light treatment. Light enhances the level of chloroplast rRNAs and the ratio of large to small ribosomal subunits becomes closer to that for functional ribosomes.

Dark grown shoots contain higher amounts of tRNA.

Presumptive chloroplast tRNA appears as a result of exposure to light.

Ribosomes increase on exposure to light. After 48h. the level reverts to pre-light treatment amounts. The polypeptide synthetic capacity of the ribosomes declines slightly with prolonged light treatment.

24. Chapter 5. II. 2 ii.

Synthesis of $1.1 \times 10^6$ rRNA is enhanced by light but synthesis also occurs in the dark.

25. Chapter 5. II. 3.

Soluble protein increases as a result of light treatment.

26. Chapter 5. II. 4.

Two patterns of response of enzymes of phosphorus metabolism to light are apparent. Type 1, exemplified by acid RNAse, acid Phosphodiesterase and alkaline pyrophosphatase, show an immediate increase in activity (2h.) followed by a decrease which is followed by an increase at 24h.

ATPase, alkaline phosphodiesterase and acid pyrophosphatase show high activity at 12h. Acid phosphatase, acid and alkaline RNAses and, debatably, alkaline pyrophosphatase continue to show an increase in activity after 70h.

27. Chapter 5. III. 2.

Ribosome content decreases following excision of the leaf after an initial increase over the first 12h. Their polypeptide-synthesizing capacity also decreases with a small increase at senescence (96h.)

28. Chapter 5. III. 3.

Soluble protein declines following excision after an initial increase over the first 12h.

29. Chapter 5. III. 4.

Most of the enzymes of phosphorus metabolism investigated show an overall increase in activity following excision with a decline only apparent beyond 96-108h. The increase in acid RNAse and ATPase at 108h. immediately follows the period of increased ribosome polypeptide-synthesizing capacity.

30. Chapter 6. 10.

Three phases of increased polypeptide-synthesizing capacity are evident in development along the leaf, at early development (point 1), mid-development (point 4-5) and at senescence (point 7-8).

31. Chapter 6. 11.

The polypeptide-synthesizing capacities of 'membrane-bound' and 'free' ribosomes differ, particularly at later stages. At points 5 and 8, 'membrane-bound' ribosomes are potentially more active than 'free' ribosomes. The 'free' ribosome population may be inflated at these points due to monoribosomes so that specific activities may be artificially low.

## APPENDIX A.

## Purification of RNAse.

I. Introduction.

II. Methods

a. Affinity chromatography procedure for RNAse purification.

b. Sephadex G-100 chromatographic procedure for RNAse purification.

III. Summary.

### I. Introduction.

Active RNAse is present at high levels in senescent leaves of F. pratensis (Figure 4.26 and Figure 5.10) and, by virtue of its specific and central role, investigation of its metabolism would provide an interesting probe into the senescence phenomenon. It is one of the few enzymes which has been shown to be synthesized at this stage (Thomas, 1975; Baumgartner et al., 1975; Sacher and Davies, 1974). There have been conflicting reports concerning the relationship between RNAse activity and RNA content (for example, Kessler and Engelberg, 1962; Phillips and Fletcher, 1969; Phillips et al., 1969) and there have been several reports implicating specific RNAses in the age-related decline in RNA (Srivastava, 1968b; Hadziyev et al., 1969). Eilam et al. (1971) proposed that only the later stages of the decline in RNA were correlated with RNAse levels, earlier stages arising by other means. The speculative nature of all these reports gave credence to the value of isolating and purifying particular RNAse isoenzymes in order to raise specific antibodies for immunochemical identification both in situ (intracellular location) and of products of F. pratensis ribosome-directed cell-free polypeptide-synthesizing systems.

Cellular location work would provide evidence of compartmentation which may be significant in the progress of development. The subtleties of compartmentation would tend to be masked by the usual rigorous extraction methods.

The following attempts were ultimately unsuccessful but important additional information was obtained as a result which is worthy of reporting here.

## II. Methods.

### a. Affinity chromatography.

The method of Baumgartner et al. (1975) for affinity chromatography as an efficient and fast purification of RNAse was adopted for F. pratensis. 3 g. cyanogen bromide-activated Sepharose 4B were allowed to swell by washing with a total of 600 ml. 1mM. HCl in a Buchner funnel. The swollen gel was mixed with a 5 mg./ml. solution of ADP in 100 mM sodium carbonate and 500 mM. sodium chloride buffer at pH 5. The solution was shaken gently at 4°C. for 48 h. The degree of coupling was measured by spectrophotometric measurement of the ADP solution at $A_{260}$ before and after the coupling period which amounted to 1.36 mg. ADP/ml. gel. The substituted gel was washed with carbonate buffer and then with ethanolamine for 2 h. at room temperature. Finally, the gel was washed alternately with carbonate buffer and 100 mM. sodium acetate with 500 mM. sodium chloride buffer at pH 4. The gel was then stored at 4°C. under carbonate buffer containing sodium cyanide (0.2% W/V).

20 g. mixed-aged leaves of F. pratensis were ground following freezing in liquid nitrogen, using a pestle and mortar. 75 ml. 20 mM. sodium acetate buffer (pH 5) were added together with 0.5 g. PVP. Following centrifugation at 16,000 g. for 15 min. the supernatant was heated at 55°C. for 1 hour. Denatured protein was removed by further centrifugation at 16,000 g. for 15 min. 42 g. ammonium sulphate were added to the supernatant which was stirred briskly for 1 h. at 4°C. The precipitate was collected by centrifugation at 16,000 g. for 15 min. and resuspended in 10 ml. 3 mM. sodium acetate

Figure A.1

Purification of RNAse by affinity chromatography. ADP-Sepharose elutions are those referred to in the text.

i.e., I. 3mM. sodium acetate.

