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The control of bud dormancy in potato tubers. Measurement of the seasonal pattern of changing concentrations of zeatin-cytokinins

C.G.N. Turnbull* and D.E. Hanke
Botany School, Downing Street, Cambridge CB2 3EA, UK

Abstract. A radioimmunoassay, combined with high-performance liquid chromatography, has been used to analyse the zeatin-type cytokinins of potato (*Solanum tuberosum* L. cv. Majestic) tubers and tuber buds throughout growth and storage. During tuber growth, zeatin riboside was the predominant cytokinin detected in all tissues. Immediately after harvest, the total cytokinin concentration fell dramatically in the storage tissue, largely as a consequence of the disappearance of zeatin riboside. During storage, levels of cytokinins in the storage tissue remained relatively constant, but increased in the tuber buds. In the buds of tubers stored at 2° C there was a 20- to 50-fold increase in total cytokinin over six weeks, coinciding with the natural break of innate dormancy. At 10° C the rise in the level of bud cytokinins was slower, correlating with the longer duration of innate dormancy. Injecting unlabelled cytokinins into tubers in amounts known to induce sprouting gave rise to increases in cytokinin concentrations in the buds of the same order as the increase associated with the natural break of dormancy. Metabolism of injected cytokinins was greater in non-dormant than in dormant tubers. The roles of cytokinin concentration and the sensitivity of the buds to cytokinin in the control of dormancy are discussed.

Key words: Bud dormancy (innate) – Cytokinin metabolism – *Solanum* (bud dormancy).

* *Present address and address for correspondence:* Department of Botany, The University, Glasgow G12 8QQ, UK

Abbreviations: CK = cytokinin; FW = fresh weight; HPLC = high-performance liquid chromatography; RIA = radioimmunoassay; tio^{6}ade = 6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)-purine = zeatin; tio^{6}adeglc^9 = 6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)-9-β-D-glucopyranosyl purine = zeatin-9-glucoside; tio^{6}ado = 6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)-9-β-D-ribofuranosyl purine = zeatin riboside; tio^{6}ado-[^{3}H]-diol = a radioactive derivative of zeatin riboside, synthesised by periodate-oxidation followed by [^{3}H]$NaBH_4$-reduction; tio^{6}AMP = 6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)-9-β-D-5′-phosphoribofuranosyl purine = zeatin riboside 5′-monophosphate; t(ioglc^4)^{6}ade = 6-(4-O-β-D-glucopyranosyl-3-methylbut-*trans*-2-enylamino)-purine = zeatin-O-glucoside

Introduction

In a previous paper (Turnbull and Hanke 1985), evidence was presented that treatment with cytokinin (CK) can break the dormancy of potato tuber buds. However, exogenous CK was effective only during short periods at specific stages in the potato life-cycle, following the beginning and preceding the end of innate dormancy. During the period in between, the innate dormancy of the tuber buds was not affected by exogenous CK.

To find out whether or not endogenous CK is involved in the natural control of potato bud dormancy, we first have to know the content of CKs in the buds at all stages in the life cycle of the tuber. Published measurements of the endogenous CKs of potatoes (Okazawa 1969; Van Staden 1976; Van Staden and Dimalla 1978; Koda 1982a, b) were all obtained by bioassays after separation using methods with low resolving power, e.g. paper- or Sephadex LH 20-chromatography, which limits the usefulness of the results. In tuber tissue, CK activity co-chromatographing with zeatin (tio^{6}ade), zeatin riboside (tio^{6}ado), zeatin riboside-5′-phosphate (tio^{6}AMP) and isopentenyladenine was reported. Nothing at all is known about CKs in unsprouted buds.

It therefore seemed necessary to analyse and estimate the endogenous CKs of tubers and their buds and to investigate CK metabolism in these tissues. The only technique sensitive enough to

measure the CKs of unsprouted buds [approx. 100 µg fresh weight (FW) per bud] is immunoassay and so a radioimmunoassay (RIA) for CKs with a zeatin-type (tio^6) side chain on N6 was developed from the method of Weiler (1980). Wherever possible the CKs in an extract were fractionated using high-performance liquid chromatography (HPLC) (Horgan and Kramers 1979) before estimation by RIA. These analytical methods were used to follow the changing pattern of CK in various tissues, including tuber buds, at different stages in the potato life cycle.

Materials and methods

Plant material. Potato (*Solanum tuberosum* L. cv. Majestic) was grown, harvested and stored as described previously (Turnbull and Hanke 1985).

Chemicals. Cytokinins were from Sigma Chem. Co., Poole, Dorset, UK, except zeatin-7-glucoside, zeatin-9-glucoside (tio^6-adeglc^9), zeatin-O-glucoside (t(ioglc^4)^{6}ade), dihydrozeatin-O-glucoside and tio^{6}AMP which were generous gifts from Dr. R. Horgan (University College of Wales, Aberystwyth). All CKs were checked for purity by reverse-phase HPLC and where necessary purified before estimating cross-reactivity in the RIA.

Feeding tubers with cytokinins. Cytokinins (200–400 µM) were injected into whole tubers as described previously (Turnbull and Hanke 1985). A radioactively labelled CK derivative (tio^{6}ado-[^{3}H]-diol, see subsequent "Radioimmunoassay" section for synthesis) was used to estimate the volume of CK solution infiltrated into the tuber tissues. From the total extractable radioactivity subsequently recovered, values of 10–20 µl per tuber were calculated, corresponding to a CK dose of 2–8 nmol per tuber.

Cytokinin extraction from plant tissues. Although a methanol/$CHCl_3$/HCOOH/H_2O mixture (Bieleski 1964) is the preferred extractant for CKs, its use gave rise to severe interference in the RIA, masking CKs detectable in 80%-ethanol extracts. The nature of this interference could not be determined. In consequence, 80% ethanol was used as extractant in the knowledge that this solvent does not completely inactivate phosphatases and so a proportion of any CK present as the nucleotide in the tissue might be detected as the nucleoside. The protocol for extraction was designed to minimise any such hydrolysis.

The outer cortex (2–3 mm depth of 'peel') of the tuber in the vicinity of the buds and the buds or sprouts themselves were used for the majority of analyses. Tissue from at least 10 tubers was taken for each extract. Bud samples were prepared by individual dissection, discarding the protective bud scale leaves to ensure that only living tissue was taken. With practice, buds could be excised at 200–300 (20–30 mg FW) per hour. The time from excision of each bud to freezing in liquid N_2 was 10–20 s.

