CHELATING AGENTS AND CELLULAR ASSOCIATION OF Ni^{2+}

INFLUENCE OF Ni(II)-BINDING LIGANDS ON THE CELLULAR UPTAKE AND DISTRIBUTION OF Ni^{2+}

BY

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ABSTRACT

The effect on Ni^{2+} uptake by human serum albumin (HSA), sodium diethyldithiocarbamate (DDC), D-penicillamine (D-Pen), ethylenediaminetetra-acetic acid [disodium salt] (EDTA), L-aspartic acid (L-Asp), L-Lysine (L-Lys), and L-histidine (L-His) was examined in three cell lines: (1) human red blood cells (RBCs), (2) cultured human B-lymphoblasts and (3) rabbit alveolar macrophages. It was found that EDTA, L-His, HSA, and D-Pen were good inhibitors of $^{63}Ni^{2+}$ uptake by cells and each was able to remove $^{63}Ni^{2+}$ previously associated with the cells. In contrast L-Lys and L-Asp, which do not bind Ni^{2+} well, were both poor inhibitors of Ni uptake and poor sequestering agents for cell-associated Ni^{2+}. Thus it seems that at physiological concentrations , L-His and HSA play a major role in regulating the association of Ni^{2+} with cells. DDC enhanced cellular uptake of $^{63}Ni^{2+}$, but was not very effective in removing $^{63}Ni^{2+}$ from cells. An increase in pH enhanced $^{63}Ni^{2+}$ uptake in the lymphoblasts, macrophages and human peripheral lymphocytes. This dependence was interpreted to indicate the existence of either: (1) an increase in membrane permeability with an increase in pH; (2) the development of a proton gradient across the cell membrane favouring the antiport transport of H^+ and Ni^{2+};

or (3) Ni^{2+}/proton competition for cellular binding groups. The cellular uptake of Ni^{2+} is interpreted in terms of an "equilbrium" model of metal-ion transport. It is concluded that since HSA and L-His can control cellular uptake and removal of Ni^{2+}, they may play a role in regulating the cellular toxicity of this ion.

It was found that L-His and D-Pen acted similarly such that at various concentrations both inhibited cellular uptake of $^{63}Ni^{2+}$ but did not change the normal distribution of Ni^{2+} within the cell. Conversely, DDC enhanced Ni^{2+} uptake by cells while simultaneously shifting the distribution of Ni^{2+} from the cell lysate to the cellular membranous pellet. Furthermore, DDC caused Ni^{2+} to become more lipophilic as shown by the increase of $^{63}Ni^{2+}$ in a chloroform extract. DDC also caused a change in Ni^{2+} distribution in whole blood by enhancing Ni^{2+} association with RBCs and lymphocytes and decreasing serum-associated Ni^{2+}. The different responses produced by D-Pen, L-His and DDC are ascribed to the hydrophilicity of the $[Ni(D\text{-}Pen)_2]^{2-}$ and $Ni(His)_2$ complexes and the lipophilicity of the $Ni(DDC)_2$ complex, and allow a rationalization of the contrasting therapeutic effects of D-Pen and DDC.

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LIST OF ABBREVIATIONS

RBCs	Red Blood Cells
DDC	Diethlydithiocarbamate
D-Pen	D-Penicillamine
L-His	L-Histidine
L-Asp	L-Aspartic Acid
L-Lys	L-Lysine
L-Cys	L-Cysteine
HSA	Human Serum Albumin
EDTA	Ethylenediaminetetra-acetic Acid (disodium salt)
cpm	Counts per minute
Tris	Tris(hydroxymethyl)aminomethane

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 Introductory Remarks

In this thesis, experimental evidence is presented which demonstrates the effects of natural and synthetic nickel sequestering agents upon the distribution, uptake and removal of cell-associated Ni^{2+}. In order for the reader to appreciate the need for such a study, this introductory chapter provides the basic background information on nickel toxicology. It is shown how common exposure to nickel is, and what consequential effects can arise from such contact. It is futher illustrated that crucial to the understanding of nickel toxicity, especially of carcinogenesis, is a knowledge of how Ni^{2+} is accumulated by cells and what factors regulate such association and the intra-cellular distribution of Ni^{2+}.

1.2 Health Effects of Exposure to Nickel and its Compounds

1.2.1 General Population. Since nickel is used in so many metal-containing products, the general population is constantly exposed to nickel in every-day life. Nickel is found in costume jewelry, as well as in coins and stainless steel products. Thus the most common type of exposure to nickel that the general population experiences is by skin contact. Those individuals who are sensitive to nickel usually have a dermatological response. Nickel dermatitis is characterized by inflammation of the skin upon continued contact with nickel-containing items (Sunderman et al.,

1975). A simple test for diagnosing individuals for nickel sensitivity is by use of a patch test. It involves placing a few drops of a water soluble nickel compound (e.g. $NiSO_4$) on a patch, and taping it to an individual's skin. Inflammation under the patch usually develops in 48 h. Unfortunately, this test is not very sensitive for persons who produce only mild reactions to nickel, or who display a very delayed test result. A complementary and perhaps more sensitive test in diagnosing nickel sensitivity in people is to look for lymphocyte transformation induced by nickel sulfate in vitro (Al-Tawil et al., 1981). This test is based on the fact that lymphocytes isolated from individuals sensitive to nickel will undergo a lymphocyte/lymphoblast transformation after 7 to 8 days incubation in the presence of a Ni(II) salt. The transformation is monitored by the increased uptake of ^3H-thymidine when the cells proliferate during blastogenesis.

1.2.2 Nickel-Related Occupations

1.2.2.1 Nickel Carbonyl Nickel carbonyl is a very lipophilic gas. Consequently, when this gas is inhaled, it passes through the lungs' membranes easily and causes nickel to be deposited in the lung and other tissues. Clinical symptoms of nickel carbonyl poisoning appear similar to those produced by severe viral or influenzal pneumonia (Vuopala et al., 1970; Sunderman Jr., 1977).

Severe exposure to nickel carbonyl can lead to death, usually by respiratory failure. A number of fatal accidents are known.

Nickel carbonyl ($Ni(CO)_4$) is produced as an intermediate in the purification of nickel in refineries (Head et al., 1976). Carbon monoxide is passed over crude nickel at high pressures to produce the $Ni(CO)_4$ complex. This gas is removed to a different chamber, and at ambient pressure upon heating this complex degrades readily to nickel powder and carbon monoxide gas. Accidental exposures to this toxic gaseous form of nickel constitutes a serious risk. Nickel carbonyl is also employed as an intermediate in the production of methyl and ethyl acrylate monomers (which are used in the making of plastics), as well as in nickel vapour-plating operations (Sunderman Sr., 1970).

1.2.2.2 Cancer Exposure to inhalation of relatively insoluble compounds was linked to an increase in cancer as far back as the 1950's (Doll, 1958; Morgan, 1958). This link has been confirmed more recently by Roberts et al. (1982) and Pederson et al. (1978). Exposure by this means usually generates an increase in lung and nasal cancer, but an increase of cancer in kidney, prostate and stomach have also been suggested (ibid). The nickel containing-dust is produced through primary refinery processes (roasting, smelting and sintering), and is also generated in welding,

the casting of alloys, grinding and polishing. The nickel compounds that are most strongly incriminated as carcinogens are nickel sulfides and perhaps nickel sulfide/oxide mixtures. Sunderman and Hopfer (1983) have shown in animal studies that alpha-Ni_3S_2 and beta-NiS can cause 100% induction of sarcomas at the injection sites, whereas Ni dust and amorphous NiS caused 55% and only 4% incidence of sarcoma development, respectively. Their work suggests that those compounds with well-defined crystal structures are the most potent carcinogens. This agrees well with work done by Kasprzak et al. (1983), who have shown that colloidal $Ni(OH)_2$ is non-carcinogenic in rats, whereas "dried" and more crystalline forms appear to be carcinogenic. Surface charge on particulates ($\leq$ 2 µm diameter) of these nickel compounds have also shown to be important in determining their relative ability to be phagocytized, induce cellular transformation and cause cellular toxicity in cultured mammalian cells (Heck and Costa, 1982). Recent evidence indicates that most likely it is not necessarily the sign of the surface charge that is important for activity, but the magnitude (Nieboer et al., 1983a). It would appear for a nickel compound to be carcinogenic it must be crystalline, it must possess surface charge of low magnitude (which appears to facilitate phagocytosis and reduce cell damage), and must have favourable dissolution properties (slow and

regulated). These characteristics appear to maximize intracellular bioavailability of Ni^{2+} which, as shown below (Section 1.5), may enhance carcinogenic action of the nickel(II) ion.

1.2.2.3. Nickel-Induced Asthma Cases have been reported of nickel-exposed workers who experience asthma attacks while at work. For such individuals, asthma could be induced by inhalation challenge with nickel salts (McConnel et al., 1973; Malo et al., 1982). In the patient studied by Malo et al. (1982), not only did the individual have asthma attacks on inhalation challenges with $NiSO_4$, he also showed a positive response to the allergy skin tests with $NiSO_4$. Upon further investigation, evidence was found that the asthmatic response was consistent with circulating IgE and IgG antibodies against the Ni-human serum albumin antigen suggesting antibodies with nickel specificity.

1.3 Nickel Essentiality

So far this report has stressed the hazardous side of nickel, but as shown in this section, nickel appears to be a required metal in a number of biological systems and is thus needed in certain biochemical functions. Rats fed on a Ni-deficient diet showed a decrease in malate and glucose-6-phosphate dehydrogenase activity, as well as reduction in growth and erythrocyte count, a decrease in hematocrit and hemoglobin level in blood, and alterations in the content of iron, copper and zinc in various body

organs (Kirchgessner and Schnegg, 1976). Nickel deficiency has also been established in other animals, namely in pigs, goats, chicks and sheep (see summary in Cecutti and Nieboer, 1981). Common deficiency symptoms were ultrastructural changes in liver, reduced oxidative ability and diminished activity of other key liver enzymes. In humans nickel has a well-defined narrow concentration range in serum and urine, implying a physiological or homeostatic control. Renal excretion is a major excretionary pathway. Ni has also been found in significant amounts in human fetal tissues (Cecutti and Nieboer, 1981). Jack-bean plant urease has has proven to be a metalloenzyme requiring Ni^{2+} for the urea/NH_3 interconversion (Dixon et al., 1980). Polocco (1977) has also demonstrated that soybean tissue cultures need Ni^{2+} for utilizing urea as the only nitrogen source. Furthermore, nickel is a growth requirement for many algae when urea is the nitrogen source (Rees and Beckheet, 1982).

Nickel is known to be essential to microbes. A decade ago, Bertrand (1974) observed that the presence of Ni^{2+} stimulated N_2-fixation in soil bacteria. Methanogenic bacteria can use methanol, acetate, or H_2 plus CO_2 as the energy source (Thompson 1982), and it has been shown that nickel was needed for the following biological reaction to take place (Diekert et al., 1981):

$$4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O + \text{energy} \qquad (1.1)$$

This reaction is catalyzed by the co-enzyme methyl reductase (CoM), the prosthetic group of which is factor F_{430}. The latter factor (which has been found in every methanogenic bacterium so far examined) has been shown to contain the tetrapyrrole moiety, as well as nickel in the Ni(III) oxidation state (Thompson, 1982). The involvement of the Ni(III)/Ni(II) redox couple in the formation of methane explains why growth of methanogenic bacteria is dependent on nickel (Thauer et al., 1980; Thauer, 1982).

In conclusion, there is very convincing evidence that nickel is an essential requirement for various biochemical processes in animal, bacterial and plant systems.

1.4 Nickel Distribution and Metabolism

The typical Canadian consumes in his diet about 350-580 µg/day of nickel, of which 1-10% is absorbed from the gastrointestinal tract (Kirkpatrick and Coffin, 1977). As mentioned previously, another form of nickel absorption is by inhalation of nickel compounds, such as documented for nickel refinery and electroplating workers. The average person dwelling in an urban center is exposed to approximately 0.025 µg Ni/m^3 of air. The amount of nickel inhaled annually would be about 0.2 mg, assuming that 20 m^3 of air is breathed daily (Cecutti and Nieboer, 1981; Barrie, 1981).

Nickel has been found in most human tissues. It

has been concluded that the highest concentration of nickel occurs in tissues exposed to exogenous factors, like the lung, large and small intestines and skin (Sumino et al., 1975). Substantial amounts of nickel have also been found in the kidney, muscle, and rib bone. It has been concluded that an average 70-kg subject would have a total nickel content of 7-10 mg. There is no evidence that nickel is stored in a particular organ.

Nickel can be detected in whole blood (3-7 µg/L), serum (1-5 µg/L), urine (0.7-5.2 µg/L), saliva (2.2±1.2 µg/L), sweat (52±36 µg/L), and feces (14.2±2.7 µg/g or 258±126 µg/day) (Cecutti and Nieboer, 1981; Sunderman Jr., 1983a). Most of the nickel that is ingested orally is excreted in the feces. Renal excretion is the principal route in rodents for the elimination of injected Ni^{2+} (Sunderman Jr., 1977). The mean concentration of nickel in sweat from healthy men is approximately 20 times greater than the mean concentration of nickel in urine. Under conditions of profuse sweating, appreaciable amounts of nickel appear in sweat, which probably accounts for the diminished concentrations of serum nickel in workmen who were chronically exposed to extreme heat (Sunderman Jr., 1977). Thus it appears that removal of absorbed Ni^{2+} occurs principally by sweating and renal excretion.

Nickel is transported in the body via serum by binding to carriers. The major Ni(II)-binding ligands have

been established to be L-histidine (L-His) and human serum albumin (HSA), and possibly a nickel metalloprotein which most likely is an alpha-macroglobulin (also called nickeloplasmin) (Sunderman, 1977; Nomoto, 1980). Although discrepancies exist about the relative affinity Ni^{2+} has for each of these carriers, free L-His appears to be the most important natural low-molecular-weight ligand. As amino acid residue 3, L-His also plays a major role in the binding site of Ni^{2+} to HSA (Lucassen and Sarkar, 1979). The amino acids L-aspartic acid (L-Asp) and perhaps L-cysteine (L-Cys) have also been shown to have some ability to bind Ni^{2+} (Abbrachio et al., 1982a; Lucassen and Sarkar, 1979; see below for additional evidence). The metalloprotein nickeloplasmin is not well characterized, and considerable doubt exists about its importance in the distribution of Ni^{2+} in serum. Indeed it is interesting to note that in older studies, it was determined that approximately 26% of serum-bound nickel was associated with a nickel metalloprotein fraction (Sunderman et al., 1972); whereas in a more recent experiment, Sarkar (1983) showed that only approximately 0.1% of Ni^{2+} may be designated as such. It is generally believed that protein-bound Ni^{2+} is in equilibrium with that complexed with free L-His. Lucassen and Sarkar (1979) showed that 96% of all the Ni^{2+} in Ni(II)-supplemented human serum was associated with albumin, and that L-His was the main Ni(II)-binding amino

acid.

Thus by binding to these carriers, Ni^{2+} may be transported, and of course, can only undergo renal excretion when complexed to low molecular ligands. Van Soestbergen and Sunderman (1972) have also provided evidence that Ni^{2+} is bound to both L-His and L-Asp in the ultrafiltrable fractions of rat serum and urine. This agrees well with the work of Lucassen and Sarkar (1979) mentioned above. Paulsen et al., (1978) have also identified the presence of a Ni^{2+}-His complex in the Ni(II)-containing ultrafilterable fraction of rabbit serum. In fact it was the only free amino acid identified prior to hydrolysis of this fraction. Furthermore, Asato et al., (1975) have studied the ultrafiltrates from rabbits that received intravenous injections of $^{63}NiCl_2$. It was found that the $^{63}Ni(II)$-ultrafilterable complexes were rapidly cleared from serum via urine and bile. Although the identity of all the ultrafilterable Ni(II)-ligands have not yet been totally established, preliminary studies indicated that $^{63}Ni^{2+}$ was again complexed with L-His, L-Cys and L-Asp, both singly or as mixed-ligand species. Additional background material on the role of ligands in nickel transport and distribution is provided in Chapter 2.

1.5 Biochemistry of Nickel

The toxic effects displayed by phagocytized Ni-containing solid compounds are believed to be the result

of the release of Ni^{2+} on dissolution. Ni^{2+} has the ability to compete with endogenous divalent cations for binding sites, such as Ca^{2+} and Mg^{2+}. Ni^{2+} can form octahedral complexes like Ca^{2+} and Mg^{2+}, and it almost has the same ion size as Mg^{2+}. Ca^{2+} and Mg^{2+} are class A ions, and both have preference for binding sites containing oxygen as the donor atom. Ni^{2+} exhibits both class A (oxygen-seeking) and B (nitrogen- and sulphur-seeking) character. Nevertheless, Ni^{2+} tends to bind with oxygen sites even better than Ca^{2+} and Mg^{2+}. Consequently it competes effectively with these class A ions for metal-ion binding centers (Nieboer and Richardson, 1980). Ni^{2+} has a much slower rate of metal complex formation in solution than Mg^{2+} and Ca^{2+}. Perhaps because of this, it may inhibit biological processes by slowing down the critical reactions. Complex formation is very rapid for Ca^{2+} (forward rate constant, k_f of 10^8-$10^9 s^{-1}$), somewhat slower for Mg^{2+} ($k_f \simeq 10^5 s^{-1}$), and slowest for Ni^{2+} ($k_f \simeq 10^4 s^{-1}$) (Nieboer, 1981). Substitution of Ni^{2+} for Ca^{2+} and Mg^{2+} can lead to inhibition, activation or inactivation of enzymes (Nielson, 1974 and 1980; Nieboer *et al.*, 1983a). Ca^{2+} has been implicated in many hormone release processes, and in some cases Ni^{2+} inhibits those processes which require Ca^{2+}. Dormer *et al.*, (1973) have studied three different Ca^{2+}-requiring systems in detail: the release of the salivary enzyme amylase from the parotid gland, the

release of insulin from mouse pancreatic islets, and the release of growth hormone from bovine pituitary slices. In each case, the presence of Ni^{2+} had inhibited the release of the hormone or enzyme in conditions which would otherwise cause stimulation. Substitution of Ni^{2+} for Ca^{2+} from isolated rat uterine strips inhibited spontaneous contractile acitivity and decreased the basal tone (Rubanyi and Balogh, 1982). Even algae have shown to be affected by Ni^{2+}, such that Ni^{2+} caused a decrease in cell division, calcification, and cell protein content in the coccolithophorid algae, Cricosphaera carterae (Stillwell and Holland, 1977). Ni^{2+} might also replace Mn^{2+} and Zn^{2+} in some cases (Van de Drift and Vogels, 1970; Cecutti and Nieboer, 1981). A summary of effects of essential ions replaced by Ni^{2+} is given in Table 1.0.

Should Ni^{2+} enter the nucleus, it can potentially cause quite detrimental effects. Mg^{2+} is the counter ion to the negatively charged phosphate groups and thus stabilizes genetic materials (Eichorn, 1975). It is also needed to regulate the incorporation of the proper nucleotides in both DNA and RNA synthesis (Spiro, 1980). Not surprisingly, if Ni^{2+} replaces Mg^{2+} a decrease in the fidelity of DNA synthesis is observed (Sirover and Loeb, 1976). Ni^{2+} can also bind to DNA and RNA polymerases and appears to deactivate them (Sirover and Loeb, 1976; Sunderman, Jr., 1979). Therefore, Ni^{2+} is a potential

Table 1.0 THE EFFECT OF THE REPLACEMENT OF ESSENTIAL METAL IONS BY Ni^{2+} (reprinted from Nieboer *et al.*, 1983a)[a]

A. Ni^{2+}/Ca^{2+} PROTAGONISM/ANTAGONISM

1. Activates (+)/Inhibits (-) Enzymes

+, human salivary alpha-amylase
-, staphylococcal nuclease

2. Stimulates (+)/Inhibits (-) Excitable Tissues

uncoupling of excitation (+) and contraction (-) in frog skeletal and cat heart muscles

+ (low levels), - (high levels), uterine contraction in rat

3. Stimulates (+)/Inhibits (-) Exocytosis

+, epinephrine in frog adrenal
+, prolongs trasmitter release in frog neuromuscular junction

-, amylase (rat parotid)
-, growth hormone (bovine pituitary)

B. Ni^{2+}/Mg^{2+} PROTAGONISM/ANTAGONISM

Activates (+)/Inhibits (-) Enzymes

+, phosphoglucomutase (rabbit muscle)
+, ribulose diphosphate carboxylase
-, yeast enolase
-, pyruvate kinase (rabbit muscle)
-, AMV and *E. coli* DNA polymerases

C. Ni^{2+}/Zn^{2+} PROTAGONISM/ANTAGONISM

Activates (+),/Inhibits (-) Enzymes

+, carboxypeptidase A (bovine)
+, aspartate transcarbamoylase (*E. coli*)
-, alkaline phosphatases (*E. coli*)
-, carbonic anhydrase B (human)

[a]A decrease in enzyme activity of >70% relative to the native enzyme was considered inhibitory.

mutagen and carcinogen.

