STUDIES OF THE HORMONAL REGULATION OF LEAF SENESCENCE

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by

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Abbreviations

ABA	=	abscisic acid
BA	=	benzyladenine
CD	=	circular dichroism
Ci	=	Curie
CO_2	=	carbon dioxide
c.p.m.	=	counts per minute
d.p.m.	=	disintegrations per minute
DNA	=	deoxyribonucleic acid
g	=	gram
GA	=	gibberellic acid
GC-MS	=	gas chromatography-mass spectrometry
GLC	=	gas liquid chromatography
hr	=	hour
HyOH	=	hyamine hydroxide
IAA	=	indole acetic acid
m	=	metre
min	=	minute
mol	=	mole (gram-molecule)
NAD	=	nicotinamide
No.	=	number
O.R.D.	=	optical rotatory dispersion
pers. comm.	=	personal communication
Pet. ether	=	petroleum ether
p.p.m.	=	parts per million
Rf	=	retardation factor (chromatograms)
RNA	=	ribonucleic acid
S.E.	=	standard error
TCA	=	trichloracetic acid
TLC	=	thin layer chromatography
u-v	=	ultra-violet
2,4-D	=	2,4-dichlorophenoxyacetic acid

SUMMARY

This thesis describes an investigation into the role of abscisic acid (ABA) in the regulation of foliar senescence in radish and bean.

The effect of ABA on the decline of protein and RNA pre-labelled with radioactive precursors, and on the loss of chlorophyll during the senescence of leaf discs of radish (Raphanus sativus L.) were studied. ABA stimulated the decline of chlorophyll during the early period of incubation, but subsequent loss of chlorophyll was retarded by ABA. ABA also enhanced the loss of protein and RNA from the leaf discs. Moreover, since the level of radioactive protein or RNA was normally lower in ABA-treated discs than in water-treated discs, it is possible that ABA was acting to stimulate protein and RNA degradation. On the other hand, the data indicate that ABA might act by limiting synthesis of radioactive protein or RNA in the post-labelling period. Thus, protein and RNA synthesis inhibitors were used in order to limit this period of incorporation. The limitations and possible disadvantages of the use of such inhibitors are discussed.

The uptake of ^{14}C-ABA by radish leaf discs was highest 1 to 2 days after excision and then declined to 6 days. This pattern of uptake was not mirrored by uptake of ^{14}C-sucrose. The uptake of ^{14}C-ABA and ^{14}C-sucrose was substantially reduced by anaerobic conditions.

Radioactive ABA is metabolised to 2 other major radioactive products by radish leaf discs, even after 6 days of pre-ageing in water. A similar pattern of metabolism for ^{14}C-ABA was observed in whole leaves of radish. Metabolism of ^{14}C-ABA in leaf discs was largely dependent on aerobic metabolism and was not affected by the addition of antibiotics to control any microbial contamination. Studies were also made on the pattern of metabolism during a 24 hour time-course and on the fate of partially purified metabolites re-supplied to radish leaf discs. The extraction and identification of the metabolic products of ABA has been attempted.

The presence of endogenous ABA in primary leaves of bean (Phaseolus vulgaris L.) has been demonstrated. Using circular dichroism procedures, quantitative determinations of ABA in bean leaves at various stages of development indicated little consistent change in the amount of ABA with the onset and progress of senescence. ABA levels in extracts of wilted and non-wilted leaves of bean were compared in a preliminary experiment.

In ultrastructural studies of radish leaf tissue, differences in the pattern of senescence-associated changes in cellular organelles were noted between leaf tissue aged in water, tissue aged in ABA and naturally senescent leaf material. The problems resulting from the possible variation in leaf material and to the asynchronous nature of senescence are discussed.

The benefits and disadvantages associated with the use of leaf discs as an assay system for leaf senescence are also discussed.

INTRODUCTION

It is a generally accepted tenet that all living organisms eventually die. The social and economic implications underlying the limited mortality of man and that of his crops and livestock have resulted in considerable attention being focused on the possible biological mechanisms controlling ageing processes. In recent years, ageing research has centred on the possible involvement of the genome in the control of ageing and on the expression of the genome as enzymic and structural proteins. Consequently, a number of theories for the control of ageing have evolved, mainly from research with animals, e.g.:-

(1) The error catastrophe theory proposes that ageing may be due to the accumulation of errors in protein synthesis leading to a gradual, but irreversible breakdown in the accuracy of the protein synthesizing machinery and finally to a lethal "error-catastrophe" (Orgel, 1963).

(2) A relationship has been suggested between the rate of somatic mutation and the rate of ageing (Szilard, 1959).

(3) The relationship between the phylogenetic "load" of deteriorative mutations and the life-span of different species may be important in determining longevity.

(4) A genetic programme of ageing has been proposed, perhaps involving changes in the expression of certain genes or the loss of specific aminoacylated transfer RNA (t-RNA) species (Strehler, 1969). Alternatively, there may be a genetic control system for the rate of repair (cited by Medvedev, 1972).

(5) The repetition of genetic information in DNA may have importance for determining the ageing rate of differentiated cells (Medvedev, 1972).

Several of these possible mechanisms for the control of ageing appear unsatisfactory for explaining the processes of ageing observed in plant tissues. In particular, the importance of random changes, implicit in some of the above theories, does not accord with the apparently precise mechanisms underlying the ageing of leaves of deciduous trees or annual plants. These discrepancies may perhaps be attributable to the presence of the chloroplast in plant tissues, as the means by which plants can exist autotrophically, as opposed to the heterotrophic existence of most animals and fungi. Furthermore, differences between animals and plants regarding the relationship between the correlative processes of growth and ageing may be of significance.

Ageing studies in plants have concentrated on the processes of senescence as the orderly and apparently programmed sequence of events leading to the

death of particular organs or of the whole plant, in contrast to the gradual decline in vigour of woody perennials, as indicated by a gradual decline in growth rate and a general debilitation (Woolhouse, 1967). The term "senescence" has been variously defined (see Leopold, 1963; Addicott, 1969; Sacher, 1973), but is generally accepted as the final stage of ontogeny when a series of normally irreversible events is initiated, leading to cellular breakdown and hence death of the organ or organism. Senescence of higher plants may be classified into 4 major types (Leopold, 1961; Simon, 1967), although any one organism may exhibit more than one type of senescence during its life cycle:-

(1) Overall or population senescence, a characteristic of monocarpic plants, in which flowering is followed by the death of the organism, the seeds being the only part of the plant to remain alive.

(2) Selective or partial senescence, in which the plant typically dies back, except for a perennating organ, which may remain viable for many years.

(3) Synchronous senescence, exhibited by the leaves of deciduous trees.

(4) Sequential or progressive senescence; the condition in a vegetative plant in which the older, lower leaves senesce first, more leaves entering senescence as fresh leaves expand nearer the shoot apex.

It is important to bear in mind the distinctions between the different types of plant senescence, outlined above. In particular, the differences in timing and control for synchronous senescence may be so distinct from those involved in sequential senescence as to suggest some basic differences in mechanism (Simon, 1967). Further, many studies, including this one, have made use of isolated leaf tissue and the senescence processes observed therein may differ in some respects from the situation in the intact plant.

Various aspects of leaf senescence have been reviewed by Varner (1961), Sax (1962), Butler and Simon (1971), Sacher (1973) and Woolhouse (1974).

Certain correlative processes, especially the diversion of nutrients, may affect plant senescence. Johnson (1862; cited by Sax, 1962) noted that the longevity of annuals can be increased by removal of the inflorescences and he attributed this to a reduction in the normal nutrient exhaustion processes. Similarly Stahl (1909) noted that leaf discs of Philadelphus remained green while the leaves, from which the discs had been derived, yellowed and died. Stahl thus proposed that leaf senescence is a function controlled by physiological events occurring in other parts of the plant. Molisch (1928) showed that if flowering and fruiting were prevented by excision, senescence was delayed; he suggested that the flowers and fruit

act as a metabolic sink, diverting both organic and inorganic nutrients from the leaf and thereby inducing leaf yellowing. Similar studies have largely substantiated the conclusion that senescence of older leaves is stimulated by the presence of developing leaves, flowers and fruit (Murneek, 1932; Crowther, 1934; Michael, 1936; Hopkinson, 1966; Cockshull and Hughes, 1967; Srivastava and Atkin , 1968). The presence of relatively high hormone concentrations in the younger, developing organs may play a role in creating a sink for nutrient diversion (Loomis, 1953; Wareing and Seth, 1967; Herner et al., 1967). At the cellular level, Fletcher (1969) described a rapid decline in the incorporation of RNA and protein precursors in primary leaves of bean at the onset of flowering. Similarly, Gupta and Chatterjee (1971) observed an increase in total chlorophyll, RNA and protein in leaves of deflowered and defruited plants of Nicotiana plumbaginifolia; furthermore, the subsequent decline of RNA and protein was slower in deflowered plants.

In detached leaves, the presence of the petiole can stimulate senescence of the lamina (Möthes and Engelbrecht, 1959; Mishra and Gaur, 1970) and this has been attributed to mobilization of assimilates from the leaf into the petiole (Chibnall, 1954; Leonard and Glenn, 1968).

Garner and Allard (1923) in their classical studies on photoperiodism pointed to the relationship between daylength and senescence. In annuals, for instance, the photoperiod favourable for flowering leads to rapid senescence of the whole plant; conversely, non-inductive conditions may indefinitely delay death.

It is clear from all these studies that the presence and development of flowers and fruit can hasten the senescence of individual leaves and the death of the whole plant. Leaf and shoot senescence may not, however, be solely a consequence of nutrient diversion to developing tissues. Petrie et al. (1939), for instance, suggested that breakdown of leaf components occurred independently of flower and fruit development and thus that mobilization to developing organs occurred as a result of leaf senescence, not as a cause of it. Similarly, Leopold et al. (1959) showed that delaying defloration or defruiting of spinach and soyabean plants shortened the life of the plant. They interpreted this as supporting the concept of a gradually intensifying signal in reproductive plants, rather than to a depletion of food reserves through mobilization into developing fruit. This was substantiated by the observation that even the removal of very small staminate flowers of spinach could delay senescence. Moreover, the artificial production of a sink by inducing stem growth did not produce the stimulus for

whole plant senescence in spinach, normally resulting from bolting and flowering (Janick and Leopold, 1961). These observations were interpreted as further evidence that mobilization alone could not cause plant death. In a study of apical senescence in peas, Lockhart and Gottschall (1961) pointed to the importance of the seeds within the fruit in hastening senescence, but, in general, they concluded that apical senescence, which occurred even in the absence of flowering, was due to some degenerative change taking place within the apex. They do, however, point out that sequential leaf senescence may be totally unrelated to apical senescence. Other studies also question the role of mobilization influences in overall senescence (Krizek et al., 1966; Marx, 1968; Trippi and Brulfert, 1973). Moreover, prevention of pollination or removal of the ears in maize caused premature senescence in leaves above the ear (Allison and Weinmann, 1970); this may be due to increased carbohydrate concentration in these leaves. Senescence of leaves below the ear was not affected. The time of senescence of individual leaves may be determined prior to floral induction. Schwabe (1970), for instance, demonstrated that the rate of senescence of Kleinia leaves was partially determined by the daylength during leaf expansion. Moreover, in red clover seedlings, there is no senescence of leaf tissue prior to the third leaf stage; the onset of senescence in the oldest leaf coincides with the attainment of sensitivity to floral induction (Jones and Stoddart, 1973).

Thus, although the presence of developing organs is certainly of significance in hastening leaf and overall senescence, other processes also appear to be involved and mobilization effects alone are unlikely to be causal in senescence.

The most easily detectable, and hence the most widely used parameter of leaf senescence is the progressive loss of chlorophyll and the consequent development of yellowing due to the increased prominence of the various carotenoid pigments.

Wolf (1956), from examination of changes in chlorophyll a and b in the autumn leaves of 25 plant species, concluded that, in general, destruction of chlorophyll a is faster than that of chlorophyll b. In oat leaves, however, there was little change in the chlorophyll a:b ratio during autumnal senescence. Chichester and Nakayama (1965) attributed the faster loss of chlorophyll a to a higher degree of chemical reactivity relative to chlorophyll b, rather than to differential physiological selection for degradation of the chlorophyll within the chloroplast. They further showed that both chlorophylls are initially converted to their respective phaeophytins, the ultimate fate for

the chlorophyll molecule apparently being oxidation. Similarly, Sanger (1971) demonstrated a faster loss of chlorophyll a than of b in several woody species and detected only traces of pheophorbide and chlorophyllide, in addition to pheophytin as the main chlorophyll degradation product.

In detached leaves of herbaceous species, chlorophyll a and b may decline at similar rates (Treffry et al., 1967; Laetsch, 1967; Goldthwaite, and Laetsch, 1968; Holden, 1972) or chlorophyll a may decline more rapidly than chlorophyll b (Sestak, 1972). These differences may be due to environmental conditions, leaf age or plant species (Sestak, 1972; Whitfield and Rowan, 1974). The mechanism by which chlorophyll is released prior to oxidation is unclear, but breakdown is clearly linked with the degradation of protein and probably also of lipids (Holden, 1972). Chlorophyllase has been implicated in chlorophyll breakdown (Holden, 1965).

Goodwin (1958) distinguished between 3 patterns of total carotenoid changes in woody species based on the speed of decline in relation to chlorophyll. Considerable disagreement still exists, however, as to the nature of carotenoid changes during senescence. Important differences, in this respect, may exist between plants of the woody and herbaceous habits. Grob (1962) could find no detectable amounts of β-carotene in yellow autumn leaves and detected no new carotenoids during yellowing; all xanthophylls became esterified. The xanthophylls may increase in relative abundance so that in autumn leaves the carotenoids consist chiefly of esters of violaxanthin and lutein (Chichester and Nakayama, 1965). In contrast, Sanger (1971) found that β-carotene and lutein were the most stable of the carotenoids in autumn leaves and that violaxanthin and neoxanthin disappeared rapidly. Again, Goodwin (1965) considered zeaxanthin to be the predominant carotenoid in yellow leaves and attributed this to greater stability rather than to synthesis from other carotenoids. In herbaceous species, leaf position and age appear to be important, but Whitfield and Rowan (1974) detected no esterification of the xanthophylls in tobacco leaves; lutein and β-carotene were relatively stable. Lichtenthaler (1969b) demonstrated that plastoquinone 45 is synthesized in mature and senescent leaves of Ficus and is deposited, together with increased carotenoids derived mainly from lutein synthesis, in the plastoglobulite of the plastid stroma.

Anthocyanin synthesis may also occur in autumn leaves, concurrent with plastid pigment degradation; these anthocyanins may be associated with sugar metabolism in dying leaves (Harborne, 1965; Sanger, 1971).

An important aspect of plant development concerns the role of proteins as enzymic and structural components in the cell. Although loss of protein

through proteolysis occurs during leaf senescence (Yemm, 1937, 1949; Wood et al., 1943), Walkley (1940) was able to prove that the decline of protein is a net effect and that as long as chlorophyll is present, protein synthesis can occur in senescent leaves. Accordingly, Chibnall and Wiltshire (1954), on the basis of experiments using heavy nitrogen as a tracer for protein synthesis, proposed that a protein cycle occurred in leaves; thus, protein synthesis would continue, albeit at a reduced rate, in spite of conditions which enhance the rate of protein decomposition (see also Racusen and Foote, 1962). Subsequently, the decline in protein levels in senescence and the concomitant decreased incorporation of amino acids into protein has been observed in a wide range of leaf tissues (see, for instance, Racusen and Aranoff, 1954; Osborne, 1962; Simon, 1967; Wollgiehn, 1967); these changes have been used as indices for the progress of senescence. The decrease in protein synthesis may be due to a reduction in the supply of amino acids as a result of translocation from the leaf to other parts of the plant or through a reduction in the efficiency of protein synthesis (Simon, 1967). These points will be discussed later in greater detail.

The investigations described above were mainly concerned with changes in gross protein levels. Some studies, however, have examined changes in particular protein fractions from specific organelles or other sub-cellular systems. Wood et al. (1943) noted that chloroplast protein was degraded more rapidly than cytoplasmic protein in detached Sudan grass tissue. In tobacco chloroplasts Fraction I protein was degraded more rapidly than Fraction II starting from the time of completion of leaf expansion (Dorner et al., 1957). These results have been generally substantiated in several other senescing systems (Kannangara and Woolhouse, 1968; Treharne et al., 1970; Tung and Brady, 1970). Brady et al. further showed that the rate of turnover of the polypeptides of Fraction I occurred at a very slow rate in wheat leaves. The pattern of decline of Fraction I, consisting largely of ribulose diphosphate carboxylase and accounting for about 40-45% of the chloroplastic soluble proteins was similar in detached and attached wheat leaves after the completion of expansion. Callow (1974) found that incorporation of radio-activity into Fraction I declined several days before the completion of leaf expansion in cucumber and before the decline in Fraction I levels. Ribulose diphosphate carboxylase activity mirrored the changes in Fraction I during leaf development but as the leaves senesced, the enzymatic activity declined at a faster rate than the level of Fraction I. These changes were apparently specific to Fraction I. Callow suggested that since the large sub-unit of Fraction I is synthesized on chloroplastic ribosomes, a decline in polysome content of the chloroplasts may repress

Fraction I synthesis. Further work is, however, necessary to determine the specificity of control of Fraction I synthesis by 80S ribosome activity as compared to other proteins synthesized in the chloroplast.

The proportion of chloroplast protein synthesis, in general, may decline relative to cytoplasmic protein, during apple leaf senescence (Spencer, 1973). In addition, the relative protein content of ribosomes in senescing barley leaves may be reduced (Srivastava and Arglebe, 1967). On the other hand, from observed changes in the specific activity of the proteins of various sub-cellular fractions, Parthier (1964) concluded that mitochondrial proteins were more influenced by senescence than were the proteins of other fractions, although the specific activity of all fractions declined with senescence. Alternatively, histone proteins may be relatively labile (Gupta and Chatterjee, 1971). Senescence of leaves and petals may also be associated with an increase in relatively small protein units (Parups, 1971).

The size of the alcohol-soluble nitrogen fraction (amino acids, amides) normally increases with senescence in detached leaves (Wood et al, 1943; Yemm, 1949; Anderson and Rowan, 1968; Martin and Thimann, 1972). The changes occurring in herbaceous species during natural sequential senescence, however, are less clear. In leaves of woody species, there is a gradual decline in the alcohol-soluble fraction due to translocation during the summer, with a large rise in soluble nitrogen as abscission is approached (Thomas, 1927; Plaisted, 1956; Spencer and Titus, 1972, 1973). It has been suggested that translocation also occurs from the older leaves of herbaceous species (Simon, 1967), possibly limiting synthesis of new protein. Protein levels, however, decline faster in detached leaves where amino acids, released from protein, are presumably still available for protein synthesis. Analysis of the situation is further confounded by the presence of several amino acid pools within the senescing leaf (Tavares and Kende, 1970), and by the observed de novo synthesis of hydrolytic and other enzymes during senescence (see Dove, 1973). Thus, differential decreases in protein synthesis efficiency may be involved, in addition to the limitation of amino acid supply resulting from increased translocation. The decline in protein may also result from a reduced availability of protein assembly sites (Osborne, 1962).

Modern views of development and differentiation place heavy emphasis on the importance of the genetic code and its expression. Accordingly, many studies in leaf senescence have looked for changes in levels, activity and synthesis of DNA and of the various RNA fractions. It has been generally noted that DNA levels change little after the early stages of leaf

development and that DNA is more stable than RNA during senescence, both in the intact leaf (Smillie and Krotkov, 1961; Phillips and Fletcher, 1969) and in excised leaf tissues (Osborne, 1962; Shaw et al., 1965; Srivastava, 1967a). Some disagreement is evident (see Srivastava and Ware, 1965; Garg and Kapoor, 1972) but in no case was DNA less stable than RNA; discrepancies may perhaps be attributable to species differences (Dyer and Osborne, 1971) or to examination of nucleic acid changes in tissues at different stages of senescence, particularly in excised tissues (Callow and Woolhouse, 1973). Srivastava and Ware (1965) noted some incorporation of precursors into DNA of mature leaves, but generally little DNA synthesis was detected (e.g. Osborne, 1967). Furthermore, little change was noted in histone levels, or chromatin or in the ratio of histone:DNA (Srivastava, 1967a, 1968), although leaves of different ages had different histone types predominating (Srivastava, 1971). DNA synthesis was not detected in mature leaves or in old rooted cuttings of Nicotiana rustica (Wollgiehn and Möthes, 1964). In view of recent work on Fraction I synthesis (Callow, 1974), re-examination of the termination of the synthesis of chloroplast DNA might prove of interest. In this connection, Osborne (1967) suggested that the decline in RNA synthesis during senescence was not due to loss of total DNA but rather to a failure of DNA to provide an effective template for RNA synthesis.

A decline in RNA levels during leaf senescence was first noted by Böttger and Wollgiehn (1958). This has subsequently been confirmed for both excised tissues (Smillie and Krotkov, 1961; Osborne, 1962; Cherry et al, 1965; Knight and Quick, 1969) and attached leaves (Hardwick and Woolhouse, 1967; Srivastava and Atkin, 1968; Lynn and Pillay, 1971). As for protein metabolism, the incorporation of precursors into RNA declines progressively during senescence, particularly in excised leaves (Osborne, 1962). The leaves do, however, retain considerable capacity for RNA synthesis (Wollgiehn, 1967) and the length of life of the leaf may depend on its ability to conserve RNA synthesis capability (Krul and Leopold, 1965). The capacity for RNA degradation by enzymes also increases in senescence (see Dove, 1973). In addition, the specific activity of the RNA fraction may increase with senescence (Cherry et al, 1965; Beevers, 1968; Srivastava and Atkin, 1968) i.e. during a phase of net decline in RNA levels.

As fractionation procedures for nucleic acids have improved, qualitative and quantitative changes in the synthesis and levels of the different RNA fractions have been studied. Oota (1964) suggested that ribosomal RNA (r-RNA) was preferentially degraded. Krul and Leopold (1965) detected little synthesis of new RNA in various fractions after completion of growth

of soyabean cotyledons. In peanut cotyledons, on the other hand, Cherry et al. (1965) suggested that the decline in RNA was mainly due to loss of r-RNA and messenger RNA (m-RNA) and demonstrated an increase in soluble RNA (s-RNA). Again, Shaw et al. (1965) detected a rapid loss of nuclear and chloroplastic RNA relative to the loss of nuclear and chloroplastic RNA relative to the loss of nuclear protein and DNA. Other reports indicate that there may be little difference in the rates of the various RNA fractions (Millikan and Ghosh, 1971; Srivastava, 1972). This has been interpreted by Srivastava as indicating control of senescence at the translational rather than at the transcriptional level.

Various messenger-like RNA fractions have been shown to vary with senescence. Wollgiehn (1967) noted a relatively fast decline in the synthesis of a rapidly labelled RNA fraction, tentatively characterised as having messenger-like activity. Srivastava and Atkin (1967) demonstrated that while there was a continuous decline in the amounts of 4S, 16S and 23S, a peak of synthesis of rapidly labelled RNA occurred in the early stages of senescence; this may be associated with a requirement for new protein species at the start of senescence. Similarly, there is an increase in rapidly labelled RNA following oat leaf excision (Udvardy et al., 1969). In contrast, the amount of a tenaciously bound RNA fraction, probably consisting mainly of a messenger-like fraction, declined with age in wheat leaves (Hadziyev et al., 1969). DNA-RNA, another messenger-like fraction, was relatively stable in senescing soyabean leaves (Lynn and Pillay, 1971). There is an obvious need for improved characterisation of these messenger-like fractions before the role of m-RNA in senescence can be critically evaluated. The presence of oligonucleotide degradation products may add further confusion in the present situation (Knight and Quick, 1969).

Wollgiehn (1967) reported that in yellow leaves of Nicotiana rustica, there is an increased incorporation of ^{32}P into a low molecular weight RNA, probably corresponding to s-RNA. Similarly, Lynn and Pillay (1971) found that the specific activity of s-RNA in yellowing leaves increased to a greater extent than did the specific activity of other fractions. Bick and co-workers (1970, 1971) have detected differential senescence- and age-related changes of the various isoaccepting species of transfer RNA (t-RNA - probably the major component of s-RNA) of leucine and tyrosine in soyabean cotyledons. They consider that their data are consistent with Strehler's hypothesis of ageing (1969) in which the initiating events in senescence would consist of the loss of certain translational capacities in the post-mitotic nature phase. These conclusions are generally substantiated by Dyer and Osborne (1971) and also by Venkataraman and De Leo (1972) who

suggest that changes in relative levels of isoaccepting species could occur either by modification of pre-existing t-RNA molecules (see also Shugart, 1972) or by selective degradation of certain types. Further investigation of changes in isoaccepting species of t-RNA in the chloroplast might prove valuable.

Ribosomal RNA has been shown to be more labile than other RNA fractions in some systems (Srivastava and Atkin, 1968; Dyer and Osborne, 1971). Thus in yellow leaves, there may only be about 5% of the r-RNA present in green leaves (Srivastava, 1971) with a consequent low level of ribosomes (Eilam et al., 1971). The correlation between loss of r-RNA and loss of ribosomes may indicate that RNA released during ribosome breakdown is degraded (Srivastava and Arglebe, 1967). Ribosome loss has also been linked with a progressive decline in the percentage of ribosomes present as polysomes (Srivastava and Arglebe, 1967; Lin et al., 1973). Eilam et al. (1971), however, demonstrated that the polysome content of cucumber leaves declined initially but did not vary further during senescence. They distinguished between free and membrane-bound populations of ribosomes and noted that there was a preferential loss of the former during ageing. Both decreased synthesis and increased breakdown of ribosomes probably contribute to the loss of r-RNA and ribosomes.

Chloroplast r-RNA appears to be synthesized only during a limited phase of leaf development, perhaps while the chloroplasts are being formed (Ingle, 1968; Treharne et al., 1970; Paranjothy and Wareing, 1971; Detchon and Possingham, 1972). Moreover, the amount of turnover of plastid r-RNA is much less than that of cytoplasmic r-RNA (Ingle, 1968), although Callow et al. (1972) could detect some turnover of the smaller chloroplastic r-RNA molecule. Preferential reduction in incorporation of precursor into plastid r-RNA (Callow, 1974) could be correlated with a decline in plastid polysome content (Eilam et al., 1971; Callow et al., 1972). The ability of yellowed leaves to regreen may depend on the presence of either an active chloroplastic genome (Dyer and Osborne, 1971) or intact r-RNA in plastid ribosomes or on the ability of these to be re-synthesized by nuclear control (Callow and Woolhouse, 1973).

Enzymes, as vital components in plant growth and development, have been examined in relation to the onset and progress of leaf senescence. Changes in the activities of many enzymes will, of course, merely reflect changes in protein metabolism as a whole. Moreover, activities of enzymes *in vitro* do not necessarily correspond to activities *in vivo*; rather, compartmentalisation and other factors may be of importance in determining actual activity.

Among the best documented groups of plant enzymes are the nucleases, DN ases and RN ases which are thought to be responsible for the degradation *in vivo* of nucleic acids. The role of RN ases in plant development has recently been comprehensively reviewed (Dove, 1973). Increases in RN ase activity have been detected in excised leaf tissue from a wide range of plant species (see, for example, Udvardy *et al.*, 1964; Srivastava, 1968a; McHale and Dove, 1969; Sodek and Wright, 1969). Dove (1973) has divided these increases into 2 phases (a) a rapid rise in RN ase shortly after excision - this is relatively purine specific; and (b) a longer term increase in RN ase which probably contributes to the death in isolated plant tissue. The short term increase is activity appears to be due to a burst of *de novo* enzyme synthesis which ceases after a few hours; this may be initiated by osmotic stress, resulting from detachment or as a direct wounding response. The longer term increase in RN ase which may be associated with the *de novo* synthesis of new RN ase types (Wyen *et al.*, 1971) appears to be more closely related to senescence processes.

Attached, senescing leaves tend to have higher RN ase activities than younger leaves (Reddi, 1959; McHale and Dove, 1968; Lazar and Farkas, 1970). The RN ase activity is progressively higher in leaves further from the apex with a plateau of activity in the oldest leaves (Yatsu and Jacks, 1972). RN ase activity associated with apple leaf senescence appears in the autumn when the photoperiod is less than 12 hours (Spencer and Titus, 1972). Kessler and Engelberg (1962) showed an inverse relationship between cytoplasmic RN ase and RNA during apple leaf senescence, whereas particle-bound RN ase increased as RNA level increased. The soluble RN ase of bean leaves declines in parallel with the loss of chlorophyll and RNA (Phillips and Fletcher, 1969). A similar correlation was demonstrated for RN ase and RNA in barley leaves (Lazar and Farkas, 1970). Phosphomonoesterase and phosphodiesterase may also have RNA degrading properties in *Avena* leaf tissue (Udvardy *et al.*, 1969). In addition some enzymes may be capable of hydrolysing both RNA and DNA (see Dove, 1973). Other DN ases active in senescence have not been widely recorded. Srivastava (1968b) noted an increase in the activities of chromatin-associated RN ase and DN ase of excised barley leaves, but it is not clear to what extent these increases are attributable to excision effects.

Changes in proteolytic enzyme activity have also been recorded during senescence. Balz (1966) noted an increase in sedimentable protease activity due to *de novo* synthesis in excised tobacco leaves. Similarly, Martin and Thimann (1972) have reported increases in 2 proteinases in detached senescing oat leaves. In attached leaves of *Perilla*, however, there was no change in

protease activity with leaf age (Kannangara and Woolhouse, 1968). The possible importance to senescence of changes in specific proteases remains apparently uninvestigated.

Peptidase increases in excised leaf tissue (Anderson and Rowan, 1968; Atkin and Srivastava, 1969). Hochkeppel (1973) has isolated an endopeptidase from yellowing tobacco leaves; no activity was detectable in green leaves. The enzyme was capable of hydrolyzing 30% of the protein of the lamellar system and thus could accelerate chlorophyll degradation.

Peroxidase has been shown to increase during the senescence of attached cucumber cotyledons (Simon, 1967) and attached and detached leaves of tobacco and barley (Kawashima et al., 1967; Lazar and Farkas, 1970). Acid phosphatase activity showed some qualitative, but little quantitative change, during tea leaf senescence (Baker and Takeo, 1973), but the enzyme increased in Rhoeo leaf tissue (De Leo and Sacher, 1970). An increase in acid pyrophosphatase was noted in the later stages of ageing in excised rice leaves (Mishra et al., 1973). In contrast, acid phosphatase in Perilla leaves declined progressively with leaf age (Kannangara and Woolhouse, 1968). Senescence-related changes have also been noted in the following hydrolytic enzymes:- esterase activity (Balz, 1966), chlorophyllase (Phillips et al., 1969), β-1,3-glucan hydrolase (Moore and Stone, 1972) and, in abscission zones, pectin methyesterase and cellulase (Osborne 1958, 1968a). Changes in the enzymes of carbohydrate metabolism have been followed by Simon (1967), Kannangara and Woolhouse (1968), Lazar and Farkas (1970), Spencer and Titus (1972) and Fair et al. (1973). Similarly, studies of changes in activities of enzymes involved in nitrogen metabolism have been made by Simon (1967), Kannangara and Woolhouse (1968), Hedley and Stoddart (1971, 1972) and Spencer and Titus (1972).

The most widely used parameters of leaf senescence are thus the loss of RNA, protein and chlorophyll. The degree of correlation between the decline of these fractions, however, is far from clear. Further, analysis is likely to be confounded by leaf excision, age and species, as well as by the possible differential loss of RNA and protein from different cellular sites. Chlorophyll and protein may decline at the same rate (Michael, 1936; Smillie and Krotkov, 1961; Martin and Thimann, 1970; Back and Richmond, 1971). Alternatively, chlorophyll may be lost faster than protein (Knight and Quick, 1969) or vice versa (Simon, 1964; Hardwick and Woolhouse, 1967; Spencer and Titus, 1972). Contrasting reports have also been presented by Osborne (1962) and Smillie and Krotkov (1961) in regard to the rate of loss of RNA relative to the decline in protein and chlorophyll.

The respiration rate of attached leaves and cotyledons tends to decline gradually during senescence (Leinweber and Hall, 1959; Yemm, 1965; Draper and Simon, 1971), although some reports suggest that respiration rate may increase (Arney, 1947) or remain more or less constant (Kannangara and Woolhouse, 1967) as leaves senesce. Some leaves may exhibit a respiratory climacteric peak in aerobic conditions (Wood et al., 1943) and this peak may be associated with biochemical events occurring immediately prior to leaf fall (Katterman and Hall, 1961). MacNicol and co-workers (1973a,b) have suggested that the climacteric rise in senescent leaves is maintained by a large increase in aerobic glycolysis; this is substantiated by a rise in respiratory quotient and in citrate level during the climacteric. The climacteric is then followed by a general collapse of cell organization. Detached leaves also show a rise in respiration rate shortly after excision, with a subsequent decline (Yemm, 1965; Udvardy et al. (1964); MacNicol, 1973). It is not clear whether this rise is of a climacteric nature, although Udvardy et al. suggest that significant changes in biochemical pathways may be involved.

Photosynthetic rate is thought to decline with leaf age from the time of completion of leaf expansion (Kannangara and Woolhouse, 1968; Hernandez-Gil and Schaedle, 1973). Vaclavik (1973), on the other hand, showed that CO_2 absorption began to decline before the completion of expansion; he attributed the decline in photosynthesis with leaf age to increased internal resistance. Fair et al. (1973) noted a decline in ribulose diphosphate carboxylase activity with leaf age and thus they generally substantiate the findings of Woolhouse and co-workers (loc. cit.) for changes in Fraction I. Back and Richmond (1971) observed a correlation between chlorophyll and total protein levels and the amount of CO_2 fixation during leaf senescence, whereas Harris and Arnott (1973) could find no such correlation - indeed, chlorophyll decline here preceded a reduction in the rate of oxygen evolution by at least a week. Hernandez-Gil and Schaedle (1973) noted a correlation between the decline of CO_2 fixation in isolated chloroplasts and the rate of ATP synthesis and thus concluded that the decline in photosynthesis resulted from changes in the soluble enzyme fraction and in the synthetic performance of the membrane-bound ATP synthesizing system.

Grob and Csupar (1967) suggested that lipid changes in autumn leaves of Acer occurred in 3 phases (1) lipid degradation, (2) formation of esters (e.g. phytyl linolenate) and (3) the accumulation of lipid intermediates. Draper (1969) noted an early loss of galactolipid derived from the chloroplasts of senescing cucumber cotyledons. This correlated with an increase in linolenic acid in the free fatty acid fraction, loss of chlorophyll and

ultrastructural changes in the chloroplast as early features of senescence. Later in senescence, a decrease in sulpholipid and an increase in sterol glycoside occurred; there was little change in phosphatidyl-choline and -ethanolamine. A decrease in lipid biosynthesis also occurred during senescence, the reduction in ^{14}C-acetate incorporation into various lipid fractions coinciding with a decline in photosynthesis (Draper and Simon, 1970). The changes in the free fatty acid fraction - also observed by Newman et al. (1973) - did not occur early enough in senescence to account for an observed decline in respiration associated with senescence (Draper and Simon, 1971). Ferguson and Simon (1973) showed that, at a late stage in senescence (after the disappearance of the chloroplastic glycolipids) the phospholipids are lost from cell membranes, probably resulting in a reduction in membrane integrity, disruption of the vacuoles and increase in apparent free space.

Similar changes in cellular free space and membrane integrity have been observed by Sacher (1957, 1959) in senescing bean endocarp and Rhoeo leaf tissue. These changes were accompanied in endocarp segments by rapid plasmolysis, conspicuous exudation on the segment surface and leakage of cellular components. The loss of respiratory activity of the segments may be associated with the breakdown of membrane systems, although Eilam (1965) indicated that changes in permeability occurring early in senescence were independent of the later pattern of respiratory decline. Increased leakage of electrolytes, and a rise in free space and sodium export have been noted in senescent bean leaves (Das, 1968; Jacoby et al., 1973). Pooviah and Leopold (1973) were able to delay the senescence-associated increases in apparent free space and hydraulic permeability by the application of calcium; they suggest that calcium may act by maintaining cell membrane integrity.

Direct observations of changes in cell membranes and of other organelles during leaf senescence have become possible using electron microscopy. Ultra-structural changes in plant senescence have recently been comprehensively reviewed by Butler and Simon (1971). Molisch (1938) observed the appearance of fat droplets during the disorganisation of the chloroplast in yellowing leaves. More recent work has examined these changes in more detail and has tried to relate temporal changes in the chloroplast to those in other parts of the cell. It is a matter of some controversy as to where in the cell the earliest ultra-structural events during senescence occur. Early changes were noted in the integrity of the endoplasmic reticulum in detached senescing wheat leaves (Shaw and Manocha, 1965). Similarly, in detached tomato leaves, changes in the endoplasmic reticulum, tonoplast and plasmalemma occurred before abnormalities in the chloroplast were detectable (Roux and McHale, 1968). More generally, however, the earliest changes

have been noted in chloroplasts (Barton, 1966; Dennis et al., 1967; Stetler and Laetsch, 1968; Dodge, 1970; Mlodzianowski and Ponitka, 1973). These changes may be associated with swellings and distortions of the thylakoids (Barton, 1966; Harris and Arnott, 1973); in particular stromal lamellae may be preferentially affected (Ljubesic, 1968; Yoshida, 1970). In addition, phytoferritin particles are laid down at an early stage of senescence in bean leaves (Barton, 1970). Chloroplasts may also shrink during senescence (Srivastava, 1967; Ljubesic , 1968; Dodge, 1970; Mlodzianowski and Kwintkiewicz, 1973) and become more spherical (Barton, 1966).

Mittelheuser and Van Steveninck (1970) detected an early loss of chloroplastic ribosomes in detached and attached senescing wheat leaves. Markedly reduced numbers of chloroplastic ribosomes in senescent tissues were also noted by De Greef et al. (1971 and Mlodzianowski and Kwintkiewicz (1973). Ribosomes in general were found to be quickly lost during senescence (Butler, 1967) although Ragetli et al. (1970) noted the persistence of both free and bound ribosomes in tobacco tissue.

A characteristic feature of chloroplast senescence is the increased prominence of the plastoglobuli (osmophilic, lipid-containing globules), first observed by Molisch (1938). Such globules are an apparently normal feature of the chloroplast stroma (Granick, 1961) and they increase in number and/or size (possibly through fusion) during senescence (Lichtenthaler, 1969a). The plastoglobuli may result from the breakdown of plastid membranes (Ikeda and Ueda, 1964; Barton, 1966); their lipid contents are similar to those occurring in the thylakoids, although plastoglobuli from senescent plastids may contain a higher proportion of carotenoids than those from fully functional plastids (Lichtenthaler, 1969b). A role for the globules in thylakoid decay is also suggested by their observed proximity to degenerating thylakoids (Barton, 1966; Dennis et al., 1967). Loss of chloroplast integrity has been correlated with the loss of chlorophyll and protein (Shaw and Manocha, 1965; Butler, 1967), although no such correlation could be detected by Roux and McHale (1968).

The nucleus has been reported to be relatively stable (Mittelheuser and Van Steveninck, 1970), although abnormalities of the nucleus and its envelope have been noted (Srivastava, 1967a; Ragetli et al., 1970). The presence of the nucleus may speed chloroplast senescence (Yoshida, 1961); the significance of this observation requires further investigation.

The mitochondria are relatively persistent (Butler, 1967; Dennis et al., 1967; Mittelheuser and Van Steveninck, 1970), although some shrinkage may occur (Butler, 1967) as well as alterations in cristae structure (Ragetli et al., 1970; De Vecchi, 1971).

Persistence of the tonoplast and plasmalemma in relation to other cellular structures (Butler, 1967; Dodge, 1970) suggests that the role of lysosomal-type hydrolytic enzymes may be restricted to a "mopping up" late in senescence (Barton, 1966). Shaw and Manocha (1965), however, point out that leakage of enzymes through the tonoplast could occur while this membrane is still apparently intact.

Light (irradiation in the visible part of the electromagnetic spectrum) has been shown to delay changes in cellular ultra-structure (Mittelheuser and Van Steveninck, 1970) and this may be associated with photosynthesis since a more rapid depletion of chloroplastic starch occurs during senescence in the dark.

It is not clear to what extent ultra-structural events in detached tissues differ from those in naturally senescent leaves, although some differences have been detected (Butler, 1967; Ragetli et al, 1970).

Related studies have examined ultra-structural changes in ageing plant embryos (Berjak and Villiers, 1972), flowers (Matile and Winkenback, 1974) and fruits (Harris and Spurr, 1966).

Senescence of leaves is also associated with changes in mineral content (Leinweber and Hall, 1959; Oland, 1963), a decrease in sugars and shikimic acid (Moore, 1965; Spencer and Titus, 1972) and an increase in total acidity (Facey, 1950) and urea content (Parups, 1971). In addition, stomatal resistance appears to increase with leaf age (Gee and Federer, 1972; Solarova, 1973). Senescent leaves may also become more susceptible to microbial attack (e.g. Balazs et al., 1973).

The symptoms of senescence described above can be stimulated or delayed by environmental conditions and exogenous hormonal and chemical factors. The effect of environmental conditions on leaf longevity has long been appreciated (Theophrastus, 285 B.C.) and, notably, variation in light quality and quantity may greatly modify senescence. As discussed above, photoperiodic conditions can alter leaf longevity in herbaceous plants both directly (Schwabe, 1970) and indirectly through induction of the reproductive phase (Garner and Allard, 1923). In woody species, the shortening daylength in autumn may be a crucial factor in determining the time of leaf fall (Olmsted, 1951); artificial illumination is capable of delaying normal leaf fall, perhaps by supplementing the natural photoperiod (Matzke, 1936). The shading of old basal leaves by younger leaves can stimulate the senescence of the former, perhaps by reducing the effective photoperiod or by reducing their photosynthetic rate (Hopkinson, 1966). The importance of photosynthesis in delaying senescence of detailed organs has been demonstrated by Goldthwaite

and Laetsch, 1967; Lewington and Simon, 1969; Mittelheuser and Van Steveninck, 1970). Light may also, however, have another role in retarding senescence (Basler, 1966; Haber et al., 1969b). The senescence of tobacco leaf discs can be delayed by red light and this effect is reversible by far-red light. Similar observations, apparently involving phytochrome, have been made using intact Marchantia thalli (De Greef et al., 1971) and detached rice leaves (Mishra and Kar, 1973). In addition, the capacity for phytochrome-mediated development of chloroplasts declines with leaf age in etiolated barley seedlings (Stobart et al., 1972).

Sucrose can retard senescence of detached leaf tissue in the dark (Goldthwaite and Laetsch, 1967; De Vecchi, 1971). Similar effects may be obtained using other sugars (Lewington and Simon, 1969; Khudairi, 1970) and these are probably due to the sugar partially substituting for light in the provision of respiratory substrate. Sucrose may stimulate senescence in the light (Moore et al., 1972).

Senescence can be artificially stimulated by the addition of NAD and nicotinic acid (Waygood et al., 1968) ascorbic acid (Garg and Kapoor, 1972) or amino acids (Shiboaka and Thimann, 1970) and delayed by calcium (Poovaiah and Leopold, 1973) or nickel ions (Mishra and Kar, 1973).

Environmental stresses can apparently induce or stimulate senescence (Shah and Loomis, 1965; Prisco and O'Leary, 1972; Itai et al., 1973). Ben-Zioni et al. (1967) suggest that this effect may be due to a reduced supply of root cytokinins. In addition, senescence can be delayed by anaerobic conditions (Wood et al., 1943; Thimann et al., 1970).

Flowers and fruits, like leaves, are determinate organs. Moreover, in the pattern of events preceding their death, there are similarities to the processes of leaf senescence. Although the importance of the climacteric in leaves is uncertain, there are obvious parallels between fruits and leaves in the selective degradation and/or synthesis of certain pigments (Katayama et al., 1971; Eilati et al., 1972), and enzymes (Dilley, 1970). Similar to leaf senescence, fruit ripening is also associated with changes in membrane integrity (Sacher, 1957), fine structure (Bain and Mercer, 1964) and hormone activity (Steward and Mohan-Ram, 1961; Maxie and Crane, 1967; Goldschmidt, 1973). Similar parallels exist between leaf and flower senescence (see, for instance, Matile and Winkenback, 1971; Mayak and Halevy, 1972; Bredermeijer, 1973).

At a later stage in the senescence of leaves - and also of fruits and flowers - abscission normally occurs at a discrete zone at the base of the organ. Under natural conditions, senescence is a pre-requisite for the

initiation of the enzymic and other changes involved in abscission (Osborne, 1968a); indeed, senescent leaf tissue may produce a factor responsible for triggering these changes (Osborne, 1955; Osborne et al., 1972). Abscission can also be modified by addition of plant growth substances; in vivo, these may affect abscission through modulation of leaf and petiole senescence processes.

The possible involvement of hormones in the regulation of leaf senescence was first suggested by the work of Chibnall (1939, 1954) who noted that the development of adventitious roots on the petioles of detached leaves delayed the loss of chlorophyll and protein from the leaf. He postulated the existence of a "root factor" which could delay senescence. In recent years, many studies have been made concerning the possible hormonal modification of leaf senescence and although species differences exist for the responses to different hormones (Fletcher and Osborne, 1965; Phillips, 1971), various mechanisms have been suggested as a basis for hormonal control of senescence (Osborne, 1967; Srivastava, 1967b; Paranjothy and Wareing, 1971). Hormones have also been implicated in the control of ageing processes in animals (see, for instance, Bellamy, 1967; Finch, 1972). Detailed correlations between critically identified endogenous plant hormones and senescence are still required, however. In addition, in interpreting the effects of applications of exogenous hormones, the uptake, metabolism and distribution of the hormone needs to be considered.

The possible role of cytokinins in modifying leaf senescence was first demonstrated by Richmond and Lang (1957) who noted that the artificial cytokinin, kinetin, could delay protein and chlorophyll loss from detached Xanthium leaves. This was substantiated in an independent investigation (Person et al., 1957) in which benzimidazole delayed the loss of chlorophyll and the increase in amino acids in detached wheat leaves.

Möthes and co-workers (1961a, b; 1963) suggested that cytokinins delayed senescence by mobilization of nutrients to cytokinin-rich (or cytokinin-treated) areas of the leaf from other parts of the leaf or plant. Accumulation of radioactive amino acids could occur against a concentration gradient and this was not directly dependent on the creation of a metabolic sink. Möthes therefore suggested that the effect of kinetin in maintaining protein synthesis was an indirect one. Similarly, Gunning and Barkley (1963) demonstrated that kinetin applied as drops to oat leaves caused the accumulation of ^{32}P and ^{14}C-glycine in the treated area. This accumulation was not observed in older leaves and only occurred in a basipetal direction. Furthermore, accumulation appeared to be accompanied by an inhibition of growth and a stimulation of senescence in untreated parts of the leaf and in other leaves (Leopold, 1963;

Leopold and Kawase, 1964). Leopold suggested that the mobilization effects due to natural cytokinins could provide endogenous stimuli for leaf senescence by restricting accessibility of nutrients. Natural cytokinins are present in root exudates (Kende, 1964) and thus a reduction in their supply to the shoot may be important in inducing senescence (Sitton et al., 1967). Alternatively, the supply of cytokinins from the roots may be diverted from the mature leaves to the growing regions, thus inducing senescence of the former (Wareing and Seth, 1967). Different types of cytokinins may differ in their mobilization effects (Van Staden, 1973).

Investigations using excised leaf tissue suggest, however, that the senescence-delaying effects of cytokinins cannot be attributed entirely to mobilization effects. In particular, kinetin can delay senescence and promote RNA and protein synthesis in leaf discs of Xanthium, i.e., tissue in which mobilization from other parts of the plant could not occur (Osborne, 1962). Furthermore, application of kinetin to one half of dumb-bell-shaped leaf discs indicated that the effects in the treated half were not dependent on mobilization from the untreated half. These results were generally substantiated by Suguira et al. (1962) and Wollgiehn and Parthier (1964).

Oota (1964), attempting to reconcile the apparently conflicting views of Osborne and Möthes and co-workers, suggested that both concepts of kinetin action could be correct if kinetin had 2 independent cellular loci. Thus, accumulation in response to kinetin treatment could be coupled with a stimulation or maintenance of protein and RNA synthesis.

It was suggested that cytokinins had little effect in delaying senescence in intact plants (Engelbrecht, 1964; Muller and Leopold, 1966; Atkin and Srivastava, 1968). Kulaeva (1962) attributed this to the presence of high levels of endogenous cytokinins derived from the roots. Fletcher and co-workers however, were able to retard senescence in primary leaves of intact bean plants by treatment with benzyladenine (BA), which delayed chlorophyll loss, prevented the decline in photosynthesis and maintained leaf growth (Adepipe et al., 1971). In particular, BA treatment was associated with an increased retention of photosynthate (Fletcher et al., 1970), accompanied by an increased turnover (Adepipe and Fletcher, 1970) and utilization of metabolites for biosynthetic processes (Fletcher, 1969). These effects, moreover, were apparently independent of the mobilization of metabolites from the untreated parts of the plant (Adepipe and Fletcher, 1970, 1971). Repeated applications of BA could, however, delay senescence of untreated trifoliate leaves (Adepipe and Fletcher, 1970); thus the possibility remains that the lack of directed transport may be due to translocation of BA away from the treated leaf.

Following the earlier reports of cytokinin effects on RNA synthesis (Osborne, 1962) the effects of cytokinin on specific RNA fractions have been examined. Carpenter and Cherry (1966) could find no effect of BA on specific RNA fractions and suggested that the effect of BA in maintaining RNA synthesis may be at the nucleotide precursor level. Similarly, Burdett and Wareing (1968) noted an increased incorporation into various RNA fractions following cytokinin treatment. Wollgiehn (1967) suggested that the important effect of cytokinin may be to maintain the normal spectrum of RNA synthesis. Osborne (1965), however, suggested that the action of kinetin could be directed through an effect on DNA-controlled RNA synthesis (possibly m-RNA). Thus the effect of kinetin may be to delay the progressive repression of genes thought to be associated with senescence. Alternatively, cytokinins may repress the synthesis of degradative enzymes (Srivastava, 1967a,b). Srivastava (1967a) also noted that kinetin delayed the loss of ribosomal components. Paranjothy and Wareing (1971), on the other hand, considered that the primary effect of kinetin was not on r-RNA, but rather on polydisperse RNA and s-RNA. In addition, cytokinin may act to delay RNA degradation. This would be in general agreement with the observation that BA can stimulate both RNA synthesis and degradation in Lemna i.e., RNA turnover is increased (Trewavas, 1970). Of unresolved significance is the observation that certain t-RNA species contain adenine derivatives with cytokinin-like properties (Zachau et al., 1966). Cherry and Anderson (1970) have postulated that free cytokinins may delay senescence by acting as competitive inhibitors of nucleases binding to cytokinin-like moieties in t-RNA molecules containing cytokinins.

Pre-labelling studies, in which the loss of radioactivity from labelled protein is studied in the presence or absence of cytokinin, appear to indicate that the primary effect of the hormone is to delay protein degradation rather than to maintain protein synthesis (Kuraishi, 1968; Tavares and Kende, 1970). This conclusion has been substantiated using other methods (Atkin and Srivastava, 1969; Trewavas, 1972; Martin and Thimann, 1972).

Cytokinins can apparently modify the activities of various enzymes thought to be associated with senescence. In particular, Srivastava (1968) showed that kinetin could reduce the senescence-linked increases in chromatin-associated nucleases in excised barley leaves. Similarly, BA delayed the increase in RN ase in excised tomato leaflets (McHale and Dove, 1969) and this was apparently associated with changes in specific nucleases in excised Avena leaves (Wyen et al., 1972). The short term increases in RN ase level, indicated earlier, may be of significance here (Dove, 1971). In contrast, in intact bean plants, cytokinin d elayed the senescence-linked decreases in

both RN ase and chlorophyllase activity, as well as of their respective substrates.

Anderson and Rowan (1968) noted that cytokinin delayed the decrease in aminoacyl-t-RNA synthetase and the increase in peptidase associated with senescence, but these changes did not occur early enough to account for the inhibition by cytokinin of changes in chlorophyll and alcohol-soluble nitrogen. Cytokinins also delayed senescence-linked changes in β1,3-glucan hydrolase (Moore and Stone, 1972) and glucose-6-phosphate dehydrogenase (Udvardy et al., 1964). Cytokinins have also been reported to competitively inhibit glycolytic kinases (Tuli et al., 1964) and to depress respiration rate (Dedolph et al., 1961; Shaw et al., 1965).

Cytokinins may act preferentially on the chloroplast to delay degenerative processes (Shaw and Manocha, 1965; Harding et al., 1968; Yoshida, 1970; Mlodzianowski and Ponitka, 1973). In addition, Mittelheuser and Van Steveninck (1970) found that kinetin can delay the loss of 70S ribosomes and starch grains. Thus the primary of cytokinins may be to maintain or stimulate protein synthesis on 70S ribosomes (Dennis et al., 1967; Brady et al., 1971). Richmond et al. (1971), on the other hand, suggest that the major effect of cytokinins on the chloroplast is on hydration and membrane permeability. Conflicting results have been obtained from studies with albino mutant plants; Shaw and Srivastava (1964) found kinetin to be ineffective in albino barley, whereas cytokinins could defer senescence in albino soyabean plants (Krul and Leopold, 1965) but they were ineffective on isolated chloroplasts unless a cytoplasmic factor were also present.

Reports of the effects of natural cytokinins in regulating senescence and of changes in cytokinin levels during senescence are limited. In addition, there may be significant differences in response of plant tissue to applications of synthetic and natural cytokinins (Varga and Bruinsma, 1973). Van Staden (1973) observed changes in butanol-soluble cytokinins prior to senescence of Streptocarpus leaves and also suggested a causal relationship between the loss of chlorophyll and the decline in cytokinins resulting from short-day treatment of Xanthium plants (Van Staden and Wareing, 1972). In addition, applications of cytokinins may influence the levels of other endogenous growth regulators (Conrad, 1965 - cited by Sheldrake, 1973; Chin and Beevers, 1970).

Shoji et al. (1951) tested auxin levels in bean leaves of different ages and found an initial sharp decline in auxin in the leaves to a plateau, which persisted until abscission. Moreover, the level of diffusible auxin from a leaf correlated with the longevity of the leaf (Wetmore and Jacobs, 1953).

It thus appears that senescence is associated with a decline in diffusible auxin levels (see also Böttger, 1970) and a reduction in the polarity of auxin transport through petioles of different ages (Jacobs et al., 1966). In contrast, however, Wheeler (1968) detected more auxin in senescent than in non-senescent bean leaves and Sheldrake and Northcote claimed that auxin levels increased after leaf detachment, possibly derived from tryptophan released on proteolysis (Sheldrake, 1973).

The ability of auxin to delay leaf senescence may be largely restricted to woody species; this may be associated with differences between the control mechanisms for sequestral and synchronous senescence (Phillips, 1971), although Sacher (1959) was able to delay the senescence of Rhoeo leaf tissue using auxin. In addition, IAA delayed the increases in cell permeability and tissue free space, as well as other changes, in bean endocarp. Similar to early work with cytokinins, application of auxins as discrete spots to leaf tissue of Euonymus and Prunus serrulata-senriko could cause accumulation of radioactive nutrients and increased nitrogen levels in the treated areas as well as stimulation of senescence in adjacent untreated areas (Osborne, 1959; Osborne and Hallaway, 1959). Similar results were reported by Booth et al. (1962). Possibly, auxin acts to maintain incorporation of amino acids into protein and that senescence proceeds when auxin is limiting (Osborne and Hallaway, 1960, 1964). Auxin also maintains the incorporation of RNA precursors (Sacher, 1967; Osborne, 1967) and this effect did not appear to be dependent on mobilization from other parts of the leaf (Osborne, 1967). Auxin may also delay the rise in RN ase in excised leaf tissue (Sacher, 1969) and is apparently effective in delaying both short and long-term increases in enzyme activity (Dove, 1971).

Brian et al. (1959) demonstrated that senescence could be delayed in some woody plants by gibberellin sprayed onto the leaves; in other species, however, gibberellin was apparently without effect. GA_3 delayed the loss of chlorophyll, protein and RNA in dandelion leaves and the action of the hormone may be to maintain the DNA fraction as a functional template for DNA-dependent RNA synthesis (Fletcher and Osborne, 1965). These findings have been largely substantiated by Beevers (1966, 1968) in studies using Nasturtium leaves; GA could also delay the loss of carbohydrate. The GA-induced delay of senescence has been used as the basis for bioassays of endogenous GA using Rumex species (Whyte and Luckwill, 1966; Fletcher and Osborne, 1966). The effects of gibberellin inhibitors (e.g. CCC, B-995 and AMO-1618) on senescence processes appear to depend on inhibitor concentration and plant species (Ruddat and Pharis, 1966; Beevers and Guernsey, 1967).

Definitive studies of changes in the levels of specific gibberellins in relation to senescence are lacking, although there is evidence to suggest that gibberellin levels fall in old or detached leaves (Fletcher et al., 1969; Chin and Beevers, 1970). Goldthwaite (1970) has expressed doubt as to whether or not the apparent endogenous concentrations of gibberellins in mature Rumex leaves are sufficient to prevent the onset of senescence.

The effects of leaf senescence on ethylene (C_2H_4) production are unclear from the literature. Zimmerman et al. (1931) and Hall (1951) suggest that C_2H_4 production by senescent leaves is reduced relative to mature or green leaves. However, more recent data of Morgan and McAfee (1970), Beyer and Morgan (1971), and Jackson et al. (1972) indicate that production of C_2H_4 by senescent leaves is several fold higher than by green leaves. It is evident, however, that exogenous C_2H_4 can stimulate the ripening of fruit and the senescence of some leaves and flowers (Maxie and Crane, 1967; Parups, 1971; Mayak and Halevy, 1972). Moreover, stimulation of senescence in certain circumstances by auxin may be mediated through increased C_2H_4 production (Maxie and Crane, 1967; Osborne, 1968b).

The plant growth hormone, abscisic acid (ABA), has been shown to occur in a wide range of species and plant tissues and has been implicated in the regulation of leaf senescence. The physiology and biochemistry of ABA, together with the history of its isolation have been the subject of reviews by Addicott and Lyon (1969) and Wareing and Ryback (1970).

Abscisic acid is the accepted trivial name (Addicott et al., 1968) for 3-methyl-5-(1'hydroxy-4'-oxo-2',6',6'-trimethyl-2'-cyclohexen-1'-yl)-cis, trans-2,4-pentadienoic acid. The natural compound has one centre of asymmetry, melts at 160°C and is designated (+)-ABA due to the dextrorotatory properties of its solution at the D-line of sodium (Cornforth et al., 1966). Its absolute configuration has now been designated as (S) (Oritani and Yamashita, 1972; Ryback, 1972). Most investigations, however, have used the synthetic acid, which, in contrast to the natural compound is a racemate RS-(±)-ABA, with a higher melting-point, 190°C, and a lower solubility than the natural form. The (+)- and (-)- enantiomers, as components of the racemic mixture differ chemically only in their effect on polarized light. In addition, ABA can exist as 2 geometrical isomers, cis, trans (2,e) and trans, trans (e,e) dependent on the configuration around the 2-double bond in the side chain (Addicott and Lyon, 1969). The natural existence of the 2-trans isomer has been demonstrated conclusively (Milborrow, 1970a) although other reports of its existence may result from

photolytic isomerisation of the cis, trans isomer which can occur as a result of exposure in vitro to ultra-violet radiation or to sunlight (Mousseron-Canet et al., 1966).

Several methods are now available for critically identifying ABA in purified extracts by chemical assay. (+)-ABA has a unique optical rotatory dispersion (O.R.D.), exhibiting a very intense cotton effect with a large, positive extremum at 289 nm, passing through zero at 269 nm and swinging to a very large negative extremum at 247 nm (Cornforth et al., 1966). The Cotton effect is so large that in a well purified solution, in which interference from other substances is negligible, O.R.D. can be used as a quantitative estimate for very small quantities of (+)-ABA (0.2 µg in 0.7 ml of solvent) by calculation from the measured amplitude (Milborrow, 1967, 1968). A similar assay technique, circular dichroism (C.D.), dependent on the specific absorption properties of optically active molecules, has also been used for determination of ABA levels and has been reported to give improved resolution relative to O.R.D. (Milborrow, 1967). Differential absorption of the 2 circular polarised beams of opposite chiralities, which compose a plane-polarised beam, results in a rotated elliptical polarised beam: C.D. is thus measured, in terms, either of the difference in absorption between the circular polarised beams or of the ellipticity to which it gives one. From the amplitude of the curve constructed by measuring one of these parameters as a function of wavelength, the quantity of non-racemic ABA present in the solution can be estimated.

Using the O.R.D. and U-V characteristics of ABA, Milborrow (1967) has developed a technique for assessing the high losses of ABA which normally occur during extraction and purification. Based on isotopic dilution methods, Milborrow's "racemate dilution" technique involves the addition of racemic ($\pm$)-ABA to the crude extract which can be measured by U-V absorption after purification. (+)-ABA remaining in the extract can be determined by O.R.D. and hence the loss of endogenous ABA from the original extract can be determined.

Addicott and Lyon (1969) have drawn attention, however, to the stringent purity requirements as a disadvantage associated with spectropolarimetric methods of determining ABA.

Davis et al. (1968) were able to detect 0.025 µg of trimethylsilyl derivatives of ABA using gas-liquid chromatography (G.L.C.) as a chemical assay for ABA after prior partial purification of the acidic ether extract on a carbon:celite column. More recently, however, the use of trimethylsilyl derivatives has been criticised (Most et al., 1970). Alternatively, methyl esters of ABA resulting from diazomethane treatment of extracts can

be used in G.L.C. (Lenton et al., 1968). The ability of G.L.C. to separate 2-cis and 2-trans isomers of ABA allowed the use of 2-trans ABA as a means of internal standardisation, both for losses of ABA during extraction and for determination of relative retention times on G.L.C. columns (Lenton et al., 1971). Development of electron capture techniques for gas chromatography of ABA has both reduced the need for such high degrees of purification and lowered the limits of detection (Seeley and Powell, 1970). Unequivocal demonstration of the presence of ABA has also been possible using mass spectrometry linked to G.L.C. (Gaskin and MacMillan, 1968).

ABA can also sometimes be identified by its U-V absorption properties, but this is dependent on a high degree of purification (Milborrow, 1967). In addition, Martin and Nishijima (1972) have used the bathochromic shift in the U-V absorption spectrum of ABA, resulting from a change in pH of the solution, as a means of identifying ABA. Mallaby and Ryback (1972) have devised a qualitative colour test for ABA, based on the intense transient violet colour produced when ABA is heated for a few minutes with a formic acid-hydrochloric acid mixture and then treated with sodium hydroxide.

Improvements to ABA extraction and determination procedures have also been suggested by Antoszewski and Rudnicki (1969). Asmundson et al. (1969), Browning et al. (1970) and Little et al. (1972).

The presence of ABA in various higher plant species and organs has been summarised by Milborrow (1967, 1968) and Wareing and Ryback (1970). More recent demonstrations of the presence of ABA, based on chemical assay(s), include flower buds of coffee (Browning et al, 1970), young stems of Pinus radiata (Jenkins and Shepherd, 1972), immature fruit and syrup of carob (Most et al., 1970), leaves of Bryophyllum daigremontianum (Gaskin et al., 1973) and of vine (Loveys and Kriedemann, 1973) and hyacinth bulbs (Nowak et al., 1973).

Many other reports of the presence and measurement of ABA have been based on bioassay techniques. In particular, bioassays involving the acceleration of abscission (Addicott et al., 1949), inhibition of the growth of coleoptile segments (Nitsch and Nitsch, 1956) and inhibition of seed germination (Gabr and Guttridge, 1968) have been most widely used. Although Milborrow (1967) was able to attribute all of the inhibiting activity in his extracts to ABA, the possibility remains, however, that other components may contribute to or mask the inhibiting effects of ABA in these bioassays.

An interesting addition to the range of available bioassay techniques has been reported by Fuchs et al. (1972), based on immunological assay.

Abscisic acid was originally isolated from cotton fruits as an abscission accelerator (Okhuma et al., 1963). Indeed, in some explants, ABA is effective in promoting the formation of apparently normal petiolar abscission (Pieniazek, 1971) and it was thus thought that ABA might have commercial potential as a regulator for defoliation and fruit drop. It is now evident, however, that ABA is not effective in explants of all species and, moreover, is frequently ineffective when used as a spray for whole plants (El-Antably et al., 1967). High and repeated doses of ABA may be needed to get constant effects in intact plants (Smith et al., 1968); positional effects of applications in relation to the abscission zone may also be important (Leopold, 1967). Nevertheless, changes in ABA levels have been correlated with the onset of fruit and leaf abscission (Smith, 1969; Davis and Addicott, 1972; Davison and Young, 1974). In addition, ABA can induce the rises in cellulase and C_2H_4 associated with abscission in explants (Cracker and Abeles, 1969). Böttger (1970) suggested that the relationship between IAA and ABA flux through the petiole may be important in regulating abscission. Furthermore, Ingersoll and Smith (1970) noted close agreement between the rate of movement of an abscission stimulus and that of ABA. Similarly, Chang and Jacobs (1973) have pointed out certain similarities between senescence factor (Osborne, 1955) and ABA. Osborne et al. (1972), however, have produced good evidence to indicate that senescence factor is not ABA. Osborne (1968b) suggested that the effect of exogenous ABA on abscission is probably mediated through a stimulation of the senescence of leaf or pulvinar tissue, perhaps with a consequent increase in ethylene production (Jackson and Osborne, 1972).

ABA is claimed to be capable of simulating the effects of short days in inducing the cessation of extension growth and the formation of resting buds in Betula pubescens, Acer and Ribes nigrum (El-Antably et al, 1967). Stewart (1969) was able to induce the formation of turions in Lemna polyrhiza by ABA treatment. The inhibitor content of buds, now attributed to ABA (Cornforth et al., 1966; Milborrow, 1967), increased in short days (Phillips and Wareing, 1958). More recently, however, Lenton et al. (1972) were unable to observe an increase in ABA levels, detected by GLC, when plants of several woody species were transferred to short days. Similarly, Hillman et al. (in press) were unable to induce bud dormancy by application of exogenous ABA. Again, Browning (1973) could detect no changes in ABA-like activity when coffee flower buds were released from dormancy by irrigation. In contrast, Davison and Young (1974) have recently shown decreases in ABA levels of xylem sap of peach at the time of bud swelling.

ABA inhibits the germination of a wide range of seeds (see Addicott

and Lyon, 1969). Some seeds (e.g. Fraxinus) require a period of stratification before germination will occur. ABA levels have been shown to decline during stratification (Sondheimer et al., 1968). Application of exogenous ABA after stratification inhibits germination (Sondheimer and Galson, 1966). ABA is also present in a variety of other seeds, suggesting that it may be the main hormonal agent directly controlling the dormancy of seeds (Milborrow, 1967). ABA may also be present in the flesh of some fruits and the level of the inhibitor has been correlated with the inhibition of germination in those species (Jackson, 1968). The inhibitory influence of ABA on seed germination is frequently transient and reversible (see e.g. McWha and Hillman, 1973), but appears to be more persistent in Chenopodium album (Wareing and Saunders, 1971). Williams et al. (1973) distinguished between the maintenance of seed dormancy, in which ABA was active, and the control of embryo dormancy in which ABA appeared to be inactive.

Inhibitor β was originally observed as a result of the inhibition of coleoptile extension in bioassays of certain regions of chromatograms (Bennet-Clark and Kefford, 1953). Subsequent studies using pure ABA indicated its effectiveness in the inhibition of elongation in a number of excised tissues (Addicott et al., 1964; Cornforth et al., 1966; Milborrow, 1966; Wareing et al., 1968b; Pilet, 1970). In addition, ABA can inhibit the growth of Lemna (van Overbeek et al., 1967; Stewart, 1969) and Wolffia (see Addicott and Lyon, 1969). Reports of the effectiveness of ABA in retarding the growth and development of whole plants are more limited; this may be due to penetration difficulties (Blumenfeld and Bukovac, 1972) or to the need for repeated applications of the hormone to overcome its endogenous inactivation (Herner et al., 1967; Van Staden and Bornman, 1969). ABA is apparently capable of inhibiting both cell extension and cell division (Wareing and Ryback, 1970; Pilet, 1971; de la Torre et al., 1972). There is a lag of about 5 minutes before ABA is effective in inhibiting elongation of sections of pea and Avena shoots (Warner and Leopold, 1971; Rehm and Cline, 1973a, b). Under certain circumstances, however, ABA can stimulate growth (Van Staden and Bornman, 1969; Takahashi, 1973) and may promote cell division (Schier, 1973; Minocha and Halperin, 1974). In addition, Mullins and Osborne (1970) demonstrated that ABA treatment could increase the number of inflorescences of vine cuttings showing continued growth and development.