II. 20mM. sodium acetate

III. 200mM. sodium acetate + 200mM. sodium chloride.

IV. 200mM. sodium acetate + 1.5M. sodium chloride.

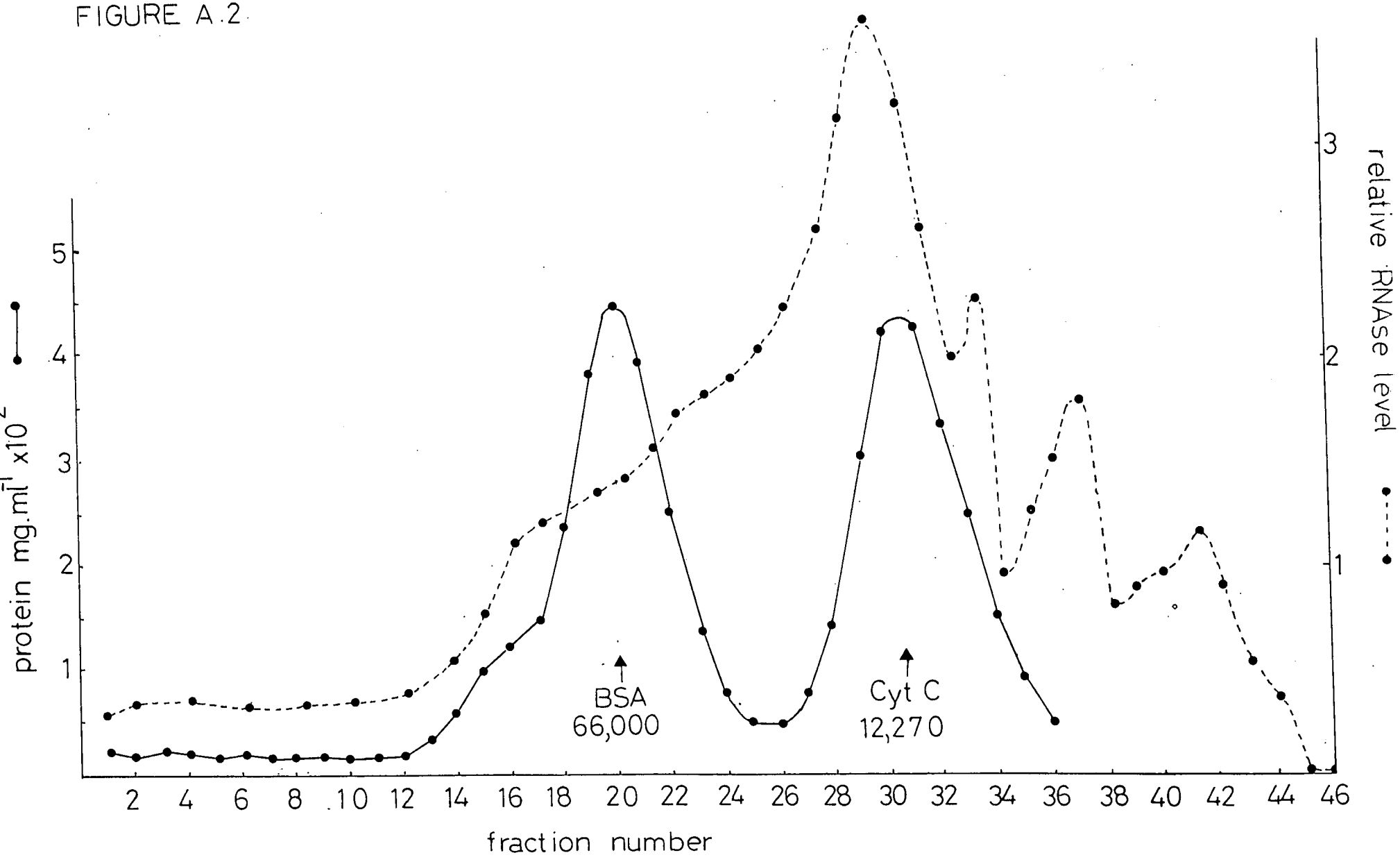
FIGURE A.1

| | step | volume | RNAse | Protein | Specific activity |
|---|---|---|---|---|---|
| | | ml | $U.ml^{-1}$ | $mg.ml^{-1}$ | $U.mg^{-1}$ |
| a | heat denaturation | 75 | 0·273 | 0·566 | 0·48 |
| b | $(NH_4)_2SO_4$ precipitation | 10 | 0·564 | 0·99 | 0·57 |
| | supernatant (from b) | 100 | 0·307 | 0·284 | 1·08 |
| c | ADP Sepharose elutions | | | | |
| | I | 10 | 0·479 | 0·566 | 0·84 |
| | II | 6 | 0·308 | 0·283 | 1·09 |
| | III | 10 | 0·273 | 0·283 | 0·96 |
| | IV | 5 | 0·273 | 0·070 | 3·89 |

Figure A.2

Sephadex G-100 column elution profile of RNAse purification.
Calibration of the column was achieved by elution of standard solution (2 ml.) containing 5 mg./ml. bovine-serum albumin (BSA) and Cytochrome C (Cyt C).

FIGURE A.2


protein mg.ml$^{-1}$ x10$^{-2}$
relative RNAse level
fraction number
BSA
66,000
Cyt C
12,270
1
2
3
4
5
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
46

buffer (pH 5) were added together with 0.5 g. PVP. Following centrifugation at 16,000 g. for 15 min. the supernatant was heated at 55°C. for 1 hour. Denatured protein was removed by further centrifugation at 16,000 g. for 15 min. 42 g. ammonium sulphate were added to the supernatant which was stirred briskly for 1 h. at 4°C. The precipitate was collected by centrifugation at 16,000 g. for 15 min. and resuspended in 10 ml. 3 mM. sodium acetate buffer (pH 5) and loaded onto the ADP-Sepharose column (1x12 cm.). Elution, without restricting flow, was carried out using 3 mM. sodium acetate (I) followed by 20 mM. sodium acetate (II) followed by 200 mM. acetic acid containing 200 mM. sodium chloride (III) and, finally, 200 mM. acetic acid containing 1.5 M. sodium chloride (IV). The results, in Figure A.1 suggest that binding was incomplete. Some purification was achieved but, even bearing in mind the possible improvement in precipitation of RNAse by raising the ammonium sulphate level, the amounts obtained would still be extremely low.

b. Sephadex G-100 chromatographic procedure for purification of RNAse.

64 g. of F. pratensis leaf tissue were extracted as outlined above. However, following the centrifugation of heat-denatured protein, 20 ml. aliquots of the resultant supernatant were loaded onto a Sephadex G-100 column (4x27 cm.) previously equilibrated with 20 mM. sodium acetate buffer (pH 5). Separation was achieved by ascending elution by means of a Perpex pump at a flow rate of 0.45 ml./min. Following a void volume of 57 ml., 5.0 ml. fractions were collected and assayed for acid RNAse activity (Figure A.2). The major active fractions (25 - 34) were pooled and concentrated by ultrafiltration in a 4.7 cm. stirred molecular filtration cell fitted with a 'Pellicon' membrane (10.5 $cm.^2$). Operating pressure was exerted via a Luer locking adaptor from a nitrogen cylinder at 69 - 105 KPa. The volume was reduced by 90%. On one occasion this concentrated extract was dialysed overnight at 4°C. against 2.5 l. distilled water prior to further purification by preparative

Figure A.3

Purification of RNAse using Sephadex G-100 chromatography.

FIGURE A.3

| | step | volume | RNAse | Protein | Specific activity |
|---|---|---|---|---|---|
| | | ml | $U.ml^{-1}$ | $mg.ml^{-1}$ | $U.mg^{-1}$ |
| a | crude extract | 200 | 0·264 | 2·830 | 0·09 |
| b | heat denaturation | 120 | 0·250 | 2·500 | 0·10 |
| c | SephadexG100 | 17·0 | 0·171 | 0·058 | 2·97 |
| d | ultrafiltration | 15·6 | 0295 | 0·256 | 1·15 |
| e | SephadexG·25 | 3·1 | 0·219 | 0·256 | 0·85 |
| f | ultrafiltration | 2·6 | 0·250 | 0·246 | 1·02 |

starch gel electrophoresis. RNAse activity was markedly reduced by dialysis and could not be located on the preparative gels by the usual staining procedure. The possibility that the RNAse had adhered to the dialysis membrane was investigated by soaking the membrane in salt solution (200 mM. acetic acid containing 1.5 M. sodium chloride). No retrieval of activity was obtained suggesting that the RNAse must have been in small enough quantity to be lost by dialysis. As an alternative to dialysis, concentrated samples were loaded onto Sephadex G-25 columns (1.5x20 cm.). Following a void volume of 13.4 ml., fractions were collected by elution with 20 mM. sodium acetate buffer, without restricting flow. The most active fractions were pooled and concentrated in a 1.3 cm. stirred molecular filtration cell using a 'Pellicon' membrane of 0.7 $cm.^2$ by a pressure of 69 KPa (Figure A.3). Following dilution of 1:1 with distilled water, the final extract was used for injection into a rabbit. Unfortunately, the Ouchterlony plates set up to confirm that antibodies had been raised in the rabbit showed no immunochemical precipitation.

Although steps were taken to eliminate small molecular weight proteins it is possible that during handling the major acid RNAse ( $\simeq$ 15,000 MW) dissociated and the criterion of collection of the most active samples (for example, from the Sephadex G-25 column) preferentially selected for these smaller molecules (perhaps similar to the minor RNAses separated by Sephadex G-100 chromatography - see Figure A.2). Evidence for active small molecules has been reported by Bryant et al. (1976) from pea leaves. A fraction with molecular weight of 3,100 retained enzymatic activity and, since this molecular weight was so much lower than any other enzyme, it was considered unlikely to be a native enzyme. They not only found that its concentration varied markedly but it was lost by dialysis. Figure A.2 shows an active RNAse with molecular weight $\simeq$ 3,000. The dialytic loss obtained during the purification procedure reported here could be explained if conditions had caused fragmentation of the major RNAse into just such active fragments. Pitt (1974) also reported an acid RNAse of low molecular weight from leaves of potato ( $\simeq$ 5,000 MW).

If these low molecular weight RNAses do represent fragments then acid RNAse is capable of retaining activity even after the loss of a large number of amino acid residues. (Bryant and Phillips (197 ) have shown that photodynamic modification of RNAse confirms this possibility). This may be of considerable significance in its degradative role inasmuch as it would seem to be an important adaptation for maximal breakdown for redeployment purposes.

