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A STUDY ON THE APPLICABILITY OF CARBON PASTE ELECTRODES TO THE DETERMINATION OF 6-BENZYLAMINOPURINE

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Electrochemical behaviour of 6-benzylaminopurine at a carbon paste electrode has been studied. The substance of interest was investigated with respect to its oxidation, reduction, and accumulation capabilities at chemically and electrolytically pretreated carbon paste surface. By evaluating the results and observations obtained, the possibilities and limitations in applicability of carbon paste-based electrodes to the determination of 6-benzylaminopurine are outlined.

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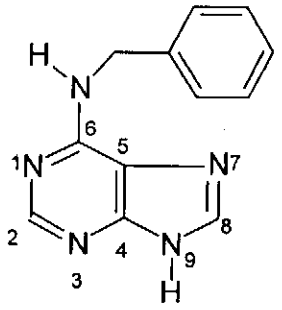
Introduction

6-Benzylaminopurine (BAP, "benzyladenine") is a naturally occurring plant hormone belonging to the group of the so-called cytokinins [1]. These substances play an important regulatory role in the plant growth development and in its individual stages such as the cell division, root and bud, or flower and fruit formation. Some plant hormones can regress certain types of mammal tumours [2,3] and, therefore, these derivatives are intensively studied for their potential applicability as anti-cancer medicaments [4–7]. At present, BAP as well as other cytokinins are applied in modern biotechnology as substances inducing cell division in media for *in vitro* cultivation of some plants that are important in agriculture or decorative greenery. Available biotechnological techniques for the transfer of genes can significantly shorten the breeding procedures and overcome some of the agronomic and environmental problems hard to solve with the use of conventional methods.

The structure of all cytokinins can be derived from adenine (6-aminopurine) by means of the substitution in amino group at the position "6" (see formula below), providing two fundamental classification groups of derivatives with either isoprenoid or aromatic side chain [1]. In the molecule of BAP, the adenine base

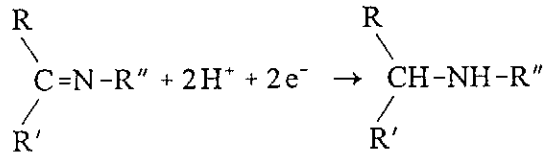


bears the benzyl group. Due to the presence of the secondary amino group, the compound exhibits a weakly basic character (pK_{a1} = 4.2 [1,8]; pK_{a2} = 9.8 [1]) and can thus be protonated. The lipophilic aromatic ring in the molecule then results in its hydrophobic character and hence in water insolubility.

In order to identify and quantify cytokinin derivatives in real samples at the endogenous levels, one has to select analytical techniques with a very good performance and operating at extremely low concentrations. Such requirements can best be fulfilled by various sophisticated methods based on HPLC combined with MS detection (e.g. [9–11]). Sometimes, effective are also some procedures employing GC-MS instrumentation [12,13]. Other approaches utilise highly

sensitive immunomethods such as enzyme-linked immunosorbent assay (ELISA [14] or radioimmunoassay (RIA [15] that, when being coupled again with HPLC [14,16], may offer reliable methods for practical analyses.

From electrochemical point of view, purine and its substituted derivatives represent substances with typically electroactive behaviour. Their electrochemical reduction has been studied at mercury electrodes in acidic media where protonated purines undergo a single irreversible six-electron process at a potential of the second purine DC-wave accompanied by elimination of the benzyl side chain [17–19]. The pathway is relatively complex and involves several reaction sequences with the reduction of the azomethine group



(1)

In the case of 6-substituted analogues like BAP, it is supposed that the whole reduction proceeds in the pyrimidine ring. First, the "$C^6=N^1$" double bond is reduced and this process is followed by reduction at the "$C^2=N^3$" bond while a side-chain at the $C^6=N^1H-$ position is eliminated. Instantaneous recombination of the "$C^6=N^1$" bond and its subsequent reduction *via* a two-electron hydrogenation then complete the whole mechanism [18,19]. Also the electrochemical oxidation of purine derivatives has been investigated in detail (e.g. [20]). Herein, it can be stated that anodic oxidation of purines at graphite electrodes is usually accompanied by a marked adsorption of reaction products at the electrode surface, which can be exploited in some determinations [21].

