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EARLY EFFECTS OF BORON DEFICIENCY ON MEMBRANE FUNCTION IN HIGHER PLANTS

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Abstract.

The transfer of plants to boron-free solutions induces rapid responses in membrane functions without necessarily affecting root growth and anatomy. In sunflowers (Helianthus annuus), root growth slows within 3-6 h. However in maize (Zea mays), no growth effects are apparent after more than 30 h without boron (-B). In both species early disturbances in ion uptake and cell wall deposition are seen.

Ultrastructural studies on sunflower root tips after 5.5 h or 3 d -B are reported. Detailed studies on the absorption of P_i and K^+ by root tips were complemented by studies on protoplasts isolated from the root tips of +B and -B plants. There were no significant differences in the protoplast yield or viability according to their B status. Ion absorption by protoplasts isolated from roots of +B and -B plants generally resembled that by intact roots of the corresponding B status.

Altering the B status of the protoplasts was expected to initiate earlier responses than in the roots where cell wall binding and diffusion times buffer the system against change; but the greater variability inherent in measuring the protoplast responses prevented the detection of subtle changes.

The activities of two membrane bound enzymes were investigated; β-glucan synthetase and a K^+-stimulated, Mg^{2+}-dependent ATPase. UDPG incorporation by protoplasts continued for over 18 h and was consistently higher in +B protoplasts and root membranes than -B. However SEM revealed no significant differences in fibre deposition around sunflower and maize protoplasts according to their boron status.

$(K^+ + Mg^{2+})$-ATPase from sunflower roots was found to be reversibly impaired by the loss of B; and preliminary investigations implied that restoration of activity when B was resupplied to the intact roots was correlated with the B content of the membrane fraction, as determined by the (n,α) method.

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Abbreviations

ABA	abscisic acid
ATP(ase)	adenosine triphosphat/e (/ase)
BSA	bovine serum albumin
CPD	critical point drying
2,6-DCB	2,6-dichlorobenzonitrile
DNP	dinitrophenol
DTT	dithiothreitol
FDA	fluorescein diacetate
IAA	indoleacetic acid
MES	2[n-morpholino]ethanesulphonic acid
P_i	inorganic phosphate
PMSF	phenylmethylsulphonylfluoride
PSM	protoplast suspension medium
PVP	polyvinylpyrrolidone
SDS	sodium dodecylsulphate (sodium lauryl sulphate)
SEM	scanning electron microscopy
SIP	stock iso-osmotic Percoll
TEM	transmission electron microscopy
UDP(G)	uridine diphosphate(glucose)
UTP	uridine triphosphate

General Introduction

"Our continued ignorance of the site of action of boron in plants is remarkable because it has been known to be essential for growth since 1923."

E.J.Underwood (1975).

Arnon and Stout (1939) listed three criteria by which the essentiality of an element could be judged. Hewitt and Smith (1975) have re-expressed these criteria as follows:

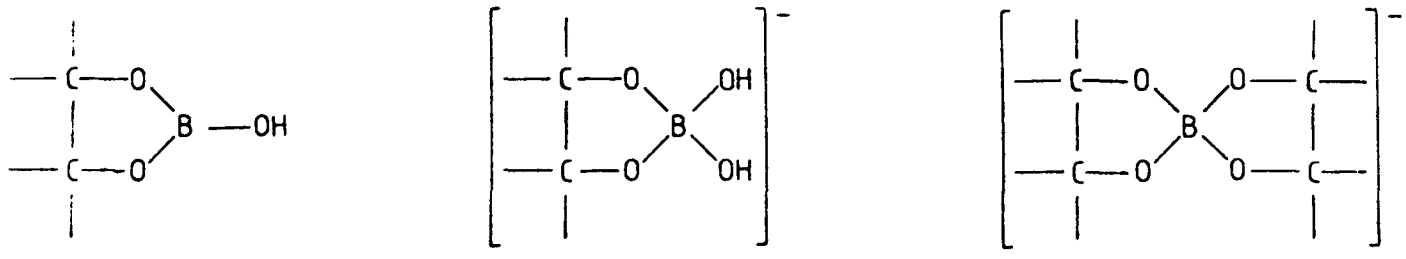
1. Omission of the element must directly cause abnormal growth or failure to complete the life cycle, or premature senescence and death.
2. The effect must be specific and not fulfilled completely by any other element.
3. The effect must be direct on some aspect of growth or metabolism and not indirect, as for example by antagonism of the effect of another element present in relative excess.

The first suggestion of the essentiality of boron and demonstration of its beneficial effects on plant growth was made by Agulhon in 1910(a,b), working with a wide variety of plants. There was considerable interest in boron at that time as a toxic contaminant of fertilisers; but reports of its beneficial nature in small quantities persisted and Mazé (1915,1919) concluded it was necessary for the growth of maize (Zea mays). Warington (1923) is regarded as the first to demonstrate the essential nature of boron in her work on broad beans (Vicia faba) and a number of other plants, describing root tip thickening and death of the growing points among the symptoms of boron deficiency.

Boron is now regarded as essential for all angiosperms and gymnosperms and all pteridophytes studied to date (reviews by Shkol'nik, 1974, Jackson and

Chapman, 1975, Augsten and Eichhorn, 1976 and Dugger, 1983). Bryophytes do not seem to have been studied but some algae and bacteria (including blue-green algae) require boron (see e.g. Eyster, 1952, Lewin, 1966 and Gerloff, 1968). Many other algae and bacteria do not require boron (e.g. Lewin, 1966; Dear and Aronoff, 1968) and a boron requirement has not been demonstrated for any fungi or animals (Bowen and Gauch, 1966; West, 1981). Angiosperms have been the most widely studied of all the plant groups and consequently have the largest body of information available on their response to boron deficiency. One dicotyledonous plant (sunflower, Helianthus annuus) and one monocotyledonous plant (maize) have been chosen for this study by virtue of their boron sensitivity within those groups. Although we still do not know the site of action of boron nine years after Underwood's quotation at the start of this section, it is accepted that the reason for boron's essentiality is connected to its ability to complex with compounds containing cis-hydroxyl groups, first described by Klein, (1878) and suggested as a mode of action by Schmucker (1934). Three classes of complex can be formed as shown below (after Boeseken, 1949):

I II III



With the transfer of a seedling from a boron sufficient (+B) to a boron free (-B) nutrient solution, 'soluble' borate will start to diffuse out of the root tissue and will no longer be available as a complexing agent. Also, B will be removed from exchangeably-bound sites in the cell wall, membranes and cytoplasm. How physiologically important these changes are, and which processes are affected first, are of course the subjects of the debate about the 'primary' site of boron's action.

The time it takes for these effects to become visible is very short and partly determined by the nature of the process under study. For example, an effect on cell division in the diatom Cylindrotheca fusiformis, which has a cell cycle time of 24 h, will take longer to become discernible than an effect on ^{86}Rb uptake (Smyth and Dugger, 1980). The root tip is the first part of an angiosperm to show deficiency symptoms and some of the earliest reported effects of boron deficiency on root tips are listed in Table 1 (p. 5).

Monocots have a lower boron requirement than dicots and so deficiency symptoms typically take longer to show. Maize is regarded as being very boron sensitive for a monocot and metabolic effects have been reported after 15 h without boron, although growth effects are not recorded until after 4 d deficiency (Parr, 1980); in other monocots, effects may not be observed until flowering (e.g. Shkol'nik, 1974).

During the early stages of boron deficiency the effects can be corrected by resupplying borate to the plant. This effect is at least as rapid as the removal - in 1928 Sommer and Sorokin reported that only 6 h resupply was needed to restore meristematic activity in the boron deficient root tip of the pea. Pollard, Parr and Loughman (1977) demonstrated that one hour's resupply was sufficient to restore the phosphate uptake capacity of maize roots.

Observations on the effect of borate resupply are open to the criticism that the flooding of tissue with a complexing agent may induce alterations unrelated to its role in the natural state. However, if the effect of such an addition is to restore a complicated metabolic or developmental process towards its pre-deficiency level then the criticism may be regarded simply as a caveat.

The search for the primary role of boron has been a matter of considerable interest from the very first suggestion of its essentiality (witness the

Table 1. Some early effects of boron deficiency on root tips

Observed effect	Time -B	Species	Ref.
Decreased root elongation	3 h	Broad bean, Vicia faba	1
	3-6 h	Sunflower, Helianthus annuus	2,3,4,5
	3-6 h	Squash, Cucurbita pepo	6,7,8,9
	6 h	Tomato, Lycopersicon esculentum	2,10
Decreased mitotic index	3-6 h	Broad bean	1
	6 h	Squash	11
Increased incorporation of P into nucleic acids	4 h	Broad bean	12
Increased incorporation of RNA precursors	6-24 h	Mung bean, Phaseolus aureus	13
Decreased incorporation of thymidine into DNA	6 h	Squash	8,9
	6 h	Sunflower	4
Cell wall abnormalities	6 h	Sunflower	5,14,15
	8 h	Tomato	16
Increased Golgi activity	8 h	Tomato	16
Increased IAA oxidase	6-9 h	Squash	6

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11. Cohen and Albert, 1974
12. Cory and Finch, 1966
13. Chapman and Jackson, 1974
14. Hirsch, Pengelly and Torrey, 1979
15. Hirsch, 1980
16. Kouchi and Kumazawa, 1976

title of Agulhon's thesis, 1910a). Three theories which could account for the effects seen in Table 1 are among those discussed in recent reviews (Dugger, 1983, Parr and Loughman, 1983, Pilbeam and Kirkby, 1983).

1. Auxin and/or phenol accumulation. Coke and Whittington (1968) and Shkol'nik (1974) are among those who advocate that the primary role of B is to prevent the toxic accumulation of these compounds.

2. Carbohydrate metabolism. Dugger (1983) continues to maintain that the presence of B is necessary for the proper functioning of carbohydrate metabolism. The possible control points at which B may act are discussed further in section 3.

3. Membrane function. Several groups now acknowledge that a direct interaction of B with glycolipids or glycoproteins in membranes could affect the activity of membrane-bound enzymes involved in the above processes. This would account for the rapid reversibility demonstrated in some cases (see Parr and Loughman, 1983, and Tanada, 1983).

The experiments reported here have not been designed to test hypotheses about the role of B. This thesis is concerned with deepening our understanding of some of the rapid responses to the removal of boron from the root tip's growth medium and then seeking to observe similar responses in protoplasts derived from that tissue.

The main processes studied are K^+ and P_i uptake and cell wall synthesis, set in the context of any growth disturbances in each plant.

The use of protoplasts offers many advantages for the study of plant cell physiology. In work on leaf protoplasts, Kuhn and Stumpf (1981) comment on the usefulness of a homogeneous population of higher plant cells with ready access of materials to their immediate environment. Factors which are closely interrelated in root tips may be separable in protoplast studies. For example in the case of ion uptake, alterations to the amount of cell wall material, the growth rate and the root tip morphology can

affect ion flux as a secondary effect. The protoplast by contrast is wall-less and accumulation of ions should provide a more direct measure of the activity of the uptake process at the cell level.

Similarly it is hard to study the process of cell wall deposition around the cells of intact roots. Protoplasts will regenerate a wall under appropriate conditions and this process lends itself to observation and manipulation.

Thus if early responses to boron deprivation can be shown for protoplasts derived from root tips, similar to those observed in the root tips themselves, the strong implication will be that the removal of boron can effect changes at the cell level and does not absolutely require an organised tissue response (although that does occur). Once that has been established, protoplast preparations could provide a means for very short term studies of supply and withdrawal of boron without the leaching time required for intact root tips and without the presence of reserves of boron bound in the cell walls. The system would thus be ideally suited to the testing of hypotheses about boron's primary role.

Protoplast research offers the capacity to study most of the earliest responses to deficiency observed in the root tip over the 0-3 h time scale required. Cell cultures have been used in the past (Heller, 1949; Koblitz, 1955; Wilson, 1961; Dutta and McIlrath, 1964; Yih, Hille and Clark, 1966; Krosing, 1978a) with similar anticipated advantages. However such cultures frequently develop polyploid cells which may alter the nature of their response to early deficiency and, of particular interest for this study, ion fluxes are still affected by the presence of the cell wall, whose synthesis may itself be affected by boron deficiency.

The first enzymatic isolation of higher plant protoplasts was from tomato roots (Cocking, 1960). However, until relatively recently protoplast research has been concentrated on source material such as leaves. The

advantages of leaves include the homogeneous tissue from which the epidermis can be readily removed and the walls readily digested. Root protoplast work has been hampered by low yields because of the 'limited activity of cellulase on root cells, when used alone and in combination with pectinase' (Xu, Davey and Cocking, 1981). Frequently, digested tissue was teased apart to promote protoplast release which resulted in the formation of large amounts of debris. It was not until the work of Lin (1980) and Senn and Pilet (1980) that high yields of debris-free protoplasts could be prepared. Their success stemmed from the inclusion of hemicellulase in the wall digesting enzymes and, in Lin's case, the laborious but effective method of slitting the epidermis of each root lengthwise before digestion to facilitate enzyme entry and protoplast release. Since then the range of plants yielding successful root protoplast systems has been extended dramatically.

The potential advantages of root protoplast systems spelt out above are countered by a major drawback. This is the question of to what extent protoplasts can be expected to behave in the same way as the cells in the root behaved before isolation (Burgess, 1978; Galun, 1981). Some of the possibly harmful steps in protoplast preparation are shared with studies using excised root tips, e.g. the initial wounding stimulus caused by cutting the root tip. Also in ion absorption studies root tips pose a similar problem to protoplasts in that the long distance source/sink involvement in the flow of nutrients has been interrupted; the problem is of course more severe in protoplasts where even short range gradients are abolished and all symplastic communication is prevented. However other problems are peculiar to the process of protoplast preparation: plasmolysis of the tissue and the digestion of the cell wall with enzymes contaminated with proteases (Morris, Linstead and Thain, 1981); the loss of some cell contents, including both ions and macromolecules, during the

isolation process when cells are known to be leaky (Taylor and Hall, 1976; Morris et al., 1981); and the onset of de-differentiation (Fleck et al., 1979; Ruzicska et al., 1979).

The loss of the cell wall and substitution of a dense osmoticum to prevent lysis place additional stresses on the protoplasts. The cell wall has more than simply a mechanical role in the intact plant- for example, it is a site for cation exchange. The concentration of ions to which the plasma membrane is normally exposed is very different from the external nutrient medium and buffered against sudden changes. This may be particularly important for boron itself. The ratio of toxic to sufficient levels of boron is the lowest of all the essential elements (Reisenauer, Walsh and Hoeft, 1973). Since boron is normally associated with the cell wall (Smith, 1944; Skok and McIlrath, 1958; Bowen and Nissen, 1976) it is quite conceivable that the amount of boron supplied to the protoplast may approach levels with toxic effects on the membrane.

Much research has been done to try to quantify the impact of the isolation process on cell physiology. Burgess (1978) concluded that the wall-less protoplast should be regarded as a highly stressed system; so for each process studied in the following sections the extent to which protoplast behaviour is believed to reflect the pre-isolation situation in the root tip will be reviewed.

As a general rule it should be apparent that comparisons between +B and -B protoplast preparations or +B and -B root preparations are much easier to interpret than comparisons between roots and protoplasts. In fact, whereas growth effects make it difficult to obtain physiologically matched roots from boron sensitive plants such as sunflower, protoplast preparations should in theory be simpler to match; a single population of protoplasts can be divided and the effect of altering the boron status studied.

Section 1. Plant growth and protoplast characteristics.

Introduction.

In order to interpret the physiological effects of boron deficiency on ion uptake and cell wall deposition some understanding of the tissue systems used to study these processes is required.

The root tip is a heterogeneous tissue with a succession of cell types from meristematic cells to mature differentiated cells in the space of a few millimetres. The physiological activity of the cells therefore depends on their position relative to the apex (e.g. Chong, Senn and Pilet, 1983). In maize this succession of cell types has been characterised in terms of distance from the apex:

0-0.5 mm	root cap
0.5-1 mm	meristem
2-4 mm	zone of maximum elongation
4-10 mm	zone of differentiation

(Senn and Pilet, 1980; Pilet and Senn, 1980)

The same succession of cell types occurs in sunflowers. The root tip is much thinner, and the meristem is open whereas it is closed in maize (Clowes, 1981).

With the onset of boron deficiency root growth slows in all dicots and some monocots (see e.g. Shkol'nik, 1974). In physiological terms this decreased growth is very important; Warington (1926) was the first to point out that cells of broad bean plants at the same position relative to the apex have differing physiological age depending on the boron status of the root.

Different workers have reported greater sensitivity of either cell division or cell elongation to boron deficiency. It can be hard to tell

whether this is a result of genuine species differences in the response, working with intact root tips. For example in squash plants cell division is reported to be more sensitive to boron deficiency than cell elongation. Cell division and the incorporation of ^{3}H-thymidine into DNA are reported to stop within 20 h of the withdrawal of boron whereas cell elongation continues into the apical meristem giving rise to long narrow cells in that region (Cohen and Albert, 1974; Cohen and Lepper, 1977). By contrast, in the broad bean, cell elongation seems to be more sensitive; meristematic cell divisions slow but cell elongation is also reduced and cells expand and divide in a radial direction (Warington, 1926; Whittington, 1959; Neales, 1960; Robertson and Loughman, 1974b). Slack and Whittington (1964) and Kouchi (1977) suggested that cell elongation had slowed before division was seen to be affected.

The broad bean and squash plants may in fact be responding identically, but reported from two different points of view. Those working with broad beans looked for the first sign of a decrease in an activity (cell elongation) whereas Cohen et al. put more weight on the cessation of an activity (meristematic cell division). Thus although cell elongation slowed in the broad bean it continued into the apex so that the cells there became longer than the controls, just as in squash. Also, as Kouchi (1977) noted, the length of the cell cycle (20 h) would delay the detection of a decreased mitotic index.

Boron deficiency appears to promote cell division further back in the dicot root and initiate lateral branching. This may be a secondary effect resulting from the disruption of normal apical activity, similar to the situation in shoots where boron deficiency appears to promote cambial tissue divisions which lead to stem swelling near the apex (Bussler, 1981). Various methods have been tried to test whether this is a direct result of the withdrawal of boron or a secondary effect. Krosing (1978a,b)

demonstrated that excised and cultured sunflower and carrot (Daucus carota) cambial tissue does not show increased cell divisions in the absence of boron, implying that an organised tissue response was required for the effect; and he demonstrated that destruction of the apical meristem with a red hot needle mimicked the effect of boron deficiency in stimulating cambial cell divisions. Similarly, Yih, Hille and Clark (1966) showed that the absence of boron inhibited rather than promoted cell division in Ginkgo pollen-derived tissue culture. Cotton (Gossypium hirsutum) appears to behave differently. Birnbaum, Beasley and Dugger (1974) observed that cotton ovules cultured without boron callused, owing to rapid cell divisions, and fibre cell elongation was markedly reduced. Jarvis, Ali and Shaheed (1983) showed that the initiation of adventitious roots by mung bean hypocotyls in the presence of exogenous IAA or IBA requires the inclusion of boron in the rooting medium. Robertson (1974) showed that none of a range of root growth inhibitors tested exactly mimicked the effect of boron deprivation on radial cell expansion, although some promoted the formation of lateral roots close to the apex. It is difficult to predict what the behaviour of protoplasts will be from these studies.

With respect to the behaviour of the plants chosen for this study, the consensus of the literature is that in sunflower roots, by 3-6 h -B root extension is impaired (Kouchi and Kumazawa, 1975a; Hirsch and Torrey, 1980). By 6 h -B mitosis is reduced (Moore and Hirsch, 1981; Hirsch et al., 1982). The weight of the boron deficient root tip increases as the tip swells (Hirsch et al., 1982) owing to radial cell expansion (Hirsch and Torrey, 1980).

Studies on sunflower roots at the ultrastructural level have been done by Starck (1963) and Hirsch and Torrey (1980). Both groups reported an increase in the thickness and/or irregularity of cortical cell walls 1 mm

behind the root tip. Hirsch and Torrey (1980) also reported increased vesicle (plasmalemmasome) formation at the cell wall/plasmalemma interface after 6 h -B. By 20 h many of the mitochondrial cristae were hypertrophied and the cells were depleted of polysomes. The ultrastructural observations on sunflower roots have been re-examined in this thesis.

In maize the depression of root growth is not nearly as severe as in sunflowers (Shkol'nik, Krupnikova and Smirnov, 1981) and takes longer to develop (Shkol'nik, Krupnikova and Dmitrieva, 1964). Parr (1980) reported no gross morphological effects on the root tips after four days of culture without boron. He also studied cortical cell ultrastructure and observed an increase in the thickness and irregularity of the cell walls by 4 d -B and an increase in the frequency of plasmalemmasomes.

Isolating protoplasts from root tips gives rise to a mixed population of cells from the different regions of the root (see e.g. Chong, Senn and Pilet, 1983). Protoplast yields from root tissue are commonly of the order of 5-10% of the cells (Lin, 1980). It is therefore apparent that differences between protoplasts isolated from boron deficient roots and protoplasts from boron sufficient roots may reflect differences in their site of origin rather than effects of boron deficiency. This should be borne in mind during the discussion of the results which follow.

Materials and methods

1. Plant growth.

Seeds were briefly (ca. 2 min) surface sterilised in 8-14% NaOCl solution and rinsed thoroughly with sterile distilled water. They were then sown into plastic trays, between four layers of moist absorbent paper, and covered with aluminium foil. The seeds were germinated at 25°C for 2-3 d. Seedlings were transplanted to metal racks in aerated 1 ℓ baths of nutrient solution at pH 6.0 (modified from 1/10 strength Arnon (1938)):

$Ca(NO_3)_2$	0.4 mM	$MnCl_2$	0.9 µM
$MgSO_4$	0.2 mM	$ZnSO_4$	0.08 µM
$NH_4H_2PO_4$	0.2 mM	Na_2MoO_4	0.05 µM
K_2SO_4	0.3 mM	$CuSO_4$	0.03 µM
$FeSO_4$	10 µM		
Na_2EDTA	10 µM	H_3BO_3	4.6 µM (='+B')

All chemicals and reagents came from BDH or Sigma unless otherwise specified.

Seedlings were cultured in a growth cabinet at 25°C and 40% relative humidity. Lighting, by a mixture of tungsten filament lamps and 'warm white' fluorescent tubes in a 16 h day/8 h night regime, provided 180 $\mu E.m^{-2}.sec^{-1}$ of photosynthetically active radiation.

To transfer plants to boron-free (-B) solutions the whole rack of plants was transferred to a fresh bath of -B solution for 30-40 minutes, rinsed with -B water and transferred again to fresh -B solution. Boron-sufficient (+B) plants were transferred to fresh +B solutions in the same way to ensure that any response could be ascribed solely to the change in boron status. Growth period after 2-3 d germination detailed in text.

2. Boron free conditions.

Boron-free water was prepared by passing de-ionised water through an Elgacan C114 mixed-bed ion exchange column (Gallenkamp) at a maximum flow rate of 1 $\ell.h^{-1}$. Conductivity of the boron-free water was always < 1 $\mu mhos.cm^{-1}$. Attention was paid to the risk of contamination by boron found in sweat, dust, reagents, filter paper and boro-silicate glass. Accordingly all work was done in plasticware; new vinyl gloves were worn for all experimental work; and all reagents were prepared in boron-free water. Chemicals were tested periodically for boron contamination.

3. Boron determination.

The boron content of solutions and plant material was determined by a modification of the simplified curcumin procedure of Dible, Truog and Berger (1954).

3.1 Plant material

About 10 mg oven-dried plant material was ground and weighed. One part of dry $Ca(OH)_2$ to two parts plant material was stirred in and the mixture was ashed in a platinum crucible over a bunsen burner or in a muffle furnace (550°C maintained for 1 h). The grey-white ash was dissolved in 1 ml 0.4 M HCl and diluted to 2 ml with boron-free water. One ml aliquots were treated as below. B standards were ashed and assayed in the same way.

3.2 Solutions

Four ml curcumin reagent (0.04 g curcumin and 5 g oxalic acid dissolved in 100 ml 95% ethanol) were added to each 1 ml test solution in plastic beakers. The solutions were mixed thoroughly and evaporated at 55°C. The dry residues were baked for exactly 15 min and then cooled. After adding 25 ml 95% ethanol, the solutions were filtered through Whatman no.2 filter paper and the absorbance read at 540 nm on a Pye-Unicam SP8-100 UV/Vis spectrophotometer. B standards were run each time.

4. Protoplast isolation

Root tips were cut into buffer (25 mM 2[N-morpholino]ethanesulphonic acid (MES), 5 mM $CaCl_2$, 0.5 mM dithiothreitol (DTT), 0.25% bovine serum albumin (BSA) and 10 µM phenylmethylsulphonyl fluoride (PMSF) in 0.01% n-propanol (final conc.), pH 6.0) and weighed. Tips were transferred to incubation medium (2% Cellulysin* (Calbiochem-Behring Corp.), 0.1% Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Japan), 5 mM $CaCl_2$, 0.5 mM DTT, 0.25% BSA, 10 µM PMSF in 0.45 M mannitol at pH 5.5) and slit (lengthwise for maize, but in 3 mm lengths for sunflower). The tissue was incubated at 30°C in the dark (20 ml incubation medium per 3 g roots), shaken continuously at 75 oscillations per minute.

Protoplasts were removed from the enzyme mix after 4 h incubation by filtration through two layers of nylon mesh (pore diameter approx. 150 µm) and centrifugation (300 'g', 5 min). The debris retained by the mesh was re-incubated for 30 min and then re-filtered to recover more protoplasts. Pooled protoplast pellets were washed with protoplast suspension medium (PSM, i.e. 25 mM MES, 5 mM $CaCl_2$, 0.5 mM DTT in 0.5 M mannitol, pH 6.0) and centrifuged at 300 'g' for 5 min. Pellets in suspension medium were mixed with stock iso-osmotic Percoll* (Pharmacia Fine Chemicals) (SIP; prepared by adding nine parts (v/v) Percoll to one part (v/v) 10x concentrated suspension medium) to yield 7 ml of a 55% by volume SIP solution. This was overlaid with three 7 ml layers (40%, 25% and 0% SIP respectively) in a transparent polycarbonate centrifuge tube. Viable protoplasts banded at the 0-25% interface after centrifugation at 300 'g' for 15 min. These were carefully collected with a Pasteur pipette, pelleted and run on a second Percoll gradient as before. Finally protoplasts were washed twice with PSM (300 'g', 5 min). (Method modified

* Registered trademarks

from Lin, 1981).

Protoplast viability was assayed with fluorescein diacetate (FDA) (Widholm, 1972). 0.1 ml of a 5 $mg.ml^{-1}$ FDA solution in acetone was added to 9.9 ml of PSM. A drop of this solution was mixed gently with a drop of protoplast suspension on a slide. The suspension was viewed 5-30 min later on a Leitz Dialux 20EB microscope using phase contrast, with normal or UV illumination (λ = 325-480 nm from an HBO 200W/4 mercury vapour lamp, fluorescence at $\lambda > 500$ nm observed). Intact, viable protoplasts fluoresced brightly and the % viability could be readily determined.

5.1 Cell counts.

Ten-15 root tips were macerated in 5 ml 5% chromic acid in 1 M HCl for 24 h at room temperature (method after Brown and Broadbent, 1951 and Lovatt, Albert and Tremblay, 1981). Cells were separated by repeatedly drawing the suspension into a syringe and expelling it vigorously. Six replicate samples were counted in a 0.2 mm deep modified Fuchs-Rosenthal counting chamber viewed in a Swift phase contrast microscope.

5.2 Protoplast counts.

Five replicates of each protoplast suspension were counted in an Improved Neubauer haemacytometer, 0.1 mm deep, viewed as above.

6. Transmission electron microscopy.

Root tips were fixed in a solution of 3% glutaraldehyde, 0.5% formaldehyde, 0.1 M sodium cacodylate, 1 mM $CaCl_2$, pH 5.8, for 4 h at 4°C (or 75 min, room temperature). Tips were then washed in three changes of sodium cacodylate buffer for a total of 1 h and post-fixed in 0.5% OsO_4, 0.1 M sodium cacodylate, 1 mM $CaCl_2$, pH 5.8 for 75 min at room temperature. Fixed tips were dehydrated in an ethanol series: 30%, 50% for

15-20 min each; 70% overnight at 0-8°C; then 95%, 100% and 100% for 30-40 min each. Tips were embedded in Spurr's medium (1:1 mix with ethanol for 1 h, 3:1 for 1 h and full strength resin for 7-10 h). Block moulds were half filled with resin and polymerised at 70°C for 11 h; then the root tips were added and oriented with the aid of a dissecting microscope. The filled blocks were polymerised for 11-15 h at 70°C. (Method from Glauert, 1975; chemicals from Agar Aids).

Blocks were sent to Dr M. Čiamporová (Slovak Academy of Sciences, Bratislava, Czechoslovakia). Transverse sections were cut 1 mm behind the apex, stained with 1% uranyl acetate for 1 h followed by lead citrate for 10 min (Venable and Coggeshall, 1965) and observed with a Tesla BS 500 microscope operating at 60 kV. Micrographs of identified regions of the transverse sections were then returned to this laboratory for analysis.

Areas of cells or organelles on the enlarged micrographs were measured by counting squares with a grid overlay marked out in 1 cm^2 units. In cases where micrographs overlapped care was taken to count areas once only.

Results and Discussion

1. Plant growth.

Earlier work in this laboratory showed that broad beans had sufficient seed reserves for the first four weeks of growth, with the exception of Ca and B (Robertson, 1974). They could therefore be grown in $Ca(NO_3)_2$ ± H_3BO_3. Preliminary experiments with 'Polestar' sunflowers demonstrated that the addition of B to $Ca(NO_3)_2$ increased shoot growth, but the effect was much more marked if plants were grown in complete nutrient solution, so this method was adopted thereafter (figs.1, 2, pp. 20, 21). The level of boron supplied to +B plants was kept deliberately low (4.6 µM) to facilitate the development of deficiency symptoms on transfer to -B media. It was still within the normal range of B concentrations in the soil solution, i.e. 1-8 µM (Mengel and Kirkby, 1978). The rate of root growth declined immediately upon the withdrawal of boron in the Single Tall sunflowers used most commonly (fig.3, p. 22). In many experiments a statistically significant 10% reduction in root length compared with controls was recorded only three hours after transfer to boron-free solutions. Another sunflower cultivar, 'Fransol', was more boron sensitive; root growth stopped almost immediately on withdrawal of B. 'LG11' maize plants, by contrast, did not show a significant reduction in the rate of root extension over a 31 h period (fig.4, p. 22).

Sunflower and maize root tip weights were recorded in many experiments. Fresh weight measurements were done after a standard blotting procedure. In the few experiments where roots could not be weighed at identical times after removal from solutions, water loss was corrected for; this was quite rapid, of the order of 60 $\mu g.min^{-1}.cm^{-1}$ of root.

Single Tall sunflower root weights were very variable. Plant age, tank aeration and planting density all contributed to the variation between

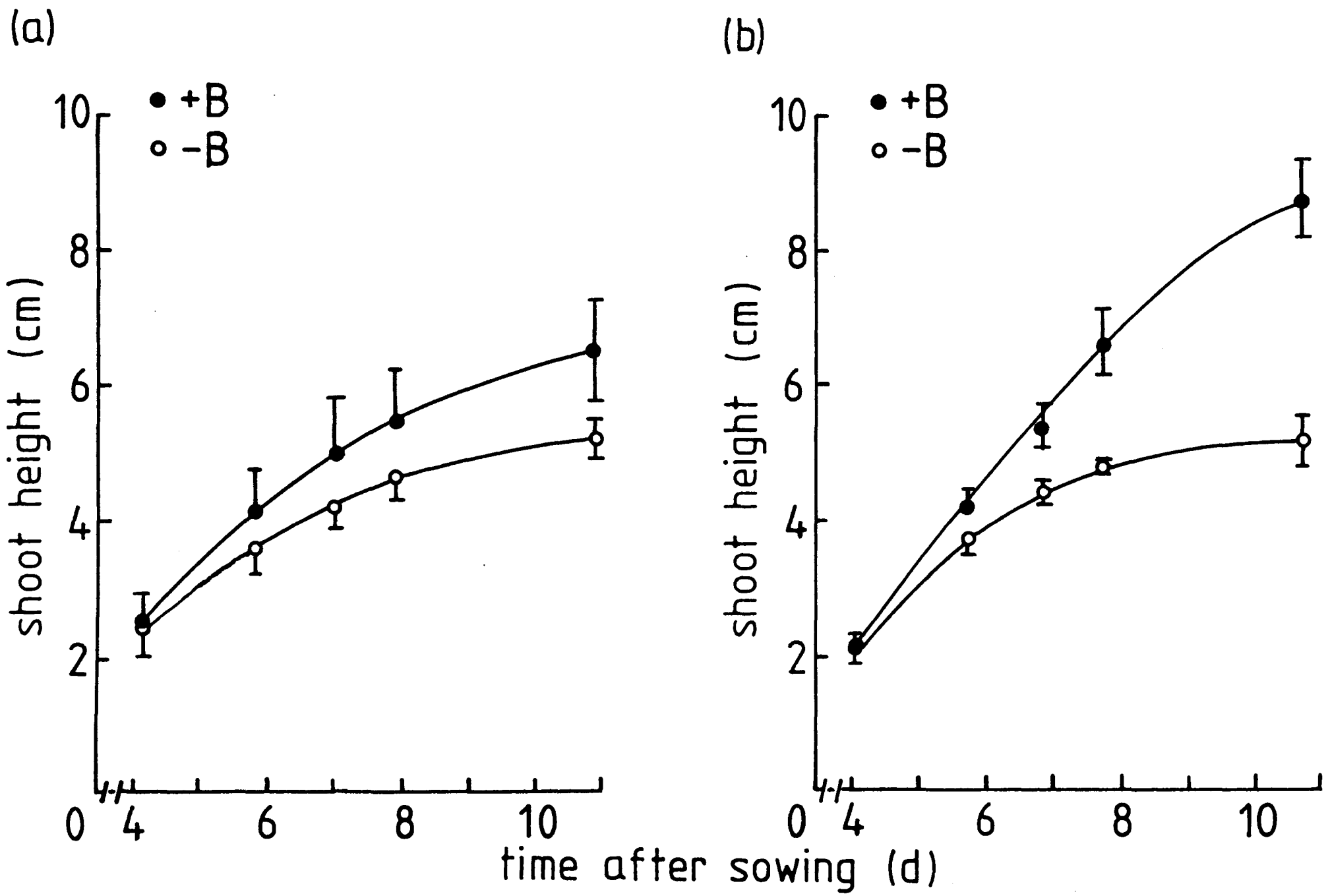


Fig. 1. Effect of boron supply and nutrient status on shoot elongation in 'Polestar' sunflowers. Plants grown in (a) 0.4 mM $Ca(NO_3)_2$ or (b) complete nutrient solution. +B plants supplied with 4.6 µM H_3BO_3.

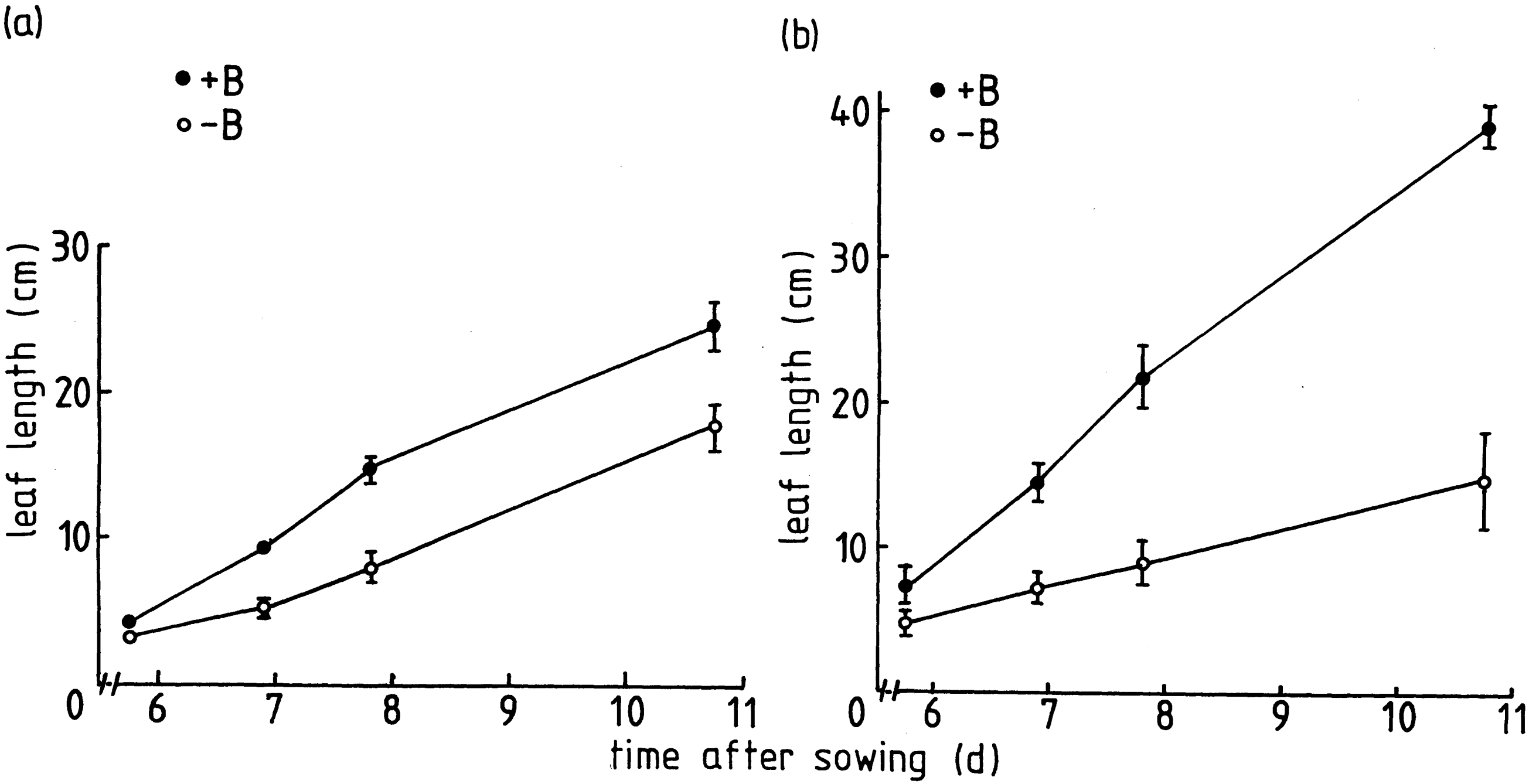


Fig. 2. Effect of boron supply and nutrient status on extension growth of the first leaf in 'Polestar' sunflowers. Plants grown in (a) 0.4 mM $Ca(NO_3)_2$ or (b) complete nutrient solution. +B plants supplied with 4.6 µM H_3BO_3.

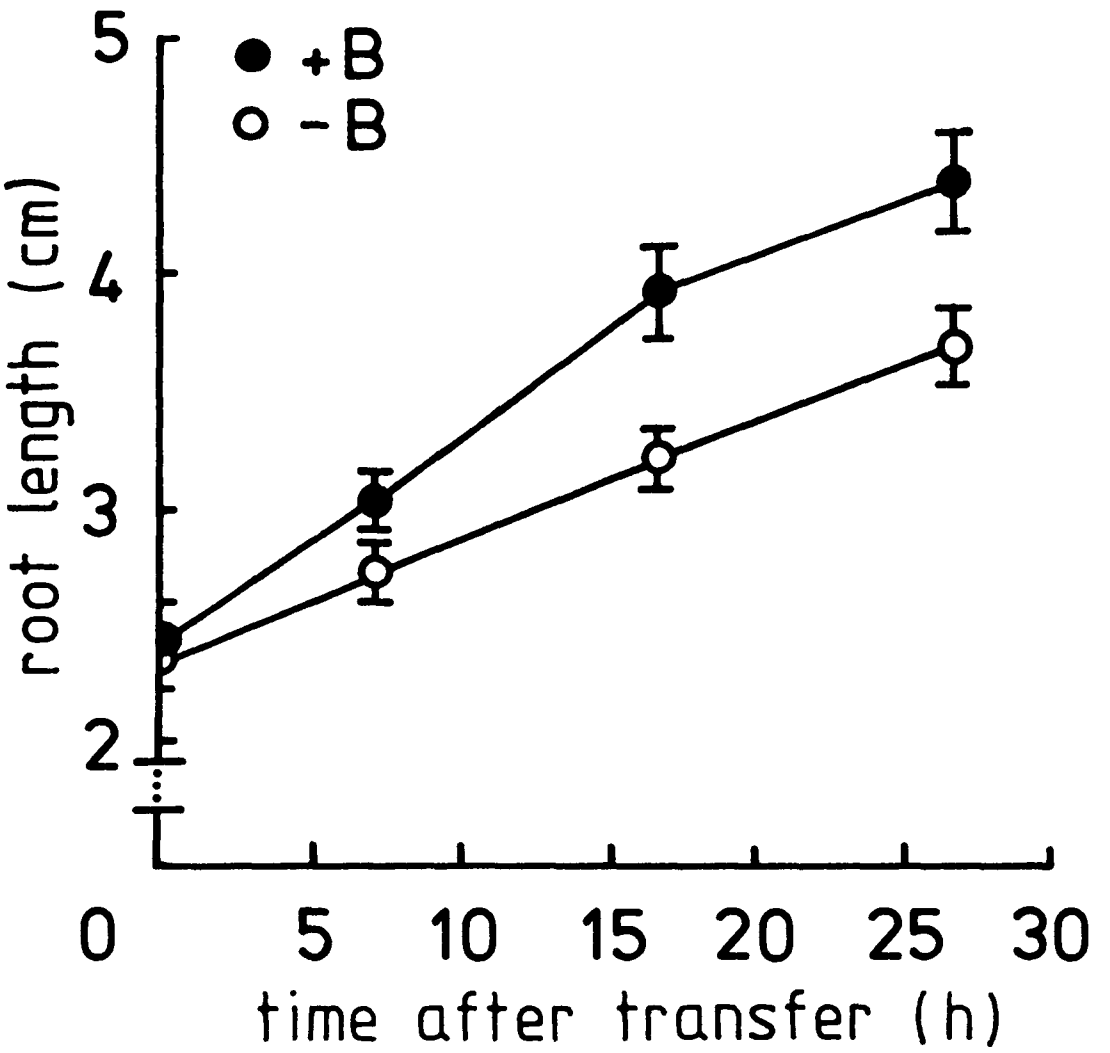


Fig. 3. Effect of boron supply on root growth in 'Single Tall' sunflowers. Plants were grown for 30 h +B (4.6 µM H_3BO_3) and then transferred to +B or -B solutions for the times shown. Root length was measured from the swelling which marked the transplantation point.

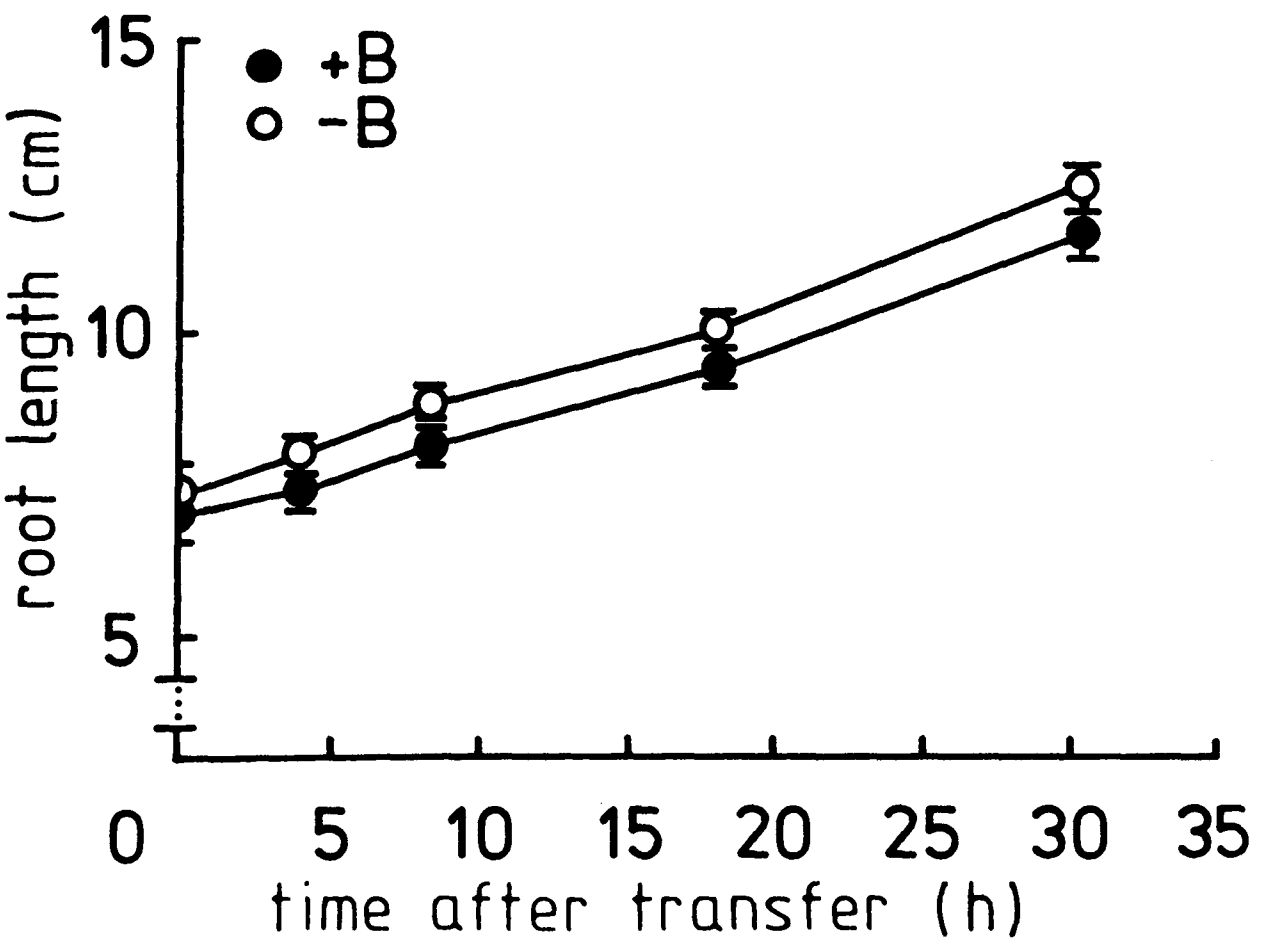


Fig. 4. Effect of boron supply on root growth in 'LG11' maize. Plants were grown for 2 d +B (4.6 µM H_3BO_3) and then transferred to +B or -B solutions for the times shown. Whole primary root length measured.

experiments, but a major source of variability was the impossibility of starting boron treatments with batches of plants matched for their root tip weights. Nevertheless, trends could be discerned. Significant weight increases of up to 95% were observed after 15 h -B in 2 cm tips; the greatest weight gains could have been due to primordia initiation but visibly branched tips were never used. When 0-1 and 1-2 cm segments were considered independently, each section was usually 20-35% heavier after 12-24 h -B. 0-5 and 0-10 mm tips appeared to show significant weight increases after 3.5-4.5 h in some experiments; but in these cases the effect was caused by unusually light +B tips. In maize, root tip weight analysis revealed no significant weight differences between +B and -B treatments over the first 24 h of deficiency, using 0-5, 5-10, 0-10, 10-20 and 0-20 mm segments.

These observations on sunflower and maize root extension growth and tip weights agree qualitatively with previously published findings (see Introduction).

Cell counts were performed (table 2, p. 24) which suggest there was little effect on cell number at these times in both sunflower and maize root tips. The greater variability in the cell counts of the -B 5-20 mm zones in sunflowers was caused by there being fewer and on average heavier cells in the -B roots in two experiments but in the other two experiments there were more, slightly lighter cells, suggesting that cell expansion had been accompanied by cell division in the latter case.

In maize, the higher variability in cell counts reflects the difficulty of obtaining satisfactory cell separation. Brief sonication in a Kerry KS101 ultrasonicating water bath was tried, but was unsuitable because the improved tissue dispersion was accompanied by some fragmentation of mature cortical cells. Gronwald and Leonard (1982) obtained an estimate for the number of cells in the cortex of maize root tips; their figure of 4 x

Table 2. Cell counts in sunflower and maize root tips.

Root zone	Cell number (10^4 cells.root tip^{-1})			
	Sunflower[a]		Maize[b]	
	+B	13-16 h -B	+B	24 h -B
0-5 mm	4.5 ± 0.1	5.0 ± 0.3	10.9 ± 1.6	10.2 ± 1.3
5-20 mm	5.6 ± 0.4	5.6 ± 1.1	2.7 ± 1.5	2.7 ± 1.0

[a] 4 experiments

[b] 3 experiments

Root tips from plants grown for 2 d +B followed by +B or -B for the times shown were weighed and digested in chromic acid (see Methods). No significant difference between B treatments.

N.B. Figures in all tables are mean (± SE where appropriate).

Table 3. Sunflower and maize root cell weights.

Root zone	Approximate cell weights (ng.cell^{-1})	
	Sunflower	Maize
0-5 mm	20	25
5-20 mm	100	300-400

Approximate cell weights for plants from Table 2.

10^6 cells per g. f.wt. is within the range of the more detailed estimates here.

An approximation of mean cell weights can be derived from these counts to obtain a very rough guide for comparison with protoplast size later (table 3, p. 24).

2. Sunflower root ultrastructure.

Ultrastructural studies were performed for the sunflower roots to complement the work of Parr (1980) on maize root ultrastructure. The cells examined were located 1 mm behind the root tip of plants which had been grown for 3 d after germination, +B or -B throughout, or transferred from +B to -B solutions 5.5 h before fixation. All the micrographs were prepared in Czechoslovakia and examined in detail here. Cells were considered in layers (epidermis, hypodermis, C2, C3, etc from the outside in). The results are tabulated in table 4 (p. 26). The following generalisations can be made.

2.1 Vacuolation increased progressively from the epidermis to C4 in +B roots. By 3 d -B vacuolation had increased in most cell layers as the less active meristem resulted in more mature cells being found 1 mm behind the apex. Similar observations were made by Wyn-Jones and Lunt (1967) and Kouchi and Kumazawa (1975b). A wide variety of vacuolar inclusions was seen in +B roots (plates 1, 3, pp. 27, 28). The more vacuolate -B cells contained fewer of the darker-staining aggregates in particular. This could represent either the use of some stored reserves, or a change in the staining properties.

2.2 Dark-staining plastids (plates 1,2,3, pp. 27, 28) occupied a larger proportion of the cytoplasmic area in hypodermal and cortical cells after 5.5 h -B or 3 d -B. A similar observation was made by Hirsch and Torrey (1980) for sunflowers and by Kouchi and Kumazawa (1976) for tomato root tips.

2.3 Spherosomes (plate 4, p. 28) were more frequently observed in cells of

Table 4. Data derived from electron micrographs of sunflower root tips.

Cell layer	Cell area studied (μm^2)	Vac'n (% cell area)	Plastids (% cyto-plasmic area)	Dictyo's	Mitoch.* (number per μm^2 of cyto-plasm)	Sphero's
Epidermis						
+B	1123	12	3.4	0.07	0.22	0.006
5.5 h -B	1869	34	3.3	0.16	0.37	0.027
3 d -B	2828	61	2.3	0.15	0.26	0.007
Hypodermis						
+B	2139	17	3.5	0.03	0.20	0.023
5.5 h -B	1575	32	4.5	0.06	0.25	0.047
3 d -B	2732	41	7.1	0.07	0.32	0.024
C 2						
+B	1856	30	2.2	0.04	0.21	0.020
5.5 h -B	1621	52	3.5	0.08	0.25	0.050
-B	1622	41	5.2	0.07	0.31	0.022
C 3						
+B	1590	38	2.2	0.03	0.19	0.022
5.5 h -B	3235	45	4.5	0.05	0.21	0.052
3 d -B	1179	52	3.0	0.05	0.26	0.002
C 4						
+B	288	73	0	0.06	0.11	0
-B	1031	23	4.0	0.04	0.29	0.002

Growth details and TEM preparative methods in text.

*Mean mitochondrial size 0.35 ± 0.03 μm^2.

Plates 1-4. Transmission electron micrographs of Polestar sunflower roots; transverse sections cut 1 mm behind the apex. Plants grown for 3 d +B, 3 d -B or 66 h +B followed by 5.5 h -B ('+/-B'). H_3BO_3 supplied at 4.6 µM.

Symbols used: CW = cell wall
D = dictyosome
ER = endoplasmic reticulum
M = mitochondrion
N = nucleus
P = plastid
S = spherosome
V = vacuole

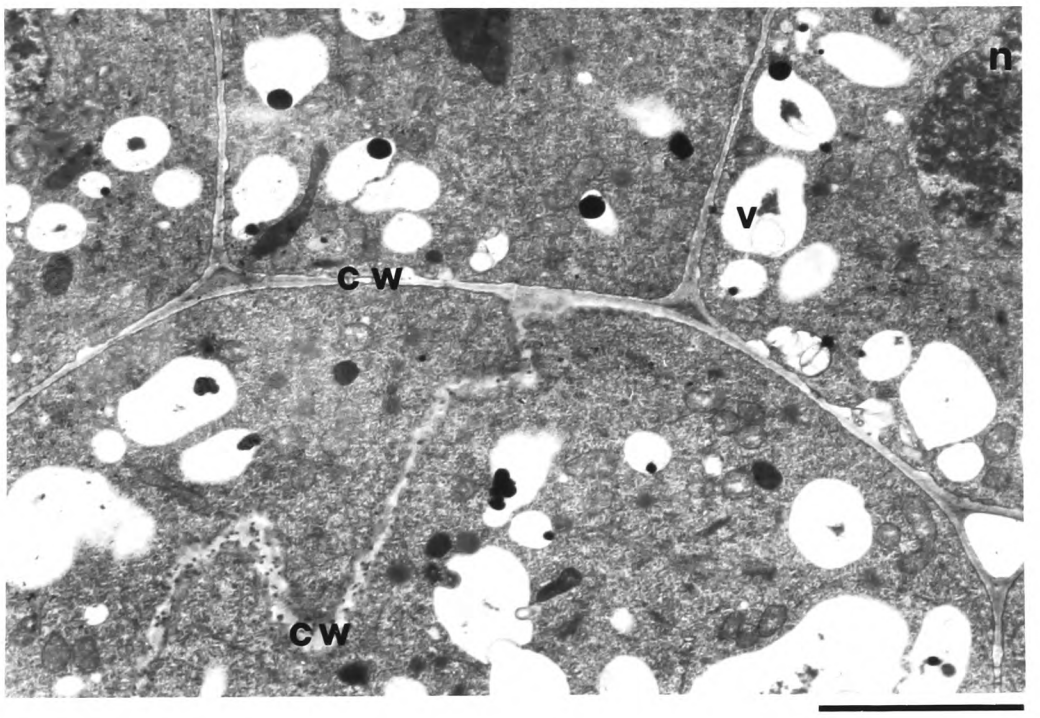


Plate 1. Hypodermal (top) and C2 cells, +B. Note vacuolar inclusions and formation of serrated cross wall. Scale bar = 5 µm.

