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PTEROYLGLUTAMATE DERIVATIVES IN THE ROOTS OF *PISUM SATIVUM**

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Abstract—The pteroylglutamate derivatives in the radicles of 3-day old pea seedlings have been isolated, and assayed microbiologically using *Lactobacillus casei* and *Streptococcus faecalis*; these derivatives were maximal in extracts prepared from the 5 mm apical segment. About 52 per cent of the pteroylglutamates in the root tip consisted of polyglutamyl derivatives. Formyl and methyl derivatives of tetrahydropteroylglutamic acid were also found. The polyglutamyl derivatives were, in large part, converted to 5-methyltetrahydropteroylglutamates on treatment with γ-glutamyl carboxypeptidases. [2-^{14}C]Pteroylglutamic acid and [methyl-^{14}C]5-methyltetrahydropteroylglutamate accumulated in the radicles after injection of these compounds into the cotyledons of 1-day old pea seedlings. Labelled pteroylglutamic acid was incorporated into the majority of the pteroylglutamate derivatives. In contrast, [methyl-^{14}C]5-methyltetrahydropteroylglutamate was incorporated into compounds to which the assay organisms did not respond.

INTRODUCTION

A LARGE number of physiological and biochemical investigations have been conducted with root tissues. As a result there is considerable information regarding rates of respiration,[1-4] metabolism of respiratory substrates,[5-9] salt uptake,[10-12] biosynthesis of amino acids and proteins[13-17] and formation of nucleic acids[17-21] in these tissues. In many species

* The abbreviations used for pteroylglutamic acid and its derivatives are those suggested by the IUPAC–IUB Commission as listed in the *Biochem. J.* **102,** 15 (1967): e.g. 5-CH_3-H_4PteGlu = 5-methyltetrahydropteroylmonoglutamate.

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it is clear that the meristematic, elongating and differentiating regions of the root can be distinguished in physiological and biochemical terms. In general, the meristematic cells have relatively low rates of respiration when expressed on a per cell basis. These rates are increased considerably on expansion and vacuolation, events which are also marked by large increases in protein, RNA and DNA contents. With maturation of these cells there is generally a gradual fall in respiratory rates and protein contents.[22]

It may be concluded, by analogy to other cellular systems, that the synthesis of DNA and consequently cell division in roots would be highly dependent on pteroylglutamate-mediated reactions. Although this important area of metabolism has not been thoroughly examined in these tissues, there are several reports in the literature which support this contention. For example, Boll,[23] working with aseptic cultures of tomato roots has shown that growth is readily inhibited by addition of sulphanilamide, a compound known to inhibit the biosynthesis of pteroylglutamates in microorganisms.[24] Root growth inhibition was reversed when *p*-aminobenzoate or PteGlu were added to the medium. Root growth is also severely inhibited by pteroylglutamate antagonists like aminopterin[25,26] and 2,4-diamino-9,10-dimethylpteroylglutamate.[27]

The endogenous levels of pteroylglutamates in pea roots have been examined in early work by Schopfer and Grob[27] but to our knowledge there have been no detailed investigations of the nature of these derivatives using recently improved methods[28] for their isolation and assay. As part of an investigation of one-carbon metabolism in plant tissues, the pteroylglutamates in the root tips of 3-day old pea seedlings have now been characterized. Possible relations between pteroylglutamates in the cotyledons[29] and those present in the apical 5-mm section of the radicle have been examined by employing ^{14}C-labelled pteroylglutamates.

RESULTS

Levels of Pteroylglutamate Derivatives in Root Segments of Increasing Age

In order to examine variations in pteroylglutamate levels in 3-day old pea radicles, 5 mm segments were removed, killed by boiling in ascorbate, and the pteroylglutamate levels determined using *L. casei*, before and after treatment of the extracts with kidney γ-glutamyl carboxypeptidase. The results of these studies are summarized in Fig. 1. It is clear that the total levels of these derivatives, when expressed on a root segment basis, did not vary appreciably in segments excised from regions 5–30 mm from the root tip. This is in contrast to the much higher levels which were present in extracts of the 5 mm tip segment. Of these totals, an appreciable percentage supported the growth of the assay organism without γ-glutamyl carboxypeptidase treatment. This suggests that pteroylglutamates with up to