For analytical purposes, however, the above-described reduction process has been shown more convenient and prevails as the principle of various methods for the determination of BAP and related compounds. The corresponding procedures are usually performed by means of electrochemical stripping analysis at mercury electrodes [22] or with carbon fibre micro- and ultramicroelectrodes [23–25]. It is worth mentioning that these miniature electrode constructions offer — besides sufficient performance and sensitivity — some interesting applications in non-destructive measurements *in vivo* that represent a very useful tool for direct monitoring of the plant hormones in living organisms [25].

One of the alternative electrode materials for designing such sensors would also be a carbon paste which, as a mixture of spectroscopic graphite with a suitable liquid binder, may offer some valuable electrochemical properties [26,27]. As shown in previous studies falling into the area of brain electrochemistry [28], fine

carbon paste mixtures can be packed in special electrode tips of micrometric dimensions and, as tiny electrode cells, then implanted into tissues for measurements *in vivo* [29,30]. However, prior to operability under such unusual arrangement, it is necessary to carry out the basic characterisation of the electrode itself to check its response to the target substance [28].

In this article, the suitability of a carbon paste as electrode material for electrochemical detection and determination of BAP has been tested. To the authors' knowledge, the resultant study represents the first attempt to perform the basic electrochemical characterisation of this compound at a carbon paste electrode (CPE). However, regarding purines as such, some reports concerning their investigation at CPEs can be traced up in the literature. For instance, electrolytically and chemically pre-oxidised carbon paste surface has recently been demonstrated as an excellent substrate for accumulating adenine and its subsequent cathodic detection [31].

Experimental

Apparatus

A polarographic analyzer (Model PAR 174, Princeton Applied Research, U.S.A.) was interfaced to a personal computer *via* a card for A/D conversion of the data (PC ADDA-14, Model FPC-011; Flytech Technology, U.S.A.). This set-up controlled *via* an "ADDA-174 A" software was combined with an SMDE electrode stand (Laboratorní přístroje Praha, Czech Republic) adapted for measurements with CPEs.

A laboratory-made platinum plate and a Ag/AgCl electrode (inner electrolyte: 1 M KCl) served as auxiliary electrode or as the reference, respectively. In accumulation experiments, stirring was performed with a Teflon®-coated magnetic bar at approx. 600 rpm. The pH values were measured using a digital pH meter (Model 420A; Orion, U.S.A.) equipped with a combined glass pH sensor (Model OP-0808P, Radelkis, Hungary).

Working Electrode

According to the previously recommended procedure and denotation [32], a silicone oil-based carbon paste, "C/SO", was prepared from 0.5 g spectroscopic graphite powder (RW-B, Ringsdorff Werke, Germany) and 0.2 ml silicone oil (Lukooil MV-15500, Lučební závody Kolín, Czech Republic). The manually homogenised paste was then packed into electrode holders equipped with a piston and the surface was renewed by smoothing with a wet filter paper.

Chemicals and Reagents

All chemicals used were of analytical reagent grade and purchased from Lachema (Czech Republic) except the substance studied, 6-benzylaminopurine (BAP), which was obtained from Sigma Aldrich. Test solution of BAP (0.001 mol l^{-1}) was prepared by dissolving an appropriate amount of solid compound in methanol. Stock solutions of the supporting electrolytes were made 1 M in concentration if not stated otherwise. Two stock solutions of Britton–Robinson buffer were prepared in a usual way [33] and contained: 1.24 g H_3BO_3 + 1.15 ml 99% CH_3COOH + 1.35 ml 85% H_3PO_4 in 500 ml (solution "A") and 4.0 g NaOH in 500 ml (solution "B"). Mixing of both solutions at the recommended ratios and measurement of the corresponding pH values were made always before use. For some experiments, also other solutions and reagents were used; their specification is given correspondingly in the text.

With respect to the fact that the whole study had not been quantitative in nature, minor variations in concentration of the individual solutions were neglected. Prior to measurement, each supporting medium used was purged with argon gas (purity 99.996%, Linde Technoplyn) for approx. 5 min. in order to remove air oxygen dissolved in the solution.

The water used throughout for the preparation of the solutions was obtained by passing deionised water through a laboratory-made distillation unit.

Procedures

Direct Voltammetry: Voltammetric curves were recorded in the supporting electrolyte from the initial potential, E_{INIT}, towards the final potential, E_{FIN}, in the differential pulse (DP) mode. The scan rate was 20 mV s^{-1}, the pulse height +/−50 mV (according to the scan direction), and the sampling rate was 5 data points per second. The individual parameters are again specified below.

