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EFFECTS OF BORON DEFICIENCY ON RNA METABOLISM IN *Phaseolus aureus* ROOT TIPS

by

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A thesis submitted for admission to the degree of
Doctor of Philosophy

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SUMMARY

1. Morphological symptoms and some physiological effects of boron deficiency have been recorded for whole plants of *Phaseolus aureus*. Effects of the deficiency on some aspects of RNA metabolism were examined in the root tip of this plant.

2. Changes in fresh weight, cell number, cell size and protein content resulting from boron deficiency have been followed in 5 mm root tips. These parameters, on which physiological and biochemical determinations are usually based, are affected differently by boron deficiency. This important aspect is discussed, with particular reference to contradictory reports in the literature concerning the biochemical effects of the deficiency.

3. The RNA content of root tips decreases under boron-deficient conditions, but only after at least 100 hr of growth in deficient medium. At the same time, the DNA content of root tips and individual cells is increased by the deficiency.

4. The decrease in RNA content may be due to increased RNase activity which occurs at approximately the same stage of boron deficiency.

5. Boron deficiency results in an increase in both the total and the specific activity of precursor incorporation into RNA after plants are grown for only 6 hr in deficient medium. This was the first effect of deficiency noted, and it occurs

much earlier than visible symptoms (inhibition of root elongation at 24-48 hr being the first). These effects on precursor incorporation become more pronounced the longer the plants are kept in boron-deficient medium.

6. During the first 144 hr, boron deficiency increases the incorporation of precursors into RNA without inducing any corresponding change in the total precursor pool. The incorporation into an acid-soluble fraction is not affected. After more than 144 hr of deficiency, incorporation into both fractions is increased.

7. Deficiencies of either iron, manganese or copper did not result in similar changes in the incorporation of nucleic acid precursors into RNA or total precursor pools.

8. Different fractions of RNA (separated by MAK chromatography and polyacrylamide-gel electrophoresis) are affected at different stages of boron deficiency. Initially (first 48 hr) the order of increased specific activity of precursor incorporation is TB-RNA > r-RNA > s-RNA, but this changes to r-RNA > TB-RNA > s-RNA (100-300 hr) and eventually to r-RNA > s-RNA > TB-RNA (more than 300 hr). RNase degradation of RNA (particularly r-RNA) complicates the separation of these species after long periods of deficiency (more than 200 hr).

9. During the first 48 hr of boron deficiency, no changes in the properties of newly synthesized (labelled) RNA could be

detected by DNA-RNA hybridization or base composition analysis. Changes could be detected at later stages (144 hr or more).

10. The activities of uridine kinase and RNA polymerase, enzymes involved in the pathway of incorporation of nucleosides into RNA, were studied in plant extracts. Some evidence was obtained suggesting that boron deficiency may result in a small increase in RNA polymerase activity, but uridine kinase was not affected after 48 or 91 hr of the deficiency.

11. Similarities between changes in nucleic acid metabolism resulting from boron deficiency and those induced by treating plants with auxins and auxin-type herbicides are discussed.

12. Effects of the accelerated rate of maturation and senescence of boron-deficient root tips on nucleic acid metabolism are also considered.

DECLARATION

The investigations described herein were carried out by myself between March 1969 and July 1972. To the best of my knowledge and belief, no material in this thesis has been previously submitted for a degree in any university, or reported by any other person, except where due reference is made in the text.

KEITH CHAPMAN

A C K N O W L E D G E M E N T S

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ABBREVIATIONS

Abbreviations recommended by 'The Biochemical Journal' (Biochem. J. 126:2) are used in this thesis and only additional or different forms are listed below.

DNase	deoxyribonuclease
RNase	ribonuclease
ATPase	adenosine triphosphatase
TB-RNA	tenaceously bound RNA
UDPG	uridine diphosphate glucose
c-AMP	cyclic 3',5'-adenosine monophosphate
IAA	indoleacetic acid
NAA	naphthaleneacetic acid
GA	gibberellic acid
2,4-D	2,4-dichlorophenoxyacetic acid
Tris	*tris*(hydroxymethyl)aminomethane
SDS	sodium dodecyl sulphate
SSC	standard saline citrate (0.15 M-NaCl, 0.015 M-sodium citrate; pH 7.0)
TCA	trichloroacetic acid
PCA	perchloric acid
MAK	methylated albumin coated Kieselguhr
O.D.	optical density
A.R.	analytical reagent

ma	milliamp
hr	hour(s)
f.w.	fresh weight
p.s.i.	pounds per square inch
-B	boron-deficient nutrient medium (or plants grown in boron-deficient nutrient medium)
+B	complete nutrient medium (or plants grown in complete nutrient medium)

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1. INTRODUCTION

Despite a great deal of work on physiological aspects of boron deficiency, the primary site of action of this element is still not known. In contrast to other trace elements, boron has not been demonstrated to be a component or activator of any enzyme system.

Many hypotheses explaining the role of boron have been presented, but most have been based on differences between normal and grossly deficient plants. Hinde *et al.* (1966) have justly criticised this approach —

> "... in attempting to determine the direct action of boron in plant growth, no reliance can be placed on comparisons between normal tissue and tissue which is at, or past the point of becoming macroscopically deficient. This criticism would apply to the majority of such studies presented in the literature to date."

Recent work has shown that even before the appearance of visible deficiency symptoms, many physiological and biochemical changes occur. Thus, only by determining the sequence of these changes can the comparison approach be used to determine the primary effect(s) of boron deficiency. This has been attempted in only a few studies, and the review of literature will emphasize this type of work. Apart from the initial comments on deficiency symptoms in root tips, this review will be confined to biochemical aspects

of boron deficiency, and only work directly relevant to the experimental section of the thesis will be discussed in detail.

1.1 Morphological and Physiological Symptoms

For reasons which are discussed later (Section 2.1.1), root tips were used as the experimental material in the work reported in this thesis. A brief discussion of the morphological symptoms of deficiency in root tips is therefore relevant.

1.1.1 Inhibition of root elongation

The earliest visible symptoms of boron deficiency have been observed in roots (Odhnoff 1957, Whittington 1959, Sarin & Sadgopal 1965). Complete inhibition of root elongation in pea and tomato plants has been reported within 24 hr of transfer to boron-deficient medium (Neales 1960, Albert & Wilson 1961, Yih & Clark 1965), but variations between species are large.

This rapid effect is probably due to the absence of any redistribution of boron within the plant. Albert & Wilson (1961) used split-plant experiments to demonstrate that boron supplied to one half of the plant does not stimulate root elongation in the other half. Vlamis & Williams (1970) have suggested that boric acid exists largely in the undissociated form, which moves passively in the transpiration stream to accumulate in the leaves. Boron is not fixed in plant tissues, but is soluble and immobile because of the unidirectional flow of water (Oertli & Ahmed 1971).

If the flow is artificially reversed, the movement of boron is also reversed. Under boron-deficient growth conditions, therefore, one would expect the roots to be the first part of the plant deprived of boron, and the leaves the last to be affected. This may explain why the first visible symptoms (and physiological and biochemical effects — Section 1.3.2) of deficiency occur in root tips.

1.1.2 Thickening of roots

Yih & Clark (1965) found that, after 72 hr in boron-deficient medium, the root tips of tomato plants were thicker than those grown in complete medium. The dry weight of the roots was also higher than that of the controls, due to enlargement of parenchyma and cambial cells (Warington 1926, Sarin & Sadgopal 1965). A rapid cessation of cell division occurs in boron-deficient field bean seedling radicles, but this is preceded by an inhibition of cell elongation (Whittington 1957, Odhnoff 1961, Slack & Whittington 1964). Cell division and elongation cease, but radial expansion continues.

1.1.3 Necrosis of root tips

The brown colour of root tips has been reported many times (Whittington 1957, Albert & Wilson 1961, Yih & Clark 1965, Lee & Aronoff 1967), and as early as 24 hr after transfer of plants to boron-deficient medium. Polyphenolic compounds, especially caffeic and chlorogenic acids, accumulate in deficient roots (Perkins & Aronoff 1956, Watanabe *et al.* 1964, Dear & Aronoff 1965,

Dear 1968) and the oxidation of these by polyphenol oxidase produces the brown colour (Shiroya *et al.* 1955, Mache 1962).

1.1.4 Lateral root growth

Within 72 hr of transferring tomato plants to deficient media, local swellings associated with the emergence of lateral roots are observed along the main roots (Whittington 1959, Albert & Wilson 1961, Yih & Clark 1965). These appear at least 5 cm from the tip in normal roots, but within 0.5 cm in deficient roots. Whittington (1957) suggested that this occurs because, although root elongation ceases in boron-deficient plants, differentiation and development continue; thus, even the root tip eventually reaches a stage of maturity, which normally only occurs 7-10 cm from the tip.

1.2 Some General Hypotheses

The ability of borate to complex with adjacent *cis*-hydroxyl groups, and the failure to demonstrate a requirement for boron in animals, have influenced the direction of much work on boron deficiency. The complexing with such substances as sugars and polyphenols (Zittle 1951, Torssell 1956, Neales 1967) has received much attention, and formed the basis of many hypotheses, but little evidence to support these has been obtained (Skok 1957, Dear 1968,

Wildes & Neales 1969). However, whatever the role and site of action of boron, such complexes may well be involved.

Gauch & Dugger (1953) considered that boron promotes sugar translocation by forming ionized sugar-borate complexes which move more readily through membranes than non-ionized sugar molecules. Boron-deficiency symptoms, such as necrosis of apical buds and root tips, were considered to be manifestations of a sugar deficiency at these sites of active growth. Although widely accepted for several years, this hypothesis has often been questioned (Neales 1959, Neales 1960, McIlrath 1960, Albert & Wilson 1961, Yih & Clarke 1965). Carbohydrate metabolism and starch-sugar balances (Dugger *et al.* 1957, Dugger & Humphreys 1960, Scott 1960, Dyar & Webb 1961, Zaleski 1964, Berzina 1965, Lee *et al.* 1966, Dani *et al.* 1970*b*), and citric acid and glyoxylic acid cycles (Lee & Aronoff 1967, Lee 1969, Timashov 1970) have been suggested as sites of action for boron.

The involvement of boron in cell-wall structure or synthesis has also received much attention. Deficiency causes vast changes in cell-wall components of root tips, e.g. lignin, pectin, protein and cellulose (Odhnoff 1957, Whittington 1959, Odhnoff 1961, Slack & Whittington 1964, Dutta & McIlrath 1964). Some of the phenolic compounds which accumulate in deficient tissue (Section 1.1.3) are precursors of lignin (McCalla & Neish 1959). The importance of these observations has been discussed by Neales (1960), Odhnoff (1961) and Slack & Whittington (1964). They agree that the vast changes in cell-wall composition are secondary effects due to the

inhibition of cell elongation and division and the resulting increased maturity of root-tip cells. Slack & Whittington (1964) claim that this may still involve effects on the synthesis of cell-wall components. UDPG (Whittington 1959) and galactans (Wilson 1961) may be involved. Boron may be a component of cell walls, binding microfibrils of pectin units (Odhnoff 1961) or polysaccharides (Slack & Whittington 1964).

Higher rates of respiration (Mache 1963, Shkol'nik *et al.* 1965, Timashov 1968*a* and 1970) and lower levels of ATP (Section 1.3.3) occur in boron-deficient plants. Because of this, Shkol'nik & Maevskaya (1962*b*) suggested that deficiency leads to an uncoupling of oxidative phosphorylation. However, 2,4-dinitrophenol (DNP), which specifically uncouples oxidative phosphorylation, does not produce morphological changes similar to boron deficiency (Shkol'nik *et al.* 1965). At early stages of deficiency the esterification of phosphorus by isolated pea-leaf and barley-root mitochondria is increased by deficiency so that the P:O ratio is not altered until after the appearance of deficiency symptoms (Maevskaya & Alekseeva 1966, Timashov 1968*a*). In contrast to this, mitochondria isolated from sunflower roots have a decreased rate of phosphate esterification and a lower P:O ratio after only 4-6 hr in deficient medium (Timashov 1968*a*). Complete uncoupling of oxidative phosphorylation, and disruption of mitochondrial structure occur when deficiency symptoms appear. Non-phosphorylating oxidation of succinate increases with deficiency, and glycolytic and hexose monophosphate respiration pathways are more active in

5-6 day deficient sunflower leaves (Timashov 1970). After 2-4 days of deficient conditions, a decrease was recorded in the esterification of orthophosphate by chloroplasts isolated from sunflower cotyledons, but not leaves (Timashov 1967*b*). Changes in the sensitivity of boron-deficient sunflower primary leaves and root segments (after 5-6 days in deficient medium) to inhibitors of respiration and phosphorylation have been reported by Timashov (1970).

Decreased levels of ATP can be explained by increased ATPase activity (Section 1.3.4.2), which could also contribute to the lower apparent phosphate esterification values in the above assays. Timashov (1968*a*) also points out that effects on the mitochondrial membrane could be important.

Effects on many oxidative processes and enzymes have been reported (MacVicar & Burris 1948, Nason *et al.* 1952, Odhnoff 1957, Mache 1963, Mathan 1965, Gupta 1966, Mache 1967, Weiser & Blaney 1967, Timashov 1968*a*).

The many effects of boron deficiency have been extensively reviewed (Skok 1958, Hewitt 1963, Nason & McElroy 1963, Shkol'nik 1967, Hewitt 1971).

1.3 Nucleic Acid Metabolism

1.3.1 Nucleic acid levels

Boron deficiency results in a reduction in the amounts of RNA in growing points (shoots and roots) of sunflower (Shkol'nik & Maevskaya 1962*a*, Timashov 1966, Jaweed & Scott 1967, Timashov 1967*a*), tomato (Albert 1965, Johnson & Albert 1967) and beans (Whittington 1959, Berzina 1965). Other plant parts are probably affected to a lesser extent (Shkol'nik & Maevskaya 1962*a*, Berzina 1965). DNA levels either do not change at all, or as much (Whittington 1959, Shkol'nik & Maevskaya 1962*a*, Albert 1965). Except for the work of Albert (1965) and Jaweed & Scott (1967), these results were obtained from grossly deficient plants. Using time-course studies, Albert demonstrated that root elongation ceased before changes in the levels of RNA could be detected. The RNA content began to decrease at the same time as the tips turned brown. This was also the stage at which deficient roots lost their ability to respond to added boron. Jaweed & Scott observed the same sequence of changes in sunflower roots and shoots.

Treatment with boric acid increases the level of RNA in germinating wheat seedlings (Dani *et al.* 1970*a*), and reduces the loss of RNA in senescing tomato-leaf discs (Parmar & Hammond 1971).

1.3.2 Incorporation of labelled precursors into nucleic acids

Two approaches, which give different results, have

been used. Cory *et al.* (1966) and Cory & Finch (1967) grew 2- to 3-day-old *Vicia faba* seedlings in complete or boron-deficient medium for time intervals between 1 and 52 hr, and then transferred the plants to 1 μM-phosphate solution containing 0.25-1.0 mCi of [^{32}Pi] for 50 min. The specific activity of ~~radioactivity~~ the ~~incorporated into~~ RNA increased in 0-2 mm root tips and the section 5-10 mm from the tip after 12-15 hr in boron-deficient medium, and after only 3-4 hr in the section 2-5 mm from the tip. The magnitude of the specific activity increased with the time of growth in the liquid medium, for both normal and deficient plants, but the increase was more rapid in the deficient roots. The difference between the specific activities of RNA from normal and deficient roots was greatest in the section 2-5 mm from the tip. Thus, these workers observed an early increase in incorporation of label into RNA, which became more pronounced with continued growth in deficient medium. The section 2-5 mm from the tip, which was affected first, probably corresponds to the immediate post-meristematic region in which Albert & Wilson (1961) and Reed (1947) observed the first anatomical symptoms of deficiency. It may also correspond to the region of most rapid nutrient uptake.

Preliminary experiments with MAK chromatography (Cory & Finch 1967) indicated that in the second section (2-5 mm from the tip), the specific activities of soluble RNA, DNA and particularly ribosomal RNA were greater in boron-deficient than normal tissue.

These changes in the incorporation of [^{32}Pi] into RNA were not due to changes in the specific activity of the total nucleotide

pool — a fact which also precludes the possibility that the changes were due to a generalized increase in the permeability of deficient radicles to radioisotope.

Shkol'nik & Kositsyn (1962), Sherstnev & Kurilenok (1962), Timashov (1967*a*) and Rapota (1970) grew pea and sunflower plants showing clear signs of boron deficiency (at least 6 days in deficient medium), in a culture solution containing [^{14}C]adenine or [^{32}Pi] for 2-4 days. A deficiency of the micronutrient resulted in a decrease in the specific activity of RNA from apical leaves and roots, and a slight increase in cotyledons and stems. The incorporation of [^{3}H]thymidine into DNA of pea roots was eliminated by deficiency (Rapota 1970). Using a 40 min pulse of [^{32}Pi], Sherstnev (1967) demonstrated the same effect on incorporation into RNA of pea roots. The TB-RNA fraction from MAK chromatography was affected more than ribosomal or soluble RNA, but less than a DNA-bound RNA fraction.

The disagreement between these two sets of results may be due to the different experimental conditions and plant species employed. The Russian workers used grossly deficient plants and studied the incorporation into the total root and leaf systems, while Cory *et al.* (1966) and Cory & Finch (1967) used only root tips after short exposure to deficient conditions. Two explanations are possible to account for these results. Either the increase in the incorporation of radioisotope into the RNA of deficient roots is confined to the root tip and the reverse is true for the remainder of the root system, or stimulation of incorporation

occurs only before visible symptoms appear and at later stages incorporation is inhibited. The first explanation seems most plausible, because the third root segment (5-10 mm from the tip) in the experiments of Cory *et al.* (1966) was affected less than the other segments, and the enhanced incorporation in the first two sections was still increasing after 30 hr, the stage when root elongation was severely inhibited. The results reported in this thesis also support this explanation.

1.3.3 Nucleotides

The only nucleotide which has been extensively studied in connection with boron-deficiency work is ATP. The level of ATP decreases in deficient sunflower plants (Shkol'nik & Maevskaya 1962*b*) but only after deficiency symptoms have appeared (8 days' growth in deficient medium). The apical bud is affected more than cotyledons or stems. This was initially attributed to an uncoupling of oxidative phosphorylation; however, it has since been shown (Maevskaya & Alekseeva 1964) that increased ATPase activity precedes and probably causes the fall in ATP content (see Sections 1.2 and 1.3.4.2). After deficiency symptoms appear, AMP also decreases, but ADP increases in the apical shoots of sunflower (Timashov 1968*a*).

Introduction of RNA (0.2 g/litre) into boron-deficient medium prevents deficiency symptoms in wheat, flax and sunflower (Shkol'nik & Troitskaya 1961, 1962*a* and 1962*b*, Shkol'nik & Maevskaya 1962*a*). At 15-20°, elimination of symptoms is complete,

but at higher temperatures, only a reduction in intensity occurs. Normal seed formation takes place and the RNA content of deficient plants receiving RNA is similar to that of normal plants. Thymidine, guanine and cytosine similarly prevent deficiency symptoms and the loss of RNA in tomato root tips, but uracil, orotic acid and adenine are less effective (Johnson & Albert 1967). As it was assumed that RNA is degraded to nucleotides, nucleosides or bases before entering the plants, both of these observations were interpreted to indicate that boron has some role in the synthesis of nucleotides. Selman *et al.* (1954), however, have pointed out the danger of boron contamination in experiments of this type, and as the specific activity of [^{32}P] incorporated into the total nucleotide pool is not affected by deficiency (Section 1.3.2), the interpretation of these experiments must be treated with caution.

Timashov (1966) suggested that boron affects nucleotides through its interaction with magnesium, which affects a cofactor of nucleotide metabolism; this role was supported by Parmar & Hammond (1971).

1.3.4 Enzymes involved in nucleic acid metabolism

1.3.4.1 RNase

RNase activity increases in the growing tips, young leaves and roots of grossly deficient sunflower and pea plants (Sherstnev & Rasumova 1965, Timashov 1967*a*). Inclusion of

heparin, an RNase inhibitor, in the deficient nutrient medium, temporarily eliminates deficiency symptoms, but does not prevent necrosis of growing points. In young leaves of sunflower, the RNase activity rises only when the plants show incipient boron-deficiency symptoms (Abrol 1966). The activity can be decreased if the plants are returned to complete medium after 4 but not 6 days of deficiency (Abrol 1966), but does not return to the normal level (Shkol'nik *et al.* 1964). The increase in activity is large (3- to 8-fold) and probably causes the reduction in RNA content (Section 1.3.1).

1.3.4.2 ATPase and other phosphatase enzymes

ATPase activity increases in deficient sunflower leaves, terminal buds, cotyledons and roots, and *Vicia faba* root tips (Maevskaya & Alekseeva 1964, Hinde & Finch 1966, Timashov 1968*b*), when assayed at various pH values (5.1, 5.4, 5.7, 7.2, 8.2). Differences can be detected after only 24 hr in deficient medium for *Vicia faba* root tips (Hinde & Finch 1966) and 30 hr in sunflower roots (Timashov 1968*b*). The differences continue to increase if the plants remain in deficient medium, particularly after visible deficiency symptoms appear. AMP, ADP and GTP are similarly degraded more rapidly in deficient sunflower leaves, cotyledons and roots (Timashov 1968*b*). The *Vicia faba* acid enzyme (pH 5.1) also hydrolyzes phenyl-phosphate and pyrophosphate with similar increases in activity, but an alkaline pyrophosphatase enzyme (pH 7.2 and 8.2) decreases in activity

after 24-48 hr of boron deficiency (Hinde & Finch 1966). Phosphatase activity is also enhanced in sunflower leaves and cotyledons after 4 days in deficient medium (Timashov 1967*b*).

Hinde & Finch (1966) observed that the changes in these enzyme activities (ATPase, phosphatase, phenyl-phosphatase, and the acid and alkaline pyrophosphatases) with increasing distance from the root tip were the same as those resulting from boron deficiency — i.e. the enzyme activities in deficient root sections were similar to those of sections further from the tip in normal roots. Because of this, they suggested that these enzyme activity changes are due to

> "... general physiological and morphological changes which are far removed in the chain of causation from the original event that is susceptible to boron deficiency".

These increases in phosphatase activity result in higher levels of inorganic phosphate in deficient plants (Reed 1947, Hinde & Finch 1966, Timashov 1967*b*), and lower organic phosphate levels (Reed 1947).

1.3.5 Base analogue treatment

Introduction of 8-azaguanine into complete nutrient medium inhibits growth of sunflower plants and produces morphological changes similar to boron deficiency (Shkol'nik *et al.* 1965). Treatment with 2-thiouracil, 6-azauracil or barbituric acid results in similar morphological changes in tomato, including

the reduction of root elongation. The RNA content is also reduced (Johnson & Albert 1967, Albert 1968). Guanine and uracil reverse these effects.

Similar induction of senescence by treatment with base analogues has been reported by others (Heyes 1963, Key 1966, Heyes & Vaughan 1967), and Kulkarni & Rege (1971) found that 8-azaguanine induces the production of different types of RNA and RNase.

Shkol'nik *et al.* (1965) considered that with boron deficiency, just as in the presence of base analogues, a synthesis of altered nucleic acids, proteins and enzymes occurs, but Johnson & Albert (1967) favoured a role in the synthesis or utilization of certain nitrogenous bases (Section 1.3.3).

1.4 Protein Metabolism

1.4.1 Protein levels

Boron deficiency decreases the level of protein and increases the amino acid and non-protein nitrogen content of many plant organs and species (Briggs 1943, Sherstnev & Kurilenok 1964, Yih & Clark 1965, Hinde *et al.* 1966, Zabalotny & Miranyenka 1967, Shiralipour *et al.* 1969). Total nitrogen has been reported to increase (Odhnoff 1957, Whittington 1959, Hinde *et al.* 1966, Shiralipour *et al.* 1969) and decrease (Briggs 1943, Sarin &

Sadgopal 1965), but the use of grossly deficient plants and calculations based on different parameters (per cell, dry weight or fresh weight) may well result in these contradictions.

1.4.2 Protein synthesis

When plants are grown in a medium containing labelled amino acids for 20-48 hr, the incorporation into proteins extracted from pea roots, and from roots, young leaves and shoots of sunflower is reduced by boron deficiency (Sherstnev & Kurilenok 1963, Timashov & Volkova 1967, Rapota 1970), but only after the appearance of visible deficiency symptoms (Borshchenko & Sherstnev 1968*a*). Cotyledons, stems, and mitochondrial and ribosomal fractions of deficient sunflower roots, however, incorporate increased amounts of radioactivity (Timashov & Volkova 1967), which the authors attribute to the faster rate of differentiation of these deficient tissues.

In vitro incorporation of $[^{14}C]$tyrosine into proteins by ribosomes isolated from the roots of pea plants showing the first signs of deficiency is reduced (Borshchenko & Sherstnev 1968*b*). The formation of polysomes appears to be interrupted in plants deprived of boron, possibly due to the increased RNase activity (Section 1.3.4.1). Ten-day deficient sunflower roots and shoots have a decreased ribosome content (Timashov 1967*a*).

Although the incorporation of labelled amino acids into protein is reduced, the *in vivo* incorporation into s-RNA (formation

of amino acyl-s-RNA) is increased by boron deficiency, even before deficiency symptoms appear (Borshchenko & Sherstnev 1968*a*). After only 3 days in deficient medium, the specific activity of the amino acyl-s-RNA is doubled. This suggests that boron deficiency interrupts normal protein synthesis, but does not interfere with the formation of amino acyl-s-RNA. The rate of this reaction has also been measured *in vitro*. Using a partially purified enzyme extract from pea roots, Borshchenko & Sherstnev (1968*a*) found no effect of boron deficiency on the incorporation of ^{14}C-labelled amino acids (tyrosine, glycine or threonine) into TCA-insoluble amino acyl-s-RNA, before or after the appearance of deficiency symptoms. Hinde *et al.* (1966) obtained similar results when measuring the amino acid-dependent ATP-pyrophosphate exchange activity of a soluble extract from field bean root tips. When 19 amino acids were added, however, the activity decreased after the plants had been grown for 48 hr, and in some cases 24 hr, in deficient medium. Since the activity also decreased up the root (i.e. away from the tip), Hinde *et al.* (1966) suggested that the decreased activity was due to accelerated maturation in deficient root tips (see discussion in Section 1.3.4.2). These results suggest that the increased incorporation of ^{14}C-labelled amino acids into amino acyl-s-RNA, and its higher specific activity, are due to a slower rate of utilization in protein synthesis, and not to an increased rate of amino acyl-s-RNA synthesis.

1.5 Plant Hormones

1.5.1 Auxins

1.5.1.1 Auxin levels

A relationship between boron and IAA has often been suggested. Early workers (Eaton 1940, Moinat 1943, MacVicar & Tottingham 1947, Torssell 1956, Odhnoff 1957, Dyar & Webb 1961, Shkol'nik & Maevskaya 1962*a*) equated boron deficiency with IAA deficiency, and suggested that boron may be involved in IAA synthesis. Most experimental work, however, has failed to substantiate this idea (see Selman *et al.* 1954).

Brandenburg (1949) and Neales (1960) suggested that boron deficiency may result in the accumulation of high levels of auxin. Slack & Coke (1965), Jaweed & Scott (1967) and Coke & Whittington (1968) have elaborated on this hypothesis. These authors realized that many of the morphological symptoms and physiological and biochemical changes induced by boron deficiency are similar to those associated with high concentrations of IAA and auxin-type herbicides. They have extensively discussed the similarities and possible relationships. The results of Dyar & Webb (1961) in fact conform to this hypothesis — although they argued the converse. NAA treatment of normal bean plants inhibited the translocation of photosynthetically incorporated $^{14}CO_2$, and produced morphological changes (reduced root growth and initiation of lateral root primordia) similar to boron deficiency. If the nutrient medium

contains 10 µM-IAA, the root growth of *Vicia faba* is inhibited to a similar extent to that resulting from a deficiency of boron (Coke & Whittington 1968). If both treatments are applied simultaneously (i.e. addition of IAA and deletion of boron), the inhibition of root growth is even greater.

Auxin levels in deficient and normal tissue have been estimated by Odhnoff (1957), Shkol'nik *et al.* (1964), Jaweed & Scott (1967) and Coke & Whittington (1968). Odhnoff's results were rather variable, but there was some evidence of slightly higher auxin levels in boron-deficient bean shoots. Shkol'nik *et al.* found decreased amounts of free and increased amounts of bound auxins in shoots, hypocotyls and roots of sunflower and maize, but the significance of the separation into bound and free forms is obscure (see Bentley 1962). After 4 days in deficient medium (the earliest time examined and 4 days before the appearance of deficiency symptoms), Jaweed & Scott found that the IAA contents (chemical estimation) of sunflower root and shoot apices were 70-80% higher than those of normal plants. This was maintained until after symptoms appeared, when the levels decreased. Coke & Whittington obtained evidence that extracts from deficient bean root tips contained twice as much IAA as normal root tips after only 24 hr of growth in deficient medium. The difference appeared to be even greater after 42 hr.

1.5.1.2 Auxin degradation

Increases in peroxidase activity in boron-

deficient buckwheat leaves (Mache 1963 and 1967) and bean root tips (Odhnoff 1957), and an "auxin oxidase" in buckwheat leaves (Mache 1967) have been reported. However, one interpretation of the auxin theory is that elevated levels of auxin are due to inhibition of auxin degradation (Slack & Coke 1965). Some evidence for a decreased rate of IAA degradation has been obtained. At the time when visible deficiency symptoms first appear, there is a decreased IAA-oxidase activity in sunflower hypocotyls and growing points (shoots and roots), and the activity almost disappears when growth ceases (Shkol'nik *et al.* 1964). Shoots and lateral roots are most severely affected. Boron-deficient bean roots absorb ^{14}C-labelled IAA more slowly than normal roots, and the rate of decarboxylation is slower in the deficient tissue (Coke & Whittington 1968). *In vitro* studies with horse-radish peroxidase have shown that the presence of boron stimulates the aerobic oxidation of IAA by this enzyme (Parish 1968). Parish suggested that boron may function by facilitating the formation of hydrogen peroxide, which is required for the reaction. This may be related to the observation by Shkol'nik & Steklova (1954) that daily applications of hydrogen peroxide can partly correct deficiency symptoms. Inactivation of IAA-oxidase activity could also result from an accumulation of phenolic compounds (Section 1.1.3, Slack & Coke 1965, Jaweed & Scott 1967, Coke & Whittington 1968, Hewitt 1971) which inhibit IAA-oxidase (Rabin & Klein 1957, Thimann *et al.* 1962, Tomaszewski 1963, Tomaszewski & Thimann 1966, Psenak *et al.* 1970, Runkova *et al.* 1972). This may be because boron binds with

and inactivates phenolic compounds (Slack & Coke 1965, Coke & Whittington 1968).

1.5.1.3 Relationship between boron, auxins and nucleic acid metabolism

Shkol'nik *et al.* (1964) suggested that a study of the relationship of boron to auxin and nucleic acid metabolism is of great interest, but so far little work has been done in this field.

IAA levels are increased by boron deficiency before decreases in RNA content can be detected (Jaweed & Scott 1967). High concentrations of auxin enhance RNase activity (see Section 4.4). The decrease in RNA content of boron-deficient tissue may therefore be indirectly due to a high IAA level. IAA treatment of plants stimulates RNA synthesis (see more detailed discussion in Section 4.4), which may be related to a similar effect of boron deficiency (Section 1.3.2). It is known that 2-thiouracil, which disrupts nucleic acid metabolism and produces morphological changes similar to boron deficiency (Section 1.3.5), also inhibits IAA-oxidase (Gaspar *et al.* 1968). These observations merely suggest lines for further investigation.

1.5.2 Gibberellic acid

Gibberellic acid-induced proliferation of debudded tobacco plants, and elongation of kidney bean and lettuce

seedlings, is reduced in the absence of boron (Skok 1958, Daniels & Struckmeyer 1970). This effect may merely be due to inhibition of growth by boron deficiency. Boron (1 μM) and GA_3 (4.33 μM), together or alone, offset the loss of RNA, DNA, protein and chlorophyll from senescing tomato leaf discs (Parmar & Hammond 1971) and the authors suggest that both boron and GA_3 are involved in nucleotide metabolism.

1.6 Outline of this Investigation

When comparing plants grown in complete medium with those grown in boron-deficient medium, the earliest effects of the deficiency should provide the best information about the primary site(s) of action of the element. The stimulation of [^{32}Pi] incorporation into the RNA of *Vicia faba* root tips (Section 1.3.2) is the earliest effect of boron deficiency yet recorded. Since many other changes in plant growth and development also involve nucleic acid metabolism, further investigations of this aspect of the deficiency were undertaken. Some of the experimental work has been modelled on approaches used to investigate the effects of plant hormones on nucleic acid metabolism. In this way, comparisons could be made between the effects of boron deficiency and those of hormones. This has involved the following lines of work: —

a. Establishing whether an increased incorporation of radioactive precursors into RNA occurs in the root tips of boron-deficient *Phaseolus aureus*, and determining the extent of this increase before and during the onset of boron deficiency. This has included a study of some factors which might affect the uptake and incorporation of precursors into RNA.

b. Studies of the properties of the labelled RNA, aimed at determining whether it is a general increase in synthesis, or whether particular (or new) species are affected more than others.

c. Studies to determine whether changes in nucleic acid metabolism can be related to changes in activity of some of the enzymes involved.

d. Extension of this work to the stage when the plants exhibited gross morphological deficiency symptoms, in an attempt to explain some of the effects of boron deficiency on nucleic acid and protein metabolism reported in such plants.

2. MATERIALS AND METHODS

2.1 Plant Material

2.1.1 Choice of plant material

Phaseolus aureus (mung bean) was chosen as the experimental plant for the following reasons:-

a. It is highly susceptible to boron deficiency.

b. It is a suitable plant to grow in nutrient culture.

c. The radicle and roots are easily harvested.

d. Much biochemical work, particularly relating to nucleic acid metabolism has been done with this plant, in this laboratory (Ong & Jackson 1969, 1970, 1971, 1972*a* and *b*, Jackson & Ong 1972) and elsewhere (e.g. Stockx & Vandendriessche 1961, Barber 1962, Loring *et al.* 1966, Laskowski 1967, Ishii *et al.* 1967).

e. Facilities were already available within this laboratory for the culture of this plant under conditions of boron deficiency.

Root tips were used because this is where the first visible symptoms (root elongation Section 1.1.1) and physiological (cell enlargement and disintegration) and biochemical (increased

incorporation of [^{32}Pi] into RNA, Section 1.3.2) effects of boron deficiency have been recorded. The first anatomical symptoms of deficiency occur in the immediate post-meristematic region of tomato and sunflower root tips (Reed 1947, Albert & Wilson 1961) and the earliest and greatest effect on [^{32}Pi] incorporation into RNA is in the 2-5 mm section of root tips of *Vicia faba* seedlings (Cory *et al.* 1966). The equivalent stages of root development in *Phaseolus aureus* are included in 5 mm root tips. Many previous boron-deficiency studies have used root tips, so that comparisons can be made with the present work.

Feeding radioactive nucleic acid precursors into excised root tips is readily carried out. Incubation solution volumes are smaller (and therefore require less radioactive precursor), and the conditions of incubation (temperature, aeration, shaking rate, etc.) can be more accurately controlled than when using intact plants. Entry of radioactive precursors into the tissue is also enhanced by excision (Ingle & Key 1965). Neales (1964) has shown that excised tomato roots have similar boron requirements to intact roots, and the incorporation of labelled precursors into different RNA species (as separated by MAK chromatography and sucrose-density-gradient centrifugation) is similar in most intact and excised plant tissues (Cherry *et al.* 1965, Ingle & Key 1965, Chen 1971). However, Loening (1965) and Rogers *et al.* (1970) have reported differences in r-RNA metabolism in excised root tips, and these will be discussed further in Section 3.5.2.

2.1.2 Culture of *Phaseolus aureus*

For all culture techniques and nutrient preparation, double glass-distilled water was further distilled in a silica apparatus, passed through a millipore filter (Gelman Instrument Company, Ann Arbor Michigan, U.S.A.; 0.2 μm pore size) and stored in sealed plastic containers. All equipment was either autoclaved or surface-sterilized with ethanol (or concentrated HCl) and exposed to u.v. light for at least 30 min.

Phaseolus aureus seeds purchased from Anderson's Seeds Ltd. (Brisbane, Queensland, Australia) were surface-sterilized with ethanol for 1 min and with 0.2% (w/v) sodium hypochlorite for 1 hr, then rinsed three times with water and soaked in water overnight. The seeds were germinated in the dark on moist blotting paper (previously irradiated with u.v. light) at 30° for 72 hr in covered containers.