Tissue samples were frozen immediately in liquid N_2 and extracted without thawing in chilled (−20° C) 80% ethanol (10:1, v/w). Storage, where necessary, was at −90° C as CK losses were detected after storage at −20° C. After 1-2 h extraction, the homogenate was centrifuged (1000 g_{av}), or filtered, and the solids re-extracted in chilled 80% ethanol. The combined extracts were reduced to <0.5 ml g^{-1} FW original tissue by rotary evaporation under vacuum at 35° C. Precipitated solids were removed by centrifugation (9000 g_{av} for 2 min in a Microfuge B (Beckman, High Wycombe, Buck., UK), or 35000 g_{av} for 20 min in a J2-21 (Beckman)). The clear supernatant, 'crude extract', was frozen in liquid N_2 and stored at −90° C if not analysed immediately.

Factors in the crude extract which interfered in the RIA could be removed in a single step using C_{18} Sep Paks (Waters, Harrow, Mddx., UK). Methanol, 4 ml, followed by 10 ml of 10 mM triethylammonium acetate (TEAA) solution, pH 7, was passed through the Sep Pak cartridge before crude extract from up to 5 g FW tissue was loaded and washed through with a total of 9 ml of 10 mM TEAA solution, pH 7. Cytokinins were retained on the cartridge and could be eluted in 6 ml 40% (v/v) methanol in 10 mM TEAA solution. Afterwards 6 ml methanol was passed through to clean the cartridge. The 40%-methanol fraction was evaporated at low pressure at 35° C to <0.2 ml g^{-1} FW original tissue, cleared by centrifugation (9000 g_{av} for 2 min) and either analysed by HPLC, or estimated by RIA, or frozen in liquid N_2 and stored at −90° C.

High-performance liquid chromatography. The equipment, from Spectra Physics, St. Albans, Herts., UK, consisted of an SP8700 solvent delivery system coupled to an SP8300 UV(254 nm) detector and an SP4100 computing integrator. Samples were loaded using a Rheodyne 7125 injector fitted with a 1000 µl sample loop. C_{18}-bonded silica reverse-phase columns were used; most analyses were carried out on an Ultrasphere 5 µm ODS column (150 mm long, 4,5 mm diameter; Anachem, Luton, Beds., UK). The mobile phase was a mixture of methanol (LC grade, Fisons, Loughborough, Leics., UK) and water (from a glass still fed with distilled water) containing 0.2 mM TEAA, pH 7. Solvents were vacuum-degassed and helium-purged before us. Gradient elution was sometimes used, but in practice all the CKs of interest could be separated in 10 min by an isocratic programme of 30% methanol at 1 ml^{-1} (Fig. 1).

Only extracts from small amounts of tissue (5–50 mg FW) were injected without prior clean-up on a C_{18}-Sep Pak. Samples of any volume up to 0.9 ml could be loaded, made up to 1 ml with H_2O. In this solvent, CKs are immobilised on the column head where they accumulate with very little loss of resolution. For preparative separations, fractions of 11 drops collected by a Redirac 2112 (LKB, Bromma, Sweden) were of equal volume. Thirty fractions were collected from 0.95 (void time) to 11 min after injection, dried in vacuo and redissolved in H_2O before RIA.

A mixture of all the CKs of interest (10–200 pmol of each compound) was run immediately before and after the fractionation of an extract, and the retention times of the standards were used to identify the peaks detected by RIA in the sample.

Radioimmunoassay. Because almost all CKs reported as occurring in potatoes co-chromatographed with members of the zeatin family of compounds, an RIA method of Weiler (1980) for CKs with the tio^6 [6-(4-hydroxy-3-methylbut-*trans*-2-enyl)] side-chain was adapted for our use.

(a) Preparation of antigen. An immunogenic CK-conjugate was synthesised by the method of Erlanger and Beiser (1964) as described by Weiler (1980): the furanose ring of tio^{6}ado is cleaved between carbons 2 and 3 by oxidation with IO^{4-}, resulting in a dialdehyde. This reacts with the primary amino groups of bovine serum albumin to form a linkage which is stabilised by reduction with $NaBH_4$. In our reaction, 70.9 mg conjugate was recovered which, from UV difference-spectra,

includes an estimated 1.6 mg tio^{6}ado, equivalent to a molar conjugation ratio of 4.2 tio^{6}ado per bovine serum albumin.

(b) Immunisation. Bovine serum albumin-tio^{6}ado conjugate, 2 mg in 0.5 ml H_2O, was emulsified with 0.75 ml Freund's complete adjuvant (Difco, West Moseley, UK) immediately before 0.4 ml of the mixture was injected subcutaneously at multiple sites on the back of New Zealand White rabbits. Blood (20 ml) was collected from a marginal ear vein, allowed to clot for 24 h at 4° C and the serum separated by 10 min centrifugation, 1000 g_{av}. Immunisation and bleeding was repeated at two to three week intervals until maximum antibody titre was obtained (25 weeks). Of two rabbits immunised, both gave high antibody titres. Antisera were stored at −20° C with no loss of activity over two years.

(c) Synthesis of tio^{6}ado-[^{3}H]-diol. As in section (a), 10 mg tio^{6}ado was oxidised, but the dialdehyde was isolated after the addition of ethan-1,2-diol by thin-layer chromatography in $CHCl_3$: methanol, 9:1 (v/v), on Silica Gel 60 plates (Merck, Darmstadt, FRG). Material at R_f 0.12 to 0.19 was eluted in ethanol and re-chromatographed in $CHCl_3$: methanol, 9:2 (v/v). Pure tio^{6}ado-dialdehyde (1.05 mg) was recovered at R_f 0.66, well separated from tio^{6}ado (R_f 0.53), and dissolved in 0.88 ml 90% ethanol. To this was added 10.6 µmol (3.7 GBq) of [^{3}H] $NaBH_4$ (Amersham International, Amersham, Bucks., UK) and after 10 min the mixture was subjected to thin-layer chromatography ($CHCl_3$: methanol, 9:2 (v/v)). Examination of the plate under UV indicated almost complete conversion of tio^{6}ado-dialdehyde to two products (R_f 0.32 and R_f 0.40) which were individually eluted in 80% ethanol and stored at −20° C. The initial specific radioactivity of the tio^6-[^{3}H]-diol (R_f 0.32) was estimated from the total radioactivity and UV spectrum to be 170 GBq mmol^{-1}, and the radiochemical purity >99% by HPLC. This compound was chemically stable in storage at −20° C for over two years.

(d) Immunoassay. Although $(NH_4)_2SO_4$ precipitation of the antibody-antigen complex (Weiler 1980) proved effective for separating 'bound' from 'free' antigen, it was time-consuming so an equally effective but more rapid assay was developed using Protein A, which specifically binds immunoglobulins, covalently linked to Sepharose beads. Up to 144 incubations could be carried out simultaneously using BAS centrifugal microfilter tubes (Anachem, Luton, Beds., UK), containing a 0.45 µm pore-size cellulose-nitrate Millipore filter over a 5 µm pore-size PTFE support to ensure that liquids remained above the filter until centrifuged. An Eppendorf 'Multipet' (Anderman, East Moseley, UK) repeating pipette was used to dispense reagents.

