



Behavior of glutathione as ligand of lead (II)

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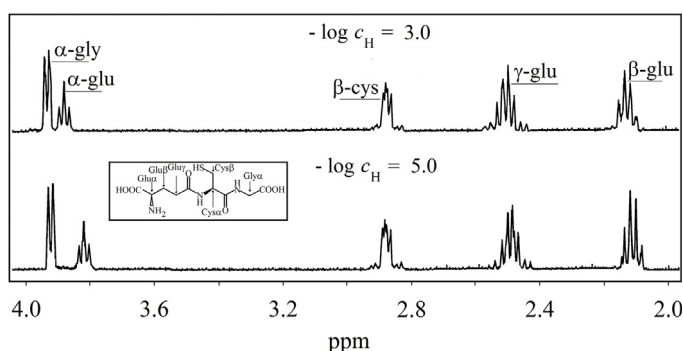
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HIGHLIGHTS

- Glutathione can work as polydentate chelating agent.
- Glutathione contained in plants can be useful for phytoremediation of lead (II) pollution.
- The chelation of glutathione with lead (II) gives rise to three 5 and 6 membered rings.

GRAPHICAL ABSTRACT



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ABSTRACT

The presence and mobilization of toxic metal cations represents under many aspects a current and important problem in the environmental field. In this research, as cation lead (II) ion was studied. The formation of complexes between glutathione and lead (II) was studied at 25 °C and in 1.00 M NaCl as ionic medium by means of measurements of electromotive force (e.m.f.) of cells containing glass and lead amalgam electrodes. In the same experimental conditions, the protonation constants of glutathione were determined potentiometrically, using a cell containing the glass electrode. In the same experimental conditions, by considering glutathione (GSH) completely deprotonated, four protonation constants were determined.

Potentiometric data could be explained by assuming the formation of 1:1 complexes between GSH and Pb²⁺ and with the participation of hydrogen ions. The stability constants of the assumed complexes were determined. The 1:1 ratio between GSH and lead (II) was confirmed by spectrophotometric investigations.

Measurements by Infrared Rays (IR) and protonic Nuclear Magnetic Resonance (¹H NMR) provide information on the structure of the found complexes.

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1. Introduction

The ecosystem can be considered as a general equilibrium between water, soil and air, influenced by light temperature, as main parameters. The right healthiness of the system is recently suffering

for several polluting agents. The cations of toxic metals are nonbiodegradable and accumulate in the environment. The consequent contamination involves risks in the feed chain, absorption from vegetal organisms and in the soil, with serious damage because often these metals are carcinogenic, mutagenic and others. In the soil, in particular, metals as lead (II) can produce toxic actions on soil microbes (Khan et al. 2010). As an example, Yoon et al. (2006) put into evidence the accumulation of Pb, Cd and Zn in native plants growing on a contaminated Florida site. Sadana and Singy (1987) studied the yield and uptake of Cd, Pb, and Zn by wheat grown in a soil polluted with these metals.

Lead (II) presents dangerous effects on human and plant health. Concerning the cellular metabolism, lead (II) can inhibit some enzymes (Barrio Diez-Caballero et al., 1985), obstructs the summary of the heme and can lead to anemia. Bassi (1991) evaluated a possible link between environment and gene expression in plants and Bassi et al. (1995) investigated on the effects of lead on the nuclear repetitive DNA of the moss *Funaria hygrometrica*. Salem et al. (2000), Padmavathamma and Li (2007), Wuana and Okieimen (2011) and Iqbal (2012) found that a lead absorption in children can produce reduction of intelligence, loss of memory, renal failure, risk of cardiovascular disease.

The interest of the problem has suggested employing different chemical methods to minimize the pollution and to protect both soil and consequently feed chain of animals and humans. Kumar et al. (1995) propose the use of phytoextraction as remedy to remove toxic metals from soil. Pulford and Watson, 2003 published an interesting review on phytoremediation applied to toxic metal contaminated land by trees.

Concepts and applications on phytoremediation of toxic metals are proposed by Ali et al. (2013), to limit the contaminant concentration in the environment. The absorption of cations can be helped by the presence in plants of chemical compounds able to bond the cation forming stable complexes.

The glutathione (GSH) is an antioxidant tripeptide constituted by cysteine and glycine connected through a normal peptide bond and glutamate, which instead is bound to cysteine with an atipic peptide bond between the carboxyl-group of the side chain of glutamate and the amino group of the cysteine. In Fig. 1 the structure of glutathione is reported.

Meister and Anderson (1983) and Meister (1988) found that the concentration of GSH in the cells of plant, animals and microorganisms is in the range $0.1-10 \times 10^{-3}$ M.

As glutathione can be a main responsible of the phytoremediation, seems very interesting to study the equilibria of complex formation between GSH and cations. Really, from an inspection of Fig. 1, it can be observed that GSH is potentially a polydentate chelator. It possesses carboxyl-, amino and amide groups and a SH group. All these chemical functions are able to bond cations to form complexes.

These observations suggest studying the reactions taking place between GSH and lead (II) to prove if the use of plants with a content of GSH can be useful to reduce the lead (II) presence in

environment.

Only few papers report the complex formation between lead (II) and glutathione. Li and Manning (1955) studied the behavior of GSH as ligand of lead (II) by employing a glass electrode (G.E.) at 25 °C and in 0.15 M KNO₃ explaining the experimental data assuming only a complex PbL (L is GSH in the completely deprotonated form) with a constant $\log \beta = 10.60$. Corrie et al. (1976) explained their experimental data obtained potentiometrically at 25 °C and in 1.00 M NaClO₄, assuming the formation of complexes in the ratio 1:1 and 1:2 and of a series of protonated and deprotonated species with relative stability constants (PbL, $\log \beta_1 = 10.57$; PbL₂, $\log \beta_2 = 15.00$ and PbHL, $\log \beta = 17.14$; PbHL₂, $\log \beta = 24.66$; PbH₂L₂, $\log \beta = 32.10$ and PbH₁L₂, $\log \beta = 4.50$). In a subsequent paper, always Corrie and Williams (1976), in the same experimental conditions as above, but from calorimetric measures, propose the formation of PbL ($\log \beta_1 = 9.91$), PbHL ($\log \beta = 16.82$), PbHL₂ ($\log \beta = 23.40$) and PbH₂L₂ ($\log \beta = 32.31$). With electrospray ionization mass spectroscopy, Burford et al. (2005) detected spectra of two species, assuming in both cases 1:1 complexes. One of them was formed with assumption of two protons. Cruz et al. (2001) explained their polarographic data by assuming the formation of mononuclear and dinuclear complexes, both with assumption of two protons. More recently, Mah and Jalilehvand (2012) performed a structural investigation of complexes between Pb²⁺ and glutathione in aqueous solutions and in the solid state, but no complex formation constants proposed, only an approach on a possible model.

