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INVESTIGATIONS ON THE BINDING OF MERCURY IONS TO ALBUMINS EMPLOYING DIFFERENTIAL PULSE VOLTAMMETRY

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Abstract: Binding of mercury to BSA and Ovalbumin was investigated by Differential Pulse Voltammetry. The method relies on the direct monitoring of peak current variation due to mercury oxidation in the presence of these two albumins. Linear calibration graphs were obtained for both BSA and Ovalbumin in concentrations ranging from 2.49 x 10^{-7} to 19.6 x 10^{-9} mol L^{-1}. The acquired data was used to quantify these two proteins independently and to calculate the dissociation constants of Hg-BSA and Hg-Ovalbumin complexes.

Key words: Differential Pulse Voltammetry, Mercury, Bovine Serum Albumin, Ovalbumin.

INTRODUCTION

Albumins are water soluble globular proteins, easily crystallizable and constituted by a high percentage of acidic amino acid residues. They can occur in body fluids, animal tissues and in the majority of plant seeds. Important representatives from the animal group are serum albumin, α-lactoalbumin and ovalbumin. Serum albumin contributes up to 80% to the colloid osmotic blood pressure, it is responsible for the maintenance of blood pH, and functions as a storage protein as well as a transport protein for a number of endogenous and exogenous compounds [1, 2]. Serum albumin performs many other functions, such as trapping oxygen free radicals and inactivating various toxic lipophilic metabolites [3]. Additionally, it forms covalent adducts with pyridoxyl phosphate, cysteine, glutathione and various cations, such as Au(I), Ag(I), Cu(II), Hg(II) and Ni(II) [1]. Both bovine serum albumin (BSA) and human serum albumin (HSA) have been extensively studied regarding their physiological importance, binding affinities and structural features [1, 2, 4-8]. BSA is a single 582 residue polypeptide chain, with 17

disulphide bridges that are arranged in three major helical domains [9, 10]. Ovalbumin comprises up to 65% of the total protein in egg-white [11], nevertheless its function is yet unknown. It has been proposed that the dephosphorylated form of Ovalbumin present in the egg-yolk may act as an amino acid source for the growing embryo [12]. Ovalbumin might also be involved in the transport of nutrients and storage of metal ions [13]. A single strong binding site for several metal ions has been found [14]. Ovalbumin is a glycoprotein with 385 amino acid residues [15-17] and its sequence includes four thiol groups with just a single disulphide bond between Cys 87 and Cys 133 [18].

Since the early 1950s, binding of metal ions to proteins has been extensively investigated, for a better understanding of biological systems in which metals are involved. Most albumins are able to associate with positively charged inorganic ions in a reversible manner [2]. A wide variety of experimental methods have been employed to characterize metal-albumin complexes, such as equilibrium dialysis [21-27], ultracentrifugation [21], circular dichroism [21, 28], UV spectroscopy [25], electron spin resonance spectroscopy [28], NMR [29, 30], ultrafiltation [31], gel filtration chromatography [32, 33], atomic absorption spectrometry [32], fluorescence [34], immobilized metal ion affinity chromatography [35, 36], x-ray photoelectron spectroscopy [37, 38], potentiometry [39], inductively coupled plasma atomic emission spectrometry [40] and electrophoresis [41]. In most cases association constants (K_{ass}) have been determined according to the Scatchard model [20] assuming that the ligand in question is bound to classes of identical, independent binding sites. All these studies have shown that binding data can be influenced by albumin concentration, pH, temperature and concentration of salts in solution.

The binding of mercury ions to albumins has been investigated by potentiometry [39], intrinsic protein fluorescence [34], ultracentrifugation [21], equilibrium dialysis [21, 43], circular dichroism [21], x-ray photoelectron spectroscopy [38] and amperometric titrations [43]. In these studies, the authors verified that mercury ions bind very strongly to BSA, HSA and α-lactoalbumin. It was also proposed that binding of Hg^{2+} to serum albumins (HSA and BSA) takes place at the deprotonated thiol group (-RS-) of the cysteinyl residue [6, 38, 39, 43] and that the K_d value is very similar to that of the complex formed between Hg^{2+} and cysteine (6.9×10^{-23} mol L^{-1}; pH 7.2) [6]. In addition to the strong binding on the –SH site (association constant ~10^7), Saroff *et. al* [43] verified other five weaker binding sites for mercury in BSA with a K value of 2.7×10^4 to 4.5×10^4.