The following reagents were added to the filter compartment of a microfilter tube in the order indicated: (i) 0.5–1.0 mg Protein A-Sepharose CL4B (Pharmacia, Uppsala, Sweden) in 200 µl of 0.1 M NaCl, 0.1 M potassium phosphate solution, pH 7.1, containing 170 Bq (1.02 pmol) of tio^{6}ado-[^{3}H]-diol; (ii) 50 µl of either tio^{6}ado standard in H_2O, or sample, or H_2O; (iii) 50 µl of either antiserum (final dilution 2500-fold or 3000-fold) or, for determination of non-specific binding, H_2O. Each tube was sealed with the cap from a Beckman 1.5-ml Microfuge tube and shaken, upside-down to ensure against transfilter leakage, for 60 min at approx. 20° C.

'Receiver' tubes (capless 1.5 ml Microfuge tubes) were fitted below the filter compartment and the tubes centrifuged at 1400 g_{filter} in an Centaur 1 centrifuge (MSE, Crawley, Sussex, UK) for 7 min, separating most of the unbound radioactivity from that bound up in Protein A-Sepharose-antibody-antigen complexes which is held back by the filter. To wash off the remaining unbound label, 150 µl 10 mM NaCl, 10 mM potassium phosphate solution, pH 7.1, was added to the filter compartment and after a brief shake, the tubes were re-centrifuged for 5 min. New receiver tubes were fitted and 150 µl 2 M acetic acid added to the filter compartment to elute the bound tracer which was recovered by a final 5-min centrifugation. The eluate was transferred to a 5-ml polythene Betavial (Hughes and Hughes, Romford, Essex, UK) and 2 ml of Beckman EP scintillation cocktail added before counting in a Beckman LS 7500 using the RIA programme. Counting efficiency was reasonably constant (43 ± 3%) and so cpm was used routinely. All samples and standards were immunoassayed in duplicate.

A standard curve was computed from the results of unlabelled tio^{6}ado, added to compete with the labelled antigen, and a new standard curve constructed for every set of assays. The curve was linearised by the 'logit' transformation:

$$y = \ln\left[\frac{\%(B/B_0)}{100 - \%(B/B_0)}\right],\ x = \lg\ [\text{moles tio}^6\text{ado added}];$$

where B = radioactivity bound in the presence of added sample or standard as a percentage of the total radioactivity, and B_0 = radioactivity bound in the absence of added sample or standard as a percentage of the total radioactivity. A straight line was fitted by least-squares regression from which the amount of cytokinin in the sample was computed.

The optimum dilution of the antiserum was determined using two parameters; first, the affinity of the antibodies for the tracer and, second, the minimum quantity of tracer that can be measured accurately using the scintillation counter. The former was calculated from a Scatchard (1949) plot of the relationship between ligand binding and ligand concentration. Maximum sensitivity of the assay is associated with near-saturation of the binding sites in the absence of competing sample or standard. For 2-min counts on the scintillation counter, a range of values for B up to 1500 cpm gave the necessary accuracy, and this corresponded to a total amount of tracer per assay tube of 170 Bq (1.02 pmol) when B_0 = 35%. To achieve this B_0 value, the final antiserum dilution required was calculated, from the affinity constant, to be 1/2500–1/3000, and these values were confirmed by experiment.

Results

Evaluation of methods

(a) Sample preparation. Cytokinin loss during extraction was estimated by recovery of an internal standard, 108 pmol (16 kBq) tio^{6}ado-[^{3}H]-diol, added to 5.9 g FW of tuber peel immediately before freezing in liquid N_2. The total content of endogenous CK in this tissue was estimated at 83 pmol from RIA measurements of other samples of the same tissue. Of the label added at the start, 98.9% was recovered, after the HPLC stage, as a single peak co-chromatographing with authentic tio^{6}ado-[^{3}H]-diol.

The Sep Pak step was evaluated by loading increasing amounts of extract spiked with tio^{6}ado-[^{3}H]-diol and estimating radioactivity in the eluate. Even when the extract from > 10 g FW was loaded, 91% of the tio^{6}ado-[^{3}H]-diol was recovered in the

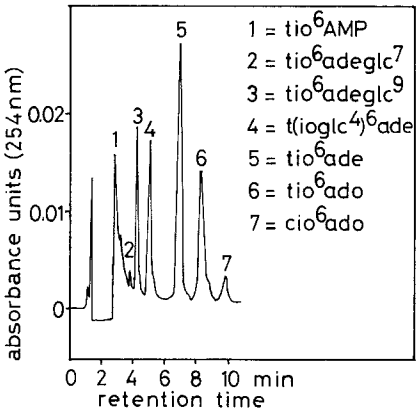

Fig. 1. Ultra-violet-absorbance trace from reverse-phase HPLC of standard mixture of cytokinins. Column: Ultrasphere 5 µm ODS, 150 mm long, 4.5 mm diameter; mobile phase: 30% methanol in 0.2 mM triethylammonium acetate, pH 7, 1 ml min^{-1}; sample: 12–600 pmol each cytokinin in 1 ml H_2O; detector: UV, 254 nm. *cio⁶ado* = 6-(4-hydroxy-3-methylbut-*cis*-2-enyl amino)-9-β-D-ribofuranosyl purine; *tio⁶adeglc⁷* = 6-(4-hydroxy-3-methylbut-*trans*-2-enyl amino)-7-β-D-glucopyranosyl purine

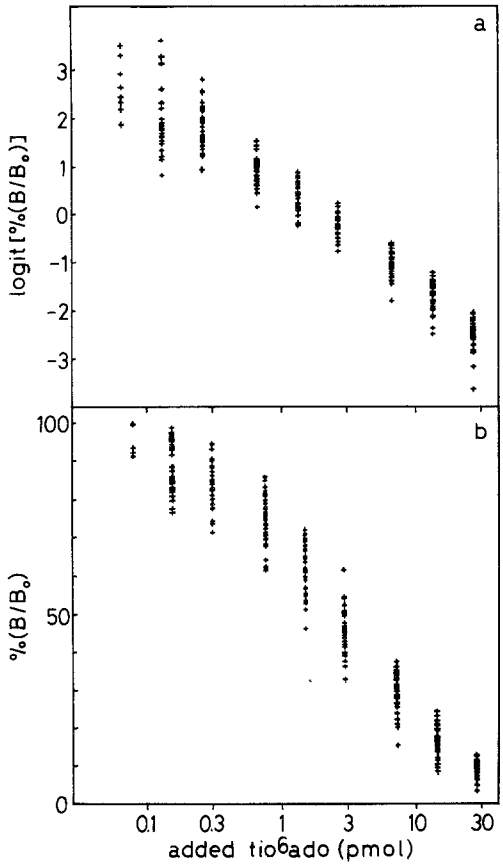

Fig. 2a, b. Standard-curve points combined from many RIAs. Each point is an average of duplicate assay incubations; data were collected over a 15-month period. **a** Linearised plot with logit-log scales; **b** sigmoid plot with linear-log scales

40%-methanol fraction, but normally only the equivalent of 5 g FW or less was loaded.

