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NITROGEN FIXATION IN NODULATED LEGUMES  
IN RELATION TO THE ASSIMILATION OF CARBON

Thesis presented by

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for the degree of

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CONTENTS

	Page
Acknowledgements	(ii)
Summary	(iii)
Introduction	1
Materials and Methods	23
Results	44
Discussion	81
Bibliography	(vi)
Errata	(xxi)



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NITROGEN FIXATION IN NODULATED LEGUMES  
IN RELATION TO THE ASSIMILATION OF CARBON

There is a considerable volume of work which suggests that photosynthetic assimilates are important for nitrogen fixation and, in the work described in this thesis, attempts have been made to clarify the relationship between nitrogen fixation and photosynthetic assimilates in the nodules of two leguminous species - Pisum sativum L. and Vicia faba L. Simultaneous measurements of acetylene-reducing activity and radioactivity of  $^{14}\text{C}$ -labelled photosynthates in the nodules have been made over a wide range of experimental conditions.

In order to verify that the conditions used in the acetylene reduction assay were suitable for the species studied, the parameters thought most likely to affect acetylene-reducing activity in the nodules - those of temperature of incubation, oxygen and acetylene concentrations and the nature of the biological material assayed - were investigated. Optimum conditions were broadly similar for both species and differences between the species were probably due to differences in the biological materials themselves, such as nodule size and whether nodules were attached to or detached from the host plant.

Nitrogenase activity changed during growth of both species and differences between the two species were thought to be due to different patterns of flowering and fruiting. Studies of pea plants during growth showed that nodules active in nitrogen fixation accumulated most  $^{14}\text{C}$ -labelled photosynthates, but that a substantial proportion was not used in nitrogen fixation, but in respiration and growth of the nodule. Both nitrogenase activity and the accumulation of  $^{14}\text{C}$ -labelled photosynthates in nodules declined when flowering and fruiting took place and this was believed to be due partly to a redirection of the photosynthetic assimilates within the plant to the developing seed. However, removal of the shoot apex did not result

consistently in increases in nitrogenase activity, even though the accumulation of photosynthates increased, and so it is believed that, in this instance, control of nitrogenase activity is exerted by some factor other than, or additional to, the supply of photosynthates to the bacteroids.

Light and photoperiod were shown to play important rôles in the regulation of nitrogenase activity of pea nodules. Darkening pea plants resulted in large reductions in both nitrogenase activity and the ethanol-soluble carbohydrates in the nodules in 24 hours, but there was no corresponding reduction in the accumulation of  $^{14}\text{C}$ -labelled photosynthates manufactured just prior to darkening. However, the accumulation in the nodules of  $^{14}\text{C}$ -labelled photosynthates manufactured after darkened plants were returned to the light was much reduced and recovery of accumulation of photosynthates preceded recovery in nitrogenase activity. Although changes in the artificial light intensity to which pea plants were exposed for a short period had little effect on nitrogenase activity, diurnal fluctuations in nitrogenase activity were observed. However, maxima in nitrogenase activity did not correspond with maxima in light intensity, but occurred in the evening due to the accumulation of photosynthates translocated to the nodules after a delay of 4 to 8 hours. An additional mid-day maximum was observed sometimes and was believed to be due to changes in ambient temperature.

In both species, a large proportion of the  $^{14}\text{C}$ -labelled photosynthates in the nodules were recovered as sugars, but there was some indication that ethanol-insoluble compounds were formed in pea nodules. Some photosynthates were rapidly utilized, as basic compounds were formed in bean nodules only 30 minutes after exposure of the shoots to  $^{14}\text{CO}_2$ . Accumulation of basic compounds in detached bean nodules exposed to  $^{14}\text{CO}_2$  did not inhibit nitrogen fixation, and it is likely that organic acids formed by carboxylation reactions were aminated directly, as sugars were unlabelled.

Microautoradiography indicated that the main site of accumulation of  $^{14}\text{C}$ -labelled photosynthates was the young infected cells near the meristematic

tip of pea nodules, whereas histochemical tests with tetrazolium salts suggested that most nitrogen fixation took place in older cells. Thus the site of maximum accumulation of  $^{14}\text{C}$ -labelled photosynthates does not apparently correspond with the site of maximum nitrogen fixation and therefore this observation supports the suggestion that a substantial proportion of the photosynthates which accumulate in the nodules are not used directly in nitrogen fixation.

It is suggested that nitrogen fixation is not supported by reserve products, such as starch and poly- $\beta$ -hydroxybutyric acid, in pea nodules, but by a supply of photosynthates which are only available when the requirements of the actively growing regions of the nodule have been met and which, after amination, are exported rapidly from nitrogen-fixing cells.

INTRODUCTION

Assessment of nitrogen fixation	1
Conditions affecting the nitrogen fixation assays	4
Oxygen concentration	
Biological material	
Plant growth and development	5
Importance of light	7
Effects of darkening	
Daylength	
Light intensity	
Temperature	9
Diurnal variations	10
Nodule carbohydrates	12
Translocation of photosynthates to the nodules	14
Carbohydrate metabolism in the nodules	15
Export of aminated compounds from the nodules	17
Nodule respiration	18
Reserve substances in the nodules.	19

## INTRODUCTION

The importance of combined nitrogen to plant life cannot be over-emphasised, as without this the proteins essential for cell protoplasm cannot be synthesised. Most of the combined nitrogen on the surface of the Earth is derived from biological nitrogen fixation by past and present micro-organisms. The process of biological nitrogen fixation has been estimated to contribute  $10^8$  tons of combined nitrogen per year to the soil (Donald, 1960) and it was suggested that most of this was contributed by legume nodules. Most of the 13,000 known leguminous species examined bear root nodules (Burris, 1965) and the value of legumes in crop rotations was known in Classical Greek times.

Dalechamps (1587) was the first to observe root nodules, on a species of Ornithopodium, and Malpighi (1679) found 'insect galls' on Vicia faba roots. Recognition of the true nature and constituents of the nodules was first made by Woronin (1867) and it was demonstrated that no nodules formed on legume roots in sterile soil (Frank, 1879; Hellriegel and Wilfarth, 1888). Beijerinck (1888) isolated a nodule-forming bacterium in pure culture and called it Bacillus radicumicola, which is now known as Rhizobium leguminosarum.

### Assessment of nitrogen fixation

Early workers employed plant growth studies and Kjeldahl analyses to demonstrate increases in the nitrogen content of nodulated legumes in nitrogen-free culture (e.g. Weber, 1898; Friedburg, 1890; Giobel, 1926). However, these methods are time-consuming and laborious, and the availability of isotopes of nitrogen made possible the use of  $^{15}\text{N}_2$ , in conjunction with mass spectrometric analyses of plant material, to show conclusively that legume nodules could remove elemental nitrogen into organic combination (e.g. Lindstrom, Newton and Wilson, 1952; Aprison and Burris, 1952; Aprison, Magee and Burris, 1954; Virtanen, Moisio and Burris, 1955; Leaf, Gardner and Bond, 1958). This method is less time-consuming and is a thousand times

more sensitive than Kjeldahl analysis, although it is still fairly laborious.

The discovery that the enzyme complex nitrogenase reduced acetylene to ethylene at a rate proportional to that of nitrogen fixation (Dilworth, 1966; Schöllhorn and Burris, 1966) and the use of gas chromatography to estimate the ethylene produced, made possible an assay for nitrogen fixation which is simple, economical, rapid and a thousand times more sensitive than  $^{15}\text{N}_2$  fixation. The acetylene-reduction test has now been used on numerous occasions to demonstrate and quantify nitrogen fixation (e.g. Koch and Evans, 1966; Hardy *et al.*, 1968; Wheeler, 1969; Engin and Sprent, 1973). Acetylene concentrations of 0.10 to 0.20 atm. are routinely used and this concentration was found to be well above the saturation point of the enzyme in soybean nodules (Bergersen, 1970; Mague and Burris, 1972).

The main disadvantage of the acetylene reduction assay is its indirect nature, and the possibility that catalysis of this reaction may not proceed at a rate proportional to that of nitrogen fixation over a wide range of experimental conditions. The theoretical ratio of  $3\text{N}_2 : 1\text{C}_2\text{H}_2$  molecules reduced is based on the equations  $\text{N}_2 + 3\text{H}_2 \rightarrow 2\text{NH}_3$  and  $3\text{C}_2\text{H}_2 + 3\text{H}_2 \rightarrow 3\text{C}_2\text{H}_4$ , in which both substrates accept six electrons, but this ratio has not always been found in critical tests. Satisfactory ratios of 3:1 to 4:1 were obtained using soybeans (Hardy *et al.*, 1968; Schwinghamer, Evans and Dawson, 1970) although variations in acetylene reduction were proportionately much greater than variations in Kjeldahl analyses. In contrast, a ratio of  $2.0 \pm 1.1$  was calculated for soybeans by Mague and Burris (1972), and Engin and Sprent (1973) observed that the ratio was not constant throughout the growth of clover plants. In addition, the acetylene reduction test tended to underestimate nitrogen fixation, especially when rates were low (Roughley and Dart, 1969; Hardy *et al.*, 1971). Bergersen (1970) found ratios varying from 2.71:1 to 4.20:1 using soybean nodules, and attributed the large variations to small unavoidable oscillations in oxygen concentration from 0.21 to 0.24 atm. in the incubating vessels, and to the different solubilities of acetylene and nitrogen in the cell sap. Correction for differences in

solubility of the two gases resulted in constancy of the ratio over a wide range of oxygen concentrations, but the author advised caution on the use of acetylene reduction tests alone in the accurate measurement of nitrogen fixation although differences in rates of fixation by one assay material were accurately reflected by its acetylene-reducing activity.

### Conditions affecting the nitrogen fixation assays

#### Oxygen concentration

The importance of oxygen concentrations favourable for nitrogen fixation has been demonstrated by plant growth studies (Fred and Wilson, 1934; Ferguson and Bond, 1954),  $^{15}\text{N}$  fixation (Bond and MacConnell, 1955; Bergersen, 1962a, 1963; Bond, 1964; Silver, 1969) and the acetylene reduction assay (Bergersen, 1970; Mague and Burris, 1972; Waughman, 1972).

*legumes.* Studies on soybean nodules suggested that nitrogen fixation was enhanced by increasing oxygen concentration up to 0.50 atm. and declined at greater concentrations (Bergersen, 1962a, 1963). In contrast, acetylene reduction was not inhibited even at oxygen concentrations greater than 0.70 atm. (Bergersen, 1970; Mague and Burris, 1972). Oxygen was shown to be a competitive inhibitor of nitrogen fixation (Bergersen, 1962a, 1963) and Bergersen (1970) suggested that the apparently high rate of acetylene reduction at high oxygen concentrations in soybeans was due to the competitive advantage of acetylene over oxygen for nitrogenase.

In contrast, the maximum nitrogen fixation and acetylene reduction rates in non-legume nodules were achieved at 0.15 to 0.20 atm. oxygen (Bond and MacConnell, 1955; Silver, 1969; Waughman, 1972). Waughman (1972) suggested that these results were artifacts of the large abnormally exposed basal areas of detached non-legume nodules. However, in nodules of whole clover plants,

Ferguson and Bond (1954) observed maximum growth in nitrogen-free culture at Bond (1961) found that  $^{15}\text{N}_2$  fixation by nodulated pea roots was optimal at 0.20 atm. oxygen 0.21 atm. oxygen. All available data indicate that low oxygen concentrations (less than 0.15 atm.) inhibit fixation in whole plants or detached nodules.



The fall in nitrogen fixation with increasing time of incubation was attributed to lack of oxygen, since regassing the incubation vessels restored the initial rate (Sprent, 1969; Mague and Burris, 1972; Waughman, 1972). Mague and Burris (1972) calculated that respiration by soybean nodules led to a fall in oxygen concentration from 0.20 to 0.18 atm. in the incubation vessels and recommended that samples of less than 20% of the gas volume of the vessels be used.

#### Biological material

It is now generally accepted that the form of the biological material assayed can affect nitrogenase activity. Thus the activity of whole soybean plants was greater than that of nodulated roots, which in turn was greater than that of detached nodules (Mague and Burris, 1972). The size of nodules assayed may also cause significant variation in observed activities, since detached soybean nodules of 5 to 6 mm. and 2 to 5 mm. diameter were most active in  $^{15}\text{N}_2$  fixation (Aprison, Mague and Burris, 1954) and acetylene reduction (Mague and Burris, 1972) respectively. Experiments with Myrica cerifera showed that young nodules were more active in acetylene reduction than older, woody nodules (Silver and Mague, 1970). Akkermans (1971) showed that only the apical 2 mm. of lobes of alder nodules were active in acetylene reduction and that activity varied with the size of the lobes.

#### Plant growth and development

Bond (1936) found that nodular efficiency in soybeans grown in nitrogen-free culture rose to a maximum during flowering but fell to one-third of this after fruiting and attributed this either to a decreasing supply of carbohydrates to the nodules or to the presence of a constantly increasing number of inactive bacteria. He found that 78% to 92% of the nitrogen fixed was "transferred" without delay from the nodules to the host plant at all times. In contrast, in field-grown soybeans Hardy et al. (1971) and Mague and Burris (1972) found that more than 90% of the combined nitrogen fixed was due to

activity during fruit formation and maturation. The total amount of nitrogen fixed depended on the age of the plants when nitrogen fixation commenced, the rate of daily increase in fixation, the age at the end of the logarithmic phase of increase in fixation and the number and life of infected cells in the nodules. These results agreed with the findings of Chen and Thornton (1940) and Nutman (1946). Removal of the maturing pods prevented the increases in acetylene reduction and nodule weight which normally occurred during fruit development in soybean, but there was no increase in nodular efficiency and it was suggested that the increase in fixation reflected the demand for nitrogen created by the plant (Hardy *et al.*, 1968).

Pate (1958a) showed that nodule development in Pisum arvense was typically associated with plant development, and that a maximum in the yield of active, red nodules occurred just prior to flowering. However, the maximum nitrogen content of the plants was reached later and this was attributed to increased nodule efficiency from 80% to 100% in the larger nodules of older plants. Nodules of Vicia sativa were most active in fixation just prior to flowering (Pate, 1958b) and removal of the flower buds resulted in continued nodule development on secondary roots; it was suggested that the nutritional demands of the reproductive processes might withdraw foodstuffs from vegetative organs and lead to starvation. Similar delays in nodule senescence were also observed on removal of the apical flower buds in P. sativum (Ropenen and Virtanen, 1968). Abnormally large nodules up to six times the normal size were produced on treated plants and these fixed eight times the normal amount of nitrogen.

The development of reproductive organs in field-grown soybeans thus stimulated nitrogen fixation, but inhibited fixation in soybeans in nitrogen-free culture, in peas and in V. sativa.

## Importance of light

### Effects of darkening

Artificial darkening had significant effects on nodulation and nitrogen fixation in all reported examinations of nodulated plants. Thornton (1930) found that nodulation and nodule growth were reduced by half in 38-day-old lucerne seedlings after 19 days in the dark, and Lindstrom, Newton and Wilson (1952) reported that  $^{15}\text{N}_2$  fixation by darkened red clover plants declined progressively to zero within 24 hours. Fixation by darkened pea plants also virtually ceased after 24 hours, and after 48 hours the nodules began to degenerate (Virtanen, Moisisio and Burris, 1955). Plants darkened for 24 hours showed only partial recovery 24 hours after their return to the light. The decline in fixation was attributed to lack of photosynthetic substrates and lack of complete recovery to partial destruction of haemoglobin. Bond (1956) ascribed the fall of 66% in  $^{15}\text{N}_2$  fixation by ringed alder plants to lack of a carbohydrate supply to the nodules.

Recent research using the acetylene reduction assay has confirmed these results of earlier workers. Hardy et al. (1971) showed that fixation in darkened soybeans was reduced to one-sixth of that of light-maintained plants after 17 hours, and ceased completely after 3 days, although there was a transient rise after 24 hours which was attributed to utilization of storage products. However, Wong and Evans (1971) reported that acetylene reduction by darkened soybeans fell by only 30% after 24 hours and that acetylene reduction was still detectable after 10 days. The rapid decline in nitrogenase activity in young alder plants during the 24 hours after darkening was correlated with low sucrose levels, but there was no significant decline in the storage carbohydrates during this initial period of darkening (Wheeler, 1971).

### Daylength

The direct effect of daylength on nitrogen fixation by previously-

nodulated legumes has not been examined but research has been carried out into its effects on nodulation and plant growth. Eaton (1931) showed that the growth, carbohydrate and nitrogen contents of nodulated "Piper" soybeans were directly proportional to daylength. Increasing the daylength from 3 to 13.5 hours increased the number and fresh weight of nodules per plant and this was attributed to increased levels of photosynthetically assimilated carbohydrates available as substrates for nitrogen fixation. By contrast, the carbohydrate and nitrogen contents of "Manchu" soybeans were both greater with 7-hour rather than 16-hour photoperiods but there was a decline in the nodule yield expressed as a percentage of the total plant weight (Hopkins, 1935). Federov and Uspenskaya (1955) reported that maxima in nitrogen fixation and nodule yield in soybeans and peas were attained with 8-hour and 16-hour photoperiods respectively, but that continuous illumination resulted in decreased nitrogenase activity. However, Sironval, Bonnier and Verlinden (1957) reported that soybeans grown with an 8-hour photoperiod developed only slightly pigmented nodules and it was proposed that the greater plant growth rate achieved with a 16-hour photoperiod was required to support nodule development and nitrogen fixation. Photoperiods greater than 8 hours also favoured healthy nodule development in Trifolium subterraneum (Gibson, 1957) and T. glomeratum (Subba-Rao, 1971).

#### Light intensity

Little research has been carried out on the effects of light intensity and quality on fixation by nodulated legumes and most studies have been concerned with their effects on nodulation and plant growth, e.g. the work of Lie (1971) on nodulation of rooted leaves. Vines (1888) showed that shaded Vicia faba plants did not nodulate and Diener (1950) found that reductions in light intensity of less than 50% had a greater effect on the size than the number of pea nodules. Low light intensities reduced nodule yields of pea, clover and lucerne plants (Van Schreven, 1959). Under temperature-controlled conditions, the nodulation, dry weight and nitrogen

<u>plant species examined</u>	<u>temperature (°C) at which fixation maximal</u>	<u>source of information</u>
<u>Pisum sativum</u>	30	Jones and Tisdale (1921) <sup>o</sup>
	20-24	Stalder (1952) <sup>o</sup>
	18-21	Mes (1959) <sup>†</sup>
<u>Vicia atropurpurea</u>	24	Pate (1962a) <sup>†</sup>
<u>V. faba</u>	20	Dart and Day (1971)*
<u>V. sativa</u>	18-21	Mes (1959) <sup>†</sup>
	20	Dart and Day (1971)*
<u>Trifolium glomeratum</u>	15-20	Subba-Rao (1971) <sup>o</sup>
<u>T. pratense</u>	25	Dart and Day (1971)*
<u>T. subterraneum</u>	20	Meyer and Anderson (1959) <sup>†</sup>
	18	Gibson (1961) <sup>†</sup>
	15	Roughley and Dart (1969)*
	30	Dart and Day (1971)*
<u>Medicago sativa</u>	35	Dart and Day (1971)*
<u>M. tribuloides</u>	24	Pate (1962a) <sup>†</sup>
<u>M. truncatula</u>	20	Dart and Day (1971)*
<u>Glycine max</u>	30	Mes (1959) <sup>†</sup>
	25	Dart and Day (1971)*
<u>Lupinus luteus</u>	18-21	Mes (1959) <sup>†</sup>
<u>Stizolobium deeringianum</u>	30	Mes (1959) <sup>†</sup>
<u>Arachis hypogaea</u>	30	Mes (1959) <sup>†</sup>
<u>Vigna sinensis</u>	37.5	Dart and Day (1971)*
<u>Alnus glutinosa</u>	25	Wheeler (1971)*
	20	Waughman (1972)*
<u>Hippophae rhamnoides</u>	20	Waughman (1972)*

Symbols indicate the method of assessment as follows:

\* acetylene reduction

† Kjeldahl analysis

o implied by colour, growth and nodule yield of plants.

content of purple vetch and barrel medic were greater at 800 ft. candles than 400 ft. candles (Pate 1961, 1962a). Reduction in supplemented natural daylight reduced nodulation and plant dry weight of cowpeas over a range of temperatures (Dart and Mercer, 1965).

The findings that nodulation and plant growth were reduced by high light intensities (Orcutt and Fred, 1935; Fred, Wilson and Wyss, 1938) were based on inadequately controlled experiments and it is likely that high soil temperatures contributed to the observed decreases.

### Temperature

The effects of temperature on fixation have been extensively investigated and a summary of results is given in the table opposite. Although the data were obtained by several means of assessment, maximum nitrogen fixation and plant growth occurred in all cases at 15 to 25°C with the exception of the tropical legumes examined by Mes (1959) and cowpeas (Dart and Day, 1971). Mes (1959) found that the temperatures at which maxima in plant growth and nitrogen fixation were achieved in these species were approximately 10°C higher than those found for temperate species and it has been suggested that this variation may reflect properties of the rhizobial species (Allison and Minor, 1940) and strains (Pate, 1962a; Gibson, 1963). It is interesting to note that the temperature maxima for nodulation frequently differ from those shown in the table for nodule development and nitrogen fixation (Mes, 1959; Dart and Mercer, 1965; Pate, 1962a).

The effects of unfavourably high temperatures throughout the day could be partially offset by lowering the night temperatures (Mes, 1959; Pate, 1962a) and Meyer and Anderson (1959) suggested that the carbohydrate supply to the nodules was reduced in high temperatures due to high respiration rates in the shoots. However, they were unable to increase fixation by the addition of sucrose and concluded that high temperatures caused a deficiency of some other metabolite required for fixation.

Plants grown at 19°C root temperature showed no decrease in fixation 24 hours after transfer to 7°C root temperature and it was proposed that root temperature determined the active life of each infected cell and the quantity of enzyme formed rather than its subsequent activity (Roughley and Dart, 1969; Roughley, 1970).

#### Diurnal variations

Diurnal fluctuations in both light and temperature have been implicated by many authors as the cause of diurnal fluctuations in nitrogen fixation. Nodulated peas were more active in  $^{15}\text{N}_2$  fixation at 1200 hours than at 0800 hours (Virtanen, Moissio and Burris, 1955) and decreased activity was associated with lower light intensities and temperatures on following days. Diurnal fluctuations in acetylene-reducing activity have been observed also in nodulated soybean roots. Greater activity occurred in field-grown soybeans between 0800 and 2000 hours than between 2000 and 0800 hours (Hardy *et al.*, 1968) and it was suggested that these variations corresponded to variations in photosynthesis in daylight and darkness. Bergersen (1970) demonstrated variations in acetylene-reducing activity, which closely followed changes in illumination, in nodules still attached to the roots, but little variation in the lower activity of detached nodules from glasshouse-grown plants. Activity reached a maximum during the day and fell to a minimum during darkness and it was proposed that activity consisted of two components: endogenous activity (as expressed in detached nodules), due to utilization of storage reserves, and additional activity supported by photosynthetic assimilates translocated to the nodules. However, Mague and Burris (1972) observed diurnal fluctuations using detached nodules from field-grown plants; activity was maximal between 1300 and 1700 hours and minimal between 2000 and 0700 hours, and was correlated with both temperature and light intensity, with a "lag period" of 1 hour.

Stewart, Fitzgerald and Burris (1967) found that detached nodules of

Comptonia peregrina collected at 1330 hours reduced acetylene at twice the rate of those collected at 0730 hours, and attributed this to differences in the availability of photosynthetic products from the plant. Wheeler (1969) observed diurnal fluctuations in acetylene-reducing activity in glasshouse-grown alders, with maxima at 1400 hours at approximately constant temperature. Exposure of the shoots to  $^{14}\text{CO}_2$  showed that the influx of newly-synthesised photosynthetic assimilates into the nodules was correlated with nitrogenase activity and it was suggested that nitrogenase activity was influenced mainly by supplies of newly-photosynthesised carbohydrates to the nodules (Wheeler, 1971). However, fluctuations in carbohydrate levels in the nodules were not correlated with fluctuations in activity and the influx of photosynthates and it was proposed that a substantial part of nodule carbohydrates were not available for use in fixation, and that increases in fixation might be caused by temporary increases in the sugar concentrations in the infected cells. Similar diurnal fluctuations were also noted in nitrogenase activity and carbohydrate content in the nodules of Myrica gale (Wheeler, 1969) and Casuarina cunninghamiana (Mackintosh, 1969), although in the latter species temperature was thought to play an important role in determining rates of fixation. In contrast, Rodriguez-Barrueco (1967) observed significantly greater activity in nodules of C. torulosa detached at 1900 hours than at 1100 hours and he attributed the evening maximum to the time required by photosynthates to reach the nodules.

Diurnal rhythms in exudation from decapitated nodulated roots of Pisum arvense were observed (Pate, 1962b; Greig, Pate and Wallace, 1962; Pate and Greig, 1964). Maxima in both the volume of liquid and the total amount of nitrogen exuded from the xylem vessels occurred around mid-day and minima were recorded during the night. However, the concentration of nitrogenous compounds in the exudate rose to a maximum between 2100 and 0500 hours, in contrast to results obtained from analyses of the exudate of non-nodulated plants of Vicia faba (Kipps and Boulter, 1973). Since nitrogenous compounds



in the exudate were derived principally from nodule metabolism (Pate and Greig, 1964), export of assimilated substances from the nodules continued actively at night as well as during the day in the absence of photosynthesis.

#### Nodule carbohydrates

The importance of carbohydrates from photosynthesis in nitrogen fixation was implicated by Ruffer (1932), who showed that uninoculated soybeans grown in nitrogen-free soil had greater carbohydrate contents than nodulated plants, but lower nitrogen contents. Allison (1935) suggested that the amount of available photosynthate entering the roots was normally the chief factor influencing nodulation and nitrogen fixation, since the addition of sucrose to alfalfa roots increased nodulation and fixation in nitrate-containing rooting media (Allison and Ludwig, 1934). However, Wilson and Fred (1939) proposed that although carbohydrates might limit infection and nodule initiation, the main factor influencing nodule development was limiting levels of nitrogen, and that the carbon:nitrogen relationship of the plant was the controlling factor in symbiosis in legume nodules. This view was refuted by Allison and Ludwig (1939) on the grounds that no such complicated explanation was required, since even a small increase in the amounts of sugars in the roots led to a large increase in nodule yield.

Other workers have confirmed that artificial application of carbohydrates to nodulated plants often increases rates of fixation. Thus Van Schreven (1959) found that spraying sugars on the leaves of peas kept in darkness or low light intensities partially restored the original rates of fixation. The addition of up to 1% glucose or sucrose to the rooting medium resulted in increases in plant dry weight, nitrogen fixation and nodule yield in clover, but decreases in lucerne (Orcutt and Fred, 1935; Van Schreven, 1959). Nodulation of isolated Phaseolus vulgaris roots increased in proportion to the concentration of sucrose supplied to the basal ends (Cartwright, 1967).

Increasing supplies of photosynthetically synthesised carbohydrates in

the host plants may also increase nitrogen fixation. The beneficial effect on nitrogen fixation of increasing the carbon dioxide concentration from 0.03% to 0.11% around the shoots of clover, alfalfa, lucerne and peas was demonstrated (Krueseler, 1885; Georgi, Orcutt and Wilson, 1933; Wilson, Fred and Salmon, 1933; Fred and Wilson, 1934). Increases in nodule yield and distribution, leaf area, plant weight and carbohydrate content were shown, but the percentage nitrogen content of the plants fell since the plant dry weight increased more than the plant nitrogen (Fred and Wilson, 1934). The reduction in growth of clover and lucerne in closed tubes was attributed to reduction of the carbon dioxide concentration inside the tubes (Gibson, 1967) and the author warned against the concealment of significant results by poor overall growth in such systems.

The presence of  $\text{CO}_2$  in the rooting medium alone increased nodulation and nitrogen fixation by clover, peas and beans (Mulder and Van Veen, 1960) and the increase, which was greatest at pH 4.8, was attributed to increased levels of keto-acids in the nodules to sequester the ammonia produced by nitrogen fixation. A similar function was reported by Carrodus (1967) for  $^{14}\text{CO}_2$  fixation in mycorrhizal roots in  $\text{NH}_4\text{Cl}$ -containing medium, as glutamine was the product with greatest activity.

The potential for photosynthesis of nodulated soybeans, as measured by the chlorophyll content of the leaves, was found to be greater than that of uninoculated plants (Gukova and Butkevich, 1941; Sironval, Bonnier and Verlinden, 1957) and was thought to result from increased demands for carbohydrates by the presence of nodules. Comparison of nodulated and non-nodulated clover plants of similar age and size showed that the "carbohydrate requirement" of nitrogen fixation as opposed to assimilation of combined nitrogen was greatest during the period of active growth and fixation by the nodules (Gibson, 1966). Assimilation of elemental as opposed to combined nitrogen also increased the sucrose requirement of Clostridium pasteurianum (Daesch and Mortenson, 1968) and it was calculated that whole cells required

20 moles ATP per mole of elemental nitrogen converted to ammonia (approximately 14 mg. sucrose per mg. nitrogen fixed).

Bond (1941) calculated that soybeans utilized 19 mg. carbohydrate per mg. nitrogen assimilated.

Minchin and Pate (1973)

showed that the assimilation of both elemental and combined nitrogen by pea nodules required 10.3 mg. carbohydrate per mg. nitrogen assimilated.

Nodules sequestered 32% of the carbohydrates photosynthesised by the plants, and of this portion 17% was used in growth, 36% in respiration and the remaining 47% was returned to the shoot as aminated compounds.

#### Translocation of photosynthates to the nodules

The rapidity of translocation of photosynthates from the shoot to active nodules has been noted by some authors. Thus autoradiography of pea and clover plants, following exposure of the shoots to  $^{14}\text{CO}_2$  for a short time, showed that the nodules were labelled within 4 hours and 1 hour respectively (Small and Leonard, 1969; Harvey, 1970). Nodules were active "sinks" for the accumulation of photosynthates equivalent to stolon, shoot and root apices, expanding leaves and developing flowers (Hoshimo, Mishimura and Okubo, 1964; Harvey, 1970). Similar direction of photosynthates from photosynthetic to infected cells was also observed in wheat leaves infected by loose smut disease (Gaunt and Manners, 1971).

The direction of translocation of photosynthates from the leaves of P. arvense was influenced by the age of the leaf (Pate, 1966). Upper leaves exported assimilates mainly to the shoot apex and lower leaves to the root, which re-exported assimilates to the shoot as aminated compounds. Low light intensities and darkness were reported to direct more assimilates into soybean roots (Nelson and Gorham, 1957) but to decrease the total amount of photosynthate exported (Wardlaw, 1969). Defoliation between the upper leaves and the root system also resulted in redirection of a large part of photosynthetic

assimilates to the roots (Thrower, 1962; Belikov, 1957). The development of flowers and fruits increased the "sink" capacity of the shoot apex in soybeans, peas and clover (Belikov, 1955; Wardlaw, 1968; Harvey, 1970) and root starvation and death was reported when lower leaves senesced in flowering plants of P. arvense (Pate, 1968).

#### Carbohydrate metabolism in the nodules

One important role of carbohydrates in the nodules is to supply the carbon skeletons required for incorporation of the ammonia produced by nitrogenase activity. Earlier workers found that the nitrogenous constituents of nodules, roots and shoots of legumes were similar except for increased amounts of basic non-amino nitrogen in the nodules (Orcutt, 1937; Umbreit and Burris, 1938) and Hunt (1951) found few qualitative differences in soluble amino-acids between legume nodules of different species or ages. Aspartic and glutamic acids, principally as their amides, contributed 50% and 20% respectively of the high level of ethanol-soluble nitrogen content in lupin nodules (Bathurst, 1954). Asparagine and glutamine together also contributed 92% of the total amino-acid nitrogen in a variety of non-legume nodules, although citrulline comprised at least 25% of the amino-acid nitrogen in Alnus species (Wheeler and Bond, 1970).

$^{15}\text{N}_2$ -tracer experiments with detached soybean nodules showed that the most highly enriched organic compound after short periods of exposure was glutamic acid (Zelitch, Wilson and Burris, 1952; Aprison, Magee and Burris, 1954) and there was relatively little enrichment in asparagine. Kennedy (1966a,b) showed that glutamic acid and glutamine were the primary products of  $^{15}\text{N}_2$  fixation by serradella nodules and that other compounds, such as aspartic acid, alanine and asparagine, were formed secondarily by active transamination. Four electrophoretically distinct forms of aspartate amino-transferase have been detected in soybean nodules (Ryan, Bodley and Fottrell, 1972). Nodules of both Myrica gale and Alnus glutinosa also showed initially

most enrichment in glutamine, with less enrichment in asparagine and glutamic acid in M. gale, and in citrulline and aspartic acid in A. glutinosa (Leaf, Gardner and Bond, 1958, 1959).

Metabolism of photosynthetic assimilates in soybean nodules was examined by exposure of the shoots of field-grown plants to  $^{14}\text{CO}_2$  for several hours, followed by "cold-chase" periods (Bach, Magee and Burris, 1958). Ethanolic extracts of plants harvested in the evening of the same day ("day" plants) or the following morning ("night" plants) showed that the specific activity of the nodules was greater than that of the roots but considerably less than that of the leaves. On analysis by ion-exchange chromatography, 60% of the activity in the nodules was recovered in the neutral fraction, mainly as glucose, although fructose was labelled in the "night" plants. Sucrose was not detected in the nodules, although it was abundant in the roots, and this was perhaps due to active invertase action similar to that observed in lupin nodules (Kidby, 1966). The amino-acid fraction contained twice the activity of the organic acid fraction after a "cold-chase" period in "day" plants, but the reverse was true in "night" plants, and it was suggested that this was due to nitrogen fixation failing to keep pace with the production of suitable acceptors for the ammonia produced. Malic acid accumulated most of the activity in the organic acid fraction, but activity was also associated, in "night" plants only, with an unidentified 4-carbon, non-keto acid. However, these results must be treated with caution, since the conditions were inadequately controlled during the experiments.

After exposure of alder shoots to  $^{14}\text{CO}_2$ , Wheeler (1971) observed that most of the radioactivity in ethanolic extracts of the nodules was recovered in the neutral fraction on ion-exchange chromatography and that the activity of the basic fraction was greater than that of the acidic fraction. Sucrose was the major component of the neutral fraction in this species.

### Export of aminated compounds from the nodules

The exchange of assimilates between nodulated roots and shoots, and the accuracy with which root exudates reflect the metabolic activity of the nodules, was studied by Wieringa and Bakhuis (1957). The presence of asparagine in the sap exuded from decapitated nodulated roots was indicative of effective nodulation.

Detailed study of the exudate from nodulated roots of P. arvense showed that the major components were aspartic acid, asparagine, glutamine and homoserine, with smaller quantities of glutamic acid, valine and leucine/isoleucine (Pate, 1962b). High specific activity was noted only in asparagine, aspartic acid and glutamine after exposure of the shoot to  $^{14}\text{CO}_2$  for 1 hour, although homoserine became labelled afterwards. The development of pigmentation in the nodules resulted in a three-fold increase in amides, especially asparagine, in the exudate, and it was suggested that this reflected economy in the utilization of carbon skeletons for transport of nitrogen (Pate and Wallace, 1964). Root exudates resembled xylem fluids but were 4 to 8 times more concentrated (Pate, Walker and Wallace, 1965) and 20% to 30% of the nitrogen exported from the root was used in protein synthesis by the shoot apex within 24 hours (Pate, Wallace and Van Die, 1964). The carbon skeletons for amination were derived mainly from lower leaves (Pate, 1966) and the amino-acid content of the exudate increased until flowering, and then declined, as did nitrogen fixation by the nodules (Pate, 1968).

However, the amino-acid composition of root and nodule exudates did not reflect that of the nodules of V. faba plants (Pate, Gunning and Briarty, 1969). Export was selectively biased towards aspartic acid, asparagine, glutamic acid, glutamine and alanine and against  $\gamma$ -amino butyric acid. It was calculated that with the observed rate of 30 to 100 mg. nitrogen fixed per gram fresh weight pea nodules, the turnover of nitrogen each day was equal to the nitrogen content of the nodules (Minchin and Pate, 1973). Fixation and export of 1 mg. nitrogen required 3 ml. oxygen and 0.35 ml. water.

### Nodule respiration

The respiratory rate of nodules active in nitrogen fixation has generally been found to exceed that of other plant parts. Thus Bond (1941) calculated that the respiration of soybean nodules was three times that of the roots and equal to 25% of that of the whole plant during flowering. Nodule and bacteroid respiration rose with increasing oxygen concentration (Allison *et al.*, 1940a,b) but reached maxima at lower oxygen concentrations than cultured bacteria (Burris and Wilson, 1939; Thorne and Burris, 1940). Bond (1941) also noted that nodule respiration rose as nitrogen fixation fell in an oxygen concentration of 0.40 atm., and Bergersen (1958) observed that nodule respiration declined during the period of active nitrogen fixation in soybean. A two-step rise in nodule respiration with increased oxygen concentration was found (Bergersen, 1962a) and the first rise was attributed to host tissue respiration and the second to bacteroid respiration, with discontinuity between them due to a permeability barrier, probably the common endodermis (Frazer, 1942). It was proposed that nitrogen fixation and respiration were two competitive branches of an electron-transport chain, utilizing the same substrates (Bergersen, 1962a).

It is probable that respiration and nitrogen fixation in Rhizobium japonicum and soybean nodules involve the TCA cycle (Keele, Hamilton and Elkan, 1969). Both respiration (Burris and Wilson, 1939; Thorne and Burris, 1940) and nitrogen fixation (Bergersen and Turner, 1967) of soybean bacteroids were stimulated more by the addition of TCA cycle acids than by sucrose. Bacteroids contained large quantities of succinate (Bergersen, 1969) although nodules contained mainly sugars, and glucose metabolism was restored only after 7 to 10 hours in washed lupin bacteroids (Bergersen, 1971). Incomplete oxidation of TCA cycle acids was reported using radiorespirometric methods in both bacteria and bacteroids (Bergersen, 1958) and it was suggested that in the bacteroids TCA cycle acids were used as acceptor molecules for ammonia formed during nitrogen fixation; in bacteria, they

were used in the production of gums (Georgi and Wilson, 1933) and poly- $\beta$ -hydroxybutyric acid (PHB) (Wong and Evans, 1971).

#### Reserve substances in the nodules

Although lipids and reserve polysaccharides other than starch may play an important part in storage of food reserves in the nodules, only two reserve substances have been studied in detail - PHB (characteristic of the bacteria) and starch (characteristic of the host plants).

The presence of PHB in R. leguminosarum and legume nodules was shown (Forsyth, Hayward and Roberts, 1958; Hayward, Forsyth and Roberts, 1959), but the gravimetric method used was highly inaccurate. Law and Slepecky (1961) used spectroscopic examination after destructive digestion of PHB by concentrated acid to crotonic acid to estimate PHB in Bacillus megasterium. It was shown that the empirical formula was  $(C_4H_6O)_n$ , the molecular weight was between 1,000 and 250,000 according to the species of bacterium used (Lundgren et al., 1965) and it was estimated that as much as 50% of the dry weight of soybean bacteroids consisted of PHB (Wong and Evans, 1971).

PHB was also reported in Rhizobium trifolii (Vincent, Humphries and North, 1962), using the gravimetric method and examination by electron microscopy, and estimated to contribute 39% to 50% of the dry weight of the bacteria. Dixon (1964) observed PHB deposits in bacteroids of pea and clover nodules in preparations for light but not electron microscopy and attributed the absence of PHB in the latter to faulty fixation techniques. However, Oppenheim and Marcus (1970) did not find PHB in electron microscope preparations of nitrogen-fixing cells of Azotobacter vinelandii, whereas cells cultured on combined nitrogen contained large deposits of PHB equivalent to half the cell volume.

PHB formation in A. beijerinckii increased to 70% of the bacterial dry weight in limiting oxygen concentrations (Senior and Dawes, 1971) and it was suggested that PHB was used as an electron and carbon "sink" which would allow



for re-oxidation of reduced pyridine nucleotides under relatively anaerobic conditions. Although  $\beta$ -hydroxybutyric acid (BOHB) supported acetylene reduction by cell-free extracts of soybean nodules (Klucas and Evans, 1968), there was no evidence of utilization of PHB to support acetylene reduction when the carbohydrate supply to soybean nodules ceased (Wong and Evans, 1971). PHB is thus probably present in rhizobia and soybean nodule bacteroids, but may not be present in the nodules of all legumes, and its precise role in legume nodules remains to be established.

The universal presence of starch grains as reserve carbohydrates in legume nodules was examined by earlier workers (Brunchorst, 1885; Stefan, 1906) and it was believed that starch grains were deposited either as parietal layers in the infected cells or in special starch-containing uninfected cells inside the infected area. However, later investigations showed that both types of deposition could occur, although one type was predominant in each species (Wendel, 1918; Dangeard, 1926; McCoy, 1929). Starch grains occurred mainly as parietal layers in infected cells of P. sativum and V. faba (Dangeard, 1926) and those situated towards the periphery of cells near the base of the nodules were only partially stained by iodine due to partial digestion and dextrin formation.

Many starch grains were found in pea nodules active in nitrogen fixation (Dangeard, 1926; Löhnis, 1930) and few in inactive basal regions of nodules, older nodules or those on darkened plants (Frank, 1890; Dangeard, 1926; Rippel and Poschenreider, 1928; Allison, 1935). In contrast, ineffective nodules contained greater than normal numbers of starch grains (McCoy, 1929; Dart and Mercer, 1965) and amounts of an unknown dextrin-like polysaccharide (Bergersen, 1957). McCoy (1929) proposed that the bacteroids were unable to attack starch grains inside the plastid membranes, since whole undigested starch grains remained at the end of nodule life. She suggested that the disappearance of starch grains in older infected cells was due to the activity of the increased numbers of mitochondria in the host cells.

Löhnis (1930) observed that both efficient and inefficient rhizobial strains would not grow on soluble starch as a carbohydrate source. However, Mukerji and Johari (1968) grew rhizobia on both soluble and potato starch in the presence of mannitol, and observed dextrinisation of starch. No detectable free sugars were produced in the medium and this was attributed to their immediate utilization in preference to mannitol.

The situation regarding the importance of starch and other polysaccharides in nodules is not clear and has not altered significantly since 1962 when Raggio and Raggio commented that "...the behaviour of starch and other polysaccharides in nodules deserves careful and systematic attention before conclusions can be drawn on the importance of these substances for nodule function".

Although many authors cited previously have implicated the importance of photosynthetic assimilates in nitrogen fixation, there have been few attempts to examine the connexion between them in detail. In the work described here, attempts have been made to clarify the relationship between nitrogen fixation and photosynthetic assimilates in the nodules of two leguminous species. Simultaneous measurements of acetylene-reducing activity and radioactivity of  $^{14}\text{C}$ -labelled photosynthates in the nodules have been made over a wide range of experimental conditions.

Natural variations during the growth cycle of plants have been examined and attempts made to explain the effects of removing shoot apices. Diurnal fluctuations have been observed and efforts have been made to elucidate the main factors responsible. Experiments involving artificial darkening of plants were carried out to study its effects on acetylene-reducing activity and the transport of photosynthates to the nodules. The nature and metabolism of the photosynthetic assimilates in the nodules have also been examined by  $^{14}\text{C}$ -tracer studies.

The distribution of  $^{14}\text{C}$ -labelled photosynthates and sites of active reduction within the nodules have been determined by microautoradiography and histochemical tests with tetrazolium salts respectively. Attempts have been made to assess the importance of the storage reserves, PHB and starch, in the support of nitrogen fixation in the nodules.

MATERIALS AND METHODS

(1) Biological material	25
(2) Assessment of growth	27
(3) Acetylene reduction assay	28
(4) Total plant nitrogen	29
(5) <sup>14</sup> C-tracer experiments	30
a) Exposure of plant material to <sup>14</sup> CO <sub>2</sub>	30
(i) Photosynthetic fixation by shoots	
(ii) Dark fixation by nodules	
b) Extraction procedures	31
(i) Extraction of <u>Pisum sativum</u> plants for direct assessment of radioactivity	
(ii) Extraction of nodules for analysis of radioactive compounds	
c) Fractionation of nodule extracts	32
(i) Ion-exchange chromatography	
(ii) Analysis of individual fractions	
a. Neutral fraction	
b. Basic fraction	
c. Acidic fraction	
d) Analysis by gas-liquid chromatography (GLC)	35
e) Analysis by gas chromatography-mass spectrometry (GC-MS)	36
f) Scintillation counting	36
(6) Respiration rate and respiratory quotient (R.Q.)	37
(7) Poly-β-hydroxy-butyric acid (PHB) content of nodules and cultured <u>Rhizobium leguminosarum</u>	37
(8) Total ethanol-soluble carbohydrates	39

(9) Autoradiography	39
a) Whole plants	
b) Microautoradiography	
(10) Histochemical tests with tetrazolium salts	40
(11) Statistical treatment of data	41
a) Replication	
b) Standard error of the mean	
c) t-test	
d) Percentage data.	

(1) BIOLOGICAL MATERIAL

Two species of legumes were used in these studies: Pisum sativum L. cv. "Alaska" (garden pea) from Alex S. Mair (Glasgow), and Vicia faba L.

"The Sutton" (dwarf broad bean) from Sutton and Sons Ltd. Both species were inoculated at planting with Rothamsted strain 1202 of Rhizobium leguminosarum.

R. leguminosarum was obtained as a freeze-dried culture in a sealed ampoule and was reconstituted in the mannitol yeast-water medium of Nutman (1946): mannitol 10.0,  $K_2HPO_4$  0.5,  $MgSO_4$  0.2, NaCl 0.2,  $CaCl_2$  0.2,  $FeCl_3$  0.01 and 'Difco' yeast extract 0.4 g.l.<sup>-1</sup>. The culture was maintained on slopes of mannitol yeast-water medium solidified with 1.5% agar in rimless tubes (150 mm. x 25 mm.) at  $28 \pm 1^\circ C$  in a dark incubator. The bacteria were subcultured at intervals not greater than 14 days to maintain an actively growing population. Liquid cultures were inoculated from tube cultures by a modification of the method of Van Schreven et al. (1953). The bacteria were scraped off the agar into sterile distilled water and the resulting suspension was added to 100 ml. mannitol yeast-water medium in 250 ml. Erlenmeyer flasks. Inoculated flasks were incubated on a Gallenkamp Orbital Incubator at 150 revs.min.<sup>-1</sup> and  $28 \pm 1^\circ C$ . Routine subculturing was carried out every 14 days using a 1% inoculum. Media and glassware were sterilised by autoclaving at  $120^\circ C$  (15 p.s.i.) for 15 minutes, and inoculation and subculturing were carried out in a chamber designed and built in the Botany Department.

P. sativum plants, inoculated with a suspension of agar-slope cultures of R. leguminosarum, were grown in groups of four in 7.5 cm. diameter plastic pots in "Peralite" (British Gypsum Ltd.). They were supplied with a nitrogen-free nutrient solution of Johnson, Evans and Ching (1966):

<u>Macronutrient salts (g.l.<sup>-1</sup>)</u>		<u>Micronutrient salts (mg.l.<sup>-1</sup>)</u>	
K <sub>2</sub> SO <sub>4</sub>	0.28	Fe EDTA	1.0
MgSO <sub>4</sub>	0.49	H <sub>3</sub> BO <sub>3</sub>	0.25
KH <sub>2</sub> PO <sub>4</sub>	0.02	MnSO <sub>4</sub> ·7H <sub>2</sub> O	0.25
K <sub>2</sub> HPO <sub>4</sub>	0.15	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.05
CaSO <sub>4</sub> ·2H <sub>2</sub> O	1.03	CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.02
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.06	Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.01
		CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.05

The nutrient solution was made up with tap water and had a pH of 6.0. It was used at one-third strength to wet the "Peralite" before planting the seeds.

After emergence of the seedlings, the plants were supplied once weekly with nutrient solution and watered with tap water as necessary for healthy growth. Plants were grown in a glasshouse with a minimum temperature of 15°C under natural daylight supplemented by 400W MBFR/U high pressure mercury vapour lamps (Thorn Lighting Ltd.), placed approximately 1 m. apart and 1 m. above the glasshouse benches to give a minimum 16-hour photoperiod.

Plants required for experiments were transferred at least 24 hours before use to a Mark I Saxcil Plant Growth Cabinet and maintained at 25 ± 1°C under fluorescent lights, unless otherwise stated. The fluorescent light was supplied by equal numbers of Mazda 60/85W "Daylight" and "Warm White" tubes to give a light intensity of 4.039 cal.m.<sup>-2</sup>.h<sup>-1</sup> (total irradiation measured on a Pye Scalamp Galvanometer using a Kipp and Zonen calibrated thermopile) or 1,000 ft. candles (measured on an EEL light-meter, which records wavelengths of light from 250 to 730 nm). Both light sensors were placed horizontally at a height of 5 cm. above the surface of the "Peralite". The ratio of "Daylight" to "Warm White" tubes was kept constant when the light intensity was adjusted. During darkening experiments, plants were placed inside a wooden light-tight container in the growth cabinet; the interior of the box remained at the same temperature as the cabinet during the experiments.

Unsuccessful attempts were made in 1970 to 1971 to grow nodulated V. faba

plants in the glasshouse in nitrogen-free culture similar to that used for P. sativum. These plants were therefore field-grown during the summer in the grounds of the Botany Department Research Laboratories at Garscube Estate, Bearsden, Glasgow, in a plot of ground which had received no fertilizer prior to planting. Seed was sown in rows approximately 35 cm. apart, from May until August 1971 and 1972, and the plants were nodulated naturally. Plants for use during the winter were grown in beds of steam-sterilized soil in a heated glasshouse from September 1972 until May 1973, with natural daylight supplemented by 400W MBFR/U high pressure mercury vapour lamps placed 1.5 m. above the soil surface. Little natural nodulation took place, and so the plants were inoculated with R. leguminosarum from liquid shake-cultures. Plants used for experiments in the growth cabinets were carefully dug up, potted in 12.5 cm. diameter plastic pots in the habitat soil and grown on in the glasshouse for one week. They were then placed in a growth cabinet for 24 hours before use under the same conditions as P. sativum.

## (2) ASSESSMENT OF GROWTH

The growth of R. leguminosarum liquid cultures was followed

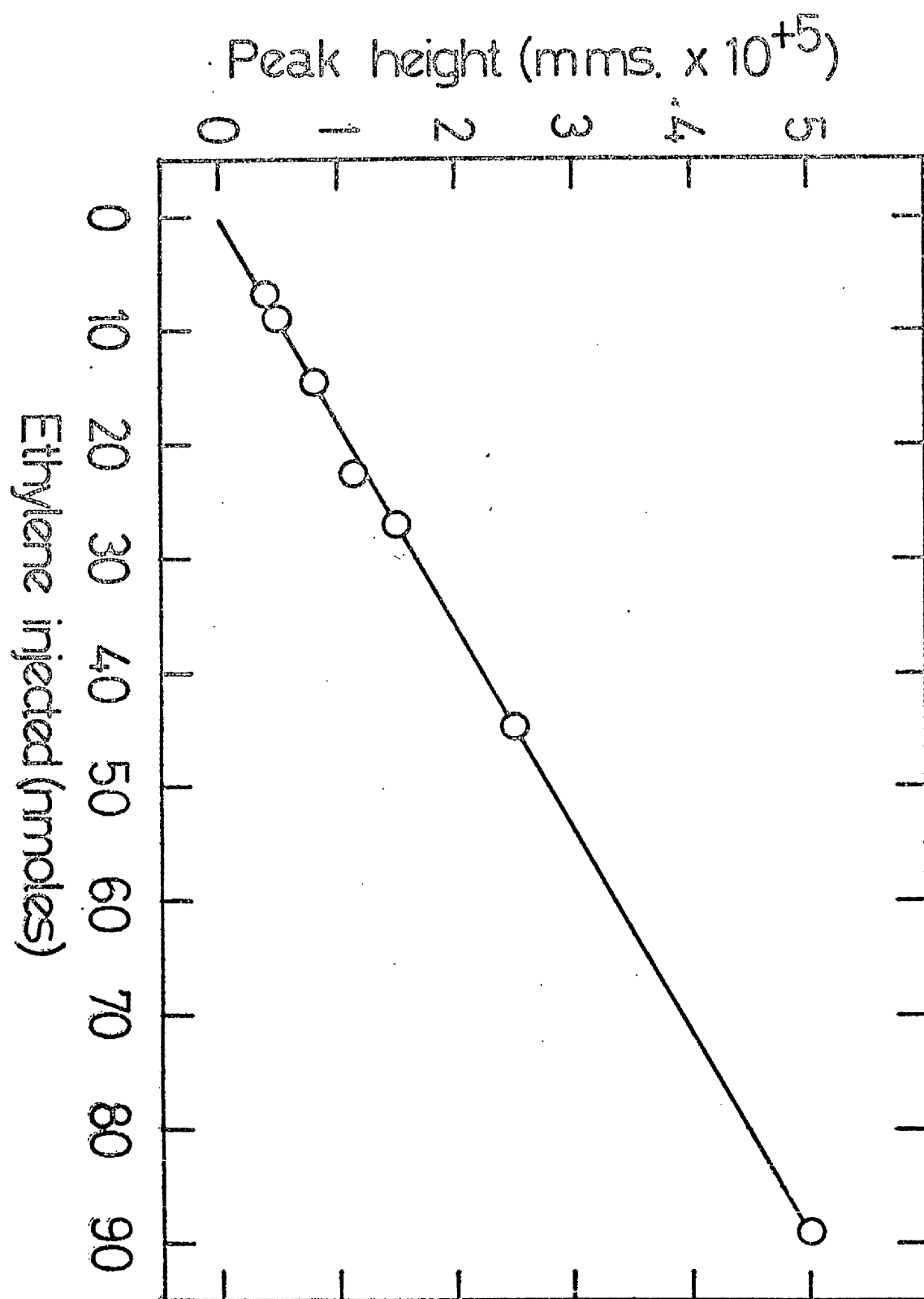
- a) by measuring the absorption at 600 nm. of samples of the inoculated liquid medium on a Pye Unicam Series 2 Ultraviolet and Visible Spectrophotometer.
- b) by determining the dry weight of bacteria in equal aliquots of inoculated liquid medium after drying at 95°C for 24 hours.

Three replicate flasks were used for each measurement and the results were highly reproducible.

The growth and development of the two plant species selected for study was monitored at weekly intervals during the growth period. Thirty pea plants and ten bean plants from each of six and three crops respectively were harvested weekly and the fresh weight of the shoot, denodulated root and nodules determined for each plant.



Fig. 1. Relationship between detector response and amount of ethylene injected. Each point is the mean of five replicate injections of standard ethylene : air mixtures into a 7 ft Porapak R column at 65°C with gas flow rates of 50 ml. min.<sup>-1</sup> N<sub>2</sub>, 50 ml.min.<sup>-1</sup> H<sub>2</sub> and 150 ml. min.<sup>-1</sup> air on a Pye 105 Gas Chromatograph.



### (3) ACETYLENE REDUCTION ASSAY

Nitrogen fixation was measured using the acetylene reduction technique. Gases were supplied from cylinders of oxygen (British Oxygen Co. Ltd.) and a mixture of 25% acetylene : 75% argon (Hilger and Watts) and were passed through wash bottles containing (i)  $\text{H}_2\text{SO}_4$  (80% w/v) to remove traces of  $\text{H}_2\text{S}$ , and (ii) distilled water to remove traces of  $\text{SO}_2$ . A small amount of ethylene was present as an impurity in the final gas mixture and control tubes without plant material were used to estimate and correct for this in all experiments. There was no detectable ethylene production by denodulated roots.

The ethylene produced by reduction of acetylene was estimated using a Pye 105 gas chromatograph with a 7 ft. column of Porapak R (Phase Separations Ltd.) at  $65^\circ\text{C}$  and gas flow rates of  $50 \text{ ml. min.}^{-1} \text{ N}_2$ ,  $50 \text{ ml. min.}^{-1} \text{ H}_2$  and  $150 \text{ ml. min.}^{-1}$  air (30, 24 and 20 p.s.i. respectively). The retention times for ethylene and acetylene were approximately two and three minutes respectively. Ethylene samples of known concentrations were prepared, by dilution if necessary, from standard ethylene:air mixtures (British Oxygen Co. Special Gases). The height of the ethylene peak was linearly related to the amount injected, and a calibration graph was constructed (Fig. 1).

Plant material was placed inside each of four 37 or 52 ml. tubes fitted with "Subaseal" stoppers and side arms. The tubes were attached to a manifold fitted with a manometer and the gas phase was evacuated and replaced by a mixture containing 20% oxygen, 20% acetylene and 60% argon to atmospheric pressure, unless otherwise stated. The sealed tubes were incubated for 30 minutes at  $25 \pm 1^\circ\text{C}$  in the dark, unless otherwise stated, and reactions were terminated by freezing the samples at  $-25^\circ\text{C}$ . Gas samples were withdrawn using 2 ml. polythene disposable syringes, and normally 1 ml. was injected in triplicate from each sample tube.

Pea plants were normally prepared for analysis by gently removing plants from their pots, shaking off the "Peralite" and washing the roots briefly under a running tap. Nodulated roots were blotted dry with paper tissues,

Fig. 2 (top). Time course of acetylene reduction by nodulated pea roots. Plant material was incubated in closed tubes in the acetylene : oxygen : argon atmosphere used for assay and samples were withdrawn at the times shown. Dashed lines indicate initial rate of acetylene reduction.

Fig. 3 (bottom). Time course of acetylene reduction by detached broad bean nodules. Plant material was incubated and assayed as stated above.

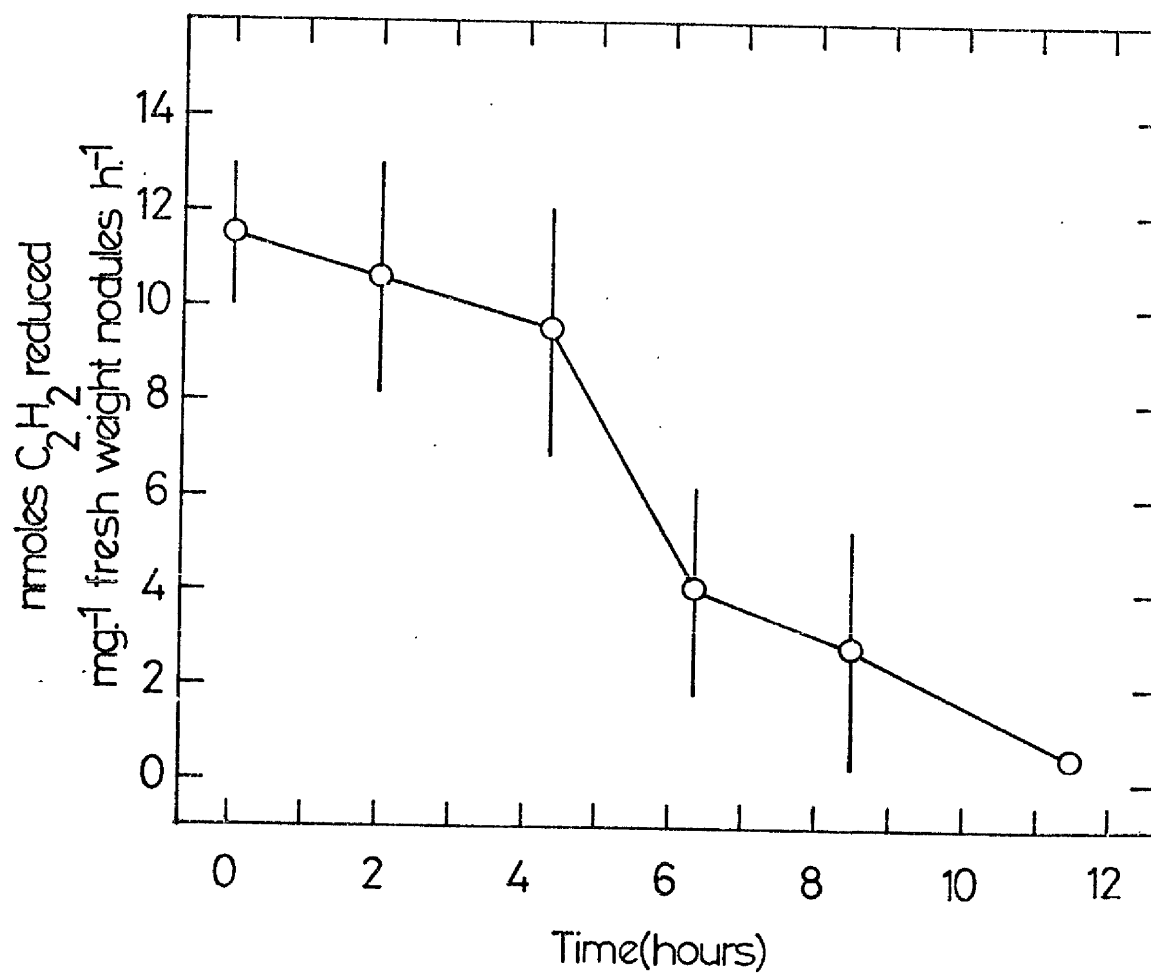
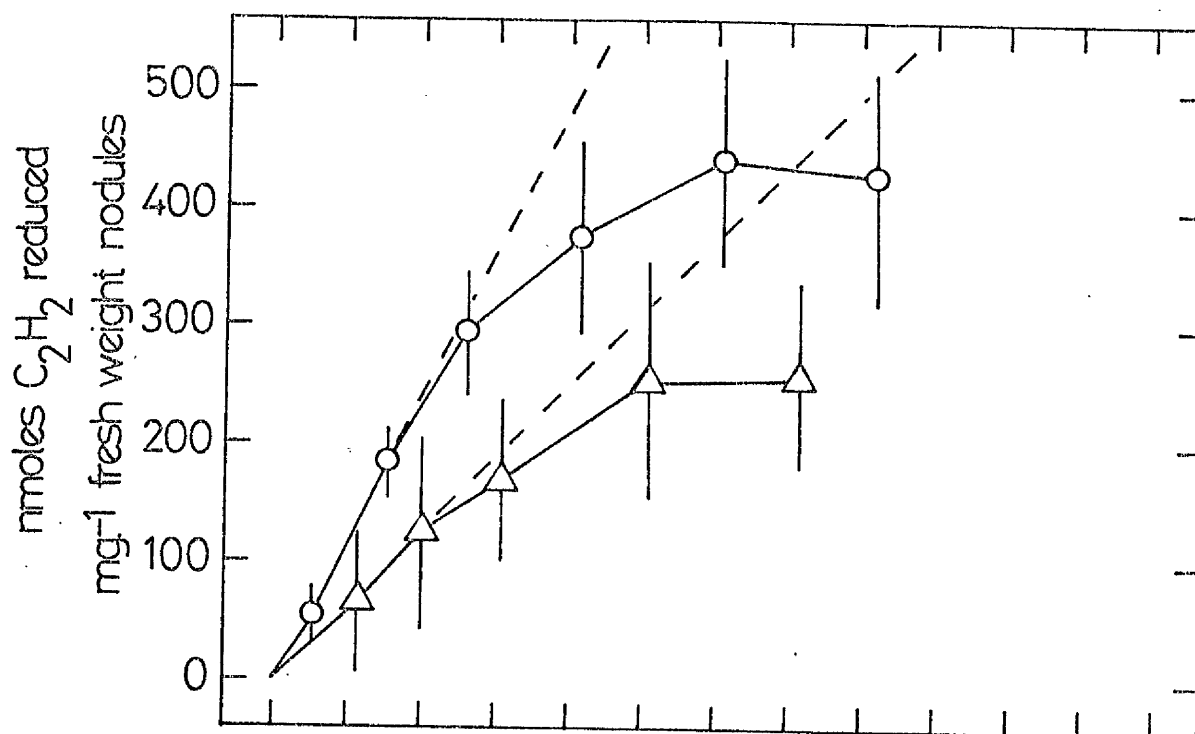
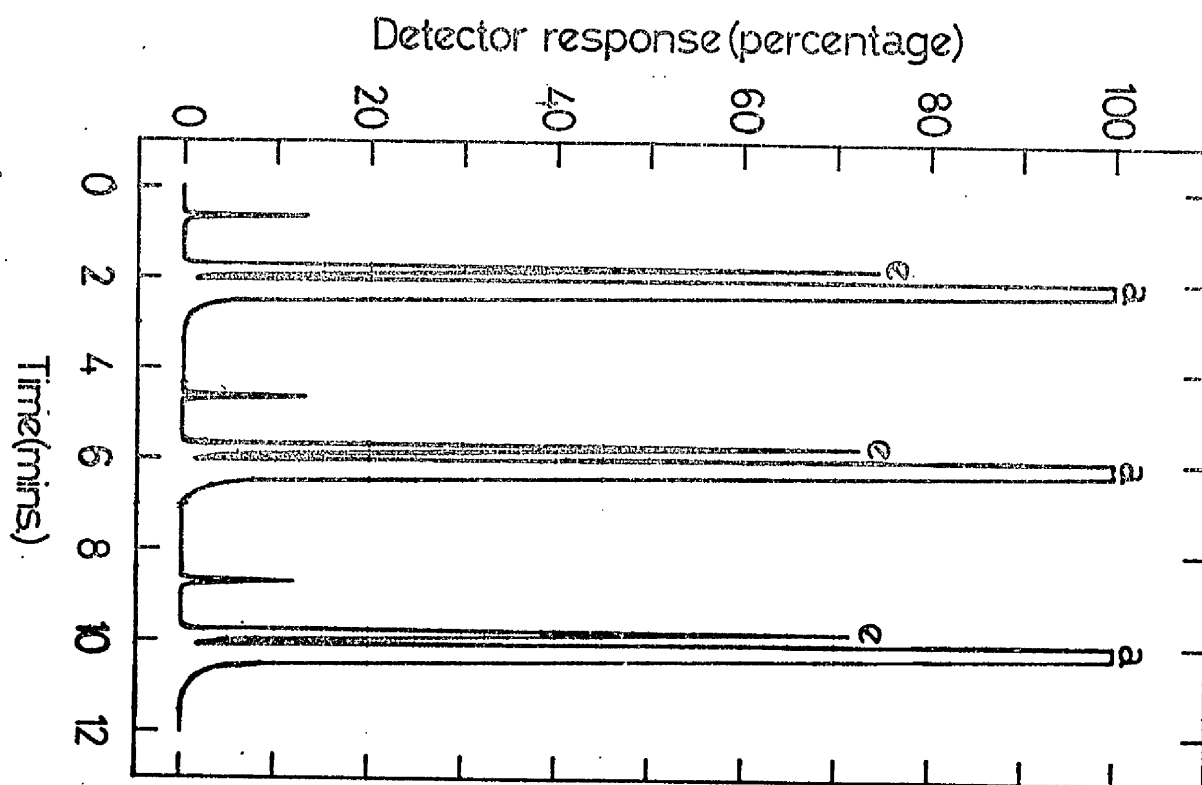


Fig. 4. Separation of ethylene (e) from acetylene (a) in 3 replicate gas samples chromatographed in a 7 ft Porapak R column operated at 65°C with gas flow rates of 50 ml.min.<sup>-1</sup> N<sub>2</sub>, 50 ml.min.<sup>-1</sup> H<sub>2</sub> and 150 ml.min.<sup>-1</sup> air.



severed from the tops and inserted in groups of four into each of four replicate tubes. After analysis, nodules were excised from the roots with forceps and weighed immediately. In some experiments, nodules were extracted in alcohol in their original sample groups for anthrone analysis (Section 8). In one experiment, nodules were excised from the roots before analysis and whole plants were also used (one plant in each tube).

