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Henkens et al.

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[54] **CONVENIENT DETERMINATION OF TRACE LEAD IN WHOLE BLOOD AND OTHER FLUIDS**

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[21] Appl. No.: **73,806**

[22] Filed: **Jun. 7, 1993**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 821,732, Jan. 15, 1992, Pat. No. 5,217,594.

[51] **Int. Cl.**[5] **G01N 27/26**; G01N 33/20

[52] **U.S. Cl.** **204/153.12**; 204/153.1; 204/403; 436/74; 436/77

[58] **Field of Search** 204/153.12, 153.1, 153.17, 204/403, 412, 415, 418; 436/74, 77

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[57] **ABSTRACT**

The invention relates to methods of determining micromolar levels of lead ion in various fluids, including blood. Detection of lead or other heavy metal ion concentrations as low as 1 μg/dL is achieved. The methods are adaptable to the detection of low levels of lead in whole blood, employing a novel separation and release of lead ion from lead chelating agents. The disclosed methods employ isocitrate dehydrogenase-based electrodes which are particularly suited for detecting nanomolar levels of lead.

11 Claims, 6 Drawing Sheets

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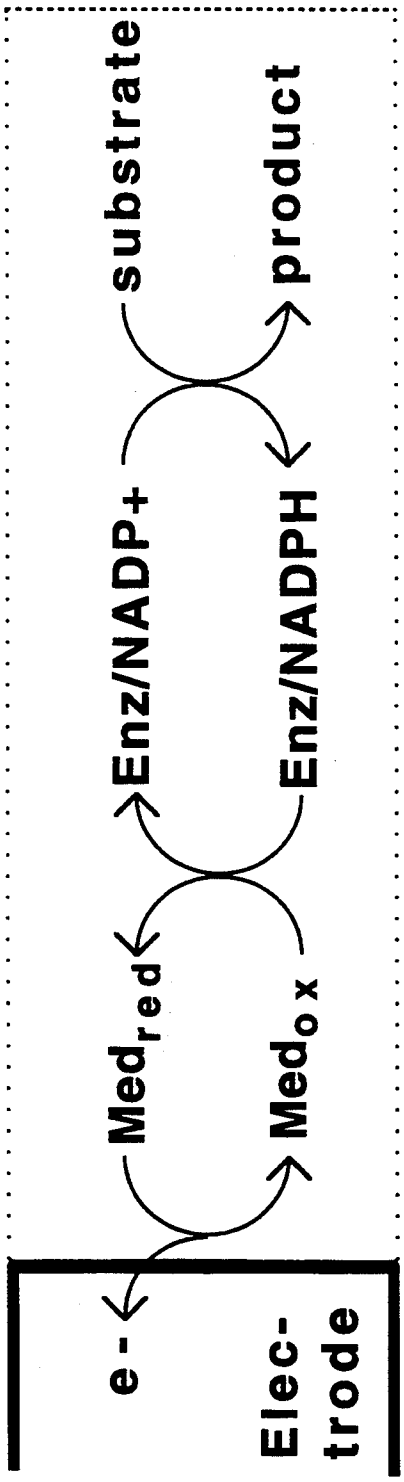
substrate
product
Enz/NADP+
Enz/NADPH
Med red
Med ox
e-
Elec-
trode