The most commonly used methods for the determination of albumins are spectrophotometric dye binding techniques for which bromocresol green, BCG [44], HABA (2-(4'-hydroxyazobenzene) benzoic acid) [45], or methyl orange [46] are selective reagents. It is also possible to determine albumins in low concentrations using voltammetric techniques [47-53] and resonance Rayleigh light-scattering [54-57].

This work describes the interaction of mercury with BSA and Ovalbumin using differential pulse voltammetry with a dropping mercury electrode at nanomolar concentration of protein. The method relies on the direct monitoring of the peak current variation due to mercury oxidation in the presence of BSA and Ovalbumin. The height of each peak is proportional to protein concentration in solution.

MATERIALS AND METHODS

Chemicals

Analytical-reagent grade chemicals and triple distilled water from a quartz still (Quartex) were used to prepare all solutions. Ammonia buffer (568 mL NH_4OH, 70g NH_4Cl /L) pH 10 was prepared from ammonium chloride and ammonium hydroxide, both purchased from Merck. Highly purified (99%) bovine serum albumin (BSA) and Ovalbumin were obtained from Sigma Chemical Co.

Apparatus

Differential pulse polarographic (DPP) measurements were carried out on a Metrohm 646 Voltammetric Analyser Processor connected to a 647 Metrohm electrochemical cell composed of a dropping mercury electrode (working electrode), a Ag/AgCl (3 mol L^{-1} KCl) (reference electrode) and a platinum electrode (auxiliary electrode). All DPP measurements were performed in the potential range –0.25 V (initial potential) to 0.1 V (final potential) at the following settings: surface area of the mercury drop 0.4 mm^2, DME drop time 600 ms, pulse amplitude 50 mV and the scan rate v = 10 mV s^{-1}.

Procedure

Prior to polarographic measurements, the concentrations of BSA and Ovalbumin solutions were determined by UV measurements at 280 nm using Lambert-Beer law.

In order to avoid measurement interference due to protein adsorption on the working surfaces of the electrode system, electrodes were submitted to periodic cleaning with sulphochromic solution under N_2 for 1 minute after each voltammetric experiment. After that, they were rinsed with 20% HNO_3 (v/v) followed by a generous wash with triple distilled water. All experiments were performed at room temperature and proceeded by a gentle N_2 bubbling to prevent oxygen diffusion into the electrochemical cell (10 minutes for the supporting electrolyte and 2 minutes after each protein addition).

Calibration curves, quantitative protein determination and dissociation constants (K_d) for BSA and Ovalbumin were obtained from the combined data of supporting electrolyte polarograms, and from polarograms produced by the additions of standard protein concentrations into the cell. Signals obtained in these experiments were considered to be the effect of the peak current produced by the Hg-protein interactions observed for both proteins under investigation.

RESULTS

Amperometric complex-formation titrations of metal ions with indication of the end-point by following the anodic wave of the excess of ligand at mercury electrodes have been described [58-59]. By means of an appropriate choice of pH and titrant (EDTA, TRIEN), selective determinations of metals ions (Cu(II), Th(IV), Zn(II), Ca(II)) at low concentration levels appear to be possible. Calcium has been determined in the submicrogram range by means of an amperometric titration with γ-thionin SIα1, the end-point being detected by following the anodic wave of mercury in the presence of an excess of this protein [60]. A similar phenomenon was observed when Ovalbumin or BSA was present in the electrochemical cell.

The voltammetric behavior of 10^{-4} mol L^{-1} Ovalbumin and 10^{-4} mol L^{-1} BSA is shown in figures 1 and 2. The curve 0 (Figures 1 and 2) represents the supporting electrolyte (ammonia buffer pH 10) and shows the oxidation of mercury in basic media.

$$Hg^{2+} + 2e^- \overset{E^0}{\longleftrightarrow} Hg^0 \qquad E^0 = 0.346 \text{ V/Ag/AgCl} \qquad \textbf{(1)}$$

Curves number 1, 2, 3 and 4 (Figures 1 and 2) represent the effect of the addition of 10, 20, 30 and 40 μL of 10^{-4} mol L^{-1} protein (Ovalbumin and BSA) to the electrochemical cell. The resulting changes in current attest to the interaction between mercury and both proteins.

