Taylor & Francis
Taylor & Francis Group

Inhibition by N′-nitrosonornicotine of the catalytic activity of glutamate dehydrogenase in α-ketoglutarate amination

YOU-AN MAO[1,2], KE-JUN ZHONG[1,2], WAN-ZHI WEI[2], XIN-LIANG WEI[1], & HONG-BING LU[1]

[1]*Postdoctorate Working Station, Changde Cigarette Factory, Changde 415000, China, and* [2]*State Key Laboratory of Chemo/Biosensing & Chemometrics, Hunan University, Changsha 410082, China*

Abstract
The effect of N′-nitrosonornicotine (NNN), one of the tobacco-specific nitrosamines, on the catalytic activity of glutamate dehydrogenase (GLDH) in the α-ketoglutarate amination, using reduced nicotinamide adenine dinucleotide as coenzyme, was studied by a chronoamperometric method. The maximum reaction rate of the enzyme-catalyzed reaction and the Michaelis-Menten constant, or the apparent Michaelis-Menten constant, were determined in the absence and presence of NNN. NNN remarkably inhibited the bio-catalysis activity of GLDH, and was a reversible competitive inhibitior with K_i, estimated as 199 $\mu mol\, l^{-1}$ at 25°C and pH 8.0.

Keywords: *N′-nitrosonornicotine (NNN), activity, glutamate dehydrogenase, inhibition, inhibition constant*

Introduction

Tobacco-specific nitrosamines (TSNAs) are a group of important compounds, which have been only found in tobacco, tobacco products and tobacco smoke. With the increase in public attention to the relation between smoking and health problems, the effect of TSNAs on human health has become a hot and important topic. Therefore, it is imperative to investigate the interaction of TSNAs with various bio-macromolecules at molecular level.

N′-Nitrosonornicotine (NNN) is one of the TSNAs. It is a carcinogen [1,2] as proved by animal experiments; NNN administered in drinking water induces esophageal tumors in rats[3] and tracheal tumors in Syrian Golden hamsters [4]. However, to the best of our knowledge, few reports about the interaction of NNN with enzymes and its effect on the activity of enzymes have been published, although there have been some studies regarding its carcinogenicity.

Glutamate dehydrogenase (GLDH) plays a pivotal role in the metabolism of most organisms because it provides a link between carbohydrate and nitrogen metabolism [5]. The reductive amination of α-ketoglutarate (α-KG) catalyzed by the enzyme is a very important reaction in nitrogen assimilation. In the reaction, reduced nicotinamide adenine dinucleotide (NADH) is used as coenzyme. The dissociation of the enzyme-coenzyme complex is the rate-limiting step in the whole catalysis reaction [6,7]. Any chemical species, affecting this equilibrium, will change the steady-state rate of the enzyme-catalyzed reaction. The effect of a wide variety of substances, including nucleotides, drugs, hormones, functional group modifiers, and amino and other carboxylic acids, on the reaction has been investigated [6–11]. Nevertheless, no study has been carried out which examines TSNA effects on the GLDH-catalyzed reaction.

The major difficulty in the enzyme assay is to measure the initial reaction rate. In the reductive amination of α-KG catalyzed by GLDH, NADH is oxidized to NAD^+ and rate measurement of the amination reaction has been usually achieved

Correspondence: Y.-A. Mao, Postdoctorate Working Station, Changde Cigarette Factory, Changde 415000, China. Fax: 86 736 7299381.
E-mail: youanmao@hotmail.com

RIGHTSLINK

by monitoring the absorbance change of NADH at 350 nm. However, there exists a lag-phase in the conventional spectrophotometric method [12]. In addition, NADH may be monitored by oxidation at an electrode surface and this oxidation reaction has well been studied [13–15]. In 1996, Xin and Gao proposed a chronoamperometric method to evaluate the initial kinetics of the enzyme-catalyzed reaction [16] and their method represents a significant advance. One of the advantages of the method is that it could be used to obtain the real initial reaction rate. Using this method, Gao and her coworkers have studied the effects of lanthanide ions, adenosine-5′-diphosphate and adenosine-5′-monophosphate on GLDH activity [16–18].