The inspection of literature shows that few and not in agreement data exist on the equilibria taking place between lead (II) and GSH, even if they were obtained in different conditions. However, we were not able to find studies performed in 1.00 M NaCl.

The aim of this paper is to study the behavior of glutathione as ligand of lead (II) in a large range of concentration of reagents in aqueous solutions, to find the type of interaction taking place, in terms of equilibrium constants.

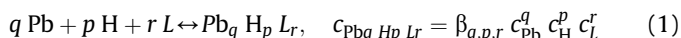
2. Materials and methods

2.1. Experimental procedure

The proposed wide variation of the reagent concentration would provoke a variation of activity coefficients of the reagents. To minimize this effect, the method of the constant ionic medium, proposed by Biedermann and Sillén (1953) was adopted. In this way, by substituting until 15% of the ionic medium with the reagents, the activity coefficients do not change appreciably, in spite of variation of concentrations. In the constant ionic medium, concentrations can substitute activities.

In this research, 1.00 M NaCl as ionic medium is adopted and all the measurements are carried out at 25 °C.

The equilibrium constant for the formation of a complex Pb_qH_pL_r (where L is GSH in form completely deprotonated) from GSH and Pb²⁺, is written as follows:



where charges are omitted and $q \geq 1$, $p \geq 0$ and $r \geq 1$. If $q > 1$, polynuclear complexes are formed, $p > 0$ indicates complexes with assumption of protons, while $p < 0$ corresponds to cession of protons or assumption of OH⁻.

In expression (1) and in the following, small *c* indicates free concentration of the reagents, while capital *C* is referred to their total concentrations.

The aim of this paper is the determination of *q*, *p*, and *r* and the corresponding $\beta_{q,p,r}$.

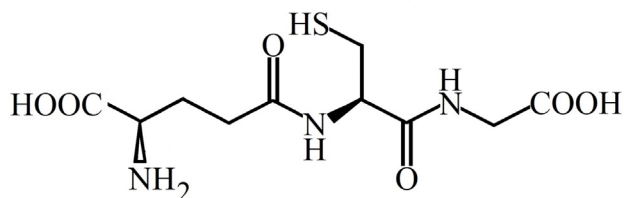


Fig. 1. Structure of glutathione.

Before studying the relation (1), it is necessary to determine in the same experimental conditions the protonation constants of GSH, defined as follows:

$$C_{HnL} = K_n C_H C_{Hn-1L} \quad (2)$$

where n can be 1, 2, 3, 4.

2.1.1. Protonation constants of GSH

Several researchers studied the glutathione protolytic equilibria. The main literature investigations are collected by Powell (2000), but they are incomplete and not agreeing each other. Mah and Jalilehvand (2012) report values of the GSH dissociation constants determined by NMR by Rabenstein et al. (1989), but no information about the experimental conditions adopted are cited. The results of Rabenstein et al. (1989) are not present in the main collection of stability constants of Powell (2000). Some authors determine only one or two constants, others have not determined the fourth. However, the reported amino-protonation and S-protonation constants suffered the alkaline error of G.E., while those of carboxyl-, determined at very high hydrogen ion concentrations, suffered very high liquid junction potential errors because many of them are carried out in ionic strength 0.1–0.5 M KNO_3 .

The protonation constants of GSH, indicated with L in its completely deprotonated form, were determined in 1.00 M NaCl and at 25 °C, by performing electromotive force (e.m.f.) measurements of the cell:

R.E. / Solution S1 / G.E. (I)

where R.E. and G.E. are reference and glass electrode, respectively.

At 25 °C, in millivolts and in constant ionic medium, the e.m.f. of the cell (I) can be expressed as follows:

$$E = E^\circ + 59.16 \log c_H + E_j \quad (3)$$

In eq. (3), E° , a constant, and $E_j = -j c_H$, according to Biedermann and Sillén (1953), were determined in the first part of each measure, where solution S1 had the following general composition:

C_H M in H^+ ; $(1 - C_H)$ M in Na^+ ; 1.00 M in Cl^-

In the second part of the measure, a solution of glutathione in the selected ionic medium was added and $-\log c_H$ gradually increased, by keeping C_{GSH} constant.

2.1.2. Complex formation between lead (II) and glutathione

The investigation on the behavior of glutathione as ligand of lead (II) was performed with several different procedures always at 25 °C. At first, the aim was to find the existent species formed in solution and to obtain the relative stability constants. For this purpose, e.m.f. measurements were performed in 1.00 M NaCl. The second investigation, carried out spectrophotometrically, was performed to obtain the stoichiometric ratio between lead (II) and GSH and the third, looking at to obtain information on the possible structure of the formed complexes, involved protonic Nuclear Magnetic Resonance (1H NMR) measurements in ionic medium 1.00 M NaCl. Finally, Infrared Rays (IR) spectra were applied to approach a hypothesis of the precipitate formation structure.

2.1.2.1. E.M.F. measurements. The e.m.f. of the following cells:

Pb(Hg)/Solution S2/R.E. (II)

R.E./Solution S2/G.E. (III)

was measured at 25 °C. In the cells (II) and (III) R.E. and G.E. are reference electrode and glass electrode, respectively, whereas Pb (Hg) is a lead amalgam electrode.

