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RELATIONSHIP BETWEEN TRIGONELLINE CONCENTRATION AND PROMOTION OF CELL ARREST IN G2 IN CULTURED ROOTS OF *PISUM SATIVUM*

WILLIAM A. TRAMONTANO, CHRISTINE M. HARTNETT, DAVID G. LYNN* and LANCE S. EVANS

Laboratory of Plant Morphogenesis, Manhattan College, The Bronx, NY 10471, U.S.A.; *Chemistry Department, University of Virginia, Charlottesville, VA 22903, U.S.A.

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Key Word Index—*Pisum sativum*; Leguminosae; pea; trigonelline; cell arrest in G2; pyridine nucleotide metabolic pathway; nicotinic acid; nicotinamide; cell cycle.

Abstract—Trigonelline, G2 Factor, present in cotyledons of *Pisum sativum* is transported to roots and shoots after germination. This hormone promotes preferential cell arrest in G2 in 40% of all root cells during normal cell differentiation. In the absence of trigonelline this cell population arrests in G1. High concentrations of other components of the pyridine nucleotide metabolic pathway for NAD production promote cell arrest in G2 when added to culture medium. The order of decreasing positive effect was trigonelline, nicotinic acid, nicotinamide, and NAD. Nicotinic acid and nicotinamide are converted to trigonelline to provide an effective concentration which promotes cell arrest in G2. Cell arrest was more closely correlated with the concentration of trigonelline in the medium than with the quantity/concentration of trigonelline in roots. Trigonelline is most effective in aseptic media and other components of the pyridine nucleotide pathway are effective only because a sufficient amount of trigonelline is synthesized to promote cell arrest in G2. Trigonelline is the first chemically identified hormone that controls the cell cycle in plants or animals.

INTRODUCTION

Recent results demonstrate that trigonelline (G2 Factor) functions as a plant hormone[1–3]. Trigonelline, found in cotyledons of dry seeds, promotes preferential cell arrest in G2 of the cell cycle[4]. During early seedling development trigonelline is transported from cotyledons to other plant tissues[3].

Arrest in the mitotic cycle by meristematic cells in cultured primary root tips after temporary carbohydrate deprivation is a non-random process[5]. Cell arrest during normal cell differentiation also occurs in a non-random process and cells arrest in both tissues (meristematic and mature root tissues) in a similar manner within each plant[6]. Few cells stop during DNA synthesis and no cells arrest during mitosis. During normal cell arrest in mature root tissues and in root meristems under temporary carbohydrate deprivation, cells arrest in G1, in G2, or become polyploid in *P. sativum* L. If trigonelline is present, 40% of the meristem cell population is pre-conditioned to arrest in G2. If sufficient trigonelline is not present, these cells arrest only in G1[2, 4, 7].

Trigonelline has been detected in many plants and animals. In plants, trigonelline has been found in several Gymnospermae, in several Monocotyledoneae, and many Dicotyledoneae [8]. Moreover, trigonelline has been found in a wide variety of animals [9]. Many mammals excrete trigonelline in urine [10].

Trigonelline is usually the most abundant molecule of the pyridine nucleotide metabolic pathway for the production of nicotinamide adenine dinucleotide (NAD) [11, 12]. This indicates trigonelline may have a regulatory role in NAD biosynthesis as well as in cell proliferation. Chen *et al.*[13] incorporated [^{14}C]nicotinic acid into trifoliate leaves of *Phaseolus vulgaris* L. Nine radioactive compounds were isolated and all were identified as possible intermediates of NAD metabolism. After 24 and 48 hr, *ca* 80% of the nicotinic acid fed into the leaves was converted into trigonelline.

Experiments with excised roots of *P. sativum* in aseptic culture were performed to determine (a) if high concentrations of other components of the pyridine nucleotide pathway promote cell arrest in G2, (b) the quantity of components of the pathway in roots and in media when various concentrations of intermediates are added to culture media and (c) if components of the pathway promote cell arrest directly or indirectly through conversion to trigonelline.