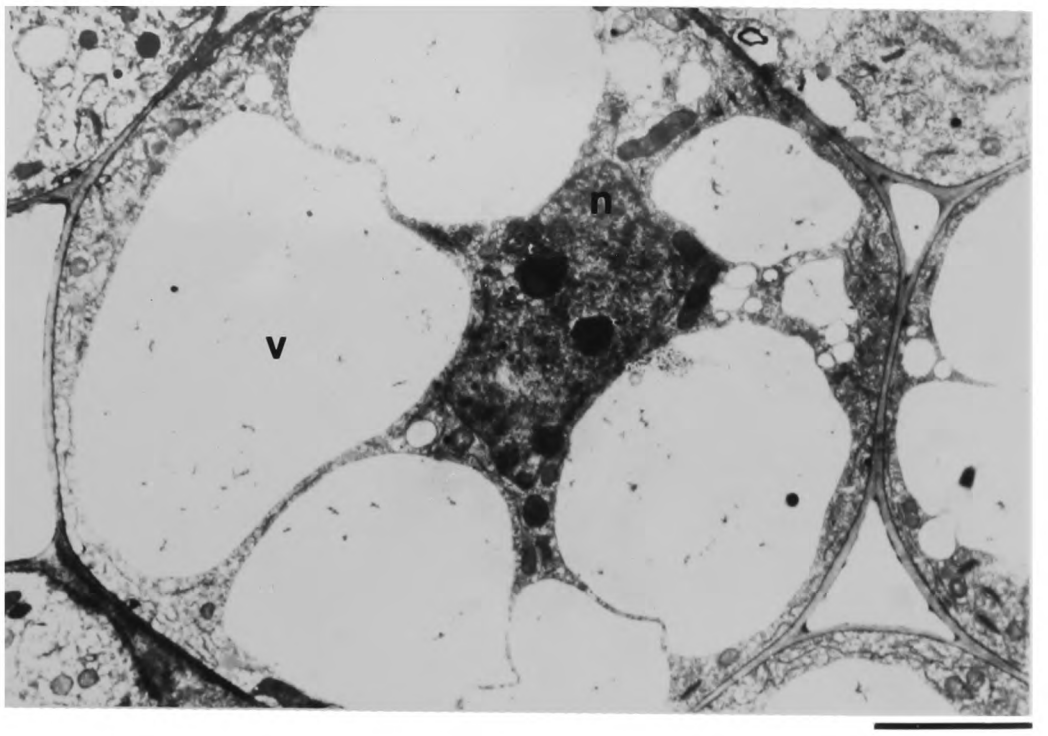


Plate 2. Highly vacuolate C2 cell, -B. Scale bar = 5 µm.

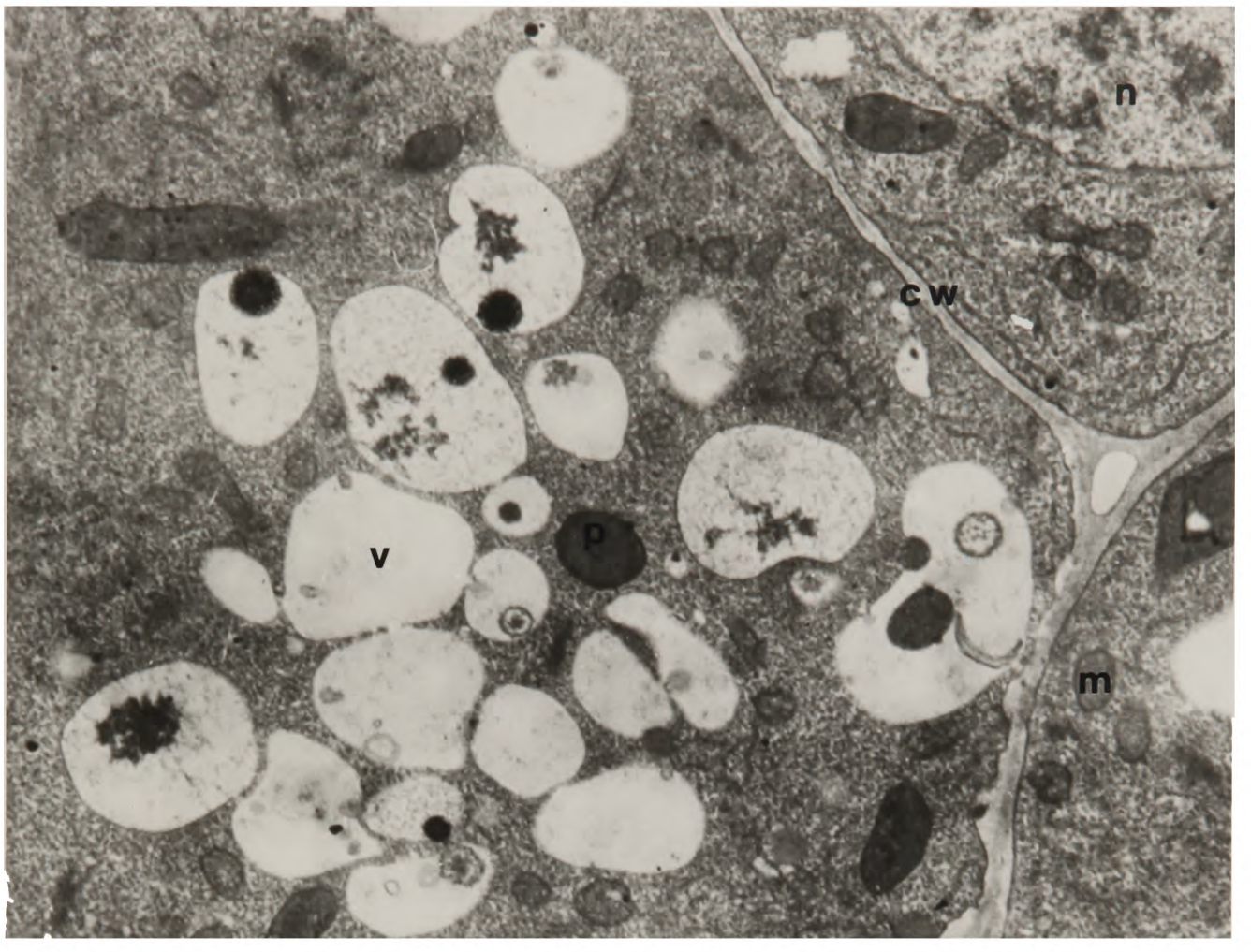


Plate 3. Parts of two hypodermal cells and a C2 cell (at right), +B, showing a variety of vacuolar inclusions. Scale bar = 1 µm.

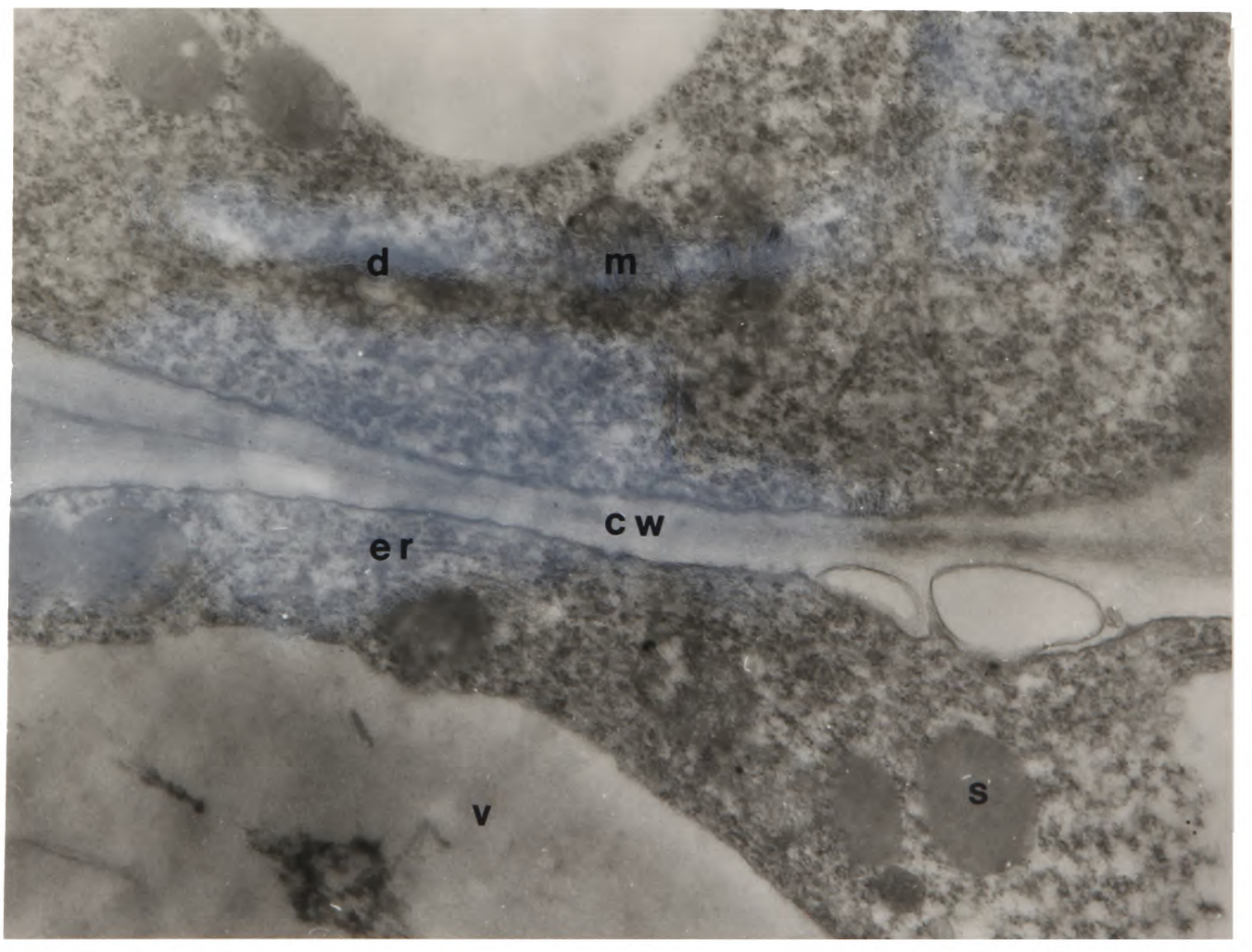


Plate 4. Part of two C2 cells, +/-B, containing spherosomes.

Scale bar = 1 µm.

each layer tested. after 5.5 h -B. By 3 d -B there were few visible in the epidermis & C3; this is symptomatic of a decline in energy reserves (Čiamporová, 1983) and is probably an indirect effect of deficiency.

2.4 Mitochondrial size and frequency were not consistently affected by deficiency although the increased vacuolation by 3 d -B meant that they occupied a smaller proportion of the area of the cell as a whole. Hypertrophy of cristae, reported by Hirsch and Torrey (1980) after 20 h -B, was not more marked than in control roots.

These observations will be supplemented by those on aspects of cell wall structure and synthesis in section 3.

An interesting observation was made of some relevance to section 2. Plasmodesmata were counted in cell walls, grouped by the nature of the cells they connected; the results are shown in table 5, (p. 30). The arrangement reflects the origin of the cells - i.e. if each epidermal cell is derived from the cell below it in a file leading back to the meristem then connections in the radial walls between epidermal cells would only be found at a point where the files divide into two. (Longitudinal sections would be required to find the density of plasmodesmatal connections between epidermal cells via their transverse walls). Nevertheless, it is clear that radial symplastic transport from the two cell layers closest to the external solution will be preferentially directed into the cortex even without a strong demand from that tissue; once within the cortex this channelling process stops.

3. Improvements in protoplast isolation

The isolation of protoplasts from root tips was initially attempted following the method of Lin (1980). Improvements to the technique were continually tested and adopted during the research. Throughout the process, careful handling of the preparations is essential (Ruesink, 1971, 1980).

Table 5. Plasmodesmatal distribution in cell walls of sunflower root tips

Cell layers linked	Length of wall studied (μm)	No. of plasmodesmata	No. μm^{-1}
epidermis - epidermis	174.5	4	0.023
epidermis - hypodermis	391.6	72	0.184
hypodermis - hypodermis	227.4	37	0.163
hypodermis - C 2	331.7	86	0.259
cortex - cortex	329.5	190	0.577

Plasmodesmata counted from transmission electron micrographs. Preparative TEM methods given in text.

3.1 Incubation medium.

3.1.1 Enzyme preparations.

Care was taken to avoid denaturing the preparation by vigorous shaking. The wall-digesting enzymes were sprinkled on top of the fully-prepared liquid medium and left to disperse. Gentle swirling was all that was required to complete the process. Enzyme preparations were made up and used immediately to avoid any diminution of activity (Kuhn and Stumpf, 1981). Initially the enzymes used were Cellulysin, and hemicellulase and pectinase from Sigma. The rate of protoplast production was speeded up by changing to Cellulysin and Pectolyase Y-23, (Lin, 1981). The latter enzyme has been shown to contain an endopolygalacturonase, endopectinlyase and an unidentified 'maceration stimulation factor' (Nagata and Ishii, 1979).

None of these enzymes was purified further as suggested by e.g. Taylor and Hall (1976) by desalting or gel filtration. Instead DTT was added to protect the disulphide bonds in the proteins; and in the later experiments, protease contaminants were effectively countered with additional substrate (BSA) and the protease inhibitor PMSF (Fahrney and Gold, 1963). BSA would also bind any phenols released into the medium during protoplast isolation (Weinbach and Garbus, 1966). The yield was not affected by the addition of BSA and PMSF but improved physiological activities were shown (see Section 2).

3.1.2 Osmoticum.

Mannitol was chosen as the non-penetrating osmoticum. It is not incorporated by higher plant protoplasts (Blaschek et al, 1981) although Berliner and Martindale (1981) observed that it will enter algal protoplasts within 6 h. Lin (1980) suggested a concentration of 0.7 M; this was decreased to 0.5 M to maximise ion uptake (Lefebvre, 1984) without effect on the yield.

3.1.3 Calcium content.

Recommended Ca^{2+} levels range from 0.2 mM (Lin, 1980) to 10 mM (see e.g. Nagata and Takebe, 1970). However, Boss and Mott (1980) showed membrane fluidity of carrot protoplasts was decreased by culture in 10 mM Ca^{2+}, but was unaffected by culture in 0.5-5 mM Ca^{2+}. Therefore 5 mM was chosen for this work; no effect on the yield of protoplasts was observed under the different Ca^{2+} levels tested.

3.1.4 Incubation time.

The wall digesting period should be kept to a minimum to limit the adverse effects of the enzymes used (Vasil, 1976). Four hours' incubation was sufficient for the 0-1 cm section of sunflower root tips. The debris retained by filtering out the protoplasts from 1-2 cm tips was reincubated for a further 30 min to complete digestion. Four hours' incubation plus 30 min for the debris was sufficient for maize too. Overnight (10 h) incubation in a half-strength medium was tried for 'Rubis' maize root cortex; the yield of 4.4 - 5.1 x 10^5 protoplasts.g^{-1} f.wt. was at the lower end of the range 4.4 - 24 x 10^5 protoplasts.g^{-1} f.wt. obtained for that tissue, so was not continued.

3.2 Tissue pretreatment.

3.2.1 Preplasmolysis.

Pretreatment of the tissue in 3% sucrose plus 20 mM cysteine was recommended to rupture plasmodesmata, loosen the plasmalemma from the cell wall and protect the membrane surface from the wall degrading enzymes (Evans, 1976; Senn and Pilet, 1980). Multinucleate protoplasts were not obtained from +B or -B, normal or preplasmolysed tissue in this thesis. Tissue browning during incubation was diminished in the presence of 5 or 20 mM cysteine-HCl (neutralised with NaOH), but protoplast release was not improved. Xu, Davey and

Cocking (1981) found 20 mM cysteine to be inhibitory to protoplast release.

In maize, preplasmolysis with or without 5 mM cysteine did not affect protoplast yield (table 6, p. 34). Gronwald and Leonard (1982) also found no increase in the yield of maize protoplasts after pre-incubation with cysteine. Effects on protoplast physiology were not investigated.

3.2.2 Root slitting.

The epidermis is very resistant to wall degrading enzymes (Kochian and Lucas (1983) used this fact to obtain root cortical protoplasts practically uncontaminated by epidermal protoplasts). The epidermis therefore had to be split to permit enzyme entry and protoplast release. A single lengthwise slit gave the best results; when maize root tips cut into short sections were compared with tips slit lengthwise, after 4.5 h incubation the latter were fully digested, but after 5.5 h the former had only been digested for 1-2 mm from each cut end. The thinner roots of sunflowers made lengthwise slitting prohibitively time consuming so they had to be cut into 3-4 mm segments.

3.3 Protoplast handling.

3.3.1 Fragility.

The fragility of protoplast preparations varies widely. Taylor and Hall (1976) noted that maize protoplasts were tougher than tobacco protoplasts. Lin (1980) obtained acceptable yields of maize protoplasts after two centrifugations at 2000 'g'. My own early experiments suggested that maize protoplasts could withstand such extreme g forces but 300-400 'g' was sufficient to precipitate them. Sunflower protoplasts suffered very high losses at 2000 'g' in agreement with most protoplast systems according to Ruesink (1980).

Table 6. The effect of preplasmolysis on maize protoplasts

Pretreatment	Duration (min)	Yield ($10^5.g^{-1}$ f.wt.)
None	-	4-7
0.45 M mannitol	15	5-6
5 mM cysteine, 0.45 M mannitol	15	5-6

Therefore all centrifugation was done below 350 'g'; Lin (1981) made the same modification to his earlier technique. Whenever protoplasts had to be pipetted, wide-bore tips were used to avoid shearing the preparation. Short-tip pasteur pipettes, graduated plastic 1 ml pipettes and shortened micropipette tips, all with an internal diameter > 1 mm, were used throughout. There was approximately a 50% loss of protoplasts during the entire purification process.

The shaking of preparations in a water bath can itself lead to a loss of protoplasts (Kuhn and Stumpf, 1981) but without shaking, protoplast release was impaired. It was found that a gentle shaking process of 75 oscillations per minute was satisfactory once the mechanism had been upgraded to prevent jerking.

3.3.2 Sterility.

Protoplast experiments were usually performed within 6 h of protoplast preparation (maximum 18 h). Protoplasts were isolated from non-sterile roots but bacterial contamination was effectively reduced by each Percoll centrifugation, in the same way as Piwowaczyk (1979) observed; bacteria were concentrated in the bottom layer whereas intact protoplasts banded at the 0-25% interface. Sucrose was omitted from culture media such as Gamborg's B5 (Gamborg, Miller and Ojima, 1968) to prevent the few remaining bacteria from dividing rapidly, as this was observed to be a problem in the earliest experiments.

3.3.3 Oxygenation.

Protoplast preparations should be held in shallow layers to improve oxygenation (Galbraith, 1981). However test tubes were found to be more convenient than Petri dishes for mixing preparations before removing samples; solution depth was therefore up to 1 cm.

4. Protoplast characteristics.

The yield of protoplasts was variable but never very high. Table 7 (p.36)

Table 7. Protoplast yield from sunflower and maize roots.

	Sunflower	Maize
Root zone	Protoplasts recovered, as % of root cells	
0-1 cm	6	3
1-2 cm	5	15
0-2 cm	5	5

Figures pooled from experiments with a range of plant ages; intended as approximate guide only.

Table 8. Protoplast yield from +B and -B sunflower and maize roots.

Root zone	Yield (10^5 protoplasts.g^{-1} f.wt.)	
'Single Tall' sunflower	+B	12-16 h -B
0-1 cm	14 ± 4	15 ± 2
1-2 cm	5.9 ± 1.4	4.3 ± 1.1
LG11 maize	+B	15-24 h -B
0-2 cm	4.4 ± 0.7	4.8 ± 0.9

Results pooled from four experiments for each species. No significant difference between boron treatments.

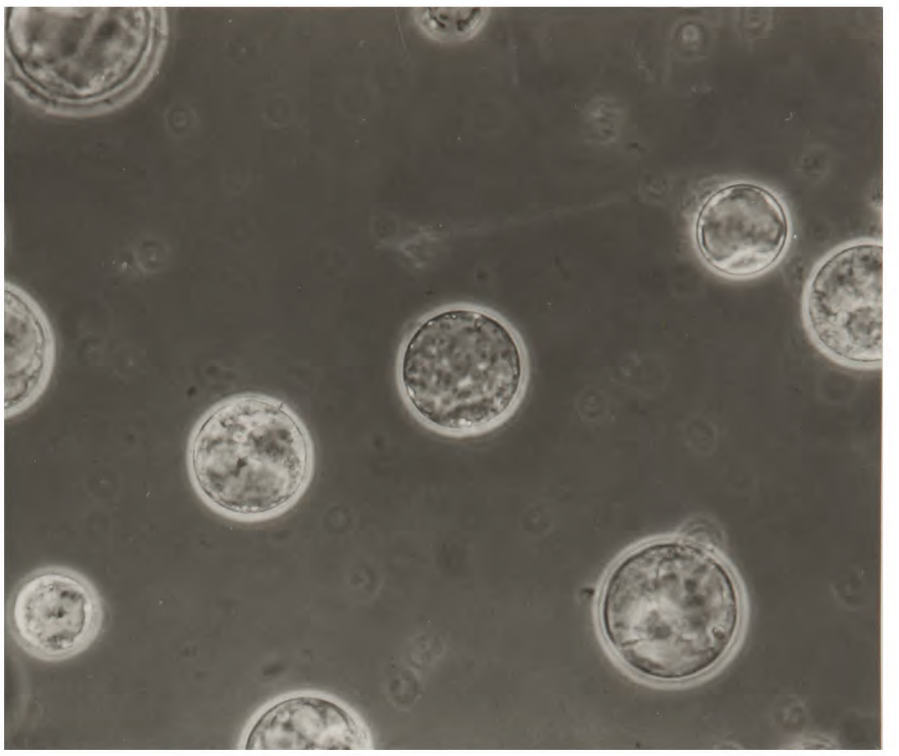
shows that sunflower and maize roots differed in their 'digestibility'; but meristematic tissue was generally left undigested in both systems. Although these figures are only approximate, they are more reliable than figures quoted by some workers based on cell counts found in the literature. The maize yield from the 1-2 cm zone is similar to the 10% quoted by Lin (1980) and the 16% yield obtained by Gronwald and Leonard (1982) for maize cortical tissue.

Yields were not affected by boron deficiency (table 8, p. 36). The yield of sunflower protoplasts for the 1-2 cm zone is almost identical with the range obtained by Lefebvre (1984).

The viability of the protoplast preparations was routinely >95% for both +B and -B preparations when freshly prepared. By the end of the experiments viability was still >80% (see plates 5 a,b, p. 38). Protoplast size was quite variable and cells from most regions of the root were represented, ranging from small, densely cytoplasmic protoplasts to large, highly vacuolate ones (plate 6, p. 38). Diameters typically ranged from 10-60 μm; and calculations based on protoplast volume, as measured with ^{3}HHO (Section 2) gave figures which agreed well (20-26 μm diameter maize; 16-40 μm diameter sunflower). Gronwald and Leonard (1982) reported a mean diameter of 24 μm for maize cortical protoplasts. Comparing these values with the rough approximation of cell size in table 3 (p. 24), and allowing for the mass of the cell walls and for water loss during plasmolysis, it would seem as though each cell of maize and sunflower roots gave rise to a single protoplast. This is in agreement with the results of Lefebvre (1984) for sunflower roots, although he suggested that up to 15 'sub-protoplasts' per cell were isolated from pea roots.

The size variability makes a considerable difference to the surface area of the protoplasts; the importance of this will be seen in Section 2.

(a)



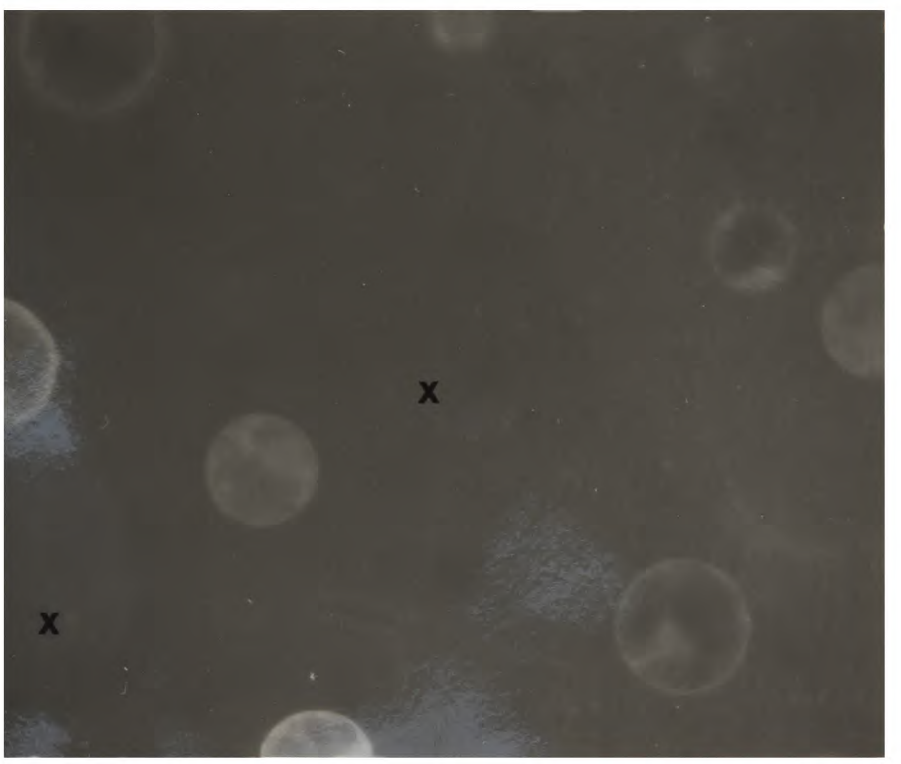
Plates 5 a and b.

Sunflower root protoplasts stained with FDA and viewed under phase contrast 6 h after isolation.

(a) normal and (b) UV light.

Note two non-viable (x) protoplasts.

Scale bar = 20 μm.



(b)

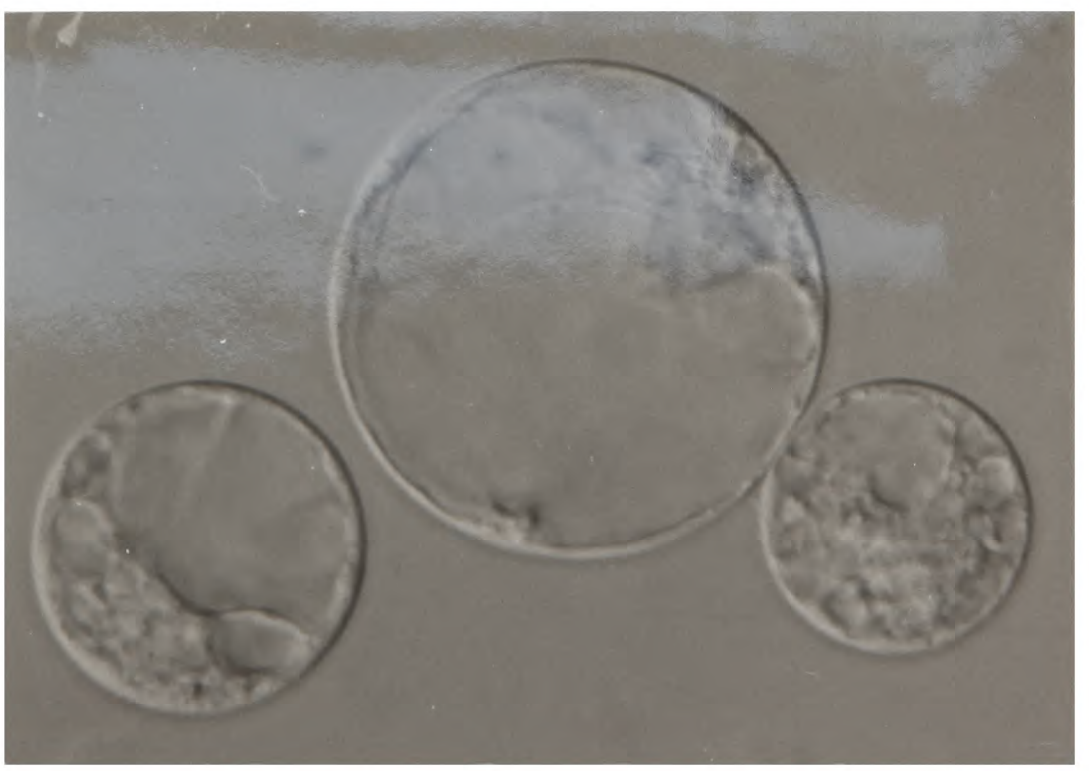


Plate 6. Sunflower root protoplasts viewed under Nomarski optics.

Scale bar = 10 μm.

5. Boron content.

B tests on the root tips after transfer to -B solutions suggested that, in maize, 20% of the B content was lost within 2 h. However this value did not drop further over the next 22 h, despite routine boron tests on the media confirming that -B solutions contained less than 0.5 μM B. A similar result was obtained by White (1983) for the 2 cm sunflower root tips used in these experiments. The remaining B in sunflower and maize root tips was probably bound as stable borate diesters in the cell walls (Thellier, Duval and Demarty, 1979) or intracellular compartments and was presumably not involved in the physiological action of B. Others have reported similar 'non-dialysable' B reserves within plant homogenates and drawn the same conclusions (e.g. Skok and McIlrath, 1958). Parr (1980) found that 60% of the B in maize root homogenates was soluble, the rest being associated with membranes and cell wall debris.

Mannitol was found to interfere with the boron assay and so the protoplast suspension media had to be ashed. Without added $Ca(OH)_2$ 90% of added H_3BO_3 was lost by volatilisation; but in the presence of $Ca(OH)_2$ recovery of added B was > 95%. Using this method, -B protoplast media were routinely confirmed to be 'boron-free'.

Section 2. Ion absorption and ATPase activity.

Introduction

Wyn-Jones and Lunt (1967) suggested that calcium and boron may be required in the external medium to maintain the selective permeability of the cell membrane. While this role is well accepted for calcium (e.g. Clarkson and Hanson, 1980) there are still those who debate whether this is a primary role for boron (e.g. Dugger, 1983). Ion uptake has been known to be affected by boron deficiency for many years. Some of these early effects are listed in table 9 (p. 41).

Roth-Bejerano and Itai (1981) showed an increase in K^+ influx and stomatal opening in epidermal strips of Commelina in the presence of 1-10 mM B. Tanada (1974, 1982) showed effects of the boron supply on the bioelectric field around mung bean hypocotyls and on nyctinastic pinnule closure in Albizzia. Pinnule closure was affected within 40 minutes of supplying 0.5-5 µM H_3BO_3, a lag which may represent the diffusion time required for boron to enter the system.

Similarly rapid, and by implication possibly direct, effects have been recorded for the reversibility of impaired ion uptake. Resupplying boron at 10-100 µM for one hour restored the uptake of P_i, K^+ and Cl^- by 3 d deficient maize roots (Pollard, Parr and Loughman, 1977) and P_i uptake by 3 d -B Vicia faba roots (but not K^+ or Cl^- in the latter case) (Robertson, 1974).

In this section more detailed studies have been performed on K^+ and P_i uptake by maize and sunflower root tips over the first few hours after transfer to -B solutions and the subsequent resupply of H_3BO_3. The 2 cm root tip, and zones within that region, have been studied, to confirm Robertson's suggestion (1974) that the meristematic region is more

Table 9. Some early effects of boron deficiency on ion absorption.

Element	Species	Time -B	-B:+B Uptake Rate	Ref.
$K^+(Rb^+)$	Cylindrotheca fusiformis	5 h	80%	1
	Sunflower	12 h	92%	2
	Field bean	40 h	46%	3
		44 h	50%*(per plant)	4
		44 h	40%*(0-1 cm tips)	5
	Maize	4 d	64%	6
P_i	C. fusiformis	5 h	100%	7
	Maize	15 h	75%	8
		4 d	51%	3
	Sunflower	40 h	69%	3
	Field bean	44 h	50%*	9
		3 d	60%*	9
	Mung bean	3 d	66%*	9
	Barley (Hordeum vulgare)	7 d	145%*	5
Cl^-	Sunflower	40 h	47%	3
	Field bean	48 h	50%*(0-1 cm tips)	5
	Maize	4 d	75%	6
SO_4^{2-}	Field bean	48 h	100%*	5

References

1. Smyth and Dugger, 1980
2. Timashov, 1969
3. Parr and Loughman, 1983
4. Robertson and Loughman, 1973
5. Robertson, 1974
6. Pollard, Parr and Loughman, 1977
7. Smyth and Dugger, 1981
8. Parr, 1980
9. Robertson and Loughman, 1974a

* Uptake expressed cm^{-1} root length in refs 4,5,9. Robertson (1974) states -B, 0-1 cm field bean root tips weigh 30% more than +B on average; no weight change in barley. Uptake expressed g^{-1} f.wt. in refs 1,2,3,6, 7,8 as in this thesis.

sensitive than more mature tissue to boron deficiency.

External ion concentrations were chosen to represent typical soil solution levels, viz. 10 μM for P_i (Bieleski, 1973) and 1 mM K^+ (Bidwell, 1974). These values fall within the range of 'system I' uptake for P_i and 'system II' for K^+; earlier experiments suggested that both systems are affected by boron deficiency (Parr, 1980). In some experiments the effect of increased plant age was studied, to follow up the observation (Parr and Loughman, 1983) that boron deficiency may prevent the increase in uptake capacity which occurs over the first five days after germination in maize root tips.

Alterations to the underlying cellular processes cannot readily be inferred from ion accumulation studies, i.e. an observed change in influx of an ion, e.g. K^+, could be ascribed to an alteration in any one or a combination of the following processes:

1. Penetration.

Some workers (e.g. Peterson and Perumalla, 1984) question the ability of ions to diffuse past the hypodermis, on the basis of studies using non-permeant cell wall stains such as Calcofluor. Kochian and Lucas (1983) concluded that although ions can penetrate the hypodermis, epidermal uptake is responsible for the bulk of uptake from low external solution concentrations; and may even act to restrict cortical uptake in some way. Therefore, uptake might be increased by alterations to the root morphology which favoured bulk solution entry, such as an increase in the intercellular spaces, cell wall thickness or hypodermal penetrability; or by a shift in the postulated epidermal control over cortical cell uptake. If the surface area for absorption is primarily the epidermis, an increase in surface area caused by increased root diameter may not be sufficient to keep pace with the increased tissue volume. Detailed knowledge of the functional surface area involved, including root hairs, would be required

to test this suggestion.

2. Adsorption on the cell walls and the outer surface of the plasmalemma. A part of the uptake measured is accounted for by the binding of ions externally to the cells. Hemicellulose is suggested as a likely K^+ binding site in maize, whereas Ca^{2+} and Na^+ are thought to bind to pectins (Stassart, Neirinckx and Dejaegere, 1981). In general monocots are richer than dicots in hemicellulose which can account for more than 50% of their cell wall; dicots are richer in pectins (Stassart et al, 1981). Thus quantitative or qualitative changes in the cell wall composition caused by boron deficiency could exert small, indirect effects on ion uptake.
The binding step to the outside of the plasmalemma is reputed to be energy requiring (Jensén, 1981); thus alterations to the energy supply could result in secondary effects here.

3. Passive influx/efflux

Jensén (1981) notes that the proportion of K^+ uptake by metabolic means is high in system I uptake whereas the non-metabolic uptake is higher in system II. There is a continuous diffusion of K^+ into or out of the cytoplasm down the electrochemical gradient set up by the range of factors below.

3.1 Artificial alterations to the external $[K^+]$.

3.2 Requirement for K^+ to counteract changes in the internal water potential caused by, e.g., decreased internal solute concentrations during cell expansion.

3.3 Requirement for K^+ to counteract changes in the internal charge balance arising from (e.g.) organic acid production, NO_3^- assimilation, or fluxes of other ions; H^+ movements at the tonoplast or plasmalemma may be particularly important here.

3.4 Alterations to the internal (cytoplasmic) $[K^+]$ arising from:

3.4.1 Compartmentation, e.g. exchange of K^+ between the cytoplasm,

its organelles and the vacuole (itself under complex control).

3.4.2 Symplastic transport to/from adjacent cells, driven by the demand for K^+ in the shoot (Baligar, Nielsen and Barber, 1979). In excised root tips this sink is lost, although there will still be movement towards the stele if the cells there are actively secreting ions to the lumen of the xylem vessels (Bowling, 1981). The vacuole may replace the shoot sink in excised root tips.

3.4.3 Active transport- see 4 below.

The overall level of passive flux will be enhanced by any treatment rendering the membrane more permeable, e.g. the action of DNP on the tonoplast (Kime, Loughman and Ratcliffe, 1982). More specific effects on the passive uptake system can be envisaged, via alterations in the number, activity or capacity of channels for facilitated diffusion (Raven, 1977).

4. Active transport.

It is now generally accepted that active K^+ uptake is controlled by a plasmalemma-bound enzyme, a K^+-stimulated, Mg^{2+}-dependent, ATPase. This is borne out by theoretical considerations (Nissen, 1977) and experimental observations (e.g Leigh and Wyn-Jones, 1975; Rasi-Caldogno, Cerano and Pugliarello, 1980 and Jensén, Lundborg and Kylin, 1983). The ATPase is thought to operate a proton extruding pump (Marré, 1979). Control of active uptake can be exerted in the following ways:

4.1 Direct effects on the activity of the enzyme complex, e.g. a conformational change.

4.2 Altering the rate of synthesis or turnover of the transport proteins. Pitman and Cram (1977) concluded that the membrane transport enzymes have a half-life of 2 h; Chastain, Lafayette and Hanson (1981) observed the blockage of H^+ efflux within 20 minutes of supplying protein synthesis inhibitors, implying an even higher

natural turnover rate. However inferences from inhibitor studies need to be interpreted cautiously; cycloheximide can act as an uncoupler and so may be exerting a direct effect on the membrane.

4.3 Altering the activity of the H^+ pump - e.g. fusicoccin increases K^+ influx by stimulating H^+ efflux (Marré, 1977); also wounding, such as excision damage, leads to a local H^+ influx and depresses the level of K^+ uptake locally (Chastain and Hanson, 1982).

4.4 Altering the supply of ATP to the enzyme, e.g. by the action of uncouplers (Rasi-Caldogno et al., 1980).

4.5 Altering the nature of the membrane in which the enzyme is located, e.g. localised microviscosity changes (Kleinfeld et al., 1981).

4.6 Allosteric control by negative feedback from the cytoplasmic K^+ concentration (Jensén et al., 1983). This has been discussed in section 3.4 above.

Plant hormones may affect passive and active uptake processes in various ways. Although auxin has not been shown to regulate ion flux in roots (Pitman and Cram, 1977), recent work suggests that auxin may be involved in localised Ca^{2+} movements (Raven and Rubery, 1982). IAA also promotes H^+ extrusion in (e.g.) coleoptile segments, leading to K^+ and Cl^- uptake (Marré, 1977). Parr (1980) demonstrated that high [IAA] decreased ion uptake by maize roots in a similar way to boron deficiency.

Abscisic acid (ABA) produced by the root cap in the light is known to affect the activity of the K^+-stimulated ATPase in maize (Chong et al., 1983) as well as the overall rate of water movement (Chastain and Hanson,1982). Tanada (1978) observed a dependence on boron for the interaction between phytochrome and gravity; so we may be approaching more than casual connections here.

It is not surprising that growth is affected rapidly in species with rapid alterations to their ion uptake; however the reverse is also true because of the controls on ion uptake exerted by the growth capacity of the plant (Glass, 1983).

Most reports suggest that Rb^+ is not discriminated against by any of the processes governing K^+ influx into higher plants (Läuchli and Epstein, 1970; Baligar et al., 1979) so that it is suitable as a tracer. Discrimination may occur within the plant; Vakhmistrov and Zakharin (1968) reported differences in the transport of K^+ and Rb^+. Similarly, Stassart et al (1981) suggested that Rb^+ may interfere with Ca^{2+} binding to pectins more than K^+ does. In bacteria and yeasts Rb^+ is not a satisfactory tracer for K^+ (Rhoads, Woo and Epstein, 1977); and recently Feichtinger and Tromballa (1983) reported that in algae Rb^+ supply may induce a K^+ leak.

Phosphate uptake involves similar processes to K^+ uptake. Pettersson (1975a) notes that phosphate uptake at the lowest external concentrations (i.e. system I) may be limited by passive uptake. Active uptake seems to be under the control of an OH^-/P_i antiport system in maize (Lin, 1979). In sunflower protoplasts, Lefebvre (1984) reported inhibition by VO_4^{3+} implying that the process is mediated by an ATPase. Bowling (1983) has suggested that P_i uptake is the major electrogenic force in sunflower roots, rather than K^+ uptake.

The first step of uptake appears to be the incorporation of P_i into ATP (Loughman and Scott-Russell, 1957). The cytoplasmic $[P_i]$ seems to be tightly regulated at a constant level despite incorporation into nucleoside triphosphates and sugar phosphates, apparently by exchange with vacuolar P_i (Kime, Loughman and Ratcliffe, 1982). Lefebvre (1984) confirmed the importance of allosteric feedback from cytoplasmic $[P_i]$ in controlling active uptake by sunflowers. However, shoot demand may take

precedence over allosteric feedback; Drew and Saker (1978) demonstrated that P_i uptake remained high in the zone of a P-deficient root system resupplied with phosphate. At the low $[P_i]$ prevailing in the soil solution almost all uptake should be accounted for by the epidermis and hypodermis, so root surface area is of great importance (Bieleski, 1973). Influx studies give an indication of only a part of the complex process of ion uptake. In order to assess the amount of binding in the free space Cram and Laties (1971) have suggested washing routines for removing ions from the various compartments of the root. These routines can also assist in studies of vacuolar accumulation of non-metabolised ions such as Cl^-. Studies of vacuolar compartmentation of metabolised ions such as P_i are not as easy; efflux is restricted once they have been incorporated into organic compounds. Lefebvre (1984) demonstrated that efflux studies for such ions can be done with appropriate controls; but provided the ions have a suitable resonance frequency, nuclear magnetic resonance (NMR) studies provide a more elegant means of studying vacuolar compartmentation in intact plant material. Parr and Loughman (1983) observed a marked decrease in vacuolar $[P_i]$ as well as a decrease in the level of 'high energy' P-containing compounds in field bean roots after 3 d -B. The spectrum produced was characteristic of less metabolically active tissue. Efflux measurements from preloaded tissue permitted Smyth and Dugger (1980) to explain the increase in internal $[K^+]$ in the diatom Cylindrotheca despite the impaired uptake caused by 5 h -B. In this case efflux was found to have decreased more than influx. However, Parr and Loughman (1983) demonstrated that Cl^- efflux from maize roots was unaffected by 4 d -B whereas uptake was severely reduced.

Experiments on the effects of boron deficiency on metabolic aspects of ion uptake include the work of Robertson and Loughman (1974a). They demonstrated that as well as the uptake of phosphate being impaired in

field beans after 3 d -B, the incorporation of P_i into organic-P compounds was altered. There was an overall decrease in incorporation; and the proportion of hexose phosphates increased with a decrease in the nucleotide-P content.

Additional information comes from Parr's observation (1980) that the characteristic increase in uptake caused by washing excised maize root tips in an aerated solution (which involves protein synthesis and an increase in the respiration rate) was not affected by 3 d -B. This may suggest that impaired uptake is not a result of a reduced rate of incorporation of active carriers into the plasma membrane (4.2).

Parr (1980) continued his studies by directly comparing ion uptake by root tips with the activity of the K^+-stimulated, Mg^{2+}-dependent ATPase in a mixed membrane fraction isolated from maize roots. K^+ uptake was impaired by 35% after 4 d -B and 65% of the control ATPase activity remained in the membranes. Resupplying 10-100 µM H_3BO_3 for 1 h to the roots or to the isolated membranes restored the activity towards the control level. There is a very clear implication that B is directly affecting the ATPase activity in the membranes. However, effects on other aspects of active uptake (e.g. 4.3, 4.4, 4.6 above) could occur as well. Protoplast studies obviate the need to consider -B effects on penetration or cell wall binding. In theory only the transport to adjacent cells (3.4.2) has been lost from the rest of the process, an effect already reduced in excised root tips as noted above.

Experimental evidence has favoured the suggestion that ion uptake by protoplasts functions in much the same way as in the intact tissue. Mettler and Leonard (1979b) have shown that in protoplasts as in root tips Ca^{2+} is required for the selective absorption of K^+; without Ca^{2+}, Na^+ does not interfere with K^+. Kochian and Lucas (1983) conclude from their results that two separate transporting mechanisms, one saturable and

the other linear, operate at the plasmalemma of cortical and epidermal cells and protoplasts derived from the roots. Mettler and Leonard (1979a), Lin (1980), Gronwald and Leonard (1982), Kochian and Lucas (1983) and Lefebvre (1984) reported characteristic effects of inhibitors affecting ATP levels, membrane permeability, protein synthesis and H^+ pumping on the uptake of K^+, P_i and Cl^- by protoplasts. Protoplasts were generally more sensitive than roots to the inhibitors, presumably because the inhibitors were directly applied to the cell's environment.

Despite this general similarity between uptake by cells and protoplasts, differences in detail have been reported. Plasmolysis reduces ion uptake; maize roots placed in 0.3-0.7 M mannitol solutions showed a decreased ion uptake (Parrondo, Smith and Lazurick, 1975). Morris and Thain (1980) made a similar observation with plasmolysed leaf cells, but noted that the influx of K^+ per unit surface area was similar in protoplasts and the unplasmolysed leaf cells from which they came. Lefebvre (1984) found that in pea protoplasts the effects of plasmolysis were partially overcome by increased uptake as a result of ageing. The protoplast uptake rate was about 50% of that of the root cells before isolation.

Other changes of importance include the loss of the negative internal potential in freshly isolated protoplasts (Kinnersley, Racusen and Galston, 1978). Morris and Thain (1980) report a higher K^+ content in protoplasts than in the leaf cells from which they came. Lin (1980) reported that uptake by maize root protoplasts was not sustained over an hour, but levelled off after 30 minutes, for which no explanation was given. Taylor and Hall (1976) suggested that cellulase induced the leak of phosphate and Rb^+ in plant tissue; Morris et al. (1981) confirmed that the presence of cellulase (even boiled cellulase) reduced uptake by leaf cells and protoplasts.

The aims of this section are to characterise more fully the early changes

in ion uptake by root tips of maize and sunflower during boron deprivation and then look for similar effects in protoplasts derived from that tissue. Additional insights can thus be gained into the way the plants are responding to boron deficiency.

Materials and Methods.

1. Ion uptake by root tips

1.1 Phosphate uptake.

Plants germinated for 2-3 d and grown +B or -B for the times shown in the text were treated as follows:

Root tips, usually 4 x 2 cm per flask, cut into 10 µM KH_2PO_4, 1 mM $Ca(NO_3)_2$, pH 6.0. (four replicate flasks per treatment.)

Blotted quickly; incubated in 5 ml of the same medium labelled with 3.7 $kBq.ml^{-1}$ carrier-free $H_3{}^{32}PO_4$, in 50 ml conical flasks, shaken at 75 oscillations per minute for 1 h at 25°C.

Separated from the uptake medium in a sintered glass funnel; washed for 2 min in successive aliquots of de-ionised water to a total of 200 ml.

Fresh weight of the tips determined; transferred to scintillation vials.

(Where required, desorbed for 30 min at 0-4°C in: 0.1 mM KH_2PO_4, 1 mM $Ca(NO_3)_2$, pH 6.0, to remove the ions bound in the free space.)

Root tips digested in scintillation vials, in 2 ml of 75% conc. HNO_3, 25% conc. H_2SO_4 (method modified from Humphries, 1956), at 170°C until all the HNO_3 had evolved. (Up to 100 vials could be digested simultaneously in an electric frying pan).

Vials allowed to cool; residue made up to 10 ml with distilled water and radioactivity counted by Cerenkov methods (Läuchli, 1969) in a Beckman Liquid Scintillation Counter. Internal standards run in each experiment.

1.2 Potassium (^{86}Rb) uptake.

As above, except: roots cut into 1 mM KCl, 1 mM $Ca(NO_3)_2$, pH 6.0. Uptake from the same medium, labelled with 1.11 $kBq.ml^{-1}$ ^{86}Rb. Desorption (when it was performed) into 10 mM KCl, 1 mM $Ca(NO_3)_2$, pH 6.0, 0-4°C. (Methods modified from Parr, 1980).

2. Ion uptake by protoplasts

Ion uptake was carried out by a modification of Lin's layer technique (1981), in 0.4 ml narrow-form polyethylene microcentrifuge tubes (fig.5, p. 53). Silicone oil was kindly provided by Wacker Chemicals, The Clock Tower, Bridge St., Walton-on-Thames, Surrey.

To start uptake, 50 µl samples containing 1-2 x 10^4 protoplasts were pipetted in to the top (uptake medium) layer. The tubes were incubated in a 25°C water bath. Uptake was stopped by 30 sec centrifugation at 25°C in a Quickfit Instrumentation Ltd microcentrifuge. To measure passive 'carry-down' of radioactivity 50 µl protoplast samples were pipetted in as a layer above the uptake medium (separated from it by an air space) and immediately centrifuged.

Centrifugation carried the protoplasts through the layers to the tube tip, which was cut off into 4 ml scintillant: 6 g 2,5-diphenyloxazole (PPO), 0.075 g 1,4-bis[5-phenyl-2-oxazolyl]benzene (POPOP) in 1 ℓ toluene. Radioactivity was counted over a 10 min interval.

The uptake media were:-

Phosphate uptake: 10 µM (final conc.) KH_2PO_4, 25 mM MES, 5 mM $CaCl_2$, 0.5 M mannitol, pH 6.0, containing 29.6 $kBq.ml^{-1}$ $H_3{}^{32}PO_4$.

Potassium uptake: 20-30 µM (K^++Rb^+), 25 mM MES, 5 mM $CaCl_2$, 0.5 M mannitol, pH 6.0, containing 55.5 $kBq.ml^{-1}$ $^{86}RbCl$.

3. Protoplast volume measurement

Narrow-form microfuge tubes were set up as for ^{32}P or ^{86}Rb ion uptake except that the upper layer contained ^3HHO or ^3H-inulin at 370 $kBq.ml^{-1}$. 50 µl samples of protoplasts were pipetted into these tubes and incubated for 1 h at 25°C. Tubes were then spun in a microfuge and the tips containing the protoplast pellet cut off into scintillant. Comparison with ^3HHO standards gave an estimate of the total pellet volume; and the non-permeant ^3H-inulin gave an estimate of the fluid volume around the

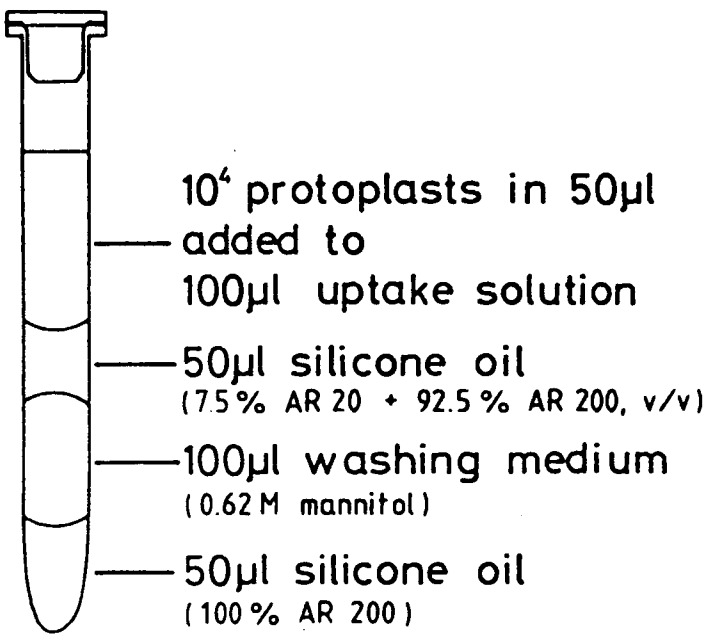


Fig. 5. 0.4 ml microcentrifuge tube used for ion uptake by protoplasts. Silicone oils numbered by their viscosities at 25°C, e.g. AR 20 = 20 $mm^2.s^{-1}$. Specific gravities: AR 20 = 0.97-1.02; AR 200 = 1.03-1.05.

protoplasts. Subtraction gave the volume of intact protoplasts present (Lefebvre, 1984).

4. Membrane isolation

Two cm root tips were cut into 4 $ml.g^{-1}$ f.wt. ice cold grinding medium (20 mM Tris, MES to pH 7.0, 0.3 M sucrose, 2.5 mM DTT and 2% w/v polyvinylpyrrolidone (PVP)) in a chilled mortar and ground for 3-4 min. The homogenate was filtered through two layers of cheesecloth and washed through with grinding medium. Suspensions were centrifuged at 10,000 'g' for 20 min at 4°C in a Beckman L2 65 ultracentrifuge.

