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The biosynthetic pathway of the S-alk(en)yl-L-cysteine sulphoxides (flavour precursors) in species of *Allium*

S. J. Edwards[1,2], G. Britton[1] & H. A. Collin[2]
[1]*Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, UK;* [2]*Department of Genetics and Microbiology, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, UK*

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Abstract

Pulse labelling experiments with $^{35}SO_4^{2-}$ fed for 24h to intact plants (shooted onion sets) of *Allium cepa* (onion) showed that > 70% of the label appeared in the S-alkenyl-L-cysteine sulphoxides within 18h, reached a maximum at 48h and thereafter decreased. The amount of label detected in the γ-glutamyl peptide fractions was below 20% of the total label at any time. It is concluded that in intact plants (at the growth stage used) the γ-glutamyl peptides are not the immediate precursors of the S-alkenyl-L-cysteine sulphoxides. The major S-alkenyl-L-cysteine sulphoxide in onion was found to be compartmentalized mainly within the endoplasmatic reticulum.

Abbreviations: AllCysSO – (+)-S-2-propenyl-L-cysteine sulphoxide; MeCysSO – (+)-S-methyl-L-cysteine sulphoxide; PrenCysSO – *trans*-(+)-S-1-propenyl-L-cysteine sulphoxide; ProCysSO – (+)-S-propyl-L-cysteine sulphoxide

Introduction

Allium species contain a high proportion (1–5% dry weight) of non-protein sulphur amino-acids (Lancaster & Shaw 1989). One class of these secondary metabolites, the S-alk(en)yl-L-cysteine sulphoxides give rise to the characteristic flavours associated with the *Allium* species. Four sulphoxides have been found to occur naturally in the *Allium* species: namely (+)-S-methyl-, (+)-S-propyl-, *trans*-(+)-S-1-propenyl-, and (+)-S-2-propenyl-L-cysteine sulphoxides (respectively Me-, Pro-, Pren- and AllCysSO). All *Allium* species are reported to contain MeCysSO (Lancaster & Shaw 1989), while ProCysSO predominates in chives, PrenCysSO in onions and AllCysSO in garlic (Edwards et al. 1994).

Flavour in *Allium* is only released when the tissue is cut or broken. Work on garlic (Cavellito & Bailey 1944; Stoll & Seebeck 1951) and on onion (Virtanen & Spåre 1961; Schwimmer 1968) established that when the plant tissue was cut the S-alk(en)yl cysteine sulphoxides were hydrolysed by the enzyme alliinase. Lancaster & Collin (1981) provided evidence that the S-alk(en)yl cysteine sulphoxides were compartmentalised within the cytoplasm and the hydrolytic enzyme alliinase located within the vacuole. The initial products of the reaction between alk(en)yl cysteine sulphoxides and alliinase are ammonia, pyruvate and alk(en)yl sulphenic acids; the last of which breaks down non-enzymatically to form a variety of disulphides, which have a much milder flavour. The quantitative and qualitative difference in the alk(en)yl cysteine sulphoxide content results in the different flavours and odours of the many *Allium* species. In onions it is particularly the presence or absence of PrenCysSO which is responsible for the lachrymatory effect of onions, and AllCysSO which produces the characteristic taste of garlic. Early work established that PrenCysSO when hydrolysed by alliinase is converted into propenyl sulphenic acid, which is chemically unstable and undergoes rearrangement to form the lachrymator compound propanal sulphoxide (Virtanen 1965).

Allium species also contain other non-volatile sulphur compounds, the γ-glutamyl peptides, which are not hydrolysed by alliinase and do not therefore contribute to flavour production. For example in the onion

bulb the majority of the PrenCysSO is bound as γ-glutamyl PrenCysSO (Lancaster & Shaw 1991), which effectively removes a large proportion of the PrenCysSO from contributing to lachrymator production (Whitaker 1976). In *Allium* species there are as many as 18 sulphur-containing γ-glutamyl peptides which have been identified by Virtanen and Suzuki and their respective co-workers (reported in Lancaster & Shaw 1989). The function of the γ-glutamyl peptides in the metabolism of the plant remains unclear. Originally they were generally considered to function as reserves of nitrogen and sulphur, and have since been implicated in the transport of amino-acids in cells (Kasai & Larson 1980). In 1989 however, Lancaster and Shaw proposed that some of the γ-glutamyl peptides (e.g. γ-glutamyl PrenCysSO) may be intermediates in the biosynthetic pathway of alk(en)yl cysteine sulphoxides.

