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PROLINE ACCUMULATION DURING ANTHER DEVELOPMENT IN PETUNIA HYBRIDA

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SUMMARY

Accumulation of free proline within anthers of *Petunia* proceeds continuously from the microspore stage to anthesis except for an intervening period when bud length is between 15 and 35 mm. The second increase in proline content coincides with the stage of anther desiccation. The metabolism of proline was studied in isolated peduncles fed [^{3}H]proline and [^{14}C]glutamine. Three factors appeared to be involved in elevating the proline content of the anthers: I. transport of proline from the leaves, II. transport of precursors and conversion to proline in the anthers and III. high stability of proline within the anthers.

1. INTRODUCTION

In the pollen of many species of higher plants the content of free proline is very high (BATHURST 1954; VIRTANEN & KARI 1955; BRITIKOV & MUSATOVA 1964). Proline has been assumed to play an important role in pollen germination, tube elongation and successful fertilization (TUPÝ 1963; DASHEK et al. 1971; BRITIKOV et al. 1965. 1970; PÁLFI et al. 1981; ZHANG et al. 1982; ZHANG & CROES 1983). However, there is little information regarding the kinetics and mechanism of proline accumulation. Much more is known about that mechanism for leaves of water-stressed plants. The conversion of proline to glutamate has been reported to be inhibited under water-stress (ASPINALL et al. 1974) as is proline oxidation in both barley leaves and isolated maize mitochondria (STEWART et al. 1977; SELLS & KOEPPE 1981). Also in stressed barley leaves, fixation of carbon dioxide by concurrent photosynthesis was found to be required for proline accumulation (AIYAR et al. 1980). The situation in anthers is more complex since either proline or its precursors must be imported because of the absence of photosynthesis. Although it seems likely that the leaves are the source of either proline or its precursors, this has not been established with certitude. Neither is it known at what stage of anther development proline accumulates.

Petunia hybrida is one of the species characterized by a large amount of proline in ungerminated pollen (LINSKENS & TUPÝ 1966; LINSKENS & SCHRAUWEN 1969; ZHANG et al. 1982). In the present paper we report on accumulation of proline

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in isolated *Petunia* branches bearing one developing bud. We asked whether proline is transported as such into the anther or is synthesized there. In the latter case, glutamate should be involved in the accumulation, as it is a direct precursor of proline. Therefore, the glutamine-proline interconversion was also examined.

2. MATERIALS AND METHODS

2.1. Plant material

Plants of *Petunia hybrida*, clone W166H, were grown at 25 °C in a greenhouse with artificial light at 15,000 lx and a photoperiod of 18 h. Only vigorous plants were used for the experiments.

2.2. Assay of amino acids in anthers

For each determination of amino acid content, at least 5–10 buds of the same corolla length were collected and all the anthers gathered. The free amino acid pool was extracted from the anthers by 70% ethanol containing 3 mmol.l^{-1} citric acid. The extract was partitioned against 2 volumes of chloroform. After either centrifugation at 1000 × *g* for 5 min or standing at 0–4 °C overnight, the water phase was collected, dried and maintained at −20 °C until analysis. All samples were assayed with an amino acid analyzer (JEOL, type JLC-6AH) Tokyo, Japan) equipped with a 6 × 600 mm column packed with LCR resin. To determine the ratio of dry to fresh weight, anthers from at least 20 buds were pooled. The anthers were weighed before and after drying in an oven at 90 °C for 24 h. All experiments on isolated branches were performed in an artificially illuminated growth chamber. Light intensity and temperature were as in the greenhouse.

2.3. Culture and labelling of isolated branches

Branches of 20–25 cm were cut from the plants and trimmed. On each branch only one bud, the corolla about 10 mm long, remained with two adjacent leaf pairs. Five cm of the lower end of the stem were removed under water and the branch was transferred into a test tube (100 × 28 mm) in the growth chamber prefilled with culture medium without radioisotopes. The medium was a modification of Hoagland's (ARNON & HOAGLAND 1940) medium. The mechanism of proline accumulation in the anther was studied by labelling branches simultaneously with [^{3}H]proline and [^{14}C]glutamine. For all labelling experiments, 1.4 MBq of [^{3}H]proline and 150 KBq of [^{14}C]glutamine were added to 50 µl of the culture medium in a tiny tube and given to the cut end of a branch. Just before the last droplet was taken up, another 100 µl of medium without isotopes was provided to the branch. The feeding with unlabelled medium was repeated twice to ensure that all isotope was taken up. The branches were then immediately placed into the culture medium in the large test tubes. Great care was taken to ensure that air could not enter the stem. All the branches were incubated up to 5 days in the test tubes in the growth chamber. At one-day intervals, the amino acids were extracted from the different parts of the branches and separated by thin-layer chromatography.