RESULTS

Fig. 1 shows results of experiments in which quantities and concentrations of trigonelline were determined in excised roots cultured at various concentrations of added trigonelline, nicotinic acid (NA), nicotinamide (NAM), and nicotinic adenine dinucleotide (NAD). Roots exposed to media trigonelline concentrations lower than 10^{-6} M did not result in a significant change in the root concentration of trigonelline. However, the concentration of trigonelline

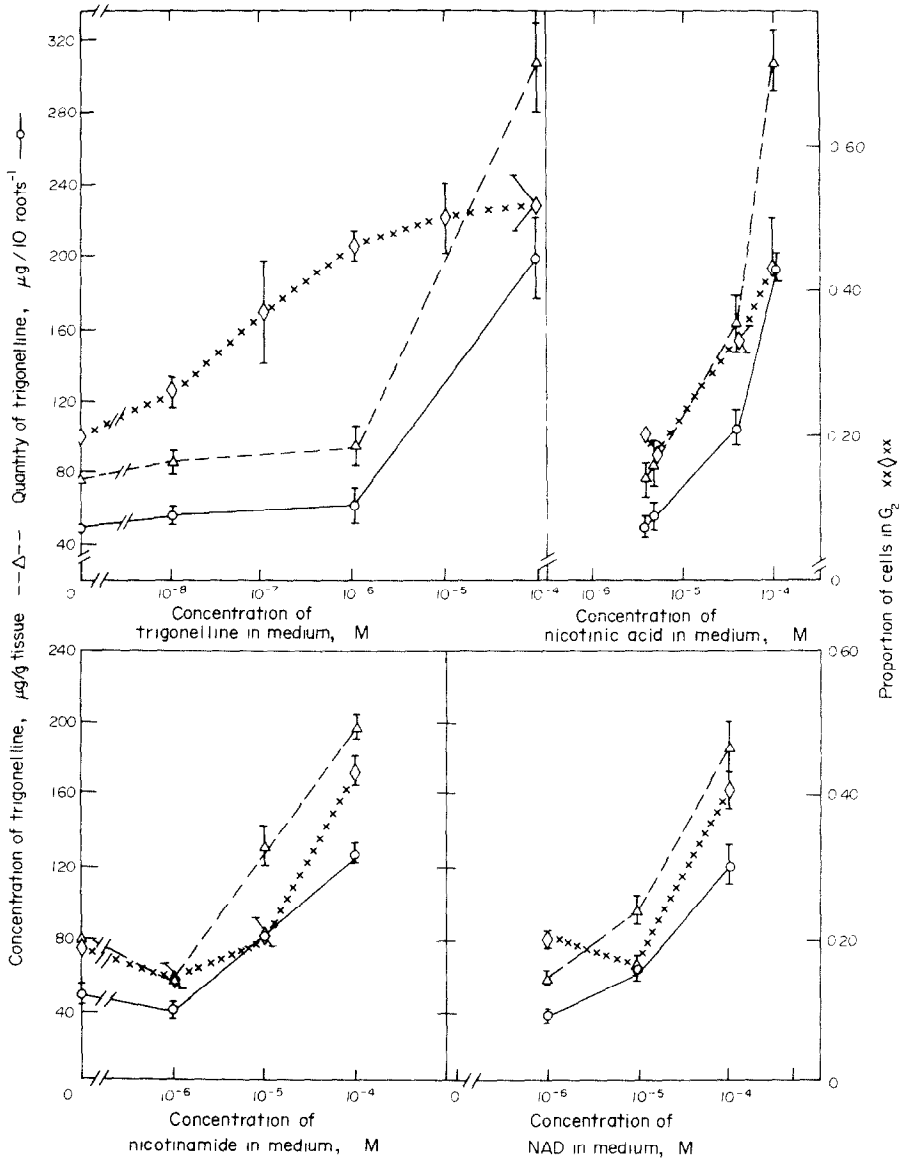

Fig. 1. Concentrations (μg/g tissue) and quantities (μg/10 roots) of trigonelline, and proportions of cells arrested in G2 in stationary phase meristems of excised roots of *Pisum sativum* exposed to various substances in aseptic culture. Excised roots from 3-day-old seedlings were exposed to various concentrations of trigonelline (upper left), nicotinic acid (upper right), nicotinamide (lower left), and nicotinamide adenine dinucleotide, NAD (lower right) while in media with sucrose. After 3 days in media with sucrose some roots were harvested to determine concentrations and quantities of trigonelline while the remainder were transferred to media without sucrose to establish a stationary phase.

