ELECTROCHEMICAL REDUCTION OF 6-BENZYLAMINOPURINE AT MERCURY ELECTRODES AND ITS ANALYTICAL APPLICATION

Danuše TARKOWSKÁ[a1,*], Milan KOTOUČEK[b] and Karel DOLEŽAL[a2]

[a] *Laboratory of Growth Regulators, Palacký University and Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic; e-mail: [1] tada@email.cz, [2] dolezal@aix.upol.cz*

[b] *Department of Analytical Chemistry, Palacký University, Třída Svobody 8, CZ-771 46 Olomouc, Czech Republic; e-mail: kotoucek@risc.upol.cz*

Received June 14, 2002
Accepted December 12, 2002

The commercial exploitation of modern, *in vitro* plant micropropagation methods, featuring synthetic cytokinins as essential components of the cultivation media, is rapidly increasing. Thus, development of rapid, inexpensive and less labour-intensive methods for monitoring cytokinin levels could help to optimise media consumption and reduce costs. Therefore, we studied the electrochemical behaviour of the highly active and widely used cytokinin, 6-benzylaminopurine (BAP), in aqueous solutions by DC polarography, cyclic and differential pulse voltammetry and constant potential coulometry. The BAP molecule undergoes a six-electron irreversible reduction process that starts with four-electron reduction of the protonated pyrimidine skeleton. As a result of elimination of the amine from the side chain, the N1=C6 electrochemically active bond is re-established and the last two-electron step follows. The intermediates of constant potential electrolysis were identified using mass spectrometric analysis. The dissociation constant (pK_a) of BAP was found, spectrophotometrically, to be 4.16. BAP concentrations were measured using two voltammetric techniques, fast-scan differential pulse (FSDPV) and adsorptive stripping voltammetry (AdSV). The relative standard deviations for these two methods were lower than 4.5% (c < 28.7 ng ml^{-1}) and 1.2% (c < 20 ng ml^{-1}), while the detection limits were 7.88 and 0.80 ng ml^{-1}, respectively. Using these techniques, BAP was determined in two types of nutrition media used for the micropropagation of plants *in vitro* (*Gerbera* and banana media). In the case of media samples containing the interfering agent adenine (*i.e. Gerbera* plant medium), the analyte was preconcentrated by ion-exchange chromatography and immunoaffinity chromatography. This preconcentration process gives 92% recovery. In contrast, it was possible to determine BAP levels in simple banana cultivation medium directly, without any pre-purification process. Both methods, reported here (FSDPV and AdSV), were found to be useful for rapidly monitoring BAP consumption by plants during their growth under *in vitro* conditions.

Keywords: 6-Benzylaminopurine; Cytokinins; Electrochemistry; Polarography; Voltammetry; Mass spectrometry; Coulometry; Purines; Phytohormones.

doi:10.1135/cccc20031076

6-Benzylaminopurine (BAP) is a naturally occurring plant hormone (phytohormone) belonging to the cytokinin group. All naturally occurring cytokinins are derived from adenine (6-aminopurine) by means of N^6-substitution, at the position of the amino group, with either an isoprenoid or an aromatic side chain. In the BAP molecule, the adenine base bears the benzyl functional group (Fig. 1). These compounds play an important regulatory role in many stages of plant development, including cell division, root and bud formation, and flower and fruit development. They also participate in sexual differentiation of the plant[1]. Some plant hormones and their synthetic derivatives are known for their potential to regress certain types of mammalian tumours[2,3], so these derivatives are being intensively studied for their possible application in anti-cancer treatments[4–7]. Recently, BAP and other cytokinins have been extensively used in modern biotechnology as components of *in vitro* cultivation media because of their ability to induce cell division during the propagation of diverse plant species grown for agricultural purposes or for their use in decorative greenery[8,9].

In order to identify and quantify cytokinins at the extremely low levels at which they occur, very sensitive analytical techniques are required. Such requirements can be fulfilled by various chromatographic techniques, such as HPLC with various detectors[10–13] and GC coupled with MS [14,15]. For the major groups of naturally occurring cytokinins highly sensitive immunomethods (ELISA, RIA) have also been developed[16,17]. However, these experimental approaches are labour-intensive and demand the use of radiolabelled standards. Thus, neither mass spectrometry nor immunomethods are very appropriate for high throughput screening or for the large-scale monitoring of cytokinin consumption in cultivation media. Thus, the development of alternative, rapid, low-cost techniques for measuring phytohormone levels would be highly desirable.

From an electrochemical perspective, purine and its substituted derivatives represent substances with typically electroactive properties. Anodic oxidation of some phytohormones at graphite electrodes has been recently

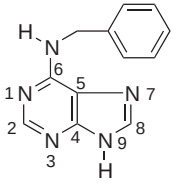


FIG. 1
Structure of 6-(benzylamino)purine (BAP)

used for analytical purposes[18–22]. The redox properties of purine and some of its 6-substituted derivatives at mercury electrodes have also been investigated in detail[23,24]. However, relatively little attention has been given to the reduction of BAP and the reduction mechanism of this compound has not yet been explained away.

The aims of the study presented here were to elucidate aspects of BAPs electrochemical behaviour, including the mechanism of its reduction at mercury electrodes in aqueous solutions, and to develop an inexpensive, time-saving electroanalytical procedure for its determination.

EXPERIMENTAL

Materials

The BAP (5×10^{-4} mol l^{-1}), purine and adenine (both 1×10^{-3} mol l^{-1}) standard stock solutions were prepared by dissolving commercial products (all from Sigma–Aldrich, St. Louis (MO), U.S.A.) in HPLC grade methanol, which was also used for further dilution. All other reagents used were of analytical reagent grade. Water purified by means of an ELGA system (ELGA, Ltd., U.K.) was used throughout for preparation of the solutions. Buffer solutions with defined ionic strength, obtained by adding solid $NaClO_4$ or KCl served as the supporting electrolytes. Because the purine compounds are poorly soluble in water, the presence of organic solvent (10% v/v methanol) in the voltammetric cells was necessary to keep the solutions homogeneous.