The seedlings were grown in 10 litre black plastic containers 40 cm long × 25 cm wide × 15 cm deep. Holes (120), 5 mm in diameter, were drilled through black Perspex sheets which were placed on top of the open containers. The radicles (3-5 cm long) were passed through these holes into the nutrient solution below. The composition of the medium was based on that of Hoagland & Arnon (1950) and is shown in Table 1. Fe-EDTA was prepared by the method of Jacobson (1951). Boron (H_3BO_3) was omitted from the boron-deficient medium. Filtered air was bubbled through four silica tubes in each container. The seedlings were grown in a

TABLE 1 : *Composition of nutrient medium*

NUTRIENT	CONCENTRATION (mg/litre)
KNO_3	255
$Ca(NO_3)_2\ 4H_2O$	590
$MgSO_4\ 7H_2O$	245
KH_2PO_4	68
K_2HPO_4	25
B (as H_3BO_3)	0.25
Mn (as $MnCl_2\ 4H_2O$)	0.25
Zn (as $ZnSO_4\ 7H_2O$)	0.025
Cu (as $CuCl_2\ 2H_2O$)	0.01
Mo (as Na_2MoO_4)	0.05
Fe (as Fe-EDTA)	1.25

phytotron (Ellis & Clark Ltd., Electrical Engineers, Adelaide, South Australia) using a day-length of 16 hr. Day temperature was 25° and night temperature 20°.

The nutrient was changed every 5-6 days. To do this, the Perspex sheets containing the plants were removed and the roots placed on, and covered with, moist, u.v.-irradiated blotting paper. The nutrient containers were rinsed three times, and refilled with nutrient medium. After thoroughly rinsing the roots with water, the plants were returned to the nutrient medium. Normally, batches of plants were grown in pairs, and at the first nutrient change (5 days after planting) one container was refilled with boron-deficient medium.

2.1.3 Root-elongation measurements

Root-tip elongation was determined by marking the main roots 10 mm from the tip with a mixture of finely ground charcoal in lanoline (Hydrous Wool Fat, Drug Houses of Australia, Adelaide, South Australia) and measuring the subsequent increase in the distance from the tip to the mark. Measurements to the nearest mm were made on 15-25 plants.

2.1.4 Harvesting of root tips

Root tips (5 mm long) were excised from the main and lateral roots with a sterile scalpel, and stored in a chilled beaker in ice until the harvest was completed. Small samples (200-500 root tips) required 30-60 min and large samples (1000-2000 root tips) 2-3 hr to complete harvesting.

2.1.5 Incorporation of labelled precursors into excised root tips

Excised root tips were incubated at 30° for 4 hr in plastic test-tubes (1.35 × 10 cm) in 1-2 ml of nutrient medium (Table 1 — with or without boron) containing the radioactive precursor, 50 units/ml of Penicillin G and 2% (w/v) sucrose. The nutrient medium was diluted 4-fold with water for use in [^{32}Pi] incorporation experiments to reduce the level of unlabelled phosphate. The levels of radioactive precursors used were —

a. [2-^{14}C]uridine, 1 μCi/ml, 40 mCi/mmol;

b. [^{3}H]uridine (generally labelled), 20 μCi/ml, 500 mCi/mmol;

c. [^{32}Pi], 500 μCi/ml, 3.0 mCi/μmol.

After incubation, the tips were washed six times with a jet of distilled water before homogenization, which, unless otherwise indicated, was by 20 plunges in a glass-glass homogenizer (10 ml).

2.2 Nucleic Acid Extraction and Determination

2.2.1 Extraction

2.2.1.1 Total nucleic acids

Nucleic acids were extracted and separated into acid-soluble, RNA and DNA fractions by the Schmidt & Thannhauser (1945) method as modified by Bonner & Zeevaart (1962). In some experiments, the acid-soluble fraction was extracted with 0.2 M-PCA instead of 5% (w/v) TCA, and in others the initial extraction in 80% (v/v) ethanol at 100° was omitted.

2.2.1.2 Nucleotides

Root tips were homogenized in 10% (w/v) TCA [1 ml/250 mg (f.w.)] and the mixture shaken by hand at 2° for 15 min. After centrifugation for 20 min at 2° and 800*g*, the pellet was re-extracted with 5% (w/v) TCA and the combined

supernatants were washed ten times with ether to remove TCA. The washed supernatant was used for chromatography on DEAE-Sephadex or Dowex-1.

The acid-insoluble pellet remaining was hydrolyzed in 0.3 M-KOH for 20 hr at 37°, then neutralized with 0.6 M-PCA. After standing at 2° for 15 min, followed by centrifugation at 400*g* for 5 min, the supernatant fraction was used for chromatography on Dowex-1.

2.2.1.3 RNA

Excised root tips (100-400 mg) were homogenized at 2° in 2 ml each of buffer [10 mM-phosphate, 50 mM-KCl, 10 mM-$MgCl_2$, 1.5% (w/v) SDS, 0.2% (w/v) bentonite; pH 7.6] and 90% (v/v) phenol [containing 0.1% (w/v) 8-hydroxyquinoline]. The mixture was shaken by hand at 2° for 20 min and centrifuged for 20 min at 2° and 1000*g*. The aqueous layer was re-extracted with phenol and bentonite, and the RNA precipitated from the final aqueous phase with 2.5 volumes of ethanol at -15° overnight. This precipitate was washed with 70% (v/v) ethanol, 95% (v/v) ethanol, and twice with ether, then dried *in vacuo*. With larger amounts of plant material, 1 ml each of buffer and phenol was used for each 200 mg of plant material.

RNA was extracted from cotyledons and young shoots by the same method, except that 1 ml each of phenol and buffer for each gram of tissue was used, and the phenol extraction was repeated

three times.

For DNA-RNA hybridization studies the RNA was further purified by dissolving the precipitate in 10 mM-Tris buffer (containing 10 mM-$MgCl_2$; pH 7.4) and incubating with 25 μg/ml DNase (RNase free) for 30 min at 37°. After deproteinization with phenol, as above, the aqueous phase was washed with ether and dialyzed for 24 hr against three changes of 2×SSC (SSC = 0.15 M-NaCl, 0.015 M-sodium citrate; pH 7.0).

2.2.1.4 DNA

DNA was prepared from 72 hr germinated seedling radicles, grown as described in Section 2.1.2, by a method based on that of Kado & Yin (1971). The radicles were homogenized for 2 min at 65° at full speed in a Sorvall Omnimixer with an equal volume of buffer (0.05 M-sodium borate, 0.01 M-EDTA; pH 9.0). Solid SDS and then NaCl were slowly added to final concentrations of 5% (w/v) and 15% (w/v) respectively, and the mixture stirred gently for 15 min at 65° after each addition. After cooling to room temperature, an equal volume of a mixture of chloroform-isoamyl alcohol (24:1, v/v) was added and swirled gently for 15 min at room temperature. After centrifuging for 10 min at 10,000*g*, the aqueous phase was removed and re-extracted with chloroform-isoamyl alcohol, and then precipitated overnight at -15° in 2.5 volumes of ethanol. The precipitate was dissolved in 0.1×SSC and reprecipitated with ethanol. After re-dissolving in 0.1×SSC, the precipitate was incubated at 37° for 60 min with

0.1 mg/ml RNase (previously incubated at 90° for 5 min), followed by 60 min with 0.4 mg/ml diastase, and 60 min with 0.8 mg/ml pronase (self-digested for 30 min at 37°). The pronase digestion was continued overnight at room temperature. The solution was deproteinized twice with an equal volume of chloroform-isoamyl alcohol and dialyzed for 24 hr against three changes of 0.1×SSC. After adjusting to 0.3 M-KOH and incubating for 5 hr at 37°, the solution was neutralized with 1 N-HCl and the DNA pelleted by centrifuging overnight at 40,000*g* in a Beckman Model L ultracentrifuge (Titanium-50 head). The DNA pellet was dissolved in 0.1×SSC and stored at -15°.

DNA prepared by this method has a maximum absorption at 258 nm (Figure 1), which is similar to that reported for DNA prepared from embryonic axes of mung bean by Chen (1971).

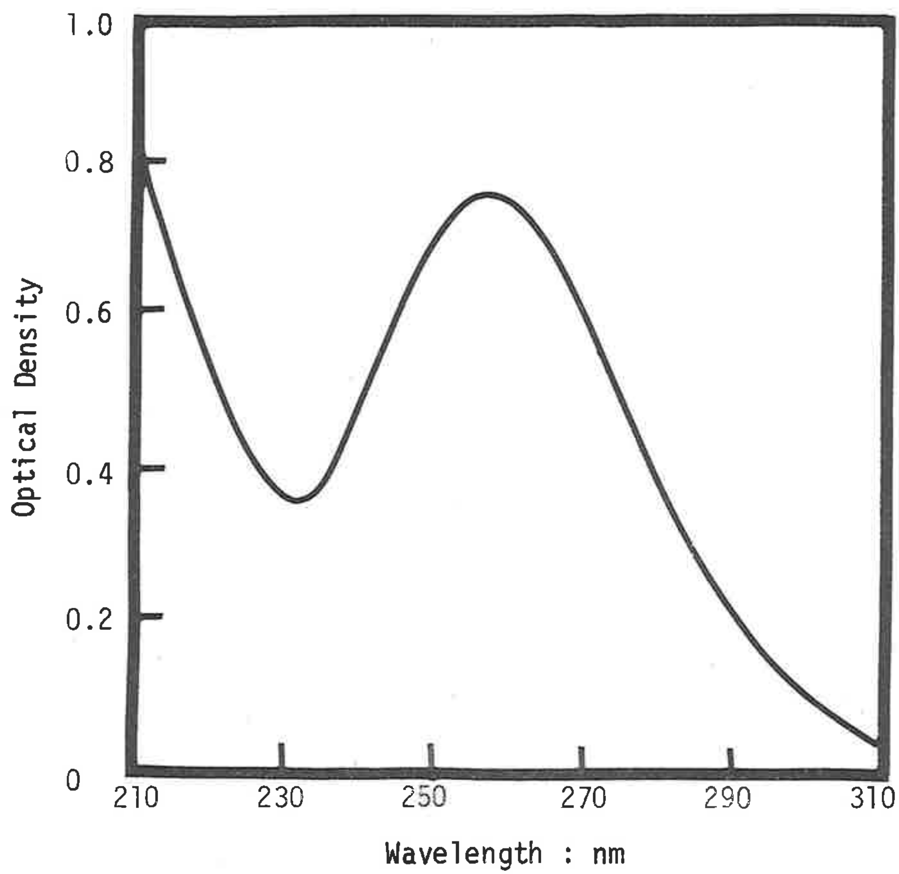
2.2.2 Determinations

2.2.2.1 Absorption spectra

The optical extinction of a neutralized aliquot was measured at 260 nm and the RNA or DNA content calculated using extinction coefficients of 6.7, 9.5 and 12.0 for double-stranded, single-stranded and hydrolyzed samples respectively.

2.2.2.2 Ribose

RNA-ribose was determined by the method of Mejbaum (Schneider 1957), using cupric chloride as the catalyst



<u>FIGURE 1</u> : *Absorption spectrum of DNA.* DNA was prepared by the method described in Section 2.2.1.4 and the spectrum was determined using a Gilford Model 2400 recording spectrophotometer.

(Lin & Schjeide 1969) and AMP as the reference standard. Pyrimidines react very poorly in this assay (Lin & Schjeide 1969). RNA contents determined by ribose assays of samples of hydrolyzed pure RNA (after MAK chromatography) were only 55-60% of the values calculated from the absorption at 260 nm. This is similar to the results of Lin & Schjeide (1969), and all values have therefore been corrected for a 60% estimation of RNA content by ribose determination.

2.2.2.3 Deoxyribose

DNA was determined as deoxyribose in an aliquot of the acid DNA fraction before neutralization (see Bonner & Zeevaart 1962) with the Dische diphenylamine reagent, using the procedure adapted by Burton (1956). The difference between optical extinction at 595 and 650 nm was measured and these values were compared with those of a d-AMP standard. Pyrimidines also fail to react significantly in this assay (Burton 1956) and values were corrected as for the ribose assay.

2.2.2.4 Phosphate

An aliquot (0.1-0.2 ml) of the RNA fraction was digested by adding a drop of 10% (w/v) $Mg(NO_3)_2$ in ethanol and heating in a test-tube over a strong flame until all the brown fumes had been expelled. When cool, 0.9 ml of HCl was added and the solution was heated in a boiling water bath for 15 min. After cooling, the solution was analyzed for phosphate by the method of

Chen *et al.* (1956). The optical extinction at 820 nm was measured and reference standards of AMP were developed at the same time.

2.3 Analysis of Nucleic Acids

2.3.1 RNA

2.3.1.1 MAK chromatography

Methylated albumin and column preparation were by the method of Mandell & Hershey (1960), as modified by Monier *et al.* (1962), but using celite (H. Selby & Co., Adelaide) instead of Kieselguhr. Columns (0.65 × 10.0 cm) were equilibrated with 0.1 M-NaCl in 0.05 M-phosphate buffer (pH 6.7) and nucleic acid samples (0.1 to 0.5 mg) were dissolved in the same buffer and applied to the column. The column was washed with buffer until no radioactive or u.v.-absorbing material could be detected and then eluted with a linear gradient of NaCl (0.2 to 1.1 M) in 0.05 M-phosphate buffer (pH 6.7). The RNA tenaciously bound to the column (TB-RNA) was eluted with 0.5% (w/v) SDS. Gradients of 60-100 ml were used, and fractions (1.5 ml) collected. The absorbance at 260 nm was measured for each fraction and the radioactivity measured by drying aliquots onto glass-paper squares (2 × 2 cm) and counting as described in Section 2.5.1.1 or, in the case of [^{32}P], by Cerenkov radiation in 10 ml of 0.1 M-phosphate

as described in Section 2.5.1.3.

The u.v. absorption of protein and other material removed from the column by SDS prevents the estimation of nucleic acids by measuring the optical extinction at 260 nm. A modified ribose assay (Section 2.2.2.2) was used. When the sample and orcinol reagent were mixed, the black precipitate formed was removed by centrifuging at 400*g* for 10 min and the clear supernatant fraction boiled for 60 min. After centrifuging again, the absorption of the clear fraction was measured at 660 nm. At low values, the RNA content is underestimated by this method, as shown in Figure 2. However, if the aliquot used in the assay contains more than 20 nmol of RNA, the resulting error in the determination is small and, by referring to Figure 2, can be easily corrected for.

2.3.1.2 Polyacrylamide-gel electrophoresis

Polyacrylamide gels cross-linked with bis-acrylamide were prepared as described by Loening (1967). The buffer used contained 36 mM-Tris, 30 mM-NaH_2PO_4 and 1 mM-EDTA (disodium salt), pH 7.8. The developing buffer also contained 0.2% (w/v) SDS. Electrophoresis was at room temperature in 2.4% (w/v) gels in 0.6 × 9.0 cm glass tubes. If the tubes were cleaned in alcoholic KOH and wetted with 0.2% (w/v) SDS before casting, the gels could be easily removed from the tube by injecting a small volume of water with a syringe between the gel and the glass surface. The gels were pre-run at 5 ma/gel for 1 hr before application of the sample, at 2 ma/gel for 10 min after loading

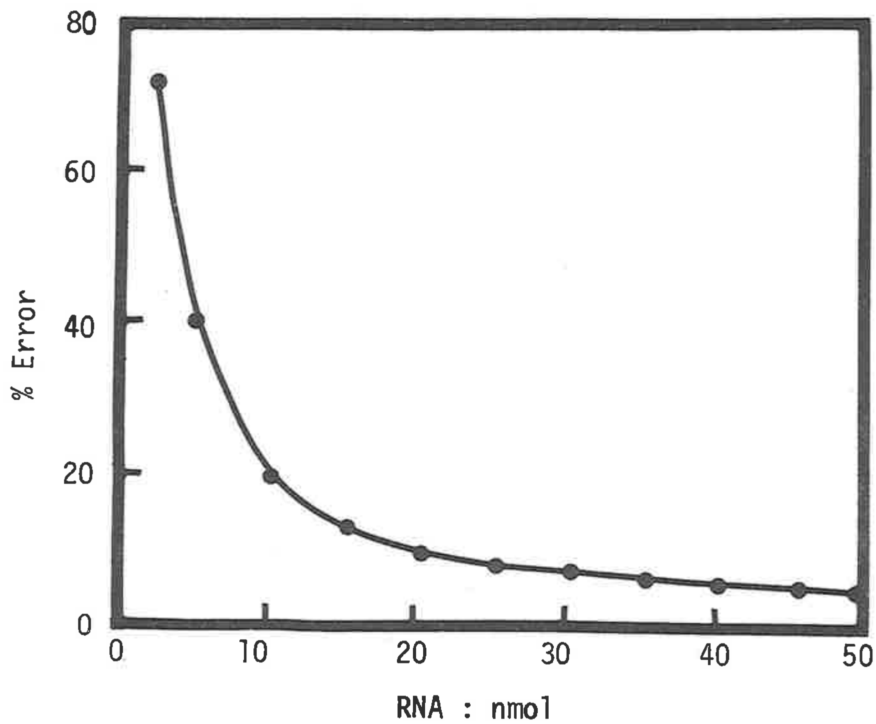


FIGURE 2 : *Error in ribose assay for RNA content of TB-RNA fraction.* Identical samples of TB-RNA containing various known amounts of r-RNA were assayed for ribose as described in Section 2.2.2.2 and the error in the determination of added r-RNA calculated.

the sample, and then at 5 ma/gel and 15 volts/cm for the times indicated in the results (Section 3.5.2). Bromophenol blue was run as a marker. Samples (less than 100 µl) dissolved in developing buffer containing 5% (w/v) sucrose were layered over the gels.

After separation of the sample, the gels were removed from the tubes and soaked in 0.1 M-phosphate buffer (pH 7.0) for 30 min, then transferred to a quartz cell (0.6 × 12.0 × 1.0 cm) and scanned in a Joyce-Loebl Chromoscan, fitted with a 265 nm interference filter. The gels were then frozen at -15° and cut into 1.5 mm slices. The slicing apparatus consisted of 70 razor blades mounted side by side with equal spacing. The frozen gels were laid onto several sheets of parafilm on a Perspex sheet and the razor blades pressed firmly down through the gel. The slices remained between the blades and could be easily removed with a spatula. These slices were dried on filter-paper squares (1.3 × 1.3 cm) and counted in toluene-based scintillation fluid as described in Section 2.5.1.1.

2.3.1.3 Base composition

Yeast RNA (1 mg/ml) was added to fractions from MAK chromatography and the RNA precipitated with 2.5 volumes of ethanol. After washing with ether and drying *in vacuo*, the precipitate was dissolved in water and reprecipitated with a final concentration of 0.2 M-PCA and this precipitate hydrolyzed in 0.3 M-KOH at 37° for 20 hr. The solution was neutralized with

1.2 M-PCA and, after centrifuging at 400*g* for 10 min, the supernatant was evaporated to dryness under a stream of cool, filtered air. The residue was then dissolved in 0.1 ml water.

Paper electrophoresis was used to separate the four monophosphates. Samples (up to 40 μl) or authentic markers (0.1 μmol of each) were spotted onto the origin points of Whatman 3 MM paper. The papers were wet with buffer (0.1 M-citrate, pH 3.6) and excess was removed with absorbent paper. Electrophoresis was carried out for 120 min at 26.5 volts/cm, with the paper immersed in a tank of carbon tetrachloride as coolant. Nucleotides moved towards the anode and were located under short-wave u.v. radiation and a contact photographic print produced with the same radiation source. The electrophoretograms were then cut into 5 mm sections and radioassayed with a Beckman Lowbeta II gas-flow planchet counter (Section 2.5.2) to determine the ratio of counts incorporated into the four nucleoside monophosphates.

2.3.1.4 DNA-RNA hybridization

The technique of Gillespie & Spiegelman (1965) was used. DNA samples (Section 2.2.1.4) were diluted to 50 μg/ml in 0.1×SSC and denatured by adjusting to pH 12.5 with 0.1 M-KOH and stirring gently for 15 min. The solution was then neutralized with 0.1 M-HCl and diluted to 5 μg/ml with 2×SSC.

Filters (Sartorius Membrane Filters, Göttingen, West Germany; MF 50, 50 mm diameter) were pre-soaked for at least 1 hr

in 2×SSC and washed with 100 ml of the same buffer. The denatured DNA solution was passed slowly through the filters, which were then washed with 50 ml of 2×SSC. After drying overnight, the filters were further dried at 80° *in vacuo* for 4 hr. Blank filters were treated similarly except that 2×SSC was used instead of DNA solution. Discs, 5 mm in diameter, were cut from the filters with a sterilized cork borer, and some checked for uniform DNA distribution by deoxyribose assay (Section 2.2.2.3). Before use, the filters were pre-incubated for 1 hr in 2×SSC at 70°. Any DNA not tightly bound to the filter was lost during this pre-incubation and further losses were very small (see Figure 33 in Section 3.6.2).

Hybrids were formed in tightly stoppered 0.8 × 7.0 cm test-tubes by immersing three DNA filter discs (0.4-1.5 μg/disc) and three blank filter discs in 0.2 ml of 2×SSC containing the amounts of RNA indicated in the results (Section 3.6.2). The tubes and solutions were pre-heated to 70° before addition of the filter discs. Incubation was for 6-8 hr at 70°. The RNA solution was then removed and the filter discs further incubated in 2×SSC for 5 min to minimize non-specific hybridization (Bishop 1970, Benedich & McCarthy 1970). The discs were washed twice in 2×SSC (standing in ice 10 min each wash), then incubated for 1 hr at 30° in 1 ml of 2×SSC containing 50 μg of RNase (pre-incubated 10 min at 90°). After washing three times with 2×SSC, the filters were dried and radioassayed as described for samples on paper in Section 2.5.1.1. The blank values were deducted from the experimental values to give the amount of hybrid formed. Under

these conditions, a high degree of specificity is achieved (Church & McCarthy 1968, Thompson & Cleland 1971*b*). Several additional DNA filters from the same batch were carried through the entire procedure and then assayed for DNA content either by the deoxyribose assay or by incubating in 1 ml 1 M-HCl at 100° for 15 min and measuring the absorption at 260 nm (Section 2.2.2.1).

Competition was achieved using the simultaneous incubation technique. Filter discs were incubated with constant amounts of labelled RNA (approaching the previously determined saturation level) and increasing amounts of unlabelled, competing RNA as indicated in the results (Section 3.6.2).

2.3.2 Nucleotides

2.3.2.1 DEAE-Sephadex

The nucleotides in the acid-soluble fraction (Section 2.2.1.2) were separated by chromatography on DEAE-Sephadex.

DEAE-Sephadex (A-25) was hydrated overnight in water and then washed by decantation, five times with 0.5 M-NaOH, four times with water, five times with 0.5 M-sodium bicarbonate and five times with water (Caldwell 1969). A slurry of the ion-exchanger was poured into columns 0.9 × 20 cm and washed with at least 200 ml water. The acid-soluble fraction (diluted 5-fold with cold water to ensure a low salt concentration) was then loaded onto the column at a flow rate of 40-50 ml/hr. Unlabelled marker

nucleotides (0.25 μmol each) were loaded with the sample. When loading was completed, the column was washed with water until no u.v.-absorbing material was present in the effluent.

The nucleotides were eluted with a linear gradient of triethylammonium bicarbonate in which the mixing vessel initially contained 400 ml of water and the reservoir 400 ml of the buffer (1.0 M, pH 8.0). The flow rate was 40-50 ml/hr. The effluent was monitored automatically at 254 nm with an LKB Uvicord II ultraviolet absorptiometer, and fractions of 4 ml were collected and radioassayed. For [^{14}C] and [^{3}H], aliquots of the fractions were spotted onto glass-paper squares and radioassayed as described in Section 2.5.1.1, and ^{32}P-containing samples were assayed by Cerenkov radiation (Section 2.5.1.3).

2.3.2.2 Dowex-1

Nucleotides in the acid-soluble and hydrolyzed acid-insoluble fractions were also separated by chromatography on Dowex-1 resin.

Dowex-1 (Bio-Rad, AG 1-×8; 200-400 mesh) was converted from the chloride to the formate form by soaking in 0.2 M-NaOH for 60 min, washing with water until approaching neutrality, then washing twice with sodium formate and once with 5 M-formic acid (Hurlbert *et al.* 1954). After again washing with water until the pH approached neutrality, the resin was stored at 2° under water.

Columns (1.2 × 10.0 cm) of the resin were prepared and

washed with 2-3 bed volumes of 88% (v/v) formic acid and then with water until the pH approached neutrality. The sample was then loaded and washed with water until no u.v.-absorbing material was present in the effluent.

Elution was achieved with a two-stage linear gradient system. A 0-4 M gradient of formic acid (100 ml) was followed by gradients of either 0-0.5 M (for separation of mononucleotides) or 0-1.0 M (total nucleotides) ammonium formate in 4 M formic acid (100 ml). The effluent was monitored and fractions collected and radioassayed as in Section 2.3.2.1.

2.4 Enzyme Activity Determinations

Only crude plant extracts were used for enzyme determinations. The rate of activity was determined from the initial linear part of the time curve if the activity was not linear for the full reaction time. Zero-time and boiled-extract controls were included with all assays.

2.4.1 RNase

Root tips were homogenized in 8 ml/100 mg (f.w.) of acetate buffer (0.1 M; pH 5.4), and the degradation of RNA to acid-soluble nucleotides and oligonucleotides was used as an assay for RNase activity (Walters & Loring 1966). The assay mixture

in a total volume of 2 ml of acetate buffer (0.05 M; pH 5.4) contained 4 O.D. units of dialyzed yeast RNA and 0.1 ml of plant extract. The reaction was initiated by adding plant extract, and terminated with 2 ml of 3 mM-uranyl acetate in 0.2 M-HCl. Incubation was at 37° and samples were taken at various periods between 2 and 30 min. After precipitating in ice and centrifuging for 10 min at 2° and 800*g*, the optical extinction of the supernatant solution was measured at 260 nm and the rate of RNA degradation calculated.

2.4.2 Uridine kinase

Root tips were homogenized at 2° in 2 ml/g (f.w.) of 50 mM-Tris (pH 8.0) containing 1 mM-glutathione, and then passed through a French Pressure Cell at 2,500 p.s.i. The conversion of [^{14}C]uridine to [^{14}C]UMP was used as a measure of uridine kinase activity. The assay mixture in a total volume of 0.4 ml contained: 50 µmol Tris (pH 8.1), 5 µmol $MgCl_2$, 1.5 µmol mercaptoethanol, 15 µmol ATP, 5.6 nmol [2-^{14}C]uridine (4.6×10^4 c.p.m./nmol) and 0.05-0.1 ml of plant extract. The reaction was initiated by adding the plant extract and, after incubating at 30° for various periods between 30 and 120 min, was terminated by acidification with 1.25 ml 10% (w/v) TCA. After standing at 2° the supernatant was removed and extracted twice with ether, and then 0.04 ml of each sample (to which was added 0.1 µmol each of uracil, uridine and UMP) was spotted onto Whatman 3 MM chromatography paper and developed by ascending chromatography in 0.25 M-formic acid. The

marker substances were located under u.v. radiation, and a contact photographic print was prepared with the same light source. The chromatogram was cut into 5 mm strips and radioassayed (Section 2.5.1.1) to determine the conversion of $[^{14}C]$uridine to $[^{14}C]$UMP.

2.4.3 RNA polymerase

Plant extract was prepared as in Section 2.4.2, and the incorporation of labelled nucleoside triphosphate into TCA-insoluble material was used as a measure of RNA synthesis. The assay mixture in a total volume of 0.15 ml contained: 1 μmol $MnCl_2$, 20 μmol $(NH_4)_2SO_4$, 0.1 μmol heat-denatured calf thymus DNA, 20 μmol Tris (pH 8.0), 0.4 μmol glutathione, 0.4 μmol CTP, 0.4 μmol GTP, 0.8 μmol ATP, 8 nmol $[^{14}C]$UTP (10^5 c.p.m.) and 0.01-0.05 ml of plant extract. The reaction was initiated by adding plant extract and incubation was at 37° for various periods between 15 and 60 min. The reaction was terminated by placing the assay tubes in ice and adding 0.2 ml of 0.5 M-PPi and 0.05 ml of 10 mg/ml yeast RNA followed by 2 ml of 10% (w/v) TCA. The mixture was centrifuged and the precipitate washed four times with 10% (w/v) TCA and finally dissolved overnight in 1.5 ml of 2N-NH_4OH. Then 1.0 ml was dried onto a planchet and radioassayed as in Section 2.5.2.

2.5 Radioisotope Determinations

2.5.1 Liquid scintillation

2.5.1.1 General

Most samples containing [^{3}H] and [^{14}C] and some containing [^{32}P] were radioassayed on paper, either as areas cut from electrophoretograms and chromatograms or as aliquots of samples dried onto squares of glass-fibre paper (2 × 2 cm; Whatman GF-83). Unless otherwise indicated, standard glass vials (Packard Instrument Co., Chicago, Illinois, U.S.A.) were used with 2 ml of scintillation fluid containing (in g/litre of A.R. toluene): 2,5-diphenyloxazole (PPO), 3.0, and 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), 0.2. Radioassays were usually done in a Packard Tricarb model 3375 liquid scintillation spectrometer, but some [^{14}C] samples were assayed in a Unilux bench-top model 6851 spectrometer. Samples containing perchloric acid (PCA) were first made alkaline with 2 M-KOH and cooled in ice to allow $KClO_4$ to precipitate, since the presence of perchloric acid results in severe and variable quenching. Channels-ratio quench-correction curves calculated using Packard Instrument Co. standards have been used to determine counting efficiency and absolute disintegration rates where indicated.

2.5.1.2 Dual-label analysis

For experiments in which [^{3}H] and [^{14}C] were

used simultaneously, samples were spotted onto glass-fibre paper squares and radioassayed in 2 ml scintillation fluid (Section 2.5.1.1) in nylon scintillation vials (Nuclear Chicago Corp., Des Plaines, Illinois, U.S.A.). Samples were assayed with the three discriminator channels of the Packard liquid scintillation spectrometer, and count rates for both [^{3}H] and [^{14}C] were corrected for quench and converted into estimates of absolute disintegration rates by means of a series of standard quench curves. These curves were derived from data obtained with a set each of [^{3}H] and [^{14}C] quench standards prepared in the same form as actual samples, as indicated in Figure 3. The numerical calculations, made with a desk-top calculator (Sharp Compet-32 fitted with a Memorizer-60 Automatic Programer) were essentially similar to those described by Hetenyi & Reynolds (1967), except that channels-ratio values (derived from the [^{14}C] present in each sample) were used for quench determination instead of an external standard channels-ratio value. The [^{3}H] standards were corrected for progressive decay.

2.5.1.3 Cerenkov radiation

Aqueous [^{32}P] samples were usually radioassayed by Cerenkov radiation. Samples were made up to constant volumes (10-14 ml) with 0.1 M-phosphate buffer (pH 7.0) and assayed using the pre-set tritium channel of the Packard liquid scintillation spectrometer. Quenching was always similar within each set of samples, so corrections for quenching were not made.

FIGURE 3 : *Quench-correction calibration curves for [^3H] + [^{14}C] dual-label experiments.* Two sets of chemically quenched isotope standards were prepared:- *Series (a)* Six samples of [2-^{14}C]uridine (91,000 d.p.m.) dried onto 2 × 2 cm squares of glass-fibre paper were sealed in nylon scintillation vials containing 2.0 ml of scintillation fluid plus graded amounts (0-20 µl) of carbon tetrachloride as quenching agent. *Series (b)* As for *(a)* using [5-^{3}H]uridine (553,000 d.p.m.) in place of [2-^{14}C]uridine.

The standards were prepared as matched pairs (one each of [^{14}C] and [^{3}H] containing identical amounts of quenching agent), so that measured [^{3}H] efficiencies could be related to the channels-ratio values obtained with the corresponding [^{14}C] series.

Standards and experimental samples were radioassayed in the Packard scintillation spectrometer with the three channels calibrated as follows:

Channel	*Discriminator limits*	*Gain (%)*	*Isotopes detected*
RED	20 - 500	100	[^{3}H] and [^{14}C]
GREEN	175 - 1000	8	[^{14}C]
BLUE	325 - 1000	8	[^{14}C]

For each sample the channels-ratio value determined for [^{14}C] (BLUE count : GREEN count; i.e. B:G) was used for count-efficiency estimation by means of the standard quench-correction curves obtained with the standards (this figure). Thus, the amounts of the isotopes (as d.p.m.) in each sample were calculated as follows:-

$$[^{14}C]\ \text{d.p.m.} = \frac{\text{GREEN c.p.m.}}{[^{14}C]\ \text{count-efficiency in GREEN}}$$

$$[^{3}H]\ \text{d.p.m.} = \frac{\text{RED c.p.m.} - ([^{14}C]\ \text{d.p.m.} \times [^{14}C]\ \text{count-efficiency in RED})}{[^{3}H]\ \text{count-efficiency in RED}}$$

The standard quench-correction curves of this figure are:-

A : [^{14}C] count efficiency in GREEN v. [^{14}C] B:G ratio

B : [^{3}H] count efficiency in RED v. [^{14}C] B:G ratio

C : [^{14}C] count efficiency in RED v. [^{14}C] B:G ratio

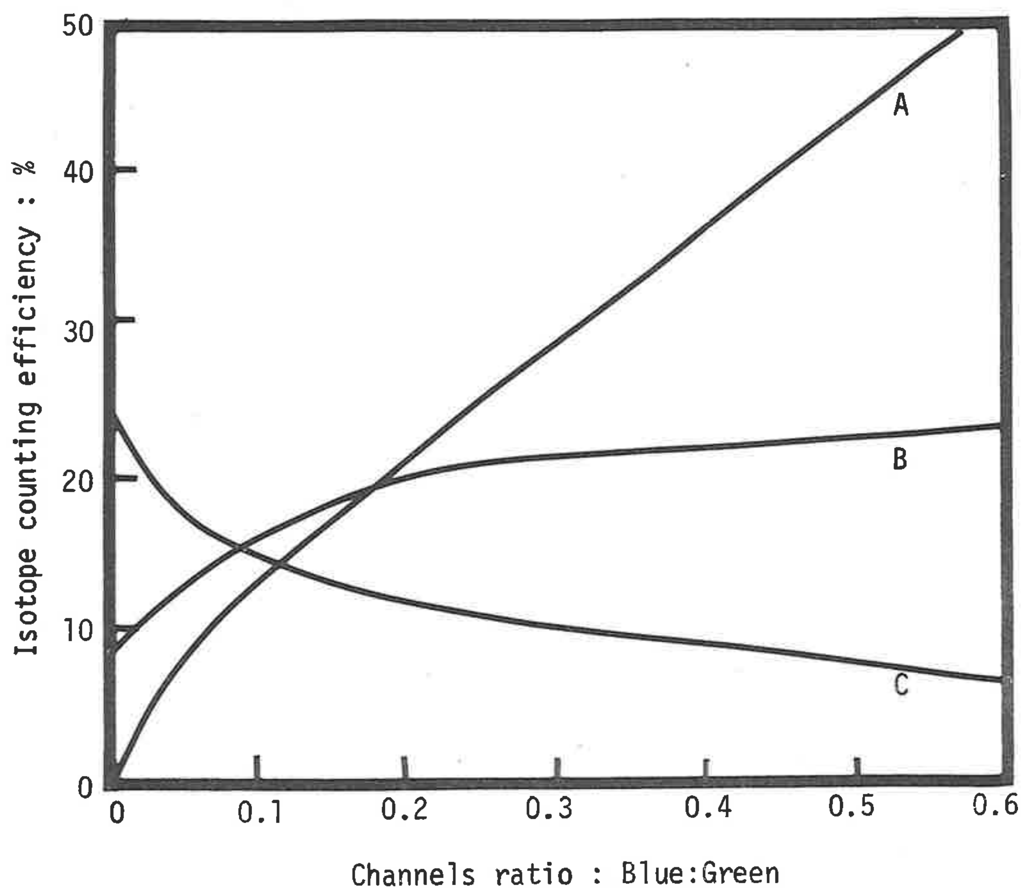


FIGURE 3 : *Quench-correction calibration curves for [^{3}H] + [^{14}C] dual-label experiments.*

2.5.2 Gas-flow analysis

Low-activity samples were often radioassayed in a Beckman Lowbeta II gas-flow planchet counter (background 3 c.p.m.). Aqueous samples were dried onto ringed planchets (5 cm diameter) and electrophoretogram sections were attached to the planchets with adhesive tape.

2.6 Other Techniques

2.6.1 Protein determinations

Protein was determined with reference to a standard solution of bovine serum albumin by the Folin procedure of Lowry *et al.* (1951) after precipitating the protein in 5% (w/v) TCA. The optical extinction was measured at 725 nm.

2.6.2 Cell counts

Thirty 5 mm root tips were incubated in 1 ml of 10% (w/v) chromic acid for 24 hr at 30° (Brown & Rickless 1949). Maceration was completed by pressing any lumps against the side of the test-tube with a glass rod and forcing the suspension through the fine hole of a pipette. Cell counts were made in duplicate on each suspension using a haemacytometer.