(b) High-performance liquid chromatography. Excellent separation of CKs was obtained in 30% methanol in 0.2 mM triethylammonium acetate, pH 7, at 1 ml min^{-1} through an Ultrasphere ODS 5 µm column (150 mm long, 4.6 mm diameter) maintained in good condition (Fig. 1).

(c) Radioimmunoassay. Antigen-binding was first detected at six weeks after the first immunisation and reached a maximum by 25 weeks. From Scatchard (1949) plots of the antigen bound at different concentrations of antigen, an affinity constant, K_a, of $2.63 \cdot 10^9$ mol^{-1} was calculated, and the concentration of binding sites in undiluted serum was estimated at 6.1 µM. The latter agrees well with a value of 6.5 µM obtained empirically by incubating serum with saturating concentrations of tio⁶ado-[³H]-diol. At a serum dilution of 1/100, 96% of the label added as 3.1 nM tio⁶ado-[³H]-diol (radiochemical purity 98.4% by HPLC) was bound.

In each 0.3-ml assay there were 0.63 pmol (1/3000 serum dilution) of binding sites, which if all were occupied would give $B_0 = 62\%$ since 1.02 pmol (170 Bq) of tio⁶ado-[³H]-diol were present. In practice the B_0 attained was 30–35%, a value consistent with the measured K_a. The total protein in each assay was estimated at 9.2 µg bovine-serum-albumin equivalents by the Amido Black method (Schaffner and Weissman 1973), insufficient to tax the IgG-binding capacity, 20–60 µg, of the Protein A-Sepharose present.

Standard curves were prepared using tio⁶ado. The practical range for CK measurement was 0.15 to 30 pmol tio⁶ado, but amounts down to 0.06 pmol were detectable. Data for about 100 standard curves obtained over a 15-month period are shown in Fig. 2. Correlation coefficients (R^2) after fitting straight lines to the logit plots by least-squares regression for individual curves were from 0.91 to 0.995. Standard curves prepared using tio⁶ade paralleled those obtained using tio⁶ado.

The values for molar cross-reactivity presented in Table 1 show that the RIA is highly specific for all members of the tio⁶ade group except for t(ioglc⁴)⁶ade (which can be hydrolysed to assayable tio⁶ade using β-glucosidase), and zeatin-7-glucoside.

(d) Combined HPLC-RIA. Although all the HPLC solvent was always evaporated before RIA,

Table 1. Molar cross-reactivities of cytokinins and related compounds in the RIA. All values were calculated relative to the binding of tio^{6}ado (= 100%)

Compound	Cross-reactivity (% mol/mol)
Zeatin riboside (tio^{6}ado)	100
Zeatin ribotide (tio^{6}AMP)	100
Zeatin (tio^{6}ade)	45
Zeatin-O-glucoside (t(ioglc^4)^{6}ade)	1.8
Zeatin-9-glucoside (tio^{6}adeglc^9)	100
Zeatin-7-glucoside	3.0
cis-Zeatin riboside	0.9
Dihydrozeatin	1.3
Dihydrozeatin-O-glucoside	(0)[a]
Isopentenyladenosine	0.44
Isopentenyladenine	0.27
Kinetin	0.15
Adenosine-5'-monophosphate	0.002
Adenosine	0.002
Adenine	0.006

[a] The very low cross-reactivity measured was contaminating dihydrozeatin

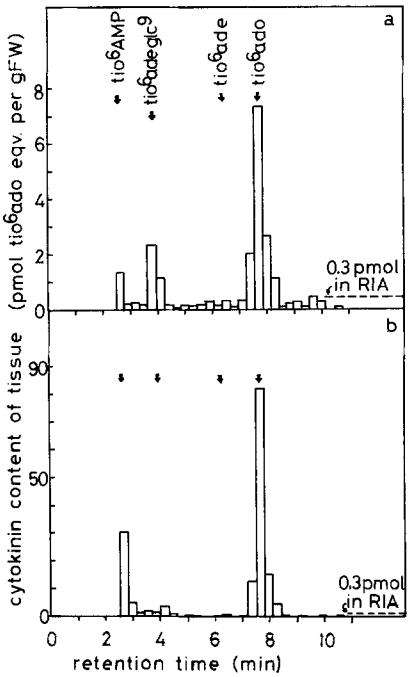

Fig. 3a, b. High-performance liquid chromatography-RIA profiles from extracts of underground tissues. Date 03/06/81: **a** roots, total cytokinin in the sample = 20 pmol tio^{6}ado eqv. g^{-1} FW; **b** underground stems, total cytokinin in sample = 150 pmol tio^{6}ado equivalents g^{-1} FW. Retention times of standards shown by *arrows*. Conditions for HPLC as in Fig. 1

methanol up to 10% (v/v) in the assay mixture did not affect the result.

Combined HPLC-RIA was evaluated using test mixtures of known amounts of tio^{6}ade, tio^{6}ado, tio^{6}adeglc^9 and t(ioglc^4)^{6}ade. The HPLC fractions containing t(ioglc^4)^{6}ade were combined, dried down, and dissolved in 300 µl H_2O containing 0.2 mg almond β-glucosidase (Sigma). After 3 h incubation at 35° C, the mixture was analysed by HPLC-RIA. Of the t(ioglc^4)^{6}ade in the original sample, 92% was recovered as tio^{6}ade at the end of the procedure. Recoveries (pmol estimated from the area of the peak of UV absorbance × 100/pmol estimated by RIA) of all other CKs in the assay were also in excess of 90%.

Measurements of CKs in potato tissues

The following limitations of the methods used in this study must be borne in mind when interpreting the results.

(i) All measurements were by RIA which, except for samples from very small (< 50 mg FW) amounts of tissue, was always preceded by HPLC fractionation. Because of the specificity of the RIA, it is not practicable to measure non-zeatin-type CKs. Therefore, throughout this section 'CK' refers to cytokinins substituted at N6 with the 4-hydroxy-3-methyl-but-*trans*-2-enyl group (tio^6).

(ii) Conversion of tio^{6}AMP to tio^{6}ado by phosphatases during extraction may have affected the relative amounts of these two compounds. Although pure tio^{6}AMP was quantitatively recovered in the 40%-methanol eluate from Sep Paks, losses of some of this highly polar CK may have occurred because of interference with its retention on the C_{18} cartridge by components of the extract.