ABA-like activity and the vigour of apple rootstocks are apparently inversely related (Yadava and Dayton, 1972). Similarly, increased inhibitor levels in tobacco are associated with decreased internode elongation

(Steadman and Sequeira, 1970). On the other hand, no differences in ABA level were detected between light- and dark-grown dwarf peas (Kende and Kays, 1971; Simpson and Saunders, 1972). On this basis Simpson and Saunders consider it unlikely that differences in the height of pea plants are associated with different ABA levels.

ABA may, however, be associated with other light-controlled processes in which phytochrome has been implicated. Pearson and Wareing (1969) noted that the pre-treatment of dark-grown radish hypocotyls with ABA reduces their capacity for extension growth. Dörffling (1973) demonstrated that growth reduction in pea shoots by red light was not, however, the result of red-light induced increase in ABA levels. ABA can also inhibit the red-light induced unrolling of etiolated cereal leaves (Poulson and Beevers, 1969, 1970) and counteracts the stimulation of unrolling in the dark resulting from GA and kinetin treatment (Beevers et al., 1970).

ABA can inhibit the induction of flowering in several long day plants including Lolium temulentum and Spinacia oleracea, growing under inductive conditions (Evans, 1966; El-Antably et al., 1967; Cathey, 1968). Thus ABA may be produced in the leaves of long day plants kept under short day conditions and act as an inhibitor of flower induction (Addicott and Lyon, 1969). However, contrary to a report by Wareing and El-Antably (1970). Zeevaart (1971) was unable to detect any lowering of ABA levels as a result of the transfer of spinach plants from short to long days; thus ABA may not act as a natural inhibitor of flowering in these species.

ABA promoted the flowering of the short day plants, Pharbitis nil, Ribes nigrum and strawberry, held under long day conditions but was ineffective in certain other typical short day plants (El-Antably et al., 1967). Plant age, species and growth and photoperiodic conditions may all affect the ability of ABA to substitute for inductive conditions in short day plants (see e.g. Addicott and Lyon, 1969; Nakayama and Hashimoto, 1973). Krekule and Horavka (1972), from work on Chenopodium, suggest that ABA certainly does not represent the only hormonal factor regulating flower induction in short day plants and further conclude that the effect of ABA may be restricted to the post-inductive period of flowering. Furthermore, Addicott and Lyon (1969) consider that the competition existing between vegetative and reproductive development in many species may be important in determining response to ABA - ABA effects on flowering may perhaps be attributable to growth retardation processes. In squash plants, ABA preferentially stimulated the initiation of female flowers (Abdel-Gawad and Ketellaper, 1969).

Fractions inhibiting the outgrowth of lateral buds have been extracted

from Vicia seedlings (Kefford, 1955) and pea stems (Dörffling, 1965, 1966). The latter fraction has been shown to contain ABA (Dörffling, 1967). Higher levels of inhibitor have been detected in axillary buds, subjected to correlative inhibition, than in those released from such inhibition (Dörffling, 1966). Thus Arney and Mitchell (1969), having demonstrated the inhibition of lateral bud outgrowth by ABA in decapitated pea plants, suggested that ABA could act as the endogenous correlative inhibitor of axillary bud outgrowth. In contrast, Hillman (1970) noted no inhibitory effect of ABA on the outgrowth of lateral buds of Phaseolus although ABA did add to the inhibitory effect of IAA and GA_3. Tucker and Mansfield (1971), noting a correlation between ABA levels and apical dominance, suggested that a hormonal balance, perhaps involving cytokinin and ABA, may control bud outgrowth.

ABA appears to be important in enabling the plant to adapt to environmental stress. Pustovoitova (1967) noted an increase in inhibitory activity in wilted apricot leaves. ABA can reduce the transpiration rate in some woody species (Little and Eidt, 1968) and, in herbaceous species, this has been shown to be due to induction of stomatal closure by the hormone (Mittelheuser and V n Steveninck, 1969). Subsequently, manifold increases in the concentration of ABA or ABA-like inhibitors have been observed as a result of water stress (see, in particular, Wright, 1969; Wright and Hiron, 1969; Zeevaart, 1971; Most, 1971). The increases in ABA levels are mainly detectable in leaf tissues (Milborrow and Robinson, 1973); increases in ABA in other parts of the plant probably originate from changes in the hormone level in the leaves (Hoad, 1973), especially since ABA levels in detached leaves can rise as a result of water stress (Most, 1971). The magnitude of the increase in ABA levels due to water stress are related to the degree of wilting (Wright, 1969) and these increses probably result from rapid synthesis of ABA since ^{3}H-mevalonic acid is rapidly converted to ^{3}H-ABA in wilted wheat leaves (Milborrow and Noddle, 1970). The site of regulation of ABA biosynthesis in vivo under conditions of water stress may be between its presumed precursor and ABA. Milborrow (1974) has recently demonstrated the synthesis of ABA in isolated chloroplasts; it may thus be of significance that the guard cells are the only cells in the leaf epidermis to possess chloroplasts. Zabadal (1974) has suggested that there may be a threshold water potential for the increased synthesis of ABA in leaves under water stress.

Mansfield and Jones (1970) demonstrated that ABA acts preferentially on the guard cells, rather than on the rest of the leaf, to effect stomatal closure. ABA effects may be linked with a lower potassium (K^+) concen-

tration and reduced starch hydrolysis in the guard cells. ABA can reduce the influx of K^+ into the guard cells of expanding leaf tissue of Vicia faba (Horton and Moran, 1972; Horton and Bruce, 1972). Kriedemann et al. (1972) consider that the ABA effect is unlikely to be due to a general change in membrane permeability; a selective effect on permeability would be substantiated by the data of Van Steveninck (1972). The onset of closure of stomata induced by ABA is apparently very rapid, occurring in some species within 3 minutes (Cummins et al., 1971; Kriedemann et al., 1972). Any species differences with regard to rate of response to ABA may be attributable to different endogenous ABA concentrations.

Jones and Mansfield (1970) noted that stomata did not reopen for a considerable time after the cessation of ABA treatment. Cummins et al. (1971) and Kriedemann et al. (1972) however, detected a more rapid recovery and suggested that ABA may be rapidly sequestered or inactivated, although recent evidence suggests that the reversal of ABA-induced closure could not apparently be attributed to catabolism (Cummins, 1973). Loveys and Kriedemann (1973) observed a rapid decline in endogenous ABA levels after rewatering stressed vine leaves, although Hiron and Wright (1973) noted a much slower rate of recovery to pre-stress levels of ABA.

ABA levels can also apparently increase in response to other types of environmental stress (see, for instance, Wright and Hiron, 1970; Mizrahi et al., 1972; Mizrahi and Richmond, 1972; Itai et al., 1973). Moreover, ABA application can reduce the effect of stress (e.g. Fletcher et al., 1972; Mizrahi et al., 1972). In addition, links have been indicated between ABA responses and pathogenic effects (Steadman and Sequeira, 1970; Squire and Mansfield, 1972), and between a mutant of tomato, flacca, exhibiting low resistance to wilting and low levels of endogenous ABA (Imber and Tal, 1970; Nevo and Tal, 1973).

The rapid responses of coleoptile and stem sections (Warner and Leopold, 1971; Philipson et al., 1973) and stomata (Cummins et al., 1971) to ABA treatment might be interpreted as indicating an effect of ABA on membrane permeability. Reed and Bonner (1974) showed that ABA reduced the uptake rate of K^+ and Cl^- by Avena coleoptile sections, but there was no simple quantitative correspondence between the inhibition by ABA of coleoptile elongation and inhibition of K^+ uptake. ABA apparently acted on specific ion uptake mechanisms in this system. These results disagree with those of Van Steveninck (1972), who found that ABA delayed development of ion uptake capacity, but stimulated net uptake once the tissue had developed that capacity. For carrot root tissue, ABA increased the hydraulic conductivity of cell membranes and increased flux, in and out;

these effects were apparently metabolically dependent (Glinka and Reinhold, 1971, 1972). Similarly, observations that ABA increased the exudation rate from decapitated roots was attributed to an increase in permeability of the root to water (Tal and Imber, 1971; Glinka, 1973; Collins and Kerrigan, 1974) although Cram and Pitman (1972) interpret their data in another way.

ABA can modify the phytochrome-mediated attachment of mung bean and barley root-tips to glass plates (Tanada, 1973a, b). This could be interpreted as an effect of the hormone on systems controlling the electric surface potentials of plant cell membranes.

ABA can apparently inhibit the development of plastid membranes and of isolated etioplasts in greening cereals (Wellburn et al., 1973). The development of chlorophyll and carotenoids and of other chloroplastic components is also delayed by ABA treatment (Sondheimer and Galson, 1966; Lichtenthaler and Becker, 1969; Beevers et al., 1970; Tietz, 1972). Similarly, Mercer and Pughe (1969) suggested that the effect of ABA is specifically inhibitory for chloroplast formation - the synthesis of plastid quinones was inhibited whereas mitochondrial ubiquinone was unaffected. Lichtenthaler and Becker (1970) suggest that these effects of ABA are achieved through a reduction in the development of thylakoids by an inhibition of protein synthesis. At the ultra-structural level, ABA has been observed to reduce the starch content and the density of the stroma in amyloplasts of Rubus hispidus tissue cultures (Pilet, 1971; Pilet and Roland, 1971). The treated cells were generally smaller, had a reduced number of ribosomes and produced abundant mucilage from the cell walls.

ABA appears to be transported through petiolar segments at a velocity of about 20-30 mm per hour (Dörffling and Böttger, 1968; Ingersoll and Smith, 1970); it is not clear whether this transport is of a polar nature, but it is apparently metabolically dependent. Little ABA is catabolised during transport through the segments (Ingersoll and Smith, 1971). Diffusible ABA from senescent leaves is considerably higher than from young or mature leaves (Böttger, 1970b). For Lens stem sections, ABA reduced the uptake and movement of ^{14}C-IAA and may also reduce its transport velocity (Pilet, 1971). Studies of ABA movement in intact plants are more limited. Hocking et al. (1972) found that 24 hours after application of ^{14}C-ABA to the primary leaf of a bean plant, radioactivity was detectable in most parts of the plant, but particularly in the growing zones and root nodules. Transport of radioactivity apparently occurred in the phloem. Shindy et al. (1973) also detected radioactivity in all parts of cotton seedlings 4 days after application of ^{14}C-ABA to coty-

ledons or the first leaf. Distribution and movement of radioactivity did not depend on leaf type or age.

A number of investigations have made use of analogues of ABA in order to elucidate the relationship between the structure of the hormone molecule and its biological activity (see, for instance, Tamura and Nagao, 1969a, b; Sondheimer and Walton, 1970; McWha et al., 1973). Different assay systems for the biological activity of the analogues may yield conflicting results (McWha et al., 1973). In addition, differential rates of uptake and penetration of the analogues (Popoff et al., 1972) and of possible conversion to ABA (Milborrow and Noddle, 1970) may confound comparisons between the various analogues; for instance, the methyl ester of ABA may be as active as ABA (Koshimizu et al., 1966; Asmundson et al., 1968) or totally inactive (Milborrow, 1966). The ring double bond is probably necessary for biological activity while the importance of the ring carbonyl and hydroxyl groups is less clear (McWha et al., 1973).

The 2-cis double bond of the side chain may exert a major effect on the biological activity of ABA (Milborrow and Garmston, 1973). The 2-trans isomer is reported to be active in some physiological processes (see Addicott and Lyon, 1969), although more recent reports suggest that 2-trans ABA is certainly not active in a wide range of tests (see, for instance, Milborrow, 1970a; Bex, 1972b; Kriedemann et al., 1972). Sondheimer et al., (1971) consider that possession of the 2-trans side chain is more deactivating in physiological tests than is a change in absolute configuration from (S) to (R). Firn et al. (1972) also found that the inhibitory activity of the natural ABA analogue, xanthoxin, was reduced in the 2-trans isomer.

Indirect comparisons of the physiological activities of S-(+)- and R-(-)- ABA using racemic mixtures suggested that only the natural form, S(+), was active (Cornforth et al., 1965; Smith et al., 1968). Preparation of R-(-)- ABA by Cornforth et al. (1967), however, has enabled direct comparisons of the enantiomers to be made. These indicate that S-(+)-, R-(-)- and RS-($^{+}$-)- ABA are equally active in the inhibition of growth (Milborrow, 1968). Results may, however, depend on the bioassay system employed and on the differential uptake rates for the 2 entiomers (Sondheimer et al., 1971; Kriedemann et al., 1972).

ABA appears to be capable, in many systems, of modifying or antagonising the activity of other hormones. Conversely, the physiological effects of ABA may be affected by the presence or addition of the other growth regulators (see, for instance, Aspinall et al., 1967; Wareing et al, 1968a). From such studies, models for the competitive or non-com-

petitive "interaction" between ABA and other hormones have been proposed (e.g. Wareing et al., 1968a). The use of the term "interaction", however, has often been imprecise (Drury, 1969, 1970) and, moreover, the validity of such studies, based on kinetic analysis is questionable in systems as complex as developing plant tissue (Addicott and Lyon, 1969). Nevertheless, the mutual modification of activity by ABA and other hormones remains of interest.

GA-induced synthesis of hydrolytic enzymes in cereal aleurone layers may be antagonised by addition of ABA (Chrispeels and Varner, 1967). Jacobsen (1973) has cautioned against using this type of system as a basis for studies of competitive interaction. In barley half-grains, ABA enhanced the uptake and metabolism of ^{3}H-GA (Stolp et al., 1973). Investigations using other systems suggest that, under certain conditions, ABA may lower GA levels (Wareing et al., 1968a; Beevers et al., 1970; Rudnicki et al., 1973), perhaps through an inhibition of GA biosynthesis. In contrast, Railton and Wareing (1973) noted that ABA increased the level of GA-like components in Solanum andigena leaves. The effect of ABA may be to control the supply of gibberellins, perhaps by regulating the equilibrium between free and bound forms of GA (Browning, 1973). Conversely, GA can prevent or inhibit the rise in levels of ABA or ABA-like inhibitors in some systems (Chin and Beevers, 1970; Kopecewicz and Rogozinska, 1972), and may influence the distribution of ABA within the plant (Tietz and Dörffling, 1969). The degree of antagonism between ABA and GA in physiological responses may be nil, complete or partial; alternatively, the 2 compounds may interact synergistically (El-Antably et al., Addicott and Lyon, 1969; Tietz, 1972; Schier, 1973).

Similar controversies and contradictions exist for the relationship between ABA and cytokinins or auxins where the interaction may be apparently competitive or non-competitive and where antagonism may be absent, partial or total (Aspinall et al., 1967; Wareing et al., 1968a; Addicott and Lyon, 1969).

In senescing and abscinding leaf tissue, addition of ABA can stimulate the production of ethylene (Abeles, 1968; Cooper et al., 1968; Lieberman and Kunishi, 1971; Jackson and Osborne, 1972). On the other hand, in tissue from ripening fruit, ABA may either stimulate ethylene production (Gertman and Fuchs, 1972) or have no effect (Cooper et al., 1968). Moreover, in ageing flower and fruit tissue, C_2H_4 may apparently promote an increase in ABA levels (Mayak et al., 1972; Goldschmidt, 1973); ABA and C_2H_4 levels may be inter-related through feedback mechanisms (Mayak et al., 1972). In growing tissue, ABA may either stimulate or inhibit C_2H_4 syn-

thesis (Gamborg and La Rue, 1971; Ketring and Morgan, 1971; Loveys and Wareing, 1971; Goto and Esashi, 1974) but, in general, ABA inhibition of growth cannot be attributed to effects on C_2H_4 production. In barley aleurone layers, C_2H_4 canaantagonize the inhibitory effect of ABA on α-amylase synthesis (Jacobsen, 1973).

The structure of ABA, as a sesquiterpene resembles the end portions of some xanthophyll nolecules. Thus, ABA may possibly be derived from the oxidation of xanthophylls; indeed, Taylor and co-workers (1967, 1968, 1970) showed that photoxidation in vitro of neoxanthin and, more particularly, of violaxanthin produced a neutral inhibitor, xanthoxin, which has an aldehyde group in place of the carboxylic acid group of ABA. Moreover, the presence of natural xanthoxin has been shown (Firn et al., 1972) and this apparently exists in only one enantiomeric form. More recently, Taylor and Burden (1973) were able to obtain ^{14}C-ABA from tissue labelled with ^{14}C-xanthoxin; phaseic acid, an apparent metabolite of ABA was also present. Isoe et al. (1969) also favour photo-oxidation of carotenoid pigments as the means of synthesis of ABA.

In contrast, good evidence is now available to suggest that ABA, synthesized in vivo, is derived from mevalonic acid (Wright, 1968; Noddle and Robinson, 1969). The incorporation of radioactivity from mevalonic acid increased sharply in wilted tissue; furthermore, incorporation from a 1',2' epoxide was also increased by wilting, suggesting that it could act as a precursor for ABA biosynthesis. The oxygen of the 1', 2' epoxy group became the tertiary hydroxyl of ABA (Milborrow and Noddle, 1970b). By contrast, the 1'4'-cis and 1',4'-trans diols of ABA were converted to ABA equally in wilted and turgid wheat leaf tissue. (Milborrow, 1970b). There was some evidence for a feedback inhibition by ABA of its biosynthesis. The 2-cis double bond of ABA may be originally formed in the trans configuration (Robinson and Ryback, 1969). Milborrow (1970a) showed that the isomerisation necessary to produce 2-cis-ABA does not occur directly from 2-trans-ABA, but must occur at an earlier stage in biosynthesis. More recently, Milborrow and co-workers (1973b; 1974) indicated that more ABA biosynthesis appeared to occur in leaf and fruit tissue than in roots and that biosynthesis was at least partially located in the chloroplast, a view supported by Railton et al. (1970).

The proposed synthesis of ABA from xanthoxin appears to require a configurational change (Milborrow and Garmston, 1973). Moreover, the use of carotenois biosynthesis inhibitors had no effect on ABA synthesis in isolated chloroplasts (Milborrow, 1974). The observation that phytoene, a precursor of carotenoids was converted only to carotenoids in avocado

fruit; and that mevalonic acid can be converted to ABA in the dark also argue against the importance of the xanthophyll photo-oxidation pathway (Milborrow, 1970b). Furthermore, $^{14}CO_2$ can be incorporated into ABA in unripe strawberry fruit, i.e., at a time when significant degradation of carotenoids is unlikely to occur (Rudnicki and Antoszewski, 1968). It thus appears that the biosynthesis of ABA as a degradation product of xanthophylls is unlikely to be of general importance. Nevertheless, the breakdown of some carotenoid pigments during leaf senescence may possibly be of significance in relation to ABA levels.

Milborrow (1968) detected the presence of 3 labelled products resulting from the treatment of bean and Acer petiole sections with ^{14}C-ABA; these products were designated as "Metabolites A, B and C" and accounted for more than 50% of the total radioactivity in bean extracts.

"Metabolite A" has been shown to correspond to methyl abscisate and is probably an artefact (Milborrow, 1970b).

"Metabolite B" has been identified, by feeding ^{14}C-ABA to tomato shoots, as abscisyl-β-D-glucopyranoside (Milborrow, 1970a), the natural occurrence of which in Lupinus luteus fruit was already known (Koshimizu et al., 1968). This compound, a glucosyl ester of ABA, has more recently also been shown to occur naturally in rose pseudocarp (Milborrow, 1970a) and citrus fruit peel (Goldschmidt et al., 1973). In addition, reports of "bound" or hydrolysable" fractions of inhibitors derived from or related to ABA may correspond to this glucosyl ester (Gil et al., 1972; Cummins, 1973; During and Alleweldt, 1973; Hiron and Wright, 1973). A "conjugate form" of ABA, tentatively identified as the glucosyl ester, may be involved in the adaptation of plants to water stress, acting perhaps as a "metabolic backstop" enabling the free ABA level to remain high for a period even when the leaves have regained turgor(Hiron and Wright, 1973). The levels of the glucosyl ester demonstrated in unstressed tissue are apparently insufficient to account for the manifold rise in ABA levels during stress (Milborrow and Robinson, 1973). Conversely, the rate of metabolism of ABA to the "hydrolysate" fraction or to other metabolites of ABA was also insufficient to account for the rapid recovery of barley leaf stomata after the removal of exogenous ABA (Cummins, 1973).

"Metabolite C" has been identified as 6'-hydroxy-methyl ABA from tomato shoots treated with ^{14}C-ABA (Milborrow, 1969). Fractions with properties similar to 6'-hydroxy-methyl ABA have been detected by Hocking et al. (1972) and Cummins (1973). Metabolite C has also been shown to re-arrange to phaseic acid (Milborrow, 1969, 1970b), a compound originally extracted and characterised by MacMillan and Pryce (1968, 1969a, b) from

Phaseolus multiflorus seeds. A structure proposed for phaseic acid (Milborrow, 1969) has been generally accepted (Durley et al., 1971). Phaseic acid has also been shown to occur in cotton fruit (Davis et al., 1972). Bryophyllum shoots and leaves (Gaskin et al., 1973) and Phaseolus vulgaris seeds (Tinelli et al., 1973). In addition, Most (1971) has isolated a fraction with a similar GLC retention time to phaseic acid. Phaseic acid exhibits a plain negative O.R.D. curve, in contrast to ABA (MacMillan and Pryce, 1969a) and possesses weak growth inhibitory activity (MacMillan and Pryce, 1968; Davis et al., 1972). Fractions extracted from embryonic bean axes treated with ^{14}C-ABA and designated as M_1 and M_2 (Sondheimer et al., 1971; Walton and Sondheimer, 1972) have now been identified as phaseic acid and 4'-dihydrophaseic acid respectively (Tinelli et al., 1973). These fractions also possessed low growth activity and were not re-converted to ABA when re-applied to plant tissue (Walton and Sondheimer, 1972). 4'-dihydrophaseic acid has been shown to occur naturally in Phaseolus vulgaris seeds and several pathways for its production from ABA have been postulated (Walton et al., 1973). If of widespread occurrence, this metabolite may represent a major inactivation or storage product for ABA. Milborrow (1969, 1970b), however, has questioned whether phaseic acid is a natural degradation product of ABA or whether, perhaps, it merely represents an extraction artefact.

When ^{14}C-(±)-2-ABA is supplied to excised tomato shoots the unnatural R-(-) enantiomer is converted to the glucose ester of ABA faster than the natural S-(+) form. 6'-hydroxy methyl ABA, on the other hand, is derived exclusively from the S(+) enantiomer (Milborrow, 1968, 1970a, b). A faster rate of metabolism for the natural enantiomer has also been noted by Sondheimer et al. (1971) and Cummins and Sondheimer (1973).

Studies, attempting to determine the mode of action of ABA, have mainly focused on the effects of the hormone on nucleic acid and protein metabolism. ABA reduced the growth of Lemna minor and inhibited ^{32}P incorporation into both DNA and RNA fractions; however, since the DNA fraction was affected first, Van Overbeek et al. (1967) suggested that the physiological effects of the hormone were explainable on the basis of selective inhibition of DNA synthesis. Similarly, Stewart and Smith (1972) showed that ABA inhibition of incorporation of ^{3}H-thymidine into DNA preceded effects on incorporation of ^{3}H-thymidine into RNA ~~preceded effects on incorporation into~~ RNA and suggested that the primary role of ABA in this system was to inhibit cell division and thereby to reduce first DNA and then RNA synthesis. In non-dividing systems (e.g. mature leaf tissue, barley aleurone layers), however, the effects of ABA are not easily explainable on the basis of

inhibition of DNA synthesis (Wareing et al., 1968a). Haber et al. (1969b) showed that inhibition by ABA of lettuce fruit germination occurred in the absence of DNA synthesis. Similarly, Walton et al. (1970) noted a reduction in DNA synthesis in the presence of ABA, but considered that this could not account for the inhibition of growth of embryonic bean axes also resulting from ABA treatment.

ABA may reduce DNA levels (Benhanafi and Collet, 1970; Leshem and Schwarz, 1972) or may interact with DNA to reduce template activity, possibly by causing cross-linking of the DNA strands (Mondal and Biswas, 1972). Similarly, Pearson and Wareing (1969) showed that ABA added prior to extraction reduced the in vitro capacity of chromatin to support RNA synthesis. Spang and Platt (1972) suggested that ABA may decrease DNA hyperchromicity. Srivastava (1968a), however, could detect no significant changes in barley leaf chromatin as a result of ABA treatment; furthermore, there was little alteration in the histone:DNA ratio. Similarly, ABA had no direct effect on histone synthesis and did not affect binding between DNA and protein (Bex, 1972d).

Early reports suggested that ABA reduced the levels of all RNA fractions and inhibited the incorporation of precursors into these fractions (Wareing et al., 1968a, b; Van Overbeek, 1968; also Stewart and Smith, 1972). It is apparent that quantitative differences exist between these fractions within the general depression of RNA metabolism resulting from ABA treatment. In particular, ABA appears to have a specific effect on r-RNA fractions and on polysome content in some systems. Wareing et al. (1968b) suggested that an early effect of ABA might be on r-RNA incorporation; further, ABA may reduce ribosome level in general, and polysome content in particular. Apparently specific effects of ABA on r-RNA levels and synthesis have been noted by Bex (1972a) and Leshem and Schwarz (1972). In addition, Khan and Heit (1969), Walton et al (1970) and Paranjothy and Wareing (1971) have observed effects of ABA on r-RNA fractions as well as on other specific RNA fractions. ABA may also decrease the percentage of ribosomes existing as polysomes ~~due to ABA~~ (Poulson and Beevers, 1970; Evins and Varner, 1972; Brown and Sun, 1973). A decrease in polysome content may not necessarily be linked, however, to hormonal effects on r-RNA or ribosomal protein; supply of m-RNA molecules may also limit polysome formation (Poulson and Beevers, 1970). ABA can also reduce total ribosome content under certain conditions (Pilet and Roland, 1971; Evins and Varner, 1972). Bonnafois et al. (1973), on the other hand, suggested that ABA did not affect the association of ribosomes into

polysomes; rather, the hormone strongly inhibited the ability of polysomes to incorporate amino acids into protein. Alternatively, ABA may control ribosomal levels by limiting phosphorylation of ribosomal proteins (Trewaras, 1973).

Inhibitory effects of ABA on s-RNA are apparently confined to a general or partially selective inhibition of RNA metabolism (Khan and Heit, 1969; Paranjothy and Wareing, 1971), although Leshem and Schwarz (1972) suggest that ABA increases the specific activity of s-RNA, while inhibiting r-RNA metabolism.

ABA may inhibit protein synthesis, in certain systems, either by limiting transcription or by inhibiting messenger translation (Villiers, 1968). Studies on the effects of ABA on specific RNA polymerase enzymes are lacking, but ABA can affect total RNA polymerase activity both *in vivo* and *in vitro* (Pearson and Wareing, 1969; Khan, 1970; Mondal and Biswas, 1970; Poulson and Beevers, 1970). Bex (1972b) also noted a reduction in RNA polymerase activity due to ABA treatment of maize coleoptiles, but considered that this effect was not rapid enough to account for the observed reduction in RNA synthesis. Paranjothy and Wareing (1971) showed that ABA could inhibit synthesis of polydisperse RNA, a messenger-like fraction. Along similar lines, ABA altered the base composition of 3 messenger-like fractions in pear embryos (Khan and Anojulu, 1970).

Evidence for the site for ABA action being subsequent to m-RNA synthesis has been presented by Osborne (1967), Gayler and Glasziou (1969), Chen and Osborne (1970), Walton *et al.* (1970) and Ihle and Dure (1970). Bonnafois *et al.* (1973) suggest that an early effect of ABA is to limit translation *via* polysome activity. However, as Bonnafois *et al.* point out, it is difficult to account for some of the observed rapid effects of ABA (Warner and Leopold, 1971; Cummins *et al.*, 1971) by either translational or transcriptional control. Thus effects of RNA metabolism may in some cases be of a secondary nature. Moreover, qualitative and quantitative effects of ABA on RNA metabolism and on individual RNA fractions may vary with tissue and species (Benhanafi and Collet, 1970; Khan and Anderson, 1970).

As well as inhibiting synthesis of RNA, ABA may also regulate the catabolism of DNA and RNA. ABA has been shown to stimulate nuclease activity in a variety of tissues (Srivastava, 1968a; De Leo and Sacher, 1970, 1971; Pilet, 1971; Leshem, 1971; Wyen *et al.*, 1972). Pearson and Wareing (1969), however, were unable to detect any differences in RNase activity as a result of treating radish hypocotyl chromatin with ABA. The effects of ABA on nuclease activity appear to be specific for

their substrates; they appear to depend on de novo synthesis of the enzymes (Leshem, 1971; Bex, 1972b; Udvardy et al., 1972). In water-stressed barley leaves, the normal effect of ABA on nuclease activity is reversed; perhaps, under conditions of water deficit, cell water supercedes hormonal regulation in changing RN ase activity (Arad et al., 1973).

The level of protein in ABA-treated tissue is generally reduced relative to control tissue (Beevers, 1968; Pilet, 1971; Colquhoun and Hillman, 1972). In addition, ABA can inhibit the incorporation of amino-acids into protein (Osborne, 1967; Poulson and Beevers, 1970; Mullins and Osborne, 1970; Paranjothy and Wareing, 1971). Villiers (1968), on the other hand, detected no inhibition of ^{3}H-leucine incorporation, although the incorporation of ^{3}H-uridine and ^{3}H-thymidine was inhibited; the condition of the plant tissue may be important in this connection (Pietro and Sacher, 1970). It is likely, however, that ABA effects on protein synthesis are of a secondary nature (Stewart and Smith, 1972).

The effects of ABA on a wide variety of enzymes have been reviewed by Addicott and Lyon (1969) and Addicott (1970). As Addicott (1970) has stated "where ABA is retarding or inhibiting a process such as growth or germination, its effect is to retard or inhibit the development of enzymes required for those processes. Conversely, when ABA is promoting a process, such as senescence or abscission, it promotes the development of the special enzymes required for those processes. At the same time, it may inhibit enzyme development and related events involved with the delay of senescence or abscission." The effects of ABA on individual enzymes may depend on plant species, tissue and state of development (compare, for instance, Pilet, 1971; Sankhla and Huber, 1974).

ABA can reduce the photosynthetic rate of leaf tissues (Mittelheuser and Van Steveninck, 1969, 1971c; Most, 1971). The effect on photosynthesis does not, however, appear to be controlled through ABA-induced stomatal closure (Cummins et al., 1971; Poskuta et al., 1972). Rather ABA may reduce ribulose diphosphate carboxylase (Wellburn et al., 1973; Loveys and Kriedemann, 1973) and thus inhibit CO_2 uptake. ABA inhibits dark respiration and, under certain conditions, can reduce the rate of photorespiration (Poskuta et al., 1972).

One of the earliest recorded physiological effects of ABA is the ability to stimulate chlorophyll loss in detached leaves (Rothwell and Wain, 1963; Addicott et al., 1965). More detailed studies indicated that ABA could stimulate senescence in detached leaves or leaf discs of a variety of species (El-Antably et al., 1967; Aspinall et al., 1967; Sankhla and

Sankhla, 1968). ABA is apparently less effective, however, in stimulating senescence in attached leaves (El-Antably et al., 1967). This may be due to penetration difficulties in intact leaves or to inactivation of the ABA applied. Sloger and Caldwell (1970) could stimulate senescence by application of ABA to leaf discs of a number of varieties of soyabean, but ABA was effective on less than half this number when applied to intact leaves; thus varietal differences, as well as species differences may be of importance. In addition, response to ABA may be modified by leaf age - leaf tissue in which senescence had apparently started showed a greater response to exogenous ABA than did tissue from expanding leaves (Osborne, 1968a; Smith et al., 1968).

Effects of ABA in stimulating senescence can be partially or totally inhibited by GA or cytokinins (Aspinall et al., 1967; Beevers, 1968; Sankhla and Sankhla, 1968; Back and Richmond, 1971).

ABA can also accelerate the decline in RNA and protein levels associated with senescence (Beevers, 1968; Wareing et al., 1968a; Colquhoun and Hillman, 1972) and can inhibit incorporation of RNA and protein precursors (Osborne, 1967; Beevers, 1968; Paranjothy and Wareing, 1971). In addition, ABA may stimulate the rise in specific nucleases and in acid phosphatase associated with senescence of detached leaf tissues (Srivastava, 1968a; Pietro and Sacher, 1970; Udvardy et al., 1972); analysis of the rise in nuclease activity, however, may be confounded by leaf excision. Pietro and Sacher (1970), moreover, think it unlikely that the stimulation of senescence by ABA can be entirely attributed to effects on nuclease activity. Rather, ABA may also affect membrane permeability in senescent tissues and accelerate the senescence-associated increase in free space (Pietro and Sacher, 1970; Brown and Sun, 1973). In addition, ABA can enhance the normal pattern of loss of fatty acids from senescing leaf tissue, with particular effects on linolenic acid, 18:3 (Newman et al., 1973). ABA induced a rapid acceleration of cell degeneration in detached wheat leaves, examined at the ultra-structural level. The pattern of senescence changes were reported to be similar to those occurring in water-treated control tissue (Mittelheuser and Van Steveninck, 1971a,b).

Wareing and Seth (1967) suggested that the synchronous senescence of leaves of woody species might be regulated by the increased levels of ABA, resulting from the shortening photperiod in late summer and sutumn. However, as they point out, high ABA levels will not necessarily cause senescence (e.g., Acer and Betula). Moreover, in herbaceous species, the content of ABA-like inhibitors may remain high even in expanding

leaves (Goodwin and Gordon, 1972). The levels of ABA or of ABA-like inhibitors may be higher in old leaves than in younger leaves (Rudnicki et al., 1968) and in aged detached leaves than in freshly excised ones (Chin and Beevers, 1970). In addition, the amounts of diffusible ABA obtainable from senescent Coleus leaves are apparently higher than from green leaves (Böttger, 1970). Osborne et al. (1972), however, were unable to detect significant differences in ABA levels between bean leaves at 2 stages of development, viz. young, expanding leaves and wholly yellow, senescent leaves.

ABA can accelerate senescence-like changes in citrus fruit (Cooper et al., 1968); orchid flowers (Arditti et al., 1971) and rose petals (Mayak and Halevy, 1972). Moreover, ABA levels of these tissues may increase with the onset of senescence (Mayak and Halevy, 1972a; Goldschmidt et al., 1973).

ABA can thus apparently stimulate the physiological processes normally associated with leaf senescence in detached leaf tissue. It is unclear, however, whether the effects of ABA merely hasten those occurring in the leaves of intact plants or whether the hormone induces fundamental changes in the pattern of senescence. Moreover much of the evidence relating to changes in ABA levels during leaf senescence is based on the use of bioassays, the specificity of which is open to doubt. In addition, if changes in the level of ABA during development are involved in the regulation of natural senescence, then it is important to relate these changes to alterations in leaf growth rate and to the onset of senescence.

The activity of ABA in any tissue, however, is not necessarily a direct function of the gross levels of the hormone; rather its rate of turnover must also be considered. Thus, in senescent tissues, the rate of de-activation of ABA may be important in determining the effective concentration of the hormone.

The investigations reported in this thesis have made use of bean and radish leaves, species which have been quite widely used in the study of senescence and which can be quickly and easily grown.

These studies have been concerned with 5 main aspects of the possible involvement of ABA in the senescence of leaf tissue:-

(1) The effect of ABA and kinetin on chlorophyll loss and on the decline of radioactive RNA and protein during leaf disc senescence.

(2) The uptake of ^{14}C-ABA, and also of ^{14}C-sucrose, during leaf disc senescence.

(3) The metabolism of ^{14}C-ABA in relation to senescence.

(4) The quantitative extraction of ABA from leaf tissue at various stages of development.

(5) The ultra-structural changes occurring during senescence in water or ABA, and their relationship to the changes occurring during natural senescence.

The significance of the leaf disc, as an assay system for senescence is also discussed.

MATERIALS AND METHODS

1. PLANT MATERIALS

Leaf material was normally obtained from fully expanded, healthy leaves of radish (Raphanus sativus L. var. "Cherry Belle") and from primary leaves of bean (Phaseolus vulgaris var. "Canadian Wonder"). Plants were grown in 4" or 5" plastic pots containing John Innes No. 2 compost in a heated greenhouse with supplementary irradiation supplied by Thorn 400 W MBFR/U high pressure mercury vapour lamps to give a photoperiod of 16 hours throughout the year. Radish plants were watered twice a week with Vitafeed 103 (Fisons Ltd., Loughborough, Leics.).

2. PREPARATION OF RADISH LEAVES

In experiments using leaf discs, radish leaves were detached, washed briefly in tap-water and then dipped for 45 seconds in a solution of sodium hypochlorate containing 5 to 10% available chlorine. The leaves were then rinsed in 6 changes of sterile distilled water. Leaf discs were obtained using a flame-sterilised 1 cm diameter cork-borer (No. 6) and immediately transferred to sterile distilled water prior to experimental use.

3. SOLUTION OF HORMONAL COMPOUNDS

Racemic abscisic acid (ABA), obtained from Hoffmann La Roche, Basle, was made into aqueous solution by dissolving the solid compound in a small volume of redistilled methanol and adding this methanolic solution, with stirring, to warm sterile distilled water; this was then made up to the required volume. ^{14}C-2-ABA, also obtained from Hoffmann La Roche, at a specific activity of 45 µCi mg^{-1}, was made into solution in the same way.

Kinetin (6-furfurylaminopurine) was obtained from British Drug Houses, Poole, Dorset, and was made into solution by dissolving the solid compound in a small volume of dimethylformamide; this was then added to a larger volume of sterile distilled water and stirred overnight, before being made up to the required volume. Adenine solutions were prepared in a similar manner.

Hormonal solutions and solutions of radioactively labelled compounds were stored in the dark at 1°C.

4. RADIOACTIVE ANALYSIS

Radioactive samples were assayed by one of the following methods:-

(1) Liquid scintillation spectrometry using a Packard Tricarb Spectrometer (model 3380) with automatic activity analyser (model 544) giving an automatic conversion to absolute radioactivity by reference to an

external standard. This machine was used for all samples quenched by colour or chemical agents.

(2) Liquid scintillation spectrometry using a Packard Tricarb Spectrometer without automatic quench correction; this machine was thus normally used for samples with low or nil quench.

Scintillation vials were obtained from Packard Ltd. and were of the low potassium type. Radioactive samples in scintillation vials were normally stored at 2°C prior to radioactive analyses. For both spectrometers, the scintillation fluid was toluene-based (Asschem Ltd., Falkirk) with 4 gm. l^{-1} of the scintillation chemical, 2,5-diphenyl oxazole (P.P.O., obtained from Fisons). In experiments involving the incorporation of radioactivity into RNA and in some experiments involving incorporation into protein, Triton X-100 (Lennig Chemical Co., London) was added to toluene in the ratio (1:2 by volume), in order to obtain an emulsive mixture.

Prior to radioactive analysis of any set of vials, background radioactivity was determined by assaying 4 clean vials containing only 10 mls of the scintillation fluid; the background values thus obtained were then automatically subtracted by the spectrometer. Vials were normally assayed for 5 mins. or 10 mins.

(3) In certain experiments, radioactivity present on chromatograms was analyzed using a Panax Radio-chromatogram Scanner with 98% argon: 2% propane as the carrier gas at 5 lbs. in^{-2}. Relative amounts of radioactivity were transformed into a trace produced on a Servoscribe RE 511 20 flat-bed recorder.

5. PRE-LABELLING EXPERIMENTS : DETERMINATION OF CHLOROPHYLL PROTEIN AND RNA, AND OF THE INCORPORATION OF RADIOACTIVITY INTO PROTEIN AND RNA

Extraction and assay procedures for chlorophyll, protein and RNA fractions were modified after Lowry *et al.* (1951) and Osborne (1962).

Radish leaf discs derived from surface-sterilised leaves were placed in 10 mls of a radioactively labelled precursor of RNA or protein in a 5 cm sterile plastic petri dish with 10 discs per dish. Leaf discs were incubated, adaxial side uppermost in 0.5 µCi of radioactive precursor (Radiochemical Centre, Amersham) for 4 hrs in a temperature controlled growth room at 25°C with 65/80 watt warm white Atlas fluorescent tubes, 71 cm above the bench surface to give a radiant flux density of 5.3 J. m^{-2} sec^{-1}.

The discs were then removed from the radioactive solution and washed 3 times in sterile distilled water, prior to flotation, adaxial side

uppermost, in 10 mls of an incubation solution, which was normally one of the following:-

(1) Sterile distilled water.

(2) 10^{-4} ABA or 2 x 10^{-5}M kinetin.

(3) The appropriate non-radioactive precursor of RNA or protein at a concentration approximately 100 times that of the radioactive precursor used during the labelling period (i.e. one of non-radioactive adenine, leucine or uracil).

(4) The non-radioactive precursor in (3) plus 10^{-4}M ABA or 2 x 10^{-5}M kinetin.

In addition in some experiments, RNA or protein synthesis inhibitors were used; these were actinomycin D, cycloheximide (both from Sigma Chemical Co., St. Louis, Missouri) and 2-(4-methyl-2,6-dinitroanilino)-N-methyl propionamide (MDMP; a personal gift from Dr R. Baxter, Shell Research Division, Sittingbourne). The inhibitors were normally added to the post-labelling incubation solutions, although, in certain tests, the inhibitors were added to the radioactive solution itself.

(a) Chlorophyll and alcohol-soluble fraction analysis

At various intervals (up to 3 or 6 days depending upon the particular experiment), leaf discs were removed from the incubation solution, washed briefly in distilled water, blotted dry and placed in 5 mls of 80% ethanol in tapered 12.5 ml glass centrifuge tubes with 10 leaf discs per tube. The alcoholic solution was boiled in a water bath for 6 mins. and then decanted into a 50 ml volumetric flask. This procedure was repeated twice with fresh volumes of ethanol, decanting the solution each time. The discs were then homogenized in a small volume of 80% ethanol using a ground-glass hand-grinder. The resulting homogenate was boiled for 6 mins., centrifuged at 2000 x g for 4 mins. in a Gallenkamp bench centrifuge and the supernatant added to the bulk alcoholic solution. The pellet was resuspended in 80% ethanol and the procedure repeated twice, decanting the alcoholic supernatant each time. The bulked alcoholic solution was made up to 50 mls and its absorbance at 665 nm determined using either a SP 500 ultra-violet and visible spectrophotometer or a SP 8000 ultra-violet recording spectrophotometer (both from Pye Unicam Ltd., Cambridge).

In addition, 1.0 ml samples were taken in triplicate, placed in scintillation and dried under vacuum for radioassay of the alcohol-soluble fraction.

The pellets remaining in the centrifuge tubes were washed twice each

with 5 mls of ice-cold 20% trichloracetic acid, ice-cold ethanol and ether: ethanol:chloroform (2:2:1) at room temperature. Between each washing, the solutions were centrifuged as before and the supernatant discarded. The pellets were then allowed to dry in the centrifuge tubes overnight at room temperature.

(b) Protein analysis

In experiments involving the use of protein precursors, the dried pellets were subjected to assay of the T.C.A.-insoluble protein by suspending in 5 mls of 1N sodium hydroxide solution and boiling in a water bath for 10 mins., taking care to avoid excessive foaming or evaporation. After centrifugation to precipitate the residue, aliquots of 0.5 ml or 1.0 ml were taken and placed in scintillation vials for radioassay. For determination of protein levels, a colorimetric mixture was prepared containing 100 parts by volume of 2% sodium carbonate in 0.1N NaOh solution: 1 part of tartrate. One ml of this mixture was added to tubes to which 0.1 ml or 0.2 ml of the extract supernatant was then also added. After incubation at 35°C for 15 mins., 0.1 ml of Folin and Ciocalteu's reagent (6.5N; B.D.H.) was added and the solutions immediately mixed. After allowing the solutions to stand for 30 minutes at room temperature, the colorimetric mixture was diluted to 5 ml or to 10 ml and the absorbance at 660 nm determined by spectrophotometry.

(c) RNA analysis

In those experiments involving the use of RNA precursors, the RNA levels in the discs were determined by suspending the T.C.A.-insoluble pellets in 5 mls of 0.3M KOH solution and incubating overnight at 35°C. The solution was then centrifuged and the supernatant decanted into a fresh tube. The pellet was washed twice with 1.0 ml of distilled water, the washings being added after centrifugation to the bulk supernatant. The pH was adjusted to 4.0 with 10% perchloric acid and the volume made up to 10 mls with distilled water. Aliquots of 1.0 ml were taken in triplicate and placed in scintillation vials for radioassay. The absorbance at 260 nm was determined using an SP 8000 ultra-violet spectrophotometer.

The various steps in the extraction and assay procedure for chlorophyll, protein and RNA are summarised in the accompanying flow-sheet (Table 1).

6. UPTAKE AND INCORPORATION PATTERNS OF RADISH LEAF DISCS

In those experiments involving the examination of uptake and incorporation patterns across the leaf disc, 1 cm discs were treated with ^{14}C-1-leucine for 4 hours as described above, washed 3 times in distilled water and then divided into 3 concentric rings of tissue by means of 0.7 cm and 0.5 cm cork borers (Nos. 4 and 2 respectively). The disc segments were then extracted either in methanol or as described above, before being analysed for radioactivity.

7. THE UPTAKE OF ^{14}C-2-ABA AND ^{14}C-SUCROSE BY RADISH LEAF DISCS

(a) Uptake of ^{14}C-2-ABA

Discs freshly excised from surface-sterilised radish leaves were incubated in the dark at 25°C in 10 mls of sterile distilled water with 5 discs per dish for periods up to 6 days. At daily intervals, discs were transferred to 10 mls of 1.4×10^{-6}M ^{14}C-2-ABA for periods up to 12 hours. (Preliminary chromatographic checks on the purity of the ^{14}C-2-ABA stock indicated the presence of at least one other radioactive compound; subsequently, therefore, ^{14}C-2-ABA stock solutions were routinely re-purified by chromatography prior to experimental use.) Infrequently, dishes of discs in water exhibited signs of microbial contamination and these were discarded. After completion of the incubation period, the discs were washed 6 times in 10 mls of distilled water and transferred individually to 2 mls of 80% redistilled methanol in scintillation vials. After extraction in the dark for 2 days at room temperature, the methanolic solutions were dried under vacuum, prior to radioassay.

Samples were taken from ^{14}C-2-ABA solutions after incubation in order to check whether or not there was any breakdown of the labelled hormone in solution. These samples were dried under vacuum, dissolved in a small volume of redistilled methanol, loaded onto 5 cm Whatman No. 1 paper chromatography strips and developed in isopropanol:ammonia:water (8:1:1) by descending chromatography. After drying, the chromatograms were divided into 20 equal zones and subjected to radioassay together with authentic samples of ^{14}C-2-ABA chromatographed in the same way.

(b) Uptake of ^{14}C-2-ABA by radish leaf discs incubated in a N_2 or air stream

Surface sterilised leaf discs, derived and incubated for periods of 0 to 6 d-ays as described above, were transferred at daily intervals to 10 mls of sterile distilled water in 5 cm plastic petri dishes in glass desiccators previously flushed through with N_2 or air (British Oxygen Company, London) for several minutes. The discs were then incubated

in the desiccators in a stream of N_2 or air for 1 hour. Each gas stream was moistened, prior to passage over the discs, by bubbling through distilled water contained in a Dreschel bottle, situated between the gas cylinder and desiccator. Any possible backflow of air into the desiccators was prevented by connecting another Dreschel bottle, containing water, to the outlet tubes of the desiccator. In experiments in which tests were made for the production of $^{14}CO_2$ in the experimental system, this second Dreschel bottle (and sometimes also a third bottle) contained 40 mls of hyamine 10X-hydroxide (10% $\frac{w}{v}$ in methanol; B.D.H.), from which 1.0 ml aliquots were taken in triplicate for radioassay. After incubation in the gas stream for 1 hour, the discs were quickly transferred (in less than 1 minute) to 10 mls of ^{14}C-2-ABA solution. The desiccators were then sealed with masking tape and the discs incubated for 8 hours. Gas flow rates were adjusted to attain an approximate rate of 1 bubble per 2 seconds in the second Dreschel bottle. Actual flow rates were determined using a Pye bubble flowmeter assembly and for different experiments varied between 130 and 220 mls. min^{-1}. After completion of the incubation period in ^{14}C-ABA, the discs were quickly washed 3 times in distilled water and transferred immediately to 2 mls of 80% methanol in scintillation vials (3 discs per vial). The discs were extracted and dried as before prior to radioassay.

(c) Uptake of ^{14}C-sucrose by radish leaf discs

Surface-sterilised radish leaf discs, incubated as above for 0 to 6 days and pre-treated for 1 hour in N_2 or air, were transferred at daily intervals to 0.5 µCi per 10 mls of uniformly labelled ^{14}C-sucrose (specific activity 9.6 $mCi.mM^{-1}$; Radiochemical Centre, Amersham). The arrangement of Dreschel bottles containing hyamine hydroxide and subsequent radioassay of the latter was as described above. After 8 hours incubation in ^{14}C-sucrose under N_2 or air stream, the leaf discs were washed, extracted in 80% methanol, dried and subjected to radioassay.

(d) Release of radioactivity as $^{14}CO_2$ from incubation solutions

The above procedures were repeated in turn for ^{14}C-2-ABA and ^{14}C-sucrose, but without any leaf tissue in the incubation solution. Aliquots of hyamine hydroxide were taken as before in order to check whether or not there was any release of radioactivity as $^{14}CO_2$ from the solutions during the incubation period.

8. EXTRACTION OF ENDOGENOUS ABA FROM LEAF MATERIAL

Primary leaves of bean were harvested at various stages of development by excising with a razor blade at the junction of the distal pulvinus and

the lamina. Leaves were normally harvested 4 to 5 hours after watering, at which stage the leaves were still apparently turgid and showed no signs of wilting. Batches of leaves were weighed and stored in a deep-freeze at -20°C prior to extraction.

From batches of plants extracted by the second procedure described below, six plants were selected at random prior to harvest, the leaves of which were weighed and then extracted in ethanol, in order to determine their chlorophyll content.

All solvents used in the extraction procedures outlined below were routinely redistilled. All glass apparatus was specially cleaned with acidified methanol and then rinsed in 6 changes of distilled water. During partition procedures, the apparatus was also rinsed with the solvent of the fraction to be retained. Extracts were shielded from light as far as possible and direct exposure to sunlight was avoided.

(a) Extraction method 1

The first method adopted for the extraction of endogenous ABA from primary leaves of bean was adapted after Lenton et al. (1971). The leaves were immersed in 80% methanol and allowed to extract in the dark. The methanolic solution was decanted and replaced by fresh 80% methanol. This was repeated at intervals until most of the chlorophyll had been extracted. The leaf material was then homogenised in a Waring blender and allowed to extract in 80% methanol for two hours more. After filtration through Whatman No. 1 filter paper, the filtrate was added to the total methanolic solution which was then reduced to an aqueous solution on a Buchi rotary evaporator under vacuum at 35°C. The aqueous extract was re-filtered under vacuum through a sintered glass Buchner filter funnel and the pH adjusted to 3.5 with 2N hydrochloric acid. The acidified aqueous extract was partitioned 3 times with equal volumes of diethyl ether and the resulting aqueous fraction discarded. The ether phase was partitioned 5 times each against one-tenth of its volume of 5% sodium bicarbonate solution and distilled water alternately. The ether fraction was discarded and the bulked sodium bicarbonate-aqueous fraction was readjusted to pH 3.5 with concentrated HCl and partitioned 3 times with equal volumes of diethyl ether. Any remaining water in the ether fraction was frozen out overnight at -20°C, the resulting ice being filtered off and washed 3 times with 50 ml volumes of chilled diethyl ether in order to remove any remaining ABA.

The combined ether or ethyl acetate fractions were reduced to dryness under vacuum at 25°C by rotary evaporation and then redissolved in a small

volume of methanol for loading on 1 mm thin-layer plates of silica gel GF 254 (E. Merck, Darmstadt, Germany). All plates were pre-eluted in methanol for at least 2 days. Extracts were developed alongside marker spots of synthetic ABA and also with ABA mixed with a little of the extract itself. Plates were developed successively in a solvent system of hexane: ethyl acetate (1:1) until examination of the marker spots of synthetic ABA, by their fluorescence properties under ultra-violet irradiation at 254 nm, indicated that the hormone had moved clear of the majority of the impurities, remaining at the origin. The corresponding zones of the plates containing the extract were eluted in methanol in 12.5 cm glass centrifuge tubes, centrifuging the silica gel 4 times and decanting the supernatant each time. The bulked supernatants were reduced to dryness by rotary evaporation.

Diazomethane was prepared by a procedure adapted after Schlenk and Gellerman (1960) in which 20 gm of NaOH pellets were dissolved in 50 mls of distilled water and 100 mls of ethylene glycol carefully added to form a layer above the NaOH solution. 25 ml of diethyl ether were then similarly added as a layer above the ethylene glycol and 3 to 4 gm of Nitrosan (Hopkin and Williams) added. The diazomethane gas produced was bubbled through a delivery tube into a volume of chilled diethyl ether. The diazomethane solution was normally used immediately but was never used after more than 24 hours storage, even if kept at $1^{o}C$. Extracts were methylated by dissolving in a small volume of diethyl ether, to which 4 to 5 mls of diazomethane solution was then added. After standing for several minutes at room temperature, the ether was removed by rotary evaporation or under nitrogen; the methylation process repeated 3 times more.

The final methylated extract was re-dissolved in a small volume of methanol and reloaded on 0.25 mm silica gel GF 254 plates (Merck) with marker spots of methylated synthetic ABA. The plates were developed once in hexane:ethyl acetate (1:1) and the marker spots located by ultra-violet fluorescence. Corresponding zones on the extract plates were eluted in methanol as before. Dried extracts were taken up in 3 ml of methanol and subjected to analysis by circular dichroism procedures on a Cary Model 6003 Spectropolarimeter with circular dichroism attachment, where the characteristic properties of ABA served as a guide to its presence (Milborrow, 1967)). According to this author, ABA has a positive peak at 262 nm, a negative peak at 230 nm and a further small negative peak at 320 nm. A further characteristic is that the ratio of the ellipticity at 262 nm (ψ 262) to the ellipticity at 230 nm (ψ 230) is 1.2. Milborrow also quotes the differential molar absorptivity (Δ E) at 262 nm as + 39.5

degrees. cm^2. d. mol^{-1} and from this the concentration of non-racemic ABA in a solution can be calculated using the determined ellipticity values as shown:-

Ellipticity $\psi = 3{,}300\ \Delta A$

where ΔA is the differential absorbance of the sample

Molar ellipticity $[\psi] = 3{,}300\ \Delta E$

where ΔE is the differential molar absorptivity

$$[\psi] = \frac{\psi}{l \times c}$$

where l = path length in cm

c = concentration in d.mol cm^{-3}

The procedures described above are summarised in the accompanying flow-sheet (Table 2).

(b) Extraction method 2

The second procedure used for the determination of ABA levels in primary bean leaves was adapted after Browning et al. (1970), and J. Good (pers. comm.).

The frozen leaves were freeze-dried in an Edwards High Vacuum centrifugal freeze-drier (Model 30-P2) at 0.12 torr, weighed and then milled to a powder. This powder was saturated with 0.1M sodium dihydrogen orthophosphate solution (pH 4.2) with 2 ml of solution per gm dry weight of tissue and then extracted overnight at 1°C in methylene dichloride with 10 ml of solvent per gm dry weight. The residue was filtered under vacuum through Whatman No. 1 filter paper and then resuspended in the same volume of the phosphate-methylene dichloride mixture. The filtration process was repeated twice more after allowing the extract to stand overnight each time. The bulked filtrates were allowed to separate and the lower methylene dichloride layer was removed and reduced to dryness by rotary evaporation at 25°C. The residue was redissolved in 100 ml of methylene dichloride and then partitioned 3 times against half its volume of 0.1M sodium phosphate buffer at pH 8.0. The combined aqueous phases were partitioned against 2 equal volumes of petroleum ether (60-80° boiling range), followed by half volumes of ethyl acetate. The aqueous fraction was adjusted to pH 3.0 with HCl and partitioned a further 5 times against half volumes of ethyl acetate. The combined ethyl acetate fractions were kept at -20°C overnight and any ice crystals filtered off and washed as described above. The ethyl acetate fraction was then reduced to dryness by rotary evaporation at 30°C, taken up in a small volume of methanol and subjected to chromatographic purification, methylation and circular

dichroism determinations as described above.

The procedures involved in this method of extraction are summarised in the accompanying flow-sheet (Table 3).

(c) Recovery efficiency of ^{14}C-ABA

The efficiency of recovery of endogenous ABA from leaf extracts was assessed by adding a small quantity of ^{14}C-ABA to the tissue at the beginning of the extraction procedure. The levels of the radioactive compound could be monitored at various stages by removing a small aliquot and subjecting it to radioactive analysis. Thus comparison could be made between the different techniques adopted and a degree of correction made to the final concentrations (demonstrated by circular dichoism) for the loss of endogenous ABA during extraction.

9. ASSESSMENT OF BEAN LEAF DEVELOPMENT

From batches of 500 bean plants, grown in the greenhouse, 20 were selected at random 10 days after planting and the growth of their primary leaves was assessed daily by measurement of the length and area of each leaf. Area determinations were performed using a perspex spot chart divided into squares of 0.25 mm^2. In addition, plants were also selected at random on alternate days for fresh weight and chlorophyll determinations. As before, care was taken to ensure that plants were only harvested in a non-wilted condition.

10. THE METABOLISM OF ABA

In non-preparative experiments, surface-sterilised radish leaf discs were incubated for 0 to 6 days in 10 ml of sterile water in the dark at 25^{o}C. Discs were then transferred either to 9 cm petri dishes containing 20 ml of 10^{-6}M ^{14}C-ABA or to 5 cm dishes containing 10 ml of ^{14}C-ABA. Discs were then incubated in the dark at 25^{o}C for periods up to 24 hours, washed 3 times in distilled water and extracted in 3 ml of 80% methanol for 2 days in the dark at room temperature (3 discs per vial). After drying under vacuum, extracts were re-dissolved in 0.5 ml of 80% methanol (0.2 ml + 0.1 ml) and loaded onto either 5 cm strips of Whatman No. 1 chromatograpny paper or 5 cm Camlab silica gel G thin layer plates (Macherey-Nagel, Germany). Paper chromatograms were loaded at a position 11 cm from the top of the paper and were developed by descending chromatography in one of the following solvent systems:-

(a) Butanol:isopropanol:ammonia:water (2:6:1:2)

(b) Butanol:acetic acid:water (5:1:2:2)

Solvent was not added to the chromatograms until an equilibration

period of at least 30 minutes had elapsed.

Thin-layer chromatograms were loaded 3.5 cm from the base of the plate and were developed in one of the following solvent systems:-

(a) Benzene:acetic acid (50:20)

(b) Benzene:ethyl acetate:acetic acid (50:5:2)

(c) Chloroform:methanol:water (75:22:3)

(d) Di-isopropyl ether:acetic acid (19:1)

Of these 4 thin-layer solvent systems, only (a) and (c) were found to ensure adequate movement of ^{14}C-ABA from the origin; thus, these systems are the only ones which have been routinely used in experiments. After developing the chromatograms, the distribution of radioactivity along the chromatograms was determined by 2 methods:-

(a) By Panax radiochromatogram scanning of the intact chromatograms.

(b) By dividing the chromatograms into 10 or 20 equal strips ('Rf' or '½ Rf' zones respectively) and placing each strip in scintillation vials and subjecting to radioassay by liquid scintillation spectrometry.

Due to a lower efficiency of the Panax scanner and to self-absorption of radioactivity by the silica gel or paper of the chromatogram itself, liquid scintillation spectrometry was found to be the more sensitive method for determining the distribution of radioactivity along the chromatogram and thus, has been routinely used. Some data are presented, however, for both methods of radioassay.

In those experiments involving the examination of the effect of antibiotics, on chlorophyll loss and ABA catabolism, either 50 µg ml^{-1} of chloramphenicol (Sigma Chemical Co.) or 0.5 µg ml^{-1} of mycostatin (Calbiochem, San Diego, California) were added to incubation solutions of water and of ^{14}C-ABA at 0, 1 and 6 days for 24 hours. Other samples were taken at these times for chlorophyll analysis by ethanol extraction. Incubation solutions were checked for microbial contamination by streaking 2 loopfuls of the solution across 9 cm plates of 4% trypticase soybean agar (Bioquest, Cockeysville, Maryland), autoclaved at 118-121°C for 15 minutes. Plates were examined for microbial contamination after 24 and 48 hours. After extraction of the discs in methanol, the extracts were spotted onto silica gel thin-layer plates and developed in chloroform: methanol:water (75:22:3) for radioassay.

In those experiments examining the effect of anaerobiosis on ^{14}C-ABA metabolism, the procedure adopted was similar to that used for examining the uptake of ^{14}C-ABA under a N_2 stream, except that after extraction in

80% methanol, samples were loaded onto silica-gel thin-layer plates and developed in chloroform:methanol:water (75:22:3) for radioassay.

11. EXTRACTION OF THE PRODUCTS OF ABA METABOLISM

Surface-sterilised radish leaf discs were incubated, with about 100 discs per 9 cm dish, in 20 ml of ^{14}C-ABA for 24 hours. The discs were then extracted in 80% methanol for 24 hours in the dark at room temperature, after which time the methanol was decanted and replaced by fresh solvent. This procedure was repeated once more and the discs were then macerated in an M.S.E. top-drive blender and filtered under vacuum. The bulked methanolic filtrate was reduced to an aqueous volume by rotary evaporation and the pH adjusted to 8.0, 7.0 and 3.0 in turn, with 5% sodium bicarbonate solution and 2N HCl as required, partitioning each time with 3 equal volumes of diethyl ether. Aliquots were taken at each stage for radioactive analysis and fractions found to contain radioactivity were chromatographed by one or more of the solvent systems described above, in order to determine the chromatographic properties of the radioactive component. The final aqueous fraction was partitioned with 2 equal volumes of petroleum ether (60^{o}-80^{o}) and the pH was then readjusted to neutrality with sodium bicarbonate prior to reduction to dryness by rotary evaporation. The use of butanol for partition against the aqueous fraction was also tested but did not give complete separation and afforded no apparent advantages in terms of purification.

In later trials of this nature, the final dried aqueous fraction was redissolved in 2 to 3 ml of 30% acetone and was then added to the top of a 12.5 x 2.5 cm charcoal:celite column ($3:2\frac{w}{w}$), contained in a Fisons chromatography column with a sintered glass filter overlaid by a ring of Whatman No. 1 filter paper and a layer of celite. Both the charcoal ("Norit OL", Hopkin and Williams) and the celite ("Celite 505", Hopkin and Williams) were washed for several days in methanol, dried and then intimately mixed in a coffee blender. The homogenous powder was suspended in 30% acetone, which was then evacuated to remove air bubbles, poured into the column and allowed to settle under its own weight.

After allowing the metabolite extract, in 30% acetone, to be absorbed, the column was eluted successively with 50 ml of 30%, 50%, 70% and 100% acetone, the eluted fractions being collected using an LKB 7000 Ultrorac fraction collector, then dried and analysed for radioactivity.

The steps outlined above are summarised in the accompanying flowsheet (Table 4).

In an attempt to identify the products of ABA metabolism, about 5000 surface-sterilised leaf discs were incubated for 24 hours, at 25^{o}C in the

dark, in 50 mls of 10^{-5}M ABA in sterile 15 cm glass petri dishes. The discs were then harvested, treated with methanol and partitioned as described above. At the same time, a number of discs were incubated in ^{14}C-ABA and then extracted in order to act as a marker for the non-radioactive metabolites. In 2 out of 3 trials of this type, the charcoal-celite column was of the type described above; in the third trial, however, the column was increased to 63.0 x 3.2 cm in order to increase the adsorption power of the system. Every tenth fraction collected in the large-scale experiments was dried and assessed for radioactivity. Fractions close to those found to contain radioactivity were dried and subjected to chromatography, first on thin-layer plates (GF_{254}) in chloroform: methanol:water (75:22:3), and then, after elution of radioactive zones in methanol, on Whatman 3MM 5 cm paper chromatography strips developed in butanol:isopropanol:ammonia:water (2:6:1:2). Eluates from radioactive zones were dried and attempts made to derivatise them for gas-liquid chromatography and mass spectrometry. Diazomethane was prepared and applied to half the extracts in the manner described above. To the other extracts, 0.2 ml of a mixture of pyridine:acetic anhydride (1:1) was added and heated for 1 hour on a hot plate at about 100°C. The extracts were allowed to stand overnight at room temperature, after which time the extracts were dried under N_2. This procedure should allow the formation of tetra-acetate derivatives of any glycosides present (Koshimizu et al., 1968). Together with samples of synthetic ABA, treated with diazomethane or with pyridine:acetic anhydride, the extracts were then analysed by gas-liquid chromatography using a Pye Series 104 gas chromatograph with hydrogen flame ionisation detection and with N_2 as the carrier gas; the gas chromatograph was fitted with a 9' 1% or 3% OV-17 column.

Purified samples were analysed using an AEI MS 12 mass spectrometer (A.E.I. Scientific Apparatus Ltd., Manchester). Samples were either introduced using a direct probe or were injected onto an OV-17 column at 195°C to 210°C in a Pye 104 gas chromatograph with an outlet to the mass spectrometer equipped with a membrane separator. The samples were carried in a flow of helium gas. Chosen peaks of activity observed by gas-liquid chromatography were introduced into the mass spectrometer and scanned.

12. METABOLISM OF ^{14}C-ABA IN WHOLE LEAVES OF RADISH

The third leaves of radish plants were excised at weekly intervals, midway along the petiole, using a razor blade and the cut ends placed immediately in 2 ml of ^{14}C-ABA in small trident bottles, the necks of which were then loosely plugged with cotton wool. After 8 hours incub-

ation in a light room at 25°C, the petioles were cut off at the base of the leaf. The laminas were then extracted in 80% methanol and the methanolic extracts developed on thin-layer plates in chloroform:methanol: water for analysis of the distribution of radioactivity.

Leaf samples were also taken at the same time for fresh weight and chlorophyll determination.

13. ELECTRON MICROSCOPY

Segments of leaf tissue were excised from the centre of surface-sterilized radish leaf discs incubated for varying periods in the dark at 25°C in sterile distilled water or 10^{-4} M ABA. Segments were also taken from mature green leaves and naturally senescent yellow leaves immediately after excision from the intact plant. The segments were fixed either in 3% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.1) for 24 hours at room temperature, prior to storage at 2°C or in Karnovsky's paraformaldehyde/glutaraldehyde fixative (Karnovsky, 1965). For both fixation procedures, the fixative was removed by washing 3 times in buffer over the course of 12 hours; the material was then post-fixed in 1% osmic acid in buffer for 2 hours and then washed as before, followed by dehydration through a graded series of ethanol. Glutaraldehyde-fixed material was then further dehydrated in propylene oxide for 1 hour and infiltrated with a propylene oxide-resin mixture overnight, followed by infiltration in pure resin for 2 to 3 hours (the resin used was either Epon or araldite + 2% plasticizer). After embedding, the material was polymerized for 24 hours at 60°C under vacuum (25 in. Hg). Material treated with the Karnovsky fixative was gradually infiltrated with Spurr resin (Spurr, 1969) added at the 100% alcohol stage of dehydration. After infiltration overnight, the material was embedded and polymerized at 70°C for 8 hours under vacuum. Sections were cut using an L.K.B. "Ultratome III", stained in aqueous uranyl acetate and lead citrate for 1 hour each and examined using an AEI electron microscope 6B.

14. STATISTICAL ANALYSIS

For some of the experimental data presented, an Olivetti Programma 101 desk-top computer was used to calculate the standard error of the mean, using the equation:-

$$\text{Standard error (SE)} = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n(n-1)}}$$

where x = the individual value of each observation

n = the number of observations

Table 1. Chlorophyll, protein and RNA determinations.