2.4. Sample fractionation

At 1-day intervals, 8–10 branches were collected and used for both amino acid and radioactivity determinations. The branches were separated into anthers, pistils, corolla, sepals, leaves and flower stalks. Except for the anthers these were cut into small pieces with a razor blade. After fresh weight determination, the floral parts and leaves were extracted with 3 mmol.l^{-1} citric acid in 70% ethanol. Because of the size of the leaves and the sepals, only parts of them were extracted.

2.5. Determination of radioactivity in proline and glutamate

The 70% ethanol extracts of the fractions were shaken with chloroform as described above. The water phase was applied to a Dowex 50WX8 resin. The amino acids adsorbed by the resin were eluted by 5 mol.l^{-1} ammonia. The ammonia was removed from the eluate by evaporation under a stream of N_2. The residue was redissolved in water and cochromatographed with 12.5 nmol of both proline and glutamic acid on 10 × 10 cm cellulose TLC plates (cut from cellulose precoated plates, 0.5 mm in thickness, 20 = 20 cm, MERCK, type PLC). Up to 10 small plates were placed in a glass rack and developed simultaneously. Amino acids are separated just as well as on large plates in about 2 h for the first and 3 h for the second dimension. The plates were developed at 25 °C in iso-propanol-methylethylketone-HCl, 1 mol.l^{-1}, (12:3:5, v/v/v) in the first dimension and phenol-H_2O-3% NH_3 (3:1:12, w/v/v) in the second. Both proline and glutamic acid were visualized with a ninhydrin reagent and the cellulose scraped into acidified water (pH 3). The samples were allowed to stand overnight, taken up in 4 ml of Aqualyte (Baker, Deventer, Netherlands) and counted in a liquid scintillation analyzer (PHILIPS PW-4540).

2.6. Radiochemicals

L-[2,3,4,5-^{3}H]proline, 4.03 TBq.mmol^{-1}, and L-[U-^{14}C]glutamine, 1.48 GBq.mmol^{-1} were purchased from the Radiochemical Centre, Amersham, UK.

3. RESULTS

3.1. Development of anthers and accumulation of proline

The lengths of the flower buds used in our experiments covered the range 5 to 55 mm, which corresponds to the stages of microspore to mature pollen grain one day prior to anthesis. This range is traversed by the developing flower bud within 7–8 days. During the first half of this period both fresh and dry weight of the anthers increased (*fig. 1*). After the 35 mm bud stage the fresh weight of the anthers dropped quickly but the dry weight remained almost constant. In this late period of pollen development the water content in the anthers decreased by 40%.

Free proline accumulates within the anthers at a high rate and the proline content increases by a factor of 3 when the flower buds grow from 5 to 15 mm (*fig. 2*). The accumulation is interrupted and the proline content decreases at a bud length between 15 and 25 mm. After this short period, accumulation is