Bean plants were dug up, the nodules removed and immediately rinsed under a running tap. The nodules were blotted dry and weighed samples were placed into each of four replicate tubes. Normally 2.5 g. nodules were placed in each tube, occupying less than 7% of the gas volume. All samples for each experiment were collected simultaneously to avoid effects of diurnal variation in acetylene-reducing activity.

The incubation time used (30 minutes) was chosen after incubation of the plant materials in closed tubes for times up to 12 hours. Gas samples were withdrawn at intervals and a graph was constructed for each plant material to show the time of incubation after which activity declined significantly (Figs. 2 and 3). This was 6 hours for both P. sativum and V. faba nodules, and 30 minutes was used as incubation time thereafter for both materials.

A typical trace from the gas chromatograph is shown (Fig. 4) and two unidentified peaks can be seen in the gas mixture. These peaks were also detectable in samples of air injected into the column and no inhibition of acetylene reduction by their presence was observed.

#### (4) TOTAL PLANT NITROGEN

For comparison with the data obtained on nitrogen fixation by acetylene reduction, one series only of four successive crops of P. sativum were planted at weekly intervals and grown in the glasshouse. All crops were harvested on two consecutive days to give a range of ages from one to four weeks old. Plants were removed from the pots and the root systems were washed and blotted dry. One-week-old plants were weighed, placed in weighed metal tins and



dried at 95°C for 48 hours to constant weight. Nodules were detached from the roots of the other crops and weighed samples of denodulated plants and nodules were dried as above. All samples were cooled in a desiccator before determining the dry weights. Nodules were finely ground in a mortar and pestle and the plant samples were finely milled and returned to the tins, which were tightly capped to prevent admittance of moisture. Three replicate 0.2 g. samples of each type of dried material were subjected to micro-Kjeldahl analyses, following the procedure outlined by Wheeler (1969), and distillations of aliquots of digests were repeated until the titres agreed to within 0.050 ml.

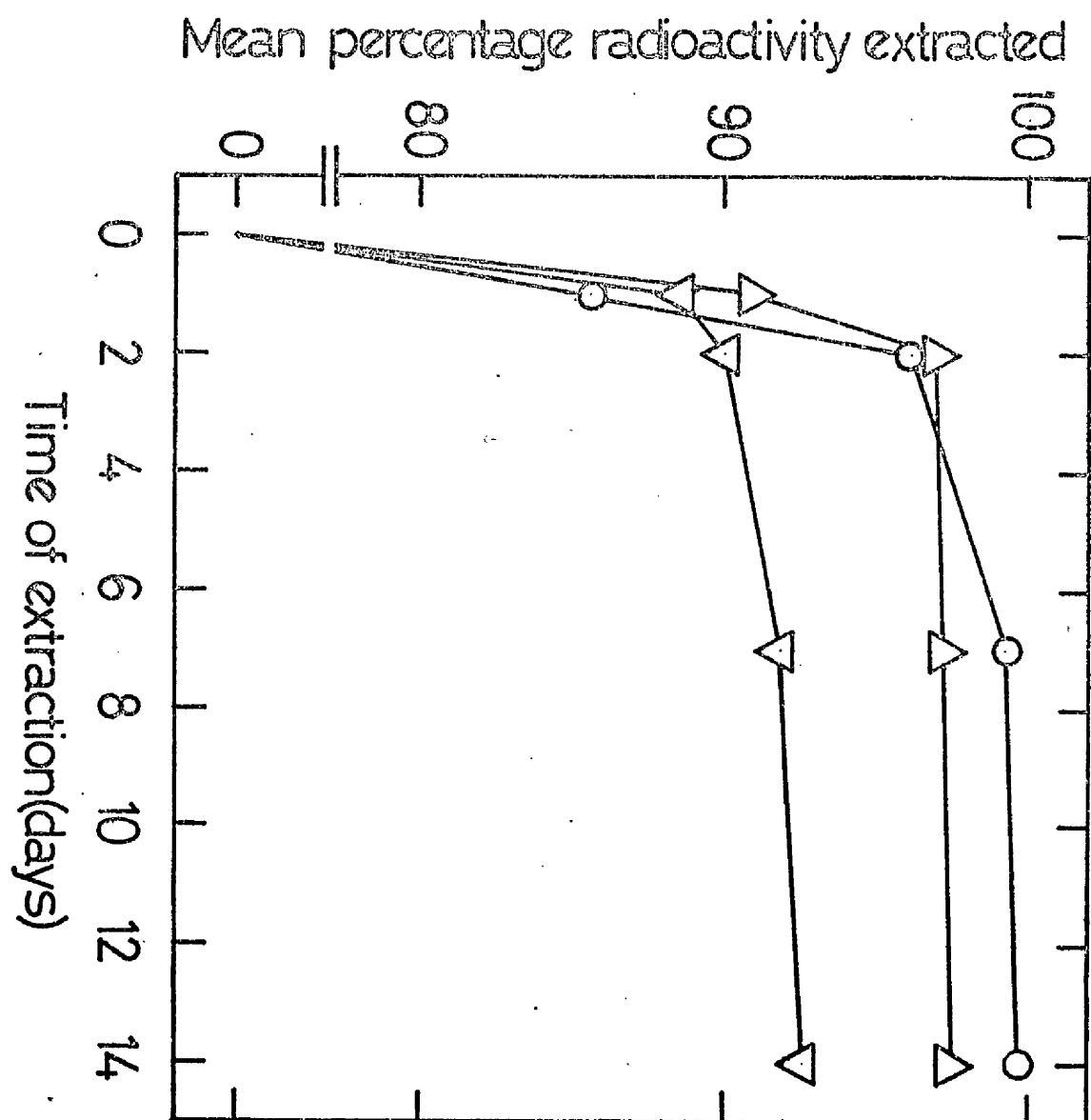
(5)  $^{14}\text{C}$ -TRACER EXPERIMENTS

a) Exposure of plant material to  $^{14}\text{CO}_2$

(i) Photosynthetic fixation by shoots

Photosynthetic assimilates accumulated by the nodules were estimated by measuring the radioactivity of the nodules after feeding  $^{14}\text{CO}_2$  to the shoot, either continuously or for 15 or 30 minutes followed by a "cold-chase" period. Pots were enclosed in polythene bags for the duration of the experiments to prevent absorption of  $^{14}\text{CO}_2$  into the rooting medium and  $^{14}\text{CO}_2$  fixation by the nodulated root. Exposure of the shoots to  $^{14}\text{CO}_2$  was made inside polythene containers of approximately 10 l. capacity by the release of 100  $\mu\text{Ci}$ .  $^{14}\text{CO}_2$  ( $4.48 \times 10^{-9}$  atm.) from  $\text{Na}_2^{14}\text{CO}_3$  solution of approximate specific activity 50  $\text{mCi. mmol.}^{-1}$  (The Radiochemical Centre, Amersham). The required quantity of  $\text{Na}_2^{14}\text{CO}_3$  was measured into gelatin capsules in a beaker and 50% perchloric acid added just before sealing the polythene containers from the atmosphere. Digestion of the capsule and release of  $^{14}\text{CO}_2$  was complete in less than one minute. After the desired exposure period, the plants were removed from the sealed containers in a fume cupboard and then returned to the growth cabinet for the required "cold-chase" period.

Fig. 5. Time course of extraction of radioactive compounds from detached pea nodules in 2 ml 95% ethanol at 0 to 4°C. Plant shoots were exposed to  $^{14}\text{CO}_2$  for 15 minutes and nodules were detached after 4 hours (○), 10 hours (Δ) and 23 hours (▽) "cold-chase".



### (ii) Dark fixation by nodules

For  $^{14}\text{CO}_2$  fixation experiments with V. faba nodules, the nodules were removed from the roots, washed and blotted dry. Samples were placed on moist filter papers inside 250 ml. conical flasks fitted with side arms and ground glass stoppers. Gelatin capsules containing the required amounts of  $\text{Na}_2^{14}\text{CO}_3$  solution were inserted in the side arms and 50% perchloric acid was added to the side arms just before the flasks were sealed from the atmosphere. Each flask contained 10 g. nodules and 100  $\mu\text{Ci. } ^{14}\text{CO}_2$  ( $1.79 \times 10^{-4}$  atm.) was released. Exposures were either continuous or for 30 minutes followed by a "cold-chase" period during which compressed air was passed through the flasks by means of a manifold to flush out any  $^{14}\text{CO}_2$  produced in respiration. All the operations were carried out in a darkened fume cupboard at room temperature (approximately  $20^\circ\text{C}$ ).

### b) Extraction procedures

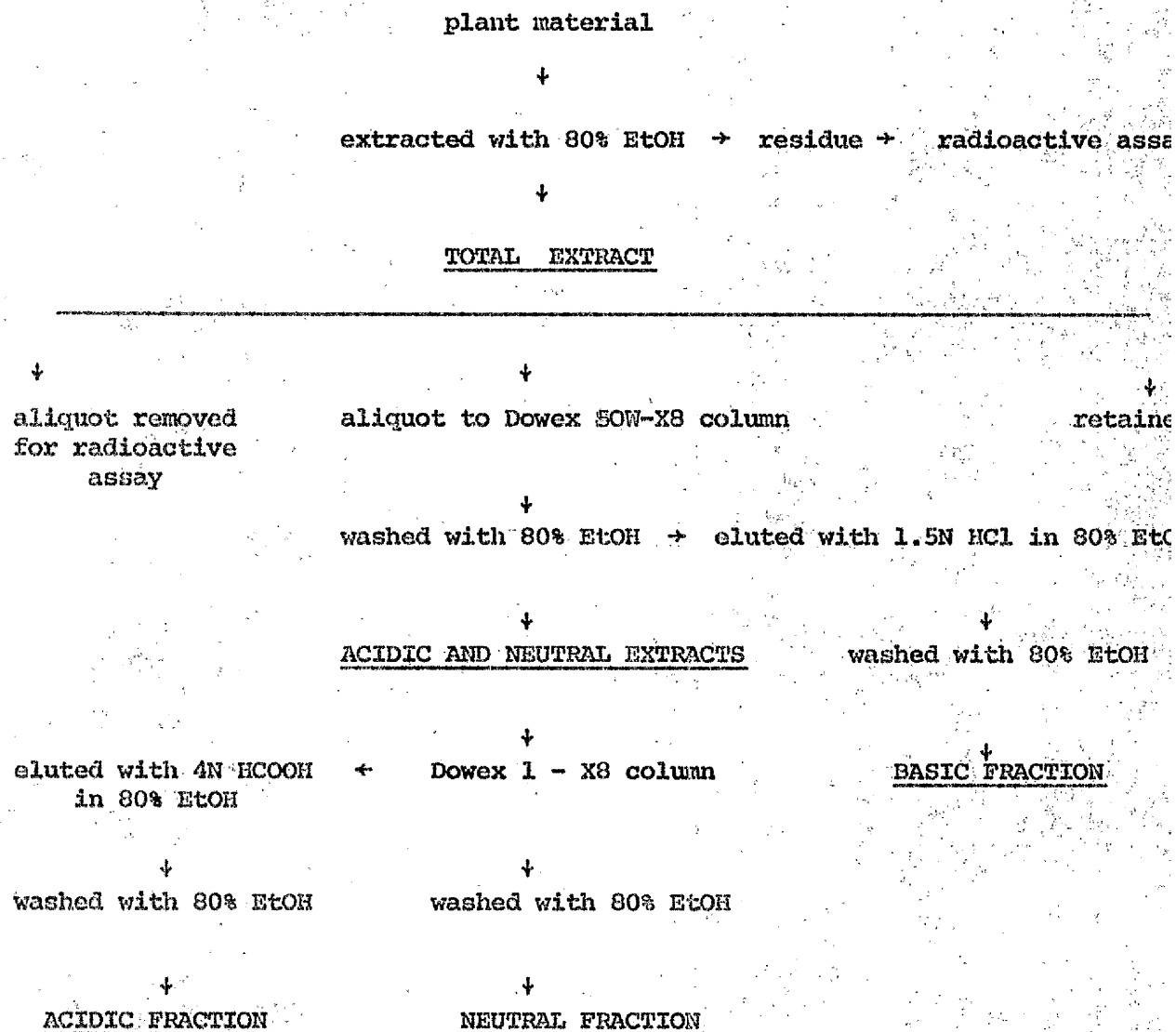
#### (i) Extraction of P. sativum plants for direct assessment of radioactivity

Plants were carefully removed from the pots after the desired "cold-chase" period and the "Peralite" shaken from the roots, which were washed briefly and blotted dry. Nodules were removed from each plant and the plant was cut into sufficiently small pieces for insertion into numbered scintillation vials. Normally ten replicate plants were used for each sample. Radioactive compounds were extracted from each plant part for scintillation counting in 2 ml. 95% ethanol per vial at 0 to  $4^\circ\text{C}$  for 48 hours, and this procedure extracted over 90% of the activity (Fig. 5).

#### (ii) Extraction of nodules for analysis of radioactive compounds

All solvents used in extraction and analytical procedures were redistilled or "Analar" quality.

P. sativum. Three replicate samples, each comprising the nodules

Summary of fractionation procedure

from 12 plants, were extracted in scintillation vials in 10 ml. 80% ethanol for 24 hours at 2°C. The extracts were then filtered under suction through Whatman No. 1 filter paper, and the residues, washed twice with 10 ml. aliquots of 80% ethanol, were retained for assessment of unextracted activity.

V. faba. In those experiments in which the shoots were exposed to  $^{14}\text{CO}_2$ , the nodules of 8 plants were combined for each sample, while 10 g. samples of nodules were used for dark fixation experiments. Nodules were ground with 80% ethanol in a mortar and pestle and the resulting slurry was extracted in 100 ml. 80% ethanol on a wrist-action shaker at room temperature for one hour. Extracts were filtered under suction through muslin and Whatman No. 1 filter paper and the residue was re-extracted as before. The extracts were combined and centrifuged at 2000 g. at 2°C to obtain clear supernatants. Samples of the residues were retained for assessment of radioactivity.

c) Fractionation of nodule extracts

(i) Ion-exchange chromatography

Extracts were dried by rotary evaporation at 35°C and redissolved in 80% ethanol. Samples of each extract were removed for radioactive assay and neutralised aliquots were fractionated into "basic" (mainly amino-acids), "neutral" (mainly sugars) and "acidic" (mainly organic acids) portions by a modification of the ion-exchange method of Wang (1960). A summary of the fractionation procedure is shown opposite.

Both resins were regenerated before use by soaking for 30 minutes in the acids used for elution and washing with 80% ethanol until neutral. The resins were poured as slurries into 10 cm. diameter glass columns fitted with taps, glass sinters and reservoirs. Aliquots of each fraction were removed for radioactive assay after rotary evaporation to a small volume. Recovery of radioactivity after fractionation was 63% to 78% (P. sativum) and not less

than 73% (V. faba).

(ii) Analysis of individual fractions

All solvent systems used were freshly prepared from "Analar" or redistilled solvents.

a. Neutral fraction

Suitable aliquots were applied in duplicate to Whatman No. 1 paper and one-dimensional chromatograms run in duplicate in the solvents shown below. Those marked \* were used as descending solvents. Authentic samples of sugars were made up as 0.5 or 0.7% solutions in 10% iso-propanol and spotted as control spots to check Rf values of unknown compounds. Solvents used were:

- a) IPrAq (iso-propanol : water = 4:1) (Smith, 1962)\*
- b) EtAcPy (ethyl acetate : pyridine : water = 12:5:4) (Smith, 1962)\* or run three times as an ascending solvent.
- c) AcW (acetone : water = 85:15) (Veiga and Chandelier, 1967) - run twice as an ascending solvent.
- d) water\*
- e) BuA (n-butanol : glacial acetic acid : water = 12:3:5) (Smith, 1962).

One of each pair of duplicate chromatograms was either cut into 20 equal strips in half-Rf zones and assayed for radioactivity by scintillation counting or was passed through a Panax Thin-Layer and Paper Chromatogram Scanner. The duplicate chromatograms of each pair were developed in one or more of the following location reagents after examination under ultraviolet light:

- a) aniline-diphenylamine (locates and colorimetrically identifies sugars) (Smith, 1962).
- b) aniline (locates sugars) (Smith, 1962).
- c) Elson-Morgan reagent (locates hexosamines) (Smith, 1962).
- d) silver nitrate reagent (locates sugars, organic acids, phenolic acids and many other compounds) (Smith, 1962).
- e) ferric chloride-ferricyanide (locates phenols) (Smith, 1962).
- f) hypochlorite (locates carbamates) (Smith, 1962).

Sugars were identified on the basis of their Rf or Rg values in solvents a) to c), cochromatography with authentic samples in three different solvent systems and their colour reaction with aniline-diphenylamine reagent.

b. Basic fraction

Suitable aliquots were applied in duplicate to Whatman No. 1 paper and one-dimensional chromatograms run in duplicate in the solvents shown below. Those marked \* were used as descending chromatographic solvents. Authentic samples of amino-acids and amides were made up as 2 mg.ml.<sup>-1</sup> solutions in 10% iso-propanol and spotted as controls to check the Rf values of unknown compounds. Solvents used were:

- a) BuA (n-butanol : glacial acetic acid : water = 12:3:5) (Smith, 1962).
- b) Ph (phenol : water = 4:1 w/v) (Smith, 1962).
- c) PhNH<sub>3</sub> (80% w/v phenol : ammonia (0.880) = 200:1) (Smith, 1962).
- d) PhA (72% w/v phenol : ammonia (0.880) = 200:1) (Pate, 1962)\*
- e) BuP (n-butanol : pyridine : water = 5:6:6) (pate, 1962)\*

One of each pair of chromatograms was assayed for radioactivity by cutting into 20 equal strips and scintillation counting as before. Amino-acids and amides were located on the other using one or both of the following reagents:

- a) ninhydrin (0.2% in acetone) (Smith, 1962).
- b) isatin (0.2% in acetone) (Smith, 1962).

Two-dimensional chromatograms were also run on Whatman No. 1 paper, firstly in BuA and secondly in PhNH<sub>3</sub> and autoradiographs were prepared using Ilford Red Seal X-ray film. The chromatograms were exposed for one or two months as required, and the films were developed in Ilford "Phenisol" X-ray developer for 5 minutes and fixed in "Kodafix" for 5 minutes in total darkness. The chromatograms were developed with the locating reagents above and spots on the films and chromatograms aligned to locate the radioactive amino-acids and amides. Two-dimensional autoradiography was also used in conjunction with cochromatography with authentic amino-acids and amides to confirm the suspected identity of compounds in the extracts.



Fig. 6. Separation of trimethylsilyl (TMS) derivatives of oxalic (o), pyruvic (p),

$\beta$ -hydroxybutyric ( $\beta$ ), malonic (m), succinic (s), fumaric (f), glutaric (g), malic (mal),  $\alpha$ -ketoglutaric ( $\alpha$ ) and citric acids and n-eicosane (n). A 7 ft 5% F-60 column was used and the temperature was programmed at 2° min<sup>-1</sup> from 75 to 210°C with gas flow rates of 50 ml.min.<sup>-1</sup> N<sub>2</sub>, 50 ml.min.<sup>-1</sup> H<sub>2</sub> and 150 ml.min.<sup>-1</sup> air.

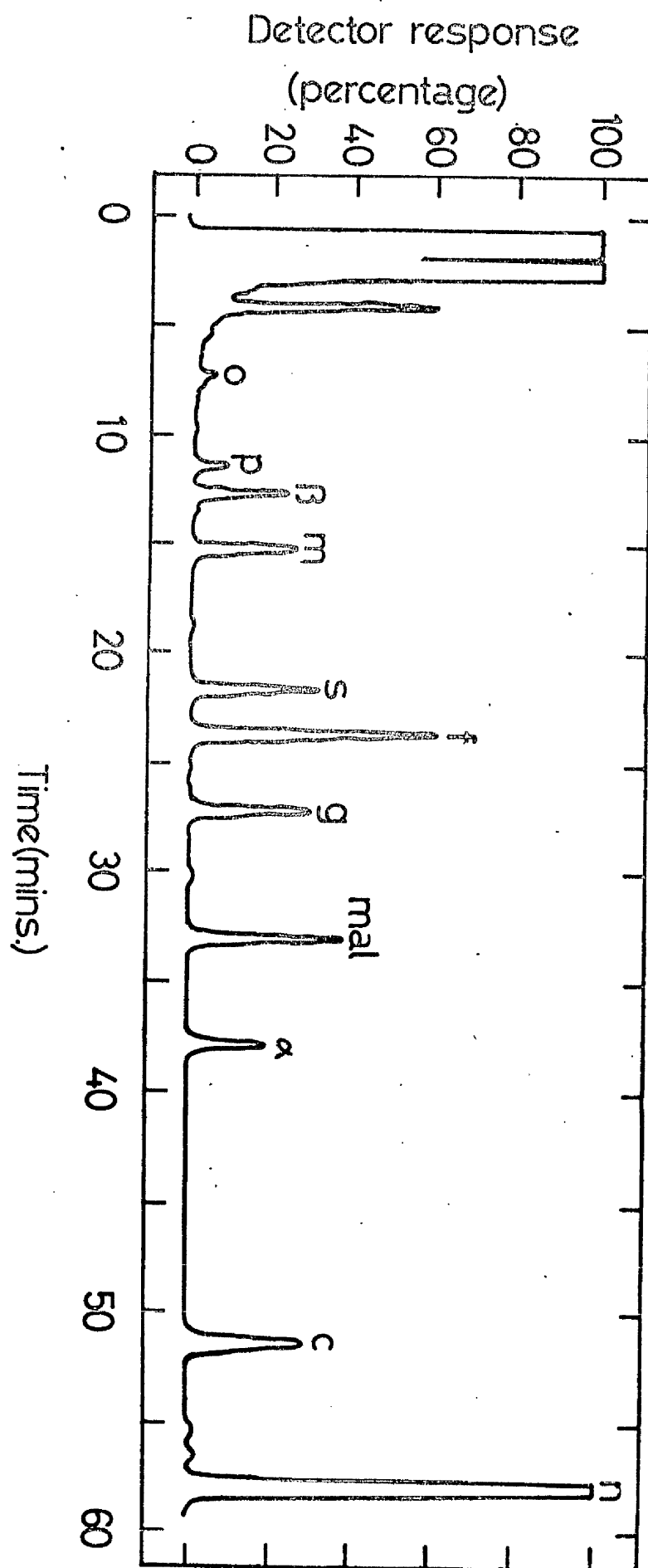
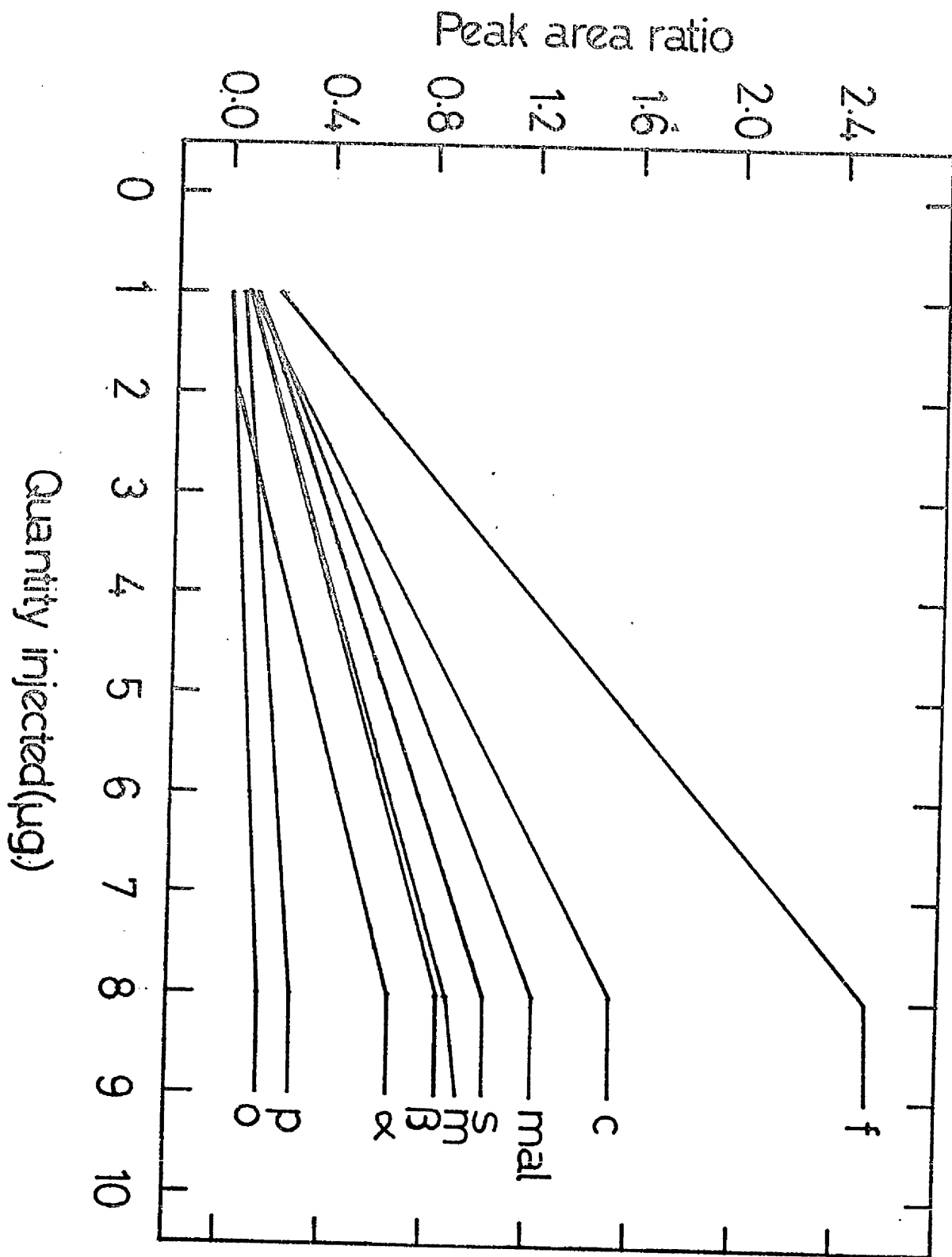


Fig. 7. Detector response to various TMS acids,  
expressed as peak area ratio, which was  
calculated from the expressions:

$$(i) \text{ peak area ratio} = \frac{\text{peak area of TMS acid}}{\text{peak area of } 8\mu\text{g TMS glutarate}}$$

$$(ii) \text{ peak area} = \text{peak height} \times \text{width at half height}$$

The abbreviations used<sup>are</sup>/as detailed in Fig. 6.



### c. Acidic fraction

Suitable aliquots were spotted in duplicate on Whatman No. 1 paper and one-dimensional chromatograms run in the solvent systems below.

Authentic organic acids were made up as 10 mg.ml.<sup>-1</sup> solutions in 10% isopropanol and control spots of a mixture of these were run on each chromatogram to check R<sub>f</sub> values of unknown compounds. The solvents used were:

- a) PrF (n-propanol : cineole(eucalyptol) : 98% formic acid = 5:5:2 [water saturated]) (Smith, 1962).
- b) EtAm (ethanol : ammonia (0.880) : water = 16:1:3) (Smith, 1962).
- c) BuA (n-butanol : glacial acetic acid : water = 12:3:5) (Smith, 1962).

One of each pair of chromatograms was cut into 20 strips and assayed for radioactivity by scintillation counting as before. Organic acids were located on the other with the following reagents:

- a) bromocresol green (0.13% in acetone) (Smith, 1962).
- b) aniline-xylose (Smith, 1962).

### d) Analysis by gas-liquid chromatography (GLC)

Analyses of suitable aliquots and eluted spots from paper chromatograms of acidic fractions of extracts were performed using a Pye 104 or 105 gas chromatograph with a 7 ft. 5% F-60 column. The temperature was programmed at 2° min.<sup>-1</sup> from 75°C to 210°C with gas flow rates of 50 ml.min.<sup>-1</sup> N<sub>2</sub>, 50 ml.min.<sup>-1</sup> H<sub>2</sub> and 150 ml.min.<sup>-1</sup> air (35, 20 and 20 p.s.i. respectively). Samples were converted to the methyloxime-trimethylsilyl (MO-TMS) esters (Horning *et al.*, 1968) by successive reaction with methyloxime hydrochloride and bis-trimethylsilyltrifluoroacetamide (BSTFA). Internal standards of n-eicosane and glutaric acid were incorporated for qualitative and quantitative purposes respectively, and a typical trace is shown in Fig. 6. A calibration graph was prepared, using authentic organic acids, of peak area ratio (peak height x width at half height) versus the amount (µg.) injected and linear relationships were found for all the acids tested (Fig. 7) and their R<sub>E</sub> values

noted relative to that of n-eicosane.

e) Analysis by gas chromatography - mass spectrometry (GC-MS)

The Rf values (retention time relative to n-eicosane) of MO-TMS esters in the samples were determined on a 3% OV-17 column for use in GC-MS analysis. Samples were investigated using a 9 ft. 1% OV-17 column temperature-programmed at  $1^{\circ}\text{min.}^{-1}$  on an LKB 9000 Gas Chromatograph - Mass Spectrometer and scans of the desired peaks taken. Scans of derivatives of authentic acids were also made for comparative purposes.

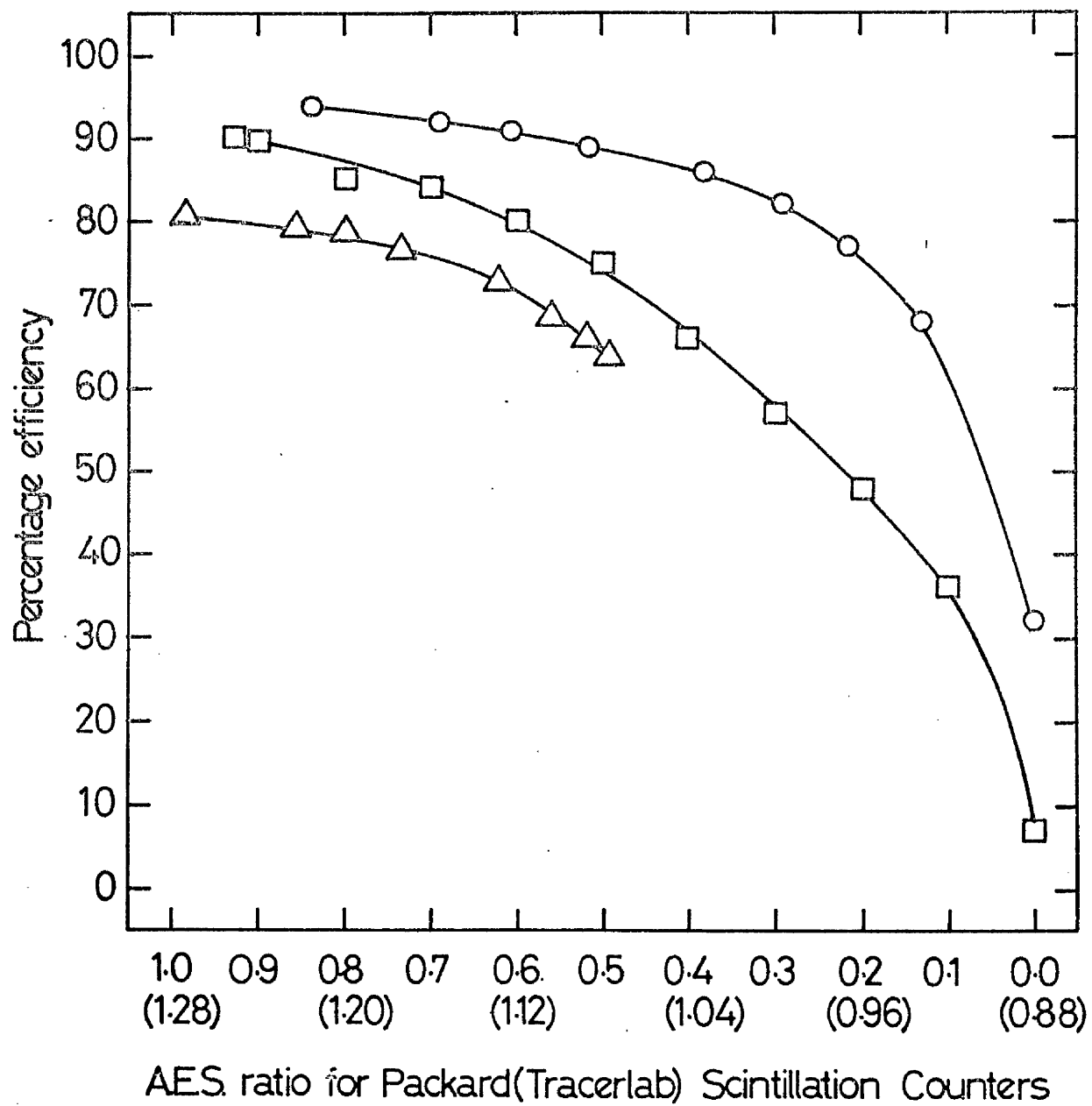
Organic acids were identified by Rf values on paper chromatograms, Rt values on GLC analysis and similarity of GC-MS scans to those of authentic samples.

f) Scintillation counting

Alcohol was removed from extracts or samples of fractions under vacuum in the presence of dry silica gel until the plant material, where present, was completely dry. As the plant material was not removed from the vials, unextracted radioactivity was also assessed, although less efficiently than that in solution. Scintillation fluid (4g. PPO[2,5-diphenyloxazole]1.  $^{-1}$  toluene) was added (10 ml. per vial) and the vials were capped tightly and stored at 0 to  $4^{\circ}\text{C}$  until counted. In some cases the scintillant used was "Unisolve" (Koch-Light Laboratories Ltd.).

Radioactivity was assessed by scintillation counting of vials on either of two Packard Tri-Carb Scintillation Spectrometers, one of which was fitted with an AAA attachment for automatic quench-correction, or on a Tracerlab Corumatic 200 Liquid Scintillation Spectrometer. The efficiency of counting unquenched  $^{14}\text{C}$ -labelled samples was greater than 90% on both Packard machines. Correction for quenching was made by the external standard-channels ratio method, using americium 241 and radium 226 as external standards. A series of acetone-quenched standards of known activity were prepared from  $^{14}\text{C}$ -

Fig. 8. Calibration curve for Packard (○ and □) and Tracerlab (△) Scintillation Spectrometers. Acetone-quenched samples of  $^{14}\text{C}$ -hexdecane of known activity were used to find the relationships between the efficiency of assessment of radioactivity and the automatic external standard (AES) ratio.





hexadecane of specific activity  $1.10 \mu\text{Ci.g.}^{-1}$  (The Radiochemical Centre, Amersham). Calibration curves were thus obtained of the  $\beta$ -channel counting efficiency (observed/expected c.p.m.) versus the external standard count rates in two different channels, expressed as a ratio (Fig. 8). Correction for loss of efficiency due to quenching could then be made using the relationship:

$$\text{absolute activity (d.p.m.)} = \frac{\text{observed activity (c.p.m.)}}{\text{efficiency}}$$

where d.p.m. = disintegrations per minute.

c.p.m. = counts per minute.

#### (6) RESPIRATION RATE AND RESPIRATORY QUOTIENT

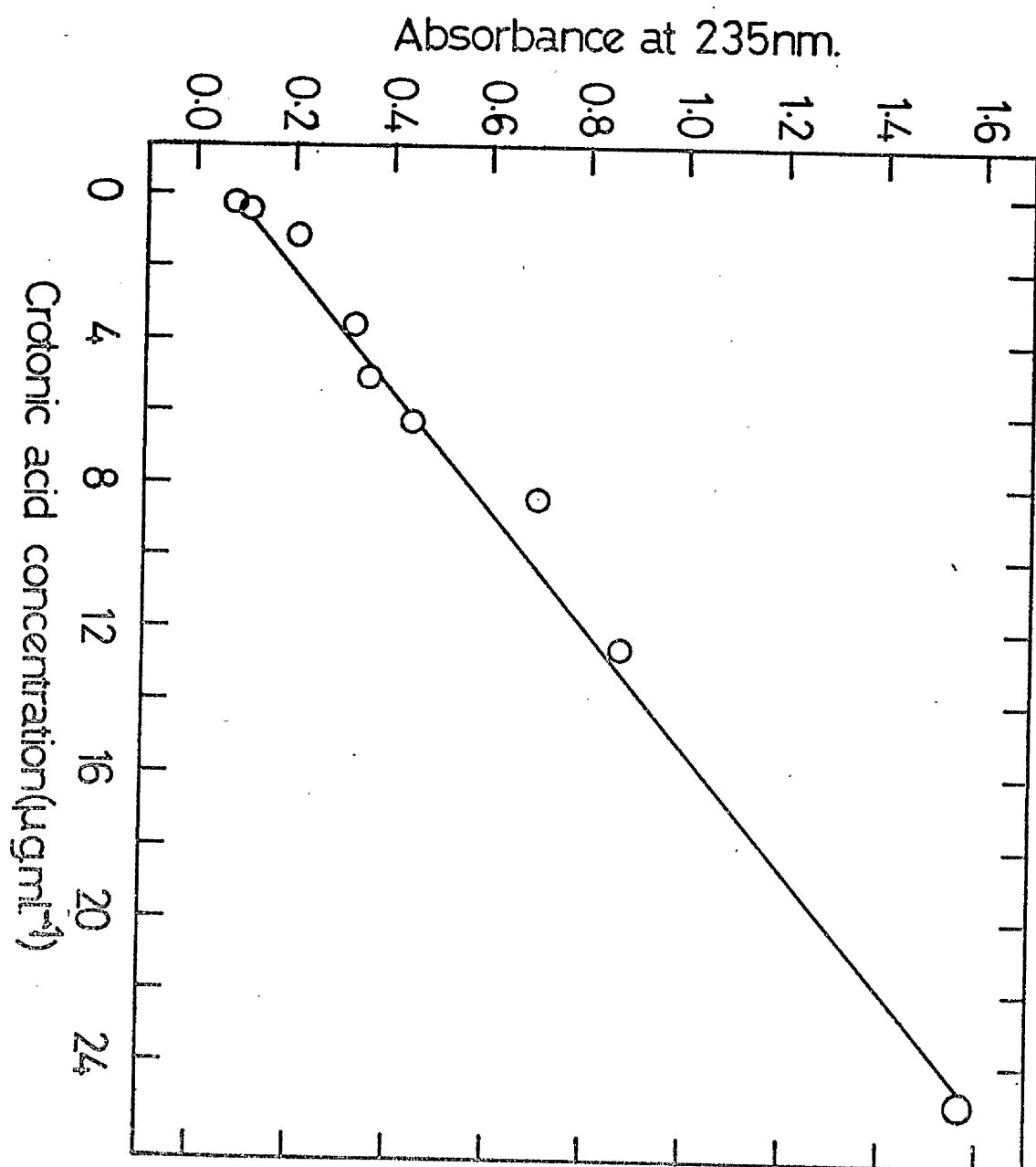
Glasshouse-grown plants were used in these experiments since it was not possible to accommodate sufficient numbers of plants in the growth cabinet. Samples were taken between 1200 and 1400 hours to minimise effects caused by possible diurnal fluctuations. Six 500 mg. samples of nodules from each age of pea plants were excised quickly from the washed roots and blotted dry, and similar samples of denodulated roots were also taken. Samples of 2-week-old nodules were not included due to their small size.

Measurements of respiration rate and  $R.Q.$  were made at  $25^{\circ}\text{C}$  using a Warburg apparatus as described by Umbreit, Burris and Stauffer (1949). Readings were taken every 10 minutes for 90 minutes, during which period the respiratory activity did not decline.

#### (7) POLY- $\beta$ -HYDROXYBUTYRIC ACID (PHB) CONTENT OF NODULES AND CULTURED RHIZOBIUM LEGUMINOSARUM

The method used for extraction and assay of PHB was that of Law and Slepecky (1961). All glassware was acid-washed, rinsed thoroughly and dried before use. PHB was converted to crotonic acid and measurements taken of

Fig. 9. Relationship between crotonic acid concentration in concentrated  $\text{H}_2\text{SO}_4$  and absorbance at 235 nm, using a concentrated  $\text{H}_2\text{SO}_4$  "blank". Each point is the mean of five separate measurements.



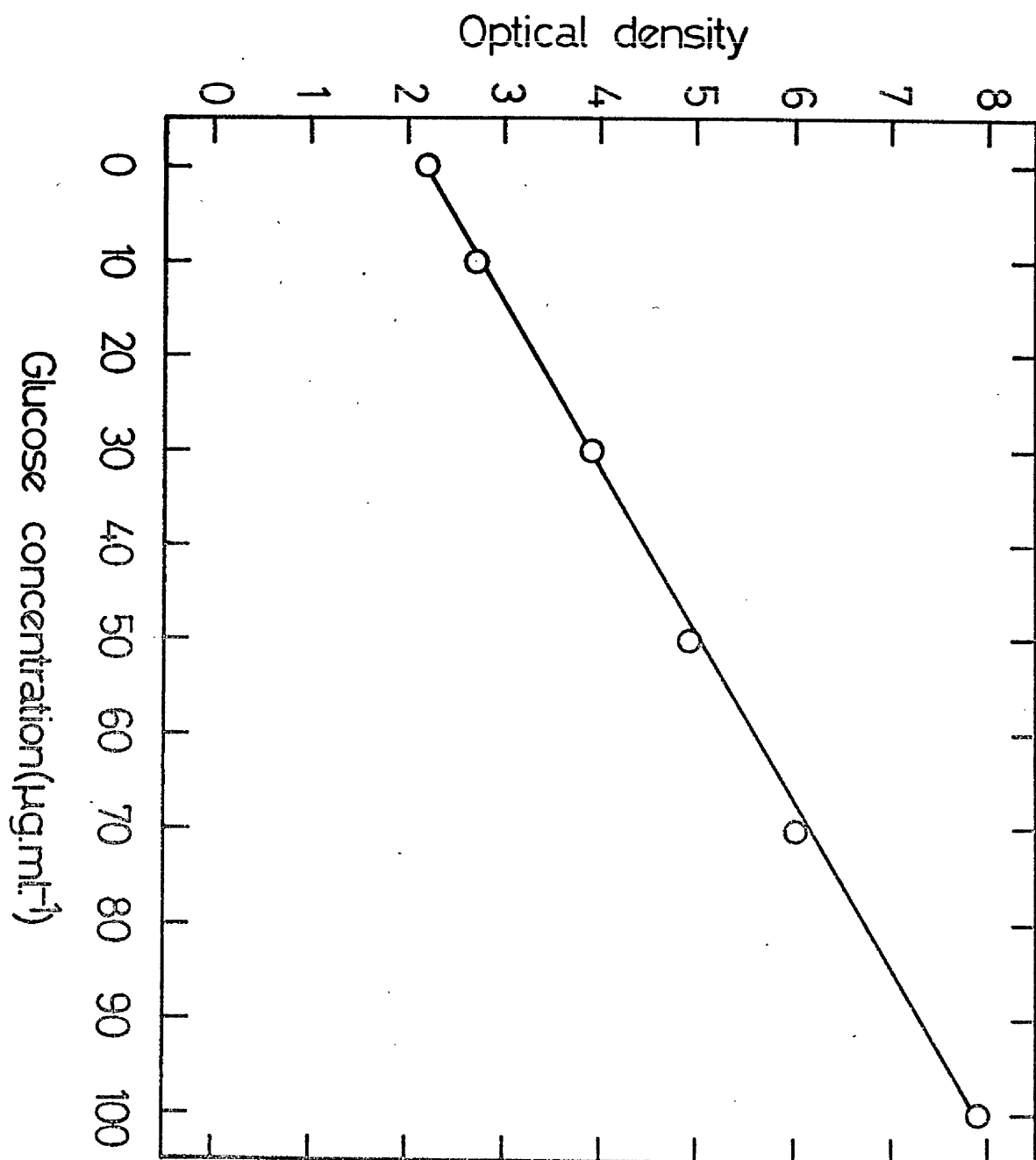
the absorbence of the solution at the absorption maximum of crotonic acid in concentrated sulphuric acid (235 nm.). A linear relationship was found between concentrations of authentic crotonic acid, from 0.05 to 25.00  $\mu\text{g}.\text{ml}^{-1}$ , and absorbence at 235 nm. and calibration graph was constructed (Fig. 9).

Triplicate 500 mg. samples of nodules and denodulated roots of P. sativum plants and triplicate sample flasks of liquid shake-cultures of R. leguminosarum were assayed at each time of collection. The experiment was performed three times with R. leguminosarum but once only in entirety with P. sativum.

Nodule and root samples were finely ground to a slurry in glass homogenizers in 0.05M tris-Cl buffer pH 8.4 at 4°C and the resulting suspensions were centrifuged at 500 g. for 5 minutes at 2°C in a Mistral 2L centrifuge to remove plant debris. The supernatants were recentrifuged in weighed 10 ml. tapered centrifuge tubes at 2000 g. at 2°C for 10 minutes to sediment the bacteroids. The resulting pellet was washed twice with cold distilled water and dried at 85°C overnight (constant weight). Bacteria were sedimented from liquid cultures by centrifugation at 2000 g. at 2°C for 10 minutes and were washed, dried and weighed as above.

Samples were hydrolysed for 16 hours with 0.2 ml. sodium hypochlorite solution (10% to 15% available Cl)  $\text{mg}^{-1}$  dried material. The pellets were resedimented, washed with cold distilled water and cold redistilled acetone and allowed to air-dry. The PHB granules were finally dissolved in boiling redistilled chloroform, cooled and filtered through pre-washed Whatman No. 1 filter papers. After evaporation of the chloroform, 10 ml. "Analar" concentrated sulphuric acid was added to each tube and the foil-capped tubes were heated for 10 minutes in boiling water to convert PHB to crotonic acid. The absorbences of the cooled solutions at 235 nm. were determined in silica cuvettes against a concentrated sulphuric acid "blank" on a Pye Unicam Series 2 Ultraviolet and Visible Spectrophotometer. The absorption spectra of all solutions were checked from 220 to 260 nm. on a Pye Unicam SP 800 Ultraviolet Spectrophotometer.

Fig. 10. Relationship between glucose concentration in distilled water and optical density (measured on an EEL photoelectric colorimeter with Orange Filter No. 607). Sample solutions were heated with anthrone reagent at 100°C for 10 minutes and cooled before the optical density was determined. Each point is the mean of 3 separate determinations.



### (8) TOTAL ETHANOL-SOLUBLE CARBOHYDRATES

Nodules from four plants of P. sativum were used for each of four replicate samples at each collection and were extracted in 2 ml. 95% ethanol at 0 to 4°C for 48 hours. The alcohol was evaporated under vacuum and each extract was redissolved in a known quantity of distilled water.

The total ethanol-soluble carbohydrate content of the diluted extracts was determined colorimetrically by the anthrone method of Dreywood (1946), as described by Morris (1948), with modifications of Yemm and Willis (1954). Sugar concentrations in the extracts were determined by reference to a calibration curve prepared for glucose (Fig. 10). Optical density was measured in matched colorimeter tubes against a distilled water "blank" using an Ilford Spectrum Orange Filter No. 607 (maximum transmission 600 nm.) with an EEL photoelectric colorimeter.

### (9) AUTORADIOGRAPHY

#### a) Whole plants

Shoots of P. sativum were exposed to  $^{14}\text{CO}_2$  for 30 minutes and, after the desired "cold-chase" periods plants were removed from the pots and the roots washed and blotted dry. The shoots were removed and the root systems mounted on stiff cards and freeze-dried for one week. Autoradiographs were prepared using Ilford Red Seal X-ray film and exposure was made for one month at -25°C, after which the films were developed and fixed as stated previously (Section 5c).

#### b) Microautoradiography

Microautoradiographs were prepared using a technique suitable for soluble compounds (Bowen et al., 1971), since 90% of the activity accumulated within the nodules was soluble in alcohol (Section 5b). Plant shoots or nodules were fed  $^{14}\text{CO}_2$  for 30 minutes and collected after the required "cold-chase"

period. Detached, washed nodules were embedded in 1 cm.<sup>3</sup> cubes of pig's liver, which had been placed previously on top of microtome chucks, and were frozen rapidly by immersion of the chuck in liquid nitrogen. Sections about 15  $\mu$  thick were cut under safelights (Kodak Series 1 - dark red) on a freezing microtome (Cambridge Scientific Instruments Ltd.) mounted in a Bright's cryostat maintained at -18 to -20°C. The sections were placed next to Kodak AR-10 stripping film, previously mounted with emulsion side outermost, on coverslips attached to glass slides for support and were maintained at -20°C.

Sections were exposed to the film for about one month at -25°C in light-tight slide boxes and microautoradiographs were developed by immersion of the slides in Kodak D-19 developer for 5 minutes and then fixed in "Kodafix" for 12 minutes. The slides were dried gently in a stream of cold air from a hair-dryer and the filmed coverslips with their attached sections eased from the slides. On occasions, some sections were stained with iodine to show the distribution of starch grains before mounting. The coverslips were permanently mounted for examination in DPX mountant with the section to the inside. Microautoradiographs were examined on a Zeiss Photomicroscope II under dark-field and phase-contrast illumination. Silver grains developed by the presence of radioactivity therefore appear as white dots, not black, in the photographs taken under dark-field illumination. Photographs were taken using Ilford FP4 low-contrast film.

#### (10) HISTOCHEMICAL TESTS WITH TETRAZOLIUM SALTS

Tetrazolium salts were used to locate sites of active reduction within the nodules by the method of Akkermanns (1971). The tetrazolium salts were used at a concentration of 0.1% in distilled water and are detailed below:



- a) INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyl tetrazolium chloride).

This became reddish-violet on reduction.

- b) NBT (nitroblue tetrazolium or 2,2'-di-4-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene)ditetrazolium chloride).

This became blue-black on reduction.

- c) TNBT (tetranitro-blue tetrazolium or 2,2',5,5'-tetra-p-nitrophenyl-3,3'-dimethoxy-4,4'-diphenylene ditetrazolium chloride). This

became blue-black on reduction.

- d) TTC (2,3,5-triphenyl tetrazolium chloride). This formed red crystals on reduction.

Washed, detached nodules from at least four plants of P. sativum of different ages were halved longitudinally to allow penetration of the tetrazolium salts. They were immediately placed with cut side down in 0.5 ml. tetrazolium solution in small vials and incubated for 2 to 4 hours in the dark at room temperature (about 20°C). The nodules were blotted dry with paper tissues, embedded in liver, frozen and sectioned as described in Section 9b. Sections (15 to 30  $\mu$  thick) were collected on glass slides at -20°, air-dried, mounted in water and examined on a Zeiss Photomicroscope II. Photographs were taken under phase-contrast and bright-field illumination using Kodachrome II Professional KPA 135 colour film.

## (12) STATISTICAL TREATMENT OF DATA

### a) Replication

The replication used within each experiment was as stated previously. Each experiment was performed in entirety at least three times unless otherwise stated and the results of each experiment were analysed separately.

### b) Standard error of the mean

The standard error of the mean (S.E.) for each sample was calculated from the relationship:

$$S.E. = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n(n-1)}} \quad \text{where } x = \text{individual values of observations}$$

$$\sum x^2 = \text{sum of (observations)}^2$$

$$(\sum x)^2 = (\text{sum of observations})^2$$

$$n = \text{number of observations.}$$

The calculations were performed on an Olivetti 101 desk-top computer. The standard error of a mean was expressed graphically as a symmetrical vertical bar above and below the mean, equal to twice the standard error of the mean, but as a bar equal to the standard error of the mean to one side only where limited by space. The standard error of the mean was used as a method of illustrating the spread of the values of individual observations around the mean.

#### c) t-test

Comparison of means of paired samples was performed by use of the t-test, calculated on an Olivetti 101 desk-top computer, using the formula:

$$t = \frac{\text{deviation of the difference between the means from zero}}{\text{standard deviation of the difference between the means}}$$

$$= \frac{\bar{x}_1 - \bar{x}_2}{\sigma_d} \quad \text{where } \sigma_d = \sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}}$$

$$\bar{x}_r = \text{mean of population } r$$

$$n = \text{number of observations}$$

$$\sigma_r^2 = \text{variance of population } r.$$

The t value is therefore a deviation of one mean from another expressed in terms of standard deviation, and is the deviation of observations from the mean of a sample of a population, corrected for the number of observations in the sample and the fact that the calculated mean and standard deviation of the population are only estimates based on that sample (Bishop, 1966). The table of distribution of t (Fisher and Yates, 1963) is entered at  $(n_1 + n_2 - 2)$  degrees of freedom. If the calculated value of t exceeded that in the table, the samples probably came from two different populations and the means were said to be significantly different. Confidence limits

were expressed as follows:

$p < 0.950$	N.S.	not significantly different.
$p \geq 0.950$	*	)
$p \geq 0.990$	**	) significantly different.
$p \geq 0.999$	***	)

c) Percentage data

The angular transformation tables of Fisher and Yates (1963) were used to compare data expressed as percentages. The t-test may only be applied to normally distributed populations in which the limits of variation for individual observations are infinite. Data expressed as percentages do not have infinitely variable values, but fixed limits of 0% and 100%. The angular transformation used restores the properties of a normally distributed population to the percentage data and t-tests may be performed on the transformed data.

RESULTSA. PISUM SATIVUM

- (1) Effect of assay conditions on acetylene reduction 47
  - a) Gas composition
  - b) Assay material
  - c) Temperature of incubation
- (2) Development of plants 49
  - a) Plant growth
  - b) Acetylene-reducing activity
  - c) Total plant nitrogen
  - d) Accumulation of  $^{14}\text{C}$ -labelled photosynthates
  - e) Respiration rate and respiratory quotient (R.Q.)
  - f) Poly- $\beta$ -hydroxybutyric acid (PHB) content of nodules and  
Rhizobium leguminosarum
- (3) Effect of light 53
  - a) Photoperiod
  - b) Light intensity
- (4) Diurnal fluctuations 55
  - a) Experiments showing a single maximum in acetylene-reducing activity
  - b) Experiments showing two maxima in acetylene-reducing activity
- (5) Effect of darkening 57
  - a) Acetylene-reducing activity
  - b) Accumulation of  $^{14}\text{C}$ -labelled photosynthates
    - (i) Plants fed  $^{14}\text{CO}_2$  prior to darkening
    - (ii) Plants darkened prior to  $^{14}\text{CO}_2$  feeding
  - c) Recovery from darkening
    - (i) Acetylene-reducing activity
    - (ii) Accumulation of  $^{14}\text{C}$ -labelled photosynthates

- (6) Distribution of  $^{14}\text{C}$ -labelled photosynthates 60
- a) Distribution in relation to plant age
  - b) Specific activity of plant parts
  - c) Effect of removal of apex on distribution of  $^{14}\text{C}$ -labelled assimilates and on the rate of acetylene reduction
    - (i) Vegetative plants
    - (ii) Flowering plants
    - (iii) Fruiting plants
    - (iv) Continuous removal of flowers
- (7) Analysis of radioactive compounds in ethanolic extracts of nodules 64
- a) Neutral fraction
  - b) Basic fraction
  - c) Acidic fraction
- (8) Autoradiography 66
- a) Whole plants
  - b) Microautoradiography
    - (i) Plants maintained in the light
    - (ii) Darkened plants
    - (iii) Distribution of starch grains
    - (iv) Accumulation of radioactivity in forked nodules
- (9) Histochemical tests with tetrazolium salts 70

B. VICIA FABA

- (1) Effect of assay conditions on acetylene reduction 71
- a) Nodule size
  - b) Gas volume
  - c) Oxygen concentration
  - d) Temperature of incubation
- (2) Development of plants 73
- a) Plant growth
  - b) Acetylene-reducing activity

(3) Microautoradiography 74

- a) Photosynthetic fixation of  $^{14}\text{CO}_2$
- b) Dark fixation of  $^{14}\text{CO}_2$  by detached nodules

(4) Analysis of radioactive compounds in ethanolic extracts of nodules

a) Photosynthetic fixation of  $^{14}\text{CO}_2$  75

- (i) Continuous exposure to  $^{14}\text{CO}_2$  (Treatment L1)
- (ii) Short exposure to  $^{14}\text{CO}_2$  (Treatment L2)
- (iii) Fractionation of ethanolic extracts
- (iv) Neutral fraction
- (v) Basic fraction
- (vi) Acidic fraction

b) Dark fixation of  $^{14}\text{CO}_2$  by detached nodules 78

- (i) Continuous exposure to  $^{14}\text{CO}_2$  (Treatment D1)
- (ii) Short exposure to  $^{14}\text{CO}_2$  (Treatment D2)
- (iii) Fractionation of ethanolic extracts
- (iv) Neutral fraction
- (v) Basic fraction
- (vi) Acidic fraction

Fig. 11. Effect of oxygen concentration on acetylene reduction by nodulated 3-week-old pea roots. Plant material was incubated for 30 minutes at 25°C at atmospheric pressure in the acetylene : argon (1:3) atmosphere with oxygen concentrations as shown.

The t-test was used to test the differences between the means for acetylene-reducing activity with 0.10 atm. oxygen and other concentrations.

		Value of t									
pO <sub>2</sub> (atm.) o-o	0.00	0.10	0.20	0.30	0.40	0.60	0.80				
	6.818***	-	5.952***	6.661***	6.561***	6.801***	6.807***				
pO <sub>2</sub> (atm.) Δ-Δ	0.00	0.05	0.10	0.15	0.20	0.25	0.30	0.50			
	21.720***	2.564*	-	1.657 <sup>NS</sup>	9.385***	16.087***	6.097***	20.890***			

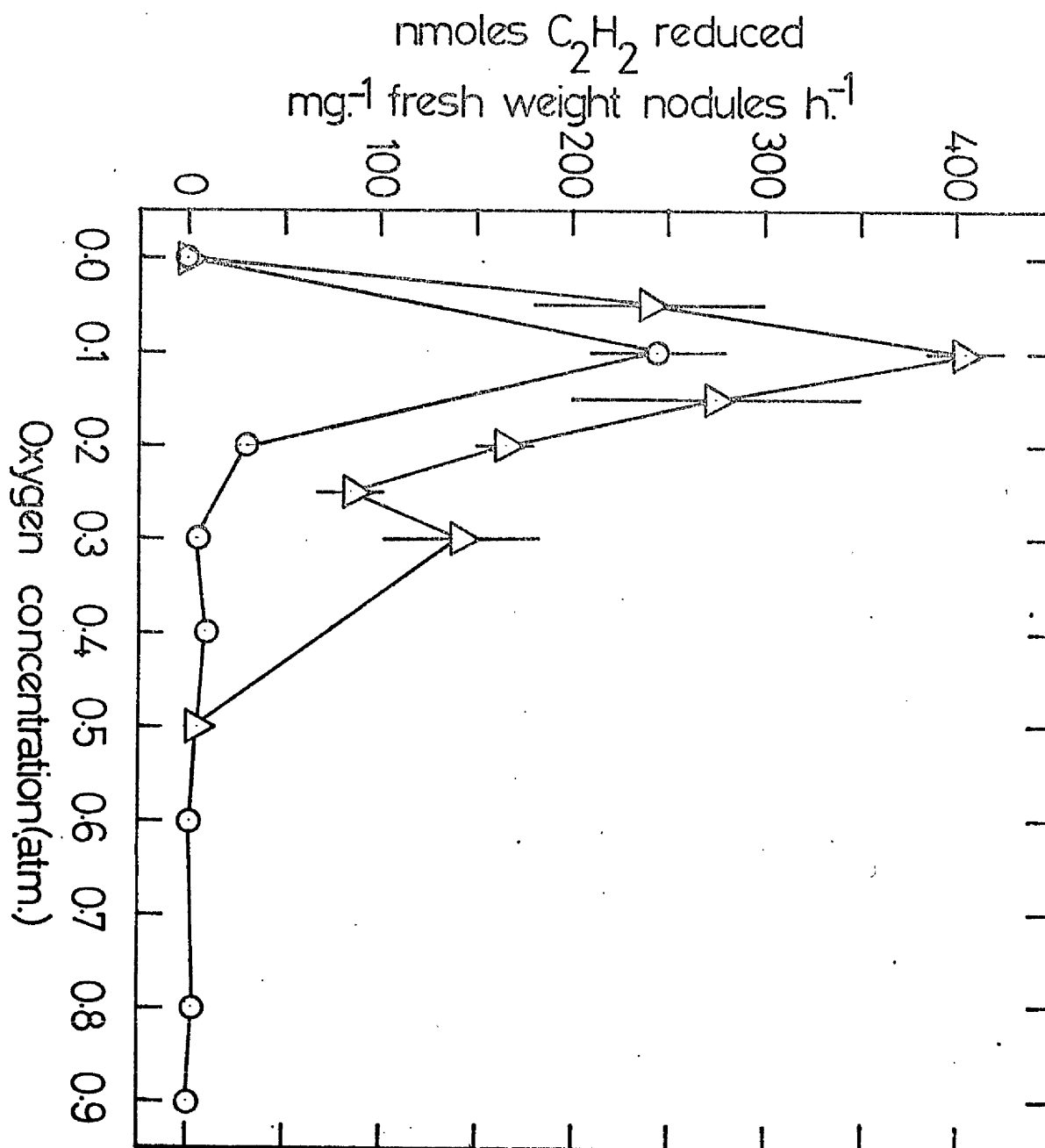




Fig. 12. Effect of acetylene concentration on acetylene reduction by nodulated 3-week-old pea roots. Plant material was incubated for 30 minutes at 25°C at atmospheric pressure with the stated concentrations of acetylene in air.

The t-test was used to test the differences between the means for acetylene-reducing activity with 0.20 atm. acetylene and other concentrations.

Value of t						
pC <sub>2</sub> H <sub>2</sub> (atm.)	0.00	0.05	0.10	0.15	0.20	0.40
	9.839***	2.514*	3.541*	1.639 <sup>NS</sup>	-	1.460 <sup>NS</sup>

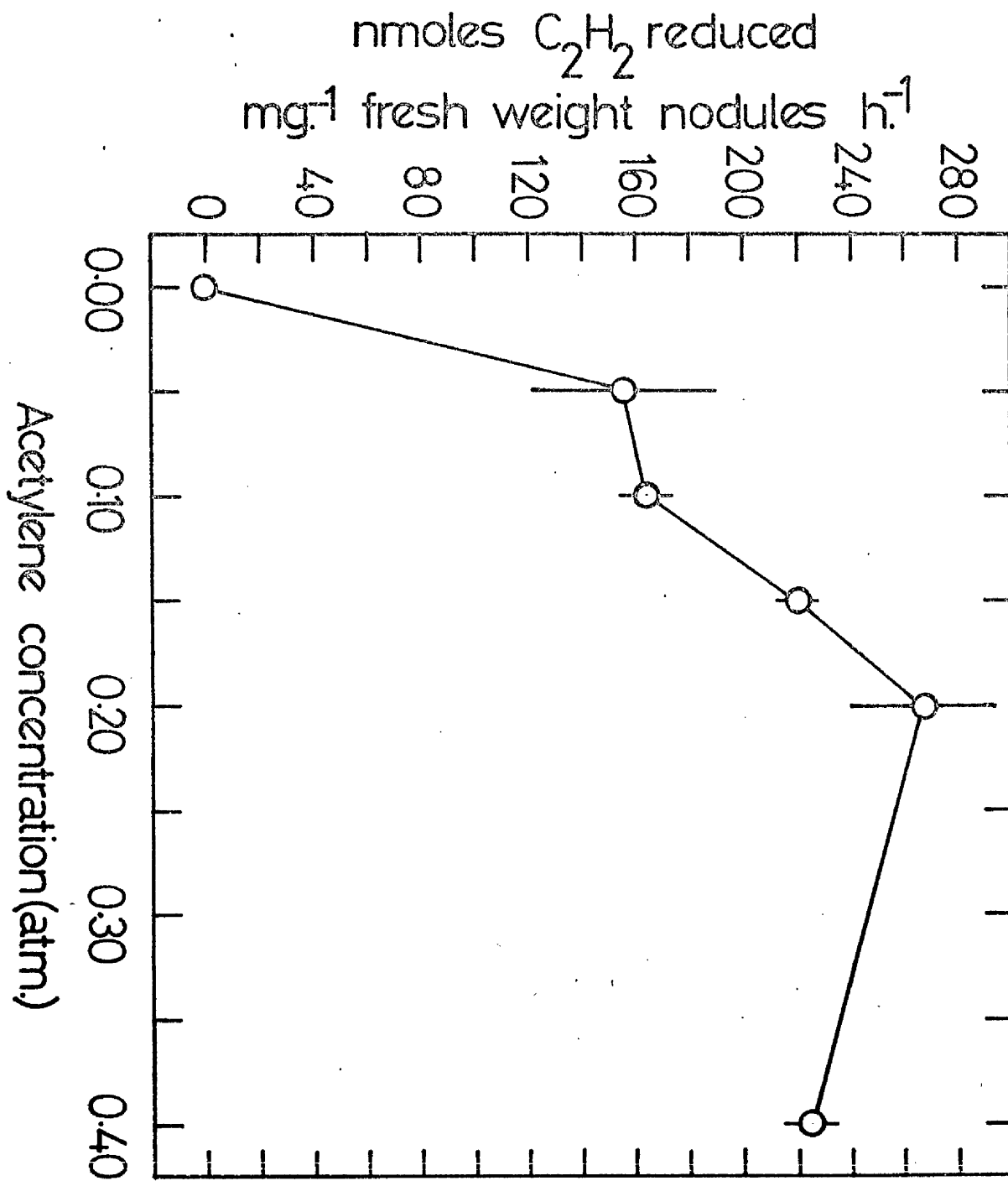


Table 1. Effect on acetylene-reducing activity of pea nodules of assaying whole plants, nodulated roots or detached nodules.

Assay material (n moles $C_2H_2$ reduced $mg^{-1}$ fresh weight nodules $h^{-1}$ )	Acetylene-reducing activity		
	Exp. 1	Exp. 2	Exp. 3
Whole plants	$295.12 \pm 44.16$	$128.34 \pm 36.88$	$129.25 \pm 43.92$
Nodulated roots	$203.51 \pm 28.23$	$139.49 \pm 27.49$	$70.10 \pm 19.58$
Detached nodules	$31.37 \pm 9.62$	$16.31 \pm 4.15$	$17.07 \pm 4.91$

Acetylene reduction assays were performed simultaneously with nodulated roots and detached nodules from 3-week-old pea plants and the whole plants were also assessed. One whole plant, 4 nodulated roots or the detached nodules from 4 plants were incubated in tubes of capacity 52 ml in the atmosphere normally used ( $C_2H_2 : O_2 : Ar = 20 : 20 : 60$ ).