$$\text{Ovalbumin} - Hg^{2+} + 2e^- \longleftrightarrow Hg^0 + \text{Ovalbumin} \qquad E_p = -70 \text{ mV/Ag/AgCl} \qquad \textbf{(2)}$$

$$\text{BSA} - Hg^{2+} + 2e^- \longleftrightarrow Hg^0 + \text{BSA} \qquad E_p = -20 \text{mV/Ag/AgCl} \qquad \textbf{(3)}$$

During the process of mercury interaction with each protein, it was observed that the measured current varied linearly with protein concentration in the electrochemical cell.

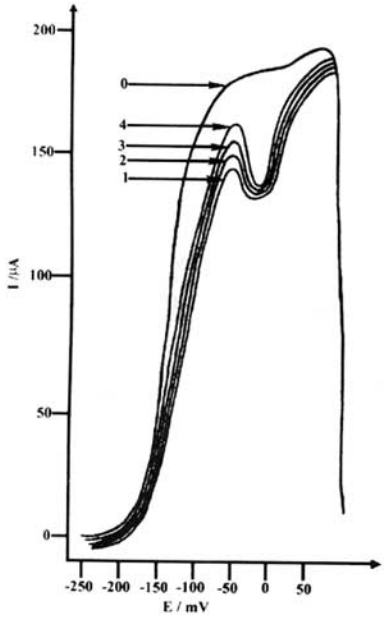

Figure 1: Voltammograms for the electro-dissolution of mercury in NH_3/NH_4Cl pH 10. Curve 0 represents the supporting electrolyte; curves 1, 2, 3 and 4 represent progressive additions of fix volume of 10 μL of ovalbumin 10^{-4} mol L^{-1} solution.

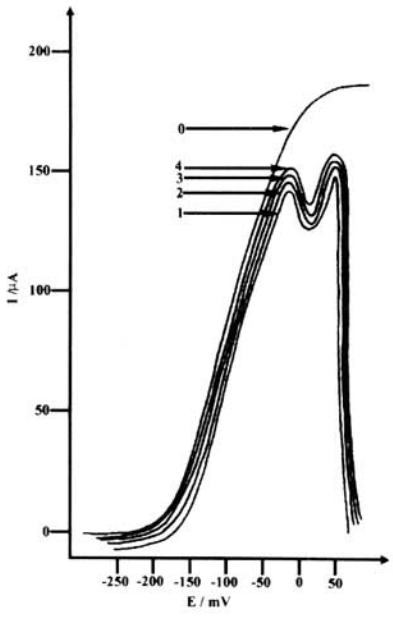

Figure 2: Voltammograms for the electro-dissolution of mercury in NH_3/NH_4Cl pH 10. Curve 0 represents the supporting electrolyte; curves 1, 2, 3 and 4 represent progressive additions of fix volume of 10 μL of BSA 10^{-4} mol L^{-1} solution.

Quantitative Determinations

Tables 1 and 2 show regression data obtained from the calibration curves of BSA and Ovalbumin and their interaction with mercury. In order to determine the linear correlation between the protein concentration and the peak current, eight experimental measurements for each protein were performed and used to build correlation graphs, which are represented by the following parameters.

Table 1: Regression data of Ovalbumin (Y = A + BX).

[Ovalbumin]range / mol L^{-1}	Param A	Sd_A	Param B	Sd_B	R	SD	N
2.49 – 19.6 x 10^{-7}	-0.17192	0.05304	1.94764	0.00427	0.99999	0.06769	8
2.49 – 19.6 x 10^{-8}	1.47887	0.41755	1.10099	0.03361	0.99722	0.5325	8
2.49 – 19.6 x 10^{-9}	-2.68056	0.71213	1.52998	0.05732	0.99582	0.90818	8

Table 2: Regression data of BSA (Y = A + BX).

