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Kinetic study of a substrate cycle involving a chemical step: highly amplified determination of phenolic compounds

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Abstract

A kinetic analysis of a substrate cycle in which one of the two steps was substituted by a chemical reaction has been made. The model is illustrated by the amplified determination, in a continuous assay, of phenolic compounds at low concentrations using the enzyme tyrosinase and β-NADH to reduce the *o*-quinone product of catalytic activity. Progress curves corresponding to β-NADH disappearance were not linear and followed first-order kinetics. Knowledge of the kinetics of the reaction has allowed us to achieve detection limits as low as 50 nM in a simple 10-min assay. There is no analytical solution to the non-linear differential equation system that describes the kinetics of the reaction, therefore, computer simulations of its dynamic behaviour are also presented, good agreement with the experimental results being obtained. The method is applicable to the measurement of any other metabolite, and its amplification capacity as well as the simplicity of determining kinetic parameters enable it to be implemented in a bioreactor for automation purposes. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The ability of metabolic processes to regulation is a consequence of the evolution of an extremely complex system of regulatory mechanisms. Thus, living organisms possess several systems of biological amplification which helps them achieve a fast response to a given stimulus, such as enzyme cascades [1,2], limited proteolysis reactions [3,4] and substrate cycles [5,6]. These mechanisms have special characteristics which make them well-suited to playing a central role in cell regulation, and some of them have been applied to the in vitro quantitative analysis of low levels of a metabolite or to the amplification of an enzymatic activity [7–10].

In the substrate cycling technique, the target metabolite is reversibly interconverted without being consumed into another chemical species by two different enzymes coupled in the opposite direction, i.e., the product of one of them is the substrate of the other and vice versa. This is accompanied by the concomitant accumulation

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of other non-cycling products. A small amount of metabolite can therefore generate a large amount of product, which can easily be measured by conventional methods. The steady-state kinetics of these systems are well known [11] and a complete kinetic analysis valid for both the transient phase and the steady-state, including equations to minimize the cost of assays has recently been reported [12].

This approach, however, is not widely used for analytical purposes because of the long incubation times required and because of the difficulty in finding appropriate recycling enzymes. One alternative is to use a chemical step, although kinetic equations describing such a mechanism have not yet been derived. In the present paper, we make a kinetic analysis of a substrate cycle in which one of the two steps is a chemical non-enzymatically catalyzed reaction, assuming a Michaelis–Menten mechanism for the enzyme involved in the cycle. The model is applied to a continuous assay to measure low levels of phenolic compounds using tyrosinase and β-NADH as reducing agent reacting with the *o*-quinone product of the enzymatic reaction. This assay has previously been proposed by other authors [13] to measure phenolic compounds, and ascorbic acid has also been used as recycling chemical reagent [14,15], although the kinetics of the reaction were not taken into account.

2. Kinetic analysis

The mechanism under study is described by the following chemical equations:

$$A + X \xrightarrow{E} B + Y \quad (1)$$

$$C + Y \rightarrow D + X \quad (2)$$

where X and Y are the recycling substrates, A and C are non-recycling substrates, (A and X being substrates of the enzyme E), and B and D are the non-recycling products accumulated at each turn of the cycle.

The following analysis is valid provided that the concentration of A in the reaction medium is high enough to be saturating or remain constant during the reaction time. Furthermore, during cycling, the concentration of X must be lower than its Michaelis–Menten constant towards E (K_{mX}), so that the reaction rate of step (1) will be proportional to its concentration. Under these conditions, the reaction turns-out with no consumption of recycling substrates, while at the same time other products are accumulated with each turn of the cycle (B and D), the system thus acts as a chemical amplifier for the determination of X and Y.