A number of pathways for the biosynthesis of the alk(en)yl cysteine sulphoxides have been proposed. Early biosynthetic studies also showed that the uptake of labelled sulphur compounds resulted in many labelled γ-glutamyl peptides as well as alk(en)yl cysteine sulphoxides. These early studies gave rise to an important question concerning the biosynthetic relationship between γ-glutamyl peptides and the alk(en)yl cysteine sulphoxides. Lancaster & Shaw (1989) used a pulse chase experiment ($^{35}SO_4$) to investigate the sequence of appearance of label in sulphur compounds in order to determine the biosynthetic relationship between flavour precursors and the γ-glutamyl peptides. Their work, performed on excised leaves, indicated that within the first 24h after the leaves were exposed to ^{35}S the label appeared predominantly in the fractions containing γ-glutamyl peptides. After 24h, as the amount of label declined in the γ-glutamyl peptides, the amount of label in the alk(en)yl cysteine sulphoxides fractions began to increase, thus indicating in this single experiment that the ^{35}S label was incorporated into the γ-glutamyl peptides *before* the alk(en)yl cysteine sulphoxides. This scheme was confirmed by Parry & Lii (1991) who showed that addition of γ-glutamyl cysteine to methacrylic acid gave rise to γ-glutamyl-S-2-carboxypropylcysteine which in onion undergoes sequential decarboxylation to γ-glutamyl-S-1-propenyl cysteine, oxidation to γ-glutamyl-S-1-propenylcysteine sulphoxide and finally cleavage by γ-glutamyl transpeptidase to PrenCysSO.

More recently however, Ohsumi et al. (1993) have shown that in differentiating tissue cultures of garlic AllCysSO is formed by the oxidation of AllCys in agreement with the pathways proposed by Suzuki et al. (1961, 1962), Sugii et al. (1963), Granroth (1970) and Turnbull et al. (1980). Thus there are two main pathways proposed so far for the synthesis of PrenCysSO. The first involves no γ-glutamyl precursors (as reported by Suzuki et al. 1961, 1962 and Sugii et al. 1963) and Granroth (1970) while the second pathway is based on the presence of γ-glutamyl alkenyl cysteine compounds as the precursors (Lancaster & Shaw 1989). It is of course possible that both biosynthetic pathways are functional.

Much of the previous work that has been performed on the biosynthetic route has been done on excised plant organs and cell tissue culture systems. To our knowledge no one has yet made a detailed study of the dynamics of the complex interaction between the two chemical groups in the intact plant. Excised leaves are effectively senescent tissue and undifferentiated tissue cultures are meristematic tissue so that the results obtained from such material may be of limited relevance to the healthy intact plant. The work presented in this paper was obtained by pulse labelling ($^{35}SO_4$) on intact growing plants in an attempt to resolve the relative importance of γ-glutamyl peptides in the biosynthesis of the sulphoxides. In addition we present data on the compartmentation of the flavour precursors and their intermediates in the cell.

Materials and methods

Plant material

Onion sets of variety Stuttgarter Riesen were obtained from Bridgemere Garden World, Cheshire, U.K. and stored at 4 °C in the dark until used. Sets were encouraged to shoot by supporting them above water for 1 week as described in Edwards et al. 1994. Water was replaced with Hoaglands and Arnon solution for 1 week then fresh culture solution was added which contained $^{35}SO_4$ (in the form $Na_2{}^{35}SO_4$, obtained from Amersham International PLC, Buckinghamshire, U.K.). When shoots were labelled prior to fractionation the plants were exposed to the same radioactive source for 7 days before being excised and the shoot material was used for preparation of protoplasts. For the pulse labelling experiments, onion sets were exposed to the label for a total of 24h, then transferred to fresh nutrient solution. Two plants were harvested at 18h, 24h, 28h, 67h and 73h after the initial exposure to radioactive solution then tissue was taken from the shoots, roots, outer brown scale, middle fleshy scale and the inner

scale immediately adjacent to the shoot and extracted as described.