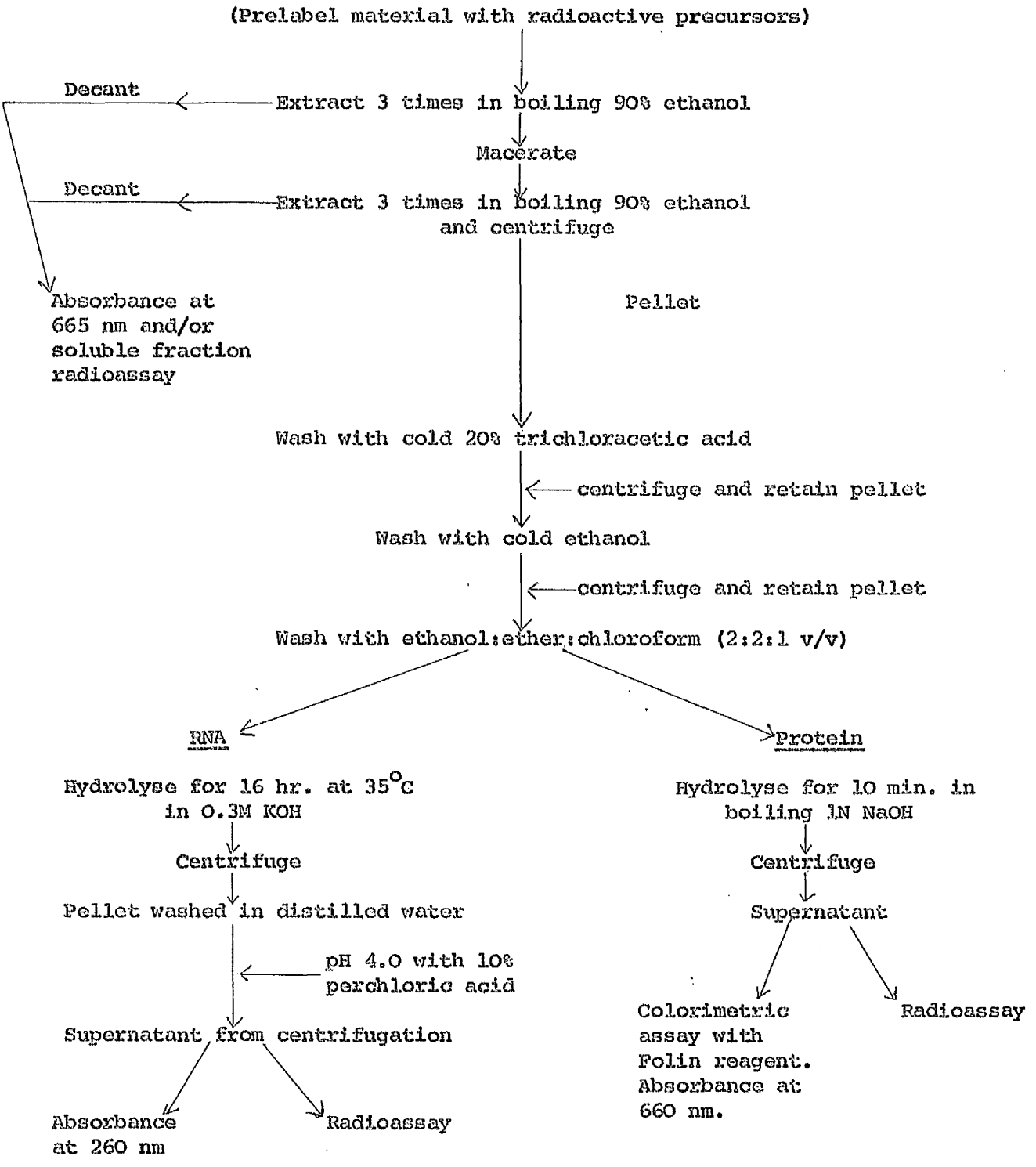


Table 2. Extraction of Endogenous ABA I (after Lenton et al. (1971))

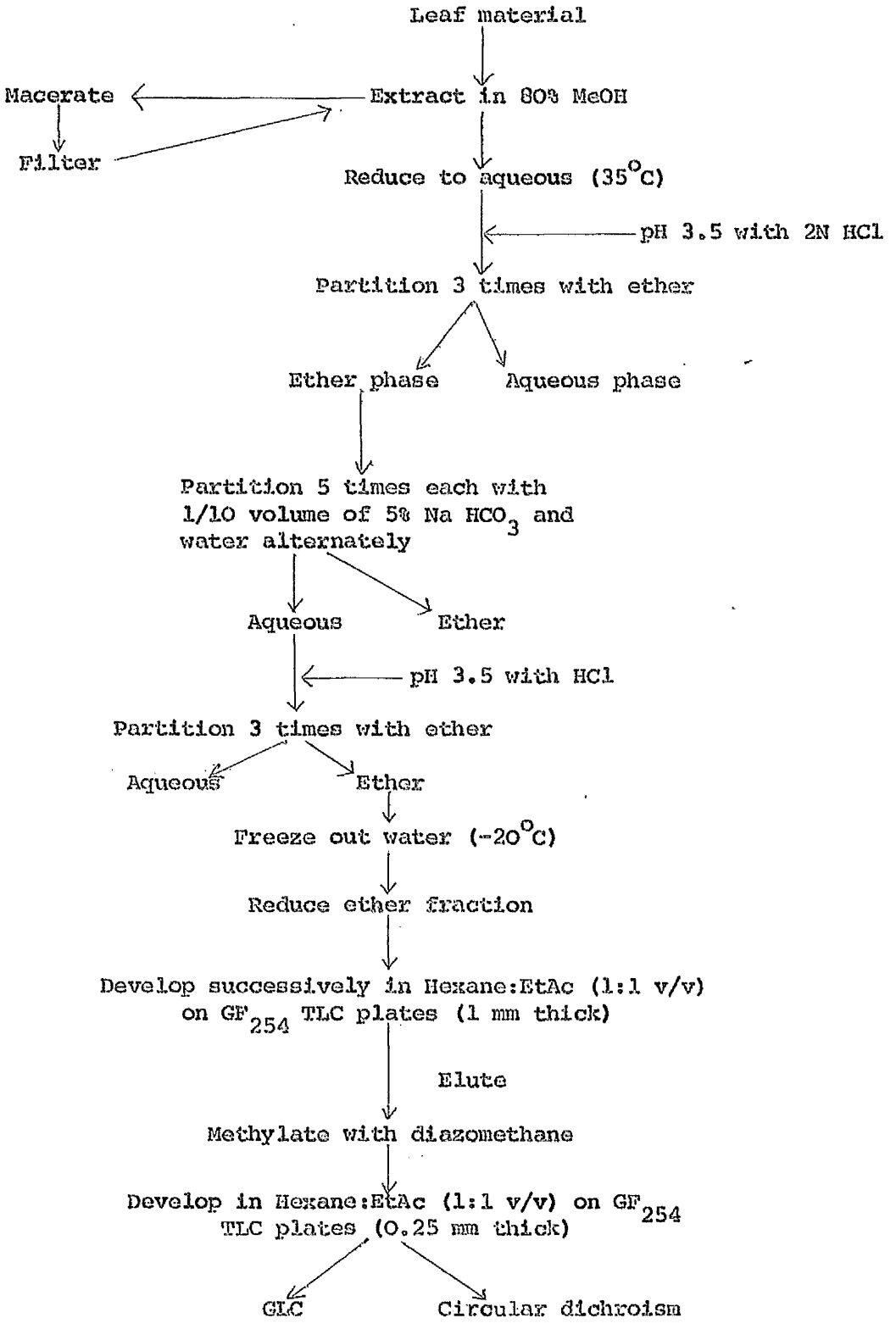


Table 3. Extraction of Endogenous ABA II (after Browning et al. (1970))

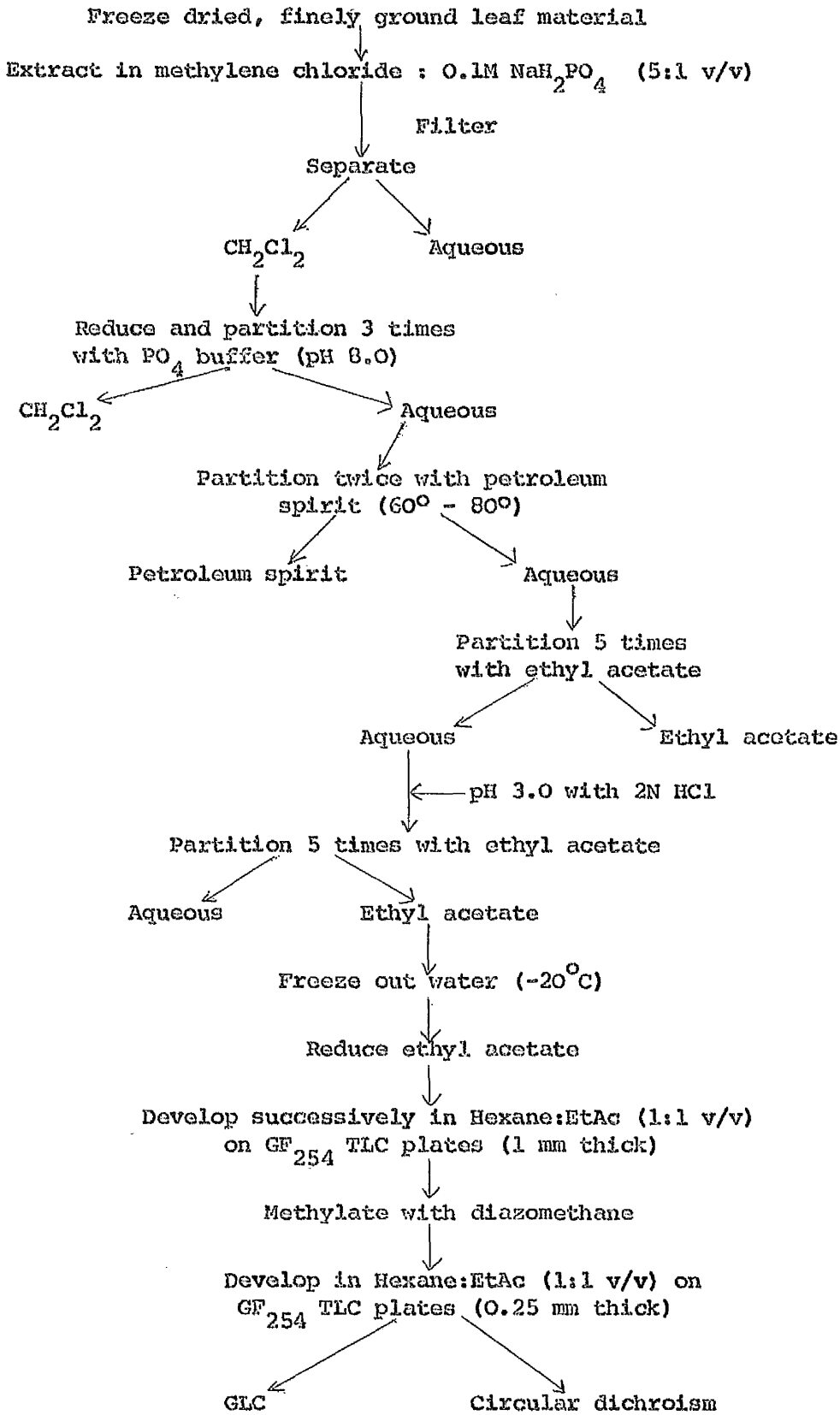
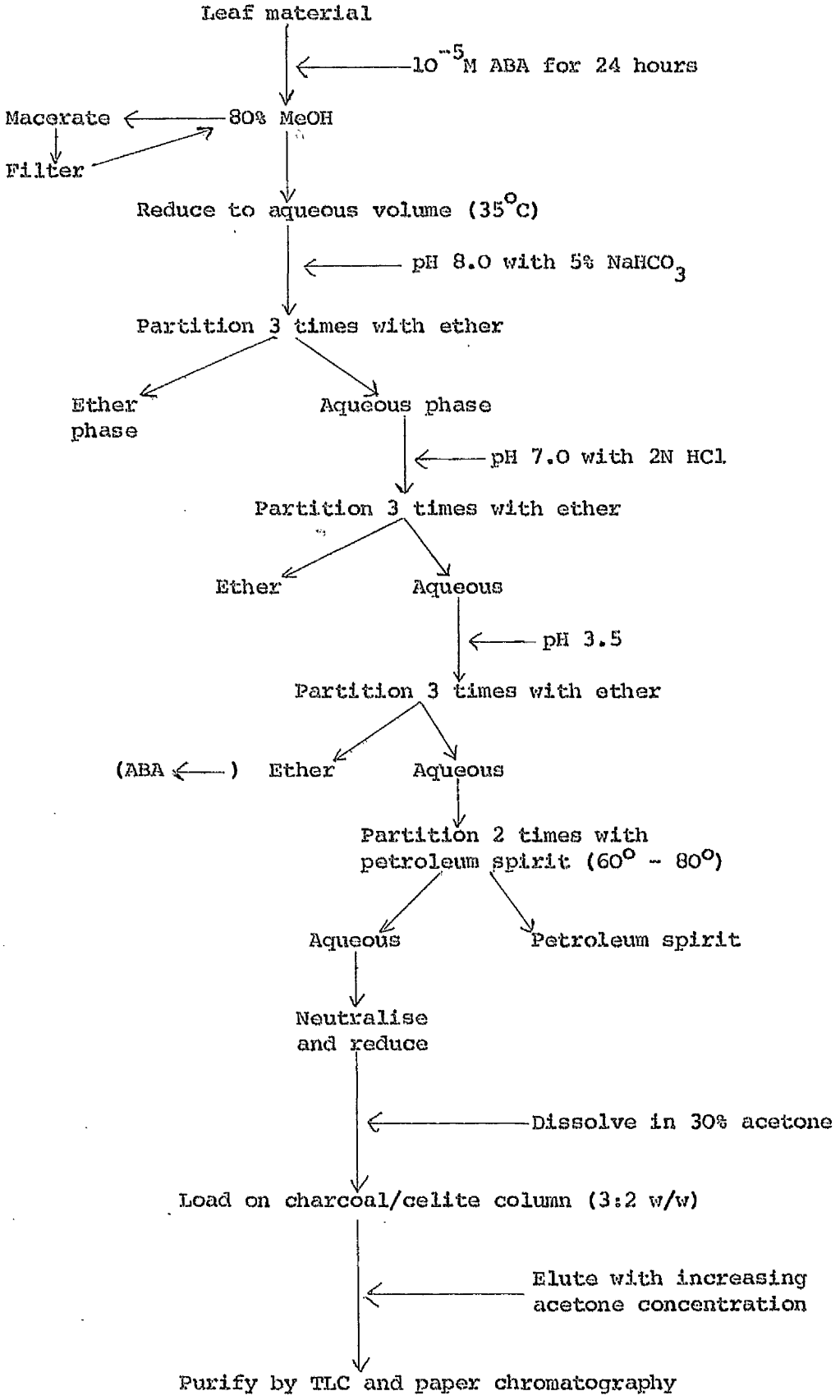


Table 4. Extraction of Metabolites of ABA



EXPERIMENTAL

SECTION I : THE ACTION OF ABA AND KINETIN ON PROTEIN AND RNA LEVELS IN SENESCING RADISH LEAF DISCS

It is known that the plant growth regulators, ABA and cytokinins, can influence the speed of senescence of leaf tissue with particular effects on protein and nucleic acid metabolism (e.g. Richmond and Lang 1957; Osborne, 1962; Beevers, 1968; Colquhoun and Hillman, 1972). It is still debatable, however, as to whether hormonal regulation of protein and nucleic acids in senescing tissue is achieved through modulation of the synthesis of these macromolecules and/or their degradation. Alternatively, these regulators may act at some other site in the cell. The pre-labelling techniques developed by Kuraishi (1968) and Tavares and Kende (1970) appeared to offer a means of resolving these controversies and have therefore been modified to investigate the influence of both ABA and the synthetic cytokinin, kinetin, on the fate of radioactivity incorporated into radish leaf discs.

(a) The effect of ABA on the fate of ^{14}C-8-adenine incorporated into RNA in senescing leaf discs

After incubation in ^{14}C-8-adenine solution for 4 hours, leaf discs were transferred either to sterile distilled water of 10^{-4} M ABA in the presence or absence of 1.3×10^{-5} M non-radioactive adenine. It was considered that the inclusion of adenine at a concentration approximately 100 times that of adenine in the radioactive solution would help to minimise turnover of the labelled molecules. The experiment was carried out 3 times.

Data are presented in Figure 1 to show the effect of the various incubation solutions on the chlorophyll levels of the discs as determined by extraction of batches of discs in ethanol at daily intervals and measurement of the absorbance at 665 nm. Values recorded for"0 days" were obtained by extracting batches of leaf discs immediately after removal from ^{14}C-8-adenine solution (i.e. 4 hours after excision). During the first 3 days of incubation, ABA caused a faster decline in the level of chlorophyll than did water. After 4 days of incubation, however, an apparent reversal of this situation occurred and by 6 days there was slightly more chlorophyll remaining in ABA-treated discs than in water-treated discs. More significantly, visual observations of the discs indicated that while water-treated discs had yellowed considerably, but were still turgid, ABA-treated discs remained relatively green but had become limp. Comparison of chlorophyll levels from discs incubated in

the presence and absence of non-radioactive adenine at this concentration had no apparent major toxic or stimulating effects. Furthermore, the influence of ABA on changes in chlorophyll level were apparently unaffected by the simultaneous presence of adenine.

Data for aliquots, taken from the ethanolic extracts and analyzed for radioactivity (the "alcohol-soluble" fraction) indicated that in the control treatments (water and adenine), the level of detectable radioactivity remained at a relatively high level throughout the 6 day incubation period at a level only slightly lower than the alcohol-soluble radioactivity present in discs extracted immediately after completion of the labelling period (Figure 2). At 1 day, the level of radioactivity in this fraction was slightly higher in treatments containing ABA than in those which did not contain the hormone. After 2 days, however, there was a sharp and continuing decline in soluble radioactivity in ABA-treated discs. The presence of non-radioactive adenine did not influence this trend. Moreover, preliminary data obtained from incubation solutions, dried and subjected to radioassay, indicated that more radioactivity was lost into the incubation medium from ABA-treated discs than from discs treated with either water or adenine alone (Table 5). There was no consistent pattern of difference in radioactivity between water treatments and adenine treatments.

In all treatments, the level of RNA, determined by absorbance at 260 nm, declined from the time of excision and the rate of decline was enhanced in those treatments containing ABA (Figure 3). Comparatively high levels of adenine, an RNA precursor, had no apparent stimulatory effect on RNA levels, regardless of the presence of ABA.

There was an initial increase in the radioactivity present in RNA in all treatments relative to fresh discs; radioactivity in this fraction was at a maximum at 1 to 2 days and then declined gradually at a similar rate in all fractions during the remainder of the 6 day incubation period (Figure 4). ABA suppressed to some extent the initial rise in radioactivity and consequently the amount of radioactive RNA was consistently lower in ABA-treated discs than in water-treated discs. An initial rise in radioactive RNA also occurred in treatments containing adenine, both with and without ABA; it was partially suppressed, however, in adenine-containing treatments relative to water control.

(b) <u>The effect of ABA on the fate of ^{14}C-2-uracil incorporated into RNA in senescing leaf discs</u>

Although ^{14}C-8-adenine has been used in previous studies of the effect of ABA on RNA metabolism (Beevers, 1968; Colquhoun and Hillman,

Figure 1. Radish leaf discs pre-labelled with ^{14}C-8-adenine. Changes in chlorophyll levels during 6 days incubation in ABA.

Data are shown with associated standard errors.

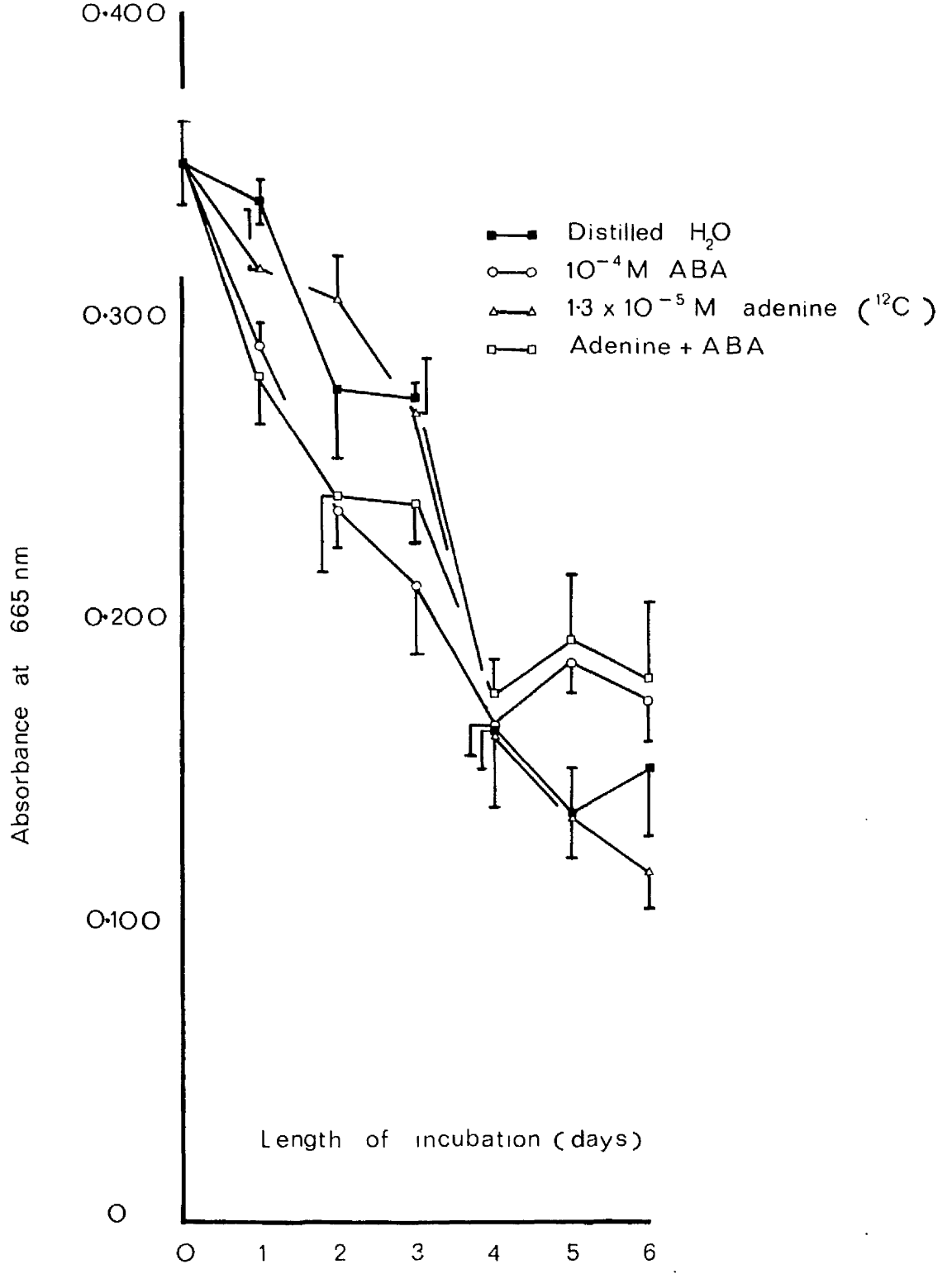

0·400
0·300
0·200
0·100
0
Absorbance at 665 nm
Distilled H_2O
10^{-4} M ABA
$1{\cdot}3 \times 10^{-5}$ M adenine (^{12}C)
Adenine + ABA
Length of incubation (days)
0
1
2
3
4
5
6

Figure 2. Radish leaf discs pre-labelled with ^{14}C-8-adenine. Changes in radioactivity of the alcohol-soluble fraction during 6 days incubation in ABA.

For key to symbols, see Figure 1.

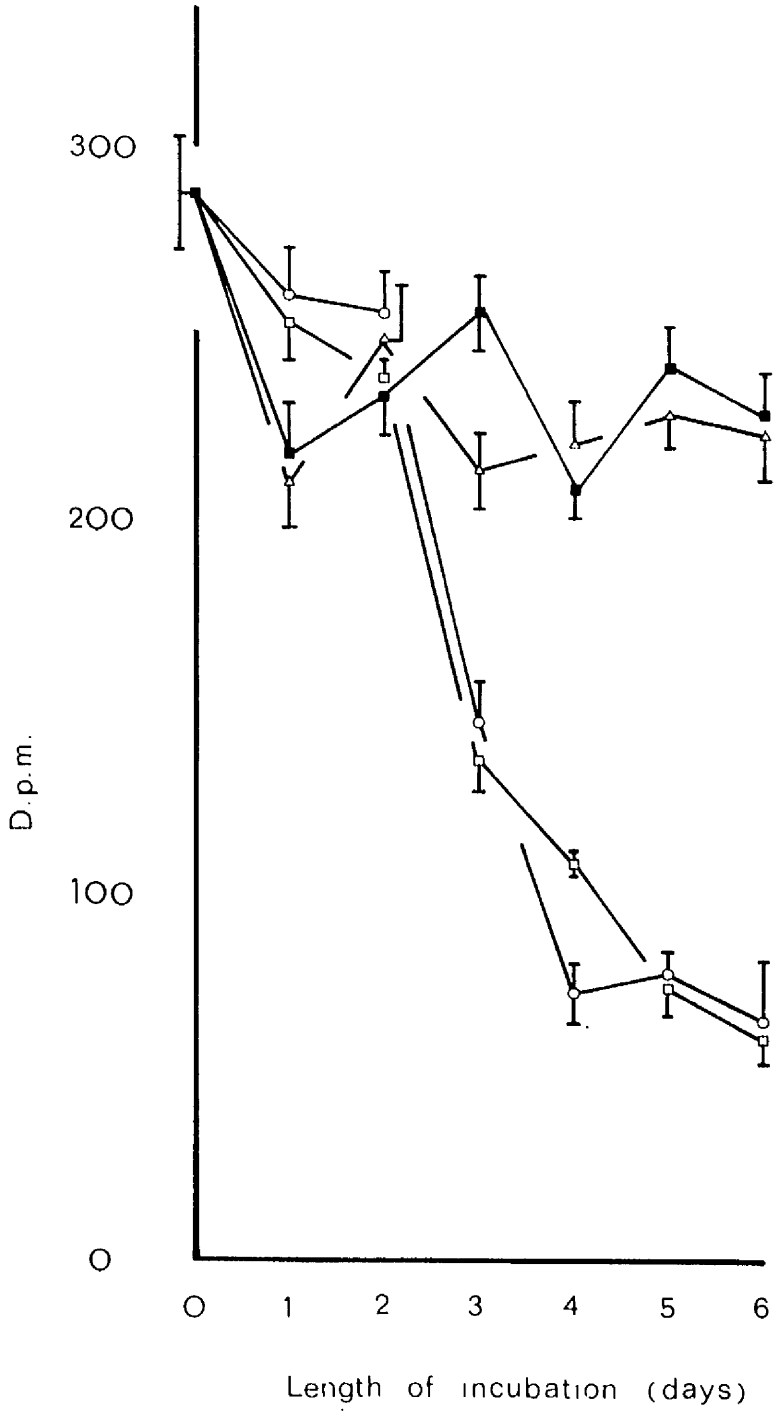

300
200
100
0
D.p.m.
0
1
2
3
4
5
6
Length of incubation (days)

Figure 3. Radish leaf discs pre-labelled with ^{14}C-8-adenine. Changes in RNA levels during 6 days incubation in ABA.

For key to symbols, see Figure 1.

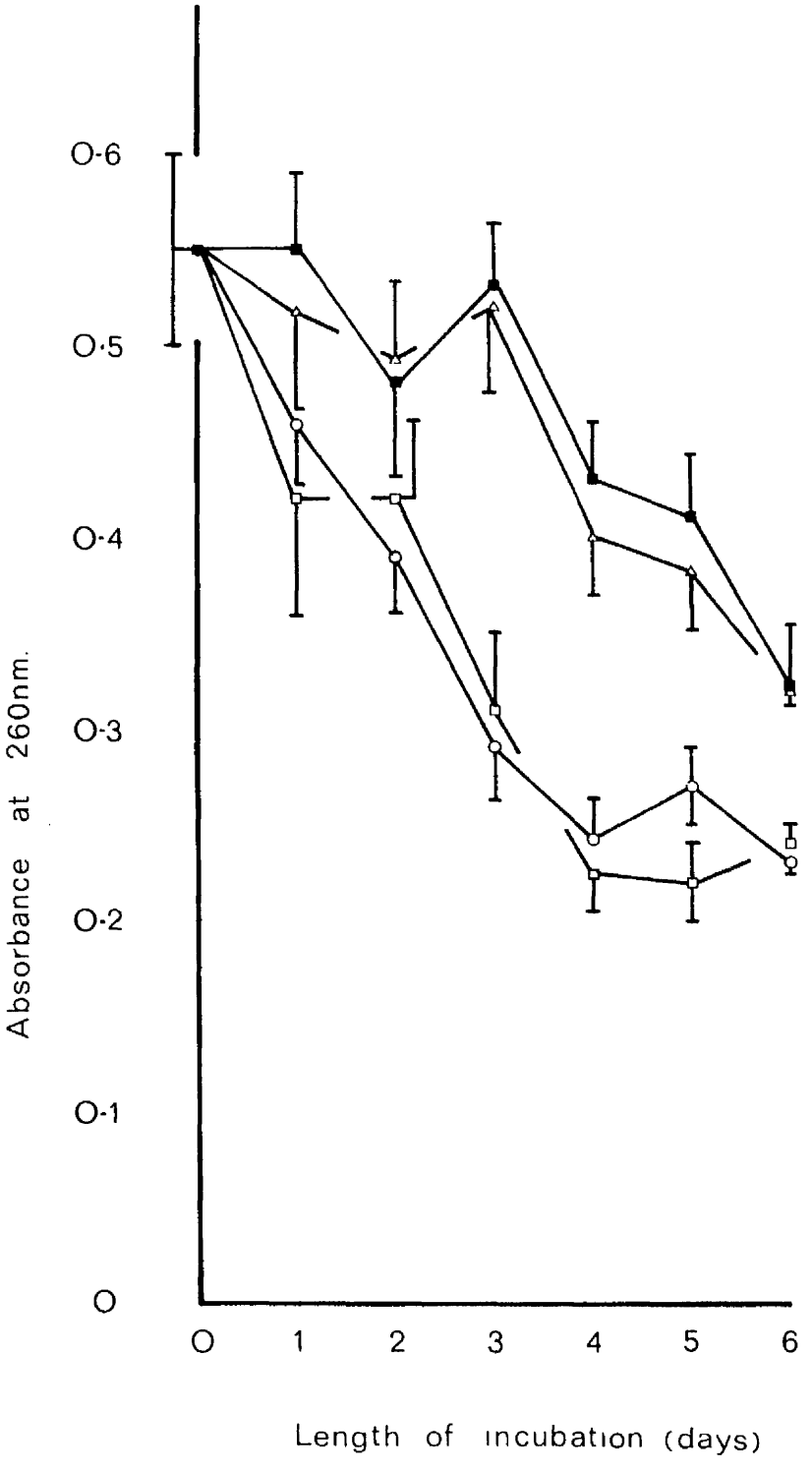

O·6
O·5
O·4
O·3
O·2
O·1
O
Absorbance at 260nm.
O
1
2
3
4
5
6
Length of incubation (days)

Figure 4. Radish leaf discs pre-labelled with ^{14}C-8-adenine. Changes in radioactivity of the RNA fraction during 6 days incubation in ABA.

For key to symbols, see Figure 1.

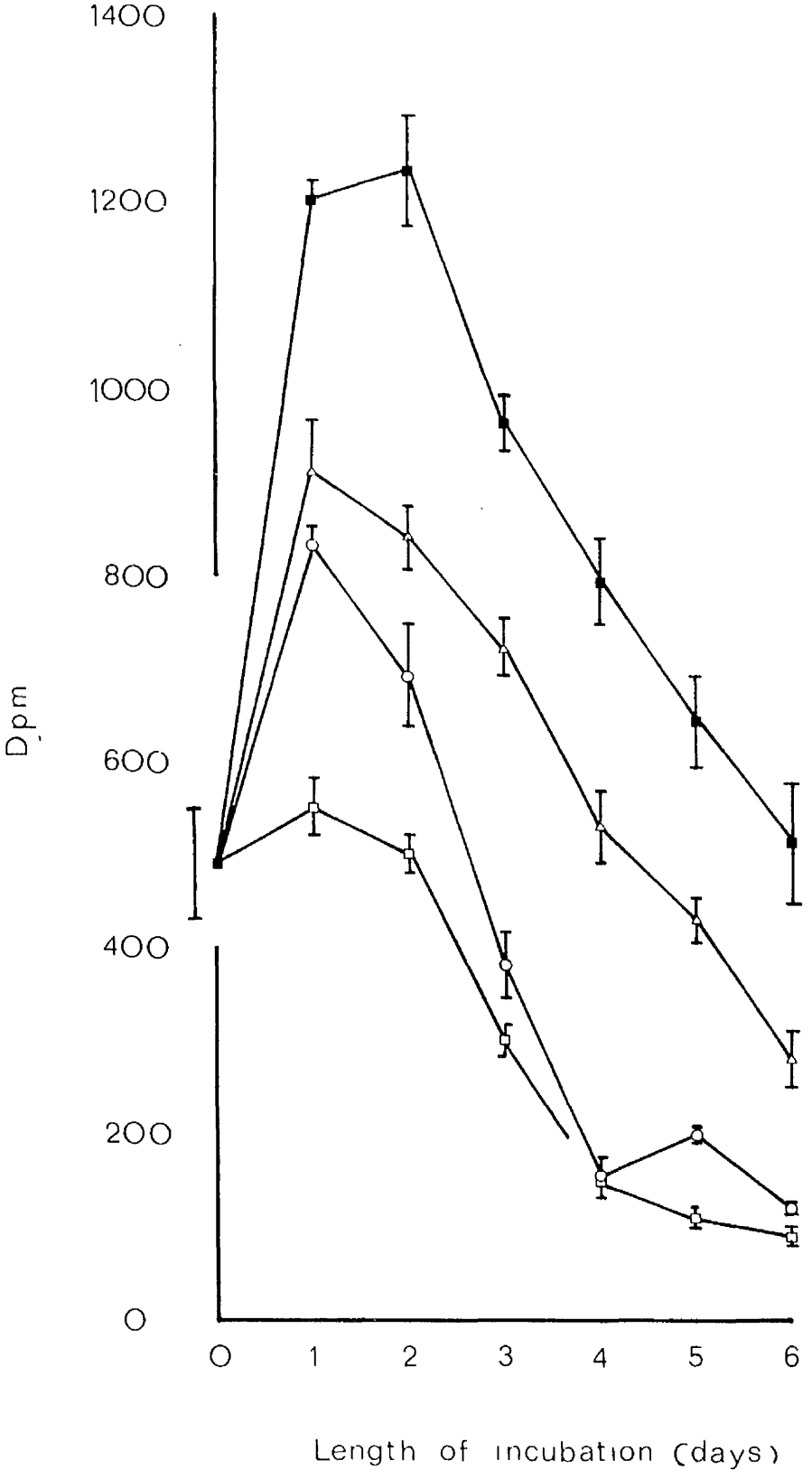

1400
1200
1000
800
600
400
200
0
Dpm
0
1
2
3
4
5
6
Length of incubation (days)

Table 5. Radioactivity in incubation solutions from leaf discs pre-labelled with ^{14}C-8-adenine

Treatment	Length of incubation (days)						Total dpm (1-6 days)
	1	2	3	4	5	6	
Distilled water	94.3	85.6	390.7	75.9	104.9	84.0	835.4
10^{-4} M ABA	211.7	410.4	1777.0	1286.0	595.3	606.0	4886.4
1.3×10^{-5} M Adenine	219.5	79.2	162.2	106.9	224.3	101.4	893.4
Adenine + ABA	479.1	595.7	787.2	665.6	485.8	506.1	3519.5

(Disintegrations per minute)

Table 6. Radioactivity in incubation solutions from leaf discs pre-labelled with ^{14}C-2-uracil

Treatment	Length of incubation (days)						Total dpm (1-6 days)
	1	2	3	4	5	6	
Distilled water	452.9	316.6	943.8	564.8	453.3	551.8	3283.2
10^{-4} M ABA	464.2	951.4	956.4	1707.2	1777.2	2105.4	7961.8
8.3×10^{-5} M Uracil	383.5	287.6	330.1	460.2	439.0	951.3	2851.7
Uracil + ABA	685.3	557.5	1184.8	1254.2	1120.5	1792.8	6595.1

(Disintegrations per minute)

1972), the previous experiment, I(a), could be criticised on the basis that adenine is an important component of many other portions of cell metabolism and that, in particular, it is a precursor for DNA as well as for RNA. It was therefore relevant to repeat the previous experiment replacing ^{14}C-8-adenine with ^{14}C-2-uracil, a compound not thought to act as a precursor for DNA. In order to minimise the turnover of ^{14}C-uracil molecules, 8.3×10^{-5}M non-radioactive uracil was added to the post-labelling incubation solutions. The experiment was carried out 3 times with similar results.

ABA, in the presence or absence of non-radioactive uracil stimulated the decline in chlorophyll level between 0 and 3 days (Figure 5). By 4 days any differences between the treatments are lost. Despite the fact that the reversal noted in Figure 1 was numerically less distinct in this instance, the visual characteristics of disc colour and texture were similar to those noted in the first experiment. The inclusion of uracil at 8.3×10^{-5} M had no apparent effect on the pattern or rate of chlorophyll loss.

In contrast to the previous experiment there was an initial sharp decline in the radioactivity in the alcohol-soluble fraction and no differences were detectable between the 4 treatments until after 4 days (Figure 6). After this time, however, ABA appeared to stimulate further the decline in radioactivity of this fraction, whether or not uracil was present. There was little difference between the radioactivity detectable in water and in uracil treatments. Moreover, the preliminary data presented in Table 6 indicate that from 3 to 4 days onwards there was more radioactivity present in those incubation solutions which contained ABA than in those which did not.

As in Figure 3, the level of RNA in all treatments declined from the time of excision and the rate of decline was enhanced by ABA (Figure 7). Little difference was detected between those treatments which contained non-radioactive uracil and those which did not.

The radioactivity present in the RNA fraction rose from the levels detected at day 0, attaining a maximum at 1 to 2 days, before declining in all treatments (Figure 8). In agreement with the data of Figure 4, ABA suppressed this rise; consequently, the amount of radioactive RNA in ABA-treated discs was consistently lower throughout the incubation period than the amount from control discs. The rates of decline in radioactive RNA, however, were similar for all treatments. In contrast to Figure 4, the rise in radioactivity in RNA at 1 to 2 days was not significantly suppressed by the presence of non-radioactive uracil; in

Figure 5. Radish leaf discs pre-labelled with ^{14}C-2-uracil. Changes in chlorophyll levels during 6 days incubation in ABA.

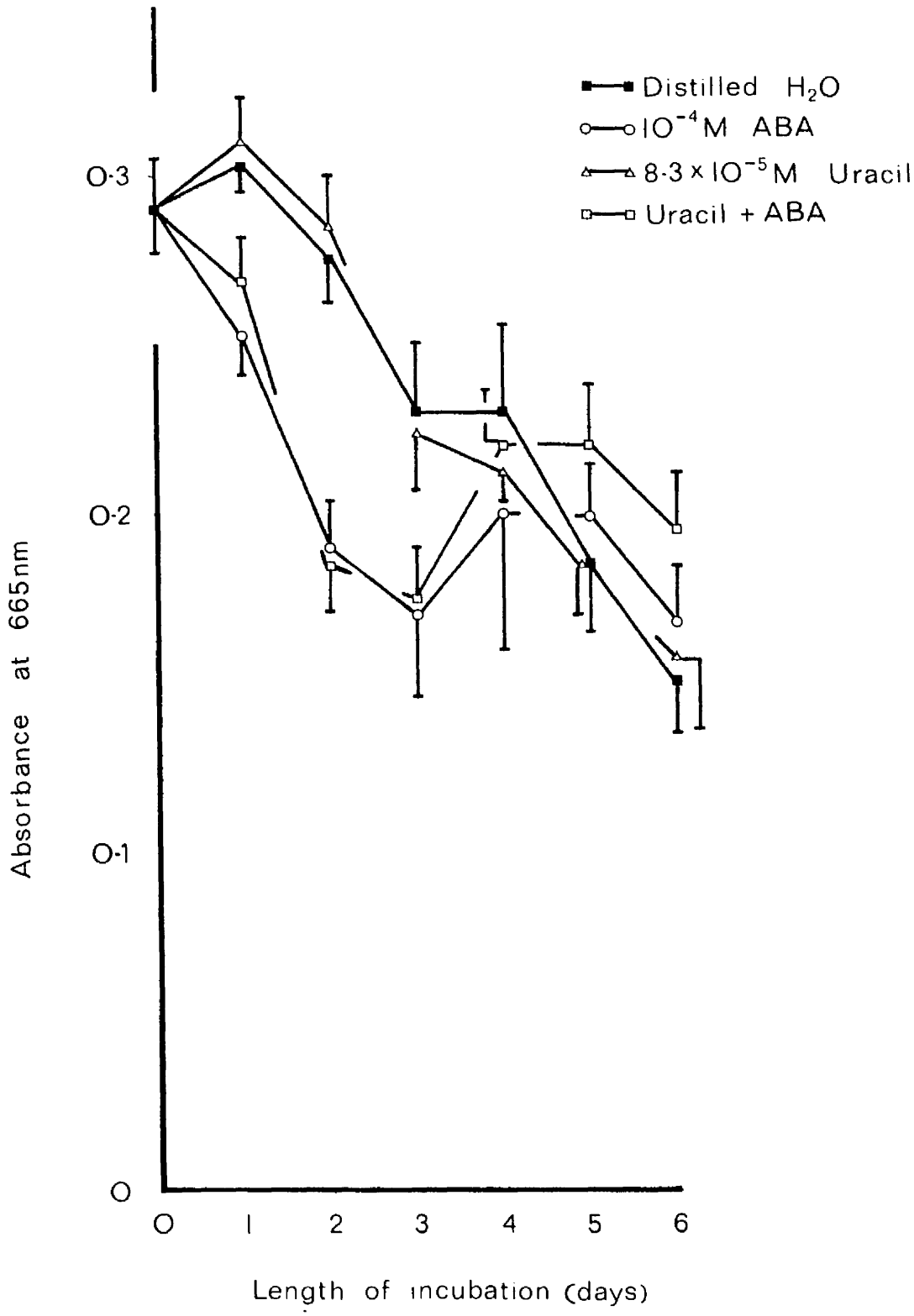

Distilled H_2O
10^{-4}M ABA
$8{\cdot}3 \times 10^{-5}$M Uracil
Uracil + ABA
0·3
0·2
0·1
0
Absorbance at 665nm
0
1
2
3
4
5
6
Length of incubation (days)

Figure 6. Radish leaf discs pre-labelled with ^{14}C-2-uracil. Changes in radioactivity of the alcohol-soluble fraction during 6 days incubation in ABA.

For key to symbols, see Figure 5.

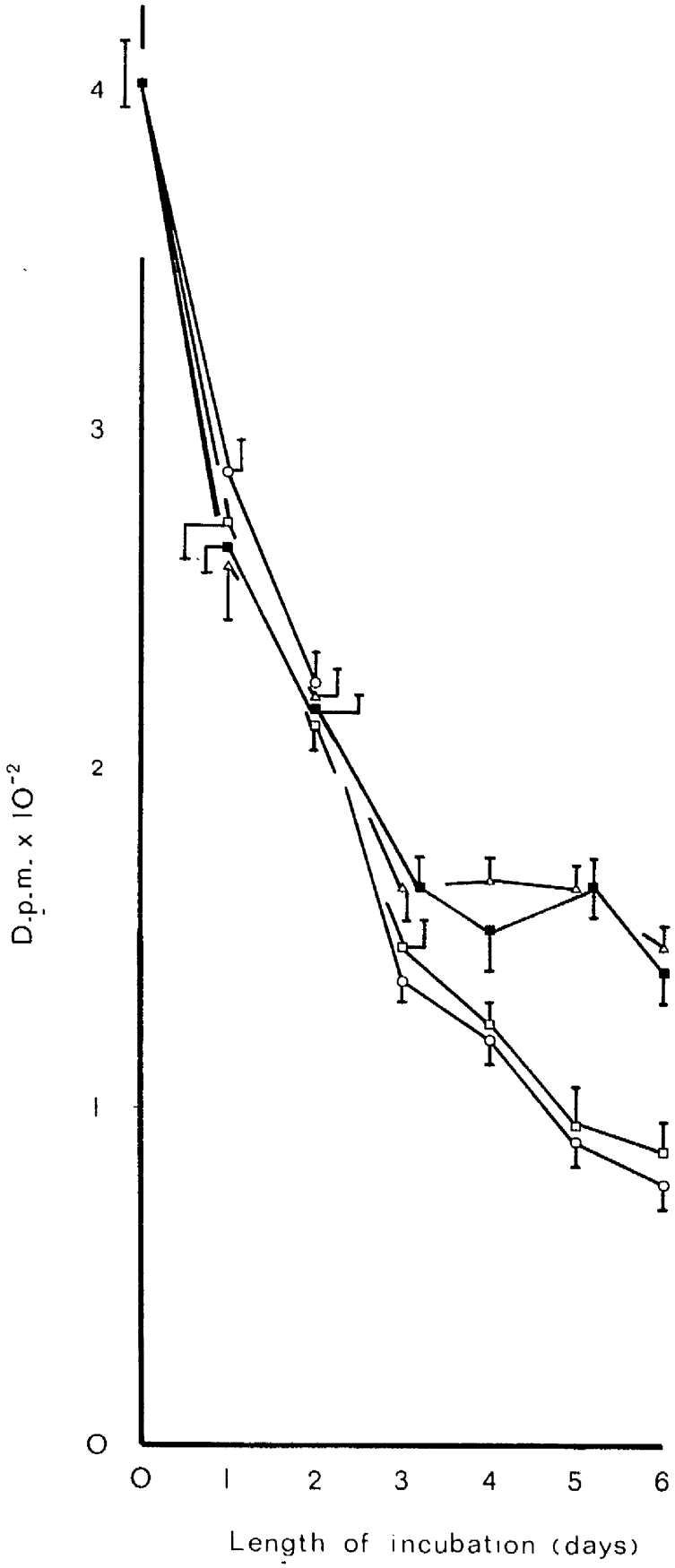

4
3
2
1
0
D.p.m. x 10^{-2}
0
1
2
3
4
5
6
Length of incubation (days)

Figure 7. Radish leaf discs pre-labelled with ^{14}C-2-uracil. Changes in RNA levels during 6 days incubation in ABA.

For key to symbols, see Figure 5.

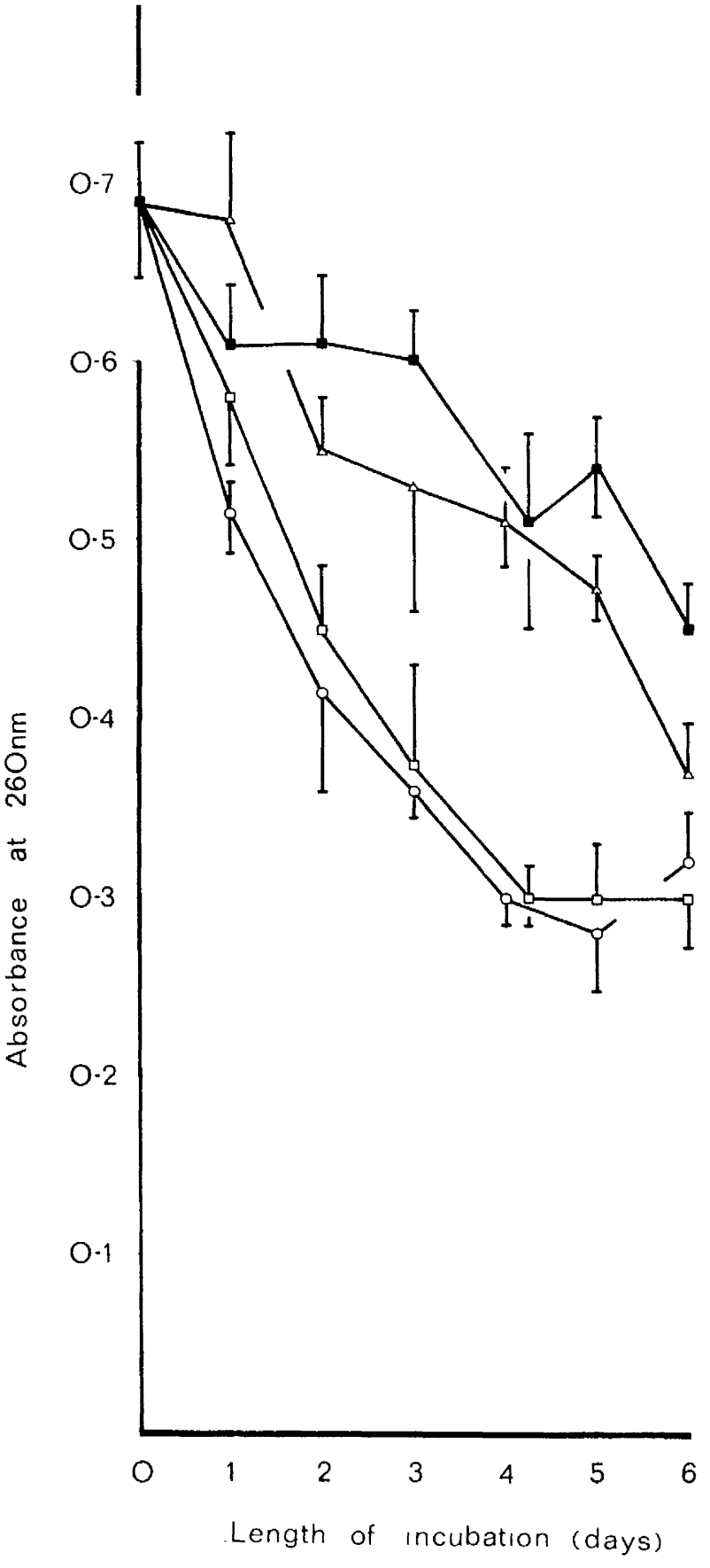
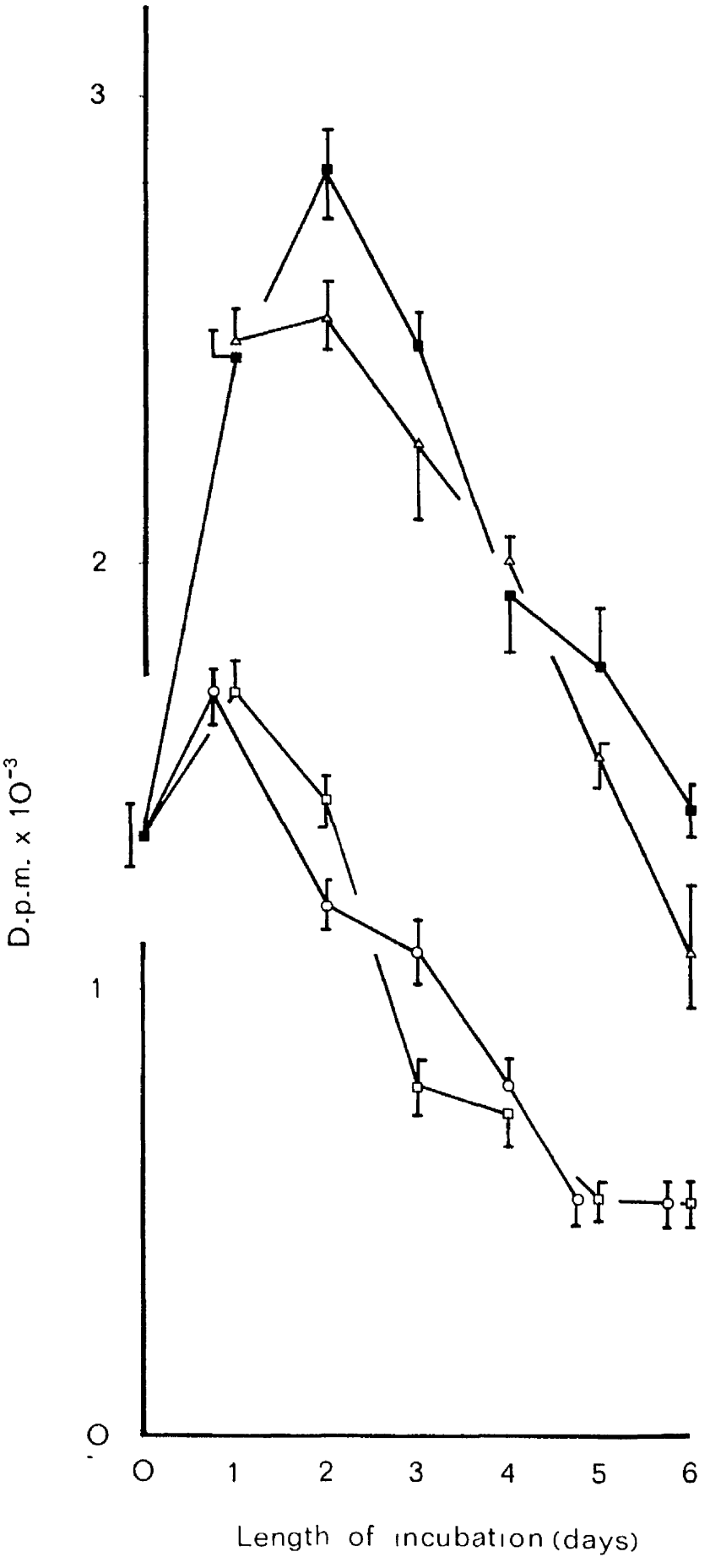

O·7
O·6
O·5
O·4
O·3
O·2
O·1
Absorbance at 260nm
0
1
2
3
4
5
6
Length of incubation (days)

Figure 8. Radish leaf discs pre-labelled with ^{14}C-2-uracil. Changes in radioactivity of the RNA fraction during 6 days incubation in ABA.

For key to symbols, see Figure 5.


3
2
1
0
D.p.m. x 10⁻³
0
1
2
3
4
5
6
Length of incubation (days)

general, uracil did not appear to affect the changes in radioactive RNA, relative to treatments which did not contain uracil.

(c) The effect of ABA on the fate of ^{14}C-1-leucine incorporated into protein in senescing leaf discs

The experiments described above examined the effect of ABA on RNA metabolism using ^{14}C-8-adenine and ^{14}C-2-uracil as RNA precursors. Although synthesis of cellular proteins is dependent on RNA metabolism, protein turnover rates and the effect of hormones on such turnover rates may not necessarily mirror changes in RNA metabolism. Indeed Kuraishi (1968) and Tavares and Kende (1970) examined the role of kinetin in regulating the loss of radioactivity from pre-labelled proteins. It was thus of interest to examine the effect of 10^{-4} M ABA on the loss of radioactivity from protein pre-labelled with ^{14}C-1-leucine and incubated in the presence or absence of non-radioactive leucine at 7.8×10^{-5} M. The experiment was carried out 3 times with similar results.

The chlorophyll levels of discs incubated in the presence of ABA declined at a faster rate than in control treatments during the first 2 to 3 days after excision (Figure 9). After this time, there was little further loss of chlorophyll in discs treated with ABA and the discs exhibited the textural and pigment differences due to ABA noted in earlier experiments. The presence of non-radioactive leucine at a concentration of 7.8×10^{-5} M did not apparently influence the rate of chlorophyll loss.

There was an early sharp decrease in the radioactivity of the alcohol-soluble fraction (Figure 10); thus these data are similar to those presented in Figure 6, but contrast with those in Figure 7. The effects of ABA were statistically apparent only after 3 days. There was little difference between treatments which contained leucine and those which did not.

In accord with the preliminary data presented in Tables 5 and 6, incubation solutions including ABA contain more radioactivity than solutions which do not (Table 7).

Examination of the changes in gross protein levels during the incubation period of 6 days indicated that the amounts of protein declined sharply following excision (Figure 11). There was little variation between the different treatments at 1 day, but between 2 and 4 days it was evident that ABA significantly stimulated protein loss relative to control treatments (water and leucine). The inclusion of leucine, a protein precursor, had little apparent effect in the pattern of protein loss, whether ABA was present or not.

Figure 9. Radish leaf discs pre-labelled with ^{14}C-l-leucine. Changes in chlorophyll levels during 6 days incubation in ABA.

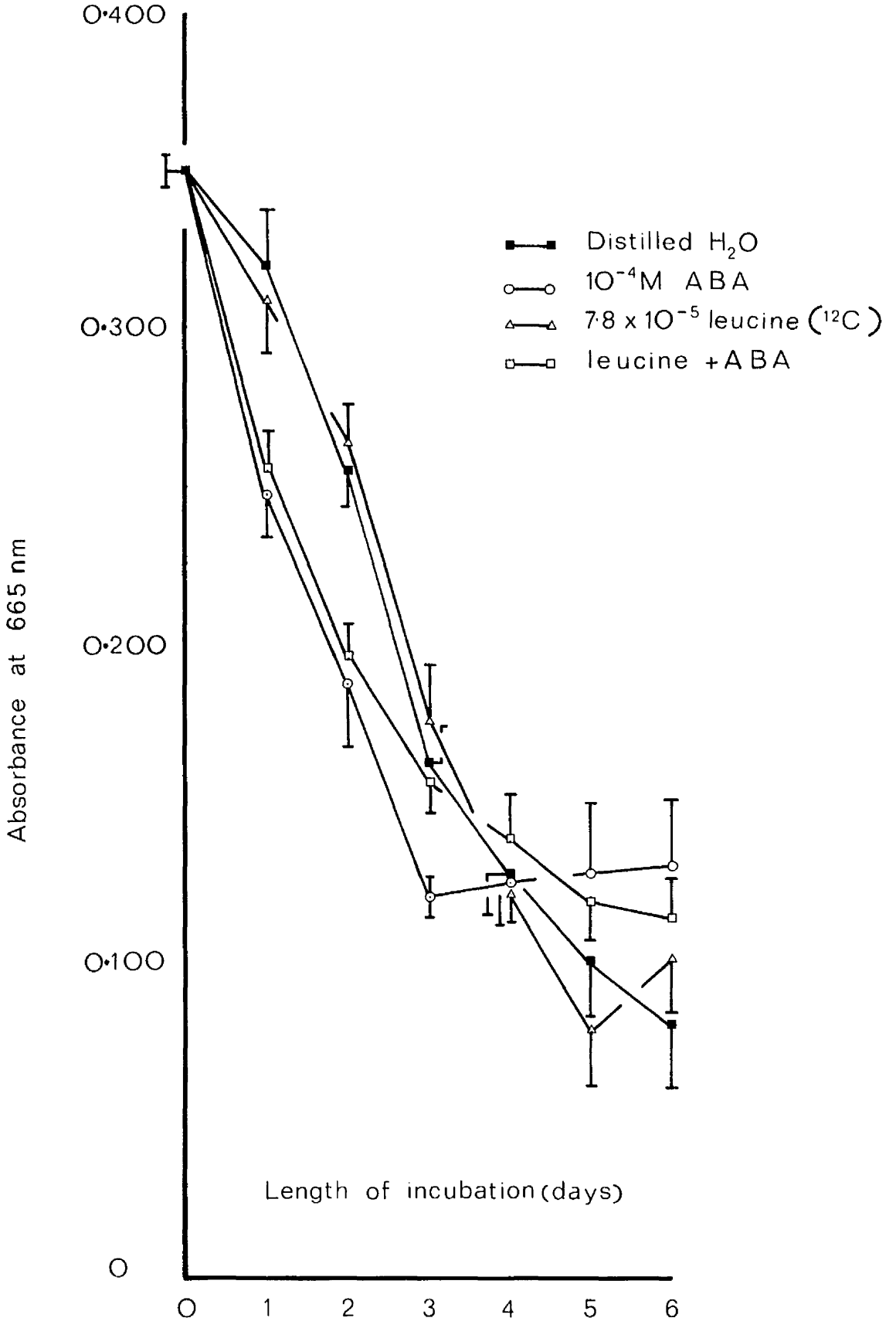

0·400
0·300
0·200
0·100
0
Absorbance at 665 nm
Distilled H_2O
10^{-4}M ABA
$7{\cdot}8 \times 10^{-5}$ leucine (^{12}C)
leucine +ABA
Length of incubation (days)
0
1
2
3
4
5
6

Figure 10. Radish leaf discs pre-labelled with ^{14}C-l-leucine. Changes in radioactivity of the alcohol-soluble fraction during 6 days incubation in ABA.

For key to symbols, see Figure 9.

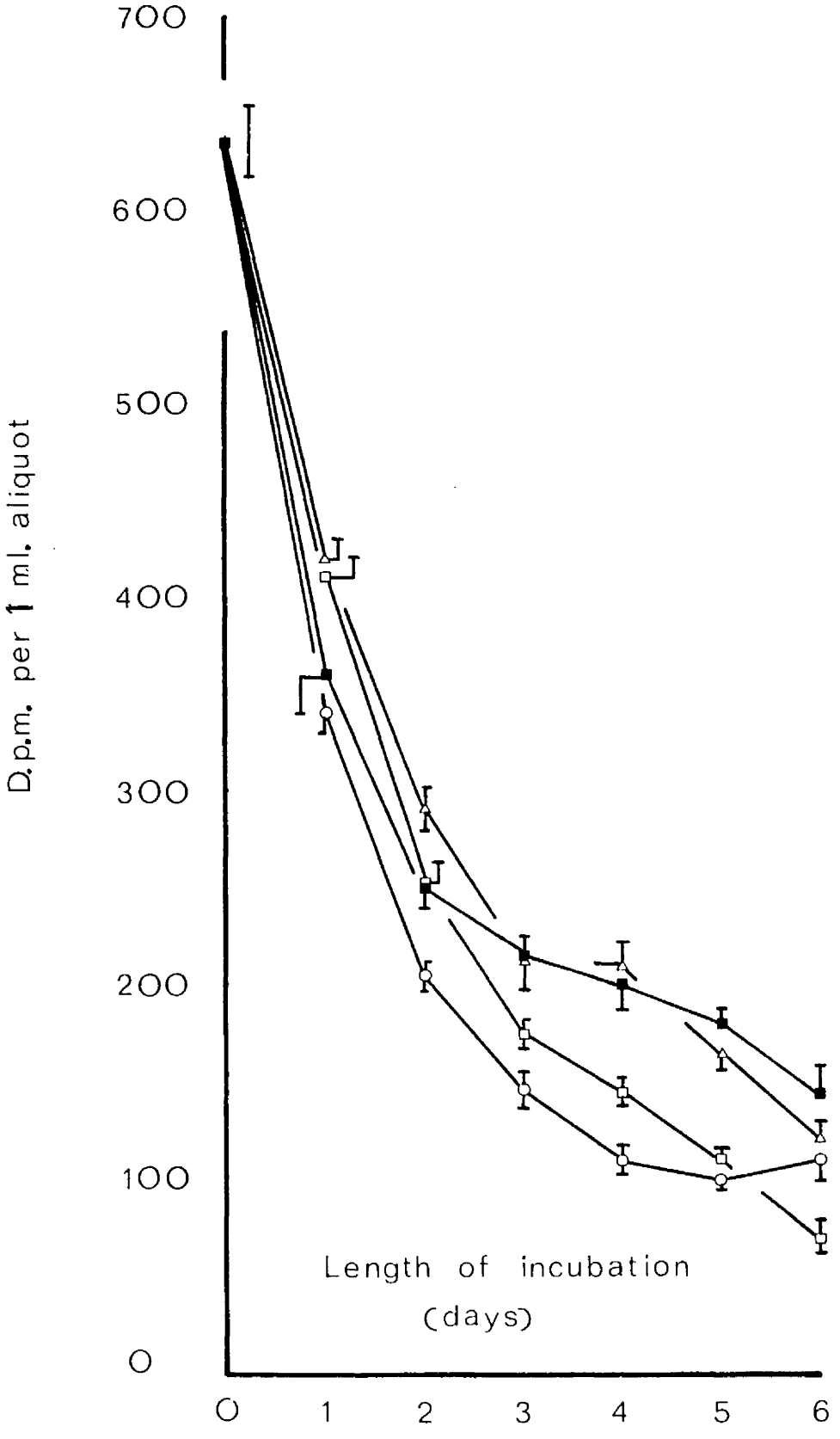

700
600
500
400
300
200
100
0
D.p.m. per 1 ml. aliquot
Length of incubation
(days)
0
1
2
3
4
5
6

Figure 11. Radish leaf discs pre-labelled with ^{14}C-l-leucine. Changes in protein levels during 6 days incubation in ABA.

For key to symbols, see Figure 9.

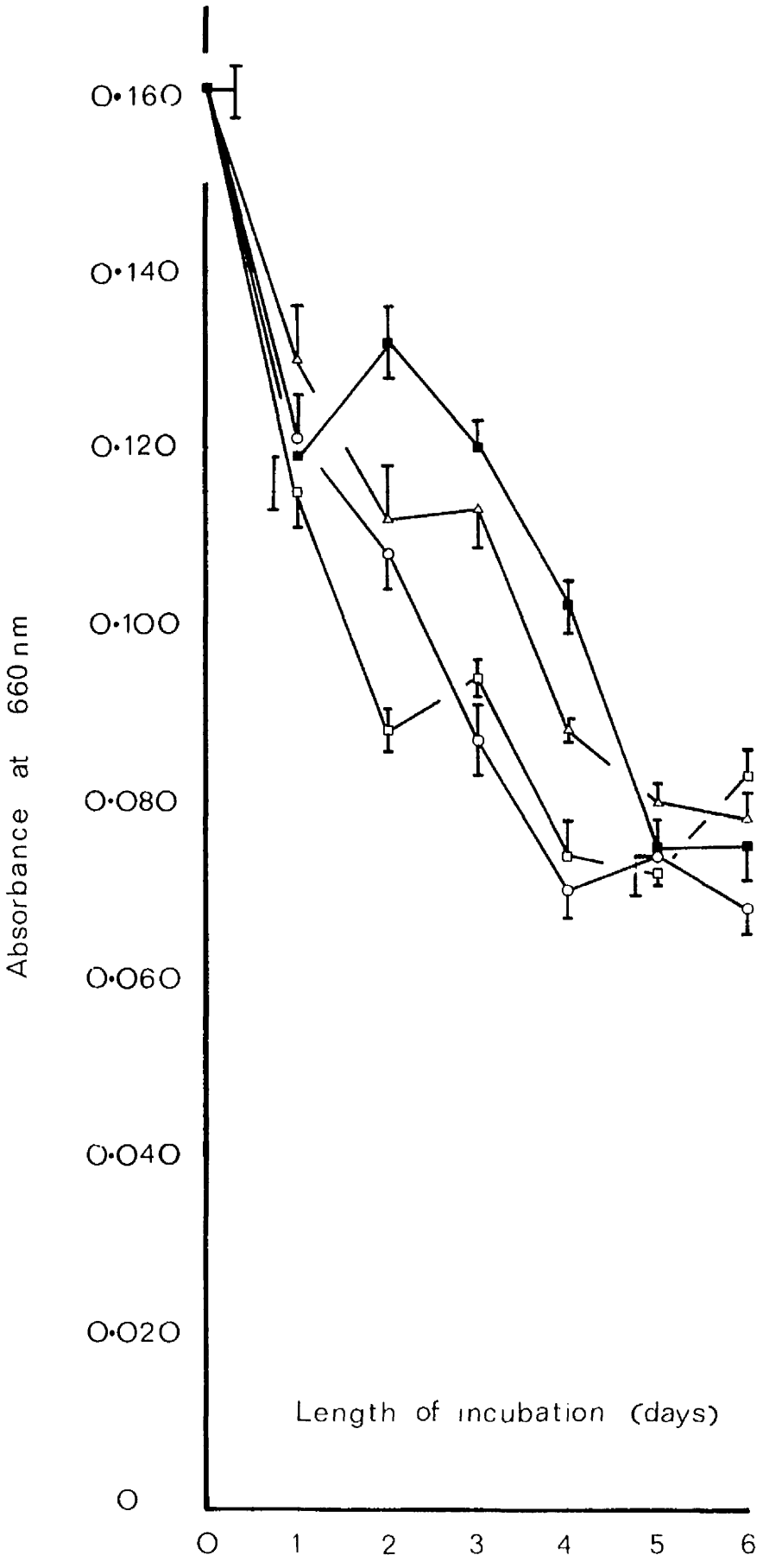

0·160
0·140
0·120
0·100
0·080
0·060
0·040
0·020
0
Absorbance at 660 nm
Length of incubation (days)
0
1
2
3
4
5
6

Figure 12. Radish leaf discs pre-labelled with ^{14}C-l-leucine. Changes in radioactivity of the protein fraction during 6 days incubation in ABA.

For key to symbols, see Figure 9.

