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BIOSYNTHESIS OF CYTOKININS BY POTATO CELL CULTURES

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; tissue culture; biosynthesis; cytokinins; zeatin riboside; zeatin.

Abstract—Potato cells grown in liquid culture incorporated mevalonic acid lactone-[2-^{14}C] into free cytokinin (zeatin riboside and zeatin) and the cytokinin of RNA (zeatin riboside). The cytokinin liberated by catabolism of RNA can account for no more than 40% of the free cytokinins.

INTRODUCTION

Substituted purines of the cytokinin type have a wide distribution, being found in *t*RNA species of a wide variety of plants, animals and micro-organisms [1, 2]. The biosynthetic pathway leading to the *t*RNA cytokinins of micro-organisms and animal tissue is well known [3–6], the key step being the transfer of isopentenyl pyrophosphate (IPP) to the adenosine of the *t*RNA to give N^6-(Δ^2-isopentenyl) adenosine (IPA).

It has been suggested that the same route is used in plants, and it has been shown that mevalonic acid is incorporated into the cytokinins of tobacco RNA [7, 8]. Recently an enzyme system has been isolated from maize, which is able to transfer the isoprene unit from IPP to the adenosine of RNA *in vitro* [9].

The occurrence of cytokinins in plant *t*RNA adjacent to the anti-codon has led to controversy over their role in the control of plant growth, particularly at the translational level [1]. One important aspect of this has been the problem of whether the 'free' cytokinins are the physiologically significant ones and if so what their relationship is to the 'bound' cytokinins of the *t*RNA. A number of authors have attempted to determine whether the turnover of *t*RNA in plant is sufficient to supply the required amount of free cytokinin [10,11] but their results have been inconclusive. Less direct evidence is available [12, 13] which suggests that the free cytokinins are synthesized by a *de novo* route.

A number of plant tissues are able to synthesize cytokinins when grown *in vitro*, one of these being potato tuber tissue. The major cytokinin produced by this system is zeatin riboside (ZR) [14,15]. This paper reports our use of suspension cultures of potato cells to incorporate mevalonic acid-[2-^{14}C] into the free and *t*RNA zeatin riboside produced by this tissue. The data obtained are used to calculate the relative rate of zeatin riboside release by RNA turnover and to compare this with the production of free cytokinin.

RESULTS AND DISCUSSION

In the aerated liquid culture the potato cells grew steadliy, if slowly, with a doubling time of approximately 11 days. The growth of tissue as represented by the cell dry weight showed no indication of synchronous growth.

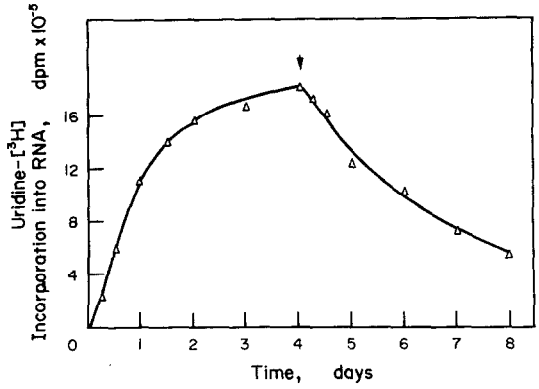

Fig. 1. Incorporation of uridine-[^{3}H] into the RNA of a 3 l. liquid culture of potato cells. Uridine-[G-^{3}H] (250 μCi) was injected into the culture at day 0. A non-radioactive chaser of 2 mg uridine was injected at day 4 as shown by the arrow. See Experimental for details.

Uridine-[U-^{3}H] was incorporated into the potato RNA with little or no lag time after the injection of either the radioactive substrate or the cold chaser (Fig. 1). The complex pattern of the incorporation suggests that the RNA into which the uridine was being incorporated consisted of a number of pools which were being turned over at different rates (see Appendix).