in roots increased markedly in 10^{-4} M trigonelline. A similar increase in trigonelline concentration was observed upon addition of other intermediates of the pyridine nucleotide metabolic pathway at concentrations greater than 10^{-5} M. The increase in trigonelline concentration in roots was correlated with the proportion of cells arrested in G2. A 10^{-4} M concentration of either NA, NAM, or NAD significantly stimulated G2 arrest[3]. The concentration of trigonelline in the medium necessary to stimulate arrest in G2 was 10^{-7} M whereas NA, NAM and NAD showed effective concentrations between 10^{-4} and 10^{-5} M. When regression analyses were performed between the proportion of cells in G2 in stationary phase meristems and the amount of trigonelline in roots, regression coefficients for nicotinic acid, nicotinamide and NAD were +0.98, +0.92, and +0.96, respectively.

Experiments with ^{14}C-labelled NA and NAM were performed to determine concentrations of newly synthesized components of the pathway in both roots and media. This experimental protocol determined the concentrations of these components produced only by roots in media during 3 days of culture.

Addition of either 10^{-4} M NA or NAM into White's media promoted cell arrest in G2. A 10^{-6} M concen-

tration of either NA or NAM did not produce this effect. These experiments provided the ability to determine whether concentrations of NA and NAM effectively promote cell arrest in G2 directly or indirectly, by being converted into trigonelline. When radioactive NA was added to White's media at 10^{-4} M, the concentration of radioactive trigonelline found in media after culturing roots of *P. sativum* for 3 days was 1.36×10^{-6} M (Table 1), which was effective in promoting cell arrest in G2 [4]. Addition of radioactive NA at 10^{-6} M yielded 6.95×10^{-8} M trigonelline, which was ineffective. Addition of 10^{-4} M radioactive NAM yielded an effective concentration of trigonelline (3.32×10^{-7} M), whereas addition of 10^{-6} M NAM yielded an ineffective concentration of trigonelline (2.60×10^{-8} M) in media after 3 days of culture.

When either radioactive NA or NAM was added to media at 10^{-4} M, the majority of total radioactivity was found in the media and not in roots after culture for 3 days. About 12% of the total radioactivity was present as trigonelline. When either NA or NAM at 10^{-6} M was added, the majority of radioactivity was found to be in roots and not in media. More than 20% of the total radioactivity existed as trigonelline when 10^{-6} M NA was added to media. When roots were cultured in 10^{-6} M NAM for 3 days 45% of the total radioactivity existed as trigonelline.

When roots were cultured in 10^{-6} M radioactive trigonelline for 3 days, there was little conversion to other components of the pathway. In media and roots, respectively 85 and 15% of the total radioactivity was found. Trigonelline comprised 65% of the total radioactivity. During the 3 days culture period the concentration of trigonelline declined to 7.2×10^{-7} M. Calculated amounts of trigonelline in (a) culture media, (b) roots before culture, (c) roots after culture, and the proportion of cells arrested in G2 in stationary phase meristems are shown in Table 2. The calculated concentration of trigonelline produced in the presence of 5×10^{-6} M NA (the usual amount of NA in White's media) is also calculated in Table 2. In this manner, the theoretical quantity of trigonelline present after culture for three days can be calculated (Table 2, column 4). These values compare well with actual amounts (Table 2, column 5).

Proportions of cells arrested in G2 in stationary phase meristems after exposure to various concentrations of trigonelline in media are also shown in Table 2. Proportions of cells arrested in G2 are compared with concentrations of trigonelline within roots after culture in media with sucrose for 3 days prior to establishment of stationary phase (Fig. 2). The proportion of cells arrested in G2 in stationary phase meristems increased markedly from 0.26 to 0.47 between trigonelline concentrations from 4.82 to 6.12 μg per root. Above a concentration of 6.2 μg trigonelline per root the proportion of cells arrested in G2 did not increase significantly.