Methods

An OH-102 polarographic analyzer (Radelkis, Budapest, Hungary) with a three-electrode cell containing a dropping mercury electrode (DME) (t = 3.6 s, m_h = 1.98 mg s^{-1}), a saturated calomel reference electrode (SCE) and a platinum auxiliary electrode was used for DC polarography. Electrocapillary curves were measured with a Novotný spindle electrode (Polaro-Sensors, Prague, Czech Republic). The voltammetric measurements (fast-scan differential pulse voltammetry, FSDPV; adsorption stripping voltammetry, AdSV; cyclic voltammetry, CV) were performed using two instruments: a PA-4 polarographic analyzer with a static mercury drop electrode (SMDE, capillary inner diameter 0.138 mm, drop size defined by opening the valve for 160 ms) and a 4105 X–Y recorder (both from Laboratorní přístroje, Prague, Czech Republic) and an Eko-Tribo polarograph with a mercury pencil minielectrode (drop size defined by opening the valve for 200 ms), a silver–silver chloride electrode in saturated KCl and a platinum auxiliary electrode (all supplied by Polaro-Sensors, Prague, Czech Republic). FSDP voltammograms were recorded at a scan rate of 50 mV s^{-1} and modulation amplitude of –50 mV. Cyclic voltammetric curves were recorded at scan rates varied between 20 and 1400 mV s^{-1} in McIlvaine buffer at pH 2.6 and 4.5 as the supporting electrolyte, with 1×10^{-4} mol l^{-1} of the substance of interest.

All samples in the voltammetric and polarographic cells were deaerated by passing a nitrogen stream through them for 10 min.

The controlled-potential coulometry measurements were carried out using an OH-404 coulometer (Radelkis, Budapest, Hungary) with a stirred mercury pool cathode (A =

26.4 cm^2). The SCE served as a reference electrode, and there was an auxiliary platinum electrode in the anodic compartment, separated by a frit. The electrolysis of BAP was carried out at a potential of –1.4 V in 20 mM ammonium acetate buffer solution at pH 4.7. The coulometric analyses of purine and adenine were performed at E = –1.45 V and pH 4.7. Before each analysis the solutions were purged by helium for 15 min. The mass spectra of coulometrically reduced solutions containing purine, adenine or BAP in 20 mM ammonium acetate were recorded by a mass detector (Micromass ZMD 2000, Manchester, U.K.) equipped with an electrospray interface (working in positive mode, (+)ESI-MS; source temperature 80 °C, capillary voltage +3.0 kV, cone voltage 40 V) using the direct injection of 10 μl sample per minute into the ion source by a syringe pump (Model 11, Harvard Apparatus, Inc., Holliston (MA), U.S.A.). Nitrogen was used both as desolvation gas (200 l h^{-1}) and cone gas (50 l h^{-1}).

Spectrophotometric data for determination of the BAP dissociation constant were recorded using a Shimadzu UV 1601 instrument with quartz cells (path length, 1 cm). Britton–Robinson buffers with different pH values, but a constant ionic strength of 0.15 (adjusted by adding solid $NaClO_4 \cdot H_2O$), deionized water and methanolic solution of BAP were mixed in volumetric flasks. The resulting solutions, containing 1×10^{-4} mol l^{-1} of BAP were used for the measurements of UV spectra and actual pH. A blank solution was prepared from Britton–Robinson buffer, deionized water and 100% methanol.

The pH measurements were carried out using a Präcitronic MV 870 pH meter (Dresden, Germany) with a combined glass and silver–silver chloride electrode. The pH-meter was calibrated using aqueous standard buffers (Institute of Sera and Vaccines, Prague, Czech Republic).

Procedures

Modified Murashige–Skoog medium (MS medium; standard composition, see Table I) for growing plants under *in vitro* conditions was used for BAP determination as follows. First, solid medium used for cultivating young *Gerbera* plants was prepared by enrichment of standard MS medium with 170 mg l^{-1} of NaH_2PO_4, 40 mg l^{-1} of adenine sulfate, 0.1 mg l^{-1} of indolyl-3-acetic acid, 9.5 mg l^{-1} of nicotinic acid, 100 mg l^{-1} of tyrosine, 24.1 mg l^{-1} of thiamine, 0.5 mg l^{-1} of pyridoxine, 8 g l^{-1} of agar and 4 mg l^{-1} of BAP. We also used standard liquid MS media for *in vitro* cultivation of banana containing 2 mg l^{-1} of BAP. These media, before being used for growing plants, were analysed to test for the possible presence of interfering substances. All samples of MS liquid media, as well as MS solid agar media, were obtained from Biolab Co. (Olomouc, Czech Republic).

The BAP was extracted from nutritional agar media for cultivating young *Gerbera* plants by dissolving the agar in a microwave oven for five minutes then adding 40 ml of the medium to 40 ml of 100% methanol. After cooling, the sample with precipitated agar was centrifuged for 30 min at 6000 rpm. The supernatant was collected and evaporated to dryness using a vacuum rotary evaporator at 37 °C. The sample was dissolved in 0.5 ml 70% (v/v) ethanol, diluted by the addition of 20 ml 40 mM ammonium acetate (pH 6.5), and purified on a DEAE-Sephadex (Sigma–Aldrich, St. Louis (MO), U.S.A.) column coupled to a SPE C18 cartridge (Waters, Milford (MA), U.S.A.), on which hydrophobic compounds including BAP and adenine were retained, before being eluted with 80% (v/v) methanol. The sample was evaporated to dryness, then dissolved in 50 μl of 70% (v/v) ethanol, diluted by the addition of 950 μl phosphate buffer (pH 7.2) and purified by passage through pre-immunoaffinity

and immunoaffinity columns containing polyclonal specific anti-cytokinin antibody (both OlChemIm Ltd, Olomouc, Czech Republic).