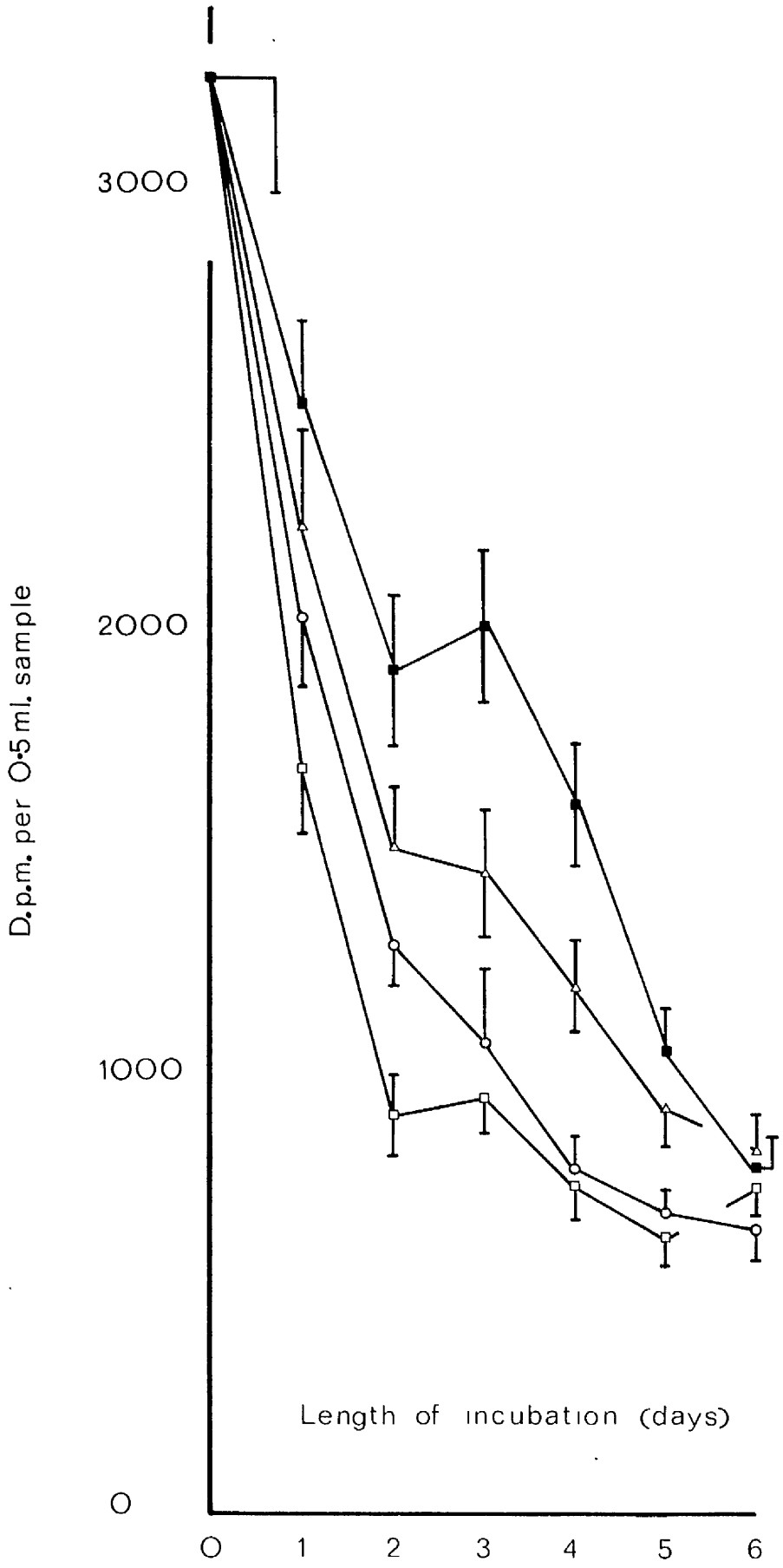

3000
2000
1000
0
D.p.m. per 0·5 ml. sample
Length of incubation (days)
0
1
2
3
4
5
6

Table 7. Radioactivity in incubation solutions from leaf discs pre-labelled with ^{14}C-1-leucine

Treatment	Length of incubation (days) 1	2	3	4	5	6	Total dpm (1-6 days)
Distilled H_2O	646.8	657.1	696.0	801.8	801.5	1947.2	5550.4
10^{-4} M ABA	1471.9	2106.4	3118.1	3286.5	4074.4	4181.6	18238.9
7.8×10^{-5} M leucine	524.1	591.8	632.1	627.0	727.0	866.8	3968.8
ABA + leucine	967.3	1279.6	1670.8	3808.7	2388.4	2904.8	13019.6

(Disintegrations per minute)

Table 8. Radioactivity in incubation solutions from leaf discs pre-labelled with ^{14}C-1-leucine

Treatment	Length of incubation (days) 1	2	3	Total radioactivity (Days 1-3)
Distilled H_2O	597.3	616.6	670.8	1884.7
2×10^{-5} M Kinetin	551.8	608.4	919.3	2079.5
7.8×10^{-5} M Leucine	475.7	468.5	522.8	1467.0
Leucine + Kinetin	476.6	672.3	317.2	1466.1

D.p.m.

In contrast to the data obtained in previous experiments in which the radioactivity of the RNA fraction increased in the early part of the post-labelling period, the amount of radioactive protein declined sharply from day 0 (Figure 12). The presence of both ABA and leucine appeared to stimulate this decline; fractions from ABA-treated discs contained significantly less radioactive protein than those from control discs. Fractions from leucine-treated discs, at no time before 6 days, contained more radioactive protein than those from treatments without leucine.

(d) The effect of kinetin on the fate of ^{14}C-l-leucine incorporated into protein in senescing leaf discs

The original pre-labelling techniques of Kuraishi (1968) and Tavares and Kende (1970) were used for examining the role of kinetin in maintaining protein levels in senescing leaf tissue. The data presented above, however, indicated that incorporation of radioactivity into RNA and protein was continuing after the completion of the pre-labelling period and that radioactivity was passing from the soluble pool into RNA and protein. The original experiments of Kuraishi and Tavares and Kende were thus re-investigated in the light of these observations.

After a period of 4 hours in ^{14}C-l-leucine, discs were transferred to solutions containing 2×10^{-5} M kinetin for 3 days. Non-radioactive leucine was included in incubation solutions, as before. The experiment was carried out 3 times with similar results.

No significant differences were detected between the 4 treatments with respect to chlorophyll levels, until 3 days, when it was evident that kinetin was delaying chlorophyll loss relative to control treatments (Figure 13). The inclusion of non-radioactive leucine had little effect on the pattern of chlorophyll loss when compared to treatments without leucine.

Alcohol-soluble radioactivity declined more rapidly during day 1 in those treatments which did not contain leucine than in those which did (Figure 14). Further, there was some indication that kinetin slightly delayed the decline in radioactivity of the alcohol-soluble fraction. At 2 and 3 days, however, there were no significant differences between the 4 treatments.

Radioassay of the incubation solutions revealed no consistent differences between the 4 treatments (Table 8).

Kinetin had little significant effect on the decline in protein levels during the first 2 days of incubation (Figure 15). At 3 days, however, there was evidence to suggest that protein levels were higher

Figure 13. Radish leaf discs pre-labelled with ^{14}C-l-leucine. Changes in chlorophyll levels during 3 days incubation in kinetin.

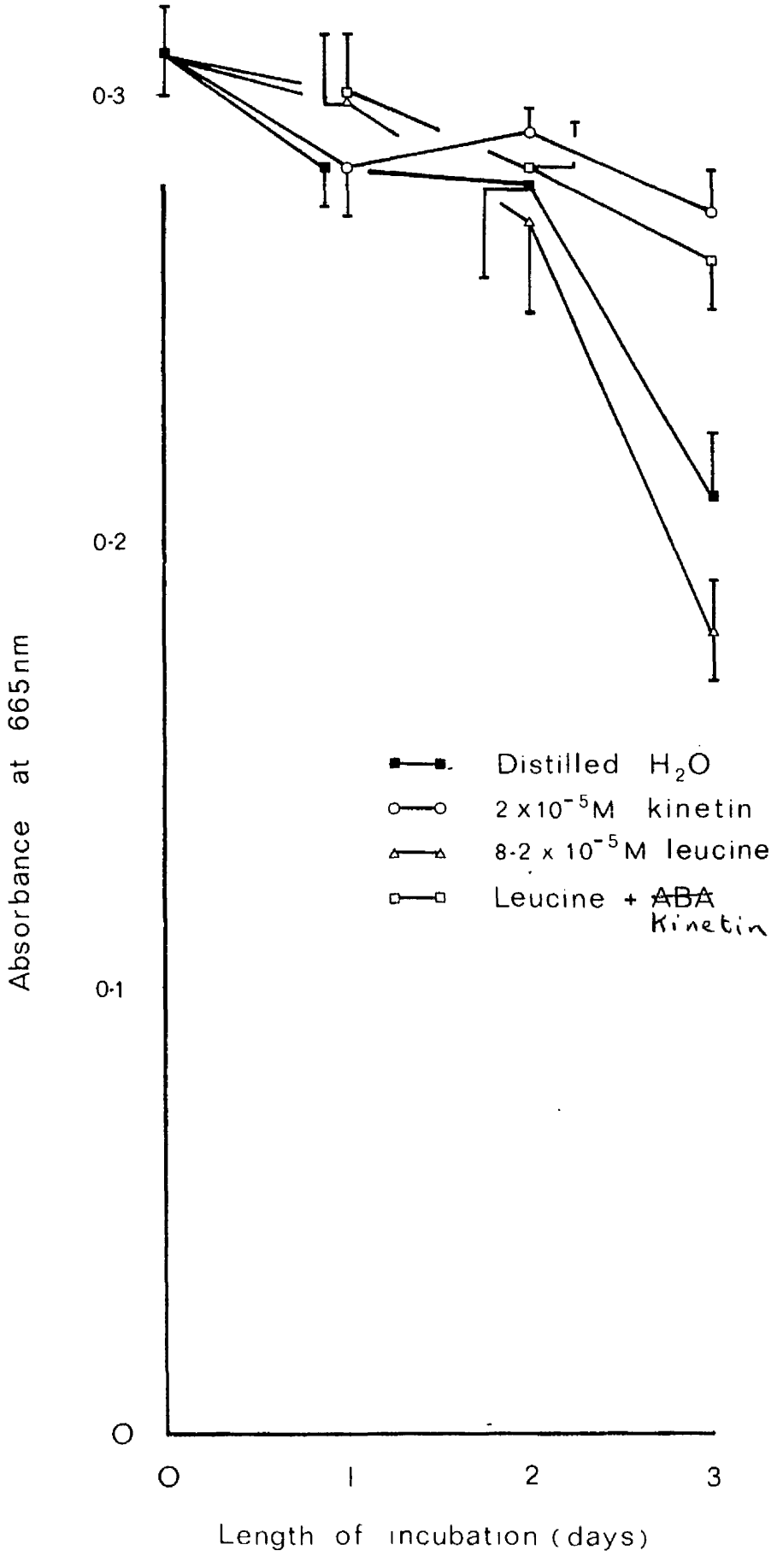

0·3
0·2
0·1
0
T
Absorbance at 665nm
Distilled H_2O
2×10^{-5}M kinetin
$8{\cdot}2 \times 10^{-5}$M leucine
Leucine + ~~ABA~~ Kinetin
0
1
2
3
Length of incubation (days)

Figure 14. Radish leaf discs pre-labelled with ^{14}C-l-leucine. Changes in radioactivity of the alcohol-soluble fraction during 3 days incubation in kinetin.

For key to symbols, see Figure 13.

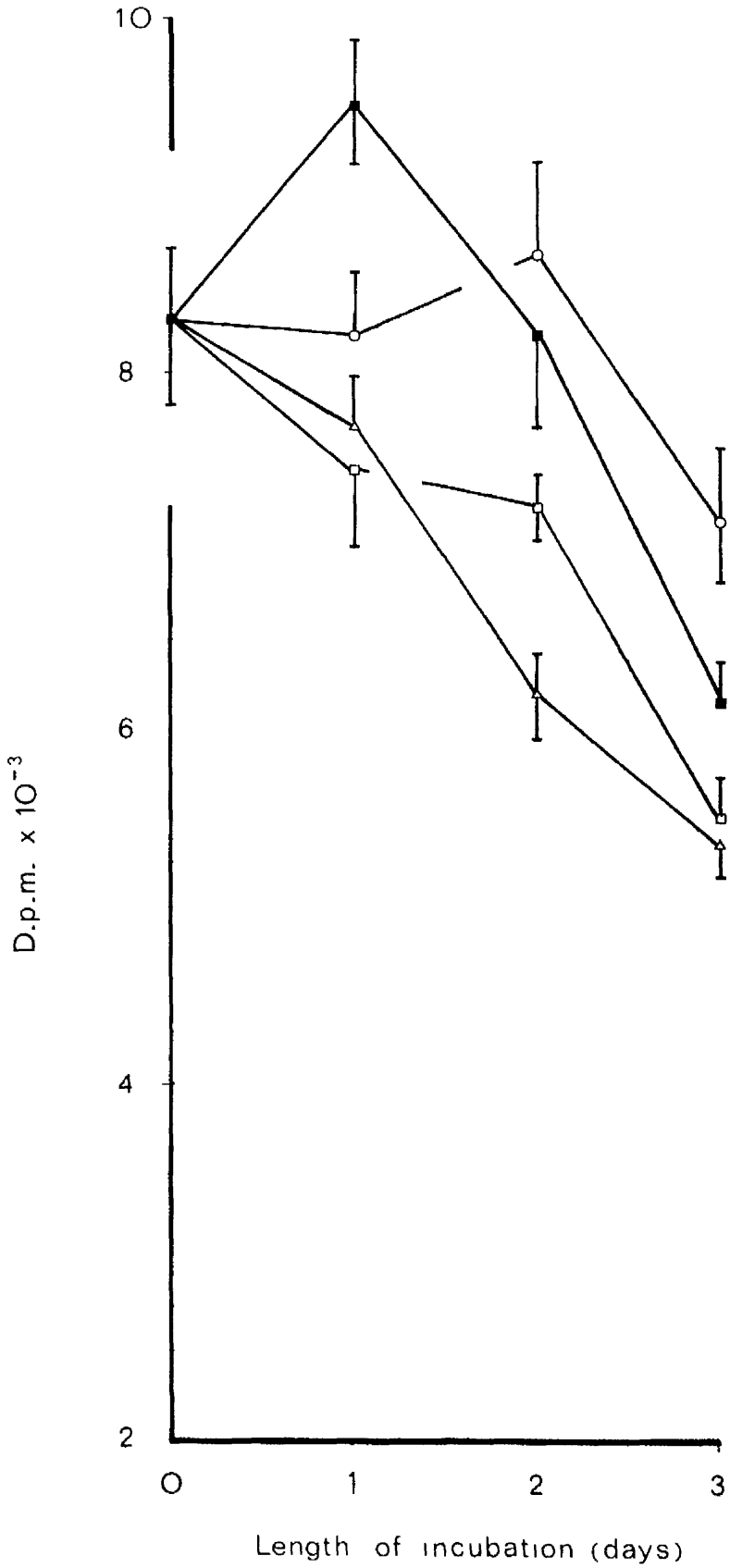

10
8
6
4
2
D.p.m. x 10^{-3}
0
1
2
3
Length of incubation (days)

Figure 15. Radish leaf discs pre-labelled with ^{14}C-1-leucine. Changes in protein levels during 3 days incubation in kinetin.

For key to symbols, see Figure 13.

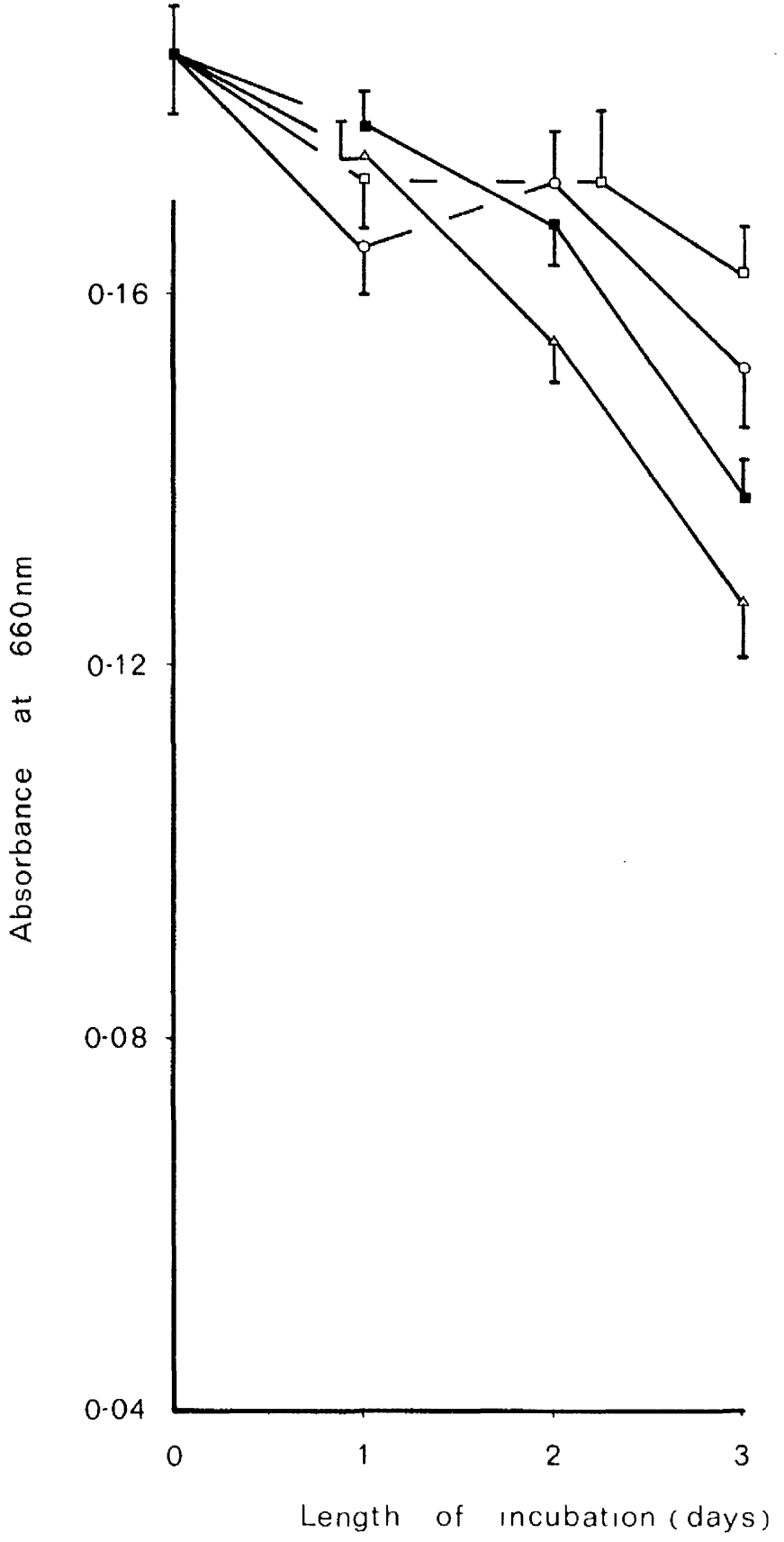
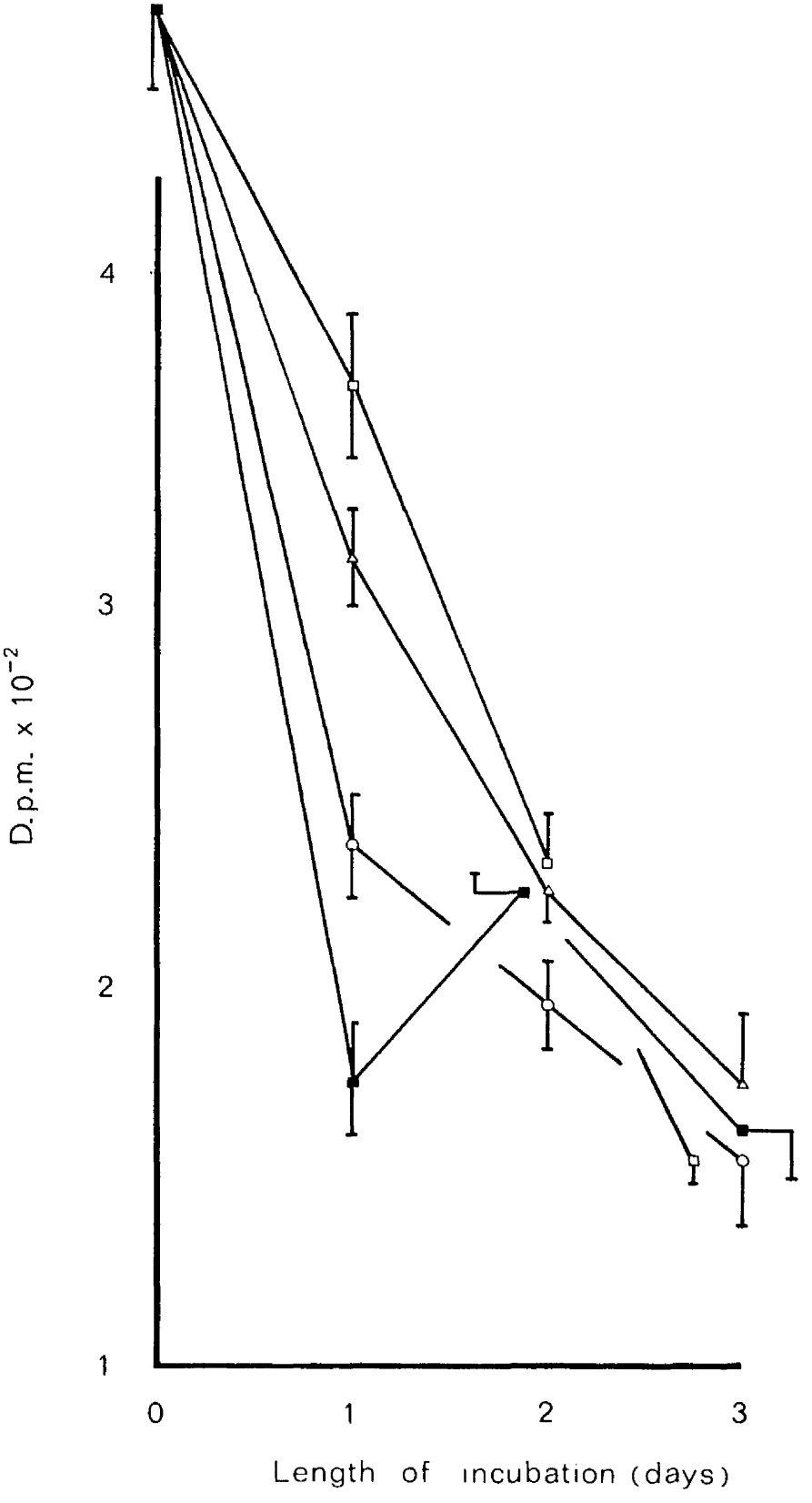

0·16
0·12
0·08
0·04
Absorbance at 660nm
0
1
2
3
Length of incubation (days)

Figure 16. Radish leaf discs pre-labelled with ^{14}C-l-leucine. Changes in radioactivity of the protein fraction during 3 days incubation in kinetin.

For key to symbols, see Figure 13.


4
3
2
1
D.p.m. x 10^{-2}
0
1
2
3
Length of incubation (days)

in treatments containing kinetin, although the differences were not particularly marked. The inclusion of leucine had little apparent effect on protein levels.

Kinetin had little consistent effect on the pattern of decline of radioactive protein relative to control treatments (Figure 16). The presence of leucine had a stimulatory effect on the decline of radioactivity.

(e) The effect of ABA and Actinomycin D on the fate of ^{14}C-8-adenine incorporated into RNA in senescing leaf discs

Data from earlier experiments suggested that incorporation of radioactivity was continuing after the removal of the discs from the solution of the radioactive precursor and transfer to non-radioactive incubation solution. It is feasible that the use of commonly accepted RNA or protein synthesis inhibitors could prevent this second period of incorporation and thus make interpretation of the data less ambiguous with respect to the effect of ABA on the radioactivity of RNA and protein. Accordingly, in experiments I(e), (f) and (g), various synthesis inhibitors have been added to incubation solutions at the time of transfer of the discs from the radioactive precursor solution to ABA. Since the inclusion of the non-radioactive precursors of RNA and protein had little effect on the pattern of senescence changes in previous experiments, they were no longer added to incubation solutions.

Actinomycin D, an RNA synthesis inhibitor, was added to dishes containing ABA or water to give a concentration of 20 µg. ml^{-1} (1.6×10^{-5}M). Changes in chlorophyll levels and RNA metabolism of discs pre-labelled in ^{14}C-8-adenine were followed during the course of a 3 day incubation period. The experiment was repeated twice with similar results.

The pattern of chlorophyll loss was unaffected by the inclusion of actinomycin D in incubation solutions (Table 9). The stimulation of chlorophyll loss due to ABA noted in previous experiments, was partially reduced, however, by actinomycin D at 1 and 2 days.

As in previous experiments, ABA stimulated the decline of radioactivity in the alcohol-soluble fraction, this stimulation becoming particularly evident at 3 days (Table 10). Actinomycin D reduced the decline in soluble radioactivity caused by ABA.

ABA stimulated the decline in total RNA levels, but, significantly, actinomycin D did not add to the effect of ABA (Table 11). It is not clear from the data whether or not actinomycin D was capable of causing RNA decline alone.

Table 9. Radish leaf discs pre-labelled with ^{14}C-8-adenine. Changes in chlorophyll level in the presence and absence of actinomycin D

	Length of incubation (days)			
	Fresh	1	2	3
Distilled H_2O	0.404 (± 0.010)	0.388 (± 0.005)	0.320 (± 0.018)	0.257 (± 0.016)
20 µg/ml actinomycin D		0.382 (± 0.003	0.332 (± 0.007)	0.258 (± 0.010)
10^{-4} M ABA		0.304 (± 0.008)	0.198 (± 0.020)	0.190 (± 0.028)
ABA + actinomycin D		0.342 (± 0.020)	0.246 (± 0.019)	0.184 (± 0.010)

(Absorbance at 665 nm)

Table 10. Radish leaf discs pre-labelled with ^{14}C-8-adenine. Changes in radioactivity of soluble fraction in the presence and absence of actinomycin D

	Length of incubation (days)			
	Fresh	1	2	3
Distilled H_2O	856.3 (± 23.4)	816.4 (±25.6)	882.8 (± 20.1)	802.6 (± 16.9)
20 µg/ml actinomycin D		896.2 (± 30.8)	934.3 (± 35.0)	924.4 (± 20.6)
10^{-4} M ABA		841.7 (± 22.2)	799.6 (± 26.6)	476.7 (± 29.6)
ABA + actinomycin D		858.1 (± 36.0)	918.1 (± 30.7)	668.0 (± 10.1)

(Disintegrations per minute)

Table 11. Radish leaf discs pre-labelled with ^{14}C-8-adenine. Changes in RNA level in the presence and absence of actinomycin D.

	Length of incubation (days)			
	Fresh	1	2	3
Distilled H_2O	0.61 (± 0.03)	0.52 (± 0.03)	0.52 (± 0.04)	0.50 (± 0.04)
20 µg/ml actinomycin D		0.43 (± 0.05)	0.50 (± 0.02)	0.44 (± 0.01)
10^{-4} M ABA		0.38 (± 0.03)	0.38 (± 0.03)	0.27 (± 0.04)
ABA + actinomycin D		0.39 (± 0.02)	0.40 (± 0.03)	0.28 (± 0.02)

(Absorbance at 260 nm)

Table 12. Radish leaf discs pre-labelled with ^{14}C-8-adenine. Changes in radioactivity in the RNA fraction in the presence and absence of actinomycin D.

	Length of incubation (days)			
	Fresh	1	2	3
Distilled H_2O	2450.4 (± 68.8)	4118.8 (± 257.7)	3433.0 (± 117.0)	2857.2 (± 131.3)
20 µg/ml actinomycin D.		2183.8 (± 125.5)	1943,0 (± 52.5)	1675.1 (± 46.4)
10^{-4} M ABA		2333.8 (± 79.3)	1509.0 (± 92.5)	801.5 (± 62.3)
ABA + actinomycin D.		1927.2 (± 40.8)	1210.4 (± 21.7)	785.5 (± 20.6)

(Disintegrations per minute)

The total amount of radioactivity increased between 0 and 1 days. Both ABA and actinomycin D suppressed, to the same extent, the early rise in radioactive RNA noted in previous experiments. Moreover, for treatments with both ABA and actinomycin D individually, the decline in the radioactivity of RNA was more rapid than in water (Table 12). Furthermore, at 1 and 2 days, a combination of the 2 compounds decreased the amount of radioactive RNA to a greater extent than either compound alone.

(f) The effect of ABA and cycloheximide on the fate of ^{14}C-l-leucine incorporated into protein in senescing leaf discs

Cycloheximide, a protein synthesis inhibitor, was added to dishes containing ABA or water to give a concentration of 20 µg. ml^{-1} (7.1×10^{-5}M). Changes in chlorophyll levels and protein metabolism in discs pre-labelled with ^{14}C-l-leucine were followed during the course of a 3 day incubation. The experiment was repeated twice with similar results.

Cycloheximide alone had little influence on the pattern of chlorophyll loss, but did reduce the chlorophyll loss induced by ABA (Table 13). The sharp decline in the radioactivity of the alcohol-soluble fraction was enhanced by both ABA and cycloheximide, the latter being the more effective in this respect (Table 14). In a combination of the 2 compounds, ABA did not further stimulate the cycloheximide-induced decline. Similarly, both ABA and cycloheximide appeared capable of stimulating the loss of protein from the discs (TAble 15). In combination, however, their effectiveness in enhancing protein loss was reduced so that the protein levels of discs treated in both ABA and cycloheximide were little different from discs treated in water. Similarly, both ABA and cycloheximide alone were capable of stimulating the loss of radioactivity from the protein fraction relative to the rate of loss from water-treated discs (Table 16), but, in combination, the effectiveness of each was reduced at 2 and 3 days.

(g) The effect of ABA and MDMP on the fate of ^{14}C-leucine incorporated into protein in senescing leaf discs

Cycloheximide is thought to inhibit protein synthesis by preventing peptide elongation on the ribosomal complex. On the other hand, MDMP, a relatively new protein synthesis inhibitor, probably acts on protein synthesis by inhibiting formation of the ribosomal complex (Baxter et al., 1973). Hence, it was of relevance to examine whether the effects of MDMP on chlorophyll loss and on protein metabolism of discs pre-labelled with ^{14}C-l-leucine were similar to those of cycloheximide. Thus, pre-labelled discs were transferred to dishes containing ABA or water to which 10^{-5}M MDMP had been added.

Table 13. Radish leaf discs pre-labelled with ^{14}C-l-leucine. Changes in chlorophyll level in the presence and absence of cycloheximide.

	Length of incubation (days)			
	Fresh	1	2	3
Distilled H_2O	0.431 (± 0.008)	0.376 (± 0.012)	0.364 (± 0.007)	0.304 (± 0.030)
20 µg/ml cyclo-heximide		0.354 (± 0.013)	0.359 (± 0.011)	0.344 (± 0.019)
10^{-4} M ABA		0.314 (± 0.015)	0.261 (± 0.007)	0.214 (± 0.015)
ABA + cyclo-heximide		0.383 (± 0.003)	0.359 (± 0.010)	0.338 (± 0.013)

(Absorbance at 665 nm)

Table 14. Radish leaf discs pre-labelled with ^{14}C-l-leucine. Changes in radioactivity of soluble fraction in the presence and absence of cycloheximide.

	Length of incubation (days)			
	Fresh	1	2	3
Distilled H_2O	756.4 (± 20.9)	226.0 (± 4.2)	204.9 (± 4.8)	163.6 (± 15.9)
20 µg/ml cyclo-heximide		94.4 (± 6.8)	65.3 (± 3.2)	41.3 (± 1.8)
10^{-4} M ABA		188.4 (± 5.8)	140.4 (± 3.9)	76.1 (± 8.8)
ABA + cyclo-heximide		116.5 (± 4.2)	69.2 (± 6.5)	80.2 (± 7.3)

(Disintegrations per minute)

Table 15. Radish leaf discs pre-labelled with ^{14}C-1-leucine. Changes in protein level in the presence and absence of cycloheximide.

	Length of incubation (days)			
	Fresh	1	2	3
Distilled H_2O	0.093 (± 0.004)	0.081 (± 0.002)	0.078 (± 0.003)	0.066 (± 0.005)
20 µg/ml cyclo-heximide		0.077 (± 0.004)	0.065 (± 0.002)	0.050 (± 0.003)
10^{-4} M ABA		0.073 (± 0.003)	0.054 (± 0.003)	0.053 (± 0.003)
ABA + cyclo-heximide		0.081 (± 0.002)	0.072 (± 0.003)	0.069 (± 0.004)

(Absorbance at 660 nm)

Table 16. Radish leaf discs pre-labelled with ^{14}C-1-leucine. Changes in radioactivity in protein fraction in the presence and absence of cycloheximide. (dpm)

	Length of incubation (days)			
	Fresh	1	2	3
Distilled H_2O	3125.9 (± 443.5)	2447.1 (± 186.6)	2419.3 (± 239.9)	1500.0 (± 172.1)
20 µg/ml cyclo-heximide		1788.9 (± 201.7)	1207.1 (± 115.9)	1169.2 (± 182.6)
10^{-4} M ABA		1997.1 (± 147.8)	1053.2 (± 120.4)	850.0 (± 86.6)
ABA + cyclo-heximide		1936.2 (± 135.0)	1596.2 (± 160.0)	1727.6 (± 212.7)

Table 17. Radish leaf discs pre-labelled with ^{14}C-l-leucine. Changes in chlorophyll level (O.D.) in the presence and absence of MDMP.

	Length of incubation (days)			
	Fresh	1	2	3
Distilled H_2O	0.348 (± 0.005)	0.327 (± 0.013)	0.326 (± 0.006)	0.231 (± 0.015)
10^{-4} M ABA		0.225 (± 0.012)	0.223 (± 0.017	0.188 (± 0.017)
10^{-5} M MDMP		0.330 (± 0.006)	0.332 (± 0.006)	0.297 (± 0.012)
ABA + MDMP		0.301 (± 0.012)	0.286 (± 0.006)	0.239 (± 0.010)

Table 18. Radish leaf discs pre-labelled with ^{14}C-l-leucine. Changes in radioactivity (Dpm) of soluble fraction in the presence and absence of MDMP.

	Length of incubation (days)			
	Fresh	1	2	3
Distilled H_2O	529.3 (± 17.1)	208.2 (± 9.3)	171.5 (± 5.2)	128.4 (± 5.1)
10^{-4} M ABA		230.8 (± 14.8)	153.3 (± 6.8)	70.7 (± 3.8)
10^{-5} M MDMP		279.3 (± 5.6)	266.3 (± 6.6)	146.3 (± 5.0)
ABA + MDMP		269.6 (± 8.9)	205.1 (± 5.2)	109.5 (± 3.4)

Table 19. Radish leaf discs pre-labelled with ^{14}C-1-leucine. Changes in protein level (O.D.) in the presence and absence of MDMP.

	Length of incubation (days)			
	Fresh	1	2	3
Distilled H_2O	0.167 (± 0.004)	0.198 (± 0.002)	0.211 (± 0.004)	0.202 (± 0.006)
10^{-4} M ABA		0.179 (± 0.012)	0.164 (± 0.006)	0.154 (± 0.006)
10^{-5} M MDMP		0.216 (± 0.004)	0.194 (± 0.008)	0.180 (± 0.006)
ABA + MDMP		0.221 (± 0.006)	0.181 (± 0.004)	0.153 (± 0.003)

Table 20. Radish leaf discs pre-labelled with ^{14}C-1-leucine. Changes in radioactivity (dpm) of protein fraction in the presence and absence of MDMP.

	Length of incubation (days)			
	Fresh	1	2	3
Distilled H_2O	1452.8 (± 54.4)	899.2 (± 50.9)	936.9 (± 51.4)	966.2 (± 45.7)
10^{-4} M ABA		821.1 (± 52.4)	460.0 (± 61.7)	454.9 (± 26.7)
10^{-5} M MDMP		906.8 (± 52.5)	773.7 (± 80.9)	830.9 (± 49.7)
ABA + MDMP		880.1 (± 23.1)	765.6 (± 69.9)	626.6 (± 42.7)

MDMP had little effect on chlorophyll loss until 3 days when it appeared to delay chlorophyll loss relative to water-treated discs (Table 17). The effect of ABA on chlorophyll decline was partially reduced by MDMP at 1 and 2 days and totally abolished at 3 days.

MDMP appeared to delay the loss of radioactivity from the alcohol-soluble fraction and it abolished the ABA-induced decline in soluble radioactivity (Table 18).

MDMP had little consistent effect on total protein levels in the leaf discs either in the presence or absence of ABA (Table 19). The decline in radioactive protein was slightly stimulated by MDMP (Table 20). In the presence of ABA, MDMP had no effect on the decline of radioactive protein until 3 days at which time the inhibitor appeared to stimulate loss of radioactivity.

(h) The efficiency of cycloheximide and MDMP in inhibiting incorporation of ^{14}C-l-leucine into protein

In the experiments using cycloheximide, MDMP and actinomycin D to limit the secondary incorporation of radioactivity into protein and RNA, interpretation of the data ultimately depends on knowledge of their efficiency and timing of action in the inhibition of protein and RNA synthesis in the radish leaf disc system. Hence, freshly excised leaf discs were incubated in MDMP or cycloheximide alone for 0, 1 or 2 hours and then transferred to ^{14}C-leucine containing MDMP or cycloheximide for 4 hours. Discs were then extracted in the normal manner and the radioactivity assessed. The experiment was repeated twice with similar results.

MDMP and cycloheximide treatments had little effect on chlorophyll or protein levels during the relatively short duration of the experiment (Table 21). MDMP, however, did increase the radioactivity of the alcohol-soluble fraction to a degree independent of the length of pre-incubation in the inhibitor. Cycloheximide, on the other hand, increased the level of soluble radioactivity when added at the same time as ^{14}C-leucine but when discs were pre-incubated in cycloheximide for 1 or 2 hours, this level was reduced relative to control treatments. All inhibitor treatments were capable of reducing incorporation of ^{14}C-leucine into protein and the data suggest that cycloheximide was more effective in this respect than MDMP. It was evident, however, that both inhibitors were more effective at reducing incorporation into protein when discs had been pre-incubated for 1 or 2 hours prior to the introduction of ^{14}C-leucine.

Table 21. The effect of pre-incubation with MDMP and cycloheximide on incorporation of ^{14}C-l-leucine into protein.

	^{14}C-leucine (-)	Length of pre-incubation (hours)					
		10^{-5} MDMP			7.1×10^{-5} M Cycloheximide		
		0	1	2	0	1	2
Chlorophyll 0.D	0.246 (± 0.011)	0.268 (± 0.003)	0.276 (± 0.003)	0.284 (± 0.004)	0.255 (± 0.007)	0.273 (± 0.004)	0.279 (± 0.013)
Protein level 0.D	0.214 (± 0.006)	0.220 (± 0.006)	0.220 (± 0.004)	0.199 (± 0.009)	0.212 (± 0.004)	0.221 (± 0.005)	0.212 (± 0.008)
Alcohol soluble dpm	197.0 (± 4.0)	258.0 (± 4.8)	252.0 (± 7.6)	250.8 (± 4.6)	235.3 (± 4.9)	162.9 (± 6.0)	138.6 (± 2.7)
Protein dpm	402.6 (± 15.6)	203.2 (± 13.5	143.9 (± 7.3)	74.1 (± 4.7)	146.2 (± 10.2)	95.4 (± 5.7)	60.5 (± 4.8)
Protein dpm % of (-) (≡ Efficiency)	100	50.5	35.7	18.4	36.3	23.7	15.0

(i) The efficiency of actinomycin D in inhibiting incorporation of ^{14}C-8-adenine into RNA

The efficiency of actinomycin D in inhibiting ^{14}C-8-adenine incorporation into RNA was determined by incubating leaf discs in actinomycin D for 0, 1 or 2 hours and then transferring to ^{14}C-8-adenine solution containing actinomycin D for a further 4 hours. Discs were extracted as before and the radioactivity in soluble and RNA fractions were analyzed (Table 22). The experiments were repeated twice with similar results.

The inhibitor treatments had little consistent effect on chlorophyll and RNA levels during the course of the experiment. There was a higher level of alcohol-soluble radioactivity of discs pre-incubated in actinomycin D for 1 or 2 hours than in the control treatments. Compared to the control, the inhibitor treatments reduced the incorporation of ^{14}C-8-adenine into the RNA fraction, the degree of reduction being dependent on the length of pre-incubation, i.e. actinomycin D was apparently more effective in inhibiting incorporation into discs pre-incubated for 1 or 2 hours.

(j) Differential uptake patterns across the leaf disc

In all of the above experiments, leaf discs excised by a cork borer have been used as the basic experimental material for labelling with various RNA and protein precursors. These experiments are possibly open to the criticism that the incorporation patterns observed were a function of stimulated metabolism in wounded tissue at the cut edge of the leaf disc. The uptake and incorporation patterns of radioactive compounds in different areas of the leaf disc were therefore investigated. Leaf discs incubated in ^{14}C-l-leucine for 4 hours in the light were divided into 3 concentric rings of tissue by successively smaller cork borers; after extraction in methanol, the radioactivity in the tissues was assessed (Table 23).

At the time of extraction 83% of the radioactivity was present in the outer ring of tissue (0.7-1.0 cm) with progressively smaller amounts in the inner portions of the disc (10% and 6% respectively). Correction of these figures for the amount of tissue present in each disc portion by using chlorophyll values in experiment I(k) below, indicated a substantially similar distribution of radioactivity. Although these data are not conclusive, it would appear that most if not all of the radioactivity is taken up via the cut edge of the original 1 cm disc. There was some difference in radioactivity between intact discs and the total

Table 22. The effect of pre-incubation in actinomycin D on incorporation of ^{14}C-8-adenine into RNA.

	^{14}C-adenine (-)	Actinomycin D 1.6 x 10^{-5} M Length of pre-incubation (hr) 0	1	2
Chlorophyll o.D.	0.214 (± 0.002)	0.211 (± 0.010)	0.233 (± 0.008)	0.216 (± 0.006)
RNA level o.D.	0.40 (± 0.02)	0.43 (± 0.03)	0.52 (± 0.03)	0.42 (± 0.03)
Alcohol-soluble dpm	373.4 (± 4.4)	361.3 (± 8.5)	429.8 (± 7.9)	486.6 (± 13.7)
RNA dpm	360.5 (± 11.4)	227.7 (± 5.5)	198.8 (± 4.7)	185.6 (± 6.6)
RNA dpm % of (-) (≡ efficiency)	100	63.2	55.1	51.5

Table 23. Uptake of ^{14}C-1-leucine by different parts of the leaf disc.

	Intact disc	Outer ring	Middle ring	Central disc
D.p.m.	4492.5 (± 393.3)	3506.3 (± 203.1)	424.9 (± 42.4)	271.1 (± 28.0)
% of total dpm	-	83.2	10.4	6.4
Corrected % of dpm	-	74.3	16.3	9.4

Table 24. Incorporation of ^{14}C-1-leucine into protein by different parts of the leaf disc.

	Intact disc	Outer ring	Middle ring	Central disc
Chlorophyll o.D.	0.211 (± 0.008)	0.092 (± 0.003)	0.053 (± 0.002)	0.056 (± 0.001)
Protein level o.D.	0.104 (± 0.003	0.060 (± 0.002)	0.045 (± 0.001)	0.038 (± 0.003)
Alcohol-soluble dpm	271.4 (± 6.0)	183.3 (± 5.5)	14.4 (± 2.0)	11.9 (± 2.1)
Protein dpm	672.3 (± 27.7)	686.8 (± 46.0)	46.2 (± 3.8)	38.2 (± 8.7)
Total soluble dpm	13,570	9,165	720	595
Total protein dpm	6,723	6,868	462	382
Total dpm	20,293	16,033	1,182	1,177
% Protein / Total	33.1	42.8	39.1	32.4

radioactivity of the 3 disc portions; this was probably due to loss of some radioactivity during the cutting procedure.

(k) Differential incorporation patterns across the leaf disc

Leaf discs, pre-labelled in ^{14}C-l-leucine and divided into concentric rings as described above, were extracted for chlorophyll and protein in order to determine if the percentage of radioactivity incorporated into the protein fraction varied across the leaf disc. The experiment was carried out three times with similar results.

The incorporation of ^{14}C-l-leucine into protein did not vary greatly across the disc and there was no evidence to suggest that protein metabolism was greatly stimulated in tissue adjacent to the original cut edge of the 1 cm disc (Table 24). Incorporation into protein was apparently higher in segmented discs but this was possibly due to preferential loss of soluble radioactivity during the cutting of the discs into the concentric portions.

SECTION II : THE UPTAKE OF ^{14}C-2-ABA AND ^{14}C-SUCROSE BY SENESCING LEAF TISSUE

Radish leaf discs incubated in ABA show an apparently anomalous pattern of chlorophyll loss and lose radioactivity from the alcohol-soluble fraction faster than from water-treated discs (Colquhoun and Hillman, 1972 and experiments reported in this thesis). In addition, ABA has no effect in further promoting senescence in discs already pre-aged in water (Colquhoun and Hillman, 1972). It is feasible that these observations may be related to alterations in the pattern of ABA uptake during the course of senescence. It is therefore relevant to determine the uptake patterns of ABA by radish leaf discs; such data are also necessary as a basis for studies on the metabolism of ABA (see Section III). Accordingly, the uptake of ^{14}C-2-ABA by radish leaf discs has been studied under both aerobic and anaerobic experimental conditions. Uptake patterns for ^{14}C-sucrose as a function of senescence have also been obtained to aid interpretation of the data for ^{14}C-2-ABA uptake.

(a) The uptake of ^{14}C-2-ABA by senescing radish leaf discs during a 12 hour time course

Radish leaf discs aged in water for 0 to 6 days were incubated in 10^{-6}M ^{14}C-2-ABA for 1 to 12 hours washed 6 times in distilled water and extracted in methanol before radioassay. Similar results were obtained from 3 such experiments.

Table 25 presents a sample of the data for the radioactivity detectable in the washings of leaf discs incubated in ^{14}C-2-ABA for 1 hour. The total amount of radioactivity and its distribution in the washings of leaf discs incubated in ^{14}C-2-ABA for 1 hour. The total amount of radioactivity and its distribution in the washings did not vary greatly with the length of pre-ageing of the discs. The pattern of distribution of radioactivity between the 6 washings indicated that most of the radioactivity occurred in the first washing and that little further radioactivity was removed by fourth and subsequent washings. It thus appeared that the radioactivity present in the washings was derived from the disc surface and not from cell leakage.

The uptake of radioactivity from ^{14}C-ABA solution by radish leaf discs pre-aged for 0 to 6 days in water was at a maximum at 1 to 2 days after excision of the discs (Table 26). Discs treated in water for more than 2 days (i.e. discs which were apparently more senescent) contained progessively less radioactivity at the time of extraction for any one length of incubation period in ^{14}C-ABA. Maximum differences in uptake

Table 25. Radioactivity detectable in 10 ml washings from leaf discs after 1 hour incubation in ^{14}C-2-ABA

Length of pre-ageing (days)	C.p.m. in 1 hour washings						Total cpm
	Washing No. 1	2	3	4	5	6	
0	1780.8	279.4	49.9	96.3	7.5	10.0	2223.9
1	2003.4	322.8	56.6	24.3	10.5	11.6	2429.2
2	1871.6	243.6	43.5	16.5	10.5	14.4	2200.1
3	1601.5	243.9	71.4	16.5	15.4	14.2	1962.9
4	2054.5	338.2	43.9	21.5	24.5	12.8	2495.4
5	1619.0	288.4	60.2	14.1	23.8	9.8	2015.3
6	2377.0	64.8	35.2	17.7	22.3	22.9	2539.9

Table 26. The uptake of radioactivity from ^{14}C-ABA solution by senescing leaf discs

Length of pre-ageing (days)	Length of incubation (hours)									
	1 hour		2 hours		4 hours		8 hours		12 hours	
	Dpm	SE	Dpm	SE	Dpm	SE	Dpm	SE	Dpm	SE
0	27.7	±3.4	28.4	±2.0	56.2	±5.1	88.0	± 9.0	126.2	±14.7
1	37.4	±3.3	68.8	±6.6	111.7	±6.2	171.4	±12.5	171.8	±11.3
2	39.3	±4.0	58.5	±3.3	92.7	±7.2	140.3	± 9.3	165.1	± 9.6
3	32.9	±2.5	59.1	±5.3	86.7	±6.0	110.1	± 8.5	130.6	± 9.3
4	31.3	±4.6	41.0	±3.3	64.0	±5.0	84.8	± 5.4	106.0	±10.5
5	29.8	±3.9	47.7	±5.3	61.0	±5.1	85.0	± 7.0	85.5	± 5.6
6	27.8	±3.7	45.5	±4.5	55.9	±5.7	81.0	± 6.5	94.0	± 8.8

between discs pre-aged for different periods of time were detected after 8 and 12 hour incubations in ^{14}C-ABA; lesser differences were detectable in discs incubated in ^{14}C-ABA for shorter periods. Examination of the data for the uptake of radioactivity at any stage of senescence indicated that there was an initial fairly rapid uptake of radioactivity during the first 2 hours of incubation, but subsequently the rate of uptake declined, i.e. uptake was apparently not linear with time.

Evidence is presented in Section III to suggest that ^{14}C-2-ABA is metabolised in leaf tissue to other radioactive compounds. Indirect evidence presented here suggests, however, that radioactivity is taken up from solution by the leaf discs as ^{14}C-2-ABA. Samples taken from radioactive solutions after completion of the incubation period, chromatographed on paper strips developed in isopropanol:ammonia:water (8:1:1) indicated that the distribution of radioactivity coincided with that of stock samples of ^{14}C-ABA. Although conclusive evidence would depend on similar chromatographic checks in a number of other solvent systems, it is considered that there was no significant breakdown of ^{14}C-ABA in the incubation solutions (Table 27).

(b) The use of hyamine hydroxide

The results of experiments on the uptake and metabolism of ^{14}C-labelled isotopes may be confounded by loss of radioactivity as $^{14}CO_2$; further complexity can also arise during experiments carried out using aerobic and anaerobic conditions. An estimate of such losses when using ^{14}C-ABA and ^{14}C-sucrose solutions was obtained by trapping $^{14}CO_2$ from these solutions by hyamine hydroxide; this was then analyzed by scintillation spectrometry. Initially, it was necessary to determine how much radioactivity was lost as $^{14}CO_2$ from ^{14}C-ABA and ^{14}C-sucrose solutions *per se* under anaerobic and aerobic conditions, i.e. in the absence of leaf tissue. Dishes, 5 cm in diameter containing 10 mls of ^{14}C-ABA (0.15 µCi) or ^{14}C-sucrose (1.0 µCi) were incubated for 9 hours in air or nitrogen (see Materials and Methods). Any $^{14}CO_2$ produced was trapped by passage through 40 mls of hyamine hydroxide and the radioactivity was subsequently assessed.

Significant amounts of radioactivity were detected in hyamine hydroxide solutions as a result of incubation with ^{14}C-ABA and ^{14}C-sucrose, although these amounts remained relatively small compared to the total radioactivity in the incubation solutions (Table 28). In all 3 experiments, more radioactivity was detected in hyamine hydroxide solutions resulting from incubation of sucrose solution in aerobic conditions than in anaerobic

Table 27. The chromatographic behaviour of radioactivity in ^{14}C-ABA incubation solutions after 8 hours

	% distribution of radioactivity in 20 equal zones																				Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	dpm
Incubation solution	0.3	0	0	0	0.1	0.1	0	0	0	0	0	0.1	3.3	29.0	48.5	17.9	0.7	0	0	0	1694.3
^{14}C-ABA	0.1	0	0	0	0	0	0	0	0	0	0	0	0	8.9	53.7	37.0	0.2	0	0	0	2021.4

Table 28. Radioactivity in hyamine hydroxide solution after incubation with ^{14}C-sucrose and ^{14}C-ABA (mean of 3 trials)

	Nitrogen		Air	
	D.p.m.	% of total dpm	D.p.m.	% of total dpm
^{14}C-ABA	699	0.21%	858	0.26%
^{14}C-sucrose	6402	0.29%	10,035	0.46%

conditions. Similarly, hyamine hydroxide derived from ^{14}C-ABA solution contained more radioactivity from air incubations than from nitrogen incubations in 2 trials out of 3.

The values for radioactivity in hyamine hydroxide solutions reported in Table 28 have been used in calculating the estimates of total radioactivity shown in Tables 29 and 30.

(c) The uptake of ^{14}C-ABA by senescing radish leaf discs in nitrogen or air

Having established that ^{14}C-ABA uptake was at a maximum in radish leaf discs aged in water for 1 to 2 days and that uptake into more senescent tissue progressively declined (Table 26), it was logical to examine whether or not this uptake pattern was a function of changes in the amount of aerobic uptake of the radioactive compound. This can be considered by determining the uptake of ^{14}C-ABA by pre-aged leaf discs in nitrogen or air in order to estimate the contribution of aerobic metabolism to active uptake. After 1 hour pre-incubation in water under aerobic or anaerobic conditions, discs aged for 0 to 6 days were transferred for 8 hours to ^{14}C-ABA, then extracted in methanol and the radioactivity monitored (Table 29). Estimates of the radioactivity in hyamine hydroxide solutions are also presented, which after subtraction of the values reported in experiment II(b) for the loss of radioactivity by the solutions alone ("solution background") have been added as radioactivity per replicate to the radioactivity detected in methanolic extracts of the leaf discs. The experiment was repeated twice.

As reported in experiment II(a), uptake of ^{14}C-ABA under aerobic conditions by leaf discs pre-aged in water for 0 to 6 days was at a maximum at 1 to 2 days after excision, thereafter declining steadily. (Table 29). Leaf discs incubated in ^{14}C-ABA under anaerobic conditions showed a much lower level of uptake of the radioactive compound and there were no consistent changes in uptake for discs at different stages of senescence. Some radioactivity was lost as $^{14}CO_2$ from the discs (beyond that lost by the incubation solution *per se*), but this at no time exceeded 0.65% of the original radioactivity in the incubation solution. The levels of radioactivity detectable in hyamine hydroxide solutions did not necessarily correlate with the amount of the radioactivity in the methanolic extracts; for example, radioactivity in the hyamine hydroxide solution for air treatment was highest at 0 days and then declined whereas radioactivity in the methanolic extracts was at a maximum at 1 day.

Table 29. Radioactivity in hyamine hydroxide solutions and methanolic extracts of senescing leaf discs incubated in ^{14}C-ABA in nitrogen or air

Nitrogen	Length of pre-ageing (day) 0	1	2	3	4	5	6
HyOH (dpm ml^{-1})	28.7	65.3	32.5	32.8	32.7	21.6	25.0
Total HyOH dpm	976	2135	1040	1181	1079	712	800
- solution background (699 dpm)	277	1456	341	482	380	13	101
Total - solution ^{b}Kg / No. of replicates (16)	17.3	91.0	21.3	30.1	23.8	0.8	6.3
Dpm / replicate (2 discs per vial)	316.6 (± 19.1)	211.9 (± 12.1)	251.2 (± 20.3)	307.4 (± 21.0)	249.4 (± 29.4)	260.8 (± 25.6)	303.0 (± 23.1)
Total uptake (dpm)	333.9	302.9	272.5	337.5	273.2	261.6	309.3
Air							
HyOH (dpm ml^{-1})	79.0	69.3	55.9	45.5	40.4	26.6	28.2
Total HyOH dpm	3002	2564	2012	1774	1333	958	987
- solution background (858 dpm)	2144	1706	1154	916	475	100	129
Total - background / No. of replicates (16)	134.0	106.6	72.1	57.3	29.7	6.3	8.1
Dpm / replicate (2 discs per vial)	947.5 (± 55.7)	1587.0 (± 97.1)	1298.0 (± 78.2)	1092.8 (± 87.0)	710.9 (± 55.5)	578.8 (± 48.9)	607.9 (± 48.0)
Total uptake (dpm)	1081.5	1693.6	1370.1	1150.1	740.6	585.1	616.0

(d) The uptake of ^{14}C-sucrose by senescing radish leaf discs in nitrogen or air

In experiments II(a) and II(c), characteristic patterns of ^{14}C-ABA uptake by leaf discs at different stages of senescence have been observed. It is not clear, however, to what extent these patterns reflect changes in the rate of cell metabolism and to what extent they are specifically characteristic of the uptake of ABA. Uptake of ^{14}C-ABA has therefore been compared with that of ^{14}C-sucrose, a compound known to be very reactive in cell metabolism. Leaf discs, pre-aged in water for 0 to 6 days, were incubated in ^{14}C-sucrose under nitrogen or air as described above. The experiment was carried out 3 times with similar results.

The data for radioactivity in the hyamine hydroxide and methanolic fractions have been calculated as described above. In freshly excised discs (0 days), anaerobic conditions reduced the level of radioactivity in methanolic extracts by 75% compared to the level in extracts derived from air treatments (Table 30). After pre-ageing in water for 1 day, however, there was a sharp rise in the radioactivity of both nitrogen and air treated discs. The data suggest that although the rise in radioactivity was greater in nitrogen treated discs, anaerobic conditions still reduced somwhat the radioactivity in this fraction relative to air treatment. After 1 day there was no further significant change in radioactivity detected in the methanolic extracts, either in air or in nitrogen. Examination of the data for radioactivity present in hyamine hydroxide solution, however, revealed a different pattern; in both nitrogen and air treatments, the level of radioactivity rose consistently to 5 days and then fell sharply. Radioactivity in the hyamine hydroxide fractions was at all times ~~higher~~ lower in the aliquots from nitrogen treatments than those from air treatments. When figures for the uptake of radioactivity from ^{14}C-sucrose solutions (methanolic extracts + hyamine hydroxide solutions) are compared it is apparent that uptake rose steadily to 5 days in air treatments and then fell sharply, whereas in nitrogen treatments there was a sharp increase at 1 day with maximum uptake occurring at 5 days. At no time did the total uptake of radioactivity in nitrogen exceed that in air.

Table 30. Radioactivity in hyamine hydroxide solutions and methanolic extracts of senescing leaf discs incubated in ^{14}C-sucrose in nitrogen or air.

Nitrogen	Length of pre-ageing (days)													
	0		1		2		3		4		5		6	
Dreschel bottle	1	2	1	2	1	2	1	2	1	2	1	2	1	2
HyOH (dpm ml^{-1})	529.8	65.7	513.7	62.5	1626.5	73.9	2946.8	44.0	3237.7	51.3	4063.3	52.5	2186.8	35.1
Total HyOH dpm	13047	2244	19195	2436	52841	2880	98402	1723	104962	2022	130636	2100	72814	1352
Σ 1+ 2 dpm	15291		21631		55721		100125		106894		132736		74166	
- solution background (6402 dpm)	8889		15299		49319		93723		100582		126334		67664	
Total - background / No. of replicates (30)	296		510		1644		3124		3353		4211		2259	
Dpm (1 disc replicate (per vial)	2108 (± 410)		11834 (± 972)		7983 (± 799)		9419 (± 774)		9300 (± 829)		10302 (± 1292)		10633 (± 1303)	
Total uptake (dpm)	2404		12344		9627		12543		12653		14513		12892	
Air														
Dreschel bottle	1	2	1	2	1	2	1	2	1	2	1	2	1	2
HyOH (dpm ml^{-1})	789.3	98.4	1156.5	48.0	3532.1	58.0	3598.8	56.6	7639.1	59.3	12975.9	204.1	4246.9	42.7
Total HyOH dpm	21878	3802	39629	1850	119032	2226	130243	2208	260220	2244	328079	5769	152853	1685
Σ 1 + 2 dpm	25680		41479		121258		132451		262464		333848		154538	
- solution background (10035 dpm)	15645		31444		111223		122416		252429		323813		144503	
(Total - background) / No. of replicates (30)	521		1048		3707		4081		8414		10794		4817	
Dpm (1 disc replicate (per vial)	8810 (± 508)		13882 (± 972)		12265 (± 985)		12736 (± 985)		10897 (± 1046)		13511 (± 1713)		10682 (± 1240)	
Total uptake (dpm)	9331		14930		15972		16817		19311		24305		15499	

SECTION III : THE METABOLISM OF ABA

If ABA is involved in the control of leaf senescence, or indeed other developmental processes in plants, it is necessary to postulate a mechanism by which the levels of ABA itself are regulated. Studies of the metabolism of ABA in relation to senescence processes may yield valuable information about such a mechanism. Further, in Section II(a) described above, characteristic patterns of uptake of ^{14}C-ABA have been observed. It is not yet clear, however, whether the radioactivity assessed still exists as ^{14}C-ABA *per se* or, alternatively, whether the radioactive hormone has been metabolised to other components. In this section, therefore, the metabolism of radioactive ABA by leaf tissues is examined.

(a) Chromatography of ^{14}C-ABA supplied to leaf discs

Surface-sterilised leaf discs of radish were pre-aged for 0 to 6 days, as before, incubated for 24 hours in 10^{-6}M ^{14}C-ABA and then extracted in 80% methanol in order to determine the proportion of radioactivity still co-chromatographing with ^{14}C-ABA.

Figure 17(a) to (d) indicates the percentage distribution of radioactivity along paper and thin-layer chromatograms developed in one of the 4 solvent systems described in Materials and Methods. Each set of chromatograms was developed with a marker of pure ^{14}C-ABA. The experiment was carried out 3 times.

Extracts of discs chromatographed in butanol:isopropanol:ammonia:water (2:6:1:2) apparently consistently contained a peak of radioactivity at Rf 0.6 to 0.7, which was thus less mobile than ^{14}C-ABA itself, occurring at Rf 0.75 to 0.85 (Figure 17(a)). Between 0 and 4 days, there was little variation in the relative amount of radioactivity present in the less mobile peak, but at 5 and 6 days, the relative size of this peak was reduced. A further minor peak of radioactivity may occur at Rf 0.4 to 0.5.

Similarly, methanolic extracts chromatographed on paper strips in butanol:acetic acid:water (5:1:2.2) contained 2 peaks of radioactivity; one occurred at Rf 0.85 to 0.95, co-chromatographing with marker spots of ^{14}C-ABA and thus was consistently more mobile than the second peak at Rf 0.7 to 0.8. The relative level of this peak did not vary greatly with the length of pre-ageing of the discs (Figure 17(b)).

Figure 17(c) illustrates the distribution of radioactivity on thin layer chromatograms developed in benzene:acetic acid (50:20). About 25% of the radioactivity remained at the origin, irrespective of the

Figure 17. Chromatography of methanolic extracts of radish leaf discs supplied with ^{14}C-ABA for 24 hours.

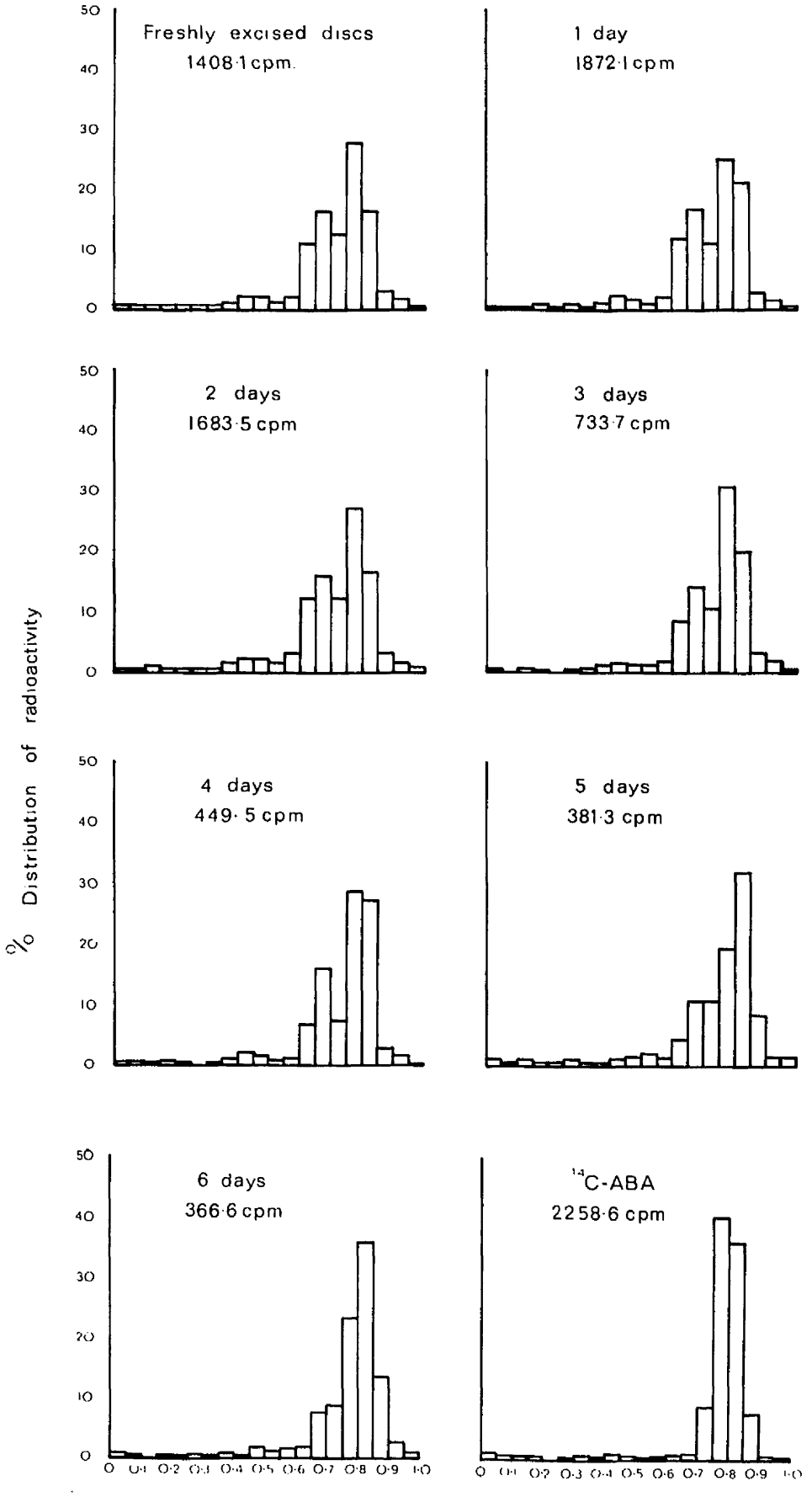
(a) Butanol:isopropanol:ammonia:water (2:6:1:2)

(b) Butanol:acetic acid:water (5:1:2.2)

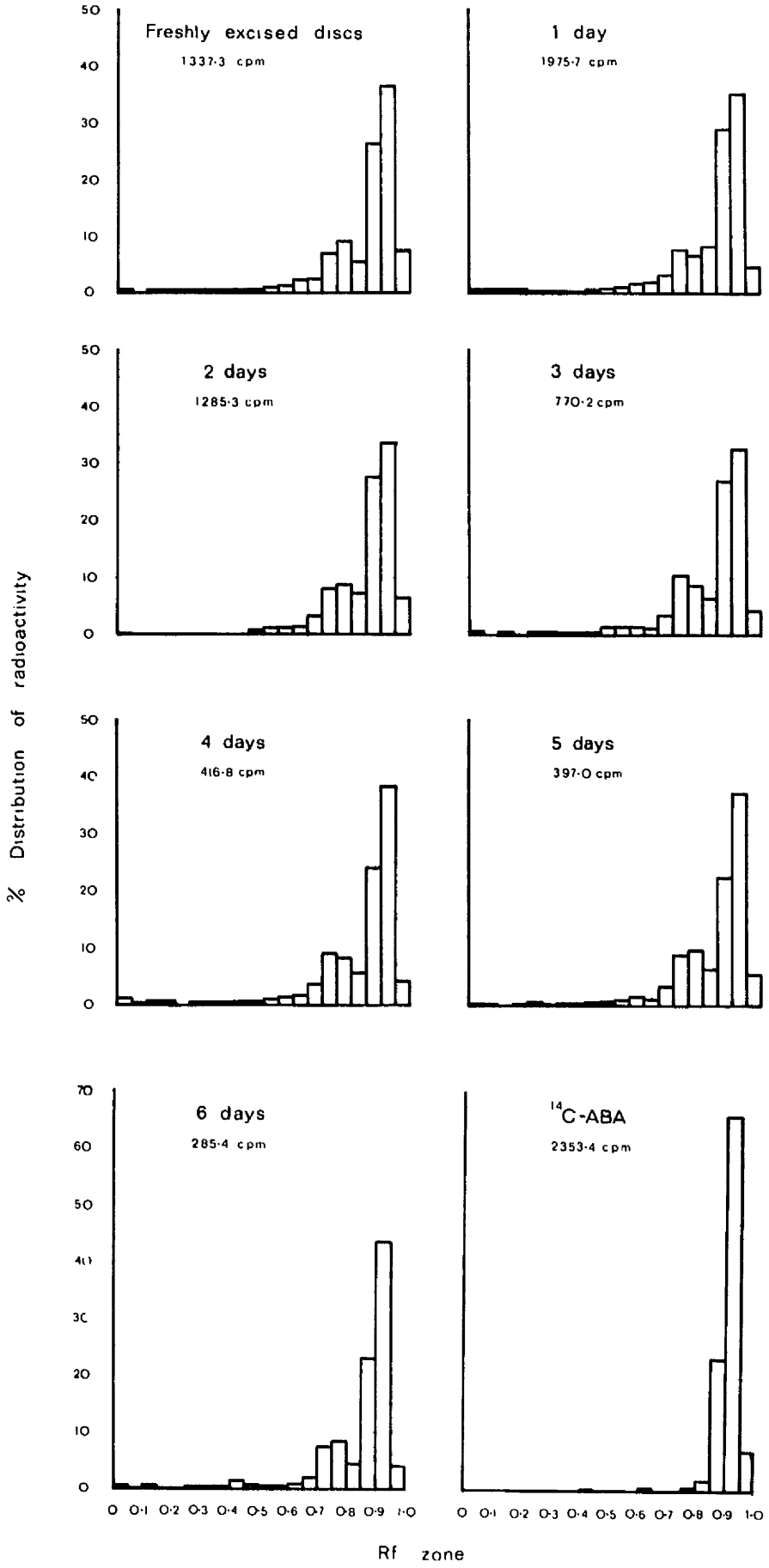
(c) Benzene:acetic acid (50:20)

(d) Chloroform:methanol:water (75:22:3)

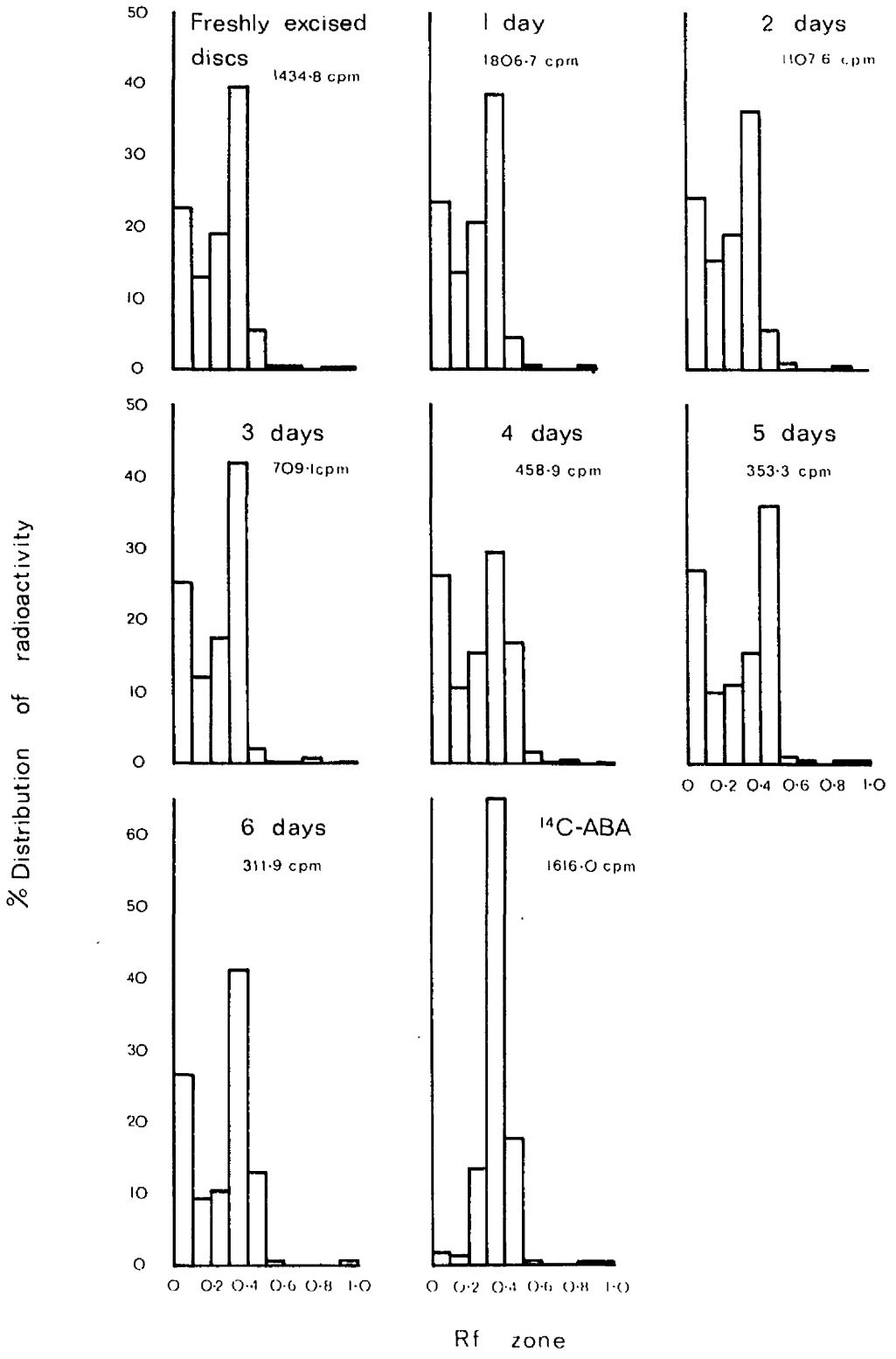
(a) Butanol : propanol : NH_3 : H_2O (2·6·1·2) - Paper.



