Cytokinin Activation of de novo Thiamine Biosynthesis in Tobacco Callus Cultures

Dzintar E. Dravnieks[1], Folke Skoog, and R. H. Burris
Departments of Biochemistry and Botany, University of Wisconsin, Madison, Wisconsin 53706

Received January 27, 1969.

Abstract. **The effect of kinetin on the *de novo* formation of thiamine in tobacco callus cultures was measured by following the isotope dilution of previously introduced ^{14}C-thiamine. Thiamine was determined by the thiochrome fluorescence assay after chromatographic purification.**

Morphological effects induced by high kinetin concentrations were visible within a week after tissue transfer, but thiamine synthesis was insignificant for 2 weeks both in cultures with high (1000 μg/l) and low (30 μg/l) kinetin treatments. Thiamine synthesis during the third week was observed at both kinetin levels, the high kinetin treatment supporting 2.5 times the thiamine synthesis of the low kinetin treatment. The kinetin induced increases in thiamine observed earlier by Digby and Skoog apparently resulted from stimulation of thiamine synthesis rather than from sparing its destruction. Thiamine synthesis is initiated when thiamine concentration reaches a minimum in the callus tissue. This suggests that kinetin is required for the synthesis, but that the activation of synthesis is under feedback control sensitive to the level of thiamine in the tissue.

Linsmaier and Skoog (5) observed an interesting cytokinin-thiamine interaction which suggested that high levels of kinetin could overcome the otherwise absolute requirement of tobacco callus tissue cultures for thiamine. This interaction was studied by Digby and Skoog (3), and their results with the *Phycomyces* bioassay for thiamine indicated that in the presence of high kinetin concentrations there is a large net increase in thiamine per culture although there is a decrease in concentration of thiamine in the tissue. With low kinetin, no net increase in thiamine was observed, and the tissue concentration decreased to levels lower than those observed under high kinetin. The effectiveness of antithiamines indicated that thiamine still was being utilized at high kinetin concentrations, and return to low kinetin media after 20 transfers (*ca.* 2 years) indicated that the callus again needed exogenous thiamine for growth; thus, the high kinetin treatment brought about some nonpermanent change in thiamine metabolism. These results suggest that kinetin activates the synthesis of thiamine. However, because the relative rates and time course of thiamine synthesis and destruction were unknown, the possibility of an effect on thiamine catabolism could not be eliminated.

This study was undertaken to determine if the observed accumulation of thiamine resulted from an activation of its synthesis or from a thiamine sparing effect.

[1] Supported by NIH Predoctoral Fellowship No. 5-F1-GM-35, 499.

Materials and Methods

Tobacco callus from the pith of *Nicotiana tabacum* var. Wisconsin No. 38 was maintained on Murashige and Skoog revised medium (8) as further modified in organic constituents by Linsmaier and Skoog (RM-1965) (5) with 2 mg IAA/l, 200 μg kinetin (6-furfurylaminopurine)/l and 400 μg thiamine/l. Callus subcultured twice on this medium containing only 30 μg kinetin/l was used for the experiments. Callus accumulated ^{14}C-thiamine while growing on a medium with 30 μg kinetin/l and 200 μg ^{14}C-thiamine/l for 17 days. Three pieces of stock callus were planted on 50 ml of medium in each 125 ml Erlenmeyer flask; thiamine was added after autoclaving and was sterilized by filtration.

After the callus had taken up the labeled thiamine, it was transferred to fresh medium containing no thiamine and either low (30 μg/l, half the flasks) or high (1000 μg/l) levels of kinetin. Cultures were grown in continuous dim light at about 28°. Eight flasks of each treatment were harvested weekly, except in the fourth week when only 3 flasks of each were harvested. Callus was weighed, frozen in a dry ice bath and stored at −20° until it was assayed.

^{14}C-thiamine was obtained from Nuclear Chicago as thiamine (thiazole-2-^{14}C) hydrochloride, specific activity 26.7 mc/mmole. It was diluted with unlabeled thiamine to give a solution of 29,700 cpm/μg. Approximately 316,000 cpm of this solution was added to each culture flask.