Here, a chronoamperometric method was used for the study of the effect of NNN on the activity of GLDH, based on the catalysis of the enzyme for the reductive amination of α-KG. The maximum reaction rate, V_{max}, of the enzyme-catalyzed reaction and the Michaelis-Menten constant, K_m, or the apparent Michaelis-Menten constant, K_m', of the enzyme was determined in the absence and presence of NNN, respectively.

Materials and methods

Reagents and chemicals

N′-Nitrosonornicotine (NNN), $M_r = 177.20$, was purchased from Toronto Research Chemicals Inc. L-Glutamate dehydrogenase (GLDH, EC 1.4.1.3) from bovine liver was obtained from Boehringer (Mannheim, Germany) as a suspension in $2.0 \, mol \, l^{-1}$ $(NH_4)_2SO_4$ solution, at a concentration of $28.57 \, mg \, ml^{-1}$, and used without further purification. Reduced nicotinamide adenine dinucleotide (NADH, coenzyme I) and α-ketoglutaric acid were biochemical-reagent grade, obtained from Shanghai Bio-chemicals Company (China). Tris, CH_3COONH_4, HCl and other reagents used were of analytical reagent grade.

All solutions were made up with super-pure water with a resistivity of $18.3 \, M\Omega \, cm^{-1}$.

Apparatus

A three-electrode cell was employed in all experiments. A glassy carbon electrode (GCE), 3.5 mm in diameter, was obtained from Jintan Electro-analytical Instrument Factory (Jiangsu, China) and used as the working electrode. It was polished with $0.06 \, \mu m$ alumina slurry and sonicated in fresh water before *each* use. The saturated calomel (SCE) and platinum wire electrodes were applied as the reference and counter electrodes, respectively.

Chronoamperometric and cyclic voltammetric experiments were performed with a CHI 660A electrochemical working station (Shanghai, China).

The experimental set-up also included an IBM-compatible computer, a super thermostat, and a magnetic stirrer.

Procedure

The cyclic voltammograms of NADH were measured at the GCE in the mixture containing $10 \, mmol \, l^{-1}$ CH_3COONH_4, $2 \, mmol \, l^{-1}$ α-KG, and $50 \, mmol \, l^{-1}$ Tris buffer (pH 8.0) with scanning scope of $-0.2 \sim +1.2 \, V$ and scanning rate of $50 \, mV \, s^{-1}$. The solution volume was controlled at 5 ml. Different NADH concentrations were used in these measurements. The data of the peak potential, E_p, and the peak current, i_p, for NADH oxidation were obtained by CHI electrochemical data analysis software.

The chronoamperomatric measurements of the reaction process for the GLDH-catalyzed α-KG amination in the absence and presence of NNN were carried out in $50 \, mmol \, l^{-1}$ Tris buffer solution (pH 8.0) containing quantitative NADH, $10 \, mmol \, l^{-1}$ CH_3COONH_4, and $2 \, mmol \, l^{-1}$ α-KG. The final volume of the mixture solution was controlled at 5 ml. The solution was stirred at a given rate during the measurements.

All experiments were conducted at a temperature $25 \pm 0.1°C$ and in triplicate and the solutions were previously deaerated with high-purity nitrogen gas for at least 15 min.

Results and discussion

### Theoretical consideration

In many electrochemical techniques, the electrode moves with respect to the solution. One of the few convective electrode systems is the rotating disk electrode. Based on the diffusion layer approach described by Bard and Faulkenr, [19] Xin and Gao [16] developed a chronoamperometric method using a disk electrode in a well-stirred solution. It is assumed that convection maintains a uniform concentration of all species that are equal to the bulk values up to a certain distance, i.e., the "diffusion" layer thickness (δ) at the electrode fed by convective transfer. Within the layer, $0 < x < \delta$, no solution moves, and the mass transfer takes place by diffusion. According to the general equation for the flux of species z for one-dimensional diffusion and convection, [19] the following expression can be derived if all experimental conditions, including stirring rate, are fixed:

$$i = \frac{nFAD_z}{P} c_z^*$$ (1)

where i is the limiting current, n the number of electron transfer, F the Faraday constant, A the area of the electrode, D_z the diffusion coefficient of

species z, c_z^* the bulk concentration of species z, and P a conditional constant, the value of which depends on the stirring rate, D_z and solution viscosity. Therefore, Equation (1) applies to the mass-transfer-limited condition at a disk electrode in a well-stirred solution and it can be predicted from this equation that i is proportional to c_z^* under fixed experimental conditions.

The GLDH-catalyzed reductive amination of α-KG, utilizing NADH as the coenzyme, can be expressed as follows:

$$\alpha - KG + NH_4^+ + NADH \; \overset{GLDH}{\rightleftharpoons} \; L$$

$$- \text{glutamate} + NAD^+ + H_2O \quad (2)$$
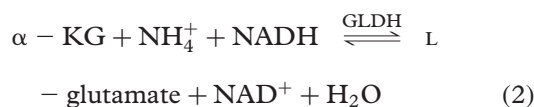
In the reaction, NADH is oxidized stoichiometrically and the rate of the reaction, ν, can be measured as the decrease in NADH concentration:

$$\nu = -\frac{dc_{t,NADH}}{dt} \quad (3)$$

Therefore, GLDH activity can be evaluated by the measurement of the change in NADH concentration during the reaction.

It is known that NADH can be *electrochemically* oxidized to NAD^+ at the electrode surface under adequate experimental conditions. If the chrono-amperometric method is applied, the limiting anodic current for the *electrochemical* oxidation of NADH can be real-time measured, and then the NADH concentration in the bulk solution can be determined according to Equation (1). When the GLDH-catalyzed amination of α-KG using NADH as coenzyme takes place, the bulk concentration of NADH will decrease, and correspondingly, the limiting current also decreases. With the combination of Equations (1) and (3), the reaction kinetics of the GLDH-catalyzed amination of α-KG may be examined. Therefore, the activity of GLDH and the effect of other chemical species, such as NNN, on the GLDH activity can be quantitatively investigated using this method.

Main experimental conditions and data reproducibility

Step potential is one of the most important experimental conditions in chronoamperometry. A series of cyclic voltammograms were recorded at GCE in the presence of different NADH concentrations at a temperature of 25°C in order to select an adequate step potential. For these experiments, the assay solution contained 50 mmol l^{-1} Tris buffer (pH 8.0), 10 mmol l^{-1} CH$_3$COONH$_4$, and 2 mmol l^{-1} α-KG in a final volume of 5 ml. A scan rate of 50 mV s^{-1} and a scan scope of $-0.2 \sim +1.2$ V was used. Experimental results showed that the peak potential of NADH oxidation was in the range of
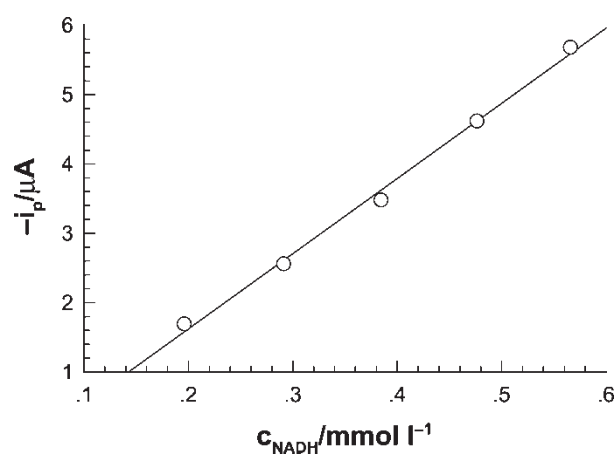

Figure 1. A plot of peak current (i_p) for NADH oxidation obtained from cyclic voltammograms against NADH concentration (c_{NADH}).