RESULTS AND DISCUSSION

Mechanism of the Electrode Reaction

6-Benzylaminopurine is electrochemically reducible at a mercury electrode only in acidic medium. A single DC-wave/DPV-peak was observed at pH values less than 6.9. The limiting current is strongest in the pH region of

TABLE I
Standard composition of the Murashige–Skoog (pH 5.6–5.8) medium for *in vitro* cultivation of plants

MS cultivation medium	Substance concentration in medium, mg l^{-1}
Inorganic compounds	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2 \cdot 2H_2O$	440
$MgSO_4 \cdot 7H_2O$	370
KH_2PO_4	170
H_3BO_3	6.2
$MnSO_4 \cdot 4H_2O$	22.3
$ZnSO_4 \cdot 7H_2O$	8.6
KI	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
$FeSO_4 \cdot 7H_2O$	13.6
Organic compounds	
Chelaton 3	18.6
Glycine	2.0
Nicotinic acid	0.5
Pyridoxine	0.5
Thiamine	0.1
Inositol	100.0
Saccharose	30 000

4.2–4.7, decreasing slightly as pH falls below this level (and markedly at pH values < 2.5). As pH rises above 4.7 the wave decreases, then disappears when pH > 6.9 (Fig. 2). These observations indicate that protonation of the molecule plays a very important role in the electrochemical reaction. It seems that protonation of the BAP molecule is more important for the electrochemical reaction of BAP at the static mercury than at the dropping mercury electrode, since a single DPV-peak height is maximal at pH < 2.5, decreases rapidly and disappears at pH ≈ 6.9 (Fig. 3). The dissociation constant, pK_a, of BAP, was found to be 4.16 ± 0.18 according to spectrophotometric determination (λ = 280 nm) and use of the modified Henderson–Hasselbach equation.

Plots of the pH dependence of the DPV-peak or DC-wave potential consist of two straight lines (dE/dpH = –0.072 and –0.096 V for DPV) and their point of intersection corresponds to the dissociation constant of the oxidized form of BAP. This constant (pK_a = 4.2) is in agreement with the spectrophotometrically determined value.

To determine the number of electrons exchanged during the reduction, the height of the BAP DC-wave was compared with that of benzile (two-electron transfer) and 3-nitrobenzoic acid (four-electron transfer) under the same conditions. It is evident from the data (Fig. 4), that the BAP wave is three times higher than the benzile wave and 1.5 time higher than the 3-nitrobenzoic acid wave, suggesting a six-electron process. This conclusion was verified by controlled-potential coulometry using a stirred mercury pool cathode at the limiting current potential of the BAP DC-wave.

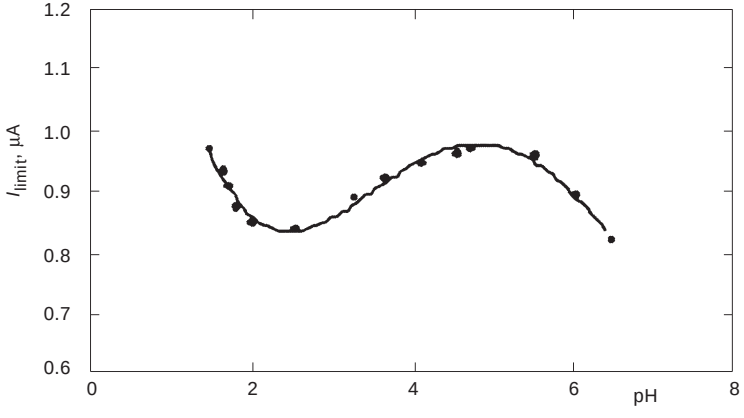


FIG. 2
Dependence of the limiting current I_{lim} of BAP (5×10^{-5} mol l^{-1}) on pH of Britton–Robinson buffers

During cytokinin reduction the N^6-side chain is eliminated, as can be seen from the MS spectrum of the hormone solution following coulometric reduction at a mercury pool electrode. A decrease in the intensity of the BAP quasi-molecular ion ($[M + H]^+ = 226$) and an increase in the intensity of the peaks corresponding with BAP degradation products was found, with an extension of the reduction time (Fig. 5). Tropilium cation ($m/z = 91$) was identified in (+)ESI spectra of coulometric reduction mixture (Fig. 5) as well

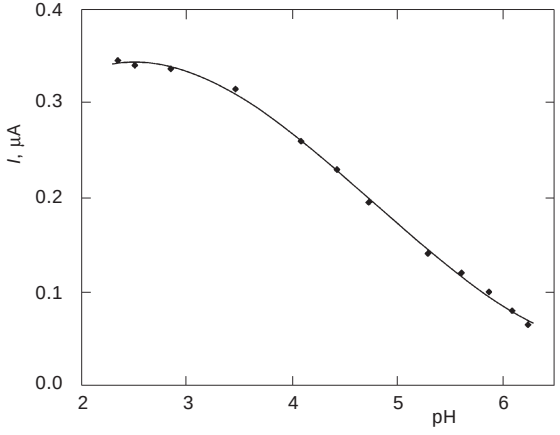


FIG. 3

Dependence of FSDPV peak current of BAP on acidity of McIlvaine buffer solution; $c_{BAP} = 5 \times 10^{-5}$ mol l^{-1}, $\upsilon = 50$ mV s^{-1}, $\Delta = -50$ mV