(iii) The low (3%) cross-reactivity of zeatin-7-glucoside in the RIA (Table 1), and the lack of any method, enzymic or chemical, by which immunoassayable CK could be quantitatively released from it, mean that this compound is only detectable if present in high concentrations.

(iv) It was discovered that, after analysing potato extracts by HPLC, almond β-glucosidase-treatment of HPLC fractions containing no O-glucosides sometimes gave rise to false-positive values by interference in the RIA. However, as there was never any convincing positive evidence for the presence of t(ioglc^4)^{6}ade in any extract, this observation does not affect the validity of the results presented here.

(v) The lower cross-reactivity of tio^{6}ade (Table 1) has been corrected for in calculating the amounts of each CK in extracts fractionated by HPLC. Where an estimate of total CK content was all that could be obtained, this is expressed as moles equivalent of tio^{6}ado.

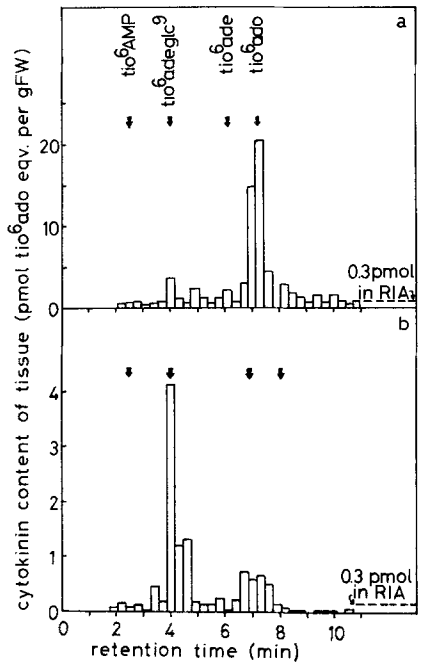

Fig. 4a, b. High-performance liquid chromatography-RIA profiles from extracts of tuber peel, 3 mm deep: **a** Dormant tubers extracted on 10/09/81 (total cytokinin in the sample = 66 pmol tio^{6}ado equivalents g^{-1} FW); **b** non-dormant tubers extracted on 21/04/82, i.e. after 31 weeks in storage at 2° C (total cytokinin in the sample = 11 pmol tio^{6}ado equivalents g^{-1} FW). Retention times of standards shown by *arrows*. Conditions for HPLC as in Fig. 1

Cytokinins in growing potato plants. To gain some idea of the levels and types of CKs in non-tuber tissues, extracts of roots, which are thought to be sources of CK for the rest of the plant, and underground stems were analysed by HPLC-RIA. The predominant CK detected was tio^{6}ado in both roots (72%) and underground stems (74%), with smaller quantities of tio^{6}AMP (9% and 24% respectively) and, in roots, some tio^{6}adeglc^9 (20%) (Fig. 3). No tio^{6}ade was detected.

Growing tubers had high levels of CK, e.g. tubers six weeks old (17 July 1981) contained 170 pmol g^{-1} FW in the outer 3 mm of peel. By the end of the phase of tuber growth, the level of CKs was lower, e.g. 66 pmol g^{-1} FW on 10 September 1981 and 40 pmol g^{-1} FW on 21 August 1982. The major CK component was tio^{6}ado throughout tuber growth (Fig. 4a).

Cytokinins in storage tissue of stored tubers. Table 2 summarises the HPLC-RIA data on CKs in outer tissues of tubers stored post-harvest at constant temperature. The most striking feature is that within four weeks of harvest there was a five- to ten-fold decrease in the total CK content, e.g. from 66 to 7 pmol g^{-1} FW in 1981 and from 40 to 4 pmol g^{-1} FW in 1982. This decrease was caused almost entirely by the disappearance of tio^{6}ado and it was independent of whether tubers were stored at 2° C or 10° C.

After the disappearance of most of the tio^{6}ado there was no major change in the level (range 6 to 15 pmol g^{-1} FW for 1981–1982 season) or types of CKs detected in tuber tissues. The predominant forms were now tio^{6}ade, tio^{6}AMP and in the last measurement made (21 April 1982) tio^{6}adeglc^9 (Fig. 4b) although the low levels detected by this stage reduce the accuracy of estimation. This period of insignificant changes in the CK content

Table 2. Cytokinins detected in extracts of storage tissue from potato tubers stored at constant temperature. Measurements of total CK were made by taking a portion of each extract for RIA prior to HPLC. + = 10–20% of total CK; + + = > 20% of total CK

Time since tuber initiation[a] (weeks)	Time in storage (weeks)	Storage temperature (° C)	Component CK				Total CK
			tio^{6}ade	tio^{6}ado	tio^{6}AMP	tio^{6}adeglc^9	(pmol tio^{6}ado eqv. g^{-1} FW)
7				+ +			170
12				+ +			40
15				+ +			66
	3	2	+ +	+ +	+ +	+	2
	3	10	+ +	+ +	+ +		4
	4	2	+ +				10
	4	10	+ +		+ +		7
	10	2	+ +	+	+ +	+	15
	10	10	+ +	+ +	+ +		6
	14	2	+ +		+ +	+ +	7
	17	2		+	+ +	+ +	8
	25	2	+ +	+	+ +	+	8
	28	2	+ +		+ +	+	9
	31	2	+ +			+ +	11

[a] Tuber age

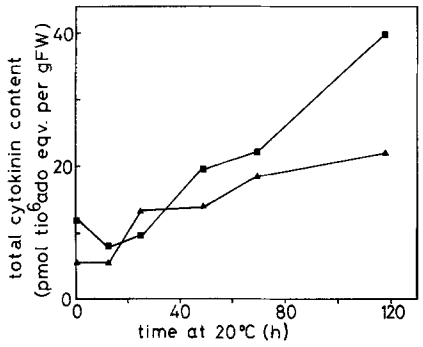

Fig. 5. Total cytokinin content of non-dormant tubers transferred to 20° C on 04/03/81 after 24 weeks in storage at 2° C. ■–■ tuber peel, 3 mm deep and 11 mm radius around apical bud; ▲–▲ tuber peel, 3 mm deep and 11 mm radius around lateral buds