The pellet containing large organelles and debris was discarded. The supernatant was spun (80,000 'g', 50 min, 4°C) to yield a mixed membrane fraction which was washed by recentrifugation as above in buffer without DTT or PVP. The membrane fraction ('10-80 K 'g'') was suspended in the latter buffer at 0.6-1.0 ml $buffer.g^{-1}$ f.wt. roots (method modified from Parr, 1980).

In later experiments two different membrane fractions were prepared. Centrifugation was at 9,000 'g', 13,000 'g' and 80,000 'g' for 20, 20 and 50 min respectively. The '9-13 K 'g'' and '13-80 K 'g'' membrane fractions were separately suspended in buffer without PVP or DTT.

5. ATPase assay

50 µl samples of membrane suspension, containing 70-100 µg of protein, were added to 5 ml assay medium: 20 mM Tris, 3 mM Tris-ATP and 2 mM $MgSO_4$, adjusted to pH 7 with MES. 60 mM KCl was added to the assay medium where appropriate; as was 100 µM Na_3VO_4 (method modified from Leigh, Williamson and Wyn-Jones, 1975 and Leonard and Hotchkiss, 1976).

The reaction was stopped by the addition of 0.8 ml 60% $HClO_4$ and P_i

release determined by the method of Allen (1940). 0.8 ml amidol/bisulphite reagent (1 g 2:4-diamino-phenol hydrochloride (amidol), 20 g $Na_2S_2O_5$ in total vol. 100 ml) were added followed by 0.4 ml 8.3% (w/v) $(NH_4)_6Mo_7O_{24}.4H_2O$. Tubes were mixed well after each addition. Samples were made up to 10 ml with water and the optical density read at 712 nm after 5 min, against reagent blanks.

K^+-stimulated ATPase activity could be calculated from the additional P_i released in the presence of K^+ ions, expressed per mg of protein in the mix. This activity should be inhibited in the presence of 100 µM vanadate; any remaining stimulation above Mg^{2+}-dependent ATPase activity would be due to a Cl^--stimulated, vanadate insensitive, ATPase such as that found in oats by Churchill, Holaway and Sze (1983), characteristic of the tonoplast.

In later experiments non-specific P_i release from ATP was reduced by changing to the method of Rathbun and Betlach (1969): 50 µl membrane sample added to 2.55 ml reaction mix as above. Reaction stopped with 0.6 ml 26.7% (w/w) TCA on ice and held for 5 min. The preparation was centrifuged at 4°C, 3,000 'g' for 5 min and 1.6 ml added to 1.1 ml acetate + formaldehyde buffer (1 ml [3 N acetic acid + 3 M sodium acetate] and 0.1 ml 37% formaldehyde), pH 4.29. To this mixture are added 0.1 ml 2.5% ammonium molybdate and 0.2 ml 6.75 mM $SnCl_2$ within 15 sec of each other. O.D. read at 735 nm after 15 min.

6. Protein assay

Preliminary experiments demonstrated that mannitol interfered with the method of Lowry et al. (1951). The method of Peterson (1977) was therefore adopted for all protoplast protein assays; membrane samples, which did not contain interfering substances, did not require the initial steps.

Samples of protoplast preparation containing 1-10 x 10^4 protoplasts in

20-50 µl of medium were made up to 1 ml and the protein solubilised with 0.1 ml 0.15 % sodium deoxycholate. 10 min later 0.1 ml 72% trichloroacetic acid was added to precipitate the protein and after 15 min on ice the preparations were spun in a microfuge for 5 min. The supernatant was decanted and aspirated off thoroughly. The blank used for protoplasts contained the same amount (20-50 µl) of medium as the samples and was treated identically.

These purified protoplast samples, or 10-20 µl membrane samples, were made up to 1 ml with distilled water and 1 ml 'Reagent A' was added (2.5% Na_2CO_3, 0.025% $CuSO_4.5H_2O$, 0.05% potassium sodium tartrate, 2.5% sodium dodecyl sulphate (SDS) in 0.2 M NaOH), mixing well. After 10 min at room temperature, 0.5 ml of 'Reagent B' was added (1:5 dilution of Folin-Ciocalteu solution in water) and immediately mixed. The absorbance of the resulting blue colour was read in a Pye-Unicam SP8-100 UV-Vis spectrophotometer at 750 nm between 30 min and 2 h later and compared with replicated BSA standards included in each experiment.

7. Detection of boron in membranes

Fifteen-20 µl membrane samples prepared as before were pipetted onto transparent polyethylene squares. The samples were dried in a vacuum desiccator over P_2O_5 and sent to Dr F.Martini in Rouen, France for assay. There the membrane preparations were bombarded with slow neutrons to produce alpha particles by the reaction:

$$^{10}_{5}B + ^{1}_{0}n \longrightarrow ^{7}_{3}Li + ^{4}_{2}\alpha \text{ particle.}$$

The alpha particles were detected and their density recorded on Kodak E115 detector film at the upper and lower face of the samples. Comparison with standards should give a measure of the boron content of the preparations (Martini and Thellier, 1980).

Results

1. Phosphate uptake

1.1 Sunflower roots.

The uptake of phosphate (P_i; expressed per gram fresh wt. of root material unless otherwise specified) from 10 µM KH_2PO_4 was linear over 1 h for both 0-1 and 1-2 cm root segments from boron sufficient or deficient roots (fig. 6 a,b, p. 58). A small proportion (2-14%) of this uptake could be removed by subsequently desorbing the root tips in unlabelled 0.1 mM KH_2PO_4 at 0-4°C for 30 min, with no difference between the boron treatments. All of the plants used in these experiments were germinated for 2-3 d in the dark at 25°C before transfer to +B or -B solutions as detailed in the figure legends.

P_i uptake was rapidly affected by the transfer of plants to -B media. Two cm root tips show an early rise in uptake (fig. 7, p. 59). Uptake was then restored to the +B level. At 15 h -B no significant difference in uptake is visible despite the marked increase in weight at this time; uptake per root tip has therefore increased. In early experiments with cultivar 'Polestar' sunflowers an increased sensitivity to boron withdrawal was seen. By 24 h -B phosphate uptake per root tip had decreased by 15-20% (table 10, p. 60) which implied a still greater reduction in uptake per gram fresh weight, since the -B tips were heavier. There seemed to be some variability in response along the roots of Single Tall sunflowers, since 15 mm tips tended towards impaired uptake after about 3 h -B in three experiments (table 11, p. 60). Accordingly, uptake by 5 mm sections of the root tip was investigated during the earliest stages of boron deficiency. Results are shown in table 12 (p. 61).

Uptake by the 0-5 mm section was impaired after 3 h -B. However, the

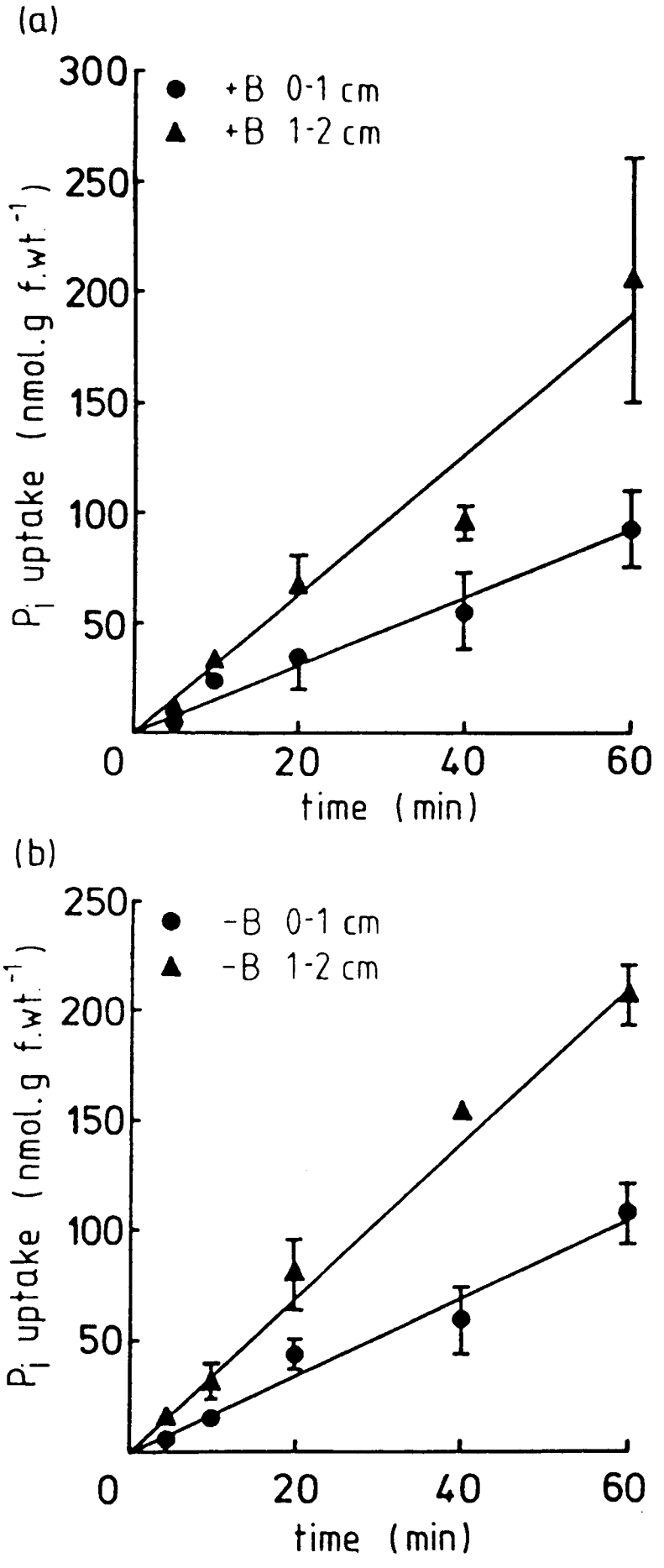


Fig. 6. P_i uptake by root segments of 'Single Tall' sunflowers. Plants were grown for 30 h +B (4.6 µM H_3BO_3) followed by (a) 18 h +B or (b) 18 h -B. Lines plotted by linear regression; all *** with no significant reduction with a quadratic term.

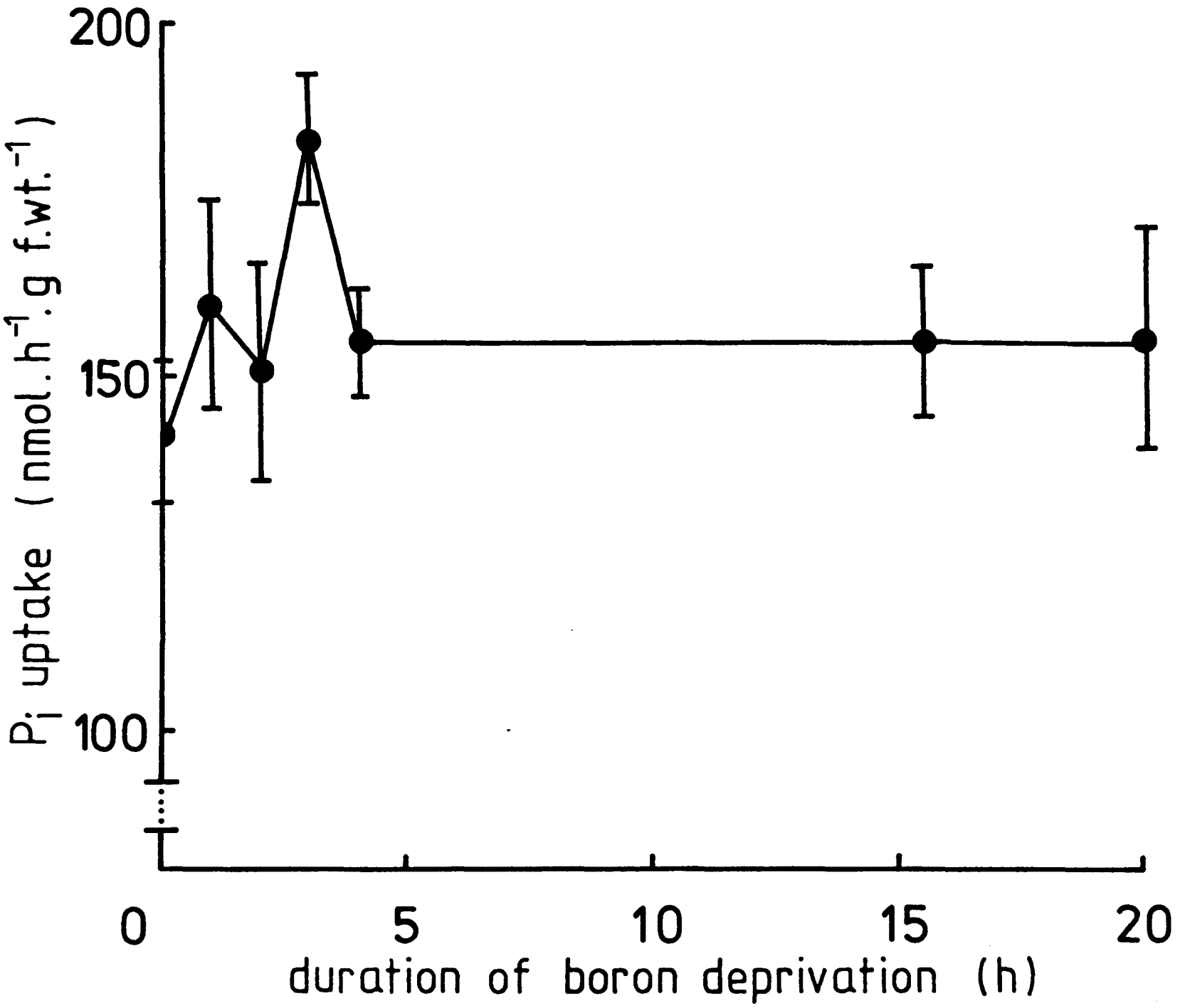


Fig. 7. Effect of boron deprivation on P_i uptake by 2 cm root tips of 'Single Tall' sunflowers. All plants were grown for a total of 40-44 h, initially +B (4.6 µM H_3BO_3) and then transferred to -B solutions for the times shown.

Table 10. P_i uptake by 'Polestar' sunflower roots

P_i uptake ($nmol.h^{-1}.(2\ cm\ tip)^{-1}$)		
24 h +B	24 h -B	24 h -B/2 h +B
1.96 ± 0.06 **	1.62 ± 0.07 **	2.36 ± 0.11

Plants grown in nutrient solution for the times shown; H_3BO_3 supplied at 4.6 µM where required. Pairwise comparisons by t tests, using convention:

* = $p < 0.05$

** = $p < 0.01$

*** = $p < 0.001$

Table 11. P_i uptake by Single Tall sunflower roots (15 mm tips).

	P_i uptake ($nmol.h^{-1}.g^{-1}$ f.wt.)		
Time	-B	+B	-B
2.5 h		337 ± 8	307 ± 15	n.s.
3 h		402 ± 29	335 ± 14	*
3.5 h		168 ± 18	141 ± 17	n.s.

Plants grown for 2-3 d +B followed by transfer to +B or -B solutions for the times shown. +B plants supplied with 4.6 µM H_3BO_3.

N.B. Higher uptake rates in 2.5 and 3 h -B experiments caused by addition of penicillin and streptomycin to the root growth medium.

Table 12.

P_i uptake by 5 mm segments of Single Tall sunflower roots

Root zone	P_i uptake ($nmol.h^{-1}.g^{-1}$ f.wt.) +B	P_i uptake ($nmol.h^{-1}.g^{-1}$ f.wt.) -B	
0-5 mm	109 ± 14	75 ± 21	n.s.
	118 ± 8	51 ± 11	**
5-10 mm	87 ± 4	106 ± 11	n.s.
	174 ± 11	165 ± 24	n.s.
10-15 mm	132 ± 3	109 ± 7	*
	152 ± 8	136 ± 1	n.s.
15-20 mm	116 ± 7	83 ± 6	**
	136 ± 11	89 ± 15	*

Results from two experiments. Plants grown for 36 h +B and then +B or -B for 3 h. H_3BO_3 supplied at 4.6 µM.

effect was complicated by changes to the root volume. In one experiment where the root tips were significantly heavier in that zone, uptake was decreased by 56%; in another where the weight increase was negligible the 30% decrease in uptake was not significant. Five to 10 mm sections showed no significant difference after 3 h -B, but both 10-15 and 15-20 mm sections showed significant decreases at that time. More complete studies were carried out on 0-1 and 1-2 cm segments over the first 24 h after boron removal (fig. 8 a,b, p. 63). Analysis of these results suggested that there is a genuine decline in uptake in the tip (0-1 cm) region from 2-4 h -B followed by a transient peak in uptake and subsequent restoration of normal uptake per g, despite a marked increase in tip weight after 12 h -B in some experiments. The 1-2 cm region does not show a significant early decline, but a transient peak occurs at 6 h -B. After 12 h -B the results are apparently complicated by the increased fresh weight; flasks containing roots which showed the biggest weight increase tended to show the largest decrease in uptake. This relationship was not observed for intact 2 cm tips mentioned above.

There was no significant effect on ion uptake attributable to the cut ends. A comparison of uptake by 15 or 20 mm root tips with the total uptake of the three or four component 5 mm segments showed no consistent pattern of increased or decreased uptake per root tip. In theory uptake could be increased as the external solution penetrates the segments with two cut ends more easily; but this would be counteracted by both a greater leaching of the segment during washing and the impairment in uptake resulting directly from wounding.

The effect of resupplying boron at the same low level (4.6 μM) as used for growth was tested. The cultivar 'Polestar' showed the classic response on uptake per root tip (table 10, p. 60) with 2 cm tips. As Parr (1980) observed, uptake capacity could be restored to a level greater than the

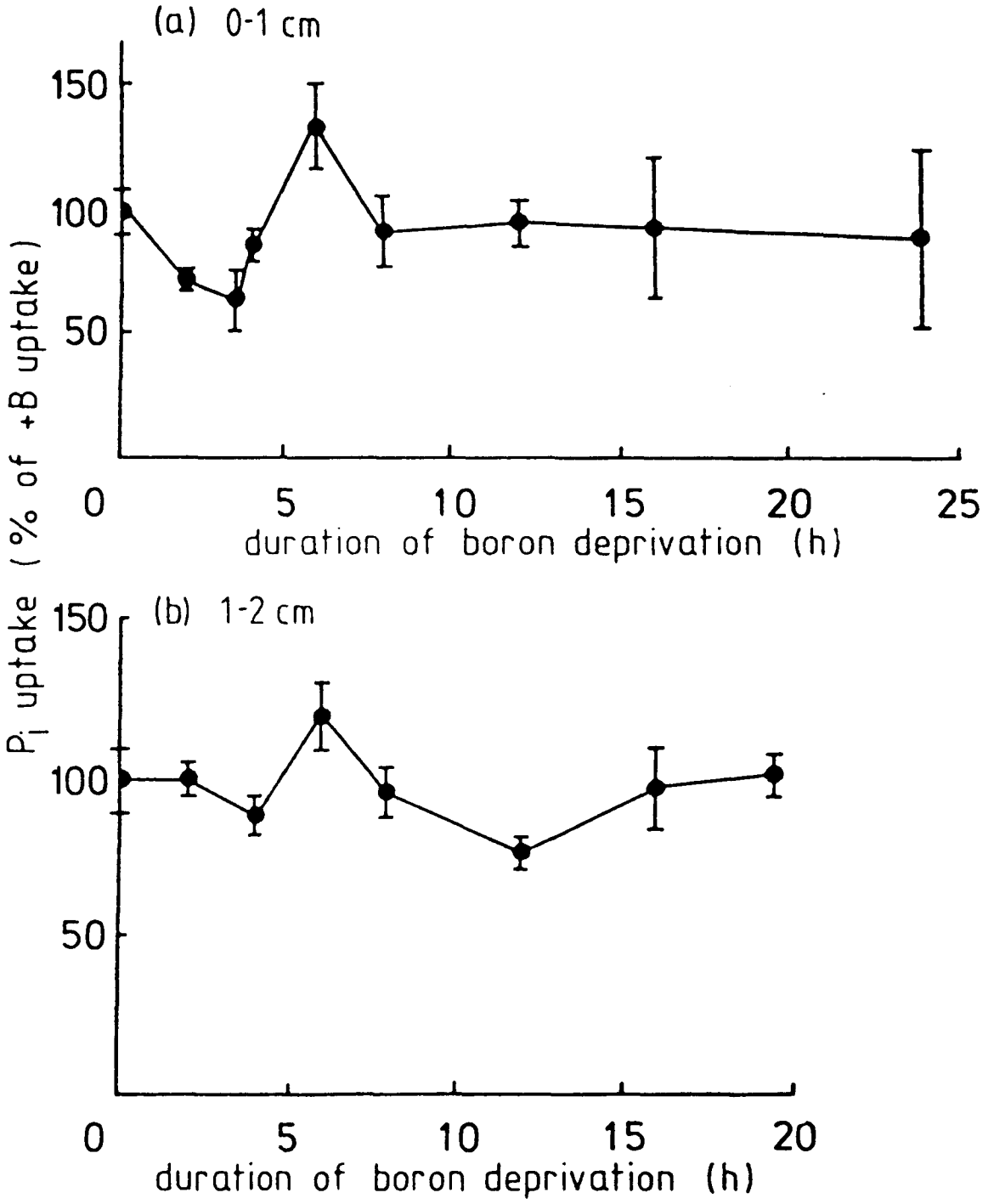


Fig. 8. Effect of boron deprivation on P_i uptake by 'Single Tall' sunflower root sections (a) 0-1 cm or (b) 1-2 cm from the apex. All plants were grown for a total of 40-44 h, initially +B (4.6 µM H_3BO_3) and then transferred to -B solutions for the times shown.

control. However the 'Single Tall' sunflowers were not as consistent in their response (fig. 9 a,b,c, p. 65).

After 4 h -B (fig. 9a) brief incubation with 4.6 µM H_3BO_3 had no significant effect on the decreased uptake of 1 cm tips. In two experiments, 16 h -B (fig. 9b) or 24 h -B (fig. 9c) followed by brief (1-4 h) resupply led to a reduction in the variability of uptake and a significant decrease in uptake compared to the +B controls. Root geometry did not seem to be involved in this effect, for in one case there had been no observed weight increase but in the other the weight had markedly increased.

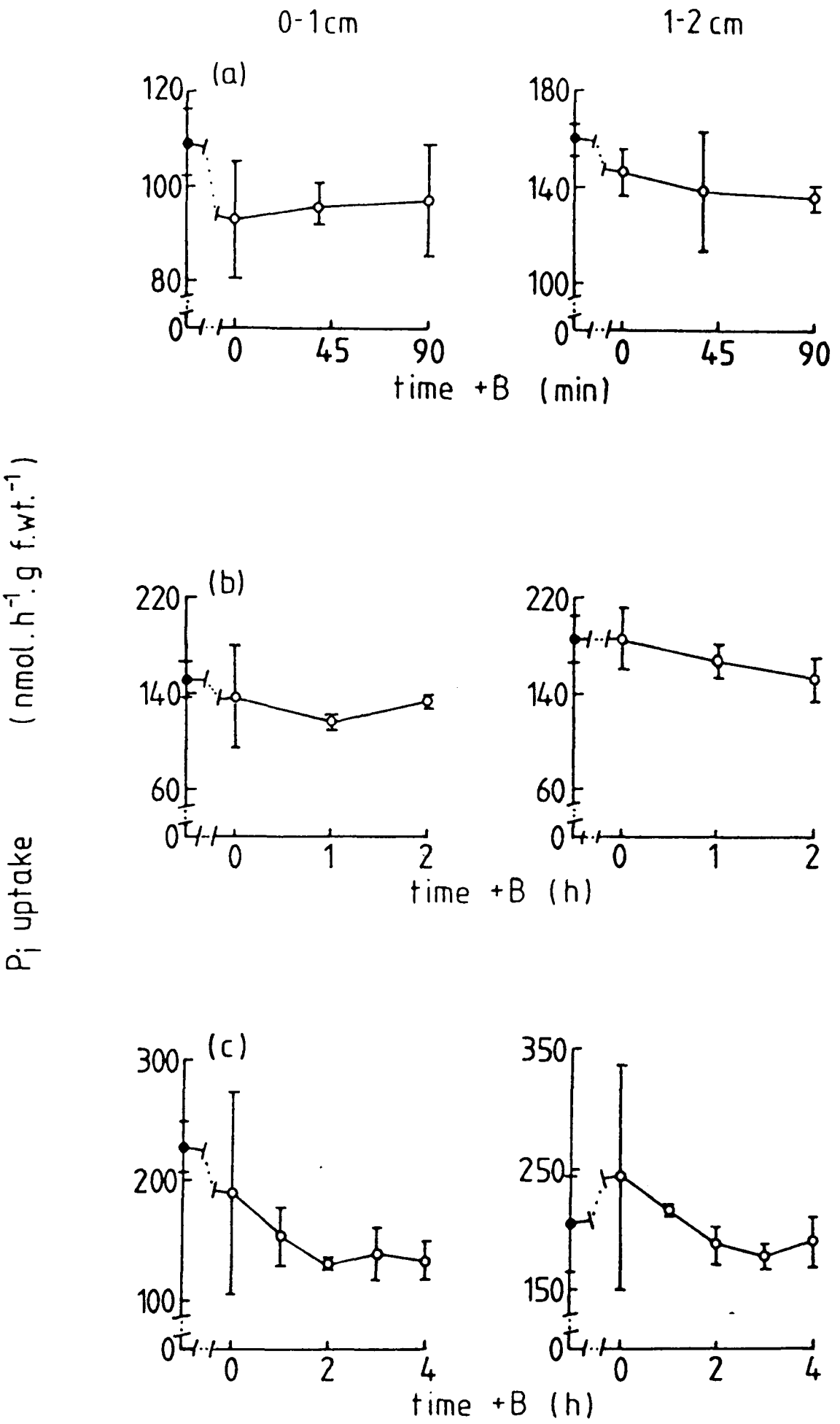
One-2 cm root segments also failed to show an increase in uptake with the resupply of boron. Instead there was a tendency towards decreased uptake with boron resupply in each case.

A rough estimate can be made of the uptake per cell; the figures are approximately 5 $fmol.h^{-1}.cell^{-1}$ from the 0-1 cm region, and 20 $fmol.h^{-1}.cell^{-1}$ from the 1-2 cm region.

1.2 Sunflower protoplasts

Protoplasts derived from 0-1 cm sunflower roots showed linear P_i uptake from 10 µM solutions over 60 min whereas protoplasts from the 1-2 cm root zone generally showed uptake which tailed off after 20-40 min (fig. 10 a,b, p. 69). The uptake per protoplast from 1-2 cm root segments was greater than the uptake per protoplast from 0-1 cm segments, which correlates with their increased size and maturity; and the range of ion uptake figures obtained was 5-20 $fmol.h^{-1}.protoplast^{-1}$ from the 0-1 cm region, and 15-100 $fmol.h^{-1}.protoplast^{-1}$ from the 1-2 cm region.

The addition of BSA during the isolation process greatly improved the ion uptake capacity of protoplasts from the 0-1 cm tips but the further addition of PMSF in 0.01% propanol (final concentration) counteracted this



P_i uptake ($nmol.h^{-1}.g\ f.wt.^{-1}$)

Fig. 9. Effect of resupplying 4.6 µM boron on P_i uptake by 'Single Tall' sunflower root sections. Time 0 on the x axis represents: (a) plants grown for 35 h +B (4.6 µM H_3BO_3) followed by 4 h -B; (b) 34 h +B/ 16 h -B; (c) 29 h +B/ 24 h -B. Control uptake shown on y axis for plants grown +B throughout; i.e. in (a), 0-0 on x axis represents 4 h.

there is no page 66

effect (table 13, p. 68). Protoplasts derived from the 1-2 cm zone benefitted from the addition of BSA alone or in conjunction with PMSF (table 13, p. 68).

Uptake by protoplasts derived from 1 cm root tips did not vary consistently with their boron treatments. Boron was supplied where appropriate at 9.2 μM. The two left-hand columns of table 14 (p. 68) show that in two experiments protoplasts from 13-17 h -B roots did not significantly differ from the controls in their uptake capacity; and in the other experiments uptake was either impaired or enhanced.

Figs. 10, 11, 12 and 13 (pp. 69-71) show the range of results obtained for protoplasts derived from the 1-2 cm root zone. Again there were inconsistent differences between the +B and -B preparations. In one experiment (fig. 13) the surface area for uptake was estimated from volume measurements and protoplast counts. The small increase in phosphate uptake per -B protoplast was entirely accounted for by the increased size of those protoplasts. This implies that the reduced uptake capacity of protoplasts derived from 14 h -B roots (fig. 11) is likely to be a genuine decrease since the protoplasts are still likely to be larger. Volume measurements were not done to confirm this; but expressing the results per mg protein left the graphs unchanged.

More reliable comparisons should be found when taking a single protoplast preparation and dividing it into batches for different B treatments. Resupplying boron for 4-5 h to protoplasts isolated from 1 cm -B root tips made little difference to their uptake capacity (right hand pair of columns, table 14, p. 68). However in the experiment with the highest uptake, resupply did lead to enhanced uptake for both 0-1 and 1-2 cm zones of origin (table 14 and fig. 12, p. 70). In the one experiment when figures could be expressed per unit surface area, uptake declined with the resupply of boron (table 15, p. 72). Inconsistent results were obtained

Table 13. P_i uptake by Single Tall sunflower protoplasts

	P_i uptake ($pmol.h^{-1}.10^4$ $protoplasts^{-1}$)	
Additive	0-1 cm origin	1-2 cm origin
None	25- 40	150- 350
BSA	150-220	500- 800
BSA + PMSF	50- 60	850-1000

Protoplasts isolated from plants grown for 61-65 h in nutrient solution.

Table 14. P_i uptake by protoplasts from 1 cm Single Tall sunflower root tips

Time	+B		-B		-/+B
13-14 h	0.66 ± 0.02	*	0.52 ± 0.03	n.s.	0.49 ± 0.01
13-14 h	0.99 ± 0.19	n.s.	0.87 ± 0.04	n.s.	0.79 ± 0.05
14 h	0.78 ± 0.06	**	1.17 ± 0.05		not done
17 h	3.12 ± 0.12	n.s.	3.08 ± 0.11	*	3.69 ± 0.29

Figures are slopes (± SE) of regression equations for uptake per 10^4 protoplasts ($pmol.(10^4$ $protoplasts)^{-1}$) vs. time, all straight lines through the origin. Significance rating applies to adjacent pairs of figures. 'Time' column refers to duration of root treatment after 2 d growth in full nutrient solution; -/+B refers to protoplasts resupplied with B for 4-5 h after isolation. H_3BO_3 supplied at 9.2 µM.

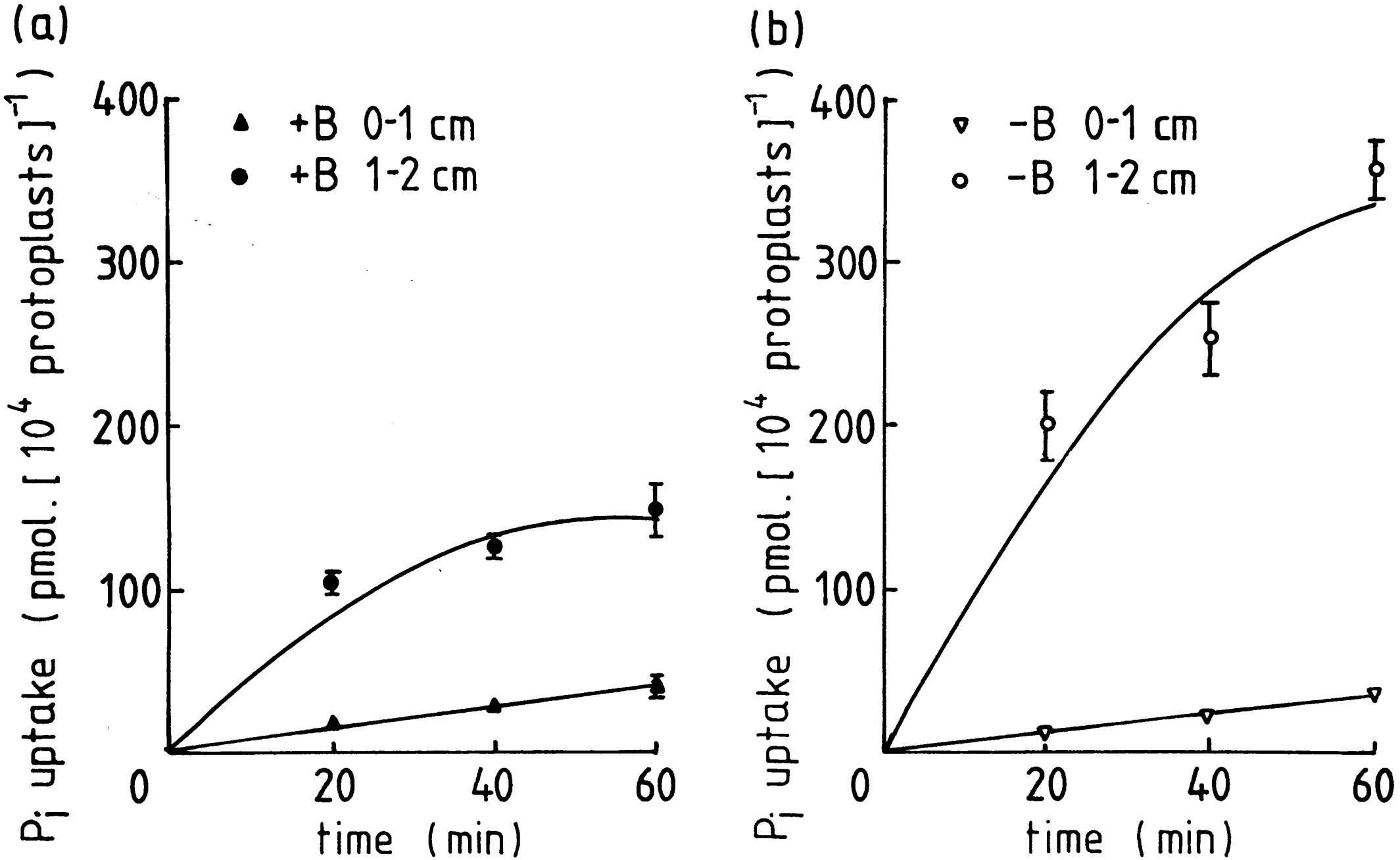


Fig. 10. P_i uptake by protoplasts from 'Single Tall' sunflower root segments. Linear regression *** for 0-1 cm zone; significant reduction with negative quadratic term for 1-2 cm zone. Plants grown for a total of 2 d, initially +B (4.6 µM H_3BO_3) and then (a) +B or (b) -B for 13-14 h before isolation of protoplasts. +B protoplasts supplied with 9.2 µM H_3BO_3.

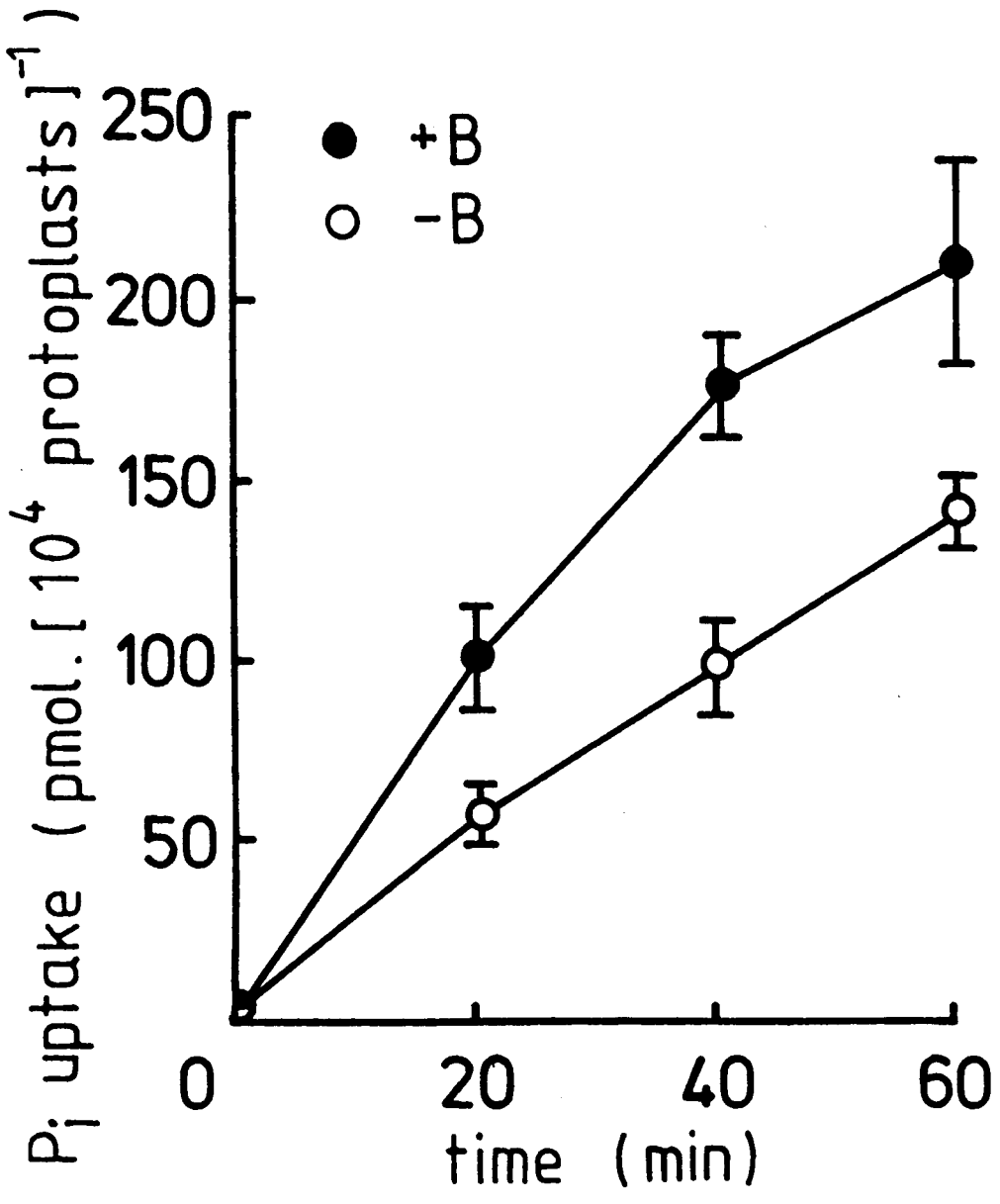


Fig. 11. Effect of B status of roots on P_i uptake by 'Single Tall' sunflower protoplasts (1-2 cm zone). Plants grown for 2 d +B (4.6 µM H_3BO_3) followed by 14 h +B or -B. +B protoplasts supplied with 9.2 µM H_3BO_3.

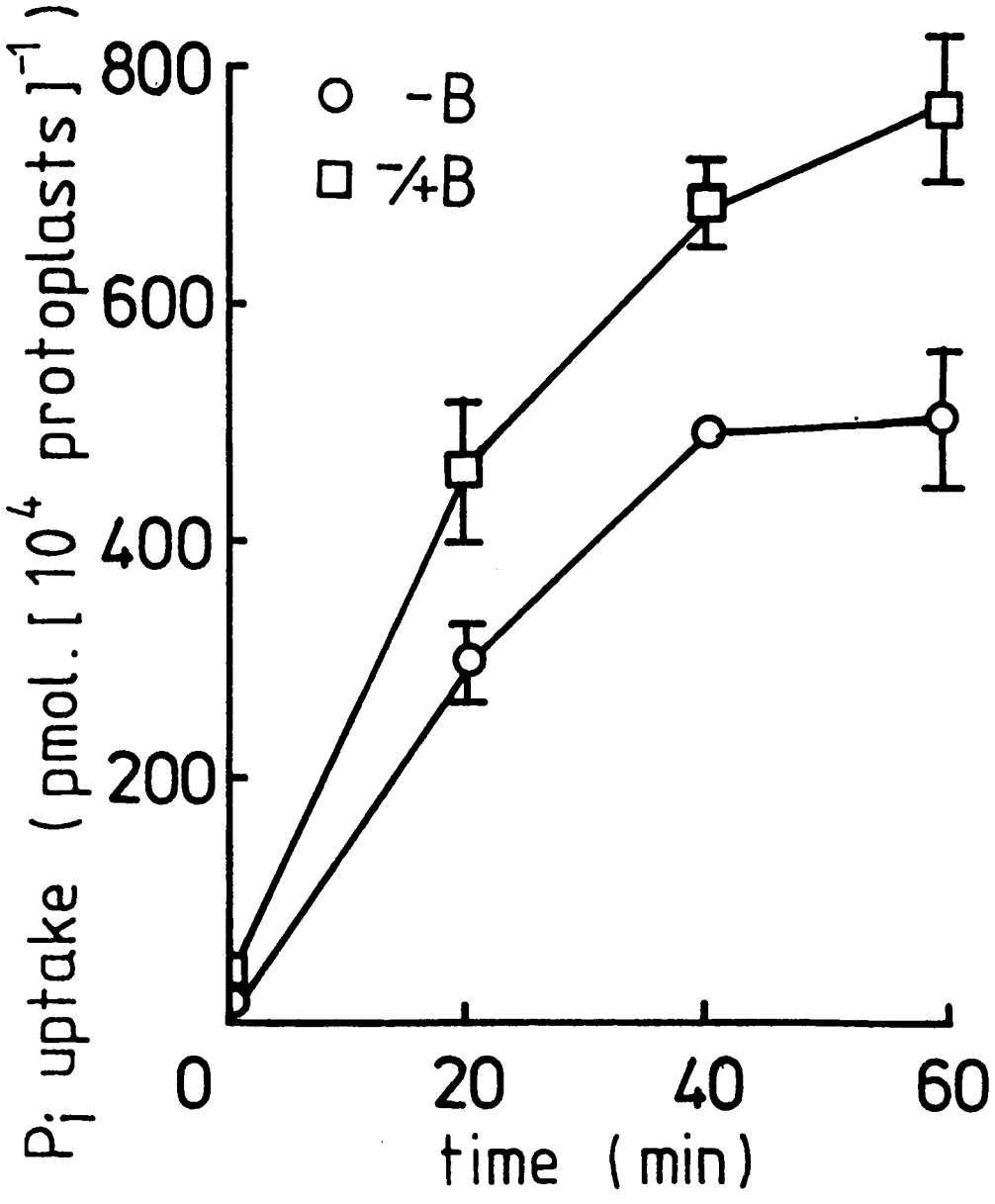


Fig. 12. Effect on P_i uptake of resupplying B to 'Single Tall' sunflower root protoplasts (1-2 cm zone). Plants grown for 2 d +B (4.6 µM H_3BO_3) followed by 17 h -B. Protoplasts isolated in the presence of BSA; -/+B protoplasts supplied with 9.2 µM H_3BO_3 for 4.5 h prior to uptake.

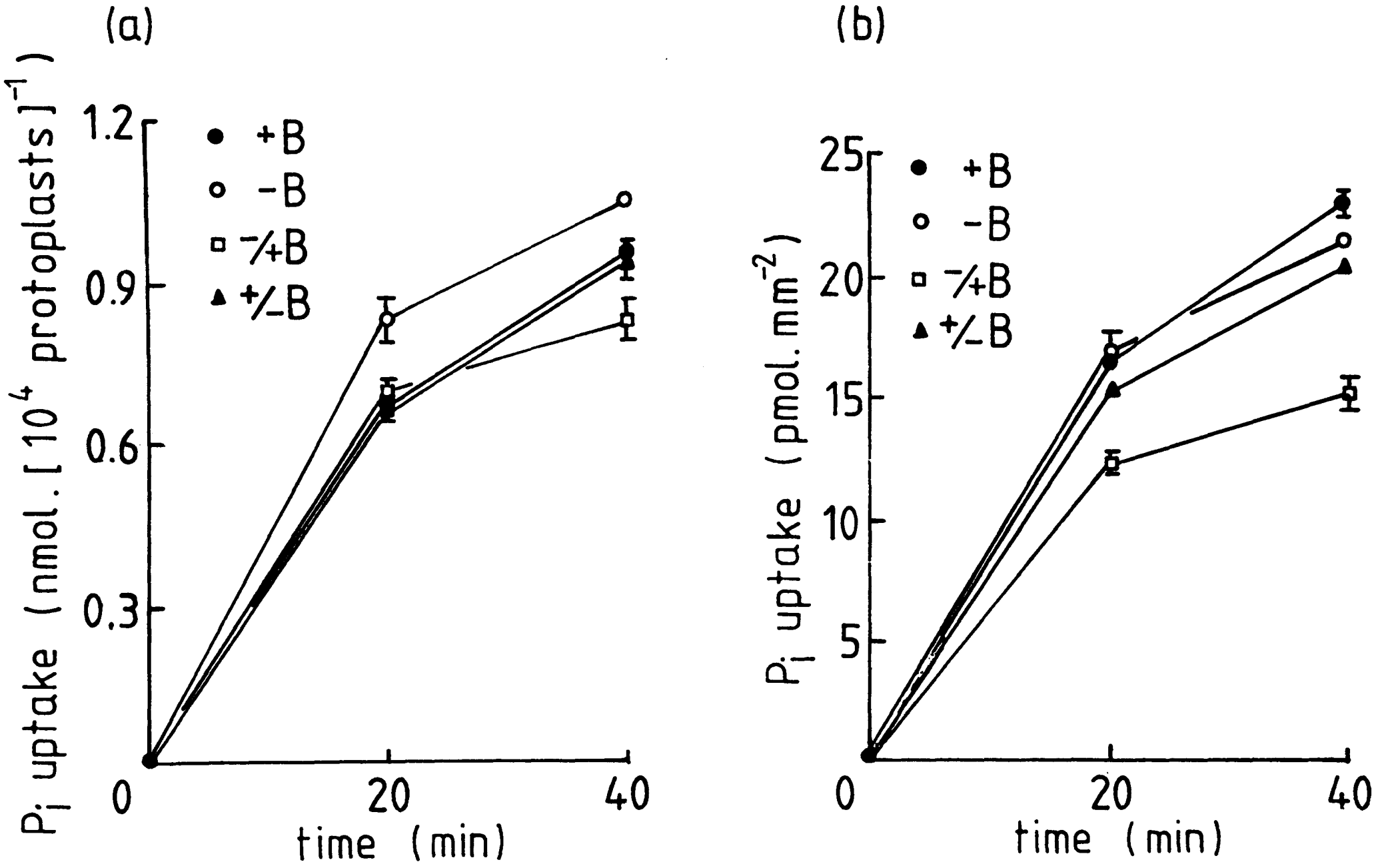


Fig. 13. Effect of B status on P_i uptake by 'Single Tall' sunflower protoplasts isolated from the 1-2 cm root zone. Plants grown for 35 h +B (4.6 µM H_3BO_3) followed by 13-14 h +B or -B. Protoplasts isolated in the presence of BSA and PMSF. Uptake expressed (a) per protoplast or (b) per unit surface area. +B protoplasts supplied with 9.2 µM H_3BO_3 throughout; +/-B and -/+B protoplasts transferred 5 h prior to uptake.

Table 15. P_i uptake by protoplasts from 1 cm Single Tall sunflower root tips

-B		-/+B
0.095 ± 0.004	***	0.054 ± 0.003

Figures are slopes (± SE) of regression equations for uptake per unit surface area (pmol.(mm^2 surface area)$^{-1}$) vs. time, both straight lines through the origin. All protoplasts were isolated from 13-14 h -B roots (see table 14); -/+B protoplasts resupplied with 9.2 µM B for 4-5 h after isolation.

Table 16. P_i uptake by protoplasts from 1 cm Single Tall sunflower root tips

	+B		+/-B
Per 10^4 protoplasts	0.99 ± 0.19	n.s.	1.26 ± 0.09
Per unit surface area	0.084 ± 0.016	*	0.154 ± 0.011

Figures are slopes (± SE) for regression equations; uptake expressed in pmol.(10^4 protoplasts)$^{-1}$ or pmol.(mm^2 surface area)$^{-1}$. All protoplasts came from plants grown for 3 d in nutrient solution; +/-B protoplasts transferred to -B media for 5 h after isolation. B supplied at 9.2 µM.

from resupplying boron to protoplasts derived from the 1-2 cm zone; no effect, a 15% decrease (fig. 13) and a 25% increase (fig. 12) in uptake per 10^4 protoplasts were obtained in separate experiments. When uptake was expressed per unit surface area in the experiment which showed a 15% decline in uptake, the decrease was more marked (fig. 13).

Short-term deprivation of boron was tried in only a single experiment. Protoplasts isolated from +B roots were observed 5 h after transfer to boron-free media. Table 16 (p. 72) shows that whereas the uptake rate per protoplast was not significantly higher after 5 h -B for protoplasts from the 0-1 cm zone of origin, the uptake rate per unit surface area was higher than in controls. Uptake by protoplasts from the 1-2 cm zone was not significantly affected by the boron treatment on either a per protoplast or per unit area basis (fig. 13).

1.3 Maize roots

Root tips from 5 d old LG11 maize plants were divided into 0-1 and 1-2 cm sections as for sunflowers. A straight line through the origin could be fitted to the uptake curves over a 60 min period for both zones of +B and -B roots (fig. 14 a,b, p. 74) but the +B uptake appeared to accelerate with time. The uptake rate is higher than in sunflowers.

The time course of onset of deficiency shows some interesting contrasts with that of sunflowers (fig. 15, p. 75). One cm root tips show a gradual decline in uptake with increasing duration of deficiency, without a significant peak in the early hours. One to 2 cm root segments show increased uptake at 4 h -B and by 15 h -B show significantly decreased uptake compared with +B plants. The uptake rate was higher in the 1-2 cm sections than the 0-1 cm sections, as in sunflowers. The approximate uptake per cell was 5-10 $fmol.h^{-1}.cell^{-1}$ in the 0-1 cm zone and 50-150 $fmol.h^{-1}.cell^{-1}$ in the 1-2 cm zone.

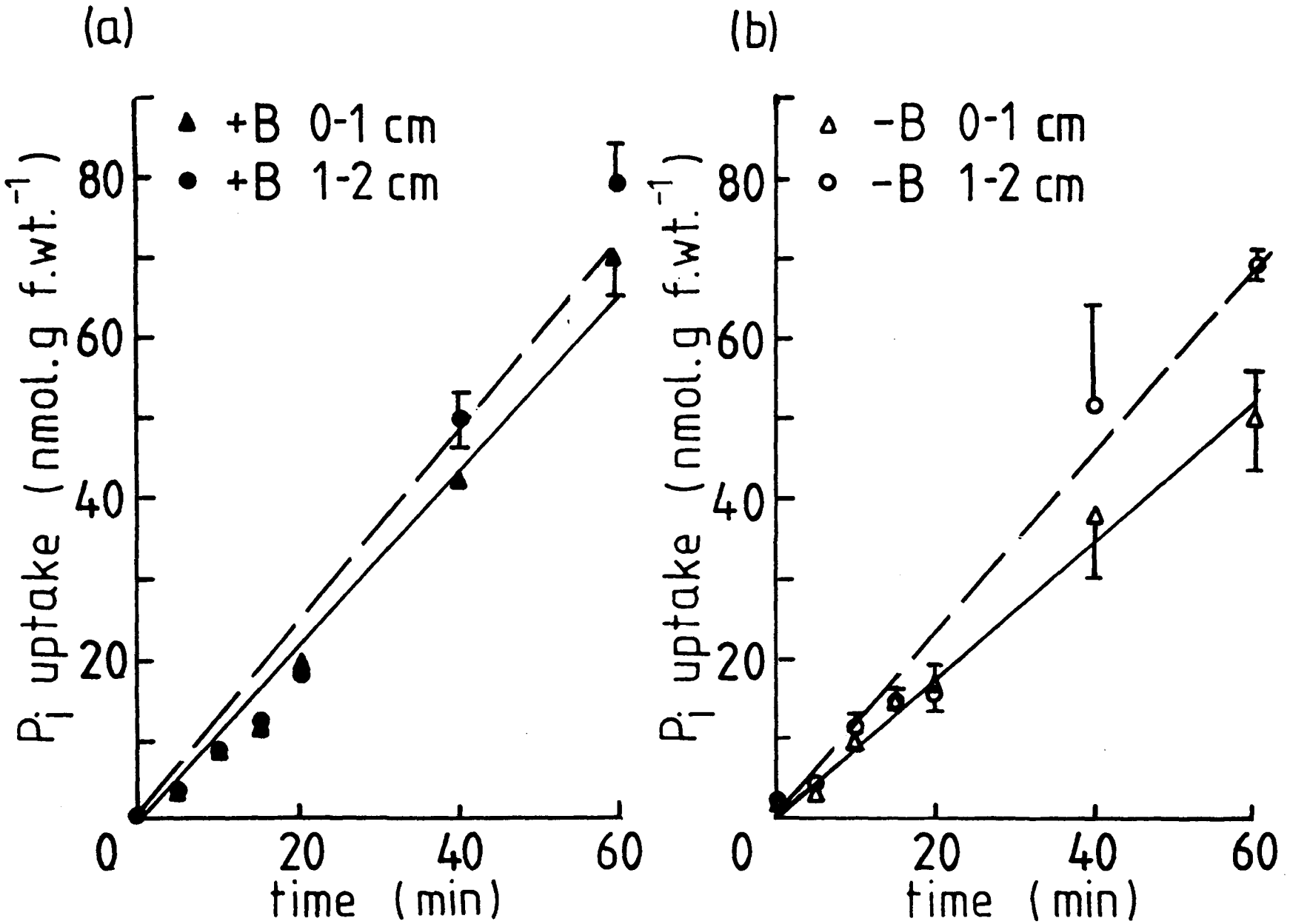


Fig. 14. Effect of B status and root zone on P_i uptake by 'LG11' maize roots. Plants grown for 45 h +B (4.6 µM H_3BO_3) followed by 24 h (a) +B or (b) -B. Linear regression *** in each case; +B curves show *** reduction with positive quadratic term.