Extraction of S-alk(en)yl cysteine sulphoxides and γ-glutamyl peptides

S-alk(en)yl cysteine sulphoxides and γ-glutamyl peptides in the plant material were extracted in methanol:chloroform:water (12:5:3) containing 10 mM hydroxylamine (Edwards et al. 1994). The mixture was partitioned between chloroform and water and the methanol:water layer kept, dried under vacuum at 40 °C and resuspended in 100 μl distilled water. In the protoplast fractionation studies, individual gradients were extracted as above then the concentrated sample was applied to an Amberlite 120(H+) ion exchange column. The purified samples resuspended in 1 ml 0.03 M HCL, filtered through a 0.45μ millipore filter and sonicated for 15 min prior to HPLC analysis (Edwards et al. 1994). In the pulse labelling experiments the plant material was extracted in the same way but the sulphoxides and γ-glutamyl peptides were separated by Dowex 1 chromatography as described by Lancaster & Shaw (1989), sulphoxides were eluted in 0.1 M acetic acid, and the γ-glutamyl sulphoxides eluted in 1 and 2 M acetic acid. All eluates were again dried down under N_2 and resuspended in 500 μl distilled water, filtered and sonicated.

Counting of radioactivity

100 μl of the sample obtained at each point of the isolation procedure into 5 ml of scintillation fluid (Optiphase 'Hisafe' 3, LKB scintillation products), and secondly injecting 15 μl for separation of individual components by HPLC analysis and subsequent scintillation counting of the individual fractions. Samples were counted through a C14 window on an LKB 1219 Rackbeta liquid scintillation counter. Confirmation of radioactivity in the alk(en)yl cysteine sulphoxides was also obtained by TLC of 100 μl of sample on 20 × 20 cm pre-coated silica gel 60 F_{254}, 0.2 mm thick. Plates were run in solvent I, butan-2-one:pyridine:water:acetic acid (70:15:15:2) and then in the same direction in solvent II, propan-1-ol:water:n-propyl acetate:acetic acid:pyridine (120:60:20:4:1). Plates were subsequently scanned for radioactivity by means of Raytest RITA (Rapid Intelligent Radio TLC Analyser), part of the plate (5 × 20 cm) was removed and sprayed with 0.5% ninhydrin solution (in absolute ethanol). The colour was developed at 128 °C for 10 min and the ninhydrin positive spots were located.

Isolation of protoplasts

Green leaf shoot material (approx. 9 g) was cut in half to expose the internal tissue of leaves and placed face downwards into protoplast osmotic buffer (3 mM MES buffer, 0.6 M sorbitol and 3 mM $CaCl_2$ at pH 5.5) containing 2% (w/w) cellulase and 0.5% (w/w) pectinase, and left to incubate for 24h. Crude protoplasts were filtered through muslin to remove plant debris, then the filtrate of protoplast suspension was spun down at low speed for 10 min. The supernatant was resuspended in 5 ml of fresh protoplast osmoticum, then layered on top of 10 ml 16% dextran T40 and spun down for 30 min at 1 rpm. Protoplasts were collected from the Dextran/buffer interface and resuspended in fresh protoplast osmoticum. Protoplasts were then spun down for 10 min at 1 rpm and the top supernatant removed and replaced with fresh protoplast osmoticum. This procedure was repeated 3 more times to ensure a clean preparation of protoplasts. After the final spin the supernatant was removed, the protoplasts were transferred to a chilled homogeniser and 5 ml of lytic buffer (0.45 M sorbitol, 25 mM tris-HCl, 1 mM DTT, 1 mM EDTA, pH 7.0) was added plus 100 μl 0.1 M hydroxylamine and a final 3 ml of lytic buffer. Protoplasts were then homogenised by 10 strokes then left to settle before being layered onto sucrose density gradients.