## III. Summary.

The existence of active fragments of RNAse as reported by Bryant et al. (1976) complicates the strategem for its purification and would explain the incomplete precipitation by addition of ammonium sulphate. The amounts of the latter two peaks of RNAse activity of approximate molecular weights of 5,000 and 3,000 (Figure A.2) were extremely variable which supports the possibility of being fragments rather than native enzymes. If such fragmentation does occur either in vivo or during early extraction then it is not unreasonable to postulate that further manipulation (particularly the exertion of pressure of 105 KPa during ultrafiltration) might cause this also, (Briggs and Rice (1972) have recorded the reduction of enzyme size by proteases during purification without affecting the activity.) At the ultrafiltration step it is also feasible that the unfragmented RNAse molecules were forced into the membranes. In fact, 10% (W/W) protein was lost on the membrane during ultrafiltration (Figure A.3 d) and 46% (W/W) protein passed through to the filtrate. Pooling of Sephadex G-25 eluants would have selected for these fragmentation products. The smallness of the RNAse molecule would also provide an explanation for the lack of immune response in the rabbit.

## REFERENCES.


ABADOM, P.N. and ELSON, D. (1970)
Biochim. Biophys. Acta, 199 528.

ACTON, G.J., LEWINGTON, R.J. and MYERS, A. (1970)
Biochim. Biophys. Acta, 204 144.

ADESNIK, M., SALDITT, M., THOMAS, W. and DARNELL, J.E. (1972)
J. Molec. Biol., 71 21.

AJTKHOZHIN, M., AKHANOV, A. and DOSHCHANOV, Kh. (1973)
F E B S Letters, 31 104.

ALLEN, S.L., MISCH, M.S. and MORRISON, B.M. (1963)
J. Histochem. Cytochem., 11 706.

ALLINSON, D.W. (1971)
Crop Sci., 11 456.

ALSCHER, R., PATTERSON, R. and JAGENDORF, A.T. (1978)
Plant Physiol., 62 88.

ANDERSON, J.M. and KEY, J.L. (1971)
Plant Physiol., 48 801.

ARCHER, K.A. and DECKER, A.M. (1977)
Agron. J., 69 601.

ARNSTEIN, H.R.V. (1961)
Biochem. J., 81 24P.

ATCHISON, B.A., BOURQUE, D.P. and WILDMAN, S.G. (1973)
Biochim. Biophys. Acta, 331 382.

ATKIN, R.K. and SRIVASTAVA, B.I.S. (1969)
Physiol. Plantarum 22 742.

AVIV, H. and LEDER, P. (1972)
Proc. natn. Acad. Sci. U.S.A., 69 1408.

BAGI, G. and FARKAS, G.L. (1967)
Phytochemistry 6 161.

BALTUS, E. and QUERTIER, J. (1966)
Biochim. Biophys. Acta, 119 192.

BARKER, G.R. and DOUGLAS, T. (1960)
Nature 188 943.

BARKER, G.R. and RIEBER, M. (1967)
Biochem. J., 105 1195.

BARKER, G.R., BRAY, C.M. and WALTER, T.J. (1971)
Biochem. J., 124 11P.

BARLOW, J. and MATHIAS, A.P. (1966)
in Procedures in Nucleic Acid Research (Edited by G.L. Cantoni and D.R. Davies) 444.

BARTLETT, G.R. (1959)
J. Biol. Chem., 234 466.

BARTON, R. (1966)
Planta 71 314.

BAUMGARTNER, B., KENDE, H. and MATILE, P. (1975)
Plant Physiol., 55 734.

BAUMGARTNER, B. and MATILE, P. (1977)
Zeits. fur Planzen 82 371.

BEACHY, R.N., THOMPSON, J.F. and MADISON, J.T. (1978)
Plant Physiol., 61 139.

BEEVERS, L., LOVEYS, B., PEARSON, J.A. and WAREING, P.F. (1970)
Planta 90 286.

BEEVERS, L. and POULSON, R. (1972)
Plant Physiol., 49 476.

BERIDZE, T.G., ODINTSOVA, M.S., CHERKASHINA, N.A. and SISSAKIAN, N.M. (1966)
Biochem. Biophys. Res. Commun., 23 683.

BHAT, S.P. and PADAYATTY, J.D. (1974)
Ind. J. Biochem. Biophys., 11 47.

BHAT, S.P. and PADAYATTY, J.D. (1975)
Nature 256 227.

BICK, M.D., LIEBKE, H., CHERRY, J.H. and STREHLER, B.L. (1970)
Biochim. Biophys. Acta, 204 175.

BIRMINGHAM, B.C. and MACLACHLAN, G.A. (1972)
Plant Physiol., 49 371.

BISHOP, D.H.L., CLAYBROOK, J.R. and SPIEGELMAN, S. (1967)
J. Molec. Biol., 26 373.

BLACK, M. and VLITOS (1971)
in Phytochrome (Edited by Mitrakos and Shropshire).

BLOBEL, G. (1971)
Proc. natn. Acad. Sci. U.S.A., 68 832.

BOARDMAN, N.K., FRANCKI, R.I.B. and WILDMAN, S.G. (1965)
Biochemistry 4 872.

BOARDMAN, N.K., FRANCKI, R.I.B. and WILDMAN, S.G. (1966)
J. Molec. Biol., 17 470.

BOARDMAN, N.K. (1967)
in "Harvesting the Sun - Photosynthesis in Plant Life"
(Edited by A. San Pietro, G.A. Greer and T.J. Army) 211
(Acad, Press, N.Y.)

BOGORAD, L. and JACOBSON, A.B. (1964)
Biochem. Biophys. Res. Commun., 14 113.

BOGORAD, L. (1967)
in "Molecular Organization and Biological Function"
(Edited by J.M. Allen) 134.

BOGORAD, L. (1968)
Develop. Biol. Suppl. 1 1.

BOGORAD, L. and WOODCOCK, C.L.F. (1971)
in "Autonomy and Biogenesis of Mitochondria and Chloroplasts"
(Edited by N.K. Boardman, A.W. Linnane and R.M. Smillie) 92.
(North Holland Publishing Co., Amsterdam)

BOLTON, E.T. (1966)
in "Procedures in Nucleic Acid Research" (Edited by
G.L. Cantoni and D.R. Davies).

BOTTGER, I. and WOLLGIEHN, R. (1958)
Flora (Jena) 146 302.

BOULTER, D. (1970)
Ann. Rev. Plant Physiol., 21 91.

BOURQUE, D.P., HAGILADI, A. and NAYLOR, A.W. (1973)
Biochem. Biophys. Res. Commun., 51 993.

BOWMAN, D.E. and LAW, A.G. (1964)
Agron. J., 56 177.

BRAWERMAN, G. and EISENSTADT (1967)
in "Organizational Biosynthesis" (Edited by H.J. Vogel, J.O. Lampen and V. Ryson). Acad. Press, N.Y.

BRAWERMAN, G. (1974)
Ann. Rev. Biochem., 43 621.

BRAY, C.M. and CHOW, T-Y. (1976)
Biochim. Biophys. Acta, 442 14.

BREEN, M.D., WHITEHEAD, E.I. and KENEFICK, D.G. (1972)
Plant Physiol., 49 733.

BRIGGS, W.R. and RICE, H.V. (1972)
Ann. Rev. Plant Physiol., 23 293.

BRITTEN, B.J. and ROBERTS, R.B. (1960)
Science 131 32.

BROWN, G.N. and NAYLOR, A.W. (1965)
Bot. Gaz., 126 167.

BROWN, R.D. and HASELKORN, R. (1971)
Proc. natn. Acad. Sci. U.S.A., 68 2536.

BRYANT, J.A., GREENWAY, S.C. and WEST, G.A. (1976)
Planta 130 141.

BURKA, E.R. and MARKS, P.A. (1964)
J. Molec. Biol., 9 439.

BURKARD, G., GUILLEMAUT, P. and WEIL, J.H. (1970)
Biochim. Biophys. Acta, 224 184.

BURR, B. and BURR, F.A. (1976)
Proc. natn. Acad. Sci. U.S.A. 73 515.

BUTLER, R.D. (1967)
J. Expl. Bot., 18 535.

BUTLER, R.D. and SIMON, E.W. (1970)
Adv. Gerontol. Res., 3 73.

CAERS, L.I., PEUMANS, W.J. and CARLIER, A.R. (1979)
Planta 144 491.