Voltammetry with Accumulation Step (Stripping Voltammetry): The accumulation was performed in stirred solution (supporting electrolyte) at selected accumulation potential, E_{ACC}, for a chosen period, t_{ACC}. After equilibration, t_{EQ}, (in quiet solution), the electrode was stripped from the E_{INIT} (always being identical with E_{ACC}) to the E_{FIN} in the manner and under experimental conditions given in the previous paragraph.

Electrolytic Pretreatment of Working Electrode [34]: The C/SO CPE was either anodised or cathodised by exposing their freshly renewed surface to a potential of +1.5 V (or − 1.5 V) vs Ag/AgCl for 60 s and, subsequently, to − 1.0 V (or +1.0 V) for 15 s in "activation solution" (0.01M phosphate buffer + 0.1 M KNO_3 + 0.01

M HNO_3, pH 7.0 [35]). After rinsing the activated surface with water and after exchange of medium, C/SO pretreated in this way could be used immediately for voltammetric measurements.

Results and Discussion

Choice of Carbon Paste and Electrode Arrangement

A carbon paste containing silicone oil ("C/SO" type) was chosen as standard carbon paste electrode with chemically inert binder [26]. In comparison with electrochemically very similar and widely used paraffin oil-containing carbon pastes [27,32], the C/SO CPE was selected due to its better stability against methanol [36] used as a solvent for preparing a stock standard solution of BAP. Throughout the work, the carbon paste was fixed in an electrode holder of typical macroelectrode construction (with surface diameter of 2 mm).

Selection of Supporting Media and of Model Concentration of 6-Benzyl-aminopurine

The supporting electrolytes and buffered media were chosen according to previous experience [32]. As a medium serving for measurements at the controlled pH, Britton–Robinson buffer was selected. In all cases, the effect of methanol upon the response of the C/SO CPE was checked by comparing the electrode base-line in blank solutions (i.e., without the substance tested) containing 5, 10, 25, and 50% CH_3OH. It was found that the C/SO CPE operated best in media with max. 10% methanol. This relatively low content (finally chosen as 7.5 % CH_3OH) then co-determined the concentration level of the substance in the supporting media for testing measurements. A concentration of 5×10^{-5} mol l^{-1} BAP was found optimal for voltammetric detection and, at the same, still sufficient to keep the compound fully soluble.

Oxidation of 6-Benzylaminopurine at a Carbon Paste Electrode

Table I surveys all important experimental data and measurements characterising this study. A relatively wide spectrum of buffering media used allowed one to obtain a representative insight at the oxidation of BAP in dependence on pH. This can also be illustrated in Fig.1 depicting the individual voltammograms obtained by oxidising BAP at the C/SO CPE in Britton–Robinson buffers with various pH. As indicated by the E_p values, a nearly linear relation was obtained between the

Table I Anodic oxidation of 6-benzylaminopurine[a] at the C/SO carbon paste electrode. Survey of experimental data

Medium[b] (supporting electrolyte)	pH	Voltammetry with the C/SO CPE		Notes
		potential range [d,e], V	E_p(BAP) [e,f], V	
0.10 M HCl	1.30	−0.7 → +1.1	-	A
0.05 M citrate buffer	4.82	−0.8 → +1.0	+0.95	B, C
0.50 M acetate buffer	4.87	−0.8 → +1.1	+0.98	D
0.05 M borate buffer	6.61	−0.9 → +1.3	+0.87	B, E
0.05 M phosphate buffer	7.22	−0.8 → +1.2	+0.82	F
	2.27	−0.8 → +1.4	+1.18	B, G
	3.51	−0.8 → +1.4	+1.07	B, G
	4.25	−0.8 → +1.4	+1.05	B, G
	5.06	−0.8 → +1.3	+0.95	G
	6.00	−0.8 → +1.3	+0.91	G
Britton–Robinson buffer (B–R B)	7.46	−0.8 → +1.3	+0.82	G
	7.97	−0.8 → +1.2	+0.80	G
	8.94	−0.8 → +1.1	+0.79	G
	9.65	−0.8 → +1.1	+0.70	G
	10.88	−0.8 → +1.0	+0.57	G
	11.95	−0.8 → +0.9	+0.62	B, G
	12.89	−0.8 → +0.7	+0.51	B, G