The t-test was used to test the differences of means between whole plants and nodulated roots or detached nodules.

Assay material	Value of t		
	Whole plants	nodulated roots	detached nodules
Exp. 1	$1.727^{NS}$		$5.835^{**}$
Exp. 2	$-0.212^{NS}$		$2.659^*$
Exp. 3	$1.230^{NS}$		$2.458^*$

## A. PISUM SATIVUM

### (1) EFFECT OF ASSAY CONDITIONS ON ACETYLENE REDUCTION

Plants 3 weeks old were used in the following experiments.

#### a) Gas Composition

The acetylene-reducing activity of nodulated roots was dependent on oxygen concentration (Fig. 11). There was no activity in the absence of oxygen and the maximum activity was attained at a  $pO_2$  of 0.10 atm. in both experiments. There was increasing inhibition with a  $pO_2$  greater than 0.10 atm. although 0.82% of the maximum acetylene-reducing activity was still detected at 0.90 atm. The  $pO_2$  of 0.20 atm. (atmospheric) normally used was thus not optimal.

Ethylene was not produced in the absence of acetylene and acetylene-reducing activity was dependent on the  $pC_2H_2$  (Fig. 12). A  $pC_2H_2$  of 0.20 atm. was optimal for ethylene production in all experiments and nitrogenase activity at a  $pC_2H_2$  of 0.10 atm. was about half the optimum rate observed. As the experiments were carried out on the same day, the rates of acetylene reduction were almost identical for each replicate experiment, and so only one set of results is shown.

#### b) Assay material

Detached nodules, nodulated roots and whole plants were tested for acetylene reduction under standard conditions. The rate of acetylene reduction by nodules was not reduced significantly when nodulated roots rather than whole plants were used. Detachment of the nodules resulted in an immediate decline in their acetylene-reducing activity to one-eighth of that of whole plants (Table 1).

#### c) Temperature of incubation

The acetylene-reducing activity of nodulated roots was affected

Fig. 13a.

Effect of temperature of incubation on acetylene reduction by nodulated 3-week-old pea roots. Plant material was incubated for 30 minutes at atmospheric pressure with the standard gas mixture

(O<sub>2</sub> : C<sub>2</sub>H<sub>2</sub> : Ar = 20 : 20 : 60) in temperatures as shown.

The t-test was used to test the differences between the means for acetylene-reducing activity at successively higher temperatures.

		value of t										
Temperature °C		0	6	10	20	25	30	35	40	60		
Δ - Δ		0.587 <sup>NS</sup>	-1.436 <sup>NS</sup>	-7.855***	-6.221***	2.310 <sup>NS</sup>	0.741 <sup>NS</sup>	-0.157 <sup>NS</sup>	4.745**			
Temperature °C		0	5	10	15	20	25	30	41			
□ - □		-0.800 <sup>NS</sup>	0.735 <sup>NS</sup>	0.406 <sup>NS</sup>	-1.244 <sup>NS</sup>	-3.471*	-2.009 <sup>NS</sup>	1.098 <sup>NS</sup>				
Temperature °C		3	8	14	21	25	30	44	60			
0 - 0		-1.885 <sup>NS</sup>	-6.641***	-1.193 <sup>NS</sup>	-0.421 <sup>NS</sup>	-0.887 <sup>NS</sup>	-0.456 <sup>NS</sup>	7.891***				

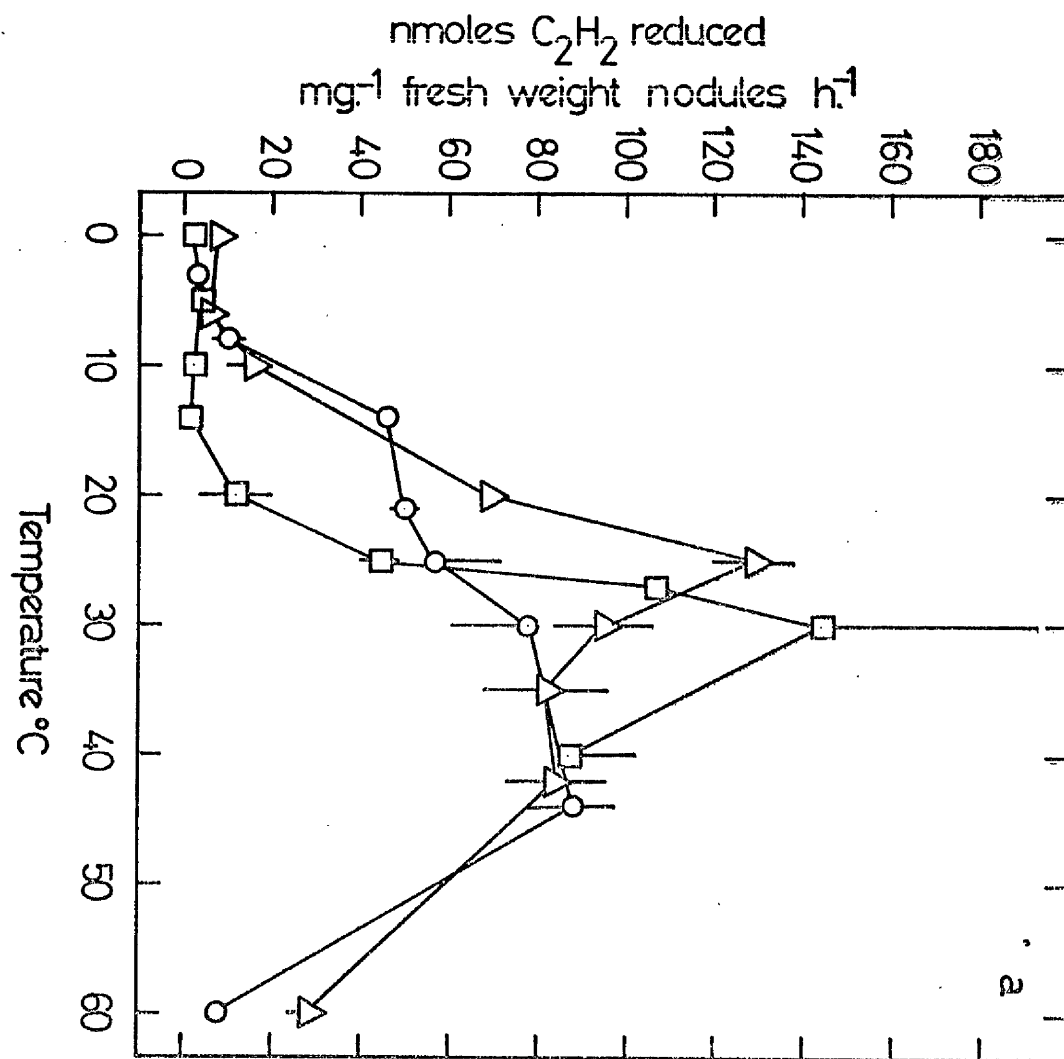


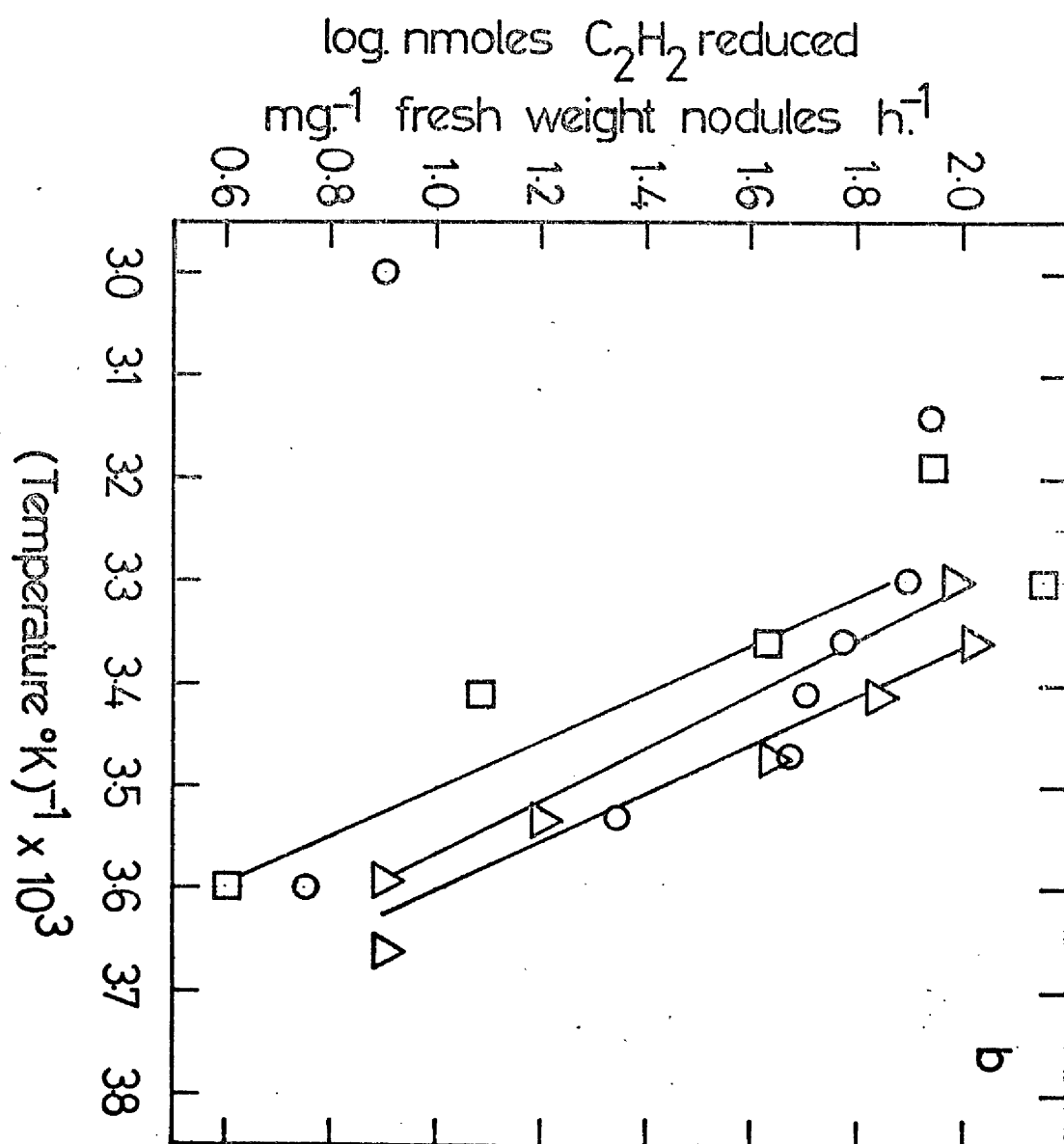
Table 2. Effect of temperature of incubation on  $Q_{10}$  value for acetylene reduction by nodulated pea roots.

Temperature range (°C)	Mean $Q_{10}$ (average of 3)
5-15	5.30
10-20	4.19
15-25	8.14
20-30	4.97
25-35	1.61
30-40	0.86

Nodulated roots of 3-week-old pea plants were assayed for acetylene-reducing activity for 30 minutes at atmospheric pressure with the standard gas mixture ( $O_2 : C_2H_2 : A = 20 : 20 : 60$ ) in temperatures between 0 and 60°C.

Fig. 13b. Arrhenius plot of acetylene-reducing activity of nodulated pea roots at different temperatures between 0 and 60°C. Plant material was incubated for 30 minutes at atmospheric pressure with standard gas mixture ( $O_2 : C_2H_2 : Ar = 20 : 20 : 60$ ) in temperatures as shown.





by the temperature of incubation during the acetylene reduction assay (Fig. 13a). Maximum rates of activity were usually achieved at 25 to 30°C and temperatures greater than 40°C decreased activity. Increasing the incubation time from 30 minutes to 1 hour did not alter the results obtained.

Mean  $Q_{10}$  values calculated over the temperature range 5 to 40°C (Table 2) showed that changes in temperature between 15 and 25°C produced the largest changes in acetylene reduction. The effect of temperature on the rate of acetylene reduction is further illustrated by an Arrhenius plot of the data (Fig. 13b). The results of all experiments fit the Arrhenius equation between 5 and 25°C, indicating a constant activation energy for the process between these temperatures. Temperatures greater than 25°C resulted in reduced rates of increase in acetylene reduction and so results obtained at these temperatures do not fit the equation.

Plate 1. Growth of pea plants in nitrogen-free culture in the glasshouse. Seeds were planted in "Peralite" and inoculated with a suspension of Rhizobium leguminosarum. The plants shown are (left to right) 1, 2, 3, 4, 5 and 6 weeks old.



———— 100mms.

Fig. 14. Changes during growth of pea plants in

- a) fresh weight of shoot of Batch 1 (o) and Batch 2 (□) plants.
- b) fresh weight of root of Batch 1 (o) and Batch 2 (□) plants.
- c) fresh weight of pink (o) and green (□) nodules of Batch 1 and pink (Δ) and green (V) nodules of Batch 2 plants.

Measurements were taken for each of 30 plants at weekly intervals from planting and plants were grown in nitrogen-free culture in 'Peralite' in a glasshouse. Dashed lines indicate times of commencement of flowering (---) and fruiting (— —).

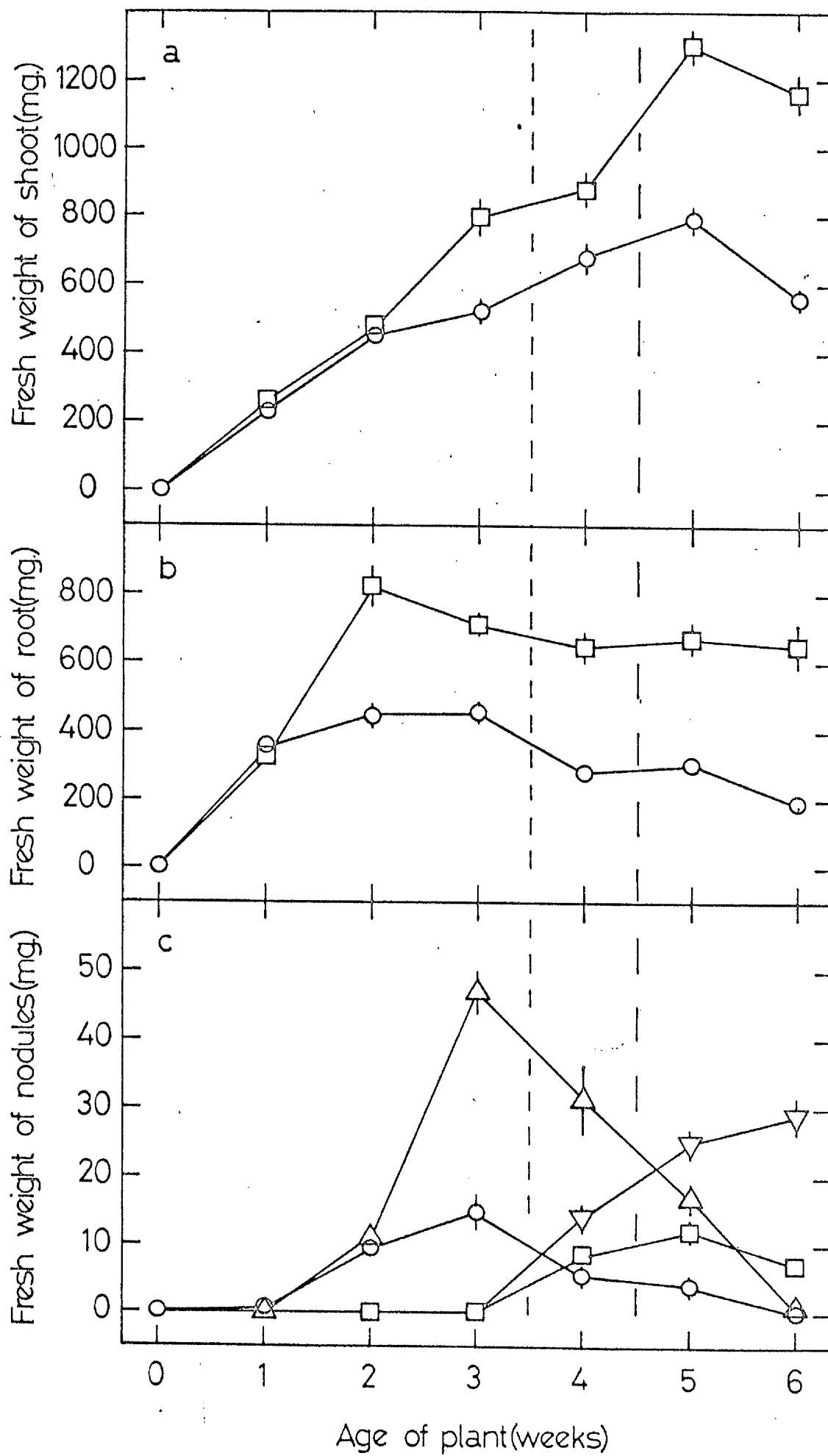
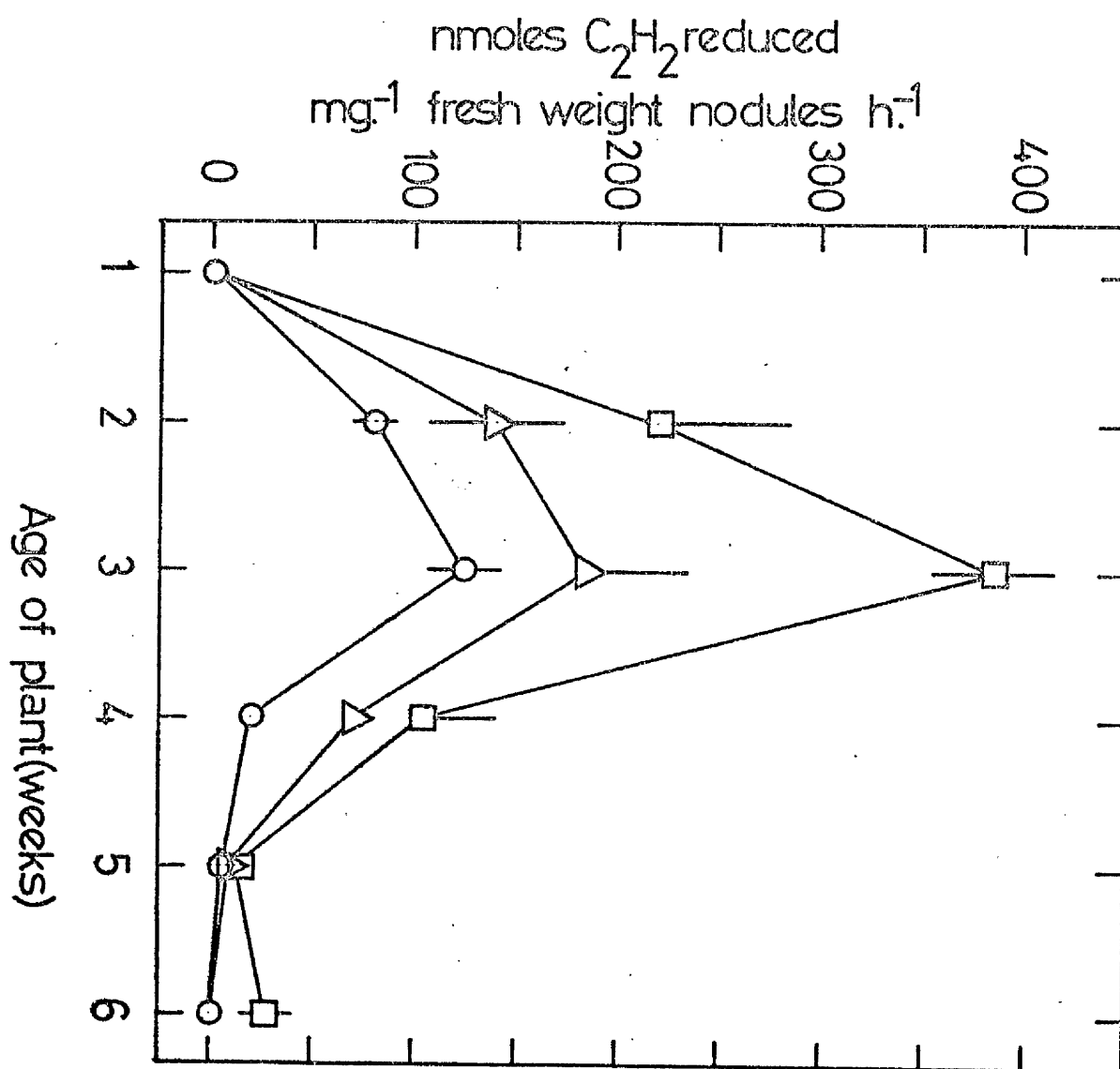


Fig. 15. Changes during growth in acetylene-reducing activity of nodulated pea roots of Batch 1 (○) and Batch 2 (□ and △) plants. All plants were placed under constant light and temperature in a growth cabinet for 24 hours before use. Samples for acetylene reduction assays were incubated for 30 minutes under standard conditions.

The t-test was used to test the differences between the means for acetylene-reducing activity at successive ages.

Age of plant (weeks)	Value of t				
	2	3	4	5	6
○	-2.784*	6.916***	2.844*	1.259 <sup>NS</sup>	
□	-2.334 <sup>NS</sup>	6.160***	7.276***	-1.358 <sup>NS</sup>	
△	-0.726 <sup>NS</sup>	2.256 <sup>NS</sup>	7.424***	7.311***	





## (2) DEVELOPMENT OF PLANTS

### a) Plant growth

The conditions of culture used in these experiments produced small plants (Plate 1) with a much shorter growth cycle than field-grown plants of the same variety. Flowering began at week 3, fruit development at week 4 and both processes were complete 6 weeks after seed germination. The fresh weight of the shoot increased until week 5, when leaf fall commenced (Fig. 14a), but the fresh weight of the roots increased rapidly until week 2 and declined slightly thereafter (Fig. 14b). The weight of pink nodules (presumably active in nitrogen fixation) on each plant increased to a maximum at week 3, after which greening commenced (Fig. 14c), until at week 6 all the nodules were green and inactive (a nodule was judged as "green" when more than half of the nodule was visibly green).

Two batches of seed were used in these experiments, and plants of Batch 2 were much more vigorous than those of Batch 1. Batch 2 plants nodulated slightly earlier and developed a greater weight of pink nodules, which remained pink 3 to 4 days longer than those of Batch 1 plants.

Variations in growth and nodulation were also noted between crops harvested in winter and summer. Summer-grown plants were larger and bore greater weights of nodules, which turned green later than winter-grown plants.

### b) Acetylene-reducing activity

Nodulated roots of plants of different ages showed marked differences in their rates of acetylene-reduction (Fig. 15). The acetylene-reducing activity increased to a maximum in plants 3 weeks old and then declined until there was no detectable activity in the nodules of 6-week-old plants. Very high rates of acetylene reduction were commonly observed in Batch 2 plants.

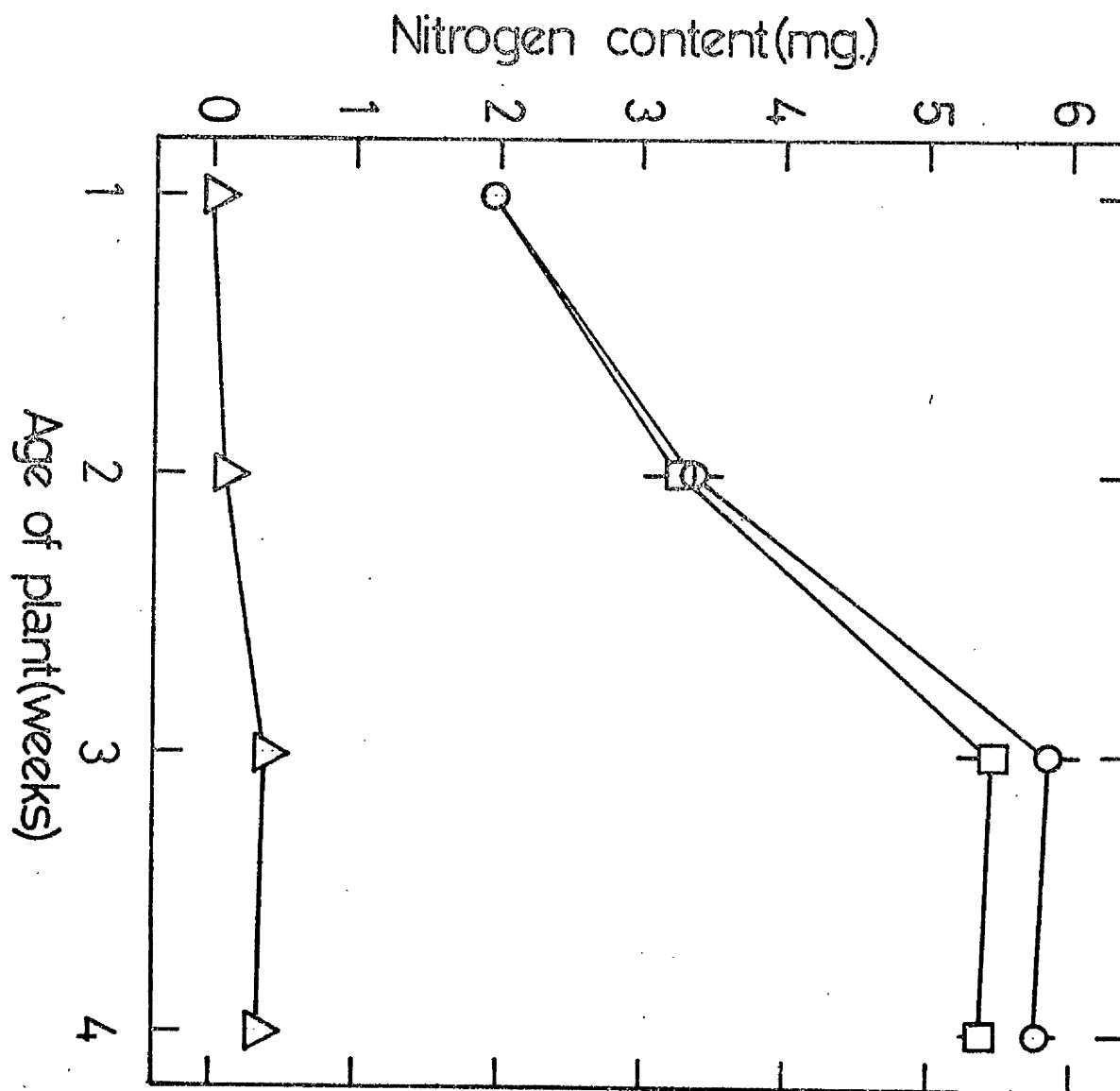
### c) Total plant nitrogen

This experiment was terminated after 4 weeks because artificial

Fig. 16. Changes during growth in the total nitrogen in nodulated pea plants of Batch 2 in nitrogen-free culture. Nitrogen contents per gram dry weight of detached nodules ( $\Delta$ ) and denodulated plants ( $\square$ ) were assessed separately by Kjeldahl analysis and the total nitrogen in nodules and denodulated plants calculated using the data of Fig. 14. The total nitrogen in nodulated plants ( $\circ$ ) was found by addition of the total nitrogen in nodules and denodulated plants.

The t-test was used to test the differences between the means for total nitrogen in different plant parts at successive ages.

Age of plant (weeks)	Value of t			
	1	2	3	4
$\circ$	-11.430***	-15.899***	0.697 <sup>NS</sup>	
$\square$	-10.939***	-15.558***	0.717 <sup>NS</sup>	
$\Delta$	-	-12.790***	0.223 <sup>NS</sup>	



(see over)

Table 3. Mean nitrogen fixed and transferred from nodules to remainder of pea plants during successive periods of growth in nitrogen-free culture.

Age of plant (weeks)	Nitrogen Content (mg. g. <sup>-1</sup> fresh weight)		Total Nitrogen (mg.) *		Nitrogen fixed per plant per week (mg.) <sup>0</sup>	Nitrogen transferred to rest of plant per week (mg.) <sup>†</sup>	Nitrogen transferred as % of nitrogen fixed
	Nodules	Denodulated plants	Nodules	Denodulated plants			
1	-	3.33	-	1.96	-	-	-
2	8.24	2.55	0.09	3.28	1.33	1.32	99.2
3	8.19	3.62	0.38	5.44	2.46	2.16	87.8
4	8.10	3.52	0.38	5.36	-	-	-

<sup>0</sup> Calculated by subtracting the mean total nitrogen per nodulated plant at each sampling from the corresponding figure at the following sampling.

<sup>†</sup> Calculated by subtracting the mean total nitrogen of the denodulated plant at each sampling from the corresponding figure at the following sampling.

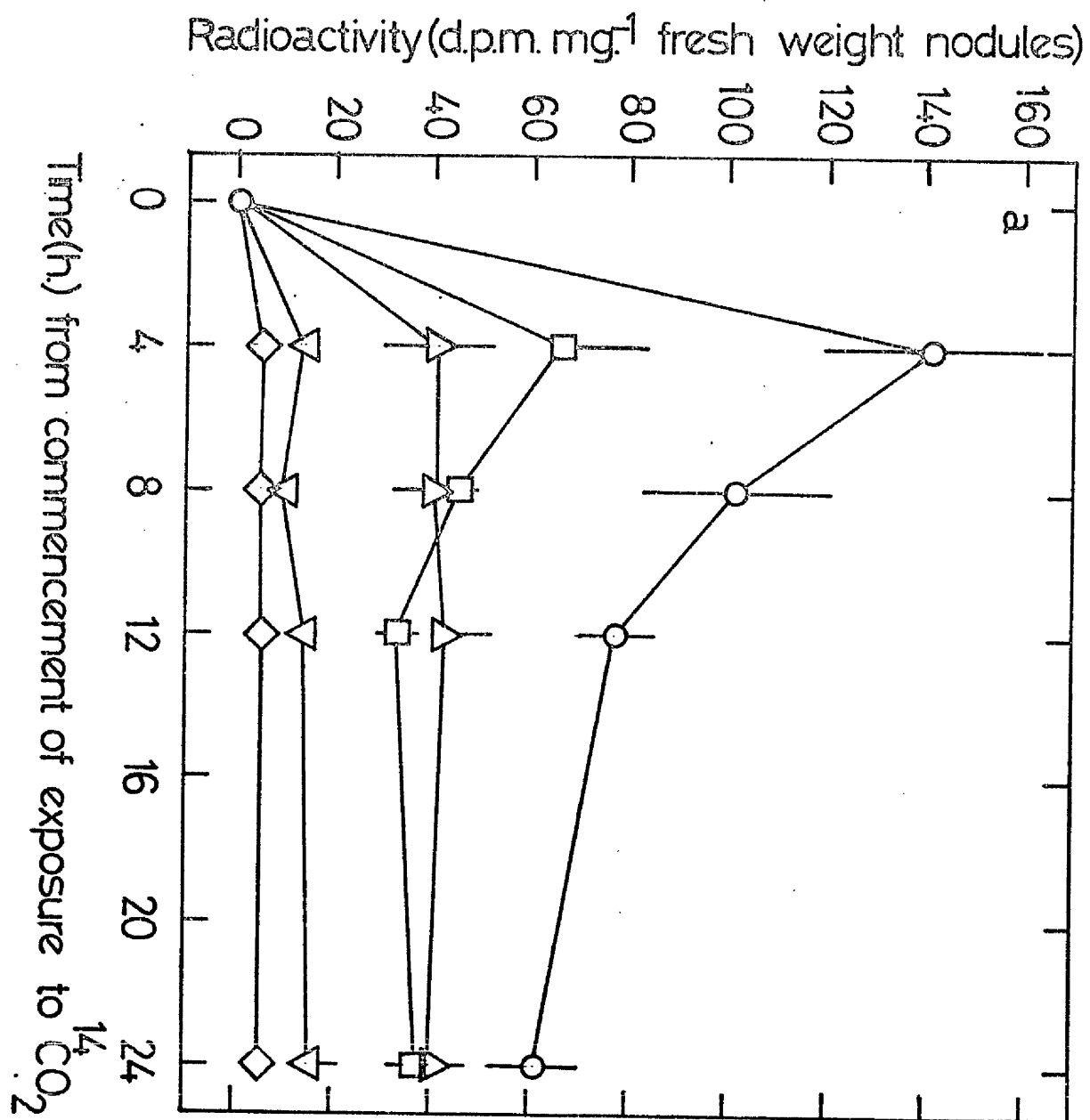
Nitrogen contents per gram dry weight of detached nodules and denodulated plants were assessed separately by Kjeldahl analysis and the total nitrogen in nodules and denodulated plants calculated using the data of Fig. 14. The total nitrogen in nodulated plants was found by addition of the total nitrogen in nodules and denodulated plants.

\* The weight of the cotyledons of the seed was not included in calculating these figures. The mean dry weight of the seeds was 127 <sup>†</sup> 5 mg. and it is estimated that the mean total nitrogen in the seed was 2.48 mg. if 20% of the seed dry weight is protein. Thus at least 3 mg. of nitrogen were fixed by the nodules during growth of the plants.

Fig. 17a. Accumulation of  $^{14}\text{C}$ -labelled photosynthates in pea nodules of plants aged 2( $\bigcirc$ ), 3( $\square$ ), 4( $\triangle$ ), 5( $\nabla$ ) and 6( $\diamond$ ) weeks after "cold-chase" periods as shown following exposure of the shoots to  $^{14}\text{CO}_2$  for 30 minutes in a growth cabinet under constant light and temperature.

The t-test was used to test the differences between the mean radioactivity in pea nodules of each age after 3.5 hours "cold-chase" and longer periods.

Time of "cold-chase" (h)	7.5	11.5	23.5
Age of plant (weeks)	Value of t		
2 weeks	1.412 <sup>NS</sup>	2.878*	3.557**
3 weeks	1.202 <sup>NS</sup>	1.937 <sup>NS</sup>	1.640 <sup>NS</sup>
4 weeks	0.022 <sup>NS</sup>	-0.112 <sup>NS</sup>	0.084 <sup>NS</sup>
5 weeks	1.184 <sup>NS</sup>	-0.027 <sup>NS</sup>	-0.227 <sup>NS</sup>
6 weeks	0.245 <sup>NS</sup>	0.081 <sup>NS</sup>	0.078 <sup>NS</sup>



illumination in the glasshouse was switched off, resulting in atypical growth of the older plants. The results are included here as a rough check on the trends shown by the acetylene reduction data and to provide information on the retention of fixed nitrogen by the nodules. Changes in the total nitrogen contents of nodules and denodulated and nodulated plants were calculated from the growth data (Fig. 14) and the data obtained from Kjeldahl analyses. Fig. 16 shows that the nitrogen content of the plants increased rapidly until they were 3 weeks old, when they contained three times the nitrogen content of 1-week-old plants. No further increase was found in 4-week-old plants, even though acetylene reduction data still showed significant nitrogenase activity at this age; this could be due to variations arising from the design of this experiment, in which successive crops from separate sowings were harvested for each sample age.

The nitrogen content per gram fresh weight nodules remained constant during growth and was almost three times that of the parent plant after denodulation (Table 3). Transfer of fixed nitrogen from nodules to the host plant was considerable - 93.5% in 2-week-old plants and 84.3% in 3-week-old plants (Fig. 16 and Table 3).

#### d) Accumulation of $^{14}\text{C}$ -labelled photosynthates

Maximum accumulation of radioactivity in the nodules of pea plants whose shoots had been fed  $^{14}\text{CO}_2$  for 15 minutes was found 4 to 8 hours after removal of the plants from the labelled atmosphere, irrespective of the age of plant (Fig. 17a). The radioactivity of nodules of plants 2 and 3 weeks old, which were most active in fixation (Fig. 15), then declined by up to one-half over the next 20 hours, but the radioactivity of the nodules from older plants showed little change during this period (Fig. 17a).

The nodules from plants 2 and 3 weeks old showed greatest accumulation of radioactivity, irrespective of the length of the "cold-chase" period (Fig. 17 a to c). In Batch 1 plants, changes in the accumulation of labelled

Fig. 17b. Changes during growth in accumulation of  $^{14}\text{C}$ -labelled photosynthates by pea nodules of Batch 1 after 5.5 hours (○) and 24.5 hours (△) "cold-chase" periods following exposure of the shoots to  $^{14}\text{CO}_2$  for 30 minutes in a growth cabinet under constant light and temperature.

The t-test was used to test the differences between the mean radioactivities of successive ages of pea nodules after each "cold-chase" period.

Age of plant (weeks)	2	3	4	5	6
Time of "cold-chase" (h)	Value of t				
5.5 h	-0.911 <sup>NS</sup>	3.268**	4.606***	2.276*	
24.5 h	-0.980 <sup>NS</sup>	3.356**	5.729***	3.076**	



Fig. 17c. Changes during growth in accumulation of  $^{14}\text{C}$ -labelled photosynthates by pea nodules of Batch 2 after 3.5 hours (○), 7.5 hours (□) and 23.5<sup>(Δ)</sup> hours following exposure of the shoots to  $^{14}\text{CO}_2$  for 30 minutes in a growth cabinet under constant light and temperature.

The t-test was used to test the differences between the mean radio-activities of successive ages of pea nodules after each "cold-chase" period.

Age of plant (weeks)	2	3	4	5	6
Length of "cold-chase" (h)	Value of t				
3.5 h	2.855*	11.262***	2.400*	2.210*	
7.5 h	3.046**	0.553 <sup>NS</sup>	3.770**	1.509 <sup>NS</sup>	
23.5 h	2.293*	-0.263 <sup>NS</sup>	2.672*	1.707 <sup>NS</sup>	

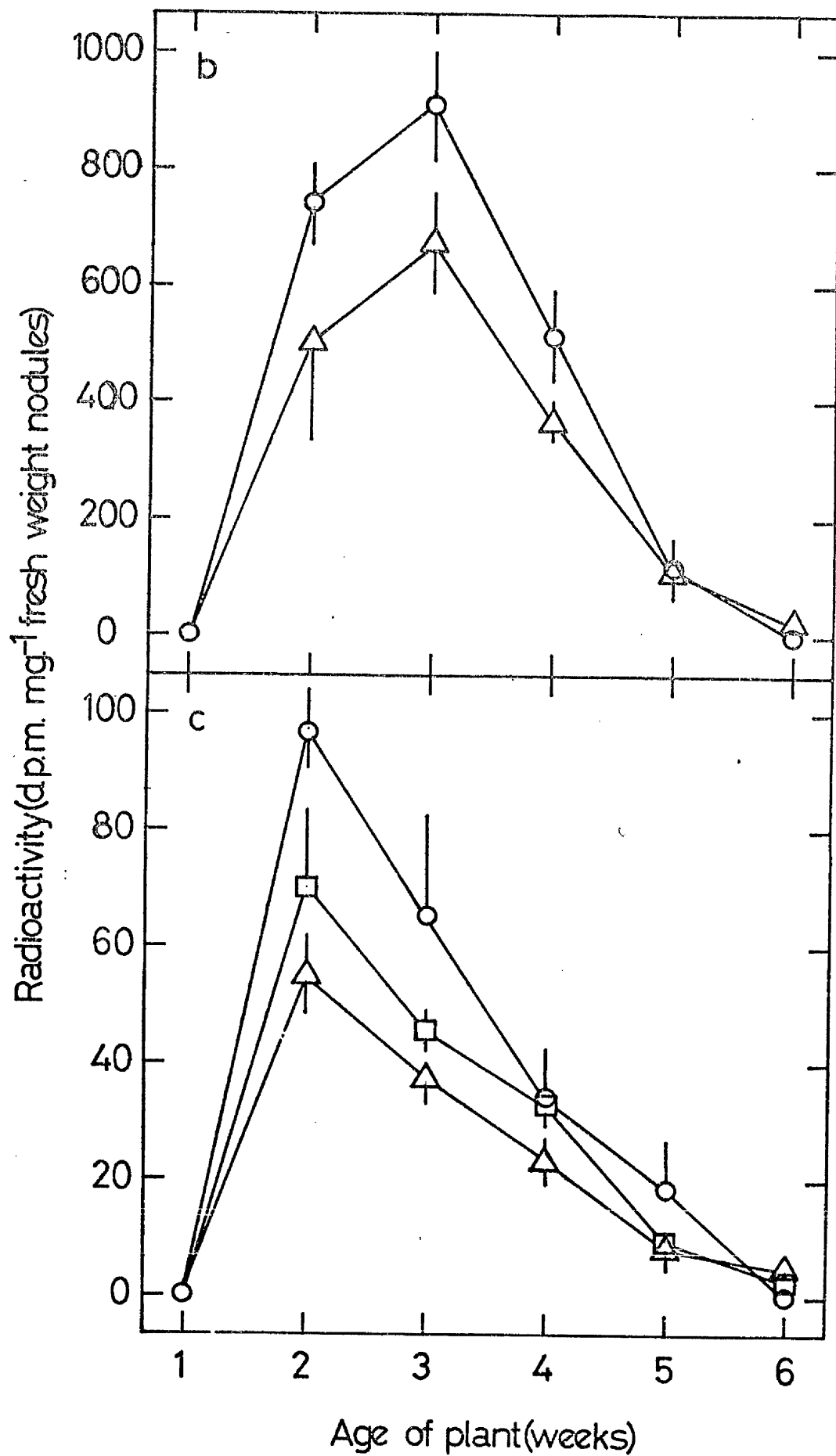


Fig. 17d. Changes during growth in total  $^{14}\text{C}$ -labelled photosynthates accumulated by nodulated pea plants following exposure of the shoots to  $^{14}\text{CO}_2$  for 15 minutes. The radioactivity of the plants was assessed immediately after exposure.

The t-test was used to test the differences between the mean radioactivities of successive ages of pea plants.

	Value of t					
Age of plant (weeks)	1	2	3	4	5	6
○	-6.784***	-0.840 <sup>NS</sup>	-6.400***	4.473***	2.951**	
□	-3.600**	-1.406 <sup>NS</sup>	-4.713***	4.438***	3.918**	
△	-5.357***	-6.407***	-3.184**	0.240 <sup>NS</sup>	3.660**	

Fig. 17e.

Accumulation of  $^{14}\text{C}$ -labelled photosynthates in pink (o) and green ( $\Delta$ ) nodules of 4-week-old plants. Plant shoots were exposed to  $^{14}\text{CO}_2$  for 30 minutes and nodules were collected after "cold-chase" periods up to 23.5 hours.

The t-test was used to test the differences between the mean radioactivities accumulated by pink and green nodules.

	value of t (pink/green)			
Time of "cold-chase" (h.)	3.5	7.5	11.5	23.5
	2.265*	3.598**	4.096***	0.856 <sup>N.S.</sup>

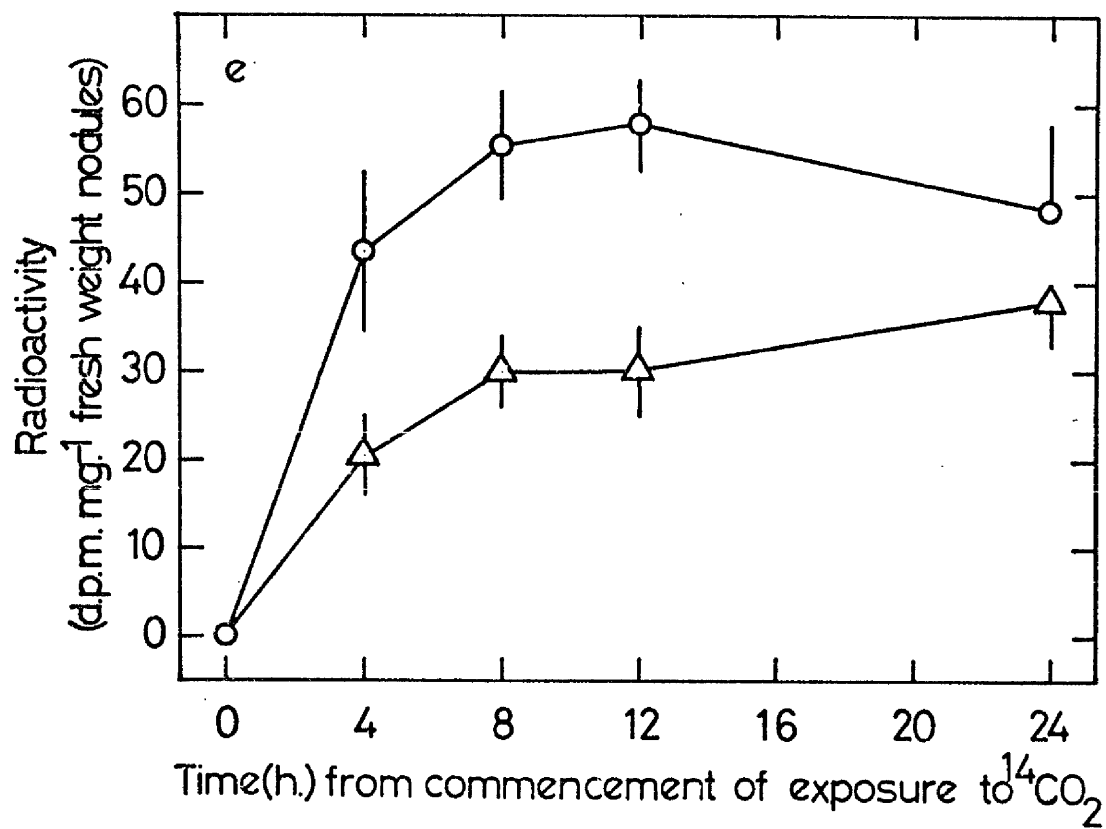
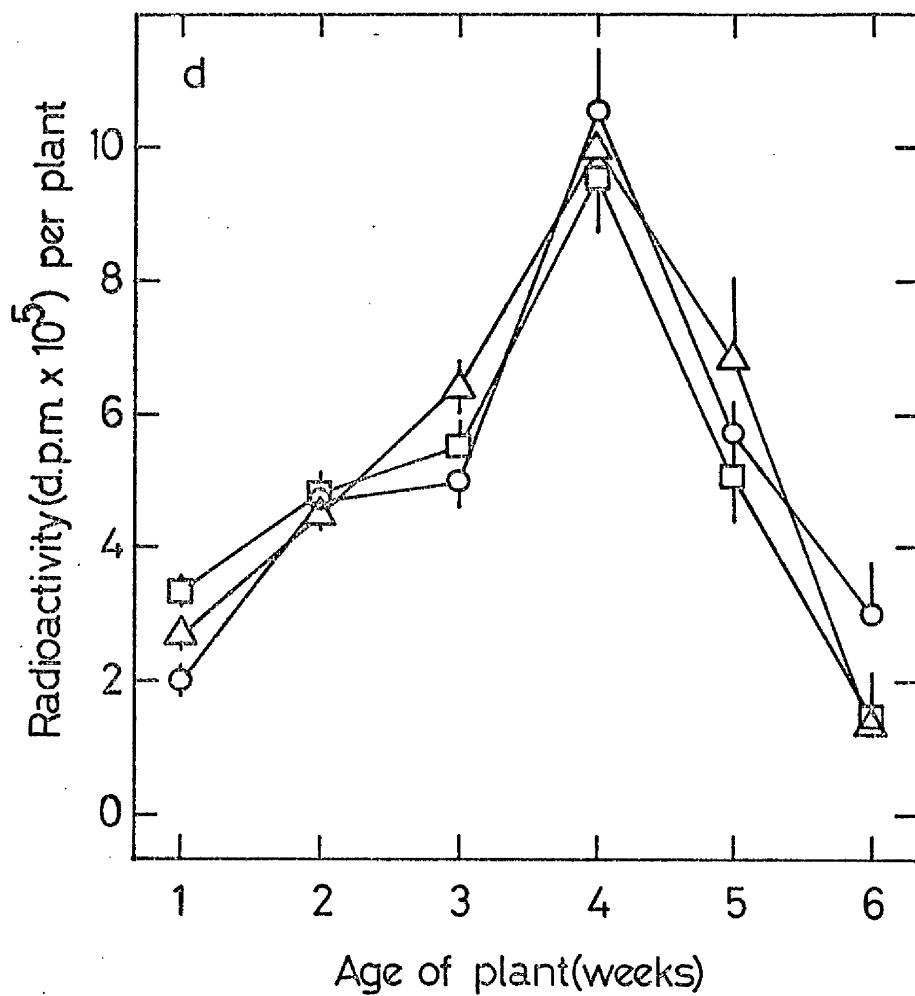


Fig. 18.

Changes during growth in oxygen uptake by detached pea nodules (o) and denodulated roots (A). Measurements were taken by Warburg respirometry and all samples were harvested between 1200 and 1400 hours from glasshouse-grown plants.

The t-test was used to test the differences between the oxygen uptake of successive ages of nodules and roots and the difference between uptake by nodules and roots at each age of plants.

Age of plant (weeks)	value of t					
	1	2	3	4	5	6
Nodules	-	-	-0.926 <sup>NS</sup>	1.030 <sup>NS</sup>	0.409 <sup>NS</sup>	
Roots	29.595***	-0.137 <sup>NS</sup>	0.627	-0.766	0.045	
Nodules/roots	-	-	2.315 <sup>NS</sup>	3.000*	1.125 <sup>NS</sup>	4.689***

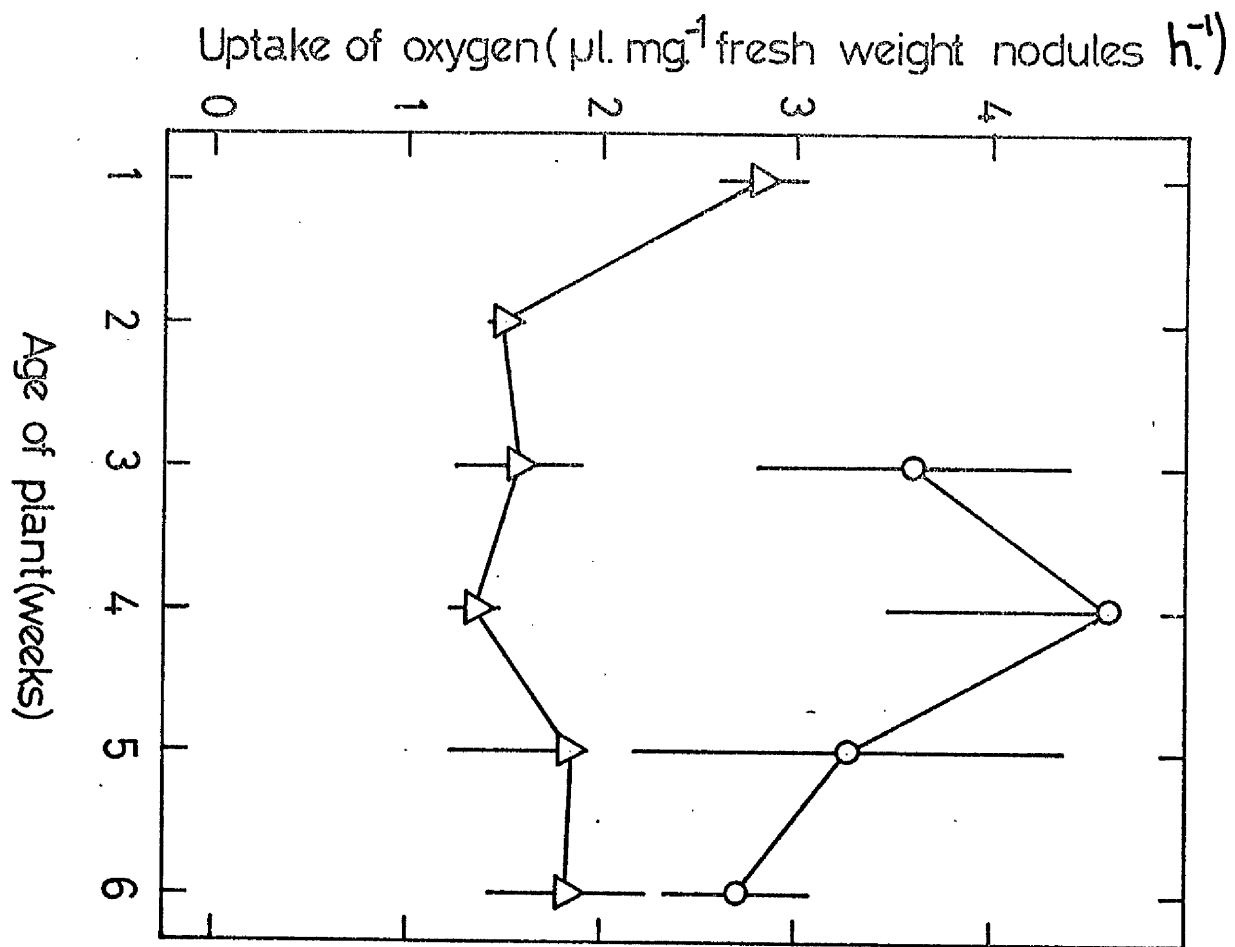


Table 4. Effect of age on R.Q. of pea nodules and roots.

Age of nodules (weeks)	1	2	3	4	5	6
R.Q.	-	-	$1.02 \pm 0.15$	$1.08 \pm 0.02$	$1.08 \pm 0.13$	$1.08 \pm 0.08$
Age of roots (weeks)	1	2	3	4	5	6
R.Q.	$0.94 \pm 0.06$	$1.01 \pm 0.11$	$1.07 \pm 0.26$	$0.99 \pm 0.08$	$0.96 \pm 0.04$	$1.15 \pm 0.08$

Measurements of respiratory activity were taken by Warburg respirometry and all samples were harvested between 1200 and 1400 hours from glasshouse-grown plants.



photosynthates by the nodules of plants of different ages closely paralleled changes in the rate of acetylene reduction (Figs. 15 and 17b). However, in Batch 2 plants, the maximum accumulation of activity was found in the nodules of 2-week-old plants, a little earlier than the maximum in acetylene-reducing activity (Fig. 17c). By contrast, the maximum net photosynthesis by the plants, as determined by the total radioactivity accumulated immediately after exposure to  $^{14}\text{CO}_2$ , was reached at week 4 in both batches (Fig. 17d).

When 4-week-old plants were fed  $^{14}\text{CO}_2$  for 30 minutes and the pink and green nodules collected separately after different "cold-chase" periods, the pink nodules were always more heavily labelled than the green nodules (Fig. 17e). Although the radioactivity accumulated by the pink and green nodules was not always significantly different, this could be due to difficulties in segregating the nodules into pink and green categories.

e) Respiration rate and respiratory quotient (R.Q.)

Measurements of the rate of respiration of nodules were not possible until the plants were 3 weeks old, due to the small size of the nodules prior to this age. The data obtained for subsequent ages of nodules were quite variable and the maximum in respiration recorded for nodules from 4-week-old plants was not statistically different from the values for nodules from plants 3 and 5 weeks old (Fig. 18). The rate of respiration of nodules from 6-week-old plants was only 60% of that of 4-week-old nodules, however, showing that there was some decline in respiratory activity in old nodules. The respiratory rate of the roots did not alter significantly during growth except for a 50% decrease at week 2 from the high rates shown by roots from 1-week-old plants.

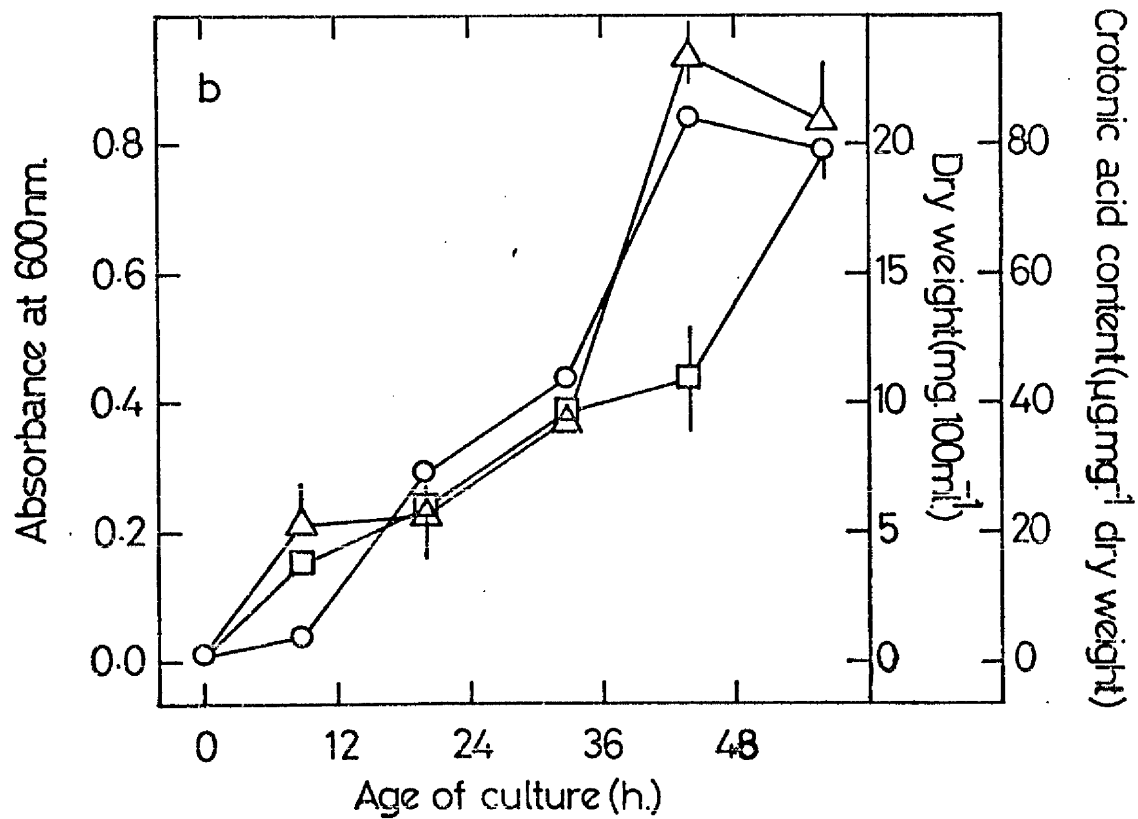
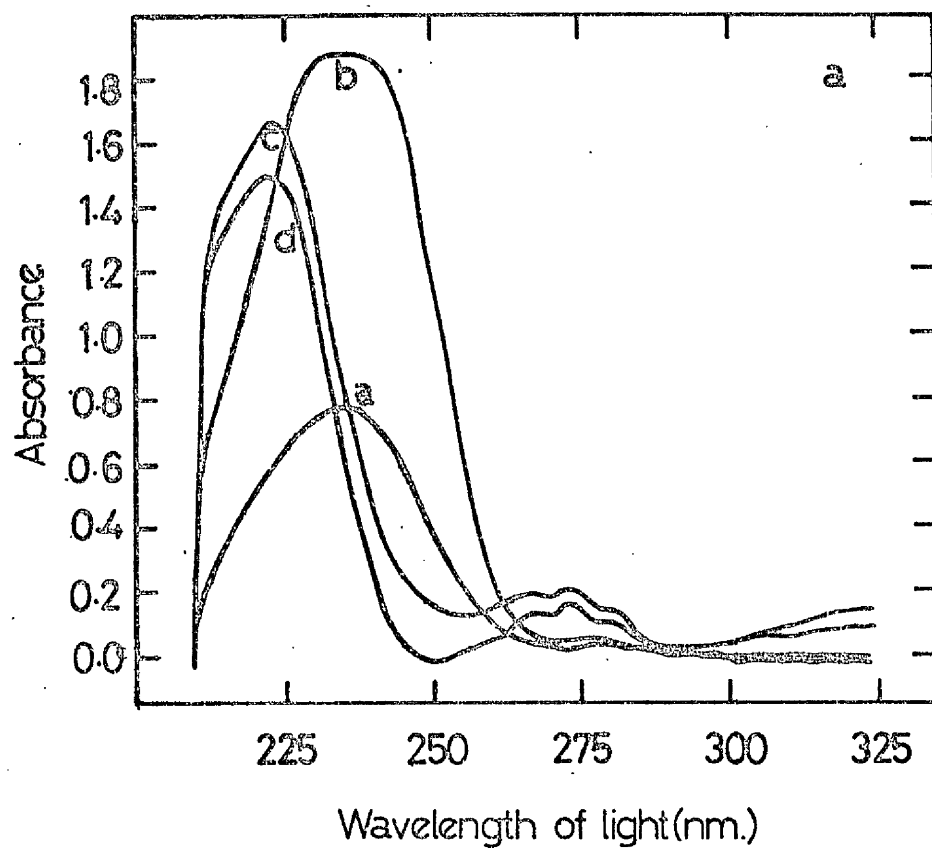
The R.Q. for both nodules and roots showed little change with age (Table 4), and was always between 0.94 and 1.15, indicating that carbohydrates were probably the main substrates for respiration.

Fig. 19a. Absorption spectra of solution of authentic crotonic acid ( $12.5 \mu\text{g ml}^{-1}$ ) (a) and extracts from Rhizobium leguminosarum (b), nodules (c) and roots (d) of 3-week-old plants. Samples of PHB were heated in concentrated sulphuric acid for 10 minutes and cooled before absorbance was measured against a sulphuric acid "blank".

Fig. 19b. Accumulation of PHB ( $\triangle$ ) by Rhizobium leguminosarum during growth, as measured by increases in optical density ( $\circ$ ) and dry weight ( $\square$ ). Bacteria were grown in liquid culture and samples collected at intervals until the end of the log phase of growth (48 hours).

The t-test was used to test the differences between the means of growth and accumulation of PHB by the bacteria at successive samplings.

Age of culture (h)	Value of t					
	0	9	21	33	45	57
Growth (optical density)	-5.345**	-41.184***	-81.283***	-118.549***	0.580 <sup>NS</sup>	
Growth (dry weight)	-387.000***	-44.714***	-19.587***	-11.789***	-1.360 <sup>NS</sup>	
PHB content	-4.340*	-0.283 <sup>NS</sup>	-2.990 <sup>NS</sup>	-20.820***	1.557 <sup>NS</sup>	



f) Poly- $\beta$ -hydroxybutyric acid (PHB) content of nodules and cultured *Rhizobium leguminosarum*

PHB was detected and measured spectrophotometrically by analysis of the crotonic acid produced by its digestion. This compound has a typical absorption spectrum with a maximum at 235 nm. Digests of nodule extracts of all ages failed to show typical absorption spectra; the spectra obtained resembled those of the roots of corresponding ages, with maximum absorption at 223 nm (Fig. 19a).

In contrast, typical spectra were obtained from liquid shake-cultures of *R. leguminosarum* (Fig. 19a) and the PHB content increased during the logarithmic phase of growth of the cultures but remained constant thereafter (Fig. 19b).

Fig. 20. Effect of photoperiod on acetylene-reducing activity (○) and accumulation of  $^{14}\text{C}$ -labelled photosynthates ( $\Delta$ ) by nodules of 3-week-old pea plants. Plants were exposed to photo-periods as shown in a growth cabinet operated at constant temperature and light for 3 days. Plant shoots were then exposed to  $^{14}\text{CO}_2$  for 30 minutes at the start of the light period and both acetylene-reducing activity and accumulation of  $^{14}\text{C}$ -labelled photosynthates were assessed after 4 hours.

The t-test was used to test differences between mean acetylene-reducing activities and radioactivities of pea nodules with successive increases in the photoperiod.