2.7 Materials

2.7.1 Buffers

Buffers and other aqueous solutions were made up in either glass-distilled water or water further distilled in a silica apparatus (Section 2.1.2). Stock solutions (1 M) of phosphate, Tris (Sigma Chemical Co., St. Louis, Missouri, U.S.A.), citrate and acetate buffers were prepared as described by Gomori (1955) and stored frozen at -15°. Measurements of pH were made at 20-25° on a Beckman pH meter standardized with 0.05 M-potassium hydrogen phthalate buffer (pH 4.0) or standard concentrated buffer (pH 7.0) produced by Beckman Instruments Inc. (Fullerton, California, U.S.A.).

2.7.2 Biological materials

Bovine serum albumin was purchased from the Commonwealth Serum Laboratories (Australia) and yeast RNA from Schwartz BioResearch Inc. (Orangeburg, New York, U.S.A.). Calf thymus DNA was prepared by Dr. J.F. Jackson as described by Jackson *et al.* (1968).

DNase (electrophoretically purified, RNase free) and RNase were obtained from Worthington Biochemicals Corporation (Freehold, New Jersey, U.S.A.), pronase from Calbiochem (Los Angeles, California, U.S.A.) and diastase from Ajax Chemicals (Auburn, New South Wales, Australia).

2.7.3 Nucleic acid components

All nucleic acid components were purchased from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.).

Stock solutions were prepared in distilled water and titrated to pH 7.0-7.5 with 0.3 M-HCl or 0.3 M-NaOH. Concentrations were calculated on the basis of molar extinction coefficients and optical extinction readings at the wavelength of maximum absorption measured on a Shimadzu spectrophotometer. Molar extinction coefficients were obtained from published sources (e.g. Schwartz BioResearch Inc., Radiochemical Catalogue, 1969).

2.7.4 Radioisotopes

Labelled nucleic acid components were obtained from the Radiochemical Centre (Amersham, England) and [^{32}Pi] (in dilute HCl) from the Australian Atomic Energy Commission, Isotope Division (Lucas Heights, Sydney, New South Wales, Australia).

2.7.5 Other materials

Solutes for liquid scintillation fluids were from Packard Instrument Co. (Chicago, Illinois, U.S.A.).

Bentonite was obtained as technical powder from British Drug Houses (Poole, England).

DEAE-Sephadex was supplied by Pharmacia Fine Chemicals (Uppsala, Sweden) and Dowex-1 by Bio-Rad Laboratories (Richmond, California, U.S.A.).

All other chemicals were of analytical reagent grade, obtained from Ajax Chemical Company (Auburn, New South Wales, Australia), May & Baker (Dagenham, England), Univar (Auburn, New South Wales, Australia) and British Drug Houses (Poole, England).

3. RESULTS AND DISCUSSION

3.1 Morphological Symptoms of Boron Deficiency

Before embarking on biochemical studies, some of the visible symptoms and general physiological effects of boron deficiency were studied. This was done for the following reasons:-

a. To determine whether *Phaseolus aureus* exhibits similar deficiency symptoms to those reported in other plant species.

b. To establish a time sequence for the initiation of visible symptoms and physiological effects of the deficiency in *Phaseolus aureus*.

c. To determine the interactions between these factors, so that the changes in biochemical parameters could be correlated with the early growth and physiological effects of the deficiency.

3.1.1 Root elongation

Boron deficiency has a rapid effect on root elongation, as shown in Figure 4. Inhibition of root elongation was usually observed after 24 hr and complete cessation occurred after 120 hr growth in deficient medium. This is somewhat longer than the cessation of root elongation after 24 hr reported by Neales (1960), Albert & Wilson (1961) and Yih & Clark (1965). Differences between species (Neales 1960) or stages of plant

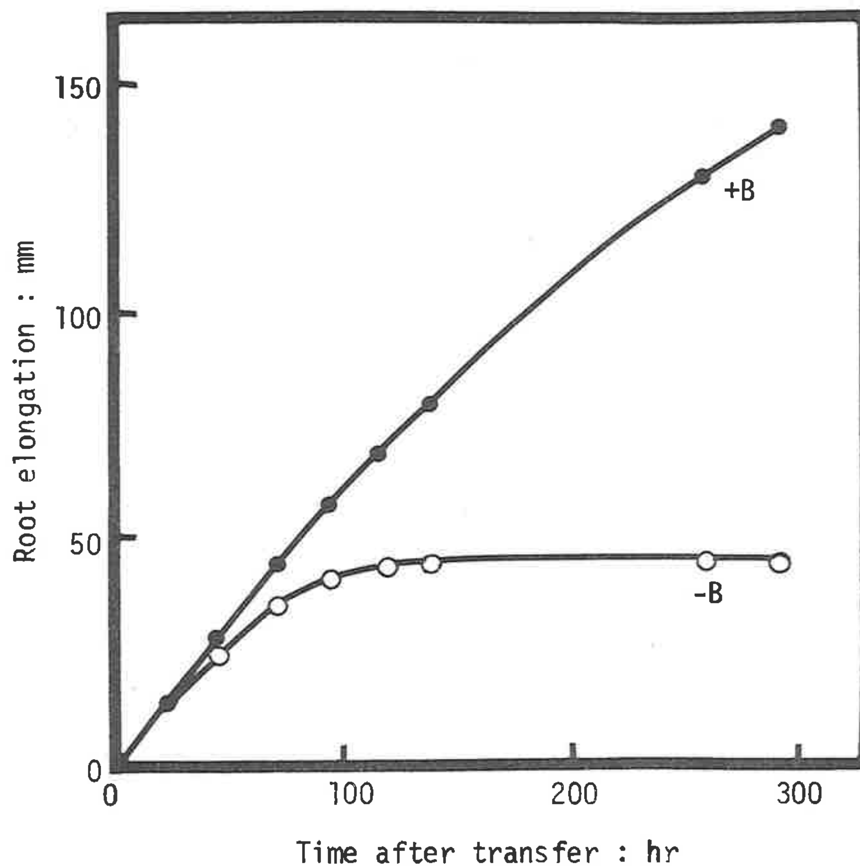


FIGURE 4 : *Effect of boron deficiency on root elongation.* Plants were grown in normal and deficient medium as described in Section 2.1.2. Roots were marked at the time of transfer to deficient medium and the subsequent elongation measured as described in Section 2.1.3. The time after the transfer of plants to boron-deficient medium is plotted on the abscissa.

growth (MacInnes & Albert 1969), or the presence of traces of boron in the nutrient medium may contribute to this slower reaction. Some variation between plant batches occurred, but only plants in which inhibition and cessation of elongation took place within 48 hr and 150 hr respectively were used for the experimental work reported in this thesis.

3.1.2 Thickening of roots

This was usually observed just prior to the cessation of root elongation. Figure 5 indicates that when measured as an increase in fresh weight, this first occurs after 50-100 hr in deficient medium. Yih & Clark (1965) observed similar changes after 72 hr. The difference between deficient and normal roots increases with the time that the plants are grown in deficient medium; this appears to be mainly due to cell enlargement, and not to an increased number of cells (Table 2). The method used for the estimation of cell number does not permit accurate determinations in meristematic regions containing large numbers of small and incompletely separated cells, and fails to differentiate between cell types. However, the results indicate that the deficiency has little if any effect on the number of cells per 5 mm root tip. The difference in weight per root tip is due largely to increased cell size. The suspension of boron-deficient root-tip cells contained many very large cells, several times the size of the largest observed in suspensions obtained from normal root tips.

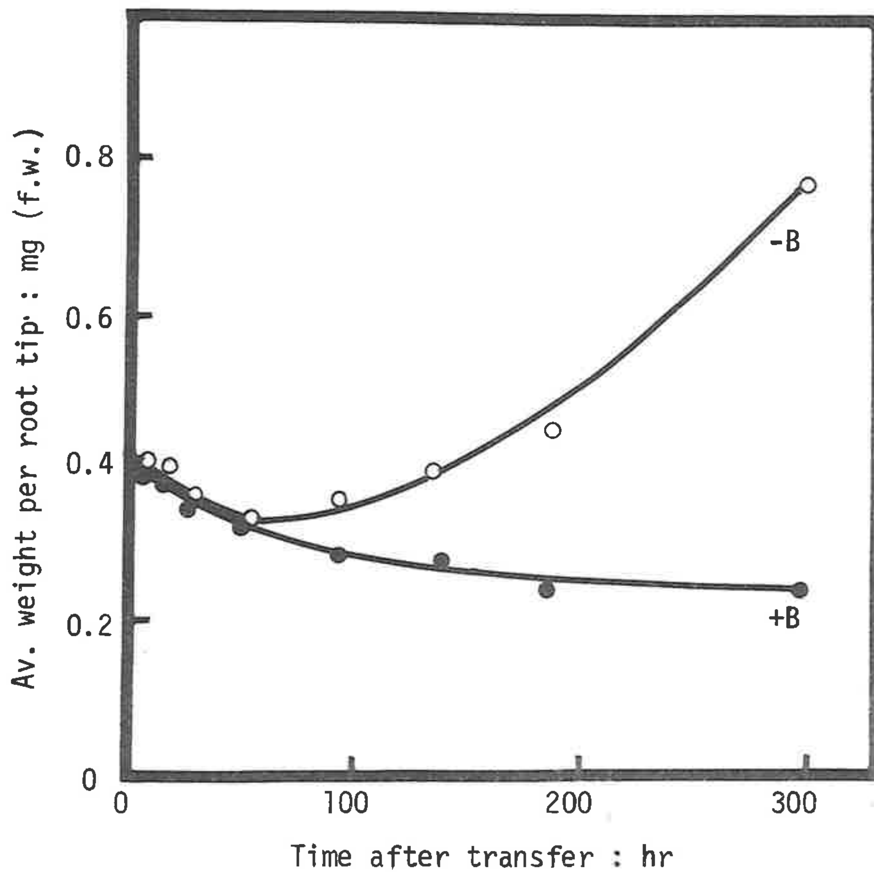


FIGURE 5 : *Effect of boron deficiency on average weight per root tip*. Plants were grown in normal and deficient medium (Section 2.1.2) and at various time intervals 200-300 root tips were excised, weighed, and the average weight per root tip calculated. The boron-deficient roots ceased elongating after 150 hr in deficient medium.

TABLE 2 : *Effect of boron deficiency on the number and average weight of cells in 5 mm root tips*

5 mm root tips were excised, weighed and macerated as described in Section 2.6.2. Each set of results was obtained from plants grown for 200-300 hr in deficient medium, and values are the means of two determinations on each sample.

Cells/tip		Cells/mg (f.w.)		Average weight (μg)/cell	
+B	-B	+B	-B	+B	-B
25,300	32,800	—	—	—	—
22,600	27,800	58,600	25,000	0.018	0.040
19,100	20,200	50,800	18,100	0.020	0.055

3.1.3 Browning of root tips

The colour of boron-deficient root tips began to change from white to brown after approximately 100 hr in deficient medium (i.e. just prior to the cessation of root elongation). The colour became darker with time and eventually affected the entire root system.

3.1.4 Lateral roots

Lateral root growth was inhibited, and eventually

stopped at approximately the same time as the main root (Figure 4). Many of the lateral roots ceased growth when only 3-5 mm long. As the deficiency progressed (250-300 hr), lateral root initials (1-2 mm long) appeared at 2-5 mm intervals right to the root tip. This then contributed to the increased weight of the root tips, and makes any comparison between normal and deficient tissue more difficult at this stage.

3.1.5 Symptoms in aerial plant parts

Symptoms of boron deficiency similar to those reported in other plants were observed, but these only occurred several days after root elongation ceased. The stem and leaf petioles became thicker and more brittle and the leaves a darker green colour than in normal plants. After 250-300 hr the terminal shoot died and fell away.

3.2 Physiological Effects of Boron Deficiency

3.2.1 Protein

When the protein content per root tip is plotted against time (Figure 6a), the changes are similar to those observed for fresh weight (Figure 5). As plants grow and the root system becomes more extensive, normal root tips become thinner and contain less protein, eventually reaching a constant

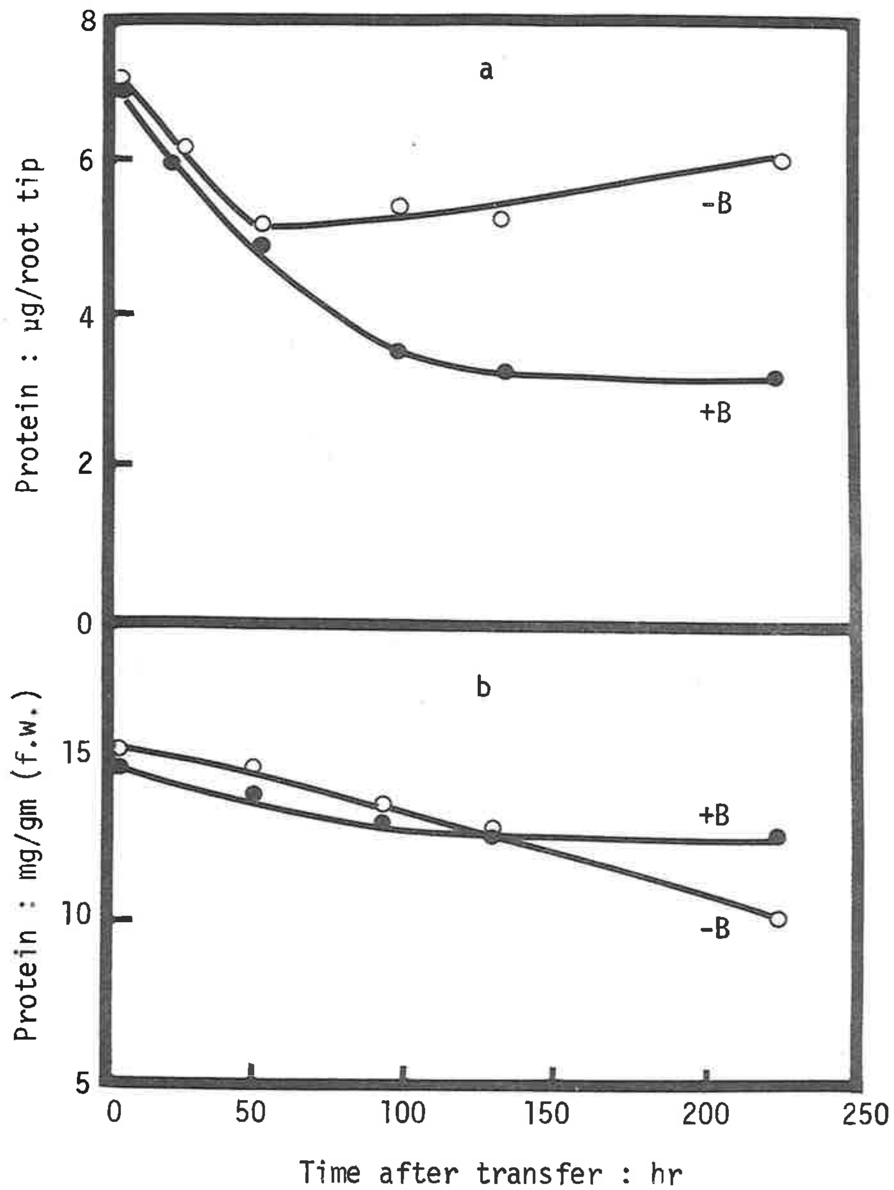


FIGURE 6 : *Effect of boron deficiency on the protein content of 5 mm root tips.* 1000-2000 root tips were excised and homogenized as described in Section 2.4.2. Protein was determined as described in Section 2.6.1.

a. Protein (µg) per root tip

b. Protein (mg) per gram (f.w.) of root tips

value 100-120 hr after the first nutrient change. Deficient root tips follow a similar pattern for the first 48 hr of deficiency, but then, as the tips become thicker, the protein content increases to a level much higher than that of normal root tips. On a fresh-weight basis (Figure 6b) the protein content of normal tips decreased slightly, until 100-120 hr after the first nutrient change, while in deficient tips it declined steadily throughout the study, reaching a slightly lower level than the normal root tips after 220 hr growth in deficient medium.

Since protein levels closely follow fresh-weight changes, some net synthesis must occur at all stages of deficiency (up to 200 hr — Figure 6). This is, however, consistent with a decreased rate of protein synthesis (Section 1.4.2) because the rate of increase in fresh weight of non-elongating boron-deficient roots is less than that of elongating normal roots (Odhnoff 1957), so that a lower rate of protein synthesis is required to maintain the protein content shown in Figure 6.

These results demonstrate the significance of the choice of parameter on which comparisons are made. Based on the content per root tip, there is an increase in protein following the withdrawal of boron from the medium, while on a fresh-weight basis a slight decrease is observed. From the results in Table 2, an increase in the protein content per cell would also be expected. These differences help explain the contradictory results discussed in Section 1.4.1.

3.2.2 RNA

Boron deficiency results in a decrease in the RNA level (Figure 7) on either basis shown (fresh-weight or root-tip), but the difference is greater on a fresh-weight basis. RNA determinations by the absorption at 260 nm and by the ribose and phosphate assays gave comparable results. Inclusion of the lipid-extraction steps recommended by Munro & Fleck (1966, p.125) or omission of the initial extraction in 80% (v/v) ethanol had little effect on these values. The effect of boron deficiency on RNA content is first observed just prior to the cessation of root elongation. This is similar to the observations of Albert (1965) and Jaweed & Scott (1967) where changes in RNA level were not detected until just after root elongation had ceased. The results in Table 2 suggest that a slight decrease in RNA per cell would also be expected.

3.2.3 DNA

Changes in DNA content also depend on the way in which the results are expressed. Boron deficiency increases the DNA content per root tip, but has little effect on a fresh-weight basis (Figure 8). This increase in DNA per root tip (and therefore probably per cell) suggests that DNA synthesis continues after cell division stops, producing an approximately doubled DNA content per cell. Thus, although the RNA content per cell falls, the DNA content is doubled under boron-deficient conditions.

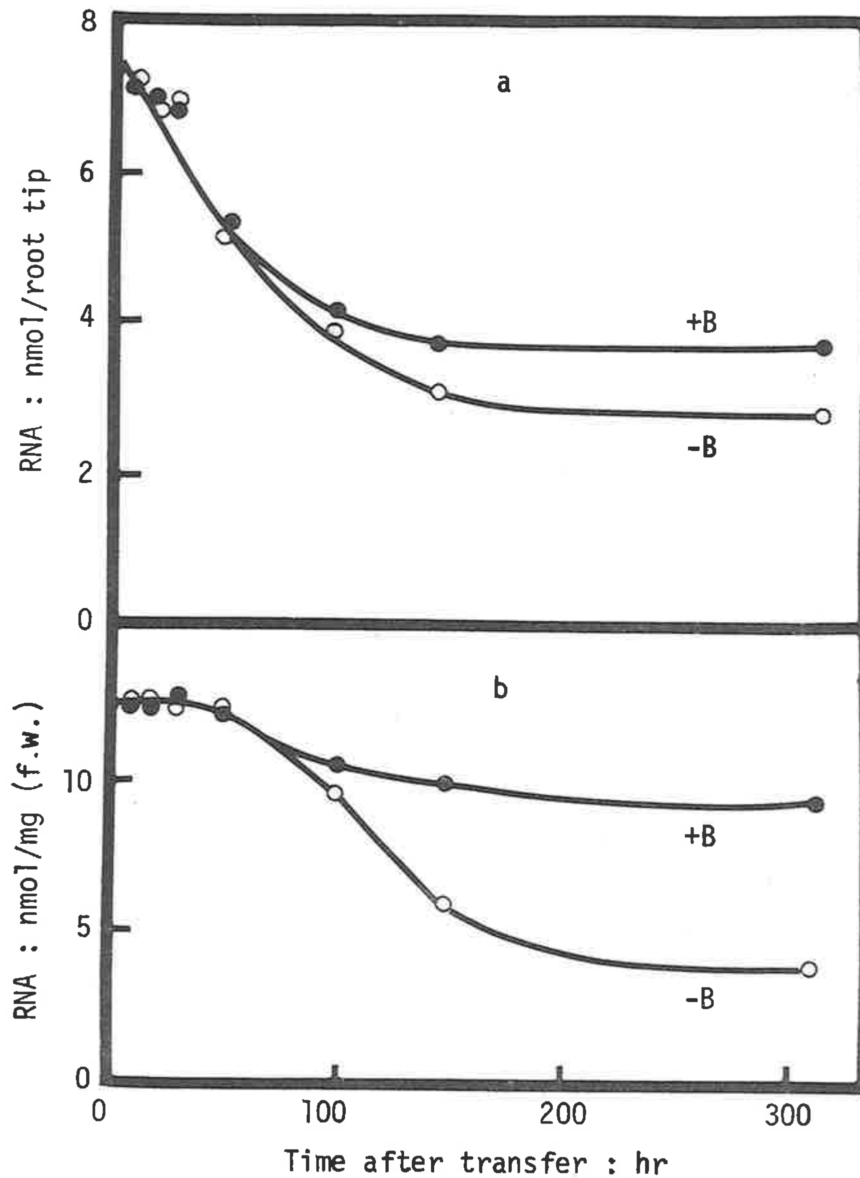


FIGURE 7 : *Effect of boron deficiency on RNA content of 5 mm root tips.* RNA was extracted from 250-300 root tips (Section 2.2.1.1) and determined by (i) absorption at 260 nm, (ii) ribose method and (iii) phosphate method (Section 2.2.2). Mean values from the three methods are plotted.

a. RNA (nmol) per root tip

b. RNA (nmol) per mg (f.w.) of root tips

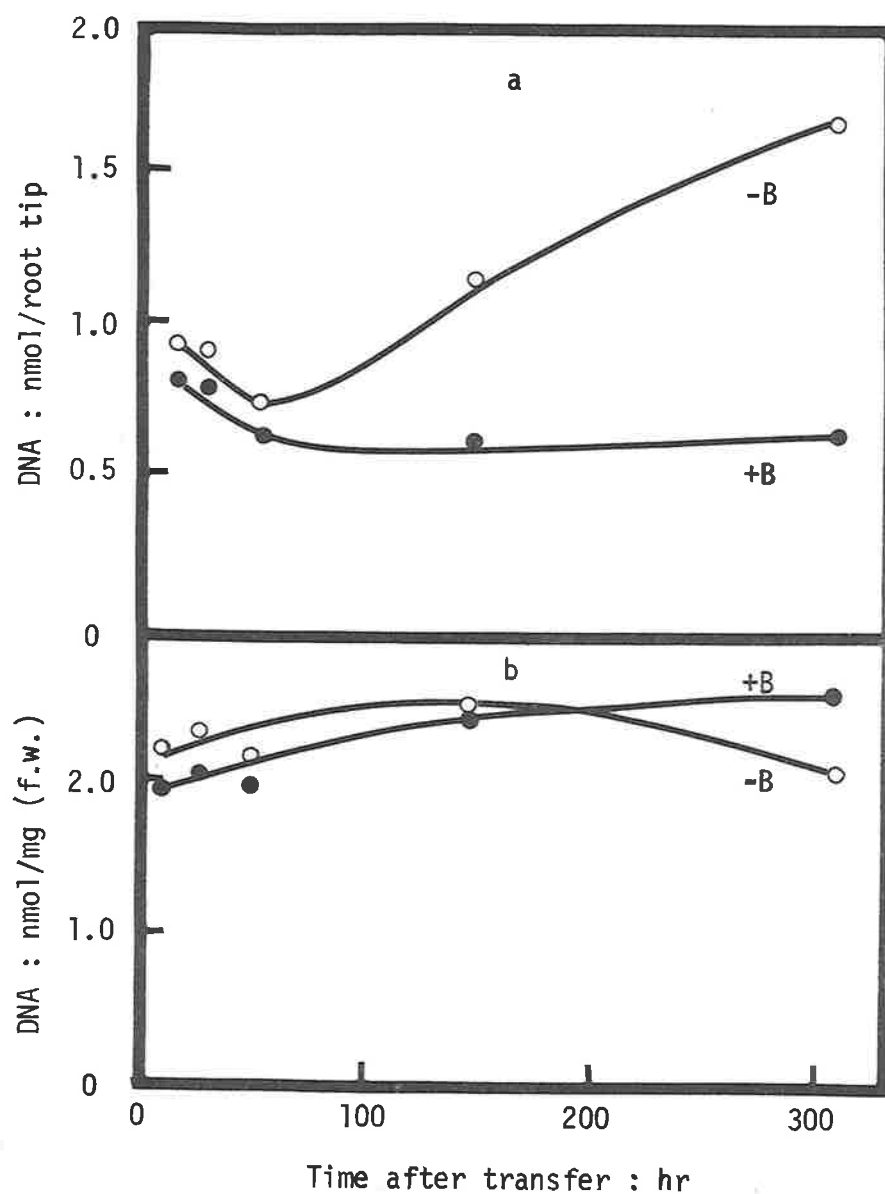


FIGURE 8 : *Effect of boron deficiency on DNA content of 5 mm root tips.* DNA was extracted from 250-300 root tips (Section 2.2.1.1) and determined by the deoxyribose assay (Section 2.2.2.3).

a. DNA (nmol) per root tip
b. DNA (nmol) per mg (f.w.) of root tips

3.2.4 Acid-soluble fraction

The acid-soluble [0.2 M-PCA or 5% (w/v) TCA] fraction extracted by the Schmidt & Thannhauser method (Section 2.2.1.1), even with the inclusion of lipid-extraction steps (Munro & Fleck 1966) before acid extraction, contains large quantities of non-nucleotide material which interferes with the various nucleotide determinations (e.g. absorption at 260 nm, ribose and phosphate). Thus, the estimation of nucleotides is not feasible on such a crude fraction, but changes in the levels of substances reacting with the above assays may be of interest. Figure 9 indicates that large increases take place in these three groups of substances (i.e. substances absorbing at 260 nm or reacting in ribose or phosphate assays) after 50-100 hr in deficient medium (at the time when root elongation is inhibited). No changes can be detected at earlier stages. If the lipid-extraction steps are included before the acid extraction, the values are reduced by 50-70% but the general effects of the deficiency are unchanged.

It is interesting to note that the RNA content of deficient tissue decreases at the same time as these substances in the acid-soluble fraction increase. This may suggest that with the onset of deficiency symptoms, either synthesis of some macro-molecular compounds ceases, or degradation rates increase, resulting in lower levels of the high molecular-weight compounds and accumulation of precursors or breakdown of products.

The measurement of nucleotide levels is discussed in Section 3.4.

FIGURE 9 : *Effect of boron deficiency on the acid-soluble fraction of 5 mm root tips.* The acid-soluble fraction was extracted from 250-300 root tips (Section 2.2.1.1) and determined by absorption at 260 nm and the ribose and phosphate assays (Section 2.2.2). The values plotted are the amounts of AMP required to produce an equivalent optical absorption or colour formation in the assay.

a. & c. Phosphate of +B (●) and -B (o) root tips

b. & d. Ribose of +B (▲) and -B (Δ) root tips

b. & d. Absorption at 260 nm of +B (●) and -B (o) root tips

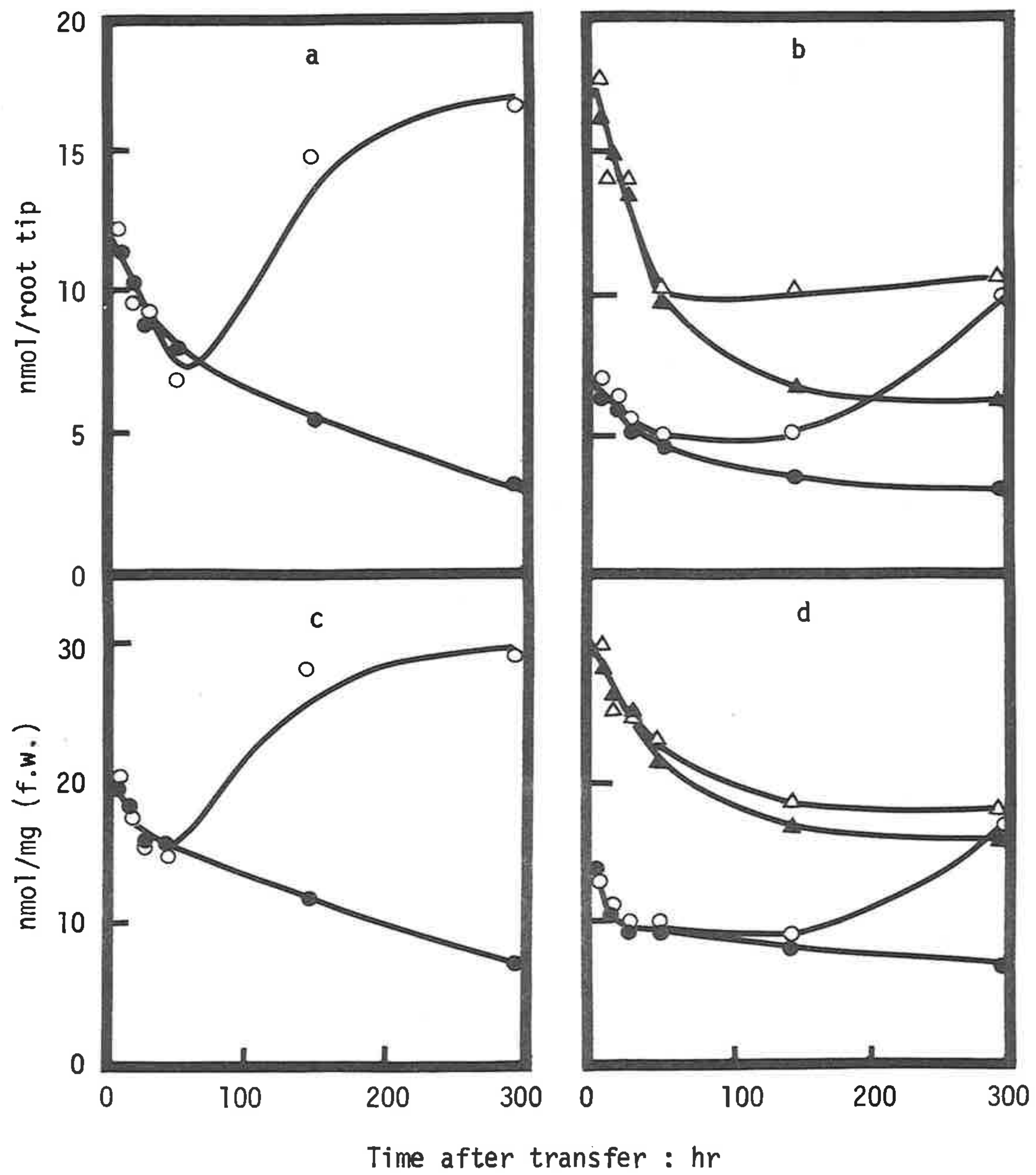


FIGURE 9 : *Effect of boron deficiency on the acid-soluble fraction of 5 mm root tips.*

3.3 Uptake and Incorporation of Labelled Precursors of Nucleic Acids

3.3.1 Effects on total uptake and incorporation

3.3.1.1 Uridine concentration in the incubation medium

Figure 10 shows the effect of the concentration of uridine in the incubation medium on both its total uptake and its incorporation into RNA. For subsequent [^{14}C]uridine experiments, 25 nmol/ml was chosen as a suitable compromise between the following factors:-

a. Economical use of both labelled and unlabelled uridine.

b. Incorporation of sufficient of the radioactive material (at the specific activity of the [^{14}C]uridine available) to give accurate results.

c. Reproducible partitioning of radioactivity between acid-soluble and RNA fractions.

[^{3}H]uridine was more readily available and the concentration was increased to 40 nmol/ml.

3.3.1.2 Incubation time

The influence of incubation time on the incorporation of [^{14}C]uridine into acid-soluble and RNA fractions is shown in Figure 11. Incorporation into the acid-soluble

FIGURE 10 : *Influence of uridine concentration in the incubation medium on uptake and incorporation of uridine.* 50 root tips from plants grown in complete nutrient medium for 7 days were incubated in 1 ml of nutrient medium as described in Section 2.1.5, and extracted and separated into acid-soluble and RNA fractions (Section 2.2.1.1).

a. Total uridine uptake into root tips
b. % of total uptake incorporated into RNA

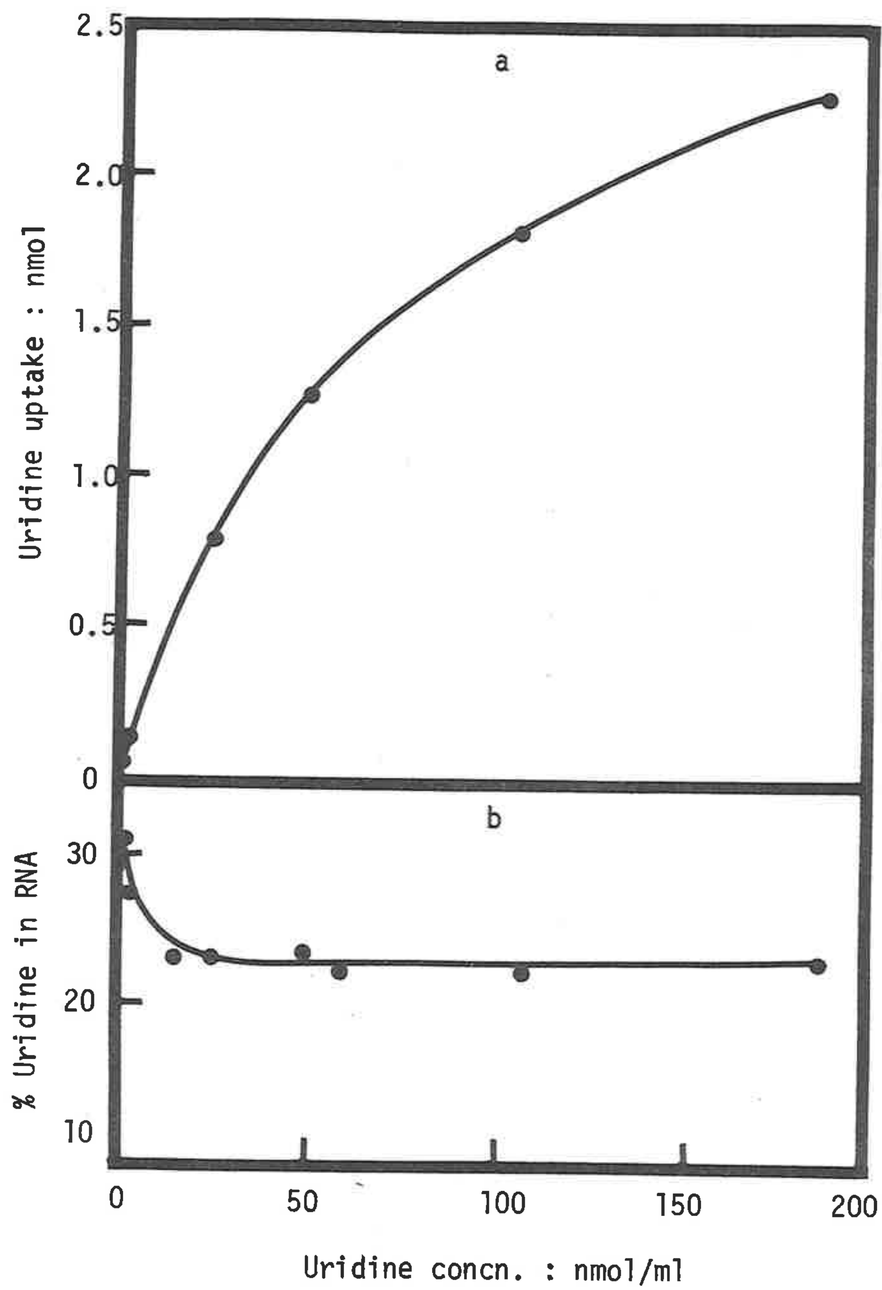


FIGURE 10 : *Influence of uridine concentration in the incubation medium on uptake and incorporation of uridine.*

FIGURE 11 : *Effect of incubation time and boron deficiency on uptake and incorporation of [^{14}C]uridine.* 100-200 root tips were incubated in [^{14}C]uridine as described in Section 2.1.5 and extracted and separated into acid-soluble and RNA fractions (Section 2.2.1.1).