a) c(BAP) = 5×10^{-5} mol l^{-1}; b) 18 ml medium + 1.5 ml CH_3OH; c) experimental value; d) evaluated as a range between anodic and cathodic potential limits where the corresponding currents exceeded an arbitrary value estimated from the mean background currents observed within this interval (for details, see Ref. [32]; e) measured against Ag/AgCl, f) computed by "ADDA-174A" software; A) peak not obtained; B) deformed peak; C) citrate buffer: 0.1 M $C_6H_8O_7$ + 0.1 M $C_6H_5O_7Na_3$, diluted 1 : 2; D) acetate buffer: 1 M CH_3COOH + 1 M CH_3COONa, diluted 1 : 10; E) borate buffer: 0.05 M $Na_2B_4O_7$ + 0.1 M HCl; F) phosphate buffer: 0.1 M KH_2PO_4 + 0.1 M Na_2HPO_4 + 0.1 M NaOH, diluted 1 : 2; G) B–R B, for composition see Experimental and Ref. [33])

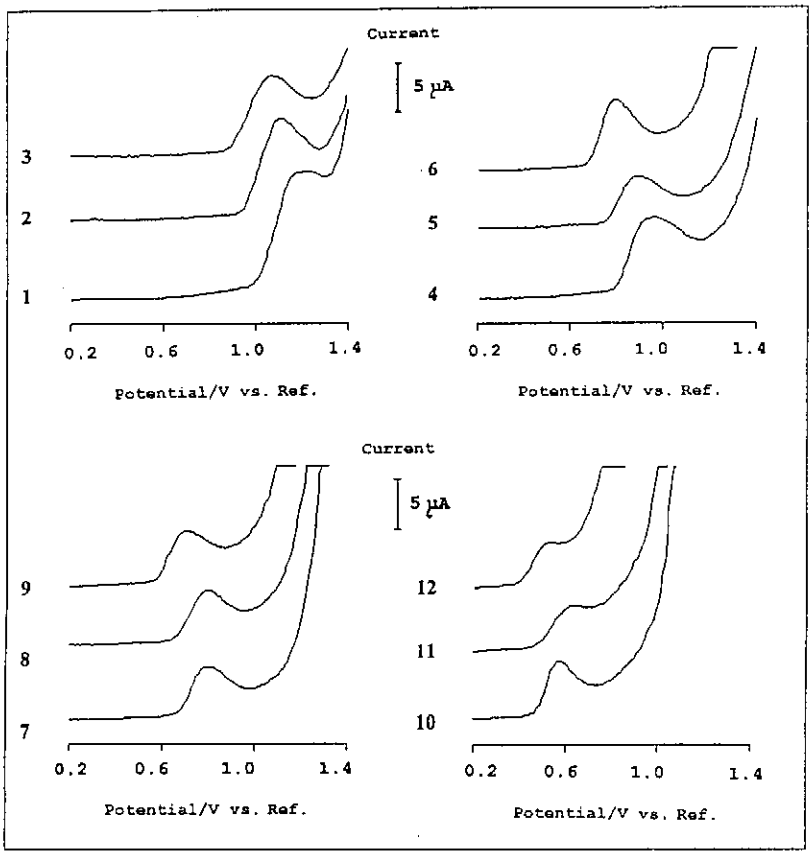


Fig. 1 Oxidation of 6-benzylaminopurine at C/SO carbon paste electrode in buffered media: 1 – Britton–Robinson buffer, pH 2.27; 2 – 3.51; 3 – 4.25; 4 – 5.06; 5 – 6.00; 6 – 7.46; 7 – 7.97; 8 – 8.94; 9 – 9.65; 10 – 10.88; 11 – 11.95; 12 – 12.89. Experimental conditions: C/SO carbon paste electrode; differential pulse voltammetry (DPV); supporting electrolyte, s.e.: 18 ml buffer (see above) + 1.5 ml MeOH; c(BAP) = 2.5×10^{-5} mol l^{-1}; initial potential, E_{IN} = −1.0 V vs Ag/AgCl; final potential, E_{FIN} = +1.5 V; scan rate, v = 20 mV s^{-1}; pulse height, ΔE = +50 mV

peak shift towards less positive potentials and the increasing pH value of the medium. In acidic solutions, the oxidation signal has always appeared as a single peak beyond +1.0 V vs Ag/AgCl, which is in accordance with previous observations made for adenine [19,31]. However, as found out, measurements in media with pH < 4 provided very deformed or even no responses for BAP due to its overlap with the background at higher positive potentials (see also Table I and Fig. 1). The observations that no other peaks were noticed over the whole potential range investigated and no new signals were registered in media with increased pH

have suggested that the oxidation mechanism of BAP is of the same character as that known for the native purine [19]. In other words, there was no evidence that the side-chain of BAP had undergone any elimination or electrochemical transformation indicated by some specific peaks.