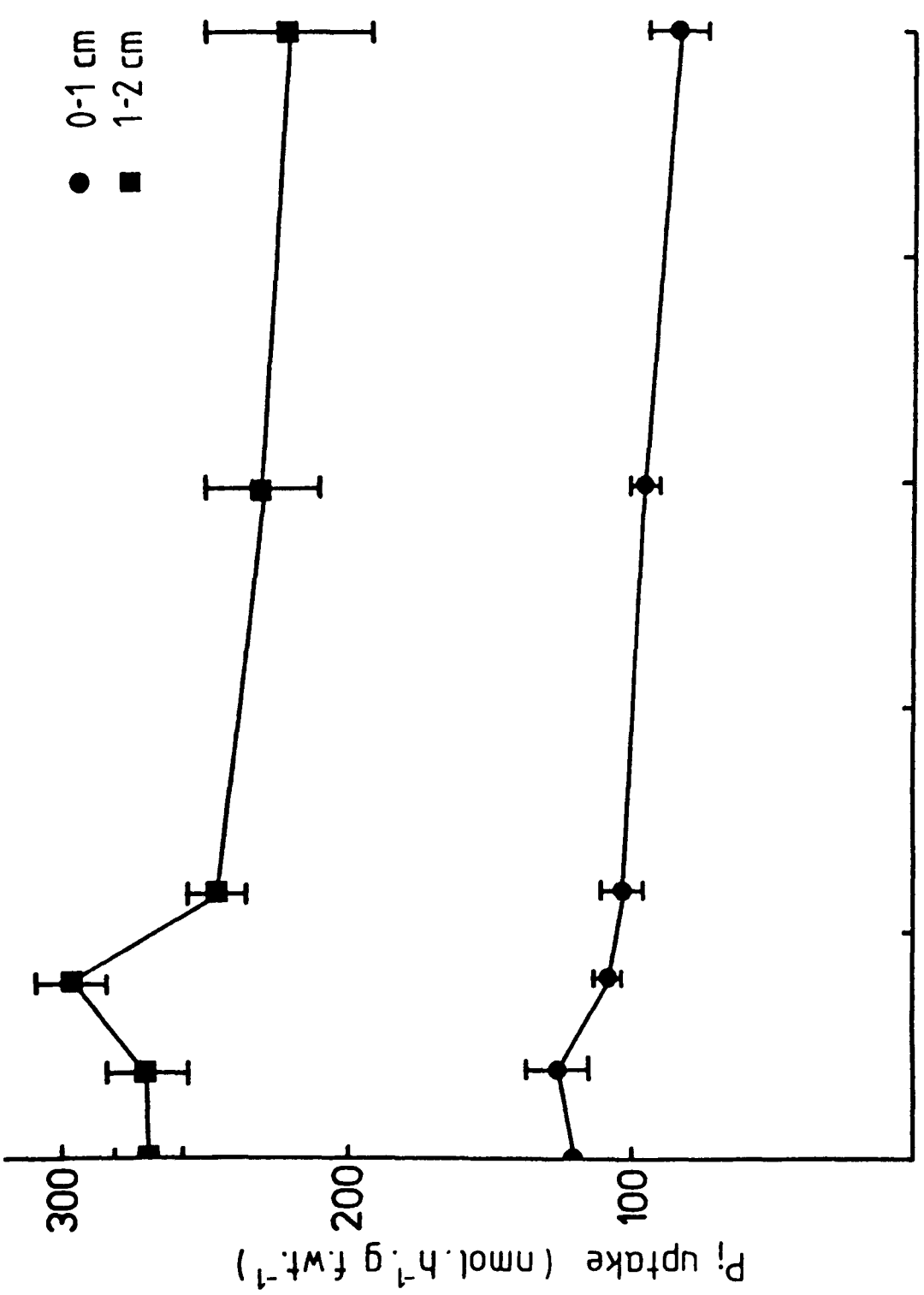


Fig. 15. Effect of boron deprivation on on P_i uptake by sections of 'LG11' maize roots. All plants grown for a total of 2 d; initially +B (4.6 µM H_3BO_3) and then transferred to -B solutions for the times shown.

Early experiments indicated that P_i uptake by cv 'Rubis' was more boron sensitive than uptake by cv 'LG11'. Uptake by 5-25 mm root segments is shown in table 17 (p. 77).

1.4 Maize protoplasts

In early experiments protoplasts were isolated from 2 cm -B root tips and held for 6-15 h before uptake was tested (with or without boron at 46 µM). Uptake was from 0.7 mM P_i; in each case uptake was linear with time over 1 h and there was no significant difference in uptake ±B. There was no difference in the rate of uptake over this time. Increasing the number of protoplasts per vial 7-fold reduced the mean uptake per protoplast by a factor of 2, so the number of protoplasts per vial was routinely kept between 0.7 and 1.5×10^4.

When protoplasts were isolated from 0-1 or 1-2 cm root sections after 2 d growth in +B nutrient solution and 24 h in ±B solutions, uptake from 10 µM P_i 5 h after isolation was as shown in fig. 16 (p. 78). The uptake capacity of protoplasts from the 1-2 cm section was greater than in the 0-1 cm section as expected and compared favourably with the uptake per cell; i.e. 15-20 $fmol.h^{-1}.protoplast^{-1}$ from the 0-1 cm zone and 20-30 $fmol.h^{-1}.protoplast^{-1}$ from the 1-2 cm zone. B was supplied at 11.5 µM where appropriate. There was no significant difference in uptake between ±B protoplasts from the 1-2 cm zone. Resupplying boron to the -B, 0-1 cm protoplasts for 3 h prior to uptake (fig. 16, p.78) marginally increased their uptake capacity. Recalculating these figures in terms of protoplast surface area made no difference to the uptake rates for protoplasts from the 1-2 cm zone and accounted for the small difference observed in the 1 cm tips.

Table 17. P_i uptake by 'Rubis' maize roots

	Boron treatment			
	24 h +B	24 h -B	48 h +B	48 h -B
Uptake (nmol. $h^{-1}.cm^{-1}$ root)	1.12 ± 0.05	0.73 ± 0.04 ***	1.19 ± 0.08	0.72 ± 0.04 **
% of 24 h +B	(100)	65	106	64

All plants grown for 2 d in full nutrient solution before experimentation. 0.5-2.5 cm tips used for uptake. B supplied at 4.6 μM .

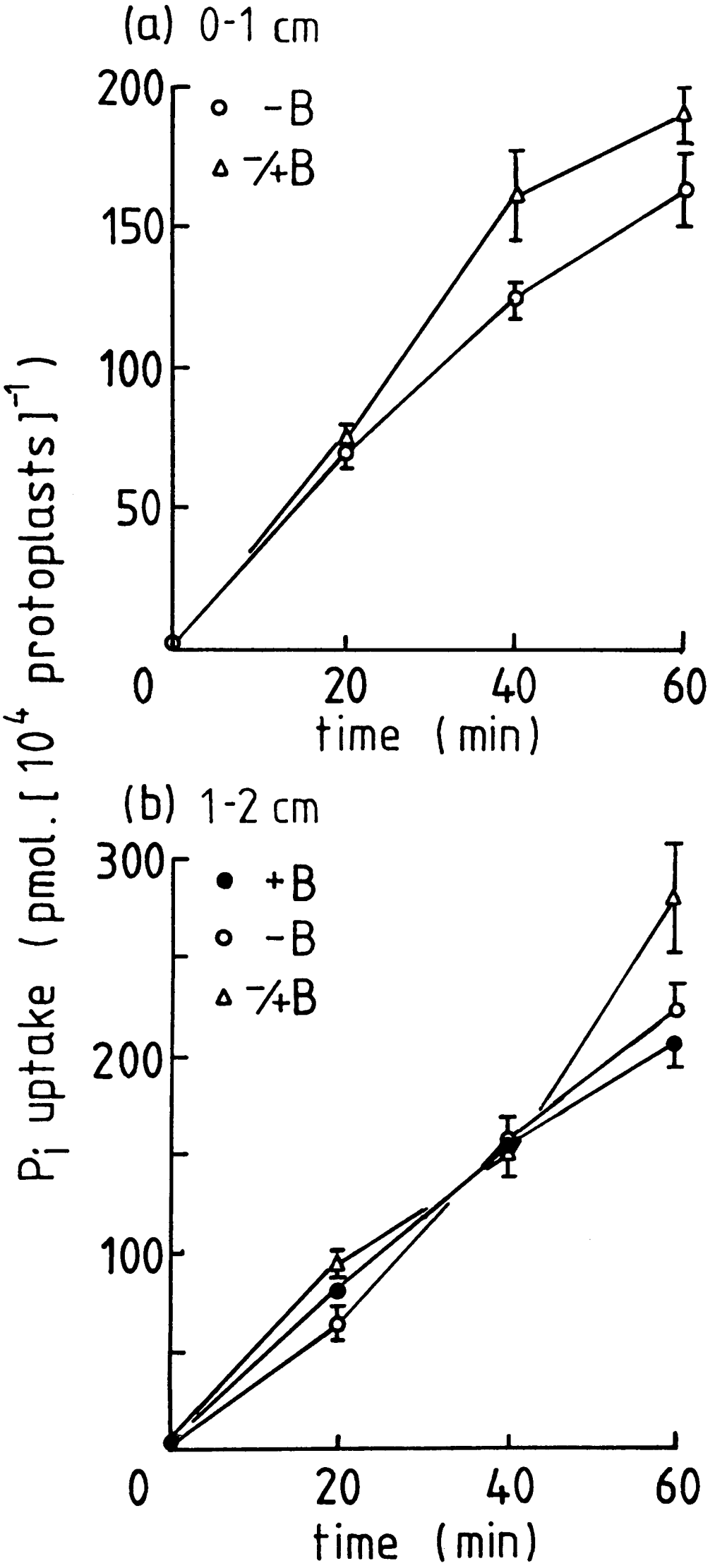


Fig. 16. Effect of B status and zone of origin on P_i uptake by protoplasts isolated from (a) 0-1 and (b) 1-2 cm zone of 'LG11' maize roots. Plants grown for 2 d +B (4.6 µM H_3BO_3) followed by 1 d +B or -B. Protoplasts isolated in the presence of BSA and PMSF. +B protoplasts supplied with 11.5 µM H_3BO_3 throughout; -/+B supplied with B for 3 h prior to uptake.

2. Potassium (^{86}Rb) uptake

2.1 Sunflower roots

Uptake studies from 1 mM KCl by 2 cm root tips gave consistent results; those from a typical experiment are shown in fig. 17 (p. 80). The effect of transfer to -B solutions was to increase uptake for the first 1-2.5 h; this increased uptake was followed by a gradual decline; uptake became significantly different from controls by 15-18 h -B. In these particular experiments the weight increase often recorded after 12-18 h -B was not significant, because the +B roots were heavier than usual; this suggests that the decreased uptake was not due to altered root geometry.

Successively shorter root sections were examined to see whether the tip region was more sensitive. The results of two experiments are shown in fig. 18 a,b (p. 81). Uptake by 15 mm tips was very variable between experiments. In one experiment (data not shown) uptake was reduced by 20% after 5 h -B; after 18 h -B it was further impaired (fig. 18b). In 10 mm root tips the mean uptake was reduced by 35% after 4 h -B (but high variability meant this was not a statistically significant reduction). Uptake was reduced by 40% after 18 h -B. However, whereas the terminal 5 mm segment showed a 60% decline in uptake after 4 h -B in an experiment where the weight and diameter of that region had also increased, uptake was restored to the control level after 18 h -B. In that experiment, the tip weights were equal.

K^+ uptake experiments were also performed with 10 µM external $[K^+]$ in joint experiments reported in White (1983). Greater boron sensitivity was demonstrated at this concentration; after 18 h -B uptake was decreased by up to 86% (fig. 19, p. 82). White (1983) also showed that uptake from 1 mM KCl could be restored to 120% of control levels after 3 h resupply of 100 µM H_3BO_3 to 18 h -B plants.

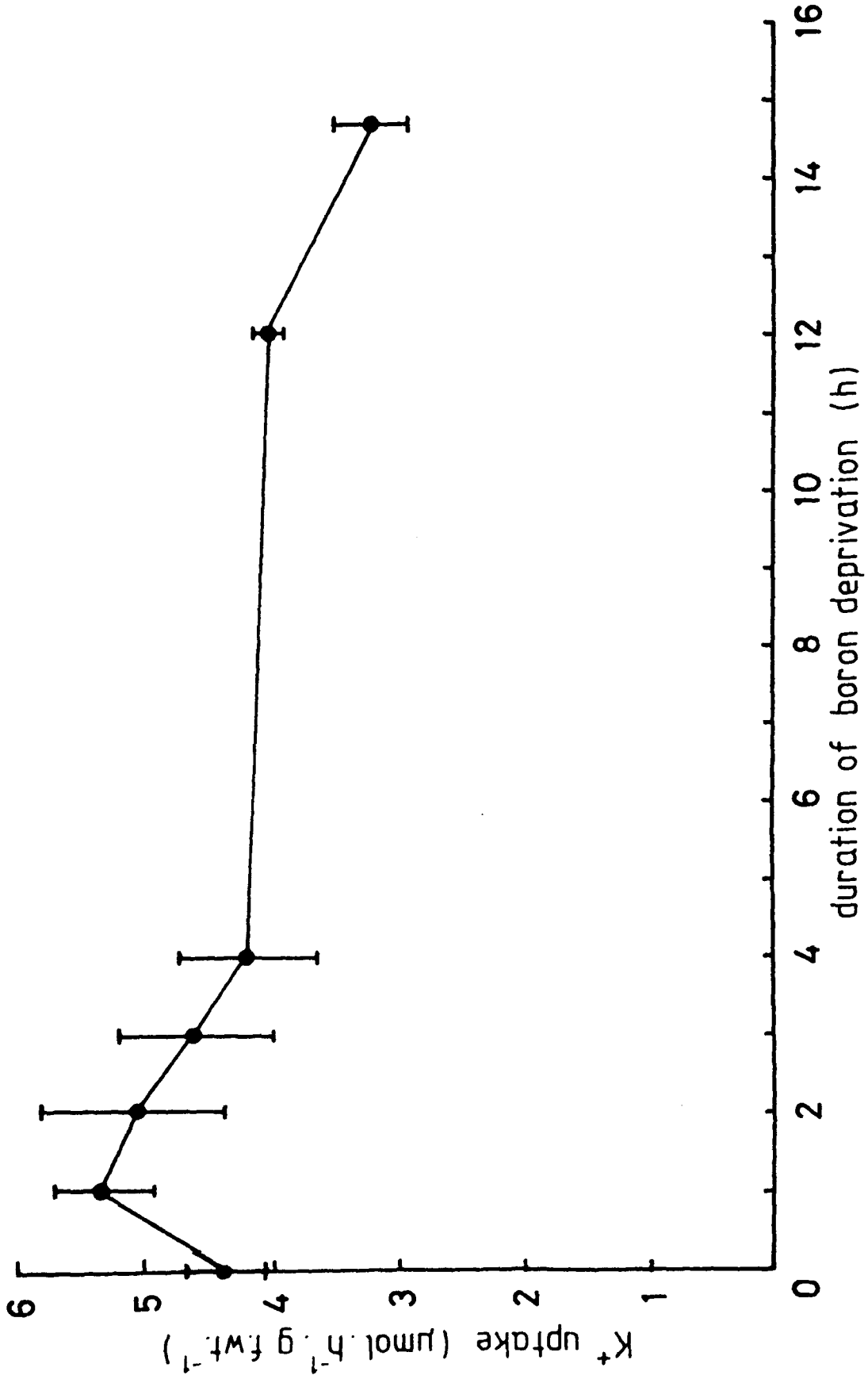


Fig. 17. Effect of boron deprivation on K^+ uptake by 2 cm 'Single Tall' sunflower root tips. Plants grown for a total of 42 h; initially +B (4.6 μM H_3BO_3) and then transferred to -B solutions for the times shown.

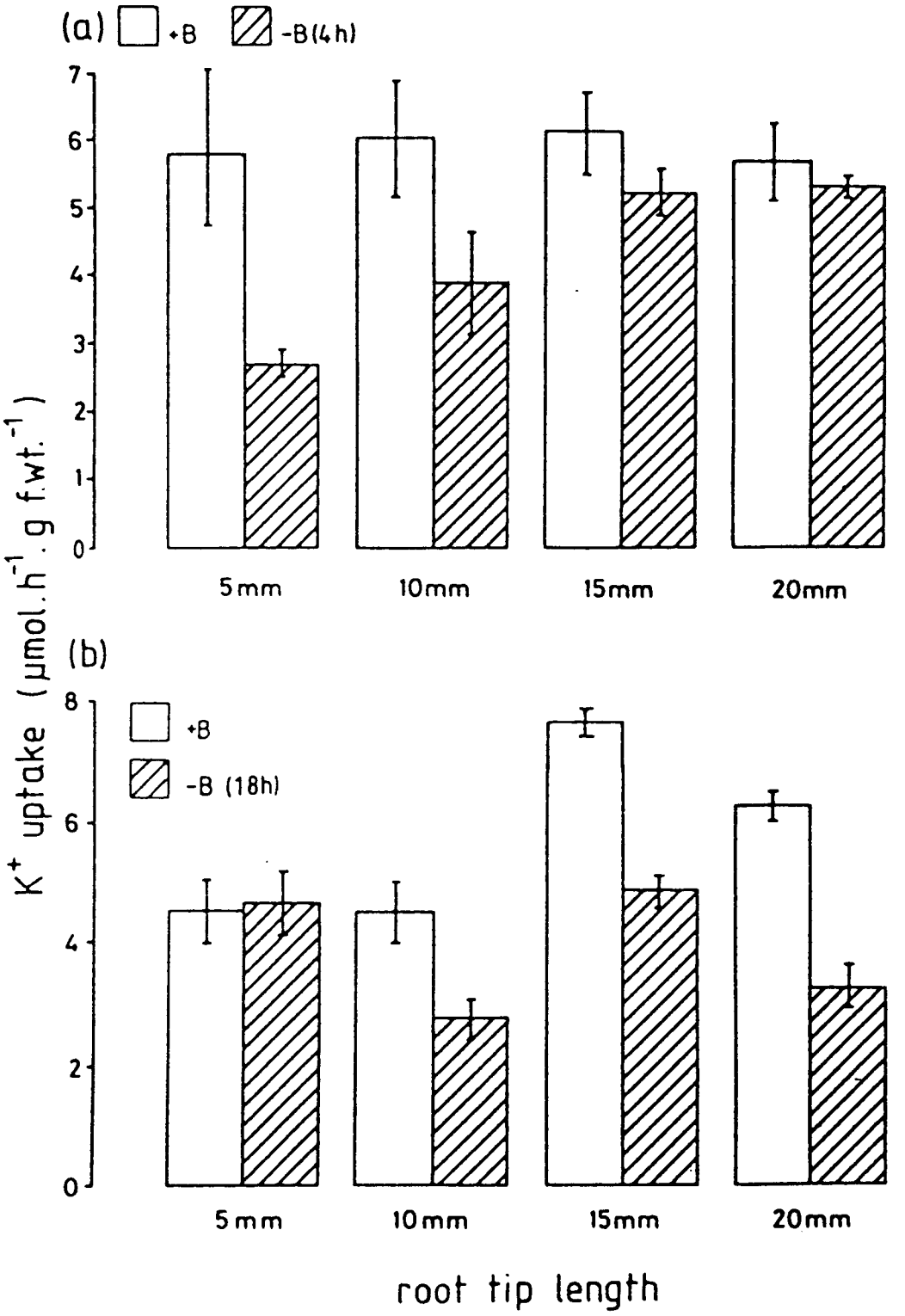


Fig. 18. Effect of boron deprivation on K^+ uptake by 'Single Tall' sunflower root tips of varying lengths. Plants grown for a total of 42 h; initially +B (4.6 μM H_3BO_3) and then +B or -B for (a) 4 h or (b) 18 h.

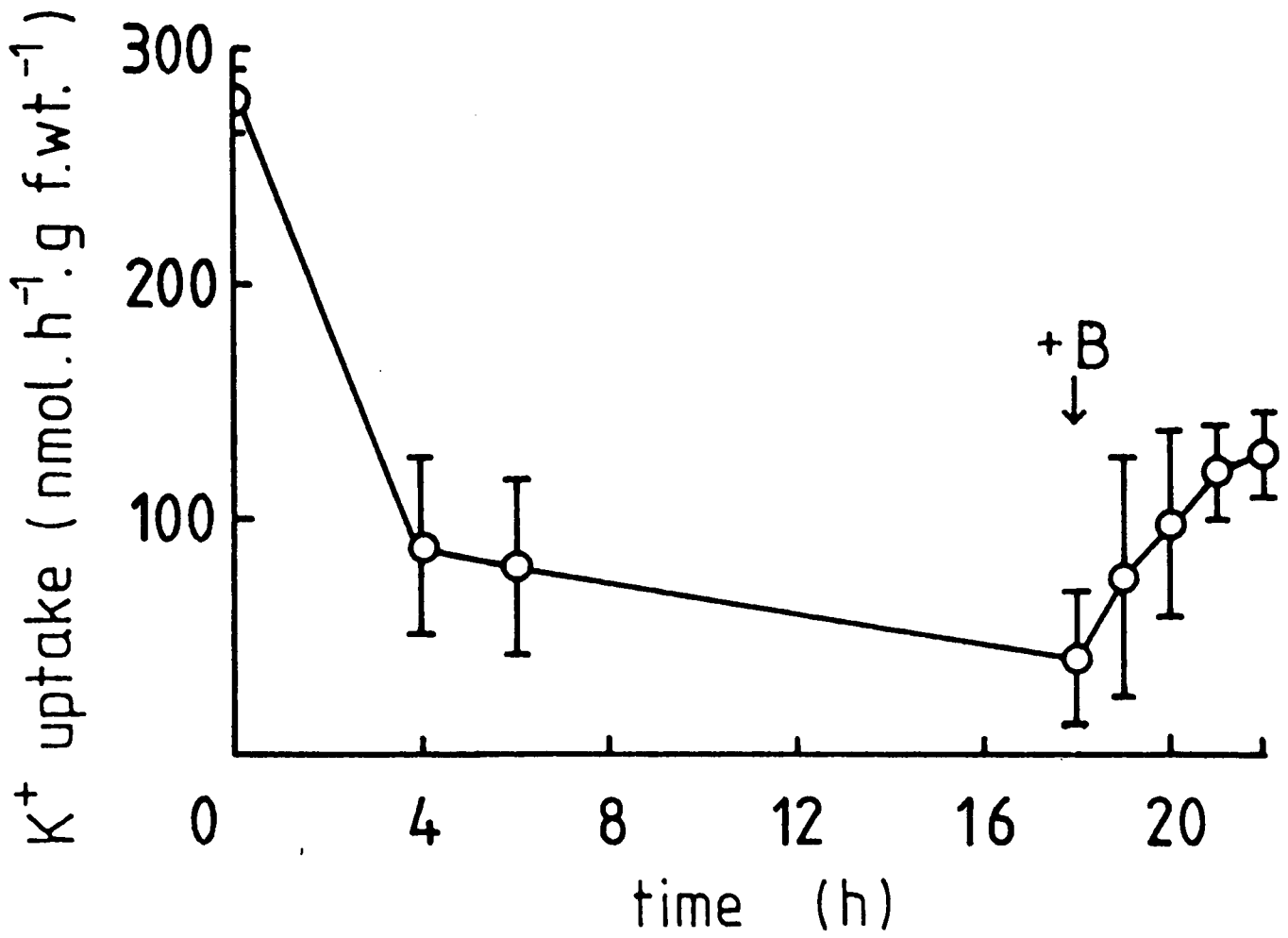


Fig. 19. Effect of boron deprivation and resupply on K^+ uptake by 2 cm 'Single Tall' sunflower root tips. Plants grown for 3 d +B (100 µM H_3BO_3) before transfer to -B solutions at time = 0 in figure. B resupplied at 100 µM after 18 h -B. Uptake from 10 µM [K^+], measured over 10 min period; root tips then desorbed in 100 µM [K^+] for 30 min.

The approximate uptake per cell in 2 cm tips was calculated as 300 $fmol.h^{-1}.cell^{-1}$ from 1 mM KCl.

2.2 Sunflower protoplasts

Preliminary experiments suggested that, as in roots (White, 1983), uptake was not directly proportional to the external $[K^+]$ because of system I and II effects; a 70-fold increase in $[K^+]$ led to about a 25-fold increase in uptake. Protoplast ion uptake experiments were therefore done with 10-20 µM KCl solutions to increase ^{86}Rb accumulation and make any deficiency effects more readily detectable. Boron was added where required at the comparatively high level of 46 µM. The uptake of K^+ by protoplasts from 2 cm tips was generally linear with time over a period of 60 min when determined 4-5 h after isolation (figs. 20 b, 21 b, pp. 84-85). The uptake rate was approximately 10-20 $fmol.h^{-1}.protoplast^{-1}$ from 20 µM (K^++Rb^+) solutions.

In some experiments uptake was tested 2-3 h after isolation as well as at 4-5 h. Figs 20, 21 (pp. 84-85) show that uptake tailed off after 30-40 min to a greater extent at the earlier time than at the later time. All K^+ and P_i uptake experiments with protoplasts were therefore routinely done after the 'recovery period' of 4-5 h.

In one experiment where there was appreciable bacterial contamination of the preparation the opportunity was taken to determine the contribution of bacterial uptake. Vials were left to settle and protoplast-free supernatant samples taken. Uptake by the bacteria alone was approximately 10% of the uptake by protoplasts in the same volume.

Identical batches of protoplasts were obtained from 2 cm root tips of 3 d old plants which had been grown in the dark on tissues and not transplanted to nutrient solutions. When these batches were divided and treated with +B or -B for 2.5-4.5 h after isolation, no effect of added B

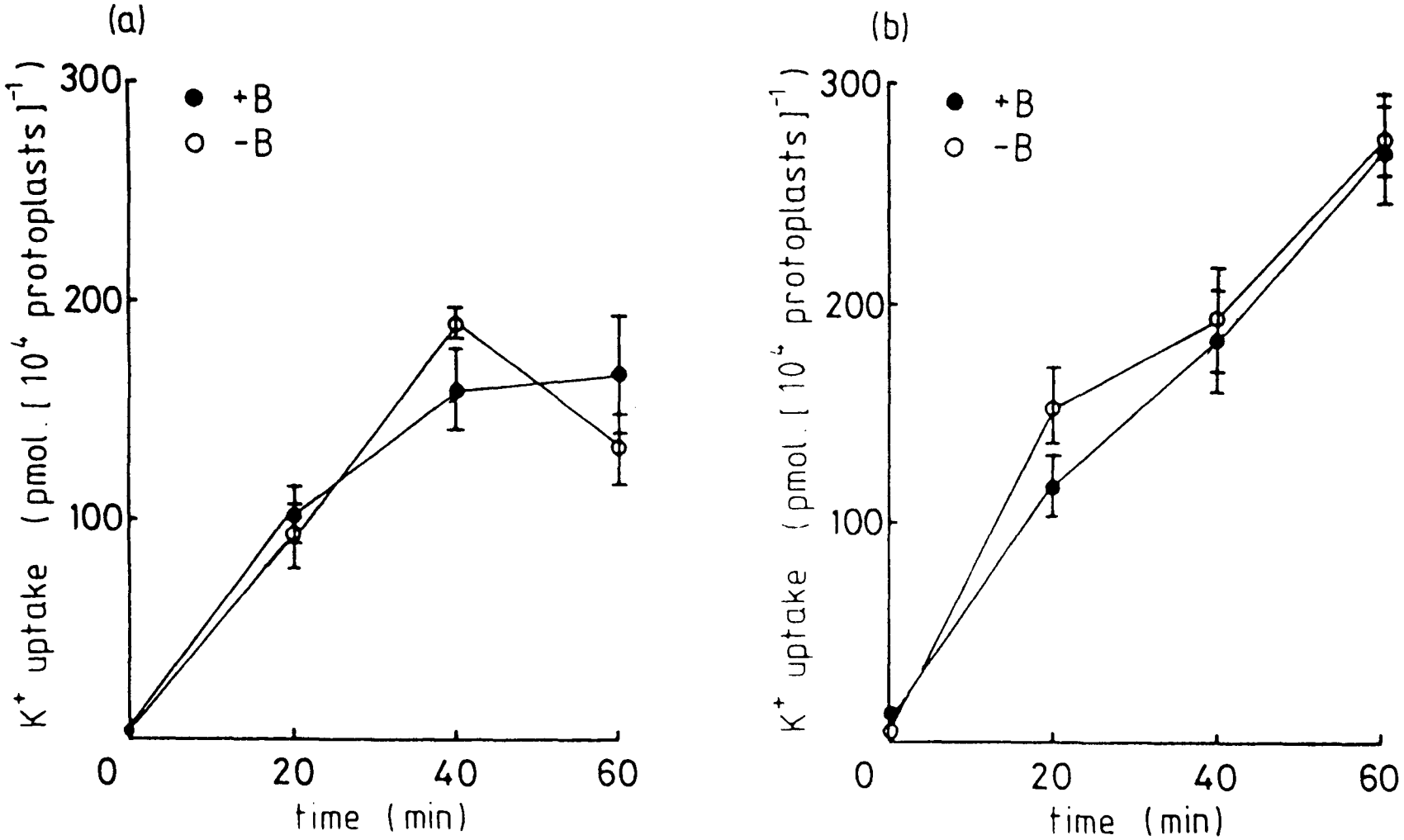


Fig. 20. Effect of boron status and recovery time on K^+ uptake by protoplasts from 2 cm 'Single Tall' sunflower root tips. Plants germinated for 3 d; 46 μM H_3BO_3 supplied to +B protoplasts after isolation. Uptake measured (a) 2.5 h or (b) 4.5 h after isolation.

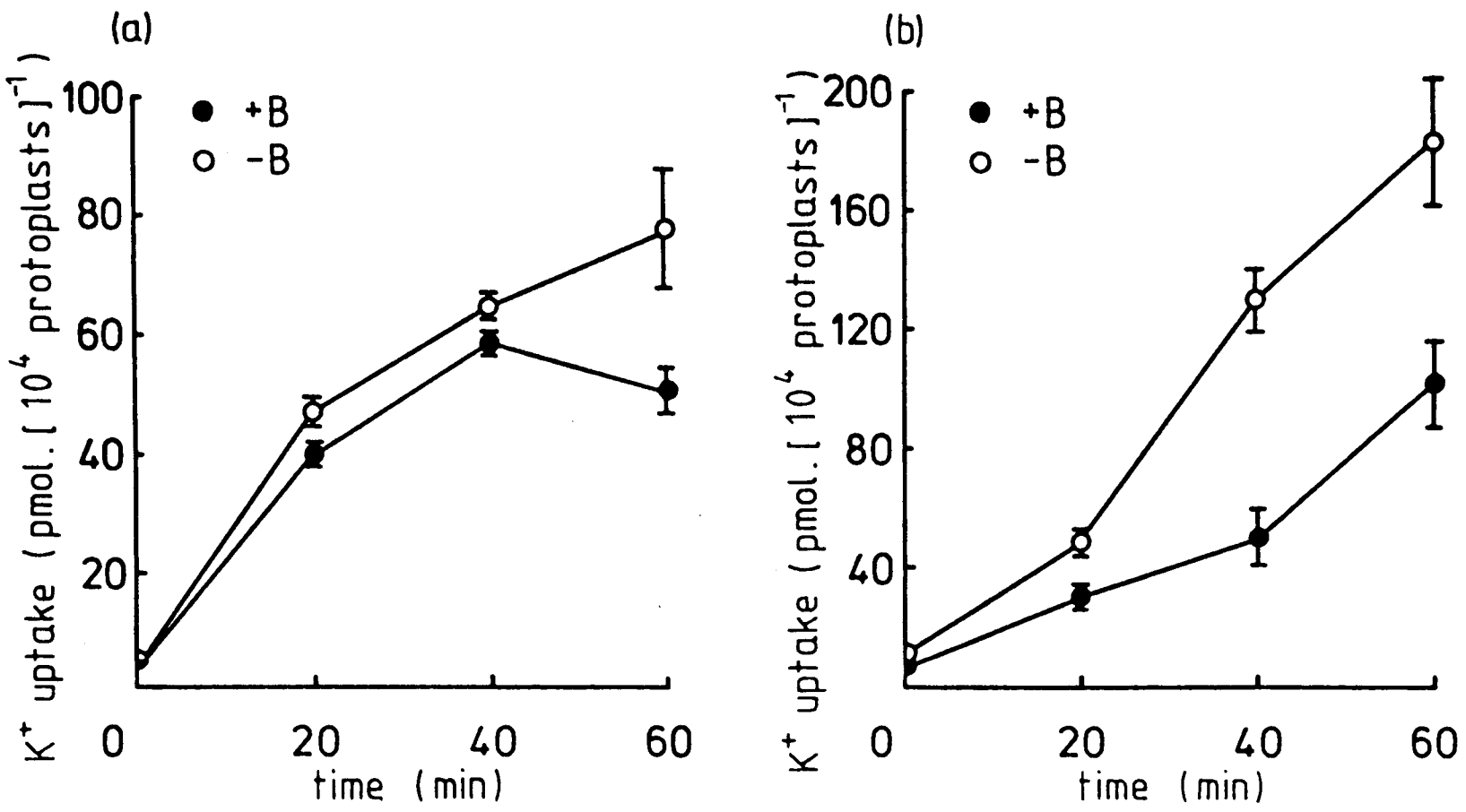


Fig. 21. Effect of boron status and recovery time on K^+ uptake by protoplasts from 2 cm 'Single Tall' sunflower root tips. Plants germinated for 3 d; 46 µM H_3BO_3 supplied to +B protoplasts throughout isolation. Uptake measured (a) 3 h or (b) 5 h after isolation.

was observed (fig. 20 a,b, p. 84). However if protoplasts were obtained from similar tissue-grown 3 d old plants but isolated in the presence of 46 μM B, the subsequent removal of boron for 3-5 h resulted in increased uptake (fig. 21 a,b, p. 85). Finally if protoplasts were isolated from the usual 5 d old plants which had been growing in +B or -B nutrient solutions for 2 days, the following points could be noted (fig. 22, p. 87).

The overall level of ion uptake was reduced and batches of protoplasts isolated from replicated batches of roots showed uptake roughly proportional to the fresh weight of the roots from which they were isolated, irrespective of the B treatment. It was this observation which highlighted the need to measure protoplast size.

Some workers express protoplast uptake per unit protein in the preparations; this method was tested for many experiments in addition to the counts. Typical uptake rates for K^+ were 2-4 $nmol.h^{-1}.mg\ protein^{-1}$. In the experiment shown in fig. 22, calculations demonstrated that all the uptake rates were n.s. different from each other; i.e. differences in the amount of protein in the preparation accounted for the variability between the preparations.

2.3 Maize roots

After 10 h -B, uptake by 1 cm root tips from 1 mM K^+ was the same as control uptake. The mean uptake rate of the older root parts (1-2 cm) seemed to be reduced by 35% but the high variability of the +B roots meant the difference was not statistically significant (fig. 23a, p. 88). Some plants from the same experiment were grown for a further 14 h +B or -B; at that time uptake by the control 1 cm tips had increased more than uptake by the -B tips. Uptake in the 1-2 cm zone had not significantly changed with time (fig. 23b, p. 88). There was no effect of boron deficiency on the proportion of exchangeable K^+ removed by 30 min desorption in ice-

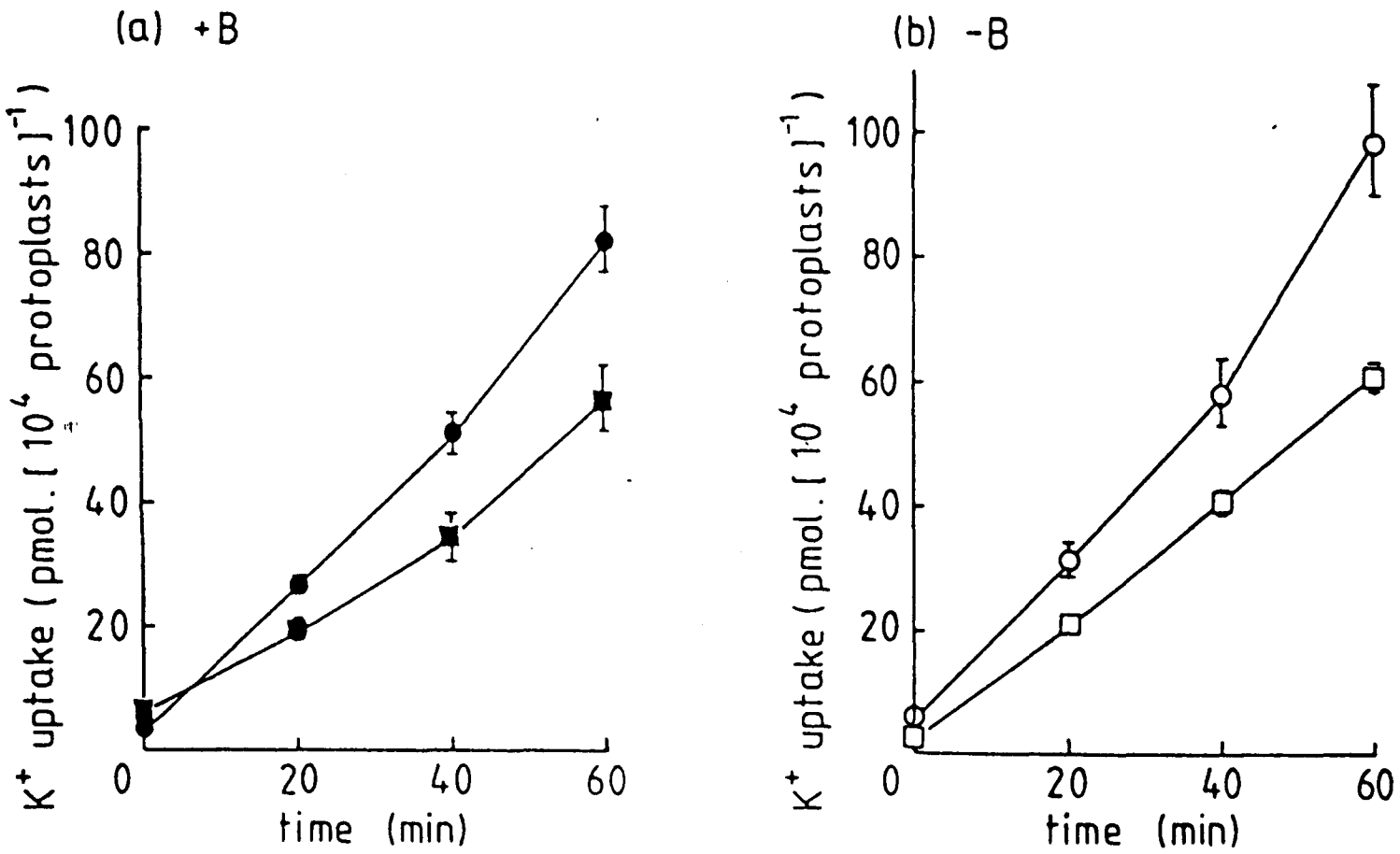


Fig. 22. K^+ uptake by protoplasts from 2 cm 'Single Tall' sunflower root tips. Plants grown for 54 h +B (4.6 µM H_3BO_3) followed by (a) +B or (b) -B for 14-15 h. Replicate root batches were kept separate throughout the isolation process. +B protoplasts supplied with 9.2 µM H_3BO_3; uptake measured 5 h after isolation. Mean f.wt. of root tip batches: +B 7.6 (●) and 5.8 (■) mg.tip^{-1}; -B 11.0 (○) and 6.7 (□) mg.tip^{-1}.

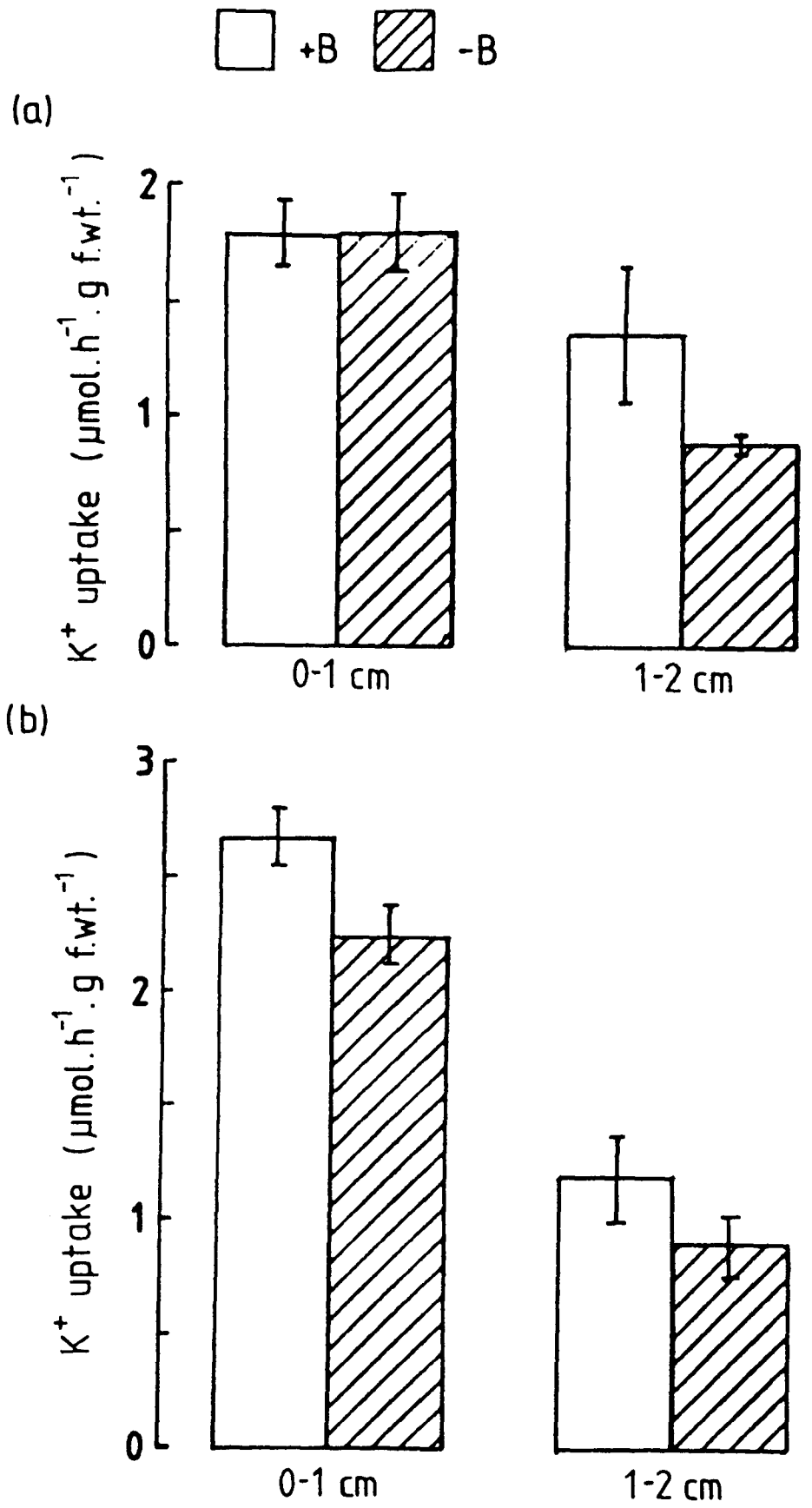


Fig. 23. Effect of B status and zone of origin on K^+ uptake by 'LG11' maize root segments. Plants grown for 37 h +B (4.6 µM H_3BO_3) and then +B or -B for (a) 10 h or (b) 24 h.

cold 10 mM KCl (table 18, p. 90).

Uptake was of the order of 200-300 fmol.h^{-1}.$cell^{-1}$ in the 2 cm tips.

2.4 Maize protoplasts

Only one experiment was done for K^+ uptake. The results are shown in fig. 24 (p. 91); uptake was linear over 60 min and protoplasts from 15 h -B plants absorbed less K^+ than protoplasts from +B plants. These results agree with those for the root tip experiment above; but uptake per unit surface area was not measured and so any protoplast size differences would affect the figures.

The uptake rate was 4-8 fmol.h^{-1}.$protoplast^{-1}$ from 30 µM (K^++Rb^+).

3. ATPase assay in sunflower roots.

The use of Allen's P_i assay (1940) poses serious problems when determining ATPase activity. The low pH treatment used to stop the ATPase activity and high molybdate concentration result in considerable breakdown of ATP and a resultant high background against which enzymic P_i release is measured. Table 19 (p. 92) shows some values for P_i released at each stage of the assay. Similar problems arise with other P_i assays commonly used in the study of ATPase activities, e.g. Fiske and Subbarow (1925). With strict attention to time and a minimal delay between stopping the ATPase reaction and recording the absorbance the assays can nevertheless give repeatable results, provided the Mg^{2+}-dependent and K^+-stimulated activities are equally affected.

Methods specifically designed for assaying enzymic P_i release in the presence of labile phosphate compounds do exist. Marsh (1959) suggested extracting the phosphomolybdate complex into butanol, with a cumbersome technique limited to less than 10 µg P_i in the assay. Lowry and Lopez

Table 18. K^+ removed by 30 min desorption of LG11 maize roots in ice-cold 10 mM KCl after 1 h uptake from 1 mM KCl

Root zone	Expt.No.	+B K^+ loss $\mu mol.(g\ f.wt)^{-1}$	+B % uptake	-B K^+ loss $\mu mol.(g\ f.wt)^{-1}$	-B % uptake
0-1 cm	1	0.20 ± 0.01	11	0.22 ± 0.02	12
	2	0.28 ± 0.02	11	0.25 ± 0.01	11
1-2 cm	1	0.21 ± 0.02	15	0.16 ± 0.02	18
	2	0.22 ± 0.03	19	0.17 ± 0.01	19

All plants grown for 2 d in full nutrient solution, followed by 10 h +B or -B (expt 1) or 24 h +B or -B (expt 2). Difference between treatments n.s.

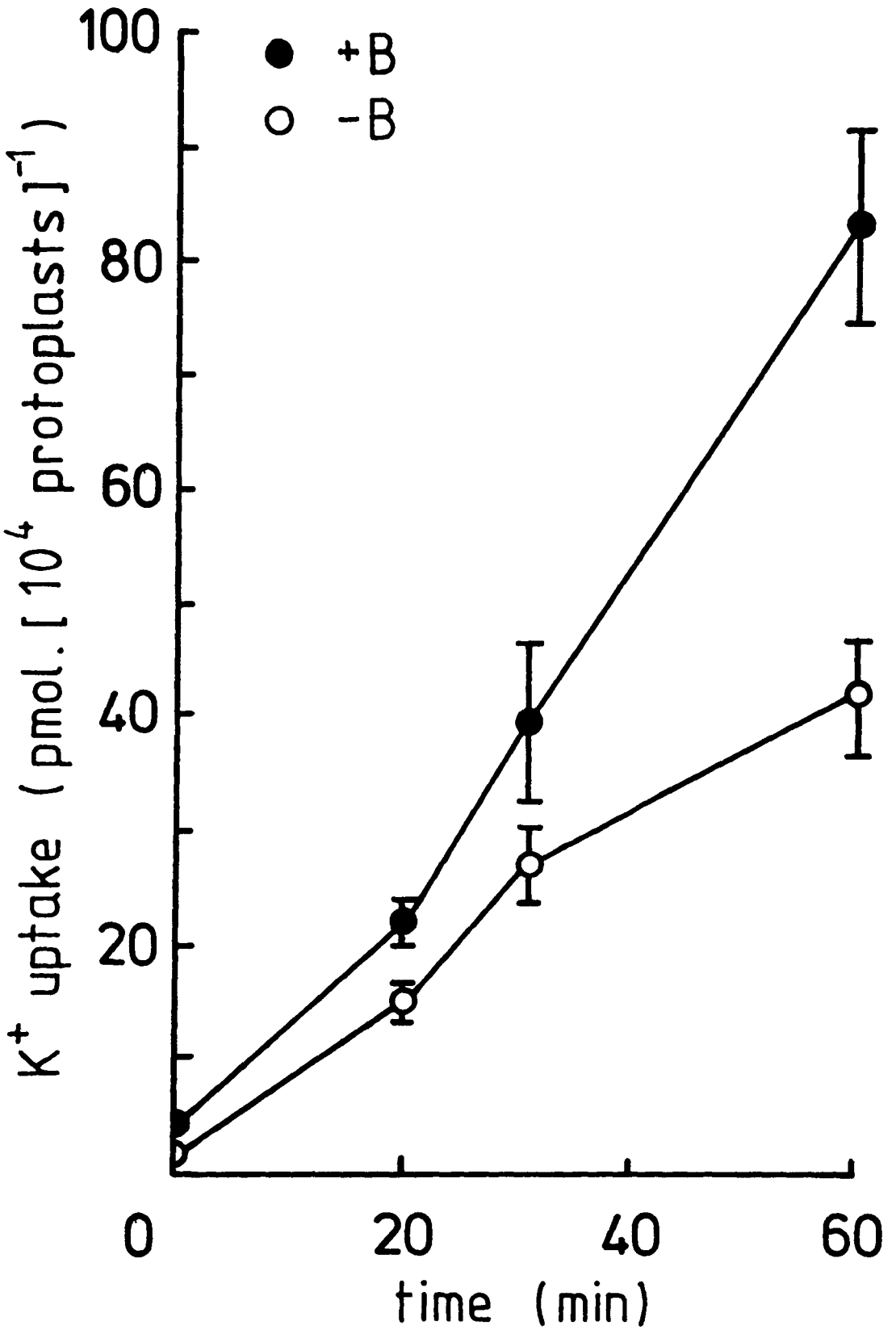


Fig. 24. Effect of B status on K^+ uptake by protoplasts from 2 cm 'LG11' maize root tips. Plants grown for 30 h +B (4.6 µM H_3BO_3) and then +B or -B for 14-15 h. +B protoplasts supplied with 9.2 µM H_3BO_3; uptake measured 5 h after isolation.

Table 19. Sources of enzymic and non-enzymic P_i release during ATPase assay

Enzyme type	Incubation temp (°C)	Stop temp (°C)	Hold temp (°C)	Time till read (min)	P_i released (nmol)
None	20	0-4	0-4	5	0[a]
None	30	0-4	0-4	5	35
None	30	20	20	5	230
Mg^{2+}-depdt.	30	0-4	0-4	5	260-370
K^+-stim.	30	0-4	0-4	5	320-440
Mg^{2+}-depdt.	30	0-4	0-4	45-70	9.6 ± 1.0[b]
K^+-stim.	30	0-4	0-4	45-70	8.5 ± 0.7[b]

a. Defined; actually 260 nmol, vs water blank.

b. $nmol.min^{-1}$, (n=7). Difference = n.s.

(1946) (modified by Peel and Loughman (1957)), and Rathbun and Betlach (1969) devised methods with lower molybdate concentrations and employing buffers to maintain the pH at around 4. The latter method was successfully applied here but was found to be restricted to a maximum of 60 µg P_i in the reaction mix.

Working with Allen's method, White (1983) characterised the ATPase activity of a membrane preparation isolated from 2 cm sunflower root tips. Ca^{2+} or Mg^{2+} were required for the enzyme activity; 2-7 mM Mg^{2+} gave maximal activity. K^+ stimulation measured over the range 0-100 mM was maximal between 50 and 80 mM at around 170% of the Mg^{2+}-dependent activity. The pH optimum for the Mg^{2+}-dependent activity showed a shallow peak at around pH 6.5 whereas the K^+-stimulated activity had a sharper peak at the same pH.

White also observed early effects of boron deficiency on this K^+-stimulated, Mg^{2+}-dependent ATPase activity. He reported a continuous decline in activity over the first 18 h -B; most significantly he also reported that this activity could be restored within 3-4 h by resupplying 100 µM H_3BO_3 to the plant roots for 1-3 h before membrane isolation; treatment of the isolated membrane preparation with 100 µM H_3BO_3 was much less effective (fig. 25, p. 94).

A preliminary investigation of the boron content of the membrane fraction used in such experiments was carried out. The boron bound to the membranes was detected with the (n,α) method; results are shown in fig. 26 (p. 95) (experiments by P. White, sample preparation by J.A.H., B determination by F. Martini in Rouen). The membrane samples provided by us were too large for accurate estimation of the B content by their method and had a gradient of B from the top to the bottom of each droplet (50 µm). The estimates obtained from the bottom detector were used to compile fig. 26, but could not be converted to a reliable concentration figure.