0.64–0.67 V (versus SCE). And a linear relationship was obtained between the anodic peak current and NADH concentration, as shown in Figure 1. Therefore, the potential was stepped from 0.20 to 0.80 V in the following chronoamperometric experiments to ensure that the NADH at the electrode surface could be oxidized rapidly and completely and the experiment was carried out in the limiting current region.

According to Equation (1), i response in the chronoamperometric method is sensitive to the system temperature because the solution viscosity and the diffusion coefficient of NADH are related to temperature. We examined the effect of temperature on the stability of i response in the range of 20–32°C to obtain an appropriate test temperature. The solution of 50 mmol l^{-1} Tris buffer (pH 8.0), 0.4 mmol l^{-1} NADH, 10 mmol l^{-1} CH$_3$COONH$_4$, and 2 mmol l^{-1} α-KG in a final volume of 5 ml was used and GLDH was not added to the experiments. Experimental results showed that a stable i response could be obtained under any temperature in that range. Thus, the system temperature was controlled to be 25 ± 0.1°C in the following experiments in order to ensure enzyme stability and to maintain the solution viscosity and the diffusion coefficient of NADH.

The stirring rate of the solution is also an important experimental condition in the chronoamperometric method. We investigated the effect of stirring rate on the i response in the range of 600–1200 rpm. The solution of 50 mmol l^{-1} Tris buffer (pH 8.0), 0.4 mmol l^{-1} NADH, 10 mmol l^{-1} CH$_3$COONH$_4$, and 2 mmol l^{-1} α-KG in a final volume of 5 ml was used and GLDH was not added to the experiments. The results showed that a stable i response could be obtained if the stirring rate was above 800 rpm. Therefore, the stirring rate of the solution was controlled at 1000 rpm in the following experiments to maintain the value of P in Equation (1) constant.

Good data reproducibility was achieved under the experimental conditions mentioned above. The values of i caused by varying the concentrations of NADH were very reproducible: the coefficients of variation (five independent experiments) were lower than 1.8% for all data from the chronoamperometric experiments in different NADH concentrations.

Chronoamperometric curve and initial rate for the GLDH-catalyzed amination of α-KG

A real-time chronoamperometric curve for the GLDH-catalyzed amination of α-KG, using NADH as coenzyme, could be obtained under the experimental conditions described above, as shown in curve 1 of Figure 2. It was shown that the anodic current of NADH oxidization reached a steady state quickly after potential stepping to 0.8 V. The steady state current, or limiting current, was proportional to the initial concentration of NADH. After the addition of GLDH to the assay solution, the enzyme-catalyzed amination reaction of α-KG immediately commenced because the solution system was in a well-stirred state and the added enzyme would be rapidly dispersed in the solution. At the same time, the NADH molecules in bulk solution were bio-chemically oxidized according to Equation (2), which resulted in a decrease in bulk NADH concentration and, correspondingly, the

Figure 2. Chronoamperometric curves for GLDH-catalyzed amination of α-KG. The arrow indicates the time of GLDH addition. The solution contained $50\,mmol\,l^{-1}$ Tris buffer (pH 8.0), $10\,mmol\,l^{-1}$ CH_3COONH_4, $2\,mmol\,l^{-1}$ α-KG, $0.1\,mmol\,l^{-1}$ NADH, and without NNN (1) or $0.05\,mmol\,l^{-1}$ NNN (2) in a final volume of 5 ml. The concentration of added GLDH after dilution in both (1) and (2) was $20\,\mu g\,ml^{-1}$.

reduction of the corresponding limiting current. With the development of the GLDH-catalyzed amination reaction, the NADH concentration in bulk solution and i response became less and less. Finally, i reached a steady value, indicating the end of the enzyme-catalyzed reaction.

