Biochem. J. (1972) **130**, 1157–1160
Printed in Great Britain

A Quantitative Limit for Cytokinin Incorporation into Transfer Ribonucleic Acid by Soya-Bean Callus Tissue

By Daphne C. Elliott and A. W. Murray
School of Biological Sciences, Flinders University of South Australia, Bedford Park, S. Austral. 5042, Australia

(*Received* 2 *October* 1972)

There has been considerable speculation in the literature that cytokinins may exert their physiological effects on growth and morphogenesis by modifying the function of specific tRNA species, in which they occur in a position adjacent to the 3′-hydroxyl end of the anticodon (see review by Skoog & Armstrong, 1970). For this to be the mode of action of cytokinins it would need to be shown that exogenous cytokinins are incorporated into tRNA and also that such incorporation results in a biochemical control mechanism. Controversy exists over the first of these requirements. Thus although Fox & Chen (1967) have reported the incorporation of 6-benzyladenine into tRNA and Burrows *et al.* (1971) have identified 6-benzyladenosine from tRNA isolated from tobacco callus grown on 6-benzyladenine, evidence against incorporation of this synthetic cytokinin into tRNA has been presented by Kende & Tavares (1968), Richmond *et al.* (1970) and Bezemer-Sybrandy & Veldstra (1971). In an attempt to resolve this question, the incorporation of ^{3}H-labelled 6-benzyladenine and 6-benzyl-9-butyladenine into tRNA during the growth of soyabean (*Glycine max*) callus tissue has been quantitatively evaluated.

The results showed no significant incorporation of intact 6-benzyladenine molecules into tRNA. Some incorporation by transbenzylation appeared to take place, but only to the extent of one molecule of 6-benzyladenosine/755–2210 molecules of tRNA. A preliminary account of these findings has been given (Elliott & Murray, 1971).

Materials and methods

Soya-bean callus tissue. The procedure of Miller (1965) was used for the growth of plant material, cultures being maintained on 5μM-6-benzyladenine.

Labelled cytokinins. 6-Benzyl-9-butyladenine was prepared as previously described (Elliott *et al.*, 1972). [G-^{3}H]6-Benzyladenine and [G-^{3}H]6-benzyl-9-butyladenine were prepared at The Radiochemical Centre (Amersham, Bucks., U.K.) by catalytic exchange in an aqueous medium. One would expect the distribution of ^{3}H in 6-benzyladenine to be very approximately as follows: methylene group of benzyl moiety > 8-position of adenine > benzyl group ≫ other positions (J. A. Spanner, The Radiochemical Centre, personal communication). The crude products were freed from labile ^{3}H and purified by crystallization from methanol followed by preparative chromatography on Whatman 3MM paper in methanol–water (17:3, v/v), in which system 6-benzyladenine and 6-benzyl-9-butyladenine have R_F values 0.69 and 0.83 respectively. The cytokinins were eluted with methanol and stored at −15°C in this solution. The specific radioactivities of [G-^{3}H]6-benzyladenine and [G-^{3}H]6-benzyl-9-butyladenine were 25.6 and 815mCi/mmol respectively. [2,8-^{3}H]Adenine (specific radioactivity 78.6mCi/mmol) was obtained from The Radiochemical Centre.

Isolation of tRNA from callus tissue. The method of Letham & Ralph (1967) was used with some modifications. These were as follows. (*a*) Pre-extraction of the tissue with hot 80% (v/v) ethanol (final concentration) containing 2mM-$MgCl_2$ followed by washing with 95% (v/v) ethanol, acetone and finally acetone–ether (1:1, v/v). These steps were introduced to permit isolation of the low-molecular-weight metabolites of the cytokinins. (*b*) Enzymic hydrolysis of the purified tRNA (Hall, 1964). Alkaline hydrolysis was found to result in considerable loss of ^{3}H. No such loss was observed with the enzymic digestion. (*c*) Unlabelled 6-benzyladenine, 6-benzyladenosine and 6-benzyladenosine monophosphate (all at 20μM) were included in solutions at three points in the purification procedure (the hot-ethanol extraction, during the phenol extraction and before the precipitation with cetyltrimethylammonium bromide). Inclusion of the unlabelled compounds was essential to remove labelled low-molecular-weight metabolites non-covalently bound to the tRNA (see below under 'Results'). It was important to include a control in which ^{3}H-labelled 6-benzyladenine was added to the tissue just before the preliminary ethanol extraction.