		Value of t					
Photoperiod (h)	0	4	8	12	16	20	24
$\text{C}_2\text{H}_2$ reduction	-0.768 <sup>NS</sup>	-1.027 <sup>NS</sup>	-1.469 <sup>NS</sup>	-0.570 <sup>NS</sup>	-3.923**	1.426 <sup>NS</sup>	
Radioactivity	0.201 <sup>NS</sup>	0.429 <sup>NS</sup>	-2.733*	-3.942***	0.750 <sup>NS</sup>	3.283**	

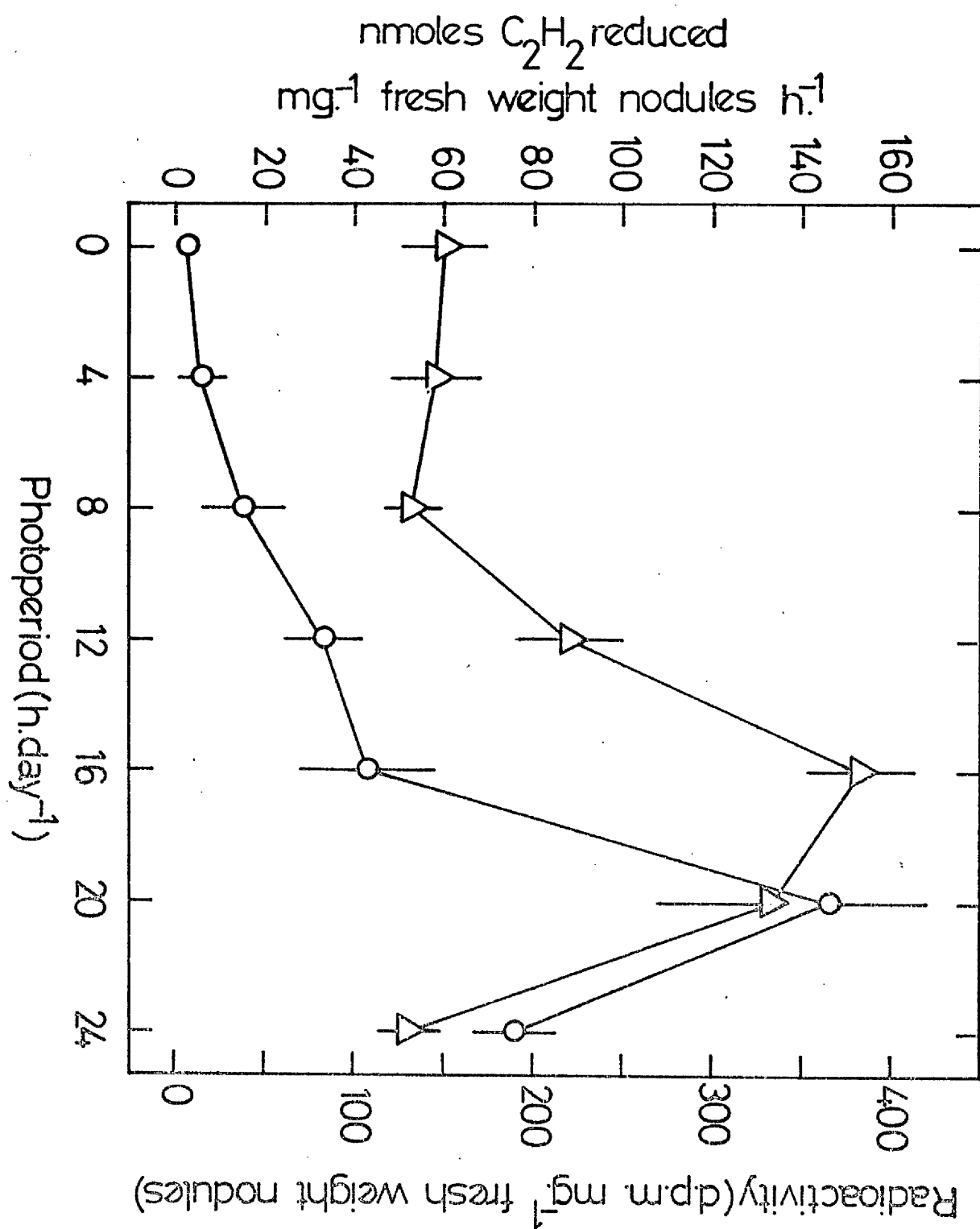
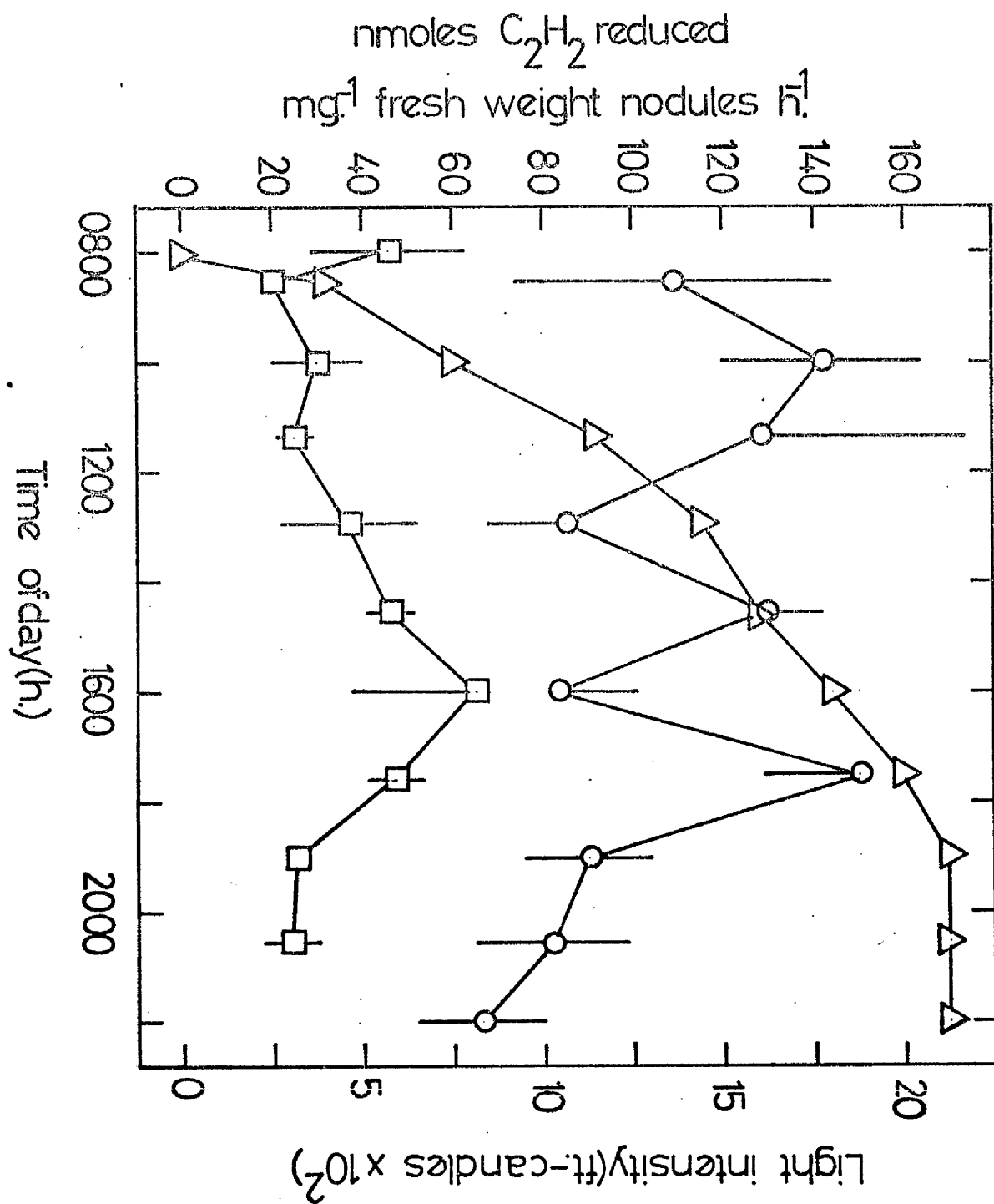


Fig. 21. Effect of light intensity ( $\Delta$ ) on acetylene-reduction ( $\square$  and  $\circ$ ) by nodules of 3-week-old pea plants. Plants were incubated in a growth cabinet for 24 hours at 25°C and 1,000 ft-candles and were then darkened for 8 hours to simulate nocturnal darkening. Light intensity was increased stepwise at 90-minute intervals until the maximum was reached and samples were collected at the end of each 90-minute period.

The t-test was used to test differences between the means of the maximum acetylene-reducing activity observed and those observed at other light intensities.

		Value of t							
Light intensity (ft-candles $\times 10^2$ )		0	4.05	7.60	11.50	14.20	16.00	18.00	
$\circ$	-	0.984 <sup>NS</sup>	0.258 <sup>NS</sup>	0.433 <sup>NS</sup>	2.396 <sup>NS</sup>	0.822 <sup>NS</sup>	2.385 <sup>NS</sup>		
$\square$	0.618 <sup>NS</sup>	1.683 <sup>NS</sup>	1.211 <sup>NS</sup>	1.476 <sup>NS</sup>	0.911 <sup>NS</sup>	1.854 <sup>NS</sup>	-		
Light intensity (ft-candles $\times 10^2$ )		20.00	21.80	21.80	21.80				
$\circ$	-	2.369 <sup>NS</sup>	2.492*	3.328*					
$\square$	1.826 <sup>NS</sup>	1.413 <sup>NS</sup>	1.486 <sup>NS</sup>						





### (3) EFFECT OF LIGHT

#### a) Photoperiod

"Photoperiod" in this context is used to describe the length of time in every 24 hours in which 3-week-old plants were exposed to fluorescent lights in a growth cabinet. No precautions were taken to prevent plants receiving the flashes of light which occurred when plants were placed in or removed from the dark wooden cabinet.

The rate of acetylene reduction and accumulation of labelled photosynthates by the nodules were both greatly affected by the length of the photoperiod after only three days' treatment. Thus lengthening the photoperiod from 4 to 16 hours resulted in a seven-fold increase in the rate of acetylene reduction, but the maximum rate of reduction attained with a 20-hour photoperiod was seven times the rate with a 16-hour photoperiod (Fig. 20). The accumulation of labelled photosynthates by the nodules 3-5 hours after exposure of the shoots to  $^{14}\text{CO}_2$  for 30 minutes was not affected by photoperiods shorter than 8 hours, but was trebled by lengthening the photoperiod to 16 hours (Fig. 20). The radioactivity accumulated in the nodules with a 20-hour photoperiod was not significantly different from that with a 16-hour photoperiod.

Growth under continuous illumination resulted in a decline in both the rate of acetylene reduction and accumulation of labelled photosynthates in the nodules.

#### b) Light intensity

Gradually increasing the light intensity to which 3-week-old plants were exposed over a 14-hour period following darkening for 8 hours resulted in one small and insignificant increase in the rate of acetylene reduction over the range 0 to 1600 ft. candles (0 to  $5.740 \text{ cal.m.}^{-2}\text{h.}^{-1}$ ) (Fig. 21). Further increases in light intensity resulted in significant decreases in the rate of acetylene reduction in the two replicate experiments.

Caution must be used in comparison of these results with those obtained under conditions of natural daylight, due to the spectral differences between daylight and the light from the fluorescent tubes in the growth cabinet.

Fig. 22. Diurnal variations in:

- (a) acetylene-reducing activity (○);
- (b) light intensity (Δ) and air temperature (□);
- (c) ethanol-soluble carbohydrate content of the nodules (▽).

The experiment was performed with 3-week-old pea plants on May 2, 1975, and samples for acetylene-reduction assays were incubated in darkness at ambient temperature in the glasshouse immediately after collection at the times shown.

The t-test was used to test the differences between the means of the evening maxima in acetylene-reducing activity and ethanol-soluble carbohydrates and those of other samples.

		Value of t						
Time of day (h)	0800	1000	1200	1400	1600	1800	2000	2200
C <sub>2</sub> H <sub>2</sub> reduction	-0.234 <sup>NS</sup>	2.284 <sup>NS</sup>	3.235*	5.246**	5.365**	3.902**	-	1.880 <sup>NS</sup>
Carbohydrates	6.458***	3.748**	3.652*	2.759*	1.961 <sup>NS</sup>	-	0.852 <sup>NS</sup>	4.940**

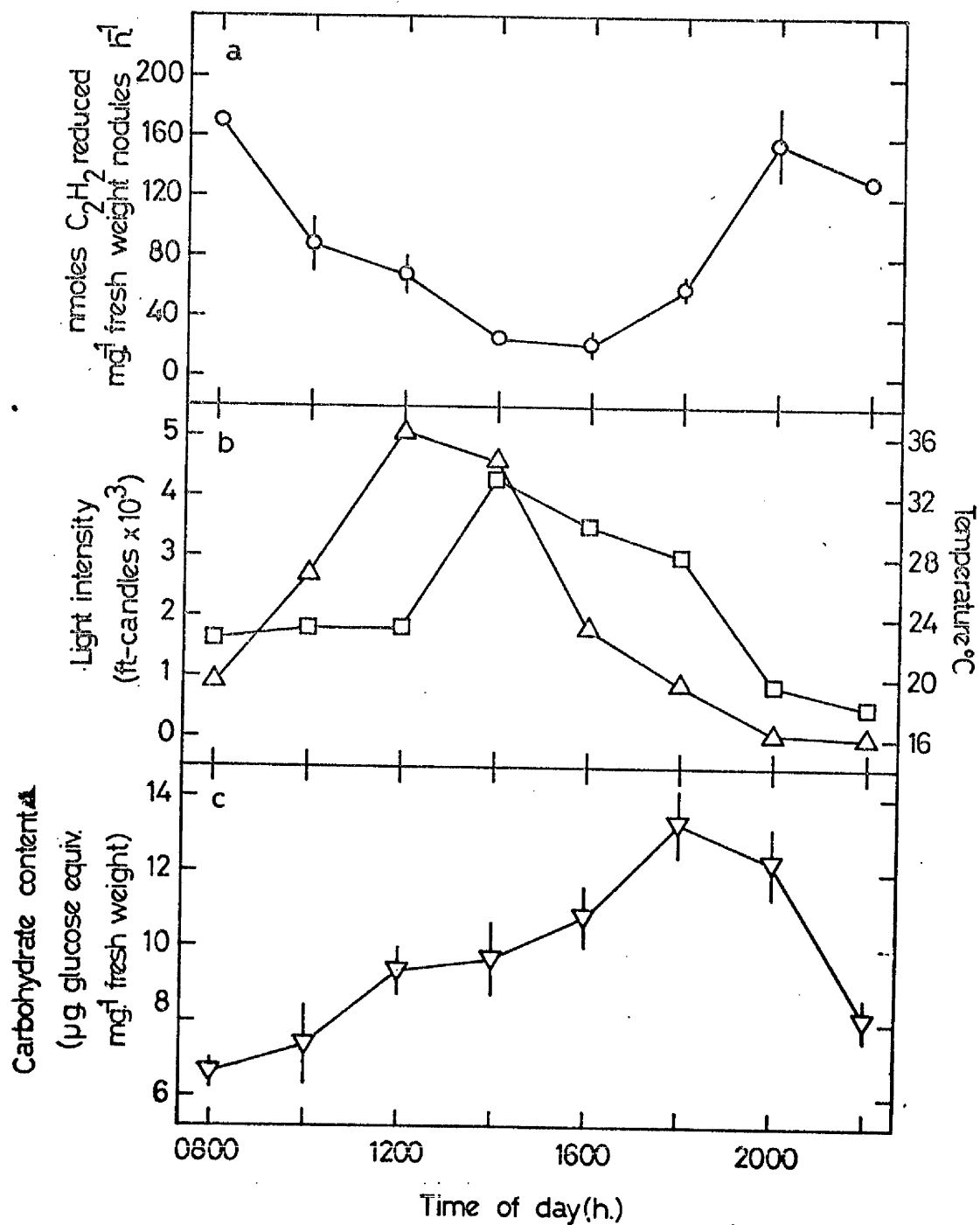
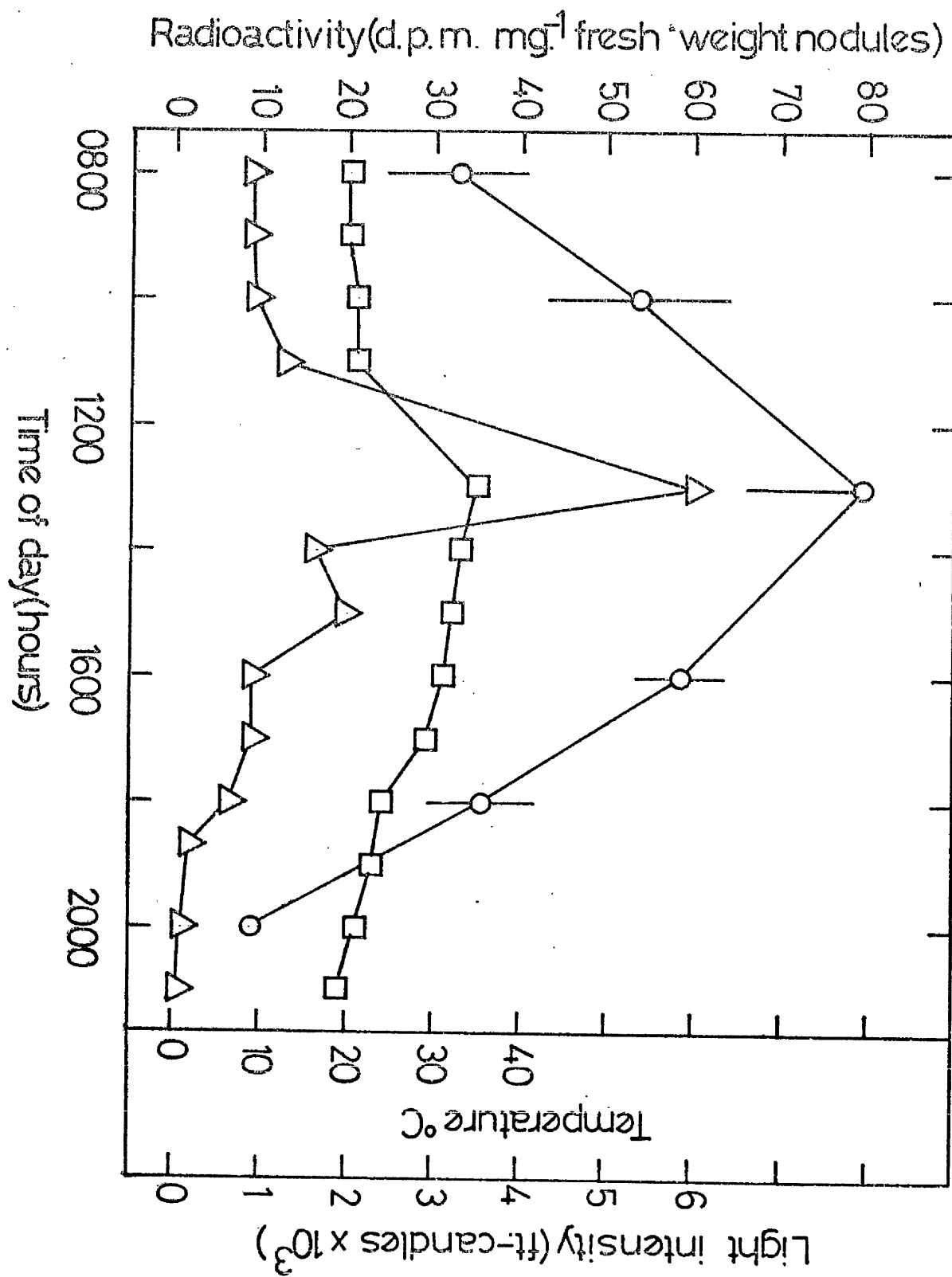


Fig. 23. Diurnal variations in accumulation of  $^{14}\text{C}$ -labelled photosynthates by pea nodules (○), light intensity (△) and air temperature (□). The experiment was performed on June 5, 1973, and the shoots of 3-week-old plants were exposed to  $^{14}\text{CO}_2$  for one hour at ambient light and air temperature at the times shown. Nodules were harvested immediately after the exposure period.

The t-test was used to test the differences between the mean radioactivities of the nodules at successive harvests.

Value of t						
Time of day (h)	0800	1000	1300	1600	1800	2000
	-1.646 <sup>NS</sup>	-1.568 <sup>NS</sup>	1.500 <sup>NS</sup>	3.070**	4.356***	



#### (4) DIURNAL FLUCTUATIONS

Two patterns of fluctuation were observed in experiments performed in a glasshouse at ambient light and temperature, using plants 3 weeks old.

##### a) Experiments showing a single maximum in acetylene-reducing activity

Maximum acetylene-reducing activity was recorded in all experiments at 2000 or 2200 hours (Fig. 22a) and was often, but not always, approximately equal to that recorded previously at 0800 hours on the same day. Minimum activity was recorded between 1400 and 1600 hours, and was only one-eighth of the maximum activity. By contrast, clear diurnal fluctuations in both light intensity, from 0 to 5100 ft. candles, and air temperature, from 23 to 33°C, were recorded in all experiments (Fig. 22b) and maxima in both occurred at 1200 to 1400 hours and minima at 2200 hours.

Anthrone determinations showed that the ethanol-soluble carbohydrate content of the nodules increased steadily during the day to reach a maximum at 1800 hours, and then declined (Fig. 22c). The maximum content was reached 5 to 6 hours after the maximum in translocation of newly-photosynthesised assimilates to the nodules (see below) and shortly before the maximum rate of acetylene reduction was achieved.

A clear diurnal fluctuation in translocation of labelled photosynthates to the nodules was observed when nodules were collected immediately after exposure of the plant shoots to  $^{14}\text{CO}_2$  for 1 hour at different times of the day. Maxima at 1300 hours (Fig. 23) or 1400 hours were recorded and changes in the accumulation of these newly-photosynthesised assimilates were similar to those in light intensity and air temperature.

##### b) Experiments showing two maxima in acetylene-reducing activity

Maxima in acetylene reducing activity were observed at 2000 or 2200 hours as before, but there was also a second, usually smaller peak at

Fig. 24. Diurnal variations in:

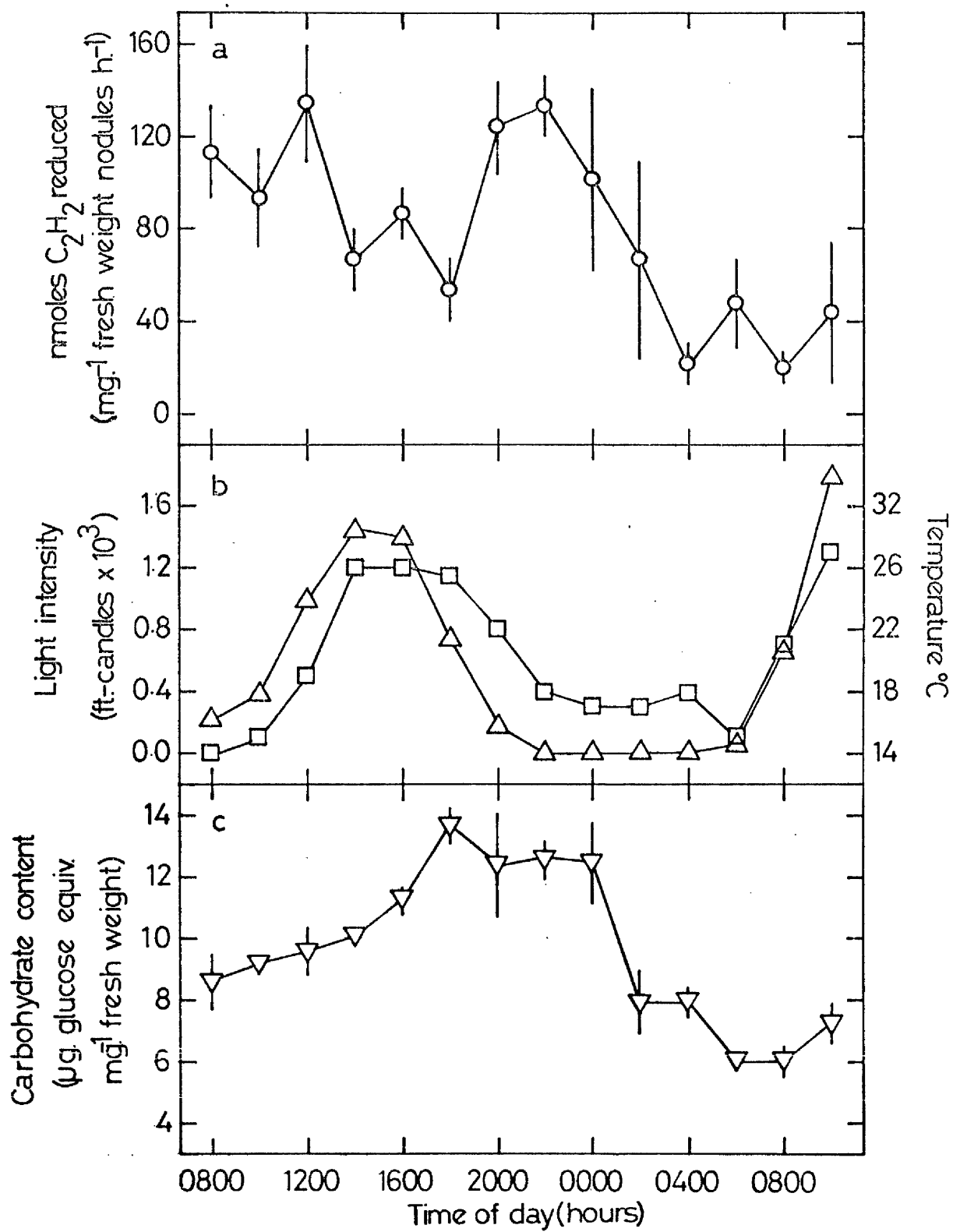
- (a) acetylene-reducing activity (○);
- (b) light intensity (△) and air temperature (□);
- (c) ethanol-soluble carbohydrate content of the nodules (▽).

The experiment was performed with 3-week-old pea plants on May 18-19, 1973, and samples for acetylene reduction assays were incubated in darkness at ambient temperature in the glasshouse immediately after collection at the times shown.

The t-test was used to test the differences between the means of the evening maxima in acetylene-reducing activity and ethanol-soluble carbohydrates and those of other samples.

		Value of t							
Time of day (h)		0800	1000	1200	1400	1600	1800	2000	
C <sub>2</sub> H <sub>2</sub> reduction	0.856 <sup>NS</sup>	1.445 <sup>NS</sup>	-0.099 <sup>NS</sup>	3.368*	2.605*	4.612**	0.343 <sup>NS</sup>		
Carbohydrates	7.207***	6.469***	3.994**	5.405**	3.090*	-	0.707 <sup>NS</sup>		
Time of day (h)		2200	0000	0200	0400	0600	0800	1000	
C <sub>2</sub> H <sub>2</sub> reduction	-	-0.730 <sup>NS</sup>	1.471 <sup>NS</sup>	7.031***	3.665**	7.507***	2.697*		
Carbohydrates	1.346 <sup>NS</sup>	0.823 <sup>NS</sup>	4.659**	7.116***	11.841***	10.921***	7.124***		





1200 or 1400 hours (Fig. 24a). Minimum activities were recorded between 1400 and 1800 hours, and between 0400 and 0800 hours, and were one-half and one-quarter respectively of the evening maxima. Mid-day maxima always occurred 1 to 2 hours before the maxima in both air temperature and light intensity. Clear diurnal variations were recorded in both light intensity, from 0 to 1500 ft. candles, and air temperature, from 14 to 26°C (Fig. 24b). Maxima in both occurred at 1300 to 1400 hours in all experiments and minima were recorded during darkness.

Anthrone determinations showed that the level of ethanol-soluble carbohydrates in the nodules reached a maximum between 1800 and 0000 hours (Fig. 24c). The carbohydrate content rose steadily by 50% from 0800 to 1800 hours, remained constant until 0000 hours and then declined rapidly to a minimum value of half the maximum at 0600 to 0800 hours.

The diurnal fluctuation in translocation of newly-photosynthesised <sup>14</sup>C-labelled assimilates was similar to that observed previously, with a maximum at 1300 to 1400 hours (Fig. 23).

In all experiments, changes in the rate of acetylene reduction followed 6 to 8 hours after changes in light intensity, air temperature and translocation of newly-photosynthesised assimilates to the nodules, and 1 to 2 hours after changes in the level of ethanol-soluble carbohydrates in the nodules. The second maximum at mid-day in acetylene-reducing activity at mid-day preceded by 1 to 2 hours maxima in light intensity and air temperature.

Fig. 25a. Effect of darkening on the acetylene-reducing activity of nodules of 5-week-old pea plants. Plants were maintained at 25°C throughout the experiments and the activity of nodules of control plants maintained in the light did not decline during this period.

The t-test was used to test the differences between means of acetylene-reducing activity prior to darkening and during the dark period.

		Value of t							
Time (h) from darkening		10	24	48	72	96			
○		1.296 <sup>NS</sup>	2.641*	2.679*	2.679*	2.679*			
Time (h) from darkening		8	12	16	20	24	48	72	96
△		5.539**	5.811**	9.076***	9.141***	9.108***	9.511***	9.478***	9.459***
□		0.294 <sup>NS</sup>	1.006 <sup>NS</sup>	1.947 <sup>NS</sup>	2.083 <sup>NS</sup>	2.837*	2.951*	2.995*	2.995*

Fig. 25b. Effect of darkening on the accumulation of  $^{14}\text{C}$ -labelled photosynthates in nodules of 3-week-old pea plants. Plant shoots were exposed to  $^{14}\text{CO}_2$  for 30 minutes immediately before darkening and assessments made of the radioactivity of nodules on darkened plants ( $\triangle$ ) and those on control plants ( $\circ$ ) maintained in the light.

The t-test was used to test the differences between the mean radioactivities of nodules on darkened and control plants.

Value of t (dark/light)					
Time (h) from darkening	10	24	48	72	96
	0.466 <sup>NS</sup>	-2.209 <sup>NS</sup>	-0.858 <sup>NS</sup>	-1.128 <sup>NS</sup>	-0.743 <sup>NS</sup>

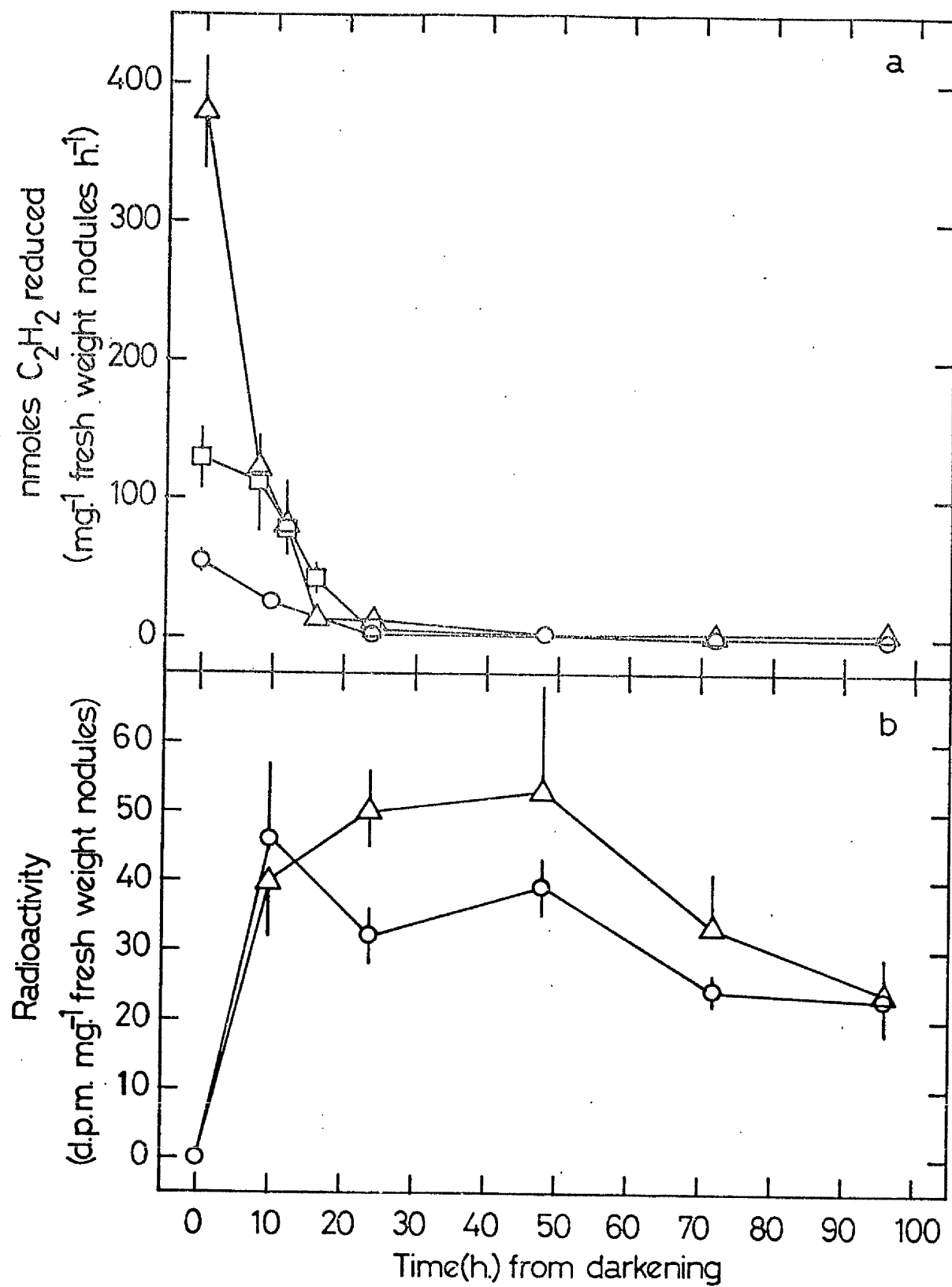


Fig. 25c. Effect of darkening on the ethanol-soluble carbohydrate content of nodules of 3-week-old pea plants. Plants were maintained at 25°C throughout the experiments and the ethanol-soluble carbohydrate content of nodules on control plants maintained in the light did not decline during this period.

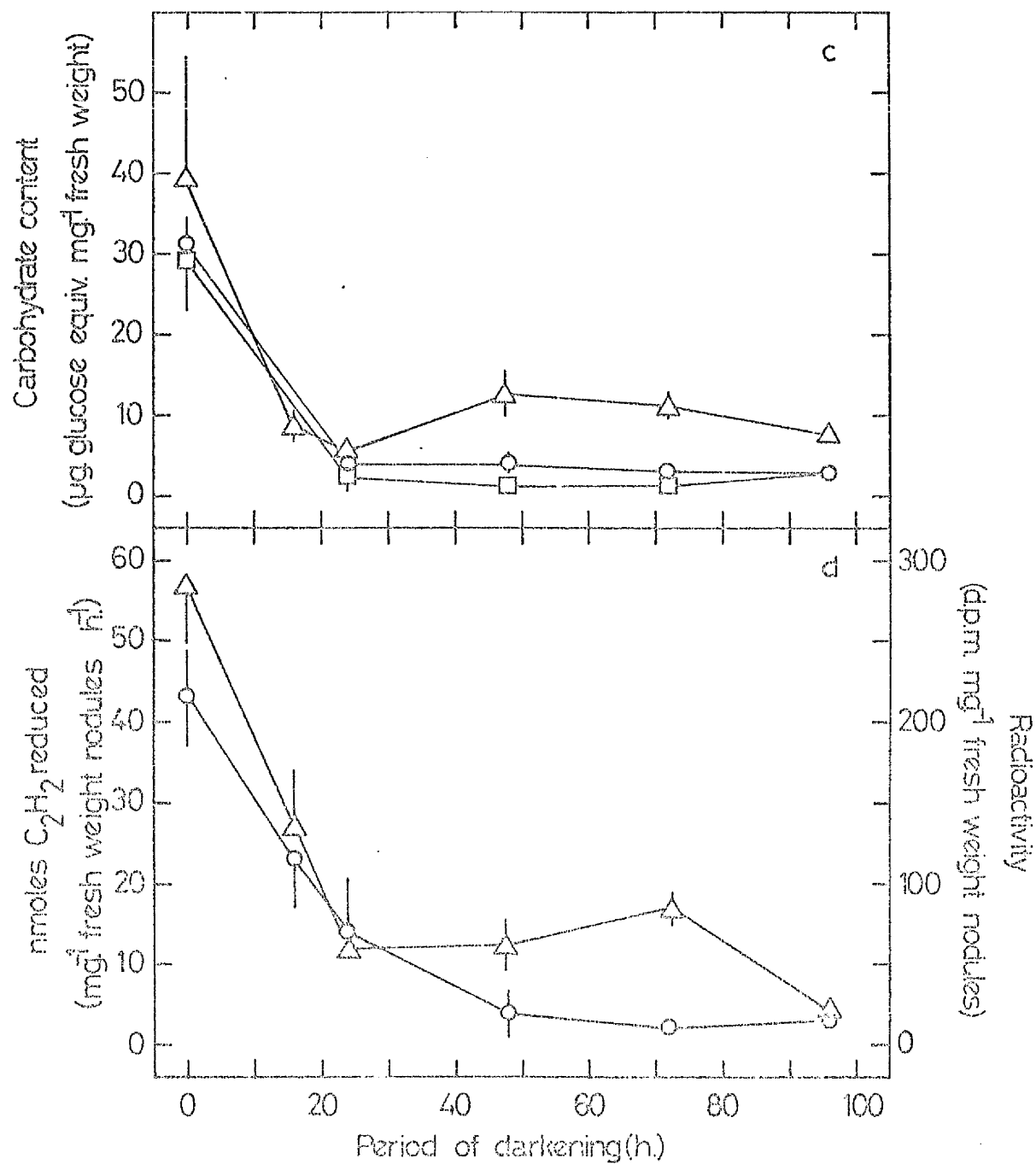
The t-test was used to test the differences between the mean ethanol-soluble carbohydrate content of nodules prior to and during darkening.

Time (h) from darkening	Value of t				
	16	24	48	72	96
△	1.943 <sup>NS</sup>	2.152 <sup>NS</sup>	1.693 <sup>NS</sup>	1.909 <sup>NS</sup>	2.033 <sup>NS</sup>
○	-	3.994**	3.864**	4.044**	4.090**
□	-	8.342***	8.530***	8.388***	7.637***

Fig. 25d. The effect of pre-darkening on acetylene-reducing activity (  $\circ$  ) and accumulation of  $^{14}\text{C}$ -labelled photosynthates (  $\Delta$  ) in nodules of 3-week-old pea plants. Plants were darkened for the times shown and the shoots were exposed to  $^{14}\text{CO}_2$  for 30 minutes immediately on return to the light. Nodules were collected for both acetylene reduction assays and assessment of radioactivity 10 hours after return to the light.

The t-test was used to test the differences between the mean acetylene-reducing activity and accumulation of radioactivity in nodules of control and darkened plants.

		Value of t				
Period of darkening (h)	16	24	48	72	96	
C <sub>2</sub> H <sub>2</sub> reduction	1.832 <sup>NS</sup>	3.050**	5.739**	6.514***	6.514***	
Radio-activity	2.957**	5.733***	5.439***	5.146***	7.022***	





(5) EFFECT OF DARKENING

The following experiments were performed with plants 2.5 weeks old.

a) Acetylene-reducing activity

The rate of acetylene reduction was greatly reduced by darkening so that little reduction was detected after only 24 hours in the dark (Fig. 25a). Exceptionally vigorous plants of Batch 2 sometimes retained activity for a longer time, but in all cases acetylene reduction had virtually ceased after 62 hours in the dark. Plants maintained in the light showed no significant decrease in activity during the experiments.

b) Accumulation of  $^{14}\text{C}$ -labelled photosynthates(i) Plants fed  $^{14}\text{CO}_2$  prior to darkening

The accumulation of labelled photosynthates in the nodules of plants fed  $^{14}\text{CO}_2$  for 30 minutes and then darkened did not differ significantly from the accumulation in nodules of plants maintained in the light (Fig. 25b). Similarly, the total radioactivity of plants either darkened or maintained in the light was not significantly different at any time of harvest and showed little change from 10 to 96 hours after exposure to  $^{14}\text{CO}_2$  (mean radioactivity =  $2.2 \pm 0.2$  (d.p.m.  $\times 10^4$ )).

Anthrone determinations showed that the ethanol-soluble carbohydrate content of the nodules of darkened plants declined to one-quarter of the original level after 20 to 30 hours in the dark, whereas there was no decline in plants maintained in the light (Fig. 25c).

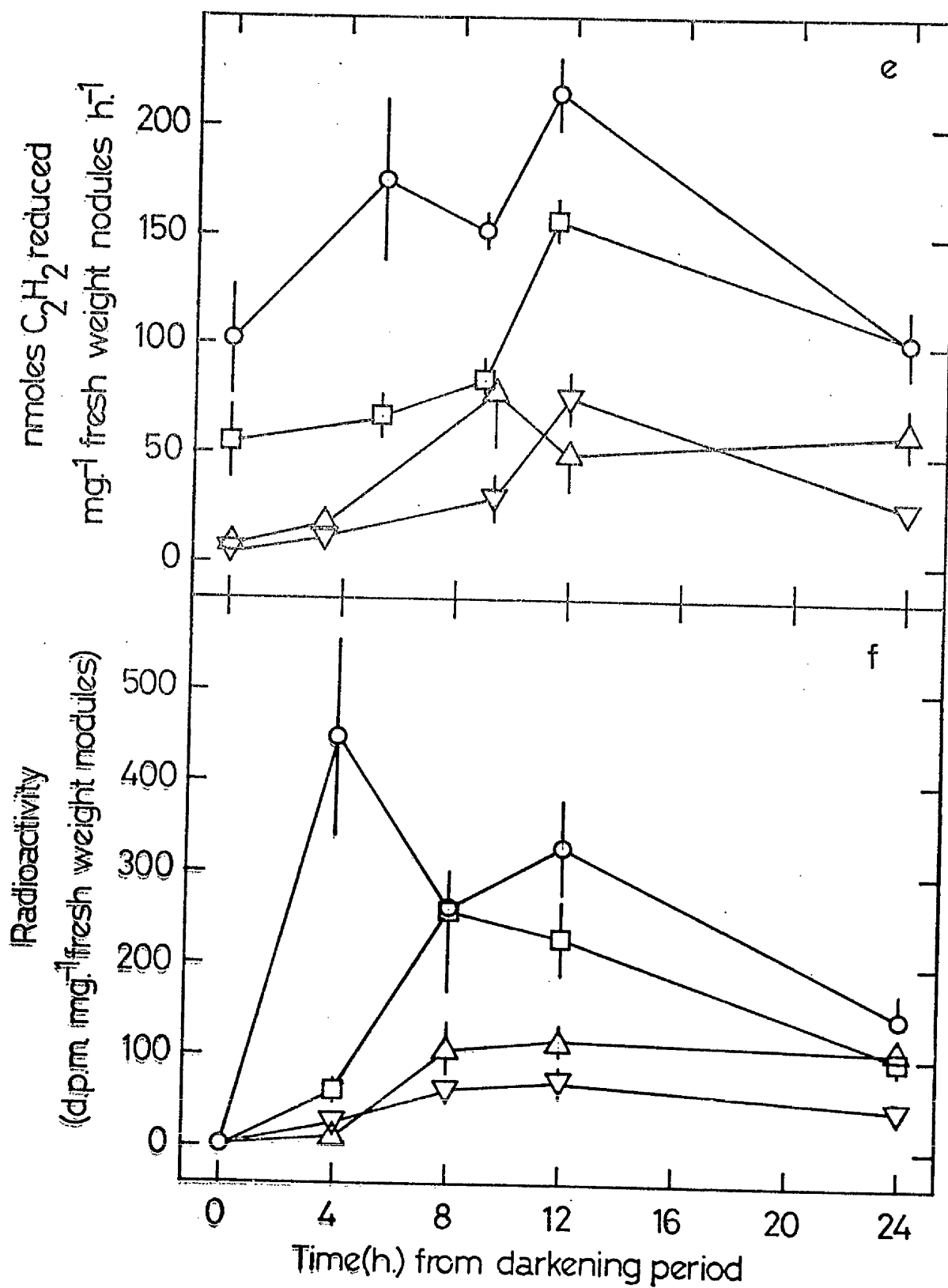
(ii) Plants darkened prior to  $^{14}\text{CO}_2$  feeding

Both the acetylene-reducing activity and the accumulation of labelled photosynthates by the nodules 10 hours after darkened plants were returned to the light and fed  $^{14}\text{CO}_2$  declined initially at similar rates (Fig. 25d). Thus, in plants darkened for 24 hours, both acetylene-reducing activity and accumulation of labelled photosynthates were only approximately

Fig. 25e. The recovery of acetylene-reducing activity (  $e$  ) and accumulation of  $^{14}\text{C}$ -labelled photosynthates (  $f$  ) in nodules of plants returned to the light after darkening. Plants were kept continually in the light (  $\circ$  ) or darkened for 38 hours (  $\square$  ), 62 hours (  $\triangle$  ) or 72 hours (  $\nabla$  ). The shoots were exposed to  $^{14}\text{CO}_2$  for 30 minutes immediately on return to the light and changes in acetylene-reducing activity and radioactivity were monitored over the following 23.5 hours.

The t-test was used to test the differences between the mean acetylene-reducing activity and radioactivity of nodules either maintained in the light or darkened.

Time (h) from return to light	Value of t				
	0	4	8	12	24
$\text{C}_2\text{H}_2$ reduction					
38 h darkened	1.492 <sup>NS</sup>	2.891*	5.065**	2.741*	0.061 <sup>NS</sup>
62 h darkened	3.531*	4.065**	2.701*	6.593***	4.917**
72 h darkened	3.686*	4.280**	8.250***	6.440***	2.174 <sup>NS</sup>
Radioactivity					
38 h darkened	-	3.701**	-0.006 <sup>NS</sup>	1.600 <sup>NS</sup>	1.491 <sup>NS</sup>
62 h darkened	-	4.210***	3.250**	4.708***	3.625**
72 h darkened	-	4.076***	5.102***	3.903**	1.243 <sup>NS</sup>



one-third of that found prior to darkening, but longer periods of pre-darkening resulted in a relatively more rapid decline in acetylene-reducing activity. By contrast, the total radioactivity of darkened plants did not differ significantly at any time from the radioactivity of plants kept in the light (mean radioactivity =  $1.7 \pm 0.1$  (d.p.m.  $\times 10^5$ )).

c) Recovery from darkening

(i) Acetylene-reducing activity

This experiment was performed twice only and the plants used on the occasion illustrated (Fig. 25e) were particularly vigorous and, after 38 hours in the dark, still retained half of the acetylene-reducing activity of plants maintained in the light. However, activity was lost almost completely after 62 hours in the dark.

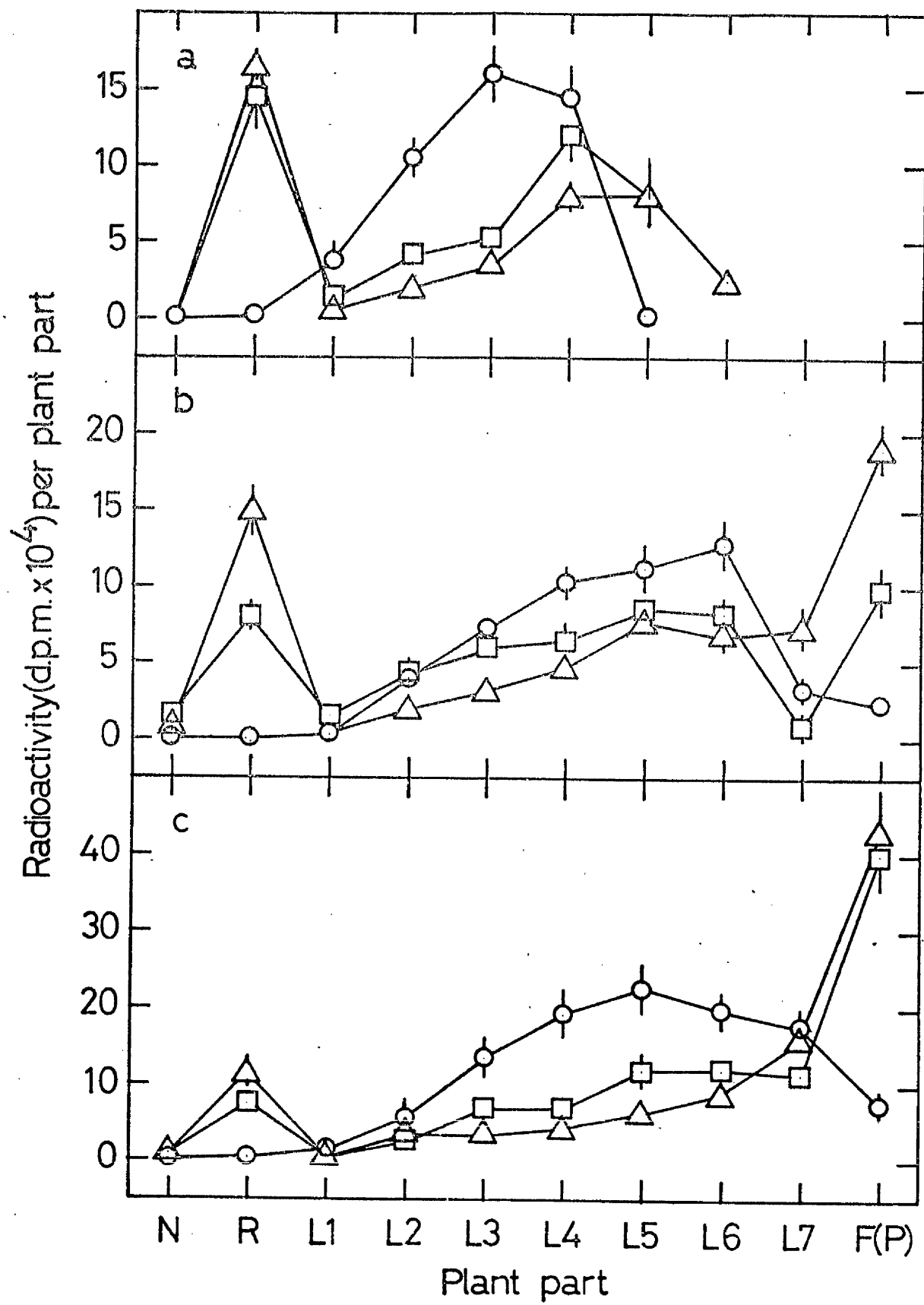
The rate of recovery of acetylene-reducing activity was dependent on the length of time the plants were darkened. The acetylene-reducing activity of 38-hour-darkened plants 12 hours after they were returned to the light was little different from that of control plants, but plants darkened for 62 hours recovered after 24 hours, and plants darkened for 72 hours had still not fully recovered after 24 hours in the light (Fig. 25e).

(ii) Accumulation of  $^{14}\text{C}$ -labelled photosynthates

The total radioactivity of whole plants immediately after exposure to  $^{14}\text{CO}_2$  for 30 minutes on return to the light was the same irrespective of the period of darkening (mean radioactivity =  $4.5 \pm 0.2$  (d.p.m.  $\times 10^5$ )). However, the rate of accumulation of labelled photosynthates in the nodules after different "cold-chase" periods was much reduced depending on the length of the pre-darkening period (Fig. 25f) and was similar to the rate of recovery of acetylene-reducing activity (Fig. 25e). Thus the accumulation of photosynthates in nodules of 38-hour-darkened plants after they had been returned to the light for 8 hours was little different from that in nodules of plants maintained in the light, but plants darkened for

62 hours did not fully recover within 24 hours. The nodules of plants darkened for 72 hours showed greater recovery than those darkened for 62 hours. In all experiments, the accumulation of labelled photosynthates in the nodules of darkened plants returned to the light always returned to the level of plants maintained in the light before there was full recovery in acetylene-reducing activity.

Fig. 26. Changes in the distribution within the plants of  $^{14}\text{C}$ -labelled photosynthates during growth. Shoots of plants 2 weeks old (a), 3 weeks old (b) and 4 weeks old (c) were exposed to  $^{14}\text{CO}_2$  for 15 minutes and the total radioactivity in each plant part was extracted immediately (o) or after 6 hours ( $\square$ ) or 25 hours ( $\Delta$ ) "cold-chase". Symbols used to designate the plant parts assessed are: N = nodules, R = root, L 1 to L 7 = leaves 1 to 7, numbered from shoot base to apex and F(P) = flower (or fruit).



(6) DISTRIBUTION OF  $^{14}\text{C}$ -LABELLED ASSIMILATES

a) Distribution in relation to plant age.

2-week-old plants. Immediately after exposure of the shoots to  $^{14}\text{CO}_2$  for 15 minutes, leaves 3 and 4 were most heavily labelled and thus were probably the most photosynthetically active leaves (Fig. 26a). Older leaves (1 and 2) showed less activity and the shoot apex (labelled as L5) root and nodules accumulated negligible activity. After 6 hours "cold-chase", the activity of the photosynthetically active leaves declined by half and the activity of the nodules, roots and shoot apex (the main "sinks" in the plant) increased to 0.3%, 30% and 17% respectively of the total radioactivity of each plant. Activity continued to decline slightly in the leaves but increased in the "sinks" for 25 hours after feeding, during which time the shoot apex expanded so that leaf 5 could be detached without damage to the apex (labelled as L6) and the activity of the roots increased to 39% of the total radioactivity of each plant.

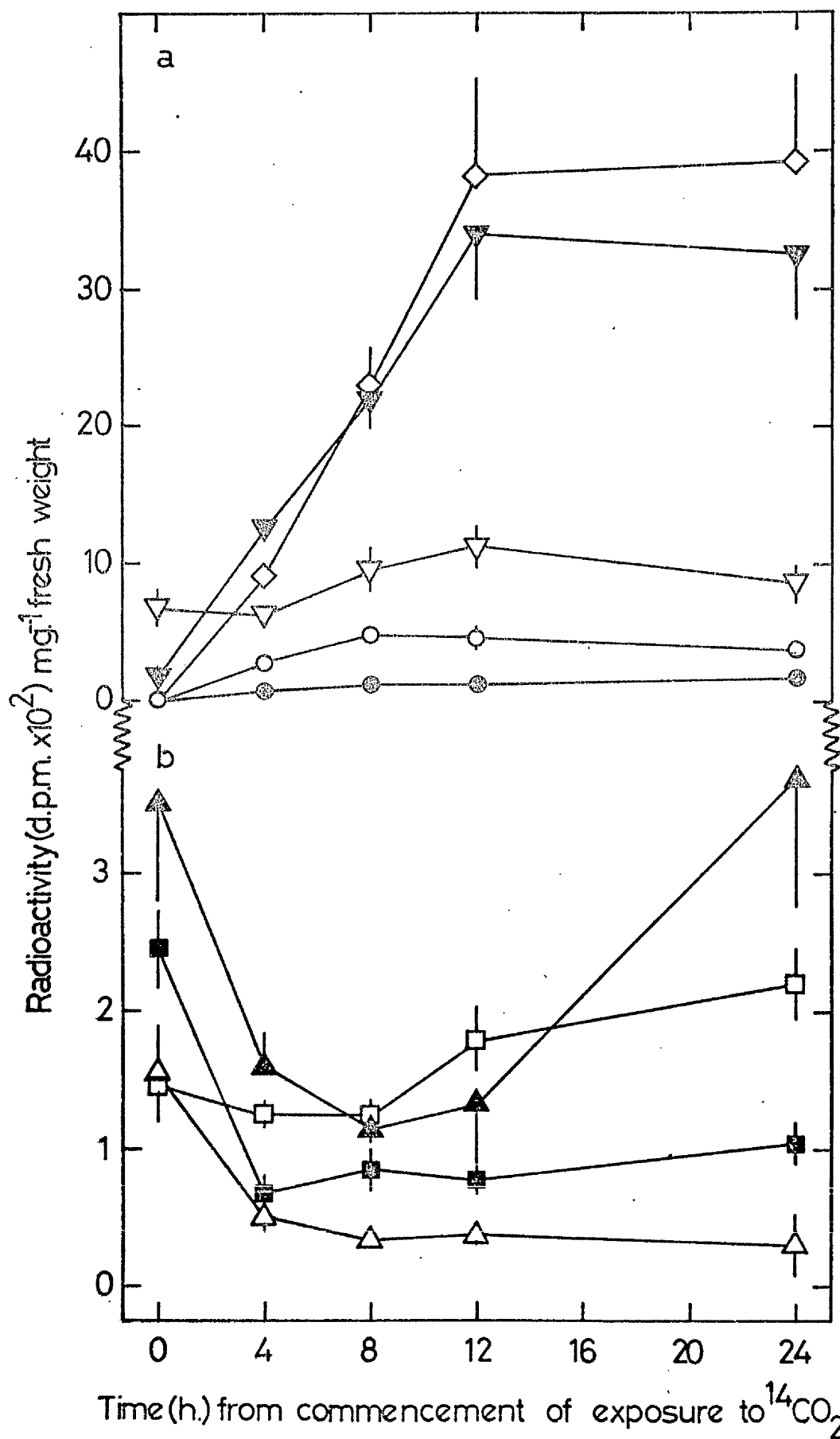
3-week-old plants. Leaves 3 to 6 were most photosynthetically active and older leaves (1 and 2) assimilated less activity (Fig. 26b). The nodules and roots contained no detectable activity immediately after feeding  $^{14}\text{CO}_2$  for 15 minutes to the shoots, but the flower and immature leaf 7 accumulated 6% and 4% respectively of the total radioactivity. After 6 hours, the activity of the leaves declined by 30% and the activity of the nodules, roots and flower increased to 3%, 14% and 18% respectively of the total radioactivity. The activity of the leaves continued to decline to half their original activity after 25 hours, and the activity of the roots and flower continued to increase until they contained 23% and 33% respectively of the total radioactivity. By contrast, the activity of the nodules declined from 3% to 1% of the total radioactivity from 6 to 25 hours after exposure of the shoot to  $^{14}\text{CO}_2$ .



7. I certify that the above is a true and correct copy of the original document.

1. The following information was obtained from the above mentioned sources:

Fig. 27. Changes in the specific activity of parts of vegetative plants 2.5 weeks old following exposure of the shoots to  $^{14}\text{CO}_2$  for 30 minutes. Plant parts assessed were the nodules (o), root (●), leaf 1 (leaves were numbered from shoot base to apex) (□), leaf 2 (■), leaf 3 (△), leaf 4 (▲), leaf 5 (∇), leaf 6 (▼) and shoot apex (◇).



4-week-old plants Leaves 3 to 7 were the most photosynthetically active and older leaves (1 and 2) assimilated relatively little activity (Fig. 26c). No activity was detected in the roots and nodules immediately after feeding the shoots  $^{14}\text{CO}_2$  for 15 minutes, but the fruits accumulated 7% of the total radioactivity of the plants. After 6 hours, the activity of leaves 4 to 7 declined by half, and the fruit was the most active "sink" for photosynthates, as it accumulated 35% of the total radioactivity of the plant. By contrast, the nodules and roots accumulated only 0.8% and 7% respectively of the total radioactivity. Even 25 hours after exposure of the shoot to  $^{14}\text{CO}_2$ , the nodules and roots contained only 0.8% and 12% respectively, whereas the fruit contained 50% of the total radioactivity of the plant.

The most striking changes in the pattern of accumulation of photosynthates as the plant aged from 2 to 4 weeks were therefore the three-fold increase in the percentage accumulation at the shoot tip as it changed from a vegetative to reproductive function and the accompanying three-fold decrease in the percentage accumulation in the roots. The activity accumulated by the nodules, expressed as a percentage of the total activity, increased 20-fold from 2- to 3-week-old plants but declined in 4-week-old plants to only twice the activity in nodules of plants 2 weeks old. The total radioactivity accumulated by the nodules never exceeded 3% of the total radioactivity of the plant at any time.

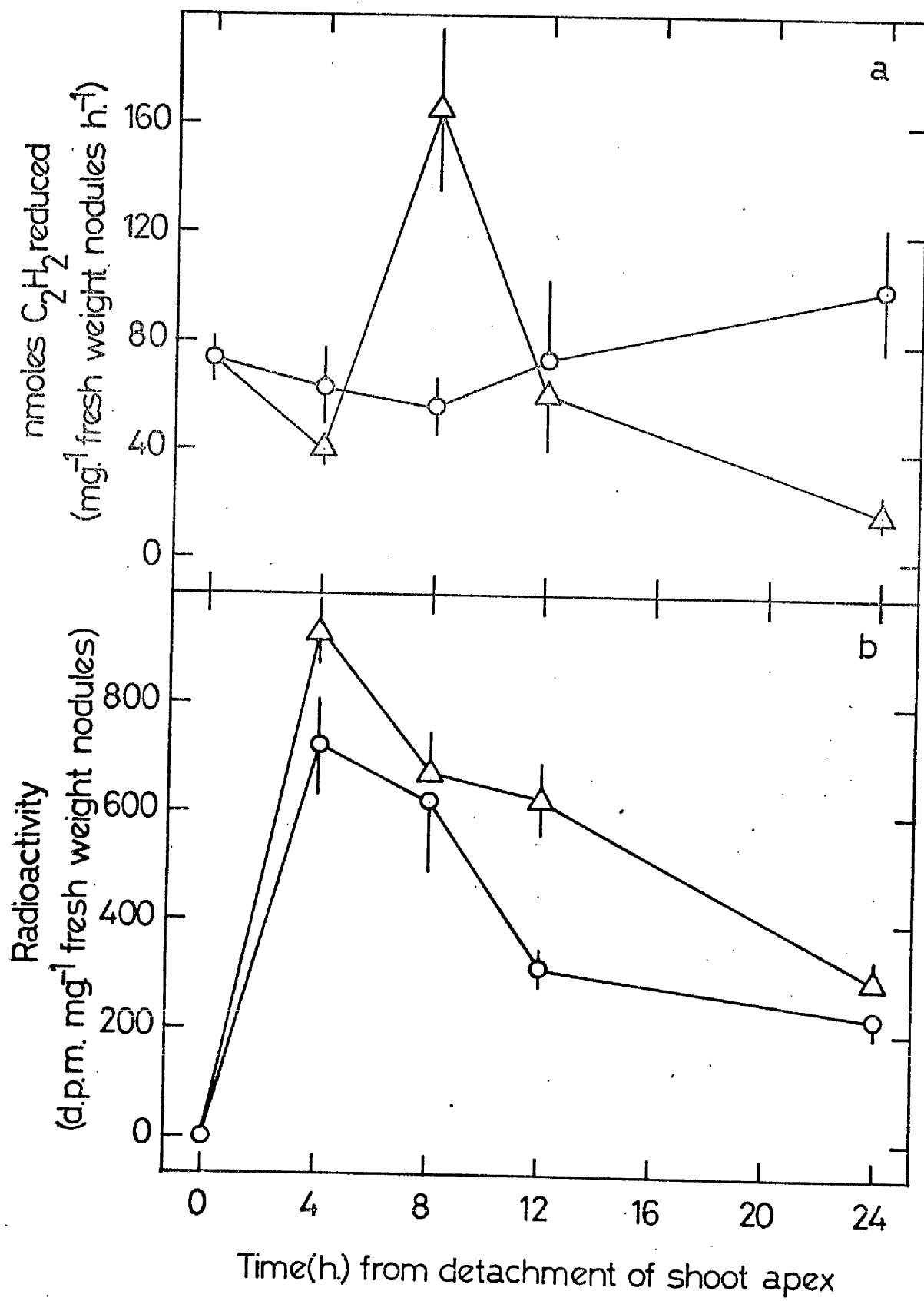
#### b) Specific activity of plant parts

The total radioactivity accumulated in the nodules of vegetative plants 2.5 weeks old did not exceed 5% of that of the whole plant at any time during the "cold-chase" period after the shoots were exposed to  $^{14}\text{CO}_2$  for 30 minutes. However, accumulation of labelled photosynthates by the nodules was such that their specific activity exceeded that of the most photosynthetically active leaves (3 and 4) only 3.5 hours after the shoots were exposed to  $^{14}\text{CO}_2$  (Fig. 27a). Although the roots accumulated up to half the

Figs 28a & b. Effect of removal of shoot apex of 2-week-old vegetative plants on acetylene reduction (a) and accumulation of  $^{14}\text{C}$ -labelled photosynthates (b) by the nodules. Acetylene-reducing activity of nodules on treated plants ( $\triangle$ ) was assessed at the same time as that of nodules on intact plants ( $\circ$ ) at intervals up to 24 hours after treatment. Plant shoots were exposed to  $^{14}\text{CO}_2$  for 30 minutes immediately before detachment of the apex and nodules of treated ( $\triangle$ ) and intact ( $\circ$ ) plants were collected after "cold-chase" periods up to 24 hours after detachment of the apex.

The t-test was used to test the differences between the mean acetylene-reducing activity and radioactivity of nodules on treated and intact plants at each time of collection.

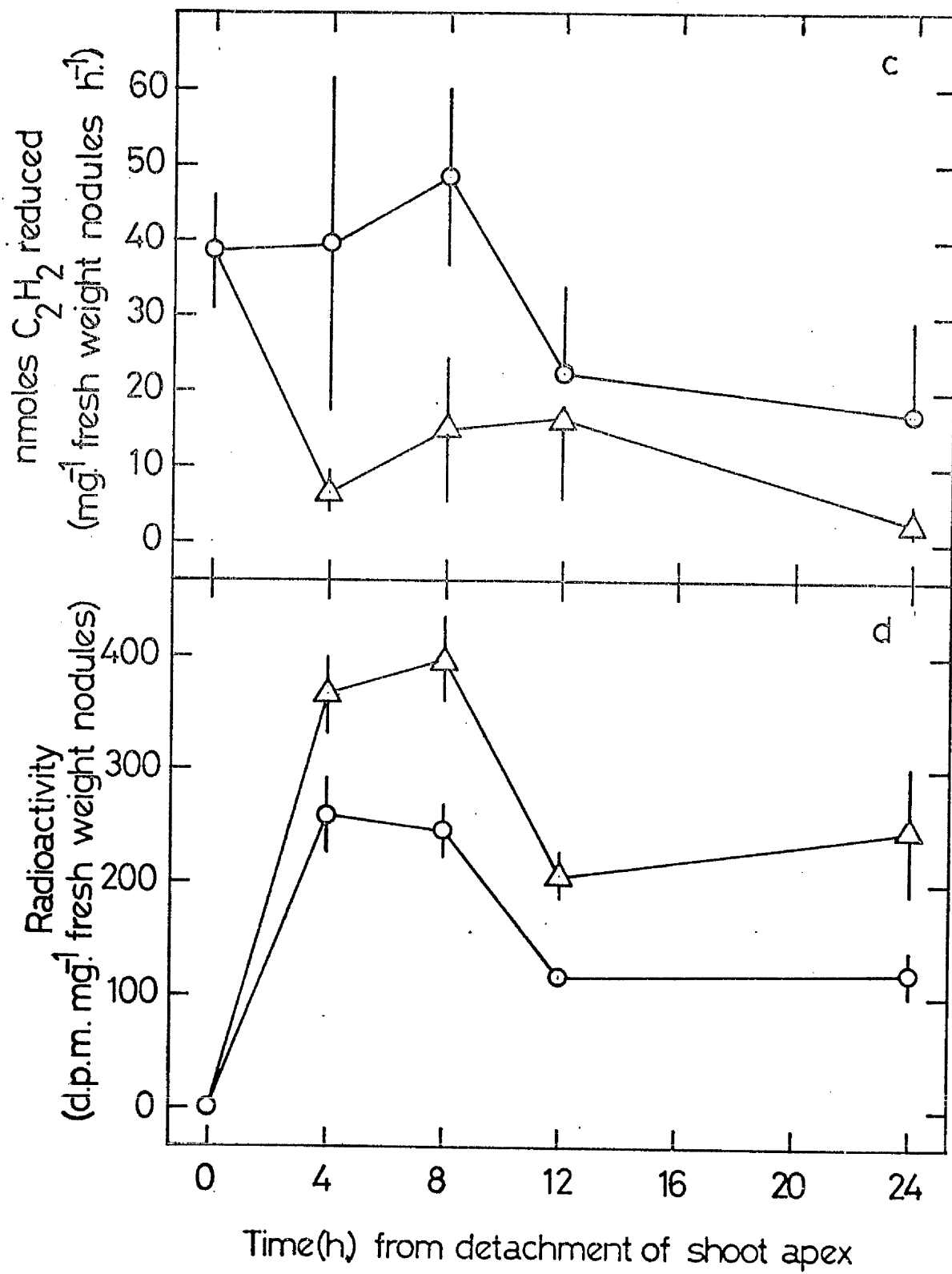
Time (h) after detachment of apex	Value of t (treated/intact)			
	4	8	12	24
$\text{C}_2\text{H}_2$ reduction	-1.741 <sup>NS</sup>	3.136*	-0.323 <sup>NS</sup>	-3.090*
Radio- activity	2.098*	0.465 <sup>NS</sup>	4.330***	1.458 <sup>NS</sup>



Figs 28c & d. Effect of removal of shoot apex of 3-week-old flowering plants on acetylene reduction ( c ) and accumulation of  $^{14}\text{C}$ -labelled photosynthates ( d ) by the nodules. Acetylene-reducing activity of nodules on treated plants ( $\Delta$ ) was assessed at the same time as that of nodules on intact plants ( $\circ$ ) at intervals up to 24 hours after treatment. Plant shoots were exposed to  $^{14}\text{CO}_2$  for 30 minutes immediately before detachment of the apex and nodules of treated ( $\Delta$ ) and intact ( $\circ$ ) plants were collected after "cold-chase" periods up to 24 hours after detachment of the apex.

The t-test was used to test the differences between the mean acetylene-reducing activity and radioactivity of nodules in treated and intact plants at each time of collection.