Regarding the signal-to-noise characteristics, the most favourably developed responses of BAP were obtained in neutral media (see Fig 1). Whereas the oxidation in more alkaline solutions already causes a decrease of the signal, the peaks of interest obtained in acidic electrolytes suffered from the above-mentioned deformations. For the neutral supporting electrolyte, a phosphate-based solution was selected for further measurements as it represents a medium suitable for biological substances measured at physiological pH [1].

A Few Remarks on Using the C/SO Carbon Paste Electrode for Cathodic Reductions

As already mentioned, the reduction of BAP is generally more advantageous for analytical applications compared to its oxidation. However, one has to employ an electrode capable of operating at highly negative potentials beyond - 1.10 V vs SCE where the reduction of BAP and related compounds usually takes place [21]. In this respect, the use of a CPE is rather problematic because of limited cathodic potential range as well as due to the presence of oxygen dissolved in the paste [26,27]. During cathodic scanning at negative potentials, oxygen is always anyhow reduced, thus giving rise to undesirable signals that may overlap the peaks of interest [32,37].

Also in this study, it was confirmed that the C/SO CPE suffered from these drawbacks and hence, it was found unsuitable for reductive detection of BAP.

Accumulation Studies with the C/SO Carbon Paste Electrode

A possibility to accumulate (pre-concentrate) an analyte onto the electrode is being utilised in many analytical methods that offer high selectivity and low detection limits. Accumulative capabilities of BAP towards the C/SO were investigated with the aid of two different approaches based on alteration of the carbon paste surface states using either chemical modification *in situ* or electrolytical activation at high potentials [32,34].

Accumulation at Surfactant-Modified Carbon Paste Surface. In these experiments, BAP was tested whether — as lipophilic and relatively voluminous molecule — it is capable of forming ion-pairs with some surfactants [26,27]. Here, special attention was paid to the effect of anion-active substances offering counter-ions to be paired [38] with the cationic form of BAP (i.e., its protonated molecules).