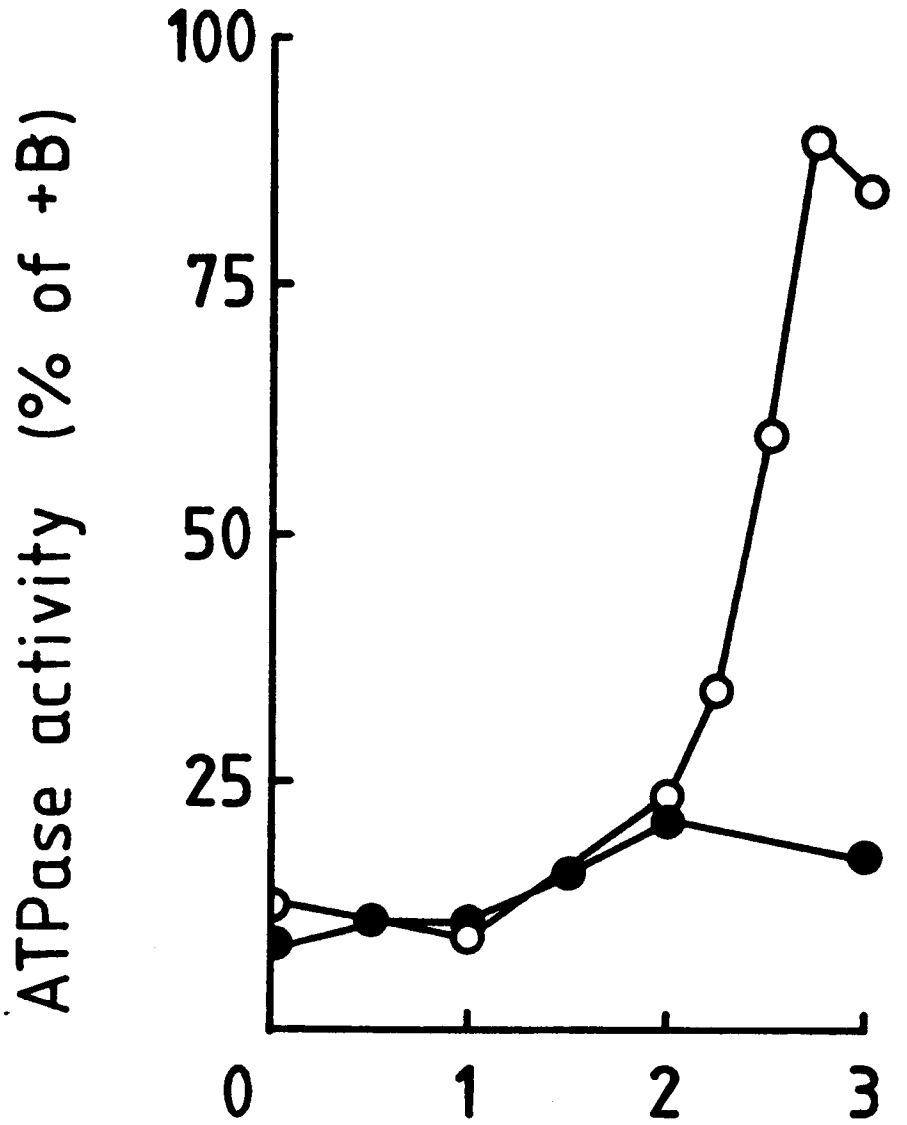


Fig. 25. Effect of resupply of 100 µM H_3BO_3 on K^+-stimulated, Mg^{2+}-dependent ATPase activity of 18 h -B 'Single Tall' sunflowers. B resupplied to intact roots (o) or isolated membranes (●). Plants initially grown for 3 d +B (100 µM H_3BO_3). (Redrawn from White, 1983.)

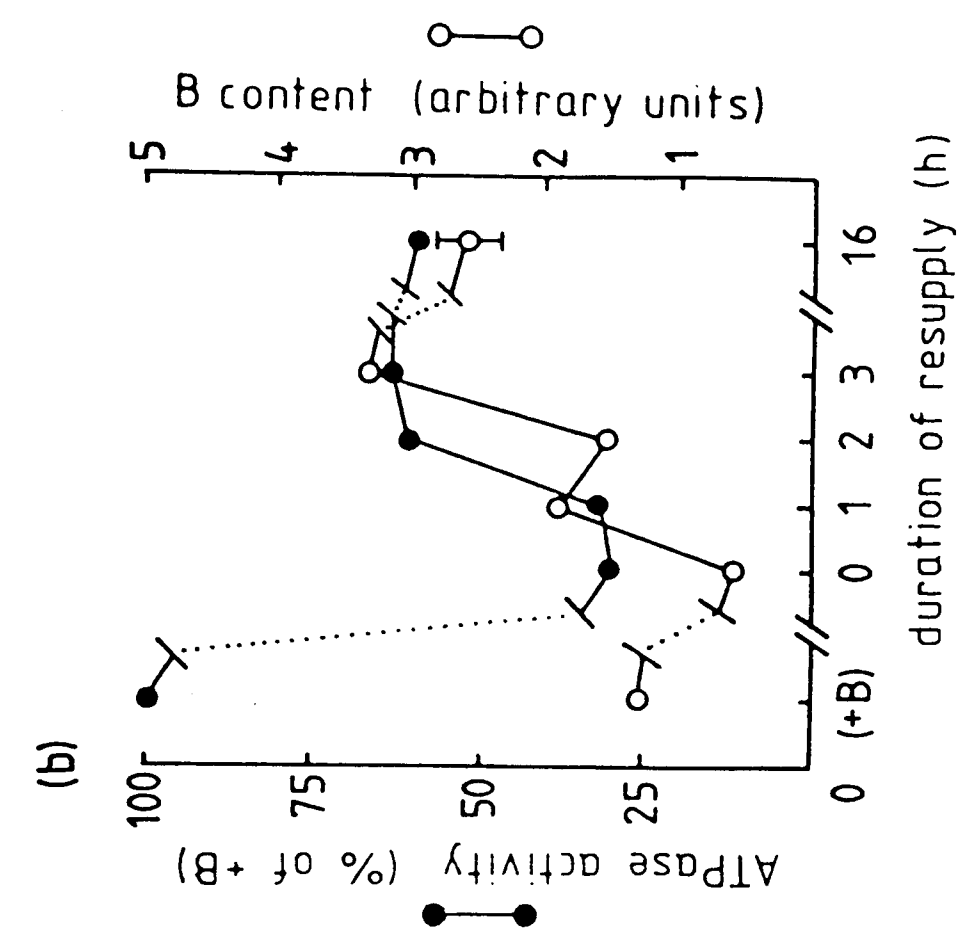


Fig. 25. B content estimated by (n,α) method.

Fig. 26. Correlation between B content and ATPase activity of membranes isolated from 18 h -B 'Single Tall' sunflower roots. B resupplied to intact roots; (a) and (b) = replicated experiments; other details as in fig. 25.

Some further experiments suggest that the specific activity of a K^+-stimulated ATPase found in a 9-13 K 'g' fraction is 2-4 times that of the 13-80 K 'g' fraction. Further work is required to identify the membrane fractions involved. All of the K^+-stimulated activity and some of the Mg^{2+}-dependent activity were inhibited by 100 μM Na_3VO_4.

Discussion

The rates of uptake for the different ions and plant species used here agree in general with published figures (table 20, p. 98).

The low rate of P_i uptake by protoplasts reported in Lin (1980) was partly caused by the incubation of 5×10^5 protoplasts in each tube. Lin's uptake system functions better with a lower number of protoplasts per tube (see e.g. p. 76). Also, Lin did not add BSA to the wall digestion mixture. The beneficial effect of adding additional substrate for the protease contaminants of the wall digesting enzymes and thereby protecting the protoplast membranes from damage is now well established (table 13, p. 68).

Similarly, because BSA or PMSF were not used in the protoplast K^+ uptake experiments, table 20 contains some much higher rates reported in the literature from experiments done with such protectants included in the incubation medium (and at higher external molarities).

The rate of ion uptake by root tips was expected to be linear over an hour (Epstein, Rains and Schmid, 1962); it is interesting to note that the rate of P_i uptake by +B maize root tips appeared to increase over the hour (fig.14, p. 74). Root tips were excised and tested within a few minutes so this may represent a recovery from the slight inhibition of uptake caused by excision; in that case the failure of -B plants to respond similarly would conform with their generally lower rate of uptake.

Ion uptake by the 1-2 cm zone of roots was regularly observed to be higher than for the 0-1 cm zone, in keeping with the greater tissue maturity in that region.

The system used here for measuring ion uptake by protoplasts seems to function very well. In all cases 'uptake' by protoplasts spun down through the layers without incubation was very low. This is more satisfactory than

Table 20. A comparison of some published uptake rates with those obtained here.

Ion	Plant species and tissue	Uptake rate ($nmol.h^{-1}.g^{-1}$ f.wt.)	External Conc.	Ref.
P_i	Sunflower roots	500-1000	2 mM	1
		300- 400	0.1 mM	2
		50	10 µM	3
		100- 200	10 µM	12
	Maize roots	80	1 mM	4
		250	0.25 mM	5
		450	0.2 mM	6
	(3 d old)	50- 160	10 µM	7
	(18 h old)	25- 70	10 µM	7
		50- 180	10 µM	12
K^+	Sunflower roots	1000-3000	2 mM	1
		4000-6000	1 mM	12
		50	0.4 mM	8
	Maize roots	6000	1 mM	9
		2500-2800	1 mM	7
		1000-3000	1 mM	12
		400	1 mM	4
		(10^6 $(nmol.h^{-1}.protoplasts)^{-1}$)		
P_i	Sunflower root protoplasts	4	30 µM	3
	(0-1 cm zone)	5- 20	10 µM	12
	(1-2 cm zone)	50-100	10 µM	12
	Maize root protoplasts	2	1 mM	10
	(cortical)	15- 20	1 mM	11
		15- 20	10 µM	12
K^+	Sunflower root protoplasts	10- 20	20 µM	12
	Maize root protoplasts	10	1 mM	10
	(low-salt roots)	700	1 mM	11
		4- 8	30 µM	12
		($nmol.h^{-1}.mg\ protein^{-1}$)		
		25-120	1 mM	9
		3- 6	30 µM	12

References

1. Pettersson, (1975b).
2. Bowling, (1983).
3. Lefebvre, (1984).
4. Morgutti et al., (1981).
5. Bravo and Uribe, (1981).
6. Lin, (1979).
7. Pollard, Parr and Loughman, (1977).
8. Timashov, (1969).
9. Kochian and Lucas, (1983)
10. Lin, (1980).
11. Gronwald and Leonard, (1982).
12. This thesis.

the high rate of 'carry-over' reported by Lin (1980), working with a higher density of protoplasts in the tubes. The uptake rate was higher for protoplasts from the more mature, larger-celled region of the root (1-2 cm) than for protoplasts from the terminal 1 cm.

If the protoplast uptake system were operating normally, one might expect linear uptake with time as in the roots from which the protoplasts were isolated. However, P_i uptake by sunflower protoplasts from the 1-2 cm root zone was not sustained for the whole hour. This is largely a result of substrate depletion; 33-50 % of the total phosphate was accumulated by the protoplast preparations, which represents an even greater local depletion because the protoplasts settle out to some extent during the uptake period. In addition, the quality of the preparation was never as good from the more mature sunflower root tissue; the larger protoplasts were more fragile and there was a general increase in contamination of the preparation with debris.

Uptake was routinely measured 4-6 hours after the last centrifugation in order to obtain a high, linear rate of uptake (see figs 20, 21, pp. 84, 85). There are two factors which contribute to the behaviour of preparations assayed shortly after isolation.

Theories of allosteric feedback suggest that protoplasts which are more depleted in their internal ion concentration will show a higher rate of ion uptake (e.g. Gronwald and Leonard, 1982). The results described in para. 2.2 (p. 86) demonstrate that protoplasts from roots which had accumulated K^+ in advance took up less K^+ than protoplasts germinated on tissues in distilled water. Lefebvre (1984) demonstrated high efflux rates for sunflower protoplasts, partly as a result of recovery from the isolation process but also perhaps as a similar response to that shown by Bowling (1983) for excised sunflower root tips; the high rate of P_i uptake was sustained after excision but efflux was observed to increase

over a 24 h period until net influx was nil. So, a few hours after isolation, protoplasts from nutrient-grown roots cultured in PSM will be depleted in their internal ion concentration and hence show a higher uptake rate. The 'tailing-off' in the earlier rate of uptake would then be interpreted as impaired uptake once the internal concentration had risen above some critical value.

Alternatively, a recovery period may be required after the protoplasts are removed from the wall digesting enzymes. Taylor and Hall (1976) found that protoplasts would not retain ^{86}Rb under their experimental conditions, when assayed immediately after isolation. If the protoplasts were allowed to 'recover' for 24 h detectable amounts of ^{86}Rb were accumulated. The lag could be due to repair of the transport system after impairment by the presence of cellulase (Morris et al., 1981).

The rate of P_i uptake by maize protoplasts did not alter between 6 and 15 h after isolation, suggesting that any recovery which occurred was complete by 6 h. This also suggests that if the increase is partly an ageing response as in washed root tips (as suggested by Lefebvre, 1984), the protoplasts are more limited than the root tips in their capacity for increasing uptake.

Maize protoplasts regularly displayed linear uptake of P_i and K^+, suggesting the system was working better than that described for maize root protoplasts by Lin (1980), despite the omission of protein protectants from the wall digestion medium in some experiments.

Further evidence of the comparability of the protoplast ion uptake with that of root tips can be found in a comparison of the relative uptake rates for the ions by sunflower and maize roots and protoplasts.

Protoplast ion uptake is commonly expressed in one of two ways - per no. of protoplasts (unit volume of suspension, 10^6 protoplasts etc) or per unit of protein. It is essential to compare uptake rates on the same

basis; thus ion uptake per root cell can be loosely compared with uptake per protoplast. There is a considerable element of approximation in this because in each case there is an assemblage of cells from different parts of the root with differing uptake capacities. Thus in the intact root, because P_i uptake from a low external concentration may be largely accomplished by the cells in the outer cortical and epidermal layers, a calculation of the mean uptake per cell would underestimate the actual rate of uptake by the particular cells responsible for the bulk of the uptake. Similarly in the case of the protoplasts the uptake capacity of each cell is affected by the cell size and its origin, e.g cortical vs stelar protoplasts.

The most useful measure of ion uptake by root tips takes into account the tip weight. There is a close relationship between uptake and the surface area for that uptake; and the internal concentration of the ions, itself affected by the tissue (particularly cytoplasmic) volume, regulates uptake as described in the introduction to this section. To compare protoplast uptake on the same basis it is necessary to determine the protoplast volume. Indeed from that measurement and a protoplast count the mean surface area per protoplast can be calculated; but the greater relevance of the last measurement is obtained only by sacrificing some precision. Whereas total protoplast sample volume can be directly measured, to convert it into units of mean surface area introduces two additional sources of error. The first is the requirement to divide by the protoplast number, with its own inherent uncertainty; and the second is the assumption that protoplasts are of a roughly similar size, which is certainly not true in preparations from large segments of root tip. An additional reason for recording protoplast volumes for each experiment lies in the fact that protoplasts may shrink or swell in response to changes in their boron status. Parr (1980) reported that liposomes

containing maize root lipids differed in their shrink and swell capacity depending on their boron status so it is quite likely that protoplasts behave similarly. However, these volume changes may or may not be accompanied by changes in the number of carriers on the membrane surface, so uptake per protoplast may still not be affected proportionally. Protoplast volumes were recorded in only two experiments. In each case resupplying 9.2 μM H_3BO_3 to the protoplasts resulted in an apparent volume increase of 50% or more for protoplasts from the 0-1 cm zone of origin, and about 20% for protoplasts from the 1-2 cm zone. In maize this swelling was accompanied by an increase in P_i uptake per protoplast; the uptake rate per unit surface area remained constant. Either existing carriers increased their activity as the internal concentration dropped, or additional active carriers were incorporated during swelling.

Sunflower protoplasts retained a constant uptake per protoplast, and thus a reduced uptake per unit surface area; it is tempting to correlate this with the known sensitivity of sunflowers to boron deprivation and to suggest that uptake is sufficiently impaired for adaptation to increased volume to be impossible; but in another experiment, where PMSF had not been included in the incubation medium and P_i uptake was higher, boron resupply increased uptake (table 14, p. 68).

Similar arguments make the comparison of root tip and protoplast uptake on a per unit protein basis difficult. In particular this method of expressing protoplast uptake is not able to discriminate between live protoplasts and any moribund protoplasts or small amounts of debris in the preparation. Nevertheless it can be a helpful comparative figure, particularly in experiments when protoplast counts had to be made from small samples with a correspondingly high variability.

An appreciation of these points is essential to the discussion which follows.

P_i uptake by sunflower roots occurs at a similar rate per g f.wt. to that of maize; therefore the thicker maize roots are accumulating more P_i per unit length. The uptake rate per cell is higher in maize than in sunflower, in keeping with the larger cell size. Sunflower and maize root protoplasts accumulate P_i at a similar rate per protoplast to the rate per cell in the excised root tips. However the similarity is not complete; maize protoplasts from the 1-2 cm segment apparently absorb P_i from the 10 μM medium at a lower rate than sunflower protoplasts from the same region, unlike the cells from which they came. Volume measurements confirmed that sunflower protoplasts from the 1-2 cm zone are larger on average than maize protoplasts, whereas mean cell size as calculated in section 1 suggested that maize cells should be larger. The most likely explanation is that the large cortical cells of maize roots were not proportionally represented in the protoplast population, either because of their greater fragility or perhaps because of difficulties during their formation. Uptake per unit surface area is very similar for sunflower and maize protoplasts from the 1-2 cm zone.

In the case of K^+ uptake, maize roots have a lower rate of uptake than sunflowers per g f.wt. but a similar rate per cell. Protoplasts from both species accumulate K^+ at a similar rate per protoplast to the rate per cell, at the same external concentration. In line with the observations above for P_i accumulation, maize protoplasts take up less K^+ than sunflower protoplasts, expressed per no. of protoplasts; volume measurements were not made, but by analogy it seems likely that the largest maize cortical cells were not proportionally represented in the protoplasts.

Bacterial contamination was usually at a low level similar to that reported in Gronwald and Leonard (1982) so should have accounted for less than 5% of the total protein content of the sample and hence a small

proportion of the uptake. Even in the exceptional case reported on p. 83 the bacterial contribution was less than 10% of the total.

Varietal differences in boron sensitivity are reported here for sunflower and maize roots; Robertson (1974) and Parr (1980) reported similar effects. However the differences observed here are not as dramatic as in some species where a genetic basis for such differences has been established; e.g. in tomato, the 'btl' genotype proved to be related to the inefficiency of transport of B from root to shoot resulting in boron deficiency symptoms such as a brittle stem even with adequate boron supplies to the root (Wall and Andrus, 1962; Wann and Hills, 1973).

Parr (1980) observed that P_i uptake by sunflower (cv Polestar) roots was reduced by 30% after 40 h -B. The results quoted here show that uptake does not simply decline steadily during boron deprivation. It is important to remember that growth effects are often visible within 3 h of boron deprivation, so it is essential to a theory which puts membrane-mediated processes in the forefront of the plant's response to boron deficiency that effects on uptake should be seen within a similar time scale. It should also be remembered that the effects of Ca^{2+} withdrawal on ion uptake can be seen within 30 min to 1 h (Lauchli and Epstein, 1970) so there is precedent for the brief time scale reported above.

The early rise in uptake seen in 2 cm tips and their component 1 cm sections is consistent with some adjustment to ion flux occurring at the same time as 'soluble' borate is removed from the tissue; parallel studies on the boron status of the roots (reported in section 1) suggest that the boron level drops within 2 h to around 80% of the control level and then changes relatively little for the next 22 h. +B plants were transferred to fresh +B solutions 4 h before measuring uptake, or simultaneously with the -B plants, wherever possible; so +B and -B differences should only be

ascribable to changes in the boron status and not to washing effects.

The apparent restoration of normal uptake after the initial disturbance may imply some ability to adapt to the loss of 'soluble' borate; an ability which is obviously limited as the overall decline ensues within about 24 h. If boron is involved in maintaining membrane structure and function, an early increase in uptake may relate to a temporary loss of control. Efflux measurements made during those hours are required to confirm increased membrane permeability. Ion efflux from pre-loaded roots was not studied in this thesis; both Robertson (1974) and Parr (1980) observed no effect of 2-4 d -B on efflux from Vicia faba and Zea mays roots respectively, but the effect is probably transitory.

Although the fluctuations are wider with 1 cm tips, the more mature cells in 1-2 cm tips are apparently affected too. This is a particularly early response of more mature tissue to -B conditions.

The inability of 'Single Tall' sunflowers to increase uptake after 1-4 h resupply of B may be because of the low B levels used throughout. However, uptake by 'Polestar' sunflowers was restored by 2 h resupply. Similar non-reversibility was reported by Robertson (1974) for K^+ uptake by Vicia faba. A tendency towards a reduction in uptake caused by the resupply has not been reported elsewhere. 'Single Tall' sunflowers generally responded in a very variable way to boron deprivation; resupply often reduced this variability, implying some restoration of control.

Sunflower root protoplasts isolated from +B or 13-17 h -B plants were expected to behave in a similar way to the roots from which they came. At this time roots show little difference in the uptake per g f.wt. Similarly uptake per unit length of root has either increased or not changed in line with the changes in tip weight. Therefore the results which show that uptake per -B protoplast may be higher, lower or equivalent to the controls, for both the 0-1 and 1-2 cm zones of origin, are not surprising.

Protoplasts from roots of different boron status could differ by virtue of preferential isolation of different cell types. Therefore more reliable comparisons are possible when a single preparation is divided and its boron status controlled for a few hours. It can be seen from table 14, (p. 68) and fig. 12, (p. 70) that in the experiment where the addition of BSA gave the highest rate of uptake the addition of B at 9.2 μM for 4-5 h increased uptake by the 0-1 cm root protoplasts by 20% and by the 1-2 cm protoplasts by 40% . This effect is in line with the results for 'Polestar' roots reported in this thesis and by Parr (1980); but not with the usual results for Single Tall sunflowers. The higher rate of boron supply and absence of a cell wall to bind the added boron presumably combined to permit the increased rate of absorption in the best-preserved system.

In experiments where the protoplasts had not been protected by BSA no increase or a decrease in uptake were observed and the same was true in the case where uptake was expressed per unit surface area (i.e. in units roughly comparable to root uptake per g f.wt.). In these cases the uptake mechanism has not been preserved intact and the absence of a response to added boron does not conflict with the suggestion of a requirement for boron for maximum uptake capability.

The short-term deprivation of boron led to an apparent increase in uptake by protoplasts from the 0-1 cm zone, just as was seen in the intact roots from which they came after the same time. Similarly the smaller response by protoplasts from the 1-2 cm zone reflects the smaller effects in the 1-2 cm zone itself; and is consistent with a more direct effect on the cells or protoplasts from the apical zone.

The effects of boron withdrawal on P_i uptake by maize roots differ in several important ways from those described for sunflower roots. The impaired ion uptake previously reported by Parr (1980) after 15 h -B,

working with very young seedlings which had not previously been supplied with boron (table 9, p. 41), has now been demonstrated for plants initially grown +B and transferred to -B media. In sunflowers the 'longer-term' impairment of uptake was not seen until around 24 h -B, despite the fact that growth was impaired within 3 h -B; in maize 6 h -B was sufficient to reduce ion uptake, well before any detectable alteration to the growth rate or root tip morphology. In maize roots as in sunflowers there was an early transient rise in uptake; however in sunflowers this effect was more marked in the 0-1 cm zone of the tip, whereas in maize it was more marked in the 1-2 cm region. There was no initial depression in uptake similar to that observed for the 0-1 cm zone of sunflower roots. The short time scale again implies that some alteration to the uptake process occurs as a result of the initial loss of 'soluble' borate from the cells and the altered equilibria which follow.

The observation by Pollard et al. (1977) of restored uptake after 1 h resupply of B was not re-examined.

Protoplasts isolated from maize roots after 24 h -B, at a time when uptake by the roots should have been impaired, took up similar amounts of P_i to control protoplasts, expressed per no. of protoplasts or per unit surface area. Resupplying B to a batch of protoplasts from the 0-1 cm or 1-2 cm zone of -B roots increased the uptake rate per protoplast; the protoplast volume increased as well so uptake per unit surface area remained constant. The small differences reported were not statistically significant, suggesting that the uptake of P_i by maize root protoplasts is insensitive to the presence or absence of boron at the concentrations used. This is quite different from the situation in the intact roots. This may imply that intact root tips or cells bounded by cell walls are required for the expression of boron deprivation effects in maize roots, or simply that the uptake process has been impaired in some way during

protoplast isolation.

K^+ uptake by sunflower roots seems to be more sensitive to B deprivation than P_i uptake. There is a similar early transient peak in absorption but the longer term decline, as reported previously by Timashov (1969), was observed after 15 h -B in these experiments, preceding the impairment of P_i uptake. Timashov also reported that K^+ transport to the shoot was more impaired by 12 h -B than influx to the root; this observation was not re-examined in this thesis.

The tip region, particularly the terminal 5 mm, appears to be more sensitive to boron withdrawal than the 1-2 cm zone, with impaired uptake observed after 5 h -B. This effect is similar to the P_i uptake behaviour of sunflower roots; and similarly variable behaviour along the root was observed by Robertson (1974) with Vicia faba roots.

It is unfortunate that the experiment in which the terminal 5 mm region appeared to adapt to -B conditions and resume normal uptake was not repeated. Further work is required to see whether this was a chance occurrence.

White (1983) observed that K^+ uptake by -B sunflower roots could be restored by 2-3 h resupply of B, which suggests a direct involvement of B in the process, as noted by Parr (1980). Similarly, at 10 μM external K^+ uptake is more dependent on active transport than at 1 mM; and is more strongly impaired by boron deficiency. This again implies an interaction between B deficiency and the active absorption process. This was pursued in the section on ATPase activity. White (1983) also investigated the early peak in uptake which seems to be a feature of the effects for each plant and each ion.

He concluded that the extra uptake was due to passive influx across the plasma membrane down an electrochemical gradient. Efflux experiments would be required to test whether the plasma membrane is rendered more permeable

over the first 0-4 h -B.

When the results are expressed per cm root, the decreased uptake after 12-18 h -B is not always detectable and this has obvious implications for protoplast K^+ uptake. When protoplasts were isolated from +B or -B roots the uptake rates varied as much between 'replicate' isolates as between B treatments; the necessary volume estimates for the protoplasts to permit comparison with root uptake rates expressed per g f.wt. were not done. It is possible to infer, cautiously, that uptake per unit surface area may be impaired as in the roots: uptake was similar in both +B and -B preparations from the heavier and lighter roots and yet the f.wt. and hence the cell size of the -B roots was greater than the controls.

When -B sunflower protoplast preparations were divided into two parts and boron resupplied to one for 2.5-4.5 h, there was no significant difference between the uptake rates per no. of protoplasts. The increased volume observed in the few experiments where it was measured suggests that this represents impaired uptake per unit surface area. However, the boron level used was higher than usual and applied directly to the protoplast surface so too high a boron level may have counteracted otherwise restored activity. When boron was removed from a batch of protoplasts for 3-5 h, the rise in uptake may imply the removal of that inhibition. However, root tips also show enhanced absorption a few hours after boron removal so it is impossible to separate these effects with the available data. Raven (1980) quoted tissue B levels at toxicity as being around 2-25 mM; allowing for up to 80% bound B, the lowest toxic level would still be about 10 x higher than that supplied here (46 μM).

Pollard et al. (1977) reported a developmental increase in K^+ uptake by maize roots which was greater in +B plants than -B. The same observation was made for the 0-1 cm zone (fig. 23, p. 88) here, although not for 1-2 cm segments.

It is possible that the addition of H_3BO_3 to roots could increase the fixed negative charges in the cell wall by complex formation. However, the results of desorbing K^+ from the cell wall confirm that there was no effect of the removal of 'soluble' borate on the cation binding capacity. Maize protoplasts isolated from 15 h -B roots absorbed less K^+ than control protoplasts, as expected on the basis of most root tip results. As noted in the results, though, such an observation is only useful in conjunction with details on protoplast size in the two preparations. Since maize root morphology changes so little over the first few days without boron the protoplasts are likely to be identical in size. This would support the suggestion that these -B effects can be seen in both excised roots and isolated protoplasts.

In summary, K^+ and P_i absorption by sunflower and maize root tips are rapidly affected by the transfer of the roots to -B media. An initial increase in uptake is usually seen, which is probably caused by an increase in the plasma membrane permeability. A longer term reduction in ion uptake was seen for both ions and both plant species within 24 h -B, with the exception of P_i uptake by 'Single Tall' sunflowers, which showed considerable variability of response. In general the terminal cm of the root is more sensitive than the 1-2 cm region, with the possible exception of P_i uptake by maize roots.

Ion absorption by protoplasts has been shown to resemble the root tip in the case of P_i uptake by sunflowers and K^+ uptake by maize. The uptake of P_i by maize protoplasts seems to be less sensitive to boron status than in the intact roots. The uptake of ions by protoplasts is affected by a number of factors identified during the course of this work and only when all are controlled or optimised as appropriate are +B or -B effects seen. This suggests that B may be involved in the maintenance of that

optimal uptake rate. Now that the methodological basis for comparing protoplast and root responses to boron deprivation has been developed future research in this field may be more fruitful. However protoplast preparations have not yielded any earlier responses to changes in their boron status than root tips because of the high variability inherent in the system; this is a drawback to realising the full potential of protoplast systems in the study of the physiology of early reponses to boron deficiency.

The ATPase characterised by White (1983) in Single Tall sunflower roots shares many common characteristics of such K^+-stimulated, Mg^{2+}-dependent ATPases, e.g. Leonard and Hotchkiss (1976). There is a good correlation between the effects of boron deprivation on the activity of the ATPase and on K^+ uptake from 10 μM solutions. Most interesting of all is fig. 26, (p. 95) which suggests that the resupply of H_3BO_3 to the roots increases the B content of the membranes in conjunction with the restoration of ATPase activity. Parr (1980) observed that resupply to the roots or to the membrane fraction could restore ATPase activity in maize; White (1983) found that only when B was resupplied to the intact plant roots did restoration occur. Parr suggested that B was not simply favouring the binding of ATP to the ATPase and thereby promoting cleavage, because supplying additional B to the +B root membranes actually reduced ATPase activity. There was a longer lag in the sunflowers before restoration; 2-3 h were required before restoration of activity, whereas Parr found restoration took only 1 h. The implication is that in sunflowers the resupply of B triggers some activation process or controls new membrane component incorporation rather than directly affecting the membrane environment of the enzymes. However, resupplying H_3BO_3 to the membranes can only activate existing carriers; so if there had already been a loss of the K^+-stimulated, Mg^{2+}-dependent ATPase after 18 h -B in the sunflowers, such a result would be expected.

Further studies over a shorter period of deficiency are required in sunflower.

The observation that the highest specific activity of the ATPase occurred in the membrane pellet from 9-13 K 'g' was unexpected. Plasma membrane is found in all parts of the gradient; Verkhovskaya and Kurkova (1980) actually obtained their membrane fraction for assaying ATPase activity in barley root homogenates by treating the cell wall fraction precipitated at 1000 'g' with detergent. Nevertheless, the specific activity is usually highest in a less dense membrane fraction (e.g. 30-60 K 'g', Lundborg, Widell and Larsson, 1981). The 9-13 K 'g' fraction would contain a range of membrane types, mitochondria and Golgi vesicles. Further studies would be required to characterise the particular membranes carrying the ATPase activity.

Hinde and Finch (1966) reported an increase in the level of ATPase activity in general with increasing age of the tissue and with -B culture. They suggested that at 24 h -B the root cells may be showing the pattern of differing maturity rather than anything directly linked to B status. There was a similar increase in Mg^{2+}-dependent ATPase activity from the 0-1 to 1-2 cm root zone in this thesis.

Tanada (1983) demonstrated a strong affinity of membranes for B and Torchia and Hirsch (1982) plan to investigate the localisation of B in subcellular compartments. It is to be expected that research in this field will be particularly fruitful if one major difficulty can be overcome. This is the uncertainty over when B may bind to subcellular fractions during isolation. In our results, membranes from roots which have been deprived of B for 18 h are essentially boron free; resupply of B to the roots yields fractions containing B. To distinguish between <u>in vivo</u>- and <u>in vitro</u>- bound B, experiments are planned using ^{10}B and ^{11}B enriched

solutions and a range of washing treatments. Membrane droplet size will be reduced to under 1 µl if possible, to permit accurate estimates of the B content to be made.

Section 3. Cell wall deposition

Introduction

A role for boron related to cell wall structure or synthesis has been suggested for many years. Borate is known to bind to cell walls (Smith, 1944; Bowen and Nissen, 1976). Tanaka (1967) demonstrated that dicot cell walls bind more borate than those of monocots. The effects of adding other complexing agents led Torssell (1956) to conclude that boron was required for maintaining cell wall plasticity. He suggested that without boron the cell wall 'crystallized' as polysaccharide chains crosslinked; an effect which could be relieved by other lipophilic complexing agents such as phenylboric acid. He suggested that these substances (including borate) permeated the membrane and controlled the deposition of fibres in such a way as to permit cell extension growth. Ginzburg (1961) suggested that boron could be involved in a different way. Borate complexes with pectins and appears to stabilise Ca^{2+} complexes in the middle lamella which aid cell to cell adhesion.

There are many reports of abnormal cell wall morphology in boron deficient plants, as observed with the light microscope (e.g. Spurr, 1957a,b; Wilson, 1961; Kouchi and Kumazawa, 1975b) or electron microscope (e.g. Lee and Aronoff, 1966; Alekseeva, 1971; Kouchi and Kumazawa, 1976; Parr, 1980; Hirsch and Torrey, 1980). Typical early effects include irregular cell wall thickening, the occurrence of plasmalemmasomes and increased activity of the Golgi apparatus. These observations have been extended in this thesis by a TEM study of sunflower root tips over the first few hours of deficiency.

Boron deficiency has also been reported to induce a wide range of effects on cell wall composition. Labelling studies or chemical analysis of the

cell walls have been interpreted as implying impaired synthesis of pectins (Skok, 1958; Rajaratnam and Lowry, 1974; Yamanouchi, 1973); and/or hemicellulose (Rajaratnam and Lowry, 1974) and/or cellulose (Dugger and Palmer, 1981) and/or non-cellulosic glucans (Spurr, 1957a,b; van de Venter and Currier, 1977; Dugger and Palmer, 1981). In secondary cell walls lignification is impaired (McIlrath and Skok, 1964; Dutta and McIlrath, 1964).

Part of this apparent variety of response is due to the comparison of non-physiologically matched tissues; differences ascribed to boron deficiency may simply represent differences in cellular maturity (Slack and Whittington, 1964). A part is also due to the range of tissue types and plant species considered.

Nevertheless, evidence for a role of boron related to cell wall deposition arises from Milborrow's work (1964) where he reported that the herbicide 2,6-dichlorobenzonitrile (2,6-DCB) induced effects on root growth markedly similar to those of boron deficiency. 2,6-DCB was later shown to specifically inhibit cell wall synthesis (Hogetsu, Shibaoka and Shimokoriyama, 1974), an observation now supported by evidence from protoplast studies (e.g. Burgess and Linstead, 1982). Indeed, its effects would seem to be restricted to wall synthesis, because it does not affect the capacity for mitosis in protoplasts, unlike coumarin (Meyer and Herth, 1978,1982). 2,6-DCB prevents cytokinesis in protoplasts and root tips because of the inhibition of cell wall synthesis (Meyer and Herth, 1982).

Protoplasts can regenerate a cell wall during culture. Numerous studies on this process have been done, and good recent reviews include Meyer and Herth (1982) and Willison and Klein (1982). The capacity to regenerate a cell wall apparently varies from plant to plant and is affected by a range of cultural conditions (e.g. Rose, 1980). It has been shown that cell wall synthesis is a prerequisite for cell division, although not for nuclear

division (Meyer and Herth, 1978; Zelcer and Galun, 1980). This may be the reason why cereals are particularly difficult to culture. Sustained divisions by protoplasts isolated from maize plants have been reported by Potrykus et al. (1977). However their results proved to be unrepeatable, although protoplasts from maize callus culture have been reported to regenerate walls and divide (Potrykus, Harms and Lörz, 1979; Chourey and Zurawski, 1981). During protoplast isolation the negative internal potential may be lost; protoplasts have been reported with a positive internal potential (Kinnersley, Racusen and Galston,1978). Bacic and Delmer (1981) found that a transmembrane potential was necessary for wall synthesis in cotton fibres; but Kinnersley et al's results suggested that the situation is not quite the same in protoplasts. They observed that in tobacco protoplasts, cell division coincided with the restoration of a negative internal potential after cell wall synthesis had occurred. They suggested that part of the role of the regenerating cell wall may be to permit the protoplast to regain this negative internal potential. Oat and maize protoplasts did not regain a negative potential, nor did they divide; and other tests implied that wall regeneration was defective.

Wall synthesis can be visualised with fluorescent brighteners which bind to cell wall material, such as Calcofluor White (Nagata and Takebe, 1970) or Tinopal CBS-X (Peterson, Emmanuel and Humphreys, 1981). Galbraith (1981) describes a quantitative microfluorimetric method for assessing the amount of Calcofluor bound and hence the progress of wall synthesis in protoplasts. The stains are not specific for cellulose (e.g. Darken, 1961) but that does not affect their usefulness here. A more serious problem is that the method is not very sensitive for determining when wall synthesis first starts. Replica studies in the TEM showed that fibre deposition around Vicia hajastana protoplasts had started within 10 min of removing protoplasts from wall-digesting medium and a loose net of fibres had

developed by 1 h; but fewer than 1% of protoplasts stained with Calcofluor after 1.5 h culture (Williamson et al., 1977).

SEM studies of fibre deposition around protoplasts have attracted considerable interest as it is not possible to study de novo synthesis around root cells in this way. A range of EM techniques have been tried to aid interpretation of the process (reviewed in Willison and Klein, 1982). In this report an SEM survey of wall synthesis is reported using conventional methods of fixation, critical point drying and coating.

More detailed comparisons are possible when the synthesis of wall polymers is studied biochemically. Cell wall synthesis is envisaged as involving cellulose synthesis at the plasma membrane. Other wall polymers appear to be synthesised in the Golgi apparatus and transported to the cell wall (Ray, 1980). Uridine diphosphate-glucose (UDPG) is commonly used as a precursor for β-glucan synthesis in studies using isolated membranes and is believed to be the substrate for callose and cellulose synthesis (Delmer, 1977). There are two known β-glucan synthetases, 'β-glucan synthetase I' which operates maximally with micromolar amounts of UDPG and 'β-glucan synthetase II', active at high (millimolar) UDPG concentrations and thought to synthesise callose (a β-1,3 linked glucan). The latter is apparently plasma membrane bound whereas the 'β-glucan synthetase I' activity is found in the Golgi vesicles, where it may synthesise xyloglucans for export to the cell wall (Ray, 1980). Alternatively it may represent cellulose synthetase which is transported to the plasma membrane and activated by the potential difference across the membrane (Bacic and Delmer, 1981). The isolated membranes used for assaying β-glucan synthetase activities have never been shown to yield pure cellulose despite the fact that it is the most abundant naturally occurring polysaccharide. It appears that the isolation process activates the β-1,3 linked glucan synthetase (see Brown, 1982 for a comprehensive review of

wall polymer synthesis).

Early work in this laboratory suggested no effect of boron status on UDPG incorporation by membranes from Vicia faba roots (Robertson, 1974). Later work by Dugger and Palmer (1980, 1981) reported that -B cotton fibres incorporated UDPG into β-1,3 linked polymers rather than the mixed β-1,3 and β-1,4 linked polymers synthesised by control fibres. Van de Venter and Currier (1977) found that callose accumulated in the sieve tubes of -B bean and cotton plants. However, excess B was also observed to promote callose synthesis; and Tighe and Heath (1982) observed the same promotion in cowpeas (Vigna sinensis). The early work of Robertson (1974) has been repeated in greater depth with membranes from sunflower and maize root tips in this thesis.

There is a problem in using a similar system to study wall synthesis by protoplasts. It has been frequently asserted that UDPG does not cross the plasma membrane, and can be incorporated only from the inside. Exogenous UDPG was incorporated only by the cut cells in slices of tissue in experiments by Raymond et al. (1978), Mueller and Maclachlan (1980,1983), Brett (1981) and Tighe and Heath (1982); Quantin et al. (1980) reported that UDPG could not penetrate within membrane vesicles without the addition of a detergent (Triton); and Bacic and Delmer (1981) did not observe any incorporation of exogenous UDPG by cotton fibres. However Dugger and Palmer (1980, 1981) did observe incorporation by cotton fibres; Bacic and Delmer (1981) observed incorporation by membrane vesicles; and most significantly, Klein, Montezinos and Delmer (1981) recorded incorporation by soybean protoplasts. They found that UDPG was incorporated into a β-1,3 linked glucan but was much less effective as a substrate for cellulose synthesis than exogenously-supplied glucose.

Tissue slice experiments can be criticised because of the possibility that UDPG is incorporated rapidly at the cut ends and may be prevented by

interactions with the cell walls from penetrating further into the slices. It has been suggested that in the experiments where UDPG did enter membrane vesicles they were inside out or inside-positive (Bacic and Delmer, 1981); isolated protoplasts have been recorded as inside-positive by, e.g. Kinnersley et al. (1978) so this may assist the passage of UDPG across the membrane. Brett (1978) observed that cell clumps in tissue culture could incorporate exogenous UDPG when they were vigorously stirred, and he suggested that UDPG was incorporated during the sealing of ruptured plasmodesmata.

The reason why UDPG was used rather than ^{14}C-glucose is that the water-insoluble products of UDPG incorporation are β-glucan wall polymers whereas glucose is incorporated into a vast array of products which may be retained on the origin by the washing procedure used here. More sophisticated analysis of the products would then be required, which inevitably requires the synthesis of greater amounts of product.

Many wall polymers synthesised by protoplasts regenerating a wall are lost to the external medium. The fibres which are deposited on the surface are not necessarily pure cellulose; analyses have been done by Takeuchi and Komamine (1978,1981), Blaschek et al (1981) with tobacco protoplasts, Klein et al (1981) with soybean protoplasts and Langebartels, Seitz and Seitz (1981) with carrot protoplasts. Mixed β-1,3-and β-1,4-linked polymers were found in all preparations in the medium and/or on the protoplast surface, whether or not these were constituents of the cell wall before isolation. Callose is commonly regarded as a wound polymer and is frequently implicated by the authors as a constituent of the protoplast wall.

UDPG is therefore incorporated by protoplasts into polymers which are constituents of the protoplast walls or are released to the external medium. The water-insoluble material assayed in these experiments will

include protoplast-bound wall polymers and any external products dense enough to co-precipitate with the protoplasts in the reaction vials and to remain on the origin during washing.

Various suggestions have been put forward to predict the effects of altering the boron supply on the endogenous UDPG content. Fig. 27 (p. 121) has been compiled from Dugger (1973) and Augsten and Eichhorn (1976) to show the involvement of boron at near-physiological concentrations in the major pathways involving UDPG. The results on which it is based have been summarised in table 21 (p. 122).

Teare (1974) reported that the UDPG content of -B bean roots was reduced and the UTP content increased in proportion as would be expected if UDPG synthesis were inhibited. Robertson (1974) observed that the incorporation of ^{32}P into nucleotides was lower in -B field beans than in controls. Parr and Loughman (1983) published an NMR trace which showed a reduced [UDPG] in 4 d deficient maize roots.

However the relationship of the [UDPG] to cell wall synthesis is not simple. As van de Venter and Currier (1977) and Tighe and Heath (1982) observed, excess B promotes callose synthesis, possibly by elevating the endogenous UDPG content to the point where callose synthesis is stimulated. Thus lowering the UDPG pool size to a certain extent may actually favour cellulose synthesis. The whole question of the relation of UDPG pool size to wall synthesis is uncertain unless the compartmentation and rate of cycling are known. For example, Loughman (pers.comm.) has suggested that the rapid deposition of callose in wounded sieve tubes may be achieved without a high [UDPG] if the high sucrose content can permit sucrose synthetase to operate in reverse.

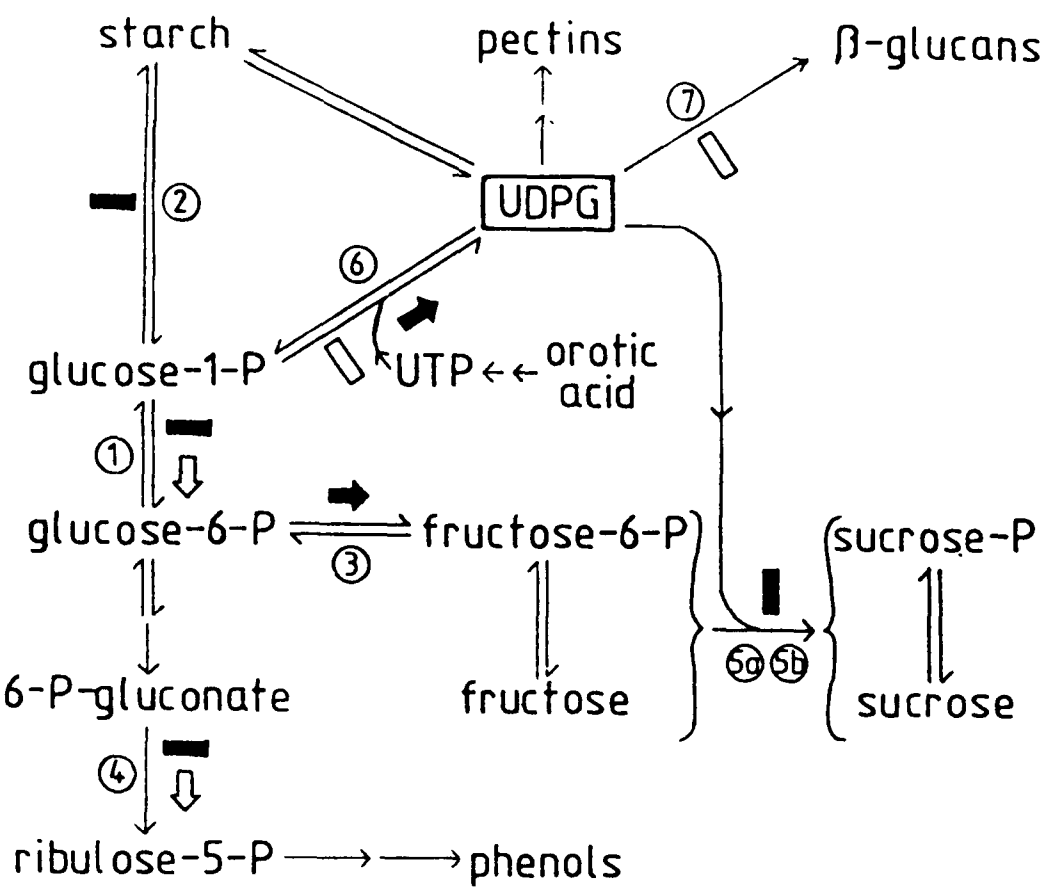


Fig. 27. Known effects of B on pathways involving UDPG. Further details and references given in table 21.

key to affected enzymes:

1. phosphoglucomutase
2. starch phosphorylase
3. glucose-phosphate isomerase
4. 6-P-gluconate dehydrogenase

5a. UDPG-fructose-6-P glucosyl transferase

5b. UDPG-fructose glucosyl transferase

6. UDPG-pyrophosphorylase
7. β-glucan synthetases

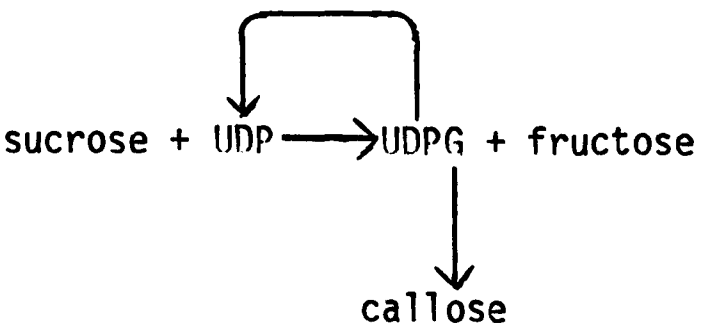
Table 21. Effects of boron supply on enzymes involved in carbohydrate metabolism

Enz. no.*	Plant material	Effective $[H_3BO_3]$	Effects	Ref.
1	pea seeds	0.2 mM	50% dec. in activity; less G-6-P and F-6-P for sucrose synth; more G-1-P for UDPG synth. UDPG channelled to wall synth.	1
2	bean leaves	1-100 mM	+B: Less starch synthesis; more sugar avail. for transport; -B: more starch	2
	sunflower leaves	40 mM		3
3	peas	2-15 mM	no effect	1,4
3	(data not given)		inc. activity	5
4	sunflowers	0.6 mM	33% dec. in activity; less phenol synth.	6
4	bean, corn	(-B)	inc. enzyme concentration	7
4	wheat	(-B)	smaller change (more tolerant sp.)	7
5	pea seeds	10-20 mM	dec. sucrose synthesis in vitro (not in vivo; see below).	4
6	pea seeds	10-20 mM	inc. activity; inc. sucrose synth.	4
6	cotton ovules	(-B)	impaired; less UDPG synthesis, less cellulose synthesis	8
6	cotton ovules	(-B)	less UDPG synthesis from orotic acid; via less cellulose synth. which increases [UDPG] (i.e. end product inhibition).	9
7	cotton ovules	(-B)	less incorp. of UDPG to β-glucans; higher proportion of β-1,3 links : β-1,4 links	10

* Enzyme numbers refer to fig. 27, (p. 121).

References:

1. Loughman, 1961
2. Dugger et al., 1957
3. Scott, 1960
4. Dugger and Humphreys, 1960
5. Chen and Aronoff, 1973
6. Lee and Aronoff, 1967
7. Shkol'nik and Il'inskaya, 1975
8. Birnbaum et al., 1977
9. Wainwright et al., 1980
10. Dugger and Palmer, 1980



A high rate of cycling would ensure callose deposition from a small pool of UDPG.

The incorporation of UDPG into water-insoluble product by isolated membranes should be solely a function of the enzyme activity. However the incorporation by protoplasts will be affected by the rate of entry into the protoplast and the endogenous [UDPG]. It is therefore difficult to predict the likely effects of ±B culture on wall resynthesis, but this method should permit a more detailed comparison of the preparations than the other methods used.

Materials and methods

1. Membrane isolation for UDPG incorporation

This method differed from that for ATPase assay in several ways. The grinding medium was: 0.1 M Tris-HCl, 1.5 mM DTT and 1% w/v PVP, pH 7.5. Centrifugation was at 1000 'g' for 5 min, 0°C in a Griffin Christ centrifuge, followed by two 15,000 'g' spins for 30 min each. The membrane pellet was suspended in 0.1 M Tris-HCl buffer at pH 7.5 at a rate of 20-40 $\mu l.g^{-1}$ f.wt. roots (Kemp, 1972). As noted in the results, in some experiments the homogenate was centrifuged at 6,000 'g' and 80,000 'g' for 20 min and 50 min respectively in a Beckman L2 65 ultracentrifuge at 4°C.

2. UDPG incorporation by root membranes and protoplasts

Ten µl of membrane suspension or 20 µl of protoplast suspension (containing 2×10^4 protoplasts) were added to incubation medium to give a final concentration of 0.1 M Tris (pH 7), 2 mM $MgCl_2$ and 10 µM or 1 mM UDPG (see text) containing 1.85 kBq ^{14}C-UDPG. The incubation medium for protoplasts was made up in PSM. Boron was added where required at concentrations specified in the text. At the end of the incubation time, tubes were plunged into a boiling water bath for 5 min and then deep frozen.

Cellulose-coated thin layer chromatography plates were prepared with a 2.5 cm 'origin' band marked off in 1.5 cm - wide strips. The thawed contents of each tube were pipetted onto these 1.5 x 2.5 cm zones and the plates were run in distilled water to wash the soluble material off the origin. Plates were air dried and re-washed; once dry, the 'origin' zones were scraped off with a razor blade into 4 ml scintillant (7.0 g 2(4'-t-butylphenyl)-5-(4"-biphenyl)-1,3,4-oxadiazole (butyl-PBD) and 25 g Cab-o-sil in 1 ℓ toluene). Counts were recorded in a Beckman Liquid Scint-

illation Counter for 10 min. Blanks were run for each experiment and standards were pipetted onto plate material and scraped into scintillant directly (Kemp, 1972).

3. Cell wall stains

Protoplasts were incubated with 0.2% Calcofluor White or 0.01% Tinopal CBS-X in 0.5 M mannitol for 10 min. The suspension was centrifuged at 3,000 'g' for 5 min and rinsed with 0.5 M mannitol 2-3 times. The stained suspension was viewed under phase contrast in a Reichert Zetopan-Binolux microscope. Illumination from an HBO 200W/4 mercury vapour lamp was filtered to λ = 325-480 nm incident light and fluorescence was observed at $\lambda > 500$ nm (Peterson et al, 1981). The percentage of stained protoplasts was recorded, with a visual estimate of the intensity.

4. Scanning electron microscopy of wall synthesis by protoplasts

Maize root protoplasts which had been incubated in PSM were fixed in 5% glutaraldehyde, 0.5 M mannitol, 5 mM $CaCl_2$ and 0.1 M sodium cacodylate, pH 6.5. Fixation was for 1 h at room temperature. Sunflower root protoplasts were fixed in the same medium with the addition of 0.4% OsO_4. Protoplasts were centrifuged out of the fixation medium at 300 'g' for 5 min and washed for 10 min in each of three changes of distilled water. Protoplasts were dehydrated through an alcohol series: 10-15 min in each of 30% and 50% ethanol, up to 8 h in 70% ethanol and then 20-30 min in 90%, 95% and 100% ethanol. This was changed for fresh absolute ethanol and the protoplasts were transferred to a plastic tubing trap (see fig. 28, p. 126) or a sintered Teflon pot (Reichert-Jung Ltd, 820 Yeovil Rd, Slough) for critical point drying (CPD).