Density gradients were centrifuged for 3h at 16,000 rpm at 4 °C and individual fractions (1.5 ml) were collected by means of a peristaltic pump. Individual fractions were then assayed for rotenone-insensitive NADH reductase, a marker for the endoplasmic reticulum (Collin et al. 1989), γ-glutamyl transpeptidase (Naftalin et al. 1969), chlorophyll as a marker for the chloroplasts (Wintermans & De Nots 1965), protein (Musker 1988), sucrose (refractometer), alkenyl cysteine sulphoxides and radioactivity (as above). Density gradient buffer was made up as follows 1 mM EDTA, 1 mM DTT, 0.1 mM $MgCl_2$, 50 mM tris-HCl pH 8.0. Gradients 60–15% (w/w) sucrose in 5% increments were prepared. Gradients were then left for 2h in a horizontal position at room temperature before being left overnight in a vertical position at 4 °C.

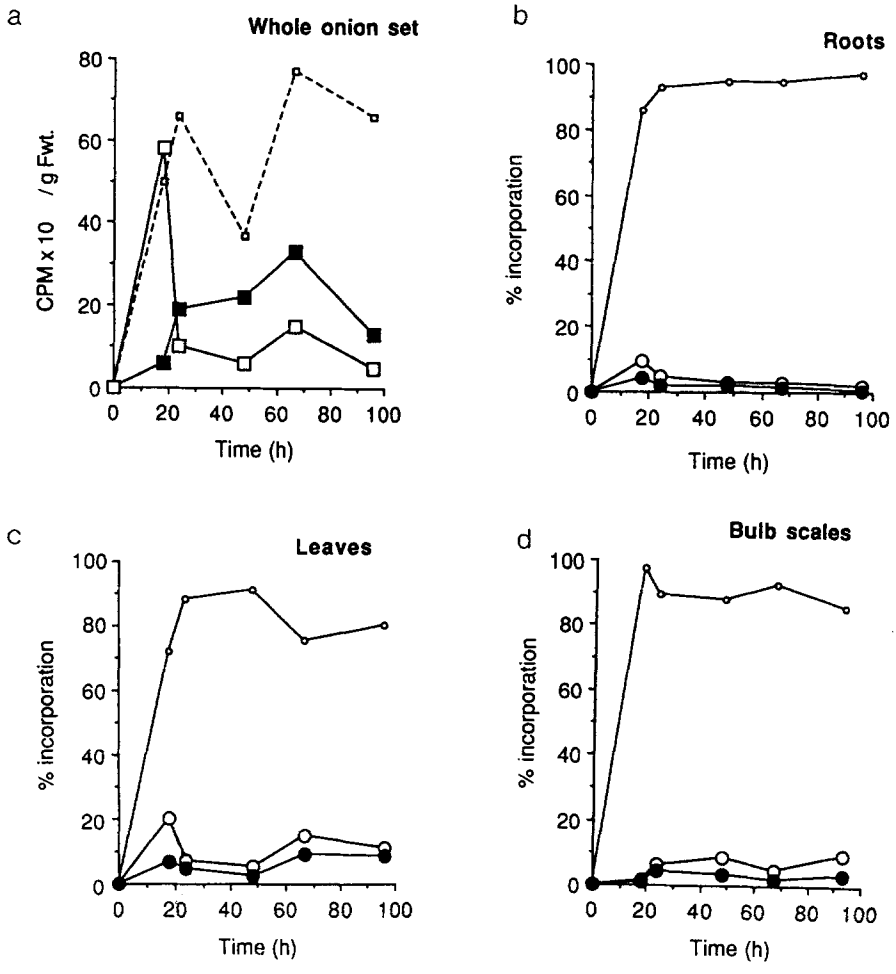

Fig. 1. Changes, with time, in incorporation of radioactivity into sulphur compounds in intact onion sets and individual plant organs after a 24h pulse of $^{35}SO_4$. Compounds were fractionated by Dowex 1 chromatography: 0.1 M acetic acid fraction (o) contains alk(en)yl cysteine sulphoxides and amino acids; 1.0 M (○) and 2.0 M acetic acid fractions (●) contain γ-glutamyl peptides of increasing acidity. a) Total amount of ^{35}S (cpm g^{-1} FW) in individual plant organs, root (□), leaves (■) and bulb scales (□); b) Percentage of ^{35}S in individual fractions extracted from root tissue; c) Percentage of ^{35}S in individual fractions extracted from leaf tissue; d) Percentage of ^{35}S in individual fractions extracted from bulb scale tissue.