Time (h) after detachment of apex	Value of t (treated/intact)			
	4	8	12	24
$\text{C}_2\text{H}_2$ reduction	-1.447 <sup>NS</sup>	-2.254 <sup>NS</sup>	-0.539 <sup>NS</sup>	-1.159 <sup>NS</sup>
Radio- activity	2.192 <sup>NS</sup>	3.565**	4.209***	2.193*





total radioactivity in the plants, their specific activity did not exceed one-third of that of the nodules, or one-half of that of the most photosynthetically active leaves. The specific activity of the shoot apex and the young developing leaf 6 both rose to seven times that of the nodules after 23.5 hours, but the radioactivities accumulated were only 2% and 5% respectively of the total radioactivity of the plant. The specific activity of leaf 5 (Fig. 27a), which was about one-third mature, was 2 to 4 times that of the most photosynthetically active leaves at all times of harvest (Fig. 27b). By contrast, the specific activity of the older photosynthetically active leaves (1 to 4) declined by half 7.5 hours after exposure to  $^{14}\text{CO}_2$  (Fig. 27b). The specific activity of leaf 4 then rose to its original level 23.5 hours after exposure.

c) Effect of removal of apex on distribution of  $^{14}\text{C}$ -labelled assimilates and on the rate of acetylene reduction

This experiment was performed twice only, with vegetative, flowering and fruiting plants (2, 3 and 4 weeks old respectively) from which the shoot apices were removed immediately after exposure of the shoots to  $^{14}\text{CO}_2$  for 30 minutes.

(i) Vegetative plants

The acetylene-reducing activity of treated vegetative plants was always higher than that of intact plants 8 hours after treatment, although differences between the means were not always statistically significant, and at some times of collection the activity of treated plants was lower than that of intact plants (Fig. 28a). The accumulation of labelled photosynthates in the nodules was at all times slightly higher than in intact plants, although differences between the means were statistically significant only 4 and 12 hours after exposure to  $^{14}\text{CO}_2$  (Fig. 28b).

(ii) Flowering plants

The acetylene-reducing activity of treated flowering plants

Figs 28e & f. Effect of removal of shoot apex of 4-week-old fruiting plants on acetylene reduction (  $\square$  ) and accumulation of  $^{14}\text{C}$ -labelled photosynthates (  $\int$  ) by the nodules. Acetylene-reducing activity of nodules on treated plants (  $\triangle$  ) was assessed at the same time as that of nodules on intact plants (  $\circ$  ) at intervals up to 24 hours after treatment. Plant shoots were exposed to  $^{14}\text{CO}_2$  for 30 minutes immediately before detachment of the apex and nodules of treated (  $\triangle$  ) and intact (  $\circ$  ) plants were collected after "cold-chase" periods up to 24 hours after detachment of the apex.

The t-test was used to test the differences between the mean acetylene-reducing activity and radioactivity of nodules on treated and intact plants at each time of collection.

Time (h) after detachment of apex	Value of t (treated/intact)			
	4	8	12	24
$\text{C}_2\text{H}_2$ reduction	3.987**	2.295 <sup>NS</sup>	0.804 <sup>NS</sup>	1.744 <sup>NS</sup>
Radio- activity	0.206 <sup>NS</sup>	2.191*	1.393 <sup>NS</sup>	2.082*

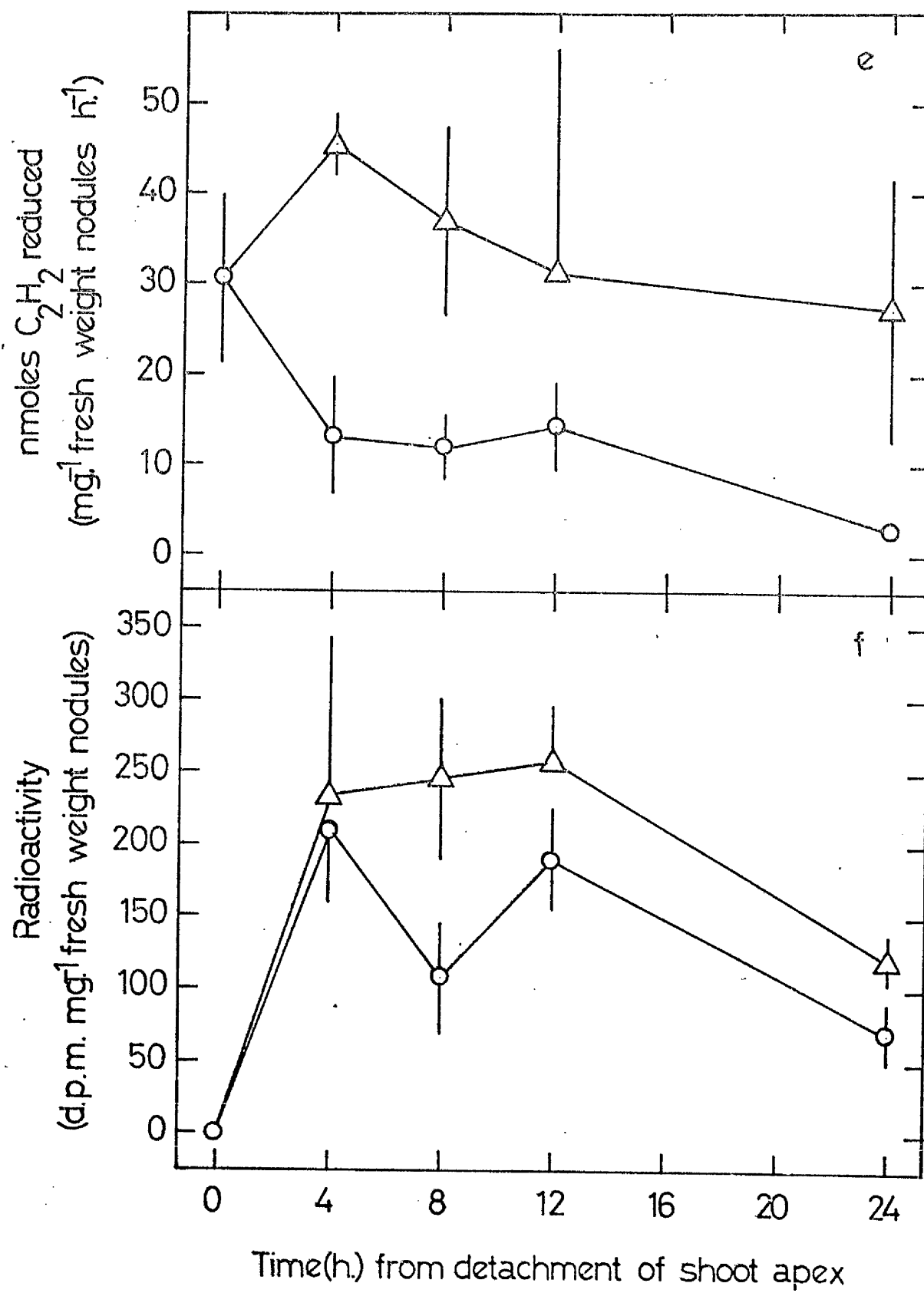


Table 5. Effect of continuous removal of flowers on the decline of acetylene-reducing activity during ageing<sup>of</sup> pea plants from 3 to 5 weeks old.

	Treated plants	Intact plants	t value of difference between means
Fresh weight of nodules per plant (mg)	36.70 $\pm$ 6.18	7.80 $\pm$ 1.87	3.687**
Acetylene-reducing activity of nodules (n moles C <sub>2</sub> H <sub>2</sub> reduced mg. fresh weight nodules h <sup>-1</sup> )	126.49 $\pm$ 28.03	42.84 $\pm$ 9.14	2.837*
Radioactivity accumulated by nodules (d.p.m. mg <sup>-1</sup> fresh weight nodules)	98.0 $\pm$ 19.4	9.3 $\pm$ 1.6	4.785***

Flowers were removed in formation from 3-week-old plants and the plants were harvested for analysis when 5 weeks old. An untreated set of plants of the same age was harvested simultaneously.

was always slightly lower than that of intact plants, and the differences found 8 hours after removal of the flower almost reached significance (Fig. 28c). By contrast, significant increases in accumulation of labelled photosynthates by the nodules of treated plants were observed at all times (Fig. 28d) and largest increases were observed 8 and 12 hours after exposure to  $^{14}\text{CO}_2$ .

#### (iii) Fruiting plants

Plants which bore young developing fruits showed significant increases in acetylene-reducing activity (Fig. 28e) and accumulation of labelled photosynthates (Fig. 28f) after detachment of the fruits. The apparently large (3-fold) stimulation in the rate of acetylene reduction following removal of the fruits must be accepted with caution, however, because of the variability of results obtained in these experiments. The increases in acetylene-reducing activity were nevertheless statistically significant 4 hours after treatment. Although the accumulation of labelled photosynthates was always slightly higher in nodules of treated rather than intact plants, only the data obtained 8 and 24 hours after detachment of the fruits reached significance.

#### (iv) Continuous removal of flowers

Continuous removal of floral apices as they were formed from plants between the ages 3 and 5 weeks prevented the normal decrease in acetylene-reducing activity (Table 5). In 5-week-old treated plants, both the fresh weight of the nodules per plant and the acetylene-reducing activity per mg. fresh weight nodules increased to three times that of untreated plants. The specific activity of the nodules 10 hours after exposure of treated plants to  $^{14}\text{CO}_2$  for 30 minutes was ten times that of nodules on untreated plants and all increases were statistically significant.

Table 6. Effect of length of "cold-chase" period on ethanol-extractable activity in pea nodules.

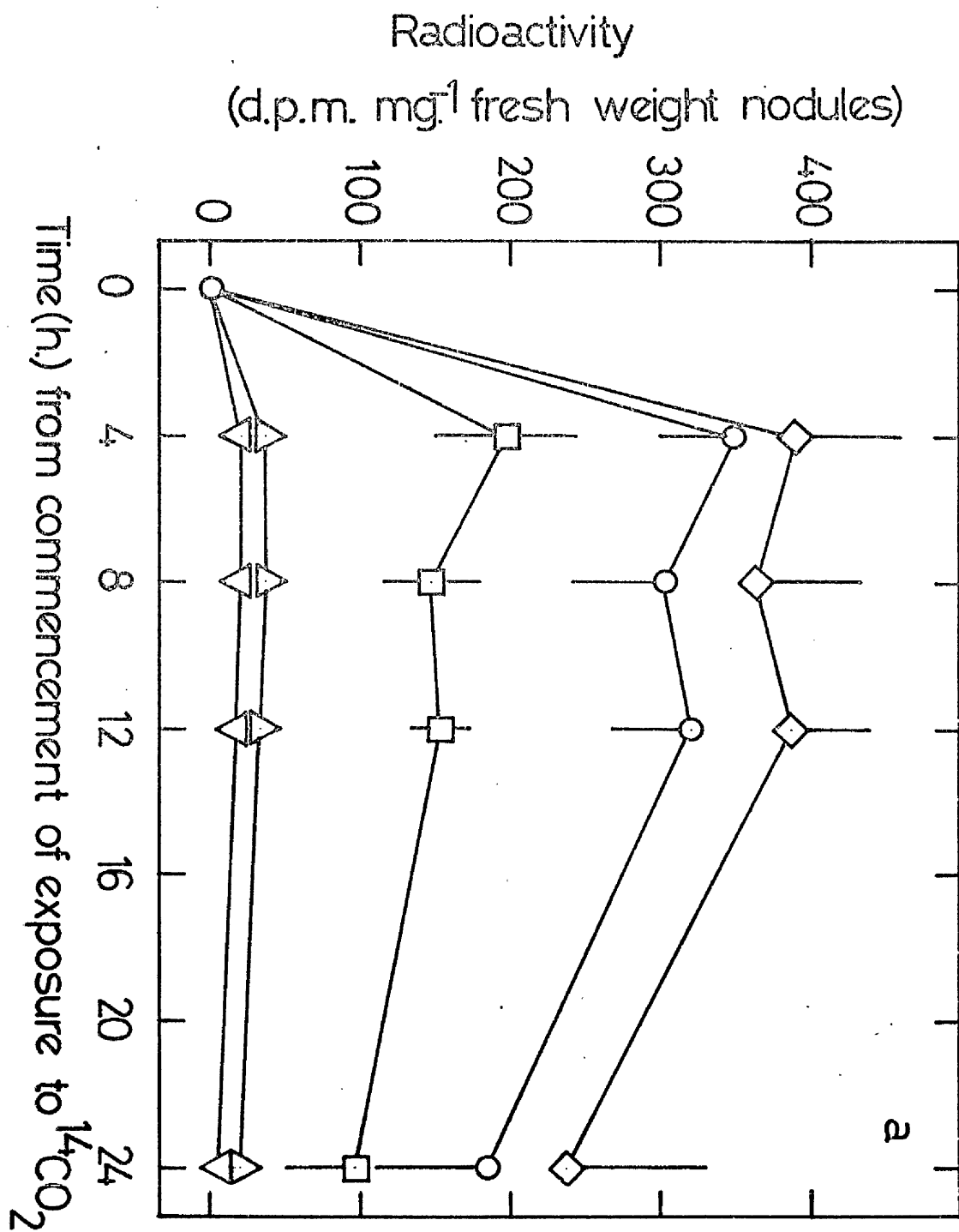
Time (hours from commencement of exposure to $^{14}\text{CO}_2$ )	Mean % radioactivity extracted	t value of difference of means from sample taken at 4 hours
4	90.9	—
8	83.7	5.189**
12	82.4	6.707**
16	79.1	10.794***
20	73.6	12.249***
24	72.5	7.703**

Shoots of 3-week-old pea plants were exposed to  $^{14}\text{CO}_2$  for 30 minutes and the nodules were collected after the "cold-chase" periods shown and extracted in 80% ethanol for 24 hours. The radioactivity in the extract and the residue left in the nodules was assessed separately.

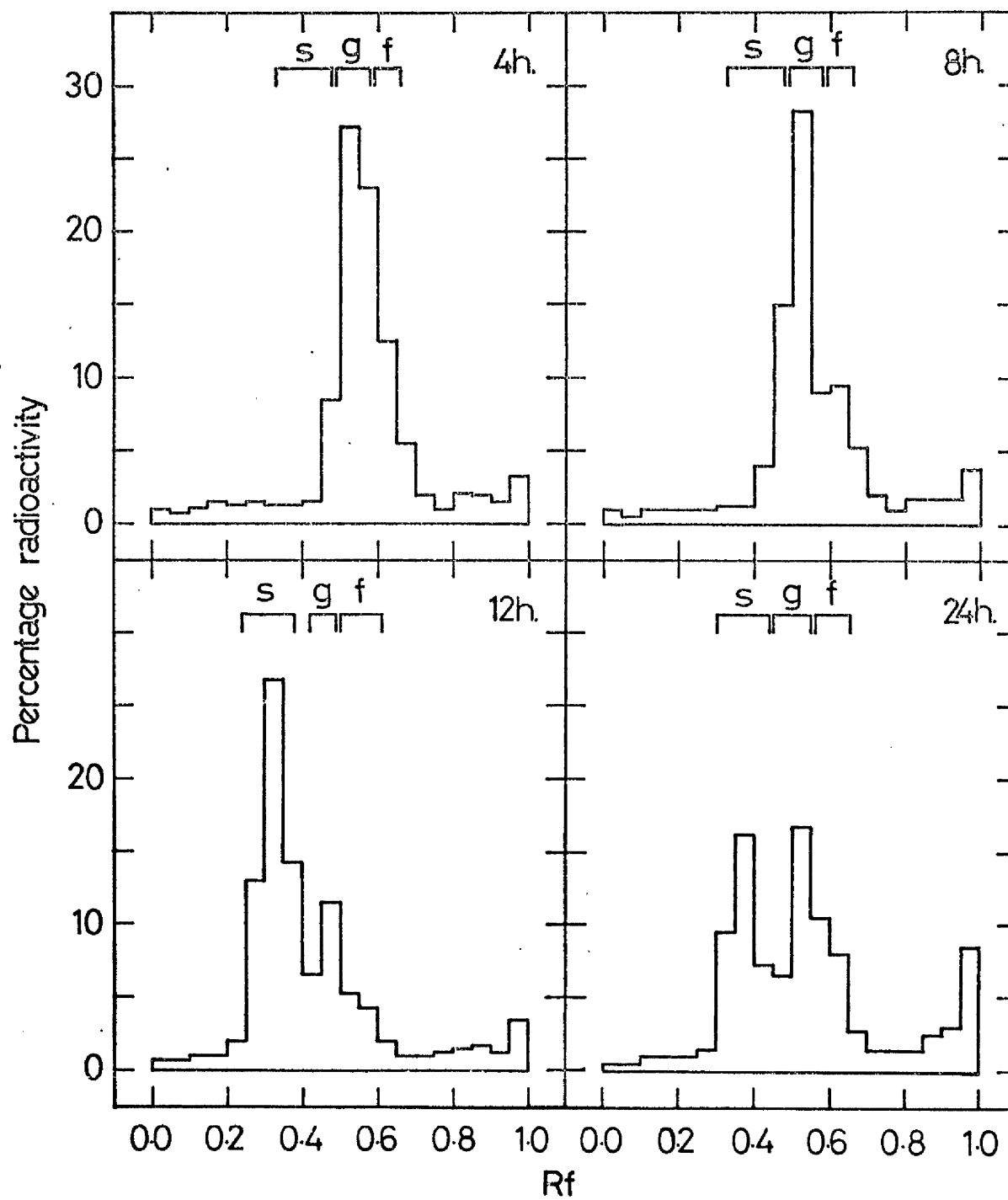
Fig. 29a. Changes in the distribution of radioactivity in ethanolic extracts (○), the neutral (□), basic (△) and acidic (▽) fractions of these extracts and the total radioactivity (◇) of nodules of 3-week-old pea plants following exposure of the shoot to  $^{14}\text{CO}_2$  for 30 minutes. Nodules were collected after "cold-chase" periods of up to 23.5 h.

The t-test was used to test the differences between the mean radioactivity of each fraction after successive increases in the "cold-chase" period.

Time (h) after commencement of exposure	Value of t			
	4	8	12	24
Total radioactivity	0.420 <sup>NS</sup>	-0.419 <sup>NS</sup>		2.335 <sup>NS</sup>
Ethanol-soluble portion	0.978 <sup>NS</sup>	-0.291 <sup>NS</sup>		2.674*
Neutral fraction	0.204 <sup>NS</sup>	0.619 <sup>NS</sup>		1.798 <sup>NS</sup>
Basic fraction	1.900 <sup>NS</sup>	-0.290 <sup>NS</sup>		1.753 <sup>NS</sup>
Acidic fraction	-0.669 <sup>NS</sup>	0.532 <sup>NS</sup>		4.562*







## (7) ANALYSIS OF RADIOACTIVE COMPOUNDS IN ETHANOLIC EXTRACTS OF NODULES

In this experiment, which was performed once only due to the time required to assay samples and chromatograms by scintillation counting, plants were fed  $^{14}\text{CO}_2$  for 30 minutes and nodules detached after various "cold-chase" periods.

The level of ethanol-insoluble activity rose steadily as the "cold-chase" period was lengthened (Table 6), but 72% of the total activity of the nodules was still in ethanol-soluble form 23.5 hours after exposure to  $^{14}\text{CO}_2$ . Initial fractionation of the ethanolic extracts by ion-exchange chromatography at times up to 24 hours after commencement of exposure to  $^{14}\text{CO}_2$  showed that the neutral fraction always contained most of the activity (71% to 77%) and the activity of the basic fraction (15% to 18%) was greater than that of the acidic fraction (6% to 11%) (Fig. 29a). The total radioactivity of the extracts and of the individual fractions showed little change between 4 and 24 hours after exposure to  $^{14}\text{CO}_2$ , except for a significant decline in the radioactivity of the acidic fraction between 12 and 24 hours after commencement of exposure to  $^{14}\text{CO}_2$ .

### a) Neutral fraction

Only the solvents IPrAg, EtAcPy and AcW were used in this analysis. The sugars sucrose, glucose and fructose accounted for more than 90% of the activity detected on paper chromatograms and no other sugars were detected (Fig. 29b). Little activity was associated with sucrose after 3.5 hours "cold-chase", but its activity then rose to 46% of the total activity 11.5 hours after exposure to  $^{14}\text{CO}_2$ . After 23.5 hours, the activity in sucrose and the reducing sugars was approximately equal. Sucrose was the only labelled sugar detected in the epicotyl a short time after feeding.

### b) Basic fraction

Several radioactive spots were seen on paper chromatograms, and

Fig. 29c. Changes in the distribution of radioactivity in basic fractions of ethanolic extracts of pea nodules from 3-week-old plants collected 4, 8, 12 and 24 hours after commencement of exposure of the shoots to  $^{14}\text{CO}_2$  for 30 minutes. Extracts were chromatographed in solvent PhA and the Rf values of aspartic acid (a), glutamic acid (g), asparagine (an), glutamine (gn), homoserine (h), valine (v) and isoleucine/leucine (i/l) in control spots run simultaneously are shown.

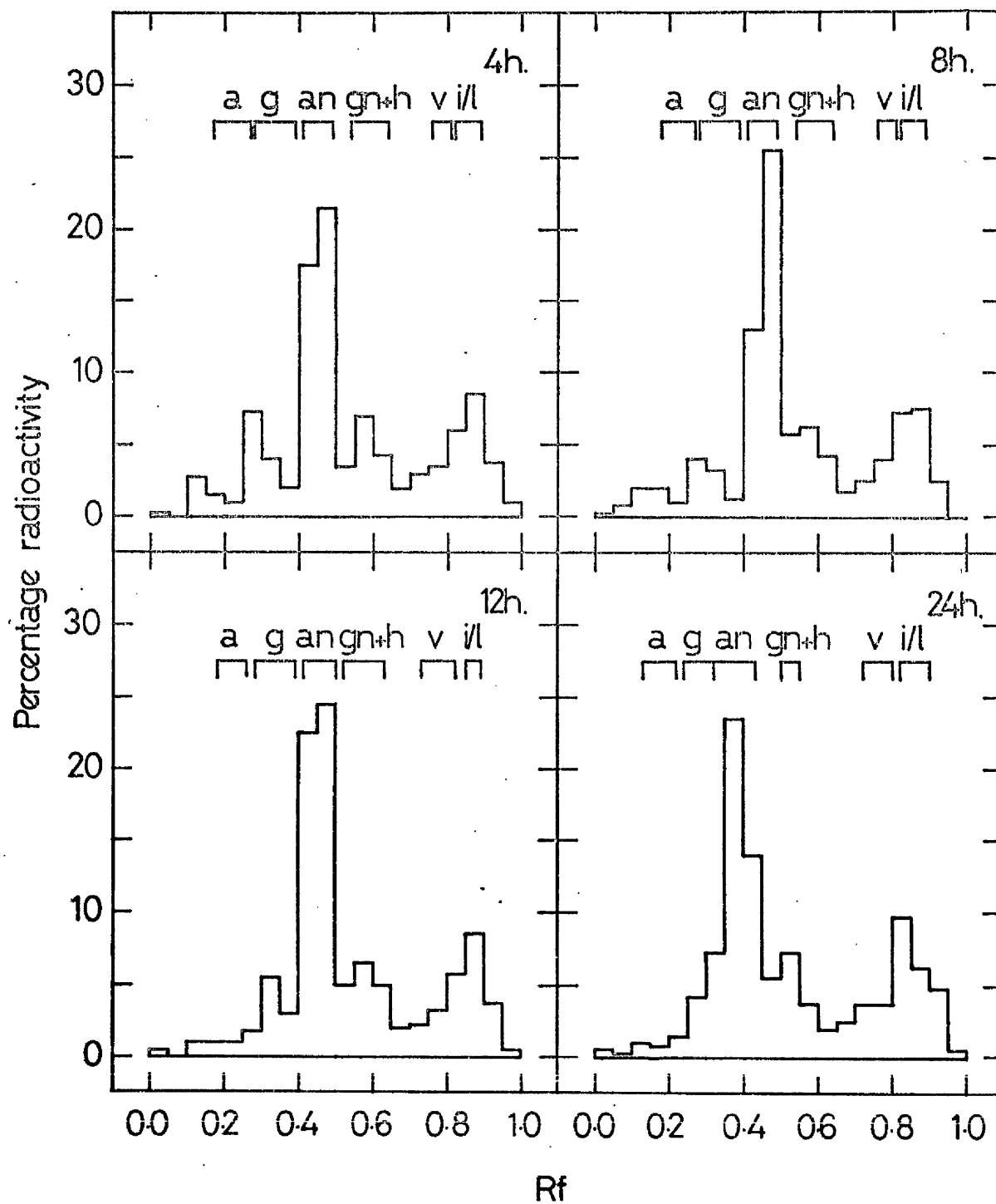
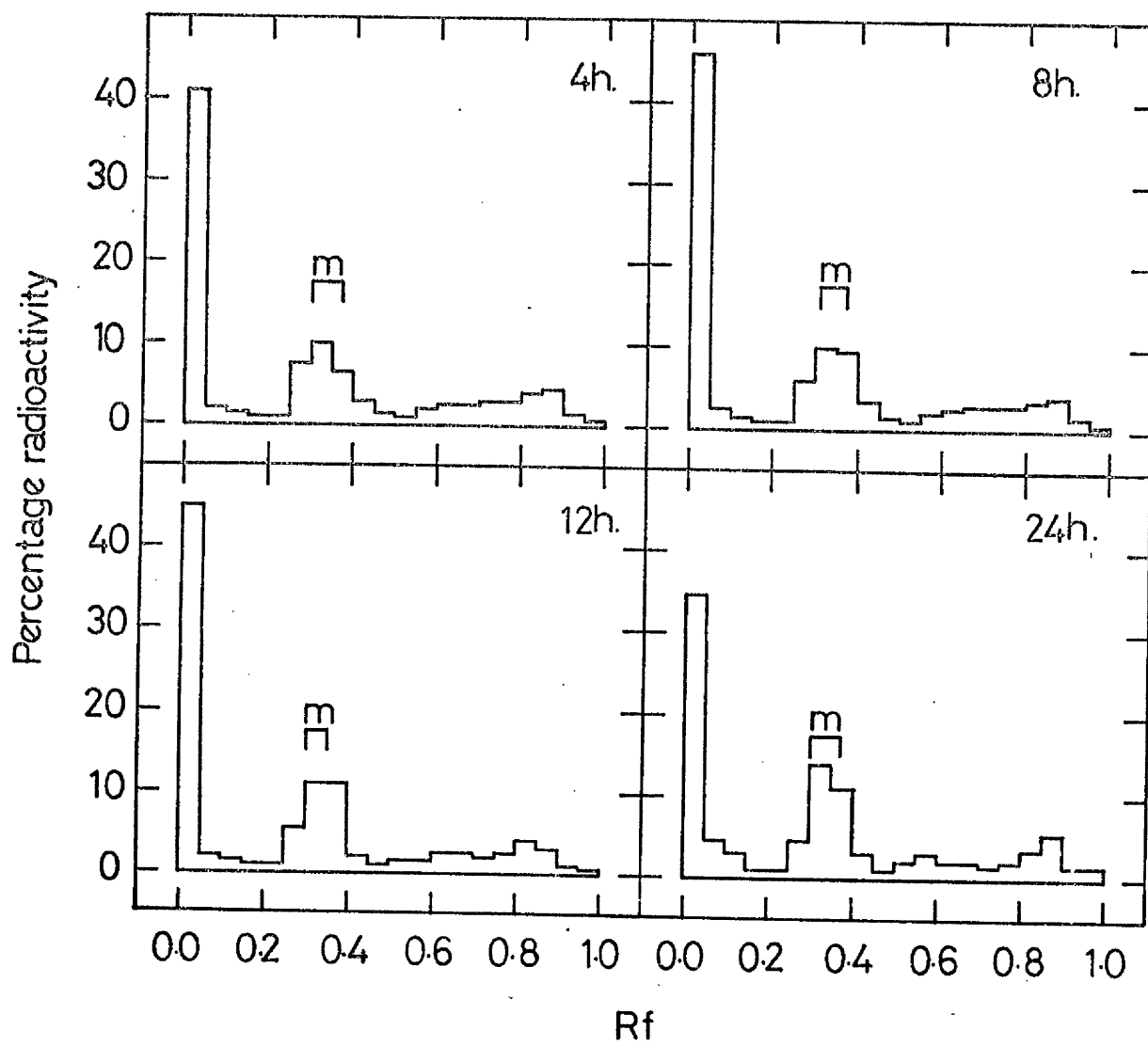


Fig. 29d. Changes in the distribution of radioactivity in acidic fractions of ethanolic extracts of pea nodules from 3-week-old plants collected 4, 8, 12 and 24 hours after commencement of exposure of the shoot to  $^{14}\text{CO}_2$  for 30 minutes. Extracts were chromatographed in solvent PrF and the Rf value of malic acid (m) in control spots run simultaneously is also shown.



asparagine was by far the most prominent spot, with 40% to 50% of the activity at all times (Fig. 29c). Radioactivity was also associated approximately equally with glutamine, glutamic acid, homoserine and leucine/isoleucine spots. Negligible radioactivity was associated with an unidentified spot on the chromatograms and no contaminating sugars were detected.

c) Acidic fraction

Only two major radioactive spots were present on the paper chromatograms (Fig. 29d). The compounds causing the large accumulation of activity at the origin in PrF were identified as contaminating sugars by their positive reaction with aniline-diphenylamine and aniline reagents, and eluted spots were similarly identified after chromatography in the solvent systems of Section A 7a. Attempts to remove this contamination by increasing the volume of ethanol used to wash the column prior to elution were unsuccessful, and the contamination accounted for approximately 45% of the radioactivity of this fraction.

The second radioactive peak comprised about 25% of the total activity and was identified as malic acid. Radioactivity associated with other organic acids was not detected. As much as 25% of the total activity was located at high Rf values, but the locating reagents used did not react in this region and no definite peak was obtained on GLC analysis. This radioactivity was probably due to low molecular weight compounds whose origin is unknown.

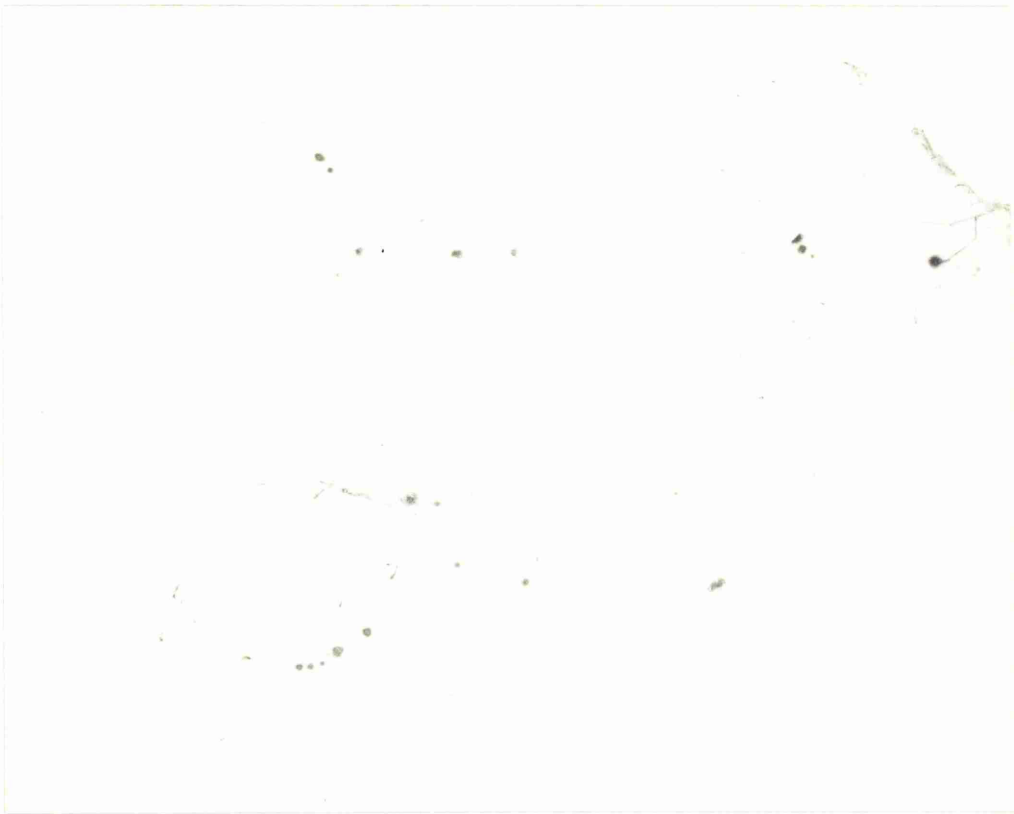
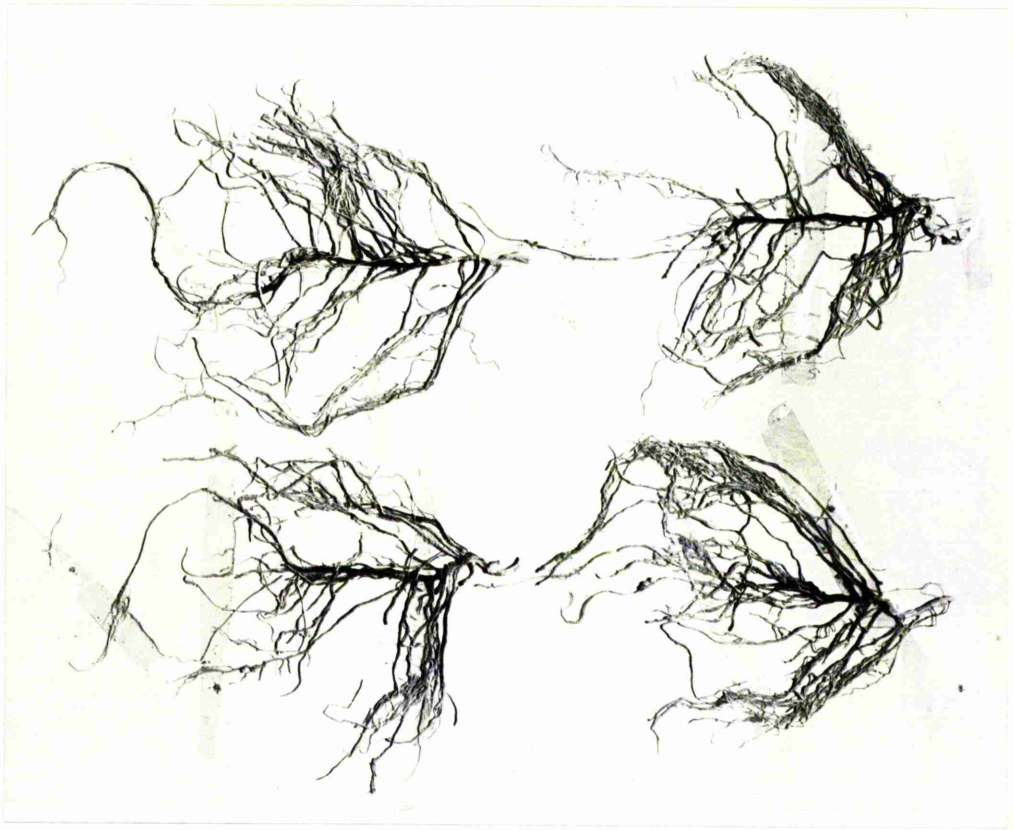
Plate 2a. Autoradiograph of 3-week-old nodulated pea roots collected 2 hours after commencement of exposure of the shoots to  $^{14}\text{CO}_4$  for 30 minutes. Some nodules are already heavily labelled.





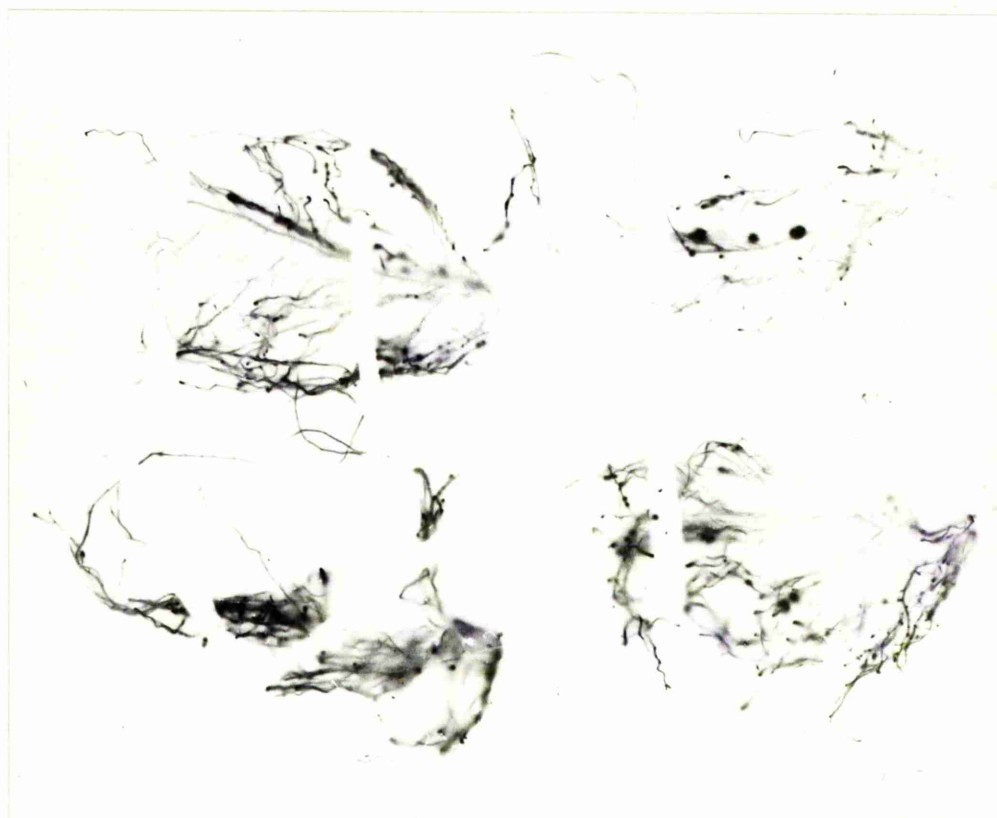
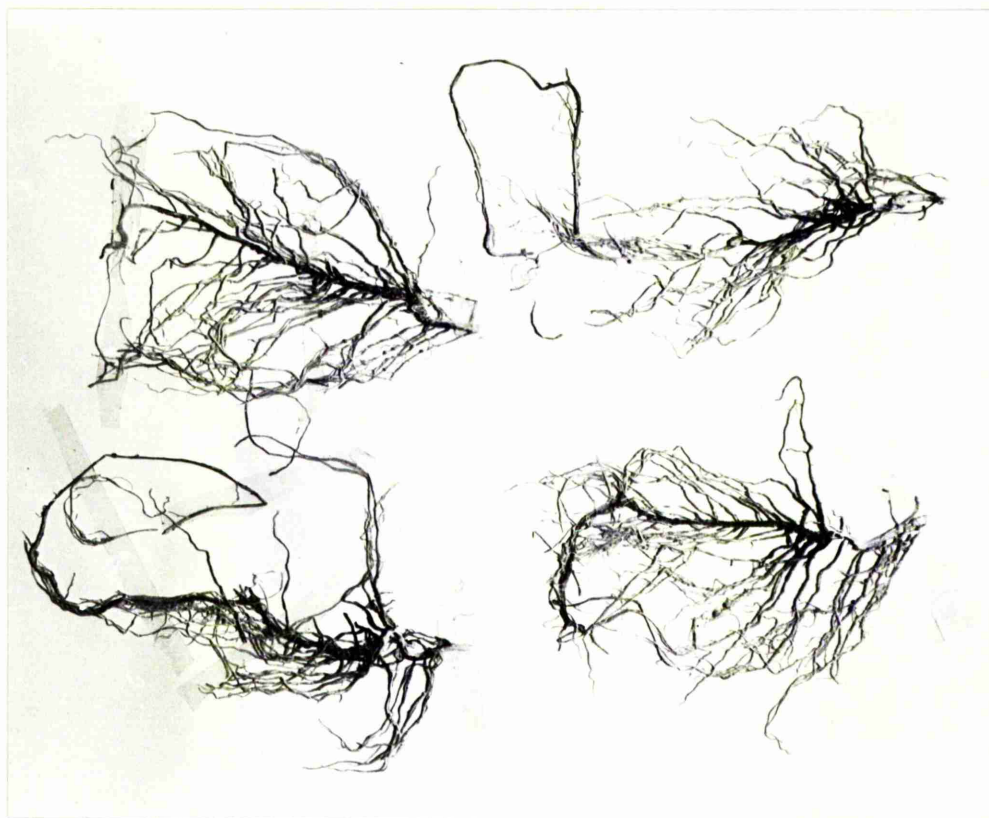
————— 100mms.

Plate 2b. Autoradiograph of 3-week-old nodulated pea roots collected 4 hours after commencement of exposure of the shoots to  $^{14}\text{CO}_2$  for 30 minutes. All nodules and some root tips are heavily labelled.



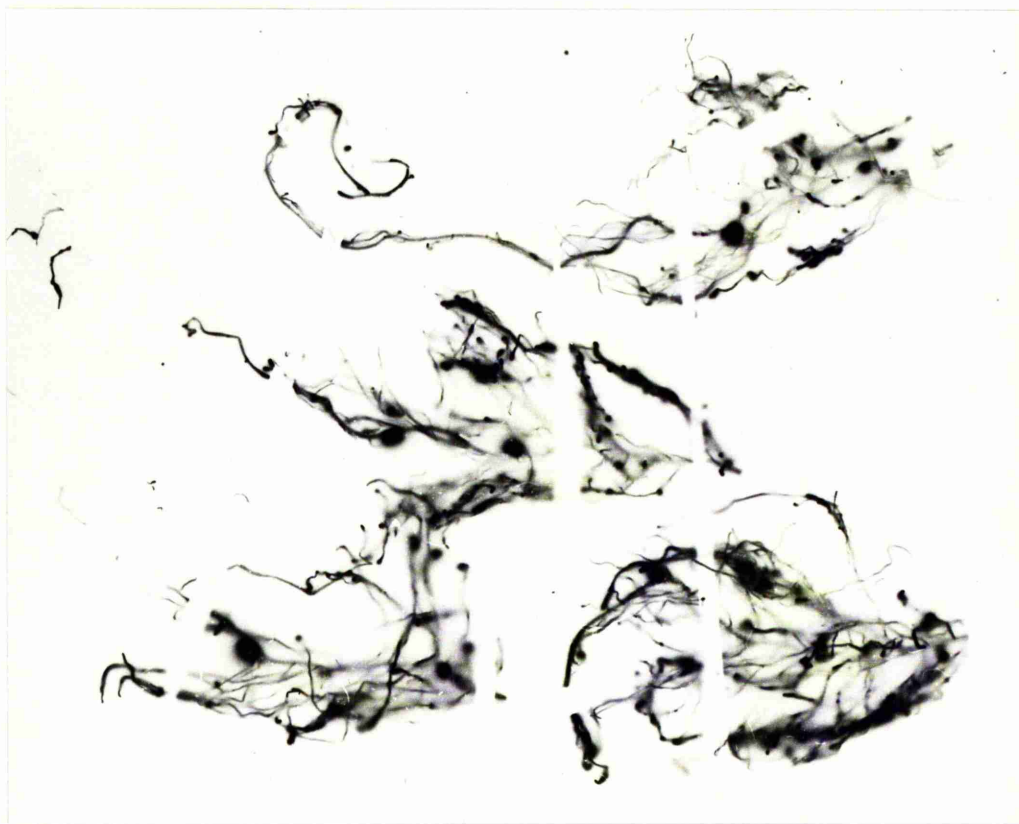
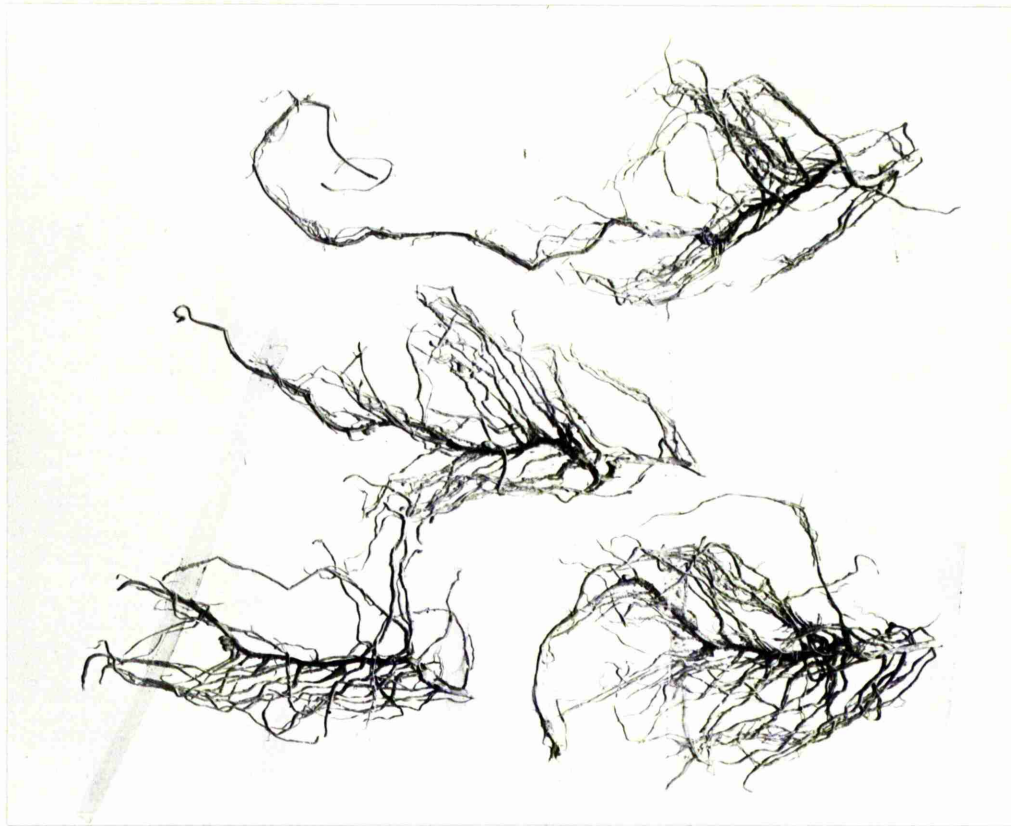
————— 100mms.

Plate 2c. Autoradiograph of 3-week-old nodulated pea roots collected 6 hours after commencement of exposure of the shoots to  $^{14}\text{CO}_2$  for 30 minutes. The roots are moderately labelled and the nodules are heavily labelled.



— 100 mms.

Plate 2d. Autoradiograph of 3-week-old nodulated pea roots collected 16 hours after commencement of exposure of the shoots to  $^{14}\text{CO}_2$  for 30 minutes. Both roots and nodules are heavily labelled.



————— 100mms.

Plate 3. Microautoradiograph of a longitudinal section of a nodule from a 3-week-old pea plant attached to the root. The nodule was collected and sectioned 6 hours after exposure of the shoot to  $^{15}\text{CO}_2$  for 15 minutes. Accumulation of  $^{14}\text{C}$ -labelled photosynthates (indicated by the white dots) is particularly evident in the infected cells (i) and the vascular tissue (v) of the nodule and root.



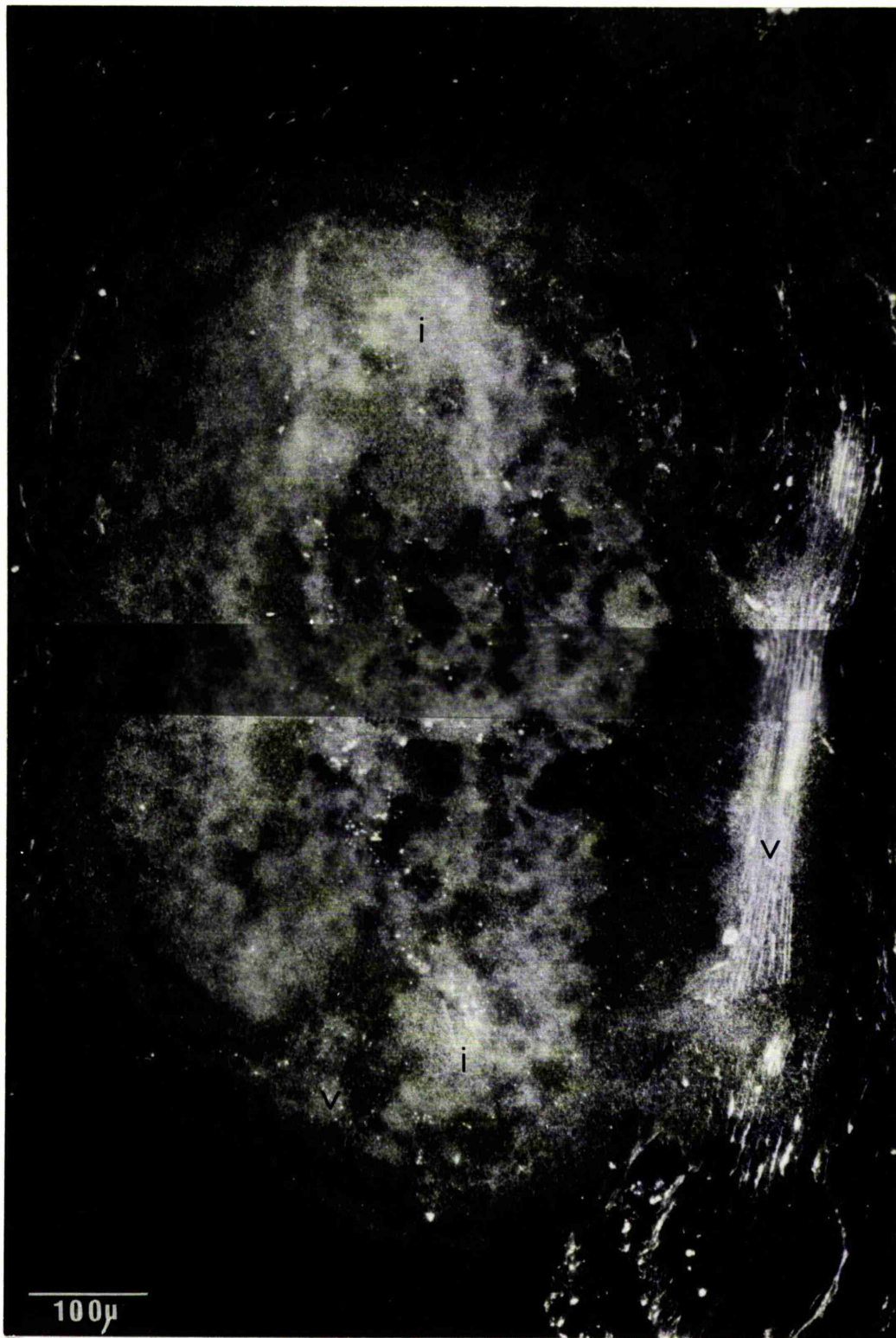
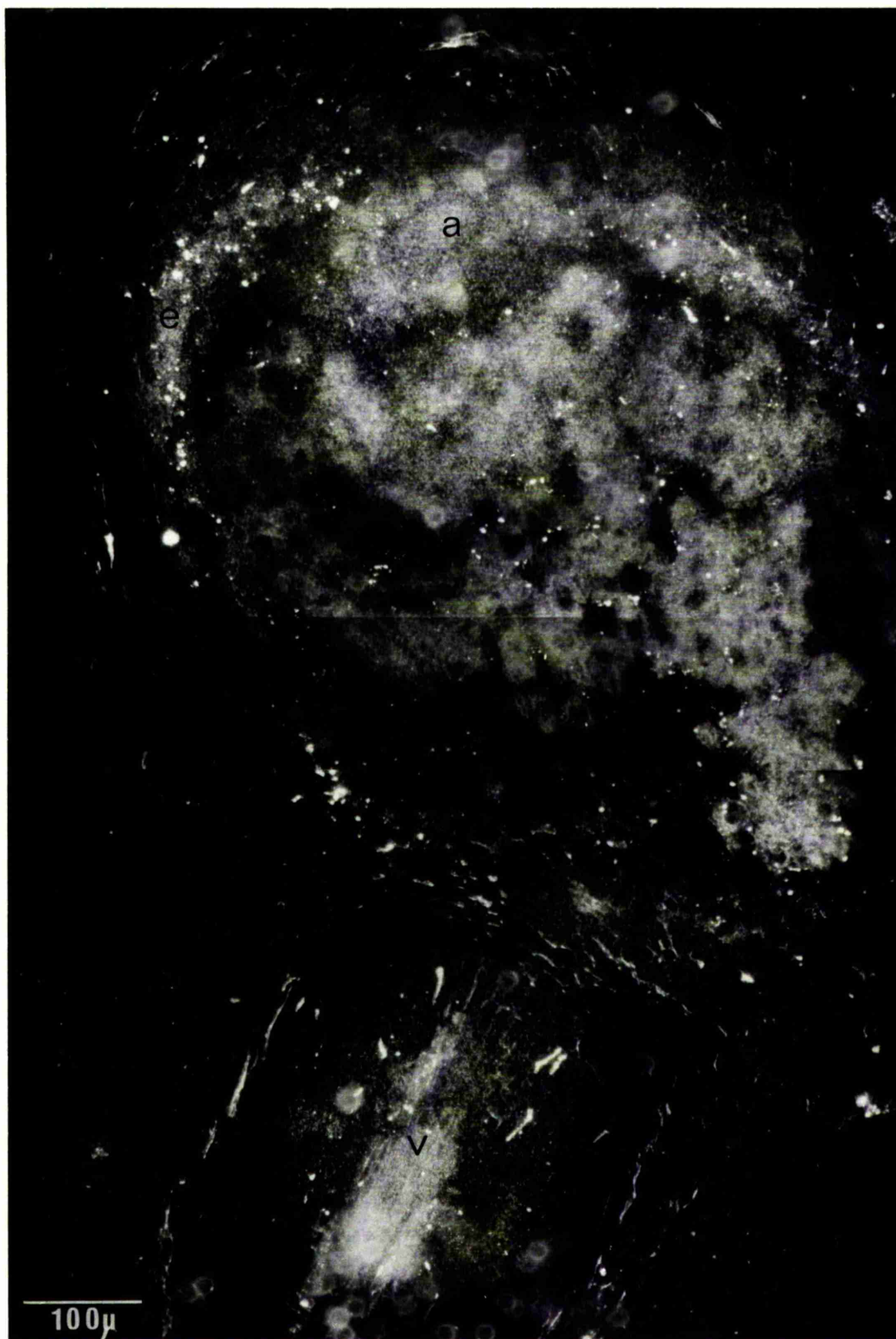


Plate 4. Microautoradiograph of a transverse section of a pea nodule from a 3-week-old plant attached to the root, which is seen in longitudinal section at the base of the photograph. The nodule was collected and sectioned 6 hours after exposure of the shoot to  $^{14}\text{CO}_2$  for 15 minutes. Accumulation of  $^{14}\text{C}$ -labelled photosynthates (indicated by the white dots) is particularly evident in the sub-apical infected cells (a) the common endodermis, (e) and the vascular tissue (v).



## (8) AUTORADIOGRAPHY

### a) Whole plants

Autoradiographs of the roots of 3-week-old plants are shown 2, 4, 6 and 16 hours after commencement of exposure of the shoots to  $^{14}\text{CO}_2$  for 30 minutes (Plate 2 a to d). Samples were also taken immediately after exposure and 1, 8, 12 and 24 hours after commencement of exposure.

There was insufficient activity in the roots and nodules after 0 and 1 hours to register on the X-ray film, but after 2 hours the nodules were visibly labelled, whereas the roots were seen only faintly (Plate 2a). Nodules on the tap root were labelled more quickly than those on lateral roots, but after 4 hours all the nodules and root tips were moderately labelled (Plate 2b). After 6 hours, all the nodules and root tips were heavily labelled, the roots were visibly labelled (Plate 2c), and the degree of labelling increased as the "cold-chase" period was lengthened (Plate 2d).

These results confirm those previously obtained by scintillation counting (Section A 1d), in that the time taken for substantial accumulation of labelled photosynthates in the nodules was 4 to 8 hours from exposure of the shoot to  $^{14}\text{CO}_2$ .

### b) Microautoradiography

#### (i) Plants maintained in the light

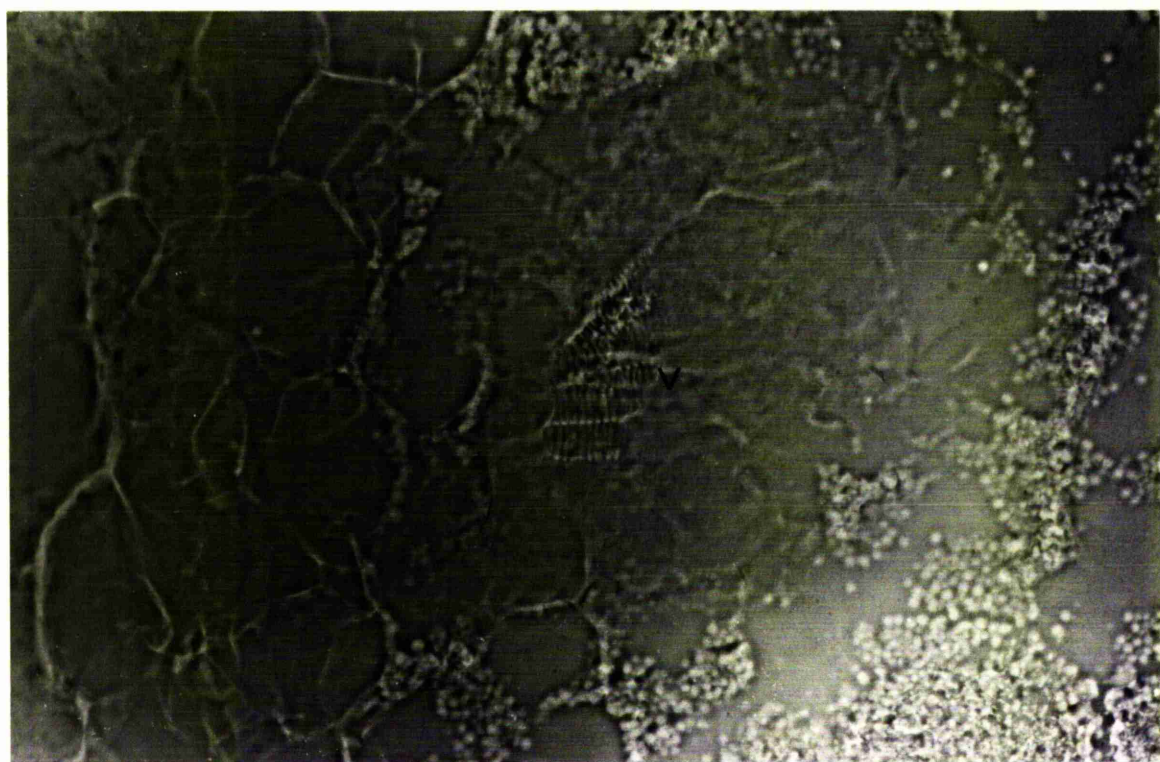
Nodules of 3- and 5-week-old plants fed  $^{14}\text{CO}_2$  for 30 minutes were collected and sectioned either immediately or at intervals up to 24 hours after exposure. No labelling was detected when the nodules were collected immediately, but 3-week-old nodules showed marked accumulation of labelled material after 6 hours "cold-chase".