The e.m.f. of the cells (II) and (III) at 25 °C in millivolt and in constant ionic medium can be expressed as follows:

$$E_{II} = E_{II}^\circ - 29.58 \log c_{Pb} - E_j \quad (4a)$$

$$E_{III} = E_{III}^\circ + 59.16 \log c_H + E_j \quad (5a)$$

In the expressions (4a) and (5a) E_{II}° and E_{III}° , two constants, and E_j , the liquid junction potential, were determined in the first part of each measure when the general composition of solution S2 was:

C_{Pb} M in Pb^{2+} ; C_H M in H^+ ;

$(1 - 2 C_{Pb} - C_H)$ M in Na^+ ; 1.00 M in Cl^-

everything on the same line

In the second part of the measure, an alkaline solution of GSH was added. Gradually, in the constant ionic medium, the concentration of glutathione and $-\log c_H$ increased, whereas for each measure C_{Pb} and C_H were kept constant.

The experimental data $-\log c_{Pb}$ and $-\log c_H$, together with the analytical knowledge of the total concentrations, C_{Pb} , C_H and C_{GSH} are the basis to obtain the formed complexes and their stability constants.

2.1.2.2. Spectrophotometric data. Spectrophotometric measurements were performed in solutions having the composition as solution S2. As shown in Fig. 2, spectra present considerable variation with changes in $-\log c_H$; it is evident the presence of isosbestic point at 270 nm, compatible with the presence of equilibria in solution.

2.1.2.3. 1H NMR measurements. The 1H NMR spectra were recorded as a function of $-\log c_H$ in 1.0 M NaCl (1% D_2O). In according to Fig. 3, it's evident a shift of α -glu proton signals with increasing $-\log c_H$ values. This result is in agreement with its involvement in the complexation.

2.1.2.4. IR analysis. The Pb-GSH precipitate solid (A) and GSH solid (B) were analyzed by FT-IR spectroscopy as reported in Fig. 4. As GSH is constituted (vide Fig. 1) by glycine, cysteine and glutamate, it

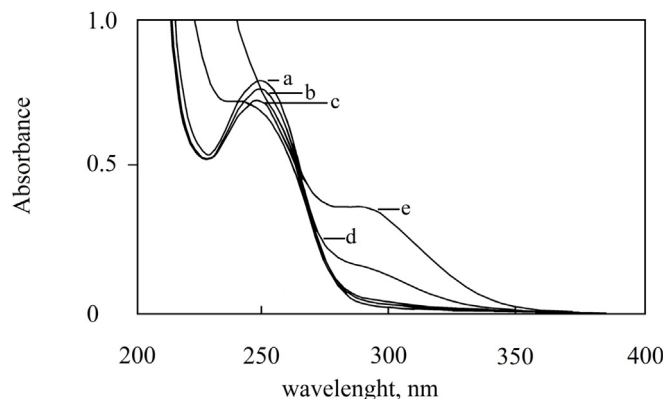


Fig. 2. UV spectra for Pb (II)–GSH system in 1 M NaCl as function of $-\log c_H$ with $C_{Pb} = 2.5 \cdot 10^{-3}$ M and $C_{GSH} = 2.5 \cdot 10^{-3}$ M ($-\log c_H$: a = 2.84, b = 3.79, c = 4.99, d = 5.50, e = 5.99).

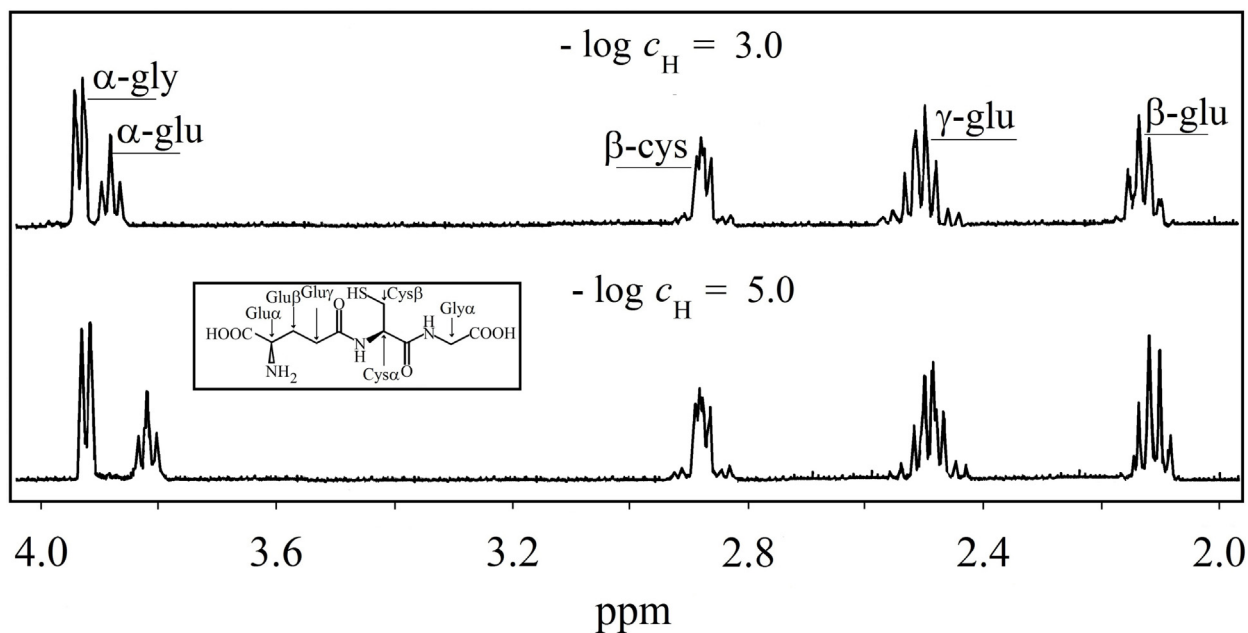


Fig. 3. ^1H NMR spectra for Pb(II)-GSH system in 1 M NaCl (1% D_2O) as function of $-\log c_{\text{H}}$ with $C_{\text{Pb}} = 2.5 \cdot 10^{-3}$ M and $C_{\text{GSH}} = 2.5 \cdot 10^{-3}$ M.