[BSA] range / mol L^{-1}	Param A	Sd_A	Param B	Sd_B	R	SD	N
2.49 – 19.6 x 10^{-7}	4.44724	0.62923	1.69228	0.05065	0.99732	0.80245	8
2.49 – 19.6 x 10^{-8}	4.67292	0.02609	0.97426	0.0021	0.99999	0.03327	8
2.49 – 19.6 x 10^{-9}	1.25604	0.55941	1.14814	0.04503	0.99542	0.71342	8

Quantitative determinations of protein concentration are shown in tables 3 and 4. Table 3 contains the results of quantitative determination of Ovalbumin in concentrations ranging from 1.48 x 10^{-6} to 7.44 x 10^{-9} mol L^{-1}, showing mean recovery yields (n=3) between 91 and 101% with a standard deviation of 5%. Table 4 shows similar results found for BSA. However, mean recovery yields for BSA were slightly lower than Ovalbumin, and were determined to be around 83 to 99%. Detection limits for both proteins were calculated according to the following equation: $y = 3S_B + Y_B$ [61], and the observed values for Ovalbumin and BSA were 2.9 x 10^{-9} mol L^{-1} and 1.4 x 10^{-9} mol L^{-1}, respectively.

K_d Determinations

Dissociation constants (K_d) for Ovalbumin-Hg^{2+} and BSA-Hg^{2+} complexes were obtained using the Nernst equation:

$$E = E^0_{Hg^{2+}/Hg^0} + \frac{0.0591}{2} \log[Hg^{2+}] \qquad \textbf{(4)}$$

Where

E= Cell potential in volts

$E^0_{Hg^{2+}/Hg^0}$ = Standard potential of the electrode in basic media (pH = 10)

$[Hg^{2+}]$ = Mercury concentration in solution (mol L^{-1})

Mercury concentration was obtained by applying the equilibrium equations for the interaction of both proteins and mercury as follows:

$$P - Hg^{2+} \underset{}{\overset{K_d}{\rightleftharpoons}} P + Hg^{2+} \qquad \textbf{(5)}$$

$$K_d = \frac{[P][Hg^{2+}]}{[P - Hg^{2+}]} \qquad \textbf{(6)}$$

$$[Hg^{2+}] = \frac{K_d[P - Hg^{2+}]}{[P]} \qquad \textbf{(7)}$$

Where

K_d = Dissociation constant (mol L^{-1})

[P] = Protein concentration in solution (mol L^{-1})

[P-Hg^{2+}] = Protein/mercury complex concentration in solution (mol L^{-1})

Substituting equation (7) into the Nernst equation leads to:

$$E = E^0_{Hg^{2+}/Hg^0} + \frac{0.0591}{2} \log \frac{K_d[P - Hg^{2+}]}{[P]} \qquad \textbf{(8)}$$

$$K_d = 10^{\left\{ \frac{\left(E - E^0_{Hg^{2+}/Hg^0}\right)2}{0.0591} - \log \frac{[P - Hg^{2+}]}{[P]} \right\}} \qquad \textbf{(9)}$$

Values of E, [P] and [P-Hg^{2+}] were determined experimentally and applied to equation 9 in order to obtain the dissociation constant values for the observed interactions. Tables 5 and 6 show the dissociation constant values of Hg-Ovalbumin and Hg-BSA complexes. An average of the K_d values for each protein was obtained from eight independent measurements, and the mean values for Hg-Ovalbumin and Hg-BSA were 6.88×10^{-16} mol L^{-1} and 5.46×10^{-14} mol L^{-1}, respectively.

Table 3: Quantitative determinations of Ovalbumin solutions.

Sample	n	Taken x 10^{-9} / mol L^{-1}	Found x 10^{-9} / mol L^{-1}	RSD / %	Recovery / %
1	3	7.44	7.20 ± 0.0036	5	97
2	3	12.3	11.9 ± 0.0059	5	97
3	3	17.2	17.3 ± 0.0086	5	101
4	3	123.0	116.0 ± 0.0580	5	94
5	3	148.0	138.0 ± 0.0690	5	93
6	3	196.0	192.0 ± 0.0960	5	98
7	3	990.0	865.0 ± 0.4325	5	87
8	3	1230.0	1230.0 ± 0.6150	5	100
9	3	1480.0	1350.0 ± 0.6750	5	91

Table 4: Quantitative determinations of BSA solutions.