The main site of accumulation was the infected cells, with little labelling over the outer uninfected cortex (Plates 3 and 4). Accumulation was also obvious over two other regions of the nodules:

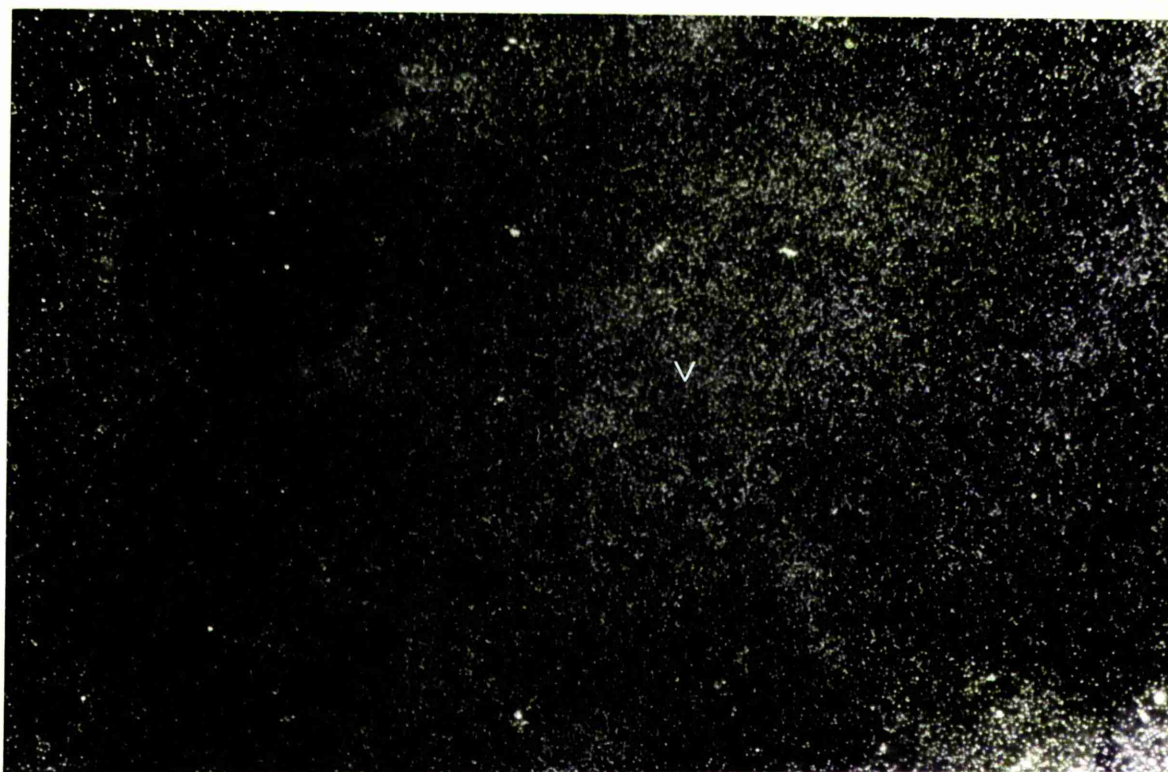
Plate 5. The vacular tissue of a nodule from a 3-week-old pea plant as seen by (a) phase contrast and (b) dark field microscopy. The accumulation of radioactivity in the vascular trace can be seen clearly 6 hours after exposure of the shoot to  $^{14}\text{CO}_2$  for 15 minutes.



a



b

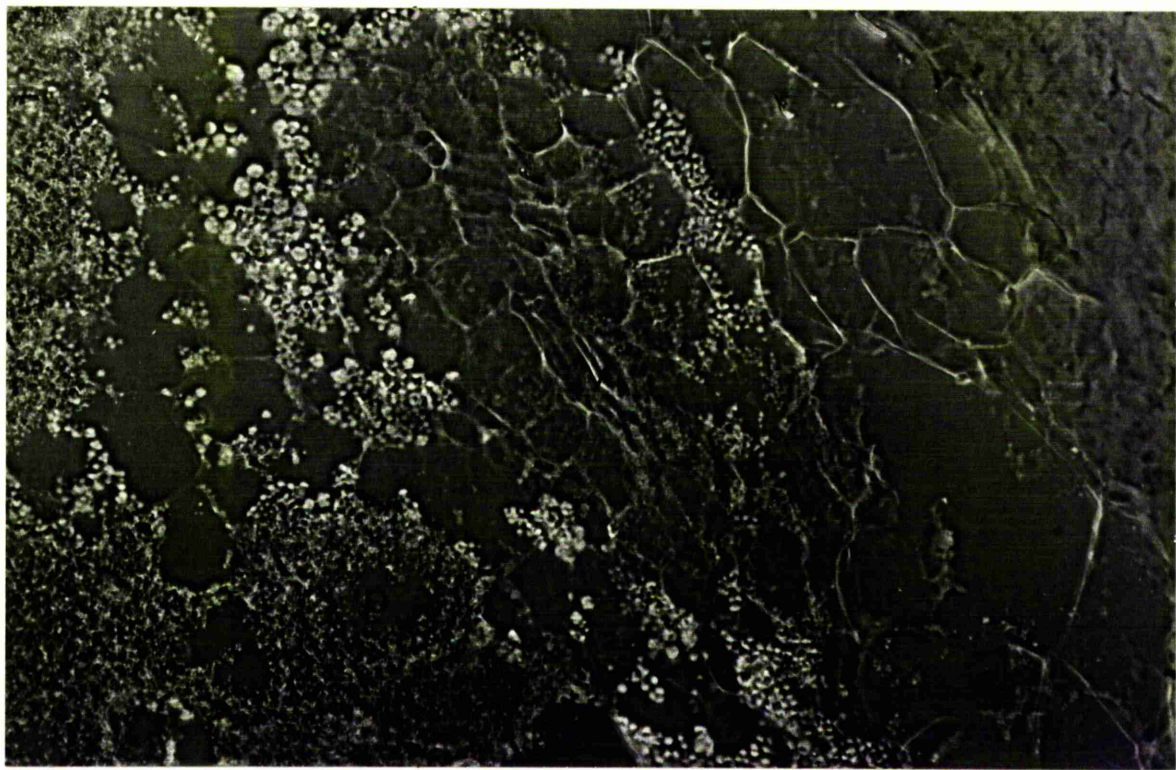


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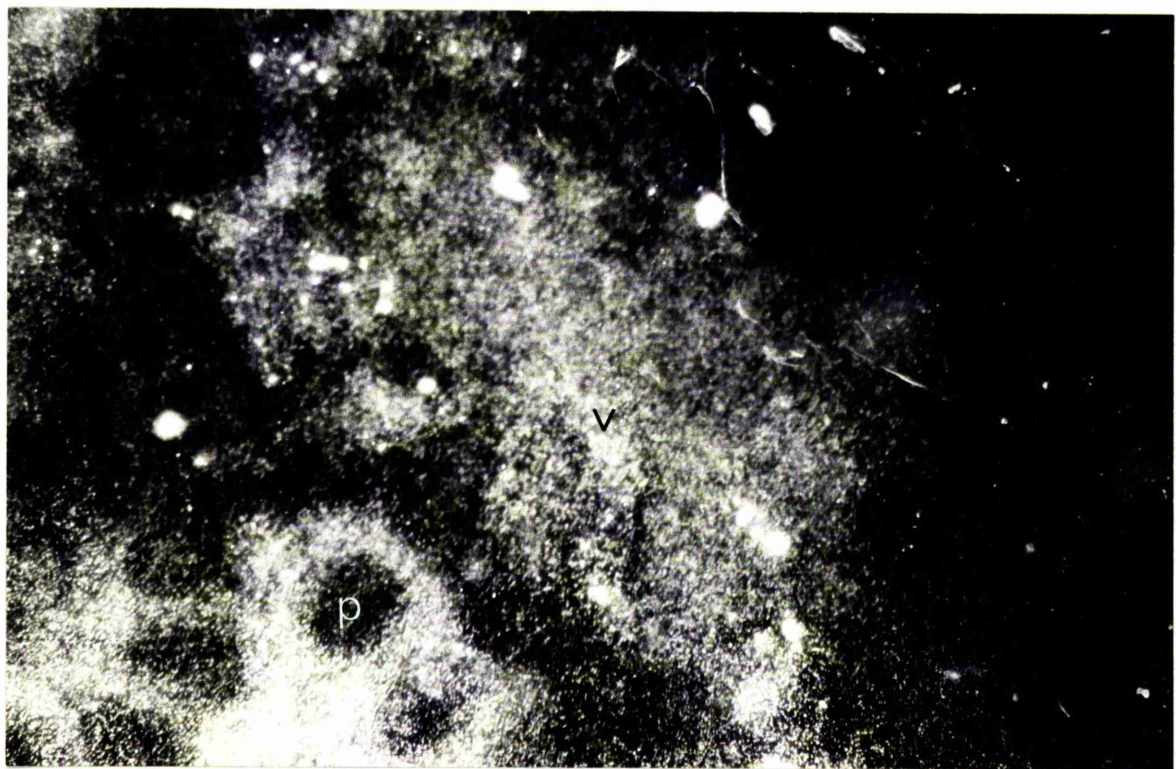
Plate 6. The outer layers of a pea nodule from a 3-week-old plant seen by (a) phase contrast and (b) dark field microscopy. Radioactivity has accumulated in the vascular trace (v) and partially infected cells (p) 6 hours after exposure of the shoot to  $^{14}\text{CO}_2$  for 15 minutes.



a



b

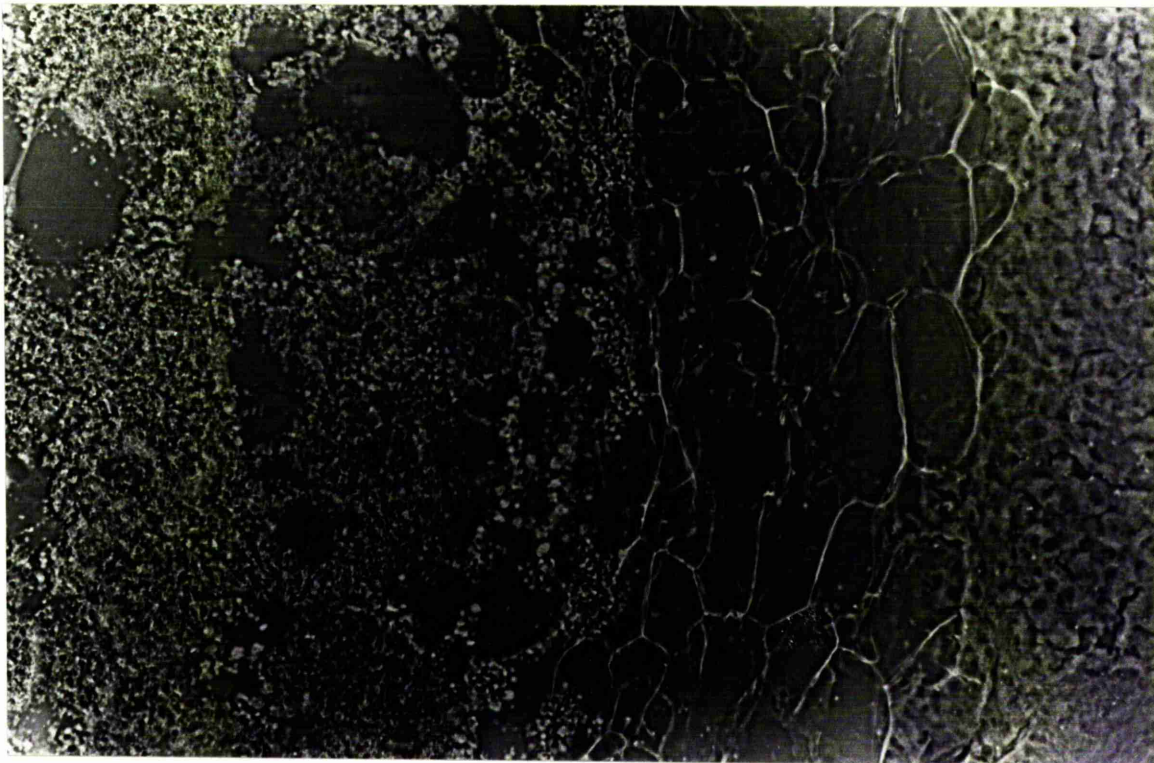


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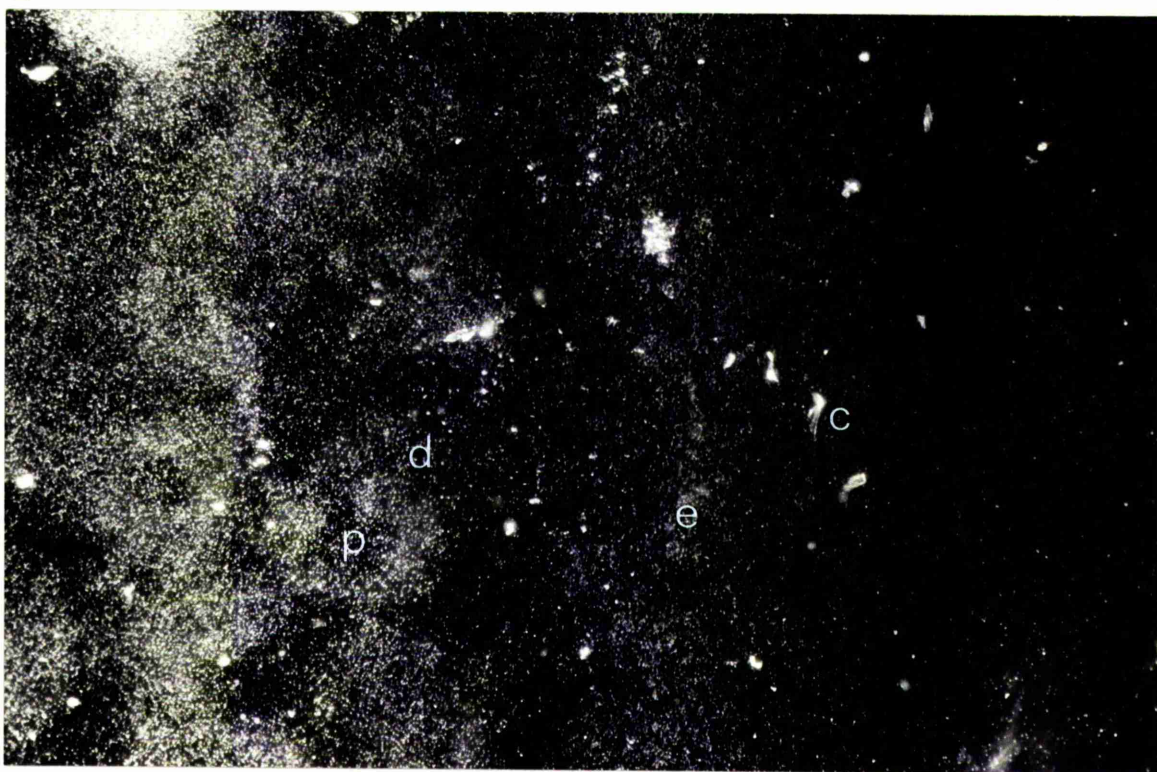


Plate 7. The outer layers of a nodule from a 3-week-old pea plant as seen by (a) phase contrast and (b) dark field microscopy. Radioactivity has accumulated in the common endodermis (e) and cells partially filled with bacteroids (p). There is little accumulation of radioactivity in the uninfected cortex (c) or cells densely packed with bacteroids (d). This nodule was collected and sectioned 6 hours after exposure of the shoot to  $^{14}\text{CO}_2$  for 15 minutes.

a



b

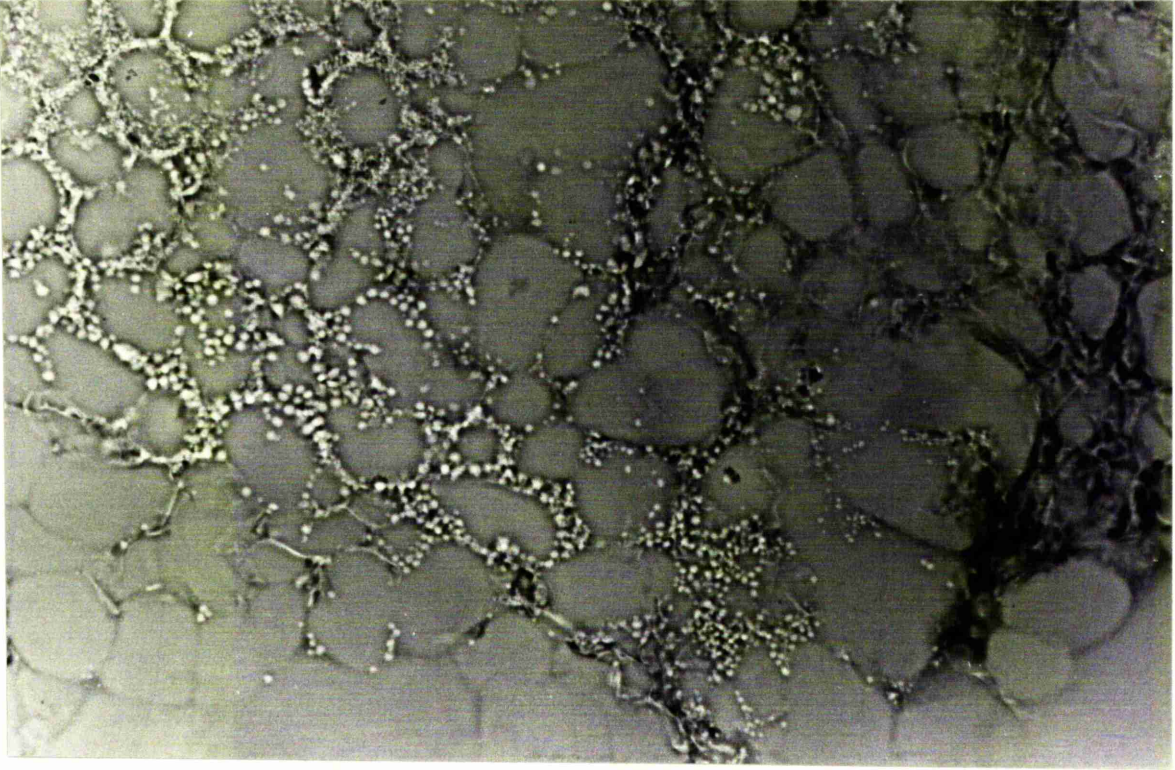


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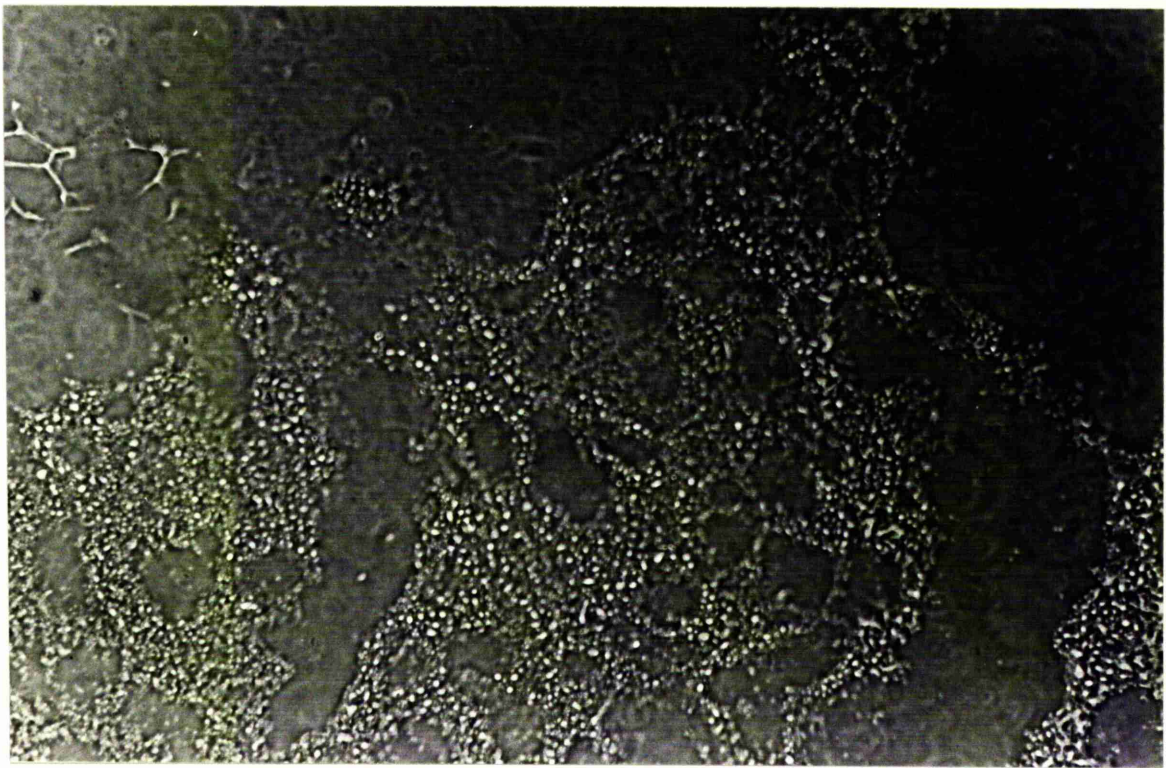
Plate 8. The increase in the number of bacteroids in each infected cell from apex to base of pea nodules of 3-week-old plants, as seen by phase contrast microscopy. There are few bacteria in sub-apical infected cells (a) but the number increases towards the centre of the nodules (b) and the basal infected cells are totally filled with bacteria (c).



a

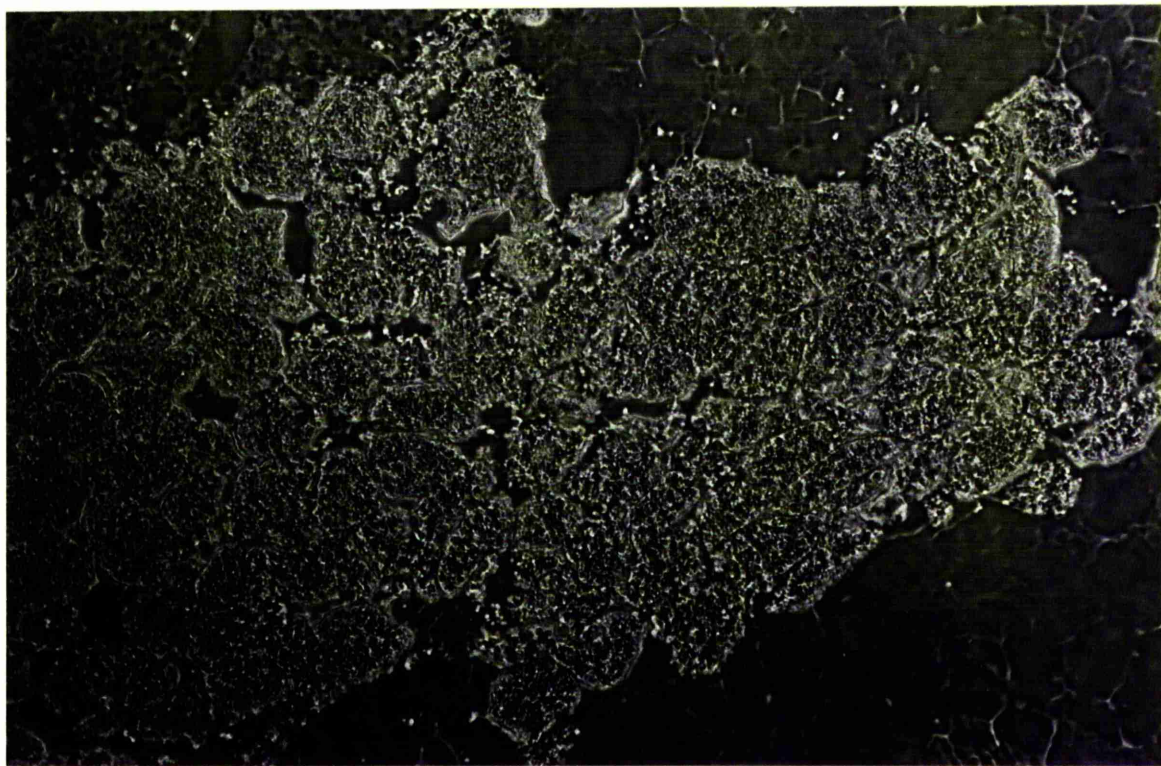


b



— 40 $\mu$

C

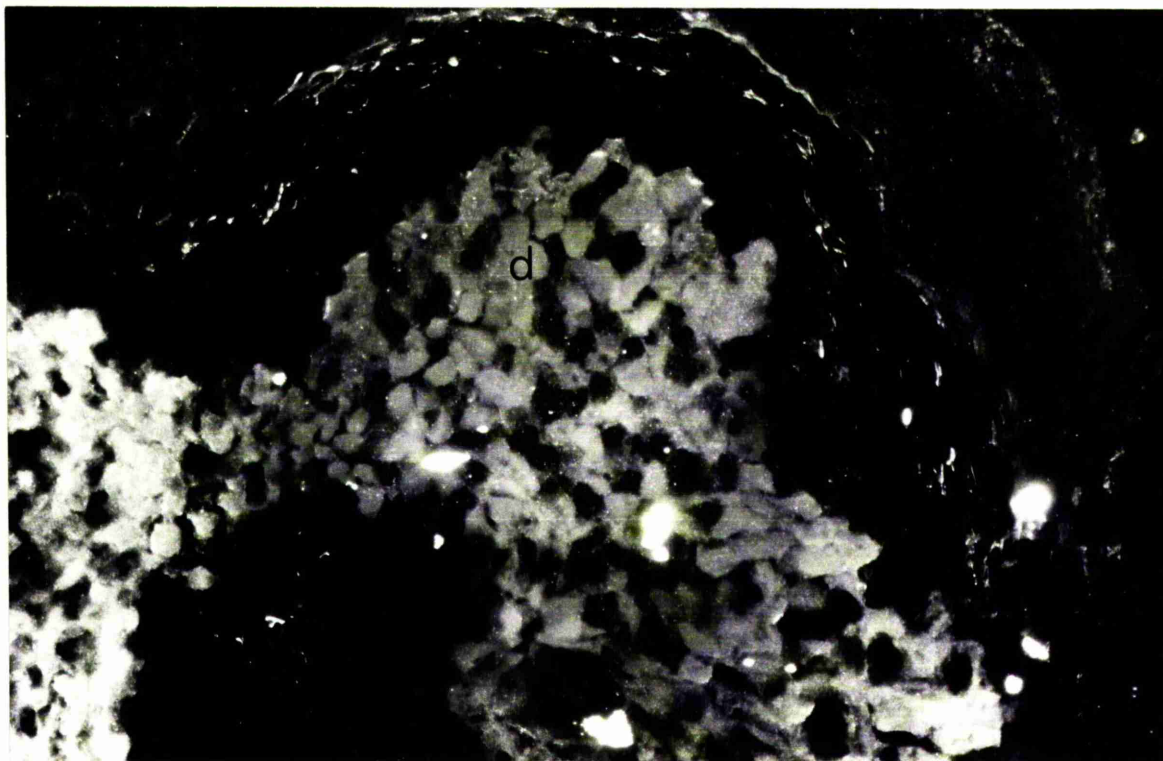


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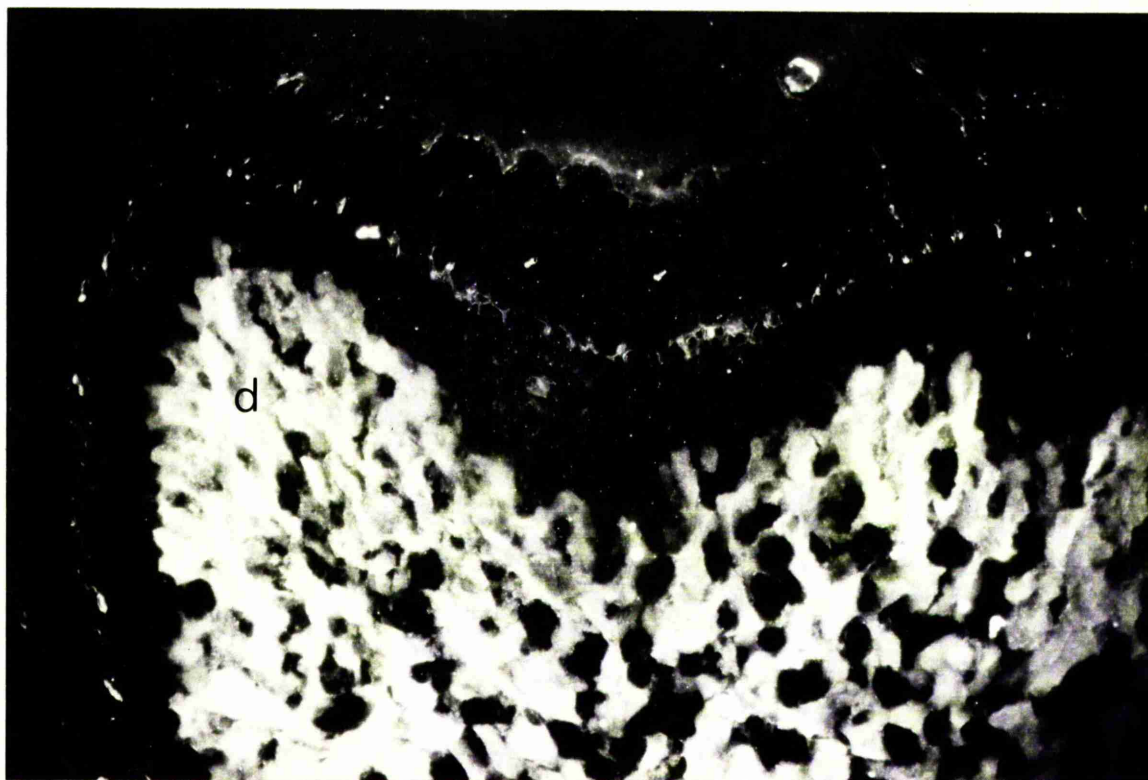
Plate 9. Microautoradiographs of sections of nodules from 5-week-old pea plants collected 6 hours after commencement of exposure of the shoots to  $^{14}\text{CO}_2$  for 30 minutes. There is no appreciable accumulation of radioactivity in these infected cells, which are densely filled with bacteroids (d).



a



b



— 100 $\mu$

a) the vascular traces (Plates 5 and 6) and b) the common endodermis between the infected tissue and the outer cortex (Plate 7).

Accumulation of labelled material was not uniform throughout the infected tissue, and there was relatively more accumulation towards the meristematic tip (Plate 4). Examination of a large number of nodule sections suggested that the number of bacteroids in each infected cell increased gradually as the cells aged until they were totally filled with bacteroids. At first the bacteroids appeared singly in the cells (Plate 8a), increased until two or three lining layers were present inside the cells (Plate 8b), and continued to increase until the infected cells were totally filled with bacteroids (Plate 8c). The inactive nodules of plants 5 weeks old consisted entirely of cells totally filled with bacteroids, and no longer accumulated labelled photosynthates, even after 6 hours "cold-chase" (Plate 9). Ageing occurred from the base to the tip of the nodules, and there was no appreciable accumulation of activity in basal cells, which were filled with bacteroids, either in nodules from 3-week-old (Plate 7) or 5-week-old plants (Plate 9). Cells which were only partially filled with bacteroids and in which the bacteroids were still increasing in number showed appreciable accumulation of activity over the bacteroids, and these cells were the primary "sink" for accumulation of photosynthates inside the nodules (Plates 6 and 7).

#### (ii) Darkened plants

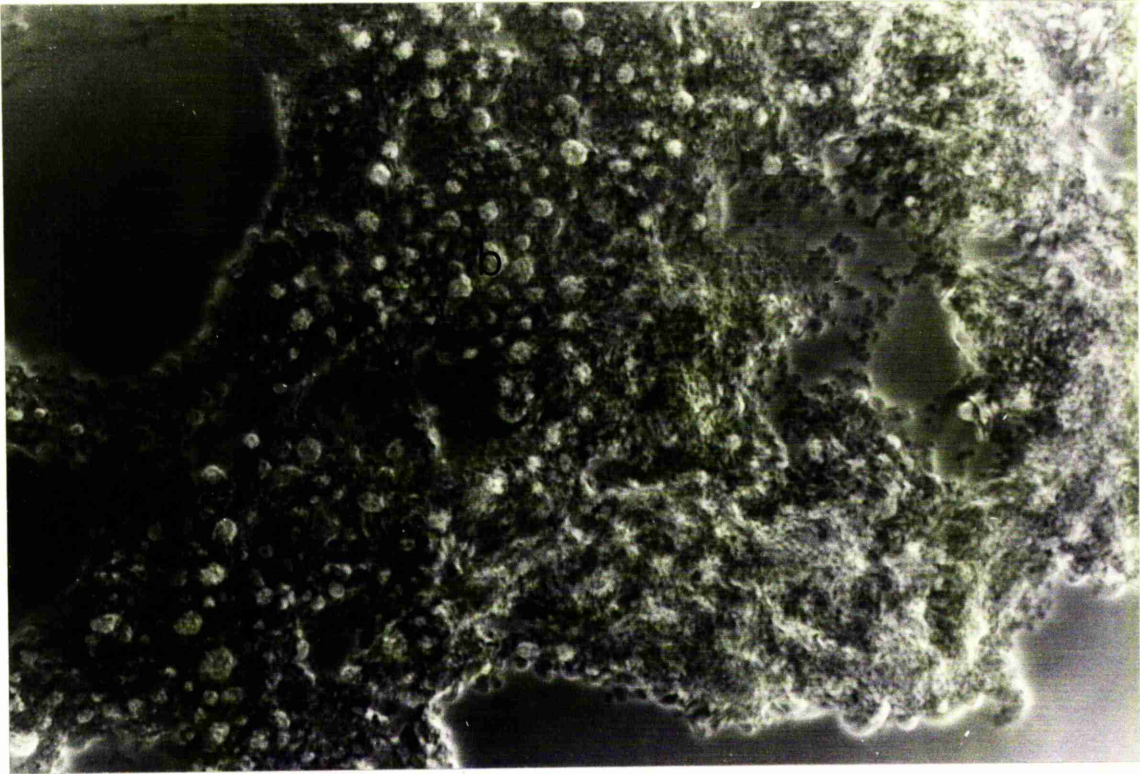
Microautoradiographs of sections of nodules harvested at intervals from plants fed  $^{14}\text{CO}_2$  for 30 minutes and then kept in the dark for periods up to 96 hours showed no difference in the pattern of accumulation of labelled material. However, there was a tendency for regions of accumulation to become less clearly defined as the darkening period was increased.

After 72 hours in the dark, the appearance of the nodule sections changed considerably due to the appearance of numerous large bodies



Plate 10. Infected cells of a nodule from a 3-week-old pea plant, collected 72 hours after the plant shoot was darkened immediately following exposure to  $^{14}\text{CO}_2$  for 30 minutes, as seen by (a) phase contrast and (b) dark field microscopy. The large bodies (b) which appear in nodules of darkened plants do not accumulate large quantities of radioactivity and are approximately the same size as starch grains(s)(Plate 10c).

a

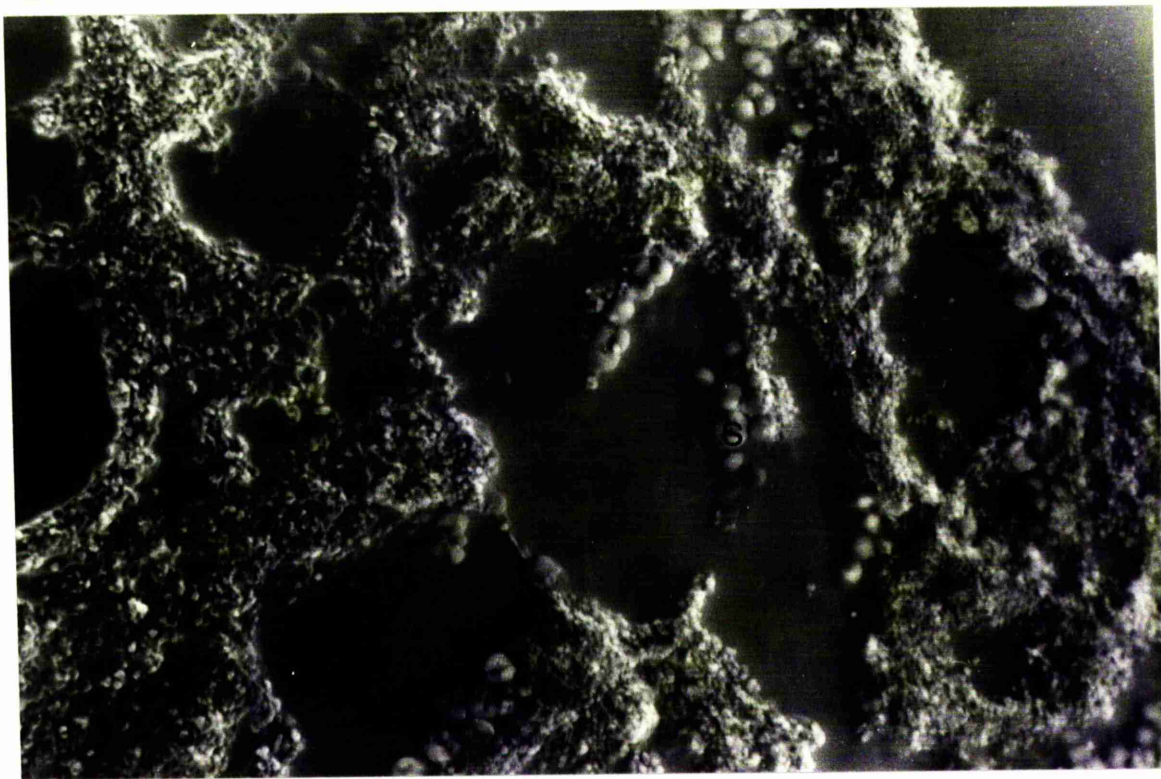


b



— 20 $\mu$

C

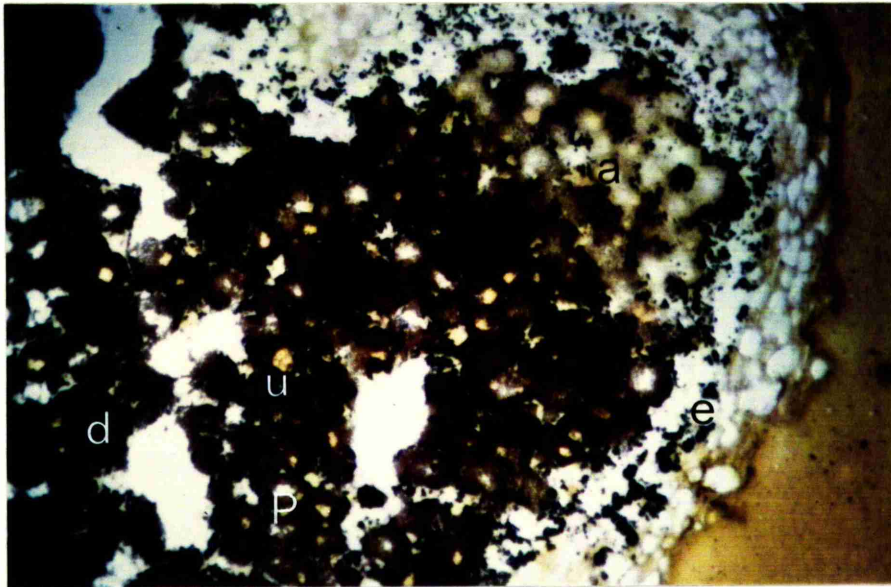


— 20μ

Plate 11 (top). Distribution of starch grains as seen by light field microscopy in section of a pea nodule from a 3-week-old plant stained with iodine. Starch grains are few in sub-apical newly infected cells (a), appear as parietal layers in cells partially filled with bacteroids (p) and are numerous in basal infected cells (d), uninfected cells (u) in the infected area and in cells near the common endodermis (e).

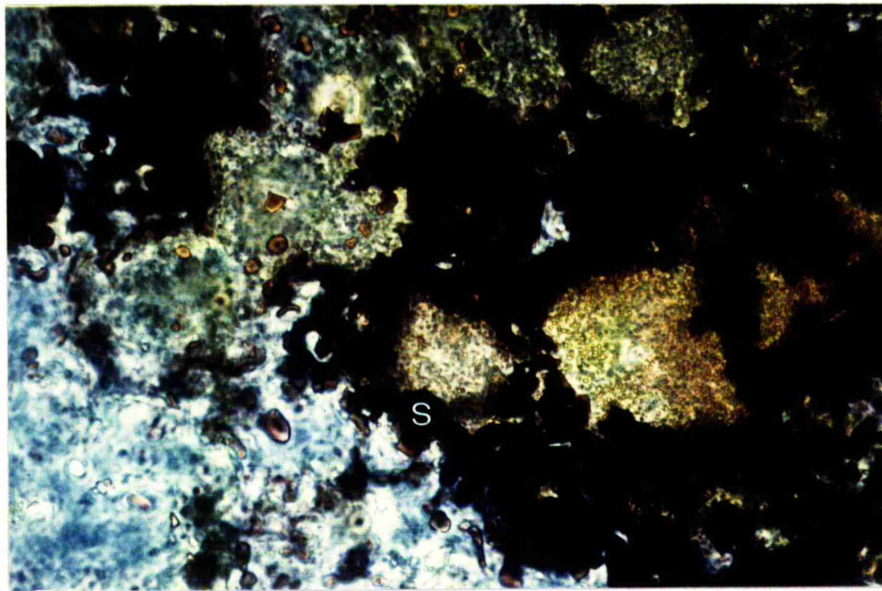
Plate 12 (bottom). Parietal distribution of starch grains (s) in cells partially filled with bacteroids, as seen by phase contrast microscopy of sections of nodules from 3-week-old pea plants stained with iodine.

11



— 100 $\mu$

12

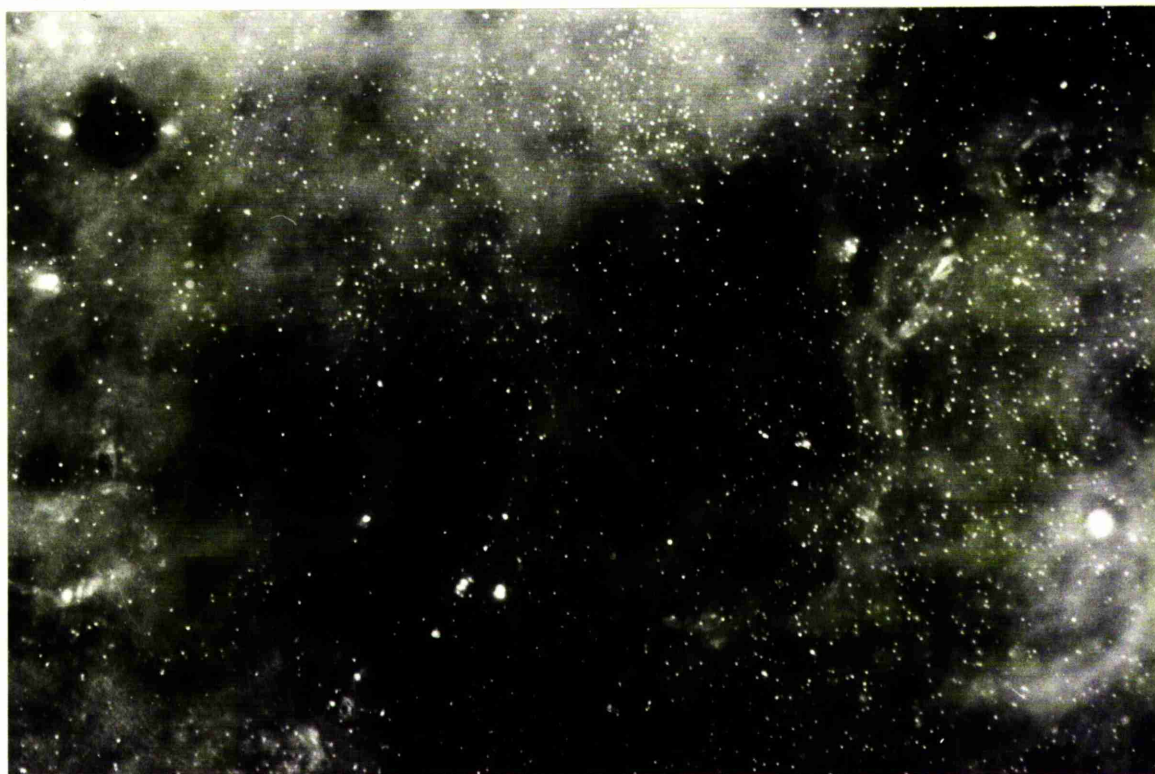


— 20 $\mu$

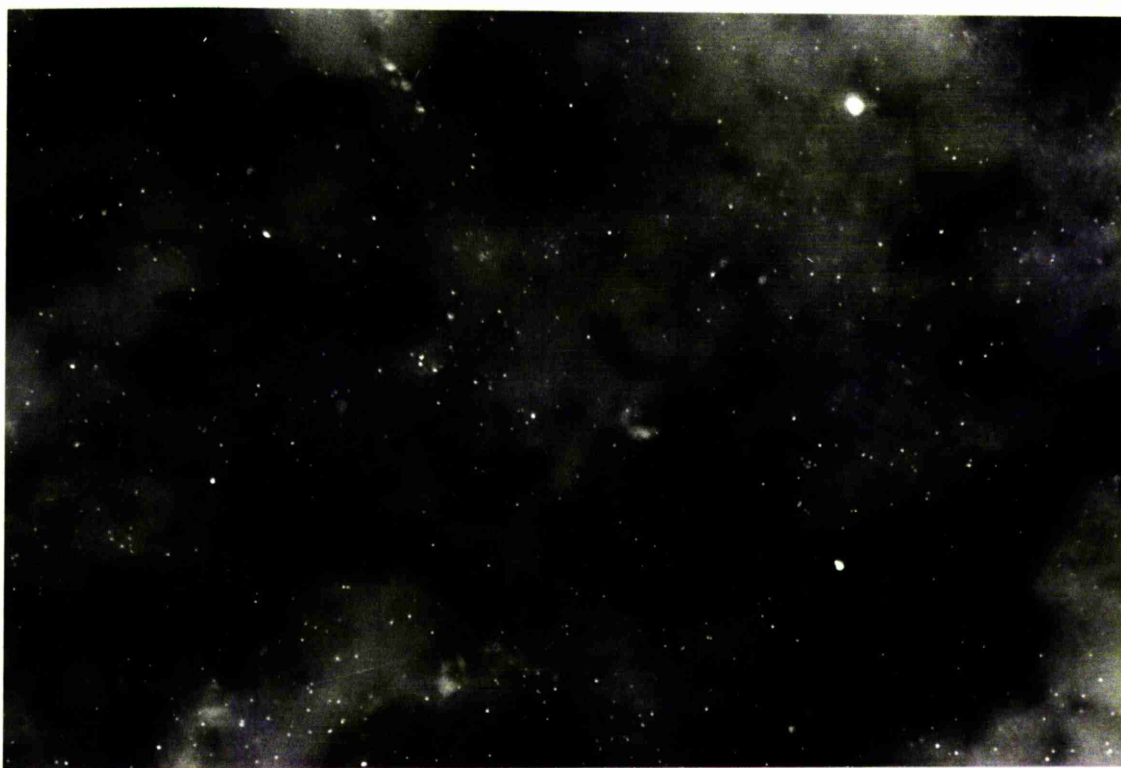
Plate 13. Microautoradiographs of infected cells in a nodule from a 4-week-old pea plant collected 6 hours after exposure of the shoot to  $^{14}\text{CO}_2$  for 30 minutes. Radioactivity has accumulated in cells of the lobe (a) but not in those of the green lobe (b).



a



b



— 40 $\mu$

which accumulated some labelled photosynthates (Plates 10a and 10b). These bodies were approximately the same size as starch grains in the nodules, but did not have the typical tetrahedral shape and did not stain black with iodine. For comparative purposes a photograph of starch grains has been included (Plate 10c).

#### (iii) Distribution of starch grains in nodule sections

Large numbers of starch grains were present in the nodules and characteristic patterns of accumulation were seen in certain cells.

In active nodules, there were few starch grains in the young infected cells at the meristematic tip and parietal layers were present in cells partially filled with bacteroids (Plates 11 and 12). Heavy deposits were normally present in the totally filled cells, the few uninfected cells in the infected region and the cells near the common endodermis (Plate 11). In older, 4-week-old nodules which were greening at the base, there was little starch in the basal cells, which were totally filled with bacteroids, whereas those cells nearer the tip still had dense deposits. Starch grains in these basal cells stained only partially with iodine and were irregular in shape. In 5-week-old nodules very little starch remained and none was present in nodules from 6-week-old plants.

There was no decline in the number of starch grains in nodules of plants darkened for 6 days and starch grains stained normally with iodine in these nodules.

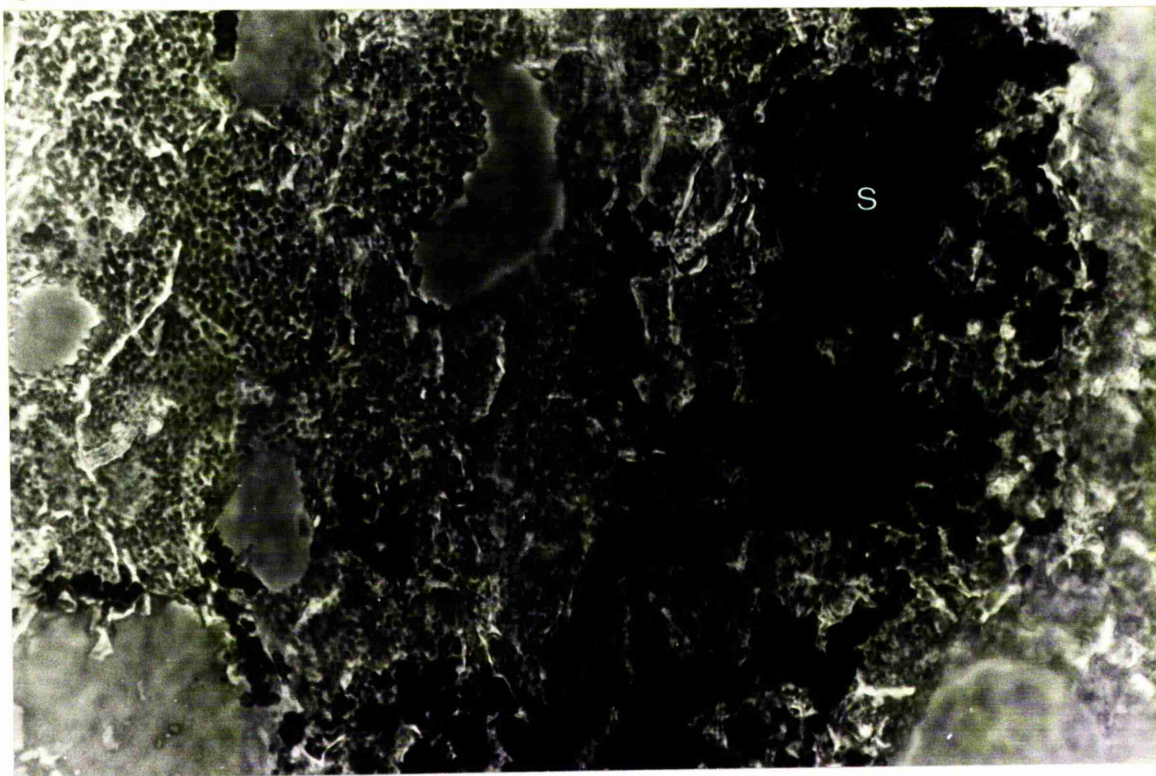
#### (iv) Accumulation of radioactivity in forked nodules

Forked nodules from 4-week-old plants exposed to  $^{14}\text{CO}_2$  for 30 minutes were collected 6 hours after exposure. Nodules were chosen so that one lobe was pink and the other was green, and sections were cut through comparable regions of both lobes. Accumulation of radioactivity was detected in infected cells in the pink lobe (Plate 13a), but there was none in cells of the green lobe (Plate 13b). Infected cells of the pink lobe were not all

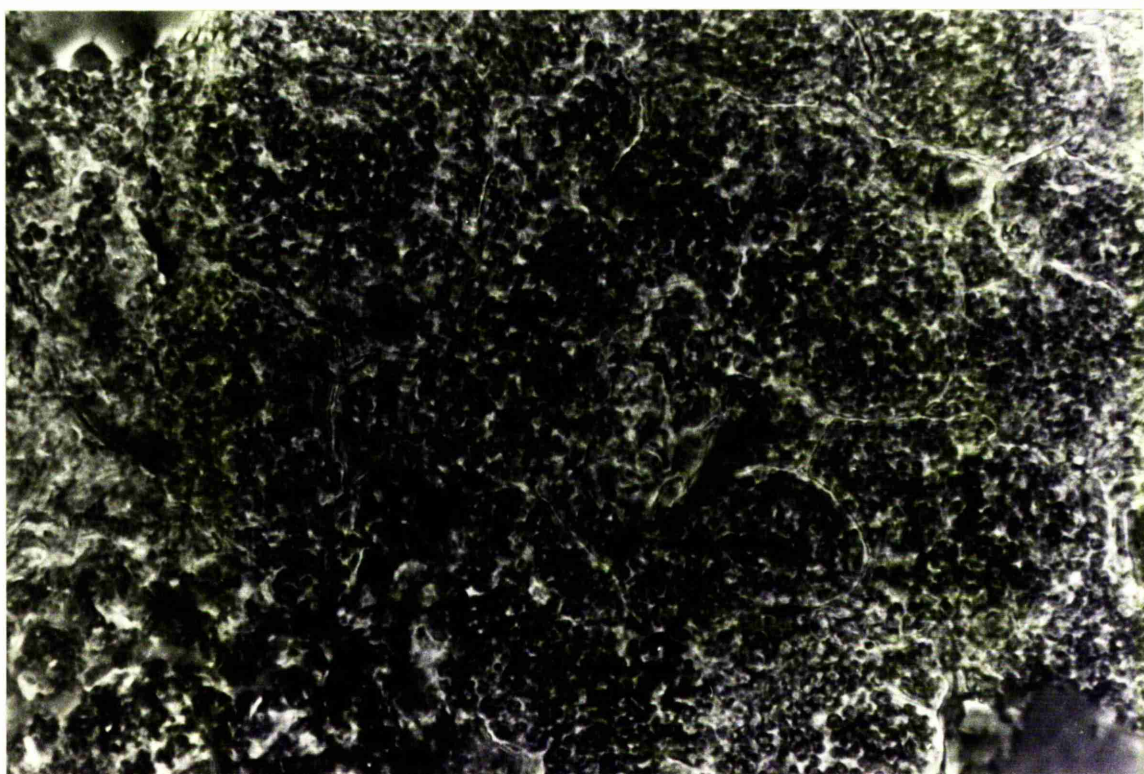


Plate 14. Distribution of starch grains (stained black in iodine) in infected cells of a nodule from a 4-week-old pea plant as seen by phase contrast microscopy. Starch grain(s) are present in cells of the pink lobe (a) but not in cells of the green lobe (b).

a



b



—20 $\mu$

totally filled with bacteroids and still retained small amounts of starch

(Plate 14a), whereas those of the green lobe were totally filled with bacteroids and did not possess starch (Plate 14b).

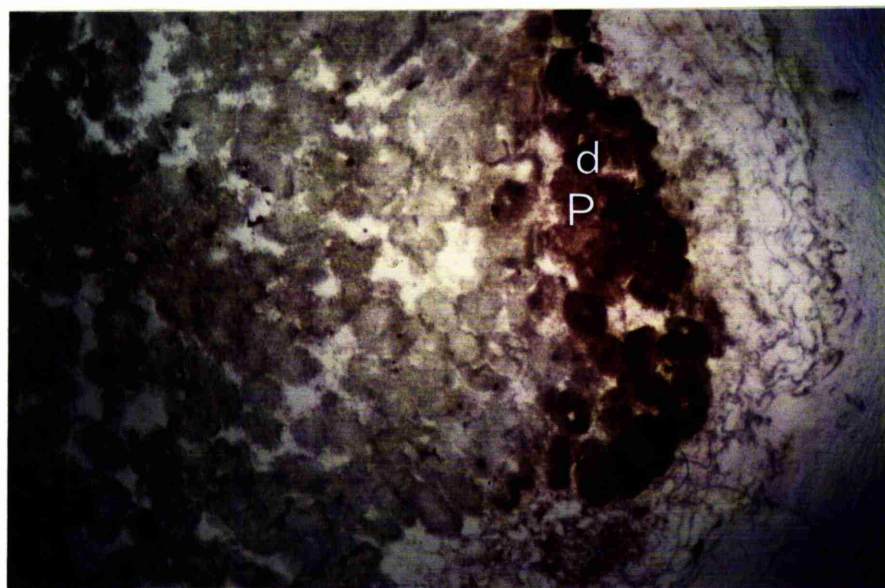


Plate 15a (top). Cells of a pea nodule from a 3-week-old plant as seen by phase contrast microscopy. Cells densely filled with bacteroids (d) show more reduction of INT than cells only partially filled (p). There is little reaction with INT to the interior of the nodule.

Plate 15b (bottom). Infected cells of a pea nodule from a 3-week-old plant as seen by phase contrast microscopy. Cells densely filled with bacteroids (d) show appreciable reduction of NBT.

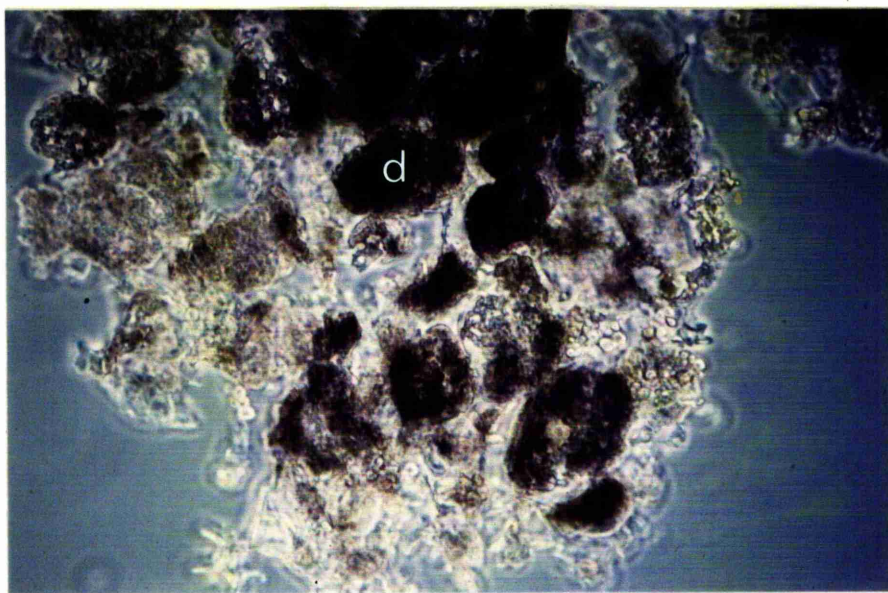


a



— 100 $\mu$

b

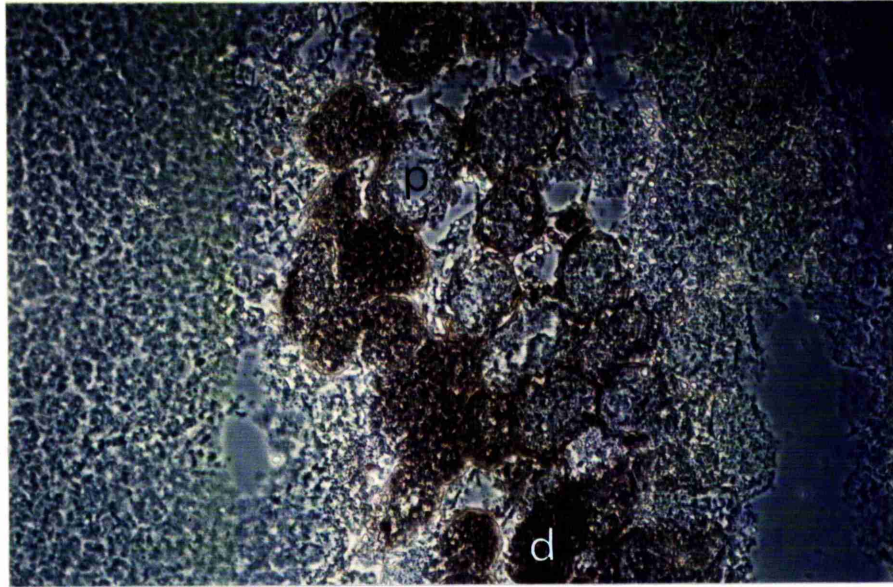


— 40 $\mu$

Plate 15c (top). Infected cells of a pea nodule from a 3-week-old plant as seen by phase contrast microscopy. Reduction of TNBT by cells densely filled with bacteroids (d) is much greater than by those only partially filled (p).

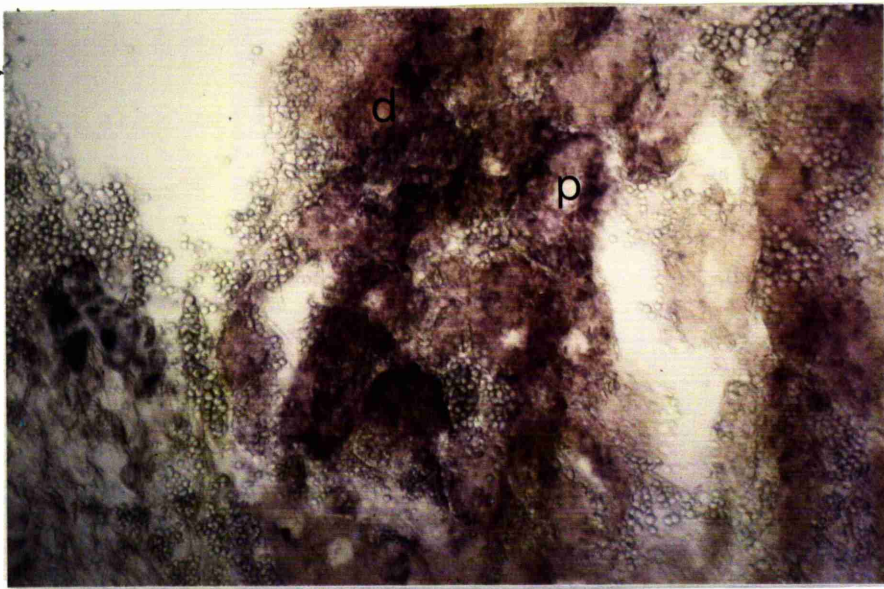
Plate 15d (bottom). Infected cells of a pea nodule from a 3-week-old plant as seen by phase contrast microscopy. Both densely (d) and partially (p) filled cells show reduction of TTC.

c



—40 $\mu$

d



—40 $\mu$



Plate 15e (top). Infected cells of a pea nodule from a 5-week-old plant as seen by phase contrast microscopy. The cells show no detectable reduction of NBT.

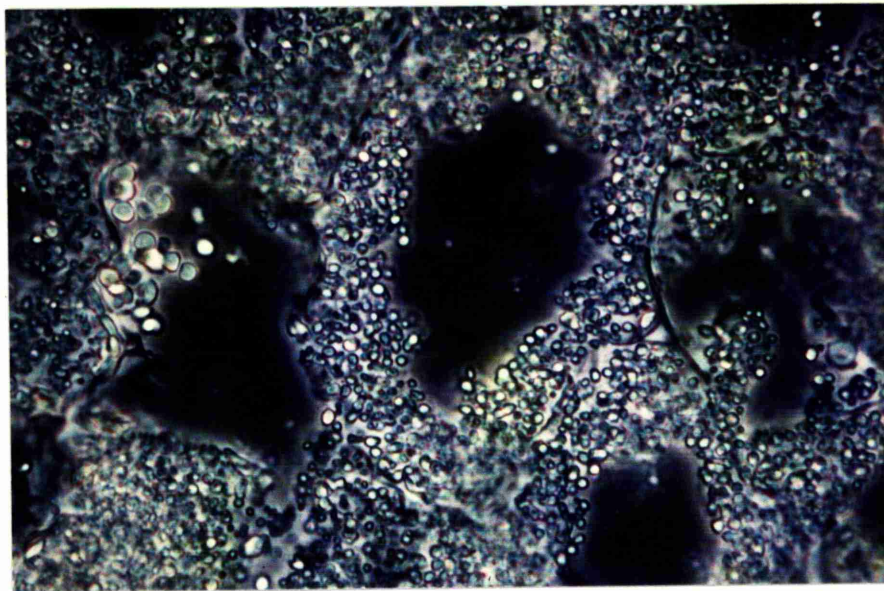
Plate 15f (bottom). Cells partially filled with bacteroids in a nodule from a 3-week-old pea plant. The cells show only slight reduction of TTC.

e



—40 $\mu$

f



—10 $\mu$

(9) HISTOCHEMICAL TESTS WITH TETRAZOLIUM SALTS

Although a number of tetrazolium salts were used to locate sites of active reduction within the nodule, penetration was slow with all the salts used and was incomplete even after 4 hours.

In active nodules, most, but not all, of the cells which were totally filled with bacteroids showed intense reduction of all tetrazolium salts (Plate 15 a to d). There was no reduction in filled cells in 5-week-old nodules, which had ceased to fix nitrogen actively (Plate 15e). Cells which were only partially filled with bacteroids showed much less reducing activity (Plate 15 a, c and f).

Table 7. Effect of nodule size on acetylene reduction by detached broad bean nodules.

Plant age (weeks)	Acetylene-reducing activity (nmoles $C_2H_2$ reduced $mg^{-1}$ fresh weight nodules $h^{-1}$ )		
	Large nodules ( 200 mg)	Medium nodules (100-200 mg)	Small nodules ( 100 mg)
9	46.97 $\pm$ 2.12	57.88 $\pm$ 7.38	41.45 $\pm$ 3.22
12	92.07 $\pm$ 25.55	128.62 $\pm$ 12.71	109.14 $\pm$ 6.37
15	37.08 $\pm$ 19.38	84.81 $\pm$ 1.87	68.18 $\pm$ 3.67

Nodules from broad bean plants were divided into large ( 200 mg fresh weight), medium (100-200 mg fresh weight) and small ( 100 mg fresh weight) categories and 2.5 g samples of each category were assessed separately for acetylene reduction. Nodules of plants 9, 12 and 15 weeks old were assessed separately.

The t-test was used to test the differences between the means for medium and small or large nodules.

Size of nodules	Large	Medium	Small
9 weeks	1.421 <sup>NS</sup>	2.041 <sup>NS</sup>	
12 weeks	1.285 <sup>NS</sup>	1.369 <sup>NS</sup>	
15 weeks	2.451*	4.025**	

Fig. 30. Effect of increasing the percentage of the gas volume occupied on acetylene reduction by detached broad bean nodules. Medium nodules from field-grown plants 9 weeks old were incubated under standard conditions for 30 minutes. Only 3 replicate samples were used in each assessment.

The t-test was used to test the differences between acetylene-reducing activities at successively greater occupation of the gas volume.

Percentage gas volume occupied	Value of t			
	10	20	30	50
○	-2.149 <sup>NS</sup>	-0.829 <sup>NS</sup>	-6.096**	
□	-3.779*	-0.639 <sup>NS</sup>	-5.952**	
△	-1.775 <sup>NS</sup>	-0.757 <sup>NS</sup>	-6.662**	

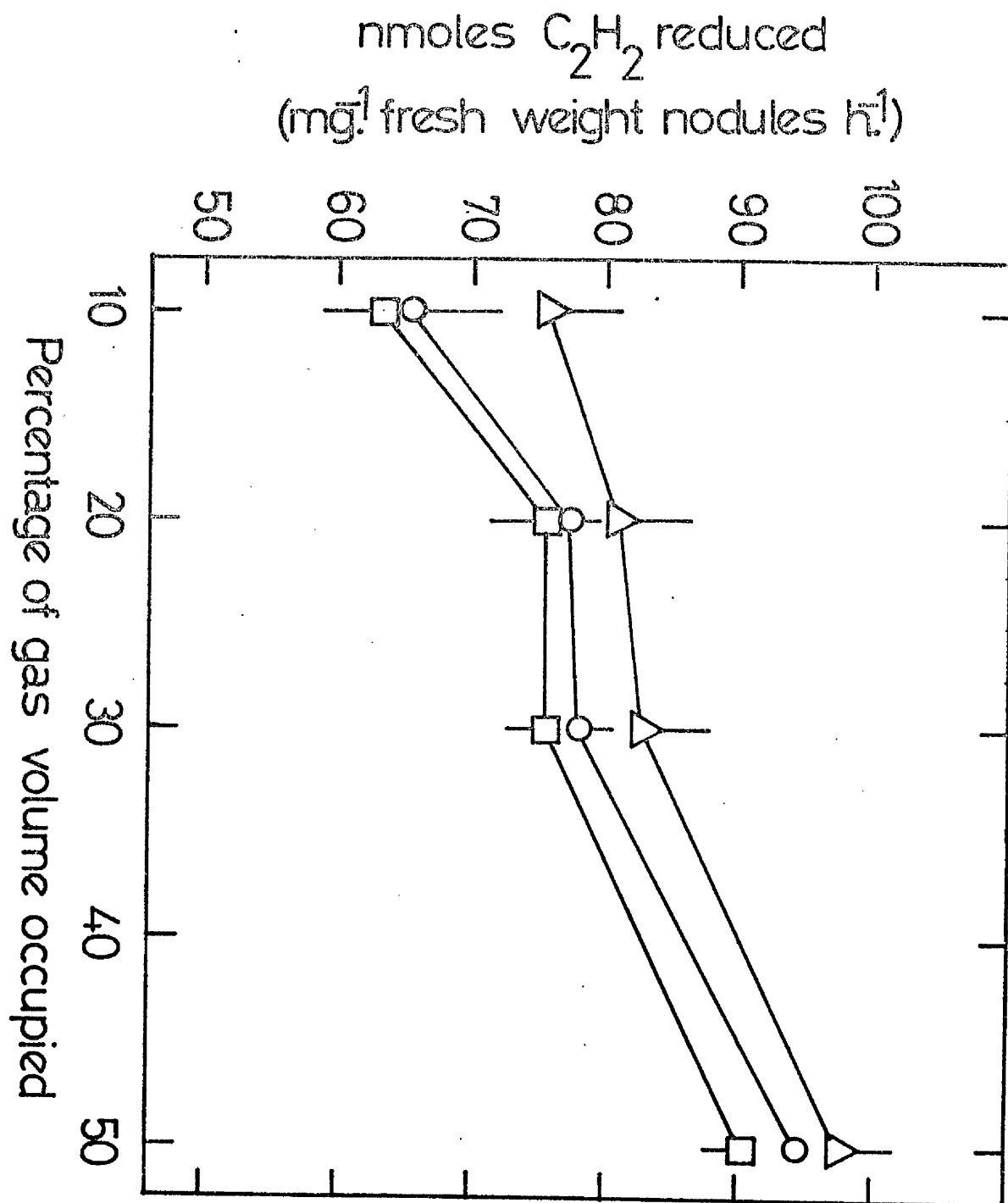
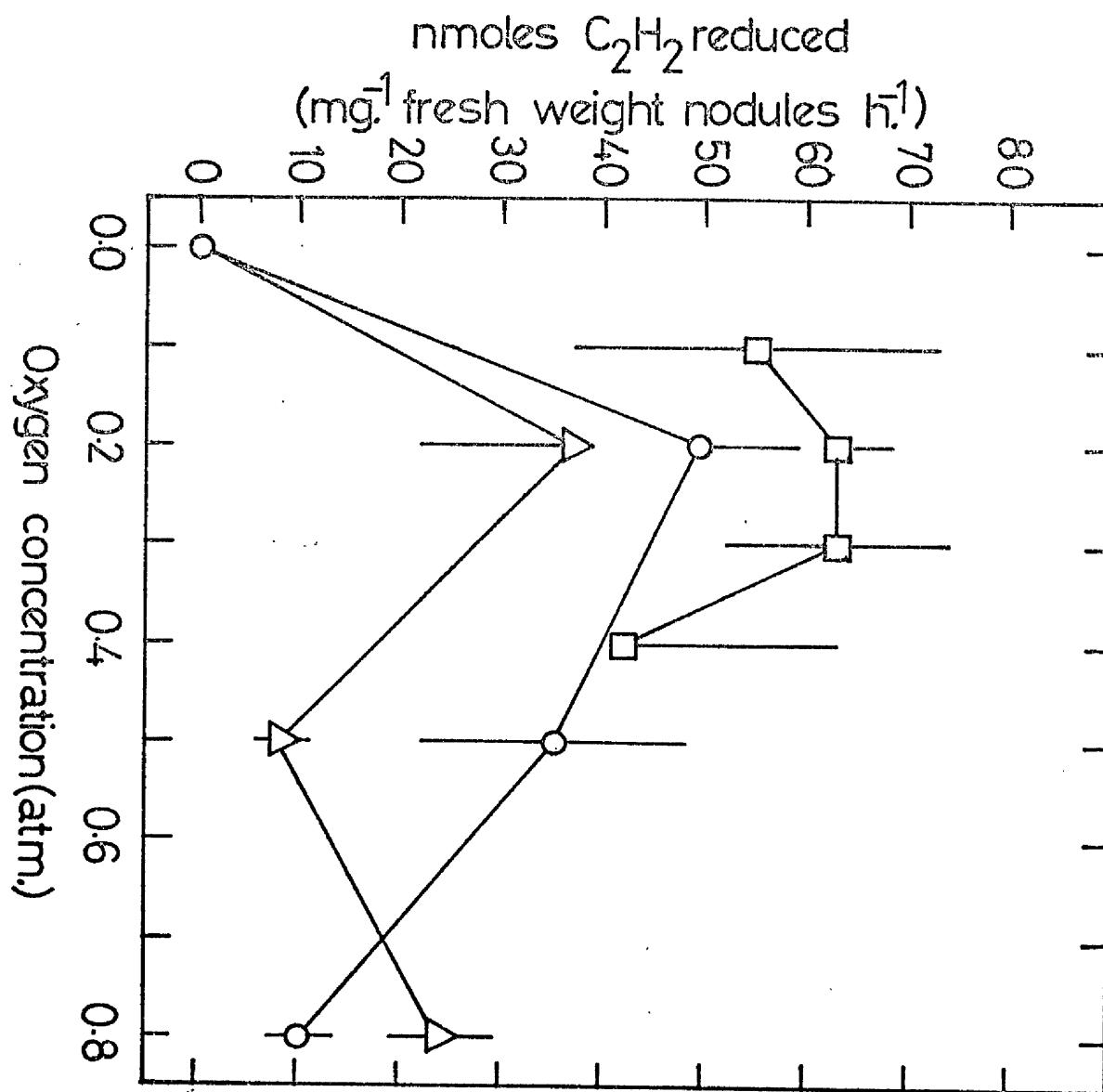


Fig. 31. Effect of oxygen concentration on acetylene reduction by detached bean nodules. Medium nodules from plants 9 weeks old were incubated for 30 minutes at 25°C atmospheric pressure in the acetylene: argon (1:3) atmosphere with oxygen concentration as stated. Only 3 replicate samples were taken at each oxygen concentration.