The C-14 label from the mevalonic acid lactone-[2-^{14}C] was incorporated into the cytokinins of potato RNA at a very low rate compared to that of the uridine. A lag period of approximately 12 hr occurred before significant incorporation was observed. This may be due to poor uptake of the mevalonic acid lactone. The ^{14}C label was found only in the zeatin riboside fraction and the pattern of incorporation (Fig. 2) was similar to that for the incorporation of uridine-[U-^{3}H], which suggests again that the label was incorporated into RNA pools with differing turnover rates. The absence of label in the IPA fraction is perhaps surprising in the light of its reported occurrence in a

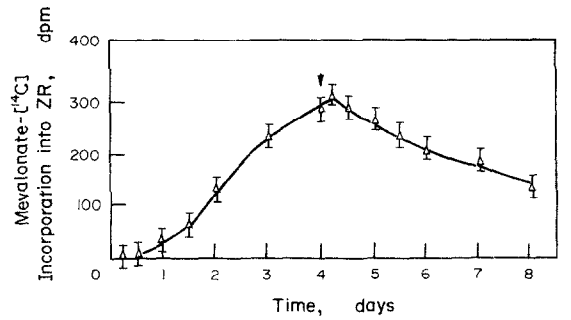

Fig. 2. Incorporation of radioactivity from mevalonolactone-[2-^{14}C] into zeatin riboside of potato cell RNA. 20 μCi were injected at day 0 followed by 2 mg of non-radioactive mevalonolactone as a chaser at day 4. Experimental details as for Fig. 1.

number of other plant *t*RNAs [13, 16]. It is however consistent with the suggestion by Vreman [17] that IPA may be associated solely with chloroplast RNA in higher plants and therefore would not appear in non-green tissue.

Bioassay of a crude butanol–ethyl acetate extract of the culture medium showed only one significant region of cytokinin activity which corresponded to zeatin riboside. This biologically active region (R_f 3.5–4.5) also contained a metabolite of the mevalonic acid lactone-[2-^{14}C]. In the isolated cytokinins the ^{14}C label was found predominantly in zeatin riboside with a small amount in *t*-zeatin (Z). No incorporation was observed into isopentenyl adenine (2IP) or IPA, which are reported to occur in sprouting potato tubers [14]. This could be due either to the absence of green tissue as discussed above, or to the low levels of incorporation being insufficient to register above background. A lag phase of similar length to that observed for the ^{14}C incorporation into the zeatin riboside of the RNA was apparent which suggests that the same limiting factor is effective in the synthesis of both groups (Fig. 3).

That the ^{14}C label of the isolated zeatin riboside was predominantly in the side chain was shown by treatment with permanganate which oxidized the cytokinin to adenine or adenosine [18, 19]. The adenosine isolated from this treatment (Table 1) contained very

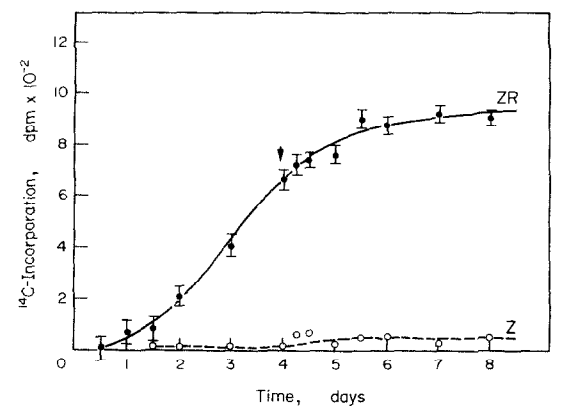

Fig. 3. Incorporation of radioactivity from mevalonolactone-[2-^{14}C] into the cytokinins found in potato culture medium. Details as for Fig. 1.