Results and discussion

Pulse labelling of sulphur compounds in intact onion sets

High levels of radiolabel were found in the root and leaf tissue (10^4–10^5 cpm g^{-1} FW), whereas relatively little label was found to have been incorporated into bulb scale tissue, except that at 18h the level of activity in the bulbs exceeded that in either the leaves or roots (Fig. 1a). These high levels of radioactivity in the shoot may indicate that there was very little transfer of biosynthetic compounds from the bulb to the shoot, confirming the view that the biosynthetic pathway in the leaves was fully functional and not relying on preformed precursors from the bulb tissue. The highest percentage incorporation of radiolabel (in all plant organs) was always seen in the fractions containing the alk(en)yl cysteine sulphoxides (Fig. 1b-d); generally this was over 70% and frequently accounted for over 90% of the total activity. The level of radioactivity detected in this fraction increased during the labelling period (0–24h) and continued to be high until 48h after the start of the experiment (= 24h after the pulse was removed), but thereafter there was a slight decrease in the level of radioactivity. Radioactivity in the fraction containing γ-glutamyl peptides (1 and 2 M) increased slightly during the first 18h of the pulse labelling period, but

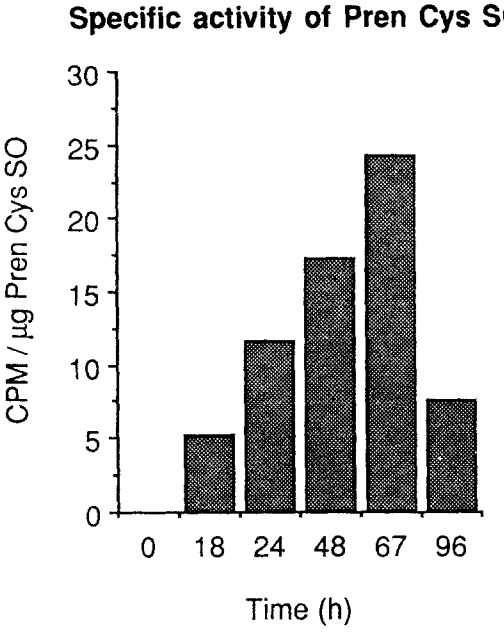

Fig. 2. Change in the amount of ^{35}S (cpm μg^{-1}) in propenyl cysteine sulphoxide isolated from intact onion leaves exposed to a 24h pulse of $^{35}SO_4$.

much less than that observed in the fractions containing sulphoxides, and thereafter declined (Fig. 1b-d). The percentage of label found in the γ-glutamyl peptide fractions (1 and 2 M acetic acid fractions) was generally less than 20%. There was little to indicate that the label had been in the γ-glutamyl peptides prior to entering the fractions containing S-alk(en)yl cysteine sulphoxides, although a shorter pulse labelling period and more intensive sampling during the pulse-labelling period may indicate otherwise. The presence of ^{35}S in the S-alkenyl cysteine sulphoxides was confirmed by TLC coupled to radioscanning. When the specific activity of PrenCysSO in leaves was measured it was found that within 18h it was already labelled and that the maximum specific activity occurred at 67h (43h after the label had been removed) after which it declined (Fig. 2). At 18 h, radioactivity in the PrenCysSO accounted for 20% of the total radioactivity recovered in the sulphoxide fraction, at 48h it accounted for 60% and at 93h it accounted for 38%.