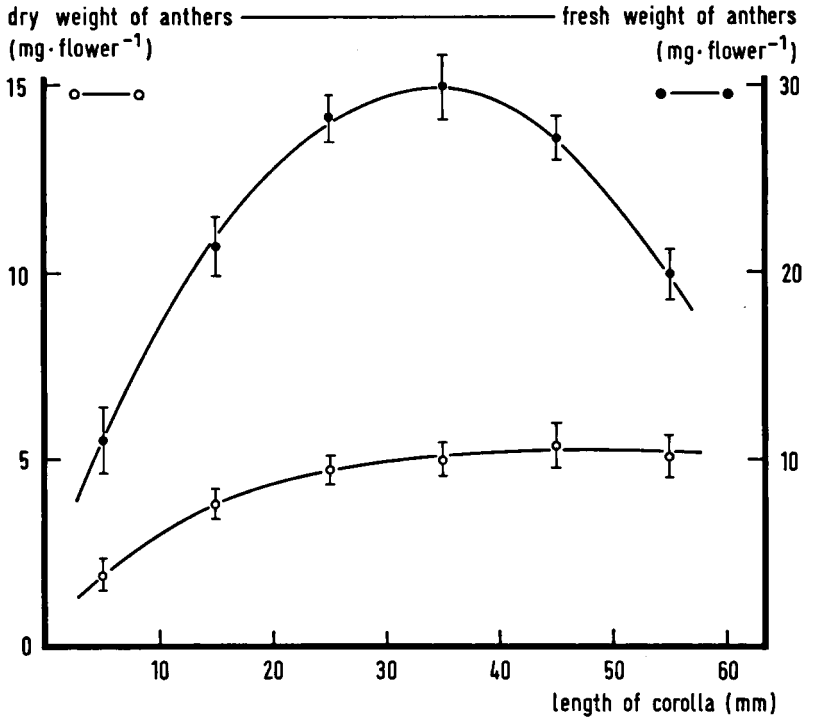

Fig. 1. Dry weight (—○—○—) and fresh weight (—●—●—) of anthers of *Petunia* during flower bud development. Determination started at the microspore stage and ended one day before anthesis. For each determination, the anthers of 20 flower buds were pooled. Vertical bars indicate standard errors of the means (n = 3).

resumed at almost the same rate as before and leads to a free proline pool in the anthers of up to 1.8% of the dry weight on the day before anthesis (55 mm). *Fig. 2* also shows the change in glutamate content, which is usually a predominant constituent of the amino acid pool. Glutamate content increases slowly up to the 25 mm bud stage and then decreases. By the end of development the glutamate content is about 0.37% of the dry weight. No substantial differences appear in the development of anthers and amino acid contents of flower parts between the isolated branches and plants grown in the greenhouse. There is, however, a slight increase in proline content (maximally not more than 1 nmol.mg^{-1} during the incubation) in the leaves of the isolated branches.

3.2. Transport of [^{3}H]proline

One important question to be answered is whether proline is transported as such from other parts of the plant. Information on this point is presented in *fig. 3*. One day after labelling in the highest radioactive proline concentration is found in the leaves. More than half of this activity disappears during the second day of incubation. At the same time the radioactive proline content increases

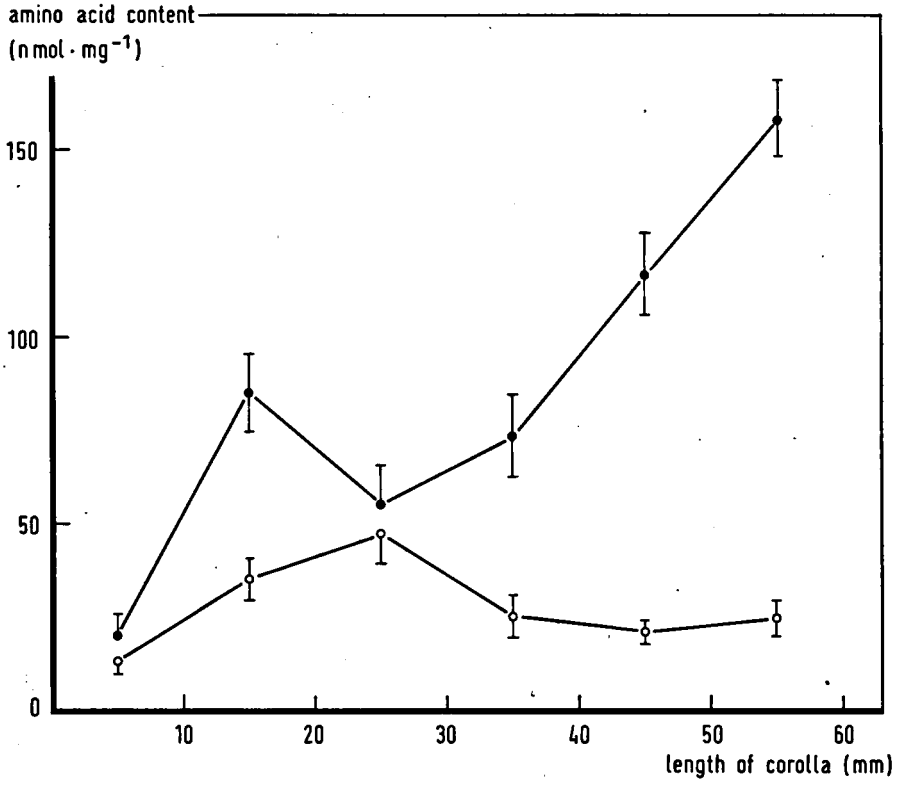

Fig. 2. Amounts of proline (—●—●—) and glutamate (—○—○—) in developing anthers of *Petunia*. The anthers of 5–10 buds were used for each determination. Data are expressed as nmol per mg fresh weight. Vertical bars indicate standard errors of the means (n = 5).