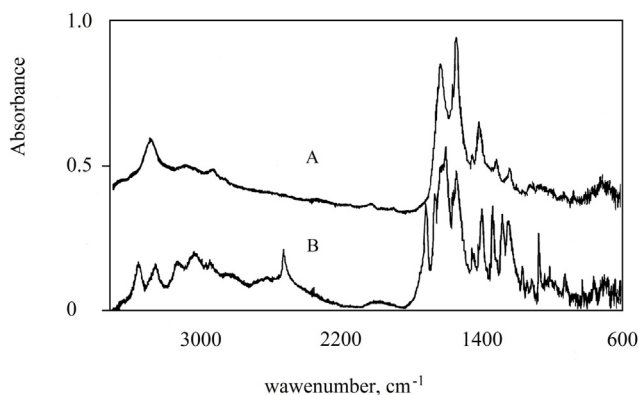


Fig. 4. FT-IR spectra of Pb-GSH solid (A) and GSH (B).

can be observed that the spectrum of GSH alone shows a peak at 1730 cm^{-1} , attributed from literature to the group $-\text{COOH}$ of glycine, a peak at 2530 cm^{-1} attributed to the group $-\text{SH}$ and an amide bond relative to the zone $3200\text{--}3400\text{ cm}^{-1}$. Instead, it is observed that in the spectrum of the precipitate Pb-GSH (A) the peaks at 1730 and 2530 cm^{-1} are absent. Furthermore, there are less peaks in A spectrum than B spectrum in the zone of amide bond absorbances.

The inspection about the IR results suggests that lead (II) can be bound to GSH through three bonds: carboxyl-, sulfur and amide nitrogen. By looking at the formula of Fig. 1, it can be formulated the hypothesis that three 5 or 6 membered rings can be formed among the three cited dents of GSH and lead (II) ion. This configuration could be particularly stable.

2.1.3. Chemical and analysis

Lead (II) chloride, sodium chloride, hydrochloric acid, stock solutions were prepared and analyzed as described previously [Bottari and Severini, (1978); Bottari et al. (1988, 2011)].

L-Glutathione (reduced form), product Sigma, $\geq 99\%$ was

crystallized twice from water. The compound dry was stored in vacuum desiccator. A sample of purified GSH, analyzed by polarography was absent of electroactive species.

Lead amalgam ($\approx 0.1\%$) was prepared by immersing metallic lead (Kooch and Light 99.9%) in bi-distilled mercury in nitrogen atmosphere. The amalgam was stored under 0.01 M HClO_4 .

All solutions were freed from O_2 and CO_2 by bubbling nitrogen from cylinders, (purity 99.999%) further purified by passing it through 10% NaOH, 10% H_2SO_4 , distilled water and 1.00 M NaCl .

2.1.4. Apparatus

The e.m.f. measurements were carried out in a thermostatic room at $25.0 \pm 0.5\text{ }^\circ\text{C}$ and in a thermostat at $25.00 \pm 0.05\text{ }^\circ\text{C}$. The cell arrangement was similar to that described by W. Forsling et al. (1952).

The reference electrode R.E. of the cells (I, II and III) was Ag, AgCl/1.00 M NaCl saturated in AgCl/1.00 M NaCl. Ag, AgCl was prepared according to Brown (1934).

E_{II} , measured with a Keathly 2000 Multimeter, provided constant values within half an hour after each addition, remaining constant for several hours. The lead amalgam electrode functioned satisfactory in our solutions and gave $E_{\text{II}} - E^\circ_{\text{II}}$ values reproducible within $\pm 0.1\text{ mV}$.

E_{I} and E_{III} were measured by employing Radiometer potentiometer mod. PHM 64 and G.E. of the same firm. Constant values were obtained in a few minutes after each addition and remained constant for several hours. The values $E_{\text{I}} - E^\circ_{\text{I}}$ and $E_{\text{III}} - E^\circ_{\text{III}}$ were reproducible within $\pm 0.2\text{ mV}$. In the range $-\log c_{\text{H}} \geq 9$, the response

Table 1

Values of the protonation constants of glutathione in the form L, at $25\text{ }^\circ\text{C}$ and in 1.00 M NaCl .

	Log K_1	Log K_1K_2	Log $K_1K_2K_3$	Log $K_1K_2K_3K_4$
Graphic method	9.55 ± 0.03	18.15 ± 0.03	21.59 ± 0.03	23.84 ± 0.04
PC	9.53 ± 0.01	18.19 ± 0.02	21.62 ± 0.03	23.81 ± 0.03

The limits of error were estimated from the maximum shift possible for the calculated curve and experimental points for which agreement was still acceptable.

of GE was corrected on the basis of that of a H₂ electrode.

Each e.m.f. measure was interrupted when a slow drift of the amalgam potential was observed. This was due to a slow precipitation. After two or three days, solutions still clear, showed light gray color whereas the precipitate appeared after standing for several days. Some measurements carried out at relatively high $-\log c_H$ (≥ 4.5) were performed on oversaturated solutions. However, it can be assumed that also such measures were carried out at equilibrium because both direct and back titrations agree well.

An UV-visible Varian Cary 50 spectrophotometer was used to obtain absorbance spectra of solutions at different wavelength. Measures were carried out in UV region with quartz cuvettes that before each use they were treated with a mixture of H₂SO₄/H₂O₂ and rinsed with plenty of bi-distilled water.

All the ¹H NMR spectra were recorded at 25 °C on a Bruker Unity spectrometer operating at a proton frequency of 400 MHz and located at the Department of Chemistry Science in Naples (Italy).

The FT-IR measurements were performed with an interferometer JASCO model 430 using KBr tablets. The KBr powder before being pressed is dried at 120 °C for overnight. The absorbance values were registered by resetting the instrument with KBr tablet as blank.