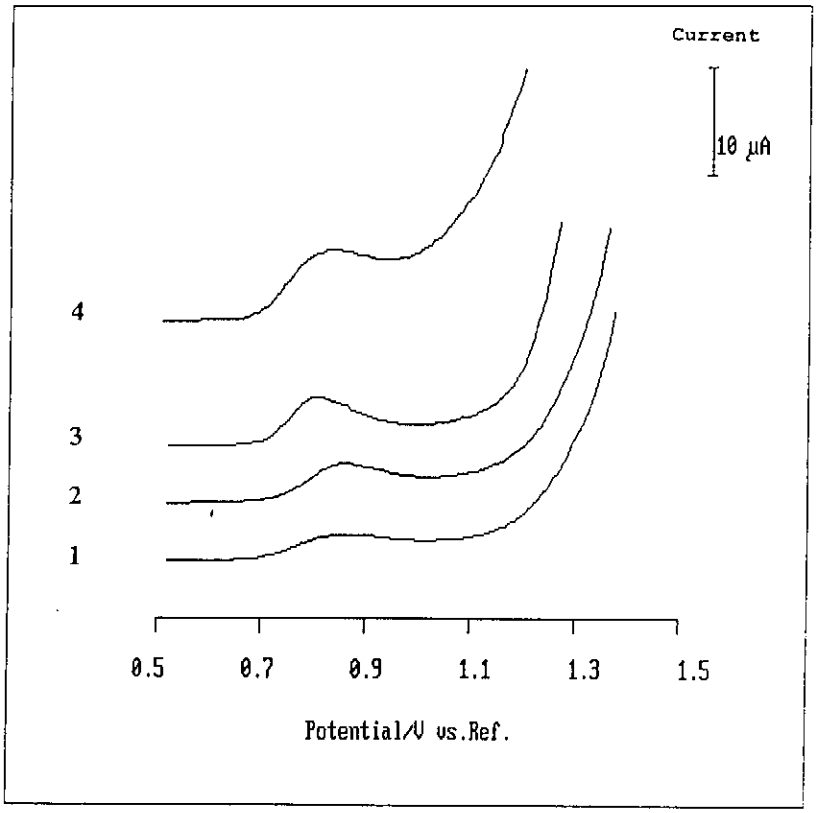


Fig. 2 Effect of the presence of a surfactant (SF) upon the response of the C/SO carbon paste electrode in weakly acidic medium: 1 – heptyl sulphonate, $C_7H_{15}-SO_3^-$; 2 – Septonex®, $R_3R'-N^+$ (1-ethoxycarbonylpentadecyltrimethylammonium bromide); 3 – Triton X-100® (t-octylphenoxy-polyethoxy ethanol); 4 – without added surfactant. Experimental conditions: DPV; s.e.: 0.5 M acetate buffer + 7.5% CH_3OH (pH 4.87); c(BAP) = 5×10^{-5} mol l^{-1}; E_{IN} = −1.0; E_{FIN} = +1.5 vs Ag/AgCl; ν = 20 mV s^{-1}; ΔE = +50 mV

Using a simple probe test, it was ascertained that model anion-active reagents, namely tetraphenyl borate, $B(C_6H_5)_4^-$, and lauryl sulphate, $C_{12}H_{25}-O-SO_3^-$, were able to precipitate BAP in a solution of ca 0.1 M HCl. It was interesting to observe that whereas the use of tetraphenyl borate led to immediate precipitation of BAP, the reaction with lauryl sulphate was markedly slower and a white insoluble product appeared after ca 10 seconds. Regardless of these kinetic aspects, both assays confirmed ion-pairing capabilities of protonated BAP.

Unfortunately, the reaction conditions of these ion-pairing processes, i.e., rather high acidity of the medium ensuring the protonation of the analyte, were in contradiction with those found convenient in voltammetric measurements. In

accordance with the results given in Table I and Fig. 1, the acidity of the supporting medium had to be lowered to min. pH 4 in order to obtain measurable voltammetric signalscorresponding to the oxidation of BAP in its cationic form. Figure 2 shows the responses of BAP obtained under such conditions with the C/SO CPE modified *in situ* with model surfactants of three fundamental types. The set of voltammograms is then completed by a curve showing the original peak of BAP in the same medium without a surfactant. Practically the same results were obtained also in neutral phosphate media (not shown).

Figure 2 documents that the modification of the C/SO CPE with anionic surfactant was ineffective and did not provide any enhancement of the response which would have indicated accumulation of oxidised BAP. In this experiment, heptyl sulphonate, $C_7H_{15}-SO_3^-$, was selected as a model anion-active compound [38] whose behaviour towards BAP was comparable to that of more lipophilic lauryl sulphate used in the probe assay. Regarding tetraphenylborate, this substance could not be used in voltammetric experiments owing to its electrochemical activity in the anodic potential range [39] where the oxidation of BAP takes place. As expected, the presence of both cation-active and non-ionic surfactants had practically no effect upon the response of interest except for a slight suppression of the background currents.

The accumulation studies proper performed in the differential pulse anodic stripping voltammetric (DPASV) mode proved that the response of BAP had remained unchanged over the whole accumulation time interval chosen (from 30 to 120 s).

Accumulation at Electrolytically Pretreated Carbon Paste. An exposition of a CPE to extremely positive or negative potentials is being reported to be another way for principal changes in the structure of the carbon paste surface [34] and its accumulation capabilities [26,27]. Such electrolytic activation has also been utilised in voltammetric detection of adenine whose distinct accumulative capabilities at the activated and chemically modified carbon paste surface have been characterised by means of the following mechanism [31]

$$CPE\ \text{surface} \xrightarrow{(\text{modifier + anodisation})} CPE\ \text{surface}_{ox} \qquad \text{... pretreatment} \qquad (2)$$

$$CPE\ \text{surface}_{ox} + ade \rightarrow ade_{ox} \rightarrow ade_{ox}^{ADS} \qquad \text{... accumulation} \qquad (3)$$

$$ade_{ox}^{ADS} \rightarrow ade_{red}^{ADS} \qquad \text{... voltammetric scan} \qquad (4)$$

where *ade* and *ADS*, *ox* or *red* abbreviate adenine and adsorptive, oxidative or reductive state, respectively.