1.6 Chelation Therapy of Ni(II)-Poisoning

As mentioned previously, there seems to be a physiologic control of Ni^{2+} *in vivo* because of the narrow concentration range found in blood. Evidence has been presented indicating that HSA and L-His may play a role in this regulation, due to their ability to bind Ni^{2+} (Lucassen and Sarkar, 1979; Nomoto, 1980; Sunderman *et al.*, 1972). Upon exposure to high levels of nickel, such as in nickel carbonyl poisoning, chelating agents are used as antidotes. Two such chelating agents currently being studied in Ni(II)-poisoning in man and animal are diethyldithiocarbamate (DDC) and D-Penicillamine (D-Pen). D-Pen is an experimental drug used in animals that have been exposed to high levels of the Ni^{2+} ion, and has proven effective in decreasing serum levels of Ni^{2+} in rats and enhancing its renal excretion (Horak *et al.*, 1976). Oskarsson and Tjälve (1983), employing autoradiography and liquid scintillation counting, have also shown that D-Pen is most efficient in decreasing $^{63}Ni^{2+}$ levels associated with tissues in rats injected with $^{63}NiCl_2$.

DDC is a therapeutic drug used in man for treatment of $Ni(CO)_4$ poisoning. Baselt *et al.*, (1977) have shown the usefulness of DDC in decreasing the toxic effects and enhancing Ni^{2+} excretion in rats exposed to $Ni(CO)_4$. Interestingly, D-Pen had little therapeutic effect when

used immediately after $Ni(CO)_4$ exposure (ibid). Futhermore, DDC proved ineffective for treatment of Ni(II)-salt poisoning. In fact, DDC could actually increase Ni(II)-associated tissue levels in rats when DDC was administered just before or just after $^{63}NiCl_2$ injections (Oskarsson and Tjälve, 1980). Thus the chemo-therapeutic action of these two drugs are obviously expressed by different mechanisms. The nickel-DDC complex is lipophilic, whereas the nickel-D-Pen complex is hydrophilic, and these differences in chemical properties may determine the different effects which these chelating agents have on the fate of Ni^{2+}. Additional detail on the nickel therapeutic use of DDC is provided in Chapter 3.

1.7 Thesis Objectives

As indicated, studies have been reported on the whole body distribution of Ni^{2+} and the effects that Ni(II)-chelation agents such as D-Pen and DDC have upon Ni^{2+} distribution and excretion. There have, however, been no studies completed on the effects of these complexing agents and of natural ligands (i.e. HSA or L-His) on the cellular uptake and intracellular distribution on Ni^{2+}. Such a study would prove beneficial, since many of the toxicological effects of nickel occur at the cellular level. For example, the nature of the interaction of Ni^{2+} in peripheral lymphocytes that induces blastogenesis is not known, nor is the role of uptake of Ni^{2+} by cells and its

concomitant intracellular compartmentalization defined in carcinogenesis. By studying the ability of natural and synthetic ligands in regulating the cellular association and distribution of Ni^{2+}, it might be possible to demonstrate their potential in controlling the toxic effects that this ion exerts in vivo. Consequently, the effect of D-Pen, DDC, ethylenediaminetetra-acetic acid (EDTA), HSA, L-His, L-Asp and L-Lys on the cellular uptake and distribution of Ni^{2+} are examined in three cell lines: (1) human red blood cells, (2) cultured human B-lymphoblasts and (3) rabbit alveolar macrophages. The effect of DDC on the distribution of Ni^{2+} in human whole blood is also studied. Appropriate rationale for selecting these cell types is provided in Chapter 2.

CHAPTER 2

THE INFLUENCE OF pH AND Ni(II)-BINDING LIGANDS UPON CELLULAR UPTAKE AND REMOVAL OF NICKEL(II)

2.1 INTRODUCTION

Exposure to nickel compounds has a variety of effects ranging from contact dermatitis to the development of fatal carcinomas. In the general population, many people have been shown to suffer from nickel hypersensitivity and to give positive patch tests to nickel. A lymphocyte response is known for nickel sensitive patients (Macleod et al, 1970; Al-Tawil et al., 1981; Thulin, 1976). Lymphocytes isolated from nickel hypersensitive patients undergo lymphoblast transformation when Ni^{2+} is added to the incubation medium, whereas lymphocytes from non-sensitive controls showed little or no stimulation (Macleod et al., 1970; Al-Tawil et al., 1981). The slight activation of the control lymphocytes may be because Ni^{2+} is a weak mitogen, as $NiSO_4$ has been demonstrated to stimulate cord blood lymphocytes from new born infants (Al-Tawil et al., 1981). Consequently in view of the above studies, a strain of cultured human B-lymphoblasts and human peripheral lymphocytes were selected for Ni^{2+} uptake studies. Other cell lines employed were human erythrocytes and rabbit alveolar macrophages. Macrophages have been shown to be sensitive to nickel inasmuch as Ni^{2+} inhibits these phagocytes, both in vitro (Castranova et al., 1980) and in vivo (Gardner, 1980). $^{63}Ni^{2+}$ transport in the body is by

the blood stream, so it would be of interest to observe the effects of $^{63}Ni^{2+}$ upon human erythrocytes since Ni^{2+} is in almost continuous contact with RBCs until excreted from the body. Another reason for using RBCs is that not much is known about Ni^{2+} distribution in blood. In fact most analyses of Ni^{2+} are performed on blood plasma or serum fractions instead of whole blood (Barton et al., 1980).

Many ligands are capable of binding Ni^{2+}, but their effect on the uptake of this ion by the mentioned cell types is not known. In this and the following chapter, it is reported how a number of natural ligands and synthetic chelating agents can regulate cellular uptake of Ni^{2+}. The rationale for selecting a number of ligand systems for study is provided in this and subsequent paragraphs. Two major natural Ni^{2+}-binding ligands in serum are HSA and L-His. As discussed in Chapter 1, at physiological ligand concentrations, 95.7% of Ni^{2+} in serum is associated with HSA while 4.2% is associated with amino acids, of which 72% is bound to L-His. The remaining 0.1% is bound to an unidentified high molecular weight (HMW) protein (Lucassen and Sarkar, 1979). Unfortunately, although physiological concentrations of HSA and L-His were employed, the amount of Ni^{2+} used by Lucassen and Sarkar was in a ratio of 1:1 with these ligands (≡35 mg Ni/l), which far exceeds in vivo levels of Ni^{2+} found in serum of 2.6 ± 0.9 µg/l (Sunderman Jr., 1977). Great discrepancies

have been reported in the actual amount of Ni^{2+} that binds to the HMW protein found in human serum. As mentioned, Sarkar (1983) found that 0.1% of the total Ni^{2+} found in human serum binds to a HMW protein, possibly an alpha-2-macroglobulin, whereas Nomoto (1980) has determined that 43% of the total Ni^{2+} found in human serum binds to an alpha-2- macroglobulin. Also Sunderman et al.(1972) have found that 26% of Ni^{2+} in human serum binds to a HMW protein, which they called nickeloplasmin. Thus the actual serum constituents and the relative affinity that Ni^{2+} has for each is still not clear. The affinity of Ni^{2+} for L-His is higher than for HSA because at equimolar concentrations of these two ligands, 70% of Ni^{2+} combines with L-His and 30% with HSA (Lucassen and Sarkar,1979). Similar conclusions may be deduced from the relative magnitudes of the equilibrium constants (Glennon & Sarkar, 1982). Interestingly, the Ni^{2+} complex of HSA is proving to be of biological importance. Antibodies have been found to Ni^{2+} in an individual with occupational asthma caused by nickel sulphate inhalation (Nieboer et al., 1983b). The actual antigen to the antibodies was found to be the Ni^{2+}-HSA complex, specifically with Ni^{2+} bound at its Cu^{2+}/Ni^{2+} transport site. Furthermore, it is believed that Ni^{2+} alone is not antigenic enough to produce a hypersensitive dermatological response. This has been demonstrated in leukocyte migration tests (Thulin, 1976).

When Ni^{2+} was used alone, leukocytes isolated from nickel sensitive patients showed no inhibition of migration when compared with those of controls. If the Ni^{2+} was first bound to a protein (HSA, bovine serum albumin, or human epidermis protein), it caused an inhibition of migration of leukocytes isolated from Ni^{2+} sensitive patients. This suggests that Ni^{2+} acts as a hapten which is inactive until bound to a protein. From this brief review, the selection of HSA and L-His for the current work evolves naturally from their relatively high abundance in serum and their known affinity for Ni^{2+} with some recognized biological consequences.

L-Lys and L-Asp were also selected, and may be considered as controls for L-His and HSA since these two amino acids have been shown to have little or no apparent binding capacity for Ni^{2+} under physiological conditions (Lucassen & Sarkar, 1979).

And finally, some synthetic nickel ligands have also been investigated in relation to nickel poisoning, of which DDC and D-Pen are the most prominent. DDC has been used as an antidote to nickel carbonyl [$Ni(CO)_4$] poisoning in man and animals, and D-Pen as an antidote to Ni^{2+} poisoning in rodents (Baselt *et al.*, 1977; Sunderman, Sr., 1979; Horak *et al.*, 1976). Thus it would be interesting to see how these therapeutic agents affect Ni^{2+}-association with cells. EDTA was also included in our experiments,

since as a chelator it is known for its high binding capacity for divalent cations.

Ion transport across membranes has been shown to be influenced by extracellular and intracellular pH (Nicholls, 1982). It should further be stressed that pH is an important factor regulating the access of ions to binding sites in biological molecules, since the proton directly competes with metal ions (Nieboer & Richardson, 1980). Thus the effects of different proton concentrations on cellular uptake of $^{63}Ni^{2+}$ merit investigation.

In all experiments performed with $^{63}Ni^{2+}$, a simple saline-veronal buffer was used so that the maximum effect of Ni^{2+} uptake and binding could be observed. If a complex medium were employed (e.g., alpha-MEM), there would be problems with Ni^{2+} binding to serum proteins and amino acids from the fetal calf serum, as well as competition by Ca^{2+} and Mg^{2+} with Ni^{2+} for cellular binding sites.

2.2 MATERIALS AND METHODS

2.2.1. Rabbit Alveolar Macrophages. Lungs were obtained from normal rabbits exsanguinated by cardiac catherization. Macrophages were removed from the lungs by 3 pulmonary washes with 40 ml aliquots of veronal buffer (pH 7.4, 5.0×10^{-3} M with respect to sodium barbital plus 5,5-diethylbarbituric acid, both obtained from Fisher

Scientific, Toronto, Ont., and 0.19M NaCl, from BDH Chemicals, Toronto, Ont.). The macrophage suspension was cleansed of red blood cells by centrifugation at 1,500 rpm for 10 min and then the resulting pellet was resuspended in 5 ml of a Tris/NH_4Cl red-cell lysing solution (pH 7.4, $1.7x10^{-2}$M Tris [tris(hydroxymethyl) aminomethane from Fisher Scientific], $5.0x10^{-4}$ M veronal buffer, 0.14M NH_4Cl [from BDH],and 0.02 M NaCl). This suspension was incubated at 37°C for 5-10 min, and then the cells were pelleted and the supernatant decanted. The macrophage pellet was subsequently resuspended in 40 ml of veronal buffer and repelleted. Again the supernatant was decanted and the cells resuspended in veronal buffer to give a final concentration of $2.0x10^7$ cells/ml, of which 100 µl was added to each incubation tube. Details of this collection procedure are provided by McGee and Myrvik (1981). Note that handling of macrophages was done in plasticware since macrophages tend to stick to glass.

2.2.2. B-lymphoblasts. The B-lymphoblast cell line was of human origin isolated at Sick Children's Hospital, Toronto, by B. Zimmerman. The lymphoblasts were cultured in an alpha-MEM medium (Stanners et al.,1971) supplemented to contain by volume: 15% fetal bovine serum, 1% of an antibiotic-antimycotic mixture, 1% L-glutamine (200mM), 1% Hepes buffer (1.0M), 1% sodium bicarbonate (7.5% w/v) and 0.2% Fungizone (amphotericin-B, 250 mcg/ml). All

ingredients were purchased from Gibco, Burlington, Ont., except the fetal bovine serum which was purchased from Flow Laboratories Mississauga, Ont. The cells were incubated under 5% CO_2 tension and near 100% humidity. On the day of an experiment, the cultured cell suspension was transferred to 50 ml centrifuge tubes, and the lymphoblasts were pelleted by centrifuging at 1,500 rpm for 10 min. The alpha-MEM supernatant was decanted and the cells were resuspended in 40 ml of veronal buffer. The cells were repelleted as described above and were then washed twice more. The resulting cell pellet was resuspended in veronal buffer to yield a cell concentration of 2.0×10^7 cells/ml. As with the macrophages, 100 µl of this stock solution of cells was used for each experiment to give a final assay concentration of 2.0×10^6 cells.

2.2.3. Human Erythrocytes. On the day of experiment, blood was taken by venipuncture into a heparinized tube and 0.5 ml was suspended in 40 ml of veronal buffer and then centrifuged at 1,500 rpm for 10 min. The supernatant was aspirated and the RBC pellet was washed and centrifuged twice more, and was then suspended in veronal buffer at a concentration of 5.0×10^7 cells/ml. For all studies, 100 µl of this suspension was used for each tube so that it would contain 5.0×10^6 cells.

2.2.4. Cell Viability. The viability of macrophages, lymphoblasts and lymphocytes was checked using the trypan

blue exclusion method (McGee and Myrvik, 1981).

2.2.5. Uptake Studies. The nickel-complexing ligands used in this assay were: HSA, L-His, L-Lys, L-Asp, DDC, D-Pen and EDTA. All were purchased from BDH, J. T. Baker (Phillipsburg, N.J.) or Sigma (St. Louis, Mo.). $^{63}NiCl_2$ was purchased from New England Nuclear Corporation (Lachine, Que.) and had a specific activity of 0.6mCi/μmol (in 0.2 ml of 0.5M HCl). The incubation vials were 15ml Falcon 2059 plastic centrifuge tubes. Each incubation tube contained (in the order added): 100 μl of $^{63}NiCl_2$ (diluted in veronal buffer to give approximately 70,000 cpm/tube and $7.0x10^{-7}$ M in the 100 μl), 100 μl of ligand (10^{-2} to 10^{-6}M dissolved in veronal buffer), 700 μl of veronal buffer, and 100 μl of the cell suspension (in the concentrations given above). Here, and throughout this study, the veronal buffer (pH 7.4) employed was $5x10^{-3}$M (sum of the acid and its conjugate base) and 0.19M in NaCl. The vials were then incubated unstoppered for 2 hr at 37°C and near 100% humidity. Following incubation, 3.0 ml of veronal buffer was added to each assay vial, which was followed by centrifugation at 1,500 rpm for 10 min. The supernatant was removed by decantation and the cells were washed twice more with 3 ml aliquots of veronal buffer. The resulting cell pellet was transferred to scintillation counting vials with 3 washes of 0.3 ml distilled water for RBCs, or 3 washes of 0.3 ml of distilled water acidified with 1 M HCl

for macrophages and lymphoblasts. The radioactivity was assessed by liquid scintillation counting of $^{63}Ni^{2+}$ using 10 ml of cocktail (either Aquasol 2 from New England Nuclear, or ACS from Amersham). The vials were allowed to sit overnight prior to counting so as to allow the $^{63}Ni^{2+}$ to stabilize, thus avoiding chemiluminescence as recommended by Kasprzak and Sunderman (1979).

2.2.6. Removal Studies. Preparation and concentrations of ligands and $^{63}Ni^{2+}$ were the same as for the uptake studies. For these assays, all incubation vials contained 100 µl of $^{63}Ni^{2+}$ (approximately 70,000 cpm). The initial incubation conditions were as described for the uptake studies. After the incubation, the cells were washed as already described. The pellets were then resuspended in 900 µl of veronal buffer, to which was added 100 µl of ligand at various concentrations (10^{-2}-10^{-6}M). The cells were allowed to incubate for 1/2 hr under the previously described conditions. Following the incubation period, 3 ml of veronal buffer was added to each tube and the cells were pelleted at 1,500 rpm for 10 min and the supernatant decanted. The cell pellet was washed twice more with 3 ml aliquots of buffer and was then transferred and counted as described for the uptake studies.

2.2.7. Effect of pH on the Uptake of Ni^{2+}. A titration curve was established by titrating barbital ($2.0x10^{-2}$M; 0.76M NaCl) into sodium barbital ($2.0x10^{-2}$M; 0.76M NaCl)

and vice versa to determine the relative volumes required for pH values from 5 to 9. Using this curve, appropriate volumes of the parent buffer solutions were combined to generate buffers of pH 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. These solutions were freshly prepared and diluted 4-fold before each experiment.

Stock $^{63}NiCl_2$ was diluted 1000 times (approximately 70,000 cpm/100 µl) in each of these buffers, then 100 µl aliquots of these dilutions were pipetted into plastic Falcon tubes in triplicate, followed by 850 µl of the appropriate buffer. Macrophages, peripheral lymphocytes or B-lymphoblasts were prepared in veronal buffer at pH 7.4, as already described and 50 µl of the cell suspension was added to each tube to yield a final concentration of 10^6 cells per ml. The cells were then incubated in loosely capped tubes for 2 hr at 37°C and 100% humidity. At the end of the incubation period the tubes plus cells were centrifuged at 1500 rpm for 10 min and the supernantant was decanted. Two ml buffer of the appropriate pH was added to each tube, and was centrifuged and decanted as before. Washing was also repeated. The cells were then transferred into counting vials with veronal buffer pH 7.4, which was followed by the addition of 10ml scintillation cocktail.

Extra tubes with cold $NiCl_2$ ($7x10^{-7}M$) replacing the $^{63}NiCl_2$, but with other conditions the same, were run

in parallel. One set was for testing cell viability at the end of the incubation period, and a second set for pH measurements. A third set of tubes without cells, but containing ^{63}Ni, was included to determine non-specific binding of label to the walls of the tubes.

2.3 RESULTS

Viability before experiments always exceeded 90%, and cell mortality by the end of the experiment usually did not exceed 20% for lymphoblasts and lymphocytes or 15% for macrophages.

Typical results for uptake and removal studies are presented in Figs. 2.1-2.6. From the data in Figs. 2.1-2.3, it is clear that EDTA, D-Pen, L-His and HSA are very effective inhibitors of the uptake of $^{63}Ni^{2+}$ by rabbit alveolar macrophages and human B-lymphoblasts. The amino acids L-Asp and L-Lys were shown to be poor blockers of $^{63}Ni^{2+}$ uptake while DDC promoted the association of $^{63}Ni^{2+}$ with these cells (as described in more detail in Chapter 3). As shown in Figs. 2.4-2.6, the ligands that proved to be good inhibitors of $^{63}Ni^{2+}$ uptake, were also good agents for the removal of $^{63}Ni^{2+}$ from cells. Again, L-Lys and L-Asp were proven to be less effective in the extraction of $^{63}Ni^{2+}$ from cells. DDC showed no significant effect in its ability to remove $^{63}Ni^{2+}$ from lymphoblasts, RBCs or

macrophages.

Tables 2.1 and 2.2 show the IC_{50} (the concentration of ligand needed to inhibit Ni^{2+} uptake by 50%) and the RC_{50} (the concentration of ligand needed to reduce cell-associated Ni^{2+} by 50%) values for all ligands and cell types tested. From the data in Table 2.1 it can be seen that EDTA is the strongest Ni^{2+} inhibitor as it has the lowest IC_{50} value. It is followed closely by HSA, D-Pen and L-His, while L-Asp is a very poor inhibitor of Ni^{2+} cellular uptake since its IC_{50} concentration is much higher than the concentrations of L-Asp normally found in vivo. DDC and L-Lys did not inhibit Ni^{2+} uptake enough to give IC_{50} values. Similar trends are shown in Table 2.2 for RC_{50} values except that in general, the RC_{50} values for ligands were higher than the corresponding IC_{50} values. The only exceptions are D-Pen, L-His and L-Asp for macrophages where the IC_{50} values are very near the RC_{50} values.