Sample	n	Taken x 10^{-9} / mol L^{-1}	Found x 10^{-9} / mol L^{-1}	RSD / %	Recovery / %
1	3	9.90	8.9 ± 0.0041	5	83
2	3	12.3	10.3 ± 0.0052	5	84
3	3	14.8	14.4 ± 0.0072	5	97
4	3	99.0	86.0 ± 0.0430	5	87
5	3	123.0	122.0 ± 0.0610	5	99
6	3	148.0	134.0 ± 0.0670	5	91
7	3	990.0	863.0 ± 0.4315	5	87
8	3	1230.0	1140.0 ± 0.5700	5	93
9	3	1480.0	1290.0 ± 0.6450	5	87

Table 5: Dissociation constants of Hg-Ovalbumin complex.

E / mV	[Ovalbumin] / mol L^{-1}	[Hg-Ovalbumin] / mol L^{-1}	K_d / mol L^{-1}
-70	1.24×10^{-7}	6.20×10^{-7}	1.67×10^{-15}
-70	1.25×10^{-7}	8.65×10^{-7}	1.21×10^{-15}
-70	1.27×10^{-7}	13.53×10^{-7}	7.84×10^{-15}
-70	6.64×10^{-9}	11.64×10^{-8}	4.77×10^{-16}
-70	1.00×10^{-8}	13.80×10^{-8}	6.06×10^{-16}
-70	4.00×10^{-9}	19.20×10^{-8}	1.74×10^{-16}
-70	2.44×10^{-10}	7.20×10^{-9}	2.83×10^{-16}
-70	4.30×10^{-10}	11.87×10^{-9}	3.03×10^{-16}
			$\overline{X} = 6.88 \times 10^{-16}$

Table 6: Dissociation constants of Hg-BSA complex.

E /mV	[BSA] / mol L^{-1}	[Hg-BSA] / mol L^{-1}	K_d / mol L^{-1}
-20	1.27×10^{-7}	8.63×10^{-7}	6.05×10^{-14}
-20	8.60×10^{-8}	11.44×10^{-7}	3.09×10^{-14}
-20	1.95×10^{-7}	$12{,}85 \times 10^{-7}$	6.24×10^{-14}
-20	1.30×10^{-8}	8.60×10^{-8}	6.22×10^{-14}
-20	1.34×10^{-8}	13.46×10^{-8}	4.09×10^{-14}
-20	1.71×10^{-9}	8.19×10^{-9}	8.58×10^{-14}
-20	2.04×10^{-9}	10.26×10^{-9}	8.17×10^{-14}
-20	4.30×10^{-10}	14.37×10^{-9}	1.23×10^{-14}
			$\overline{X} = 5.46 \times 10^{-14}$

DISCUSSION

Differential pulse voltammetry proved to be a suitable technique to investigate the interaction of mercury with macromolecules such as BSA and Ovalbumin. It has long been observed that voltammetric studies of proteins were operationally difficult to be performed due to the strong adsorption problems that proteins produce when they are in contact with the electrochemical cell [62]. The experimental conditions developed in this work allowed qualitative and quantitative studies of the interactions between mercury ions and BSA or Ovalbumin. Quantitative determinations of BSA and Ovalbumin, at nanomolar concentrations, were obtained successfully and the methodology proposed to investigate the mercury-albumin interaction could be further used in the development of a sensitive procedure to determine the concentration of other proteins with high affinity for mercury in basic media [60]. The observed interactions between mercury and the two albumins investigated proved to be highly specific with K_d values lower than 10^{-13} mol L^{-1}. Such K_d values are considerably higher than those found for classical metal chelating substances such as EDTA (7.94×10^{-23} mol L^{-1}; pH 7), Ethylenediamine (6.31×10^{-24} mol L^{-1}; pH 7), and free cysteine (7.67×10^{-23} mol L^{-1}; pH 10.2). However, these data imply that both BSA and Ovalbumin may still play an important physiological role regarding mercury chelation in the organism, supporting the wildly accepted claim that serum albumins may work as “scavengers” for many organic substances and metal ions [1, 2]. Especially in the case of BSA, Cys34 has been considered the major coordinating center in the binding site for mercury [6, 38, 43], the discrepancy between the experimental K_d values for free cysteine and BSA obtained here, might be explained by the differences in accessibility of the metal to Cys34 in BSA, compared to the free amino acid in solution. The observed one hundred fold difference in K_d values between BSA (5.46×10^{-14} mol L^{-1}) and Ovalbumin (6.88×10^{-16} mol L^{-1}) might also be related to structural peculiarities of each one of these proteins and the way in which the free thiol groups of the cysteine residues present in Ovalbumin [16-18] and BSA [1, 2] are involved in the process of complex formation for each protein. In addition to that, the electrostatic, stereochemical and solvation effects may also constitute important factors to establish the binding environment of each site.