3. Results and discussion

3.1. Protonation constants of the ligand

Measures were performed at 0.010 and 0.020 M glutathione concentrations. From the material balance of the analytical excess of hydrogen ion, C_H, by taking in to account the mass action law, it can be written:

$$C_H = c_H + \sum n K_n c_H^n c_L \quad (4b)$$

and, from the glutathione material balance:

$$C_{GSH} = c_L + \sum K_n c_H^n c_L \quad (5b)$$

By plotting the protonation function $\mathbf{p} = (\sum n K_n c_H^n) (1 + \sum K_n c_H^n)^{-1}$ versus $-\log c_H$, it can be observed that as points obtained at two different concentration of glutathione fall on the same curve, polynuclear species can be excluded. As \mathbf{p}_{\max} tends to four, four protonation constants have to be determined.

The value of these constants is obtained both applying the graphic method proposed by Sillèn (1956) and by elaborating the primary data by the PC program HYPERQUAD (1996). The results collected in Table 1 show an excellent agreement each other.

The values of the protonation constants collected in Table 1 are used to calculate the distribution percentages of GSH as a function of $-\log c_H$.

In Fig. 5 the distribution of the differently protonated species of GSH versus $-\log c_H$ is shown. The forms H₄L⁺, H₃L and H₂L⁻ predominate in the range $1 \leq -\log c_H \leq 5$, where this investigation is performed.

3.2. Complex formation

Experimental data were obtained for C_H = 0.025; 0.035; 0.050; 0.070 and 0.100 M and in the same time C_{Pb} varied from (0.25–2.00) × 10⁻³ M. The GSH concentration increased to about 0.025 M.

To study the reaction (1), the material balance of lead (II), C_{Pb}, and of the analytical excess of H⁺, C_H, by taking in to account the

law of mass action, can be written:

$$\eta = \log(C_{Pb} / c_{Pb}) = \log\left(1 + \sum \sum \sum q \beta_{q,p,r} c_{Pb}^{q-1} c_H^p c_L^r\right) \quad (6)$$

$$C_H = c_H + \sum n K_n c_H^n c_L + \sum \sum \sum p \beta_{q,p,r} c_{Pb}^q c_H^p c_L^r \quad (7)$$

In eq.s (6 and 7) hydrolytic species of lead (II) are neglected. This is permissible in the c_{Pb} and c_H ranges studied as estimated from results by Olin (1960) and Carell and Olin (1962).

The function η is plotted versus $-\log c_H$ in Fig. 6, without any hypothesis. Measures were carried out about to $-\log c_H \approx 4.5$.

The plot of Fig. 6 shows that points obtained at constant C_H, but at different C_{Pb} fall on the same curve. The function η is independent of C_{Pb} and then $q = 1$. Thus, only species mononuclear in lead(II), PbH_pL_r are present. Eq. (6) becomes:

$$\eta = \log\left(1 + \sum \sum \beta_{1,p,r} c_H^p c_L^r\right) \quad (8)$$

L must be italicus

To study the complex formation between lead (II) and glutathione, it is necessary to know the free concentration of glutathione, c_L, which can be calculated from the material balance of the analytical excess of hydrogen ion, eq. (7).

In eq. (7), K_n are the protonation constants previously determined in the same experimental conditions (vide Table 1) and the last term can be neglected, because in all investigated solutions, there are C_{Pb} ≤ 0.02 C_H.

The dependence of η on $-\log c_L$ is shown in Fig. 7, where main of the points is plotted. As points obtained at C_H different fall on different curves, mixed complexes with assumption of protons are formed because η is an increasing function of C_H.

To find the values of p and r and the relative stability constants, relationships are derived between two of the variables (at constant value of the others) which are examined by two variable methods according to Sillèn (1956).

Several series of values at constant $-\log c_H$ are obtained from the data of Fig. 6, and rearranging eq. (8) we have:

$$\phi = \log\left\{(10^\eta - 1)c_L^{-1}\right\} = \log\left\{\sum \gamma_r c_L^{r-1}\right\},$$

$$\text{where the functions } \sum \gamma_r = \sum \beta_{1,p,r} c_H^p \quad (9)$$

H is not italicus

In Table 2 the values obtained for ϕ at each C_H are collected. It can be seen that constant values of ϕ are obtained for each $-\log c_H$. The function ϕ is independent of c_L, so that it can be deduced that $r = 1$, i.e. complexes with only one glutathione per lead (II) are present. The values of γ_1 are dependent on $-\log c_H$, according to equation (9): $\sum \gamma_1 = \sum \beta_{1,p,1} c_H^p$.

Mathematically, the values of p could start from zero, so that the following equation can be written $\log \gamma_1 = \log (\beta_{1,0,1} + \beta_{1,1,1} c_H + \beta_{1,2,1} c_H^2 + \beta_{1,3,1} c_H^3)$, but it seems reasonable to exclude the presence of GSH completely deprotonated in this work investigated range. As it can be observed in Fig. 5, the form of GSH completely deprotonated is present at appreciable concentration in solution only at $-\log c_H \geq 8.5$. Furthermore, as the intercepts of a plot γ_1 versus c_H is zero, the complex PbL is not present in appreciable concentration in the here investigated range of $-\log c_H$. The above equation can be written as follows:

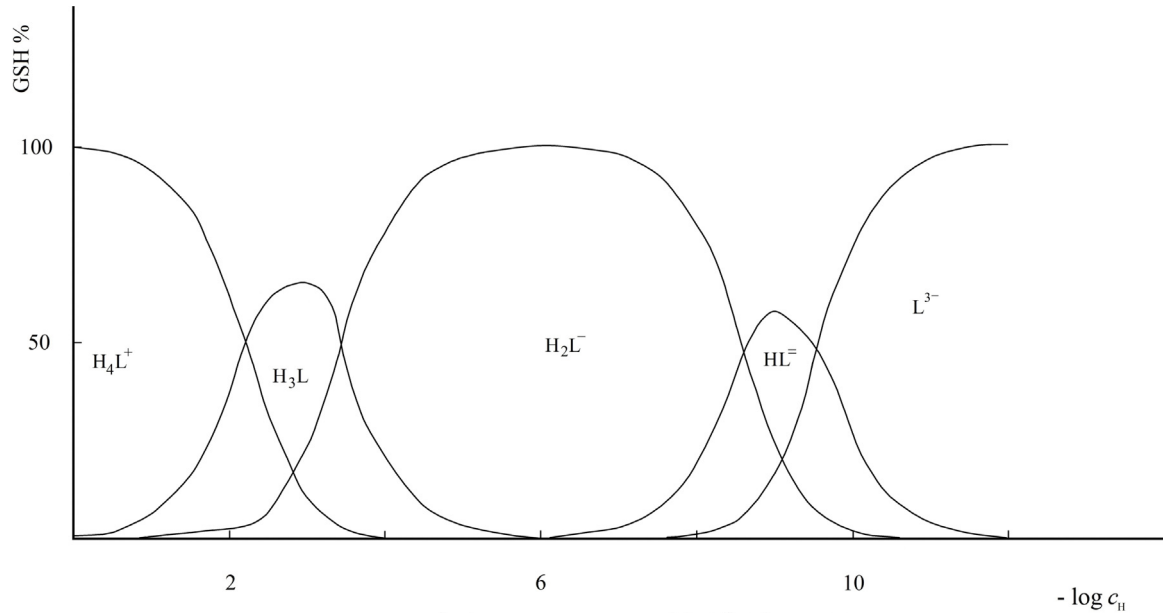


Fig. 5. Distribution of glutathione as a function of $-\log c_H$. Total concentration $C_{GSH} = 0.010$ M.

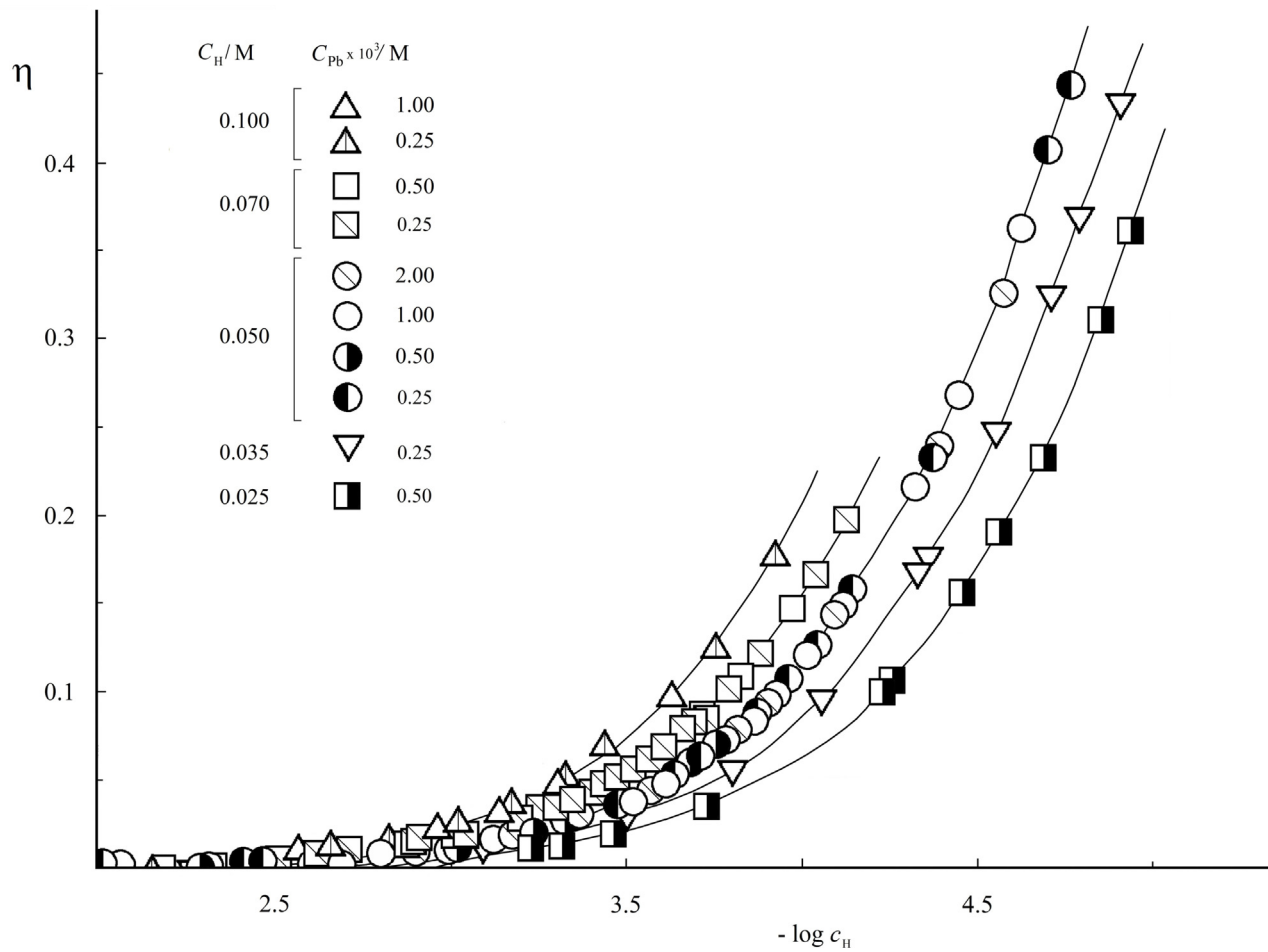


Fig. 6. The experimental data in the form η versus $-\log c_H$. The curves are calculated by the constants of Table 3 (vide below).