CALLOW, M.E. (1971)
Ph.D. Thesis, University of Sheffield.

CALLOW, J.A., CALLOW, M.E. and WOOLHOUSE, H.W. (1972)
Cell differentiation 1 79.

CAMMARANO, P., ROMEO, A., GENTILE, M., FELSANI, A. and GAULERZI, C. (1972)
Biochim. Biophys. Acta, 281 597.

CARR, D.J. and PATE, J.S. (1967)
Symp. Soc. Exp. Biol., 21 559.

CHELM, K., HOBEN, P.J. and HALLICK, R.B. (1977)
Biochemistry 16 776.

CHEN, D., SCHULTZ, G. and KATCHALSKI, E. (1971)
Nature 231 69.

CHEN, S. and WILDMAN, S.G. (1970)
Biochim. Biophys. Acta, 209 207.

CHERRY, J.H. (1967)
Symp. Soc. Exp. Biol., 21 247.

CHIBNALL, A.C. and WILTSHIRE, G.H. (1954)
New Phytol., 53 38.

CHOE, H.T. and THIMANN, K.V. (1974)
Planta 121 201.

CHRISPEELS, M.J. and VARNER, J.E. (1967)
Plant Physiol., 42 398.

CLARK, M.F., MATTHEWS, R.E.F. and RALPH, R.K. (1964)
Biochim. Biophys. Acta, 91 289.

CLICK, R.E. and HACKETT, D.P. (1963)
Proc. natl. Acad. Sci. U.S.A. 50 243.

COHEN, D. and SCHIFF, J. (1976)
Arch. Biochem. Biophys., 177 201.

COOPER, H.L. and GIBSON, E.M. (1971)
J. Biol. Chem., 246 5059.

COVEY, S.N. and GRIERSON, D. (1976)
Europ. J. Biochem., 63 599.

DAHLBERG, A.E., DINGMAN, C.W. and PEACOCK, A.C. (1969)
J. Molec. Biol., 41 139.

DAHLBERG, A.E., LUND, E. and KJELDGAARD, N.O. (1973)
J. Molec. Biol., 78 627.

DAVIES, E., LARKINS, B.A. and KNIGHT, R.H. (1972)
Plant Physiol., 50 581.

DAVIES, J.W. and KAESBERG, P. (1973)
J. Virol., 12 1434.

DE-DEKEN GRENSON, M. (1954)
Biochim. Biophys. Acta, 14 203.

DE GREEF, J.A. and CAUBERGS, R. (1972)
Societe Belge de Biochimie 961.

DE LEO, P. and SACHER, J.A. (1970)
Plant Physiol., 46 806.

DENIC, M., EHRENBERG, L. FEDORCSAK, I. and SOLYMOSY, F. (1970)
Acta Chem. Scand., 24 3753.

DOBRZANSKA, M., TOMASZEWSKI, M., GRZOLEZAK, Z., REJMAN, E. and BUCHOWICZ, J. (1973)
Nature 244 507.

DOSHCHANOV, Kh., AJTKHOZHIN, M. and DARKANBAEV, T. (1975)
Sov. Plant Physiol., 22 305.

DOVE, L.D. (1967)
Plant Physiol., 42 1176.

DOVE, L.D. (1971)
New Phytol., 70 397.

DOVE, L.D. (1973)
Phytochemistry 12 2561.

DYER, T.A., MILLER, R.H. and GREENWOOD, A.D. (1971)
J. Exp. Bot., 22 125.

DYER, T.A. and OSBORNE, D.J. (1971)
J. Exp. Bot., 22 552.

DYER, T.A. and PAYNE, P.I. (1974)
Planta 117 259.

EILAM, Y., BUTLER, R.D. and SIMON, E.W. (1971)
Plant Physiol., 47 317.

ELLIS, R.J. (1975)
in "Membrane Biogenesis" (Edited by A Tzagoloff)

ELLIS, R.J. (1976)
in "Perspectives in Experimental Biology" Vol. 2 (Edited by N. Sunderland).

ELLIS, R.J. (1969)
Science 163 477.

EVANS, M.I., CROY, R.R.D., HUTCHINSON, P., BOULTER, D., PAYNE, P.I. and GORDON, M.E. (1979)
Planta 144 455.

EYTAN, G. and OHAD, I. (1970)
J. Biol. Chem., 245 4297.

FAIRBANKS, G., STECH, T. and WALLACH, D. (1971)
Biochemistry 10 2606.

FEDORCSAK, I. and EHRENBERG, L. (1966)
Acta Chem. Scand., 20 107.

FEDORCSAK, I., and TURTOCZKY (1966)
Nature 209 830.

FEDORCSAK, I., NATARAJAN, A.T. and EHRENBERG, L. (1969)
Eur. J. Biochem., 10 450

FILNER, B. and KLEIN, A.O. (1968)
Plant Physiol., 43 1587.

FISKE, C.H. and SUBBAROW, Y. (1925)
J. Biol. Chem., 66 375.

FRASER, R.S.S. (1969)
Molec. Gen. Genetics 106 73.

FRASER, R.S.S. (1975)
Eur. J. Biochem., 60 477.

FRASER, R.S.S. and CARTER, B.L.A. (1976)
J. Molec. Biol., 104 223.

FURANO, A.V. and HARRIS, M.I. (1971)
Biochim. Biophys. Acta 247 291.

GAGNON, C. and DE LAMIRANDE, G. (1972)
Arch. Biochem. Biophys., 150 573.

GALSTON, A.W. (1968)
Proc. natn. Acad. Sci. U.S.A., 61 454.

GEIGER, P.J. and BESSMAN, S.P. (1972)
Anal. Biochem., 49 467.

GIBSON, R.A. and PALEG, L.G. (1972)
Biochem. J., 128 367.

GILES, A.B., GRIERSON, D. and SMITH, H. (1977)
Planta 136 31.

GOLDSTEIN, J.L. and SWAIN, T. (1965)
Phytochemistry 4 185.

GORDON, M.E., LETHAM, D.S. and BEEVER, J.E. (1975)
Physiologia Pl., 35 27.

GORDON, M. and PAYNE, P.I. (1976)
Planta 130 269.

GOULD, H. (1966)
Biochemistry 5 1103.

GRAHAM, D., GRIEVE, A.M. and SMILLIE, R.M. (1968)
Nature 218 89.

GRANICK, S. (1959)
Plant Physiol., 34 xviii.

GRAY, R.E. and CASHMORE, A.R. (1976)
J. Molec. Biol., 108 595.

GREENBERG, H. and PENMAN, S. (1966)
J. Molec. Biol., 21 527.

GREGORY, F.G. and SEN, P.K. (1937)
Ann. Bot., 1 521.

GREGORY, P. and BRADBEER, J.W. (1975)
Biochem. J., 148 433.

GRELLETT, F., JULIEN, R. and MILHOMME, H. (1968)
Physiol. Veg., 6 11.

GRIERSON, D., ROGERS, M.E., SARTIRANA, M.L. and LOENING, U.E. (1970)
Cold Spring Harbour Symp. Quant. Biol., 35 589.

GRIERSON, D. (1972)
Ph.D. Thesis, University of Edinburgh.

GRIERSON, D. and LOENING, U.E. (1972)
Nature 235 80.

GRIERSON, D. (1974)
Eur. J. Biochem., 44 509.

GRIERSON, D. and LOENING, U.E. (1974)
Eur. J. Biochem., 44 501.

GRIERSON, D. and COVEY, S. (1975)
Planta 127 77.

GRIERSON, D. (1975)
Planta 127 87.

GRIERSON, D. (1976)
in "Molecular Aspects of Gene Expression" (Edited by J.A. Bryant).

GRIERSON, D., COVEY, S.N. and GILES, A.B. (1976)
in "Light and Plant Development" (Edited by H. Smith) p. 229.

GROOT, G.S.P., FLAVELL, R.A., VAN OMMEN, G.J.B. and GRIVELL, L.A. (1974)
Nature 252 167.

GROSSBACH, U. and WEINSTEIN, I.B. (1968)
Anal. Biochem., 22 311.

GUALERZI, C. and CAMMARANO, T. (1970)
Biochim. Biophys. Acta 199 203.