(b) Butanol : acetic acid : H_2O (5 : 1 : 2·2) - Paper



(c) Benzene:acetic acid (50:20) -T.L.C.



(d) Chloroform : methanol : H_2O (75:22:3) - T.L.C.

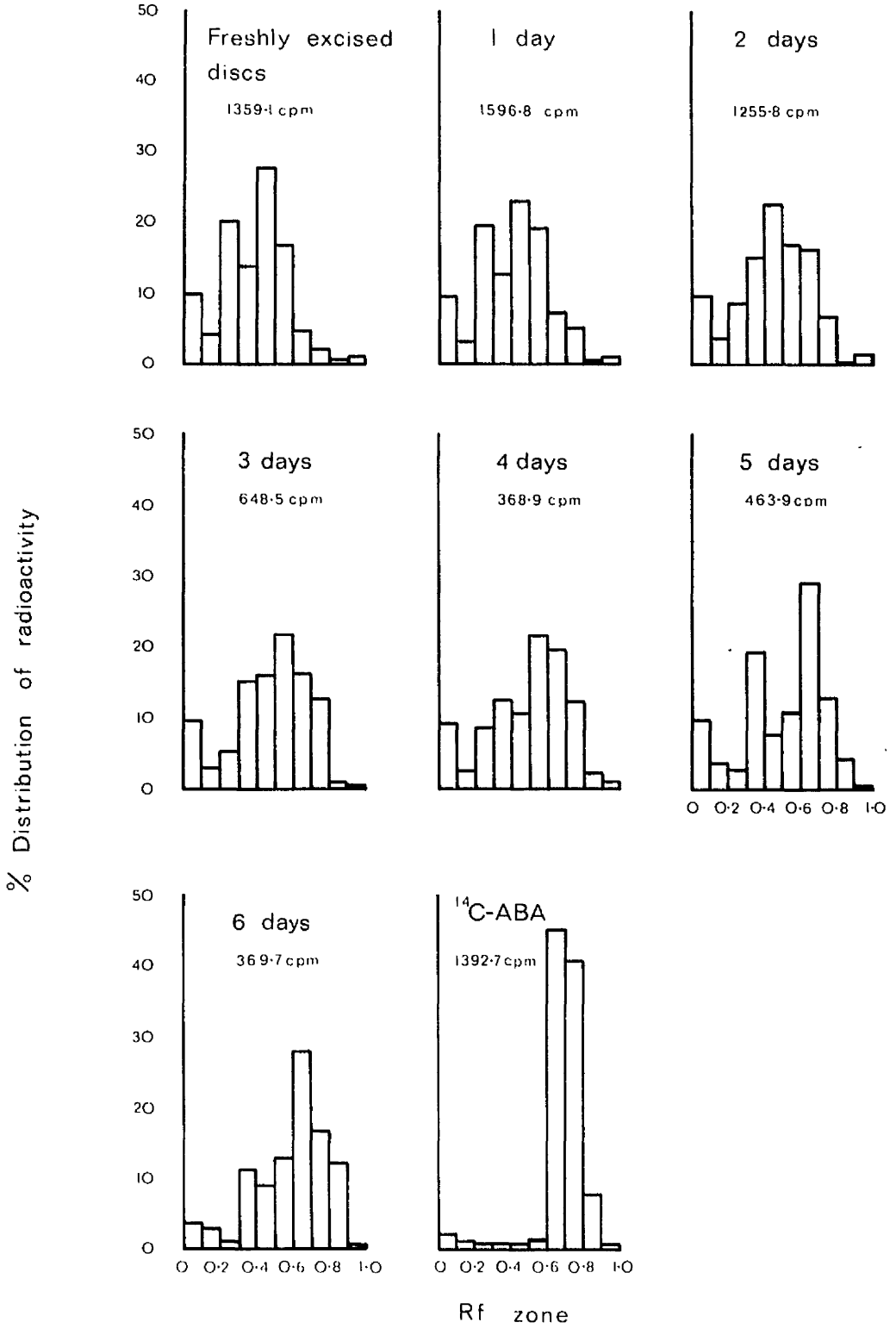
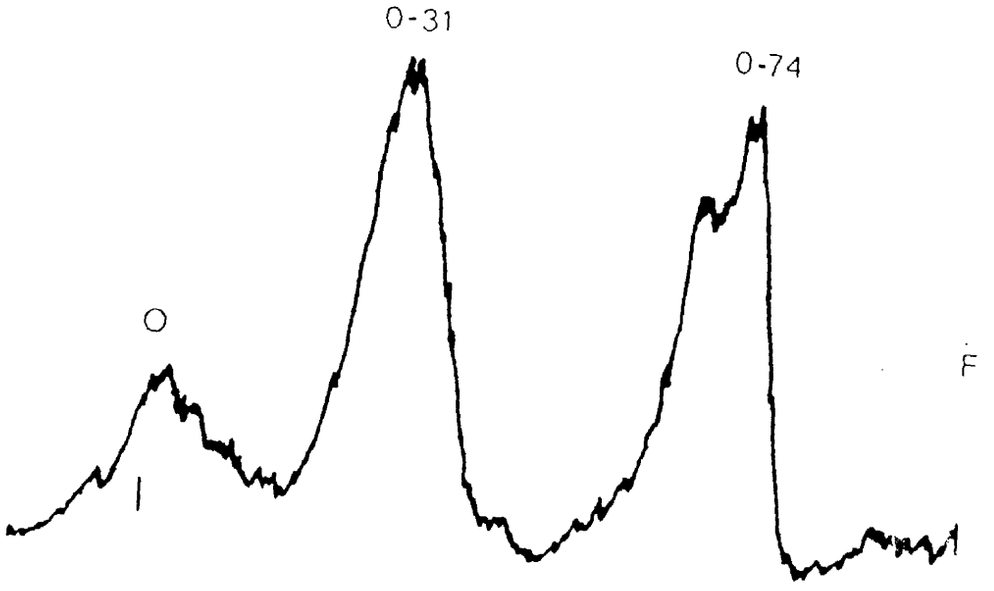


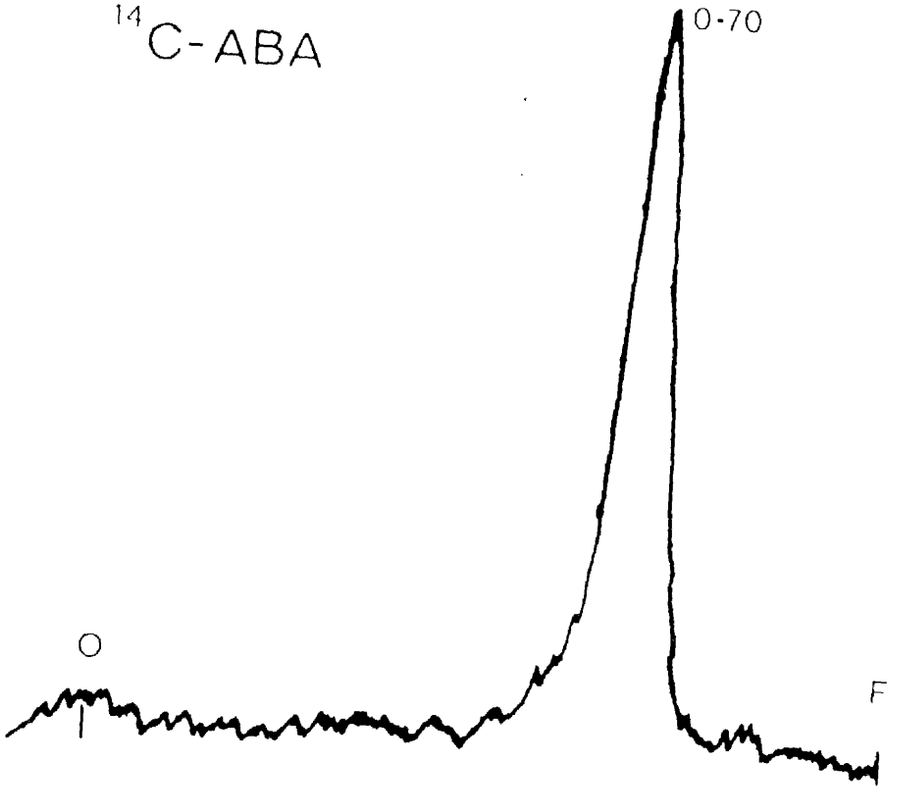
Figure 18. Chromatography of methanolic extracts of radish leaf discs supplied with ^{14}C-ABA for 24 hours and analyzed by radiochromatogram scanning.

Chloroform:methanol:water (75:22:3).

Methanolic
extract



^{14}C-ABA



Sensitivity - 3 cps
Chart speed - 120 mm.hr^{-1}

stage of senescence, and was thus distinct from ^{14}C-ABA, which was more mobile and occurred at Rf 0.3 to 0.5. It is of note that the level of radioactivity remained high between these peaks (i.e. in the region Rf 0.1 to 0.3).

Three peaks of radioactivity were present in methanolic extracts developed on thin-layer plates in chloroform:methanol:water (75:22:3). One peak remained at the origin (Rf 0 to 0.1) and contained about 10% of the total radioactivity on the chromatogram for all pre-ageing periods, except 6 days (Figure 17(d)). The second peak occurred at Rf 0.2 to 0.3 or 0.3 to 0.4, and contained up to 20% of the total radioactivity, although this proportion varied somewhat with the length of pre-ageing. The third peak of radioactivity is presumed to be ^{14}C-ABA, although there was not always exact correspondence between the position of this peak and the peak of ^{14}C-ABA markers (see also Section III(e)).

The typical distribution of radioactivity in thin-layer chromatograms, analyzed by use of a Panax radiochromatogram scanner, after development in chloroform:methanol:water (75:22:3), are presented in Figure 18. These data show that the apparent peaks of radioactivity observed by scintillation analysis of chromatograms cut into Rf or ½ Rf zones do, in fact, represent discrete areas of radioactivity.

In conclusion, therefore, exogenous radioactive ABA is metabolised within 24 hours of exposure to leaf tissue.

(b) <u>The effect of antibiotic treatment on the metabolism of ^{14}C-ABA and on the loss of chlorophyll from leaf discs</u>

It is conceivable that the apparent changes of the ^{14}C-ABA molecule, implicit in the data presented in Section III(a), were due to the action of contaminating microbial organisms and were thus not a function of the leaf tissue <u>per se</u>. It was therefore important to determine whether or not ^{14}C-ABA was still metabolised when the growth of any microbial organisms present was chemically controlled. Accordingly, mycostatin and chloramphenicol were added both to the incubation solutions, used for preageing the discs, and to the ^{14}C-ABA solution. Discs, pre-aged for 0, 1 and 6 days in water, were transferred to ^{14}C-ABA for 8 hours and methanolic extracts of the discs were subsequently chromatographed on thin-layer plates in chloroform:methanol:water (75:22:3) and the distribution of radioactivity analyzed. In addition, leaf discs incubated with the antibiotics were extracted for chlorophyll as a measure of the effect of the antibiotics on senescence. This experiment was repeated 3 times.

Figure 19. Chromatography of methanolic extracts of radish leaf discs supplied with ^{14}C-ABA in the presence and absence of antibiotics.

Chloroform:methanol:water (75:22:3)

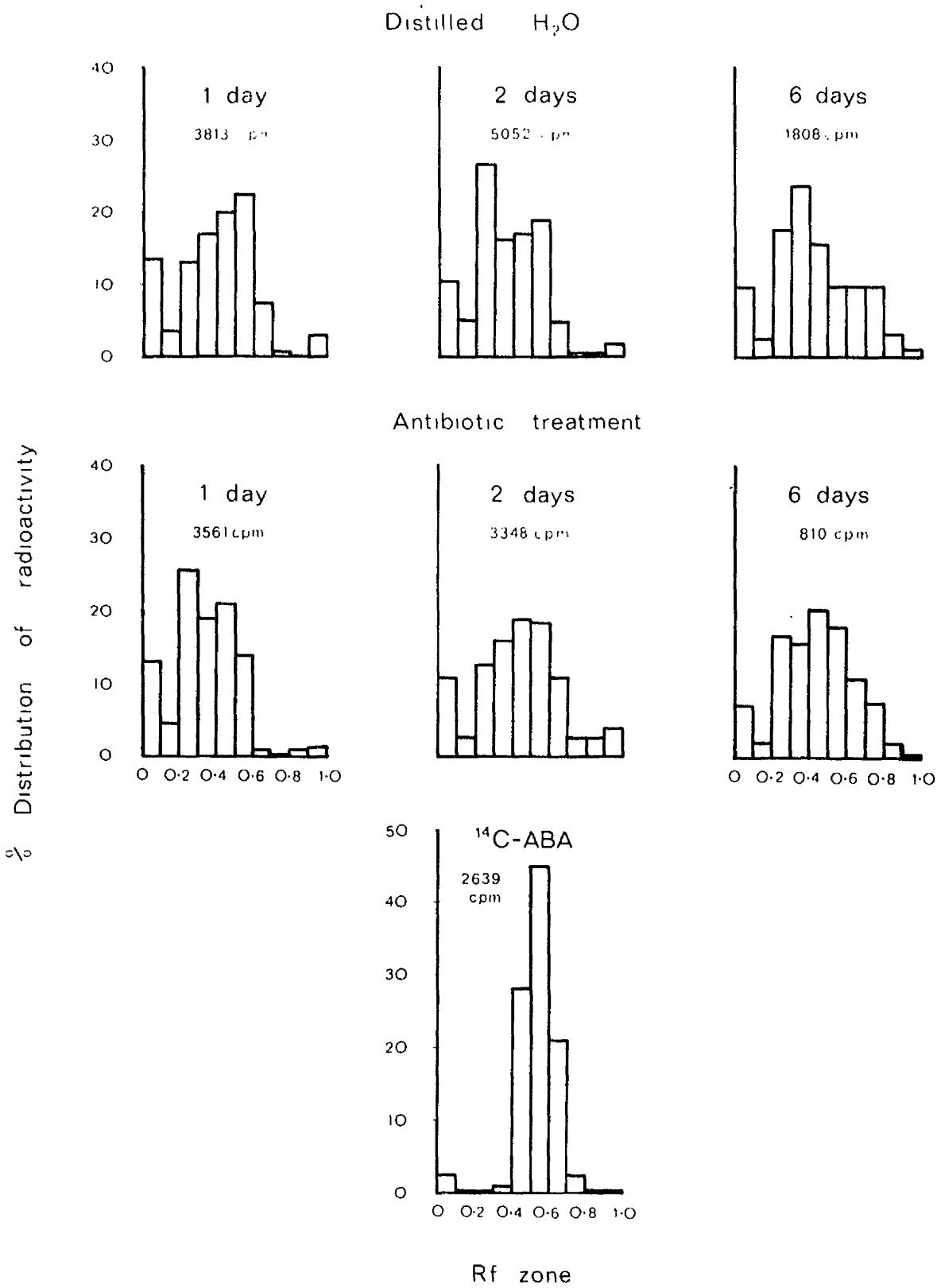

Distilled H2O
1 day
3813 cpm
2 days
5052 cpm
6 days
1808 cpm
Antibiotic treatment
1 day
3561 cpm
2 days
3348 cpm
6 days
810 cpm
% Distribution of radioactivity
14C-ABA
2639 cpm
0 0·2 0·4 0·6 0·8 1·0
Rf zone

Table 31. The effects of mycostatin and chloramphenicol on chlorophyll levels (O.D.*) of radish leaf discs.

	Length of incubation (days)			
	Fresh	1	2	6
Distilled H_2O	0.244 (± 0.008)	0.229 (± 0.009)	0.189 (± 0.012)	0.071 (± 0.014)
Mycostatin ($0.05\ \mu g.\ ml^{-1}$)		0.208 (± 0.003)	0.194 (± 0.010)	0.075 (± 0.012)
Chloramphenicol ($50\ \mu g.\ ml^{-1}$)		0.216 (± 0.010)	0.190 (± 0.016)	0.054 (± 0.017)
Mycostatin + Chloramphenicol		0.217 (± 0.005)	0.189 (± 0.013)	0.055 (± 0.011)

Table 32. Distribution of radioactivity in the partition phases from extracts of leaf discs incubated in ^{14}C-ABA.

Stage	pH	Phase	% of radio-activity (152,000 cpm)
Before partition 1	8.0	Aqueous	100
After partition 1	8.0	"	99.5
		Ether	0.5
After partition 2	7.0	Aqueous	99.7
		Ether	0.3
After partition 3	3.0	Aqueous	76.3
		Ether	0
After partition 4	3.0	Pet. Ether	0
		Aqueous	100

Table 33. Distribution of radioactivity in chromatograms of acidic aqueous and ether fractions.

	% Distribution of radioactivity in 10 equal zones										Total cpm
	0	1	2	3	4	5	6	7	8	9	
Acidic aqueous	13.1	20.4	21.0	24.5	11.3	1.7	3.5	3.5	1.0	0	107.0
Acidic ether	0.2	0.4	1.0	1.6	9.9	1.1	0.8	24.5	59.7	0.6	715.4
^{14}C - ABA	3.2	0.9	0.5	0.2	0.6	0.7	0.5	77.2	14.2	1.9	2787.0

Examination at 24 and 48 hours after inoculation of trypticase soybean agar plates with the pre-ageing incubation solution, followed by incubation at 25°C, indicated that although the mixture of mycostatin and chloramphenicol did not kill all microbial organisms in the incubation medium, the numbers of such organisms were severely reduced; furthermore the development of those organisms that were still present was delayed.

The antibiotic treatments appeared to have little effect on chlorophyll loss at 1, 2 and 6 days. (Table 31).

The presence of the antibiotic mixture in the pre-ageing incubation solutions and in the ^{14}C-ABA solutions had little apparent effect on the metabolism of ^{14}C-ABA in leaf discs; for example, the level of the metabolite occurring in Rf zone 0 to 0.1 was very similar in antibiotic and control treatments (Figure 19). There is some indication that the level of this metabolite fell with increasing length of pre-ageing period, but there were still considerable amounts of the metabolites present at 6 days. The presence of the ~~metabolites~~ antibiotics, however, did appear to inhibit the uptake of ^{14}C-ABA: the total radioactivity in extracts of discs incubated in the radioactive hormone in the presence of the antibiotics was lower than in extracts of discs not treated with the antibiotics.

(c) The metabolism of ^{14}C-ABA under anaerobic conditions

The evidence presented in Section III(a) indicated that a significant proportion of the ^{14}C-ABA supplied to radish leaf discs for 24 hours was metabolised to at least 2 other radioactive substances. It was thus of interest to determine whether this process was dependent on aerobic metabolism or whether the metabolism of ^{14}C-ABA could still occur in an anaerobic atmosphere; moreover, such an experiment might provide additional information concerning the different patterns of uptake of ^{14}C-ABA, observed in air and nitrogen treatments in Section II(c). Thus, leaf discs pre-aged for 0 to 6 days in water were incubated in ^{14}C-ABA in nitrogen or air for 8 hours. Methanolic extracts of the discs were chromatographed on thin-layer plates in chloroform:methanol:water, prior to analysis of the distribution of radioactivity. This experiment was carried out twice.

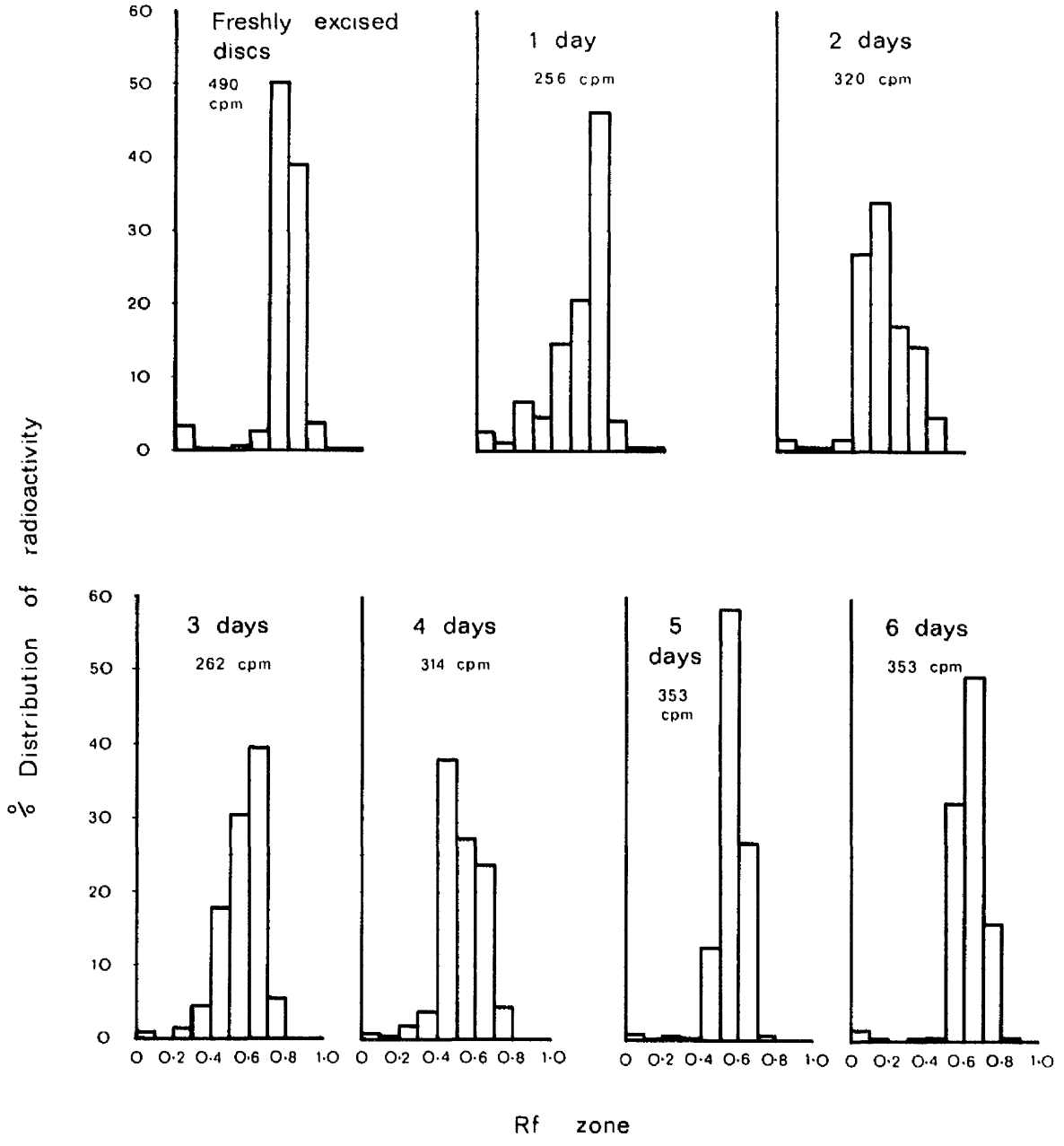
As in earlier experiments, the total uptake of radioactivity from ^{14}C-ABA solutions was inhibited by nitrogen treatment; further, the peak of uptake at 1 to 2 days, evident in the air treatment, did not occur in nitrogen (Figure 20). Nitrogen treatment also markedly reduced the metabolism of ^{14}C-ABA relative to air treatment. In discs pre-aged

Figure 20. Chromatography of methanolic extracts of radish leaf discs supplied with ^{14}C-ABA under aerobic and anaerobic conditions.

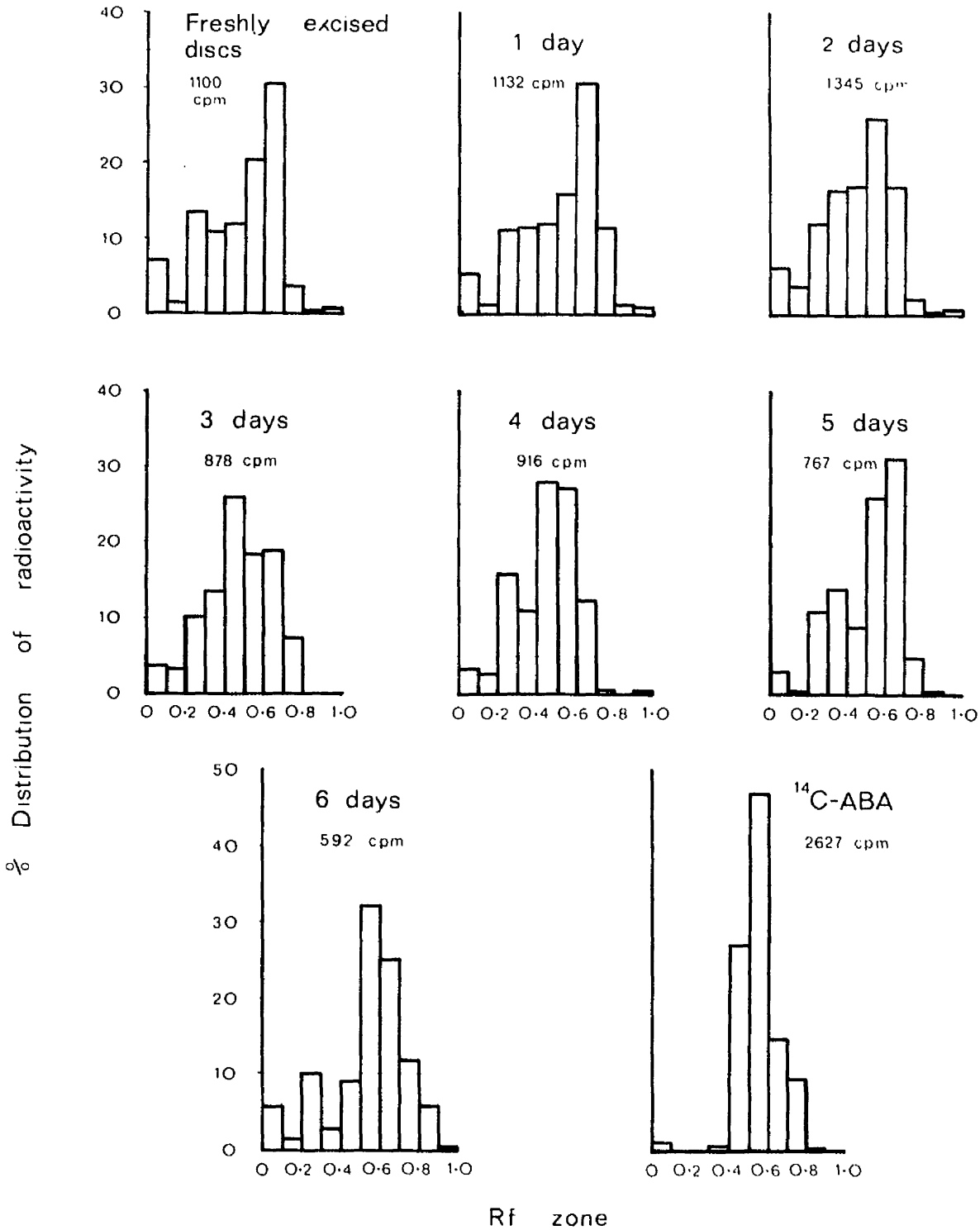
(a) Nitrogen

(b) Air

Chloroform:methanol:water (75:22:3)



(a) N_2 treatment



(b) Air treatment

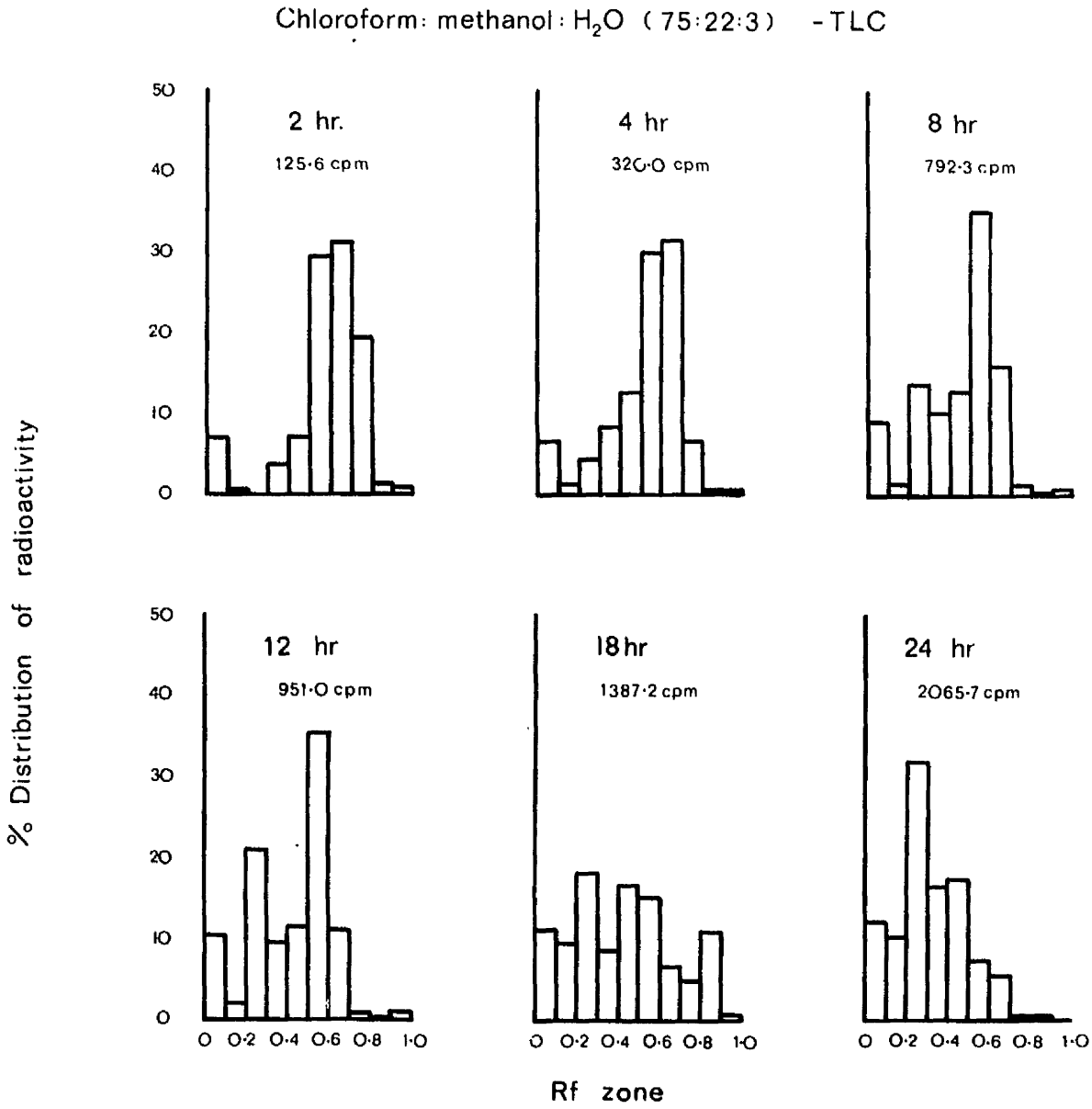
Figure 21. Chromatography of methanolic extracts of radish leaf discs supplied with ^{14}C-ABA for periods up to 24 hours.

(a) Freshly excised discs

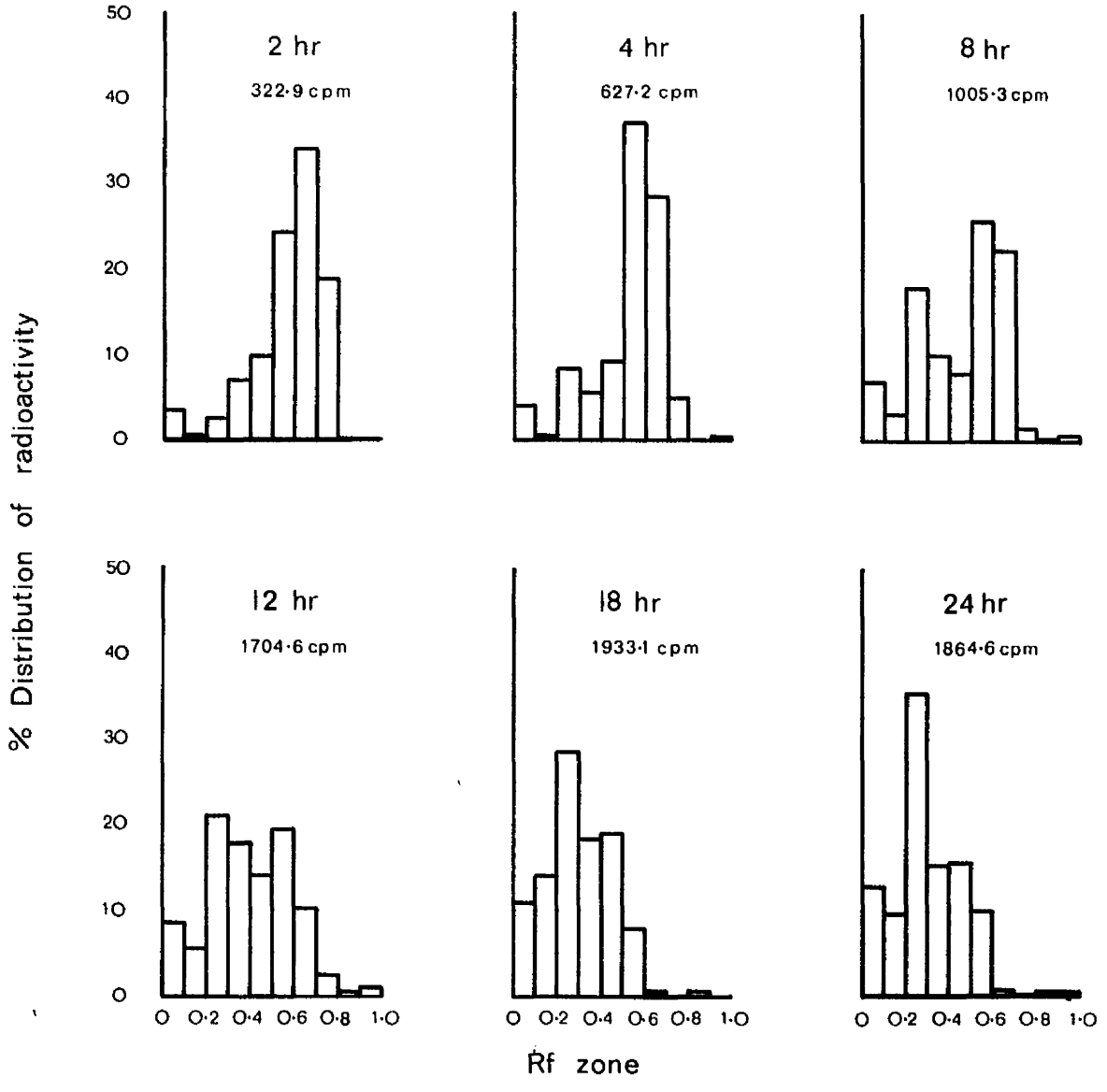
(b) 1 day

(c) 6 days

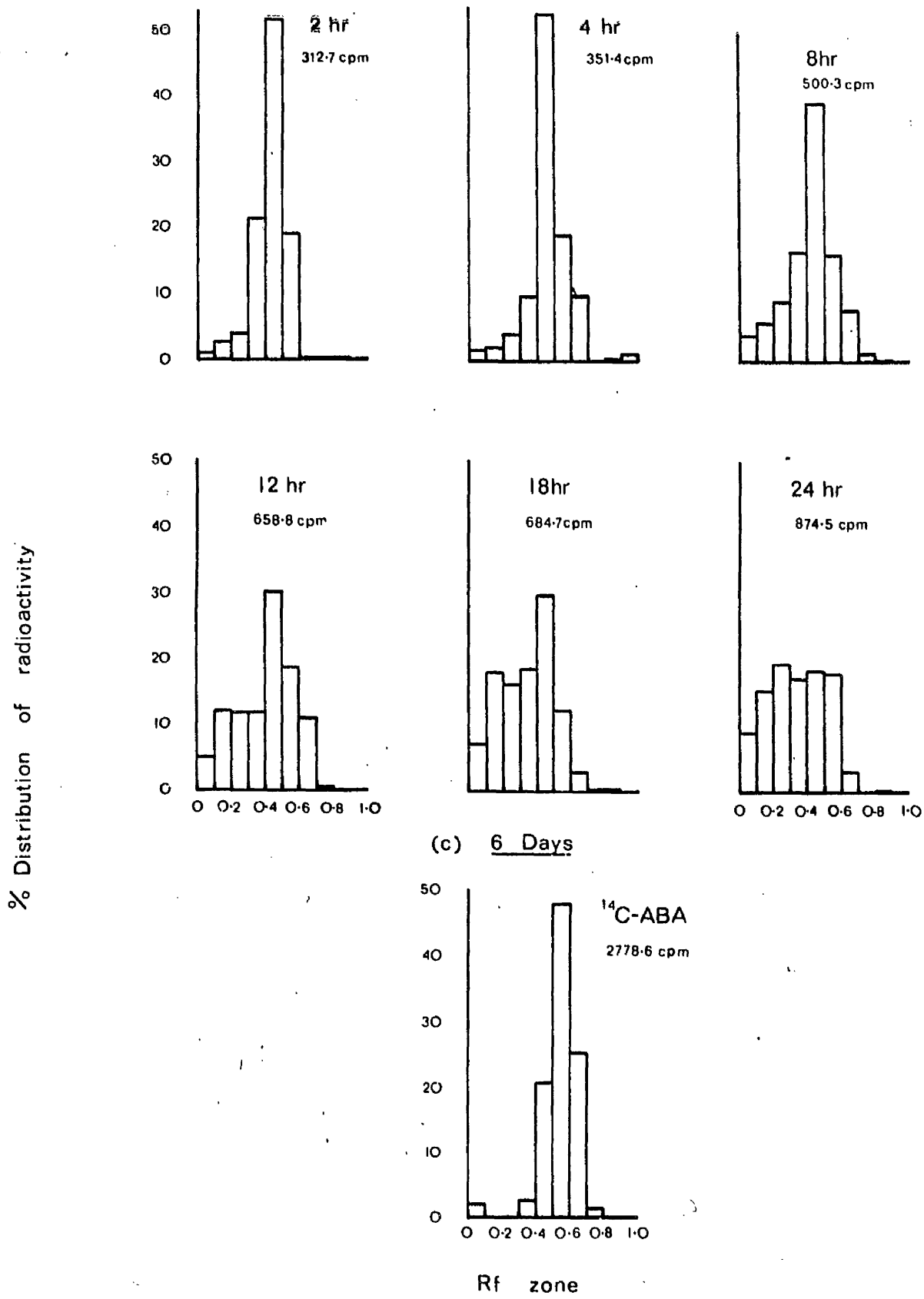
Chloroform:methanol:water (75:22:3)



(a) Freshly excised discs


% Distribution of radioactivity
2 hr
322·9 cpm
4 hr
627·2 cpm
8 hr
1005·3 cpm
12 hr
1704·6 cpm
18 hr
1933·1 cpm
24 hr
1864·6 cpm
0
10
20
30
40
50
0 0·2 0·4 0·6 0·8 1·0
Rf zone

(b) 1 Day


2 hr
312·7 cpm
4 hr
351·4 cpm
8hr
500·3 cpm
12 hr
658·8 cpm
18hr
684·7 cpm
24 hr
874·5 cpm
% Distribution of radioactivity
0
10
20
30
40
50
0 0·2 0·4 0·6 0·8 1·0
(c) 6 Days
^{14}C-ABA
2778·6 cpm
Rf zone

for longer periods (4, 5, 6 days), the nitrogen treatment apparently entirely inhibited the metabolism of ^{14}C-ABA, whereas in discs at an earlier stage of senescence a reduced level of metabolism was detectable.

(d) Time-course for the metabolism of ^{14}C-ABA in radish leaf discs

The synthetic relationship between ^{14}C-ABA and its radioactive metabolic products remains unclear; several possible synthetic sequences could be envisaged. It was decided to examine the relative rates of production of the metabolites over a time-course of 24 hours. Use of leaf discs at different stages of senescence might also provide evidence on possible differences in metabolic capacity between green and senescent leaf tissue. Leaf discs aged for 0, 1 or 6 days in water were transferred to ^{14}C-ABA for 2, 4, 8, 12, 18 or 24 hours. Methanolic extracts of the discs were developed on chloroform:methanol:water and the distribution of radioactivity analyzed (Figure 21). This experiment was carried out on 2 occasions, with a total of 10 replicate chromatograms for each treatment.

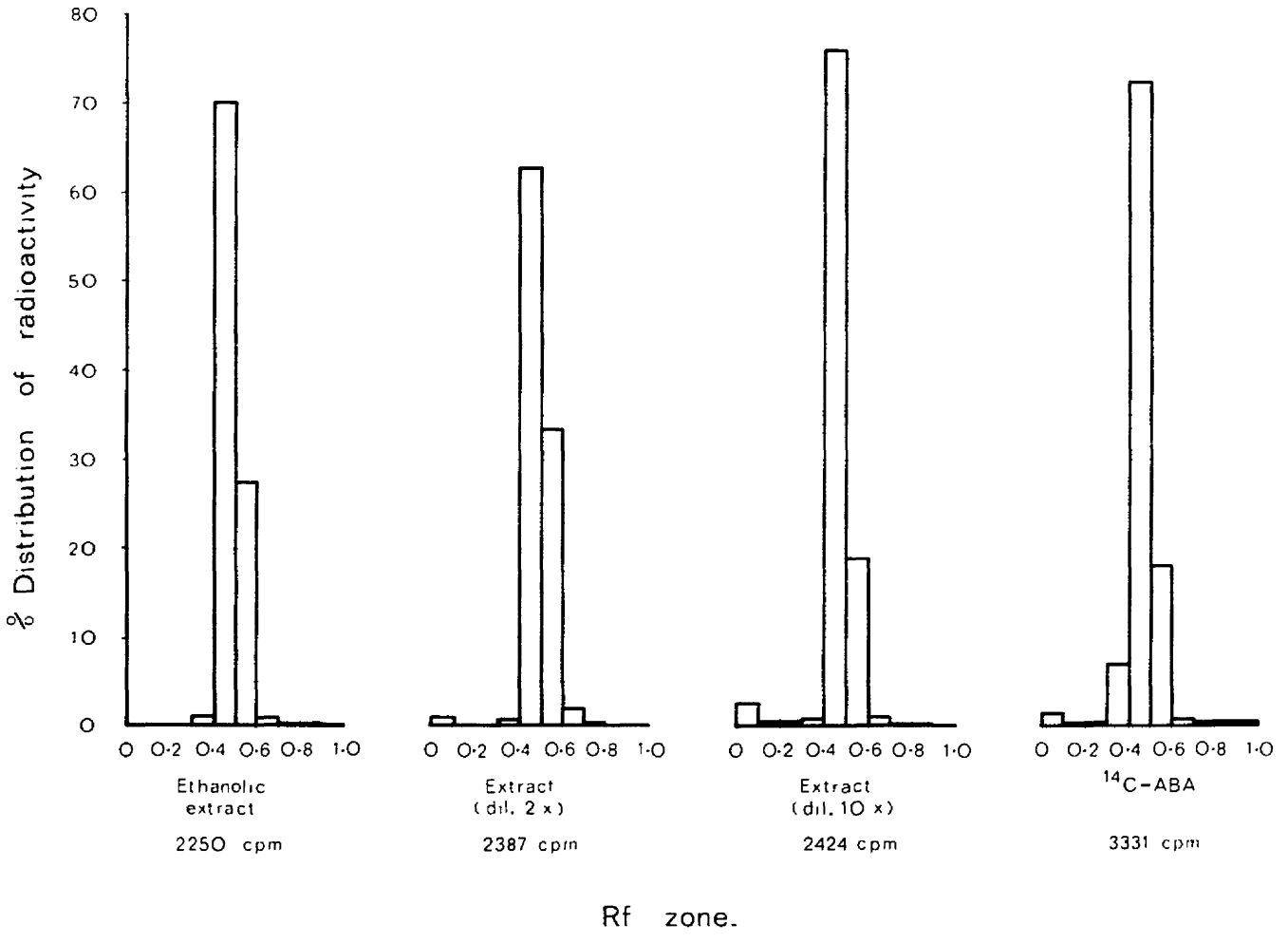
For discs pre-aged for 0 and 1 days, there was an apparent steady increase with length of incubation in ^{14}C-ABA in the level of the metabolite chromatographing at Rf 0 to 0.1. At 2 hours, the shortest incubation used, there was more of this metabolite present in freshly excised discs (6.8% of the total radioactivity) than in discs pre-aged for 1 day (3.5%); by 18 and 24 hours, however, there was little difference between these 2 ageing periods for the level of this metabolite. Little of the metabolite chromatographing at Rf 0.2 to 0.3 was present at 2 hours in discs pre-aged for 0 and 1 days; by 24 hours, however, this zone contained 35% of the total radioactivity present. The situation in discs incubated for 6 days was less clear, but a similar pattern of production of the 2 metabolites was evident. The levels of the metabolites at 24 hours were somewhat less than in discs pre-aged for shorter periods.

(e) Chromatographic behaviour of ^{14}C-ABA in tissue extracts

During the course of studies using the chloroform:methanol:water (75:22:3) system for thin-layer chromatography the chromatographic behaviour of the more mobile components, including ^{14}C-ABA, was found to be rather variable: on different chromatograms, developed at different times, the major peak of radioactivity for ^{14}C-ABA occurred in the range Rf 0.4 to 0.7. It is possible that the presence of pigmented and other impurities in the extracts may contribute to the variability observed by affecting the chromatographic behaviour of

Figure 22. Chromatography of ^{14}C-ABA added to ethanolic extracts of radish leaf tissue.

Chloroform:methanol:water (75:22:3)


% Distribution of radioactivity
80
70
60
50
40
30
20
10
0
0 0·2 0·4 0·6 0·8 1·0
Ethanolic extract
2250 cpm
Extract (dil. 2 x)
2387 cpm
Extract (dil. 10 x)
2424 cpm
^{14}C-ABA
3331 cpm
Rf zone.

^{14}C-ABA contained in the extracts. To test this possibility, 1.95 gm of fresh radish leaves were extracted in ethanol to give a solution with an absorbance of 3.20 in 50 mls; this solution was then diluted 1, 2 and 10 times. To 5 ml aliquots of the diluted solutions, 0.1 ml of ^{14}C-ABA was added and the whole extract was then loaded onto thin-layer plates and developed in chloroform:methanol:water. Examination of the distribution of radioactivity along the chromatograms indicated that the presence of alcohol-soluble impurities did not appear to influence the chromatographic behaviour of ^{14}C-ABA (Figure 22). It can be concluded however, that the patterns of metabolism noted in previous experiments in this Section are not a function of the methanolic extraction procedure or of the chromatographic techniques, since no breakdown of ^{14}C-ABA occurred when added to extracts.

(f) The extraction and attempted identification of the products of ^{14}C-ABA metabolism

To enable a comparison to be made with published findings (Koshimizu et al., 1968; Milborrow, 1970 a, b; Tinelli et al., 1973), the isolation and identification of the products of the metabolism of ^{14}C-ABA was attempted. A purification procedure for separating the metabolites from the other leaf components was devised using 7 small-scale trials of 100 or 200 radish leaf discs, incubated in ^{14}C-ABA for 24 hours; the techniques adopted here have been described in Materials and Methods, Section II. The passage of radioactivity through the various extraction, partition and chromatography stages was monitored and aliquots from solutions shown to contain significant levels of radioactivity were chromatographed in order to determine the distribution of radioactivity. Table 32 presents the data for the passage of radioactivity through the various partition procedures for one such small-scale trial. When partitioned against diethyl ether at pH 8.0 and 7.0successively, very nearly all the radioactivity remained in the aqueous fractions, which had been derived from reduction of the original 80% methanolic extract. When partitioned against ether at pH 3.0, however, the majority of the radioactivity then passed into the ether fraction and this mainly co-chromatographed with ^{14}C-ABA when developed on thin-layer plates in chloroform:methanol:water. The radioactivity remaining in the aqueous fraction remained closer to the origin than did ^{14}C-ABA and was distributed in the Rf zone 0 to 0.4 (Table 33).

After adjusting the pH of the aqueous fraction to 7.0 and reducing the fraction to dryness, a portion of the extract was re-dissolved in a small volume of 30% acetone and applied to the top of a charcoal-celite column

(Materials and Methods, paragraph 11) and eluted in increasing concentrations of acetone. In the small-scale trials, all the resulting fractions were reduced to dryness and analyzed for radioactivity, one fraction representing the drops collected in 1 1/3 hours.

The distribution of radioactivity between the fractions collected from a typical column loaded with an extract from the leaf discs or with ^{14}C-ABA is shown in Table 34; the fractions have been grouped into tens for convenience of presentation. There was some variation between different columns in the elution characteristics for the various fractions, but in general little radioactivity was eluted by 30% or 50% acetone; rather, the radioactive components of tissue extracts tended to be eluted in 2 partially separated fractions by 70% and 100% acetone. In the small-scale trials, most of the impurities in the extracts were adsorbed by the column; in large-scale feeding experiments, however, it was necessary to increase the amount of adsorbent and hence the size of the column in order to improve the purification properties of the system. Little ^{14}C-ABA was eluted from the smaller columns except by 100% acetone and even at this concentration less than half of the original radioactivity could be recovered. It would thus appear probable that any ABA remaining in extracts after the partition procedures would tend to be adsorbed by the charcoal or to be eluted in later fractions than the metabolites. Chromatographic checks of column fractions containing significant amounts of radioactivity indicated little contamination by residual ^{14}C-ABA. The Rf values of the radioactive compounds that were present corresponded with those of the metabolites noted in earlier experiments (Table 35).

In order to extract sufficient metabolites for identification by mass spectrometry, it was considered necessary to use larger quantities of leaf material than was employed in the small-scale trials described above. Thus, in 3 trials, approximately 5000 leaf discs were punched and then incubated as before, in 10^{-5}M ABA. Several hundred discs were also incubated in 10^{-6}M ^{14}C-ABA in order to obtain radioactive metabolites to act as markers during extraction. After 24 hours incubation, the discs were extracted and partitioned as before and then passed through the charcoal-celite chromatography system, before being purified further by thin-layer and paper chromatography. The purified extracts were derivatised by acylation or methylation (Materials and Methods, paragraph 11), and then analyzed by GLC and GC-MS. Although all peaks present on GLC traces were scanned by mass spectrometry, no ions were detected corresponding to those observed in other studies of known ABA metabolites. In addition, no other ions were detected as occurring consistently.

Table 34. Distribution of radioactivity of eluted fractions from a charcoal-celite column.

		Disc extract		^{14}C-ABA	
		Change of acetone concentration	% of cpm	Change of acetone concentration	% of cpm
% of recovered radioactivity in fractions	1-10	30%	0.7	30%	0
	11-20		0.3		0.8
	21-30		0		0.3
	31-40	50% at 32	0.2		1.1
	41-50	70% at 48	0.1		0.2
	51-60		0.1	50% at 59	1.1
	61-70		12.4		0.8
	71-80		16.1		2.5
	81-90		6.6		0.8
	91-100		15.7		1.6
	101-110	100% at 103	28.5		4.7
	111-120		18.6		4.4
	121-130		0.7		1.1
	131-140		0		1.7
	141-150		0		1.3
	151-160			70% at 153	4.2
	161-170				6.8
	171-180				12.2
	181-190			100% at 184	16.2
	191-200				41.0
Total radio-activity recovered		38,000 cpm		1260 cpm	

Table 35. Distribution of radioactivity in chromatograms developed in chloroform: methanol:water of fractions eluted from a charcoal-celite column

Fraction No.	% Distribution of radioactivity in 10 equal zones										Total cpm
	0	1	2	3	4	5	6	7	8	9	
72	35.3	9.4	25.3	29.9	0.1	0	0	0	0	0	189.8
93	23.0	10.9	7.0	36.2	14.8	4.1	0.9	0.4	0	2.6	509.6
107	12.9	8.7	3.1	22.5	35.4	10.7	1.0	2.1	0.4	3.2	921.2
113	26.8	12.3	2.1	23.0	22.8	9.3	1.2	0	1.2	1.1	261.6
^{14}C-ABA	1.2	0.2	0.2	0.1	0.4	0.8	67.7	29.9	1.2	0.3	5300.6

Table 36. The distribution of radioactivity on chromatograms developed in chloroform: methanol;water of discs treated with partially purified metabolite fractions.

Fraction	% Distribution of radioactivity in 10 equal zones										Total cpm
	0	1	2	3	4	5	6	7	8	9	
I	87.9	12.0	0.1	0	0	0	0	0	0	0	82.2
Solution I	82.8	16.7	0.1	0.2	0	0	0	0.2	0	0	1168.7
II	5.1	1.0	0.1	14.4	79.4	0	0	0	0	0	215.9
Solution II	3.6	0.2	0.5	18.1	76.6	0.7	0.5	0	0	0	3002.9
III	4.6	0	0	40.4	42.1	0.3	9.8	0	0	0	26.3
Solution III	9.6	0.8	0	0	3.5	3.4	60.7	22.1	0	0	639.8
^{14}C-ABA	1.4	0.2	0.1	0.1	0.2	0.6	73.7	23.0	0.5	0.2	28000.0

(g) Feeding of partially purified metabolites to leaf discs

The synthetic relationships between ABA and its metabolic products remains unclear. Valuable information on these relationships might be obtained by treating leaf discs with extracts of the metabolites and examining any consequent changes in the chromatographic distribution of radioactivity. Thus several hundred radish leaf discs were incubated in ^{14}C-ABA for 24 hours and the radioactive metabolic products extracted by the partition procedures described above and then further purified by thin-layer and paper chromatography. It was evident, however, that some impurities remained in the eluates from the chromatograms; further purification was not attempted, however, due to the loss of radioactivity which would be involved.

Dried extracts of the radioactive fractions were dissolved in 10 ml of water, and 4 radish leaf discs were incubated in this solution for 8 hours in the dark. The discs were then extracted in methanol and, together with 0.1 ml of the incubation solution as a marker, were developed in chloroform:methanol:water.

Analysis of the distribution of radioactivity indicated little difference between the incubation solutions and the methanolic extracts for Fractions I and II (Table 36). For fraction III, however, the radioactivity of the incubation solution co-chromatographed with ^{14}C-ABA, whereas the radioactivity of the methanolic extract occurred in the Rf zone 0.3 - 0.5, thus co-chromatographing with the radioactivity in fraction II. Possibly, fraction III consisted of ^{14}C-ABA which had passed through the partition procedures; alternatively, it may have represented ^{14}C-ABA which had arisen by reversion of one of the metabolite fractions. The level of radioactivity in the extract was very low, however.

(h) The metabolism of ^{14}C-ABA in whole leaves of radish

The results of experiments reported above (I(j) and (k)) indicated that the uptake and incorporation of protein precursors appeared to occur mainly via the cut edge of radish leaf discs. It is thus conceivable that the metabolism of ^{14}C-ABA, observed in leaf discs in previous experiments, is a result of wounding effects at the disc edge. In order to investigate whether similar patterns of metabolism of ^{14}C-ABA could also occur in whole leaves of radish, ^{14}C-ABA was supplied via the cut surface of the petiole to third leaves of radish, detached at weekly intervals from the time of 3rd leaf emergence approximately 5 weeks after planting, until abscission. Methanolic extracts of these leaves

Figure 23. Chromatography of methanolic extracts of whole leaves of radish supplied with ^{14}C-ABA.

Chloroform:methanol:water (75:22:3)

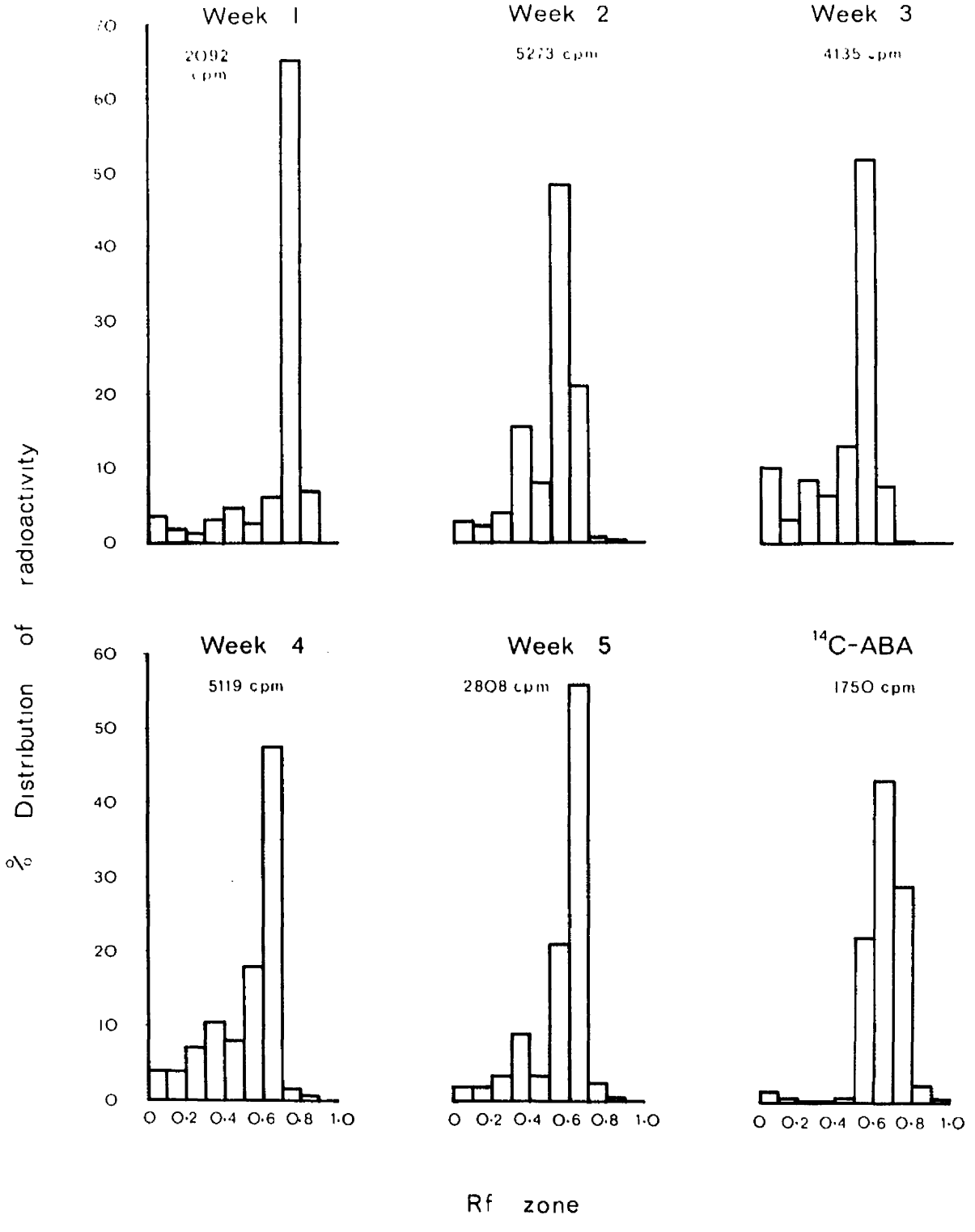

Week 1
2092 cpm
Week 2
5273 cpm
Week 3
4135 cpm
Week 4
5119 cpm
Week 5
2808 cpm
14C-ABA
1750 cpm
% Distribution of radioactivity
Rf zone
0
10
20
30
40
50
60
0·2
0·4
0·6
0·8
1·0

Table 37. Fresh weight and chlorophyll content of third leaves of radish from the time of leaf emergence

Week	Fresh weight (gm)	Absorbance 65 nm (50 ml)	Chlorophyll Fresh wt.
1	0.098	0.145	1.48
2	0.472	0.655	1.39
3	0.358	0.447	1.25
4	0.334	0.201	0.60
5	0.232	0.088	0.38

Table 38. Circular dichroism determinations (Ψ 262.5 nm) of the level of endogenous ABA in primary leaves of bean at 2 stages of development.

	Total batch size (kg)	Concentration of ABA ($\mu g\ Kg^{-1}$ F.wt.)
2 weeks	3.09	0.7
5 weeks	3.14	5.5

N.B. only one determination for each age.

were developed on thin-layer chromatograms in chloroform:methanol:water and the distribution of radioactivity analyzed. The experiment was carried out 3 times.

Weekly fresh weight and chlorophyll analyses of batches of 5 randomly selected leaves indicated that the third leaf attained maximum size and chlorophyll content at week 2, after which time both declined until abscission (Table 37). The loss in fresh weight in weeks 3 to 5 was probably attributable to variation between samples; examination of chlorophyll:fresh weight ratios indicate that chlorophyll values still fell during this period when corrected for weight differences (i.e., senescence was apparently occurring).

Some metabolism of ^{14}C-ABA occurred at week 1 (i.e. just after leaf emergence) as indicated by the presence of small peaks of radioactivity at Rf 0 to 0.1 and 0.3 to 0.5 (Figure 23). An increased level of metabolism occurred at week 2 (Rf 0.3 to 0.4) and week 3 (Rf 0 to 0.1), before declining somewhat in weeks 4 and 5.

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SECTION IV : THE EXTRACTION OF ENDOGENOUS ABA

If ABA is involved in the regulation of the natural senescence of leaves, then changes in the level of endogenous ABA during leaf development might be important in regulating the onset and/or the rate of senescence. Although ABA can apparently stimulate the senescence of leaf tissue, no detailed studies appear to have been made of any changes in the level of ABA during leaf development, based on critical identification and measurement of the hormone (as opposed to examination of changes in the biological activity of ABA-like inhibitors). In this study therefore quantitative determinations have been made of the endogenous ABA present in leaf tissue at various stages of development between the phases of rapid expansion and abscission. For this study, it was decided to use the primary leaves of the dwarf French bean, Phaseolus vulgaris: uniform plants of this species could be readily obtained within 14 to 35 days from planting, whereas comparable experiments using radish would take up to 10 weeks. Moreover, the tissue yield on a per leaf basis is greater in bean than in radish.

(a) The extraction of endogenous ABA using extraction method 1

Preliminary experiments, attempting to demonstrate the presence of ABA in extracts of the primary leaves from batches of approximately 200 bean plants, were carried out using method 1, outlinedin Materials and Methods, Section 8, and adapted after Lenton et al. (1971). No ABA could be detected by this means, however, even though a significant portion of the ^{14}C-ABA added at the beginning of the extraction procedures could be recovered after the various purification procedures. The failure to detect any endogenous ABA in the leaves may have been due to a very low level of the hormone in the tissue. Thus larger batches of tissue might permit a demonstration of the presence of the hormone. Accordingly, a preliminary trial was carried out using 2 batches of approximately 3000 bean plants each, grown at a density of about 500 plants per m^2 in soil plots in a greenhouse. The primary leaves of one batch of plants were harvested and extracted 2 weeks after sowing, i.e. during the period of rapid leaf expansion. The second batch was harvested and extracted at 5 weeks, i.e. when the leaves were yellowing, shortly prior to abscission.

The levels of endogenous ABA as determined by circular dichroism analysis are presented in Table 38.