Taking into account these assumptions and supposing that reaction (2) undergoes by only one step, the differential equation system that describes the mechanism shown by Eqs. (1) and (2) is:

$$\frac{d[X]}{dt} = -k_E[X] + k_Q[C][Y] \quad (3)$$

$$\frac{d[Y]}{dt} = k_E[X] - k_Q[C][Y] \quad (4)$$

$$\frac{d[B]}{dt} = k_E[X] \quad (5)$$

$$\frac{d[C]}{dt} = -\frac{d[D]}{dt} = -k_Q[C][Y] \quad (6)$$

where k_E is an apparent first-order rate constant, $k_E = V_{max}/K_m$ (V_{max} is the reaction rate of step (1) at saturating concentrations of A and X; K_m is a function of Michaelis–Menten constants of A and X towards E and of the initial concentration of A, with $K_m \simeq K_{mX}$ if A is saturating), and k_Q is the rate constant of chemical step (2).

Eqs. (3)–(6) do not admit any analytical solution. The steady-state corresponding to this system would be reached when the rate of formation of Y (Eq. (1)) equals the rate of regeneration of X (Eq. (2)), i.e., $k_E[X] = k_Q[C][Y]$. The concentration of X and Y in the steady-state would then be constant ($[X]_{ss}$ and $[Y]_{ss}$, respec-

tively). However, one can only talk about a pseudo steady-state, because C is consumed. Following the steady-state approach, Eqs. (5) and (6) may be rewritten as:

$$\frac{d[B]}{dt} = \lambda_E \quad (7)$$

$$\frac{d[C]}{dt} = -\lambda_Q[C] \quad (8)$$

where

$$\lambda_E = k_E[X]_{ss} \text{ and } \lambda_Q = k_Q[Y]_{ss}. \quad (9)$$

Integrating Eqs. (7) and (8) under the initial conditions $[B] = 0$ and $[C] = C_0$ at $t = 0$ gives:

$$[B]_{ss} = \lambda_E t \quad (10)$$

$$[C]_{ss} = C_0 e^{-\lambda_Q t} \quad (11)$$

and assuming that $[D] = 0$ at $t = 0$:

$$[D]_{ss} = C_0(1 - e^{-\lambda_Q t}). \quad (12)$$

These equations indicate that B will be accumulated linearly in the steady-state, whereas the disappearance of C and the appearance of D will be of uniexponential shape, and follow first-order kinetics.

3. Experimental

3.1. Materials

Catechol, phenol, β-NADH and tyrosinase (EC 1.14.18.1) from mushroom were purchased from Sigma. All other buffers and reagents were of analytical grade and used without further purification.

3.2. Methods

Unless otherwise stated, the cycling reagent consisted of 100 mM sodium phosphate buffer, pH 7.0, 160 μM β-NADH, 20.85 μg of tyrosinase and different volumes of a stock solution of 5 μM catechol. The reaction was started by the addition of enzyme, the final volume being 1 ml. The time course of the reaction was followed by measuring the disappearance of β-NADH at 340 nm ($\epsilon = 6270\ M^{-1}\ cm^{-1}$), at 30°C.

k_E was determined by a set of experiments in which the catechol concentration was varied from 50 to 265 μM, in 100 mM sodium phosphate buffer, pH 7.0, and in the presence of 0.208 μg of tyrosinase. The reaction was started by adding enzyme to a final volume of 1 ml. The time course of the reaction was followed by measuring the appearance of *o*-benzoquinone at 390 nm ($\epsilon = 1050\ M^{-1}\ cm^{-1}$), at 30°C. k_E was directly obtained from the slope of a plot of the reaction rate vs. catechol concentration, fitting data by linear regression analysis, and multiplying the value thus obtained by the corresponding dilution factor (100).

Spectrophotometric readings were obtained on a Uvikon 940 spectrophotometer from Kontron Instruments.

The reaction mechanism for catecholase activity was simulated by numerical integration of the non-linear set of differential equations (Eqs. (3)–(6)) which describe the kinetics of the reaction mechanism. To simulate cresolase activity, the following differential equation was included into the system: $d[F]/dt = -k_1[F]$, and Eq. (3) was modified as follows: $d[X]/dt = k_1[F] - k_E[X] + k_Q[C][Y]$, being F a monophenolic substrate. Computer simulations were performed for several sets of kinetic constants and initial concentrations of substrates and recycling agent. This numerical integration was carried out by using the Adams–Moulton predictor–corrector algorithm, starting with the fourth order Runge–Kutta method. The algorithm for the numerical integration and the computer program were implemented and compiled in TURBO PASCAL 6.0 [16].