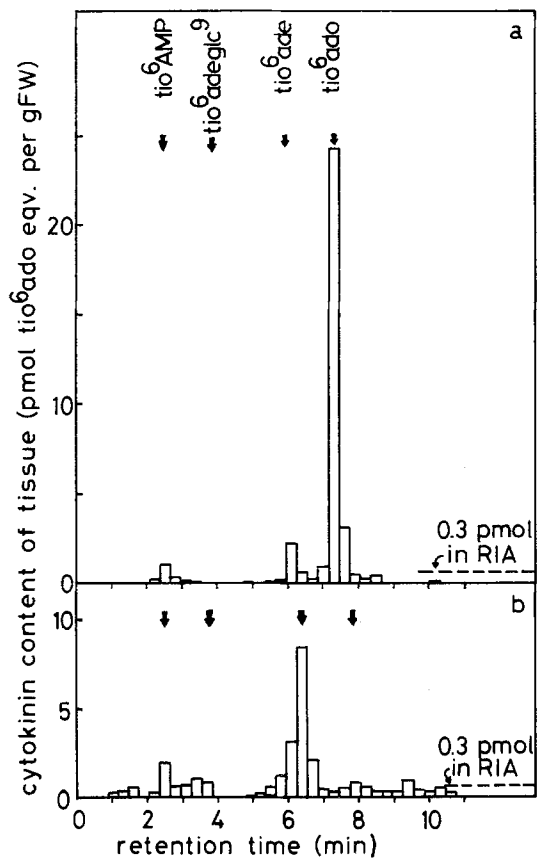

Fig. 6a, b. High-performance liquid chromatography-RIA profiles from extracts of dormant tubers after injection with cytokinin. **a** After six weeks in storage at 10° C, tubers were transferred to 20° C, injected with 200 µM tio^{6}ado on 23/10/81, and extracted 7 d later (total cytokinin in sample = 41 pmol tio^{6}ado equivalents g^{-1} FW); **b** after one week in storage at 2° C, tubers were transferred to 20° C, injected with 200 µM tio^{6}ade on 23/09/81, and extracted 8 d later (total cytokinin in sample = 11 pmol tio^{6}ado equivalents g^{-1} FW). Retention times of standards shown by *arrows*. Conditions for HPLC as in Fig. 1

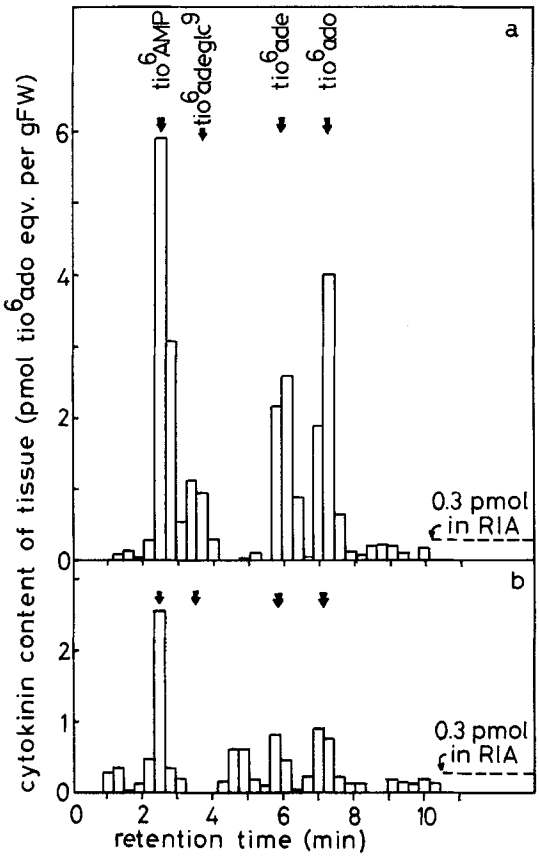

Fig. 7a, b. High-performance liquid chromatography-RIA profiles from extracts of non-dormant tubers with or without cytokinin injection. **a** After two weeks in storage at 2° C, tubers were transferred to 20° C and injected with 400 µM tio^{6}ade on 29/09/81 (total cytokinin in sample = 48 pmol tio^{6}ado equivalents g^{-1} FW); **b** as above, but injected with H_2O (control) (total cytokinin in sample = 8 pmol tio^{6}ado equivalents g^{-1} FW). Retention times of standards shown by *arrows*. Conditions for HPLC as in Fig. 1

of the tuber tissue includes the termination of innate dormancy of the buds of all tubers at 2° C and 10° C.

Cytokinins in storage tissue of sprouting tubers. In contrast to the constant level of total CK in tuber tissue during prolonged storage at 2° C, on transfer to 20° C the concentration rose markedly in the outer 3 mm of peel from tubers in which innate dormancy had terminated (Fig. 5). An increase was detectable after 1–2 d, reaching three- to four-fold at 5 d, and the effect was marginally (40%) more pronounced in apical as compared with lateral tissue. However, since new growth occurred in the buds within 24 h of transfer to 20° C (Turnbull and Hanke 1985), almost all of the increase in CKs is too late to be involved in the initiation of sprouting.

Metabolism of CKs injected into tuber tissue. Figures 6 and 7 show results from experiments in

which the fate of CK injected into tubers on transfer to 20° C was investigated by HPLC-RIA. In tubers with buds still innately dormant at the time of injection, the majority of the CK measured in the storage tissue was as the compound injected (86 and 96% as tio^{6}ade, 78% as tio^{6}ado) indicating very little conversion to other CKs, even over 7–16 d. The corresponding values for tubers with non-dormant buds were always much lower (42–52% as tio^{6}ade) since a substantial fraction of the CK detected was as compounds different from that injected. This difference in cytokinin-metabolising activity is apparent on comparing the HPLC-RIA profiles of Fig. 6 (tubers with innately dormant buds) with those of Fig. 7 (tubers with non-dormant buds). The CKs detected in the tissue of tubers with non-dormant buds 4 d after CK-injection, compared with those detected in the controls, indicates there had been substantial conversion of the injected tio^{6}ade to tio^{6}ado, tio^{6}AMP and possibly tio^{6}adeglc^9 (Fig. 7).

The significance of this result is subject to the following limitations. First, metabolism to compounds which do not cross-react with the antibody (see Table 1) will not have been detected. Second, the difference in CK-metabolising activity of the tuber might be related either to the difference in the initial state of dormancy of its buds (innately dormant versus non-dormant), or to the difference in the subsequent response of the buds to 20° C treatment (non-growing versus growing). Nevertheless, an increased ability of the tuber to interconvert CKs is clearly associated with increased activity of the buds.

Cytokinins in the buds and sprouts of potato tubers. Typical fresh weights of individual unsprouted apical and lateral buds are 300 µg and 100 µg, respectively. The total bud tissue accounts for approx. 0.001% of the total fresh weight of the tuber. Quantitative measurements of the total CK content were made by RIA of extracts from buds at all stages of the life cycle, from tuber initiation to long after the end of innate dormancy in storage. The values for total CK content of unsprouted buds made during the season 1981–1982 (storage at 2° C) are presented in Fig. 8). Measurements made in other years show the same pattern. The most striking feature was the enormous increase in the concentration of CK in the buds between harvest (mid-September) and the end of all innate dormancy (early November). The minimum level of 25–60 pmol g^{-1} FW rose to a plateau (most obvious on the log-linear plot) of approx. 1300 pmol g^{-1} FW which was maintained for at least eight months. The average increase was, therefore, 20- to 50-fold and since neither cell division nor cell expansion occur in the buds of tubers stored at 2° C (Turnbull and Hanke 1985) this increase is neither a consequence of, nor associated with, growth.