+B (▲) and -B (Δ) RNA

+B (●) and -B (o) Acid-soluble

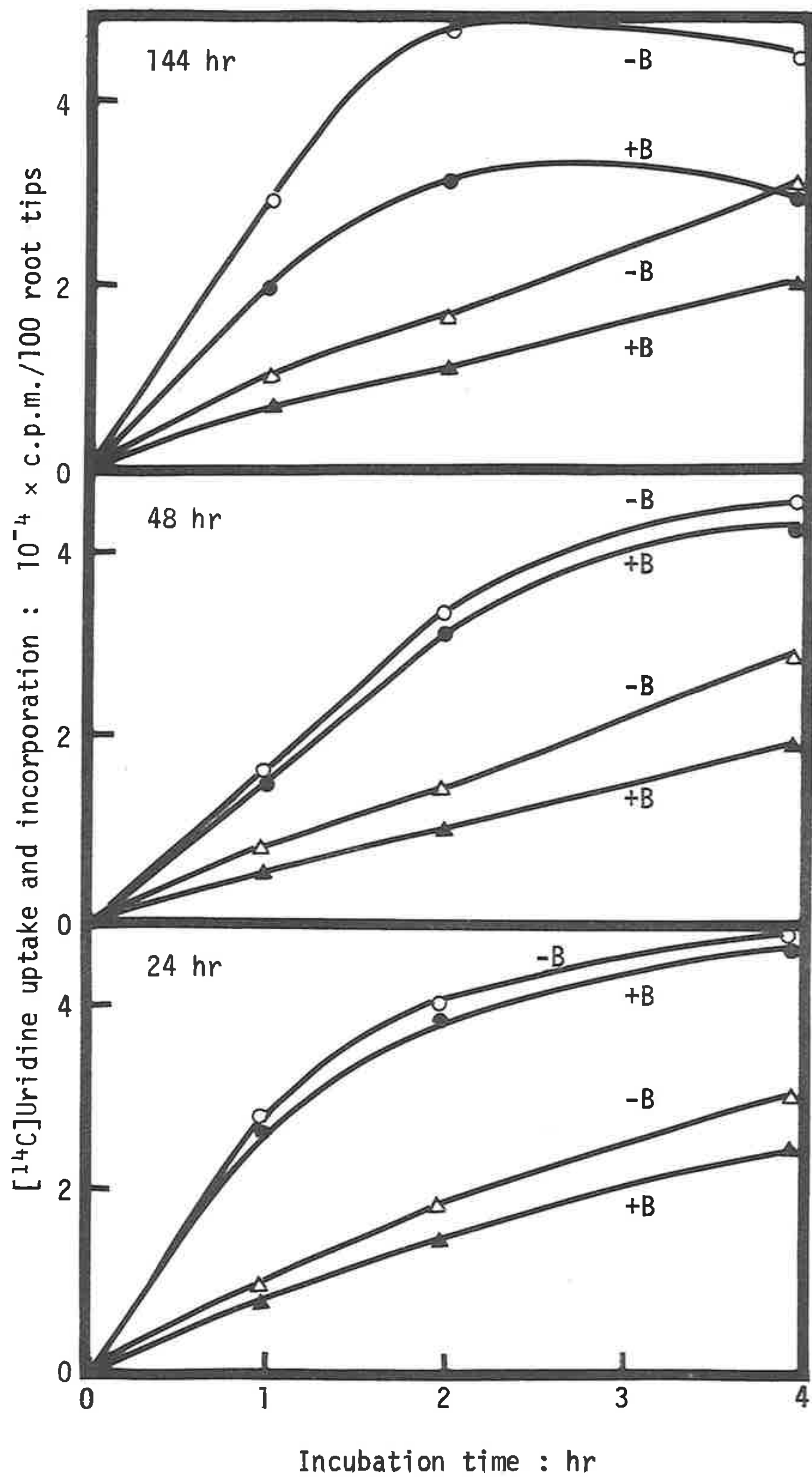


FIGURE 11 : *Effect of incubation time and boron deficiency on uptake and incorporation of [^{14}C]uridine.*

fraction is rapid over the first 2 hr, then slows down in young root tips (24 and 48 hr of deficiency) or reaches a plateau in the older roots (144 hr). Radioactivity is incorporated into the RNA fraction at an approximately linear rate throughout the 4 hr incubation.

Figure 11 also demonstrates the effect of boron deficiency on these factors. Increased incorporation into the RNA fraction occurs at all stages, and at a steady rate throughout the incubation period. During the early stages of deficiency there is little effect on the acid-soluble fraction, but after 144 hr the incorporation into deficient root tips is much higher than that into normal tips.

If these results are expressed on a fresh-weight basis, the 24 and 48 hr results are unchanged, but after 144 hr of deficiency the incorporation into RNA and acid-soluble fractions is not affected by boron deficiency.

The total uptake of [^{32}Pi] follows a similar pattern, except that the plateau is reached more rapidly (Figure 12).

3.3.1.3 Boron deficiency

During the first 48 hr of growth in boron-deficient medium, deficiency increases the incorporation of [^{14}C]uridine into RNA without influencing that into the acid-soluble fraction (Figure 13). Beyond this stage, incorporation into both fractions is increased by deficiency.

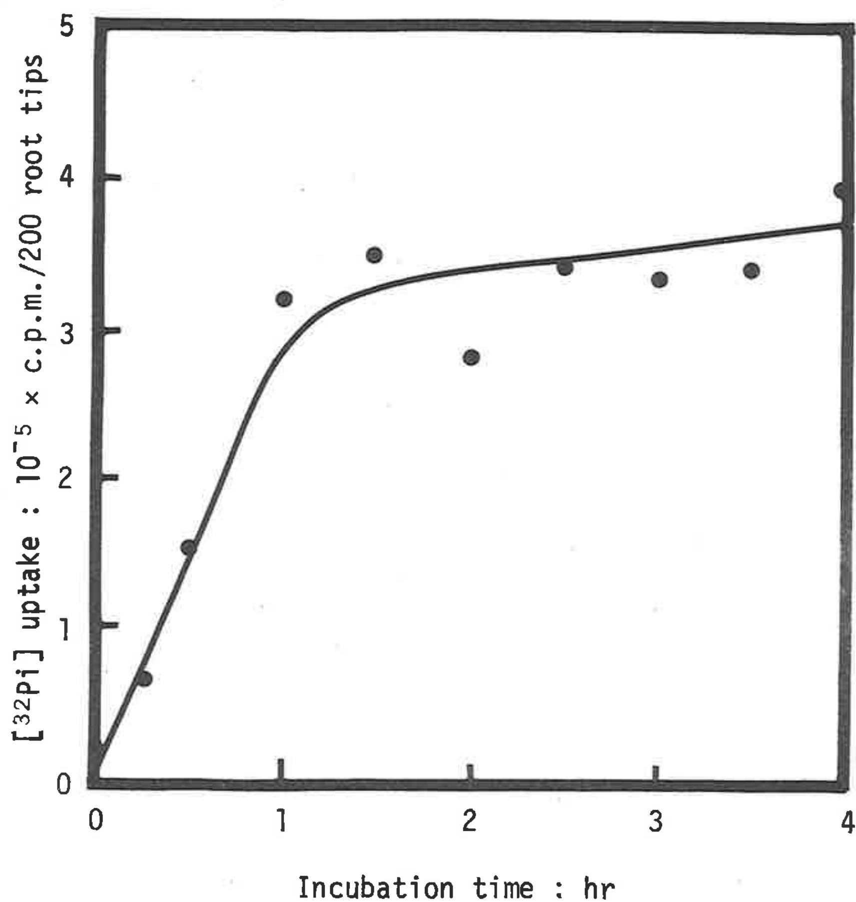


FIGURE 12 : *Effect of incubation time on total uptake of* [^{32}*Pi*]. 200 root tips from plants grown for 7 days in complete medium were incubated in [^{32}Pi] as described in Section 2.1.5. The uptake was calculated from the rate of removal of [^{32}Pi] from the incubation medium, by radioassaying (Section 2.5.1.3) aliquots at the indicated time intervals.

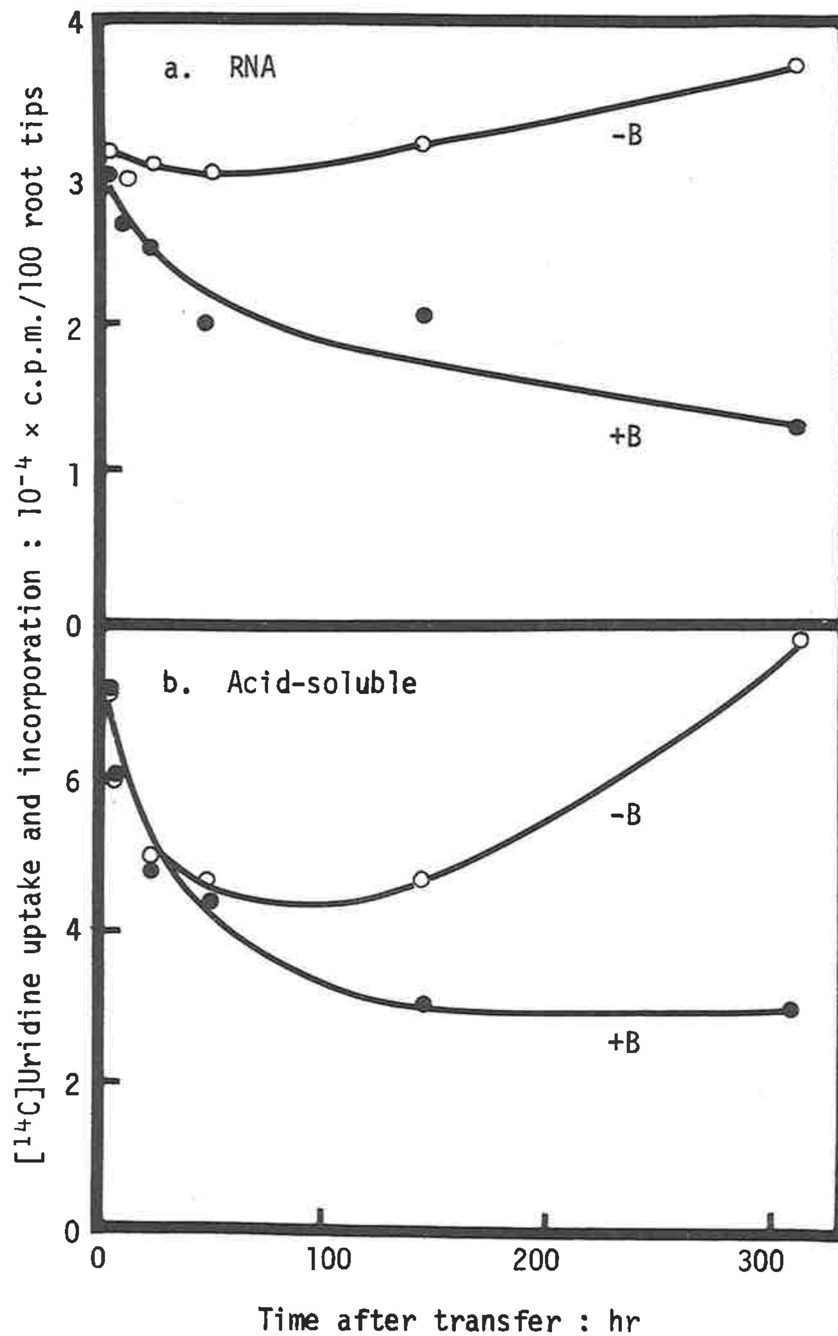
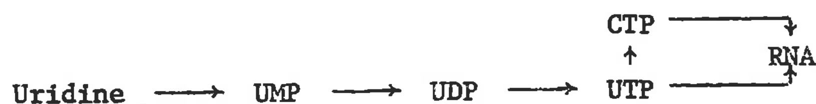


FIGURE 13 : *Effect of boron deficiency on uptake and incorporation of [^{14}C]uridine.* 100-200 root tips were incubated in [^{14}C]uridine (Section 2.1.5) and extracted and separated into acid-soluble and RNA fractions (Section 2.2.1.1).

3.3.1.4 Base distribution of [^{14}C] in RNA

[^{14}C]Uridine taken up by the plant can be incorporated into RNA as UMP or CMP as shown below:-



This is illustrated in Table 3.

TABLE 3 : *Effect of boron deficiency on the base distribution of [^{14}C] in RNA*

Root tips were incubated in [^{14}C]uridine (Section 2.1.5) and the acid-insoluble (RNA) fraction extracted and hydrolyzed in 0.3 M-KOH as described in Section 2.2.1.2. CMP and UMP were separated by chromatography on Dowex-1 (Section 2.3.2.2).

Time after transfer to boron-deficient medium (hr)	Treatment	% of total radioactivity incorporated into RNA	
		CMP	UMP
24	+B	39.7	60.3
	-B	42.0	58.0
48	+B	41.4	58.6
	-B	39.8	60.2
144	+B	38.6	61.4
	-B	37.7	62.3

Approximately 40% of the [^{14}C] enters RNA as CMP, and this proportion is not significantly influenced by boron deficiency. No radioactivity was detected in AMP or GMP.

3.3.1.5 Other trace element deficiencies

It was important to determine whether the increased incorporation of radioactive precursors into RNA is a specific effect of boron deficiency. Thus, the effects of several other trace elements were investigated. The results given in Table 4 show that, during the first 48 hr, manganese and copper deficiencies result in increased uptake of [^{14}C]uridine into the acid-soluble fraction, but without significantly increasing incorporation into RNA. At later stages of the deficiencies, incorporation into both fractions is increased. Iron deficiency does not affect the uptake of [^{14}C]uridine into the acid-soluble fraction until more than 144 hr of the deficiency, but results in an early inhibition of incorporation into RNA. The effects of deficiencies of these trace elements on [^{14}C]uridine uptake and incorporation are very different from those of boron deficiency. This is particularly interesting in the case of iron, because the effects of iron deficiency on growth, cell division and DNA and RNA contents of pea root tips are similar to those of boron deficiency (Abbott 1972).

TABLE 4 : *Effects of trace element deficiencies on [^{14}C]uridine uptake and incorporation*

Deficient and normal root tips (150 of each) were incubated in [^{14}C]uridine for 4 hr (Section 2.1.5) and the acid-soluble and RNA fractions extracted and separated as described in Section 2.2.1.1. Results are expressed as the ratio:-

$$\frac{\text{counts incorporated into 150 deficient root tips}}{\text{counts incorporated into 150 normal root tips}} .$$

All samples incorporated at least 10^4 c.p.m. into each fraction.

Deficient element	Time after transfer to deficient medium (hr)				
	12	24	48	144	336
		Acid-soluble			
Iron	1.00	1.21	0.97	0.90	1.85
Manganese	—	1.34	1.42	1.58	1.10
Copper	—	1.08	1.27	1.77	1.33
Boron	1.00	1.02	1.08	1.57	2.62
		RNA			
Iron	0.75	0.55	0.60	0.62	1.06
Manganese	—	0.98	0.92	1.30	1.56
Copper	—	0.88	1.10	1.32	1.08
Boron	1.07	1.24	1.50	1.52	3.23

3.3.2 Effects of boron deficiency on the specific activity of labelled precursor incorporation

3.3.2.1 RNA

Figure 14 shows that there is an early and very substantial increase in the specific activity of the RNA fraction following [^{14}C]uridine incorporation. After 150 hr of deficiency, the reduced RNA content of boron-deficient root tips (Figure 7) contributes to the increased specific activity. Similar results were obtained with the incorporation of [^{32}Pi] into RNA (Table 5).

TABLE 5 : *Effect of boron deficiency on the specific activity of RNA following [^{32}Pi] incorporation*

400-800 root tips were incubated in [^{32}Pi] (Section 2.1.5) and RNA was extracted by the method described in Section 2.2.1.3. Radioactivity was assayed by Cerenkov radiation (Section 2.5.1.3) and RNA by optical absorption at 260 nm (Section 2.2.2.1). Each sample incorporated at least 10^5 c.p.m.

Time after transfer to boron-deficient medium (hr)	Increase in specific activity (%)
24	53
48	69
144	100
312	202

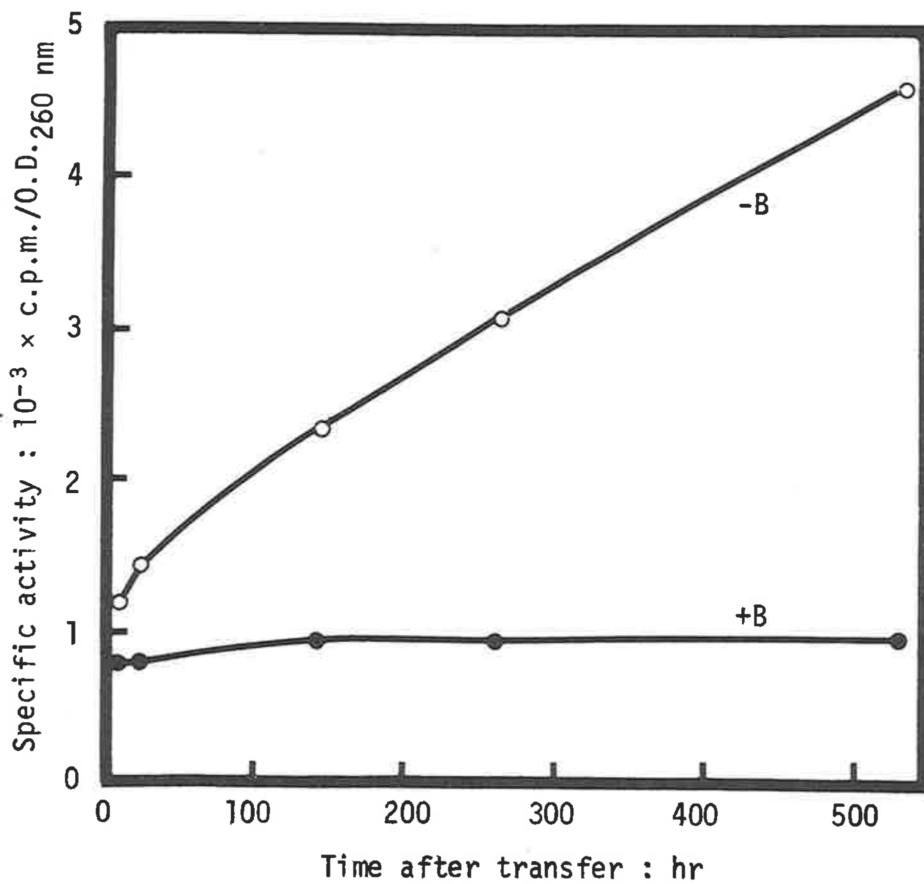


FIGURE 14 : *Effect of boron deficiency on the specific activity of RNA following [^{14}C]uridine incorporation.* 400-600 root tips were incubated in [^{14}C]uridine (Section 2.1.5) and RNA was extracted by the method described in Section 2.2.1.3.

3.3.2.2 Acid-soluble fraction

In this series of experiments, nucleotides were not separated from the acid-soluble fraction for further analysis. Accurate specific activities could not therefore be determined, but values calculated on the basis of the amount of material reacting with the ribose assay may be useful. Since many other substances give a positive result with this assay (Munro & Fleck 1966), the results must be treated with caution. On this basis, as shown in Figure 15, it is only after at least 150 hr in deficient medium that the specific-activity value is influenced by boron deficiency. Cory & Finch (1967) obtained similar results with accurate specific-activity measurements of the total nucleotide pool (see Section 1.3.2).

3.4 Nucleotides

In initial experiments, when the acid-soluble extract (Section 2.2.1.2) of 300-500 mg (f.w.) of root tips was chromatographed on Dowex-1 or DEAE-Sephadex, no nucleotides or nucleosides could be detected. If the root tips were incubated in [^{14}C]uridine, 85-95% of the [^{14}C] eluted with added uridine and only small amounts were associated with nucleotide fractions (Figure 16). Three explanations are possible to account for these results:-

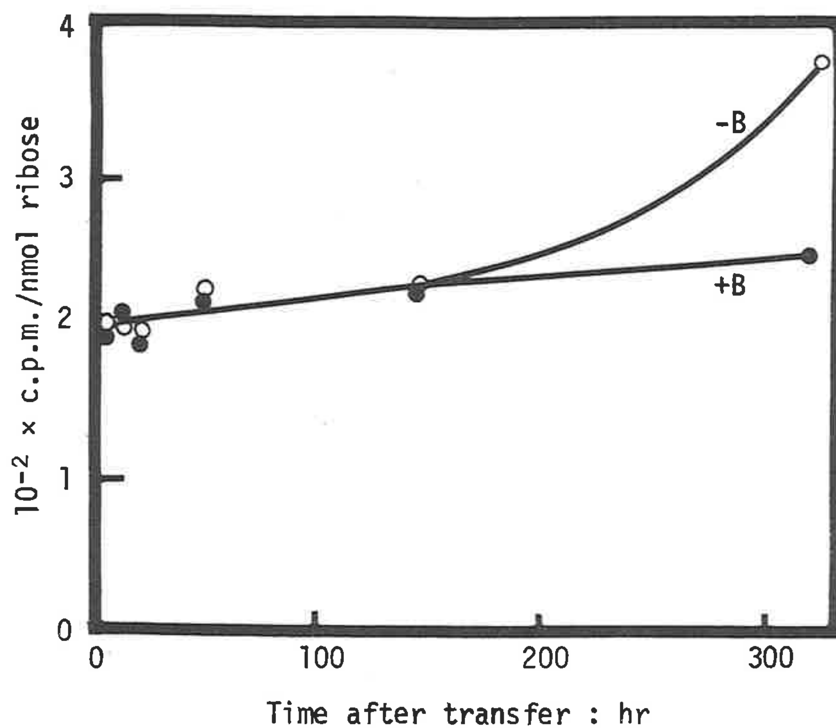


FIGURE 15 : *Effect of boron deficiency on the specific activity of [^{14}C] in the acid-soluble fraction.* 100-200 root tips were incubated in [^{14}C]uridine as described in Section 2.1.5 and the acid-soluble fraction extracted (Section 2.2.1.1). Ribose was assayed as described in Section 2.2.2.2.

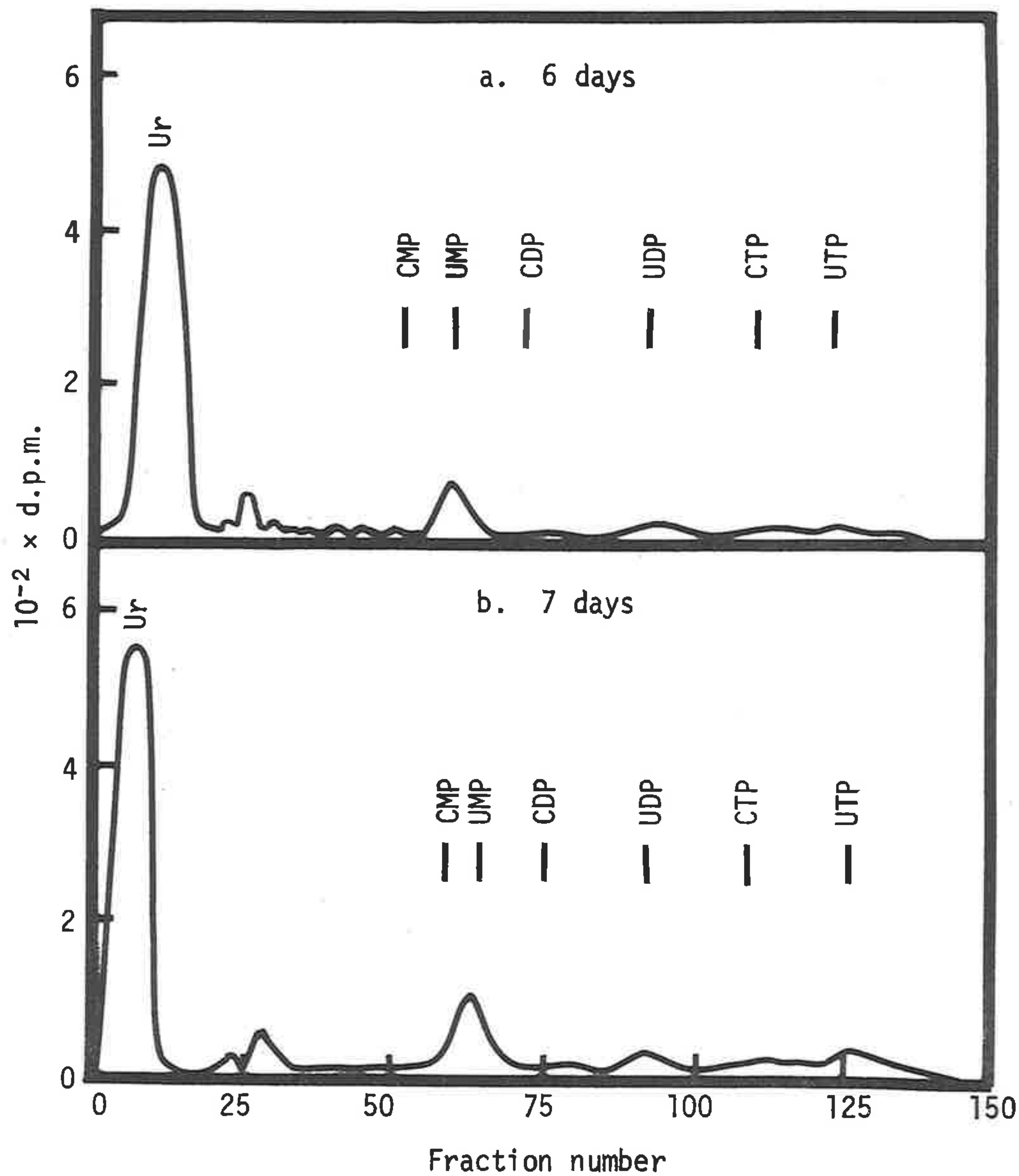


FIGURE 16 : *Incorporation of [^{14}C]uridine into nucleosides and nucleotides of root tips excised from plants grown 6 or 7 days in nutrient medium.* 900 root tips were incubated in [^{14}C]uridine (Section 2.1.5) and nucleotides extracted as described in Section 2.2.1.2 and chromatographed on DEAE-Sephadex (Section 2.3.2.1). The positions of authentic markers are shown. In this system UDPG elutes just prior to UMP.

a. Nucleotides are incompletely extracted from the tissue.

b. Nucleotides are degraded during extraction.

c. The young root tips contain low amounts of nucleotides.

The first explanation is unlikely, because other extraction procedures (e.g. 0.2 M-PCA) did not extract higher levels of nucleotides, and much higher amounts could be extracted from mature root tissue by the same methods. Thus, approximately 100 nmol of ATP (with a maximum optical absorption of 0.20 per fraction eluted from a Dowex-1 column) was extracted from one gram of mature roots.

[^{14}C]UTP included in the extraction medium is only slightly degraded during extraction (Table 6). Only 9.3 pmol of UTP (10.8% of added UTP) is degraded. Even less UDP (1.8 pmol; 7.5% of that added) and no UMP, both of which were contaminants in the [^{14}C]UTP sample, were degraded. This suggests that degradation of nucleotides is not an important factor.

Thus, it is concluded that young *Phaseolus aureus* root tips contain very low levels of nucleotides, which precludes the determination of the specific activity of the label incorporated into individual nucleotides. Unequivocal identification of nucleotides is also impossible, and their identification has only been achieved by co-chromatography with authentic standards (both ^{14}C-labelled and unlabelled). Thus, although peaks are named according to the standard with which they correspond, other minor nucleotides may contribute to the radioactivity within the peak

TABLE 6 : *Degradation of ^{14}C-labelled nucleotides during nucleotide extraction*

0.05 ml of a [^{14}C]UTP standard (44 d.p.m./pmol), also containing [^{14}C]UDP, [^{14}C]UMP and [^{14}C]uridine, was added to the buffer before extraction of nucleotides from 1000 root tips. The acid-soluble extract (Section 2.2.1.2) was chromatographed on Dowex-1 (Section 2.3.2.2) — *Test columns*. A sample (0.05 ml) of the [^{14}C]UTP standard was also chromatographed directly on Dowex-1 — *Control columns*.

Nucleotide or Nucleoside	Control			Test		
	d.p.m.	pmol	% total	d.p.m.	pmol	% total
Uridine	62	1.4	1.2	63	1.4	1.2
UMP	269	6.1	5.2	347	7.9	6.8
UDP	1060	24.1	20.5	1330	31.0	26.0
UTP	3780	85.9	73.1	3373	76.6	66.0
Total	5171	117.5	100.0	5113	116.9	100.0

(Brown 1962, Caldwell 1969). This applies particularly to [^{32}Pi] incorporation experiments.

3.4.1 [^{14}C]Uridine incorporation

At early stages of deficiency (24 and 48 hr) the amount of [^{14}C]uridine incorporated into nucleotides is very small. The only significant incorporation of radioactivity was

into a peak which appears to be associated with UDPG. This peak elutes in similar positions to those reported for UDPG from both Dowex-1 and DEAE-Sephadex (Hurlbert *et al.* 1954, Caldwell 1969, Brown and Cassells 1971), but has not been positively identified. Deficiency did appear to slightly increase the incorporation of the tracer into this peak, but the values were too low to permit an accurate assessment of the effect. This possible increase may be related to the increased incorporation of [^{14}C]glucose into pectic substances (*via* UDPG) by boron-deficient field bean root tips (Slack & Whittington 1964).

After 144 hr of the deficiency (when the plants are 4 days older) significant amounts of [^{14}C]uridine are incorporated into the nucleotides of both normal and deficient plants (Figure 17). From the analysis in Table 7 it can be seen that the deficiency causes only small changes in the distribution of radioactivity, the most significant being that a decreased proportion of the total incorporation is into triphosphates (40.8% of nucleotides in normal and 35.0% in deficient tissue) and an increased proportion is into the UDPG and UMP peak. At this late stage of deficiency there also appears to be an increase in incorporation into all uridine nucleotides and a slight reduction of incorporation into cytidine nucleotides. The deficiency does not, however, change the ratio of the corresponding bases in RNA (Table 3 in Section 3.3.1.4), suggesting that the effect is due to changes in size rather than specific activity of the uridine and cytidine nucleotide pools.

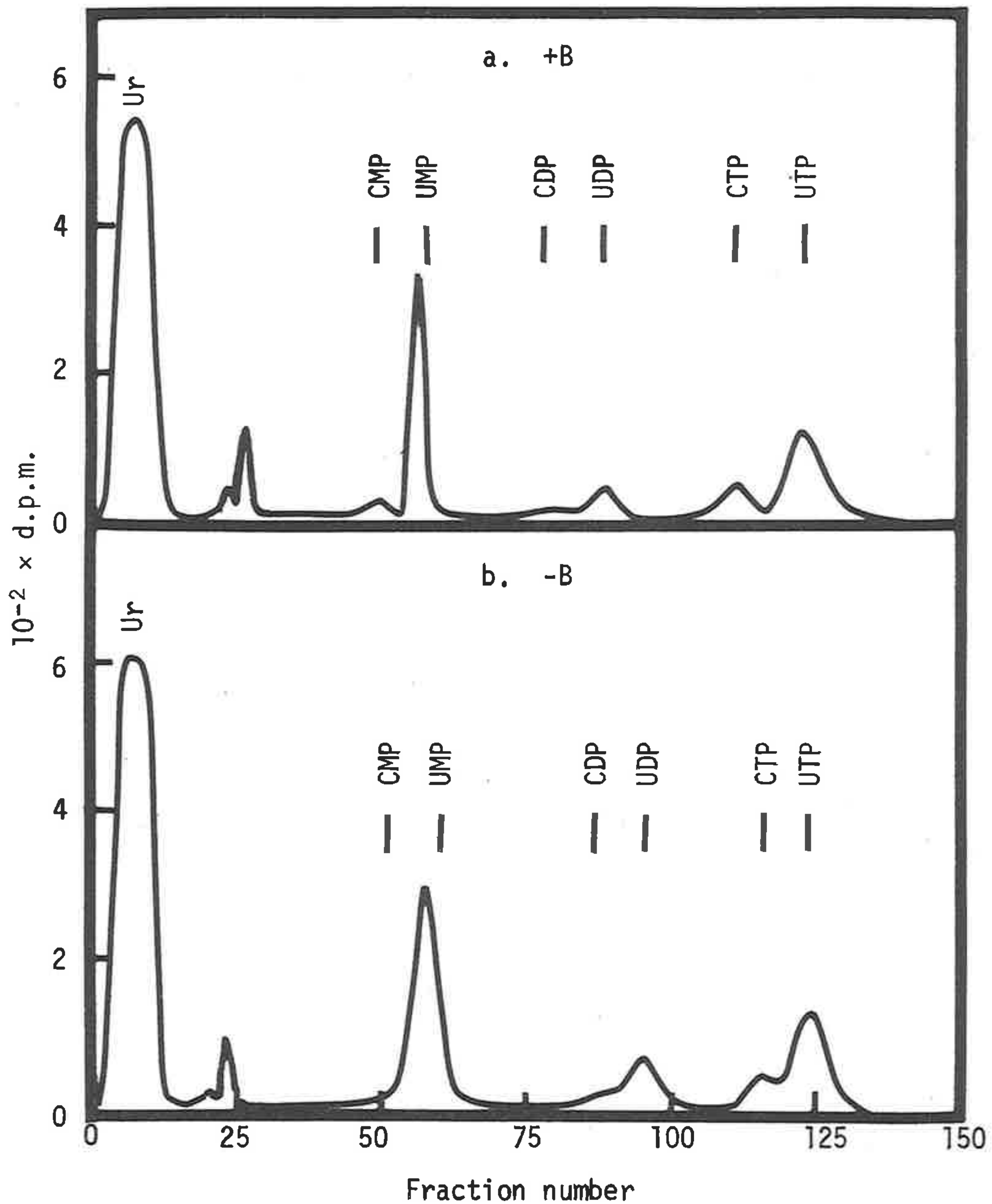


FIGURE 17 : *Incorporation of [^{14}C]uridine into nucleosides and nucleotides after 144 hr growth in deficient medium.* 1000 root tips were incubated in [^{14}C]uridine (Section 2.1.5) and nucleotides extracted as described in Section 2.2.1.2 and chromatographed on DEAE-Sephadex (Section 2.3.2.1). The positions of authentic markers are shown. In this system UDPG elutes just prior to UMP.

TABLE 7 : *Distribution of [^{14}C] in nucleosides and nucleotides of 144 hr deficient root tips following [^{14}C]uridine incorporation*

The data were obtained from the same set of results used in Figure 17.

Nucleotide or Nucleoside	Radioactivity (c.p.m.)		% of Total		% of Nucleotides	
	+B	-B	+B	-B	+B	-B
Nucleosides	7,838	8,398	74.0	72.1	—	—
CMP	95	57	0.9	0.5	3.5	1.7
UMP + UDPG	1,279	1,620	11.1	14.0	42.8	50.0
CDP	64	50	0.6	0.4	2.3	1.5
UDP	293	383	2.8	3.3	10.6	11.8
CTP	237	188	2.2	1.6	8.6	5.8
UTP	884	947	8.4	8.1	32.2	29.2

3.4.2 [^{32}Pi] incorporation

Using [^{32}Pi], the incorporation of radioactivity into nucleotides is much greater (Figures 18 and 19) but, as with [^{14}C]uridine, the incorporation increases with plant age. [^{32}Pi] is incorporated into all nucleotides, but with the simple chromatographic method used, complete separation was not achieved. Mononucleotides are very poorly separated, since CMP, UMP, AMP, UDPG and Pi are all eluted together. Based on the separation

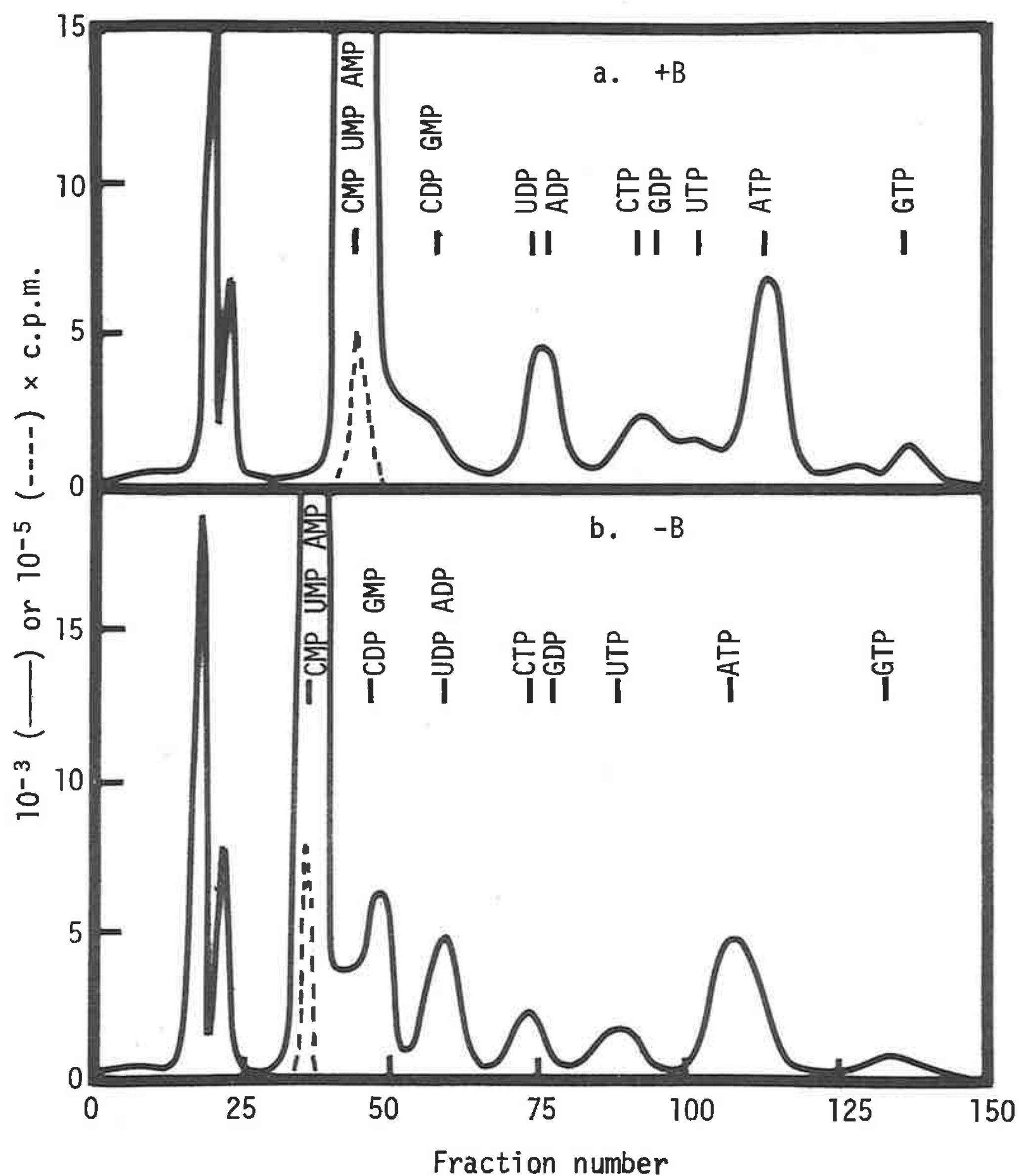


FIGURE 18 : *Incorporation of [^{32}Pi] into nucleotides of 48 hr deficient root tips.* 500 root tips were incubated in [^{32}Pi] (Section 2.1.5) and nucleotides extracted as described in Section 2.2.1.2 and chromatographed on DEAE-Sephadex (Section 2.3.2.1). The positions of authentic markers are shown. In this system UDPG elutes with UMP.