The data of both experimental and simulated progress curves corresponding to the disappearance of β-NADH or C, respectively, were fitted to first-order kinetics by linear regression using the SigmaPlot Scientific Graphing System.

4. Results and discussion

In this section, the experimental results obtained in the continuous amplified assay of low levels of catechol are shown. They illustrate the validity of the proposed model and of the equations obtained. According to the notation used, X and Y are catechol and *o*-benzoquinone, respectively, A, B, C and D are O_2, H_2O [17], β-NADH and β-NAD^+, respectively, E being the enzyme tyrosinase (catecholase activity).

The time course of the enzymatic-chemical cycling reaction was followed by monitoring the decrease in absorbance at 340 nm caused by the consumption of β-NADH. Fig. 1A shows a selection of progress curves obtained for different catechol concentrations under the conditions described in Section 3. It can be seen that the progress curves are not linear, although their corresponding semilogarithmic plots are (Fig. 1A, inset), suggesting that the disappearance of β-NADH follows a first-order fallout, according to Eq. (11). These data were, therefore, fitted by linear regression to obtain the corresponding first-order constant, λ_Q, from the slope of the straight lines. Data thus obtained are shown in Fig. 1B, in which the linearity and sensitivity of the amplified assay can be appreciated. Under the conditions described, good linearity was obtained over a range of 0.05 to 3 nmol of catechol. The continuity of the assay and the possibility of directly obtaining the chemical constant from progress curves data permitted the sensitivity of the assay to be increased five fold with respect to data reported by other authors [13], and to obtain a detection limit as low as 50 nM for catechol.

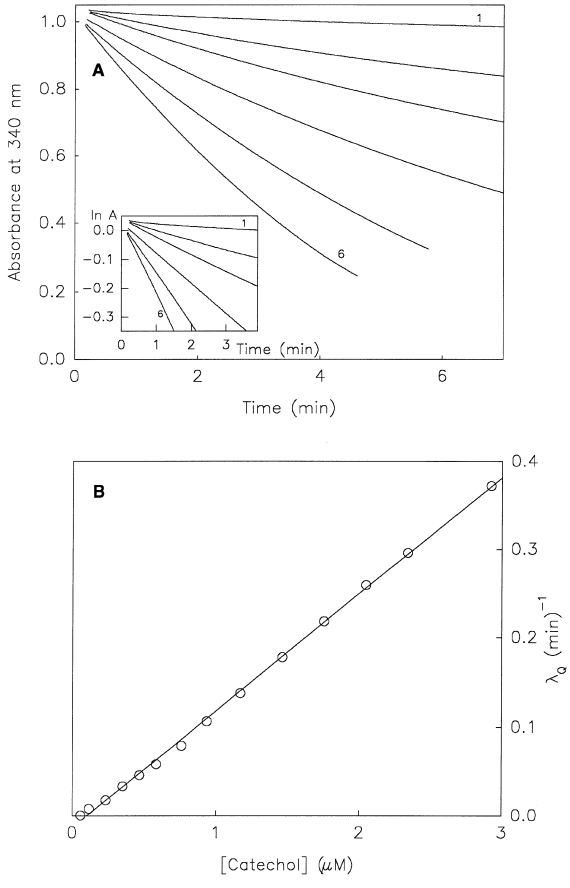

Fig. 1. (A) Progress curves corresponding to β-NADH disappearance at different catechol concentrations. The inset shows their corresponding semilogarithmic plots. The concentrations of catechol were 0.12, 0.35, 0.58, 0.94, 1.46 and 2.05 μM for curves 1–6, respectively. (B) Calibration straight line for the determination of catechol. The points represent experimental data, and the straight line corresponds to regression analysis ($y = -0.01364 + 0.13157x$, being $R = 0.99947$).