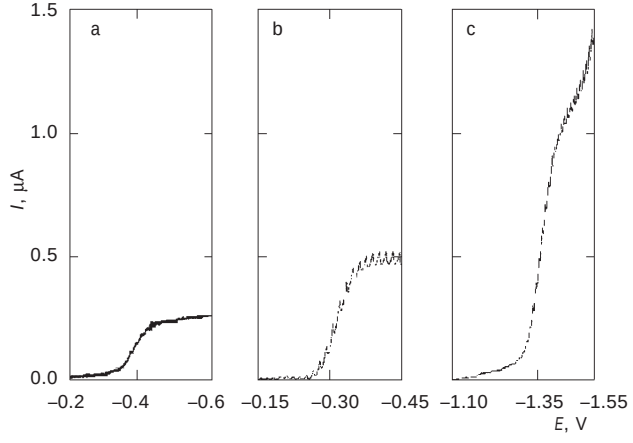
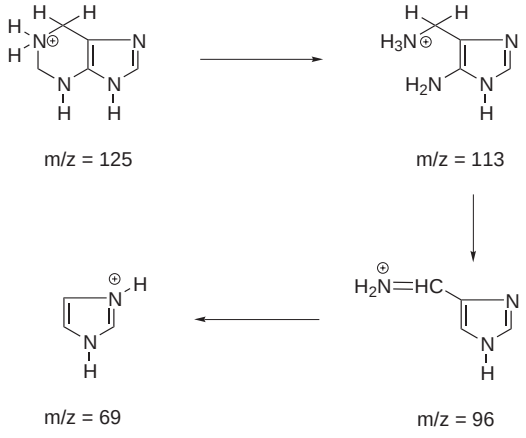


FIG. 4

DC waves of benzil (a), 3-nitrobenzoic acid (b) and BAP (c) in 0.1 M acetate buffer solution at pH 4.7 and equimolar concentration of analytes (5×10^{-5} mol l^{-1})

as major peak in the spectra of benzylamine, recorded in the same experimental conditions (data not shown). This indicates benzylamine is one of the BAP reduction products. In addition, we have detected the fragment *m/z* = 96 when scanning spectra of reduced cytokinin solutions. In the case of purine and adenine ([M + H]$^+$ = 121; 136), this fragment was also re-



SCHEME 1
Proposed scheme of electrolytic degradation pathway of BAP after coulometric analysis at mercury pool electrode at –1.4 V, studied by (+)ESI-MS in 20 mM ammonium acetate buffer solution at pH 4.7

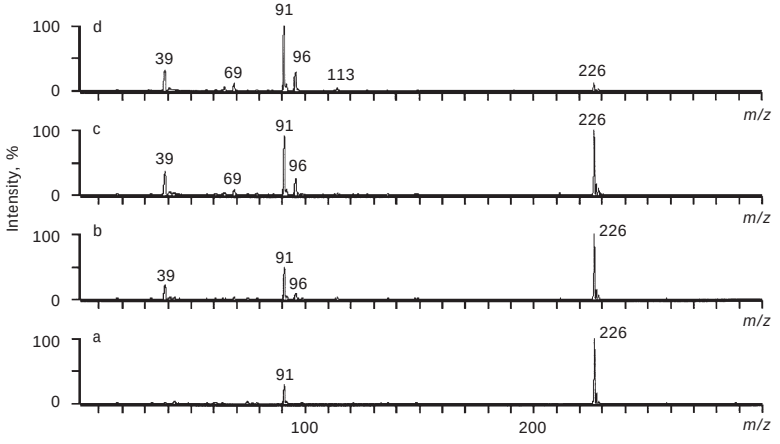
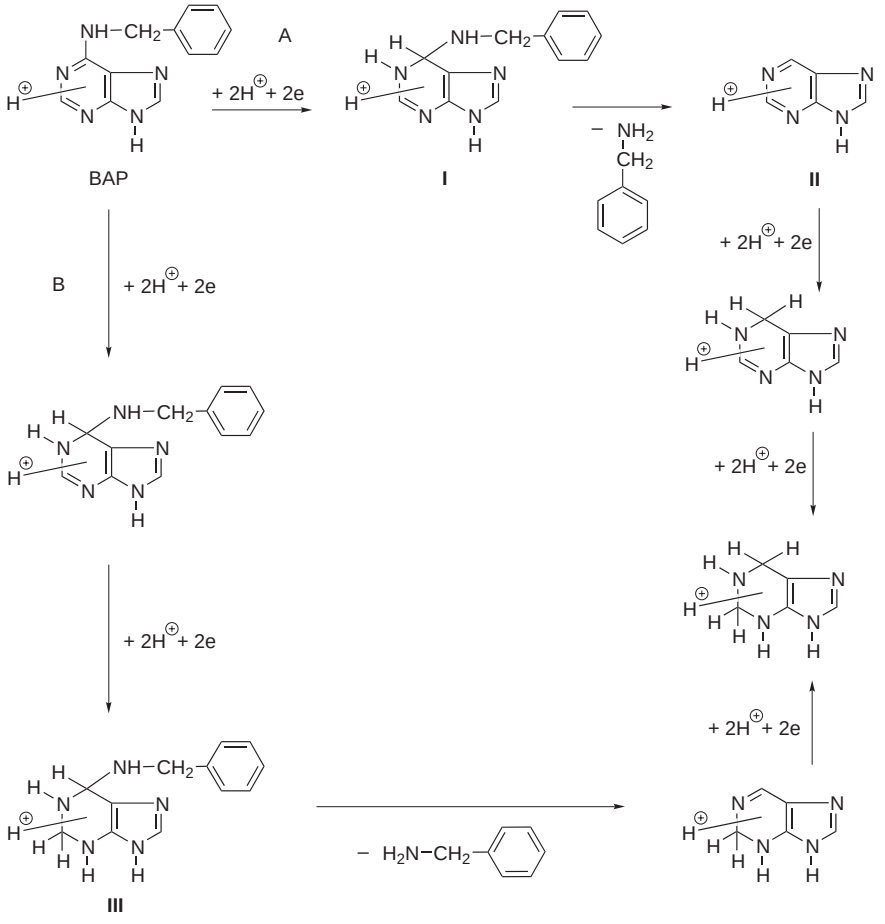


FIG. 5
(+)ESI-MS spectra of coulometrically reduced BAP recorded in 20 mM ammonium acetate at time of reduction t_{red} (in s): 0 (a), 60 (b), 180 (c) and 1800 (d)

corded (data not shown). Hence, this ion corresponds to a secondary electrolytic decomposition product of the tetrahydropurine molecule (Scheme 1) as well as ions with *m/z* values of 69 and 113.