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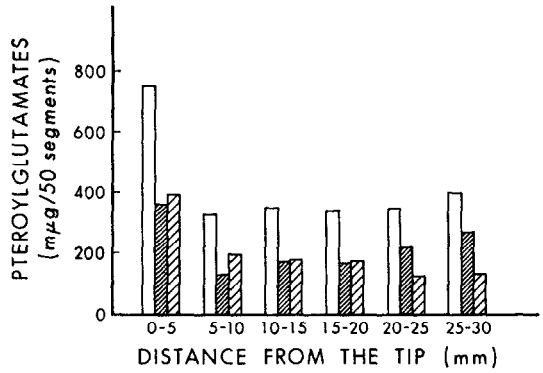

FIG. 1. PTEROYLGLUTAMATES IN PEA ROOT SEGMENTS OF INCREASING AGE.
□, Total levels after hog kidney γ-glutamyl carboxypeptidase treatment; ▨, levels before carboxypeptidase treatment; ▨, levels of polyglutamyl derivatives.

three glutamic acid residues[30] form a large part of the pteroylglutamate pool of pea root tissues. For example, this fraction represented 48 per cent of the total pteroylglutamates present in the 0–5 mm segment and 50 and 67 per cent of the derivatives present in the 15–20 mm and 25–30 mm segments respectively. Extracts of all root segments gave considerably higher values when assayed after γ-glutamyl carboxypeptidase treatment, indicating the presence of polyglutamyl derivatives.[30] These latter derivatives formed the bulk of the pteroylglutamates present in the youngest parts of the root.

Chromatography of Pteroylglutamates in Pea Root Tips

To investigate the nature of individual pteroylglutamates in the 5-mm apical segment, samples of freshly excised root tips were homogenized in ice-cold ascorbate as described by Bird *et al.*[31] This method, which did not involve killing the tissue before extraction of the derivatives, gave total pteroylglutamate contents which were very similar to those shown in Fig. 1. However the level of polyglutamyl derivatives was greatly reduced suggesting that extensive hydrolysis of these compounds had occurred during extraction. These differences were further evident when the extracts were chromatographed on DEAE-cellulose (Figs. 2 and 3). In all cases individual compounds were identified on the basis of criteria widely used by other workers.[28] These included the ability of the compound to support the growth of each assay organism, co-chromatography with the authentic derivative and re-chromatography of the 'peak' area after treatment with γ-glutamyl carboxypeptidases. If the tissues were homogenized prior to the 95° treatment (Fig. 2) the major pteroylglutamate present was 5-CH_3H_4PteGlu. In addition, a derivative collected in fractions 35–43 was identified as 10-HCO-H_4PteGlu. Other derivatives included H_4PteGlu, 5-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu$_2$. A small 'peak' occurred when fractions 50–53 were inoculated with *L. casei* and this compound was tentatively identified as 10-HCO-H_4PteGlu$_2$.

Separation of pteroylglutamate derivatives in root tips killed prior to extraction is shown in Fig. 3. The elution sequence of derivatives recovered after treatment with pancreatic

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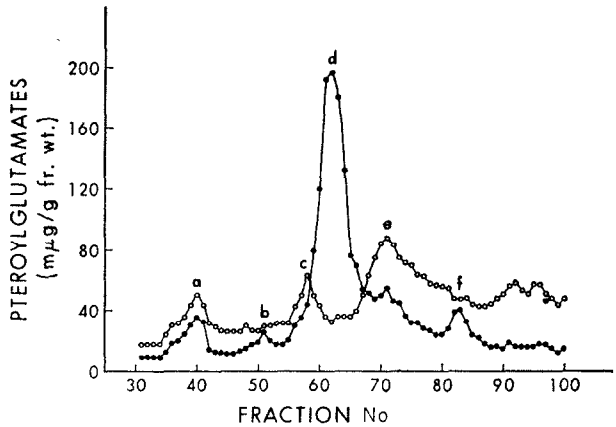

FIG. 2. SEPARATION OF THE PTEROYLGLUTAMATE DERIVATIVES PRESENT IN PEA ROOT TIPS. The tissue was extracted without prior heat treatment. The derivatives were assayed using *L. casei* (●—●) and *S. faecalis* (○—○). Individual derivatives are designated as follows: (a) 10-HCO-H_4PteGlu; (b) 10-HCO-H_4PteGlu$_2$; (c) 5-HCO-H_4PteGlu, (d) 5-CH_3H_4PteGlu; (e) H_4PteGlu; (f) 5-CH_3H_4PteGlu$_2$.