The t-test was used to test the differences between successive mean acetylene-reducing activities as the oxygen concentration increased.

		Value of t			
Oxygen concentration (atm.)	0.000	0.20	0.50	0.80	
○		-1.636 <sup>NS</sup>	0.461 <sup>NS</sup>	3.016*	
△		-4.986*	3.824*	-3.517*	
Oxygen concentration (atm.)	0.10	0.20	0.30	0.50	
□		-0.765 <sup>NS</sup>	0.029 <sup>NS</sup>	1.573 <sup>NS</sup>	





## B. VICIA FABA

### (1) EFFECT OF ASSAY CONDITIONS ON ACETYLENE REDUCTION

#### a) Nodule size

Nodules from plants 9, 12 and 15 weeks old were tested for acetylene-reducing activity after division of each age group into 3 sizes: large (greater than 200 mg. fresh weight), medium (100 to 200 mg. fresh weight) and small (less than 100 mg. fresh weight). The effect of nodule size on acetylene reduction was dependent on age (Table 7). In both 9- and 12-week-old plants, medium nodules were slightly more active than large or small nodules although differences were not significant. In 15-week-old plants the activity of medium nodules was significantly greater than that of both small nodules and large nodules. Medium nodules were always the most active irrespective of the age of the plant and in all the experiments with Vicia faba only medium nodules from plants 9 weeks old were used.

#### b) Gas volume

Nodules were inserted into assay tubes so that 10, 20, 30 or 50% of the volume of each tube was occupied and acetylene reduction tests were performed as usual. Increasing the volume of the tubes occupied from 10% to 30% did not result in significant increases in activity except in one experiment (Fig. 30). However, a further increase in the volume occupied to 50% resulted in a statistically significant increase in activity of about one-third.

#### c) Oxygen concentration

The acetylene-reducing activity of nodules, incubated with different oxygen concentrations in the acetylene/argon mixture described previously (Section A 1a), was dependent on  $pO_2$ . There was no activity in the absence of oxygen and maximum activity was found at a  $pO_2$  of 0.20 atm. (Fig. 31), which was the  $pO_2$  normally used for assay and equals that in the atmosphere.

Fig. 32a. Effect of temperature of incubation on acetylene reduction by detached bean nodules. Medium nodules from field-grown plants 9 weeks old were incubated for 30 minutes at atmospheric pressure with the standard gas mixture ( $O_2 : C_2H_2 : Ar = 20 : 20 : 60$ ) at the temperatures shown. Only 3 replicate samples were taken at each temperature.

The t-test was used to test the differences between the mean acetylene-reducing activities at successively higher temperatures.

Value of t				
Temperature °C	5	15	25	35
○	-2.640 <sup>NS</sup>	-2.644 <sup>NS</sup>	-0.977 <sup>NS</sup>	
□	-5.626**	-1.603 <sup>NS</sup>	0.681 <sup>NS</sup>	
△	-0.879 <sup>NS</sup>	-3.700*	0.397 <sup>NS</sup>	

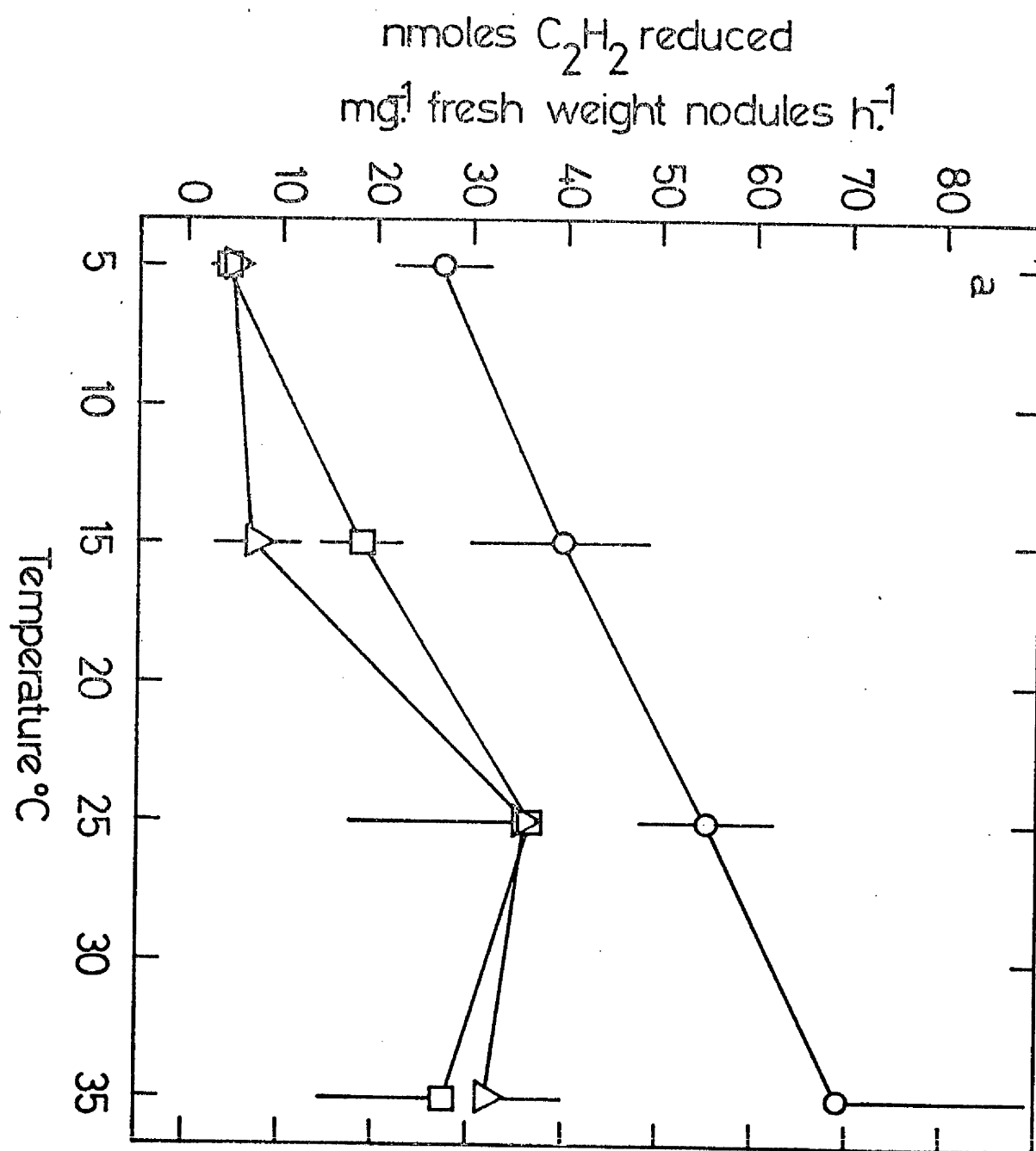
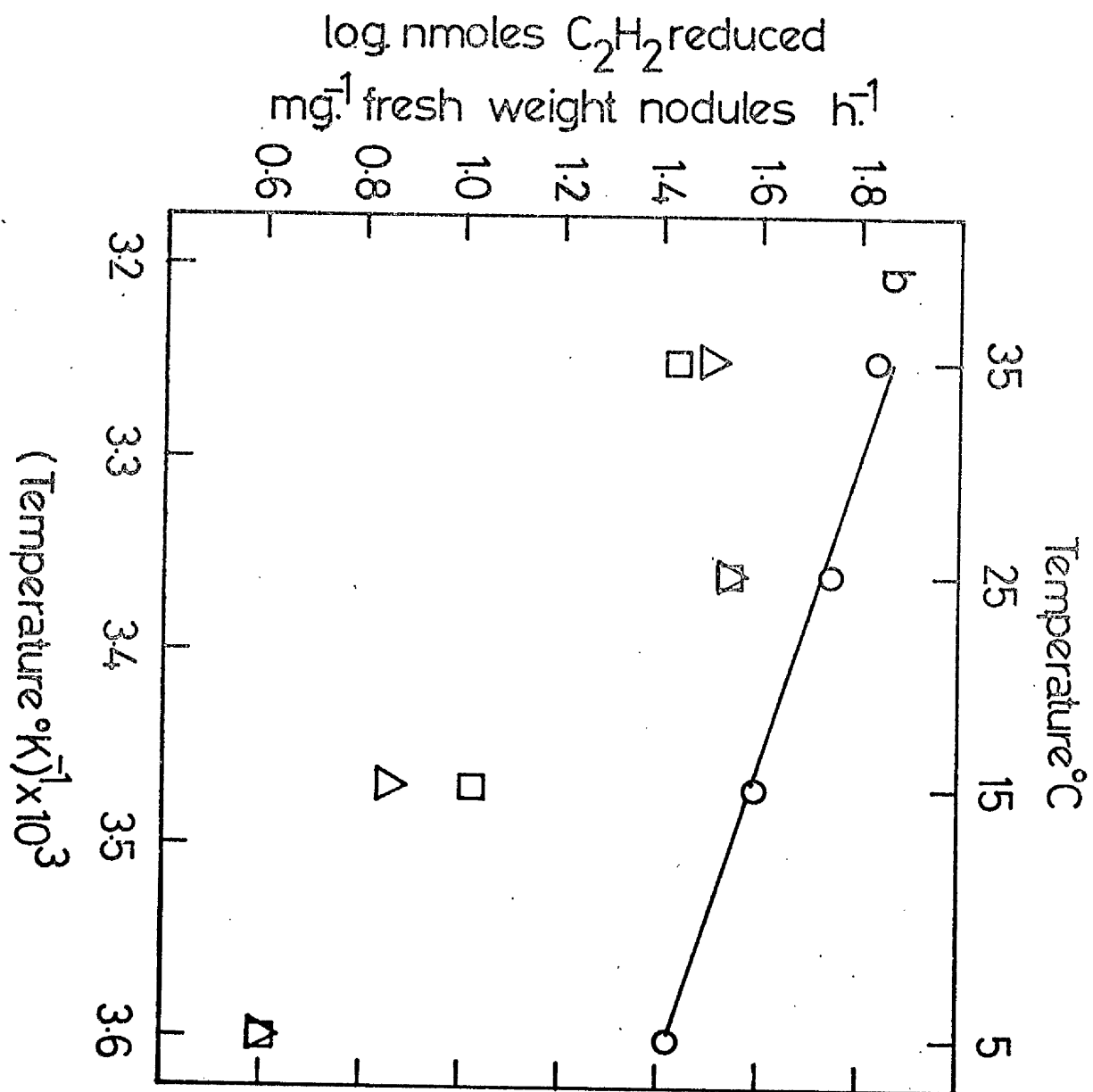


Fig. 32b. Arrhenius plot of acetylene-reducing activity of detached bean nodules at different temperatures between 5 and 35°C. Medium nodules from plants 9 weeks old were incubated for 30 minutes at atmospheric pressure with the standard gas mixture ( $O_2 : C_2H_2 : Ar = 20 : 20 : 60$ ) at the temperatures shown.



In one experiment, activity declined to approximately half the maximum at a  $pO_2$  of 0.80 atm.

d) Temperature of incubation

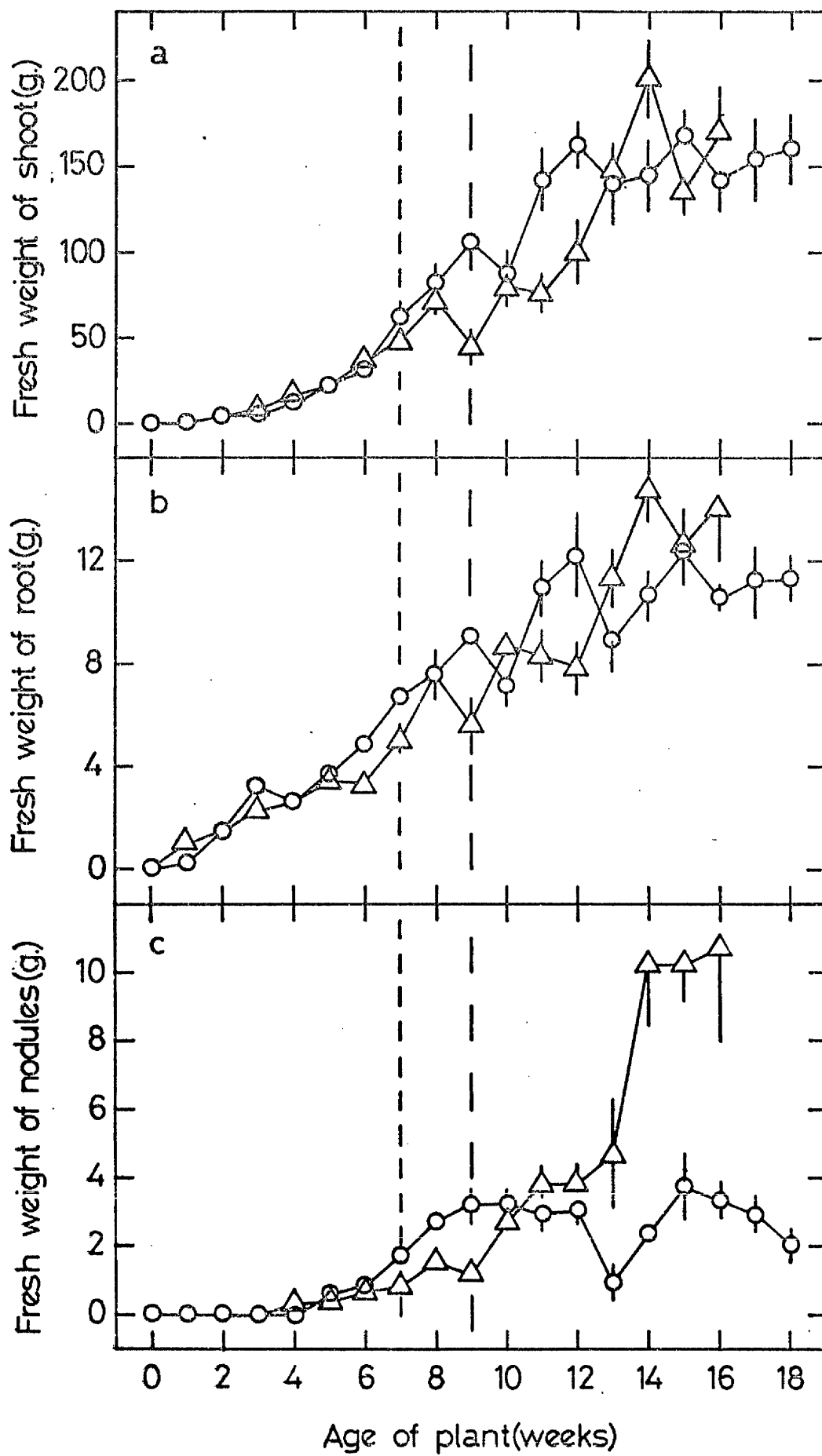
Temperature had a small but significant effect on acetylene-reducing activity over the range 5 to 35°C (Fig. 32a). Maximum activity was achieved at 25 to 35°C and was up to 7 times that at 5°C. The  $Q_{10}$  values for the temperature ranges 5 to 15°C, 15 to 25°C and 25 to 35°C were not consistently different in any experiment.

The effect of temperature on the velocity of acetylene reduction is illustrated by an Arrhenius plot (Fig. 32b). The results of only one experiment fit the Arrhenius equation, indicating a constant activation energy for the reaction between 5 and 35°C. Lines were not drawn for the other experiments because of the wide scatter of points. The slope of the line was much less than for P. sativum (Fig. 13b), indicating that less activation energy was required for the acetylene reduction process in V. faba nodules.

Fig. 33a. Changes during growth of broad bean plants  
in:

- a) fresh weight of shoot;
- b) fresh weight of root;
- c) fresh weight of nodules.

Measurements were taken for each of 10  
plants at weekly intervals from  
planting. The plants were field-  
grown and crops were planted on July 1,  
1972 (  $\circ$  ) and July 15, 1972 (  $\Delta$  ).





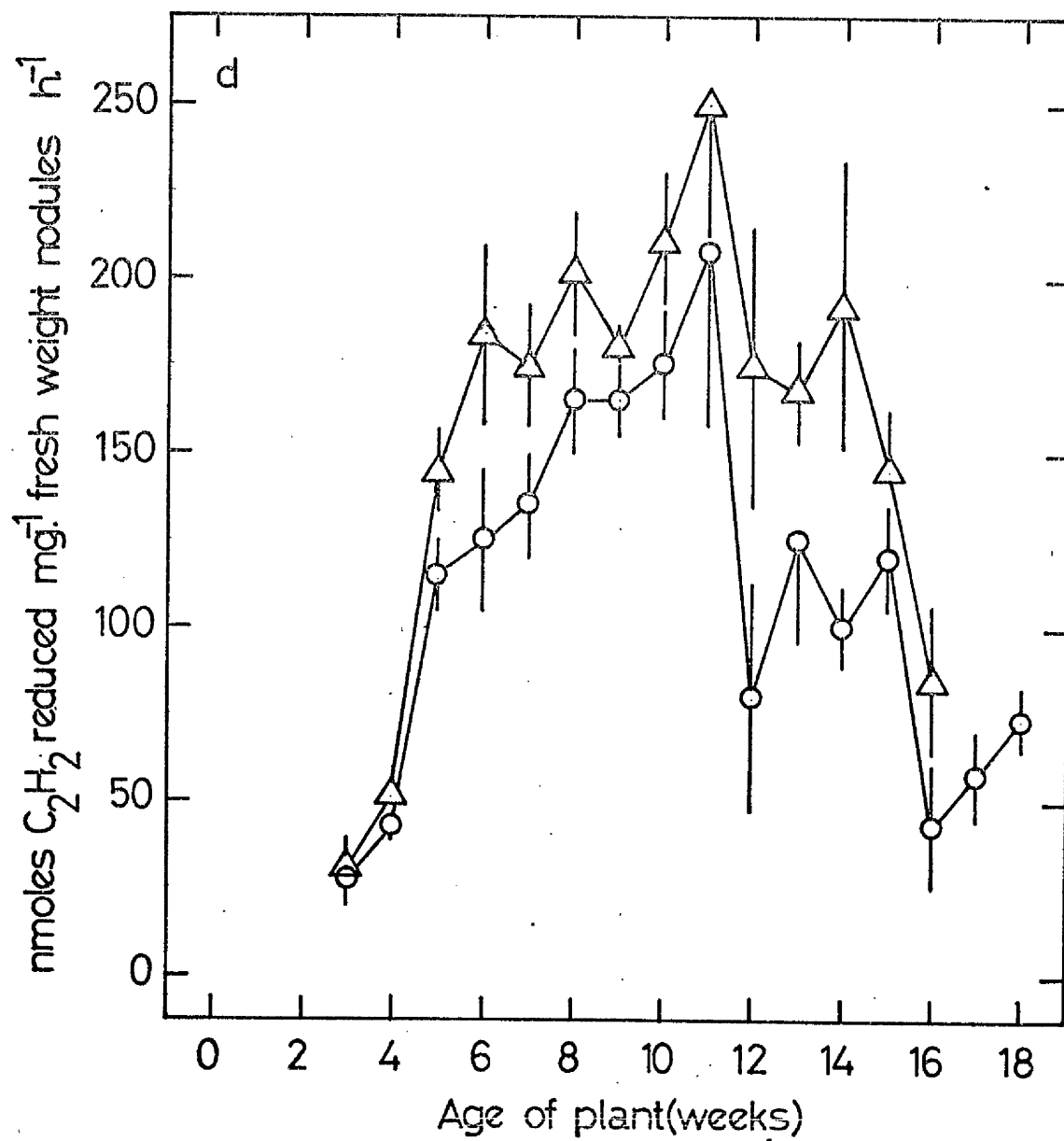
Changes during growth in acetylene-releasing activity

of *Chlorella* and *Scenedesmus* in relation to the growth phase

of the cultures. *Chlorella* (A) and *Scenedesmus* (A).

1972 (A) and 1973 (A).

Fig. 33b. Changes during growth in acetylene-reducing activity  
of detached broad bean nodules from field-grown plants.  
Crops were planted on 1st July, 1972 (o)  
and 15th July, 1972 ( $\Delta$ ).



## (2) DEVELOPMENT OF PLANTS

### a) Plant growth

The growth of V. faba in the field continued over an 18-week period, and increases in the fresh weights of shoots and roots were rapid until week 10 to week 12, but slower thereafter (Fig. 33 a to c). Flowering commenced at week 7 and fruiting at week 9, but as growth as monopodial, unlike that of P. sativum, flowers and fruit continued to form until plants were killed by frost. The weight of nodules borne by each plant was influenced markedly by the time at which the crops were planted and the weights of nodules borne by later crops were up to 4 times those of earlier crops (Fig. 33c). Basal greening of the nodules increased with age but never exceeded more than half of a given nodule at any age.

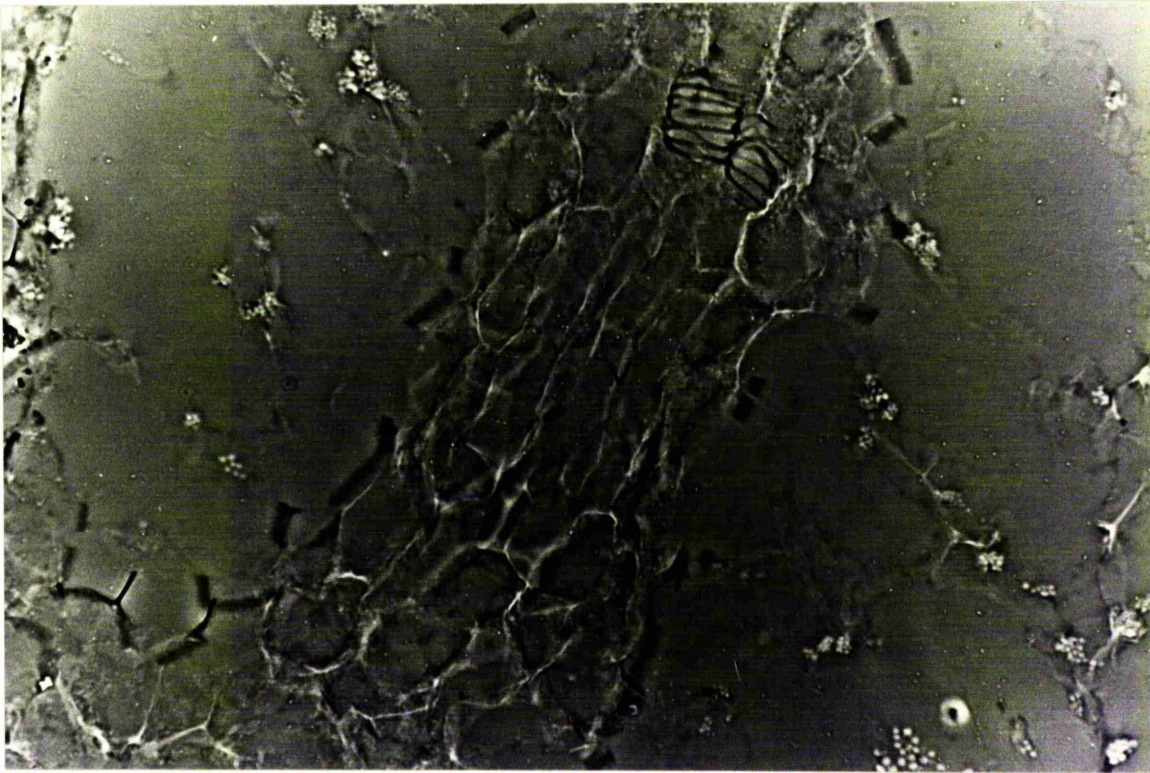
As the nodules were easily broken from the roots when plants were removed from the soil, the variation in the fresh weight of nodules borne by each plant was rather large (S.E. of mean = 10% in older plants).

### b) Acetylene-reducing activity

Nodules from plants of different ages were collected for 16 to 18 successive weeks on similarly bright days at 1200 hours to reduce variation due to diurnal fluctuations. Between weeks 3 and 7, when flowering commenced, the rate of acetylene reduction increased by 300% (Fig. 33d). There was a further increase of 30% to reach maximum activity at week 10 to 11 and then the rate decreased. It is interesting to note that acetylene-reducing activity never fell below 20% of the maximum rate even after flowering, unlike the results obtained with P. sativum.

Plate 16. The vascular tissue of a nodule from a 7-week-old bean plant as seen by (a) phase contrast and (b) dark field microscopy. Radioactivity has accumulated in the vascular trace 5 hours after commencement of exposure of the shoot to  $^{14}\text{CO}_2$  for 30 minutes.

a



b

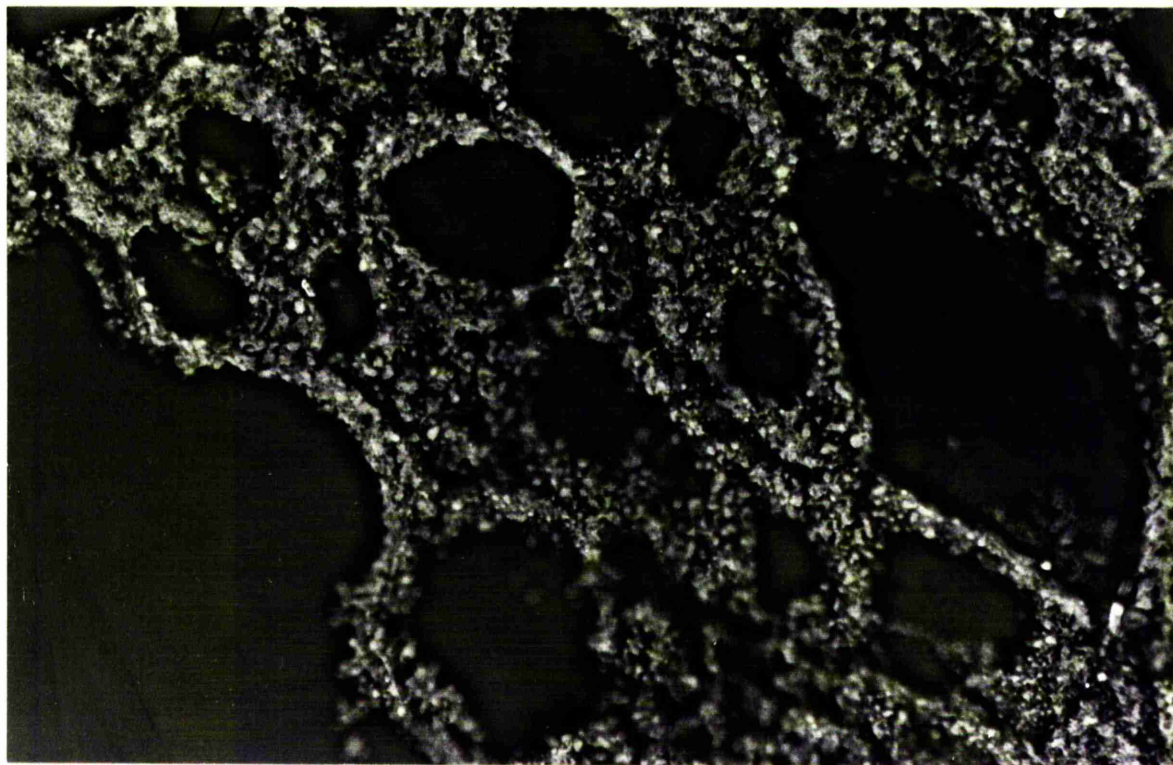


—20 $\mu$

Plate 17 (opposite). Infected cells of a nodule from a 7-week-old bean plant as seen by (a) phase contrast and (b) dark field microscopy. Radioactivity has accumulated in all cells, which contain extremely swollen bacteroids, 5 hours after commencement of exposure of the shoot to  $^{14}\text{CO}_2$  for 30 minutes.

Plate 18 (over). Swollen bacteroid forms in a nodule from a 7-week-old bean plant as seen by phase contrast microscopy.

a



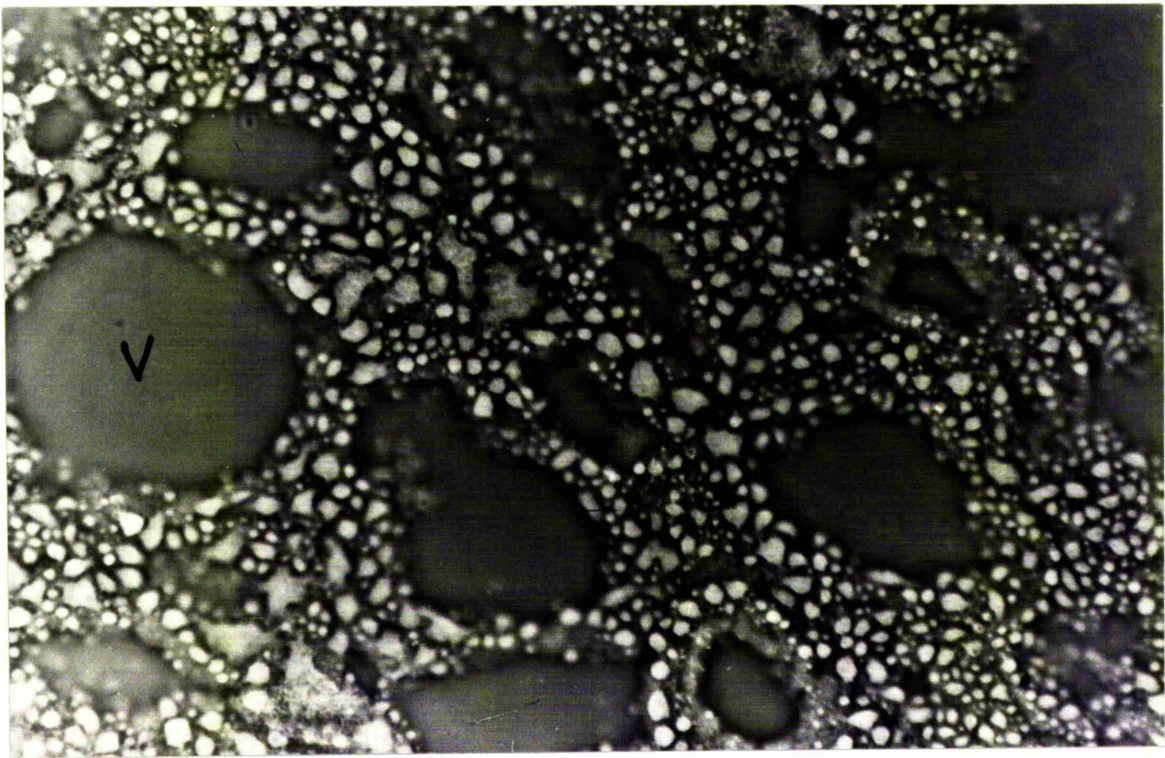
b



— 20 $\mu$



18



— 10μ

v=vacuole

(3) MICROAUTORADIOGRAPHY

a) Photosynthetic fixation of  $^{14}\text{CO}_2$

The radioactivity of nodules collected immediately after plants were exposed to  $^{14}\text{CO}_2$  for 30 minutes was insufficient to be detected by microautoradiography. Nodules detached after 4.5 hours "cold-chase" showed accumulation of labelled photosynthates in the same regions as P. sativum nodules, except that there was little accumulation at the common endodermis. The main regions of accumulation were thus the vascular traces (Plate 16) and the infected cells (Plate 17), which were only partially filled with bacteroids which were more swollen than in P. sativum nodules (Plate 18). The infected cells all showed uniform accumulation of radioactivity and there was no localization of activity towards the tips of the nodules.

b) Dark fixation of  $^{14}\text{CO}_2$  by detached nodules

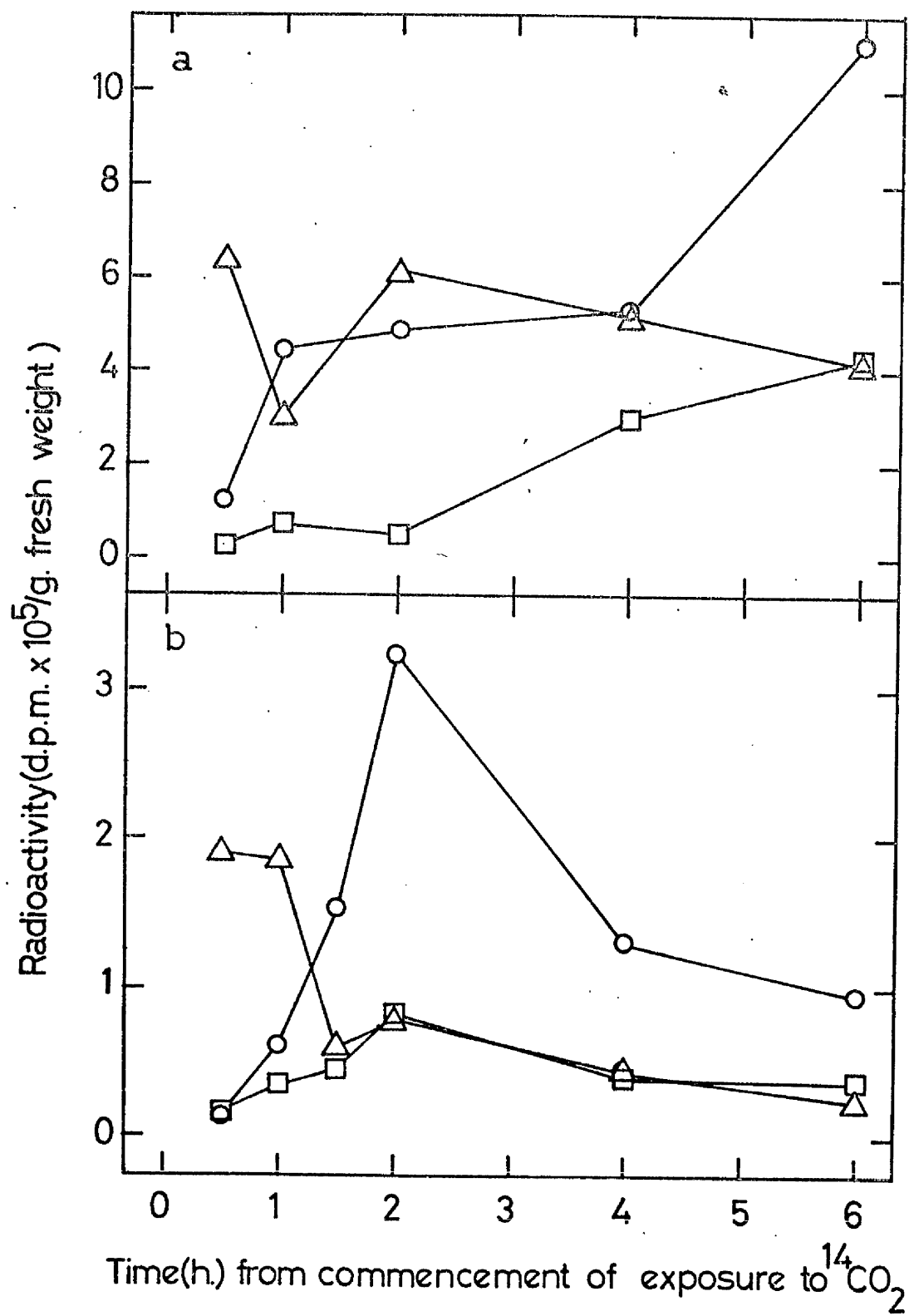
Nodules were highly labelled immediately after exposure to  $^{14}\text{CO}_2$  for 30 minutes and radioactivity remained constant for 5 hours after exposure. Labelling resembled that of nodules detached from intact plants, as described above, except that there was no accumulation of labelled material in the vascular traces when nodules were sectioned either immediately or 4.5 hours after exposure.

Fig. 34a.

Changes in radioactivity of ethanolic extracts of leaves ( $\Delta$ ), roots ( $\square$ ) and nodules (o) during continuous exposure of 6-week-old broad bean plant shoots to  $^{14}\text{CO}_2$  in a growth cabinet. Samples were collected at intervals up to 6 hours from commencement of exposure and were extracted immediately in redistilled 80% EtOH. Each point is the mean of four replicate samples.

Fig. 34b.

Changes in radioactivity of ethanolic extracts of leaves ( $\Delta$ ), roots ( $\square$ ) and nodules (o) after exposure of the shoots of 6-week-old broad bean plants to  $^{14}\text{CO}_2$  for 30 minutes. Samples were collected at intervals up to 5.5 hours "cold-chase" and extracted immediately in redistilled 80% EtOH. Each point is the mean of four replicate samples.



(4) ANALYSIS OF RADIOACTIVE COMPOUNDS IN ETHANOLIC EXTRACTS OF NODULESa) Photosynthetic fixation of  $^{14}\text{CO}_2$ 

Nodules were detached from plants 6 weeks old after exposure of the shoots to  $^{14}\text{CO}_2$  either continuously or for 30 minutes followed by "cold-chase" periods of up to 5.5 hrs. These experiments were performed once only because of the time required to assay samples by scintillation counting, but it is believed that nodule samples were sufficiently large to give results which accurately portray nodule metabolism. For comparative purposes extracts were also made of leaves and roots at each time of harvest, using the same extraction procedure as for nodules. It was calculated that 88% to 95% of the total activity of the nodules was extracted by the extraction procedure used.

(i) Continuous exposure to  $^{14}\text{CO}_2$  (Treatment L1)

Although the specific activity of the leaves showed a small decline during the 6 hour exposure to  $^{14}\text{CO}_2$ , the largest fall observed was the anomalous 50% drop after 1 hour's exposure. By contrast, the specific activity of the nodules rose rapidly over the 6 hour period to exceed that of both roots and leaves after 4 hours; the specific activity of the roots rose also, to equal that of the leaves after 6 hours. However, the total activity extracted from the nodules and roots did not exceed 5% and 18% respectively of the total activity extracted from the whole plants even after 6 hours (Fig. 34a)

(ii) Short exposure to  $^{14}\text{CO}_2$  (Treatment L2)

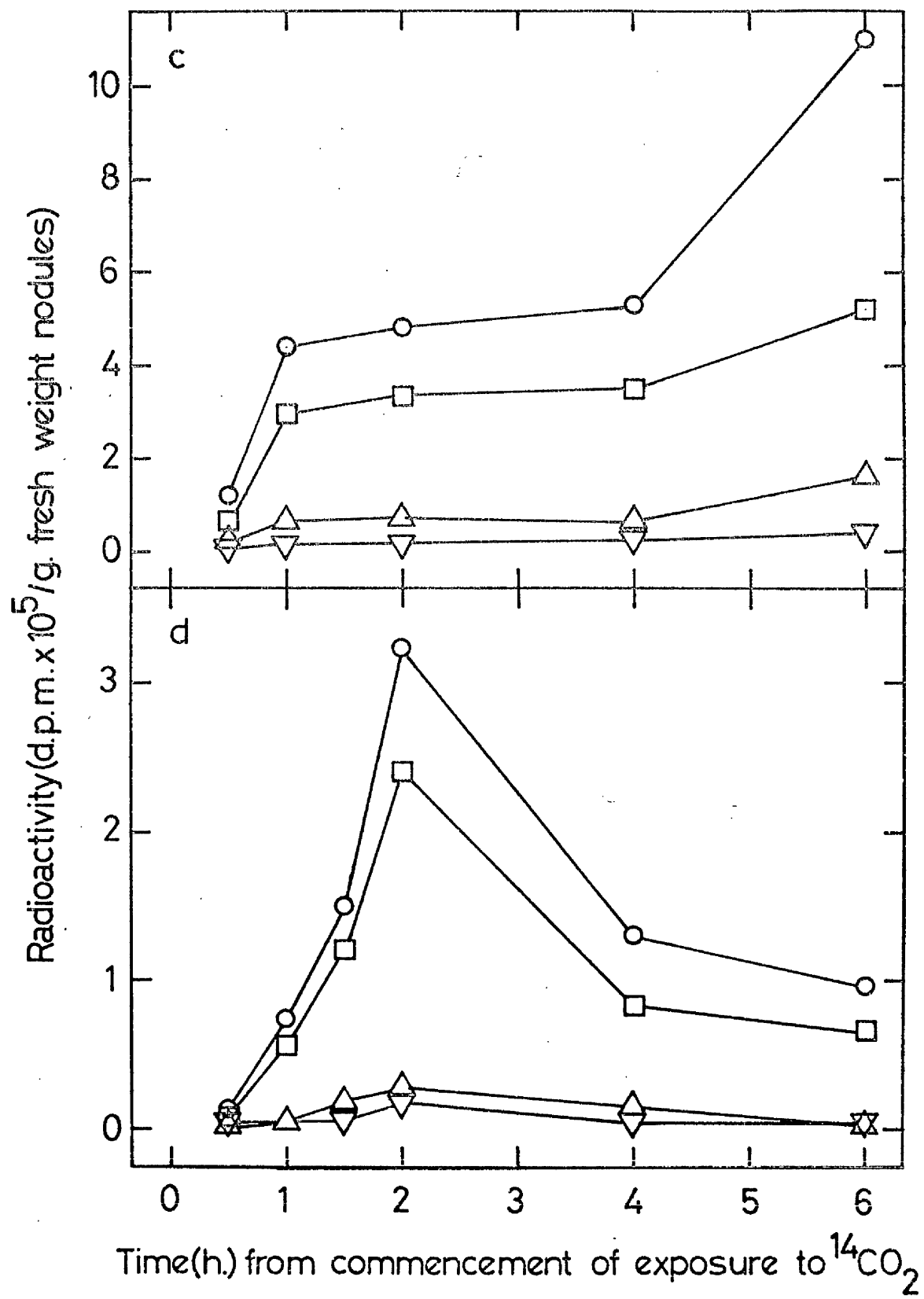
When plant shoots were exposed to  $^{14}\text{CO}_2$  for 30 minutes, followed by "cold-chase" periods, there was a rapid rise in the specific activity of the nodules to exceed that of both roots and leaves after a 1 hour "cold-chase" period. (Fig. 34b). The specific activity of the nodules reached a maximum after 90 minutes "cold-chase" and declined rapidly to one-third of the maximum value 4 hours later. The specific activity of the roots rose

Fig. 34c.

Changes in the distribution of radioactivity in ethanolic extracts (o) and the neutral (□), basic (Δ) and acidic (▽) fractions of these extracts of broad bean nodules, during continuous exposure of the shoot of 6-week-old plants to  $^{14}\text{CO}_2$ . Each point is the mean of four replicate samples.

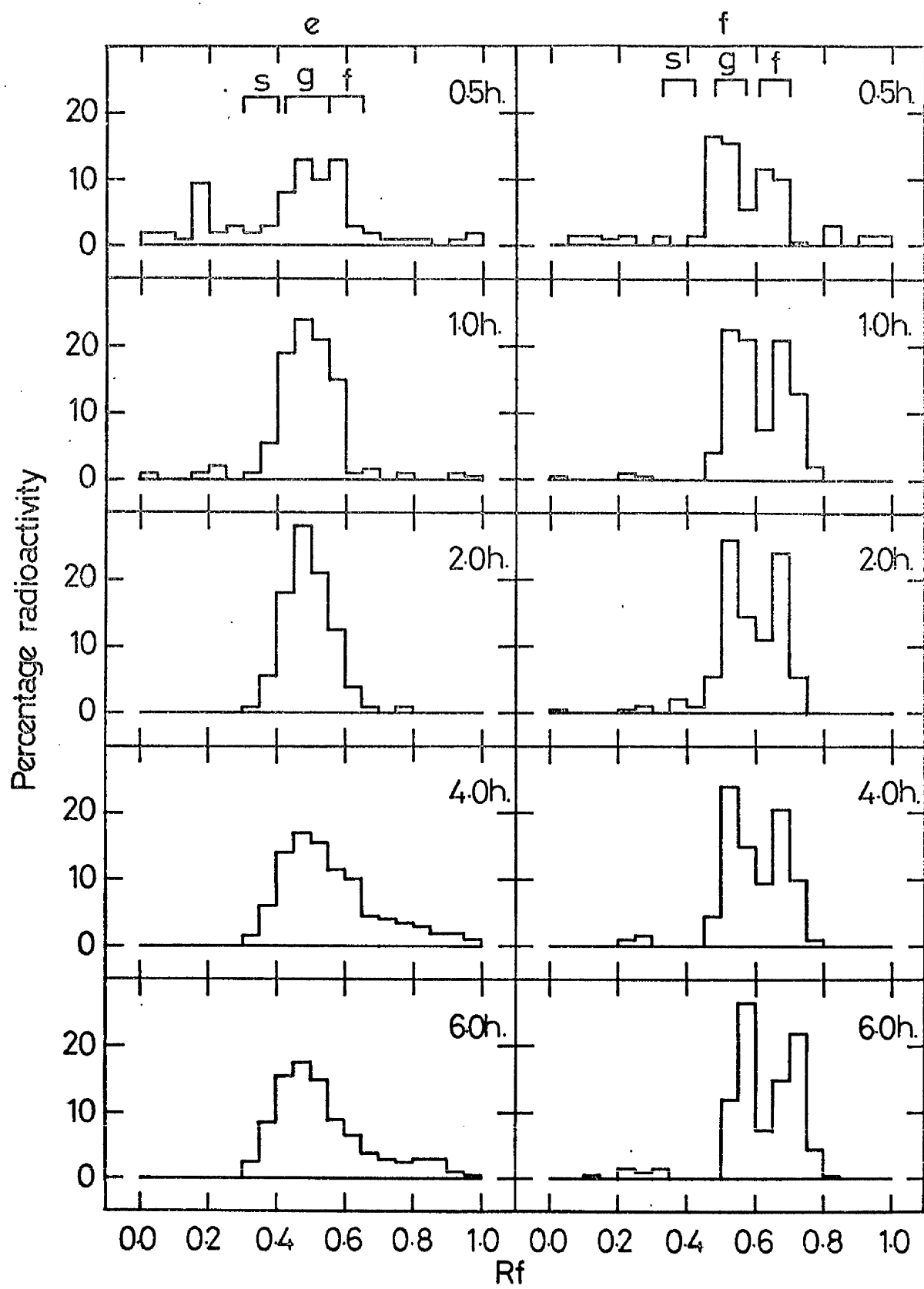
Fig. 34d.

Changes in the distribution of radioactivity in ethanolic extracts (o) and the neutral (□), basic (Δ) and acidic (▽) fractions of these extracts of broad bean nodules after exposure of the shoots of 6-week-old plants to  $^{14}\text{CO}_2$  for 30 mins, followed by "cold-chase" periods. Each point is the mean of four replicate samples.



Figs. 34e and f. Changes in the distribution of radioactivity in the neutral fractions of ethanolic extracts of broad bean nodules from plants 6 weeks old exposed to  $^{14}\text{CO}_2$  either continuously (e) or for 30 minutes only (f). Nodules were collected at the times shown after commencement of exposure and extracts were chromatographed in solvents IPrAq (e) and EtAc (f). The Rf values of sucrose (s), glucose (g) and fructose (f) in control spots run simultaneously are also shown.





and declined in a similar manner to that of the nodules, although the specific activity was much lower and did not exceed that of the leaves. The leaves declined in specific activity to one-quarter of their original value after 5.5 hours "cold-chase" and this fall was greater than that recorded in Treatment L1.

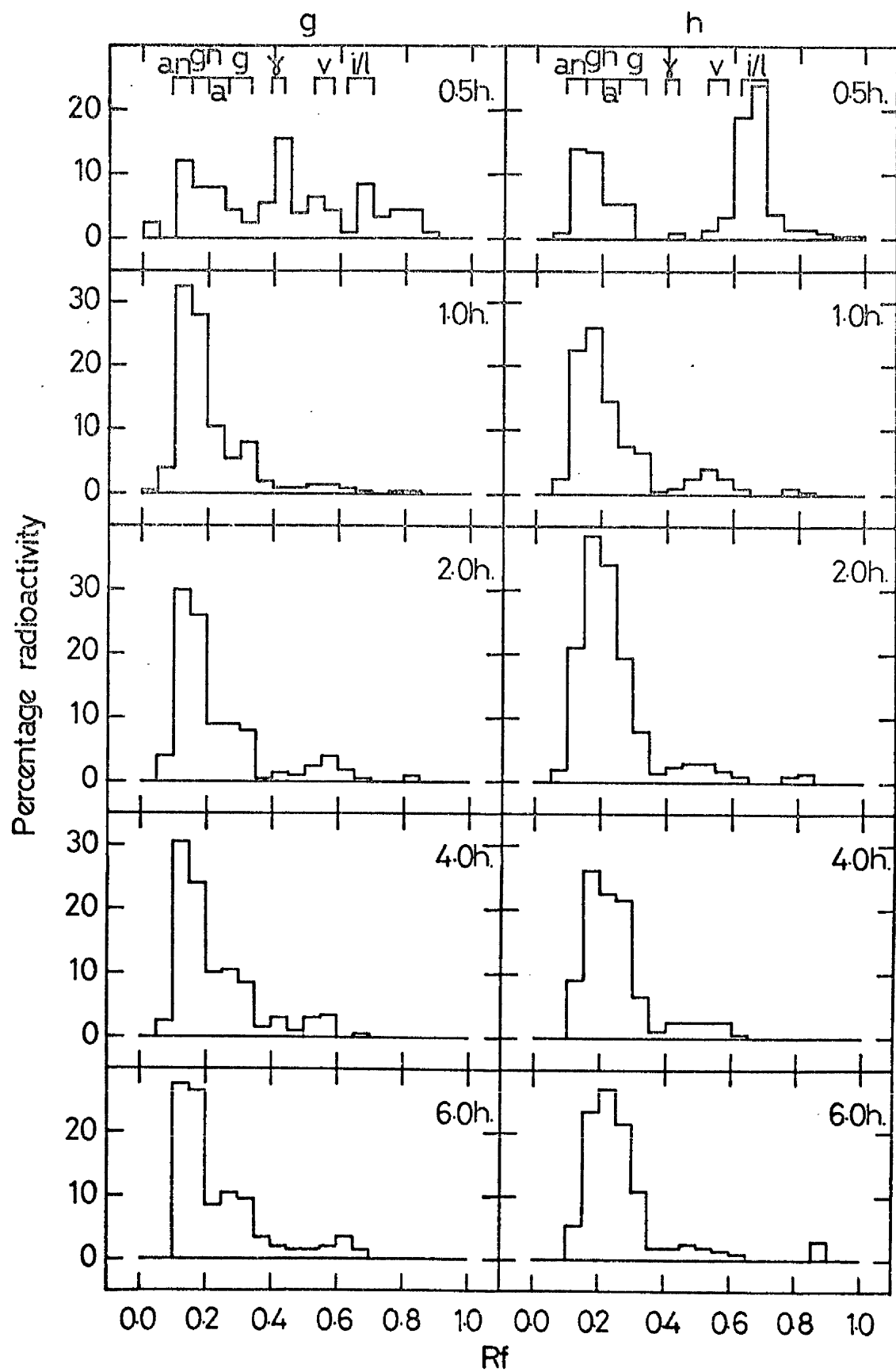
#### (iii) Fractionation of ethanolic extracts

In both treatments, the neutral fraction always contained more than 60% of the total radioactivity extracted from the nodules, with the exception of the 5.5 hr. "cold-chase" period in Treatment L1 (Figs. 34 c and d). The basic fraction accumulated 16% of the total radioactivity in the nodule extracts after continuous exposure of the shoots to  $^{14}\text{CO}_2$  for 6 hours (Fig. 34c), but reached a maximum of 12% of the total activity after 90 minutes "cold-chase" (Fig. 34d). The acidic fraction accumulated less than 9% of the total activity of the nodule extracts in both treatments. In both shoot and root extracts, the neutral fraction contained 80% to 90% of the activity at all times and the basic and acidic fractions each accumulated 5% to 10% of the total activity. In all samples the relative proportions of labelled material incorporated into neutral, basic and acidic fractions remained fairly constant, and accumulation of activity in each fraction reflected changes in the accumulation of activity in the total nodule extract at all times of collection.

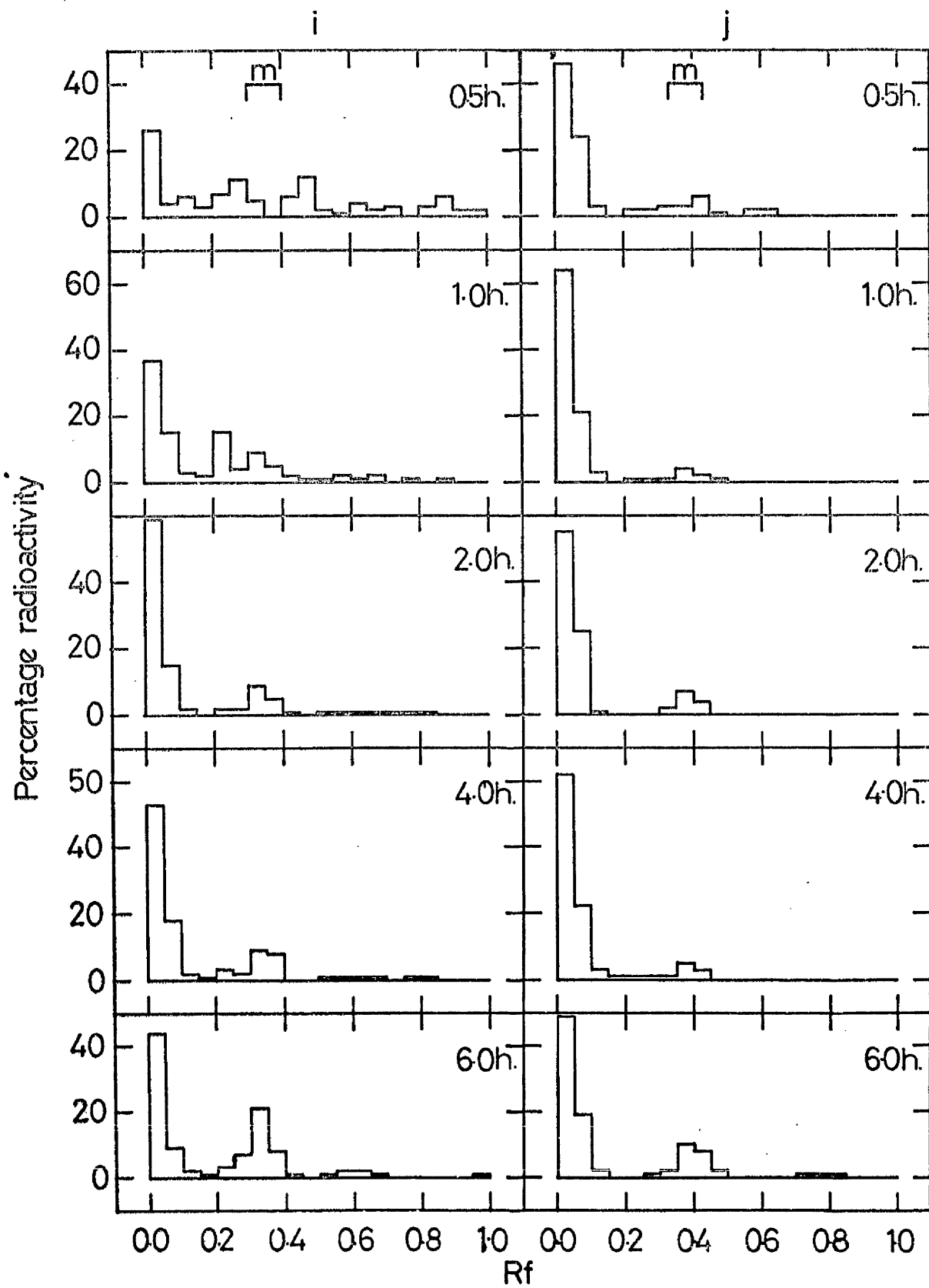
#### (iv) Neutral fraction

The solvents IPrAg, EtAcPy and AcW were used for paper chromatography and the only sugars detected in nodule extracts were glucose and fructose, which together accounted for more than 90% of the activity of this fraction (Fig. 34 e and f). Sucrose was not detected and little activity was associated with the appropriate areas of paper chromatograms. By contrast, root and shoot extracts contained large amounts of  $^{14}\text{C}$ -labelled sucrose.

Figs. 34g and h. Changes in the distribution of radioactivity in the basic fractions of ethanolic extracts of nodules from broad bean plants 6 weeks old exposed to  $^{14}\text{CO}_2$  either continuously (g) or for 30 minutes only (h). Nodules were collected at the times shown after commencement of exposure and extracts were chromatographed in the solvent BuA. The Rf values of aspartic acid (a), glutamic acid (g), asparagine (an), glutamine (gm),  $\gamma$ -aminobutyric acid ( $\gamma$ ), valine (v) and isoleucine/leucine (i/l) in control spots run simultaneously are also shown.



Figs. 34 i and j. Changes in the distribution of radioactivity in the acidic fractions of ethanolic extracts of nodules from broad bean plants 6 weeks old exposed to  $^{14}\text{CO}_2$  either continuously (i) or for 30 minutes only (j). Nodules were collected at the time shown after commencement of exposure and extracts were chromatographed in the solvent PrF. The Rf value of malic acid (m) in control spots run simultaneously is also shown.



(v) Basic fraction

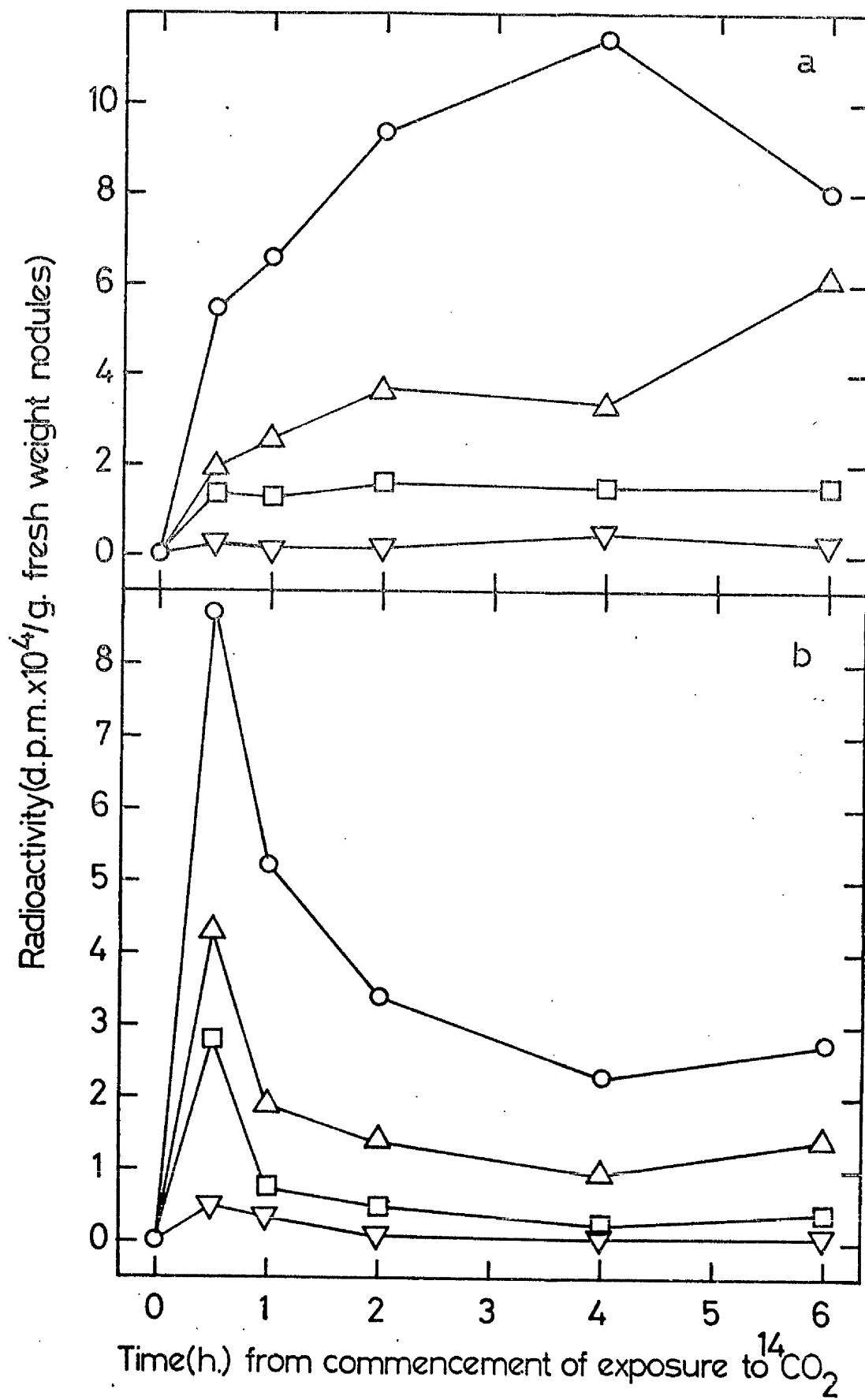
Only three major radioactive compounds were detected on paper chromatograms from both treatments and together these contained 55% to 75% of the total radioactivity of this fraction. Asparagine was by far the most prominent spot and accumulated about 20% of the activity, glutamic acid contained 20% to 30% and aspartic acid contained 16% to 25% of the activity in this fraction (Figs. 34 g and h). The proportion of the total activity associated with glutamic acid was slightly greater in Treatment L2 than in Treatment L1. Radioactive spots of leucine/isoleucine, valine and  $\gamma$ -amino butyric acid were present and a large number of other amino acids were also detected as minor constituents. The relatively large radioactive peaks apparently associated with  $\gamma$ -amino butyric acid in Treatment L1 and with leucine/isoleucine in Treatment L2 after 30 minutes' exposure were not associated with the appropriate regions of these extracts in other chromatographic solvents. Asparagine contained 50% of the activity of this fraction in root extracts, and in extracts of the leaves glutamine and aspartic acid were most heavily labelled and together contained 40% to 60% of the total activity.

(vi) Acidic fraction

A large proportion (60% to 80%) of the radioactivity in this fraction was due to contaminating sugars, which were identified as before (Section A 3c). Malic acid was the only organic acid detected on the chromatograms and contained the remainder of the activity (Figs. 34 i and j). Plants continuously exposed to  $^{14}\text{CO}_2$  accumulated up to 40% of the activity of this fraction in malic acid, but plants exposed to  $^{14}\text{CO}_2$  for 30 minutes only accumulated up to 20% of the activity as malic acid. There was no detectable activity associated with the larger quantities of malic acid which were present in root extracts. Leaf extracts contained large amounts of unlabelled fumaric acid and smaller quantities of labelled malic acid.

Figs. 35a and b. Changes in the distribution of radioactivity in ethanolic extracts (o) and the basic (M), neutral (□) and acidic (V) fractions of these extracts of detached broad bean nodules from 6-week-old plants after exposure to  $^{14}\text{CO}_2$  either continuously (a) or for 30 minutes only (b). Samples of nodules from 6-week-old plants were extracted in 80% EtOH at the times shown after commencement of exposure. The data shown are from one experiment only but are typical of the results obtained; each point is the mean of 3 replicate samples.





b) Dark fixation of  $^{14}\text{CO}_2$  by detached nodules

Detached nodules exposed to  $^{14}\text{CO}_2$  either continuously or for 30 minutes followed by "cold-chase" periods of up to 5.5 hours were extremely active in  $^{14}\text{CO}_2$  fixation and labelled assimilates were detected after only 15 minutes' exposure to  $^{14}\text{CO}_2$ .

(i) Continuous exposure to  $^{14}\text{CO}_2$  (Treatment D1)

The radioactivity of the nodules rose steadily for 4 hours after the commencement of exposure to  $^{14}\text{CO}_2$  to a level which was twice that observed after 30 minutes (Fig. 35a). The activity then declined by 25% between 3 and 6 hours after commencement of exposure.

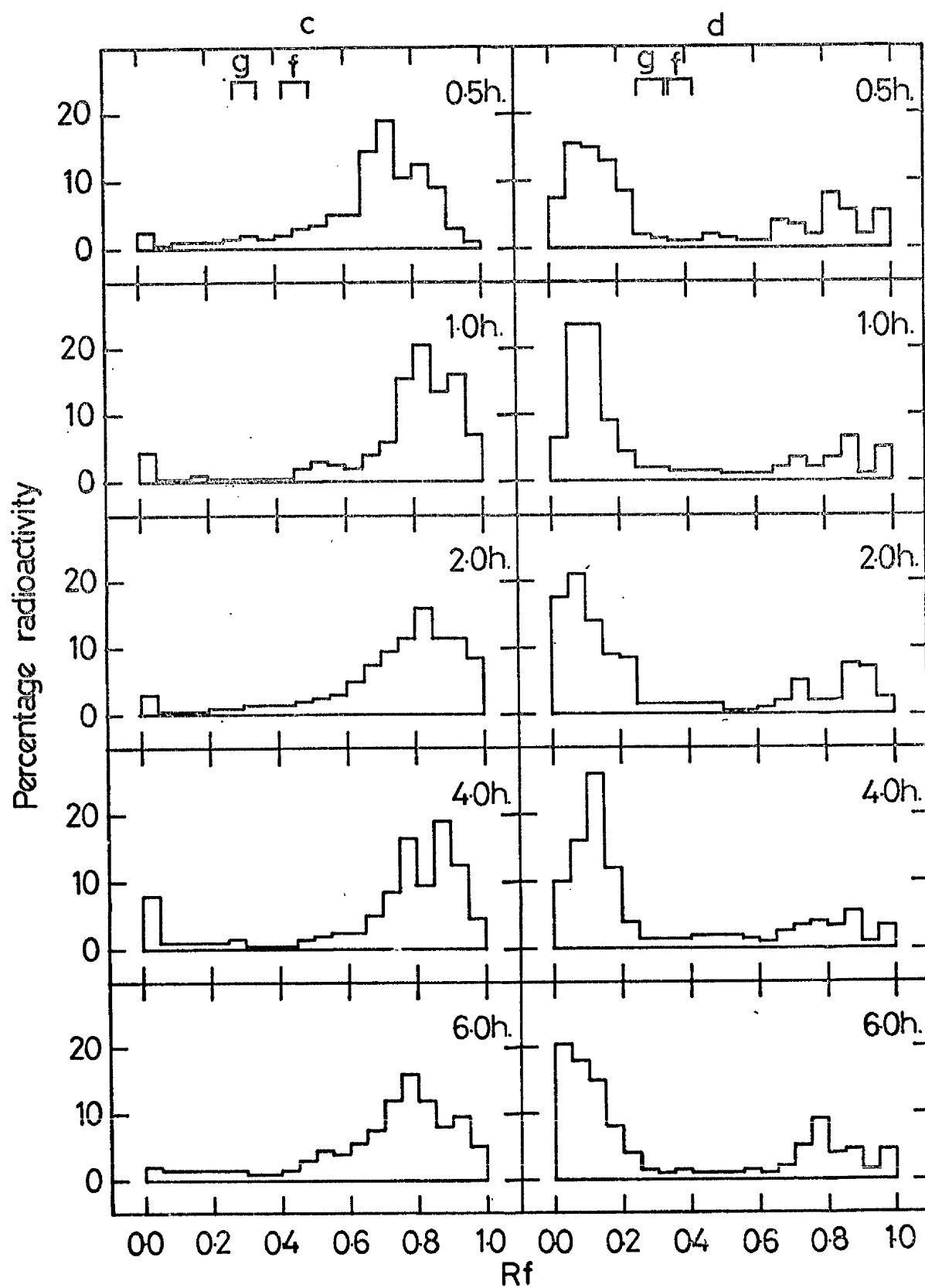
(ii) Short exposure to  $^{14}\text{CO}_2$  (Treatment D2)

The activity of the nodules was highest immediately after the exposure period and declined steadily to 26% of this activity after 5.5 hours "cold-chase" (Fig. 35b).