considerably in the anthers, the pistil and the corolla. In both the pistil and the corolla the increase is transient, since radioactive proline drops sharply between the second and the third day. In contrast, the accumulation of [^{3}H]proline continues in the anthers until the end of the experiment. Calculation of [^{3}H]proline distribution shows that on the first day 99.2% of the total [^{3}H]proline within the floral parts and leaves is present in the leaves and only 0.1% is in the anthers. After 5 days leaf [^{3}H]proline declines to 81.9% but in contrast it increases continuously in anthers to 9.4%. Only 7.1% of the total amount of [^{3}H]proline is then present in pistil, corolla and sepals although the total weight of these parts of the flower bud is 3.8 times that of the anthers. These data support the idea that proline is transported from the leaves into the anthers during flower bud development. The amount of proline transported will be discussed below.

3.3. Proline-glutamate interconversion

The above conclusion does not exclude other possible mechanisms including part of the proline to be synthesized within the anther from a precursor molecule

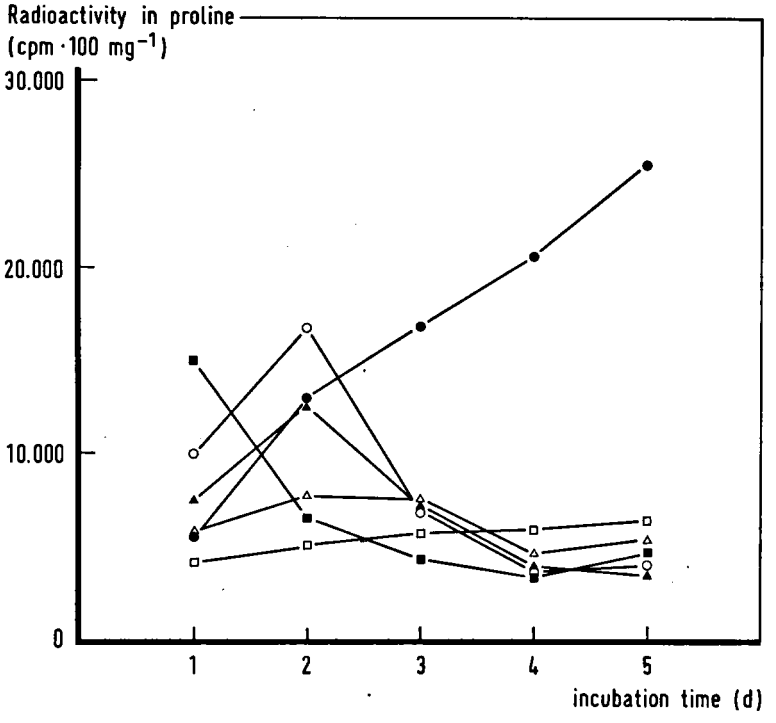

Fig. 3. Distribution of [^{3}H]proline in different parts of isolated branches of *Petunia* fed [^{3}H]proline, 1.4 MBq.branch^{-1}, at zero time. (—●—●—) anthers; (—○—○—) pistil; (—▲—▲—) corolla; (—△—△—) sepals; (—■—■—) leaves and (—□—□—) flower stalk. The proline count was measured on ethanol extracts separated by 2-dimensional thin-layer chromatography. Data are expressed as cpm per 100 mg fresh weight.

and leaf proline to be converted to another compound and resynthesized in the anther. In this case, no proline would be transported at all. In order to detect a possible proline turnover the conversion of prolinee to glutamate and vice versa were analyzed. The ratio of the [^{3}H]proline to [^{3}H]glutamate was determined after different incubation periods. These data were compared to the ratio between the pool sizes of proline and glutamate, respectively. The rationale for this calculation is that if the [^{3}H]proline is extensively degraded, the ratio of the [^{3}H]count in proline and glutamate would approach the ratio of their respective pool sizes. The results are shown in *fig. 4a–d.* The situation is most straightforward for leaves where the [^{3}H] ratio greatly exceeds the ratio of the pools. This indicates that the proline to glutamate conversion is only a minor process within the leaves, suggesting that proline itself and not a proline metabolite is transported from the leaves into the anthers.