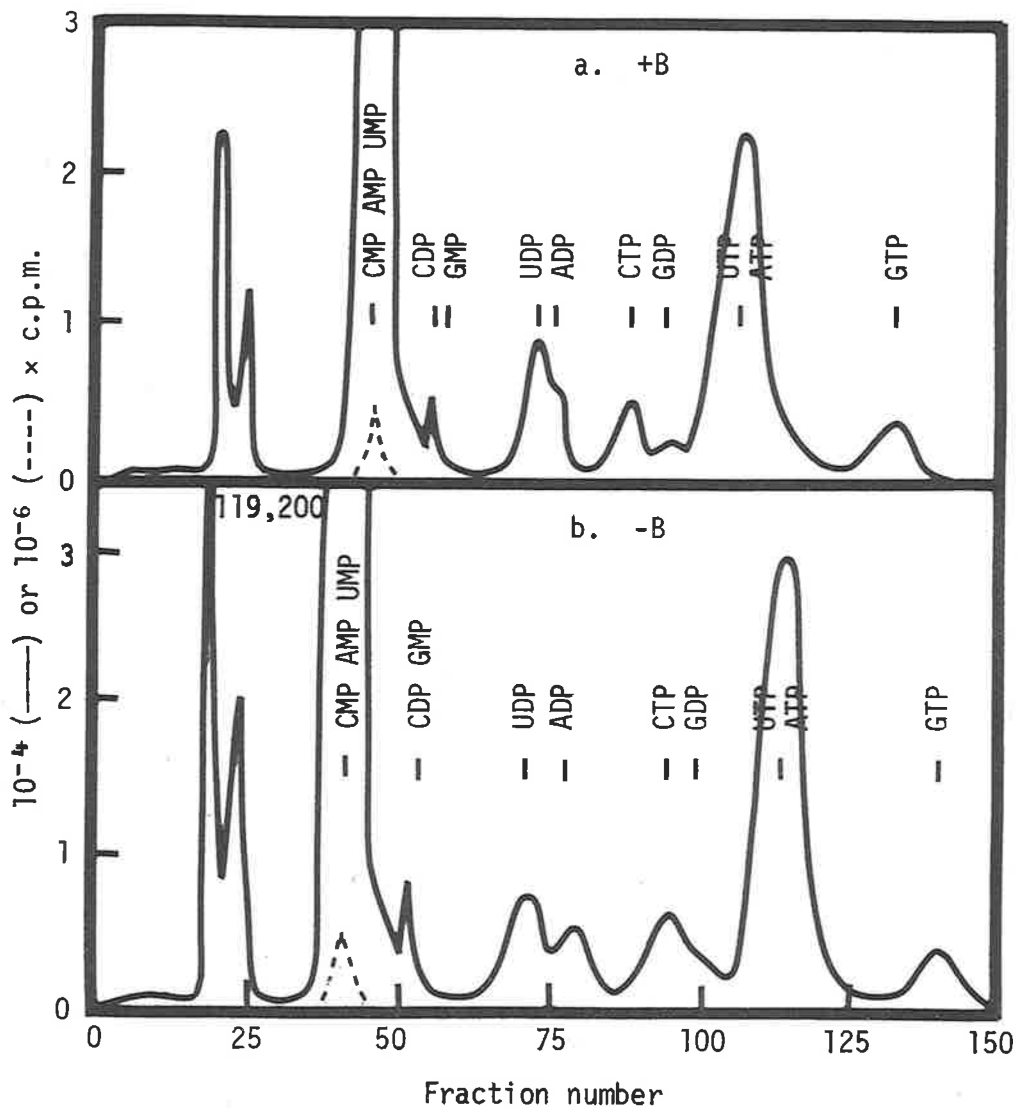


FIGURE 19 : *Incorporation of [^{32}Pi] into nucleotides of 144 hr deficient root tips.* 500 root tips were incubated in [^{32}Pi] (Section 2.1.5) and nucleotides extracted as described in Section 2.2.1.2 and chromatographed on DEAE-Sephadex (Section 2.3.2.1). The positions of authentic markers are shown. In this system UDPG elutes with UMP.

achieved with di- and tri-phosphates, boron deficiency appears to have little or no effect on the distribution of radioactivity between the nucleotides. This is demonstrated for adenosine and uridine nucleotides in Table 8. After 48 hr of deficiency, the ratio of UDP + ADP to UTP + ATP is not affected, while after 144 hr there is a slight increase (of doubtful significance) in the triphosphates relative to diphosphates. The slight variance

TABLE 8 : *Distribution of [^{32}P] between adenosine and uridine nucleotides*

The data were obtained from the same sets of results used in Figures 18 and 19.

Time after transfer to boron-deficient medium (hr)	Treatment	ADP + UDP		ATP + UTP	
		c.p.m.	% total	c.p.m.	% total
48	+B	39,633	38.2	63,991	61.8
	-B	33,807	38.3	54,516	61.7
144	+B	71,068	26.5	197,996	73.5
	-B	93,327	23.9	297,604	76.1

between this result and the effect of the deficiency on [^{14}C]uridine incorporation (Table 7) may be due to the different experimental conditions or plant batches employed.

The total amount of [^{32}Pi] incorporated into these nucleo-

tides (Table 8) is altered by boron deficiency. After 48 hr of growth in deficient medium, the slightly reduced incorporation may be due to increased rates of utilization of nucleotides in RNA synthesis, thus resulting in lower levels of nucleotides. Both changes observed after 144 hr of deficiency can be attributed to the increased maturity of deficient root tips. Total incorporation into nucleotides and the ratio of incorporation into triphosphates: diphosphates increase with both boron deficiency and plant age. These results are consistent with the observation of Shkol'nik & Maevskaya (1962*b* — Section 1.3.3) that the ATP level does not fall until after the appearance of deficiency symptoms.

Incorporation of radioactivity into the total acid-soluble fraction of normal root tips decreases with plant age (Figure 13 in Section 3.3.1.3), but the incorporation into nucleotides increases (Figures 16, 17, 18 and 19; Table 8). This is because in young root tips most of the radioactivity taken up by the root tips is associated with nucleosides, bases and non-nucleotide material, but as the plant ages much more radioactivity is associated with the nucleotide fractions. This may be due to changes in growth (Figure 4 in Section 3.1.1) and differentiation rates. The change with plant age is much greater than any effect of boron deficiency.

3.5 Fractionation of RNA

3.5.1 MAK chromatography

3.5.1.1 Appraisal of the technique

To investigate further the early effect of boron deficiency on the incorporation of precursors into RNA, MAK chromatography (Section 2.3.1.1) was employed. The major peaks separated by this technique have been well characterized (Monier *et al.* 1962, Ellem & Sheridan 1964, Cherry 1964, Key *et al.* 1965, Cherry *et al.* 1965, Chroboczek & Cherry 1966, Ellem 1967). Under ideal conditions, the fractions shown in Figure 20 can be separated, and the major components of each are as follows:-

(a) Nucleotides and oligonucleotides (Ewing & Cherry 1967);

(b) 4s(t-RNA) and 5s(r-RNA), which are sometimes separated, and are usually referred to as s-RNA;

(c) DNA and DNA-RNA hybrids;

(d) Light ribosomal RNA (lr-RNA);

(e) Heavy ribosomal RNA (hr-RNA);

(f) Messenger RNA (m-RNA);

(g) RNA which is tightly bound to the column, and eluted with SDS or alkali (TB-RNA).

Much work has been done to characterize fractions (d), (e) and (f), particularly (f). Ingle *et al.* (1965), using differential extraction techniques, showed that Fraction (f) contains RNA which

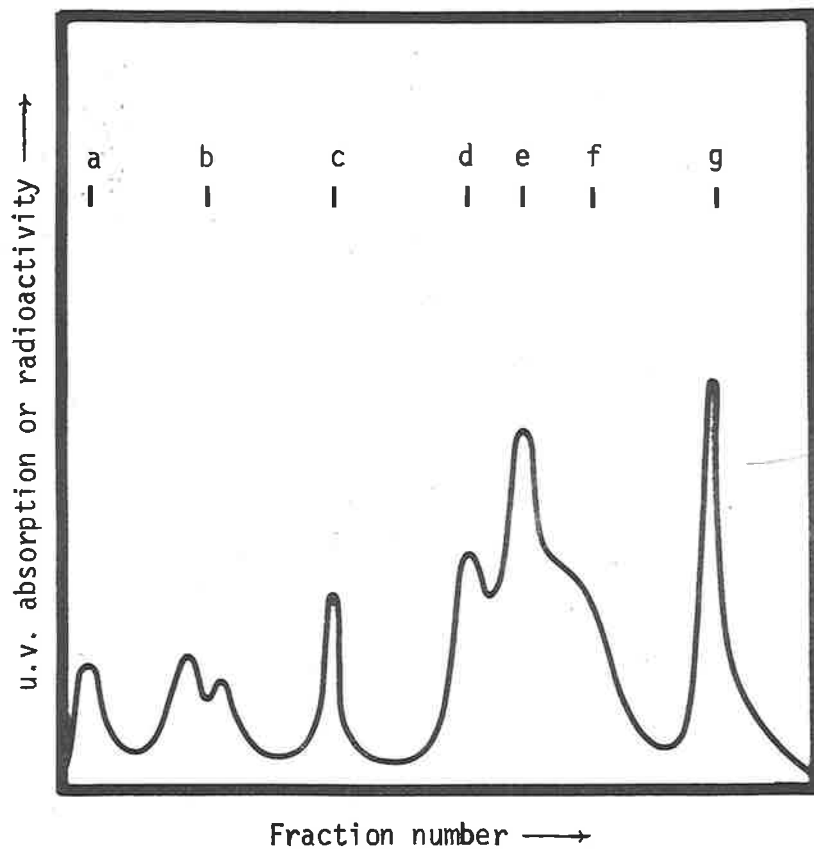


FIGURE 20 : *Fractionation of RNA by MAK chromatography.* Ideal separation by MAK chromatography from Cherry *et al.* (1965), Ewing & Cherry (1968), Ingle & Key (1968) and Johri & Varner (1970).

is different from the hr-RNA peak in base composition, rate of synthesis and half-life. Because the base composition was similar to that of DNA and the half-life approximately 2 hr, they suggested that it may be messenger RNA. On sucrose gradient centrifugation this fraction is heterogeneous (see also Johri & Varner 1970) and not coincident with the optical density patterns. Others have confirmed the high AMP content (Key & Ingle 1964, Chroboczek & Cherry 1966) and shown that this fraction hybridizes more readily with homologous DNA than any other fraction (Van Huystee & Cherry 1966), is most sensitive to Actinomycin D (Chroboczek & Cherry 1966), is associated most with and is most active in the formation of polysomes (Lin *et al.* 1966, Jachymczyk & Cherry 1968, Johri & Varner 1970) and is most capable of supporting protein synthesis *in vitro* (Jachymczyk & Cherry 1968). This fraction therefore possesses many of the characteristics expected of messenger RNA and has usually been referred to as m-RNA. The poor separation of peaks (d), (e) and (f) led Ingle & Key (1968) to fractionate them further using polyacrylamide-gel electrophoresis. Aggregation of ribosomal and other RNA species results in each peak being a complex mixture of RNA species. After a 4 hr [^{32}Pi]-labelling period, much of the radioactivity of peak (f) was associated with 18s and 25s r-RNA. With the simplified chromatographic technique used in the work reported in this thesis, the aggregation is even more severe, resulting in the elution of one r-RNA + m-RNA fraction (referred to as r-RNA).

TB-RNA [peak (g)] is known to consist of several subfractions

(Ewing & Cherry 1968, Miassod *et al.* 1970). At least one of these is similar to m-RNA [peak (f)] and rechromatography and base-composition analysis indicate that r-RNA (or a ribosomal precursor) is also a major component (Key 1967, Johri & Varner 1970). A rapidly labelled AMP-rich RNA fraction separated from total RNA by counter-current distribution is fractionated almost entirely into m-RNA and TB-RNA by MAK chromatography (Ewing & Cherry 1968). Johri & Varner (1970) have suggested that these two rapidly labelled fractions have an open structure and are separated because of their different base compositions. Those species with a high adenine content (low guanine and cytosine) have little secondary structure and interact strongly with the methylated albumin and are therefore eluted as TB-RNA, while those species with a low adenine content interact with r-RNA and elute as m-RNA. If the inability to chase label from TB-RNA is due to the accumulation of label by other subfractions (e.g. ribosomal RNA — Ingle *et al.* 1965), it is then possible that the rapidly labelled components of both m-RNA and TB-RNA represent plant messenger-RNA differing only in base composition.

The fractions will be referred to by the above names, but it must be emphasised that they are only radioactive and u.v.-absorbing material eluting in this part of the elution pattern and in some cases, especially in grossly deficient tissue (Figures 22 and 28), the identification of the RNA species may not be strictly correct.

3.5.1.2 [^{14}C]Uridine incorporation

Figure 21 shows the elution patterns for normal and deficient samples of RNA from root tips after 24 hr growth in deficient medium. The incorporation is increased into all RNA species, but particularly TB-RNA (fractions 80-90) and r-RNA (fractions 40-60). This pattern changes progressively, and after 512 hr of boron deficiency the situation depicted in Figure 22 is reached. There is almost a complete breakdown of r-RNA (shown by absorbance at 260 nm) in deficient tissue. Some of this appears to elute in the t-RNA region, but the total RNA level is considerably lower than that of the normal root tips (see Figure 7 in Section 3.2.2). There is also a vast difference in the distribution of label from [^{14}C]uridine incorporation studies. TB-RNA still incorporates approximately twice as much radioactivity, but the ratios between incorporation into r-RNA and s-RNA are reversed.

The incorporation into the s-RNA, r-RNA and TB-RNA fractions during the development of deficiency is shown in Figure 23. During the first 50 hr the effects shown in Figure 21 prevail — i.e. incorporation into all fractions is increased, but TB-RNA to a much greater extent than r-RNA, which in turn is slightly greater than s-RNA. During the period 50-120 hr, changes occur, the most important being that an even higher incorporation into the s-RNA and TB-RNA fractions is observed compared to that in normal tissues. The effect on r-RNA becomes less noticeable, eventually reverting almost to the same level of incorporation as that of

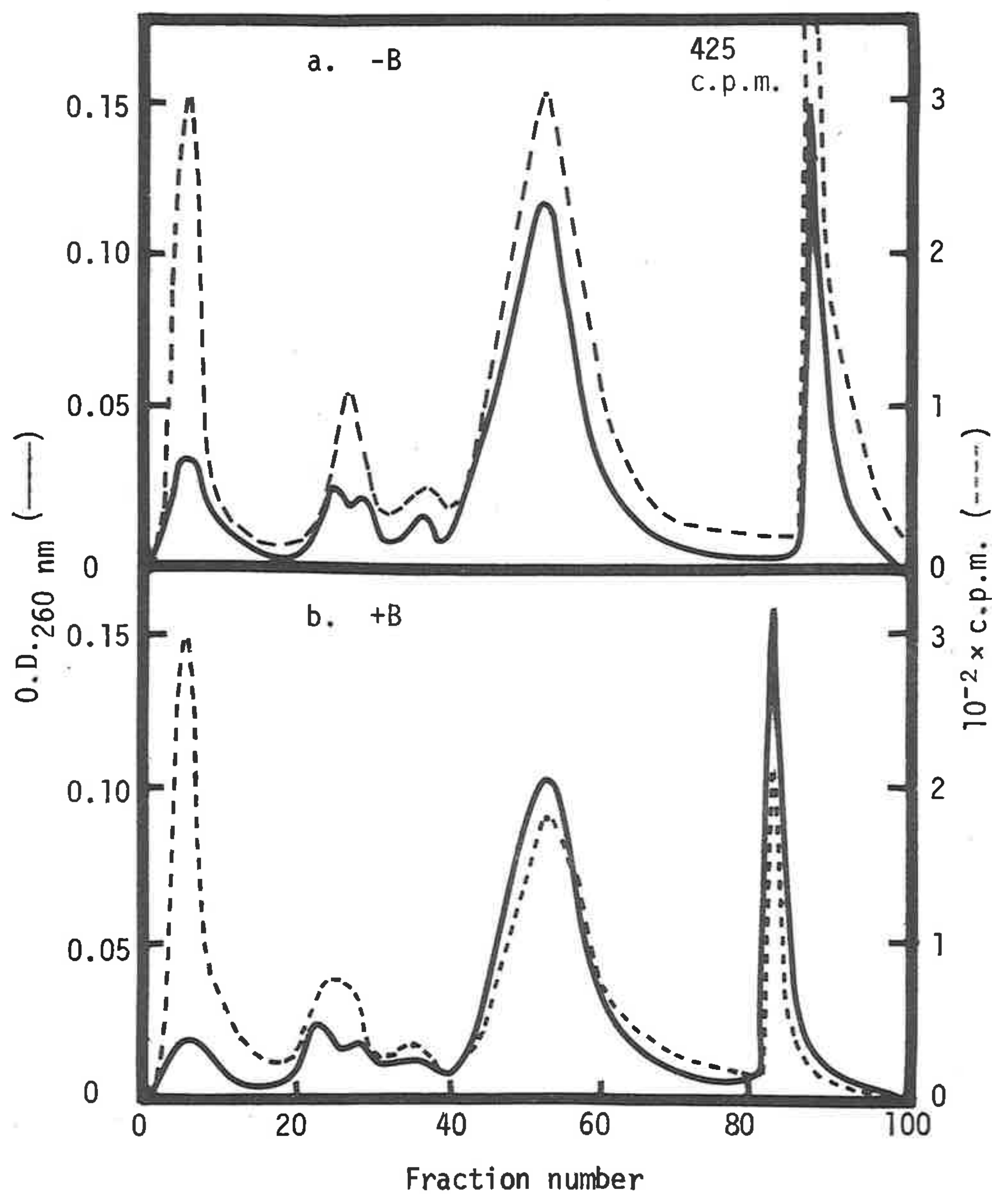


FIGURE 21 : *MAK chromatography of RNA extracted from 24 hr deficient root tips.* 400 root tips were incubated in [^{14}C]uridine (Section 2.1.5), and RNA extracted (Section 2.2.1.3) and fractionated by MAK chromatography (Section 2.3.1.1).

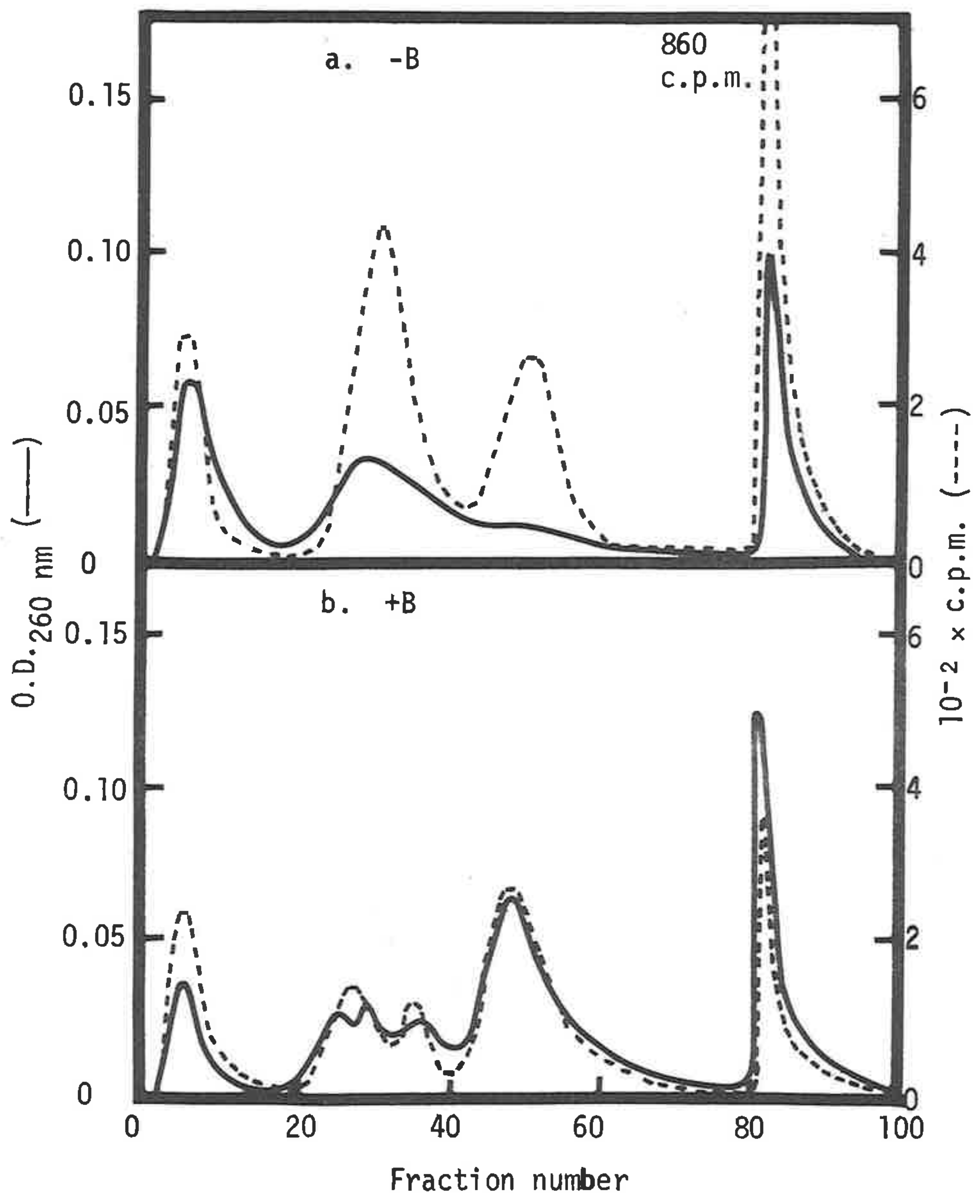
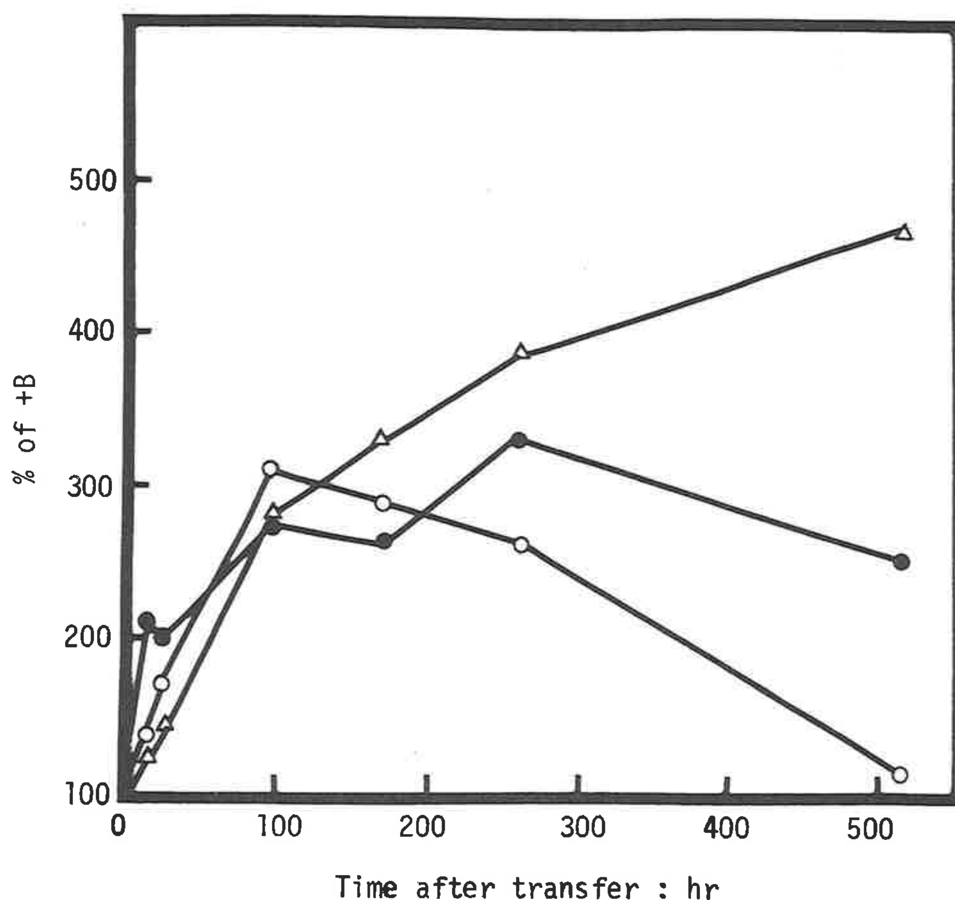


FIGURE 22 : *MAK chromatography of RNA extracted from 512 hr deficient root tips.* 400 root tips were incubated in [^{14}C]uridine (Section 2.1.5) and RNA extracted (Section 2.2.1.3) and fractionated by MAK chromatography (Section 2.3.1.1).



<u>FIGURE 23</u> : *Effect of boron deficiency on [^{14}C]uridine incorporation into RNA fractions separated by MAK chromatography.* 300-500 root tips were incubated in [^{14}C]uridine, and RNA extracted and fractionated by MAK chromatography as for Figures 21 and 22. The incorporation into deficient root tips is expressed as a percentage of the incorporation into normal root tips on the ordinate.

△ s-RNA

○ r-RNA

● TB-RNA

normal root tips. It is important to note that this is the time (50-100 hr) when inhibition and then cessation of elongation occur, and the relevance of changes in RNase activity will be discussed in Section 3.7.1.

During the first 100 hr in deficient medium the effects of the deficiency on the specific activity of these fractions (Figure 24) is the same as that on total counts, but beyond this stage different effects of boron deficiency on the RNA content of each fraction (Figure 25) change the specific-activity pattern. Because of the greatly reduced level of r-RNA but continued incorporation of radioactivity, the specific activity of this fraction continues to increase, eventually (512 hr) reaching a value almost 16 times that of the normal root-tip r-RNA. Changes in s-RNA and TB-RNA are similar to total counts, because the RNA content of these fractions does not change as much.

3.5.1.3 Dual-labelling studies

Degradation of r-RNA in deficient root tips (Figures 22 and 25) is probably due to increased RNase activity (Section 1.3.4.1) but degradation could occur either *in vivo* during the incubation period (i.e. soon after label incorporation) or during the extraction procedure. To determine whether artifacts of the extraction procedure were affecting the differences between deficient and normal tissue, mixing experiments employing dual-labelled samples were used.

FIGURE 24 : *Effect of boron deficiency on the specific activity of RNA fractions separated by MAK chromatography.* 300-500 root tips were incubated in [^{14}C]uridine and RNA extracted and fractionated by MAK chromatography as for Figures 21 and 22. The specific activity of deficient root tips is expressed as a percentage of the specific activity of normal root tips on the ordinate.

a. 0 to 170 hr in boron-deficient medium

b. 170 to 500 hr in boron-deficient medium

Δ s-RNA

o r-RNA

● TB-RNA

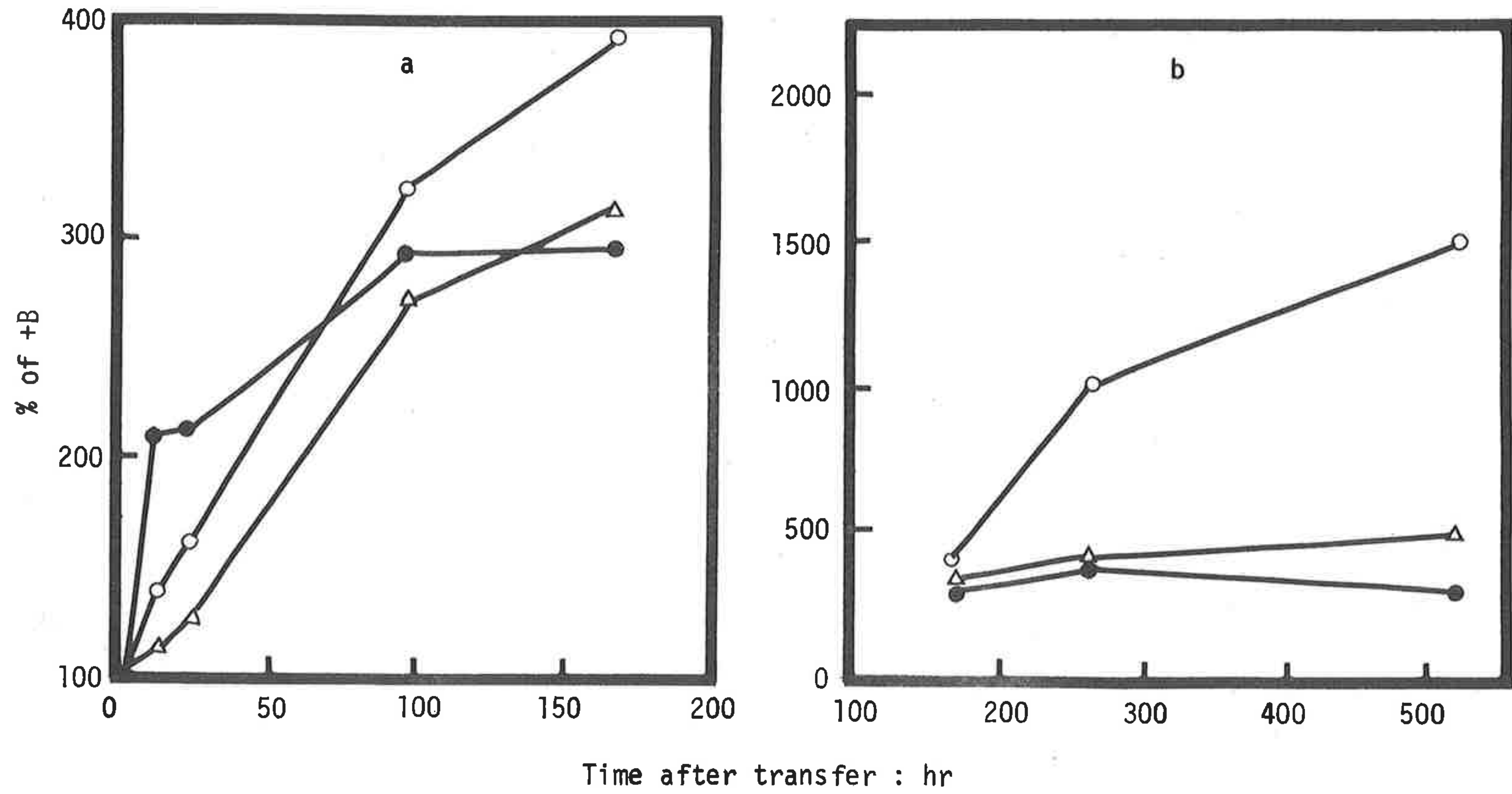


FIGURE 24 : *Effect of boron deficiency on the specific activity of RNA fractions separated by MAK chromatography.*

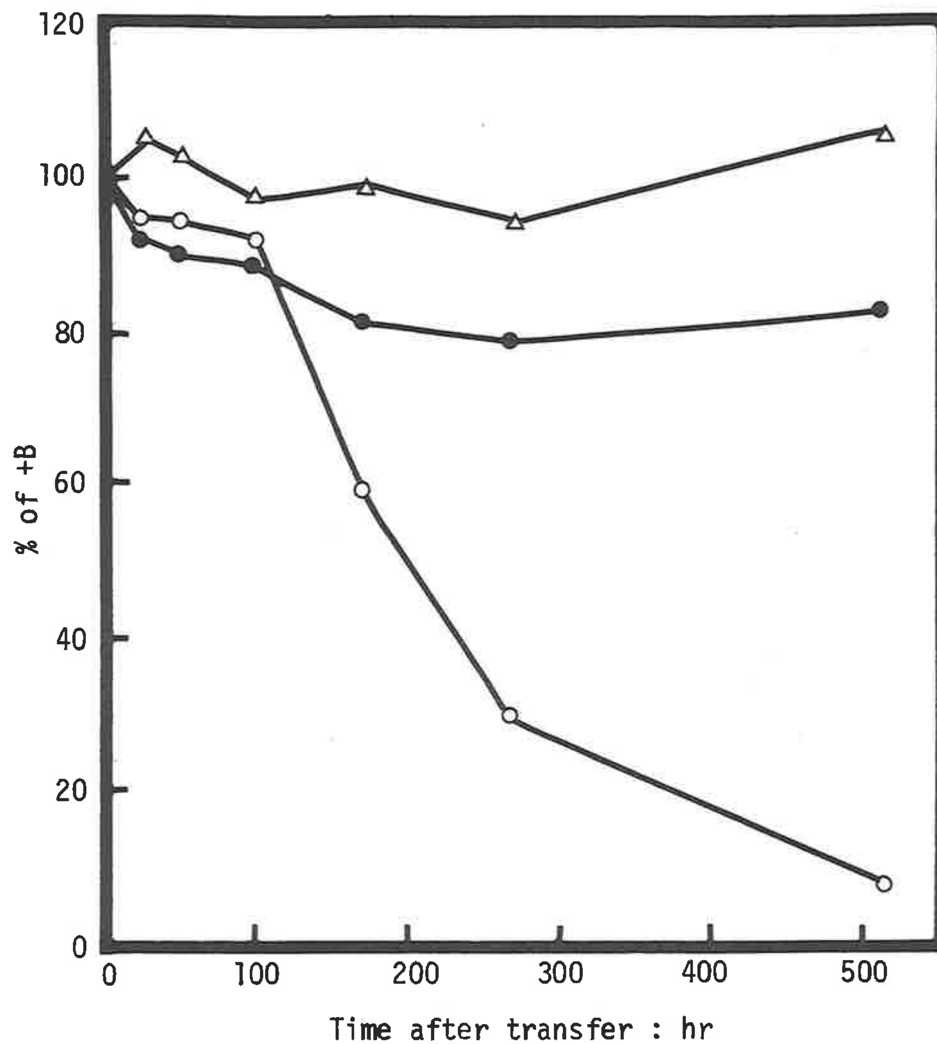


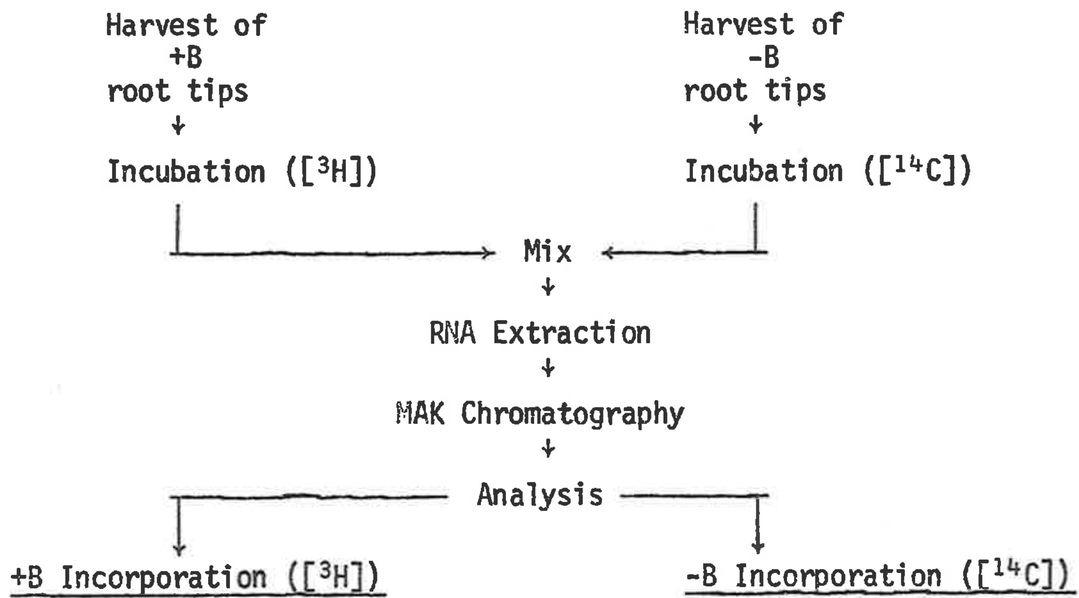
FIGURE 25 : *Effect of boron deficiency on RNA content of fractions separated by MAK chromatography.* 300-500 root tips were incubated in [^{14}C]uridine, and RNA was extracted and fractionated by MAK chromatography as for Figures 21 and 22. The RNA content of deficient tips is expressed as a percentage of the content of normal root tips on the ordinate.