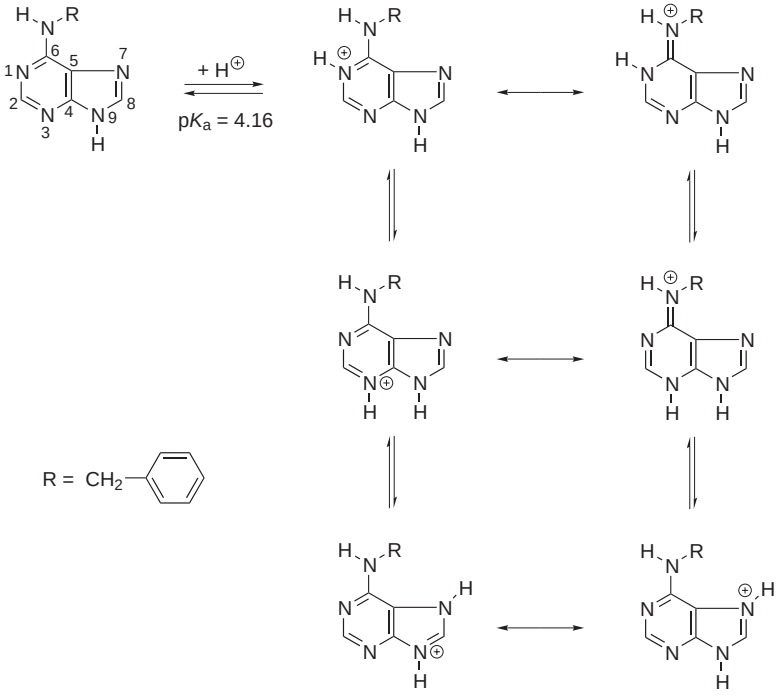
Based on the above information, the reduction mechanism of BAP can be deduced to be as follows (Scheme 2). In medium where pH < pK_a, BAP gives a well-developed cathodic wave and the distribution of species is shifted away from the protolytic towards the protonated form. At pH > 6.9, the wave disappears from the polarogram, and the content of the protonated form of BAP falls to less than 0.2%. This implies that only the protonated



SCHEME 2
Proposed scheme of electrochemical reduction of BAP at mercury electrode

form of BAP, stabilized by mesomeric and tautomeric effects (Scheme 3), is reduced at the mercury electrode surface. Therefore, the electrode reaction is initiated with the reception of an electron by the N1 nitrogen atom of the pyrimidine ring, which is the most basic position in the molecule[25,26]. Moreover, the electron density on N1 is increased due to the presence of the exocyclic N^6-secondary amine in the molecule, which is an efficient electron donor. In media where pH > pK_a the rate of the electrode process is limited by the speed of protonation. Based on the results of the electron transfer studies, it can be concluded that BAP undergoes a single six-electron reduction. This seems to involve a two-electron hydrogenation of the 1,6 double bond, two-electron reduction of the 2,3 double bond and side chain elimination at the 6-position associated with regeneration of the 1,6 double bond and finally its two-electron reduction.

In our opinion, pathway A (see Scheme 2) is the most likely, because the formation of the energetically unstable compound (**I**) is further stabilized by elimination of the N^6-side chain. No second DC-wave attributable to the



SCHEME 3
Mesomeric and tautomeric stabilization of the protonated form of BAP

alternative elimination pathway (B) was observed during the reduction. The irreversible electroreduction mechanism of BAP is consistent with the functional dependence of $E_{1/2}$ or E_p on pH, as defined by Eq. (*1*)

$$E_{1/2} = E^f - p\frac{0.0592}{\alpha z}\,\mathrm{pH}, \qquad (1)$$

where p is the number of hydrogen ions involved in the electrode process per molecule of cytokinin. The value of E^f (E^f = 0 V *vs* the silver–silver chloride electrode) was found from the plot as the point of intersection (pH 0). The product αz was calculated from the DC-waves recorded in the pH region between 2 and 7 at 25 °C on the basis of Eq. (*2*) - see Table II.

$$E_{1/2} - E = \frac{0.0592}{\alpha z}\log\frac{2x(3-x)}{5(1-x)}, \qquad (2)$$

where x is the ratio of the current at a given potential, E, and the limiting current[24]. Straight line slopes of the dependence $E_{1/2}$ or $E_p = f(\mathrm{pH})$ correspond to slopes found for particular lines according to Eq. (*3*)

$$\mathrm{d}(E_{1/2}) / \mathrm{d}(\mathrm{pH}) = -0.0592\,p / \alpha z. \qquad (3)$$

Cyclic voltammograms of the tested compound, BAP, recorded in the potential region where DC-waves appear, exhibited a single peak in the cathodic direction, while no peaks were observed in the anodic direction. The log I_p dependence on log scan rate was linear, with slopes of around 0.6 (0.58 at pH 2.6; 0.66 at pH 4.5), for polarization up to 1400 mV s^{-1}. This indicates that adsorption processes participate in the electrode reaction (15.9% at pH 2.6). Stronger adsorption of BAP molecules on the mercury electrode was observed in solutions containing more of the BAP in its deprotonated form (31.5% at pH 4.5). Unfortunately, the electrode response is not strong enough for analytical purposes in this pH region. The adsorption phenomenon was also confirmed by measurement of BAP electrocapillary curves, which are deformed in the presence of 1×10^{-4} M BAP (Fig. 6). Thus, the adsorption process can be used for AdSV in acidic media, where the DPV-peaks are high and well-defined.