(b) The extraction of endogenous ABA using extraction method 2

Although endogenous ABA was successfully extracted from bean leaves in the trials outlined above, these procedures required large quantities of leaf material which presented considerable practical problems, both in the growth of plant material and in the subsequent extraction and purification techniques. Hence, for more detailed studies than those described in Section IV(a) above, the techniques of Browning et al. (1970) and Good (pers. comm.) were adapted for the extraction of endogenous ABA from the primary leaves of batches of approximately 500 bean plants. Circular dichroism determinations of ABA levels in bean leaves harvested from plants at various stages of development are presented in Figure 24, together with chlorophyll:fresh weight values. No consistent pattern of changes in the level of endogenous ABA (µg. Kg^{-1} fresh weight) with leaf age was observable. Most extracts contained between 0 and 6 µg. Kg^{-1} of ABA, although several extracts contained amounts of ABA considerably in excess of this range. Chlorophyll levels, corrected for differences in weight between the different samples were at a maximum at 18 days and then declined as the leaves senesced.

A typical trace from circular dichroism determinations is presented in Figure 25, illustrating the positive extremum at 262.5 nm and the negative extremum at approximately 230 nm.

(c) Analysis of development of primary leaves of bean

In order to determine the stage of development of the primary leaves at the various stages after planting, a batch of 20 bean plants was selected at random from 500 plants at 10 days after planting and the changes in area and length assessed (Figure 26). In addition, on alternate days, batches of 12 randomly selected plants were assessed for chlorophyll and fresh weight values (Figure 26). The data presented represent one trial out of three carried out. Between 10 and 17 days, there was a period of rapid increase both in length and in area of the leaves, reaching a maximum at 20 to 21 days for leaf area and 22 days for leaf length. The subsequent decline in leaf area is probably largely attributable to necrosis at the edge of some of the leaves as they senesced. Chlorophyll levels were at a maximum at 12 to 14 days and then subsequently declined sharply. The values for chlorophyll:fresh weight ratios declined from 10 days and from 16 days, the rate of decline closely followed that of chlorophyll (i.e. the fluctuations, observed in chlorophyll levels during the period of decline, were attributable to variation in sample size).

Figure 24. Endogenous ABA extracted from primary leaves of bean and chlorophyll:fresh weight levels at various stages of development.

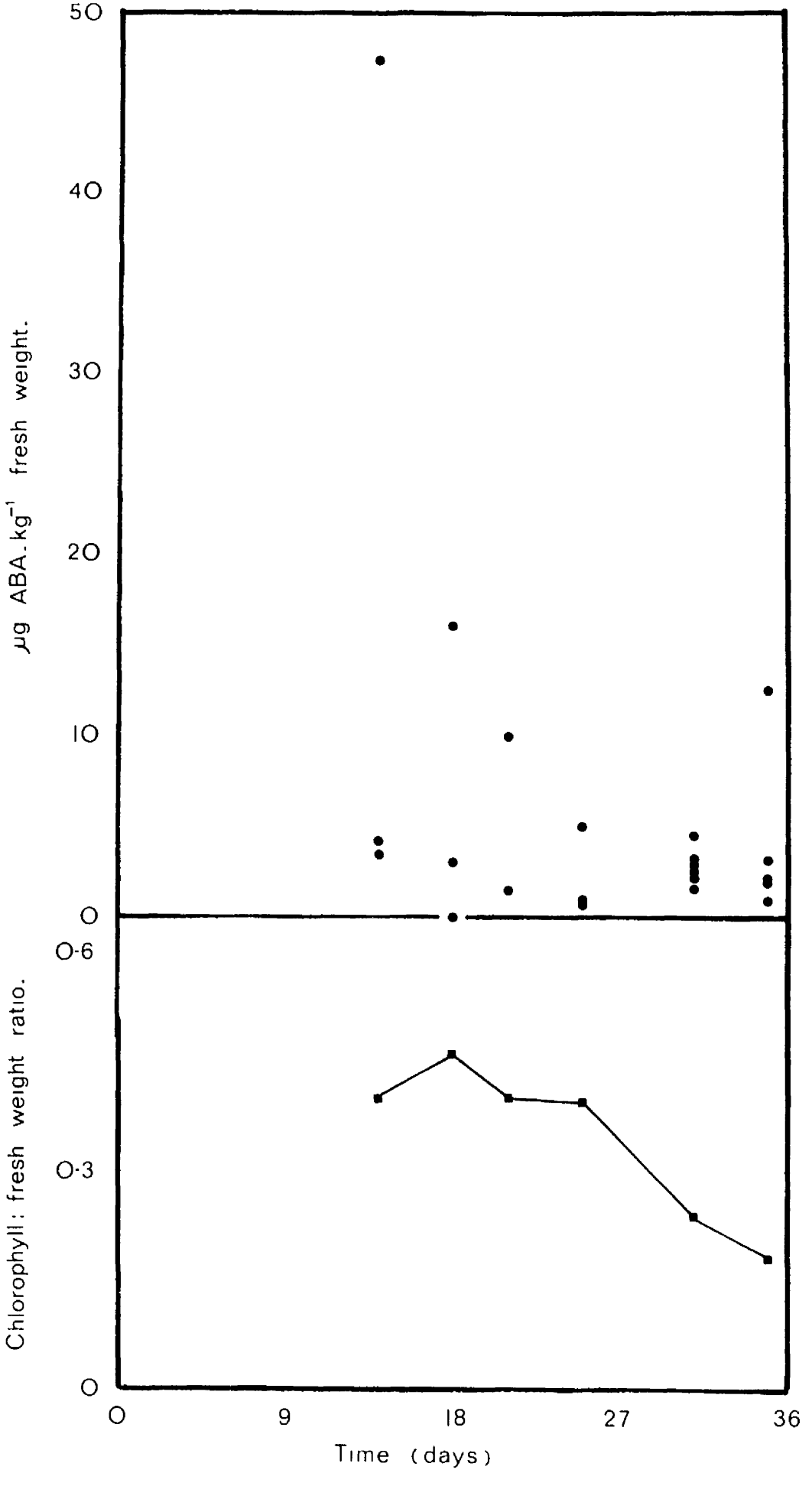

50
40
30
20
10
0
µg ABA.kg⁻¹ fresh weight.
0·6
0·3
0
Chlorophyll: fresh weight ratio.
0
9
18
27
36
Time (days)

Figure 25. Circular dichroism traces of endogenous ABA extracted from primary leaves of bean and of racemic ABA.

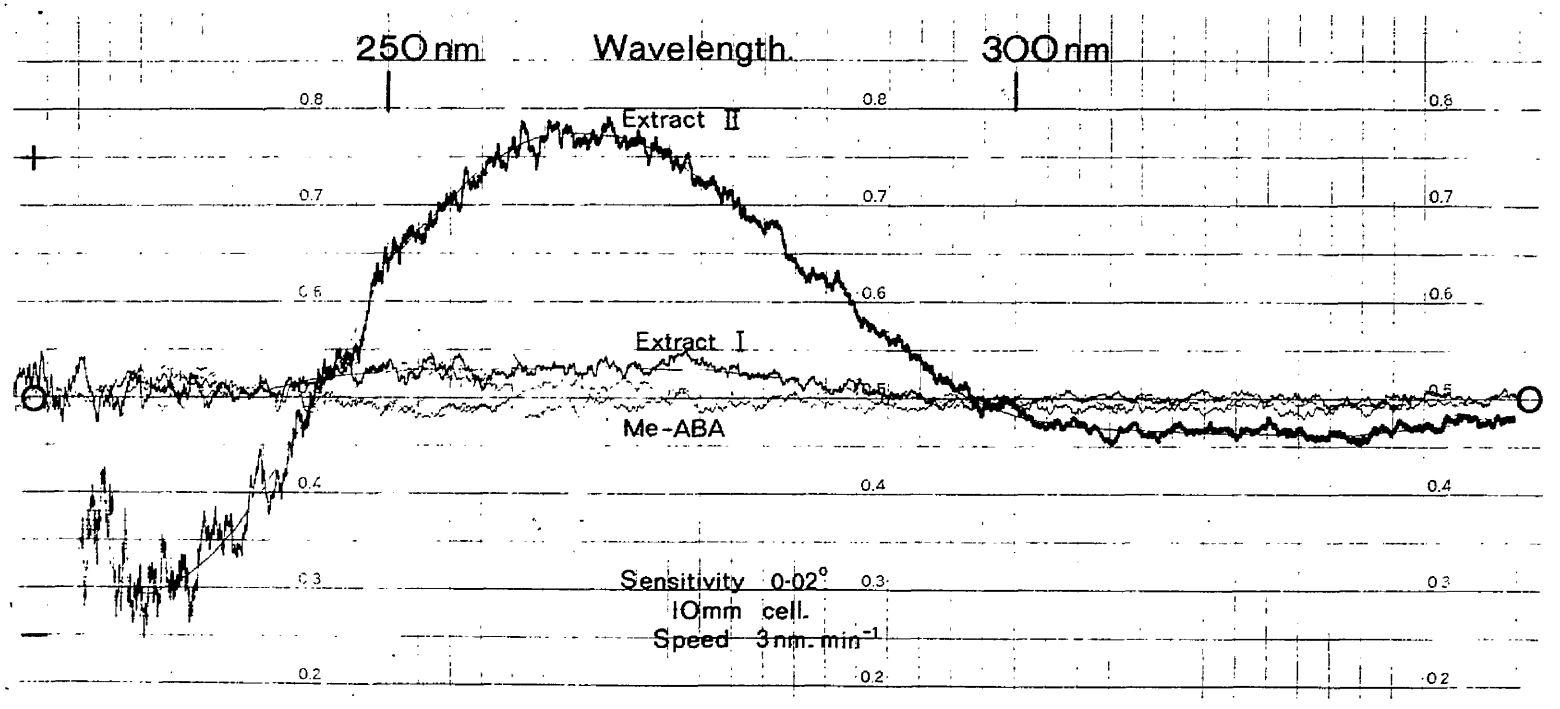
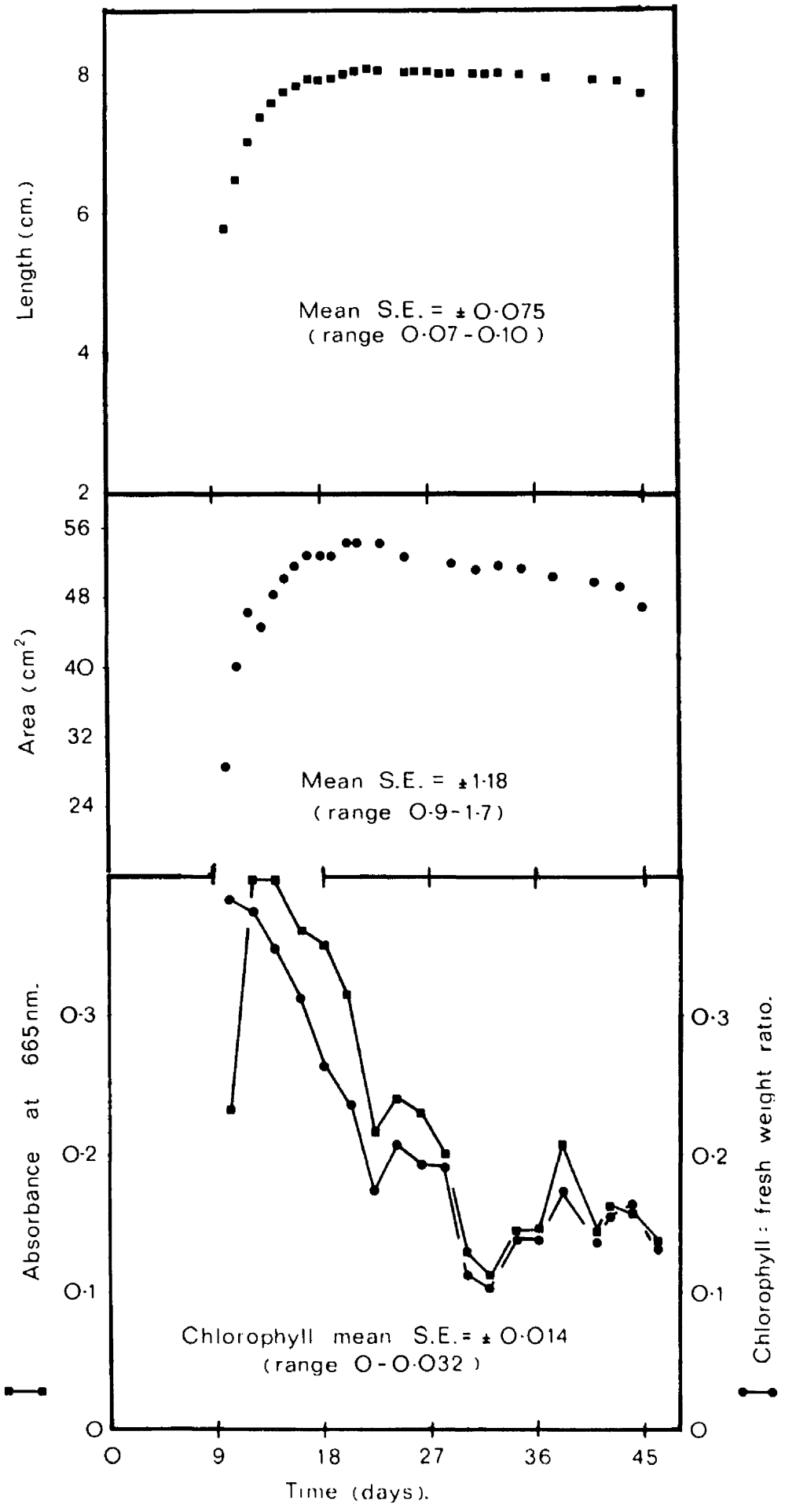

250nm
Wavelength
300nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
Extract II
Extract I
Me-ABA
Sensitivity 0-02°
10mm cell.
Speed 3nm. min⁻¹

Figure 26. Analysis of the development of primary leaves of bean by length, area, chlorophyll and fresh weight determinations.


Length (cm.)
8
6
4
2
Mean S.E. = ± 0·075
(range 0·07 - 0·10)
Area (cm²)
56
48
40
32
24
Mean S.E. = ± 1·18
(range 0·9 - 1·7)
Absorbance at 665 nm.
0·3
0·2
0·1
0
Chlorophyll mean S.E. = ± 0·014
(range 0 - 0·032)
Chlorophyll : fresh weight ratio.
0·3
0·2
0·1
0
0
9
18
27
36
45
Time (days).

(d) Determination of the efficiency of recovery of ^{14}C-ABA added to plant extracts

The levels of endogenous ABA detected in most of the extracts detected above in Section IV(b) are considerably lower than those reported for plant tissues in general (Milborrow, 1968) and for primary leaves of bean in particular (Osborne et al., 1972). This apparent discrepancy may be attributable to the losses of endogenous ABA associated with the extraction and purification procedures adopted here. Accordingly, in order to determine the efficiency of recovery by extraction method 2, 10 ml aliquots of 140,000 cpm of ^{14}C-2-ABA were added to 2 extracts after the initial infiltration stages (i.e. prior to the first partition stage). Small aliquots were then removed from each phase after each partition stage and from chromatography eluates, dried and subjected to radioassay (Table 39).

The data indicate that considerable losses of ^{14}C-ABA occur both during the partition and the chromatography stages of purification. Indeed, if these values are assumed to correspond to the extraction efficiency for endogenous ABA by this method, then the values obtained by circular dichroism determinations may represent approximately a 12-fold under-estimate. The data in Figure 24 have been corrected to allow for losses during purification and are presented in Table 40. Obviously, these corrected data do not alter the conclusions made in Section IV(b).

(e) A preliminary comparison of the endogenous ABA present in wilted and non-wilted bean leaf tissue.

The presence of ABA in primary leaves of bean has been confirmed and little consistent change in the level of ABA during leaf development and senescence could be detected (Section IV(b)). Published evidence, however, indicates that the level of endogenous ABA in leaf tissue can rise due to increased synthesis in response to environmental stress (see, for instance, Wright and Hiron, 1970; Zeevaart, 1971). It was therefore of interest to determine whether ABA levels could increase in response to water stress in bean leaf tissue in which senescence had already apparently started. Hence, a batch of 500 bean plants was divided approximately into half, 25 days after planting. Water was then withheld from one half for 3 days while the normal watering regime was maintained for the other half. After this time, when the unwatered plants were severely wilted, the primary leaves were harvested and extracted in the normal way (Materials and Methods, Section 8).

Table 39. Recovery of radioactivity from extracts of bean leaf tissue

Extraction stage		pH	Phase	% of radioactivity at each stage	% of original radioactivity (140,000 cpm)
After partition	1	4.0	CH_2Cl_2	*	
			Aqueous	*	
After partition	2	8.0	CH_2Cl_2	*	
			Aqueous	*	
After partition	3	8.0	Pet. ether	0	
			Aqueous	100	
After partition	4	8.0	Ethyl acetate	12.9	
			Aqueous	87.1	
After partition	5	3.0	Ethyl acetate	100	53.2
			Aqueous	0	
After methyl- and chromatography					8.2

* No data available since CH_2Cl_2 samples too heavily quenched.

Data are presented as the mean of duplicate trials.

Table 40. Endogenous ABA levels corrected for losses during extraction

Replicate	Concentration of ABA ($\mu g\ kg^{-1}$ F. wt.)					
	14 days	18 days	21 days	25 days	31 days	35 days
1	42.4	37.9	121.0	11.3	20.6	38.1
2	579.7	195.2	21.0	9.2	55.2	23.7
3	52.2	0		61.5	35.3	12.7
4					34.0	26.0
5					37.4	154.0
6					32.9	

Table 41. Circular dichroism determinations of endogenous ABA in wilted and non-wilted bean leaf tissue

	Concentration of ABA ($\mu g\ kg^{-1}$ f.wt.)	Corrected ABA values ($\mu g\ kg^{-1}$ f.wt)	Chlorophyll / Fresh weight ratio
Wilted	95.7 *	1167.7	2.17**
Non-wilted	6.6 *	81.0	2.14**

* One determination only for each treatment.

** Determined from random batches of 6 plants for each treatment.

Table 42. Approximate size of plastoglobuli from sections of water-aged and ABA-aged radish leaf tissue.

Length of ageing (days)	Size of plastoglobuli (μm)	
	H_2O	ABA
Fresh	0.10 (± 0.01)	
1	0.22 (± 0.03)	0.42 (± 0.02)
2	0.10 (± 0.002)	0.14 (± 0.01)
6	0.28 (± 0.03)	0.32 (± 0.02)

The wilted leaves contained approximately 14 times more ABA than the non-wilted leaves, calculated on a fresh weight basis (Table 41). The wilting treatment did not apparently affect chlorophyll levels calculated on a fresh weight basis.

SECTION V : ULTRA-STRUCTURAL STUDIES OF SENESCING LEAF TISSUE

The apparently anomalous pattern of chlorophyll loss from ABA-treated leaf discs has been described in Section I. In addition, the texture of discs aged in water was noted to differ from those aged in ABA. Reports of ultra-structural studies of the effect of ABA on the pattern of leaf senescence, however, are rather limited (Mittelheuser and Van Steveninck, 1971 a, b). It was thus of interest to examine the fine structural changes occurring in radish leaf discs during ageing in ABA and to compare those changes with the situation in fresh green leaf material, in discs aged in water and in naturally senescent, yellowed leaf material.

(a) Freshly excised leaf tissue

Leaf discs were excised as before from inter-veinal regions of mature green leaves and segments for examination by electron microscopy were selected from the central area of the discs in order to avoid any wounded tissue at the edges. Despite these precautions, however, examination of the 260 plates produced revealed considerable variation within any one treatment, even in freshly excised tissue. In particular, 2 types of cell were observed in the sections. One cell type, probably occurring in small vascular bundles, was densely packed with a relatively large nucleus and a large number of mitochondria and was characterised by a low level of vacuolation (Plates 1, 2). These cells typically had relatively few chloroplasts and were considerably smaller than the second type of cell which showed characteristics thought to be more typical of leaf mesophyll cells. In these cells, there was generally a single large vacuole, with the cytoplasm confined to a relatively thin peripheral layer adjacent to the plasmalemma. The elliptical chloroplasts were contained within the cytoplasmic strip with their long axis and stromal thylakoids parallel to the plasmalemma and the cell wall (Plate 3). Starch grains were sometimes present, situated between the thylakoids, but no more than 2 grains per chloroplast were observed (Plate 4). Starch grains were not detected in any aged tissue. Stacks of thylakoids arranged into grana were present in the chloroplasts (Plates 3, 4). A small number of plastoglobuli (lipophilic vesicles, osmiophilic globules) were distributed throughout the chloroplast stroma. The following organelles were also detected in the cytoplasmic layer:- (i) mitochondria, oval to round in shape with intact envelopes and irregularly arranged internal cristae (Plate 3); (ii) the nucleus with a distinct nuclear envelope, granular densely staining areas, probably corresponding to nucleoli or chromatin and nuclear pores, occasionally

Plate 1. Freshly excised radish leaf tissue (G = glutaraldehyde fixative)

x 20,000

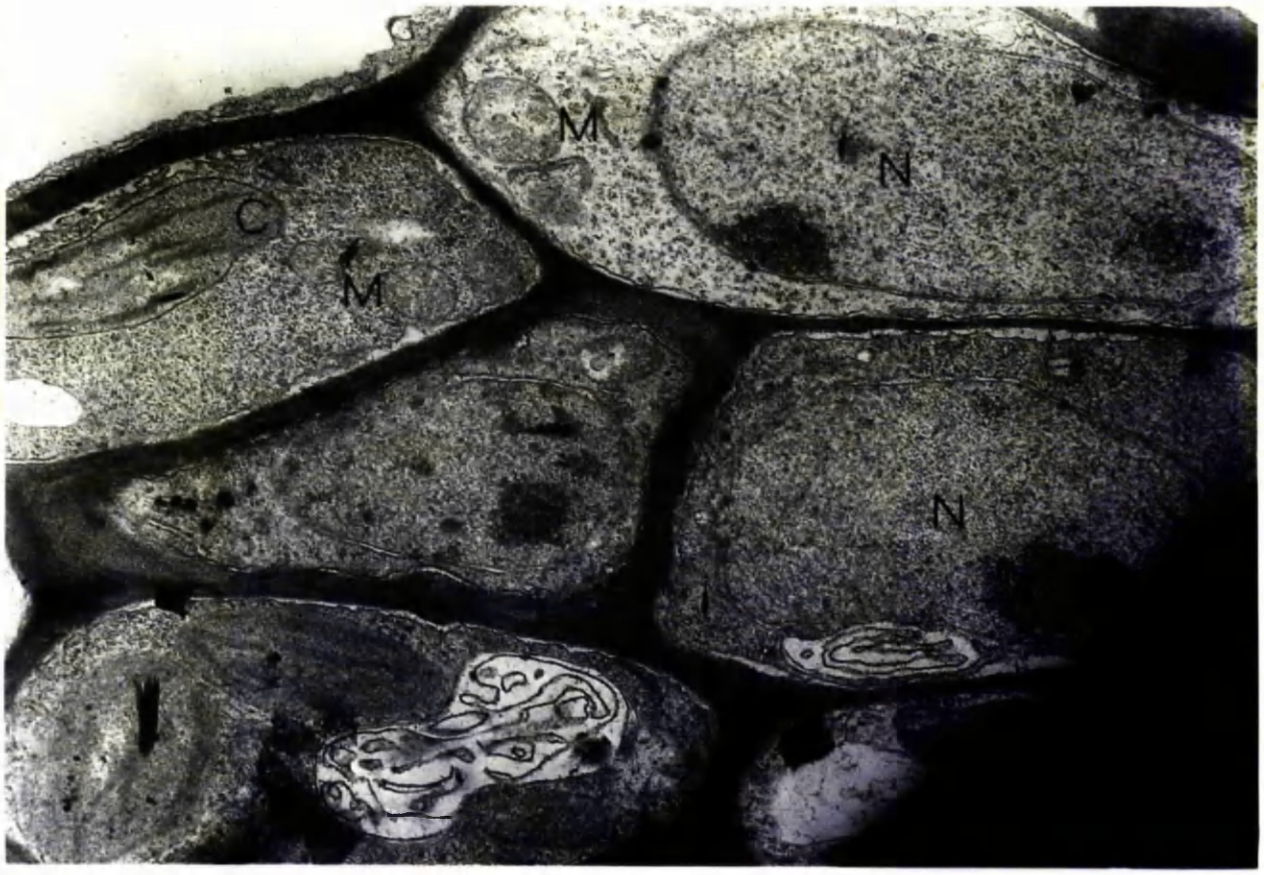
C = chloroplast

M = mitochondria

N = nucleus

Plate 2. Freshly excised radish leaf tissue (G)

x 15,000


M
N
C
M
N

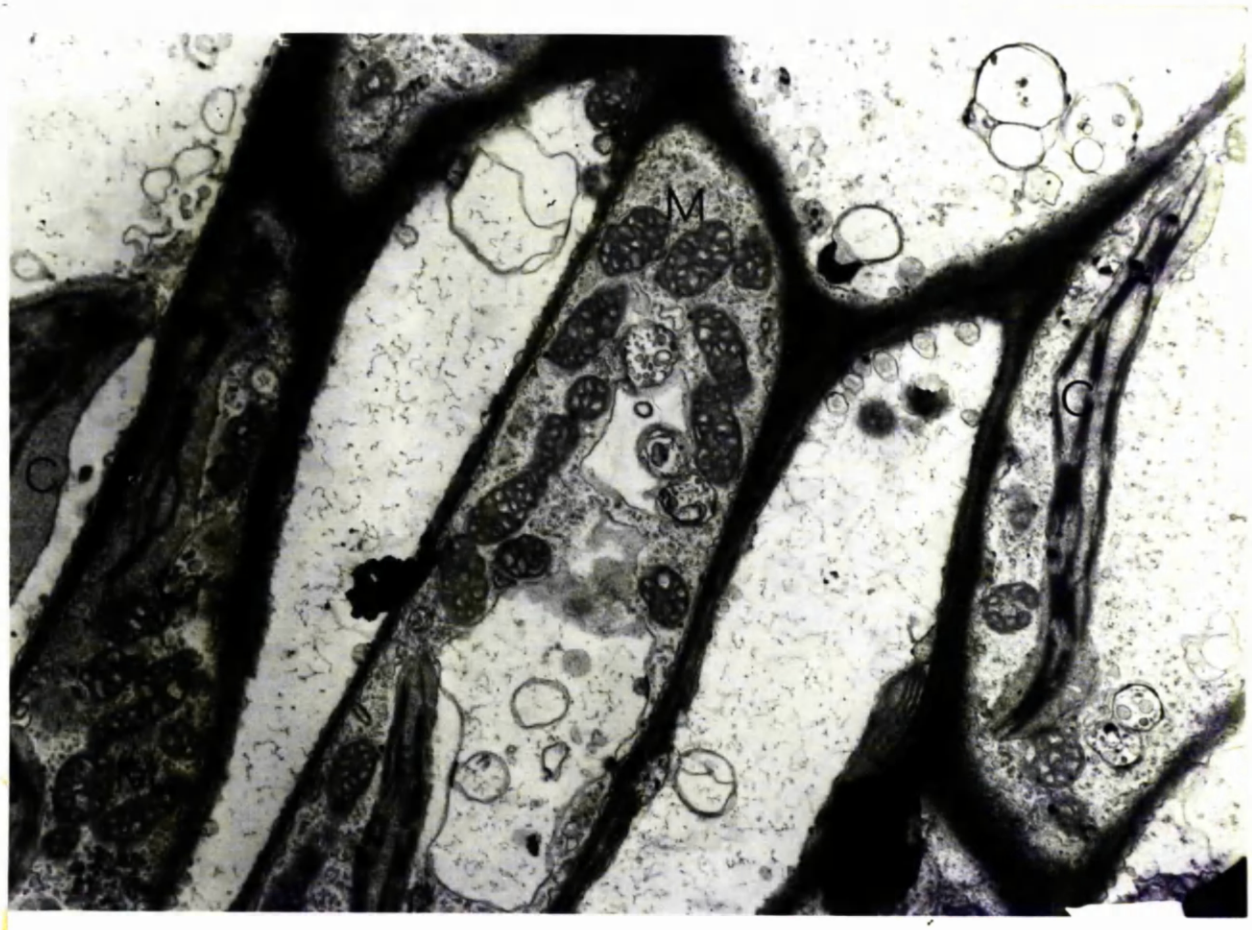

M
C
C

Plate 3. Freshly excised radish leaf tissue (K = Karnovsky fixative)

x 10,000

Cy = cytoplasm

Gr = granum

I = inter-cellular space

P = plastoglobuli

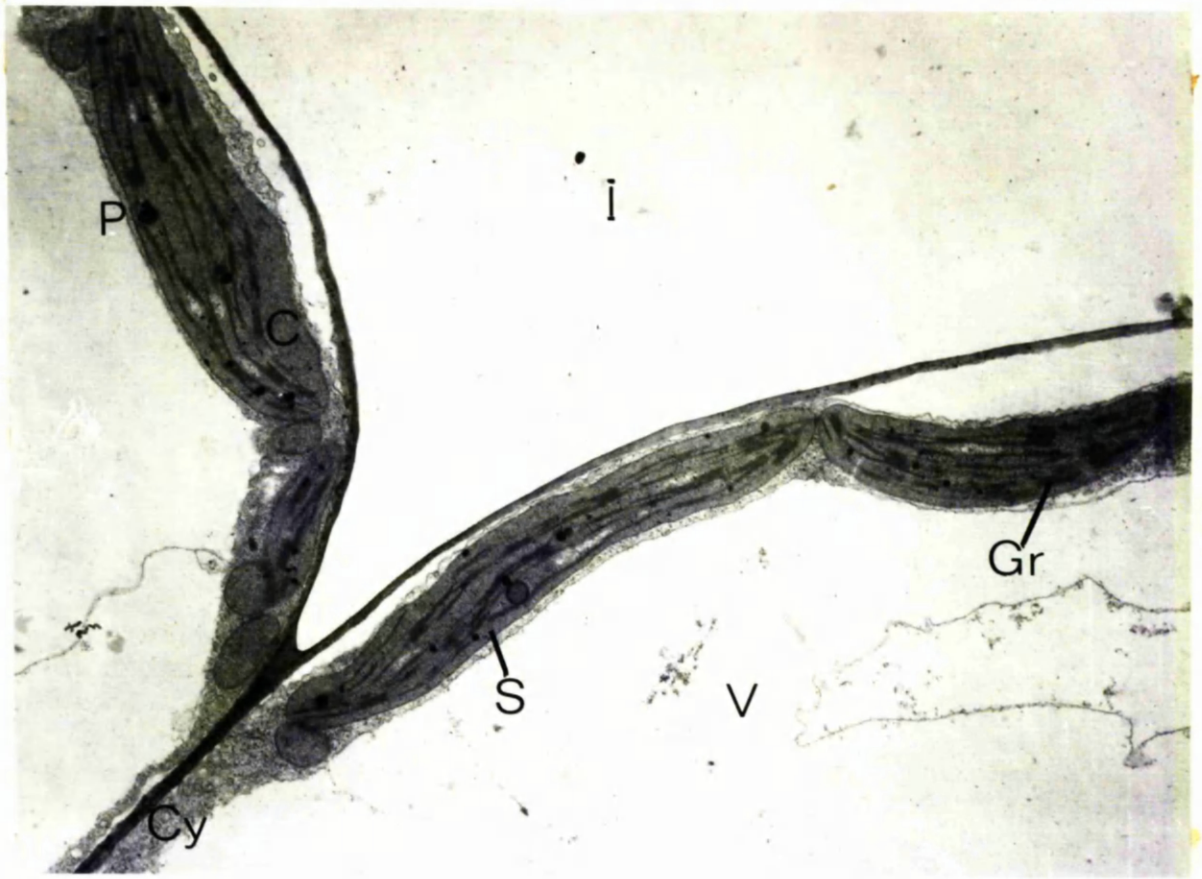
S = stroma

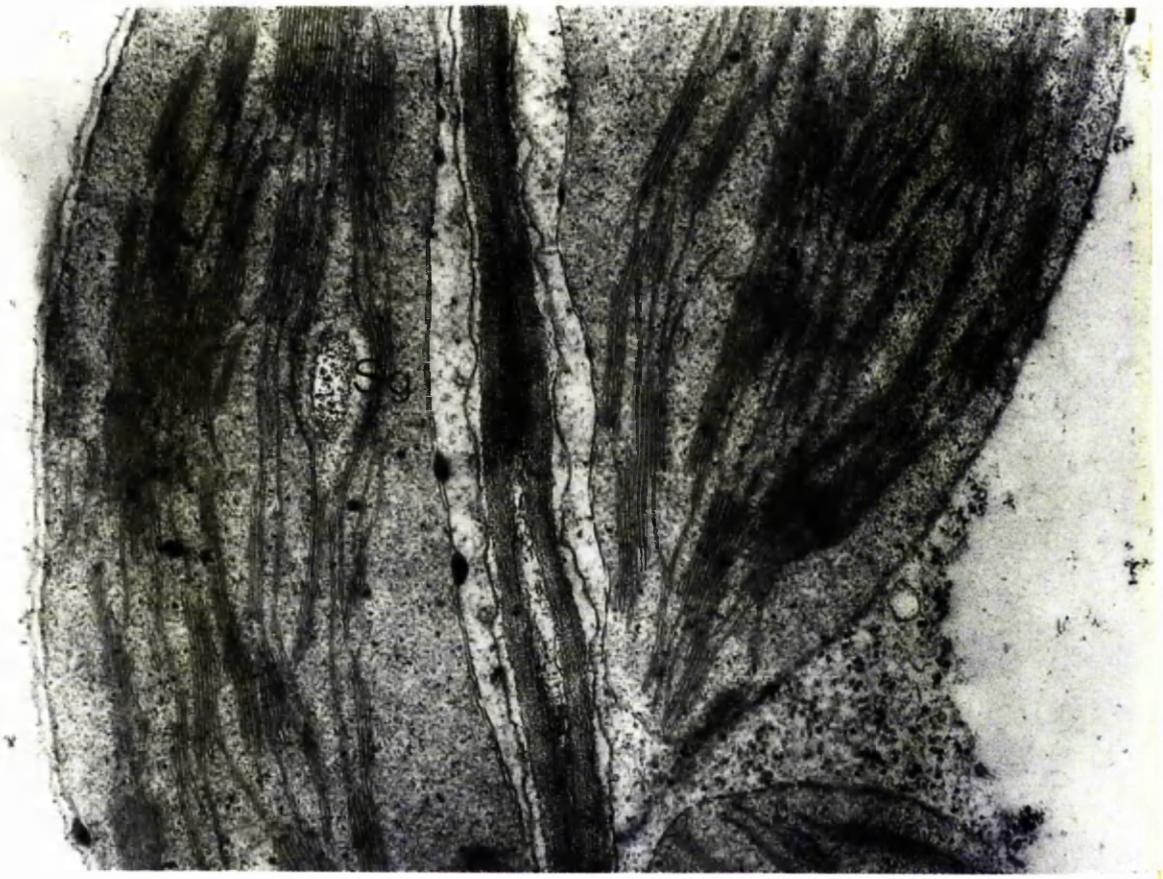
V = vacuole

Plate 4. Freshly excised radish leaf tissue (G)

x 40,000

Sg = starch grain


P
C
I
Gr
S
V
Cy


SG

visible (Plate 5); and (iii) isolated areas of membranous vesicles similar to Golgi bodies (Plate 5) or endoplasmic reticulum (Plate 6). Some variation, however, was observed from different sections of mesophyll tissue, with regard to the shape and size of the chloroplasts and their position relative to the rest of the cell (Plates 7, 8). In some cases the chloroplast envelope had been lost, apparently as part of the general disintegration of the cell (Plate 7). Alternatively, in other cells, some chloroplasts, at the cell perphery, were elliptical and were arranged parallel to the plasmalemma, while other chloroplasts towards the centre of the same cell were more rounded, both in outline and in disposition of the thylakoids; such chloroplasts often contained irregular areas of lower electron density than the normal chloroplast stroma (Plate 8). In these cells, several smaller vacuoles apparently replaced the single large vacuole generally observed. In some cells, there was evidence that the plasmalemma had retreated from certain areas of the cell wall. Large intercellular spaces, characteristic of mesophyll tissue, were evident in many sections (Plates 3, 5).

(b) Tissue aged in water for 1 day

After ageing in water for 1 day, the nuclei were still intact and retained the densely staining areas, observed in fresh tissue. The mitochondria were also apparently unaffected, when compared to those noted in freshly excised leaves (Plate 9). Some deformation of the chloroplast structure was often evident, however. Chloroplasts were more rounded than those seen in fresh tissue and the thylakoids of some chloroplasts had apparently been rearranged to follow the altered contours of the chloroplast envelope (Plate 10). Alternatively the thylakoid structure sometimes became "bowed" within the rounded chloroplast (Plates 10, 11). Granal structures were still present, but the size of the plastoglobuli had increased (Table 42) and these were apparently localised in those regions of the chloroplast which contained thylakoids. Membrane-bound areas of low electron density were present in the stroma of some chloroplasts (Plates 10, 11). Some cells contained unidentified vesicles, with a regular internal structure; these were similar in size to mitochondria (Plate 9). A myelin-like body, apparently composed of concentric membranous structures, was also present in one cell (Plate 9). Starch grains were not observed in chloroplasts of any section.

(c) Tissue aged in ABA for 1 day

Chloroplasts from discs treated in ABA for 1 day were swollen and circular in cross-section, if rather irregular, relative to fresh tissue (Plates 12, 13). The plastoglobuli in ABA-treated tissue were somewhat

Plate 5. Freshly excised radish leaf tissue (K)

x 10,000

Go = Golgi body

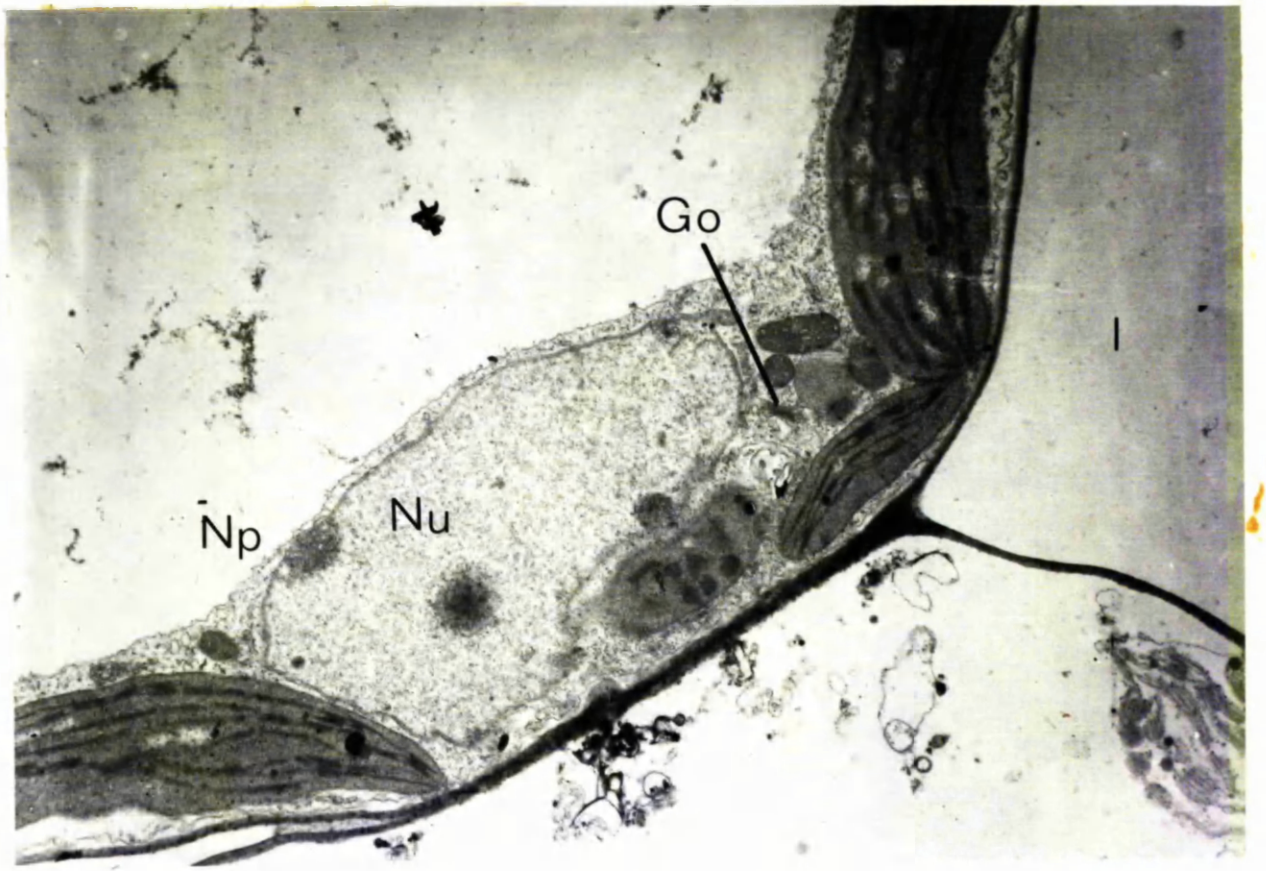
Np = nuclear pore

Nu = nucleolar areas or chromatin

Plate 6. Freshly excised radish leaf tissue (K)

x 20,000

Er = endoplasmic reticulum


Go
I
Np
Nu

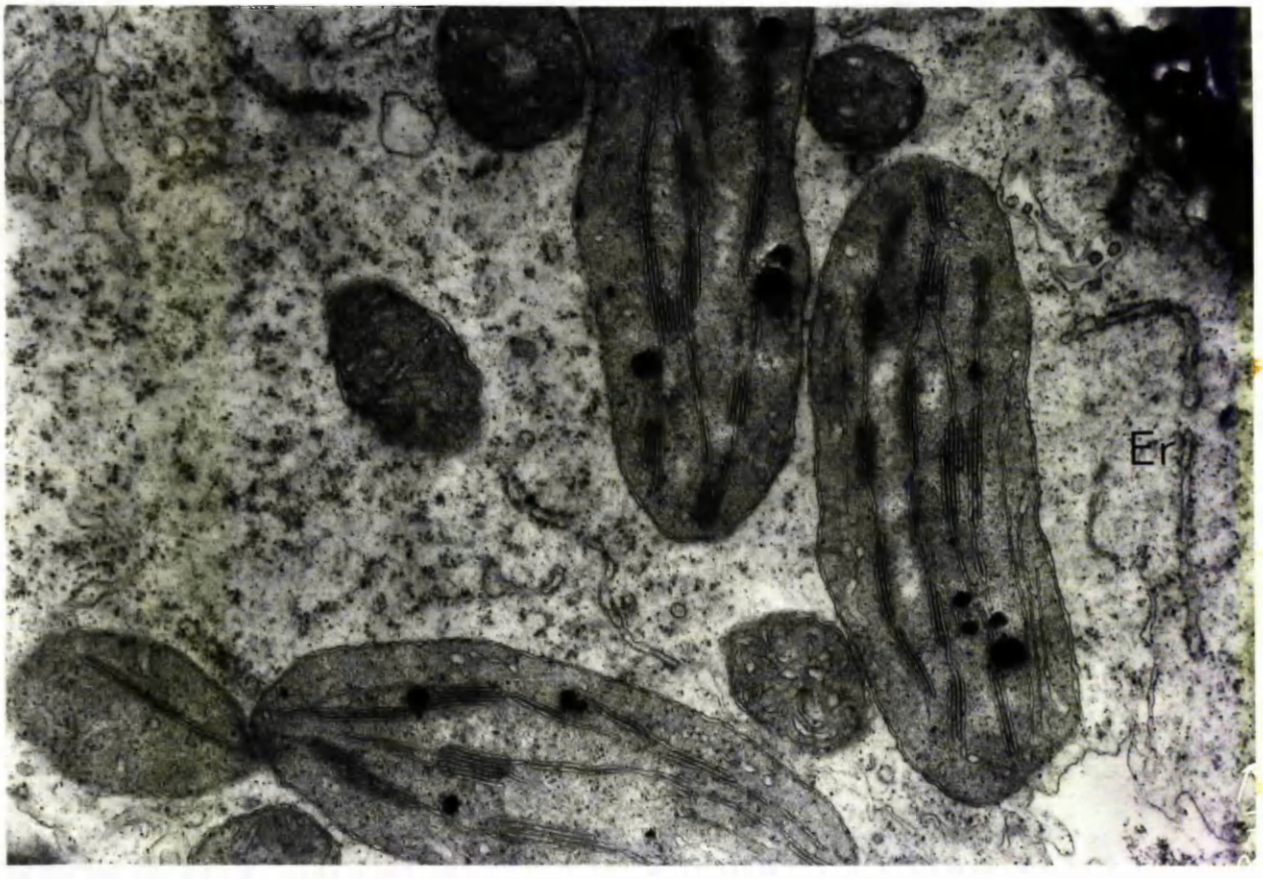

Er

Plate 7. Freshly excised radish leaf tissue (K)

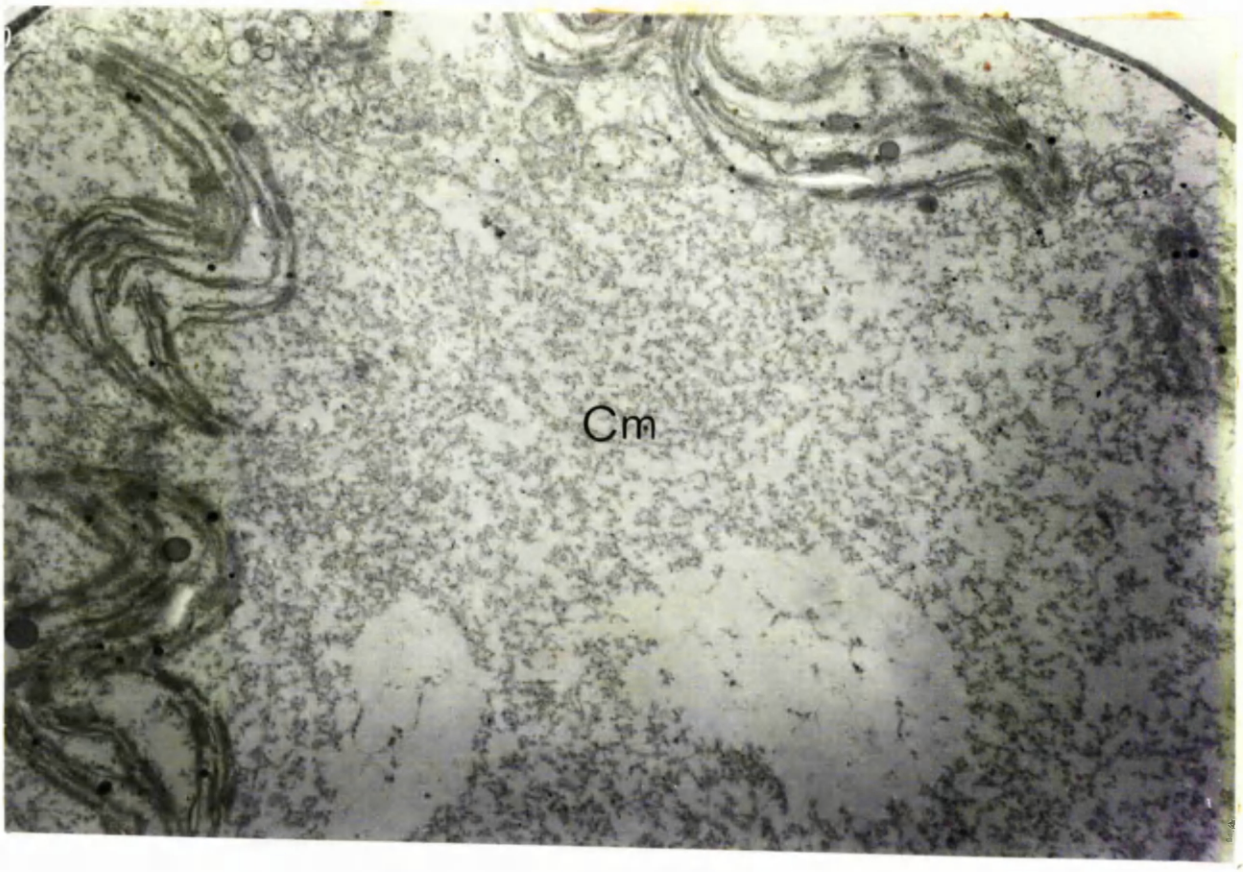
x 10,000

Cm = cytoplasmic matrix

Plate 8. Freshly excised radish leaf tissue (K)

x 10,000

A = chloroplastic area of low-electron density


Cm

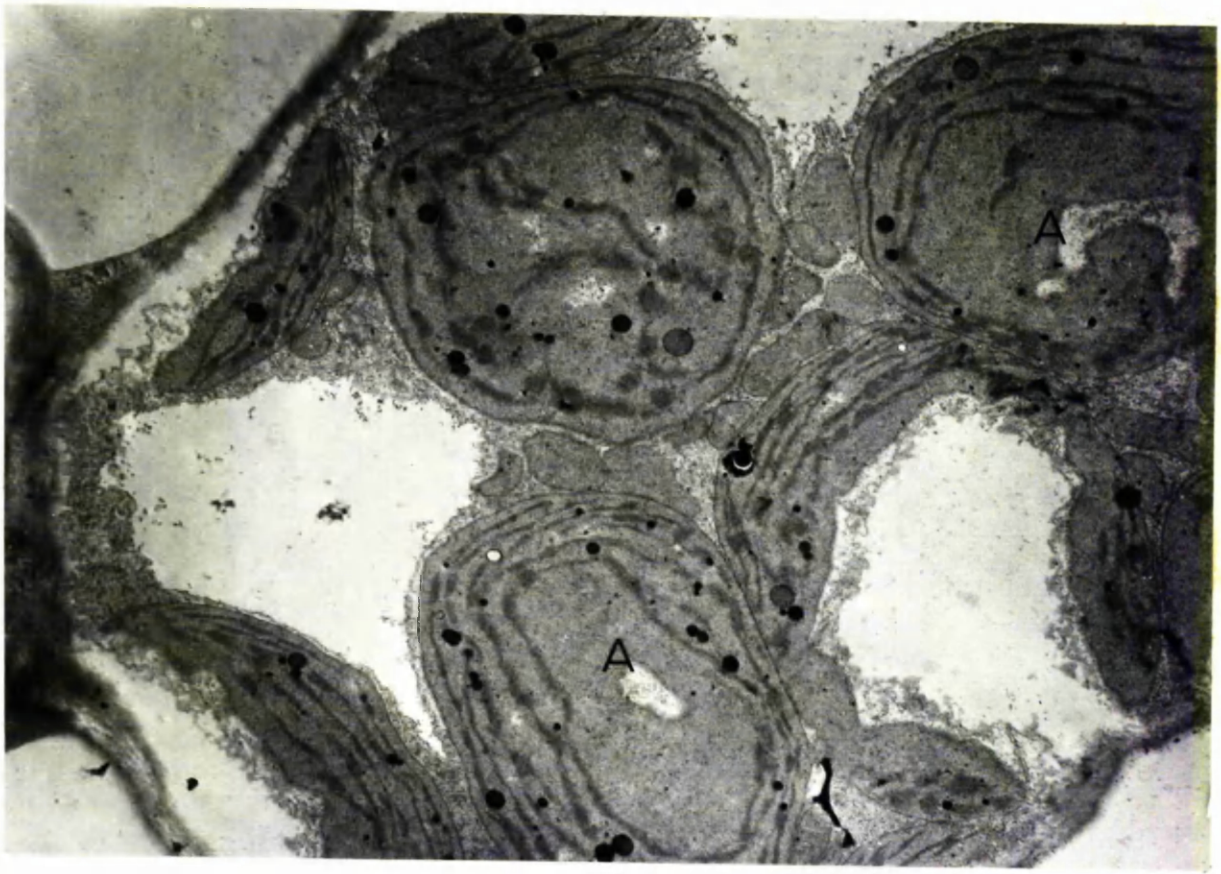

A
A

Plate 9. Radish leaf tissue aged for 1 day in water (K)

x 10,000

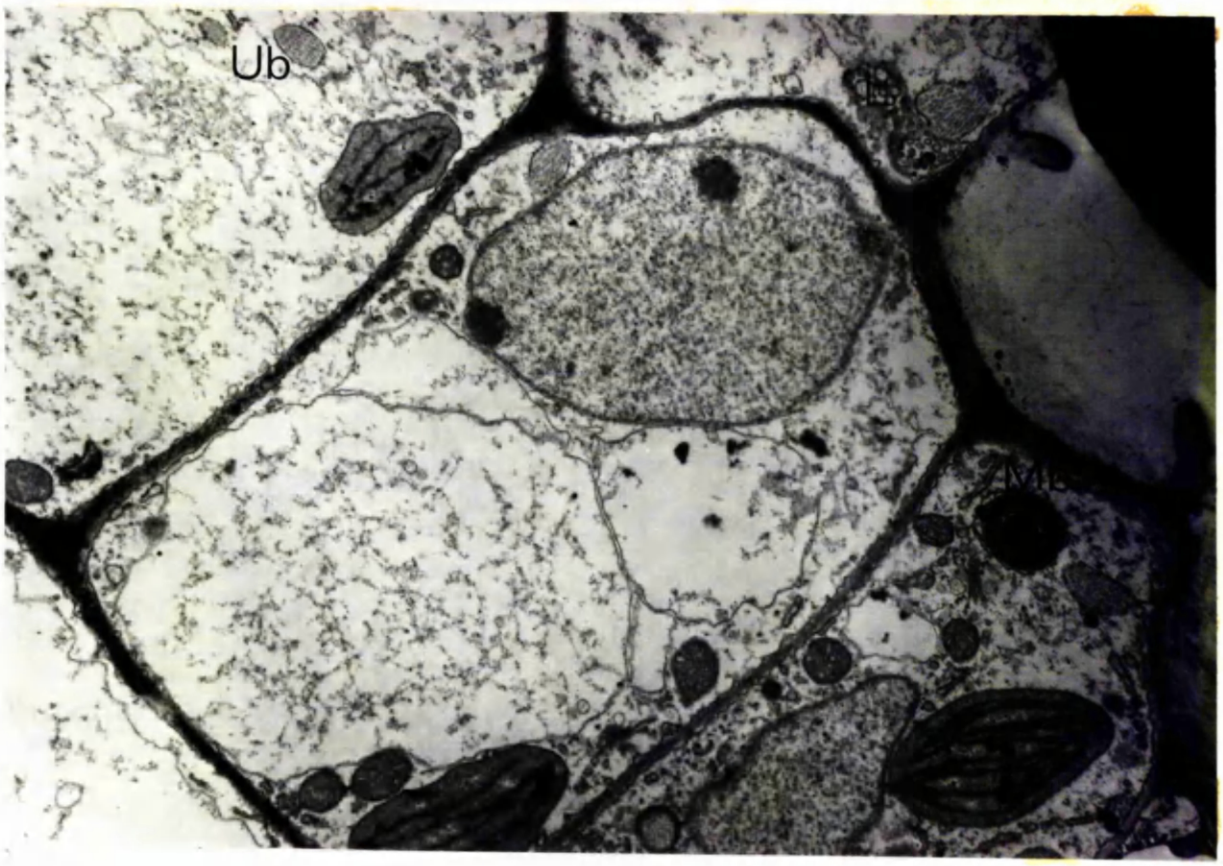
L = lomasome

Mb = myelin-like body

Ub = unidentified body

Plate 10. Radish leaf tissue aged for 1 day in water (K)

x 10,000


Ub

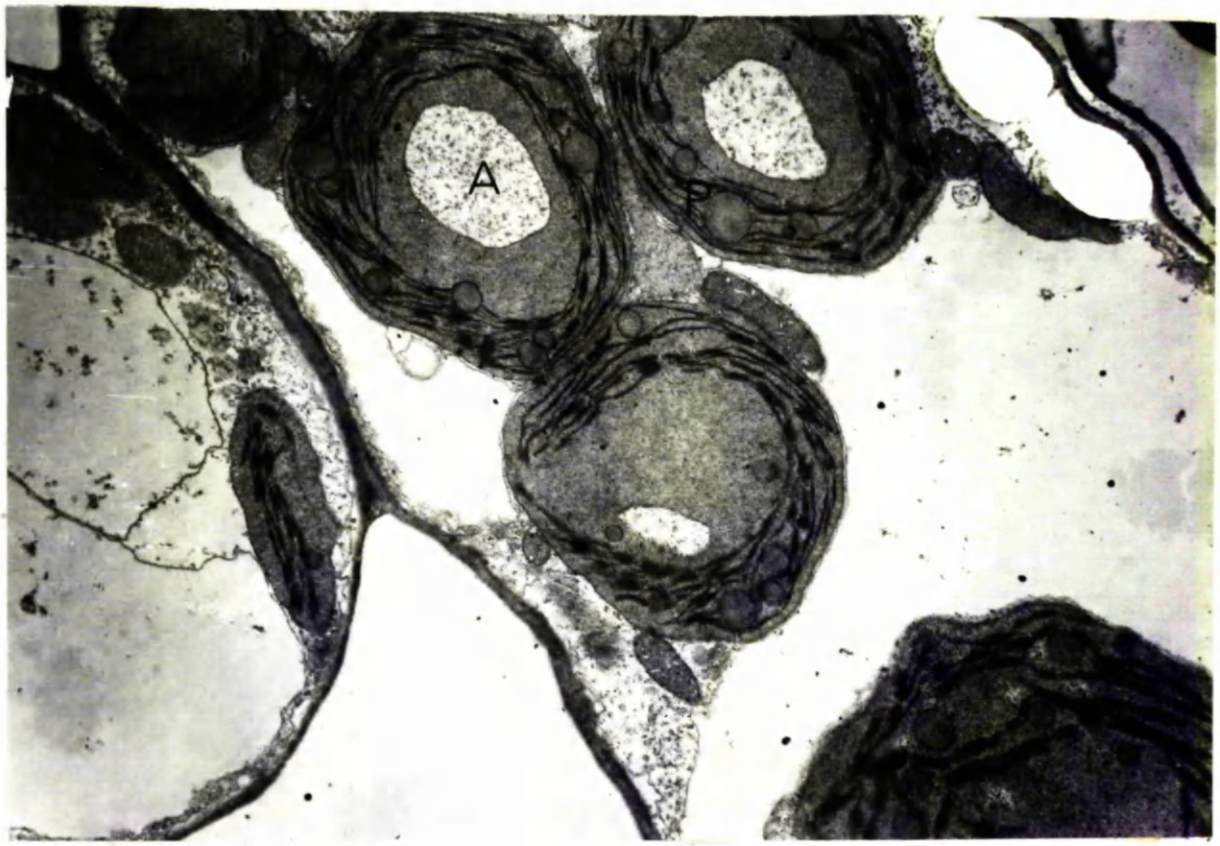

A

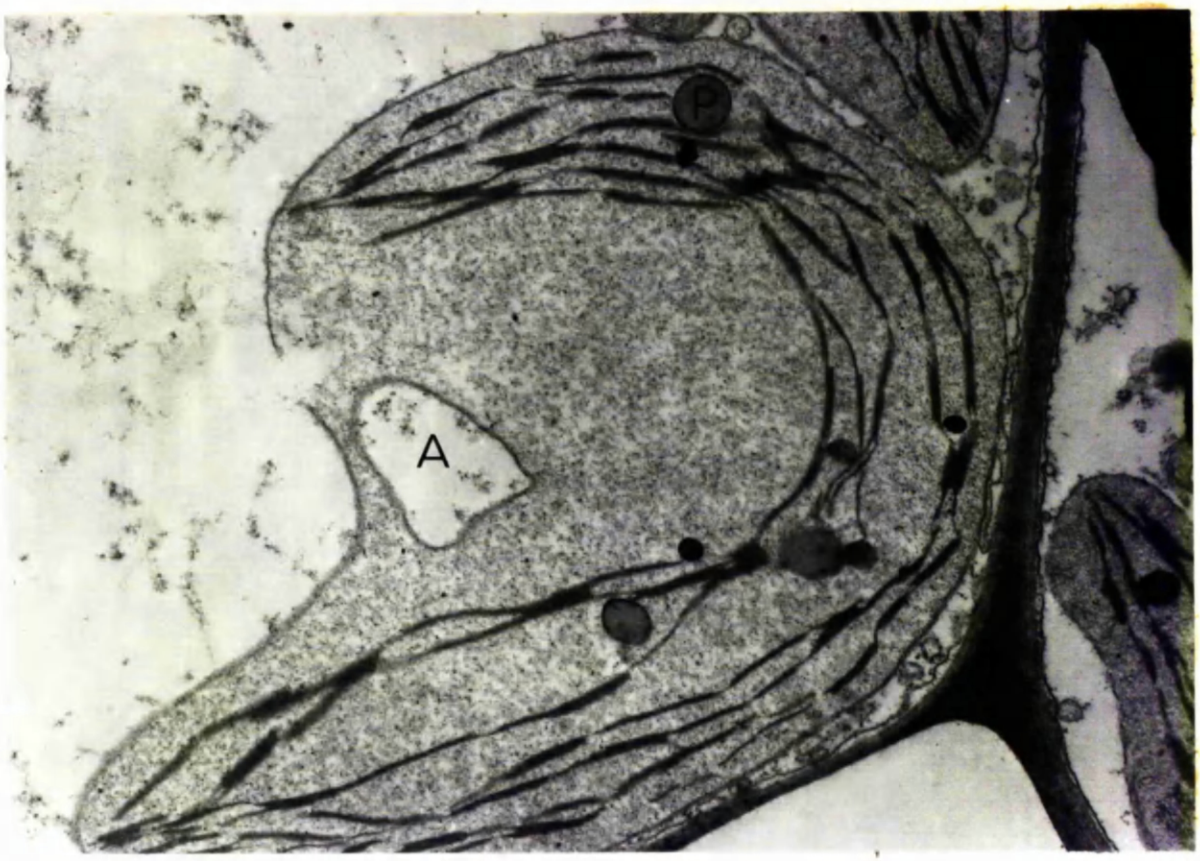
Plate 11. Radish leaf tissue aged for 1 day in water (K)

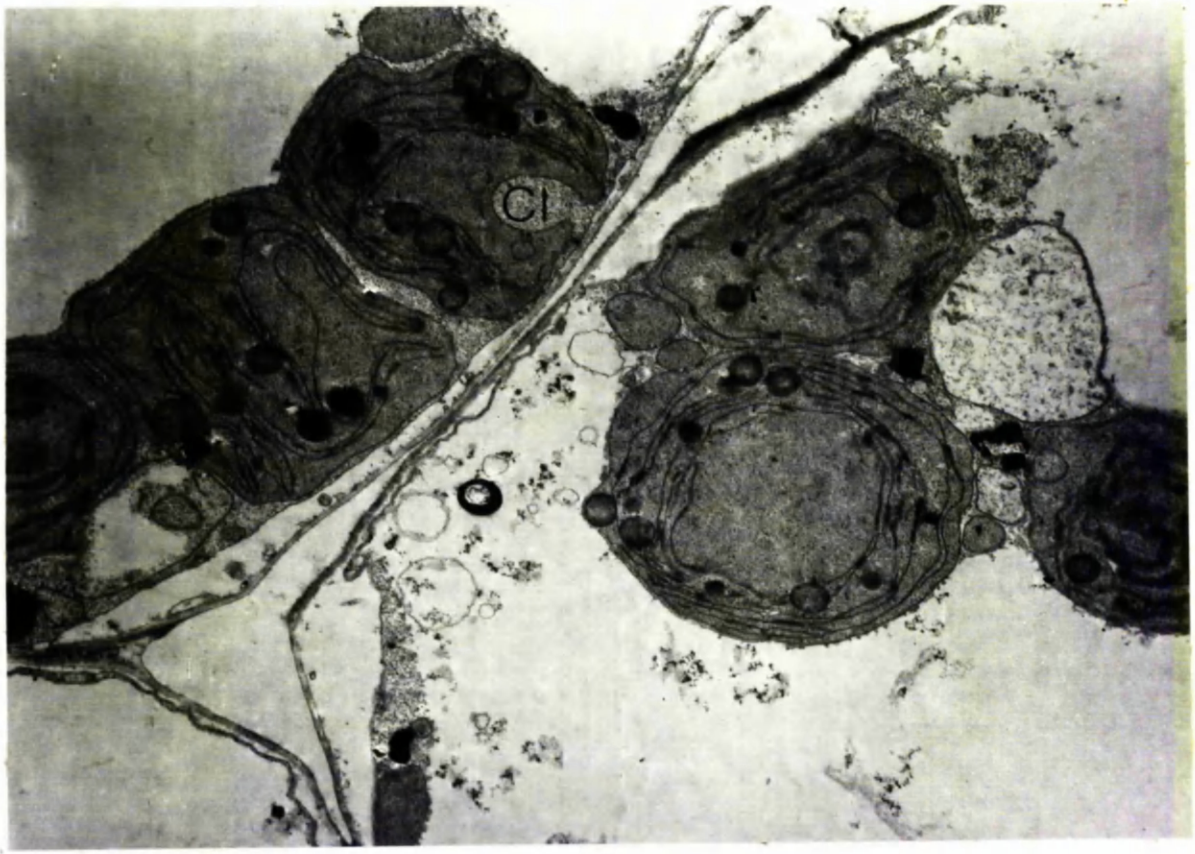
x 20,000

Plate 12. Radish leaf tissue aged for 1 day in ABA (K)

x 10,000

CI = chloroplastic invagination


P
A


Cl

larger than those noted in water-aged tissue (Table 42). ABA tended to reduce the thylakoid structure of the chloroplasts in general and the granal structure in particular. In addition, the orientation of the thylakoids frequently appeared to bear little relation to the contours of the chloroplast envelope. The structure and frequency of the mitochondria were apparently unaffected by ABA treatment. Invaginations, both of the chloroplast envelope and of the plasmalemma, were occasionally observed (Plates 12, 14). As described above, there was some variation in the degree of senescence of individual cells; in some cases, the chloroplast envelope had been lost and the remaining thylakoids appeared to be swollen (Plate 15).

The density of staining of the plastoglobuli appeared to vary, even within the same chloroplast (Plate 12).

(d) Tissue aged in water for 2 days

In some respects, the sections examined from tissue aged for 2 days in water appeared to be less senescent than tissue aged for only 1 day in water. There was little apparent deformation of the chloroplasts, or of the thylakoids which generally remained parallel to the long axis of the chloroplasts (Plates 16, 17, 18); in addition, the plastoglobuli tended to be less prominent and smaller than in tissue aged for 1 day (Table 42). On the other hand, invaginations of the plasmalemma were observed (Plate 19); these frequently contained small vesicles and thus may correspond to structures designated as lomasomes. Moreover, the membrane-bound, low-density areas, previously observed within the chloroplasts, were often present and appeared to contain structures resembling mitochondria (Plate 16). The structure of the mitochondria was otherwise unaffected. In addition, electron-dense, lipid-like globules were present in the cytoplasm of some cells (Plate 17). The nuclei and plasmalemma were apparently intact (Plate 18). Isolated pieces of Golgi bodies were detectable and irregular membranous structures were present which may represent the early stages in the formation of myelin-like bodies (Plate 17).