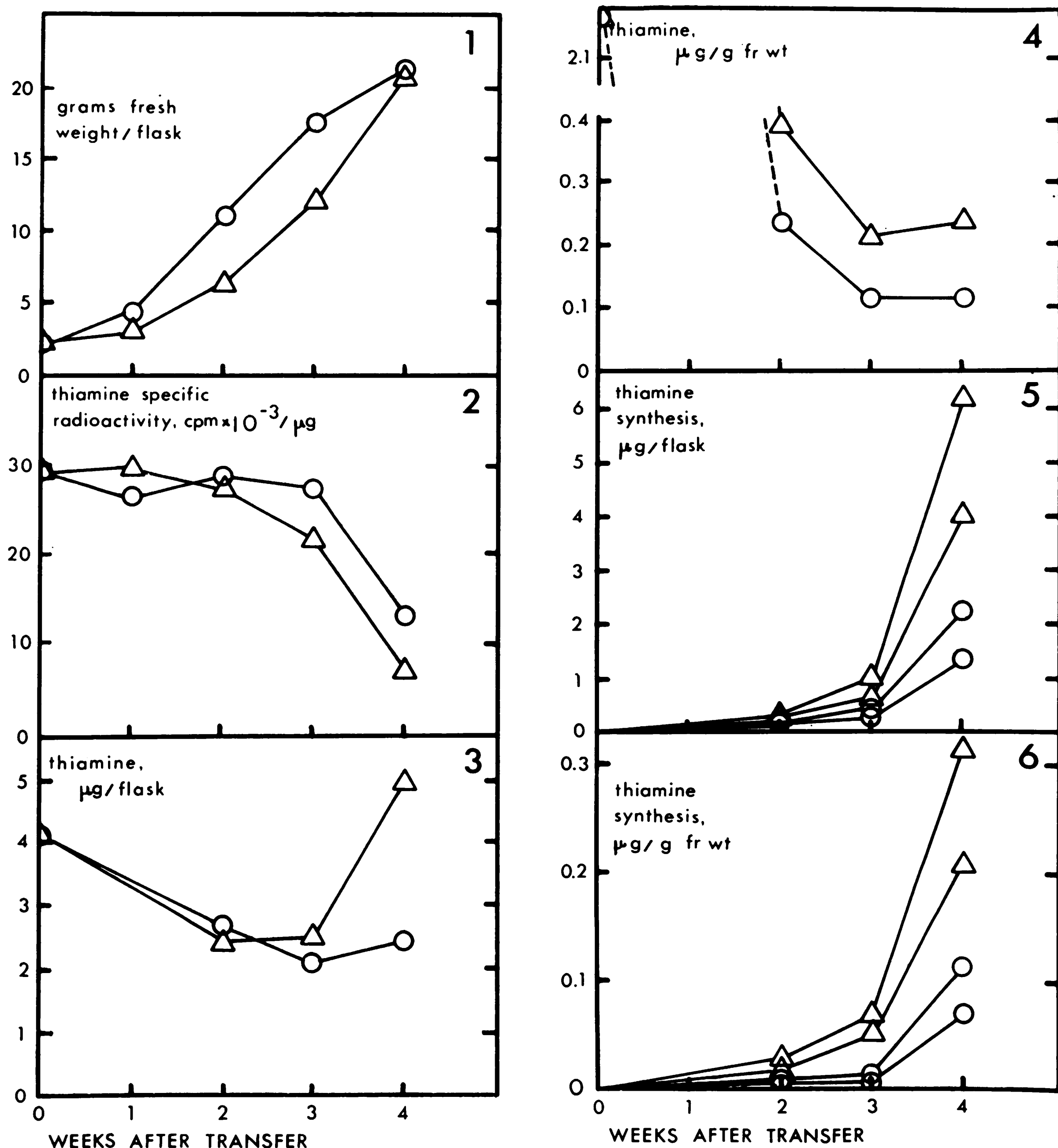

FIG. 1. Growth of cultures of tobacco callus on low kinetin (30 µg/l, 0-0) and high kinetin (1000 µg/l, △—△). The symbols are the same for all figures.