Determination of BAP

The preliminary studies of BAP behaviour at mercury electrodes using direct current polarography and differential pulse voltammetry showed that the pulse techniques can be used in order to determinate low amounts of BAP in multi-component systems, such as cultivation media for plants. In order to design an optimal determination procedure for FSDPV, as well as the AdSV method, the dependence of peak height on the solution pH, duration and potential of accumulation, and concentration of the depolariser, were carefully studied.

The influence of the pH of the supporting electrolyte on the strength of the analytical signal was studied mainly for the AdSV method, since it had already been found that the highest FSDPV-peaks of BAP occur in acidic media with pH < 2.5. The highest current response of the phytohormone was found in 0.05 M citric acid (pH 2.2; E_p = –1.07 V *vs* Ag/AgCl/KCl), so this solution was used in further experiments as the supporting electrolyte for FSDPV determination of BAP.

The dependence of the electrode response on the pH of the supporting electrolyte was investigated for the adsorptive stripping technique in the following solutions: Britton–Robinson, acetate and McIlvaine buffers, 0.1 M HCl and 0.05 M citric acid. A single DPV-reduction peak was obtained at all tested pH levels. The strongest current response induced by BAP was

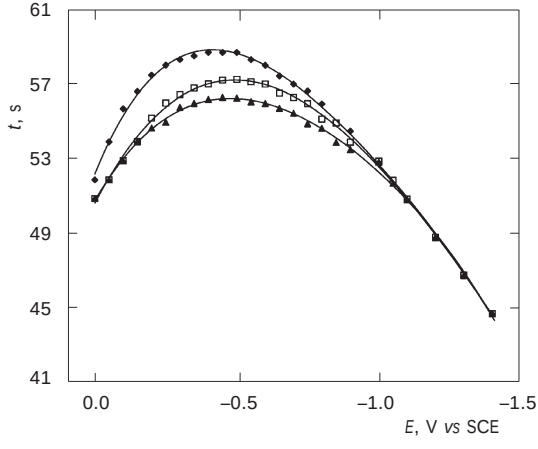


FIG. 6

Electrocapillary curves of BAP obtained in McIlvaine buffer (pH 4.5) + deionized H_2O (▲), McIlvaine buffer (pH 4.5) + deionized H_2O + 10% (v/v) methanol (□) and McIlvaine buffer (pH 4.5) + deionized H_2O + 1×10^{-4} M BAP in methanol (◆)

recorded at pH 2.3 in McIlvaine buffer (scan rate 20 mV s^{-1}, t_{ac} = 40 s and E_{ac} = –0.6 V *vs* Ag/AgCl/KCl).

The adsorptive accumulation was investigated using a range of BAP concentrations in McIlvaine buffer at pH 2.3 and by varying accumulation times at a constant potential, E_{ac}, of –0.6 V (maximum potential of electrocapillary curve, see Fig. 6) with stirring. The stripping step was performed using FSDPV. For short periods of accumulation, there is a considerable increase in the peak current and the concentration dependence is linear. For longer accumulation times the current peak decreases, reflecting saturation of the electrode surface. At a BAP concentration of 570 ng ml^{-1} in the voltammetric cell, the electrode becomes saturated at an accumulation time of around 40 s. For BAP concentrations ranging from 4 to 300 ng ml^{-1}, the current intensity is in agreement with the following equation (t_{ac} = 40 s, E_{ac} = –0.6 V *vs* Ag/AgCl/KCl):

$$I_p = 1.0995 + 0.5551 c_{BAP}, \quad r = 0.9998, \qquad (4)$$

where the peak current is in nA and the concentration in ng ml^{-1}.

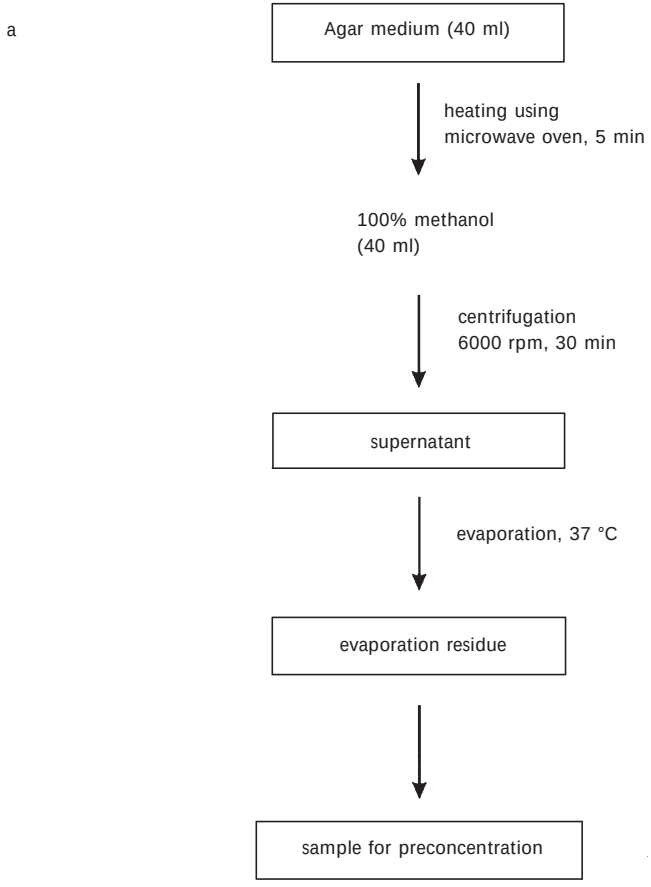
The BAP concentration of the 10.0 ng ml^{-1} sample prepared by diluting the standard stock solution was determined by AdSV, under the optimised conditions described above, to be 10.914 ng ml^{-1} (mean of five parallel determinations; relative error of average (ε), 1.20%; relative standard deviation (s_r), 1.2%). The determination limit, determined from the experimental measurements by successively reducing the concentration, was 4.51 ng ml^{-1} for this relative error. The limit of detection (L.D. = 0.80 ng ml^{-1}) was calculated from the equation L.D. = $3s_a/b$, where s_a and b are parameters of the linear regression calibration line (c_{BAP} = 4–300 ng ml^{-1}).