FIGURE 1

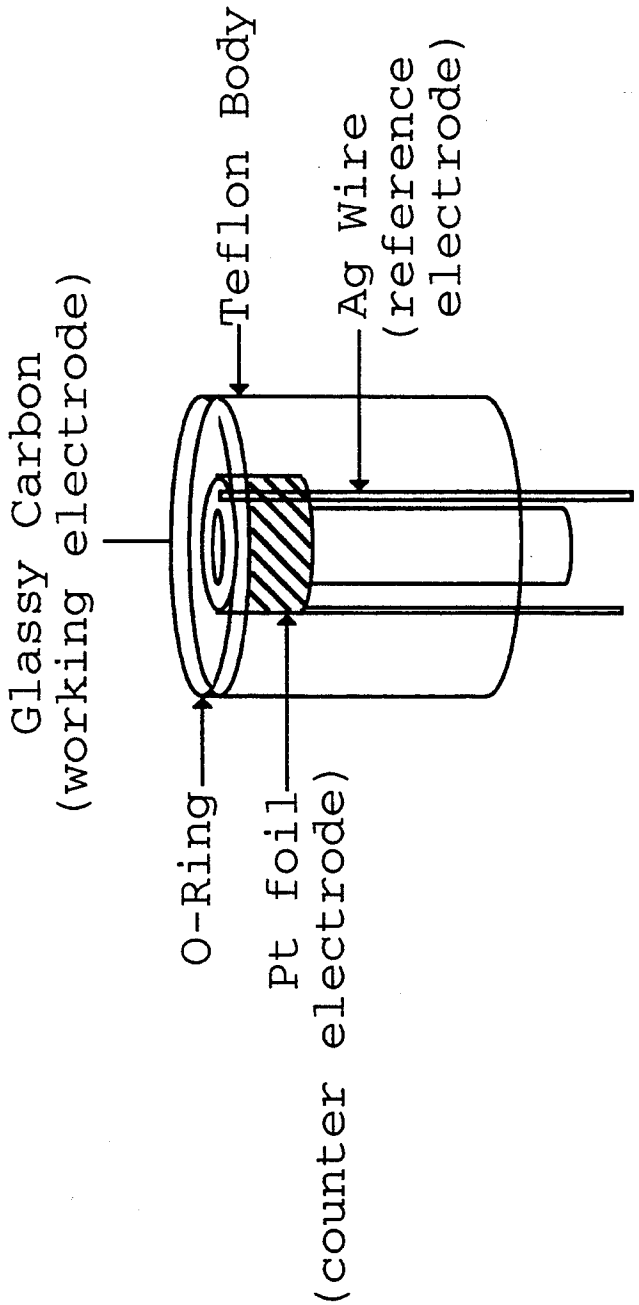
Glassy Carbon
(working electrode)
Teflon Body
Ag Wire
(reference
electrode)
O-Ring
Pt foil
(counter electrode)