FIG. 2. Decrease in the specific radioactivity of ^{14}C-thiamine in tobacco callus during growth of cultures on low and high concentrations of kinetin.

FIG. 3. Change of the total thiamine in tobacco callus during growth of cultures on low and high concentrations of kinetin.

FIG. 4. Change in thiamine concentration in tobacco callus during growth on low and high concentrations of kinetin.

FIG. 5. Thiamine synthesis by tobacco callus during growth on low and high concentrations of kinetin. The two lines for each treatment represent possible extremes of synthesis per flask calculated as described under Materials and Methods.

FIG. 6. Capacity of tobacco callus for thiamine synthesis when supplied low or high concentrations of kinetin. The 2 lines for each treatment represent possible extremes of capacity for synthesis per g average fresh weight calculated as described under Materials and Methods.

Radioactivity was measured in a Packard model 3003 Tri-Carb scintillation spectrometer. Radioactivity on paper chromatograms was located with a Vanguard Autoscanner 880 or with a Geiger tube fitted with an aluminum foil slit.

Frozen callus was homogenized with one-tenth its weight of 1 N HCl in a Potter-Elvehjem homogenizer. The homogenate was heated for 30 min in a steamer, cooled, and centrifuged 40 min at about 35,000*g*. The supernatant was adjusted to pH 4.25 with 15 % NaOH and centrifuged again in the same manner. Triplicates of this supernatant were counted for total soluble radioactivity. The fraction of total radioactivity attributable to thiamine was determined by separating the pH 4.25 extract by paper chromatography and comparing the counts under the thiamine peak with the total counts on the chromatogram. Ascending paper chromatograms on Whatman No. 3 filter paper were run for 16 hr in *n*-propanol:H_2O:acetate buffer (1 M, pH 5.0), 7:2:1 v/v (11). The radioactive spots were located and quantitated by cutting the strip into 4 pieces, 1 of which was the thiamine peak, and measuring their radioactivity by liquid scintillation.

The thiamine was purified by running the pH 4.25 extract through a Decalso column; the eluant was desalted by the phenol method (4) and purified twice by paper chromatography in the system described. The thiamine area was cut out and eluted with 4 ml of 0.1 N HCl, and six 0.5 ml aliquots were used for thiochrome assay (1); an internal standard curve was included for each assay. The fluorescence of the thiochrome was determined with an Aminco-Keirs Spectrophosphorimeter. Duplicate 0.2 ml aliquots of the eluted thiamine peak were assayed by liquid scintillation, so the specific radioactivity could be calculated from these data combined with the results of the thiochrome assay. The values for total soluble radioactivity, the fraction of the total due to thiamine, and the specific radioactivity of the recovered thiamine, permitted calculation of the quantity of thiamine in the callus and the extent of its synthesis.

To calculate the synthesis or loss of thiamine during the week between samplings, specific radioactivity must be used; if the specific radioactivity does not change, then there is a unique answer indicating no synthesis. Under conditions of change, the extreme cases are represented by the situations in which all loss occurs before any synthesis and in which all synthesis occurs before any loss. Both cases are calculated with the data presented.

Results

Growth curves (Fig. 1) indicate that about the same final weight was reached at each kinetin level, but with the high kinetin treatment there was more lag in growth. The effect of high kinetin also was reflected in morphological changes within the first week.

Table I shows the total soluble radioactivity as cpm/flask and the radioactivity in thiamine. Except for the 2HK value (last column) there seemed to be no differential effect of kinetin concentration on the rate of decrease in cpm/flask contributed by thiamine. This supports the position that the kinetin effect is on the synthesis and not on the breakdown of thiamine.

Table I. *Total Soluble Radioactivity in Tobacco Callus Tissue Cultures*

Week after transfer	Cpm $\times 10^{-3}$ per flask	% of cpm in thiamine	Cpm $\times 10^{-3}$ per flask from thiamine
0	124.0	96.6	120.0
1 LK[1]	99.4	81.8	81.3
1 HK[2]	96.4	83.7	80.1
2 LK	97.5	79.0	77.1
2 HK	89.1	78.1	96.6
3 LK	69.0	81.9	56.5
3 HK	68.4	79.1	54.1
4 LK	68.7	47.3	32.5
4 HK	60.4	56.8	34.3

[1,2] LK and HK represent high (1000 μg/l) and low (30 μg/l) kinetin treatments.