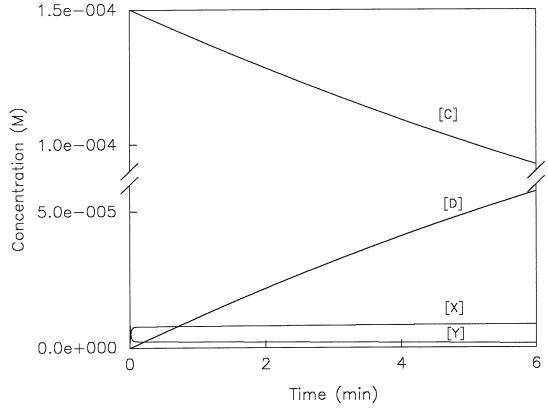

Fig. 2. Simulated curves obtained for the species involved in chemical Eqs. (1) and (2). The values of the rate constants used were $k_E = 50$ min^{-1}, $k_Q = 10^5$ M^{-1} min^{-1}, at the following initial conditions: $X_0 = 10^{-6}$ M, $Y_0 = 0$, $B_0 = 0$, $C_0 = 1.5 \times 10^{-4}$ M and $D_0 = 0$. Curves corresponding to X and Y have been multiplied by 10 for greater clarity.

The results obtained when the time course of the reaction was simulated by numerical integration of the non-linear set of differential Eqs. (3)–(6) are shown in Fig. 2 for a chosen set of

kinetic constants and initial conditions. Good agreement with the experimental results can be observed. The progress curves of C follow first-order kinetics, their corresponding semilogarithmic plots being linear ($\ln[C]_{ss}$ vs. t; data not shown). This indicates that the steady-state approach may be considered valid (it can be seen that X and Y concentrations attain an approximately constant level).

The chemical constant λ_Q also depends on the recycling chemical agent and enzyme initial concentrations, since it is dependent on the Y concentration attained at the steady-state (Eq. (9)). Fig. 3A and B show the variation of λ_Q with respect to β-NADH and tyrosinase initial concentrations, respectively, obtained experimentally. An inverse dependence on the initial

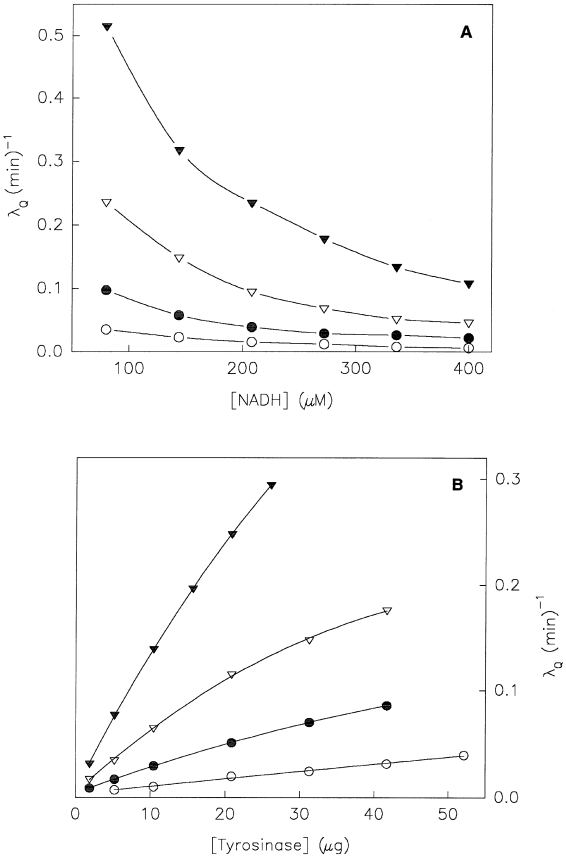

Fig. 3. Effect of β-NADH initial concentration (A) and tyrosinase concentration (B) on λ_Q, at different catechol concentrations. In (A): (○) 0.23, (●) 0.58, (Δ) 1.17 and (▲) 2.34 μM catechol. In (B): (○) 0.23, (●) 0.5, (Δ) 1 and (▲) 2 μM catechol.