DISCUSSION

Will high concentrations of components of the pyridine nucleotide pathway promote cell arrest in G2?

Addition of high concentrations of other components of the pathway besides trigonelline promoted cell arrest in G2. All members of the pathway tested promoted cell arrest in G2 in roots of *P. sativum* at concentrations of 10^{-4} M[3]. These results indicate that other components of the pathway can, at relatively high concentrations, produce the same effect as trigonelline. They do not, however, promote cell arrest in G2 at concentrations of 10^{-7} M, a concentration at which trigonelline is effective.

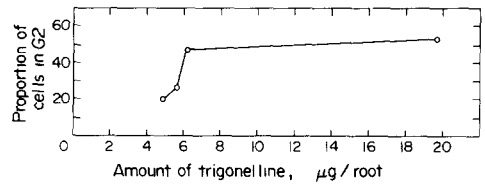

Fig. 2. Relationship between proportion of cells arrested in G2 in stationary phase meristems of *Pisum sativum* compared with the actual concentration of trigonelline in cultured roots before establishment of the stationary phase. Roots were cultured in various concentrations of trigonelline. Values taken from Table 2.

Do components of the pyridine nucleotide pathway cause cell arrest in G2 directly or indirectly, by first being converted to trigonelline?

When either NA or NAM 10^{-4} M were added to culture media, the resulting concentration of trigonelline in media was greater than 10^{-7} M which is sufficient to promote predominant cell arrest in G2[4]. Moreover, the quantity of trigonelline found in roots was also greater than the quantity necessary to cause cell arrest in G2. Components of the pathway are converted to trigonelline. These results show that it is the concentration of trigonelline and not the concentration of other components of the pathway in either roots or media which promotes cell arrest in G2 phase. Data obtained from addition of 10^{-6} M trigonelline also support this conclusion. The majority of radioactivity remained as trigonelline in cultures exposed to trigonelline. Little trigonelline was converted to other pathway components. These results agree with those of Joshi and Handler[14], which show little demethylation of trigonelline in intact seedlings of peas. Therefore, it is concluded that promotion of cell arrest in G2 is caused by a concentration of at least 10^{-7} M trigonelline present in culture media within 3 days of culture.

What are the percentages of conversion of these components to trigonelline in both roots and media?

Under high concentrations (10^{-4}) of NA and NAM a sufficient amount (12.3 and 11.8%, respectively) is converted to trigonelline, so that the concentration of trigonelline is greater than 10^{-7} M after 3 days of culture. This concentration is adequate to promote cell arrest in G2. This threshold trigonelline concentration of 10^{-7} M was not obtained if either 10^{-6} M NA or NAM were supplied in aseptic media. This threshold concentration was not reached even though 21 and 45% of the NA and NAM, respectively, at 10^{-6} M was converted to trigonelline. Only 8.1 and 4.0% NA and NAM, respectively, are produced if roots are exposed to radioactive trigonelline for 3

Table 1. Molarity and percentage of radioactivity within media and concentration and percentage of radioactivity within roots of various components of the pyridine nucleotide pathway when excised roots were cultured for 3 days in the presence of nicotinic acid, nicotinamide, or trigonelline