The change in specific radioactivity, as shown in Fig. 2, indicates a considerable decrease at both kinetin levels, the specific radioactivity of thiamine in the high kinetin treatment decreasing sooner and more rapidly than in the low kinetin treatment. The relationship between synthesis and decreased specific radioactivity is exponential, so that changes index a quantitatively greater change as the specific radioactivity approaches zero. In another thiochrome assay of the samples taken after 1 week, no difference in specific radioactivity was found, so the decrease at 1LK probably represents an aberrant result. Calculations were started with the 2 week sample in order that apparent "negative synthesis" (positive slope for plot of specific radioactivity of thiamine *vs.* time) would not complicate comparison of results. Fig. 3 and 4 show the thiamine content per flask and per g fresh weight, respectively. On a per flask basis there is a gradual decline in thiamine content for the first few weeks. Then, with the high kinetin treatment, there is a rapid accumulation of thiamine to a level higher than the initial level; with the low kinetin, there is a slight increase over the previous week but no significant accumulation.

The possible upper and lower limits for thiamine synthesis and synthetic capacity per g average fresh weight are shown in Fig. 5 and 6. Synthesis is indicated at both kinetin levels, but synthesis in the high kinetin treatment begins earlier and is always about twice as rapid as with the low kinetin treatment. A similar result is seen if synthetic capacity

is measured per g average fresh weight of tissue (Fig. 6) so the increased synthesis is not dependent upon an increase in total callus. In no case do the ranges of values for thiamine synthesis calculated for the 2 kinetin levels overlap.

Discussion

Synthesis of thiamine is indicated by a decrease in specific radioactivity both in high and low kinetin treatments and by a net increase in thiamine per flask and per g tissue in the high kinetin treatment. In another series of experiments, the low kinetin control showed no synthesis of thiamine for 6 weeks: growth was moderate and the high kinetin treatment showed about 5 % the thiamine synthesis reported here. The probability that thiamine "represses" its own synthesis and the necessity for keeping the thiamine level high enough for reasonable growth during the thiamine accumulation period make it difficult to get a control showing no thiamine synthesis. ^{14}C-Thiamine must be taken up by the callus during the 17 days before transfer; if kinetin is too low during this period growth will be poor and little thiamine will be taken up. Even if the thiamine is accumulated at moderate kinetin levels and the tissue is then transferred to kinetin-free medium, the callus becomes necrotic during the weeks following. Absence of thiamine synthesis under such circumstances cannot be attributed specifically to lack of kinetin. On the other hand, accumulating high levels of thiamine into the callus would tend to prevent thiamine synthesis at any kinetin level, if as our results suggest, thiamine "represses" its own synthesis.

The results follow the same pattern found by Digby and Skoog (3); there is a rapid early decrease in thiamine concentration and this decrease levels off between the second and third week. The callus from the high kinetin treatment has a 2-fold higher minimum level of thiamine per unit callus weight than does tissue from the low kinetin treatment. On a per flask basis, there is an initial decrease in thiamine for both kinetin levels, but during the fourth week the thiamine present in each treatment shows an increase. The increase of thiamine in the high kinetin treatment results in the accumulation of distinctly more thiamine than is present in the zero week sample, but the increase in the low kinetin treatment is much less.

Calculations of thiamine synthesis indicate that substantial synthesis does not begin until several weeks after tissue transfer. This cannot be due to delayed kinetin uptake, since morphological signs of the presence of high kinetin are evident within 1 week after transfer. If synthetic capacity (thiamine synthesis per unit weight of callus) is measured, the same marked increase as per flask is seen during weeks 3 and 4. Increases in synthesis and capacity for thiamine synthesis are seen at both kinetin levels, however, the increase starts sooner and is about 2.5 times as large with high kinetin. This time course for thiamine synthesis suggests that the kinetin effect on the synthesis is indirect through an effect on the operation of a control mechanism. The initiation of thiamine synthesis shows no obvious correlation with changes in the tissue growth rate; the time of initiation, however, does correlate well with the point of minimum thiamine concentration in the callus. This would be expected if thiamine synthesis was under a negative feedback control sensitive to the thiamine level. Newell and Tucker (9, 10) demonstrated such a mechanism in *Salmonella typhimurium;* high levels of thiamine repressed thiamine synthesis until the concentration fell below the critical level of 30 $\mu g/g$ dry weight. At this level the synthesis was de-repressed, and the thiamine concentration rose to 40 $\mu g/g$ dry weight before the synthesis was again repressed.