It is clear from the data in Fig. 2.7 that Ni^{2+} uptake is a pH-dependent process. Both lymphocytes and lymphoblasts seem to accumulate the maximum amount of $^{63}Ni^{2+}$ at pH 8.0, whereas macrophage uptake did not show evidence of levelling off with increasing pH. There was an increase in cell mortality at both extremes of pH (5.9% at pH 7.4, to 30% at pH 8.5 and 18% at pH 6.0 for lymphocytes and lymphoblasts, and 15% at pH 7.4 to 47.3% at pH 8.5 and

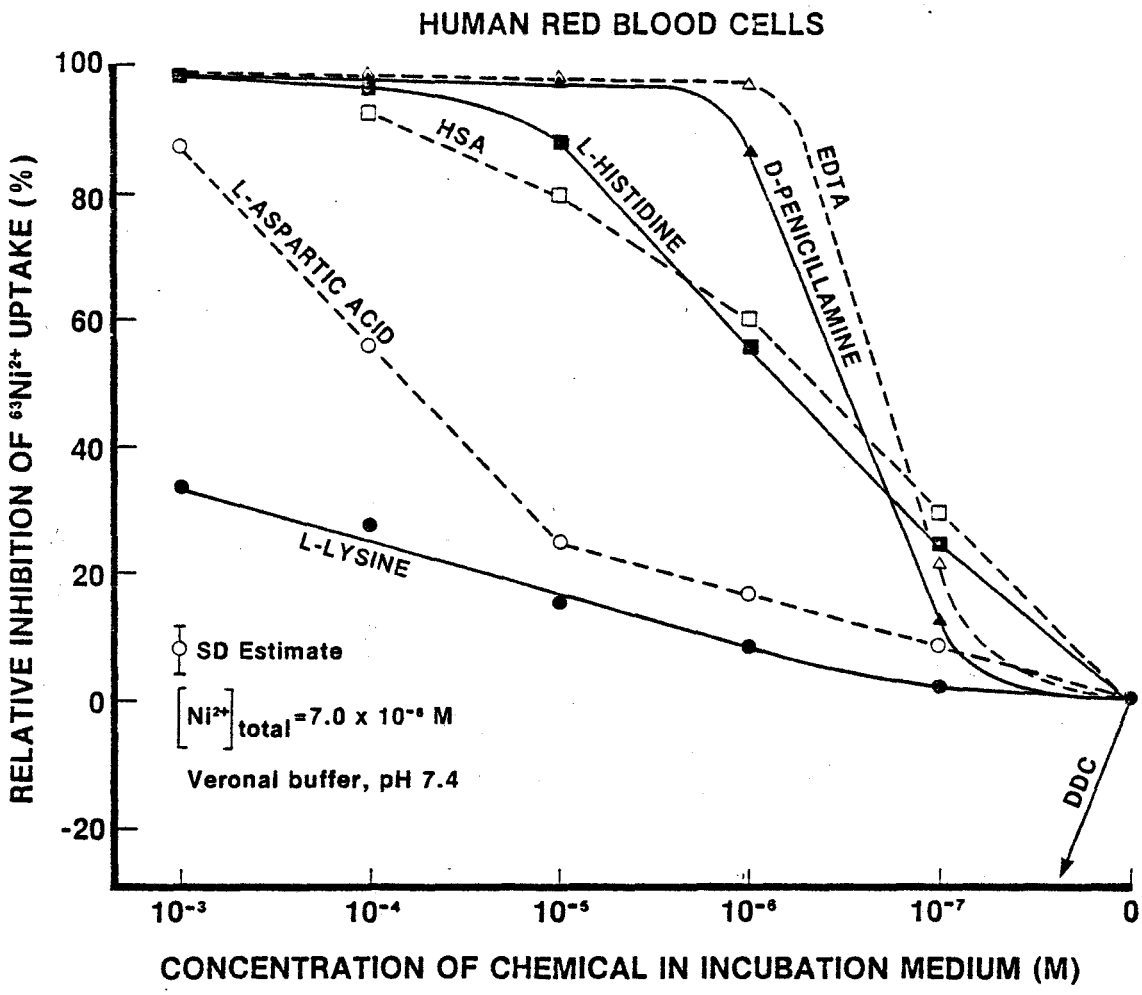

Fig. 2.1. The effect of Ni^{2+}-complexing agents upon the uptake of $^{63}Ni^{2+}$ by human red bood cells. Labelled nickel and the ligands were both present in the incubation medium. The estimate of the standard deviation corresponds to a pooled value for duplicate samples. See text for additional comments on the DDC enhancement effect.

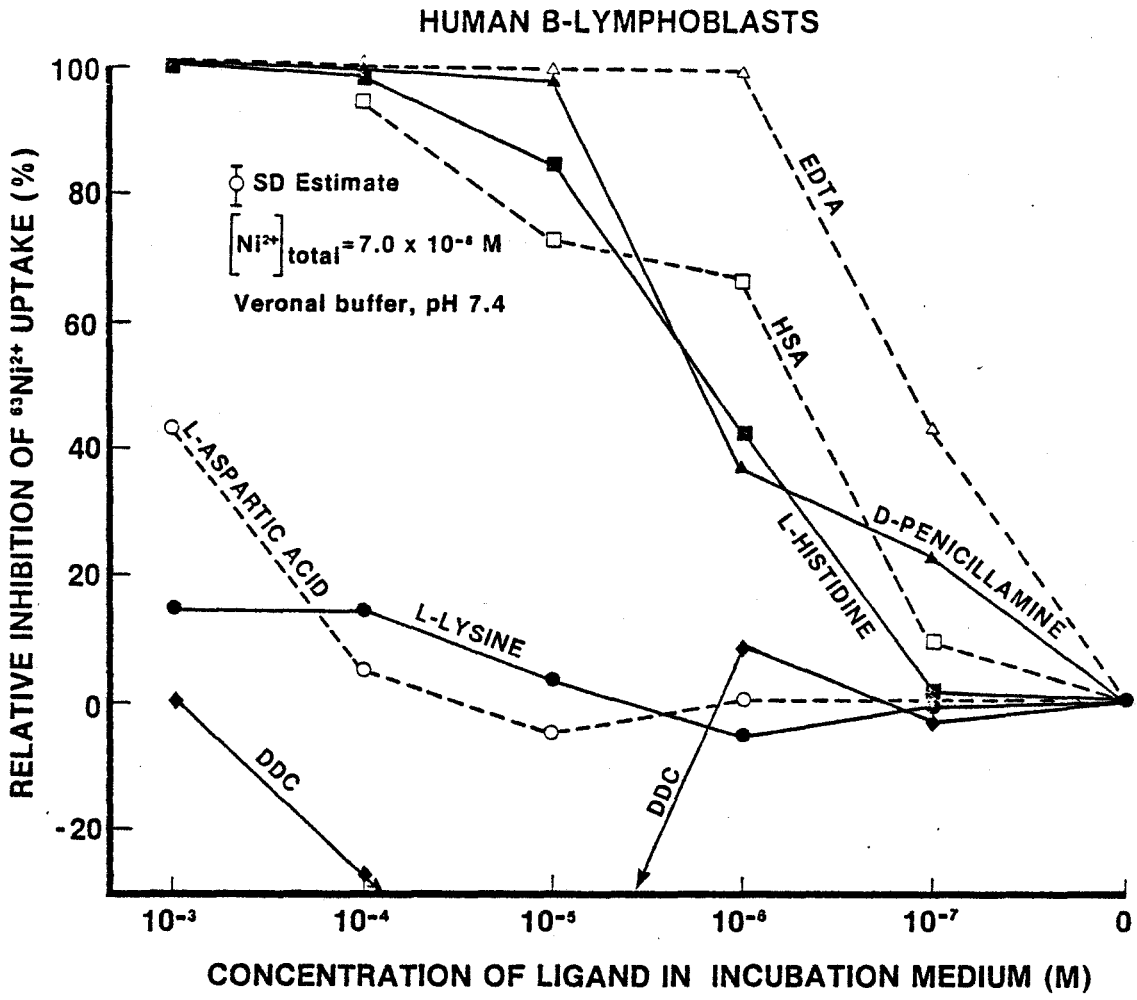

Fig. 2.2 The influence of Ni^{2+}-complexing agents on the uptake of $^{63}Ni^{2+}$ by cultured human B-lymphoblasts. Labelled nickel and the ligands were both present during incubation. The estimate of the standard deviation corresponds to a pooled value from duplicte samples. See text for additional comments on the DDC enhancement effect.

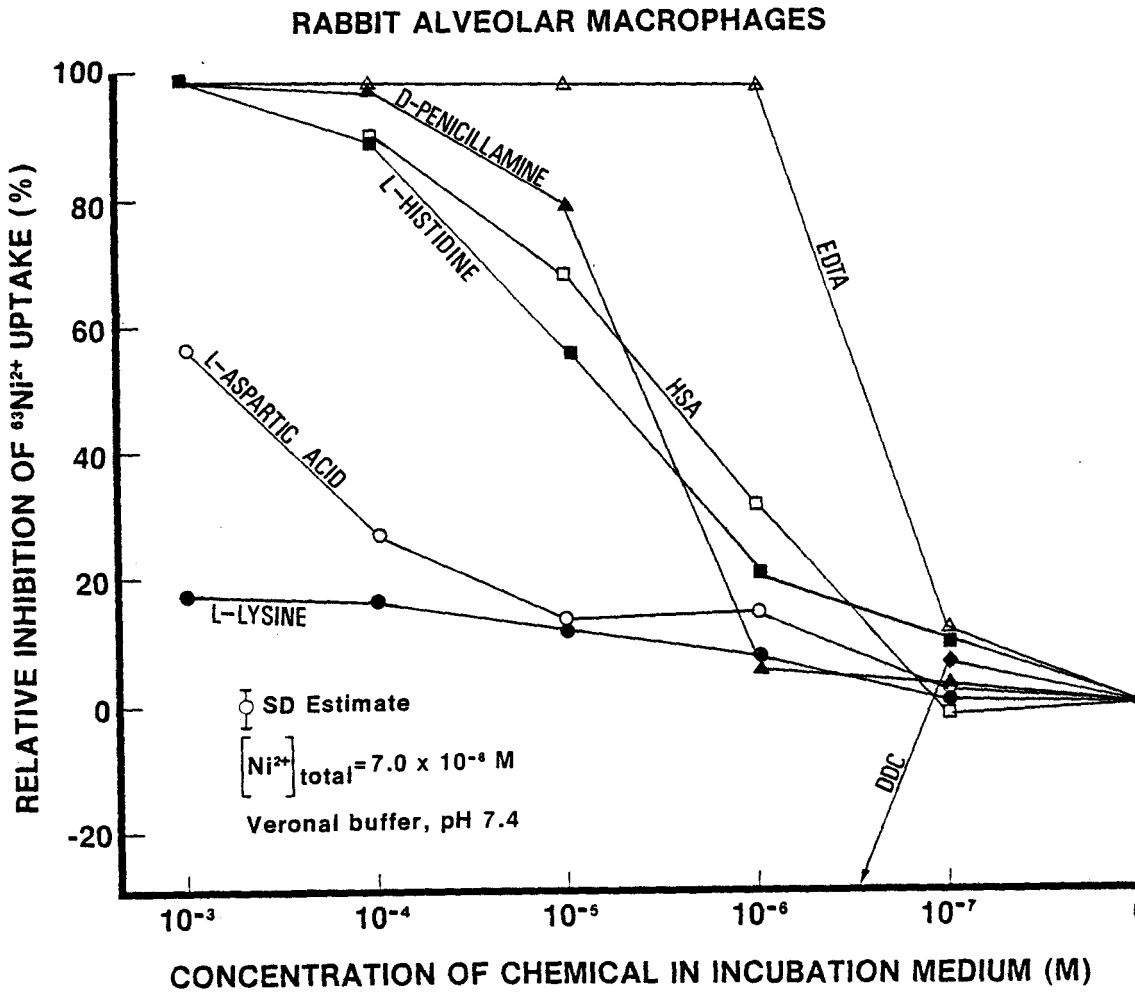

Fig. 2.3. The effect of Ni^{2+}-complexing agents upon the uptake of $^{63}Ni^{2+}$ by rabbit alveolar macrophages. Labelled nickel and the ligands were both present in the incubation medium. The estimate of the standard deviation corresponds to a pooled value for duplicate samples. See text for additional comments on the DDC enhancement effect.

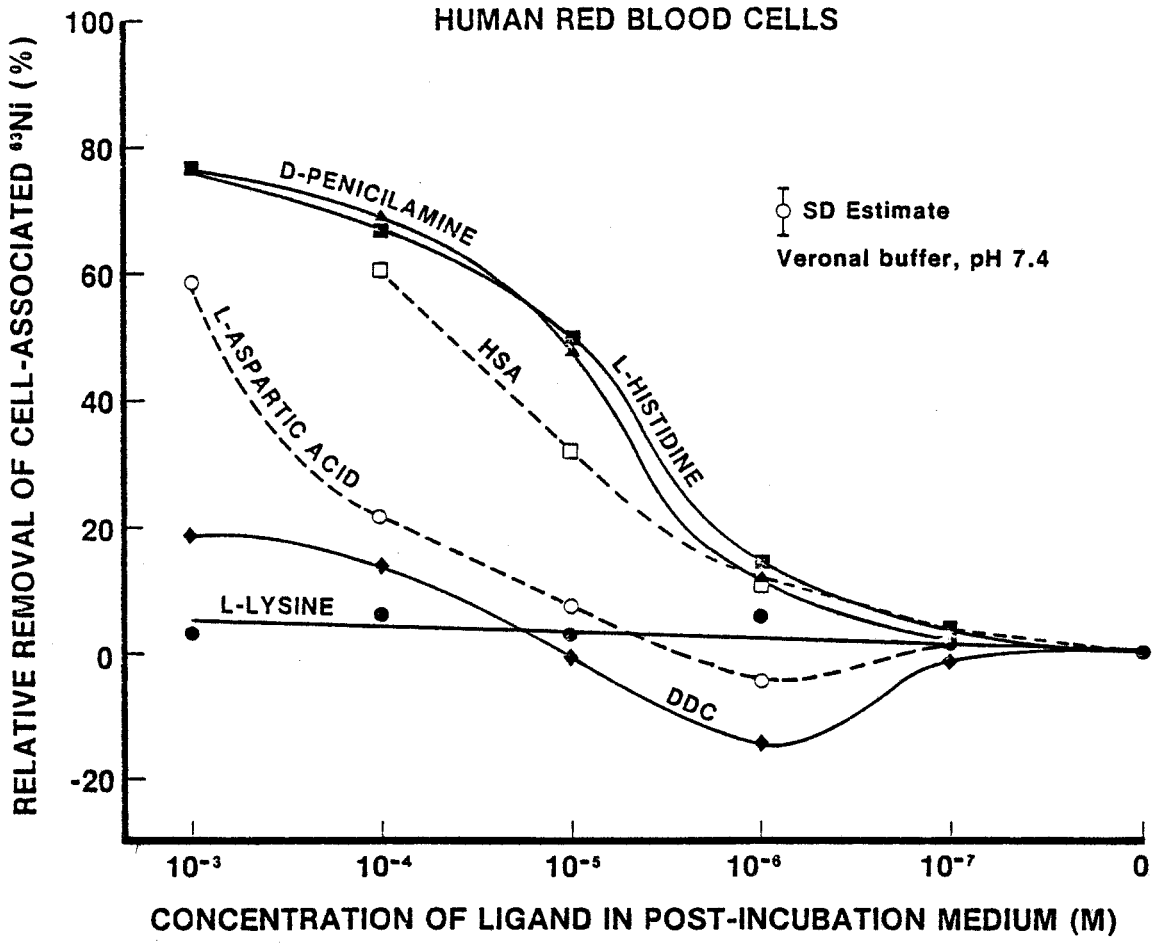

Fig. 2.4. The relative ability of chelating agents in extracting $^{63}Ni^{2+}$ from human red blood cells preloaded with $^{63}Ni^{2+}$. The standard deviation shown corresponds to a pooled value from duplicate samples.

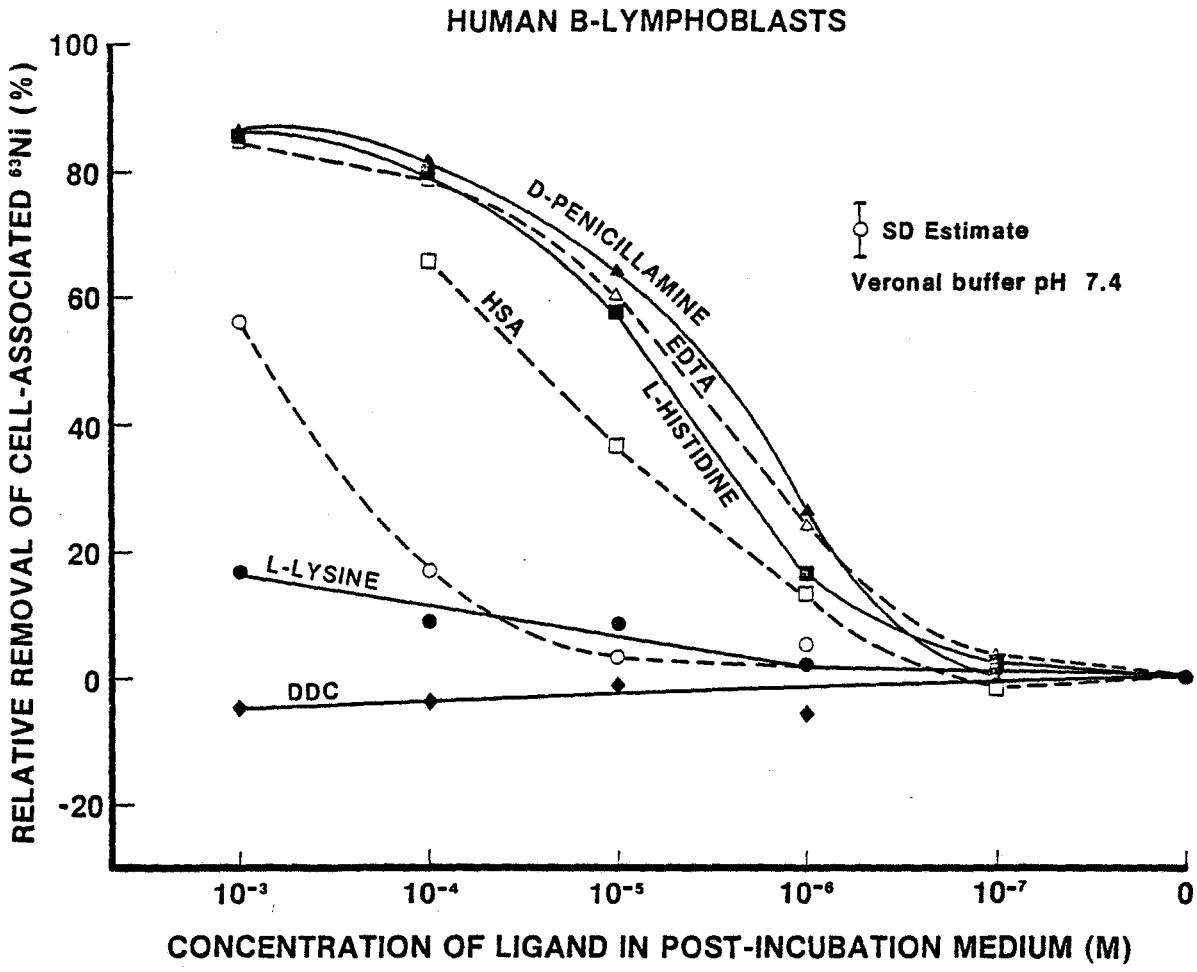

Fig. 2.5. The ability of Ni^{2+}-chelating ligands to remove $^{63}Ni^{2+}$ from preloaded human B-lymphoblast cells. The standard deviation shown corresponds to a pooled value from duplicate samples.