△ s-RNA
○ r-RNA
● TB-RNA

Two samples of normal root tips (300-400 tips each) were incubated in [^{3}H]uridine as described in Section 2.1.5 and two samples of deficient tips treated similarly in [^{14}C]uridine. After incubation, one sample from each was combined and the RNA extracted (Section 2.2.1.3) from the combined deficient (^{14}C-labelled) and normal (^{3}H-labelled) sample. After extraction, the RNA was applied to a MAK column and eluted. Analysis of the dual-labelled fractions was performed as described in Section 2.5.1.2 to separate the radioactivity into that derived from deficient (^{14}C-labelled) and normal (^{3}H-labelled) RNA. The remaining samples of normal and deficient root tips were extracted separately, then applied to a MAK column and eluted and analysed as above. The procedure is outlined in Figure 26. If degradation or any other artifact of the extraction method is affecting deficient and normal tissue differently, the samples mixed during RNA extraction would produce labelling patterns different from those which were mixed after extraction. If degradation of RNA was occurring during extraction of deficient but not normal tissue, both should be degraded in the mixed experiment.

Identical patterns were obtained with mixed and unmixed samples, indicating that changes in chromatographic elution patterns of RNA extracted from deficient root tips are due to changes *in vivo* and not artifacts of the extraction procedure. Only the mixed samples are shown in Figures 27 and 28.

Differences between columns are also eliminated by this procedure. This permits the detection of much smaller changes in



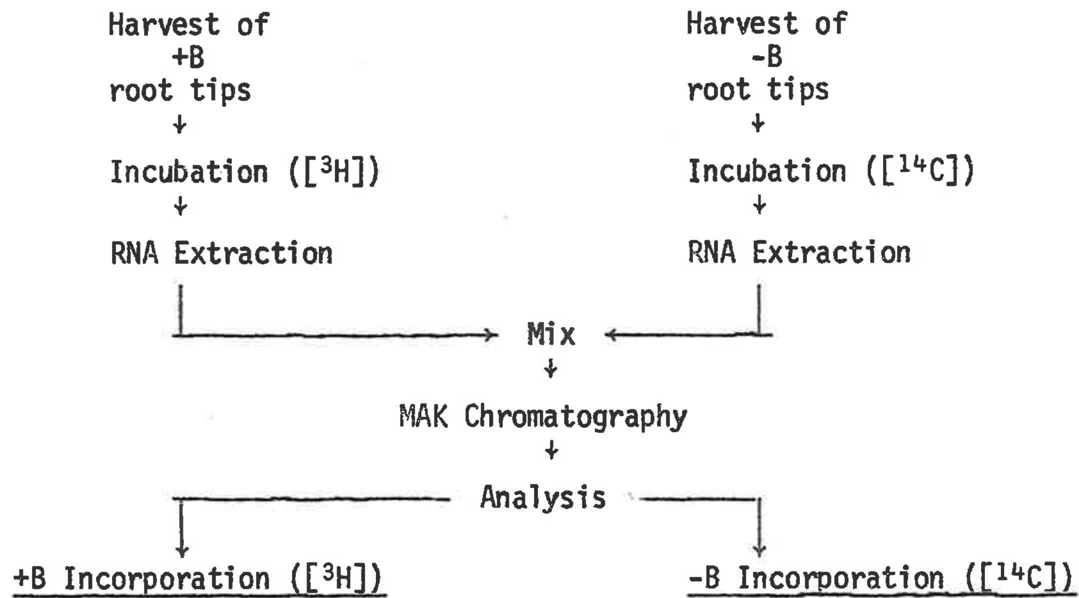


FIGURE 26 : *Procedure for dual-label experiments*

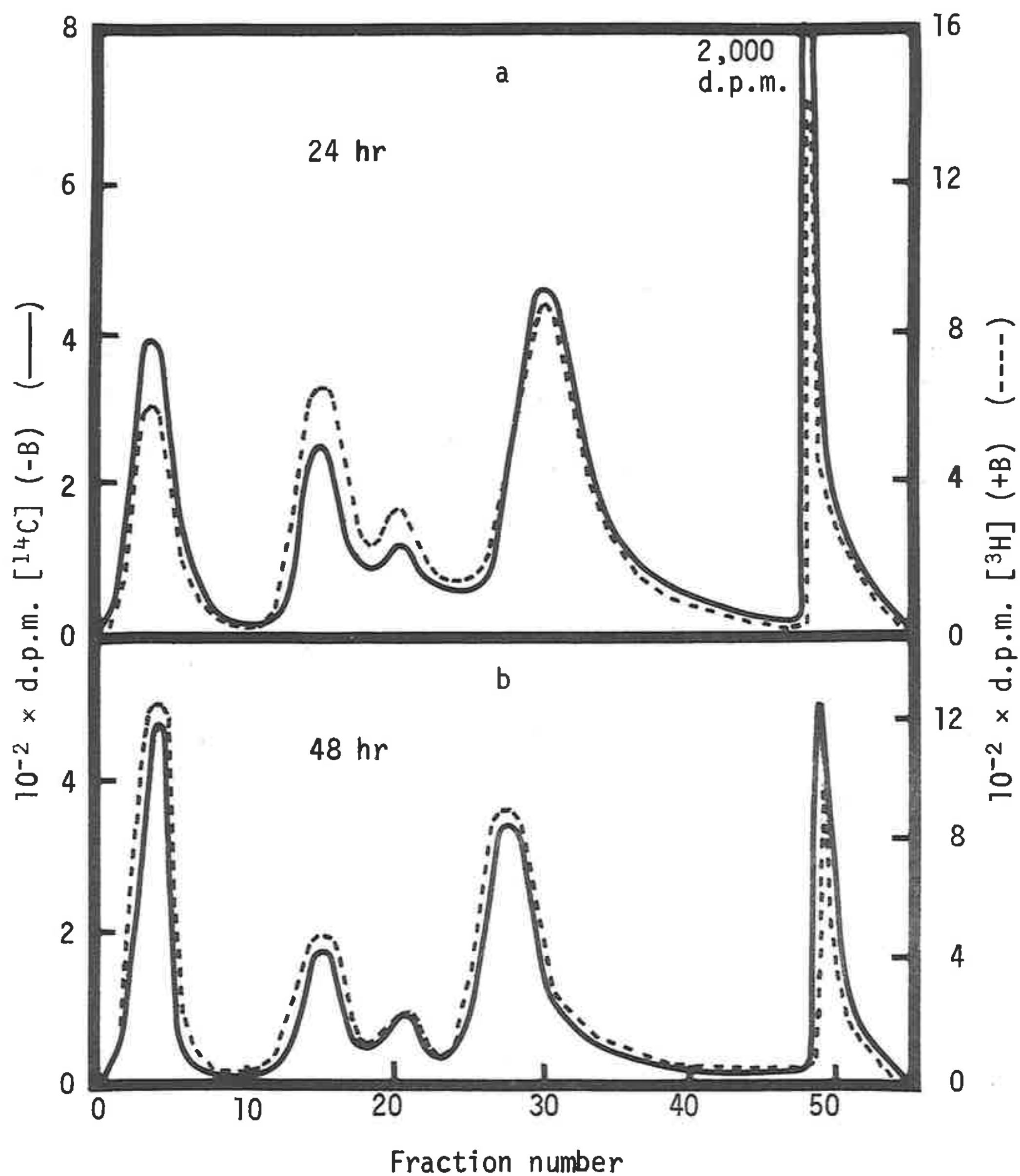


FIGURE 27 : *MAK chromatography of dual-labelled RNA samples after 24 and 48 hr of deficiency.* Root tips were incubated in ^{14}C- or ^{3}H-labelled uridine, and the RNA extracted and chromatographed as described in the text (Section 3.5.1.3) and Figure 26.

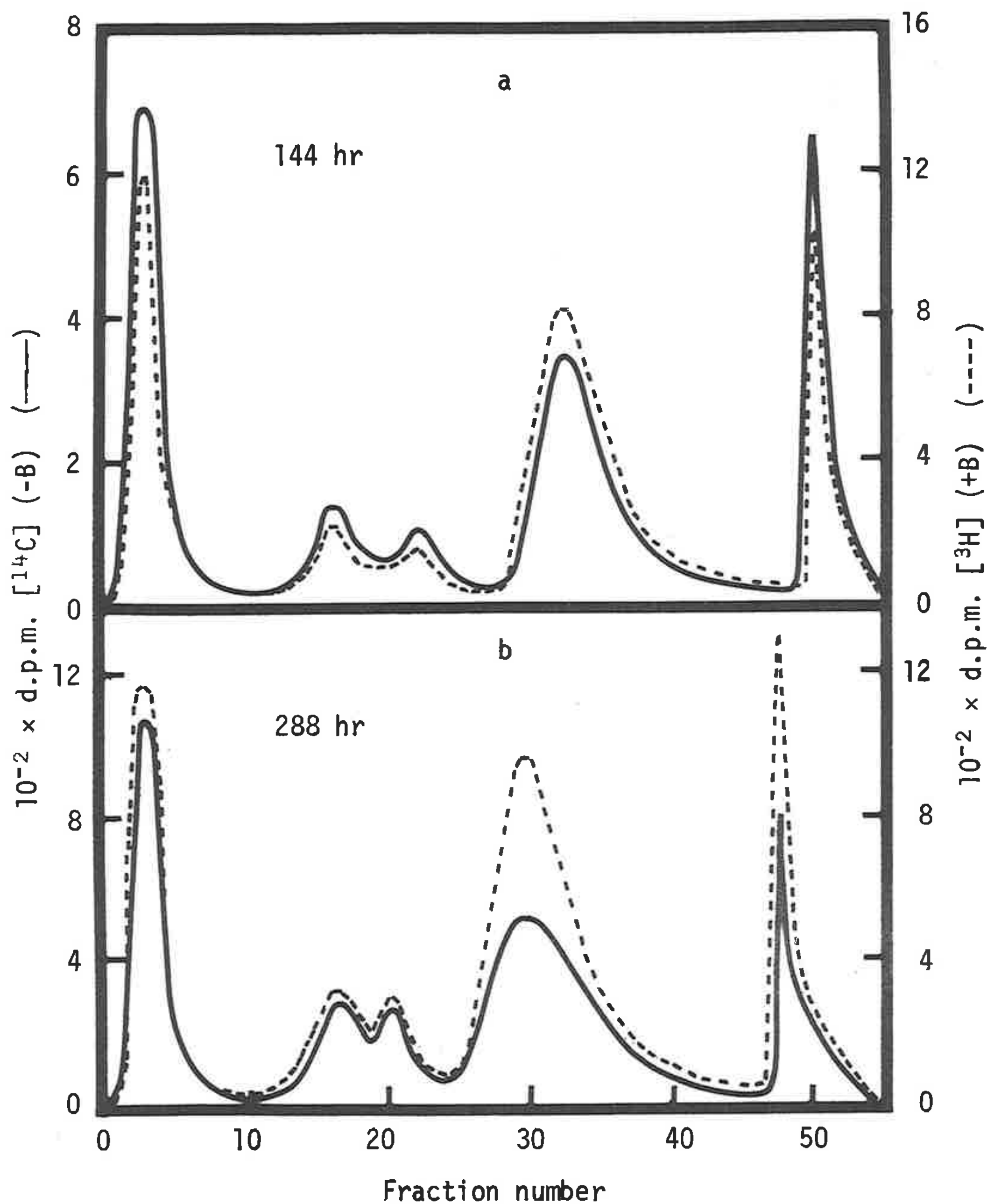


FIGURE 28 : *MAK chromatography of dual-labelled samples after 144 and 288 hr of deficiency.* Root tips were incubated in ^{14}C- or ^{3}H-labelled uridine and the RNA extracted and chromatographed as described in the text (Section 3.5.1.3) and Figure 26.

the incorporation patterns, and confirms the differences found in the previous section (Section 3.5.1.2). After 24 hr of the deficiency (Figure 27) incorporation of label into TB-RNA and r-RNA is increased more than that into s-RNA, but after 48 hr s-RNA and r-RNA are equally affected. After longer periods of deficiency (Figure 28) the effect on s-RNA becomes greater. This is more clearly demonstrated in Figure 29. The incorporation into TB-RNA is increased most by deficiency until almost 250 hr, but although incorporation into r-RNA is increased more than into s-RNA at early stages, there is a cross-over at approximately 44 hr. Thus, the effect of boron deficiency on precursor incorporation in the first 44 hr is distinctly different from that at later stages. This change in the s-RNA and r-RNA curves began at 24 hr, the time when a restriction of elongation was first observed.

Instead of using the reversed-label method to eliminate differences due to differential incorporation of the two radio-isotopes (Ellem 1967), the differences were determined directly, using two samples from plants grown with sufficient boron. These are plotted in Figure 29 as 0 hr of deficiency.

3.5.1.4 [^{32}Pi] incorporation

Incorporation of [^{32}Pi] into RNA gave similar results for the s-RNA and r-RNA fractions (Figure 30). However, the initial increase in incorporation into the TB-RNA fraction was not as great as that for labelled uridine (Figure 29),

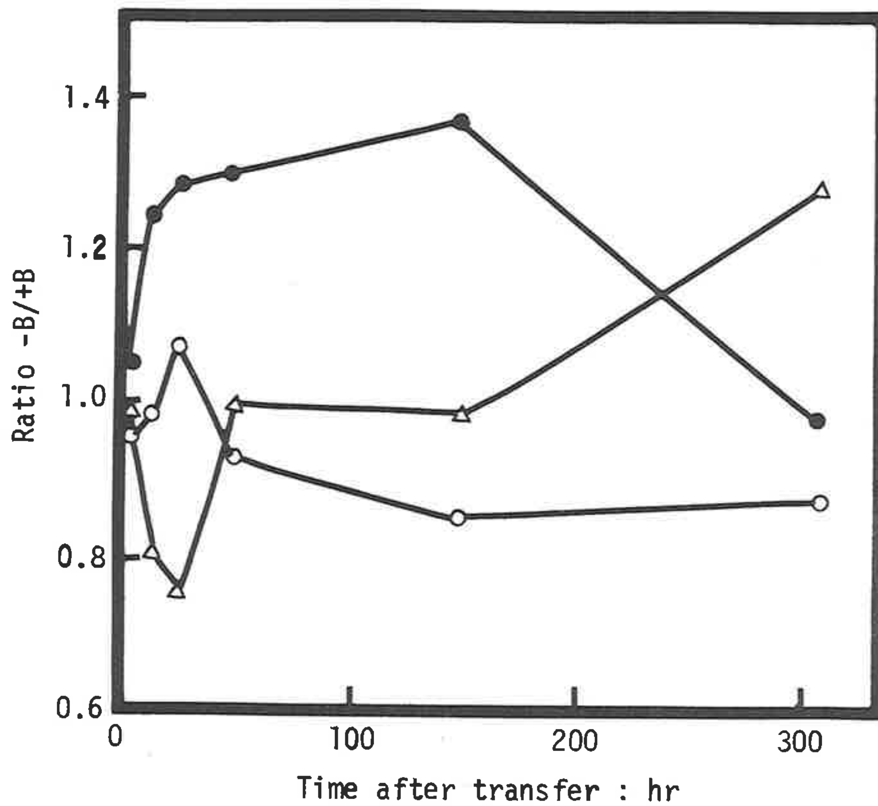


FIGURE 29 : *Effect of boron deficiency on precursor incorporation into dual-labelled RNA fractions separated by MAK chromatography.* Root tips were incubated in ^{14}C- and ^{3}H-labelled uridine and the RNA extracted and chromatographed as described in the text (Section 3.5.1.3) and Figure 26. The values plotted on the ordinate are:-

$$\frac{\%\ \text{Total -B radioactivity}}{\%\ \text{Total +B radioactivity}}$$

for each RNA species.

△	s-RNA
○	r-RNA
●	TB-RNA

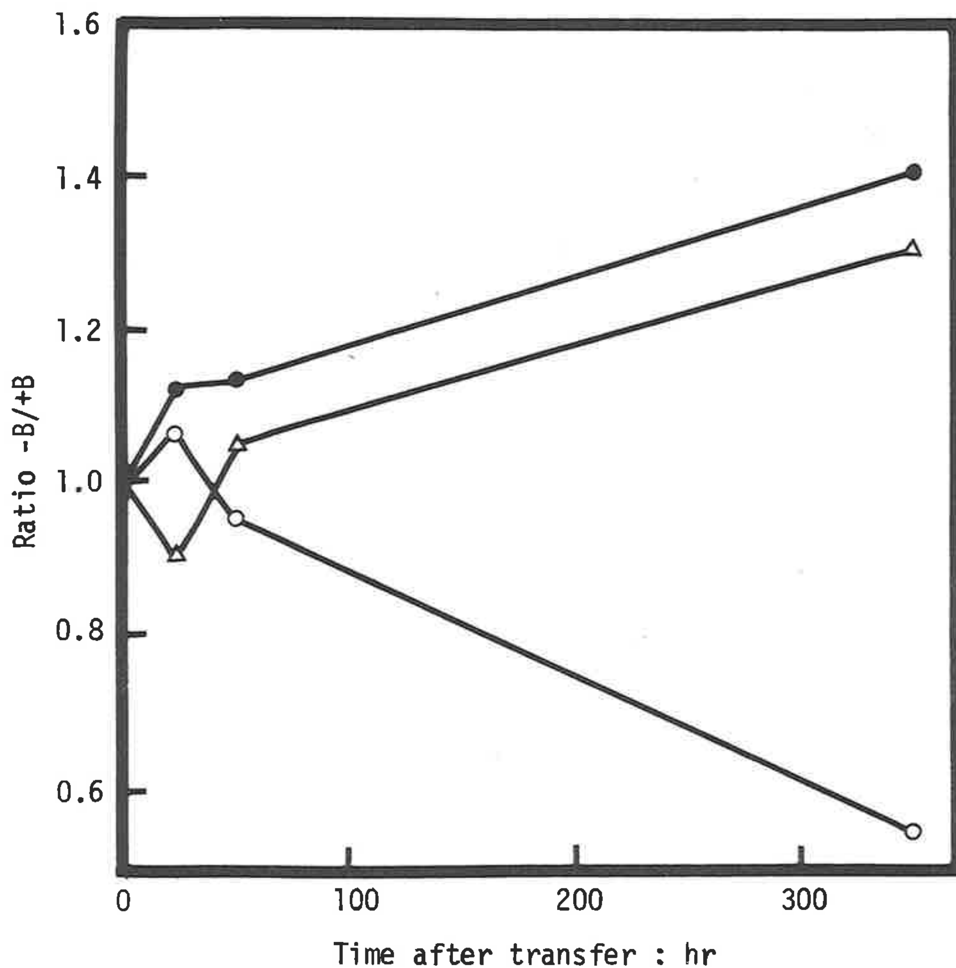


FIGURE 30 : *Effect of boron deficiency on [^{32}Pi] incorporation into RNA fractions separated by MAK chromatography.* 1000 root tips were incubated in [^{32}Pi] (Section 2.1.5) and RNA extracted (Section 2.2.1.3) and fractionated by MAK chromatography (Section 2.3.1.1). Aliquots of the collected fractions were radioassayed by Cerenkov radiation (Section 2.5.1.3). The values plotted on the ordinate are:-

$$\frac{\text{\% Total -B radioactivity}}{\text{\% Total +B radioactivity}}$$

for each RNA species.

△	s-RNA
○	r-RNA
●	TB-RNA

and after 350 hr the increased incorporation into TB-RNA was still greater than for either s-RNA or r-RNA.

3.5.2 Polyacrylamide-gel electrophoresis

The superior separation of RNA species by polyacrylamide-gel electrophoresis was used to follow the incorporation of labelled precursors into r-RNA species and to determine the apparent molecular weight of the labelled species. With the extraction method used, root-tip RNA preparations did not readily enter the gels, and only with small amounts (2-5 μg) of RNA sample was complete entry achieved. At higher loadings, only slightly more RNA entered the gels, and low molecular-weight species appeared to enter more readily than those with high values. This was insufficient RNA for a reliable densitometer trace. The entry of RNA prepared from cotyledons and young leaves of *Phaseolus aureus* by a similar method (Section 2.2.1.3) was not inhibited and the densitometer traces for this preparation are shown in Figure 31. The reason for the difference between these two RNA preparations has not been determined. It may be due to slight differences in the extraction technique (i.e. ratio of buffer to plant material during homogenization, and number of phenol extractions), or to the presence of a higher concentration of some interfering substance in root tips (e.g. polyphenols).

Electrophoresis of the RNA prepared from cotyledons and leaves revealed a number of high molecular-weight species, corresponding to those reported for green plant tissue by Loening

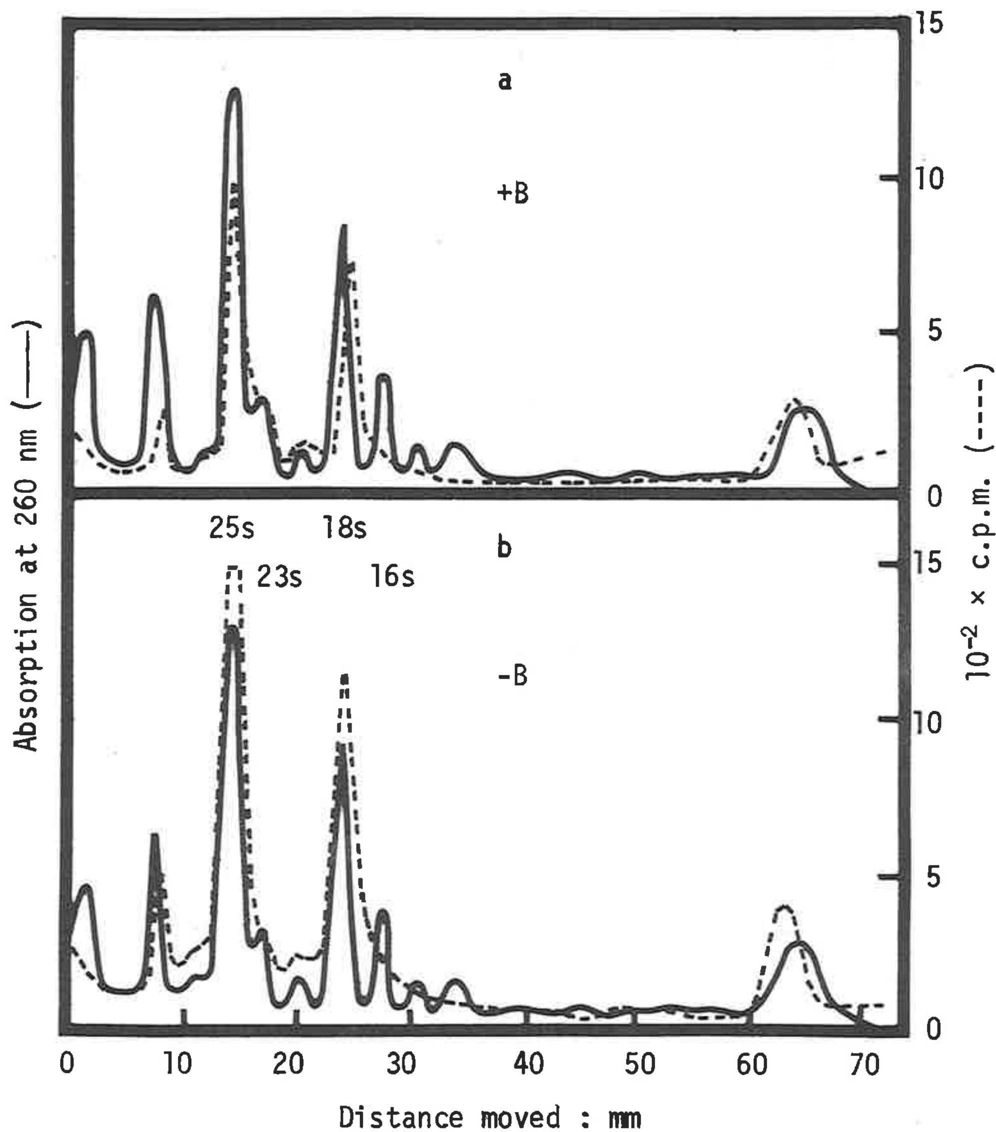


FIGURE 31 : *Polyacrylamide-gel electrophoresis of ^{32}P-labelled RNA extracted from 24 hr deficient root tips.* 400 root tips were incubated in [^{32}Pi] (Section 2.1.5) and RNA extracted as described in Section 2.2.1.3. 5 μg were loaded onto the gels, together with 40 μg of RNA extracted from cotyledons and young leaves (Section 2.2.1.3). Electrophoresis (Section 2.3.1.2) was for 115 min.

& Ingle (1967) and Ingle (1968). In Figure 31, 5 μg of ^{32}P-labelled root-tip RNA was run with 40 μg of RNA extracted from cotyledons and young leaves. The densitometer trace is therefore primarily that of green-tissue RNA, and the radioactivity curve is due to the [^{32}Pi] incorporated into the RNA of normal and boron-deficient root tips. The deficiency increases the incorporation of [^{32}Pi] into all species of RNA, but particularly into r-RNA, affecting the two r-RNA species equally.

The radioactivity traces for later stages of deficiency are shown in Figure 32. The increases in [^{32}Pi] incorporation are similar to those observed with MAK chromatography and the two r-RNA species are again equally affected.

Loening (1965) and Rogers *et al.* (1970) have shown that root tips excised from 2- to 3-day-old pea seedlings and cultured in 2% (w/v) sucrose, synthesize 18s and 25s r-RNA slowly, resulting in an accumulation of ribosomal precursors. This situation was not observed in the present work. However, Chen (1971), using excised *Phaseolus aureus* root tips incubated in a complete mineral medium in addition to sucrose, also obtained labelled 18s and 25s r-RNA after incubating for 18 hr in [^{3}H]uridine.

Unlike the 2- to 3-day-old pea seedlings germinated on vermiculate moistened with water used by Loening (1965) and Rogers *et al.* (1970), the root tips used in this work were excised from mature plants (at least 14 days after germination) growing in a complete mineral medium. In this study, the root tips were longer

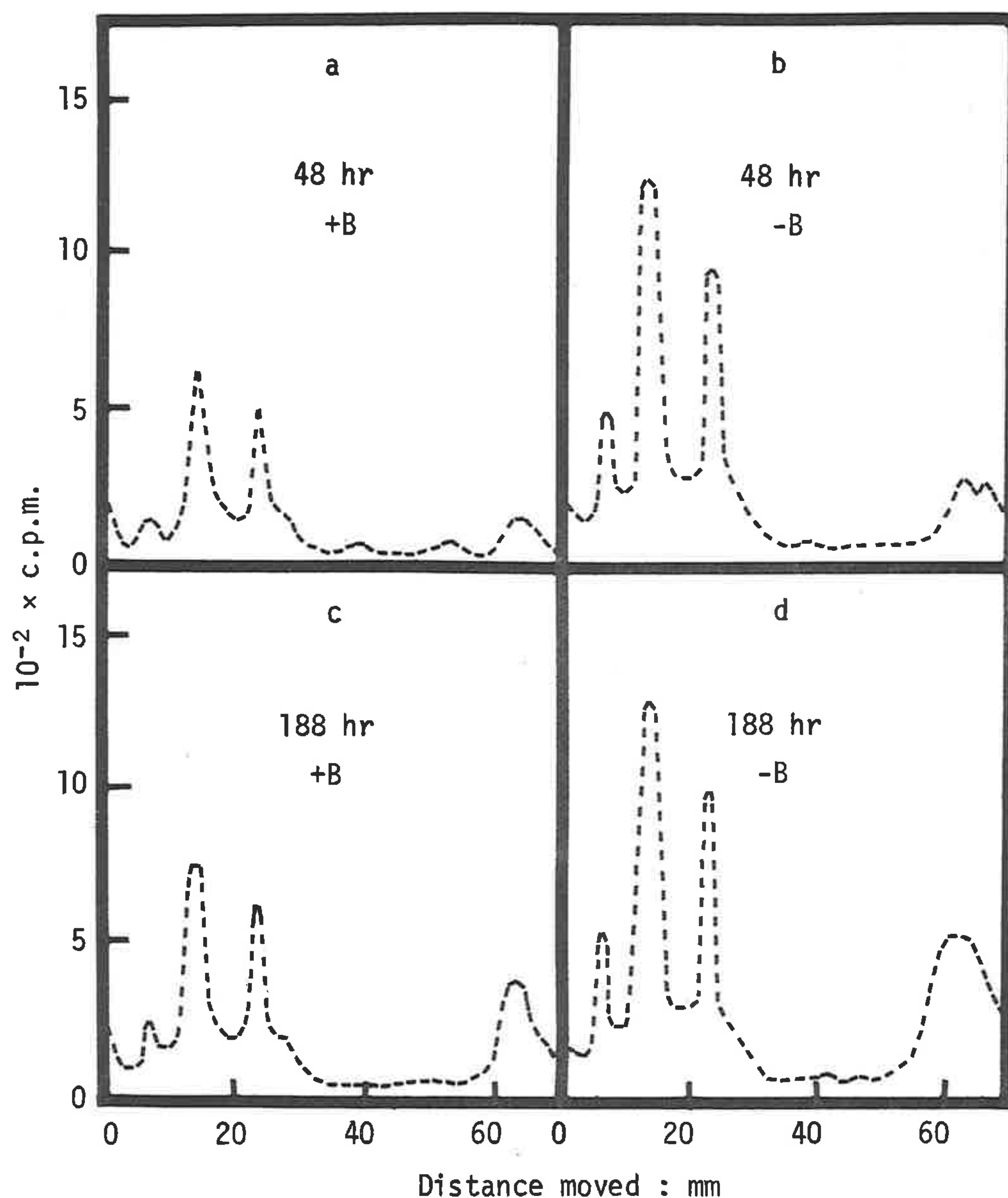


FIGURE 32 : *Polyacrylamide-gel electrophoresis of ^{32}P-labelled RNA extracted from 48 (a & b) and 188 (c & d) hr deficient root tips.* 400 root tips were incubated in [^{32}Pi] (Section 2.1.5) and RNA extracted as described in Section 2.2.1.3. 2.5 μg were loaded onto the gels and electrophoresis (Section 2.3.1.2) was for 105 min.

than those reported by the above authors, and incubation was in nutrient medium of similar or identical composition to that in which the root tips had been growing (and similar to that used by Chen 1971). The root tips are therefore not subjected to such a severe step-down in nutritional conditions as those of Loening (1965) and Rogers *et al.* (1970). In addition, the incubation period is longer than that employed by these authors, and Loening (1965) has indicated that longer incubation periods result in the formation of more ribosomal RNA (as observed by Chen 1971). Repeated re-extraction of the interphase layer in the phenol method (Loening *et al.* 1969) is necessary to obtain a good yield of the labile (Rogers *et al.* 1970) r-RNA precursors. Thus, with the present extraction technique, much of this RNA fraction would remain in the unextracted interphase layer, and any in the aqueous layer would probably be degraded. Consequently, in the system used here, some synthesis of 18s and 25s r-RNA could be expected and even if there is an accumulation of ribosomal precursors, these would not be extracted without being degraded. It must be emphasized, however, that the rates and types of RNA synthesis in the excised root tissue may not accurately reflect the situation in the intact tissue. This does not decrease the importance of the differences observed between normal and boron-deficient tissue.

3.6 Properties of RNA

The increased incorporation of precursors into RNA may be due to a general increased rate of synthesis, or synthesis of particular (or new) RNA base sequences. If the increase is not general, but is restricted to particular base sequences, this may involve changes in the properties of the newly synthesized RNA. To investigate this, base composition (of incorporated radioactive precursors) and DNA-RNA hybridization studies were undertaken.

3.6.1 Base composition

The effect of boron deficiency on the nucleotide distribution of [^{32}P] in the RNA species separated by MAK chromatography is shown in Table 9. While there are differences between RNA species, the base composition of individual species is not significantly altered by growth in deficient medium for 24 or 48 hr. After 360 hr there are some changes, the most important being that the s-RNA fraction becomes more like the r-RNA fraction. This is consistent with the suggestion that in grossly deficient tissue much of the radioactivity eluting as s-RNA is due to degraded r-RNA (Section 3.5.1.2). TB-RNA is not affected at any stage of deficiency.

Effects of boron deficiency on the base distribution of label in total RNA were studied by alkaline hydrolysis of the ^{32}P-labelled acid-insoluble (RNA) fraction (Section 2.2.1.2) followed by chromatographic separation of the mononucleotides on Dowex-1.

TABLE 9 : *Distribution of [^{32}P] among component nucleotides of RNA species separated by MAK chromatography*

300-400 root tips were incubated in [^{32}Pi] (Section 2.1.5), RNA was extracted (Section 2.2.1.3) and the RNA species were separated by MAK chromatography (Section 2.3.1.1). After RNA was recovered and hydrolysed, the nucleotide distribution of [^{32}P] was analysed by paper electrophoresis as described in Section 2.3.1.3. Values are expressed as the percentage of the radioactivity associated with each nucleotide.

Time after transfer to boron-deficient medium (hr)	Treatment	CMP	AMP	GMP	UMP
	s-RNA fraction				
24	+B	21.4	28.8	28.9	21.9
	-B	20.3	28.4	27.0	24.3
48	+B	20.8	25.7	31.3	22.2
	-B	21.5	25.6	28.4	24.5
360	+B	22.1	28.7	27.2	22.0
	-B	18.0	29.6	31.1	21.3
	r-RNA fraction				
24	+B	16.6	28.7	29.4	25.3
	-B	16.2	28.1	30.5	25.2
48	+B	17.5	29.3	30.4	22.8
	-B	18.2	28.2	29.7	23.9
360	+B	15.2	29.0	31.7	24.1
	-B	19.9	27.7	29.6	22.8
	TB-RNA fraction				
24	+B	23.0	28.0	25.9	23.1
	-B	23.3	28.5	26.2	22.0
48	+B	21.2	31.2	25.3	22.3
	-B	21.5	30.4	26.0	22.1
360	+B	21.6	29.9	25.7	22.8
	-B	22.0	30.5	25.2	22.3

This also indicated that boron deficiency does not affect the nucleotide distribution of [^{32}P] after 48 hr growth in deficient medium, but AMP and GMP increase slightly after 144 hr (Table 10).

TABLE 10 : *Distribution of [^{32}P] among component nucleotides of total RNA*

900-1000 root tips were incubated in [^{32}Pi] (Section 2.1.5), and the acid-insoluble fraction was extracted as described in Section 2.2.1.1 and hydrolysed in 0.3 M-KOH at 37° for 16 hr. The mononucleotides were separated on Dowex-1 (Section 2.3.2.2). Values are expressed as the percentage of the total radioactivity associated with each RNA fraction.

Time after transfer to boron-deficient medium (hr)	Treatment	CMP	AMP	GMP	UMP
48	+B	23.3	25.3	26.7	24.6
	-B	23.5	25.4	26.6	24.5
144	+B	23.0	25.2	28.1	23.7
	-B	21.9	26.5	28.6	23.0

Thus, differences in base distribution of [^{32}P] within total or MAK-fractionated RNA can be detected only after long periods of growth in boron-deficient medium. However, the sensitivity of these methods is only sufficient to detect large changes in composition, and changes in RNA base sequences need not necessarily

involve changes in base composition.

3.6.2 DNA-RNA hybridization

With the complex RNA preparations obtained from higher organisms, it is difficult to obtain sufficient locus specificity in DNA-RNA hybridization reactions to enable simple interpretation of the results (Church & McCarthy 1968, Paul & Gilmour 1968, Bishop 1969, Bishop *et al.* 1969, McCarthy & Church 1970). Studies involving saturation determinations are particularly difficult to interpret (McCarthy & Church 1970). Although still subjected to the same criticisms, competitive hybridization experiments can with the use of discriminatory annealing conditions (Church & McCarthy 1968) provide useful information. Competition between identical RNA preparations is always greater than that between preparations from different organs or organisms (see Paul & Gilmour 1966, Church & McCarthy 1968). It is only the sensitivity of the technique which can be questioned. Within an RNA population, individual base sequences anneal with DNA at vastly different rates (due mainly to differences in concentration) and some RNA base sequences synthesised from unique DNA base sequences fail to react. These factors result in an underestimation of the extent of homology between the RNA and DNA samples, and thus will lead to under-estimation of differences between RNA populations. The technique cannot be used to *prove* identity, since this may be readily confused with lack of resolution. Qualitative differences are

not easily distinguished from quantitative distortions in the population distribution (McCarthy & Church 1970). Thompson & Cleland (1971*b*) have recently discussed and improved the application of the technique to plant nucleic acids.