In the study with BAP, the C/SO CPE was activated using anodisation and the subsequent cathodisation according to Baldwin [35]. When comparing the

corresponding responses given in Fig. 3, it is evident that neither anodic nor cathodic activation stimulated any accumulation of BAP and the corresponding enhancement of the signal. On the contrary, both responses are substantially deformed and much smaller than that obtained with unactivated C/SO CPE.

It can be stated that, under conditions used, the molecules of BAP exhibit a minimal willingness to be accumulated onto the C/SO electrode and this behaviour remains the same despite special modifications of the carbon paste surface. This suggests that less acidic solutions which had to be used in voltammetric measurements did not permit an effective protonation of BAP and its accumulation at the carbon paste surface *via* ion-pairs with alkyl sulphonate or sulphate. Also, the presence of methanol in the electrolytes (even when introduced

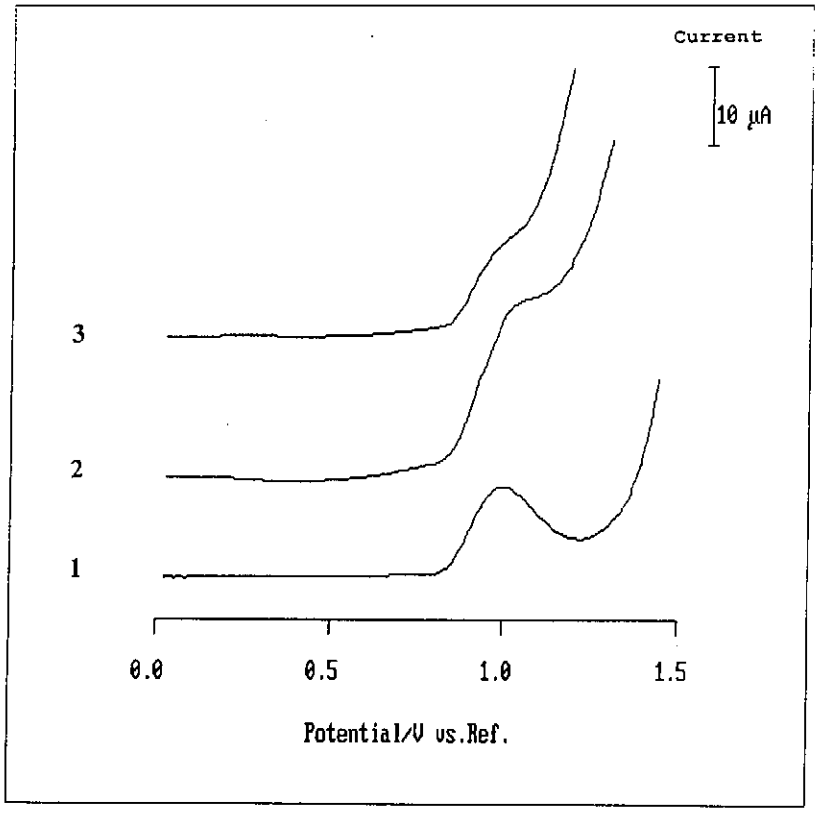


Fig. 3 Effect of electrolytic activation upon the response of the C/SO carbon paste electrode: 1 – unactivated surface; 2 – activated surface [in "activation solution", a.s. (See Experimental) at +1.5 V for 60 s and −1.0 V for 15 s]; 3 – activated surface [a.s. −1.5 V (60 s) and +0.5 V (15 s)] Experimental conditions: DPV; s.e.: 0.1 M acetate buffer + 7.5% CH_3OH (pH 4.71); c(BAP) = 5×10^{-5} mol l^{-1}; E_{IN} = 0; E_{FIN} = +1.5 V; ν = 20 mV s^{-1}; ΔE = +50 mV