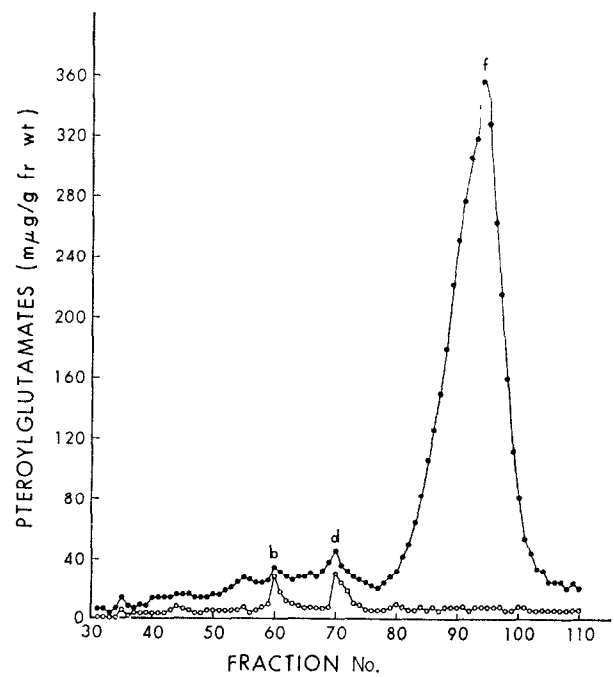

FIG. 3. CHROMATOGRAPHY OF DERIVATIVES BEFORE AND AFTER CARBOXYPEPTIDASE TREATMENT. Extracts of the 0–5 mm apical segment prepared after heat treatment, chromatographed before (○—○) and after (●—●) incubation with chicken pancreas γ-glutamyl carboxypeptidase. Fractions were assayed for pteroylglutamate with *L casei*; derivatives are designated as in FIG. 2.

γ-glutamyl carboxypeptidase is due to compounds present in the root extract as polyglutamates. Before enzyme treatment 10-HCO-H_4PteGlu$_2$ and 5-CH_3H_4PteGlu were found. No evidence was obtained for the other derivatives shown in Fig. 2. After peptidase treatment an additional derivative (fractions 78–103) was observed. This derivative did not support the growth of *S. faecalis* and was largely hydrolyzed to 5-CH_3H_4PteGlu on treatment with kidney carboxypeptidase. On the basis of these observations it is concluded that the major derivative present after pancreatic carboxypeptidase treatment is 5-CH_3H_4-PteGlu$_2$. Treatment of the extracts with the mammalian enzyme also produced 10-HCO-H_4PteGlu and H_4PteGlu together with smaller quantities of 10-HCO-H_4PteGlu$_2$ and 5-CH_3H_4PteGlu$_2$.

The Presence of γ-Glutamyl Carboxypeptidase Activity in Root Extracts

The hydrolysis of polyglutamyl derivatives which occurred during extraction of root tissues without prior 95° treatment, suggested that an active γ-glutamyl carboxypeptidase was present. To investigate this, cell-free extracts of the 5 mm apical segment were assayed using yeast extract as a source of polyglutamyl derivatives. Pteroylglutamates capable of supporting the growth of *L. casei* were rapidly produced on addition of root extract. These derivatives were not produced in the absence of the cell-free extract or in the presence of boiled cell-free extracts. The reaction was essentially complete as 95 per cent of the total polyglutamyl derivatives were hydrolyzed after 60 min of incubation.