	10^{-4} M nicotinic acid		10^{-6} M nicotinic acid		10^{-4} M nicotinamide		10^{-6} M nicotinamide		10^{-6} M trigonelline	
	Media*	Root	Media*	Root	Media	Root	Media	Root	Media*	Root
Component	final molarity %†	μg/g tissue %†	molarity %†	μg/g tissue %†	molarity %†	μg/g tissue %†	molarity %†	μg/g tissue %†	molarity %†	μg/g tissue %†
NAM	5.2×10^{-8}	1.75	6.9×10^{-9}	0.94	8.7×10^{-6}	4.40	3.83×10^{-8}	0.44	1×10^{-7}	0.05
	0.28	0.77	0.32	4.61	11.8	0.70	4.78	5.97	7.69	0.37
NAD	—	120	—	3.0	2.47×10^{-5}	29.80	7.0×10^{-9}	0.38	5.8×10^{-8}	0.54
		1.28	—	2.47	0.33	0.60	0.96	5.07	4.31	0.79
NA	1.08×10^{-4}	236	5.9×10^{-7}	0.97	2.41×10^{-7}	139.60	1.0×10^{-7}	0.14	5.1×10^{-8}	0.003
	57.6	14.0	27.7	4.94	34.8	20.00	12.8	1.43	3.75	0.24
Trigonelline	1.36×10^{-6}	285	6.95×10^{-8}	4.70	3.32×10^{-7}	110.00	2.6×10^{-8}	4.32	7.16×10^{-7}	1.93
	0.73	11.6	3.26	17.5	0.44	12.40	3.28	41.8	52.4	11.2
All others‡	—	—	—	—	—	—	—	—	—	—
	6.93	6.85	7.58	31.6	7.26	11.70	9.25	14.6	16.9	2.26
Total	—	—	—	—	—	—	—	—	—	—
	65.5	34.5	38.9	61.1	54.6	45.40	31.1	68.9	85.1	14.9

*All calculations are corrected for 1 l. of media and 200 roots that would be grown in 1 l. of media. In all experiments ten excised roots were placed in 50 ml of media.

†Percentage of radioactivity in each component recovered from either media or roots was calculated on the basis of total radioactivity in media plus roots.

‡The phrase 'all others' refers to all other areas on the thin-layer plates not related to the four components assayed.

Table 2. Calculation of the theoretical quantity of trigonelline, actual quantity of trigonelline, and proportion of cell arrest in G2 in stationary phase meristems of excised cultured roots of *Pisum sativum*

Concentration of trigonelline in media (M)	Amount of trigonelline added to media (μg/flask)*	Amount of trigonelline incorporated from media/ cultured root* (μg)	Theoretical amount of trigonelline cultured root† (μg)	Actual amount/ root‡ (μg)	Proportion of cells in G2‡
10^{-4}	870	12.87	17.14	19.72	0.53
10^{-5}	87	1.29	5.56	—	0.51
10^{-6}	8.7	0.13	4.40	6.12	0.47
10^{-7}	0.87	0.01	4.28	—	0.37
10^{-8}	0.087	0.00	4.27	5.55	0.26
0	0	0	4.27	4.82	0.20

*Calculated quantities based upon the incorporation percentage (11.2%) at 10^{-6} M trigonelline concentration (see Table 1).
†The amount of trigonelline in excised roots before culture is 3.73 μg per root. The amount of trigonelline produced from exposure of excised roots to 5×10^{-6} M nicotinic acid is 0.54 μg per root. These values are used in these calculations.
‡Values obtained from Fig. 1.

days. The event common to all situations for positive results involves a concentration of 10^{-7} M trigonelline in the media. These results suggest that other intermediates of the pyridine nucleotide pathway are converted to trigonelline, which in turn, is responsible for promoting cell arrest in G2.

Is promotion of cell arrest in G2 related more to the trigonelline concentration in roots or in media?

Promotion of cell arrest in G2 was more correlated with concentration (or quantity) or newly synthesized trigonelline in media than in roots themselves. The reason(s) for this better correlation is unknown. In the non-radioisotope experiments the quantity of trigonelline in roots was correlated with the proportion of cells in G2. As long as the concentration of trigonelline in media was at least 10^{-7} M, promotion of cell arrest in G2 occurred. Results suggest that NAM and NA are first converted to trigonelline, which provides the signal for preferential cell arrest in G2.

These experimental results show that trigonelline is the effective member of the pyridine nucleotide pathway which promotes cell arrest in G2 in root tissues of garden peas and demonstrate the relationship between components of the pyridine nucleotide pathway and cell arrest in G2.