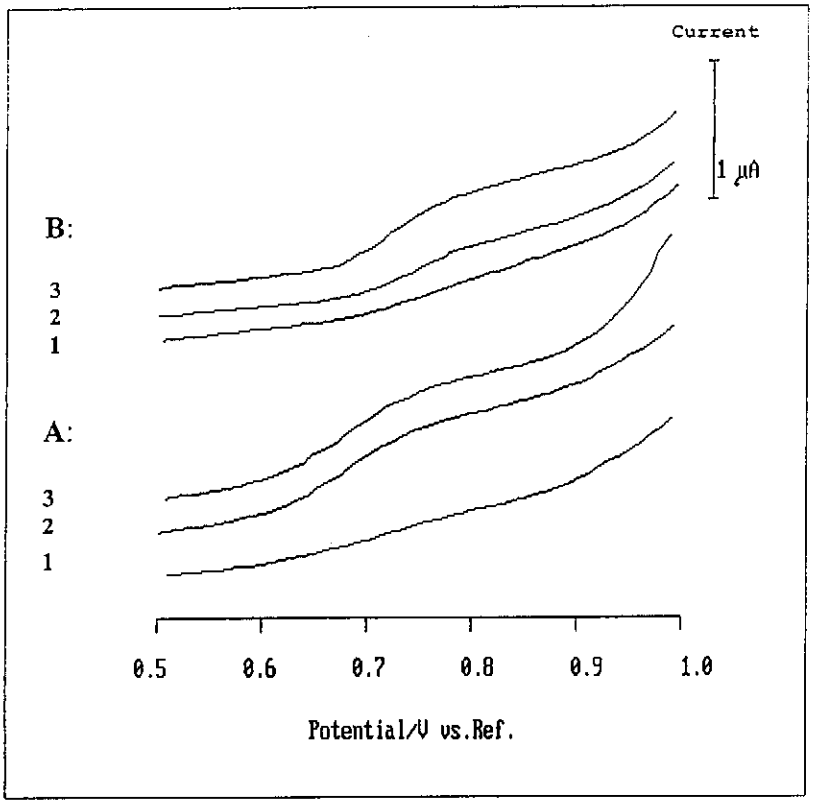


Fig. 4 Detection of 6-benzylaminopurine at a carbon paste electrode in acidic and neutral media: A – 0.1 M acetate buffer + 7.5% CH_3OH (pH 4.71); B – 0.1 M phosphate buffer + 7.5% CH_3OH (pH 7.22). 1 – base-line; 2 – 1×10^{-6} mol l^{-1} BAP; 3 – 2.5×10^{-6} mol l^{-1} BAP. Experimental conditions: DPV; C/SO, E_{IN} = 0; E_{FIN} = +1.5 V; v = 20 mV s^{-1}; ΔE = +50 mV

in the form of small amount as the stock solution of the compound) could play a certain negative role.

Finally, accumulation unwillingness of BAP can be interpreted — when considering markedly adsorptive behaviour of the native adenine at similarly pretreated CPE [31] — by a steric effect of voluminous benzyl group in the molecule. At CPEs, such structural hindrances may complicate the accumulation of even highly lipophilic substances [36].

In consequence of impossibility to utilise accumulation onto the carbon paste surface as well as sensitive cathodic reduction of the adenine ring [21], CPEs have been found in this study not very promising for monitoring of the endogenous levels of BAP. In such experiments, the concentration of this substance is typically at the low nanomolar or even sub-nanomolar level [1,21,25] whereas, as seen in Fig. 4, direct voltammetry with the C/SO in the DPV mode could detect only about 1 μM BAP.

Nevertheless, it seems that even this relatively high detection limit would still be sufficient in some measurements *in vitro* designed as time-monitoring of hormone consumption by a plant in model cultivation solutions [1]. Among others, similar simulations may considerably reduce the expenses associated with the use of commercially marketed cultivation media [19].

In perspective, there is still a possibility to test some other approaches for obtaining carbon paste electrodes with a better analytical performance. For example, some special modifiers admixed in the carbon paste can favourably influence the kinetics of the oxidation of organic compound at the carbon paste surface, which is reflected in significantly improved signal-to-noise characteristics [32]. Such a modified CPE is usually applicable in more acidic media, thus providing a way to utilise effectively even already discussed protonation of an analyte and its subsequent ion-pairing with suitable agent, e.g., with another modifier. A retrospective insight into the electrochemistry with modified carbon paste electrodes shows clearly that such attempts are often successful [26,27]. Also, a choice of appropriate measuring mode may improve significantly the analytical performance of a CPE. This is the case of a very recent work reporting on square-wave voltammetric determination of 6-furfurylaminopurine ("kinetin"), which is a plant hormone found in nature very similar to BAP [40].

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