FIGURE 2

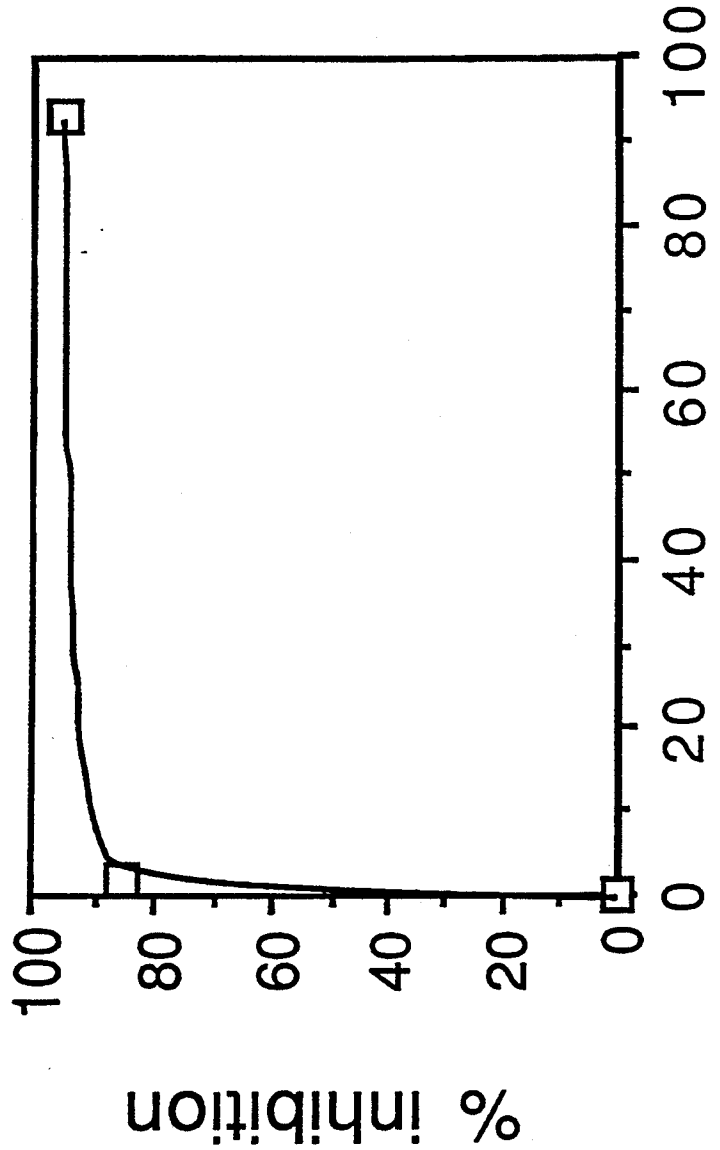

FIGURE 3

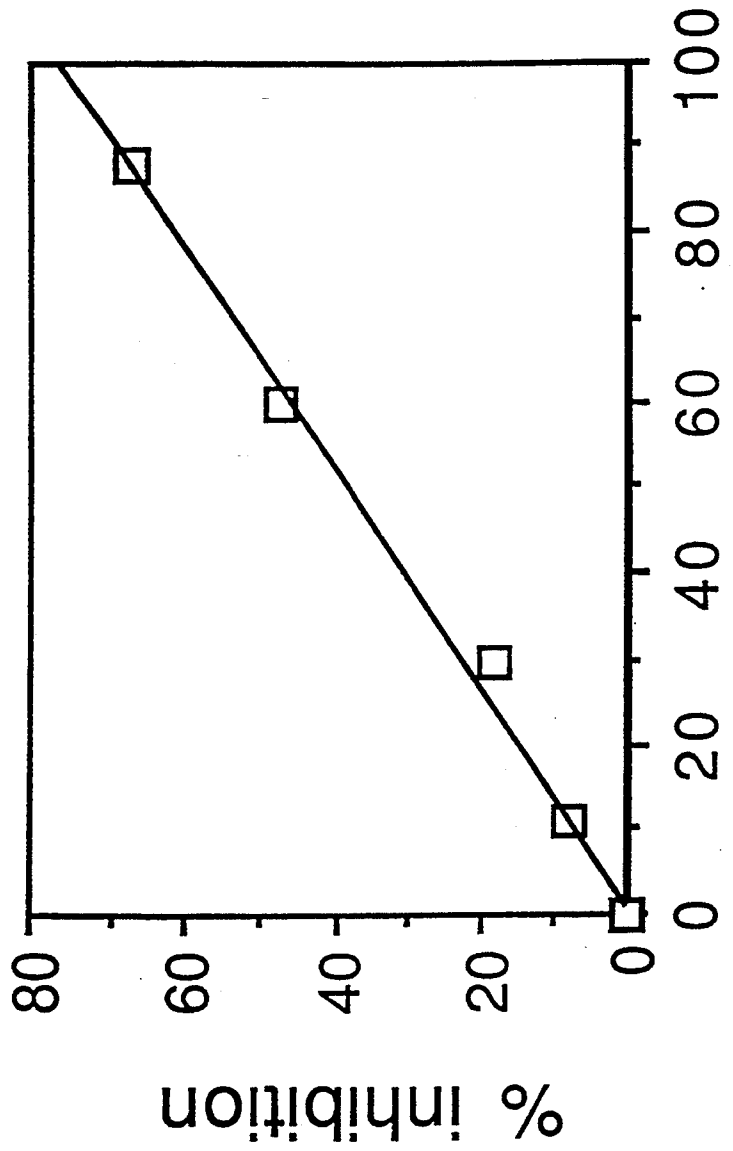

FIGURE 4

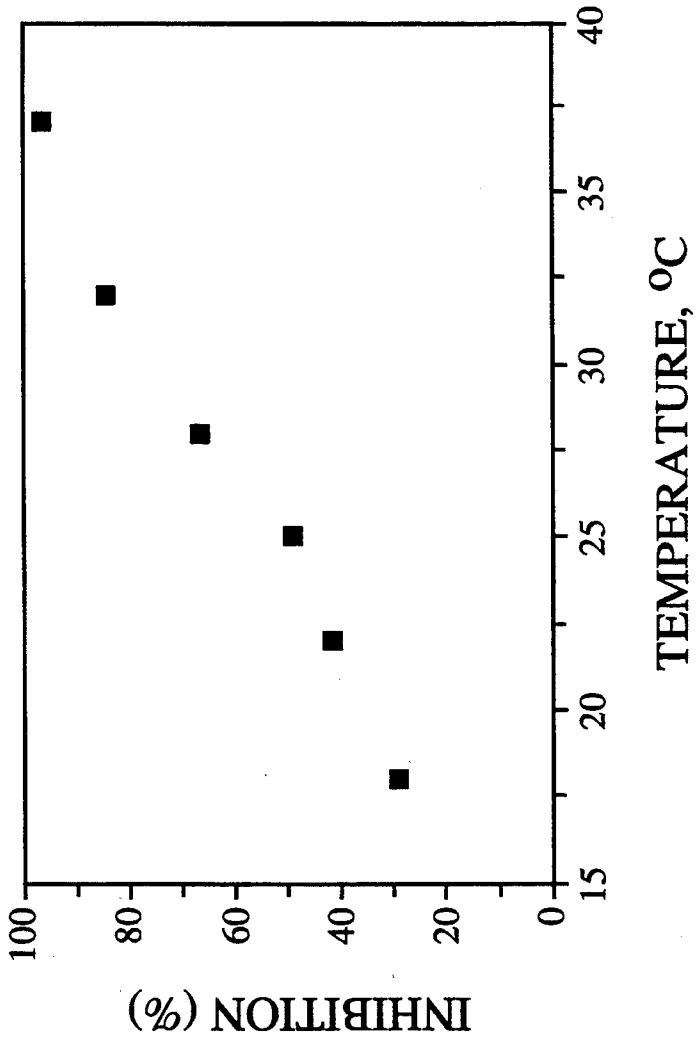

FIGURE 5

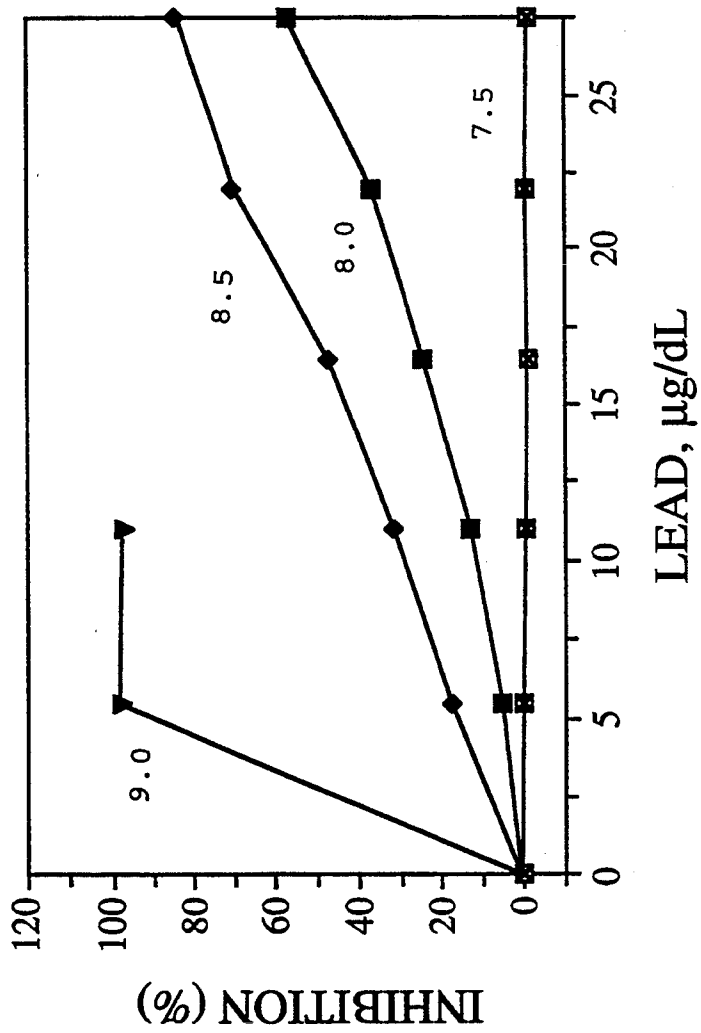
INHIBITION (%)
120
100
80
60
40
20
0
LEAD, µg/dL
0
5
10
15
20
25
9.0
8.5
8.0
7.5

FIGURE 6

ods of detecting lead in blood and other fluids. The methods take advantage of the irreversible inhibition of isocitrate dehydrogenase or other enzymes selectively inhibited by lead ions to determine micromolar levels of lead in various fluids, including blood. In one aspect of the invention, the method employs novel bioelectrodes constructed from adsorbed enzymes. Several versions of biosensors may be employed, including biosensors constructed from novel colloidal gold based bioelectrodes to measure changes in current generated in the presence of an oxidizable substrate and an enzyme cofactor.