Table 1. The specificity of incorportion of mevalonic acid-[2-^{14}C] with zeatin riboside in potato tissue RNA

	Amount isolated (μM)	Radioactivity (dpm)	Specific activity (dpm/μM)
Zeatin riboside (ZR)	160	129	0.81
Adenosine	41.6	3	0.07

Potato cells were grown in liquid culture in the presence of mevalonic acid-[2-^{14}C] for 8 days. ZR was isolated from the culture filtrate and a portion was oxidized with potassium permanganate and the resultant adenosine isolated and purified by TLC [18]. The ZR and adenosine were measured by their UV adsorption at 270 and 260 nm, respectively.

little of the original ^{14}C activity of zeatin riboside, indicating that only a small amount of randomization of the label had occurred during the eight day period.

A superficial comparison of Figs. 2 and 3 does not give an immediate answer to the question of the origins of the free cyotkinins. The similarity of lag period, in conjunction with low levels of incorporation in the inital stages does not allow any definite conclusion on sequence to be drawn. However the correspondence between the amount of radioactivity accumulated in the free zeatin riboside during days 7 and 8 (60 dpm, Fig. 3) and the decrease in radioactivity in the RNA zeatin riboside over the same period (60 dpm, Fig. 2) suggests that RNA degradiation does contribute to the free cytokinin pool. However, if we consider the situation from the time that the effect of the cold chase was observed in day 5 and through day 6, then the lack of correspondence (115 dpm, Fig. 2 and 260 dpm, Fig. 3) suggests another source for zeatin riboside in conjunction with the RNA.

Further support for this possibility was provided if the amount of labelled cytokinin released by RNA turnover was calculated for days 1 to 4 (see Appendix for details). The release of zeatin riboside-[^{14}C] as calculated from the turnover of the RNA was less than 40% of that released into the culture medium by the potato cells (Fig. 4). If allowance is made for the degradation of zeatin and zeatin riboside in the cells and culture medium [20] and also for the zeatin riboside retained in the cells, then it can be seen that the

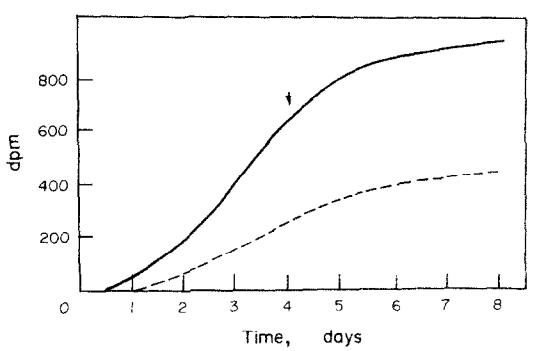

Fig. 4. Cytokinin production by potato cells in liquid culture. (——) The radioactivity of the zeatin riboside in the culture medium (Fig. 3). (. . . .) The release of radioactivity calculated from the degradation of cellular RNA (data from Fig. 2).

cytokinin released from the RNA will be a small fraction only of the total amount synthesized by the potato cells. This indicates that the plant has two sources of cytokinin, in degradation of RNA which supplies a base level of the hormone and a second source which can be manipulated by the internal control systems in order to respond to environmental or physiological changes. This second pathway would therefore allow manipulation of hormone metabolism without interference with the fundamental RNA metabolism.

This second route could be either one in which the cytokinin is synthesized *de novo* by transfer of the isopentenyl side chain to some simple adenine derivative, or, following the observations of Holtz and Klämbt [9], that the cytokinin is initially synthesized by isopentenylation of an adenine residue of oligo-RNA and released by breakdown of these units. An investigation into these possibilities is in progress.

EXPERIMENTAL

Callus from *Solanum tuberosum* var. King Edward was initiated and maintained according to the published procedure [15]. Suspension cultures were established by aseptic transfer of the callus to the liquid medium (100 ml in each 250 ml flask). Aeration of the suspension cultures was achieved by shaking them on an oscillating bed (10 cycles/min). After 3 weeks the contents of eight flasks were pumped into 2.2 l. of fresh medium in the 3 l. culture vessel of a Biotec-100 fermentor. The cell culture was maintained at pH 5.6 and 28°; aerated with filtered air at the rate of 3 l./min and stirred at 80 rpm. The culture vessel was modified to allow samples to be pumped from the culture.