Chromatography of nucleosides. The tRNA hydrolysis products were chromatographed on Whatman 3MM paper in either butan-1-ol–acetic acid–water (20:3:7, by vol.) or methanol–water (17:3, v/v).

Radioactivity determinations. For analysis of the tRNA hydrolysis products, nucleosides were first eluted from the chromatograms. Adenosine, uridine, guanosine and cytidine were eluted in 50mM-HCl and the absorptions at 260 and 290nm were measured,

for calculation of tRNA, before they were added to Bray's solution (Bray, 1960) for counting of radioactivity. 6-Benzyladenosine and 6-benzyl-9-butyladenine (identified from internal markers) were eluted from the paper with 85% (v/v) methanol and the radioactivity was counted directly in Bray's solution. The following efficiencies of counting of ^{3}H radioactivity under the different conditions were obtained: for 50mM-HCl eluates in Bray's solution, 16%, and for 85%-methanol eluates in Bray's solution, 33%.

Purity of isolated tRNA. The purified tRNA was analysed by polyacrylamide-gel electrophoresis (Loening, 1967): 95% of the radioactivity was recovered in the position of a marker of purified *Escherichia coli* tRNA.

Calculation of tRNA concentrations. The amount of tRNA applied to each chromatogram as nucleosides was calculated from the absorptions at 260nm and 290nm of 50mM-HCl eluates of the combined adenosine + uridine areas. An extinction coefficient of 12.1 was used, this being derived from the molar ratio of adenosine and uridine ($\epsilon_{mM(260)}-\epsilon_{mM(290)}$ = 13.9 and 9.92 respectively; Dawson *et al.*, 1969) in wheat-germ tRNA (see below). The amount of tRNA was calculated by using the nucleoside content of wheat-germ tRNA (Glitz & Dekker, 1963) of 15.9μmol of adenosine and 13.4μmol of uridine (excluding pseudouridine)/μmol of tRNA (assuming an average content of 70 nucleotides/molecule).

Results

Contamination of tRNA with low-molecular-weight metabolites. Preliminary experiments were carried out with [G-^{3}H]6-benzyl-9-butyladenine, which cannot be incorporated into nucleotides without prior dealkylation, but which is a potent cytokinin (Elliott *et al.*, 1972). These studies were discontinued, as soya-bean callus converted the alkylated derivative into 6-benzyladenine. However, these initial experiments with [G-^{3}H]6-benzyl-9-butyladenine indicated that non-specific binding of labelled materials to isolated tRNA was a potential source of error in incorporation experiments. Hydrolysates of tRNA, isolated from callus tissue that had been grown on [G-^{3}H]6-benzyl-9-butyladenine, contained radioactivity associated with the 6-benzyl-9-butyladenine marker spot when unlabelled cytokinins were not included during the tRNA isolation. This point was established by chromatography of the hydrolysate in methanol–water (4:1, v/v); in this solvent 6-benzyl-9-butyladenine (R_F 0.83) separates cleanly from 6-benzyladenosine (R_F 0.69). Since 6-benzyl-9-butyladenine cannot be incorporated as such into tRNA, this material must have been carried through the whole isolation procedure as a contaminant, and this raised the possibility that some of the radioactivity associated with the 6-benzyladenosine area had similarly been carried through either as 6-benzyladenosine (which is an early metabolite of 6-benzyladenine) or as 6-benzyladenine, which is difficult to separate well from 6-benzyladenosine.