The methods of the present invention concern detection of very low levels of heavy metal ions, particularly lead ions, employing isocitrate dehydrogenase based biosensors. The biosensors typically include a reference electrode, a counter electrode and a working electrode, or optionally, a counter electrode combined with the reference electrode. The appropriate enzyme is located on or near the surface of the working electrode. In the presence of a suitable substrate the enzyme catalyzes a redox reaction. Current generated by the redox reaction is adversely affected by metal ions that inhibit the enzyme. In particular, lead ion concentrations in the 1 μg/dL range are detectable when the disclosed methods are employed.

The disclosed methods, particularly those employing the disclosed biosensors, may be used to detect lead as well as a wide variety of nontoxic and toxic metal ions including mercury, cadmium, silver, zinc, copper, calcium, manganese, thallium, etc. However, sensitivity and selectivity of the biosensor will depend on the enzyme selected. In order to be effectively inhibited by low metal ion concentrations, it is preferable that the enzyme, or enzymes, selected is strongly and irreversibly inhibited by the metal ion. In most situations, one will desire inhibition in the micromolar or lower ranges of metal ion concentration. Mercury ion, for example, is detected in the nanomolar range employing colloidal gold adsorbed alcohol dehydrogenase biosensors. Lead ion may be quantitatively determined employing immobilized isocitrate dehydrogenase.

A conditional stability or formation constant is an equilibrium constant characterizing the ability of a ligand to bind to a metal ion under particular solution conditions. Unlike the thermodynamic constants, which should be only temperature and ionic strength dependent, the conditional constants include (a) the effect of solution pH on the availability of free ligand for binding (protonation of the ligand), (b) the effect of solution pH on the availability of free metal ion for binding (formation of hydroxide complexes of the metal), and (c) the effect of other competing ligands present in the solution. According to convention, the symbol K'_{PbX} represents the conditional stability constant for PbX complex (where X is a component that binds with Pb^{+2}) with inclusion of effect (a) only, while symbol K''_{PbX} denotes the conditional stability constant for PbX complex with inclusion of effects (a) and (b). The concept of conditional stability constant is well known to those of skill in the art and is found in conventional analytical chemistry textbooks.

It will be recognized that enzyme inhibition constants of enzymes selected for special use biosensors may differ depending the physical state of the enzyme. Constants may be quite different for immobilized species compared with the same species in solution. While solution inhibition constants in the nanomolar range may suggest suitability of an enzyme as a detection agent for low levels of inhibition, the constant may change after immobilization, thus requiring some degree of experimentation after potentially suitably enzymes have been selected on the basis of solution inhibition constants. The inventors have found, however, that solution inhibition of isocitrate dehydrogenase by lead is comparable to inhibition by the immobilized enzyme. The disclosed methods of determining low lead concentrations thus provide the option of employing amperometric determination of current inhibition for reactions catalyzed by isocitrate dehydrogenase in solution or by immobilized forms such as the particular colloidal gold biosensors developed by the inventors.

Enzymes useful in the practice of the present invention include a wide variety of redox enzymes. Cofactors typically associated with such enzymes include NADP and NAD. During the oxidation process, NADP or NAD is reduced to NADPH or NADH respectively. While it is not necessary to employ mediators with the methods of the present invention as oxidation currents are detectable without added mediators, mediators may be optionally employed and may under some conditions enhance efficiency. Suitable mediators include ferrocene and its derivatives, ferricyanide, N-methylphenazine methosulfate and related compounds such as N-ethyl phenazinium, phenoxazine and the like. Generally, mediators will be selected based on the electrochemical properties of the bioelectrode which depend on the enzyme and substrates chosen. Mediators are typically utilized in their oxidized forms initially to reduce background signal.

The inventors also contemplate alternate embodiments which employ a working electrode surface-coated with a membranous or gelatinous film. The redox enzyme may be dispersed within the gelatin or membrane material and then applied to the surface of the working electrode. Alternatively, the enzyme applied to the electrode may be covered with a membranous film. Enzyme cofactors such as NADP or NAD may also be included in the gelatinous film with the enzyme. Alternatively, such cofactors may be present in the bulk solution where they may freely enter and exit the membrane material with access to the electrode surface and to the gelatinous film immobilized enzyme. Suitable film materials include substances that are compatible with the enzyme selected. These include any of a variety of carrageenans, such as k-carrageenan, hydrophilic polymers or hydrophilic gels such as agar.

The working electrode surface of the biosensor of the present invention is typically a conducting material such as gold, platinum, or carbon. A preferred surface is carbon.