It was also shown from Figure 2 that in a short time span after the start of the enzyme-catalyzed reaction, i response decreased linearly with time. That was because the bio-chemically oxidized amount of NADH was proportional to time and the NADH concentration reduced linearly with time in the initial short period. Therefore, the initial reaction rate of the enzyme-catalyzed amination of α-KG, v_0, could be measured with the initial slope of the chronoamperometric curve, that is,

$$v_0 = -\left(\frac{dc_{t,NADH}}{dt}\right)_{t=0} \qquad (4)$$

According to Equation (1), the above equation can be expressed as follows:

$$v_0 = c_{0,NADH}\frac{i_0 - i_t}{(t - t_0)i_0} \qquad (5)$$

where i_t and i_0 are as indicated in Figure 2.

Effect of NNN on the activity of GLDH

The chronoamperometric curve of the reductive amination reaction in the presence of NNN is shown as curve 2 in Figure 2. It is seen from the comparison of curve 2 with curve 1 in Figure 2 that the slope of the initial linear part in curve 2 is evidently smaller than that in curve 1, indicating that the presence of NNN reduces the initial rate of the GLDH-catalyzed reaction. In other words, NNN could inhibit the bio-catalytic activity of GLDH.

In order to investigate the link between the inhibition and NNN concentration, we recorded the chronoamperometric curves of GLDH-catalyzed amination of α-KG at different concentrations of NNN (curves are not shown) and determined the corresponding initial reaction rates. Figure 3 shows the effect of NNN at different concentrations on the initial rate of the GLDH-catalyzed reaction, or on the activity of GLDH and it was evident that when the concentration of NNN was enhanced the inhibition was increased. Inhibition by NNN of GLDH activity exhibited a tendency to be saturated when the inhibitor concentration became larger.

Estimation of kinetic parameters for the enzyme-catalyzed reaction

In order to study further the inhibition of NNN on the activity of GLDH, the enzyme-catalyzed reaction with
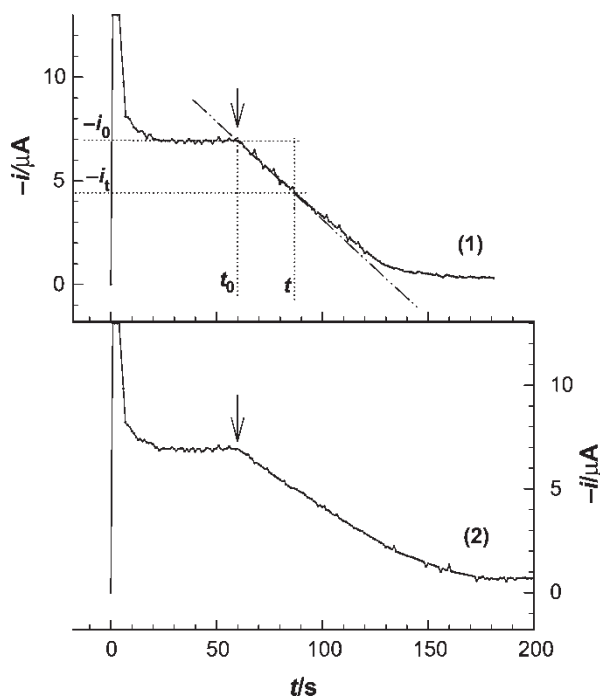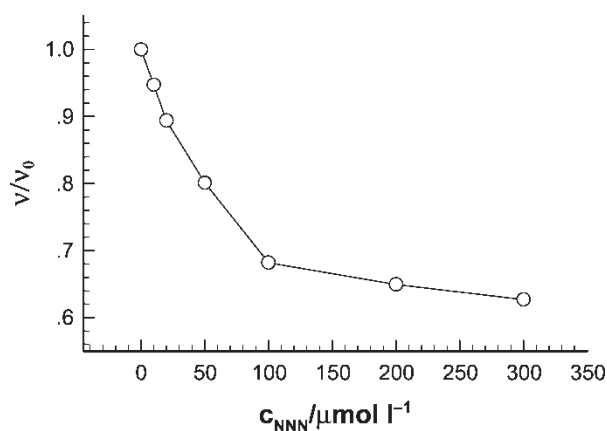
Figure 3. Effect of NNN on the initial rate of the GLDH catalyzed amination of α-KG. v and v_o are the initial reaction rates in the presence and absence of NNN, respectively. The solution contained $50\,mmol\,l^{-1}$ Tris buffer (pH 8.0), $10\,mmol\,l^{-1}$ CH_3COONH_4, $2\,mmol\,l^{-1}$ α-KG, and $0.1\,mmol\,l^{-1}$ NADH in a final volume of $5\,ml$. The concentration of added GLDH after dilution was $20\,\mu g\,ml^{-1}$.