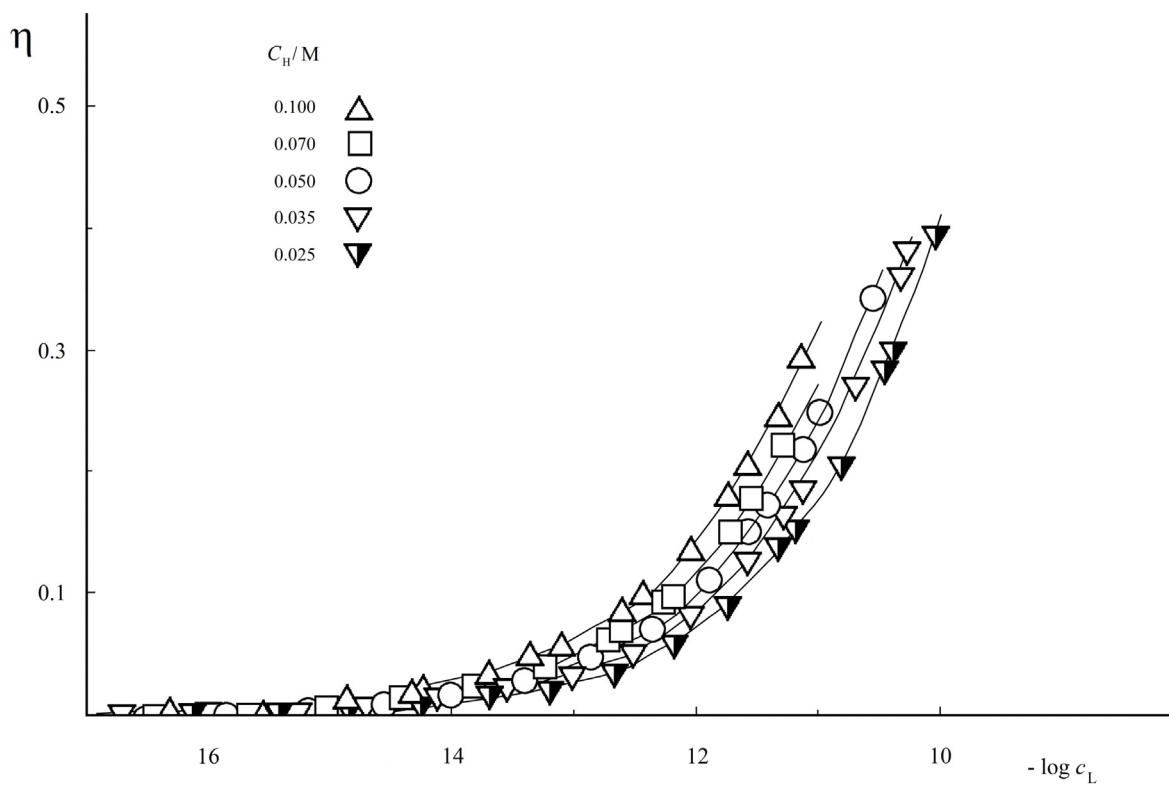


Fig. 7. Main of experimental data at different C_H , in the form η versus $-\log c_L$. Curves are calculated using the protonation constants of Table 1 and the stability constant of Table 3 (vide below).

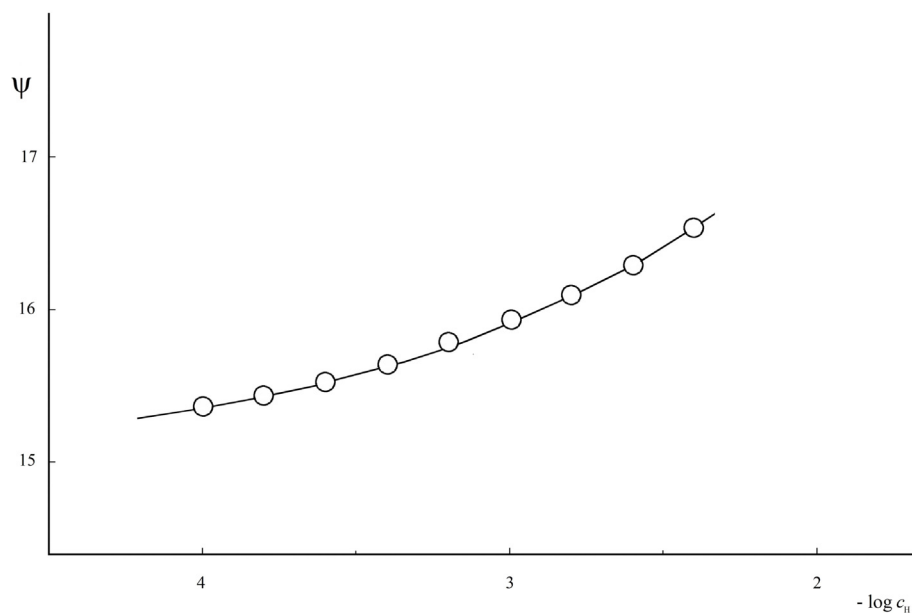


Fig. 8. Dependence of $\psi = \log \{(\sum \gamma_i) c_H^{-1}\}$ on $-\log c_H$. The curve is the normalized one in the position of best fit.

$$\psi = \log \left\{ \left(\sum \gamma_1 \right) c_H^{-1} \right\}$$

$$= \log \beta_{1,1,1} + \log \left(1 + \beta_{1,2,1} \beta_{1,1,1}^{-1} c_H + \beta_{1,3,1} \beta_{1,1,1}^{-1} c_H^2 \right)$$

in gamma 1, the number must be deponent
The formulas must be written as in the above lines

(10)

Eq. (10) can be normalized, according to Sill en (1956) in the following:

$$\psi = \log \left(1 + \alpha u + u^2 \right)$$
(11)

where $\Psi - \psi = \log \beta_{1,1,1}$; $\log u = \log c_H + 1/2 \log \beta_{1,3,1} \beta_{1,1,1}^{-1}$ and $\log \beta_{1,2,1} = \log \alpha + 1/2 \log \beta_{1,3,1} + 1/2 \log \beta_{1,1,1}$.

In Fig. 8 the theoretical curve Ψ of eq. (11) is superimposed on the data ψ versus $-\log c_H$ and the two graphics are shifted parallel to both axes till the best fit is reached. In correspondence of the best fit and on the basis of the mathematical positions, the following values are obtained:

$$\log \beta_{1,1,1} = 15.25 \pm 0.05;$$

$$\log \beta_{1,3,1} = 20.7 \pm 0.06 \text{ and}$$

$$\log \beta_{1,2,1} = 18.8 \pm 0.08$$
(12)

The limits of error represent the maximum deviation possible with a still acceptable agreement between experimental data and normalized curve.