GYLDENHOLM, A.O. (1968)
Hereditas 59 142.

HADZIYEV, D., MEHTA, S.L. and ZALIK, S. (1969)
Can. J. Biochem., 47 273.

HAFF, L.A. and BOGORAD, L. (1976)
Biochemistry 15 4110.

HALL, T.C. and COCKING, E.C. (1966)
Biochim. Biophys. Acta 123 163.

HALLAM, N.D., ROBERTS, B.E. and OSBORNE, D.J. (1972)
Planta 105 293.

HAMKALO, B.A. and MILLER, O.L. (1973)
Ann. Rev. Biochem., 42 379.

HARDWICK, K., WOOD, M. and WOOLHOUSE, H.W. (1968)
New Phytol., 67 79.

HAREL, E. and BOGORAD, L. (1973)
Plant Physiol., 51 10 .

HARTLEY, M. R. (1976)
Personal communication.

HEDLEY, C.L. (1971)
Ph.D. Thesis (Welsh Plant Breeding Station).

HEDLEY, C.L. and STODDART, J.L. (1971 a.)
J. Exp. Bot., 22 239.

HEDLEY, C.L. and STODDART, J.L. (1971 b.)
J. Exp. Bot., 22 249.

HEDLEY, C.L. and STODDART, J.L. (1971 c.)
Planta 100 309.

HEDLEY, C.L. and STODDART, J.L. (1972 a.)
J. Exp. Bot., 23 502.

HEDLEY, C.L. and STODDART, J.L. (1972 b.)
J. Exp. Bot., 23 490.

HEIZMANN, P. (1970)
Biochim. Biophys. Acta 224 144.

HENDRICKS, S.B. and BORTHWICK, H.A. (1967)
Proc. natn. Acad. Sci. U.S.A. 58 2125.

HENRIQUES, F. and PARK, R.B. (1975)
Plant Physiol., 55 763.

HIGGINS, T.J.V., MERCER, J.F.B. and GOODWIN, P.B. (1973)
Nature 246 68.

HJERTENS, S., JERSTEDT, S. and TISELIUS, A. (1965)
Anal. Biochem., 11 211.

HOAGLAND, M.B., STEPHENSON, M.L., SCOTT, J.F., HECHT, I. and ZAMECNIK, P.C. (1958)
J. Biol. Chem., 231 241.

HSIAO, T.C. (1968)
Plant Physiol., 43 1355.

HUEZ, G., MARBAIX, G., HUBERT, E., LECLERCQ, M., NUDEL, U., SOREQ, H., SALOMON, R., LEBLEU, B., REVEL, M. and LITTAVER, U.Z. (1974)
Proc. natn. Acad. Sci. U.S.A. 71 3143.

HUVOS, P. and SOLYMOSY, F. (1971)
Acta Biochim. Biophys. Acad. Sci. Hung., 6 317.

IHLE, J.N. and DURE, L. (1969)
Biochem. Biophys. Res. Commun., 36 705.

INFANTE, A.A. and BAIERLEIN, R. (1971)
Proc. natn. Acad. Sci. U.S.A. 68 1780.

INGLE, J. (1968 a.)
Plant Physiol., 43 1448.

INGLE, J. (1968 b.)
Plant Physiol., 43 1850.

INGLE, J. and BURNS, R.G. (1968)
Biochem. J., 110 605.

INGLE, J., POSSINGHAM, J.V., WELLS, R., LEAVER, C.J. and LOENING, U.E. (1970)
Symp. Soc. Exp. Biol., 24 303.

INGLE, J., WELLS, R., POSSINGHAM, J.V. and LEAVER, C.J. (1971)
in "Autonomy and Biogenesis of mitochondria and chloroplasts" (Edited by N.K. Boardman, A.W. Linnane and R.M. Smillie).

ISHIHAMA, A., MIZUNO, N., TAKAI, M., OTAKA, E. and OSAWA, S. (1962)
J. Molec. Biol., 5 251.

JACHYMCZYK, W.J. and CHERRY, J.H. (1968)
Biochim. Biophys. Acta 157 368.

JACHYMCZYK, W.J., ZAWIERUCHA, R. and SIELIWANOWICZ, B. (1971)
Acta Biochim. Polon., 18 129.

JACHYMCZYK, W.J., SIELIWANOWICZ, B. and CHLEBOWICZ, E. (1974)
Acta Biochim. Polon., 21 137.

JACKSON, A.O. and LARKINS, B. (1976)
Plant Physiol., 57 5.

JACOBSON, A.B., SWIFT, H. and BOGORAD, L. (1963)
J. Cell. Biol., 17 557.

JAFFE, M.J. and GALSTON, A.W. (1966)
Plant Physiol., 41 1152.

JOHNSON, M.W. and MARKHAM, R. (1962)
Virology 17 276.

KABAT, D. and RICH, A. (1969)
Biochemistry 8 3742.

KAGAWA, T., McGREGOR, D.I. and BEEVERS, H. (1973)
Plant Physiol., 51 66.

KANEKO, I. and DOI, R.H. (1966)
Proc. natn. Acad. Sci. U.S.A. 55 564.

KAR, M. and MISHRA, D. (1975)
Can. J. Bot., 53 503.

KEISTER, D.L., JAGENDORF, A.T. and SAN PIETRO, A. (1962)
Biochim. Biophys. Acta 62 332.

KELLER, E.B. and ZAMECNIK, P.C. (1956)
J. Biol. Chem., 221 45.

KESSLER, B. and ENGELBERG, N. (1962)
Biochim. Biophys. Acta 55 70.

KEY, J.L. (1964)
Plant Physiol., 39 365.

KIDSON, C., KIRBY, K.S. and RALPH, R.K. (1963)
J. Molec. Biol., 7 213.

KIDSON, C. and KIRBY, K.S. (1964)
J. Molec. Biol., 10 187.

KIRBY, K.S. (1962)
Biochim. Biophys. Acta 55 545.

KIRBY, K.S. (1965)
Biochem. J., 96 266.

KIRBY, K.S. (1968)
in "Methods in Enzymology" 12 B. (Edited by L. Grossman and K. Moldave) p. 87.

KISBAN, C., HORVATH, M., DEZSI, L., UDVARDY, J. and FARKAS, G.L. (1964)
Acta Bot. Acad. Sci. Hung., 10 275.

KOERNER, J.F. and SINSHEIMER, R.L. (1957)
J. Biol. Chem., 228 1048.

KONDOROSI, A., SVAB, Z., SOLYMOSY, F. and FEDORCSAK, I. (1972)
J. Gen. Virol., 16 373.

KORNBERG, A. (1962)
in "Horizons in Biochemistry" (Edited by M. Kasha and B. Pullman).

KRIZEK, D.T., McILRATH, W.J. and VERGARA, B.S. (1966)
Science 151 95.

KURAISHI, S. (1968)
Physiologia Pl., 21 78.

LARKINS, B.A. and DAVIES, E. (1973)
Plant Physiol., 52 655.

LARKINS, B.A., BRACKER, C.E. and TSAI, C-Y. (1976)
Plant Physiol., 57 740.

LARSON, L.A. and BEEVERS, H. (1965)
Plant Physiol., 40 424.

LAZAR, G., FEDORCSAK, I. and SOLYMOSY, F. (1969)
Phytochemistry 8 2353.

LAZAR, G. and FARKAS, G.L. (1970)
Acta biol. Acad. Sci. Hung., 21 389.

LEAVER, C.J. and KEY, J.L. (1970)
J. Molec. Biol., 49 671.

LEAVER, C.J. and INGLE, J. (1971)
Biochem. J., 123 235.

LEAVER, C.J. (1973)
Biochem. J., 135 237.

LEAVER, C.J. and DYER, T.A. (1974)
Biochem. J., 144 165.

LEDOIGT, G., CURGY, J.J., STEVENS, B.J. and ANDRE, J. (1975)
Biochim. Biophys. Acta 407 222.

LEDOUX, L., GALAND, D. and HUART, R. (1962)
Biochim. Biophys. Acta 55 97.

LEECH, R.M. (1976)
in "Plant Structure, Function and Adaptation" (Edited by M.A. Hall) p. 126.

LEONARD, N.J., McDONALD, J.J. and REICHMANN (1970)
Proc. natn. Acad. Sci. U.S.A. 67 93.