In a previous report[3] the proportion of cells in G2 in stationary phase meristems was highly correlated with concentrations of trigonelline in intact seedling meristems of *P. sativum*. In culture experiments reported here, the proportion of cells arrested in G2 was correlated with concentrations of trigonelline in excised roots exposed to elevated concentrations of NA, NAM and NAD. However, promotion of cell arrest in G2 is more highly correlated with concentrations of trigonelline in media rather than trigonelline concentrations in excised roots.

EXPERIMENTAL

General culture conditions. Seeds of *Pisum sativum* L. were surface-sterilized with undiluted Clorox®, which contains 5.25% Na hypochlorite, stirred frequently for 10 min, washed with sterile H_2O to remove bleach and germinated in sterile vermiculite. Under aseptic conditions, excised root tips 33 mm long from 3-day-old seedlings were cultured in White's[15] medium with sucrose plus a specific concn of a radioactive or non-radioactive component of the pyridine metabolic pathway. Five different experiments were conducted with one of the following radioactive components added to the medium: 10^{-4} or 10^{-6} M [carboxyl-^{14}C]NA (0.25 μCi/ml; sp. act. 50 mCi/mmol), 10^{-4} M or 10^{-6} M [carbonyl-^{14}C]NAM (0.25 μCi/ml; sp. act. 50 mCi/mmol), or 10^{-6} M [carboxyl-^{14}C]trigonelline. All were purchased from Amersham Corporation except [carboxyl-^{14}C]trigonelline, which was synthesized from [carboxyl-^{14}C]NA.

Bioassay for promotion of cell arrest in G2. General culture techniques to determine the ability of plant-derived extracts which promote cell arrest in G2 by a standardized bioassay have been described[16]. Under aseptic conditions excised root meristems were placed in culture medium with sucrose for 3 days before temporary carbohydrate deprivation (establishment of a stationary phase meristem). A stationary phase meristem may be defined as a meristem (0–2 mm portion) in which progression through the cell cycle has ceased temporarily[17]. In some experiments an intermediate of the pyridine nucleotide metabolic pathway was added to 50 ml sucrose medium, which normally supports growth of ten roots. With the eventual establishment of a stationary phase by temporary carbohydrate deprivation, cells were arrested in G1 and in G2 (2C and 4C contents respectively) within the terminal meristem (2 mm). If roots from 3-day-old seedlings are placed in sucrose medium alone before establishment of stationary phase, only 20% cells arrest in G2. However, if a sufficient concentration of trigonelline is present in medium with sucrose before establishment of stationary phase, then a larger proportion (*ca* 40–60%) of cells arrest in G2[16].

DNA measurements. Measurements of relative DNA per nucleus of Feulgen stained nuclei were obtained via microfluorimetry. The method is modified from that of Alvarez and Truitt[18] and is described elsewhere[4].

Extraction procedures. After 3 days in culture, roots were harvested and 0–10 mm meristems were excised and extracted through an EtOH series [11]. After three extractions with $CHCl_3$, extract vols were reduced to 1 ml/g of tissue. Extracts were plated on Analtech Si Gel GF thin-layer

plates, 250 μm thickness. 200 μl of extract was applied to each plate along with non-radioactive NA, NAM, trigonelline and NAD. Plates were developed in a solvent system of isobutyric acid–18 M NH_4OH–H_2O (66:1:33), pH 3.8[11]. Known vols of media were also plated along with standards and developed. An analysis of all plates (both root extracts and medium) was performed from origin to solvent front. This was done by scraping silica from plates after component identification. Each component band was placed in a separate liquid scintillation vial with Aquasol®. All vials were counted in a Beckman LS-150 Liquid Scintillation Counter. The amount of radioactivity in each component was calculated from quench curves and corrections of specific activity. Only bands of NAD, trigonelline, NA and NAM could be accurately identified. Since a majority of the radioactivity was associated with these four, components and since the other components of the pathway could not be identified positively, no estimates of the percentages of radioactivity in the other members of the pathway were made. The total amount of radioactivity on each plate was determined by combining all other areas except areas taken for the components above.