Although specific activities for all the individual compounds in the extract are at present not available it is possible to consider radioactivity associated with each broad group of compounds as that in the alk(en)yl cysteine sulphoxides and the γ-glutamyl peptides. The results shown here obtained from intact plants differed markedly from those presented from work with excised leaves. Thus Lancaster & Shaw (1989) found that the ^{35}S label was rapidly taken up and incorporated into the fraction containing γ-glutamyl peptides and remained in the γ-glutamyl peptides for 24 h. During this period the amount of label in the alk(en)yl cysteine sulphoxides was very low but after the first 24h the percentage of label in the γ-glutamyl peptides had declined and the amount in the alk(en)yl cysteine sulphoxides began to increase. It is difficult to explain the difference between our results and those of Lancaster & Shaw (1989). One suggestion is that the different results obtained from intact leaves and detached leaves may be due to the fact that the developmental stage and physiological condition of the experimental material influences activity of the separate biosynthetic routes. In the intact plant when leaves began to senesce during bulb filling there was reported to be a decline in total alk(en)yl cysteine sulphoxide content and an increase in γ-glutamyl peptides. Conversely when the leaves were actively growing prior to bulb formation, the concentration of alk(en)yl cysteine sulphoxides was high relative to the amount of γ-glutamyl peptides present (Lancaster et al. 1984; Lancaster & Shaw 1991). The reason for changeover in the pattern of accumulation between the two chemical groups (i.e. free alkenyl cysteine sulphoxides and γ-glutamyl peptide) is not known, nor is it known whether synthesis or transformation of one to the other is responsible for their fluctuating levels. Lancaster proposed that during the early stages of shoot formation γ-glutamyl peptides were metabolised to form the free alk(en)yl cysteine sulphoxide and in support of this she was able to detect an increase in the levels of γ-glutamyl transpeptidase at this time (Lancaster et al. 1991).

The labelling of the γ-glutamyl peptides as observed by ourselves, Granroth (1970) and Suzuki et al. (1961, 1962) is not unexpected. When $^{35}SO_4$ is fed to plants, over 90% of SO_4 taken up by plants is assimilated into cysteine and over 35% of this is immediately channelled into reactions of the γ-glutamyl cycle via glutamyl cysteine and glutathione (Giovanelli et al. 1980). Further work is required to understand the full significance of the relationship between the γ-glutamyl peptides and the alk(en)yl cysteine sulphoxides.

To our knowledge no one has yet done a detailed study on the dynamics of the complex interaction between the two chemical groups, or on whether one group could be precursors to the other depending on the developmental and physiological state of the plant. Information that is available has been obtained with individual bulbs destructively harvested at different