Incorporation studies. 24 hr after the 3 l. suspension culture had been established mevalonic acid lactone-[2-^{14}C] (20 μCi) and uridine-[G-^{3}H] (250 μCi) were injected into the culture through a sterile bacterial filter. A chaser of mevalonic acid lactone (2 mg) and uridine (2 mg) was injected into the vessel 96 hr later. Samples (180 ml) were removed for analysis at 6 or 12 hr intervals as indicated in Fig. 1. The cells were separated from the medium by centrifugation, washed with H_2O and lyophilized. The culture medium and the washings were combined and IPA, 2IP, Z and ZR (4.0 OD_{260} units each) were added to act as carriers. The cytokinins were extracted with EtOAc–BuOH [15, 21], purified by ion exchange chromatography [21] and sequential TLC as indicated in Table 2.

The RNA was extracted from the lyophilized cells by a modification of the method of ref. [22]. The dried cells were homogenized in cold 0.025 M Tris–HCl (pH 7.3) containing 1% SDS and poly-uridine (0.65 mg), and extracted 2× with an equal vol. of buffer-saturated phenol. The RNA was precipitated as the EtOH-insoluble potassium salt [23]. This was dialysed against 0.01 M Tris–HCl (pH 7.0) and the amount of poly-uridine recovered was calculated from the OD_{260}. The ribosides were released from the RNA by hydrolysis with snake venom and bacterial alkaline phosphatase [24]. Carrier IPA and ZR were added to the incubation mixture and the cytokinins and ribosides were purified by sequential TLC as before. Radioactivity was measured in a toluene/Triton X-100 scintillant [25]. Losses during the purification were corrected by calculating the recovery of the non-radioactive carriers from their UV absorption at 270 nm.

Bio-assay. The extracts were assayed by the *Amaranthus caudatus* procedure [18].

Table 2. Purification of the crude cytokinin extract: TLC characteristics

	R_f		
	System 1	System 2	System 3
2IP	0.69	0.68	0.43
IPA	0.66	0.57	0.45
t-Zeatin	0.53	0.36	0.15
c-Zeatin	0.45	0.45	0.15
t-Zeatin Riboside / *c*-Zeatin Riboside	0.51	0.28	0.13
Uracil	0.44	0.37	0.43
Uridine	0.32	0.10	0.11
Mevalonic acid lactone	0.61	0.69	0.73

The cytokinins were purified by sequential TLC on Si gel GF_{254} in system (1) H_2O-saturated BuOH; (2) $CHCl_3$–MeOH (17:3) and (3) H_2O-saturated EtOAc.

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APPENDIX

Calculation of the rate of degradation of RNA

The curve presented in Fig. 2 can be divided into two sections:
(a) the labelling phase (days 1–4);
(b) the post-chase phase (days 5–8).
In the labelling phase the amount of label in the RNA will be a function of both the rate of synthesis of RNA (rate of incorporation of label) and rate of degradation of RNA (rate of release of labelling zeatin riboside). In the post chase phase the amount of label in the RNA will be a function only of the degradation of RNA.

Post-chase phase

After injection of the unlabelled substrate the amount of ^{14}C incorporated into the RNA will be negligible and the amount of radioactive label in pool x at time t can be described by the exponential decay function $q_{x(t)} = q_{x(0)}e^{-F_x/Q_x \cdot t}$ where $q_{x(0)}$ is the amount of radioactivity in pool x at time 0; F_x is the rate of degradation of the RNA at time t and Q_x is the size of the pool of RNA species x at time t. The total amount of radioactivity in the RNA at time t will be the sum of label in the various pools

$$q_{T(t)} = q_1 + q_2 + q_3 + q_4 + \ldots = q_{1(0)} \cdot e^{-F_1/Q_1 \cdot t} + q_{2(0)} \cdot e^{-F_2/Q_2 \cdot t} + \ldots \quad (1)$$

By means of the 'Peel-back procedure' [26] this function can be solved to give information on the number of pools; the amount of radioactivity in them at $t_{(0)}$ (day 4) and the rate of turnover of each pool (F_x/Q_x).