In spite of these limitations, competitive DNA-RNA hybridization is the most sensitive technique available for comparing RNA samples. So long as it is realized that any measured differences will be minimal estimates of the real extent of dissimilarity, the technique can be applied to complex RNA samples.

Bishop *et al.* (1969) have pointed out that straight-forward interpretation of competitive experiments is dependent on the following conditions: (1) Use of a concentration of labelled RNA sufficient to saturate the DNA, and (2) use of a concentration of unlabelled RNA sufficient to dilute out identical labelled RNA sequences completely. In practice, however, these conditions cannot be met, and only approximations are possible.

Preliminary experiments with the technique demonstrated the following facts:-

a. Some DNA (up to 20%) is lost from the filters during incubation, but this only occurs during the first hour, so that after the 1 hr preincubation (see Section 2.3.1.4) further losses are small (Figure 33).

b. The amount of hybrid formed reaches a maximum after 10-12 hr of annealing (Figure 34), a time course

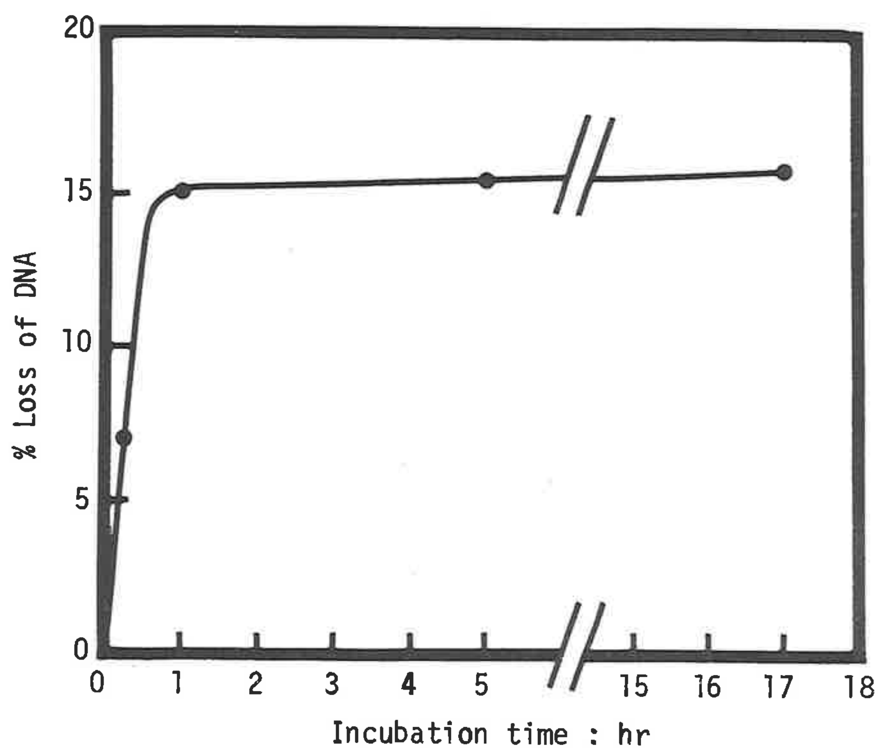


FIGURE 33 : *Rate of DNA loss from nitrocellulose filter-discs.* 5 discs each containing 1.0 μg of DNA were incubated in 0.2 ml of 2×SSC at 70° for the times indicated and the DNA remaining on the filters was determined by the deoxyribose assay (Section 2.2.2.3).

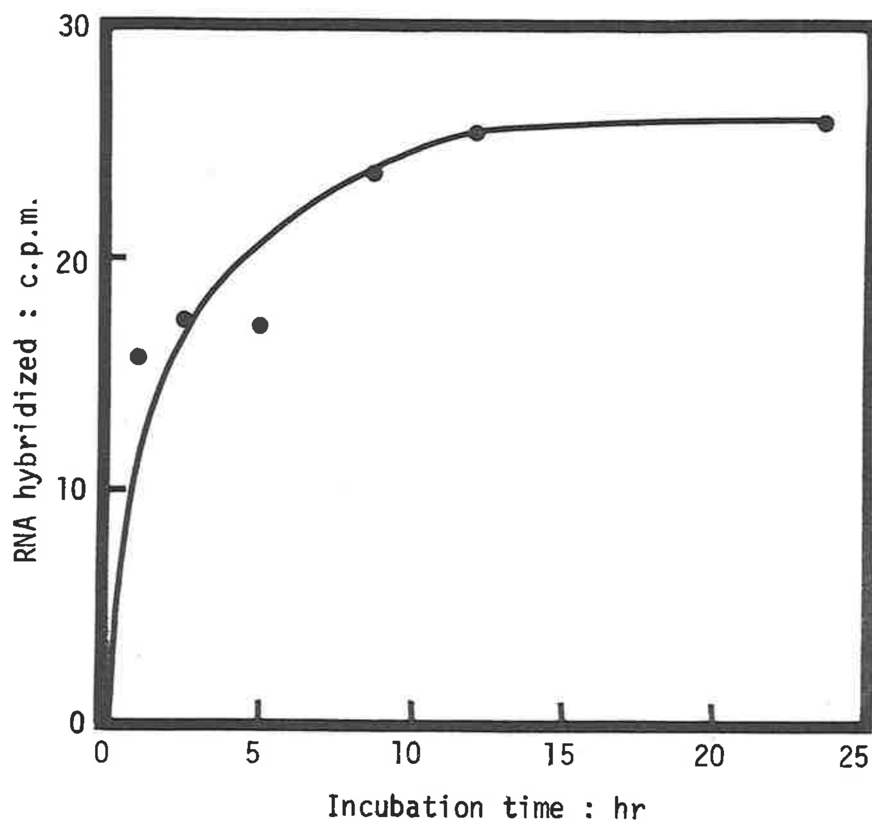


FIGURE 34 : *Rate of DNA-RNA hybrid formation.*
4 discs each containing 1.0 μg of DNA were incubated in 0.2 ml of 2×SSC containing 10 μg of ^{32}P-labelled RNA at 70° for the times indicated. The amount of hybrid formed was determined as described in Section 2.3.1.4. The specific activity of the RNA used was 150 cpm/μg (approx.).

which is similar to that reported for nucleic acids from higher organisms under similar annealing conditions (Melli & Bishop 1969, Chen 1971).

c. Treatment of DNA-containing discs with DNase I before annealing almost eliminated the formation of hybrid. Under the conditions used [100 μg DNase/ml in 10 mM-$MgCl_2$ and 10 mM-Tris (pH 7.4) and incubated for 60 min at 37°] it was found that DNase I removes most of the DNA from the filter discs.

d. DNA-loaded filter discs have negligible RNase activity (Table 11). The importance of this has been discussed by Gillespie & Spiegelman (1965).

TABLE 11 : *Degradation of RNA during annealing*

Filter discs containing 1.5 μg of DNA were incubated in 0.2 ml of 2×SSC containing 37 μg of yeast RNA for 12 hr at 70°. RNA degradation was determined by the method described in Section 2.4.1.

Filter discs	RNA degraded (μg)	RNA degraded (%)
-	0.80	2.2
-	0.48	1.3
2	0.96	2.6
2	1.68	4.5

In attempting to fulfil the first condition of Bishop *et al.* (1969) (i.e. the use of a concentration of labelled RNA sufficient to saturate the DNA), saturation curves were obtained for each RNA preparation (Figures 35, 37 and 39). Because of the small amounts of RNA available, complete saturation could not be achieved, and only curves approaching saturation were possible. In each case, the curve for RNA from boron-deficient root tips is higher than that from normal tissue because of the higher specific activity of the former (e.g. the 144 hr samples had specific activities of 1816 and 1040 c.p.m./μg for RNA from deficient and normal plants respectively). The quantities of RNA available required that values far from the saturation level be used in competitive hybridization studies, and restricted dilution with unlabelled RNA to only 10- to 15-fold (Figures 36, 38 and 40).

After 24 and 48 hr of growth in deficient medium, no differences in the competition curves could be detected (Figures 36 and 38). Thus, at these early stages of deficiency, within the limits of sensitivity discussed earlier in this section, the two RNA preparations (from normal and deficient plants) do not differ in their base sequence composition.

After 144 hr of boron-deficient growth, unlabelled RNA from both normal and deficient tissues competes equally well with labelled RNA from normal plants (Figure 40a) but not with labelled RNA from deficient plants (Figure 40b). RNA from normal tissue does not compete as successfully with labelled RNA from deficient tissue as does the homologous RNA, indicating that there may be

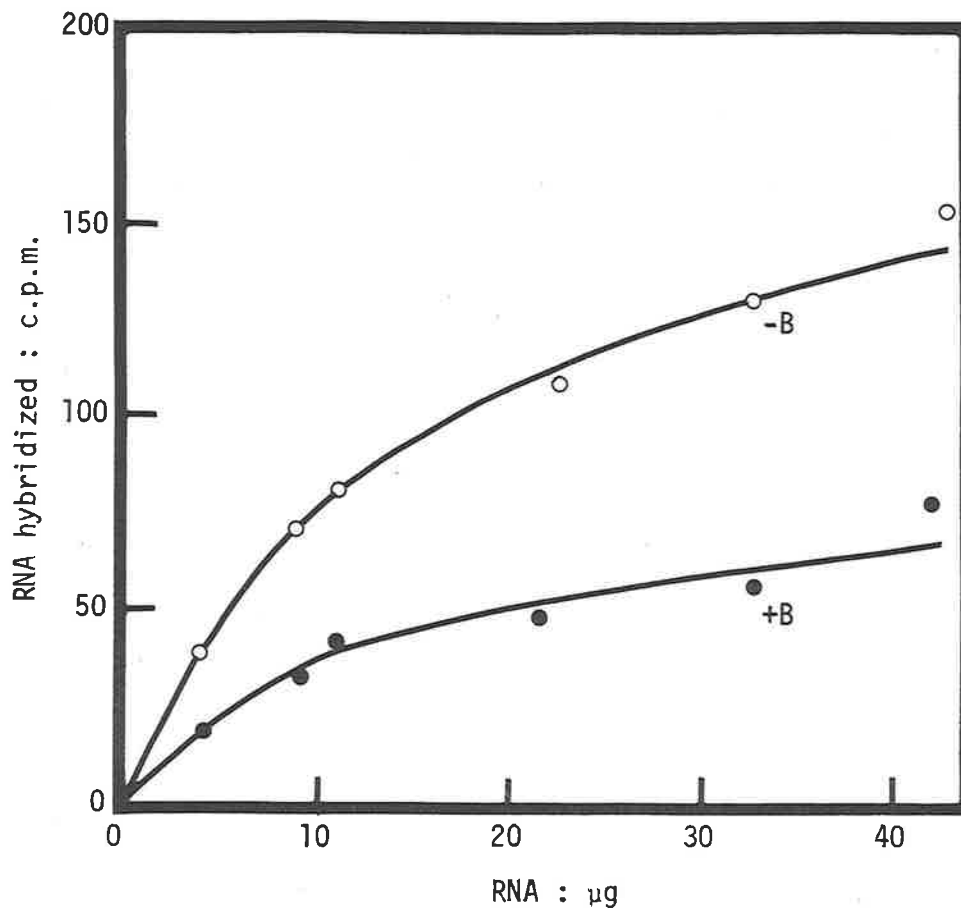


FIGURE 35 : *Saturation curves for DNA-RNA hybrid formation after 24 hr of deficient growth.* 3 discs each containing 0.4 μg of DNA were annealed (Section 2.3.1.4) with the amounts of ^{32}P-labelled RNA indicated and the hybrid formation determined as described in Section 2.3.1.4. The specific activity of the -B RNA is higher than that for +B RNA (see Fig 39).

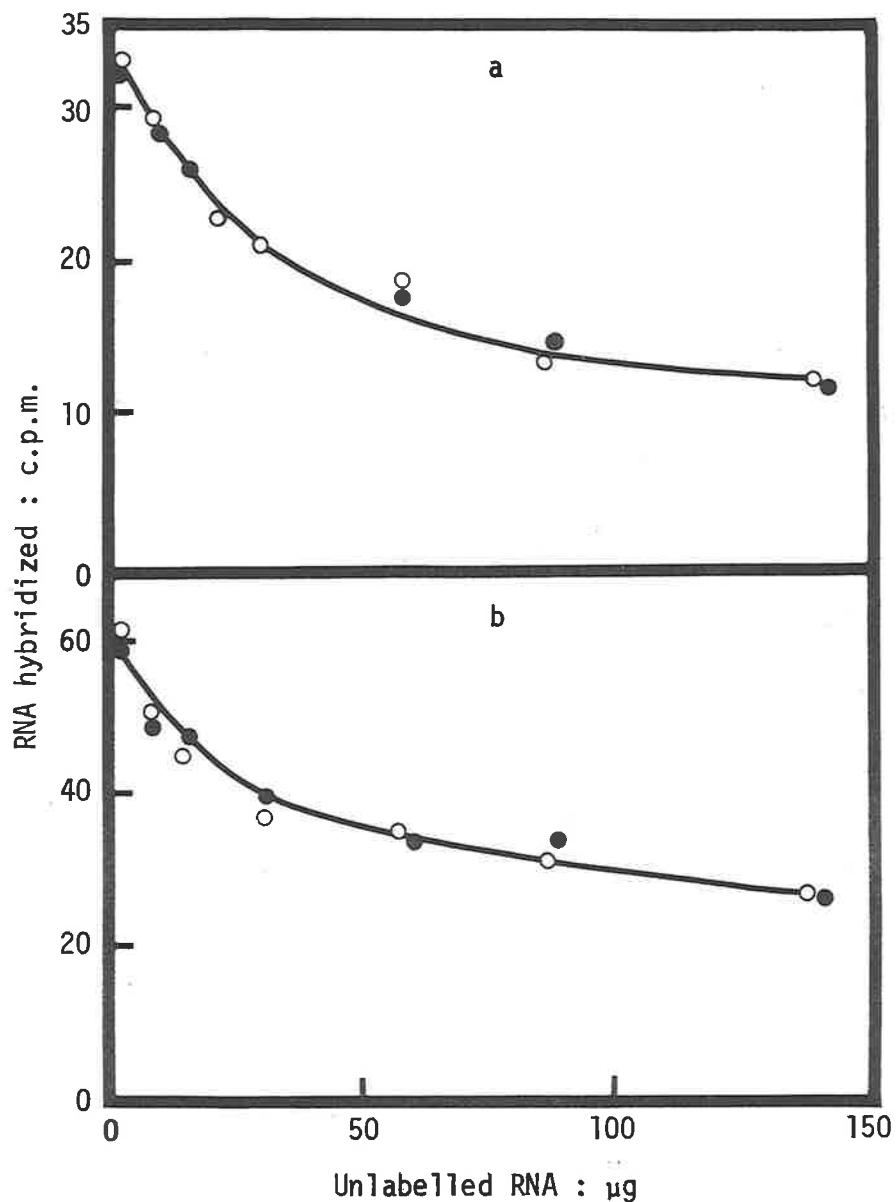


FIGURE 36 : *Competitive DNA-RNA hybridization after 24 hr of deficient growth.* 3 discs each containing 0.4 µg DNA were annealed with 11 µg of ^{32}P-labelled +B (a) or -B (b) RNA in the presence of various amounts of competing +B (●) or -B (O) unlabelled RNA. Hybrid formation was determined as described in Section 2.3.1.4.

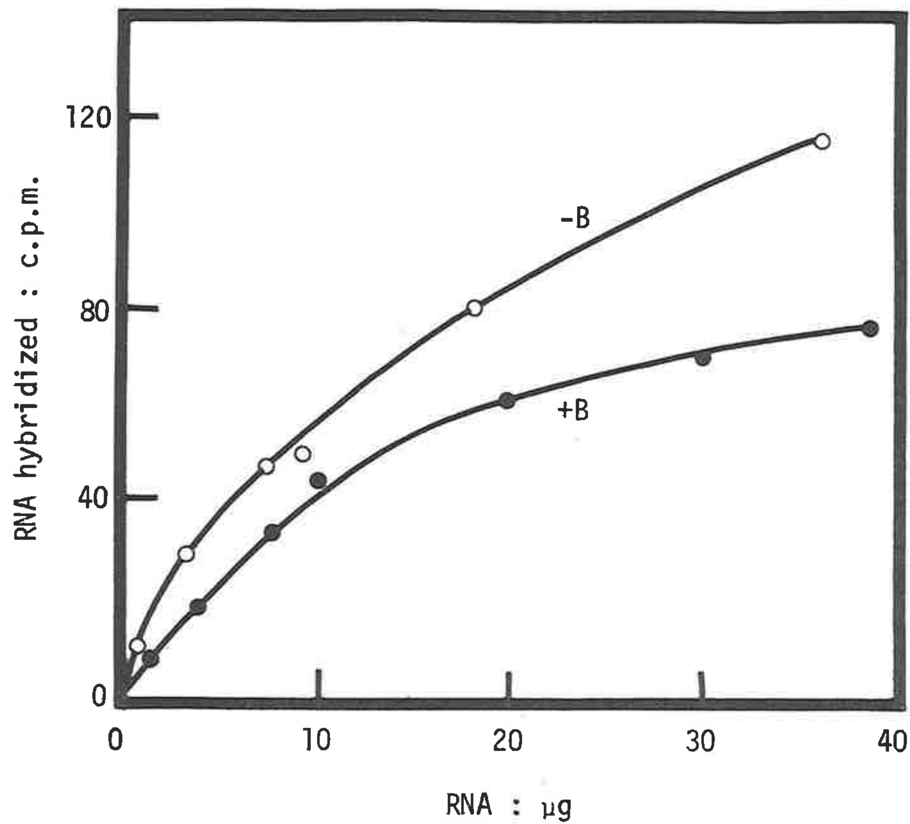


FIGURE 37 : *Saturation curves for DNA-RNA hybrid formation after 48 hr of deficient growth.* 3 discs each containing 0.7 µg of DNA were annealed (Section 2.3.1.4) with the amounts of ^{32}P-labelled RNA indicated and the hybrid formation determined as described in Section 2.3.1.4. The specific activity of the -B RNA is higher than that for +B RNA (see fig. 39).

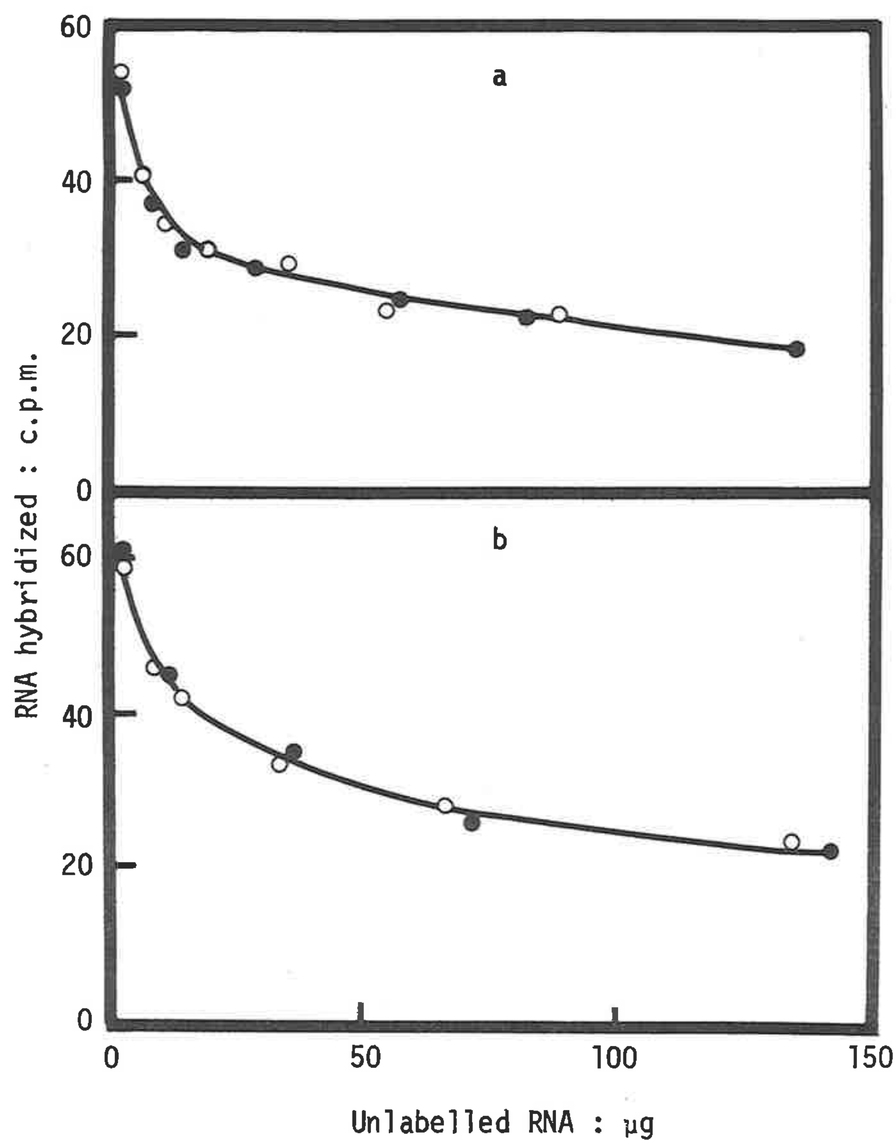


FIGURE 38 : *Competitive DNA-RNA hybridization after 48 hr of deficient growth.* 3 discs each containing 0.75 μg of DNA were annealed with 15 μg of ^{32}P-labelled +B (a) or -B (b) RNA in the presence of various amounts of competing +B (●) or -B (○) unlabelled RNA. Hybrid formation was determined as described in Section 2.3.1.4.

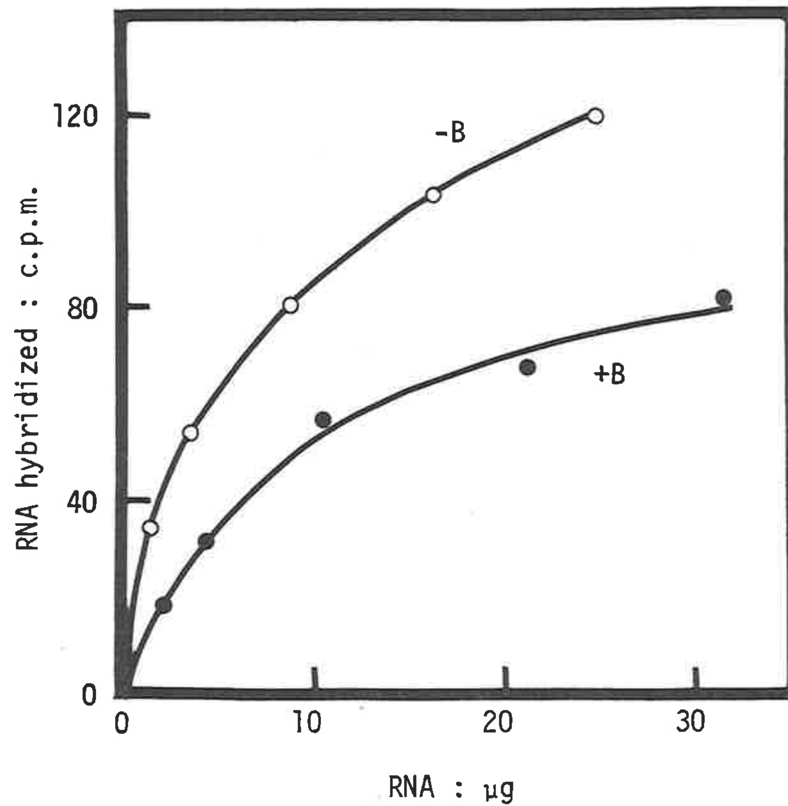


FIGURE 39 : *Saturation curves for DNA-RNA hybrid formation after 144 hr of deficient growth.* 3 discs each containing 0.5 µg of DNA were annealed (Section 2.3.1.4) with the amounts of ^{32}P-labelled RNA indicated and the hybrid formation determined as described in Section 2.3.1.4. The specific activity of RNA used was 1816 and 1040 cpm / µg for RNA from deficient & normal plants respectively.

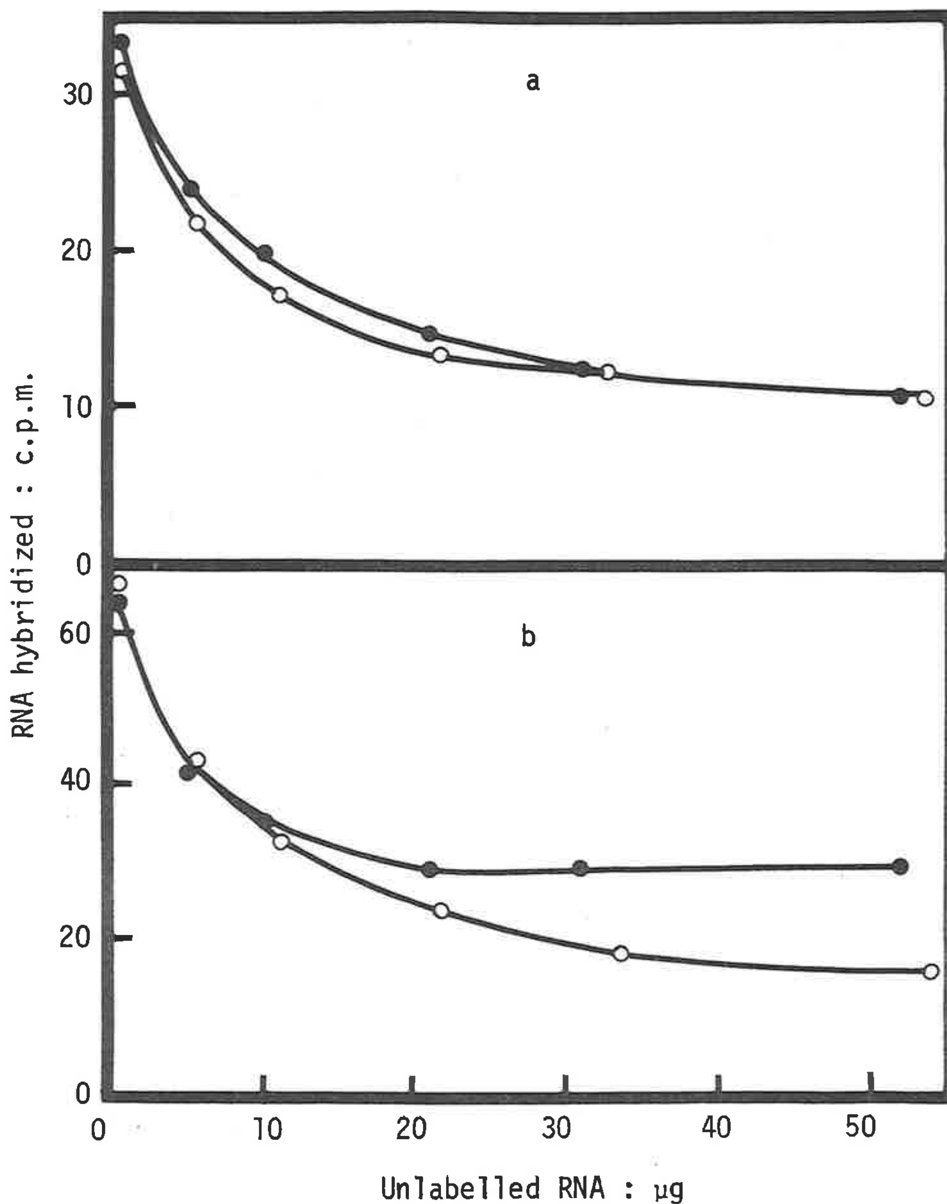


FIGURE 40 : *Saturation curves for DNA-RNA hybrid formation after 144 hr of deficient growth.* 3 discs each containing 0.5 µg of DNA were annealed with 5 µg of ^{32}P-labelled +B (a) or -B (b) RNA in the presence of various amounts of competing +B (●) or -B (O) unlabelled RNA. Hybrid formation was determined as described in Section 2.3.1.4.

some RNA base sequences in the sample from deficient tissue with which RNA from normal tissue does not compete. The possibility that degradation of r-RNA into lower molecular-weight material at this stage of deficiency permits more rapid annealing and produces the differences observed (Figure 40b) is unlikely, because such degradation should produce a similar effect with labelled normal RNA (Figure 40a). The presence of any ^{32}P-labelled contaminant in the RNA preparation should also effect competition with both normal and deficient labelled RNA. The differences could be due to changes in the concentration of particular base sequences within the RNA population, or to the synthesis of additional RNA types in deficient root tips. These differences indicate that the technique is capable of detecting at least some changes within the RNA preparations.

Within the limits of sensitivity of the technique, these results suggest that early stages of boron deficiency (up to 48 hr) do not result in any changes in the type or distribution of newly synthesized, hybridizable RNA. After 144 hr of deficiency, some base sequences are produced in deficient root tips which either are not present or are present in different concentrations in normal root tips.

Attempts to carry out competitive hybridization studies with the RNA species separated by MAK chromatography were unsuccessful because of the limited quantities of RNA available from the system.

3.7 Enzyme Activity Determinations

Enzyme activities of crude extracts were determined as described in Section 2.4. RNase activity has been shown to increase in grossly deficient tissue (Section 1.3.4.1), but it is of interest to determine whether this increase can be correlated with the decreased RNA levels observed in this and other studies (Sections 1.3.1 and 3.2.2).

It is also important to determine whether increased incorporation of labelled precursors into RNA can be explained by increases in the activity of enzymes involved. Uridine kinase and RNA polymerase were chosen for study because they may represent the first and last enzymes in the pathway of incorporation of uridine into RNA.

3.7.1 RNase

RNase activity was determined as described in Section 2.4.1, and the results are shown in Figure 41. On either basis (per mg of protein or per root tip), boron deficiency results in a sharp increase in activity, but only after at least 100 hr of growth in deficient medium. This is the stage at which the RNA level begins to decline (Figure 7 in Section 3.2.2) and slightly precedes the cessation of root elongation (Figure 4 in Section 3.1.1). The fall in RNA content induced by boron deficiency may therefore be due to increased RNase activity. The decrease in RNase activity per root tip in both normal and deficient plants

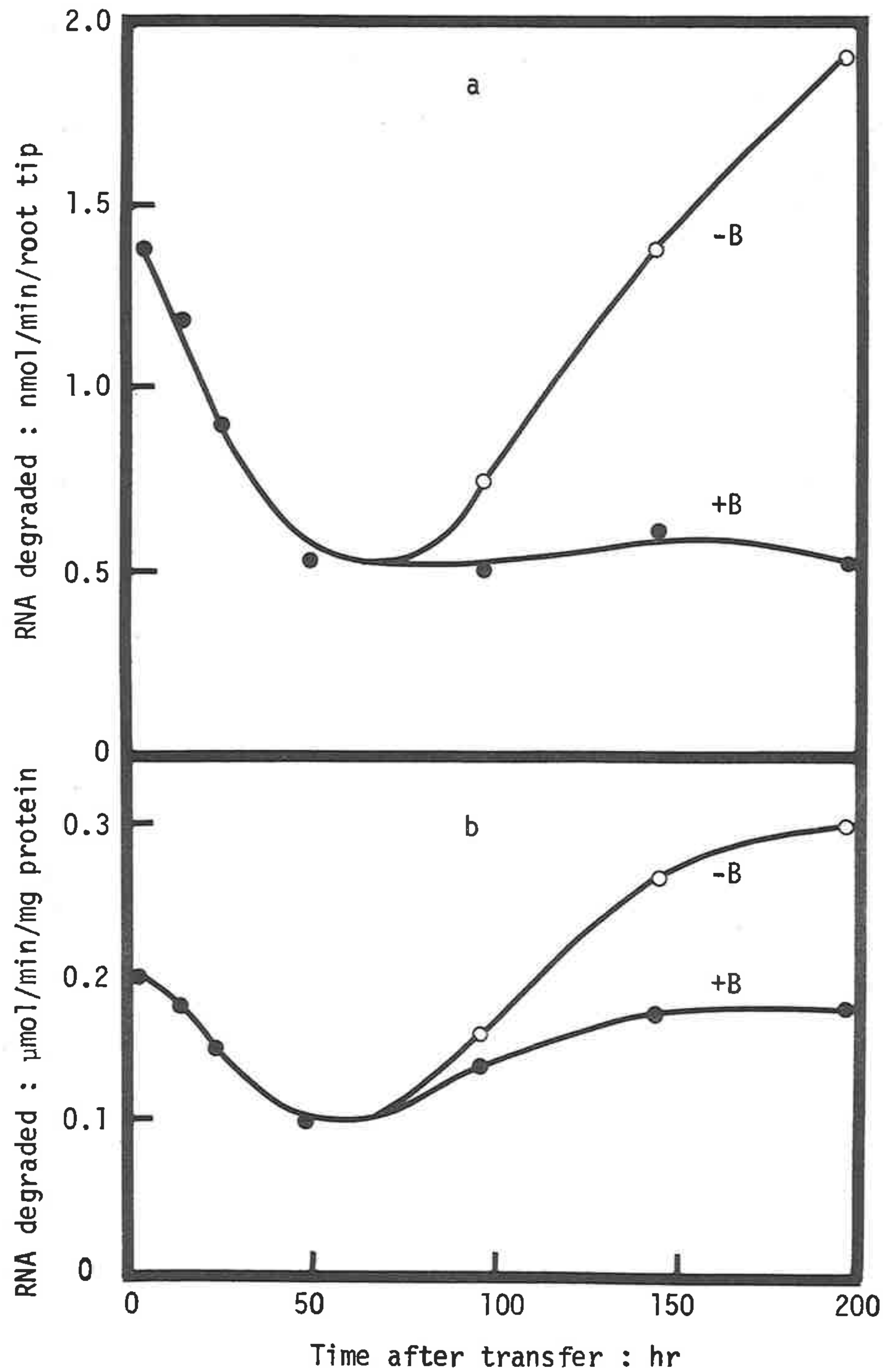


FIGURE 41 : *Effect of boron deficiency on RNase activity.* 100 root tips were harvested (Section 2.1.4) and the protein content (Section 2.6.1) and RNase activity (Section 2.4.1) determined.

over the first 50 hr (Figure 41a) is largely due to the decrease in fresh weight of these tips (Figure 5 in Section 3.1.2), but the decrease per mg of protein (Figure 41b) has not been explained. It may be due to some metabolic change in seedling growth, particularly in lateral root growth, which is rapid at this stage.

3.7.2 Uridine kinase

Table 12 shows that only when calculated on a per root-tip basis does boron deficiency affect the uridine kinase activity. Even on this basis, it is only after 91 hr growth in deficient medium that a major effect is observed, suggesting that the increased incorporation of precursors into RNA is not due to increased rates of precursor synthesis. This was also indicated in Section 3.4. At all stages of deficiency, a large proportion of [^{14}C] within the acid-soluble fraction is still in the form of [^{14}C]uridine (or uracil — Figures 16 and 17 and Table 7 in Section 3.4), possibly because this is a rate-limiting step in the pathway of nucleotide synthesis. After 48 hr of growth in deficient medium, the rates of incorporation of exogenous uridine (from the incubation medium) into RNA of deficient and normal root tips are 0.8 and 0.4 pmol per mg of root tips (f.w.) per hr respectively. This is less than the uridine kinase activity measured *in vitro* (Table 12), but as endogenous uridine is also incorporated into RNA, the total rate of uridine utilization may be as great as the *in vitro* rate of UMP synthesis. Without determining the specific activity of [^{14}C] in specific nucleotide

TABLE 12 : *Effect of boron deficiency on uridine kinase activity*

2000 root tips were harvested (Section 2.1.4) and the protein content (Section 2.6.1) and uridine kinase activities (Section 2.4.2) determined.

Time after transfer to boron-deficient medium (hr)	Treatment	pmol UMP produced/hr		
		/mg protein	/mg tissue (f.w.)	/root tip
49	+B	943	14.3	5.18
	-B	1040	16.2	6.24
91	+B	1455	19.9	5.82
	-B	1292	20.4	8.02

pools, the total rate of utilization cannot be calculated. The accumulation of [^{14}C]uridine could also occur because uridine is taken up into parts of the cell or tissue lacking uridine kinase activity.