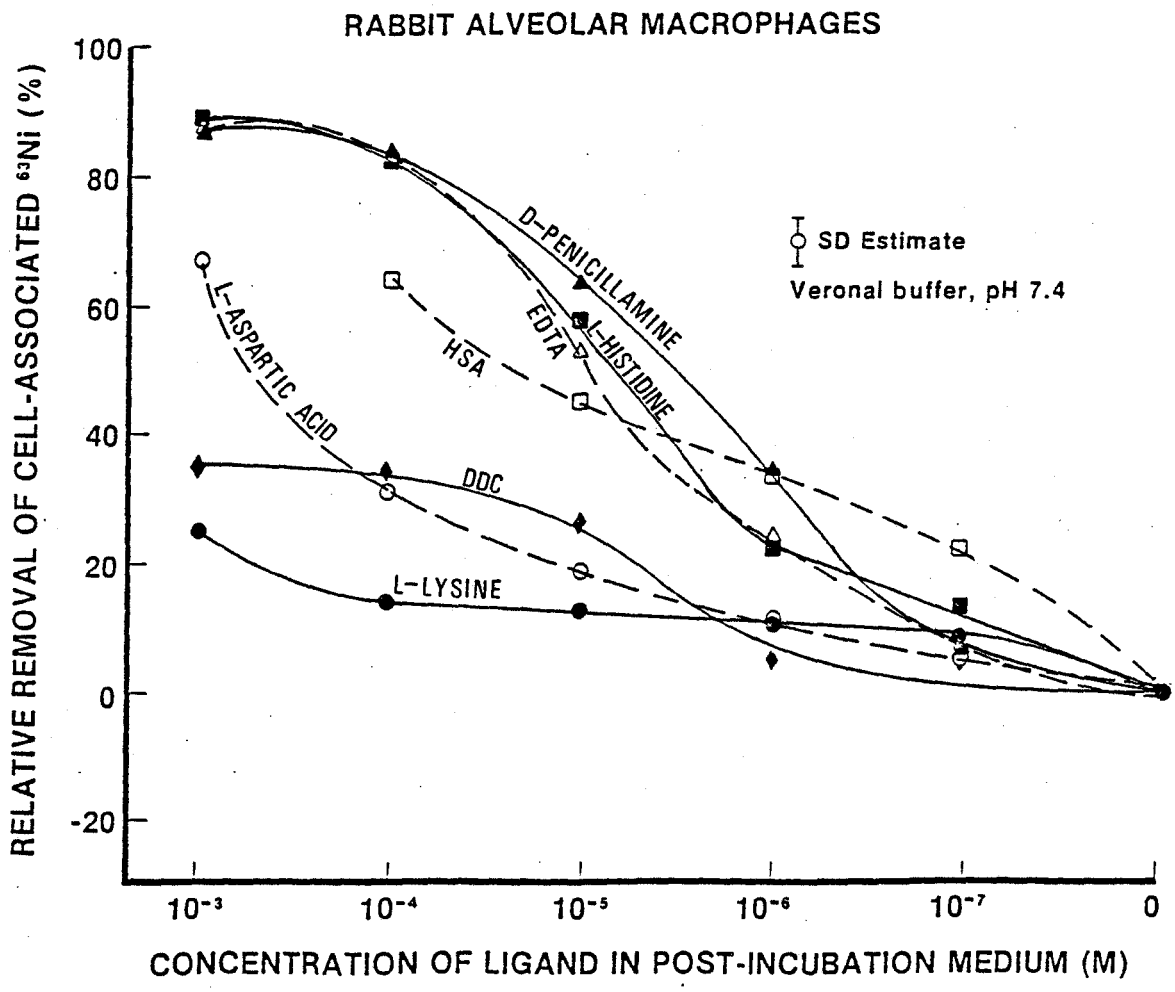

Fig. 2.6. The relative ability of chelating agents in extracting $^{63}Ni^{2+}$ from rabbit alveolar macrophages preloaded with $^{63}Ni^{2+}$. The standard deviation shown corresponds to a pooled value from duplicate samples.

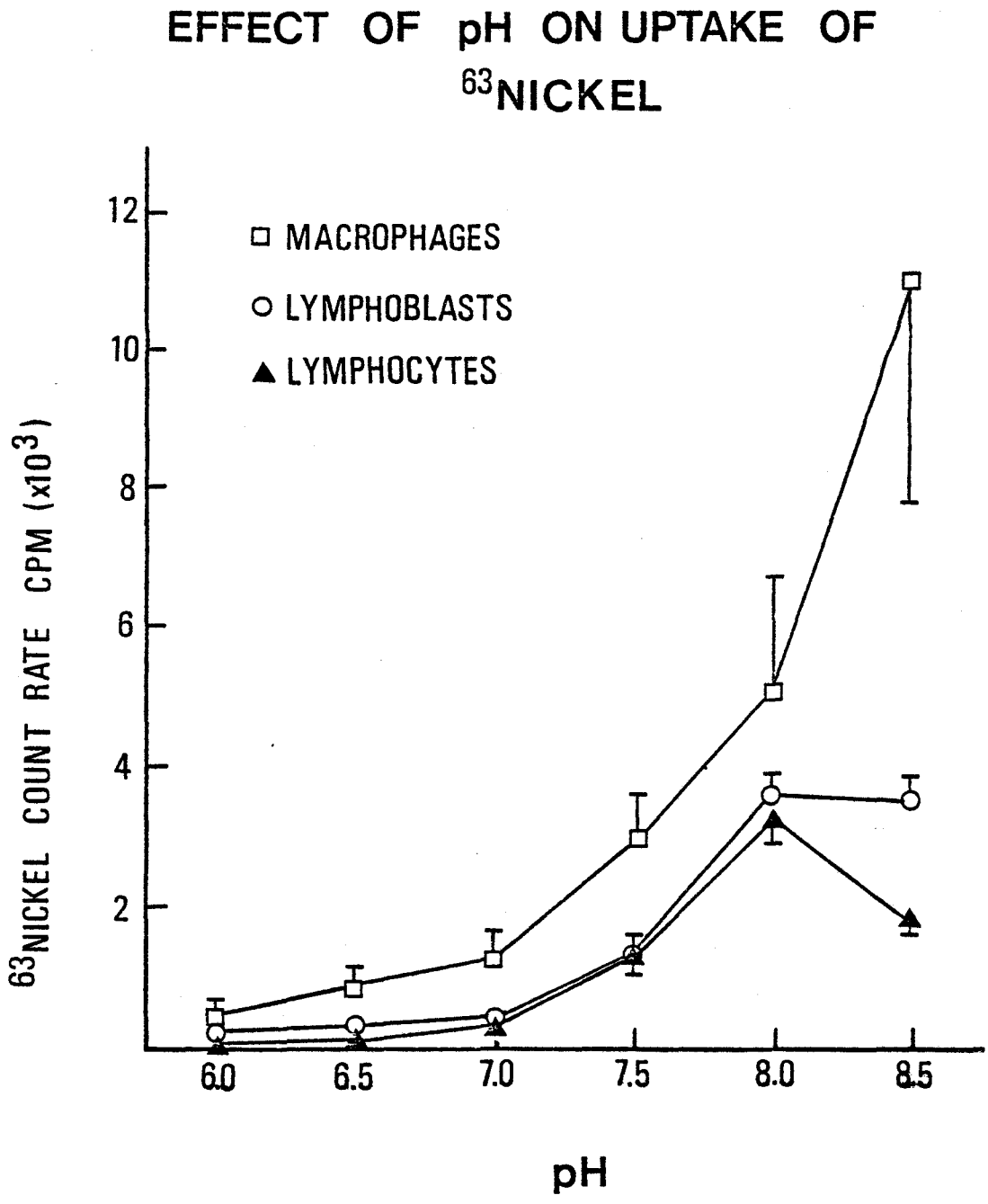

Fig. 2.7. The effect of pH upon the uptake of $^{63}Ni^{2+}$ by rabbit alveolar macrophages, human periperal lymphocytes and cultured human B-lymphoblasts. The standard deviation is calculated from each sample done in triplicate.

Table 2.1. Concentration of ligand that inhibits $^{63}Ni^{2+}$ uptake by 50% (IC_{50})[a].

Ligand	IC_{50} for RBCs	IC_{50} for Macrophages	IC_{50} for Lymphoblasts
EDTA	[b] $(3\pm1)\times10^{-7}$	[c] $(2.8\pm0.5)\times10^{-7}$	[c] $(1.3\pm0.4)\times10^{-7}$
HSA	[b] $(5\pm1)\times10^{-7}$	[c] $(2\pm1)\times10^{-6}$	[c] $(5.2\pm0.5)\times10^{-7}$
D-Pen	[b] $(3.3\pm0.3)\times10^{-7}$	[b] $(3\pm1)\times10^{-6}$	[b] $(1.6\pm0.2)\times10^{-6}$
L-His	[b] $(1.0\pm0.4)\times10^{-6}$	[b] $(9\pm3)\times10^{-6}$	[b] $(1.5\pm0.1)\times10^{-6}$
L-Asp	[b] $(3\pm2)\times10^{-4}$	[c] $(6\pm1)\times10^{-4}$	-------
L-Lys	--------	--------	-------
DDC	--------	--------	-------

[a] In case of L-Asp, L-Lys and DDC, 50% inhibition was not attained and thus no IC_{50} could be determined. IC_{50} values are expressed in mol/l.

[b] Estimate $\pm$SD; evaluated from the intersection of the horizontal line y=50% with the inhibition curve and the associated error-band branches. The inhibition curves were constructed by pooling data for two separate experiments, each with duplicate samples. The lines joining the tips of the error bars constituted the error-band branches.

[c] Calculated as per footnote b, but employing data for a single experiment with duplicate samples.

Table 2.2. Concentration of ligand that removes 50% of $^{63}Ni^{2+}$ $(RC_{50})^{a,b}$.

Ligand	RC_{50} for RBCs	RC_{50} for Macrophages	RC_{50} for Lymphoblasts
EDTA	$(5.1\pm0.4)\times10^{-6}$	$(7.7\pm0.3)\times10^{-6}$	$(1.0\pm0.3)\times10^{-5}$
HSA	$(2.8\pm0.5)\times10^{-5}$	$(1.9\pm0.5)\times10^{-5}$	$(4.4\pm0.6)\times10^{-5}$
D-Pen	$(4.2\pm0.5)\times10^{-6}$	$(3.6\pm0.7)\times10^{-6}$	$(1\pm2)\times10^{-5}$
L-His	$(6.4\pm0.6)\times10^{-6}$	$(6\pm1)\times10^{-6}$	$(9\pm3)\times10^{-6}$
L-Asp	$(7.0\pm0.9)\times10^{-4}$	$(3.3\pm0.1)\times10^{-4}$	$(6.3\pm0.4)\times10^{-4}$
L-Lys	------	------	------
DDC	------	------	------

[a]In case of L-Lys and DDC, 50% removal was not attained and thus no RC_{50} could be determined. RC_{50} values are expressed in mol/l.

[b]Estimate $\pm$SD; evaluated from the intersection of the horizontal line Y=50% with the removal curve and the associated error-band branches. The removal curves were constructed by employing data for a single experiment with duplicate samples. The lines joining the tips of the error bars constituted the error-band branches.

Table 2.3. % Inhibition (IP) of $^{63}Ni^{2+}$ uptake at physiological concentrations of ligand.

Ligand	Mean Serum Concentration(M)	IP for RBCs	IP for Macrophages	IP for Lymphoblasts
HSA[a]	6.0×10^{-4}	[b]97±4	[c]99.0±0.9	[c]99.0±0.1
L-His	7.4×10^{-5}	[b]94±2	[b]84±3	[b]96±2
L-Asp	7.5×10^{-6}	[b]18±9	[c]14±3	[c]-4±5
L-Lys	1.5×10^{-4}	[b]20±9	[c]17±5	[c]14±3

[a]The HSA_4 values were obtained by extrapolating its uptake curves from 10^{-4}M to 6.0×10^{-4}M.

[b]Estimate ± SD; evaluated from the intersection of the vertical line X=physiological concentration with the inhibition curve and the associated error-band branches. The inhibition curves were constructed by pooling data for two separate experiments, each with duplicate samples. The lines joining the tips of the error bars constituted the error-band branches.

[c]Calulated as per footnote b, but employing data for a single experiment with duplicate samples.

Table 2.4. % Removal (RP) of $^{63}Ni^{2+}$ from cells at physiological concentrations.[b]

Ligand	Mean Serum Concentration (M)	RP for RBCs	RP for Macrophages	RP for Lymphoblasts
HSA[a]	6.0×10^{-4}	70.0±0.6	79±3	84±1
L-His	7.4×10^{-5}	65±2	79±1	76.7±0.4
L-Asp	7.5×10^{-6}	6±4	17.8±0.6	3.9±0.8
L-Lys	1.5×10^{-4}	5±2	16±6	11±4

[a]The HSA values were obtained by extrapolating its removal curves from 10^{-4}M to 6.0×10^{-4}M.

[b]Estimate ± SD; evaluted from the intersection of the vertical line X=physiological concentration with the removal curve and the associated error-band branches. The removal curves were constructed by employing data for a single experiment with duplicate samples. The lines joining the tips of the error bars constituted the error-band branches.

Table 2.5. Relative uptake of $^{63}Ni^{2+}$ by cells.

Cell Type	% $^{63}Ni^{2+}$ Uptake per 10^6 cells[a]	Relative Affinity
RBCs	0.4±0.1	0.13
Lymphocytes	3.0±1.3	1.0
Macrophages[b]	5.6±4.3	1.3
B-Lymphoblasts[c]	9.6±1.4	3.2

[a] In veronal buffer, pH 7.4, no ligand added; added $[Ni^{2+}]=7.0\times10^{-8}$M. Average ± SD for five or more experiments.

[b] The large standard deviation reflects that the magnitude of the nickel uptake depended on the lung specimens, although the trends reported (e.g. dependence on pH, and ligand concentration) were reproducible.

[c] The mean value reported for B-Lymphoblasts are for studies involving cells harvested and used in their mid-log growing phase. Use of end-of-growing-phase cells resulted in lower Ni^{2+} accumulations and higher cell mortality. Nevertheless, the trends reported (e.g., dependence on pH, and ligand concentration) were reproducible.

23% at pH 6.0 for macrophages). A slight change in pH towards more acidic values was observed by the end of the incubation period.

The compilations in Tables 2.3 and 2.4 show the relative abilities in blocking the association of Ni^{2+} with cells at physiological concentrations of HSA and the three amino acids tested. It is clear that HSA and L-His were very good inhibitors and extractors of cell-associated Ni^{2+}, while L-Asp and L-Lys were relatively ineffective.

2.4 DISCUSSION

The concentrations of HSA and amino acids used (10^{-3} to 10^{-7}M) were chosen so as to encompass the range found *in vivo*. The experimental concentration of added Ni^{2+} was 7.0×10^{-8}M (=4.1 µg/l; $^{63}Ni^{2+}$ was 5-20% of total nickel in the labelled reagent) which is comparable to the levels in serum (2.6±0.9 µg/l) for individuals not occupationally exposed to nickel (Sunderman Jr., 1977). The mean concentrations in adult plasma or serum of the three amino acids are: L-Asp, 7.5×10^{-6}M (range $0-2.4 \times 10^{-5}$M); L-Lys, 1.5×10^{-4}M (range $8.3 \times 10^{-5}-2.4 \times 10^{-4}$M); and L-His, 7.4×10^{-5}M (range $3.2 \times 10^{-5}-1.1 \times 10^{-4}$M). For comparison, the mean serum/plasma concentration of albumin is 6.0×10^{-4}M (Dickinson *et al.*, 1965), with a range of 5.4×10^{-4} to 7.7×10^{-4}M (Cannon *et al.*,1974). From the

plots in Figs. 2.1-2.6 and the data in Tables 2.1-2.4, it can be seen how effective physiological concentrations of L-His and HSA are in controlling association and removal of $^{63}Ni^{2+}$ from cells. The inhibitory effect of these ligands is comparable to that exhibited by EDTA and D-Pen which, like L-His and HSA (Glennon and Sarkar, 1982), have a high capacity for binding nickel (Laitinen and Harris, 1975; Laurie et al., 1979). The high (and in some cases undetected) values of IC_{50} and RC_{50} for L-Lys and L-Asp were expected, since these amino acids have apparent low affinities for Ni^{2+} (Lucassen & Sarkar, 1979). DDC, however, has a relatively high capacity for binding Ni^{2+} (Sandell and Onishi, 1978), and thus it might have been expected to yield similar IC_{50} and RC_{50} values to EDTA and D-Pen. Instead DDC enhanced Ni^{2+} association with the cells, an unexpected action for a therapeutic drug whose activity was believed to be the removal of Ni^{2+} from tissues. In Chapter 3 it is demonstrated that this behavior of DDC is related to the lipophilicity of its nickel complex.

Lucassen and Sarkar (1979) determined that the only amino acid that could compete with HSA for Ni^{2+} binding was L-His, as no other amino acid had any substantial binding capacity for Ni^{2+}. This was shown at a 1:1 ratio of HSA to each amino acid as well as at physiological concentrations. More recently, cysteine has been suggested to be an

efficient inhibitor of Ni^{2+} uptake by chinese hamster ovary cells (Abbracchio et al., 1982a), but the cysteine concentrations needed to block effectively Ni^{2+} uptake ($2.5x10^{-4}$ to $5x10^{-3}$M) were much higher than normal in vivo concentrations (the mean adult blood plasma level of cysteine is $4.4x10^{-5}$M, with a range of $8.3x10^{-6}$-$8.4x10^{-5}$M, Dickinson et al., 1965). In Figs. 2.1-2.6, it is shown that L-Asp also had some nickel regulating effect at concentrations well above the in vivo levels. Thus, it seems that neither cysteine nor aspartic acid contribute greatly to cellular Ni^{2+} regulation.

Recently, Hansen and Stern (1983) have formulated an important toxicological principle for nickel: namely, that equal transformation rates of baby hamster kidney cells (BHK-21) occurred at equal toxicities, regardless of the source of nickel. Both soluble and insoluble nickel compounds were studied. Equal toxicity, as measured by survival rates, was interpreted to correspond to equal intracellular concentrations of Ni^{2+}. Consequently, nickel compounds with high phagocytic indices (Kuehn et al., 1982) and relatively favourable dissolution rates (Kuehn & Sunderman, 1982), may be expected to have significant toxicity because of high bioavailablilty of Ni^{2+}. A prerequisite for nickel carcinogenesis appears to be the accumulation of Ni^{2+} in the cell nucleus, where it has the potential of interacting with DNA and presumably inducing

unwanted mutations (Costa et al., 1982). It seems clear from the present work, that the removal of Ni^{2+} from the cell by the sequestering action of extracellular ligands such as HSA and L-His may influence the intracellular compartmentalization of Ni^{2+}, and may thus constitute an important detoxification mechanism.

It is clear from the data plotted in Fig. 2.7 that an increase in pH produces an increase in cellular association of Ni^{2+}. It is interesting to speculate about the basis for this observation. Increasing the extracellular pH above 7.4 causes significant proton gradients to be established across membranes. Such gradients are implicated in the regulation of many biochemical processes, including the translocation of ions and neutral metabolites (Nicholls, 1982; Nieboer et al., 1983c). Consequently, the observed dependence on pH implies that either cell membrane permeability increases with pH, or that extrusion of protons accompanies Ni^{2+} uptake. Interestingly, proton/Ni^{2+} co-transport has been reported in the bacterium Methanobacterium bryantii (Jarrell and Sprott, 1982). Futhermore, it is likely that another contributing factor is a reduction in the competition of protons with Ni^{2+} for binding sites at higher pH values. This occurs because most functional groups that bind metal ions are weak acids, and thus metal complex formation reactions in aqueous media are

effectively proton displacements. Some degree of saturation at high pH values ($\geq$8) was evident for lymphocytes and lymphoblasts, but not for macrophages (see Fig. 2.7). The increase in cell mortality at pH 8.0 to 8.5 may have contributed to this "leveling off" effect by lymphocytes and lymphoblasts and/or the greater increase of cellular Ni^{2+} uptake in macrophages.

The percentage uptake of $^{63}Ni^{2+}$ under the experimental conditions varied with cell type. The $^{63}Ni^{2+}$ uptake per 10^6 cells was: 0.4% for human RBCs, 5.6% for rabbit alveolar macrophages, 9.6% for cultured human B-lymphoblasts, and 3.0% for human peripheral blood lymphocytes (as summarized in Table 2.5). The physiological and biological significance of such differences in Ni^{2+} uptake capacity is still to be elucidated, but no doubt is of relevance to our knowledge of the distribution of Ni^{2+} among the cellular components of whole blood, and of the mechanism underlying the cellular response in individuals with nickel dermatitis.

CHAPTER 3

THE EFFECT OF NI(II)-BINDING LIGANDS UPON CELLULAR AND WHOLE BLOOD DISTRIBUTION OF NICKEL(II)

3.1 INTRODUCTION

Nickel has various forms to which man can be exposed. Commonly found compounds of nickel in industry are: metallic Ni, Ni-containing sulfur compounds (e.g. alpha Ni_3S_2), nickel oxides (e.g. NiO), and the gas nickel carbonyl ($Ni(CO)_4$). Some of these compounds (especially alpha Ni_3S_2) have proven in animal studies to be carcinogenic (Sunderman & Maenza, 1976; Sunderman, 1981, 1983b) and epidemiological studies have implicated their carcinogenicity in man (Doll, 1958; Morgan, 1958; Doll et al., 1977; Pedersen et al., 1978; Roberts, et al., 1982). As discussed in Chapter 2, an attractive model of nickel toxicity is that intracellular bioavailability is the most important determinant. Once Ni^{2+} is introduced into the cellular cytosol, it appears to enter the nucleus causing chromosomal damage, such as increasing the amount of DNA single strand breaks, which lead to DNA crosslinkages and an increase in chromosomal abberations. It is also postulated that Ni^{2+} may lead to a decrease in the fidelity of DNA synthesis resulting in mutations, transformations and ultimately carcinogenesis (Costa et al., 1982; Sirover and Loeb, 1976).