Biosensors useful in practicing methods of the present invention may optionally include both a cofactor such as NADP or NAD and a mediator. Mediators may be associated with the enzyme through hydrophobic association, ionic interactions or by covalent bonding. Alternatively, mediators or cofactors may also be included within a film used to immobilize selected enzymes near the working electrode surface. It is also possible to coat a mediator on the electrode surface, e.g., an insoluble compound on a surface that will slowly dissolve to provide a relatively low but constant amount of mediator or cofactor.

The invention provides novel methods of detecting lead ion in fluids, particularly with regard to the measurement of lead ion in whole blood samples. A sample

there was no significant inhibition at 27.6 μg/dL lead at pH 7.5.

TABLE 2

INHIBITION OF ISOCITRATE DEHYDROGENASE[1] BY LEAD ION

	pH 7.5		pH 8.0		pH 8.5		pH 9.0	
Pb^{+2} μg/dL	Current (nA)	Lead Inhibition (%)	Current (nA)	Lead Inhibition (%)	Current (nA)	Lead Inhibition (%)	Current (nA)	Lead Inhibition (%)
0	751	0	713	0	776	0	832	0
5.5	749	0	673	6	636	18	4	99
11.1	754	0	613	14	522	33	21	98
16.6	760	−1	532	25	406	48		
22.1	750	0	439	38	241	69		
27.6	761	−1	303	58	99	87		

[1]1.0 U/mL in 50 mM tris buffer

EXAMPLE 8

A major problem in the measurement of lead in blood is the interference by various blood proteins and, as in the majority of clinical samples, the presence of excess amounts of EDTA. The inventors have discovered that lead chelated with EDTA is exchangeable with cobalt-(II). As shown in this example, lead Pb(II) is displaced from EDTA by cobalt(II) and detected by its inhibition of isocitrate dehydrogenase (ICDH).

Computer simulations were developed to assess lead binding to blood components. These calculations showed that Co(II) displaced lead complexed with EDTA under conditions of the blood lead assay.

Micromolar concentrations of lead were determined through ICDH inhibition in the presence of 1 mM $CoSO_4$. Inhibition of the enzyme at several lead concentrations is shown in Table 3.

TABLE 3

Lead Ion Concentration (μM)	Inhibition (%)
0	0
2.4	24
4.8	46
12	71

[1]$CoSO_4$ concentration was 1 mM

Micromolor concentrations of lead were detected by ICDH inhibition in the presence of 2 mM EDTA and 2.5 mM $CoSO_4$. In the absence of $CoSO_4$, no inhibition was observed. Inhibition at several lead ion concentrations is shown in Table 4.

TABLE 4

Lead Ion Concentration (μM)	Inhibition(%)
0	0
12	23
36	32
121	47

[1]Concentration of EDTA was 2 mM; concentration of $CoSO_4$ was 2.5 mM

EXAMPLE 9

The activity of isocitrate dehydrogenase and its response is affected by several factors. The following data indicate effect of heating on activity and rates.

Heating

A solution of isocitrate dehydrogenase (10 nM) was incubated for 15 min at various temperatures between 18° C. and 37° C. and rate of lead inhibition measured. Inhibition rates increased approximately linearly from about 25% to 85% at a lead concentration of 4 μM (FIG. 5).

pH

The effect of pH and lead ion concentration on inhibition of ICDH is shown in FIG. 6.

EXAMPLE 10

Several compounds were tested in combination with Co^{2+} for ability to displace Pb(II) from Pb^{2+}EDTA complexes, employing computer simulations.

$$Pb^{2+}EDTA+Co^{2+}=Co^{2+}EDTA+PbX$$

Selection was based on thermodynamic considerations of stability constants to assure that equilibrium would lie to the right, that is, the compound would bind more tightly to Pb(II) than binding to Co(II).

Conversion of PbEDTA to PbX

The compounds shown in Table 5 were set up in a computer simulation as added to a buffered solution containing 1 μM Pb^{2+}, 4 mM EDTA, 5 mM Co^{2+} and the selected compound at 1 mM. Displacement of Pb^{2+} ranged from 38% for dimercaptosuccinic acid to virtually complete for cysteine.

TABLE 5

X	$\log"K_{PbX}$	$\log K"_{CoX}$	Percent Pb released
EGTA	12.07	10.86	58
MEDTA	13.66	11.85	85
DMSA	12.48	10.48	38
Pyrocatechol violet	6.75	3.60	94
Cysteine	9.14	5.93	99
Penicillamine	8.95	6.72	94

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