LEONARD, R.T. and HOTCHKISS, C.W. (1978)
Plant Physiol., 61 175.

LEOPOLD, A.C. (1961)
Science 134 1727.

LEWINGTON, R.J., TALBOT, M. and SIMON, E.W. (1967)
J. Exp. Bot., 18 526.

LEWINGTON, R.J. and SIMON, E.W. (1968)
J. Exp. Bot., 20 138.

LIAUTARD, J-P. and LIAUTARD, J. (1977)
Biochim. Biophys. Acta 474 588.

LIN, C.Y., TRAVIS, R.L., CHIA, S.Y. and KEY, J.L. (1973)
Phytochemistry 12 515.

LIN, C.Y., CHEN, Y-M., GUILFOYLE, T.J. and KEY, J.L. (1976)
Plant Physiol., 58 614.

LINDNER, R.C., KIRKPATRICK, H.C. and WEEKS, T.E. (1956)
Plant Physiol., 31 1.

LINEWEAVER, H. and BURK, D. (1934)
J. Am. Chem. Soc., 56 658.

LITTAUER, U.Z. and SELA, M. (1962)
Biochim. Biophys. Acta 61 609.

LODISH, H.F., JACOBSON, A. FIRTEL, R., ALTON, T. and TUCHMAN, J. (1974)
Proc. natn. Acad. Sci. U.S.A. 71 5103.

LOENING, U.E. (1967)
Biochem. J., 102 251.

LOENING, U.E. and INGLE, J. (1967)
Nature 215 363.

LOENING, U.E. (1968)
Ann. Rev. Pl. Physiol., 19 37.

LOENING, U.E. (1969)
Biochem. J., 113 131.

LONSDALE, D.M. and BOULTER, D. (1973)
Phytochemistry 12 39.

LOVETT, J.S. and LEAVER, C.J. (1969)
Biochim. Biophys. Acta 195 319.

LOWRY, O.H. and LOPEZ, J.A. (1946)
J. Biol. Chem., 162 421.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. (1951)
J. Biol. Chem., 193 265.

LYNDON, R.F. (1966)
Biochim. Biophys. Acta 113 110.

LYTTLETON, J.W. (1962)
Exptl. Cell Res., 26 312.

McCARTY, R.E. and RACKER, E. (1968)
J. Biol. Chem., 243 129.

McGOWN, E., RICHARDSON, A., HENDERSON, L.M. and SWAN, P.B. (1971)
Biochim. Biophys. Acta 40 358.

McHALE, J.S. and DOVE, L.D. (1968)
New Phytol., 67 505.

MACHE, R., JALLIFIER-VERNE, M., ROZIER, L. and LOISEAUX, S. (1978)
Biochim. Biophys. Acta 517 390.

MACNICOL, P.K. (1976)
Plant Physiol., 57 80.

MANS, R.J. and NOVELLI, G.D. (1961)
Arch. Biochem. Biophys., 94 48.

MARCU, K. and DUDOCK, B. (1974)
Nucleic Acids Research 1 1385.

MARCUS, A. (1960)
Plant Physiol., 35 126.

MARCUS, A. and FEELEY, J. (1964)
Proc. natn. Acad. Sci. U.S.A. 51 1075.

MARCUS, A. and FEELEY, J. (1965)
J. Biol. Chem., 240 1675.

MARCUS, A., BEWLEY, J.D. and WEEKS, D.P. (1970)
Science 167 1735.

MARTIN, C. and THIMANN, K.V. (1972)
Plant Physiol., 49 64.

MASUDA, Y., SETTERFIELD, G. and BAYLEY, S.T. (1966)
Pl. Cell Physiol., 7 243.

MATILE, P.H. (1966)
Z. Naturforsch., 21 b 871.

MATILE, P.H. and MOOR, H. (1968)
Planta 80 159.

MATHEW, T., GAUR, B.K. and DAVE, I.C. (1976)
Plant Physiol., 57 suppl. 102.

MATSUSHITA, S. and IBUKI, F. (1960)
Biochim. Biophys. Acta 40 358.

MATTHEWS, M.B. (1973)
in "Essays in Biochemistry 9" (Edited by P.N. Campbell and F. Dickens) 59. Acad. Press.

MEGO, J.L. and JAGENDORF, A.T. (1961)
Biochim. Biophys. Acta 53 237.

MELERA, P.W., CHET, I. and RUSCH, H.P. (1970)
Biochim. Biophys. Acta 209 569.

MEYER, H., MAYER, A.M. and HAREL, E. (1971)
Physiol. Plant., 24 95.

MIKULOVICH, T.P. (1978)
Biochem. Physiol. Planz., 172 93.

MILCAREK, C., PRICE, R. and PENMAN, S. (1974)
Cell., 3 1.

MISRA, G. and BISWAL, U.C. (1973)
Bot. Gaz., 134 5.

MITTELHEUSER, C.J. and VAN STEVENINCK, R.F.M. (1970)
in "Plant Growth Substances" (Edited by D.J. Carr) 618.

MORRILL, G.A. and REISS, M.M. (1969)
Biochim. Biophys. Acta 179 43.

MOTHES, K. (1926)
Planta 1 472.

MOTHES, K. and ENGELBRECHT, L. (1956)
Flora, Jena 143 428.

MÜLLER, K. and LEOPOLD, A.C. (1966)
Planta 68 167.

MUNTZ, K. (1978)
in "Regulation of Developmental Processes in Plants"
(Edited by H.R. Schutter and D. Gross).

NADLER, K. and GRANICK, S. (1970)
Plant Physiol., 46 240.

NAGANNA, B. and SRIPATHI, C.E. (1954)
Nature 174 593.

NEMER, M., GRAHAM, M. and DUBROFF, L.M. (1974)
J. Molec. Biol., 89 435.

NIERLICH, D.P. (1967)
Science 158 1186.

NIERLICH, D.P. (1968)
Proc. natn. Acad. Sci. U.S.A. 60 1345.

NOLL, H. (1969)
Analyt. Biochem., 27 130.

OBERG, B. (1970)
Biochim. Biophys. Acta 204 430.

OBERG, B. (1971 a)
Eur. J. Biochem., 19 496.

OBERG, B. (1971 b)
Biochim. Biophys. Acta 232 107.

OCHOA, S. (1968)
Naturwissenschaften 55 505.

OPIK, H. (1968)
J. Exp. Bot., 19 64.

ORGEL, L.E. (1970)
Proc. natn. Acad. Sci. U.S.A. 67 1476.

ORTWERTH, B.J. (1971)
Biochim. Biophys. Acta 246 344.

OSBORNE, D.J., DOBRZANSKA, M. and SEN, S. (1977)
Symp. Soc. Expt. Biol., 31 177.

OXELFELT, P. and ARSTRAND, K. (1970)
Biochim. Biophys. Acta 217 544.

PARANJOTHY, K. and WAREING, P.F. (1971)
Planta 99 112.

PARISH, R.W. (1968)
Planta 82 14.

PARTHIER and WOLLGIEHN,

PARUPS, E.V. (1976)
Can. J. Pl. Sci., 56 525.

PATTERSON, M.S. and GREEN, R.C. (1965)
Analyt. Chem., 37 854.

PAYNE, P.I. and LOENING, U.E. (1970)
Biochim. Biophys. Acta 224 128.

PAYNE, P.I. and BOULTER (1969)
Planta 84 263.

PAYNE, P.I. and BOULTER, D. (1974)
Planta 117 251.

PAYNE, P.I. (1976)
Biol. Rev., 51 329.

PEACOCK, A.C. and DINGMAN, C.W. (1967)
Biochemistry 6 1818.

PEACOCK, A.C. and DINGMAN, C.W. (1968)
Biochemistry 7 668.

PEARSON, J.A. (1969)
Expl. Cell Res., 57 235.

PEARSON, J.A. and WAREING, P.F. (1970)
Planta 93 309.

PERRY, R.P., LATORRE, J., KELLEY, D.E. and GREENBERG, J.R. (1972)
Biochim. Biophys. Acta 262 220.

PETERMAN, M.L. (1964)
"The Physical and Chemical Properties of Ribosomes."