β-NADH concentration was obtained since, as it increases, the chemical reaction rate (Eq. (2)) becomes faster and so the level of *o*-benzoquinone in the steady-state ($[Y]_{ss}$) falls. The dependence of λ_Q on enzyme concentration was not linear, as can be seen from Fig. 3B, since increasing the rate of the enzymatic step rate involves that chemical step is also faster (owing to more amount of Y is produced), which means that the level of *o*-benzoquinone attained at the steady-state cannot linearly vary with tyrosinase concentration. The results obtained by computer simulation showed a similar behaviour for the system (data not shown), which supports the kinetic model proposed.

It was not possible to design a kinetic analysis of the model to determine the kinetic parameters which characterize the cycle (k_E and k_Q), since no analytical equations exist to allow it. Therefore, these constants must be individually evaluated, by another type of experiments. k_E is an apparent first-order rate constant (V_{max}/K_m), as has been mentioned above, and so can be evaluated by determining its corresponding kinetic parameters, V_{max} and K_m, in a conventional way. However, it is not necessary to know the individual V_{max} and K_m, and this constant may be determined directly from a set of experiments at several substrate concentrations very much smaller than the Michaelis–Menten constant. By this means, we obtained a $k_E = 13.86\ min^{-1}$ in the example here illustrated (data not shown, conditions as specified in Section 3). When this value was introduced into the simulation experiments, catechol oxidation was seen to be the probable rate-limiting step, and that reduction of *o*-benzoquinone to regenerate catechol takes place very rapidly, although the specific value of k_Q could not be experimentally determined owing to the instability of *o*-benzoquinone at this pH [18].

4.1. Other phenolic substrates

The reaction scheme here described is applicable to the quantitative determination of all

o-diphenolic compounds which are substrates of catecholase activity of tyrosinase. The detection limit and the cycling rate in each particular case will depend on the catalytic efficacy of the enzyme, k_E, and on the capacity of the corresponding *o*-quinone to oxidize β-NADH, which is reflected in k_Q.

The enzyme tyrosinase also shows another catalytic activity, the so-called cresolase activity, which consists of the *o*-hydroxylation of monophenols to *o*-diphenols, also in the presence of molecular oxygen. Therefore, an analytical determination of similar levels of monophenolics may also be carried out by this amplification approach. The expression of this activity is characterized by a lag period, which can be shortened or eliminated by addition of reducing agents or *o*-diphenols which act as co-substrates [19–21]. Experiments were performed using phenol as the monophenolic substrate of cresolase activity of tyrosinase and also by computer simulation, including the differential equation corresponding to this first step (see Section 3). The progress curves obtained both experimental and theoretically showed two well differentiated zones (data not shown): (a) an initial lag period, typical of cresolase activity, as has been mentioned above, due to the slow conversion of phenol into catechol and the subsequent conversion of this to *o*-benzoquinone, which reacts with the electron donor β-NADH to regenerate catechol, and (b) a pseudo steady-state phase, in which all the monophenolic substrate has been depleted and during which β-NADH decay follows first-order kinetics, as could be checked by performing the corresponding semilogarithmic plots. The first-order constant, λ_Q, was obtained in this case from the slope of the linear portion of semilogarithmic β-NADH disappearance plots. Fig. 4 shows the dependence of λ_Q on phenol concentration, which was obtained both experimentally and theoretically by simulation in the computer. It can be seen that an upwardly concave calibration curve was obtained, instead of a straight line as was expected, which may be explained by the fact that an important charac-