In our case since the growth of the cells was linear with time it is reasonable to assume that the size of each RNA pool will keep pace with the growth of the cell culture and that the rate of degradation of the RNA will be linear with time: as a consequence of this F_x/Q_x will be constant with time.

Now as time t increases the pools which are rapidly degraded will be depleted of radioactivity and $q_{x(t)} \to 0$.
$\therefore$ at some time t: $q_2 + q_3 + q_4 + \ldots \to 0$.
$\therefore$ from equation (1);

$$q_{T(t)} \Rightarrow q_{1(0)}e^{-F_1/Q_1 \cdot t} = q_{1(t)}$$

and

$$\ln q_T \Rightarrow \ln q_{1(0)} - F_1/Q_1 \cdot t \ldots \quad (2)$$

If $\ln q_T$ is plotted against t, at some point $t > 0$ the curve becomes linear corresponding to equation (2). Extrapolation of this straight line back to $t_{(0)}$ will give $q_{1(0)}$ and F_1/Q_1 can be calculated.

If the values $q_{1(t)}$ determined by the straight line are substracted from the experimental curve; a new curve will be obtained in which

$$q_{T'(t)} \Rightarrow q_{2(0)}e^{-F_2/Q_2 \cdot t} = q_{2(t)}$$

The substraction process can be repeated for q_3 and q_4 etc. In this way it can be shown that only two pools of RNA contribute significantly to the zeatin riboside-[^{14}C] pools. The characteristics of these pools are shown in Table 3.

Table 3. Independent pools of zeatin riboside (ZR) in potato tissue RNA

Pool	Turnover rate, F/Q (day^{-1})	ZR-[^{14}C] in each pool at day 4 $\frac{q_{x(0)}}{q_{T(0)}} \times 100$
F	2.1	8.5
S	0.5	91.5

Potato cells were grown in liquid culture in the presence of mevalonic acid-[2-^{14}C], after 4 days a pulse of mevalonic acid-[2-^{14}C] was added. The ZR in the RNA of the potato cells was isolated at 6 hr intervals as described in the Experimental and its ^{14}C content was measured. The two pools F (fast turnover) and S (slow turnover) were identified by 'Peel-back' analysis [26] of the rate of loss of ^{14}C from the ZR. The rate of turnover (F/Q) of each pool, and the amount of ZR-[^{14}C] ($q_{x(0)}$) in each pool after 4 days of exposure to mevalonic acid-[2-^{14}C] were calculated from

$$q_{(T)} \Rightarrow q_{x(0)} \cdot e^{-F_x/Q_x \cdot t}$$

as described in the Appendix. The figures given in the table are the average of 3 experiments.

Labelling phase

The turnover rates of the two pools (F/Q) are known, but to calculate the amount of zeatin riboside-[^{14}C] liberated at time t it is necessary to know the amount of radioactivity in the individual pools.

The rate of change of radioactivity in each pool is described by the function

$$\frac{dq_x}{dt} = R_{x(t)} - \frac{F_x q_x}{Q_x}$$

where $R_{x(t)}$ is the rate of incorporation of ^{14}C into the RNA at time t, and $R_{x(t)} = R_0 kt$. Where R_0 is the rate of incorporation at the begining of the experiment (t_0). The calculation can be simplified by observing that since the turnover rate F/Q of pool F is 2.1 day^{-1} then this pool will be rapidly saturated and the amount of radioactivity in this pool at $t > 0$ will be equivalent to that calculated for day 4 when corrected for the change in pool size. The amount of radioactivity in pool S may then be obtained from $q_S = q_T - q_F$ and the amount of zeatin riboside-[^{14}C] released by degradation of these pools may be calculated from

$$\frac{dq'_x}{dt} = \frac{-F_x}{d_x} \cdot q_x$$

where dq'_x/dt is the rate of change in radioactivity due to degradation. Combination of the figures derived from the two pools will then give total release of zeatin riboside-[^{14}C] (Fig. 3).

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