For determination of the BAP standard solution by the FSDPV method (50 mV s^{-1}) in 0.05 M citric acid (the most suitable supporting electrolyte tested), we obtained a determination limit of 7.88 ng ml^{-1} (measured in the concentration range 11.3–28.7 ng ml^{-1}; relative standard deviation, 4.5%) experimentally by successive reduction of the BAP concentration.

Application of the Methods

Both methods, FSDPV and AdSV, were used to determine BAP in real samples, *i.e.* in media used in the *in vitro* cultivation of plants.

The FSDPV method was tested for the determination of BAP in solid nutrition medium used for growing young *Gerbera* plants *in vitro*. This medium contained 40 mg l^{-1} of adenine sulfate, which was found to be an interfering agent. Therefore, it was necessary to preconcentrate the BAP before quantifying it. BAP was isolated by ion-exchange chromatography and immunoaffinity chromatography (based on the specific interaction of 6-substituted purines and immobilised anti-cytokinin antibodies) according to Scheme 4. The preconcentration process was found empirically to give

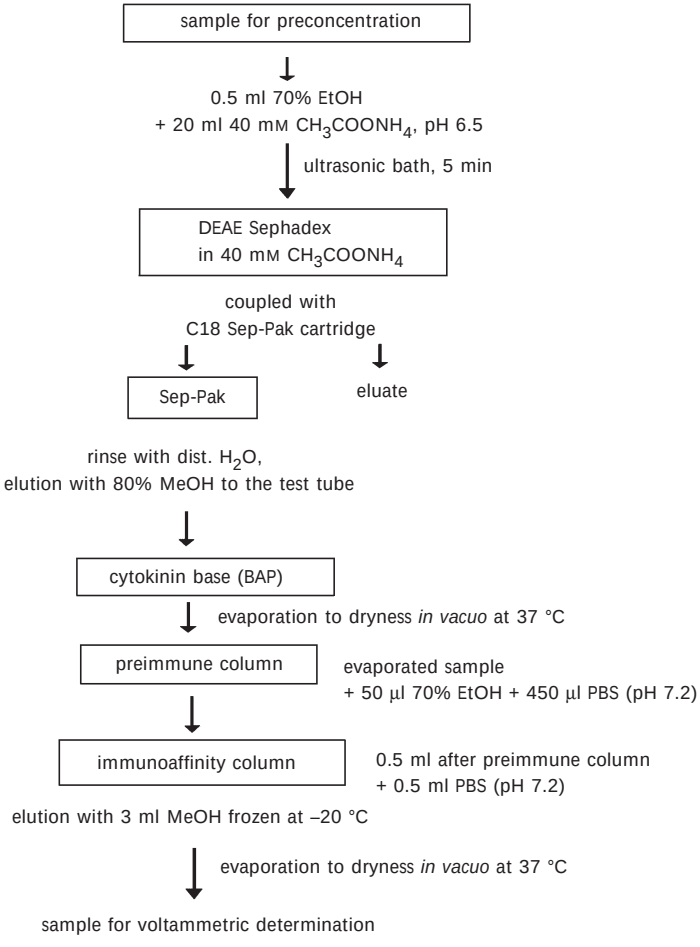


SCHEME 4
The first stage of BAP preconcentration procedure (a) from cultivation medium for *Gerbera* plants: preparation of sample before use of chromatographic techniques; the second stage of BAP preconcentration (b), use of ion-exchange chromatography (DEAE Sephadex) and immunoaffinity chromatography before BAP determination by FSDPV

an overall recovery of 92%. After evaporation of the immunoaffinity column eluate containing only BAP, the sample was dissolved in 1 ml methanol with 9 ml of 0.05 M citric acid and determined by use of FSDPV. The results of the BAP determinations in medium samples, quantified by the calibration curve method, are given in Table III.

The adsorptive stripping method thus developed was successfully applied to the determination of BAP in liquid media being used for the *in vitro* cultivation of banana plants. This medium did not contain adenine, so BAP could be analysed directly without prior medium purification. Metal cat-

b



SCHEME 4
(*Continued*)

ions (*e.g.* Zn^{2+} and Co^{2+}) were chelated with EDTA to prevent them from interfering with the measurements. The liquid cultivation medium (100 μl) was added to 10 ml of the supporting electrolyte (McIlvaine buffer solution at pH 2.3). The obtained peak of BAP was quantified by the standard addition method. The results of BAP determination in banana cultivation medium are shown in Table IV.