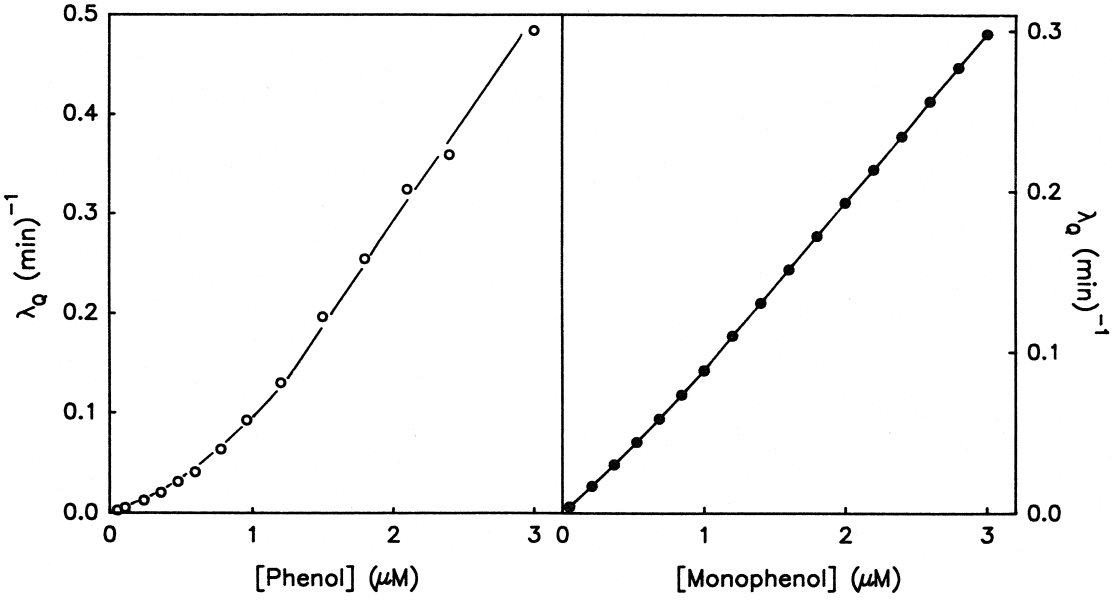

Fig. 4. Calibration curves for the determination of phenol, obtained experimentally (○) and theoretically by computer simulation (●). Experimental conditions were the following: 100 mM sodium phosphate buffer, pH 7.0, 160 μM β-NADH, 29.4 μg of tyrosinase and different volumes of a stock solution of 6 μM phenol. The reaction was started by the addition of the enzyme, the final volume being 1 ml. The values of the rate constants used were the same that for catecholase activity, being $k_1 = 0.5\ \text{min}^{-1}$. Initial conditions were: $X_0 = 0$, $Y_0 = 0$, $B_0 = 0$, $C_0 = 1.5 \times 10^{-4}$ M, $D_0 = 0$, and different values of F_0 from 5×10^{-8} to 3×10^{-6} M.

teristic of the cresolase activity of tyrosinase is its need for a certain amount of *o*-diphenol in the steady-state in order to maintain a constant hydroxylation rate. Reducing agents, such as β-NADH, shorten the lag period because they reduce the *o*-quinone product of catalytic activity to the corresponding *o*-diphenol, and so its necessary level is attained earlier. Some of the reducing agent is, therefore, consumed during the lag period, the more the greater the monophenol concentration is. The lag period lengthens and the necessary level of *o*-diphenol in the steady-state increases at increasing monophenolic substrate concentrations. Thus, at the end of the lag period, when the monophenol is totally deplete and β-NADH consumption becomes a first-order decay, the level of β-NADH is smaller than at the onset and, according to Fig. 3A, λ_Q is greater than expected. This explains why Fig. 4 is not a straight line but an upwards curve. This explanation was corroborated by computer simulation, assuming that C concentration during the lag period was constant since the λ_Q obtained agreed well with the value expected if the plot were a straight line (data not shown).

4.2. Concluding remarks

The described enzymatic-chemical cycling assay procedure for amplification may be used for the quantitative determination of low levels of a metabolite. It has been shown that the progress curves fit a first-order process, which demonstrated the importance of continuous measurement in these systems. The constant characterizing the first-order process, λ_Q, is the parameter directly related to the initial metabolite concentration, since in these systems the cycling rate is not constant with time.