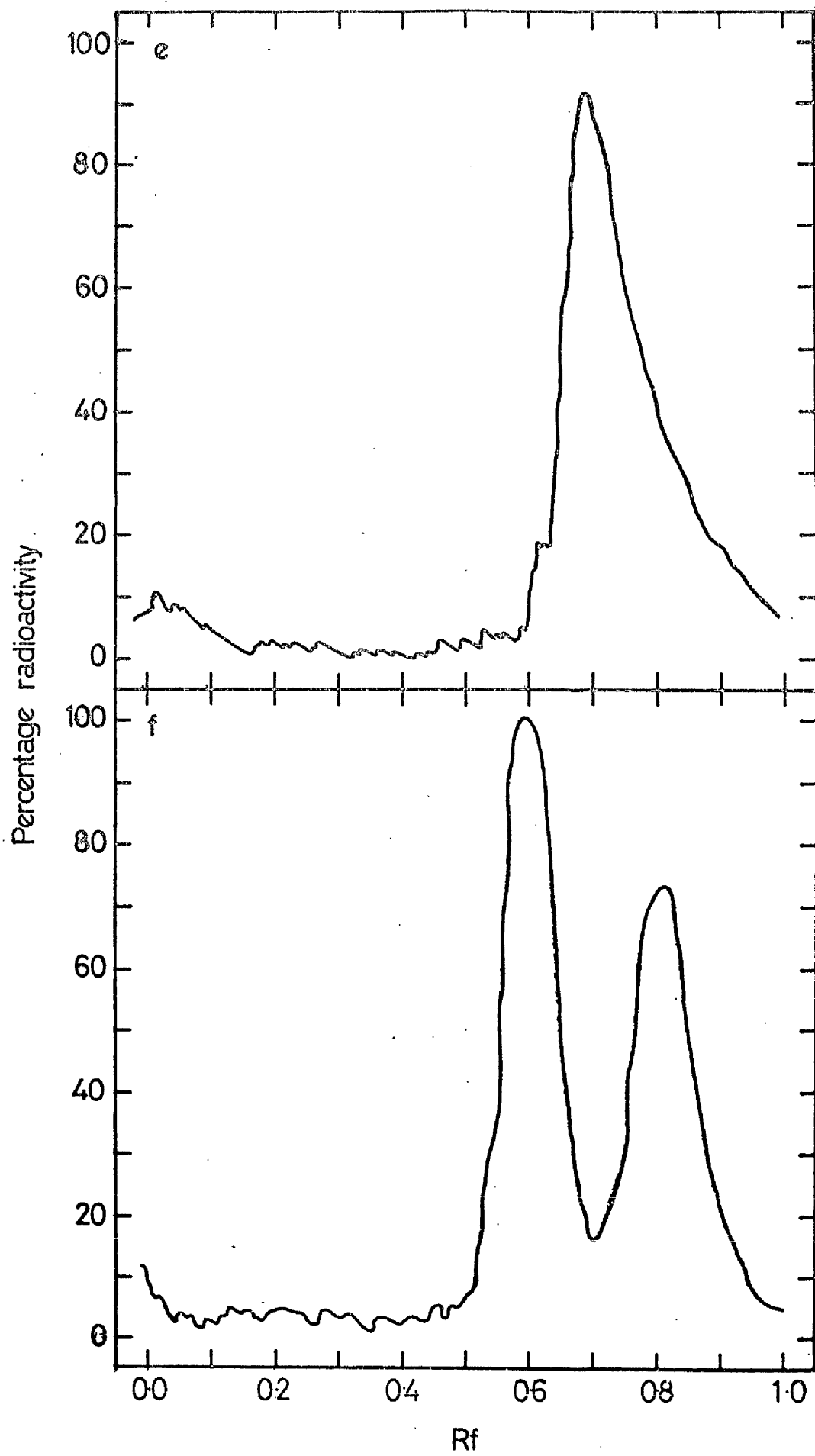
(iii) Fractionation of ethanolic extracts

The activity of the basic fraction in both treatments was greater than that of the neutral fraction, in contrast to results obtained with whole plants (Figs. 35 a and b). The basic fraction contained 28% to 76%, the neutral fraction 10% to 32% and the acidic fraction 2% to 7% of the total ethanol-extractable activity fixed by the nodules. In Treatment D1, activity incorporated into the basic fraction increased with time, whereas the activity in the neutral and acidic fractions changed little after the first 30 minutes of exposure to  $^{14}\text{CO}_2$ . In Treatment D2, the activity of the basic fraction decreased by 50%, the neutral fraction by 70% and the acidic fraction by 30% in the first 30 minutes following exposure to  $^{14}\text{CO}_2$ . The radioactivity of the basic and neutral fractions then declined slowly for a further 5 hours, but the activity of the acidic fraction fell to 16% of the original activity after only 90 minutes "cold-chase" and showed little change

Figs. 35c and d. Changes in the distribution of radioactivity in the neutral fractions of ethanolic extracts of nodules (from broad bean plants 6 weeks old) exposed to  $^{14}\text{CO}_2$  either continuously (c) or for 30 minutes only (d). Nodules were collected at the times shown after commencement of exposure and extracts were chromatographed in the solvents AcW (c) and EtAc (d). The Rf values of glucose (g) and fructose (f) in control spots run simultaneously are also shown.

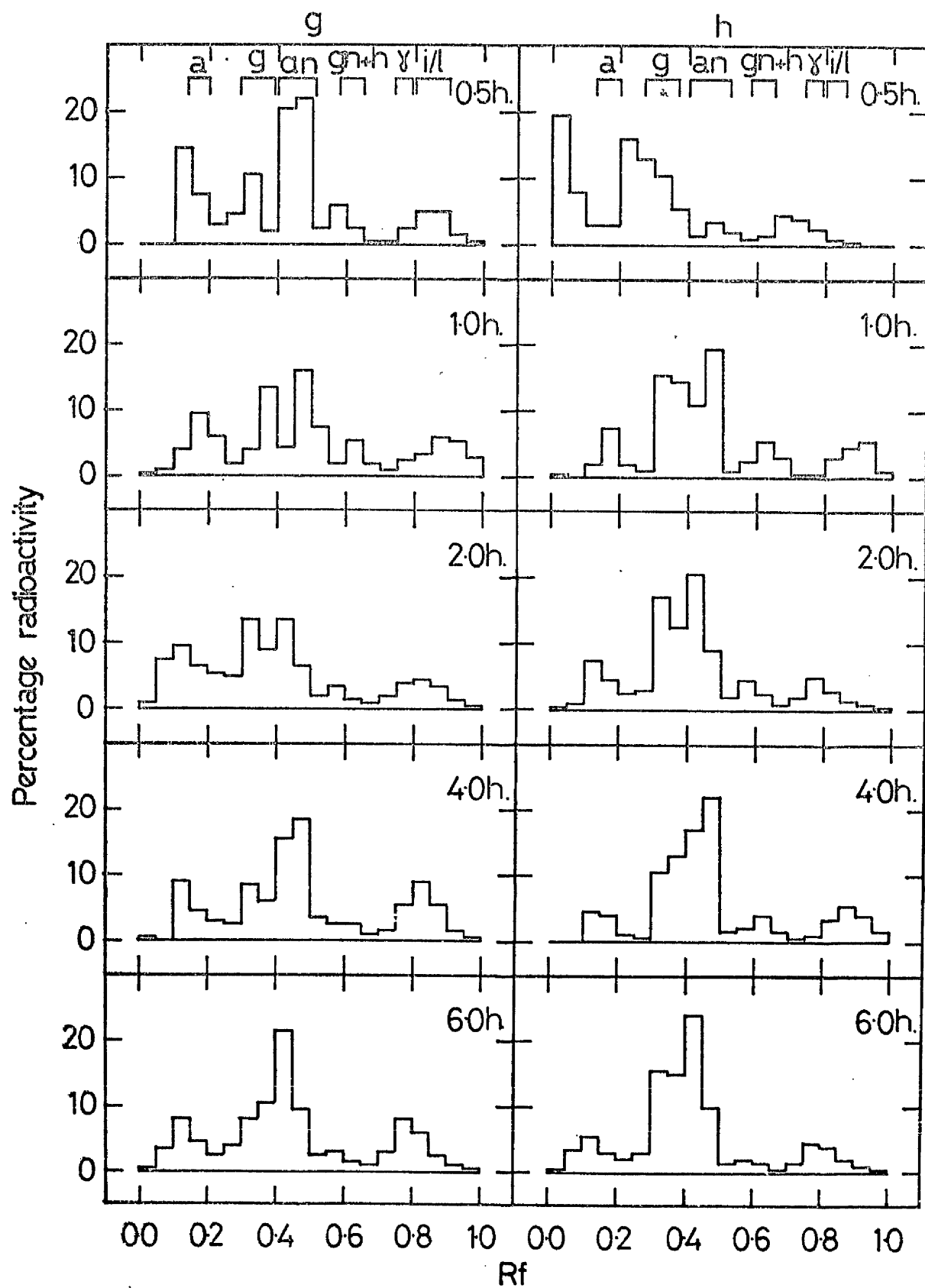


Figs. 35e and f. The distribution of radioactivity in the neutral fraction of ethanolic extracts of broad bean nodules (from 6-week-old plants) 30 minutes after exposure to  $^{14}\text{CO}_2$ . Extracts were chromatographed in distilled water (e) and the solvent BuA (f). Similar distributions of radioactivity were obtained at all times of collection.



Figs. 35g and h.

Changes in the distribution of radioactivity in the basic fraction of ethanolic extracts of broad bean nodules (from plants 6 weeks old) exposed to  $^{14}\text{CO}_2$  either continuously (g) or for 30 minutes only (h). Nodules were collected at the times shown after commencement of exposure and extracts were chromatographed in the solvent PhA. The Rf values of aspartic acid (a), glutamic acid (g), asparagine (an), glutamine (gh), homoserine (h),  $\gamma$ -aminobutyric acid ( $\gamma$ ) and isoleucine/leucine (i/l) in control spots run simultaneously are also shown.





thereafter. In all samples the activity of the acidic fraction was less than 25% of that of the neutral fraction, and less than one-fifth of that of the basic fraction.

(iv) Neutral fraction

The sugars glucose and fructose were present on all chromatograms, but there were no corresponding peaks of radioactivity (Figs. 35 c and d). Extracts were chromatographed in a variety of solvents to separate the radioactive compounds from the sugars, and the following values were obtained for the radioactive compounds:

- a) one spot R<sub>f</sub> 0.90 to 1.10 in IPRAq.
- b) one spot R<sub>f</sub> 0.67 to 0.78 in distilled water (Fig. 35e).
- c) two spots R<sub>f</sub> 0.57 to 0.61 and 0.78 to 0.86 in BuA (Fig. 35f).

The radioactive compounds were visible as purple spots under ultraviolet light and produced black spots with silver nitrate, but no reaction occurred with the other locating reagents used. The compounds involved were probably of high molecular weight and were unlikely to be involved directly in providing carbon skeletons for amination, and so were not investigated further.

(v) Basic fraction

Aspartic acid, asparagine, glutamic acid and leucine/isoleucine were the major labelled compounds detected and increasing amounts of activity were associated with asparagine (Figs. 35 g and h). The asparagine spot on paper chromatograms contained 40% of the activity in this fraction 6 hours after the commencement of exposure to  $^{14}\text{CO}_2$  in both treatments, and there was little difference between corresponding samples in both treatments. Aspartic acid and glutamic acid each declined from 30% to 15% of the total activity from 30 minutes to 6 hours after the beginning of exposure, and the activity in the leucine/isoleucine spot contributed 20% of the total activity at all times.

Figs. 35 i and j. Changes in the distribution of radioactivity in the acidic fraction of ethanolic extracts of broad bean nodules (from plants 6 weeks old) exposed to  $^{14}\text{CO}_2$  either continuously (i) or for 30 minutes only (j). Nodules were collected at the times shown after commencement of exposure and extracts were chromatographed in the solvent PrF. The Rf values of malic acid (m) in control spots run simultaneously and of the unknown compound (x) are also shown.

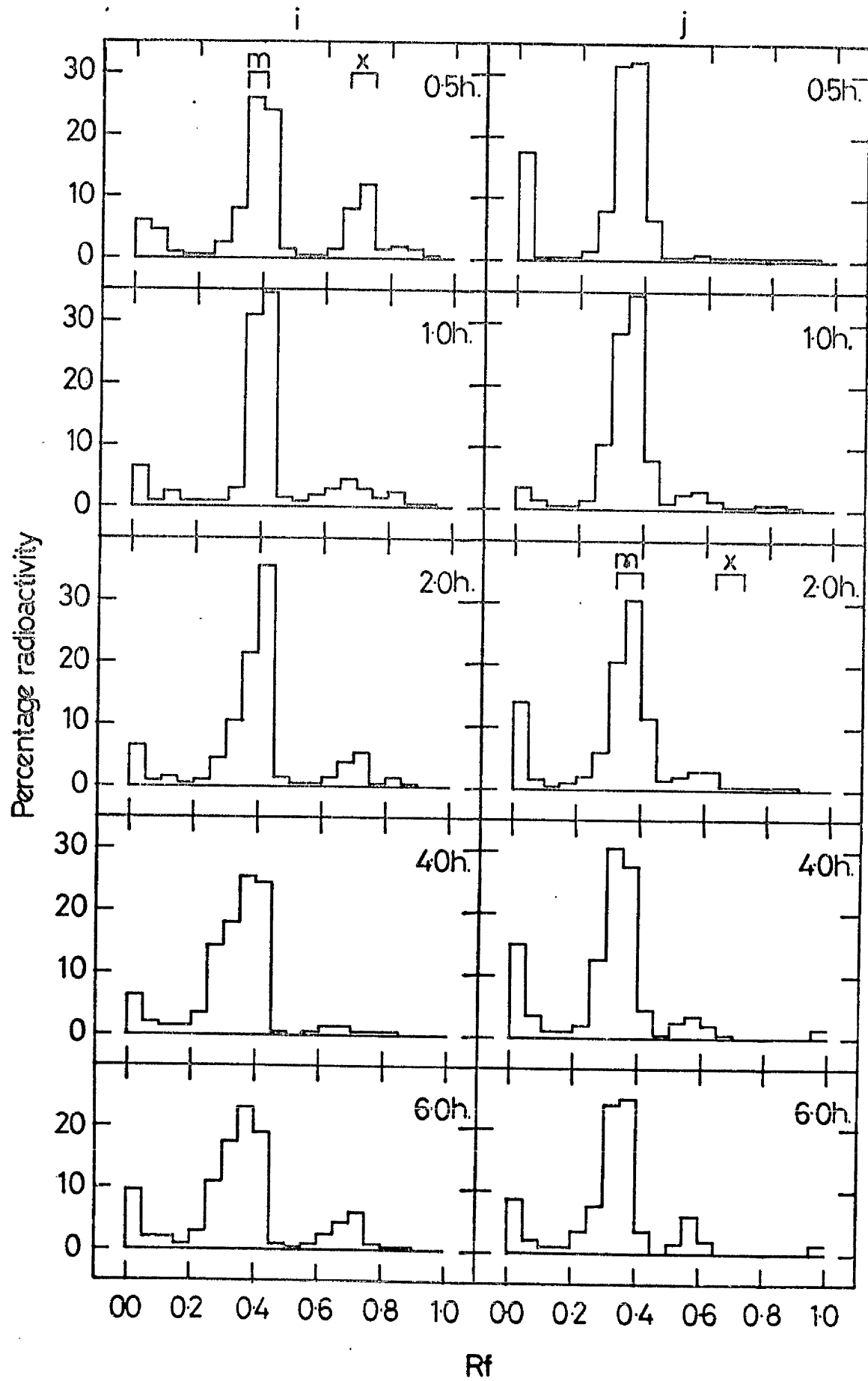
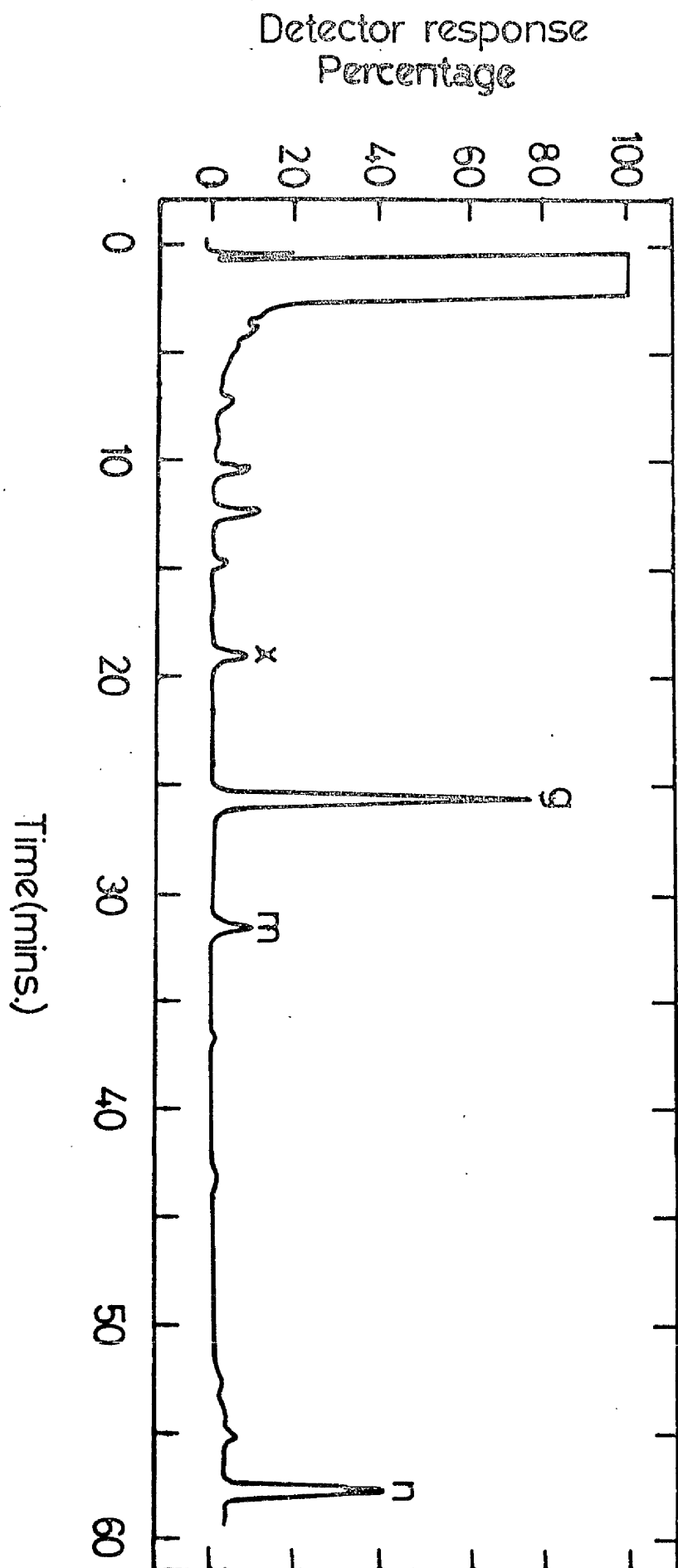


Fig. 35 1.

Separation of compounds obtained after formation of TMS derivatives in the acidic fractions of ethanolic extracts of broad bean nodules (from 6-week-old plants) exposed continuously to  $^{14}\text{CO}_2$ .

A 7 ft. 5% F-60 column was used and the temperature was programmed from 75 to 210°C with gas flow rates of 50 ml.  $\text{min.}^{-1}$   $\text{N}_2$ , 50 ml.  $\text{min.}^{-1}$   $\text{H}_2$  and 150 ml.  $\text{min.}^{-1}$  air. The peaks are believed to be due to eicosane (n) and the TMS derivatives of malic (m) itaconic (x) and glutaric (g) acids.

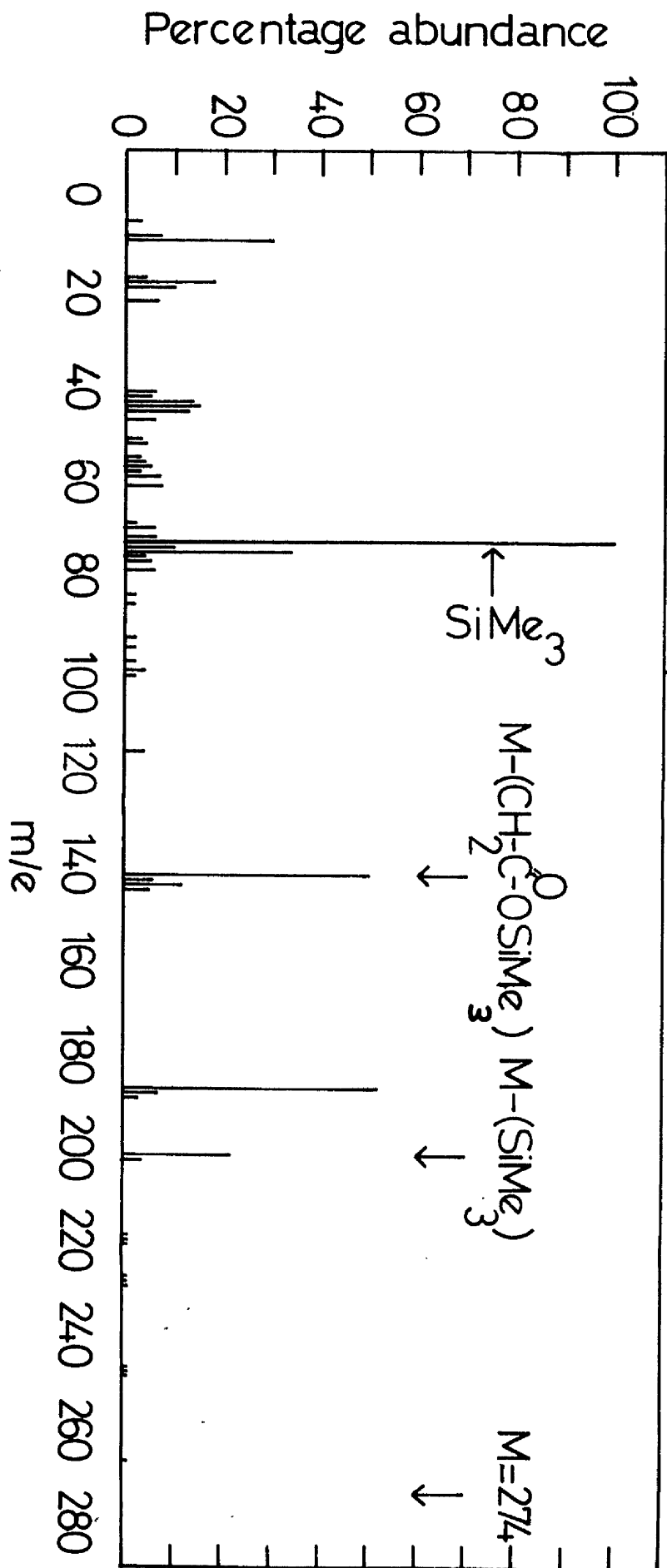


100-1000

These specimens of the same material are being submitted to the Bureau of Standards for analysis. The results of the analysis will be reported to the Bureau of Standards.

Fig. 35m.

Mass spectrum of the "TMS itaconate" peak obtained on GIC analysis of the acidic fractions of ethanollic extracts of broad bean nodules (from 6-week-old plants) exposed continuously to  $^{14}\text{CO}_2$ . Scans were taken using a 1s OV-17 column on an LKB 9000 Gas Chromatograph-Mass Spectrometer.





(vi) Acidic fraction

Two radioactive compounds were detected on paper chromatograms (Fig. 35 i and j). One of these was identified conclusively as malic acid by comparison of the mass spectrum of the MO-TMS derivative with that of authentic MO-TMS malate (Fig. 35k) and malic acid always contained 60% of the total activity in this fraction.

The activity associated with the other compound comprised most of the remainder of the activity of this fraction (up to 25%) and was greater in Treatment D1 than Treatment D2. The following values were determined for this labelled compound on paper chromatograms:

- a) Rf 0.72 in PrF (Figs. 35 i and j).
- b) Rf 0.44 in EtAm.
- c) Rf 0.63 in BuA.

The compound reacted positively with the locating reagents used and GLC analysis gave an  $R_t$  value of 0.347 relative to n-eicosane for the MO-TMS derivative on the 5% F-60 column used (Fig. 35 l). Reaction of the compound with BSTFA alone produced no change in  $R_t$  and the compound was thought to be a 4- or 5-carbon, non-keto acid. Examination of the mass spectrum produced on GC-MS analysis (Fig. 35m) suggested that the unknown compound was itaconic acid (methylene succinic acid or methylene butanedioic acid).

### DISCUSSION

## DISCUSSION

### Assay conditions affecting nitrogenase activity

Although conditions used in the acetylene reduction assay in these experiments resembled those employed by other authors (Hardy *et al.*, 1968; Sprent, 1969; Wheeler, 1969; Waughman, 1972), it was thought desirable to verify that they were suitable for the species studied. Both pea and bean nodules showed similar responses when assay conditions were varied, and it is possible that differences which did occur may be explained in terms of differences in the nature of the nodule samples. In particular, the average size of the bean nodules was about ten times that of pea nodules and detached bean nodules were used, whereas nodulated pea roots were used in the assays because of the small size of the nodules. The parameters thought most likely to influence acetylene-reducing activity - those of temperature of incubation, oxygen and acetylene concentration and the nature of the biological material assayed - were investigated individually.

### Temperature of incubation

Both species showed maximum nitrogenase activity at 25 to 35°C (Sections A 1c and B 1d), which is slightly higher than the optimum temperature recorded for other temperate legumes previously mentioned, such as *Vicia* spp. and *Trifolium* spp. (see Introduction). However, the effects of prolonged exposure to the optimum temperatures found in these assays is not known and therefore these results do not portray accurately the long-term effects of growth at the temperatures examined. Nevertheless, they do show that 25°C was a suitable incubation temperature for the acetylene reduction assays.

The temperature maxima of the two species were not markedly different, even though pea plants were glasshouse-grown whereas bean plants were field-grown and consequently experienced lower mean temperatures during growth than the peas. Roughley and Dart (1969) found that changing the

root temperature of Trifolium subterraneum plants did not alter nitrogenase activity within 24 hours, but both results obtained here and by other authors (Dart and Day, 1971; Wheeler, 1971; Waughman, 1972) indicate that nitrogenase activity is sensitive to ambient temperature.

The response of both pea and bean nodules differed in magnitude, with the increase in activity of pea nodules twice that of bean nodules over the range 5 to 25°C. Arrhenius plots for acetylene reduction by the two species were linear to 25°C (pea) and 35°C (bean) (Figs. 13b and 32b), showing that the apparent activation energy of pea nodules was four times that of bean nodules between 5 and 25°C. Two explanations may be advanced to explain these differences in response. Firstly, it is possible that not all of the infected cells in the bean nodules had reached the ambient temperature in the incubation period used, as bean nodules were about 10 times the volume of pea nodules. However, this seems unlikely, as the activities of random samples incubated for one hour (twice the normal incubation period) were not significantly different from those incubated for half this time. Secondly, increased temperatures could have increased the rate of translocation of photosynthetic assimilates from the pea roots to the nodules, in which case the greater increase in nitrogenase activity in pea nodules could result from increased availability of carbohydrates. In detached bean nodules, nitrogenase activity was dependent on energy sources and carbon skeletons already within the nodules, and so the effects of temperature on activity might be smaller. Although the importance of the temperature on the rate of diffusion of acetylene into the nodules is unknown, it is likely that the effects would be greater on the activity of pea nodules, due to their relatively small mass. The nodules cannot be considered as simple systems for gaseous diffusion, however, as the living cells must exert some control over the movement of gases, such as carbon dioxide, nitrogen and oxygen.

### Oxygen concentration

Nitrogenase activity was greatest in pea and bean nodules when the oxygen concentrations were 0.10 atm. and 0.20 atm. respectively in the acetylene/argon atmosphere used (Sections A 1a and B 1c). In these experiments, when the oxygen concentration was varied, atmospheric pressure was restored to the incubation tubes by appropriate adjustments of the amount of the acetylene/argon mixture added. The simultaneous variations in acetylene concentration may have exerted some influence on the results obtained, and so the effect on nitrogenase activity of varying the acetylene concentration in air was examined. When the oxygen concentration was less than 0.20 atm., the concentration of acetylene increased, up to a maximum of 0.25 atm. There was no significant decrease in the activity of pea nodules with this acetylene concentration in air (Section A 1a), in keeping with the results of Hardy *et al.* (1968), who found that an acetylene concentration of 0.50 atm. did not inhibit nitrogenase activity in Clostridium pasteurianum. In oxygen concentrations greater than 0.20 atm., the acetylene concentration would not be reduced to a level at which it would affect nitrogenase activity (0.10 atm.) until the oxygen concentration exceeded 0.60 atm. (calculated from the data of Fig. 12). Thus it is unlikely that the variations in nitrogenase activity of pea nodules observed over the range 0.00 to 0.20 atm. oxygen were due to simultaneous fluctuations in acetylene concentration. However, the effects on nitrogenase activity in bean nodules is not known, although both the results discussed above and those obtained by other authors (Hardy *et al.*, 1968; Wheeler, 1971; Waughman, 1972) for a variety of species suggest that the acetylene concentration of 0.20 atm. employed would be satisfactory with this species also.

It appears that these results are in general agreement with those for clover (Ferguson and Bond, 1954) and non-legumes (Bond and MacConnell, 1955; Silver, 1969; Waughman, 1972) in that oxygen concentrations of less than

0.20 atm. resulted frequently in maximum nitrogenase activity. This is in contrast to the results obtained for soybeans, where maximum nitrogenase activity was found at a  $pO_2$  of 0.50 atm. (Bergersen, 1962a, 1963, 1970; Mague and Burris, 1972). Possible explanations for this difference are considered below.

Frazer (1942) observed that the common endodermis in legume nodules considerably restricted the penetration of non-toxic dyes into the central infected tissue and concluded that gas exchange between the interior and exterior of the nodules was considerably reduced. Dyes penetrated only to the inner infected cells of those legume nodules with apical meristems, such as those of pea and bean. Although results obtained from liquid diffusion may not be applied strictly to gaseous behaviour, it is likely that penetration of oxygen to the interior of soybean nodules is more restricted than in pea and bean nodules. It is possible that the small size of the pea nodules (less than one-tenth of that of the bean nodules) may have contributed to the greater sensitivity of nitrogenase activity to oxygen, due to increased oxygen penetration to the interior of the nodules. However, Burris, Magee and Bach (1955) showed that nitrogenase activity was maximal in sliced soybean nodules at 0.50 atm. oxygen and Bergersen (1963) found greatest activity in both sliced and whole soybean nodules at 0.50 atm. It is probable that the oxygen permeability barrier is to the interior of the endodermis of soybean nodules. Bergersen (1962b) showed that nitrogenase activity in soybean nodules declined with age and increase in the external  $pO_2$  and attributed decreases to increased oxygenation of the haemoglobin. It is probable that oxygenation of haemoglobin above a favourable level for nitrogenase activity took place in pea and bean nodules at lower oxygen concentrations than 0.50 atm.

The oxygen concentration in closed incubation tubes must be reduced by nodule respiration (Mague and Burris, 1972). Experiments with bean nodules showed that the nitrogenase activity increased slightly when 50% of the gas volume in the assay tube was occupied (Fig. 30). These results contrast

with those of Mague and Burris (1972), who found reduced activity when soybean nodules occupied more than 20% of the gas volume, which they ascribed to lack of oxygen. No similar explanation can be advanced to explain the results obtained here.

#### Biological material

Mechanical damage and oxygen penetration into the nodules are probably the main causes of the reduction in activity of pea nodules on detachment from the roots (Section A 1b), as observed by Bergersen (1970) and Mague and Burris (1972) for soybeans. Waughman (1972) suggested that exposure of the basal ends of non-legume nodules may produce artifacts due to oxygen penetration and it is unlikely that deprivation of supplies of assimilates from the shoots immediately affects activity, since the effect of detachment was immediate and the activity of nodulated roots was not significantly different from that of whole plants. By contrast, Mague and Burris (1972) observed that whole soybean plants were twice as active as nodulated roots, but their assay was different from that used here in that the plant shoots were exposed to light and the atmosphere and so were actively photosynthetic during the assay. Schwinghamer, Evans and Dawson (1970) observed a steady decline over 3 hours in the nitrogenase activity of detached pea nodules to one-fifth of their original activity and they ascribed this fall to lack of assimilates from the host plant. It is clear that assays must be conducted as soon as possible after removal of the plant material from the rooting medium and that serious underestimations of nitrogenase activity may occur if detached nodules rather than nodulated roots are used. Nevertheless, the use of nodulated roots is not always possible, as often plant size makes it necessary to use detached nodules. In the bean plants used in these experiments, the nodules were very large and, because they were broken off both main and lateral roots in handling, it was thought that more reproducible results would be obtained with detached nodules.

The greater nitrogenase activity found in medium rather than small or large nodules shows the importance of uniformity in nodule size in assays with detached nodules (Section B 1a). This agrees with the findings of Aprison, Magee and Burris (1954) and Mague and Burris (1972) for soybean nodules, while Akkermans (1971) noted also that alder nodules with lobes 3.5 to 5.0 mm. long were five times more active than those with lobes 1.0 to 1.5 mm. long. The reasons for the dependence of nitrogenase activity on nodule size in beans have not been examined. However, Bond (1941) proposed that the decreasing activity in older, large soybean nodules may be due to increasing numbers of inactive infected cells, and this may explain results obtained here, as greening had commenced at the base of some large nodules. Pate (1958a) noted that nodule development preceded haemoglobin formation and thus nitrogenase activity in Pisum arvense. Although no such obvious period of maturation was noted in broad bean nodules, it is probable that newly-formed infected cells are relatively inactive, as the apical 1 to 2 mm. of the nodules are white. Thus it is likely that a peak of nodule activity is reached in nodules of medium size and age, when a balance is achieved between loss of activity due to the number of inactive basal cells and immature apical cells with little activity, as discussed by Chen and Thornton (1940).

#### Plant growth and development

Data from total plant nitrogen analyses (Section A 2c) indicate that the acetylene reduction assay reflects accurately total nitrogen fixation by peas from 1 to 4 weeks old, as greatest increases in nitrogen content of plants occurred between weeks 1 and 3, when acetylene-reducing activity rose rapidly. Little additional nitrogen was fixed between weeks 3 and 4 during the rapid decline in acetylene-reducing activity. No attempt was made to correlate more closely the nitrogen fixed with acetylene-reducing activity by the method of Hardy et al. (1968), as was done by Bergersen (1970), Mague



and Burris (1972) and Engin and Sprent (1973), since the acetylene-reducing activities of plants of different ages were determined under standard conditions in a growth cabinet, and these prevailed only transiently in the glasshouse.

The close association between plant development and nitrogenase activity in both pea and bean nodules (Sections A2 and B2) showed that seed production was inhibitory to activity in both species, as shown for soybean (Bond, 1936), Pisum arvense (Pate, 1958a) and Vicia sativa (Pate, 1958b). However, there were differences between the two species in the stage at which inhibition commenced and its subsequent intensity. In peas, activity declined when the flower buds opened and continued to decline to virtually zero thereafter as the nodules became totally green. By contrast, the activity of bean nodules declined only when fruit formation was advanced, the nodules never became totally green and still retained 20% of their maximum activity until the plants were killed by frost. These differences could be due to the different reproductive patterns in the two species, since continued flowering and fruiting took place in beans, while fruiting in peas ended plant life. Differences in nitrogenase activity between soybeans with indeterminate and determinate flowering patterns were observed by Hardy et al. (1971) and it was noted that the former fixed only 10% of its total nitrogen before flowering, whereas the latter fixed 25% of its total nitrogen at this time. It is thus possible that the flowering process itself does not inhibit nitrogen fixation, but that fruit development may be the main factor responsible for inhibition of nitrogenase activity and that continued growth by the shoot apex during fruiting may reduce the inhibition. Although some inhibition of nitrogenase activity was noted in peas as soon as the flower buds opened, this may be due to their habit of self-pollination, thus shortening the interval between flowering and fruit formation. It is also possible that the differences observed were due to the fact that peas were glasshouse-grown, whereas beans were field-grown, and this may partially

explain the different results obtained by Bond (1936) and Hardy *et al.* (1968) with glasshouse-grown and field-grown soybeans respectively. Growth in nitrogen-free culture may result in an artificially rapid and large inhibition of fixation after flowering, but it is thought unlikely that the differences in intensity of inhibition between the two species studied in these experiments were totally explicable in this way, since Pate (1958a) observed total greening in nodules of field-grown Pisum arvense plants after fruiting.

In peas, the close parallel between nitrogenase activity and the accumulation of labelled photosynthates in the nodules (Section A2) during the growth cycle of plants of Batch 1 suggests that the most active nodules (on 3-week-old plants) receive the largest quantities of photosynthates and that their activities may be dependent on the quantities of photosynthates received. The presence of haemoglobin in nodules indicates active nitrogen fixation (Virtanen, 1945, 1947; Jordan and Garrard, 1951) and the greater accumulation of photosynthates in active (pink) nodules than in inactive (green) nodules (Fig. 17e) provides confirmation of these suggestions. The maximum in accumulation of <sup>14</sup>C-labelled photosynthates in the nodules of Batch 2 plants at week 2, a week earlier than the maximum in nitrogenase activity, suggests that a large proportion of the photosynthesized carbohydrates accumulating in the nodules is not used in nitrogen fixation, but in other nodule functions such as growth and respiration, as suggested by Wheeler (1971) for young alder nodules. This is in agreement with the findings of Minchin and Pate (1973), who estimated that, in pea nodules, 17% of the photosynthate translocated to the nodules was consumed in growth, 36% in respiration (including support of nitrogen fixation) and 47% returned to the shoot as aminated compounds. Thus at least 17% of the photosynthates reaching the nodules, plus an unknown proportion of the respired photosynthate is not used to support nitrogenase activity and subsequent metabolism of the ammonia formed.

There were no significant differences in the respiration rates of

nodules of plants 3 to 5 weeks old (Section A 2e), as shown by Minchin and Pate (1973), even though both nitrogenase activity and accumulation of  $^{14}\text{C}$ -labelled photosynthates by nodules showed significant decreases over this period. It is likely, therefore, that changes in the latter resulted from a decline in carbohydrate requirements for growth and nitrogenase activity rather than from changes in respiration. However, the nitrogenase activity of detached pea nodules fell to one-eighth of that of attached nodules immediately after detachment from the root, and similar effects on nodule respiration could well hide otherwise significant differences.

Further information on the respiration of the nodules was obtained by "pulse - cold-chase"  $^{14}\text{CO}_2$  feeding experiments carried out with pea plants of different ages. Analysis of the radioactivity of the nodules following exposure of the shoots to  $^{14}\text{CO}_2$  for 30 minutes showed that the maximum accumulation of labelled photosynthates in nodules of 2-week-old plants always occurred 4 to 8 hours after exposure and was followed by a usually significant decline of up to 50% in the following 16 to 20 hours (Fig. 17a). By contrast, the decline in radioactivity between 4 and 24 hours after exposure of the shoots of 3-week-old plants to  $^{14}\text{CO}_2$  was not always significant, although these nodules were most active in nitrogen fixation. This suggests that nodule respiration, rather than export of aminated compounds to the shoot, is the activity mainly responsible for the decrease in  $^{14}\text{C}$ -labelled photosynthates in nodules from 2-week-old plants, although the respiration rate of these nodules was not assessed due to their small size. Minchin and Pate (1973) calculated that 47% of the carbohydrates translocated to the nodules were re-exported to the shoot as aminated compounds, and the relatively small decline in the radioactivity of the nodules on 3-week-old plants suggests that such compounds do not accumulate in the nodules, but that there is a continual flow of carbohydrates into the nodules and of aminated carbon skeletons from the nodules to the shoot. In plants 4 to 6 weeks old, which are relatively inactive in nitrogen fixation, the small

change in radioactivity of the nodules from 4 to 24 hours after exposure of the shoot to  $^{14}\text{CO}_2$  (Fig. 17a) suggests that the rate of influx of carbohydrates from the shoot equals their consumption for respiration or that there is a large inactive "pool" of carbohydrates within the nodules.

The high respiration rate of nodules (nodules from 3-week-old plants were three times as active as the corresponding roots) suggests immediately that the nodules must be one of the most active "sinks" for photosynthates in the plant. However, determination of the specific activity of the different plant parts shows that they were not so active as the shoot tips and young expanding leaves of vegetative plants, although it is likely that analyses of the radioactivity in the nodules after exposure of the shoot to  $^{14}\text{CO}_2$  will underestimate their potential as a "sink" because the nodules are continually exporting aminated compounds. It is clear from autoradiographs (Section A 8a) that root tips were also powerful "sinks" for photosynthates within the plants, and thus the actively meristematic regions of the plants constituted the most active "sinks" for photosynthates, as reported by Harvey (1970) for clover. However, the relative proportions of the total radioactivity of the plants accumulated by the different plant parts showed that the nodules never accumulated more than 5% of the total activity in the plants (Section A 6a). The roots of 2-week-old plants accumulated 39% of the total, however, and this may be of importance in the support of early nodule development. As the plants aged, the proportion of the total radioactive photosynthate accumulated by the roots fell to 33% (3-week-old plants) and 25% (4-week-old plants) and this may be one of the causes of the reduction in root growth and nodulation in plants older than 3 weeks.

The decrease in the proportion of assimilates accumulated by the roots is directly related to increases in accumulation by the shoot tip as it changes from vegetative (2-week-old plants) to flowering (3-week-old plants) and fruiting (4-week-old plants). The two-fold and four-fold increases in accumulation of photosynthates by the shoot tip when plants flowered and

fruited respectively must be powerful factors in reducing the quantities of photosynthates available for root and nodule development and metabolic activity. Certainly the specific activity of the nodules decreased by 60% during the period from vegetative plant growth to fruiting, whereas the total accumulation of photosynthates by the whole plants doubled. This implies that the distribution of photosynthates within the plant was influenced greatly by its stage of development, i.e. vegetative or reproductive growth.

It might be expected, therefore, that detachment of the shoot tips of vegetative plants would have less effect on the accumulation of photosynthates in the nodules than detachment of fruiting apices, since a smaller proportion of the total photosynthates would be available for redirection into other plant "sinks". The nitrogenase activity would be expected to show similar changes if it were primarily dependent on the amount of photosynthates available to the nodules. Significant increases in accumulation of  $^{14}\text{C}$ -labelled photosynthates were achieved in nodules of vegetative, flowering and fruiting plants after decapitation, but only in fruiting plants were there always significant increases in nitrogenase activity (Section A 6c), although the large variations in experimental samples may have concealed some changes. For example, the accumulation of labelled photosynthates by the nodules of treated vegetative plants was significantly greater than that of nodules of intact plants 4 and 12 hours after removal of the apex, whereas their nitrogenase activity was significantly greater only 8 hours after treatment in one experiment (Figs. 28 a and b). Moreover, detachment of the flowers resulted consistently in an almost significant decline in the nitrogenase activity of treated plants, even though their nodules accumulated significantly more photosynthates than those of intact plants (Figs. 28 c and d). However, it is unknown how much of the photosynthates normally directed to the shoot apex were redirected to other "sinks" in the plant in this and other treatments, such as to axillary buds and root tips. Lovell, Oo and Sagar (1972) reported that removal of the shoot apex of pea plants reduced export from the leaves and suggested that export of assimilates from the

leaves was governed mainly by the activity of the "sinks" of the plant. This observation supports the proposal of Hardy et al. (1968) that nitrogenase activity is governed mainly by the demand of the plant for nitrogen so that "sink" demand governs the activity of the source. This may explain some of the results obtained in these experiments in that removal of the apex demand for nitrogen may negate possible increases in nitrogenase activity resulting from increased availability of photosynthates. However, the fact that increased levels of nitrogenase activity and  $^{14}\text{C}$ -labelled photosynthates in the nodules of fruiting plants (Figs. 28 e and f) were produced within only 24 hours of detachment of the apex suggests that the shoot apex exerts some control over nodule activity by its accumulation of photosynthates.

In long-term experiments, removal of the flower buds from 3-week-old plants for 2 weeks resulted in increases in nitrogenase activity and nodule yield, as reported by Ropenen and Virtanen (1968), and accumulation of  $^{14}\text{C}$ -labelled photosynthates by the nodules of treated as compared with untreated plants (Section A b,c). In such long-term experiments, however, it is possible that many factors are involved in the control of nodule metabolism. Thus removal of the shoot tip alters the hormonal balance within the plant, resulting in the production of abnormally thick stems and leaf blades and the development of axillary buds. The effects of hormonal changes on nitrogenase activity have not been investigated thoroughly although high levels of free auxins and gibberellic acid have been reported in nodules of Pisum arvense (Pate, 1958c), alder (Dullaart, 1970b) and lupin (Dullaart, 1970a; Dullaart and Duba, 1973) and at least part of the auxin in alder nodules is produced by the plant in response to infection (Dullaart, 1970b). It has also been shown that the auxin content of nodules falls towards the end of plant life (Thimann, 1963) in several species, although no significant changes in auxin levels in alder nodules were found during one year's growth (Dullaart, 1970b). The possibility of fluctuations in the hormone content of nodules during flowering and fruiting, such as those observed by Pate (1958c) in

Pisum sativum nodules, affecting carbohydrate supply and nitrogenase activity cannot be eliminated.

### The Importance of Light

#### Darkening

The decline in nitrogenase activity of pea plants by as much as one-third after only 8 hours in the dark (Section A 5a) suggests that this was primarily due to a reduction in the translocation of photosynthates to the nodules, as proposed by Virtanen, Moisio and Burris (1955), Hardy et al. (1968) and Wheeler (1971). Fewer carbon skeletons and consequently less photosynthate should be required for removal of ammonia into organic combination, but this is not apparent from  $^{14}\text{CO}_2$  feeding experiments, which showed no difference after 10 hours "cold-chase" in the accumulation of  $^{14}\text{C}$ -labelled photosynthates in nodules of plants maintained in the light or darkened after exposure to  $^{14}\text{CO}_2$ . This suggests that nitrogenase activity may be one of the first metabolic activities in the nodules to be affected by a reduction in carbohydrate supply and that when photosynthates are in short supply they are used preferentially to support other aspects of nodule metabolism. After 16 hours in the dark, the continued absence of photosynthesis resulted in a reduction in the carbohydrate content of the nodules and in further reductions in nitrogenase activity, which were paralleled by significant reductions in the accumulation of photosynthates in nodules of darkened plants exposed to  $^{14}\text{CO}_2$ . These results suggest that nitrogenase activity is supported primarily by newly-photosynthesised carbohydrates and that their accumulation in the nodules is determined by the nitrogenase activity of the bacteroids. However, it must be remembered that the carbohydrate requirements for nodule growth and respiration may also alter under these conditions, influence greatly the "sink" value of the nodules and thus alter their accumulation of photosynthates.

Nodules of darkened plants returned to the light showed an earlier

recovery of accumulation of photosynthates than nitrogenase activity (Section A 5c) and the time taken for recovery of both activities was dependent on the length of darkening. It is possible that the bacteroids must be returned to an active metabolic state by ingress of photosynthates before nitrogenase activity is recommenced. However, Virtanen, Moisio and Burris (1955) ascribed the incomplete recovery of peas darkened for 24 hours to partial destruction of haemoglobin and it is possible that this also may have affected the plants used here, although greening of the nodules was not noticed until the plants had been darkened for 48 hours.

Although active pea nodules contain large numbers of starch grains (Section A 8b) (Dangeard, 1926; Löhnis, 1930), and PHB granules have been observed (Dixon, 1964), there is little evidence to suggest that these reserve substances are utilized extensively to support nitrogen fixation when supplies of photosynthates are removed. The continued presence of starch grains in the nodules during the period when nitrogenase activity was lost in darkened pea plants (Section A 8b) suggests that starch was little utilized in support of fixation, although no quantitative estimates were made. These observations are supported by the data obtained by Wheeler (1971), who did not find a significant decline in the level of storage carbohydrates during the initial fall in nitrogenase activity in darkened alder plants.

PHB was not detected as a reserve substance in nodules (Section A 2f), but Wong and Evans (1971) could not obtain evidence for its utilization in support of nitrogenase activity in darkened soybeans even when it formed 50% of the dry weight of the bacteroids. It seems unlikely, therefore, that small amounts which might have remained undetected during analysis of the pea nodules could support nitrogenase activity in the absence of photosynthates, and the decline in nitrogenase activity to virtually zero in 24 hours supports this suggestion.

Thus evidence presented here supports the suggestion that lack of carbohydrates from photosynthesis is the primary factor leading to the decline of



nitrogenase activity in darkened plants and there is no evidence that either starch or PHB as reserve substances in the nodules supported nitrogenase activity when supplies of photosynthates were withdrawn.

#### Photoperiod

The influence of photoperiod on the nitrogenase activity of pea nodules after only 3 days of treatment (Section A 3a) was similar to that reported by Federov and Uspenskaya (1955), who proposed that the 16 hour photoperiods suitable for flowering favoured nodule development. Although the accumulation of  $^{14}\text{C}$ -labelled photosynthates in the nodules was also affected by photoperiod, changes did not occur within the same range as changes in nitrogenase activity. Thus, whereas increases in accumulation of  $^{14}\text{C}$ -labelled photosynthates were found only with photoperiods between 8 and 16 hours, nitrogenase activity was affected over the whole range from 4 to 20 h. However, it must be emphasised that accumulation of photosynthates was measured after only 3.5 hours "cold-chase" and the final accumulation which occurred in each treatment is not known. The doubling in both the nitrogenase activity and accumulation of  $^{14}\text{C}$ -labelled photosynthates when the photoperiod was increased from 8 to 16 hours suggests that there is a certain "proportional range" within which nitrogenase activity and accumulation of  $^{14}\text{C}$ -labelled photosynthates are mutually related. The decline in nitrogenase activity when plants were kept in continuous illumination was attributed by Federov and Uspenskaya (1955) to premature ageing of the bacteroids, due to carbohydrate deficiency, and in these experiments there was a corresponding decline in accumulation of photosynthates.

#### Light intensity

Gradually increasing the light intensity within a growth cabinet to simulate normal daily changes in sunlight had no effect on the nitrogenase activity of pea nodules, apart from significant decreases in activity with light intensities greater than 2000 ft. candles (Section A 3b). These

results contrast with those of Wheeler (1969), who found that the nitrogenase activity of alder nodules could be correlated with changes in the intensity of natural daylight in a glasshouse kept at approximately constant temperature. It would be incorrect to compare the data directly, however, since the spectra of the fluorescent tubes used in these experiments differ markedly from that of natural daylight, and it is possible that insufficient differences in photosynthetically useful light were created to cause variations in nitrogenase activity. In addition, the reduction in nitrogenase activity when light intensities were increased above 2000 ft. candles suggests that some component in the light sources used was inhibitory to nitrogenase activity.

#### Diurnal variations

The diurnal fluctuations in nitrogenase activity in peas (Section A4) are thought to be the result of the cumulative effects of temperature and light intensity, the latter of which affects photosynthesis and hence availability of carbohydrates as energy sources and carbon skeletons for amination in the nodules. The evening maxima at 2000 to 2200 hours in nitrogenase activity closely succeeded maxima in the ethanol-soluble carbohydrate concentration in the nodules at 1800 hours. It is probable that nitrogenase activity was determined by light intensity through photosynthesis, with a delay of 6 to 8 hours, which was the time established for translocation of a substantial quantity of photosynthates from the 30 minute period of photosynthesis to the nodules (Section A 2c). Although clear maxima in the translocation of newly-photosynthesized assimilates to the nodules were seen at 1300 to 1400 hours (Fig. 23), it appears that the quantity of photosynthates translocated during this one hour period was only a small fraction of the total photosynthate accumulating in the nodules, as the total ethanol-soluble carbohydrate content did not rise until 1800 hours (Figs. 22 and 24).

Changes in air temperature were not responsible for the evening maxima in nitrogenase activity, but it is possible that they are the cause of the

mid-day maxima observed in some experiments (e.g. Fig. 24), and Mackintosh (1969) claimed that fluctuations in the nitrogenase activity of Casuarina cunninghamiana could be explained wholly in terms of its response to fluctuations in air temperature. In the experiments illustrated, the air temperature varied from 0800 until 1400 <sup>hours</sup> over the range 14 to 26°C in the experiment with a mid-day maximum (Fig. 24), whereas temperature varied over the range 23 to 33°C in the experiment with no mid-day maximum (Fig. 22). Nitrogenase activity was maximal at 25 to 30°C in pea nodules (Fig. 13b) and increasing the incubation temperature during the acetylene reduction assay from 15 to 25°C resulted in an increase in nitrogenase activity more than four times that of increasing the temperature from 25 to 35°C. Thus it is to be expected that the observed increase in air temperature from 15 to 26°C would have increased the nitrogenase activity eight-fold, while the increase from 23 to 33°C would have had little effect on activity (Table 2). However, the mid-day maximum observed in Fig. 24 was less than would be expected from these calculations and it is probable that at this time lack of carbohydrates was limiting nitrogenase activity.

It is likely, therefore, that the primary factor influencing nitrogenase activity in pea nodules in these glasshouse experiments was the substantial quantity of photosynthates translocated to the nodules with a delay of 6 to 8 hours after the time of maximum light intensity, around mid-day, and that changes in temperature in the range 15 to 25°C caused additional mid-day maxima. These results appear to contrast with the mid-day maxima in nitrogenase activity found by many authors cited in the Introduction (Hardy et al., 1968; Wheeler, 1969; Bergersen, 1970; Mague and Burris, 1972), but to agree with those of Rodriguez-Barrueco (1967) for Casuarina torulosa using  $^{15}\text{N}_2$ . Mid-day maxima in nitrogenase activity could result from an increased availability of photosynthates at this time if sufficient quantities of photosynthates were transported without delay to the nodules. However, if there is a delay in the translocation to the nodules of effective quantities

of photosynthates, as observed here, than the maximum in nitrogenase activity will not correspond to the maximum in light intensity.

#### The metabolism of carbohydrates in the nodules

Information on the metabolism of photosynthates in the pea and bean nodules was obtained by analysis of ethanolic extracts of the nodules harvested after exposure of the plant shoots to  $^{14}\text{CO}_2$  (Sections A7 and B 4a). Ion-exchange chromatography was employed to fractionate the extracts into "neutral" (mainly sugars), "basic" (mainly amino-acids and amides) and "acidic" (mainly organic acids) fractions.

The neutral fraction always contained more than 60% of the total ethanol-soluble activity (Figs. 29b and 34 e and f). Similar results were found by Bach, Magee and Burris (1958) in soybean nodules and by Wheeler (1971) in alder nodules, and the latter author suggested that a large proportion of the photosynthates in the nodules is not available for use in nitrogen fixation. The fall in ethanol-soluble activity as a percentage of the total radioactivity in pea nodules with increasing time of "cold-chase" is probably due to the formation of insoluble compounds and incorporation of radioactivity into structural elements in the cells. Sucrose was a prominent constituent of the neutral fraction of pea nodules although little radioactivity was associated with this sugar until 11.5 hours after exposure of the shoot to  $^{14}\text{CO}_2$ , and this indicated that sucrose was actively metabolised on arrival in these nodules. The presence of  $^{14}\text{C}$ -labelled sucrose in the nodules after longer periods of "cold-chase" (11.5 and 23.5 hours) suggests that sucrose translocated some time after its photosynthesis to the nodules may not be metabolised so rapidly as that translocated with little delay. In bean nodules (Section B 4a) and soybean nodules (Bach, Magee and Burris, 1958), photosynthates translocated as sucrose to the nodules were metabolised immediately to glucose and fructose, as sucrose was not detected. This is in agreement with the reports that

there was at least one active invertase in nodules of serradella (Kidby, 1966) and lupin (Robertson and Taylor, 1973).

The rapid utilization of carbon skeletons from sugars newly-arrived in the nodules is shown by the occurrence of radioactivity in the basic fraction of bean nodules after only 30 minutes exposure of the shoot to  $^{14}\text{CO}_2$  (Figs. 34 c and d). A similar rapid incorporation of activity into the basic fraction of alder nodules was reported by Wheeler (1971). Asparagine was the most prominent compound in both pea and bean and accumulated up to half the total radioactivity in the basic fractions of pea nodules (Figs. 29b and 34 g and h). It is probable that this is the form in which most of the fixed nitrogen was transported to the plants, as it was reported in large quantities in root exudates of pea (Weiringa and Bakhuis, 1957) and broad bean (Pate, Gunning and Briarty, 1969). A considerable amount of radioactivity was associated also with glutamic acid, glutamine and aspartic acid, especially in collections made soon after exposure to  $^{14}\text{CO}_2$ , and it is probable that in both species the primary product of nitrogen fixation is glutamic acid, as found for soybean nodules (Zelitch, Wilson and Burris, 1952), non-legume nodules (Leaf, Gardner and Bond, 1958, 1959) and serradella nodules (Kennedy, 1966a,b).

The acidic fractions of extracts of both pea and bean nodules contained only one labelled organic acid - malic acid - which accumulated up to 40% of the total activity of this fraction (Figs. 29d and 34 i and j).  $^{14}\text{C}$ -malic acid was also detected in soybean nodules (Bach, Magee and Burris, 1958) and it is possible that, as suggested by these authors, this is indicative of the rate of removal of other organic acids suitable as acceptors for ammonia. Bean nodules were metabolically more active than the roots, in which large amounts of completely unlabelled malic acid were detected, but malic acid commonly accumulates in large amounts in plant cells (James, 1973).

In addition to studies on the metabolism of photosynthates translocated to both pea and broad bean nodules, the metabolism of assimilates produced

as a result of dark fixation of  $^{14}\text{CO}_2$  by detached bean nodules was studied (Section B 4b). The experiments are referred to as follows:

- Treatment L1.    Photosynthetic fixation by bean shoots exposed continuously to  $^{14}\text{CO}_2$ .
- Treatment L2.    Photosynthetic fixation by bean shoots exposed to  $^{14}\text{CO}_2$  for 30 minutes only.
- Treatment D1.    Dark fixation by detached bean nodules exposed continuously to  $^{14}\text{CO}_2$ .
- Treatment D2.    Dark fixation by detached bean nodules exposed to  $^{14}\text{CO}_2$  for 30 minutes only.

Most of the activity in extracts of nodules from Treatments D1 and D2 was recovered in the basic fraction, in contrast to results obtained in Treatments L1 and L2 where most of the activity was in the neutral fraction. The increase in activity of the basic fraction was presumably due to the retention of aminated  $^{14}\text{C}$ -labelled acceptor molecules within the nodules. As the radioactivity of the basic fraction continued to increase even 6 hours after detachment of the nodules (Fig. 35a) and nitrogenase activity did not decline significantly during this time (Fig. 3), it seems unlikely that the presence of a larger than normal concentration of amino-acids and amides in the nodules was inhibitory to nitrogenase activity. It is unlikely that suitable acceptor molecules for ammonia were derived from reducing sugars as in Treatments L1 and L2, since sugars were not detectably labelled (Figs. 35 c-f), and it is probable that the acceptor molecules were formed directly from organic acids produced by  $^{14}\text{CO}_2$  fixation. As malic acid contained more than 75% of the total radioactivity in this fraction (Figs. 35 i and j), it is probable that this and other acids were formed by carboxylation reactions and that suitable acceptor molecules were rapidly aminated. This resulted in the observed pattern of accumulation of firstly  $^{14}\text{C}$ -labelled aspartic and glutamic acids, and then increasing amounts of  $^{14}\text{C}$ -labelled asparagine (Figs. 35 g and h).

Up to 25% of the radioactivity in the acidic fraction was present in Treatments D1 and D2 as an acidic compound which is believed to be itaconic acid. This acid was also found by Coic and Lesaint (1960) in small quantities in broad bean nodules immediately after detachment. No traces of "itaconic" acid were found in nodules freshly detached from the roots in these experiments, but the quantity found in nodules fed  $^{14}\text{CO}_2$  for 30 minutes equalled that of malic acid (as estimated from Fig. 35.1). It is probable that itaconic acid is formed by decarboxylation of  $^{14}\text{C}$ -labelled cis-aconitic acid under acid conditions and high concentrations of sugars, as in Aspergillus terreus (Bentley and Thiessen, 1957). Although little information is available in the literature on the occurrence of this acid, it may be a product of bacterial metabolism rather than that of the host plant.

It is clear that initially formation of aminated compounds from suitable acceptor molecules was extremely rapid, as the basic fraction was the most radioactive after only 30 minutes exposure to  $^{14}\text{CO}_2$  and always contained twice as much activity as the acidic fraction. The contrast between the continued increase in total radioactivity in Treatment D1 and the decrease in Treatment D2 after the exposure period of 30 minutes to  $^{14}\text{CO}_2$  was probably due to respiratory loss of the fixed  $^{14}\text{CO}_2$ .

It is clear that nodule bacteroids can use suitable acceptor molecules derived from  $^{14}\text{CO}_2$  fixation to provide carbon skeletons for nitrogen fixation when the carbohydrate supply from the plant is removed. There may be a similar function for  $^{14}\text{CO}_2$  fixation in nodules still attached to the plants, as proposed by Mulder and Van Veen (1960), and Minchin and Pate (1973) stated that carboxylation reactions in nodulated pea roots led to the formation of malate and aspartate. The pattern of labelling in the basic fraction closely resembled that of nodules still attached to the plant, suggesting that fixation and metabolism of  $^{14}\text{CO}_2$  was performed mainly by the infected cells.

Microautoradiography

Similar conclusions may be drawn from microautoradiographic examination of sections of bean nodules from both Treatments L2 and D2 (Section B3), as radioactivity was associated almost exclusively with the infected cells. The heavy labelling of vascular traces in Treatment L2, and its absence in Treatment D2, illustrates the normal mode of translocation of photosynthates to the nodules.

More detailed studies carried out with pea nodules indicated that translocation of  $^{14}\text{C}$ -labelled compounds took place through the heavily labelled vascular traces and that the infected cells were the main "sink" for photosynthates in the nodules, since the uninfected cortex was almost entirely unlabelled (Section A 8b). Marked accumulation was also associated with cells of the common endodermis, which contained numerous starch grains, and this suggested that these cells acted as barriers to outward movement of photosynthetic assimilates at which excess photosynthate was converted to starch as a storage reserve.

The pattern of accumulation of radioactivity (Section A 8b) differed from the distribution of starch grains (Section A 8b) and nitrogenase activity, as determined by the reduction of tetrazolium salts (Section A9) (Stewart, Haystead and Pearson, 1969; Akkermans, 1971). Whereas most accumulation of  $^{14}\text{C}$ -labelled photosynthates took place in younger infected cells in which the bacteria were still multiplying, most reducing activity and starch deposition occurred in older cells which were totally filled with bacteroids. These results therefore provide further support for the suggestion that a large part of the nodule carbohydrates are not available for use in the support of nitrogenase activity. The accumulation of photosynthates by younger, growing cells which are concentrated near the meristematic tip and are less active in nitrogen fixation than older cells more densely filled with bacteroids would result in most of the photosynthates accumulating in the nodules being unavailable to the cells which contribute



most to the overall fixation of nitrogen. It is proposed that nitrogen fixation in the cells densely filled with bacteroids, in which growth and multiplication have virtually ceased, is supported by a rapid undetected flux of photosynthates, since there was little accumulation in the cells, although the supply of photosynthates exerted a marked influence on nitrogenase activity. It is possible that these mature infected cells, in which nitrogen fixation takes place, are supplied with photosynthates only when the requirements of the younger infected cells have been met. This suggestion is supported by the fact that when plants were darkened immediately after exposure of the shoots to  $^{14}\text{CO}_2$  (Section A 5b), there was no difference in the pattern of accumulation of  $^{14}\text{C}$ -labelled photosynthates in the nodules while nitrogenase activity declined. In such conditions, almost all the photosynthates in the nodules would be sequestered for use in respiration, growth and multiplication of meristematic cells and of the bacteroids in young infected cells and little, if any, would be available to support nitrogen fixation.

This rapid utilization of photosynthates suggests that the formation of the large numbers of starch grains associated with cells densely filled with bacteroids until greening begins (Section A 8b) is unlikely to occur when the cells are active in nitrogen fixation. It is possible that starch formation takes place when there is a temporary excess of photosynthates in the nodules, as may occur when the infected cells begin to restrict the growth and multiplication of the bacteroids. It is obvious that these starch grains do not support nitrogen fixation when supplies of photosynthates are limiting, such as during darkening, and McCoy (1929) proposed that the bacteroids were unable to metabolize starch. Its disappearance from pea nodules in greening tissue and its accumulation in uninfected cells in the infected area suggest that control over its deposition and digestion is exerted by the host plant, perhaps to regulate bacterial growth by removal of excess photosynthate. The digestion of starch in greening tissue does

not prevent the fall in fixation and McCoy (1929) suggested that the host cell mitochondria took part in its digestion. This seems unlikely, since recent research has indicated that the enzymes associated with starch metabolism are associated with the outer membrane of the plastid, but it must not be forgotten that the infected cells are still alive - for instance, Muecke and Wiskich (1969) reported that the mitochondria of infected soybean nodule cells were still capable of respiratory activity.

In conclusion, it appears that nitrogenase activity within pea nodules is normally dependent on maintenance of adequate supplies of photosynthates from the shoots. However, a large proportion of the photosynthates entering the nodules is not used to support nitrogen fixation, but to support growth of the meristematic regions of the nodules and growth and multiplication of the bacteroids in cells not active in nitrogen fixation. Therefore, nitrogen fixation is probably supported by supplies of photosynthates which do not accumulate in the nodules, are exported from actively nitrogen-fixing cells soon after amination and are only available when the requirements of the actively growing regions of the nodules have been met.

Because the main storage reserves in the nodules do not appear to be used to support nitrogen fixation, maintenance of the supply of photosynthates is clearly extremely important to the maintenance of nitrogenase activity in the pea plants used in these experiments.

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ERRATA

The references cited as Smith (1962) and Virtanen (1947) should be Smith (1960) and Virtanen et al. (1947) respectively.