Translocation of ^{14}C-labelled Pteroylglutamates to the Root Tip

Since many of the pteroylglutamic acid derivatives present in pea root tips (Fig. 3) are also present in the cotyledons of 3-day old pea seedlings,[29] it was of interest to determine whether components of the cotyledon pool were translocated into the root. To examine this possibility, micromolar quantities of [2-^{14}C]PteGlu were injected into the cotyledons of 1-day old pea seedlings, and in other experiments dry pea seeds were allowed to imbibe the labelled solution. Extracts of the developing embryos were prepared during a 4-day germination period and assayed for radioactivity. Progressive accumulations of ^{14}C were observed over this period. For example, 1·3 per cent of the supplied ^{14}C was recovered from the embryos, 24 hr after injection. The figures for 72 hr and 96 hr were 7·1 and 7·5 per cent respectively. In order to determine the nature of the labelled compounds present, extracts of the 0–5 mm and 25–30 mm segments of 3-day old radicles were treated with pancreatic γ-glutamyl carboxypeptidase and subjected to column chromatography. Both regions of the root contained appreciable levels of ^{14}C, the greater part of which was attributable to pteroylglutamate derivatives (Fig. 4). A large peak of radioactivity was present in fractions 135–160 which was identified, by co-chromatography, as PteGlu. Due to the high specific radioactivity (55·3 μc/μmole) of the supplied PteGlu these fractions did not give a detectable growth response with *L. casei* (Fig. 4b). Extracts of the 0–5 mm segment (Fig. 4a) contained considerably more labelled PteGlu. Both segments of the radicle contained labelled 5-CH_3H_4PteGlu and 5-CH_3H_4PteGlu$_2$. The latter compound, not present in the radicle before carboxypeptidase treatment (Fig. 3), indicates that polyglutamyl derivatives were also labelled in these experiments. Two other conspicuous radioactive peaks were eluted from the columns and collected in fractions 33–45 and 54–61 respectively. The position of the first peak corresponds to that of authentic 10-HCO-H_4PteGlu, although the absence

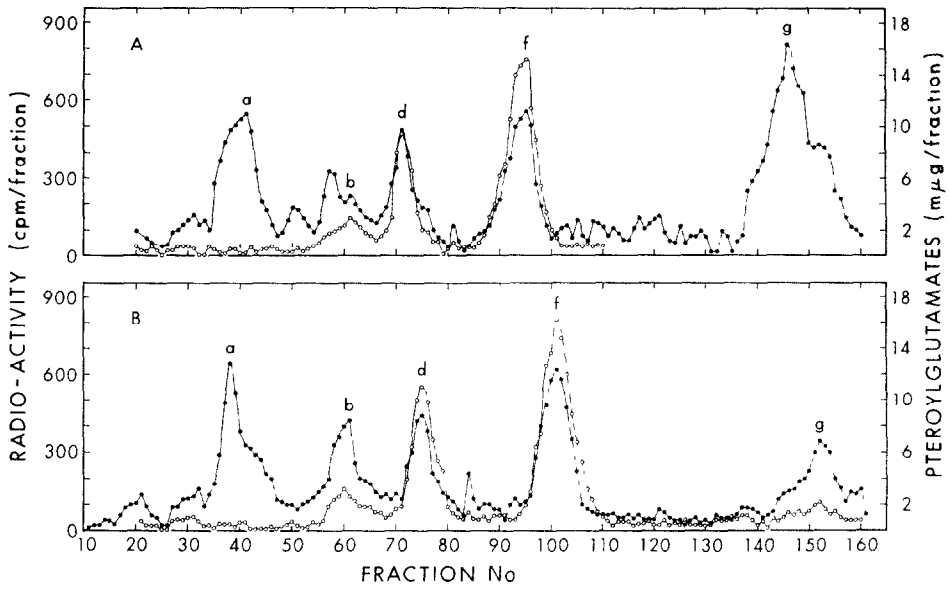

FIG. 4. OCCURRENCE OF LABELLED PTEROYLGLUTAMATES IN ROOT SEGMENTS AFTER ADMINISTRATION OF [2-^{14}C]PteGlu TO THE COTYLEDONS.
(a) Separation of derivatives in extracts of the 0–5-mm segment. (b) Separation of derivatives in extracts of the 25–30-mm segment. ●—●, Radioactivity; ○—○, *L. casei* active pteroylglutamates. Derivatives designated as in Fig. 2 (g) PteGlu.

of any detectable growth response by *L. casei* makes identification of this compound uncertain. The labelled peak collected in fractions 54–61 appeared to coincide with the bacterial growth response for 10-HCO-H_4PteGlu$_2$.