The applicability of this method was demonstrated by the continuous measurement of low levels of phenolic compounds (particularly catechol and phenol) with the cycling system tyrosinase/β-NADH. A knowledge of the kinetic behaviour of the cyclic reaction and the use of the complete progress curves allowed to us to increase the sensitivity of the assay five times for catechol and ten times for phenol compared with the values reported by other authors for the same assay [13]. In general, the relative sensitivity for other phenolic substrates will be related to their reactivities with tyrosinase and the relative values of the rate constant of the chemical step. The great sensitivity of the method, as well as the numerous advantages of using a continuous assay, mean it can be applied to the determination of phenolic compounds in a wide variety of samples, such as environmental, food and clinical analysis. Furthermore, it may be implemented in a bioreactor using tyrosinase immobilized for automation purposes. The system can also be exploited for the determination of salicylate, an important compound in clinical diagnostic, by converting it to catechol in the presence of the enzyme salicylate hydroxylase.

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References

[1] P.B. Chock, E.R. Stadtman, Proc. Natl. Acad. Sci. U.S.A. 74 (1977) 2766.
[2] R. Varón, M. Molina, M. García-Moreno, F. García-Sevilla, E. Valero, Biol. Chem. Hoppe-Seyler 375 (1994) 365.
[3] H. Holzer, P.C. Heinrich, Annu. Rev. Biochem. 49 (1980) 63.
[4] B.H. Havsteen, M. García-Moreno, E. Valero, M.C. Manjabacas, R. Varón, Bull. Math. Biol. 55 (1993) 561.
[5] E.A. Newsholme, R.A.J. Challiss, B. Crabtree, Trends Biochem. Sci. 9 (1984) 277.
[6] A. Sorribas, R. Bartrons, Eur. J. Biochem. 158 (1986) 107.
[7] A. Johannson, D.H. Ellis, D.L. Bates, A.M. Plumb, C.J. Stanley, J. Immunol. Methods 87 (1986) 7.
[8] J.V. Passonneau, O.H. Lowry, Enzymatic analysis: a practical guide, in: J.V. Passonneau, O.H. Lowry (Eds.), Humana Press, Totowa, NJ, 1993, p. 85.

[9] S. Harbron, M. Fisher, B.R. Rabin, Biochim. Biophys. Acta 1161 (1993) 311.
[10] E. Valero, R. Varón, F. García-Carmona, Biochem. J. 309 (1995) 181.
[11] J.V. Passonneau, O.H. Lowry, Principles of enzymatic analysis, in: H.U. Bergmeyer (Ed.), Verlag Chemie, Weinheim, NY, 1978, p. 86.
[12] E. Valero, F. García-Carmona, Anal. Biochem. 239 (1996) 47.
[13] R.S. Brown, K.B. Male, J.H.T. Luong, Anal. Biochem. 222 (1994) 131.
[14] S. Uchiyama, Y. Hasebe, H. Shimizy, H. Ishihara, Anal. Chim. Acta 276 (1993) 341.
[15] Y. Hasebe, Y. Tanaka, S. Uchiyama, Anal. Lett. 27 (1994) 41.
[16] C. Garrido del Solo, R. Varón, F. García-Cánovas, An. Quím. 88 (1992) 633.
[17] K. Lerch, Metal ions in biological systems, in: H. Sigel (Ed.), Marcel Dekker, New York, 1981, p. 143.
[18] C.R. Dawson, W.B. Tarpley, Ann. New York Acad. Sci. 100 (1963) 937.
[19] S.H. Pomerantz, M.C. Warner, J. Biol. Chem. 242 (1967) 5308.
[20] H.W. Duckworth, J.E. Coleman, J. Biol. Chem. 245 (1970) 1613.
[21] F. García-Carmona, E. Pedreño, J.D. Galindo, F. García-Cánovas, Anal. Biochem. 95 (1979) 433.