Evidence for a non-covalent binding of 6-benzyladenine to tRNA was obtained in the following experiment. Callus tissue was grown for 14 days on 4.5μM-6-benzyladenine, transferred to agar medium without cytokinin for 5 days and then used for the preparation of tRNA. [G-^{3}H]6-Benzyladenine

Table 1. *Incorporation of ^{3}H into nucleoside hydrolysis products of tRNA isolated from soya-bean callus tissue grown on [G-^{3}H]6-benzyladenine or [2,8-^{3}H]adenine*

Callus tissue used for this experiment was grown for 14 days on 4.5μM-6-benzyladenine, transferred to agar medium without cytokinin for 5 days and then to agar medium containing the additions specified below for 8 days. The amounts of tissue used for isolation of tRNA were 18.6g, 18.7g, 16.5g and 18.0g for Expts. 1, 2, 3 and 3a respectively. See the text under 'Materials and methods' for isolation and hydrolysis of tRNA, chromatography and counting of the radioactivity of nucleosides. These results are from a chromatogram developed in butan-1-ol–acetic acid–water (20:3:7, by vol.); R_F values with this system are: 6-benzyladenosine, 0.81; adenosine, 0.34; uridine, 0.29.

Expt. no.	Callus grown for 8 days on	Increase in fresh weight in 8 days (%)	Radioactivity (c.p.m.)*		Incorporation (pmol/μmol of tRNA)	
			6-Benzyladenosine	Adenosine+uridine	6-Benzyladenosine	Adenosine+uridine
1	4.5μM-[G-^{3}H]6-Benzyladenine	188	0†	294	0	18900
2	32μM-[2,8-^{3}H]Adenine	22	0†	2287	0	244000
3	32μM-[2,8-^{3}H]Adenine+4.5μM-6-benzyladenine	177	62	19988	1325	792000
3a	32μM-[2,8-^{3}H]Adenine+4.5μM-6-benzyladenine	168	13	11215	453	724000

* The radioactivity of each sample was counted for 100min and is corrected for background.
† The standard error resulting from machine variability was ±2c.p.m.

[9 nmol (510000 d.p.m.)/g fresh wt. of tissue] was added to each of 20 g samples of callus just before extraction. tRNA was purified from one sample by using non-radioactive carriers as described above under 'Materials and methods'; the other sample was extracted without the addition of carriers. A total of 594 d.p.m. (1160 pmol/μmol of tRNA) was associated with purified tRNA prepared in the absence of added carriers and 18 d.p.m. (35 pmol/μmol of tRNA) when non-radioactive carriers were included.

In all subsequent experiments unlabelled cytokinins were included at three stages in the procedure for isolation of tRNA [see modification (*c*) under 'Materials and methods'].

Quantitative limit for the incorporation of 6-*benzyladenine into tRNA*. When suitable precautions were taken to prevent contamination of the isolated tRNA with low-molecular-weight metabolites, no detectable radioactivity was associated with 6-benzyladenosine in hydrolysates of tRNA isolated from tissue grown on [G-^{3}H]6-benzyladenine (Expt. 1 in Table 1). Some radioactivity was associated with adenosine, presumably resulting from the degradation of 6-benzyladenine to adenine, which was then incorporated into tRNA. Experiments in which [2,8-^{3}H] adenine and unlabelled 6-benzyladenine were included in the growth medium showed a small incorporation of label into 6-benzyladenosine. The radioactivities measured were low, but were reproducibly obtained and suggest the existence of a transbenzylation reaction from 6-benzyladenine to adenosine residues in tRNA.

Although no radioactive 6-benzyladenosine was detectable in hydrolysates of tRNA isolated from callus grown on [G-^{3}H]6-benzyladenine, the presence of 2 c.p.m. or less may not have been measured (limit of machine sensitivity). With this value as an upper limit, one molecule of 6-benzyladenine may have been incorporated/16400 molecules of tRNA (Expt. 1 in Table 1). This calculation on the extent of possible incorporation from [G-^{3}H]6-benzyladenine assumed that intact 6-benzyladenine molecules were incorporated and will underestimate incorporation if transbenzylation occurs, particularly if the [G-^{3}H]-6-benzyladenine is not uniformly labelled. Attempts to estimate the distribution of label in the 6-benzyladenine molecule have not been successful, but grossly uneven labelling of the purine and benzyl moieties seems most unlikely (see above under 'Materials and methods'). Maximum incorporations were 1 molecule of 6-benzyladenosine/755 and 2210 molecules of tRNA in Expts. 3 and 3a, in which callus was grown in the presence of [2,8-^{3}H]adenine and unlabelled 6-benzyladenine (see Table 1). Two estimates of the cytokinin content of plant tRNA have shown the presence of 1 molecule of natural cytokinin/47 and 178 molecules of tRNA (Hall, 1970).