Entry of nickel into the cells is usually by one of

several means. The simplest is the direct uptake of Ni^{2+}; while facilitated transport by suitable ligands also is possible as demonstrated in the present studies. Considerable evidence exists that phagocytosis of nickel containing particulates (Costa et al., 1981; Kuehn et al., 1982). Presumably, Ni^{2+} ions are subsequently released into the cytosol by intracellular dissolution of the phagocytized material (Abbracchio et al., 1982b). From a mechanistic point of view, and as already alluded to, the intracellular accumulation and compartmentalization of Ni^{2+} may thus be critical. In Chapter 2 it was shown that natural and synthetic nickel chelating agents were able to regulate the amount of nickel associated with cells. Since ligands such as L-His and HSA can readily remove Ni^{2+} ions from cells, a detoxification mechanism may exist. A related question is whether ligands are able to influence the compartmentalization of cell-associated Ni^{2+}. This matter is examined in some detail in the current work.

Two therapeutic agents used in Ni poisoning are diethyldithiocarbamate (DDC) and D-Penicillamine (D-Pen). Although they have been proven to be effective in preventing injury caused by exposure to nickel compounds in animals, the exact mechanisms by which they act have not yet been determined. It has been shown that DDC has the best antidotal effects in $Ni(CO)_4$ poisoning, especially when administered just before or just after exposure to the

gas $Ni(CO)_4$. D-Pen did not show any antidotal effect until used at least 6 hr after exposure (Baselt et al,1977). In contrast, for Ni^{2+} toxicity in rats (administered as $NiCl_2$), D-Pen was shown to be more effective in preventing death than was DDC (Horak et al,1976). It appears therefore that these reagents act by quite different mechanisms. Interestingly, it has been shown that DDC appears to enhance nickel deposition in such organs as the lung and the brain after injection of $NiCl_2$, while D-Pen appears to remove Ni^{2+} from all tissues (Oskarsson and Tjälve, 1980). This observation agrees with results obtained in Chapter 2 where it was found that DDC enhanced association of Ni^{2+} with cells; D-Pen, on the other hand, prevented such Ni^{2+} uptake. Cell distribution studies described below indicate that these different experimental patterns may be correlated with the lipophilic character of the resulting nickel complexes.

3.2 MATERIALS AND METHODS

3.2.1. Distribution Studies. Isolation and purification of RBCs, rabbit alveolar macrophages and cultured human B-lymphoblasts was as described in the previous chapter. The nickel-complexing ligands used in this assay were: L-histidine and D(-)-penicillamine (from Sigma) and sodium diethyldithiocarbamate (from J. T. Baker). $^{63}NiCl_2$ was

purchased from New England Nuclear Corporation (Lachine, Que.) and had an activity of 2mCi in 0.2 ml of 0.5M HCl. The incubation vials were 15ml Falcon 2059 plastic centrifuge tubes. Each incubation tube contained (in the order added): 100 µl of $^{63}NiCl_2$ (diluted in veronal buffer to give approximately 70,000 cpm/tube and $7.0x10^{-7}$ M in the 100 µl), 100 µl of ligand (10^{-2}-10^{-6}M dissolved in veronal buffer), 700 µl of veronal buffer, and 100 µl of the cell suspension (in the concentrations given above). The vials were then incubated unstoppered for 2 hr at 37°C and near 100% humidity. Following incubation, 3.0 ml of veronal buffer was added to each assay vial, followed by centrifugation at 1,500 rpm for 10 min. The supernatant was removed by decantation and the cells were washed twice more with 3 ml aliquots of veronal buffer. At this point the cells were lysed. For RBCs this was accomplished by adding 0.4 ml of distilled water to the cell pellet and briefly vortexing the vial. In order to lyse lymphoblasts and macrophages, 0.4 ml of distilled water was added to the cell pellet, which was then vortexed briefly, and frozen quickly in a methanol/dry ice bath, and subsequently was thawed. The suspensions were checked by means of a microscope to confirm lysis. If lysis was not complete, the freeze-thaw procedure was repeated. The cell pellet was isolated by centrifugation at 4°C for 40 min at 20,000 x g (Clark and Switzer, 1977). The supernatant

containing the cell lysate was decanted and saved. The lymphoblast and macrophage membrane pellets were each washed twice with 0.3 ml distilled water followed by centrifugation at 4°C for 30 min at 20,000 x g. The supernatants were decanted and added to the supernatants collected earlier. The RBC membranes were washed and treated in a similar manner except that the 2 washes of distilled water were followed by 2 washes of 0.01M Tris-HCl buffer (pH 7.4) to remove all membrane-associated haemoglobin (Harris, 1969). The pooled lysates were counted in 10 ml of scintillation cocktail. The RBCs membrane pellet was transferred to scintillation vials with 3 washes of 0.3 ml of distilled water followed by 30 s of vortexing. The lymphoblast and macrophage pellets were transferred in a like manner except that the distilled water was heated and had been acidified with 1M HCl. Results are reported only for those samples for which the sum of the radioactive counts for the residual pellet and lysate equalled those of unlysed cells.

3.2.2. Extraction of Protein and Lipid from Lymphoblasts. Lipid and protein were extracted from cells by the method of Ong and Lee (1980a & 1980b). Lymphoblasts were incubated with $^{63}Ni^{2+}$ and DDC, then washed as described previously for uptake studies (see Section 2.2.5). The washed cell pellet was suspended in 0.3 ml of veronal buffer followed by the addition of 0.3 ml of

chloroform and 0.3 ml of methanol. The mixture was shaken vigorously for 30 s, then another 0.3 ml chloroform and 0.3 ml of distilled water were added, and shaken vigorously for an additional 10 min. The extraction mixture was then centrifuged at 2,000 rpm for 20 min which resulted in the formation of two distinct layers. The upper aqueous layer contained the water-soluble $^{63}Ni^{2+}$ ions, while the lower chloroform layer contained the phospholipids and associated lipophilic $^{63}Ni^{2+}$ complexes. The interface between the two layers consisted of insoluble protein and protein-bound $^{63}Ni^{2+}$ ions. The methanolic phase and the insoluble protein layer were removed together. The methanol/protein and chloroform layers were each analysed for $^{63}Ni^{2+}$ by scintillation counting.

3.2.3. Distribution of $^{63}Ni^{2+}$ in Whole Blood. The effect DDC has upon $^{63}Ni^{2+}$ distribution between serum, lymphocytes and RBCs in whole blood was examined. Aliquots of 0.4 ml fresh heparinized whole blood were added to falcon tubes (type 2063). Next was added 50 µl of $^{63}Ni^{2+}$ with known activity (48,000 cpm/50 µl) and 50 µl of DDC (10^{-2}-10^{-6}M). The contents were mixed with gentle shaking and were incubated as previously described (2h, 37°C, 100% humidity). After incubation, 0.5 ml of buffer was added to each vial, mixed and centrifuged at 1,500 rpm for 10 min. So as not to disturb the white buffy coat layer, the serum supernatant was removed carefully. The cells were washed

twice more in the same manner using 1 ml buffer, and the supernatants were removed and counted with the serum fraction. Next 1 ml of veronal buffer was mixed with the cells followed by the addition of 1.0 ml Ficoll Hypaque (1.075 g/ml). After centrifugation at 1,700 rpm for 25 min, the lymphocytes (as the white buffy coat) were removed from the Ficoll-buffer interface and counted in scintillation cocktail. Since the red cells remaining had too high a quench factor to be counted directly, the counts associated with the RBCs were calculated by:

Counts added - (Serum counts + Lymphocyte counts) = RBC counts (3.1)

3.3 RESULTS

The results of the distribution studies are summarized in Figs. 3.1-3.8. It is clear that although L-His and D-Pen had an effect upon the relative amount of $^{63}Ni^{2+}$ associated with cells, they essentially had no influence upon the pattern of distribution of $^{63}Ni^{2+}$ between the cell lysate fraction and the membranous cell pellet fraction. This was demonstrated for all cell types tested. By contrast, as shown Figs. 3.6-3.8, increasing concentrations of DDC caused a shift in $^{63}Ni^{2+}$ distribution from the cell lysate to the cell pellet.

Also noteworthy is the distribution of $^{63}Ni^{2+}$ in

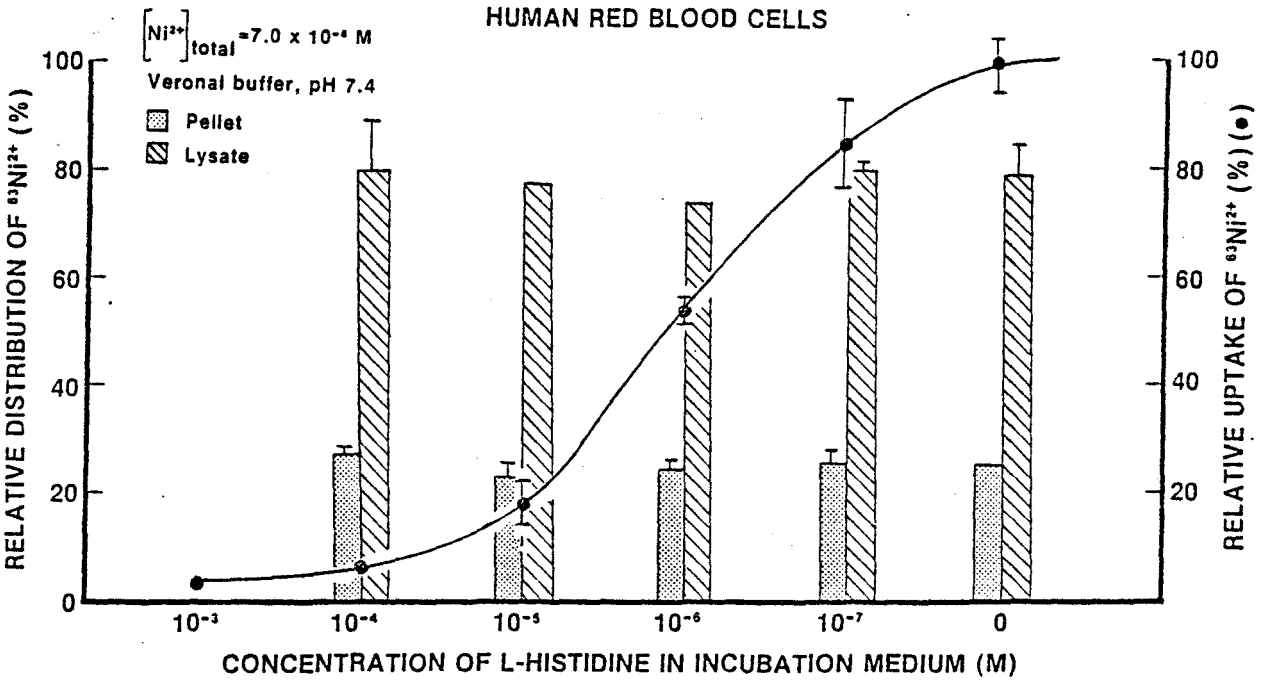

Fig. 3.1. The effect of L-histidine on the uptake and cellular distribution of $^{63}Ni^{2+}$ by human red blood cells. Error bars denote the mean standard deviation for duplicate samples; the absence of error bars indicates that the variance was small.

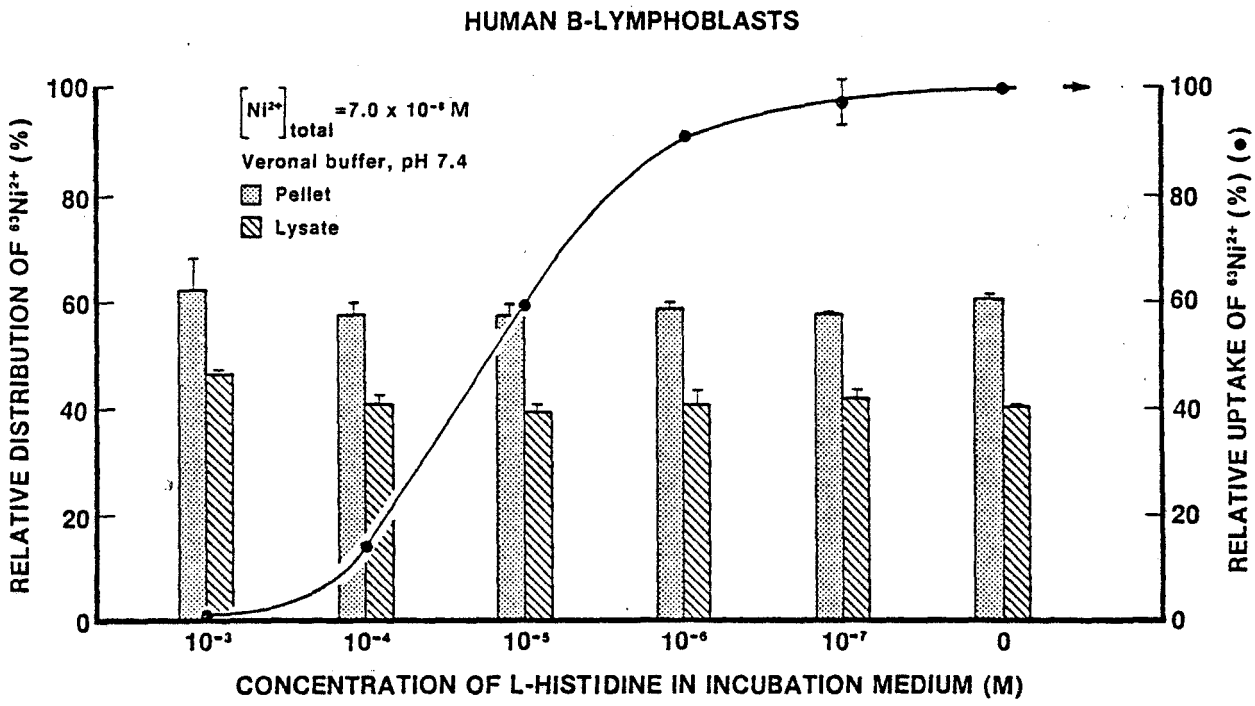

Fig. 3.2. The influence of L-histidine on the uptake and cellular distribution of $^{63}Ni^{2+}$ by cultured human B-lymphoblasts. Error bars denote the mean standard deviation for duplicate samples; the absence of error bars indicates that the variance was small.

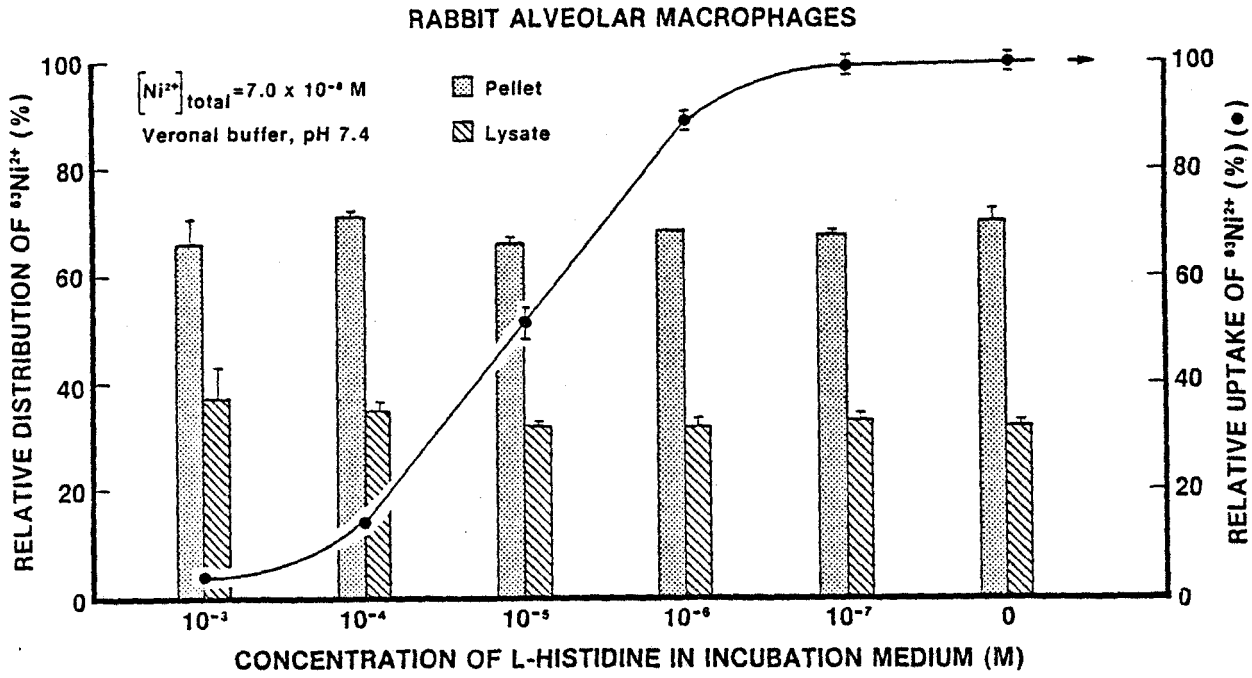

Fig. 3.3. The influence of L-histidine on the uptake and cellular distribution of $^{63}Ni^{2+}$ by rabbit alveolar macrophages. Error bars denote the mean standard deviation for duplicate samples; the absence of error bars indicates that the variance was small.

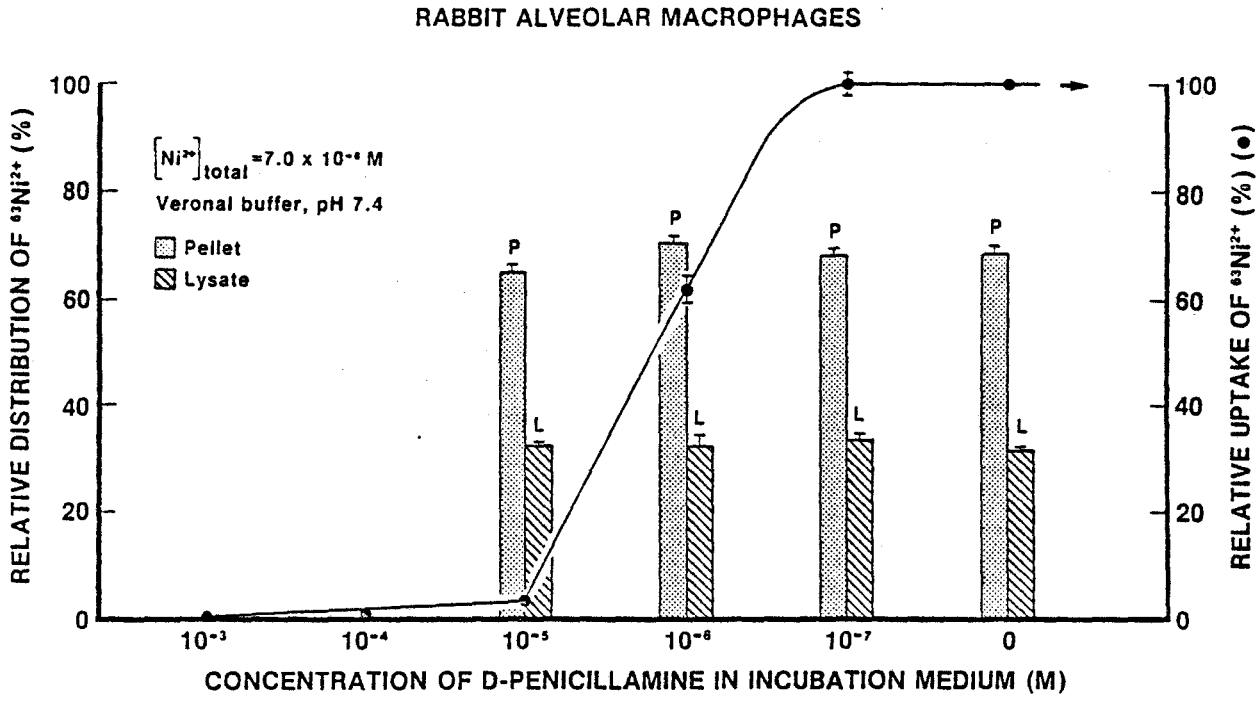

Fig. 3.4 The effect of D-penicillamine on the uptake and cellular distribution of $^{63}Ni^{2+}$ by rabbit alveolar macrophages. Error bars denote the mean standard deviation for duplicate samples; the absence of error bars indicates that the variance was small.