The studies by Mann *et al.* (7) and Steinhart *et al.* (12) on the activation by kinetin of tyramine methylpherase in germinating barley illustrate a similar situation. As with thiamine biosynthesis, kinetin did not influence the time of initiation of synthesis but only its intensity.

The change in shape of the synthetic capacity curve with time (Fig. 6) suggests but certainly does not establish a *de novo* synthesis of the thiamine synthesizing enzymes rather than an activation of preformed enzymes. Observations of Linsmaier-Bednar and Skoog (6) indicate that "mutant" strains which produce adequate levels of cytokinin for their own growth also grow without addition of thiamine in the light, and that one mutant did so even in darkness. Bergmann and Bergmann (2) found enhanced thiamine synthesis in tissue grown in light and suggest that photosynthesis may be required to raise endogenous cytokinin levels. The increased cytokinin levels then could stimulate the biosynthesis of thiamine.

Our results indicate that the thiamine level can be limited by the cytokinin supply, and since thiamine is an essential cofactor in many metabolic pathways, cytokinins may modulate general cellular activity through control of thiamine synthesis as well as by other means.

Acknowledgments

We thank Jim Chickering for his skillful assistance with the tissue cultures.

Literature Cited

1. Association of Vitamin Chemists. 1951. Methods of Vitamin Assay. Interscience. New York-London. p 107–27.
2. BERGMANN, L. AND A. L. BERGMANN. 1968. Aktivierung der Biosynthese von Thiamin in Calluskulturen von *Nicotiana tabacum* im Licht. Planta 79: 84–91.

3. DIGBY, J. AND F. SKOOG. 1966. Cytokinin activation of thiamine biosynthesis in tobacco callus cultures. Plant Physiol. 41: 647–52.

4. IACONO, J. M. AND B. C. JOHNSON. 1957. Thiamine metabolism. I. The metabolism of thiazole-2-^{14}C-thiamine in rat. J. Am. Chem. Soc. 79: 6321–24.

5. LINSMAIER, E. M. AND F. SKOOG. 1965. Organic growth factor requirements of tobacco tissue cultures. Physiol. Plantarum 18: 100–27.

6. LINSMAIER-BEDNAR, E. M. AND F. SKOOG. 1967. Thiamine requirement in relation to cytokinin in "normal" and "mutant" strains of tobacco callus. Planta 72: 146–52.

7. MANN, J. D., C. E. STEINHART, AND S. H. MUDD. 1963. Alkaloids and plant metabolism. V. The distribution and formation of tyramine methylpherase during germination of barley. J. Biol. Chem. 238: 676–81.

8. MURASHIGE, T. AND F. SKOOG. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plantarum 15: 473–97.

9. NEWELL, P. C. AND R. G. TUCKER. 1966. The de-repression of thiamine biosynthesis by adenosine; a tool for investigating this biosynthetic pathway. Biochem. J. 100: 512–16.

10. NEWELL, P. C. AND R. G. TUCKER. 1966. The control mechanism of thiamine biosynthesis; a model for the study of control of converging pathways. Biochem. J. 100: 517–24.

11. SILIPRANDI, D. AND N. SILIPRANDI. 1954. Separation and quantitative determination of thiamine and thiamine phosphoric esters and their preparation in pure form. Biochim. Biophys. Acta 14: 52–61.

12. STEINHART, C. E., J. D. MANN, AND S. H. MUDD. 1964. Alkaloids and plant metabolism. VII. The kinetin-produced elevation in tyramine methylpherase levels. Plant Physiol. 39: 1030–38.