Burrows *et al.* (1971) have reported the presence of three 'natural' cytokinin ribonucleosides as well as a smaller amount of 6-benzyladenosine in cytokinin-dependent tobacco callus tissue grown on 6-benzyladenine. However, a similar quantitative estimate cannot be obtained from their experiment because of the possibility that some free 6-benzyladenosine has been carried through the purification procedure. Similarly, incorporation of radioactive cytokinins observed in the past should be treated with some reservation in view of the demonstrated difficulty in freeing tRNA from non-covalently bound low-molecular-weight metabolites.

Discussion

It can be concluded from these results that no incorporation of intact 6-benzyladenine molecules into callus tRNA takes place (within the experimental limits). Some incorporation of the N-6 side chain by transbenzylation to adenosine appears to take place but only into approx. 2–23% of the tRNA species that normally contain cytokinins. These results were obtained from callus tissue grown for 8 days on 6-benzyladenine; metabolic changes are observable within 6 h of the addition of 6-benzyladenine (e.g. ^{32}P incorporation into nucleic acid, phosphoprotein and phospholipid; D. C. Elliott & A. W. Murray, unpublished work). In view of findings showing that mevalonate and not 6-isopentenyladenosine is the precursor of 6-isopentenyladenosine residues in tRNA (Chen & Hall, 1969), the very small incorporation of 6-benzyladenine observed in the present work may not be significant.

Any theory of cytokinin action based on their presence in certain tRNA species must take into account the quantitative limits established in the present experiments. Theories based on these limits would also need to assume that all transbenzylation has occurred specifically on to the adenosine residues next to the anticodon in specific tRNA species. Although it is conceivable that incorporation of exogeneous cytokinin into an extremely small fraction of tRNA may control growth, this does not seem to be a very satisfying hypothesis. Until a connexion can be demonstrated to link incorporation of cytokinins with growth, alternative mechanisms of action such as specific modification of gene activity by cytokinin base, nucleoside or nucleotides should be considered more strongly.

This work was supported by the Rural Credits Development Fund of The Reserve Bank of Australia.

Bezemer-Sybrandy, S. M. & Veldstra, H. (1971) *Physiol. Plant.* **25**, 1–7

Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285

Burrows, W. J., Skoog, F. & Leonard, N. J. (1971) *Biochemistry* **10**, 2189–2194

Chen, C. M. & Hall, R. H. (1969) *Phytochemistry* **8**, 1687–1695

Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1969) *Data for Biochemical Research*, pp. 170, 176, Oxford University Press, Oxford

Elliott, D. C. & Murray, A. W. (1971) *Proc. Aust. Biochem. Soc.* **4**, 44

Elliott, D. C., Murray, A. W., Saccone, G. T. & Atkinson, M. R. (1972) in *Plant Growth Substances 1970* (Carr, D. J., ed.), pp. 359–466, Springer-Verlag, Heidelberg

Fox, J. E. & Chen, C. M. (1967) *J. Biol. Chem.* **242**, 4490–4494

Glitz, D. G. & Dekker, C. A. (1963) *Biochemistry* **2**, 1185–1192

Hall, R. H. (1964) *Biochemistry* **3**, 769–773

Hall, R. H. (1970) *Progr. Nucleic Acid Res. Mol. Biol.* **10**, 57–86

Kende, H. & Tavares, J. E. (1968) *Plant Physiol.* **43**, 1244–1248

Letham, D. S. & Ralph, R. K. (1967) *Life Sci.* **6**, 387–394

Loening, U. E. (1967) *Biochem. J.* **102**, 251–257

Miller, C. O. (1965) *Proc. Nat. Acad. Sci. U.S.* **54**, 1052–1058

Richmond, A., Back, A. & Sachs, B. (1970) *Planta* **90**, 57–65

Skoog, F. & Armstrong, D. J. (1970) *Annu. Rev. Plant Physiol.* **21**, 359–384