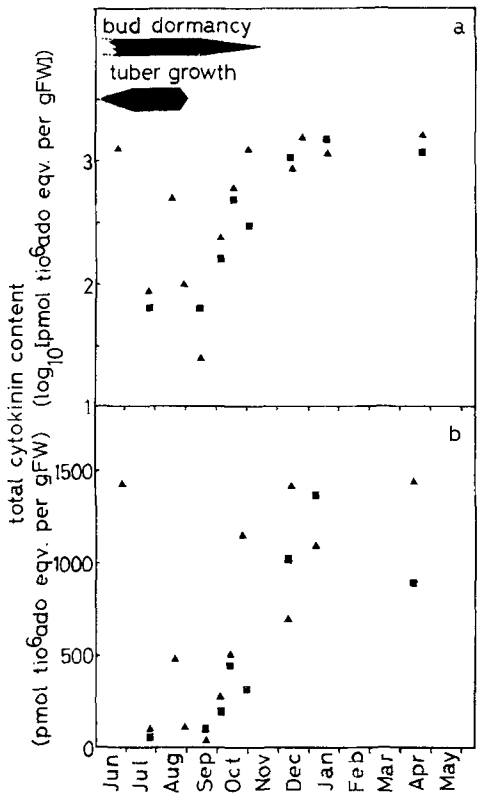

Fig. 8a, b. Total cytokinin content of non-growing buds through a single season (1981–1982). After harvest (15/09/81), storage was at 2° C. ■ Apical buds; ▲ lateral buds. **a** Log-linear plot; **b** linear plot

For tubers stored at 10° C, the concentration of CK in the buds also increased during storage, but more slowly, e.g. after four weeks storage, the level in buds from tubers stored at 10° C was 210–260 pmol g^{-1} FW, whereas in buds from tubers stored at 2° C it had reached 460–510 pmol g^{-1} FW. As soon as innate dormancy had terminated in tubers stored at 10° C, the buds started to grow rapidly and therefore could no longer be compared with buds on tubers stored at 2° C.

For buds collected from tubers before harvest, i.e. during tuber growth and maturation, the pattern of changes in CK content was less clear. The concentration of CK was always very high shortly after tuber initiation when CK levels in all tissues were high, and repeatedly found to be very low in the buds of newly mature tubers (e.g. 30 pmol g^{-1} FW on 14 October 1980, 25–60 pmol

Table 3. Cytokinins detected in extracts of potato tuber buds and growing sprouts. Measurements of total CK were made by taking a portion of each extract for RIA prior to HPLC. + = 10–20% of total CK; + + = > 20% of total CK

Date	Period at 20° C (d)	Component CK				Total CK (pmol tio^{6}ado eqv. g^{-1}FW)
		tio^{6}ade	tio^{6}ado	tio^{6}AMP	tio^{6}adeglc^9	
Buds:						
17/08/82	0 (dormant)	+ +	+ +			1070
15/12/81	0 (not dormant)		+ +	+ +		700
Growing sprouts:						
22/09/82	5	+ +	+ +	+ +		154
07/12/81	10	+ +	+ +	+ +		260
11/12/81	10	+ +	+ +			108
26/01/82	41	+ +	+ +			60

g^{-1} FW on 19 September 1981). However, there was a great deal of variation in the timing of the fall to this low level (Fig. 8). Some of this variation may be a consequence of sampling tubers from only a small number of plants for each analysis, since individual plants differed in relative maturity by as much as four weeks. Since all the tubers are mixed at harvest, variability caused by poor sampling will be reduced post harvest. Note that these dramatic changes in CK concentration in buds of growing tubers are not accompanied by any alteration in the state of dormancy, since all buds normally remained innately dormant throughout the period of tuber growth.

On a few occasions, the samples from bud or very young sprout tissue were large enough to be fractionated by HPLC before RIA. Recoveries of CKs from such small samples were variable. This limited information (Table 3) indicated that the same CKs as occur in the tuber, tio^{6}ade, tio^{6}ado and tio^{6}AMP, were also present in buds and young sprouts, with the notable exception of tio^{6}adeglc^9 which is absent from all extracts of these tissues although frequently detected in stored tubers (Table 2). Note that since tio^{6}adeglc^9 is the only biologically inactive CK detected by the RIA, its absence from buds means that RIA measurements of total CK in this tissue represents only biologically active CKs.

In some cases, the level of CKs in very young sprouts was determined by RIA 1–10 d after CK solution or H_2O had been supplied to the tuber (Table 4). The measured content of CK in sprouts from tubers injected with CK was always higher than that of sprouts from tubers injected with H_2O, both per unit fresh weight and per sprout (sprouts of CK-treated tubers were larger than those of the controls).

An experiment was carried out to test the correlation of innate dormancy with endogenous CK levels in the bud cells. After nine weeks storage at 10° C (buds still innately dormant) or 2° C (no innate dormancy) tubers were transferred to 20° C. After 24 h, i.e. just before growth started in non-dormant buds, some of the buds were assayed for total CK content. The others were used for determinations of sprout volume and cell number (Fig. 2 of Turnbull and Hanke 1985). The level of CKs in buds from tubers stored at 2° C was three to four times higher than in buds from tubers stored at 10° C, whether expressed per unit fresh weight, per sprout, or per cell (Table 5). It is possible to estimate mean numbers of CK molecules per cell of a lateral bud from the data. The values are $2.5 \cdot 10^6$ per cell after storage at 2° C and $8 \cdot 10^5$ per cell after storage at 10° C. The corresponding value for lateral buds immediately after harvest nine weeks before (on 18 September 1981) was $8 \cdot 10^4$ CK molecules per cell. The increase during nine weeks storage at 10° C was therefore ten fold and, for buds at 2° C, greater than 30-fold.