(e) Tissue aged in ABA for 2 days

In leaf discs treated with ABA for 2 days, 2 alternative patterns of senescence were detected. In some cells, the chloroplast envelope remained intact, although the thylakoids were in various stages of disorganization (Plate 20). Some grana were present and the plastoglobuli were prominent, if somewhat smaller than those observed at 1 day (Table 42). Nuclei with possible nucleolar areas and mitochondria with detectable

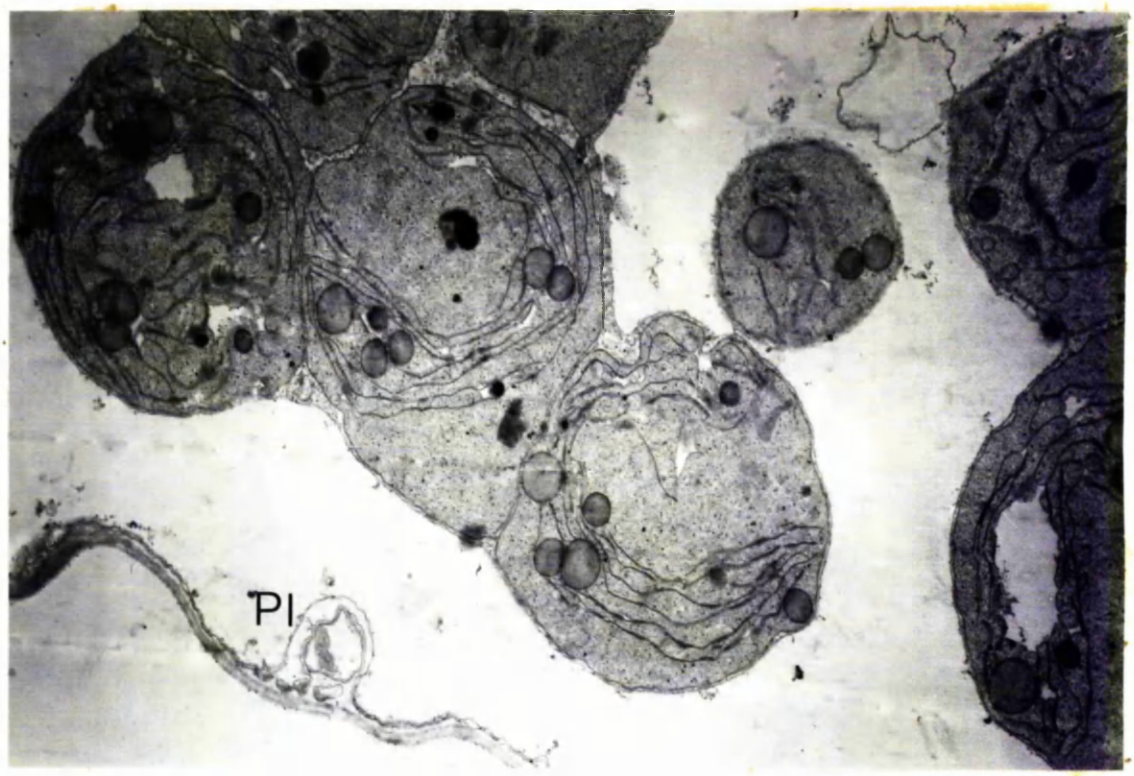
Plate 13. Radish leaf tissue aged for 1 day in ABA (K)

x 10,000

PI = plasmalemma invagination

Plate 14. Radish leaf tissue aged for 1 day in ABA (K)

x 20,000


Pl

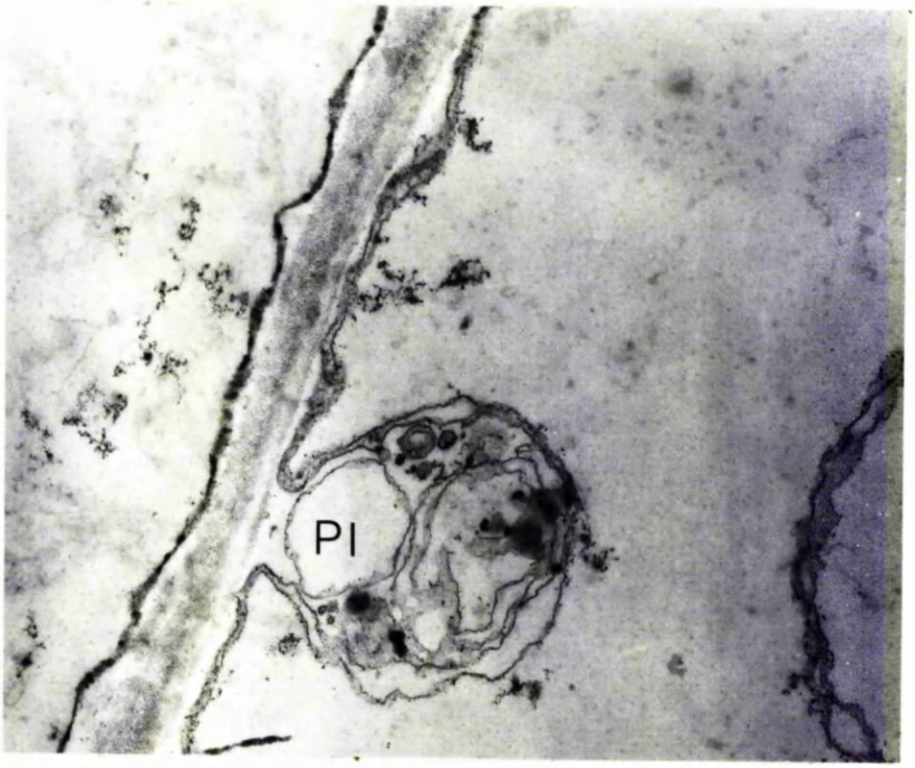
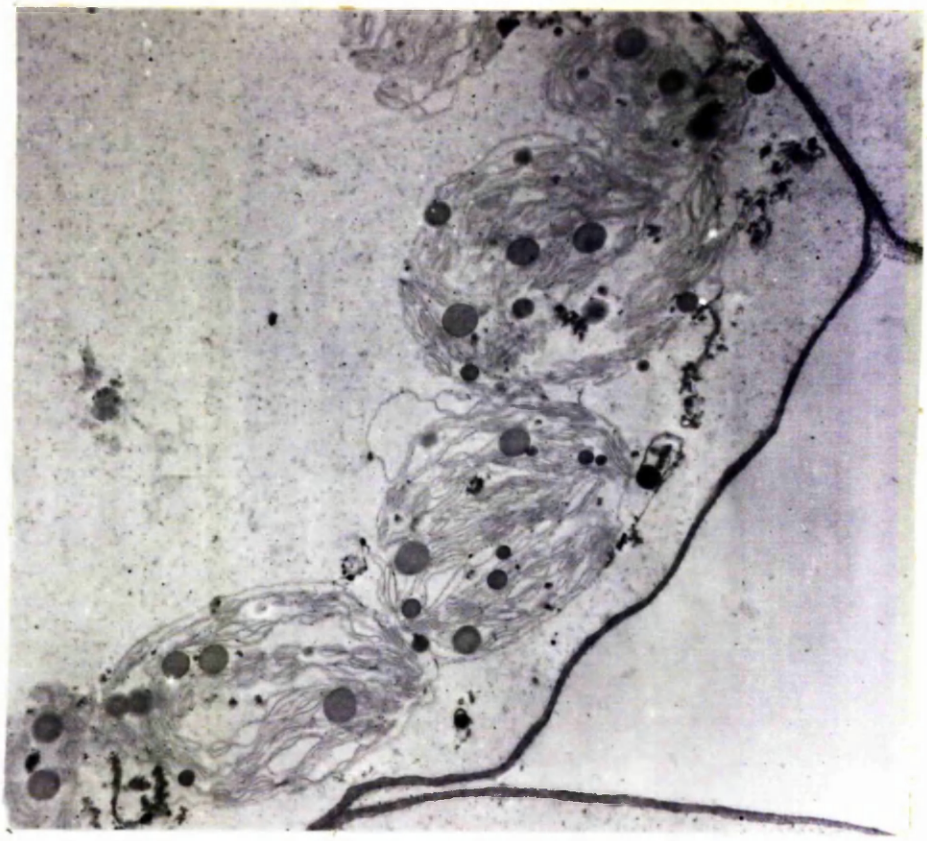

Pl

Plate 15. Radish leaf tissue aged for 1 day in ABA (K)

x 10,000

Plate 16. Radish leaf tissue aged for 2 days in water (K)

x 10,000



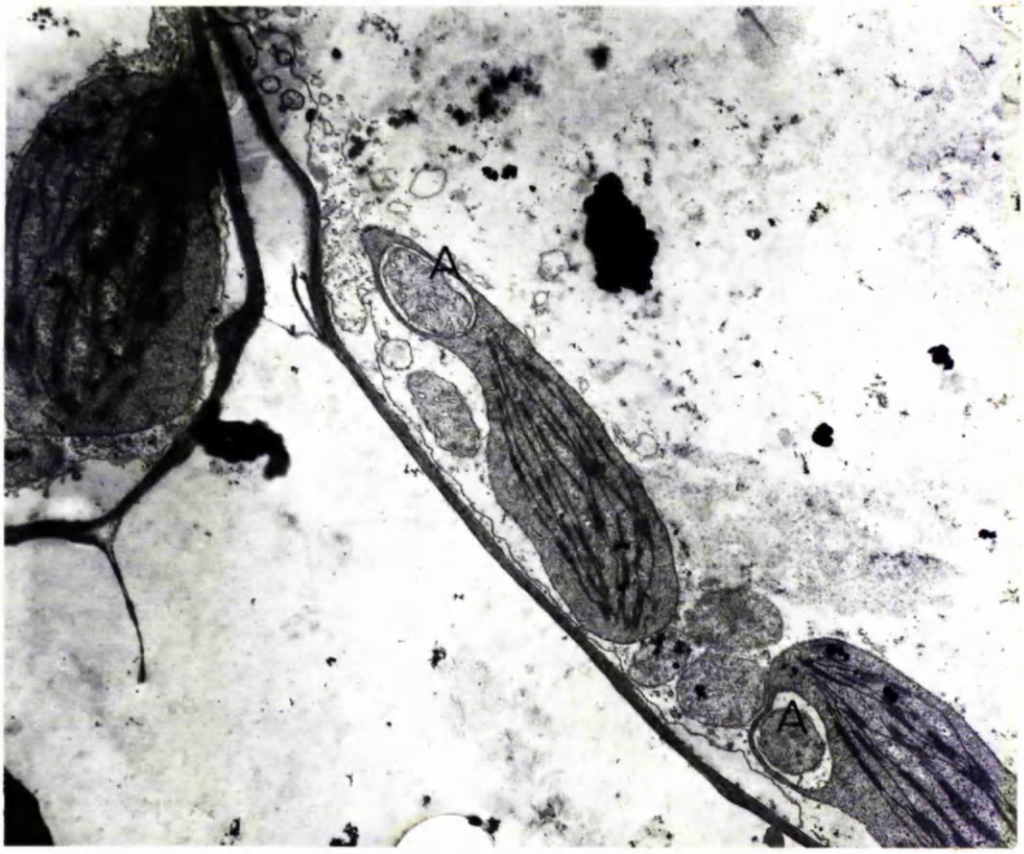

A
A

Plate 17. Radish leaf tissue aged for 2 days in water (K)

x 20,000

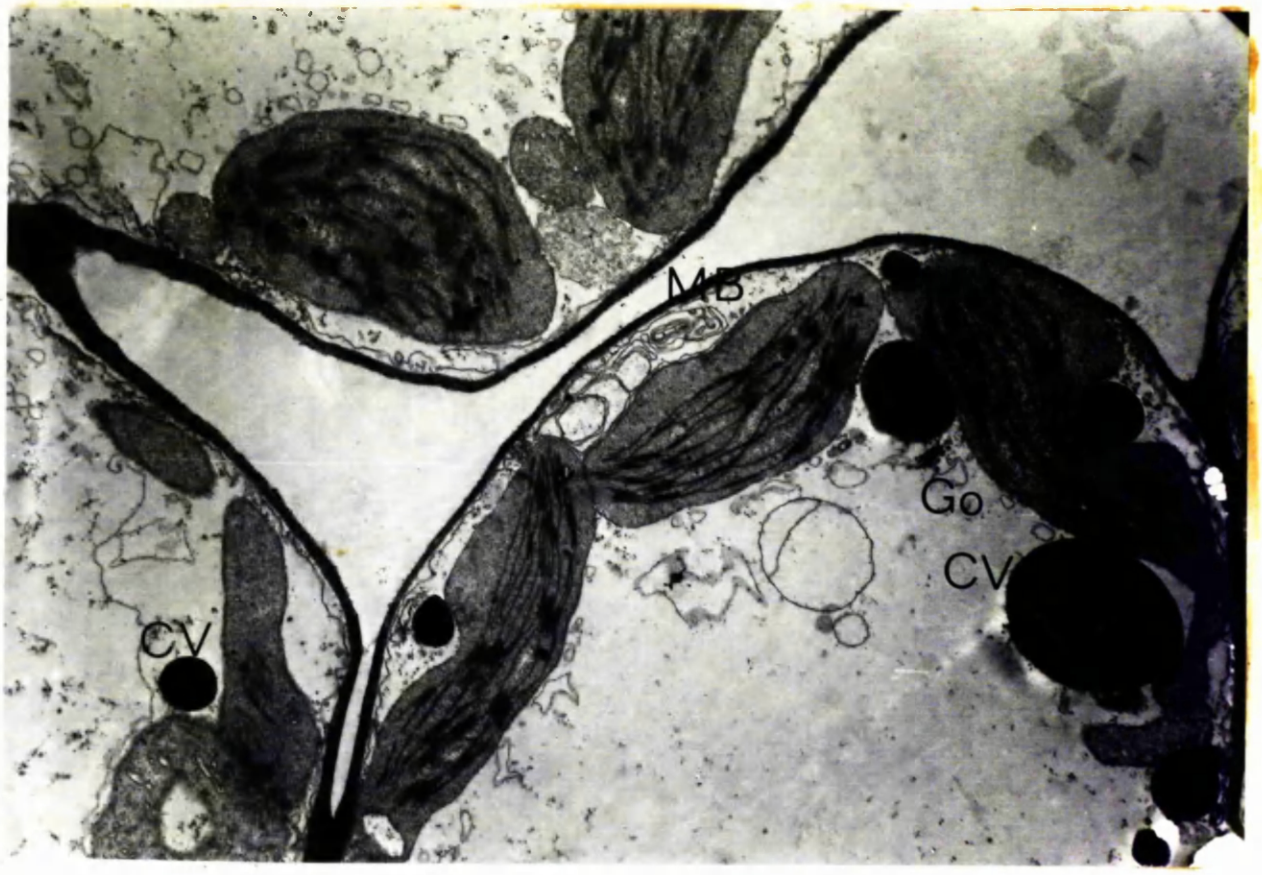
CV = cytoplasmic vesicle

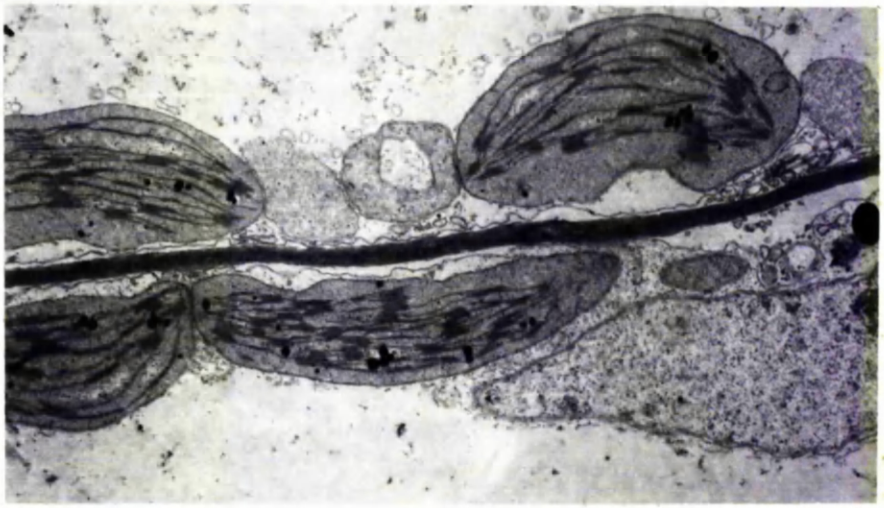
Plate 18. Radish leaf tissue aged for 2 days in water (K)

x 10,000

Plate 19. Radish leaf tissue aged for 2 days in water (G)

x 15,000


MB
Go
CV
CV



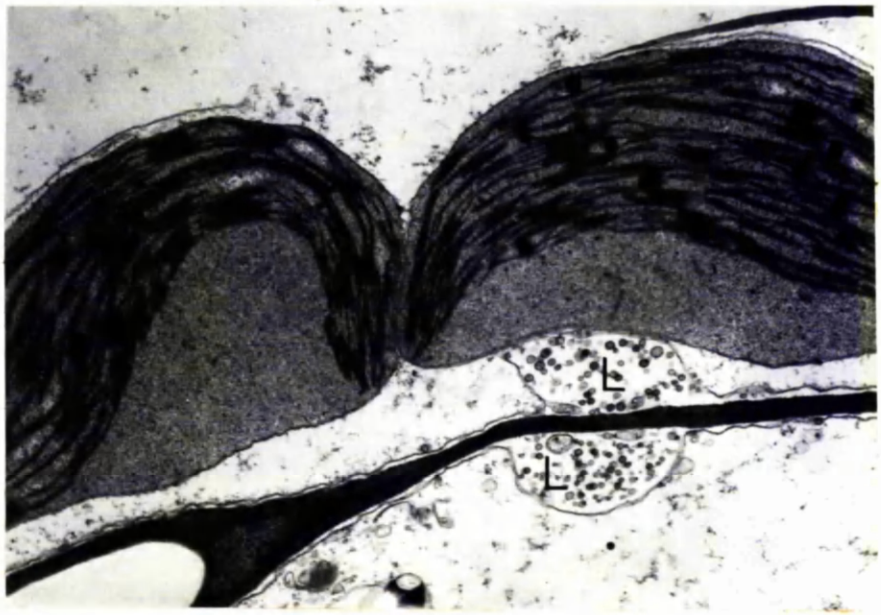
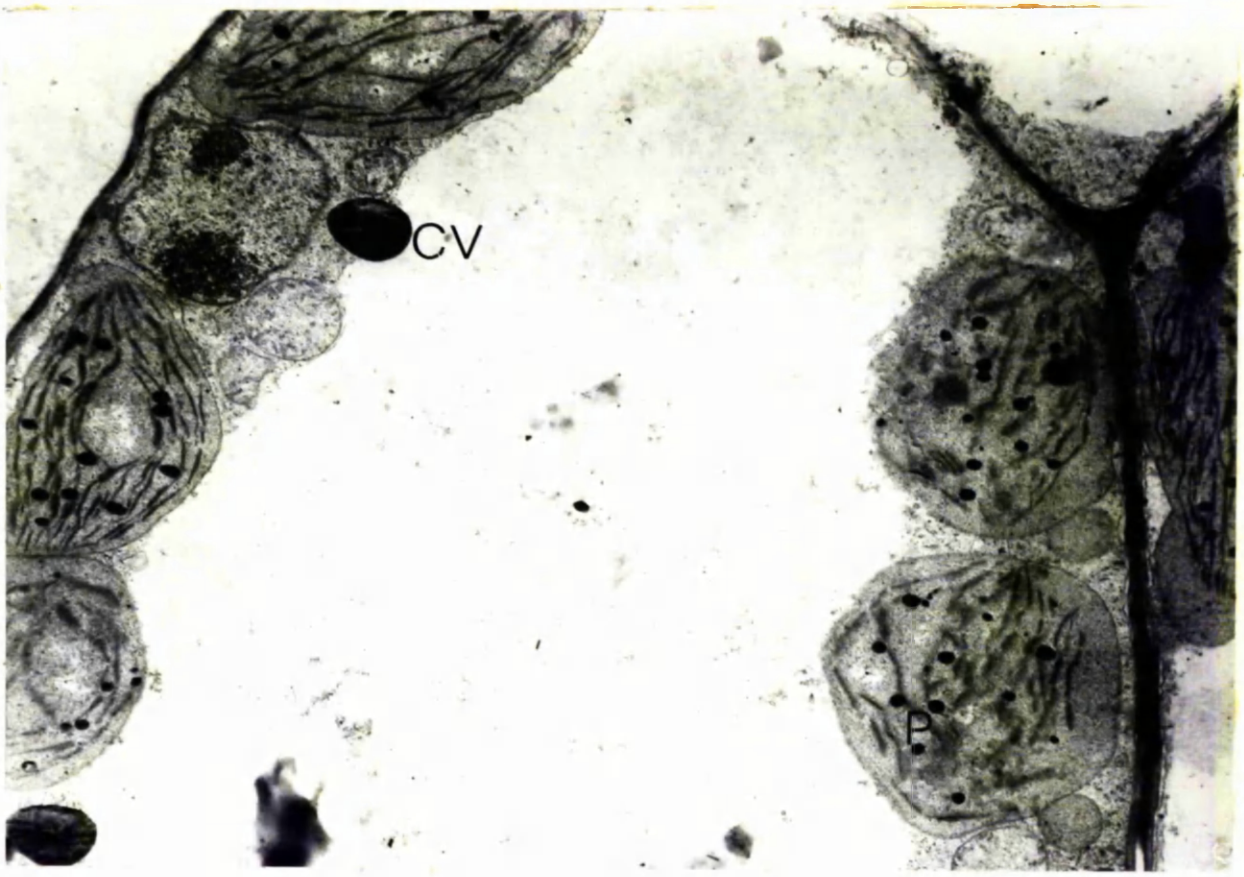

L
L

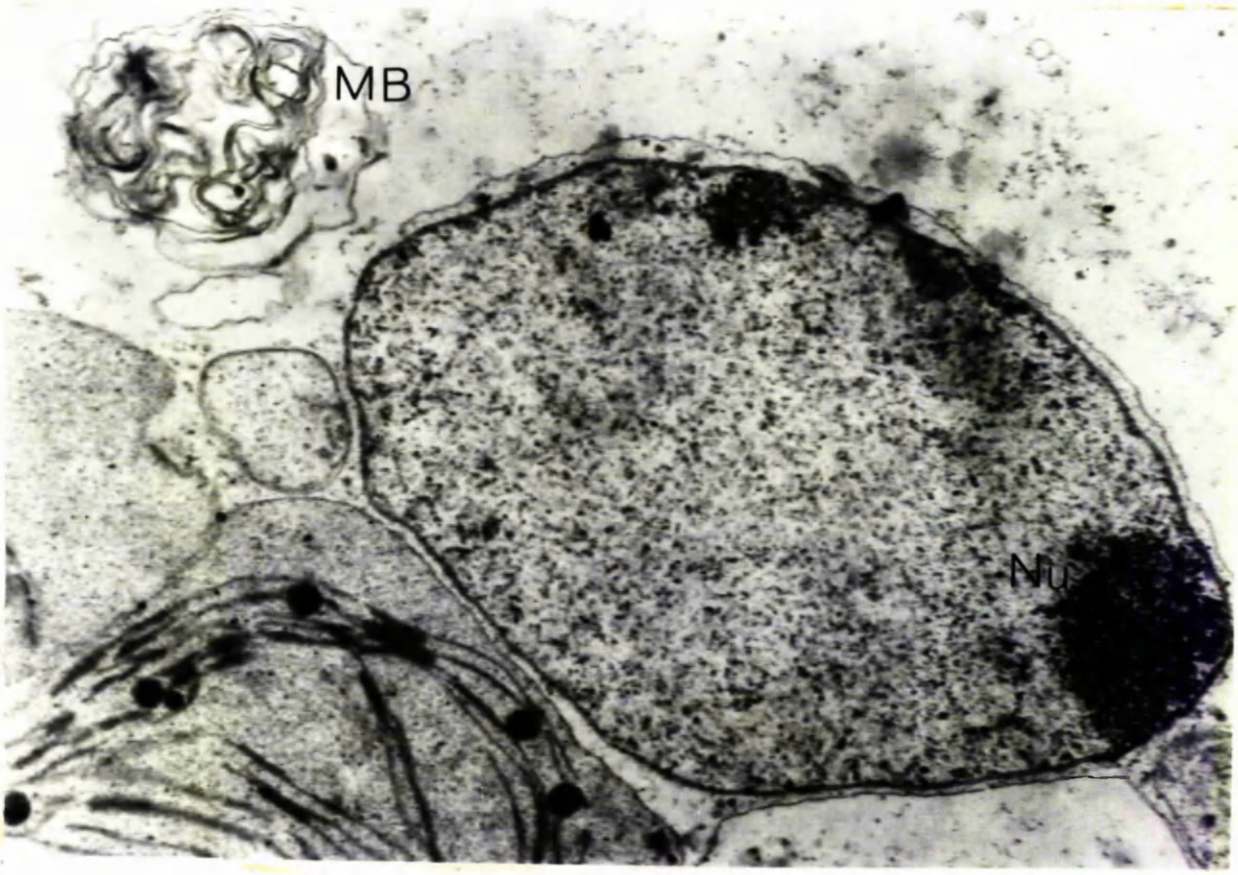
Plate 20. Radish leaf tissue aged for 2 days in ABA (G)

x 10,000

Plate 21. Radish leaf tissue aged for 2 days in ABA (G)

x 20,000


CV
P


MB
Nu

internal cristae were discernible (Plates 20, 21). As in tissue aged for 2 days in water, lipid-like, cytoplasmic globules (Plate 20) and irregular membranous structures were also present (Plate 21). In the second pattern of senescence, the chloroplast envelope was absent, although the thylakoids were swollen in the presumptive granal areas and remained close together (Plates 22, 23). The plasmalemma and tonoplast were not persistent in these cells and the remaining cellular structures, including disintegrating mitochondria, appeared to be surrounded by a matrix of low electron-density (Plates 22, 23). The advanced state of cellular disintegration in this tissue may be related to the occasional presence of bacteria in the inter-cellular spaces (Plate 23).

(f) Tissue aged in water for 6 days

After 6 days of ageing in water, the thylakoids, if still present, were completely disoriented in most cells (Plates 24, 25). The plastoglobuli tended to be more prominent than at earlier stages of ageing, although there was some variation in the density of staining of the globules (Plates 25, 26). The chloroplast envelope generally remained intact. Nuclei, with prominent densely staining areas and intact nuclear envelopes, mitochondria with detectable cristae and the plasmalemma were all still evident at this stage. Myelin-like bodies were also present (Plate 26). Some cells, however, retained chloroplasts very similar to those observed in freshly excised tissue, i.e. the chloroplasts were elliptical with their long axes and thylakoids parallel to the plasmalemma. The chloroplasts of these cells also still possessed definite granal structures and had few prominent plastoglobuli (Plate 27).

(g) Tissue aged in ABA for 6 days

Leaf discs aged in ABA for 6 days were in an advanced state of degeneration. The plasmalemma and tonoplast were no longer present and the remains of the chloroplasts were distributed throughout the cellular matrix, away from their normal peripheral position in the cell (Plate 28). The chloroplast envelope was also absent, although the swollen thylakoids remained together as membranous masses. There were a large number of densely staining globules present, mainly localised in the area of the decaying thylakoids. These globules probably correspond to plastoglobuli or possibly to cytoplasmic lipid-like bodies or to a mixture of both. No nuclei or mitochondria could be detected. Cells of the smaller type described above (from vascular tissue) were in a similar state of disintegration.

Plate 22. Radish leaf tissue aged for 2 days in ABA (K)

x 20,000

CM = cytoplasmic matrix

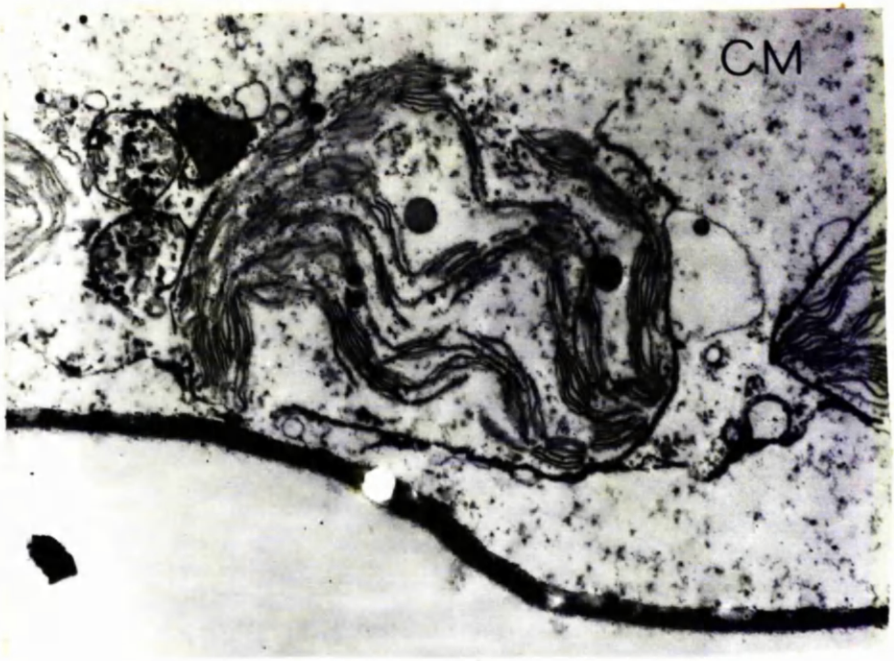
Plate 23. Radish leaf tissue aged for 2 days in ABA (K)

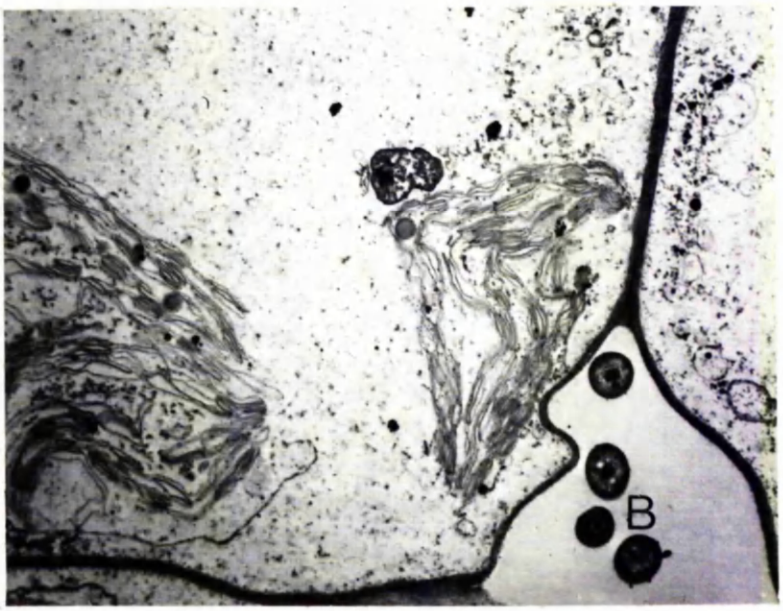
x 10,000

B = bacteria

Plate 24. Radish leaf tissue aged for 6 days in water (G)

x 10,000


CM


B

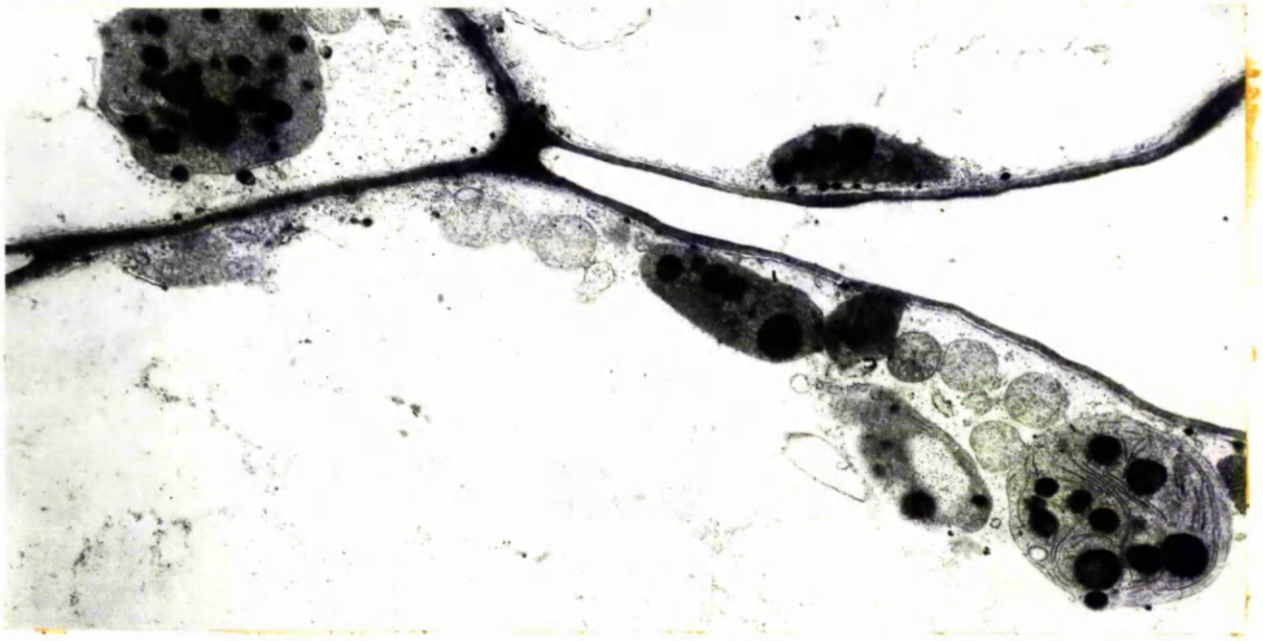
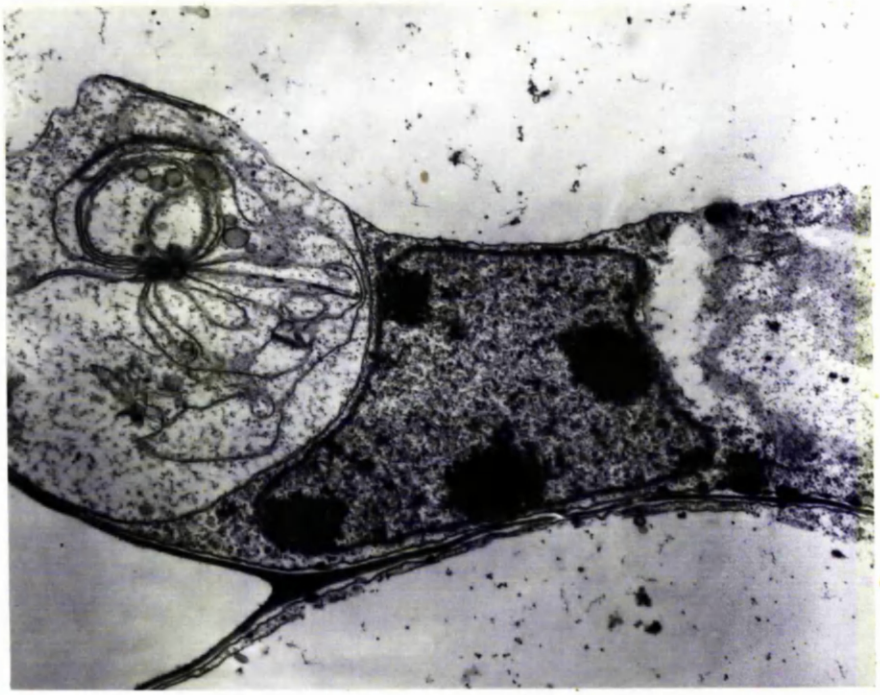


Plate 25. Radish leaf tissue aged for 6 days in water (K)

x 15,000

Plate 26. Radish leaf tissue aged for 6 days in water (G)

x 10,000



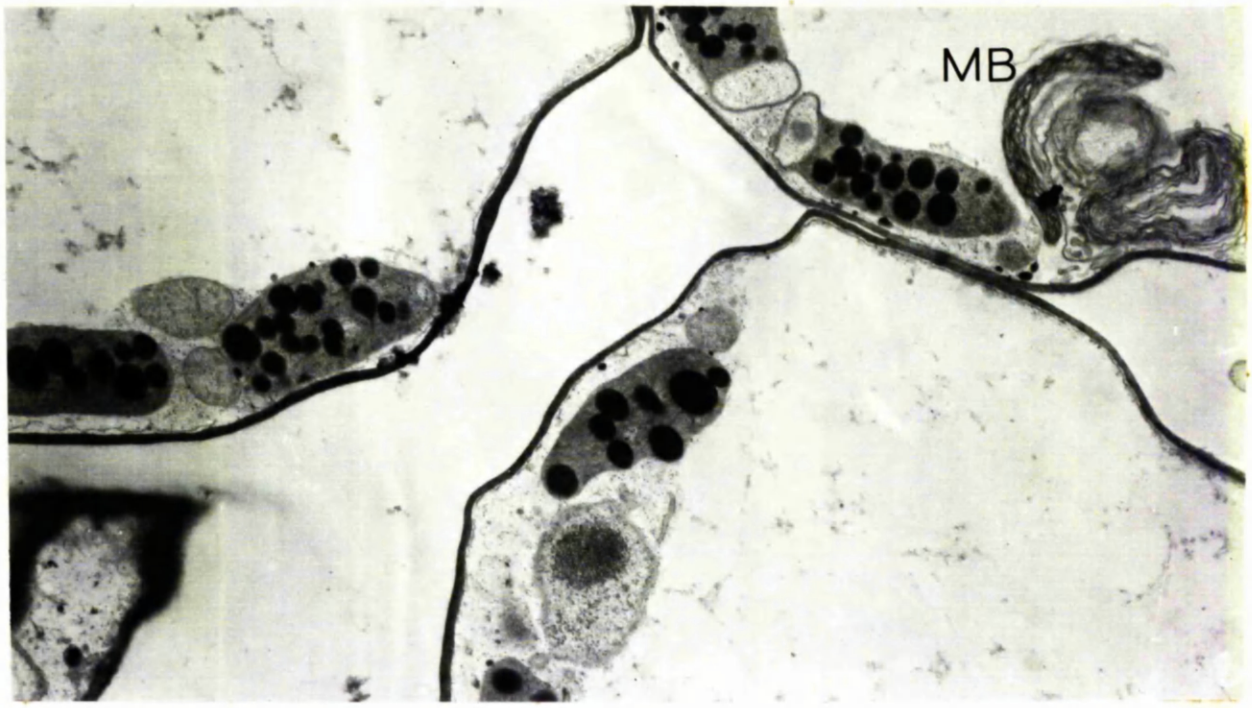

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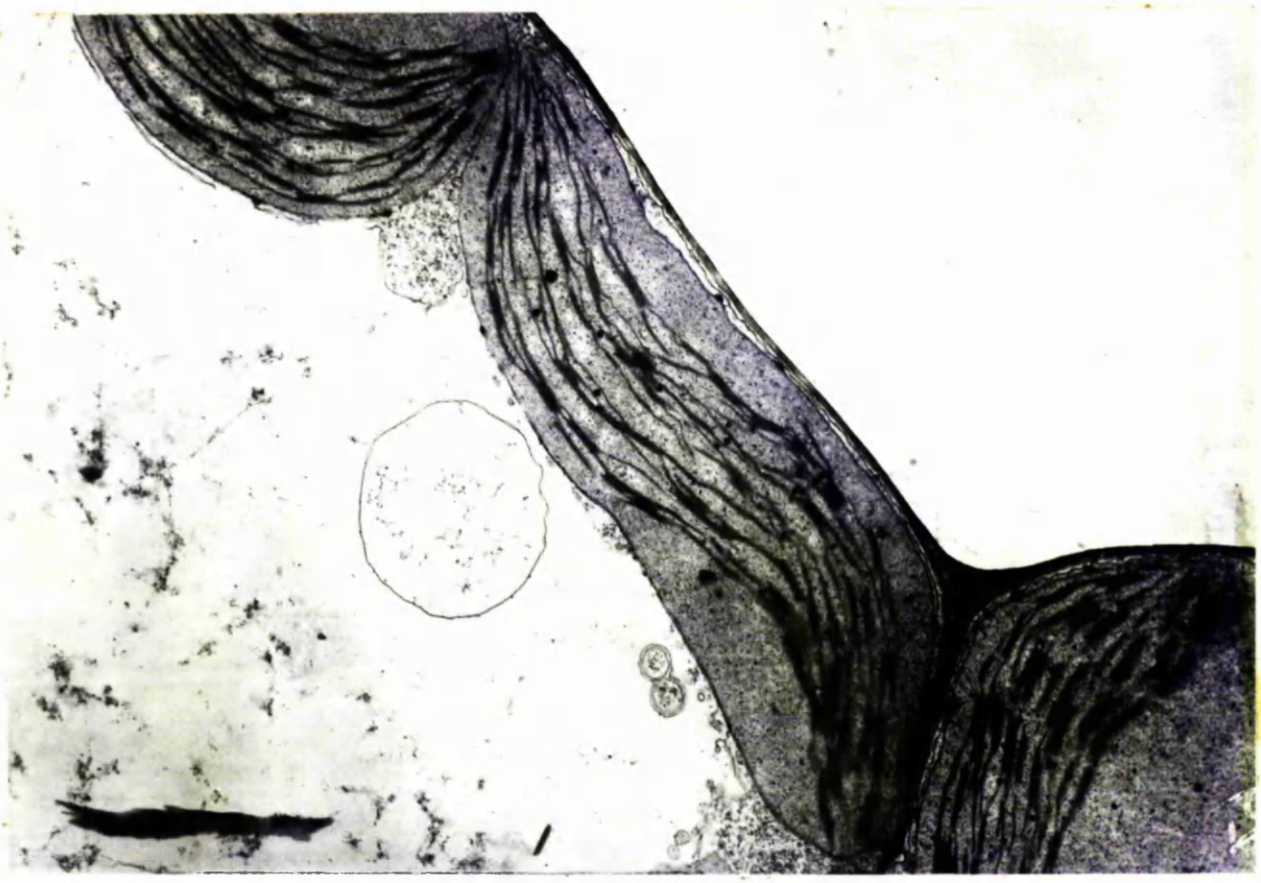
Plate 27. Radish leaf tissue aged for 6 days in water (G)

x 15,000

Plate 28. Radish leaf tissue aged for 6 days in ABA (G)

x 10,000



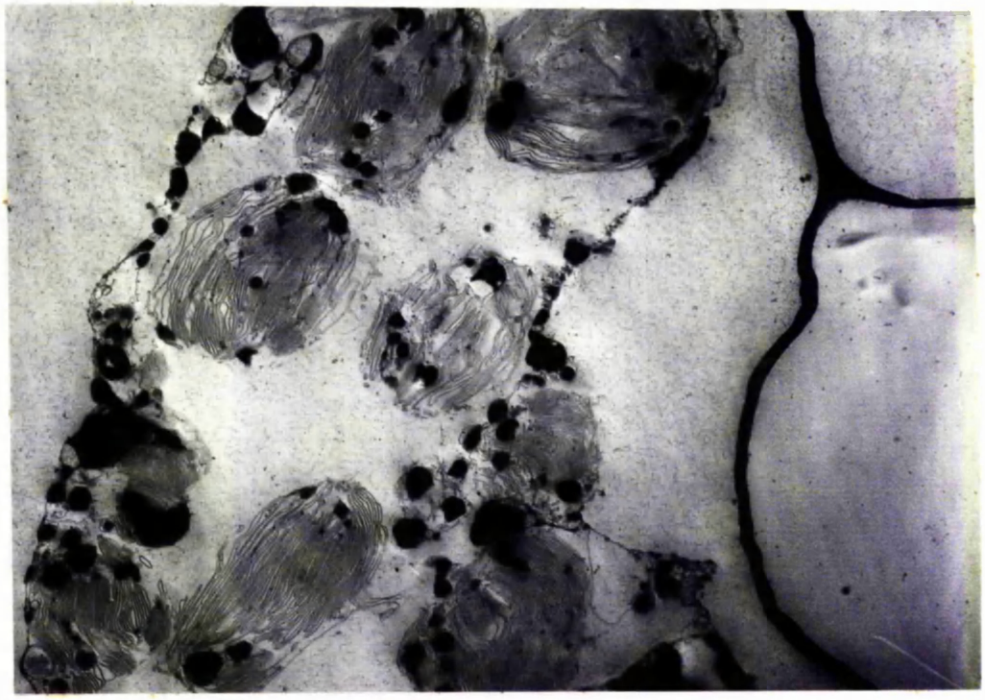
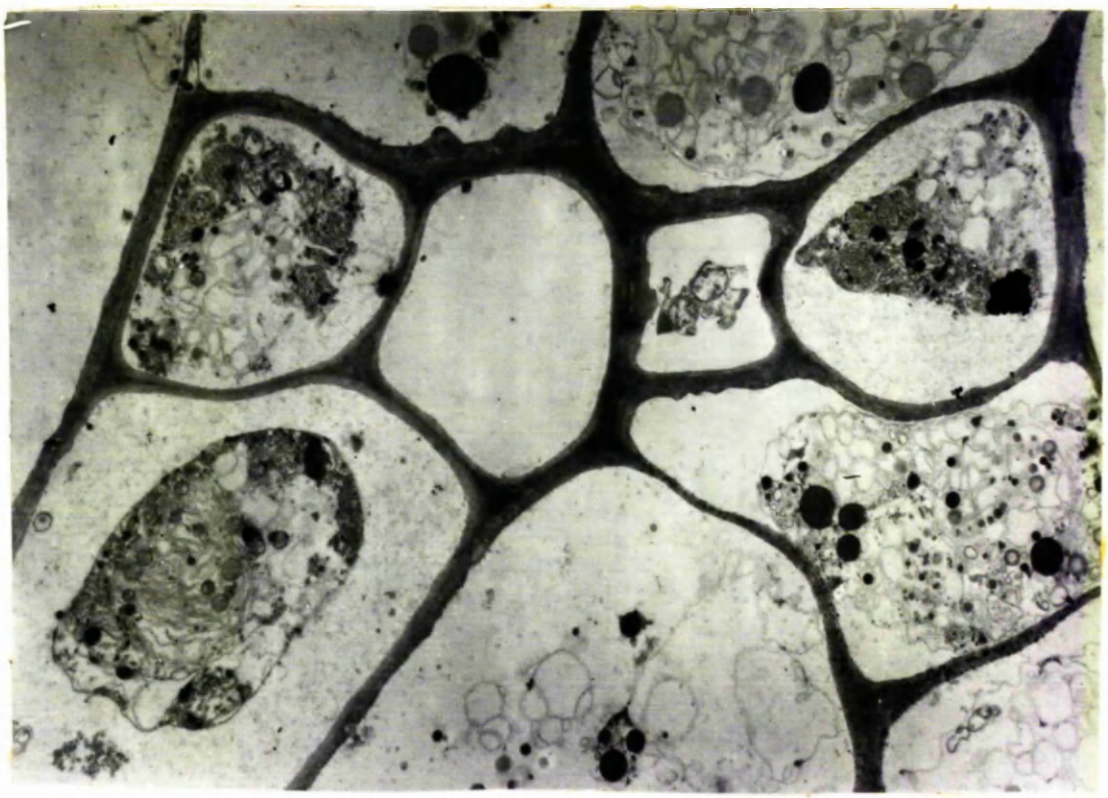


Plate 29. Radish leaf tissue aged for 6 days in ABA (K)

x 10,000

Plate 30. Radish leaf tissue aged for 12 days in water (G)

x 15,000



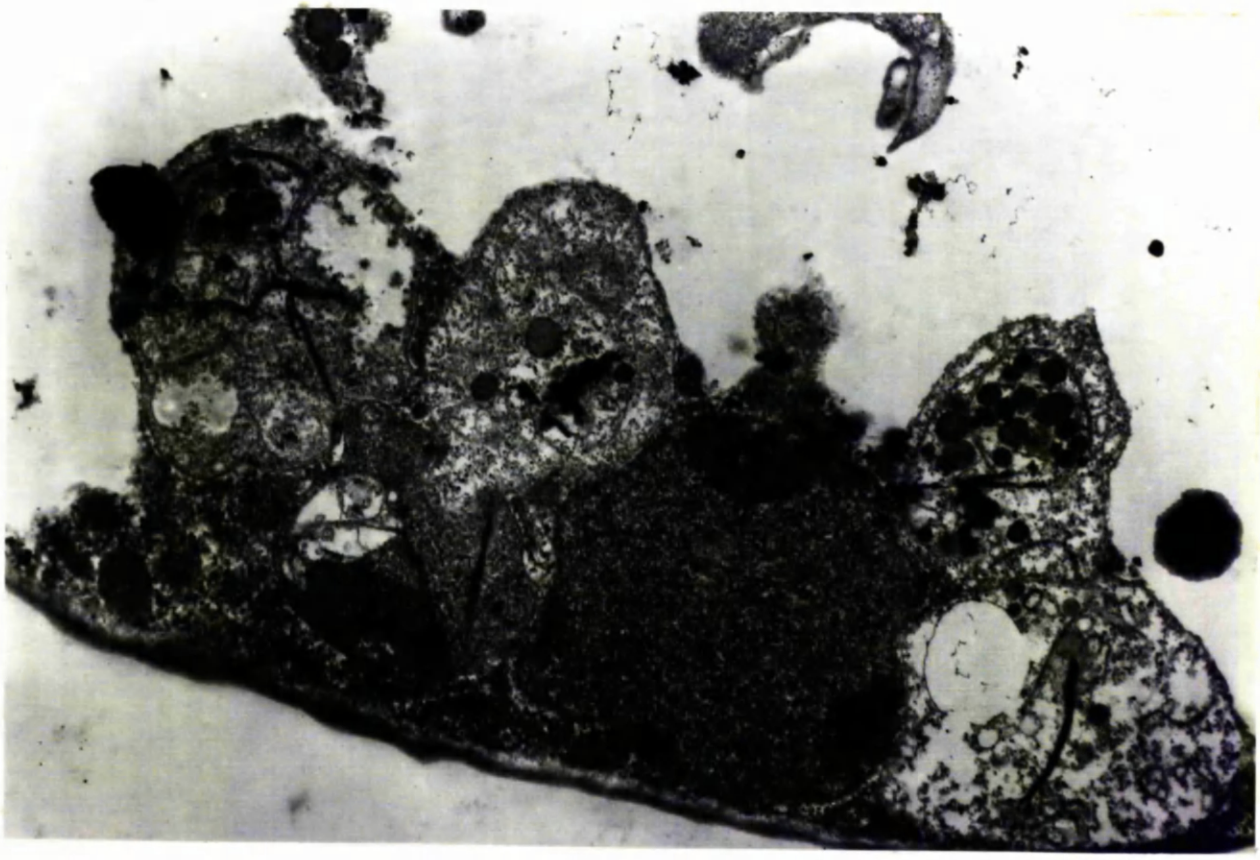


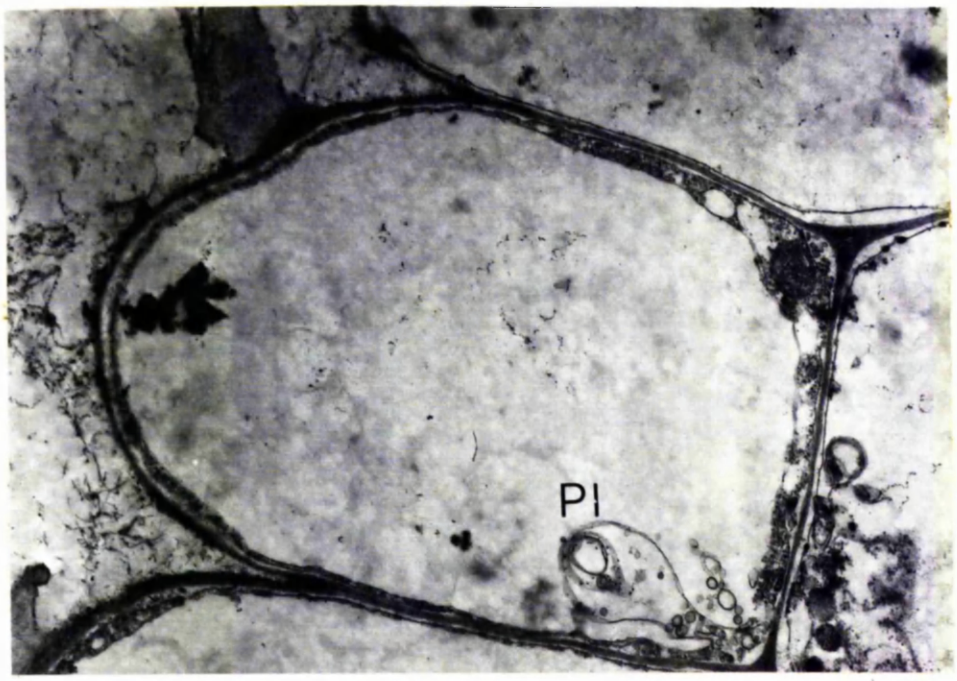
Plate 31. Radish leaf tissue aged for 12 days in water (G)

x 15,000

Plate 32. Radish leaf tissue aged for 12 days in water (G)

x 15,000

DC = disintegrating chloroplasts


PI

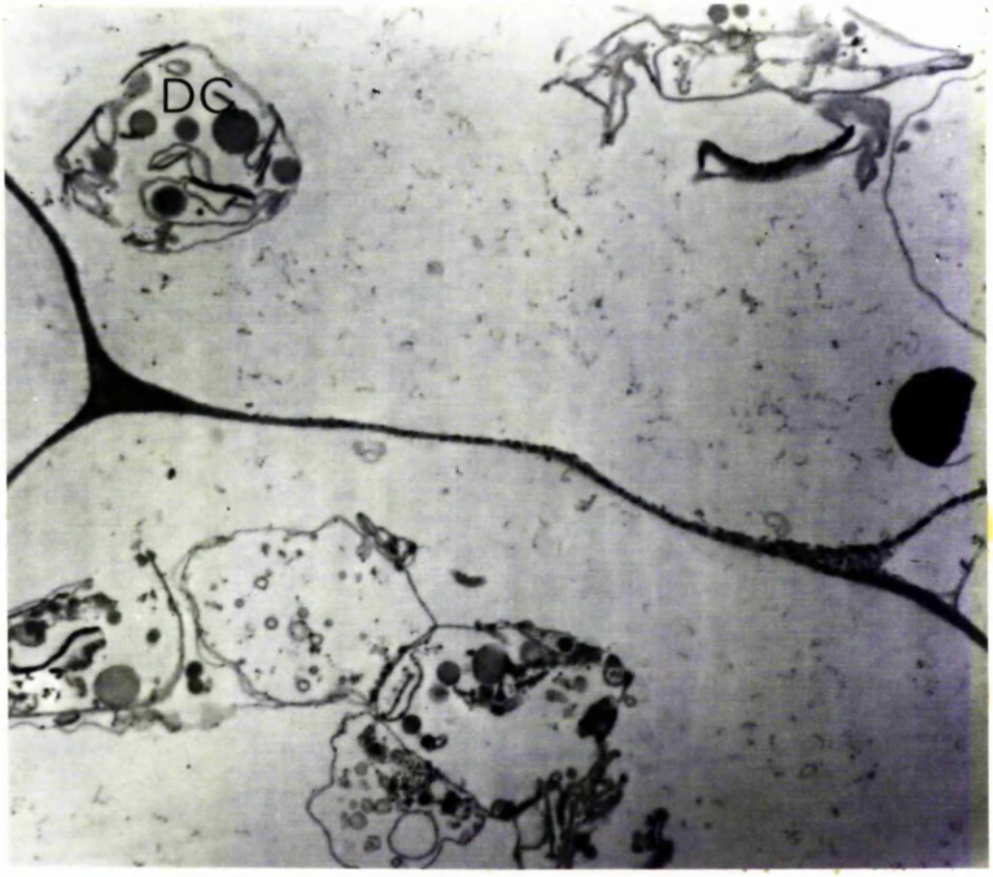
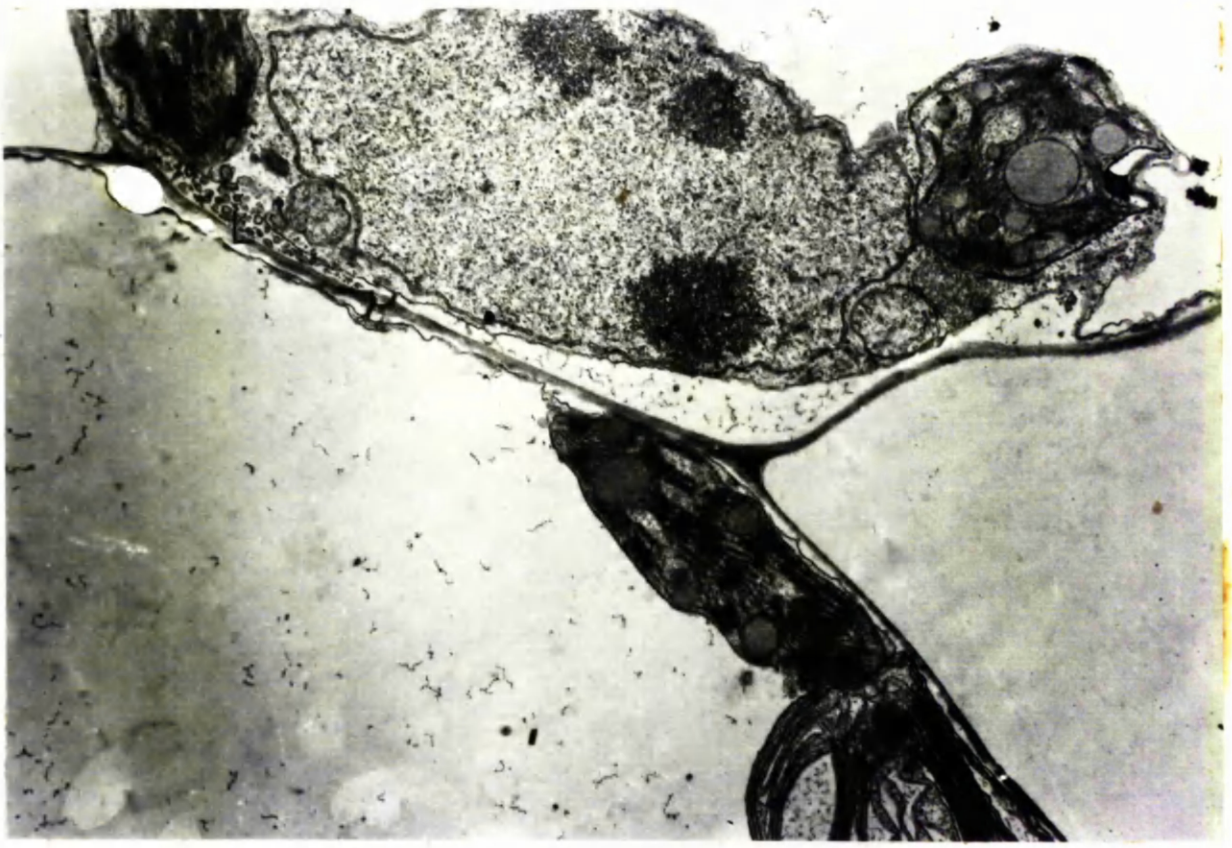

DG

Plate 33. Naturally senescent radish leaf tissue (G)

x 15,000

Plate 34. Naturally senescent radish leaf tissue (G)

x 15,000



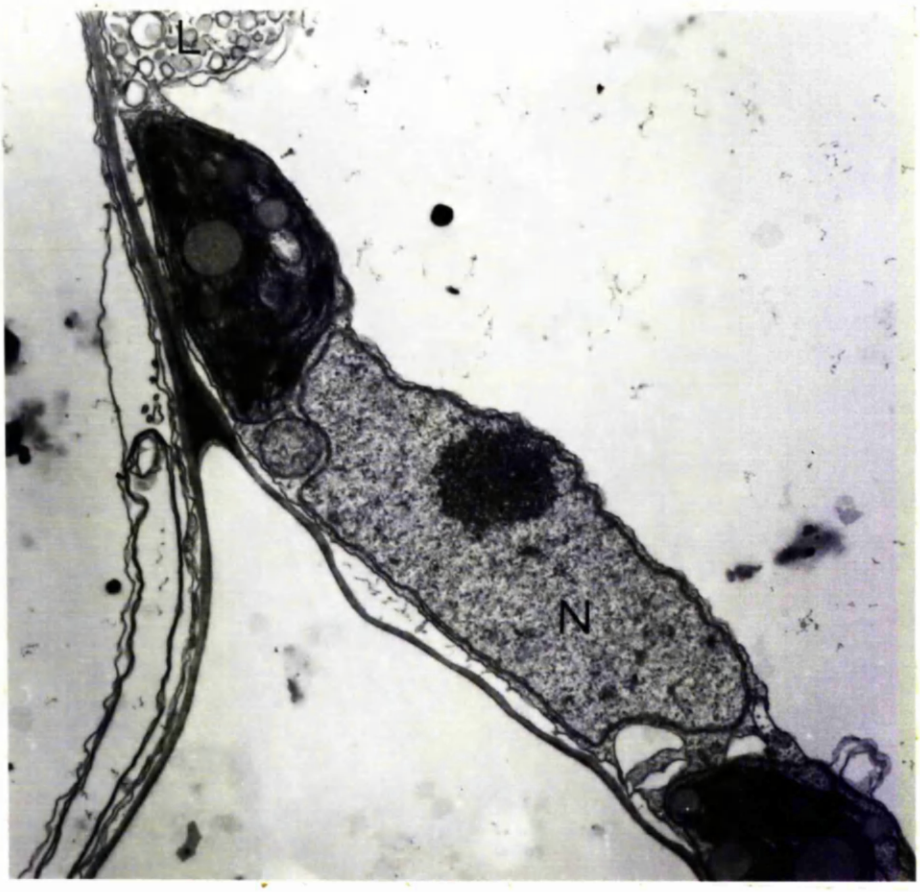
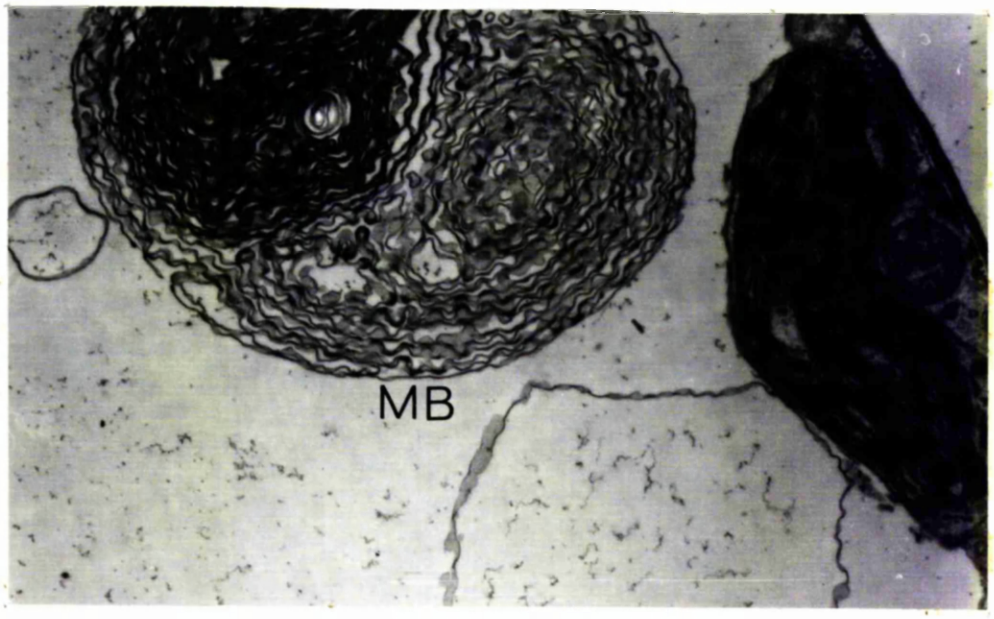

L
N

Plate 35. Naturally senescent radish leaf tissue (G)

x 15,000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(h) Tissue aged in water for 12 days

After 12 days of ageing in water, all cells were degenerating; there was some variation, however, in the degree and type of degeneration. Some cells had retained the tonoplast and plasmalemma and, in these cells, nuclei and mitochondria could be detected (Plates 30, 31), together with chloroplasts in an advanced state of disintegration, and associated plastoglobuli. In other cells, the plasmalemma and tonoplast were not persistent and disintegrating chloroplasts were evident in the matrix, resulting from vacuolar rupture (Plate 32).

(i) Naturally senescent tissue

Cells from naturally senescent, yellow leaf material apparently retained a high degree of membrane integrity. The plasmalemma and tonoplast were still present (Plates 33, 34, 35) and the peripheral layer of cytoplasm contained intact nuclei, with densely staining areas and mitochondria, with discernible cristae (Plates 33, 34). Chloroplast envelopes were detectable and the thylakoid system retained many granal areas. Signs of senescence, however, were evidenced by the presence of large plastoglobuli; some chloroplasts were also somewhat deformed from the more normal elliptical shape (Plate 33). A separation between the plasmalemma and the cell wall was evident in some cells and lomasome-like structures were frequently observed in the resulting gap. In an isolated case, a large and complex myelin-like body was present (Plate 35).

DISCUSSION

In stimulating the senescence of detached leaf tissue, ABA has been shown to hasten the decline of chlorophyll, protein and RNA (Beevers, 1968; Wareing et al., 1968; Colquhoun and Hillman, 1972). In some biological systems, ABA can apparently inhibit the synthesis of RNA and protein (Osborne, 1967; Wareing et al., 1968; Stewart and Smith, 1972). On the other hand, ABA may be able to depress RNA and protein metabolism through enhanced degradation of these fractions: ABA is known to be capable of stimulating nuclease activity (Srivastava, 1968; De Leo and Sacher, 1970, 1971), particularly in excised tissues (Wyen et al., 1972). Thus, ABA may enhance the decline of the RNA and protein fractions, either by inhibition of their synthesis or by stimulating their degradation or both. In order to distinguish between these possibilities, the pre-labelling techniques of Kuraishi (1968) and Tavares and Kende (1970) were adapted for examining the effect of ABA on the decline of radioactive RNA and protein in senescing radish leaf discs. These techniques were originally devised for distinguishing between the possible effects of cytokinin on protein synthesis and degradation in maintaining protein metabolism in senescing leaf tissue. They were based on the supposition that if exogenous cytokinins were delaying protein degradation, then the rate of decline of radioactive protein, labelled with a protein precursor before the addition of the hormone, should be faster in control treatments than in cytokinin treatments. Alternatively, if the effect of the hormone were to maintain or enhance protein synthesis, the rate of decline of radioactivity should be the same for radioactive protein in both the cytokinin and control treatments.

In experiments examining the effect of ABA on the decline of RNA levels, the leaf discs were pre-labelled with either ^{14}C-8-adenine or ^{14}C-2-uracil. ^{14}C-adenine has been used in previous studies of the effects of ABA on RNA metabolism (Beevers, 1968; Colquhoun and Hillman, 1972), but is subject to the criticism that it may act as a precursor for DNA synthesis as well as for RNA synthesis, even though DNA synthesis is likely to be slight in mature leaf tissue and DNA levels have been shown to be relatively stable during leaf senescence (Osborne, 1962; Shaw et al., 1965; Srivastava, 1967a). Moreover, adenine may enter into a wide range of reactions in intermediary metabolism. The pre-labelling experiments were therefore repeated using ^{14}C-uracil instead of ^{14}C-adenine. Little substantial difference was noted between experiments using the 2 different precursors, however.

In agreement with previous findings (Beevers, 1968; Wareing et al., 1968; Colquhoun and Hillman, 1972), ABA was found to stimulate the decline in total RNA levels. In addition, during the course of a 6 day experiment, ABA also stimulated the decline in the amount of radioactive RNA. Thus according to the rationale of Kuraishi (1968) and Tavares and Kende (1970), it could be concluded that ABA acted to stimulate net RNA degradation, rather than inhibiting RNA synthesis. Closer examination of the data, however, reveals that the amount of radioactive RNA rose in the period of 1 to 2 days after completion of the 4 hour labelling period, before declining sharply to 6 days. The increase in the amount of radioactive RNA was particularly marked in those treatments which did not contain ABA; furthermore, there was some evidence, especially from the experiment using ^{14}C-adenine, as the precursor of RNA, that the presence of the non-radioactive precursor (^{12}C-adenine or ^{12}C-uracil) reduced the rise in radioactive RNA during this period. It could thus be concluded that the rise in radioactive RNA is due to a period of continued RNA synthesis after the completion of the labelling period and that this could only be derived from the passage of radioactivity in RNA from the large soluble pool (the alcohol-soluble fraction) still existing in the discs. Thus, if ABA were inhibiting RNA synthesis, then the increase in radioactive RNA would, as has been observed here, be lower in ABA-treated discs than in discs of control treatments. Further, an inhibition of RNA synthesis by ABA could also account for the higher level radioactivity in the alcohol-soluble fraction in ABA treatments at 1 day than in control treatments in the experiment using ^{14}C-adenine.

It is thus apparent that the data for the decline in radioactive RNA obtained from these experiments could be interpreted as being due to an effect of ABA either in stimulating net RNA degradation or inhibiting net RNA synthesis. It may be significant, however, that the rate of loss of radioactive RNA, after the peak in the period of post-labelling synthesis, was approximately similar in all treatments. Using the original rationale of Kuraishi (1968) and Tavares and Kende (1970), this could be interpreted as indicating that ABA had no net effect on RNA degradation; rather, the observed differences between the treatments may be attributable to the inhibition of RNA synthesis during the first 2 days after labelling.