A Samdri-780 critical point drier was used; three purges of 2 min each were adequate to remove all the ethanol, with 5 min soaking in liquid

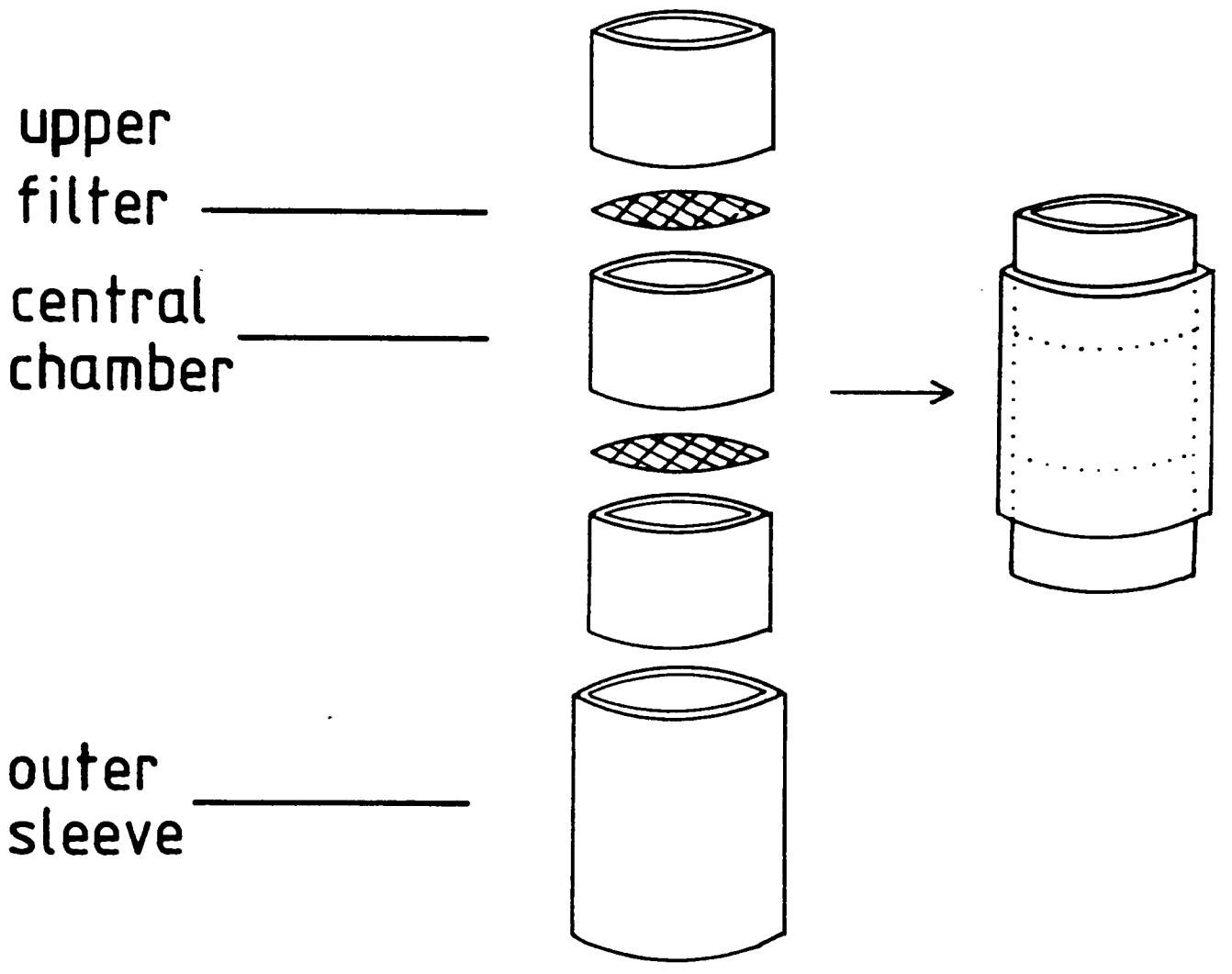


Fig. 28. Components of, and assembled, 'trap' for critical point drying of protoplasts. Inner diameter of tubing segments 9 mm; of outer sleeve, 12 mm. Filters made from Whatman no. 1 filter paper. Partially disassembled trap is immersed in absolute alcohol; protoplasts pipetted into central chamber and upper filter secured with uppermost tubing segment. (Modified from Robards (1978).)

CO_2 between each. The total cycle time was approximately 45 min. Dried protoplasts were immediately transferred in their containers to a desiccator before coating.

Double-sided sellotape was stuck to the SEM stub and the upper adhesive surface revealed immediately prior to the addition of the protoplasts. These were tipped out of their container onto the tape; two stubs were prepared from each batch and they were gently brushed together to ensure firm adhesion of the protoplasts; loose protoplasts were blown off with compressed air. The stubs were coated with gold at 1.2 kV, 40 mA for 2 min and stored in desiccators until observation (method modified from Robards, 1978).

The scanning electron microscope used was a Cambridge Stereoscan 150, operated at 5-10 kV.

Results

1. TEM of sunflower root tips.

The electron micrographs prepared for section 1 revealed a number of characteristic effects of early deficiency related to cell wall structure and synthesis. The outer epidermal cell wall often shows a less densely stained region adjacent to the plasma membrane after 5.5 h -B (+/-B) and 3 d -B (plate 7, p. 130) which does not seem to be just an artefact of plasmolysis; +B sections showed uniformly-stained fibrillar material external to the plasma membrane.

Abnormal vesicular inclusions in the middle lamella or intercellular spaces were observed in some sections (plate 8, p. 130) from +/-B and -B sections. Most radial cell walls in +B sections resembled that in plate 9 (p. 131); however irregular thickening was occasionally seen. Plate 10 (p. 131) shows a radial cell wall from a -B root typical of the irregularities found more commonly in the +/-B and -B sections; particularly in epidermal and cortical cells. The mean cell wall thicknesses calculated for each type of cell wall (e.g 'radial, hypodermal' or 'tangential, C2-C3') did not appear to be significantly altered by 3 d -B; but a more extensive survey would permit more detailed comparisons.

The tangential cell walls between the epidermis and hypodermis, and between the hypodermis and C2, contained artefactual membranous structures caused by shrinkage of the tissue during the fixation and embedding process (plates 11, 12, p. 132). Similar plasmalemmasomes were observed more frequently and without concomitant shrinkage in sections of -B roots; after 5.5 h -B their number had increased beside the cortical cell walls and after 3 d -B they were observed beside most radial and tangential walls of the epidermis, hypodermis and cortex.

The activity of the Golgi apparatus appeared to be promoted by deficiency;

Plates 7-12. Transmission electron micrographs of Polestar sunflower roots; transverse sections cut 1 mm behind the apex. Plants grown for 3 d +B, 3 d -B or 66 h +B followed by 5.5 h -B ('+/-B'). H_3BO_3 supplied at 4.6 μM.

Symbols used:

CW = cell wall	N = nucleus
D = dictyosome	PL = plasmalemmasome
ER = endoplasmic reticulum	S = spherosome
ML = middle lamella	V = vacuole

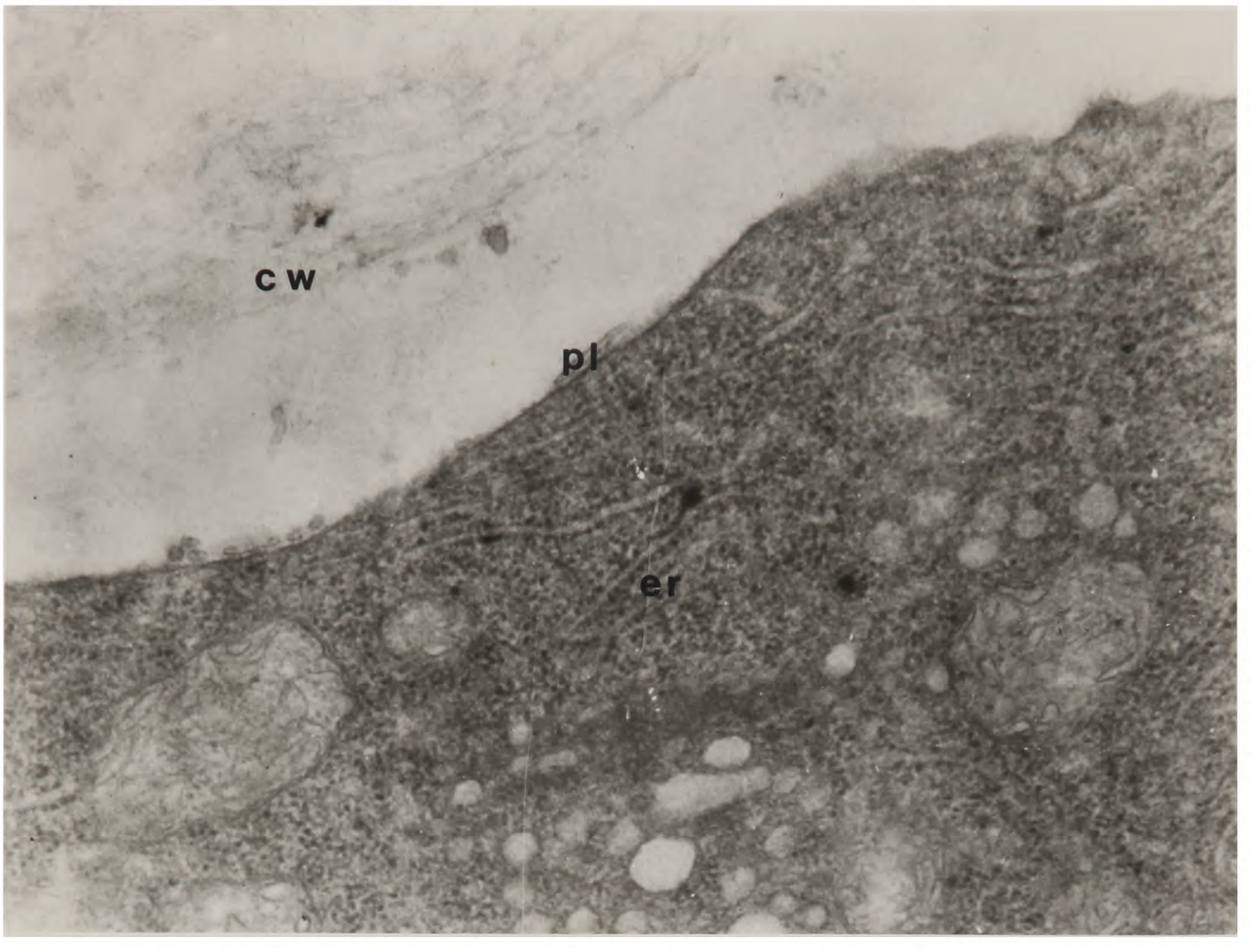


Plate 7. Outer epidermal cell wall, +/-B. Scale bar = 500 nm.

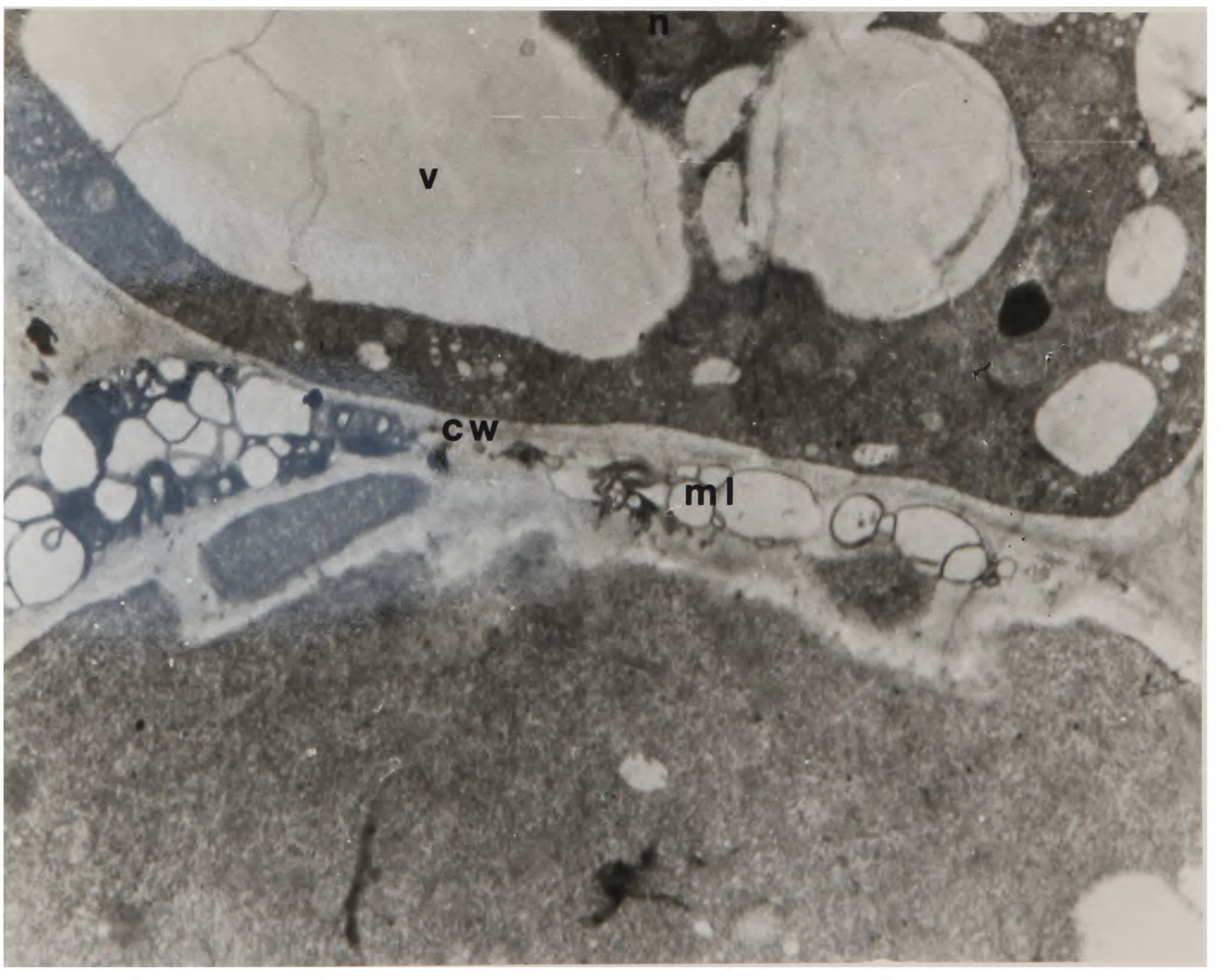


Plate 8. Abnormal vesicles in middle lamella of cortical cell walls, +/-B.

Scale bar = 1 µm.



Plate 9. Cell wall between two hypodermal cells, +B. Scale bar = 200 nm.

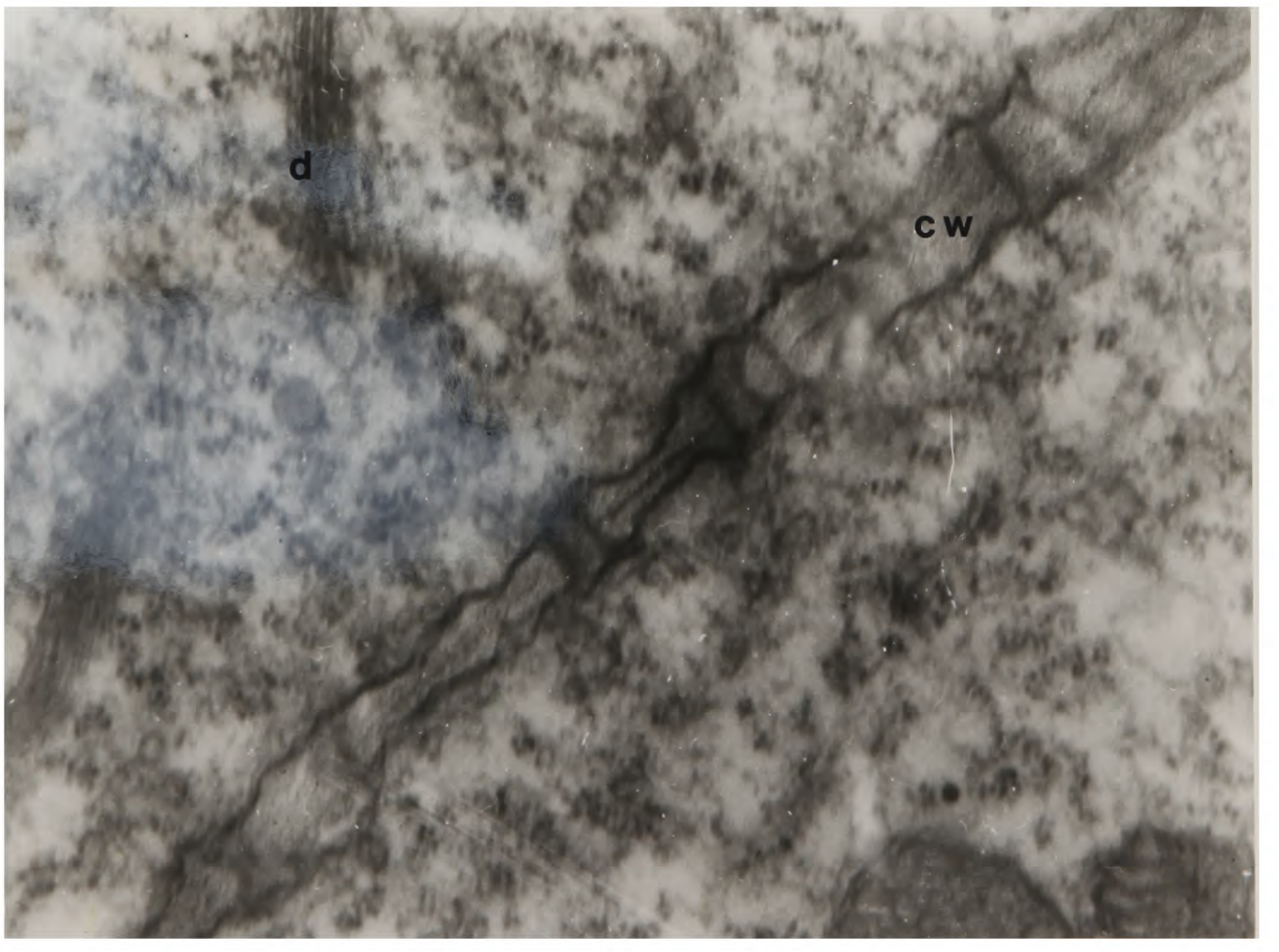


Plate 10. Radial cell wall between two C2 cells, -B. Scale bar = 200 nm.

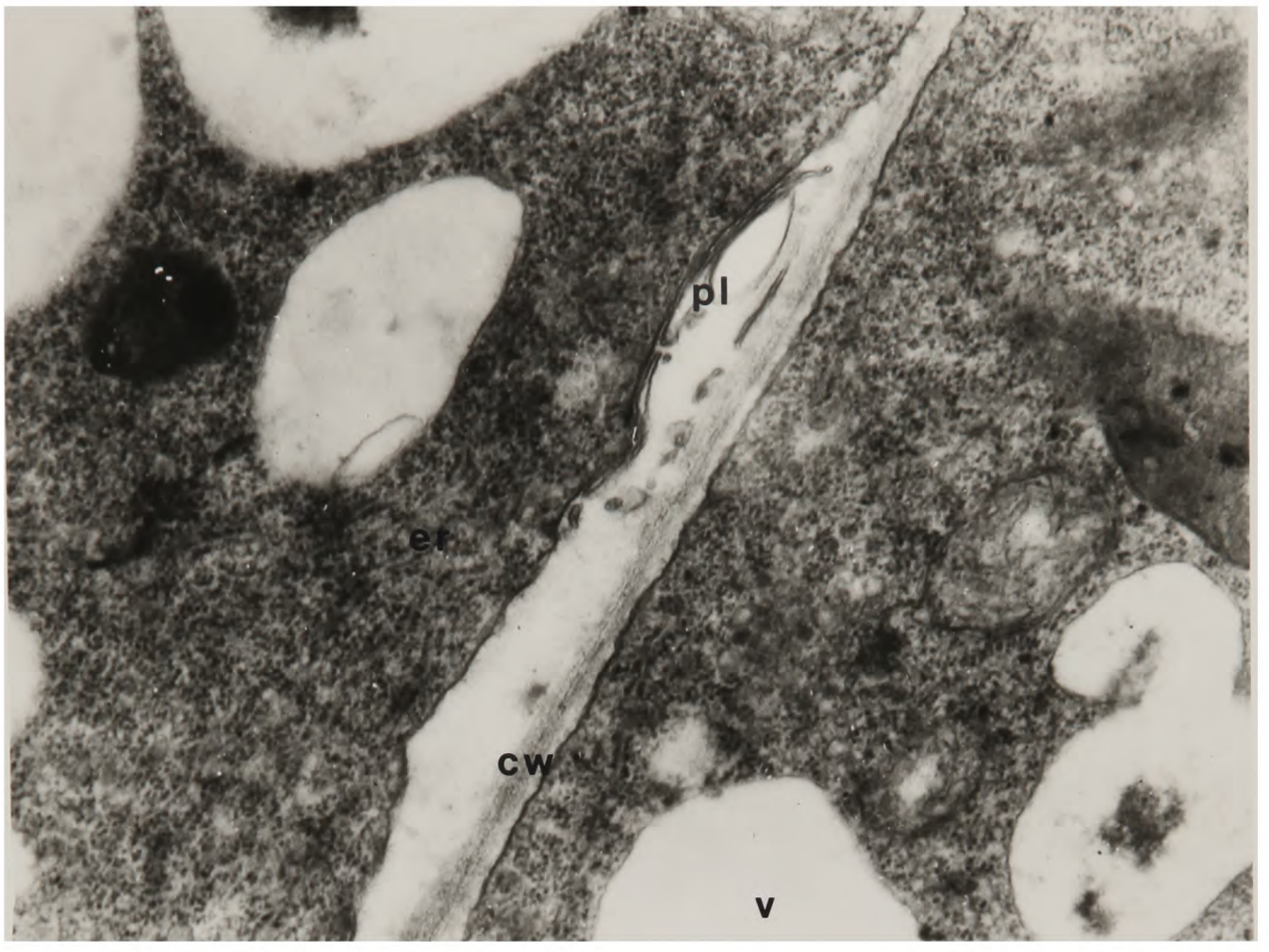


Plate 11. Tangential cell wall between epidermal and hypodermal cells, +B; hypodermis to left. Scale bar = 200 nm.

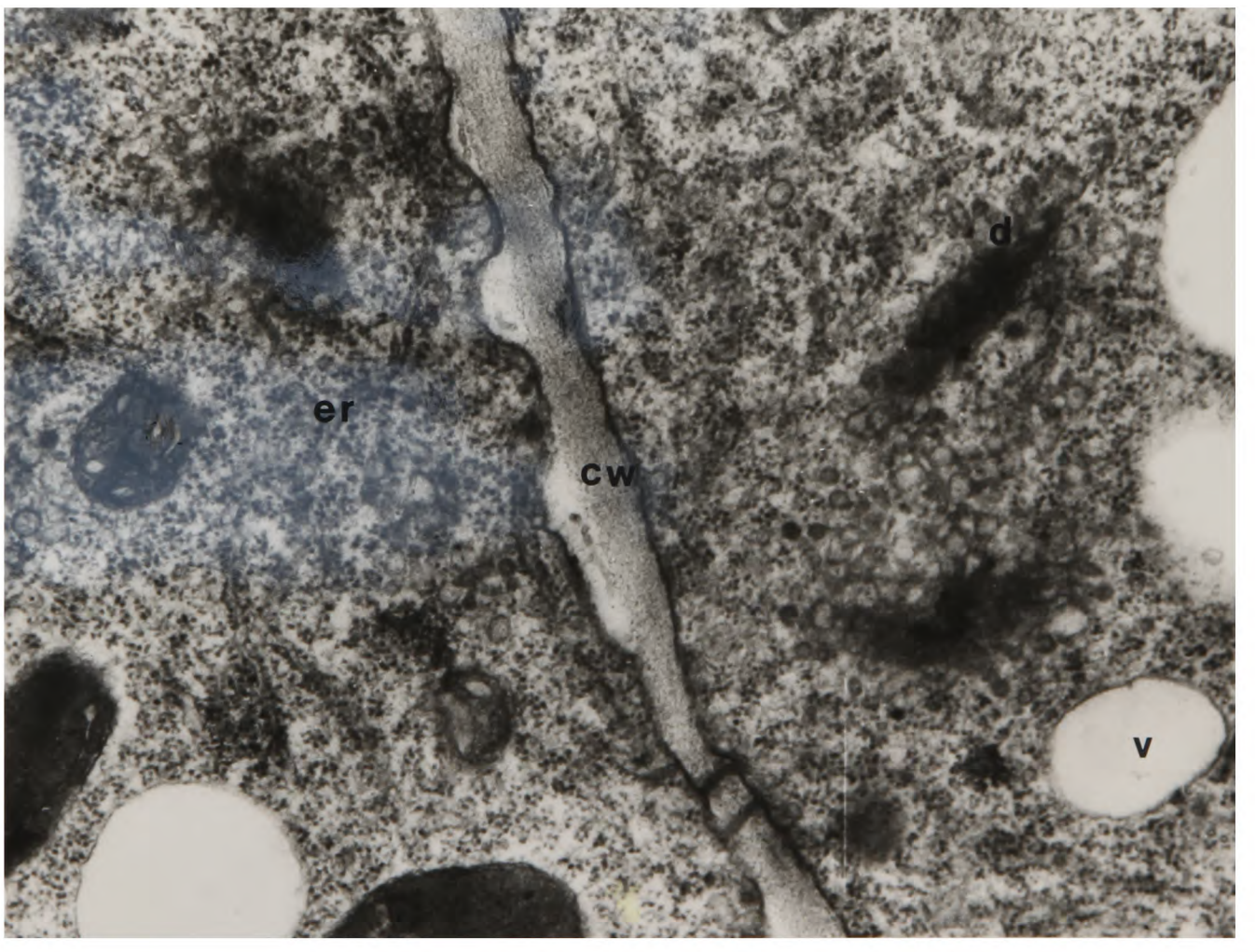


Plate 12. Tangential cell wall between hypodermal and C2 cells, -B; hypodermis to left. Scale bar = 200 nm.

vesicle release from dictyosomes (plates 10, 12, pp. 131-132) was enhanced after 5.5 h -B and by 3 d -B there were more dictyosomes per unit of cytoplasmic area.

2. Cell wall synthesis around protoplasts

2.1 Cell wall stains.

Calcofluor White and Tinopal CBS-X were used as fluorescent brighteners for visualising wall resynthesis. Results can be briefly summarised as follows:

2.1.1 Sunflower

Protoplasts were observed at a wide range of times 20-96 h after isolation. Fluorescence varied considerably at all times, from faint to intense staining. Calcofluor and Tinopal were equally effective at rendering the wall visible, but Tinopal was more soluble and left no flecks of fluorescent material in the preparation. In batches of protoplasts observed from 20-48 h after isolation there was some increase in the intensity of staining but accurate counts were not attempted, and there was no visible difference between +B and -B preparations.

2.1.2 Maize

Protoplasts were observed 20 h and 40 h after isolation. Fluorescence with Calcofluor was faint at both times.

2.1.3 Pea

Protoplasts examined 48 h after isolation fluoresced brightly with Calcofluor. There was no detectable difference between +B and -B preparations.

2.2 SEM of wall resynthesis

2.2.1 Sunflower

There were considerable protoplast losses incurred during the fixation and

CPD processes. Nevertheless given the fragility of the protoplasts, it is perhaps more surprising that many intact protoplasts were found than that there were considerable numbers of damaged protoplasts in each preparation. Sunflower protoplasts were found to vary considerably in appearance. From 4-18 h after removal from the wall-digesting enzymes naked protoplasts could be found in all preparations. Some of these had fine membranous protrusions on the surface (e.g. plates 13-15, p. 135). Others appeared to be smooth (plates 16-18, p. 136). The protoplast surface seen at higher magnification was typically covered in small protrusions, but their profusion was not related to the boron status of the samples or the time of sampling.

Some protoplasts were seen to be synthesising a new cell wall. Plate 19 a, (enlarged in 19 b; p. 136) shows a single fibre on the protoplast surface about 60 nm thick, apparently linking several of the membrane protuberances mentioned above. Protoplasts from similar 12 h -B roots but resupplied with 4.6 μM B were also found with fibres on the surface, e.g. plate 20 a,b, (p. 137); and a similar stage in wall synthesis is shown for a protoplast from +B roots in plate 21 a,b, (p. 137). Some contaminating bacteria can also be seen in this micrograph.

As wall synthesis progressed a mesh of fibres was formed as in plates 22-26, (pp. 137-138). The fibres sometimes appear to splay out at their ends implying that these are actually 'ropes' of several strands. Some +B and -B protoplasts showed what may be later stages in wall regeneration (plates 27, 28, p. 139) where the mesh is becoming progressively infilled. In some areas the mesh appears to merge with the protoplast surface.

The later stages of wall synthesis were less commonly observed on +B protoplasts. However some -B protoplasts appeared to have similar walls at the first time of examination, i.e. 4 h after removal from the wall-digesting medium. The possibility that in some cases the cell wall was not

Plates 13-30. Scanning electron micrographs of fixed, critical point dried protoplasts from 'Single Tall' sunflower roots. Plants grown for 2 d +B then 12 h +B or -B. All times shown beneath plates measured from removal from wall digestion medium. '-/+B' protoplasts resupplied with 4.6 µM H_3BO_3 after 4 h. Protoplasts fixed after 4 h, 7 h and 18 h; 1 mM UDPG supplied to all preparations after 4 h.

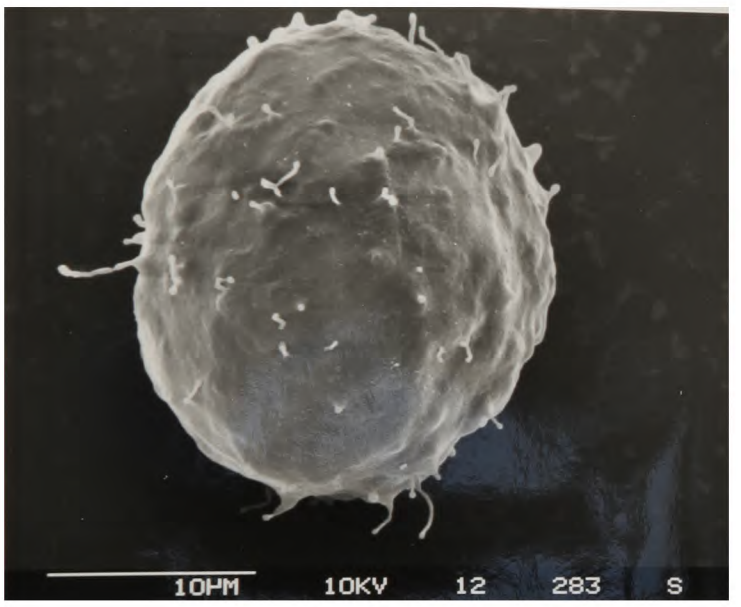


Plate 13. -/+B, 7 h.

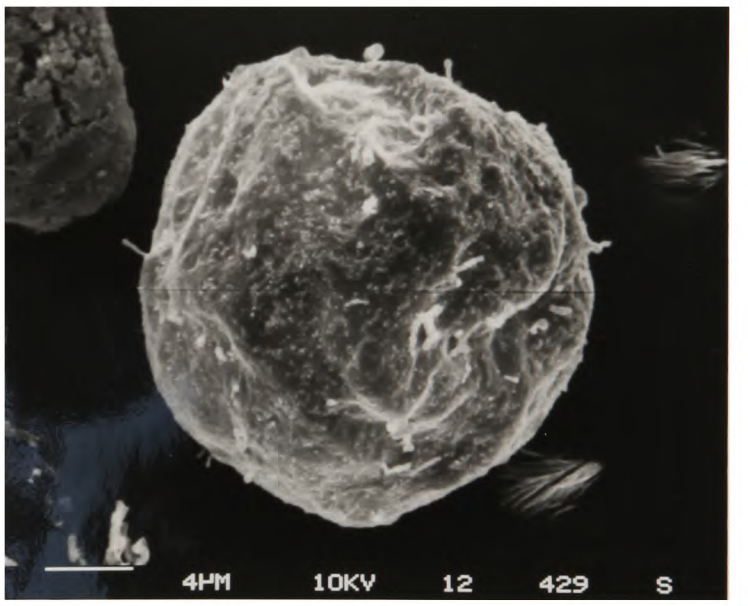


Plate 14. +B, 4 h.

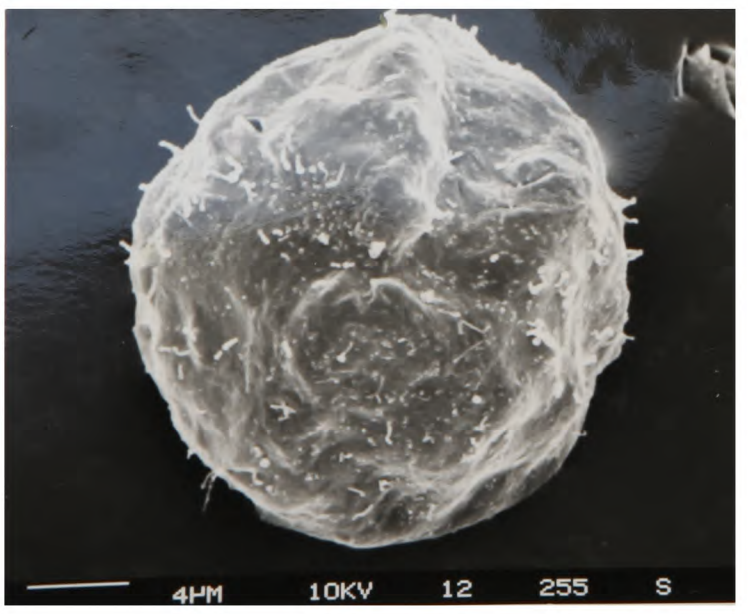


Plate 15a. -B, 7 h.

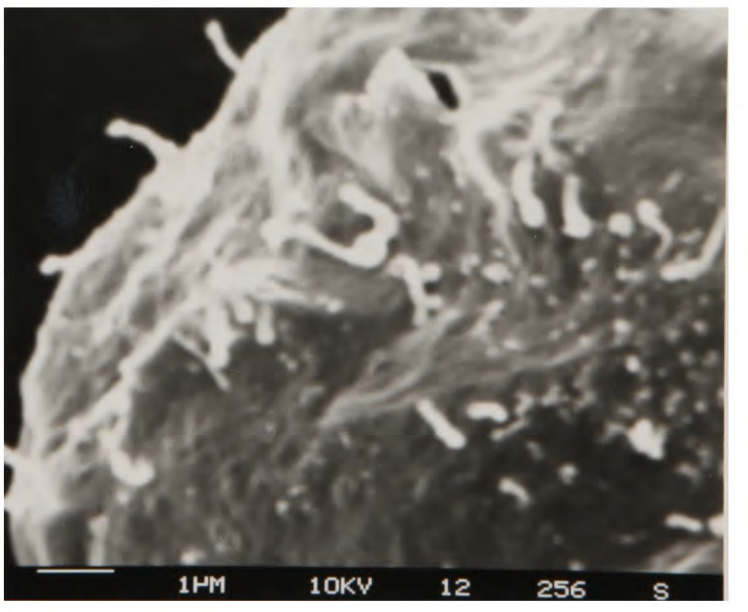


Plate 15b. -B, 7 h.

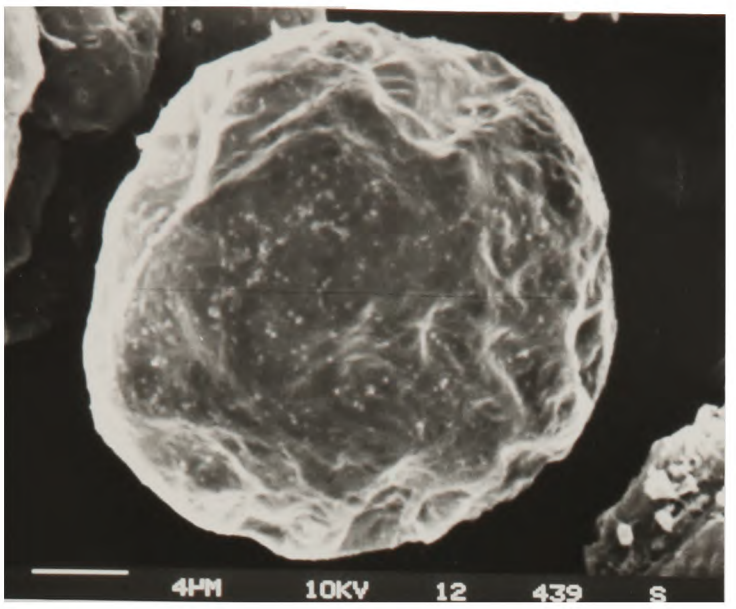


Plate 16a. +B, 4 h.

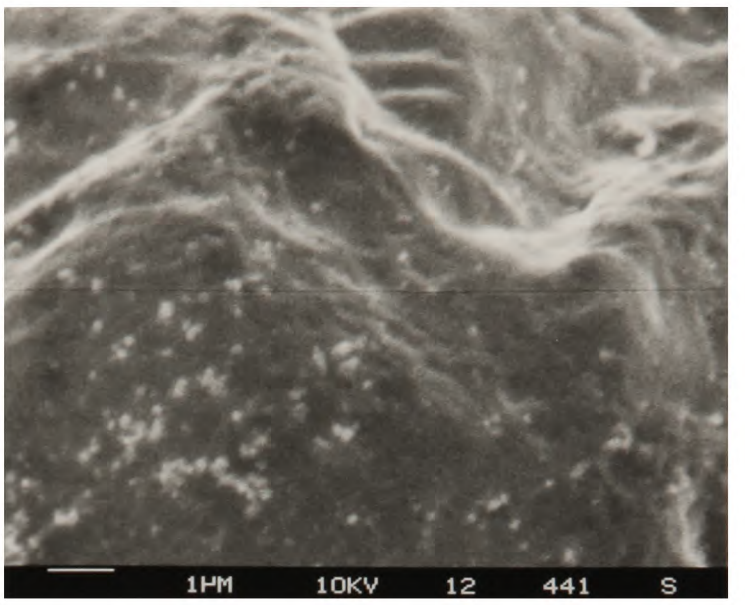


Plate 16b. +B, 4 h.

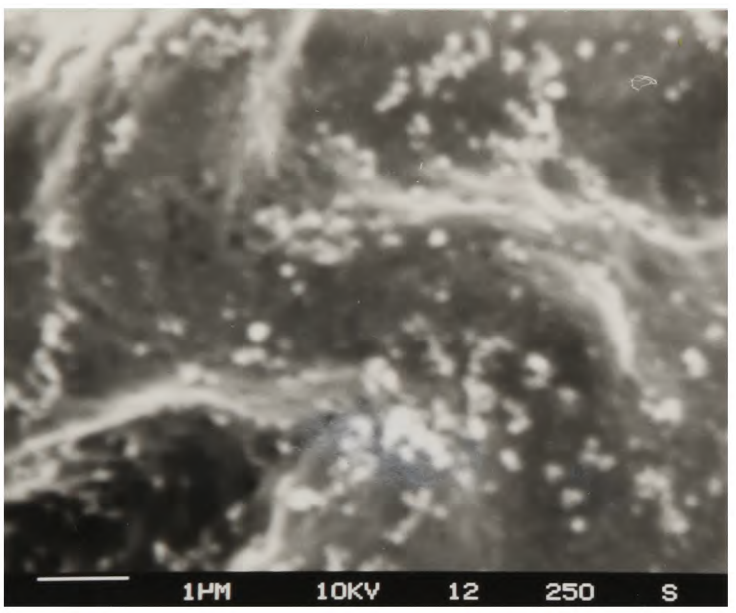


Plate 17. -B, 7 h.

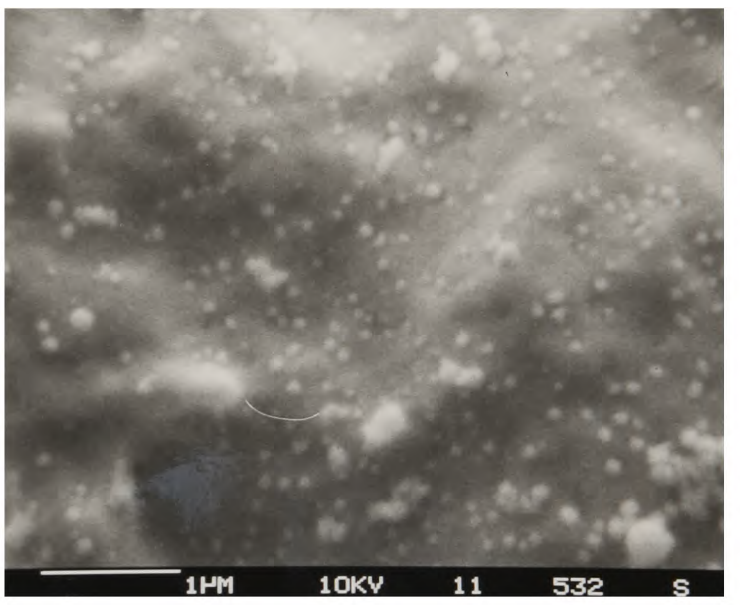


Plate 18. -B, 4 h.

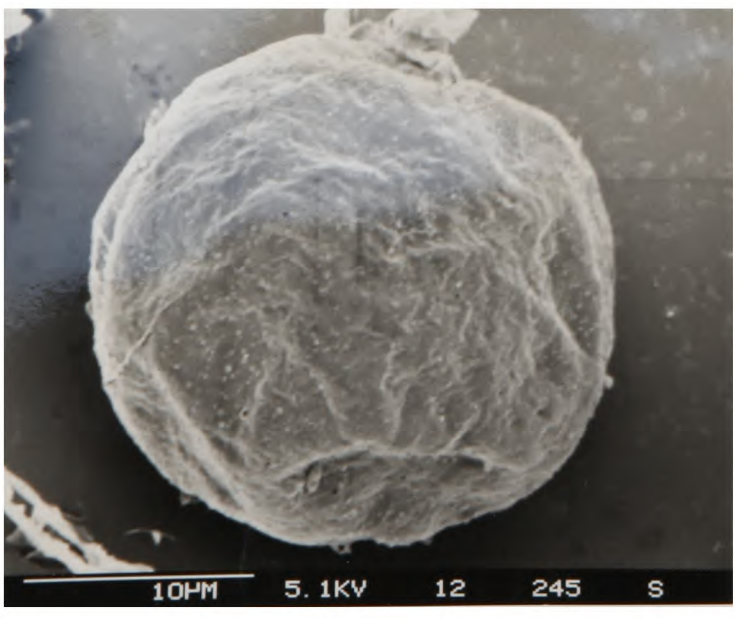


Plate 19a. -B, 4 h.

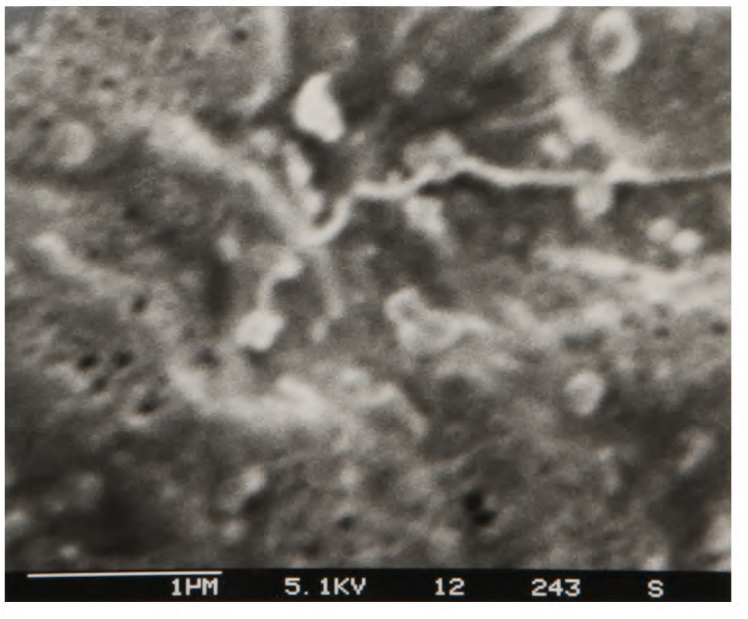


Plate 19b. -B, 4 h.

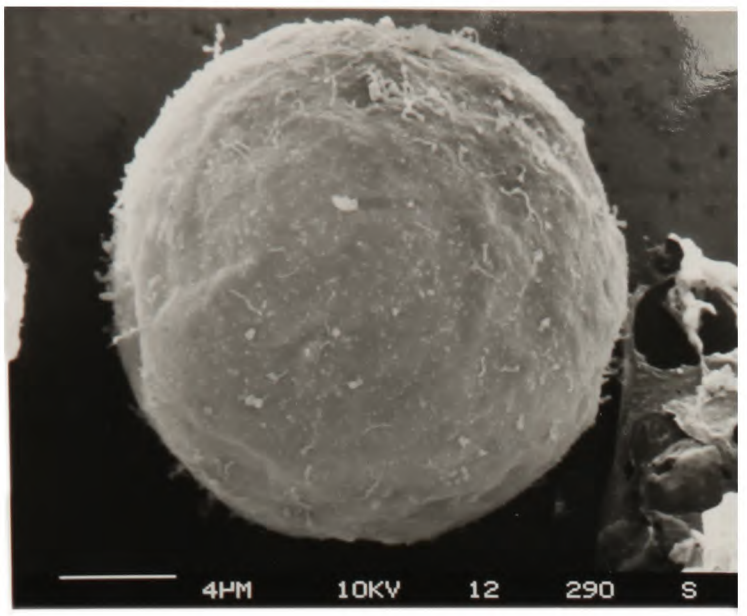


Plate 20a. -/+B, 18 h.

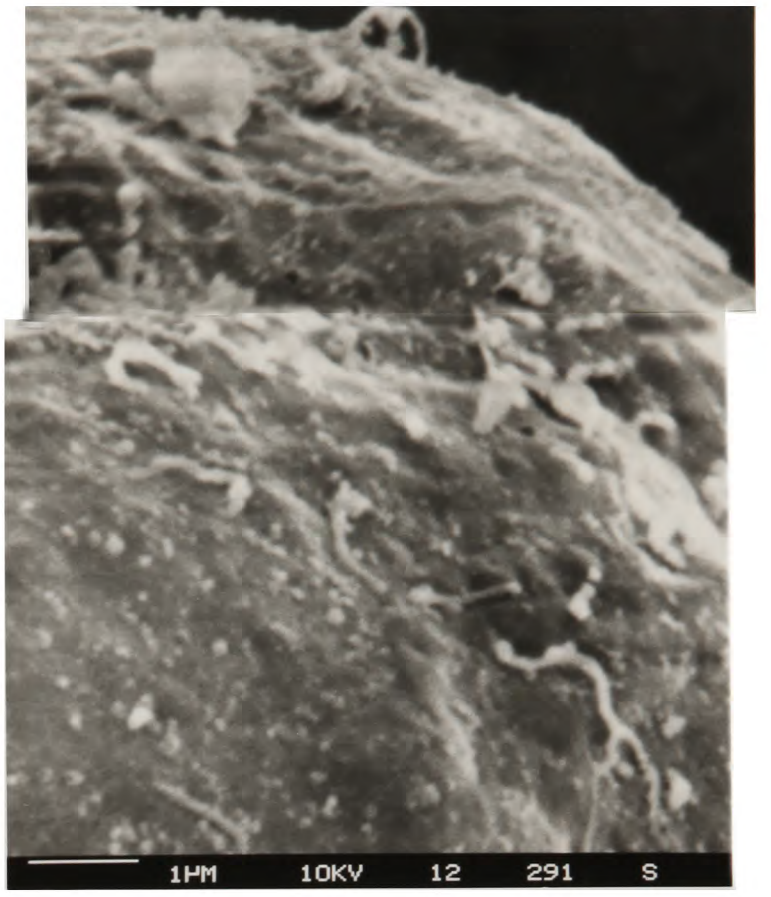


Plate 20b. -/+B, 18 h.

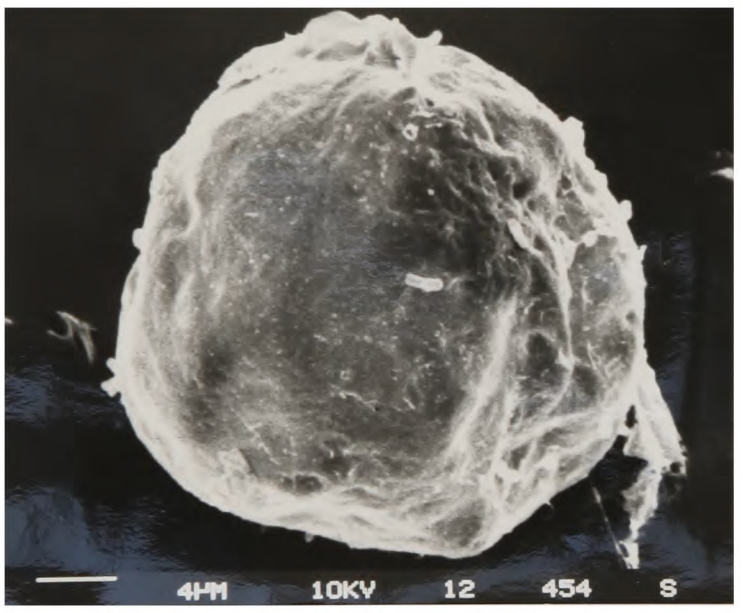


Plate 21a. +B, 7 h.

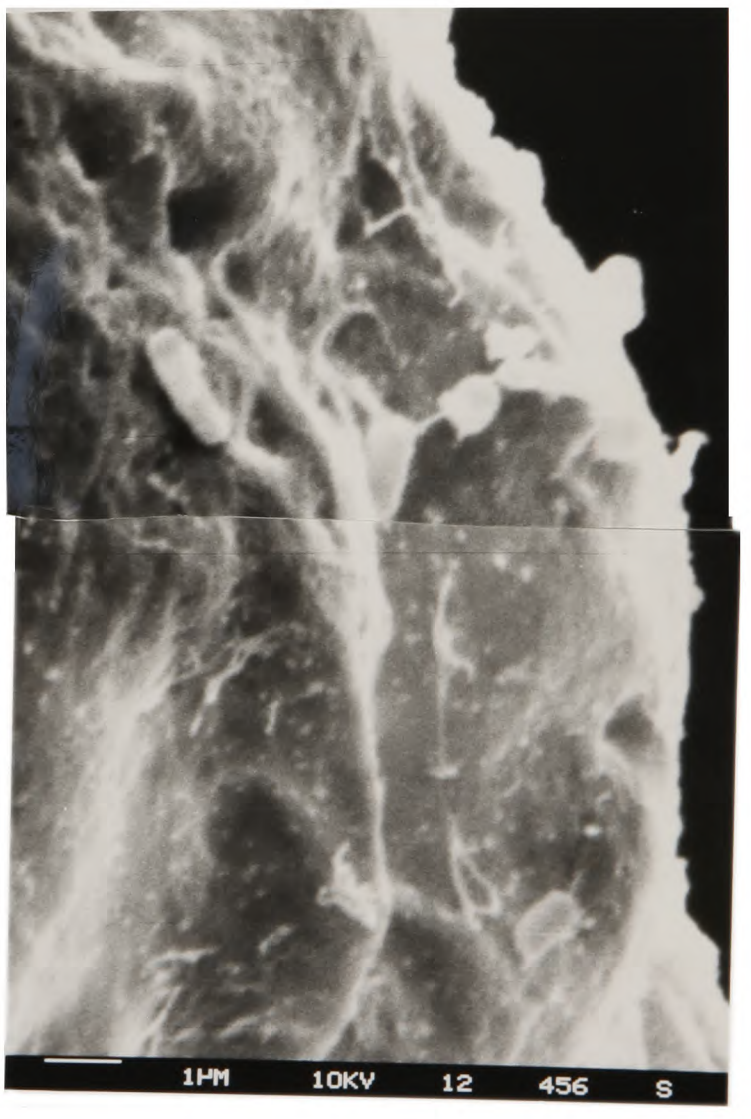


Plate 21b. +B, 7 h.

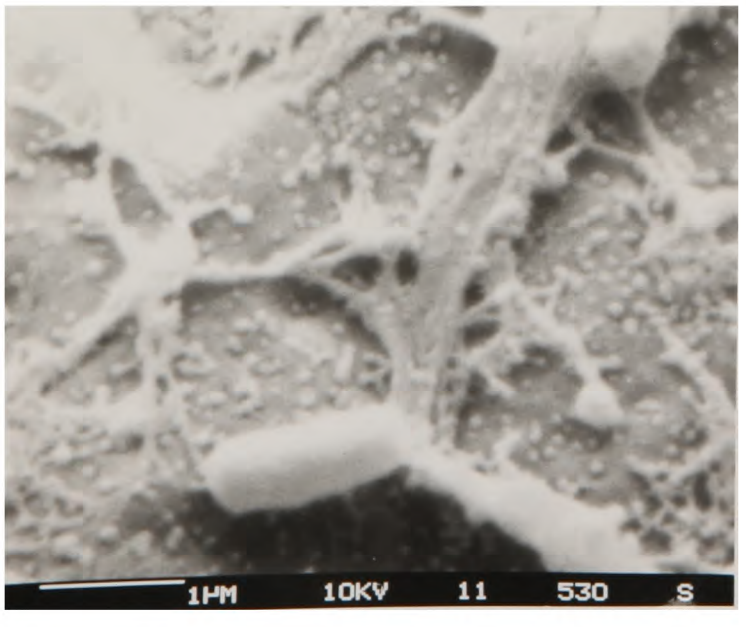


Plate 22. -B, 4 h.

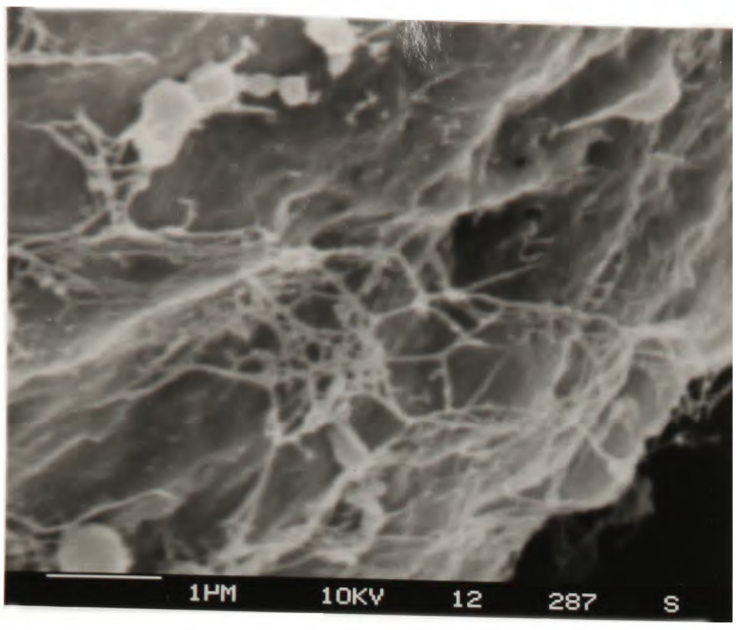


Plate 23. -/+B, 7 h.