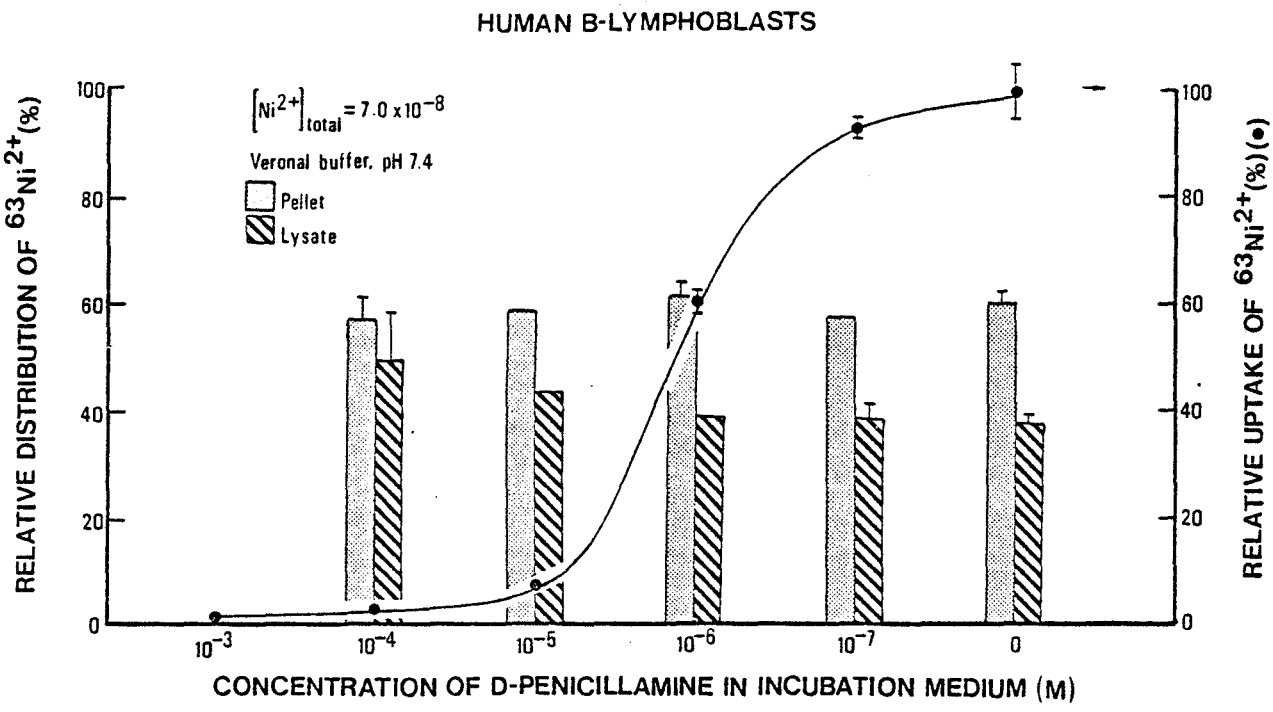

Fig. 3.5 The effect of D-penicillamine on the uptake and cellular distribution of $^{63}Ni^{2+}$ by human B-lymphoblasts. Error bars denote the mean standard deviation for duplicate samples; the absence of error bars indicates that the variance was small.

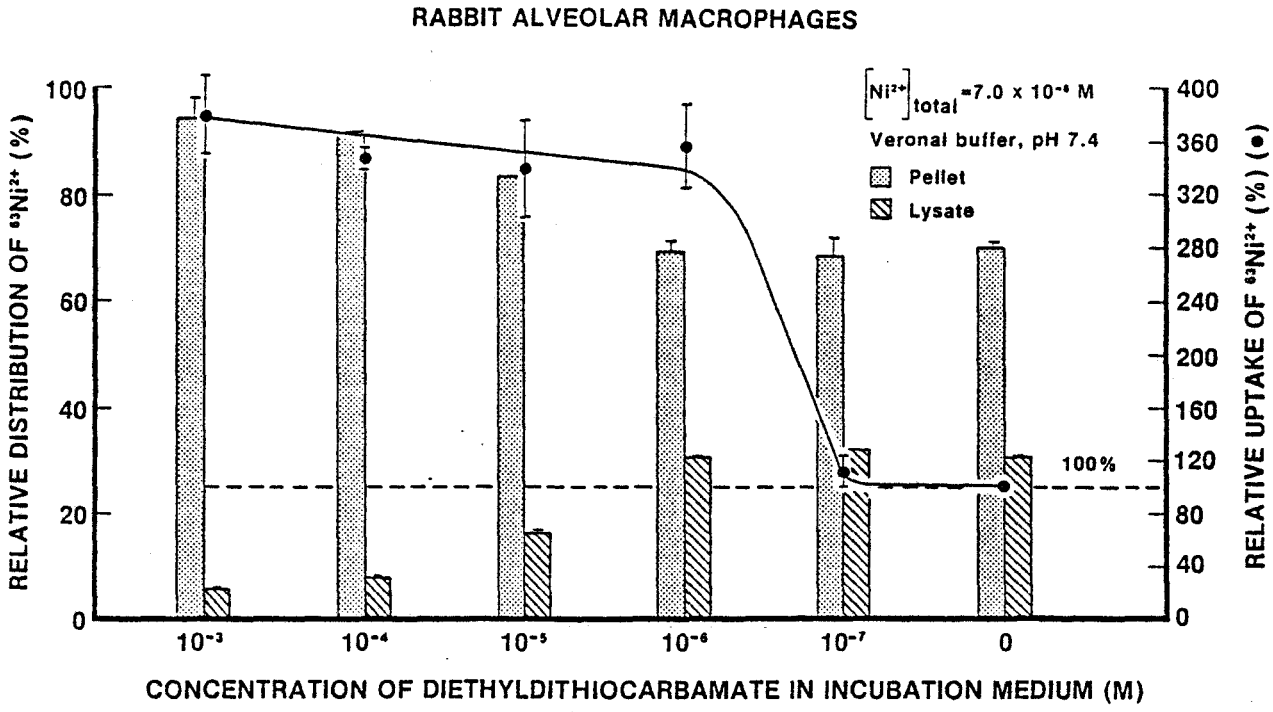

Fig. 3.6. The influence of DDC on the uptake and cellular distribution of $^{63}Ni^{2+}$ by rabbit alveolar macrophages. Error bars denote the mean standard deviation for duplicate samples; the absence of error bars indicates that the variance was small.

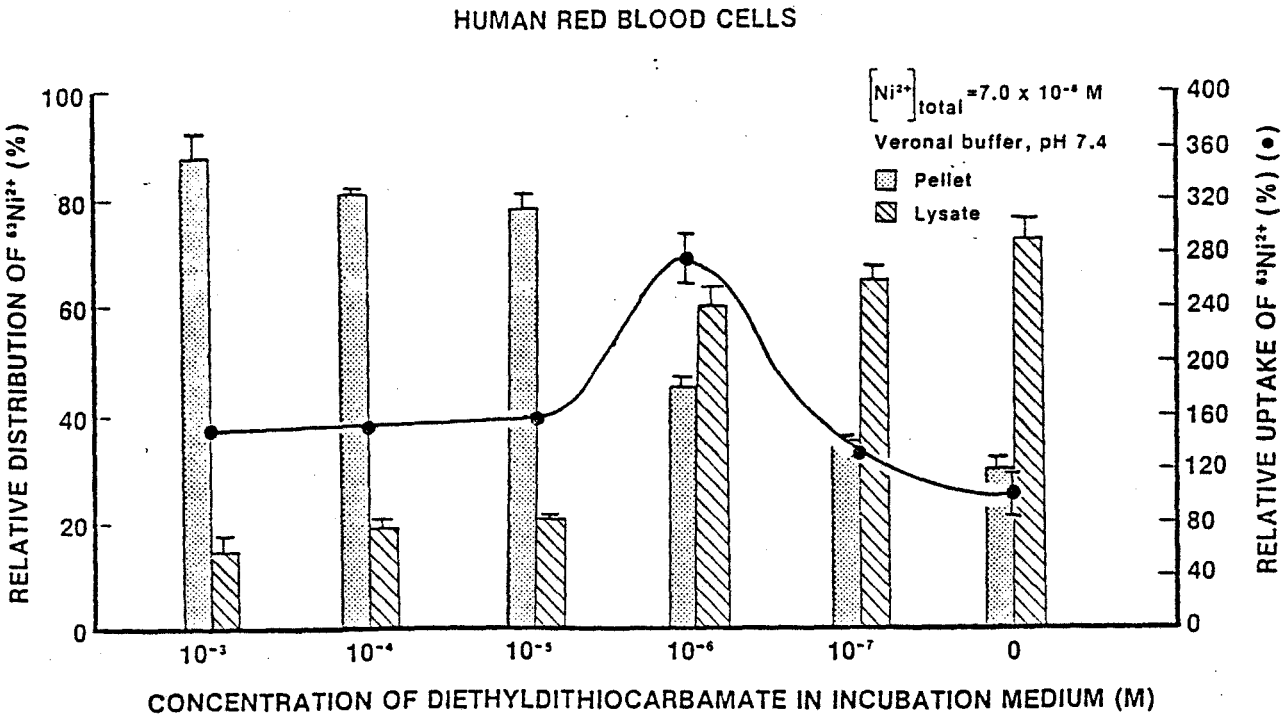

Fig. 3.7. The effect of DDC upon the uptake and cellular distribution of $^{63}Ni^{2+}$ by human RBCs. Error bars denote the mean standard deviation for duplicate samples; the absence of error bars indicates that the variance was small.

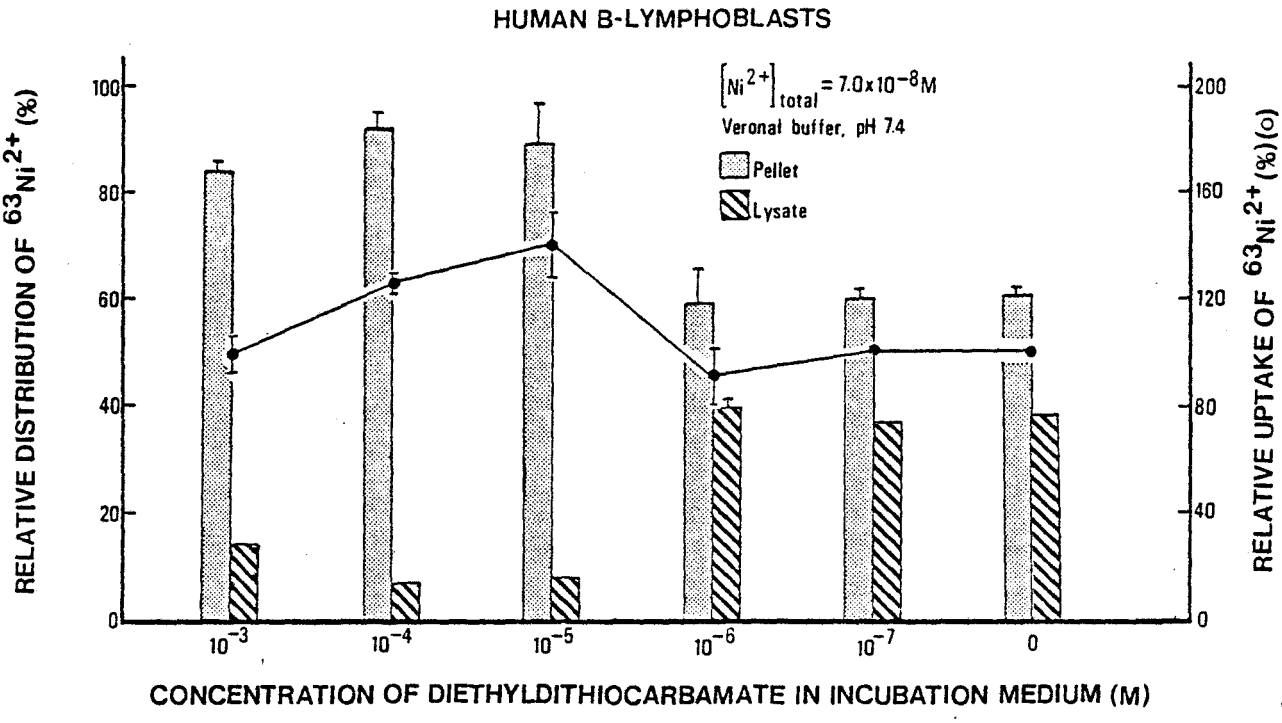

Fig. 3.8. The effect of DDC upon the uptake and cellular distribution of $^{63}Ni^{2+}$ by human B-lymphoblasts. Error bars denote the mean standard deviation for duplicate samples; the absence of error bars indicates that the variance was small.

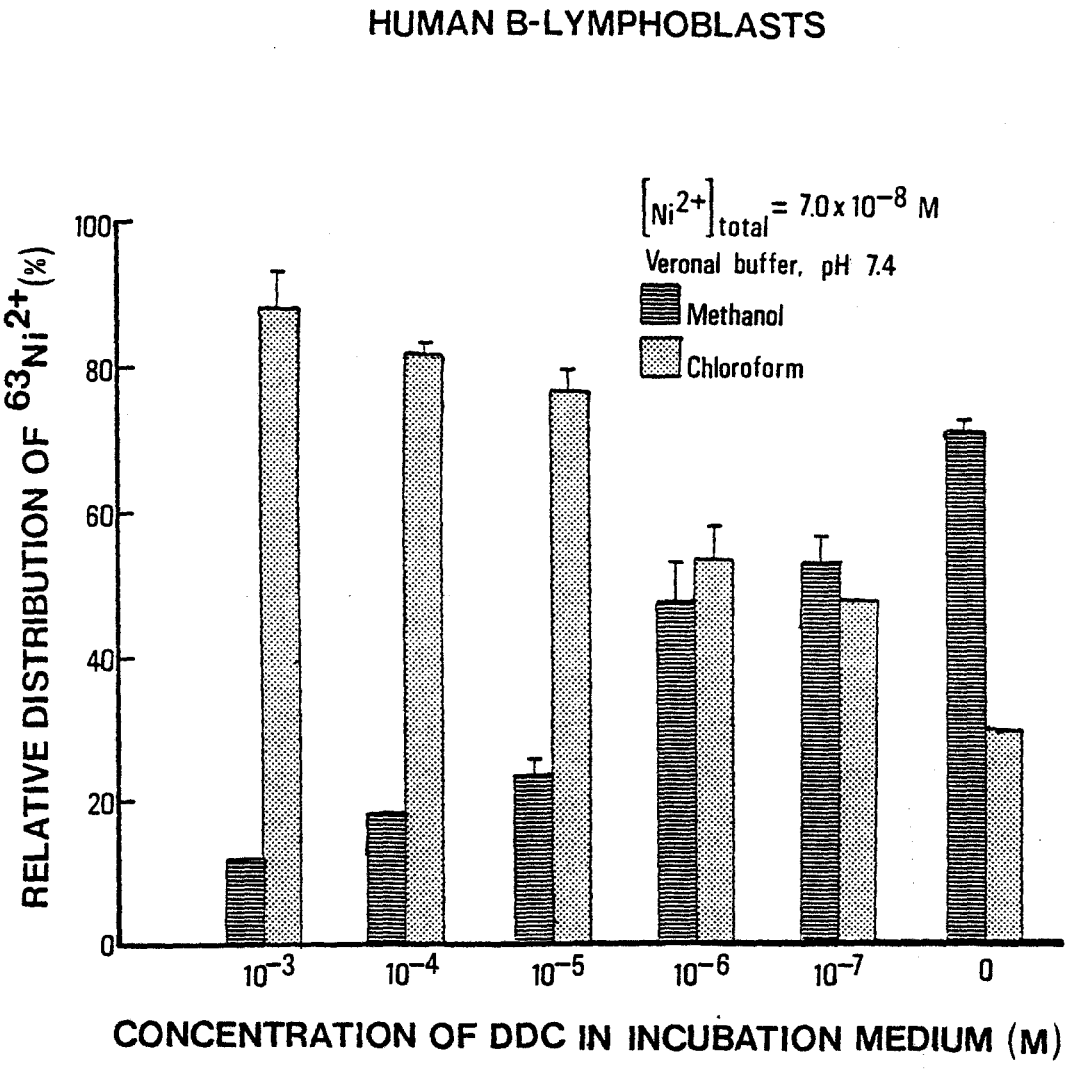

Fig. 3.9. The effect of DDC upon the distribution of $^{63}Ni^{2+}$ between protein (methanol soluble fraction) and lipid (chloroform soluble fraction) components of B-lymphoblasts cells. Error bars denote the mean standard deviation for duplicate samples; the absence of error bars indicates that the variance was small.

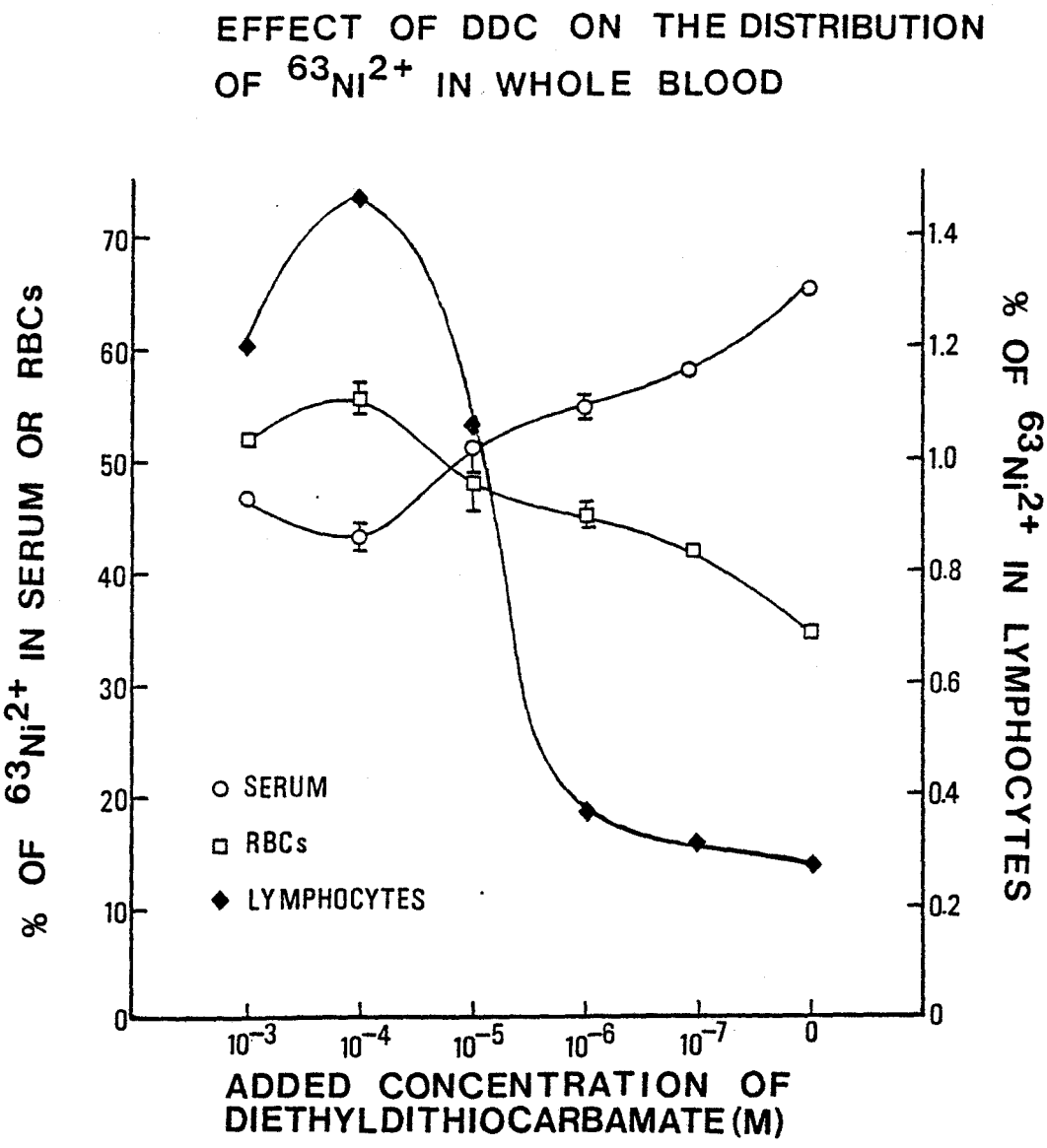

Fig. 3.10. The effect of DDC upon the distribution of $^{63}Ni^{2+}$ in human whole blood as partitioned between RBCs, lymphocytes, and serum. Error bars denote the mean standard deviation for duplicate samples; the absence of error bars indicates that the variance was small.

Table 3.1. The cellular distribution of $^{63}Ni^{2+}$ in absence of ligand.

Fraction	RBCs	Macrophages	B-Lymphoblasts
% in lysate	75.9±3.1	30.4±1.6	39.9±2.7
% in pellet	25.6±3.1	68.8±1.1	59.3±2.5

the absence of ligand by the three different cell types. The fraction of radiolabel associated with the membranous pellet increased in the order: RBCs <B-lymphoblasts <rabbit alveolar macrophages (see Table 3.1).