These values are considered of first approximation. To refine them, theoretic values of η_c are calculated for each point and compared with the corresponding η_{ex} (experimental data of Figs. 6 and 7). The values of the constants (12) are varied within the reported error till to obtain an acceptable $\eta_c - \eta_{ex}$ difference. The refinement was concluded when it was obtained $\eta_c - \eta_{ex}$ within ± 0.002 , for nearly all points. Positive and negative deviations were comparable. The obtained values were considered refined stability

Table 2
Average values obtained for $\log \gamma_1 = \phi - \log c_H$.

$-\log c_H$	C_H	0.100	0.070	0.050	0.035	0.025	$\log \gamma_1$ average
Examples of values of ϕ calculated for each C_H and $-\log c_H$.							
2.40	$\phi =$	14.15	14.15	14.15	14.15	14.17	16.55 ± 0.01
2.60	$\phi =$	13.72	13.76	13.65	13.72	13.75	16.35 ± 0.02
2.80	$\phi =$	13.32	13.32	13.32	13.32	13.28	16.07 ± 0.04
3.00	$\phi =$	12.94	12.94	12.94	12.93	13.00	15.95 ± 0.04
3.20	$\phi =$	12.58	12.57	12.58	12.58	12.67	15.80 ± 0.07
3.40	$\phi =$	12.24	12.24	12.24	12.24	12.26	15.66 ± 0.07
3.60	$\phi =$	11.92	11.93	11.93	11.93	12.08	15.56 ± 0.03
3.80	$\phi =$	11.64	11.64	11.64	11.64	11.66	15.48 ± 0.02
4.00	$\phi =$	11.38	11.38	11.38	11.38	11.37	15.42 ± 0.01

The limits of error were estimated from the difference between the minimum and maximum calculated value.

Table 3
Complexes with refined constants assumed to explain the experimental data at 25 °C and in 1.00 M NaCl.

Species	First approximation constants	Refined constants
PbHL	$\log \beta_{1,1,1} = 15.23 \pm 0.05$	$\log \beta_{1,1,1} = 15.20 \pm 0.03$
PbH ₂ L	$\log \beta_{1,2,1} = 18.8 \pm 0.08$	$\log \beta_{1,2,1} = 18.80 \pm 0.06$
PbH ₃ L	$\log \beta_{1,3,1} = 20.7 \pm 0.06$	$\log \beta_{1,3,1} = 20.73 \pm 0.05$

The limits of error were estimated from the maximum shift possible for the calculated curve and experimental points for which agreement was still acceptable.

constants and are reported in Table 3. The constants collected in Table 3 are used to calculate the curves of Figs. 6 and 7. The good agreement between points and curves supports the validity of the applied procedure and allows assuming that the proposed species explain well the experimental data.

Furthermore, the results obtained from e.m.f. data, were confirmed by spectrophotometric measurements carried out by means of the Job method of continuous variation (1928) for finding the ratio between lead (II) and GSH in the complex formula.

4. Conclusions

This paper shows the investigation performed on solutions with ionic medium 1.00 M NaCl in a wide range of reagent concentration. In particular, the cation concentration varied in the range $(0.25-2.00) \times 10^{-3}$ M and the GSH concentration was increased from 0 to 0.025 M, so that the concentration of lead (II) varied 8 times and the ratio between cation and ligand arrived to about 100 times.

In the studied concentration range, polynuclear complexes are absent, while complexes with the assumption of 1, 2 or 3 protons are present. No species with assumption of OH^- are present. The assumption of the presence of only mononuclear complexes with ratio 1:1 between cation and ligand is supported by the evidence of the data of Figs. 6 and 7 and by the spectrophotometric results. Glutathione not protonated is not able to form complexes in the here investigated range. Its not protonated form (L) is present at appreciable concentration (about 10%) only at $-\log c_H \geq 9$, where HL is still about 60% and H₂L is about 25%.

On the basis of the experimental values obtained and the researches carried out by Carell and Olin (1962) and Olin (1960) hydrolytic species of lead (II) are absent in appreciable concentration.

By taking into account that GSH is essentially composed of glycine, cysteine and glutamate, a comparison between the behavior of GSH and the glycine and glutamate as ligand seems interesting.

Both glycine and glutamate form till $-\log c_H \approx 5$ (Bottari and Severini, 1978; Bottari et al., 1989) only protonated complexes, with lead (II), so that, this is bound in protonated form (PbHL) only to 10% even with a ratio 100:1, ligand:cation. This means that lead (II) is bound only to the carboxylic group. It seems reasonable to suppose that the presence of the $-\text{SH}$ group together with glycine and glutamate forming the 5 and 6 membered rings favor the stability of the chelate compound.

We have never observed the formation of a white precipitate in the range $2 \leq -\log c_H \leq 2.2$, successively soluble in an excess of NaOH, as described by Mah and Jalilehvand (2012). The suspect that the formation of $\text{Pb}(\text{OH})_3$ could take place according the researches of Carell and Olin (1962) could represent a possible hypothesis.

The conclusion of this paper seems particular interesting on the botanic point of view. From the botanic literature (Buchanan et al. 2000–2003) the toxic metallic cations are absorbed by plant organisms, penetrating inside the cellulose where the cytosol (solution at $\text{pH} \approx 7$, containing also glutathione) is present and they accumulate mainly in vacuoles containing the vacuolar solution characterized of $4 \leq -\log c_H \leq 5$.

This information can be useful to propose GSH as chelating agent for toxic metal cations taking into account the formation of complexes formed own in the range of concentrations of lead (II) and GSH here studied and the suitable $-\log c_H$ range.

Author contribution section

Author E. Bottari: Planing of the work and program of measurements, control of references. Author G. De Tommaso: spectra

Uv visible and deduction of results. Author M.R. Festa: General conduction of the work, collection of e.m.f. data. Author M. Iuliano: N.M.R. measures and relative interpretations. Author G. Zennaro: Performances of e.m.f. measurements.

Declaration of interest statement

Declaration of interest none.

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