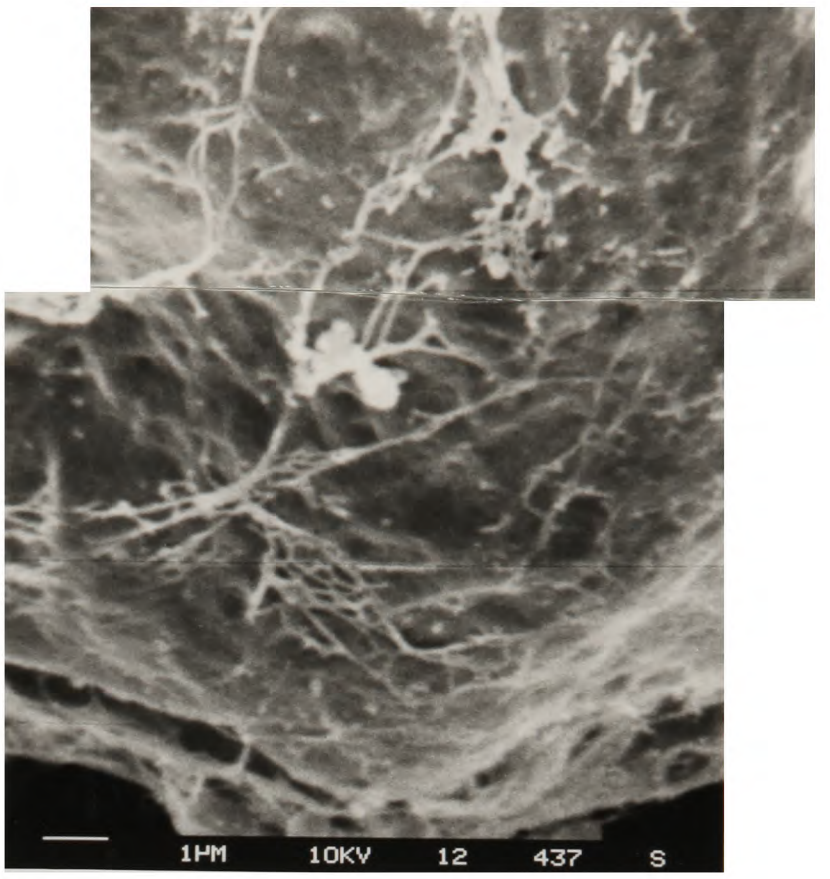


Plate 24. +B, 4 h.

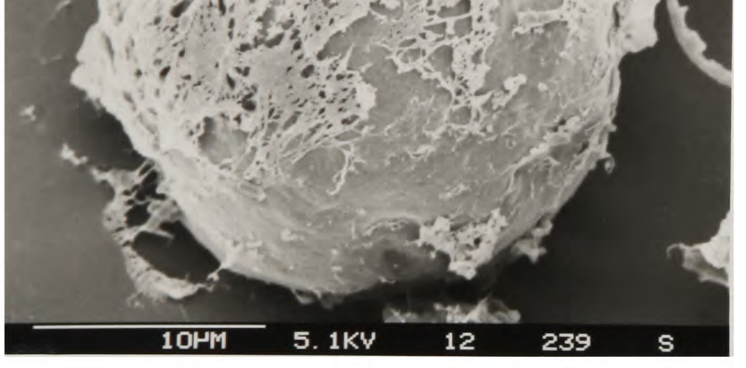


Plate 25. -B, 4 h.

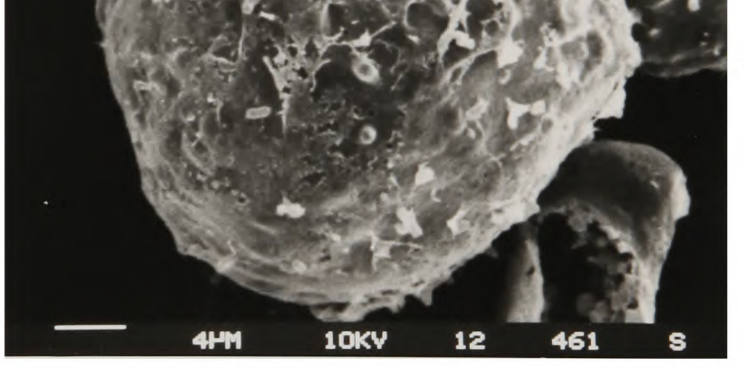


Plate 26a. +B, 7 h.

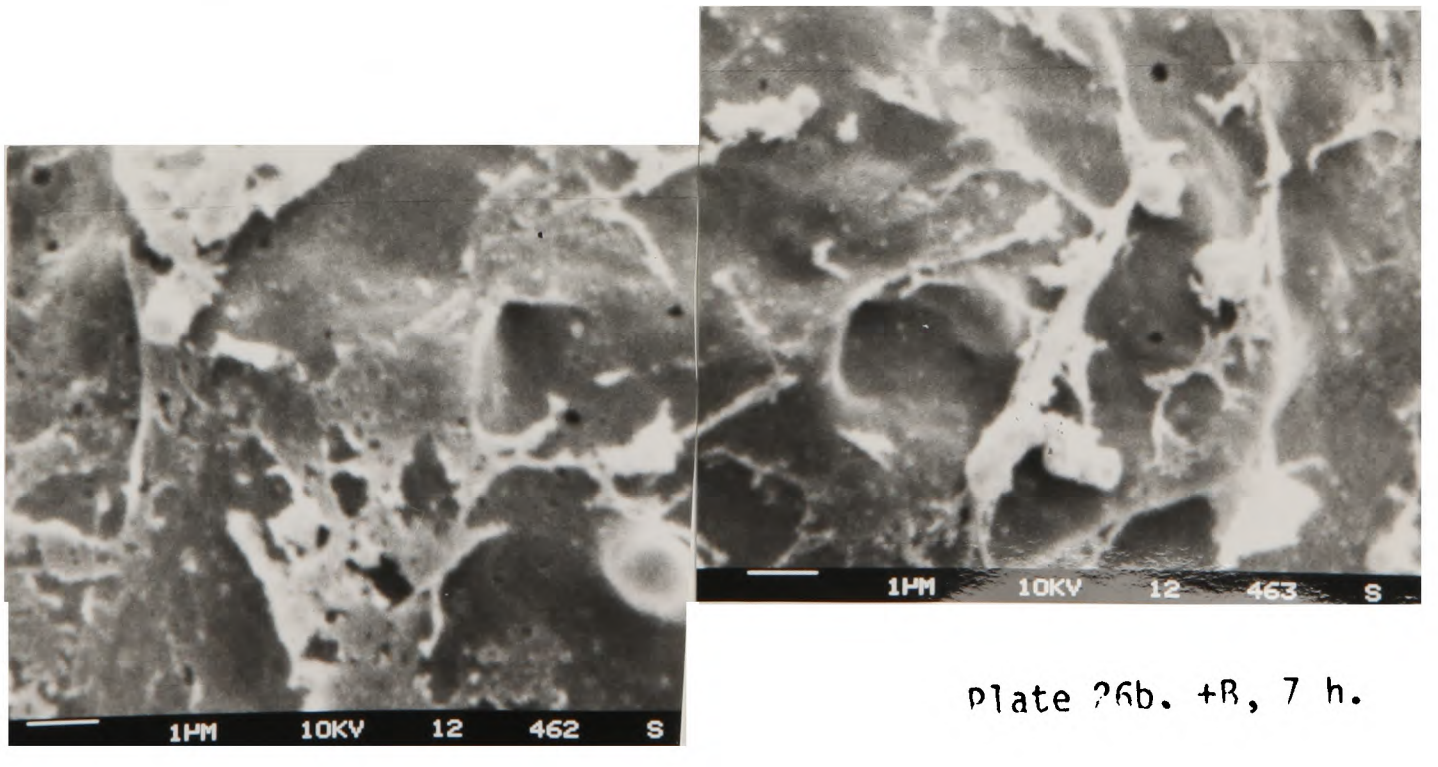


Plate 26b. +B, 7 h.

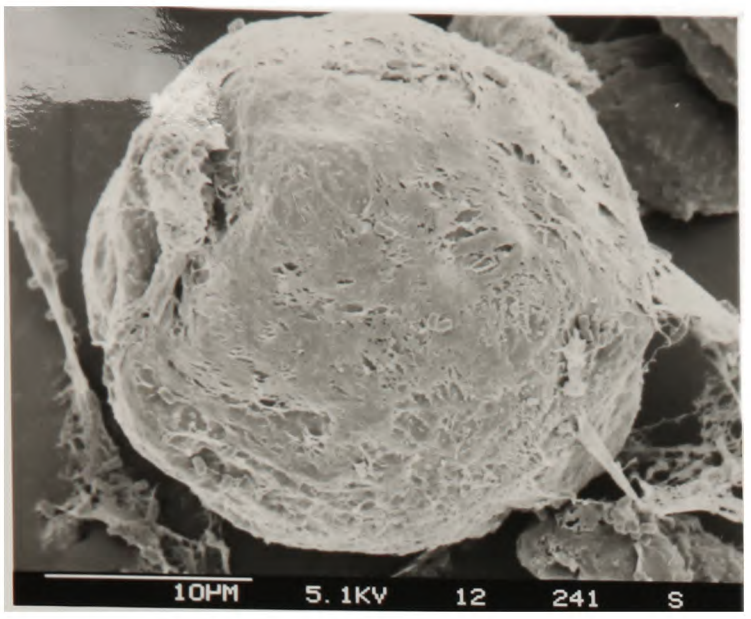


Plate 27a. -B, 4 h.

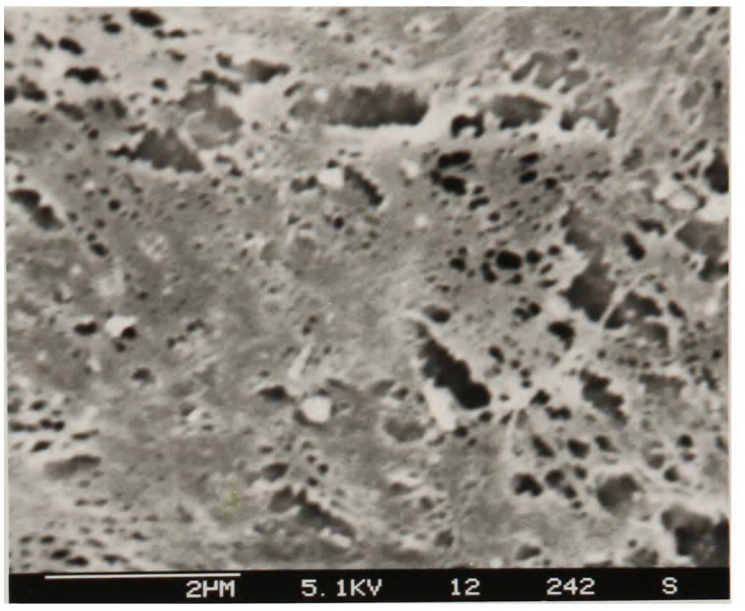


Plate 27b. -B, 4 h.

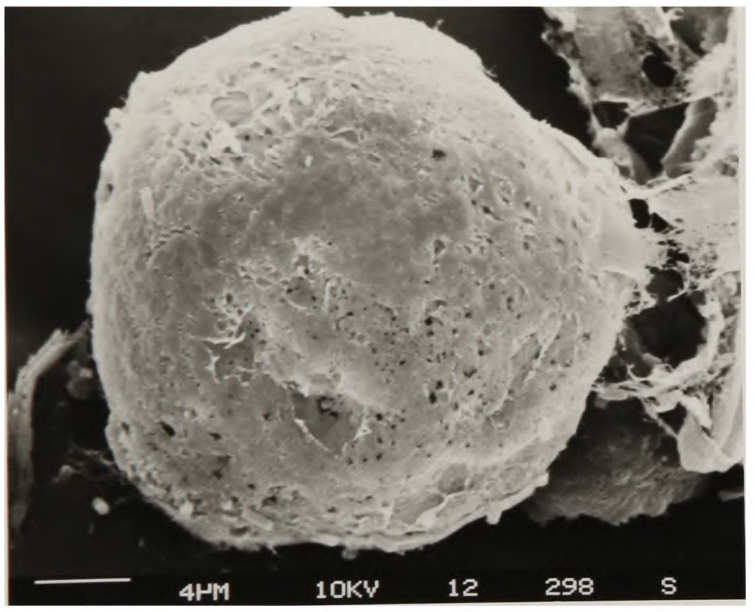


Plate 28a. -B, 18 h.

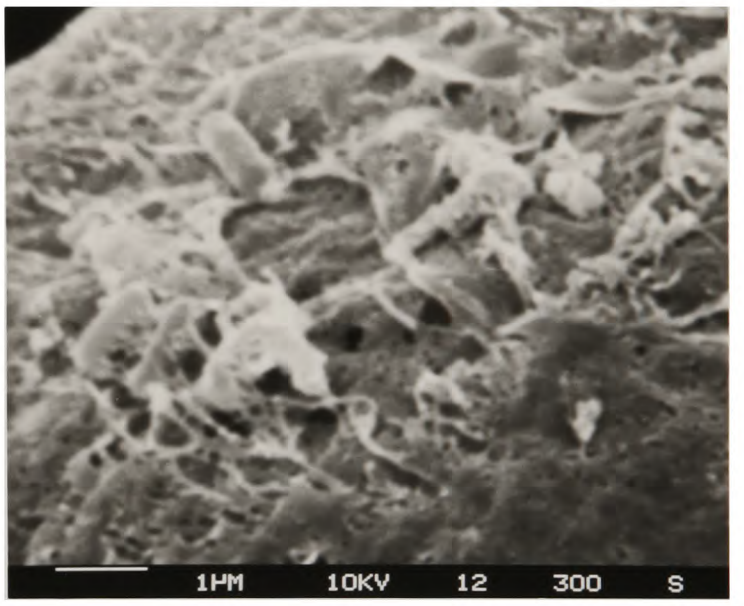


Plate 28b. -B, 18 h.

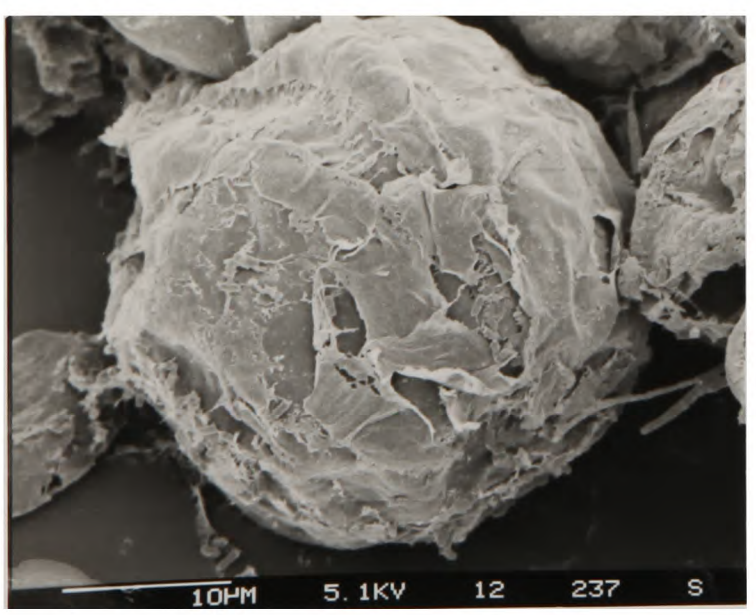


Plate 29. -B, 4 h.

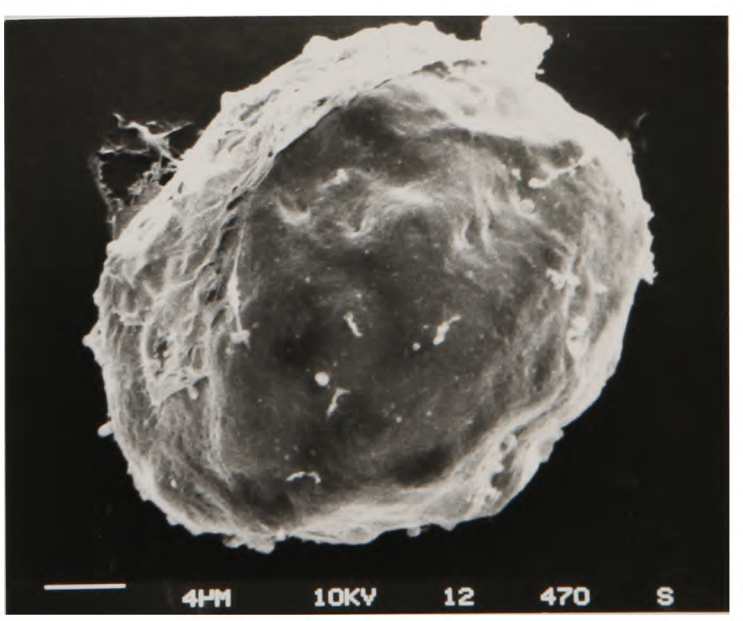


Plate 30. +B, 7 h.

fully removed by the fungal enzymes was considered and some walls were tentatively identified as remnants of pre-existing walls by their damaged appearance (plate 29, p. 139) or irregular deposition on a protoplast (plate 30, p. 139). The meshes shown in plates 22-28 (pp. 137-139) clearly differ from these but that does not prove de novo synthesis. It is unlikely that remnants of the original cell walls would resemble the spherical, closely appressed meshes seen here, since the plasmolysed protoplasts are smaller than the cells from which they were isolated.

Plates 18 & 22 (pp. 136-137) show the surface nature of -B protoplasts more clearly than in the other plates. These protoplasts were fixed in glutaraldehyde and post-fixed in OsO_4 (and the micrographs are focussed better). Wall fibres in plate 22 again appear to end at the protruberances on the protoplast surface.

During observation of the preparations, protoplast descriptions were recorded as 'naked', 'occasional fibres', 'more substantial mesh' and 'wall residue'. Although proportionally more -B protoplasts were seen to be synthesising new walls, the difference was not statistically significant (table 22, p. 141). Two counts made with the same +B stubs six weeks apart gave similar results (χ^2 = 5.8, 6 d.f., $p > 0.1$), suggesting that the scoring and sampling system was reliable.

2.2.2 Maize

Wall synthesis by maize protoplasts was not as readily observed as in sunflowers. Naked protoplasts with membranous surfaces were seen in all preparations from 4-17 h after removal from the wall-digestion medium (plates 31-33, p. 142). The protoplast in plate 33 (a and b) shows remarkable contortions of the surface, reinforcing the interpretation that the surfaces are wall-less. Occasionally artefactual holes in the membrane could be seen; similar observations occur in the literature for

Table 22. Wall resynthesis by sunflower protoplasts

	+B		-B		-/+B		
	O	E	O	E	O	E	T
S	60	50.8	17	21.7	5	9.5	82
F	21	25.4	13	10.8	7	4.8	41
M	5	10.5	7	4.5	5	2.0	17
R	10	9.3	4	4.0	1	1.7	15
T	96	96	41	41	18	18	155

$\chi^2 = 16.15$
10 d.f.; $p < 0.1$, n.s.

Key: S = smooth (i.e. naked) protoplasts
F = fibre deposition
M = mesh formed
R = remnant of old wall still encloses protoplast
O = observed
E = expected (proportion of row and column totals)
T = total

Protoplasts were isolated from 2 cm tips of roots grown for 2 d +B post-germination followed by 12 h + or - B. '-/+B' protoplasts isolated from -B plants and resupplied 4.6 μM B after isolation, during the 4-17 h incubation period. UDPG supplied at 1 mM; protoplasts fixed, dried and examined in the SEM as in text.

Plates 31-36. Scanning electron micrographs of fixed, critical point dried protoplasts from 'LG11' maize roots. Plants grown for 29 h +B, followed by 15 h +B or -B. H_3BO_3 supplied at 4.6 µM. Protoplasts fixed 4, 7 and 17 h after removal from wall digestion medium; 1 mM UDPG supplied to all preparations after 4 h.

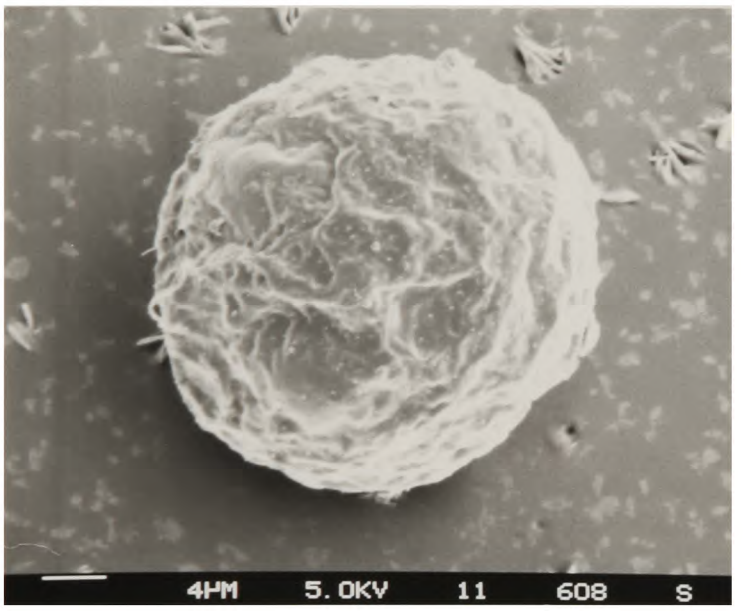


Plate 31. -B, 7 h.

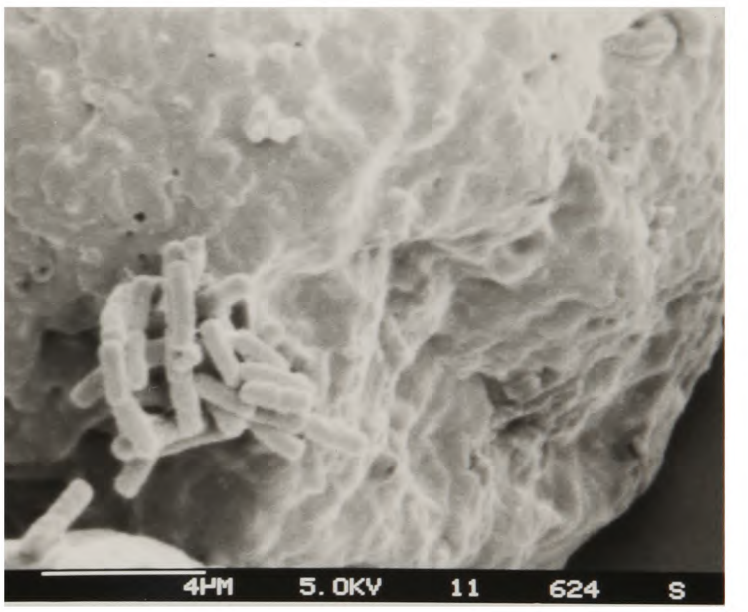


Plate 32. +B, 7 h.

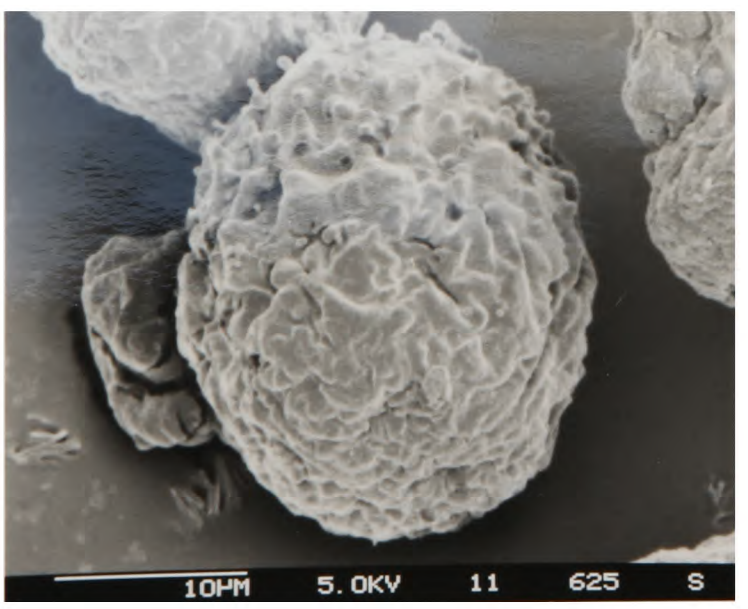


Plate 33a. +B, 7 h.

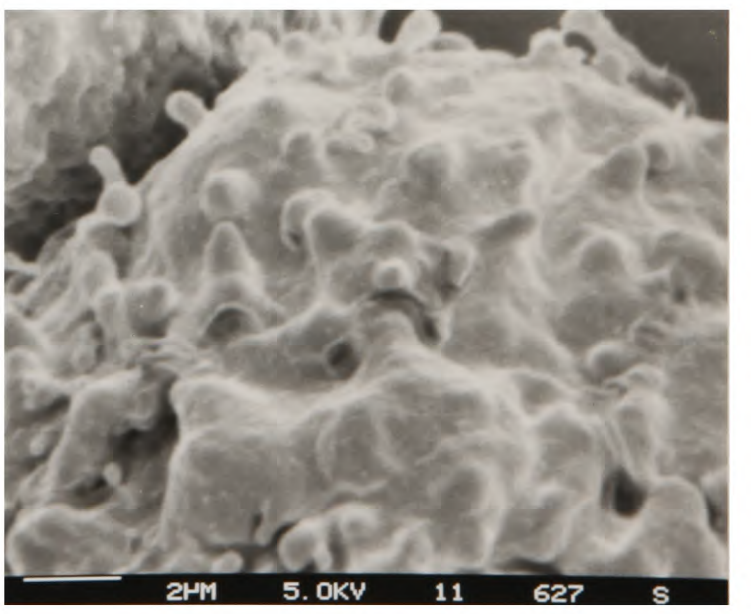


Plate 33b. +B, 7 h.

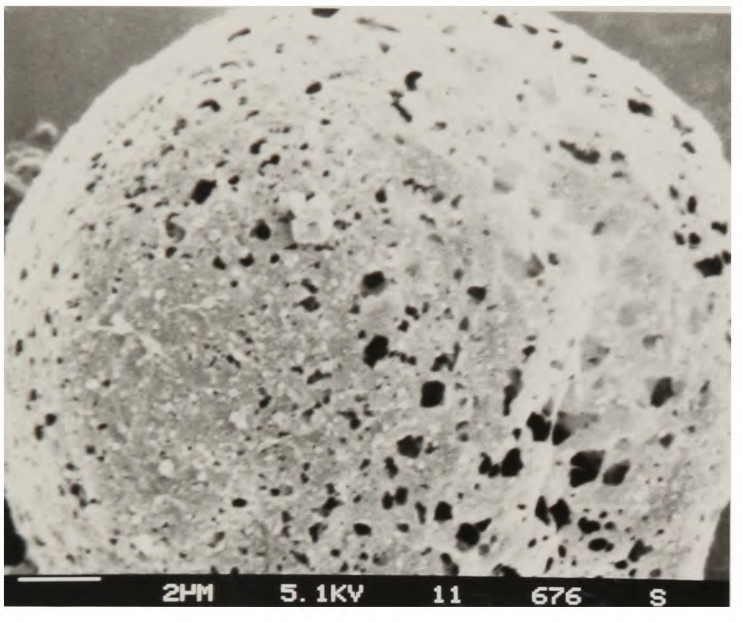


Plate 34. -B, 17 h.

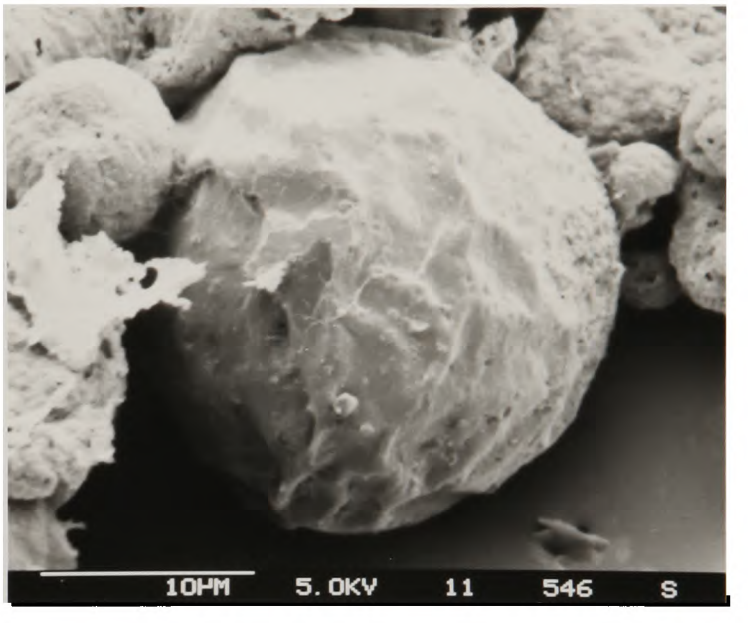


Plate 35. -B, 7 h.

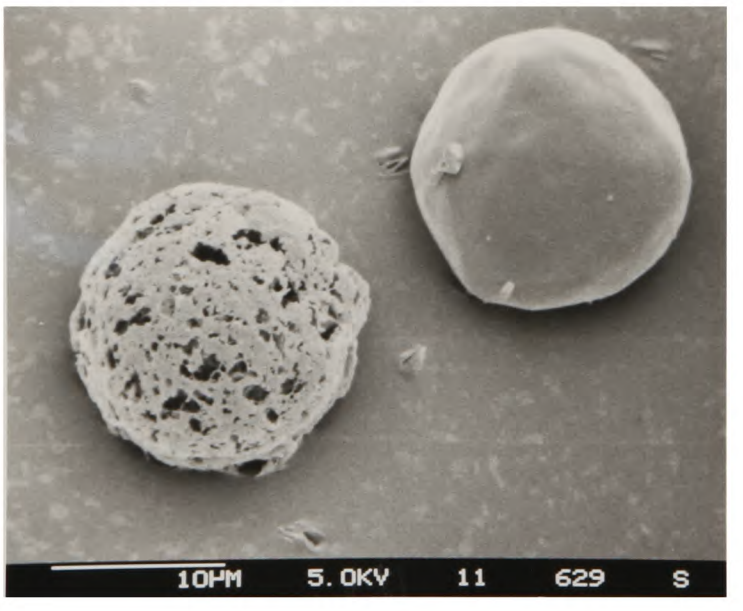


Plate 36. +B, 17 h.

protoplasts, like the maize protoplasts here, which were not post-fixed in OsO_4 (e.g. Burgess, Linstead and Harndon, 1977). After 7 and 17 h some protoplasts seemed to have a more robust surface perforated by larger holes. Plate 34 (p. 143) shows that in fact these holes are often traversed by fibres and the micrographs are therefore interpreted as showing an advanced stage of wall formation with the underlying surface insufficiently coated to be seen. However intermediate stages of fibre deposition were not observed, with the possible exception of the micrograph in plate 35 (p. 143); the 'fibres' were not seen on the microscope screen so the protoplast was not examined at a higher magnification. Plate 36 (p. 143) shows the striking difference between a 'robust' protoplast after 17 h of culture and an isolated vacuole.

3. UDPG incorporation by roots and protoplasts.

3.1 Pea protoplasts

The system for studying UDPG incorporation into water-insoluble material was initially tested with pea (Pisum sativum cv Meteor) root protoplasts. When UDPG was supplied at 1 mM it was incorporated into water insoluble product at a rate of around 200 $pmol.h^{-1}.(10^4$ protoplasts$)^{-1}$. There was no significant difference between the rate of incorporation by freshly isolated protoplasts over a 15 h period, and the rate shown by protoplasts held for 10 h and then tested over a 3 h period. The total amount of incorporation was roughly proportional to the number of protoplasts incubated in the vials (fig. 29, p. 145); maximal incorporation occurred with around 2×10^4 protoplasts. In these early experiments protoplasts were suspended in a medium based on Gamborg's 'B5' (Gamborg et al., 1968) containing 0.7 M mannitol, nutrient salts, vitamins and sucrose. The sucrose content resulted in an enormous population of microorganisms after 15 h incubation of an initially non-sterile preparation. To investigate the amount of incorporation for which any broken protoplasts and the

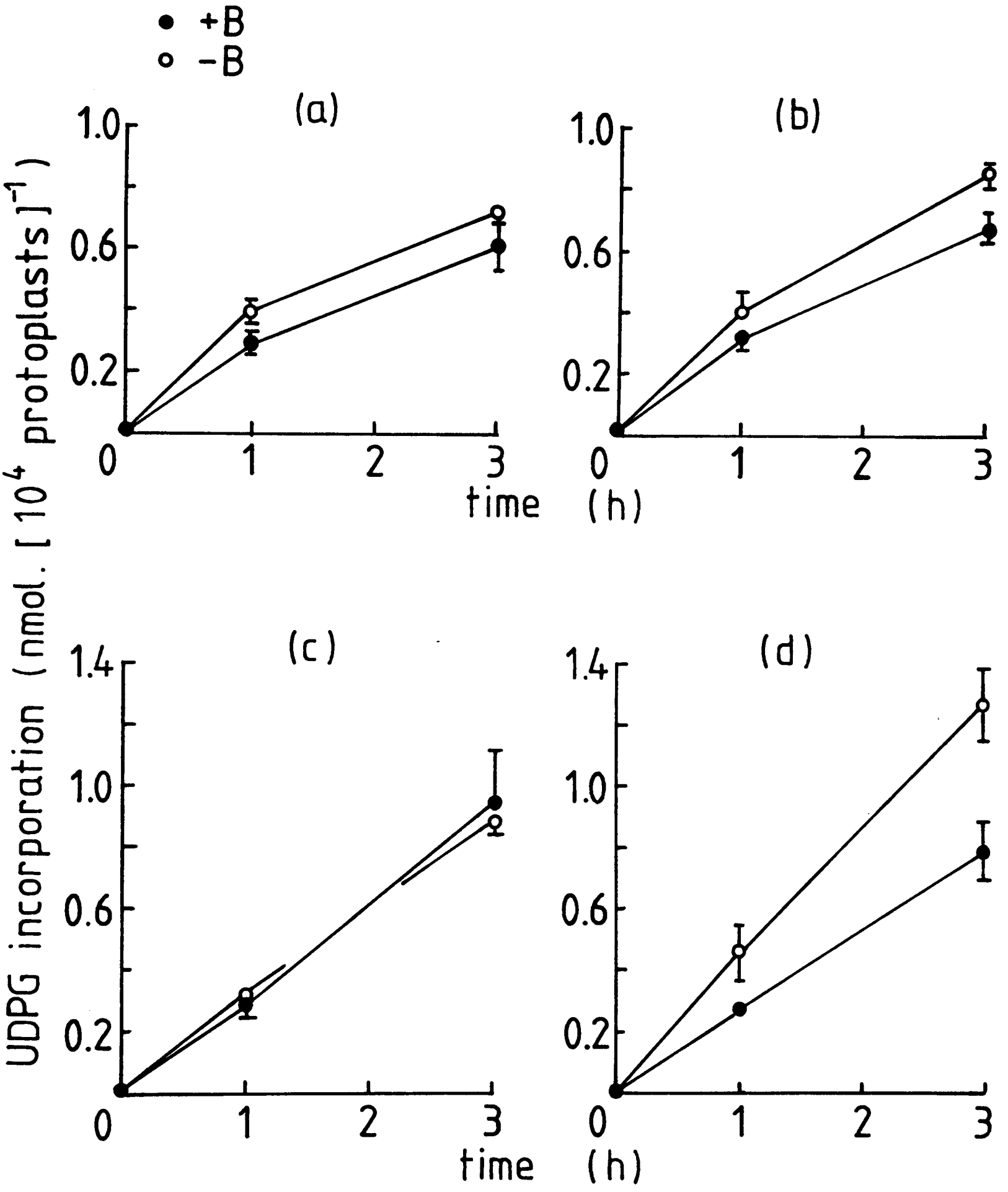


Fig. 29. Effect of protoplast density and B status on UDPG incorporation by protoplasts isolated from 2 cm 'Meteor' pea root tips. Plants germinated for 5 d. Protoplasts suspended in +B or -B 'B5' medium after isolation at (a) 10^5 protoplasts per vial; (b) 5.3 x 10^4; (c) 3.4 x 10^4 or (d) 1.7 x 10^4. B supplied at 9.2 µM where required.

contaminants were responsible, a preparation was ruptured with an ultrasonicating probe (3 x 10 s bursts, cooled on ice between each). No protoplasts survived intact, so the preparation consisted of membrane fragments and bacteria. UDPG incorporation by this preparation was lower than by untreated samples of intact protoplasts (fig. 30, p. 147).

3.2.1 Maize protoplasts

Initial experiments on the incorporation of UDPG by root protoplasts isolated from tissue-grown, 3 d old, -B maize seedlings showed linear incorporation of UDPG for 9 h, with no significant effect of 46 µM H_3BO_3 added during the incorporation period.

Protoplasts isolated from 2 cm root tips of plants (grown for 2 d +B followed by 15 h +B or -B) incorporated UDPG at a constant rate for 18 h (fig. 31, p. 148). +B protoplasts in the presence of 9.2 µM B incorporated more UDPG than -B protoplasts, per mg protein.

The response of the system to UDPG concentrations from 10 µM to 10 mM was also measured. Protoplasts were isolated from 2 cm root tips of plants grown for 4 d +B after germination and then +B or -B for the last 15 h. At each concentration tested protoplasts from +B roots incorporated more UDPG than -B protoplasts, and -B incorporation tailed off over the 3-6 h incorporation period. Eadie-Hofstee plots were drawn from the initial 0-1 h rates (0-3 h for 10 and 100 µM) (fig. 32, p. 149) and suggest a single enzyme system may be operating from 1-10 mM UDPG. Below 1 mM UDPG the values do not lie on the same line; a separate system may be involved at that concentration. 'Km' and 'V max' were calculated for the 1-10 mM results (table 23, p. 150), as though only a single enzyme were involved; in fact the system is likely to be more complex.

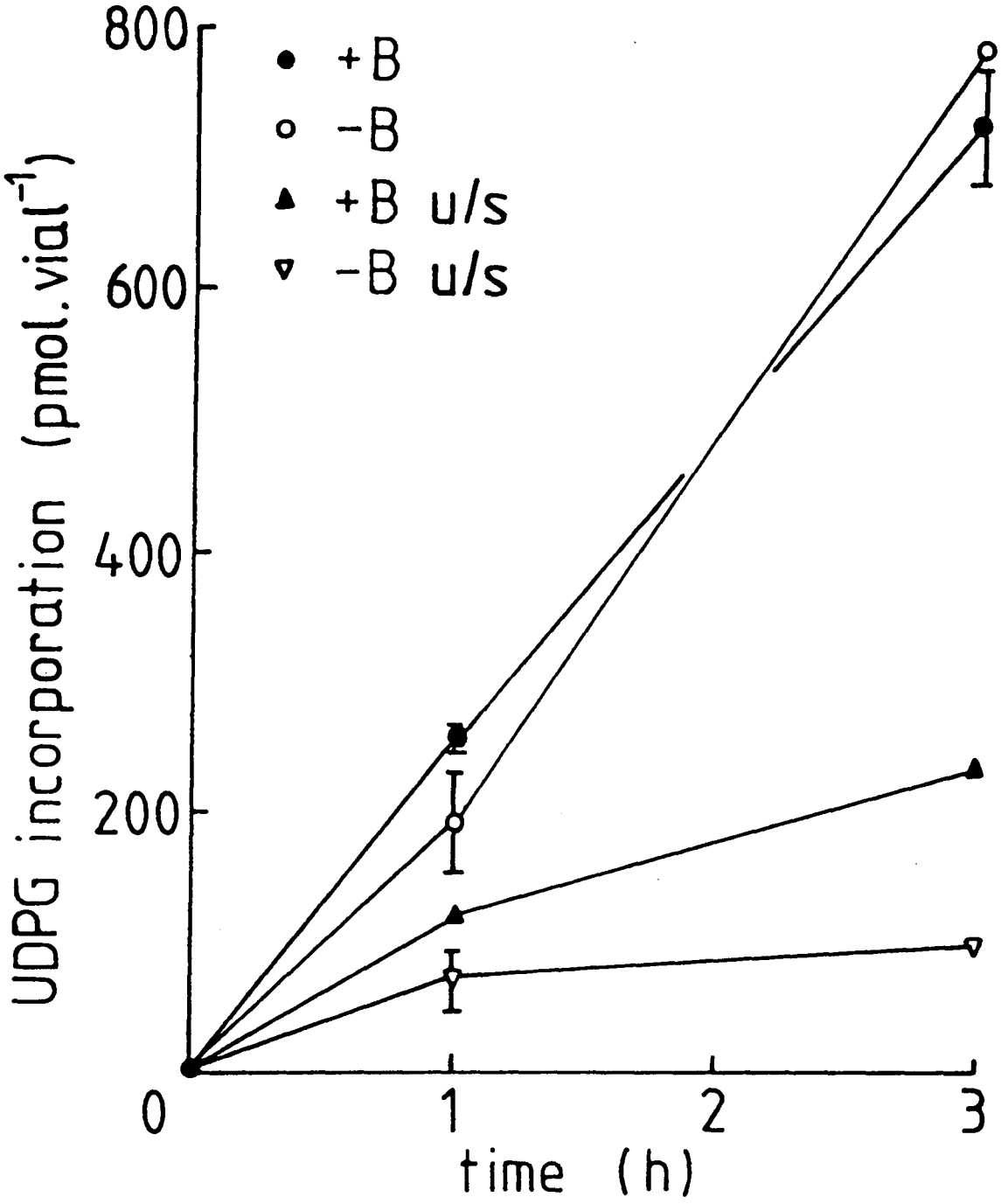


Fig. 30. Effect of B status and ultrasonication on UDPG incorporation by protoplasts isolated from 2 cm 'Meteor' pea root tips. Plants germinated for 6 d. Protoplasts suspended in +B or -B 'B5' medium after isolation; B supplied at 9.2 μM where required. Suspension held for 18 h before experimentation. 'u/s' = ultrasonicated. Protoplast incorporation rate around 400 pmol.h^{-1}.(10^4 protoplasts)$^{-1}$.

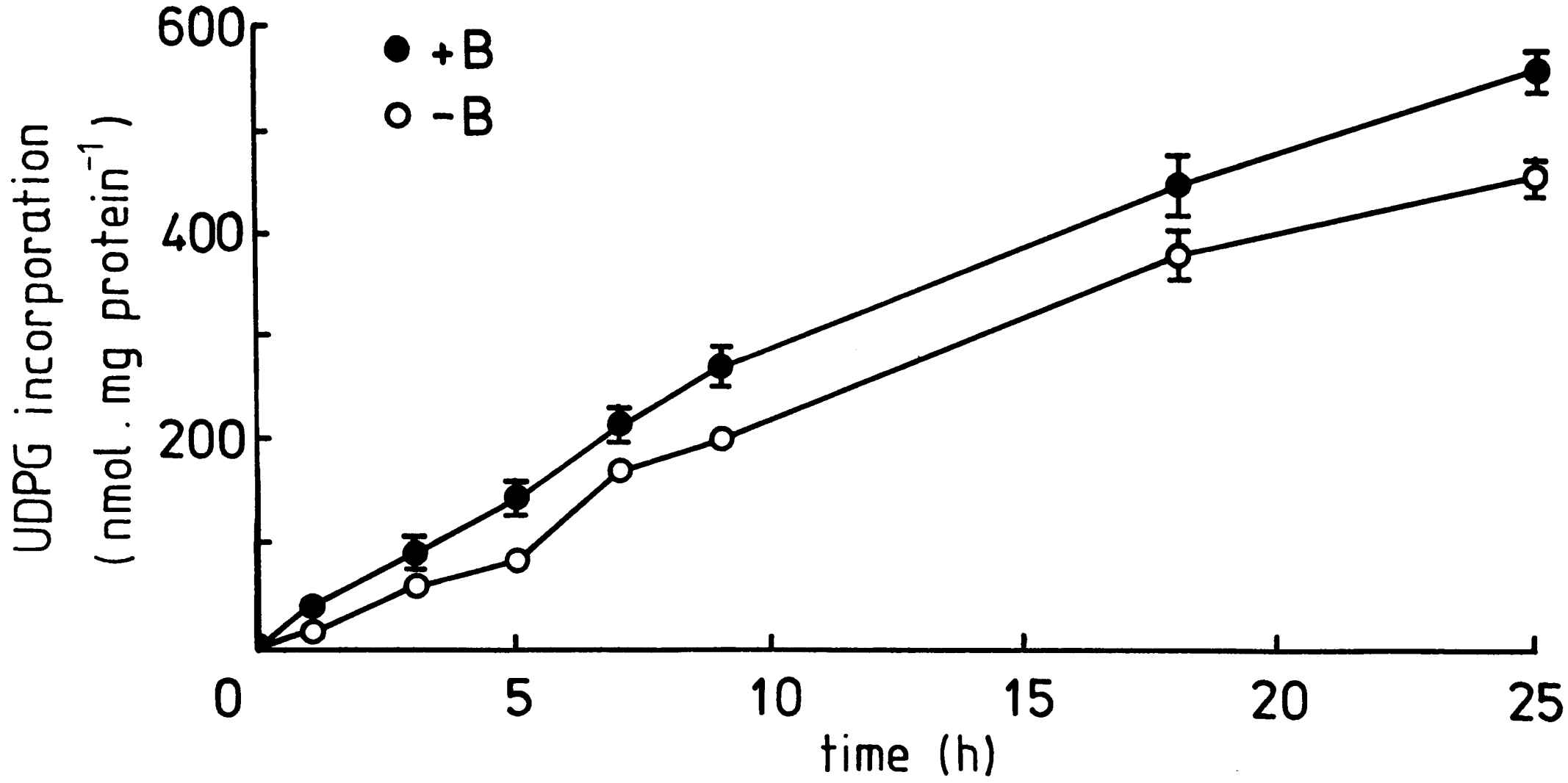


Fig. 31. Effect of B status on UDPG incorporation by protoplasts isolated from 2 cm 'LG11' maize root tips. Plants grown for 30 h +B (4.6 µM H_3BO_3) followed by 15 h +B or -B. +B protoplasts supplied with 9.2 µM H_3BO_3.

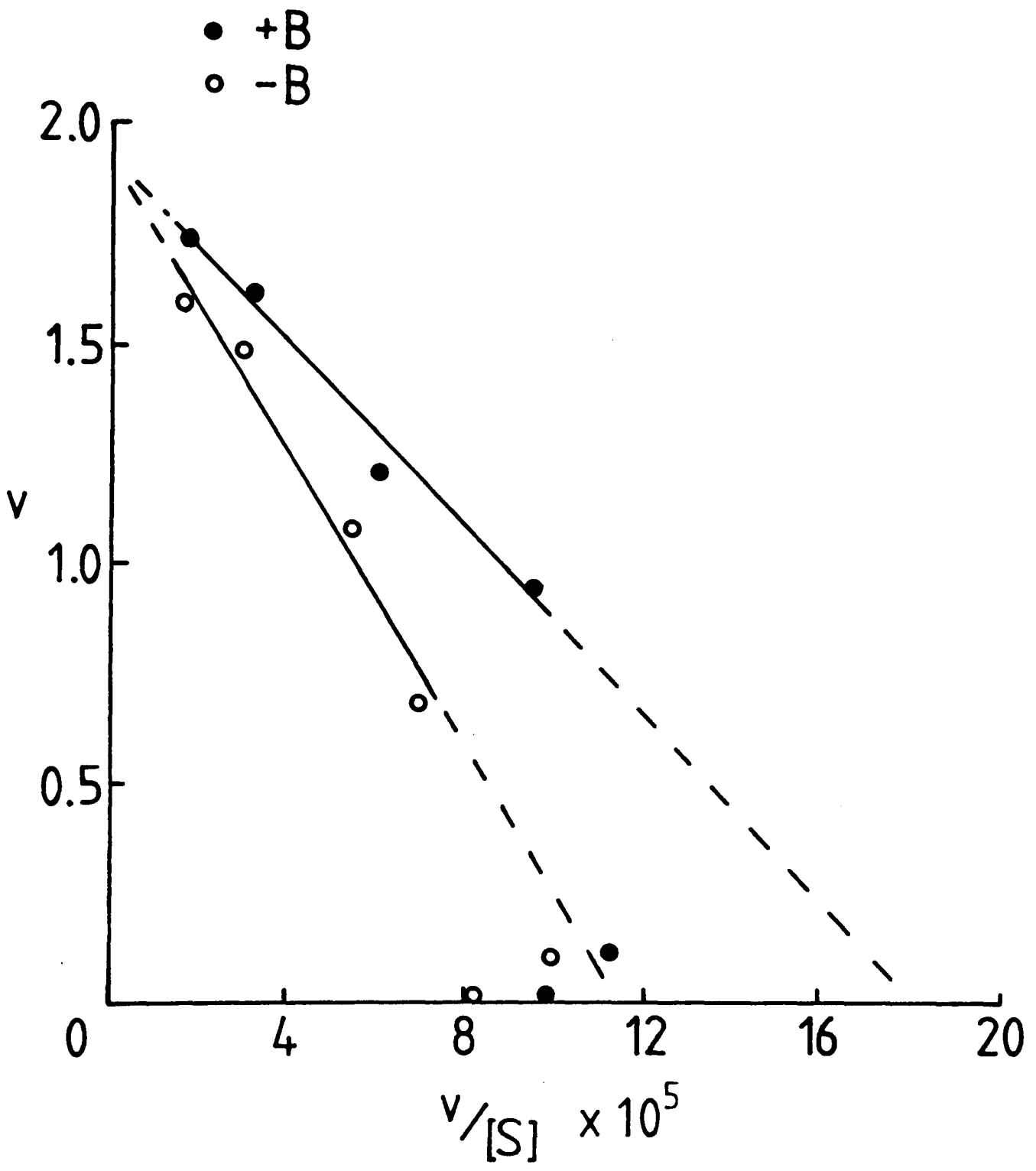


Fig. 32. Eadie-Hofstee plot of UDPG incorporation by protoplasts from 2 cm 'LG11' maize root tips. Plants grown for 30 h +B (4.6 µM H_3BO_3) followed by 15 h +B or -B; +B protoplasts supplied with 9.2 µM H_3BO_3. v = initial velocity (0-1 h or 0-3 h; see text). [S] = UDPG concentration.

Table 23. Kinetics of UDPG incorporation by maize protoplasts

Boron status	K_m (mM)	V_{max} (nmol.h^{-1}. (10^4 protoplasts)$^{-1}$)
+B	1.05	0.95
-B	1.71	0.96

Protoplasts isolated from 2 cm root tips of plants grown for 4 d +B followed by 15 h + or - B. UDPG supplied at 1-10 mM; initial incorporation rates (0-1 h) used. K_m and V_{max} calculated from linear regression of Eadie-Hofstee plots. +B protoplasts supplied with 9.2 µM H_3BO_3.

3.2.2 Maize root membranes

Membrane fractions were isolated from 2 cm root tips of plants grown for 1 d +B after germination and then +B or -B for 15 h. Borate was supplied to the +B membrane preparations at 4.6 µM. Ten µl aliquots of a 1000-15,000 'g' membrane fraction prepared as described in the methods section were incubated with a similar UDPG medium to that used for protoplasts but made up in water instead of PSM. Incorporation was roughly linear over 1 h and then tailed off (fig. 33, p. 152). Holding the preparation on ice for 30 min slowed or stopped incorporation (data not shown). There was little difference between the rate of incorporation by membranes from +B or -B roots, expressed per g f.wt. or per mg protein.

The experiment was repeated with membranes isolated in a different way. The activity of the cell wall/cell debris fraction precipitated at 6 K 'g' was compared with the 6-80 K 'g' membrane pellet. Root tips from plants grown for 3 d after germination were separated into 0-1 and 1-2 cm segments before membrane isolation. UDPG was supplied at 10 µM or 1 mM and B at 4.6 µM where required. With UDPG supplied at 1 mM (fig. 34a, p.153), the activity of the 0-1 cm zone was generally higher than that of the 1-2 cm zone. There was no effect of boron status on the membranes from the 1-2 cm zone but incorporation by 0-1 cm +B membranes was higher than by -B membranes. Qualitatively similar results were obtained at 10 µM UDPG (fig. 35, p.154) with an overall reduction in incorporation.

The 6 K 'g' pellet incorporated UDPG at about 75% of the rate of the 6-80 K 'g' pellet per mg protein (fig. 34 b, p. 153). The protein content of the preparation was high, thus any contamination of membrane preparations obtained at higher g forces with this material could significantly affect their activity.