PETERSON, L.W. and HUFFAKER, R.C. (1975)
Plant Physiol., 55 1009.

PEUMANS, W. and CARLIER, A. (1977)
Planta 136 195.

PEUMANS, W., CARLIER, A. and CAERS, L. (1978)
Planta 140 171.

PHILLIPS, D.R. and FLETCHER, R.A. (1969)
Physiol. Plant., 22 764.

PHILLIPS, L.A., HOTHAM-IGLEWSKI, B. and FRANKLIN, R.M. (1969)
J. Molec. Biol., 45 23.

PINE, K. and KLEIN, A.O. (1972)
Develop. Biol., 28 280.

PIRIE, N.W. (1959)
Ann. Rev. Pl. Physiol., 10 33.

PITT, D. and GALPIN, M. (1971)
Planta 101 317.

PITT, D. (1974)
Planta 117 43.

POLLACK, R.W. and DAVIES, P.J. (1970)
Phytochemistry 9 471.

POLLARD, G.J., STEMLER, A. and BLAYDES, D.F. (1966)
Plant Physiol., 41 1323.

POULSON, R. and BEEVERS, L. (1970)
Plant Physiol., 46 315.

POULSON, R. (1973)
in "The Ribonucleic Acids" (Edited by P.R. Stewart and D.S. Letham) p. 243.

PRATT, M.J. and MATTHEWS, R.E.F. (1971)
Planta 99 21.

PRICE, G.H. (1968)
Lab. Pract., 17 467.

QUAIL, P. (1975)
Planta 123 223.

RABSON, R. and NOVELLI, G.D. (1960)
Proc. natn. Acad. Sci. U.S.A. 46 484.

RAGHAVENDRA, A.S. and DAS, V.S.R. (1978)
J. Exp. Bot., 29 39.

RANDLES, J.W. (1968)
Virology 36 556.

RAUSER, W.E. (1971)
Can. J. Bot., 49 311.

RAWSON, J.R. and STUTZ, E. (1969)
Biochim. Biophys. Acta 190 368.

REDDI, K.K. (1959)
Proc. natn. Acad. Sci. U.S.A. 45 293.

REDDI, K.K. (1966)
in "Procedures in Nucleic Acid Research" (Edited by G.L. Cantoni and D.R. Davies) p. 71.

REDDI, K.K. and MAUSER, L.J. (1965)
Proc. natn. Acad. Sci. U.S.A. 53 607.

RHODES, M.J.C. and YEMM, E.W. (1963)
Nature 200 1077.

RICHARDS, E.G., COLL, J.A. and GRATZER, W.B. (1965)
Analyt. Biochem., 12 452.

RICHMOND, A.E. and LANG, A. (1957)
Science 125 650.

ROBERTS, B. and PATTERSON, B. (1973)
Proc. natn. Acad. Sci. U.S.A. 70 2330.

ROBERTS, B.E., PAYNE, P.I. and OSBORNE, D.J. (1973)
Biochem. J., 131 275.

ROGERS, G.T., ULBRICHT, T.L.V. and SZER, W. (1967)
Biochem. Biophys. Res. Commun., 27 372.

ROGERS, M.E., LOENING, U.E. and FRASER, R.S.S. (1970)
J. Molec. Biol., 49 681.

ROHEIM, J.R., KNIGHT, R.H. and VAN ETTEN, J.L. (1974)
Dèvelop. Biol., 41 137.

ROLLESTON, F.S. (1974)
Sub-Cell Biochem., 3 91.

ROSÉN, C.G. and FEDORSCSAK, I. (1966)
Biochim. Biophys. Acta 130 401.

ROSENTHAL, A.L. and LACKS, S.A. (1977)
Analyt. Biochem., 80 76.

ROSS, E and SCHATZ, G. (1973)
Analyt. Biochem., 54 304.

RUSHIZKY, G.W., GRECO, A.E., HARTLEY, R.W. and SOBER, H.A. (1963)
Biochem. Biophys. Res. Commun., 10 311.

RYLE, G.J.A. and POWELL, C.E. (1972)
Ann. Bot, 36 363.

SACHER, J.A. (1973)
Ann. Rev. Plant Physiol., 24 197.

SACHER, J.A. and DAVIES, D.D. (1974)
Plant Cell Physiol., 15 157.

SACHER, J.A. and DELEO, P. (1977)
Plant Cell Physiol., 18 161.

SAGER, R. and HAMILTON, M.G. (1967)
Science 157 709.

SAGHER, D., EDELMAN, M. and JAKOB, K.M. (1974)
Biochim. Biophys. Acta 349 32.

SAHULKA, J. (1971)
Biol. Plant., 13 243.

SANCHEZ, R.A. and COGLIATI, D. (1975)
Bot. Gaz., 136 281.

SCHECHTER, I. (1973)
Proc. natn. Acad. Sci. U.S.A. 70 2256.

SCHIFF, J.A. (1974)
in "Proceedings 3rd. Intern. Congress on Photosynthesis"
Vol. III (Edited by M. Auron) p. 1691.

SCHOPFER, P. and MOHR, H. (1972)
Plant Physiol., 49 8.

SCHULTZ, G.A., CHEN, D. and KATCHALSKI, E. (1972)
J. Molec. Biol., 66 379.

SCHWABE, W.W. (1970)
Ann. Bot., 34 43.

SCOTT, N.S., GRAHAM, J., SMILLIE, R.M. and MUNNS, R. (1971)
in "Autonomy and Biogenesis of Mitochondria and Chloroplasts"
(Edited by M. Boardman) p. 382.

SEDAT, J.W. and SINSHEIMER, R.L. (1970)
Cold Spring Symp. Quant. Biol., 35 163.

SEMADENI, E.G. (1967)
Planta 72 91.

SENGER, D.R. and GROSS, P.R. (1976)
Develop. Biol., 53 125.

SETTERFIELD, G. (1961)
Can. J. Bot., 39 469.

SHAIN, Y. and MAYER, A.M. (1968)
Phytochemistry 7 1491.

SHAW, C.R. and PRASAD, R. (1970)
Biochem. Genetics 4 297.

SHAW, M. and MANOCHA, M.S. (1965)
Can. J. Bot., 43 747.

SHEINESS, D. and DARNELL, J.E. (1973)
Nature 241 265.

SHULMAN, R.W., HARTWELL, L.H. and WARNER, J.R. (1973)
J. Molec. Biol., 73 513.

SIDDELL, S.G. and ELLIS, R.J. (1975)
Biochem. J., 146 675.

SIMON, E.W. (1967)
Symp. Soc. Exp. Biol., 21 215.

SIMON, E.W. (1974)
New Phytol., 73 377.

SINCLAIR, T.R. and DE WIT, C.T. (1975)
Science, 189 565.

SINGH, P.W. (1975)
Physiol. Plant., 32

SISSAKIAN, N.M., FILIPPOVICH, I.I., SVETAILO, E.N. and ALIYEV, K.A. (1965)
Biochim. Biophys. Acta 95 474.

SKOREY, R. and BEEVERS, L. (1978)
Plant Physiol., 61 494.

SMILLIE, R.M. and KROTOV, G. (1961)
Can. J. Bot., 39 891.

SMILLIE, R.M. (1969)
Abstr. XI. Int. Bot. Cong., 203

SMITH, H. (1970)
Phytochemistry 9 965.

SMITH, H. and ATTRIDGE, T.H. (1970)
Phytochemistry 9 487.

SMITH, H. (1976)
Europ. J. Biochem., 65 161.

SODEK, L. and WRIGHT, S.T.C. (1969)
Phytochemistry 8 1629.

SOLYMOSY, F., FEDORCSAK, I., GULYAS, A., FARKAS, G.L. and EHRENBERG, L. (1968)
Europ. J. Biochem., 5 520.

SOLYMOSY, F., LAZAR, G. and BAGI, G. (1970)
Analyt. Biochem., 38 40.

SOLYMOSY, F., GULYAS, A., KONDOROSI, A., LAZAR, G., BAGI, G. and FEDORCSAK, I. (1972)
Symp. Biol. Hung., 13 85.

SPENCER, D. and WHITFIELD, P.R. (1966)
Arch. Biochem. Biophys., 117 337.

SPENCER, D. and WHITFIELD, P.R. (1967)
Arch. Biochem. Biophys., 121 336.