On the basis of the experiments shown in Fig. 4, it is possible that 5-CH_3H_4PteGlu, the major pteroylglutamate derivative in pea cotyledons,[29] was transported to the radicle and there conjugated with glutamic acid residues. Experiments employing [methyl-^{14}C]5-CH_3-H_4PteGlu using the same feeding procedure showed that ^{14}C was distributed along the radicle, most occurring in the 5 mm segment adjoining the cotyledons. Chromatography of the 0–5 mm and 25–30 mm root segments revealed labelled 5-CH_3H_4PteGlu but not the corresponding diglutamate even after pancreatic carboxypeptidase treatment. In addition, several radioactive peaks which did not support growth of *L. casei* were present, indicating that the labelled substrate had been extensively incorporated into a number of other compounds.

DISCUSSION

In earlier work using *S. faecalis* and *Pediococcus cerevisiae*, Schopfer and Grob[27] reported pteroylglutamates in various parts of pea seedlings. Their data for root tissues expressed on a dry weight basis, are lower than the lowest values obtained in the present work. For example, whole pea roots were found to contain 3·49 μg pteroylglutamates/g dry wt., whereas data in Fig. 1 reveal a range of these derivatives between 6·8–16·8 μg/g dry wt. depending on the segment analyzed. This difference can be largely explained in two

ways. Firstly, the technique used, in the present work,[32] prevents oxidative decomposition of labile pteroylglutamates.[28] Secondly, the use of *L. casei*, an effective assay organism for a wide variety of pteroylglutamates including methylated derivatives,[30] has allowed measurement of the major components of the pteroylglutamate pool in these tissues.

The levels of pteroylglutamates in root segments of increasing age has not, to our knowledge, been previously examined. From such studies (Fig. 1) it is clear that the total level of these compounds is highest in the youngest parts of the root when expressed on a per segment basis. Histological examination of the 0–5 mm segment of such roots revealed meristematic and elongating cells, but vascular tissues were absent. It is concluded that the apical segments used in the present work may in general be compared to regions of the root which have high metabolic rates,[22] involving amino acid, protein[13–17] and nucleic acid synthesis.[17–21] Such cells would also have high levels of one-carbon metabolism. The observation (Fig. 1) that the actively growing region of the root contains the highest level of pteroylglutamate derivatives is consistent with this suggestion.

In common with other plant tissues[28] pea radicles have high levels of polyglutamyl derivatives. Although these compounds were not separated as conjugates, the results of carboxypeptidase treatments suggest that they occur mainly as 5-methyl derivatives (Fig. 3). The physiological significance of these compounds is still not clear. In *Clostridium sticklandii* evidence has been obtained for participation of polyglutamate derivatives in the interconversion of glycine and serine.[33] However in the majority of other species the folate derivatives participating in one-carbon metabolism are generally mono, di and triglutamates.[28] If this also applies to pea root tissues it is possible that the polyglutamates are a store of one-carbon units. Such units could be made available by the action of γ-glutamyl carboxypeptidase. It is of interest that several workers have demonstrated this enzyme in a wide variety of organisms[28] including several species of higher plants.[34–38]

Translocation occurs from the cotyledons to the growing seedling during germination, and although it is not possible to assess what proportion of the pteroylglutamates in the seedling have been transported from the cotyledons, the possibility remains that such movement may have considerable physiological significance. It is not known whether the inability of the growing regions to synthesize pteroylglutamates necessitates this translocation. Although excised roots of several species may be maintained in culture without addition of pteroylglutamic acid derivatives[39] this does not necessarily imply that these compounds are synthesized by root tissues in the intact plant.

Another question posed by the translocation experiments is the nature of the relationship existing between 5-CH_3H_4PteGlu and its polyglutamate derivatives. When [2-^{14}C PteGlu was supplied, both 5-CH_3H_4PteGlu and polyglutamyl forms of this compound were heavily labelled (Fig. 4). In contrast, when [methyl-^{14}C]5-CH_3H_4PteGlu was used, no evidence was obtained for incorporation of ^{14}C into polyglutamyl derivatives despite the fact that 5-CH_3H_4PteGlu was extensively translocated to the root. It appears that pea root tissues do not have ability to synthesize polyglutamyl derivatives from 5-CH_3H_4PteGlu

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but can use PteGlu for this purpose. Furthermore, this observation implies that glutamic acid residues are added to the polyglutamyl precursor prior to formation of the methyl group.