When the data for anthers, pistil and corolla are compared, the highest [^{3}H]proline – [^{3}H]glutamate ratio is found in the anthers. The value gradually decreases with time which suggests that proline is slowly degraded in the anther.

However, even at the end of the experiment this ratio exceeds that of the pool sizes by a factor of approximately 2. The lower [^{3}H]proline over [^{3}H]glutamate in the corolla and the pistil points to a more extensive proline degradation in these organs. This result is in agreement with the observation that proline is only transiently accumulated in these organs (*fig. 3*).

A similar calculation was made for the distribution of the [^{14}C] counts from glutamine over proline and glutamate. Again the most clear-cut results are obtained for the leaves (*fig. 4a*). Over the whole period of investigation the [^{14}C] count ratio coincides with the ratio of the pool sizes. This means that glutamate is rapidly converted into proline and that the pathway of synthesis of proline via glutamate is operative in the leaves. A comparison of the [^{14}C] ratio in proline and glutamate within the floral parts (*fig. 4b–d*) shows that this value is highest in the anthers and much lower in both the pistil and corolla. Thus, the higher ratio in the anthers might indicate that synthesis of proline proceeds faster in this organ than in other parts of the flower. However, there are other possible interpretations. In anthers, proline degradation is low and hence barely affects the ratio of [^{14}C]proline to [^{14}C]glutamate. In the corolla and pistil, proline is more easily degraded which would reduce the [^{14}C]count ratio. A third possibility is that the [^{14}C]proline is not synthesized in the anthers but in the leaves and transported afterwards. Therefore it is significant to know the extent of [^{3}H]proline transport. Evidence concerning this point was obtained by analyzing the [^{14}C]proline of [^{14}C]glutamate ratio in the flower stalk, the organ that connects the flower to the leaves. The results (*table 1*) show that this ratio is lower than those in the leaves and in the anthers (*fig. 4a–b*) over the whole period of investigation. As far as proline and glutamate are concerned, glutamate is obviously the preferred transportation form. These data taken together provide evidence for a second mechanism of proline accumulation in the anthers involving proline synthesis *in situ* either from or via glutamate.

4. DISCUSSION

The experiments on the mechanism of proline accumulation have shown that 10 mm buds of *Petunia* actively accumulate both proline and glutamate at least during the first part of the incubation period (*fig. 2*). There is little doubt that the leaves serve as proline sources and the anthers as proline sinks (*fig. 3*). The transport of proline is one factor involved in its accumulation in anthers. Proline once synthesized, is relatively stable in the leaves as well as in the anthers (*fig. 4a–b*). This lack of utilization is a second important element in the accumulation mechanism. A similar accumulation and high stability of proline have been reported for water-stressed leaves (BARNET & NAYLOR 1966; ASPINALL et al. 1974; SINGH et al. 1972; STEWART et al. 1977; SELLS & KOEPPE 1981). Another analogy between anthers and water-stressed leaves is the dramatic loss of water during the second part of pollen maturation (*fig. 1*). There is, however, no evidence relating the increase in proline content during this period (*fig. 2*) to dehydration of the anthers.