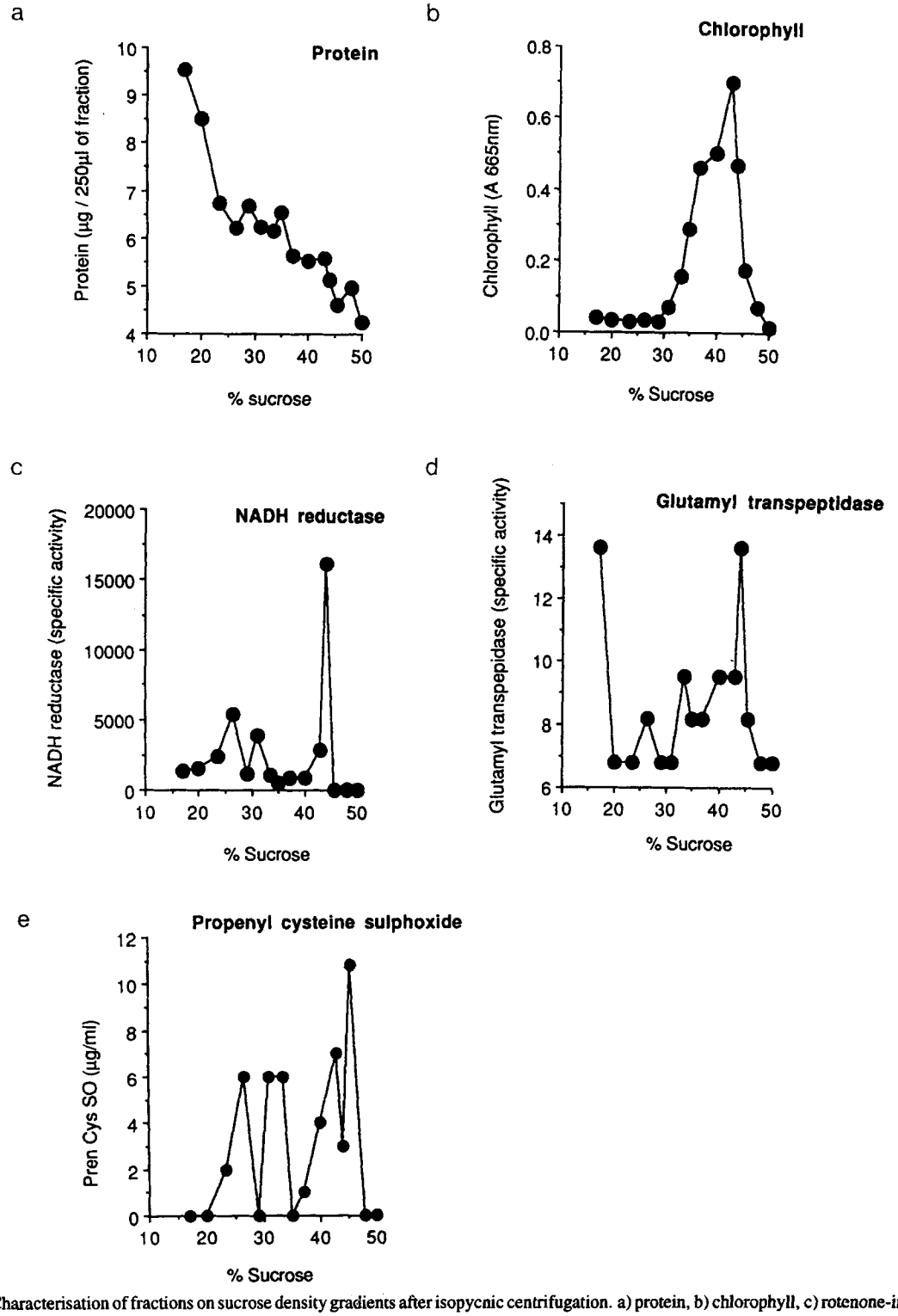

Fig. 3. Characterisation of fractions on sucrose density gradients after isopycnic centrifugation. a) protein, b) chlorophyll, c) rotenone-insensitive NADH reductase, d) γ-glutamyl transpeptidase and e) concentration of propenyl cysteine sulphoxide.

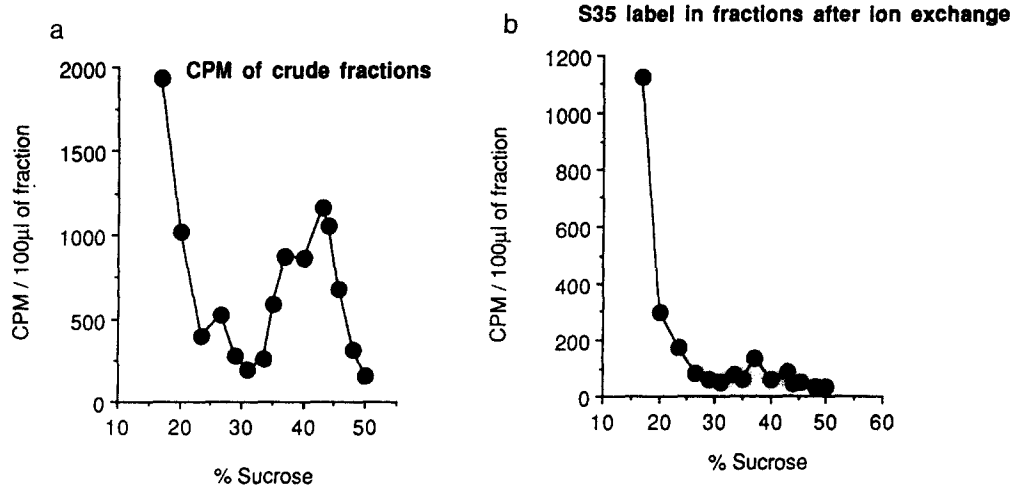

Fig. 4. Incorporation of radioactivity (^{35}S) in fractions on a sucrose density gradient after isopycnic centrifugation a) crude fraction before ion exchange and b) individual fractions after ion exchange.

times in the growing cycle and on excised leaves which may represent senescing tissue. Results obtained from such material may only provide results that describe only part of the story.