Table 4. Total cytokinin content of growing sprouts from potato tubers incubated at 20° C after injection of cytokinin or water

Time in storage (weeks)	Storage temperature (° C)	Compound injected	Period at 20° C (d)	Sprout type	Sprout FW (mg)	Total CK (pmol tio^{6}ado eqv. g^{-1} FW)	CK per sprout (fmol tio^{6}ado eqv.)
2.5	2	H_2O	3.5	apical	0.50	140	70
				lateral	0.16	108	17
		400 µM tio^{6}ade	3.5	apical	0.85	333	280
				lateral	0.23	410	94
12	10	H_2O	10	lateral	0.46	72	33
		400 µM tio^{6}ade	10	lateral	2.49	114	284

Table 5. Total cytokinin content of sprouts from stored potato tubers transferred to 20° C. Tubers were stored for 10 weeks then, on 25 November 1981, transferred to 20° C for 24 h before the sprouts were extracted

Storage temp. (° C)	Sprout type	Total CK content (pmol tio^{6}ado eqv. g^{-1} FW)	CK per sprout (fmol tio^{6}ado eqv.)	CK per cell (amol)
2	apical	402	157	5.40
2	lateral	971	109	4.10
10	apical	~40[a]	~22	~0.65
10	lateral	222	25	1.32

[a] Barely detected in RIA

Discussion

Because the RIA used in this work was specific only for the zeatin-type CKs, any CKs with dihydrozeatin- or isopentenyladenine-type side-chains would not have been detected. We are in the process of developing immunoassays for these other CKs. However we feel that some discussion of the relevance of the results obtained so far is appropriate, especially since the literature indicates that members of the zeatin family may be important CKs in potato.

Cytokinin levels during tuber growth. In the early stages of tuber growth, the total level of CK in tubers and their buds was very high: 150 and 1300 pmol g^{-1} FW, respectively (Table 2, Fig. 8). The principal CK in all tissues at this time was tio^{6}ado; this CK predominated throughout tuber growth, although by the end of the growth phase, the concentration of total CK in the tuber tissue had fallen to one third (Table 2), and in the buds to as little as one-fiftieth (Fig. 8), of that associated with the early stages. The decrease in the tuber tissue may be caused by simple dilution in consequence of the three-fold increase in tuber volume over the same period. Alternatively, the decrease may be the result of differential rates of synthesis, interconversion, degradation, import or export of CKs by the tuber, and some mechanism of this sort must be invoked to explain the dramatic decrease in CK concentration in the buds over the latter stages of tuber growth, because the volume of the buds does not change.

During the later stages of tuber growth, when levels of endogenous bud CK were low, exogenous CK did not induce sprouting. However, in young tubers, exogenous CK induced sprouting of buds which were dormant even though, paradoxically, their endogenous CK level was very high. Clearly, the concentration of CK in the tissue is by itself no guide to the state of dormancy of the tissue or its responsiveness to CK.

Cytokinin levels during tuber storage. Immediately after harvest, regardless of storage temperature, there was a rapid five- to ten-fold decrease in the concentration of CK in the tuber cortex (Table 2). This decrease was the consequence of the disappearance of tio^{6}ado, until then the major CK in this tissue. Thereafter, the total concentration of CKs in the tuber tissue remained low.

At harvest, buds of mature tubers contained very low levels of CKs (Fig. 8). In storage at 2° C, the concentration of CK in the buds increased 20- to 50-fold over six weeks (Fig. 8). Levels of CK in buds also increased during storage at 10° C, but more slowly (Table 5). These increases could be the result of import from the tuber tissue. The buds are such a small proportion of the total volume that even when the CK content of the tuber tissue has reached its post-harvest low, there is more than enough to generate the observed increase in the buds. The quantity of CK involved (an increase of approx. 1500 pmol g^{-1} FW in 2 mg of bud per tuber = 3 pmol per tuber) is inadequate to account for the decrease observed in the tuber tissue (approx. 50 pmol g^{-1} FW in a 200-g tuber = 10000 pmol per tuber) indicating that the early-storage loss of tio^{6}ado from the tuber must be the consequence of an alteration in the balance of CK synthesis and breakdown. The increase in bud CK content is almost certainly unrelated to the decrease in the tuber because it occurs much later and shows temperature dependency. The mechanism for this is unknown but could involve temperature sensitivity of synthesis, breakdown, import or export mechanisms.

Physiological importance of changes in CK concentration in tuber buds. Attempts to establish whether the availability of plant growth substances ever controls a developmental transition are often complicated by gross physiological changes which usually accompany such transitions, e.g. in the case of tropism, where changes in growth substance distribution might be caused by the altered pattern of growth or generated independently by the processes responsible for the altered pattern of growth. In contrast, the increase in bud CK in tubers stored at 2° C is not accompanied by any growth or cell division and cannot be a product of these developmental processes.

In a companion paper to this (Turnbull and Hanke 1985), we presented results which showed

that at certain stages in the growth cycle, exogenous CK can specifically convert innately dormant buds to the non-dormant condition. Here, it has been shown that treating tubers with exogenous CK resulted in an increase in the CK level in the buds (Table 4), and it was of the same order as natural increases in endogenous bud CK (Table 5, Fig. 8). The inevitable conclusion is that an increased concentration of CK in an innately dormant bud *can* break dormancy. The question then is: does this ever occur naturally?

The rapid and substantial rise in bud CK detected in potatoes stored at 2° C coincided with the timing of the natural transition from innate dormancy to the non-dormant, but non-growing state. When tubers were stored at 10° C, the natural break of innate dormancy was delayed and the increase in the bud CK level was slower. Although it is possible that this correlation is only a coincidence, there is a strong association between a dramatic rise in endogenous bud CK and the natural transition out of innate dormancy. Furthermore, towards the end of the phase of innate dormancy of buds of tubers stored at 10° C, break of dormancy could be induced as a consequence of an exogenous increase in bud CK. Note that without the results of experiments on tubers stored at an artificially warm temperature (10° C), there would have been no grounds for supposing that CKs are involved at all in the control of dormancy since at 2° C no phase of CK-sensitivity could be detected. We consider this to be analogous to the response of artificially isolated pieces of coleoptile tissue to auxin which reveals the permissive role of auxin in the control of cell expansion although the same tissue in the intact plant does not respond to auxin (Trewavas 1981).

In a necessarily preliminary way our investigations have touched upon some of the interactions in a complex web linking environment, growth substances, tissue responsiveness and developmental transitions. By ignoring much of the evidence it is possible to select a series of interactions which fits the 'classical' concept of growth substances as intermediaries between the environment and the plant's developmental response to it, e.g. temperature *can* influence the CK concentration which *can* break bud dormancy. However, the changes in the levels of endogenous CKs in buds of *immature* tubers are not related to changes in innate dormancy or to the effectiveness of exogenous CK at inducing sprouting. The level of CKs can only be considered to be physiologically relevant when the tissue is able to respond to CK. The evidence, as discussed in Turnbull and Hanke (1985), indicates that the pattern of changing CK-responsiveness of the buds – also influenced by temperature – normally plays a major part in controlling the transition out of innate dormancy. From the results presented in this paper it appears that the pattern of changes in the level of endogenous CK in the buds is also part of the control process.

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