TABLE II
Values of αn and number of exchanged protons p calculated from DC-waves of BAP

pH	αn	α	p
1.6–4.2	1.28±0.08	0.22±0.04	1.52±0.07
4.2–6.9	1.05±0.08	0.18±0.01	1.94±0.14

TABLE III
Determination of BAP in real samples of previously preconcentrated media for *Gerbera* cultivation (before use) by the calibration curve method

Sample	Number of measurements	Mean content mg l^{-1}	Relative standard deviation s_r, %	Recovery %
1	4	3.7265	1.97	93.16
2	4	3.5910	1.16	89.78

TABLE IV
Determination of BAP in real samples of banana liquid cultivation media

Plant No./days of cultivation	Number of measurements	Mean content mg ml^{-1}	Relative standard deviation s_r, %
0[a]	3	2.1623	0.92
1/1	3	1.8734	0.53
2/1	3	1.4754	0.97
1/4	3	1.2106	0.68
2/4	3	1.0270	0.83
1/5	3	0.9343	1.07
2/5	3	0.6133	1.63
1/8	3	0.5238	1.91
2/8	3	0.3630	2.75

[a] Before cultivation.

CONCLUSIONS

The electrochemical behaviour of 6-benzylaminopurine (BAP) at a mercury electrode has been investigated. The electrode reaction of this compound is irreversible and proceeds only with the protonated form of the molecule. MS analysis of the products of coulometric reduction at the mercury pool electrode confirmed that a six-electron reduction process, accompanied by elimination of the side chain, is involved. The FSDPV method was used to determine BAP in solid media for the *in vitro* cultivation of young *Gerbera* plants after preconcentration.

It was also found that the adsorption process at the HMDE provides an effective preconcentration step for determination of BAP at low concentrations in samples of adenine-free liquid nutrition media (without any prior purification). Thus, the AdSV procedure may be a convenient method for rapidly, and directly, monitoring BAP in medium in which bananas are being cultivated and, thus, its uptake by the plants. This may be a very useful approach for controlling of plant growth times and medium consumption in biotechnological laboratories.

The authors gratefully acknowledge financial support from the Grant Agency of the Czech Republic (grants No. 203/99/1511 and No. 522/01/0275) and from the Ministry of Education, Youth and Sports of the Czech Republic (No. VS 96021 and No. MSM 153100013). The authors wish to thank Mr J. Holub from the Biolab Company (Olomouc, Czech Republic) for supplying cultivation media, valuable comments and information. The authors appreciate Dr J. Blackwell for his careful and critical editing of this manuscript.

REFERENCES

1. Takahashi N.: *Chemistry of Plant Hormones*. CRC Press, Boca Raton 1986.
2. Gallo R. C., Whang-Peng J., Perry S.: *Science* **1969**, *165*, 400.
3. Suk D., Simpson C. L., Mihich E.: *Cancer Res.* **1970**, *30*, 1429.
4. Havlíček L., Hanuš J., Leclerc S., Meijer L., Shaw G., Strnad M.: *J. Med. Chem.* **1997**, *40*, 408.
5. Hajdúch M., Kolář Z., Novotný R., Hanuš J., Mihál V., Hlobílková A., Nosková V., Strnad M.: *Anti-Cancer Drugs* **1997**, *8*, 1007.
6. Kovářová H., Hajdúch M., Kořínková G., Halada P., Krupičková S., Gouldsworthy A., Zhelev N., Strnad M.: *Electrophoresis* **2000**, *21*, 3757.
7. Otyepka M., Kryštof V., Havlíček L., Siglerová V., Strnad M., Koča J.: *J. Med. Chem.* **2000**, *43*, 2506.
8. Nandi S. K., Letham D. S., Palni L. M. S., Wong O. C., Summons R. E.: *Plant Sci. (Shannon, Irel.)* **1989**, *61*, 189.
9. Nandi S. K., Palni L. M. S., Letham D. S., Wong O. C.: *Plant Cell Environ.* **1989**, *12*, 273.
10. Bennet-Clark T. A., Tambiah M. S., Keford N. P.: *Nature* **1953**, *17*, 1645.

11. Law D. M., Hamilton R. H.: *Biochem. Biophys. Res. Commun.* **1982**, *106*, 1035.
12. Renu Agra C. L., Mandelaer-Gulati A.: *Mycopathologia* **1990**, *109*, 177.
13. Sjut V., Palmer M. V.: *J. Chromatogr.* **1983**, *270*, 309.
14. Hocard C. H., Wong O. C., Letham D. S., Tay S. A. P., MacLeod J. K.: *Anal. Biochem.* **1986**, *15*, 385.
15. Brenner M. L.: *Ann. Rev. Plant Physiol.* **1981**, *32*, 511.
16. Strnad M., Vaněk T., Binarová P., Kamínek M., Hanuš J.: *Molecular Aspects of Hormonal Regulation of Plant Development*, p. 41. SPB Academic Publishers, The Hague 1990.
17. Strnad M., Vereš K., Hanuš J., Sieglerová V.: *Physiology and Biochemistry of Cytokinins in Plants*, p. 437. SPB Academic Publishers, The Hague 1992.
18. Hernández L., Hernández P., Rica M., Galán F.: *Anal. Chim. Acta* **1995**, *315*, 33.
19. Hernández P., Paton F., Ballesteros Y., Hernández L.: *Electroanalysis (N. Y.)* **1997**, *9*, 235.
20. Hernández P., Paton F., Hernández L.: *Electroanalysis (N. Y.)* **1997**, *9*, 1372.
21. Blanco M. H., Quintana M. D., Hernández L.: *Fresenius' J. Anal. Chem.* **1999**, *364*, 254.
22. Blanco M. H., Quintana M. D., Hernández L.: *Electroanalysis (N. Y.)* **2000**, *12*, 147.
23. Smith D. L., Elving P. J.: *J. Am. Soc.* **1962**, *84*, 1412.
24. Smith D. L., Elving P. J.: *J. Am. Soc.* **1969**, *116*, 1087.
25. Raszka M.: *Biochemistry* **1974**, *13*, 4616.
26. Buchner P., Maurer W., Ruterjans H.: *J. Magn. Reson.* **1978**, *29*, 45.