The lipophilicity of the Ni/DDC complex is demonstrated by the data in Fig. 3.9. There is a definite DDC concentration-dependent transfer of $^{63}Ni^{2+}$ to the lipid components of the cell.

The effect of DDC on $^{63}Ni^{2+}$ in whole blood is illustrated in Fig. 3.10. Again there is a DDC concentration-dependent transfer of nickel from serum to RBCs and lymphocytes which peaks at 10^{-4} M DDC.

3.4 DISCUSSION

It is evident from the distribution studies shown in Figs. 3.1-3.8 that D-Pen and L-His differ from DDC in their mechanisms of action upon Ni^{2+}. L-His and D-Pen have the ability to block $^{63}Ni^{2+}$ uptake into cells without changing the distribution of $^{63}Ni^{2+}$ between the cell lysate and pellet. DDC, on the other hand, causes an increase in cell-associated $^{63}Ni^{2+}$ and brings about a DDC concentration-dependent shift of $^{63}Ni^{2+}$ from the cell lysate to the pellet (Figs. 3.6-3.8). This divergence in behaviour is probably due to the difference in the solubility properties of the corresponding ligand-Ni^{2+}

complexes. L-His and D-Pen form hydrophilic complexes with Ni^{2+}, whereas the Ni^{2+}/DDC interaction results in the formation of a lipophilic complex. The predominant complex with D-Pen is $[Ni(D\text{-}Pen)_2]^{2-}$, which because of its relatively high negative charge is hydrophilic (Laurie et al., 1979; Jones et al., 1980). Lack of charge alone does not guarantee lipophilicity. This is illustrated by comparing the complexes $[Ni(L\text{-}His)_2]^{o}$ and $[Ni(DDC)_2]^{o}$, which are the dominant forms at physiological pH values. The latter complex is readily soluble in organic solvents (Sandell and Onishi, 1978), while the work of Glennon and Sarkar (1982) indicates that the former species exists in aqueous solution. Presumably the polar nature of the L-His ligand renders its neutral 2:1 nickel complex hydrophilic. Thus it is not surprising to find that DDC increased the cell pellet concentration of $^{63}Ni^{2+}$, as shown in Figs. 3.6-3.8, since the membranous cell pellet is high in lipids. This is further confirmed by the data in Fig. 3.9 which show an increase of $^{63}Ni^{2+}$ deposition in the lipophilic chloroform extract as the dose of DDC increases. These results are consistent with autoradiographic studies by Oskarsson and Tjälve (1980), who showed that DL-Pen treatment of Ni^{2+} poisoning (by $NiCl_2$) caused a decrease in nickel levels in mouse tissues, whereas DDC brought about an increase in nickel deposition in all tissues. In a related study with mice exposed to $Ni(CO)_4$, the Ni^{2+} was

transferred from the lung to lipid-rich tissues in the body on treatment with DDC (Oskarsson and Tjälve, 1979,1983). These observations are analogous to our whole-blood distribution findings. The data summarized in Fig. 3.10, show that DDC increased the association of $^{63}Ni^{2+}$ with RBCs and lymphocytes of whole blood, and from the results in Figs. 3.6-3.9 it may be deduced that this corresponds to the depostion of Ni^{2+} into the lipid structures of these cells.

The decline in the enhanced uptake observed for the B-lymphoblasts (Fig. 2.2, Chapter 2), RBCs (Fig. 3.7) and B-lymphoblasts (Fig. 3.8) at high DDC concentrations ($\geq 10^{-5}$M) suggest that the external ligand-incubation solution begins to compete effectively for the Ni^{2+}. This phenomenon was not observed for the alveolar macrophages (Fig. 3.6), which implies that the accessible lipid compartments proportionally constitute a larger fraction in this cell type.

Since the concentration of the $^{63}Ni^{2+}$ label in our experiments was comparable to that occurring naturally in blood (5 µg/l, Sunderman, 1977), the data in Fig. 3.10 (in the absence of DDC) should reflect the actual distribution of Ni^{2+} among the cellular components of blood. Thus about 65% occurs in serum, with 35% associated with the RBCs, and <<1% with the lymphocytes. This distribution pattern predicts that the whole blood Ni^{2+} concentration may be

expected to be comparable or slightly lower than serum levels. This conclusion is in conflict with the reported normal values of 2.6±0.9 µg/l for serum compared to 4.8±1.9 µg/l for whole blood (Sunderman, 1977). The quoted average concentration for Ni^{2+} in whole blood should be considered the least reliable because, unlike serum, no consensus exists about a dependable method for its analysis (Brown et al., 1981). There is evidence that adventitious contamination may have artificially elevated the reported whole blood results (Nieboer & Jusys, 1983), especially since the removal of iron required large sample size and extensive manipulation (Nomoto & Sunderman, 1970). Confirmation of this hypothesis and concern awaits the development of more reliable methods of whole-blood Ni^{2+} analysis.

The success of using DDC in the treatment of animals poisoned by $Ni(CO)_4$ has prompted its therapeutic use in man for the same purpose (Sunderman Jr., 1977; Sunderman Sr., 1979, 1981). The findings here and the whole body autoradiographic data reviewed suggest a possible mode of action. Ni^{2+} released from $Ni(CO)_4$ either by decomposition or ligand displacement (Nieboer et al., 1983c) presumably complexes with DDC, and in this form is temporarily stored in the lipid components of tissues. Since extracellular ligands such as HSA and L-His can remove Ni^{2+} from cells, equilibration with these ligands

results in transfer of Ni^{2+} to the blood which facilitates its renal excretion (Sarkar, 1983). Since the toxicologic impact of this partitioning into cellular lipid components has not been studied, the use of DDC in man in chelation therapy should be carefully investigated. It is known, for example, that DDC and $NiCl_2$ act synergistically to cause superinduction of microsomal heme oxygenase activity in rat liver and kidney (Sunderman et al., 1983). Because there is some evidence that Ni^{2+} is embryotoxic (Leonard et al., 1981), and since DDC may promote placental transport of Ni^{2+}, treatment of women of child-bearing years with DDC or its dimer Antabuse as practiced in the treatment of nickel hand dermatitis (Menne and Kaaber, 1978; Christensen and Kristensen, 1982) seems ill-advised.

CHAPTER 4

MODEL OF CELLULAR UPTAKE OF Ni^{2+}

4.1 Introductory Remarks

In Chapters 2 and 3, experimental evidence was summarized for the effects of ligands and pH on the cellular association and/or uptake of Ni^{2+}. In this Chapter, a model of Ni^{2+} uptake by cells is proposed and this ion's behaviour is compared to other metal ions.

4.2 Basis for Model of Ni^{2+} Uptake

The data reported in Figs. 2.1-2.6 illustrate the relative ease of Ni^{2+} passage in and out of a number of cell types. In the absence of ligands (other than the buffer), significant amounts of nickel were able to associate with cells. Roughly speaking, 50%$\pm$25% of the Ni^{2+} was incorporated into the lysate, depending on cell type, and this clearly confirms that intracellular accumulation had occurred. Formation of polar Ni(II)-complexes effectively blocked Ni^{2+} uptake. In the presence of the corresponding ligands, the sequestering of Ni^{2+} from preloaded cells was also highly efficient. This suggests that Ni^{2+} readily diffuses across cell membranes, and this appears not to depend on cell energy processes such as known for the ions K^+, Na^+ and Ca^{2+}. Further proof that Ni^{2+} translocation involves simple diffusion derives from the work of Evans, Dolovich and Nieboer (unpublished results), which showed that Ni^{2+} uptake by cells was relatively unaffected by oubain or iodoacetamide, which are known to disrupt cellular energy processes. With reference

to Figures 4.1 and 4.2, it may be concluded that Ni^{2+} uptake may be labelled as "equilibrium" uptake.

4.3 Model of Metal-Ion Uptake

The ability of Ni^{2+} to pass through membranes of cells places Ni^{2+} in a class different from most other cations. The endogenous metal cations fall into 1 of 2 classes. The first category consists of those cations (such as H^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Mn^{2+} and to some extent Fe^{2+}) which are actively transported across the membrane as the free ion by energy dependent processes (Williams, 1983; also see Fig. 4.1). A good example would be the ions whose transport is coupled to Na^+/K^+ ATPase. The Na^+/K^+-activated ATPase of most higher organisms expels 3 Na^+ from the cell with the consumption of 1 ATP and the influx of 1-2 K^+. This cycle of reaction is Mg^{2+} dependent. Furthermore, many processes depend on intracellular Ca^{2+} concentrations being kept very low. A Na^+/Ca^{2+} exchange pump, which uses the normal Na^+ concentration gradient across the plasma membrane to expel Ca^{2+}, is thought to operate in nerve cells, muscles of all types, and the gut (Harrison and Hoare, 1980). The passage of ions of this type through membranes is therefore under strict cellular control.

The second category consists of metal ions which must first bind to a carrier before they can be transported across membranes (formation of ML_1 in Fig. 4.1). These

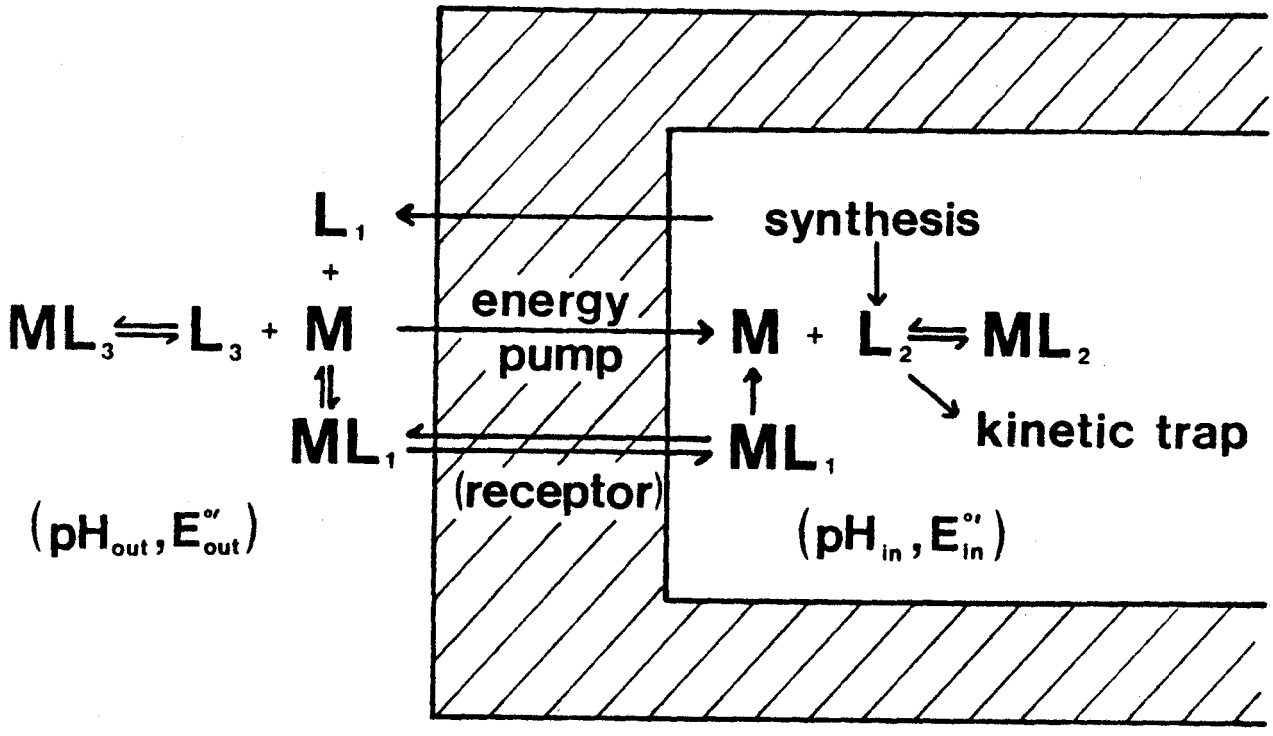

Figure 4.1 Diagram illustrating the various pathways of metal-ion uptake by cells. Ligand L_3 is an extracellular ligand that assists in the delivery of the metal ion, M(or its removal from) the extracellular compartment, while L_1 and L_2 are intracellular ligands that combine with M. Ligand L_1 may facilitate trans-membrane transport of M, and is shown to compete for M with L_3 in the extracellular fluid (or on the membrane surface). While the complex ML_1 passess through the membrane, there can be coupling with energy from unrelated energy-generating biochemical reactions. Membranes can also have highly selective channels with gates that by-pass the need for complex formation, and such direct transfer of M may or may not require energy. Non-mediated transport is possible because the electric potential across membranes at rest is negative. This potential gradient promotes the inward diffusion of cations, providing the concentration gradient is favourable.

Formal charges on M and L and ML are omitted for convenience, and the membrane is shaded. $E^{o'}$ refers to the redox potential at physiologic pH. Adapted from Williams (1981).

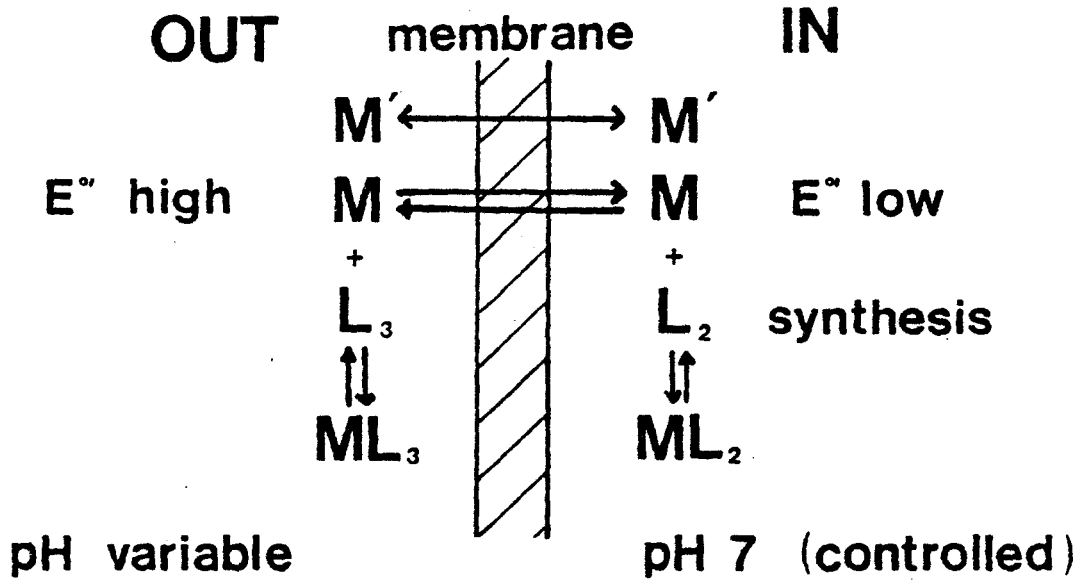

Figure 4.2. The "equilibrium" metal-ion uptake model. In contrast to phases in solvent extraction (or similar processes), the extracellular phase, the membrane, and the intracellular phase of cells are not in equilibrium. This is so because the uptake of M is energized in an indirect way. Many energized steps exist , owing to the impermeability of the membrane to many species, and to the metabolism and synthetic activities of cells. However, if its assumed that a steady state prevails (e.g., of pH and potential gradients, or ligand concentrations in the various phases), then the uptake of M can be treated as one in which M distributes between two very different phases containing L_3 and L_2 (or L_1), respectively. Only M itself can equilibrate, and no membrane processes are involved. The magnitude of the binding constants (of ML_2, ML_3; or ML_1) then determines partitioning of M between the outer aqueous phase and either the inner aqueous phase or the membrane. Often, to maintain charge balance, concomitant with the uptake of M another cation (M') is released. High redox potentials (E^{o}') favour metal ions in high oxidation states. Adapted with minor modifications from Williams (1981).

include Mo, Cu, Zn, Co, and Fe (Williams, 1983). For example it is well known that iron can easily be transported across membranes when bound to transferrin, haem, citrate or hydroxamic acids (Williams, 1983). This often involves receptor sites with either the surface release and subsequent transport of ions, or the direct uptake of the complex (e.g. transferrin by endocytosis). From the data reported in Chapters 2 and 3, it is evident that of the ligands examined, only DDC facilitated the intracellular uptake of Ni^{2+}. As explained (Section 3.4), the lipid solubility of the $Ni(DDC)_2$ complex appears to account adequately for this observation. Although the uptake of polar neutrally-charged nickel complexes such as $Ni(His)_2$ was not observed, such a mechanism cannot be ignored, *in vivo* (c.f., the iron(III)-citrate system). Studies with Cr(III)-complexes also emphasize this. The chromium glucose tolerance factor, CrGTF, which has been identified in Brewer's yeast as a chromium(III) dinicotinic acid-glutathione complex, is characterized by enhanced intestinal and placental absorption (Mertz, 1983; Nieboer *et al.*, 1983d). Synthetic analogues behaved similarly, in contrast to the Cr^{3+} ion which is not accumulated by cells (Langard, 1982).

4.4 The "Equilibrium" Model of Ni^{2+} Uptake

For all ligands examined except DDC, the equilibrium distribution of Ni^{2+} appeared to be controlled

by the binding capacities and concentrations of the extracellular ligand in the incubation medium. Since L-His, HSA, D-Pen and EDTA were very effective, these ligands can be said to bind Ni^{2+} better than binding sites on the cell surface and inside the cell. In contrast, L-Asp and L-Lys appear to exhibit lower apparent affinities than intracellular ligands. Like extracellular ligands, those inside the cell are mainly proteins and amino acids, although Ni^{2+} also has high affinity for the phosphate moieties of polynucleotides. Because of its boderline character, the Ni^{2+} would seek out oxygen-, nitrogen- and perhaps sulphur-containing functional groups (Nieboer and Richardson, 1980; Williams, 1981, also see section 1.5). Interestingly, and by analogy to Ni^{2+} (see Table 3.1), about 85% of Pb^{2+} occurred in the cell cytosol of RBCs and 15% in the corresponding membrane fraction (Ong and Lee, 1980a,b). The cellular distributions of Ni^{2+} and Pb^{2+} are therefore very similar. Fractionation experiments indicated that about 90% of the membrane Pb^{2+} was associated with protein, as was the case for Ca^{2+}. And like Ca^{2+}, chemical blocking of carboxylate groups on the membrane proteins showed that 70% of the Pb^{2+} appeared to be bound to these oxygen-type binding centers. Since Pb^{2+} also is a borderline metal ion, the binding of Ni^{2+} might be expected to parallel the Pb^{2+} complex-formation patterns described.

It was noted that the flux of Ni^{2+} ions was pH dependent (Fig. 2.7; Section 2.3). This was explained as originating from either: (1) an increase in membrane permeability with increasing pH; (2) the development of a proton gradient across the cell membrane favouring the antiport transport of H^+ and Ni^{2+}; or (3) Ni^{2+}/proton competition for cellular binding groups. Explanations (2) and (3) are consistent with the "equilibrium" model depicted in Fig 4.2. Thus one possibility is that M' in this figure represents the proton. The other interpretation is the expected competition between M and H^+ for L_3 in Fig. 4.2. This may be illustrated by a specific example if L_3 were ammonia (Williams, 1981). Ni^{2+} binds to ammonia molecules with a 1:1 binding constant of logK=3.0. However, the proton binds to NH_3 with a pK_a of 9.0. From these data it may be shown that at pH<6, nickel ions do not bind NH_3 while protons do. It is conceivable, therefore, that Ni^{2+} and H^+ compete for functional groups at the cell surface. Consequently at pH values <7, it would appear that it was difficult for Ni^{2+} to compete with the proton as little accumulation occurred (see Fig. 2.7).

And finally, the illustrated models in Figs. 4.1 and 4.2 facilitate an understanding of the additional events that must occur under simulated physiological and <u>in vivo</u> conditions. As summarized in Chapter 2, L-His and HSA added to veronal buffer in physiological concentrations

inhibited Ni^{2+} uptake by 85-95%, depending on cell type. The whole blood distribution data summarized in Fig. 3.10 indicated that about 0.3% of the added $^{63}Ni^{2+}$ became associated with peripheral lymphocytes, 35% with RBCs, and 65% with serum. The unexpected high association of Ni^{2+} with the RBCs in whole blood may originate for one or more of the following reasons. (1) Endogenous cations might compete with Ni^{2+} for extracellular ligands, which would increase the free Ni^{2+} concentration and thus would enhance uptake. (2) The presence of chelating agents suitable for promoting uptake of Ni^{2+} by facilitated diffusion might occur. And (3), a higher concentration ratio of cells to Ni^{2+} than employed in the simple buffer systems exists *in vivo* and should promote additional association of Ni^{2+} with RBCs in whole blood.