Trigonelline concentrations. To determine the concn of trigonelline in roots, 0–1 cm terminal root segments of 3-day-old seedling roots were excised and extracted in an EtOH series[11]. Each extract was concd, spotted on Analtech Si gel TLC UV plates, 250 μm thickness (Analtech Corp., Newark, Delaware, U.S.A.) and developed in Me_2CO–H_2O (1:1). Plates were allowed to air-dry and trigonelline was eluted. Trigonelline concns were determined using HPLC[19]. A Spectra Physics Chromatronix Liquid Chromatograph Model 3500 equipped with a Waters Differential Ultraviolet (254 nm) Detector and a Waters Injector System with a Whatman Partisil–Sax 10 column was employed. KPi buffer, (pH 5.8) 0.007 M, was used at a flow rate of 1 ml/min.

Synthesis of [carboxyl-^{14}C]trigonelline. Carboxyl-labelled NA (0.01 mM, 50 mCi/mM) was dissolved in dry MeOH (10 ml) containing 0.015 mmol of H_2SO_4 and refluxed under N_2 for 6 hr. The reaction mixture was cooled, neutralized with dry $NaHCO_3$ and taken to dryness *in vacuo*. The Me ester of [carbonyl-^{14}C]NA was recovered quantitatively from this residue by trituration with Et_2O. Methylation of this ester with MeI (0.1 mmol) in C_6H_6 (10 ml) produces the methiodide salt. Passage of an aq. soln of this salt through Dowex I-X8 (OH^- form, 15 mg) produces [carboxyl-^{14}C]trigonelline, which crystallizes from a minimum amount of EtOH as the monohydrate (71%).

REFERENCES

1. Lynn, D. G., Nakanishi, K., Patt, L. S., Occolowitz, J. L., Almeida, M. S. and Evans, L. S. (1978) *J. Am. Chem. Soc.* **100**, 7759.
2. Evans, L. S., Almeida, M. S., Lynn, D. G. and Nakanishi, K. (1979) *Science* **203**, 1122.
3. Evans, L. S. and Tramontano, W. A. (1981) *Am. J. Botany* **68**, 1282.
4. Evans, L. S. (1979) *Am. J. Botany* **66**, 880.
5. Van't Hof, J. and Kovacs, C. J. (1972) in *The Dynamics of Meristem Cell Population* (Miller, M. W. and Keuhnert, C. C., eds.) pp. 13–33. Plenum Press, New York.
6. Evans, L. S. and Van't Hof, J. (1974) *Am. J. Botany* **61**, 1104.
7. Evans, L. S. and Van't Hof, J. (1973) *Exp. Cell Res.* **82**, 471.
8. Willeke, U., Heeger, V., Meise, M., Neumann, H., Schindelmeiser, I., Vordemfelde, K. and Barz, W. (1979) *Phytochemistry* **18**, 105.
9. Beers, J. R. (1967) *Comp. Biochem. Physiol.* **21**, 11.
10. Handler, P. and Dean, W. J. (1942) *J. Biol. Chem.* **146**, 357.
11. Godavari, H. R. and Waygood, E. R. (1973) *Can J. Botany* **48**, 2267.
12. Preiss, J. and Handler, P. (1957) *J. Am. Chem. Soc.* **79**, 4246.
13. Chen, S. C., Godavari, H. R. and Waygood, E. R. (1973) *Can. J. Botany* **52**, 707.
14. Joshi, J. G. and Handler, P. (1962) *J. Biol. Chem.* **237**, 3185.
15. White, P. R. (1943) *A Handbook of Plant Tissue Culture.* Cattell, Lancaster, PA.
16. Evans, L. S. and Van't Hof, J. (1974) *Exp. Cell Res.* **87**, 259.
17. Van't Hof, J. (1973) *Brookhaven Symp. Biol.* **25**, 152.
18. Alvarez, M. R. and Truitt, A. J. (1977) *Exp. Cell Res.* **106**, 105.
19. Miksic, J. R. and Brown, P. R. (1977) *J. Chromatogr.* **142**, 641.