3.7.3 RNA polymerase

With the crude extract used, it was impossible to obtain an accurate estimation of RNA polymerase activity. Figures 42, 43 and 44 show that the reaction rates are not linear with either incubation time or amount of plant extract. This is probably because high levels of nuclease and phosphatase enzymes

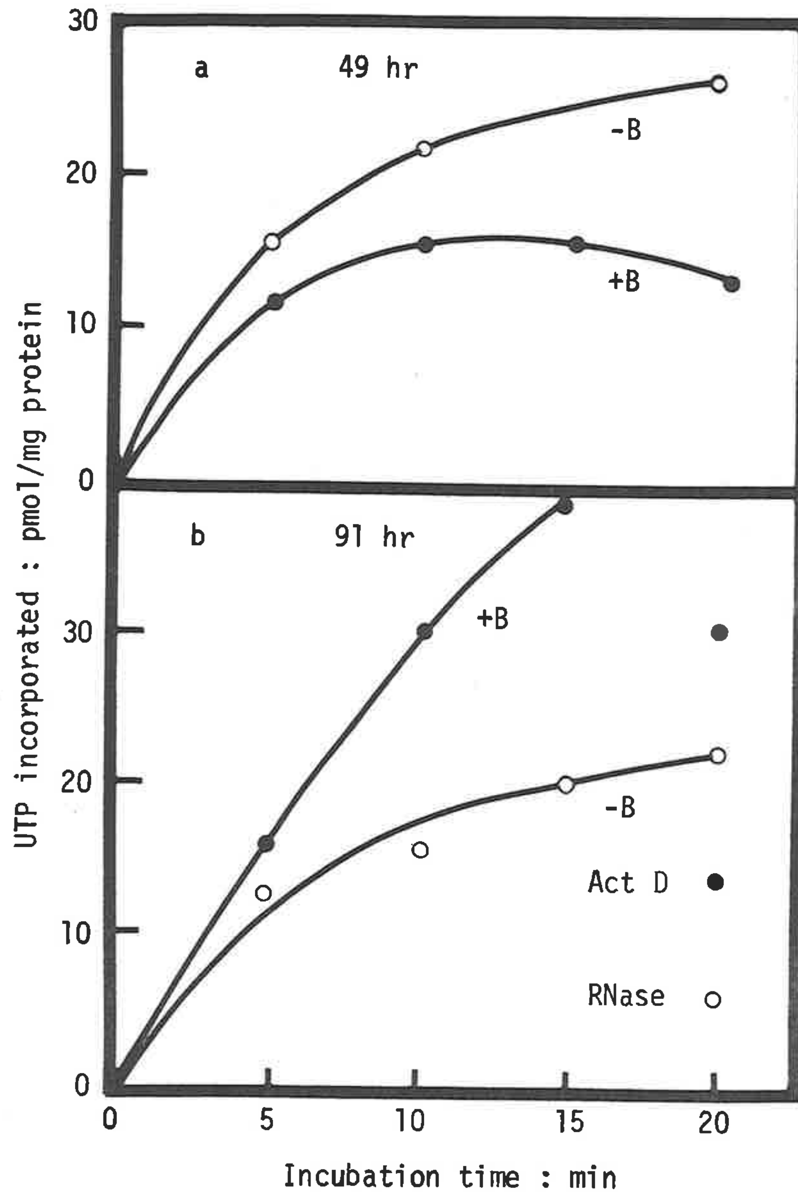


FIGURE 42 : *Effect of incubation time on RNA polymerase activity after 49 (a) and 91 (b) hr of growth in deficient medium.* 2000 root tips were harvested (Section 2.1.4) and the protein content determined (Section 2.6.1). RNA polymerase activity was determined (Section 2.4.3) using 0.02 ml of plant extract. The effects of Actinomycin D (10 μg/ml) and RNase (150 μg/ml) included in the incubation mixture are shown in (b).

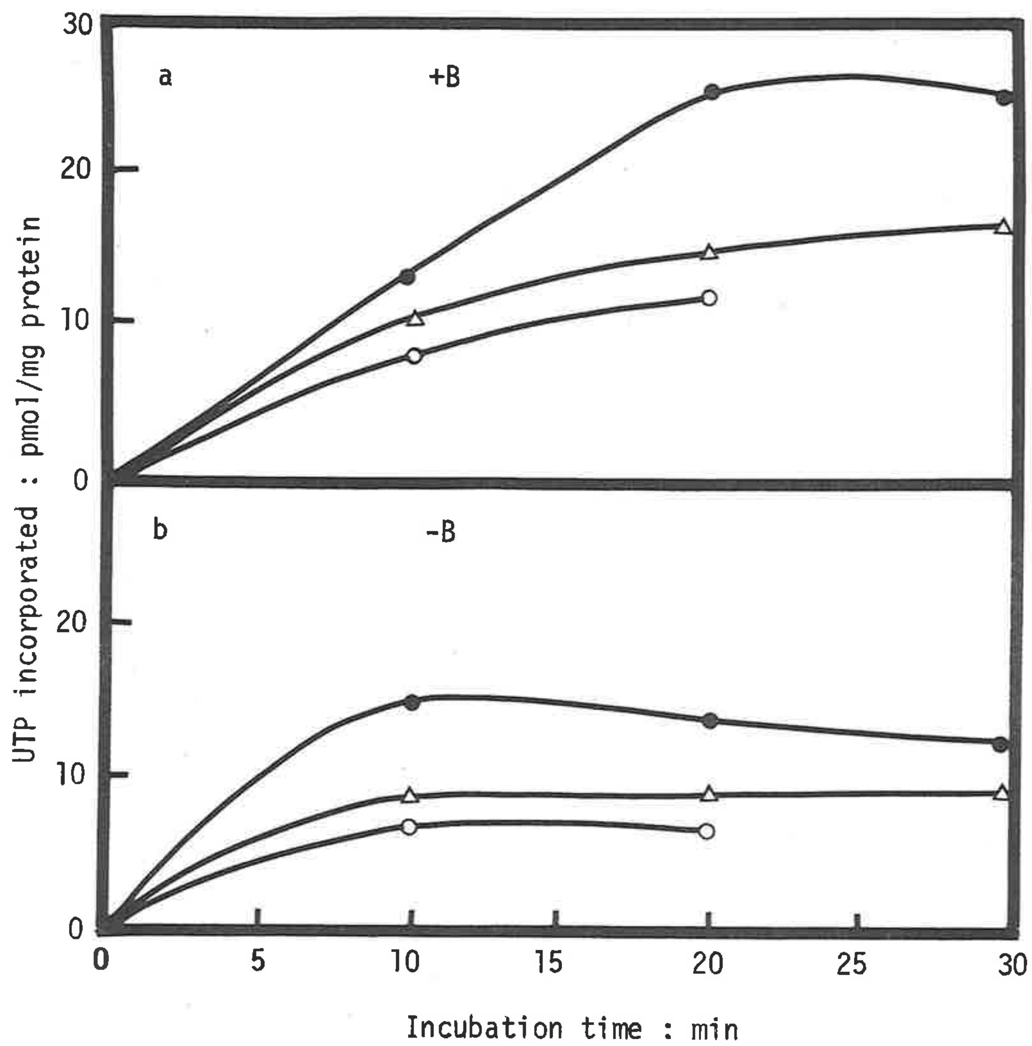


FIGURE 43 : *Effect of incubation time on RNA polymerase activity after 216 hr of growth in deficient medium.*
2000 root tips were harvested (Section 2.1.4) and the protein content determined (Section 2.6.1). RNA polymerase activity was determined (Section 2.4.3) using 0.01 ml (●), 0.02 ml (△) or 0.05 ml (○) of plant extract.

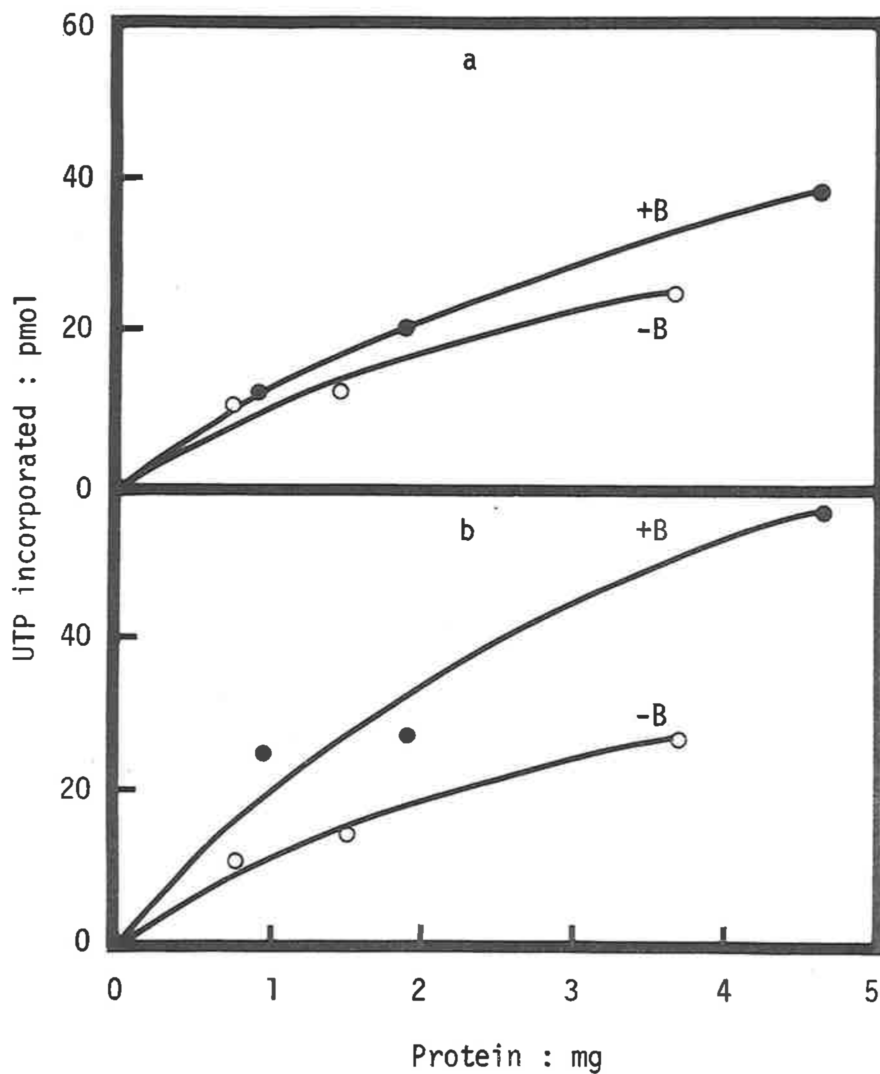


FIGURE 44 : *Effect of varying the amount of extract used in the RNA polymerase assay.* 2000 root tips were harvested (Section 2.1.4) after 216 hr of growth in deficient medium, and the protein content determined (Section 2.6.1). RNA polymerase activity was determined (Section 2.4.3) after incubating for 10 min (a) or 20 min (b).

in the extract (especially that from grossly deficient tissue) interfere with the assay. However, under boron-deficient conditions, the activities of many phosphatase and nuclease enzymes are increased (Sections 1.3.4.1, 1.3.4.2 and 3.7.1) so that the reduction of the measured RNA polymerase activity would be greatest in the assay of deficient tissue. In view of this, the results suggest that there may be an increased *in vivo* RNA polymerase activity in deficient root tips. If the results are calculated on a per root-tip basis, the activity in deficient tissue is equal to, or greater than, that of normal tissue at all stages of deficiency.

Inclusion of Actinomycin D (10 μg/ml) or RNase (150 μg/ml) in the reaction mixture reduced the incorporation of UTP by 50-70% (Figure 42b), indicating that the product formed is predominantly RNA.

4. GENERAL DISCUSSION

4.1 Morphological and Physiological Effects of Boron Deficiency

The morphological and physiological lesions of boron deficiency reported here agree closely with previous reports in the literature (see Section 1.1). The sequence of development of these symptoms is also similar. It is clear that after 80-100 hr, the deficiency influences many morphological and physiological characteristics. Some of these are parameters on which biochemical calculations are based, e.g. protein, fresh weight, cell number and size. Because boron deficiency affects these parameters in different ways and to a different extent, calculations based on different parameters may indicate different effects of the deficiency. This is demonstrated many times in Section 3, when results are expressed in different ways (e.g. Figures 6, 7, 8, 9 and 41, and Table 12). This must be taken into account in evaluating many reported effects of boron deficiency. During the first 50-70 hr of deficiency, these parameters are not affected and the basis used to express results is therefore less important. Most of the results in this thesis are expressed on a root-tip basis. This was chosen because it approximates to a per cell basis and is the most convenient parameter to assess. However, by reference to Figures 5 or 6, or Table 2, the results may readily be converted to other forms.

4.2 RNA Degradation

The decrease in the level of RNA reported by previous workers (Section 1.3.1) was confirmed, but time-course studies indicated that this only occurred at or just before the stage when root elongation ceased. The RNase activity increases at approximately the same stage of the deficiency, suggesting that the lower level of RNA is due to a higher rate of degradation by RNase. The fractionation of RNA by MAK chromatography provides further evidence for this. At advanced stages of the deficiency, the level of r-RNA is reduced and the amount of RNA in the s-RNA region increases slightly. The base composition of ^{32}P-labelled RNA in the s-RNA region suggests that this is due to degradation of r-RNA into lower molecular-weight material, some of which will fractionate with s-RNA on MAK columns. This degradation of r-RNA also explains the inhibition of protein synthesis discussed in Section 1.4.2. Interruption of polysome formation and decreased ribosome content were reported, but only after the appearance of visible deficiency symptoms, i.e. at the stage when a decrease in the r-RNA content was observed in the present work. Thus, increased RNase activity results in degradation of RNA (particularly r-RNA), leading to an interruption of protein synthesis. The increased RNase activity could therefore be a key factor in inducing the late symptoms of boron deficiency.

These changes in RNA content and RNase activity are similar to those occurring in maturing and senescing tissue. Several

different types of RNase enzymes having different intracellular distribution have been reported (Kessler & Engelberg 1962, Wilson 1963*a* and *b*, Ingle & Hageman 1965, Srivastava 1968). In general, it appears that total RNase activity, and particularly the soluble cytoplasmic enzymes increase with the maturation of plant tissue (Kessler & Engelberg 1962, Shannon & Hanson 1962, Ingle & Hageman 1965, McHale & Dove 1968, Phillips & Fletcher 1969, Millikan & Ghosh 1971). At the same time the RNA level decreases. Detached leaves and leaf discs also show loss of RNA and increased total, cytoplasmic and chromatin-bound RNase activity during the onset of senescence (Cherry 1963, Dove 1967, McHale & Dove 1968, Srivastava 1968, Ecklund & Moore 1969, Atkin & Srivastava 1969). Decreased RNA levels of attached and excised senescing leaves are primarily due to degradation of r-RNA. Srivastava & Atkin (1968), using sucrose density gradient centrifugation, and Cherry *et al.* (1965), using MAK chromatography, demonstrated a rapid decline in r-RNA, with a smaller decrease (Srivastava & Atkin) or slight increase (Cherry *et al.*) in s-RNA. Srivastava & Atkin found no change in the base composition of 4 hr ^{32}P-labelled r-RNA, but the base composition of s-RNA became more like r-RNA during the advanced stages of senescence. Both groups suggested that the s-RNA peak is contaminated with degradation products of r-RNA and m-RNA. These changes are similar to those induced by boron deficiency (see Figure 25). Degradation of ribosomes and polysomes in senescing tissue has been reported by Oota (1964) and Srivastava & Arglebe (1967). RNase activity also increases, and

RNA content decreases with increasing distance from the tip (i.e. increasing maturity) of broad bean (Robinson & Cartwright 1958) and *Lens* (Pilet & Braun 1970) roots.

Elongation ceases in boron-deficient roots, but maturation continues, so that the tissue within the 5 mm root tips eventually reaches a stage of maturity usually only found much more distant from the tip. Later, senescence takes place. The boron-deficient root tip therefore resembles the maturing and senescing tissue discussed above, and the changes in RNA content and RNase activity are similar. Thus, the increased RNase activity and resulting degradation of RNA and interruption of protein synthesis can be explained by the accelerated maturation and senescence of deficient root tips.

The induction of boron-deficiency symptoms by base analogue treatment (Section 1.3.5) could be due to the induction of senescence by these compounds which disrupt RNA and protein synthesis (Sugiura 1963).

4.3 RNA Synthesis

During the early stages of boron deficiency, there is an increased rate of incorporation of radioactive precursors into RNA without any apparent effect on the total precursor pools. The total level of radioactivity in the acid-soluble fraction and its

distribution between the different nucleotides and nucleosides is not significantly changed until at least 48 hr after transferring plants to deficient medium. The absence of any significant effect on uridine kinase activity or the distribution of [^{14}C] (introduced as [^{14}C]uridine) between UMP and CMP within the newly synthesized RNA also suggests that deficiency does not affect the total nucleotide pool. Thus, increased incorporation of radioactivity into RNA must be due either to changes in the properties of small specific nucleotide pools or to an increased rate of RNA synthesis.

MAK chromatography indicated that at these early stages of deficiency, the incorporation of precursors into TB-RNA is stimulated more than the incorporation into other RNA fractions. As the rapidly labelled components of TB-RNA have properties (e.g. base composition and half-life) similar to those expected for m-RNA (Tester & Dure 1967), these results may be due to an initial stimulation of m-RNA synthesis. If m-RNA is the major labelled component of the RNA fraction, this could occur without causing a detectable change in base composition. After a 4 hr labelling period, m-RNA would probably be a minor component of the r-RNA peak (Ingle & Key 1968), and a slightly greater increase in the incorporation into this fraction than r-RNA would not significantly change the base composition of the peak. Thus, the fact that the incorporation of precursors into TB-RNA is increased more than r-RNA or s-RNA may reflect a greater effect of deficiency on m-RNA synthesis at these early stages of deficiency.

At later stages of deficiency (after 144 hr or more), the

increased specific activity of incorporation of precursors into RNA may be due to the increased level of radioactivity within the precursor pools. If, however, nucleotide and nucleoside levels are increased by the deficiency, as are many other polymer precursors [e.g. amino acids and ammonia (Section 1.4.1), inorganic phosphate (Sections 1.3.4.2 and 3.2.4) and components of the acid-soluble fraction (Section 3.2.4)], the increased amount of radioactivity within this fraction may be due to an increased level and not an increased specific activity of nucleosides and nucleotides (see Figure 15). The different rates of RNA turnover (due to increased degradation by RNase) and the many other physiological and biochemical changes induced by boron deficiency make any comparison of RNA synthesis rates during these late stages of deficiency difficult to interpret.

During these later stages in the development of boron deficiency, the incorporation of precursors into ribosomal RNA (r-RNA peak and degraded r-RNA in the s-RNA peak) is increased more than TB-RNA. The two subfractions of r-RNA separated by polyacrylamide-gel electrophoresis are equally affected at all stages of deficiency.

Without further characterization of the labelled RNA within each fraction, it is not possible to state unequivocally which RNA species are affected by boron deficiency. The results are consistent with the hypothesis that initially m-RNA synthesis is increased, but after 50-100 hr of growth in deficient medium, the synthesis of r-RNA is stimulated most. It is certain, however,

that different RNA fractions are affected at different stages in the development of the deficiency. If, as suggested by Ingle & Key (1965), these RNA species are synthesised from different precursor pools, the distribution of labelled precursors between specific pools, rather than their incorporation into RNA, may be influenced by the deficiency to produce the different labelling patterns. The similar effects of the deficiency on [^{32}Pi], [^{14}C]uridine and [^{3}H]uridine incorporation provide indirect evidence against this. If different pools are used, they must have similar equilibrium properties.

Thus, the effects of boron deficiency on the incorporation of precursors into RNA can be divided into two distinct stages. Within the first 48 hr, the incorporation is stimulated without any apparent effect on the total nucleotide and nucleoside pool. The increase is mostly into TB-RNA and r-RNA (possibly both due to m-RNA). Beyond this stage the incorporation into nucleotides and nucleosides also increases and that into r-RNA is stimulated more than into TB-RNA. This change occurs between 40-50 hr after transferring plants to boron-deficient medium (Figures 13, 29 and 30).

Like changes in RNA content and RNase activity, the later effects of boron deficiency on RNA precursor incorporation (second stage described above) may be due to the accelerated rate of maturation and senescence in deficient root tips. Senescence of attached or excised leaves results in increased incorporation of [^{32}Pi] into RNA (Cherry *et al.* 1965, Srivastava & Atkin 1968,

Ecklund & Moore 1969) and changes in the distribution of label between RNA fractions are very similar to those observed in boron-deficient root tips *after the cessation of root elongation* (Cherry *et al.* 1965). Thus, after 80-100 hr growth in boron-deficient medium, the increased incorporation of precursors into RNA, the changes in RNase activity, RNA content and protein synthesis and possibly many other symptoms of the deficiency appear to be associated with maturation and senescence of the deficient tissue, and are therefore far removed from the initial site of action of boron deficiency.

Within the first 48 hr, the changes in RNA synthesis could be due to a direct effect of boron on the synthesis of particular RNA species, but the absence of any detectable changes in the properties of the newly synthesised RNA (DNA-RNA hybridization and base composition) do not support this idea. An increased RNA polymerase activity could produce the increased precursor incorporation but may not account for the different effects on different RNA species. In the absence of any evidence supporting a primary effect of boron on the control of RNA synthesis, it seems most likely that although this is an early effect of the deficiency, it is still a secondary effect. Several possible explanations for this are discussed in this and the following sections.

The initiation of adventitious roots of *Cicer arientinum* involves an increase in the incorporation of precursors into RNA (Jalouzot 1971). In pea roots, a lower proportion of t-RNA is

associated with rapidly dividing cells (Vanderhoef & Key 1971); when cells change from a dividing to a non-dividing state, the amount of 4s t-RNA expressed as a percentage of total RNA increases from 5.6% to 11.1%. Brown & Mangat (1970) have also shown that with the initiation of rooting in petioles of *Phaseolus vulgaris*, there is a 3.5-fold increase in r-RNA concentration, while s-RNA and DNA are not affected. The level of triphosphates and particularly UDPG increased just prior to the rooting of this tissue. Thus, an increased incorporation of precursors into RNA, affecting particularly r-RNA and TB-RNA, and the slightly increased incorporation of precursors into nucleotides (Tables 7 and 8) could be associated with the actively dividing cells of adventitious root initiation. It is not known when these are initiated in deficient root tips, but if initiation were induced by deficiency during the first few hours, this may well explain the increased RNA precursor incorporation. The absence of any effect on the total nucleotide pool at 48 hr may however suggest that lateral-root initiation is not responsible for differences in precursor incorporation at this early stage. This possible interpretation emphasizes the difficulties associated with studying changes in complex tissues. The changes may be due to only a small group of specialized cells within the tissue and may even be masking important changes in other groups of cells. If the increased precursor incorporation into RNA were due to the initiation of adventitious roots, it may explain the different results obtained with the two systems discussed in Section 1.3.2.

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In whole root or plant systems, adventitious roots would be relatively unimportant and an overall reduction in precursor incorporation could be recorded in spite of the increase in tissue where initiation occurs.

4.4 Plant Hormones

The similarities between auxin treatment of plant tissue and boron deficiency have been extensively reviewed by Jaweed & Scott (1967) and Coke & Whittington (1968). Some additional points are relevant to the root-tip system. The inhibition of cell division and elongation but continued radial enlargement of 2,4-D treated apical soybean hypocotyls observed by Key *et al.* (1966) and the swelling of chicory root tissue (Flood *et al.* 1970) are similar to the effects of boron deficiency. Following the auxin-induced swelling of root tissue, root primordia are initiated in the pericycle, leading to the appearance of adventitious roots (Goldacre 1959, Fan & McLachlan 1967, Jackson & Harney 1970, Grant & Fuller 1971).

IAA applied to pea or bean shoots or stems is rapidly transported unchanged to root tips and lateral-root primordia, where it is degraded to inactive forms at such a rate as to maintain a constant level of IAA in the root system (Morris *et al.* 1969, Iversen *et al.* 1971). Thus, any change in auxin metabolism would have an early effect on root-tip growth. Inhibition of

auxin degradation would rapidly result in auxin toxicity in root tips as discussed in Section 1.5.1.2.

Following the work reported in this thesis, further comparisons can be made. Auxins (2,4-D and IAA) increase the total (Shannon *et al.* 1964) and microsomal (Birmingham & McLachlan 1972) RNase activity but reduce the soluble cytoplasmic enzyme activity (Pilet 1970, Pilet & Braun 1970). In maturing tissue, however, the increase in the activity of the latter enzyme continues independently of hormone treatment (Birmingham & McLachlan 1972) so that in IAA-treated, maturing and senescing tissue the activity of both ribonuclease enzymes increases.

Auxin treatment (IAA, 2,4-D, NAA) increases RNA levels in most intact plant systems and reduces or prevents the loss of RNA in most excised systems (see Key 1969). Ribosomal RNA increases more than s-RNA (Key *et al.* 1966, Fites *et al.* 1969). Incorporation of nucleic acid precursors ([^{14}C]nucleosides or [^{32}Pi]) into RNA is also enhanced by auxin treatment in both types of systems (Key & Shannon 1964, Sacher 1967, Trewavas 1968*a*, Key 1969, Haissig 1971). Most auxin-responsive tissues show an enhanced precursor incorporation after a lag period of 5-120 min (Click & Hackett 1964, Masuda & Kamisaka 1969, Key 1969). Uptake of precursors into the total nucleotide pool is enhanced in some tissues (Nooden 1968), but the levels of incorporation into RNA are such that this cannot account for the effect of auxin on the apparent RNA synthesis (Trewavas 1968*a* and *b*).

The application of auxins to intact tissue promotes or inhibits growth, depending upon the auxin concentration, but RNase activity and protein and nucleic acid levels increase with applied auxin, even into the herbicidal concentration range. Herbicidal action appears to be due to an over-production of RNA and protein, leading to massive tissue proliferation, disorganized growth and eventually death of the plant (Shannon *et al.* 1964, Key *et al.* 1966, Key 1969). This is similar to the situation occurring under boron deficiency. Growth is inhibited, but cell expansion continues, while RNase activity and RNA synthesis increase, and the tissue eventually dies. At late stages of boron deficiency, RNA levels fall, but this is probably due to the increased cytoplasmic RNase activity resulting from the increased rate of maturation and senescence of deficient root tips.

Further characterization of the newly synthesized (labelled) RNA by sucrose density gradient separation (Hamilton *et al.* 1965), after incubating sub-apical *Avena* coleoptile segments in [^{3}H]uridine and IAA for 8 hr, indicated that all classes of RNA were equally affected. However, after shorter treatment periods and separation by MAK chromatography, changes similar to those induced by boron deficiency have been noted. Initially, incorporation into m-RNA is stimulated by auxin treatment of elongating and dividing tissue (soybean hypocotyl, pea internode and oat coleoptile) but later the incorporation into r-RNA is stimulated most (Key & Ingle 1964, Ingle *et al.* 1965, Jachymczyk & Cherry 1968). At early stages, the incorporation into TB-RNA

is stimulated more than any other fraction (Tester & Dure 1967, Obrien *et al.* 1968, Tanimoto & Masuda 1969). The base composition (of incorporated [^{32}P]) is also slightly altered, the AMP:UMP ratio decreasing. The similarity between the changes with time after auxin treatment and boron deficiency is shown by comparing the plot of Tanimoto & Masuda's (1969) results (Figure 45 of this thesis) with Figure 24. Different species within the TB-RNA fraction are affected at different stages after auxin treatment (Key 1969, Miassod *et al.* 1970). If, as has been suggested by Tester & Dure (1967), the rapidly labelled TB-RNA is mostly m-RNA (see also Section 3.5.1.1), auxin treatment appears to initially stimulate m-RNA synthesis. This hypothesis is supported by the observation that auxin treatment rapidly induces polysome formation (Trewavas 1968*b*, Fites *et al.* 1969).

Thus, from the available data, the following suggestion by Fites *et al.* (1969) could apply equally well to either auxin treatment or boron deprivation:-

> "In the initial hours after treatment there is an apparent burst of messenger-RNA synthesis such that monoribosomes are converted to polyribosomes. Presumably this response is to transitory low auxin concentrations. Following closely onto this period there is an increase in ribosome production which appears independent of DNA synthesis and cell division."

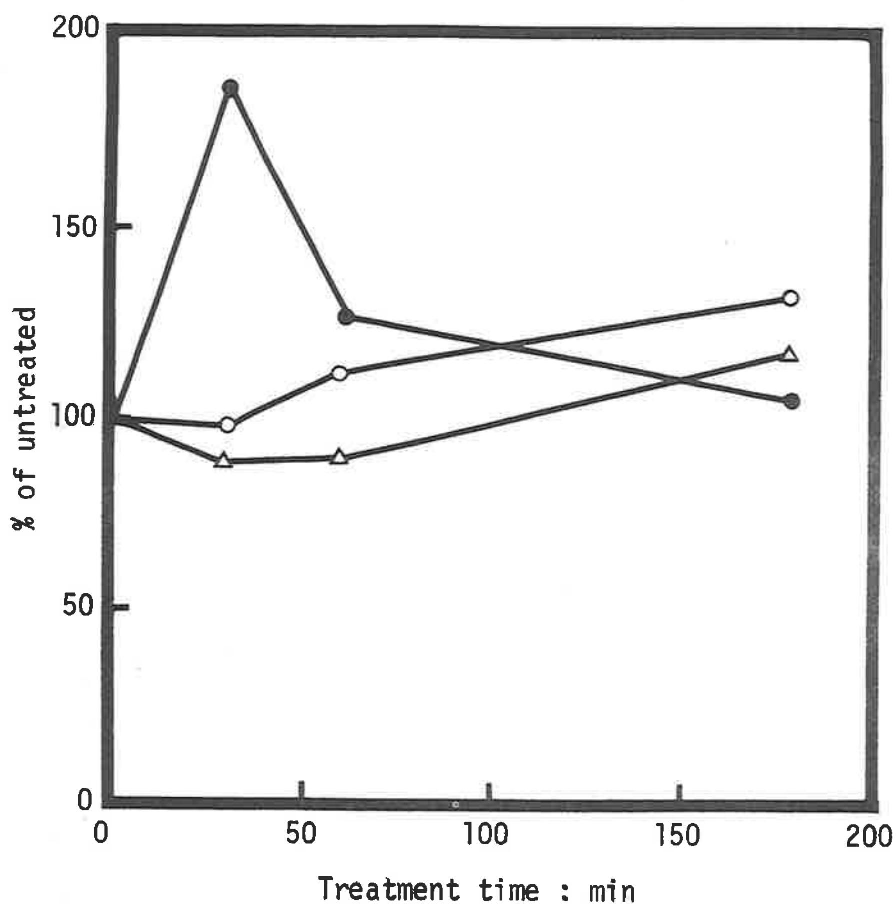

FIGURE 45 : *Effect of 2,4-D treatment on the specific activity of [^{32}P] incorporated into different RNA fractions.* Values were calculated from Table 1 of Tanimoto & Masuda (1969). Fractions are those separated by MAK chromatography. The specific activity of 2,4-D treated pea stems is expressed as a percentage of the specific activity of normal tissue on the ordinate.

● TB-RNA

○ r-RNA

△ s-RNA

Elongation-stimulating concentrations of auxin do not produce any detectable changes in the competitive DNA-RNA hybridization curves of total RNA extracted from excised pea-stem tissue. Higher concentrations of 2,4-D result in differences between hybridizable RNA species similar to those shown here to be induced by boron deficiency (Thompson & Cleland 1971*b*).

Thus, the early effects of boron deficiency on RNase activity, the uptake and incorporation of precursors into RNA, and the properties of the newly synthesized RNA are similar to the effects of auxin treatment. All of these deficiency-induced changes could be explained by an accumulation of auxin, passing through growth-promoting to inhibiting to herbicidal concentrations. The same effect could be achieved, however, if boron and auxins were operating independently or together through the same site of action or biochemical process.

The mode of action of auxins is still not clear. Direct effects on RNA synthesis have been suggested, and direct-binding with chromatin (Fellenberg 1969 and 1970) and s-RNA (Kobayashi & Yamaki 1972) reported. Reduced thermal stability of reconstituted nucleoproteins (Fellenberg 1971) and increased chromatin-associated RNA polymerase without increased template availability (Obrien *et al.* 1968) have also been demonstrated. Although continued RNA synthesis is essential for auxin-induced growth in a variety of systems (see Key 1969), the importance of this as an initial site of action is uncertain (Nelson & Ilan 1969, Neumann & Palmer 1971). Recent reports indicate that other factors may be important in

mediating the auxin effect (Hardin *et al.* 1970). Proteins (Matthysse & Phillips 1969, Venis 1971), other hormones (Thompson & Cleland 1971*b*) and c-AMP (Salomon & Mascarenhas 1971) have been suggested. IAA stimulates the incorporation of [^{14}C]adenine into c-AMP (Azhar & Krishna Murti 1971, Salomon & Mascarenhas 1971), resulting in a 5- to 10-fold increase in the basal level of c-AMP within 1-2 min after auxin treatment of *Avena* coleoptile sections (Salomon & Mascarenhas 1972*b*). The auxin (2,4-D) induced cell expansion in Jerusalem Artichoke tuber tissue is synergistically enhanced by c-AMP (Kamisaka & Masuda 1970), and the presence of c-AMP and IAA during chromatin extraction also results in an increased rate of RNA synthesis by the isolated chromatin (Salomon & Mascarenhas 1972*a*). Boron could influence nucleic acid metabolism *via* some effect on c-AMP, but the regulatory role of c-AMP *in vivo* has not yet been unequivocally established in plants (Ockerse & Mumford 1972).

Treatment of plant tissue with gibberellic acid (Chandra & Varner 1965, Fletcher & Osborne 1966, Johri & Varner 1968, Broughton 1969, Poulson & Beevers 1970) and cytokinins (Osborne 1962, Oota 1964, Carpenter & Cherry 1966, Burdett & Wareing 1968, Kulaeva *et al.* 1971) also increases the incorporation of radioactive precursors into RNA of both excised and intact plant tissue. Cytokinins affect all RNA fractions (separated by MAK chromatography) equally, but gibberellins stimulate the incorporation of precursors into r-RNA and TB-RNA more than s-RNA. RNA polymerase activity associated with isolated chromatin (McComb *et al.* 1970)

and RNase activity in isolated barley aleurone layers (Chrispeels & Varner 1967*a*) are also increased by gibberellin treatment. GA_3 treatment does not induce any differences in readily hybridizable RNA species in epicotyls of dwarf pea seedlings (Thompson & Cleland 1971*a*), but unlike boron deficiency, GA and kinetin induce changes in nucleotide patterns (Brown & Cassells 1971, Collins *et al.* 1972). c-AMP may also be involved in the action of gibberellins (Galsky & Lippincott 1969, Pollard 1970). Because of the similar effects of boron and GA_3 in breaking dormancy in *Themeda triandra* seeds, Cresswell & Nelson (1972) have recently suggested that boron may be implicated in the synthesis of GA_3.

Many interactions between plant hormones have been reported (e.g. Van Overbeek *et al.* 1967, Chrispeels & Varner 1967*b*, Vanderhoef & Key 1968, Srivastava 1968, Khan & Heit 1969, Pilet 1970) and it is possible that boron may influence these. Interactions between kinetin and auxins (Goldacre 1959, Key *et al.* 1966, Birmingham & McLachlan 1972), and particularly the kinetin inhibition of auxin-induced [^{32}Pi] incorporation into RNA (Vanderhoef & Key 1968), could be influenced by boron deficiency to produce the changes observed in nucleic acid metabolism.

4.5 Conclusions

Boron deficiency affects several distinct aspects of RNA metabolism. The increased incorporation of radioactive precursors,

which is observed in plant roots after only 6 hr growth in deficient nutrient, is the earliest effect of the deficiency yet recorded. This occurs without any significant changes in the total precursor pool. Much later in the development of the deficiency, increased rates of RNA degradation result in lower levels of RNA and may also contribute to the changes in the total nucleotide pool which are observed at this stage. These effects on RNA metabolism, which are not induced by deficiencies of iron, manganese or copper, appear to be specific to boron.

Many symptoms and effects of boron deficiency give the appearance of being associated with changes in growth rates and the state of maturity of deficient tissues. Only changes which precede effects on growth and development can be attributed to a more direct effect of the deficiency. The increased incorporation of precursors into RNA, as shown in the present work, is one of the few changes which have been demonstrated during this early stage of boron-deficient growth. So far, no evidence is available to suggest that the primary role(s) of boron involves some aspect of nucleic acid metabolism. However, correlations between the effects on nucleic acid metabolism of boron deficiency and other factors (e.g. hormone action or other types of disturbances to the normal biochemical events taking place during growth) may provide clues for the primary site of boron action. For this reason, further work on RNA polymerase for example may be useful, but in this case some form of purification would be necessary prior to enzyme activity measurements to obtain meaningful results.

Further investigation of a possible involvement of plant hormones in the expression of boron deficiency symptoms is also required, but until more information is available about the biochemistry of hormone action, interpretation of the results would be difficult.

Most work on boron deficiency has employed whole plant or complex tissue systems. With such material, the control of experimental conditions and the interpretation of results is difficult. Further progress will probably require the development of some simplified system. The elongation of pollen tubes (Whittington 1959, Bamzai & Randhawa 1967) and the growth of pollen-derived tissue cultures (Yih *et al.* 1966) could be useful model systems. Thus, the reported effects of plant hormones on the grape pollen system (Bamzai & Randhawa 1967) provide preliminary information for the study of possible hormone-boron interactions in this material. The demonstration of boron requirements for some algae (Eyster 1952, McIlrath & Skok 1958, Lewin 1966, Gerloff 1968, Boyd 1970), particularly unicellular species, and *Azotobacter chroococcum* (Gerretsen & De Hoop 1954) suggests that these could also be used for biochemical studies.

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