α-KG at different concentrations of NADH, with fixed α-KG and GLDH concentrations, was monitored by the chronoamperometric method. The initial rates were measured, and the Lineweaver-Burk double-reciprocal plots [20] with NADH as the varied substrate were constructed in the presence and absence of NNN. The plots of reciprocal initial rate, $1/v_0$, vs. the reciprocal of NADH concentration, $1/c_{NADH}$, are shown in Figure 4. From the slopes and intercepts of the plots, the maximum initial reaction rate, V_{max}, and the Michaelis-Menten constant, K_m, or the apparent Michaelis-Menten constant, K_m', for GLDH in the absence and presence of NNN were obtained. The results at 25°C and pH 8.0 were as follows: $V_{max} = 0.3918\,mmol\,l^{-1}\,min^{-1}$
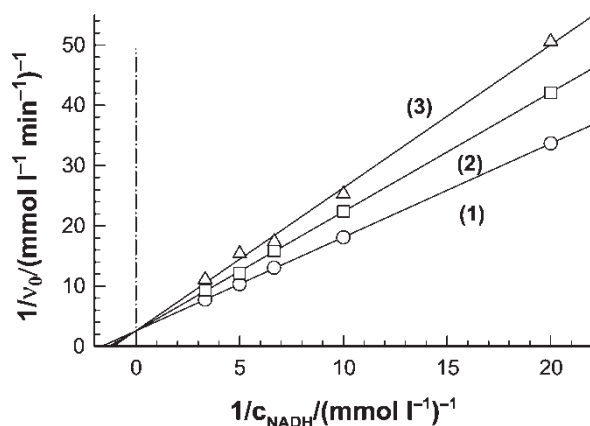

Figure 4. Lineweaver-Burk plots with NADH as the substrate. The solution contains $50\,mmol\,l^{-1}$ Tris buffer (pH 8.0), $10\,mmol\,l^{-1}$ CH_3COONH_4, $2\,mmol\,l^{-1}$ α-KG, and without NNN (1), or $0.05\,mmol\,l^{-1}$ NNN (2), or $0.10\,mmol\,l^{-1}$ NNN (3) in a final volume of $5\,ml$. The concentration of added GLDH after dilution was $20\,\mu g\,ml^{-1}$.

and $K_m = 0.619\,mmol\,l^{-1}$ when NNN was absent; $V_{max} = 0.3916\,mmol\,l^{-1}\,min^{-1}$ and $K_m = 0.774\,mmol\,l^{-1}$ when $0.05\,mmol\,l^{-1}$ NNN was present; $V_{max} = 0.3915\,mmol\,l^{-1}\,min^{-1}$ and $K_m' = 0.930\,mmol\,l^{-1}$ when $0.10\,mmol\,l^{-1}$ NNN was present.

The obtained apparent Michaelis-Menten constant K_m' after the introduction of NNN into the GLDH-catalyzed amination system of α-KG was larger than K_m, further showing the inhibition by NNN of the catalytic activity of GLDH. On the other hand, the value of V_{max} showed little change after NNN was introduced. And it was also indicated from Figure 4 that the effect of NNN on the GLDH activity was greatly reduced when the concentration of NADH was higher. These observations suggested that the inhibition by NNN of GLDH activity might be attributed to reversible competitive inhibition [21].

The main functional group in NADH, when it is used as coenzyme is the nicotinamide ring. The structure of the pyridine ring in NNN is similar to that of the nicotinamide ring in NADH so that, the NNN molecule can compete with NADH to bind to the active site of GLDH during the enzyme-catalyzed reaction which explains why NNN is a competitive inhibitor of GLDH.