Intracellular localisation of PrenCysSO

The purpose of the second part of the work was to identify the intracellular location of PrenCysSO and intermediates in its formation. Analysis of cell fractions of leaf protoplasts showed that intact chloroplasts (measured as chlorophyll) were at a maximum on the gradient at 43% sucrose with the band extending from 30% sucrose to 45% sucrose (Fig. 3b). Endoplasmic reticulum membranes (measured as rotenone-insensitive NADH reductase) were located as two small peaks at 26% and 31% and a large peak at 44% sucrose (Fig. 3c). The peak at 26% sucrose coincided with the position of smooth ER, and the second at 31% overlapped with the beginning of the chloroplast band and the third at 44% sucrose with the chloroplast band. Each overlap of chloroplast and ER fractions had also been previously noted by Collin et al. (1989). Previously Lancaster et al. (1989) had located onion chloroplasts at 42% w/w while Collin et al. (1989) had identified onion chloroplasts at 35%, ER at 27% and 38% w/w sucrose. The distribution of PrenCysSO in the fractions showed peaks at 25, 32 and 45% w/w sucrose which coincided with the position of the ER and chloroplasts (Fig. 3e).

There was no peak which coincided with the presence of small vesicles detected at 55% w/w sucrose by Collin et al. (1989). The continuous sucrose gradient used in the present work did not extend beyond 50% sucrose. These present results showed further differences since they suggest a greater accumulation of PrenCysSO in the chloroplasts than shown by Collin et al. (1989). The enzyme, γ-glutamyl transpeptidase, which reportedly (Lancaster et al. 1991) controls the release of PrenCysSO from its bound form, γ-glutamyl PrenCysSO, was also found to be associated with the ER and the chloroplasts as well as the soluble fraction (Fig. 3d). In contrast, Lancaster et al. (1989) located γ- glutamyl transpeptidase predominantly in the soluble fraction and only a small fraction with the ER and none in the chloroplasts. Later workers suggested that γ-glutamyl transpeptidase is membrane bound (Schneider et al. 1992). The difference in the results may reflect the different physiological condition of the plant material used for the protoplast isolation. The protoplasts used in the present study were prepared on a similar time scale to that of Collin et al. (1989) except that the latter used seedling leaves as a source of material, Lancaster et al. (1989) prepared protoplasts from leaves that had been excised for 24h before protoplast isolation. The age and stage of development of leaves used for protoplast isolation might well have an effect on the activity of the cell and its components. Electron microscope examination of the protoplasts isolated directly from leaves of intact plants indicated that such protoplasts were already under stress since the protoplasts showed multiple vesicle formation and thylakoid disintegration. Care must be taken to stan-

dardise the starting material before direct comparisons can be made.

Radioactivity of the cell fractions showed the highest level in the soluble and chloroplast fractions (Fig. 4). The entry of radioactivity into specific components within the ER and chloroplasts needs to be resolved before conclusions can be drawn over sites of synthesis of PrenCysSO. Nevertheless the PrenCysSO, and the other alk(en)yl cysteine sulphoxides, by their association with membranes and organelles (vesicles, ER and chloroplasts) indicated compartmentation of sites of synthesis within the cytoplasm. The earlier work (Collin et al. 1989) and present data suggest that PrenCysSO synthesis may occur in stages in intact tissue with the first stage in the chloroplast from where intermediates are exported to the ER, possibly the smooth ER, then its products transferred to ER derived cytoplasmic vesicles for long term storage. These stages will be confirmed by the analysis of radioactivities of specific compounds in the different fractions.

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