EXPERIMENTAL

Plant Material

Seeds of *Pisum sativum* L. cv. Homesteader, were soaked in distilled water for 6 hr at room temp. The seeds were then transferred to sterile vermiculite and germinated at 22° for 3 days in darkness. After this period of germination, the seedlings were thoroughly washed in distilled water and the radicles were cut into 5-mm sections using a sharp razor blade.

Chemicals

[2-^{14}C]PteGlu (55·3 μc/μmole) and [methyl-^{14}C]-5-CH_3H_4PteGlu (61 μc/μmole) (Amersham–Searle) were dissolved in 0·05% K-ascorbate (pH 6·0) and used without carrier. Other authentic pteroylglutamates were synthesized chemically.[29]

Extraction and Chromatography of Pteroylglutamate Derivatives

Root segments (100) were rapidly transferred to 5 ml of 2% K-ascorbate (pH 6·0) at 95° and held at this temperature for 10 min. After cooling to 4° the tissue samples were homogenized in a hand blender and centrifuged at 10,000 *g* for 10 min. The supernatant was stored at −20° before chromatography. Aliquots of the root extracts containing approximately 200 mμg of total pteroylglutamates were applied to 20 × 1·5 cm columns of DEAE-cellulose and eluted in 3 ml fractions using a gradient of phosphate buffer in the presence of ascorbate.[40]

Microbiological Assay of Pteroylglutamate Derivatives

Pteroylglutamates were assayed using *Lactobacillus casei* (A.T.C.C. 7469) and *Streptococcus faecalis* (A.T.C.C. 8043).[32] Growth was measured by titration of the lactic acid produced after 72 hr incubation at 37°, using authentic pteroylglutamic acid for calibration. Polyglutamyl derivatives were assayed with *L. casei* after the initial root extracts had been incubated with the γ-glutamyl carboxypeptidases from hog kidney[41] and chicken pancreas.[42]

Assay of γ-Glutamyl Carboxypeptidase Activity in Root Extracts

Root tip segments (100, each 5 mm long) were ground at 4° in 5 ml of 0·1 M K-phosphate buffer containing 0·5% (w/v) K-ascorbate (pH 6·0). After centrifugation at 18,000 *g* for 10 min the clear supernatant was dialyzed for 18 hr at 4° against 2 l. of the same buffer. Enzyme activity was assayed in reaction systems containing; 0·5 ml plant extract (0·3 mg protein), 0·5 ml of yeast extract containing 1·9 μg of pteroylglutamate derivatives, and 4 ml of 0·1 M Na-acetate buffer (pH 4·7). The reaction systems were incubated at 37° for periods up to 4 hr, the reaction terminated at 95°, and after dilution with 0·5% K-ascorbate (pH 6 0) the levels of pteroylglutamates were assayed with *L. casei*. Protein was assayed colorimetrically[43] using crystalline bovine serum albumin as standard.

Feeding Experiments Employing ^{14}C-Labelled Pteroylglutamates

(a) *Injection of* [2-^{14}C]*PteGlu.* Pea seeds were allowed to imbibe distilled water for 18 hr at 22°. A 2-μl aliquot (0·02 μc of ^{14}C) was injected into each cotyledon. The seeds were then transferred to sterile petri dishes containing moist filter paper and germinated in darkness for 4 days at 22°.

(b) *Imbibition of labelled pteroylglutamates.* Dry pea seeds (30) were allowed to imbibe 2-ml samples of [2-^{14}C]PteGlu (6·2 × 10^6 counts/min) and [methyl-^{14}C]5-CH_3H_4PteGlu (13·5 × 10^6 counts/min) in sterile petri dishes for 2 hr, and to complete imbibition sterile distilled water (2 × 2 ml) was added. The seeds were then transferred to sterile petri dishes containing moist filter paper and germinated as before. During germination, samples of the developing plumules and radicles were extracted using K-ascorbate as described above

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and examined by DEAE-cellulose chromatography. Radioactivity was assayed in a liquid scintillation counter[44] (70 per cent efficiency).

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