5. REFERENCES

Abbracchio, M. P., Evans, R. M., Heck, J.D., Cantoni, O. and Costa, M. (1982a). The regulation of ionic nickel uptake and cytotoxicity by specific amino acids and serum components. Biol. Trace Element Res., 4, 289-301.

Abbracchio, M. P., Simmons-Hansen, J. and Costa, M. (1982b). Cytoplasmic dissolution of phagocytized crystalline nickel sulfide particles: A prerequisite for nuclear uptake of nickel. J. Toxicol. Environ. Health, 9, 663-676.

Al-Tawil, N. G., Marcusson, J. A. and Möller, E. (1981). Lymphocyte transformation test in patients with nickel sensitivity: an aid to diagnosis. Acta Derm. Venereol. (Stockholm), 61, 511-515.

Asato, N., Soestbergen, M., Van, and Sunderman, F. W., Jr. (1975). Binding of ^{63}Ni(II) to ultrafiltrable constituents of rabbit serum in vivo and in vitro. Clin. Chem., 21, 521-527.

Barrie, L. A. (1981). Atmospheric nickel in Canada., In: NRCC No. 18568, Effects of Nickel in the Canadian Environment., Ottawa, pp. 55-75.

Barton, R.T., Andersen, I. and Høqetveit, A. C. (1980). Distribution of nickel in blood fractions. In: Brown, S. S. and Sunderman, F. W., Jr., eds., Nickel Toxicology., Toronto, Academic Press, pp. 85-88.

Baselt, R. C., Sunderman, F. W., Jr., Mitchell, J. and Horak, E. (1977). Comparisons of antidotal efficacy of sodium diethyldithiocarbamate, D-Penicillamine and triethylenetetramine upon acute toxicity of nickel carbonyl in rats. Res. Commun. Pathol. Pharmacol., 18, 677-688.

Bertrand, D. (1974). Microbioligie du sol. Le nickel, oligoelement pour les micro-organismes fixateurs de l'azote de l'aire. C. R. Acad. Sci. Paris, Ser. D., 278, 2231-2235.

Brown, S. S., Nomoto, S., Stoeppler, M. and Sunderman, F. W., Jr. (1981). IUPAC reference method for analysis of nickel in serum and urine by electrothermal atomic

absorption spectrometry. Pure. Appl. Chem., 53, 773-781.

Cannon, D. C., Olitzky, I. and Inkpen, J. A. (1974). Proteins. In: Henry, R. J., Cannon, D. C. and Winkelman, J. W., eds., Clincal Chemistry. New York, Harper and Row., pp. 405-502.

Castranova, V., Bowman, L., Miles, P. R. and Reasor, M. J. (1980). Toxicity of metal ions to alveolar macrophages. Am. J. Ind. Med., 1, 349-357.

Cecutti, A., and Nieboer, E. (1981). Nickel Metabolism and Biochemistry., In: NRCC No. 18568, Effects of Nickel in the Canadian Environment., Ottawa, pp 193-216.

Christensen, O. B. and Kristensen, M. (1982). Treatment with disulfiram in chronic nickel hand dermatitis. Contact Dermatitis, 8, 59-63.

Clark, J. M. and Switzer, R. L. (1977). Experimental Biochemistry, 2nd ed., San Francisco, W.H. Freeman and Company, pp. 191-194.

Costa, M., Heck, J. D. and Robison, S. H. (1982). Selective phagocytosis of crystalline metal sulfide particles and DNA strand breaks as a mechanism for the induction of cellular transformation. Cancer Res., 42, 2757-2763.

Costa, M., Simmon-Hansen, J. and, Bedrossian, C. W. M., Bonura, J. and Caprioli, R. M. (1981). Phagocytosis, cellular distribution, and carcinogenic activity of particulate nickel compounds in tissue culture. Cancer Res., 41, 2868-2876.

Dickinson, J. C., Rosenblum, H. and Hamilton, P. B. (1965). Ion exchange chromatography of the free amino acids in the plasma of the newborn infant. Pediatrics, 36, 2-13.

Diekert, G., Konheiser, U., Piechulia, K. and Thauer, R. (1981). Nickel requirement and factor F_{430} content of methanogenic bacteria. J. Bact., 148, 459-464.

Dixon, N. E., Gazzola, C., Asher, C. J., Lee, D. S. W., Blakeley, R. L. and Zerner, B. (1980). Jack bean urease (EC 3.5.1.5). II. The relationship between nickel, enzymatic activity, and the "abnormal" ultraviolet spectrum. The nickel content of jack beans. Can. J. Biochem., 58, 474-488.

Doll, R. (1958). Cancer of the lung and nose in nickel workers. Br. J. Ind. Med., 15, 217-223.

Doll, R., Mathews, J. D. and Morgan, L. G. (1977). Cancers of the lung and nasal sinuses in nickel workers: a reassessment of the period of risk. Br. J. Ind. Med., 34, 102-105.

Dormer, R. L., Kerbey, A. L., McPherson, M., Manley, S., Ashcroft, S. J. H., Schofield, J. G. and Randle, P. J. (1973). The effect of nickel on secretory systems. Studies on the release of amylase, insulin and growth hormone. Biochem. J., 140, 135-142.

Eichhorn, G. L. (1975). Complexes of polynucleotides and nucleic acids. In: Eichhorn, G. L., ed., Inorganic Biochemistry., Elsevier Pub. Co., Amsterdam, pp. 1210-1243.

Gardner, D. E. (1980). Dysfunction of host defenses following nickel inhalation. In: Brown, S. S. and Sunderman, F. W., Jr., eds., Nickel Toxicology, Toronto, Academic Press, pp. 121-124.

Glennon, J. D. and Sarkar, B. (1982). Nickel (II) transport in human blood serum. Biochem. J., 203, 15-23.

Hansen, K. and Stern, R. M. (1983). Toxicity and transformation potency of nickel compounds in BKH cells in vitro. In: IARC Monograph on Nickel in the Human Environment., in press.

Harris, J. R. (1969). The isolation and purification of a macromolecular protein component from the human erythrocyte ghost. Biochim. Biophys. Acta, 188, 31-42.

Harrison, P. M. and Hoare, R. J. (1980). Metals in Biochemistry., London, Chapman and Hall, 80 pp.

Head, M. D., Englesakis, V. A., Pearson, B. C. and Wilkinson, D. H. (1976). Nickel Refining by the TBRC Smelting and Pressure Carbonyl Route., International Nickel Company (Canada) Ltd., Copper Cliff, Ontario, 60 pp.

Heck, J. D. and Costa, M. (1982). Surface reduction of amorphous NiS particles potentiates their phagocytosis and subsequent induction of morphological transformation in Syrian hamster embryo cells. Cancer Lett., 15, 19-26.

Horak, E., Sunderman, F. W., Jr. and Sarkar, B. (1976). Comparisons of antidotal efficacy of chelating drugs upon acute toxicity of Ni(II) in rats. Res. Commun. Chem. Pathol. Pharmacol., 14, 153-165.

Jarrell, K. F. and Sprott, G. D. (1982). Nickel transport in Methanobacterium bryantii. J. Bacteriol., 151, 1195-1203.

Jones, D. C., May, P. M. and Williams, D. R. (1980). Computer simulation models of low-molecular-weight nickel(II) complexes and therapeuticals in vivo. In: Brown, S. S. and Sunderman, F. W., Jr., eds., Nickel Toxicology, Toronto, Academic Press, pp. 73-76.

Kasprzak, K., Gabryel, G. and Jarczewska, K. (1983). Carcinogenicity of nickel(II)hydroxides and nickel(II)sulfate in Wistar rats and its relation to the in vitro dissolution rates. Carcinogenesis, 4, 275-279.

Kasprzak, K. S. and Sunderman, F. W., Jr. (1979). Radioactive ^{63}Ni in biological research. Pure Appl. Chem., 51, 1375-1389.

Kirchgessner, M. and Schnegg, A. (1976). Malate dehydrogenase and glucose-6-phosphate dehydrogenase activity in livers of Nideficient rats. Bioinorg. Chem., 6, 155-161.

Kirkpatrick, D. C., and Coffin, D. E. (1977). The trace metal content of a representative Canadian diet in 1972. Can. J. Public Health, 68, 162-164.

Kuehn, K., Fraser, C. B. and Sunderman, F. W., Jr. (1982). Phagocytosis of particulate nickel compounds by rat peritoneal macrophages in vitro. Carcinogenesis., 3, 321-326.

Kuehn, K. and Sunderman, F. W., Jr. (1982). Dissolution half-times of nickel compounds in water, rat serum, and renal cytosol. J. Inorg. Biochem., 17, 29-39.

Laitinen, H. A. and Harris, W. E. (1975). Chemical Analysis. 2nd ed., McGraw-Hill Book Co., Toronto, pp 190-191.

Langard, S. (1982). Absorption, transport and excretion of chromium in man and animals. In: Langard, S. ed., Biological and Environmental Aspects of Chromium., Elsevier Biomedical Press, pp. 149-170.

Laurie, S. H., Prime, D. H. and Sarkar, B. (1979). Analytical potentiometric and spectroscopic study of the equilibria in the aqueous nickel(II)-triethylenetetramine and nickel(II)-D-penicillamine systems. Can. J. Chem., 57, 1411-1417.

Leonard, A., Gerber, G. B. and Jacquet, P. (1981). Carcinogenicity, mutagenicity and teratogenicity of nickel. Mut. Res., 87, 1-15.

Lucassen, M. and Sarkar, B. (1979). Nickel(II)-binding constituents of human blood serum. J. Toxicol. Environ. Health, 5, 897-905.

Macleod, T. M., Hutchinson, F. and Raffle, E. J. (1970). The uptake of labelled thymidine by leucocytes of nickel sensitive patients. Br. J. Derm., 82, 487-492.

Malo, J. L., Cartier, A., Doepner, M., Nieboer, E., Evans, S. and Dolovich, J. (1982). Occupational asthma caused by nickel sulfate. J. Allergy Clin. Immunol., 69, 55-59.

McConnell, L. H., Fink, J. N., Schlueter, D. P. and Schmidt, M. G., Jr. (1973). Asthma caused by nickel sensitivity. Ann. Intern. Med., 78, 888-890.

McGee, M. P. and Myrvik, Q. N. (1981). Collection of alveolar macrophages from rabbit lungs. In: Herscowitz, H. B., Holden, H. T., Bellanti, J. A. and Ghaffar, A., eds., Manual of Macrophage Methodology. New York, Marcel Dekker Inc., pp. 17-22.

Menne, T. and Kaaber, K. (1978). Treatment of pompholyx due to nickel allergy with chelating agents. Contact Dermatitis, 4, 289-290.

Mertz, W. (1983). Chromium: An ultra-trace element. Chemica Scripta, 21, 145-150.

Morgan, J. G. (1958). Some observations on the incidence of respiratory cancer in nickel workers. Br. J. Ind. Med., 15, 224-234.

Nicholls, D. G. (1982). Bioenergetics. Toronto, Academic Press, 199 pp.

Nieboer, E. (1981). The physical and chemical properties of nickel. In: NRCC No. 18568, Effects of Nickel in the Canadian Environment., Ottawa, pp. 261-265.

Nieboer, E., Evans, S. L. and Dolovich, J. (1983b). Occupational asthma from nickel sensitivity: II, Factors influencing the interactions of Ni^{2+}, HSA, and serum antibodies with nickel related specificity. Br. J. Ind. Med., 40, 325-329.

Nieboer, E. and Jusys, A. A. (1983). Contamination control in routine ultratrace analysis of toxic metals., In: Brown, S. S. and Savory, J., eds., Clinical Chemistry and Chemical Toxicology of Metals, Academic Press, London, in press.

Nieboer, E., Maxwell, R. I. and Stafford, A. R. (1983a). Chemical and biological reactivity of insoluble nickel compounds and the bioinorganic chemistry of nickel. In: IARC Monograph on Nickel in the Human Environment., In press.

Nieboer, E. and Richardson, D. H. S. (1980). The replacement of the nondescript term "heavy metals" by a biologically and chemically significant classification of metal ions. Environ. Pollut. (Series B)., 1, 3-26.

Nieboer, E., Yassi, A. and Haines, T. (1983d). Chromium and Human Health. Special document, McMaster University, Hamilton, Ont.

Nieboer, E., Yassi, A., Jusys, A. A. and Muir, D. C. (1983c). Technical Feasibility and Usefulness of Biological Monitoring in the Nickel Producing Industry, Technical Report, McMaster University, Hamilton, Ontario, 286 pp.

Nielson, F. H. (1974). Essentiality and function of nickel. In: Hoekstra, W. G., Suttie, J. W., Gauther, H. E. and Mertz, W., eds., Trace Element Metabolism in Animals-2, Maryland University Park Press, Baltimore, Md., pp. 381-395.

Nielson, F. H. (1980). Nutrients and growth regulators: chemistry and physiology-nickel., In: CRC Handbook of Nutrition and Food., Chemical Rubber Company Press, Boca Raton, Florida, USA.

Nomoto, S. (1980). Fractionation and quantitative determination of alpha-2 macroglobulin-combined nickel in serum by affinity column chromatography. In: Brown, S. S. and Sunderman, F. W., Jr., eds., Nickel Toxicology, Toronto, Academic Press, pp. 89-90.

Nomoto, S. and Sunderman, F. W., Jr. (1970). Atomic absorption spectrometry of nickel in serum, urine, and other biological materials. Clin. Chem., 16, 477-485.

Ochiai, E. I. (1977). Bioinorganic Chemistry. Allyn and Bacon Inc., Boston, U.S.A., 515 pp.

Ong, C. N. and Lee, W. R. (1980a). Interaction of calcium and lead in human erythrocytes. Br. J. Ind. Med., 37, 70-77.

Ong, C. N. and Lee, W. R. (1980b). Distribution of lead-203 in human peripheral blood in vitro. Br. J. Ind. Med, 37, 78-84.

Oskarsson, A. and Tjälve, H. (1979). The distribution and metabolism of nickel carbonyl in mice. Br. J. Ind. Med., 36, 326-335.

Oskarsson, A. and Tjälve, H. (1980). Effects of diethyldithiocarbamate and penicillamine of the tissue distribution of $^{63}NiCl_2$ in mice. Arch. Toxicol., 45, 45-52.

Oskarsson, A. and Tjälve, H. (1983). Nickel mobilization by sodium diethyldithiocarbamate in nickel carbonyl treated mice. In: IARC Monograph on Nickel in the Human Environment. in press.

Paulsen, G., Hofsoy, H. and Jonsen, J. (1978). Isolation of a possible nickel-binding peptide in rabbit serum. Abstracts of Conference on Nickel Toxicity., Kristiansand, Norway, May 22-24, 1978.

Pedersen, E., Andersen, A. and Høgetveit, A. C. (1978). Second study of the incidence and mortality of cancer of respiratory organs among workers at a nickel refinery. Ann. Clin. Sci., 8, 503.

Polacco, J. C. (1977). Nitrogen metabolism in soybean tissue culture. II. Urea utilization and urea synthesis require Ni(2+). Plant Physiol., 59, 827-830.

Rees, T. A. V. and Bekheet, I. A. (1982). The role of nickel in urea assimilation by algae. Planta, 156, 385-387.

Roberts, R. S., Julian, J. A. and Muir, D. C. F. (1982). An Analysis of Mortality from Cancer. The JOHC-INCO Mortality Study, McMaster University, Hamilton, Ont., 37 pp.

Rubanyi, G. and Balogh, I. (1982). Effect of nickel on uterine contraction and ultrastructure in the rat. Am. J. Obstet. Gynecol., 142, 1016-1020.

Sandell, E. B. and Onishi, H. (1978). Photometric Determination of Trace Metals: General Aspects. In: Elving, P. J., Winefordner, J. D. and Kolthoff, I. M., eds., Chemical Analysis. Vol. 3, Part 1, fourth ed., New York, John Wiley, pp. 512-526.

Sarkar, B. (1983). Biological specificity of the transport process of copper and nickel. Chemica Scripta, 21, 101-108.

Sirover, M. A. and Loeb, L. A. (1976). Infidelity of DNA synthesis in vitro: screening for potential metal mutagens or carcinogens. Science, 194, 1434-1436.

Spiro, T. G. ed. (1980). Nucleic Acid-Metal Ion Interactions., New York, John Wiley and Sons, pp. 599-684.

Stanners, C. P., Eliceiri, G. L. and Green, H. (1971). Two types of ribosome in mouse-hamster hybrid cells. (Nature) New Biol., 230, 52-54.

Stillwell, E. F. and Holland, J. R. (1977). Nickel effects on cell division, calcification and cell protein in the Coccolithophorid, Cricosphaera carterae. Sci. Biol. J., 3, 401-408.

Sumino, K., Hayakawa, K., Shibata, T. and Kitamura, S. (1975). Heavy metals in normal Japanese tissues. Arch. Environ. Health, 30, 487-494.

Sunderman, F. W., Jr. (1977). A review of the metabolism and toxicology of nickel. Ann. Clin. Lab. Sci., 7, 377-398.

Sunderman, F. W., Jr. (1979). Mechanisms of metal carcinogenesis, In: Fleicher, M., ed., Proc. 2nd Arnold O. Beckman Conference in Clinical Chemistry, San Antonio, Texas, September 6-8, 1978., Am. Assoc. Clin. Chem., Washington, D. C., pp. 265-297.

Sunderman, F. W., Jr. (1983b). Carcinogenicity of nickel compounds in animals. In: IARC Monograph on Nickel in the Human Environment. in press.

Sunderman, F. W., Jr. (1983a). Nickel derermination in body fluids, tissues, excretion and water. IARC Manual

Series: Environmental Carcinogens - Selected Methods of Analysis, Vol. 7: Some Elements and their Compounds., 24pp.

Sunderman, F. W., Jr., Coulston, F., Eichorn, G. I., Fellows, J. A., Mastromatteo, E., Reno, H. T. and Samitz, M. H. (1975). Nickel. A report of the committee on medical and biologic effects environmental pollutants., U.S. Nat. Acad. Sci., Washington, D. C., 277 pp.

Sunderman, F. W., Jr., Decsy, M. I. and McNeely, M. D. (1972). Nickel metabolism in health and disease. Ann. N. Y. Acad. Sci., 199, 300-312.

Sunderman, F. W., Jr. and Hopfer, S. M. (1983). Correlation between the carcinogenic activities of nickel compounds and their potencies for stimulating erythropoiesis in rats. In: Sarkar, B., ed., Biological Aspects of Metals and Metal-Related Diseases, Raven Press, New York, in press.

Sunderman, F. W., Jr. and Maenza, R. M. (1976). Comparisons of carcinogenicities of nickel compounds in rats. Res. Commun. Chem. Path. Pharmacol., 14, 319-330.

Sunderman, F. W., Jr., Reid, M. C., Bibeau, L. M. and Linden, J. V. (1983). Nickel induction of microsomal heme oxygenase activity in rodents. Toxicol. Appl. Pharmacol., 68, 87-95.

Sunderman, F. W., Sr. (1970). Nickel poisioning., In: Sunderman, F. W., Sr. and Sunderman, F. W., Jr., eds. Laboratory Diagnosis of Diseases Caused by Toxic Agents, W. H. Green Inc., St. Louis, USA, pp. 387-396.

Sunderman, F. W., Sr. (1979). Efficacy of sodium diethyldithiocarbamate (Dithiocarb) in acute nickel carbonyl poisoning. Ann. Clin. Lab. Sci., 9, 1-10.

Sunderman, F. W., Sr. (1981). Chelation therapy in nickel poisoning. Ann. Clin. Lab. Sci., 11, 1-8.

Thauer, R. K., Diekert, G. and Schonheit, P. (1980). Biological role of nickel. TIBS, 5, 304-306.

Thomsom, A. J. (1982). Proteins containing nickel. Nature, 298, 602-603.

Thulin, H. (1976). The leukocyte migration test in nickel contact dermatitis. Acta Derm. Venereol. (Stockholm), 56, 377-380.

Van de Drift, C. and Vogels, G. D. (1970). Effect of metal and hydrogen ions on the activity and stability of allantoicase. Biochem. Biophys. Acta., 198, 339-352.

Van Soestbergen, M. and Sunderman, F. W., Jr. (1972). ^{63}Ni complexes in rabbit serum and urine after injection of $^{63}NiCl_2$. Clin. Chem., 18, 1478-1484.

Vuopala, V., Huhti, E., Takkunen, J. and Huikko, M. (1970). Nickel carbonyl poisoning: report of 25 cases. Ann. Clin. Res., 2, 214-222.

Williams, R. J. P. (1983). Inorganic elements in biological space and time. Pure and Appl. Chem., 55, 1089-1100.