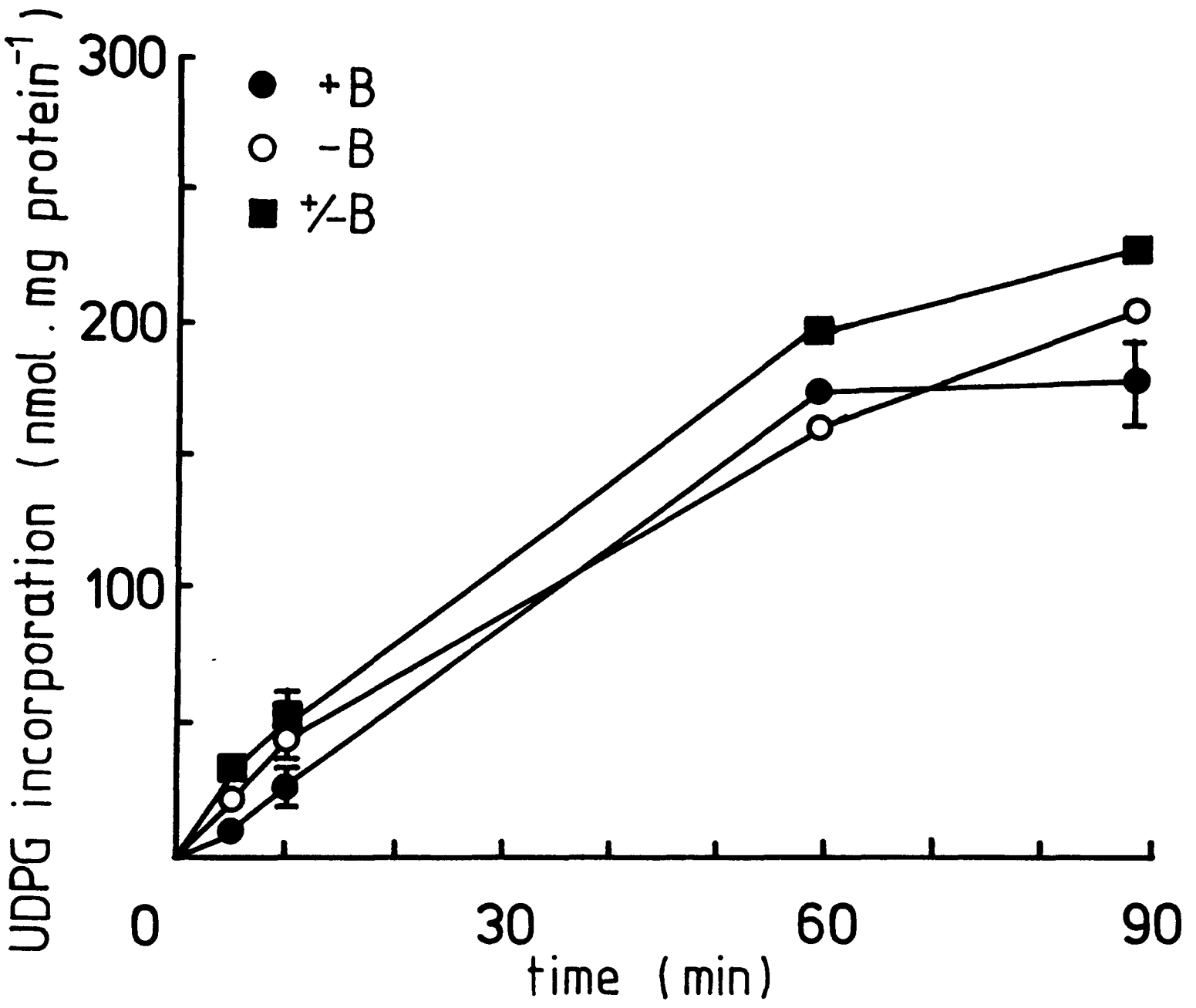


Fig. 33. Effect of B status on UDPG incorporation by a 1-15 K 'g' membrane fraction isolated from 2 cm 'LG11' maize root tips. Plants grown for 1 d +B (4.6 µM H_3BO_3) followed by 15 h +B or -B. +B preparation supplied with 4.6 µM H_3BO_3; '+/-B' preparation isolated from +B plants but washed and suspended -B.

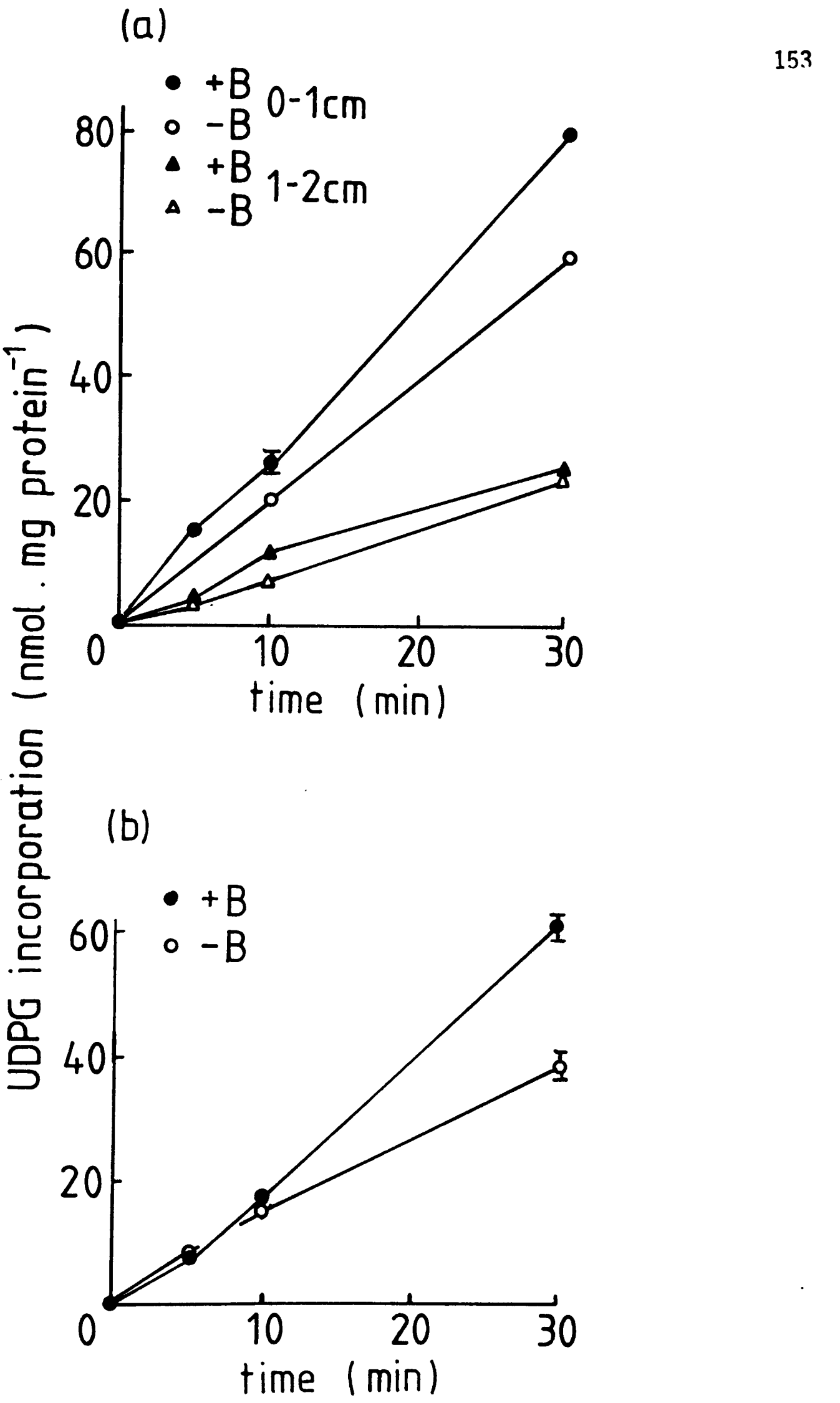


Fig. 34. Effect of B status and root zone on UDPG incorporation by membrane fractions isolated from 2 cm 'LG11' maize root tips. Plants grown for 2 d +B (4.6 μM H_3BO_3) followed by 26 h +B or -B. +B membrane fractions supplied with 4.6 μM H_3BO_3; UDPG supplied at 1 mM. (a) 6-80 K 'g' fraction; (b) 0-6 K 'g' fraction.

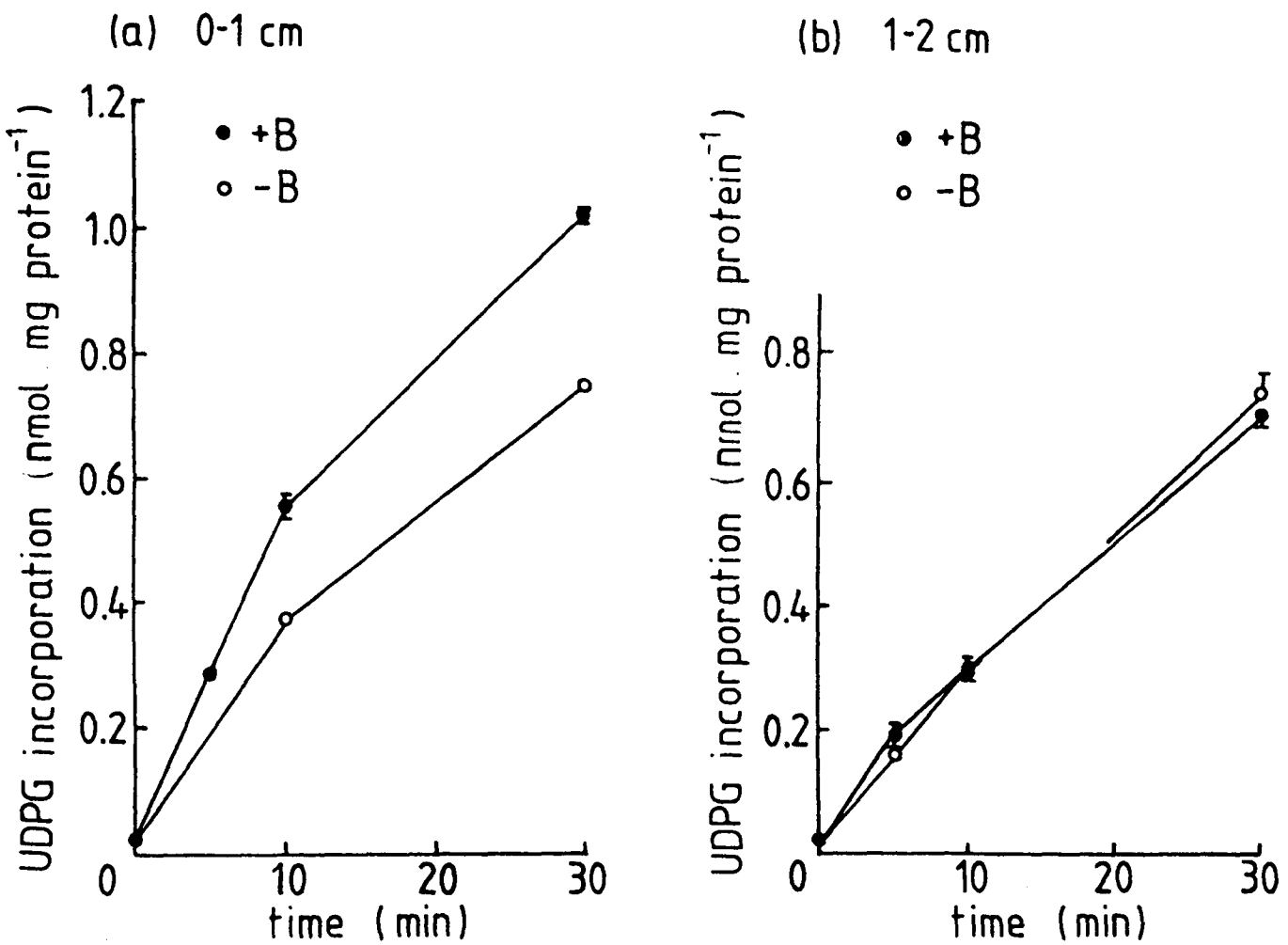


Fig. 35. Effect of B status and root zone on UDPG incorporation by 6-80 K 'g' membrane fraction isolated from 2 cm 'LG11' maize root tips. Details as in fig. 34; except UDPG supplied at 10 µM.

3.3.1 Sunflower protoplasts

An initial experiment suggested that the rate of incorporation by protoplasts isolated from 2 cm root tips of plants (grown for 2.5 d +B after germination) was relatively constant for 7-8 h at around 40 $nmol.h^{-1}.mg\ protein^{-1}$. The rate was similar for protoplasts held for a 'recovery period' of 5 h. B was supplied to the protoplasts at 46 µM.

In later experiments with protoplasts isolated from 2 cm tips of plants grown for 2 d +B after germination, followed by 12 h +B or -B, incorporation by protoplasts from +B roots was greater than by protoplasts from 12 h -B roots at each time of sampling and was roughly linear for 18 h (fig. 36, p. 156). The protein content of each batch was very similar at around 2 $\mu g.(10^4\ protoplasts)^{-1}$; so the same relative positions were maintained when the results were expressed per mg protein. The rates were around 80 $nmol.h^{-1}.mg\ protein^{-1}$ +B and 55 $nmol.h^{-1}.mg\ protein^{-1}$ -B. Incorporation by protoplast preparations macerated in a glass homogeniser was compared with that by intact protoplasts. The rate per sample of suspension decreased but the protein content decreased proportionally, suggesting tissue losses during the homogenisation process. The rates per mg protein proved to be very similar to the intact rate for each sample, (50-80 $nmol.h^{-1}.mg\ protein^{-1}$). Examination of the macerated preparation showed that approximately 10% of the protoplasts had remained intact (data not shown).

Protoplast incorporation from 10 µM UDPG was also tested. Protoplasts were obtained from the 0-1 and 1-2 cm zones of root tips from plants grown for 16 h +B or -B. Incorporation by protoplasts from the 1-2 cm zone was higher than from the 0-1 cm zone, expressed per protoplast (fig. 37, p. 157). Incorporation by protoplasts from the 0-1 cm zone of +B roots was higher than from -B roots, per protoplast or per mg protein. The protein contents of the preparations were similar and the rate of incorporation

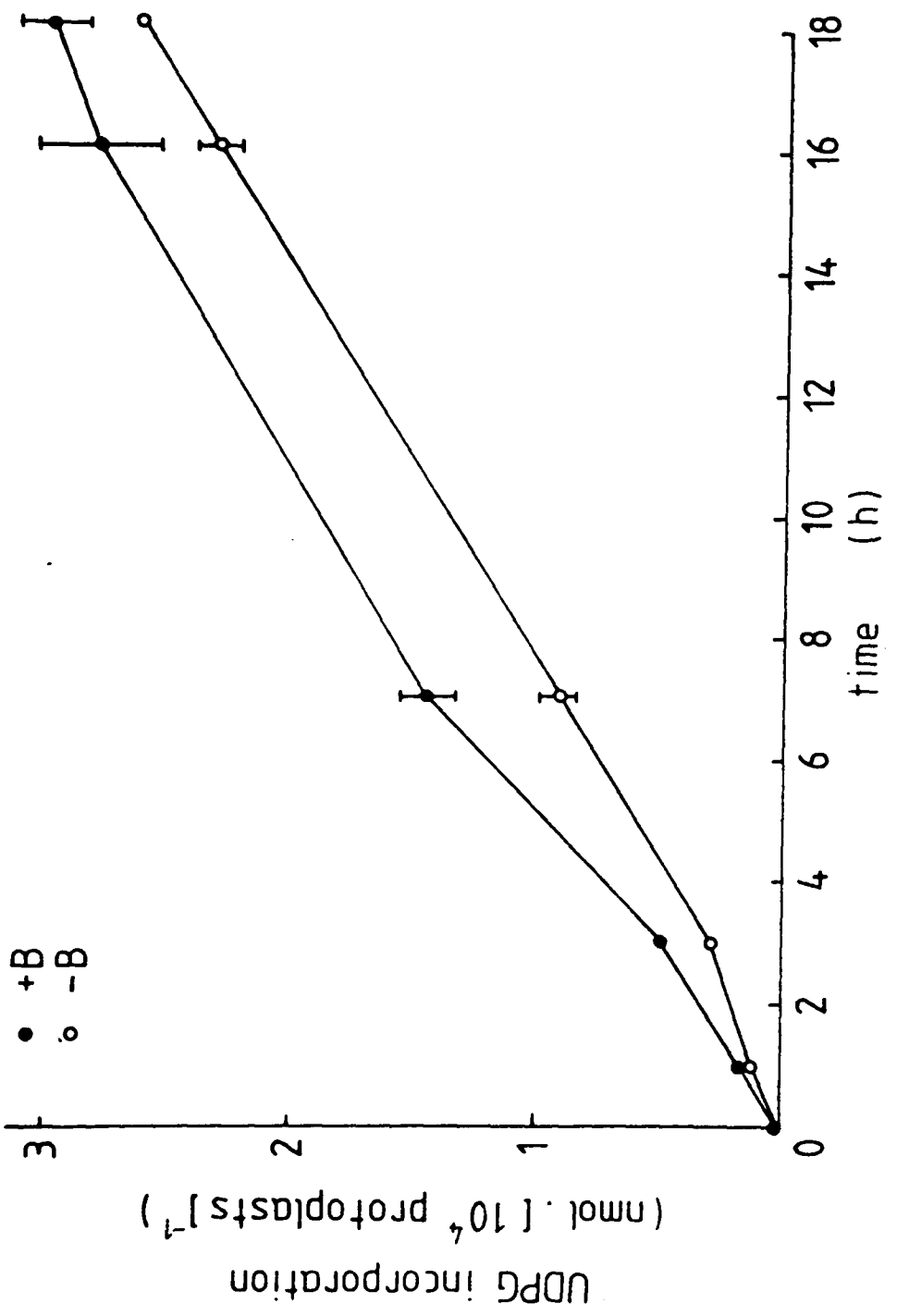


Fig. 36. Effect of B status on UDPG incorporation by protoplasts isolated from 2 cm root tips of 'Single Tall' sunflowers. Plants grown for 2 d +B (4.6 µM H_3BO_3) followed by 12 h +B or -B. +B protoplasts supplied with 9.2 µM H_3BO_3; UDPG supplied at 1 mM.

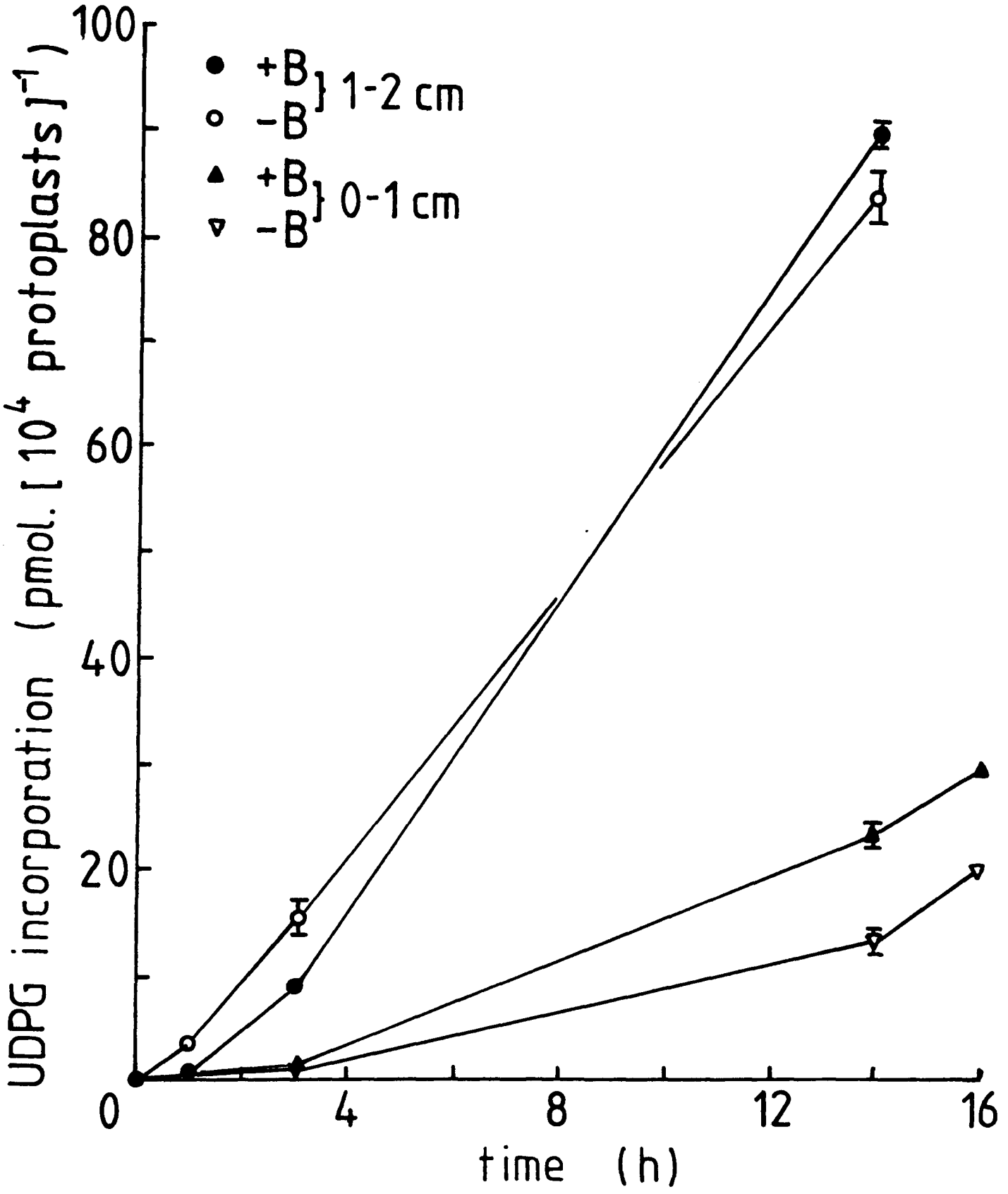


Fig. 37. Effect of B status on UDPG incorporation by protoplasts isolated from root segments of 'Single Tall' sunflowers. Plants grown for 2 d +B (4.6 µM H_3BO_3) followed by 16 h +B or -B. +B protoplasts supplied with 9.2 µM H_3BO_3; UDPG supplied at 10 µM.

was 1.1-1.3 nmol.h^{-1}.mg protein^{-1}. Incorporation by protoplasts from the 1-2 cm zone was not affected by their boron status, although there was some suggestion that the rate of incorporation by +B protoplasts increased with time. The rate of incorporation by protoplasts from the 0-1 cm zone increased after the first 3 h.

3.3.2 Sunflower root membranes

Membranes were isolated from 2 cm tips of plants grown +B or -B for 15 h. Borate was added at 4.6 µM where required. In an experiment where the roots were homogenised without PVP membranes from the 1-15 K 'g' pellet were allowed to incorporate UDPG from a 1 mM solution for 3 h. Results are shown in fig. 38 (p. 159). Incorporation was not directly proportional to the amount of tissue present; doubling the tissue concentration tripled the incorporation rate. In later experiments PVP was included in the homogenisation buffer, and the 1-15 K 'g' pellet was suspended in washing buffer without PVP and resedimented. Results are shown in fig. 39 (p. 160) for roots previously grown for 12 h +B or -B. The difference between +B and -B root membranes was very small and the rate was higher than before at around 250 nmol.h^{-1}.mg protein^{-1}. However the suspensions were quite dilute so only 10% of the added substrate was incorporated in each experiment.

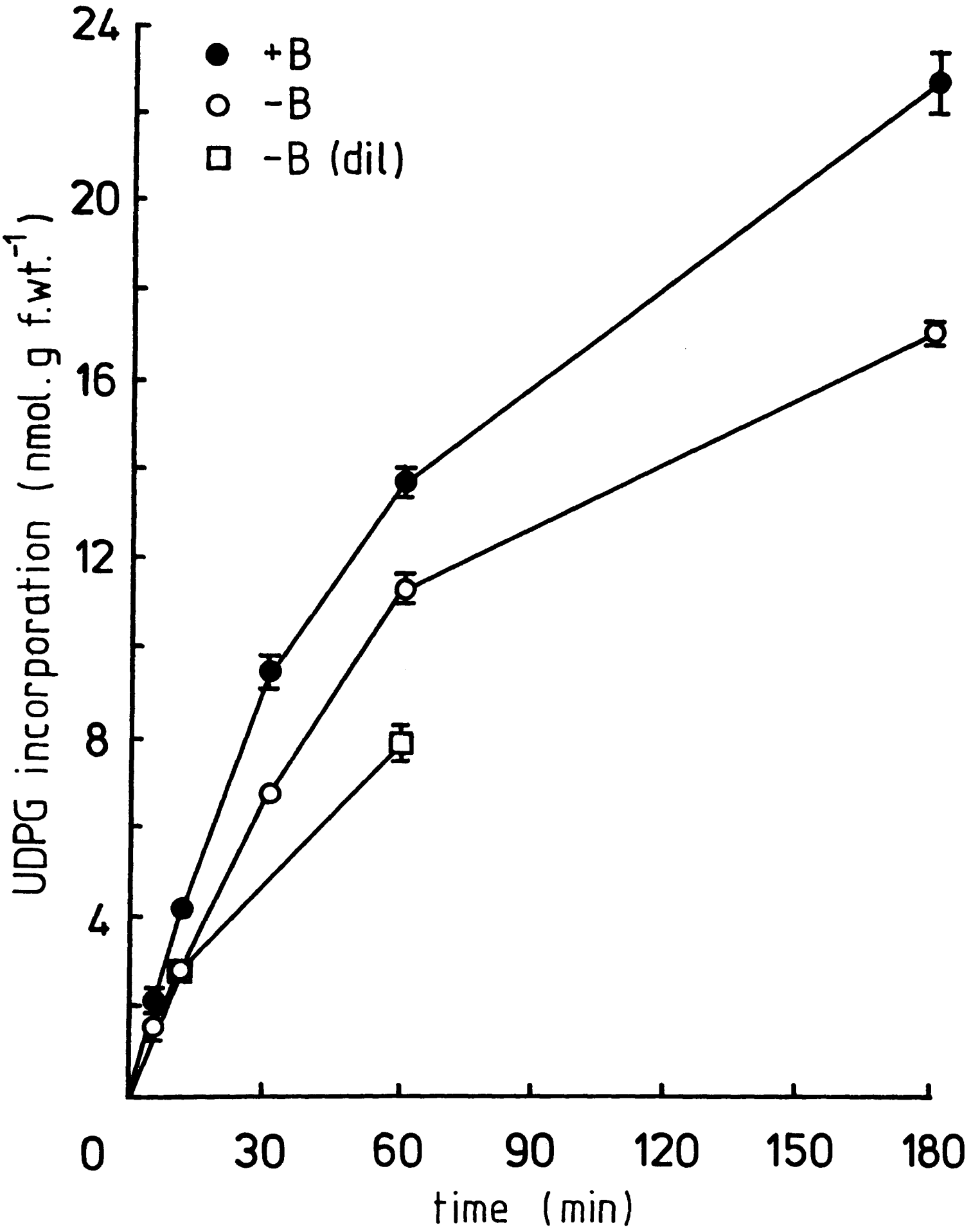


Fig. 38. Effect of B status on UDPG incorporation by a 1-15 K 'g' membrane fraction isolated from 2 cm root tips of 'Single Tall' sunflowers. Plants grown for 2 d +B (4.6 µM H_3BO_3) followed by 15 h +B or -B. +B preparation supplied with 4.6 µM H_3BO_3; UDPG supplied at 1 mM.

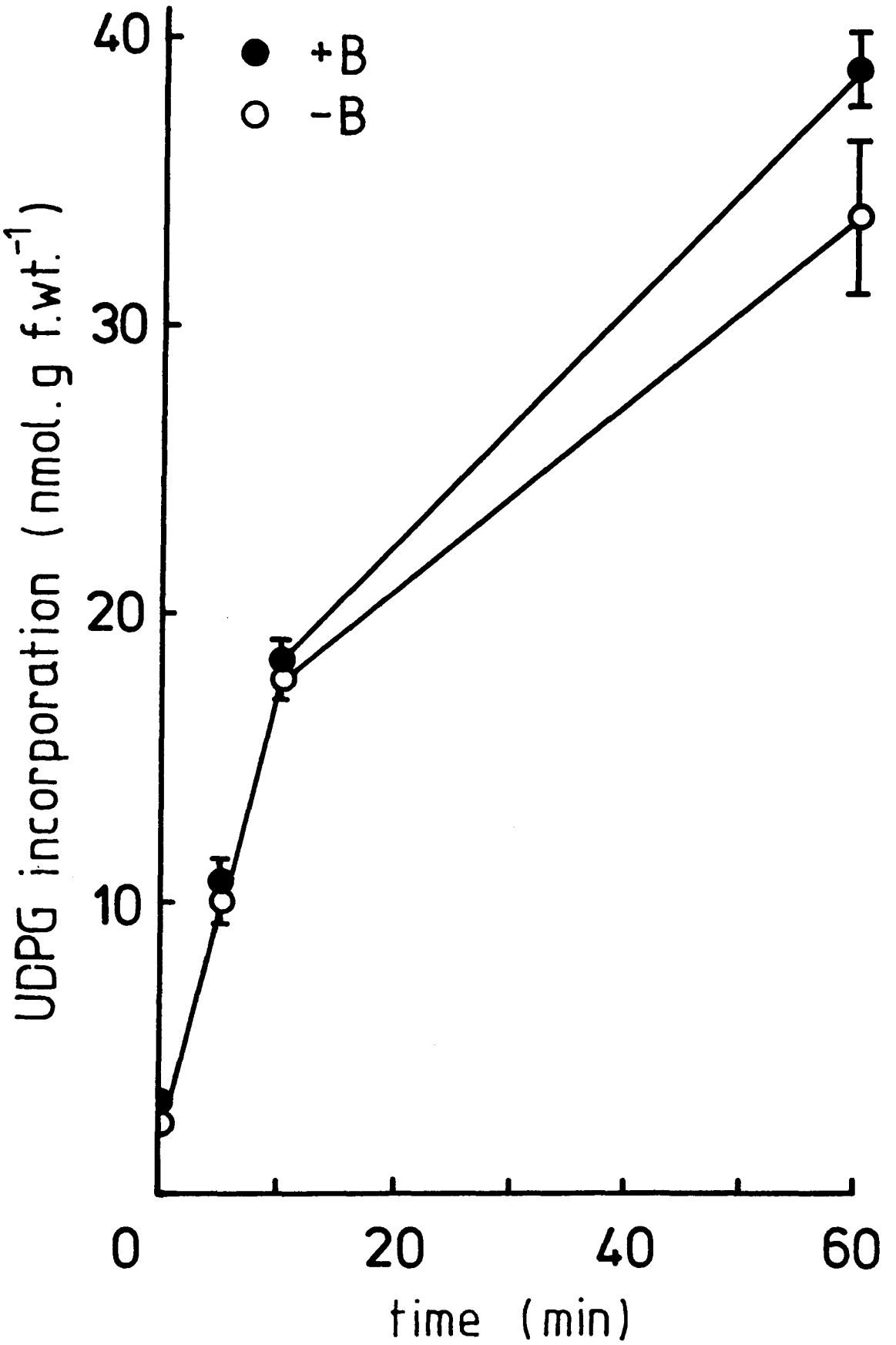


Fig. 39. Effect of B status on UDPG incorporation by a 1-15 K 'g' membrane fraction isolated in the presence of PVP from 2 cm root tips of 'Single Tall' sunflowers. Plants grown for 1 d +B (4.6 µM H_3BO_3) followed by 12 h +B or -B. +B preparation supplied with 4.6 µM H_3BO_3; UDPG supplied at 1 mM.

Discussion

The effects of growth in the absence of boron on cell wall deposition by sunflowers were similar to those reported by Hirsch and Torrey (1980). Several effects such as irregular thickening of the cell walls were seen in this study to be enhanced in -B roots rather than restricted to -B roots. Similarly Parr (1980) reported that new cell walls became markedly serrated; but noted minor serration in +B cell walls too. The formation of new serrated cross walls was seen in all preparations here. Parr also reported a loss of order in the deposition of outer epidermal cell walls and the presence of less electron dense regions in those -B walls, similar to those seen here.

The early promotion of Golgi activity (vesicle release) could arise in a similar way to that proposed by Hertel (1983) for auxin action; he suggests that the release of Ca^{2+} from the vacuole by elevated auxin levels stimulates vesicle release from the Golgi and permits enhanced growth. Since auxin levels may rise shortly after boron withdrawal, the promotion could be a result of increased auxin levels. Alternatively if B is involved in maintaining plasma membrane integrity there is no reason why early deficiency should not increase the tonoplast permeability to Ca^{2+} directly. As deficiency progresses one would expect reduced growth and therefore impaired vesicular formation; but the opposite seems to occur. Either some kind of wound polymer is being made; or else it is possible that the movement of vesicles to the plasmalemma is impaired, resulting in an apparent increase in synthesis.

Extracellular plasmalemmasomes in the outer epidermal cell wall were generally similar in size to Golgi vesicles and may contain cell wall precursors (Robards, 1978); elsewhere a wide variety of membranous objects were seen adjacent to the outside of the plasma membrane. That some of

these were artefactual was inferred from the appearance of the surrounding cytoplasm and the evidence of low magnification views of the tissue; there had been some shrinking of the tissue during root tip fixation, particularly at the hypodermal/epidermal cell wall. The abnormal structures seen in the middle lamella after 5.5 h -B may not be a response to boron deficiency; it is difficult to imagine their deposition occurring in such a short time. Possibly they too are artefacts caused by the influx of fixatives through intercellular spaces.

If the membrane properties were affected by -B growth, it is possible that the disturbances seen in -B preparations may represent an increased susceptibility to such artefacts, rather than in vivo processes. For our purposes their presence is enough to confirm that differences do exist between the preparations.

The results for Calcofluor and Tinopal staining conform to the established suggestion that monocot protoplasts do not synthesise cell walls as readily as dicot protoplasts (Willison and Klein, 1982). Since the stains are known to be inadequate to visualise the earliest stages of wall synthesis (Williamson et al, 1977) it was not anticipated that they would reveal any subtle differences between +B and -B preparations, so only preliminary investigations were made.

The method of SEM analysis used here is not the most sensitive for observing details of fibre deposition but has the advantage of simplicity for the non-specialist and does provide a directly comparable view of the surface of +B and -B protoplasts. The fibres observed on sunflower protoplasts are very similar to those described and depicted in Burgess, Linstead and Harnden (1977). The great thickness of these fibres and their apparent termination at membrane protuberances were shown to be artefactual in that article and in Willison and Grout (1978). It appears that in regions where the fibres project above the surface artefactual

thickening occurs during coating which increases the fibre diameter from the 8-10 nm found by deep-etched freeze etching to the >40 nm seen in these micrographs. Similarly in regions where the mesh appears to merge with the surface artefactual thickening may not have occurred because the mesh is closely appressed to the surface. Where artefactual thickening has not occurred the fibres are too fine to be resolved by this SEM technique. Although the differences between the wall formation capacities of protoplasts from +B and -B roots were n.s. different there appeared to be a tendency for more complete wall formation by -B protoplasts during the time span of the observations. Given the nature of the artefacts described above it is possible that a greater number of surface projections on -B protoplasts could serve to render wall synthesis more readily detectable. However the micrographs do not support the theory of cell wall deposition around protoplasts from -B roots being more 'visible'. There is no gross effect of B status on the appearance of the surface. Both ±B protoplasts may be covered in 'long flexuous membrane projections' (closed residual plasmodesmata ruptured during plasmolysis) (Burgess, Linstead and Fisher, 1977) or with small surface projections, thought to be evaginations of the plasma membrane rather than particles on or in the membrane (Burgess and Linstead, 1977; Burgess et al., 1978).

Literature reports have differed over the duration of any lag before wall synthesis. Burgess et al. (1978) investigated the possible reasons for lags in wall synthesis. They found that, once initiated, a dense mat of fibres could be found around tobacco protoplasts within 2.5 h. However there was a lag of 7-10 h before any deposition occurred; but when the new walls were digested with dilute cellulase, wall synthesis resumed immediately afterwards. They suggested that the lag was caused either by the necessity to assemble the wall-synthesising 'machinery' in protoplasts isolated from cells which were not actively synthesising walls; or by the

trauma of the plasmolysis step in protoplast isolation. Willison and Klein (1982) summarise the literature by suggesting that protoplasts from rapidly growing tissue cultures or meristematic cells begin to regenerate walls immediately after isolation; mesophyll protoplasts typically show a lag of 8-45 h before microfibrils are seen; and grasses or ageing callus cultures are apparently slow to start synthesising walls. It would therefore seem quite feasible to find some walls at the advanced stage seen in plate 27 (p. 139) after only 4 h of culture.

Some of the protoplasts were still naked after 18 h and yet appeared intact in the SEM; the medium used was not ideal for wall synthesis or long-term culture of protoplasts. It is also possible that the larger protoplasts from more mature cells may have been less actively synthesising walls at the time of isolation.

In maize the less thorough SEM investigation also failed to show significant differences between +B and -B protoplasts. Wall removal appeared to be complete as all protoplasts fixed 3 h after isolation had a membranous surface; the more robust-looking protoplasts were found only in preparations fixed after 7-17 h had elapsed.

The absence of convincing intermediate stages of wall synthesis may be directly due to the artefactual thickening of such fibres required before they are visualised. Small surface projections comparable to those on sunflower protoplasts were not seen so it is conceivable that fibres were deposited closely appressed to the surface and wall structures could only be seen when the wall had become sufficiently robust for the protoplast to shrink away from it during the CPD process.

The results of feeding the protoplasts ^{14}C-UDPG provide the most detailed comparisons for the assessment of differences between +B and -B wall synthesis.

The pea protoplast results were used solely to test the protoplast incorporation system, e.g. the optimum protoplast number per vial and the duration and extent of incorporation. The results supported the suggestion that protoplasts were able to utilise externally-supplied UDPG and incorporate it into insoluble products. As was stated in the introduction, this has been found by others also; and one is tempted to reassess Brett's results (1978) as implying that in the tissue culture system he used, vigorous stirring was sufficient to activate wound-induced UDPG incorporation, not just at ruptured plasmodesmata but around the cell surface. Either UDPG is crossing the membrane and being incorporated from the inside or the enzyme can accept UDPG directly from the outside. A third possibility exists, of UDPG being broken down exogenously by e.g. bacterial action; and the ^{14}C- glucose portion then diffusing across the membranes. Should such breakdown occur, the liberated glucose would be readily metabolised and the size of any labelled glucose pool would be small; thus, although free radioactive glucose is not seen in the membrane samples after an incorporation period, the pathway is still a possibility. Our results cannot distinguish between these suggestions; if it is movement across the membrane that is favoured by protoplast isolation, by comparison with the intact cells, the reason may lie in the altered potential difference across the membranes. The observations that the rate of incorporation of 1 mM UDPG was the same for freshly isolated protoplasts and those allowed a recovery period suggests that the UDPG was not just penetrating the protoplasts when they are known to be 'leaky' and being subsequently incorporated.

The ultrasonication treatment was expected to permit direct access of the UDPG to the inside of the membrane and thereby increase incorporation. Instead it appears that the polymer synthesising system functions in the intact protoplasts as well as or better than in membranes isolated from

them; although still at a lower rate than membranes directly isolated from the roots in the presence of a range of protectants.

The observed decrease may however imply that the ultrasonication treatment was too harsh for the enzyme complexes involved in wall polymer synthesis. Assays of the ability to incorporate UDPG by membranes which have been ultrasonicated would be required to see if the ability to incorporate UDPG was itself affected by the ultrasonication treatment.

In order to resolve the question of whether intact protoplasts are actually responsible for incorporation, experiments like those of van der Valk and Wessels (1977) could be tried. They observed the site of precursor incorporation by fungal protoplasts using microautoradiography. If protoplasts were attached to poly-L-lysine coated glass, allowed to incorporate and then fixed, dehydrated and CPD *in situ* it might be possible to compare the areas of incorporation with an SEM view and confirm that intact protoplasts were responsible. This was not attempted in this thesis.

Maize protoplasts incorporated UDPG into insoluble product over an 18 h period, in line with the observed wall synthesis in the SEM. The kinetic analysis suggested that a single system may operate at and above 1 mM UDPG but the results do not support a single system operating below that level. Thus the high-[UDPG] stimulated, plasma membrane bound glucan synthetase activity apparently accepts UDPG more readily than the low-[UDPG] glucan synthetase activity. It is likely that the 'wound-induced' β-1,3 glucan synthesising machinery is stimulated by the isolation process, whereas the mixed β-1,3 and β-1,4 glucan synthesising complex may be inhibited. The activity of the latter may however increase with time (see below).

The apparently higher affinity for UDPG shown by the +B protoplasts and the increased amount of incorporation were not sufficiently marked to conflict with the SEM view of generally similar wall synthesis; and were

consistent with the higher incorporation shown by membranes from the 0-1 cm zone of +B roots. The rate of incorporation per mg protein was around 25 $nmol.h^{-1}.mg\ protein^{-1}$, which is lower than that of the isolated membranes.

The activity of maize root membranes was about 50-200 $nmol.h^{-1}.mg\ protein^{-1}$. Incorporation seemed to be sustained much better in the protoplast preparations but substrate depletion by the membranes in the first experiment amounted to 30% after 1 h which probably caused the decreased rate thereafter. The effect of lowering the temperature suggested that incorporation was active. The difference between the membrane preparations and sonicated protoplasts may be partly a result of degradation of the protoplast preparation when the vacuolar contents are released; in root membrane isolation these contaminants are counteracted by the addition of various protectants.

The specific activity of the membranes from the 6 K 'g' pellet was similar to that of the 6-80 K 'g' pellet. The 6 K 'g' pellet would be expected to contain intact cells, large organelles and membrane fragments attached to cell walls as in the ATPase preparation; density gradient centrifugation of membrane samples would be required to detect zones of enriched activity.

Sunflower protoplasts incorporated UDPG into insoluble product at a slightly higher rate per mg protein than the maize protoplasts. Since more gentle maceration than the sonication tried for maize still resulted in no increase in the incorporation rate, it is possible that no additional sites for incorporation were revealed by the treatment. Similar arguments to those above still apply; the release of the vacuolar contents and damage directly attributable to the homogenisation process may have counteracted any increase caused by exposing internal sites.

protoplasts isolated from the 1-2 cm zone incorporated more UDPG per

protoplast than those from the 0-1 cm zone. This is partly because of their larger size and hence surface area for incorporation. However there may also be an input from the pool sizes within the protoplasts. The root membranes showed higher rates of incorporation by membranes from the 0-1 cm zone; and yet the smaller protoplasts seemed to be restricted in their incorporation. Internal UDPG concentrations would be expected to be higher in the 0-1 cm protoplasts; and so any labelled UDPG crossing the membrane would be diluted by the larger pool size and underrepresented in the product. The increasing incorporation rate with time, when 10 μM UDPG was supplied, could be a result of the synthesis of additional β-glucan synthetases; Langebartels et al. (1981) found protein synthesis inhibitors prevented a similar increase in β-glucan synthetase activity in carrot protoplasts. Such a synthesis would conform to the suggestion that a recovery period is required before the system operating at low UDPG becomes active; whereas the high UDPG, wound-promoted system is already operating maximally after protoplast isolation. Incorporation at 10 μM was approximately 50-fold less than at 1 mM, a similar result to that obtained with maize root membranes and protoplasts.

Sunflower root experiments highlighted the need to protect the enzyme preparation from degradation; the addition of PVP to the homogenisation medium increased the incorporation rate by 3-4 fold. The incorporation rate per mg protein was considerably higher than for sunflower protoplasts or for maize roots and protoplasts.

The effects of boron deficiency on UDPG incorporation are very consistent. -B root preparations incorporated less UDPG into insoluble product than those from +B preparations in the case of maize protoplasts, maize root membranes from the 0-1 cm zone and sunflower protoplasts from the same region. The work of Teare (1974) and Parr and Loughman (1983) suggest that the pool size of UDPG is lower in -B plants; this would be expected to

favour the incorporation of labelled UDPG by protoplasts, but is apparently outweighed by a genuine decrease in incorporation capacity. Without accurate measurements of the pool size and compartmentation, such a discussion is purely speculative; Wainwright, Palmer and Dugger (1980) proposed that internal UDPG concentrations in cotton ovules should rise in the absence of B as cellulose synthesis is impaired and UDPG therefore accumulates.

The crude sunflower membrane preparation also showed lower incorporation in the absence of B; but once the membranes were protected by PVP during isolation this difference was lost. This is the only situation where an increase in the activity of the system as methods were optimised failed to increase the +B/-B differential. Further experiments would be required to test whether the reduction in activity in -B sunflower root tips is caused by interference from phenolics released during the isolation process. These would be removed by the addition of PVP. In maize roots the decreased activity was observed despite the presence of PVP in the isolation medium in each experiment.

The decreased incorporation by so many -B preparations agrees with the results of Dugger and Palmer (1980), working with cotton ovules. However Timashov (1977) found increased ^{14}C-glucose incorporation into wall fractions by -B sunflower root tips. The SEM results suggest that the fibre synthesis seen is not directly related to the UDPG incorporation results. However a relatively small number of protoplasts were examined in the SEM and the relationship between the fibres visualised around the protoplasts and incorporated UDPG is unknown.

General Discussion.

Boron withdrawal induces characteristic changes in the root anatomy of the sunflower and maize cultivars used here. In sunflowers the rapid cessation (cv. 'Fransol') or slowing down ('Single Tall') of root elongation is coupled with radial cell expansion and an increased fresh weight of the root tips. Cells mature closer to the tip, as shown by the increased vacuolation. Energy reserves are consumed (spherosome depletion) and a dark-staining substance accumulates in the cortical cells. Cell wall deposition is disturbed; the activity of the Golgi apparatus is stimulated and plasmalemmasomes become more frequent.

In maize there is no change in the growth rate of the tips over the first 30 h and Parr (1980) reported less marked ultrastructural changes than those reported here for sunflowers. Effects on cell wall deposition were, however, seen within 4 d -B.

In contrast to this apparent dissimilarity of response, the physiological effects of impaired ion uptake and altered β-glucan synthetase activity are seen over a similar time scale in both species. A similar observation by Parr (1980), working with maize and field beans (which behave like sunflowers in this respect) led him to conclude that impaired ion uptake was not a consequence of the impaired growth. It would seem that the reverse should also be emphasised; the altered cell wall deposition and ion uptake need not directly impair growth. Impaired cellulose synthesis can impair growth- Milborrow (1964) noted the similarity between the effects of 2,6-DCB and boron deficiency, and 2,6-DCB was later shown to be a specific cellulose synthesis inhibitor (Hogetsu et al., 1974); yet we have seen here that the β-glucan synthetase activity in maize is apparently as sensitive to -B conditions as in sunflowers, yet the growth rate is unaffected.

Thus the differences between the responses of plant species are still likely to be ascribable to such properties as their pattern of lignification (Lewis, 1980), or the IAA/IAA oxidase ratios, or their phenolic content (Shkol'nik and Maevskaya, 1977). These differences may account for the different sensitivities of the plants to boron deprivation; but the primary role of boron must underlie these secondary responses.

In the first few hours of deficiency, regardless of the plant species involved, any response is caused by the removal of borate from the tissue. Meristematic cells would be expected to react more rapidly; the cell wall is thin and vacuolation minimal, so the loss of B directly disturbs cytoplasmic metabolism or membrane functions. More mature tissue has larger reserves of insoluble or membrane bound B which may continue to be available by slow exchange. Because of its high lipid permeability (Raven, 1980) membrane bound borate would be expected to be lost by diffusion fairly readily unless it is complexed - e.g. in the case of the vacuole, to phenolics.

As the period of deprivation increases, the nature of the plant response becomes more dependent on the effects of altered growth or metabolism. Thus in sunflowers within 3-4 h growth is impaired which makes it difficult to compare physiologically matched tissue in +B and -B plants. This complication does not arise in maize where cell size and root growth are not affected for some considerable time.

Where growth continues in the absence of exogenous B, albeit at a reduced rate, the root tip develops into a system whereby the mature cells have reserves of 'bound-B' in their cell walls and complexed within the cell; at the apex, by contrast, are cells with practically no B and which have not had to adjust to the removal of B. A minor complication is introduced by the presence of the quiescent centre and root cap, both of which were

present when the plant was growing +B; but the contribution of these cells to the B status of the neighbouring cells is probably small.

The work on root tips in this thesis has described in greater detail the pattern of -B effects on root growth, ion uptake and cell wall deposition by sunflower and maize seedlings. The other aim of the work was to devise a protoplast system which could be used to study these effects on isolated, wall-less cells, where B effects on membranes would be unhindered by cell walls. This protoplast system was developed and found to give acceptable yields and quality of preparations, comparable to other root protoplast systems. There was no evidence that growth -B affected the yield or viability of the protoplasts; but the low yield of root cells meant that there can be no guarantee that the preparations came from identical 'sub-populations' of the root. Protoplasts isolated from -B root tips continued to show physiological effects of B deficiency with respect to many different properties. The situations where this was not observed illustrated the nature of the problems involved in protoplast physiology.

One of the first questions which requires an answer is, can borate supplied to protoplasts exert any effect in the presence of 0.5 M mannitol? The equilibrium constant for the reaction:

$$H_3BO_3 + 2\ \text{Mannitol} \rightleftharpoons [\text{Borate-mannitol diester}]^{-1} + H_3O^+ + 2\ H_2O$$

is 10^{-4} (Raven, 1980). However, evidence from apple (Malus sylvestris) leaves which contain sorbitol suggest that the total B content at deficiency, sufficiency and toxicity are relatively low despite the sequestration of borate as sorbitol esters. Raven (1980) concludes from such evidence: 'The available data suggest that complexed B is active in satisfying the growth requirements and the toxic reactions of B'.

The evidence of the protoplast studies is that borate supplied with mannitol is available for the cells. If this were not so, then the strong complexing ability of PSM and the high lipid solubility of B would lead to extensive loss of B from exchangeable mono-esters or internal compartments, and all cells would be boron deficient. One final consideration on this theme is that mannitol has not been shown to cross the plasma membrane of higher plant protoplasts. Either the effective supply of borate is obtained by the cells by a process of exchange; or B is effective at the outside of the plasma membrane.

The differences between the growth response of the tissue to boron deprivation account for many of the apparent uncertainties in the results obtained for protoplasts.

The response of phosphate uptake by 'Single Tall' sunflower roots to a lack of boron proved to be very variable. Longer term deficiency did not consistently lead to a significant decrease in uptake per g f.wt.; and the variable growth response meant that weight (and hence cell volume) increases were not always seen. Phosphate uptake by protoplasts from those roots is therefore bound to be variable. Only around 5% of the cells were isolated as protoplasts so the response depends on the particular population of cells isolated. The inference drawn from the volume measurements was that -B protoplasts were, as expected, larger than +B protoplasts, and their uptake was roughly equivalent per unit surface area. Thus where decreased uptake was seen in one preparation, a genuine decrease in uptake capacity may be inferred. The effect of short term resupply of B to the roots appeared to reduce variability in general, implying some restoration of control.

Potassium uptake by the sunflower roots was rather more consistent. There was a trend towards decreased uptake with 15-24 h deficiency. Marked effects on the activity of a K^+-stimulated, Mg^{2+}-dependent ATPase

imply a direct involvement in active uptake. Protoplasts isolated from this tissue still gave ambiguous results. K^+ uptake by the protoplasts seems to be proportional to the root size of the preparations from which the protoplasts were isolated, rather than to B status per se.

Better comparability of root and protoplast behaviour was expected from maize because of the absence of complicating differences in initial cell size. Should protoplasts fail to respond in the same way as intact tissue there may be more serious grounds for considering the protoplasts to be responding in a different way from the intact roots to boron deprivation. P_i uptake by the roots is impaired over a 24 h period - yet the protoplasts do not show a marked difference between their uptake rates. The protoplasts appear to swell in response to B resupply with ion uptake increasing also; there is no suggestion in these results with protoplasts of the impaired uptake seen for intact root tips.

K^+ uptake is impaired in maize roots; and there is a close relationship with the activity of a membrane bound ATPase (Parr, 1980); White (1983) reported the same in sunflowers. The lower uptake -B appears to be related to the inability of the roots to increase absorptive capacity with age; and protoplasts show the same impaired uptake -B.

Studies on cell wall deposition were unequivocally assisted by the protoplast methods used here. TEM of the root tips suggested that disruptions in the process may be occurring; but the protoplasts provide a range of new insights into the overall problem.

Membrane studies implied that the 0-1 cm zone was more sensitive than the 1-2 cm zone to disturbances in UDPG incorporation. PVP abolished the differences between +B and -B preparations from sunflower roots, but not from maize; again there is a situation where in sunflowers the B effects may be secondary to altered growth patterns or the release of phenolics and yet the same effects are seen in maize to be genuine early responses

to deficiency.

The protoplast results confirm that differences are confined to the 0-1 cm region, where cell wall deposition is apparently most vulnerable to disruption by a shortage of B. The effects occur at both low (10 μM) and high (1 mM) UDPG concentrations with certain notable differences in detail. Incorporation at or above 1 mM is impaired in -B protoplasts and yet SEM studies at 1 mM imply that -B protoplasts synthesise more complete walls. In sunflowers the -B protoplasts should be larger, and yet this is not seen in the SEM. The implication of these two facts is that -B protoplasts which do not regenerate a wall are less able to withstand the fixation process and hence are not as well represented in the SEM preparation.

At 10 μM UDPG it is interesting to note that incorporation by protoplasts increases with time. The suggestion is that the 'wound polymer' synthesising system is already operating maximally shortly after isolation whereas the putative β-1,4-linked glucan synthetase, active at lower UDPG concentrations is synthesised after isolation or becomes more active. Characterisation of the products made is required to test these hypotheses.

In the maize protoplasts assayed at a range of molarities the low affinity of the preparation at low UDPG concentrations may thus be ascribed in part to the early time of assay after isolation, before this system had recovered (or developed). Wound-induced polymer synthesis continued unabated. The poor capacity for maize protoplast wall synthesis suggests that the activity at low external [UDPG] may never reach appreciable levels in these protoplasts.

The possibility of observing early -B effects better in protoplasts than in the roots was not fully realised. The greater variability of the protoplast preparations meant that subtle changes in activities were not

detectable. Steps have been identified which could be taken to make the system more likely to yield insights into early deficiency. These include:

a. The routine use of protein protectants such as BSA in the isolation medium to preserve the surface proteins in the membranes.

b. Measuring ion uptake by protoplasts after a 5 h recovery period and expressing that uptake per unit volume of the preparation (or per unit surface area), so figures are comparable with root uptake per g f.wt. and any effects of B status on protoplast volume are accounted for.

c. Isolating protoplasts from well defined source tissue; high yields and relatively uniform cell size would suggest that cortical tissue 0.5-1.0 cm behind the apex would be a good source, but that would still necessitate the use of large numbers of roots to obtain sufficient material for well replicated experiments.

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