SPENCER, P.W. and TITUS, J.S. (1972)
Plant Physiol., 49 746.

SPENCER, P.W. and TITUS, J.S. (1973)
Plant Physiol., 51 89.

SPIEGEL, S. and MARCUS, A. (1975)
Nature 256 228.

SPIEGEL, S., OBENDORF, R. and MARCUS, A. (1975)
Plant Physiol., 56 502.

SPIRIN, A.S. (1969)
Europ. J. Biochem., 10 20.

SRIVASTAVA, B.I.S. (1968 a)
Biochem. Biophys. Res. Commun., 32 533.

SRIVASTAVA, B.I.S. (1968 b)
Biochem. J., 110 683.

SRIVASTAVA, B.I.S. and ATKIN, R.K. (1968)
Biochem. J., 107 361.

SRIVASTAVA, B.I.S. (1972)
New Phytol., 71 93.

STANTON, M. (1968)
Analyt. Biochem., 22 27.

STODDART, J.L., THOMAS, H. and ROBERTSON, A. (1973)
Planta 112 309.

STRANGEWAY, G. (1977)
Ph.D. Thesis, University of Durham.

STREHLER, B.L. (1967)
Symp. Soc. Exp. Biol., 21 149.

STUTZ, E. and NOLL, H. (1967)
Proc. natn. Acad. Sci. U.S.A. 57 774.

SUN, S-M.M., BUCHBINDER, B.U. and HALL, T.C. (1975)
Plant Physiol., 56 780.

TAKEGAMI, T. (1975)
Plant and Cell Physiol., 16 417.

TAKEGAMI, T. and YOSHIDA, K. (1975)
Biochem. Biophys. Res. Commun., 67 782.

TANADA, T. (1968)
Proc. natn. Acad. Sci. U.S.A. 59 376.

TANAKA, K, ONO, T. and YANAGITA, T. (1966)
J. Gen. Appl. Microbiol., 12 329.

TANEJA, S. and SACHAR, R. (1976)
Phytochemistry 15 1589.

TAVARES, J. and KENDE, H. (1970)
Phytochemistry 9 1763.

TESTER, C.F. (1977)
Physiol. Plant., 41 305.

TESTER, C.F. and DURE, L. (1966)
Biochem. Biophys. Res. Commun., 23 287.

THIEN, W. and SCHOPFER, P. (1975)
Planta 124 215.

THOMAS, H. (1973)
Exptl. Cell Res., 77 298.

THOMAS, H. (1974)
Z. Pflanzenphysiol., 74 208.

THOMAS, H. (1975)
Report of the Welsh Plant Breeding Station.

THOMAS, H. and STODDART, J.L. (1975)
Plant Physiol., 56 438.

THOMAS, H. (1976 a)
Plant Sci. Lett., 6 369.

THOMAS, H. (1976 b)
in "Plant Structure, Function and Adaptation"
(Edited by M.A. Hall)

THROWER, S.L. (1967)
Symp. Soc. Exp. Biol., 21 483.

TOBIN, E.M. and KLEIN, A.O. (1975)
Plant Physiol., 56 88.

TRAVIS, R.L., HUFFAKER, R.C. and KEY, J.L. (1970)
Plant Physiol., 46 800.

TRAVIS, R.L., LIN, C.Y. and KEY, J.L. (1972)
Biochim. Biophys. Acta 277 606.

TREHARNE, K.J., STODDART, J.L., PUGHE, J., PARANJOTHY, K. and WAREING, P.F. (1970)
Nature 228 129.

TREWAVAS, A. (1970)
Plant Physiol., 45 742.

TREWAVAS, A. (1976)
Phytochemistry 15 363.

UDVARDY, J., FARKAS, G.L., MARRE, E. and FORTI, G. (1967)
Physiol. Plant., 20 781.

UDVARDY, J., FARKAS, G.L. and MARRE, E. (1969)
Plant Cell Physiol., 10 375.

VALLEJO, C.G. and LAGUNAS, R. (1970)
Analyt. Biochem., 36 207.

VANYUSHIN, B.F. and DUNN, D.B. (1967)
Biochim. Biophys. Acta 134 91.

VERMA, D.P.S., NASH, D.T. and SCHULMAN, H.M. (1974)
Nature 251 74.

VERMA, D.P.S., MACLACHLAN, G.A., BYRNE, H. and EWINGS, D. (1975)
J. Biol. Chem., 250 1019.

VICKERY, H.B., PUCHER, G.W., WAKEMAN, A.J. and LEAVONWORTH, C.S. (1937)
Conn. Agric. Exp. Stn. New Haven Bull. 399 757.

VICKERY, H.B., PUCHER, G.W., SCHOENHEIMER, R. and RITTENBERG, D. (1940)
J. Biol. Chem., 135 531.

VILLIERS, T.A. (1968)
Planta 82 342.

WALBOT, V. (1972)
Planta 108 161.

WALKLEY, J. (1940)
New Phytol., 39 362.

WALTER, K. and SCHÜTT, C. (1974)
in "Methods of Enzymatic Analysis 2" (Edited by H.U. Bergmeyer)

WAREING, P.F. and PHILLIPS, I.D.J. (1970)
in "The Control of Growth and Differentiation in Plants"

WARNER, J.R. (1974)
in "Ribosomes" (Edited by Nomura, Tissieres and Lengyel, Cold Spring Harbor Lab.)

WEBER, K. and OSBORN, M. (1969)
J. Biol. Chem., 244 4406.

WEEKS, D.P. and MARCUS, A. (1969)
Plant Physiol., 44 1291.

WEEKS, D.P. and MARCUS, A. (1971)
Biochim. Biophys. Acta 232 671.

WEIDNER, M. and MOHR, H. (1967)
Planta 75 99.

WETTSTEIN, F.O., STAEHELIN, T. and NOLL, H. (1963)
Nature 197 430.

WHITFIELD, P.R. (1973)
in "Ribonucleic Acids" (Edited by P.R.Stewart and D.S. Letham)

WILSON, C.M. and SHANNON, J.C. (1963)
Biochim. Biophys. Acta 68 311.

WILSON, C.M. (1968)
Plant Physiol., 43 1332.

WILSON, C.M. (1969)
Analyt. Biochem., 31 506.

WILLIAMS, G.R. and NOVELLI, G.D. (1964)
Biochem. Biophys. Res. Commun., 17 23.

WILLIAMS, G.R. and NOVELLI, G.D. (1968)
Biochim. Biophys. Acta 155 183.

WINTER-SLUITER, E., LAUCHLI, A. and KRAMER, D. (1977)
Plant Physiol., 60 923.

WOLF, B., LESNAW, J.A. and REICHMANN, M.E. (1970)
Europ. J. Biochem., 13 519.

WOLLGIEHN, R. and PARTHIER, B. (1964)
Phytochemistry 3 241.

WOLLGIEHN, R. (1967)
Symp. Soc. Exp. Biol., 21 231.

WOLLGIEHN, R., RUESS, M. and MUNSCHE, D. (1966)
Flora Allg. Bot. Zeitung. (Jena) 157 92.

WOLLGIEHN, R. and PARTHIER, B. (1977)
in "Plastid Differentiation" (Edited by J. Reinert)

WOODSTOCK, L.W. and SKOOG, F. (1960)
Ann. J. Bot., 47 713.

WOOLHOUSE, H.W. (1967)
Symp. Soc. Exp. Biol., 21 178.

WRIGHT, R.D., PILLAY, D.T.N. and CHERRY, J.H. (1973)
Mech. Ageing Dev., 1 403.

WRIGHT, S.T.C. (1963)
Symp. Soc. Exp. Biol., 17 18.

WYEN, N.V., UDVARDY, J. SOLYMOSY, F., MARRE, E. and FARKAS, G.L. (1969)
Biochim. Biophys. Acta 191 588.

WYEN, N.V., ERDEI, S. and FARKAS, G.L. (1971)
Biochim. Biophys. Acta 232 472.

YARWOOD, A., PAYNE, E.S., YARWOOD, J.N. and BOULTER, D. (1971)
Phytochemistry 10 2305.

YATSU, L.Y. and JACKS, T.J. (1968)
Arch. Biochem. Biophys., 124 466.