The increase in the amount of radioactive RNA occurred even in the presence of a large excess of the appropriate non-radioactive precursor, included in order to dilute the radioactive precursor pool; this

increase thus represents a considerable amount of RNA synthesis. Yet during this period (i.e. 1 to 2 days after excision), the level of RNA did not increase - rather, depending on the treatment the amount of RNA either declined or remained approximately constant. It must be concluded therefore that the turnover of RNA during this period increased sharply. Increases in the specific activity of RNA in detached senescing leaf tissue have also been noted by Cherry et al. (1965), Srivastava and Atkin (1968) and Colquhoun and Hillman (1972). Increased RNA turnover may accompany periods of new or increased enzyme synthesis, but it is not possible from the evidence presented here to determine whether or not the period of increased RNA turnover here is a direct result of disc excision. Published evidence, however, suggests that excision of leaf tissues can stimulate nuclease activity (see Dove, 1973). Thus, the increased degradation of RNA, which must accompany the increase in RNA turnover observed here, may result from an increased activity of RN ase or other hydrolytic enzymes in the radish leaf discs. This could occur either through release from sequestration or through removal of an enzymic inhibitor or, more probably (Dove, 1973), through de novo enzyme synthesis. An increase in RNA synthesis may, in turn, result from increased activity of synthetic enzymes, although the possibilities of a reduction in the repression of the genome or the removal of some other restraint on RNA synthesis should also be considered. If de novo synthesis of nucleases is occurring, part of the RNA synthesis may be attributable to the synthesis of messengers and/or other RNA components necessary for the formation of these enzymes. Alternatively, the increase in RNA synthesis may represent the synthesis of "nonsense" RNA species, possibly as a result of wounding or osmotic disruption due to leaf excision. This possibility would appear to require further investigation. It is known that ABA can stimulate a rise in the activity of specific nucleases, particularly in excised tissues (Srivastava, 1968; Pietro and Sacher, 1970; Udvardy and Farkas, 1972). Thus, if ABA were stimulating nuclease activity during the period immediately after completion of labelling, the hormone may through the action of the enzymes, effectively depress the level of radioactive RNA detected at 1 day. Such a possibility further confounds any attempt to analyse the effects of ABA on RNA levels.

Many of the difficulties which prevent unequivocal determination of the effects of ABA on the decline of radioactive RNA in the radish leaf disc system are also evident in experiments attempting to examine the effects of ABA on the loss of protein, pre-labelled with ^{14}C-leucine. Radioactive leucine has been widely used for studies in protein metabolism

since it is unlikely to enter intermediary metabolism as readily as some other amino acids. Radioactive leucine (^{14}C) was used as a protein precursor in the pre-labelling studies of Kuraishi (1968) and Tavares and Kende (1970).

In general agreement with previous findings (Beevers, 1968; Colquhoun and Hillman, 1972), ABA slightly stimulated the decline in protein levels between 1 and 4 days after excision, although at 5 and 6 days, there was no difference between the treatments. Moreover, ABA stimulated the decline in radioactive protein throughout most of the incubation period; thus, according to the original rationale for pre-labelling experiments, it might be concluded that ABA was stimulating net protein breakdown. Unlike the experiments examining the loss of radioactive RNA, the amount of radioactive protein declined from immediately after completion of the labelling period. There was thus no period of net incorporation into protein; there may, however, have been some synthesis of individual enzymes (e.g. nucleases) within the general pattern of protein decline. Indeed, since the amount of radioactive protein was lower, between 0 and 5 days, in treatments which contained non-radioactive leucine than in those which did not, it may be concluded that some synthesis of protein, and hence incorporation of radioactivity did occur in the post-labelling period. Thus, if ABA were inhibiting protein synthesis either directly at the translational level or indirectly through RNA synthesis (or by some other means), the level of radioactive protein would be lower in ABA treatments than in control treatments (i.e. as observed in this experiment).

The use of non-radioactive precursors of RNA and protein to minimise any re-incorporation of radioactivity released after hydrolysis of radioactive RNA or protein and to reduce the passage of radioactivity from the soluble pool into RNA or protein was apparently only partially effective, even though the concentration of the non-radioactive compound was 100 times greater than that of the radioactive precursor. Possibly the uptake of the non-radioactive compound was relatively low; alternatively, the radioactive precursor may have been preferentially incorporated as a result of isotopic discrimination during synthesis in the post-labelling period. The amount of synthesis during this period appears to have been particularly high for RNA; it is feasible therefore that a further increase in the relative concentration of the non-radioactive precursor may have reduced the re-incorporation of the radioactive molecules. It is probable, however, that even then, these precautions would not be totally effective in preventing secondary incorporation,

especially in the case of RNA. Moreover, although no toxic or stimulating effects of the non-radioactive precursor were noted in chlorophyll, protein and RNA levels were noted at the concentrations used here, such effects may well have become apparent at higher concentrations. In their studies of the decline of pre-labelled protein, Kuraishi (1968) and Tavares and Kende (1970) also used ^{12}C-leucine to limit the turnover of the radioactive molecules. In view of the evidence presented here, the effectiveness of such precautions must be questioned, especially since other evidence presented by Kuraishi showed that considerable incorporation of ^{14}C-leucine could still occur 48 hours after leaf excision.

An alternative method for reducing the incorporation of the radioactive precursors during the post-labelling period is to include RNA and protein synthesis inhibitors in the incubation medium at the same time as ABA. Hence, any effects of ABA in inhibiting incorporation of radioactivity in the post-labelling period will be severely reduced, if not totally abolished.

Actinomycin D was originally devised for use in animal cells, in which it apparently acted by inhibiting cellular RNA synthesis in a specific selective and total manner (Reich et al., 1963). The compound has subsequently been used in many studies in plant tissues (see, for example, Leaver and Key, 1967; Ihle and Dure, 1970). Actinomycin D did not delay chlorophyll loss in control treatments, but did partially reduce the effect of ABA. Perhaps the stimulation of chlorophyll loss by ABA is dependent on the synthesis of new species of RNA and protein, e.g. hydrolytic enzymes; thus, actinomycin D may reduce the effect of ABA by blocking, at least partially, the synthesis of these components. A similar interpretation could be applied to the effect of actinomycin D in reducing the decline of radioactivity of the alcohol-soluble fraction induced by ABA. If, as suggested later, one effect of ABA is to increase membrane leakiness, this may also be mediated through hydrolytic enzymes; inclusion of actinomycin D would then reduce the effect of ABA on cell membranes and hence maintain the level of radioactivity in the alcohol-soluble fraction. Actinomycin D and ABA individually inhibited the early rise in radioactive RNA and, in combination, the 2 substances increased its subsequent decline, both relative to each substance alone and to water controls. This could possibly be interpreted as indicating that ABA is still apparently capable of stimulating RNA loss at a time when RNA synthesis is already apparently inhibited by actinomycin D. Thus, ABA might be concluded to be acting on RNA degradation. This

interpretation would not be substantiated, however, by the data for changes in total RNA level in which the effects of ABA and actinomycin D were not apparently ~~effective~~ additive.

Cycloheximide is believed to inhibit the elongation of polypeptide chains by blocking the transfer of amino acids from t-RNA to polypeptide chains; its action may be specific for 80S ribosome complexes (Ennis and Lubin, 1964). In the experiments reported here, cycloheximide was used to inhibit secondary incorporation of ^{14}C-leucine into protein. In contrast to previous findings, cycloheximide did not appear to delay chlorophyll from radish leaf discs (Martin and Thimann, 1972; Spencer, 1973); tissue age may be important, however, in determining the nature of the response to the inhibitor (Spencer, 1973). The effects of other protein and RNA synthesis inhibitors on leaf senescence is also variable and is presumably dependent on the type of inhibitor action, as well as tissue age (Wollgiehn and Parthier, 1964; Srivastava, 1967a; Paranjothy and Wareing, 1971). Cycloheximide did, on the other hand, totally abolish the effect of ABA in stimulating chlorophyll loss, an observation which could be interpreted in the same way as the effects of actinomycin D on ABA-induced chlorophyll loss, viz. an inhibition of the synthesis of hydrolytic enzymes through which the effects of ABA may be normally mediated. Cycloheximide did not stimulate further the decline in both total protein and radioactive protein induced by ABA alone; indeed, the effectiveness of each substance was somewhat reduced in combination. Again, this could be explained if the normal action of ABA was to stimulate synthesis of hydrolytic enzymes.

MDMP is thought to inhibit protein synthesis by preventing the formation of the 80S ribosomal complex (Weeks and Baxter, 1972; Baxter et al., 1973). In the radish leaf disc system, this inhibitor reduced the stimulation of chlorophyll loss induced by ABA. The effects of MDMP are thus similar to those observed for cycloheximide and actinomycin D and hence may be interpreted in the same way. It is of interest that cycloheximide and MDMP, which are believed to be specific for cytoplasmic protein synthesis should reduce the effect of ABA in stimulating chlorophyll loss, a process which occurs in the chloroplast. If these inhibitors are indeed specific for synthesis on 80S ribosomes, ABA effects on chlorophyll loss may be dependent on nuclear controlled RNA protein synthesis rather than on control by the chloroplastic genome (see Yoshida, 1961, 1970). ABA stimulated the loss of radioactive protein to a much greater extent than did MDMP. In combination, however, the effects of the 2 compounds were not additive; rather, MDMP tended to

reduce the effectiveness of ABA. One interpretation of this observation would be that ABA was acting to stimulate protein degradation.

If the use of RNA and protein synthesis inhibitors is to aid in resolving the apparent ambiguities resulting from the pre-labelling experiments, the efficiency of the synthesis inhibitors must be total or virtually so. Determinations of inhibitor efficiencies in the radish leaf disc system indicated that if the inhibitors were added at the same time as the radioactive precursor, incorporation of the precursor was reduced, but that a considerable percentage of the incorporation, relative to control tissue, still occurred. Treatment with the inhibitor 1 or 2 hours prior to the addition of the radioactive precursor reduced the level of incorporation further. However, even after a 2 hour lag, actinomycin D only reduced incorporation to about 50% of the control. The apparent efficiency of the protein synthesis inhibitors was somewhat greater, but considerable incorporation still occurred even after a 2 hour lag. It is thus probable that, in the pre-labelling experiments, considerable synthesis of protein or RNA could occur in the post-labelling period, even in the presence of the appropriate synthesis inhibitor. It is feasible that the efficiency of the inhibitors may increase further with time (i.e., beyond the 2 hour lag period used here); ABA may have acted, however, to inhibit protein or RNA synthesis before this higher efficiency is attained. The observed increased efficiency of the inhibitors with time possibly represents the time required for sufficient uptake of the inhibitor or for its action to be effective or both.

It is conceivable that a considerable portion of any protein synthesis occurring in the post-labelling period takes place in the chloroplast; this portion of synthesis will thus be unaffected by cycloheximide or MDMP, both of which are apparently specific for 80S ribosomes.

The concentration of the inhibitors used in these experiments were the same as those used in previous investigations (see, for example, Leaver and Key, 1967; Spencer, 1973; Baxter et al., 1973). Nevertheless, the specificity of the inhibitors has not been demonstrated here and thus it is possible that they had other effects in the cell, besides the inhibition of protein or RNA synthesis. Notably, cycloheximide reduced the total uptake of ^{14}C-leucine in the efficiency determinations and stimulated the loss of radioactivity from the alcohol-soluble fraction in the pre-labelling experiment. The effect, if any, of cycloheximide on the uptake of ABA by the discs has not been investigated. It is also not clear whether or not cycloheximide and the other synthesis inhibitors can affect membrane permeability.

The use of inhibitors in this study are perhaps also subject to the criticism that they distort the normal pattern of senescence changes in leaf discs by altering the sequence of changes in enzyme activity; this may further confound analysis of the effects of ABA in the senescing system.

The conclusions drawn from the prelabelling experiments reported by Kuraishi (1968) and Tavares and Kende (1970) concerning the role of cytokinins in protein metabolism should perhaps be questioned in the light of the ambiguities discussed above. Accordingly, their experiments were repeated using the radish leaf disc system. Kinetin had little effect on senescence until 3 days, but then delayed chlorophyll, and perhaps protein, loss. Kinetin had no apparent consistent effect on the loss of radioactive protein; consequently, although the variation in these data is relatively high, it does not appear that kinetin was effective in delaying protein degradation, in this species and under the experimental conditions employed. It should not necessarily be assumed from this, however, that kinetin does not delay protein breakdown. Rather, all that can be concluded from these data is that the pre-labelling system does not permit an unequivocal distinction to be drawn between the possible effects of kinetin on protein synthesis or degradation. Indeed, the hypothesis that kinetin does act to delay protein breakdown in plant tissues, including senescing leaves, has been substantiated using other techniques (Atkin and Srivastava, 1969; Trewavas, 1972; Martin and Thimann, 1972).

The possibility that, in pre-labelling studies, incorporation of radioactive precursors into protein or RNA does not occur through de novo synthesis, but, rather, results from end-group addition to pre-existing macromolecules or from exchange reactions, is a factor apparently not previously considered. The possible presence of more than one amino acid pool in leaf tissues is a further complicating factor (Tavares and Kende, 1970). If amino acids can exist either in protein precursor pools or in metabolically inactive pools, then different experimental treatments in the post-labelling period may affect the size of the protein precursor pool, thus changing the specific activity and incorporation rate of a labelled amino acid in this pool. In addition, different amino acid pools may exist for the synthesis of proteins within the various cellular organelles. The possible presence of radioactivity in the T.C.A.-soluble fraction has not been monitored, either in this or in previous studies and may account for the observed discrepancies in total radioactivity. Loss of radioactivity as $^{14}CO_2$

and $^{14}C_2H_4$ may also be significant.

The value of pre-labelling experiments for total protein and RNA fractions may perhaps be questionable. As Addicott (1970) has pointed out, ABA-regulated senescence is likely to involve an inhibition of synthesis of some enzymes and an increased synthesis of others. Furthermore, it is probable that similar changes may be occurring in different RNA fractions. Pre-labelling studies of total fractions will only examine the net effect of hormonal treatments, within which a more complex situation may therefore exist. More detailed studies of the effect of ABA on individual fractions may yield further information on the mode of action of ABA; on the other hand, analysis may still be complicated by ambiguities similar to those discussed above, particularly if ABA can affect both synthesis and degradation of individual protein and RNA fractions.

The experiments examining uptake of ^{14}C-leucine by different areas of the leaf indicate that most, if not all, the radioactivity is taken up via the cut edge. Further preliminary data, not presented here, suggests that little redistribution of this radioactivity occurs within 24 hours after completion of the labelling period (cf Hardwick and Woolhouse, 1968). Thus, the changes in protein and RNA metabolism reported here for leaf discs could result from biochemical events at or adjacent to the wounded tissue of the cut edge. It is known, for instance, that excision of leaf tissue can promote the synthesis of specific nucleases (see Dove, 1973) and that ethylene is frequently produced as a result of wounding and other stress effects (see Pratt and Goeschl, 1969). It was thus important to determine whether the incorporation of amino acids into protein was different at the edge of the disc from other areas of the disc. The data suggest that the percentage incorporation of amino acids into protein did not vary substantially across the disc. It could therefore be concluded that the observations made on protein (and hence probably RNA) metabolism are not a function of wounding effects. This conclusion is substantiated by the similar findings of Spencer (1973). The observations reported here do not, of course, preclude wounding effects on individual protein components, which might not have been detected here, or on other portions of cell metabolism.

Although the physiological effects of ABA in excised plant tissues have been widely studied (see, e.g., Addicott and Lyon, 1969), these investigations have not always been related to the uptake of ABA by the tissue in question. Studies of the uptake of ABA are important

for evaluating the timing of the physiological response where this is being used to study the mechanism of action of the hormone; knowledge of the uptake of ABA are also of relevance in tissues in which the rapid metabolism of the hormone or changes in membrane permeability may modify the effects of the hormone.

In the studies reported here of the uptake of ^{14}C-ABA by radish leaf discs, the uptake of the hormone into different regions of the disc has not been investigated. It can probably be assumed, however, that most of the radioactivity is taken up via the cut edge of the disc - i.e. in the same way as radioactive amino acids (Hardwick and Woolhouse, 1968; Spencer, 1973; see also Experimental, Section I(j)).

Bex (1972a) noted a declining rate of uptake of ^{14}C-ABA with time from 4 hours onwards in maize coleoptiles. Similarly, the rate of uptake of ^{14}C-ABA by radish leaf discs tended to decline with time during a 12 hour time course. By 12 hours, however, a considerable portion of the ^{14}C-ABA supplied to radish leaf discs is apparently converted to other components also soluble in 80% methanol; these products will also thus contribute to the radioactivity detected. Walton and Sondheimer (1972) demonstrated that, while the increase in the amount of radioactivity was approximately linear with time in embryonic bean axes supplied with ^{14}C-ABA, the amount of ^{14}C-ABA *per se* increased little after 2 hours, due to metabolism to other methanol-soluble radioactive products. In addition, ^{14}C-ABA uptake may be affected by conversion to methanol-insoluble components or to $^{14}CO_2$. It is known that ABA can be oxidized *in vitro* (Milborrow and Robinson, 1973); thus, perhaps, the small amounts of $^{14}CO_2$ released from incubation solutions of ^{14}C-ABA may result from oxidation of the hormone. In addition, however, some radioactivity derived from ^{14}C-ABA was apparently lost from the discs as $^{14}CO_2$; thus, ^{14}C-ABA may also be oxidized *in vivo*. Since the radioactive carbon of the ^{14}C-ABA used in these experiments is located at the 2-position in the side chain, such an oxidation would imply a greater degree of degradation of the hormone molecule than is involved in the production of those metabolites of ABA which have been noted before (Milborrow 1970a, b; Tinelli *et al.*, 1973).

In radish leaf discs, pre-aged in water for up to 6 days, the maximum uptake of ^{14}C-ABA under aerobic conditions occurred 1 to 2 days after excision of the discs; after this time, uptake declines steadily to 6 days. The uptake of ^{14}C-ABA in nitrogen was substantially reduced relative to air treatment; moreover, uptake in nitrogen did not vary greatly with the length of ageing, i.e. there was no peak in uptake.

The uptake of ^{14}C-ABA, in general, and the peak of uptake at 1 to 2 days in particular, thus appears to be at least partially dependent on aerobic metabolism, although the presence of some radioactivity in nitrogen treated discs may indicate the operation of both diffusive and anaerobic metabolic processes. It is of interest that the uptake of ^{14}C-ABA was still considerably reduced by nitrogen even 5 to 6 days after excision, thus indicating that aerobic metabolism apparently persists until a late stage in senescence of the discs; the relative stability of mitochondria and their presence after 6 days ageing in water may be of significance here (see Section V).

It was of interest to determine to what extent the observed pattern of uptake was characteristic for ^{14}C-ABA. Similar experiments using ^{14}C-sucrose, a compound of approximately similar molecular weight to ABA indicated that the uptake of sucrose by pre-aged radish leaf discs was also partially dependent on aerobic metabolism: uptake was reduced by nitrogen treatment. Moreover, again similar to ABA, there was a sharp rise in the total uptake of radioactivity 1 day after excision. Unlike the uptake of ^{14}C-ABA, however, the rise in total radioactivity at 1 day occurred in nitrogen as well as in air. It is feasible that this observation may reflect on the efficiency of the experimental conditions used for maintaining a state of anoxia; very similar procedures were used for ^{14}C-ABA uptake, however, when no peak occurred in nitrogen. Thus, the mechanisms of uptake for ^{14}C-sucrose and ^{14}C-ABA in nitrogen may be fundamentally different in some respects. Moreover, unlike ^{14}C-ABA, the uptake of ^{14}C-sucrose did not decline after the peak at 1 to 2 days. In nitrogen treatments, total uptake of ^{14}C-sucrose showed little consistent change with time except for a possible peak at 5 days; the radioactivity of the hyamine hydroxide fraction, however, increased steadily until 5 days and then fell sharply. The steady increase in total uptake in air was largely attributable to an increase in the radioactivity of the hyamine hydroxide fraction between 0 and 5 days, with a sharp decline to 6 days.

The data for ^{14}C-sucrose uptake may represent an under-estimate of the true uptake capacity for ^{14}C-sucrose, since after 8 hours incubation at 5 days, for example, the discs had removed nearly 20% of the radioactivity from the incubation solution; the consequent reduction in ^{14}C-sucrose concentration may thus have reduced uptake.

It is of note that for both ^{14}C-ABA and ^{14}C-sucrose, there was little correspondence between radioactivity in methanolic extracts of the discs and in hyamine hydroxide fractions; the reasons for such differences

are not immediately apparent. Since the corrected radioactivity in the hyamine hydroxide fraction consists mainly, if not entirely, of $^{14}CO_2$ released from the discs, it might be assumed that the radioactivity in this fraction could be used as a measure of the changes in the rate of respiration of the discs during ageing. The amount of radioactivity in the hyamine hydroxide fraction resulting from ^{14}C-sucrose uptake, however, does not correspond to that from ^{14}C-ABA. Moreover, it is probably fair to suppose that ^{14}C-sucrose will enter the glycolytic and other pathways of respiration much more readily than will ^{14}C-ABA; thus, the output of $^{14}CO_2$ from ^{14}C-sucrose incubation is probably more directly related to respiration rate of the discs, especially since ABA may be able to inhibit leaf respiration (Poskuta et al., 1972). Although the respiration rate of attached leaves generally declines with leaf age (Yemm, 1965), there may be a climacteric rise in respiration rate (Wood et al., 1943), perhaps prior to abscission. In detached leaves, there may be a climacteric-like change in respiration rate shortly after leaf excision and this may coincide with increased nuclease activity and other apparent wounding responses (Udvardy et al., 1964; Dove, 1973). In detached tobacco leaves, the rise in respiration occurred at 1.5 days after excision, followed by a subsequent gradual decline from 3 days (MacNicol, 1973); the rise was associated with a large increase in aerobic glycolysis (MacNicol et al., 1973). In radish leaf tissue a similar pattern was noted for the $^{14}CO_2$ output from ^{14}C-sucrose incubations, although there were differences in the timing of the changes. Alternatively it could be argued that the changes in respiration rate noted by MacNicol (1973) also coincide closely with the pattern of aerobic uptake of ^{14}C-ABA observed in radish leaf discs. Direct measurements of the respiration rate of the discs during ageing would help to clarify the relationship, if any between ^{14}C-ABA and ^{14}C-sucrose uptake and the rate of cell metabolism. It can be concluded, however, that the uptake of both ^{14}C-ABA and ^{14}C-sucrose is partially dependent on aerobic metabolism, possibly for active transport into the cell or for usage within the cell or both. The differences in uptake pattern between the 2 radioactive compounds make it unlikely, however, that the changes in ABA uptake during ageing can be entirely explained in these terms.

It is known that during senescence of intact and excised tissues, the permeability of cell membranes generally increases (Sacher, 1957, 1959) with a consequent increase in apparent free space (Eilam, 1965; Pietro and Sacher, 1970; Ferguson and Simon, 1973); thus, possibly, ^{14}C-ABA which is taken up during the later stages of ageing is not retained in the discs: leakage of electrolytes has been noted from discs of senescing

cucumber cotyledons (Ferguson and Simon, 1973). The loss of radioactivity from the radish discs incubated in ^{14}C-ABA, as a result of the washing procedures did not vary, however, with length of ageing. Moreover, if the apparent free space of the discs increases with ageing, it might be expected that the uptake of ^{14}C-ABA would also be increased because the resistance of the cell membranes to passive entry of small molecules would be lowered; the uptake of ^{14}C-acetate, for instance, by detached cucumber cotyledons (Draper and Simon, 1970) and of ^{14}C-glycine by Perilla leaf discs (Hardwick and Woolhouse, 1968) increased with age, although these investigations did not relate these changes to increases in apparent free space. Non-specific changes of this nature in membrane permeability cannot account for the differences in uptake pattern with ageing between ^{14}C-sucrose and ^{14}C-ABA. Direct measurements of changes in apparent free space and membrane permeability with senescence might prove valuable.

The peak in uptake of ^{14}C-ABA under aerobic conditions observed at 1 to 2 days may result from a stimulation of general metabolic rate shortly after excision (see, e.g., MacNicol, 1973; Dove, 1973). Again, however, it is unlikely that excision effects alone could account for the differences in uptake pattern between ^{14}C-ABA and ^{14}C-sucrose.

It is claimed that the level of endogenous ABA-like components increases during the senescence of detached Nasturtium leaves (Chin and Beevers, 1970). If significant increases in ABA occur in radish leaf discs, perhaps as a result of stresses induced by disc excision, it is possible that the diffusive component of ABA uptake would be reduced as a consequence of the reduction in the concentration gradient between the incubation solution and the cells of the discs.

The ability of radish leaf tissue to metabolise ^{14}C-ABA tended to decline slightly with ageing (Section III). If, as the results suggest, a significant portion of ^{14}C-ABA supplied to radish leaf discs is rapidly metabolised, then a reduction with ageing in the rate of conversion of ^{14}C-ABA might tend to reduce further uptake, perhaps either through a feedback effect or by a reduction in the concentration gradient. Similarly, the observation that the metabolism of ^{14}C-ABA was sharply reduced by anaerobic conditions might also partially explain the reduction in ^{14}C-ABA uptake under nitrogen. Changes in the rate of metabolism of ^{14}C-ABA, however, cannot account for the peak of ^{14}C-ABA uptake observed at 1 to 2 days after excision, since the rate of metabolism of ^{14}C-ABA did not vary greatly at this stage of ageing.

The biological activity of a hormone is not necessarily a direct function of its concentration in any tissue; rather, its activity may

also depend on its rate of synthesis and on its rate of inactivation, sequestration or other means of removal from the receptor site. Thus, if ABA is involved in the regulation of leaf senescence, the biological activity of ABA in senescence processes may depend on its rate of conversion to inactive derivatives; e.g. a reduction in the rate of such a conversion, at or prior to the onset of senescence, could raise the effective cellular concentration of ABA. Although metabolites of ABA have been characterised in a range of plant tissues (Koshimizu et al., 1968; Milborrow, 1970a; Tinelli et al., 1973), and the functions of certain metabolites of ABA have been examined in relation to stomatal closure and water stress in leaf tissues (Cummins, 1973; Hiron and Wright, 1973), the metabolism of ABA has not apparently been previously studied in relation to leaf senescence.

The length of pre-ageing in water did not basically affect the pattern of production of the metabolites from ^{14}C-ABA supplied to radish leaf discs. In some experiments, however, the proportion of ^{14}C-ABA converted to other radioactive components was slightly lower in discs aged for 5 and 6 days in water than in discs aged for shorter periods. This may, of course, merely reflect a decline in the general metabolism of the discs as they senesce as opposed to a specific decline in the rate of conversion of ABA to other products. Moreover, previous experiments (see Experimental, Section I) indicated that ABA was effective in speeding the senescence of radish leaf discs as little as 1 day after excision; thus, if a reduction in the metabolism of ABA were important in the initiation or stimulation of senescence, then it would be observable in the first day or so after excision. The rate of metabolism of ^{14}C-ABA in freshly excised discs, however, is similar to that in discs pre-aged for 1 to 3 days. In addition, in other experiments, whole leaves of radish, which exhibited a qualitatively similar pattern of metabolism of ^{14}C-ABA to leaf discs, yellowed senescing leaves were still capable of metabolising ^{14}C-ABA. It thus appears unlikely that a reduction in rate of metabolism of ^{14}C-ABA is important for determining the initiation or stimulation of radish leaf senescence. Perhaps, if changes in the concentration of ABA are important for regulating senescence, these changes are controlled via the biosynthetic pathway for ABA.

The rate of conversion of ^{14}C-ABA to its metabolic products tended to be somewhat lower for whole leaves than for leaf discs. This may reflect possible differences in pool size between intact leaves and leaf discs. Alternatively, disc excision may have a stimulatory effect on the general metabolic activity of the leaf tissue with a consequent increase in the rate of turnover of ^{14}C-ABA. The observed similarities

in the pattern of metabolism for ^{14}C-ABA in both whole leaves and leaf discs indicate, however, that ^{14}C-ABA metabolism in radish leaves cannot be totally attributed to excision effects. The possibility that the metabolites observed in whole leaf experiments were produced at the cut edge of the petiole and then translocated into the lamina, however, has not been excluded.

Possibly the persistence of the metabolism of ^{14}C-ABA in naturally senescent leaves and in discs aged for long periods in water is associated with the asynchronous nature of cellular senescence (Moore, 1965). Thus, in any leaf tissue, the capacity to metabolise ^{14}C-ABA may be confined to non-senescent cells or cells in the early stages of senescence. Further investigation of the capacity to metabolise ^{14}C-ABA, and indeed other hormones, in the various tissues of the leaf, both senescent and non-senescent, might prove of interest. Moreover, the recent demonstrations of the synthesis of ABA (Milborrow, 1974) and its presence (Railton et al., 1974) in isolated chloroplasts point to the possible existence of different sites of synthesis for ABA within any one cell and hence, possibly, to different pools for the natural metabolites of ABA. That exogenous abscisyl glucopyranoside is rapidly metabolised by plant tissue whereas the endogenous compound is relatively stable ' provides indirect evidence for the existence of several metabolite pools, at least for this derivative of ABA (Milborrow, 1970a). This observation, however, also points to the possibility that the metabolites observed as a result of supplying ^{14}C-ABA may be artefacts, produced by abnormal metabolic pathways, resulting perhaps from disturbance of the normal pool sizes. Milborrow (1970b) has suggested that phaseic acid represents such an artefact. Moreover, Walton and Sondheimer (1972) noted that the capacity to convert their metabolites $M_1 \rightarrow M_2$ only develops during incubation of the tissue (embryonic bean axes). Demonstrations of the natural occurrence of several metabolites of ABA, however, argue against the artefactual origin of these compounds (Koshimizu et al., 1968; MacMillan and Pryce, 1968; Walton et al., 1973). Furthermore, the rapid conversion of ^{14}C-ABA to its metabolites observed both here and in other studies (Walton and Sondheimer, 1972; Cummins, 1973) adds further evidence for the in vivo importance of ABA metabolites.

In certain experiments reported here, an antibiotic mixture containing chloramphenicol was employed in order to demonstrate that the metabolism of ^{14}C-ABA in leaf discs was not a function of microbial action. Chloramphenicol, at certain concentrations can inhibit protein synthesis on 70S ribosomes. Thus, possibly the occurrence of the metabolism of

^{14}C-ABA in the presence of chloramphenicol might be interpreted as indicating either that metabolism of the hormone could occur outside the chloroplast or that metabolism of the hormone in the chloroplast was not dependent on new protein synthesis. Studies of the synthesis and metabolism of ABA in the presence of selective protein synthesis inhibitors might yield valuable information concerning the sites of the various hormonal pools.

It is perhaps arguable that the inclusion of antibiotics is desirable in all senescence experiments in order to limit microbial action. It should be appreciated, however, that like chloramphenicol, many antibiotics act by inhibiting the synthesis of protein by the micro-organisms and that consequently protein synthesis by the plant cells may also be adversely affected. In addition, certain antibiotics may have senescence delaying effects (Sharma and Sen, 1970) or may perhaps interact chemically with ABA or other hormones, distorting their biological activity.

It has not yet been possible to identify, by mass spectrometry, the metabolites of ABA produced in radish leaf discs. None of the ions observed in mass spectra, obtained by scanning the various peaks from gas chromatography, corresponded to those expected for known metabolites of ABA (see Koshimizu et al., 1968; Milborrow, 1969; Tinelli et al., 1973). Moreover, no other ions could be detected as occurring consistentently to indicate the presence of previously unknown metabolites. Perhaps larger quantities of leaf material need to be used in order to obtain sufficient amounts of the metabolite to be easily detectable by mass spectrometry.

Indirect evidence concerning the structures of the radish leaf metabolites can be obtained from their partition and chromatographic properties. Both metabolites remained in the aqueous phase at basic, neutral and acidic pH's; this observation thus rules out 6'-hydroxymethyl-ABA (Milborrow, 1968) and phaseic acid (Walton and Sondheimer, 1972; Tinelli et al., 1973), both of which pass into the ethereal phase at pH 3.0 . The chromatographic and partition characteristics reported for 4'-dihydrophaseic acid (Walton and Sondheimer, 1972; Tinelli et al., 1973), however, resemble those for the more mobile metabolite observed in radish leaf tissue (i.e. the component chromatographing at Rf 0.2 to 0.4 in chloroform:methanol:water). Thus, it is possible that this metabolite is in fact 4'-dihydrophaseic acid. This compound apparently occurs as a metabolite of ^{14}C-ABA in several plant species (Walton et al., 1973) and may correspond to the metabolite remaining in the aqueous fraction reported by Cummins (1973).

The other metabolite observed in radish leaf tissue remained at the origin in both thin-layer chromatography systems used and was less mobile than ABA in the 2 paper systems, thus indicating a more polar compound than ABA, possibly corresponding to the glucose ester, abscisyl glucopyranoside. There is some correspondence between the Rf values reported in some solvent systems for this compound (Milborrow, 1968) and for the component observed in radish leaf tissue. Similarly the Rf values for this component in some systems also resembled those reported for an unidentified metabolite of ABA in lettuce fruits (McWha and Hillman, 1973). In chloroform:methanol:water, however, the glucose ester moved away from the origin (Koshimizu et al., 1968; Milborrow, 1970a), whereas in the experiments reported here, the radish leaf component remained at the origin. It is thus not possible from these data to conclude definitely that the less mobile component observed here is abscisyl-B-D-glucopyranoside. Alkaline hydrolysis of this metabolite might yield further information as to its identity. In addition, comparison of the growth inhibitory activity of both metabolites to published data for known metabolic products of ABA might yield further indirect evidence on their structures (e.g. Koshimizu et al., 1968; Davis et al., 1972).

The ambiguities concerning chromatographic properties of the metabolites, discussed above, illustrate clearly the difficulties of using Rf values for comparing results from different laboratories, even for known compounds: the Rf values reported for ABA by Milborrow (1968), Walton and Sondheimer (1972) and McWha and Hillman (1972) vary considerably, even in relatively non-volatile systems. Moreover, as the results reported here suggest some variation in Rf values can occur for chromatograms developed at different times under apparently similar conditions, particularly when using volatile chromatographic systems. This variability was apparently not attributable to the presence of pigmented and other impurities, which under certain circumstances can enhance or reduce the mobility of other compounds present on the chromatograms. Possibly, greater attention needs to be paid to rigorous control of temperature and other conditions for the development of thin-layer chromatograms in volatile systems. In addition, although laborious, the use of internal standards with every chromatogram may aid subsequent interpretation. That the most mobile component observed in the various chromatographic systems is in fact ^{14}C-ABA is evidenced by the observations (1) of correspondence to external standards of ^{14}C-ABA, (2) that it was the largest component in terms of radioactivity, and (3) that it declined as a proportion of total radioactivity with time during a 24 hour time course.

Possible synthetic relationships have been suggested for ABA, 6'-hydroxymethyl-ABA, phaseic acid and 4'-dihydrophaseic acid (Milborrow, 1969; Walton et al., 1973). From the data for the time-course experiment in which ^{14}C-ABA was supplied for varying periods up to 24 hours, there was some indication that the metabolite remaining at the origin on chromatograms may develop before the other, more mobile component, but these data are certainly not sufficient to suggest a catenary relationship between the 2 components. Again, the experiment in which partially purified metabolites were resupplied to leaf discs, afforded little evidence for such a relationship. Indeed, if the metabolites observed in radish leaf tissue do correspond to 4'-dihydrophaseic acid and abscisyl glucopyranoside, no catenary relationship would be expected, especially if, as has been suggested, both compounds represent terminal storage or inactivation products for ABA (Milborrow, 1970a; Walton et al., 1973). If 4'-dihydrophaseic acid is produced in radish leaf tissue supplied with ^{14}C-ABA, it might be expected that 6'-hydroxymethyl-ABA and phaseic acid would also be present as intermediates between ABA and 4'-dihydrophaseic acid (Walton et al., 1973). 6'-hydroxymethyl-ABA, however, has been reported to be unstable on thin-layer plates (Milborrow, 1970a) and thus perhaps was not detectable by the methods used here. Analysis of acidic ether extracts of radish leaf extracts supplied with ^{14}C-ABA indicated that approximately 10% of the radioactivity was slightly less mobile than ^{14}C-ABA (Table 33); this may correspond to phaseic acid, but it was not detected in any other experiments. The possibility that other undetected minor metabolites co-chromatographed with ^{14}C-ABA or its major metabolites should also be considered, as should the possible occurrence of methanol-insoluble radioactive products. The possible loss of radioactivity as $^{14}CO_2$ or $^{14}C_2H_4$ has been discussed above.

It is of significance that less than 50% of the radioactivity present in methanolic extracts of leaf discs incubated for 18 or 24 hours in ^{14}C-ABA could be ascribed to ^{14}C-ABA per se despite the presence of a continued supply of the radioactive hormone. It can thus be concluded that both the natural (+) and the unnatural (-) enantiomers of ABA are metabolised, although not necessarily at the same rate. Milborrow (1970b), however, indicated that the (+) form is preferentially converted to 6'-hydroxymethyl-ABA and phaseic acid when supplied with racemic ^{14}C-ABA, whereas ^{14}C-abscisyl glucopyranoside is derived preferentially from the (-) form. It is not clear whether a similar selectivity operates in radish leaf tissue supplied with ^{14}C-ABA.

If ABA is involved in the regulation of natural senescence of leaves, it might be expected that the levels of ABA present in the leaf would be higher during the initiation and progress of senescence than during the phase of rapid leaf expansion. Increases in the amounts of ABA-like inhibitors have been noted during the senescence of strawberry leaves (Rudnicki et al., 1968) and detached Nasturtium leaves (Chin and Beevers, 1970). Moreover, the amounts of diffusible ABA obtained from senescent Coleus leaves are apparently greater than from green leaves (Böttger, 1970). In addition, increases in the levels of ABA or of ABA-like inhibitors have been observed during the senescence of petals (Mayak et al., 1972) and the ripening and senescence of fruit tissues (Coombe and Hale, 1973; Goldschmidt et al., 1973). Osborne et al. (1972), on the other hand, were unable to detect significant differences in the total ABA levels of rapidly expanding and fully senescent primary leaves of bean. No detailed studies appear to have been made, however, of the changes in the levels of ABA, critically identified by chemical assay, during the growth and senescence of leaf tissue. Bioassays for ABA, on which most previous investigations have relied, tend to be based on inhibitory activity, the specificity of which must be open to doubt. Milborrow (1967) demonstrated that, in extracts from several plant species, all the inhibitory activity observed in bioassays could be accounted for by ABA. More recent data, however, question the general validity of this observation and point to the existence of other inhibitory components co-chromatographing with ABA in many solvent systems (Barnes and Light, 1969; Jenkins and Shepherd, 1972; Gaspar et al., 1972). Circular dichroism analysis offers a convenient method for the critical determination of ABA levels and has been used for assaying the amounts of ABA in bean leaves reported in this study. No consistent pattern of changes could be observed in the concentration of ABA in the leaves extracted at various stages of development between rapid expansion and abscission. Our results therefore largely substantiate the less detailed investigation of Osborne et al. (1972), in that senescent and expanding bean leaves apparently contained similar amounts of ABA, although the possible release of bound ABA by alkaline hydrolysis of the tissue has not been investigated here. It is not clear whether these observations can be extended to other species, including radish, but if these observations have general validity, then the hypothesis that changes in ABA levels are important in the regulation of leaf senescence should be questioned. The possibility of differential extractability of ABA between young, mature and senescent leaves should perhaps be considered, however.

The amount of ABA or of ABA-like components reported to be present

in unstressed leaves varies considerably, e.g. between 8 µg. Kg^{-1} for strawberry leaves (Rudnicki et al., 1968) and 1240 µg. Kg^{-1} fresh weight for Betula (Lenton et al., 1971); values reported for the same species may also vary widely (see, for instance, for Betula - Milborrow, 1967; Lenton et al., 1971). Part of these discrepancies may be attributable to the use of different assay techniques, particularly where bioassays have been used. Moreover, it is not clear from some investigations whether or not the values presented for ABA concentrations have been corrected for the losses involved during the extraction and purification procedures. Milborrow's data (1967) indicates that for different tissues the loss of ABA, as determined by racemate dilution analysis varied between 60 and 77%. Similarly, Hoad (1973) reported a recovery percentage of between 52 and 68% using t-ABA as an internal standard for extracts of Ricinus tissue. Thus, it is evident that high losses of ABA are involved in the extraction of the hormone. The recovery efficiency of the extraction technique devised by Browning et al. (1970) has not apparently been previously tested. The data presented here based on the recovery of ^{14}C-ABA indicate that although the losses of ABA associated with this method are considerable, the final recovery percentage did not vary greatly. Improved recovery efficiency determinations could have been achieved by the addition of ^{14}C-ABA as an internal standard to each extract. It should, of course, be appreciated that recovery efficiency of the exogenous hormone does not necessarily equal extraction efficiency for the endogenous hormone. Thus, corrected values for ABA concentrations may still represent under-estimates of the true level of endogenous ABA. In summary, however, although there is some variation between different extracts for the corrected levels based on the recovery efficiency, the amount of ABA in most extracts was of the same order as the total levels of ABA (free ABA and ABA released by alkaline hydrolysis) reported by Osborne et al (1972) and the levels reported by Hiron and Wright (1973) for the primary leaves of bean.

In the preliminary experiment reported here, the ABA concentration was 14.5 times higher in wilted than in non-wilted bean leaves as a result of a 3 day water-stress treatment. This observation confirms a number of previous investigations (Wright and Hiron, 1969; Most, 1971; Zeevaart, 1971; Hoad, 1973), including one using bean leaf tissue (Hiron and Wright, 1973). The extent of the rise in ABA levels appears to vary in these investigations, but is presumably dependent on both the ABA concentration before the stress and the severity of the stress. The increase in ABA is apparently dependent on increased synthesis of the hormone, rather than on release from a storage compound (Milborrow, 1970b; Milborrow and

Noddle, 1970). The concentration of ABA can also rise as a response to a number of other environmental stresses (e.g. Mizrahi and Richmond, 1972; Hiron and Wright, 1973; Itai et al., 1973). These responses illustrate clearly the need for rigorous control of the environmental conditions for growth and harvest of the plant material. Failure to control all environmental parameters satisfactorily may account for some of the variation in ABA levels, noted both here and in studies of Ricinus (Hoad, 1973). This variation, however, tends to question the importance of any relatively small changes observed in ABA levels in regulating developmental processes such as senescence, abscission, dormancy, etc.: if short term fluctuations in environmental conditions can cause large increases in the level of ABA, it is difficult to envisage a controlling system for senescence in which changes in ABA concentration alone regulate the initiation and rate of senescence. Certainly, the genetic programming of the tissue and the levels of other hormones would also need to be important. If ABA is involved in senescence initiation, however, then release of the hormone from sequestration or a change in the distribution of ABA between different cellular pools may be involved: changes in total ABA concentration would not then be needed. Alternatively, the rise in ABA concentration in response to environmental stress may be confined to the guard cells of the epidermis, in which case small changes in ABA concentration in the rest of the leaf tissue - undetected here - might still be important in regulating senescence of the mesophyllous cells. Although in this study, wilting of the bean leaves did not apparently affect chlorophyll levels (corrected on a fresh weight basis), despite a large increase in ABA levels, other studies indicate that long-term water and osmotic stress can induce leaf senescence (Shah and Loomis, 1965; Most, 1971; Prisco and O'Leary, 1972). Investigation of the relationship between stress-induced senescence and ABA may prove of interest, particularly in relation to the different tissues of the leaf and their capacity to synthesize ABA. In this study, ABA synthesis could apparently occur as a result of water stress in a tissue in which senescence had already apparently started: tissue age did not appear to act as a constraint on ABA biosynthesis. The later stages of leaf senescence are often accompanied by partial desiccation of the tissue. It appears unlikely that desiccation results from a significant decline in stomatal control; rather, epidermal cells tend to become senescent before the stomatal guard cells, which can apparently remain turgid and retain green chloroplasts, even after the death of the leaf (Meidner and Mansfield, 1968). The relationship between ABA levels, desiccation of the leaf and senescence of the epidermis and guard cells remains unclear.

In the pre-labelling experiments, ABA enhanced the rate of chlorophyll loss in radish leaf discs during the earlier stages of senescence (up to 3 to 4 days), in general agreement with previous reports (El-Antably et al., 1967; Aspinall et al., 1967; Beevers, 1968). Subsequently, however, there was little further loss of chlorophyll in ABA-treated tissue so that by 6 days there was sometimes more chlorophyll remaining in ABA treatments than in control treatments (see also Colquhoun and Hillman, 1972). Probably more significant than any numerical differences at this stage, however, was the observation that the water-treated discs remained turgid, but had yellowed considerably, whereas the ABA-treated discs were still green, but had become limp. This difference may perhaps be attributed to an effect of ABA on membrane integrity. ABA also apparently stimulated the decline in radioactivity from the alcohol-soluble fraction, especially after 3 to 4 days. Moreover, preliminary data suggested that incubation solutions resulting from ABA treatments contained more radioactivity than those from control treatments. These observations could also be taken to indicate an increased leakiness of cell membranes due to ABA treatment. Other studies suggest that ABA may have a selective effect on guard cell permeability (Kriedemann et al., 1972) and on ion flux in beetroot tissue (Van Steveninck, 1972). In root tissues, however, ABA can increase the hydraulic conductivity of cell membranes and increase the influx and efflux of water (Glinka and Reinhold, 1971, 1972). Thus the effects of ABA in the later stages of disc ageing may perhaps be to induce water-logging and to disrupt the normal osmotic control, which is apparently still maintained in water treated tissue. It was considered that, further, more direct evidence on those observations could be obtained from ultra-structural studies of the effects of ABA on disc senescence. Although ABA can apparently stimulate the senescence of excised leaf tissue, it is not clear whether ABA acts merely to hasten the normal sequence of ageing processes or whether the pattern of senescence is fundamentally altered by the hormone. Certainly, ABA has been shown here to alter the pattern of chlorophyll loss from radish leaf discs. It was thus of relevance to examine the ultra-structural changes induced by ABA in cells of radish mesophyll tissue, which contain the majority of the chlorophyll in radish leaves, and to compare these changes with those occurring in water-aged tissue and in naturally senescent leaves.

It has become evident from these studies that the use of leaf material for determining the sequence of fine-structural changes during senescence must take account of several sources of possible variation which have been shown to occur in the tissue examined. Despite the fact that leaf tissue was only excised from inter-veinal areas of mature green

leaves and that wounded tissue at the edge of discs was avoided, the cell population obtained, as detedmined by occurrence in tissue section, showed a considerable degree of heterogeneity, even in freshly excised tissue. In particular, 2 types of cell were observed, one of which exhibited the characteristics typical of mesophyll tissue. The other type probably corresponded to cells from small vascular bundles, which would not be excluded by the excision process adapted, avoiding as it does, only the larger leaf veins. Variation of this nature cou-ld be excluded, in practical terms, by only examining cells of the particular type which is of interest. Nevertheless, more careful selection of material in terms of both developmental state and relative location within the leaf could perhaps reduce possible variation of this nature.

A further and more serious form of variation occurred in the degree of degeneration that was observable between different mesophyll cells, even in freshly excised tissue. Some cells were obviously in advanced stages of degeneration, whereas the majority of cells maintained a high degree of organization and contained apparently functional chloroplasts, cell membranes and other organelles. Such variation which inevitably confounds determination of the normal pattern of senescence, presumably results from the asynchronous senescence of cells within any leaf. It has already been noted in various species that cellular senescence is not a synchronous process within any tissue (Moore, 1965; Treffry et al., 1967; Ragetli et al., 1970). This may be due to unequal distribution of essential metabolites, perhaps resulting from differential hormone effects or from mobilization. Differences in the state of degeneration of adjacent cells and tissues have also been observed in other ageing systems (Matile and Winkenbach, 1971; Berjak and Villiers, 1972). Dennis et al. (1967) have suggested that environmental conditions during the growth of leaves may be a further source of variation in the pattern of senescence.

The data reported here suggested that, in some respects, tissue aged for 2 days was apparently less senescent than tissue aged for only 1 day; these observations may perhaps be attributable to variations of the type discussed above.

In general agreement with observations reported here for radish, Mlodzianowski and Ponitka (1973) have distinguished between 2 types of degenerating plastids in detached parsley leaves. In addition, variations in the structure of plastids within any one cell have been observed here, both in freshly excised and aged radish leaf tissue. In particular, chloroplasts at the edge of mesophyll cells were elliptical and were normally arranged with their long axis and stromal thylakoids parallel to

the cell membrane. Chloroplasts towards the centre of the cell, on the other hand, frequently appeared rounded with consequent distortion in the arrangement of the thylakoids. Such apparent differences may in reality relate to the plane of sectioning of the chloroplasts although a similar distinction between the shape of chloroplasts at different locations in the cell has been made in detached oat leaves (De Vecchi, 1971).

Ragetli et al. (1970) noted the formation of chloroplastic vacuoles during the senescence of detached tobacco leaves; these vacuoles were bounded by a double membrane and appeared to result from the enclosure of portions of cytoplasm, as a consequence of evagination of the chloroplast. Other vacuoles, with single membranes, were also detected in the chloroplasts of tobacco and these resulted from the hypertrophy of intracellular spaces of the thylakoids. Similarly, Hernandez-Gil and Schaedle (1973) described the occurrence of anomalous electron-opaque areas in the stroma of some senescing plastids of Populus leaves and suggested that these areas may have been produced by the action of hydrolytic enzymes. Similar regions have been noted in the plastids of radish leaves; these occurred both in some freshly excised leaves and in tissue aged for 1 or 2 days in water. In some cases, distinct membranes could be detailed as de-limiting those areas of low-electron density. In tissue, aged for 2 days in water, bodies closely resembling mitochondria were detected, apparently enclosed in these regions. This would suggest that these areas are unlikely to arise either by the action of hydrolytic enzymes or by intra-cellular separation. They may, on the other hand, arise by vacuolation, with resultant enclosure of portions of cytoplasm and perhaps of cytoplasmic organelles (cf. Ragetli et al., 1970). Invagination of the chloroplasts was noted in a few cases in radish tissue. Alternatively, if the chloroplasts could exist in a concave form, then sections through the chloroplast would occasionally show apparent vacuoles, with delimiting membranes, existing in the chloroplast stroma. Tulett et al. (1969) noted the occurrence of similar structures in the stroma of plastids of artichoke tubers and suggested that they result from the plane of section of the tissue.

The retreat of the plasmalemma from the cell wall observed in some parts of some cells is perhaps another feature of intra- and inter-cellular variation. These apparent abnormalities in freshly excised tissue (i.e. in shape and disposition of some chloroplasts, in the possible existence of chloroplastic vacuoles and the observation that the cell membrane is not always oppressed against the cell wall) may result from fixation and processing artefacts. Alternatively, they may indicate the occurrence

of "sub-clinical" viral infection(s) in the ostensibly healthy leaf tissue; i.e. viral particles in the tissue may be distorting normal cellular structures without producing overt disease symptoms. Such a possibility could only be eradicated by the use of clonal plant material proved to be virus-free.

Some studies of the ultrastructural changes occurring during leaf senescence suggest that the chloroplast is not the first organelle affected; rather, early changes have been noted in the endoplasmic reticulum, plasmalemma and tonoplast (Shaw and Manocha, 1965; Roux and McHale, 1968). In general, however, the chloroplast has been found to exhibit the first symptoms of senescence (Barton, 1966; Dennis et al., 1967; Stetler and Laetsch, 1968; Mittelheuser and Van Steveninck, 1970). Similarly in the studies reported here, the earliest changes observed were the enlargement of the plastoglobuli and the disappearance of starch grains from the chloroplast stroma. In addition, the chloroplasts apparently swelled and became more spherical; a concomitant deformation of the thylakoids was also observed.

An early increase in size of the plastoglobuli during leaf senescence has been widely reported (see, for instance, Barton, 1966; Roux and McHale, 1968; Lichtenthaler, 1969). The number of plastoglobuli have been generally reported not to increase greatly during senescence and the number may, in fact, decrease as a result of fusion of individual globules. Other studies indicate that the number and size of plastoglobuli may both increase during senescence (Ljubesic, 1968). It has been suggested that the plastoglobuli contain the lipids and carotenoids released as a result of thylakoid breakdown. Their observed proximity to the thylakoids, both in the studies reported here and in those of Barton (1966) and Dennis et al. (1967), would tend to substantiate this suggestion.

The rapid loss of starch, especially from detached tissue aged in the dark, has been observed in other species (Yoshida, 1961; Harding et al., 1968; Mittelheuser and Van Steveninck, 1970) and presumably results from the mobilization of carbohydrate reserves, required to replace respired carbohydrate.

Some swelling of the chloroplasts was observed during the early stages of ageing. Other reports have suggested that as the chloroplastic components are degraded there is a consequent shrinkage of plastid volume (Barton, 1966; Dodge, 1970; Mlodzionowski and Kwintkiewicz, 1973), although Srivastava (1967) did note a transient increase in plastid volume

in aged tissue. Treffry et al. (1967) attributed a similar apparent increase in the volume of the plastids of germinating soybean cotyledons to a fixation response and doubted whether the increase in volume observed represented the in vivo situation. It was also noted in the studies reported here that the radish chloroplasts became more spherical during the early stages of ageing. This observation is substantiated by the studies of Barton (1966) and Yoshida (1970). Other changes in chloroplast shape have been noted by Dodge (1970), De Vecchi (1971) and Mlodzianowski and Kwintkiewicz (1973). In addition, De Vecchi has observed the "bowing" of the chloroplast thylakoids noted in some of the chloroplasts of radish.

The main effect of ABA, after 1 day of ageing, was apparently to reduce the thylakoid structure of the chloroplasts in general, and the granal content in particular. The decomposition and distortion due to swelling of the thylakoids have been noted as early changes in leaf senescence (Barton, 1966; De Vecchi, 1971; Harris and Arnold, 1973). In addition the concensus of opinion indicates that stromal thylakoids are normally preferentially lost, relative to granal thylakoids (Ljubesic, 1968; Yoshida, 1970; Mlodzianowski and Ponitka, 1973), although Dennis et al. (1973) and Harding et al. (1968) suggest that granal structure is degraded first in Brassica tissue. Thus the observation that ABA apparently preferentially stimulated granal loss may represent a deviation from the normal pattern of senescence. On the other hand, Mittelheuser and Van Steveninck (1970) found that ABA hastened the normal preferential loss of stromal thylakoids in detached wheat leaves; thus, species differences may be of significance in respect of thylakoid degradation. Lichthenthaler and Becker (1970) postulated that ABA could limit thylakoid formation in etiolated barley tissue. If this suggestion were extended for other phases of development, perhaps ABA may stimulate thylakoid loss by limiting synthesis of those thylakoid components which undergo turnover.

The relationship of the orientation of the thylakoids to the external chloroplast membrane appeared to be reduced in tissue treated in ABA for 1 day. This change did not appear to be specific for ABA treatment, however, but was also observed in some chloroplasts in tissue aged for longer periods in water. The means of control, if any, of the apparent relationship between the orientation of the thylakoids and of the chloroplast envelope is not clear. Some cells aged in ABA for 1 day were in an advanced state of degeneration and thus resembled some of the cells observed in freshly excised tissue. The chloroplasts of these cells had lost their envelopes although the thylakoids devoid of grana remained in

close association in the matrix resulting from the evident rupture of the tonoplast and plasmalemma. The plastoglobuli of ABA-aged tissue tended to be larger than those of water-aged tissue; this may be associated with the reduction in granal structure noted in ABA-aged tissue.

Great emphasis has been placed in previous studies on detecting early changes in ultra-structural features during leaf senescence. Valuable though such determinations may be, they should perhaps be treated with caution. In particular, the sources of variation discussed above make unequivocal determination of sequential processes difficult. Moreover, detachment of leaf tissue may distort the pattern of changes to a greater or lesser degree (Butler, 1967; Ragetli et al., 1970). Furthermore, changes detectable at the fine structural level are likely in molecular terms to be gross ones. If earlier changes at the molecular level occur, differences in fine structure may be of a secondary nature. Observations of changes in ribosome content have not been made in this study, although it has been suggested that loss of chloroplastic ribosomes is an early feature of senescence in some species (Mittelheuser and Van Steveninck, 1970). The significance of early changes noted here could be further questioned on the basis that observations of aged tissue were not made until 24 hours after leaf excision; significant changes at the ultra-structural level might well be observable after shorter periods of incubation. This criticism could, however, also be levelled at most other studies of this type.

Comparison of senescent tissue from the 3 types of ageing procedure used in these studies (i.e. tissue aged in water for 6 or 12 days and in ABA for 6 days and naturally senescent, yellow leaf material) reveals somewhat different patterns of cellular degeneration. Although some variation between different cells was evident, cells from tissue aged in water generally retained their nuclei and mitochondria, and the tonoplast and plasmalemma were normally present. Moreover, although there were prominent plastoglobuli in the chloroplasts and despite the fact that the normal parallel thylakoid arrangement had been disrupted, the envelope of the chloroplast generally remained intact. In tissue aged in ABA, on the other hand, the thylakoids were swollen and all granal structure was lost, together with the plastid membrane, the tonoplast and the plasma-lemma. Large numbers of irregular lipophilic vesicles were present in the region of the decaying chloroplasts, which were marked by the mass of degenerating thylakoids, surrounded by the matrix of low electron-density extending throughout the cell. No other cellular organelles were detected. Examination of naturally senescent material indicated yet

another pattern of senescence changes in which there was a high degree of membrane and organelle integrity. The chloroplasts retained many granal areas although the plastoglobuli were large and prominent. It is thus evident that the sequence of changes in these 3 types of tissue appear to have been fundamentally different. Such differences must inevitably reflect on the validity of the usage of detached leaf material, including segments and discs, for senescence studies; Butler (1967) and Ragetli et al. (1970) have also noted ultrastructural differences in patterns of senescence between detached and attached leaves. Moreover, the differences observed between ABA- and water-aged material suggest that the hypothesis that ABA merely acts to hasten the sequence of changes in senescent leaves (Mittelheuser and Van Steveninck, 1970) should be questioned. It is possible, however, that the material designated as naturally senescent actually represented a relatively early stage in cell degradation and that examination of tissue in a more advanced state of decay, perhaps even after natural abscission, would more closely resemble tissue aged in water. This possibility would appear to require further investigation.

The observations reported here can give little further indication as to the cause of the anomalous pattern of chlorophyll loss occurring in ABA-treated tissue. The retention of pigments in chromoplasts developed from chloroplasts in fruit skins has been associated with their deposition in lipid globules (Harris and Spurr, 1969; Goldschmidt, 1973). Goldschmidt has shown that ABA levels in citrus peel rise as a result of ethylene treatment. Thus possibly the transformation of chloroplast to chromoplast, which Goldschmidt has suggested is controlled by ethylene, may in fact be regulated by changes in ABA level. The presence of large numbers of lipophilic vesicles has been shown in ABA-treated radish leaf discs. Thus it is feasible that an analogous process to chromoplast formation has occurred during the degeneration of the leaf chloroplasts with deposition of chlorophyll pigments in the lipid globules. The evidence obtained here, however, does not permit a firm parallel to be drawn with chromoplast formation. Nevertheless, it is clear that the effects of ABA in apparently retarding chlorophyll loss in later stages of ageing is not related to the re-greening processes observed in some ageing tissues (Ljubesic, 1968; Callow and Woolhouse, 1973).

Bacteria were occasionally noted in the inter-cellular spaces of aged tissue. It is perhaps of significance that the presence of bacteria was generally related to the pattern of cell degeneration most frequently observed in tissue aged in ABA (i.e. cells in which the tonoplast, plasma-

lemma, and chloroplast envelopes had been lost). It is possible that bacteria and other micro-organisms stimulated the degeneration of these cells and that the effects of ABA on cell degeneration are, in fact, a result of an interaction between ABA and microbial organisms. Alternatively, the growth of bacteria may have been enhanced in the area of those cells by products of cellular senescence (e.g. leakage of sugars). Again, the presence of the bacteria may be totally unrelated to the speed of senescence. It is not possible with the available evidence to distinguish between these various possibilities. It is probably significant, however, that cells in an advanced state of degeneration were seen in fresh leaves, tissue in which microbial contamination would be expected to be minimal; moreover, even in aged tissue the occurrence of bacteria was relatively low. Thus, microbial contamination may well be a coincidental or, indeed, a natural occurrence in leaf senescence.

Roux and McHale (1968) considered that rupture of the tonoplast and plasmalemma were early features in detached tomato leaves. In general, however, these membranes appear to be relatively persistent in senescing leaf tissue (Shaw and Manocha, 1965; Barton, 1966; Butler, 1967; Mittelheuser and Van Steveninck, 1970). This was substantiated by the observations reported here, both for tissue aged in water and for naturally senescent material. Barton (1966) considered that the relative stability of these membranes argued against an early involvement of hydrolytic enzymes of vacuolar origin in leaf senescence.

The nucleus has also been shown to be relatively long-lasting in these studies; nuclei with distinct nucleolar or chromatin areas and nuclear membranes were detectable in both water-aged and naturally senescent tissue. These observations are in general agreement with the published literature (e.g. Mittelheuser and Van Steveninck, 1970). Whether or not these nuclei were still active in terms of RNA production is beyond the scope of this work; it may be noteworthy, however, that the nucleus has been shown to exert some control over the senescence of the chloroplast (Yoshida), 1961).

Similarly, in agreement with most of the literature (Butler, 1967; Dennis et al., 1967), the mitochondria of senescing radish leaf tissue persisted at a time when the chloroplasts were in an advanced state of degeneration. Furthermore, the mitochondria appeared to retain their internal cristae structure. Again, it is not possible from the present data to determine whether or not these mitochondria retain their activity in senescent cells. Certainly, respiration normally declines with senescence (Leinweber and Hall, 1959), although in intact leaves of some

species there may be a climacteric peak in respiration immediately prior to abscission (Katterman and Hall, 1961) for which functional mitochondria would presumably be required.

A number of other organelles have been detected in ageing radish leaf tissue. The low frequency of their occurrence, however, makes it difficult to determine whether or not they are related to the progress of senescence. Certainly, the fact that such bodies did not occur in a large number of cells would suggest that their importance in terms of senescence is probably limited. These bodies include structures designated as lomasomes, myelin-like bodies, cytoplasmic lipophilic-vesicles and an unidentified cytoplasmic body. Lomasome-like structures were seen as membranous vesicles at the plasmalemma-cell wall interface. Similar structures have also been seen in senescing wheat leaf tissue, although the possible artefactual nature of these vesicles should be considered (Mittelheuser and Van Steveninck, 1971b). The myelin-like bodies (Bowes, 1969), a striking example of which was seen in a cell from naturally senescent material, appeared to be composed of masses of concentric membranes and these were situated in the cytoplasm or in the matrix of degenerate cells. The occurrence of cytoplasmic lipid bodies has been noted in previous studies. Lipid bodies in the cytoplasm of tobacco leaf cells, designated as plastosomes, were apparently derived from the chloroplasts being released into the cytoplasm by rupture of the chloroplast membrane (Harris and Arnott, 1973). This process, however, apparently occurred in cells before the completion of leaf expansion and thus appears to be unrelated to senescence processes. Mlodzianowski and Kwintkiewicz (1973) noted the occurrence of osmiophilic masses on the surface of chloroplasts and mitochondria. Probably of more relevance to the observations reported here are the cytoplasmic lipid bodies noted by Mittelheuser and Van Steveninck (1970), which appeared and grew in size in naturally senescent wheat leaves. Similar to the bodies reported here, these were more or less round, devoid of internal structure and apparently were not membrane bound.

Little consistent difference was observed between the sections resulting from fixation by the two procedures. The Karnovsky fixative should reduce any distortion occurring between the time of leaf excision and the time that the fixative becomes effective, since the formaldehyde component of this fixative will penetrate the cell rapidly, relative to the penetration rate of glutaraldehyde. It is pertinent here to draw attention to the increased delicacy of senescent leaf tissue compared to that of fresh leaves (see also, Barton, 1966; Roux and McHale, 1968). Although it is possible to reduce damage during processing to some extent (see, for instance, Mittelheuser and Van Steveninck, 1971a), the possibility

remains that the observations made on senescent tissues are distorted by artefactual effects occurring during the processing of the material.

Many studies of leaf senescence, including this one, have used whole detached leaves and excised leaf discs or leaf sections as an assay system for investigating the manifestations and regulation of senescence processes (see, for instance, Osborne, 1962; El-Antably et al., 1967; Mizrahi et al., 1970). By using excised leaf tissue, the correlative influences of other parts of the plant or, indeed, of the same leaf can be minimised. Moreover, leaf discs or segments can provide large numbers of apparently reproducible experimental units and thus are frequently more convenient in experimental terms than attached leaves, especially since excised tissue often senesces more rapidly than leaves remaining on the plant (although exceptions do exist - see, for instance, Simon, 1967; Spencer and Titus, 1973). In studies of the hormonal regulation of leaf senescence, it has been observed that exogenous hormones do not always penetrate the surface of the intact leaf successfully (El-Antably et al., 1967; Sloger and Caldwell, 1970); the use of excised leaf tissue permits greater uptake of these and other compounds via the cut edge.

If leaf discs and other excised tissues are to be used as assay systems in plant physiology, it is necessary to demonstrate that the normal sequence of cellular events is not greatly altered, relative to those occurring in the tissue in situ. Current evidence suggests that senescence changes in excised or detached leaf tissue may be fundamentally different in some respects from the pattern of changes associated with natural senescence in attached leaves (see, for instance, Simon, 1967; Hardwick and Woolhouse, 1968; Lewington and Simon, 1969; Dove, 1973).

At the ultrastructural level, differences between the senescence processes of detached and attached leaves have been noted (Butler, 1967; Ragetli et al., 1970; Mittelheuser and Van Steveninck, 1971a,b). Similarly, the pattern of cellular changes observed in radish leaf discs differed somewhat from those occurring during natural senescence.

Leaf detachment may disrupt the normal respiratory metabolism of the leaf (Yemm, 1965). In addition, excision may induce a climacteric-like rise in respiration rate (MacNicol, 1973); this may be associated with the increase in uptake of ^{14}C-ABA noted for radish leaf discs. As suggested above, excision may increase the specific activity of RNA fractions (see also Cherry et al., 1965; Srivastava and Atkin, 1968) and may also increase the content of lysosomes and of certain species of RNA (Udvardy et al., 1969). Moreover, leaf detachment may distort the size of the precursor pools for protein synthesis: amino acids released

by proteolysis, instead of being translocated to other parts of the plant (Simon, 1967), tend to accumulate in detached leaf tissue (Wood et al., 1943; Anderson and Rowan, 1968). Although the pattern of changes for some enzymes is similar in excised and attached senescing leaves (Lazar and Farkas, 1970; Moore and Stone, 1972), leaf excision can enhance the synthesis of particular enzymes and may induce the de novo synthesis of others (Udvardy et al., 1964; Wyen et al., 1971; Dove, 1971, 1973). Notably, the enhanced activity of specific nucleases as an apparent consequence of leaf excision may result from a stimulation of metabolism by a direct response to wounding or by disruption of normal osmotic control at the cut edge of the tissue (Dove, 1971, 1973). The uptake of radioactive precursors occurs via the cut edge of leaf discs (Hardwick and Woolhouse, 1968), although the data presented here (see Section I(k)) and the data of Spencer (1973) indicate that there is no marked stimulation of incorporation into total protein as a result of disc excision.

The role of C_2H_4 in the processes of senescence in excised leaf tissue is unclear, although C_2H_4 production by plant tissues can be stimulated by wounding (see Pratt and Goeschl, 1969).

In summary, therefore, the use of excised leaf tissue and detached leaves for the study of leaf senescence should perhaps be questioned. Although as an assay system, these types of tissue offer considerable experimental advantages, these are perhaps outweighed by the distortion of cell metabolism induced by detachment or excision of the leaf.

The leaf is a heterogeneous organ with several other types of cells in addition to mesophyllous tissue. In discussing the ultrastructural aspects of senescence of radish leaves, the asynchronous nature of cellular degeneration was considered. Possibly, therefore, the degree of senescence of the different types of leaf tissue is also unsynchronized. Certainly, the supply of nutrients and hormones to different tissues within the leaf is likely to vary; this may be critical in determining the onset and speed of senescence. In addition, in experimental use, the different rates of uptake incorporation and metabolism of exogenous compounds by different tissues may be important. It may thus be concluded that the heterogeneous nature of the leaf tends to confound analysis of changes at the cellular level during leaf senescence. At the organelle level, further investigation of the relative rates of senescence of the various organelles in leaf tissues may prove of interest.

The ability to control ageing, both of animals and plants, could be of fundamental social and economic importance. In crop plants, growth hormones and their synthetic derivatives may offer opportunities for the chemical regulation of senescence and ripening and for understanding the natural mechanisms governing ageing processes. Although ABA can promote the senescence of excised leaf tissue and stimulate the decline in chlorophyll, RNA and protein, it remains uncertain whether or not ABA can be claimed to be an important endogenous factor for controlling natural senescence. Certainly it appears unlikely that the rate of inactivation of ABA plays a major role in regulating ABA levels prior to and during radish leaf senescence. Indeed, little consistent change could be observed in the concentration of ABA in bean leaves during development; furthermore, the large rises in ABA observed as a result of environmental stress must inevitably question the significance of any small changes in ABA associated with senescence and other developmental processes. Ultrastructural studies suggest that the pattern of cellular changes observed in ABA-treated excised leaf tissue differ in certain important respects from those of water-aged tissue. Moreover, the differences observed in the pattern of senescence between excised leaf tissue and naturally aged material must cast further doubt on the use of excised tissue in senescence studies. If plant development is viewed as a continuous and genetically programmed sequence of biochemical events, the role of ABA and other plant hormones may be to act as rate-regulating factors, rather than as prime causes of entirely new cellular processes.

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