According to the kinetic equation in the presence of a competitive inhibitor [21], we can estimate the inhibition constant, K_i, for NNN with GLDH from the values of K_m and K_m' obtained above and the NNN concentration used. Our results showed that $K_i = 200\,\mu mol\,l^{-1}$ when $C_{NNN} = 0.05\,mmol\,l^{-1}$ and $K_i = 198\,\mu mol\,l^{-1}$ when $C_{NNN} = 0.10\,mmol\,l^{-1}$. Thus, the average value of K_i was $199\,\mu mol\,l^{-1}$.

To date, there has been no report about the effect of NNN on the activity of enzymes. As to other TSNAs, such studies are also very few. El-Bayoumy *et al.* [22] reported that 4-(*N*-nitroso-*N*-methylamino)-1-(3-pyridyl)-1-butanone, another type of TSNAs, could induce an increase in the activity of cyclooxygenase in rat lung when the rat was fed a high-fat diet; the excessive expression of cyclooxygenase may give rise to many types of diseases. Here, we first studied the effect of NNN, one of TSNAs, on the activity of GLDH and obtained the inhibition constant of NNN. Our results showed that NNN could remarkably inhibit the bio-catalysis activity of GLDH, which might have a potential influence on human health because GLDH is an important enzyme in the body. In our view, more attention should be paid to the study of the interaction between TSNAs and enzymes with consideration of the relation between smoking and health problems.

References

[1] Hecht SS, Hoffmann D, Carcinogensis 1988;9:875–884.
[2] Hoffmann D, Hecht SS, Cancer Res 1995;45:935–944.

[3] Hoffmann D, Raineri R, Hecht SS, Maronpot RR, Wynder EL, J Natl Cancer Inst 1975;55:977–981.

[4] Hilfrich J, Hecht SS, Hoffmann D, Cancer Lett 1977; 2:169–175.

[5] Veronese FM, Boccu E, Conventi L, Biochim Biophys Acta 1975;377:217–228.

[6] Singh N, Fisher HF, Arch Biochem Biophys 1994; 312:38–44.

[7] Pazahanisamy S, Maniscalco SJ, Singh N, Fisher HF, Biochemistry 1994;33:10381–10385.

[8] Diprisco G, Strecher HJ, Eur J Biochem 1969;9:507–511.

[9] Dieter H, Koberstein R, Sund H, Eur J Biochem 1981; 115:217–226.

[10] Reincke S, Bauler M, Bell E, FASEB J 1997;11:A1147.

[11] Sood CR, Chanda SV, Singh YD, J Plant Nutr 1999; 22:1351–1364.

[12] Smith TJ, Bell JE, Arch Biochem Biophys 1985;239:63–73.

[13] Moiroux J, Elving PJ, Anal Chem 1978;50:1056–1062.

[14] Jaegfeldt H, J. Electroanal Chem Interfacial Electrochem 1980;110:295–302.

[15] Samec Z, Elving PJ, J. Electroanal Chem Interfacial Electrochem 1983;144:217–234.

[16] Xin WK, Gao XX, Analyst (Lond.) 1996;121:687–690.

[17] Dai H-C, Zhuang Q-K, Li N-Q, Luo H-X, Gao X-X, Bioelectrochemistry 2000;51:35–39.

[18] Zhuang Q-K, Dai H-C, Gao X-X, Xin W-K, Bioelectrochemistry 2000;52:37–41.

[19] Bard AJ, Faulkner LR. Electrochemical Methods: Fundamentals and Applications. New York: John Wiley and Sons; 1980.

[20] McKee T, McKee JR. Biochemistry: An Introduction., 2nd Ed., New York: McGraw-Hill; 1999.

[21] Zhou A-R, Zha X-L. Biological Chemistry., 5th Ed., Beijing: People's Health Press; 2001.

[22] El-Bayoumy K, Iatropoulos M, Amin S, Hoffmann D, Wynder EL, Cancer Res 1999;59:1400–1403.