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REFERENCES

[1] Carter, D.C. and Ho, J.X. (**1994**) *Adv. Prot. Chem.*, *45*, 153.

[2] Kragh-Hansen, U. (**1981**) *Pharm. Reviews*, *33*, 17.

[3] Emerson Jr., T.E. (**1989**) *CRC Crit. Care Med.*, *17*, 690.

[4] Cecil, R. and McPhee, J.R. (**1959**) *Adv. Prot. Chem.*, *14*, 255.

[5] Fogh-Andersen, N. (**1977**) *Clin. Chem.*, *23*, 2122.

[6] Foster, J.F. (**1960**) In *The Plasma Proteins* (Putnam, F.W., Ed.). pp. 179-239. Academic Press, New York.

[7] Foster, J.F. (**1977**) In *Albumin Structure, Function and Uses*, (Rosenoer, V.M., Oratz, M. and Rothschild, M.A., Eds.), Pergamon, Oxford.

[8] Peter Jr., T. (**1985**) *Adv. Prot. Chem., 37*, 161.

[9] Brown, J.R. (**1977**) In *11th F.E.B.S. Meeting, Albumin Structure, Biosynthesis, Function* (Peters T. and Sjöholm, I., Eds.), Pergamon, Oxford.

[10] Reed, R.G., Putman, F.W. and Peters Jr., T. (**1980**) *Biochem. J., 191*, 867.

[11] Fevold, H.L. (**1951**) *Adv. Prot. Chem., 6*, 188.

[12] Saito, Z and Martin, W.G. (**1966**) *Canad. J. Biochem., 44*, 293.

[13] Taborsky, G. (**1974**) *Adv. Prot. Chem., 28*, 1.

[14] Goux, W.J. and Venkatasoubramanian, P.N. (**1986**) *Biochemistry, 25*, 84.

[15] McReynolds, L., O'Malley, B.W., Nisbel, A.D., Fothergill, J.E., Givol, D., Fields, S., Robertson, M. and Brownlee, G.G. (**1978**) *Nature, 273*, 723.

[16] Woo, S.L.C., Beattie, W.G., Catterall, J.F., Dugaiczyk, A., Staden, R., Brownlee, G.G. and O'Malley, B.W. (**1981**) *Biochemistry, 20*, 6437.

[17] Nisbet, A.D., Saundry, R.H., Moir, A.J.G., Fothergill, L.A. and Fothergill, J.E. (**1981**) *Eur. J. Biochem., 115*, 335.

[18] Thompson, E.O.P. and Fisher, W.K. (**1978**) *Aust.J.Biol.Sci., 31*, 433.

[19] Stein, P.E., Leslie, A.G.W., Finich, J.T. and Carrell, R.W. (**1991**) *Biochemistry, 221*, 941.

[20] Scatchard, G. (**1949**) *Ann N. Y. Acad. Sci. USA, 51*, 600.

[21] Lau, S. and Sarkar, B. (**1979**) *J. Toxicol. Environ. Health, 5*, 907.

[22] Pedersen, S.M. (**1987**) *Biochem. Pharmacol., 36*, 2661.

[23] Singh, H., Flynn, A. and Fox, P.F. (**1989**) *J. Dairy Res., 56*, 235.

[24] Bentley, P.J. (**1991**) *Comp. Biochem. Phisiol. C, 100*, 491.

[25] Predki, P.F., Harford, C., Brar, P. and Sarkar, B. (**1992**) *Biochem. J., 287*, 211.

[26] Masuoka, J., Hegenauer, J., Van Dyke, B.R and Saltman, P. (**1993**) *J. Biol. Chem., 268*, 21533.

[27] Masuoka, J. and Saltaman, P. (**1994**) *J. Biol. Chem., 269*, 25557.

[28] Bal, W., Christodoulou, J., Sadler, P.J. and Tucker, A. (**1998**) *J. Inorg. Biochem., 70*, 33.

[29] Danchin, A. (**1969**) *J. Theor. Biol., 25*, 317.