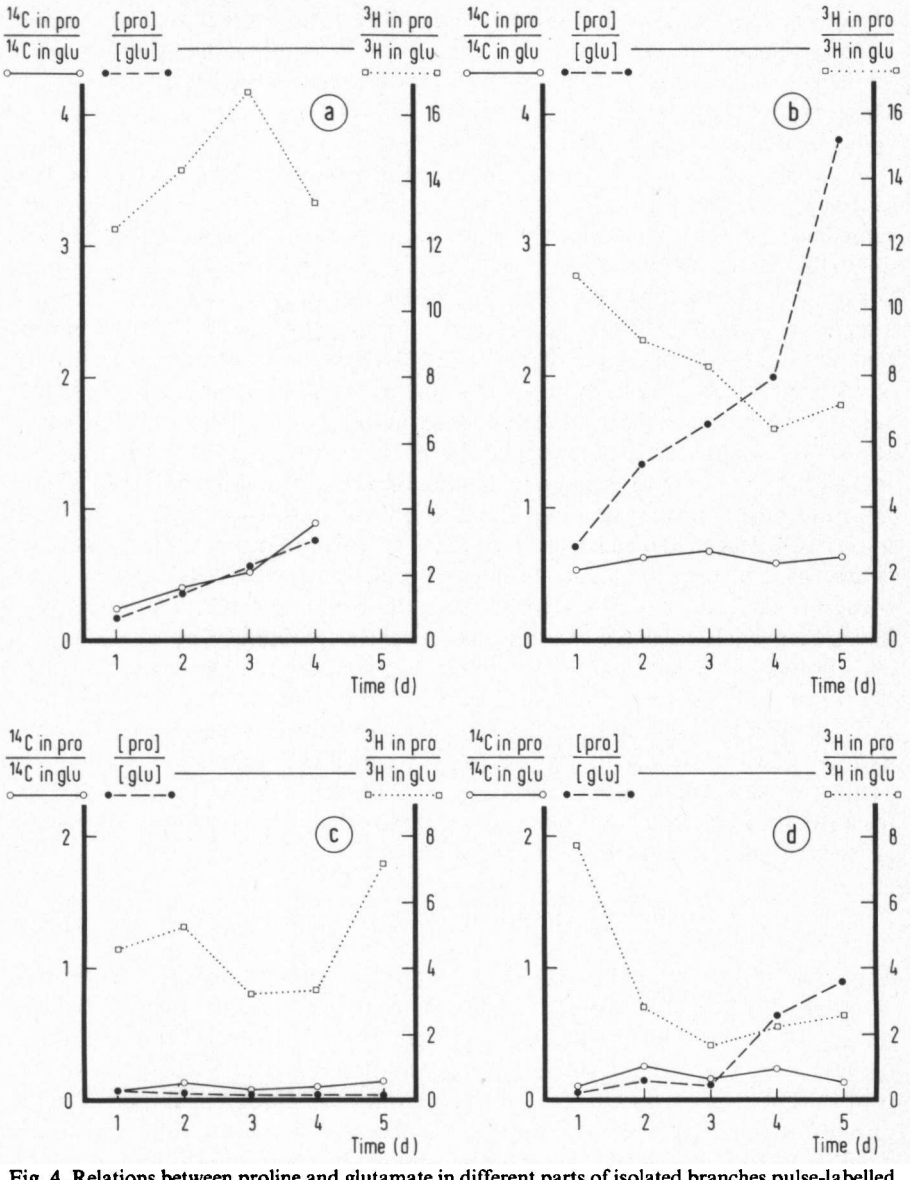

Fig. 4. Relations between proline and glutamate in different parts of isolated branches pulse-labelled with [^{3}H]proline, 1.4 MBq.branch^{-1} and [^{14}C]glutamine, 150 KBq.branch^{-1}: (a) leaves, (b) anthers, (c) pistil and (d) corolla. The proline to glutamate ratio was determined with respect to three parameters: (---●---●---) chemical concentration; (....□....□....) [^{3}H]radioactivity; (—○—○—) [^{14}C]radioactivity.

The extent of proline transport can be roughly estimated from the proline count (*fig. 3*) and content in the anthers and the specific activity of the amino

Table 1. Ratio of [^{14}C]proline over [^{14}C]glutamate in flower stalks on isolated branches pulse-labelled with [^{14}C]glutamine. At the onset of the experiment 150 KBq of [^{14}C]glutamine was given to the cut end of a branch bearing one developing bud. The pool of the free amino acids was isolated on five successive days after labelling.

time (d)	[^{14}C]proline/[^{14}C]glutamate (cpm.cpm^{-1})
1	0.17
2	0.22
3	0.11
4	0.18
5	0.10

acid in the leaves. The estimate is that 10–15% of the proline accumulated results from proline transport. The low figure is not completely unexpected as there is indirect evidence that proline is slowly transported (*table 1*). The obvious conclusion is that the anther possesses a high capacity for proline synthesis. This situation is analogous to the biochemistry of water-stressed leaves.

In anthers there are indications that this synthesis proceeds via glutamate (*fig. 4*) but the actual precursor is unknown. Our findings conflict with the observations of BRITIKOV & MUSATOVA (1964) who did not find a glutamate to proline conversion in anthers of *Amaryllus*. The difference may be due to the different labelling procedure used by these investigators. They immersed anthers in a medium containing the radioisotope. The way of entering of the glutamate via the anther wall may have influenced the extent to which it was metabolized.

In conclusion, three factors have been found to lead to proline accumulation in anthers: transport of proline from the leaves, synthesis of proline in the anthers either from or via glutamate and finally a low degradation rate of the proline accumulated.

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