[30] Sun, H., Li, H., Mason, A.B., Woodworth, R.C. and Sadler, P.J. (**2001**) *J. Biol. Chem., 276*, 8829.

[31] Rao, N. and Feldman, S. (**1990**) *Pharm. Res., 7*, 188.

[32] King, S.W., Wills, M.R. and Savory, J. (**1979**) *Res. Commun. Chem. Pathol. Pharmacol., 26*, 161.

[33] Lucassen, M. and Sarkar, B. (**1979**) *J. Toxicol. Environ. Health, 5*, 897.

[34] Veprintsev, D.B., Permyakov, E.A., Kalinichenko, L.P. and Berliner, L.J., (**1996**) *Biochem. Mol. Biol. Int., 39*, 1255.

[35] Andersson, L., Sulkowski, E. and Porath, J. (**1991**) *Bioseparation, 2*, 15.

[36] Hutchens, T.W., Yip, T.T. and Porath, J. (**1998**) *Anal. Biochem., 170*, 168.

[37] Millard, M.M. (**1974**) *Adv. Exp. Med. Biol., 48*, 589.

[38] Zhou, Y., Hu, X., Dou, C., Liu, H., Wang, S. and Shen, P. (**1992**) *Biophys. Chem., 42*, 201.

[39] Berg, G.G. and Miles, E.F. (**1978**) *Membr. Biochem., 2*, 117.

[40] Sandier, A., Amiel, C., Sébille, B., Rouchaud, J.C., Fedoroff, M. and Soltes, L., (**1997**) *J. Chromatogr A*, 776, 93.

[41] Waldmann-Meyer, H. (**1960**) *J. Biol. Chem.*, 235, 3337.

[42] Fang, S.C. and Fallin, E. (**1976**) *Bull. Environ. Contam. Toxicol.*, 15, 110.

[43] Saroff H. A. and Mark, H.J. (**1953**) *J. Am. Chem. Soc.*, 75, 1420.

[44] Doumas, B.T. and Biggs, H.G. (1972) *Stand. Methods Clin. Chem.*, 7, 175.

[45] Ruststein, D.D., Ingenito, E.F. and Reynolds, W.E. (**1954**) *J. Clin. Invest.*, 33, 211.

[46] Wren, H.T. and Fetchmeyer, T.V. (**1956**) *Am. J. Clin. Pathol.*, 26, 960.

[47] Brdicka, R. (**1933**) *Collection Czech. Chem. Commun.*, 5, 112.

[48] Palecek, E. and Pechan, Z. (**1971**) *Anal. Biochem.*, 42, 59.

[49] Kolthoff, I.M. and Kihara, S. (**1977**) *Anal. Chem.*, 49, 108.

[50] Alexander, P.W. and Shah, M.H. (**1980**) *Anal. Chem.*, 52, 1896.

[51] Hertl, W., (**1987**) *Anal. Biochem.*, 164, 1.

[52] Qiu, J. and Song, J. (**1996**) *Anal. Biochem.*, 240, 13.

[53] Suznjevi, D., Erceg, M. and Vucelic, D. (**2001**) *Microchemical J.*, 69, 59.

[54] Liu, S.P. and Liu, Q. (**2001**) *Anal. Sci.*, 17, 239.

[55] Liu, S., Yang, R. and Liu, Q. (**2001**) *Anal. Sci.*, 17, 243.

[56] Cao, Q.E., Ding, Z., Fang, R. and Zhao, X. (**2001**) *Analyst*, 126, 1444.

[57] Li, Y.F., Huang, C.Z. and Li, M. (**2002**) *Anal. Sci.*, 18, 177.

[58] Freese, F., Jasper, H.J. and Den Boef, G. (**1970**) *Talanta*, 17, 945.

[59] Dieker, J.W., Van der Linden, W.E. and Den Boef, G. (**1977**) *Talanta*, 24, 321.

[60] Castro, C.S.P., Souza, J.R. and Bloch Jr., C. (**2002**) *Protein and Peptide Letters*, 9, 45.

[61] Miller, J.C.; Miller, J.N. (**1989**) in *Statistics for Analytical Chemistry*, John Wiley and Sons, pp. 115-117 Chichester.

[62] Castro, C.S.P., Souza, J.R. and Bloch Jr., C. (**2001**) *Ciência e Cultura*, 53, 88.

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